PUCRS

ESCOLA DE CIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR
DOUTORADO EM BIOLOGIA CELULAR E MOLECULAR

ANA PAULA AQUISTAPASE DAGNINO

CARACTERIZAÇÃO DO SISTEMA NOCICEPTINA/ORFANINA FQ-RECEPTOR NOP NA MODULAÇÃO DA FIBROMIALGIA EXPERIMENTAL

Porto Alegre
2019

PÓS-GRADUAÇÃO - *STRICTO SENSU*

Pontifícia Universidade Católica
do Rio Grande do Sul

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Ficha Catalográfica

D126c Dagnino, Ana Paula Aquistapase

Caracterização do sistema nociceptina/orfanina FQ receptor NOP na modulação da fibromialgia experimental / Ana Paula Aquistapase Dagnino . – 2019.

148.

Tese (Doutorado) – Programa de Pós-Graduação em Biologia Celular e Molecular, PUCRS.

Orientadora: Profa. Dra. Maria Martha Campos.

1. Fibromialgia. 2. Reserpina. 3. Nociceptina/orfanina FQ. 4. Receptor da nociceptina/orfanina FQ. 5. UFP-101. I. Campos, Maria Martha. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da PUCRS com os dados fornecidos pelo(a) autor(a).

Bibliotecária responsável: Salete Maria Sartori CRB-10/1363

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Porto Alegre
2019

Dedico este trabalho as pessoas mais importantes na minha vida:

Minha mãe, dentre todas as mulheres, a mais guerreira.

Meu pai, dentre todos os homens, o mais generoso.

Meu amor, Luis, o mais amoroso e gentil que conheço.

O convívio é uma benção. Amo demais vocês!!!

AGRADECIMENTOS

Aos meus pais, Rodolfo e Graciela Dagnino. Sinto muita gratidão de tê-los como pais, sempre tão amorosos e presentes em minha vida. Muitas vezes estive distante devido ao doutorado, mas sempre com vocês dentro do meu coração. Obrigada pela minha vida.

Ao meu amado Luis Fernando Kanan. Como é possível olhar lá no alto e ter os pés no chão? Nos momentos de risada que escondiam desespero. Homem inteligente, dedicado, bondoso e educado, um *gentleman*. Um suspiro de alento em minha insanidade. Minha alegria e meu bem querer.

À minha orientadora, Maria Martha Campos, muitas vezes amiga, muitas vezes mãe. O que melhor descreve esta mãe científica é o cuidado, o carinho, o capricho e a generosidade com seus alunos. Cientista íntegra, correta, de caráter. Totalmente dedicada à ciência, que não somente é um trabalho, mas uma vida. Bom Prof. Martha, acho que tu és a cereja do bolo do nosso laboratório. Uma vez um colega me desejou “Boa sorte”. E hoje vejo, sorte esta, que tive quando cheguei neste laboratório e aprendi tudo que aprendi com você.

Ao meu amigo e colega Pedro Chagastelles, que traz leveza para o dia-dia do laboratório, que muitas vezes é exaustivo. Cientista astuto, com humor a flor da pele. Descontraído, sem mesmices, criativo. As melhores pessoas na minha vida chegaram e se foram com muita facilidade. Espero encontrar esta alma em outras vidas, se possível ainda nesta, na Itália...

Ao meu amigo e colega Rodrigo Braccini, defensor de suas crenças e ideologias. Me acompanhou desde o começo da vida de doutorado, quando não sabia nem como pegar um camundongo. Organizado, regrado, entusiasta e cientista obstinado.

As minhas queridas amigas e colegas de laboratório, Priscilla Pail, Raquel Dal Sasso e Renata Medeiros, pela colaboração em experimentos, pelas risadas e por compartilhar dos mesmos sentimentos inerentes à busca pela Pós-graduação, tão almejada. Em nenhum momento esta caminhada foi individual.

Aos professores e colaboradores deste projeto, Dr. Samuel Greggio, Dra. Gianina Teribele, Dr. Jaderson Costa da Costa, Dr. Maurício Bogo e Dra. Talita Pereira, sem os quais este trabalho não teria sido tão completo e elegante. Sem dúvida a contribuição de vocês foi imensa para que este trabalho fosse promissor.

Aos técnicos de laboratório Rafaela Rubim, Juliano Soares, Moema Queiroz Vieira e Janaína Pasetti Nunes, extremamente solícitos e empenhados em auxiliar no que fosse preciso no laboratório, sempre nos passando seus conhecimentos nas técnicas utilizadas.

À equipe do CeMBE, em especial as queridas Priscila Alves da Silveira e Andressa Vargas Ribeiro, pelo bom humor e profissionalismo, sempre muito criteriosas no manejo com os animais.

“Às vezes todos os nossos pensamentos são inquietantes”

Led Zeppelin

RESUMO

A fibromialgia é caracterizada por dor generalizada, sendo acompanhada por distúrbios funcionais e afetivos. Este estudo avaliou a implicação do receptor do peptídeo nociceptina/ orfanina FQ (NOPr) em um modelo murino de fibromialgia. Os protocolos experimentais foram aprovados pela Comissão de Ética no Uso de Animais (CEUA/PUCRS 15/00487). A fibromialgia foi induzida em camundongos fêmeas CF-1 (20-24 g, 4 semanas de idade) pela administração de reserpina (0,25 mg/kg; via subcutânea), uma vez ao dia, durante 3 dias consecutivos. Grupos controle receberam veículo. No quarto dia, os camundongos foram tratados com uma dose única de nociceptina (N/OFQ) ou, do antagonista peptídico seletivo, UFP-101, administrados por via intraperitoneal (i.p., 0,3-5 nmol/kg), intracerebroventricular (i.c.v., 0,3 -1 nmol/sítio) ou intratecal (i.t., 0,3-5 nmol/sítio), 30 min antes das sessões experimentais. Em outra série de experimentos, os animais foram tratados com o antagonista UFP-101 (1 nmol/kg) ou, com o antagonista não peptídico, SB-612111 (6,6 µmol/kg), administrados por via intraperitoneal, durante três dias consecutivos, 30 min após a injeção diária de reserpina. No 4º dia, os animais também receberam um dos antagonistas, 30 min antes das avaliações comportamentais. Os animais foram submetidos aos testes de Von Frey, placa quente, nado forçado, labirinto em cruz elevado, rotarod e de preensão palmar. A expressão da pré-pró-nociceptina ppN/OFQ e do NOPr foi determinada por RT-qPCR e imunoistoquímica. O microPET [^{18}F]-FDG foi utilizado para avaliar os padrões de ativação cerebral em camundongos tratados com reserpina. A distribuição do tamanho das fibras musculares do masseter e do gastrocnêmio foi avaliada através de análise histológica. A área e densidade das mitocôndrias no músculo esquelético foram analisadas por microscopia eletrônica de transmissão. Nos protocolos de tratamentos agudos, a administração i.t. ou i.p. de N/OFQ (1 nmol/sítio ou 1 nmol/kg, respectivamente) reduziu significativamente a alodínia mecânica induzida pela reserpina. Contudo, a administração i.p. de N/OFQ, na dose de 5

nmol/kg, teve efeito oposto, induzindo hipernocicepção. Em relação aos efeitos agudos do UFP-101, este antagonista peptídico, administrado pelas vias i.c.v. (1 nmol/sítio), i.t. (3 e 5 nmol/sítio) ou i.p. (1, 3 e 5 nmol/kg), reduziu significativamente a hipersensibilidade mecânica em camundongos tratados com reserpina. O tratamento agudo com N/OFQ ou UFP-101 não alterou significativamente a hipersensibilidade térmica, pelas vias i.c.v. ou i.p. A administração i.t. de N/OFQ (3 nmol/sítio) ou de UFP-101 (5 nmol/sítio) teve um efeito inibitório significativo na nocicepção térmica. No teste da natação forçada, a reserpina elevou o tempo de imobilidade, e este foi inibido de forma significativa pela N/OFQ, administrada pelas vias i.c.v (1 nmol/sítio) ou i.t. (3 nmol/sítio). N/OFQ e UFP-101 não modificaram nenhum parâmetro relacionado à ansiedade. Os tratamentos repetidos com UFP-101 e com SB-612111 reduziram a alodínia mecânica (37 ± 8% e 43 ± 15,2%), a hipernocicepção térmica (32,2 ± 5% e 45 ± 17,5%), melhoraram a coordenação motora no rotarod (aumento de 7 e 2 vezes no tempo de permanência) e a força de preensão palmar (15 ± 16% e 9 ± 5.5%), respectivamente. A administração de ambos antagonistas não foi capaz de alterar parâmetros de ansiedade ou depressão. A fibromialgia induzida por reserpina foi associada ao aumento na expressão de RNAm para a ppN/OFQ na medula espinhal lombar (dia 3) e no masseter (dias 1 e 2), enquanto a expressão do RNAm do NOPr foi aumentada no músculo masseter (dia 1). Alternativamente, a expressão de RNAm do NOPr foi reduzida no tálamo/hipotálamo (dia 3). A análise por imunoistoquímica revelou expressão aumentada do NOPr no gânglio da raiz dorsal (dia 4). O UFP-101 causou uma diminuição no metabolismo de [^{18}F]-FDG no giro do cingulado, no colículo superior, no mesencéfalo esquerdo, no colículo inferior esquerdo e no colículo inferior direito de camundongos tratados com reserpina. Além disso, o UFP-101 preveniu as alterações induzidas pela reserpina na distribuição do tamanho das fibras musculares, de acordo com a avaliação dos cortes histológicos do masseter e do gastrocnêmio. Tanto a indução da fibromialgia pela reserpina, quanto o tratamento crônico com UFP-101,

não alteraram a área mitocondrial. Em resumos, os dados do presente estudo indicam que o bloqueio farmacológico do NOPr reduziu os sintomas de dor, fadiga e adinamia, recuperando também os padrões de ativação cerebral e as alterações musculares esqueléticas no modelo de fibromialgia experimental induzido pela reserpina. Além disso, expressão da ppN/OFQ e do NOPr foi alterada pela indução de fibromialgia, tanto em sítios centrais, quanto periféricos, reforçando a relevância do sistema N/OFQ-NOPr na patofisiologia da fibromialgia.

Palavras-chave: fibromialgia; reserpina; nociceptina/orfanina FQ; receptor da nociceptina/orfanina FQ; UFP-101; SB-612111; nocicepção; fadiga.

ABSTRACT

Fibromyalgia is characterized by widespread pain, being accompanied by functional and affective disorders. This study evaluated the implication of nociceptin/orphanin FQ peptide receptor (NOPr) in a mouse model of fibromyalgia. The local Animal Ethics Committee approved the experimental protocols (15/00487). Fibromyalgia was induced in female CF-1 mice (20-24 g, 4 week-old) by reserpine administration (0.25 mg/kg; subcutaneous route), once a day, during 3 consecutive days. Control groups received vehicle. On the fourth day, mice were acutely treated with the selective NOP agonist nociceptin (N/OFQ), or with the selective peptide antagonist UFP-101, given by intraperitoneal (i.p., 0.3-5 nmol/kg), intracerebroventricular (i.c.v., 0.3-1 nmol/site), or intrathecal (i.t., 0.3-5 nmol/site) routes, 30 min before the experimental sessions. In a separate set of experiments, the animals were treated with the peptide UFP-101 (1 nmol/kg) or the non-peptide SB-612111 (6.6 μmol/kg) antagonists, given by intraperitoneal route, during three consecutive days, 30 min after daily reserpine injection. At the 4^{th} day, mice also received the antagonist, dosed 30 min before evaluations. The animals were subjected to Von Frey, hot-plate, forced swimming, elevated plus-maze, rotarod and grasping tests. Pre-pro-nociceptin (ppN/OFQ) and NOPr expression was determined by RT-qPCR and immunohistochemistry. The [^{18}F]-FDG microPET imaging was used to assess the brain activation patterns in reserpine-treated mice. The fiber size distribution of masseter and gastrocnemius muscles was evaluated by histological analysis. The mitochondria area and density in the skeletal muscle were analysed by transmission electron microscopy (TEM). In the acute protocols of treatment, the i.t. or i.p. administration of N/OFQ (1 nmol/site or 1 nmol/kg, respectively) significantly reduced the mechanical allodynia. However, i.p. treatment with N/OFQ at the dose of 5 nmol/kg had an opposite effect, leading to hypernociception. Concerning the UFP-101 effects, this peptide antagonist given i.c.v. (1 nmol/site), i.t. (3 and 5 nmol/site) or i.p. (1, 3 and 5 nmol/kg)

significantly reduced the mechanical hypersensitivity in mice treated with reserpine. The acute treatment with N/OFQ or UFP-101 did not significantly alter the thermal hypersensivity, when given by i.c.v. or i.p. routes. The i.t. administration of N/OFQ (3 nmol/site) and UFP-101 (5 nmol/site) had a significant inhibitory effect on the thermal nociception. The immobility time was significantly inhibited by N/OFQ, given by i.c.v (1 nmol/site) or i.t. (3 nmol/site) routes. N/OFQ and UFP-101 did not modify any anxiety-related parameter. The chronic treatment with UFP-101 and SB-612111 reduced the mechanical allodynia (37 ± 8% and 43 ± 15.2%) and the thermal hypernociception (32.2 ± 5% and 45 ± 17.5%), besides improving the motor coordination in the rotational apparatus (7 and 2-fold increase in permanence time) and the grasping strength (15 ± 16% and 9 ± 5.5%), respectively. None of the antagonists altered the parameters of anxiety or depression. Reserpine-induced fibromyalgia was associated with an increase in ppN/OFQ mRNA expression in the lumbar spinal cord (day 3) and masseter (days 1 and 2), whereas NOPr mRNA expression was increased in the masseter muscle (day 1). Alternatively, NOPr m RNA expression was reduced in the thalamus/hypothalamus (day 3). The immunohistochemistry analysis revealed an increased expression of NOPr in the dorsal root ganglion (DRG; on day 4). UFP-101 led to a decrease in the [^{18}F]-FDG metabolism in cingulate gyrus, superior colliculus, left midbrain, left inferior colliculus and right inferior colliculus of reserpine-treated mice. Additionally, UFP-101 prevented reserpine-induced changes in fiber size distribution, according to the assessment of masseter and gastrocnemius histological sections. TEM analysis revealed that either the induction of fibromyalgia by reserpine, or the chronic treatment with UFP-101, did not alter the mitochondrial area or density. The expression of nociceptin and NOPr was altered in the mouse model of fibromyalgia induced by reserpine. Remarkably, UFP-101 improved the symptoms of pain, fatigue and adinamia, also recovering the brain activation patterns and the muscle fiber changes in this experimental paradigm. Our

data shed new lights on the mechanisms underlying the fibromyalgia pathogenesis, supporting a role for NOPr in this syndrome.

Keywords: fibromyalgia; reserpine; nociceptin/orphanin FQ; nociceptin/orphanin FQ receptor; UFP-101; SB-612111; nociception; fatigue.

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LISTA DE ABREVIATURAS

ACR – Colégio Americano de Reumatologia

ACTH – Hormônio adrenocorticotrófico

BDNF – Fator neurotrófico derivado do cérebro

CCI – Injúria crônica do nervo ciático

CCL2 – Ligante 2 de CC quimiocina

CCL17 - Ligante 17 de CC quimiocina

CCL22 - Ligante 22 de CC quimiocina

CRH – Hormônio liberador de corticotrofina

CXCL1 – Ligante 1 de quimiocina CXC

CXCL9 - Ligante 9 de quimiocina CXC

CXCL11 - Ligante 11 de quimiocina CXC

DRG – Gânglio da raiz dorsal

ERK – Quinase regulada por sinal extracelular

FDA – *Food and Drug Administration*

GABA – Ácido gama-aminobutírico

GHB – Gama-hidroxibutirato

HPA – Eixo hipotálamo-pituitária-adrenal

IASP – *International Association for the Study of Pain*

ICS – Frio intermitente induzido pelo estresse

IFN-γ – Interferon-gama

JNK – Proteína Jun N-terminal quinase

LDH – Lactato desidrogenase

LPS – Lipopolissacarídeo

MadCAM-1 – Molécula de adesão celular de adressina da mucosa 1

MAPK – Proteínas quinases ativadas por mitógeno

MIF – Fator de inibição de migração de macrófagos

MuRF1 - Muscle RING-finger protein-1

N/OFQ – Peptídeo nociceptina/orfanina FQ

NDR – núcleo dorsal da rafe

NF-κB – Fator de transcrição nuclear κB

NGF – Fator de crescimento do nervo

NOPr – Receptor do peptídeo nociceptina/orfanina FQ

NRM – núcleo magno da rafe

PGD_2 – prostaglandina D_2

PGE_2 – Prostaglandina E_2

PKC – Proteína quinase C

PLA_2 – Fosfolipase A_2

PLC – Fosfolipase C

ppN/OFQ – Precursor pré-pró-N/OFQ

SNC – Sistema nervoso central

SNL – Modelo de ligação do nervo espinhal

SNRI – Inibidores da recaptação de serotonina e noradrenalina

SSRI – Inibidores seletivos da recaptação de serotonina

STAT3 – Transdutor de sinal e ativador da transcrição 3

TCC – Terapia cognitivo-comportamental

TNBS – Colite induzida por ácido trinitrobenzeno sulfônico

TNF – Fator de necrose tumoral

vlPAG – Substância cinzenta periaquedutal ventrolateral

LISTA DE SÍMBOLOS E UNIDADES DE MEDIDAS

ºC Celsius

cm Centímetro

h Hora

kg Quilograma

M Molar

min Minuto

mg Miligrama

mL Mililitro

µg Micrograma

µL Microlitro

pmol Picomol

s Segundo

± mais ou menos

α Alpha

β Beta

γ Gamma

δ Delta

SUMÁRIO

1 FUNDAMENTAÇÃO TEÓRICA

1.1 Sistema nociceptina/orfanina FQ-receptor NOP

Este sistema é composto pelo peptídeo nociceptina/orfanina FQ (N/OFQ) e pelo receptor de N/OFQ (NOPr) (1). O receptor da N/OFQ foi classificado como um membro da família de receptores opioides, sendo denominado como receptor opioide do tipo 1 (ORL-1). Posteriormente, a IUPHAR (International Union of Basic & Clinical Pharmacology) denominou o receptor de OP4 e, subsequentemente, como receptor do peptídeo nociceptina/orfanina FQ (NOPr) (2). O peptídeo N/OFQ é produzido a partir do precursor pré-pró-N/OFQ (ppN/OFQ), que é constituído por 176 aminoácidos (3). A N/OFQ contém 17 aminoácidos (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) e foi descrita pela primeira vez em 1995, como o ligante endógeno do NOPr. De acordo com a sua estrutura, o peptídeo possui similaridade com peptídeos opioides, como a dinorfina. Por isto, geralmente é relatado como um peptídeo opioide. Entretanto, não possui afinidade pelos receptores opioides MOP (μ), DOP (δ) e KOP (κ) (1, 4, 5). De maneira semelhante, os opioides endógenos não se ligam ao NOPr, pois apresentam baixa afinidade pelo mesmo (6). Esta seletividade distinta tem sido atribuída a três resíduos do NOPr que diferem dos demais receptores opioides, Ala216 de Lys, Gln280 de His e, Thr305 de Ile, respectivamente. Isto está intimamente relacionado com os motivos estruturais dos peptídeos ligantes, sendo que as partes hidrofóbicas e hidrofílicas das bolsas de ligação de receptores de opioides diferem do NOPr (7, 8).

1.2 Transdução de sinal e heterodimerização do receptor NOP

O NOPr é acoplado à proteína G (GPCR) e é um membro não-opioide da família de receptores opioides, sendo insensível ao antagonista naloxona (6). Quando o NOPr é ativado pelo peptídeo N/OFQ, desencadeia uma cascata de eventos, incluindo a inibição de canais de Ca^{+} e da proteína adenilato ciclase, além da ativação do canal de K^{+} retificador de corrente, reduzindo assim,

a excitabilidade neuronal e a liberação de neurotransmissores na fenda sináptica (4, 5, 9-12). A redução na liberação de neurotransmissores, como catecolaminas (dopamina, e noradrenalina), serotonina (5-HT), acetilcolina e glutamato é observada após a ativação do NOPr (13-16). Esta ação é a base para a modulação de transtornos como ansiedade, depressão e dor.

Os mecanismos pelos quais o NOPr interage com os canais de cálcio ainda não estão bem elucidados. Altier e colaboradores (17) mostraram que a ativação prolongada do receptor por N/OFQ leva à internalização dos canais de cálcio do tipo N (neuronal) em vesículas e, à redução da entrada de cálcio, sendo que este efeito é revertido pelo bloqueio do receptor com o antagonista peptídico III-BTD. Estes dados indicam que ocorre a remoção dos canais da membrana plasmática. Outro trabalho demonstrou que há a heterodimerização do NOPr com o receptor opioide MOP e que a posterior ativação de MOP leva à internalização dos canais de cálcio, somente na presença de NOP. No entanto, esta inibição é menor quando comparada com aquela mediada por NOP apenas (18). Contudo, Murali e cols. (19) verificaram que não houve a internalização destes canais no corpo celular e nos terminais nervosos centrais de neurônios do gânglio da raiz dorsal de ratos. Vale ressaltar que este mecanismo é fundamental no controle da transmissão de sinais nociceptivos.

A participação do NOPr também tem sido descrita em outras vias importantes, como na ativação da proteína quinase C (PKC) (20), da fosfolipase A_2 (PLA_2) (21) e da fosfolipase C (PLC) (20), na modulação das proteínas quinases ativadas por mitógeno (MAPK), que incluem a quinase regulada por sinal extracelular (ERK) e a proteína Jun N-terminal quinase (JNK), além do fator de transcrição nuclear κB (NF-κB) (20, 22-26). Mais recentemente, o transdutor de sinal e ativador da transcrição 3 (STAT3) tem sido implicado nas vias de transdução deste receptor (27).

1.3 Ligantes do receptor NOP

1.3.1 Agonistas e antagonistas peptídicos

O [F/G]N/OFQ(1–13)-NH_2 foi descrito como o primeiro ligante do NOPr com eficácia reduzida, sendo gerado a partir de mudanças na ligação entre Phe^1 e Gly^2 (28). Já, o primeiro antagonista seletivo para o NOPr ([$Nphe^1$] N/OFQ (1-13) NH_2) surgiu das modificações realizadas na porção N-terminal de N/OFQ, através da mudança de Phe^1 da cadeia lateral do átomo C para o átomo N (29). Contudo, este peptídeo apresentou uma baixa potência. Outra modificação foi realizada em N/OFQ [Arg^{14}, Lys^{15}] e forneceu um agonista bastante potente (30X mais potente que N/OFQ) (30). Foi observado um efeito pró-nociceptivo após a administração intracerebroventricular (i.c.v.) deste peptídeo, como avaliado no teste de retirada da cauda, além de produzir inibição da atividade locomotora de camundongos (31).

O antagonista [$Nphe^1$, Arg^{14}, Lys^{15}] N/OFQ-NH_2 foi produzido a partir da junção das duas modificações citadas acima em uma única molécula. Também chamado de UFP-101, este peptídeo é um antagonista altamente seletivo para o NOPr (32, 33), demonstrando várias atividades biológicas, incluindo ação anti-inflamatória (34) e efeitos antiarrítmicos (35), dentre outras.

1.3.2 Antagonistas não peptídicos

O J-113397 foi descrito como o primeiro antagonista não peptídico com uma alta potência (pA2 7.5–8.0) e uma seletividade aceitável para o NOPr (36). No entanto, outro antagonista, o SB-612111, apresentou potência mais elevada (pA_2 8.0–8.5) que o J-113397, com seletividade comparável ao mesmo (37). Ademais, outra molécula foi caracterizada como um antagonista com melhores propriedades farmacológicas, apresentando alta potência (pA2 8.5–9.0), denominado composto 24 (C-24) (38-42).

1.3.3 Moléculas com atividade bifuncional

Em uma tentativa de desenhar novas moléculas com atividade bifuncional, alguns grupos têm utilizado a integração de farmacóforos que têm afinidade por diferentes receptores, como NOP e MOP, simultaneamente (43, 44). Este conceito de ligantes com múltiplos alvos é baseado na terapia com opioides, com a utilização, por exemplo, de buprenorfina, sendo este um agonista de MOP e antagonista de KOP (45, 46). Sobczak e cols. (47) verificaram ação analgésica e inibição da motilidade gastrointestinal para o agonista BU08070, um análogo de buprenorfina, em um modelo de síndrome do intestino irritável. Este agonista possui atividade bifuncional, pois é ligante dos receptores NOP e MOP. Neste estudo, o efeito antinociceptivo do BU08070 foi mediado pelo receptor MOP, enquanto que sua ação sobre o trato gastrointestinal foi mediada pelo NOPr.

1.4 Padrão de expressão do receptor NOP e do peptídeo N/OFQ

O peptídeo N/OFQ e o NOPr são amplamente expressos em células imunes, no sistema nervoso central (SNC) e, em diversos órgãos periféricos (48). Em particular, o NOPr é expresso no sistema aminérgico (núcleos adrenérgico, colinérgico, dopaminérgico e serotoninérgico). Este também é encontrado no sistema límbico, incluindo o complexo amigdaloide, o hipocampo, vários núcleos do hipotálamo, a banda diagonal de Broca, dentre outras áreas envolvidas no processamento das emoções. O receptor também está distribuído pelas vias olfatória, auditiva e visual, da percepção somatossensorial e no controle motor (49).

Nos tecidos periféricos, foi detectada a expressão do NOPr na mucosa intestinal humana (50) e de ratos (51), no miocárdio humano (52) e nos gânglios neuronais simpáticos e sensoriais periféricos de cobaias (53) e ratos (54). Os receptores da nociceptina são encontrados amplamente distribuídos nos neurônios (grandes e pequenos, mielinizados e não mielinizados) do gânglio da raiz dorsal (DRGs), onde 43% de todos os neurônios expressam o NOPr (55). Estes receptores estão presentes tanto nos neurônios peptidérgicos, como não peptidérgicos, importantes para a dor aguda

pelo calor e para a dor mecânica, respectivamente (56-60). Os receptores da nociceptina também estão co-localizados com os receptores opioides MOP nas fibras C peptidérgicas (55, 61).

Em condições patológicas, como no modelo de dor neuropática de ligação do nervo ciático (SNL), a expressão do RNAm da N/OFQ e do NOPr está diminuída no tálamo e no hipotálamo e aumentada no córtex cingulado anterior (62). Ainda neste estudo, um aumento na expressão do RNAm da N/OFQ também foi observado na amígdala. Neste mesmo modelo, Ozawa et al. (2018) relataram a diminuição da imunoreatividade ao NOP-eGFP nas lâminas I e II externas na medula e nos aferentes primários nos DRGs de L4 (lombar 4), sem alteração da expressão na borda ventral da lâmina II interna (63). No modelo induzido por injúria crônica do nervo ciático (CCI), a expressão do NOPr está elevada no núcleo magno da rafe (NRM), substância cinzenta periaquedutal ventrolateral (vlPAG) e núcleo dorsal da rafe (NDR) (64). Em adição, os níveis de proteína do NOPr estão aumentados na medula espinal dorsal ipsilateral de ratos com CCI (65). Outros estudos demonstram a modulação do NOPr em diferentes modelos de dor (66-68). Quanto à localização de NOPr em tecidos humanos, um estudo demonstrou o aumento de fibras positivas para o receptor no sub-urotélio da bexiga de pacientes com bexiga hiperativa e com síndrome da bexiga dolorosa (69). Anand e cols. (2016) mostraram também que 75% a 80% dos neurônios pequenos/médios nos DRGs lombares e sacrais humanos eram positivos para NOPr, e que a imunorreatividade do NOPr foi diminuída nos nervos periféricos lesados e nos neuromas dolorosos (69). Stamer et al. (2011) também demonstrou a modulação da expressão da N/OFQ e do NOPr em células sanguíneas periféricas de pacientes com câncer ou sépticos (70) e Sobczak et al. (2011) do NOPr em amostras do cólon de pacientes com doença inflamatória intestinal (71). Entretanto, a modulação da expressão da N/OFQ e de seu receptor no DRG ainda não foi investigado em modelos de dor disfuncional, como a fibromialgia.

1.5 Ação biológica do Sistema N/OFQ-receptor NOP

O sistema N/OFQ- NOPr afeta uma variedade de sistemas biológicos (1) (Figura 1). Assim, muitos grupos têm estudado agonistas e antagonistas do NOPr como potenciais ferramentas terapêuticas. Experimentos *in vivo* demonstram a modulação de uma diversidade de funções biológicas, como vasodilatação (72). Os efeitos dos ligantes desses receptores também têm sido caracterizados na dor, depressão e na ansiedade (1, 7, 73-81). Além disto, a sua ação sobre a aprendizagem e memória foi também descrita (82, 83).

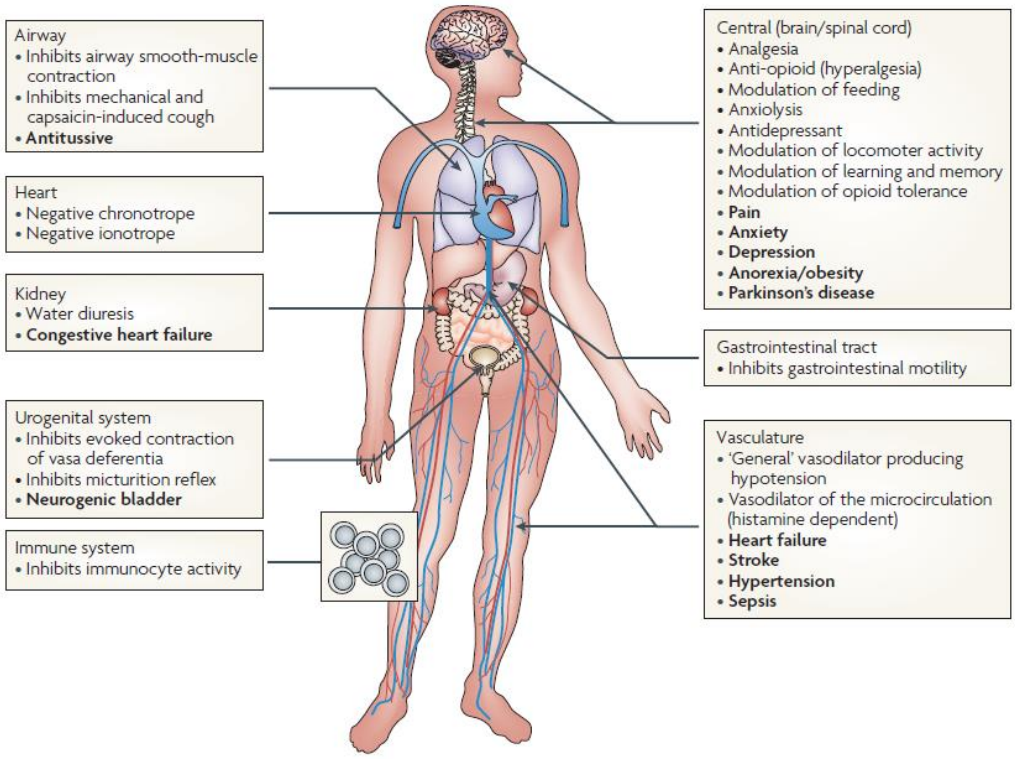

Figura 1: Efeitos pleiotrópicos de nociceptina/orfanina FQ (N/OFQ) nos principais sistemas. Indicações clínicas potenciais estão em negrito.

Extraído de (1) – *Publicação aprovada pela revista (Número de licença: 3681510776945).*

1.5.1 Ação biológica da N/OFQ sobre o sistema imune

Mais recentemente, estudos *in vitro* e *in vivo* mostraram o efeito do sistema N/OFQ-NOP na modulação de funções imunes, sendo que o RNA mensageiro (RNAm) do NOPr foi detectado em linfócitos T citotóxicos e T-*helper* de camundongos (84), em linhagens de linfócitos T e monócitos

(U937) e, em monócitos e linfócitos humanos (85, 86). A expressão de RNAm também foi relatada em órgãos linfoides de suínos, nos linfonodos, no timo e no baço (87). Além da expressão nos órgãos linfoides, a N/OFQ e o NOPr são expressos na epiderme e na mucosa intestinal, importantes sítios para reconhecimento de antígenos (50, 88, 89).

A N/OFQ modula diferentes respostas imunes, induzindo a quimiotaxia de neutrófilos (90), e de monócitos humanos (91), promovendo a migração de leucócitos (90), aumentando a permeabilidade vascular (92), além de induzir hipotensão, rolamento de leucócitos e vazamento macromolecular (34, 72). O peptídeo também estimula a liberação de histamina pelos mastócitos (92).

Vários trabalhos demonstraram que agonistas e antagonistas do NOPr induzem efeitos pró- e anti-inflamatórios, respectivamente, em vários modelos de sepse. Carvalho e cols. (93) utilizaram o modelo de sepse induzida por punção e ligadura cecal em ratos Wistar e verificaram que o antagonista peptídico do NOPr, o UFP-101, administrado por via subcutânea (s.c.), na dose de 0,03 mg/kg, reduziu a mortalidade em 50%. Em adição, houve a diminuição do infiltrado inflamatório no fluído broncoalveolar e no exsudato peritoneal, prevenção da disseminação bacteriana e, diminuição da concentração plasmática das citocinas fator de necrose tumoral (TNF) e interleucina-1 beta (IL-1β). Foram identificados efeitos anti-inflamatórios para o UFP-101 no modelo de sepse induzida por lipopolissacarídeo (LPS) (34). Neste estudo, o extravasamento plasmático e o rolamento de leucócitos induzidos por LPS foram reduzidos pela co-administração endovenosa de 286,23 µg/kg do antagonista.

No modelo de colite em camundongos, foi observada a inibição da produção dos mediadores inflamatórios interferon-gama (IFN-γ), TNF-α, IL-1β e ligante 1 de quimiocina CXC (CXCL1), após o tratamento com o antagonista não-peptídico, SB-612111, na dose de 30 mg/kg (94). De forma interessante, camundongos deficientes para NOPr apresentaram diminuição nos níveis da molécula de adesão celular de adressina da mucosa 1 (MadCAM-1) e, do número de células inflamatórias na mucosa do cólon (95).

Ao contrário dos efeitos anti-inflamatórios dos antagonistas, a administração de N/OFQ pela via endovenosa, nas doses de 1,085 µg/kg a 108,54 µg/kg, em ratos Wistar, ocasionou vasodilatação, hipotensão e adesão de leucócitos (72). Além disto, a aplicação intradérmica do agonista endógeno aumentou a permeabilidade vascular na pele dos ratos, por um mecanismo dependente do receptor H1 de histamina (92).

Na literatura, também são encontrados resultados que discordam dos achados acima citados, como o efeito anti-inflamatório de agonistas não-peptídicos do NOPr. Sobczak e cols. (71) demonstraram atividade anti-inflamatória e antinociceptiva para o SCH 221510, um agonista não peptídico e altamente seletivo para o NOPr (96).

Abaixo estão descritos os efeitos pró-inflamatórios de ligantes do NOPr (48) (Tabela 1).

Tabela 1:

Table 1 Available evidence for a proinflammatory effects of NOP activation

***In vivo* and *in vitro* studies**	**Effects of NOP activation or NOP blockage**	**References**
C57BL/6J mice (normal and ppN/OFQ knockout)	• The administration of N/OFQ (55 nmol/kg, i.p.) 30 min prior to Staphylococcal enterotoxin A increased the expression of TNF-α and IFN-γ on the spleen • N/OFQ-deficient mice displayed attenuated TNF-α and IFN-γ mRNA levels triggered by antigen challenge	Goldfarb, Reinscheid, and Kusnecov (2006)
Rat astrocytes	• The expression of N/OFQ mRNA and protein was increased by proinflammatory mediators such as TNF-α, IL-1β, and LPS	Buzas, Rosenberger, Kim, and Cox (2002)
Rat splenocytes	• TNF-α and IL-1-β increased the N/OFQ secretion by splenocytes *in vitro*	Miller and Fulford (2007)
Anesthetized Wistar rats	• Administration of N/OFQ (0.6–60 nmol/kg i.v.) caused hypotension, vasodilatation, macromolecular leak, and leukocyte adhesion	Brookes et al. (2007)

Anesthetized Wistar rats	• Administration of N/OFQ (0.6–60 nmol/kg i.v.) caused hypotension, vasodilatation, macromolecular leak, and leukocyte adhesion	Brookes et al. (2007)
Wistar rats and isolated mast cell	• Intradermal application of N/OFQ increased vascular permeability in rat skin by a mechanism dependent of histamine H1 receptor • *In vitro* N/OFQ stimulated the release of histamine by rat peritoneal mast cells	Kimura et al. (2000)
ICR mice and C57BL/6 NOP-deficient mice	• Intradermal inoculation of N/OFQ presented pruritogenic effect in normal but not in NOP-deficient mice. The leukotriene B_4 receptor antagonist inhibited the itch • N/OFQ stimulated the production of leukotriene B_4 by keratinocytes	Andoh et al. (2004)
Monocytes and neutrophils obtained from healthy subjects	• NOP activation stimulated the chemotaxis of human monocytes and increased the release of lysozyme by neutrophils	Trombella et al. (2005)
Neutrophils obtained from healthy volunteers	• N/OFQ exhibited a potent chemoattractant activity *in vitro*	Serhan et al. (2001)
BALB/c mice (air pouch model)	• N/OFQ at low doses (10 ng) induced significant leukocyte recruitment into the air pouch	Serhan et al. (2001)
Human neutrophils	• Neutrophils stimulated by fMLP quickly secreted N/OFQ upon exocytosis of granules	Fiset et al. (2003)
Septic rats (CLP model)	• Pharmacological blockade of NOP receptor with UFP-101 enhanced the bacterial control and decreased systemic inflammation and mortality of animals, while N/OFQ administration increased animal mortality	Carvalho et al. (2008)

Colitic mice (DSS model)	• The NOP receptor antagonist (SB612111—30 mg/kg) ameliorated the clinical signs of colitis and inhibited the production of CXCL1, IFN-γ, TNF-α, IL-1β, IL-6, and TNF-α	Alt et al. (2012)
Colitic mice (DSS model: wild-type and NOP-deficient C57BL/6 mice)	• NOP-deficient animals developed attenuated DSS-induced colitis and expressed decreased levels of mucosal addressin (MadCAM-1) and significant reduction in the number of inflammatory cells in colonic mucosa	Kato et al. (2005)

CXCL1, chemokine (C-X-C motif) ligand 1; fMLP, proinflammatory peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; IFN-γ, interferon-gamma; IL-1β, interleukin 1 beta; LPSs, lipopolysaccharides; MadCAM-1, mucosal addressin cell adhesion molecule-1; N/OFQ, nociceptin/orphanin FQ peptide; NOP, N/OFQ receptor; UFP-101, University of Ferrara Peptide-101; TNF-α, tumor necrosis factor-alpha.

Extraído de (48) – *Publicação aprovada pela revista (Número de licença: 4524251386841).*

1.5.2 Ação biológica do sistema N/OFQ-NOPr no sistema nervoso central

1.5.2.1 Nocicepção

A sensação dolorosa inicia com um estímulo nocivo, detectado na periferia (pele e outros tecidos fora do SNC) pelos nociceptores, que possuem seus corpos celulares no DRG. Estas fibras nervosas sensoriais aferentes são ativadas na maioria das alterações dolorosas (fibras de alto limiar C e Aδ). Os nociceptores transmitem o impulso para os neurônios do corno dorsal da medula espinhal, e estes, conduzem o impulso até o cérebro como potencial de ação (57, 97). As principais alterações sensoriais são a alodínia (dor provocada por um estímulo não nocivo) e a hiperalgesia (sensibilidade aumentada para um estímulo doloroso) (98), presentes na fibromialgia.

Em relação às ações centrais do sistema N/OFQ–NOP, os principais estudos se concentram no seu papel sobre o processamento da dor. De acordo com o trabalho de Meunier e cols. (4), a denominação da nociceptina foi baseada em seus efeitos hiperalgésicos, após sua administração por via i.c.v. Lambert (2008) demonstrou que a morfina inibe as células ON na medula ventromedial

rostral, sendo incapazes de inibir as células OFF, que reduzem a informação ascendente nociceptiva ao nível medular, originando assim, um efeito de analgesia. É importante destacar que este efeito é revertido pela ação de N/OFQ. Desta maneira, a N/OFQ possui um efeito nociceptivo quando administrado ao nível supra-espinhal, mediado pela inibição de células ON (primárias) e células OFF (secundárias) na medula ventromedial rostral. Por outro lado, a analgesia opioide clássica, decorrente da administração da N/OFQ por via intratecal (i.t.), parece estar relacionada com a inibição do afluxo aferente nociceptivo, que ocorre em tecidos periféricos. A inibição deste fluxo aferente também pode ocorrer perifericamente, sobre a pele, a bexiga e/ou sobre células sanguíneas mononucleares (1). A figura abaixo demonstra os prováveis sítios de ação da N/OFQ na modulação da nocicepção (Figura 2).

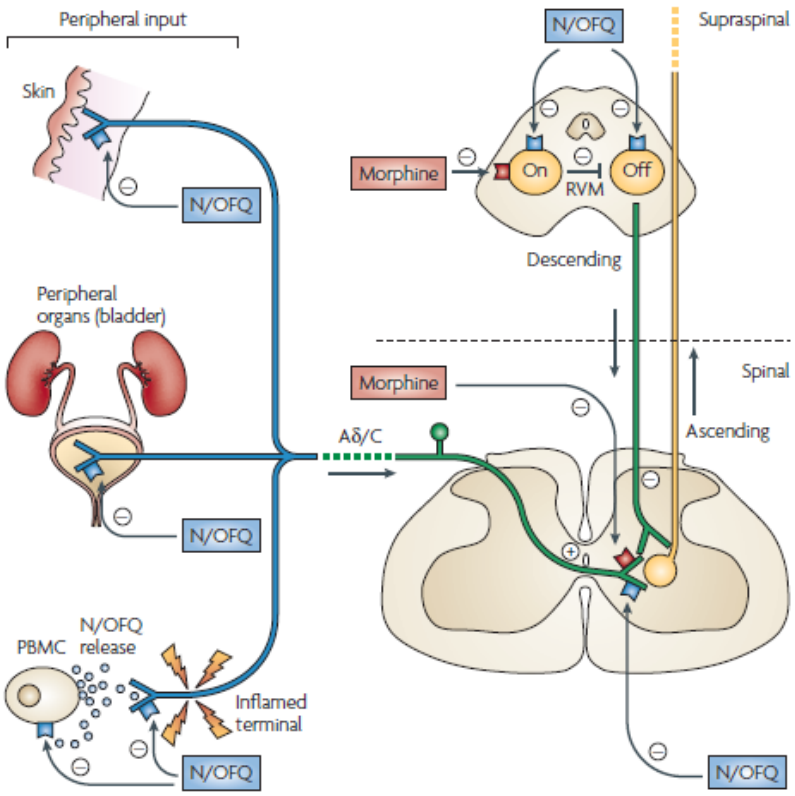

Figura 2: Esquema para descrever a relação entre locais anatômicos subjacentes às ações do N/OFQ na nocicepção.

RVM: medula ventromedial rostral. PBMC: células sanguíneas mononucleares periféricas.

Extraído de (1) – *Publicação aprovada pela revista (Número de licença: 3682510662258*).

Os efeitos de agonistas do NOPr sobre a dor podem levar tanto à nocicepção quanto à antinocicepção em roedores, dependendo das diferentes vias de administração, das doses utilizadas e da modalidade da dor (7, 73). Com relação à dor aguda, quando da administração de N/OFQ periférica em camundongos, foi observada redução significativa da nocicepção induzida por capsaicina (99). Em contrapartida, o antagonista não peptídico, SB-612111, administrado por via intravenosa, antagonizou a atividade antimorfina e a hiperalgesia térmica induzidas pela N/OFQ (100). É importante ressaltar que os efeitos da administração sistêmica em roedores de agonistas do NOPr são dependentes da soma dos efeitos supra-espinhal e espinal, relativos à sinalização do sistema N/OFQ- NOPr (73).

Este mesmo efeito de analgesia foi verificado em ratos no teste de retirada da cauda, com a administração i.t. ao nível da cauda equina de [Phe1psi(CH2-NH)Gly2]N/OFQ(1-13)-NH2, um análogo de N/OFQ (101). Em adição, Tian e cols. (102) demonstraram que a administração i.t. de N/OFQ produziu efeito antinociceptivo em ratos no teste de retirada da cauda, além de potencializar os efeitos da morfina. Corroborando estes achados, Tsai et al. (2018) demostraram que a injeção i.t. de N/OFQ (1, 2 ou 5 nmol/sítio) levou ao aumento da latência no teste de retirada da cauda (103), sendo este efeito antagonizado por UFP-101 (10 nmol/sítio). Por outro lado, a administração de N/OFQ (10 nmol/sítio) na vlPAG de ratos bloqueou a analgesia provocada por DAMGO (um agonista do receptor MOP, na dose de 1,9 nmol/sítio), enquanto UFP-101 (10 nmol/sítio) potencializou a ação do opioide (0,5 nmol/sítio) (104). Em adição, após a injeção de [Phe1psi(CH2-NH)Gly2]N/OFQ(1-13)-NH2, por via i.c.v., foi observada resposta pró-nociceptiva no teste de retirada da cauda em ratos (101) e camundongos (5). Além disso, baixas doses do peptídeo, administradas perifericamente ou por via i.t., induziram algesia em camundongos (105, 106). Neste mesmo teste, Calo e cols. (2002) (32) demonstraram que o antagonista UFP-101 (3 nmol/sítio, i.c.v.) preveniu o efeito pró-nociceptivo da N/OFQ (1 nmol/sítio, i.c.v.). Em adição, o antagonista produziu efeito antinociceptivo *per se*, apenas na dose de 10 nmol. Quando co-injetados (N/OFQ 1 nmol/sítio + UFP-101 10 nmol/sítio), o efeito pró-nociceptivo do peptídeo natural e o efeito

antinociceptivo do antagonista foram abolidos, atingindo valores aproximados aos observados nos animais controle. O antagonista SB-612111, administrado por via i.p. (3 mg/kg), também foi capaz de antagonizar as ações pró-nociceptiva e antinociceptiva da N/OFQ (1 nmol), por via i.c.v. e i.t., respectivamente (107). Este antagonista, na dose de 10 mg/kg, por via s.c., também foi capaz de potencializar os efeitos antinociceptivos de agonistas de NOP/MOP, a buprenorfina (1 e 3 mg/kg; via s.c.), SR16435 e (3 – 30 mg/kg; via s.c.) e SR16507 (0.3 e 3 mg/kg; via s.c.) (108). Outro agonista bifuncional, o cebranopadol, apresentou efeito antinociceptivo no teste de retirada da cauda nas doses de 0.01 – 1 mg/kg, por via i.v. (109).

Em modelos de dor inflamatória em roedores, agonistas do NOPr, quando administrados por via i.t. ou supra-espinhal, induzem ações antinociceptiva e pró-nociceptivas, respectivamente (7). Agostini e cols. (51) demonstraram que a administração periférica de N/OFQ (2 nmol/kg) teve efeito anti-hiperalgésico no modelo de colite induzida por ácido trinitrobenzeno sulfônico (TNBS) em ratos Wistar. Neste mesmo estudo, a administração periférica de UFP-101 (10 nmol/kg) inibiu a ação da N/OFQ. No teste de formalina em camundongos, a administração de UFP-101, na dose de 10 nmol/sítio, pelas vias i.c.v. e i.t., exerceu efeitos analgésico e algésico, respectivamente (110). Em contrapartida, nos modelos de dor neuropática, efeitos analgésicos são verificados tanto pela via i.t., quanto supra-espinhal. Isto pode ser explicado pela ativação do NOPr nas duas vias (i.t. e supra-espinhal), após administração sistêmica de N/OFQ, onde foi observado um potente efeito analgésico (7). Exemplo disto foi observado com a injeção s.c. dos agonistas não peptídicos, SR14150 (10 mg/kg; via s.c.) e SR16835 (30 mg/kg; via s.c.), que induziram ação anti-alodínica no modelo SNL em camundongos ICR (111). Neste estudo, os autores demonstraram que o circuito envolvido na dor crônica e aguda são diferentes, onde SB-612111 (10 mg/kg; via s.c.) bloqueou o efeito anti-alodínico dos dois agonistas não peptídicos, mas não preveniu a ação antinociceptiva de SR14150 (3 e 10 mg/kg; via s.c.). O SB-61211 também foi responsável pela potencialização dos efeitos da morfina de antinocicepção (3 mg/kg; via s.c.) e inibiu a alodínia (1 e 3 mg/kg; via s.c.) (111). Um estudo mais recente, relatou os efeitos espinhais nociceptivos para o agonista PWT2-N/OFQ (2,5,

25 e 250 pmol/site) e para a N/OFQ (0.1, 1 e 10 nmol/site), em modelos de dor neuropática e nociceptiva, sendo estes efeitos bloqueados por SB-612111 (112).

A administração i.t. de N/OFQ inibiu a alodínia no modelo de dano por constrição crônica do nervo ciático em ratos (113). Neste mesmo modelo, a infusão do antagonista UFP-101 intra-vlPAG, na dose de 18 µg/1 µl/rato, reverteu a diminuição do limiar alodínico (114). Um estudo recente com o modelo de SNL, relatou o papel do sistema N/OFQ-NOPr na diminuição da hiperalgesia induzida pelo dano. Esta ação é resultante da inibição direta dos neurônios na medula espinhal (63). Outro estudo demonstrou que a administração periodontal de UFP-101 aliviou a dor orofacial induzida pela movimentação dentária experimental em ratos. Este mesmo efeito não foi visto para a administração intraperitoneal do antagonista (115). Além dos efeitos observados para o antagonista UFP-101 sobre modelos de dor crônica, também foram descritos efeitos benéficos para agonista AT-200 (10 mg/kg; s.c.) sobre a hiperalgesia musculoesquelética térmica e mecânica em camundongos com anemia falciforme, sendo esta atividade revertida por SB-612111 (10 mg/kg; s.c.) (116).

Em contrapartida, diferentemente do observado em roedores, a administração i.t. de agonistas do NOPr, independentemente da dose, levam à somente à antinocicepção, em modelos de dor em primatas, como revisado por Kiguchi et al. (2016) (73). Em um modelo de dor aguda (retirada da cauda da água morna à 50 ºC, como estímulo nocivo), a administração espinal de N/OFQ (10-100 nmol/sítio) ou PWT2-N/OFQ (doses de 0,3 – 3 nmol/sítio) induziu efeitos antinociceptivos em macacos (112, 117). Este mesmo efeito foi demonstrado para o agonista não peptídico Ro 64-6198 (0,001–0,06 mg/kg, s.c.), além de uma ação anti-alodínica no modelo induzido por capsaicina. Neste estudo, o antagonista J-113397 (0,01–0,1 mg/kg, s.c.) foi capaz de reverter o efeito de Ro 64-6198 (118). As ações supraespinhais da N/OFQ em primatas levam à antinocicepção, diferentemente do que é visto em roedores (73). Ding e cols. (2015) demonstraram que a administração intracisternal da N/OFQ induziu efeitos antinociceptivos de maneira dose-dependente, sendo estes efeitos bloqueados pelo antagonista J-113397 do NOPr (117). As ações

sistêmicas de agonistas do NOPr, de maneira geral, levam à analgesia, independente da modalidade da dor, como visto em roedores (73). Estudos demonstram que a administração sistêmica dos agonistas do NOPr, Ro64-6198 e SCH 221510, induz efeitos anti-alodínico e anti-hiperalgésico em modelos de dor induzidos por capsaicina e por carragenina (73, 118-121).

Abaixo estão descritos os principais efeitos de agonistas e antagonistas do NOPr em modelos de nocicepção (73) (Tabela 2).

Tabela 2

Multiple effects of NOP receptor-related ligands on regulating pain processing.

NOP receptor-related ligands	Findings in rodents	Findings in primates
NOP Receptor Agonists (Peptides)		
N/OFQ	Spinal, Acute pain ↓ (Xu et al., 1996) (Erb et al., 1997) (King et al., 1997) (Yamamoto et al., 1997a) Spinal, Acute pain ↑ (Inoue et al., 1999) (Sakurada et al., 1999) Spinal, Inflammatory pain ↓ (Yamamoto et al., 1997b) (Hao et al., 1998) (Chen & Sommer, 2007) Spinal, Neuropathic pain ↓ (Yamamoto & Nozaki-Taguchi, 1997) (Corradini et al., 2001) (Courteix et al., 2004) Supraspinal, Acute pain ↑ (Meunier et al., 1995) (Reinscheid et al., 1995) Supraspinal, Inflammatory pain ↑ (Zhu et al., 1997) (Wang et al., 1999a)	Spinal, Acute pain ↓ (Ko et al., 2006) (Ko & Naughton, 2009) Supraspinal, Acute pain ↓ (Ding et al., 2015b)

[Phe$^1\psi$(CH_2-NH)Gly^2]N/OFQ-(1–13)-NH_2	Supraspinal, Acute pain ↑ (Calo et al., 1998) (Wang et al., 1999b) Supraspinal, Inflammatory pain ↑ (Bertorelli et al., 1999)	
UFP-112	Spinal, Acute pain ↓ (Rizzi et al., 2007) (Calo et al., 2011)	Spinal, Acute pain ↓ (Hu et al., 2010) Spinal, Capsaicin-induced allodynia ↓ (Hu et al., 2010)
PWT2-N/OFQ	Spinal, Acute pain ↓ (Rizzi et al., 2015) Spinal, Neuropathic pain ↓ (Rizzi et al., 2015)	Spinal, Acute pain ↓ (Rizzi et al., 2015)
NOP Receptor Agonists (Non-peptides)		
Ro64-6198	Spinal, Neuropathic pain ↓ (Obara et al., 2005) Systemic, Acute pain ↓ (Reiss et al., 2008) Systemic, Acute pain ↑ (Reiss et al., 2008)	 Systemic, Acute pain ↓ (Ko et al., 2009) Systemic, Inflammatory pain ↓ (Sukhtankar et al., 2014) Systemic, Capsaicin-induced allodynia ↓ (Ko et al., 2009)
Ro65-6570	Supraspinal, Neuropathic pain ↓ (Schiene et al., 2013) Systemic, Inflammatory pain ↓ (Schiene et al., 2013) Supraspinal, Neuropathic pain ↓ (Schiene et al., 2013)	
GRT-TA2210	Supraspinal, Neuropathic pain ↓ (Linz et al., 2013) Systemic, Inflammatory pain ↓ (Linz et al., 2013) `	
SCH 221510	Systemic, Inflammatory pain ↓ (Sobczak et al., 2013) (Sobczak et al., 2014)	Systemic, Acute pain ↓ (Cremeans et al., 2012) Systemic, Inflammatory pain ↓ (Wladischkin et al., 2012) Systemic, Capsaicin-induced allodynia ↓ (Wladischkin et al., 2012)

NOP receptor-related ligands	**Findings in rodents**	**Findings in primates**

NOP Receptor Antagonist		
UFP-101	Supraspinal, Acute pain ↓ (Rizzi et al., 2006) Supraspinal, Inflammatory pain ↓ (Scoto et al., 2009) Supraspinal, Neuropathic pain ↓ (Scoto et al., 2009)	
Mixed NOP/MOP Receptor Agonists		
[Dmt^1]N/OFQ(1–13)-NH_2	Spinal, Acute pain ↓ (Calo et al., 2012)	Spinal, Acute pain ↓ (Molinari et al., 2013)
SR16435	Spinal, Inflammatory pain ↓ (Sukhtankar et al., 2013) Spinal, Neuropathic pain ↓ (Sukhtankar et al., 2013) Systemic, Acute pain ↓ (Khroyan et al., 2009)	
SR14150	Systemic, Neuropathic pain ↓ (Khroyan et al., 2011b)	
SR16835	Systemic, Neuropathic pain ↓ (Khroyan et al., 2011b)	
BU08028	Spinal, Inflammatory pain ↓ (Sukhtankar et al., 2013) Spinal, Neuropathic pain ↓ (Sukhtankar et al., 2013) Systemic, Acute pain ↓ (Khroyan et al., 2011a)	Systemic, Acute pain ↓ (Ding et al., 2015a) Systemic, Capsaicin-induced allodynia ↓ (Ding et al., 2015a)
Cebranopadol	Systemic, Acute pain ↓ (Linz et al., 2014) Systemic, Inflammatory pain ↓ (Linz et al., 2014) Systemic, Neuropathic pain ↓ (Linz et al., 2014)	

NOP receptor-related ligands	**Findings in rodents**	**Findings in primates**

↓, antinociception or antihypersensitivity; ↑, pronociception or hypersensitivity

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1.5.2.2 Ansiedade, estresse e depressão

Estudos indicam que os efeitos de N/OFQ são comparáveis aos produzidos por estressores, agindo diretamente sobre o eixo HPA e o SNC (77). Vários estudos apontam para um aumento do hormônio adrenocorticotrófico (ACTH) e corticosterona no plasma, após a administração i.c.v. de N/OFQ em ratos (122, 123). O aumento de corticosterona também foi detectado no plasma de ratos, após administração endovenosa do antagonista não peptídico, JTC-801, em condições de repouso (124). A administração por via i.c.v. do antagonista peptídico UFP-101 preveniu o aumento da corticosterona induzido pela injeção central de N/OFQ (125). Estes estudos sugerem resultados controversos, mas os efeitos que prevalecem na literatura são de que antagonistas de NOPr previnem o efeito estimulatório da N/OFQ sob o eixo HPA (48).

Vários trabalhos demonstram, de forma consistente, que antagonistas do NOPr produzem melhora dos sintomas da depressão (81, 126). Okawa e cols. (2001) demonstraram a diminuição da liberação de noradrenalina do córtex pré-frontal do cérebro de ratos, após a injeção de 1,825 µg do agonista N/OFQNH2 dentro do *locus coeruleus*, sendo parcialmente revertida por 138,16 µg do antagonista [Nphe1]N/OFQ(1-13)NH2 (127). Além disso, ratos e camundongos com deleção gênica do NOPr tiveram redução no tempo de imobilidade no teste de nado forçado (107, 128-131). Contudo, Witkin e cols. não identificaram este mesmo efeito (132). Gavioli e cols. (2003, 2004) (129, 130) relataram que o bloqueio do NOPr pelo antagonista UFP-101, pela via i.c.v. (3-10 nmol/sítio), induz efeitos do tipo antidepressivo em camundongos e ratos. De acordo com Gavioli et al. (2003), a co-injeção com a N/OFQ, na dose de 1 nmol pela mesma via, levou a reversão dos efeitos do UFP-101 (3 e 10 nmol/sítio) (129). Em adição, o antagonista peptídico, UFP-101, apresentou efeito antidepressivo em camundongos nos testes do nado forçado e da suspensão da cauda, com a infusão de 5,72 µg, no hipocampo dorsal (133). A administração de UFP-101, pela via i.c.v., também resultou no aumento do consumo de sacarose por animais com estresse crônico moderado (134, 135). O antagonista SB-612111, pela via i.p., nas doses de 1-10 mg/kg, diminuiu o tempo de imobilidade no teste do nado forçado, sendo este efeito prevenido pela N/OFQ,

administrada centralmente na dose de 1 nmol (107). Importante ressaltar que Holanda et al. (2016) e Medeiros et al. (2015) também relataram efeitos antidepressivos para UFP-101 (3–10 nmol; i.c.v.) e SB-612111 (3–10 mg/kg; i.p.) (136, 137). Ademais, outros antagonistas do NOPr apresentaram ações antidepressivas em modelos de depressão, [Nphe1]-nociceptin (1–13)-NH2 e J-113397 (138), LY2940094 (139), assim como agonistas parciais, UFP-113 e [F/G]N/OFQ(1–13)NH2 (140). Por outro lado, os agonistas N/OFQ e Ro65-6570 do NOPr são capazes de prevenir efeitos do tipo-antidepressivo da nortriptilina e da fluoxetina, mas se apresentaram inativos *per se* em camundongos naive (141).

Abaixo estão descritos as ações de antagonistas do NOPr sobre modelos de depressão (81) (Tabela 3).

Tabela 3

Effects of NOP receptor antagonists in preclinical models of depression

Assay	Compound	Species and strain	Effects	References
Forced swimming test	UFP-101	Swiss and NOP(−/−) mouse	↓ Immobility time; no effects in NOP(−/−) mice	Gavioli et al. (2003, 2004)
	UFP-101	Wistar rat	↓ Immobility time	Gavioli et al. (2004)
	[Nphe^1]N/OFQ(1–13)-NH_2	CD-1 mouse	↓ Immobility time	Redrobe et al. (2002)
	UFP-113	Swiss mouse	↓ Immobility time	Asth et al. (2016)
	[F/G]N/OFQ(1–13) NH_2	Swiss mouse	↓ Immobility time	Asth et al. (2016)
	J-113397	CD-, Swiss, and NOP(−/−) mouse	↓ Immobility time; no effects in NOP(−/−) mice	Redrobe et al. (2002) and Gavioli and Calo' (2006)
	SB-612111	Swiss and NOP(−/−) mouse	↓ Immobility time; no effects in NOP(−/−) mice	Rizzi et al. (2007)
	LY2940094	NIH-Swiss and NOP(−/−) mouse	↓ Immobility time; no effects in NOP(−/−) mice	Post et al. (2016) and Witkin et al. (2016)

Tail suspension test	UFP-101	Swiss mouse	↓ Immobility time	Gavioli et al. (2004)
	SB-612111	Swiss mouse	↓ Immobility time	Rizzi et al. (2007)
DRL-72	LY2940094	SD rat	No effect	Witkin et al. (2016)
Chronic mild stress	UFP-101	Wistar rat	↑ Sucrose solution intake and ↓ immobility time after 21 days of treatment	Vitale et al. (2009, 2017)
Learned helplessness	SB-612111 and UFP-101	Swiss mouse	↑ Escapes and ↓ escape latencies	Holanda et al. (2016, 2018)
LPS-induced depressive-like behavior	SB-612111 and UFP-101	Swiss and CD-1 mouse	↓ Immobility time	Medeiros et al. (2015)

DRL differential reinforcement of low rate schedule, *J-113397* 1-[(3R,4R)-1-(cyclooctylmethyl)-3-(hydroxymethyl)-4-piperidinyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one, *LPS* bacterial lipopolysaccharide, *LY2940094* [2-[4-[(2-chloro-4,4-difluoro-spiro[5Hthieno[2,3-c]pyran-7,4′-piperidine]-1′-yl)methyl]-3-methylpyrazol-1-yl]-3-pyridyl]methanol, *SB-612111* (5S,7S)-7-[[4-(2,6-dichlorophenyl)-1-piperidinyl]methyl]-6,7,8,9-tetrahydro-1-methyl-5H-benzocyclohepten-5-ol, *UFP-101* [Nphe^{1}, Arg^{14}, Lys^{15}] N/OFQ-NH_2

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Com relação à ansiedade, a administração central de N/OFQ induziu o comportamento de ansiedade em ratos (142, 143). Contudo, esses dados são exceção à literatura e a maioria dos estudos apontam ausência de efeitos para os antagonistas de NOPr. Desta maneira, a administração central de UFP-101 (1-10 nmol), em ratos Wistar, reduziu a latência na esquiva inibitória no teste do labirinto em T elevado, indicando um efeito ansiolítico (144). Em adição, o antagonista, LY2940094, atenuou a imobilidade condicionada ao medo e a hipertermia induzidas pelo estresse (132). Mesmo com os dados que sugerem o impacto negativo do agonista natural N/OFQ sobre a ansiedade, a maior parte dos trabalhos se opõem a estes achados, demonstrando de forma consistente que a administração central de N/OFQ resultou na redução da ansiedade em roedores (140, 145-152). Assim, muitos ligantes não peptídicos do NOPr têm sido desenvolvidos como potenciais candidatos para o tratamento da ansiedade (55). Desta maneira, efeito ansiolítico foi verificado para agonistas em diferentes modelos de ansiedade e em diferentes espécies de roedores (81). Por exemplo, diferentes estudos revelaram um efeito do tipo-ansiolítico para os agonistas Ro

64-6198, Ro 65-6570, SCH 221510, SR-8993, AT-090 e Compostos 1, 1c e 3c, com aumento do tempo no braço aberto no labirinto em cruz elevado (96, 140, 153-158).

Abaixo estão listados os principais efeitos de agonistas do NOPr sobre modelos de ansiedade (81) (Tabela 4).

Tabela 4

Effects of non-peptide NOP receptor agonists in preclinical models of anxiety

Assay	Compound	Species and strain	Effects	References
Elevated plus-maze test	Ro 64-6198	Wistar and SD rat	↑ Time spent and distance moved in open arms	Jenck et al. (2000) and Dautzenberg et al. (2001)
	SCH 221510	CD-1 mouse	↑ Time spent in open arms	Varty et al. (2008)
	Compound 1c	Rat	↑ Time spent and distance moved in open arms	Wichmann et al. (1999)
	Compound 3c	Rat	↑ Time spent and distance moved in open arms	Wichmann et al. (2000)
	SCH 221510	Gerbil	↑ Time spent in open arms	Varty et al. (2008)
	Compound 1	Long-Evans and Hooded rat	↑ Time spent in open arms	Ross et al. (2015)
	Ro 65-6570	CD-1 mouse and NOP (−/−) mouse	↑ Time spent and number of entries in open arms; no effects in NOP(−/−) mice	Asth et al. (2016)
	SR-8993	Wistar rat	↑ Time spent in open arms in naive and after chronic alcohol consumption	Aziz et al. (2016)
	AT-090	CD-1 mouse and NOP (−/−) mouse	↑ Time spent and number of entries in open arms; no effects in NOP(−/−)	Asth et al. (2016)
Isolation-induced vocalizations	Ro 64-6198	CD-1 mouse	↓ Number and duration of vocalization	Varty et al. (2005)
	Ro 64-6198	Hartley guinea pig	↓ Number of vocalization	Varty et al. (2005)
	SCH 221510	Dunkin-Hartley guinea pig	↓ Separation-induced vocalizations in pups	Varty et al. (2008)
	Compounds 15 and 16	Hartley guinea pig	↓ Number of vocalization	Yang et al. (2009)
	SCH 655842	Dunkin-Hartley guinea pig	↓ Separation-induced vocalizations in pups	Lu et al. (2011)

Conditioned lick suppression	Ro 64-6198	CD-1 mouse	↑ Number of punished licks	Varty et al. (2005)
	SCH 221510	CD-1 mouse	↑ Number of punished licks	Varty et al. (2008)
	Compounds 15 and 16	Rat	↑ Number of punished licks	Yang et al. (2009)
	Compound 24	Rat	↑ Number of punished licks	Ho et al. (2009)
	SCH 655842	CD-1 mouse	↑ Number of punished licks	Lu et al. (2011)
	Ro 64-6198	Wistar rat	↑ Number of punished responses	Jenck et al. (2000)
	Ro 64-6198	SD rat	↑ Drinking time	Goeldner et al. (2012)
	SCH 221510	CD-1 mouse	↑ Number of punished licks	Varty et al. (2008)
	MCOPPB	ddY mouse	↑ Number of punished responses	Hirao et al. (2008b)
	PCPB	ddY mouse	↑ Number of punished responses	Hirao et al. (2008a)
Marble-burying test	Ro 64-6198	C57BL/6J mouse	↓ Marble-burying behavior	Nicolas et al. (2006)
	SCH 655842	C57BL/6J mouse	↓ Marble-burying behavior	Lu et al. (2011)
Ultrasound-induced defensive behaviors	Ro 64-6198	Lister-hooded rat	↓ Freezing behavior	Nicolas et al. (2007)
Fear-potentiated auditory startle	Ro 64-6198	Wistar rat	↓ Startle responses	Jenck et al. (2000)
Panic-like anxiety test	Ro 64-6198	Wistar rat	No effects	Jenck et al. (2000)
Open-field test	Ro 64-6198	Mouse	↑ Time spent in the center	Chang et al. (2015)
Social approach-avoidance	Ro 64-6198	Lewis rat	↑ Time spent in the social compartment	Goeldner et al. (2012)
Novelty-induced hypophagia	Ro 64-6198	C57BL/6J mouse	↓ Latency to drink and increase milk intake	Goeldner et al. (2012)
Stress-induced hyperthermia	Ro 64-6198	NMRI mouse	↓ Stress-induced hyperthermia	Goeldner et al. (2012)

AT-090 1-(1-((1s,4s)-4-iso-propylcyclohexyl)piperidin-4-yl)indoline-2,3-dione, *MCOPPB* 1-[1-(1-methylcyclooctyl)-4-piperidinyl]-2-[(3R)-3-piperidinyl]-1H-benzimidazole, *PCPB* 2-(3,5-dimethylpiperazin-1-yl)-1-[1-(1-methylcyclooctyl)piperidin-4-yl]-1H-benzimidazole, *Ro 64-6198* (1S,3aS)-8-(2,3,3a,4,5,6-hexahydro-1H-phenalen-1-yl)-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one, *Ro 65-6570* (RS)-8-(1,2-dihydro-1-acenaphthylenyl)-1-phenyl-1,3,8-triazaspiro [4.5]decan-4-one, *SCH 221510* 3-endo-8-[bis(2-methylphenyl)methyl]-3-phenyl-8-azabicyclo[3.2.1]octan-3-ol, *SCH 655842* endo-8-[bis(2-chlorophenyl) methyl]-3-phenyl-8-azabicyclo[3.2.1]octane-3-carboxamide, *SR-8993* 1-[1-(cyclooctylmethyl)-4-piperidinyl]-5-fluoro-2-(3R)-3-pyrrolidinyl-1H-benzimidazole

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1.5.2.3 Ligantes do NOPr na clínica

De maneira interessante, os ligantes do NOPr demonstraram eficácia em ensaios clínicos com pacientes (2, 78). Dentre estes estudos, potenciais para a utilização na clínica, destaca-se o antagonista LY2940094, um novo medicamento disponível na forma oral, para o tratamento da transtorno depressivo maior (139). Este antagonista também foi testado em pacientes com dependência alcóolica, reduzindo os dias de consumo pesado e aumentando os dias de abstinência (159). O agonista bifuncional cebranopadol foi conduzido em um ensaio clínico em fase II para o tratamento de pacientes com dor lombar crônica, revelando eficácia analgésica (160). Outro agonista, o SER100, em um tratamento por via s.c., induziu diminuição da pressão sanguínea diastólica e sistólica em pacientes com hipertensão sistólica isolada (161). Outros efeitos clínicos têm sido descritos para a N/OFQ, como ações benéficas para a incontinência urinária neurogênica. O agonista SCH486757 se mostrou efetivo na tosse subaguda, enquanto o JNJ-19385899 reduziu os sintomas de depressão e da ansiedade (78, 162). Além disto, foram descritos efeitos analgésicos para o antagonista, JTC-801, na dor neuropática e pós-operatória. Finalmente, o antagonista MK-5757 produziu melhora cognitiva dos sintomas esquizofrenia (78).

1.6 Fibromialgia

A fibromialgia é uma doença crônica, caracterizada principalmente por dor generalizada. Esta dor é iniciada por um estímulo habitualmente indolor, definindo assim, a alodínia presente na fibromialgia (163). A fibromialgia é uma dor disfuncional, ou seja, não é provocada por uma inflamação periférica, nem por uma lesão no sistema nervoso (164). Além disso, é uma dor presente mesmo sem estímulo, evocada por alta e baixa intensidade, sendo caracterizada por amplificação sensorial (164). Esta síndrome é acompanhada por sintomas como fadiga, dor musculoesquelética, distúrbios do sono, disfunções cognitivas e depressão (165-167). De acordo com a Associação

Internacional para o Estudo da Dor (IASP), a fibromialgia é definida como uma dor nociplástica, ou seja, relacionada com o processamento anormal da nocicepção (168).

Embora o diagnóstico da doença tenha embasamento no critério de classificação do Colégio Americano de Reumatologia (ACR), ainda permanece de difícil resolução (169). Os critérios utilizados em 1990 (ACR 1990) compreendiam: história de dor crônica generalizada (dor afetando os lados esquerdo e direito do corpo, acima e abaixo da cintura, e no esqueleto axial) e, pontos sensíveis à palpação digital (positivo para a doença quando ≥11 dos 18 sítios dolorosos) (170). No critério de 2010 (ACR 2010), os pontos sensíveis à palpação digital foram eliminados e um questionário com duas escalas foi adicionado: (i) o índice de dor generalizada e, (ii) o escore da gravidade dos sintomas (166). Este último critério sofreu modificações no ano de 2011 (ACR 2010 modificada), onde a estimativa de sintomas somáticos foi eliminada e as duas escalas foram expandidas (171). Em 2016, houve a revisão dos critérios de 2010/2011, sendo a fibromialgia diagnosticada de acordo com os seguintes critérios (1-4) (167):

(1) Dor generalizada, definida como dor em pelo menos 4 das 5 regiões (região superior esquerda, região superior direita, região inferior esquerda, região inferior direita e região axial), está presente.

(2) Os sintomas estão presentes em um nível semelhante há pelo menos 3 meses.

(3) Índice de dor generalizada (WPI) ≥ 7 e escore de gravidade dos sintomas (SSS) ≥ 5 ou WPI de 4-6 e escore SSS ≥ 9.

(4) Um diagnóstico de fibromialgia é válido independentemente de outros diagnósticos. Um diagnóstico de fibromialgia não exclui a presença de outras doenças clinicamente importantes.

A prevalência mundial de fibromialgia é de 2,1%, com uma proporção de mulheres para homens de 4: 1 (172). Contudo, estes dados são de difícil caracterização, pois a maioria dos estudos epidemiológicos utiliza diferentes critérios de diagnóstico. Além disto, indivíduos com idade entre 40 e 60 anos são os mais afetados (173-175).

1.6.1 Diagnóstico da fibromialgia

O diagnóstico da fibromialgia demonstra dificuldades, pois não existem achados laboratoriais que permitam a caracterização específica da síndrome. Contudo, estudos de neuroimagem mostram anormalidades no sistema nervoso de pacientes com fibromialgia, como estrutura cerebral alterada (176, 177), atividade metabólica (178) e conectividade funcional de estado de repouso (179), em regiões envolvidas no processamento da dor. Em adição, há um aumento na resposta a uma variedade de estímulos dolorosos (180-182). López-Solà et al. (183) verificaram a existência de uma "assinatura cerebral" em pacientes com fibromialgia, utilizando a ressonância magnética funcional. Nesse estudo, pacientes e grupo controle (indivíduos saudáveis) foram expostos a estímulos dolorosos (pressão) e indolores (táteis, auditivos e visuais) e foi demonstrado que os sujeitos fibromiálgicos apresentaram respostas aumentadas nas regiões de integração sensorial (ínsula/opérculo) e auto-referência (por exemplo, pré-frontal medial) e respostas reduzidas no córtex frontal lateral. A ressonância magnética permite elucidar o que está acontecendo em nível cerebral nos pacientes com fibromialgia, refletindo a individualidade da sensação dolorosa inerente a estes sujeitos (183).

1.6.2 Fisiopatologia da doença

A patogênese da doença não é clara, mas sabidamente, o processamento anormal da dor é influente na fibromialgia (184, 185). Em pacientes com a doença, uma pressão mecânica menor é necessária para desencadear a mesma atividade neuronal induzida por dor em relação a um indivíduo saudável. Em pacientes com fibromialgia, esta atividade também é maior (181). Isto se deve à sensibilização central, onde há uma resposta aumentada ao estímulo através da amplificação de sinal no SNC (185-187).

Além da sensibilização central, outras alterações fisiopatológicas têm sido sugeridas, incluindo uma influência genética no controle da dor, que pode ser modulada por fatores como ansiedade, depressão, estresse, trauma, adversidades na infância e/ou infecções. Fatores periféricos

também têm influência, como a dor contínua induzida por co-morbidades. Desequilíbrios de neurotransmissores e mudanças no HPA também podem ser detectados (188) (Figura 3).

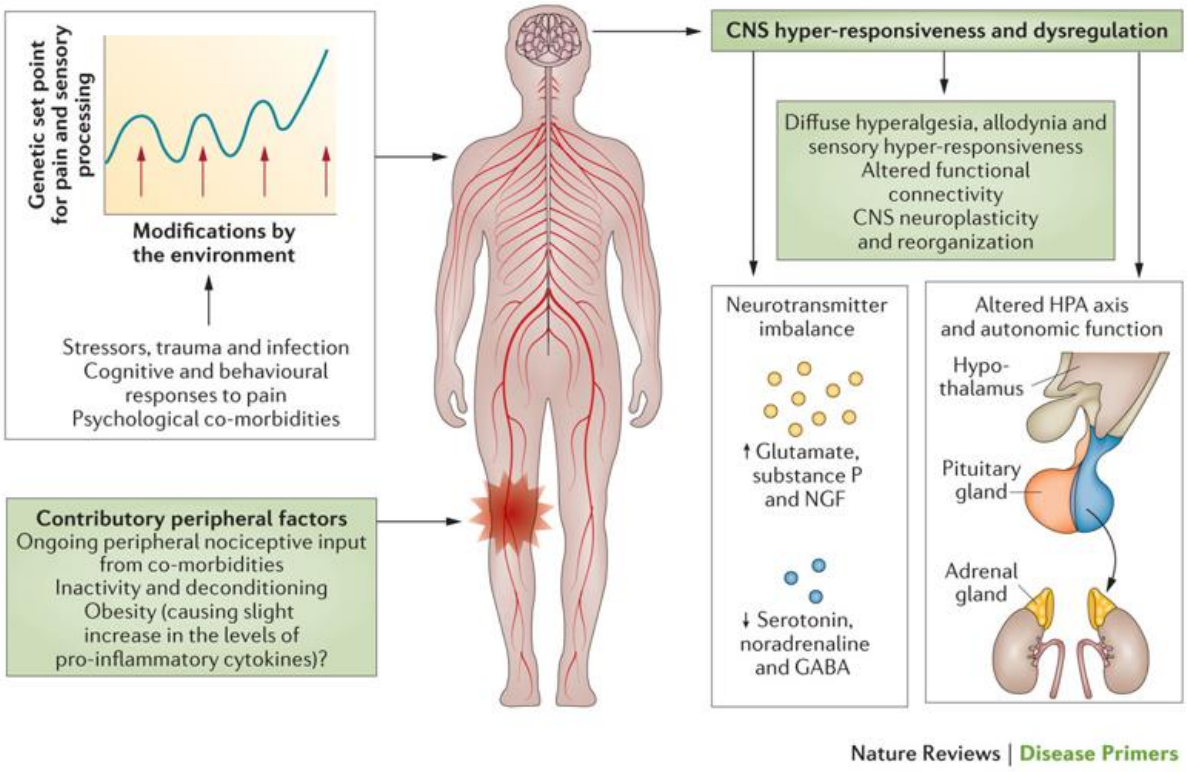

Figura 3: Potenciais processos fisiopatológicos na fibromialgia.

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Na via ascendente da dor, os neurônios aferentes primários trazem os estímulos nociceptivos da periferia até o corno dorsal da medula espinhal, onde ocorre a sinapse com neurônios de segunda ordem que se projetam centralmente. Estes estímulos chegam ao hipotálamo e sua percepção ocorre no córtex somato-sensorial. Os neurotransmissores que facilitam a transmissão da dor são a substância P (SP), o glutamato e o fator de crescimento do nervo (NGF). Estes neuropeptídeos estão com os níveis aumentados no fluído cerebroespinhal de pacientes com fibromialgia, estando implicados na excitabilidade dos neurônios da medula espinhal (189-192).

A inibição da transmissão da dor no corno dorsal ocorre através das vias descendentes: a medial, que tem origem na medula a partir de neurônios que contêm 5-HT, glutamato e ácido γ-aminobutírico (GABA) e, a via lateral, que se origina do tronco cerebral superior de neurônios que

contêm noradrenalina. No fluído cerebroespinhal de indivíduos com a síndrome, são encontrados níveis reduzidos de 5-HT e noradrenalina (189, 190, 193-195).

Os opioides endógenos também parecem estar envolvidos na fibromialgia, pois suas concentrações estão aumentadas no fluído cerebroespinhal de pacientes com fibromialgia (196). Além disto, estudos demonstram que pacientes com a doença apresentam diminuição da disponibilidade do receptor opioide MOP. Sugere-se que este receptor pode estar altamente ocupado por opioides endógenos, numa tentativa de reduzir a dor, ficando sua disponibilidade reduzida após a estimulação prolongada (197).

Lucas e cols. (198) sugeriram que mastócitos estão envolvidos na fibromialgia, e estas células estão aumentadas na camada papilar da derme de pacientes que tem a doença (199). Além disto, os mastócitos estão envolvidos na urticária crônica, condição frequente em indivíduos com fibromialgia (200). Os mastócitos secretam o peptídeo bradicinina, juntamente com o hormônio liberador de corticotrofina (CRH), histamina, interleucina-1 (IL-1), interleucina-6 (IL-6), prostaglandina D_2 (PGD_2) e TNF, e estas moléculas podem ativar os nervos sensoriais periféricos diretamente ou, chegar ao cérebro através da circulação sistêmica, criando um circuito de dor auto-sustentável. Estas moléculas são liberadas em resposta ao estímulo dos mastócitos pelos peptídeos do estresse: CRH, NGF, neurotensina (NT) e SP (201) (Figura 4). O CRH encontra-se elevado no fluído cerebroespinhal de pacientes com fibromialgia e está associada com a dor (202).

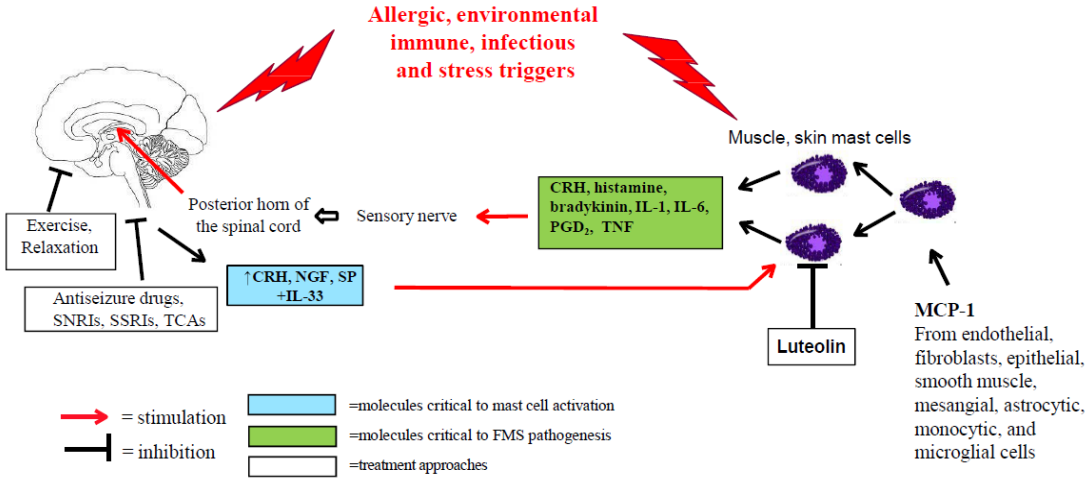

Figura 4: Representação dos mecanismos envolvidos na patogênese da fibromialgia e alvos para o tratamento. Extraído de (201) - *Publicação aprovada pela revista (carta de autorização para utilização do doi:10.1124/jpet.115.227298)*.

1.6.3 Fibromialgia e marcadores inflamatórios

Vários estudos sugerem uma relação entre a sintomatologia da fibromialgia e a ação de diferentes citocinas, pois estas atuam sobre o eixo HPA, linfócitos T e sobre o sistema nervoso simpático (203). As citocinas pró-inflamatórias estão envolvidas na amplificação da dor, através da sensibilização dos neurônios periféricos, aumentando as respostas ao óxido nítrico e à prostaglandina E_2 (PGE_2). As células gliais ativadas pela SP, pelo glutamato e pelo fator neurotrófico derivado do cérebro (BDNF) liberam citocinas pró-inflamatórias e vários neuropeptídeos, todos os quais podem contribuir para a amplificação da dor (204, 205) (Figura 5).

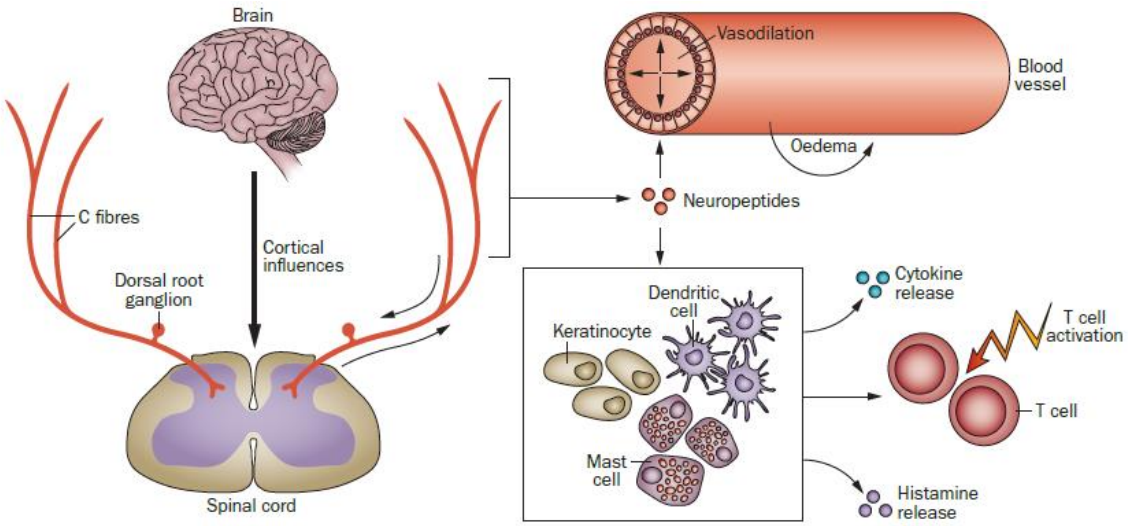

Figura 5: Efeitos centrais e periféricos associados com a liberação de neuropeptídios pelas fibras C terminais.

Extraído de (204) – *Publicação aprovada pela revista (Número de licença: 3713771070231).*

Sistemicamente, pacientes com fibromialgia, apresentam níveis elevados das citocinas pró-inflamatórias IL-6 e interleucina-8 (IL-8), níveis reduzidos ou inalterados das citocinas anti-inflamatórias interleucina-4 (IL-4) e interleucina-10 (IL-10) e concentrações normais de TNF. Além de níveis diminuídos da citocina pró-inflamatória IL-1β no plasma de pacientes fibromiálgicos. Contudo, muitos dos estudos nesta área apresentam problemas como amostras pequenas e qualidade metodológica baixa e, não levam em consideração que outras doenças (co-morbidades) podem influenciar no resultado da liberação de citocinas (206-210).

Com relação às citocinas pró-inflamatórias, foram detectados altos níveis de IL-1Ra (anticorpo do receptor de IL-1) e níveis inalterados de IL-1β e IL1 em pacientes com fibromialgia (211-213). Diferenças entre o grupo controle e indivíduos com a doença não foram encontradas para TNF (212, 213). .

Outros estudos demonstram um aumento de TNF e IL-8 e IL-10 no plasma e soro de pacientes com fibromialgia (208, 211, 214). A concentração elevada de IL-8 no fluído cérebro-espinhal medeia a dor simpática, através da ativação de células gliais (215). Quanto à concentração de IL-6, também há discordância entre os estudos, mas níveis aumentados desta interleucina têm sido associados com pacientes que têm a doença (206, 208, 211, 214, 216). Um estudo de

Pernambuco e cols. (217) mostrou níveis aumentados de interleucina-17A (IL-17A), interleucina-2 (IL-2), IL-4, TNF e IFN-γ no plasma de pacientes com fibromialgia. A análise de amostras de pele de pacientes com a doença não demonstrou diferenças significativas nas concentrações de IL-10, quando comparadas com o grupo controle (218). Em adição à IL-10, outras citocinas anti-inflamatórias têm sido pouco estudadas, quando comparadas as citocinas pró-inflamatórias (207). Uma concentração reduzida de IL-4 foi encontrada no sangue total (219) e no plasma (220) de pacientes com fibromialgia, além da diminuição de outras citocinas de respostas do tipo Th2, como interleucina-5 (IL-5) e interleucina-13 (IL-13) (220). Yigit e cols. (221) observaram associação entre polimorfismo no gene da IL-4 e fibromialgia.

Alguns autores observaram o aumento de quimiocinas pró-inflamatórias em indivíduos com a síndrome, como altos níveis do ligante 2 de CC quimiocina (CCL2) e eotaxina no plasma (222) e uma concentração elevada de ligante 9 de quimiocina CXC (CXCL9), ligante 22 de CC quimiocina (CCL22), ligante 11 de quimiocina CXC (CXCL11), ligante 17 de CC quimiocina (CCL17) e eotaxina no soro (223). Um trabalho recente (2016), demonstrou que monócitos em repouso ou ativados de pacientes com fibromialgia secretam mais CCL11, CCL22 e CXCL1 quando comparados ao grupo controle (224).

1.6.4 Tratamento

Grupos na Europa, América do Norte e no Oriente Médio enfatizam que o tratamento da fibromialgia deve englobar terapias farmacológicas e não farmacológicas adaptadas a cada paciente (225). Além disto, por causa da heterogeneidade dos sintomas, um único tratamento não é eficaz para todos os indivíduos, sendo necessária, em alguns casos, uma combinação farmacológica (226). Dentre os tratamentos não famacológicos, cabe citar a educação do paciente para a realização de atividades físicas e a terapia cognitivo-comportamental (TCC), que são benéficas em relação à dor e ao humor (227-229). A melhora na eficácia da TCC ocorre quando esta faz parte de um programa de tratamento multidisciplinar (educação, exercício e terapia psicológica). Nesse contexto, a

combinação de milnaciprano e TCC e a monoterapia com TCC foram igualmente benéficos para diminuir os sintomas da fibromialgia, sendo que o medicamento em pouco acrescentou no alívio dos sintomas (230, 231).

O antidepressivo tricíclico, amitriptilina, administrado em baixas doses, é efetivo para os sintomas da dor, distúrbios do sono e fadiga, mas tem efeitos limitados devido ao desenvolvimento de taquifilaxia e efeitos adversos anticolinérgicos e anti-histamínicos (232). A amitriptilina e a ciclobenzaprina apresentam efeitos como boca seca e constipação (233, 234). Outros antidepressivos, como os inibidores seletivos da recaptação de serotonina (SSRI), têm efeito positivo sobre a dor (235), mas medicamentos como a fluoxetina, a paroxetina e a sertralina podem ocasionar disfunção sexual, distúrbios do sono e náuseas (234). Já, os inibidores da recaptação de serotonina e noradrenalina (SNRI), a duloxetina e o milnaciprano, apresentam efeitos como dor de cabeça, náuseas, palpitações, hipertensão e taquicardia (234).

A venlafaxina é um medicamento que tem demonstrado efeitos positivos ou neutros para a fibromialgia; também é bem tolerado pelos pacientes e apresenta um baixo custo (236-239). Entretanto, estudos com o uso deste medicamento na depressão revelaram reações adversas, como insônia, tonturas, sonolência, prisão de ventre, sudorese e aumento ligeiro da pressão arterial (240-242).

Um dos fármacos mais prescritos para tratar casos mais resistentes é o tramadol, um agonista fraco do receptor MOP (243). Contudo, este medicamento apresenta efeitos adversos (hiperalgesia induzida por opioides e sedação), não tendo efeito benéfico sobre a qualidade de vida dos indivíduos, com eficácia moderada sobre a dor (244, 245). Os analgésicos anti-inflamatórios não esteroidais são frequentemente utilizados por pacientes com fibromialgia, mas não há evidências claras da sua eficácia (175, 246, 247).

Os sedativos benzodiazepínicos, assim como, os agentes hipinóticos não benzodiazepínicos, são empregados para o tratamento de distúrbios do sono e ansiedade em pacientes com fibromialgia, mas o uso crônico pode ocasionar tolerância (248, 249). Estudos controlados não

conseguiram demonstrar efeitos benéficos suficientes do clonazepam para a sua utilização na fibromialgia (246, 250-252). Outra classe estudada para o tratamento da fibromialgia tem sido a dos agonistas dopaminérgicos, que melhoraram os sintomas da doença, como o pramipexol (253).

O oxibato de sódio (um sal de gama-hidroxibutirato; GHB) se mostrou eficaz para o controle dos sintomas da fibromialgia, mas apresentou problemas como vômitos, tontura, diarreia, dor de cabeça, ansiedade e sinusite (254). Por motivos de segurança (potencial droga de abuso) e por não apresentar resultados equivalentes aos medicamentos já aprovados para uso, o oxibato de sódio não foi aprovado pela Food and Drug Administration (FDA) e não é utilizado no Brasil para o tratamento da fibromialgia (249, 255, 256).

Os anticonvulsivantes também são utilizados no tratamento da fibromialgia, mas fármacos como a pregabalina apresentam pouco efeito sobre a fadiga (249). Os fármacos, gabapentina e pregabalina, apresentam baixa eficácia para a fibromialgia e ação limitada para a fadiga, a ansiedade e a depressão (257). Efeitos adversos importantes como sonolência, tontura, ganho de peso e edema são observados com estes medicamentos (258).

Em um estudo duplo-cego, a nabilona (canabinoide sintético) diminuiu significativamente a dor de pacientes com fibromialgia, após um tratamento de quatro semanas. Contudo, após oito semanas os seus efeitos desapareceram (259). Além disto, efeitos adversos como sonolência, boca seca e vertigem foram verificados (260). Outro estudo demonstrou diminuição dos distúrbios do sono, assim como efeitos adversos para este medicamento, incluindo, tonturas, náuseas, boca seca e sonolência (261). Lynch e cols. (262) descreveram que a utilização de canabinoides para indivíduos com fibromialgia apresentam efeitos modestos, porém seguros.

1.6.5 Fibromialgia e alterações musculares

A fibromialgia é caracterizada por dor musculoesquelética, fadiga e perda de força muscular. Esses sintomas têm sido correlacionados com disfunções sensoriais e motoras, envolvendo alterações centrais e periféricas (263). Srikuea e colaboradores descreveram que pacientes com

fibromialgia apresentam maior variabilidade de tamanho e alteração na distribuição do tamanho das fibras musculares, quando comparados com indivíduos saudáveis (264). Alterações na morfologia mitocondrial dos músculos gastrocnêmio e sóleo têm sido investigadas no modelo de fibromialgia induzida por estresse, após indução ao frio intermitente (ICS) (265). Neste estudo, os autores demonstraram diminuição na área transversal das fibras musculares de camundongos machos submetidos ao modelo de fibromialgia por ICS. Além disto, também foi relatada a perda de fibras do tipo IIa no sóleo destes animais. No gastrocnêmio, houve aumento na densidade de células positivas para marcadores inflamatórios e atrogênicos, com a presença de mitocôndrias danificadas. Neste mesmo modelo de fibromialgia, Oezel e cols. (266) observaram aumento da atividade da enzima lactato desidrogenase (LDH) nos extratos mitocondriais dos músculos de camundongos induzidos por ICS.

1.6.6 Sistema nociceptina/orfanina FQ-NOPr na fibromialgia

Poucos trabalhos têm investigado a relação entre o peptídeo N/OFQ e a fibromialgia. Anderberg e colaboradores (267) descreveram diminuição dos níveis plasmáticos de N/OFQ em mulheres fibromiálgicas, na fase lútea do ciclo menstrual, quando comparadas ao grupo controle. No trabalho de Baraniuk et al. (196), verificou-se que não houve diferença significativa nas concentrações de N/OFQ no fluído cérebro-espinhal, em pacientes com fibromialgia, comparados com indivíduos controle ou, com diagnóstico de dor lombar crônica.

2 JUSTIFICATIVA

Vários estudos têm demostrado atividades antinociceptivas para agonistas e antagonistas do NOPr, em modelos de dor aguda, inflamatória e neuropática, tanto em roedores quanto em primatas não-humanos. Ademais, os ligantes do NOPr apresentam efeitos sobre alterações comportamentais relacionadas com ansiedade e depressão em roedores. A fibromialgia, representa uma síndrome dolorosa, com co-morbidades, sendo que os pacientes apresentam quadros depressivo-ansiosos. Por outro lado, diversos efeitos adversos têm sido demonstrados pelos tratamentos existentes para controlar as alterações relacionadas à fibromialgia.

Com base no que foi descrito acima, justifica-se a relevância de investigar os possíveis efeitos farmacológicos exercidos por agonistas e antagonistas do NOPr em modelo de fibromialgia. Dessa forma, o presente estudo permitirá obter evidências sobre o papel do NOPr no modelo de fibromialgia induzido por reserpina em camundongos, a fim de identificar novas alternativas potenciais para a compreensão e tratamento desta doença, contribuindo para o avanço científico na área de Farmacologia e Terapêutica Experimental.

3 OBJETIVOS

3.1 Objetivo Geral

O presente estudo teve como objetivo avaliar o efeito dos ligantes do NOPr em um modelo de fibromialgia em camundongos, bem como, investigar a plasticidade do sistema N/OFQ-NOPr nesse modelo.

3.2 Objetivos Específicos

- Investigar os efeitos do tratamento agudo com N/OFQ ou com o antagonista peptídico, UFP-101, administrados por diferentes vias, sobre a alodínia mecânica, nocicepção térmica e comportamentos do tipo depressivo-ansioso, em camundongos, no modelo de fibromialgia induzido por reserpina;
- Determinar os níveis N/OFQ em amostras de saliva de pacientes com fibromialgia e, em amostras de cérebro e soro, em animais submetidos ao modelo de fibromialgia;
- Investigar os efeitos do tratamento agudo com a naloxona (antagonista do receptor μ) em combinação com N/OFQ;
- Investigar os efeitos do tratamento agudo da combinação de N/OFQ, com o antagonista UFP-101;
- Investigar os efeitos do tratamento repetido com os antagonistas NOPr, UFP-101 e SB-612111, sobre a alodínia mecânica, nocicepção térmica, e comportamentos do tipo depressivo-ansioso e na fadiga, em camundongos, no modelo de fibromialgia induzido por reserpina;
- Avaliar os efeitos da reserpina e do bloqueio farmacológico do NOPr sobre o tamanho das fibras musculares através de avaliação histológica;

- Avaliar os efeitos da reserpina e dos tratamentos com os antagonistas NOPr sobre a morfologia mitocondrial do músculo, através de microscopia eletrônica de transmissão;
- Avaliar os níveis cerebrais e espinhais de 5-HT e glutamato após o tratamento repetido com o antagonista UFP-101, através de LC-MS/MS;
- Analisar a ativação cerebral, através do microPET/CT, após a indução de fibromialgia por reserpina, avaliando o tratamento repetido com o antagonista UFP-101;
- Avaliar a expressão da ppN/OFQ e do NOPr no cérebro, na medula espinhal e no músculo, através de PCR em tempo real, em camundongos, no modelo de fibromialgia induzido por reserpina;
- Avaliar a expressão do NOPr em amostras de cérebro, DRG, medula e músculo, através de imunoistoquímica em camundongos, no modelo de fibromialgia induzido por reserpina;
- Medir os níveis substância P em amostras de cérebro, medula e músculo, através de ELISA após o tratamento repetido com o antagonista UFP-101;
- Avaliar os efeitos dos tratamentos sobre os níveis de citocinas pró-infamatórias (TNF e IL-1β) e da citocina anti-inflamatória IL-10, em amostras de cérebro, medula, músculo e soro, através de ELISA, após o tratamento repetido com o antagonista UFP-101;
- Determinar os níveis de glutationa em amostras de cérebro, medula e músculo, após o tratamento crônico com o antagonista UFP-101, em animais submetidos ao modelo de fibromialgia;
- Determinar a atividade da LDH em amostras de soro e músculo, após o tratamento repetido com o antagonista UFP-101.

4 MANUSCRITO DO TRABALHO EXPERIMENTAL

Nociceptin/orphanin FQ receptor modulates painful and fatigue symptoms in a mouse model of fibromyalgia

Short running title: NOP receptor and fibromyalgia

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ABSTRACT

Generalized pain and fatigue are both hallmarks of fibromyalgia, a syndrome with an indefinite aetiology. The treatment options for fibromyalgia are currently limited, probably due to its intricate pathophysiology. Thus, further basic and clinical research on this condition is currently needed. This study investigated the effects of nociceptin/orphanin FQ (N/OFQ) receptor (NOPr) ligands and the modulation of the NOP system in the preclinical mouse model of reserpine-induced fibromyalgia. The effects of administration of the natural agonist N/OFQ and the selective NOPr antagonists (UFP-101 and SB-612111) were evaluated in fibromyalgia-related symptoms in reserpine-treated mice. The expression of preproN/OFQ (ppN/OFQ) and NOPr was assessed in central and peripheral sites at different time-points after reserpine administration. N/OFQ displayed dual effects in the behavioural changes in the reserpine-elicited fibromyalgia model. The peptide NOPr antagonist UFP-101 produced analgesic and anti-fatigue effects, by preventing alterations of brain activity and skeletal muscle metabolism, secondary to fibromyalgia induction. The non-peptide NOPr antagonist SB-612111 mirrored the favourable effects of UFP-101 in painful and fatigue alterations induced by reserpine. A time-related up- or down-regulation of ppN/OFQ and NOPr was observed in supraspinal, spinal and peripheral sites of reserpine-treated mice. Our data shed new lights on the mechanisms underlying the fibromyalgia pathogenesis, supporting a role for N/OFQ-NOP receptor system in this syndrome.

Introduction

The worldwide prevalence of fibromyalgia is 2.1%, with a female to male ratio of 4:1 [13]. Patients with a fibromyalgia diagnosis display widespread pain, usually evoked by painless stimuli, thus defining the allodynia present in this syndrome [70]. It is accompanied by comorbidities such as fatigue, sleep disturbances, cognitive dysfunctions and depression [83]. A recent note by the International Association for the Study of Pain (IASP) described fibromyalgia as a nociplastic pain, i.e. “pain that arises from altered nociception” [6]. The fibromyalgia pathogenesis is not well defined, but abnormal pain processing is patently present [18]. Besides the involvement of monoamine deficits, the symptoms of fibromyalgia likely rely on the release of neuropeptides and cytokines, which causes central and peripheral alterations [46].

Nociceptin/orphanin FQ (N/OFQ) is a 17-amino acid peptide that exerts its biological actions by activating the opioid-related G protein-coupled nociceptin/orphanin FQ receptor (NOPr) [2]. The effects of NOPr ligands have been characterized in pain, depression and anxiety, among a series of pathophysiological alterations [17,25,39,44,61,65,82,86,87]. Regarding the painful responses, NOPr agonists can elicit either nociception or antinociception, depending on the experimental paradigm [39]. The actions of NOPr ligands have been examined in a series of rodent models of long-lasting pain, as reviewed by Kiguchi et al., 2016 [39]. Notably, chronic neuropathic pain states have been correlated with a plasticity of the N/OFQ-NOPr system. The mRNA levels of the N/OFQ precursor prepronociceptin and the NOPr were decreased in the thalamus and hypothalamus [59], whereas the NOPr mRNA expression was increased in the dorsal root ganglia (DRG) and spinal cord [12] of mice submitted to the chronic constriction injury (CCI) model. In rats, the CCI led to an upregulation of N/OFQ levels in DRG, periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) [16]. Additionally, the spinal nerve ligation induced a decrement of NOPr immunoreactivity in the ipsilateral L4-L5 spinal cord and dorsal root ganglion (DRG) of mice [58].

Despite the substantial evidence showing a relevant role for the N/OFQ-NOPr system in neuropathic pain, only a few studies examined its participation in fibromyalgia. Anderberg and cols. [3] described a decrease of N/OFQ plasma levels in fibromyalgic women in the luteal phase of the menstrual cycle, when compared to controls. Otherwise, Baraniuk and cols. [7] did not identify any significant difference of N/OFQ concentrations in the cerebrospinal fluid of patients with fibromyalgia, in relation to control health subjects or individuals with chronic low back pain. Accordingly, the literature data regarding the role of the NOP system in fibromyalgia is controversial, indicating the needing of additional studies. Considering the previous evidence showing a relevant participation of the NOP system in fibromyalgia-related symptoms and

comorbidities (such as pain, depression and anxiety), we judged relevant to perform additional investigations on this matter. To our knowledge, no previous study assessed the effects of NOP ligands and/or the plasticity of this system in experimental fibromyalgia, justifying the premise of the study.

The biogenic amine depletion induced by reserpine has been firstly validated in rats and after in mice, as an animal model of fibromyalgia. In this experimental paradigm, the repeated administration of reserpine to rodents leads to fibromyalgia-related painful and depression changes (face validity), leading to reduced levels of neurotransmitters (construct validity), being sensitive to the treatment with pregabalin, duloxetine and pramipexole (predictive validity) [5,8,11,21,41,43,54,55]. The present study evaluated the site-specific modulatory effects of NOPr ligands in a mouse model of reserpine-induced fibromyalgia, investigating the plasticity of the N/OFQ-NOPr system at the supraspinal, spinal and peripheral sites.

Methods

Animals

The experimental procedures followed the current Brazilian guidelines for the care and use of animals for scientific and didactic procedures, from the National Council for the Control of Animal Experimentation (CONCEA, Brazil, 2014). The local Animal Ethics Committee evaluated and approved all of the protocols (CEUA 15/00487). The animal studies are reported in compliance with the ARRIVE guidelines [40,49].

Female CF-1 specific-pathogen-free mice (2-month old, 20-24 g, N = 596) were obtained from the Centre for Experimental Biological Models (CeMBE, PUCRS, Porto Alegre, RS, Brazil). Reserpine leads to fibromyalgia-related symptoms with similar effects in female and male mice [33]. The experimental *n* was determined based on previous literature data [43]. The *n* per group is indicated within the figures. The dose-response experiments were independently replicated three to four times, explaining the variations of the *n*. We initiated with the systemic administration of NOP ligands, by i.p. route, with the dose of 3 nmol/kg (for N/OFQ and UFP-101), including the appropriate negative and positive controls. To complete the dose-response curves, we tested additional doses of NOP ligands, but we included more animals treated with the 3-nmol/kg dose, besides the negative and positive control groups, for purposes of comparison. For this reason, the *n* presents such a great variation in this experimental set. We performed an analysis to determine the power of a completed experiment (GraphPad StatMate 2.00; by GraphPad Software Inc.), considering a significance level (alpha=0.05; two-tailed). The analysis revealed power values ranging from 80% to 95% for this experimental set.

The mice were kept in micro-isolator cages (4 per cage), equipped with inlet/outlet air filters, under controlled temperature (22 ± 1°C) and humidity (50 - 70%), and a light-dark cycle of 12 h (lights on at 7 a.m., lights off at 7 p.m.). The cages were filled with autoclaved wood chip bedding. The animals received pelleted feed and sterile water *ad libitum*. During the experimental procedures, the laboratory temperature was maintained at 22 ± 1°C. A period of adaptation to the new environment of at least one hour was used. All the experiments were performed between 7 a.m. and 7 p.m.

The animals were allocated into the experimental groups (with or without fibromyalgia induction) considering their basal responses to Von Frey stimulation before fibromyalgia induction. The mice were treated and assessed in the behavioural tests in the following order: vehicle/saline (negative control group), reserpine/saline (positive control group), mice treated with reserpine/NOPr ligands, one mice from each group per turn. When possible, the collection of samples for biochemical, molecular and histochemical studies was performed after the behavioural

assessments, to reduce the total number of animals included in the study. The investigators were blinded to the experimental groups in either *in vivo* or *ex vivo* assessments. The animals were euthanized by sevoflurane inhalation.

Drugs

Reserpine and naloxone were purchased from Sigma Aldrich Chemical Company (St. Louis MO, USA); N/OFQ and UFP-101 were purchased from Tocris Bioscience (Bristol, UK). Pregabalin (Lyrica) was obtained from Pfizer (Tadworth, UK). SB-612111 was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). N/OFQ and UFP-101 (1 mM) were diluted in saline solution (0.9% NaCl) and stored at - 20°C. SB-612111 (10 mM) was diluted in 1.2% dimethyl sulfoxide (DMSO) in saline solution (V/V), and stored at - 20°C. Reserpine was dissolved in 0.5% vehicle solution in distilled water (V/V). Pregabalin and naloxone were diluted in saline solution (0.9% NaCl). Reserpine, pregabalin and naloxone were diluted immediately before use. The NOPr ligands were prepared at the desired concentration just before treatments.

Reserpine-induced fibromyalgia

The fibromyalgia mouse model was accomplished as described before [43]. Briefly, an amine depletion was induced by reserpine administration via subcutaneous route (s.c.; 0.25 mg/kg), once a day, for three consecutive days. The control group received the vehicle (0.5% acetic acid). The behavioural tests were carried out on the fourth day (Supplementary Figure 1A). The samples were collected from one to four days after the onset of the induction protocol, depending on the analysis.

Dose-related effects of NOPr ligands by different routes of administration

This part of the study evaluated the acute dose-dependent effects of N/OFQ (NOPr agonist) and UFP-101 (selective peptide NOPr antagonist) on painful-, depression- and anxiety-like changes, in the mouse model of reserpine-induced fibromyalgia. To assess the sites of action of NOPr ligands, N/OFQ or UFP-101 were administered by the intracerebroventricular (i.c.v.), intrathecal (i.t.) or intraperitoneal (i.p.) routes, 15 min (i.c.v. and i.t) or 30 min (i.p.) before the experimental sessions, on the fourth day after the onset of the fibromyalgia induction protocol (Supplementary Figure 1A). Mice were evaluated in behavioural tests, for assessing mechanical and thermal hypersensitivity, depression-related immobility time, and anxiety parameters, as described in the next sections. The *n* for this experimental set was 303 animals (Supplementary Figure 1B).

The doses of N/OFQ and UFP-101 were determined based on prior publications [15,51,56,63,85]. For N/OFQ, the doses were 1 nmol/site (i.c.v.); 0.3, 1 and 3 nmol/site (i.t.); and 0.3, 1, 3 and 5 nmol/kg (i.p.). The doses of UFP-101 were 0.3 and 1 nmol/site (i.c.v.); 1, 3 and 5 nmol/site (i.t.); and 0.3, 1, 3 and 5 nmol/kg (i.p.). An additional dose of N/OFQ was tested by i.c.v. route (3 nmol/site), but the animals presented unspecific central side effects, such as shivering and shaking (n = 3). The animals in this group were euthanized, and they were excluded from the study.

For the i.t. injections, a volume of five μl of saline containing N/OFQ or UFP-101 was injected between the L5 and L6 vertebral spaces. For the i.c.v injections, a volume of two μl of saline containing N/OFQ or UFP-101 was injected directly into the lateral ventricle (coordinates from bregma: 1 mm lateral, 1 mm rostral, 3 mm vertical), of animals slightly anesthetized with sevoflurane (3%) and oxygen (97%). The control groups received the corresponding volumes of vehicle (0.9% NaCl solution) [43].

Assessment of N/OFQ selectivity and site of action of UFP-101

To exclude the participation of opioid receptors in the effects of N/OFQ, the animals were pre-treated with the MOP/DOP/KOP antagonist naloxone (5 μmol/kg; i.p.) [74], administered 5 min prior to N/OFQ treatment. To assess whether UFP-101 might revert the N/OFQ effects, separate groups of animals received (i) UFP-101 (1 nmol/kg; i.p.) plus N/OFQ (1 nmol/kg or 5 nmol/kg; i.p.; co-treatment); (ii) UFP-101 (1 nmol/kg; i.p.; 15 min prior) plus N/OFQ (1 nmol/site; i.c.v.); or (iii) UFP-101 (1 nmol/kg; i.p.; 15 min prior) plus N/OFQ (1 nmol/site; i.t.). The doses of NOPr ligands were chosen from the dose-response experiments. The behavioural changes were evaluated at the fourth day after the beginning of the protocol for induction of fibromyalgia. One hundred and seven animals were used in this experimental set.

Repeated treatment with NOPr antagonists

Based on the dose-response experiments, the effects of the selective peptide NOPr antagonist UFP-101 (1 nmol/kg; i.p.) were evaluated in a protocol of repeated administration. The animals received UFP-101, for three consecutive days, 30 min after the daily reserpine injection. On the fourth day, the animals also received UFP-101, 30 min before the behavioural evaluation. Control animals received saline (0.9% NaCl, 10 ml/kg; i.p.). Pregabalin, an inhibitor of the α2δ subunit of voltage-gated calcium channels, was used as a positive control drug (188 μmol/kg, i.p.), and it was administered at the same schedule of treatment. The pregabalin dose was selected based on a previous publication [71]. Mice were assessed for mechanical and thermal hypersensitivity,

depression-related immobility time, and anxiety parameters. The animals were also submitted to additional tests to analyse fatigue-associated symptoms (*n* for this experimental set of 76 mice).

The effects of the repeated treatment with the selective non-peptide NOPr antagonist SB-612111 (6.6 µmol/kg, i.p.) were also investigated. The antagonist or the vehicle was dosed, for three consecutive days, 30 min after the reserpine injection. On the fourth day, the animals also received SB-612111, 30 min before the behavioural testing. Separate experiments were performed to test the effects of different doses of SB-612111 (2.2, 6.6 and 22 µmol/kg) on the fatigue symptoms evoked by reserpine. The doses of SB-612111 were selected from previous studies [48,62,85]. An experimental *n* of 56 animals was used for this part of the study. A general presentation of the repeated schedule of treatment and the behavioural tests for this experimental set is depicted in the Supplementary Figure 1B, C, D.

Behavioural tests

Mechanical hypersensitivity

The animals were placed in individual Plexiglas compartments on a metal screen. An adaptation period of 60 min before testing was used. The mechanical allodynia was evaluated using a 0.4-g Von Frey hair filament. The results were expressed as the withdrawal response frequency (%) [24]. The filament was applied ten times to the plantar surface of the right hind paw, with three seconds between each application. The withdrawal response frequency was evaluated before (baseline records), and at the fourth day after the onset of fibromyalgia induction. A significant increase in the response frequency compared to the baseline was considered as an indicative of mechanical hypersensitivity.

Hot-plate test

The thermal hypersensitivity following heat stimulation was assessed in the hot-plate apparatus (Ugo Basile, Italy), as described previously [42]. The surface of the hot-plate was heated at a constant temperature of 50 ± 1°C. After the appropriate treatments, the animals were placed in the apparatus, which consists of a metal plate surrounded by a transparent acrylic cylinder. The latency to respond to heat stimulus (hind paw licking, withdrawal of the hind paw, or a jump) was measured before (baseline records) and at the fourth day after initiating the induction of fibromyalgia. The tests were finalised if the animals did not respond within 30 s, to avoid tissue damage.

Forced swimming test

The experiments were performed using a cylinder (18.5 cm in diameter and 25 cm in height) filled with water at a height of 17 cm. The water was maintained at 23-25°C. The animals were placed in the water and the immobility was defined as the absence of any movements, except those necessary to keep the mouse's head above the water [47]. The time that the mice remained immobile was quantified over a period of 6 min, at the fourth day after initiating the protocol of reserpine treatment, and it was used as an indication of depressive-like behaviour.

Elevated plus maze

The mice were placed in the intersection of the four arms of an elevated plus maze and their behaviour was recorded for 5 min [78]. The parameters recorded were the total number of entries, the number of entries in the open arms, and the percent of time spent in the open arms.

Fatigue evaluation on the rotarod

A rotarod apparatus (Insight, Ribeirão Preto, Brazil) was used to assess the fatigue-like symptoms in reserpine-treated mice, in the protocols of repeated treatment with the NOPr antagonists. The mice were trained three times, for one min, at 20 rpm, on the day before the first reserpine injection (baseline records). At the fourth day, the mice were submitted to two exercise sections (1 min each) and afterwards, the fatigue analysis was assessed at a speed of 20 rpm. The duration for which the mouse remained on the rod was recorded. A 60-min cut-off time was used [80].

Grasping strength measurement

The mice were submitted to the grasping test, as an indication of the grip strength [19]. For this purpose, the animals were repeatedly treated with UFP-101 or SB-612111 as described before. After 30 min of the last treatment, they were lifted by the tail and allowed for grasping a grid connected to an electronic balance. When the first signs of active finger flexion were noted, the grasping strength was registered (in grams). The baseline records were acquired before the first reserpine injection. On this occasion, the mice were submitted to three training assessments. On the fourth day, the mean of three readings was used to calculate the individual grasping strength.

Kondziela's inverted screen test

The Kondziela's inverted screen test [20] was performed on day 1 (baseline records) and on day 4, in the groups submitted to the repeated treatment with NOPr antagonists. The mice were placed in the centre of the wire mesh screen and the apparatus was inverted. The time elapsed before the mouse fell from the screen was recorded. A one min cut-off time was used.

Determination of neurotransmitters by LC-MS/MS

The levels of serotonin (5-HT) and glutamate were analysed in thalamus/hypothalamus, prefrontal cortex and lumbar spinal cord of animals submitted to the repeated treatment with UFP-101 (1 nmol/kg; i.p) in the mouse model of reserpine-induced fibromyalgia, according to the method described by [43]. Tissues were collected on the fourth experimental day. The results were expressed as ng/g tissue.

MicroPET imaging

These experiments were performed as described previously [67], to assess the supraspinal changes related to fibromyalgia induction, as well as, the effects of NOPr modulation on brain activity. Saline or UFP-101 (1 nmol/kg; i.p) were repeatedly administered for three consecutive days, 30 min after the daily reserpine injection, and 10 minutes after [^{18}F]-FDG (250 μCi, i.p.), at the fourth day. After [^{18}F]-FDG administration, the animal remained isolated and conscious for 40 min (uptake). For the scanning in Triumph microPET (LabPET-4, TriFoil Imaging, Northridge, CA, USA), the rodent was anesthetized with inhalatory isoflurane and medical oxygen (3-4% induction and 2-3% maintenance dose) and placed in a supine position in the imaging chamber, maintained at a constant temperature of 36°C. The animals were scanned for 10 min, with the brain region positioned in the centre of the microPET field-of-view (FOV). At the end of the scan, the mouse was removed from the device, and kept on a heating surface until complete recovery. The animals were scanned on the fourth day after initiating the protocol for fibromyalgia induction. The reconstruction algorithm for image processing was the MLEM-3D, and the capture of brain [^{18}F]-FDG was quantified through the PMOD v3.5 software and Fusion Toolbox (PMOD Technologies, Zurich, Switzerland). Each mouse's uptake of [^{18}F]-FDG was normalised to a reference brain region, avoiding unwanted sources of variation related to differences in mouse body weights. Glucose uptake in all brain regions was normalized by the cerebellum and expressed as relative standardized uptake value (SUVr) [84].

Dorsal root ganglion (DRG) isolation

This protocol was accomplished as described before [68], with minor modifications. The spinal cords of vehicle- and reserpine-treated mice were isolated on the fourth experimental day; the surrounding muscles, fat, spinal nerves and other soft tissues were removed. To avoid the damage of DRG, transverse cuts were made through the vertebrae between the discs. Each DRG was collected individually, by using a fine tip scissor. The spinal cord was maintained on ice thoroughly. Once dissected and cleaned, the DRGs of lumbar spinal cord were collected, and fixed in 10%-buffered formalin solution until the immunohistochemistry analysis.

Histological analysis of skeletal muscle

To assess the effects of UFP-101 on fibromyalgia-related skeletal muscle changes, the masseter, the gastrocnemius and the soleus were collected on the fourth experimental day. The samples were fixed in 10%-buffered formalin solution for 24 h, and embedded in paraffin after dehydration. The histological analysis was performed using haematoxylin-eosin (H&E) staining. The cross-sectional area (CSA) and the frequency (%) of fibres with different diameters were determined using the NIH Image J 1.36b Software. The slides were acquired with a Zeiss AxioImager M2 light microscope (Carl Zeiss, Gottingen, Germany). The images were captured at × 200 magnification.

Transmission electron microscopy (TEM) for mitochondria evaluation

Masseter muscles were collected from vehicle and reserpine treated-mice at fourth day, and a mitochondrial analysis was performed according to the method described previously [45]. The samples were cut into small pieces and fixed in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde, buffered with 0.1 M phosphate (pH 7.3), at room temperature. Then, the samples were post-fixed in osmium tetroxide in the same buffer for 45 min. The dehydration was performed in a graded acetone series (30–100%) and embedded in araldite (Durcupan^{TM} ACM, Fluka), for 72 h, at 60°C. Thin sections (70 nm) were stained with 2% uranyl acetate, followed by an immersion in lead citrate. Ultrastructural analysis was performed using the Tecnai G2 T20, FEI transmission electron microscopy and Image Pro Plus software (Image Pro Plus 6.1, Media Cybernetics, Silver Spring, USA). The mitochondrial density and area were analysed as described by [45], with minor modifications. Squares measuring 27.27 μm^2 were considered as the full microscopic image of each analysed muscle area (field), and mitochondria located inside each square or intersected by the upper and/or right edges of the squares were counted. Mitochondria intersected by the lower and/or left edges of the squares were not considered. Six to ten muscle regions (field) were analysed per

mouse, with an original magnification x 8,900. A grid mask (a grid of crosses with equidistant intervals) with an area/point value of 0.0311418 μm^2 was placed over the mitochondria images.

ppN/OFQ and NOPr RT-qPCR analysis

Thalamus/hypothalamus, prefrontal cortex, lumbar spinal cord and masseter muscle were isolated on days 1, 2, or 3 after reserpine induction. The structures were stored in 300 µl of TRIzol Reagent® (Sigma, St. Louis, MO, USA). The total RNA isolated was then quantified by spectrophotometry and the cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA), in accordance with manufacturer's instructions, after DNAse I treatment (DNase I Amplification Grade; Sigma-Aldrich, EUA). Quantitative PCR was performed using SYBR® Green I (Invitrogen, Carlsbad, CA, USA). Reactions were performed in a volume of 25 µl using 12.5 µl of diluted cDNA (1:50) and 200 nM of each reverse and forward primers (ppN/OFQ-F 5′-AGCACCTGAAGAGAATGCCG-3′; ppN/OFQ-R 5′-CATCTCGCACTTGCACCAAG-3′; NOPr-F 5′-ATGACTAGGCGTGGACCTGC-3′; NOPr-R 5′-GATGGGCTCTGTGGACTGACA-3′ [31]. PCR cycling conditions followed an initial 5 min at 95˚C polymerase activation step, plus 40 cycles of 15 s at 95˚C for denaturation, 35 s at 60˚C for annealing and 15 s at 72˚C for elongation. Finally, a melting curve analysis was included with fluorescence measures from 60 to 99˚C. The Cq values were obtained with 7500 Fast Real-Time PCR System v.2.0.6 (Applied Biosystems, Carlsbad, CA, USA) and relative expression levels were determined using *ppia* and *hprt* as reference genes [60] through $2^{-\Delta\Delta Cq}$ method. The efficiency per sample was calculated using LinRegPCR 11.0 software (http://LinRegPCR.nl).

Immunohistochemistry

The expression of NOPr was evaluated by immunohistochemistry, according to the method described previously [67]. The brain, the lumbar spinal cord (L1-L6), the masseter muscle and the DRGs were collected on the fourth day, from reserpine- or vehicle-treated mice, and fixed in 10%-buffered formalin solution, for 24 h. The immunopositivity to NOPr was assessed in paraffin-embedded tissue sections (4 µm), using a polyclonal rabbit anti-NOPr antibody (1:400; Alomone, Jerusalem, Israel, Catalog Number AOR-015). Images were examined with a Zeiss AxioImager M2 light microscope (Carl Zeiss, Gottingen, Germany). For analysis, the images were captured in × 100 (brain, spinal cord and masseter), in × 200 (DRG) or in × 400 (masseter) magnification. The schematic representations of brain and spinal cord were captured in × 8 and × 32 magnification, respectively (ZEISS Stemi DV4 Stereo Microscope). The number of NOPr positive cells was quantified in lumbar spinal cord (laminas I – VI in the dorsal horn) and in brain areas (thalamus and

agranular insular cortex). For each mouse, three images were taken. To determine the NOPr positive neurons in DRGs, digitized RGB (24-bit) images were analysed by using the NIH ImageJ 1.36b Software. A specific macro was created to quantify the positive areas in DRG and masseter based on pixel colour, according to a previous study [24]. For this purpose, an image from the vehicle-injected group, without reserpine induction was chosen and this macro was applied to all images from the experimental groups, as presented in the Supplementary Figure 2. To confirm the selectivity of the anti-NOPr antibody, we have used an internal antigen control (2.5 μg/ml; nociceptin receptor 337-352 peptide; Alomone, Jerusalem, Israel, Catalog Number AOR015AG0140), by using brain, spinal cord and DRG slides, according to the manufacturer`s instructions. The co-incubation of the internal antigen with the primary antibody blocked the immunolabelling in all the evaluated anatomical structures, indicating the specificity of the tested antibody (Supplementary Figure 3). Furthermore, no immunolabelling was observed when the primary antibody was omitted (results not shown).

Biochemical parameters

The biomarkers described in the next sections (cytokine and substance P levels, glutathione (GSH) and lactate dehydrogenase (LDH) contents) were selected based on the pathophysiology of fibromyalgia [10,23,57,64,76]. These experiments were performed to gain further insights into the beneficial effects of UFP-101 in painful- and fatigue-like symptoms in the reserpine mouse model of fibromyalgia.

Cytokine levels

Brain, spinal cord, masseter muscle and serum were collected from animals that received a repeated treatment with UFP-101 (1 nmol/kg) or pregabalin (188 μmol/kg), given i.p., and the respective controls. The samples were stored in an ultra-freezer for analysis of the levels of TNF, IL-1β and IL-10. The tissues were homogenised according to the methodology described previously [67]. The blood was collected from the abdominal aorta, centrifuged, and the serum was frozen. Cytokine levels were measured by ELISA (sandwich enzyme-linked immunosorbent assays) kits according to the manufacturer's recommendations (R&D Systems; Minneapolis, USA) and expressed in pg/100 mg tissue (brain, spinal cord and masseter muscle) or in pg/ml (serum).

Substance P (SP) levels

An ELISA assay for SP detection was performed using a commercially available kit, according to the manufacturer's recommendations (Cayman Chemical, Michigan, USA). Samples of brain, spinal cord and masseter muscle were homogenised, and further processed by using a SPE

(C-18) purification protocol prior to the ELISA assay, as described in the instructions accompanying the kit. For this experimental set, the samples were obtained from the experimental groups submitted to the chronic treatment with UFP-101 or pregabalin, and the respective control groups on the fourth day.

Fibromyalgia induction and oxidative stress

GSH contents in brain, spinal cord and muscles were determined as described before [66], as a measure of oxidative stress. The samples were collected from mice chronically treated with saline or UFP-101 (1 nmol/kg, i.p.), at the fourth day. The tissues were homogenised in saline (1:10 w/v) on ice with an Ultra Turrax (brain and spinal cord) or with a glass-Teflon homogeniser (masseter muscle). Homogenates were centrifuged at 3000 rpm for 10 min. Next, 250 µl of 4% sulfosalicylic acid were added to 250 µl of the supernatant, and centrifuged at 3000 rpm for 10 min. The supernatant (250 µl) was mixed with one ml of 0.1 M phosphate buffer (pH 8) and 5 µl of 0.01 M 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma-Aldrich, USA). The absorbance was read at 412 nm in a spectrophotometer (GENESYS™ 10S UV-Vis, Thermo Fisher Scientific, USA). The protein content was determined by the biuret method (total protein monoreagent kit, Bioclin, Brazil) and the values of GSH contents were normalised by the protein content of the same sample.

Fibromyalgia induction and lactate dehydrogenase activity in serum and mitochondrial extracts of muscle

LDH contents in serum and mitochondrial extracts of muscles were determined as described before [26], as a biochemical marker for fatigue. The samples were collected from mice chronically treated with saline or UFP-101 (1 nmol/kg, i.p.), at the fourth day. Then, the serum was separated from the blood sample by centrifugation at 3000 rpm at 4°C for 15 min. The masseter muscle was homogenised in Isolation Buffer 1 on ice with a glass-Teflon homogeniser. Homogenates were centrifuged at 700×g for 10 min at 4°C. Next, the supernatant was centrifuged at 10,500 ×g for 10 min at 4°C. The pellet was re-suspended in 500 µl of Isolation Buffer 2 and was again centrifuged at 10,500×g for 10 min at 4°C. The final mitochondrial pellet was re-suspended in 100 µl of Isolation Buffer 2. The LDH activity in the serum and muscle were measured using a commercially available kit, according to the manufacturer's recommendations (Labtest, Minas Gerais, Brazil). The absorbance was read at 340 nm in a spectrophotometer (SpectraMax M2/M2e Microplate Readers, Molecular Devices, USA). The protein content was determined by the biuret method (total protein monoreagent kit, Bioclin, Brazil) and the values of LDH were normalised by the protein content of the same sample.

N/OFQ levels

The N/OFQ levels were analysed in serum and brain of vehicle and reserpine-treated mice (collected on the fourth day), or in the saliva of fibromyalgia patients included in a major study designed to investigate the salivary levels of inflammatory mediators in chronic pain states, such as fibromyalgia and temporomandibular disorder (Human Research Ethical Committee: 844208) by ELISA assay. The *n* of control and fibromyalgia patients was 10 per group. The EIA kit [reference number EK-021-55; Nociceptin/Orphanin FQ (Human, Rat, Mouse, OX); Phoenix Pharmaceuticals, INC, USA] presents a sensitivity of 0.18 ng/ml. Brain samples were homogenised, and an ELISA assay was performed using a commercially available kit, according to the manufacturer's recommendations. The absorbance was read at 450 nm and the results were calculated from a standard curve ranging from 0.01 to 100 ng/ml.

Data and statistical analysis

The results were expressed as the mean ± standard error of the mean (SEM). Brown-Forsythe and Bartlett tests were used for checking normality of data. Statistical analysis was performed by Student *t* test (comparison between the vehicle group *vs.* the reserpine treated-group). Kruskal Wallis (non-parametric data) and one-way ANOVA (parametric data) were used for comparison of the vehicle group *vs.* reserpine treated-group *vs.* NOPr ligand treated-group. Two-way ANOVA was used for comparison of the vehicle group *vs.* reserpine treated-group *vs.* NOPr ligand treated-group, by analysing data at the baseline and on the fourth day. When the interaction of factors was statistically significant (*P* values less than 0.05), pairwise comparisons were conducted by using Dunn’s or Bonferroni’s *post-hoc* tests, after Kruskal Wallis and ANOVA, respectively. ELISA assays (N/OFQ, substance P and cytokine levels) were run in duplicate (for brain samples) or in a single reaction (for serum, spinal cord and masseter muscle), with an experimental *n* = 5-6 per group. All tests were performed using GraphPad Software® version 6.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Dose-related acute effects of N/OFQ and UFP-101 administered by different routes

Previous studies to validate the reserpine-induced fibromyalgia model revealed the development of mechanical and thermal hypersensitivity, associated with depression-like behaviour in mice [43]. The first part of the study was designed to evaluate the dose-response effects of N/OFQ and UFP-101 on these reserpine-elicited behavioural changes. The possible sites of action of NOPr ligands were investigated by dosing the agonist or the antagonist by i.c.v., i.t. or i.p. routes of administration. As expected, the fibromyalgia induction by the repeated administration of reserpine evoked a significant increase of the frequency withdrawal after the stimulation with the 0.4-g von Frey hair (Figure 1). The i.c.v. administration of N/OFQ (1 nmol/site) produced a slight, but not significant reduction of this response (Figure 1A). However, the i.t. (Figure 1B) or i.p. (Figure 1C) administration of N/OFQ (1 nmol/site or 1 nmol/kg) significantly reduced the fibromyalgia-related mechanical hypersensitivity (by 62 ± 10% and 36 ± 11%, respectively). Instead, reserpine-induced mechanical allodynia was significantly enhanced by the i.p. treatment with N/OFQ at the dose of 5 nmol/kg (Figure 1C; by 27 ± 6%). Concerning the UFP-101 effects, this peptide antagonist given i.c.v. (1 nmol/site; Figure 1D) or i.t. (3 and 5 nmol/site; Figure 1E) significantly reduced the mechanical hypersensitivity in mice treated with reserpine (by 46 ± 14%, 53 ± 14% and 51 ± 13%, respectively). All the tested i.p. doses of UFP-101 (1, 3 and 5 nmol/kg), apart from the 0.3 nmol/kg dose, significantly prevented the mechanical hypernociception in mice submitted to the fibromyalgia model (Figure 1F). In this case, the effects of UFP-101 lacked a typical dose-related profile (inhibition percentages of 43 ± 10%, 33 ± 5% and 28 ± 10%, respectively).

The induction of fibromyalgia by the acute administration of reserpine significantly reduced the latency time to reaction after heat stimulation, in the hot-plate apparatus (Figure 2). The acute treatment with N/OFQ or UFP-101 did not significantly alter the thermal hypersensivity, when given by i.c.v. (Figure 2A, D) or i.p. (Figure 2C, F) routes. Noteworthy, the spinal administration of N/OFQ (3 nmol/site; i.t.) had a significant inhibitory effect on the thermal nociception (by 49 ± 13%; Figure 2B). Additionally, the higher tested dose of UFP-101 given i.t. (5 nmol/site) significantly prevented the thermal hypernociception in reserpine-treated mice (by 69 ± 13%; Figure 2E).

Reserpine-elicited fibromyalgia led to a slight increase of immobility time in the forced-swimming test, as an indicative of depressive-like behaviour (Figure 3). This parameter was significantly inhibited by N/OFQ, given by i.c.v (1 nmol/site; Figure 3A) or i.t. (3 nmol/site; Figure 3B) routes, with inhibition percentages of 27 ± 7% and 31 ± 5%, respectively. The systemic

administration of N/OFQ (Figure 3C) or all the routes of administration and doses tested for UFP-101 failed to significantly affect the immobility time of animals submitted to the reserpine fibromyalgia model (Figure 3D, E, F).

Considering the relevance of NOPr in anxiety, we decided to assess the effects of NOP ligands in the plus maze paradigm in the mouse model of fibromyalgia induced by reserpine. Overall, the induction of fibromyalgia led to a diminished locomotor activity, as indicated by a significant reduction in the total number of entries in open and close arms. However, the NOPr ligands, given by i.c.v., i.t. or i.p. routes, at different doses, did not elicit any significant alteration of the locomotor index. The anxiety-related parameters, namely the entries and the time spent in open arms, were also unaffected (Supplementary Table 1).

To assess whether the effects of N/OFQ might involve the activation of opioid receptors under fibromyalgia induction by reserpine, we performed a separate series of experiments in which the animals received naloxone in combination with N/OFQ. The mice were evaluated in the same behavioural tasks as described above. The treatment with naloxone (5 μmol/kg; i.p.) did not significantly alter the effects of N/OFQ (1 nmol/kg; i.p.) in any of the evaluated parameters (Supplementary Figure 4).

The co-treatment with UFP-101 (1 nmol/kg; i.p.) significantly prevented the pro-nociceptive effect of N/OFQ (5 nmol/kg; i.p.), according to the assessment of mechanical hypersensitivity in animals subjected to the fibromyalgia model induced by reserpine (Supplementary Figure 5). However, the pre-treatment with UFP-101 (1 nmol/kg, i.p.), dosed 15 min before, failed to significantly alter the effects of N/OFQ given by i.c.v. or i.t. routes, as evaluated by von Frey and hot plate tests (Supplementary Figure 6).

The chronic administration of UFP-101 improves fibromyalgia-related pain and fatigue

Based on the dose-response experiments, UFP-101 (1 nmol/kg), dosed by i.p. route, was tested in a repeated treatment scheme, in which the peptide NOPr antagonist was administered daily during the fibromyalgia induction protocol. The effects of UFP-101 were compared to those displayed by the positive control drug pregabalin (188 μmol/kg, i.p.). The administration of reserpine led to mechanical and thermal hypersensitivity in saline-treated control mice (Figure 4A, B). The repeated treatment with UFP-101 significantly reduced the mechanical allodynia (37 ± 8%, Figure 4A), and increased the latency time in the hot-plate test (32.2 ± 5%, Figure 4B). UFP-101 failed to alter the depression-like behaviour in the forced swimming test (Figure 4C). The administration of pregabalin resulted in a similar inhibition of mechanical and thermal

hypernociception secondary to fibromyalgia induction (45.5 ± 10% and 32 ± 7%, respectively), without affecting the immobility time (Figure 4A, B, C).

Fatigue is a frequent complaint of patients with fibromyalgia diagnosis [77]. Thus, we tested the effects of repeated administration of the NOPr antagonist UFP-101 (1 nmol/kg; i.p.) on fatigue-related symptoms in reserpine-treated mice. The induction of fibromyalgia by reserpine led to a reduction in the time that animals remained in the rotarod apparatus adjusted for provoking a fatigue status. Noteworthy, the treatment with UFP-101 significantly improved the permanence time in the apparatus (with a 7-fold increase). However, the repeated scheme of treatment with the clinically used drug pregabalin (188 µmol/kg; i.p.) lacked any significant effect in this experimental paradigm (Figure 4D). The chronic treatment with UFP-101, but not pregabalin, partially improved the grasping strength of mice subjected to fibromyalgia induction by reserpine (15 ± 16%; Figure 4E). There was no difference among the experimental groups regarding the latency to fall in the Kondziela's inverted screen test (Figure 4F). In relation to the anxiety parameters, the reserpine administration decreased the total number of entries when compared to the vehicle control group, without differences for the UFP-101 and pregabalin treatments (Figure 4G, H, I).

Skeletal muscle changes related to fibromyalgia are restored by NOPr inhibition

Considering the favourable effects of NOPr antagonism on fatigue and reduced grip strength in the reserpine fibromyalgia model, we carried out a histological analysis of masseter, gastrocnemius, and soleus sections. The induction of fibromyalgia by reserpine led to a decrease in the frequency of fibres with 301-400 μm^2 and 401-500 μm^2, associated with an elevation in the frequency of fibres with 601-700 μm^2 in the masseter muscle (Figure 5A). In the gastrocnemius muscle, we observed an increase in the frequency of fibres with 401-500 μm^2 and 501-600 μm^2, and a reduction of fibres with 901-1000 μm^2 and 1001-1100 μm^2 (Figure 5C). Notably, the repeated treatment with UFP-101 (1 nmol/kg; i.p.) significantly rescued the changes elicited by reserpine in the frequency of fibres with 301-400 μm^2 (masseter) and 401-500 μm^2 (gastrocnemius) (Figure 5A, C). No change was found in the cross-section area (Figure 5B, D) or weight (data not shown) of masseter and gastrocnemius muscles. The soleus muscle was also analysed for these parameters and there was no difference among the experimental groups (data not shown).

Mitochondrial analysis in reserpine-elicited fibromyalgia

To evaluate the effects of the repeated treatment with UFP-101 (1 nmol/kg; i.p.) on the area and density of mitochondria in the skeletal muscle, we performed an ultrastructural analysis of the masseter muscle (Supplementary Figure 7). The examination of transmission electron microscopy

images revealed that either the induction of fibromyalgia by reserpine, or the chronic treatment with UFP-101, did not alter the mitochondrial area (Supplementary Figure 7D) and density (Supplementary Figure 7E).

5-HT depletion and glutamate levels are not altered by UFP-101

As demonstrated before [43], the induction of fibromyalgia by reserpine elicits a marked reduction of 5-HT levels in brain and spinal cord. Accordingly, reserpine-evoked fibromyalgia was associated with a diminishment of 5-HT levels in the pre-frontal cortex, thalamus/hypothalamus, and lumbar spinal cord, when compared with vehicle control mice. The repeated i.p. treatment with UFP-101 (1 nmol/kg) or pregabalin (188 µmol/kg) did not prevent the reduction of 5-TH in reserpine-injected mice (Figure 6A, B, C). There was no significant alteration of glutamate levels in the same anatomical structures (Figure 6D, E, F).

Assessment of brain activity by microPET imaging

Considering the marked behavioural changes associated with the fibromyalgia model induced by reserpine, we performed a microPET scan to evaluate the brain metabolism. An analysis of the whole brain did not show any significant difference in the glucose metabolism among the experimental groups (Figure 7A). However, the induction of fibromyalgia by reserpine caused a significant increment of glucose metabolic rates, according to the assessment of specific cerebral areas, such as cingulate gyrus (CG), superior colliculus (SC), and left or right inferior colliculus (LIC and RIC). The repeated i.p. administration of UFP-101 (1 nmol/kg), but not pregabalin (188 µmol/kg), reduced the glucose metabolism toward the values observed in the vehicle-treated control group (Figure 7B, C). There was no difference of brain activity among the experimental groups, in the following brain structures: striatum (RSTR and LSTR), cortex (CTX), hippocampus (RHIP and LHIP), thalamus (THA), basal forebrain/septum (BFS), hypothalamus (HYP), amygdala (RAMY and LAMY), brainstem (BS), olfactory areas (OLF) and right midbrain (RMID) (Figure 7B).

ppN/OFQ and NOPr expression

To further examining the relevance of the NOP system in fibromyalgia, we evaluated the time-related central and peripheral expression of ppN/OFQ and NOPr mRNA in mice submitted to the reserpine model. Reserpine-induced fibromyalgia was associated with an increase in ppN/OFQ mRNA expression in the lumbar spinal cord on day three (Figure 8C) and in the masseter on days one and two (Figure 8D), whereas NOPr mRNA expression was increased in the masseter muscle on day one (Figure 8H). Alternatively, the NOPr mRNA expression was reduced in the

thalamus/hypothalamus on day three (Figure 8F). The ppN/OFQ or NOPr mRNA expression in the pre-frontal cortex, the ppN/OFQ mRNA expression in the thalamus/hypothalamus, or the NOPr mRNA expression in the lumbar spinal cord were not significantly altered by the protocol of fibromyalgia induction used in this study (Figure 8A, B, E).

To extend the PCR data, the immunopositivity for NOPr was assessed in the agranular insular cortex, thalamus, lumbar spinal cord, lumbar DRGs and masseter muscle, according to the evaluation at the fourth day after initiating the protocol of fibromyalgia induction. There was a significant decrease in the immunolabelling for NOPr in the agranular insular cortex of reserpine-treated mice when compared to the vehicle control group (Figure 9A, B, C). Otherwise, the immunohistochemistry analysis revealed an increased expression of NOPr in the lumbar DRGs of reserpine-treated mice (Figure 9G, H, I). Additionally, reserpine slightly reduced the immunopositivity for NOPr in thalamus (Supplementary Figure 8A, B, C), while it failed to significantly affect NOPr distribution in the lumbar spinal cord (Figure 9D, E, F) or masseter (Supplementary Figure 8D, E, F).

Analysis of inflammatory changes

An inflammatory status has been correlated with the pathophysiology of fibromyalgia [46]. Thus, the variations of some inflammatory mediators were examined in the different experimental groups. No significant differences in SP levels were observed between reserpine- and vehicle-treated mice, in brain, spinal cord, and masseter, on the fourth day (Supplementary Figure 9A, B, C). Concerning the UFP-101 effects (1 nmol/kg; i.p.; four days), this NOPr antagonist decreased the SP levels in the masseter, when compared to the vehicle control group or to the reserpine-treated mice (by 42 ± 1.5% and 45 ± 1.5%, respectively) (Supplementary Figure 9C).

In a separate experimental set, the levels of TNF, IL-1β, and IL-10 were evaluated in brain, spinal cord, masseter and serum, at the fourth experimental day. The induction of fibromyalgia by reserpine did not cause any evident change of cytokine levels, in all the analysed samples. The repeated scheme of treatment with UFP-101 failed to significantly altering the production of cytokines, except by a reduction of the IL-1β levels in brain when compared to the pregabalin treatment. Unexpectedly, pregabalin (188 μmol/kg; i.p.) caused an elevation in the levels of TNF and IL-10 in brain homogenates (Supplementary Table 2).

Effects of treatment with UFP-101 on glutathione levels

The glutathione levels were measured in brain (Supplementary Figure 10A), spinal cord (Supplementary Figure 10B), and masseter muscle (Supplementary Figure 10C) of mice, as an

indicative of oxidative stress. There were no significant differences in the levels of glutathione when comparing reserpine- and vehicle-treated mice, in any of the tested samples. In addition, the repeated treatment with UFP-101 (1 nmol/kg; i.p.) failed to affect the glutathione levels of mice submitted to the reserpine fibromyalgia model.

Effects of treatment with UFP-101 on lactate dehydrogenase activity

LDH activity was quantified in serum (Supplementary Figure 11A) and mitochondrial extracts from muscle tissue (Supplementary Figure 11B). There were no significant differences in the LDH activity when comparing reserpine- and vehicle-treated mice, in any of the tested samples. Its activity was increased in mitochondrial extracts of UFP-101-treated mice, when compared with reserpine and vehicle groups (Supplementary Figure 11B).

N/OFQ concentrations in fibromyalgia mice and patients

To obtain additional evidence on the relevance of the N/OFQ-NOP system in fibromyalgia, we assessed the N/OFQ levels in brain or serum obtained from mice subjected to the fibromyalgia model induced by reserpine, as well as in the saliva of a small sample of patients with fibromyalgia diagnosis. There was a slight but not statistically significant decrease of the N/OFQ levels in the serum of reserpine-treated mice in comparison to the vehicle control animals, whereas this peptide was undetectable in brain samples (Supplementary Figure 12A). N/OFQ was detected in the saliva of control or fibromyalgia patients, without any evident difference between the groups (Supplementary Figure 12B).

Effects of the non-peptide NOPr antagonist SB-612111 on fibromyalgia signs

We performed a separate set of experiments to test the effects of SB-612111, a selective non-peptide NOPr antagonist in the reserpine-elicited fibromyalgia model. As described above, the reserpine administration led to an increase of the response frequency to mechanical stimulation, associated with a decrease in the latency to respond to heat stimulus, compared to the vehicle control groups (Figure 10A, B). The repeated treatment with SB-612111 (6.6 µmol/kg; i.p.) reduced both the mechanical allodynia (43 ± 15.2%, Figure 10A) and the thermal hypernociception (45 ± 17.5%, Figure 10B) induced by reserpine. The induction of fibromyalgia by reserpine evoked depression-like behaviour, an effect that was not altered by SB-612111, at 6.6 µmol/kg, given i.p. (Figure 10C). The repeated treatment with SB-612111 (2.2 µmol/kg; i.p.) recovered the fatigue (with a 2-fold increase) and the loss of grip strength (9 ± 5.5%) caused by reserpine, according to the evaluation in the rotarod (Figure 10D) and the grasping tests (Figure 10E). The upper doses of

SB-611211 (6.6 and 22 µmol/kg; i.p.) failed to significantly alter both fatigue-related symptoms (Figure 10D, E). There was no difference among the experimental groups in the latency to fall in the Kondziela's inverted screen test (Figure 10F). Regarding the anxiety parameters, the reserpine administration diminished the total number of entries when compared to the vehicle control group, without any significant effect for the SB-612111 treatment (Figure 10G, H, I).

Discussion

Herein, we evaluated the dose-related effects of the natural agonist N/OFQ, administered acutely by different routes (i.c.v., i.t and i.p), after completing the protocol of fibromyalgia induction by reserpine. This experimental set showed that i.c.v. administration of N/OFQ (1 nmol/site) did not markedly affect the mechanical allodynia or the thermal hypersensitivity secondary to fibromyalgia induction. When dosed spinally (0.3 to 3 nmol/site), N/OFQ exhibited a U-shaped profile regarding its inhibitory effects on mechanical hypersensitivity, whereas only the higher dose (3 nmol/site) prevented the thermal nociception. Spinal analgesic effects for N/OFQ have been described in rodents elsewhere [56,75]. Finally, the systemic administration of N/OFQ (i.p.; 0.3 to 5 nmol/kg) failed to alter the thermal hypersensitivity, although it potentiated the mechanical allodynia at 9 $\mu g.site^{-1}$. These results suggest that N/OFQ exhibits dual effects in the nociplastic fibromyalgia-like pain caused by reserpine in mice, extending and confirming the previous notion that N/OFQ might produce pro- or antinociceptive effects, depending on the dose and the route of treatment [1,14,39,52,63,65].

Next, we tested the dose-related effects of the peptide antagonist UFP-101, given acutely by different routes of administration. The supraspinal treatment with UFP-101 (1 nmol/site) prevented the mechanical allodynia, without any change of the thermal nociception. A previous study demonstrated analgesic effects for UFP-101, dosed i.c.v., in the thermal tail-flick test - in this case, a higher dose was used (10 nmol/site) [15]. Another study showed that UFP-101, given i.c.v., inhibited the second phase of formalin-induced nociception, at 10 nmol, but not at 1 nmol/site, supporting our results [63]. The same publication demonstrated pronociceptive effects for UFP-101 (10 nmol/site), administered spinally, in the second phase of the formalin model [63]. Herein, when given i.t., either dose of UFP-101 (3 or 5 nmol/site) reduced the mechanical allodynia, whereas only the higher dose (5 nmol/site) inhibited the thermal hypersensitivity. The acute i.p. treatment with UFP-101 (1 to 5 nmol/kg) markedly prevented the tactile allodynia, without altering the thermal hypernociception, regardless of the tested dose.

To assess the possible site (s) of action of UFP-101 when given systemically, the i.p. effects of this antagonist were tested against N/OFQ, dosed by different routes. UFP-101 (1 nmol/kg; i.p.) significantly prevented the pro-nociceptive systemic effects of N/OFQ (5 nmol/kg), whereas it failed to modify the analgesic effects of N/OFQ given i.t. or i.c.v. (1 nmol/site). Thus, UFP-101, given systemically, probably acts peripherally to induce analgesia in the reserpine-induced fibromyalgia model. In our study, naloxone failed to interfere with N/OFQ peripheral analgesic actions, discarding an interference with opioid receptors.

UFP-101 (i.p.; 1 nmol/kg) was also tested in a schedule of repeated administration, throughout the four-day protocol of fibromyalgia induction. In this protocol, UFP-101 reduced both the mechanical and the thermal hypernociception, at a similar inhibition grade as observed for the reference drug pregabalin. The different effects observed for UFP-101 in thermal nociception, when given in acute or repeated schemes, might be explained by a time-related sensitisation of the N/OFQ-NOPr system in fibromyalgia [72].

The pharmacological or genic inhibition of NOPr induced antidepressant-like effects in control rodents, whereas N/OFQ lacked any effect *per se* [34,50,62]. Herein, the administration of N/OFQ, at 1 nmol/site (i.c.v.) or 3 nmol/site (i.t.), recovered the depressive-like behaviour induced by reserpine, whereas UFP-101 failed to alter the immobility time in any of the tested schemes. Literature data showed antidepressant effects for UFP-101 administered i.c.v., in doses ranging from 1 to 10 nmol/site in naïve animals, with consistent effects only for the higher tested dose [27,28,29,50]. This might partly explain the absence of antidepressant effects for UFP-101 in our experimental model. This discrepancy might also be explained by the modulation of the N/OFQ-NOPr system after fibromyalgia induction.

The N/OFQ levels were decreased in the plasma of patients with fibromyalgia [3]. We found a slight decrement of N/OFQ levels in the serum of reserpine-treated mice, whereas the N/OFQ levels were undetectable in brain samples from vehicle- or reserpine-treated mice. The analysis of N/OFQ levels by radioimmunoassay (RIA) also detected very low levels of the peptide in brain (in fmol) [22,32,79], partly supporting our data, considering the sensitivity of the ELISA kit used by us (0.18 ng/ml). Strikingly, N/OFQ was detected in the saliva of control or fibromyalgia patients, without any evident difference between the groups. Previous studies on chronic pain demonstrated a good correlation between plasma and salivary levels of calcitonin gene related peptide (CGRP) and SP, with higher levels of both peptides in saliva [36].

Fibromyalgia encompasses musculoskeletal pain and fatigue, which is correlated with an impaired sensory-motor function [35,69]. The NOPr inhibition improved the motor disorders in the Parkinsonism model induced by high doses of reserpine [4]. Reserpine-induced fibromyalgia was associated with a marked reduction of the permanence time in the rotarod apparatus, adjusted for inducing fatigue, an effect that was prevented by the repeated treatment with UFP-101 (1 nmol/kg; i.p.). The daily UFP-101 administration also improved the grip strength, supporting its anti-fatigue effects. The non-peptide NOPr antagonist SB-612111 paralleled the favourable effects of UFP-101 in fibromyalgia-related pain and physical distress. Conversely, the reference drug pregabalin failed to rescue the fatigue or the grip strength.

The animals submitted to the reserpine model presented a mild reduction of the total number of entries in the plus-maze, an effect that was unaltered by the acute treatment with UFP-101 (0.3 to 5 nmol/kg), irrespective of the administration route. The repeated systemic treatment with UFP-101 (1 nmol/kg) or pregabalin (188 µmol/kg) also failed to modify the same parameter. Additionally, no experimental group displayed changes in the Kondziela's inverted screen test. It seems that UFP-101 is able to alleviate pain and fatigue alterations featuring the fibromyalgia symptomatology, not simply by altering the locomotor activity of mice.

Srikuea et al. (2013) [69] described an altered distribution of skeletal muscle fibre sizes in fibromyalgia women. Fibromyalgia induction by reserpine led to changes in the fibre size distribution, and the daily treatment with UFP-101 (1 nmol/kg; i.p.) partially prevented these muscle morphological changes. We also performed an ultrastructural analysis of the muscle mitochondrial areas and densities, but there was no difference of these parameters between control and fibromyalgic mice, irrespective of UFP-101 treatment. The tested experimental groups did not exhibit any difference in glutathione levels, which is a measure of oxidative stress. Nonetheless, the repeated administration of UFP-101 led to an increase in muscle LDH activity, what might partly support the preventive effects of NOPr blockade in fibromyalgia-related muscle dysfunction. The induction of fibromyalgia by reserpine did not evoke changes of SP levels in the brain, spinal cord or masseter, but the daily treatment with UFP-101 led to a reduction of SP production in the masseter. This might be an additional mechanism to explain the UFP-101 effects on fibromyalgia-related fatigue. Interestingly, a randomized clinical trial correlated the benefits of acupuncture in alleviating the fibromyalgia symptoms with a reduction of SP serum levels [38]. Central or peripheral changes in cytokine production have been described in fibromyalgia patients [73]. Furthermore, the N/OFQ-NOPr system has been suggested to influence the balance between pro- and anti-inflammatory cytokines [9]. Herein, we did not detect any significant alteration of TNF, IL-1β or IL-10 in the brain, spinal cord or muscle samples of reserpine-treated mice, regardless of the administration of UFP-101.

The animals submitted to the reserpine model presented a reduction of the brain and spinal 5-HT levels, an event that was not modified by UFP-101. The same brain and spinal cord samples were used for analysis of glutamate levels, lacking any significant difference. A recent study [41] showed reduced levels of 5-HT, noradrenaline, and dopamine, without any alteration of glutamate and GABA, in the spinal cord reserpine-treated rats. The authors showed a recovery of monoamine levels by duloxetine, whereas pregabalin failed to modify these changes. Apparently, UFP-101, as pregabalin, displays beneficial effects on fibromyalgia symptoms, independent on the ability to restore the monoamine levels.

A functional MRI study demonstrated different patterns of brain activation following innocuous or noxious stimulation in rats submitted to the reserpine model of fibromyalgia [81]. Herein, a micro-PET scanning analysis demonstrated an increased brain activity in the mouse CG, SC, LIC and RIC, secondary to the induction of fibromyalgia, an effect that was restored by UFP-101 treatment. Neuroimaging studies revealed a reduction of fibromyalgia-related brain hyperactivity in patients submitted to pharmacological and non-pharmacological treatment protocols [37,53]. Thus, the inhibition of brain hyperactivity by UFP-101 might underlie its beneficial effects in fibromyalgia-related painful and fatigue symptoms.

The analgesic effects observed for daily UFP-101 or SB-612111 might be explained by the increased NOPr immunoreactivity in the DRG of reserpine-treated mice. The small unmyelinated DRG neurons are peptidergic and non-peptidergic C nociceptors, primordial to heat and mechanical pain, respectively [72]. An upregulation of NOPr in DRG has also been demonstrated in other chronic pain models [12,16]. The induction of fibromyalgia by reserpine led to an increase of ppN/OFQ mRNA in the spinal cord, further supporting that changes in the N/OFQ-NOPr tonus underlie dysfunctional pain. Alternatively, reserpine-treated mice showed a reduction of immunolabelling for NOPr in the agranular insular cortex, which is a pivotal region for pain control [30]. A decrease of NOPr expression was previously demonstrated in thalamus and hippocampus in a mouse model of neuropathic pain caused by sciatic nerve lesion [59]. Curiously, the levels of ppN/OFQ mRNA were significantly increased in masseter, from the first to the third day after reserpine injection, what might further support the anti-fatigue effects of the daily treatment with NOPr antagonists.

Collectively, these results help to pave the way on the pathophysiology of fibromyalgia, indicating that NOPr antagonism might be an alternative for managing of fibromyalgia-related pain and tenderness.

Acknowledgements

We would like to thank Mrs. Janaína Pasetti Nunes for her valuable technical assistance in histological processing (Laboratory of Oral Pathology/PUCRS). The authors are grateful to Mrs. Moema Queiroz Vieira (Central Laboratory of Microscopy and Microanalysis; LabCEMM/PUCRS) for the technical assistance in transmission electron microscopy experiments. We also thank the staff of the Centre of Experimental Biological Models (CeMBE/PUCRS) for animal care. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior – Brasil (CAPES) Finance Code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), in addition to a Financiadora de Estudos e Projetos (FINEP) research grant "Expansão da Infraestrutura Multiusuária de Pesquisa na PUCRS (MULTIPUCRS)" # 01.13.0292-00 (Brazil). A.P.A.D. is a PhD student receiving grants from CAPES and T.C.B.P is recipient of PNPD/CAPES fellowship. M.M.C. (CNPq, 303842-2014-8) and M.R.B. (CNPq, 303776/2013-7) are Research Career Awardees of the National Research Council of Brazil (CNPq).

Conflict of interest statement

The authors declare no conflict of interest.

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Figure Legends

Figure 1: Dose-related effects of N/OFQ or UFP-101 on the mechanical hypersensitivity in the mouse model of fibromyalgia induced by reserpine. Effects of acute treatment with N/OFQ (A-C) or UFP-101 (D-F), dosed by intracerebroventricular (i.c.v.; A and D), intrathecal (i.t.; B and E) or intraperitoneal (i.p.; C and F) routes, on hindpaw withdrawal threshold (response frequency in percentage) to tactile stimulation. The mechanical hypersensitivity was assessed by using the Von Frey filaments before (baseline), and at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). The NOPr ligands were administered in a single dose, 15 min (i.c.v. and i.t.) or 30 min (i.p.) before the behavioural evaluations. Each column represents the mean ± SEM. $^{*}p < 0.05$ when compared to the control vehicle/saline group, indicating the development of mechanical allodynia; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni's *post hoc* test (A-F). (A) n = 6-8 mice/group; (B) n = 8-12 mice/group; (C) n = 8-25 mice/group; (D) n = 8-11 mice/group; (E) n = 8-12 mice/group; (F) n = 8-26 mice/group.

Figure 2: Dose-related effects of N/OFQ or UFP-101 on the thermal hypersensitivity in reserpine-treated mice. Effects of acute treatment with N/OFQ (A-C) or UFP-101 (D-F), dosed by intracerebroventricular (i.c.v.; A and D), intrathecal (i.t.; B and E) or intraperitoneal (i.p.; C and F) routes, on the latency time (s) in response to hot thermal stimulation. Thermal hypersensitivity was assessed in the hot-plate test, at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). The NOPr ligands were administered in a single dose, 15 min (i.c.v. and i.t.) or 30 min (i.p.) before the behavioural evaluations. Each column represents the mean ± SEM. $^{*}p < 0.05$ when compared to the control vehicle/saline group, indicating the development of thermal sensitivity; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's *post hoc* test (A-F). (A) n = 6-8 mice/group; (B) n = 8-12 mice/group; (C) n = 8-25 mice/group; (D) n = 8-11 mice/group; (E) n = 8-12 mice/group; (F) n = 8-26 mice/group.

Figure 3: Dose-related effects of the acute treatment with N/OFQ (A-C) or UFP-101 (D-F), dosed by intracerebroventricular (i.c.v; A and D), intrathecal (i.t.; B and E) or intraperitoneal (i.p.; C and F) routes, on depression-like behaviour in reserpine-treated mice submitted to the forced swimming test. The immobility time (s) was assessed at fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). The NOPr ligands were administered in a single dose, 30 min before the behavioural evaluations. Each column represents the mean ± SEM.

*p < 0.05 when compared to the control vehicle/saline group, indicating the development of depressive behaviour; $^{\#}p$ < 0.05 when compared to the reserpine/saline group. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's *post hoc* test (B, C, E and F) or one-way ANOVA followed by Bonferroni's *post hoc* test (A and D). (A) n = 6-8 mice/group; (B) n = 8-12 mice/group; (C) n = 8-25 mice/group; (D) n = 8-11 mice/group; (E) n = 8-12 mice/group; (F) n = 8-26 mice/group.

Figure 4: Effects of the repeated treatment with UFP-101 or pregabalin on painful-, fatigue-, depressive- and anxiety-like behaviours in reserpine-treated mice. The effects of both drugs were also assessed on the fatigue and grip strength in the fibromyalgia model elicited by reserpine. Effects of the repeated administration of UFP-101 (1 nmol/kg, intraperitoneal) or pregabalin (188 µmol/kg, intraperitoneal), on hind paw mechanical allodynia (A), latency time (s) in response to hot thermal stimulation (B), immobility time in the forced swimming test (C), rotating time (D), grasping strength (E), latency to fall (F), and on plus maze parameters (G-I). All the tests were performed at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). UFP-101 or pregabalin were dosed for three consecutive days, 30 min after reserpine injection. At the fourth day, mice also received UFP-101 or pregabalin, 30 min before evaluations. Each column represents the mean ± SEM. *p < 0.05 when compared to the control vehicle/saline group; $^{\#}p$ < 0.05 when compared to the reserpine/saline group. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's *post hoc* test (D, F, H and I), one-way (C and G) or two-way (A, B and E) ANOVA followed by Bonferroni's *post hoc* test. (A-C) n = 8-11 mice/group; (D-E) n = 8-10 mice/group; (F-I) n = 8 mice/group.

Figure 5: Histological analysis of masseter (A and B) and gastrocnemius (C and D) muscles of reserpine-treated mice. Effects of intraperitoneal (i.p.) repeated treatment with UFP-101 (1 nmol/kg) on fibre size distribution (A and C) and mean fibre cross-sectional area (B and D). UFP-101 was administered once a day, for four consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days) and 30 min before the muscle collection, at the fourth day. Each point or column represent the mean ± SEM. *p < 0.05 when compared to the control vehicle/saline group; $^{\#}p$ < 0.05 compared to reserpine/saline group. Statistical analysis was performed by one-way (cross sectional area) or two-way (frequency of fibres) ANOVA followed by Bonferroni's *post hoc* test. (A-D) n = 5 mice/group.

Figure 6: Serotonin and glutamate levels in prefrontal cortex (A and D), thalamus/hypothalamus (B and E) and lumbar spinal cord (C and F) of reserpine-treated mice. UFP-101 (1 nmol/kg, intraperitoneal) or pregabalin (188 µmol/kg, intraperitoneal) were administered once a day, for four consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days), and 30 min before tissue collection at the fourth day. Serotonin (A-C) and glutamate (D-F) levels are expressed in ng/g tissue. Each bar represents the mean ± SEM. *p < 0.05 when compared to the control vehicle/saline group. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's *post hoc* test (A and C) or one-way ANOVA followed by Bonferroni's *post hoc* test (B, D, E and F). (A and D) n = 7-8 mice/group; (B, C, E and F) n = 8 mice/group.

Figure 7: Effects of the repeated treatment with UFP-101 or pregabalin on the [^{18}F]-FDG hypermetabolism in the whole brain (A) or in several brain structures (B) in the fibromyalgia-like model induced by reserpine in mice. UFP-101 (1 nmol/kg, intraperitoneal) or pregabalin (188 µmol/kg, intraperitoneal) were administered once a day, for four consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days), and 30 min before tissue collection at the fourth day. Representative images of the coronal plane of the vehicle control group (CI), the reserpine-treated group (CII), the group treated with UFP-101 (CIII), or the group treated with pregabalin (CIV). Each column represents the mean ± SEM. *p < 0.05 when compared to the control vehicle/saline group. Differences in the standardised uptake value ratio (SUVr) per areas of brain and in whole brain were determined by one-way (A) or two-way ANOVA (B), followed by Bonferroni's *post hoc* tests, respectively. (A-B) n = 7-8 mice/group. Abbreviations: RSTR, right striatum region; LSTR, left striatum region; CTX, cortex; RHIP, right hippocampal region; LHIP, left hippocampal region; THA, thalamus; BFS, basal forebrain/septum; HYP, hypothalamus; RAMY, right amygdala; LAMY, left amygdala; BS, brainstem; CG, cingulate gyrus; SC, superior colliculus; OLF, olfactory areas; RMID, right midbrain; LMID, left midbrain; LIC, left inferior colliculus; RIC, right inferior colliculus.

Figure 8: The ppN/OFQ (A-D) and NOP receptor (E-H) mRNA expression was measured by RT-qPCR in prefrontal cortex (A and E), thalamus/hypothalamus (B and F), lumbar spinal cord (C and G) and masseter muscle (D and H) tissues, at days 1, 2 and 3 after the administration of reserpine (0.25 mg/kg; subcutaneous). Each scatter dot plot represents the mean ± SEM of 6-8 samples. *p < 0.05 when compared to the control vehicle/saline group. Statistical analysis was performed by unpaired Student t test.

Figure 9: Quantitative immunohistochemistry analysis for NOP receptor in the agranular insular cortex (A-C), the lumbar spinal cord (D-F) and the dorsal root ganglia (DRG; G-I) of vehicle- and reserpine-treated mice. The samples were collected at the fourth day after the onset of reserpine administration. NOP receptor immunopositivity was quantified in the regions corresponding to the laminas I to VI (D) of the spinal cord. Representative images for NOP receptor immunolabelling in the agranular insular cortex of the vehicle/saline control (B) or reserpine-treated group (C); in the spinal cord of the vehicle/saline control (E) or reserpine-treated group (F); and the DRG of the vehicle/saline control (H) or reserpine-treated group (I). The DRG images were acquired in 200-x magnification. The schematic representations of brain and lumbar spinal cord were captured in × 8 and × 32 magnification, respectively. Red continuous lines delimit the regions of interest analysed in the brain and lumbar spinal cord. Scale bar (—) represents 2 mm, 1 mm and 100 µm, for brain, spinal cord and DRG, respectively. *$p < 0.05$ when compared to the vehicle/saline control group. Statistical analysis was performed by Student *t* test. (A and D) $n = 5$ mice/group; (G) $n = 5$-6 mice/group.

Figure 10: Effects of the repeated treatment with SB-612111 on fibromyalgia-like symptoms in reserpine-treated mice. Effects of the repeated administration of SB-612111 (6.6 µmol/kg; intraperitoneal) on hind paw mechanical allodynia (A), latency time in response to heat stimulation (B), immobility time (C), and on plus maze parameters (G-I). Effects of the repeated administration of SB-612111 (2.2, 6.6 and 22 µmol/kg; intraperitoneal) on the rotating time (D), grasping strength (E), and latency to fall (F). All the tests were performed at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). SB-612111 was dosed for three consecutive days, 30 min after reserpine injection. At the fourth day, mice also received SB-612111, 30 min before evaluations. Each bar represents the mean ± SEM. *$p < 0.05$ when compared to the control vehicle/saline group; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by Kruskal-Wallis (F, H), one-way (C, D, G and I) or two-way (A, B and E) ANOVA followed by Bonferroni's *post hoc* test. (A-C, G- I) $n = 10$ mice/group; (D-F) $n = 8$-14 mice/group.

Figures and Legends

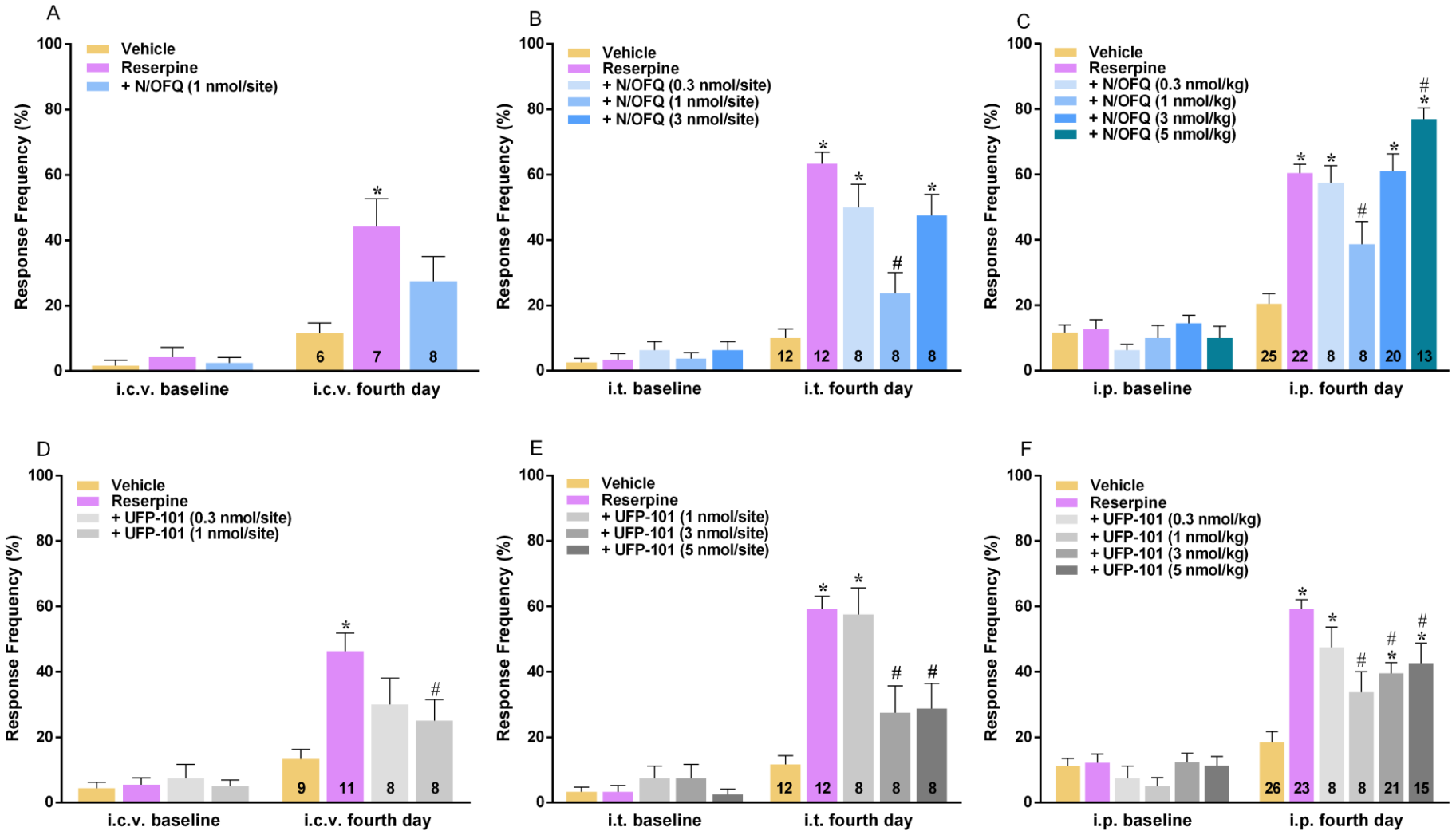
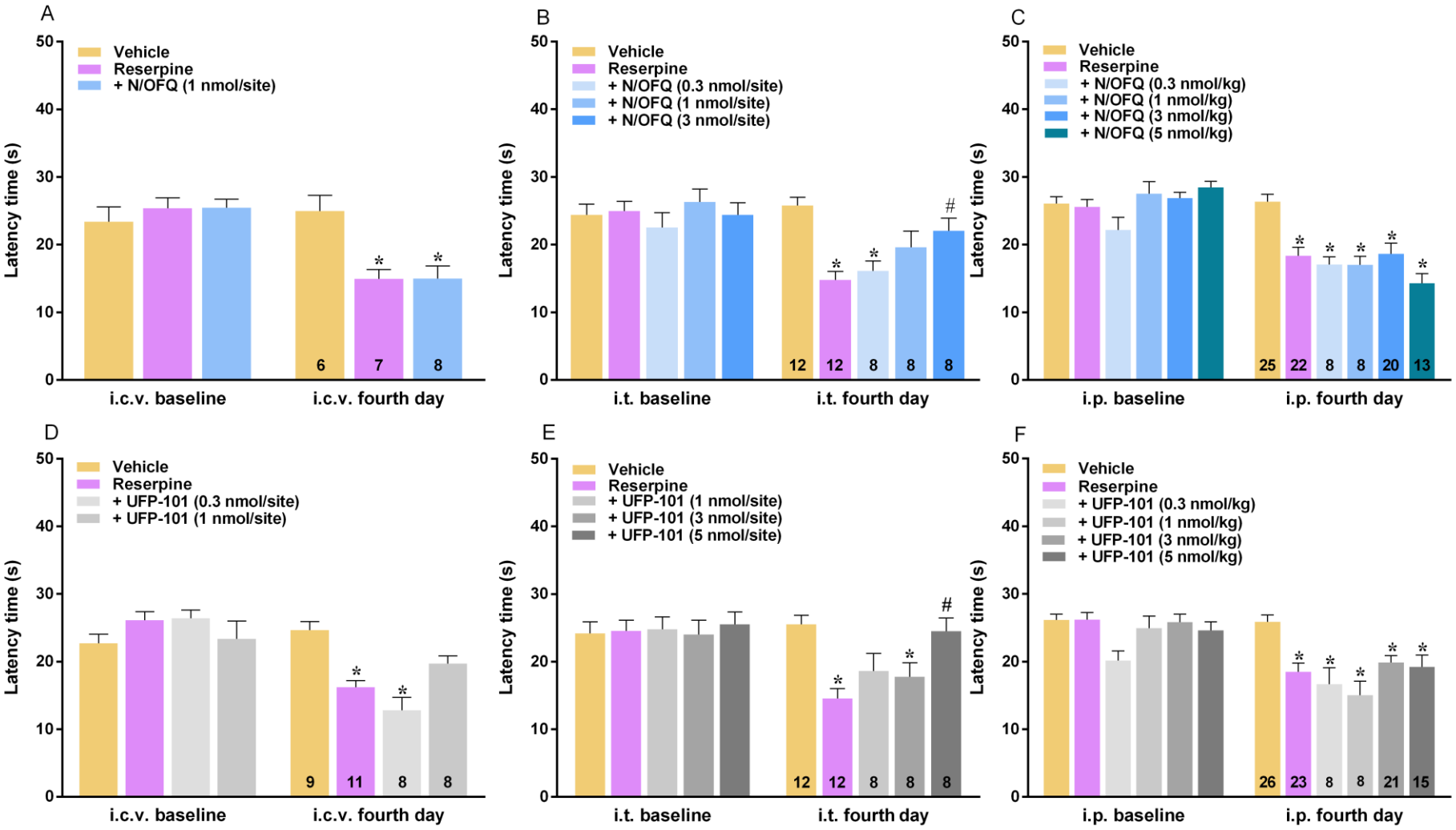


Figure 1: Dose-related effects of N/OFQ or UFP-101 on the mechanical hypersensitivity in the mouse model of fibromyalgia induced by reserpine. Effects of acute treatment with N/OFQ (A-C) or UFP-101 (D-F), dosed by intracerebroventricular (i.c.v.; A and D), intrathecal (i.t.; B and E) or intraperitoneal (i.p.; C and F) routes, on hindpaw withdrawal threshold (response frequency in percentage) to tactile stimulation. The mechanical hypersensitivity was assessed by using the Von Frey filaments before (baseline), and at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). The NOPr ligands were administered in a single dose, 15 min (i.c.v. and i.t.) or 30 min (i.p.) before the behavioural evaluations. Each column represents the mean ± SEM. $^{*}p < 0.05$ when compared to the control vehicle/saline group, indicating the development of mechanical allodynia; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni's *post hoc* test (A-F). (A) n = 6-8 mice/group; (B) n = 8-12 mice/group; (C) n = 8-25 mice/group; (D) n = 8-11 mice/group; (E) n = 8-12 mice/group; (F) n = 8-26 mice/group.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i.c.v. fourth day
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Vehicle
Reserpine
+ N/OFQ (0.3 nmol/site)
+ N/OFQ (1 nmol/site)
+ N/OFQ (3 nmol/site)
i.t. baseline
i.t. fourth day
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+ N/OFQ (1 nmol/kg)
+ N/OFQ (3 nmol/kg)
+ N/OFQ (5 nmol/kg)
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21
15

Figure 2: Dose-related effects of N/OFQ or UFP-101 on the thermal hypersensitivity in reserpine-treated mice. Effects of acute treatment with N/OFQ (A-C) or UFP-101 (D-F), dosed by intracerebroventricular (i.c.v.; A and D), intrathecal (i.t.; B and E) or intraperitoneal (i.p.; C and F) routes, on the latency time (s) in response to hot thermal stimulation. Thermal hypersensitivity was assessed in the hot-plate test, at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). The NOPr ligands were administered in a single dose, 15 min (i.c.v. and i.t.) or 30 min (i.p.) before the behavioural evaluations. Each column represents the mean ± SEM. $^{*}p < 0.05$ when compared to the control vehicle/saline group, indicating the development of thermal sensitivity; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's *post hoc* test (A-F). (A) n = 6-8 mice/group; (B) n = 8-12 mice/group; (C) n = 8-25 mice/group; (D) n = 8-11 mice/group; (E) n = 8-12 mice/group; (F) n = 8-26 mice/group.

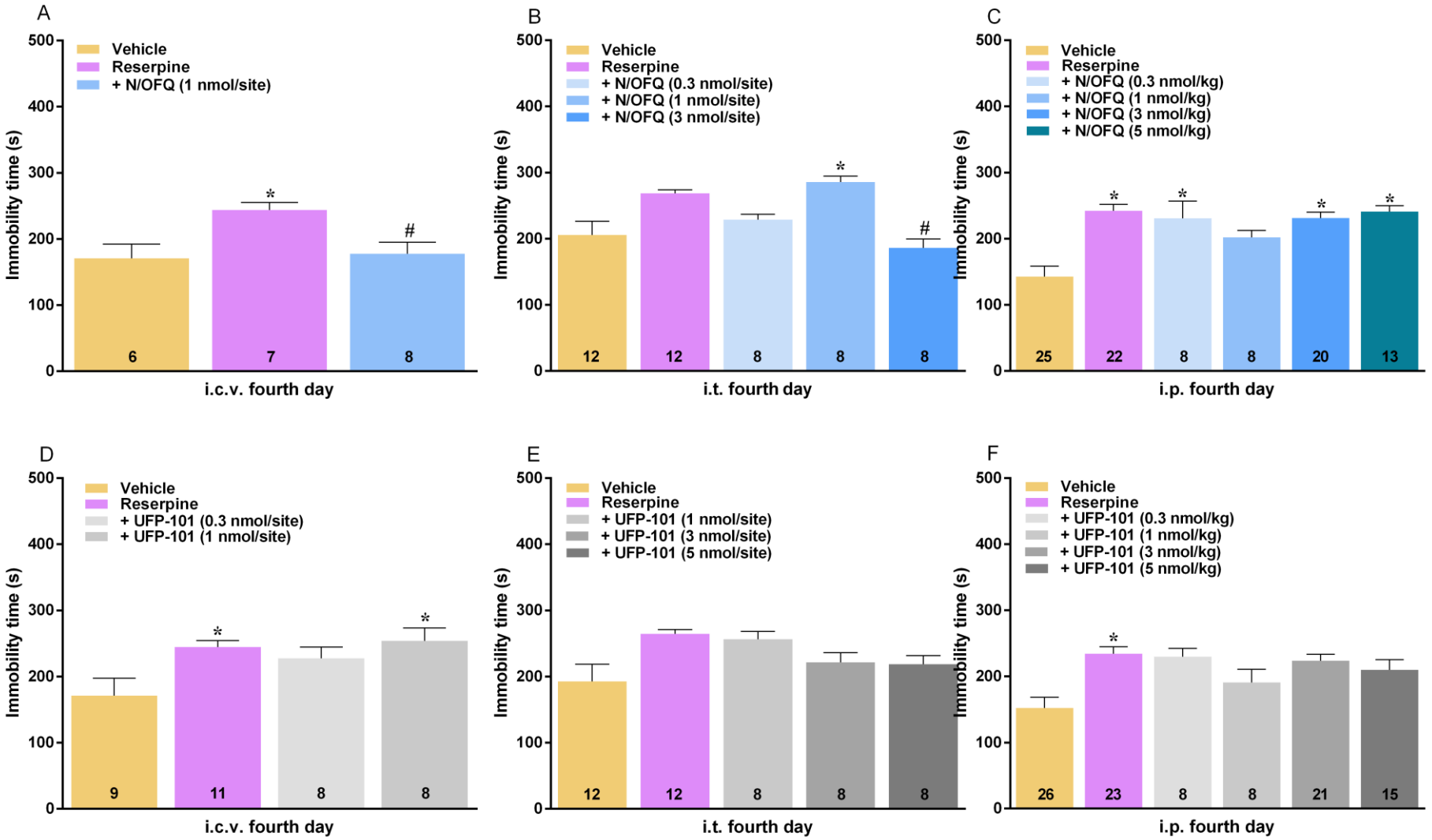

A
Vehicle
Reserpine
+ N/OFQ (1 nmol/site)
Immobility time (s)
500
400
300
200
100
0
6
7
8
*
#
i.c.v. fourth day
B
Vehicle
Reserpine
+ N/OFQ (0.3 nmol/site)
+ N/OFQ (1 nmol/site)
+ N/OFQ (3 nmol/site)
12
12
8
8
8
i.t. fourth day
C
Vehicle
Reserpine
+ N/OFQ (0.3 nmol/kg)
+ N/OFQ (1 nmol/kg)
+ N/OFQ (3 nmol/kg)
+ N/OFQ (5 nmol/kg)
25
22
8
8
20
13
i.p. fourth day
D
Vehicle
Reserpine
+ UFP-101 (0.3 nmol/site)
+ UFP-101 (1 nmol/site)
9
11
8
8
i.c.v. fourth day
E
Vehicle
Reserpine
+ UFP-101 (1 nmol/site)
+ UFP-101 (3 nmol/site)
+ UFP-101 (5 nmol/site)
12
12
8
8
8
i.t. fourth day
F
Vehicle
Reserpine
+ UFP-101 (0.3 nmol/kg)
+ UFP-101 (1 nmol/kg)
+ UFP-101 (3 nmol/kg)
+ UFP-101 (5 nmol/kg)
26
23
8
8
21
15
i.p. fourth day

Figure 3: Dose-related effects of the acute treatment with N/OFQ (A-C) or UFP-101 (D-F), dosed by intracerebroventricular (i.c.v; A and D), intrathecal (i.t.; B and E) or intraperitoneal (i.p.; C and F) routes, on depression-like behaviour in reserpine-treated mice submitted to the forced swimming test. The immobility time (s) was assessed at fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). The NOPr ligands were administered in a single dose, 30 min before the behavioural evaluations. Each column represents the mean ± SEM. $^{*}p < 0.05$ when compared to the control vehicle/saline group, indicating the development of depressive behaviour; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's *post hoc* test (B, C, E and F) or one-way ANOVA followed by Bonferroni's *post hoc* test (A and D). (A) n = 6-8 mice/group; (B) n = 8-12 mice/group; (C) n = 8-25 mice/group; (D) n = 8-11 mice/group; (E) n = 8-12 mice/group; (F) n = 8-26 mice/group.

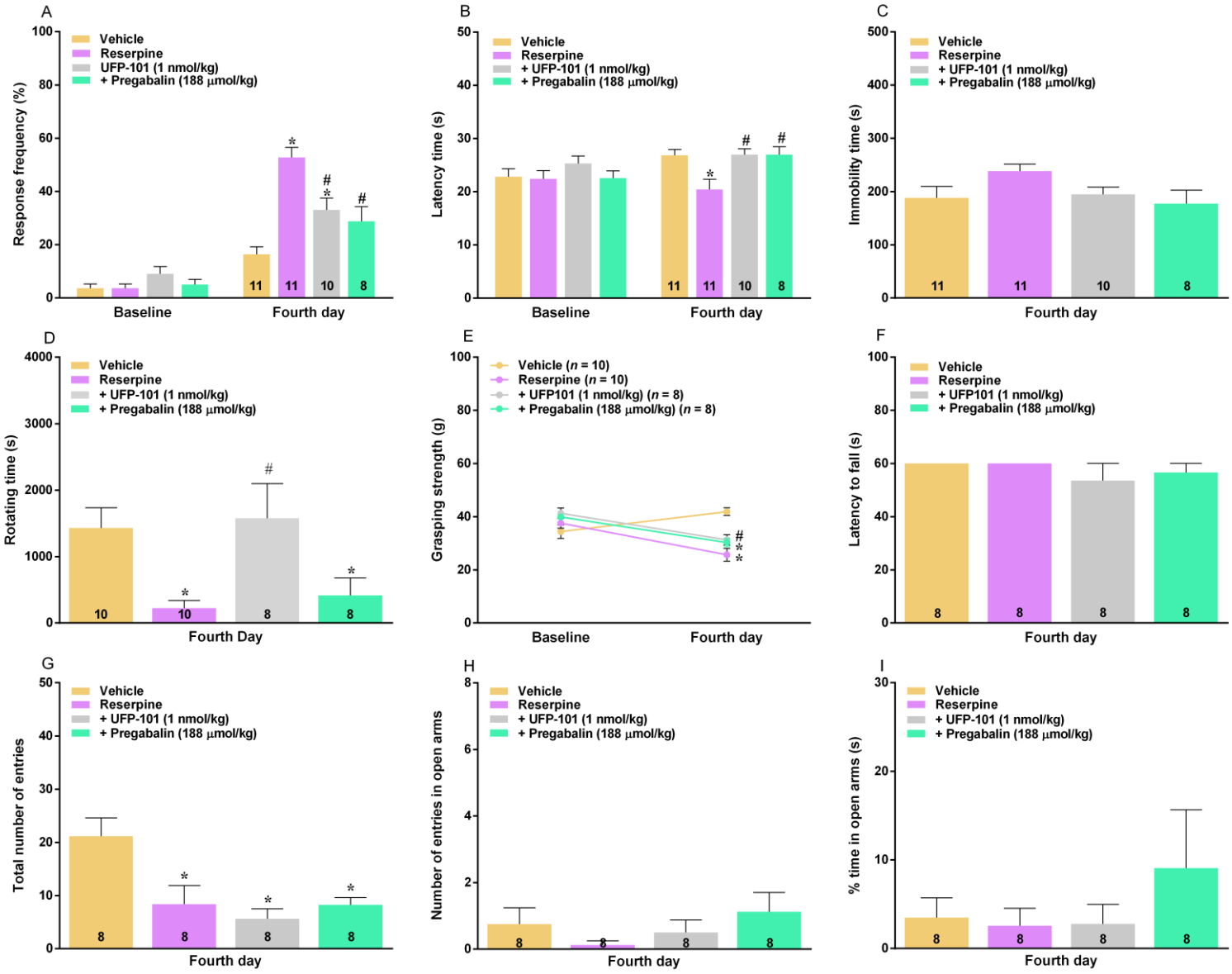

A
Response frequency (%)
Vehicle
Reserpine
UFP-101 (1 nmol/kg)
+ Pregabalin (188 μmol/kg)
Baseline
Fourth day
B
Latency time (s)
+ UFP-101 (1 nmol/kg)
C
Immobility time (s)
D
Rotating time (s)
Fourth Day
E
Grasping strength (g)
Vehicle (n = 10)
Reserpine (n = 10)
+ UFP101 (1 nmol/kg) (n = 8)
+ Pregabalin (188 μmol/kg) (n = 8)
F
Latency to fall (s)
G
Total number of entries
H
Number of entries in open arms
I
% time in open arms (s)

Figure 4: Effects of the repeated treatment with UFP-101 or pregabalin on painful-, fatigue-, depressive- and anxiety-like behaviours in reserpine-treated mice. The effects of both drugs were also assessed on the fatigue and grip strength in the fibromyalgia model elicited by reserpine. Effects of the repeated administration of UFP-101 (1 nmol/kg, intraperitoneal) or pregabalin (188 µmol/kg, intraperitoneal), on hind paw mechanical allodynia (A), latency time (s) in response to hot thermal stimulation (B), immobility time in the forced swimming test (C), rotating time (D), grasping strength (E), latency to fall (F), and on plus maze parameters (G-I). All the tests were performed at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). UFP-101 or pregabalin were dosed for three consecutive days, 30 min after reserpine injection. At the fourth day, mice also received UFP-101 or pregabalin, 30 min before evaluations. Each column represents the mean ± SEM. $*p < 0.05$ when compared to the control vehicle/saline group; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's *post hoc* test (D, F, H and I), one-way (C and G) or two-way (A, B and E) ANOVA followed by Bonferroni's *post hoc* test. (A-C) n = 8-11 mice/group; (D-E) n = 8-10 mice/group; (F-I) n = 8 mice/group.

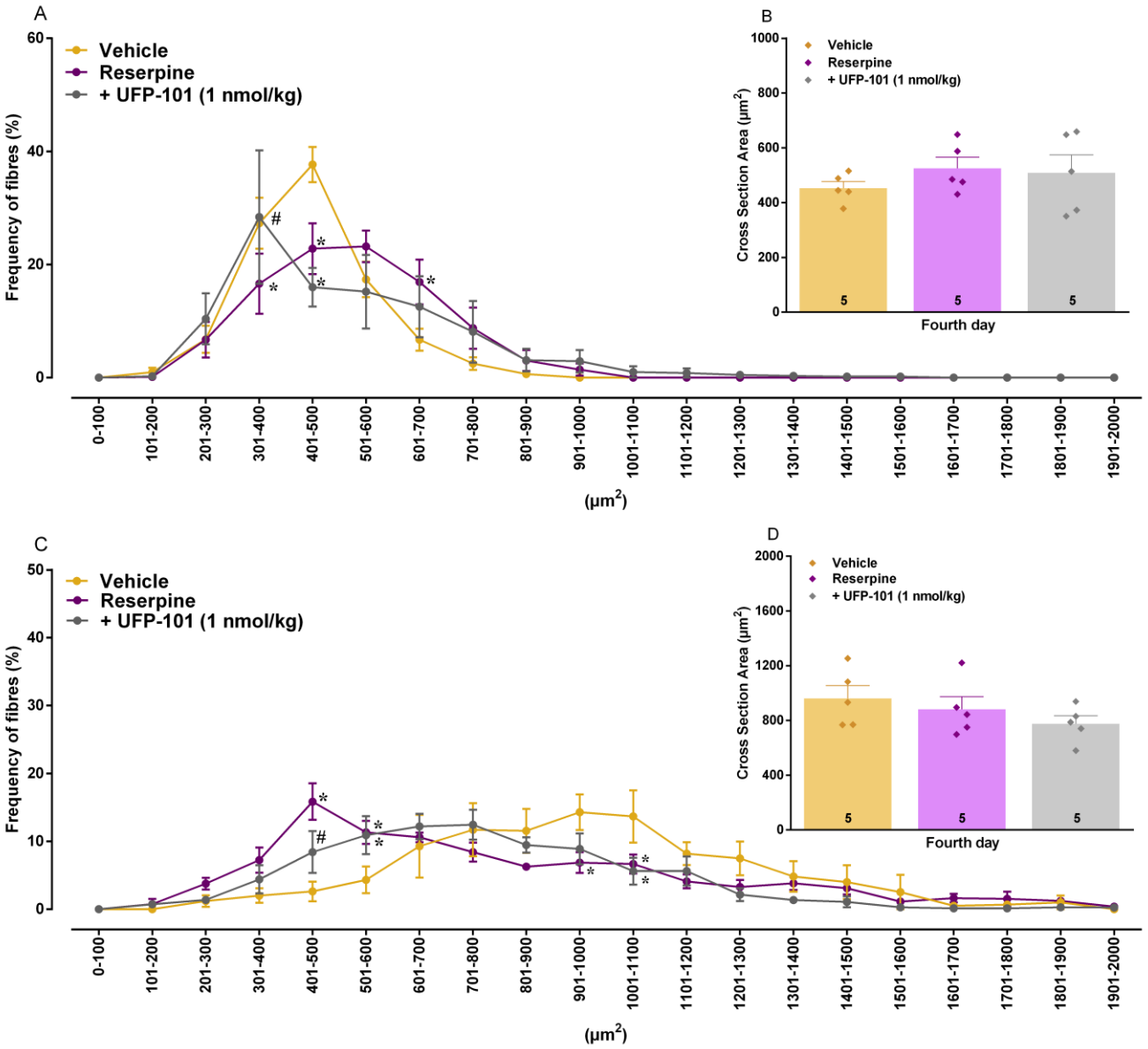

A
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
Frequency of fibres (%)
0-100
101-200
201-300
301-400
401-500
501-600
601-700
701-800
801-900
901-1000
1001-1100
1101-1200
1201-1300
1301-1400
1401-1500
1501-1600
1601-1700
1701-1800
1801-1900
1901-2000
(µm²)
B
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
Cross Section Area (µm²)
Fourth day
C
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
Frequency of fibres (%)
(µm²)
D
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
Cross Section Area (µm²)
Fourth day

Figure 5: Histological analysis of masseter (A and B) and gastrocnemius (C and D) muscles of reserpine-treated mice. Effects of intraperitoneal (i.p.) repeated treatment with UFP-101 (1 nmol/kg) on fibre size distribution (A and C) and mean fibre cross-sectional area (B and D). UFP-101 was administered once a day, for four consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days) and 30 min before the muscle collection, at the fourth day. Each point or column represent the mean ± SEM. $^{*}p < 0.05$ when compared to the control vehicle/saline group; $^{\#}p < 0.05$ compared to reserpine/saline group. Statistical analysis was performed by one-way (cross sectional area) or two-way (frequency of fibres) ANOVA followed by Bonferroni's *post hoc* test. (A-D) $n = 5$ mice/group.

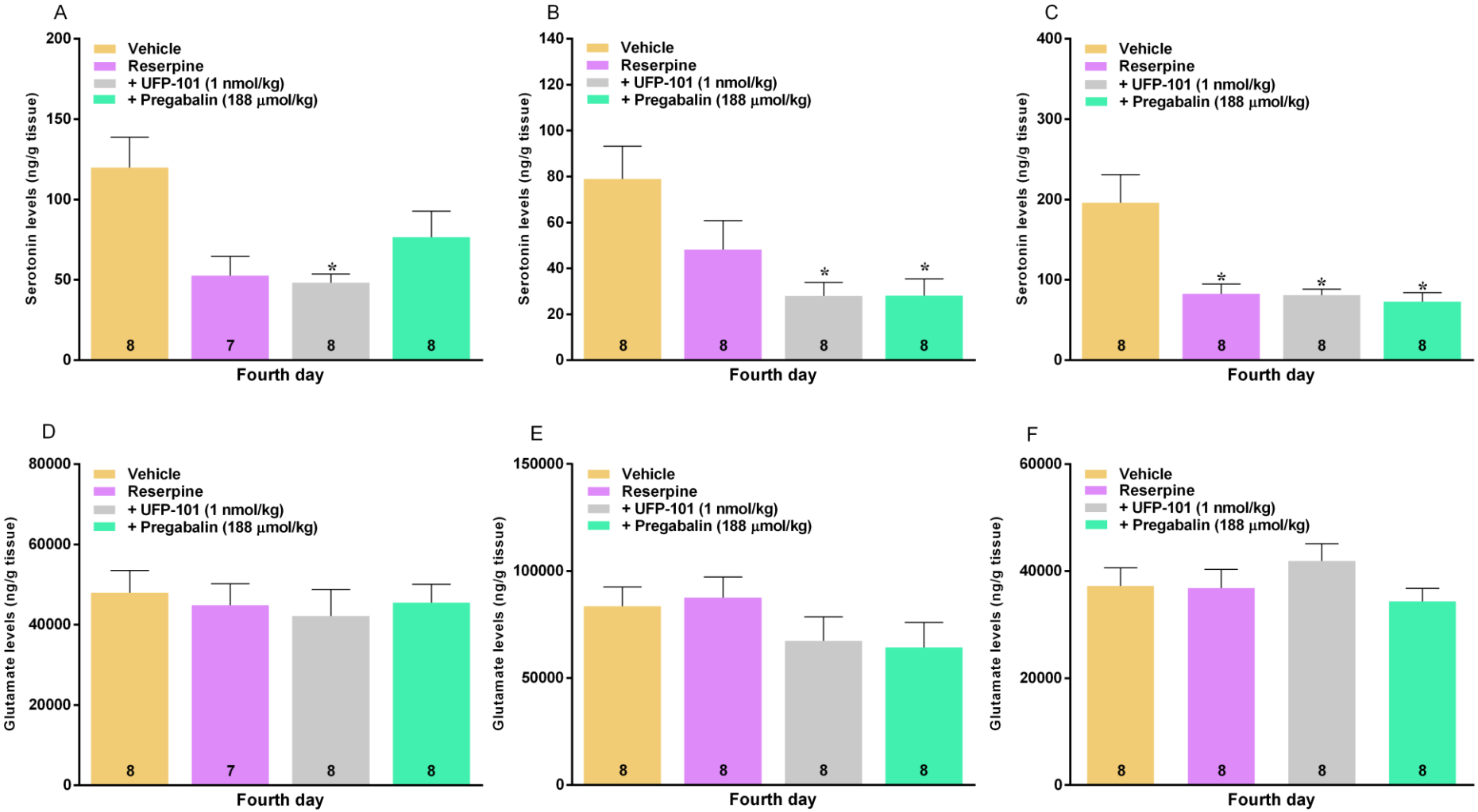

A
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
+ Pregabalin (188 μmol/kg)
Serotonin levels (ng/g tissue)
200
150
100
50
0
8
7
8
8
*
Fourth day
B
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
+ Pregabalin (188 μmol/kg)
Serotonin levels (ng/g tissue)
140
120
100
80
60
40
20
0
8
8
8
8
*
*
Fourth day
C
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
+ Pregabalin (188 μmol/kg)
Serotonin levels (ng/g tissue)
400
300
200
100
0
8
8
8
8
*
*
*
Fourth day
D
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
+ Pregabalin (188 μmol/kg)
Glutamate levels (ng/g tissue)
80000
60000
40000
20000
0
8
7
8
8
Fourth day
E
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
+ Pregabalin (188 μmol/kg)
Glutamate levels (ng/g tissue)
150000
100000
50000
0
8
8
8
8
Fourth day
F
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
+ Pregabalin (188 μmol/kg)
Glutamate levels (ng/g tissue)
60000
40000
20000
0
8
8
8
8
Fourth day

Figure 6: Serotonin and glutamate levels in prefrontal cortex (A and D), thalamus/hypothalamus (B and E) and lumbar spinal cord (C and F) of reserpine-treated mice. UFP-101 (1 nmol/kg, intraperitoneal) or pregabalin (188 µmol/kg, intraperitoneal) were administered once a day, for four consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days), and 30 min before tissue collection at the fourth day. Serotonin (A-C) and glutamate (D-F) levels are expressed in ng/g tissue. Each bar represents the mean ± SEM. *p < 0.05 when compared to the control vehicle/saline group. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's *post hoc* test (A and C) or one-way ANOVA followed by Bonferroni's *post hoc* test (B, D, E and F). (A and D) n = 7-8 mice/group; (B, C, E and F) n = 8 mice/group.

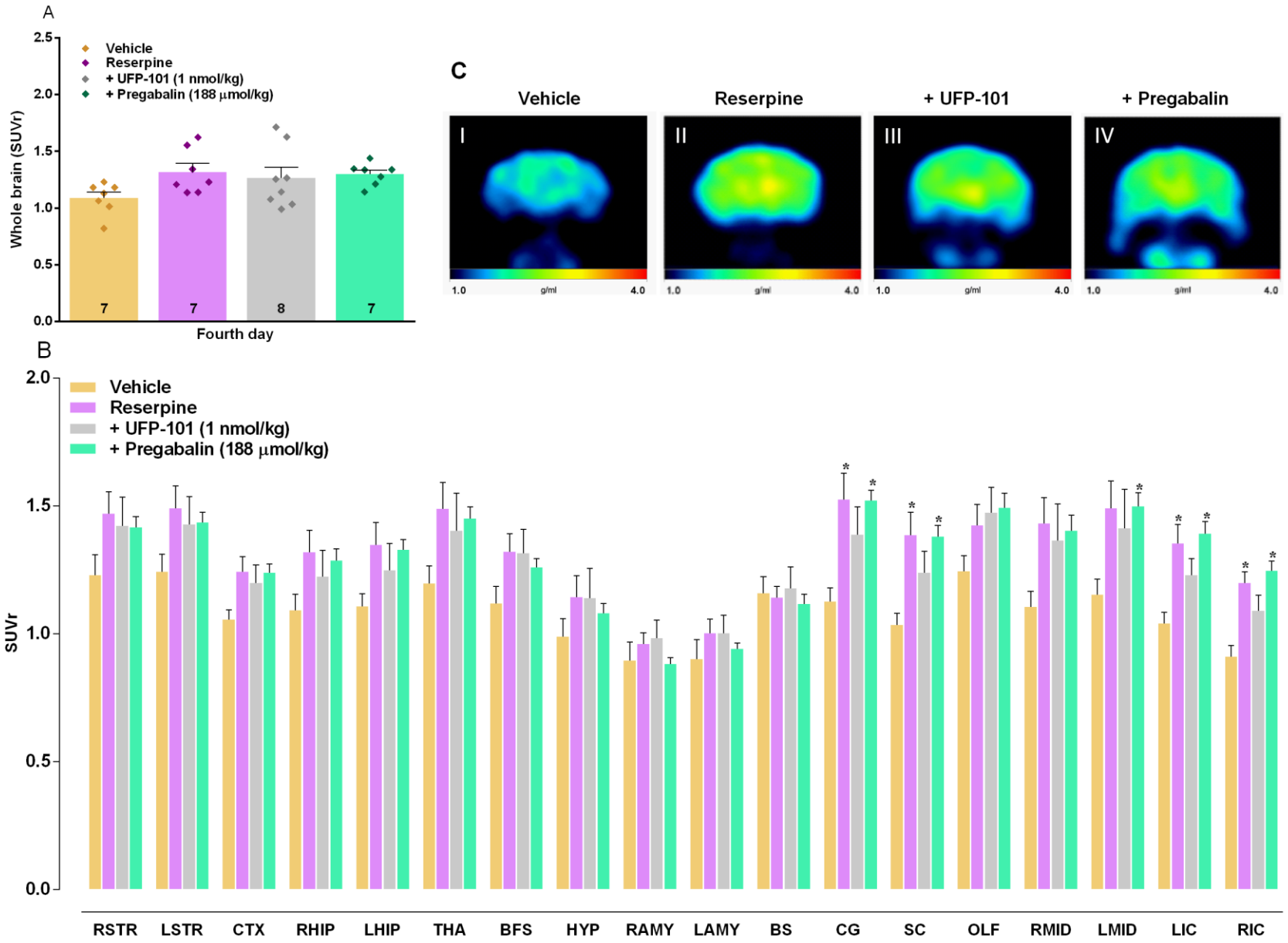

A
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
+ Pregabalin (188 μmol/kg)
Whole brain (SUVr)
2.5
2.0
1.5
1.0
0.5
0.0
7
7
8
7
Fourth day
C
Vehicle
Reserpine
+ UFP-101
+ Pregabalin
I
II
III
IV
1.0
g/ml
4.0
B
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
+ Pregabalin (188 μmol/kg)
SUVr
2.0
1.5
1.0
0.5
0.0
RSTR
LSTR
CTX
RHIP
LHIP
THA
BFS
HYP
RAMY
LAMY
BS
CG
SC
OLF
RMID
LMID
LIC
RIC

Figure 7: Effects of the repeated treatment with UFP-101 or pregabalin on the [^{18}F]-FDG hypermetabolism in the whole brain (A) or in several brain structures (B) in the fibromyalgia-like model induced by reserpine in mice. UFP-101 (1 nmol/kg, intraperitoneal) or pregabalin (188 µmol/kg, intraperitoneal) were administered once a day, for four consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days), and 30 min before tissue collection at the fourth day. Representative images of the coronal plane of the vehicle control group (CI), the reserpine-treated group (CII), the group treated with UFP-101 (CIII), or the group treated with pregabalin (CIV). Each column represents the mean ± SEM. *$p < 0.05$ when compared to the control vehicle/saline group. Differences in the standardised uptake value ratio (SUVr) per areas of brain and in whole brain were determined by one-way (A) or two-way ANOVA (B), followed by Bonferroni's *post hoc* tests, respectively. (A-B) n = 7-8 mice/group. Abbreviations: RSTR, right striatum region; LSTR, left striatum region; CTX, cortex; RHIP, right hippocampal region; LHIP, left hippocampal region; THA, thalamus; BFS, basal forebrain/septum; HYP, hypothalamus; RAMY, right amygdala; LAMY, left amygdala; BS, brainstem; CG, cingulate gyrus; SC, superior colliculus; OLF, olfactory areas; RMID, right midbrain; LMID, left midbrain; LIC, left inferior colliculus; RIC, right inferior colliculus.

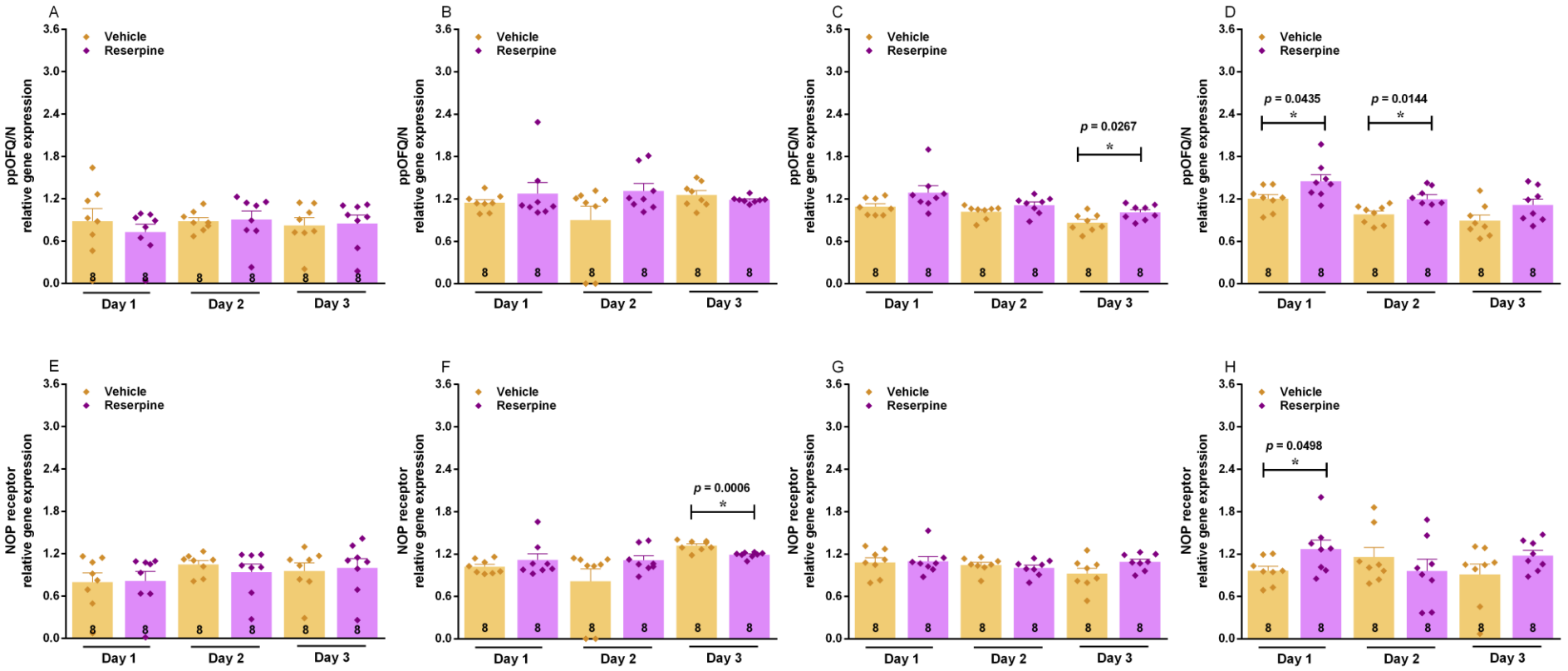

A
B
C
D
E
F
G
H
Vehicle
Reserpine
ppOFQ/N
relative gene expression
NOP receptor
relative gene expression
Day 1
Day 2
Day 3
p = 0.0435
p = 0.0144
p = 0.0267
p = 0.0006
p = 0.0498
*
8

Figure 8: The ppN/OFQ (A-D) and NOP receptor (E-H) mRNA expression was measured by RT-qPCR in prefrontal cortex (A and E), thalamus/hypothalamus (B and F), lumbar spinal cord (C and G) and masseter muscle (D and H) tissues, at days 1, 2 and 3 after the administration of reserpine (0.25 mg/kg; subcutaneous). Each scatter dot plot represents the mean ± SEM of 6-8 samples. *$p < 0.05$ when compared to the control vehicle/saline group. Statistical analysis was performed by unpaired Student *t* test.

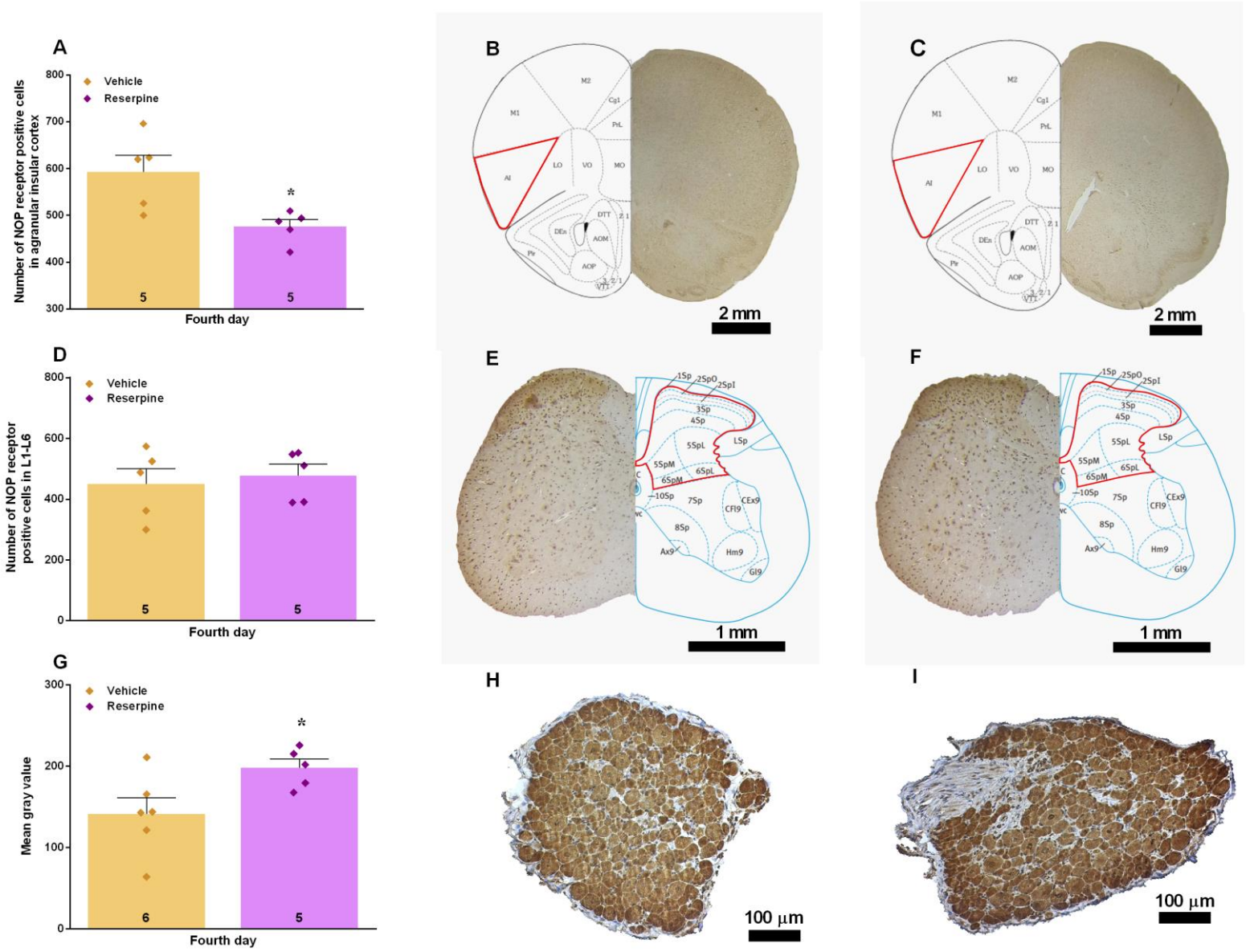

A
Vehicle
Reserpine
Number of NOP receptor positive cells in agranular insular cortex
800
700
600
500
400
300
5
5
*
Fourth day
B
M2
M1
Cg1
PrL
Al
LO
VO
MO
DTT
DEn
AOM
Pir
AOP
2 mm
C
2 mm
D
Vehicle
Reserpine
Number of NOP receptor positive cells in L1-L6
800
600
400
200
0
5
5
Fourth day
E
1Sp
2SpO
2SpI
3Sp
4Sp
5SpL
LSp
5SpM
6SpL
6SpM
10Sp
7Sp
CEx9
CFl9
8Sp
Ax9
Hm9
Gl9
1 mm
F
1 mm
G
Vehicle
Reserpine
Mean gray value
300
200
100
0
6
5
*
Fourth day
H
100 µm
I
100 µm

Figure 9: Quantitative immunohistochemistry analysis for NOP receptor in the agranular insular cortex (A-C), the lumbar spinal cord (D-F) and the dorsal root ganglia (DRG; G-I) of vehicle- and reserpine-treated mice. The samples were collected at the fourth day after the onset of reserpine administration. NOP receptor immunopositivity was quantified in the regions corresponding to the laminas I to VI (D) of the spinal cord. Representative images for NOP receptor immunolabelling in the agranular insular cortex of the vehicle/saline control (B) or reserpine-treated group (C); in the spinal cord of the vehicle/saline control (E) or reserpine-treated group (F); and the DRG of the vehicle/saline control (H) or reserpine-treated group (I). The DRG images were acquired in 200-x magnification. The schematic representations of brain and lumbar spinal cord were captured in × 8 and × 32 magnification, respectively. Red continuous lines delimit the regions of interest analysed in the brain and lumbar spinal cord. Scale bar (—) represents 2 mm, 1 mm and 100 µm, for brain, spinal cord and DRG, respectively. *$p < 0.05$ when compared to the vehicle/saline control group. Statistical analysis was performed by Student *t* test. (A and D) $n = 5$ mice/group; (G) $n = 5$-6 mice/group.

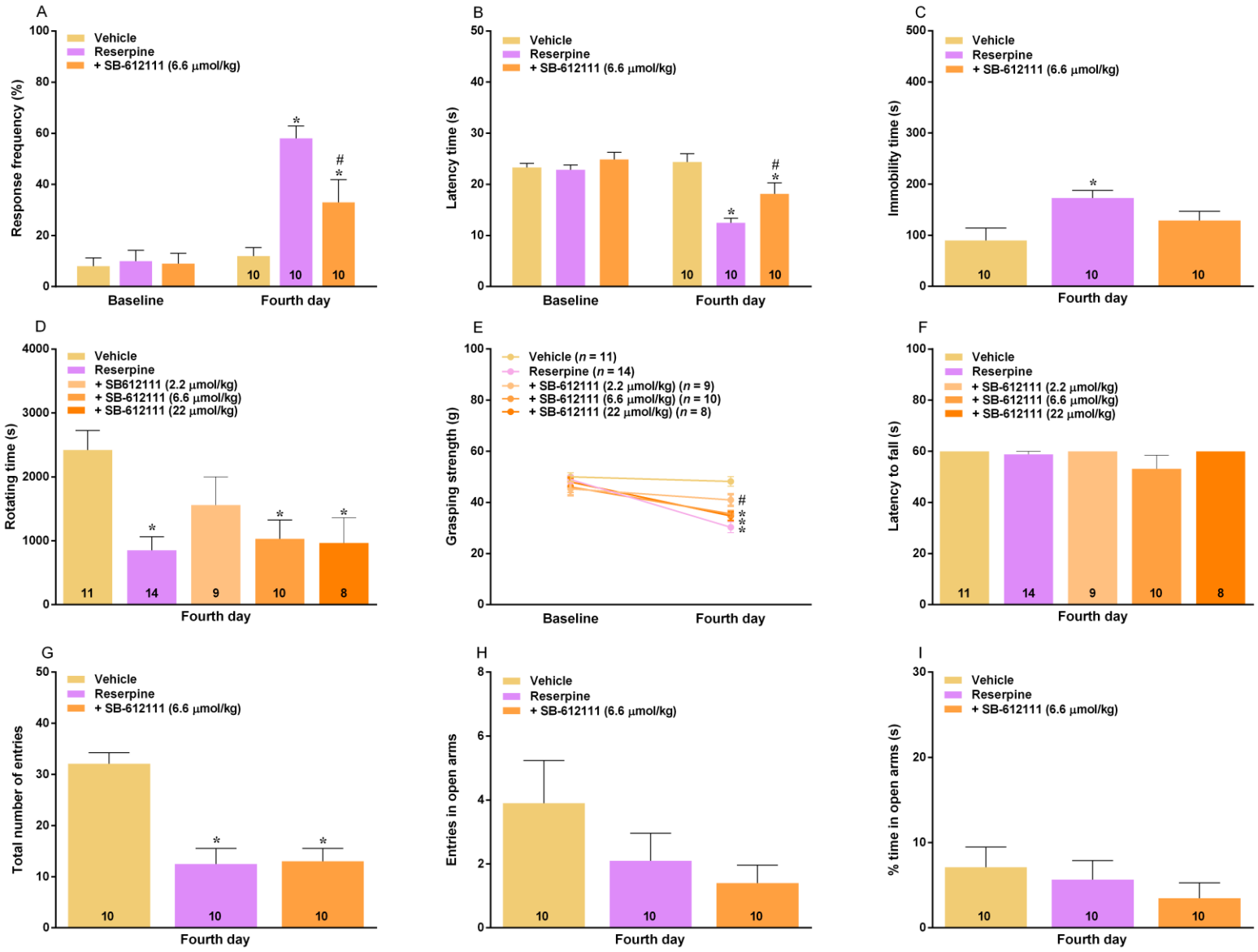

A
Vehicle
Reserpine
+ SB-612111 (6.6 μmol/kg)
Response frequency (%)
Baseline
Fourth day
B
Latency time (s)
C
Immobility time (s)
D
+ SB612111 (2.2 μmol/kg)
+ SB-612111 (6.6 μmol/kg)
+ SB-612111 (22 μmol/kg)
Rotating time (s)
E
Vehicle (n = 11)
Reserpine (n = 14)
+ SB-612111 (2.2 μmol/kg) (n = 9)
+ SB-612111 (6.6 μmol/kg) (n = 10)
+ SB-612111 (22 μmol/kg) (n = 8)
Grasping strength (g)
F
Latency to fall (s)
G
Total number of entries
H
Entries in open arms
I
% time in open arms (s)

Figure 10: Effects of the repeated treatment with SB-612111 on fibromyalgia-like symptoms in reserpine-treated mice. Effects of the repeated administration of SB-612111 (6.6 µmol/kg; intraperitoneal) on hind paw mechanical allodynia (A), latency time in response to heat stimulation (B), immobility time (C), and on plus maze parameters (G-I). Effects of the repeated administration of SB-612111 (2.2, 6.6 and 22 µmol/kg; intraperitoneal) on the rotating time (D), grasping strength (E), and latency to fall (F). All the tests were performed at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). SB-612111 was dosed for three consecutive days, 30 min after reserpine injection. At the fourth day, mice also received SB-612111, 30 min before evaluations. Each bar represents the mean ± SEM. $^{*}p < 0.05$ when compared to the control vehicle/saline group; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by Kruskal-Wallis (F, H), one-way (C, D, G and I) or two-way (A, B and E) ANOVA followed by Bonferroni's *post hoc* test. (A-C, G- I) $n = 10$ mice/group; (D-F) $n = 8$-14 mice/group.

Supporting Information

Supplementary Table 1. Effects of the acute treatment with N/OFQ or UFP-101 on the locomotor activity and anxiety parameters in reserpine-treated mice. The values represent the mean ± SEM.

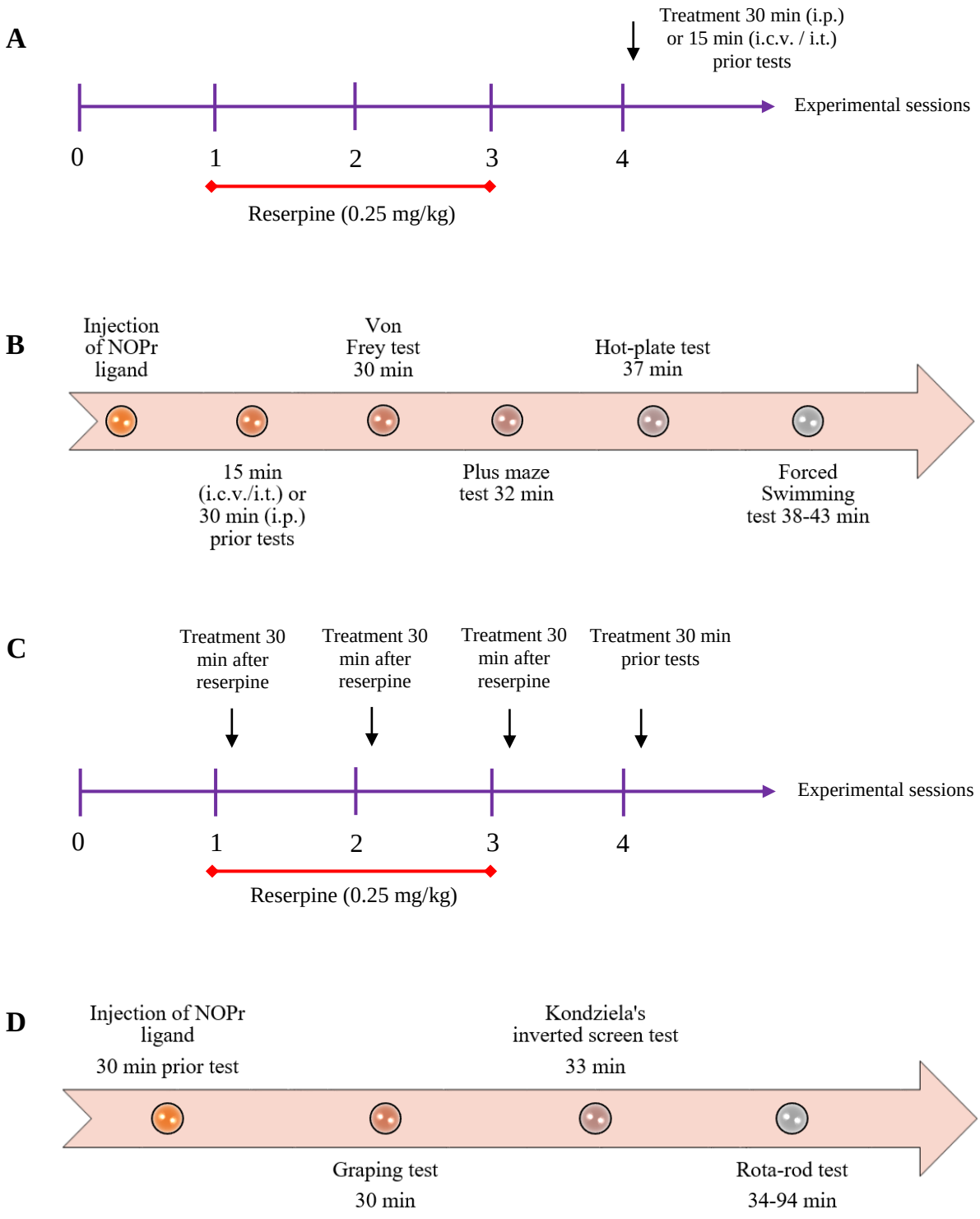
I.C.V.	**Vehicle**	**Reserpine**	**N/OFQ (1 nmol/site)**			
Total number entries	20 ± 1.1	8.4 ± 3.6 *	8 ± 1.4 *			
Entries in open arms	0.8 ± 0.5	1.3 ± 1	1.5 ± 0.8			
%Time in open arms	1.6 ± 0.9	12.9 ± 9.6	3.8 ± 1.7			
I.C.V.	**Vehicle**	**Reserpine**	**UFP-101 (0.3 nmol/site)**	**UFP-101 (1 nmol/site)**		
Total number entries	16.3 ± 3.1	6.7 ± 2.6 *	8.6 ± 2	3.9 ± 1.5 *		
Entries in open arms	0.8 ± 0.3	0.4 ± 0.4	1.1 ± 0.5	1.5 ± 0.8		
%Time in open arms	1.7 ± 0.5	0.4 ± 0.2	3.5 ± 1.5	14 ± 9.2		
I.T.	**Vehicle**	**Reserpine**	**N/OFQ (0.3 nmol/site)**	**N/OFQ (1 nmol/site)**	**N/OFQ (3 nmol/site)**	
Total number entries	23.2 ± 1.7	11.6 ± 3.1 *	10.5 ± 2.4 *	14.4 ± 3.3	8.1 ± 1.6 *	
Entries in open arms	1.1 ± 0.6	2 ± 0.9	1.2 ± 0.4	2.9 ± 1.5	1.9 ± 0.7	
%Time in open arms	3.3 ± 1.6	9.2 ± 4.7	3 ± 1.1	6.2 ± 3.8	1.7 ± 0.7	
I.T.	**Vehicle**	**Reserpine**	**UFP-101 (1 nmol/site)**	**UFP-101 (3 nmol/site)**	**UFP-101 (5 nmol/site)**	
Total number entries	20.2 ± 2.6	11.4 ± 3.2	13.6 ± 3.3	10.5 ± 1.5	13.2 ± 4.2	
Entries in open arms	0.5 ± 0.3	1.9 ± 0.9	2 ± 1	0.9 ± 0.4	5.6 ± 1.8	
%Time in open arms	1.9 ± 0.9	4.9 ± 2.1	3.1 ± 1.5	4.2 ± 1.8	35.7 ± 12.9	
I.P.	**Vehicle**	**Reserpine**	**N/OFQ (0.3 nmol/kg)**	**N/OFQ (1 nmol/kg)**	**N/OFQ (3 nmol/kg)**	**N/OFQ (5 nmol/kg)**
Total number entries	22.5 ± 2	14 ± 2 *	6.4 ± 2.3 *	13.1 ± 2.8	8.4 ± 1.9 *	14.4 ± 1.8
Entries in open arms	2.1 ± 0.5	2.6 ± 0.7	0.9 ± 0.9	1.4 ± 0.6	0.9 ± 0.3	2.8 ± 0.7
%Time in open arms	3.4 ± 1	6.7 ± 1.5	3.2 ± 2.8	4.3 ± 2	2 ± 1	7 ± 2
I.P.	**Vehicle**	**Reserpine**	**UFP-101 (0.3 nmol/kg)**	**UFP-101 (1 nmol/kg)**	**UFP-101 (3 nmol/kg)**	**UFP-101 (5 nmol/kg)**
Total number entries	23.8 ± 2	12.1 ± 2.1 *	13 ± 1.8	4.2 ± 1.5 *	16.2 ± 4	12.7 ± 2.2 *
Entries in open arms	2.4 ± 0.6	2.3 ± 0.7	0.1 ± 0.1	1.2 ± 0.6	3.3 ± 1.3	3.1 ± 0.5
%Time in open arms	4.7 ± 1.4	6.2 ± 1.6	0.4 ± 0.2	8 ± 4.4	9.3 ± 2.9	12 ± 5.4

N/OFQ and UFP-101 were dosed by intracerebroventricular (i.c.v.), intrathecal (i.t.) or intraperitoneal (i.p.) routes, at the fourth day after the onset of fibromyalgia induction, 30 min before the plus maze test. *$p < 0.05$ when compared to the control vehicle/saline group. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's *post hoc* test, or by one-way ANOVA followed by Bonferroni's *post hoc* test. (N/OFQ i.c.v. treatment) n = 6-8 mice per group; (UFP-101 i.c.v. treatment) n = eight mice per group; (N/OFQ i.t. treatment) n = 8-10 mice per group; (UFP-101 i.t. treatment) n = 7-10 mice per group; (N/OFQ i.p. treatment) n = 8-17 mice per group; (UFP-101 i.p. treatment) n = 8-18 mice per group.

Supplementary Table 2. Cytokine levels (TNF, IL-1β and IL-10) in brain, spinal cord and masseter muscle (pg/100 mg tissue), or in serum (pg/ml) in the fibromyalgia model induced by reserpine in mice. The values represent the mean ± SEM.

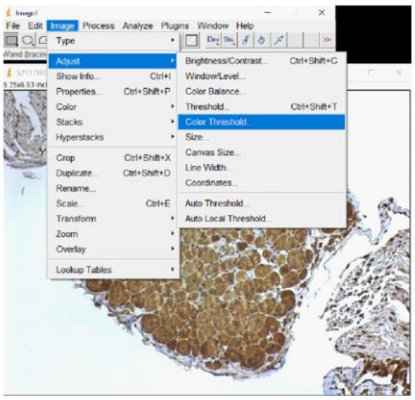
Brain	**TNF**	**IL-10**	**IL-1β**
Vehicle	1573 ± 159.7	1430 ± 195.8	209.4 ± 43.4
Reserpine	1599 ± 142.9	1426 ± 217.6	210.2 ± 58.5
UFP-101 (1 nmol/kg)	1576 ± 233.6	1297 ± 132.8	18 ± 9
Pregabalin (188 µmol/kg)	5707 ± 299.4*	2522 ± 155*	692 ± 103.5$^{\#}$
Spinal cord	**TNF**	**IL-10**	**IL-1β**
Vehicle	10600 ± 2115	3958 ± 138.9	2934 ± 176
Reserpine	11602 ± 2077	5193 ± 609	2636 ± 381.1
UFP-101 (1 nmol/kg)	13463 ± 1902	4212 ± 371.2	2966 ± 266.1
Pregabalin (188 µmol/kg)	13051 ± 1157	4485 ± 292.2	2338 ± 264.7
Masseter	**TNF**	**IL-10**	**IL-1β**
Vehicle	22769 ± 2601	4442 ± 556.2	1090 ± 144.6
Reserpine	23173 ± 2745	4207 ± 387.7	1357 ± 225.4
UFP-101 (1 nmol/kg)	20900 ± 2496	4221 ± 393.2	1118 ± 146.8
Pregabalin (188 µmol/kg)	16922 ± 2698	3569 ± 436.8	1189 ± 110
Serum	**TNF**	**IL-10**	**IL-1β**
Vehicle	† ND	† ND	2569 ± 151
Reserpine	† ND	† ND	1856 ± 460.3
UFP-101 (1 nmol/kg)	† ND	† ND	1767 ± 444
Pregabalin (188 µmol/kg)	† ND	† ND	2615 ± 65.2

UFP-101 (1 nmol/kg, intraperitoneal) or pregabalin (188 µmol/kg, intraperitoneal) were administered for three consecutive days, 30 min after daily reserpine injection. On the fourth day, mice also received UFP-101 or pregabalin, 30 min before of sample collection. *$p < 0.05$ when the pregabalin group was compared to the other groups. $^{\#}p < 0.05$ when the pregabalin group was compared to the UFP-101-treated group. The statistical analysis was performed by Kruskal-Wallis followed by Dunn's *post hoc* test or by one-way ANOVA followed by Bonferroni's *post hoc* test. n = 5-6 mice per group. † ND = not detectable.


A
Treatment 30 min (i.p.)
or 15 min (i.c.v. / i.t.)
prior tests
Experimental sessions
0
1
2
3
4
Reserpine (0.25 mg/kg)
B
Injection
of NOPr
ligand
Von
Frey test
30 min
Hot-plate test
37 min
15 min
(i.c.v./i.t.) or
30 min (i.p.)
prior tests
Plus maze
test 32 min
Forced
Swimming
test 38-43 min
C
Treatment 30
min after
reserpine
Treatment 30
min after
reserpine
Treatment 30
min after
reserpine
Treatment 30 min
prior tests
Experimental sessions
0
1
2
3
4
Reserpine (0.25 mg/kg)
D
Injection of NOPr
ligand
30 min prior test
Kondziela's
inverted screen test
33 min
Graping test
30 min
Rota-rod test
34-94 min

Supplementary Figure 1: Schedule of acute treatment with N/OFQ or UFP-101, administered 15 min (intracerebroventricular, i.c.v. and intrathecal, i.t.) or 30 min (intraperitoneal, i.p.) prior behavioural tests, at the fourth day (Panel A). Timeline for the behavioural tests after acute (N/OFQ or UFP-101) or repeated (UFP-101 or SB-612111) treatment (Panel B). Schedule of repeated treatment by i.p. injection of UFP-101 or SB-612111 administered once a day, for 4 consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days) and 30 min prior behavioural tests, at the fourth day (Panel C). Timeline for the fatigue-related tests after the repeated treatment with UFP-101 or SB-612111 (Panel D).

A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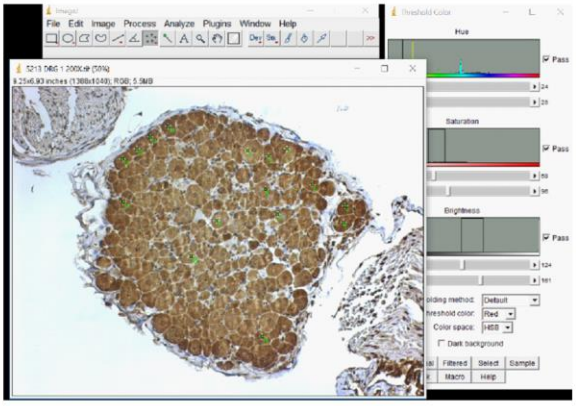
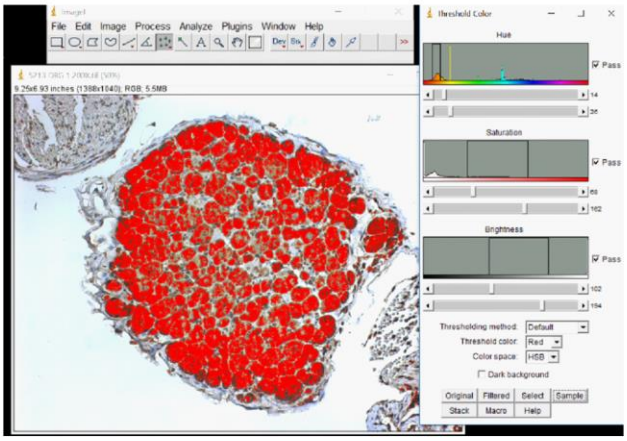
B


Hue
Saturation
Brightness
Pass

C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Saturation
Brightness
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Dark background
Original Filtered Select Sample
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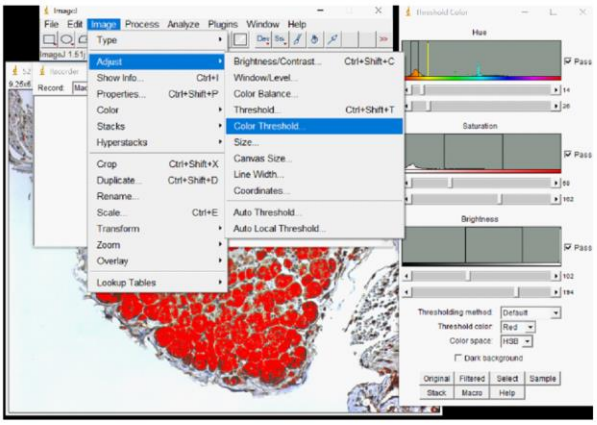
D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Window/Level...
Color Balance...
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Size...
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Coordinates...
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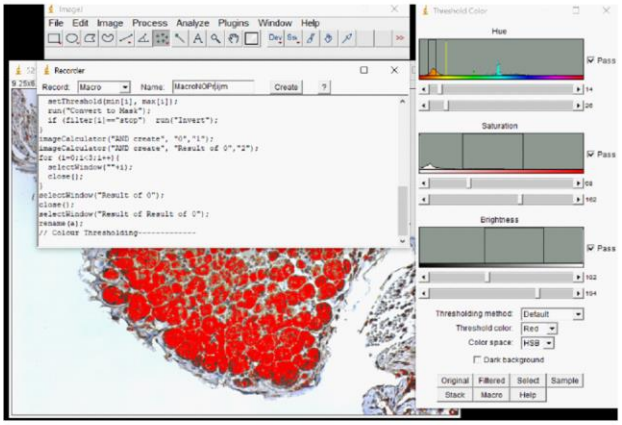
Auto Local Threshold...
Hue
Saturation
Brightness

E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Name: MacroNOPéjm
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Brightness
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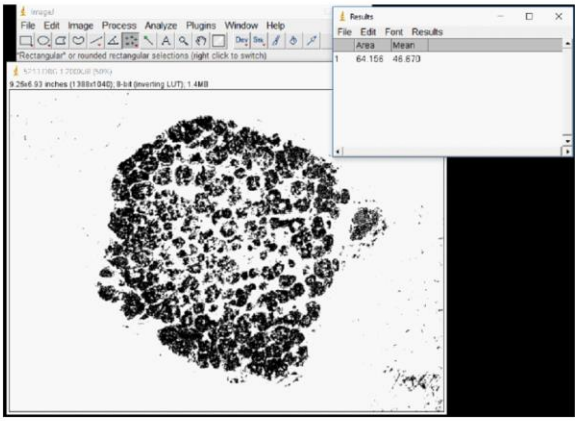
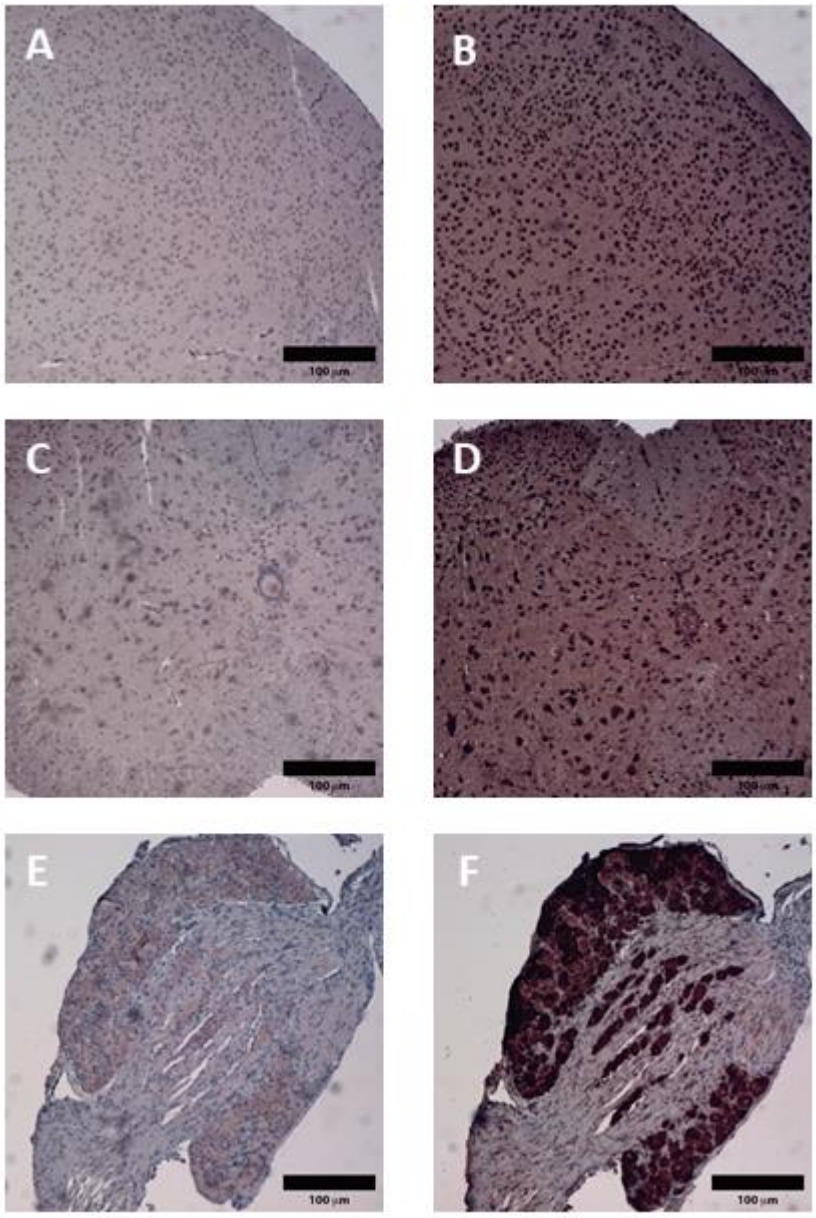
Color space: HSB
Dark background
Original Filtered Select Sample
Stack Macro Help

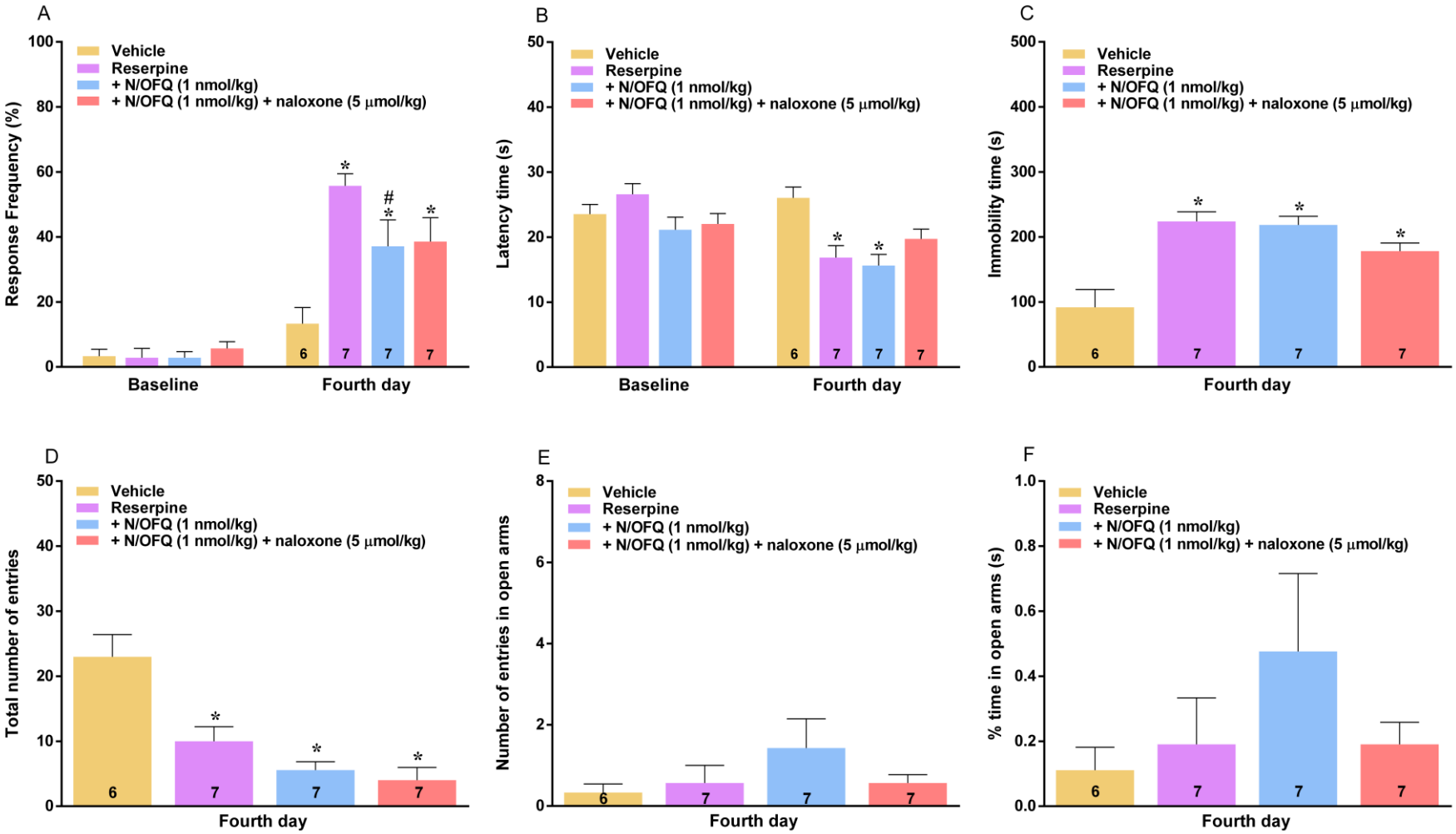
F


Results
File Edit Font Results
Area Mean
1 64.156 46.670

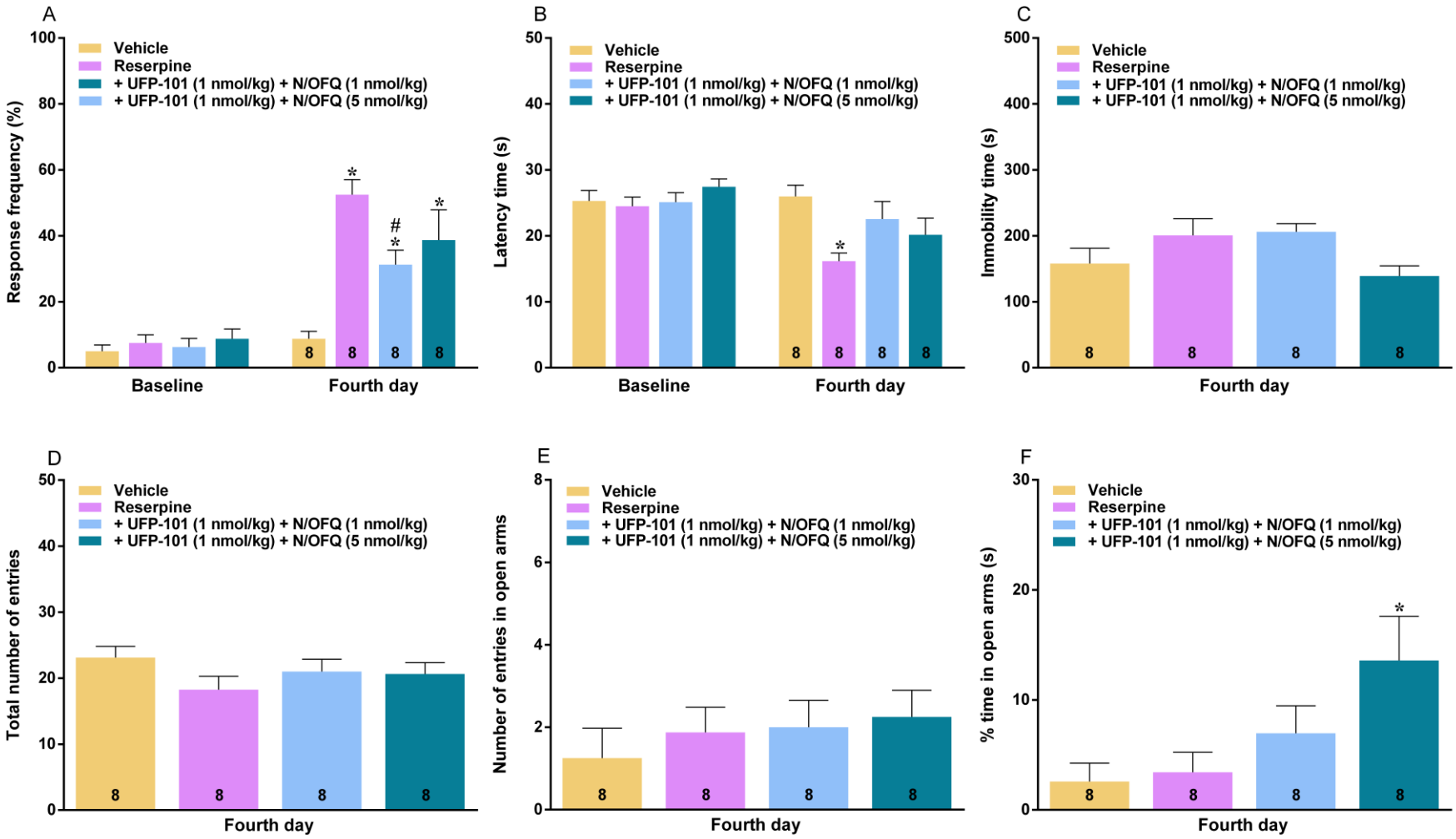
Supplementary Figure 2: Representative DRG images showing the creation of the macro used for the analysis of NOPr immunopositivity by ImageJ Software. (A) An image reference of negative control (vehicle-treated mice) was used for the macro creation. (B) The dark-to-medium brown areas (considered as immunopositive neurons for NOPr) were selected (16 small green multi-points) to determine the pixels for the macro creation. (C) Sampling the range of colours from the selected regions. (D) Determination of colour threshold following the next steps: (i) Plugins → macros → record; (ii) Image → adjust→ colour threshold. (E) Creation of macro "MacroNOPr": Save and install macro in Plugins → macros. (F) Resulting black-and-white image after the analysis of the RGB image. The small window on the right shows the value of the mean grey value of the black regions. The macro "MacroNOPr" was used for analysis of the other images as a reference macro.



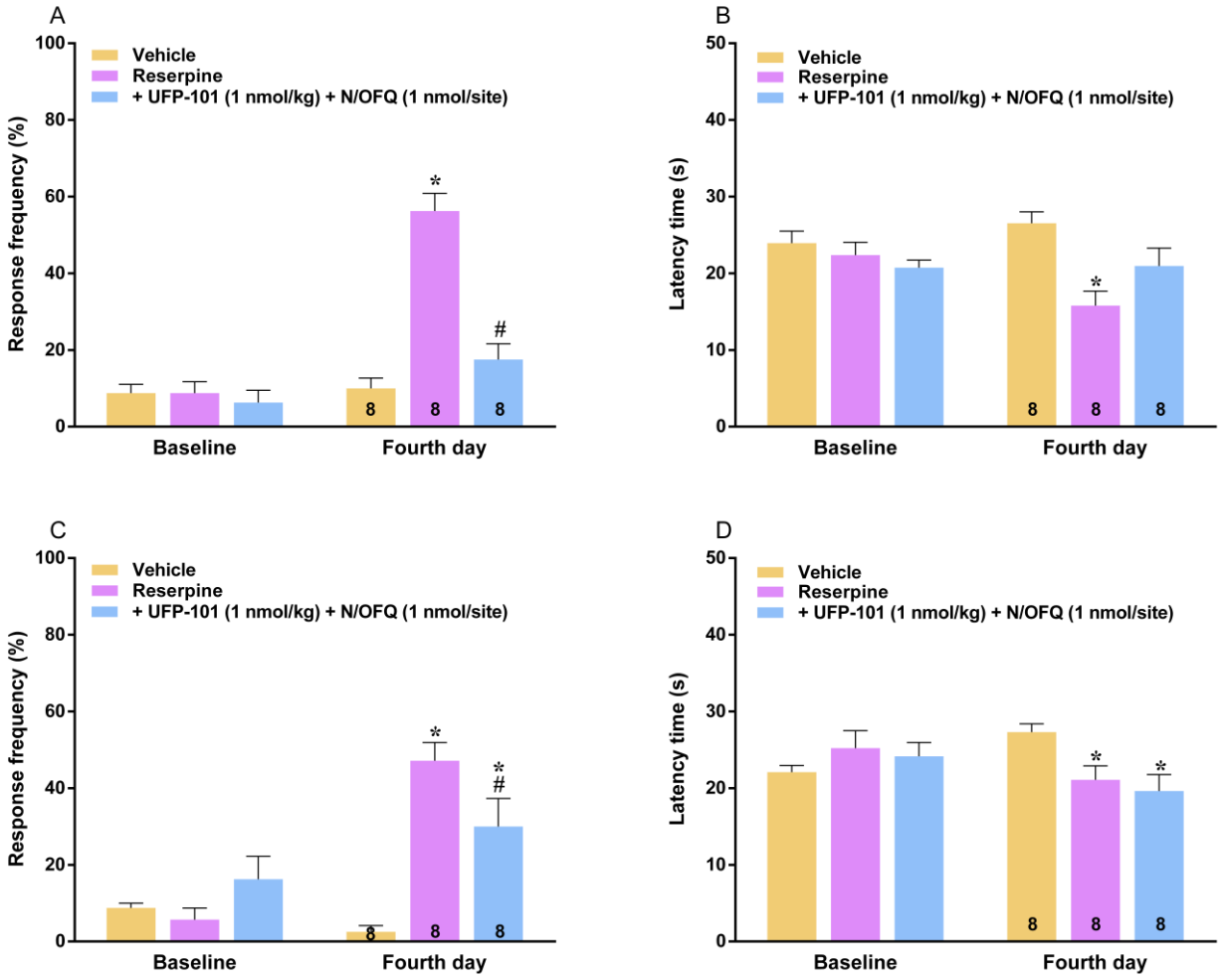
Supplementary Figure 3: Immunohistochemistry analysis to confirm the selectivity of the anti-NOPr antibody (A-F). Representative images for NOP receptor immunolabelling in the brain, spinal cord or DRG slides, with (A, C and E) or without (B, D and F) the co-incubation of the internal antigen with the primary antibody. The schematic representations were captured in ×40 magnification. Scale bar (—) represents 100 µm


A
Response Frequency (%)
Vehicle
Reserpine
+ N/OFQ (1 nmol/kg)
+ N/OFQ (1 nmol/kg) + naloxone (5 μmol/kg)
Baseline
Fourth day
B
Latency time (s)
C
Immobility time (s)
D
Total number of entries
E
Number of entries in open arms
F
% time in open arms (s)

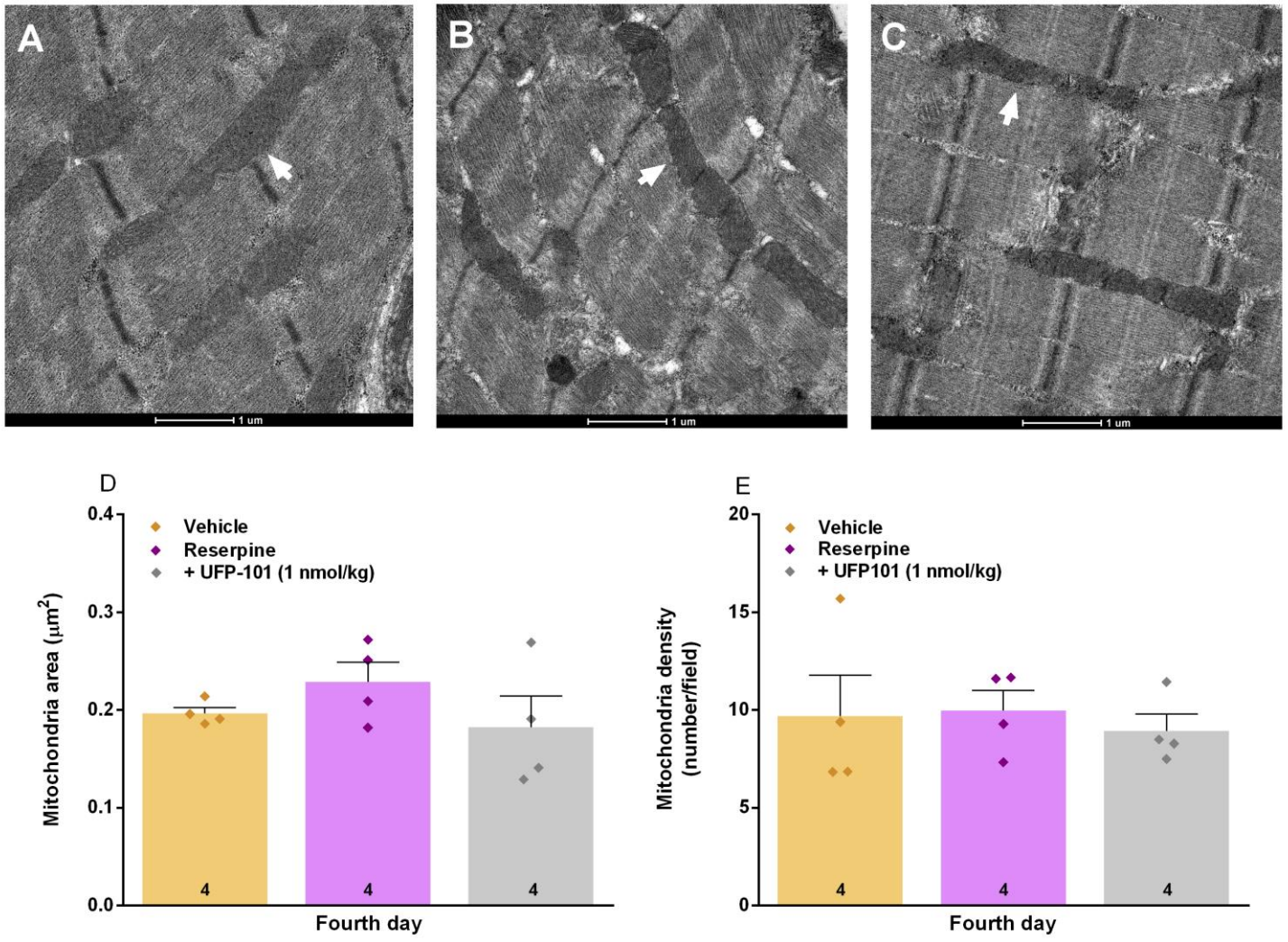
Supplementary Figure 4: Effects of N/OFQ alone or after the pre-treatment with naloxone on painful-, depressive- and anxiety-like behaviours in reserpine-treated mice. Effects of N/OFQ (1 nmol/kg) administered acutely, by intraperitoneal (i.p.) route, alone or after the pre-treatment with naloxone (5 µmol/kg, dosed i.p.; 5 min before the agonist treatment), on hind paw mechanical allodynia (A), latency time in response to the hot thermal stimulation (B), immobility time (C), and on plus maze parameters (D-F). All of the tests were performed at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). Each bar represents the mean ± SEM. *$p < 0.05$ when compared to the control vehicle/saline group; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by Kruskal-Wallis (E and F), one-way (C and D) or two-way (A and B) ANOVA followed by Bonferroni's *post hoc* test. (A-F) n = 6-7 mice per group


A
Response frequency (%)
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg) + N/OFQ (1 nmol/kg)
+ UFP-101 (1 nmol/kg) + N/OFQ (5 nmol/kg)
Baseline
Fourth day
B
Latency time (s)
C
Immobility time (s)
D
Total number of entries
E
Number of entries in open arms
F
% time in open arms (s)

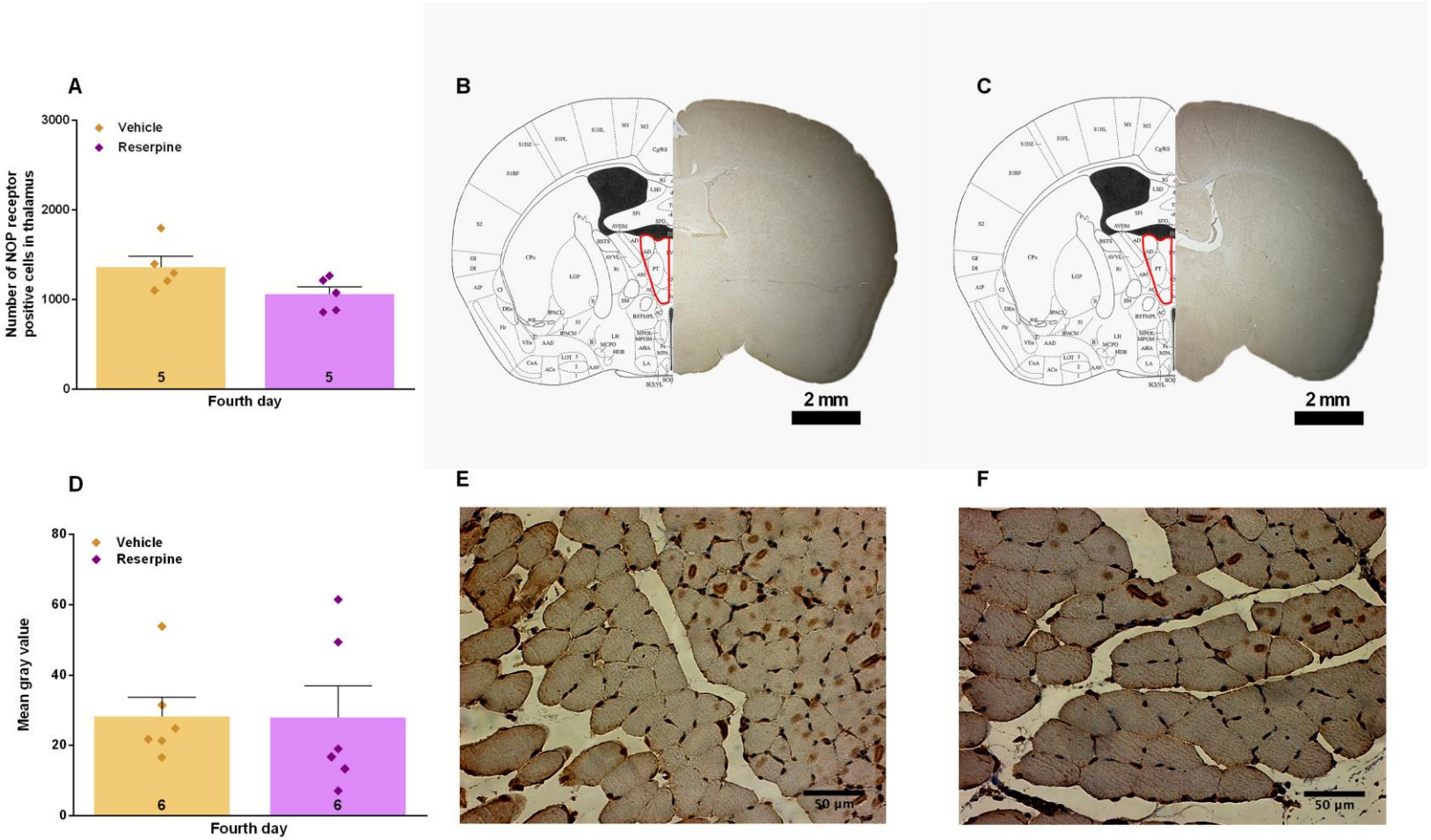
Supplementary Figure 5: Effects of UFP-101 combined with N/OFQ, both dosed i.p., on painful-, depressive- and anxiety-like behaviours in reserpine-treated mice. Effects of UFP-101 (1 nmol/kg) administered acutely, by intraperitoneal (i.p.) route, in combination with N/OFQ (1 nmol/kg or 5 nmol/kg, i.p.), on hind paw mechanical allodynia (A), latency time in response to hot thermal stimulation (B), immobility time (C) and on plus maze parameters (D-F). All of the tests were performed at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). Each column represents the mean ± SEM. *$p < 0.05$ when compared to the control vehicle/saline group; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by one-way (C-F) or two-way (A and B) ANOVA followed by Bonferroni's *post hoc* test. (A-F) *n* = eight mice per group.


A
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg) + N/OFQ (1 nmol/site)
Response frequency (%)
100
80
60
40
20
0
*
#
8
8
8
Baseline
Fourth day
B
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg) + N/OFQ (1 nmol/site)
Latency time (s)
50
40
30
20
10
0
*
8
8
8
Baseline
Fourth day
C
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg) + N/OFQ (1 nmol/site)
Response frequency (%)
100
80
60
40
20
0
*
*
#
8
8
8
Baseline
Fourth day
D
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg) + N/OFQ (1 nmol/site)
Latency time (s)
50
40
30
20
10
0
*
*
8
8
8
Baseline
Fourth day

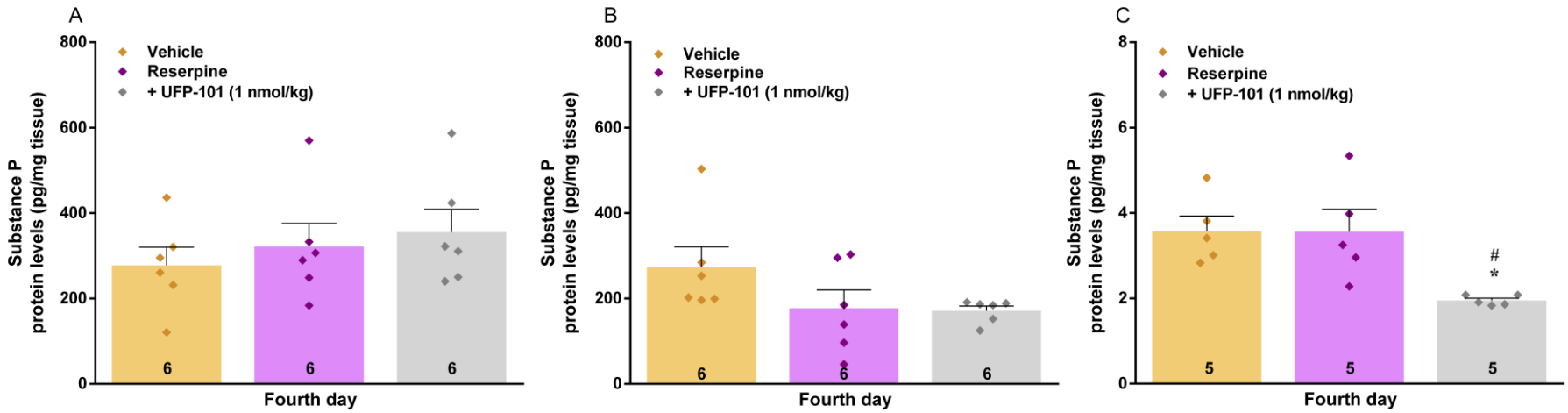
Supplementary Figure 6: Effects of systemic treatment with UFP-101 combined with N/OFQ (i.c.v. or i.t.) on mechanical and thermal nociception in reserpine-treated mice. Effects of UFP-101 (1 nmol/kg) administered acutely, by intraperitoneal (i.p.) route, in combination with N/OFQ (1 nmol/kg, i.c.v.; A and B) or N/OFQ (1 nmol/kg, i.t.; C and D), on hind paw mechanical allodynia (A and C) and latency time in response to hot stimulation (B and D). All of the tests were performed at the fourth day after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days). Each column represents the mean ± SEM. $^{*}p < 0.05$ when compared to the control vehicle/saline group; $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by two-way (A-D) ANOVA followed by Bonferroni's *post hoc* test. (A-F) n = eight mice per group.


A
B
C
1 um
1 um
1 um
D
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
Mitochondria area (μm²)
0.4
0.3
0.2
0.1
0.0
4
4
4
Fourth day
E
Vehicle
Reserpine
+ UFP101 (1 nmol/kg)
Mitochondria density (number/field)
20
15
10
5
0
4
4
4
Fourth day

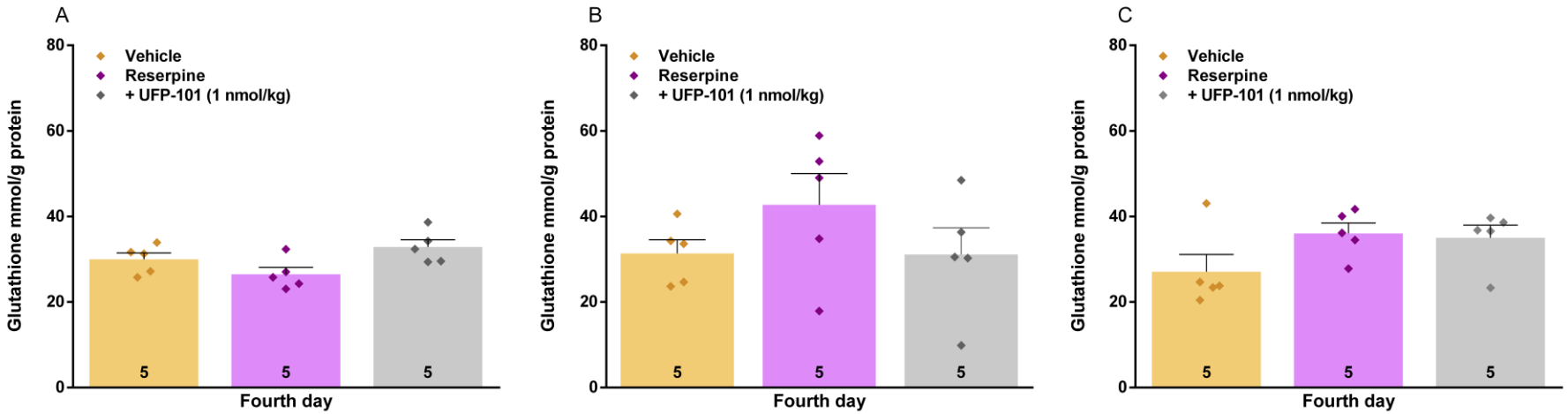
Supplementary Figure 7: Ultrastructural analysis of the masseter muscle in reserpine-treated mice. Representative transmission electron microscopic (TEM) images of masseter muscle in vehicle (A), reserpine (B) and UFP-101-treated mice (C). Effects of the repeated administration of UFP-101 (1 nmol/kg, intraperitoneal on mitochondrial area in μm^2 (D) or number of mitochondria/field (E) in masseter muscle. Scale bars represent one μm. Original magnification x 8,900. White arrows identify the mitochondria. (A, B) *n* = four mice per group.


A
Vehicle
Reserpine
Number of NOP receptor positive cells in thalamus
3000
2000
1000
0
5
5
Fourth day
B
2 mm
C
2 mm
D
Vehicle
Reserpine
Mean gray value
80
60
40
20
0
6
6
Fourth day
E
50 µm
F
50 µm

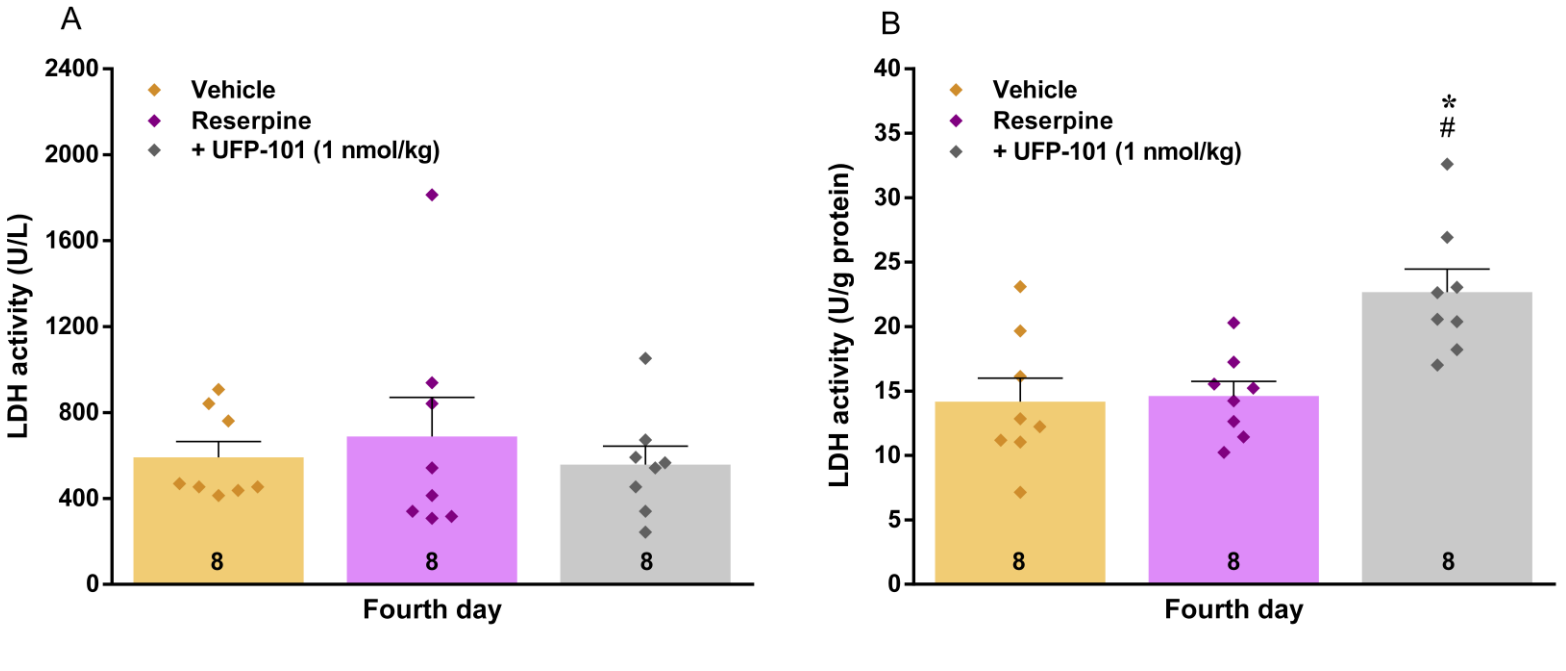
Supplementary Figure 8: Quantitative immunohistochemistry analysis for NOP receptor in the thalamus and masseter muscle of vehicle- and reserpine-treated mice (A-F). The samples were collected at the fourth day after the onset of reserpine administration. Representative images for NOP receptor immunolabelling in the thalamus of the vehicle/saline control (B) or reserpine-treated group (C), and in masseter of the vehicle/saline control (E) or reserpine-treated group (F). The schematic representations of brain and masseter were captured in ×8 and ×400 magnification, respectively. Red continuous lines delimit the region of interest analysed in the brain. Scale bar (—) represents 2 mm for brain and 50 μm for muscle. Statistical analysis was performed by Student *t* test. (A-C) *n* = five mice per group; (D-F) *n* = six mice per group.


A
B
C
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
Substance P
protein levels (pg/mg tissue)
Fourth day
800
600
400
200
0
8
6
4
2
6
5
#
*

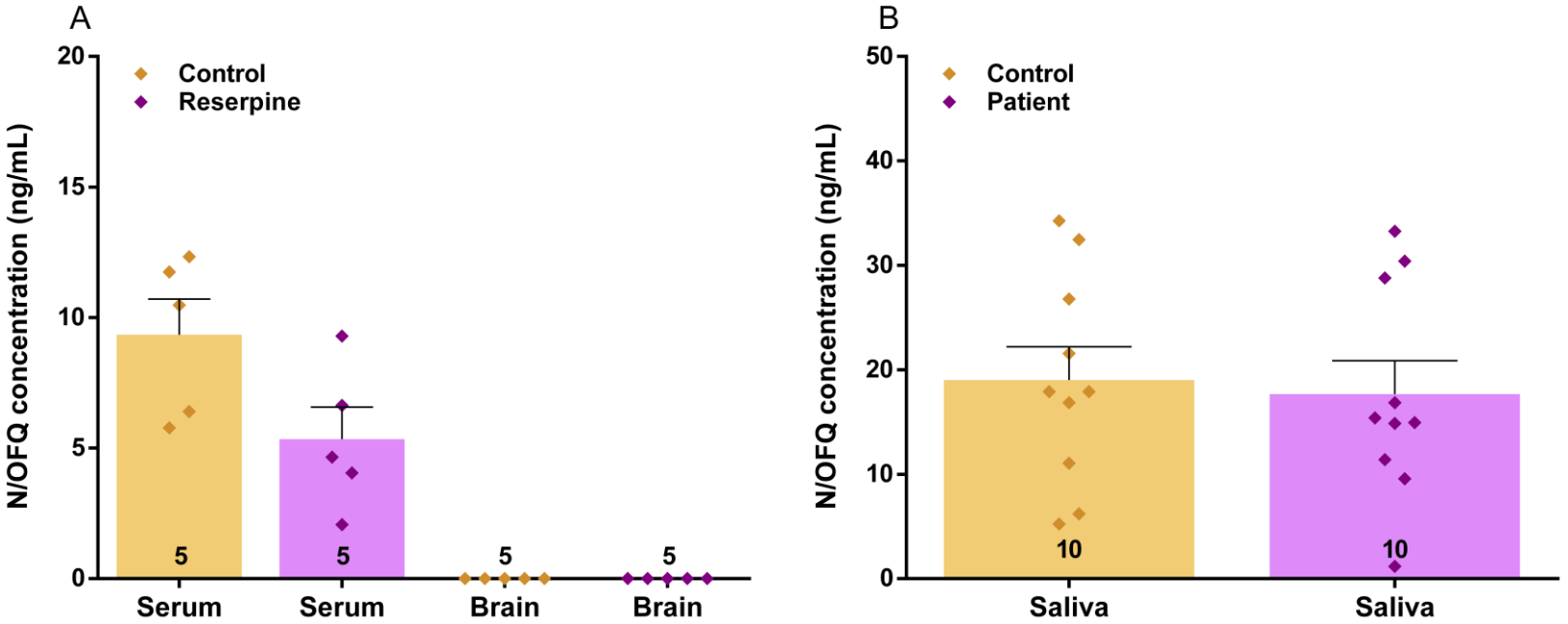
Supplementary Figure 9: Substance P levels in brain (A), spinal cord (B) and masseter muscle (C) of vehicle- and reserpine-treated mice. UFP-101 (1 nmol/kg, intraperitoneal) was administered once a day, for four consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days), and 30 min before the tissue collection, at the fourth day. Each column represents the mean ± SEM. *$p < 0.05$ when compared to the control vehicle/saline group. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's *post hoc* test (B and C) or by one-way ANOVA (A). (A-B) $n = 6$ mice per group; (C) $n = 5$ mice per group.


A
B
C
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
Glutathione mmol/g protein
0
20
40
60
80
5
5
5
Fourth day

Supplementary Figure 10: Tissue concentrations of glutathione in brain (A), spinal cord (B) and masseter muscle (C) of vehicle- and reserpine-treated mice. UFP-101 (1 nmol/kg, intraperitoneal) was administered once a day, for four consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days), and 30 min before tissue collection, at the fourth day. Each column represents the mean ± SEM. Statistical analysis was performed by one-way ANOVA. (A-C) n = 5 mice per group.


A
LDH activity (U/L)
2400
2000
1600
1200
800
400
0
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
8
8
8
Fourth day
B
LDH activity (U/g protein)
40
35
30
25
20
15
10
5
0
Vehicle
Reserpine
+ UFP-101 (1 nmol/kg)
*
#
8
8
8
Fourth day

Supplementary Figure 11: Lactate dehydrogenase (LDH) activity in serum (A) and mitochondrial extracts of masseter (B) of vehicle- and reserpine-treated mice. UFP-101 (1 nmol/kg, intraperitoneal) was administered once a day, for four consecutive days, 30 min after the onset of reserpine administration (0.25 mg/kg, subcutaneous, once a day for three days), and 30 min before tissue collection, at the fourth day. Each column represents the mean ± SEM. *$p < 0.05$ when compared to the control vehicle/saline group. $^{\#}p < 0.05$ when compared to the reserpine/saline group. Statistical analysis was performed by Kruskal-Wallis (A) or one-way (B) ANOVA followed by Bonferroni's *post hoc* test. (A, B) n = eight mice per group.


A
Control
Reserpine
N/OFQ concentration (ng/mL)
20
15
10
5
0
5
5
5
5
Serum
Serum
Brain
Brain
B
Control
Patient
N/OFQ concentration (ng/mL)
50
40
30
20
10
0
10
10
Saliva
Saliva

Supplementary Figure 12: N/OFQ concentration in serum and brain samples of vehicle- and reserpine-treated mice (A), and in saliva of female control subjects and fibromyalgia patients (B). The levels of endogenous N/OFQ were measured at the fourth day after of the onset of reserpine administration (0.25 mg//kg, subcutaneous, once a day for three days) in mice. Statistical analysis was performed by Student *t* test. (A) *n* = five mice per group; (B) *n* = 10 subjects per group.

5 CONSIDERAÇÕES FINAIS

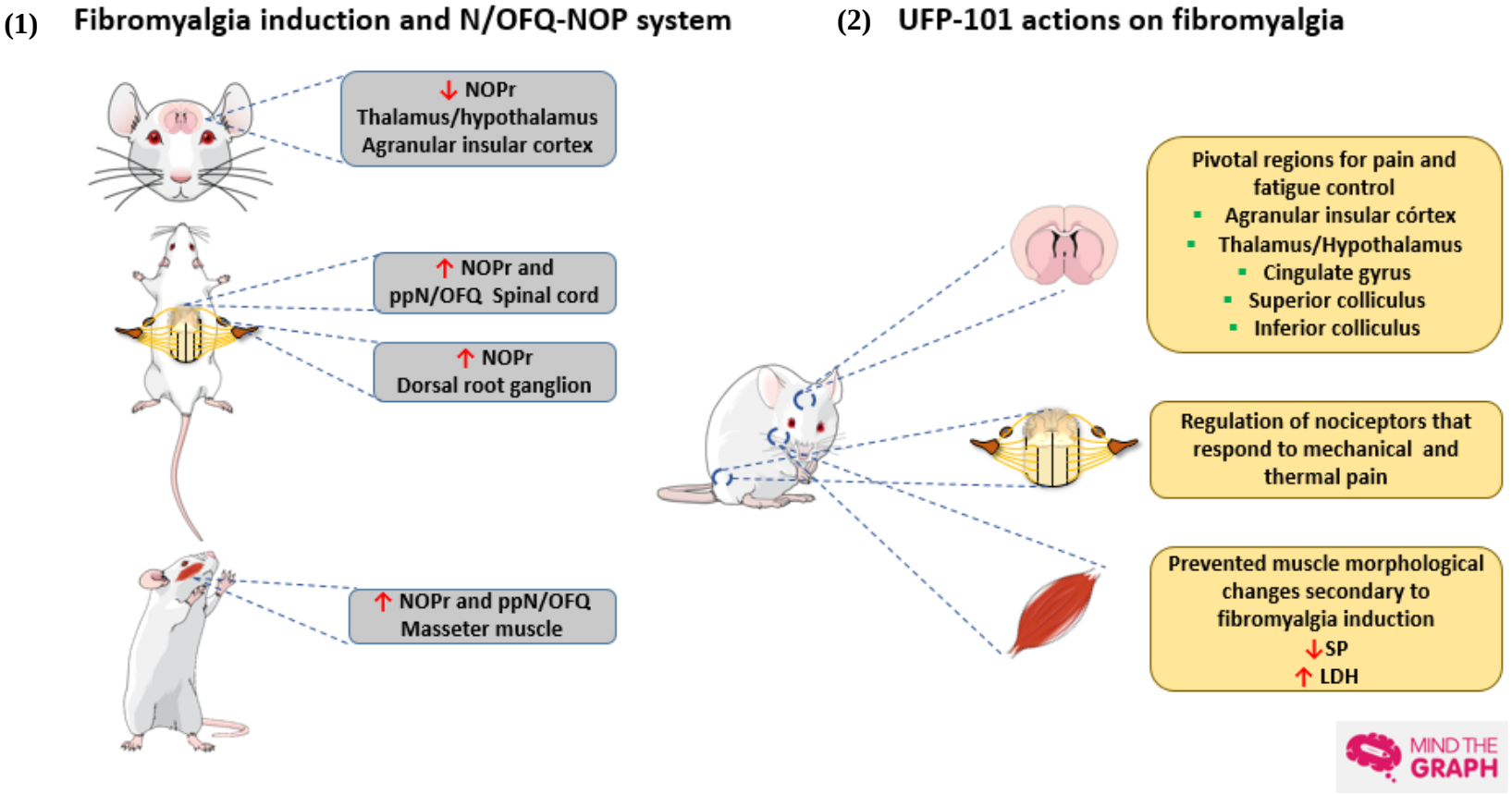
A fibromialgia é uma doença crônica que afeta mais de 2% da população ao redor do mundo. Além disto, as dores musculoesqueléticas crônicas estão no ranking das 10 principais enfermidades globais. É importante ressaltar que a dor crônica gera grandes custos e impacta negativamente na qualidade de vida do paciente, pois é acompanhada por depressão, ansiedade, problemas cognitivos e no sono, além de fadiga e perda de função física. Todas estas co-morbidades associadas, resultam na perda de produtividade e diminuição na habilidade de manter as atividades do dia-dia. Além disto, os fármacos existentes para o tratamento da fibromialgia geram em seus pacientes efeitos adversos e, muitas vezes, perda de eficácia ou tolerabilidade.

Diante deste cenário, a fibromialgia é uma doença que necessita de novos estudos para desvendar os mecanismos da sua patofisiologia e para a descoberta de possíveis tratamentos. O presente trabalho demonstrou a relação entre o sistema N/OFQ-NOPr e o modelo de fibromialgia induzido por reserpina. A modulação da expressão do RNAm da ppN/OFQ e do NOPr, além do padrão de ativação cerebral induzidos pela reserpina ocorreram em regiões importantes para o controle da dor e da fadiga (córtex insular agranular, tálamo/hipotálamo, giro do cingulado, colículos superior e inferior esquerdo e direito). Estas regiões advêm como possíveis pontos chaves para os efeitos anti-alodínico, anti-hipernociceptivo térmico e anti-fadiga encontrados para o antagonista UFP-101, na dose de 1 nmol pela via intraperitoneal. Estes achados visam caracterizar o sistema N/OFQ-NOPr nesta condição patológica. Como a causa da doença ainda é pouco estabelecida, é de extrema importância elucidar os circuitos centrais e periféricos envolvidos nos efeitos do antagonista UFP-101 do NOPr.

O efeito encontrado para o antagonista UFP-101 pode ser devido à sua ação sobre os nociceptores que respondem à dor térmica e mecânica que chegam até a medula espinhal,

regulando, por consequência, as vias ascendentes e descendentes da dor. A partir de sua ação periférica, sobre os músculos esqueléticos, este potencial pode alcançar regiões centrais, que respondem aos estímulos periféricos diminuindo a dor e a fadiga. A melhora proporcionada por UFP-101 nos sintomas de fadiga pode ser em decorrência de uma ação indireta da diminuição da dor ao nível central, levando a maior atividade motora. O antagonista UFP-101 pode estar atuando perifericamente sobre mediadores inflamatórios, neurotransmissores e/ou neuropeptídios, primordiais para a patofisiologia da fibromialgia induzida por reserpina, agindo sobre os seus receptores NOP no músculo. Consequentemente, o antagonista pode, indiretamente, modular os disparos nociceptivos a nível medular, inibindo a via ascendente da dor ou ativando a via descendente inibitória da dor.

Também é válido ressaltar que a prevenção dos sintomas de fadiga pode ser explicado por uma ação direta sobre os NOPr nas fibras musculares, modulando parâmetros bioquímicos, como a LDH e a SP. De maneira interessante, os efeitos anti-fadiga de UFP-101 se sobrepuseram aos efeitos do fármaco pregabalina, amplamente utilizado para o tratamento da fibromialgia. Somando-se a isto, estão os estudos que demonstram a eficácia de ligantes do NOPr em ensaios clínicos para o tratamento da dor neuropática e crônica. Diante deste contexto, onde pela primeira vez descrevemos o efeito de um antagonista peptídico do NOPr para o tratamento de sintomas de dor e fadiga na fibromialgia experimental, se torna claro que este ligante é promissor para futuros ensaios clínicos. Abaixo é demonstrada uma representação esquemática para os principais achados deste estudo.



Representação esquemática da relação entre o modelo da fibromialgia induzido por reserpina em camundongos e o sistema N/OFQ-NOPr e os efeitos do tratamento sistêmico com o antagonista peptídico UFP-101. (1) A injeção subcutânea (s.c.) com reserpina (0.25 mg/kg, por três dias consecutivos) modula a expressão do RNAm da ppN/OFQ e do NOPr ao nível central e periférico. (2) O tratamento repetido intraperitoneal (i.p.) com UFP-101 (1 nmol/kg) produz efeito anti-alodínico, previne a hipernocicepção térmica e reduz a fadiga em camundongos tratados com reserpina. Estes efeitos podem ser em decorrência de sua ação (direta e/ou indireta) em regiões centrais e periféricas fundamentais para o controle da dor e da fadiga. SP = substância P; LDH = lactato desidrogenase.

6 PERSPECTIVAS

- Investigar os efeitos do tratamento repetido com o antagonista seletivo do NOPr, UFP-101, sobre a anedonia, no modelo de fibromialgia induzido por reserpina em camundongos, através do teste de ingestão de sacarose;
- Investigar os efeitos do tratamento repetido com o antagonista seletivo do NOPr, UFP-101, sobre parâmetros de ansiedade, no modelo de fibromialgia induzido por reserpina em camundongos, utilizando outros paradigmas experimentais;
- Investigar os efeitos do tratamento repetido com o antagonista seletivo do NOPr, UFP-101, sobre a memória, no modelo de fibromialgia induzido por reserpina em camundongos, no teste de memória espacial;
- Avaliar o peptídeo relacionado ao gene da calcitonina (CGRP) em amostras de cérebro, medula e músculo, após o tratamento repetido com o antagonista seletivo do NOPr, UFP-101;
- Avaliar os níveis cerebrais, espinhais e musculares de dopamina e noradrenalina, após o tratamento repetido com o antagonista seletivo do NOPr, UFP-101;
- Avaliar e diferenciar os tipos de fibras musculares no masseter de camundongos submetidos ao modelo de fibromialgia induzido por reserpina e tratados com o antagonista seletivo do NOPr, UFP-101;
- Avaliar a expressão de MIF, MuRF e Fbxo32 no masseter de camundongos submetidos ao modelo de fibromialgia induzido por reserpina e tratados com o antagonista seletivo do NOPr, UFP-101;
- Quantificar indicadores de estresse oxidativo (catalase e superóxido dismutase) no masseter de camundongos tratados com reserpina e com o antagonista seletivo do NOPr, UFP-101;

- Quantificar os níveis de IL-8, centralmente e perifericamente, em camundongos tratados com reserpina e com o antagonista seletivo do NOPr UFP-101;
- Quantificar os níveis de Ca^{+} em cultivo *in vitro* de neurônios, para elucidar o possível mecanismo de ação do antagonista seletivo do NOPr, UFP-101.

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ANEXO A – Aprovação da CEUA

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 98/2015 - CEUA

Porto Alegre, 03 de dezembro de 2015.

Prezado Sr(a). Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 15/00487 intitulado **"Caracterização do sistema nociceptina/orfanina FG-receptor NOP na modulação da fibromialgia experimental".**

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

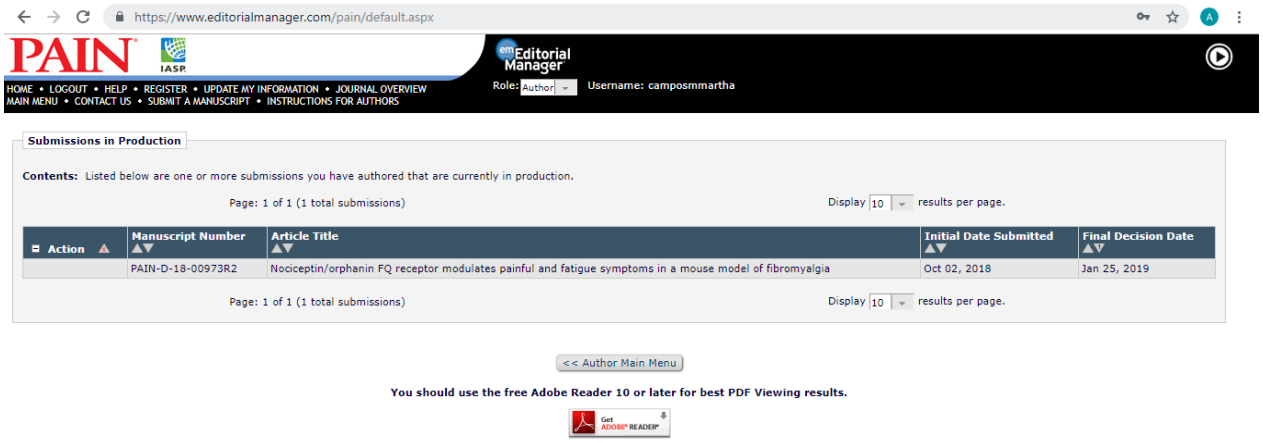
Nº de Animais	Espécie	Duração do Projeto
548	Mus musculus	03/2015 – 03/2019

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilma. Sra.
Profa. Dra. Maria Martha Campos
FABIO

ANEXO B – Aceite do manuscrito – artigo em produção



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Page: 1 of 1 (1 total submissions) Display 10 results per page.

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	PAIN-D-18-00973R2	Nociceptin/orphanin FQ receptor modulates painful and fatigue symptoms in a mouse model of fibromyalgia	Oct 02, 2018	Jan 25, 2019

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Journal: PAIN
Title: Nociceptin/orphanin FQ receptor modulates painful and fatigue symptoms in a mouse model of fibromyalgia
ID: PAIN-D-18-00973R2
Format: Research Paper
Authors: Ana Paula Aquistapase Dagnino, Master; Rodrigo Braccini Madeira da Silva, PhD; Pedro Cesar Chagastelles, PhD; Talita Carneiro Brandão Pereira, PhD; Gianina Venturin Teribele, PhD; Samuel Greggio, PhD; Jaderson Costa da Costa, PhD; Maurício Reis Bogo, PhD; Maria Martha Campos, Ph.D.

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