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Avaliação da participação de canais de cálcio voltagem-dependentes sobre a resposta pruritogênica em camundongos

IZAQUE DE SOUSA MACIEL

Porto Alegre, 2014

Izaque de Sousa Maciel

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Dissertação apresentado ao Programa de Pós-Graduação em Medicina e Ciências da Saúde, com ênfase em Farmacologia Bioquímica e Molecular, da Pontifícia Universidade Católica do Rio Grande do Sul, como parte dos requisitos para a obtenção do Título de Mestre.

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Banca Examinadora:

Dr. Robson Costa – (UFRJ)

Dr. Jarbas Rodrigues Oliveira – (PUCRS)

Dr. Ivan Carlos F. Antonello– (PUCRS)

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“A humildade é o primeiro degrau para a sabedoria”

“Dê-me, Senhor, agudeza para entender, capacidade para reter, método e faculdade para aprender, sutileza para interpretar, graça e abundância para falar. Dê-me, Senhor, acerto ao começar, direção ao progredir e perfeição ao concluir”

“O estudioso é aquele que leva aos demais o que ele compreendeu: a Verdade”

Santo Tomás de Aquino

RESUMO

Foram avaliados os efeitos da inibição farmacológica dos canais de cálcio voltagem-dependentes (CCVD) no prurido em camundongos. A administração intratecal (i.t.) dos bloqueadores CCVD do subtipo P/Q (MVIIA ou PhTx3.3), subtipo L (verapamil), subtipo T (NNC 55-0396) ou do subtipo R (SNX-482) não alterou o comportamento de coçar induzido pela tripsina (agonista do receptor PAR-2), aplicada na região dorsal do pescoço de camundongos. Entretanto, o comportamento de coçar induzido pela tripsina foi significativamente diminuído pela administração i.t. dos bloqueadores de CCVD do subtipo N (MVIIA e Phα1β). A toxina MVIIA, derivada do *C. magus*, apresentou efeitos significativos quando administrada entre 1 a 4 horas antes da aplicação de tripsina. Por outro lado, a toxina Phα1β derivada do veneno da aranha *P. nigriventer* demonstrou atividade, quando injetada até 12 h antes da tripsina. O pré-tratamento com MVIIA ou Phα1β foi efetivo em inibir o comportamento de coçar induzido pela aplicação intradérmica (i.d.) de SLIGRL-NH2, composto 48/80 ou cloroquina; entretanto, as toxinas não inibiram o comportamento de coçar induzido pela injeção i.d. de H_2O_2. A co-injeção de MVIIA ou Phα1β inibiu o comportamento de coçar induzido pela aplicação i.t. do peptídeo liberador de gastrina (GRP), mas não inibiu o prurido causado pela morfina. Relevantemente, a administração i.t. da toxina MVIIA ou Phα1β inibiu a coceira crônica induzida pelo modelo de pele seca em camundongos. A atividade anti-pruritogênica de ambas as toxinas não parece estar relacionada com a modulação do processo inflamatório na pele, uma vez que as toxinas MVIIA ou Phα1β não foram capazes de inibir o edema e a migração de neutrófilos induzidos pela aplicação i.d. de tripsina. A aplicação i.t. de MVIIA ou Phα1β não modificou a expressão do receptor para o GRP (GRP-R) na medula espinhal. Por outro lado, as duas reduziram a expressão de c-Fos aos níveis do grupo controle, de acordo com a avaliação na medula dos camundongos. Finalmente, a incubação de MVIIA ou Phα1β preveniu o influxo de cálcio estimulado pelo agonista do receptor PAR-2 AC264613 em sinaptossomas de medula de camundongos. Estes resultados trazem uma nova perspectiva acerca dos mecanismos envolvidos na sinalização da coceira, indicando os inibidores de CCVD do subtipo N como possíveis estratégias para o tratamento do prurido, especialmente nas condições onde há ausência de resposta à terapia atual.

ABSTRACT

We assessed the effects of pharmacological spinal inhibition of voltage-gated calcium channels (VGCC) in mouse pruritus. The epidural administration of P/Q-type MVIIC or PhTx3.3, L-type verapamil, T-type NNC 55-0396 or R-type SNX-482 VGCC blockers failed to alter the scratching behavior caused by the PAR-2 activator trypsin, injected into the mouse nape skin. Otherwise, trypsin-elicited pruritus was markedly reduced by the spinal administration of preferential N-type VGCC inhibitors MVIIA and Phα1β. *C. magus*-obtained toxin MVIIA displayed significant effects when dosed from 1 to 4 h before trypsin, whereas the effects of *P. nigriventer*-derived Phα1β remained for up to 12 h. MVIIA or Phα1β also prevented the itching elicited by intradermal (i.d.) injection of SLIGRL-NH2, compound 48/80 or chloroquine, although they did not affect H_2O_2-induced itching. Furthermore, the co-administration of MVIIA or Phα1β markedly inhibited the pruritus caused by the spinal injection of gastrin-releasing peptide (GRP), but not morphine. Notably, spinal MVIIA or Phα1β greatly prevented chronic pruritus allied to dry skin. However, either toxin failed to alter the edema formation or neutrophil influx caused by trypsin. In addition, epidural MVIIA or Phα1β did not modify the expression of GRP receptor (GRP-R) in the spinal cord, whilst they brought c-Fos activation to control levels. Finally, the *in vitro* incubation of MVIIA or Phα1β prevented the calcium influx evoked by the synthetic PAR-2 agonist AC264613 in spinal cord synaptosomes. Data brings novel evidence on itching transmission mechanisms, pointing out the therapeutic relevance of N-type VGCC inhibitors to control refractory pruritus.

ABREVIAÇÕES

5-HT – Serotonina

Ach – Acetilcolina

AEW – Acetona-Dietl-éter-Água

CCVD – Canais de Ca^{2+} Voltagem-Dependentes

CGRP – Peptídeo Relacionado ao Gene da Calcitonina

COX-2 – Ciclooxigenase-2

DA – Dermatite Atópica

DRG – Gânglio da Raiz Dorsal

GPCR – Receptor Acoplado à Proteína G

GRKs – Quinases relacionadas aos GPCR

GRP – Peptídeo Liberador de Gastrina

GRP-R – Receptor do Peptídeo Liberador de Gastrina

i.d. – intradérmica

i.t. – intratecal

IL-31 – Interleucina-31

IP3 – Inositol trifosfato

MPO – Mieloperoxidase

mRNA – RNA mensageiro

NKR – Receptor de Neurocinina

P. nigriventer – Phoneutria Nigriventer

PAR-2 – Receptor Ativado por Protease-2

PhTx3 – Phoneutriatoxina-3

PLC – Fosfolipase C

SNC – Sistema Nervoso Central

SNP – Sistema Nervoso Periférico

SP – Substância P

TRP – *Transient Receptor Potential*

TRPA1 – *Transient Receptor Potential Ankirin A1*

TRPV1 – *Transient Receptor Potential Vanilloid 1*

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1. Introdução

A coceira (ou prurido) é definida como uma sensação desagradável que evoca o desejo ou o reflexo de coçar e, serve como um mecanismo de autoproteção contra agentes que geram danos ao organismo (Ikoma *et al.*, 2006; Metz, Grundmann e Stander, 2011; Raap, Stander e Metz, 2011). A coceira aguda é frequentemente induzida por agentes químicos irritantes à pele ou por picadas de insetos, os quais induzem o reflexo de coçar (Ikoma *et al.*, 2006; Dhand e Aminoff, 2013). O ato de coçar leva ao aumento dos danos da pele, bem como, a alterações inflamatórias, que por sua vez, aumentam ainda mais a sensação de coceira, mediada pela liberação de alguns mediadores intracelulares (Gilchrest, 1982; Hanifin, 1986; Patel e Dong, 2010; Liu e Ji, 2013).

A coceira não é uma doença *per se*, mas um sintoma presente em muitas doenças crônicas, sendo um dos fatores relacionados com a diminuição da qualidade de vida dos pacientes (Kini *et al.*, 2011; Yosipovitch e Bernhard, 2013). O prurido crônico é classificado como uma coiceira que persiste por mais de seis semanas (Weisshaar *et al.*, 2012), estando presente em muitas doenças de pele, como a dermatite atópica (DA), pele seca ou xerose (Gilchrest, 1982; Ikoma *et al.*, 2011). O prurido também é relatado em outros estados patológicos, como em doenças sistêmicas, câncer, infecções e desordens metabólicas (Ikoma *et al.*, 2006; Dhand e Aminoff, 2013).

A coceira pode ser desencadeada por diversos mediadores, conhecidos coletivamente como agentes pruritogênicos (Ikoma *et al.*, 2006), sendo a histamina, um dos mais bem caracterizados. Esta substância é liberada principalmente por mastócitos residentes, em resposta a estímulos externos, como por exemplo, picadas de insetos (Ikoma *et al.*, 2006; Liu *et al.*, 2009). A vontade de coçar é desencadeada pela ligação da histamina aos seus receptores, especialmente aos subtipos H_1 e H_4, que são acoplados à proteína G e, que estão presentes nos neurônios sensoriais e, em células da pele (Liu *et al.*, 2009). Em humanos, a coceira mediada pela histamina pode ser efetivamente bloqueada por antagonistas seletivos dos receptores H_1 de histamina. Entretanto, estudos recentes têm demonstrado que algumas condições crônicas de coceira não respondem ao tratamento com anti-histamínicos (Davidson e Giesler, 2010). Em testes pré-clínicos, o comportamento de coçar induzido em animais pelo degranulador de mastócitos, o composto 48/80, pelo agente antimalárico cloroquina ou, pela tripsina, não foi revertido pelo bloqueio dos receptores H_1 de histamina (Ikoma *et al.*, 2006; Costa *et al.*, 2008; Davidson e Giesler, 2010; Liu e Ji, 2013), indicando a implicação de vias e mediadores pruritogênicos, independentes de histamina (Tey e Yosipovitch, 2011; Wilson *et al.*, 2011). Corticóides tópicos ou sistêmicos também podem diminuir o prurido em

consequência de uma redução da resposta inflamatória, embora não sejam efetivos em várias situações (Greaves, 2007).

Desde a identificação de vias independentes da histamina, diversos trabalhos buscam investigar outros receptores e vias de sinalização, relacionados com a coceira. Sabe-se que as fibras nervosas não-mielinizadas do tipo C, que estão localizadas nas junções dermoepidermais, são responsáveis pela transmissão da coceira (Raap, Ikoma e Kapp, 2006; Davidson e Giesler, 2010; Akiyama e Carstens, 2013). Ademais, diferentes tipos de receptores acoplados à proteína G, sensíveis a agentes pruritogênicos são expressos nessas fibras nervosas (Nakano *et al.*, 2008; Liu *et al.*, 2009; Raap, Stander e Metz, 2011; Dhand e Aminoff, 2013).

As sensações de coceira e dor parecem ser mediadas através de mecanismos distintos. Sabe-se que a evocação da dor é capaz de inibir a sensação de coceira (o ato de coçar ativa vias nociceptivas), enquanto que a inibição da dor pode desencadear coceira (Ikoma *et al.*, 2006; Liu *et al.*, 2009; Davidson e Giesler, 2010; Miyamoto e Patapoutian, 2011; Dhand e Aminoff, 2013). Essa relação é observada em pacientes tratados com morfina, os quais apresentam prurido marcante como efeito adverso (Phan *et al.*, 2010). Nesse contexto, antagonistas dos receptores opioides do tipo μ, como naloxona, naltrexona e nalmefeno são efetivos contra a coceira causada pela morfina (Mansour-Ghanaei *et al.*, 2006). Por outro lado, em estudos pré-clínicos, o agonista κ-opioide inibe a coceira induzida por morfina, bem como, tem demonstrado efetividade em tratar a coceira em pacientes que são submetidos à diálise renal (Togashi *et al.*, 2002; Kumagai *et al.*, 2012). Recentemente, foi demonstrado que a coceira induzida por morfina é mediada especificamente por um subtipo dos receptores μ-opioides, denominado MORD1. (Liu, X. Y. *et al.*, 2011).

Outros receptores e mediadores apresentam papéis relevantes na sinalização da coceira (Schulz *et al.*, 2007). Por exemplo, níveis elevados de interleucina 31 (IL-31) foram encontrados em lesões na pele e também no gânglio da raiz dorsal de pacientes com DA em comparação a outros tecidos. A IL-31 parece exercer atividade pruritogênica pela ligação direta ao seu receptor nas fibras nervosas (Schulz *et al.*, 2007). Estudos demonstraram que o anticorpo anti-IL-31 é capaz de reduzir o comportamento de coçar em modelos animais de DA (Grimstad *et al.*, 2009).

Os TRPVs (*transient receptor potential vanilloid*) são canais iônicos ativados por diversos estímulos como calor, alterações de pH e substâncias pungentes exógenas (Bodo *et al.*, 2004). O TRPV1, um dos membros mais bem caracterizados desta família, é ativado por

capsaicina, um composto ativo encontrado na pimenta (Papoiu, Yosipovitch e Shihabi, 2010). De forma interessante, foi demonstrado que a capsaicina é efetiva no tratamento local de doenças crônicas associadas com prurido, particularmente aquelas de origem neuropática, como coceira pós-herpética, prurido braquio-radial e notalgia paraestésica (Papoiu, Yosipovitch e Shihabi, 2010). Entretanto, estudos recentes têm demonstrado a participação do TRPV1 na sinalização da coceira induzida por diversos mediadores pruritogênicos (Roberson *et al.*, 2013). Outro membro da família dos TRPs, o TRPA1 (*transient receptor potential Ankirin A1*) é ativado por isocianato ou, compostos pungentes presente no óleo de mostarda induzindo nocicepção. A ativação do TRPA1 está diretamente relacionada com a cascata de ativação de receptores acoplados à proteína G, no qual controla a liberação e ativação de diversos mediadores intracelulares, envolvidos na sinalização da coceira (Wilson *et al.*, 2011; Liu e Ji, 2012). A substância P (SP) é um mediador peptídico que se liga aos receptores de neurocininas NKR-1, NKR-2 e NKR-3, apresentando maior afinidade pelos receptores NKR-1. Estes receptores estão expressos nos terminais dos neurônios aferentes primários, em neurônios do gânglio da raiz dorsal (DRG) e nos neurônios ascendentes do corno dorsal da medula espinhal, tendo sido relacionados com a transmissão de coceira em ratos (Carstens *et al.*, 2010). Mais recentemente, outro neuropeptídeo, o GRP (peptídeo liberador de gastrina) e, o seu receptor (GRP-R), tem sido implicado na sinalização da coceira, trazendo novas perspectivas sobre a distinção entre vias de ativação da coceira e da dor (Sun e Chen, 2007; Sun *et al.*, 2009). Foi demonstrado que o GRP é expresso no DRG, enquanto que o GRP-R é encontrado no corno dorsal da medula espinhal. Animais *knockout* para GRP-R, bem como aqueles tratados com antagonista para GRP-R, apresentaram uma redução marcante do comportamento de coçar induzido por diferentes agentes pruritogênicos. Por outro lado, as respostas aos estímulos dolorosos não foram alteradas nesses animais (Sun e Chen, 2007; Sun *et al.*, 2009).

O receptor PAR-2 (*proteinase-activated receptor-2*), o qual é altamente expresso em pacientes com DA, tem sido descrito como um importante ativador da sensação de coceira, através de estímulos cutâneos em primatas (Davidson *et al.*, 2007). A caracterização destes receptores na transmissão pruritogênica tem contribuído para uma melhor compreensão dos mecanismos relacionados à coceira (Lamotte *et al.*, 2009). As proteases (ou proteinases) são um grupo enzimas responsáveis pela quebra das ligações peptídicas e possuem diversas funções biológicas (Cottrell *et al.*, 2003; Paszcuk *et al.*, 2008). Por muito tempo, elas eram vistas como enzimas de degradação; mais recentemente foi demonstrado que as proteases

também podem atuar como moléculas de sinalização, desempenhando importantes papéis na cascata de coagulação e no controle do ciclo celular (Cottrell *et al.*, 2003; Cirino e Vergnolle, 2006; Paszcuk *et al.*, 2008).

Algumas proteases são geradas ou liberadas durante o trauma e estão envolvidas em processos patológicos, como, inflamação, dor, câncer e aterosclerose (Koshikawa *et al.*, 1998; Vergnolle *et al.*, 2001). Uma das serino-proteases mais bem caracterizadas é a tripsina, cuja produção ocorre nas células acinares do pâncreas, em forma de zimogênio (tripsinogênio). É secretada no duodeno e se torna ativa devido à ação de enteroquinases, sendo uma enzima essencial para a digestão (Koshikawa *et al.*, 1998; Paszcuk *et al.*, 2008). Foram caracterizados quatro genes que codificam a tripsina em humanos: tripsinogênio I, II, III e IV, enquanto os três primeiros são as principais formas presentes no suco pancreático, o IV é expresso no cérebro humano (Koshikawa *et al.*, 1998; Cottrell *et al.*, 2003). A tripsina é expressa em diversos tecidos e células de mamíferos, incluindo humanos (Koshikawa *et al.*, 1998). Entretanto, ainda se sabe pouco sobre as funções da tripsina em cada tipo de célula e em tecidos normais (Koshikawa *et al.*, 1998; Knecht *et al.*, 2007). Recentemente, tem sido demonstrado que a tripsina é capaz de evocar os sinais clássicos da resposta inflamatória, respostas nociceptivas e pruriginosas, especialmente através da ativação de receptor PAR-2 e da produção secundária de diversos mediadores da inflamação (Costa *et al.*, 2008; Pereira *et al.*, 2011).

O receptor PAR-2 é membro da família de receptores ativados por proteases, que são receptores acoplados à proteína G (GPCR), com sete domínios transmembrana (Hollenberg, 2005; Sanchez-Hernandez *et al.*, 2008). Onde foram identificados quatro membros desta família (PAR-1, PAR-2, PAR-3 e PAR- 4) (Hirano *et al.*, 2005). De forma interessante, estes receptores são ativados de maneira irreversível por um único processo que envolve o reconhecimento do receptor pela protease, seguidos da clivagem hidrolítica de um sítio específico localizado na alça N-terminal do receptor. Finalmente, ocorre a exposição de um novo sítio N-terminal que atua como o ligante (geralmente composto por seis ou mais aminoácidos). Uma vez exposto, o ligante endógeno interage com a alça extracelular do segundo domínio transmembrana do receptor, iniciando a ativação de diversas cascatas de sinalização intracelular (Schmidlin e Bunnett, 2001; Vergnolle *et al.*, 2001; Hirano *et al.*, 2005; Paszcuk *et al.*, 2008). A ativação destes receptores resulta em ligação a diferentes proteínas G. Os receptores mais estudados, PAR-1 e PAR-2, acoplam-se preferencialmente às proteínas Gi, Gq e G12/13, promovendo a ativação direta ou indireta de eventos intracelulares

(Sabri *et al.*, 2000; Macfarlane *et al.*, 2001; Schmidlin e Bunnett, 2001; Kanke *et al.*, 2005; Costa *et al.*, 2008; Paszcuk *et al.*, 2008). Essa atividade é altamente regulada e rapidamente finalizada por um mecanismo de dessensibilização e internalização do receptor. Depois de ativados, os receptores PARs sofrem fosforilação por quinases ligadas aos GPCRs (GRKs) e pela proteína quinase C. Minutos depois, as β-arrestinas são deslocadas do citosol até a membrana plasmática para interagirem com os receptores fosforilados, o que medeia o desacoplamento e a dessensibilização, pelo bloqueio da associação dos receptores com as proteínas G. As β-arrestinas acoplam os receptores PARs às clatrinas, que são necessárias para a endocitose destes. Depois de internalizados, os PARs são degradados pelos lisossomos (Schmidlin e Bunnett, 2001; Paszcuk *et al.*, 2008). O receptores PAR-2, além de serem ativados pela tripsina de fonte pancreática ou extra-pancreática, também podem ser ativados por fatores de coagulação, como VIIa e Xa (Hollenberg, 2005; Kanke *et al.*, 2005; Costa *et al.*, 2008; Sanchez-Hernandez *et al.*, 2008). Outros agentes como Cowhage (protease) presente na espícula da planta *Macuna puriens,* ativam os receptores PAR-2 e PAR-4 produzindo prurido (Reddy *et al.*, 2008; Lamotte *et al.*, 2009). Por outro lado, o desenvolvimento de agonistas peptídicos e não peptídicos seletivos para o receptor PAR-2 permitiram avaliar a participação seletiva deste receptor na sinalização da inflamação e, também dos processos celulares que desencadeiam a sensação de coceira (Costa *et al.*, 2008; Gardell *et al.*, 2008; Paszcuk *et al.*, 2008; Liu, Q. *et al.*, 2011).

A ativação do PAR-2 em neurônios aferentes primários estimula a secreção de substância P (SP) e do peptídeo relacionado com o gene da calcitonina (CGRP) na medula espinhal. Esta ativação depende do aumento da concentração intracelular de íons cálcio (Gardell *et al.*, 2008; Oikawa *et al.*, 2013), a partir de suas projeções em tecidos periféricos e medula espinhal, causando inflamação neurogênica (Cottrell *et al.*, 2003). O receptor PAR-2 parece desempenhar um papel mais importante em situações patológicas do que em condições fisiológicas, estando envolvido tanto no início, quanto na manutenção de processos inflamatórios e dolorosos (Hirano *et al.*, 2005; Kanke *et al.*, 2005; Costa *et al.*, 2008; Pereira *et al.*, 2011). Ademais, como descrito anteriormente, este tipo de receptor tem sido apontado como um efetor importante de processos pruriginosos (Costa *et al.*, 2008; Liu, Q. *et al.*, 2011; Pereira *et al.*, 2011; Akiyama e Carstens, 2013).

A tripsina é capaz de promover prurido através da ativação dos receptores PAR-2 localizados nas fibras C, fibroblastos, queratinócitos ou mastócitos, levando à liberação de diferentes moléculas, tais como proteases, serotonina (5-HT), histamina, SP, CGRP,

prostaglandinas (via ativação de COX-2) e cininas (Costa *et al.*, 2008; Paszcuk *et al.*, 2008). Além disso, Chen e colaboradores (2009) demonstraram que a coceira induzida pelo agonista seletivo do PAR-2 (SLIGRL-NH2) é dependente do receptor GRP-R (Sun *et al.*, 2009). Estes mediadores atuam em conjunto para ativar e/ou sensibilizar nervos sensoriais, transmitindo a sensação de prurido para estruturas centrais (Sun e Chen, 2007; Costa *et al.*, 2008; Sun *et al.*, 2009). Entretanto, poucos mecanismos de ativação intracelular envolvendo a estimulação do PAR-2 foram elucidados, especialmente no que diz respeito às alterações pruriginosas. De fato, o desconhecimento dos mecanismos exatos envolvidos na coceira contribui para a baixa efetividade do tratamento farmacológico deste sintoma, na prática clínica.

Os venenos de animais como aranhas, caracóis, cobras e escorpiões, contêm uma grande quantidade de substâncias com a capacidade de ativar ou inibir canais iônicos, podendo representar precursores para o desenvolvimento de drogas (Estrada, Villegas e Corzo, 2007). A aranha *Phoneutria nigriventer* é encontrada desde o sul do Rio de Janeiro até o Uruguai, onde é a aranha mais comumente envolvida em envenenamentos humanos no Brasil (Lucas, 1988). É muito agressiva e é conhecida como aranha armadeira pela posição que toma ao se sentir ameaçada, possuindo hábitos noturnos e permanecendo refugiada durante o dia. A aranha *Phoneutria nigriventer* não constrói teia e seu sucesso como predadora pode ser explicado pela potência das diversas toxinas presentes em seu veneno (Gomez *et al.*, 2002). Picadas de *P. nigriventer* podem causar dor severa e outros sintomas, como câimbras, tremores, convulsões tônicas, paralisia espástica, priapismo, arritmias, distúrbios visuais e sudorese (Lucas, 1988). Esses sintomas são mais severos em crianças e idosos e a intoxicação pode levar à morte se não for devidamente tratada.

Os peptídeos produzidos pela glândula da aranha *P. nigriventer* têm sido extensivamente investigados. Já foram descritos 17 peptídeos ativos, com peso molecular entre 3500 a 9000 Daltons (Gomez *et al.*, 2002), atuando principalmente no funcionamento de canais de sódio (Na^+), cálcio (Ca^{2+}) e potássio (K^+), assim como em receptores do sistema nervoso (Grishin, 1999; Gomez *et al.*, 2002).

Um dos efeitos principais do veneno de *P. nigriventer* é a sua ação sobre os diferentes subtipos de canais de Ca^{2+} voltagem-dependentes (CCVD) (Gomez *et al.*, 1995; Prado *et al.*, 1996; Leao *et al.*, 2000). Os CCVD são uma família de canais iônicos, classificados por propriedades farmacológicas e eletrofisiológicas. São divididos em canais de baixo (canais subtipo T) e de alto limiar de ativação (subtipos L, N, P/Q e R) (Lai e Jan, 2006; Zamponi *et al.*, 2009; Park e Luo, 2010; Pringos *et al.*, 2011).

A contribuição dos diferentes CCVD para processos nociceptivos e inflamatórios tem alcançado um considerável interesse nos últimos anos (Bourinet e Zamponi, 2005; Yaksh, 2006; Rahman e Dickenson, 2013). De fato, sua atividade modulatória na resposta nociceptiva em áreas como a medula espinhal, DRG e tronco cerebral, indicam um papel essencial para os CCVD no processamento de informações nociceptivas (Westenbroek, Hoskins e Catterall, 1998; Heinke, Balzer e Sandkuhler, 2004; Murakami *et al.*, 2004; Park e Luo, 2010). Todavia, os CCVD participam da sensibilização da dor central, que ocorre em nervos lesionados e durante os estados inflamatórios (Matthews e Dickenson, 2001; Matthews *et al.*, 2007; Rahman e Dickenson, 2013). Em modelos animais de lesão nervosa, foi visto que o bloqueio dos CCVD dos subtipos N e P/Q reduziu os sinais comportamentais de nocicepção de origem neuropática (Matthews e Dickenson, 2001; Dalmolin *et al.*, 2011; Nimmrich e Gross, 2012). Os CCVD do subtipo N estão presentes nos terminais pré-sinápticos de neurônios nociceptivos no corno dorsal da medula espinhal, regulando a liberação de neurotransmissores pró-nociceptivos, como o glutamato e a substância P (Wen *et al.*, 2005; Park e Luo, 2010; Adams, Callaghan e Berecki, 2012).

A fração proteica Phoneutriatoxina-3 (PhTx3) isolada do veneno da *P. nigriventer*, induz paralisia flácida quando injetada em roedores (Rezende Junior *et al.*, 1991), provavelmente, devido a sua ação inibitória sobre a liberação de neurotransmissores (Gomez *et al.*, 1995; Prado *et al.*, 1996). A partir desta fração, seis diferentes isoformas foram purificadas (Tx3-1 a 6) (Cordeiro *et al.*, 1993). Pelo menos três delas (Tx3-3, Tx3-4 e Tx3-6) produzem bloqueio do influxo de Ca^{2+} induzido por KCl em terminais nervosos (Prado *et al.*, 1996; Guatimosim *et al.*, 1997; Miranda *et al.*, 1998; Vieira *et al.*, 2003), sugerindo que essas toxinas bloqueiam canais de Ca^{2+} de maneira semelhante ao que é observado para toxinas derivadas de diferentes espécies do gênero Conus.

A primeira toxina bloqueadora de canais de Ca^{2+} isolada do *pool* PhTx3 foi a toxina Tx3-3. Estudos farmacológicos prévios mostraram que essa toxina inibe a liberação de acetilcolina em fatias de córtex e, em neurônios do plexo mientérico. Porém, essa ação não possuía um efeito aditivo com a ω-agatoxina IVA na liberação de ACh (Gomez *et al.*, 1995). Além disso, a PhTx3-3 é bloqueadora eficaz da liberação de glutamato (Prado *et al.*, 1996). Através do uso dos marcadores fluorescentes Fura2-AM para avaliação do cálcio interno e FM1-43 para avaliar a liberação de vesícula em sinaptossomas cérebro-corticais, foi demonstrado que a PhTx3-3 inibe com alta potência (IC_{50} 0,9 nM) seletivamente canais iônicos do subtipo P/Q e R que regulam a entrada de Ca^{2+} em sinaptossomas, (Guatimosim *et*

al., 1997; Leao *et al.*, 2000). A isoforma Tx3-6, que foi patenteada e denominada Phα1β, foi capaz de bloquear com mais afinidade os CCVD do subtipo N (Vieira *et al.*, 2005). Ademais, pesquisas demonstraram que a Phα1β é tão potente quanto a ω-conotoxina MVIIA sobre as respostas nociceptivas, apresentando um índice terapêutico maior em ensaios pré-clínicos (Souza *et al.*, 2008; De Souza *et al.*, 2011; Rigo *et al.*, 2013). Entretanto não há nenhum estudo avaliando a participação de CCVD no modelo experimental de prurido.

2. Objetivos

2.1. Objetivo Geral

O presente trabalho teve como objetivo investigar os efeitos da administração intratecal de diferentes bloqueadores seletivos de canais de cálcio voltagem-dependentes em modelos de coceira em camundongos.

2.2. Objetivos Específicos

1. Avaliar a atividade dos bloqueadores de CCVD do subtipo N (MVIIA e Phα1β); P/Q (MVIIA e PhTx3-3); L (verapamil); T (NNC 55-0396) e R (SNX-482), administrados por via intratecal, no modelo de coceira causada pela tripsina;
2. Avaliar a atividade dose- e tempo-resposta dos bloqueadores do subtipo N (MVIIA e Phα1β) no modelo de coceira causada pela tripsina;
3. Verificar os efeitos do pré-tratamento por via intratecal com as toxinas MVIIA e Phα1β sobre os modelos agudos de coceira induzidos pela aplicação intradérmica do agonista peptídico do receptor PAR-2 (SLIGRL-NH2), composto 48/80, cloroquina ou H_2O_2;
4. Verificar os efeitos da coadministração das toxinas MVIIA e Phα1β sobre a coceira induzida pela aplicação intratecal de GRP ou morfina;
5. Analisar os efeitos do pré-tratamento com as toxinas MVIIA e Phα1β no modelo crônico de coceira associada à pele seca em camundongos;
6. Avaliar os efeitos das toxinas MVIIA e Phα1β sobre a resposta edematogênica e sobre a atividade da MPO, induzidas pela tripsina na pele de camundongos;
7. Determinar os efeitos da tripsina e das toxinas MVIIA e Phα1β nos níveis de expressão do mRNA para GRP-R na medula de camundongos;
8. Avaliar os efeitos das toxinas MVIIA e Phα1β sobre o influxo de cálcio induzido pelo agonista não-peptídico e seletivo para o receptor PAR-2 (AC-264613) em preparações de sinaptossomas obtidos de medulas espinhais de camundongos;
9. Investigar os efeitos toxinas MVIIA e Phα1β sobre os níveis de c-Fos ativada, após a aplicação intradérmica de tripsina, na medula espinhal de camundongos.

3. MANUSCRITO

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The spinal inhibition of N-type voltage-gated calcium channels selectively prevents scratching behavior in mice

Izaque S. Maciel [a,b], Vanessa M. Azevedo [b,c], Talita C. Pereira [a,d], Maurício R. Bogo [a,d,e], Alessandra H. Souza [f], Marcus V. Gomez [f], Maria M. Campos [a,b,g,*]

[a]Postgraduate Program in Medicine and Health Sciencies, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre/RS, Brazil.

[b]Institute of Toxicology and Pharmacology, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre/RS, Brazil.

[c]Faculty of Pharmacy, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre/RS, Brazil.

[d]Laboratory of Genomics and Molecular Biology, Faculty of Biosciences, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre/RS, Brazil.

[e]Postgraduate Program in Cellular and Molecular Biology, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre/RS, Brazil.

[f]Laboratory of Neuroscience, Federal University of Minas Gerais, Belo Horizonte/MG, Brazil.

[g]Faculty of Dentistry, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre/RS, Brazil.

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*Corresponding Author: Maria Martha Campos, School of Dentistry and Institute of Toxicology and Pharmacology, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681, Partenon, 90619-900, Porto Alegre, Brazil. Phone number: 55 51 3320 3562; Fax number: 55 51 3320 3626; E-mail: camposmmartha@yahoo.com; maria.campos@pucrs.br

Abstract

We assessed the effects of pharmacological spinal inhibition of voltage-gated calcium channels (VGCC) in mouse pruritus. The epidural administration of P/Q-type MVIIC or PhTx3.3, L-type verapamil, T-type NNC 55-0396 or R-type SNX-482 VGCC blockers failed to alter the scratching behavior caused by the PAR-2 activator trypsin, injected into the mouse nape skin. Otherwise, trypsin-elicited pruritus was markedly reduced by the spinal administration of preferential N-type VGCC inhibitors MVIIA and Phα1β. *C. magus*-obtained toxin MVIIA displayed significant effects when dosed from 1 to 4 h before trypsin, whereas the effects of *P. nigriventer*-derived Phα1β remained for up to 12 h. MVIIA or Phα1β also prevented the itching elicited by intradermal (i.d.) injection of SLIGRL-NH2, compound 48/80 or chloroquine, although they did not affect H_2O_2-induced itching. Furthermore, the co-administration of MVIIA or Phα1β markedly inhibited the pruritus caused by the spinal injection of gastrin-releasing peptide (GRP), but not morphine. Notably, spinal MVIIA or Phα1β greatly prevented chronic pruritus allied to dry skin. However, either toxin failed to alter the edema formation or neutrophil influx caused by trypsin. In addition, epidural MVIIA or Phα1β did not modify the expression of GRP receptor (GRP-R) in the spinal cord, whilst they brought c-Fos activation to control levels. Finally, the *in vitro* incubation of MVIIA or Phα1β prevented the calcium influx evoked by the synthetic PAR-2 agonist AC264613 in spinal cord synaptosomes. Data brings novel evidence on itching transmission mechanisms, pointing out the therapeutic relevance of N-type VGCC inhibitors to control refractory pruritus.

Keywords: scratching behavior, N-type voltage-gated calcium channels, trypsin, PAR-2, mice.

1. Introduction

Itch is a distressful sensation that induces an urgent desire to scratch the affected site. The scratching reflex can be generated by chemical agents, insect bites, or some drugs, and it can be alleviated by scrubbing the surrounding area [9,14]. Scratching serves as a self-protective mechanism, but this reflex might induce the additional release of inflammatory mediators in the skin, resulting in the itch-scratch cycle and perpetuation of pruritus [16,19,28]. Of clinical relevance, chronic pruritus is a symptom present in many chronic skin alterations (dry skin and psoriasis), systemic diseases (chronic renal failure, chronic liver disease and hyperthyroidism) or disorders affecting the central or peripheral nervous system (brain tumors or peripheral neuropathies) [14,16]. Nevertheless, chronic pruritus is often refractory to the current available medications, seriously compromising the life quality of patients [16,48].

The mechanisms implicated in pruritus have been target of extensive investigation during the last years. There is a close relationship between itch and pain, as they share common peripheral and central pathways of sensitization [28]. However, pain and itching are likely related to the activation of different mechanisms, as painful stimulus on the skin suppresses itch sensation [7,19]. For instance, the scratching allied to therapy with the anti-malarial drug chloroquine or the opioid morphine was demonstrated to rely on the activation of G protein-coupled receptors MrgprA3 and μ-opioid (isoform MOR1D), respectively, via mechanisms independent on nociceptive transmission [17,20]. Moreover, it has been shown that gastrin-releasing peptide (GRP) found in the dorsal root ganglion (DRG), and its receptor (GRP-R) expressed in the lamina I of the spinal cord are quite specific for mediating itching sensation, without implications on pain-related transmission [40-41].

Voltage-gated calcium channels (VGCC) are expressed in most excitable cells, playing an important role in neuronal excitability and neurotransmitter release. They are

classified as high-voltage L (CaV 1.1–1.3), N (CaV 2.2), P/Q(CaV 2.1), and R-type (CaV 2.3), or low voltage T-type (CaV 3.1–3.3) activated channels [31-32,43,49]. Calcium influx has been widely implicated in pain transmission, and the blockage of VGCC by animal-derived peptide toxins, such as *C. magus* MVIIA and *P. nigriventer* Phα1β, has been demonstrated to display marked analgesic effects in a series of acute and chronic pain models [45,47,49]. Of note, the synthetic form of the conopeptide MVIIA, namely ziconotide, was approved to be used clinically as a spinally-delivered analgesic to control untreatable pain [36]. Notwithstanding, evidence showing the relevance of VGCC in pruritus remains scarce. Some few studies demonstrated that inhibition of VGCC by gabapentinoids is able to alleviate chronic pruritus in clinical trials or in pre-clinical mouse models of itching [2,44]. These results point out a potential role for VGCC in pruritus, although additional studies on this regard are still required. The present study examined, for the first time, whether the spinal inhibition of VGCC by selective pharmacological tools might be able to modulate the scratching behavior in mice. Our results bring novel evidence on the mechanisms related to itching transmission, opening new avenues for the control of refractory chronic pruritus.

2. Methods

2.1. Drugs and reagents

The following drugs and reagents were used: PhTx3.3 and Phα1β from *Phoneutria nigriventer* venom were provided by Ezequiel Dias Foundation, MG, Brazil; MVIIA and MVIIC from *Conus magus* (Latoxan, Valence, France); trypsin, SLIGRL-NH2, compound 48/80, chloroquine, GRP, morphine, EDTA, EGTA, Hepes, glucose, sucrose, SDS, Fura-2-AM, $CaCl_2$, H-TAB, NaCl, KCl, Na_3PO_4, $MgSO_4$, $NaHCO_3$, KH_2PO_4 (all from Sigma Chemical Company, St. Louis, U.S.A); verapamil, NNC 55-0396 dihydrochloride, AC 264613, SNX-482 from *Tarantula Hysterocrates gigas* (all from Tocris bioscience, Bristol, UK); H_2O_2, acetone and diethyl-ether (all from Merck, Darmstadt, Germany).

2.2. Animals

Swiss male mice (25 to 30 g) were used in this study. Animals were housed under conditions of optimum light, temperature and humidity (12 h light-dark cycle, 22 ± 2 °C, under 50 to 70 % humidity), with food and water provided *ad libitum*. Mice were obtained from Central Animal House of the Federal University of Pelotas (UFPEL, Brazil). Experiments were conducted in accordance with current guidelines for the care of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals [50]. All the experimental procedures were approved by the local Animal Ethics Committee (protocol number 12/00293).

2.3. Induction of scratching behavior

The experimental N was 6 to 10 animals/group for all behavioral tests. Induction of scratching behavior was accomplished as described previously [5,29]. Two days before the experiments, the back of the mouse nape was shaved. In most experiments, itching was

elicited by an intradermal (i.d.) injection of saline (50 µl), containing the PAR-2 activator trypsin (200 µg/site). On the day of experiments, the animals were individually placed into glass cylinders 20-cm in diameter, for at least 30 min, in order to acclimatize them to the environment. After this period, each mouse was briefly removed from the cylinder and received trypsin injection. Immediately after, they returned to the cylinders and the number of scratches with forepaws and hindpaws close to the injected site was registered during 40 min. Scratching behind the ears, but not on the face, was also counted and included in the analysis.

In separate series of experiments, the scratching behavior was induced by an i.d. injection of saline (50 µl) containing the peptide SLIGRL-NH2 (100 µg/site), the mast cell depletor compound 48/80 (10 µg/site), the antimalarial drug chloroquine (200 µg/site) or hydrogen peroxide (H_2O_2; 0.3%). Alternatively, itching behavior was evoked by an intrathecal (i.t.) injection of saline (5 µl) containing gastrin-releasing peptide (GRP; 1 nmol/site) or the opioid morphine (1 nmol/site). The scratching behavior was quantified as described above, during 40 min after induction, for SLIGRL-NH2, compound 48/80 and chloroquine [5], whereas the itching counts were registered during 30 min for morphine and H_2O_2[18], or 60 min for GRP [40].

To assess chronic itching, the dry skin model was used, according to the methodology described before [23]. Briefly, a mixture of acetone and diethyl-ether (1:1; v/v) was applied on back of the shaved mouse nape during 15 s, followed by the application of distillated water, for 30 s (AEW group). This procedure was carried out twice a day, for up to 5 days. The control group received distillated water only, for 45 s (W group), at the same schedules of application. After the second application, at the 5^{th} day of dry skin induction, the mice were placed in glass cylinders to assess the spontaneous scratches, during a period of 30 min.

2.4. Effects of different VGCC blockers on trypsin-induced scratching behavior

This set of experiments was addressed to assess whether the spinal administration of different classes of VGCC inhibitors might affect trypsin (200 μg)-evoked itching in mice. For this purpose, mice received an i.t. injection (5 μl/site) of one of the following drugs, 1 h before the application of trypsin: the preferential N-type VGCC inhibitors MVIIA (10 pmol) or Phα1β (100 pmol); the P/Q-type blockers MVIIC or PhTx3.3 (both 100 pmol); the L-type blocker verapamil (50 nmol); the T-type blocker NNC 55-0396 (2 nmol) or the R-type inhibitor SNX-482 (222 pmol). The doses of inhibitors were selected on the basis of literature data [4,8,22,24]. Free hand i.t. injections were performed between the L5 and L6 vertebral spaces, as described before [29], by means of a 10-μl microsyringe coupled to a 0.45 x 13 (26G 1/2") gauge needle. Control groups received phosphate-buffered saline (PBS) at the same schedules of administration.

2.5. Dose- and time-related effects of N-type VGCC inhibitors on trypsin-induced itching

As a next step, we evaluated the effects of different doses of either N-type VGCC inhibitors MVIIA (0.3, 1, 10 and 30 pmol/site) or Phα1β (10, 30, 50, 100 and 300 pmol/site), when given by i.t. route, 1 h before the induction of scratching behavior by trypsin in mice. The doses were selected on the basis of previous literature data [4,8,22,24] and pilot experiments. Doses superior to 30 pmol/site of MVIIA or 300 pmol/site of Phα1β were not included in this study due to the occurrence of unspecific central effects [13,38].

Separately, the spinal effects of MVIIA (10 pmol/site) or Phα1β (50 pmol/site) on trypsin-elicited scratching behavior were examined at different intervals of time. Accordingly, the inhibitors were administered intrathecally at 1, 2, 4, 6, 12 and 24 h before trypsin injection. On the basis of time-related curves for both N-type VGCC inhibitors, the time-point of 2 h was adopted for the next experiments. Control groups received PBS at the same schedules of administration.

2.6. Effects of selective inhibition of N-type VGCC in additional models of itching

To extend the experimental evidence on the anti-pruritogenic effects of MVIIA and Phα1β in the trypsin model, we tested these inhibitors in an additional series of mouse models of scratching behavior. For this purpose, MVIIA (10 pmol/site) or Phα1β (50 pmol/site) was administered 2 h before the induction of scratching behavior by i.d. injection of the selective PAR-2 agonist SLIGRL-NH2 (100 µg/site), compound 48/80 (10 µg/site), chloroquine (200 µg/site) or hydrogen peroxide (H_2O_2; 0.3%). In separate groups, MVIIA (10 pmol) or Phα1β (50 pmol) was co-injected intrathecally together with GRP (1 nmol) or morphine (1 nmol), in total volume of 5 µl. Finally, N-type VGCC blockers MVIIA (10 pmol) or Phα1β (50 pmol) were administered 2 h before the last application of AEW, in the chronic pruritus model of dry skin. Control groups received PBS at the same schedules of administration for all experimental groups. The scratching behavior was analyzed as described in the item 2.3.

2.7. N-type VGCC inhibition and trypsin-induced c-Fos activation

It was demonstrated before that scratching behavior is allied to a marked increase of c-Fos activation in the mouse lumbar spinal cord [5,29]. To gain further insights into the mechanisms involved in the anti-pruritogenic effects of N-type VGCC inhibitors, the animals were pretreated i.t. with MVIIA (10 pmol/site) or Phα1β (50 pmol/site), 2 h before trypsin (200 µg/site) application into the mouse neck. Following additional 40 min, the animals were euthanized by deep inhalation of isofluorane and lumbar spinal cords (L3 to L6 region) were collected for c-Fos immunohistochemistry analysis. Saline-treated mice were used as negative controls. c-Fos expression was assessed on paraffin tissue sections using the polyclonal rabbit anti-c-Fos (1:700, Abcam, Cambridge, MA, USA). High-temperature antigen retrieval was performed by immersion of the slides in a water bath at 95–98º C in 10 mM trisodium citrate buffer, pH 6.0, for 45 minutes. The nonspecific binding was blocked by incubating sections

for 1 h with goat normal serum diluted in PBS. After overnight incubation at 4ºC with primary antibodies, the slides were washed with PBS and incubated with the secondary antibody Envision plus (Dako Cytomation, Houston, TX, USA), ready to use, for 1 h at room temperature. The sections were washed in PBS, and the visualization was completed by using 3, 3'-diaminobenzidine (Dako Cytomation) in chromogenic solution and counterstained lightly with Harris's Hematoxylin solution. Immunoreactivity was visualized by a dark-brown nucleus staining. Images were taken with a microscope (Axio Imager A1) coupled to an image capture system (Axio Vision Rel. 4.4 Software Multimedia), both from Imaging Solution Carl Zeiss (Hallbergmoos, Germany). Control and experimental tissues were placed on the same slide and processed under the same conditions. Settings for image acquisition were identical for control and experimental samples. For each mouse, five images were taken, in order to contemplate most of the spinal cord area. The images were captured in 50-x (for schematic representation) and 200-x (for analysis) magnification, and evaluated through the Image NIH Image J 1.36b Software. The number of c-Fos positive cells was quantified and expressed as the % positive cells/total area. For immunohistochemistry analysis, the N was 3-4 animals per group.

2.8. Influence of N-type VGCC inhibitors on neutrophil migration and edema formation

Neutrophil recruitment to the mouse nape skin was quantified indirectly by tissue myeloperoxidase (MPO) activity, according to the method described previous, with minor modifications [29]. Mice were pre-treated with MVIIA or Phα1β (10 and 50 pmol, respectively), 2 h before trypsin injection. The control group was treated with PBS, at the same schedule of treatment. After 40 min of trypsin injection, the animals were euthanatized by deep isofluorane inhalation. One cm^2 of the nape skin was removed and weighted to assess the edema index (expressed in mg tissue/cm^2). Immediately after, the tissues were

homogenized at 5% (w/v) in ethylenediaminetetraacetic acid (EDTA)/NaCl buffer (pH 4.7) and centrifuged (4,400 *g*, for 15 min, 4 ºC). The pellet was re-suspended in 0.5% hexadecyltrimethyl ammonium bromide buffer (HTAB, pH 5.4). Upon thawing, the samples were re-centrifuged under the same conditions mentioned above (4,400 *g*, 15 min, 4 ºC), and 25 µl of the supernatant was used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM Na_3PO4, and 0.3 mM hydrogen peroxide. The absorbance was measured at 650 nm, and the results are expressed as optical density per milligram of tissue.

2.9. Assessment of GRP-R expression by real-time PCR after scratching behavior induced by trypsin in mice

After 40 min of trypsin injection, the animals were euthanized and their lumbar spinal cords were removed for assessment of GRP-R mRNA expression. Total RNA was isolated using Trizol^{LS} reagent in accordance with the manufacturer's instructions. The amount of total RNA was quantified by spectrophotometry, and treated with DNase I Amplification Grade (Invitrogen) to avoid contamination with gDNA. cDNA species were synthesized using the ImProm-II™ Reverse Transcription System (Promega) from 1 µg total RNA in accordance with the manufacturer's instructions. Quantitative PCR was performed using SYBR Green I (Invitrogen) to detect double-strand cDNA synthesis. Reactions were performed in a final volume of 25 µl with 12.5 µl of diluted cDNA (1:20), 0.2 × SYBR Green I (Invitrogen), 100 µM dNTP, 1 × PCR buffer, 3 mM MgCl2, 0.25 U Platinum Taq DNA Polymerase (Invitrogen) and 200 nM of each reverse and forward primer.

Primers for *GRP-R* (forward 5'-CCTCTTTGCCTTCTGCTGGCTCC-3' and reverse 5'-CAGCAGATAAAGAGCAAAGGGGTTC-3') were designed using Oigos 9.6, producing a 163 bp PCR fragment. As described previously [30], *hprt* and *tbp* were used as reference

genes. PCR cycling conditions were: initial polymerase activation step for 5 min at 94 °C; 40 cycles of 15 s at 94 °C for denaturation; 10 s at 60 °C for annealing; 15 s at 72 °C for elongation. At the end of the cycling protocol, a melting curve analysis was included; the fluorescence was measured at temperatures ranging from 60 to 99 °C. Relative expression levels were determined with 7500 Fast Real-Time System Sequence Detection Software v.2.0.5 (Applied Biosystems). The efficiency per sample was calculated using LinRegPCR 11.0 Software (http://LinRegPCR.nl). Relative RNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method.

2.10. Measurement of $[Ca^{2+}]_i$ in spinal cord synaptosomes

As another approach, we tested the effects of *in vitro* incubation of N-type VGCC inhibitors MVIIA and Phα1β on calcium influx in mouse synaptosomes stimulated with the non-peptide selective PAR-2 agonist AC 264613. Trypsin was not used in this protocol due to the possible interference with the integrity of synaptosomes.

The method for measurement of $[Ca^{2+}]_i$ was similar to that described previously, with minor adaptations [13,38]. The spinal cords of mice (a pool of 4 samples for each analysis) were removed and immediately placed in aerated (95% O^2/5% CO^2), ice-cold, calcium-free Krebs-Ringer Hepes buffer (KRH; composition in mM: NaCl 135, KCl 4.8, $MgSO_4$ 1.2, Hepes 12.5, glucose 10, $NaHCO_3$ 25 and KH_2PO_4 1.2; adjusted to pH 7.4). For preparation of synaptosomes, the tissues were homogenized in 14 ml of ice-cold 0.32 M sucrose and the homogenate was centrifuged (1000 x g, for 5 min at 4ºC). The pellet 1 (P1) was discarded and the supernatant 1 (S1) was centrifuged (15,000 x g for 20 min at 4ºC). The supernatant 2 (S2) was discharged and the pellet 2 (P2) was resuspended in 2.4 ml of aerated KRH buffer. Immediately after, the synaptosomes were loaded with 5 µM of Fura-2 AM (from 1 mM stock solution, in DMSO), and 1 ml of KRH was incubated for additional 30 min. After that, the

synaptosomes were centrifuged (9,500 x g, 45 s), and the pellet was resuspended with 2 ml of KRH. The assays were performed using a spectrofluorimeter (SpectraMax M2®). Synaptosomes were stirred in a cuvette maintained at 37^{o}C. $CaCl_2$ (1 mM final concentration) was added to synaptosomal suspension at the beginning of each fluorimetric assay. MVIIA or Phα1β (either 100 nM) was added to the synaptosomal suspension, 150 s prior to stimulation with AC 264613 (5 µM) [12]. Fluorescence emission was monitored at 510 nm, whilst continually switching excitation between 340 and 380 nm (between 300 s and 540 s after initiating the assay). For each experiment, the maximum and the minimum fluorescence values were evaluated by addition of 20 µL of SDS (10% w/v final solution; 600 s after the onset of assay), followed by addition of 40 µL of EGTA (400 mM final solution; 750 s after the onset of assay), respectively. The results are expressed as calcium concentrations (test minus basal values) in nM, of n=3-4 repetitions.

2.11. Statistical analysis

The results are presented as the mean ± standard error mean. The percentages of inhibition were calculated as the mean of inhibitions obtained for each individual experiment or, on the basis of the area under the curve (time-dependent experiments). Statistical comparison of the data was performed by one-way analysis of variance (ANOVA) followed by Tukey test. *P*-values less than 0.05 ($p < 0.05$) were considered significant.

3. Results

3.1. Screening of a series of VGCC inhibitors in trypsin-induced scratching

Initially, we tested the effects of spinal administration of a set of different VGCC blockers in the scratching behavior caused by trypsin injection into the mouse nape. Interestingly, trypsin (200 μg)-elicited itching was markedly inhibited by i.t. pretreatment with both selective N-type VGCC inhibitors MVIIA (10 nmol/site; Figure 1A) and Phα1β (50 nmol/site; Figure 1B), administered 1 h before. The obtained percentages of inhibition were 73 ± 7% and 58 ± 8%, respectively. Conversely, P/Q-type blockers MVIIC or PhTx3.3 (both 100 pmol/site) (Figure 1C and 1D), the L-type blocker verapamil (50 nmol/site; Figure 1E), the T-type blocker NNC 55-0396 (2 nmol/site; Figure 1F) or the R-type inhibitor SNX-482 (222 pmol/site; Figure G), all failed to significantly affect the scratching behavior induced by trypsin in mice.

3.2. Dose- and time related effects of N-type VGCC inhibitors

On the basis of previous results, we decided to assess dose- and time-related effects of either MVIIA or Phα1β in the scratching behavior evoked by trypsin in mice. For both inhibitors, it was not possible to observe classical dose-related effects. Accordingly, the i.t. administration of MVIIA, at the dose of 0.3 nmol/site, did not significantly alter trypsin-elicited scratching. Otherwise, i.t. MVIIA produced marked reductions of trypsin responses, when administered at 1, 10 and 30 pmol/site, with inhibition percentages of 66 ± 7%, 75 ± 7% and 65 ± 11%, respectively (Figure 2A). A similar profile was seen for the i.t. administration of Phα1β, which displayed striking reductions of trypsin-induced itching, at the doses of 30 (53 ± 8%), 50 (76 ± 12%), 100 (59 ± 8%) and 300 pmol/site (52 ± 7%), whilst the dose of 10 pmol/site did not display significant effects (Figure 2B).

Concerning the time-related effects, the treatment with MVIIA (10 pmol/site) produced significant inhibitions of trypsin-elicited pruritus for up to 4 h. This N-type VGCC antagonist produced comparable percentages of inhibition, when administered 1 h (77 ± 6%) and 2 h (79 ± 5%) before the experimental sessions, with a visible reduction of inhibitory actions at 4 h (37 ± 11 % of reduction). In relation to Phα1β (50 pmol/site), this toxin displayed significant and long-term anti-pruritogenic effects in the trypsin model, with inhibition percentages of 68 ± 12%, 58 ± 10%, 62 ± 5%, 46 ± 6% and 53 ± 10%, for 1, 2, 4, 6 and 12 h of pretreatment, respectively. Both N-type VGCC blockers were not effective when dosed 24 h prior (Figure 2C). The evaluation of AUC from 1 h to 4 h showed similar percentages of inhibition for MVIIA (10 pmol/site) and Phα1β (50 pmol/site) (60 ± 6 % and 66 ± 6%, respectively; Figure 2D). However, AUC analysis from 6 h to 12 h revealed a reduction percentage of 54 ± 6% for Phα1β (50 pmol/site), without significant differences when comparing MVIIA (10 pmol/site) to control group (Figure 2E), corroborating the time-related differences between both toxins.

3.3. MVIIA and Phα1β inhibit the scratching behavior induced by other pruritogenic agents

To get further evidence on the anti-pruritogenic effects of MVIIA and Phα1β, these inhibitors were evaluated in a series of different models of itching in mice. As expected, MVIIA (10 pmol) and Phα1β (50 pmol), given i.t., 2 h before, markedly reduced the scratching behavior caused by i.d. injection of the selective PAR-2 agonist SLIGRL-NH2 (100 µg) (78 ± 13% and 68 ± 11%, respectively) (Figure 3A). Both N-type VGCC blockers were also effective in reducing the scratching behavior induced by i.d. injection of CP48/80 (10 µg; Figure 3B) and chloroquine (200 µg; Figure 3C), although they did not elicit any significant effect on H_2O_2 (0.3 %)-induced itching (Figure 3D). Besides, the spinal administration of MVIIA and Phα1β, given 2 h before the experimental sessions, produced

marked inhibitory rates of the scratching behavior caused by CP 48/80 (64 ± 7 % and 74 ± 6%) or chloroquine (75 ± 11% and 69 ± 12%), respectively.

MVIIA (10 pmol) or Phα1β (50 pmol) were also markedly effective when they were co-injected intrathecally, combined with GRP (1 nmol/site), with inhibition percentages of 81 ± 13% and 78 ± 15%, respectively (Figure 4A). However, neither of the tested toxins was able to significantly modify the scratching behavior caused by the opioid morphine, when co-injected by i.t. route (Figure 4B).

To complement this set of experiments, the effects of MVIIA (10 pmol/site) and Phα1β (50 nmol/site) were evaluated in the dry skin chronic model of pruritus. In this experimental paradigm, the repeated application of AEW in the mouse nape skin led to marked spontaneous scratching behavior. Interestingly, both N-type VGCC modulators were able to greatly diminish the itchy behavior allied to dry skin, reaching levels comparable to those seen in the negative control group (water application only; Figure 4C), when the toxins were administered in a single injection, 2 h before the last application of AEW.

3.4. Actions of N-type VGCC inhibitors do not rely on inhibition of peripheral inflammation

Our next aim was to verify to what extent the anti-pruritus effects of MVIIA or Phα1β might be related to the inhibition of peripheral skin inflammation. Data depicted in Figure 5A clearly demonstrate that i.d. trypsin injection (200 µg/site) was associated to edema formation, according to assessment of skin weight. However, the treatment with MVIIA (10 pmol/site) or Phα1β (50 pmol/site) failed to significantly affect this parameter, although a slight reduction was seen. Furthermore, an analysis of MPO activity on the mouse nape skin did not reveal any statistical significant difference among the groups (Figure 5B).

3.5. Analysis of spinal GRP-R expression and the effects of N-type VGCC inhibitors

GRP and its receptor GRP-R have been pointed out as pivotal components of itching transmission [40]. Therefore, we decided to examine the levels of expression of GRP-R in the spinal cord of trypsin-injected mice, as well as the possible interference of N-type VGCC inhibitors on this parameter. Our results showed that trypsin i.d. injection into the mouse nape skin (200 µg/site) did not change the relative expression of GRP-R mRNA, in comparison to the PBS-injected control group, and the expression of this receptor is constitutive. Besides, the pre-treatment with MVIIA (10 pmol/site) or Phα1β (50 pmol/site), 2 h prior, did not alter GRP-R expression in the spinal cord (Figure 5 C).

3.6. Measurement of $[Ca^{2+}]_i$ in spinal cord synaptosomes

The selective activation of PAR-2 receptors is associated to increased calcium influx [1,33]. Thus, we assessed whether the non-peptide selective PAR-2 agonist AC264613 might modulate intracellular calcium concentrations in spinal cord synaptosomes, as well as the effects of N-type VGCC blockage in this experimental condition. We found that incubation of AC264613 (5µM) induced a visible increase of calcium concentrations (black columns), when compared to the control group (white column). Noteworthy, the pre-incubation of either MVIIA or Phα1β (both, 100 µM), 150 s before the AC264613 addition, virtually brought $[Ca^{+2}]_i$ to control levels (Figure 6 A). However, statistical comparisons did not reveal any significant difference among the groups.

3.7 Influence of N-type VGCC on c-Fos expression in spinal cord from mice

Previous data from our group demonstrated that trypsin injection into the mouse nape resulted in a significant increase of c-Fos immunopositivity [5,29]. Herein, we corroborate this evidence showing that i.d. injection of trypsin caused a marked increase of c-Fos immunolabelling, as assessed in lumbar spinal cord sessions (Figure 6D). Of note, the spinal

administration of the selective N-type VGCC inhibitors MVIIA (10 pmol/site; Figure 6E) or Phα1β (50 pmol/site; Figure 6F), given 2 h before trypsin injection, visibly reduced c-Fos activation to control values (Figure 6C), although statistical significance was not observed (Figure 6B).

4. Discussion

The last decade witnessed a great progress in the comprehension of the mechanisms underlying itching transmission (for review see: [48]). However, most investigations on pruritus are still required, due to the urgent necessity of novel therapeutic options for unmanageable refractory itching. This study examined, for the first time, whether the spinal pharmacological modulation of different classes of VGCC might affect the scratching behavior in mice. We bring novel evidence indicating that N-type VGCC appears to be of higher relevance for itching transmission.

Trypsin is a potent inducer of pruritus, via stimulation of proteinase-activated receptor 2 (PAR-2) and the secondary release of inflammatory mediators [6-7]. PAR-2 is found up-regulated in the epidermis of individuals with atopic dermatitis [39], and PAR-2 overexpression in the mouse skin causes dermatitis and itching [11]. We assessed the effects of a series of VGCC inhibitors when dosed by the epidural route, in the mouse model of pruritus induced by trypsin injection into the nape skin. Surprisingly, the spinal administration of L-type verapamil, T-type NNC 55-0396, R-type SNX-482, or P/Q-type MVIIC or PhTx3-3 selective VGCC blockers failed to alter trypsin-induced scratching behavior in mice. However, trypsin-elicited pruritus was markedly diminished by the spinal administration of the preferential N-type VGCC inhibitors MVIIA or Phα1β. The doses of VGCC inhibitors used in the present study were selected on the basis of previous literature data, considering their analgesic effects in pre-clinical models of nociception, allied to the absence of adverse motor effects [3-4,22,38]. It is well recognized that all VGCC subtypes are found expressed throughout the DRG and the spinal cord, but N-type VGCC expression is prominent, with a profuse distribution within neurons at DRG [24,26,45]. Relevantly, a previous report demonstrated that trypsin-evoked itching is likely dependent on neurogenic inflammation [6], and the anti-pruritus effects of MVIIA or Phα1β might be explained by the selective

inhibition of pre-synaptic N-type VGCC and the consequent reduction of neuropeptides release.

To gain further insights into the effects of *C. magus*-derived MVIIA, or Phα1β from *P. nigriventer* in the trypsin-induced scratching model, we carried out dose- and time-related analysis for both inhibitors. An overall evaluation of the dose-response curves revealed an absence of significant differences among the percentages of inhibition achieved for spinally administered MVIIA (from 1 pmol to 30 pmol) or Phα1β (from 30 pmol to 300 pmol), characterized by a bell-shaped profile. A similar pharmacological outcome was seen for the spinal treatment with MVIIA and Phα1β (in doses between 30 and 200 pmol/site), according to assessment in the mouse model of formalin-induced nociception [38]. Otherwise, clear dose-related effects were seen for MVIIA or Phα1β (3 to 300 nmol/site, i.t.) in acute or chronic rat models of mechanical hyperalgesia elicited by paclitaxel [34]. Remarkably, both studies detected motor and sensorial side effects for MVIIA at doses higher than 30 pmol/site, whereas Phα1β administration was allied to adverse reactions in rodents at doses superior to 300 pmol/site [34,38]. In the light of literature data, these results indicate that both toxins displayed anti-pruritus effects in the trypsin model, when administered intrathecally, at doses unlikely related to collateral effects. Concerning the time-course experiments, our results are indicative of long-lasting effects for Phα1β (50 nmol/site), in comparison to MVIIA (10 pmol/site). This conclusion is based on data showing that percentages of inhibition were similar for both toxins until 4 h, whereas only Phα1β displayed significant inhibitory effects when dosed either 6 h or 12 h before trypsin injection. Souza et al. (2011) [8] showed convincing evidence on the long-term effects of Phα1β, in relation to MVIIA, in a model of post-operative pain following plantar incision in mice. The time-related differences observed between the selective N-type VGCC blockers Phα1β and MVIIA in pruritus might be explained by several different reasons, such as distinct pharmacokinetic profiles or alternative

docking sites, as proposed beforehand [34,38]. Nevertheless, the long-term actions displayed by Phα1β from *P. nigriventer* might represent a clear advantage for the control of refractory pruritus in the clinical set.

Next, we tested the effects of spinal administration of both toxins in a series of distinct models of itching in mice. Data revealed that spinal pre-treatment with MVIIA and Phα1β was capable of reducing the itchy behavior caused by i.d. injection of SLIGRL-NH2, chloroquine or compound 48/80 into the mouse nape skin. Of note, the scratching evoked by both SLIGRL-NH2 and chloroquine are likely mediated by the activation of Mrgprs family of receptors, via non-histaminergic mechanisms [17,35]. Otherwise, compound 48/80 is a classical inducer of mast cell degranulation that evokes pruritus by means of histamine release [15]. This is in accordance with our data on trypsin-induced pruritus, which is characterized by involving histaminergic and non-histaminergic components [6]. Otherwise, both tested toxins failed to affect the pruritus behavior elicited by i.d. injection of the oxidant agent H_2O_2, which has been characterized to cause scratching by mechanisms involving the activation of TRPA1 and μ-opioid receptors [18]. Our results also revealed that MVIIA or Phα1β, co-injected with morphine by i.t. route, did not significantly modify the scratching behavior caused by this agent, further discarding the relevance of opioid receptors for the anti-pruritus actions of tested toxins. Strikingly, the scratching behavior induced by i.t. injection of GRP was widely prevented by the co-injection of MVIIA or Phα1β. This evidence indicates that GRP-R-mediated pruritus relies on the activation of N-type VGCC, which we believe to be co-localized in DRG. We have also tested the effects of a single epidural administration of MVIIA or Phα1β in the chronic model of pruritus allied to dry skin in mice, in which the disruption of cutaneous barrier was induced by repeated application of AEW [23]. Dry skin is a condition clinically related to chronic itching, being present in atopic dermatitis, and in

elderly populations [37,46]; thus, our results might be of higher clinical interest, considering the limitations to treat chronic pruritus.

Trypsin has also been demonstrated to cause marked edema formation, as well as to induce neutrophil migration, as indicated by increased MPO activity in the mouse paw tissues [27]. Both MVIIA and Phα1β failed to significantly alter trypsin-induced increase of nape skin weight, although a slight reduction of this parameter had been observed. Furthermore, MPO activity was not changed in any of the tested groups, according to evaluation at 40 min following trypsin injection, discarding the possible relevance of neutrophil migration at this time-point. This experimental set suggests that anti-pruritic effects of MVIIA or Phα1β, given by epidural route of administration, are not dependent on the modulation of trypsin-caused peripheral inflammation.

As discussed above, the co-injection of MVIIA or Phα1β was able to prevent the scratching behavior caused by the i.t. application of GRP. We examined whether this effect might be related to the modulation of GRP-R expression in the spinal cord. Confirming and extending literature data [40-41], we detected constitutive GRP-R mRNA expression in the mouse spinal cord of PBS-injected control animals. However, we failed to demonstrate any significant alterations of GRP-R mRNA expression in trypsin-injected mice, or even in the groups pre-treated with MVIIA or Phα1β. We surmise that both VGCC inhibitors are able to modulate GRP-R activity, rather than its spinal expression.

Spinal c-Fos expression has been widely used as a marker of neuronal activation following a series of different peripheral stimuli [10,21]. The pharmacological inhibition of R-type VGCC, by i.t. administration of SNX-482, produced a great inhibition of spinal c-Fos expression, in the rat model of formalin-induced nociception, whereas P/Q-type VGCC blocker ϖ-agatoxin IVA was devoid of effects in this model [42]. We provide interesting data showing that trypsin injection into the mouse nape skin was allied to an increase of c-Fos

expression in the spinal cord, an effect that was reduced to the control values by i.t. administration of MVIIA or Phα1β. A similar increase of c-Fos expression was observed beforehand in the trypsin mouse model of pruritus, being largely prevented by the selective phosphatidylinositol-3 kinase γ (PI3Kγ) inhibitor, namely AS605240, given orally [29].

Finally, we evaluated whether MVIIA or Phα1β modulate calcium influx caused by the selective PAR-2 activation in mouse spinal cord synaptosomes, following the incubation of the synthetic agonist AC264613 [12]. Both toxins were effective in reducing intrasynaptosomal free calcium concentrations after capsaicin incubation, according to evaluation of rat spinal cord synaptosomes [38]. Furthermore, it was previously shown that activation of PAR-2 receptors leads to increase of $[Ca^{+2}]_i$ in acinar cells of rat lacrimal glands [25]. Herein, the selective activator of PAR-2 AC264613 led to a slight increase of calcium influx, an effect that was virtually abolished by MVIIA or Phα1β. When analyzed in concert with functional studies, this result might extend the notion that spinal modulation of N-type VGCC reduces the release of neuropeptides from nerve terminals, thus preventing scratching behavior.

Our study demonstrates, for the first time, the relevance of N-type VGCC in the transmission of either acute or chronic pruritus. It contributes for better understanding the mechanisms implicated in itching sensation, besides opening new possibilities for treating patients with chronic pruritus, unresponsive to available therapies.

5. Acknowledgments and Conflict of Interest Statement

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6. Figure Captions

Figure 1.

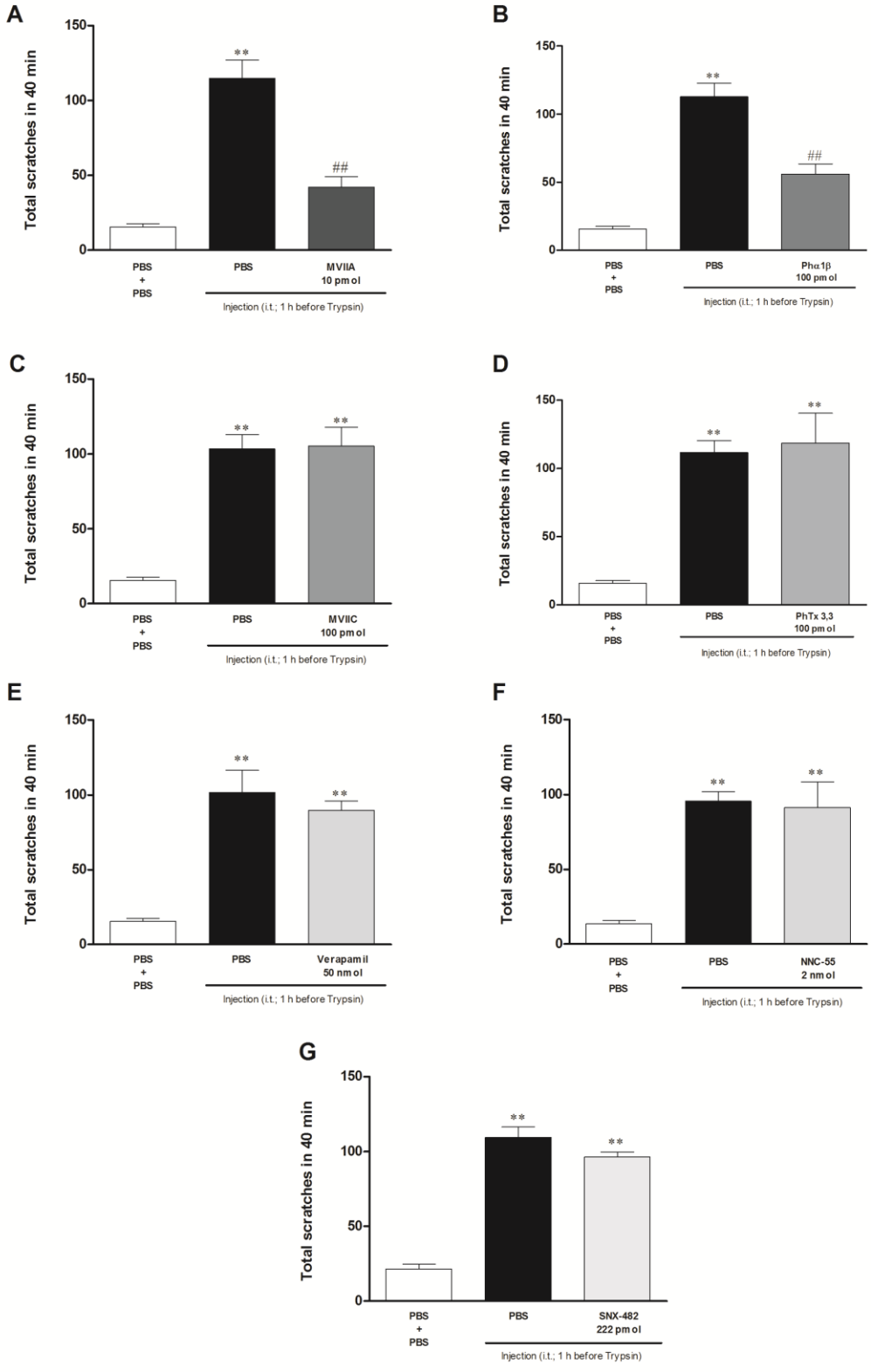

Figure 1. Effects of treatment with different VGCC blockers on trypsin-induced scratching. The i.d. injection of trypsin (200 µg/site, 50 µl) elicited elevated indexes of scratching behavior in mice (black columns). Effect of i.t. pretreatment with selective N-type VGCC inhibitors (A) MVIIA (10 pmol/site) and (B) Phα1β (100 pmol/site); P/Q-type VGCC inhibitors (C) MVIIC (100 pmol/site) and (D) PhTx3.3 (100 pmol/site); L-type VGCC inhibitor (E) verapamil (50 nmol/site); T-type VGCC inhibitor (F) NNC 55-0396 (2 nmol/site) and R-type VGCC inhibitor (G) SNX-482 (222 pmol/site). All the VGCC blockers were dosed 1 h before induction of scratching behavior by trypsin. Each column represents the mean ± SEM of 8-10 animals per group. **p<0.01 significantly different from PBS/PBS group and $^{\#\#}$p<0.01 significantly different from PBS/trypsin group (ANOVA followed by Tukey post-hoc test).

Figure 2.

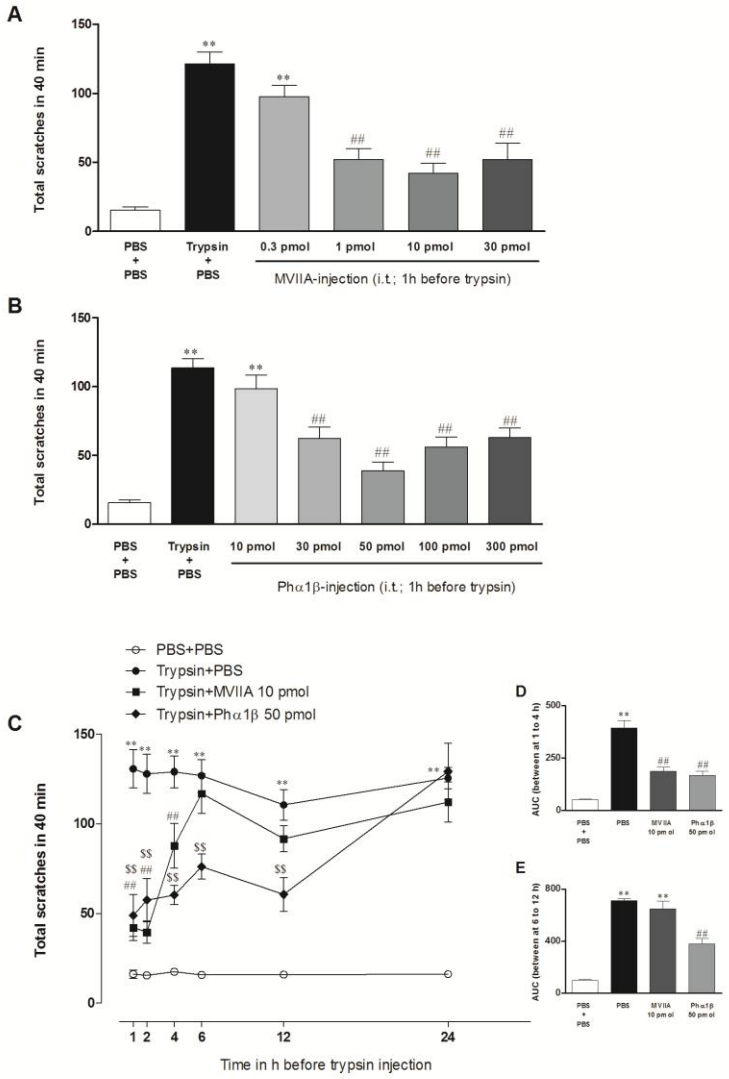

Figure 2. Dose- and time-related effects of N-type VGCC blockers on trypsin-induced scratching. The i.d. injection of trypsin (200 µg/site, 50 µl) elicited elevated indexes of scratching behavior in mice (black columns). Dose-response assessment for the i.t. treatment with the selective N-type VGCC inhibitors (A) MVIIA (0.3; 10 and 30 pmol/site; 1 h before

trypsin injection) and (B) Phα1β (10; 30; 50; 100 and 300 pmol/site; 1 h before trypsin injection). Time-related activity for i.t. treatments with the selective N-type VGCC inhibitors (C) MVIIA and Phα1β (10 and 50 pmol/site, respectively, from 1 h to 24 h before trypsin injection). AUC for the time-related activity after treatment with the selective N-type VGCC inhibitors MVIIA and Phα1β (10 and 50 pmol/site; respectively): (D) 1 h to 4 h before trypsin injection and (E) 6 h to 12 h before trypsin injection. Each column or point represents the mean ± SEM of 8-10 animals per group. **p<0.01 significantly different from PBS/PBS group and $^{\#\#}$p<0.01 or $^{\$\$}$p<0.01 significantly different from PBS/trypsin group (ANOVA followed by Tukey post-hoc test).

Figure 3.

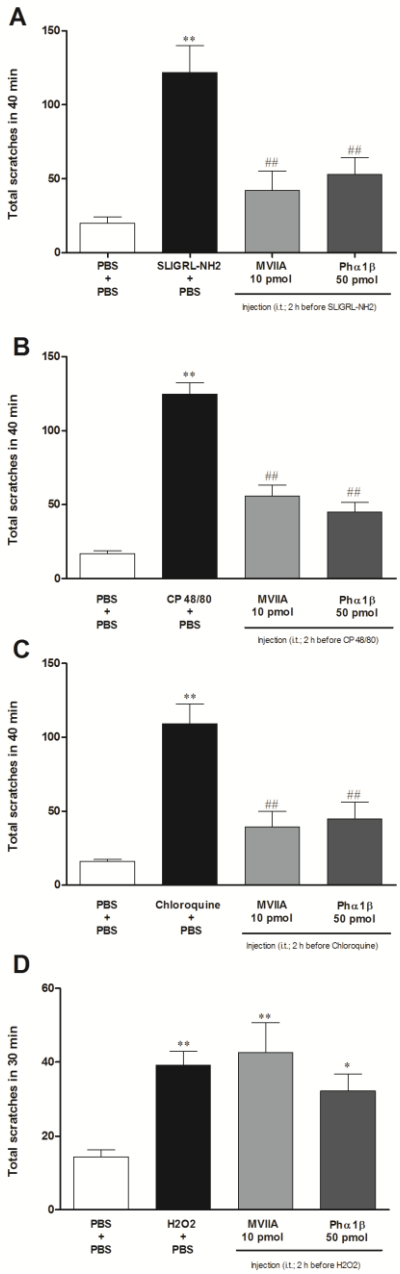

Figure 3. MVIIA and Phα1β inhibit the scratching behavior elicited by other pruritogenic agents. Effects of i.t. pretreatment with the selective N-type VGCC inhibitors MVIIA or Phα1β (10 and 50 pmol/site; respectively), given 2 h before, on the scratching behavior induced by i.d. injection of (A) selective PAR-2 agonist SLIGRL-NH2 (100 µg/site); (B) CP48/80 (10 µg/site); (C) chloroquine (200 µg/site) and H_2O_2 (0.3 % in 50 µl/site). Each column represents the mean ± SEM of 8-10 animals per group. *p<0.05 and **p<0.01 significantly different from PBS/PBS group and $^{\#\#}$p<0.01 significantly different from PBS/SLIGRL-NH2; PBS/CP48/80 or PBS/chloroquine group (ANOVA followed by Tukey post-hoc test).

Figure 4.

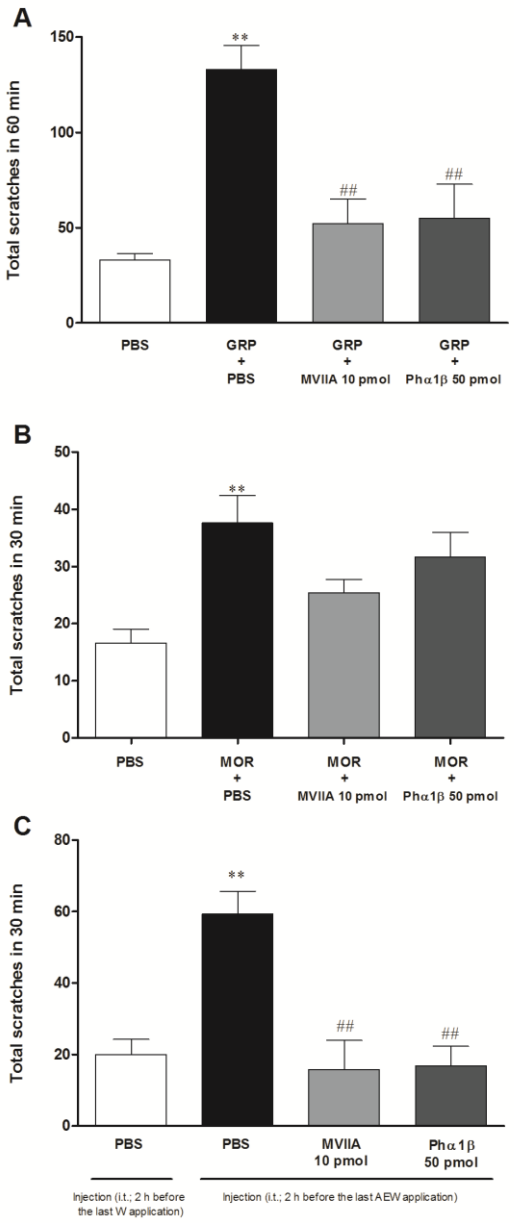

Figure 4. N-type VGCC blockage prevents the scratching behavior elicited by intrathecal injection of GRP or dry skin model. The i.t. injection of GRP or morphine (both 1 nmol/site) elicited elevated indexes of scratching behavior in mice (black columns). Effects of co-administration of MVIIA (10 pmol/site) or Phα1β (50 pmol/site) with GRP (A); effects of co-administration of MVIIA (10 pmol/site) or Phα1β (50 pmol/site) with morphine (B). The dry skin chronic model of pruritus induced by acetone and diethyl-ether (AEW group) elicited marked scratching behavior (black columns): effects of pretreatment with MVIIA or Phα1β (10 and 50 pmol/site; respectively), given 2 h before the last AEW application. Each column represents the mean ± SEM of 8-10 animals per group. **p<0.01 significantly different from PBS or PBS/W group and $^{\#\#}$p<0.01 significantly different from GRP/PBS or PBS/AEW group (ANOVA followed by Tukey post-hoc test).

Figure 5.

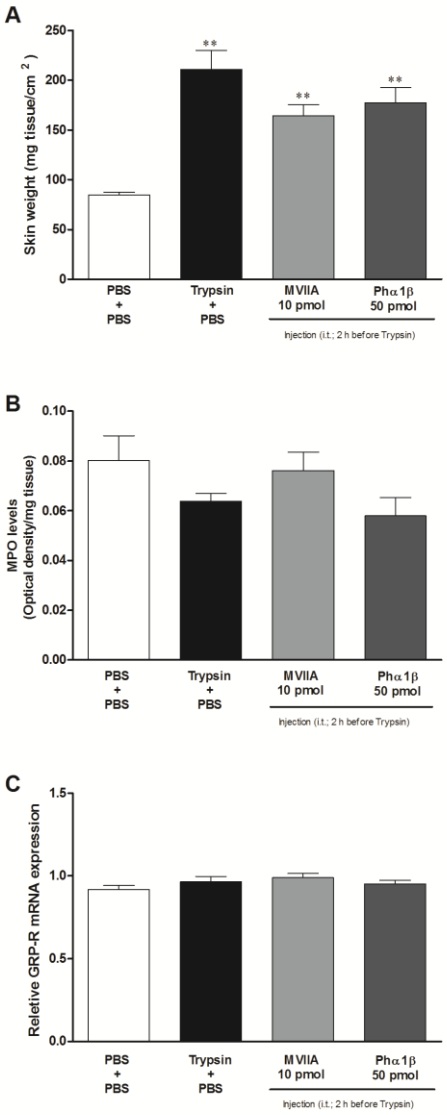

Figure 5. Scratching behavior induced by trypsin appears to be independent on skin inflammatory changes or spinal GRP-R mRNA expression. Effects of pretreatment with MVIIA or Phα1β (10 and 50 pmol/site; respectively; 2 h before trypsin injection) on trypsin-induced edema formation (A) or MPO levels in the mouse nape skin, according to assessment at 40 min. (C) GRP-R mNRA expression in spinal cords after 40 min of trypsin injection and the effects of pretreatment with MVIIA or Phα1β (10 and 50 pmol/site; respectively; 2 h before trypsin injection). Each column represents the mean ± SEM of 8-10 animals per group. **p<0.01 significantly different from PBS/PBS group (ANOVA followed by Tukey post-hoc test).

Figure 6.

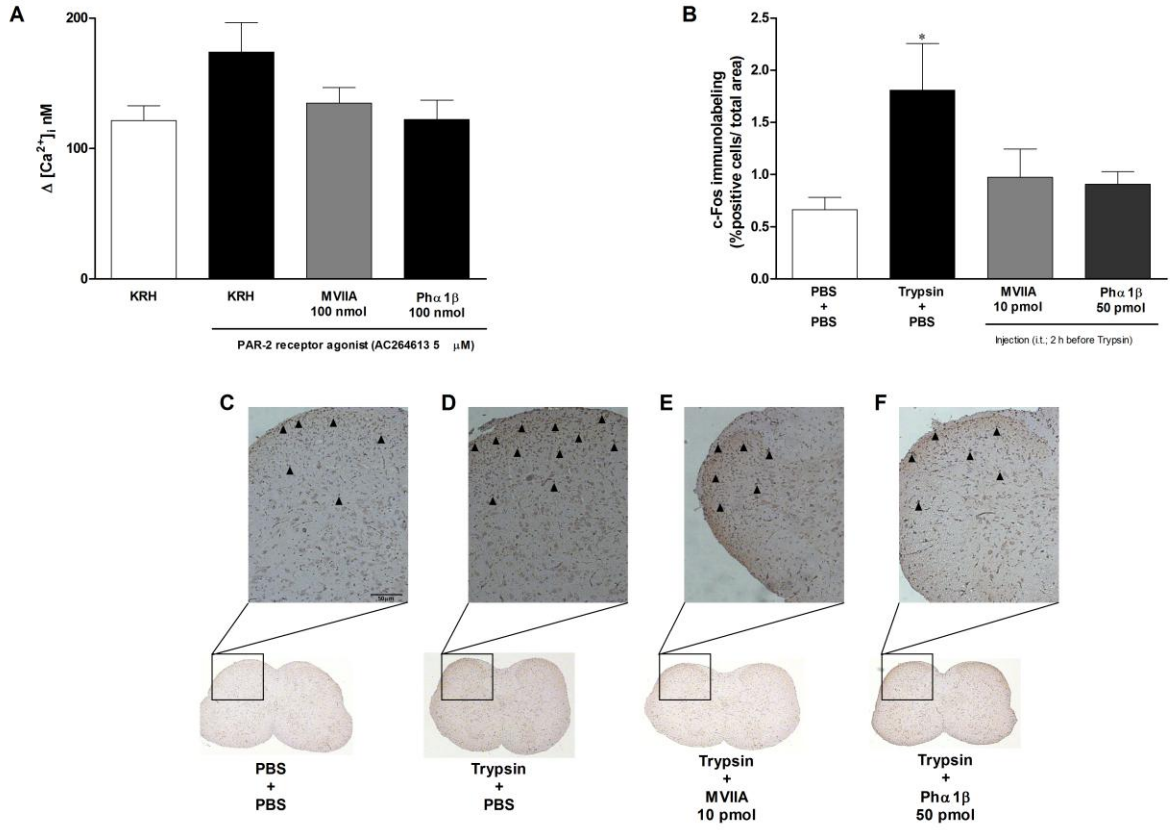

Figure 6. Blockage of N-type VGCC inhibits the increased calcium concentration and c-Fos immunolabelling in the mouse spinal cord. Effects of incubation with MVIIA or Phα1β (both 100 μM) on calcium influx elicited by the selective PAR-2 agonist AC264613 (5μM) in synaptosomes obtained from mouse spinal cord; each column represents the mean ± SEM of 3-4 repetitions (A). Immunohistochemistry analysis for c-Fos in the mouse lumbar sapinal cord, after 40 min of trypsin injection (200 μg/site; i.d.). Arrowheads (▲) indicate positive immunostaining for c-Fos. Representative images for c-Fos immunolabelling in the mouse spinal cord: (C) PBS/PBS group, (D) PBS/trypsin group, (E) MVIIA/trypsin group (10 pmol/site; 2 h before trypsin injection) and (F) Phα1β/trypsin group (50 pmol/site; 2 h before trypsin injection); all images were made in 50-x and 200-x magnification. Graphic showing

the percentage of positive cells/total area of immunostaining for c-Fos (B). Scale bar (—) represents 50 µm. Each column represents the mean ± SEM of 3-4 animals per group. *$p<0.05$ significantly different from PBS/PBS group (ANOVA followed by Tukey post-hoc test).

4. Considerações Finais

Nos últimos anos, diversos pesquisadores têm se empenhado em elucidar os mecanismos específicos envolvidos na sinalização do prurido (Ikoma *et al.*, 2006; Akiyama e Carstens, 2013; Dhand e Aminoff, 2013). Esses trabalhos trouxeram alguns esclarecimentos sobre a coceira, um sintoma que se apresenta em diversas doenças agudas ou crônicas, diminuindo a qualidade de vida dos pacientes (Ikoma *et al.*, 2006; Kini *et al.*, 2011). O prurido crônico é um sintoma presente em diferentes doenças, incluindo transtornos psiquiátricos (depressão e ansiedade) ou, doenças sistêmicas (hipertireoidismo, doença renal crônica e doença hepática/ colestase) (Patel e Dong, 2010). Por muito tempo, o prurido era visto como um subtipo de dor; entretanto, achados recentes têm contribuído para o esclarecimento das diferenças entre prurido e dor e, seus respectivos mecanismos de sinalização (Sun e Chen, 2007; Sun *et al.*, 2009; Dhand e Aminoff, 2013). Porém, ainda são necessários mais estudos, a fim de definir os possíveis mecanismos que diferem a dor e do prurido, podendo permitir o desenvolvimento de novas abordagens farmacológicas, tendo em vista os processos pruritogênicos que não respondem às terapias disponíveis.

Nosso estudo é o primeiro trabalho na literatura voltado à avaliação dos efeitos de diferentes bloqueadores seletivos para os CCVD, tendo sido demonstrada uma participação específica do subtipo N dos CCVD em diversos modelos de coceira aguda e crônica em camundongos. Este estudo abre uma nova perspectiva para o entendimento dos mecanismos celulares envolvidos na coceira. Do ponto de vista clínico, o presente estudo apresenta novas oportunidades para o desenvolvimento de estratégias farmacológicas para o tratamento do prurido, especialmente, para pacientes que não respondem a terapia atual.

Peptídeos derivados de venenos de animais são importantes ferramentas para o estudo de receptores farmacológicos e canais iônicos (Pringos *et al.*, 2011). Diversas fontes de toxinas têm sido utilizadas para a definição dos subtipos de CCVD e sua participação em processos patológicos (Gomez *et al.*, 2002; Vieira *et al.*, 2005; Souza *et al.*, 2008; De Souza *et al.*, 2011; Pringos *et al.*, 2011; Rigo *et al.*, 2013). Um exemplo clássico é a ω-conotoxina MVIIA, derivada do veneno do molusco *Conus magus* e, sua versão sintética, denominada Ziconotida (Prialt®) e, aprovada pelo FDA (Food and drugs Administration), em 2004, para o uso clínico em pacientes com dor crônica, refratários a opioides. Porém, testes pré-clínicos e clínicos têm demonstrado que o Prialt® apresenta uma estreita janela terapêutica e, diversos efeitos colaterais dose-dependentes, limitando a sua utilização (Penn e Paice, 2000; Staats *et al.*, 2004; Hama e Sagen, 2009). Os CCVD têm um papel importante na comunicação

neuronal e na liberação de neuromoduladores, exercendo um grande efeito nos processos patológicos (Hama e Sagen, 2009; Pringos *et al.*, 2011; Rahman e Dickenson, 2013). Os CCVD têm sido implicados na transmissão da dor aguda ou crônica, especialmente através da avaliação dos efeitos de inibidores seletivos em modelos animais (Yaksh, 2006; Pringos *et al.*, 2011). Entretanto, há poucas evidências sobre a relevância destes canais iônicos no prurido.

Frações peptídicas derivadas dos venenos da aranha *Phoneutria Nigriventer*, com seletividade para os subtipos P/Q e N de CCVD, tais como PhTx3.3 e Phα1β, têm sido alvo de diversos estudos, sendo demonstrada a eficácia dos mesmos na inibição da nocicepção em ratos ou camundongos e, sua atividade em bloquear a liberação de neurotransmissores *in vitro*, com menos efeitos adversos e maior tempo de ação, em comparação com toxinas originadas do molusco *C. magus* (Vieira *et al.*, 2005; Souza *et al.*, 2008; Dalmolin *et al.*, 2011; De Souza *et al.*, 2011; Rigo *et al.*, 2013).

Através de abordagens funcionais e, pelo uso de inibidores seletivos, o presente estudo demonstrou o envolvimento principal do subtipo N dos CCVD na sinalização da coceira aguda e crônica. A ativação dos CCVD do tipo N parece representar um dos mecanismos fundamentais para a sinalização da coceira, tanto dependente, quanto independente de vias histaminérgicas. Os efeitos anti-pruritogênicos das toxinas MVIIA e Phα1β foram associados a uma redução da expressão do marcador c-Fos na medula espinhal, sem alterar a expressão dos receptores GRP-R. Ademais, a atividade inibitória apresentada pela administração epidural das toxinas MVIIA e Phα1β não foi relacionada com a modulação da inflamação periférica.

Este é o primeiro estudo a relatar a importância das toxinas MVIIA e Phα1β como ferramentas farmacológicas para a modulação da coceira. Contudo, mais estudos, possivelmente empregando métodos eletrofisiológicos, ainda são necessários para melhor caracterizar o envolvimento dos CCVD na sinalização da coceira.

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ANEXO I

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 0122/12 – CEUA

Porto Alegre, 11 de outubro de 2012.

Senhora Pesquisadora:

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 12/00293, **"Avaliação da atividade das frações PhTx3-3 e PhTx3-6 da toxina da aranha Phoneutria nigriventer no modelo de coceira aguda induzida por tripsina em camundongos".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora da CEUA/PUCRS

Ilmo. Sra.
Profª Maria Martha Campos
INTOX
Nesta Universidade

PUCRS
Campus Central
Av. Ipiranga, 6690 – Prédio 60, sala 314
CEP: 90610-000
Fone/Fax: (51) 3320-3345
E-mail: ceua@pucrs.br

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

AVALIAÇÃO DE PROJETOS DE PESQUISA

TITULO DO PROJETO
Avaliação da atividade das frações PhTx3-3 e PhTx3-6 da toxina da aranha Phoneutria nigriventer no modelo de coceira aguda induzida por tripsina em camundongos
Projeto nº 12/00293
Pesquisador: Maria Martha Campos
AVALIAÇÃO GERAL DO PROJETO
(X) Aprovado ( ) Aprovado com recomendação ( ) Pendente ( ) Não aprovado
Questões levantadas pelo CEUA – PUCRS
Todas as questões levantadas pelo CEUA foram atendidas

PUCRS
Campus Central
Av. Ipiranga, 6690 – Prédio 60, sala 314
CEP: 90610-000
Fone/Fax: (51) 3320-3345
E-mail: ceua@pucrs.br

ANEXO II

Documento de confirmação de submissão de manuscrito

On Thursday, December 19, 2013 5:36 PM, Pain <painjournal@iasp-pain.org> wrote:
Journal: Pain
Title: The spinal inhibition of N-type voltage-gated calcium channels selectively prevents scratching behavior in mice
Format: Full-Length Article
Authors: Izaque S Maciel; Vanessa M Azevedo; Talita C Pereira; Maurício R Bogo; Alessandra H Souza; Marcus V Gomez; Maria Martha Campos, Ph.D.

Dear Dr Campos,

Your submission has been received by journal PAIN.

You will be able to check on the progress of your paper by logging on to EES as an author. The URL is http://ees.elsevier.com/pain/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

Editorial Staff Pain

ANEXO III

Ata de aprovação da dissertação de mestrado

FAMED
PUCRS

Pontifícia Universidade Católica do Rio Grande do Sul
FACULDADE DE MEDICINA
PÓS-GRADUAÇÃO EM MEDICINA E CIÊNCIAS DA SAÚDE

ATA DE APRESENTAÇÃO DE DISSERTAÇÃO Nº 355

Aos vinte e quatro dias do mês de janeiro do ano de dois mil e quatorze, no Curso de Mestrado em Medicina e Ciências da Saúde, área de concentração em Farmacologia Bioquímica e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul foi concluído o processo de avaliação da dissertação intitulada **"Avaliação da participação de canais de cálcio voltagem-dependentes sobre a resposta pruritogênica em camundongos"** de autoria do pós-graduando **Izaque de Sousa Maciel** sob orientação da Professora Doutora **Maria Martha Campos**. A comissão examinadora foi constituída pelos professores: Dr. Robson Costa (UFRJ), Dr. Jarbas Rodrigues de Oliveira (PUCRS), Ivan Carlos Ferreira Antonello (PUCRS) e Dr. Domingos Otavio Lorenzoni d' Avila, suplente (PUCRS). O aluno foi **APROVADO**. Para constar, lavrou-se esta ata que deverá ser anexada à documentação exigida para posterior expedição do diploma. A presente ata foi assinada pelo Coordenador em Exercício do Programa de Pós-Graduação em Medicina e Ciências da Saúde. Porto Alegre, aos vinte e quatro dias do mês de janeiro do ano de dois mil e quatorze.

Prof. Dr. Alexandre Vontobel Padoin