UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOQUÍMICA E IMUNOLOGIA

Ana Cristina Nogueira Freitas

Estudo da atividade antinociceptiva do peptídeo sintético PnPP-19

Belo Horizonte
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Tese submetida ao Programa de Pós-Graduação em Bioquímica e Imunologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do grau de Doutora em Bioquímica e Imunologia.

Orientadora: Profª. Drª. Maria Elena de Lima Perez Garcia
Co-Orientadora: Profª. Drª. Daniela da Fonseca Pacheco

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Universidade Federal de Minas Gerais
Curso de Pós-Graduação em Bioquímica e Imunologia ICB/UFMG
Av. Antônio Carlos, 6627 – Pampulha
31270-901 – Belo Horizonte – MG
e-mail: pg-biq@icb.ufmg.br (31)3409-2615

ATA DA DEFESA DA TESE DE DOUTORADO DE ANA CRISTINA NOGUEIRA FREITAS. Aos vinte e três dias do mês de fevereiro de 2018 às 14h00 horas, reuniu-se no Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, a Comissão Examinadora da tese de Doutorado, indicada *ad referendum* do Colegiado do Curso, para julgar, em exame final, o trabalho intitulado "" Estudo da atividade antinociceptiva dopeptídeo sintético PnPP-19 "", requisito final para a obtenção do grau de Doutor em Ciências: Bioquímica. Abrindo a sessão, a Presidente da Comissão, Profa. Maria Elena de Lima Perez Garcia, da Universidade Federal de Minas Gerais, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações: Dra. Márcia Renata Mortari (Universidade de Brasília), aprovada; Dr. Célio José de Castro Júnior (Instituto de Ensino e Pesquisa Santa Casa BH), aprovada; Dra. Janetti Nogueira de Francischi (Universidade Federal de Minas Gerais), aprovada; Dra. Fabiana Simão Machado (Universidade Federal de Minas Gerais), aprovada; Dr. Daniela da Fonseca Pacheco - Coorientadora (Centro Universitário Newton Paiva), aprovada; Dr. Maria Elena de Lima Perez Garcia - Orientadora (Universidade Federal de Minas Gerais), aprovada. Pelas indicações a candidata foi considerada:

(X) APROVADA
( ) REPROVADA

O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente da Comissão encerrou a reunião e lavrou a presente Ata que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 23 de fevereiro de 2018.

Márcia Renata Mortari
Dra. Márcia Renata Mortari (Universidade de Brasília)

Célio José de Castro Junior
Dr. Célio José de Castro Júnior (Instituto de Ensino e Pesquisa Santa Casa BH)

Dra. Janetti Nogueira de Francischi (UFMG)

Dra. Fabiana Simão Machado (UFMG)

Daniela Pacheco
Dr. Daniela da Fonseca Pacheco - Coorientadora (Centro Universitário Newton Paiva)

Dr. Maria Elena de Lima Perez Garcia - Orientadora (UFMG)

Jader dos Santos Cruz
Profº Jader dos Santos Cruz
Sub Coordenador de Curso de Pós Graduação
em Bioquímica e Imunologia
ICB - UFMG

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RESUMO

A toxina PnTx2-6, isolada da peçonha da aranha *Phoneutria nigriventer*, é muito tóxica (DL_{50} = 0,7 µg/camundongo) e tem sido estudada como uma molécula potenciadora da função erétil. Entretanto, esta toxina induz hiperalgesia local e sistêmica em baixas doses (3 µg/animal). O peptídeo PnPP-19 representa um epitopo descontínuo da estrutura primária da toxina PnTx2-6. Este peptídeo, assim como a toxina nativa, potencia a função erétil de ratos e camundongos. O presente estudo propôs a investigação do peptídeo PnPP-19 na modulação da via nociceptiva, uma vez que a toxina que lhe deu origem, PnTx2-6, induz hiperalgesia. Os resultados deste trabalho demonstram que o peptídeo PnPP-19 tem atividade antinociceptiva central e periférica, dose e tempo-dependentes. Esta antinocicepção deve-se à ativação concomitante dos receptores µ- e δ-opioides, e dos receptores canabinoides do tipo CB_1. O efeito anti-hiperalgésico do peptídeo parece também envolver a liberação de endocanabinoides, uma vez que a administração de doses não-analgésicas de inibidores da recaptação de endocanabinoides e da degradação da anandamida pontencializaram o efeito antinociceptivo de uma baixa dose do peptídeo. O aumento dos níveis de opioides endógenos também parece estar correlacionado com o efeito antinociceptivo de PnPP-19, uma vez que este pode atuar como inibidor da enzima neprilisina. Esta enzima é responsável por hidrolisar vários peptídeos no espaço extracelular, dentre eles o opioide endógeno encefalina. Além disso, a administração do peptídeo ativa a isoforma neuronial e endotelial da óxido nítrico sintase, aumentando os níveis de óxido nítrico nos tecidos. Ainda, a antinocicepção desencadeada por PnPP-19 parece depender dos níveis de GMPc e da ativação dos canais para potássio sensíveis ao ATP. PnPP-19 pode também atuar como um agonista de receptores µ-opioides e modular o influxo de cálcio em neurônios DRG pela ativação destes receptores. A ativação dos receptores µ-opioides desencadeada por PnPP-19 parece não induzir o recrutamento de β-arrestina2. Este trabalho demonstrou o papel do peptídeo sintético PnPP-19 na via nociceptiva, detalhando seu mecanismo de ação. Sugere-se que o PnPP-19 possa ser utilizado como uma possível ferramenta para o desenvolvimento de novos fármacos analgésicos.

Palavras chave: antinocicepção, PnTx2-6, PnPP-19, *Phoneutria nigriventer*, canabinoides, opioides, óxido nítrico, neprilisina.

ABSTRACT

The toxin PnTx2-6, isolated from the venom of the spider *Phoneutria nigriventer*, is very toxic (LD_{50} = 0.7 μg/mouse) and has been studied as a molecule that improves erectile function. Besides this effect, this toxin induces local and systemic hyperalgesia in low doses (3 μg/animal). The synthetic peptide PnPP-19 represents a discontinuous epitope of the primary structure of the spider toxin PnTx2-6. PnPP-19 improves erectile function of rats and mice. This effect is similar of what is observed for the native toxin PnTx2-6. The present study investigated the role of PnPP-19 in the nociceptive pathway, since the toxin, of which the peptide originates from, induces hyperalgesia. Our data demonstrate that PnPP-19 induces a dose and time-dependent central and peripheral antinociception. This antinociception is mediated by activation of CB_1 cannabinoid receptors and μ- and δ-opioid receptors. The antihyperalgesic effect of the peptide may also involve the release of endocannabinoids, since the administration of non-analgesic doses of inhibitors of endocannabinoid reuptake and anandamide degradation have potentialized the antinociceptive effect of a low dose of the peptide. Increased levels of endogenous opioids might also be correlated with the antinociceptive effect of PnPP-19. This peptide may act as an inhibitor of the enzyme neprilysin, which is responsible for hydrolyzing several peptides in the extracellular site, among them, the endogenous opioid enkephalin. In addition, PnPP-19 administration may activate the neuronal and endothelial isoform of nitric oxide synthase, which causes an increase of nitric oxide levels. Furthermore, the antinociception triggered by PnPP-19 seems to be depending on cGMP levels and on the activation of ATP sensitive potassium channels. PnPP-19 may also act as a μ-opioid receptor agonist and it might modulate the influx of calcium in DRG neurons by the activation of these receptors. Activation of μ-opioid receptors induced by PnPP-19 does not appear to stimulate β-arrestin recruitment. Our results have shown the role of the synthetic peptide PnPP-19 in the nociceptive pathway, demonstrating at least part of the mechanism of action by which the peptide induces antinociception. Taking into account all our data, we suggest that PnPP-19 might be a useful tool for the development of new drug cadidates for pain treatment.

Keywords: antinociception, PnTx2-6, PnPP-19, *Phoneutria nigriventer*, cannabinoids, opioids, nitric oxide, neprilysin.

LISTA DE ILUSTRAÇÕES (FIGURAS)

LISTA DE ABREVIATURAS

2-AG: 2-araquidonoil glicerol;

AM251: (1-(2,4-diclorofenil)-5-(iodofenil)-4-metil-N-(1-piperidil)pirazol-3 carboxamida);

AM630: ([6-iodo-2-metil-1-[2-(4-morfolinil)etil]-1H-indol-3-il](4etoxifenil) metanona);

AMPc: monofosfato cíclico de adenosina;

ASIC: canal iônico sensível a ácido;

ATP: trifosfato de adenosina;

CaMKII: Ca^{2+}/calmodulina proteína cinase II;

CAPES: Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior;

Cav: canal para cálcio sensível a voltagem;

CB_1: receptores canabinoides do tipo 1;

CB_2: receptores canabinoides do tipo 2;

GMPc: monofosfato cíclico de guanosina;

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico;

DAG: diacilglicerol;

DL_{50}: dose letal média;

DOCA- SAL: desoxicorticosterona e salina;

DRG: gânglio da raiz dorsal;

eNOS: óxido nítrico sintase endotelial;

FAAH: amida hidrolase de ácidos graxos;

FAPEMIG: Fundação de Amparo à Pesquisa de Minas Gerais;

FDA: *Food and Drug Administration;*

Funed: Fundação Ezequiel Dias;

GDP: bifosfato de guanosina;

GTP: trifosfato de guanosina;

$5\text{-}HT_4$: receptor 5-Hidroxitriptamina tipo 4;

IASP: Associação Internacional para o Estudo da Dor;

i.c.v.: intracerebroventricular;

iNOS: óxido nítrico sintase induzida;

i.t.: intratecal;

K_{ATP}: canal para potássio sensível ao ATP;

K_{cat}: constante de catálise;

K_i: constante de inibição;

L-NOARG: L-NG-nitro arginina;

M: massa molecular;

MAFP: (metil araquidonil fluorofosfonato) (ácido (5Z,8Z,11Z,14Z)-eicosatetraenil-fosfonofluorídrico metil ester);

MAPK: proteíno-cinases ativadas por mitógenos;

MGL: monoacilglicerol lipase;

n-Ach: receptor nicotínico para acelticolina;

Nav: canal para sódio sensível a voltagem;

NArPE: *N*-araquidonoil fosfatidiletanolamida;

NEP: neprilisina;

NMDA: N-metil-D-aspartato;

nor-BNI: norbinaltorphimine;

NPS: nitroprussiato de sódio;

nNOS: óxido nítrico sintase neuronial;

NO: óxido nítrico;

NOS: óxido nítrico sintase;

ODQ: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one;

PAG: substância cinzenta periaquedutal;

PDE_5: fosfodiesterase tipo 5;

PGE_2: prostaglandina E_2;

PIP_2: fosfatidil inositol bifosfato;

PKA: proteína cinase A;

PKB: proteína cinase B;

PKG: proteína cinase G;

PLCβ: fosfolipase C-β;

SEA: *Similarity Ensemble Approach*;

SNC: sistema nervoso central;

SNP: sistema nervoso periférico;

TRPA: canal de potencial transiente da subfamília A;

TRPV: canal de potencial transiente do tipo vanilóide;

THC: Δ9-tetrahidrocanabinol;

VGCCs - canais para cálcio sensíveis à voltagem.

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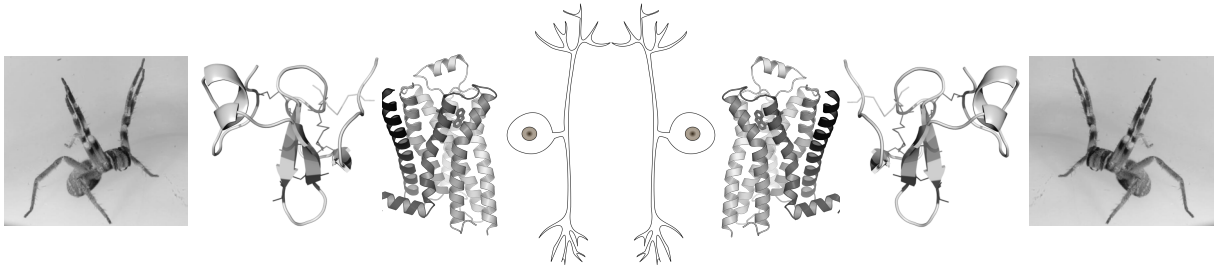
Introdução



1 INTRODUÇÃO

1.1 **Aranha** *Phoneutria nigriventer*

As aranhas de importância médica no Brasil correspondem a três gêneros distintos: *Phoneutria* (aranha armadeira), *Loxosceles* (aranha-marrom) e *Latrodectus* (viúva-negra). Segundo o ministério da saúde, os acidentes envolvendo os gêneros *Phoneutria* e *Loxoceles* representam o tipo mais frequente do país. Em 2016, ocorreram no Brasil cerca de 28.809 casos registrados de acidentes com aranhas, sendo que 25 resultaram em óbito (Ministério da Saúde). Acidentes com o gênero *Phoneutria* têm sua maior incidência nas regiões sul e sudeste e ocorrem em sua maioria nos meses de abril e maio (Cardoso et al., 2004).

O gênero *Phoneutria* foi primeiramente descrito em 1833 por Perty e os espécimes desse gênero pertecem a família Ctenidae, subordem Araneomorphae e ordem Araneae. Estas aranhas possuem ampla distribuição no continente americano, sendo encontradas nas áreas de floresta da América Central (Costa Rica) e em toda região da América do Sul, entre o leste dos Andes e o norte da Argentina (Simó e Brescovit, 2001; de Lima et al., 2015). Particularmente, a espécie *Phoneutria nigriventer*, descrita em 1891 por Keyserling, é a mais encontrada em território brasileiro, principalmente nos estados de Minas Gerais, Goiás, Mato Grosso do Sul, Rio de Janeiro, São Paulo, Paraná, Santa Catarina e Rio Grande do Sul (Lucas, 1988).

A aranha *Phoneutria nigriventer* é conhecida por "aranha armadeira" por adotar uma posição típica de defesa, na qual ela se apoia nas pernas traseiras, ergue as pernas dianteiras e os palpos, deixando as quelíceras bem evidentes (Figura 1). Esta espécie de aranha possui o corpo todo coberto por cerdas, sendo as quelíceras cobertas por cerdas avermelhadas. A região dorsal do abdômen apresenta uma coloração que pode variar do amarelo-marrom até o marrom escuro e a região ventral apresenta cor negra para as fêmeas e cor alaranjada para os machos. Esses animais não constroem teias geométricas, possuindo hábito errante e noturno (Eickstedt, 1981; Lucas, 1988).

Figura 1: Posição típica de defesa adotada por um espécime de *Phoneutria nigriventer*. Foto: Bruno Figueiredo, Fundação Ezequiel Dias (FUNED) (Magalhães, 2013, p.28).

1.2 **A peçonha da aranha** *Phoneutria nigriventer*

As aranhas possuem o maior número de espécies dentre os animais peçonhentos. Até dezembro de 2017, 47.061 espécies foram descritas, o que provavelmente ainda representa um número subestimado da diversidade taxonômica deste clado (Platnick, 1997; World Spider Catalog). Baseando-se no número de espécies de aranhas descritas em 2009 e na complexidade de suas peçonhas, foi estimado um número superior a 12 milhões de peptídeos diferentes existentes nas peçonhas destes artrópodes (Escoubas e King, 2009).

A peçonha da aranha *Phoneutria nigriventer* é altamente neurotóxica e é a mais estudada dentre as peçonhas correspondentes as outras espécies do gênero *Phoneutria*. Esta peçonha é composta por uma diversidade de peptídeos, enzimas e potentes neurotoxinas que interagem com canais iônicos, dentre outros alvos farmacológicos. Apesar da peçonha apresentar um potencial neurotóxico bastante potente, a quantidade desta peçonha inoculada em seres humanos é muito pequena para induzir um efeito letal, dessa forma, acidentes com essa aranha raramente levam o indivíduo a óbito. Entretanto, o envenenamento ocasionado por este animal em crianças pode induzir manifestações clinicas típicas de uma intoxicação sistêmica, que pode incluir os seguintes sintomas: priapismo, taquicardia, arritmias, perturbações visuais, convulsões tônicas, paralisia espástica, sialorréia, sudorese, câimbras dolorosas e tremores (Fontana, 1990; de Lima et al., 2015).

Inicialmente, por métodos cromatográficos, Rezende Jr. e colaboradores (1991) isolaram 3 frações tóxicas da peçonha, denominadas PhTx1, PhTx2 e PhTx3, e uma não tóxica,

denominada PhM. Posteriormente, Figueiredo e colaboradores (1995) isolaram uma quinta fração tóxica, denominada PhTx4.

As frações isoladas da peçonha da aranha *P. nigriventer* diferem tanto em sua composição quanto nos seus potenciais efeitos farmacológicos. A fração PhTx1 contém somente uma toxina, denominada PnTx1. Quando esta toxina é injetada via intracerebroventricular (i.c.v.), ela é capaz de induzir excitação, paralisia espástica e elevação da cauda de camundongos, assim como, também tem efeito letal para estes animais, DL_{50} (dose letal): 45 μg/Kg (Rezende et al., 1991). Hoje sabe-se que esta toxina atua inibindo vários tipos de canais para sódio voltagem dependentes (Nav) (Martin-Moutot et al., 2006; Silva et al., 2012a).

A fração PhTx2 é constituída por várias toxinas distintas e corresponde a fração mais tóxica isolada da peçonha, DL_{50}: 1,7 μg/kg (Rezende et al., 1991). A PhTx2 será discutida em maior detalhe na próxima seção. A fração PhTx3, também apresenta efeito tóxico, sendo sua DL_{50} correspondente a 137 μg/Kg. Quando injetada via i.c.v. induz paralisia flácida dos membros de camundongos por 24h ou mais. Em 1993, Cordeiro e colaboradores identificaram 6 toxinas diferentes presentes na fração PhTx3, denominadas de PnTx3-1 a PnTx3-6. Em experimentos posteriores, quando estas toxinas foram injetadas separadamente via i.c.v., as mesmas induziram diferentes efeitos neurológicos, entretanto, todas induziram paralisia, de algum nível, nos camundongos testados.

A fração PhM não apresenta efeito tóxico e foi primeiramente isolada juntamente com as frações PhTx1, PhTx2 e PhTx3. Administração de 0,1-0,3 mg via i.c.v. em camundongos não induziu efeito letal. Todavia, esta fração apresenta um efeito farmacológico interessante, pois é capaz de ativar a contração muscular em preparações de íleo de cobaia (Rezende et al., 1991). Somente mais tarde, em 2005, a fração PhM foi melhor caracterizada. Foi demonstrado que esta fração é constituída de um *pool* de isoformas peptídicas parecidas de massas moleculares menores que 2.000 Da (Pimenta et al., 2005). Ao todo, foram sequenciadas 15 isoformas destes peptídeos, que possuem entre 7 e 14 resíduos de amino ácidos em sua sequência, sendo que todos os peptídeos apresentam no seu N-terminal um piroglutamato. Os peptídeos desta fração são relacionados estruturalmente com com as taquininas, sendo assim, estas moléculas foram denominadas *Phoneutria nigriventer tachykinin peptides* (PnTkP) (Pimenta et al., 2005). Entretando, ainda são necessários mais estudos para uma completa caracterização farmacológica dos PnTkPs.

Enquanto as frações citadas acima foram isoladas em 1991, a fração PhTx4, e suas respectivas características, foram demonstradas somente 4 anos mais tarde, em 1995

(Figueiredo et al., 1995). A DL_{50} desta fração é 480 μg/Kg, portanto, dentre as frações ativas, esta é a que apresenta menor toxicidade para camundongos. Porém, PhTx4 é altamente tóxica e letal para insetos, fazendo com que esta fração seja conhecida como a "fração inseticida" da peçonha. Três toxinas com atividade inseticida foram isoladas desta fração, estas foram denominadas PnTx4(6-1), PnTx4(5-5) e PnTx4-3 (Figueiredo et al., 1995; Figueiredo et al., 2001; Oliveira et al., 2003).

Tendo como ponto de partida as diferentes frações isoladas da peçonha, vários autores têm investigado as moléculas bioativas isoladas destas frações, demonstrando o grande potencial das toxinas em desencadear diferentes efeitos farmacológicos, tanto em ensaios *in vitro* quanto *in vivo*. As toxinas descritas atuam em diversos alvos farmacológicos, o que pode levar a distintos efeitos biológicos. Como, por exemplo, estas toxinas podem ativar ou bloquear diferentes canais iônicos (Na^+, Ca^{2+} e K^+) em preparações de mamífero ou de inseto (Rash e Hodgson, 2002; Gomez et al., 2002; de Lima et al., 2007; Borges et al., 2009). Alguns outros efeitos causados por estas toxinas incluem: aumento da permeabilidade vascular da pele de mamíferos, induzindo formação de edema (Marangoni et al., 1993); indução de priapismo em modelos murinos (Andrade et al., 2008; Nunes et al., 2008); estimulação da liberação de acetilcolina e catecolaminas em terminações nervosas de cobaias (Costa et al., 1998); inibição da captação de L-glutamato em sinaptosomas de cérebro de rato (Mafra et al., 1999) e da corrente gerada pela ativação de receptores NMDA em neurônios hipocampais de rato (Figueiredo et al., 2001), dentre outros.

1.3 **A fração PhTx2 e a Toxina PnTx2-6**

A fração PhTx2 foi isolada da peçonha da aranha *P. nigriventer* por uma combinação de cromatografias de filtração em gel e fase reversa. Quando esta fração foi administrada por via i.c.v. em camundongos, a mesma induziu sintomas incluindo salivação, lacrimejamento, priapismo, convulsões e paralisia espástica das patas (Rezende et al., 1991). Estes sintomas são bastantes semelhantes aos induzidos pela administração da peçonha bruta pela mesma via de administração.

Além disso, como já citado acima, PhTx2 é a fração mais tóxica dentre todas as frações isoladas da peçonha, e, além do mais, ela é capaz de mimetizar o efeito da peçonha bruta também em ensaios *in vitro*: assim como a peçonha, esta fração foi capaz de induzir, por exemplo, uma despolarização não uniforme da membrana de fibras do músculo do diafragma de camundongos (Rezende et al., 1991). Posteriormente, Araújo e colaboradores (1993) constataram que a fração PhTx2 tem um potencial efeito excitatório, uma vez que ela diminui

a velocidade de inativação dos canais para sódio voltagem dependentes, consequentemente, deixando estes canais abertos por mais tempo. Além disso, foi demonstrado que a fração também estimula o aumento da entrada de Ca^{2+} em sinaptosomas de córtex cerebral de rato e induz a liberação de L -glutamato nesta mesma preparação (Romano-Silva et al.,1993).

A fração PhTx2 é composta por 9 peptídeos distintos denominados PnTx2-1, PnTx2-2, PnTx2-3, PnTx2-4, PnTx2-5, PnTx2-6, PnTx2-7, PnTx2-8 e PnTx2-9. Apenas 5 destas toxinas foram sequenciadas e tiveram suas massas moleculares (*M*) determinadas. A toxina PnTx2-1 possui 53 resíduos de aminoácidos e sua massa molecular corresponde a 5.838,8 Da; a PnTx2-3 têm 40 resíduos e *M* 6.015 Da; PnTx2-5 corresponde a uma sequência de 49 resíduos e *M* 5.116,6 Da; PnTx2-6 têm 48 resíduos e *M* 5.291,3 Da e a PnTx2-9 possui 32 resíduos e *M* 3.742,1 Da (Cordeiro et al., 1992).

Quando injetada via i.c.v. na dose de 0,79 μg/camundongo, a toxina PnTx2-6 causa prurido, lacrimejamento, salivação, sudorese, agitação seguida por paralisia espástica das patas e morte (Cordeiro et al., 1992), semelhante aos efeitos causados pela fração de sua origem administrada pela mesma via (Rezende et al, 1991). Adicionalmente, assim como a fração PhTx2, PnTx2-6 afeta a cinética dos canais para sódio voltagem dependentes, diminuindo sua velocidade de inativação e aumentando assim a condutância para os íons sódio (Matavel et al., 2002). Além disso, a toxina ainda é capaz de induzir a liberação de L-glutamato em sinaptosomas de córtex cerebral de rato (Nunes, 2012), semelhante à PhTx2.

Um efeito bastante interessante descrito para a toxina PnTx2-6 foi sua capacidade de induzir o priapismo, definido como uma ereção prolongada, dolorosa e involuntária (Van Der Horst et al., 2003). Quando esta toxina foi administrada diretamente no corpo cavernoso de camundongos (6 ng/Kg), a mesma induziu este efeito (Andrade et al., 2008). Posteriormente foi constatado que esta molécula é capaz de potencializar a função erétil em ratos e camundongos normotensos e de ratos DOCA-sal hipertensos via óxido nítrico (Nunes et al., 2008) e também de recuperar a função erétil de ratos com lesão bilateral do nervo cavernoso (Jung et al., 2014).

Tendo em vista o potencial uso da toxina PnTx2-6 como ferramenta para o desenvolvimento de um possível fármaco para tratamento da disfunção erétil, fez-se necessário pesquisar sobre possíveis efeitos colaterais negativos desencadeados pela administração desta molécula. Portanto, em 2008, foi investigado o efeito da toxina PnTx2-6 na via nociceptiva, uma vez que, como dito anteriormente, essa toxina atua modulando canais para sódio voltagem dependentes, aumentando a condutância dos íons sódio através destes canais. Vale ressaltar que a atividade dos canais para sódio voltagem dependentes está intimamente relacionada com a

transmissão de estímulos nociceptivos, sendo que as isoformas mais relacionados com a sinalização da via nociceptiva são $Na_v1.3$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$ e $Na_v1.9$ (Dib-Hajj et al., 2017).

Através de ensaios comportamentais, foi observado que a toxina PnTx2-6 é capaz de causar hiperalgesia. Quando a toxina foi administrada por via intraplantar na dose de 3,0 µg/animal, foi possível observar uma diminuição significativa do limiar nociceptivo dos animais decorridos 40 min após a administração da mesma (Figura 2A). Ainda, esta mesma dose de PnTx2-6 foi capaz de causar um efeito hiperalgésico sistêmico, pois a toxina administrada apenas na pata direita foi capaz de induzir hiperalgesia na pata contralateral (Figura 2B) (Nunes, 2008). Interessantemente, foi descrito posteriormente que a toxina PnTx2-6 é capaz de modular os canais $Na_v1.3$, $Na_v1.6$ e $Na_v1.8$, subtipos envolvidos em processos de sinalização da dor (Silva et al., 2015; Dib-Hajj et al., 2017).

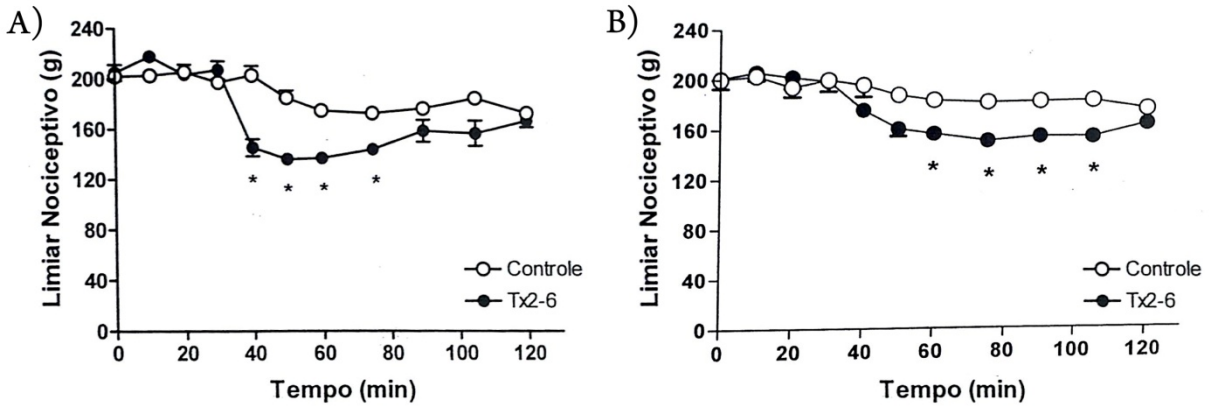


Figura 2: Efeito da PnTx2-6 (3µg/pata) sobre o limiar nociceptivo aferido na pata de ratos. Os animais (ratos wistar machos aldultos) foram submetidos ao teste algesimétrico de compressão da pata descrito por Randall & Selitto. (A) Hiperalgesia induzida pela administração da toxina na pata direita, sendo esta submetida ao teste. (B) Efeito hiperalgésico sistêmico induzido pela administração de PnTx2-6 na pata direita e limiar nociceptivo aferido na pata esquerda. *P<0,05 (*Two-way* ANOVA seguido de pós-teste de Bonferroni), n=5. (retirado de Nunes, 2008, p. 86 e 87).

1.4 **Peptídeo PnPP-19**

A sequência do peptídeo PnPP-19, "Ac- GERRQYFWIAWYKLANSKK -NH2" (sequência descrita do N para o C-terminal), que é a molécula de interesse do presente trabalho, foi obtida utilizando-se o programa de bioinformática denominado PEPOP, que propôs um epítopo descontínuo de 19 resíduos de aminoácidos a partir da sequência da toxina PnTx2-6 (Figura 3A). Este programa prediz as regiões de uma determinada proteína, neste caso a toxina, que são mais susceptíveis a interagir ou serem reconhecidas por uma outra proteína (receptores

ou canais iônicos, por exemplo). O objetivo inicial desta análise era a obtenção de um peptído antigênico usando como modelo a toxina PnTx2-6. A sequência gerada pelo programa foi então sintetizada, entretanto o resíduo de cisteína presente no C-terminal da toxina foi substituído por um resíduo de serina no peptídeo e, durante a síntese química, o N-terminal foi acetilado e o C-terminal amidado. As modificações do N e do C-terminal foram feitas visando-se aumentar a solubilidade da molécula, visto que a sequência apresenta muitos resíduos hidrofóbicos, e também como forma de diminuir a propensão do peptídeo em sofrer degradação enzimática. A massa molecular do peptídeo é igual a 2.484,97 Da, possuindo em sua sequência a região correspondente a α-hélice prevista na estrutura terciária da toxina PnTx2-6, obtida por modelagem molecular (Figura 3B) (Fleury, 2009; Matavel et al., 2009; Silva, 2012; Silva et al., 2015).

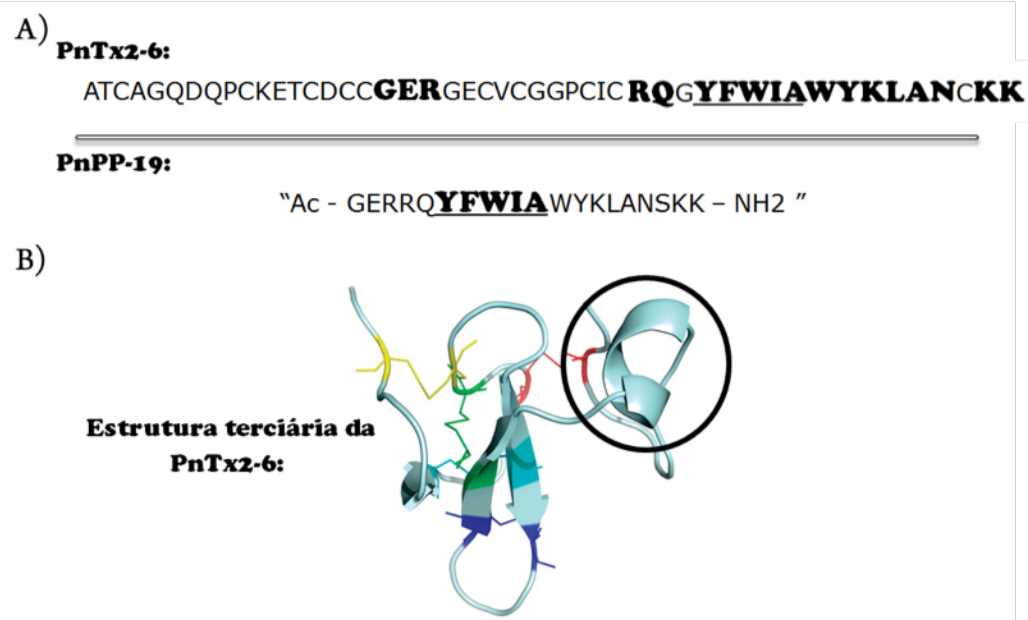


Figura 3: Estrutura primária correspondente ao peptídeo PnPP-19, um derivado sintético da toxina PnTx2-6. Em (A) estão mostradas as estruturas primárias tanto da toxina, quanto do peptídeo. Os aminoácidos em negrito na sequência da toxina representam a sequência originária do peptídeo PnPP-19. A sequência sublinhada, encontrada tanto na toxina, quanto no peptídeo, corresponde a alfa-hélice (em destaque) representada na estrutura terciária da toxina PnTx2-6 obtida por modelagem molecular utilizando-se o programa Modeler 9v3 (B) (Modificado de Matavel et al., 2009).

Utilizando-se o peptídeo PnPP-19, obtido por síntese química, demonstrou-se que, assim como a toxina PnTx2-6, este peptídeo foi capaz de induzir a liberação de L-glutamato em sinaptosomas de córtex cerebral de ratos. Entretanto, foi necessário para os testes uma

concentração de peptídeo cerca de 1000 vezes maior do que a utilizada para toxina nativa induzir um efeito semelhante (Silva, 2012).

O possível efeito do PnPP-19 na função erétil também foi explorado, visto que a toxina nativa atua como potenciador desta função. Foi observado que o peptídeo, em uma concentração relativamente baixa (12 μg/kg), é capaz de potenciar a função erétil de ratos em ensaios *in vivo* e induz um significativo relaxamento de tiras do corpo cavernoso isoladas de camundongos (10^{-8} M) em ensaios *ex vivo*. O papel do PnPP-19 na função erétil parece ser dependente do sistema nitrérgico, uma vez que o peptídeo induziu aumento dos níveis de GMPc nas tiras de corpo cavernoso e o tratamento dessas tiras com o inibidor específico da óxido nítrico sintase neuronial preveniu o relaxamento das mesmas (Silva et al., 2015).

O próximo passo foi investigar se o peptídeo sintético teria qualquer atividade sobre os canais para sódio voltagem dependentes, uma vez que a toxina PnTx2-6 tem uma atividade promíscua, modulando diversas isoformas destes canais. Utilizando-se a técnica de *Whole Cell Patch Clamp*, o peptídeo foi testado tanto em neurônios do gânglio da raiz dorsal (DRG) quanto em cardiomiócitos. Não foi observada nenhuma alteração das correntes para sódio quando estas células foram incubadas com diferentes concentrações de PnPP-19. Além disso, a atividade do peptídeo sobre ovócitos de *Xenopus laevis* superexpressando diferentes isoformas de canais para sódio (Na_v1.2-1.6 e 1.8) foi testada. Mais uma vez, todos os resultados foram negativos e nenhuma atividade do peptídeo sobre estes canais foi detectada (Silva et al., 2015).

Ensaios para analisar a possível toxicidade do PnPP-19 em modelos *in vivo* foram realizados. Nestes ensaios, a dose de 5 mg/kg de peptídeo foi injetada por via intraperitoneal em camundongos. Análises hispatológicas dos tecidos provenientes do rim, coração (ventrículo), pulmões, fígado, cérebro e pênis não detectaram qualquer sinal de toxicidade aparente induzida pelo peptídeo. Ademais, ensaios de imunogenicidade também foram realizados. Nestes experimentos, PnPP-19 (5 mg/kg ou 0,5 mg/kg) foi administrado em camundongos via subcutânea por quatro vezes consecutivas (intervalo de cerca de 2 semanas entre as administrações). Foi então possível observar que PnPP-19 não induziu morte ou reações de hipersensibilidade nos animais experimentais. A produção de anticorpos induzida por PnPP-19, nas condições experimentais utilizadas, foi considerada baixa pelos autores. Sendo assim, para os testes realizados até o momento, PnPP-19 é considerado não tóxico e induz baixa imunogenicidade (Silva et al., 2015).

1.5 **A peçonha da aranha** *Phoneutria nigriventer* **e a via nociceptiva**

Acidentes com aranhas do gênero *Phoneutria* podem causar uma série de sintomas, entretanto, dor intensa é um dos principais dentre eles. Cerca de 92.1% dos pacientes relatam dor intensa no local da picada (Bucaretchi et al., 2000). A peçonha da aranha *P. nigriventer* é constituída por uma grande variedade de componentes, portanto, a atividade nociceptiva da peçonha pode ser devida a ativação de vários alvos moleculares por toxinas distintas. De fato, a administração de 3 μg/pata da peçonha bruta, na superfície plantar de camundongos, induziu hiperalgesia nestes animais por até 4 h. Foi demonstrado que a hiperalgesia induzida pela peçonha é mediada pela estimulação das fibras aferentes através da ativação/estimulação dos receptores de bradicinina B_2, TRPV1, receptor de serotonina 5-HT_4, ASIC e dos canais para sódio voltagem dependentes sensíveis a tetrodotoxina (Gewehr et al., 2013).

Apesar da peçonha bruta da *P. nigriventer* induzir hiperalgesia, já foram isoladas algumas toxinas da mesma com efeito antinociceptivo. Por exemplo, a fração PhTx3 contém 5 toxinas distintas (descritas a seguir) que apresentam efeito anti-hiperalgesico, sendo que grande parte delas atua bloqueando diferentes isoformas de canais iônicos.

A toxina PnTx3-1 atua como um bloqueador de canais para potássio voltagem dependentes do tipo-A (Kushmerick et al., 1999), e, por isto, esta toxina é também denominada PhKv. Recentemente, foi demonstrado que esta toxina, quando administrada por via intratecal (i.t.), induz antinocicepção em diferentes modelos de dor. Este efeito anti-hiperalgésico da toxina parece ser dependente da ativação do sistema colinérgico, uma vez que foi demonstrado que o mesmo é atenuado quando os animais são pré-tratados com antagonistas de receptores nicotínicos e muscarínicos para acetilcolina. Além disso, PnTx3-1 também é capaz de inibir a atividade da acetilcolinesterase, enzima responsável pela degradação de acetilcolina. Assim, a administração desta toxina no sistema nervoso central pode induzir um aumento das concentrações de acetilcolina, fazendo que a mesma esteja mais biodisponível para se ligar e ativar os receptores da via colinérgica (Rigo et al., 2017).

A toxina PnTx3-3 induz antinocicepção quando injetada via i.t. ou i.c.v. no modelo de nocicepção térmica, e foi bastante eficaz no tratamento de dor neuropática (causada por constrição do nervo ciático) (Dalmolin et al., 2011). Esta toxina atua bloqueando diferentes isoformas de canais para cálcio voltagem dependentes (Leão et al., 2000), e, desta maneira, possivelmente interfira na liberação de neurotransmissores no sistema nervoso central.

A toxina PnTx3-4 também é um inibidor de canais para cálcio voltagem dependentes (dos Santos et al., 2002) e é bastante eficaz no tratamento de dor inflamatória (induzida pelo teste de

formalina), no modelo de dor pós-operatória (causada por incisão na superfície plantar dos animais testados) e no modelo de dor induzida pela administração i.t. de NMDA. Mesmo sendo um inibidor bastante potente de canais para cálcio, esta toxina não induz nenhuma alteração motora quando administrada por via i.t. nas doses que induzem antinocicepção (da Silva et al., 2015).

Mais recentemente, o papel da PnTx3-5 também foi investigado sobre a via nociceptiva. Esta toxina foi capaz de reverter a hiperalgesia induzida em modelos de dor pós-operatória, de dor neuropática e de dor induzida por câncer. Interessantemente, PnTx3-5 foi muito eficaz em todos os modelos testados quando administrada por via i.t em uma dose consideravelmente baixa (30 fmol/animal), sugerindo a grande potência da molécula e a provável alta afinidade pelo seu alvo molecular específico. Além disso, em doses possivelmente terapêuticas, a toxina não induziu efeitos adversos (Oliveira et al., 2016).

A toxina PnTx3-6, também conhecida como Phα1β, é a mais estudada na via nociceptiva, sendo seu efeito antinociceptivo demonstrado em vários modelos de dor. O primeiro trabalho demonstrando a atividade antinociceptiva da toxina Phα1β foi publicado em 2008. Neste trabalho mostrou-se que Phα1β é eficaz no tratamento de dor nociceptiva, inflamatória e neuropática, apresentando, em alguns aspectos, mais vantagens em relação a administração de ω-conotoxina MVIIA (Souza et al., 2008). Vale ressaltar que atualmente o medicamento conhecido como Prialt® (Ziconotide), utilizado no tratamento de dor crônica, é um derivado sintético desta conotoxina (Mcgivern, 2007). Nos anos subsequentes, vários trabalhos demonstraram que a administração intratecal de Phα1β, nas doses em que se observam atividade antinociceptiva, não induz efeitos colaterais consideráveis. Além disso, esta toxina administrada tanto via i.t. quanto inintraplantar também apresentou efeito antinociceptivo, sendo que, dependendo da via de administração, Phα1β é eficaz no tratamento de dor induzida pelo agente quimioterápico paclitaxel e por capsaicina, e em modelos de dor relacionados com câncer e fibromialgia (De Souza et al., 2011; Castro-Junior et al., 2013; De Souza et al., 2013; Rigo, et al., 2013a; Rigo, et al., 2013b; De Souza et al., 2014; Diniz et al., 2014). A toxina Phα1β tem como mecanismo de ação o bloqueio de canais para cálcio voltagem dependentes (Vieira et al., 2005), entretanto, foi descoberto mais recentemente, que esta toxina além de inibir os canais para cálcio, também pode atuar como inibidor de canais TRPA1 (Tonello et al., 2017).

Toxinas com ação antinociceptiva isoladas da fração Phtx4 já foram também caracterizadas. A toxina PnTx4(5-5) foi capaz de reverter a hiperalgesia induzida pela administração de prostaglandina E_2, carragenina e glutamato na superfície plantar de ratos.

Entretanto, ainda não foi esclarecido o mecanismo pelo qual esta toxina induz efeito antinociceptivo, apesar de já ser descrito que esta toxina pode atuar inibindo receptores NMDA (de Oliveira, 2010; Silva et al., 2016). Por outro lado, a toxina PnTx4(6-1), induz antinocicepção em modelos de dor inflamatória, nociceptiva e neuropática supostamente pela de ativação de receptores canabinoides CB_1 e receptores μ- e δ-opioides. Porém, ainda não foi demonstrado se esta toxina de fato se liga e ativa diretamente os receptores mencionados (Emerich et al., 2016). Recentemente, um peptídeo sintético de 13 resíduos de aminoácidos foi sintetizado a partir da estrutura primária da toxina PnTx4(6-1). Este peptídeo mantém todas as propriedades antinociceptivas da toxina nativa (Emerich, 2017).

1.6 **Via nociceptiva**

1.6.1 ***Dor e nocicepção***

Em 1986, a Associação Internacional para Estudo da Dor (IASP, *International Association for the Study of Pain*) definiu a dor como uma experiência sensorial e emocional desagradável associada a um dano tecidual real, potencial, ou descrita em termos de tais lesões. Portanto, a dor é uma experiência sensorial subjetiva que inclui o componente emocional e só pode ser seguramente determinada após ser relatada. Em modelos animais obviamente não ocorre o relato da dor e nem há como mensurar o componente emocional relacionado a ela, entretanto, os animais experimentais são capazes de exibir respostas comportamentais que possibilitam sua percepção por parte do experimentador. Por essas peculiaridades, em 1906, Sherrington propôs o termo *nocicepção* para modelos animais. Este termo foi definido como a percepção de um estímulo potencialmente danoso ao organismo, sem levar em consideração o componente emocional. Assim, o termo nocicepção é o que melhor substitui o termo dor para experimentação animal, pois abrange exclusivamente o componente fisiológico da via em questão.

1.6.2 ***Mecanismos de transmissão ascendente da dor***

Estímulos nocivos percebidos como dolorosos podem ser de três tipos: térmico, mecânico ou químico. Esses estímulos podem ativar nociceptores (Mcmahon & Koltzenburg, 1990) presentes nos terminais axonais não encapsulados de neurônios do gânglio da raiz dorsal (DRGs) ou do gânglio trigeminal. Essas terminações livres contendo nociceptores são amplamente distribuídas na pele ou em tecidos internos como vísceras, vasos sanguíneos,

superfícies articulares, dentre outros (Guyton & Hall, 2006), cujos corpos celulares estão localizados em gânglios trigeminais ou gânglios da raiz dorsal do nervo espinal.

Até que ocorra a percepção da dor como evento final, a transmissão do estímulo doloroso compreende 4 passos: 1) transdução: corresponde à ativação dos nociceptores, que se dá pelo reconhecimento de um estímulo potencialmente danoso ao organismo, gerando um potencial de ação; 2) transmissão: compreende uma série de eventos que possibilitam a passagem do potencial de ação gerado no nociceptor e condução ao sistema nervoso central (SNC); 3) modulação: envolve os mecanismos e vias responsáveis por suprimir ou facilitar a condução do estímulo, que então desencadeia a 4) percepção da dor (Porto, 2004).

Para que haja ativação da via nociceptiva é necessário que o potencial de ação percorra primeiramente os neurônios do gânglio da raiz dorsal. Estes neurônios podem originar quatro tipos de fibras, Aα, Aβ, Aδ e C, que exibem função, calibre e grau de mielinização distintos. As fibras Aα e Aβ são mielinizadas, de alto calibre, conduzem o estímulo rapidamente e são responsáveis pela transmissão de estímulos inócuos, embora possam contribuir indiretamente para a percepção da dor. A ativação dessas fibras pode levar a ativação de interneurônios inibitórios na medula, que por sua vez contribuem inibindo a passagem do potencial de ação pela medula, interferindo na percepção da dor (Kandel, 2000). As fibras Aδ são de médio calibre e pouco mielinizadas, sendo responsáveis pela rápida condução de estímulos nocivos e pela sensação de dor mais localizada e aguda. As fibras C são amielínicas e de pequeno calibre. Elas são responsáveis pela condução do estímulo nociceptivo de maneira mais lenta, gerando uma sensação de dor mais difusa e tardia (Julius e Basbaum, 2001). Cada uma das fibras citadas acima atinge diferentes regiões do corno da raiz dorsal, chamadas laminas, ativando diferentes grupos de neurônios na medula espinal (Dubin e Patapoutian, 2010). O corno da raiz dorsal da medula espinal é uma importante região de integração entre várias fibras aferentes com os neurônios do SNC (Kandel, 2000).

Resumidamente, a transmissão ascendente da dor ocorre quando os neurônios do gânglio da raiz dorsal transmitem o impulso nervoso para os neurônios do corno dorsal da medula espinal mediante a liberação de neurotransmissores, sendo o glutamato um dos neurotransmissores excitatórios mais importantes desta via. Após a ativação dos neurônios de segunda ordem na medula, esses ascendem contralateralmente pelo trato espinotalâmico conduzindo o potencial de ação até o tálamo. A partir daí os neurônios de terceira ordem são ativados e conduzem o impulso nervoso até o córtex somatossensorial, onde o estímulo é processado e interpretado (Figura 4) (Furst, 1999).

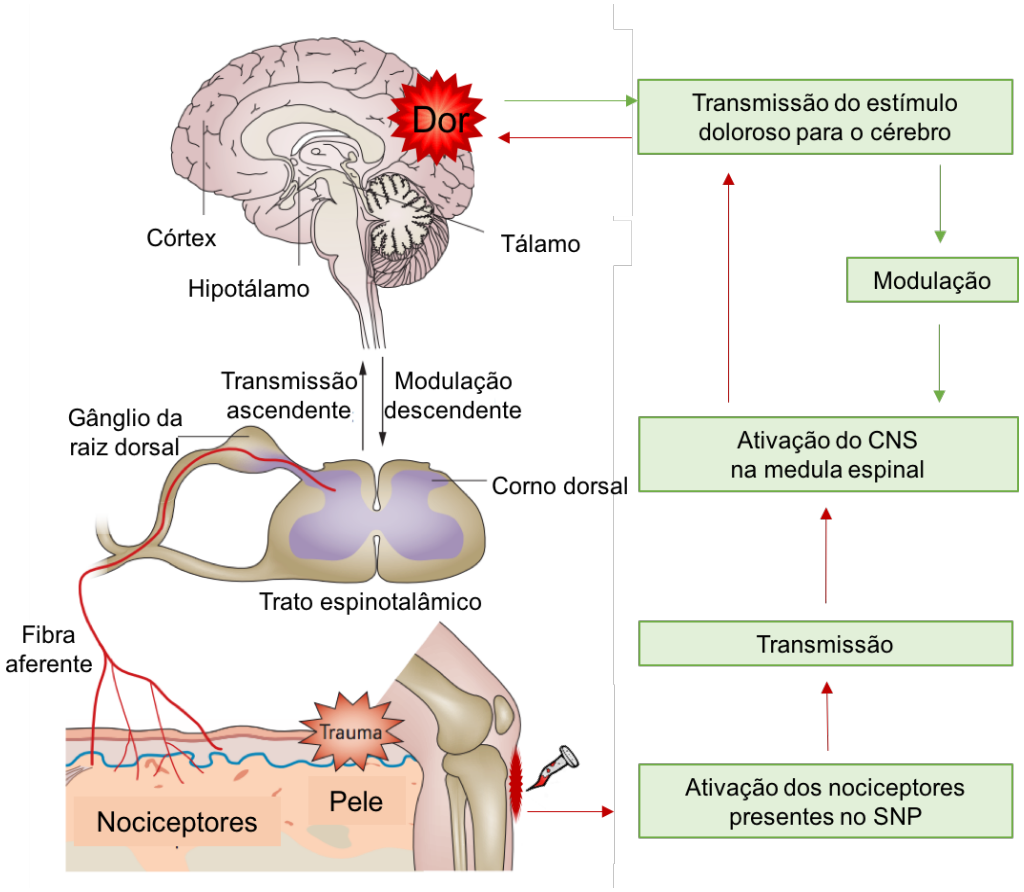


Figura 4: Transmissão do estímulo nociceptivo. A ativação dos nociceptores por estímulos nocivos gera um potencial de ação que é conduzido pelo neurônio do gânglio da raiz dorsal até corno dorsal da medula espinal. Do corno dorsal, os potenciais de ação são transportados ao longo da via de transmissão ascendente da dor através do trato espinotalâmico para o tálamo e o córtex. A via nociceptiva pode ainda ser sujeita a modulação pela via descentedente da dor, no qual sinais originados nos centros supraespinais podem modular a transmissão da dor a nível espinal. Abreviação: Sistema nervoso central (SNC); Sistema nervoso periférico **(**SNP) (Modificado de Bingham et al., 2009).

1.6.3 *Modulação do estímulo nociceptivo*

A transmissão do estímulo nocivo originado nos tecidos é modulada de diversas maneiras. Um dos mecanismos de modulação endógena é denominado "Teoria do portão da dor", e foi descrito originalmente em 1965 por Melzack e Wall. De acordo com essa teoria, a região da medula inervada pelas fibras aferentes contém interneurônios inibitórios, que são ativados pelas fibras Aβ (inibindo a transmissão do impulso nociceptivo) e inibidos pelas fibras Aδ e C (facilitando a transmissão do impulso nervoso que chega até a medula).

A modulação da condução do estímulo nociceptivo vai muito além da teoria do "portão da dor". Hoje em dia já se sabe que existem muitas influências supra-espinais no controle da dor, compondo o que é chamado de modulação descendente da dor. Várias estruturas supra-

espinais podem interferir com a sinalização na medula, estas estruturas são: tronco cerebral, hipotálamo, tálamo, córtex, núcleo magno da rafe, substância cinzenta periaquedutal e estruturas adjacentes da medula rostroventromedial. A ação coordenada de todas essas estruturas modula a sinalização da via nociceptiva através de projeções descendentes que atingem a medula espinal. Este sistema de modulação pode ter efeitos tanto anti- quanto pro-nociceptivos, portanto a transmissão da dor pode ser inibida ou facilitada, respectivamente (Figura 4) (Millan, 2002). Um exemplo clássico de modulação descente inibitória por uma estrutura supra-espinal foi a demonstração de que a estimulação elétrica da substância cinzenta periaquedutal (PAG) pode causar uma analgesia tão profunda, que permitiu que procedimentos cirúrgicos fossem realizados em ratos sem indução de nocicepção. Posteriormente, a estimulação da região da PAG (*Deep brain stimulation*) foi adotada como método para o tratamento de dor crônica em humanos (Reynolds et al., 1969; Duncan et al., 1991).

Vários neurotransmissores estão envolvidos com os processos de modulação endógena da via nociceptiva. As próximas sessões abordarão em detalhes a via dos opioides, canabinoides e óxido nítrico.

1.6.4 ***Via opioide***

Agonistas opioides endógenos estão entre os principais mediadores químicos envolvidos na modulação da via descendente inibitória. Estas moléculas são liberadas em diferentes regiões do SNC onde podem inibir a transmissão do estímulo nociceptivo (Argoff, 2011). Estruturas supra-espinais enviam projeções descentes para o corno dorsal da medula espinal, e modulam a atividade neuronial nestas regiões pela liberação de opioides, dentre outas moléculas (Budai e Fields, 1998). Entretanto, os receptores opioides também são altamente expressos no sistema nervoso periférico e em células não-neuroniais, e a ativação deles nestas regiões pode modular a transmissão do estímulo doloroso também por estes sistemas (Sehgal et al., 2011).

Os três principais tipos de receptores opioides são mu, delta e kappa, ainda existindo o receptor de nociceptina (também chamado de receptor para orfanina FQ) não muito explorado. A estrutura destes receptores compreende sete domínios transmembrana, o domínio N-terminal é extracelular, já a porção C-terminal é intracelular (Figura 5) (Law et al, 2000). Os 3 genes correspondentes aos receptores mu-, delta e kappa opioides possuem cerca de 50-70% de homologia. *Splicing* alternativo, modificações pós-traducionais e dimerização entre receptores podem gerar uma grande variação estrutural na população de receptores opioides, o que pode influenciar na farmacocinética dos seus respectivos agonistas (Waldhoer et al., 2004).

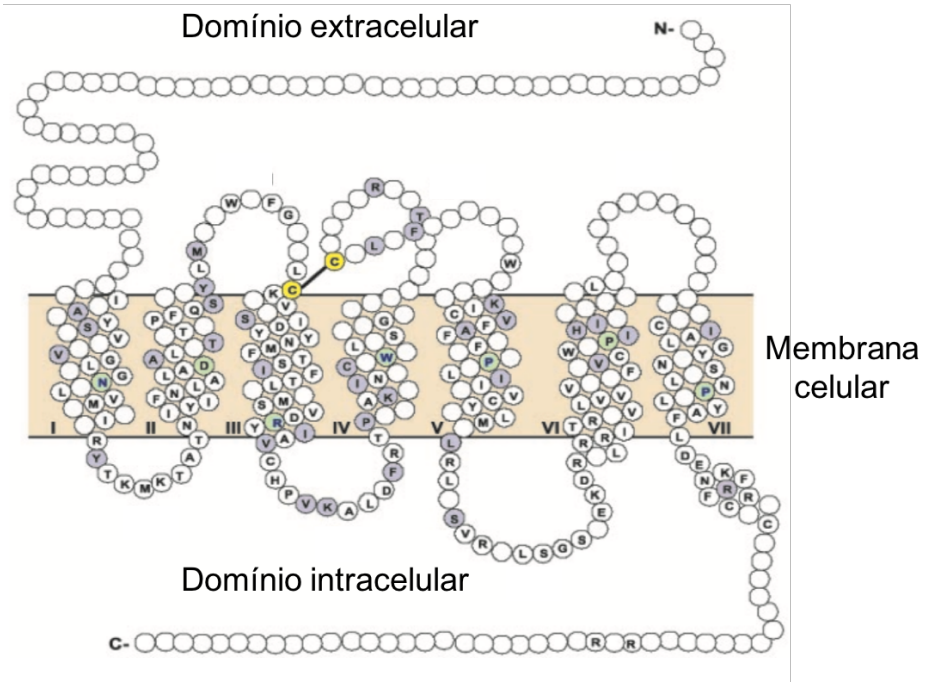


Figura 5: Estrutura dos receptores opioides. Os círculos brancos vazios representam aminoácidos não conservados entre os receptores mu, delta, kappa e o receptor de nociceptina. Os círculos brancos com letras representam os grupos de aminoácidos conservados entre os quatro receptores opioides citados acima. (Modificado de Waldhoer et al., 2004)

Dentre os principais opioides endógenos, são descritas as β-endorfinas, encefalinas, endomorfinas, dinorfinas e a orfanina FQ. As endomorfinas e β-endorfinas se ligam principalmente aos receptores mu-opioides e a encefalina tem maior afinidade pelos receptores delta-opioides. Por sua vez, as dinorfinas têm como principal alvo os receptores kappa-opioides e a orfanina FQ tem maior afinidade pelos receptores de nociceptina (Goldstein & Naidu, 1989; Meunier et al., 1995; Zaveri et al., 2001). Estes peptídeos endógenos são derivados principalmente de quatro precursores: a β-endorfina é derivada da pró-opiomelanocortina, a encefalina é sintetizada a partir da pró-encefalina, a dinorfina se origina da pró-dinorfina e a orfanina é derivada da pró-orfanina FQ. Ainda não foram identificados os precursores das endomorfinas, que são tetrapeptídeos com alta afinidade para receptores μ-opioides. Com exceção da orfanina FQ e das endomorfinas, todos os opioides endógenos citados possuem em sua estrutura primária a sequência TyrGlyGlyPheMet/Leu (YGGFM/L) (Waldhoer et al., 2004). Entretanto, somente a orfanina FQ possui o resíduo de aminoácido fenilalanina no seu N-terminal ao invés de tirosina, sendo que a tirosina nesta posição é importante para a afinidade entre os peptídeos opioides e os clássicos receptores opioides (Lapalu et al., 1997).

Os receptores opioides são pertencentes ao grupo de receptores acoplados a proteína G, e pertencem a subfamília G_i/G_o. Após a ativação dos receptores opioides por ligantes endógenos,

ou exógenos (morfina), ocorrem alterações conformacionais do receptor que permitem o desacloplamento do GDP da subunidade α e a ligação de GTP nesta subunidade do receptor. A partir daí a subunidade $G_{i\alpha}$ se dissocia da subunidade $\beta\gamma$. A subunidade $G_{i\alpha}$ vai inibir a atividade da adenilato ciclase, o que acarretará na diminuição dos níveis de monofosfato cíclico de adenosina (AMPc), e, portanto, redução da ativação da proteína cinase A (PKA) (Sharma et al., 1977). Por sua vez, a subunidade $\beta\gamma$ vai interagir diretamente com diferentes canais iônicos presentes na membrana celular, como os canais para cálcio voltagem dependentes e os canais para potássio do tipo GIRK (*G protein-gated inwardly rectifying potassium*) (Hescheler et al., 1987; North et al, 1987; Rhim e Miller, 1994; Tedford e Zamponi, 2006; Lüscher e Slesinger, 2010). Todos os três tipos de receptores opioides são capazes de levar à abertura dos canais para potássio do tipo GIRK e de induzir a inibição de diferentes isoformas dos canais para cálcio voltagem dependentes (Figura 6). A ativação de receptores opioides pode inibir também TRPV1 e ASICs (Endres-Becker et al, 2007; Cai et al., 2014). O resultado de toda essa cascata de sinalização é a hiperpolarização celular, com consequente diminuição da condução do estímulo nociceptivo e percepção da dor (Stein, 2016).

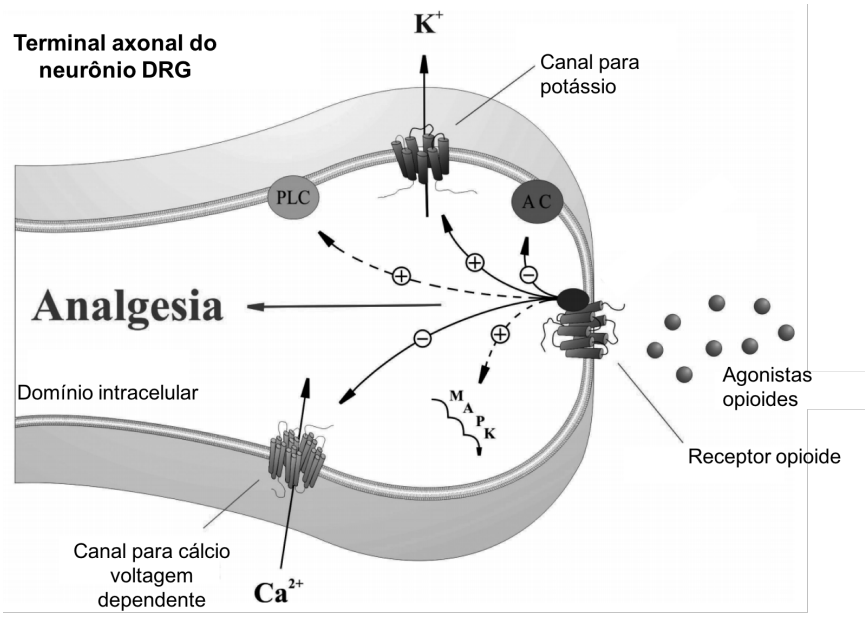


Figura 6: Sinalização via ativação de receptores opioides. A ativação dos receptores opioides por agonistas endógenos ou exógenos leva a ativação dos canais para potássio (GIRK), inibição dos canais para cálcio voltagem dependentes e inibição da adenilato ciclase (AC). A ativação destes receptores induz também a ativação indireta da fosfolipase C (PLC) e da cascata de sinalização das proteíno-cinases ativadas por mitógenos (MAPK). (Modificado de Kapitzke et al., 2005).

1.6.5 **Neprilisina (NEP)**

A neprilisina (EC 3.4.24.11) também conhecida como encefalinase, endopeptidase neutra e CD10, é uma glicoproteína integral de membrana tipo-II da família das zinco-metalopeptidases. Esta enzima foi primeiramente isolada da membrana do tecido renal de coelhos, sendo sua massa molecular equivalente a 90-100 kDa (Kerr e Kenny, 1974). A mesma possui cerca de 700 resíduos de aminoácidos, um pequeno domínio citoplasmático constituído pela porção N-terminal, uma região hidrofóbica transmembrana, e um extenso domínio extracelular que contém o sítio ativo da enzima (Devault et al., 1987).

A NEP está localizada na superfície celular de vários tecidos, e é responsável por catalisar a hidrólise de peptídeos na região extracelular. A enzima apresenta clara especificidade em clivar a porção N-terminal de peptídeos, sempre próximo a aminoácidos aromáticos e hidrofóbicos (Hersh e Morihara, 1986). A proteína apresenta dois domínios em sua estrutura que restringem o sítio ativo a pequenos substratos, desta maneira a enzima interage melhor com peptídeos pequenos, atuando como uma oligopeptidase (Oefner et al., 2000). Embora a NEP tenha sido primeiramente descrita como uma endopeptidase (Kerr e Kenny, 1974), estudos *in vitro* demonstraram que esta enzima apresenta preferencialmente atividade de carboxidipeptidase quando as duas situações são possíveis (Malfroy e Schwartz, 1982, 1985; Matsas et al., 1984)

Os primeiros substratos descobertos da NEP foram a encefalina e a substância P (Matsas et al., 1983), o que levou a uma busca pelo papel da NEP em modular sinais transmitidos por neuropeptídeos no cérebro. Posteriormente foi descrito que esta enzima é responsável por hidrolisar vários peptídeos que possuem função fisiológica importante, como o peptídeo natriurético atrial, bradicinina e peptídeo β-amilóide (Roques et al., 1993). A partir daí o papel da NEP tem sido estudado em importantes condições patológicas e fisiológicas, como, por exemplo, na hipertensão arterial (Molinaro et al., 2002), na doença de Alzheimer (Iwata et al., 2000, 2001) e em processos de analgesia (Wisner et al., 2006). Além disso, a neprilisina tem sido utilizada como marcador biológico para leucemia linfóide aguda (Letarte et al., 1988), e sua detecção em células do endométrio tem sido proposta como ferramenta para diagnóstico de endometriose (Groisman e Meir, 2003). Recentemente, foram desenvolvidos inibidores específicos da NEP para tratamento de transtornos sexuais femininos (dificuldade de excitação) (Pryde et al., 2006).

Interessantemente, foram identificados dois peptídeos endógenos que atuam como inibidores da neprilisina *in vivo*, um foi denominado sialorfina (Rougeot et al., 2003) para modelos murinos, e o seu homólogo em humanos foi denominado opiorfina (Wisner et al.,

2006). Já existem muitas evidências demonstrando que tanto a sialorfina, quanto a opiorfina, são importantes moléculas para a modulação da função sexual em mamíferos e da via nociceptiva.

O papel destes inibidores endogénos na função sexual tem sido bastante estudado recentemente. Já foi demonstrado, por exemplo, que nas glândulas submandibulares de ratos machos são expressos cerca de 1000 vezes mais sialorfina do que nas fêmeas, o que representa uma especificidade de gênero para a expressão da molécula (Rosinski-Chupin et al., 2001). Além disso, quando a sialorfina foi administrada também em ratos machos, os mesmos se mostraram muito mais susceptíveis ao acasalamento (Messaoudi et al., 2004). A expressão dos genes correspondentes a sialorfina em ratos e a opiorfina em humanos é baixa em indivíduos com disfunção erétil. Ainda, a administração de plasmídeos contendo genes correspondentes a sialorfina foi capaz de melhorar a função erétil nos animais testados (Tong et al., 2006, 2007; Davies et al., 2007).

Na via nociceptiva, os inibidores endógenos da neprilisina fazem com que uma quantidade maior do opioide encefalina permaneça circulante nos tecidos, uma vez que a encefalina é hidrolisada pela neprilisina (também chamada encefalinase). A encefalina desempenha um importante papel no controle da percepção da dor ao se ligar e ativar receptores μ- e δ-opioides. Já foi demonstrado que a sialorfina induz antinocicepção (Rougeot et al., 2003) e que a opiorfina efetivamente previne a hidrolise de met-encefalina pela neprilisina em modelos *in vitro* (Wisner et al., 2006). A opiorfina é capaz de induzir analgesia via ativação de receptores opioides (μ-opioide), mas não induz tolerância como a morfina (agonista μ-opioide) (Rougeot et al., 2010; Popik et al., 2010).

Endopeptidases que hidrolisam neuropeptídeos e possuem propriedades enzimáticas características da neprilisina expressa em humanos são encontradas em vários outros grupos: moluscos (Zappulla et al., 1999), insetos (Isaac et al., 1988), nematódeos (Sajid et al., 1995) e anelídeos (Laurent e Salzet, 1995). Em todos esses grupos os neuropeptídeos estão envolvidos em processos fisiológicos e de comportamento. Comparadas a NEP de mamíferos, essas enzimas encontradas nestes grupos são pouco caracterizadas, porém já existem dados experimentais suficientes para concluir que muitas das propriedades enzimáticas da NEP foram conservadas ao longo do curso da evolução (Turner et al., 2001), o que reitera o importante papel fisiológico da enzima neprilisina para os organismos vivos.

1.6.6 *Via canabinoide*

O sistema endocanabinoide é constituído por diferentes receptores, ligantes endógenos e enzimas reponsáveis pela síntese e degradação das moléculas que atuam como agonistas desse sistema. Os componentes desta via são expressos quase que de maneira ubíqua na via nociceptiva. Desta forma, a ativação desta via pode regular a transmissão do estímulo nociceptivo em diferentes regiões (sistema nervoso periférico, medula e estruturas supraespinais), e também pode influenciar tanto na transmissão ascendente, quanto na via inibitória descendente da dor (Woodhams et al., 2017).

O primeiro agonista canabinoide indentificado foi isolado da planta *Cannabis sativa* e foi denominado Δ^9-tetrahidrocanabinol (THC) (Figura 7A) (Gaoni e Mechoulam, 1964). A descoberta deste agonista na década de 60 impulsionou a descoberta, mais tarde, dos receptores canabinoides CB_1 e CB_2 (Matsuda et al., 1990; Munro e Abu-Shaar, 1993). Além da descoberta dos receptores canabinoides na década de 90, foram descritos também os dois endocanabinoides de maior relevância e mais estudados até hoje, são eles a *N*-araquidonoil etanolamina (anandamida) (Figura 7B) e o 2-araquidonoil glicerol (2-AG) (Figura 7C) (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). Hoje em dia já se sabe que alguns endocanabinoides, além de atuarem como agonistas dos clássicos receptores canabinoides, podem também ativar/inibir diferentes canais iônicos, como canais para cálcio, potássio, 5-HT_3, α7-nACh, TRPV1, TRPV4, dentre outros (Oz, 2006).

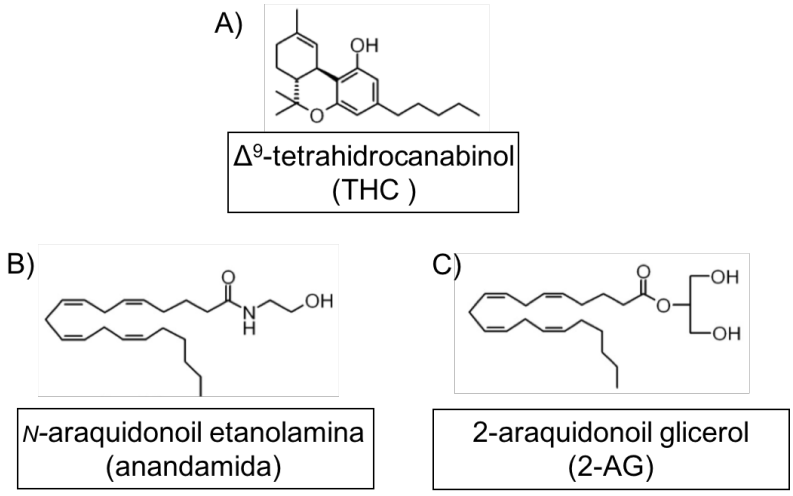


Figura 7: Estrutura química dos agonistas canabinoides. (A) Δ^9-tetrahidrocanabinol, agonista exógeno; (B) *N*-araquidonoil etanolamina, agonista endógeno; (C) 2-araquidonoil glicerol, agonista endógeno (Modificado de Howlett et al., 2002)

Os endocanabinoides são produzidos sobre demanda, sendo sua síntese estimulada pelo aumento do Ca^{2+} intracelular no terminais pós-sinápticos em resposta a estimulação sináptica contínua. Tanto a anandamida, quanto o 2-AG, são sintetizados a partir da hidrólise de precursores derivados de fosfolipídeos. A biossíntese da anandamida é catalisada pela enzima N-acil fosfatidiletanolamida fosfolipase D (NAPE-PLD) e ocorre pela hidrólise do precursor lipídico *N*-araquidonoil fosfatidiletanolamida (NArPE) (Figura 8) (Di Marzo et al., 1994). A forma majoritária que 2-AG é produzido no sistema nervoso central é via conversão de fosfatidilinositol 4,5-bifosfato (PIP_2) em diacilglicerol (DAG) pela enzima fosfolipase C-β (PLCβ). A partir daí a síntese de 2-AG é catalisada pela enzima diacilglicerol lipase (DAGL) e se dá pela clivagem do DAG (Figura 8) (Murataeva et al., 2014).

Atualmente, novas estratégias para o tratamento da dor têm sido desenvolvidas no sentido que, com o tratamento adequado, haja a estimulação do sistema canabinoide sem que haja necessidade da administração de canabinoides exógenos. Desta forma, a administração de fármacos que inibam a degradação de endocanabinoides tem sido empregada. As enzimas responsáveis pela degradação de anandamida e 2-AG são a amida hidrolase de ácido graxo (FAAH) e a monoacilglicerol lipase (MGL), respectivamente (Figura 8) (Cravatt et al., 1996; Dinh et al., 2004). A administração de tais inibidores tem se mostrado eficaz para o tratamento da dor em diferentes modelos (Woodhams et al., 2017). Além da inibição da degradação dos endocanabinoides, uma outra maneira utilizada farmacologicamente para se aumentar o nível destes compostos na fenda sináptica e induzir antinocicepção é a administratação de compostos que inibam a recaptação dos mesmos (de Lago et al., 2004).

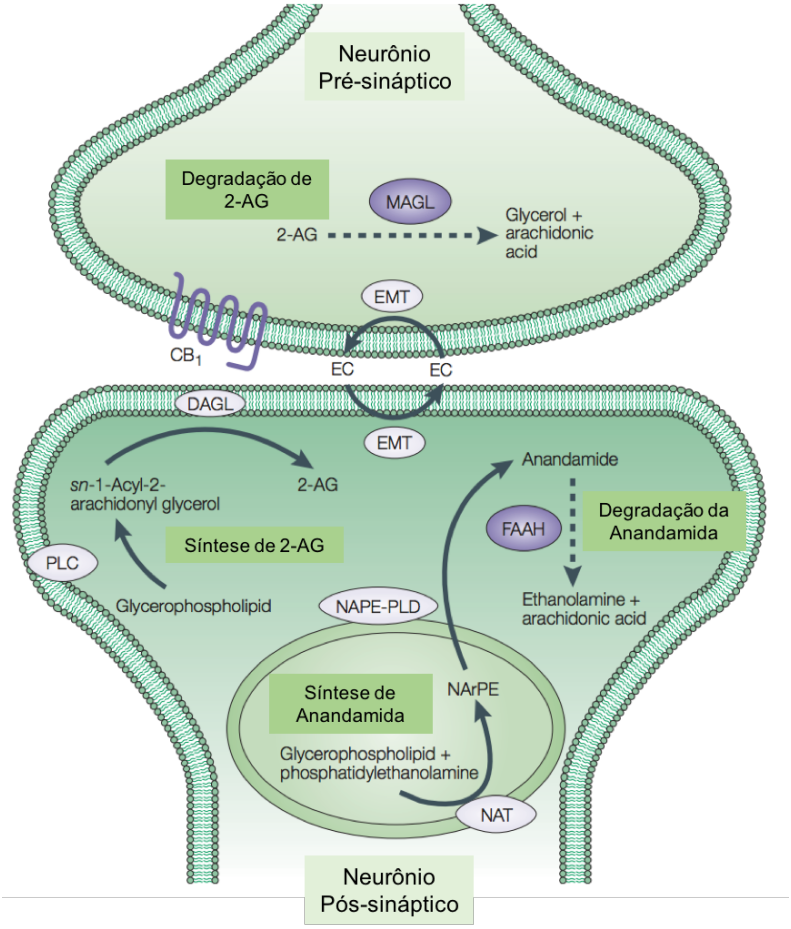


Figura 8: Síntese e degradação dos endocanabinoides (EC) anandamida e 2-AG. A formação do precurssor da anandamida, *N*-araquidonoil fosfatidiletanolamida (NArPE), é catalisada pela enzima N-aciltransferase (NAT), sendo que a síntese da anandamida se dá pela hidrólise da NArPE pela enzima N-acil fosfatidiletanolamida fosfolipase D (NAPE-PLD). A degradação da anandamida é catalisada pela enzima amida hidrolase de ácido graxo (FAAH). Esta enzima é mais encontrada nos neurônios pós-sinápticos, o que indica que a anandamida possivelmente age majoritariamente nos receptores canabinoides encontrados nesta região. Para a produção de 2-AG, a enzima fosfolipase C (PLC) converte glicerofosfolipídeos em sn-1-acyl-2- arachidonoyl-glycerol (DAG). O DAG é então clivado pela enzima diacilglicerol lipase (DAGL), originando o endocanabinoide 2-AG. A síntese de 2-AG ocorre majoritariamente nos neurônios pós-sinápticos, enquanto a sua degradação ocorre nos neurônios pré-sinápticos pela ação da enzima monoacilglicerol lipase (MGL). EMT: transportador de endocanabinoides (Modificado de Di Marzo et al., 2004).

Os receptores canabinoides, assim como os receptores opioides, também fazem parte do grupo de receptores acoplados a proteína $G_{i/o}$. O receptor canabinoide CB_1 é um dos receptores acoplados a proteína G mais expressos no sistema nervoso central, sendo encontrado no neocortex, hipocampo, gânglio basal, cerebelo, tronco encefálico e medula espinal. Este receptor também é expresso no sistema nervoso periférico (neurônios DRG) e em tecidos não neuroniais, como no endotélio vascular e no baço (Herkenham et al., 1990; Hohmann et al.,

1999; Kendall et al., 2016). Os receptores do tipo CB_2, apesar de serem expressos também no SNC, são encontrados predominantemente nas células e tecidos do sistema imune, mediando processos inflamatórios (Mackie, 2006).

A cascata de sinalização desencadea pela ativação dos receptores canabinoides se assemelha, de maneira geral, àquela gerada pela ativação dos receptores opioides. Portanto, a ativação de receptores canabinoides leva à inibição da atividade da adenilato ciclase, reduzindo assim os níveis de AMPc (Rhee et al., 1998). Ainda, seguindo a via de sinalização, o influxo de cálcio é inibido devido ao bloqueio de diferentes tipos de canais para cálcio (Mackie e Hille, 1992; Gebremedhin et al., 1999). Diferentemente dos canais para cálcio, a ativação de receptores canabinoides induz ativação de determinados canais para potássio (Mackie et al., 1995), e também interfere na cascata de sinalização das proteíno-cinases ativadas por mitógenos (MAPK) (Wartmann et al., 1995). Desta forma, a ativação dos receptores canabinoides contribue para a hiperpolarização celular e bloqueio da transmissão do estímulo nociceptivo (Figura 9).

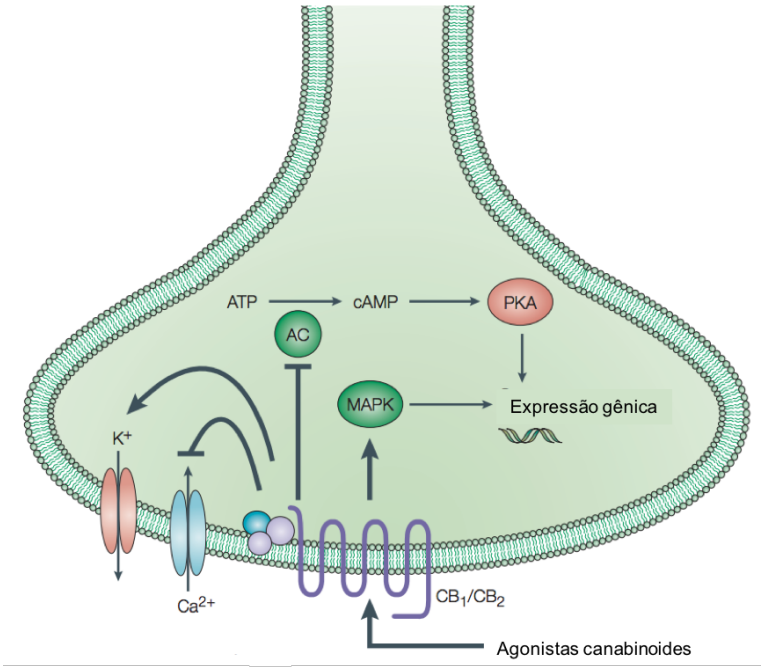


Figura 9: Sinalização via ativação de receptores canabinoides. A ativação dos receptores canabinoides CB_1 e CB_2 por agonistas endógenos ou exógenos leva a inibição da adenilato ciclase (AC) e dos canais para cálcio voltagem dependentes. A ativação destes receptores modula de maneira positiva os canais para potássio e a cascata de sinalização das proteíno-cinases ativadas por mitógenos (MAPK), portanto, fazendo com que estes dois componentes sejam ativados (Modificado de Di Marzo et al., 2004).

1.6.7 *Via do óxido nítrico*

O óxido nítrico está envolvido em inúmeros processos fisiológicos no SNC e SNP (Schuman et al., 1994). Esta molécula é um produto da catálise da L-arginina a L-citrulina efetuada pela enzima óxido nítrico sintase (NOS). A enzima NOS possui três isoformas, a neuronial (nNOS), a endotelial (eNOS) e a induzida (iNOS) (Alderton et al., 2001).

A isoforma neuronial da enzima foi a primeira a ser descoberta e é predominante em tecidos neuroniais, sendo o óxido nítrico atuante em processos de neurotransmissão. A óxido nítrico sintase endotelial modula a regulação da pressão sanguínea e é bastante encontrada em células endoteliais (Dominiczak e Bohr, 1995). Estas duas isoformas dependem do complexo cálcio-calmodulina para sua ativação. Já a isoforma denominada iNOS é mais amplamente encontrada em células do sistema imune, como macrófagos e células da glia. Esta isoforma, diferente das outras duas, não depende do complexo cálcio-calmodulina para sua ativação (Jacobs et al., 1997). Diferentemente do que se acreditava antigamente, a expressão de todas as três isoformas pode ser induzida por diferentes estímulos ou ser expressa constitutivamente em diferentes células e tecidos (Weiner et al., 1994; Lundberg et al., 1995; Geller e Billiar, 1998; Forstermann et al., 1998).

O papel do oxido nítrico na via nociceptiva é bastante discutido, visto que esta molécula pode desencadear desde hiperalgesia até analgesia dependendo das circunstâncias (Miclescu e Gordh, 2009). Este efeito dual do óxido nítrico tem sido atribuído a diversos fatores, dentre eles, a sua concentração nos tecidos. Baixos níveis de NO agindo localmente causam um efeito antinociceptivo, já em altas concentrações, como em processos tardios de inflamação, pode induzir efeito hiperalgésico (Figura 10) (Duarte et al., 1990; Aley et al., 1998).

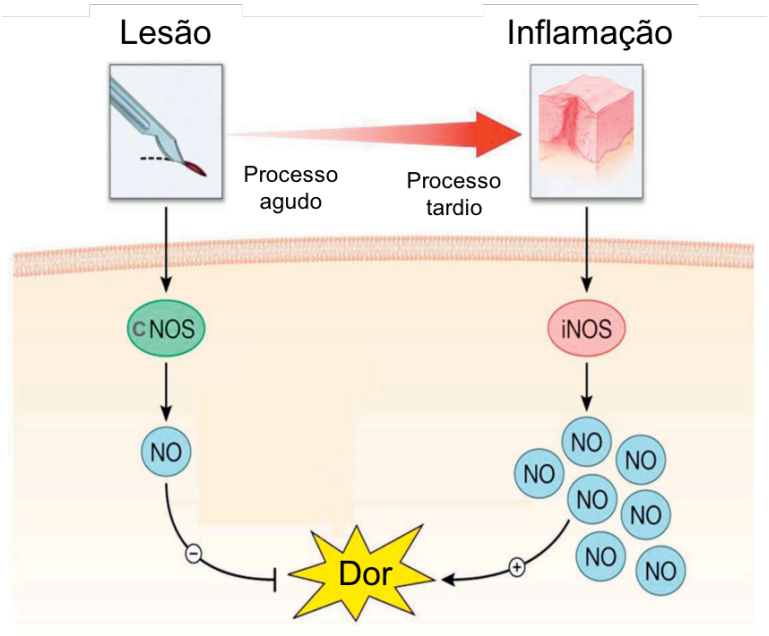
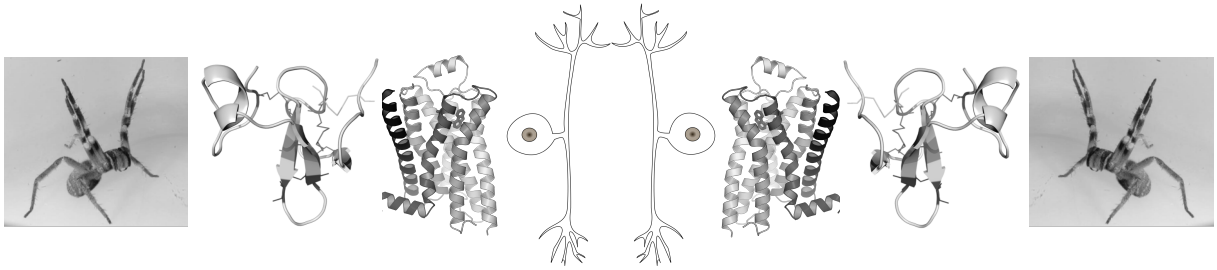


Figura 10: Efeito dual do óxido nítrico na via nociceptiva. A lesão tecidual, ou outro estímulo nocivo, pode provocar um aumento na produção de NO, possivelmente via ativação de eNOS e/ou nNOS, na fase inicial. Desta forma, isso acarretará num aumento relativamente pequeno nos níveis teciduais de NO, desencadeando um efeito antinociceptivo. No entanto, na fase tardia, a isoforma iNOS pode estar superexpressa, o que poderá acarretar num aumento acentuado dos níveis de NO nos tecidos, o que induziria um efeito hiperalgésico. cNOS: eNOS e/ou nNOS (Modificado de Hamza et al., 2010).

Em 1990 foi demonstrado o efeito antinociceptivo do NO no SNP por Duarte e colaboradores. A administração de nitroprussiato de sódio (NPS), um doador de NO, foi capaz de induzir efeito antinociceptivo de maneira dose-dependente frente a hiperalgesia causada por prostaglandina E_2 (PGE_2). A sinalização pelo NO se dá pela estimulação da guanilato ciclase, o que aumenta os níveis de monofosfato cíclico de guanosina (GMPc). Neste estudo, a administração de um inibidor da guanilato ciclase bloqueou o efeito antinociceptivo induzido pelo óxido nítrico, demonstrando que a antinocicepção ocasionada por esta molécula é mediada pelo aumento dos níveis de GMPc (Duarte et al., 1990). Uma vez que os níveis de GMPc estão aumentados, essa molécula pode se ligar e ativar a proteína cinase G (PKG). A PKG ativada poderá então estimular a abertura de canais para potássio, especificamente, o canal para potássio sensível ao ATP (K_{ATP}) (Knowles et al., 1989; Han et al., 2001; Han et al., 2002). Dentre os canais para potássio voltagem dependentes, os ativados por cálcio e os sensíveis ao ATP, somente o último (K_{ATP}) está envolvido na antinocicepção induzida por NO (Soares et al., 2000). Dessa forma, a cascata de sinalização induzida por NO na via nociceptiva pode induzir a hiperpolarização celular, tornando as células menos susceptíveis a despolarização, e, portanto, interrompendo a transmissão do estímulo nociceptivo.

A ativação do sistema nitrérgico tem sido demonstrada como um dos mecanismos responsáveis pela antinocicepção desencadeada por várias substâncias distintas, tais como a noradrenalina, angiotensina-(1–7), xilazina, ketamina, agonistas de receptores μ-, δ- e κ-opioides e agonistas dos receptores canabinoides CB_1 e CB_2 (Ferreira et al., 1991; Amarante and Duarte, 2002; Pacheco et al., 2005; Reis et al., 2009; Romero and Duarte, 2009; Romero et al., 2011a; Romero et al., 2012; Costa et al., 2014).

Justificativa



2 JUSTIFICATIVA

Milhões de pessoas no mundo sofrem de dor crônica ou aguda, o que faz com que a dor seja considerada um problema de saúde global com altos custos associados (Phillips, 2009). A maior parte dos pacientes que buscam auxílio médico descrevem a sensação de dor como a principal causa pela busca de atendimento (Droes, 2003). Desse modo, a via nociceptiva é alvo de muitas pesquisas que visam elucidar os mecanismos de transmissão e percepção envolvidos na dor.

Pesquisas nesta área são de grande relevância para os profissionais da saúde e para a população em geral, visto que, muitos fármacos disponíveis para uso clínico não são eficazes para todos os quadros de dor e, comumente, estão associados a reações adversas, o que pode levar à suspensão da terapia. Portanto, nota-se a grande importância dos estudos envolvendo novos compostos que possam ser utilizados clinicamente no tratamento da dor, sendo mais eficazes, de menor custo e com menos efeitos adversos.

Devido a grande diversidade de toxinas animais que desencadeiam diferentes respostas fisiológicas, o número de estudos focados em toxinas provenientes da peçonha de animais, que representam potenciais modelos para fármacos, vem crescendo bastante. As toxinas ganharam esse foco graças à alta especificidade ao se ligarem aos seus alvos biológicos e também, muitas vezes, a alta afinidade pelos mesmos (Rajendra et al., 2004).

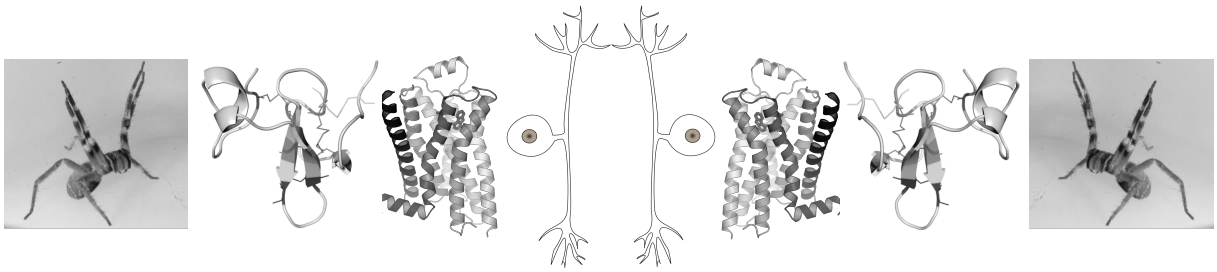
Atualmente, algumas toxinas, ou seus derivados, são utilizadas para o tratamento de doenças ou nas pesquisas sobre os mecanismos de ação das mesmas. Um exemplo da aplicação de derivados de toxinas para o tratamento de patologias é a utilização do analgésico não-opioide Prialt® (Ziconotide) para o tratamento de dor crônica. Este fármaco é um derivado sintético da toxina ω-conotoxina MVIIA, isolada da peçonha do gastrópode marinho *Conus magus*. A utilização do Prialt foi aprovada pelo FDA *(Food and Drug Administration)* em 2004, e seu mecanismo de ação consiste no bloqueio seletivo dos canais para cálcio do tipo N (Mcgivern, 2007). Apesar de todos os aspectos positivos a cerca da utilização deste fármaco para o tratamento clínico da dor, sua administração ainda é restrita por via i.t. e a utilização deste medicamento pode induzir uma série de efeitos adversos, como tontura, cefalgia, náusea e até mesmo induzir alguns distúrbios psiquiátricos, como depressão e propensão ao suicídio (Maier *et al.*, 2011).

Toxinas que apresentam atividade antinociceptiva já foram encontradas em uma grande variedade de grupos de animais peçonhentos, dentre eles os cnidários, gastrópodes, aranhas, escorpiões, abelhas, vespas, formigas, cobras e quilópodes (Rajendra *et al.*, 2004; Gazerani e

Cairns, 2014). Sendo assim, estudos com toxinas animais buscando-se o desenvolvimento de novos fármacos que atuem na via nociceptiva é bastante promissor, uma vez que pesquisas nessa área podem levar ao desenvolvimento de fármacos com maior especificidade, menos efeitos adversos, e até mesmo podem levar ao descobrimento de novos alvos farmacológicos para o tratamento da dor.

Tendo em vista que o peptídeo sintético PnPP-19 vêm sendo estudado como um candidato a modelo de fármaco para o tratamento da disfunção erétil, fez-se necessário estudá-lo quanto a sua ação na modulação da via nociceptiva, dentre outros, para se verificar possíveis reações adversas indesejáveis. PnTx2-6, a toxina nativa que deu origem ao peptído PnPP-19, apresentou, em estudos anteriores, ação hiperalgésica periférica e sistêmica em baixas concentrações (Nunes, 2008). Além disso, é importante ressaltar que o peptídeo PnPP-19 induz a liberação de L-glutamato em sinaptosomas (Silva, 2012), sendo que o glutamato é o principal neurotransmissor excitatório do SNC de mamíferos (Collingridge e Lester, 1989). Dessa forma, consideramos de grande importância que o papel do peptídeo PnPP-19 fosse investigado, sobretudo, na via nociceptiva.

Objetivos



3 OBJETIVOS

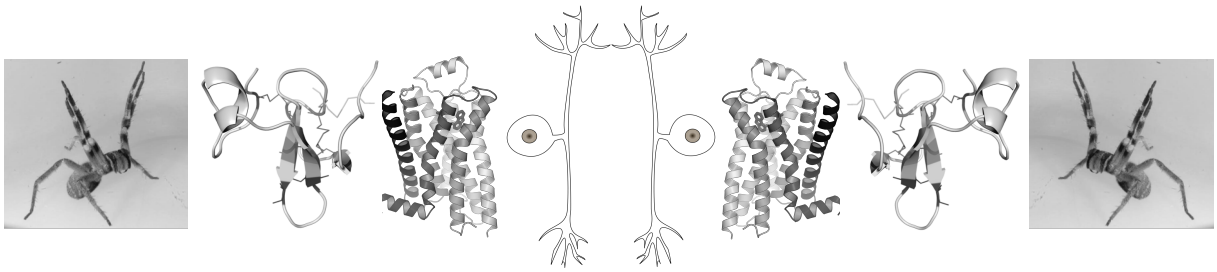
3.1 Objetivo Geral

Este trabalho teve como objetivo principal investigar a possível atividade central e periférica do peptídeo sintético PnPP-19 na via nociceptiva, bem como elucidar os mecanismos de ação envolvidos.

3.2 Objetivos Específicos

a) Verificar, por estudos de bioinformática, a possível similaridade "funcional" do peptídeo PnPP-19 com outras moléculas já conhecidas;
b) Detectar a possível modulação da via nociceptiva no SNC mediada pelo peptídeo PnPP-19;
c) Detectar a possível modulação da via nociceptiva no SNP mediada pelo peptídeo PnPP-19;
d) Estabelecer os parâmetros experimentais de curvas de tempo e de doses para o peptídeo PnPP-19;
e) Investigar a possível participação da via dos opioides na ação central e periférica do peptídeo PnPP-19;
f) Verificar a possível participação da via dos canabinóides na ação central e periférica do peptídeo PnPP-19;
g) Identificar a possível participação do sistema nitrérgico na ação periférica do peptídeo PnPP-19;
h) Elucidar a possível interação do peptídeo com a enzima neprilisina;
i) Verificar se PnPP-19 pode modular o influxo de cálcio em neurônios DRG;
j) Propor um modelo para o mecanismo de ação do peptídeo PnPP-19.

Resultados



4 Resultados

Os artigos a seguir apresentam os resultados obtidos até o presente com o peptídeo PnPP-19 na via nociceptiva.

4.1 Artigo I

A spider derived peptide, PnPP-19, induces central antinociception mediated by opioid and cannabinoid systems

Neste artigo foi investigado o papel do peptídeo sintético PnPP-19 sobre a via nociceptiva quando administrado diretamente no SNC (via i.c.v.). Após estabelecidas as doses com potencial antinociceptivo e o pico de ação do PnPP-19, a participação dos sistemas opioide e canabinoide na indução deste efeito foram avaliados. Verificou-se que a antinocicepção induzida por PnPP-19 é mediada pela ativação dos receptores canabinoides CB_1 e dos receptores μ- e δ-opioides.

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RESEARCH Open Access

A spider derived peptide, PnPP-19, induces central antinociception mediated by opioid and cannabinoid systems

Daniela da Fonseca Pacheco[1], Ana Cristina Nogueira Freitas[2], Adriano Monteiro C. Pimenta[2], Igor Dimitri Gama Duarte[1] and Maria Elena de Lima[2*]

Abstract

Background: Some peptides purified from the venom of the spider *Phoneutria nigriventer* have been identified as potential sources of drugs for pain treatment. In this study, we characterized the antinociceptive effect of the peptide PnPP-19 on the central nervous system and investigated the possible involvement of opioid and cannabinoid systems in its action mechanism.

Methods: Nociceptive threshold to thermal stimulation was measured according to the tail-flick test in Swiss mice. All drugs were administered by the intracerebroventricular route.

Results: PnPP-19 induced central antinociception in mice in the doses of 0.5 and 1 μg. The non-selective opioid receptor antagonist naloxone (2.5 and 5 μg), μ-opioid receptor antagonist clocinnamox (2 and 4 μg), δ-opioid receptor antagonist naltrindole (6 and 12 μg) and CB_1 receptor antagonist AM251 (2 and 4 μg) partially inhibited the antinociceptive effect of PnPP-19 (1 μg). Additionally, the anandamide amidase inhibitor MAFP (0.2 μg), the anandamide uptake inhibitor VDM11 (4 μg) and the aminopeptidase inhibitor bestatin (20 μg) significantly enhanced the antinociception induced by a low dose of PnPP-19 (0.5 μg). In contrast, the κ-opioid receptor antagonist nor-binaltorphimine (10 μg and 20 μg) and the CB_2 receptor antagonist AM630 (2 and 4 μg) do not appear to be involved in this effect.

Conclusions: PnPP-19-induced central antinociception involves the activation of CB_1 cannabinoid, μ- and δ-opioid receptors. Mobilization of endogenous opioids and cannabinoids might be required for the activation of those receptors, since inhibitors of endogenous substances potentiate the effect of PnPP-19. Our results contribute to elucidating the action of the peptide PnPP-19 in the antinociceptive pathway.

Keywords: Peptide PnPP-19, Central antinociception, *Phoneutria nigriventer*, μ-opioid receptor, δ-opioid receptor, CB1 receptor, CB2 receptor

Background

PnPP-19 is a synthetic peptide that contains 19 amino acid residues [1]. It represents a part of the primary structure of the native toxin PnTx2-6, also known as δ-ctenitoxin-Pn2a [2], which was isolated from the venom of the spider *Phoneutria nigriventer* [3]. Some peptides purified from the venom of this spider have been identified as potential sources of drugs for pain treatment. For example, PnTx3-3, renamed ω-ctenitoxin-Pn2a [2], showed an antinociceptive effect in different models of neuropathic pain [4]. Additionally, Phα1β neurotoxin, another toxin isolated from that same venom, induced antinociception in models of neuropathic and inflammatory pain [5].

Cannabinoids and opioids are two separate groups of psychoactive drugs that exhibit several similar pharmacological effects, including analgesia, sedation, hypothermia and inhibition of motor activity [6–8]. In recent years, our group has demonstrated the involvement of endogenous

* Correspondence: lima.mariaelena@gmail.com; melenalima@icb.ufmg.br
[2]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Av. Antônio Carlos, 6627, Belo Horizonte, MG CEP 31.270.901, Brazil
Full list of author information is available at the end of the article

opioids and cannabinoids in the antinociceptive action of several substances [9, 10]. Receptors for both drugs are coupled to similar intracellular signaling mechanisms and the interaction between cannabinoid and opioid systems in the nociceptive pathway has been the focus of much attention [9, 11–15].

Interestingly, it has been shown that endogenous opioids are involved in antinociception induced by a scorpion toxin [16]. Therefore, it is hypothesized that pain relief induced by alpha- or beta- scorpion toxins may implicate the activation of an endogenous opioid system. The analgesic effect of those toxins might be partially due to the activation of diffuse noxious inhibitory controls of supra-spinal origin, which are linked to a counter-irritation phenomenon and release of endogenous opioids [16]. Thus, opioid peptides may be involved in the action mechanism of other toxins, particularly toxins from other arthropods, such as the spider *Phoneutria nigriventer*.

Recently we have shown that PnPP-19 induces antinociception in the peripheral nervous system [17]. We suggested that this effect is attributable to an inhibition of the neutral endopeptidase (neprilysin), which may lead to an increase of enkephalin levels and may cause activation of both μ- and δ-opioid receptors. In addition, we showed evidence that the receptor CB1 is implicated in the antinociceptive effect induced by PnPP-19.

Given the lack of information concerning the antinociceptive effect of PnPP-19 on the central nervous system (CNS), the aim of the present study was to determine the possible effect of this peptide on the CNS and investigate whether there is an involvement of the cannabinoid and opioid systems.

Methods

Animals

The experiments were performed on 25–30 g male Swiss mice ($n = 4$ per group) provided by the CEBIO ("Centro de Bioterismo", the Animal Center) of the Universidade Federal de Minas Gerais (UFMG). The mice were housed in a temperature-controlled room (23 ± 1 °C) on an automatic 12-h light/dark cycle (06:00–18:00 h of light phase). All tests were carried out during the light phase (08:00–15:00 h). Food and water were freely available until the onset of the experiments. The algesimetric protocol was approved by the Committee for Ethics in Animal Experimentation (CETEA) of UFMG, with the protocol number 131/2014.

Algesimetric method

The tail-flick test used in the present study was conducted in accordance with the procedure described by D'Amour and Smith [18] with a slight modification. The test consists of restraining the mouse by one of the experimenter's hands and positioning the distal portion of the mouse's tail (about 2 cm from the tip of the tail) under a helical nickel-chrome resistance. When the device is turned on, an electric current starts to flow through the resistance, which may lead to a rise of its temperature. In addition, the moment that the equipment is turned on, a timer is activated. The time required for the animal to perceive the nociceptive stimulus and execute the tail withdrawal reflex is measured and expressed in seconds. The intensity of the heat reached by the resistance was adjusted, so the baseline latencies required to observe the withdrawal reflex of the mouse's tail were between 3 and 4 s (the thermal stimulus applied increased from 0.297 calories/s). To avoid tissue damage, the cutoff time was established at 9 s [19]. The baseline latency was obtained for each animal before drug administration (zero time) by calculating an average of three consecutive trials. To reduce stress, mice were habituated to the apparatus one day prior to conducting the experiments.

Intracerebroventricular injection (i.c.v.)

Animals were constrained by an acrylic tube-shaped device (Insight, Brazil). To facilitate the i.c.v. injection, the animals were placed inside this tube, which immobilizes their body, except for their head. With one hand, the experimenter restrained the animal's head and then injected the drugs into its right lateral ventricle, by the intracerebroventricular route, using a Hamilton syringe of 5 μL. The site of injection was 1 mm from either side of the midline of a line drawn through the anterior base of the ears (modified from Haley and McCormick, [20]). The syringe was inserted perpendicularly through the skull into the brain at the depth of 2 mm, and 2 μL of solution was injected. To determine whether drugs were injected correctly into the brain ventricular system, they were diluted in a solution containing Evans blue 0.5%. Once the experiment was finished, the animals were euthanized with an overdose of anesthesia and their brains were sectioned for confirmation of the side of injection.

Experimental protocol

All drugs were i.c.v. administered into the right lateral ventricle. Naloxone, clocinnamox, naltrindole, nor-binaltorphimine, AM251, AM630, MAFP, VDM11 and bestatin were administered 1 min prior to administration of PnPP-19. The protocol to determine the best moment for the injection of each substance was assessed in pilot experiments and literature data [10, 15].

The nociceptive threshold was always represented by the time, in seconds, required for the animal to exhibit the tail withdrawal reflex. The measurements were

performed before any drug administration and after 5, 10, 15 and 30 min after drug injection.

Statistical analysis

Data were analyzed statistically by Repeated Measures ANOVA with post-hoc Bonferroni's test for multiple comparisons. Probabilities less than 5% ($p < 0.05$) were considered to be statistically significant.

Chemicals

The following drugs and chemicals were used: PnPP-19 (synthesized by China Peptides, China), the opioid receptor antagonist naloxone (Sigma, USA), the μ-opioid receptor antagonist clocinnamox (Tocris, USA), the δ-opioid receptor antagonist naltrindole (Tocris, USA), the κ-opioid receptor antagonist nor-binaltorphimine (Sigma, USA), the aminopeptidase inhibitor bestatin (Sigma, USA), AM251 [N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide; Tocris, USA], AM630 {6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl(4-ethoxyphenyl) methanone; Tocris, USA}, MAFP [(5*Z*,8*Z*,11*Z*,14*Z*)-5,8,11,14-eicosatetraenyl-methyl ester phosphonofluoridic acid, Tocris, USA] and VDM11 [(5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-Hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide, Tocris, USA].

The drugs were dissolved as follows: PnPP-19 (saline), naloxone (saline), clocinnamox (saline), naltrindole (saline), nor-binaltorphimine (saline), bestatin (saline), AM251 and AM630 (12% DMSO in saline), MAFP (10% DMSO in saline), VDM11 (10% in saline) and injected at a volume of 2 μL into the lateral ventricle. The saline used for dilution of all drugs contained 0.5% Evans Blue.

Results

Antinociceptive effect of PnPP-19

Since the peptide PnPP-19 is known to induce peripheral antinociception, we decided to investigate whether it could also interact with the central nervous system and induce antinociception mediated by activation of central signaling related to the nociceptive pathway [17]. Firstly, PnPP-19 was injected intracerebroventricularly. Then it was observed that the doses of 0.5 and 1 μg/per animal induced a significant delay of the tail withdraw reflex of the mice. This result may indicate that at those doses, PnPP-19 leads to an antinociceptive response in a dose-dependent manner (Fig. 1). The dose of 0.25 μg/per animal was ineffective whereas the control group of mice injected only with vehicle (saline) remained unaltered. The dose of 1 μg was chosen for the following experiments in the present study since it had almost reached the cutoff time of the test (9 s).

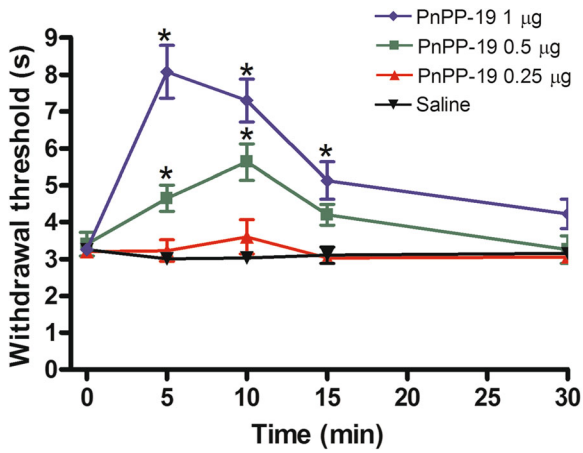

Fig. 1 Central antinociception induced by intracerebroventricular administration of PnPP-19 in mice. PnPP-19 (0.25, 0.5 and 1 μg) was administered 5 min prior measurement in the tail-flick test. Each line represents the mean ± SEM for four mice per group. *Significant difference compared to the Saline-injected group (ANOVA + Bonferroni test, $p < 0.05$). Saline (0.5% of Evans Blue)

Antagonism of PnPP-19-induced antinociception by naloxone, clocinnamox, naltrindole and AM251

To investigate whether opioid or cannabinoid receptors were involved in PnPP-19-induced antinociception, the peptide was co-administered with non-specific and specific opioid antagonists and also with specific cannabinoid antagonists. As shown in Fig. 2, the intracerebroventricular administration of naloxone (2.5 and 5 μg) (Fig. 2a), clocinnamox (2 and 4 μg) (Fig. 2b), naltrindole (6 and 12 μg) (Fig. 2c) and AM251 (2 and 4 μg) (Fig. 2d) partially inhibited the antinociceptive response induced by 1 μg of PnPP-19. Taken together, these data suggest the participation of μ- and δ-opioid receptors and the CB_1 cannabinoid receptor in the effect elicited by the peptide. The highest effective dose of the antagonists did not significantly modify the nociceptive threshold in control groups (Fig. 2a, b, c and d).

Effect of nor-binaltorphimine and AM630 on PnPP19-induced antinociception

The intracerebroventricular administration of nor-binaltorphimine (10 and 20 μg) and AM630 (2 and 4 μg) did not block the central antinociception of PnPP-19 (1 μg; Fig. 3a and b), suggesting that the activation of either κ-opioid receptors or CB_2 cannabinoid receptors does not contribute to the peptide's effect on the central nociceptive pathway. These drugs did not significantly modify the nociceptive threshold in control groups.

Increase of PnPP-19-induced antinociception by bestatin, MAFP and VDM11

Because PnPP-19 induces activation of both opioid and cannabinoid receptors, we used the aminopeptidase

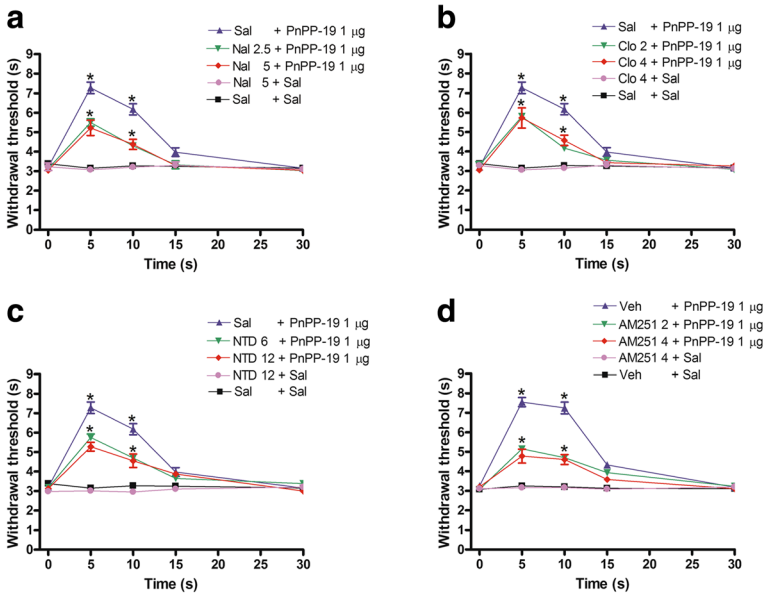

Fig. 2 Partial antagonism induced by intracerebroventricular administration of **a** naloxone, **b** clocinnamox, **c** naltrindole or **d** AM251 in the central antinociception induced by PnPP-19. Naloxone (Nal; 2.5 and 5 µg), clocinnamox (Clo; 2 and 4 µg), naltrindole (NTD; 6 and 12 µg) or AM251 (2 and 4 µg) was administered 1 min prior to PnPP-19 injection (1 µg). These antagonists did not significantly modify the nociceptive threshold in the control group. Each line represents the mean ± SEM for four mice per group. *Significant difference compared to the control group (ANOVA + Bonferroni's test). Sal: saline (0.5% of Evans Blue); Veh: vehicle (20% DMSO in saline 0.5% of Evans Blue)

inhibitor bestatin, the anandamide amidase inhibitor MAFP and the anandamide uptake inhibitor VDM11 to verify the possible involvement of the endogenous opioid and cannabinoid systems on PnPP-19-induced antinociception.

In this experiment the PnPP-19 dose of 0.5 µg, instead of 1 µg, was employed to allow the observation of the potentiation effect that the selected inhibitors could induce. Therefore, at this time the ability of the aforementioned inhibitors to potentiate a lower dose of PnPP-19 (0.5 µg) was tested. Bestatin (20 µg, Fig. 4a), MAFP (0.2 µg, Fig. 4b) and VDM11 (4 µg, Fig. 4c) enhanced the antinociception induced by a low dose of PnPP-19 (0.5 µg). No significant modification of the nociceptive threshold was observed when bestatin, MAFP, VDM11 or vehicle were injected alone.

Discussion

Spider venoms have been used as a potential source of new compounds with specific pharmacological properties. Some peptides extracted from the venom of the spider *Phoneutria nigriventer* have been suggested as potential sources of drugs for pain treatment. For instance, PnTx3-3 (ω ctenitoxin Pn2a) and Phα1β induce an antinociceptive effect in neuropathic pain models [4, 5]. More recently, we have shown that the synthetic peptide, PnPP-19, firstly characterized as a potentiator of erectile function, also produces antinociception in rats when peripherally injected [1, 17]. We also showed that this peripheral effect involves inhibition of neutral endopeptidase (NEP) (EC 3.4.24.11), and activation of CB_1, μ- and δ-opioid receptors [17]. Therefore, the next issue to be investigated was whether PnPP-19 presents a possible central activity on nociception.

Our results demonstrate a dose-dependent central antinociceptive effect induced by PnPP-19 in the tail-flick test and reinforce the role of PnPP-19 as an analgesic drug candidate. We also investigated the possible participation of opioids and cannabinoids in the PnPP-19-induced central antinociception. In recent years, our group has shown the relationship between opioid and cannabinoid systems, as well as their involvement in the central and peripheral action mechanisms of different substances [9, 10, 15, 21, 22].

Interestingly, it was demonstrated that some animal toxins induce antinociception by activation of the opioid system. The analgesic effects of the neurotoxin from the king cobra's venom (*Ophiophagus hannah*), the crude venom of the snake *Micrurus lemniscatus* and two scorpion toxins, AmmVIII (*Androctonus mauritanicus mauritanicus*) and LqqIT2 (*Leiurus quinquestriatus quinquestriatus*), were antagonized by administration of the opioid receptor antagonist naloxone [16, 23, 24]. Given the aforementioned information about the participation of opioids as at least part of the action mechanism of some toxins, and especially considering our previous results with PnPP-19 on peripheral nervous system, our experiments showed that naloxone partially inhibits the central antinociception induced by PnPP-19. As observed with a higher dose, a

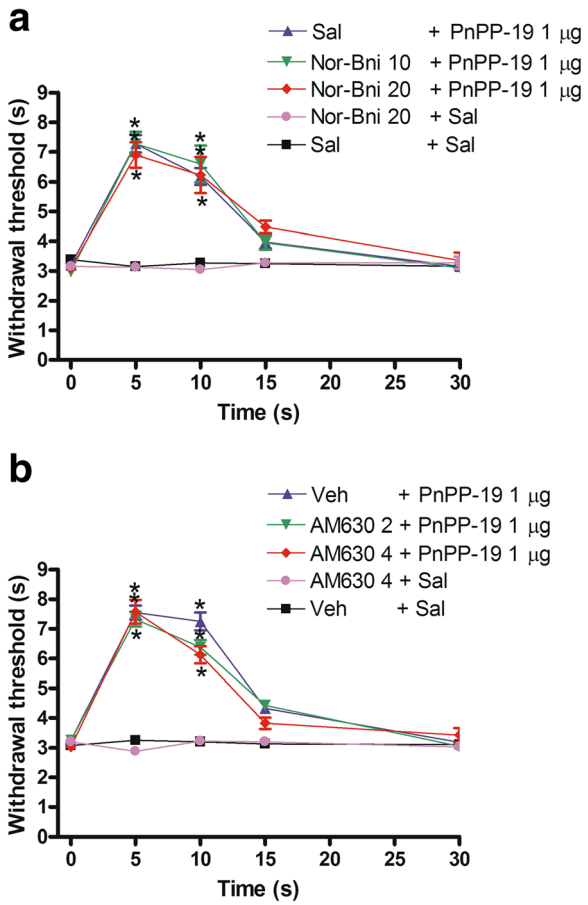

Fig. 3 Intracerebroventricular administration of **a** nor-binaltorphimine or **b** AM630 on the central antinociception produced by PnPP-19. Nor-binaltorphimine (Nor-Bni; 10 and 20 μg) or AM630 (2 and 4 μg) was administered 1 min prior to PnPP-19 (1 μg) injection. These antagonists did not significantly modify the nociceptive threshold in the control group. Each line represents the mean ± SEM for four mice per group. *Significant difference compared to Sal + Sal or Veh + Sal injected groups (ANOVA + Bonferroni's test). Sal: saline (0.5% of Evans Blue); Veh: vehicle (20% DMSO in saline 0.5% of Evans Blue)

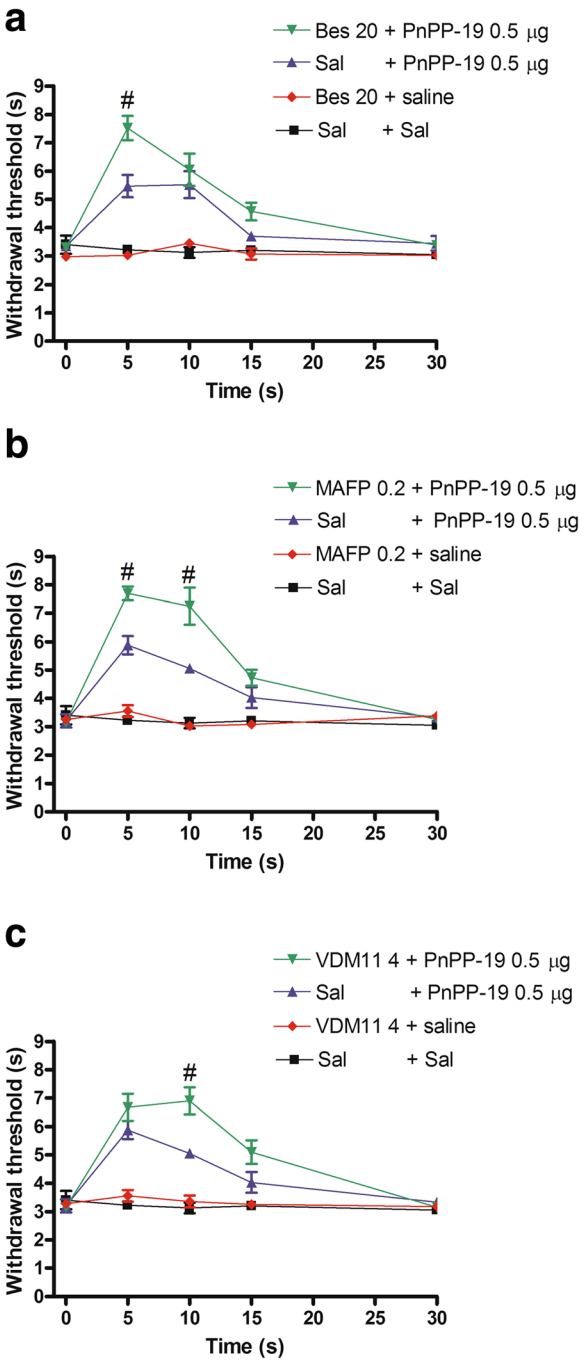

Fig. 4 Potentiation of PnPP-19-induced antinociception by **a** bestatin, **b** MAFP or **c** VDM11. The bestatin (Bes; 20 μg), MAFP (0.2 μg) or VDM11 (4 μg) was administered 1 min prior to PnPP-19 (0.5 μg) injection. These drugs administered alone did not induce any effect. Each line represents the mean ± SEM for four mice per group. #Significant difference compared to the Sal + PnPP-19-injected group (ANOVA + Bonferroni' test; $p < 0.05$). Sal: saline (0.5% of Evans Blue)

complete antagonism was not observed. This is the first report of opioid participation in the central antinociceptive mechanism of peptides derived from toxins purified from *Phoneutria nigriventer* venom.

Since naloxone interacts with μ-, κ- and δ-opioid receptors, highly selective antagonists were used to clarify which receptor subtype would be involved in the central antinociceptive effect of PnPP-19. Clocinnamox is an irreversible μ-opioid receptor antagonist with K_i values of 0.7, 1.9 and 5.7 nM for mouse μ-, δ- and κ-opioid receptors, respectively [25]. Naltrindole has 223- and 346-fold greater activity for δ- than for μ- and κ-opioid receptors, whereas nor-binaltorphimine shows 27- to 29-fold less potency, respectively, for μ and δ binding sites compared with κ receptors binding sites [26, 27].

Our results showed that clocinnamox and naltrindole, but not nor-binaltorphimine, partially antagonized PnPP-19-induced central antinociception, suggesting

the participation of μ- and δ-opioid receptors in this effect, which is in accordance with previous findings on the peripheral nervous system [17]. In contrast, κ-opioid receptors appear to be involved in the antinociception induced by the crude venom of the snake *Micrurus lemniscatus* and the potent analgesic peptide isolated from the venom of the South American rattlesnake *Crotalus durissus terrificus*, crotalphine [24, 28]. The antinociception of crotalphine was blocked by pretreatment with selective antagonist of κ opioid receptors [28], an effect not observed in the present study when we tried to inhibit the antinociception of PnPP-19 by administration of a selective antagonist of the same opioid receptor.

In relation to opioid signaling, we applied the strategy of increasing the opioidergic system potency through opioid peptide catabolism inhibition. We observed that the administration of the aminopeptidase inhibitor bestatin significantly enhanced the central antinociception produced by a low dose of PnPP-19, providing evidence of the involvement of endogenous opioids in this effect. In vivo, enkephalins appear to be degraded by enzymes such as neutral endopeptidase and aminopeptidase [29]. Other opioid peptides, such as endorphin and dynorphin, appear to be resistant to neutral endopeptidase catabolism and, to a lesser extent, aminopeptidase [30].

Several studies have demonstrated reciprocal interactions between opioid and cannabinoid systems, suggesting a common underlying mechanism. For example, the cannabinoid Δ^9-THC produces an increase in morphine antinociception by inducing the release of the endogenous opioid dynorphin [13]. On the other hand, the administration of the CB_1 receptor antagonist AM251 inhibited morphine-induced antinociception [9, 15]. The synergy in the analgesic effects of these compounds is attributed to a crosstalk between these two signaling pathways mediated by simultaneous activation of opioid and cannabinoid receptors [31].

Recently, it was shown that peripheral interactions between cannabinoid and opioid systems contribute to the antinociceptive effect of the peptide crotalphine [32]. These authors demonstrated that crotalphine-induced antinociception stimulates local release of dynorphin A, which is dependent on CB_2 receptor activation [32]. In contrast, we observed the participation of the CB_1 receptor in PnPP-19-induced central antinociception, and as previously reported, μ and δ opioids receptors are also involved [17].

It has been suggested that CB_1 and μ- and δ-opioid receptors form heterodimers [33]. These structures are necessary for the functioning of certain G-protein-coupled receptors, such as the $GABA_B$ receptor [34]. A previous study demonstrated the important role for the heterodimer CB_1-δ in neuropathic pain where cortical functions of δ opioid receptors were altered [35]. On the other hand, μ opioid receptors and CB_1 receptors form a functional heterodimer and may transmit a signal through a common G protein mechanism [36].

As a consequence of this work, the identification of the endocannabinoid involved in pain modulation was assessed indirectly by administration of pharmacological agents that regulate uptake or degradation of anandamide. This endocannabinoid is an agonist of CB_1 and CB_2 receptors, but presents greater affinity for the former [37, 38]. The results demonstrated that the anandamide amidase inhibitor MAFP and anandamide uptake inhibitor VDM11 increase the central antinociception produced by a low dose of PnPP-19, suggesting the release of endocannabinoids and subsequent activation of CB_1 receptors.

Conclusions

In conclusion, our results show that PnPP-19 induces antinociception via the central nervous system and suggest that this effect is associated with the activation of μ–, δ – opioid and CB_1 cannabinoid receptors. The release of endogenous opioids and endocannabinoids that might be acting on these receptors appears to be involved in the antinociceptive mechanism of the peptide. The results of this work contribute to elucidating the central antinociceptive effect of PnPP-19; however, more research is required to elucidate the interaction between opioid and cannabinoid systems in this effect.

In summary, our data together with the results obtained in the peripheral nervous system [17] show that PnPP-19 has a broad antinociceptive effect and thus constitutes a potential lead compound for the development of novel analgesic drugs.

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Authors' contributions

ACNF and DFP performed the research, analyzed data and wrote the first draft of the manuscript. AMP, IDGD and MEL designed research, contributed with essential reagents or tools and reviewed the paper. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval

The algesimetric protocol was approved by the Committee for Ethics in Animal Experimentation (CETEA) of UFMG, under the protocol number 131/2014.

Author details
[1]Departmento de Farmacologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, MG, Brazil. [2]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Av. Antônio Carlos, 6627, Belo Horizonte, MG CEP 31.270.901, Brazil.

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4.2 Artigo II

PnPP-19, a spider toxin peptide, induces peripheral antinociception through opioid and cannabinoid receptors and inhibition of neutral endopeptidase

Uma vez que o efeito antinociceptivo do PnPP-19 foi caracterizado no SNC, este estudo foi focado em estudar o efeito deste peptídeo no SNP. A investigação da participação da via dos opioides e canabinoides na indução do efeito antinociceptivo por PnPP-19 também foi realizada neste estudo. Adicionalmente, por métodos de bioinformática, a enzima neprilisina (endopeptidase neutra) foi apontada como um possível alvo farmacológico do peptídeo. Portanto, experimentos afim de se investigar o potencial do PnPP-19 em inibir esta enzima foram conduzidos. Os resultados mostraram que PnPP-19 inibe a enzima neprilisina. Além disso, mostrou-se que a ação antinociceptiva periférica do peptídeo envolve a ativação dos receptores canabinoides CB_1 e dos receptores μ- e δ-opioides.

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RESEARCH PAPER

PnPP-19, a spider toxin peptide, induces peripheral antinociception through opioid and cannabinoid receptors and inhibition of neutral endopeptidase

Correspondence Maria E. de Lima, Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. E-mail: melenalima@icb.ufmg.br

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A C N Freitas[1], D F Pacheco[1,2], M F M Machado[3], A K Carmona[3], I D G Duarte[2] and M E de Lima[1]

[1]Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, [2]Departamento de Farmacologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil, and [3]Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, SP, Brazil

BACKGROUND AND PURPOSE

The synthetic peptide PnPP-19 has been studied as a new drug candidate to treat erectile dysfunction. However, PnTx2–6, the spider toxin from which the peptide was designed, induces hyperalgesia. Therefore, we intended to investigate the role of PnPP-19 in the nociceptive pathway.

EXPERIMENTAL APPROACH

Nociceptive thresholds were measured by paw pressure test. PnPP-19 was administered intraplantarly alone or with selective cannabinoid or opioid receptor antagonists. The hydrolysis of PnPP-19 by neutral endopeptidase (NEP) (EC 3.4.24.11), an enzyme that cleaves enkephalin, was monitored by HPLC and the cleavage sites were deduced by LC–MS. Inhibition by PnPP-19 and Leu-enkephalin of NEP enzyme activity was determined spectrofluorimetrically.

KEY RESULTS

PnPP-19 (5, 10 and 20 μg per paw) induced peripheral antinociception in rats. Specific antagonists of μ opioid receptors (clocinnamox), δ opioid receptors (naltrindole) and CB_1 receptors (AM251) partly inhibited the antinociceptive effect of PnPP-19. Inhibition of fatty acid amide hydrolase by MAFP or of anandamide uptake by VDM11 enhanced PnPP-19-induced antinociception. NEP cleaved PnPP-19 only after a long incubation, and K_i values of 35.6 ± 1.4 and 14.6 ± 0.44 $\mu mol \cdot L^{-1}$ were determined for PnPP-19 and Leu-enkephalin respectively as inhibitors of NEP activity.

CONCLUSIONS AND IMPLICATIONS

Antinociception induced by PnPP-19 appears to involve the inhibition of NEP and activation of CB_1, μ and δ opioid receptors. Our data provide a greater understanding of the antinociceptive effects of PnPP-19. This peptide could be useful as a new antinociceptive drug candidate.

Abbreviations

K_i, inhibitory constant; NEP, neutral endopeptidase

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Tables of Links

TARGETS
GPCRs[a]
CB_1 receptors
CB_2 receptors
δ receptors
κ receptors
μ receptors
Enzymes[b]
FAAH, fatty acid amide hydrolase
NEP, neutral endopeptidase

LIGANDS
AM251
AM630
MAFP
Naltrindole
Nor-binaltorphimine
Naloxone
PGE_2

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander *et al.*, 2015a,b).

Introduction

Animal venoms have been used as sources of new compounds with specific pharmacological properties, constituting potential tools for neurobiological studies and also potential new drug candidates. Particularly, the venom of the Brazilian armed spider *Phoneutria nigriventer* contains a large range of peptide toxins, which have several activities in biological systems. Toxins from this venom have been described to be acting on many different targets, such as sodium, calcium and potassium voltage-gated ion channels (Matavel *et al.*, 2002; de Lima *et al.*, 2015; Silva *et al.*, 2015). Some of these toxins are also able to induce an increase in vascular permeability (Marangoni *et al.*, 1993) and potentiate penile erection (Nunes *et al.*, 2008) Furthermore, other venom components can inhibit glutamate uptake by brain synaptosomes (Mafra *et al.*, 1999), while others inhibit NMDA-evoked currents in rat hippocampal neurons (Figueiredo *et al.*, 2001).

Interestingly, two toxins isolated from the venom of *P. nigriventer* have been suggested as potential drug sources for pain treatment. These toxins, PnTx3–3 and PnTx3–6, inhibit voltage-activated calcium channels and induce antinociceptive effect (Souza *et al.*, 2008; Dalmolin *et al.*, 2011). However, to the best of our knowledge, none of the toxins from the venom of this spider have been assessed for analgesic activity due to activation of opioid or cannabinoid receptors.

Recently, our group has shown the involvement of the opioid and endocannabinoid systems in the mechanism of action of different substances, such as ketamine and xylazine (Romero *et al.*, 2013b; Pacheco *et al.*, 2014). There are two types of cannabinoid receptors, CB_1 receptors that are expressed primarily in central and peripheral neurons and CB_2 receptors mainly found in immune cells (see Pertwee, 2006). For the opioid receptors, there are three types, μ, δ and κ receptors, and all of them are expressed in both central and peripheral nervous system (Peng *et al.*, 2012). In addition, our group has demonstrated that the opioidergic and endocannabinoidergic systems are strongly linked and the activation of one pathway is mediated by the other (Pacheco *et al.*, 2008; Pacheco *et al.*, 2009; Reis *et al.*, 2009).

The toxin PnTx2–6 was isolated from PhTx2 fraction of the *P. nigriventer* venom (Cordeiro *et al.*, 1992), and induces a different range of biological effects from those of PnTx3–3 and PnTx3–6. The toxin PnTx2–6 causes neuronal depolarization by slowing down the inactivation of Na^+ channels (Matavel *et al.*, 2002), and it also induces penile erection (Nunes *et al.*, 2008). When this toxin was injected s.c. into the rat hind paw (0.57 nmol per paw) and the nociceptive threshold measured by the paw pressure test (Randall and Selitto, 1957), it induced hyperalgesia in both the toxin-treated and the saline-injected hind paw. For this reason, the authors concluded that this toxin, PnTx2–6, had a systemic nociceptive effect, even when administered at low doses (K P Nunes, unpublished data).

The peptide PnPP-19 represents a discontinuous epitope of the primary structure of the toxin PnTx2–6 and it was proposed as the most likely region of the toxin to interact with its molecular target, the sodium channel (Silva *et al.*, 2015). Our group has synthesized the peptide PnPP-19 and has shown that, similar to the native toxin PnTx2–6, PnPP-19 potentiates erectile function in rats. However, it no longer acts on any sodium channel subtypes (Silva *et al.*, 2015).

Given the potential use of PnPP-19 as a drug to treat erectile dysfunction and the lack of information concerning its effect in the nociceptive pathway, the present work aimed to determine the effects of PnPP-19 on nociception. Our results have shown that the peptide exhibited antinociceptive activity, mediated by activation of both opioid and cannabinoid receptors in the peripheral nervous system. We also demonstrated the involvement of the endocannabinoid system, because inhibitors of anandamide metabolism, by fatty acid amide hydrolase (FAAH), and of its uptake potentiated the antinociceptive effect induced by the peptide. In addition, PnPP-19 inhibited the neutral endopeptidase (NEP) (EC 3.4.24.11), which is responsible for the cleavage of many endogenous peptides, among them, the opioid enkephalin (see Roques *et al.*, 1993).

Methods

Animals

All animal care and experimental protocols were approved by the local Ethics Committee on Animal Experimentation (CETEA) of UFMG (Protocol number: 131/2014) and are reported in accordance with ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015). Efforts were made to minimize suffering and reduce the number of animals used in the experiments.

Male Wistar rats (170–220 g) provided by the CEBIO (The Animal Centre) of Universidade Federal de Minas Gerais (UFMG) were used in the experiments. The rats were housed in groups of a maximum of four animals per cage at a temperature-controlled room (23 ± 1°C) on an automatic 12 h light/dark cycle (06:00–18:00 h of light phase). All testing was carried out during the light phase (08:00–15:00 h). Food and water were freely available until the onset of the experiments. In this work, all the tested groups comprised 4 animals and a total of 152 animals were used to provide all the data.

Algesimetric method

Rats were injected with PGE_2 (2 μg) in the plantar surface (s.c.) of the right hind paw and measured by the paw-pressure test described by Randall and Selitto (1957). An analgesimeter (Ugo-Basile, Italy) with a cone-shaped paw presser with a rounded tip was used to apply a linearly increasing force to the hind paw. The weight in grams required to elicit the nociceptive response, paw withdrawal, was determined as the nociceptive threshold. A cut-off value of 300 g was used to prevent damage to the paws. The nociceptive threshold was measured in the right paw and determined by the average of three consecutive trials recorded before (zero time) and 3 h after PGE_2 injection (peak of effect). The results were calculated by the difference between these two averages (Δ of nociceptive threshold) and expressed as grams. To reduce stress, the rats were habituated to the apparatus for one day prior to the experiments.

Experimental protocol

Dose–response curves were obtained by injecting the peptide PnPP-19 (50 μL) 3 h after local administration of PGE_2 (100 μL) into the hind paw and the nociceptive response was measured every 5 min, for 30 min. In the protocol used to determine whether the drug was acting outside the injected paw, PGE_2 (100 μL) was injected into both hind paws (left and right), while PnPP-19 (50 μL) was administered into the right paw; only in this experiment, were both right and left paws assessed. After determination of the dose and the peak of action of PnPP-19, the next experiments were carried out using the injection of the peptide, concomitantly with opioid or cannabinoid antagonists as follows: PnPP-19 (50 μL) was administered s.c. in the right hind paw 2:55 h after local injection of PGE_2 (100 μL). Naloxone, clocinnamox, naltrindole or nor-BNI (50 μL) was intraplantarly injected into the right hind paw, 35 min prior to the measurement of hyperalgesia (3 h). AM251, AM630, MAFP and VDM11 (50 μL) were intraplantarly injected 15 min prior to the measurement of hyperalgesia (3 h). The nociceptive threshold was always measured in the right hind paw. This protocol was assessed in pilot experiments and published data was used to determine the dose and optimal time point for the injection of each substance (Pacheco *et al.*, 2008; Reis *et al.*, 2009; Galdino *et al.*, 2014; Veloso *et al.*, 2014).

Hydrolysis of PnPP-19 by NEP

A recombinant soluble form of human NEP was prepared as previously described (Lemay *et al.*, 1989; Fossiez *et al.*, 1992), and it was kindly donated by Dr. Guy Boileau from the University of Montreal (Montreal, Canada). PnPP-19 (20 $\mu mol \cdot L^{-1}$) was incubated with recombinant NEP (0.2875 $nmol \cdot L^{-1}$) in Tris–HCl (25 $mmol \cdot L^{-1}$) buffer containing 0.1 $mol \cdot L^{-1}$ NaCl, pH 7.0, 37°C. Aliquots from the incubated solutions were taken at appropriate time points (15 min, 30 min, 1 h and overnight), and the reaction was stopped in 5% *v*/v TFA (trifluoroacetic acid) solution. The samples were analysed by HPLC (Shimadzu CBM-20 A) with UV detection at 220 and 280 nm, using RP-18E column [C18-Hewlett Packard (Palo Alto, CA, USA)]. The column was eluted with a two-solvent system: solvent A, TFA/H_2O (1:1000, *v*/v) and solvent B, TFA/acetonitrile/H_2O (1:900:100, *v*/v/v), at a flow rate of 1 $mL \cdot min^{-1}$ with 10–80% gradient of solvent B over 20 min.

Determination of PnPP-19 cleavage sites

For the determination of PnPP-19 cleavage sites, a HPLC system connected to a MS detector (LC/MS), model LCMS 2010 EV (Shimadzu Inc, Nakagyo-ku, Kyoto, Japan) was used. Electrospray ionization probe was used for data analysis. Fragments resulting from NEP hydrolysis of PnPP-19 were isolated by HPLC using a C_{18} column (CLC – ODS Shimadzu 4, 6 × 150 mm). The column was eluted with the two-solvent system: solvent A, TFA/H_2O (1:1000, *v*/v) and solvent B, TFA/ acetonitrile /H_2O (1:900:100, *v*/v/v) at a flow rate of 1 $mL \cdot min^{-1}$ with 10–80% gradient of solvent B over 20 min.

Determination of the inhibitory activity of PnPP-19 and Leu-enkephalin towards NEP

The inhibition of NEP activity by PnPP-19 and Leu-enkephalin was assessed by determining the inhibitory constant (K_i), using the selective NEP fluorogenic substrate Abz-(d)Arg-Gly-Leu-Eddnp (Barros *et al.*, 2007) and appropriate concentrations of PnPP-19 or Leu-enkephalin, as the inhibitors. The hydrolysis was monitored using a spectrofluorimeter (Shimadzu-RF-5301pc) calibrated with wavelengths of λ_{em} = 420 nm and λ_{ex} = 320 nm. The assays were carried out in Tris–HCl (25 $mmol \cdot L^{-1}$) buffer containing NaCl (100 $mmol \cdot L^{-1}$), pH 7.0 at 37°C. The enzyme concentration used was 0.23 $nmol \cdot L^{-1}$ and the FRET substrate concentration was 4.95 $\mu mol \cdot L^{-1}$. The substrate solution was kept in a thermostatic chamber at 37°C for 5 min before the addition of NEP. After the determination of NEP activity in the absence of inhibitors, cumulative concentrations of PnPP-19 or Leu-enkephalin were added every 1 min during the experiment in order to induce a decrease of the hydrolysis rate of the fluorescent substrate. The fluorescence was continuously followed, and the apparent inhibition constant (K_{iapp}) values were obtained using the equation:

$$\frac{v_0}{v_1} = 1 + \frac{[I]}{K_{iapp}}$$

where v_o and v_i are the velocity of less than 2% of substrate hydrolysis in absence and in presence of different inhibitor concentrations [*I*]. The assays were performed in duplicate, and the K_i parameters were obtained from the equation:

$$K_i = \frac{K_{iapp}}{1 + \frac{[S]}{K_m}}$$

The K_i values for the NEP inhibitors were calculated by the tight-binding titration data analysis GRAFIT version 5 programme (Erithacus Software Ltd., Horley, UK).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Results are presented as means ± SEM, unless otherwise stated. Statistical analysis was carried out using GRAPHPRISM software. Our data were distributed normally and analysed statistically by one-way ANOVA with *post hoc* Bonferroni's test for multiple comparisons. Probabilities less than 5% ($P < 0.05$) were considered to show statistically significant differences between means.

Materials

The following drugs and chemicals were used: PGE_2 (Enzo Life Science, Farmingdale, NY, USA); PnPP-19 (synthesized by ChinaPeptides, Shanghai, China); naloxone (Sigma, USA); clocinnamox (Tocris, Ellisville, MO, USA); naltrindole (Tocris); nor-binaltorphimine dihydrochloride (Nor-BNI) (Tocris), *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 H-pyrazole-3-carboxamide (AM251) (Tocris), [6-iodo-2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]-(4-methoxyphenyl)methanone (AM630) (Tocris), (5*Z*,8*Z*,11*Z*,14*Z*)-5,8,11,14-eicosatetraenyl-methyl ester phosphonofluoridic acid (MAFP) (Tocris) and (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide (VDM11) (Tocris). The drugs were dissolved as follows: PGE_2 (ethanol 2% in saline); naloxone, clocinnamox, naltrindole and nor-BNI (saline); AM251 and AM630 (12% DMSO in saline), MAFP and VDM11 (10% DMSO in saline) and injected in a volume of 50 µL per paw. The FRET substrate Abz-(d)RGL-EDDnp, containing *ortho*-aminobenzoyl (Abz) and *N*-(2,4-dinitrophenyl)ethylenediamine (EDDnp) as donor/acceptor pair, was purchased from Amino Tech (São Paulo, Brazil).

Results

PnPP-19 exhibited peripheral antinociceptive effects

First, to investigate the role of PnPP-19 in nociception, the peptide was injected (5, 10 and 20 µg per paw) into rat paws-that were hyperalgesic following the administration of PGE_2. PnPP-19 induced a dose-dependent antinociceptive response, with the maximal effects at 10 or 20 µg per paw (Figure 1A). Further assays showed that the antinociceptive effect of PnPP-19 (10 µg per paw) was peripheral, because its effect was restricted to the peptide-treated paw (right paw; Figure 1B).

PnPP-19-induced peripheral antinociception is mediated by µ and δ opioid receptors

The bioinformatic assay chemoinformatic similarity ensemble approach (SEA) data bank was used as a tool to predict a molecular target for the PnPP-19 peptide. This programme generated many possible targets. However, the enzyme NEP was shown as the most likely target for the peptide with the opioid receptors also among the first group of possibilities.

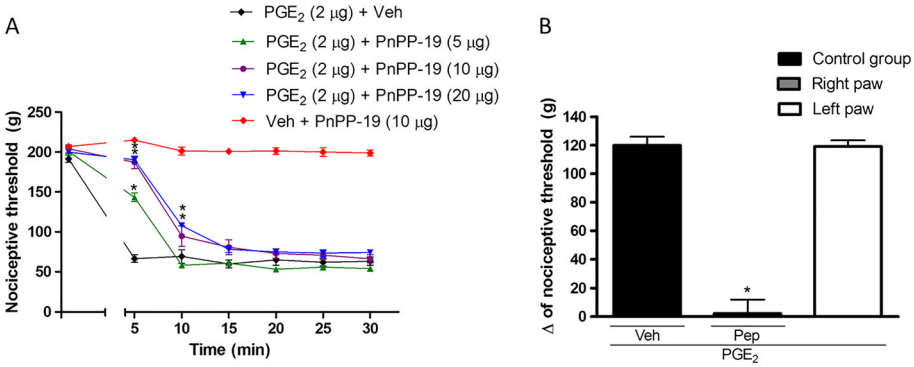

Figure 1

Peripheral antinociceptive effect of PnPP-19 on PGE_2 – induced hyperalgesia in rats. (A) PnPP-19 (5, 10 and 20 µg) was administered 3 h after local administration of PGE_2 (2 µg per paw), and the nociceptive or antinociceptive response was measured every 5 min, for 30 min. Injection of 10 µg of PnPP-19 alone did not affect the nociceptive threshold. (B) PGE_2 (2 µg) was administered in both right and left hind paws, followed by an injection of PnPP-19 (Pep; 10 µg) only into the right paw (only in this experiment, both right and left paw were measured). Both peptide and saline (as vehicle; Veh) were administered at 2 h and 55 min after local administration of PGE_2. The nociceptive or antinociceptive response was followed in both paws. The response in both assays was measured through the paw pressure test, as described in Material and Methods. Data shown are the means ± SEM ($n = 4$). *$P < 0.05$ compared with PGE_2 + Veh (ANOVA + Bonferroni's test). Veh: saline.

In addition, because the opioid pathway is associated with the mechanism of action of various analgesic drugs, we investigated the participation of this pathway in the antinociceptive response induced by PnPP-19.

Intraplantar administration of the non-specific opioid receptor antagonist naloxone (100 and 200 μg per paw; Figure 2A), the μ receptor antagonist clocinnamox (40 and 80 μg per paw; Figure 2B) or the δ receptor antagonist naltrindole (60 and 120 μg per paw; Figure 2C) partly inhibited the antinociceptive effect of PnPP-19 (10 μg per paw). On the other hand, administration of the κ receptor antagonist nor-BNI (100 μg per paw; Figure 2D) did not modify the antinociception elicited by the peptide. The effect of the highest effective dose of all tested antagonists did not differ from the hyperalgesic control (2 μg per paw of PGE_2 + vehicle; Figure 2A–D).

NEP enzymatic activity over PnPP-19

Following the results from the SEA data bank, we investigated whether PnPP-19 could act as a substrate or as an inhibitor of NEP. When the peptide was incubated with recombinant NEP for 1 h, the enzyme did not cleave PnPP-19 at any site (data not shown). On the other hand, when the PnPP-19 was incubated with NEP for a longer period (overnight), the enzyme cleaved the peptide at six different sites (Figure 3), all of them with a hydrophobic amino acid residue at the P_1' position (according to the Schechter and Berger nomenclature – Schechter and Berger, 1968).

Next, we determined the inhibitory constant (K_i) of Leu-enkephalin and PnPP-19 as inhibitors of NEP catalytic activity using the fluorogenic synthetic substrate Abz-(d) RGL-EDDnp (Figure 4A and B). The K_i values obtained for Leu-enkephalin and PnPP-19 were 14.6 ± 0.44 and 35.6 ± 1.4 μmol·L^{-1} respectively.

Ac-GERRQ↓Y↓F↓W↓IA↓W↓YKLANSKK-NH_2

Figure 3

Neutral endopeptidase (NEP) cleavage sites (arrows) in PnPP-19. PnPP-19 (20 μmol·L^{-1}) was incubated overnight at 37°C with NEP (0.2875 nmol·L^{-1}). Samples were analysed by LCMS for the determination of cleavage sites.

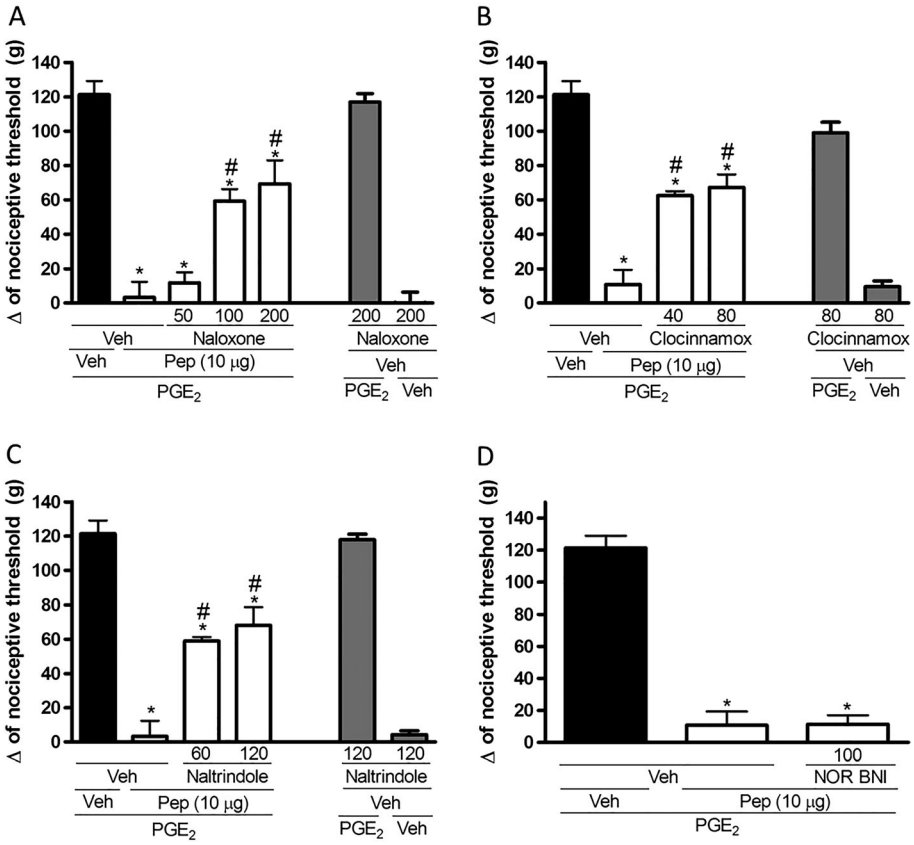

Figure 2

Effect of s.c. administration of opioid receptor antagonists on the peripheral antinociception produced by PnPP-19. (A) Non-specific opioid receptor antagonist naloxone (50, 100, 200 μg per paw), (B) μ-opioid receptor antagonist clocinnamox (40 and 80 μg per paw), (C) δ-opioid receptor antagonist naltrindole (60 and 120 μg per paw) and (D) κ opioid receptor antagonist nor-binaltorphimine (100 μg per paw) were administered 30 min before the injection of PnPP-19 (10 μg per paw). PnPP-19 was administered at 2 h and 55 min after local administration of PGE_2 (2 μg per paw). The response was measured by the paw-pressure test. Data are shown as the mean ± SEM ($n = 4$); *$P < 0.05$ compared with PGE_2 + Veh + Veh and $^{\#}P < 0.05$ compared with PGE_2 + Veh + PnPP-19 (10 μg per paw) (ANOVA + Bonferroni's test). Veh: saline; Pep: PnPP-19.

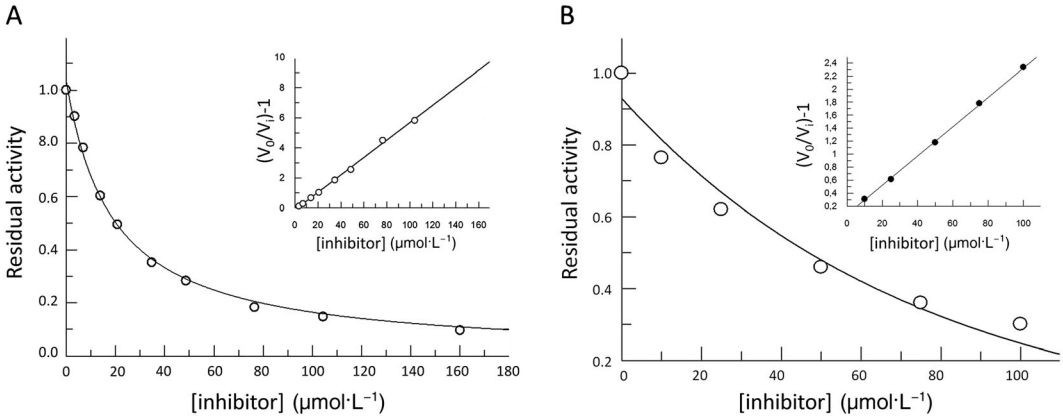

Figure 4

Determination of the inhibitory constant (K_i) of Leu-enkephalin (A) or PnPP-19 (B) on the hydrolytic activity of neutral endopeptidase (NEP). The assays were performed using the FRET-substrate Abz-(d)Arg-Gly-Leu-Eddnp. Inset: Residual activity in the presence of different inhibitors concentrations.

The cannabinoid CB_1 receptor is involved in the peripheral antinociception induced by PnPP-19

Because the opioid and cannabinoid pathways are known to interact (Befort, 2015), we investigated whether the activation of cannabinoid receptors was also involved in the antinociceptive response induced by PnPP-19 (10 μg per paw). Intraplantar administration of the CB_1 receptor antagonist AM251 (80 and 160 μg per paw) partly inhibited PnPP-19-induced peripheral antinociception (Figure 5A). However, the CB_2 receptor antagonist AM630 (100 μg per paw) did not modify the peripheral antinociceptive effects of PnPP-19 (Figure 5B). The antagonists by themselves did not significantly modify the nociceptive threshold of the control groups when injected together with PGE_2 or vehicle.

Increase of PnPP-19-induced antinociception by MAFP and VDM11

Because PnPP-19 induces activation of CB_1 receptors and the endogenous cannabinoid anandamide is slightly selective for these receptors (Lin *et al.*, 1998), we used MAFP, an inhibitor of the major anandamide metabolizing enzyme, fatty acid amide hydrolase (FAAH) and the anandamide uptake inhibitor VDM11 to confirm the potentiation of the effects of PnPP-19 on the nociceptive pathway. Both MAFP (2 and 4 μg per paw; Figure 6A) and VDM11 (20 μg per paw; Figure 6B) enhanced the antinociception induced by a low dose of PnPP-19 (5 μg per paw). MAFP and VDM11 given alone did not induce any effect.

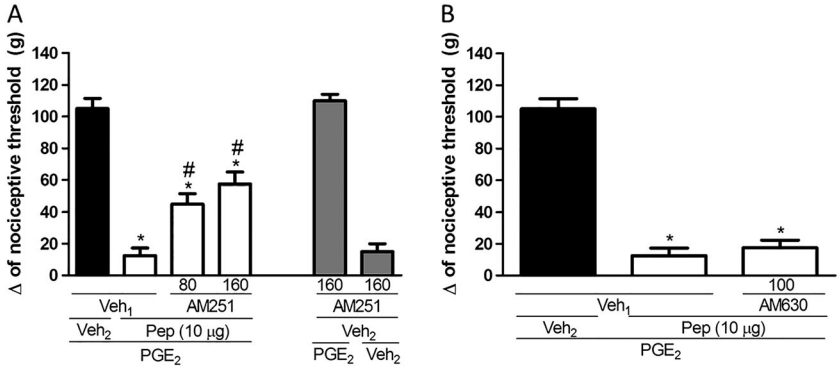

Figure 5

Effect induced by intraplantar administration of AM251 (A) or AM630 (B) on the peripheral antinociception produced by PnPP-19. AM251 (80 and 160 μg per paw) or AM630 (100 μg per paw) were administered 10 min prior to injection of PnPP-19 (10 μg per paw). PnPP-19 was administered at 2 h and 55 min after local administration of PGE_2 (2 μg per paw). The response was measured by the paw pressure test. Data are shown as the mean ± SEM ($n = 4$); *$P < 0.05$ compared with PGE_2 + Veh_1 + Veh_2 and $^{\#}P < 0.05$ compared with PGE_2 + Veh_1 + PnPP-19 (10 μg per paw) (ANOVA + Bonferroni's test). Veh_1: 12% DMSO in saline; veh_2: saline; pep: PnPP-19.

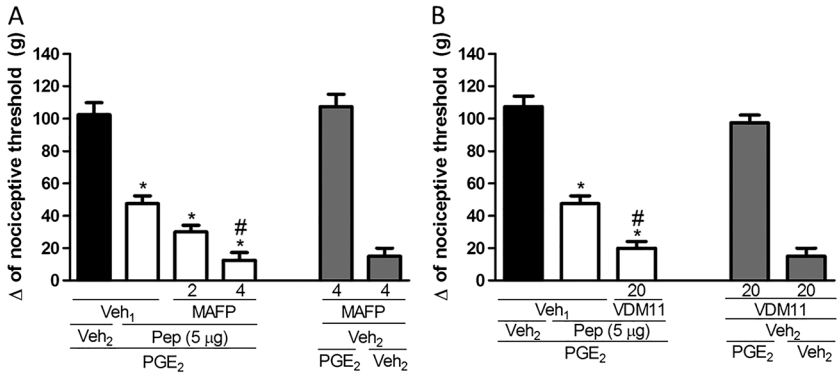

Figure 6

Potentiation of PnPP-19-induced antinociception by the FAAH inhibitor MAFP and anandamide uptake inhibitor VDM11. The MAFP (2 and 4 μg per paw) and VDM11 (20 μg per paw) were administered 10 min prior to PnPP-19 (5 μg per paw). PnPP-19 was administered at 2 h and 55 min after local administration of PGE_2 (2 μg per paw). The response was measured by the paw pressure test. Data are expressed the mean ± SEM ($n = 4$); $^{*}P < 0.05$ compared with PGE_2 + Veh1 + Veh2 and $^{\#}P < 0.05$ compared with PGE_2 + Veh1 + PnPP-19 (5 μg per paw) (ANOVA + Bonferroni's test). Veh_1: 10% DMSO in saline; veh_2: saline; pep: PnPP-19.

Discussion and conclusions

Because PnPP-19 has been suggested as a treatment for erectile dysfunction (Silva *et al.*, 2015) and also taking into account that PnTx2–6 (the toxin used as a model to obtain PnPP-19) showed nociceptive effects in rats (K Nunes, unpublished data), we decided to investigate if PnPP-19 could induce a hyperalgesic response, similar to the native toxin. However, instead of eliciting pain, PnPP-19 induced a dose-dependent antinociception in our rat model.

Initially, the ability of PnPP-19 to induce peripheral antinociception was investigated. To achieve this, we decided to use PGE_2 to induce hyperalgesia. PGs are considered as a prototype of potent direct sensitizers in animal models by stimulating a decrease of primary sensory neurons resting potential through activation of G_s protein-coupled receptors. The activation of such receptors sensitizes sodium and calcium channels and suppresses outward potassium currents (Meves, 2006). According to Ferreira (1972), a single injection of PGE_2 is capable of sensitizing nociceptors to mechanical and chemical stimuli. The use of such substance as an inducer of hyperalgesia presents, over other models of hyperalgesic induction, such as the use of the inflammatory molecule carrageenan, the advantage of eliminating the possibility that the peripheral effects of the tested compound are the results of its interaction and modulation of the mediators produced during the inflammatory process. Our results are in agreement with previous studies, which demonstrate that PGE_2 produces an intense nociceptive effect when administered peripherally, at a dose of 2 μg per paw (Pacheco *et al.*, 2008; Veloso *et al.*, 2014). Therefore, using PGE_2 to induce hyperalgesia in our model, we demonstrated that PnPP-19 produced a peripheral antinociceptive effect, in a dose-dependent manner.

Among the venomous animals, spiders comprise the group containing the largest number of species (Platnick, 1997). Many spider toxins induce antinociceptive effects, mainly by the interaction with ion channels. However, some toxins exert their antinociceptive activity by affecting glutamatergic neurotransmission or by inhibiting P2X3 receptors (see Gazerani and Cairns, 2014). On the other hand, up to date, none of the spider toxins have been described as interacting with opioid or cannabinoid systems.

The involvement of opioid receptors in central and peripheral antinociception has been extensively studied over the last few decades. Only a few animal toxins are known to induce an antinociceptive effect due to activation of the opioid system, including a neurotoxin from the venom of the king cobra (*Ophiophagus hannah*), the crude venom of the snake *Micrurus lemniscatus* and two scorpion toxins, AmmVIII and LqqIT2 (Pu *et al.*, 1995; Martin-Eauclaire *et al.*, 2010; Leite dos Santos *et al.*, 2012).

The opioid receptors belong to the superfamily of GPCRs and they are coupled to G_i/G_o proteins. Many studies have focused on elucidating the molecular mechanisms triggered by opioid receptor signalling. These include the reduction of neuronal excitability by inhibition of EPSCs evoked by NMDA receptors, calcium channels and adenyl cyclase activity, in conjunction with a stimulation of potassium channels (see Law *et al.*, 2000). Therefore, opioid peptides inhibit the sensitization of primary afferent neurons promoted by PGE_2 through activation of those receptors. Several molecules, which do not bind to opioid receptors, are still able to induce antinociception, indirectly, via activation of this pathway. Examples of the indirect analgesics are xylazine, an agonist at the α_2-adrenoceptor, and ketamine, a NMDA receptor antagonist (Romero *et al.*, 2013b; Pacheco *et al.*, 2014).

In this work, the SEA data bank suggested that the opioid pathway and NEP would be the main targets for PnPP-19. None of the spider toxins described to elicit pain relief act on these receptors nor is there any spider toxin known to interact with NEP (Gazerani and Cairns, 2014). In agreement with the results generated from the SEA data bank, we found that the antinociceptive effects of PnPP-19- were partly due to the activation of μ and δ opioid receptors. It is well established that these two types of receptors will form heterodimers and the activation of one receptor of the heterodimer can affect the signalling pathway of the other, which is in accordance with our results (Gupta *et al.*, 2010; Gomes *et al.*, 2011). Interestingly, sildenafil, a drug currently used to treat erectile

dysfunction, also induces antinociception through the activation of the same receptors (Yoon *et al.*, 2008).

We also found that PnPP-19 inhibited NEP, an enzyme responsible for the cleavage of many endogenous peptides, among them, the opioid peptide enkephalin (see Roques *et al.*, 1993). The inhibitory constants of PnPP-19 and Leu-enkephalin towards NEP catalytic activity were similar. However, NEP only cleaved PnPP-19 after a long period of incubation (overnight). Thus, although PnPP-19 is a substrate for NEP, it might have a low catalytic constant (k_{cat}). Therefore, we suggest that when PnPP-19 is administered *in vivo*, it competes with the endogenous Leu-enkephalin for the catalytic site of NEP, thereby increasing the levels of the endogenous opioid and causing the antinociceptive response. Leu-enkephalin is known to activate both μ and δ receptors (Hruby, 2002), the receptors that appeared to be involved in the peripheral antinociception induced by PnPP-19. In addition, NEP is a zinc metallopeptidase, which has specificity for cleaving substrates containing hydrophobic aliphatic or aromatic amino acids in the P_1' position (Turner *et al.*, 1985; Hersh and Morihara, 1986). In agreement with this specificity, we found the NEP to cleave PnPP-19 at six different sites, all of them close to hydrophobic amino acid residues.

The endogenous inhibitor of NEP in humans is called opiorphin (Wisner *et al.*, 2006), and the one found in rats (*Rattus norvegicus*) is called sialorphin (Rougeot *et al.*, 2003). Both of these endogenous inhibitors exhibit antinociceptive effects mediated by activation of μ and δ receptors (Rougeot *et al.*, 2003; Wisner *et al.*, 2006), as observed with PnPP-19. In addition, the gene expression of opiorphin is down-regulated in patients reporting erectile dysfunction (Tong *et al.*, 2007; Tong *et al.*, 2008). It reinforces our previous results showing that PnPP-19 potentiates erectile function (Silva *et al.*, 2015) and also highlights the role of NEP on this pathway.

The interaction of cannabinoid and opioid pathways has been extensively reported. The close vicinity of CB_1 receptors with μ or δ receptors at the neuronal level has been shown (Befort, 2015), and the heterodimerization of cannabinoid and opioid receptors has been described (Rios *et al.*, 2006; Bushlin *et al.*, 2012). In addition, activation of cannabinoid receptors stimulates the release of endogenous opioid peptides (Ibrahim *et al.*, 2005). Our group demonstrated that the central and peripheral antinociceptive effect induced by the exogenous μ receptor agonist, morphine, was mediated by activation of CB_1 receptors (Pacheco *et al.*, 2008; Pacheco *et al.*, 2009). There is also evidence that the antinociception elicited by anandamide is mediated by activation of opioid receptors (Reis *et al.*, 2009). In this study, the observed interaction between both systems could also explain part of the mechanism of action of PnPP-19 on the nociceptive pathway, because the peptide PnPP-19 induced peripheral antinociception partly through the activation of μ and δ receptors and CB_1 receptors. We also investigated the possible involvement of CB_2 receptors in PnPP-19-induced antinociception. Administration of a high dose (100 μg per paw) of the selective CB_2 receptor antagonist AM630 (Romero *et al.*, 2013a) did not inhibit the effect of the peptide. Because AM251, a selective CB_1 receptor antagonist, partly inhibited the antinociception induced by the peptide, and that cannabinoid peripheral antinociception is mainly mediated by activation of CB_1 receptors (Agarwal *et al.*, 2007), we concluded that the activation of CB_2 receptors might not be required for the antinociception elicited by PnPP-19.

The CB_1 receptor is expressed both in central and peripheral nervous systems (Herkenham *et al.*, 1990; Hohmann *et al.*, 1999; Fox *et al.*, 2001) and it is the main target for endocannabinoids and exogenous cannabinoids in the peripheral nervous system (Agarwal *et al.*, 2007). Interestingly, besides the analgesic effect of cannabinoids, they are also involved in erectile function. For instance, cannabinoids are involved in priapism (Matta *et al.*, 2014), and the endogenous cannabinoid anandamide induced relaxation of cavernosal tissue (Ghasemi *et al.*, 2006). Among the cannabinoid receptors, only CB_1 receptors are expressed in rat corpus cavernosum tissue (Ghasemi *et al.*, 2006).

The involvement of endocannabinoids in pain modulation might be assessed indirectly by administration of pharmacological agents that inhibit endocannabinoid uptake or metabolism (Hohmann and Suplita, 2006) and such inhibitors have been used as a pharmacological strategy to maximize the effects of the endogenously released cannabinoids. The endogenous cannabinoid anandamide is an agonist of both CB_1 and CB_2 receptors although it presents marginally greater affinity for CB_1 receptors (K_i: 61.0 nM) than for CB_2 receptors (K_i: 1930 nM) (Lin *et al.*, 1998). In addition, the peripheral antinociception induced by anandamide injected into the rat hind paw is mainly elicited by activation of CB_1 and not by CB_2 receptors (Reis *et al.*, 2009). It has been proposed that the biological action of anandamide is rapidly terminated by a re-uptake system, the anandamide membrane transporter, which transports anandamide into the cell where it is hydrolyzed (Di Marzo *et al.*, 1994). The enzyme primarily responsible for the hydrolysis of anandamide is FAAH (Hohmann and Suplita, 2006). In this study we administered an inhibitor of this enzyme, as well as a potent and selective inhibitor of the anandamide membrane transporter, in order to evaluate the involvement of endogenous cannabinoids in the peripheral antinociceptive effect induced by an injection of a low dose of PnPP-19 (5 μg/paw). Inhibition of FAAH and of the anandamide membrane transporter potetntiated the peripheral antinociception produced by PnPP-19. These data suggest that the peripheral antinociceptive effect of PnPP-19 is associated with anandamide release, which then could activate CB_1 receptors. These findings are in accordance with our data that show the involvement of only the CB_1 receptor in the peripheral antinociception induced by PnPP-19.

Among the analgesic animal toxins described so far, there is one peptide, crotalphine, obtained from the venom of the South American rattlesnake *Crotalus durissus terrificus*, which induces antinociception due to the activation and interaction of both opioid and cannabinoid systems (Konno *et al.*, 2008; Machado *et al.*, 2014). This is very comparable with what was found for PnPP-19. However, the exact pathways involved in the action of crotalphine and its molecular target are still not well understood.

The data presented here reveal at least part of the mechanism of action underlying the peripheral antinociceptive effect induced by the synthetic peptide PnPP-19. Our results suggest that such effects were due to activation of CB_1, μ and δ opioid receptors. In addition to that, the peptide could

inhibit the enzyme NEP, which would increase enkephalin levels, potentiating the activation of these opioid receptors. However, further studies are required to test whether PnPP-19 acts as an exogenous agonist of opioid or cannabinoid receptors, and if so, to determine its affinity for these receptors. Moreover, the release of the endogenous cannabinoid anandamide may modulate the peripheral antinociceptive effect induced by the peptide. Experiments to evaluate the possible role of PnPP-19 in the CNS are being developed.

Our current data are useful for the analysis of antinociceptive effects induced by inhibition of NEP, interactions between opioid and cannabinoid systems and for a better understanding of the role of PnPP-19 on erectile function and nociceptive pathways. In addition, our results may contribute to the consideration of PnPP-19 as a potential lead compound for the development of new drug candidates.

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Author contributions

A.C.N.F., D.F.P. and M.F.M.M. performed the research. M.E. de L., D.F.P. and I.D.D. designed the research study. M.E. de L.,I. D. D. and A.K.C. contributed essential reagents or tools. A. C.N.F., D.F.P., M.F.M.M., A.K.C., I.D.D. and M.E. de L. analysed the data and reviewed the manuscript. A.C.N.F. and D.F.P. wrote the paper.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

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4.3 Artigo III

The synthetic peptide PnPP-19 induces peripheral antinociception via activation of NO/cGMP/KATP pathway: Role of eNOS and nNOS

Foi demonstrado anteriomente que o efeito antinociceptivo central e periférico desencadeado por PnPP-19 é dependente da ativação de receptores canabinoides e opioides. Já é descrito na literatura que a ativação destes receptores leva a ativação do sistema nitrérgico (Ferreira et al., 1991; Amarante et al., 2002; Pacheco et al, 2005). Sendo assim, neste artigo foi investigado o envolvimento deste sistema na antinocicepção periférica induzida pelo peptídeo sintético PnPP-19. Mostrou-se que o efeito antinociceptivo periférico induzido pelo peptídeo resulta da ativação da via NO-cGMP-K_{ATP}. A ativação tanto da óxido nítrico sintase endotelial, quanto da óxido nítrico sintase neuronial, parecem estar envolvidas no mecanismo de ação do PnPP-19.

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The synthetic peptide PnPP-19 induces peripheral antinociception via activation of NO/cGMP/K_{ATP} pathway: Role of eNOS and nNOS

A.C.N. Freitas [a], G.C. Silva [b], D.F. Pacheco [a, b], A.M.C. Pimenta [a], V.S. Lemos [b], I.D.G. Duarte [b], M.E. de Lima [a, *]

[a] Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, M.G., Brazil
[b] Departamento Farmacologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, M.G., Brazil

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Abbreviations:
NO
nitric oxide
nNOS
Neuronal nitric oxide synthase
eNOS
Endothelial nitric oxide synthase
K_{ATP}
ATP-Sensitive potassium channels
cGMP
Cyclic guanosine monophosphate
PGE_2
Prostaglandin E_2

ABSTRACT

Background: and purpose: The peptide PnPP-19, derived from the spider toxin PnTx2-6 (renamed as δ-CNTX-Pn1c), potentiates erectile function by activating the nitrergic system. Since NO has been studied as an antinociceptive molecule and PnPP-19 is known to induce peripheral antinociception, we intended to evaluate whether PnPP-19 could induce peripheral antinociception through activation of this pathway.
Experimental approach: Nociceptive thresholds were measured by paw pressure test. PGE_2 (2 μg/paw) was administered intraplantarly together with PnPP-19 and inhibitors/blockers of NOS, guanylyl cyclase and K_{ATP} channels. The nitrite concentration was accessed by Griess test. The expression and phosphorylation of eNOS and nNOS were determined by western blot.
Key results: PnPP-19 (5, 10 and 20 μg/paw) induced peripheral antinociception in rats. Administration of NOS inhibitor (L-NOarg), selective nNOS inhibitor (L-NPA), guanylyl cyclase inhibitor (ODQ) and the blocker of K_{ATP} (glibenclamide) partially inhibited the antinociceptive effect of PnPP-19 (10 μg/paw). Tissue nitrite concentration increased after PnPP-19 (10 μg/paw) administration. Expression of eNOS and nNOS remained the same in all tested groups, however the phosphorylation of nNOS Ser852 (inactivation site) increased and phosphorylation of eNOS Ser1177 (activation site) decreased after PGE_2 injection. Administration of PnPP-19 reverted this PGE_2-induced effect.
Conclusions and implications: The peripheral antinociceptive effect induced by PnPP-19 is resulting from activation of NO-cGMP-K_{ATP} pathway. Activation of eNOS and nNOS might be required for such effect. Our results suggest PnPP-19 as a new drug candidate to treat pain and reinforce the importance of nNOS and eNOS activation, as well as endogenous NO release, for induction of peripheral antinociception.

1. Introduction

There are an increasing number of studies focusing on peptide toxins, isolated from venomous animals, as a potential source of novel pharmacological compounds. Peptide toxins acquired this interest because of the high potency and selectivity in which they act on their targets [38]. Nowadays a myriad of pharmaceutical companies are interested in the development of drugs based on toxins. Furthermore, several toxins are currently used for treatment or as tools in research concerning pathological mechanisms of many disorders, such as pain [15].

Peptide toxins able to induce antinociception are found in different groups of venomous animals, being among them cnidarians, cone snails, spiders, scorpions, bees, wasps, ants, centipedes and snakes [15,38]. Interestingly, the co-administration of nitric oxide synthase inhibitors blocked the antinociceptive effect of two snake toxins: crotalphine (isolated from the venom of *Crotalus durissus terrificus*), and hannalgesin (obtained from *Ophiophagus hannah*'s venom). Therefore, the levels of nitric oxide (NO) might be important for the mechanism of action of the aforementioned toxins [18,37].

* Corresponding author.
E-mail addresses: melenalima@icb.ufmg.br, lima.mariaelena@gmail.com (M.E. de Lima).

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NO was first described as able to induce a peripheral antinociceptive effect in 1990 [10], and since then, its role in the nociceptive pathway has been extensively studied. The intraplantar administration of a nitric oxide donor, sodium nitroprusside (SNP), induces antinociception in rat's paw made hyperalgesic with prostaglandin E_2. The co-administration of an inhibitor of guanylate cyclase blocked the antinociception induced by SNP [10]. In addition, it is described that among potassium channels, such as calcium-activated, voltage-gated and ATP-sensitive potassium channels (K_{ATP}), only the last one is involved in the peripheral antinociceptive effect induced by NO [54]. Therefore, the activation of nitric oxide-cGMP-K_{ATP} pathway has been investigated as part of the mechanism of action of many substances [11], [4,49], [7]), and it is described to be involved in the antinociception induced by different analgesic drugs, such as xylasine and ketamine [44–46,48,49].

Besides pain, the release of endogenous NO is also important for a myriad of different physiological aspects, such as erectile funtion. The toxin PnTx2-6, also known as δ-CNTX-Pn1c [27], is isolated from the venom of the spider *Phoneutria nigriventer* [3]. This spider toxin is known to be a potent potentiator of erectile function and its activity is dependent on NO and cyclic guanosine monophosphate (cGMP) levels [31]. The synthetic peptide PnPP-19 comprises 19 amino acid residues and it is a discontinuous epitope of the primary structure of δ-CNTX-Pn1c toxin. Previous histopathological experiments showed that this peptide does not induce any sign of toxicity in various tissues (kidney, heart, liver, lung and brain), and does not cause death or hypersensitivity reactions, as well, shows only low immunogenicity in mice [53]. In addition, PnPP-19 induces peripheral antinociception in rats [14] and, similar as the native toxin, it also potentiates erectile function [53]. However, the peptide apparently acts through a different mechanism of action of δ-CNTX-Pn1c toxin.

Currently, PnPP-19 has been studied as a drug model to treat both pain and erectile dysfunction. The action of this peptide in erectile function involves the activation of the nitric oxide synthase (NOS) and induction of cGMP levels augmentation [53]. On the other hand, the relationship between activation of the nitrergic system triggered by the peptide and its effect in the nociceptive pathway has never been studied.

Taking into account that the role of the peptide PnPP-19 in erectile function might be partially dependent on NO/cGMP levels [53] and that the peptide itself induces peripheral antinociception [14], we decided to investigate whether PnPP-19 also modulates the nociceptive pathway via nitrergic system.

2. Methods

2.1. Animals

Male Wistar rats of 170–220 g provided by the CEBIO (The Animal Centre) of Universidade Federal de Minas Gerais (UFMG) were used in the experiments. Animals were housed in groups of a maximum of 4 animals per cage at a temperature-controlled room (23 ± 1 °C) on an automatic 12-h light/dark cycle (06:00–18:00 h of light phase). All testing was carried out during the light phase (08:00–15:00 h) and animals were selected randomly. Food and water were freely available until the onset of the experiments. For the conclusion of this work, a total of 157 animals were used to collect all the data. All the animal care and experimental protocols are in accordance to ARRIVE guidelines [26,29], U.K. Animals (Scientific Procedures) Act_1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments and the study was also approved by the local Ethics Committee on Animal Experimentation (CETEA) of UFMG (Protocol number: 131/2014). Efforts were made to minimize suffering and reduce the number of animals used for the accomplishment of the experiments.

2.2. Algesimetric method

Rats were injected with prostaglandin E_2 (PGE_2, 2 μg) into the plantar surface (subcutaneous) of its hindpaw and measured by the paw pressure test described by Ref. [40]. This model of mechanical stimulation in rats has been in use for several years to evaluate nociceptive thresholds [24]. An analgesimeter (Ugo-Basile, Italy) with a cone-shaped paw-presser with a rounded tip was used to apply a linearly increasing force to the rat's right hindpaw. The weight in grams required to elicit nociceptive response, the paw withdrawal, was determined as the nociceptive threshold. A cut-off value of 300 g was used to prevent damage to the paws. The nociceptive threshold was measured in the right paw and determined by the average of three consecutive trials recorded before (zero time) and 3 h after PGE_2 injection (peak of effect). Results were calculated by the difference between these two averages (Δ of nociceptive threshold) and expressed as grams. To reduce stress, rats were habituated to the apparatus one day prior to the experiments.

2.3. Chemicals

The following drugs and chemicals were used: the hyperalgesic agent prostaglandin E_2 (PGE_2) (Enzo Life Science, USA); PnPP-19 (synthesized by Genone, Rio de Janeiro, Brazil); nonselective NO synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NOarg; RBI, USA); selective neuronal NO synthase inhibitor N^W-propyl-L-arginine (L-NPA; Sigma, USA); specific soluble guanylyl cyclase enzyme inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; RBI) and the ATP-sensitive K^+ channel (KATP) blocker glibenclamide (TOCRIS, USA). Drugs were dissolved as follows: PGE_2 (ethanol 2% in saline); PnPP-19 (saline); L-NOarg, L-NPA and ODQ (10% DMSO in saline); glibenclamide (2% Tween 80 in saline) and injected in a volume of 50 μl per paw.

2.4. Experimental protocol

PnPP-19 (10 μg-volume of 50 μl) was administered subcutaneously in the right hindpaw 2:55 h after local injection of PGE_2 (100 μl) [14]. In the protocol used to determine whether the drug was acting outside the injected paw, PGE_2 (100 μl) was injected into both hind paws (left and right), while PnPP-19 (50 μl) was administered into the right paw (only in this experiment both right and left paw were measured). L-NOarg and L-NPA (50 μl) were intraplantarly injected 35 min prior to the measurement of hyperalgesia (3 h). ODQ and glibenclamide (50 μl) were intraplantarly injected 15 min and 10 min, respectively, prior to the measurement of hyperalgesia (3 h). The nociceptive threshold was always measured in the right hindpaw. All the behavioral experiments were performed using a group size (n) of at least 4 animals. The group size used for the analysis of the paw pressure test has been in use for years and various literature data were assessed and published by using it [7], [2,9,14,52]. The above-mentioned protocol was assessed in pilot experiments and previous literature data to determine the dose and optimal time point for the injection of each substance [4,41,42]; de Carvalho [7], [14].

2.5. Statistical analysis

Results are presented as means ± SEM, unless otherwise stated. Statistical analyses were carried out using GraphPrism software. Our data were distributed normally and analyzed statistically by

one-way analysis of variance (ANOVA) with post-hoc Bonferroni's test for multiple comparisons. Probabilities less than 5% ($P < 0.05$) were considered to be statistically significant.

2.6. Nitrite determination

Nitric oxide (NO) levels were determined indirectly by measuring the concentration of nitrite using Griess methodology [17,48]. PnPP-19 (10 μg/paw) or saline (control groups) were administered 2:55 h after local injection of PGE_2 or saline. After 5 min, animals were euthanized and the muscular tissue located under the skin of their paw's plantar surface ($n = 6$) was collected. Tissues from each animal were homogenized in 900 μL of homogenization buffer (mM): 30 Tris-HCl, 5 EDTA, 250 sucrose, 30 KCl, 2% β -mercaptoethanol, 0.01 PMSF, 0.05 benzamidine, 0.02 aprotinin, 0.02 leupeptin and pH 6.8. Samples were then centrifuged (12,000x g, 4 °C, 15 min). 100 μL of the homogenate were applied to a microliter plate well, followed by 100 μL of Griess reagent [0.2% (w/v) naphthyleneethylenediamine and 2% (w/v) sulfanilamide in 5% (v/v) phosphoric acid]. After 10 min at room temperature, the absorbance was measured with a microplate reader (Epoch Microplate Spectrophotometer, BioTek, USA) at a wavelength of 545 nm. Each sample was assayed in duplicate. The NO_2^- standard reference curves were made with sodium NO_2^- in distilled water at concentrations of 100, 50, 25,12.5, 6.25, 3.13, and 1.56 μmol. The detection threshold of the assay was ~1.5 μM in distilled water. The total amount of protein found at the collected muscular tissue was estimated by Bradford reagent (Bio-Rad), and the nitrite release was normalized per μg of it.

2.7. Western blot analysis

Western blottings were made as previously described by Rezende and co-authors with adaptations [43]. Briefly, frozen plantar surface of the rats paws ($n = 5$) were homogenized in lysis buffer (mM): 150 NaCl, 50 Tris-HCl, 5 Na_2EDTA, and 1 $MgCl_2$ containing 0.5% SDS-plus protease inhibitors (SigmaFAST®; Sigma, St. Louis, MO, USA) and 1% Triton X-100. Proteins were denatured and separated in a denaturing 7.5% SDS-polyacrylamide gel and transferred (25 μg) onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, MA, USA). Blots were blocked at 18 °C temperature with 2.5% BSA in PBS containing 0.1% Tween 20 prior to incubation with rabbit polyclonal *anti*-nNOS (diluted 1:1000; Santa Cruz Biotechnology, Inc., CA, USA; cat. nº SC-5302), mouse monoclonal *anti*-nNOS Ser852 (diluted 1:1000; Santa Cruz Biotechnology, Inc., CA, USA, cat. nº SC-19826), rabbit polyclonal *anti*-eNOS (diluted 1:1000; Sigma, St. Louis, MO, USA; cat. nº SC-654), goat polyclonal *anti*-eNOS Ser1177 (diluted 1:1000; Sigma, St. Louis, MO, USA, cat. nº 12972), or rabbit polyclonal anti-alfa-actin (diluted 1:5000; Santa Cruz Biotechnology, Inc., CA, USA, cat. nº SC-55529) at room temperature. Antibodies were detected by chemiluminescent reaction (ECL + kit; Amersham, Les Ulis, France) followed by densitometric analyzed with ImageQuant software.

3. Results

3.1. Peripheral antinociceptive effect induced by PnPP-19

PnPP-19 (5, 10 and 20 μg/paw) induced antinociception when injected into the rat's right hindpaw made hyperalgesic with prostaglandin E_2 (Fig. 1A). As it was demonstrated by previous literature data [14], there was no difference between PnPP(19)-induced peripheral antinociception elicited by the doses of 10 μg/paw and 20 μg/paw. Therefore, the dose of 10 μg/paw was chosen for the following experiments. In addition, the dose of 10 μg/paw acts only in the treated paw (Fig. 1B), which may excludes any systemic effect or direct involvement of components of the central nervous system induced by this specific dose.

3.2. Involvement of the L-Arginine/NO/cGMP pathway in peripheral antinociception induced by PnPP-19

Intraplantar administration of the nonselective NOS inhibitor L-NOarg (48 and 96 μg/paw) partially inhibited the antinociceptive effect induced by PnPP-19 (10 μg/paw) (Fig. 2A). Likewise, the selective neuronal NOS inhibitor L-NPA (48 and 96 μg/paw), and the specific soluble guanylyl cyclase enzyme inhibitor ODQ (50, 100 and 150 μg/paw), partially antagonized PnPP-19-induced antinociception (Figs. 2B and 3A). The effect of the highest effective dose of all tested inhibitors did not differ from the hyperalgesic control (2 μg/paw of PGE_2 + vehicle; Figs. 2A–B and 3A).

3.3. Involvement of ATP-sensitive potassium channel in peripheral antinociception induced by PnPP-19

Once we confirmed that PnPP-19-induced antinociception involves the nitrergic system, we decide to investigate whether activation of ATP-sensitive potassium channels (K_{ATP}) could be also

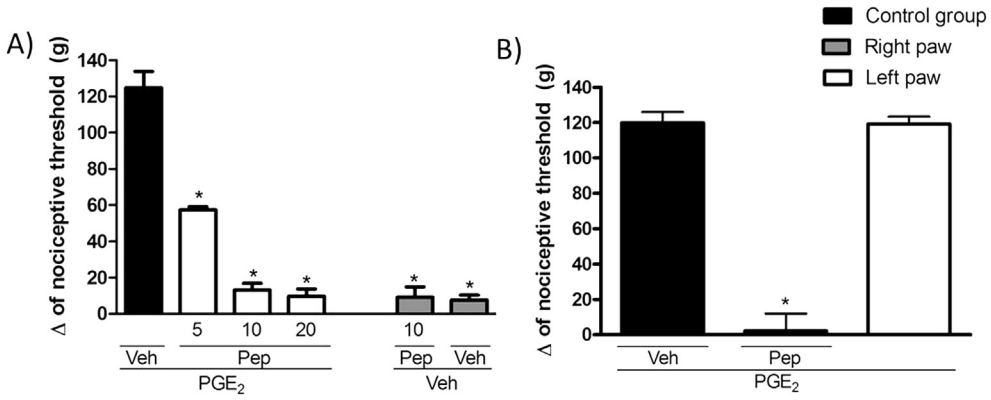

Fig. 1. Peripheral antinociceptive effect of PnPP-19 (Pep) on Prostaglandin E_2 (PGE_2) – induced hyperalgesia in rats. (A) PnPP-19 (5, 10 and 20 μg) was administered 2 h and 55 min after local administration of PGE_2 (2 μg/paw) and the antinociceptive response was measured after 5 min of peptide's administration. (B) PGE_2 (2 μg) was administered in both right and left hindpaws, followed by an injection of PnPP-19 (10 μg) only into the right paw, and vehicle into the left paw. Both peptide and vehicle were administered at 2 h and 55 min after local administration of PGE_2. Antinociceptive responses were followed in both paws. Responses in both assays were measured by means of the paw pressure test, as described in Material and Methods. Data are shown as the mean ± SEM (n = 4) and $^*P < 0.05$ compared with PGE_2 + Veh (ANOVA + Bonferroni's test). Veh: saline.

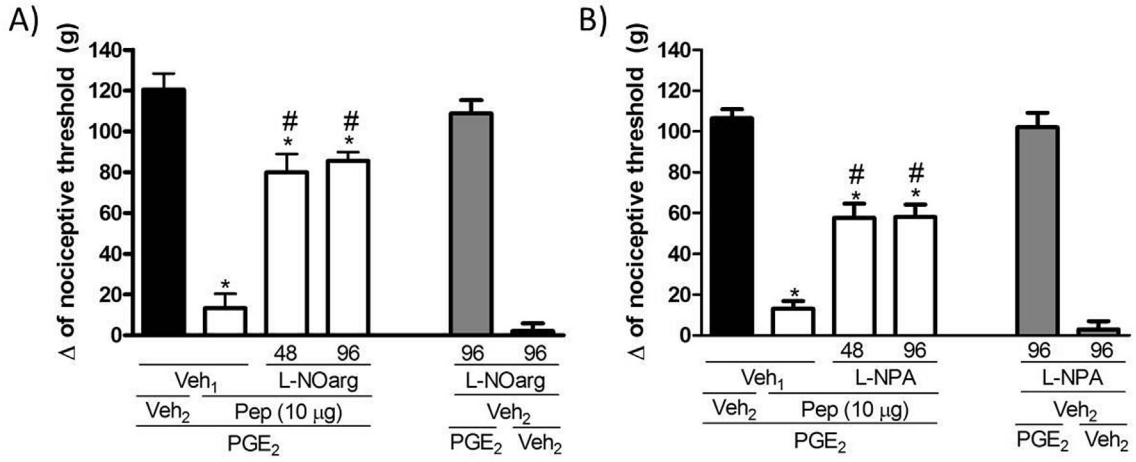

Fig. 2. Effect of subcutaneous administration of inhibitors of nitric oxide synthase on the peripheral antinociception produced by PnPP-19. (A) nonselective NOS inhibitor L-NOarg (48 and 96 μg/paw) or (B) selective neuronal NOS inhibitor L-NPA (48 and 96 μg/paw) were administered 30 min before the injection of PnPP-19 (10 μg/paw). PnPP-19 was administered at 2 h and 55 min after local administration of PGE_2 (2 μg/paw). Response were measured by the paw pressure test, as described in Material and Methods. Data are shown as the mean ± SEM (n = 4); *P < 0.05 compared with PGE_2 + Veh_1 + Veh_2 and #P < 0.05 compared with PGE_2+Veh_1+Pep (10 μg/paw) (ANOVA + Bonferroni's test). Veh_1: 10% DMSO in saline; veh_2: saline; pep: PnPP-19.

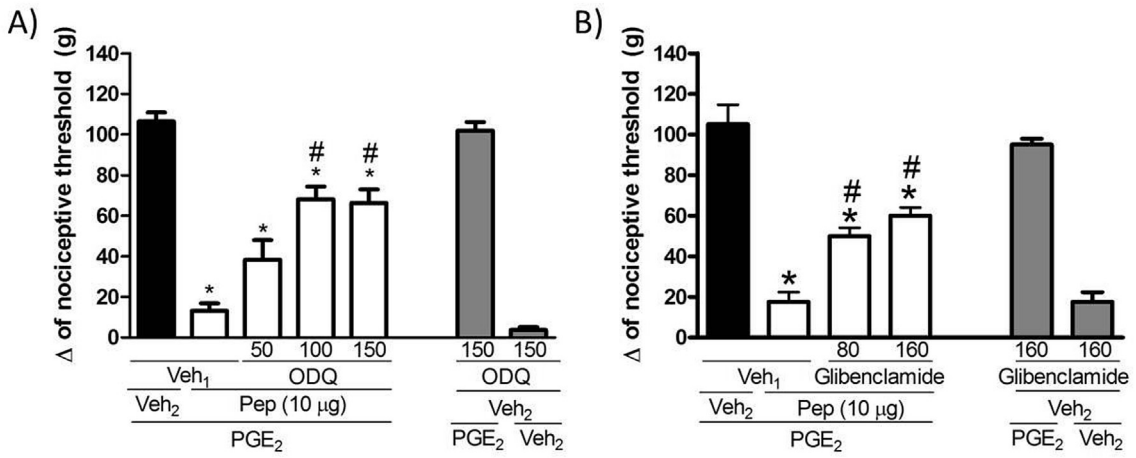

Fig. 3. Effect of subcutaneous administration of (A) a specific soluble guanylyl cyclase enzyme inhibitor and (B) an ATP-sensitive K^+ channel (K_{ATP}) blocker on the peripheral antinociception produced by PnPP-19. (A) ODQ (50, 100 and 150 μg/paw) was administered 10 min before the injection of PnPP-19 (10 μg/paw) and (B) Glibenclamide (80 and 160 μg/paw) was administered 5 min before the injection of the peptide (10 μg/paw). PnPP-19 was administered at 2 h and 55 min after local administration of PGE_2 (2 μg/paw). Responses were measured by the paw pressure test, as described in the Material and Methods. Data are shown as the mean ± SEM (n = 4); *P < 0.05 compared with PGE_2 + Veh_1 + Veh_2 and #P < 0.05 compared with PGE_2+Veh_1+Pep (10 μg/paw) (ANOVA + Bonferroni's test). (A) Veh_1: 10% DMSO in saline; veh_2: saline; pep: PnPP-19. (B) Veh_1: 2% Tween 80 in saline; veh_2: saline; pep: PnPP-19.

stimulated after peptide injection. Intraplantar administration of the K_{ATP} blocker glibenclamide (80 and 160 μg/paw) partially inhibited the antinociceptive effect induced by PnPP-19 (10 μg/paw) (Fig. 3B). Glibenclamide did not significantly modify the nociceptive threshold of control groups when injected together with prostaglandin or vehicle (Fig. 3B).

3.4. Effect of intraplantar administration of PnPP-19 on nitrite levels in homogenized plantar surface of rat's hindpaw

Since we observed that administration of inhibitors of NOS could partially prevent antinociception induced by PnPP-19, we decided to investigate whether injection of the peptide could induce a rise of nitrite concentration in rat's paw tissue. Our results show that PnPP-19 stimulated an increase in nitrite (NO_2^-) production in rat's paw made hyperalgesic with prostaglandin E_2 compared to the groups injected with vehicle or vehicle + PGE_2 (Fig. 4). Additionally, the nonselective NOS inhibitor L-NOarg (48 μg/paw) completely blocked the rise of tissue nitrite concentration induced by injection of PnPP-19 (Fig. 4).

3.5. Expression and functioning of different isoforms of nitric oxide synthase induced by PnPP-19

Considering that injection of PnPP-19 stimulates an augmentation of nitrite levels in rat's paw tissue and also that administration of inhibitors of NOS partially inhibited PnPP-19-induced antinociception, we decided to investigate whether the peptide could induce any alteration on expression and activation of both neuronal (nNOS) and endothelial (eNOS) isoforms of nitric oxide synthase. The expression of total nNOS and eNOS remained unchanged among all tested groups. Neither PnPP-19 nor PGE_2 altered the expression of both nNOS and eNOS in any situation (Figs. 5A and 6A). However, the injection of PGE_2 decreased the functioning of

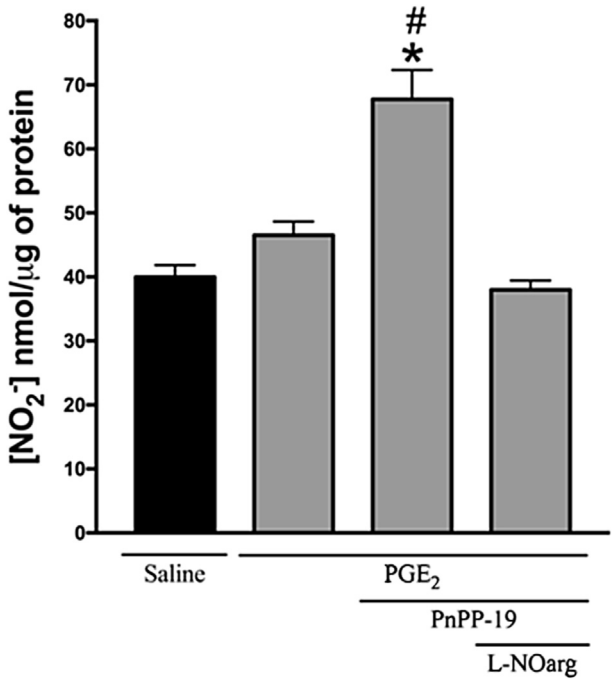

Fig. 4. Effect of PnPP-19 injection on nitrite concentration [NO2-] in the homogenized paw tissue. In the control group, saline was administered at two different time points: one injection occurred at 2 h and 55 min after the previous administration saline. In the other groups, saline or PnPP-19 (10 μg/paw) was administered at 2 h and 55 min after local administration of PGE_2 (2 μg/paw). L- NOarg (48 μg/paw) was administered 30 min before the injection of PnPP-19 (10 μg/paw). The tissue of the plantar surface of the rat hindpaw was collected 3 h after local administration of PGE_2. Each column represents the mean ± SEM (n = 6). *P < 0.05 compared with saline, #P < 0.05 compared with PGE_2 (ANOVA + Newman-Keuls).

nNOS and eNOS by causing a reduction of the phosphorylation level on the activation site of eNOS (Ser1177) [30] (Fig. 5B), and by stimulating an increase of phosphorylation level at the inactivation site of nNOS (Ser852) [39] (Fig. 6B). Interestingly, administration of PnPP-19 prevented the decrease in nNOS and eNOS functioning and led to activation of those two isoforms of NOS by inducing phosphorylation of eNOS Ser1177 (Fig. 5B) and impairing phosphorylation of nNOS Ser852 (Fig. 6B).

4. Discussion

Considering that the antinociceptive synthetic peptide PnPP-19 improves erectile function assumebly by activation of the nitrergic system [14,53], and that nitric oxide induces a peripheral antinociceptive effect by activation of both guanylate cyclase and ATP-sensitive potassium channel (K_{ATP}) [10]; [54], we aimed to investigate whether PnPP-19 could activate nitric oxide-cGMP-K_{ATP} pathway, and whether this peptide could elicit peripheral antinociception through modulation of this signal route. Our study demonstrates that the peptide PnPP-19 indeed elicits peripheral antinociceptive effect, at least partially dependent on the activation of different subtypes of nitric oxide synthase, guanylate cyclase and K_{ATP}.

Intracellularly, the enzyme nitric oxide synthase (NOS) generates nitric oxide (NO) by the catabolism of L-arginine to L-citrulline. There are three different isoforms of NOS: the neuronal (nNOS) and endothelial (eNOS) isoforms, which are regulated by intracellular calcium concentration, and the inducible isoform (iNOS), which is not dependent of calcium levels [13]. NO is involved in many physiological and pathophysiological processes, and it has a remarkable role in the nociceptive pathway [6].

Interestingly, nitric oxide has a dual effect: it may positively mediate pain in the central and peripheral nervous system, and it might also induce antinociception in both of those systems. Some

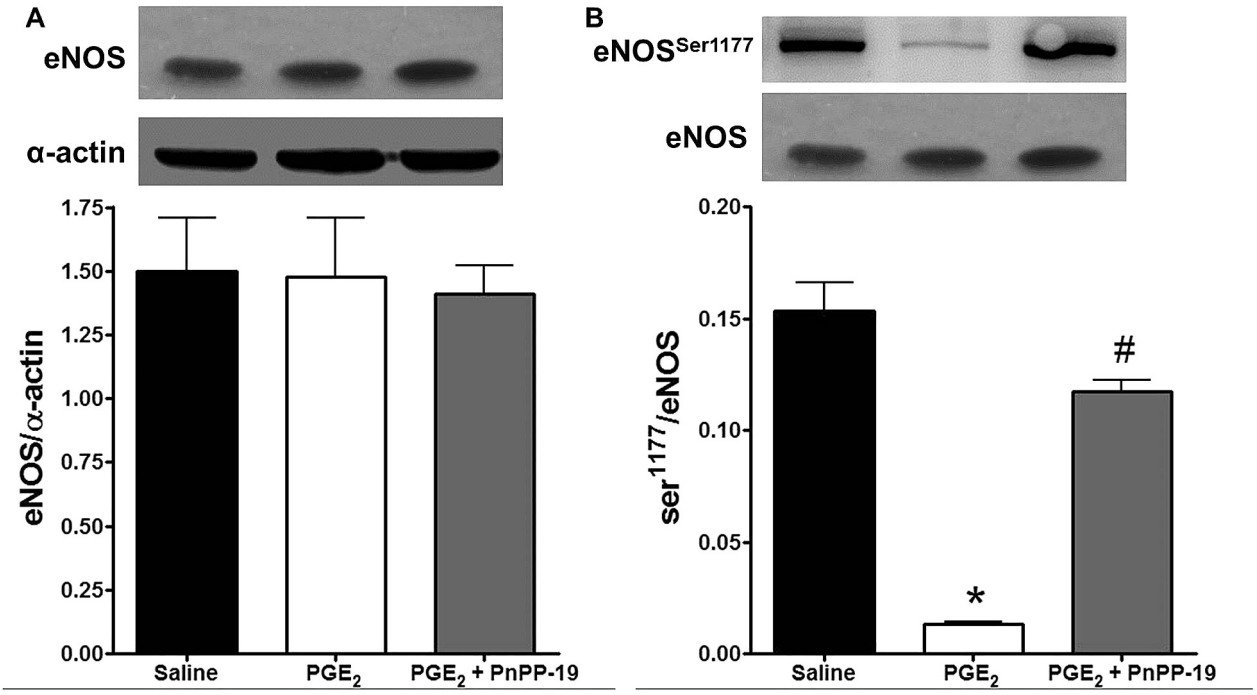

Fig. 5. Western-blot analyses of the expression of eNOS, and phosphorylation level of the activation site of eNOS ser^{1177} in the rat hindpaw injected with saline, PGE_2 and PGE_2 + PnPP-19. In the control group, saline was administered at two different time points: one injection occurred at 2 h and 55 min after the previous administration saline. In the other groups, saline or PnPP-19 (10 μg/paw) was administered at 2 h and 55 min after local administration of PGE_2 (2 μg/paw). Results are expressed as the mean ± S.E.M of five experiments. *P < 0.01 *versus* saline and #P < 0.01 *versus* PGE_2.

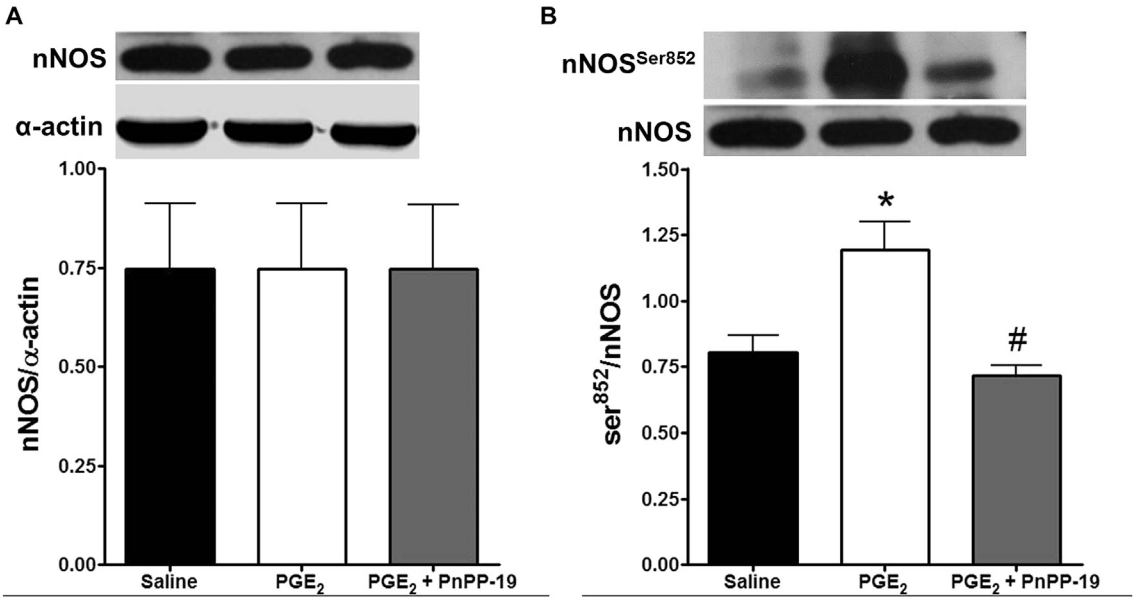

Fig. 6. Western-blot analyses of the expression of nNOS, and the phosphorylation level of the inhibition site of nNOS ser^{852} in the rat hindpaw injected with saline, PGE2 and PGE2 + PnPP-19. In the control group, saline was administered at two different time points: one injection occurred at 2 h and 55 min after the previous administration saline. In the other groups, saline or PnPP-19 (10 μg/paw) was administered at 2 h and 55 min after local administration of PGE2 (2 μg/paw). The results are expressed as the mean ± S.E.M of five experiments. *P < 0.01 *versus* saline and #P < 0.01 *versus* PGE2.

authors described that this dual effect is dependent on NO levels. Administration of high doses of substances that directly increase NO levels, such as NO donors, commonly elicits nociception and lower doses of these molecules may induce antinociception [25,36,55]. However, the effect by which NO would induce a pro- or antinociceptive effect depends on other factors, such as the type of the nociceptive stimuli (mechanical or thermal, for example) [6].

It is well established that many endogenous and exogenous substances induces peripheral antinociception due activation of nitrergic system, being among them, noradrenaline, angiotensin-(1–7), xylasine, ketamine, agonists of μ-, δ- and κ-opioid receptors and agonists of both CB_1 and CB_2 cannabinoid receptors [1,4,12,34,41,44,47–49]. In accordance to that, our data demonstrate that the antinociceptive effect elicited by the peptide PnPP-19 is at least in part dependent of NO concentration, since the inhibition of NOS activity partially antagonized the effect of the peptide.

It is difficult to determine the levels of NO in biological systems, since endogenous NO in presence of oxygen has a short half-life. Intracellular NO may be readily oxidized in nitrite (NO_2^-) and nitrate (NO_3^-), which are more stable compounds. However, in the absence of hemoproteins, L-arginine-derived NO in aqueous solution is spontaneously oxidized in nitrite [22]. For this reason, the measurement of nitrite concentration is a reasonable method to infer indirectly whether there is any alteration of NO levels after drug administration.

As mentioned above, the antinociceptive effect of some substances, such as angiotensin-(1–7), ketamine and N-palmitoylethanolamine, is dependent on NOS activity [4,47,48]. As consequence, the level of NO might increase after administration of those substances. In accordance to that, it has been demonstrated that local administration in rat's paw of those antinociceptive molecules causes an increase of nitrite levels in the plantar surface tissue. Likewise, we also observed that the nitrite concentration increases in rat's paw after injection of PnPP-19, which corroborates our data showing that eNOs and nNOS are more activated after injection of the synthetic peptide and that inhibition of NOS activity partially blocked PnPP-19-induced antinociception in *in vivo* assays.

The synthetic peptide PnPP-19 is part of the primary structure of the spider toxin δ-CNTX-Pn1c (previously known as PnTx2-6). This native toxin potentiates erectile function and this effect is dependent on the activity of nNOS isoform. However, the activation of eNOS does not seem to be involved [31]. In contrast, administration of selective nNOS inhibitor did not abolished totally rat cavernosal relaxation induced by PnPP-19, suggesting that, differently from the native toxin, the activation of other NOS isoforms might be required to elicit this effect of the peptide [53]. Correspondingly, we have demonstrated that nNOS becomes more activated after administration of PnPP-19. We have also shown that injection of a selective nNOS inhibitor partially antagonized the antinociceptive effect of the peptide. Besides that, our data indicate that PnPP-19 may induce activation of eNOS, which was previously suggested by Silva and co-authors as an additional effect of the peptide in cavernosal tissue, since eNOS has an important role in erectile function [21,53]. However, it should be considered that PnPP-19 might have differential influence on NOS activation in different tissues.

Considering the action of PnPP-19 as a potentiator of erectile function, literature data points out that some of the drugs used to treat erectile dysfunction might also induce antinociception. Vardenafil, Sildenafil and Tadalafil, inhibitors of phosphodiesterase type 5 (PDE5), are already known for their additional effect on the nociceptive pathway. All of them induce an *anti*-hyperalgesic effect, and the activation of the nitric oxide-cGMP pathway seems to contribute to their modulatory effect on pain signaling [23], [16], [32]. The relationship between activation of the NO-cGMP pathway and antinociception may be expected for those drugs, since they are PDE5 inhibitors and their use might increase cGMP levels. Interestingly, antinociception induced by Sildenafil may be associated with activation of opioid receptors, among them the μ- and δ-opioid receptors subtypes [57], which are the same receptors involved in the peripheral antinociception induced by PnPP-19 [14].

The activity of nNOS in the nociceptive pathway has been described as at least part of the mechanism of action of many

substances. The crude snake venom of *Crotalus durissus terrificus* induces antinociception mediated by activation of nNOS. Likewise, the peptide crotalphine, isolated from that same venom, elicits antinociception dependent of nNOS activity [18,35]). Using the algesimetric method of paw pressure test, many other antinociceptive molecules, such as acetylcholine; anandamide; morphine; agonists of δ- and κ-opioid receptors; agonist of α_{2C} adrenoceptor and the nonsteroidal analgesic drugs dipyrone and diclofenac, may selectively stimulate nNOS isoform to elicit peripheral antinociception [50], which highlights the importance of nNOS in the modulation of pain pathway. We showed here the possible involvement of eNOS in the antinociceptive pathway, since administration of the hyperalgesic molecule PGE_2 decreased the functioning of eNOS and injection of PnPP-19 stimulated its activation. This effect contrasts with previous literature data that demonstrated only the selective importance of nNOS to antinociception elicited by other analgesic drugs. This could be due to the lack of available potent selective eNOS inhibitors, which impairs the evaluation of its involvement in behavioral models.

Nitric oxide formed by NOS stimulates the soluble guanylate cyclase [28], and, as a consequence, it induces an increase of cGMP (cyclic guanosine monophosphate) levels. cGMP might phosphorylate and activate protein kinase G (PKG), which may stimulates and causes the opening of ATP-sensitive potassium channels (K_{ATP}) [19,20]. Once K_{ATP} is open, K^+ permeability increases and the cell membrane becomes hyperpolarized. It has been demonstrated that the activation of L-arginine/NO/cGMP pathway stimulates antinociception by inducing the opening of K_{ATP} and many analgesic molecules act through this mechanism of action [4,5,41,42,44–46,48,49,56]. Our results reinforce this data since administration of ODQ (specific soluble guanylyl cyclase enzyme inhibitor) and glibenclamide (selective K_{ATP} blocker) partially inhibited the antinociceptive effect induced by PnPP-19.

Recently, our group described that PnPP-19 induces peripheral antinociception through activation of μ-, δ-opioid and CB_1 cannabinoid receptors [14]. In accordance to our results, the interaction between nitrergic system and activation of these receptors is reported in many studies. When the μ-opioid agonist morphine is injected intracerebroventricular, it induces stimulation of the serine/threonine protein kinase Akt, which activates nNOS by Ser1417 phosphorylation [51]. Besides stimulating nNOS, once Akt is stimulated, it may also lead to an activation of eNOS by phosphorylating it at the Ser1177 site [8]. In the peripheral nervous system, morphine also activates the nitric oxide pathway culminating in stimulation of K_{ATP} channels [5], the same mechanism of action that we suggest in the present study. Also, peripheral administration of K_{ATP} blockers, NO synthase and guanylate cyclase inhibitors blocked the antinociception induced by the δ-opioid receptor agonist SNC80 [33,34]. In addition, it has been demonstrated that anandamide, a cannabinoid receptor agonist, also induces antinociception dependent of NOS and guanylate cyclase activity [41].

5. Conclusions

In conclusion, here we show for the first time that the peripheral antinociceptive effect induced by PnPP-19 is due to activation of the nitrergic system. We suggest that administration of PnPP-19 causes an increase of NO levels through activation of both eNOS and nNOS. NO may stimulate guanylate cyclase, which will cause an augmentation of cGMP concentration. After that, cGMP might indirectly induces K_{ATP} opening by activating PKG. However, the hypotheses that PKG is truly activated still need to be tested. The data presented herein reinforce our previous work [14] suggesting that PnPP-19 might be considered as a new drug candidate to treat pain. In addition, our results highlight the possible role of eNOS activity in the pain pathway, since most of the research is focused on the selective involvement of just the nNOS isoform as part of the mechanism of action of various analgesic substances.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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4.4 Artigo IV

The peptide PnPP-19, a spider toxin derivative, activates μ-opioid receptors and modulates calcium channels

Tendo em vista que os ensaios comportamentais demonstraram a importância da ativação dos receptores opioides na antinocicepção central e periférica induzida por PnPP-19, este artigo foi focado na possível caracterização do PnPP-19 como agonista opioide. Além disso, o efeito do peptídeo na modulação do influxo de cálcio em neurônios DRG foi testado, uma vez que a ativação de receptores opioides leva a inibição de diferentes canais para cálcio voltagem dependentes (Rhim et al., 1994; Law et al., 2000). Foi demonstrado que, dentre os receptores opioides testados (μ-, δ- e κ-), PnPP-19 ativa seletivamente os receptores μ-opioides. A ativação destes receptores pelo peptídeo induz o bloqueio do influxo de cálcio em neurônios DRG. Curiosamente, PnPP-19 parece não induzir o recrutamento de β-arrestina2 via ativação de receptores μ-opioides.

toxins

MDPI

Article

The Peptide PnPP-19, a Spider Toxin Derivative, Activates μ-Opioid Receptors and Modulates Calcium Channels

Ana C. N. Freitas [1], Steve Peigneur [2], Flávio H. P. Macedo [1], José E. Menezes-Filho [1], Paul Millns [3], Liciane F. Medeiros [4], Maria A. Arruda [4,5], Jader Cruz [1], Nicholas D. Holliday [4], Jan Tytgat [2], Gareth Hathway [3] and Maria E. de Lima [1,*]

1 Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte 31270-901, Brazil; acnfreitas@gmail.0100com (A.C.N.F.); flavio.hpmacedo@gmail.com (F.H.P.M.); menezesfilho10@gmail.com (J.E.M.-F.); jadercruzytrio@gmail.com (J.C.)
2 Toxicology and Pharmacology, KU Leuven, 3000 Leuven, Belgium; steve.peigneur@pharm.kuleuven.be (S.P.); jan.tytgat@pharm.kuleuven.be (J.T.)
3 Arthritis Research UK Pain Centre, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK; paul.millns@nottingham.ac.uk (P.M.); gareth.hathway@nottingham.ac.uk (G.H.)
4 Cell Signaling Research Group, School of Life Sciences, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK; licimedeiros@gmail.com (L.F.M.); Maria.Arruda@nottingham.ac.uk (M.A.A.); nicholas.holliday@nottingham.ac.uk (N.D.H.)
5 Farmanguinhos, Fiocruz, Brazilian Ministry of Health, Rio de Janeiro 22775-903, Brazil
* Correspondence: melpg@icb.ufmg.br; Tel.: +55-31-3409-2638

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Abstract: The synthetic peptide PnPP-19 comprehends 19 amino acid residues and it represents part of the primary structure of the toxin δ-CNTX-Pn1c (PnTx2-6), isolated from the venom of the spider *Phoneutria nigriventer*. Behavioural tests suggest that PnPP-19 induces antinociception by activation of CB1, μ and δ opioid receptors. Since the peripheral and central antinociception induced by PnPP-19 involves opioid activation, the aim of this work was to identify whether this synthetic peptide could directly activate opioid receptors and investigate the subtype selectivity for μ-, δ- and/or κ-opioid receptors. Furthermore, we also studied the modulation of calcium influx driven by PnPP-19 in dorsal root ganglion neurons, and analyzed whether this modulation was opioid-mediated. PnPP-19 selectively activates μ-opioid receptors inducing indirectly inhibition of calcium channels and hereby impairing calcium influx in dorsal root ganglion (DRG) neurons. Interestingly, notwithstanding the activation of opioid receptors, PnPP-19 does not induce β-arrestin2 recruitment. PnPP-19 is the first spider toxin derivative that, among opioid receptors, selectively activates μ-opioid receptors. The lack of β-arrestin2 recruitment highlights its potential for the design of new improved opioid agonists.

Keywords: *Phoneutria nigriventer*; opioid receptor; spider toxin; antinociception

Key Contribution: The spider toxin derivative PnPP-19 activates μ-opioid receptors and blocks calcium channels in DRG neurons. Our data highlights the possible use of PnPP-19 for the development of new drug candidates for pain treatment.

1. Introduction

The venom of *Phoneutria nigriventer* has been the focus of intensive research in recent years since it is of interest for discovering novel pharmaceutical bioactive peptides. This venom has a potent

neurotoxic effect and many of its toxins have been already isolated and studied in detail [1]. One of the best characterized toxins, δ-CNTX-Pn1c, also known as PnTx2-6 [2], exerts interesting pharmacological effects and it was originally studied as a modulator of voltage-gated sodium channels [1]. Recently, this toxin has been studied as a potentiator of erectile function. δ-CNTX-Pn1c improves erectile function of normotensive and DOCA-salt hypertensive rats [3] and it also ameliorates the erectile function of rats with bilateral cavernous nerve crush injury [4].

The synthetic peptide PnPP-19 comprehends 19 amino acid residues and it represents part of the primary structure of the spider toxin δ-CNTX-Pn1c. This peptide has been suggested to be a promising drug candidate for the treatment of both erectile dysfunction and pain. Through histopathological experiments [5], it was shown that PnPP-19 does not induce any sign of toxicity in different tissues (brain, heart, lung, liver and kidney) and it no longer modulates Nav channels [5]. Furthermore, it does not cause death or hypersensitivity reactions and it induces only low immunogenicity in mice. However, similar to the native toxin δ-CNTX-Pn1c, PnPP-19 does potentiate erectile function. The exact molecular target through which PnPP-19 improves erectile function still awaits elucidation [5]. Regarding the pain pathway, PnPP-19 induces both peripheral and central antinociception. This antinociceptive effect elicited by the peptide seems to involve the activation of opioid and cannabinoid receptors along with the activation of the NO/cGMP/K_{ATP} pathway [6–8].

Millions of people suffer from acute or chronic pain every year, which makes pain a serious global public health problem. Chronic pain, for instance, may cause an enormous socioeconomic impact with associated costs in treatment and reduced levels of productivity [9]. Nowadays, there is an urge for the development of novel potent and more selective analgesic drugs that elicit less undesirable side effects [10].

The opioid receptors belong to the superfamily of GPCRs and they are coupled to G_i/G_o proteins. The activation of these receptors may contribute to cellular hyperpolarization, and might impair neurotransmitters release, by suppressing calcium influx and stimulating potassium channels. The activation of the three different opioid receptor subtypes (μ-, δ- and κ-) might inhibit different calcium channels in various mammalian tissues [11]. Therefore, measuring calcium currents could be a complementary way for verifying opioid activation. In addition, the direct inhibition of calcium channels by exogenous substances may also induce per se antinociception. This is the case, for example, of two antinociceptive *P. nigriventer* toxins, PnTx3-3 and PnTx3-6 [12,13], and the well-known Food and Drug Administration (FDA) approved analgesic drug Prialt® (Ziconotide) [14]. Regarding the potassium channels, it has been shown that opioid receptor activation leads to opening of different potassium channels, among which are inward rectifying potassium channels (GIRK) [11]. As such, measuring the alteration of potassium flux through the cell membrane might also be an alternative way of investigating opioid receptor activation.

Since the peripheral and central antinociception induced by PnPP-19 involves opioid activation [6,8], the aim of this work was to identify whether this synthetic peptide could directly activate opioid receptors and investigate the possible subtype selectivity for μ-, δ- and/or κ-opioid receptors co-expressed with GIRK1/GIRK2 and RGS4. Furthermore, we also studied the modulation of calcium influx driven by PnPP-19 in dorsal root ganglion (DRG) neurons, and analyzed whether this modulation was opioid-mediated. Our data show that PnPP-19 may selectively activate μ-opioid receptors, however with low potency. Interestingly, activation of opioid receptors induced by the PnPP-19 does not stimulate the recruitment of β-arrestin2. However, it does induce indirectly inhibition of calcium channels and, consequently, impairs calcium influx in DRG neurons.

2. Results

2.1. Electrophysiological Characterization of Direct Activation of Opioid Receptors Induced by PnPP-19 Using Two-Electrode Voltage-Clamp

Each receptor was individually co-expressed with GIRK1/GIRK2 channels and RGS4, mimicking the native neuronal G-protein-mediated pathway of K^+ channel activation. We used the two-microelectrode

voltage-clamp technique to measure the opioid receptor-activated GIRK1/GIRK2 channel response as the increase of the inward K^+ current at −70 mV, evoked by the application of increasing concentrations of opioid ligands. The potency of PnPP-19 on human μ-opioid receptor (hMOR), human κ-opioid receptor (hKOR) and human δ-opioid receptor (hDOR) was investigated (Figure 1). Concentrations up to 10 μM could not evoke currents from oocytes expressing hKOR or hDOR. However, PnPP-19 could activate hMOR, albeit with low potency. Oocytes co-expressing only GIRK1/GIRK2 and RGS4 were used as a control to verify that PnPP-19 indeed interact with the opioid receptor and not the inward rectifying potassium channels. No activity was seen when PnPP-19 was applied to oocytes expressing only GIRK channels and RGS4 (Figure S1).

To confirm the interaction of PnPP-19 with the μ-opioid receptor, the activity of PnPP-19 in the presence of naloxone was investigated (Figure 2). First, expression of GIRK1/GIRK2/RGS4/hMOR was verified by applying 1 μM morphine as a control. Next, 1 μM PnPP-19 was applied as reference current. Application of 1 μM of the well characterized opioid antagonist naloxone was subsequently followed by another pulse of 1 μM PnPP-19. No PnPP-19 evoked current could be observed in the presence of naloxone (Figure 2). A similar experiment, investigating the activation of hMOR by 1 μM morphine in the presence of naloxone was performed as a control (Figure S2).

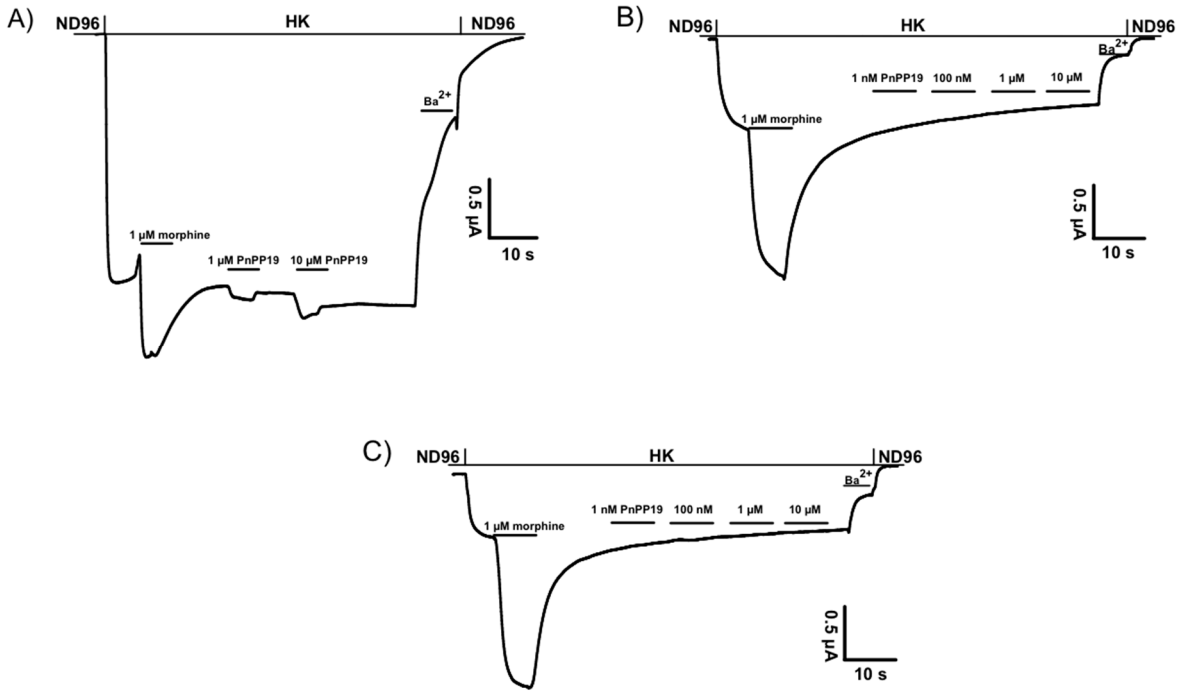

Figure 1. (**A**) Shows representative current traces of agonist-gated currents evoked from oocytes expressing human μ-opioid receptor (hMOR) by 1 μM morphine and 1 or 10 μM PnPP-19. PnPP-19 could not activate human δ-opioid receptor (hDOR) (**B**) or human κ-opioid receptor (hKOR) (**C**) up to a concentration of 10 μM.

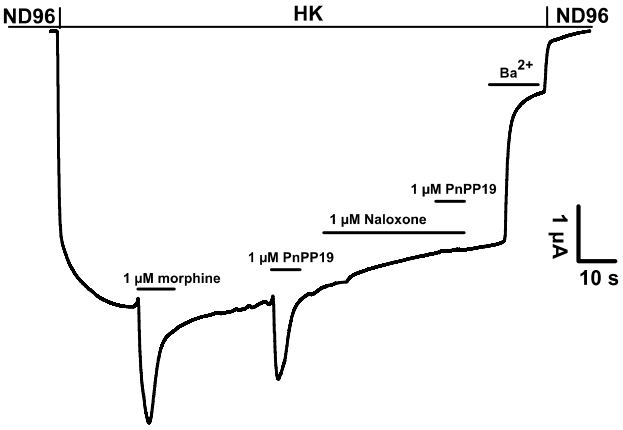

Figure 2. Representative current traces evoked from *X. laevis* oocytes co-expressing GIRK1/GIRK2 channels and RGS4 with hMOR. In addition, 1 µM naloxone inhibits the agonistic activity of PnPP-19.

2.2. Inhibition of Calcium Current Induced by PnPP-19

Intact neurons of rat dorsal root ganglia (DRG) were used for whole-cell patch-clamp recordings. PnPP-19, morphine or naloxone were added separately to the bath solution to give a final concentration of 1 µM, 1 µM and 10 µM, respectively. Figure 3 shows that PnPP-19 induced a reduction of the calcium evoked current density, with an efficacy comparable to morphine (MOR). Therefore, we could demonstrate that incubation of DRG neurons with the opioid agonist morphine (1 µM) or with PnPP-19 (1 µM) induced inhibition of calcium channels. Furthermore, pre-incubation of the cells with naloxone (10 µM) completely blocks the activity of PnPP-19, suggesting that the inhibition of calcium channels induced by the synthetic peptide is through activation of opioid receptors. Application of naloxone alone has no significant effect on the current density.

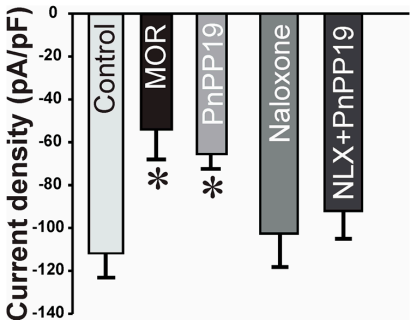

Figure 3. Effect of PnPP-19 and morphine on calcium current density evoked in dorsal root ganglion (DRG) neurons. Calcium currents were evoked by depolarizing pulses to 10 mV (200 ms) from a holding potential of −90 mV in DRG neurons incubated with 1 µM morphine, 1 µM PnPP-19 or 10 µM naloxone. Control group: cells incubated only with external/bath solution. Group NLX + PnPP-19: cells were previously incubated for 30 min with 10 µM naloxone and then PnPP-19 was added reaching a final concentration of 1 µM. MOR: morphine and NLX: naloxone. Data shown are the means ± SEM (n = 8 cells, 5 animals). * $p < 0.05$ compared with control (one-way ANOVA + Bonferroni's test).

2.3. Inhibition of Calcium Influx Induced by PnPP-19

DRG neurons were isolated and the calcium influx was evaluated using fluorescence microscopy. Cell stimulation with KCl (30 mM) induced calcium influx and consequently an increase of intracellular calcium concentration. The perfusion of the cells for 5 min with buffer did not alter the profile of calcium influx induced by KCl during the second course of stimulation if compared with the first

set of stimuli (Figures 4 and 5). On the other hand, incubation of DRG neurons with PnPP-19 (1 μM) for 5 min induced a decrease of approximately 20% of calcium influx during the second course of stimulation with KCl. In addition, PnPP-19 (1 μM) did not cause any alteration of intracellular calcium concentration when the cells were not stimulated; therefore, no change of calcium concentration was observed during the 5 min period of cell perfusion with PnPP-19 (Figure 4).

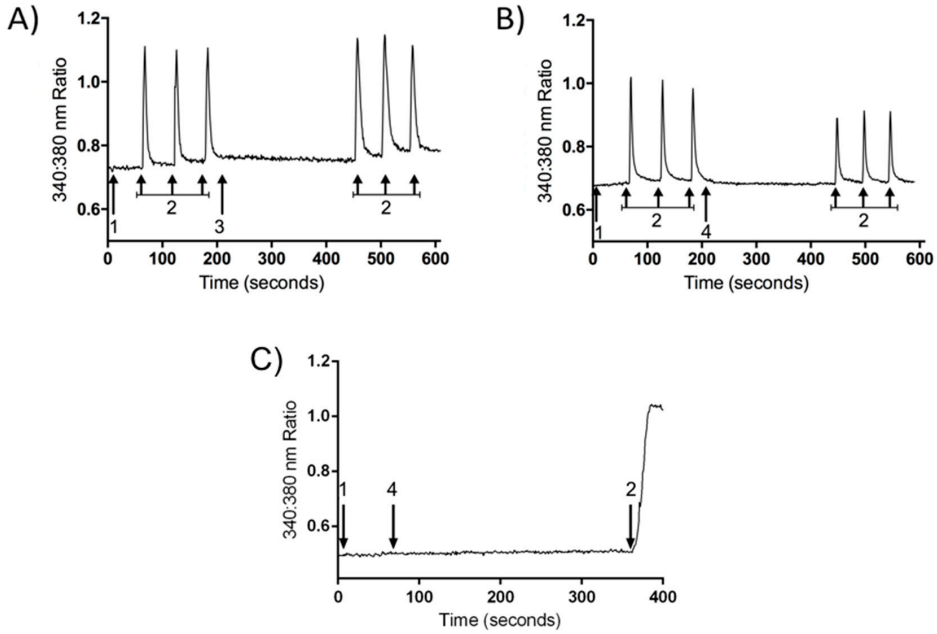

Figure 4. Representative trace showing calcium influx (changes in 340:380 nm ratios) in a single DRG neuron stimulated by KCl before and after 5 min incubation with buffer or PnPP-19. A perfusion system was used to incubate DRG neurons with buffer for 1 min (1) followed by consecutive KCl (30 mM) stimulations (2). After that, cells were perfused with buffer (3) or PnPP-19 (1 μM) (4) for 5 min, and again depolarized by KCl (30 mM) at three different time points (2). Control cells incubated only with buffer after first set of KCl stimulations do not show a significant difference in calcium influx during the second set of stimulations (**A**); However, cells incubated with PnPP-19 display a decrease in calcium influx during the second set of KCl stimulations (**B**); As a negative control, PnPP-19 does not influence calcium influx on its own (**C**).

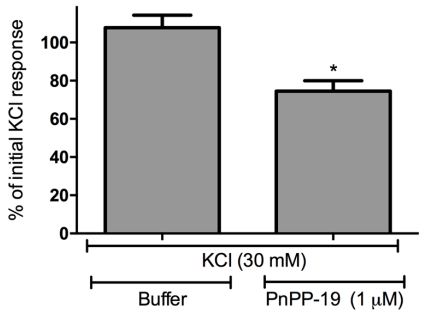

Figure 5. Effect of pre-incubation with PnPP-19 (1 μM) on KCl-evoked (30 mM) responses. The bars represent the percentage of the maximum amplitude response during the second set of KCl stimuli corresponding to the initial KCl stimulations (100%). The peak of response in each situation was calculated, and the amplitude was assessed by diminishing this value of the baseline. The baseline corresponds to the pre-incubation of cells with buffer, before any KCl stimulation. Data shown are the means ± SEM (n = 5). * $p < 0.05$ compared with KCl (30 mM) + Buffer (two-tailed t-test).

2.4. β-Arrestin2 Recruitment Induced by DAMGO and PnPP-19

Here, we intended to evaluate whether stimulation of HEK293T cells, coexpressing μ-opioid receptor-Yc and β-arrestin2-Yn, by PnPP-19 or the selective μ-opioid agonist [D-Ala^2, MePhe^4, Gly-ol^5] encephalin (DAMGO) would induce recruitment of β-arrestin2 by activating μ-opioid receptors. Incubation of the cells with DAMGO could clearly induce β-arrestin2 recruitment (Figure 6). However, different concentrations of PnPP-19 could not induce any μ-opioid receptor-β-arrestin2 association (Figure 6). In addition, pre-incubation of the cells with 10 μM of PnPP-19 was not able to prevent the binding and activation of μ-opioid receptors by the agonist DAMGO (Figure 6).

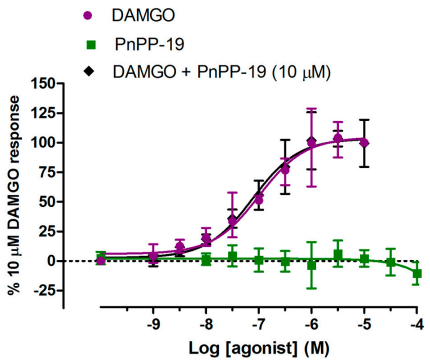

Figure 6. Recruitment of β-arrestin2 by activation of μ-opioid receptors. Stably transfected HEK293 cells coexpressing μ-opioid receptors and β-arrestin2 were pretreated for 60 min with DAMGO or PnPP-19 at the indicated concentrations. The group "DAMGO + PnPP-19" represents prior incubation of the cells with 10 μM of PnPP-19 for 30 min. β-arrestin2 recruitment was quantified by high content imaging complementation assay as described in Materials and Methods (n = 5).

3. Discussion

Previous literature data, obtained using behavioral tests, suggested that the peripheral and central antinociceptive effect induced by PnPP-19 is partially because of μ- and δ-opioid receptors activation [6–8]. Therefore, we verified whether this synthetic peptide could indeed directly bind and activate the different isoforms of opioid receptors (μ-, δ- and κ-). Moreover, since it was already described that activation of opioid receptors suppresses calcium influx through inhibition of different voltage-gated calcium channels, we also investigated the modulation of calcium influx induced by PnPP-19 in DRG neurons. Our data demonstrates that, among the μ-, δ- and κ-opioid receptor subtypes, PnPP-19 may selectively activate, with relatively low potency, the μ-opioid receptor subtype. Remarkably, it seems that the peptide does not induce the recruitment of β-arrestin2 by activating opioid receptors and, most likely, PnPP-19 binds to a different binding site of the opioid receptor than DAMGO (a selective opioid agonist). In addition, PnPP-19 induced an inhibition of calcium channels, very likely through activation of opioid receptors, in the whole-cell patch-clamp assay; it also diminished the calcium influx observed by fluorescence microscopy.

Among all the toxins isolated from the venom of *Phoneutria nigriventer* and its derivatives, only two of them are able to elicit antinociception via activation of opioid receptors. The antinociceptive effect of the toxin PnTx4(6-1), also known as δ-Ctenitoxin-Pn1a [2], is partially blocked when selective antagonists of both μ- and δ-opioid receptors are administered [15]. Likewise, antinociception of PnPP-19, a δ-CNTX-Pn1c derivative [5], occurs also through activation of those very same opioid receptors [6,8]. On the other hand, it was never investigated whether these peptides may directly bind and activate opioid receptors. Our present data show that PnPP-19 might selectively activate, with low potency, only the μ-opioid receptor subtype. However, through behavioral experiments, it was shown that the antinociception induced by PnPP-19 also involves activation of δ-opioid receptors. Here, we demonstrated that PnPP-19 is incapable of activating directly δ-opioid receptors. Therefore,

activation of this specific subtype of receptor in vivo may occur via an indirectly pathway, as previously suggested by Freitas and collaborators [6].

PnPP-19 is the first synthetic peptide, derived from a spider toxin, proven to act directly on opioid receptors, and more specifically, on μ-opioid receptor subtype. Novel ligands of the μ-opioid receptor are of clinical and social importance since the common used analgesic drugs, such as morphine, fentanyl and oxymorphone, elicit both their beneficial pharmacological effect and undesirable side effects through activation of opioid receptors [16]. One of the very serious and life-threatening conditions developed following the use of the usual opioid agonist medicines is respiratory paralysis [16,17]. It has been demonstrated that the induction of respiratory paralysis, as well as other side effects, after the use of opioids may be linked with the recruitment of the β-arrestin pathway, which is stimulated downstream following activation of μ-opioid receptor [18–22]. Since opioid receptors are still one of the most relevant targets for pain treatment, great effort is being put in the development of new opioid agonists that elicit fewer negative side effects [22,23]. In this way, the lack of β-arrestin2 recruitment by PnPP-19 underlines the potential of this peptide as a possible lead compound in the development of improved opioid agonists. Recently, a very selective and potent μ-opioid agonist was developed and named PZM21. Despite its great potency and selectiveness against μ-opioid receptors, administration of PZM21 induced minimal β-arrestin2 recruitment. Therefore, the use of PZM21 induced a long-lasting analgesia along with decreased respiratory depression and constipation when compared to morphine [10]. For this reason, studies concerning the exact mechanism of action of PnPP-19 in the pain pathway are of interest since PnPP-19 showed no induction of β-arrestin2 recruitment in cell culture. It thus seems that the peptide has a site of interaction different from the DAMGO binding site, since in our experiments the presence of PnPP-19 has no influence on DAMGO-induced β-arrestin2 recruitment. However, one other hypothesis for the lack of β-arrestin2 recruitment by PnPP-19 could be the low potency of which PnPP-19 might bind to μ-opioid receptors. Therefore, further investigation is required in order to elucidate the exact mechanism of PnPP-19 interaction with the opioid receptors. Moreover, a better characterization of the target of this synthetic peptide in erectile function is required in order to develop a PnPP-19 derived drug without unwanted side effects.

The interaction between opioid receptors and ion channels has been a subject of much interest during decades. Various studies suggest that activation of opioid receptors causes hyperpolarization of the cell and consequently prevents neurotransmitter release by inducing an inhibition of calcium channels [11,24,25] and activation potassium channels [11,26–28]. According to in vitro studies, incubation of a selective agonist of μ-opioid receptors with HEK293 cells co-expressing μ-opioid receptors together with voltage-gated N-type calcium channels (Cav2.2) or R-type (Cav2.3) channels induced an inhibition of both calcium channels tested [29]. Moreover, experiments, conducted with primary culture of vestibular afferent neurons and DRG neurons, suggest that selective stimulation of μ-opioid receptors may inhibit T-, L- and N-type calcium channels supposedly through activation of a $G\alpha_{i/o}$ protein [30–32]. Accordingly, our results demonstrate that PnPP-19 inhibits calcium influx in DRG neurons and that this inhibition is suppressed by the unspecific opioid antagonist naloxone. These data show that inhibition of calcium influx induced by PnPP-19 is mediated by activation of opioid receptors. Recently, it was demonstrated that DAMGO (selective μ-opioid agonist) induces inhibition of calcium influx and action potential-evoked Ca^{2+} fluorescent transients in individual peripheral nociceptive fiber free nerve endings from trigeminal ganglion. The authors have shown that activation of "big conductance" Ca^{2+}-activated K^+ channels (BK_{Ca}) mediates this inhibition of calcium influx induced by DAMGO. Furthermore, the activation of this subtype of potassium channel plays a major role on μ-opioid induced antinociception in a behavioral test for trigeminal nociception. Therefore, it is likely that PnPP-19 might also modulate potassium channels, since this synthetic peptide may act as an opioid agonist. However, further experiments are needed to investigate whether PnPP-19 indeed interferes with potassium channel activity.

In conclusion, the data we present here shows for the first time a spider toxin derivative that may act as a selective μ-opioid agonist. PnPP-19 directly binds and activates, albeit with low potency,

only the μ-opioid receptor subtype. In DRG neurons, the activation of μ-opioid receptors induced by PnPP-19 generates an inhibition of calcium channels, consequently reducing or even eliminating calcium influx. This modulation of calcium channels appears to follow activation of opioid receptors, confirming once again the role of PnPP-19 as an opioid agonist. Interestingly, notwithstanding the activation of opioid receptors, PnPP-19 does not induce β-arrestin2 recruitment. This could be due its low potency; however, it may also be a consequence of a differential opioid activation mechanism in which β-arrestin2 recruitment is not stimulated. Further studies with PnPP-19 could lead to the development of new and more potent opioid agonists that in turn could elicit antinociception with possibly less side effects by not inducing recruitment of β-arrestin2.

4. Materials and Methods

4.1. Expression of Voltage-Gated Potassium Channels in Xenopus laevis *Oocytes*

Xenopus laevis oocytes were isolated as previously described [33]. Oocytes were co-injected with 0.5 ng/50 nL of GIRK1, 0.5 ng/50 nL of GIRK2, and 10 ng/50 nL of RGS4 cRNA, with the addition of 10 ng/50 nL of either hMOR, hKOR, hDOR, hMORW318L, or hMORW318Y/H319Y cRNA. Injected oocytes were maintained in ND-96 solution (composition: 2 mM KCl, 96 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) supplemented with 50 μg/mL of gentamicin sulfate.

4.2. Electrophysiological Recordings: Xenopus laevis *Oocytes*

Whole-cell currents from oocytes were recorded from 1 to 2 days after injection using the two-microelectrode voltage-clamp technique. Resistances of voltage and current electrodes were kept between 0.7 and 1.5 MΩ and were filled with 3 M KCl. Currents were filtered at 20 Hz, using a 4-pole low-pass Bessel filter. To eliminate the effect of the voltage drop across the bath-grounding electrode, the bath potential was actively controlled. All experiments were performed at room temperature [19–23]. At the start and the end of each experiment, oocytes were superfused with low-potassium (ND-96) solution (composition: 2 mM KCl, 96 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). During application of increasing concentrations of ligands, oocytes were superfused with high-potassium (HK) solution (composition: 96 mM KCl, 2 mM NaCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5). In HK solution, the K^+ equilibrium potential is close to 0 mV and enables K^+ inward currents to flow through inwardly rectifying K^+ channels at negative holding potentials. A gravity-controlled fast perfusion system was used to ensure rapid solution exchanges. Analysis of un-injected cells (n = 3), under the same experimental conditions as injected oocytes, revealed an endogenous current that amounted maximally 1% as compared with the current measured in injected oocytes. Application of opioid ligands did not evoke an increase of the conductance in un-injected oocytes. In each experiment, oocytes were clamped at a holding potential of −70 mV and super-fused with ND-96 solution. Next, the super-fusion solution was switched from ND-96 to HK solution, after which increasing concentrations of morphine or peptide were applied. Each concentration was applied for as long as needed to achieve a steady-state GIRK1/GIRK2 current activation. Each ligand concentration was washed out by super-fusing it with an HK solution. During this washout period, the channels return to the control current level as a result of a deactivation process that is accelerated dramatically in the presence of RGS4, as previously described [34]. At the end of each experiment, the oocyte was super-fused with HK solution containing 300 μM $BaCl_2$, causing a blockage of the net GIRK1/2-gated inward current. Finally, the super-fusion was switched back to ND-96 solution to confirm complete reversibility.

4.3. Data Analysis of Two-Microelectrode Voltage-Clamp

The pCLAMP program (Axon Instruments, pCLAMP, Sunnyvale CA, USA) was used for data acquisition, and data files were directly imported, analyzed, and visualized with a custom-made add-in for Microsoft Excel (Redmond, WA, USA). The percentage-activated current was calculated

using the equation: percentage activation = activated current amplitude control current amplitude $\times$ 100 $-$ 100 and 0% was taken as the control current level. Current percentages were then used for the calculation of concentration–response curves, using the Hill equation $I = I_{max}/[1 + (EC_{50}/A)^{nH}]$, where I represents the current percentage, I_{max} the maximal current percentage, EC_{50} the concentration of the agonist that evokes the half-maximal response, A the concentration of agonist, and n_H the Hill coefficient. Averaged data are indicated as means $\pm$ SEM and were calculated using n experiments, where n indicates the number of oocytes tested. For each experiment, the number of oocytes tested was at least 6 ($n > 6$) For each experiment, averaged current percentages were normalized to 100%, and an averaged concentration–response curve was drawn using the average EC_{50} values and Hill coefficients of n experiments. Statistical analysis of differences between groups was carried out with Student's *t*-test, and a probability of 0.05 was taken as the level of statistical significance.

4.4. DRG Culture

DRGs were isolated from adult Wistar rats (200 $\pm$ 300 g) and neurons cultured as described by Lindsay (1988) [35] with minor modifications. The neurons were isolated and washed by gravity in phosphate-buffered saline (PBS). The cells were than incubated with collagenase type IV (sigma, St. Louis, MO, USA) solution (5 mL of Dulbecco's modified Eagle's medium—DMEM; 10% *v*/*v* fetal bovine serum; penicillin 200 units/mL—streptomycin 200 µg/mL; 12.5 mg of collagenase type IV) for 90 min at 37 °C. After that, ganglions were washed 3 times by gravity in PBS and trypsin solution (2500–6000 BAEE U/ML, sigma, St. Louis, MO, USA) was added. In order to dissociate the DRG neurons, the ganglions were taken up and down with the use of a fine tipped transfer pipette. Cells were then incubated with the trypsin solution for 10 min at 37 °C. After the incubation period, 1 mL of bovine serum albumin (BSA) solution (16% *v*/*v* in PBS) was added and cells were more firmly dissociated. The cell suspension was added on the top of 3 mL BSA solution and centrifuged at 500$\times$ *g* for 6 min. The supernatant was discarded and the pellet was resuspended in complete media (DMEM media; 10% *v*/*v* fetal bovine serum; 1% *v*/*v* penicillin/streptomycin; 0.1% *v*/*v* NGF). Cells were plated on poly-L-lysine and laminin coated cover slips and incubated at 37 °C with 5% CO_2 in a humidified incubator. The study was approved by the local Ethics Committee on Animal Experimentation (CETEA) of UFMG (Protocol number: 233/2013).

4.5. Whole-Cell Voltage-Clamp

DRG neurons were used for the measurements after 48 h of cell culture. The calcium current recordings were obtained by using the Patch Clamp amplifiers type EPC-9/EPC-10 (HEKA Instruments, Lambrecht/Pfalz, Germany) and the PULSE/PATCHMASTER data acquisition program (HEKA Instruments, Lambrecht/Pfalz, Germany) adjusted for the Whole Cell Voltage-Clamp configuration. Low resistance patch electrodes (3–4 MΩ) were filled with solution containing (in mM): 130 CsCl, 2.5 $MgCl_2$, 10 HEPES, 5 EGTA, 3 Na_2-ATP and 0.5 Li_3-GTP, pH 7.4 adjusted with 1 M CsOH. The external/bath solution contained (in mM): 125 CsCl, 10 $BaCl_2$, 1 $MgCl_2$, 10 HEPES and 60 Glucose, pH 7.4 adjusted with 1 M CsOH. An Ag-AgCl electrode was used as reference. The recordings were filtered with a Bessel low-pass filter set at 2.9 kHz and digitalized at a 10 kHz rate (100 µs interval) through an AD/DA interface (ITC 1600). Capacitive currents were electronically compensated and a P/4 protocol was used to correct the linear leakage current and to subtract residual capacity (BEZANILLA, ARMSTRONG, 1977) [36]. After establishing the Whole Cell configuration, the calcium current was evoked from negative holding potential of $-$90 mV to 10 mV (200 ms). Once the calcium current showed stable amplitude values, PnPP-19, morphine or naloxone were added separately to the bath solution to give a final concentration of 1 µM, 1 µM and 10 µM, respectively. To test whether the effect of PnPP-19 was through opioid receptors, cells were prior incubated with 10 µM of naloxone for 30 min. The experiments were performed on 35 mm diameter acrylic Petri dishes using inverted microscope (Axiovert 20, Carl Zeiss, Jena, Germany or Nikon TMF-100, Nikon, Chiyoda-Ku, Japan).

4.6. Calcium Imaging

The experiments were performed after 24 h of DRG neurons dissociation. On the day of the experiments, cells were incubated with Fura 2-AM (5 mM, 30 min, 37 °C). Intracellular Ca^{2+} concentrations ([Ca^{2+}]i) in individual neurons were estimated as the ratios of peak fluorescence intensities (measured at 500 nm) at excitation wavelengths of 340 and 380 nm, respectively (Bundey & Kendall, 1999) [37], using an Improvision imaging system. DRG neurons were superfused (2 mL min^{-1}) with buffer (NaCl 145 mM; KCl 5 mM; $CaCl_2$ 2 mM; $MgSO_4$ 1 mM; HEPES 10 mM; glucose 10 mM) for 1 min followed by three consecutive KCl (30 mM) stimulations. After that, cells were perfused with buffer (control group) or PnPP-19 (1 μM) dissolved in buffer for 5 min, and again depolarized by KCl (30 mM) at three different time points. Representative traces of calcium influx in a single DRG neuron are shown. Results are presented as means ± SEM and indicate the percentage of calcium influx related to the peak of calcium influx during the first course of activation with KCl (100%). Statistical analyses were carried out using GraphPrism software (version 7.0a, GraphPad Software, La Jolla, CA, USA, 2016). Our data were distributed normally and analyzed statistically by two-tailed *t*-test. Probabilities less than 5% ($p < 0.05$) were considered to be statistically significant.

4.7. Beta-Arrestin2 Recruitment

HEK293T were cultured in DMEM (Sigma-Aldrich) supplemented with 10% *v*/*v* fetal bovine serum. These cells were coexpressing μ-opioid receptor-Yc and β-arrestin2-Yn (Yc and Yn are complementary fragments of yellow fluorescent protein-YFP). To analyze whether activation of μ-opioid receptor would induce recruitment of β-arrestin2, the Bimolecular fluorescence complementation (BiFC) based detection of μ-opioid receptor-β-arrestin2 association was conducted. The cells were seeded at 33,000 cells/well onto poly (D-lysine)-coated Greiner 655,090 imaging plates. Plates were kept in a humidified incubator at 37 °C filled with 5% CO_2 for 24 h. HEK293T were stimulated with the selective opioid agonist DAMGO (Tocris, Minneapolis, MN, USA) or the synthetic peptide PnPP-19 in HEPES-buffered saline solution (HBSS) including 0.1% *v*/*v* BSA (10^{-10} M–10^{-4} M) for 60 min at 37 °C. In the experiment where we investigated whether PnPP-19 could impair the binding of DAMGO to μ-opioid receptors, cells were preincubated with PnPP-19 10 μM (30 min, 37 °C). After that, cells were fixated with 3% paraformaldehyde in PBS for 10 min at room temperature. Then, cells were washed once with PBS and the cell nuclei were stained for 15 min with H33342 (2 μg/mL in PBS, Sigma, St. Louis, MO, USA). H33342 was then removed by a final PBS wash. Images (4 central sites/well) were acquired automatically on the IX Ultra confocal plate reader, using 405 nm/488 nm laser lines for H33342 and complemented YFP excitation, respectively. Data was analyzed by the use of MetaXpress software (version 5.3, Sunnyvale, CA, USA, 2013) as described by Liu and co-authors [38] and normalized by 10 μM of DAMGO (100%).

Supplementary Materials: The following are available online at www.mdpi.com/2072-6651/10/1/43/s1, Figure S1: Representative current traces evoked from *X. laevis* oocytes co-expressing GIRK1/GIRK2 channels and RGS4. PnPP-19 does not interact with GIRK channels; Figure S2: Representative current traces evoked from *X. laevis* oocytes co-expressing GIRK1/GIRK2 channels and RGS4 with hMOR.

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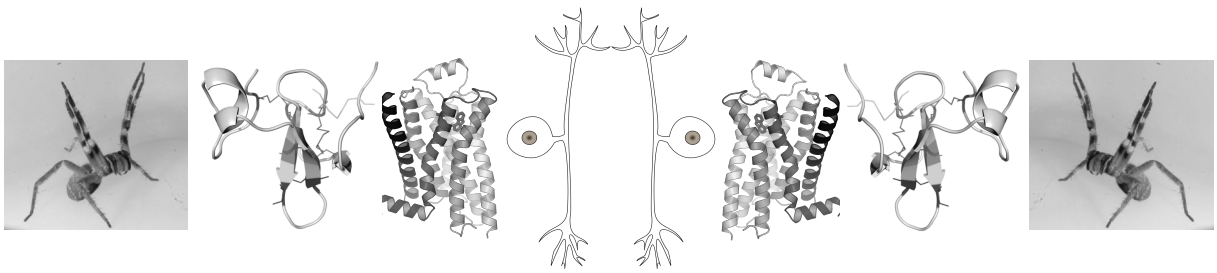
Discussão



5 DISCUSSÃO

O presente estudo propôs investigar o efeito do peptídeo PnPP-19 na via da dor, uma vez que sua toxina de origem tem efeito hiperalgésico em modelo murino (Nunes, 2008). Este trabalho demonstrou que o peptídeo sintético PnPP-19 induz antinocicepção central e periférica. Em ensaios *in vivo*, o efeito antinociceptivo induzido pelo peptídeo parece ser dependente da ativação do sistema nitrérgico, dos receptores canabinoides CB_1 e receptores μ- e δ-opioides (Freitas et al., 2016; Pacheco et al., 2016; Freitas et al., 2017). PnPP-19 parece também atuar como um inibidor da enzima neprilisina, enzima esta envolvida na sinalização da via nociceptiva (Freitas et al., 2016). Ademais, foi sugerido que o peptídeo sintético possa atuar como um agonista μ-opioide e, por ativar este receptor, pode modular o influxo de cálcio em neurônios DRG (Freitas et al., 2018).

Nossos resultados monstraram que a administração i.c.v. do peptídeo PnPP-19 foi capaz de induzir antinocicepção de maneira dose e tempo dependentes no teste algesimetrico do *Tail-Flick*. A maior dose testada (1 μg/animal) praticamente triplicou o limiar nociceptivo dos animais, sem causar morte ou qualquer alteração comportamental significativa. Vale ressaltar que a administração de uma dose significativamente menor da toxina PnTx2-6 pela mesma via (0,79 μg/animal) é capaz de causar uma série de reações adversas, tais como: coceira, lacrimejamento, salivação, sudorese e agitação seguidas de morte (Cordeiro et al., 1992).

Na sequência aqui apresentada, investigamos o efeito do peptídeo em nível periférico, utilizando o modelo de hiperalgesia induzida pela administração intraplantar de prostaglandina E_2 (PGE_2). A interação da PGE_2 com seu receptor específico, acoplado à proteína G_s, em neurônios aferentes primários ativa a adenilato ciclase, que por sua vez aumenta os níveis de AMPc. A partir daí, ocorre ativação da proteína cinase A (PKA) (Ferreira e Nakamura, 1979), que fosforila domínios intracelulares de canais para sódio e potássio voltagem dependentes, modulando suas cinéticas. A ativação de PKA leva ao aumento da condutância para íons sódio (Vijayaragavan et al., 2004 ; Chatelier et al., 2008) e diminuição da condutância para íons potássio (Nicol et al., 1997, Besana et al., 2005), levando a uma maior excitabilidade celular, o que diminui o limiar de ativação neuronial. Tem sido descrito, por exemplo, que a administração de análogos do AMPc causa diminuição do limiar nociceptivo de animais em ensaios *in vivo* (Taiwo et al., 1989) e sensibilização de neurônios aferentes primários em ensaios *in vitro* (Kress et al., 1996). De acordo com Ferreira (1972), uma única injeção de PGE_2 é capaz de sensibilizar nociceptores a estímulos mecânicos e químicos.

O peptídeo PnPP-19 foi capaz de induzir efeito antinociceptivo periférico, de maneira dose e tempo-dependente, no teste de hiperalgesia induzida por PGE_2. O pico de efeito foi observado 5 minutos após a administração do peptídeo, não sendo observado diferença estatística entre as doses de 10 μg e 20 μg/animal. Desse modo, a fim de se confirmar o efeito local da dose de 10 μg/animal, realizamos o experimento de exclusão do efeito sistêmico da dose em questão. Constatamos que o efeito antinociceptivo acontece perifericamente, uma vez que a administração de 10 μg/animal do peptídeo na pata esquerda do animal não alterou a hiperalgesia avaliada na pata contralateral (direita). Com este experimento mostramos que PnPP-19 não exibe as propriedades hiperalgésicas de sua toxina de origem, a PnTx2-6 (Nunes, 2008). A administração de apenas 3 μg (0,57 nmol) desta toxina, pela mesma via de administração do PnPP-19, foi capaz de induzir hiperalgesia local e sistêmica. Ao contrário do que se supunha, o peptídeo PnPP-19 não foi capaz de induzir hiperalgesia, e nem desencadeou resposta sistêmica através da administração periférica de uma dose maior (10 μg = 4 nmol) do que aquela responsável pela hiperalgesia sistêmica ocasionada pela toxina PnTx2-6.

Os receptores opioides são amplamente expressos no SNC e a ativação destes receptores pelos seus agonistas específicos em diferentes regiões deste sistema pode inibir a transmissão e percepção do estímulo nociceptivo (Law et al., 2000; Argoff, 2011). Já foi demonstrado que a administração i.c.v. de agonistas μ-, δ- e κ-opioides induz antinocicepção (Pacheco et al., 2009). Além da ativação do sistema opioidérgico por agonistas opioides, algumas subtâncias agonistas de outros receptores, quando administradas por esta via, podem induzir antinocicepção via ativação de receptores opioides. Dentre essas moléculas, estão listadas a xilazina e a ketamina, por exemplo (Romero et al., 2013; Pacheco et al., 2014). Por outro lado, a participação do sistema opioidérgico na antinocicepção periférica também vem sendo bastante estudada no decorrer das últimas décadas (Smith, 2008). Por exemplo, peptídeos opioides inibem a sensibilização dos neurônios aferentes primários promovida pela PGE_2 ao inibir a atividade da adenilato ciclase (Sharma et al., 1977). Assim, a ativação de receptores opioides inviabiliza a ativação de proteínas cinases A (PKA) (Ferreira e Nakamura, 1979), que seriam ativadas mediante a administração de PGE_2. Portanto, o sistema opioidérgico impede que haja alteração da cinética de canais para sódio e potássio voltagem dependentes no sentido de aumentar a excitabilidade neuronial. Além disso, foi descrito que a injeção de PGE_2 na superfície plantar de ratos (mesma via de administração de PGE_2 utilizada nesta tese) tem um efeito direto na sinalização do sistema opioidérgico, uma vez que esta induziu uma expressão diferencial de receptores opioides tanto no tecido da pata do animal, quanto nos neurônios DRG

(Zambelli et al., 2014). Desta maneira o estudo da ativação da via dos opioides no sistema nervoso central e periférico se torna um alvo bastante interessante para análise do mecanismo de ação envolvido na indução da antinocicepção desencadeada pelo PnPP-19.

Para avaliar o envolvimento dos opioides no efeito antinociceptivo do PnPP-19, o antagonista opioide inespecífico naloxona foi utilizado. Observamos que a naloxona reverteu parcialmente o efeito antinociceptivo central e periférico desencadeado pelo peptídeo. Isso demonstra que a ativação do sistema opioidérgico está envolvida na antinocicepção evocada pelo peptídeo, porém é provável que ocorra a ativação concomitante de outras vias, já que a reversão pelo antagonista foi apenas parcial.

Com o intuito de se estabelecer os subtipos específicos de receptores opioides envolvidos na antinocicepção evocada pelo peptídeo, foram administrados antagonistas específicos para os receptores μ-, δ- e κ-opioides. Os resultados obtidos mostraram que o antagonista μ-opioide e o antagonista δ-opioide reverteram parcialmente o efeito antinociceptivo central e periférico do peptídeo, porém a administração do antagonista κ-opioide não foi capaz de antagonizar o efeito do PnPP-19 no SNC e SNP. Portanto, provavelmente, a ativação da via opioidérgica pelo peptídeo se dá pela ativação de receptores μ- e δ-opioides, sem a participação dos receptores κ-opioides. Esses achados adicionam evidências para a participação da via dos opioides na antinocicepção central e periférica induzida por PnPP-19, entretanto, outras vias também podem estar envolvidas no mecanismo de ação, visto que, de maneira semelhante a naloxona, o antagonismo observado foi parcial.

Os dois receptores envolvidos na antinocicepção do PnPP-19, μ- e δ-opioides, são capazes de formar heterodímeros, e a ativação dos receptores δ-opioides, por exemplo, afeta a interação de agonistas μ-opioides com seu receptor específico, bem como a sinalização desencadeada pela ativação de receptores μ (Gupta et al., 2010; Gomes et al., 2011). Além disso, dentre os peptídeos opioides endógenos, as Leu-encefalinas e as Met-encefalinas se ligam tanto a receptores μ quanto δ, sem se ligar a receptores κ. (Rang et al., 1995; Mcdonald & Lambert, 2005). Portanto, uma hipótese levantada é que PnPP-19 poderia estar atuando como um agonista μ- e/ou δ-opioide ou o peptídeo poderia estar modulando os níveis de encefalina, ou outros opioides endógenos, nos tecidos.

Somente poucas toxinas isoladas de peçonhas animais induzem antinocicepção através da ativação de receptores opioides. Dentre elas estão a neurotoxina isolada da peçonha da serpente *Ophiophagus hannah*, a peçonha bruta da serpente *Micrurus lemniscatus*, duas toxinas de escorpião (AmmVIII e LqqIT2), alguns peptídeos sintéticos baseados em conotoxinas e a crotalfina, peptídeo isolado da peçonha da serpente *Crotalus durissus terrificus* (Pu et al., 1995;

Martin-Eauclaire et al.,2010; Leitedos Santos et al., 2012; Machado et al., 2014; Zambelli et al., 2014; Deuis et al., 2015; Brust et al., 2016). Esta última toxina, crotalfina, tem tido o seu efeito bastante caracterizado na via nociceptiva. O efeito antinociceptivo periférico desta molécula é revertido totalmente pela administração do antagonista específico de receptores κ-opioides (Zambelli et al., 2014). De maneira interessante, o efeito antinociceptivo deste peptídeo parece envolver tanto o sistema opioide, quanto o canabinoide. A administração do antagonista específico para receptores canabinoides CB_2 bloqueou o efeito antinociceptivo da crotalfina, sendo que por estudos de imunohistoquímica houve a confirmação de que crotalfina induz ativação de receptores κ-opioides e canabinoides CB_2 (Machado et al., 2014).

Nos últimos anos tem sido bastante descrita a ativação concomitante e interdependente da via dos opioides e dos canabinoides (Maldonado e Valverde, 2003; Bushlin et al., 2010). Alguns estudos têm mostrado, por exemplo, que a administração de agonistas canabinoides pode levar a liberação de peptídeos opioides endógenos (Welch & Eads, 1999). Por outro lado, tem sido mostrado que a antinocicepção central e periférica produzida pela morfina envolve a liberação de endocanabinoides que, por sua vez, ativam receptores CB_1 (Pacheco et al., 2008, Pacheco et al., 2009). Diante dos achados de que opioides estão envolvidos no mecanismo de ação de PnPP-19, testamos a participação da via dos canabinoides.

Os resultados mostraram que o antagonista para receptores canabinoides do tipo CB_1, mas não o antagonista para os receptores canabinoides CB_2, foi capaz de reverter parcialmente o efeito antinociceptivo do peptídeo PnPP-19 no SNC e SNP. Assim, a ação antinociceptiva do PnPP-19 parece ser dependente da ativação concomitante da via dos opioides e dos canabinoides.

Há evidência significativa de que ocorre associação entre receptores μ-opioides e receptores CB_1 (Rios et al., 2006). Já foi também demonstrado que há a formação de heterodímeros entre receptores δ-opioides e receptores canabinoides do tipo CB_1 (Bushlin et al., 2012; Rozenfeld et al., 2012). A interação entre os receptores δ-opioides e CB_1 permite que a ligação de um agonista CB_1 ao seu receptor aumente a ligação do agonista δ-opioide ao seu receptor opioide específico, aumentando assim a sinalização via opioide. Portanto, a ativação dos receptores CB_1 pode influenciar diretamente a sinalização via receptor μ- e δ-opioide, e vice e versa. Todos esses fatos combinados demonstram a alta interação de receptores μ- e δ-opioides com os receptores CB_1, evidenciando que os resultados gerados na análise do mecanismo de ação do PnPP-19 estão em conformidade com os dados da literatura.

Dentre os receptores canabinoides, os receptores do tipo CB_1 são os mais expressos em todo o corpo, sendo encontrados tanto no SNC (Herkenham et al., 1990; Hohmann et al., 1999),

quanto no SNP (Fox et al., 2001). O receptor CB_1 é o alvo principal de agonistas canabinoides exógenos e endógenos, sendo que a antinocicepção induzida por canabinoides é mediada, principalmente, pela ativação destes receptores (Agarwal et al., 2007). O endocanabinoide anandamida ativa preferencialmente os receptores CB_1 (Lin et al., 1998), e a antinocicepção induzida por este canabinoide endógeno é dependente da ativação de receptores opioides (Richardson et al., 1998; Reis et al., 2009). Nossos resultados mostram que a administração de inibidores da recaptação de endocanabinoides e da degradação da anandamida, em doses sem potencial antinociceptivo, potencializam o efeito de uma baixa dose de PnPP-19. Isto sugere que o peptídeo possa induzir a liberação de canabinoides endógenos, e, mais especificamente, provavelmente induz liberação de anandamida, deixando esta mais biodisponível para ativar receptores CB_1. Entretanto, não podemos descartar a hipótese de que outros endocanabinoides estejam participando na indução do efeito anti-hiperalgésico desencadeado por PnPP-19, ou que este último possa atuar como um agonista canabinoide.

De maneira interessante, além do efeito analgésico, os canabinoides também estão envolvidos na função erétil, podendo até contribuir para a ocorrência de priapismo em determinadas situações (Matta et al., 2014). Somente os receptores do tipo CB_1 são expressos no tecido do corpo cavernoso, e o endocanabinoide anandamida é capaz de potencializar o relaxamento deste tecido em ensaios *in vitro* (Ghasemi et al., 2006). Vale lembrar que o peptídeo PnPP-19 vem sendo bastante estudado como modelo de fármaco para o tratamento da disfunção erétil (Silva et al., 2015).

Muitos estudos têm mostrado que a ativação da via do óxido nítrico (NO) está envolvida no mecanismo de ação pelo qual receptores opioides (Ferreira et al., 1991; Amarante e Duarte, 2002; Pacheco et al., 2005) e canabinoides (Reis et al., 2009; Negrete et al., 2011) induzem antinocicepção. Dessa forma, como a antinocicepção do PnPP-19 é mediada pela ativação destes receptores, foram realizados experimentos para avaliar a participação do óxido nítrico na atividade antinociceptiva do PnPP-19.

Os dados obtidos mostraram que o inibidor não seletivo da NOS e o inibidor seletivo de nNOS foram capazes de antagonizar parcialmente o efeito antinociceptivo do PnPP-19, sugerindo que este efeito se deve, pelo menos parcialmente, ao aumento da concentração de NO. É muito difícil determinar os níveis de NO em tecidos, pois o NO intracelular é rapidamente oxidado em nitrito (NO_2^-) e nitrato (NO_3^-) (Ignarro et al., 1993). Sendo assim, os níveis de nitrito foram analisados afim de que pudéssemos inferir indiretamente se o tratamento com PnPP-19 induziu ou não um aumento dos níveis de NO. Os resultados mostram que o grupo tratado com PnPP-19 tem um aumento significativo dos níveis de nitrito nos tecidos,

sendo que a indução deste aumento das concentrações de nitrito é bloqueada pela administração do inibidor não seletivo da NOS. Desta maneira, comprovamos que o aumento dos níveis de NO_2^- é via ativação da NOS.

A ativação de receptores opioides e canabinoides pode modular a produção de NO via ativação de proteínas cinases, tais como a cálcio calmulina cinase (CaMKII) e a Akt, também conhecida como proteína cinase B (PKB) (Gómez Del Pulgar et al., 2002; Sánchez-Blázquez et al., 2010; Pacher e Mackie, 2012). Estas são serina/treonina cinases capazes de modular a atividade de diferentes isoformas de NOS (Michell et al., 1999; Rameau et al., 2004). Nossos resultados sugerem que a expressão da nNOS e eNOS em todos os grupos tratados se mantém a mesma, sendo que a modulação da atividade das NOS se dá pela fosforilação dos seus sítios de ativação e inibição. A administração de PGE_2 induz uma inibição da atividade da eNOS e nNOS. Entretanto, o tratamento dos animais com PnPP-19 faz com que este estado de inativação seja revertido e as enzimas passem a ficar mais ativas. Estes resultados estão de acordo com com ensaios comportamentais, que mostram o bloqueio da atividade antinociceptiva do PnPP-19 pela administração de inibidores da NOS, e com os resultados que demonstram o aumento na concentração de nitrito induzido nos tecidos após administração do peptídeo.

O papel da nNOS na via nociceptiva já foi descrito como parte do mecanismo de ação de diversas substâncias. A peçonha bruta da serpente *Crotalus durissus terrificus* e a toxina crotalfina, isolada desta mesma peçonha, induzem antinocicepção mediada pela ativação de nNOS (Picolo e Cury, 2004; Gutierrez et al., 2012). Usando o método algesimétrico de compressão da pata, pode-se observar que muitas outras substâncias antinociceptivas, tais como acetilcolina; anandamida; morfina; agonistas δ- e κ-opioides e os fármacos analgésicos não-esteroidais dipirona e diclofenaco, estimulam seletivamente a isoforma nNOS para induzir antinocicepção periférica (Romero et al., 2011b). Neste trabalho, mostramos o possível envolvimento da eNOS na sinalização da via nociceptiva, uma vez que a administração da molécula hiperalgésica PGE_2 diminuiu a fosforilação do sítio de ativação da eNOS e a injeção de PnPP-19 estimulou a ativação desta enzima. Este efeito está, de certa maneira, em desacordo com dados da literatura, pois os mesmos demonstraram apenas a importância seletiva de nNOS na antinocicepção. Isto pode ser resultado da falta de potentes inibidores seletivos de eNOS, o que prejudica a avaliação do seu envolvimento na via nociceptiva em ensaios comportamentais.

Seguindo a cascata de sinalização na via nociceptiva desencadeada pelo aumento dos níveis de NO, este estimula a guanilato ciclase, aumentando assim os níveis de GMPc, que por sua vez induzem a ativação da proteína cinase G (PKG). Esta proteína fosforila sítios

específicos de canais para potássio sensíveis ao ATP (K_{ATP}), ativando-os e diminuindo a excitabilidade neuronial (Han et al., 2001). Desta maneira, muitos estudos já demonstraram que substâncias analgésicas que causam a ativação da via NO-GMPc induzem antinocicepção exatamente por estimular a abertura de K_{ATP}. Um exemplo é o agonista μ-opioide morfina que induz efeito antinociceptivo periférico dependente da ativação da via NO/K_{ATP} (Cunha et al., 2010). Outros trabalhos também demonstraram que a ativação de receptores canabinoides CB_1 estimulam a produção de GMPc em células neuroniais (Jones et al., 2008) e que a antinocicepção causada pelo agonista deste receptor é mediada pela ativação da via NO/GMPc (Reis et al., 2009). PnPP-19 induz ativação de receptores canabinoides e opioides, e nossos resultados estão de acordo com os dados da literatura na medida que a administração do inibidor específico da enzima guanilato ciclase e do bloqueador de K_{ATP} inibiram, mesmo que parcialmente, a antinocicepção induzida pelo peptídeo sintético.

Considerando a atividade do PnPP-19 como potenciador da função erétil, alguns medicamentos utilizados para o tratamento de disfunção erétil podem induzir antinocicepção, assim como o peptídeo. Vardenafil, Sildenafil e Tadalafil, inibidores da enzima fosfodiesterase tipo 5 (PDE_5), já tiveram o seu efeito na via nociceptiva investigado. Todos eles induzem um efeito anti-hiperalgésico, e a ativação da via NO-GMPc parece mediar sua atividade na via da dor (Jain et al., 2001; Gediz et al., 2015; Otari e Upasani, 2015). A correlação entre a ativação da via NO-GMPc e antinocicepção era de ser esperada para estes medicamentos, uma vez que eles são inibidores da enzima PDE_5, enzima esta responsável pela degradação de GMPc. Curiosamente, a antinocicepção induzida pelo Sildenafil parece estar associada à ativação de receptores μ- e δ-opioides (Yoon et al., 2008). Estes são os mesmos receptores envolvidos na antinocicepção periférica induzida por PnPP-19.

Assim como a toxina nativa, PnTx2-6, o peptídeo PnPP-19 foi capaz de induzir a liberação de L-glutamato em sinaptosomas de córtex cerebral de ratos (Silva, 2012). Acredita-se que a ativação do receptor NMDA por este neurotransmissor excitatório seja essencial para a sensibilização espinal e desenvolvimento de um estado hiperalgésico (Meller e Gebhart, 1993). Ainda, a administração de L-glutamato via intraplantar em modelos murinos é capaz de induzir hiperalgesia (Leem et al., 2001; de Oliveira, 2010). Portanto, era de se esperar que o peptídeo PnPP-19 tivesse ação hiperalgésica, no entanto já foi demonstrado que o óxido nítrico é capaz de provocar liberação de glutamato em sinaptosomas de cérebro de rato (Mcnaught e Brown, 1998). Como demonstrado, o peptídeo ativa a via do óxido nítrico, sendo assim, a liberação de glutamato observada nos sinaptosomas pode ser resultado do aumento dos níveis de óxido nítrico na preparação.

A ferramenta bioinformática conhecida como SEA (*similarity ensemble approach*) vêm sendo utilizada para a predição de alvos moleculares de substâncias de interesse farmacológico (Gregori-Puigjané et al., 2012). Estudos sugerem que o mecanismo de ação de muitas das drogas utilizadas atualmente, cerca de 7%, ainda permanecem desconhecidos. Para tentar solucionar este problema, Gregori-Puigjané e colaboradores, em 2012, sugeriram a utilização do programa SEA para predição de alvos moleculares, com subsequentes ensaios *in vitro,* para confirmação do alvo predito. Neste estudo foram escolhidas 7 drogas já aprovadas para uso terapêutico, porém com seus respectivos alvos moleculares ainda desconhecidos. O programa SEA foi capaz de predizer alvos para todas elas, o que, posteriormente, pôde ser confirmado em ensaios *in vitro.* Portanto, a capacidade do programa SEA em predizer de maneira rápida e pouco dispendiosa alvos moleculares, que podem ser confirmados experimentalmente, apoia a ideia de que esse método é uma interessante ferramenta de auxílio na identificação dos mecanimos de ação de determinadas moléculas.

Quando o peptídeo PnPP-19 foi submetido a análise pelo programa SEA, os mesmos resultados foram gerados pelos dois bancos de dados utilizados. No *ranking* gerado, o segundo melhor alvo predito era correspondente a enzima Neprilisina (*E-value* = 5.45 x 10^{-39}). Esta enzima, também conhecida como encefalinase, é responsável por clivar o opioide endógeno encefalina (Matsas et al, 1983). Sendo assim, a administração de inibidores para a Neprilisina é descrita por induzir atividade analgésica via opioides (Rougeot et al., 2003; Wisner et al., 2006; Yang et al., 2011). Por outro lado, os inibidores endógenos desta enzima estão intimamente envolvidos com os processos da função erétil (Wisner et al., 2006; Davies, 2009). Como já dito anteriormente, além da atividade antinociceptiva do PnPP-19 demonstrada nesta tese, este peptídeo tem sido estudado por colegas em nosso Laboratório como potenciador da função erétil (Silva et al., 2015). Dessa maneira, a enzima Neprilisina foi escolhida para os ensaios subsequentes, *in vitro,* a fim de se verificar uma possível atividade inibitória do peptídeo sobre a enzima.

O primeiro objetivo foi avaliar se o peptídeo atua como substrato da enzima. Foi possível observar que a Neprilisina é capaz de hidrolisar o PnPP-19 apenas em tempos de incubação muito longos, o que não é o caso dos ensaios *in vivo* (pico de ação do peptídeo ocorre cinco minutos após a sua administração em modelos animais). Não houve hidrólise do peptídeo pela enzima durante um período de incubação de até duas horas, indicando que o mesmo não é um bom substrato para a enzima, e, provavelmente, deve apresentar um k_{cat} (constante de catálise: número de moléculas do substrato que são convertidas pela enzima por unidade de tempo) muito baixo.

A enzima foi capaz de hidrolisar o peptídeo em seis regiões distintas somente quando incubada com o mesmo por um período relativamente longo, *overnight*. A sequência primária do peptídeo possui vários resíduos hidrofóbicos, e a neprilisina foi capaz de clivar o PnPP-19 sempre em regiões próximas a resíduos aromáticos (tirosina, fenilalanina e triptofano). Estes dados corroboram o que já é descrito na literatura, pois a enzima tem especificidade em clivar sempre próximo a aminoácidos aromáticos e hidrofóbicos (Hersh e Morihara, 1986).

Nos ensaios de inibição enzimática, o peptídeo PnPP-19 foi capaz de inibir a enzima Neprilisina apresentando uma constante de inibição (K_i) de 35,63 ± 1,4 μM. Estes ensaios foram realizados por um período máximo de dez minutos, portanto, neste tempo, é possível afirmar que o peptídeo atuou exclusivamente como inibidor, não sendo clivado. O Tiorfan, inibidor sintético não peptídico da neprilisina, é descrito na literatura por apresentar um K_i de aproximadamente 3,5 nM (Roques et al., 1983; Roques, 1985). Em comparação, a afinidade aparente do PnPP-19 pela neprilisina é muito mais baixa do que a afinidade do tiorfan pela mesma enzima. Entretanto, a constante de afinidade da enzima pelo seu substrato endógeno encefalina varia muito na literatura, entre 61,4 μM, 125 μM, 137 μM e 200 μM, dependendo da metodologia utilizada (Akasaki e Tsuji, 1991; Iwamoto et al., 1991; Shimamura et al., 1991; Lian et al., 1996). Dessa forma, decidimos calcular o K_i para a encefalina utilizando a mesma metologia que empregamos para análise do PnPP-19 como inibidor da neprilisina. O K_i gerado para a encefalina nos nossos testes foi de 14.6 ± 0.44 μM, indicando que os valores de K_i gerados para o PnPP-19 e para a encefalina são bastantes próximos. Considerando que o PnPP-19 é clivado pela neprilisina somente depois de um longo período de incubação com a mesma, sugerimos que quando o peptídeo é administrado nos animais, este pode competir com a encefalina pelo sítio catalítico da enzima neprilisina. Desta maneira, o tratamento dos animais com PnPP-19 pode causar uma diminuição da degradação da encefalina, deixando-a mais disponível para se ligar aos receptores μ- e δ-opioides e desencadear uma resposta antinociceptiva.

Os inibidores peptídicos endógenos da neprilisina apresentam homólogos em humanos e ratos. Em humanos este inibidor é chamado de opiorfina (Wisner et al., 2006) e em ratos (*Rattus norvegicus*) sialorfina (Rougeot et al., 2003). Ambos possuem atividade antinociceptiva mediada por opioides e sua administração causa ativação de receptores μ- e δ-opioides (Rougeot et al., 2003; Wisner et al., 2006; Yang et al., 2011), assim como o PnPP-19. A expressão dos genes correspondentes a estes inibidores é baixa em indivíduos com disfunção erétil (User et al., 2003; Tong et al., 2008; Davies, 2009), demonstrando o importante papel de inibidores da neprilisina na função erétil. Como citado anteriormente, o peptídeo PnPP-19

também atua em processos relacionados a ereção (Silva et al., 2015). Além disso, já foi demonstrado que a utilização de inibidores desta enzima é capaz de aumentar a produção de óxido nítrico (Zhang et al., 1999). Mais uma vez, estes dados estão de acordo com nosso trabalho, pois o efeito antinociceptivo do peptídeo PnPP-19 é dependente do aumento dos níveis de óxido nítrico.

Outro possível alvo molecular sugerido pelo programa SEA para o PnPP-19 foi o receptor μ-opioide (quarto lugar no *ranking*, *E-value* = 1,14 x 10^{-36}). Nossos resultados corroboram a hipótese gerada pelo programa, uma vez que, dentre os receptores μ-, δ- e κ-opioides, demonstramos que PnPP-19 é capaz de se ligar e ativar somente os receptores μ-opioides. Dentre todas as toxinas isoladas da peçonha da aranha *Phoneutria nigriventer*, somente uma induz antinocicepção mediada por receptores opioides. A toxina PnTx4(6-1) tem seu efeito antinociceptivo mediado pela ativação dos receptores μ- e δ-opioides (estes receptores são os mesmos ativados após administração de PnPP-19 em ensaios *in vivo*). Entretanto, nenhum ensaio foi realizado até o momento para verificar se esta toxina consegue, de fato, atuar como um agonista direto dos receptores opioides mencionados acima (Emerich et al., 2016).

Apesar de existirem atualmente vários agonistas opioides utilizados no tratamento da dor, o desenvolvimento de novos agonistas para estes receptores é de grande importância clínica. Uma boa parte dos fármacos analgésicos utilizados, como a morfina, o fentanil e o oximorfona, provocam tanto o seu efeito farmacológico benéfico como seus efeitos colaterais indesejáveis através da ativação dos receptores μ-opioides (Benyamin et al., 2008). A utilização de medicamentos constituídos por agonistas opioides pode induzir diversos efeitos colaterais, sendo que um efeito colateral muito grave pode ser a indução de depressão respiratória (Wilson e Saukkonen, 2004; Benyamin et al., 2008). Uma vez que os receptores opioides ainda são um dos alvos mais relevantes para o tratamento da dor, muitas pesquisas têm focado no desenvolvimento de novos agonistas opioides que induzam uma potente analgesia, porém, causando menos efeitos colaterais graves (Dewire et al., 2013; Soergel et al., 2014). Atualmente, já se sabe que a indução de paralisia respiratória, bem como outros efeitos colaterais induzidos pelo uso de opioides, pode estar ligado ao recrutamento da via da β-arrestina. O recrutamento desta via pode ocorrer como consequência da ativação dos receptores μ-opioides (Bohn et al., 1999, 2000, 2002; Raehal et al., 2005). Desta forma, o fato de o PnPP-19 não induzir o recrutamento da via da β-arrestina2 destaca o uso deste peptídeo como ferramenta para o possível desenvolvimento de um fármaco opioide com características mais

favoráveis. Recentemente, um agonista μ-opioide bastante seletivo e potente foi desenvolvido e denominado PZM21. Apesar de sua grande potência e seletividade em se ligar e ativar os receptores μ-opioides, a administração de PZM21 induziu um recrutamento mínimo de β -arrestina2. Sendo assim, o uso de PZM21 induziu uma analgesia de longa duração, porém, alguns efeitos colaterais, como depressão respiratória e constipação, foram bastantes reduzidos se comparados ao uso da morfina (Manglik et al., 2016).

Além disso, nossos resultados sugerem que o peptídeo possa se ligar a um sítio do receptor diferente daquele que o agonista μ-opioide seletivo DAMGO se liga, uma vez que a presença de 10 μM de PnPP-19 não reduziu o recrutamento de β-arrestina2 induzido por DAMGO. No entanto, uma outra hipótese que pode explicar a falta de recrutamento de β-arrestina2 induzida por PnPP-19 é a provável baixa afinidade pela qual este peptídeo se liga aos receptores μ-opioides. Portanto, é necessária uma investigação mais aprofundada para elucidar o mecanismo exato da interação do PnPP-19 com estes receptores.

A interação entre receptores opioides e canais iônicos tem sido assunto de grande interesse durante décadas. Vários estudos sugerem que a ativação de receptores opioides causa hiperpolarização celular e, consequentemente, previne a liberação de neurotransmissores ao induzir a inibição dos canais para cálcio (Rhim e Miller, 1994; Piros et al., 1996; Law et al., 2000) e estimular a abertura dos canais para potássio (North et al., 1987; Schneider et al., 1998; Law et al., 2000; Marker et al., 2005).

A contribuição da ativação dos canais para cálcio voltagem dependentes (VGCCs) na transmissão e modulação da dor é de extrema importância. A transmissão da dor começa a partir da ativação de nociceptores presentes nos terminais axonais periféricos, e, a partir daí, os potenciais de ação gerados se propagam ao longo das fibras aferentes dos neurônios DRG. O estímulo é então conduzindo para a terminação axonal dos neurônios DRG presentes no corno dorsal da medula espinal. Quando o potencial de ação atinge esta região, ocorre a despolarização da membrana e ativação dos VGCCs. A ativação destes canais permitirá o influxo de cálcio e consequente liberação de neurotransmissores excitatórios na fenda sináptica. Estes neurotransmissores podem, então, causar a ativação de neurônios e interneurônios presentes na medula. Além disso, os VGCCs podem contribuir para a modulação tanto ascendente, quanto descendente da transmissão do estímulo nociceptivo no sistema nervoso central. Assim, as mudanças na expressão e na cinética dos VGCCs podem ter consequências dramáticas na transmissão da dor (Park e Luo, 2010). Os VGCCs são classificados como dos tipos L- (Cav1.1-1.3), P/Q- (Cav2.1), N- (Cav2.2), R-(Cav2. 3) e do tipo T- (Cav3.1-3.3). Todos

estes tipos estão envolvidos em algum nível nos processos de sinalização da via nociceptiva, entretanto, os canais do tipo N- e T- são considerados os principais alvos para o desenvolvimento de novos fármacos analgésicos (Zamponi et al., 2009).

Com relação à interação entre a via dos opioides e os VGCCs, já foi demonstrado, por exemplo, que a incubação de células HEK293, que co-expressavam receptores μ-opioides e canais para cálcio do tipo N (Cav2.2) ou do tipo R (Cav2.3), com um agonista seletivo μ-opioide induziu uma inibição de ambos os canais para cálcio testados (Berecki et al., 2016). Além disso, experimentos utilizando cultura primária de neurônios aferentes vestibulares e neurônios DRG sugerem que a estimulação seletiva dos receptores μ-opioides pode inibir os canais para cálcio do tipo T, L e N. Este evento ocorre, supostamente, através da ativação dos receptores opioides e não por um bloqueio direto do agonista opioide sobre os canais (Schroeder et al., 1991; Rusin e Moises, 1995; Sesena et al., 2014). Nossos resultados demonstram que o PnPP-19 inibe o influxo de cálcio em neurônios DRG e que essa inibição é suprimida pelo antagonista opioide naloxona, mostrando a participação dos receptores opioides neste efeito. Porém, mais experimentos são necessários para a determinação de quais isoformas dos VGCCs estão de fato envolvidas no efeito do PnPP-19 na via nociceptiva.

Recentemente, demonstrou-se que o DAMGO (agonista μ-opioide seletivo) induz inibição do influxo de cálcio e do potencial de ação em fibras aferentes individuais provenientes do gânglio trigeminal. Os autores também demonstraram que a ativação dos canais para potássio ativados por cálcio de "alta condutância" (BK_{Ca}) medeia essa inibição do influxo de cálcio induzido por DAMGO. Sugeriu-se, como mecanismo adicional, que a ativação dos receptores μ-opioides levaria a abertura dos canais para potássio, e, dessa maneira, causaria uma hiperpolarização da célula, o que induziria o bloqueio dos canais para cálcio. Portanto, o bloqueio dos canais para cálcio seria mediado também pela ativação dos canais para potássio. Alguns experimentos comportamentais para avaliação do papel de BK_{Ca} na nocicepção induzida pela ativação dos neurônios do gânglio trigeminal foram realizados. Estes experimentos comprovaram que a ativação destes subtipos de canais para potássio desempenha um grande papel na antinocicepção induzida pela administração de agonistas μ-opioides (Baillie et al., 2015). Os resultados desta tese, obtidos por ensaios comportamentais, demonstram que a atividade antinociceptiva do PnPP-19 é dependente da ativação de canais para potássio K_{ATP}. Entretanto, é provável que outros tipos de canais para potássio possam estar envolvidos na antinocicepção induzida por PnPP-19. Ademais, é possível que consigamos observar também a modulação dos canais para potássio induzida pelo peptídeo em ensaios *in*

vitro, como, por exemplo, em experimentos de *whole cell patch clamp*. No entanto, novos experimentos são necessários afim de se investigar a influência do PnPP-19 na cinética dos canais para potássio e determinar quais são as isoformas que de fato podem ser moduladas pelo peptídeo.

Concluindo, os resultados deste estudo demonstram, pela primeira vez, o efeito antinociceptivo central e periférico desencadeado pelo peptídeo sintético PnPP-19. O mecanismo de ação proposto para o peptídeo está descrito na Figura 11: PnPP-19 pode se ligar diretamente aos receptores opioides e ativa-los (1). Como mecanismo adicional, o peptídeo pode atuar também inibindo a enzima neprilisina, e, consequentemente, induzir um aumento dos níveis do opioide encefalina no domínio extracelular (2). O aumento dos níveis de encefalina seguida da ativação dos receptores opioides por estes opioides endógenos (3) e por PnPP-19 (1) pode estimular a liberação de canabinoides endógenos (4). É provável que a ativação dos receptores CB_1 seja provocada pelo aumento dos níveis de anandamida, visto que foi demonstrado que a inibição da degradação da anandamida potencializa o efeito do peptídeo. Além disso, este canabinoide endógeno tem maior afinidade por receptores CB_1, receptor este envolvido na antinocicepção induzida por PnPP-19 (4). A ativação concomitante da via dos opioides e canabinoides pode ser facilitada também pela heterodimerização entre os receptores destas duas vias (não mostrado). Aliado a isso, a ativação de receptores opioides e canabinoides provoca a inibição da atividade da adenilato ciclase (5), o que diminue os níveis de AMPc e PKA (5). Adicionalmente, a ativação dos receptores μ-opiodes por PnPP-19 leva ao bloqueio dos canais para cálcio voltagem dependentes, o que diminui o influxo de cálcio (6). No que concerne ao sistema nitrérgico, a ativação dos receptores opioides e canabinoides possivelmente provoca um aumento dos níveis de óxido nítrico por causar ativação da eNOS e nNOS. O aumento da produção de óxido nítrico provoca, por sua vez, o aumento da atividade da guanilato ciclase, aumentando assim a concentração de GMPc, o que causa ativação de PKG. Esta proteína cinase é a responsável por fosforilar K_{ATP}, induzindo o aumento da condutância de íons potássio pela membrana celular (7). Toda essa cascata de sinalização leva à diminuição da excitabilidade neuronial e bloqueio da liberação de neurotrasmissores (8).

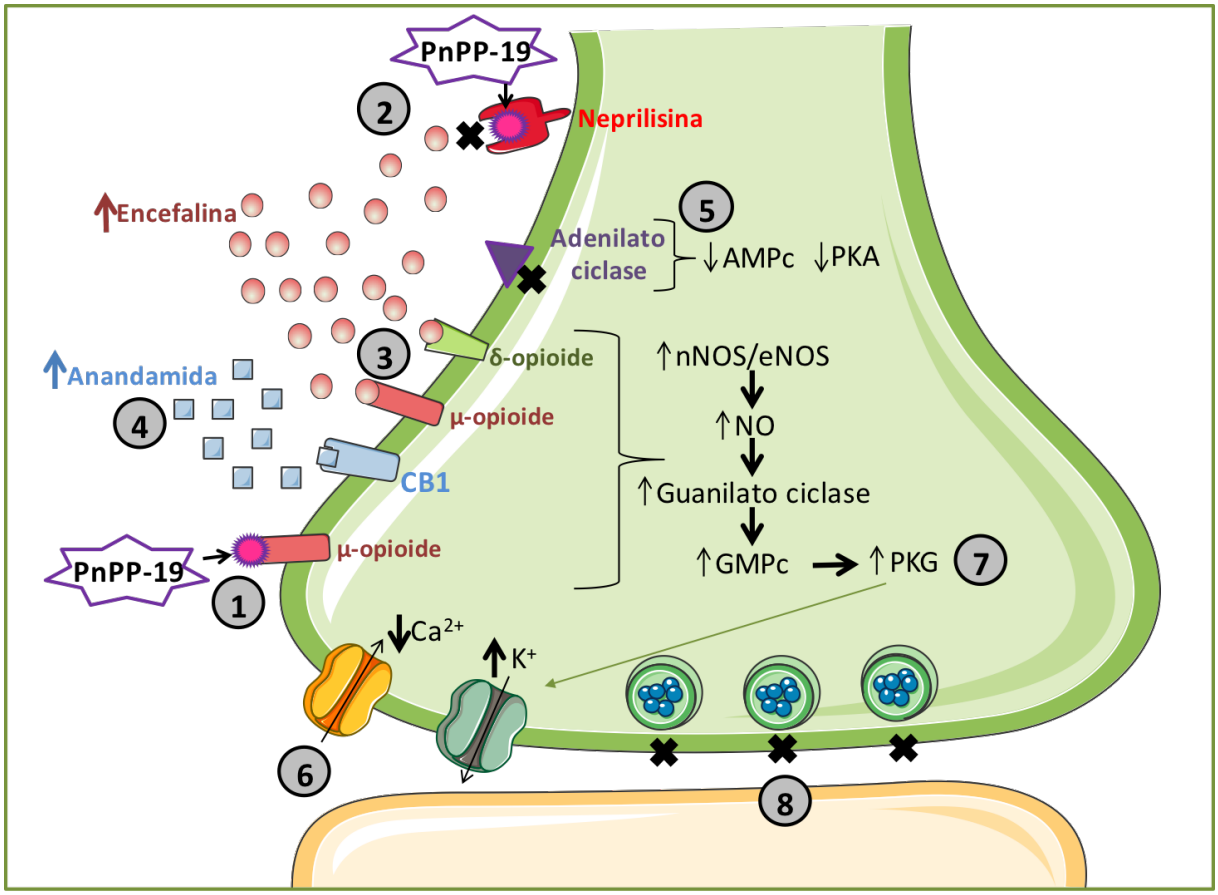
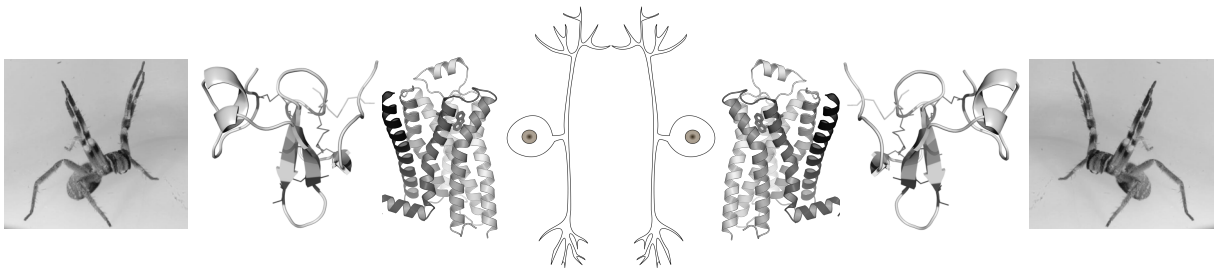


Figura 11: Desenho esquemático dos mecanismos de ação sugeridos para o peptídeo sintético PnPP-19.

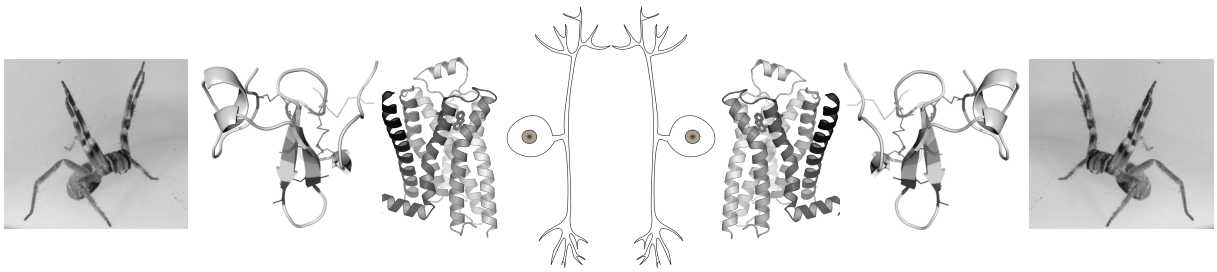
Conclusão



6 CONCLUSÃO

Há uma grande variedade de medicamentos analgésicos no mercado, dentre eles muitos têm ação nos sistemas opiode/canabinoide. Entretanto, a busca por novos fármacos que apresentem menos efeitos colaterais é de grande interesse, principalmente considerando-se os problemas decorrentes do uso dos opioides convencionais. O peptídeo PnPP-19, estudado neste trabalho, tem potencial para ser utilizado como ferramenta no desenvolvimento de novos medicamentos, visto que o mesmo induz antinocicepção central e periférica, e, possivelmente, poderá induzir menores efeitos colaterais quando comparado ao uso de outros agonistas opioides (Patente: INPI-BR1020140102680). Além do mais, o conhecimento do efeito antinociceptivo deste peptídeo e de seu mecanismo de ação dão suporte, pelo menos em parte, ao seu possível uso como fármaco para o tratamento da disfunção erétil.

Perspectivas



7 PERSPECTIVAS

- Analizar se o peptídeo PnPP-19 é capaz de induzir resposta antinociceptiva em outros modelos de dor;
- Verificar se a administração prolongada do peptídeo causa tolerância/dependência;
- Comparar a intensidade do efeito antinociceptivo desencadeado pelo peptídeo com fármacos comerciais;
- Dosar os níveis de encefalina após exposição do animal ao peptídeo;
- Dosar os níveis de anandamida após exposição do animal ao peptídeo;
- Determinar se ocorre alteração da cinética de canais para potássio induzida pelo peptídeo em experimentos de eletrofisiologia;
- Sintetizar novos peptídeos derivados da sequência primária do PnPP-19 afim de se obter peptídeo menores e com maior potência.

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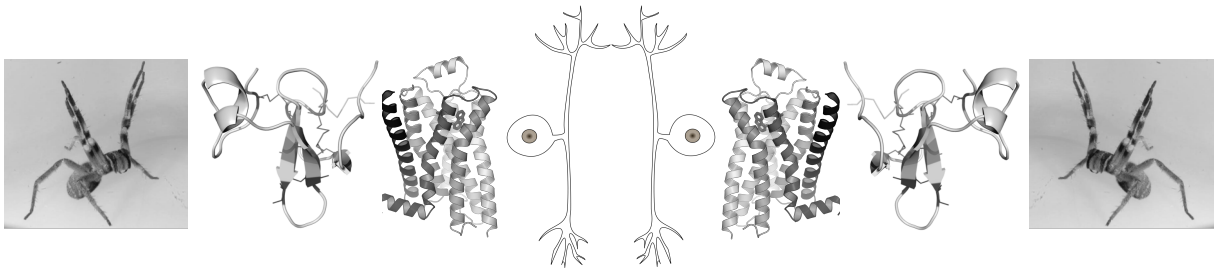

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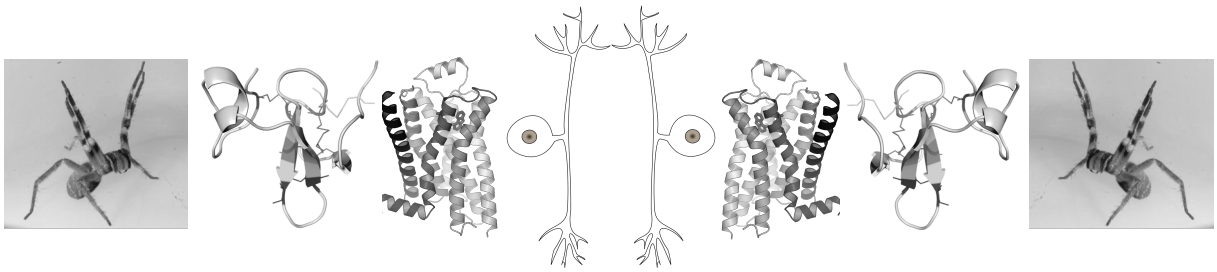
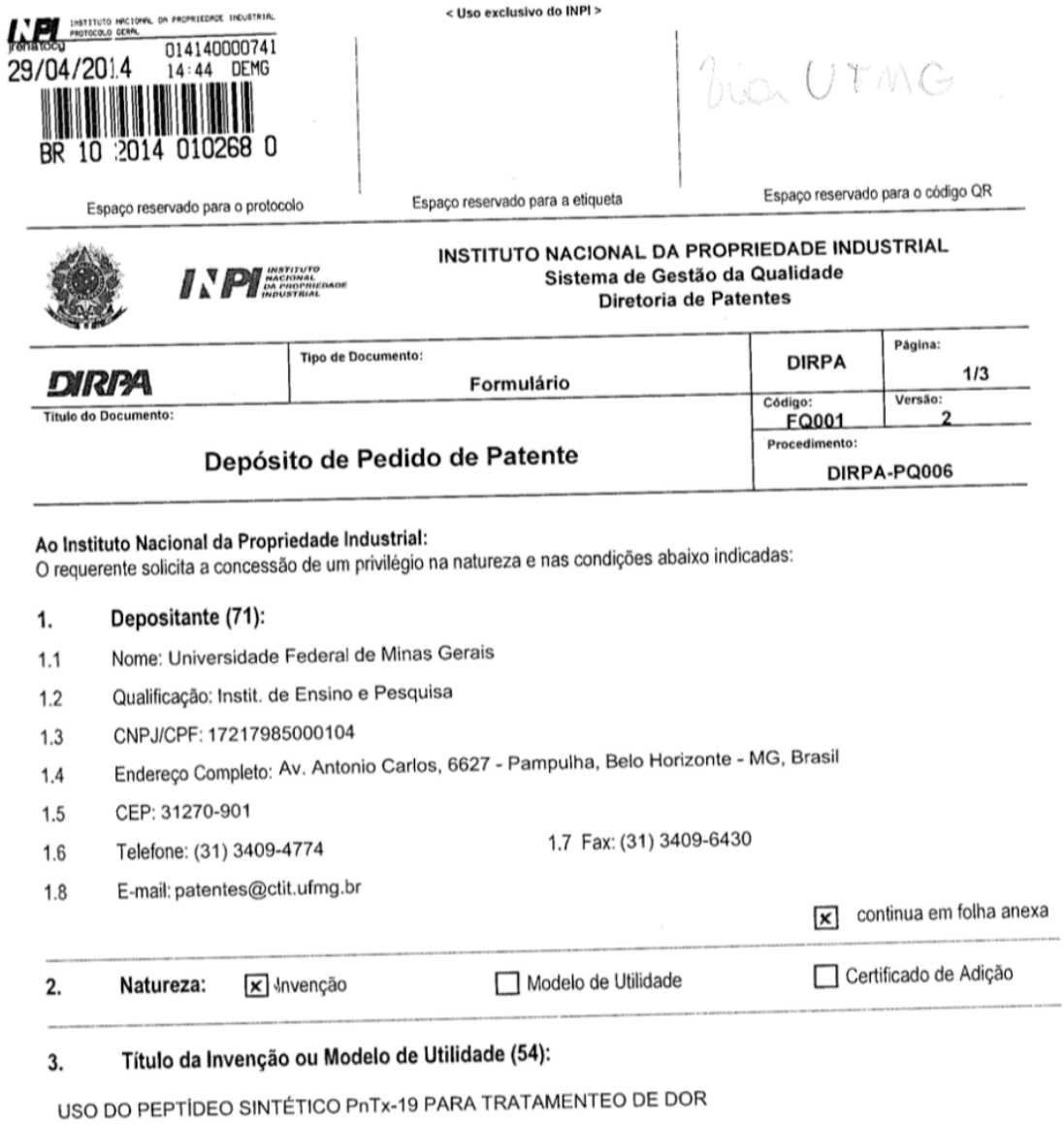
Anexos



ANEXO A- DEPÓSITO DE PATENTE.

Freitas ACN, Pacheco DF, Duarte IDG, de Lima ME. Patente: Privilégio de Inovação. Número do registro: BR1020140102680, título: " Uso do peptídeo sintético PnTx-19 para tratamento de dor". Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial, Depositante (s): Maria Elena de Lima Perez Garcia; Universidade Federal de Minas Gerais, Depósito: 29/04/2014. Instituições financiadoras: FAPEMIG; CNPq.



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		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: Universidade Federal de Minas Gerais
1.2 Qualificação: Instit. de Ensino e Pesquisa
1.3 CNPJ/CPF: 17217985000104
1.4 Endereço Completo: Av. Antonio Carlos, 6627 - Pampulha, Belo Horizonte - MG, Brasil
1.5 CEP: 31270-901
1.6 Telefone: (31) 3409-4774 1.7 Fax: (31) 3409-6430
1.8 E-mail: patentes@ctit.ufmg.br

☒ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

USO DO PEPTÍDEO SINTÉTICO PnTx-19 PARA TRATAMENTEO DE DOR

ANEXO B- CAPÍTULO DE LIVRO PUBLICADO DURANTE O DOUTORADO.

De Lima ME, Torres FS, Magalhães BLE, Freitas ACN. Perspecivas inovadoras para o uso terapêutico de toxinas da aranha "armadeira" Phoneutria nigriventer (KEYSERLING, 1891) na dor e na disfunção erétil. Biotecnologia aplicada à saúde: fundamentos e aplicações. 1ed. São Paulo: Blücher, v. 1, p. 532, 2015.

18 CAPÍTULO

PERSPECTIVAS INOVADORAS PARA O USO TERAPÊUTICO DE TOXINAS DA ARANHA "ARMADEIRA" *Phoneutria nigriventer* (KEYSERLING, 1891) NA DOR E NA DISFUNÇÃO ERÉTIL

Maria Elena de Lima
Fernanda Silva Torres
Bruna Luiza Emerich Magalhães
Ana Cristina Nogueira Freitas.

18.1 INTRODUÇÃO

Uma grande diversidade de animais evoluiu utilizando a peçonha como estratégia para defesa e predação. Muitos destes animais apresentam um aparato especializado (dente, ferrão, aguilhão) para a inoculação de peçonha.

ANEXO C- TRABALHO DE DOUTORADO APRESENTADO EM FORMA DE POSTER NO "15TH WORLD CONGRESS ON PAIN" EM 2014.

CERTIFICATE OF PRESENTATION

The following poster was submitted and presented at the 15th World Congress on Pain, held at the La Rural Convention Center in Buenos Aires, Argentina, October 6-11, 2014.

PW016

PNTX-19 INDUCES PERIPHERAL ANTINOCICEPTION MEDIATED BY MU- AND DELTA-OPIOID RECEPTORS

A. C. FREITAS, D. F. PACHECO, I. D. DUARTE, M. E. DE LIMA

The 15th World Congress on Pain is organized by the International Association for the Study of Pain.

Presented: 8 Octorber-2014

Srinivasa N. Raja, M.D.

Chair, Scientific Program Committee

Sponsored by:

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ANEXO D- TRABALHO DE DOUTORADO APRESENTADO EM FORMA DE POSTER NO "XIV CONGRESS OF THE BRAZILIAN SOCIETY OF TOXINOLOGY" EM 2017.

Toxinology in a comprehensive approach: from animal biology to toxins, envenomation and treatment

27th to 30th August - Santa Catarina, Brazil Costão do Santinho Resort

CERTIFIED

TOXINOLOGY 2017 XIV CONGRESS OF THE BRAZILIAN SOCIETY OF TOXINOLOGY

We hereby certify that

Ana Cristina Nogueira Freitas

has successfully attended and participated in the **XIV Congress of the Brazilian Society of Toxinology**, presenting the **poster**:

STUDY OF THE MECHANISM OF ACTION INVOLVED IN THE ANTINOCICEPTIVE EFFECT INDUCED BY THE SYNTHETIC PEPTIDE PNPP-19 AND ITS DERIVATIVE

With the authors
Ana Cristina Nogueira Freitas; Steve Peigneur; Marcelo Ferreira Marcondes Machado; Adriana Karaoglanovic Carmona; Jan Tytgat; Gareth Hathway; Maria Elena de Lima

Denise V. Tambourgi
President of the Brazilian Society of Toxinology

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