D 2016

U.PORTO
FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

Activating transcriptional factor 3 in joint inflammatory pain

Exploring mechanisms at the sensory ganglia

DIANA SOFIA MARQUES NASCIMENTO

TESE DE DOUTORAMENTO EM NEUROCIÊNCIAS

FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO

Dissertação apresentada à Faculdade de Medicina da Universidade do Porto para candidatura ao grau de Doutor no âmbito do Programa Doutoral em Neurociências.

A candidata realizou o presente trabalho com o apoio de uma bolsa de doutoramento de quatro anos, concedida pela Fundação para a Ciência e Tecnologia (FCT; SFRH/BD/79497/2011).

Supervisor/Orientador: Prof. Doutora Fani Lourença Moreira Neto (U. Porto, Portugal)

Co-Supervisor/Co-orientador: Prof. Doutora Marzia Malcangio (King's College, London)

Evaluation Committee/ Constituição do Juri

President/ Presidente: Reitor Universidade do Porto

Members/Vogais

Doutora Stefania Maria Ceruti, professora associada da Universidade de Milão

Doutor Carlos Manuel Gomes Reguenga, professor auxiliar da Faculdade de Medicina da Universidade do Porto

Doutora Fani Lourença Moreira Neto, professora auxiliar da Faculdade de Medicina Universidade do Porto e orientadora da tese

Doutora Filipa Santos Costa Pinto Ribeiro de Lacerda, professora auxiliar da Escola de Ciências da Saúde da Universidade do Minho

Doutora Iva Humberta Oliveira Brito; professora auxiliar convidada da Faculdade de Medicina da Universidade do Porto

Doutora Ana Paula Gomes Moreira Pêgo; investigadora prinicipal do Instituto de Engenharia Biomédica da Universidade do Porto

Artigo 48º, parágrafo 3: “A Faculdade não responde pelas doutrinas expendidas na dissertação”

Regulamento da Faculdade de Medicina da Universidade do Porto

Decreto-Lei nº 19337 de 29 de Janeiro de 1931

Corpo Catedrático da Faculdade de Medicina do Porto da Universidade do Porto

Professores Efetivos

Alberto Manuel Barros da Silva
Altamiro Manuel Rodrigues Costa Pereira
António Albino Coelho Marques Abrantes Teixeira
Deolinda Maria Valente Alves Lima Teixeira
Francisco Fernando Rocha Gonçalves
Isabel Maria Amorim Pereira Ramos
João Francisco Montenegro Andrade Lima Bernardes
Joaquim Adelino Correia Ferreira Leite Moreira
José Agostinho Marques Lopes
José Carlos Neves da Cunha Areias
José Eduardo Torres Eckenroth Guimarães
José Henrique Dias Pinto de Barros
José Manuel Lopes Teixeira Amarante
José Manuel Pereira Dias de Castro Lopes
Manuel Alberto Coimbra Sobrinho Simões
Manuel Jesus Falcão Pestana Vasconcelos
Maria Amélia Duarte Ferreira
Maria Dulce Cordeiro Madeira
Maria Fátima Machado Henriques Carneiro
Maria Leonor Martins Soares David
Patrício Manuel Vieira Araújo Soares Silva
Raquel Ângela Silva Soares Lino
Rui Manuel Almeida Mota Cardoso
Rui Manuel Marques

Professores Jubilados/Aposentados

Abel Vitorino Trigo Cabral
Alexandre Alberto Guerra Sousa Pinto
Álvaro Jerónimo Leal Machado de Aguiar
António Augusto Lopes Vaz
Antonio Carlos de Freitas Ribeiro Saraiva
António Carvalho Almeida Coimbra
António Fernandes Oliveira Barbosa Ribeiro Braga
António José Pacheco Palha
António Manuel Sampaio de Araújo Teixeira
Belmiro dos Santos Patrício
Cândido Alves Hipólito Reis
Carlos Rodrigo Magalhães Ramalhão
Cassiano Pena de Abreu e Lima
Daniel Filipe de Lima Moura
Daniel Santos Pinto Serrão
Eduardo Jorge Cunha Rodrigues Pereira
Fernando Tavarela Veloso
Henrique José Ferreira Gonçalves Lecour de Menezes
Jorge Manuel Mergulhão Castro Tavares
José Carvalho de Oliveira
José Fernando Barros Castro Correia
José Luís Medina Vieira
José Manuel Costa Mesquita Guimarães
Levi Eugénio Ribeiro Guerra
Luís Alberto Martins Gomes de Almeida
Manuel António Caldeira Pais Clemente
Manuel Augusto Cardoso de Oliveira
Manuel Machado Rodrigues Gomes
Manuel Maria Paulo Barbosa
Maria da Conceição Fernandes Marques Magalhães
Maria Isabel Amorim de Azevedo
Mário José Cerqueira Gomes Braga
Serafim Correia Pinto Guimarães
Valdemar Miguel Botelho dos Santos Cardoso
Walter Friedrich Alfred Osswald

À Professora Doutora Fani Neto

À Professora Doutora Marzia Malcangio

Aos meus pais, Amândio e Conceição

Ao meu irmão Miguel

Em obediência ao disposto no Decreto-Lei 388/70, Artigo 8º, parágrafo 2, declaro que efetuei o planeamento e execução do trabalho experimental, observação do material e análise dos resultados e redigi as publicações que fazem parte integrante desta dissertação.

Publication I. Diana Nascimento, Daniel Pozza, José Manuel Castro-Lopes, Fani Moreira Neto. Neuronal injury marker ATF-3 is induced in primary afferent neurons of monoarthritic rats. NeuroSignals. 2011;19(4):210-21. doi: 10.1159/000330195

Publication II. Diana Nascimento, José Manuel Castro-Lopes, Fani Moreira Neto. Satellite glial cells surrounding primary afferent neurons are activated and proliferate during monoarthritis in rats: is there a role for ATF3? PLoS One. 2014 Sep; 23;9(9):e108152. doi: 10.1371/journal.pone.0108152.

Publication III. The expression of P2X7 and P2X3 receptors is altered in sensory ganglia of monoarthritic rats (submitted)

Publication IV. HSP90 inhibition alleviates pain in monoarthritic rats and alters the expression of relevant pain molecules at the DRG (in preparation for submission)

A reprodução destas publicações e a utilização de imagens foi feita com autorização das respetivas editoras.

Table of Contents

Agradecimentos

Há coisas na vida que parecem realmente escritas, predestinadas, e o meu percurso académico é um desses exemplos. Lembro-me perfeitamente da altura em que descobri o gosto pela Química. Foi quando conheci a Doutora Isilda que o "bichinho" começou a crescer e quando entrei na Faculdade já sabia que queria ser investigadora. Não sabia qual seria o meu caminho e muito menos poderia imaginar que aquilo que faço hoje já pouco tem a ver com a Química pela qual me apaixonei no início deste processo. No entanto, não posso deixar de lhe agradecer o facto de me ter orientado, de ter visto potencial e por ter colocado esta semente em mim, que no fim de contas foi o princípio de tudo. Ainda durante a licenciatura em Bioquímica encontrei o antigo departamento de Histologia e Embriologia da Faculdade de Medicina. Sabia que queria aplicar os meus conhecimentos à área da saúde e as neurociências atraíram-me desde muito cedo. Foi neste laboratório que conheci a minha orientadora e amiga Fani Neto, e muitas outras pessoas que são hoje parte integrante da minha vida. À Professora Doutora Fani Neto, agradeço a orientação desde há 8 anos (licenciatura, mestrado e doutoramento), a sua amizade e dedicação. Ao Professor Doutor José Manuel Castro-Lopes por me ter aceitado no seu grupo de investigação e, como diretor do atual Departamento de Biologia Experimental, por todo o seu apoio. À Professora Doutora Deolinda Lima, como diretora do Programa Doutoral em Neurociências (PDN) mas também pela profissional de garra e inspiração que é para as jovens mulheres na ciência. A todos os outros colegas do laboratório, sem exceção, um muito obrigada por toda a ajuda e apoio. É claro que este percurso não teria sido o mesmo sem os amigos que fiz. São tantos e tão bons que posso até afirmar serem a melhor herança desta fase da minha vida. Gisela Borges, arquirrival mais doce do mundo, Ana Coelho, Isabel Regadas, Diana

Sousa, Maria Ângela, Raquel Oliveira, Lígia Almeida, Mariana Matos, Margarida Oliveira, Zé Pedro, trago-vos para sempre, no coração. Sara Adães, Joana Gomes, Catarina Potes, coleguinhas de grupo, o meu muito obrigado. Um especial agradecimento também ao Carlos Reguenga pela ajuda, paciência e discussões científicas, assim como ao LAIMM (Miguel, António e todos os restantes membros) não só pela ajuda mas pela simpatia. Aos amigos da faculdade que compreendem melhor do que ninguém a dureza deste trajeto, em especial à Tichinha uma amiga para a vida.

I will be forever grateful for the wonderful nine months spent in London, at the King's College. It was more than a professional experience; it was "the time of my life". Thank you so much for receiving me in such a kind way. Dr. Marzia Malcangio thank you so much for your guidance, for the scientific discussions and for receiving me. Rie Rikke, I will never forget the nicest danish girl who taught me everything in the lab; thanks for your friendship and warm smile during my stay at the rainy London. To all Marzia's group, for the hard work, for being so helpful, for the pints and the pubs, my thanks

Hoje vejo-me praticamente doutorada, olho para trás e sinto que não podia ter mais orgulho do percurso que fiz. Apesar de todas as intempéries, de todos os obstáculos, desafios e muitas vezes desalento que são inerentes a uma tese de doutoramento, digo com toda a certeza que adoro aquilo que faço e vou continuar a tentar conquistar o meu lugar.

Por terem estado sempre do meu lado, não posso deixar de agradecer aos meus pais e ao meu irmão. Passamos por momentos difíceis mas cá estamos sempre com força para continuar, persistir. Mesmo nos piores momentos não baixaram os braços e nunca, em condição alguma, deixaram de me apoiar nesta luta. O meu irmão, apesar de bastante mais novo, consegue ser uma inspiração e um motor na minha vida. Tenho um orgulho danado na pessoa que é e não tenho qualquer dúvida do sucesso que a vida lhe reserva.

Espero poder estar sempre por perto. Não posso deixar de agradecer também à minha madrinha por nunca se ter ausentado, por ter sempre apoiado os meus sonhos. Aos meus avós, porque o colo deles é igual a mais nenhum, e porque aquele café, avó Rosa, cura qualquer mal que carregue no coração. Sou grata por vos ter cá a todos e por poder ver a vossa alegria na conquista de mais esta vitória. Gu e Dulce, tenho o vosso percurso como um exemplo. Obrigada pelo apoio constante e pelo conforto que sempre encontrei nas vossas palavras. A toda a minha família um muito obrigada. Aos meus amigos da Póvoa, que embora não sendo da área, são sempre um porto de abrigo (especialmente à Nini, ao Zé, à Postiga ☺ vocês sabem).

Por fim, ao André, companheiro de toda esta etapa. Umas vezes mais perto, outras mais longe, mas sempre no meu coração. Incrivelmente, a pessoa que mais acredita em mim e me encoraja. Se não é amor, não sei o que será.

"Quem deseja ver o arco-íris, precisa aprender a gostar da chuva"

Paulo Coelho *in* "O Aleph"

Abbreviations

17-DMAG	17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin
ATF3	activating transcriptional factor 3
ATP	adenosine triphosphate
BrdU	bromodeoxyuridine
CaV	voltage-dependent calcium channel
CCI	chronic constriction injury
CFA	complete freund's adjuvant
CGRP	calcitonin gene-related protein
CNS	central nervous system
COX	cyclooxygenase
CREB	camp responsive element binding
DAMPs	damage-associated molecular patterns
DMARDs	disease-modifying antirheumatic drugs
DRG	dorsal root ganglion/ganglia
ERK	extracellular signal-regulated kinase
FC	fluorocitrate
FRAP	fluoride-resistant acid phosphatase
GAP43	growth-associated protein 43
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GPCRs	metabotropic g-protein coupled receptor
GS	glutamine synthase
HSF1	heat shock factor 1
HSP	heat shock protein
HTM	high-threshold mechanical (nociceptors)
IASP	international association for the study of pain
IB4	isolectine B4
IHC	immunohistochemistry
IL	interleukin
IR	immunoreactivity
JNK	c-jun N-terminal kinase
LPS	lypopolyssacharyde

MA	monoarthritis
MAPK	mitogen-activated protein kinase
MIA	mono-iodoacetate
MIAs	mechano-insensitive afferents
mRNA	messenger RNA
NaV	voltage-dependent sodium channel
NF-200	neurofilament 200
NF-κB	nuclear factor κappa b
NGF	nerve growth factor
NO	nitric oxide
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
P2XR	purinergic ligand gated ion channel receptors
P2YR	purinergic G protein-coupled receptors
PAMPs	pathogen-associated molecular patterns
PGE2	prostaglandin E2
PNS	peripheral nervous system
RT-qPCR	real-time quantitative polymerase chain reaction
RA	rheumatoid arthritis
ROS	reactive oxygen species
RTX	resiniferatoxin
SNI	spared nerve injury
SNL	spinal nerve ligation
SOM	somatostatin
SP	substance p
STAT3	signal transducer and activator of transcription 3
TG	trigeminal ganglia
TLR	toll-like receptor
TNF	tumor necrosis factor
TrkA	tyrosine-kinase receptor a
TRPV1	transient receptor potential vanilloid 1
WB	western blot

1. Abstract/Resumo

English Version

Pain arising from joint inflammatory conditions is an incapacitating, serious clinical problem affecting millions of people worldwide and representing a huge economic burden for the governmental entities. Mostly due to the lack of more knowledge concerning the underlying neurobiological mechanisms, diagnoses are still poor and undifferentiated while the current treatments are often ineffective. In this context, chronic animal models exhibiting a full spectrum of pathological changes comparable to those found in humans, are very relevant tools.

In these studies, by using the monoarthritis (MA) model, induced by complete Freud's adjuvant (CFA) injection in the tibiotarsal joint, we explored several molecular and cellular mechanisms at the dorsal root ganglia (DRG). Indeed, the DRG are important "pain structures", containing the cell bodies of nociceptors, where the information arising from the periphery is firstly processed. Thus, in **Publication I,** we show that the neuronal injury marker activating transcriptional factor 3 (ATF3) is induced in DRG of MA rats particularly at day 4 of disease evolution. This evidence suggests the activation of "neuronal damage programs" during this inflammatory condition. Moreover, we demonstrate that ATF3 is majorly expressed in peptidergic neurons, putatively C-fiber nociceptors already shown to be relevant in persistent pain processing mechanisms. Therefore, data made us hypothesize about a role for ATF3 in pain processing

Indeed, some authors had previously suggested that injury markers (like ATF3) could be the triggers of signaling cascades involved in neuron-glia communication. Activation of glial cells and their interaction with neurons (in bidirectional crosstalk) have been greatly associated with the development of pain states. In **Publication II**, we show that satellite glial cells (SGCs) surrounding primary afferents, are activated and proliferate

after 1 week of MA. Moreover, we also demonstrate that the activation of SGCs occurs preferentially around ATF3-expressing neurons, which suggested a possible association of these two events (and again a role of ATF3 in pain processing).

Activation of SGCs is mostly attributed to the stimulation of the purinergic receptor P2X7 (expressed only in SGCs) and indeed, in **Publication III**, we demonstrate an up-regulation of this receptor around 7d of MA, corresponding to the temporal profile of SGCs activation. Down-regulation of P2X3R (expressed only in neurons) was also observed after this timepoint. These data suggested that a negative feedback control of P2X7R over P2X3R expression, previously reported by other authors, was activated during MA; possibly to regulate excessive damage. Moreover, these results presuppose a crosstalk between neurons and SGCs within the sensory ganglia.

Data pointed to a role of ATF3 in the MA pathophysiology, possibly associated with pain mechanisms. Thus, in order to find novel targets under ATF3 regulation and better dissect its signaling pathways, we then suppressed ATF3 expression in DRG cell cultures. Interestingly, we detected a significant decrease in the mRNA levels of the heat shock protein 90 (HSP90), another stress inducible gene implicated in the inflammatory response **(Publication III)**. Indeed, in the DRG of inflamed animals, we then found increased levels of HSP90, indicating a role for this chaperone in MA pathophysiological mechanisms **(Publication IV)**. In this study, we also demonstrated that HSP90 is massively cleaved during MA and we propose this might be a relevant event in the pathophysiology of this disease.

Interestingly, besides reducing the inflammatory response, HSP90 inhibition had been shown to alleviate pain. In order to better evaluate the role of HSP90 in MA, we then intrathecally administered 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG, an HSP90 inhibitor) to inflamed animals. Thus, in **Publication IV**, we demonstrate that 17-DMAG attenuated MA-induced allodynia which was accompanied by a reversion in HSP90 up-regulation and cleavage. Also, the expression of P2X3R and GFAP

(typically augmented in MA) significantly decreased following HSP90 inhibition, while ATF3 expression was even more exacerbated. Thus, the observed antinociceptive effect induced by HSP90 inhibition is likely to result from the attenuation of neuronal sensitization (P2X3R) and glial activation (GFAP), as well as of a possible protective role of ATF3. Moreover, 17-DMAG seemed to effectively protect HSP90 from cleavage. We suggest that the reduced cleavage of the protein might somehow correlate with the molecular changes observed, although HSP90 is still not functional as a chaperone after 17-DMAG treatment. Indeed, this event should be further investigated as it might also dictate the efficacy of HSP90 blockers that seem to be promising drugs for pain management.

Altogether, we believe our studies contributed to the better understanding of MA pathophysiology. Hopefully, by showing the activation of "neuronal damage programs" in this inflammatory condition, we sustained a new mechanistic perception that considers the convergence of neuropathic and inflammatory events overtime. Better knowing these mechanisms is crucial for the development of more efficient treatments. In this context, ATF3 might be one important key molecule in many of the underlying signaling pathways. Our studies also support that SGCs are critical players in pain conditions and thus, considering only neuronal activity no longer provides a complete understanding of these events. Finally, we unveiled novel molecules and signaling cascades (e.g. HSP90) that can be targeted not only to ameliorate the inflammatory response but also to control pain associated with joint inflammation

.

Portuguese Version

A dor associada a inflamações articulares, muitas vezes incapacitante, é uma condição clínica grave que afeta milhões de pessoas em todo o mundo e que representa um enorme encargo económico para as entidades governamentais. Os diagnósticos são ainda pouco completos e indiferenciados e os tratamentos muitas vezes ineficazes. Isto deve-se, em grande parte, ao considerável desconhecimento dos mecanismos neurobiológicos associados a estas doenças. Neste contexto, os modelos animais crónicos que exibem muitas das alterações patológicas observadas no humano constituem ferramentas muita valiosas.

Neste trabalho, usámos como modelo animal a Monoartrite (MA) induzida por injeção de adjuvante completo de Freund's (ACF) na articulação tibiotársica, para estudarmos vários mecanismos moleculares e celulares que ocorrem nos gânglios raquidianos. De fato, estes gânglios são importantes estrututras envolvidas no processamento da dor pois contêm os corpos celulares dos nociceptores. É aqui que a informação que vem da periferia é primeiramente processada. Assim, na **Publicação I**, demonstrámos que a expressão do fator de ativação de transcrição 3 (ATF3), um marcador de lesão neuronal, é induzida nos DRG de ratos com MA, mais significativamente aos 4 dias de doença. Estes resultados sugerem que durante esta condição inflamatória ocorre ativação de "programas de dano neuronal". Demonstrámos também que o ATF3 é maioritariamente expresso em neurónios peptidérgicos, presumidamente em nociceptores com fibras C, cuja ativação se mostrou relevante na dor persistente. Assim sendo, hipotetizámos que o ATF3 pudesse ter um papel nos mecanismos de processamento de dor.

De fato, alguns autores já tinham sugerido que seria a expressão de fatores de lesão (como o ATF3) que levaria à ativação de cascatas de sinalização envolvidas na comunicação neurónio-glia. A ativação das células da glia e a sua interação com neurónios

(numa comunicação bidirecional) são mecanismos fundamentais ao desenvolvimento de estados de dor. Tendo estes dados em consideração, na **Publicação II**, mostrámos que as células gliais satélite (SGCs) que circundam os corpos celulares dos aferentes primários são ativadas e proliferam, especialmente 1 semana após indução da MA. Para além disso, demonstrámos que a ativação destas células ocorre preferencialmente em redor de neurónios que expressam ATF3 o que sugere uma possível associação destes dois eventos (e mais uma vez que o ATF3 poderá ter um papel no processamento da dor).

A ativação das SGCs é em grande parte atribuída à estimulação dos recetores purinérgicos P2X7 (expressos unicamente nas SGCs). De acordo, na **Publicação III**, demonstrámos a sobre-expressão deste recetor, especialmente a partir do dia 7 de MA, o que é coincidente com o pico da ativação das SGCs. Também observámos a sob-expressão do recetor P2X3 (expresso unicamente nos neurónios) a partir deste mesmo tempo. Estes resultados sugerem que a regulação negativa do P2X7R sobre a expressão de P2X3R, descrita previamente por outros autores, é ativada durante a MA, possivelmente de forma a controlar danos excessivos. Estes dados pressupõem também que durante a MA são ativados mecanismos de comunicação neurónio-glia nos gânglios sensitivos.

Estes estudos apontam assim para um papel do ATF3 na patofisiologia da MA, possivelmente associado a mecanismos de dor. De forma a identificar novos alvos sob a regulação do ATF3, de seguida silenciámos a expressão deste gene em culturas primárias de DRG. Surpreendentemente, detetámos uma diminuição significativa nos níveis do ARNm da proteína de choque térmico 90 (HSP90), um gene também extremamente induzido pelo *stress* e envolvido na resposta inflamatória **(Publicação III)**.

Posteriormente confirmámos que a expressão de HSP90 está significativamente aumentada em DRG de animais inflamados o que indica um possível envolvimento desta proteína nos mecanismos da MA **(Publicação IV)**. Neste estudo também mostrámos que a HSP90 é altamente clivada, o que nos parece ser um fenómeno relevante na patofisiologia desta condição inflamatória.

De facto, para além dos seus conhecidos efeitos na redução da resposta inflamatória, o uso de inibidores da HSP90 revelou-se recentemente eficaz no alívio de dor. Assim sendo e por forma a melhor compreendermos o papel da HSP90 na MA, inibimos esta proteína por administração intratecal de 17-DMAG (17-(Dimetilaminoetilamino)-17-demetoxygeldanamicina, um inibidor de HSP90) a animais inflamados. Na **Publicação IV**, demonstrámos que o inibidor consegue atenuar a alodínia inerente à condição monoartrítica e que tanto a sobreexpressão de HSP90 como a sua clivagem são revertidas. Também a expressão de P2X3R e GFAP (tipicamente aumentadas na MA) diminuíu significativamente após a inibição da HSP90, enquanto que a expressão de ATF3 aumentou ainda mais. Desta forma, é provável que o efeito anti-nociceptivo da droga resulte de uma atenuação da sensitização neuronal (P2X3R) e da activação de células da glia (GFAP), assim como de um possivel papel protector do ATF3. Para além disso, o 17-DMAG parece evitar a clivagem do HSP90. Mediante estes resultados, sugerimos que a menor clivagem da proteina possa de alguma forma correlacionar-se com os efeitos moleculares observados, muito embora a HSP90 não restitua as suas funcionalidades como chaperone após o tratamento com 17-DMAG. Assim, é de extrema relevância investigar e melhor perceber este mecanismo de clivagem já que este pode inclusivamente limitar a eficácia dos inibidores da HSP90, cujo potential no controlo da dor parece ser inegável.

Assim sendo, acreditamos que os nossos estudos contribuíram para uma melhor compreensão dos mecanismos patofisiológicos da MA. Esperamos que, ao mostrar a ativação de "programas de dano neuronal" numa condição inflamatória, tenhamos contribuído para fortalecer a recente teoria de convergência de mecanismos neuropáticos e inflamatórios ao longo da progressão da doença. Conhecer estes mecanismos é então crucial para que se desenvolvam tratamentos mais eficazes. Neste contexto, o ATF3 parece ser uma molécula chave estando envolvida em muitas das vias de sinalização ativadas nestas condições. Os nossos estudos mostram também que as SGCs são intervenientes cruciais em condições de dor, e portanto, considerar apenas a atividade neuronal já não é

suficiente para que se possam compreender integralmente estes fenómenos. Por fim, acreditamos ter desvendado algumas novas moléculas e cascatas de sinalização (como por exemplo o HSP90) que podem ser alvos terapêuticos relevantes não só na atenuação da resposta inflamatória, mas também no combate à dor inerente à inflamação articular

Introduction

2. Introduction

2.1 Pain as a disease

Pain is postulated by the International Association for the Study of Pain (IASP) as "*an unpleasant sensation and an emotional experience associated with a real or a potential tissue damage or described in terms of such damage".* It is a physiological protective mechanism that acts as a warning signal to any kind of threat to the body integrity. However, it can become a pathological condition when it persists without biological significance. In these cases, there is a chronification of the underlying mechanisms turning pain into a serious clinical problem. Therefore, and contrarily to acute pain that is characterized as a short duration, phasic and intense physiological event, chronic pain is a long-lasting, tonic, persistent pathological event characterized by its spontaneous nature and lack of evident biological reason (Tracey, I and Bushnell, MC 2009).

It is highly relevant to further elucidate pain processing mechanisms since millions of people continue suffering due to lack of more efficient treatments and knowledge in this field. In fact, chronic pain is highly prevalent in developed countries (Breivik, H *et al.* 2006, Azevedo, LF *et al.* 2012, Breivik, H *et al.* 2013) and in Portugal it is estimated that about 37% of the population suffers from this pathological state (Azevedo, LF *et al.* 2012). This condition has serious consequences, such as the patient's incapacity to perform the normal daily tasks, which also affect the family and social environment (Reid, KJ *et al.* 2011, Gorczyca, R *et al.* 2013). Therefore, chronic pain also has an enormous economic impact to nations since it is a burden to the government due to considerable direct (like health-related services) and indirect (like lower productivity of these patients and family) costs (Reid, KJ *et al.* 2011, Breivik, H *et al.* 2013). It has been estimated that chronic pain in the Portuguese population is associated with a total of 2,000 million euros per year in

direct costs which include visits to health care professionals, treatments and medical tests, while the total annual indirect costs were underestimated to be around 2,600 million euros, mostly concerning early retirement, job loss and absenteeism (Azevedo, LF *et al.* 2014). Being such a relevant and serious clinical problem, understanding chronic pain is crucial for the development of better therapeutic approaches which would solve several of the above mentioned issues.

2.2 Physiology of the nociceptors and pain processing

Pain transmission is initiated by the activation of nociceptors, a specialized sub-population of sensory neurons of the peripheral somatosensory nervous system capable of transducing and encoding noxious stimuli (Gold, M and Caterina, M 2008). Sensory neurons or primary afferents have their cell bodies (perikarya or somas) located in the dorsal root ganglia (DRG), or in the trigeminal ganglia in case of innervation from the head. Their axons are T-shaped, bifurcating into a longer branch that extends to the peripheral tissues (skin, muscle and other organs) and another branch extending to the dorsal horn of the spinal cord, where the axonal terminal synapses with the second order neurons. DRG are also constituted by non-neuronal cells, the satellite glial cells (SGCs) that envelop the cell bodies of these primary neurons (Fig. 1). SGCs can also be activated by intense stimuli, having a crucial role in intra-ganglionic communication, as will be later explored (please refer to chapter 2.5 and 2.6).

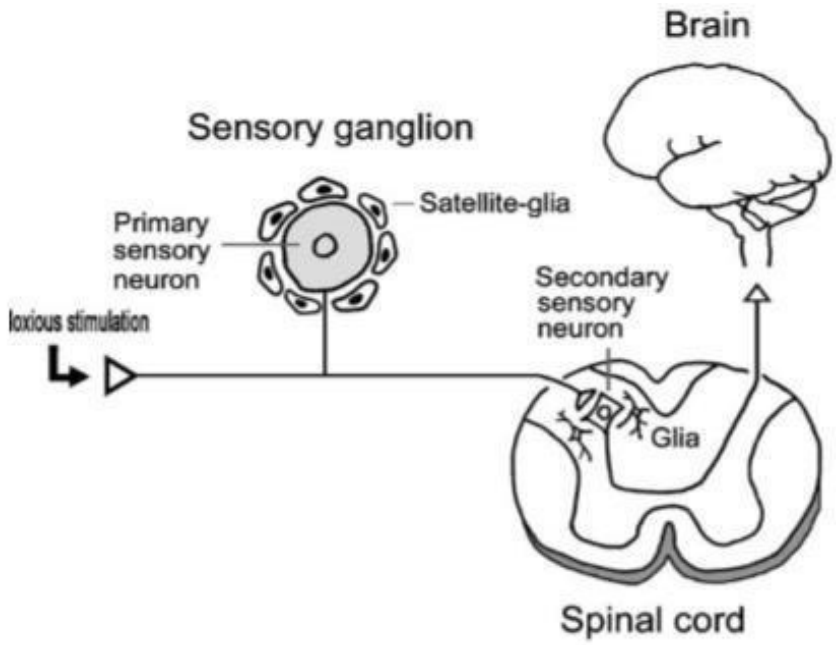


Fig. 1 - Schematic representation of the primary sensory neurons. Their cell bodies are enveloped by satellite glial cells and altogether form the dorsal root ganglia (DRG). One of the branches from these neurons extend to the peripheral tissues and the other connects to a second neuron in the spinal cord, allowing centralization of a stimulus (from (Takeda, M *et al.* 2009).

The term nociceptor distinguishes afferents capable of responding to stimuli that are potentially dangerous to tissue from those that normally only encode innocuous stimuli. Nociceptors convert environmental stimuli into nerve impulses (action potentials) in a process called transduction. During this process, the stimuli induces conformational changes in the structure of proteins located at the nociceptor peripheral terminals, which ultimately leads to the opening/closure of ionic channels resulting in the generation of an action potential (Messlinger, K 1997). These neurons codify not only the type of the stimulus but also its intensity and location. Localization depends on the somatotopic distribution of the central terminals at the dorsal horn of the spinal cord while the intensity will depend on the number and frequency of the action potentials generated. Perception of pain usually results from the sum of several successive action potentials or

the activation of various nociceptors simultaneously, which is known as spatial and temporal summation (Reichling, DB and Levine, JD 1999). Lastly, pain perception is generated if these firings are propagated to the central terminal of the nociceptor leading to successful synapses with the second order spinal cord neurons (Treede, RD 1999).

However, an interesting feature of the nociceptors is that they can also generate outgoing signals towards their peripheral terminals which may alter the peripheral tissues they innervate and contribute to the aggravation and perpetuation of the pathological states (Carlton, SM 2014). Consequently, the terminals of these neurons release a number of mediators produced in their cell bodies that will increase the vascular permeability, thus resulting in edema. Following trauma, immune cells are recruited and triggered to release inflammatory mediators at the injury site leading to the formation of an inflammatory milieu. These released mediators act directly on receptors located at the primary afferents terminals, activating several intracellular signaling cascades. In this process, called neurogenic inflammation, neuronal excitation will alter the sensitivity of these cells to subsequent stimuli (Cervero, F 2008, Basbaum, AI *et al.* 2009), ultimately resulting in phenotypic changes that largely contribute for the development of chronic pain states (Cervero, F 2008, Gold, M and Caterina, M 2008).

2.2.1 Classification of the nociceptors

Nociceptors are known to be anatomically, electrophysiologically and neurochemically heterogeneous, which results in distinct sensitivities to different stimuli. For example, the cutaneous sensory fibers can be categorized according to the diameter and degree of myelination of their axons, and conduction velocity (Table 1). This classification is usually applied to the generality of the fibers reaching other tissues/organs. Briefly, A-beta (Aβ) fibers have the largest axon diameter, are highly myelinated and have higher conduction velocities. A-delta (Aδ) fibers are thinner than Aβ

fibers, are thinly myelinated, and have lower conduction velocities. Finally, C fibers have the smallest axon diameter, are unmyelinated, and have the lowest conduction velocities (Alvarez, FJ and Fyffe, RE 2000, Gold, M and Caterina, M 2008)

Table 1 - Classification of cutaneous sensory fibers

Fiber type	Diameter (μm)	Myelination	Conduction velocity (m/s)	%
Aβ	>10	Thick	30-100	20
Aδ	2-6	Thin	12-30	10
C	0.4-1.2	None	0.5-2	70

Under normal physiological conditions, any of these subtypes may conduct innocuous information, but the majority of nociceptive afferents have C and Aδ fibers. When a nociceptive stimulus is applied to the skin, the Aδ nociceptors are the ones responsible for transmitting well-localized, immediate, acute pain, which is then followed by a more diffuse, poorly localized, slow pain caused by activation of C fibers. Activation of C nociceptors is assumed as a cause for the clinically relevant persistent pain (Baron, R 2000, Kleggetveit, IP *et al.* 2012, Weng, X *et al.* 2012). On the other hand, most Aβ fibers respond to innocuous mechanical stimulation. During tissue inflammation or peripheral nerve lesion, structural, neurochemical and physiological changes may occur in Aβ neurons that will facilitate the transduction and encoding of nociceptive stimuli by these primary afferents (Baron, R 2000).

Nociceptors are also classified taking into account the type of the stimulus they respond to which can be chemical (C), thermal (T), or mechanical (M) (Table 2). Type I Aδ nociceptors, also called high-threshold mechanical nociceptors (HTM), predominantly respond to mechanical stimuli under physiological conditions but may also respond to

chemical stimuli. Even though they have relatively high heat thresholds (>50ºC) they can be sensitized by heat stimuli of long duration such that they will start responding to lower temperatures. Tissue injury may also sensitize these fibers lowering both their heat and mechanical thresholds. On the other hand, Type II Aδ nociceptors are mainly sensitive to thermal stimuli under physiological conditions, although they may also become sensitive to chemical stimuli. On the contrary, they have very high thresholds or are unresponsive to mechanical stimuli. C nociceptors are also categorized into polymodal nociceptors which are sensitive to thermal, mechanical and chemical stimuli, comprising most of the type C nociceptors, and mechano-insensitive afferents (MIAs) C-fibers which are responsive only to thermal and chemical stimuli (Table 2) (Alvarez, FJ and Fyffe, RE 2000).

Table 2 - The most consensual categorization of the fiber types according to the stimulus they respond to.

Fiber type	Type of stimulus	Nomenclature/classification
Aδ	Mechanical (chemical and high heat)	Type I (HTM)
Aδ	Thermal (chemical); mainly unresponsive to mechanical stimuli	Type II (A-MIAs)
C	Mechanical, thermal and chemical	Polymodal
C	Mainly unresponsive to mechanical stimuli	C-MIAs

Nociceptors can also be classified according to the molecular markers they express. Among these are neuropeptides, enzymes, receptors and growth factors. Larger nociceptors are positive for neurofilament 200 (NF-200), while smaller cells, likely representing unmyelinated slow conducting neurons, are negative for this protein. The sub-population of smaller nociceptors are generally classified as peptidergic if they express Substance P (SP), calcitonin gene-related protein (CGRP) or somatostatin (SOM) or classified as non-peptidergic cells if they contain fluoride-resistant acid phosphatase

(FRAP) and bind to the plant isolectin B4 (IB4) from *Griffonia simplicifolia* (Fig. 2) (Alvarez, FJ and Fyffe, RE 2000, Priestley, JV 2009).

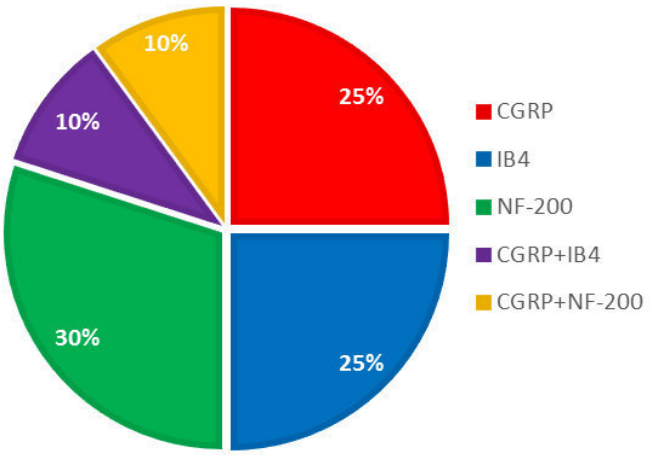


Fig. 2 – Summary of the main neurochemical populations of the DRG (modified from (Priestley, JV 2009). CGRP- Calcitonin gene-related protein; IB4 - isolectin B4 from *Griffonia simplicifolia;* NF-200 – neurofilament 200.

In rats, around 50% of sensory neurons are peptidergic cells and they also express tyrosine kinase receptor A (TrkA), the receptor for nerve growth factor (NGF). These cells also express the transient receptor potential vanilloid 1 (TRPV1, also known as capsaicin receptor) that is activated by heat stimuli. Peptidergic neurons project to lamina I and the outer lamina II of the dorsal horn of the spinal cord (Fig. 2 and 3). On the other hand, non-peptidergic IB4-positive cells express glial cell-derived neurotrophic factor (GDNF). Additionally, these cells are the ones normally expressing P2X3, a purinergic ligand-gated ionic channel for adenosine triphosphate (ATP). These neurons terminate in the inner part of lamina II (Fig. 3). Although this neurochemical classification of primary afferent neurons is widely accepted, it is important to recognize that there is sometimes an overlap in the expression of these markers, even though this is limited to a very small neuronal population (Fig. 2). Moreover, the expression of these markers changes during

development and after injury/inflammation, a fact that is also necessary to take into consideration (Fig. 3) (Alvarez, FJ and Fyffe, RE 2000, Priestley, JV 2009).

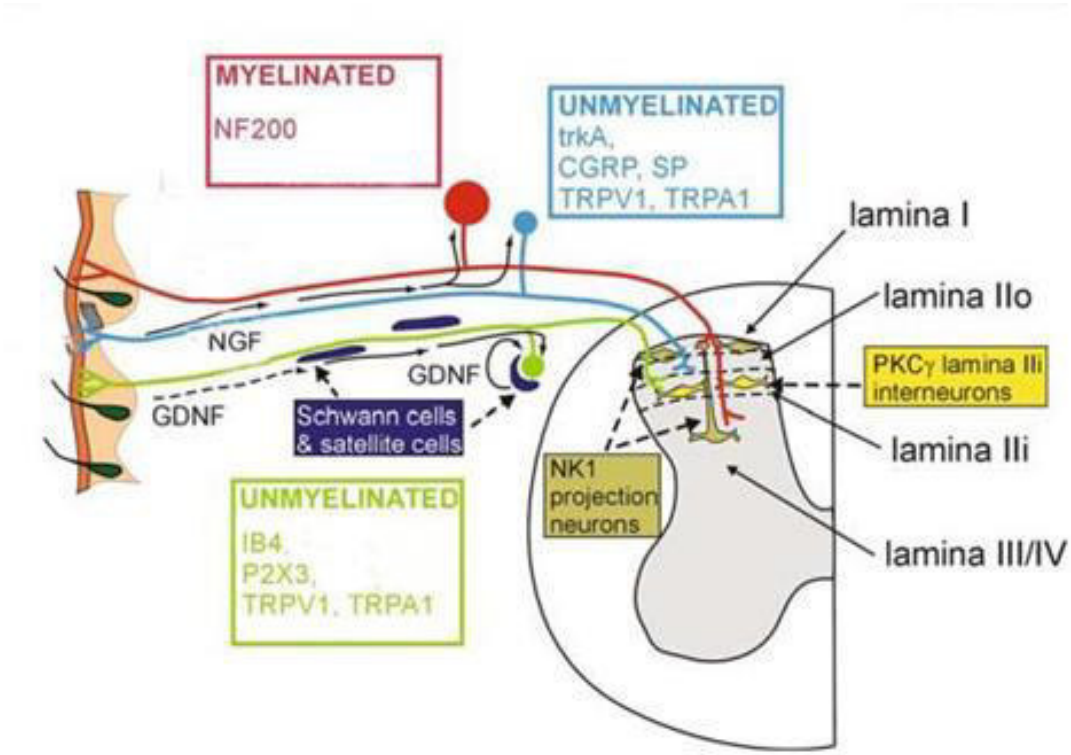


Fig. 3 – Representation of the different neuronal populations in the DRG, according to their size, myelination and projection to the spinal cord (modified from (Priestley, JV 2009). Unmyelinated peptidergic neurons express neuropeptides such as substance P (SP) and calcitonin gene-related protein (CGRP). Moreover, they express tyrosine kinase receptor A (TrkA), the receptor for nerve growth factor (NGF), and the channel transient receptor potential vanilloid 1 (TRPV1). Peptidergic neurons project to lamina I and the outer lamina II of the dorsal horn of the spinal cord. Non-peptidergic neurons, positive for isolectin B4 from *Griffonia simplicifolia* (IB4) express glial cell-derived neurotrophic factor (GDNF) and the purinergic receptor P2X3. These neurons terminate in the inner part of lamina II. Larger myelinated nociceptors are positive for neurofilament 200 (NF-200) and project to deeper dorsal horn layers. PKCγ - protein kinase C gamma; NK1 - neurokinin 1 receptor of SP.

The sensory neurons express a wide range of cell surface proteins which are commonly used as markers of the neuronal sub-populations (as shown above in Fig. 3). Additionally, and more importantly than that, these proteins are crucial mediators in signaling processes. Among these, we can outline three subclasses; ion channels, metabotropic G protein-coupled receptors (GPCRs) and receptors for neurotrophins and cytokines. It is the activation of these receptors on the cell surface that triggers the activation of distinct nociceptors and leads to different responses according to the environmental stimuli. Thus, they are qualitatively and quantitatively responsible for the conversion of a generated potential into a signal. Among the ligand-gated ion channels, the purinergic receptors (P2XR) are highly involved in the transduction of extracellular signals in response to ATP (Gold, M and Caterina, M 2008); please refer to chapter 2.5.1 for further detail).

2.3 Neuropathic versus inflammatory painful conditions

Physiological pain is a protective signal needed for survival whose mechanisms can be easily described as consisting on the transmission of impulses from the peripheral nociceptors to the central structures. However, when nerve injury or tissue damage occurs (including inflammation) a different pain state is generated. In those cases, there is nociceptor sensitization and amplification of the general neuronal excitability with greater spontaneous and evoked firing. If this overwhelming state persists in time, pain becomes pathological and its perception is modified. Chronic pain states might have different origins but it is a consensus that in all types of pain the hypersensitization and higher firing of the neurons is occurring (Gold, M and Caterina, M 2008).

Neuropathic pain is caused by a lesion or disease of the somatosensory nervous system. According to the IASP definitions, the term *lesion* is commonly used when

diagnostic investigations (e.g. imaging, neurophysiology, biopsies, laboratory tests) reveal an abnormality or when there was obvious trauma. The term *disease* is commonly used when the underlying cause of the lesion is known (e.g. stroke, vasculitis, diabetes mellitus, genetic abnormality)(Merskey, H and Bogduk, N 1994); updated by the IASP taxonomy working group). Indeed, there is some heterogeneity in the causes of neuropathic pain since it can develop following trauma (like transection, compression…), metabolic disorders (such as diabetes), infections (like HIV), exposure to chemicals (for example chemotherapy) and immune diseases (like multiple sclerosis). This fact certainly contributes to the lack of more knowledge concerning the molecular mechanisms underlying neuropathic pain. Clinical and experimental evidence suggests that not only the initiation but also the maintenance of neuropathic pain is a result of an aberrant activity of the afferent neurons (Gascon, E and Moqrich, A 2010). In fact, upon nerve injury or nerve disease, peripheral nerve fibers develop ectopic discharges originating from the site of the nerve lesion or the cell body of damaged fibers (Schaible, HG 2007).

On the other hand, **nociceptive pain** (designated to contrast with neuropathic pain) arises from actual or threatened damage to non-neural tissue and results from the activation of nociceptors (Merskey, H and Bogduk, N 1994) updated by the IASP taxonomy working group). **Inflammatory pain** presumes the occurrence of tissue damage and the recruitment of different immune cells along with the release of inflammatory molecular mediators at the lesion site , that are also capable of activating specific receptors at the peripheral terminals. Following activation, these receptors induce an increase in the nociceptor excitability that, among others, leads to lower pain thresholds. Besides pain, the typical symptoms of an inflammatory condition also include redness in the affected area, heat and swelling. There is an acute phase of inflammation characterized by tissue healing normally in a short-period. However, prolonged inflammatory states lead to

adaptive changes in the central nervous system (CNS) that result in continuous and intense pain sensation (Ji, RR *et al.* 2009).

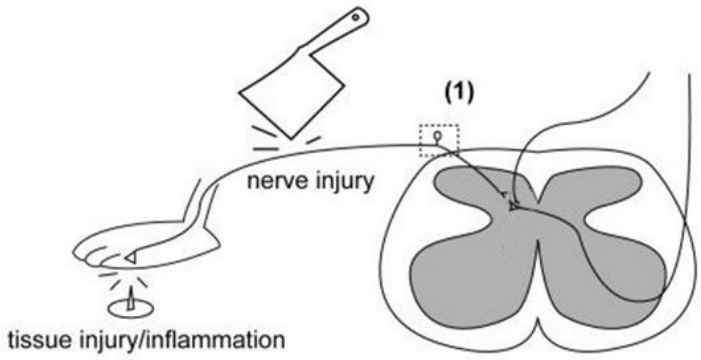
In both the neuropathic and inflammatory conditions, the altered sensitivity of these neurons normally results in the manifestation of two characteristic phenomena, hyperalgesia and allodynia. **Hyperalgesia** refers to increased pain on suprathreshold stimulation (resulting from a stimulus that normally provokes pain), and is therefore an increased response at a normal threshold, or at an increased threshold (Merskey, H and Bogduk, N 1994) updated by the IASP taxonomy working group). It refers directly to more pain in response to the same stimulus and must not be confused with the term "sensitization" that refers to an increased response of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs (Merskey, H and Bogduk, N 1994)updated by the IASP taxonomy working group). Primary hyperalgesia is confined to the site of injury while secondary hyperalgesia occurs in uninjured tissue nearby the site of the lesion. Primary hyperalgesia, in response to both heat and mechanical stimuli, is produced by activation of Aδ and C fibers that trigger pain pathways in the CNS, while secondary hyperalgesia, in response only to mechanical stimuli, is produced by activation of Aβ fibers that trigger tactile pathways (Cervero, F and Laird, JM 1996, Cervero, F 2008, Sandkuhler, J 2009). The development of **allodynia**, which is the occurrence of a pain following an innocuous stimulus (that does not normally provoke pain(Merskey, H and Bogduk, N 1994)updated by the IASP taxonomy working group) is also a consequence of the changes in the excitability thresholds of these neurons and is also a common feature in chronic pain states (Cervero, F and Laird, JM 1996, Cervero, F 2008, Sandkuhler, J 2009).

2.3.1 Common events and converging mechanisms

Although the etiologies of neuropathic and inflammatory conditions are different, there are several common events in the generation of these pain states (Xu, Q and Yaksh, TL 2011). It has been extensively shown that there is immune (i.e. recruitment and activation of immune cells) and inflammatory modulation (i.e. release of pro-inflammatory mediators) in response to nerve injury (the referred neurogenic inflammation) (Moalem, G and Tracey, DJ 2006). Others have also shown that excessive inflammation in both the peripheral nervous system (PNS) and CNS is one of the causes for the initiation and maintenance of a neuropathic pain condition (Ellis, A and Bennett, DL 2013). Indeed, recent findings suggest that neuropathic and inflammatory conditions tend to mechanistically converge along disease progression.

Among the several mechanisms that can be observed in both tissue and nerve injury pain states, one interesting and important aspect is the **altered gene expression** of **receptors**, **mediators** and **transcriptional factors** at the DRG level, as summarized in Fig. 4 (Xu, Q and Yaksh, TL 2011). One of the most relevant mediators whose expression is changed in both pain conditions is **tumor necrosis factor α (TNF-α)** which is involved, for instance, in inflammatory diseases like rheumatoid arthritis (RA) (Taylor, PC and Feldmann, M 2009) and in neuropathic pain states as inferred by studies in the spared nerve ligation (SNL) model (Schafers, M *et al.* 2003). Additionally, **voltage-gated sodium and calcium channels** (NaV and CaV, respectively) are also altered in the DRG during both conditions, playing a critical role in the control of nerve impulses and neurotransmitters release, respectively (Xu, Q and Yaksh, TL 2011). One intriguing point of convergence between both pain types is the expression of neuronal injury markers, like that of the **activating transcriptional factor 3 (ATF3),** which is found not only in nerve injury conditions but also in inflammatory pain states, as will be later detailed (please refer to section 2.6) (Fig. 4). Lastly, in both these conditions there is **activation of glial cells** (Xu, Q

and Yaksh, TL 2011) (Fig. 4) which are critical players in the continued neuronal sensitization, known today to be crucial for the development of pain states (detailed in sections 2.4/2.5).



Common features (DRG)	Time after injury	
	neuropathic	inflammatory
TNF; TNFr	Hours	Hours
Immune cells invasion	Hours/days	Hours/days
Nav 1.7,1.8	Hours/days	Hours/days
ATF3	Days (extending per weeks)	Days (transient expression)
SGCs activation	Hours/days	Hours/days

Fig. 4 – Changes at the DRG that result in persistent pain, after non-neural tissue injury and/or nerve injury. The altered expression of genes in the DRG (TNF and its receptor TNFr, and voltage-gated sodium channels or NaV are the most frequently implicated) and the activation of glial cells are common events in both neuropathic and inflammatory pain (modified from (Xu, Q and Yaksh, TL 2011)).

This mechanistic convergence might explain why the resolution of the original injury in many cases of inflammatory pain, does not reverse persistent pain. Indeed, tissue resection, herniorrhaphy and joint repair were shown to be ineffective approaches for pain control in arthritic patients (Xu, Q and Yaksh, TL 2011). In animals with rheumatoid arthritis, amelioration of the inflammatory component did not alleviate persistent

allodynia (Christianson, CA *et al.* 2010). Accordingly, a shift to a more "neuropathic pain phenotype" has been suggested for osteoarthritis, as a consequence of the activation of "damage-related programs" (Ferreira-Gomes, J *et al.* 2012, Su, J *et al.* 2015). Therefore, understanding how inflammatory and neuropathic pain mechanisms converge overtime will hopefully help develop more efficient treatments and better targeted approaches.

2.4 Joint inflammatory pain

According to the World Health Organization, musculoskeletal disorders are the most frequent cause of disability, the number of cases having increased dramatically in the past decade. Chronic or episodic pain is assumed as the main cause for loss of joint mobility and function which can deeply result in impaired quality of life. The current treatments used for the management of joint pain have limited effectiveness and one of the major reasons for this is the lack of knowledge concerning the mediators and mechanisms involved in those conditions (McDougall, JJ 2006).

2.4.1 Innervation of the joints and articular pain

Joints are innervated by branches descending from main nerve trunks or their muscular, cutaneous and periosteal branches. A typical joint nerve contains all the three types of fibers already mentioned, namely the thick myelinated Aβ, thinly myelinated Aδ, and a high proportion (~80%) of unmyelinated C fibers, the latter being either sensory afferents or sympathetic efferents (each ~50%). The Aβ fibers are not nociceptive while numerous articular Aδ and C fibers are, terminating as non-encapsulated or "free" nerve endings in the fibrous capsule, adipose tissue, ligaments, menisci and periosteum. The major neuropeptides in joint nerves are SP, CGRP and somatostatin. Neuropeptide Y has also been localized in joint afferents (Schaible, HG *et al.* 2002).

Pain in the joints can be elicited when noxious mechanical or chemical stimuli are applied to the fibrous structures, such as ligaments and fibrous capsule, while no pain is elicited by stimulation of cartilage as it is not innervated. Stimulation of normal synovial tissue rarely evokes pain, and innocuous mechanical stimulation of fibrous structures can evoke pressure sensations (Kidd, BL *et al.* 1996, Ebersberger, H-GSaA 2009). Therefore, joint pain arises from peripheral sensitization of joint afferents, being characterized by hyperalgesia and persistent pain at rest, while allodynia might be present in movements within the working range or during gentle pressure (Schaible, HG *et al.* 2009).

It is still unclear how a mechanical stimulus in the joint is converted into a noxious electrical signal and propagated along sensory nerves to the CNS. So far, it is assumed that movement of the joint generates shear stresses on the axolemma of the 'free' nerve endings which results on the opening of mechano-gated ion channels (McDougall, JJ 2006). The generated action potentials are then decoded into a mechanosensation, increasing the firing rate of the afferent nerve upon a noxious movement of the joint and leading to pain. The factors that may alter joint mechanosensitivity and promote nociception can be divided into mechanical factors and inflammatory mediators (McDougall, JJ 2006).

Following joint injury or during inflammation, the synovial blood vessels become increasingly permeable to plasma proteins resulting in fluid accumulation into the joint with subsequent edema. This effusion causes a dramatic increase in the intra-articular pressure as the joint is an enclosed space. Studies in animal models have shown that an elevation in intra-articular pressure results in burst firing of articular afferents in a rate proportional to the level of pressure incurred. Thus, the increased intra-articular pressure and edema formation in arthritic joints seems to activate joint nociceptors, leading to pain. On the other hand, following injury or pathogenic infection, a typical inflammatory response is often triggered in the joints, as part of an innate healing process initiated to repair the damaged tissues. However, these same inflammatory mediators released in this

healing process also act on joint sensory nerves, leading to either excitation or sensitization. So far, many mediators were shown to be crucial in these processes (such as cytokines and prostaglandins production or the expression of P2X purinergic receptors) but it is still unclear how joint afferents phenotypically differ from other peripheral nociceptors, which would certainly help understand the mechanisms underlying joint diseases (Grubb, BD 2009). Moreover, revealing the inflammatory agents that induce noxious stimulation and the respective molecular mechanisms is of major therapeutic value (McDougall, JJ 2006), as this will help the development of novel and successful approaches.

2.4.2 The Monoarthritis (MA) model

Among several diseases, monoarthritis (MA) is a condition characterized by the inflammation of one joint (Byng-Maddick, R *et al.* 2012). Symptoms resolving within 4 weeks are described as acute, whereas those persisting beyond 3 months are considered chronic. The causes for the development of such conditions can be either inflammatory or not and the overlapping of the general symptoms frequently leads to incorrect diagnoses which consequently results in poor responses to conventional pharmacological treatments like non-steroidal anti-inflammatory drugs (NSAID's) (Byng-Maddick, R *et al.* 2012) (Table 3). In the treatment of diseases like osteoarthritis, cyclooxygenase (COX) inhibitors (especially COX-2), a sub-class of NSAID's, are frequently used (Kivitz, A *et al.* 2008). Pharmacological inhibition of COX results in the impairment of prostanoids production and release (including prostaglandins), providing pain relief and amelioration of the inflammatory process (Laveti, D *et al.* 2013). Even though these drugs are normally effective in some joint inflammatory conditions, a high percentage of cases still remain without a successful treatment.

Table 3. Differential diagnoses for monoarthritis in humans and the typical protocol for the treatment (modified from (Byng-Maddick, R *et al.* 2012).

	Causes	**Such as**	**treatment algorithm**
Inflammatory	Infection	bacterial, fungal, viral	NSAID's ↓ disease-modifying antirheumatic drugs (DMARDs) and corticosteroids ↓ biological therapies (anti-TNF) ↓ synovectomy (chemical or surgical) ↓ others in the future
	systemic inflammatory arthritis	rheumatoid arthritis	
	Spondyloarthritis	psoriatic and reactive arthritis	
	connective tissue disease	Lupus	
	crystal arthritis	Gout	
	Neoplasia	chondrosarcoma, synovioma, osteoma	
non-inflammatory	Trauma	stress fractures, lipomas	
	Degeneration	Osteoarthritis	
	Haemarthroses	anticoagulation disorders	

In this context, animal experimental models are exceptional tools to bring light into the pathophysiological molecular and cellular mechanisms of these diseases. Monoarthritis can be induced in rats by the injection of complete Freund's adjuvant (CFA - a solution containing *Mycobacterium butyricum*) into the tibiotarsal joint, constituting a well-established model of inflammatory articular pain firstly described by Butler *et al* (Butler, SH *et al.* 1992).CFA injection not only in the joint but also into the tail, paw or muscle has been consistently used to mimic chronic inflammatory pain conditions (more severe than carrageenan) that might occur in humans along with rheumatoid arthritis or tendonitis (Gregory, NS *et al.* 2013).

In MA, intra-articular injection of CFA produces an anatomically limited arthritic process in rats, stable over 6 weeks and suitable for behavioral and neurochemical studies along the disease progression related to the condition and with the outcome of various chronic pain treatment methods. It is confined to a single joint making it possible to use the contralateral paw as an internal control. Animals normally gain weight and remain active which indicates this model has little systemic disturbance, in opposition to polyarthritis which can be very aggressive to animals. Sometimes MA may develop into a polyarthritic condition, characterized by observable swelling in the contralateral paw and sometimes the tail, but in those cases animals are excluded from the study (Butler, SH *et al.* 1992). It is a model widely used in the pain field for which the associated physiological, morphological, neurochemical and behavioral changes have been extensively explored (Neto, FL *et al.* 1999, Schadrack, J *et al.* 1999, Lourenco Neto, F *et al.* 2000, Neto, FL and Castro-Lopes, JM 2000, Neto, FL *et al.* 2001, Ferreira-Gomes, J *et al.* 2004, Cruz, CD *et al.* 2005, Ferreira-Gomes, J *et al.* 2006, Potes, CS *et al.* 2006, Neto, FL *et al.* 2008, Pozza, DH *et al.* 2010, Borges, G *et al.* 2014).

2.5 Role of glial cells in chronic pain

Pathological states were initially believed to be confined only to neuronal mechanisms, although considering only neuronal activity provides an incomplete understanding of these phenomena. Indeed, glial cells, as non-conducting cells, were firstly proposed to give only nutritional and mechanical support to neurons, but nowadays they are assumed as key modulators of neurotransmission at the synaptic level and as having a role in promoting and controlling the homeostasis of the nervous system (Vallejo, R *et al.* 2010). During dysfunctional pain signaling, as it happens in chronic pain states, glial cells are abundantly activated, proliferate and may also suffer several biochemical

modifications (Milligan, ED and Watkins, LR 2009). They are key modulators of these events and potent enhancers of neuronal sensitization, therefore emerging as new targets for drug development (Watkins, LR and Maier, SF 2003).

Indeed, glial cells in the CNS have long been recognized for their responses to injury, as well as having a critical role in the genesis and persistence of pain (Watkins, LR and Maier, SF 2003). For a long time, the activation of astrocytes and microglia has been proposed as a common mechanism underlying several pathological painful conditions from different origins (Milligan, ED and Watkins, LR 2009). In contrast, only over the last two decades, the peripheral SGCs that remained in the shadow for many years, emerged as crucial players in pain modulation. Their unique location in the sensory ganglion was shown to strongly contribute to pain facilitation, turning SGCs into promising new targets for the development of analgesic drugs (Jasmin, L *et al.* 2010)

2.5.1 Satellite glial cells: properties and functions

In the peripheral sensory ganglia, the cell bodies of primary afferents are surrounded by SGCs (Fig. 5). Each sensory neuron has its own SGCs sheath, therefore forming a distinct morphological and functional unit, separated by regions containing connective tissue. In some cases, these units can aggregate forming clusters that, however, are more prevalent in young organisms. Space between neurons is minimal and, in addition, they have fine processes that sometimes fit into invaginations of SGCs (Hanani, M 2005). This special localization and physical contact between SGCs and neurons allows a perfect communication that is functionally very relevant.

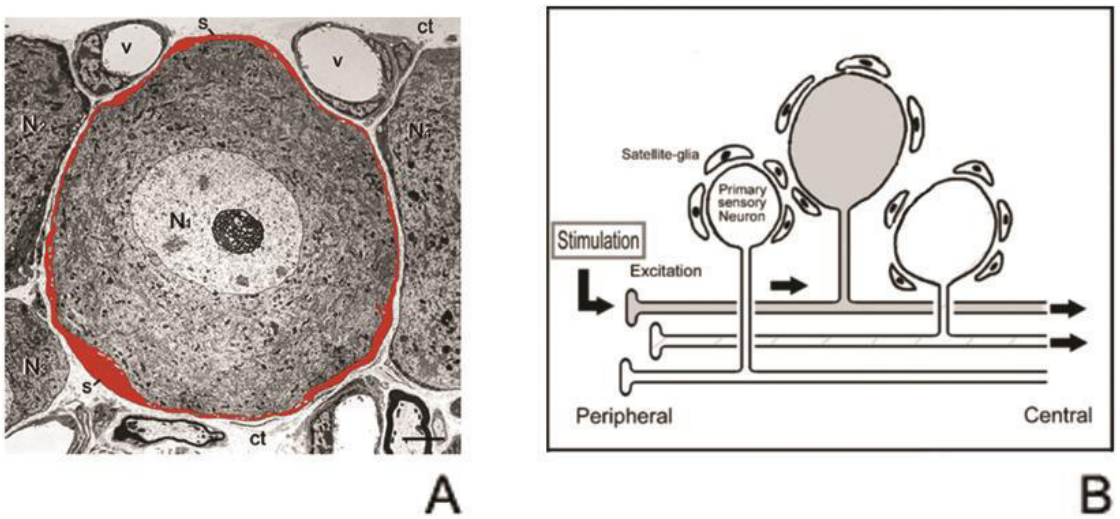


Fig. 5– A – Electron micrograph of mouse DRG showing the cell body of a sensory neuron (N1) surrounded by a SGCs sheath (in red) (from (Hanani, M 2015). B - Schematic representation of cell bodies of primary afferent neurons enwrapped by SGCs, which altogether make part of a dorsal root ganglion (modified from (Takeda, M *et al.* 2009).

SGCs can be easily identified by the presence of several proteins. Just like astrocytes, they express glial fibrillary acidic protein (GFAP) and S100, which are both part of its cytoskeleton, and glutamine synthetase (GS). GFAP is often used as a marker of SGCs activation, since in normal physiological conditions it is barely detectable by immunohistochemistry, but increases dramatically after inflammation and/or neuronal injury (contrarily to what happens in astrocytes of the CNS where GFAP is readily detectable in the cells resting state) (Ohara, PT *et al.* 2009)). Even though SGCs are often compared to astrocytes, there are many other characteristics, as for example their embryonic origin, that are completely distinct (Ohara, PT *et al.* 2009). S100 remains unexplored in sensory ganglia and is not a good marker for these cells since it can be also expressed by Schwann cells and a subpopulation of sensory ganglia neurons. GS is so far the most useful marker, while GFAP is used to identify activated SGCs (Hanani, M 2005, Jasmin, L *et al.* 2010).

Evidence shows that SGCs play an active role in the neuronal changes occurring during pathological states and therefore they are not just bystanders of these conditions. Indeed, these cells were shown to be crucial for the development of chronic pain states, in many different experimental models. Upon inflammation or neuronal damage, not only primary sensory neurons but also the surrounding SGCs undergo characteristic changes. SGCs become activated expressing higher levels of GFAP (Dublin, P and Hanani, M 2007, Gunjigake, KK *et al.* 2009, Liu, FY *et al.* 2012) and their proliferation is significantly increased (Elson, K *et al.* 2004, Elson, K *et al.* 2004). Moreover, upon nerve injury or inflammation the number of gap junctions are increased not only between SGCs of the same sheath (the only communication observed in physiological states) but also between SGCs surrounding neighboring distinct neurons (Huang, LY *et al.* 2013). These events greatly contribute to the propagation of an excitatory state in the sensory ganglia and the continued neuronal sensitization (Takeda, M *et al.* 2007, Takeda, M *et al.* 2009) which is highly associated with pain sensation.

Since these changes are common features in both neuropathic and inflammatory pain states, exploring SGCs activation and the associated events becomes crucial to better understand the pathomechanisms of these conditions at the sensory ganglia level (Xu, Q and Yaksh, TL 2011). Indeed, it is nowadays believed that inhibiting SGCs activation/proliferation or disrupting their communication might be excellent strategies to alleviate pain, in some pathological conditions. In fact, the administration of the SGCs metabolic inhibitor fluorocitrate (FC) to neuropathic pain animals, reversed the typical pain-induced behaviors (Liu, FY *et al.* 2012, Cao, J *et al.* 2014). Moreover, gap junction blockers were shown to decrease the spontaneous activity of neurons in injured DRG and also reduce pain-induced behaviors, which supports a role of gap junctions in the ectopic discharges that contribute to chronic pain states (Hanani, M 2005, 2012, Huang, LY *et al.* 2013).

Altogether these findings strongly support that communication among SGCs is a key mechanism in pain processing. However, it is not only important how SGCs communicate with each other but also how they communicate with neurons (and vice-versa), within the sensory ganglia, and how they contribute for pain states.

2.6 Neuron-glia interactions in sensory ganglia

Normally, neurons communicate directly with each other through the release of neurotransmitters and activation of receptors. However, in the DRG, a synaptic contact between neurons rarely occurs since they are completely wrapped and isolated by SGCs. Therefore, it is nowadays assumed that communication between primary afferents is majorly mediated by SGCs. Indeed, some authors have proposed a model of "transglial transmission" in the communication between a stimulated and a passive neuron. In these studies, they showed the formation of trimers (neuron-SGCs-neuron) wherein communication is majorly done via the SGC in a "sandwich synapse" mode (Rozanski, GM *et al.* 2013, Rozanski, GM *et al.* 2013) Fig. 6).

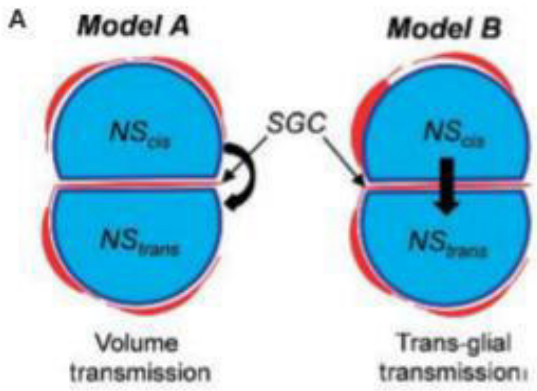


Fig. 6 – Two models of possible communication between neurons within the sensory ganglia. In Model A, designated as volume transmission, there is a direct communication between two neighbor neurons by the release of chemical mediators (NS for neuronal somata). In Model B, the

released transmitters activate the surrounding SGCs instead, which in their turn release other molecules that will activate the second neuron. This transglial activation was proposed to be the major mechanism of communication within the DRG and to be a finer modulatory mechanism of the neuronal activity (Rozanski, GM *et al.* 2013, Rozanski, GM *et al.* 2013) (Figure from (Rozanski, GM *et al.* 2013).

In sensory ganglia, the release of mediators from neuronal somata is crucial for the communication between the different cells. Many studies have demonstrated that ATP is the major mediator released from primary afferent neurons, capable of activating SGCs (Wirkner, K *et al.* 2007). In their turn, these glial cells exert a complex excitatory and inhibitory modulation of the neuronal activity. Hence, SGCs are actively involved in afferent signaling and therefore in pain processing. This bidirectional communication between neuronal somata and SGCs, under injurious conditions, implicates the participation of a number of receptors, of which the purinergic receptors are those having a dominant role (Gu, Y *et al.* 2010).

2.6.1 Purinergic system in neuron-SGCs communication

ATP is one of the major transmitters released by stimulated/sensitized neurons. The purinergic receptors, activated in response to purine nucleotides and nucleosides (such as ATP), can be divided into metabotropic and ionotropic, the P2Y and P2X being respectively the most widely studied receptors in each subfamily. Several subtypes of P2Y receptors have been implicated in nociception and are expressed in the DRG (Gerevich, Z and Illes, P 2004). Among these, P2Y1 and P2Y2 are highly expressed in rat sensory neurons while only low levels of mRNA can be found for P2Y4 and P2Y6 (neither receptor has been localized in sensory neurons; (Ruan, HZ *et al.* 2005).

However, it is widely accepted that P2X are more relevantly involved in pain transmission (Gerevich, Z and Illes, P 2004), being found in both neurons and SGCs within the sensory ganglia. To date, seven mammalian P2X receptor subunits (P2X1–P2X7) have been identified in the form of homotrimers, heterotrimers or multimers (Habermacher, C *et al.* 2015). The different subunits share the same general structure with an intracellular N- and C-termini, two membrane-spanning domains and a large extracellular loop containing 10 conserved cysteine residues. They differ in their affinity to ATP (and other analogues) as well as in the ATP-evoked currents (Dunn, PM *et al.* 2001). These receptors are non-selective channels with considerable permeability to Ca^{2+} and Na^{+} ions. Interestingly, when activated, some (but not all) might even expand their pores allowing larger molecules to pass (Khakh, BS and North, RA 2006). Among the seven subclasses known so far, P2X2–P2X6 mRNAs were found in DRG neurons (data on P2X1R are controversial (Kobayashi, K *et al.* 2005)) while P2X7 and P2X4 were the only subtypes detected in SGCs (North, RA 2002, Kobayashi, K *et al.* 2005) (Table 4). P2X3R is particularly relevant in sensory ganglia as it is the most abundant subclass found in primary afferents. Almost all neurons express P2X3R at embryonic stages, however at day 14 post-natal only 50% of the small and medium primary afferents express this receptor (Ruan, HZ *et al.* 2004). In fact, P2X3 is majorly expressed in non-peptidergic C-fiber nociceptors being frequently used as a marker of this neuronal population (Fig. 3; (Chizh, BA and Illes, P 2001, Puchalowicz, K *et al.* 2014, Beamer, E *et al.* 2015).

Table 4 - Expression of P2X receptors in primary afferent neurons (modified from (Dunn, PM *et al.* 2001) and SGCs in the sensory ganglia.

	Neuronal cell body	SGCs around cell bodies
Protein	P2X3 - high level of expression in non-peptidergic small- to medium-diameter neurons; many bind IB4	
	P2X2 - present in many small and large neurons	
	P2X1; P2X4,5,6 variable low level of expression in some cells	P2X7 abundantly expressed
mRNA transcripts	P2X1-6 - all present; the highest level was found for the P2X3 transcript (others could not find P2X1R mRNA in the DRG)	P2X7 abundantly expressed P2X4 less extent

Remarkably, a unique feature of sensory ganglia is that P2X7Rs are abundantly and exclusively expressed in SGCs (not in DRG neurons) (Chen, Y *et al.* 2008), while P2X3Rs can only be found in DRG neurons (Chen, Y *et al.* 2008) (Table 4). This distinct expression suggests that ATP acts differently on neurons and on SGCs (Gu, Y *et al.* 2010) and supports the relevance of P2XR in the communication between these two cell types.

At the DRG level, even though neurons almost do not communicate chemically with each other, there is evidence that they release chemical signals, which might affect the excitability of SGCs instead. Besides the release of mediators such as SP, CGRP or nitric oxide (NO), it has also been reported that the electrical stimulation of DRG neurons induces robust vesicular ATP release from the somata, in a process that requires entry of Ca^{2+} (Zhang, X *et al.* 2007) (Fig. 7). The ATP released from sensitized neurons is then capable of activating the P2X7R found exclusively in the SGCs, therefore mediating the communication between the neuronal soma and glial cells. In fact, P2X7R is assumed as a crucial key for the neuron-glia communication in the sensory ganglia and one of the major

triggers for SGCs activation and proliferation, during pathological conditions (Villa, G *et al.* 2010, Hanani, M 2012). Moreover, P2X7R activation is intimately associated with the production and release of pro-inflammatory mediators, such as tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β), by activated SGCs, which highly contributes for the continued neuronal sensitization, in these conditions (Alves, LA *et al.* 2013). Interestingly, ATP is also released by activated SGCs exerting a similar effect on neurons. Furthermore, the glial release of TNFα and ATP, by independent signaling pathways, is capable of increasing the activity of P2X3R in the soma (Takeda, M *et al.* 2009, Gu, Y *et al.* 2010, Huang, LY *et al.* 2013), which is also reported to increase neuronal excitability (Xu, GY and Huang, LY 2002); Fig. 7).

However, in addition to the above mentioned excitatory effect, activation of P2X7R in SGCs was also found to exert inhibitory actions on DRG neurons. It has been demonstrated that blocking P2X7Rs and their mediated ATP release resulted in increased P2X3R expression in DRG neurons and that reducing P2X7R expression (by using small interference RNA, siRNA) also increased P2X3R expression. These findings highly suggest that P2X7R activation tonically suppresses the expression of P2X3Rs in DRG neurons (Chen, Y *et al.* 2008). Additionally, this suppression was reverted by treating ganglia with an antagonist for the metabotropic P2Y1 receptor, which supports that the activation of P2Y1Rs in neurons is required and sufficient for the inhibitory P2X7R-P2X3R control (Chen, Y *et al.* 2008, Chen, Y *et al.* 2012) Fig. 7).

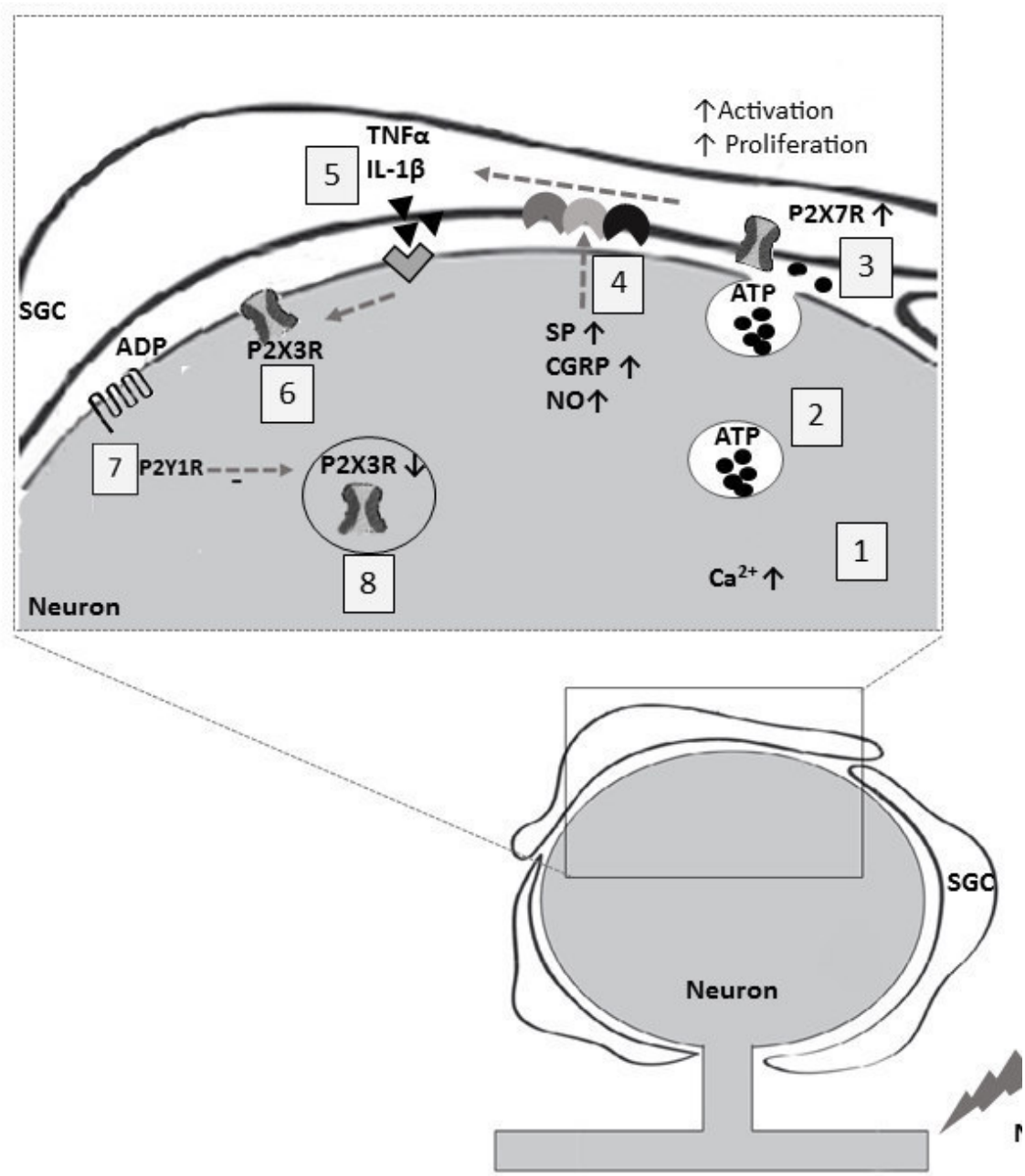


Fig. 7 – Summary of the most relevant mechanisms in neuron-glia communication in the sensory ganglia, upon nerve stimulation. Calcum entry (1) is needed for the release of intravesicular ATP (2) upon an electrical stimulus of DRG neurons. Released ATP exerts an effect on the excitability of SGCs, through activation of P2X7R (found exclusively in these cells) (3). Besides ATP, other mediators such as CGRP, SP and NO are also released from hypersensitized neurons, acting on specific receptors on the SGCs membrane (4). P2X7R activation is closely associated with the production and release of the pro-inflammatory mediators TNFα and IL-1β, from the activated SGCs

(5). Both ATP and TNFα directly and indirectly increase neuronal P2X3R activity (6), while ADP in a P2Y1-mediated cascade (7) will down-regulate P2X3R expression in a inhibitory mechanistic control (8) (modified from (Costa, FA and Moreira Neto, FL 2015).

Many studies show that disruption of these signaling cascades, namely the activation of these receptors and ATP release, results in a lower neuronal hypersensitization and reduced nociceptive behavior, turning P2X receptors into novel and appealing targets for more effective pain treatments. In the case of sensory ganglia, the fact that P2X7R is exclusively expressed in SGCs while P2X3R can only be found in neurons, along with the known negative regulation of P2X7R over P2X3R expression, makes them even more interesting targets to be evaluated in pain conditions.

2.6.2 P2X receptors in pain processing

The release of ATP from damaged or inflamed tissue was assumed as a nociceptive stimulus after it was found that administration of this molecule induced pain in humans (Bleehen, T and Keele, CA 1977). This supports per se the involvement of P2XR in nociception. In fact, the genetic disruption of P2X3R resulted in the total abolishment of transient ATP-evoked currents in cultured sensory neurons (Cockayne, DA *et al.* 2005). Numerous reports show the antinociceptive effect of P2X3R antagonists in both inflammatory (Prado, FC *et al.* 2013) and neuropathic pain models (Jarvis, MF *et al.* 2002). Additionally, null-P2X3R mice show decreases in nociceptive behavior, comparing to naïve animals, when injected with ATP (known to induce pain-like behavior) (Cockayne, DA *et al.* 2000). Alterations in P2X3R mRNA and protein levels have also been extensively documented for different painful pathological conditions (Tsuzuki, K *et al.* 2001, Xu, GY and Huang, LY 2002). However, data are sometimes inconsistent concerning the direction

of these changes and therefore further investigation is needed to better understand the role of P2X3R in pathological states and nociception.

On the other hand, P2X7R, expressed in SGCs instead, appears to be crucial for the development of normal inflammation and hyperalgesia (Dell'Antonio, G *et al.* 2002, Chessell, IP *et al.* 2005). Activation of P2X7R is directly associated with the release of cytokines like TNFα and IL-1β from glial cells, upon injury or inflammation. In fact, P2X7R knock-out mice lack the ability to release IL-1β in response to ATP stimulation (Solle, M *et al.* 2001) and therefore do not develop swollen paws or joint cartilage lesions as wild-type arthritic animals do (Labasi, JM *et al.* 2002). Additionally, many studies demonstrate that P2X7R expression is up-regulated in these pathological conditions (Chen, Y *et al.* 2008) and that it has a functional role in pain processing. Indeed, in a model of CFA induced hyperalgesia, the nociceptive behavior was reverted by the administration of oxidized ATP, an irreversible inhibitor of P2X7R (Dell'Antonio, G *et al.* 2002). Furthermore, P2X7R knock-out mice do not show thermal and mechanical hypersensitivity following inflammation, nerve ligation or lipopolysaccharide (LPS) treatment (Clark, AK *et al.* , Chessell, IP *et al.* 2005).

Both P2X3 and P2X7 receptors are undoubtedly involved in nociception and as a result they are pointed as novel targets for the relief of chronic pain (Chizh, BA and Illes, P 2001). However, the available data are still inconsistent concerning the expression of these receptors (and their contribution to pain processing) in different pathological conditions or distinct stages of the diseases progression. Additionally, the complexity of the signaling cascades involved, namely the P2X7R-P2X3R inhibitory control, might contribute to the apparently controversial data. Thus, in order to develop successful analgesic drugs, it is crucial to better elucidate the purinergic signaling cascades and underlying mechanisms, in painful conditions.

2.7 Activating Transcriptional Factor 3 (ATF3) – the stress inducible gene

Activating Transcriptional Factor 3 (ATF3) is a member of the mammalian activating transcription factor /cAMP responsive element binding (ATF/CREB) family of transcription factors. In what regards to the nervous system, ATF3 is assumed and commonly used as neuronal injury marker (Tsujino, H *et al.* 2000) as it is significantly induced in primary afferents, in several models of neuropathy (Tsujino, H *et al.* 2000, Liang, L *et al.* 2010, Hunt, D *et al.* 2012). However, more recently, it has also been found in sensory ganglia neurons upon inflammatory conditions that are believed to reach a neuropathic state (Xu, Q and Yaksh, TL 2011). Even though some of their functions and signaling cascades are known, its exact role in the nervous system is still poorly understood. Indeed, ATF3 is an interesting gene known to initiate a wide range of signaling cascades that, depending on the cellular context, might inclusively result in opposite cell fates. Therefore, it seems crucial to evaluate ATF3 role in different painful conditions, namely during inflammation where data are more inconclusive. Identifying the ATF3 signaling cascades in these conditions will help better understand the convergence of inflammatory and neuropathic mechanisms over time and the eventual relevance of this factor in nociception (regulating, for example, neuron-SGC communication).

2.7.1 Gene variants, induction, regulation and function

Interestingly, ATF3 is a transcriptional factor that represses rather than activates transcription from promoters with ATF sites. There are different isoforms and alternatively spliced forms of ATF3 that differ in their mechanism of action (Fig. 8).

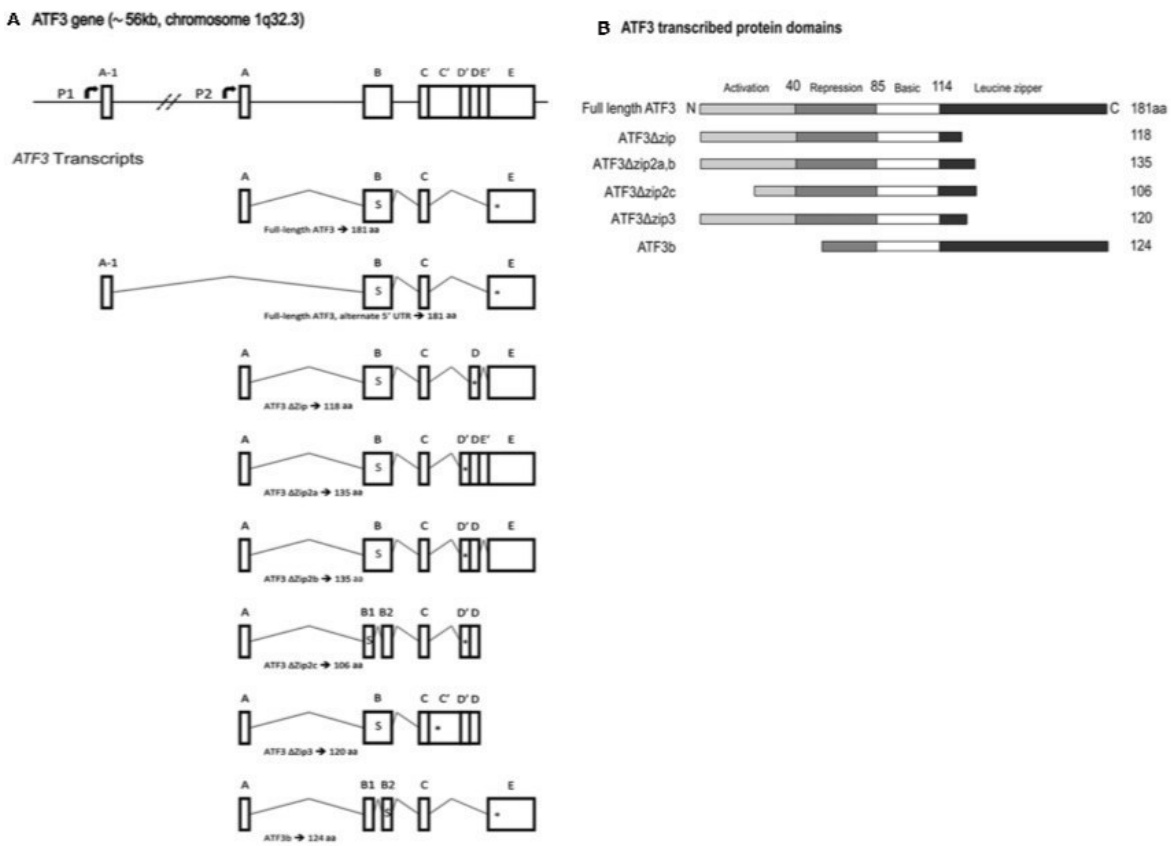


Fig. 8 – Activating transcription factor 3 (ATF3) gene and protein structure. A schematic representation of the ATF3 gene structure and transcript splice variants (Hunt, D *et al.* 2012).

In contrast to ATF3 that is a repressor, the ATF3ΔZip isoform lacks a leucine zipper domain and does not bind to DNA, therefore promoting transcription with or without ATF sites, possibly by sequestering inhibitory co-factors away from the promoter (Fig. 9) (Chen, BP *et al.* 1994). ATF3 has been described as an immediate early gene, a stress inducible gene and an adaptive response gene (Thompson, MR *et al.* 2009). The regulation of ATF3 expression appears to take place mainly at the translational level and ATF3 promoters contain transcription factor binding sites consistent with its expression being induced by stressful stimuli (Hunt, D *et al.* 2012). Other research groups, interested in the molecular mechanisms of ATF3, showed that the repression of the ATF3 promotor can be done by the ATF3 gene itself which provides a possible mechanisitic explanation for the

transient expression of ATF3 upon stress induction (Wolfgang, CD *et al.* 2000). This auto-repression shortens the period of its expression in response to stressful stimuli which is an important regulation of the stress response itself (Hunt, D *et al.* 2012); Fig. 9).

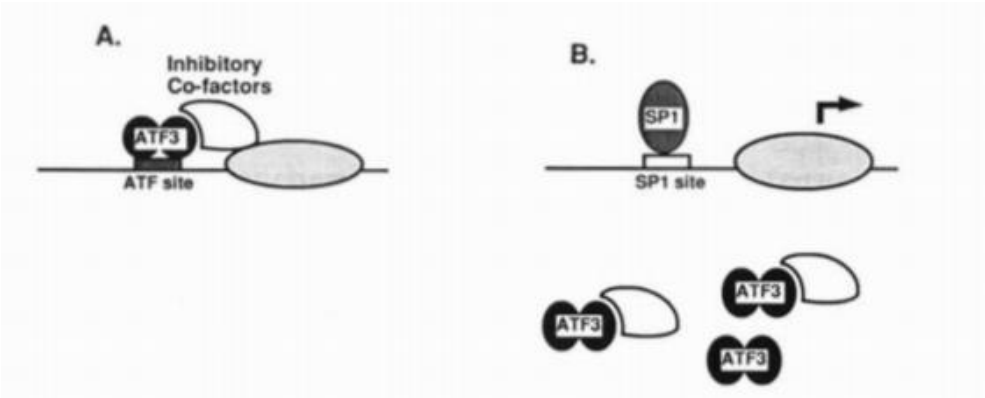


Fig. 9 – Mechanisms of inhibition (A) and activation (B) of transcription by ATF3. ATF3 represses transcription from a promoter with ATF sites by stabilizing inhibitory co-factors at the promoter (A). Other isoforms might activate transcription from a promoter without ATF sites by sequesterig the inhibitory co-factors away from the promoter (Chen, BP *et al.* 1994).

ATF3 is expressed in a wide range of cells from different tissues such as cardiomyocytes (Nobori, K *et al.* 2002), adipocytes (Jang, MK and Jung, MH 2015), myeloid-derived cells and parenchymal cells from the lung ((Shan, Y *et al.* 2015), retinal ganglia cells (Saul, KE *et al.* 2010) or immune cells (Thompson, MR *et al.* 2009), among others. In general, it has a low expression level (if at all) in the quiescent cells of healthy tissues, but it is increased/induced under stress conditions, such as injury, ischemia, ischemia/reperfusion or chemical toxin (Hai, T and Hartman, MG 2001). The ATF3 expression that occurs after an insult was shown to be either detrimental or protective depending on the different stimuli (Zhou, H *et al.* 2014) and therefore ATF3 is considered to be **an adaptive-response gene** (Hai, T and Hartman, MG 2001). In fact, it has already been associated to apoptosis, for example, in human colorectal cancer cells (Lee, JR *et al.*

2014). On the other hand and controversially to its pro-apoptotic characteristics, it has been referred several times as a survival promoter. In fact, many studies have already shown how ATF3 expression acts as a protective mechanism and cellular improvement promoter (Seijffers, R *et al.* 2014, Xie, JJ *et al.* 2014).

This discrepancy may be due to the fact that ATF3 can be either a repressor or an activator, as already mentioned. Therefore, it is possible that, when induced during stress responses, ATF3 activates some target genes but represses others, depending on the **promoter** and **cellular context** (Hai, T *et al.* 1999). In conclusion, ATF3 regulates multiple targets and may play different roles. In 2010, Tsonwin Hai, expanded these concepts referring to ATF3 as "a hub of the cellular adaptive-response network to respond to signals perturbing homeostasis" (Hai, T *et al.* 2010) please refer to section 2.7 for details on the related signaling pathways).

Despite the evident induction of ATF3 by stress signals, the inherent consequences of its activation during a stress response are not clear and even less is known about its functional significance in physiological states (Hai, T *et al.* 1999). This reinforces the fact that continue studying ATF3 is crucial as it is a key regulator of some pathological signaling cascades.

2.7.2 ATF3 expression in the nervous tissue in physiological and pathological conditions

ATF3 can be found in both the central and peripheral nervous systems, including neurons and glial cells. Centrally, it has been detected in areas of peri-infart cortex and thalamus cortical neurons, after middle cerebral artery occlusion (model cerebral ischemia) (Ohba, N *et al.* 2003), and up-regulated after intracortical axotomy (Mason, MR *et al.* 2003). Unlike peripheral neurons, neurons from the CNS do not regenerate and

therefore the presence of ATF3 is found exclusively after damage and in very specific cases, confined to very small regions near the lesion.

In the PNS, ATF3 is mainly expressed in the cell bodies of the primary afferent neurons in the sensory ganglia. As it happens in other tissues, ATF3 is either not expressed or expressed at very low levels in physiological conditions (intact neurons *in vivo*). Indeed, some detected ATF3 in the nuclei of a very small percentage of primary sensory neurons in uninjured rats (Averill, S *et al.* 2002) but others failed to find ATF3-positive immunoreactivity in the same uninjured cells (Tsuzuki, K *et al.* 2001). On the other hand, the undoubtable induction of ATF3 expression in primary afferents (but not spinal cord), in many models of **neuronal injury**, strongly suggests it plays a role in these damaged neurons. In fact, ATF3 up-regulation was found in DRG neurons after peripheral nerve compression (Isacsson, A *et al.* 2005), chronic constriction injury (CCI;(Obata, K *et al.* 2003, Pavel, J *et al.* 2013), spinal nerve ligation (SNL; (Fukuoka, T *et al.* 2012), spared nerve injury (SNI; (Cachemaille, M *et al.* 2012) or spinal nerve transection (Tsujino, H *et al.* 2000). The indirect development of peripheral neuropathy, like that induced by the chemotherapy treatment with Paclitaxel, also leads to the upregulation of ATF3 in primary sensory neurons in both DRG and trigeminal ganglia of experimental animals (Jimenez-Andrade, JM *et al.* 2006, Peters, CM *et al.* 2007). Diabetic peripheral neuropathy also caused ATF3 upregulation in mouse DRG neurons (Wright, DE *et al.* 2004). Altogether these findings largely contributed to the use of ATF3 as a neuronal injury marker.

On the other hand, ATF3 expression during **inflammatory conditions** is more inconsistent and controversial. Capsaicin, formalin, mustard oil or menthol injected into the plantar surface of the hind paw of mice also induced expression of ATF3 in distinct subpopulations of sensory neurons (Braz, JM and Basbaum, AI 2010). The injection of formalin into the footpad of rats induced an inflammatory response accompanied by induction of ATF3 expression in some DRG neurons and even a few motor neurons in the

spinal cord (Tsujino, H *et al.* 2000). Additionally, ATF3 was upregulated in DRG neurons in rat models of osteoarthritis induced by monoiodoacetate (MIA) or collagenase injection in the knee joint, where the extent of axonal injury is unknown (Ivanavicius, SP *et al.* 2007, Ferreira-Gomes, J *et al.* 2012, Adaes, S *et al.* 2015). However, ATF3 is not always induced or expressed in the same way, during an inflammatory condition. For example, CFA did not produce ATF3 upregulation when injected into the rat footpad, although it induced a profound inflammatory response (Braz, JM and Basbaum, AI 2010). It seems then that ATF3 is induced in particular inflammatory conditions proposed to concur with some extent of neuronal damage and shift to a "neuropathic phenotype" (Braz, JM and Basbaum, AI 2010). Different agents and the severity of the local damage might dictate the existence or not of a neuropathic component in these cases (triggered by an inflammatory initial condition).

Also controversial is the expression of ATF3 in peripheral glial cells. Some showed that ATF3 immunoreactivity was present not only in the sensory neurons' cell bodies but also in the SGCs of all ganglia after paclitaxel treatment in rats, while the same was not observed in ganglia of vehicle-treated animals. The highest number of ATF3-immunoreactive (IR) SGCs was observed in lumbar ganglia as well as the greatest number of nodules of Nageotte (Jimenez-Andrade, JM *et al.* 2006). Others also showed expression of ATF3 in Schwann cells during sciatic nerve repair in diabetic rats (Stenberg, L *et al.* 2012) and sciatic nerve compression (Isacsson, A *et al.* 2005). However, this has not been generally reported, perhaps because the expression is much weaker than in the sensory neurons (Hunt, D *et al.* 2012) or due to the different mechanisms triggered upon different initial stimuli. Therefore, the relevance of glial expression of ATF3 in a pathological conditions is still unknown

Taking all this in consideration, it seems of extreme relevance to further investigate the role of ATF3 in both inflammatory and neuropathic conditions in order to better

understand how these underlying mechanisms converge over time. Consequently, unraveling the ATF3 signaling pathways will also shed light into the transition from acute to persistent pain and into how the cell fate of the neurons is decided in each case (between survival, regeneration, apoptosis…). Moreover, it is also important to evaluate the ATF3 possible effect(s) on pain processing since these signaling pathways might act as regulating events with relevant contributions for the nociception-related mechanisms, such as those implicated in neuron-glia communication (SGCs activation/proliferation) or neuronal regeneration.

2.8 ATF3 signaling pathways: interactions with other proteins

Peripheral injury or inflammation elicits a sequence of molecular, cellular and ultrastructural responses at the DRG level. Among these, the **expression of ATF3** in sensory neurons was curiously observed in both conditions. Interestingly, evidence suggests that ATF3 is much more than a marker of neuronal injury, but its complex interactions with other transcription factors and proteins make it hard to evaluate the role of ATF3 in the injured nervous structures (Hunt, D *et al.* 2012). As previously mentioned, ATF3 can be part of a wide range of signaling pathways that, according to the cellular context (extracellular signals), may decide the cell fate (Hai, T *et al.* 2010, Hunt, D *et al.* 2012). The existence of activation and repression isoforms also expands the regulatory functions of this factor. However, the majority of the studies showing that ATF3 is an adaptive gene involved in the pathogenesis of several diseases were carried out in non-neuronal cells (Hai, T *et al.* 1999, Hai, T *et al.* 2010). Thus, the role of ATF3 in the nervous system still remains to elucidate and part of these signaling cascades still need *in vivo* validation.

Further investigating these events seems crucial to understand the transition from acute to chronic pain as well as identifying the "programs" triggered at each stage of a

painful condition, that basically decide the cell fate. In this context, it is important to overview the ATF3 most important signaling pathways, and its association with other relevant molecules.

Neuroprotection and regeneration pathways

ATF3 expression in many tissues is associated with dysfunction and disease progression, but interestingly, in neurons its expression is normally correlated with axonal regeneration and neuroprotection. Indeed, ATF3 was proposed to promote cell survival and enhance neurite outgrowth, in a c-Jun dependent pathway involving heat shock protein 27 (HSP27) and Akt activation (Nakagomi, S *et al.* 2003). Moreover, ATF3 was shown to induce the regeneration of sensory axons. In transgenic mice, the constitutive expression of ATF3 in DRG neurons enhanced the rate of nerve regeneration, following sciatic nerve injury (Seijffers, R *et al.* 2007). This phenomenon was again accompanied by an increased expression of regeneration-associated genes such as small-proline rich protein 1A (SPRR1A), HSP27 and c-Jun. In that study, no changes in GAP-43 or the signal transducer and activator of transcription 3 (STAT3), a regeneration-related transcription factor, were observed (Seijffers, R *et al.* 2007). However, others have suggested a parallel increase in the expression of ATF3 and GAP-43 in a model of osteoarthritis (Su, J *et al.* 2015) and increased GAP-43 expression in ATF3-positive neurons (Ferreira-Gomes, J *et al.* 2012), which supports a relation between these proteins and a role for ATF3 in neuronal regeneration (Ferreira-Gomes, J *et al.* 2012).

In fact, due to their high co-expression following traumatic injury and other stressful stimuli, c-Jun is proposed as a prime candidate for controlling ATF3. However, increases in c-Jun are not required for ATF3 upregulation in primary sensory neurons (Tsujino, H *et al.* 2000). Mitogen-activated protein kinase (MAPK) pathways are also involved in the cellular response to many of these external stimuli. Among these, the p38 pathway,

extracellular signal-regulated kinase (ERK) and the c-Jun N-terminal kinase (JNK) pathways are also known to regulate ATF3 expression (Hai, T *et al.* 1999, Lu, D *et al.* 2007, Hai, T *et al.* 2010, Lee, JR *et al.* 2014).

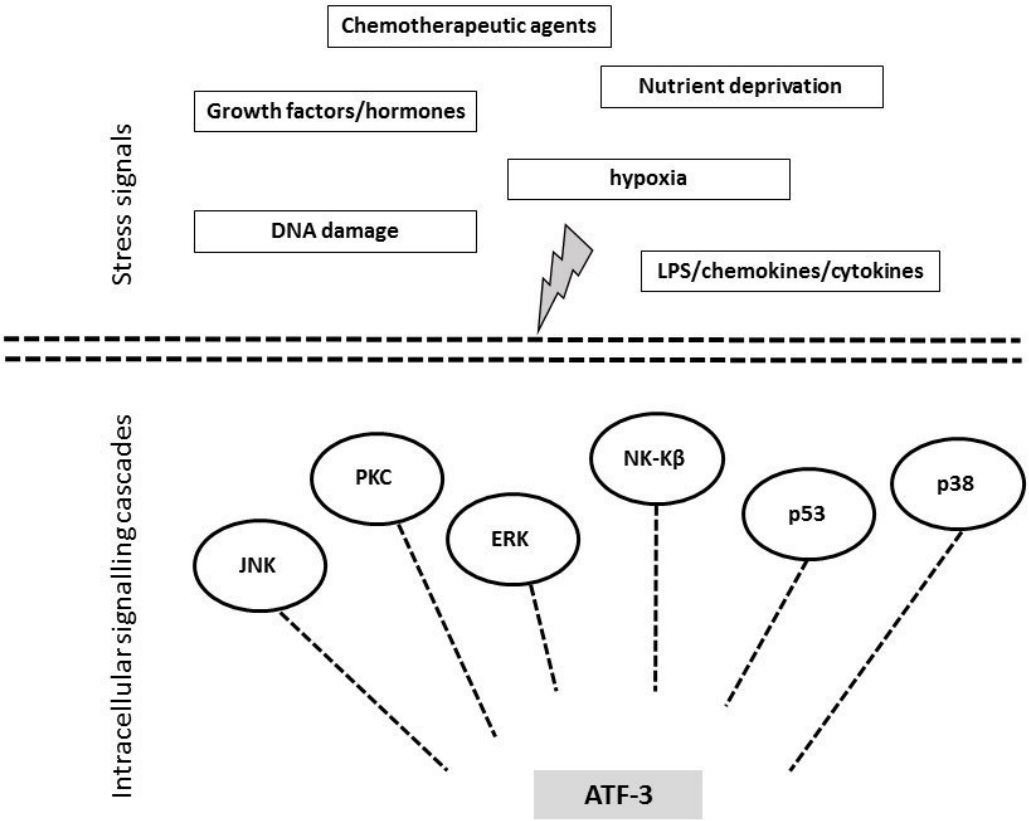


Fig. 10 – ATF3 as a hub of the cellular adaptive-reponse network. ATF3 is involved in the pathogenesis of diseases, being triggered by diverse extracellular stress signals and involved in several signaling pathways (modified from Hai, 2010 #234).

Inflammatory signaling pathways

Interestingly, ATF3 is known to be involved in the resolution of the inflammatory response by negatively regulating the toll-like receptor 4 (TLR4) pro-inflammatory signalling pathway (Gilchrist, M *et al.* 2008). TLRs are pathogen recognition receptors activated during innate immune system responses and known to identify both pathogen–

associated molecular patterns (PAMPs) and danger–associated molecular patterns (DAMPs; (Li, J *et al.* 2013). There are several subfamilies according to the type of host they recognize. TLR4 is one of these receptors highly involved in inflammatory responses, activating signaling pathways that result in the production of proinflammatory cytokines and chemokines. LPS, a membrane component typical of Gram-negative bacteria, is one of its well-known ligands. Besides microglia in the CNS and macrophages, TLR4 is also expressed in primary afferents of the sensory ganglia. (Barajon, I *et al.* 2009). Curiously, and contrarily to what happens in the CNS, TLR4 cannot be found in glial cells (SGCs) at the DRG.

Activation of TLR4 during inflammation induces ATF3 expression through stimulation of the JNK, p38 MAPK and nuclear factor κB (NF-κB) pathways, while ATF3, in its turn, exerts a negative feedback control on this cascade (Whitmore, MM *et al.* 2007, Suganami, T *et al.* 2009, Lai, PF *et al.* 2013, Park, HJ *et al.* 2014). In this negative control, ATF3 down-regulates pro-inflammatory mediators like TNFα and also IL-1β (Suganami, T *et al.* 2009), possibly to control the extent of the tissue damage. Indeed, evidence strongly suggest that ATF3 expression in the nervous system is majorly protective (Hunt, D *et al.* 2012).

Moreover, in mouse embryonic fibroblasts and macrophages, ATF3 was shown to mediate a mechanism of IL-6 down-regulation via the heat shock transcription factor 1 (HSF1;(Takii, R *et al.* 2010). HSF1 is a highly conserved transcription factor that coordinates stress-induced transcription (of genes like ATF3) and strongly induces transactivation of heat shock proteins (HSPs; (Vihervaara, A and Sistonen, L 2014). HSPs are abundant and highly conserved chaperones that, likewise ATF3, are dramatically increased in cells upon stress (supposedly as “danger signals” to promote protection; (Osterloh, A and Breloer, M 2008)). They are crucial for the survival of eukaryotic cells by promoting the correct folding of client proteins. Although ATF3 has been associated with

other HSPs (Nakagomi, S *et al.* 2003), its relation with HSP90 recently stand out. Indeed, ATF3 was shown to be regulated by HSP90 at the mRNA stage (Sato, A *et al.* 2014) while in cancer derived cell lines HSP90 inhibition induced ATF3 expression (Hackl, C *et al.* 2010). Interestingly, HSP90 is an attractive chaperone in the context of inflammation because it induces the production of proinflammatory cytokines (by the monocyte-macrophage system) via the TLR4 signal transduction pathways (Tsan, MF and Gao, B 2004), while HSP90 inhibitors are known to attenuate these responses (Hutchinson, MR *et al.* 2009, Yun, TJ *et al.* 2011, Qi, J *et al.* 2014). Therefore, besides their well-documented effects in cancer treatment (Neckers, L and Neckers, K 2002, Soti, C *et al.* 2005, Neckers, L 2007), recent studies started exploring the potential anti-inflammatory properties of HSP90 inhibitors. Even though this is not clear, evidence suggest that ATF3 could be part of the TLR4-HSP90 signalling pathway in inflammation.

Pain-related pathways

It is still unknown whether ATF3 plays a relevant role in nociception or not, and probably the major reason has been the lack of efficient transgenic mice for a long time (Hunt, D *et al.* 2012). However, many studies suggest that ATF3 regulates signaling pathways that are, directly or indirectly, associated with pain processing. Indeed, in TLR4 deficient mice, both the cisplatin-evoked allodynia and the induced ATF3 expression in DRG neurons were reduced in comparison to wild type mice. This not only suggests that the neuronal ATF3 is regulated by the activation of the TLR4 cascades in the DRG, but also that those events might be correlated with pain processing (Park, HJ *et al.* 2014). However, others have shown that TLR4 activation was not sufficient to induce pain sensation and that second mediators were necessary. Indeed, they demonstrated that

HSP90 is required, as part of the TLR4-mediated pathway, for enhancement of CCI-induced allodynia (Hutchinson, MR *et al.* 2009).

Despite the lack of more knowledge concerning the role of chaperones in the nociception, HSP90 was interestingly shown to be one of the 11 molecules that make part of a P2X7 receptor-protein complex (Adinolfi, E *et al.* 2003). Since P2X7 is highly associated with pain states, being the major responsible for the activation of SGCs in these conditions (Chen, Y *et al.* 2008, Arulkumaran, N *et al.* 2011, Alves, LA *et al.* 2013), the presence of HSP90 in this complex might also support a role for this chaperone in nociception. Moreover, the reported association of ATF3 with HSP90 (Hackl, C *et al.* 2010) might again suggest the involvement of this transcriptional factor in pain signaling cascades.

Also curious is that some studies suggest that ATF3 and P2XR might be included in the same signaling cascades, contributing to the development of pain states. Indeed, in a model of neuropathy induced by resiniferatoxin (RTX), a capsaicin analog, the number of $P2X3^+/ATF3^+$ sensory neurons was linearly correlated with increased mechanical thresholds (Hsieh, YL *et al.* 2012). As previously mentioned, P2X3R is intimately correlated with pain states (Wirkner, K *et al.* 2007) and implicated in neuron-SGCs interactions, in a P2X7R-mediated mechanism (Chen, Y *et al.* 2012). Indeed, some authors defend that it is the expression of injury factors (like ATF3) in damaged neurons that might signalize and initiate these neuron-glia communication events (Elson, K *et al.* 2004).

Therefore, although evidence might suggest the involvement of ATF3 in nociception (possibly through P2X receptors, TLR4, HSP90), not much is known so far. Particularly in DRG neurons, it is still not fully understood how ATF3 expression is correlated with pain processing events and which are the principal signaling cascades.

Aims

3. Aims

Joint inflammatory conditions are one of the major causes for chronic debilitating pain with an alarming increase in the number of cases worldwide. In fact, due to their complexity, pain processing mechanisms are still not fully understood. The lack of more knowledge might account for the substantial inefficacy of the most common treatments. Taking this in consideration, **the main aim of this work was to explore the pathophysiological mechanisms underlying the MA inflammatory pain condition, at the DRG level, namely the involvement of ATF3.** In order to do so, several tasks were designed, each devoted to a specific objective.

Increasing evidence showing that inflammatory and neuropathic conditions mechanistically converge along disease progression, together with studies suggesting the occurrence of neuronal damage in joint pain, were the first triggers that led us to explore these events in MA. Thus, as a first objective we aimed at investigating the expression of the neuronal injury marker ATF3 in primary afferents of MA rats along the disease progression, as an indication of a possible transition to a "neuropathic phenotype" in the pathophysiology of the disease. To better understand the possibility of a neuropathic component in this condition, ATF3 expression was also evaluated in DRG of MA animals treated with ketoprofen, a NSAID used for the amelioration of the inflammatory component (**Publication I**). We then aimed at identifying the DRG neuronal population(s) expressing ATF3 in order to find out the type of nociceptors being injured which would allow us not only to infer about the expression of other molecules but also about ATF3 involvement in nociception (**Publication I**).

While studying MA pathophysiological mechanisms at the DRG, the emerging role of SGCs in pain processing as well as their active intervention in neuron-glia communication within the sensory ganglia, soon stand out. Thus, we evaluated both the activation (by

quantifying the expression of GFAP) and the proliferation (by quantifying the incorporation of bromodeoxyuridine - BrdU) of SGCs in the DRG of MA animals, along the disease progression (**Publication II**). Moreover, since some suggested it is the expression of injury factors that initiate neuron-glia crosstalk mechanisms, we speculated about a role of ATF3 in regulating these events .Therefore, to understand if activation of SGCs was preferentially occurring around damaged neurons, and find some sort of correlation between these events, we also evaluated the SGCs activation around ATF3-expressing neurons (**Publication II**).

Interestingly, P2X receptors had been greatly associated with activation of SGCs and proposed as the most relevant players in neuron-glia communication (Chizh, BA and Illes, P 2001). Thus, we then evaluated the temporal profile of P2X7R and P2X3R expression in sensory ganglia of MA animals **(Publication III)**. However, whether ATF3 was regulating the expression of these receptors was still not clear. To do so, we silenced ATF3 by using siRNA in primary cell cultures of DRG and assessed the expression of P2X7R and P2X3R (at mRNA level). To also investigate the effect of ATF3 knock-down on SGCs activation, GFAP expression was equally quantified **(Publication III).** Moreover, in this study, we also evaluated the expression of HSP90, upon ATF3 suppression. Indeed, HSP90 is also a stress inducible gene expressed upon inflammation (via TLR4-signaling pathway) (Tsan, MF and Gao, B 2004, 2009) but poorly explored at the DRG. Besides being previously associated with ATF3 (Hackl, C *et al.* 2010, Sato, A *et al.* 2014), HSP90 had been described to be part of a P2X7R-protein complex (Nollen, EA and Morimoto, RI 2002, Adinolfi, E *et al.* 2003), which altogether prompted us to investigate the mRNA levels of the HSP90 chaperone in ATF3-silenced cell cultures.**(Publication III).**

Taking in consideration the results from these *in vitro* experiments, as well as the known great involvement of HSP90 in the inflammatory response, we hypothesized about a role of HSP90 in MA pathophysiological mechanisms. To test this, the expression of the

two isoforms of HSP90 was then evaluated in the DRG of MA animals by real time quantitative polymerase chain reaction (RT-qPCR), along with the expression of ATF3, GFAP, P2X3 and P2X7, to confirm previous data **(Publication IV)**.

In order to better explore the role of HSP90 in MA, we then intrathecally administered an HSP90 inhibitor, 17-(Dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) to inflamed animals and assessed the effect of this drug on nociceptive behavior **(Publication IV)**. Indeed, besides the successful use of these drugs in the treatment of cancer (Neckers, L and Neckers, K 2002), neurodegenerative diseases (Waza, M *et al.* 2006) and inflammatory conditions (Madrigal-Matute, J *et al.* 2010), HSP90 inhibition had also been shown to alleviate pain in a neuropathic model (Hutchinson, MR *et al.* 2009) suggesting a novel role of this chaperone in nociception.

Subsequently, we investigated the effect of this inhibition on the gene expression of ATF3, since its association with HSP90 had been previously reported (Hackl, C *et al.* 2010). Moreover, HSP90 had been found to promote glial activation while HSP90 inhibition resulted in suppression of these mechanisms (Kakimura, J *et al.* 2002, Lisi, L *et al.* 2013). Therefore, after 17-DMAG administration to MA animals, we also analyzed the expression of ATF3, GFAP, P2X3 and P2X7 at the DRG **(Publication IV)**. Furthermore, the level of HSP90 cleavage was assessed as a functional relevant event since it is highly promoted by reactive oxygen species (ROS) that are increased in inflammatory conditions like MA **(Publication IV).**

Results

4.1 Publication I

Neuronal injury marker ATF-3 is induced in primary afferent neurons of monoarthritic rats. Neurosignals (2011)

Original Paper

Neuro-Signals

Neurosignals 2011;19:210–221
DOI: 10.1159/000330195

Received: April 7, 2011
Accepted after revision: June 16, 2011
Published online: September 6, 2011

Neuronal Injury Marker ATF-3 Is Induced in Primary Afferent Neurons of Monoarthritic Rats

Diana Nascimento Daniel Humberto Pozza José Manuel Castro-Lopes
Fani Lourença Neto

Departamento de Biologia Experimental, Faculdade de Medicina do Porto e Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Porto, Portugal

Key Words
Activating transcription factor 3 · Calcitonin gene-related peptide · Isolectin B4 · pAkt · Dorsal root ganglia · Joint inflammatory pain · Neuronal damage · Immunohistochemistry

Abstract
Activating transcription factor 3 (ATF-3) expression has been associated with several signaling pathways implicated in cellular stress response in many cell types and is usually regarded as a neuronal damage marker in dorsal root ganglia (DRG). We investigated ATF-3 expression in primary afferents in the monoarthritic (MA) model of chronic inflammatory joint pain. Immunohistochemistry revealed that ATF-3 is highly induced mainly in small and medium neurons, especially at 2 and 4 days of MA in L_5 DRGs. Colocalization with calcitonin gene-related peptide (CGRP) and isolectin B4 (IB4) demonstrated that ATF-3-immunoreactive cells are mainly peptidergic. The lack of significant differences in ATF-3 and pAkt colocalization indicated that ATF-3 is probably not involved in a pAkt-mediated survival pathway. Anti-inflammatory (ketoprofen) administration failed to reverse ATF-3 induction in MA rats, but significantly increased CGRP expression. These data suggest that ATF-3 expression is definitely involved in MA, actually marking injured neurons. Some degree of neuronal damage seems to occur right from the first days of disease, mainly affecting small-to-medium peptidergic neurons. The intra-articular injection of complete Freund's adjuvant and the generation of a neuroinflammatory environment seem to be the plausible explanation for the local nerve damage.

Introduction

Joint inflammation is a major clinical problem and a main cause of debilitating chronic pain, characterized by pronounced mechanical hyperalgesia and persistent pain at rest [1]. Pain mechanisms require the sensitization of primary sensory neurons, whose cell bodies are located in dorsal root ganglia (DRG), and involve several mediators that trigger particular signal-transduction pathways. A great part of these pathways imply the activation of transcription factors, in which gene expression becomes altered. In particular pathological conditions, DRGs are actually responsible for the synthesis of signaling molecules involved in reaction cascades that can ultimately lead to phenomena like survival or regeneration. Elucidation of these related molecular mechanisms is crucial to understand pain transmission processing in an inflammatory condition, bringing into light possibilities for new treatment approaches [2].

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1424–862X/11/0194–0210$38.00/0

Accessible online at:
www.karger.com/nsg

Fani Lourença Moreira Neto
Departamento de Biologia Experimental
Faculdade de Medicina da Universidade do Porto
Alameda Prof. Hernâni Monteiro, PT–4200-319 Porto (Portugal)
Tel. +351 22 551 3654, E-Mail fanineto@med.up.pt

Activating transcription factor 3 (ATF-3) has been suggested as acting as an 'adaptive response' due to its ability to respond differently accordingly to the cellular context [3]. Its inclusion in anti- and pro-apoptosis mechanisms [4], cell survival [5], regeneration [6] and neuroprotection [7] signaling events has been reported. In a pAkt-mediated survival pathway the ATF-3/c-Jun heterodimer has been suggested to promote nerve elongation and inhibit apoptosis in neurons under death stress such as nerve injury [8]. In pain models, a marked increase in pAkt levels was observed following peripheral nerve injury, whereas carrageenan-induced inflammation induced only slight increases in the number of pAkt-positive DRG neurons without affecting the activated (phosphorylated) protein levels. Despite the significance of these results, a very limited effect of the intradermal carrageenan injection, and the associated inflammatory mechanisms, was demonstrated [9]. However, the role of these biomolecules in pain pathophysiology is still to be unmasked.

Nowadays, ATF-3 is mostly assumed as a neuronal injury marker, after its expression was found to be highly induced in several models of neuropathic pain [10–12]. Interestingly, in the collagen-induced arthritis and monoiodoacetate-induced osteoarthritis joint pain models, ATF-3 was also expressed in DRG neurons [13, 14], suggesting some degree of neuronal damage is occurring. However, data on ATF-3 expression in primary afferents in different models of inflammatory pain have not always been consistent [15–17]. For example, intraplantar injection of complete Freund's adjuvant (CFA) did not induce any ATF-3 expression, suggesting there is no neuronal damage associated [15, 16].

Taking into account these controversial data and the sparse information available on the role of ATF-3 in pain processing, we aimed to investigate its expression pattern in primary afferents during different timepoints of monoarthritis (MA), a well established model of chronic joint inflammatory pain induced by injection of CFA into the tibiotarsal joint [18]. In order to characterize the neuronal populations most implicated, we further analyzed the ATF-3-expressing cells' size distribution and colocalization with isolectin B4 (IB4) or calcitonin gene-related peptide (CGRP) [19]. CGRP has early been described to potentiate pain signaling from primary sensory neurons to the spinal cord, functioning as a mediator of neurogenic inflammation at the periphery [20]. It has been recognized as a nerve regeneration-promoting peptide, after neuronal damage [21]. More recently, its expression was shown to be either increased or decreased depending on the sciatic nerve injury, indicating that the nature of the peripheral injury has an impact on CGRP expression [22] even affecting different neuronal populations [23]. Thus, possible changes in CGRP expression during MA were also evaluated. In order to investigate the trigger of a possible survival pathway associated to ATF-3 induction in MA, pAkt expression was also analyzed. Additionally, we administered an anti-inflammatory non-selective cyclooxygenase (COX) inhibitor (ketoprofen) in order to evaluate its effect on ATF-3 and CGRP expression during establishment of MA. Parts of this work have been published in abstract form [24, 25].

Materials and Methods

Animal Handling and MA Induction

Experiments were carried out in adult male Wistar rats (Charles River Laboratories, France) weighing between 200 and 300 g. A total of 42 animals were used in this study but 2 of them were excluded since they developed signs of polyarthritis (see Results). Animals were housed 2–3 animals per cage under controlled conditions of lighting (12-hour light/12-hour dark cycle) and temperature as well as water and food ad libitum. Efforts were made in order to minimize pain and distress and reduce the number of animals used. All procedures were carried out according to the European Communities Council Directive 86/609/EEC amended by the Directive 2003/65/CE of July 22, 2003 and to the ethical guidelines for investigation of experimental pain in animals [26]. MA was induced by injecting 50 μl of CFA into the left tibiotarsal joint [18] under isoflurane anesthesia (5% for induction, 2.5% for maintenance). CFA was prepared as previously described [27] and MA animals were allowed to survive for 2, 4, 7 and 14 days. Control animals were injected with 50 μl of CFA vehicle (composed by the same reagents as CFA except for *Mycobacterium butyricum*) and allowed to survive for 2 days. To minimize fear-motivated behaviors, animals were habituated to the experimenter for several days before CFA injection and during the progression of MA. The evolution of the inflammatory reaction was monitored daily and was scored according to Castro-Lopes et al. [28]. This score takes in consideration the inflammatory signs of the injected ankle and reduction of the locomotor activity. Score 0 means no inflammatory signs. Score 1 indicates the presence of minor changes such as redness and swelling and score 2 denotes more intense swelling and some avoidance of passive movements. In score 3, additionally, rats show reluctance to place weight over the affected limb. In score 4, there is severe inflammation with persistent flexion of the affected limb and repercussion over the motor activity of the animal. In order to better evaluate the severity of inflammation, the diameter of the animals' affected paw was also measured, right before sacrificing them.

Immunohistochemistry

All animals were perfused through the ascending aorta with 250 ml of oxygenated Tyrode's solution followed by 750 ml of paraformaldehyde 4% in phosphate buffer (PB) 0.1 M, after intraperitoneal anesthesia with chloral hydrate 35% (0.1 ml/100 g of animal weight). The ipsi- and contralateral DRGs corresponding to spinal segments L_3, L_4 and L_5 were removed and post-fixed in the same

fixative solution for 4 h and then cryoprotected overnight (sucrose 30% in phosphate buffer saline (PBS) 0.1 M). The DRGs, either belonging to CFA vehicle-injected controls or inflamed CFA-injected animals with 2, 4, 7 and 14 days of MA, were cut sequentially into 14-μm sections in a freezing cryostat (–20°C). The material was collected into poly-L-lysine-coated slides, air dried and stored at –20°C until immunohistochemistry was performed.

After the blocking step in the correct normal serum (normal goat serum for ATF-3 single and ATF-3/pAkt double immunohistochemistry and normal horse serum for triple immunoreaction against ATF-3, CGRP and IB4), in order to avoid unspecific bindings, slides containing every fifth section of each DRG were incubated for 48 h at 4°C in appropriate primary antibodies. Sections were then incubated for 1 h at room temperature in the suitable secondary antibodies. For the single immunolabeling against ATF-3, L_3, L_4 and L_5 ipsi- and contralateral DRG sections (n = 5 rats for each experimental group) were incubated in polyclonal rabbit anti-ATF-3 (1:500; C-19: sc-188; Santa Cruz Biotechnology, Inc.). Detection was achieved by Alexa 594 goat-anti-rabbit (1:1,000; A11012; Molecular Probes, USA). For the double immunolabeling against ATF-3 and pAkt, L_5 ipsilateral DRG sections (n = 4 rats for controls; n = 5 rats for each of the other experimental groups) were incubated in polyclonal rabbit anti-ATF-3 (1:500) and monoclonal mouse anti-pAkt (1:1,000; 5106S; Cell Signalling). Detection was achieved by Alexa 594 goat-anti-rabbit (1:1,000) for ATF-3, and Alexa 488 goat-anti-mouse (1:1,000; A11029; Molecular Probes) for pAkt. For the triple immunolabeling against ATF-3, CGRP (neuronal marker for peptidergic primary afferents) and IB4 (neuronal marker for non-peptidergic primary afferents), L_5 ipsilateral DRG sections (n = 5 rats for controls and the 4d MA; n = 4 rats for each of the other experimental groups) were firstly incubated in polyclonal rabbit anti-ATF-3 (1:500) and polyclonal sheep anti-CGRP (1:4,000; ab22560; Abcam, Cambridge, UK). Detection was achieved using Alexa 488 donkey anti-rabbit (1:1,000; A21206; Molecular Probes) for ATF-3, and Alexa 568 donkey anti-sheep (1:1,000; A21099; Molecular Probes) for CGRP. Slides with the DRG sections were afterwards incubated in the biotin-conjugated IB4 from *Bandeiraea simplicifolia* (1:1,000; L2140; Sigma-Aldrich) specially diluted in a PBS with Triton X-100 (PBST) solution (without normal serum), containing magnesium chloride ($MgCl_2$), manganese chloride ($MnCl_2$) and calcium chloride ($CaCl_2$) in a 1:50 final concentration, for one night, at room temperature. Detection was achieved by incubation in Streptavidin 350 (1:200; Jackson ImmunoResearch Laboratories, Inc.). As specificity controls for each immunoreaction, slides were processed in a similar way as described above but without incubation in primary antibody.

At the time of analysis, glass slides containing the immunoreacted sections were coverslipped with glycerol prepared with PBS 0.4 M and visualized under fluorescence microscope.

Ketoprofen Treatment

MA was induced in rats as described above, and ketoprofen, a non-steroidal anti-inflammatory COX inhibitor drug, was daily administered subcutaneously (5 mg/kg of rat/24 h). In a first experimental group, ketoprofen daily treatment started at day 0, when MA was induced by CFA injection, to diminish the development of the neuroinflammatory environment right from the beginning. In a second experimental group, ketoprofen daily treatment started only after day 2 of MA, as we knew that ATF-3 was already significantly induced in L_5 ipsilateral DRGs at this timepoint. A control group of rats was injected with CFA into the tibiotarsal joint to induce MA, and received a daily subcutaneous saline injection. All animals were perfused at 4 days of MA (n = 5 rats for each experimental group), the peak of ATF-3 expression, and the L_5 ipsi- and contralateral DRGs were dissected and further processed for immunohistochemistry against ATF-3 (n = 4 rats for each experimental group) and CGRP (n = 5 rats for each experimental group) as described above. All animals were evaluated for the severity of inflammatory symptoms by using an appropriate score [28] and by measuring the diameter of the inflamed paw as described above.

Data Analysis

Cell Counting

Immunohistochemistry analysis was performed in a blinded manner using a fluorescence microscope (AxioImager Z1; Zeiss) coupled to a digital camera (AxioCam MRm) and computer image software (AxioVision 4.6) to grab the images. For the photomicrographs, the acquisition conditions such as amplification of the objective, light intensity, contrast and hue were maintained constant.

For ATF-3 single labeling, all immunoreactive (IR) cells were counted in every fifth section of the ganglion and divided by the total of tissue sections containing neuronal cell bodies present in the respective glass slides (approximately 9–12 sections per DRG). ATF-3 nuclear labeling is very clear and undoubtedly easy to detect, so only cells where nuclei were visualized were considered. For quantification of pAkt-IR cells in ATF-3+pAkt double labeling, a threshold was established based on labeling for fibers, and only cells with a signal above that threshold were considered as positive. This was performed with the help of the ImageJ® version 1.37 (free access software) computer program. At least 350 total cells with visible nuclei per rat (corresponding more or less to a sample of 1,500 cells per experimental group) were randomly chosen from 8 to 9 (every fifth) sections of the same DRG and were counted, as similarly described before [23, 29]. pAkt immunoreactivity was expressed as the percentage of pAkt-IR cells in the total number of counted neurons. Colocalization between pAkt and ATF-3 was expressed as the percentage of double IR cells in the total number of ATF-3-IR cells (ATF-3 neuronal population). For the triple immunolabeling against ATF-3, CGRP and IB4, quantification was done also randomly in 8–9 sections by using an approach similar to that described for pAkt. Depending on the marker this corresponded to 400–750 labeled neurons per experimental group. Thus, in order to evaluate the expression of ATF-3 and the two markers individually, IR cells (for each of the three molecules) were counted and divided by the total number of selected neurons. To obtain percentages of colocalization between ATF-3 and CGRP or ATF-3 and IB4, double labeled cells were also counted and divided by the total number of ATF-3-expressing neurons [15]. This same random selection of at least 350 total cells in 8–9 sections of the same DRG and a similar calculation of percentage values was used in single CGRP labeling for both untreated MA and ketoprofen-treated MA animals.

Cell Size Distribution

Areas of all the ATF-3-IR cells were measured in all experimental groups (n = 4 rats for 7d MA; n = 5 rats for each of the other experimental groups), in order to evaluate possible switches in ATF-3-positive neuronal population(s) along MA progression. CGRP-positive neuron areas were measured in the same IR cells

randomly selected for cell counting (n = 5 rats for controls and the 4d MA; n = 4 rats for each of the other experimental groups). IR cells with visible nuclei were manually outlined using a computer mouse and the cross-sectional area was determined using the ImageJ version 1.37 (free access software) [29, 30]. Cell areas were grouped into three categories: small (<600 μm^2), medium (600–1,200 μm^2) and large neurons (>1,200 μm^2) as described by Noguchi's group [31].

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 5® (GraphPad Software) and SPSS 13.0. One-way analysis of variance (one-way ANOVA) was performed for all data to investigate significant differences between experimental groups, followed by the appropriate post-hoc tests when the level of significance was considered as $p < 0.05$.

Bonferroni post-hoc test was used to discriminate differences between all experimental groups in ATF-3 and pAkt single immunolabeling, ATF-3 and pAkt colocalization and also in triple immunoreactions against ATF-3, CGRP and IB4. This post-hoc test was also used to evaluate differences in paw diameter. The Newman-Keuls multiple comparison test was used to discriminate differences in cell size distribution of ATF-3- or CGRP-positive cells. In order to find differences in ATF-3 or CGRP expression between the non-treated 4d MA rats (non-ketoprofen group), the 4d MA rats treated during 2 days (2d ketoprofen) and the 4d MA rats receiving ketoprofen from day 0 (4d ketoprofen), the Newman-Keuls multiple comparison post-hoc test was also used.

Results

MA Was Successfully Induced

All CFA-injected rats showed severe inflammatory symptoms such as swelling, redness and avoidance to put weight over the inflamed paw. This was reflected in mean inflammatory scores near 4 (maximum score), right after the second day of MA (3.67 ± 0.12), maintaining this condition up to the 14th day (3.75 ± 0.13) in accordance with our previous work [32, 33]. Controls showed insignificant mean scores (0.67 ± 0.11), probably due to local trauma caused by the injection procedure. MA animals' inflamed paws showed also significantly ($p < 0.001$) greater diameters at all inflammatory timepoints (1.24 ± 0.03 cm for 4d MA, for example) than controls (0.59 ± 0.02 cm). Therefore, MA was successfully and homogenously induced in all animals injected with CFA, as they were all showing similar physiological responses at each timepoint of disease.

Some animals in the latter phases of the condition (14 days of MA majorly) may develop polyarthritis, with the contralateral non-injected paw and sometimes the tail starting to show inflammatory signs, as described by Butler et al. [18]. In our study, all animals showing signs of polyarthritis (n = 2 rats) were immediately excluded.

ATF-3 Is Highly Induced in Primary Afferents during MA

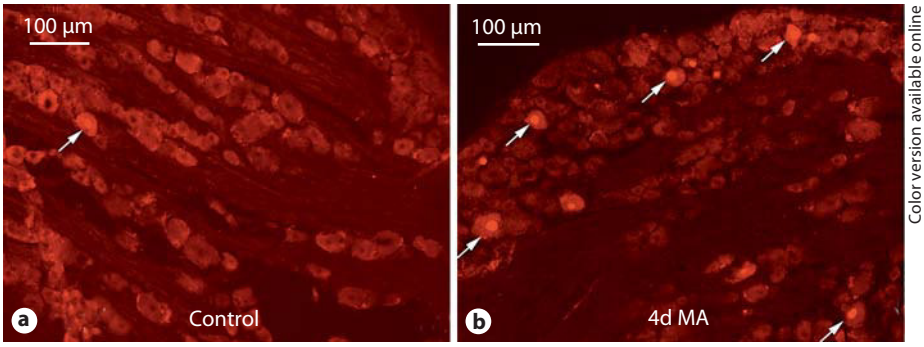
ATF-3 expression was induced in the ipsilateral DRG neurons of MA rats at all timepoints of inflammation (fig. 1b, c). The number of ATF-3-IR cells per tissue slice was significantly increased at 2 and 4 days of MA and it started diminishing after that period, although at 7 and 14 days of inflammation ATF-3 was still induced. Increases were observed in all DRG levels studied (L_3, L_4 and L_5), but were more considerable in L_4 and L_5 ganglia, while DRGs from control rats showed no significant ATF-3 expression (fig. 1c). Statistical significant differences were reached at 4 days of MA for the L_4, and at 2 and 4 days of MA for the L_5 DRGs ($p < 0.05$; one-way ANOVA followed by Bonferroni post-hoc test; fig. 1c). No significant expression of ATF-3 was observed in either controls or contralateral DRGs in any experimental group (fig. 1a).

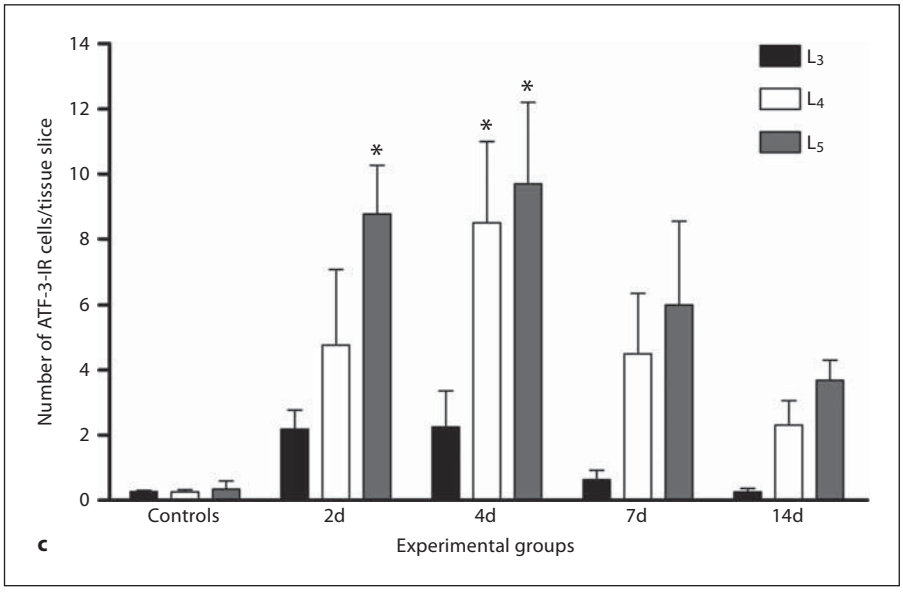
ATF-3-Expressing Cells Are Mainly Small-to-Medium Neurons

Measurement of the cells' areas indicated that ATF-3 is majorly expressed by small- to medium-sized neurons during MA. This is especially obvious for the later stages of disease, since the number of ATF-3-expressing cells belonging to the large-sized group of neurons (>1,200 μm^2) is much lower than that of small (<600 μm^2) and medium cells (600–1,200 μm^2) in the 7 and 14d MA rats (fig. 1d). Statistical analysis of significant differences in the number of ATF-3-IR cells between each of the three distinct sizes within the same experimental group revealed that they were only significant for the 14d MA animals (1.3 ± 0.2, 2.2 ± 0.4 and 0.3 ± 0.1 ATF-3-IR cells/tissue slice for sizes <600, 600–1,200 and >1,200 μm^2, respectively). Greater statistical significance was reached for differences between the number of medium- and large-sized ATF-3-IR cells ($p < 0.001$, one-way ANOVA followed by Newman-Keuls multiple comparison test), reflecting the lower number of ATF-3-expressing cells with a cross-sectional area >1,200 μm^2 (fig. 1d).

Expression of ATF-3 Is Higher in Peptidergic than in Non-Peptidergic Neurons

Analysis of ATF-3 single labeled cells (fig. 2a) corroborated data previously obtained confirming that MA induces ATF-3 expression in ipsilateral primary afferent neurons (table 1). Thus, the ATF-3 expression was always higher in MA animals when compared to controls which showed no ATF-3 expression. As in the previous data, the 2d (with 6.1 ± 1.0% ATF-3-IR cells) and 4d (8.0 ±





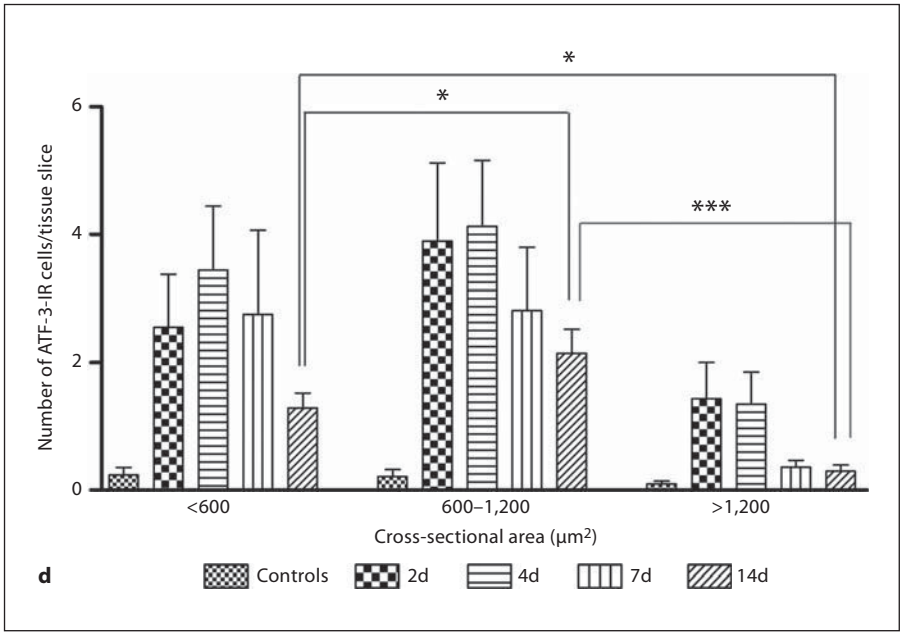
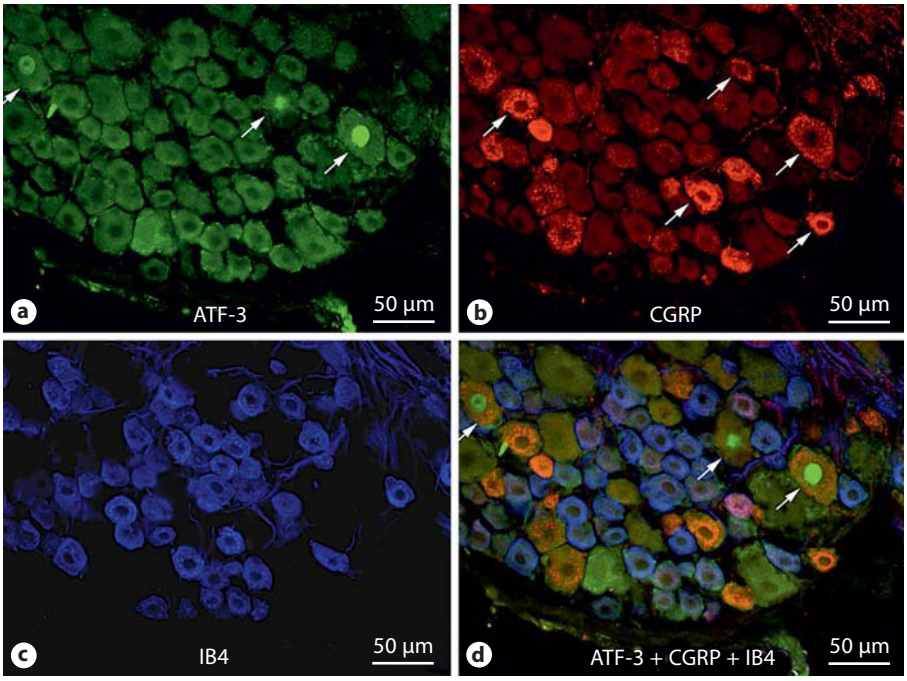


Fig. 1. ATF-3 expression in primary afferents of MA rats. **a**, **b** Fluorescent microscope photo images depicting single immunolabeling for ATF-3 in L_5 ipsilateral DRG neurons from a non-inflamed control rat (**a**) and from a CFA-injected rat with 4 days of MA (**b**). ATF-3-expressing cells are shown with the red-labeled nuclei (white arrows). Scale bar represents 100 µm. **c** The number of ATF-3-IR cells/tissue slice was significantly increased (in comparison to controls) at 4d MA for the L_4 ganglia and at 2d and 4d MA for the L_5 ganglia. Although statistical significances were not reached for other MA timepoints, inflamed animals showed always higher expression than controls. Values shown as mean ± SEM. * represents $p < 0.05$ (one-way ANOVA followed by Bonferroni post-hoc test). n = 5 rats per experimental group. **d** Cell size distribution of ATF-3-expressing cells, as described in Fukuoka et al. [31], revealed they were mainly small (<600 μm^2) and medium-sized (between 600 and 1,200 μm^2) neurons at all timepoints of MA. This is especially evident for the later stages of disease (14d MA) where significant differences were found for different size categories. * represents $p < 0.05$ and *** represents $p < 0.001$. One-way ANOVA followed by Newman-Keuls multiple comparison test for evaluating differences in the numbers of IR cells between each size within each experimental group. n = 5 rats for all groups except for 7d MA with n = 4 rats.



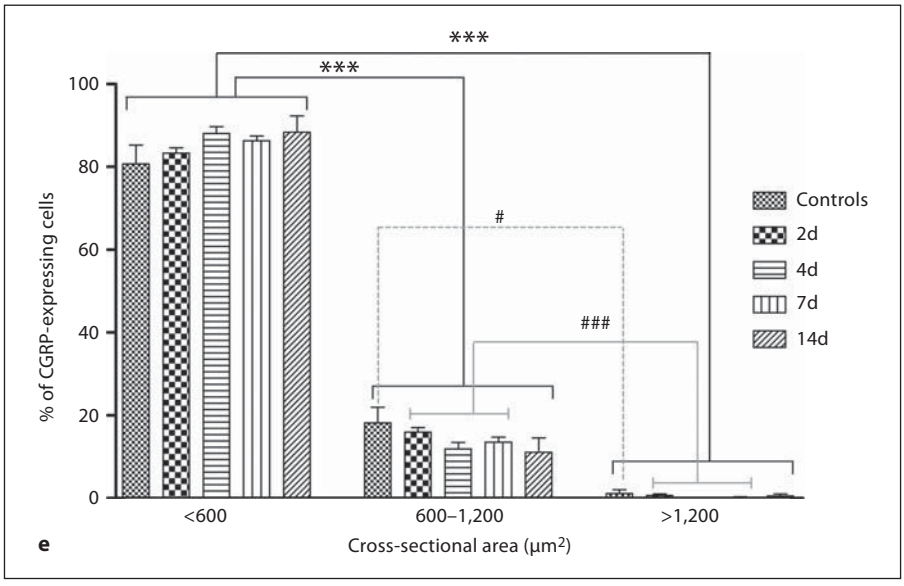


Fig. 2. ATF-3 colocalization with CGRP and IB4, and CGRP expression pattern. **a–d** Fluorescent microscope photo images depicting single immunolabeling for ATF-3 (green nucleus) (**a**), CGRP (red cytoplasms) (**b**), IB4 (blue cytoplasms) (**c**) and triple immunolabeled cells (**d**), in a L_5 ganglia of a 4d MA animal (20× magnification). Scale bars represent 50 µm. **e** Cell size distribution of CGRP-expressing cells in the whole DRG neuronal population revealed no neuronal population switch during MA, also reinforcing that these peptidergic neurons are mainly small-sized. Values shown in percentages as mean ± SEM. * were used to point significant differences between small-sized neurons and the other two size categories (medium and large); # were used to point significant differences between medium- and large-sized neurons. # represents $p < 0.05$ and ***/### represents $p < 0.001$. One-way ANOVA followed by Newman-Keuls multiple comparison test. n = 5 rats for controls and 4d MA, and n = 4 for each of the other experimental groups.

2.4% ATF-3-IR cells) MA animals were the ones showing greater and significantly different values in comparison to vehicle-injected rats ($p < 0.01$ and $p < 0.001$ for the 2d and 4d MA, respectively; one-way ANOVA followed by Bonferroni post-hoc test; table 1).

Colocalization percentages for CGRP (marker of peptidergic primary afferents) and ATF-3 within the ATF-3-positive neuronal population (fig. 2d; table 1) were low in MA rats, especially at later stages of disease (low at 7 days and no colocalization at 14 days; table 1). As expected, no colocalization was found for controls, where no ATF-3 expression was observed. However, statistically significant differences were detected for the population of ATF-3 cells also expressing CGRP. Indeed, colocalization between ATF-3 and CGRP was significantly different for 2d MA (16.0 ± 3.7% CGRP+ATF-3-IR cells in the ATF-3 neuronal population) and 4d MA (26.7 ± 2.4%) when comparing either to 7d or to 14d MA rats (table 1). These

Table 1. Percentage (mean ± SEM) of ATF-3-, CGRP- and IB4-expressing cells in the total DRG neuronal population, and of ATF-3-IR cells also expressing CGRP or IB4 (double labeling), in L_5 DRGs from controls (vehicle-injected) and MA rats at 2, 4, 7 and 14 days post-CFA intra-articular injection

	Controls	n	2d MA	n	4d MA	n	7d MA	n	14d MA	n
% DRG cells expressing ATF-3	0	5	6.1 ± 1.0*	4	8.0 ± 2.4**	5	2.1 ± 0.6	4	1.7 ± 0.4	4
% DRG cells expressing CGRP	33.0 ± 2.6	5	32.0 ± 4.0	4	31.6 ± 1.3	5	27.4 ± 2.4	4	25.7 ± 2.6	4
% ATF-3 cells expressing CGRP	N/A	5	16.0 ± 3.7$^{\#, \S\S}$	4	26.7 ± 2.4$^{\#\#\#, \S\S\S}$	5	3.1 ± 3.1	4	0	4
% DRG cells expressing IB4	50.8 ± 2.9	5	45.1 ± 4.1	4	36.8 ± 2.7	5	47.1 ± 4.5	4	52.3 ± 7.3	4
% ATF-3 cells expressing IB4	N/A	5	3.8 ± 1.5	4	7.0 ± 2.6	5	11.5 ± 7.9	4	4.2 ± 4.2	4

Colocalization between ATF-3 and the neuronal markers used was lower than expected; however, significant differences were found in the colocalization with CGRP for the 2d and 4d MA rats. Similar differences were never found for colocalization of ATF-3 with IB4. * Significant differences to control. # Significant differences to 7d MA. § Significant differences to 14d MA. * or # represents $p < 0.05$; ** or §§ represents $p < 0.01$; ### or §§§ represents $p < 0.001$. One-way ANOVA followed by Bonferroni post-hoc test.

enhanced colocalizations for the 2d and 4d MA reflect the increases in ATF-3 expression during MA at these timepoints, as described above (fig. 1c; table 1). On the other hand, colocalization percentages of ATF-3 and IB4 (marker of non-peptidergic primary afferents) within the ATF-3-positive neuronal population were very low (much lower than ATF-3 and CGRP colocalization) and did not show any significant differences (fig. 2d; table 1).

Quantification of CGRP or IB4 (fig. 2b, c; table 1) single labeled IR cells in ipsilateral ganglia revealed no statistically significant differences in their expression during MA. Also, no noticeable changes in CGRP labeling intensity were seen.

CGRP-Expressing Cells Do Not Undergo a Neuronal Population Switch during MA

The majority of CGRP-expressing cells, in respect to the total CGRP-positive neuronal population, were small-sized neurons (above 80% for all experimental groups), and a smaller portion were medium-sized neurons (around 15%; no significant differences between experimental groups). On the other hand, the percentage of larger CGRP neurons was nearly insignificant (fig. 2e). This pattern for the size distribution of CGRP-IR cells was also observed when analyzing in respect to the total DRG neuronal population (data not shown).

Possible changes in the pattern of size distribution of CGRP-expressing neurons in response to MA were also investigated, but these were not found. In fact, percentage values (both in respect to CGRP-positive or to the total DRG neuronal populations) were very similar when comparing all the different experimental groups (fig. 2e; table 1).

ATF-3 and pAkt Colocalization Has Not Changed during MA

The number of pAkt-IR cells was relatively high at baseline, with ipsilateral L_5 DRGs from control animals showing 52.3 ± 3.0% pAkt-IR cells in the total DRG neuronal population (fig. 3b, d). This number was maintained quite constant throughout the earlier timepoints of MA (50.2 ± 2.0 and 55.0 ± 1.3% for the 2 and 4 days of MA, respectively; fig. 3d), and showed a minor decrease in the later timepoints (44.2 ± 3.5 and 48.0 ± 4.3% for the 7 and 14 days of MA, respectively; fig. 3d), that did not reach statistical significance ($p > 0.05$, one-way ANOVA).

In what concerns pAkt colocalization with ATF-3 (fig. 3c, e), the quantification revealed that the percentage of double-labeled cells in respect to the total population of ATF-3-positive neurons achieved values around 55% (50.4 ± 4.3, 55.8 ± 5.6, 47.8 ± 14.2 and 64.6 ± 4.4 for the 2, 4, 7 and 14 days of MA rats, respectively). This value has not significantly changed between MA inflamed animals, as can be easily observed in figure 3e. No colocalization was found for controls, where none ATF-3 expression was observed.

Ketoprofen Treatment Did Not Affect ATF-3 Induction during MA but Increased CGRP Expression

Ketoprofen-treated MA rats showed significantly ($p < 0.01$) smaller paw diameters (1.1 ± 0.1 and 1.0 ± 0.0 cm for 4dMA+2dKet and 4dMA+4dKet, respectively) in comparison with respective untreated MA animals (1.2 ± 0.0 cm for 4d MA), although main inflammatory signs, as some swelling, redness and still some reluctance to place weight over the affected limb, remained. How-

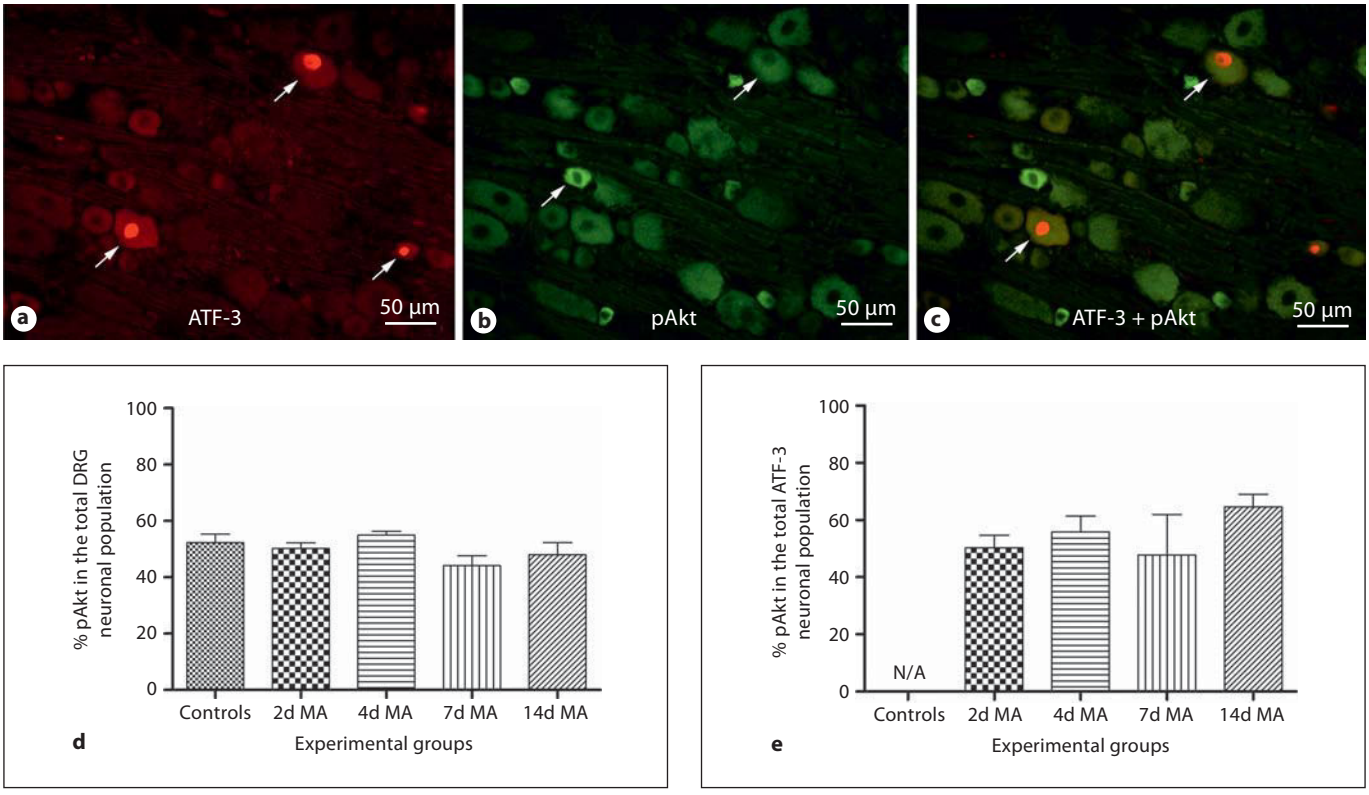


Fig. 3. pAkt expression in primary afferents of MA rats and colocalization with ATF-3. **a–c** Fluorescent microscope photo images depicting single immunolabeling for ATF-3 (red nuclei pointed with white arrows) (**a**), pAkt (green cytoplasms pointed with white arrows) (**b**), and double immunolabeled cells (red nuclei and green cytoplasms pointed with white arrows) (**c**) in a L_5 ganglia from a 4d MA rat. Scale bar represents 50 µm. **d** Quantification of the percentage (%) of single labeled cells for pAkt (pAkt-IR cells) in the total DRG neuronal population, in L_5 ipsilateral DRGs, revealed no statistically significant differences among the experimental groups. **e** Percentage (%) of colocalization between ATF-3 and pAkt did not show any significant difference during disease progression. All values shown as mean ± SEM. One-way ANOVA followed by Bonferroni's test. n = 5 rats for each of the MA experimental groups and n = 4 rats for the control group.

ever, no differences for ATF-3 expression were found (fig. 4g) in MA animals subjected either to 2 (fig. 4c) or 4 days (fig. 4e) of ketoprofen daily injection (6.4 ± 1.2 and 8.1 ± 1.4% for 4dMA+2dKet and 4dMA+4dKet, respectively) when compared to MA animals with no anti-inflammatory treatment (5.7 ± 0.7%; fig. 4a). Contralateral ATF-3 expression in L_5 DRGs from the ketoprofen-treated MA experimental groups was null in accordance to what we had previously observed for MA animals (data not shown).

Regarding CGRP expression (fig. 4h), MA untreated animals showed significantly lower values (31.6 ± 1.3%; fig. 4b) when compared to MA animals treated for both 2 (43.5 ± 2.2%; fig. 4d) or 4 days with ketoprofen (37.8 ± 2.3%; fig. 4f).

Discussion

In this study, it is reported for the first time that CFA intra-articular injection induces an immediate and transient ATF-3 expression in ipsilateral DRGs. ATF-3 expression occurs mainly in small-to-medium peptidergic neurons. No relevant colocalization was found for ATF-3 and pAkt, suggesting ATF-3 is not implicated in a survival pathway involving pAkt during MA. Finally, administration of an anti-inflammatory drug did not reverse ATF-3-induced expression in MA.

As ATF-3 is regarded as a marker of neuronal lesion [12], data suggest that some degree of neuronal damage is probably occurring, at least in an initial phase of the disease. This is supported by studies in the MIA-induced osteoarthritis model (in the knee joint) where a great ex-

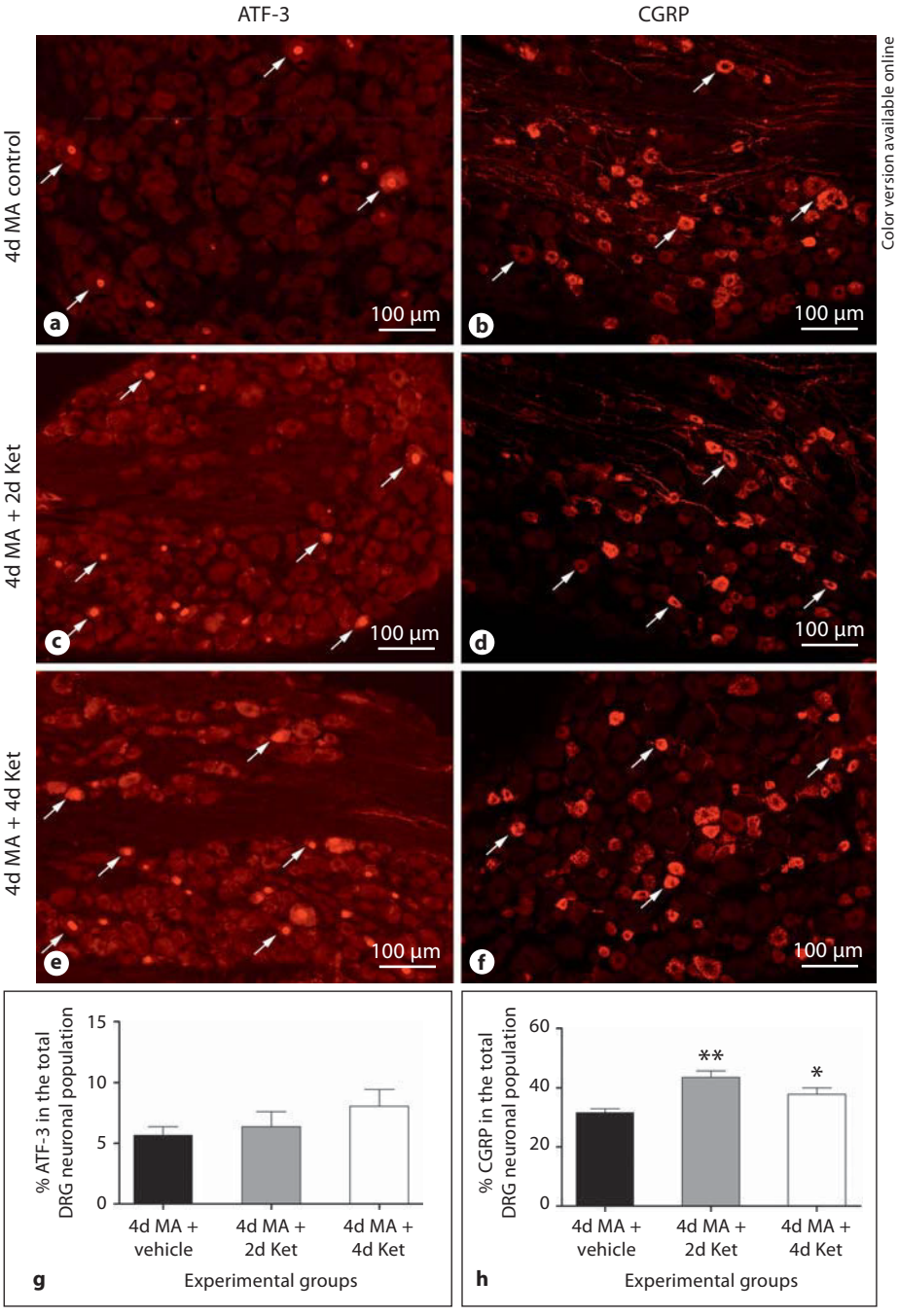


Fig. 4. Effect of ketoprofen treatment on ATF-3 and CGRP expression. **a–f** Fluorescent microscope photo images depicting single immunolabeling for ATF-3 (red-labeled nuclei pointed with white arrows) (**a**, **c**, **e**) and single immunolabeling for CGRP (red-labeled cytoplasms pointed with white arrows) (**b**, **d**, **f**), in L_5 DRGs of 4d MA rats injected with ketoprofen vehicle (**a**, **b**), 4d MA rats with 2d of ketoprofen administration (**c**, **d**) and 4d MA rats with 4d of ketoprofen administration (**e**, **f**). **g** ATF-3 expression was still induced in MA animals upon treatment with the anti-inflammatory drug. No significant differences were found between any groups. Values shown as mean ± SEM. One-way ANOVA followed by Newman-Keuls multiple comparison. n = 4 for each of the three experimental groups. **h** The number of CGRP-IR cells was significantly increased in the L_5 ganglia belonging to both experimental groups with anti-inflammatory treatment. Values shown as mean ± SEM. * represents $p < 0.05$ and ** represents $p < 0.01$. One-way ANOVA followed by Newman-Keuls multiple comparison. n = 5 for each of the three experimental groups.

pression of ATF-3, suggestive of possible neuropathy in the early phase of the disease, was observed [14]. Controversially, others showed that antigen-induced arthritis in the knee did not induce ATF-3 expression, although there was great macrophage infiltration, implying that profound inflammation by itself cannot trigger ATF-3 induction [17]. It seems that peripheral injection of different chemical stimuli induced a stable, time- and dose-dependent significant ATF-3 expression in DRG neurons, while intraplantar injection of CFA failed to evoke ATF-3 induction [15]. Considering our data in MA, it appears that CFA only triggers ATF-3 expression in primary afferents when injected in joints. The intra-articular injection procedure per se does not seem to induce nerve damage,

since no ATF-3 expression was detected in controls. Thus, the only possible neuronal damage inducer is likely to be CFA itself. This may also be related to specific sensitization of joint nociceptors, different for those found in skin [1]. In fact, most studies focus on cutaneous nociception, while joint pain mechanisms are not fully clarified. Chronic pain incidence in deeper tissues and joints might actually reflect enhanced vulnerability of the anatomical structures involved [1]. Additionally, as ATF-3 expression is triggered by TNF-α and IL-1 [3, 34], it is likely that the neuroinflammatory environment generated in response to CFA leads to local nerve damage in the joint and consequently induces ATF-3 expression in primary afferent neurons. In support, Dilley et al. [35] found ATF-3 was upregulated in DRGs following local nerve inflammation of intact sciatic nerves induced by wrapping oxidized cellulose saturated in CFA around the nerve. The transient ATF-3 expression also found in MA is consistent with other studies [36, 37]. This temporal pattern is probably due to an ability of ATF-3 to act as a transcriptional autorepressor [38]. Alternatively, the transient pattern might be explained by the occurrence of regeneration mechanisms, which in fact have already been associated with ATF-3 expression [39, 40].

Cell size measurement of ATF-3-expressing neurons indicated they are mainly distributed among small-to-medium populations in L_5 DRGs from MA rats. This size distribution was relatively constant throughout disease progression. Significant differences within each experimental group were found only at 14 days, indicating that in later phases of the disease ATF-3 is induced mainly in medium-sized neurons. However, triple immunoreactions against ATF-3 and the CGRP and IB4 neuronal markers did not show the expected colocalization with ATF-3 suggested by cell size analysis. The number of IB4-IR cells did not significantly change along MA or when compared to controls. Moreover, the ATF-3 and IB4 colocalization did not suffer any changes between experimental groups, and, besides, was very low. Considering that a high number of ATF-3-positive neurons were small-sized, this low colocalization with IB4 might seem surprising. However, others found no IB4/ATF-3 double labeled cells following severe nerve damage [29].

The ATF-3 and CGRP colocalization was significantly increased at 2 and 4 days of MA, which reflects the significantly increased ATF-3 expression we found at these timepoints of disease. However, the same was not found for colocalization with IB4. Thus, this increased colocalization with CGRP probably means ATF-3 is mainly induced in peptidergic primary afferents at the early timepoints of MA. However, the values of this colocalization were lower than expected if considering that a high number of ATF-3-positive neurons during MA were small-to-medium-sized. Indeed, ATF-3 expression has been associated with reduced CGRP mRNA expression after nerve crush, with CGRP being expressed only in uninjured neurons neighboring injured sensory neurons, which may paradoxically alter how CGRP is expressed in intact neurons [41]. This is a plausible explanation for the low colocalization found for ATF-3 and CGRP.

CGRP is normally released under painful stimulation potentiating nociceptive signaling [42], and plays important roles in the maintenance of both neuropathic and inflammatory pain states [22, 23, 43, 44]. In MA, no significant differences in CGRP expression were found. Although increased CGRP levels are usually driven by inflammation, our data are actually in accordance with previous reports where CGRP expression in DRGs was not significantly altered until the later timepoints (21 days) in arthritic pain [43]. Additionally, there is ATF-3 expression in DRGs during MA which indicates neuronal injury is most likely occurring. As discussed earlier, it has been proposed that injured neurons may alter CGRP expression in intact neurons [41]. Furthermore, the nature of the peripheral nerve injury seems to have an effect on CGRP expression dynamics [22]. Others observed a considerable increase in CGRP release from DRGs during the development of inflammation and hyperalgesia, therefore explaining the significantly decreased number of CGRP-IR cells in primary afferents 2 days after CFA subcutaneous injection [44]. Cell size measurement of CGRP-expressing cells showed they are mostly small and that no neuronal population switch occurs, meaning CGRP is expressed in neurons with identical size profiles during MA, similar to other studies [44].

In the nervous tissue, ATF-3 has been found to enhance neurite outgrowth [39] and to increase the intrinsic growth state of injured neurons [40]. Overexpression of ATF-3 induces neurite elongation and inhibits apoptosis via Akt activation [8]. Therefore, we hypothesize that ATF-3 is induced during MA as a neuronal injury/stress factor, in order to drive cells into a survival/regeneration pathway. The percentage of pAkt-IR cells in the total neuronal population was relatively high even in control animals. This is in accordance with Pezet et al. [45] who reported that pAkt is present in almost every DRG neuron of the rat, although in low levels. pAkt expression reached values around 50% which is also supported by the study of Hökfelt's group [9], although this was performed in mice. Other studies in rat revealed this percentage only

achieves values around 10% [46], but a lot of controversy still remains. Besides possible species differences, the difficulty in detecting pAkt expression and the sensitivity of the methodological approaches might also be implicated [9]. pAkt individual expression did not show any significant difference among any experimental groups. While capsaicin- or carrageenan-induced inflammation seems to induce increases in pAkt activation [9, 45], one of the few studies on pAkt expression after peripheral nerve injury showed a strong reduction 7 days after the model induction [46]. Although MA is mainly an inflammatory model, some degree of peripheral neuronal damage seems to occur judging from ATF-3 expression, and additionally the time frame of the inflammation is distinct from that observed upon carrageenan or capsaicin. Thus, a possible overlap between inflammatory and neuropathic events might be balancing Akt phosphorylation levels in MA. Also pAkt and ATF-3 colocalization, around 55%, did not change during MA progression. Thus, it is not probable that a survival pathway involving both Akt and ATF-3 is being activated during MA.

The upregulation of COX-2 and production of prostanoids are the central mechanisms for the higher hyperalgesia found in many models of peripheral inflammation [47]. In MA rats, administration of ketoprofen, a COX inhibitor anti-inflammatory drug, could not reverse ATF-3 expression. Thus, prostanoid production is unlikely to trigger ATF-3 induction, and consequent neuronal injury, in the MA model. Ketoprofen-treated MA animals showed slightly reduced paw diameters than untreated MA rats, though this was not reflected in ATF-3 expression (therefore suggesting neuronal damage is still present). Additionally, ketoprofen-treated rats showed higher CGRP expression, particularly in the 4d MA+2d Ket group. Staton et al. [48] observed decreased CGRP expression following oral administration of rofecoxib to CFA knee-injected rats between days 13 and 17. Separated quantification of small- or medium-sized cells, but particularly the differing treatments (ways of administration and timepoints) may account for the disagreement with our data in MA. Of note, our treatments started at the peak of ATF-3 induction, when apparently neuronal injury is occurring. With ketoprofen treatment, although inflammation is partially subsided, some degree of neuropathy still remains, since ATF-3 expression did not change. As discussed above, CGRP expression under these conditions might show distinct dynamics [22]. Additionally, considering Galeazza et al.'s studies [44] it is possible that the anti-inflammatory drug is blocking CGRP release from DRGs. These two conditions may lead to the greater values of CGRP immunoreactivity found in DRGs of ketoprofen-treated animals.

In conclusion, ATF-3 is expressed in DRGs in early stages of MA, suggesting neuronal damage is occurring. This is probably due to the neuroinflammatory environment induced by CFA intra-articular injection.

Acknowledgements

The work was supported by Doctoral Programme in Neurosciences, Faculty of Medicine, University of Porto. The authors wish to thank Prof. António Avelino for his expert help with the triple immunoreactions, and are grateful to Elisa Nova for skilled technical assistance during cryosectioning of DRGs.

Disclosure Statement

There are no personal or financial conflicts of interest in publishing these data.

References

1 Schaible HG, Richter F, Ebersberger A, Boettger MK, Vanegas H, Natura G, Vazquez E, Segond von Banchet G: Joint pain. Exp Brain Res 2009;196:153–162.
2 Woolf CJ, Costigan M: Transcriptional and posttranslational plasticity and the generation of inflammatory pain. Proc Natl Acad Sci USA 1999;96:7723–7730.
3 Lu D, Chen J, Hai T: The regulation of ATF3 gene expression by mitogen-activated protein kinases. Biochem J 2007;401:559–567.
4 Nobori K, Ito H, Tamamori-Adachi M, Adachi S, Ono Y, Kawauchi J, Kitajima S, Marumo F, Isobe M: ATF3 inhibits doxorubicin-induced apoptosis in cardiac myocytes: a novel cardioprotective role of ATF3. J Mol Cell Cardiol 2002;34:1387–1397.
5 Ohba N, Maeda M, Nakagomi S, Muraoka M, Kiyama H: Biphasic expression of activating transcription factor-3 in neurons after cerebral infarction. Brain Res Mol Brain Res 2003;115:147–156.
6 Francis JS, Dragunow M, During MJ: Over expression of ATF-3 protects rat hippocampal neurons from in vivo injection of kainic acid. Brain Res Mol Brain Res 2004;124:199–203.
7 Chen HM, Wang L, D'Mello SR: Inhibition of ATF-3 expression by B-Raf mediates the neuroprotective action of GW5074. J Neurochem 2008;105:1300–1312.
8 Nakagomi S, Suzuki Y, Namikawa K, Kiryu-Seo S, Kiyama H: Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. J Neurosci 2003;23:5187–5196.
9 Shi TJ, Huang P, Mulder J, Ceccatelli S, Hökfelt T: Expression of p-Akt in sensory neurons and spinal cord after peripheral nerve injury. Neurosignals 2009;17:203–212.

10 Obata K, Yamanaka H, Fukuoka T, Yi D, Tokunaga A, Hashimoto N, Yoshikawa H, Noguchi K: Contribution of injured and uninjured dorsal root ganglion neurons to pain behavior and the changes in gene expression following chronic constriction injury of the sciatic nerve in rats. Pain 2003;101:65–77.
11 Peters CM, Ghilardi JR, Keyser CP, Kubota K, Lindsay TH, Luger NM, Mach DB, Schwei MJ, Sevcik MA, Mantyh PW: Tumor-induced injury of primary afferent sensory nerve fibers in bone cancer pain. Exp Neurol 2005;193:85–100.
12 Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, Miki K, Yonenobu K, Ochi T, Noguchi K: Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: a novel neuronal marker of nerve injury. Mol Cell Neurosci 2000;15: 170–182.
13 Inglis JJ, Notley CA, Essex D, Wilson AW, Feldmann M, Anand P, Williams R: Collagen-induced arthritis as a model of hyperalgesia: functional and cellular analysis of the analgesic actions of tumor necrosis factor blockade. Arthritis Rheum 2007;56:4015–4023.
14 Ivanavicius SP, Ball AD, Heapy CG, Westwood FR, Murray F, Read SJ: Structural pathology in a rodent model of osteoarthritis is associated with neuropathic pain: increased expression of ATF-3 and pharmacological characterisation. Pain 2007;128:272–282.
15 Braz JM, Basbaum AI: Differential ATF3 expression in dorsal root ganglion neurons reveals the profile of primary afferents engaged by diverse noxious chemical stimuli. Pain 2010;150:290–301.
16 Inglis JJ, Nissim A, Lees DM, Hunt SP, Chernajovsky Y, Kidd BL: The differential contribution of tumour necrosis factor to thermal and mechanical hyperalgesia during chronic inflammation. Arthritis Res Ther 2005; 7:R807–R816.
17 Segond von Banchet G, Boettger MK, Fischer N, Gajda M, Brauer R, Schaible HG: Experimental arthritis causes tumor necrosis factor-α-dependent infiltration of macrophages into rat dorsal root ganglia which correlates with pain-related behavior. Pain 2009;145:151–159.
18 Butler SH, Godefroy F, Besson JM, Weil-Fugazza J: A limited arthritic model for chronic pain studies in the rat. Pain 1992;48:73–81.
19 Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV: Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur J Neurosci 1995;7:1484–1494.
20 Ryu PD, Gerber G, Murase K, Randic M: Actions of calcitonin gene-related peptide on rat spinal dorsal horn neurons. Brain Res 1988;441:357–361.
21 Blesch A, Tuszynski MH: GDNF gene delivery to injured adult CNS motor neurons promotes axonal growth, expression of the trophic neuropeptide CGRP, and cellular protection. J Comp Neurol 2001;436:399–410.
22 Zheng LF, Wang R, Xu YZ, Yi XN, Zhang JW, Zeng ZC: Calcitonin gene-related peptide dynamics in rat dorsal root ganglia and spinal cord following different sciatic nerve injuries. Brain Res 2008;1187:20–32.
23 Ruiz G, Banos JE: The effect of endoneurial nerve growth factor on calcitonin gene-related peptide expression in primary sensory neurons. Brain Res 2005;1042:44–52.
24 Nascimento D, Pozza DH, Castro-Lopes JM, Neto FL: Altered expression of ATF-3 in primary afferent neurons during inflammatory pain. Eur J Pain 2009;13:S52.
25 Nascimento D, Pozza DH, Castro-Lopes JM, Neto FL: Induced expression of the neuronal injury marker ATF3 in primary afferents during monoarthritis: characterization of the neuronal populations involved; in Society for Neuroscience, 2010. Online. 2010. San Diego/CA: Program No. 586.3/ZZ9. 2010 Neuroscience Meeting Planner.
26 Zimmermann M: Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109–110.
27 Lourenco Neto F, Schadrack J, Platzer S, Zieglgansberger W, Tolle TR, Castro-Lopes JM: Expression of metabotropic glutamate receptors mRNA in the thalamus and brainstem of monoarthritic rats. Brain Res Mol Brain Res 2000;81:140–154.
28 Castro-Lopes JM, Tavares I, Tolle TR, Coito A, Coimbra A: Increase in GABAergic cells and GABA levels in the spinal cord in unilateral inflammation of the hindlimb in the rat. Eur J Neurosci 1992;4:296–301.
29 Averill S, Michael GJ, Shortland PJ, Leavesley RC, King VR, Bradbury EJ, McMahon SB, Priestley JV: NGF and GDNF ameliorate the increase in ATF3 expression which occurs in dorsal root ganglion cells in response to peripheral nerve injury. Eur J Neurosci 2004; 19:1437–1445.
30 Ferreira-Gomes J, Adaes S, Sarkander J, Castro-Lopes JM: Phenotypic alterations of neurons that innervate osteoarthritic joints in rats. Arthritis Rheum 2010;62:3677–3685.
31 Fukuoka T, Kondo E, Dai Y, Hashimoto N, Noguchi K: Brain-derived neurotrophic factor increases in the uninjured dorsal root ganglion neurons in selective spinal nerve ligation model. J Neurosci 2001;21:4891–4900.
32 Neto FL, Carvalhosa AR, Ferreira-Gomes J, Reguenga C, Castro-Lopes JM: Delta opioid receptor mRNA expression is changed in the thalamus and brainstem of monoarthritic rats. J Chem Neuroanat 2008;36:122–127.
33 Schadrack J, Neto FL, Ableitner A, Castro-Lopes JM, Willoch F, Bartenstein P, Zieglgansberger W, Tolle TR: Metabolic activity changes in the rat spinal cord during adjuvant monoarthritis. Neuroscience 1999;94: 595–605.
34 Inoue K, Zama T, Kamimoto T, Aoki R, Ikeda Y, Kimura H, Hagiwara M: TNFα-induced ATF3 expression is bidirectionally regulated by the JNK and ERK pathways in vascular endothelial cells. Genes Cells 2004; 9:59–70.
35 Dilley A, Lynn B, Pang SJ: Pressure and stretch mechanosensitivity of peripheral nerve fibres following local inflammation of the nerve trunk. Pain 2005;117:462–472.
36 Chen BP, Liang G, Whelan J, Hai T: ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms. J Biol Chem 1994;269:15819–15826.
37 Liang G, Wolfgang CD, Chen BP, Chen TH, Hai T: ATF3 gene. Genomic organization, promoter, and regulation. J Biol Chem 1996; 271:1695–1701.
38 Wolfgang CD, Liang G, Okamoto Y, Allen AE, Hai T: Transcriptional autorepression of the stress-inducible gene ATF3. J Biol Chem 2000;275:16865–16870.
39 Seijffers R, Allchorne AJ, Woolf CJ: The transcription factor ATF-3 promotes neurite outgrowth. Mol Cell Neurosci 2006;32:143–154.
40 Seijffers R, Mills CD, Woolf CJ: ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. J Neurosci 2007;27:7911–7920.
41 Li XQ, Verge VM, Johnston JM, Zochodne DW: CGRP peptide and regenerating sensory axons. J Neuropathol Exp Neurol 2004;63: 1092–1103.
42 Seybold VS: The role of peptides in central sensitization. Handb Exp Pharmacol 2009; 194:451–491.
43 Calza L, Pozza M, Zanni M, Manzini CU, Manzini E, Hokfelt T: Peptide plasticity in primary sensory neurons and spinal cord during adjuvant-induced arthritis in the rat: an immunocytochemical and in situ hybridization study. Neuroscience 1998;82:575–589.
44 Galeazza MT, Garry MG, Yost HJ, Strait KA, Hargreaves KM, Seybold VS: Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation. Neuroscience 1995;66:443–458.
45 Pezet S, Spyropoulos A, Williams RJ, McMahon SB: Activity-dependent phosphorylation of Akt/PKB in adult DRG neurons. Eur J Neurosci 2005;21:1785–1797.
46 Xu JT, Tu HY, Xin WJ, Liu XG, Zhang GH, Zhai CH: Activation of phosphatidylinositol 3-kinase and protein kinase B/Akt in dorsal root ganglia and spinal cord contributes to the neuropathic pain induced by spinal nerve ligation in rats. Exp Neurol 2007;206: 269–279.
47 Marchand F, Perretti M, McMahon SB: Role of the immune system in chronic pain. Nat Rev Neurosci 2005;6:521–532.
48 Staton PC, Wilson AW, Bountra C, Chessell IP, Day NC: Changes in dorsal root ganglion CGRP expression in a chronic inflammatory model of the rat knee joint: differential modulation by rofecoxib and paracetamol. Eur J Pain 2007;11:283–289.

4.2 Publication II

Satellite glial cells surrounding primary afferent neurons are activated and proliferate during monoarthritis in rats: is there a role for ATF3? PlosOne (2014)

OPEN ACCESS Freely available online

PLOS | ONE

Satellite Glial Cells Surrounding Primary Afferent Neurons Are Activated and Proliferate during Monoarthritis in Rats: Is There a Role for ATF3?

Diana Sofia Marques Nascimento[1,2], José Manuel Castro-Lopes[1,2], Fani Lourença Moreira Neto[1,2]*

1 Departamento de Biologia Experimental, Centro de Investigação Médica (CIM), Faculdade de Medicina do Porto, Universidade do Porto, Porto, Portugal, **2** Morphophysiology of the Somatosensory System Group, Instituto de Biologia Molecular e Celular (IBMC), Porto, Portugal

Abstract

Joint inflammatory diseases are debilitating and very painful conditions that still lack effective treatments. Recently, glial cells were shown to be crucial for the development and maintenance of chronic pain, constituting novel targets for therapeutic approaches. At the periphery, the satellite glial cells (SGCs) that surround the cell bodies of primary afferents neurons in the dorsal root ganglia (DRG) display hypertrophy, proliferation, and activation following injury and/or inflammation. It has been suggested that the expression of neuronal injury factors might initially trigger these SGCs-related events. We then aimed at evaluating if SGCs are involved in the establishment/maintenance of articular inflammatory pain, by using the monoarthritis (MA) model, and if the neuronal injury marker activating transcriptional factor 3 (ATF3) is associated with these SGCs' reactive changes. Western Blot (WB) analysis of the glial fibrillary acidic protein (GFAP) expression was performed in L4-L5 DRGs from control non-inflamed rats and MA animals at different time-points of disease (4, 7, and 14d, induced by complete Freund's adjuvant injection into the left hind paw ankle joint). Data indicate that SGCs activation is occurring in MA animals, particularly after day 7 of disease evolution. Additionally, double-immunostaining for ATF3 and GFAP in L5 DRG sections shows that SGCs's activation significantly increases around stressed neurons at 7d of disease, when compared with control animals. The specific labelling of GFAP in SGCs rather than in other cell types was also confirmed by immunohistochemical labeling. Finally, BrdU incorporation indicates that proliferation of SGCs is also significantly increased after 7 days of MA. Data indicate that SGCs play an important role in the mechanisms of articular inflammation, with 7 days of disease being a critical time-point in the MA model, and suggest that ATF3 might be involved in SGCs' reactive changes such as activation.

Citation: Nascimento DSM, Castro-Lopes JM, Neto FLM (2014) Satellite Glial Cells Surrounding Primary Afferent Neurons Are Activated and Proliferate during Monoarthritis in Rats: Is There a Role for ATF3? PLoS ONE 9(9): e108152. doi:10.1371/journal.pone.0108152

Editor: Ken Arai, Massachusetts General Hospital/Harvard Medical School, United States of America

Received May 19, 2014; **Accepted** August 25, 2014; **Published** September 23, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are included within the paper.

Funding: Cátedra em Medicina da Dor Fundação Grünenthal/Faculdade de Medicina da Universidade do Porto partially granted this study. The first author received a doctoral grant(SFRH/BD/79497/2011) by Fundação para a Ciência e a Tecnologia (FCT), Portugal. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: fanineto@med.up.pt

Introduction

Inflammation of the joint is characterized, among others, by debilitating mechanical hyperalgesia and persistent pain at rest. It is one of the major causes of chronic pain and therefore a relevant clinical problem in need of better therapeutic approaches. In spite of the great advances in the study of articular inflammatory painful conditions and the existence of reliable experimental models, the nociceptive neuronal mechanisms behind these pathologies are still vague and lack investigation [1].

In the peripheral nervous system (PNS), pain mechanisms involve sensitization of primary afferents neurons whose cell bodies are located in the dorsal root ganglia (DRG). In fact, the thermal and mechanical sensations captured at the skin, viscera and joints are conveyed into the CNS through the DRGs, implying that they are the first relay centers for sensory input transmission from periphery [2] and an important site for the processing of neural information [3].

In the DRGs, the cell bodies of these primary afferents are anatomically surrounded by satellite glial cells (SGCs) forming distinct functional units [4]. SGCs may be identified by the expression of several glial markers such as glutamine synthetase (GS) and S100β. The immunoreactivity against glial fibrillary acidic protein (GFAP), an intermediate filament protein, is not readily detectable in SGCs at a resting state or under normal physiological conditions. However, following nerve injury, inflammation or viral infection, GFAP becomes detectable in the SGCs that become activated by the pathological insult. Thus, in the PNS, GFAP expression is commonly used as a marker of SGCs activation [4–6]. Although SGCs' properties and functions have not yet been fully studied, it is now clear that these cells take an important part in the "intercellular communication" [3] with the neuronal cells they are in contact with.

The role of SGCs has been underestimated for a long time [7], but the available data reveal that they are important in the establishment and maintenance of pathological conditions, largely contributing to the development of chronic pain states. In fact, the

SGCs' unique localization around neuronal cell bodies allows a bidirectional crosstalk [4] known to strongly influence nociceptive processing [3,8]. Thus, under a pathological condition, neurons are known to release specific mediators, such as ATP, nitric oxide, and neuropeptides as calcitonin gene-related protein (CGRP) and substance P, that are able to activate SGCs. Activated SGCs may also release pro-inflammatory agents that contribute to continued neuronal sensitization [9]. There is also strong evidence pointing to the occurrence of morphological and biochemical changes in SGCs as a response to pathological conditions. Accordingly, both activation [11,12] and proliferation [7,13] of these cells have been described as a response to nerve injury and/or inflammation, and consequent pain development. However, the exact factors and the associated mechanisms leading to these reactive morphological and biochemical changes in SGCs, during a pathological condition, are still partially unknown. Additionally, the onset of those alterations in relation to disease progression has not either been thoroughly investigated in the majority of the studies.

Using a model of chronic articular inflammatory pain, the monoarthritis (MA) induced by Complete Freund's Adjuvant (CFA) injection in the tibiotarsal joint, we investigated if SGCs might also be playing a role in this pathological condition. In order to evaluate SGCs activation, we quantified GFAP expression in the DRGs of MA animals by Western Blot (WB). We also confirmed by immunohistochemistry (IHC) that GFAP expression is specifically occurring in SGCs. To evaluate the time course pattern of such changes in relation to the progression of the inflammatory condition we used different time-points of the disease (4, 7 and 14d after CFA injection), that allowed us to correlate the data with our previous studies in the same pain model [14]. We have previously found a significantly increased expression of the neuronal injury marker activating transcriptional factor 3 (ATF3) in the DRGs at the initial time-points of MA [14], with a peak of expression at day 4, which suggested that some degree of neuronal damage is occurring in the early stages of this disease. Moreover, it has been suggested that the expression of injury factors might trigger part of the neuron-SGCs communication events [15]. Thus, with the aim of evaluating if activation of SGCs occurs preferentially around damaged/stressed neurons, we also performed co-immunolabeling assays for GFAP and ATF3 in the DRGs of controls and MA animals. Lastly, we also analyzed the incorporation of bromodeoxyuridine (BrdU) as a way to investigate if proliferation of SGCs is also occurring during MA.

Materials and Methods

Animal handling and Monoarthritis (MA) induction

All the procedures were carried out according to the European Communities Council Directive of September 22, 2010 (2010/63/EC) and to the ethical guidelines for investigation of experimental pain in animals [16], and were authorized by the animal welfare body (ORBEA) of the Faculty of Medicine of the University of Porto, where the experiments were performed. Animals used for Western Blot (WB) purposes (section 2.3) were decapitated after light volatile anesthesia with isoflurane. Those animals that were perfused through the ascending aorta for IHC assays (section 2.4), were deeply anesthetized with sodium pentobarbital. The humane endpoints defined for this project were always respected. Efforts were made in order to minimize pain and distress and reduce the number of animals used. Experiments were carried out in a total of 44 adult male Wistar rats (Charles River Laboratories, France) weighing between 200 and 300 g. Animals were housed 2–3 animals per cage under controlled conditions of lighting (12 h light/12 h dark cycle) and temperature as well as water and food *ad libitum*.

Monoarthritis (MA) was induced by injecting 50 µL of complete Freund's adjuvant (CFA), into the left tibiotarsal joint [17] under isoflurane anesthesia (5% for induction, 2.5% for maintenance). The CFA solution (5,45 mg/mL) was prepared as previously described [18] and monoarthritic animals were sacrificed at 4, 7 or 14 days of inflammation. Control (non-inflamed) animals were similarly injected with 50 µL of CFA vehicle and were allowed to survive for 2 days, as previously described [14]. Habituation of the animals to the experimenter was performed for several days before CFA injection and during the progression of MA, to minimize fear-motivated behaviors. The evolution of the inflammatory reaction was monitored daily and was scored taking in consideration the inflammatory signs of the injected ankle and reduction of the locomotor activity [19]. The severity of the inflammation was further evaluated by measuring the diameter of the animals' affected paw just before sacrifice [14]. One of the animals that had been injected with CFA to be used in the BrdU experiments developed polyarthritis, characterized by inflammatory signs in the contralateral non-injected paw and tail, as described before [17], and therefore was immediately excluded from the study.

Bromodeoxyuridine (BrdU) administration

Bromodeoxyuridine (BrdU-B5002, Sigma-Aldrich) was intraperitoneally (i.p.) injected (50 mg/Kg of animal weight) immediately after the preparation of a solution of 50 mg/mL, 10% in dimethyl sulfoxide (DMSO) [8]. Injections were performed twice daily, beginning at the day of CFA or CFA vehicle intra-articular injection (day 0), until 24 h prior to animals sacrifice (to allow BrdU clearance) by intracardiac perfusion, as described below. The following experimental groups were used: controls (CFA-vehicle non-inflamed rats injected with BrdU until day 3 and sacrificed at day 4; N = 6 rats), 4d MA (CFA-inflamed rats injected with BrdU until day 3 and sacrificed with 4d of disease; N = 5 rats) and 7d of MA (CFA-inflamed rats injected with BrdU until day 6 and sacrificed with 7d of disease; N = 6 rats). Prior to these experiments, a group of naive animals was injected twice a day, with 10% DMSO solution i.p., for 6 days and no toxic effects or signs of peritoneal inflammation were found (data not shown).

Analysis of GFAP expression by Western Blotting

In order to investigate SGCs activation, the expression of glial fibrillary acidic protein (GFAP) was evaluated by WB analysis of freshly harvested DRGs from MA (with 4, 7 and 14 days of disease, N = 5 animals per group) and control animals (N = 6) that had been sacrificed by decapitation under light anesthesia with isoflurane. To correlate data with the previous studies [14], DRGs from 2d MA animals were also analyzed but significant changes were not found (data not shown). Thus, this time-point was excluded from the following experiments.

For each animal, the L4 and L5 ganglia were pooled, separately for the ipsi and contralateral sides, and then were lysed and homogenized in 70 µL of radio immuno precipitation assay (RIPA) buffer containing sodium chloride 150 mM, triton X-100 1%, sodium deoxycholate 0.5%, sodium dodecyl sulphate (SDS) 0.1% and Tris pH 8.0 50 mM. Cocktails of protease and phosphatase inhibitors (1:100, Sigma-Aldrich P8340, P5726 and P0044) were also added to the buffer. The samples were sonicated and centrifuged (20 minutes at 20,000 g), the pellets were discarded and the supernatants were used for analysis. The proteins were quantified by the bicinchoninic acid (BCA) protein assay. After heating at 94°C, 30 µg of protein were loaded for each lane and separated on 14% sodium dodecyl sulphate-polyacrylamide (SDS/

PAGE) gels. The proteins were then transferred into nitrocellulose membranes which were blocked with non-fat milk (5% milk powder diluted in tris buffer saline tween20; TBST buffer), for one hour, at room temperature, to prevent non-specific bindings. In order to detect GFAP, the membranes were incubated in monoclonal mouse anti-GFAP antibody (Mab360, Chemicon-Millipore) diluted 1:500 in TBST with 2% of normal goat serum (NGS), for 24 hours at 4°C. As a loading control, the detection of β-actin (polyclonal rabbit anti-β-actin antibody, Ab8227 ABCAM, Cambridge, UK) diluted 1:4000 in TBST with 2% of normal horse serum (NHS) was also performed.

Detection of GFAP was achieved by incubation in goat anti-mouse secondary antibody conjugated with horseradish peroxidase (HRP; sc-2031, Santa Cruz Biotechnology, Inc), diluted 1:5000 in TBST with 5% milk powder, for 1 hour, at room temperature. β-actin was also detected using a donkey anti-rabbit secondary antibody conjugated with HRP (711-035-152, Jackson Laboratories), diluted 1:5000 in TBST with 5% milk powder. Antibody binding was visualized with the SuperSignal West Pico Chemiluminescent Substrat kit (Thermo Scientific; 34080) and the bands were detected by exposing the membranes to X-ray films (KODAK XOMAT Blue (XB) Film, Perkin Elmer, USA; NEF586001EA). Each blot, containing independent samples, was run in triplicates and means were used as raw values.

Double Immunohistochemistry against GFAP-ATF3 or BrdU-GS

After deep anesthesia with sodium pentobarbital (Eutasil, Ceva, Sante Animale, France; i.p., 75 mg/kg of animal body weight), the animals were perfused through the ascending aorta with 250 mL of oxygenated Tyrode's solution followed by 750 mL of paraformaldehyde (PFA) 4% in phosphate buffer saline 0.1 M (PBS 0.1 M). The ipsi- and contralateral DRGs corresponding to spinal segment L5 were removed and post fixed in the same fixative solution for 4 h and then cryoprotected over night (in sucrose 30% in phosphate buffer 0.1 M). The DRGs were cut into 14 μm sections in a freezing cryostat (−20°C). The tissue was collected sequentially into 5 different poly-L-lysine coated slides, was air dried and stored at −20°C until immunohistochemistry was performed.

To confirm if activation of SGCs is possibly occurring in cells surrounding damaged/stressed neurons (ATF3-positive profiles), double immunoreactions against GFAP and ATF3 were performed. Each slide (containing every fifth section of each L5 DRG) from both controls non-inflamed and 7d MA animals was first thawed and washed in PBS 0.1 M and then PBS containing 0.3% Triton X-100 (PBST). In order to avoid unspecific bindings, sections were incubated for 1 hour in a blocking solution containing 10% of NGS in PBST. Afterwards, slides were incubated for 48 h at 4°C in the primary antibodies rabbit anti-GFAP (ab7260, Abcam, 1:1000), and mouse anti-ATF3 (ab58668, Abcam, 1:200), diluted in PBST containing 2% of NGS. After several washes in PBST with 2% of NGS, slides were finally incubated, for 1 hour, at room temperature, in goat anti-rabbit 568 (A11011, Molecular Probes, 1:1000) and donkey anti-mouse 488 (A21202, Molecular Probes, 1:1000) secondary antibodies diluted in a solution of PBST with 2% of NGS.

To evaluate SGCs proliferation, sections from perfusion-fixed L5 DRGs of control non-inflamed, 4d and 7d MA animals, previously injected with BrdU, were double immunoreacted against BrdU (which marks proliferating cells) and GS. Slides containing every fifth section of each DRG were treated following a protocol similar to that described above, except that slides were firstly incubated in HCl at 60°C for 30 minutes and then 5 minutes in Borax 0.1 M, for antigen retrieval. Blocking was done in a solution of 10% normal swine serum (NSS) in PBST with 7.5 mg/mL of glycine. Slides were afterwards incubated in sheep anti-BrdU (BP2295, Acris, 1:100) and mouse anti-GS (MAB302, Millipore, 1:500), in a PBST solution with 2% of NSS. Detection was achieved by incubation in a biotinilated donkey anti-sheep secondary antibody (B-7390, Sigma Aldrich), 1:200 diluted in PBST with 2% of NSS, for 1 hour at room temperature. After thorough washes in PBST, the slides were incubated in streptavidin 488 (S32354, Molecular Probes) and Alexa 568 donkey-anti-mouse (A10037, Molecular Probes), both 1:1000 in PBST with 2% of NSS.

After the immunoreaction, the slides with the stained sections were stored in PBS 0.1 M at 4°C until they were mounted for visualization under a fluorescent microscope. For microscopic analysis, the slides were coverslipped with a mounting media (solution containing 3 parts of glycerol and 1 part of PBS 0.4 M).

Data analysis

Quantification of band intensity in Western Blotting. The protein levels were obtained by densitometric analysis of the signal intensity in the blots, in pixels, using the image computer software ScionImageR (Scion Corporation). Both the areas of the lanes and the background signal were used for values normalization. β-actin was used as loading control and a ratio between GFAP/β-actin protein levels was calculated. Additionally, ratios between the ipsi and contralateral levels were calculated for comparison between the different MA groups and controls. The assays were typically performed three times on samples obtained from independent groups of rats and means of these triplicates were used as raw values.

Immunoreactivity detection and cell counting. The immunohistochemistry analysis was performed by using a fluorescence microscope (AXIO Imager.Z1, Zeiss), coupled to a digital camera (Axiocam MRm) and a computer image software (Axiovision 4.6) to grab the images. For the photomicrographs the acquisition conditions, such as amplification of the objective, light intensity, contrast and hue, were maintained constant.

The expression of GFAP in SCGs was confirmed by immunodetection. SCGs were distinguished from nerve cell soma and other perineuronal cells by their shape, position, orientation and nuclear characteristics [20]. Neurons surrounded by GFAP-positive SGCs in half or more than half of their circumference were assumed as positive neuronal profiles. The total number of these immunolabeled GFAP-positive neuronal profiles ($\mathbf{GFAP^{+}_{total}\ NP}$) was quantified. The total number of cells bodies of primary afferents analyzed was defined here as $\mathbf{NP_{total}}$ and counted for each slide (corresponding to an animal and containing every fifth section of each L5 DRG). For normalization $\mathbf{GFAP^{+}_{total}\ NP}$ was divided by $\mathbf{NP_{total}}$ ($\mathbf{GFAP^{+}_{total}\ NP/NP_{total}}$), and presented as percentage.

The total number of double labeled neuronal profiles (GFAP-positive neuronal profiles also expressing nuclear ATF3; $\mathbf{Double^{+}_{total}}$) was also counted and divided by the total number of analyzed neurons ($\mathbf{Double^{+}_{total}/NP_{total}}$), and the final value is presented as percentage. Additionally, we calculated the percentage of double labeled neuronal profiles in the total ATF3-positive population ($\mathbf{Double^{+}_{total}/ATF3^{+}_{total}}$).

To evaluate the proliferation of SGCs, the total number of double-labeled cells against BrdU and GS ($\mathbf{SGCs^{+}_{total}}$) was counted in each slide (containing every fifth section of each L5 DRG). For normalization, a ratio between $\mathbf{SGC^{+}_{total}/NP_{total}}$ was calculated so that values of different animals could be compared. In order to calculate the mean of proliferating SGCs ($\mathbf{SGC^{+}}$)

around neurons, we divided the **SGCs^{+} $_{total}$** by the total number of neuronal profiles surrounded by at least one positively labeled SGC (**Mean SGC^{+} around NP**) [7]. Neuronal profiles surrounded by SGC^{+} in half or more than half of their circumference were also counted and denominated as **NP^{+}** [21]. Again, for means of standardization, a ratio between **NP$^{+}_{total}$/NP$_{total}$** was calculated to allow comparison between different animals and experimental groups.

Statistical analysis. Statistical analysis was performed by using GraphPad Prism 5 (GraphPad Software) and SPSS 13.0. One-way analysis of variance (one-way ANOVA) was performed to investigate significant differences between the different experimental groups. For the WB data, ANOVA was followed by the Tukey's Multiple Comparison post-hoc test. In this case, the values were calculated as ratios between the ipsi and contralateral sides after normalization against the loading control, β-actin. Results were displayed as mean±SEM (N = 6 for controls; N = 5 for all the other experimental groups). Data from immunohistochemical GFAP detection was analyzed using ANOVA followed by the Bonferroni post-hoc test. Results (**GFAP$^{+}_{total}$;NP/NP$_{total}$**) were shown as mean±SEM (N = 5 for all the experimental groups). The GFAP-ATF3 double-labeling data was analyzed using one-tailed Student's t-test analysis between the controls and 7d MA groups. Results (**Double$^{+}_{total}$/NP$_{total}$** and **Double$^{+}_{total}$/ATF3$^{+}_{total}$**) were displayed as mean±SEM (N = 5 for 7d MA and N = 4 for controls). For BrdU quantification, ANOVA was followed by the Newman Keuls Multiple Comparison test, for all the three different displayed results. All values (**SGC$^{+}_{total}$/NP$_{total}$**; **NP$^{+}_{total}$/NP$_{total}$**; **Mean SGC^{+}/NP**) were shown as mean±SEM (N = 6 for controls and 7d MA; N = 5 for 4d MA). In all the statistical analyses, a level of significance of $P<0.05$ was assumed.

Results

SGCs are activated during MA

MA was successfully and homogenously induced in all the animals injected with CFA, as they were all showing severe inflammatory symptoms with swelling, redness and avoidance to put weight over the inflamed paw at each time-point of disease. This was reflected in mean inflammatory scores near 4 (maximum score), immediately after the second day of MA. This condition was maintained up to the 14th day, as well as increased paw volumes (data not shown), in accordance with our previous work [14]. Controls showed insignificant mean scores.

Western blot analysis showed that the GFAP levels in MA animals were always higher in the ipsilateral (lanes 3, 5, 7 of Fig. 1A) than in the contralateral DRGs (lanes 4, 6, 8 of Fig. 1A). Consequently, ratios between ipsi and contralateral GFAP levels were significantly increased at day 7 (2.71±0.35; $p<0.05$) and 14 of disease (2.91±0.47; $p<0.01$), when compared with controls (1.13±0.08) (Fig. 1A and B). Controls showed a non-significant basal expression in both ipsi- and contralateral sides, as expected.

In order to confirm that the GFAP expression detected by Western blot was actually occurring in SGCs, we immunoreacted perfusion-fixed L5 DRG sections of control, 4d, 7d and 14d MA animals against GFAP. The specific labeling, the morphology and the unique localization around the cell bodies of DRGs neurons confirmed that GFAP expression is actually occurring in SGCs (Fig. 1C) [4]. Quantification of the total number of positive GFAP neuronal profiles (**GFAP$^{+}_{total}$ NP/NP$_{total}$**) revealed that there are significant increases for 7d MA animals (34.45±1.95%; $p<0.01$) when compared with controls (13.09±2.95%) (Fig. 1D). Animals with 14d MA presented also an increased number of GFAP-positive neuronal profiles in comparison to non-inflamed

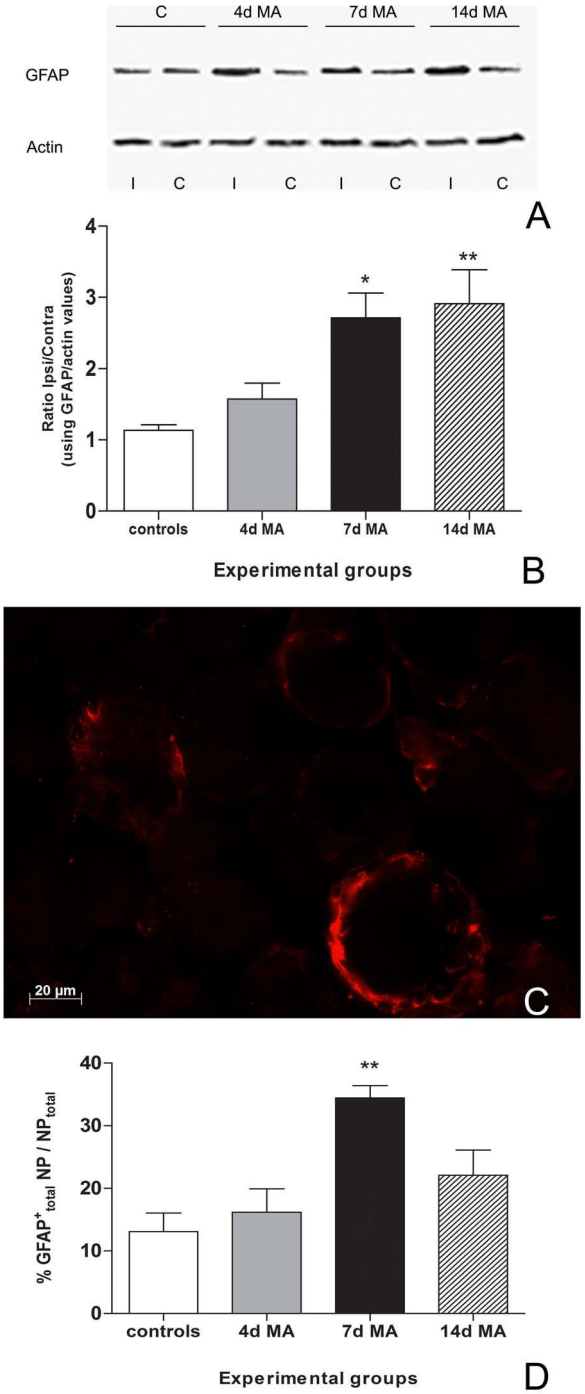


Figure 1. GFAP overexpression during MA. (**A**) GFAP levels in 4d, 7d and 14d MA animals were always higher in the ipsilateral DRGs (lines 3, 5, 7) when comparing to DRGs from the contralateral side (lines 4, 6, 8). As expected, control values were similar for both ipsi and contralateral sides (lanes 1 and 2). (**B**) The ratios between Ipsi and Contralateral GFAP levels (GFAP/actin values) were significantly increased at day 7 and 14 days of MA which suggests activation of SGCs at around 1 week after disease induction. (**C**) Single immunolabeling for GFAP (red) specifically in SGCs, in a L5 DRG from a 7d MA animal (bar represents 20 µm). **D**) The percentage of the total number of GFAP-positive neuronal profiles in the total neuronal population (**GFAP$^{+}_{total}$ NP/NP$_{total}$**) significantly increases at 7d MA. All values are shown as Mean±SEM, In B) N = 6 for controls and N = 5 for all the other experimental groups. * represents $p<0.05$ relatively to controls. One-way ANOVA was followed by Tukey's Multiple Comparison post-

hoc test. In D) N = 5 for all experimental groups.** represents $p<0.01$, relatively to controls. One-way ANOVA was followed by Bonferroni post-hoc test.
doi:10.1371/journal.pone.0108152.g001

controls, although statistical significance was not achieved (Fig. 1D).

Activation of SGCs increases around stressed neurons, in MA

The total number of neurons counted as positive for both ATF3 and GFAP (**$Double^{+}_{total}/NP_{total}$**) (Fig. 2A and B) was significantly increased at 7 days of MA (5.76±2.12%) when compared with controls (0.63±0.18%, $p<0.05$) (Fig. 2C). Also, the percentage of double labeled cells in the total ATF3-positive neuronal population (**$Double^{+}_{total}/ATF3^{+}_{total}$**) increased at 7d MA (43.09±2.37%, $p<0.05$) in comparison with controls (17.38±5.68%).

SGCs proliferate during MA

BrdU was injected in controls (non-inflamed) and in 4 and 7d MA animals (Fig 3D, E, F). In order to confirm BrdU incorporation in SGCs, a double immunocolocalization with GS was performed (Fig. 3A, B, C for GS immunoreactivity; Fig. 3G, H, I for colocalization of BrdU with GS). The **$SGC^{+}_{total}/NP_{total}$** significantly increased at 7d of MA (1.00±0.11), when compared with both controls (0.53±0.07; $p<0.01$) and 4d MA (0.49±0.15, $p<0.05$) animals (Fig 3J and Table 1). Not only the overall number of SGC^{+} increased in the ganglia along disease progression, but, in addition, the number of proliferating SGCs around a specific neuron also augmented. In fact, the **Mean SGC^{+} around NP** was also significantly higher at 7d MA (2.30±0.13) than in controls (1.75±0.08; $p<0.05$) and 4d MA (1.75±0.23; $p<0.05$) animals (Fig 3K and Table 1). Moreover, as the number of proliferating SGCs around a neuron increased, more positive neuronal profiles were also found. Thus, **$NP^{+}_{total}/NP_{total}$** was also significantly higher in 7dMA (1.55±0.29) than in controls (0.29±0.21; $p<0.01$) and 4d MA (0.51±0.28; $p<0.05$) (Fig 3L and Table 1). In summary, in all three types of

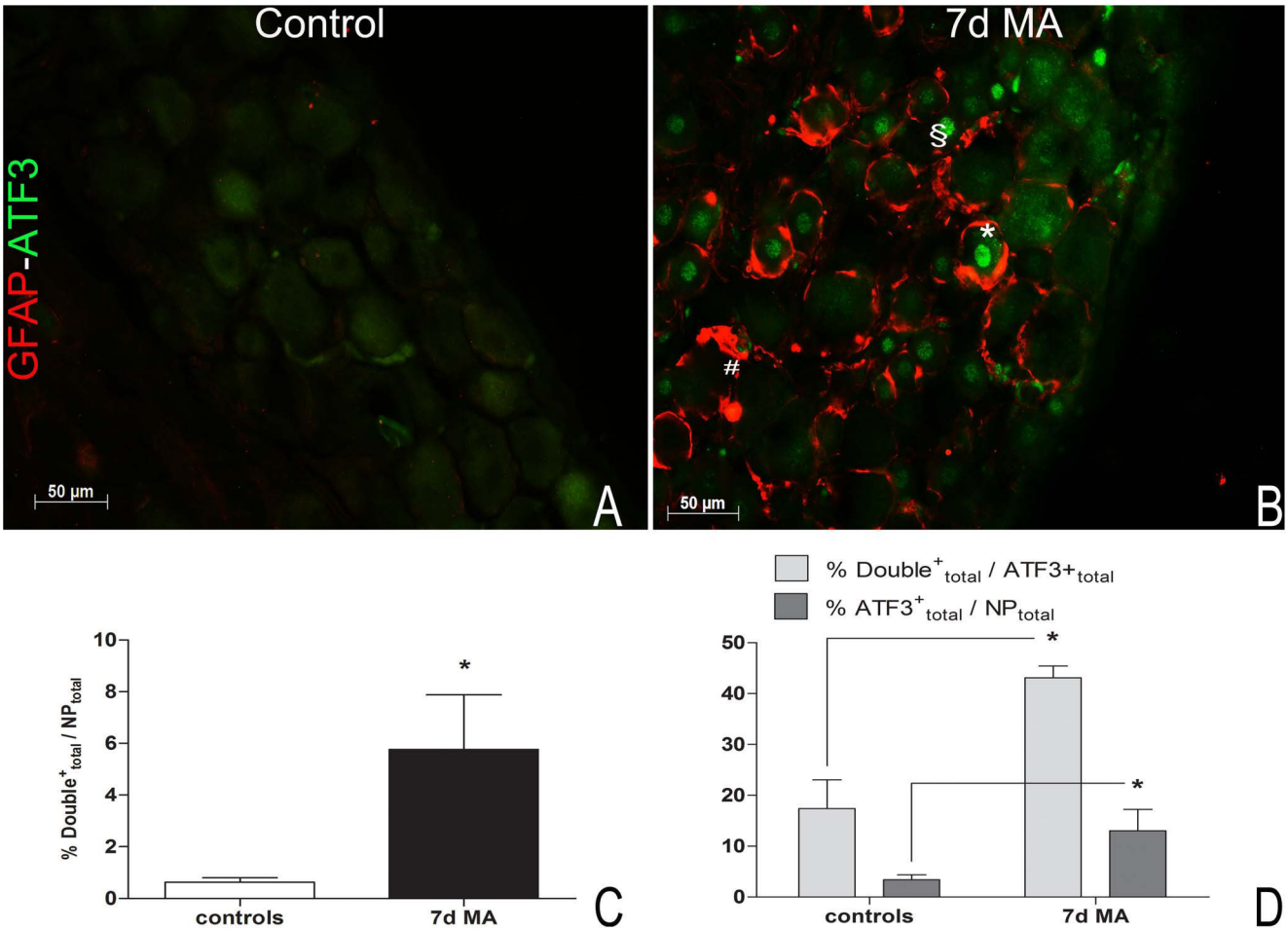


Figure 2. GFAP labeling in SGCs surrounding ATF3 positive neurons increases at 7d MA. (**A, B**) Double labeling for ATF3 (green) and GFAP (red), in L5 DRGs sections from a control (**A**) and a 7d MA animal (**B**). (**C**) The percentage of double labeled neuronal profiles in the total neuronal population (**% $Double^{+}_{total}/NP_{total}$**) increases at 7d MA. (**D**) The percentage of double labeled neuronal profiles in the total ATF3-positive neuronal population (**$Double^{+}_{total}/ATF3^{+}_{total}$**) also increases after 7d MA, even though ATF3-positive neurons represent a small portion of the total neuronal population of the DRG (**%$ATF3^{+}_{total}/NP_{total}$**). In A and B, the bar represents 50 µm, # identifies a single labeled GFAP-positive neuronal profile, § identifies a single labeled ATF3-positive neuron and* identifies co-labeling of both GFAP and ATF3. In C and D, all values are shown as Mean±SEM with N = 5 for 7dMA and N = 4 for controls. * Represents $p<0.05$ relatively to control animals. One-tailed Student's t-test analysis.
doi:10.1371/journal.pone.0108152.g002

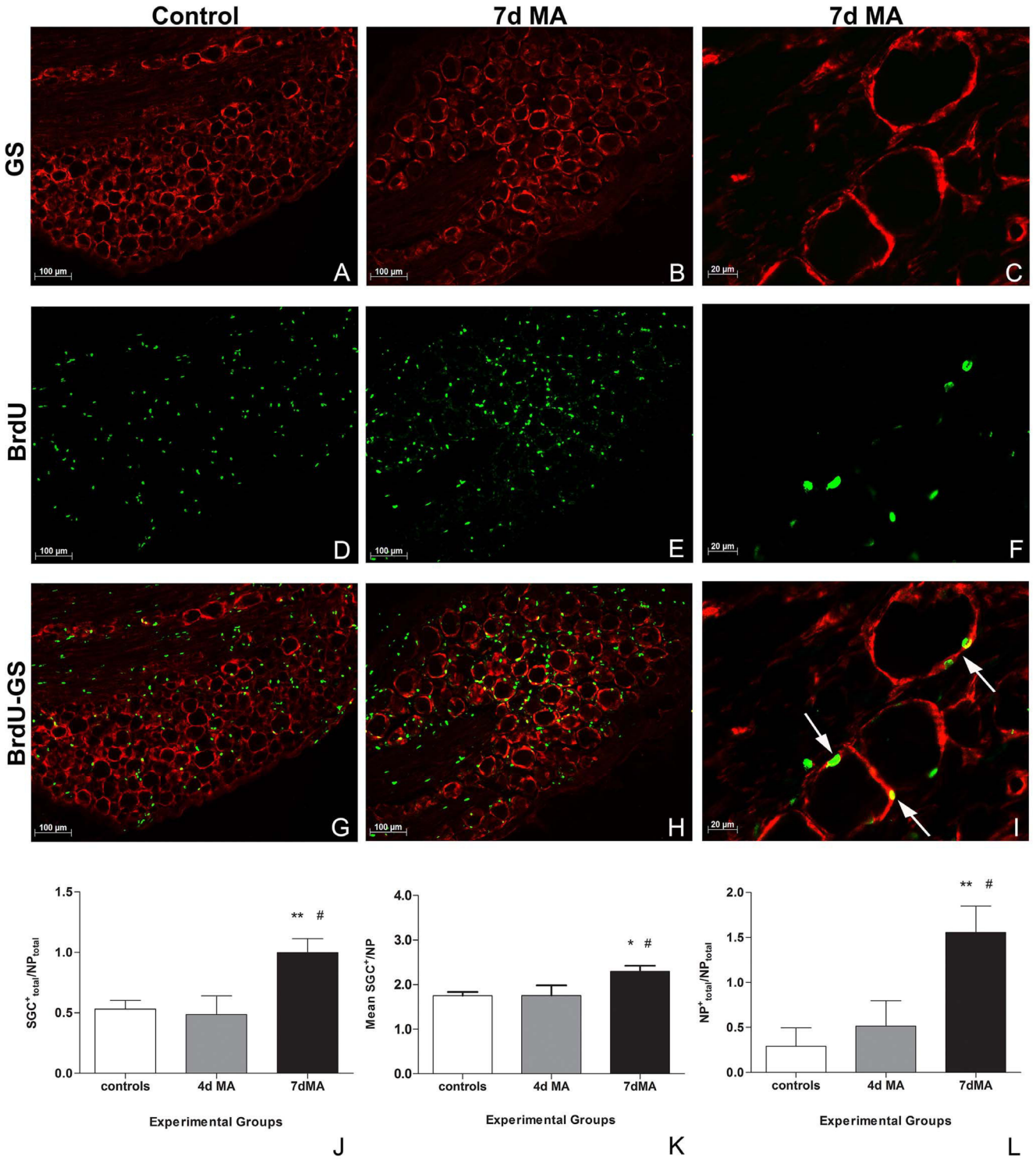


Figure 3. BrdU incorporation increases during MA. (**A–I**) Immunofluorescence labeling for GS (red) (**A, B, C**), BrdU (green) (**D, E, F**) and respective colocalization between both (**G, H, I**), in a L5 DRG of a control and a 7d MA animal (bar represents 100 µm). **A**rrows point to well visible double-labeled SGCs. An amplified image from a L5 DRG of a 7d MA animal shows BrdU labeling in detail (bar represents 20 µm) (**C, F, I**). (**J**) The number of proliferating SGCs (SGCs^{+}), in the total number of neuronal profiles (**SGC$^{+}_{total}$/NP$_{total}$**), significantly increases at 7d MA. (**K**) The mean number of proliferating SGCs around a specific neuron (**Mean SGC^{+}/NP**) also increases at 7d MA. (**L**) The number of positive neuronal profiles (**NP$^{+}_{total}$/NP$_{total}$**) is also significantly higher in 7d MA, when compared with both control non-inflamed and to 4d MA animals. All values shown as Mean±SEM. N = 6 for controls and 7d MA, and N = 5 for 4d MA experimental group.* Significant differences relatively to control. # Significant differences relatively to 4d MA.* or # represents $p<0.05$; ** represents $p<0.01$. One-way ANOVA was followed by Newman-Keuls Multiple Comparison post-hoc test.

doi:10.1371/journal.pone.0108152.g003

Table 1. SGCs significantly proliferate at 7d of MA.

	Total $SGCs^{+}_{total}/NP_{total}$	Mean SGCs around NP	% ($NP^{+}_{total}/NP_{total}$)
Controls	0.53±0.07	1.75±0.08	0.29±0.21
	(6485/12715)		**(21/12715)**
4dMA	0.49±0.15	1.75±0.23	0.51±0.28
	(4401/9117)		**(28/9117)**
7dMA	1.00±0.11**#	2.30±0.13*#	1.55±0.29**#
	(13500/14048)		**(212/14048)**

Significant increases in the total number of proliferating SGCs (**$SGC^{+}_{total}/NP_{total}$**), in the mean number of SGC^{+} surrounding a specific NP (**Mean SGC^{+} around NP**), and in the total number of positive neuronal profiles (NP surrounded by half or more than half of their circumference by SGC^{+} - **$NP^{+}_{total}/NP_{total}$**), were found at 7dMA. Values shown as Mean±SEM. In brackets, the total number of cells analyzed for each ratio is displayed. N = 6 for controls and 7d MA; N = 5 for 4d MA. * Significant differences relatively to controls. # Significant differences relatively to 4d MA.* or # represents $p<0.05$; ** represents $p<0.01$. One-way ANOVA followed by Newman-Keuls Multiple Comparison post-hoc test.

doi:10.1371/journal.pone.0108152.t001

quantification, the controls and 4d MA animals showed very similar values, both being statistically different from 7d MA (Table 1).

Discussion

In this study, we show for the first time in the CFA-induced monoarthritis model of chronic joint inflammation that SGCs are activated and proliferate, with a specific temporal profile. Moreover, significant increases in the GFAP labeling in activated SGCs surrounding ATF3 positive (stressed) neurons were also found. This fact suggests that neuronal ATF3 might be involved in the reactive biochemical and morphological changes occurring in SGCs during a chronic pathological state.

Western blot analysis showed that GFAP levels in the ipsilateral DRGs of MA rats are higher than in the contralateral ganglia, and that this ipsi/contra ratio is significantly increased at 7 and 14 days of disease induction, when compared with control non-inflamed animals. Immunohistochemical quantification of GFAP-positive neuronal profiles in the sections of L5 DRGs also showed significantly increased levels at 7 days of MA. At 14d of MA, although statistical significances were not found, the values were still higher than in controls. The slight differences between WB and IHC data at 14 days of MA are certainly due to the distinct methodological approaches. In the WB assay we measured the total amount of protein in the whole DRGs, which contain both neurons, SGCs and Schwann cells. It is possible that Schwann cells, that also express GFAP [22–24], have a small contribution to the proteic levels measured in the WB. On the other hand, the IHC data represent the number of neurons surrounded by GFAP-positive SGCs, and it is unlikely that this quantification has been biased by considerable Schwann cells' contribution since these cells are morphologically distinct from SGCs. Thus, altogether the data from these two different experiments indicate that SGCs are significantly activated after 7days of MA and at least until 2 weeks of disease induction, and that the number of positive neuronal profiles increases around day 7, suggesting a higher number of sensitized neurons. In fact, activated SGCs are known to release several pro-inflammatory and other mediators that promote neuronal sensitization [9,25]. Accordingly, it is expectable that neurons surrounded by a higher number of activated SGCs are also in a higher level of excitability [3,4,10].

These data are in accordance with several recent studies proposing that, after peripheral injury and/or inflammation, SGCs undergo relevant reactive biochemical and phenotypic changes (such as activation, proliferation and hypertrophy) that might be related to the establishment/maintenance of certain pathological and painful states [6,9,11,26–28]. In fact, GFAP expression was found to be increased in inflamed DRGs, at 7 days of model induction (chromic gut suture application onto the DRG) [6], as well as in the trigeminal ganglia of rats with orofacial inflammatory pain [27]. Additionally, two days post-CFA injection into the whisker pad area, the mean percentage of trigeminal ganglia neurons encircled by GFAP and IL-1beta-immunoreactive cells was significantly increased compared with controls [25]. These data corroborate with our results for the GFAP expression in MA animals and indicate that the first week of disease progression seems to be crucial for the events associated to SGCs activation. The slight differences in the temporal expression pattern of GFAP are probably due to the pathophysiological differences of the models under study. As observed, SGCs activation occurs in the initial time-points of disease progression in inflammatory conditions, while little is known about the more prolonged time-points [6,25–27]. Conversely, it seems that nerve damage provokes a more demarked and prolonged effect on SGCs activation. Indeed, in neuropathic pain models, such as in chemically-induced neuropathy, GFAP levels were also significantly higher after 1 week, followed by a decrease to control values only 1 month later [28]. In the spinal nerve ligation (SNL) neuropathy model, GFAP expression increased immediately after 4 hours, gradually increasing up to 7 days and staying high until the end of the experiment at day 56 [21]. In our studies, we observed that the activation of SGCs is significantly higher than in non-inflamed animals at least until 14 days of MA. We have previously proposed the occurrence of a neuropathic component in MA, possibly triggered by the initial inflammatory milieu at the joint cavity [14]. Actually, we reported that ATF3, a neuronal injury marker, is induced in primary afferent neurons, with a peak of expression at 4 days of MA [14], a fact that has not been described frequently in studies using other inflammatory models [1,29,30]. Therefore, the fact that neuronal damage is possibly occurring during MA, might be one of the reasons for the still significantly increased GFAP levels that we found at day 14. For time-points of disease evolution longer than this it is hard to speculate since the information available in the literature is limited. However, it is possible that GFAP levels do not remain high for too long, as it happens in a neuropathy, since MA is still a model triggered by an inflammatory insult.

Many studies are nowadays devoted to the identification of possible inducers of SGCs activation, in different conditions. Recently, some authors suggested a novel mechanism mediated by

fractalkine as the trigger for SGCs' activation in the carrageenan-induced inflammation model [26]. Many other molecules were shown to be released by neurons with their receptors being found in SGCs [31,32], therefore constituting possible mediators in neuron-glia crosstalk and triggers of SGCs' activation. Some authors also suggested that the expression of injury factors in stressed neurons might be one possible trigger for the activation and proliferation of SGCs as well as for the augmented intraganglionar communication [7]. Considering our previous data [14], we asked if ATF3 could be one of the injury factors involved in SGCs activation and in communication within neurons, during MA. Interestingly, we also found significant increases in the number of ATF3 positive neurons surrounded by GFAP-positive cells, in both the total neuronal (**$\mathbf{Double^{+}_{total}/NP_{total}}$**) and ATF3-positive populations (**$\mathbf{Double^{+}_{total}/ATF3^{+}_{total}}$**), at 7d MA, which supports our hypothesis of a possible role of neuronal ATF3 in the reactive changes occurring in SGCs during articular inflammation. After 7d of MA, more than 40% of the ATF3-positive neurons were surrounded by GFAP-positive SGCs, even though the ATF3-positive population represents a small portion of all DRG neurons in the CFA-induced MA model, as we have previously described [14]. Our data are in accordance with other studies showing that the number of ATF3-immunoreactive (IR) neurons enclosed by GFAP-IR SGCs increased in a time-dependent manner in the maxillary nerve region of the trigeminal ganglia [12], in a model of molar extraction in the rat. Also, after chronic constriction injury of the infraorbital nerve, SGCs proliferation was observed preferentially around ATF3-positive neurons of the trigeminal ganglia, although GFAP expression was associated with both ATF3 IR and immunonegative neurons [13]. In a pathological condition, the number of gap junctions between SGCs increase and this phenomenon is intimately related to SGCs activation. Interestingly, gap junctions promote communication between adjacent SGCs enveloping neighboring neurons [3,4,10]. This might result in GFAP labeling around adjacent ATF3-negative neurons, suggesting that it is highly possible to have activated SGCs surrounding non-stressed neurons. Indeed, Gunjigake *et al*. also demonstrated in the model of rat molar extraction that SCGs' activation spread to uninjured neurons in the maxillary nerve region, as well as to the mandibular nerve region [12]. In these studies, as it happened in our case, it has been shown that there is a basal expression of GFAP in control animals, which is probably not labelling activated SGCs [12,33]. Yet, the fact that these increases in GFAP labeling around ATF3 positive neurons are statistically different at 7 days of MA points to a possible relation between ATF3 expression and SGCs-related events.

In the MA animals, the number of SGCs proliferating in the whole DRG was also significantly higher at day 7 of disease when comparing with both controls and 4d MA. Not only the overall number of BrdU-positive SGCs in the DRG increased but also the number of SGCs proliferating around a specific neuron. Moreover, we found significantly more positive neuronal profiles in 7d MA animals, which is in accordance with other studies. There are few reports regarding the proliferation of SGCs, but early in the nineties other authors already showed that these cells proliferated after L5 nerve transection, with maximum activity of the incorporated radioactive marker 1 week after the model induction. In this case, proliferation started decreasing after this time-point [34]. Later, other BrdU incorporation studies showed that SGCs proliferate during Herpes Simplex virus infection, with increases up to 5 days of disease, the latest time point evaluated [35]. This was proposed to be part of a mechanism of neuronal survival during the disease [35]. The same group also found proliferation of SGC in an animal model of scarification of the skin, considered to be a model of minor tissue trauma [7]. BrdU incorporation increased by a 10 times fold 5 days after model induction, when compared with controls. Just recently, peaks of SGCs' proliferation were also observed nearly 4 days after model induction by chronic constriction injury of the infraorbital nerve [13]. Our results are in agreement with these previous studies, indicating that, also in MA, a significant proliferation of SGCs occurs. Also, they suggest that 7d of disease is a triggering time-point for this event.

The reactive changes observed in SGCs appear to be correlated with hypersensitivity to noxious stimuli, although the related mechanisms and their players still remain to be explored. In fact, it has been proved in several models that the administration of fluorocitrate, a metabolic inhibitor of SGCs, not only abolishes GFAP labeling in the DRGs but also alleviates pain [21,26]. MA animals display increased allodynia and hyperalgesia in the ipsilateral paw, after 1 week of CFA injection, as we have already reported [36]. Therefore, it seems that the temporal profile of the biochemical changes found in the ipsilateral DRGs of these animals matches with the painful behavior. Although further studies are needed, data suggest that SGCs might be involved in the MA nociceptive mechanisms, as, in fact, found for other chronic pain models [21].

In summary, this study indicates that SGCs are not bystanders to MA, but that they are crucial in the mechanisms underlying articular inflammation. The reactive changes involving SGCs, namely their activation and proliferation, seem to be particularly active in the early phases of MA development, with peaks around the 7th day, when the expression of the neuronal injury marker ATF3 is already subsiding, and allodynia and hyperalgesia are already obvious in the ipsilateral paws of inflamed animals. The exact functional implications of this early onset for the progression of the disease are still unknown. Additionally, ATF3 might be one potential target for the control of SGCs-mediated mechanisms. Thus, in the future, it will be important to unravel these key mechanisms which will be crucial for the development of new drugs targeting SGCs. This might help to overcome the inefficacy of certain pain-alleviating therapies [8], that have been traditionally devoted to target primary afferent neurons. This is highly relevant since pain associated with joint inflammatory diseases is still a challenge in the clinical practice.

Author Contributions

Conceived and designed the experiments: DSMN FLMN. Performed the experiments: DSMN. Analyzed the data: DSMN FLMN. Contributed reagents/materials/analysis tools: FLMN JMCL. Wrote the paper: DSMN FLMN JMCL.

References

1. Schaible HG, Richter F, Ebersberger A, Boettger MK, Vanegas H, et al. (2009) Joint pain. Exp Brain Res 196: 153–162.
2. Gascon E, Moqrich A (2010) Heterogeneity in primary nociceptive neurons: from molecules to pathology. Arch Pharm Res 33: 1489–1507.
3. Hanani M (2012) Intercellular communication in sensory ganglia by purinergic receptors and gap junctions: implications for chronic pain. Brain Res 1487: 183–191.
4. Hanani M (2005) Satellite glial cells in sensory ganglia: from form to function. Brain Res Brain Res Rev 48: 457–476.
5. Ohara PT, Vit JP, Bhargava A, Romero M, Sundberg C, et al. (2009) Gliopathic pain: when satellite glial cells go bad. Neuroscientist 15: 450–463.
6. Siemionow K, Klimczak A, Brzezicki G, Siemionow M, McLain RF (2009) The effects of inflammation on glial fibrillary acidic protein expression in satellite cells of the dorsal root ganglion. Spine (Phila Pa 1976) 34: 1631–1637.

7. Elson K, Simmons A, Speck P (2004) Satellite cell proliferation in murine sensory ganglia in response to scarification of the skin. Glia 45: 105–109.
8. Jasmin L, Vit JP, Bhargava A, Ohara PT (2010) Can satellite glial cells be therapeutic targets for pain control? Neuron Glia Biol 6: 63–71.
9. Takeda M, Takahashi M, Matsumoto S (2009) Contribution of the activation of satellite glia in sensory ganglia to pathological pain. Neurosci Biobehav Rev 33: 784–792.
10. Hanani M, Huang TY, Cherkas PS, Ledda M, Pannese E (2002) Glial cell plasticity in sensory ganglia induced by nerve damage. Neuroscience 114: 279–283.
11. Zhang H, Mei X, Zhang P, Ma C, White FA, et al. (2009) Altered functional properties of satellite glial cells in compressed spinal ganglia. Glia 57: 1588–1599.
12. Gunjigake KK, Goto T, Nakao K, Kobayashi S, Yamaguchi K (2009) Activation of satellite glial cells in rat trigeminal ganglion after upper molar extraction. Acta Histochem Cytochem 42: 143–149.
13. Donegan M, Kernisant M, Cua C, Jasmin L, Ohara PT (2013) Satellite glial cell proliferation in the trigeminal ganglia after chronic constriction injury of the infraorbital nerve. Glia 61: 2000–2008.
14. Nascimento D, Pozza DH, Castro-Lopes JM, Neto FL (2011) Neuronal injury marker ATF-3 is induced in primary afferent neurons of monoarthritic rats. Neurosignals 19: 210–221.
15. Elson K, Ribeiro RM, Perelson AS, Simmons A, Speck P (2004) The life span of ganglionic glia in murine sensory ganglia estimated by uptake of bromodeoxyuridine. Exp Neurol 186: 99–103.
16. Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16: 109–110.
17. Butler SH, Godefroy F, Besson JM, Weil-Fugazza J (1992) A limited arthritic model for chronic pain studies in the rat. Pain 48: 73–81.
18. Lourenco Neto F, Schadrack J, Platzer S, Zieglgansberger W, Tolle TR, et al. (2000) Expression of metabotropic glutamate receptors mRNA in the thalamus and brainstem of monoarthritic rats. Brain Res Mol Brain Res 81: 140–154.
19. Castro-Lopes JM, Tavares I, Tolle TR, Coito A, Coimbra A (1992) Increase in GABAergic Cells and GABA Levels in the Spinal Cord in Unilateral Inflammation of the Hindlimb in the Rat. Eur J Neurosci 4: 296–301.
20. Pannese E (1981) The satellite cells of the sensory ganglia. Adv Anat Embryol Cell Biol 65: 1–111.
21. Liu FY, Sun YN, Wang FT, Li Q, Su L, et al. (2012) Activation of satellite glial cells in lumbar dorsal root ganglia contributes to neuropathic pain after spinal nerve ligation. Brain Res 1427: 65–77.
22. Hunt D, Hossain-Ibrahim K, Mason MR, Coffin RS, Lieberman AR, et al. (2004) ATF3 upregulation in glia during Wallerian degeneration: differential expression in peripheral nerves and CNS white matter. BMC Neurosci 5: 9.
23. Hunt D, Raivich G, Anderson PN (2012) Activating transcription factor 3 and the nervous system. Front Mol Neurosci 5: 7.
24. Isacsson A, Kanje M, Dahlin LB (2005) Induction of activating transcription factor 3 (ATF3) by peripheral nerve compression. Scand J Plast Reconstr Surg Hand Surg 39: 65–72.
25. Takeda M, Tanimoto T, Kadoi J, Nasu M, Takahashi M, et al. (2007) Enhanced excitability of nociceptive trigeminal ganglion neurons by satellite glial cytokine following peripheral inflammation. Pain 129: 155–166.
26. Souza GR, Talbot J, Lotufo CM, Cunha FQ, Cunha TM, et al. (2013) Fractalkine mediates inflammatory pain through activation of satellite glial cells. Proc Natl Acad Sci U S A 110: 11193–11198.
27. Stephenson JL, Byers MR (1995) GFAP immunoreactivity in trigeminal ganglion satellite cells after tooth injury in rats. Exp Neurol 131: 11–22.
28. Warwick RA, Hanani M (2013) The contribution of satellite glial cells to chemotherapy-induced neuropathic pain. Eur J Pain 17(4): 571–580.
29. Braz JM, Basbaum AI (2010) Differential ATF3 expression in dorsal root ganglion neurons reveals the profile of primary afferents engaged by diverse noxious chemical stimuli. Pain 150: 290–301.
30. Inglis JJ, Notley CA, Essex D, Wilson AW, Feldmann M, et al. (2007) Collagen-induced arthritis as a model of hyperalgesia: functional and cellular analysis of the analgesic actions of tumor necrosis factor blockade. Arthritis Rheum 56: 4015–4023.
31. Ceruti S, Villa G, Fumagalli M, Colombo L, Magni G, et al. (2011) Calcitonin gene-related peptide-mediated enhancement of purinergic neuron/glia communication by the algogenic factor bradykinin in mouse trigeminal ganglia from wild-type and R192Q Cav2.1 Knock-in mice: implications for basic mechanisms of migraine pain. J Neurosci 31: 3638–3649.
32. Chen Y, Li G, Huang LY (2012) P2X7 receptors in satellite glial cells mediate high functional expression of P2X3 receptors in immature dorsal root ganglion neurons. Mol Pain 8: 9.
33. Ajima H, Kawano Y, Takagi R, Aita M, Gomi H, et al. (2001) The exact expression of glial fibrillary acidic protein (GFAP) in trigeminal ganglion and dental pulp. Arch Histol Cytol 64: 503–511.
34. Lu X, Richardson PM (1991) Inflammation near the nerve cell body enhances axonal regeneration. J Neurosci 11: 972–978.
35. Elson K, Speck P, Simmons A (2003) Herpes simplex virus infection of murine sensory ganglia induces proliferation of neuronal satellite cells. J Gen Virol 84: 1079–1084.
36. Borges G, Neto F, Mico JA, Berrocoso E (2014) Reversal of Monoarthritis-induced Affective Disorders by Diclofenac in Rats. Anesthesiology 120(6): 1476–1490.

4.3 Publication III

The expression of P2X7 and P2X3 receptors is altered in sensory ganglia of monoarthritic rats (submitted)

.

The expression of P2X7 and P2X3 receptors is altered in sensory ganglia of monoarthritic rats

Diana Sofia Marques Nascimento[1,2,3], Miguel Luz Soares[2,3,4], António Carlos Ferreira[2,3,4], José Manuel Castro-Lopes[1,2.3], Marzia Malcangio[5], Fani Lourença Moreira Neto[1,2,3]

[1]Departamento de Biologia Experimental, Centro de Investigação Médica (CIM), Faculdade de Medicina do Porto, Universidade do Porto, Portugal; [2]Pain Group, Instituto de Biologia Molecular e Celular (IBMC), Porto, Portugal; [3]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal; [4]Laboratório de Apoio à Investigação em Medicina Molecular (LAIMM), Centro de Investigação Médica (CIM), Departamento de Biologia Experimental, Faculdade de Medicina do Porto, Universidade do Porto, Portugal; [5] Wolfson Centre for Age Related Diseases, King's College London, London, UK.

Purinergic ionotropic P2X receptors are highly implicated in pain processing and may constitute novel analgesic therapeutic targets. Purinergic signaling is involved in the activation of satellite glial cells (SGCs) surrounding neurons in dorsal root ganglia (DRG) and in neuron-glia communication. We have previously shown that SGCs undergo activation and proliferation after 7 days of Monoarthritis (MA), a model of joint painful inflammation. Moreover, SGCs activation occurred preferentially around damaged neurons expressing activating transcription factor 3 (ATF3). Here, the expression profile of P2X7R and P2X3R was evaluated in sensory ganglia of MA rats. Western blot showed that P2X7R protein levels increase in ipsilateral DRGs after 1 week of disease, while P2X3R immunoreactivity decreases around the same timepoint. We have knocked-down ATF3 by RNAi in DRG cell cultures to evaluate its effects on the expression of P2X receptors and heat shock protein 90 (HSP90). This chaperone is part of a P2X7R-protein complex and was recently proposed to have a role in nociception. ATF3 knockdown had no effect on the expression of P2X7R, P2X3R or glial fibrillary acidic protein (GFAP – marker of SGCs activation) as determined by qRT-PCR. Conversely, HSP90 levels were dramatically decreased upon ATF3 downregulation. Our data suggest that P2X7R/P2X3R signaling is activated during MA, possibly triggering the activation of SGCs. Additionally HSP90 emerges as a novel protein under ATF3 regulation. Since ATF3 is greatly induced in MA, it is conceivable that HSP90 is also involved in MA pathophysiology. Further investigation is needed to confirm the biological significance of these findings.

Key words: Primary afferent neurons, Satellite glial cells, Purinergic signaling, receptors ATF3, HSP90

Acknowledgements

The study was supported by the Chair on Pain Medicine of the Faculty of Medicine, University of Porto and by the Grünenthal Foundation – Portugal. The first author received a doctoral grant (SFRH/BD/79497/2011) by Fundação para *a Ciência e a Tecnologia* (FCT), Portugal. The authors would also like to acknowledge Rikke Rie Hansen for the help in the optimization of cell cultures protocols.

Corresponding author:

Fani Lourença Moreira Neto

Departamento de Biologia Experimental, Centro de Investigação Médica (CIM) Faculdade de Medicina da Universidade do Porto, Alameda Prof. Hernâni Monteiro, 4200 – 319, Porto, Portugal;

Telephone: 00 351 220426769; Fax: 00 351 5513655; E-mail: fanineto@med.up.pt

1. Introduction

P2X purinoceptors (P2XR) are a family of cation-permeable fast acting ion channels that open in response to the binding of extracellular adenosine 5'-triphosphate (ATP) [1]. Many studies indicate that these receptors are involved in pain processing mechanisms, constituting possible targets for analgesic drugs [2]. P2XR function in the form of homotrimers (P2X1–7), heterotrimers or multimers [1] and are differently distributed in the nervous tissue, including in many relevant pain neuronal structures. With the exception of P2X1R (data is controversial [3]), expression of all the other subtypes was found in the dorsal root ganglia (DRG) [4,5], which contain the cell bodies of a subpopulation of primary afferent neurons, the nociceptors, that are capable of encoding and transmitting nociceptive stimuli [2].

In the rat DRG, P2X3 is the most abundant P2XR subtype, being predominantly expressed in non-peptidergic C-fiber nociceptors [6,2,7]. The anti-nociceptive effect of P2X3R antagonists has been observed in both inflammatory [8] and neuropathic pain models [9], and null-P2X3R mice show decreases in nociceptive behavior, comparing to naïve animals, when injected with ATP (known to induce pain-like behavior) [10]. Moreover, alterations in P2X3R mRNA and protein levels have been documented for different painful pathological conditions [11,12]. However, data are somehow inconsistent concerning the direction of these changes and, therefore, further investigation is needed to better understand the role of P2X3R in pathological states and nociception, and to evaluate their potential as targets for more effective treatments.

In the DRG, P2X7R are not found in primary afferent neurons, but in satellite glial cells (SGCs) [13]. SGCs enwrap the cell bodies of these neurons, forming individual functional units [13], which are nowadays known to be crucial for the development of chronic pain states [14]. It has been demonstrated that, upon pathological conditions such as inflammation or nerve damage, P2X7 receptors can mediate the activation of SGCs [15] that occurs in response to the ATP released from the sensitized neuronal somata. This evidence supports a role for P2X7R in nociception, but also indicates that they are key players in neuron-glia communication mechanisms [16-19]. Additionally, it has been demonstrated that P2X7R expression is up-regulated in these pathological conditions [20] and that it has a functional role in pain processing. Indeed, in a model of Complete Freund's Adjuvant (CFA) induced hyperalgesia, the nociceptive behavior was reverted by the administration of oxidized ATP, an irreversible inhibitor of P2X7R [21].

The activation of P2X7R triggers various signaling cascades. Heat shock protein 90 (HSP90) appears to be one of the 11 molecules that makes part of a P2X7 receptor-protein complex, responsible for the activation of the different signaling pathways. HSP90 is a homodimer that belongs to the chaperone family, which are abundant and conserved proteins dramatically increased in cells upon stress, including inflammation. Indeed, similarly to the activating transcriptional factor 3 (ATF3), HSP90 is also referred as a "danger signal" [22] being an important regulator of inflammation pathways [23]. Therefore, HSP90 inhibition has been used in order to suppress the inflammatory response in diseases like rheumatoid arthritis [24]. Curiously, a few papers show that HSP90 inhibitors can also alleviate pain [25], although very little is known about the underlying mechanisms and the possible role of HSP90 in the nervous system, in general, and in nociception, in particular.

We have previously demonstrated that SGCs are activated and proliferate after 1 week of Monoarthritis (MA), a joint inflammatory pain model in the rat [26]. SGCs activation occurred preferentially around neurons positively labelled for ATF3 [26], which is a neuronal injury marker also significantly induced in primary afferents of MA animals [27]. To better understand the mechanisms of neuron-SGC crosstalk in this painful inflammatory condition, and partly elucidate the signaling cascades involved, we have evaluated the expression of P2X7R and P2X3R in glial and neuronal cells of the DRG, respectively, at different timepoints of MA. As the expression of injury factors (like ATF3) is also proposed to be involved in mechanisms of neuron-glia communication [18], we then silenced ATF3 in DRG primary cell cultures using specific small

interference RNA (siRNA). Besides evaluating the effect of ATF3 silencing on the purinergic system and on the activation of SGCs, we also assessed HSP90 expression, as some evidence suggest that ATF3 may be regulated by or regulate chaperones like HSP90 in response to stress [28,29].

2. Materials and methods

2.1. Monoarthritis (MA) induction

Monoarthritis (MA) was induced as previously described [26] in male Wistar rats weighing between 200 and 300g (Charles River Laboratories, France). Briefly, animals were injected with 50 µL of complete Freund's adjuvant (CFA), into the left tibiotarsal joint [30] under isoflurane anesthesia. The CFA solution (5.45mg/mL of *mycobacterium butyricum*) was prepared as previously described [31] and MA animals were sacrificed at different timepoints after intraarticular injection, in accordance with each experiment. Control (non-inflamed) animals were similarly injected with 50 µL of CFA vehicle and were sacrificed after 2 days, as also previously described [26]. After injections, animals were monitored daily and scored according to the inflammatory signs and the guarding behavior towards the inflamed paw (in detail in [32,27,26])

All the procedures in animals were carried out according to the European Communities Council Directive of September 22, 2010 (2010/63/EC) and the ethical guidelines for investigation of experimental pain in animals [33]. The experiments were authorized by the animal welfare body (ORBEA) of the Faculty of Medicine of the University of Porto. The humane endpoints defined for this project were always respected. Efforts were made in order to minimize pain and distress and reduce the number of animals used. Habituation to the experimenter was done several days prior to the experiments and animals were daily monitored after the interventions. Animals were housed 2-3 animals per cage under controlled conditions of lighting (12 h light/12 h dark cycle) and temperature, with water and food *ad libitum*.

2.2. DRG primary cell cultures and transfection with siRNAs

Primary cell cultures of DRG were obtained following a similar protocol as in Delree, *et al* [34]. Briefly, all DRGs were freshly harvested from each adult naive rat (between 200-300g) and dissociated by incubation for 1 hour in a cocktail of dispase (3mg/mL) and collagenase (100µg/mL), at 37°C in a 5% CO_2 atmosphere. Prior to mechanical dissociation with glass Pasteur pipettes, cells were also incubated with trypsin (0.25%) in order to better digest the connective tissue between neurons and SGCs. Cells were plated in 6-well plates previously coated with poly-D-lysine. Cultures were maintained at 37°C in a 5% CO_2 atmosphere, in F12 Ham's media supplemented with 50 µg/mL penicillin and streptomycin, 10% heat-inactivated horse serum and Nerve Growth Factor (NGF) at a final concentration of 50 ng/mL. Growth medium was changed after 2 days. Cell cultures grew for 4 days in order to allow the establishment of interactions between SGCs and neurons. When plated the proportion between neurons and SGCs was around 50%-50%, as previously described for normal mixed DRG cell cultures [35].

After 4 days in culture, ATF3 silencing was done according to Schmutzler, *et al* [36]. Briefly, the transfection agent Metafectine Pro (Biontex Laboratories, Martinsried, Planegg, Germany) was diluted in Optimem reduced serum media (Invitrogen, Carlsbad, CA) to a titer of 1:250. In separate eppendorfs, the siRNA molecules (smart pool of 4 sequences from Dharmacon, CO, USA) were also diluted in Optimem and left at room temperature for two minutes. The Metafectine and siRNA dilutions were then mixed at a 1:1 ratio and incubated at room temperature for more 20 minutes. The final mixture was added to each well so that the final concentration of siRNA was 100 nM. Cells from each animal that were previously divided in 3 wells received either a control scramble sequence of siRNA (**siCT**), a specific siRNA for ATF3 (**siATF3**) or only the transfection reagent (**TR**). After 24h, cells were stimulated with Lipopolysaccharide (Ultra pure LPS from E. coli 0111:B4 strain, Invivogen, 1µg/mL)[35] for more 24h before lysis, meaning that siRNAs were maintained in culture for 48h. Cells were then

washed twice with PBS, scraped in 100µL of TRI® reagent (T9424, Sigma) and frozen for later analysis by real time quantitative polymerase chain reaction (RT-PCR).

Stimulation with LPS was used in order to trigger inflammatory signaling cascades (through activation of TLR4) [37]. Additionally, LPS-induced pathways are involved in the upregulation of ATF3 [37], as well as in P2X7 activation and the release of HSP90 [6]. ATF3 expression occurs in around 90% of the neurons in culture without any treatment, due to the nerve axotomy induced when harvesting the DRGs (data not shown). By using LPS stimulation we intended to better control ATF3 expression in these cultures and to induce typical inflammatory pathways, so that the effects of ATF3 silencing could be studied in an activated system.

2.3. Real-time quantitative polymerase chain reaction (RT-qPCR)

For RT-qPCR, samples were homogenized in TRI® reagent and processed for RNA extraction according to manufacturer's instructions. Briefly chloroform was added to the cell lysates and, following centrifugation, the RNA-containing upper phase was recovered for subsequent total RNA extraction.

Total RNA was isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. Quantification was performed using a Nanodrop 2000 and RNA integrity was assessed using the Agilent 2100 Bioanalyzer. All samples had a RNA Integrity Number (RIN) ≥ 7. The RevertAid H Minus cDNA synthesis kit (Fermentas) was used to reverse transcribe 1 µg of total RNA with random primers, and the resulting cDNA was diluted 1:20, aliquoted and stored at -20 ºC for subsequent use.

The expression levels of selected genes were measured by qPCR using the StepOnePlus Real-Time PCR System (Applied Biosystems). Triplicates were performed for each reaction, using Maxima SYBR Green/ROX qPCR Master Mix (Fermentas), 400 nM of primers (except where noted, Table S1) and 3µL of 20x diluted cDNA (described above), in a 12.5µL final volume. A standard curve made up of 1/2 dilutions of pooled cDNA of all samples was run on each plate for each primer set assay for relative quantification. Target gene expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The estimated efficiency of all qPCR assays ranged between 90-100%. Primer sequences and annealing temperatures are shown in Table 1.

Table 1. Primer sequences and annealing temperatures for quantitative PCR

Transcript	Primers	Annealing (ºC)
Atf3	F: CCAGAACAAGCACCTTTGCC R: GTTTCGACACTTGGCAGCAG	60
Gfap	F: AATTGCTGGAGGGCGAAGAA R: TTGAGGTGGCCTTCTGACAC	60
P2rx3	F: TTCCTTCACTCGGCTGGATG R: TGCCAGCGTTCCCATATACC	60
P2rx7	F: GCACATGACCGTCTTTTCCT R: CAAAGGGAGGGTGTAGTCGG	60
Hsp90aa1	F: CTGCGTATTTGGTTGCTGAGA R: ACCTTTGTTCCACGACCCAT	60
Hsp90ab1	F: AAATTGCCCAGCTGATGTCC R: ACTTGGAAGGGTCAGTCAGG	60
Gapdh	F: CCATCACCATCTTCCAGGAG R: GCATGGACTGTGGTCATGAG	60

F: forward primer; R: reverse primer

2.4. Western Blotting (WB)

In order to investigate P2X7R expression during MA, animals were sacrificed by decapitation under light anesthesia with isoflurane after 4, 7 and 14 days of MA induction. Control non-inflamed animals were sacrificed 2 days after vehicle injection. For each animal, freshly harvested L4 and L5 ganglia were pooled together but separately for the ipsi and contralateral sides. DRGs were then lysed and homogenized in 70µL of radio immuno precipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors, as previously described [26]. Proteins were quantified by the bicinchoninic acid (BCA) protein assay. After heating at 94ºC, 20µg of protein were loaded for each lane and separated on

12% sodium dodecyl sulphate-polyacrylamide (SDS/PAGE) gels. Proteins were then transferred into nitrocellulose membranes and blocked with non-fat milk (5% milk powder diluted in tris buffer saline tween20; TBST buffer), for one hour, at room temperature. Membranes were then incubated in polyclonal rabbit anti-P2X7R antibody (APR-004, Alomone Labs, Jerusalem, Israel) diluted 1:500 in TBST with 2% of normal goat serum (NGS), for 24 hours at 4 ºC. Incubation in rabbit anti-β-tubulin (Ab6046 ABCAM, Cambridge, UK) diluted 1:10,000 in TBST with 2% of NGS, overnight at 4 ºC, was also performed as a loading control. Lastly, blots were incubated in donkey anti-rabbit secondary antibody conjugated with HRP (711-035-152, Jackson Laboratories), diluted 1:5000 in TBST with 5% milk powder. Antibody binding was visualized with the SuperSignal West Pico Chemiluminescent Substrat kit (Thermo Scientific; 34080) and chemiluminescent signals were detected by exposure in the ChemiDoc™ system (BioRad).

The protein levels were obtained by densitometric analysis of the band signal intensity using the image computer software ScionImageR (Scion Corporation). The protein density was determined for each band after subtracting the background of the surrounding region and normalizing for the selected area. This density was corrected against the loading control signal, for each blot (ratio P2X7R/β-tubulin). Assays were performed twice on samples obtained from independent groups of rats.

ATF3 detection by WB was also performed in DRG cell cultures samples to confirm the knockdown of this protein in cells treated with siRNA for ATF3. Briefly, cells were scraped in 100 µL of RIPA buffer and after quantification of the total protein, 10µg of each sample was loaded. Basically, WB was performed exactly as described above, except that these blots were incubated in polyclonal rabbit anti-ATF-3 (diluted 1:100 in TBST with 2% of NGS; C-19: sc-188; Santa Cruz Biotechnology, Inc.), overnight at room temperature, and afterwards incubated in HRP-donkey anti-rabbit (1:5000 as described above).

2.5. Immunohistochemistry (IHC)

In order to evaluate P2X3R expression in primary afferent neurons during MA, we performed immunohistochemistry (IHC) assays in DRG slices of inflamed (4, 7 and 14d of MA) and control non-inflamed animals. The protocol for IHC was previously described in detail in Nascimento *et al.*, 2011 [27]. Briefly, rats were perfused with 4% paraformaldehyde (PFA) and the dissected biological material was post-fixed in the same solution before cryoprotection in 30% sucrose. The ipsi- and contralateral DRGs corresponding to the L5 spinal segment were then cut into 14 µm sections in a freezing cryostat (-20ºC) and tissue sections were collected sequentially into 5 different poly-L-lysine coated slides.

Each slide was firstly thawed and washed in PBS 0.1M and then in PBS containing 0.3% Triton X-100 (PBST). The blocking step was done in 10% of NGS in PBST for 1 hour. Afterwards, slides were incubated for 48h at 4ºC in the rabbit anti-P2X3R primary antibody (RA10109, Neuromics), diluted 1:4000 in PBST containing 2% of NGS. After several washes in PBST with 2% of NGS, slides were incubated for 1 hour at room temperature, in goat anti-rabbit 568 (A11011, Molecular Probes) secondary antibody diluted 1:1000 in a solution of PBST with 2% of NGS. To allow visualization under a fluorescent microscope, slides were coverslipped with a mounting media (solution containing 3 parts of glycerol and 1 part of PBS 0.4M).

Images were obtained in a fluorescence microscope (AXIO Imager.Z1, Zeiss), coupled to a digital camera (Axiocam MRm) and a computer image software (Axiovision 4.6). Acquisition conditions, such as amplification of the objective, light intensity, contrast and hue, were maintained constant.

2.6. Data and statistical analysis

Statistical analyses were performed using GraphPad Prism 5® (GraphPad Software). One-way analysis of variance (one-way ANOVA) was used to evaluate significant differences between the different experimental groups and Student's t-test to evaluate differences between only two

different conditions. Data passed the normality Kolmogorov-Smirnov test.

In the **WB assays**, the density of **P2X7R** bands was corrected against the loading control (ratio **P2X7R/β-tubulin**) and values from MA experimental groups (4, 7 and 14d) were calculated relatively to control non-inflamed samples (assumed as the reference experimental group and normalized to 1). The heavier band (77KDa) assumed as a glycosylated form of the receptor [38] showed a completely distinct profile of expression and therefore was quantified in separate. The other two bands (65-69KDa, the predicted weight for this protein taking in consideration its sequence of aminoacids) always showed identical profiles and were similar in size, possibly resulting from other smaller post-translational modifications [39], although this has not been consistently reported. Therefore they were quantified as one. Data from both quantifications was analyzed using ANOVA followed by the Dunnett's post-hoc test. Results are displayed as mean±SEM (N=4 for 7dMA and N=5 for controls, 4 and 14d MA).

For the **IHC assays against P2X3R,** each slide containing every fifth sequential section of the L5 DRG was entirely photographed. For each image, cell counting was done using a grid which allowed a random selection of the areas used for quantification. The total number of P2X3R-positive neurons was counted throughout the ganglia, normalized for the total number of neurons in the same area and calculated as percentages **(% Total P2X3R+ neurons/Total neurons),** in accordance with [27]. Statistical analysis of P2X3R immunodetection was done using ANOVA followed by Dunnett's post-hoc test. Results are shown as mean±SEM (N=5 for controls and 14d MA; N=6 for 4d MA and N=7 for 7d MA).

For the data analysis of **RT-qPCR performed in the DRG cell cultures**, we assumed normalized **siCT** as a reference experimental condition (set to 1) and the normalized values from **siATF3** cells were compared to this control. No significant differences in gene expression were found between cells in the **TR** condition in comparison to those from the **siCT** control condition (data not shown) indicating that the sequences of negative control siRNA had no apparent effect on the cells. Statistical analysis was done using two-tailed Paired Student's T-test to evaluate differences between these two groups. Results are shown as mean±SEM and percentages are also displayed. Biological replicates (N=4 for both siCT and siATF3 groups of treated cells) were analyzed in triplicate for all genes investigated.

For all the statistical analyses, a level of significance of $P < 0.05$ was assumed.

3. Results

P2X7R expression increases during MA

The WB detection of P2X7R resulted in 3 distinct bands one around 77KDa and the other two around 65-69KDa (Fig. 1a), which were quantified separately. The quantification of the 77KDa and 65-69KDa bands (Figs. 1b and 1c), was also done separately for ipsi (Fig. 1b) and contralateral DRGs (Fig. 1c), assuming non-inflamed controls as the reference group. P2X7R expression was increased in ipsilateral DRGs homogenates of MA animals, after 1 week of disease induction. Thus, quantification of the P2X7R lighter bands (65-69KDa) intensity showed statistically significant increases in the ipsilateral DRGs of 7d MA (2.55±0.27, $p<0.05$) and 14d MA (2.69±0.37, $p<0.05$) animals, when comparing to the respective controls (1.00±0.26) (Fig. 1b). Conversely, in contralateral DRGs, expression of the 65-69KDa peptides was also upregulated in the experimental group but not significantly (Fig. 1c). The glycosylated and heavier form (77KDa) of P2X7R remained unchanged, for either the ipsi or contralateral DRGs (Figs. 1b and 1c), suggesting that glycosylation of this receptor is not a crucial event in MA.

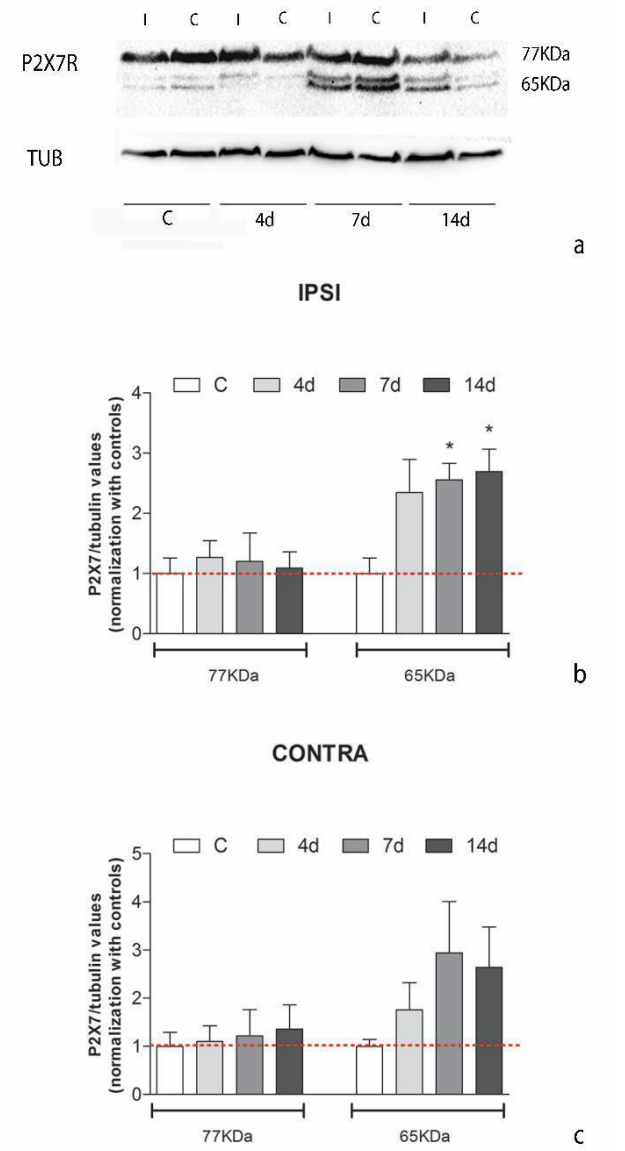


Fig. 1 P2X7R expression increases during MA. P2X7R detection in the Western blots resulted in three bands, one at 77KDa proposed to be a glycosylated form and two more at 65-69KDa (the predicted weight for the receptor), assumed and quantified as one. The increases in P2X7R expression are easily observed especially for the 65-69KDa band, after 7days of MA induction **(a)**. The 77KDa and 65-69KDa bands were quantified separately for both the ipsilateral **(b)** and contralateral **(c)** DRGs. After correction for the loading control (P2X7R/β-tubulin ratio), data from MA animals were normalized against the respective control group. The 65-69KDa forms showed significant increases in the ipsilateral DRGs of MA animals after 7 and 14d of disease when comparing to controls **(b)**. In the contralateral DRGs of MA animals no significant changes were found in comparison to controls for any of the detected forms **(c)**. All values are shown as Mean±SEM. N=4 for 7d MA and N=5 for all the other experimental groups. * represents $p < 0.05$ relatively to control non-inflamed animals. One-way ANOVA was followed by Dunnett's Multiple Comparison post-hoc test. I=Ipsilateral; C=Contralateral

P2X3R expression decreases at later timepoints of MA

P2X3R expression in L5 DRGs, as assessed by immunohistochemistry, decreased along the progression of MA, as shown in figures 2a and 2b. The percentage of P2X3R-positive cells was significantly reduced in the L5 ipsilateral ganglia of 7d (35.78±0.96%, $p<0.05$; Fig. 2c) and 14d (30.98±1.90%, $p<0.001$; Figs. 2b and 2c) MA animals, when compared with non-inflamed controls (42.08±2.05%; Figs. 2a and 2c). These data suggest a downregulation in neuronal P2X3R expression at those timepoints of MA.

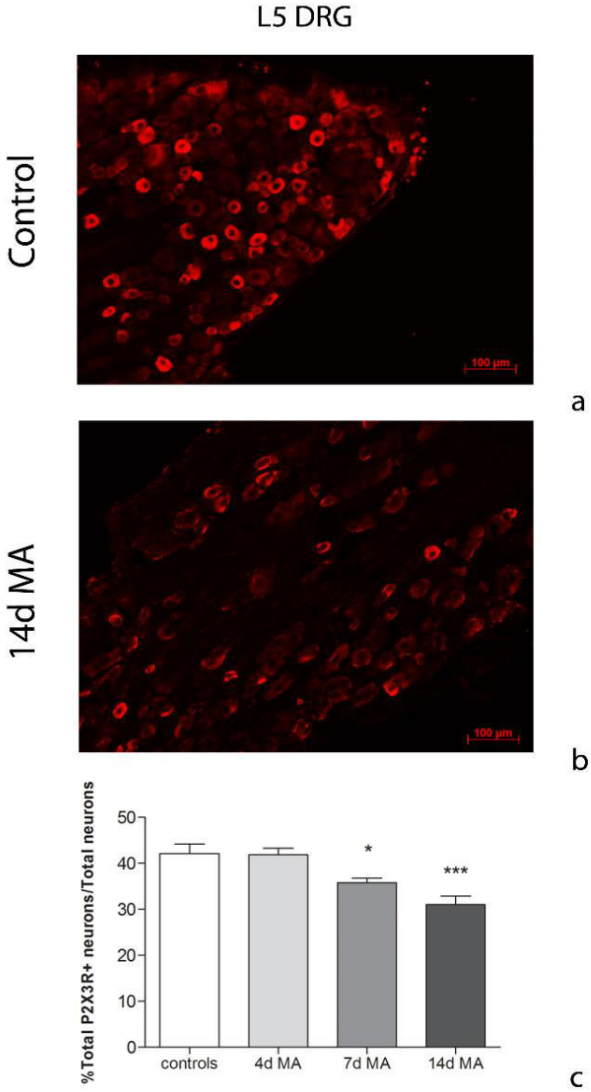


Fig. 2 P2X3R expression decreases along MA progression. (a, b) Immunolabeling for P2X3R (red), in L5 DRGs sections from a control **(a)** and a 14d MA animal **(b). (c)** The percentage of P2X3R-positive neurons in the total neurons counted (**% Total P2X3R+ neurons/Total neurons**).decreased in L5 ipsilateral DRGs of animals with

7d and 14d of MA. All values are shown as Mean±SEM. N=5 for controls and 14dMA; N=6 for 4dMA and N=7 for 7dMA. * Represents $p < 0.05$ and *** represents $p<0.001$, relatively to control non-inflamed animals. One-way ANOVA was followed by Dunnett's post-hoc test

ATF3 knockdown does not affect P2X3R/P2X7R or GFAP expression

Transfection of DRGs primary cell cultures with siRNAs specifically targeting ATF3 (siATF3) resulted in a significant knockdown of ATF3. Messenger RNA levels were decreased by approximately 60% in siATF3 –treated cells (siATF3 cells: 0.41±0.10, $p<0.01$; Fig. 3a), relative to those transfected with scramble sequences (siCT), used as a negative control for the silencing (siCT cells: 1.00±0.14). This was confirmed by WB analysis, which showed a similar reduction at the protein level in siATF3 -treated cells (Fig. 3a, top right insert). However, this level of ATF3 silencing did not induce any significant changes in GFAP gene expression (1.12±0.27 for siATF3 treated cells vs. 1.00±0.11 for siCT treated cells; Fig. 3b). Moreover, knockdown of ATF3 did not significantly affect the expression of P2X7 (1.13±0.43 for siATF3 vs. 1.00±0.24 for siCT; Fig. 3c) or P2X3 (0.82±0.21 for siATF3 vs. 1.00±0.11 for siCT; Fig. 3d) purinergic receptors in the cultured DRG cells.

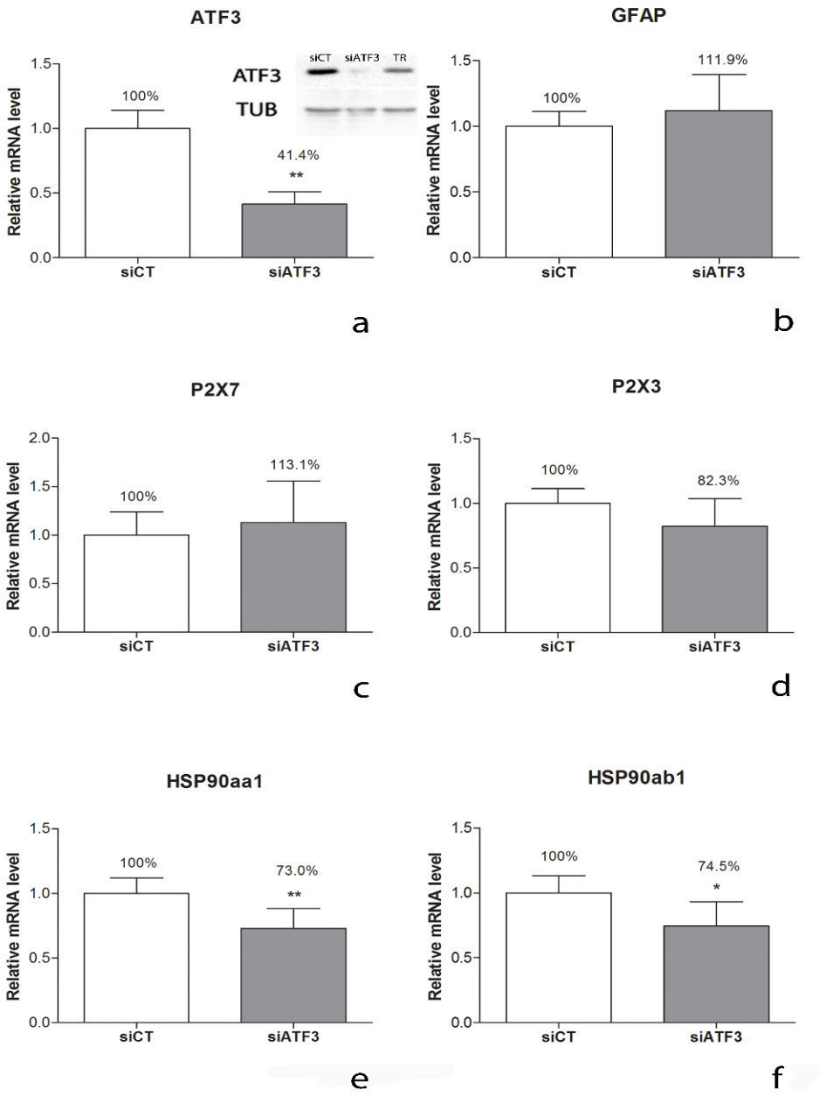


Fig. 3 ATF3 knockdown in DRG primary cell cultures does not alter P2X7R/P2X3R or GFAP gene expression but induces a significant decrease in HSP90 gene expression. RT-PCR analysis showed that ATF3 gene expression was knocked-down at around 60% in DRG cell cultures treated with ATF3 siRNAs (siATF3), when comparing to cells treated with a negative control scrambled sequence (siCT). This reduction was also observed in the protein expression by WB analysis **(a)**. However, this knockdown in ATF3 did not induce changes in the gene expression of GFAP, or of the P2X7 and P2X3 purinergic receptors **(b, c, d)**. Surprisingly, the gene expression of both the highly inducible HSP90aa1 and the constitutive HSP90ab1 isoforms of HSP90 was decreased in cell cultures after ATF3 knockdown **(e, f)**. Values from siATF3 cells were calculated comparatively to siCT, assumed as a reference condition and normalized to 1. All values shown as Mean±SEM. * Represents $p < 0.05$ and ** $p<0.01$. N=4 for both siCT and siATF3 experimental conditions, for all genes investigated. Statistical analysis was done using two-tailed Paired Student's T-test to evaluate differences between the two groups. Percentages are also displayed. TR=transfection reagent.

ATF3 knockdown significantly decreases HSP90 gene expression

The knockdown of ATF3 expression in the DRG cells transfected with siATF3 resulted in a significant reduction in the expression of both HSP90 chaperone isoforms (HSP90aa1 and HSP90ab1), with decreases of 27% for HSP90aa1 (0.73±0.15, $p<0.01$; Fig. 3e) and 25.5% for HSP90ab1 (0.74±0.19, $p<0.05$; Fig. 3f), relative to the expression found in siCT-treated cells (1.00±0.12 for HSP90aa1 and 1.00±0.13 for HSP90ab1). HSP90aa1 encodes for the HSP90α isoform which is highly inducible and HSP90ab1 encodes for HSP90β which is the constitutive form [40].

4. Discussion

We show for the first time that P2X7R expression increases in the DRGs of rats after one week of Monoarthritis and that, around the same timepoint, the expression of P2X3R is significantly decreased in primary afferent neurons. This suggests that the P2X7R/P2X3R-mediated purinergic signaling is implicated in the pathophysiology of MA. We also show that ATF3 expression directly regulates the expression of the inducible and constitutive isoforms of the HSP90 chaperone in the DRG neurons. Thus, it is conceivable that these molecules may be part of the same signaling cascade triggered during the MA condition, although the subsiding mechanisms require further investigation.

In this study, WB analysis revealed significant increases of P2X7R expression in ipsilateral DRGs of monoarthritic animals. In fact, 3 distinct bands for P2X7R were found in the western blots, corresponding most probably to different post-translational modifications. The *N*-linked glycosylation is a very important modification that promotes P2X receptors trafficking to the cell surface [41]. In human cell lines, P2X7R is N-linked glycosylated on five residues and this post-translational modification appears to be important for P2X7 signaling and pore formation [42]. However, in the present study, we detected no changes in protein levels of the heavier (77 KDa) band, proposed as the glycosylated form of PX7R, in the DRGs of MA animals comparing to non-inflamed controls. In contrast, the non-glycosylated forms with 65-69KDa (possibly cytosol stored protein) were significantly increased in the ipsilateral DRGs of MA animals.

Although very little is known about these P2X7R post-translational modifications, which would allow us to better understand their biological role in physiological an pathological states, it is interesting to note that P2X7R levels were significantly increased in animals with 7 and 14d MA. This time pattern of changes in P2X7R expression is particularly interesting as it is in accordance with our previous studies showing that SGCs are activated and proliferate around 7 days of MA [26]. In the DRG, P2X7R are located exclusively in the SGCs, implying that these receptors exert their effects almost exclusively through glia–neuron interactions [20], and possibly contributing to pain processing mechanisms [43-45,21]. Indeed, upon inflammation and/or nerve injury, neurons in the sensory ganglia release ATP which may bind to P2X7R located in SGCs and consequently lead to SGCs activation [43,45,46]. Thus, the timecourse of increased expression of P2X7R throughout the monoarthritic condition is consistent with the timecourse of SGCs activation/proliferation events we had previously reported in the same joint inflammatory model [26], strongly suggesting that P2X7R might be implicated in SGCs activation during MA. Moreover, our data is in accordance with other studies showing that P2X7R is upregulated in DRGs of animals inflamed with CFA in the plantar surface of the paw [20], while others have shown increased levels of P2X7R mRNA in a model of inflammatory bowel disease [47]. Interestingly, P2X7R was also upregulated in DRGs of neuropathic pain patients [48].

Chen and colleagues demonstrated that P2X7R upregulation exerts a negative feedback control mechanism over neuronal P2X3R expression [49,50,20]. Accordingly, we show that the number of P2X3R-positive neurons in the ipsilateral DRG of MA animals was significantly decreased after 7 days of disease duration, and this was even more pronounced at day 14 of MA. Therefore, it is likely that a similar P2X7R/P2X3R negative control is also occurring in MA. Since P2X3R is highly involved in nociception [51], by reducing its

expression the P2X7R-negative control can effectively prevent allodynia in inflamed rats acting as a protective mechanism [20]. Considering this, even though P2X7R are still relevant targets to be studied in the MA condition, its inhibition might not be a good strategy to alleviate pain, as in fact proposed by others.

Our results are also in accordance with previous studies showing that the initial expression of P2X3R in around 35% of the L4 and L5 DRG neurons dropped more than 50% after sciatic nerve axotomy [52]. In contrast, in a model of trigeminal neuropathic pain, the initial decrease of P2X3R expression in the first two weeks (in agreement with our results) was followed by increases not only in P2X3R expression but also in the number of new neuronal branches in the affected areas [53]. Moreover, both P2X3R and the neuropeptide galanin (involved in neuroprotection/ neurogenesis) were significantly increased in the DRG of mice with 15 and 47 days of collagen antibody-induced arthritis (CAIA) [54]. Thus, it seems that at later stages of the disease, a shift in the expression profile of P2X3R can occur and different signaling pathways (e.g. neuroprotection/regeneration cascades) may be activated.

A shift in the mechanisms and signaling cascades to a more "neuropathic pain phenotype", with the activation "damage-related programs" has also been proposed in models of joint pain (like in osteoarthritis - OA) [55,54]. Interestingly, we have previously found a dramatic increase in the expression of the neuronal injury marker ATF3, in the DRG of MA animals which prompted us to suggest that some degree of neuronal damage is occurring in this condition [27]. Thus, like in OA, plastic changes might be occurring during MA leading to the development of a more neuropathic-like phenotype [55,54]. These shifts might explain, at least in part, the changes in the expression profile of P2X3R in these conditions. Accordingly, it has also been reported that P2X3 mRNA decreased in ATF3-expressing neurons, after nerve injury [56]. This indicated that P2X3R was increased specifically in intact neurons, which is suggestive of a protective role of this receptor [11]. Even though P2X3R do not necessarily co-localize with ATF3, this factor seems to be extremely relevant in these events.

ATF3 is quite a particular molecule since it can trigger different signaling pathways according to the cellular environment [57]. In the nervous tissue, besides being recognized as a neuronal injury marker [56], it promotes neurite outgrowth and enhances peripheral nerve regeneration after axotomy [58,59]. In a previous study, we have also demonstrated that an augmented activation of SGCs at day 7 of MA occurs preferentially around ATF3-positive sensory neurons [26]. Curiously, some authors have proposed that the expression of injury factors might possibly be one of the triggers for the initiation of the neuron-glia communication upon a pathological condition [18]. Thus, ATF3 could be one of the factors implicated in SGCs activation, through mechanisms involving P2X3R and P2X7R. If confirmed, ATF3 could be triggering different signaling pathways along MA progression. To test this hypothesis, we knocked-down ATF3 in primary cell cultures of DRG. ATF3 knockdown did not induce any changes in the expression of P2X7R and/or P2X3R or even in the activation of SGCs, as inferred by no changes in the levels of GFAP. This suggests that either the achieved degree of silencing (~60%) is insufficient to disrupt the activity of ATF3 in inducing significant and quantifiable changes in the purinergic system (threshold effect) or, at least *in vitro*, ATF3 expression is not implicated in these events. However, before completely excluding a relation between ATF3 expression and the activation of SGCs through these purinergic receptors it is necessary to consider the methodological limitations inherent to the use of *in vitro* systems. Indeed, the disruption of the SGC-neuron functional units during the DRG dissociation may have prevented the occurrence of particular events that are dependent on this structural organization and tight communication between the neuronal cell bodies and their satellite cells.

Interestingly, even in the absence of a total silencing of ATF3, we found significant decreases in gene expression of both the inducible (alpha-α) and constitutive (beta-β) isoforms of the HSP90 chaperone in the DRG cell cultures. An ATF3 upregulation has been observed after HSP90

inhibition [28,29], but little is known about the regulation of HSP90 by ATF3. It has been demonstrated that ATF3 negatively regulates the TLR4 inflammatory signaling cascade [37] while HSP90 is a ligand of this receptor [60] contributing to the inflammatory response. Although the information about its function in the nervous system is scarce, our results showing a HSP90 upregulation in DRG cultures following ATF3 suppression may suggest an involvement of this chaperone in the MA pathophysiology (where ATF3 is significantly induced). Moreover, HSP90 was shown to be part of a P2X7-protein complex [61], a receptor whose expression was also up-regulated in MA. Interestingly, besides reducing inflammation [24], HSP90 inhibitors reversed neurodegeneration [62] and alleviated allodynia in a model of neuropathic pain [23,25].

In summary, our data demonstrates the involvement of P2X7R and P2X3R in MA pathophysiology. It is likely that P2X7R is involved in the activation of SGCs, being a key mediator in the SGCs-neuron communication occurring in this pathology. We also propose that glial P2X7R induces a negative feedback control over P2X3R expression in the MA condition. In this study, we also show that the expression of HSP90, involved in the P2X7R-protein complex, is under ATF3 regulation in DRG neurons. However, further studies are still needed to understand if these mechanisms are possibly mediated by ATF3 in the MA condition (as evidence suggests) and to evaluate the role of these proteins in the nociceptive behavior.

5. Conflict of interest

The authors certify that there are not any personal or financial conflicts of interests related with the presented data.

6. Ethical approval

All procedures involving animals were in accordance with the ethical standards of the institution at which the studies were conducted (experiments authorized by the animal welfare body (ORBEA) of the Faculty of Medicine of the University of Porto). All applicable international, national and/or institutional guidelines for the care and use of animals were followed (European Communities Council Directive of September 22, 2010 - 2010/63/EC - and the ethical guidelines for investigation of experimental pain in animals).

7. References

1. Habermacher C, Dunning K, Chataigneau T, Grutter T (2015) Molecular structure and function of P2X receptors. Neuropharmacology 104:18-30. doi:10.1016/j.neuropharm.2015.07.032

2. Chizh BA, Illes P (2001) P2X receptors and nociception. Pharmacological reviews 53 (4):553-568

3. Kobayashi K, Fukuoka T, Yamanaka H, Dai Y, Obata K, Tokunaga A, Noguchi K (2005) Differential expression patterns of mRNAs for P2X receptor subunits in neurochemically characterized dorsal root ganglion neurons in the rat. J Comp Neurol 481 (4):377-390. doi:10.1002/cne.20393

4. Magni G, Ceruti S (2014) The purinergic system and glial cells: emerging costars in nociception. BioMed research international 2014:495789. doi:10.1155/2014/495789

5. Krames ES (2014) The role of the dorsal root ganglion in the development of neuropathic pain. Pain medicine 15 (10):1669-1685. doi:10.1111/pme.12413

6. Beamer E, Goloncser F, Horvath G, Beko K, Otrokocsi L, Kovanyi B, Sperlagh B (2015) Purinergic mechanisms in neuroinflammation: An update from molecules to behavior. Neuropharmacology 104:94-104. doi:10.1016/j.neuropharm.2015.09.019

7. Puchalowicz K, Tarnowski M, Baranowska-Bosiacka I, Chlubek D, Dziedziejko V (2014) P2X and P2Y receptors-role in the pathophysiology of the nervous system. International journal of molecular sciences 15 (12):23672-23704. doi:10.3390/ijms151223672

8. Prado FC, Araldi D, Vieira AS, Oliveira-Fusaro MC, Tambeli CH, Parada CA (2013) Neuronal P2X3 receptor activation is essential to the hyperalgesia induced by prostaglandins and sympathomimetic amines released during inflammation. Neuropharmacology 67:252-258. doi:10.1016/j.neuropharm.2012.11.011

9. Jarvis MF, Burgard EC, McGaraughty S, Honore P, Lynch K, Brennan TJ, Subieta A, Van Biesen T, Cartmell J, Bianchi B, Niforatos W, Kage K, Yu H, Mikusa J, Wismer CT, Zhu CZ, Chu K, Lee CH, Stewart AO, Polakowski J, Cox BF, Kowaluk E, Williams M, Sullivan J, Faltynek C (2002) A-317491, a novel potent and selective non-nucleotide antagonist of P2X3 and P2X2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat. Proc Natl Acad Sci U S A 99 (26):17179-17184. doi:10.1073/pnas.252537299

10. Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, Ford AP (2000) Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. Nature 407 (6807):1011-1015. doi:10.1038/35039519

11. Tsuzuki K, Kondo E, Fukuoka T, Yi D, Tsujino H, Sakagami M, Noguchi K (2001) Differential regulation of P2X(3) mRNA expression by peripheral nerve injury in intact and injured neurons in the rat sensory ganglia. Pain 91 (3):351-360

12. Xu GY, Huang LY (2002) Peripheral inflammation sensitizes P2X receptor-mediated responses in rat dorsal root ganglion neurons. J Neurosci 22 (1):93-102

13. Hanani M (2005) Satellite glial cells in sensory ganglia: from form to function. Brain research Brain research reviews 48 (3):457-476. doi:10.1016/j.brainresrev.2004.09.001

14. Jasmin L, Vit JP, Bhargava A, Ohara PT (2010) Can satellite glial cells be therapeutic targets for pain control? Neuron Glia Biol 6 (1):63-71. doi:10.1017/S1740925X10000098

15. Hanani M (2012) Intercellular communication in sensory ganglia by purinergic receptors and gap junctions: implications for chronic pain. Brain Res 1487:183-191. doi:10.1016/j.brainres.2012.03.070

16. Gu Y, Chen Y, Zhang X, Li GW, Wang C, Huang LY (2010) Neuronal soma-satellite glial cell interactions in sensory ganglia and the participation of purinergic receptors. Neuron Glia Biol 6 (1):53-62. doi:10.1017/S1740925X10000116

17. Zhang X, Chen Y, Wang C, Huang LY (2007) Neuronal somatic ATP release triggers neuron-satellite glial cell communication in dorsal root ganglia. Proc Natl Acad Sci U S A 104 (23):9864-9869. doi:10.1073/pnas.0611048104

18. Elson K, Simmons A, Speck P (2004) Satellite cell proliferation in murine sensory ganglia in response to scarification of the skin. Glia 45 (1):105-109. doi:10.1002/glia.10294

19. Gunjigake KK, Goto T, Nakao K, Kobayashi S, Yamaguchi K (2009) Activation of satellite glial cells in rat trigeminal ganglion after upper molar extraction. Acta histochemica et cytochemica 42 (5):143-149. doi:10.1267/ahc.09017

20. Chen Y, Zhang X, Wang C, Li G, Gu Y, Huang LY (2008) Activation of P2X7 receptors in glial satellite cells reduces pain through downregulation of P2X3 receptors in nociceptive neurons. Proc Natl Acad Sci U S A 105 (43):16773-16778. doi 10.1073/pnas.0801793105

21. Dell'Antonio G, Quattrini A, Dal Cin E, Fulgenzi A, Ferrero ME (2002) Antinociceptive effect of a new P(2Z)/P2X7 antagonist, oxidized ATP, in arthritic rats. Neuroscience letters 327 (2):87-90

22. Osterloh A, Breloer M (2008) Heat shock proteins: linking danger and pathogen recognition. Medical microbiology and immunology 197 (1):1-8. doi:10.1007/s00430-007-0055-0

23. Hutchinson MR, Ramos KM, Loram LC, Wieseler J, Sholar PW, Kearney JJ, Lewis MT, Crysdale NY, Zhang Y, Harrison JA, Maier SF, Rice KC, Watkins LR (2009) Evidence for a role of heat shock protein-90 in toll like receptor 4 mediated pain enhancement in rats. Neuroscience 164 (4):1821-1832. doi:10.1016/j.neuroscience.2009.09.046

24. Rice JW, Veal JM, Fadden RP, Barabasz AF, Partridge JM, Barta TE, Dubois LG, Huang KH, Mabbett SR, Silinski MA, Steed PM, Hall SE (2008) Small molecule inhibitors of Hsp90 potently affect inflammatory disease pathways and exhibit activity in models of rheumatoid arthritis. Arthritis and rheumatism 58 (12):3765-3775. doi:10.1002/art.24047

25. Urban MJ, Li C, Yu C, Lu Y, Krise JM, McIntosh MP, Rajewski RA, Blagg BS, Dobrowsky RT (2010) Inhibiting heat-shock protein 90 reverses sensory hypoalgesia in diabetic mice. ASN neuro 2 (4):e00040. doi:10.1042/AN20100015

26. Nascimento DS, Castro-Lopes JM, Moreira Neto FL (2014) Satellite glial cells surrounding primary afferent neurons are activated and proliferate during monoarthritis in rats: is there a role for ATF3? PloS one 9 (9):e108152. doi:10.1371/journal.pone.0108152

27. Nascimento D, Pozza DH, Castro-Lopes JM, Neto FL (2011) Neuronal injury marker ATF-3 is induced in primary afferent neurons of monoarthritic rats. Neurosignals 19 (4):210-221. doi:10.1159/000330195

28. Hackl C, Lang SA, Moser C, Mori A, Fichtner-Feigl S, Hellerbrand C, Dietmeier W, Schlitt HJ, Geissler EK, Stoeltzing O (2010) Activating transcription factor-3 (ATF3) functions as a tumor suppressor in colon cancer and is up-regulated upon heat-shock protein 90 (Hsp90) inhibition. BMC cancer 10:668. doi:10.1186/1471-2407-10-668

29. Sato A, Nakama K, Watanabe H, Satake A, Yamamoto A, Omi T, Hiramoto A, Masutani M, Wataya Y, Kim HS (2014) Role of activating transcription factor 3 protein ATF3 in necrosis and apoptosis induced by 5-fluoro-2'-deoxyuridine. The FEBS journal 281 (7):1892-1900. doi:10.1111/febs.12752

30. Butler SH, Godefroy F, Besson JM, Weil-Fugazza J (1992) A limited arthritic model for chronic pain studies in the rat. Pain 48 (1):73-81. doi:0304-3959(92)90133-V

31. Lourenco Neto F, Schadrack J, Platzer S, Zieglgansberger W, Tolle TR, Castro-Lopes JM (2000) Expression of metabotropic glutamate receptors mRNA in the thalamus and brainstem of monoarthritic rats. Brain research Molecular brain research 81 (1-2):140-154. doi:S0169328X00001765

32. Castro-Lopes JM, Tavares I, Tolle TR, Coito A, Coimbra A (1992) Increase in GABAergic Cells and GABA Levels in the Spinal Cord in Unilateral Inflammation of the Hindlimb in the Rat. The European journal of neuroscience 4 (4):296-301. doi:ejn_04040296

33. Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16 (2):109-110

34. Delree P, Leprince P, Schoenen J, Moonen G (1989) Purification and culture of adult rat dorsal root ganglia neurons. Journal of neuroscience research 23 (2):198-206. doi:10.1002/jnr.490230210

35. Tse KH, Chow KB, Leung WK, Wong YH, Wise H (2014) Primary sensory neurons regulate Toll-like receptor-4-dependent activity of glial cells in dorsal root ganglia. Neuroscience 279:10-22. doi:10.1016/j.neuroscience.2014.08.033

36. Schmutzler BS, Roy S, Pittman SK, Meadows RM, Hingtgen CM (2011) Ret-dependent and Ret-independent mechanisms of Gfl-induced sensitization. Mol Pain 7:22. doi:10.1186/1744-8069-7-22

37. Whitmore MM, Iparraguirre A, Kubelka L, Weninger W, Hai T, Williams BR (2007) Negative regulation of TLR-signaling pathways by activating transcription factor-3.

Journal of immunology 179 (6):3622-3630. doi:179/6/3622

38. Sanchez-Nogueiro J, Marin-Garcia P, Miras-Portugal MT (2005) Characterization of a functional P2X(7)-like receptor in cerebellar granule neurons from P2X(7) knockout mice. FEBS letters 579 (17):3783-3788. doi:10.1016/j.febslet.2005.05.073

39. Stojilkovic SS, Leiva-Salcedo E, Rokic MB, Coddou C (2014) Regulation of ATP-gated P2X channels: from redox signaling to interactions with other proteins. Antioxidants & redox signaling 21 (6):953-970. doi:10.1089/ars.2013.5549

40. Beck R, Dejeans N, Glorieux C, Creton M, Delaive E, Dieu M, Raes M, Leveque P, Gallez B, Depuydt M, Collet JF, Calderon PB, Verrax J (2012) Hsp90 is cleaved by reactive oxygen species at a highly conserved N-terminal amino acid motif. PloS one 7 (7):e40795. doi:10.1371/journal.pone.0040795

41. North RA (2002) Molecular physiology of P2X receptors. Physiological reviews 82 (4):1013-1067. doi:10.1152/physrev.00015.2002

42. Lenertz LY, Wang Z, Guadarrama A, Hill LM, Gavala ML, Bertics PJ (2010) Mutation of putative N-linked glycosylation sites on the human nucleotide receptor P2X7 reveals a key residue important for receptor function. Biochemistry 49 (22):4611-4619. doi:10.1021/bi902083n

43. Alves LA, Bezerra RJ, Faria RX, Ferreira LG, da Silva Frutuoso V (2013) Physiological roles and potential therapeutic applications of the P2X7 receptor in inflammation and pain. Molecules 18 (9):10953-10972. doi:10.3390/molecules180910953

44. Antonioli L, Giron MC, Colucci R, Pellegrini C, Sacco D, Caputi V, Orso G, Tuccori M, Scarpignato C, Blandizzi C, Fornai M (2014) Involvement of the P2X7 purinergic receptor in colonic motor dysfunction associated with bowel inflammation in rats. PloS one 9 (12):e116253. doi:10.1371/journal.pone.0116253

45. Arulkumaran N, Unwin RJ, Tam FW (2011) A potential therapeutic role for P2X7 receptor (P2X7R) antagonists in the treatment of inflammatory diseases. Expert opinion on investigational drugs 20 (7):897-915. doi:10.1517/13543784.2011.578068

46. Takeda M, Takahashi M, Matsumoto S (2009) Contribution of the activation of satellite glia in sensory ganglia to pathological pain. Neurosci Biobehav Rev 33 (6):784-792. doi:10.1016/j.neubiorev.2008.12.005

47. Liu S, Shi Q, Zhu Q, Zou T, Li G, Huang A, Wu B, Peng L, Song M, Wu Q, Xie Q, Lin W, Xie W, Wen S, Zhang Z, Lv Q, Zou L, Zhang X, Ying M, Li G, Liang S (2015) P2X(7) receptor of rat dorsal root ganglia is involved in the effect of moxibustion on visceral hyperalgesia. Purinergic signalling 11 (2):161-169. doi:10.1007/s11302-014-9439-y

48. Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, Grahames CB, Casula MA, Yiangou Y, Birch R, Anand P, Buell GN (2005) Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain. Pain 114 (3):386-396. doi:10.1016/j.pain.2005.01.002

49. Chen Y, Li G, Huang LY (2012) P2X7 receptors in satellite glial cells mediate high functional expression of P2X3 receptors in immature dorsal root ganglion neurons. Mol Pain 8:9. doi:10.1186/1744-8069-8-9

50. Chen Y, Li G, Huang LY (2015) p38 MAPK mediates glial P2X7R-neuronal P2Y1R inhibitory control of P2X3R expression in dorsal root ganglion neurons. Mol Pain 11 (1):68. doi:10.1186/s12990-015-0073-7

51. Wirkner K, Sperlagh B, Illes P (2007) P2X3 receptor involvement in pain states. Molecular neurobiology 36 (2):165-183. doi:10.1007/s12035-007-0033-y

52. Bradbury EJ, Burnstock G, McMahon SB (1998) The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. Molecular and cellular neurosciences 12 (4-5):256-268. doi:10.1006/mcne.1998.0719

53. Taylor AM, Ribeiro-da-Silva A (2011) GDNF levels in the lower lip skin in a rat model of trigeminal neuropathic pain: implications for nonpeptidergic fiber reinnervation and parasympathetic sprouting. Pain 152 (7):1502-1510. doi:10.1016/j.pain.2011.02.035

54. Su J, Gao T, Shi T, Xiang Q, Xu X, Wiesenfeld-Hallin Z, Hokfelt T, Svensson CI (2015) Phenotypic changes in dorsal root ganglion and spinal cord in the collagen antibody-induced arthritis mouse model. J Comp Neurol 523 (10):1505-1528. doi:10.1002/cne.23749

55. Ferreira-Gomes J, Adaes S, Sousa RM, Mendonca M, Castro-Lopes JM (2012) Dose-dependent expression of neuronal injury markers during experimental osteoarthritis induced by monoiodoacetate in the rat. Mol Pain 8:50. doi:10.1186/1744-8069-8-50

56. Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, Miki K, Yonenobu K, Ochi T, Noguchi K (2000) Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. Molecular and cellular neurosciences 15 (2):170-182. doi:10.1006/mcne.1999.0814

57. Hai T, Wolford CC, Chang YS (2010) ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component? Gene expression 15 (1):1-11

58. Seijffers R, Allchorne AJ, Woolf CJ (2006) The transcription factor ATF-3 promotes neurite outgrowth. Molecular and cellular neurosciences 32 (1-2):143-154. doi:10.1016/j.mcn.2006.03.005

59. Seijffers R, Mills CD, Woolf CJ (2007) ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. J Neurosci 27 (30):7911-7920. doi:10.1523/JNEUROSCI.5313-06.2007

60. Tsan MF, Gao B (2009) Heat shock proteins and immune system. Journal of leukocyte biology 85 (6):905-910. doi:10.1189/jlb.0109005

61. Adinolfi E, Kim M, Young MT, Di Virgilio F, Surprenant A (2003) Tyrosine phosphorylation of HSP90 within the P2X7 receptor complex negatively regulates P2X7 receptors. The Journal of biological chemistry 278 (39):37344-37351. doi:10.1074/jbc.M301508200

62. Waza M, Adachi H, Katsuno M, Minamiyama M, Tanaka F, Sobue G (2006) Alleviating neurodegeneration by an anticancer agent: an Hsp90 inhibitor (17-AAG). Annals of the New York Academy of Sciences 1086:21-34. doi:10.1196/annals.1377.012

4.4 Publication IV

HSP90 inhibition alleviates pain in monoarthritic rats and alters the expression of relevant pain molecules at the DRG (in preparation for submission)

.

HSP90 inhibition alleviates pain in monoarthritic rats and alters the expression of relevant pain molecules at the DRG

Diana Sofia Marques Nascimento[1,2,3], Catarina Soares Potes [1,2,3], Miguel Luz Soares[2,3,4], António Carlos Ferreira[2,3,4] , José Manuel Castro-Lopes[1,2,3], Fani Lourença Moreira Neto[1,2,3]

[1]Departamento de Biologia Experimental, Centro de Investigação Médica (CIM), Faculdade de Medicina do Porto, Universidade do Porto, Portugal [2]Pain Group, Instituto de Biologia Molecular e Celular (IBMC), Porto, Portugal [3]Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal [4]Laboratório de Apoio à Investigação em Medicina Molecular (LAIMM), Centro de Investigação Médica (CIM), Faculdade de Medicina do Porto, Universidade do Porto, Portugal

Abstract

Heat shock protein 90 (HSP90) inhibitors have recently been shown to ameliorate neurodegenerative diseases and inflammatory conditions. They also alleviated pain in a neuropathic model suggesting a novel role of this chaperone in nociception. We have previously demonstrated that activating transcriptional factor 3 (ATF3) is significantly induced in dorsal root ganglia (DRG) neurons of Monoarthritic (MA) rats. Interestingly, when suppressing ATF3 in DRG cell cultures, we observed a significant decrease in HSP90 expression. Altogether data suggested an involvement of HSP90 in MA pathophysiological mechanisms, which we further investigated. Here, the mRNA levels for both the inducible and constitutive HSP90 isoforms, evaluated by qRT-PCR, were considerably increased in the ipsilateral DRG of MA animals, as well as the cleavage of its N-terminal, analyzed by western blot. The intrathecal administration of 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), an HSP90 inhibitor, attenuated MA-induced mechanical allodynia, assessed by the ankle bend test, mainly 1h-post injection. The drug also induced significant decreases in the expression of the HSP90 isoforms and reversed the high levels of the cleaved protein. The expression of the P2X3 purinergic receptor (but not P2X7) as well as of glial fibrillary acidic protein (GFAP, marker of satellite glial cells activation) was also decreased after 17-DMAG administration. Oppositely, ATF3 expression, already known to be induced in MA, was even higher in 17-DMAG-treated animals. Data suggest that HSP90 plays a role in MA and that its cleavage is a key event to understand these mechanisms. Interestingly, even though HSP90 chaperoning functions are most likely compromised, 17-DMAG attenuates glial activation (GFAP) and neuronal sensitization (P2X3R) which might correlate with the pain alleviation observed in the MA animals. Data suggest that 17-DMAG prevents the cleavage of HSP90, which can possibly relate with the amelioration of the pathological condition. However, further investigation is needed to clarify these mechanisms

Key words: HSP90inhibition, joint inflammatory pain, DRG neurons, N-terminal cleavage, ATF3, P2X receptors, SGCs activation

Corresponding author:
Fani Lourença Moreira Neto
Departamento de Biologia Experimental, Centro de Investigação Médica (CIM) Faculdade de Medicina da Universidade do Porto, Alameda Prof. Hernâni Monteiro, 4200 – 319, Porto, Portugal;
Telephone: 00 351 220426769
Fax: 00 351 5513655
E-mail: fanineto@med.up.pt

.

1. Introduction

Heat shock protein 90 (HSP90) belongs to a family of conserved molecular chaperones that play important roles for the basic function of the cell like signal transduction, intracellular transport and protein stabilization/degradation [1]. They are triggered by various cellular stresses (rather than heat) protecting the cells against damage [2]. In eukaryotic cells, there are two major isoforms of this chaperone, HSP90α that is inducible under stress conditions and HSP90β that is constitutively expressed. Both isoforms contain three main domains: an N-terminal and a C-terminal that include an ATP-binding pocket and a middle domain responsible for the binding to client proteins [3].

The use of HSP90 blockers was primarily investigated as a cancer treatment [4,5] and indeed many of these compounds have already reached phase I clinical trials, while their properties (such as pharmacokinetics, bioavailability, tissue distribution and metabolism) are being expansively evaluated. Since many events (such as aberrant cytokine production, receptors signaling and cellular invasion) are common to both cancer and inflammation, HSP90 inhibition has also been evaluated in the treatment of inflammatory diseases, including rheumatoid arthritis [6] and atherosclerosis [7]. In the nervous system, HSP90 inhibitors reduce neurodegeneration [8] and promote neuroprotection [9] being pointed as possible therapies for neurodegenerative diseases. Interestingly, intrathecal and systemic administration of HSP90 inhibitors can alleviate pain in chronic constriction injury (CCI) neuropathic animals [10], suggesting a role for HSP90 in painful states. However, still very little is known about its involvement in pain processing, mainly because HSP90 signaling pathways and functions in neurons remain unclear.

In a couple of studies, HSP90 expression has been associated to activating transcriptional factor 3 (ATF3) [11], a molecule whose expression is also triggered in stressing conditions and considered a marker of neuronal injury [12]. However, these have been conducted in other cell types rather than neurons and by using distinct approaches [11]. Interestingly, by silencing the expression of ATF3 in primary cell cultures of dorsal root ganglia (DRG) neurons, we found a significant decrease in the expression of both the inducible and constitutive forms of HSP90 [13] which suggests a regulation of ATF3 over HSP90 gene expression in primary afferent neurons. This data, together with our previous studies showing ATF3 is significantly induced during monoarthritis (MA) [14], a model of joint inflammatory pain induced by injection of complete Freund's adjuvant (CFA) in the tibiotarsal joint of the rat [15], pointed to a possible role of HSP90 in MA pathophysiology.

Moreover, a significant activation of satellite glial cells (SGCs), preferentially surrounding ATF3-positive neurons in the DRG, was also detected around 1 week of MA [16]. Indeed, HSP90 is also involved in glial activation [17,18], one of the major events implicated in pain processing [19-21]. However, it is still unknown whether the HSP90-mediated regulation of glial cells activation also occurs at the periphery (in SGCs) since the few reports available were conducted mainly in the spinal cord. In fact, SGCs activation during painful conditions is majorly mediated by P2X7R, a purinergic receptor that is highly associated with pain states [19,21,22]. Due to their selective localization, P2X7R (found exclusively in SGCs) and P2X3R (expressed only in neurons) [23] are known to be involved in mechanisms of neuron-glia crosstalk which also explain their relevant role in pain processing. Moreover, activation of P2X7R is known to down-regulate the activity of the neuronal P2X3R as a sort of protective mechanism to prevent further neuronal sensitization and damage [24-26]. In MA animals, not only we have shown P2X7R up-regulation in the DRG of MA animals, but also observed this negative feedback control resulting in P2X3R down-regulation in neurons [13]. Excitingly, HSP90 was found to be part of a P2X7R-protein complex [27,28].

Altogether, these evidences led us to hypothesize a role for HSP90 in the pathophysiological mechanisms underlying the MA condition. Therefore, in order to study the role of HSP90 in MA, we analyzed changes in HSP90 at the mRNA and protein levels in MA animals. Additionally, we intrathecally administered the HSP90 inhibitor, 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), to

inflamed animals. We then evaluated the effect of 17-DMAG treatment in their nociceptive behavior and HSP90 expression levels, as well as in the expression of ATF3, glial fibrillary-acidic protein (GFAP) (used as marker of SGCs activation) and the purinergic receptors P2X7R and P2X3R. With this study, we hope we contributed to clarify the role of HSP90 in the MA condition as well as of the use of HSP90 inhibitors in similar inflammatory conditions.

2. Materials and methods

2.1. Animal handling

The experiments were authorized by the animal welfare body (ORBEA) of the Faculty of Medicine of the University of Porto. Procedures were carried out according to the European Communities Council Directive of September 22, 2010 (2010/63/EC) and to the ethical guidelines for investigation of experimental pain in animals [29]. Measures were taken in order to minimize pain and distress as well as to reduce the number of animals used. Accordingly, animals were housed 2-3 animals per cage under controlled conditions of lighting (12h light/12h dark cycle) and temperature as well as water and food *ad libitum*. They were habituated to the experimenter and the equipment used for behavioral assessment for several days before initiating the experiments. After the first intervention they were monitored daily. The humane endpoints defined for this project were always taken into consideration.

2.2. Monoarthritis (MA) induction

Monoarthritis (MA) was induced in male Wistar rats (Charles River Laboratories, France) weighing between 200 and 300g, as previously described [16]. Briefly, animals were injected with 50µL of complete Freund's adjuvant (CFA), into the left tibiotarsal joint [15] under isoflurane anesthesia. The CFA solution (5,45mg/mL of *mycobacterium butyricum*) was prepared as previously described [30]. Control (non-inflamed) animals were similarly injected with 50µL of CFA vehicle (detailed in [14,16]).

2.3. HSP90 inhibition by 17-DMAG administration

In order to study the possible regulation of HSP90 on the expression of specific targets as well as on the nociceptive behavior of the animals with a joint inflammatory condition, we administered 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG-Calbiochem, San Diego CA, USA), an HSP90 inhibitor, to MA animals. The choice of 17-DMAG (a benzoquinone ansamycin, second generation geldanamycin derivate), among other available HSP90 inhibitors in phase I clinical testing, was based on its improved properties. It is water-soluble, has greater oral bioavailability, is widely distributed to tissues and quantitatively much less metabolized and hepatotoxic than other similar derivatives [31].

Animals were divided into 4 different experimental groups: non-inflamed animals (intra-articular injection of vehicle) receiving an intrathecal (i.t.) injection of either saline **(Control+saline)** or 17-DMAG **(Control+17DMAG)**, and 4d MA inflamed animals (intra-articular injection of CFA) receiving either i.t saline **(MA+saline)** or 17-DMAG **(MA+17DMAG).** At day 4 after CFA/vehicle intraarticular injections, 17-DMAG or saline were intrathecally administered in order to reach the lumbar spinal segments, using a non-surgical approach according to a protocol adapted from Ossipov *et al*, 1988 [32] and De la Calle *et al*, 2002 [33]. Basically, 26G needles were used to perform an injection through the intervertebral disc of the L5-L6 or L6-S1 segment while animals were kept under light isoflurane anesthesia (5% for induction, 2% for maintenance). Briefly, the lower half of the animal's back was shaved and disinfected with ethanol 70%. Using the anterior part of the iliac crest as a tactile landmark for the L6 vertebra, the animal was firmly held by the hip bones with one hand in order to lift the spinal column at the L5-S1 vertebral level and create a slight curvature, and the L5-L6 and the L6-S1 intervertebral spaces were identified using the index finger. Animals were injected either at the L5-L6 or L6-S1 intervertebral space by introducing the 26-gauge needle connected to a 100µL Hamilton syringe through the widest intervertebral space, lowering it until contact with

the vertebral body and penetrating into the intrathecal space, perceived by a change of resistance. Correct dura puncture and position of the tip of the needle was verified by a reflexive flick of the tail or a hind paw flinch.

Animals were injected with 30µL of either 17-DMAG solution (10µg dissolved in saline [10]) or saline, daily, during 4 days (that is, on day 4 after CFA/vehicle injections and until day 7 as shown in Fig. 1A diagram). Animals were sacrificed at day 7, 2 hours after the last injection, and the ipsi and contralateral L3-L5 DRG were freshly harvested and instantly frozen for later real-time quantitative PCR (RT-qPCR) analysis.

2.4. Nociceptive behavioral analysis

A habituation period of 7 days, to both the experimenter and the behavior protocols, preceded the tests. Nociceptive behavioral evaluation was performed by an experimenter blind to the solutions previously intrathecally injected by using the Ankle-Bend (AB) test. Tests were performed in all experimental groups but data were analyzed only for MA animals (**MA+saline** vs **MA+17DMAG)** since control non-inflamed animals did not show any significant pain symptoms (in accordance with our previous studies [34-37]). AB baseline tests were performed at day 0 (before MA induction) and at day 4 of MA (before intrathecal injections). Then, to evaluate the effect of the drug at day 4, AB tests were performed 1, 3 and 4 hours after the intrathecal injections of 17-DMAG or saline. To assess the possibility of an accumulative effect, animals were similarly tested on days 5 and 6 of MA, but only at 3h after injection. This timepoint was chosen to avoid the more acute effects by taking in consideration the behavior responses obtained at day 4. More prolonged timepoints were not assessed as the drug's concentration in plasma drops considerably after the 3 hours [38,39]. At day 7, the animals were similarly intrathecally injected but no behavior analysis was performed, as they were sacrificed 2h post-injection instead (Fig. 1A). The timepoints for sacrifice were also chosen taking in consideration the drug's pharmacokinetics and the effects observed on day 4. Behavioral tests were always performed in both the ipsilateral inflamed and contralateral non-inflamed paws, as the latter were used as internal controls.

The **ankle-bend test** was used to evaluate physiological movement-induced nociception (allodynia)[40]. Five alternate flexions and extensions of the ankle joint were performed and the animals' response (squeaks and struggle reactions) was scored according to an established scale, as previously described [40]. Basically, the higher scores (score 2) indicate squeak responses to moderate manipulations of the inflamed joint, whereas lower scores (score 0) indicate the absence of a response to manipulation. A mild response is reflected by the retraction of the paw without squeak (score 1). Each extension/flexion is scored and in the end a sum of the scores from the ten maneuvers is calculated (maximum possible score is 20), meaning that higher ankle-bend scores are indicative of allodynia (as in [41]).

2.5. Real-time quantitative polymerase chain reaction (RT-qPCR)

Animals were sacrificed by decapitation under light anesthesia with isoflurane. For each animal, freshly harvested L3, L4 and L5 ganglia were pooled together but separately for the ipsi- and contralateral sides. Snap frozen DRG tissue specimens were homogenized in 750µL of TRI® reagent using a MagNA Lyser System (Roche). Following addition of chloroform and centrifugation, the RNA-containing upper phase was retrieved for subsequent total RNA extraction. The organic phase was stored at 4°C for subsequent isolation of proteins.

RNA extraction and cDNA synthesis

Total RNA was isolated using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. Quantification was performed using a Nanodrop 2000 and the RNA integrity was assessed using the Agilent 2100 Bioanalyzer; all samples had a RIN $\geq$ 7. The RevertAid H Minus cDNA synthesis kit (Fermentas) was used to reverse transcribe 1µg of total RNA with random primers, and the resulting cDNA was diluted 1:20, aliquoted and stored at -20ºC for subsequent use.

Gene expression studies

The expression levels of the selected genes were measured by RT-qPCR using the StepOnePlus Real-Time PCR System (Applied Biosystems). Each reaction was performed in triplicate, with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas), 400nM of primers (except where noted, Table S1) and 3µL of 20x diluted cDNA (described above), in a 12.5µL final volume. A standard curve made up of 1/2 dilutions of pooled cDNA of all samples was run on each plate for each primer set, to assay for relative quantification. Target gene expression was normalized to the expression of Gap3dh (GAPDH). The estimated efficiency of all qPCR assays ranged between 90-100%.Primer sequences and annealing temperatures are shown in Table 1.

Table 1. Primer sequences and annealing temperatures for quantitative PCR

Transcript	Primers	Annealing (ºC)
Atf3	F: CCAGAACAAGCACCTTTGCC R: GTTTCGACACTTGGCAGCAG	60
Gfap	F: AATTGCTGGAGGGCGAAGAA R: TTGAGGTGGCCTTCTGACAC	60
P2rx3	F: TTCCTTCACTCGGCTGGATG R: TGCCAGCGTTCCCATATACC	60
P2rx7	F: GCACATGACCGTCTTTTCCT R: CAAAGGGAGGGTGTAGTCGG	60
Hsp90aa1	F: CTGCGTATTTGGTTGCTGAGA R: ACCTTTGTTCCACGACCCAT	60
Hsp90ab1	F: AAATTGCCCAGCTGATGTCC R: ACTTGGAAGGGTCAGTCAGG	60
Gapdh	F: CCATCACCATCTTCCAGGAG R: GCATGGACTGTGGTCATGAG	60

F: forward primer; R: reverse primer

Protein Extraction

The precipitation and extraction of total protein from the organic phase of DRG tissue homogenized in TRI reagent was conducted according to the manufacturer's instructions (Sigma). Briefly, the precipitate was obtained by adding 2-propanol and allowing the samples to rest for 10min at room temperature. After centrifugation (12000g at 4ºC), the supernatants were discarded and pellets were successively washed in ammonium acetate 0.1M (80% in methanol). Lastly, the pellets were dried using ethanol and dissolved in SDS 1%, until the precipitate was completely dissolved. Protein fractions were stored at -20ºC prior to use.

2.6. Western Blotting (WB)

Protein fractions of the DRG homogenates that have been processed for gene expression analysis were used to evaluate the expression of HSP90, in the protein form. The double extraction of RNA and protein from the same samples allowed for a correlation of data from behavior, gene and protein expression.

The protein fractions were quantified by the bicinchoninic acid (BCA) protein assay. After heating at 94ºC, between 20-30µg of protein were loaded for each lane and separated on 10% sodium dodecyl sulphate-polyacrylamide (SDS/PAGE) gels. The proteins were then transferred into nitrocellulose membranes and blocked with non-fat milk (5% milk powder diluted in Tris buffer saline with tween20; TBST buffer), for one hour, at room temperature. Membranes were then incubated in polyclonal rabbit anti-HSP90 (ab13495, Abcam) diluted 1:2500 in TBST with 2% of normal goat serum (NGS), for 24 hours at 4ºC. Incubation in mouse anti-γ-actin (A8481, Sigma) diluted 1:10,000 in TBST with 2% of NGS, overnight at 4ºC, was also performed as a loading control. Lastly, blots were incubated in donkey anti-rabbit secondary antibody conjugated with HRP (711-035-152, Jackson Laboratories) or goat anti-mouse secondary antibody conjugated with HRP (sc-2005, SantaCruz Biotechnology), diluted 1:5000 in TBST with 5% milk powder. Antibody binding was visualized with the SuperSignal West Pico Chemiluminescent Substrat kit (Thermo Scientific; 34080) and chemiluminescent signals were detected by exposure in the ChemiDoc™ system (BioRad).

The protein levels were obtained by densitometric analysis of the band signal intensity using the image computer software ScionImageR (Scion Corporation). The protein density was determined for each band after subtracting the

background of the surrounding region and normalizing for the selected area. This density was corrected against the loading control signal, for each blot. Assays were performed twice on samples obtained from independent groups of rats and averages were used for analysis.

Visualization in the Odyssey system

In order to identify the 70KDa band found in the blots for HSP90 detection, two more WB assays were performed to visualize the membranes with the Odyssey® CLx infrared system (LI-COR, Biosciences). The Odyssey system allows the detection of two targets whose antibodies have been made in different species. By using secondary antibodies linked to fluorescent dyes that have distinct absorption and emission wavelengths, it is possible to visualize both targets simultaneously, in distinct colors. By also allowing the overlapping of the signals it gives additional information when comparing to the traditional WB detection with chemiluminescent substrates.

The WB protocol was similar to that described above except for a few modifications in order to reduce background. Briefly, blots were blocked in a solution of 5% bovine serum albumin (BSA) in TBST instead, and no detergent was used prior to the blocking step (washes in TBS only). Primary antibodies were also diluted in the 5% BSA in TBST solution and secondary antibodies diluted in 0.5% milk in TBST. After the incubations, membranes were washed in TBS and dH_2O in order to remove any trace of detergent. In a first experiment, the blots were incubated in polyclonal rabbit anti-HSP90 (1:2500, ab13495, Abcam) together with monoclonal mouse anti-HSP90 (1:1000, ab82395, Abcam) specific for N-terminal residues. In a second experiment, the membranes were incubated again in rabbit anti-HSP90, but now together with monoclonal mouse anti-HSP70 (1:2500, ab6535, Abcam). Detection was done using, for both experiments, IRDye® 800CW Donkey anti-Rabbit (926-32213, LI-COR Biosciences) and IRDye® 680LT Donkey anti-Mouse (926-68022, LI-COR Biosciences), both at 1:15,000 in 0.5% milk in TBST. Only a qualitative analysis of this data was performed.

2.7. Data and statistical analysis

Statistical analyses were performed by using STATISTICA 10.0 (StatSoft, Tulsa, U.S.A.) or GraphPad Prism 5® (GraphPad Software). All data are presented as mean±standard error of the mean (SEM). The normality of all data was analyzed by the Kolmogorov-Smirnov test.

To investigate whether MA induced pain-like behaviors (model validation), the ipsilateral AB scores of all MA animals were compared between day 0 (before model induction) and day 4 of MA (prior to drug administration) using a two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni post-hoc tests (N=17). Animals were then randomly divided to be injected either with saline (MA+saline) or the drug (MA+17DMAG). To evaluate the effect HSP90 inhibition on pain behavior, differences between these two experimental groups, for each timepoint, were assessed by two-way ANOVA followed by the least significant difference (LSD) post-hoc test (N=6 for MA+saline and N=11 for MA+17DMAG).

To evaluate changes in gene expression of these same animals, data from the RT-qPCR performed in DRG were normalized against the control+saline group (defined as 1). Statistical analysis was done using one-way ANOVA followed by Newman-Keuls post-hoc test (N=7 for control+saline; N=5 for Control+17DMAG; N=6 for MA+saline and N=11 for MA+17DMAG).

In the WB assays, two bands were identified for HSP90 detection, one around 90KDa which is the predicted weight for the full length protein, and another one around 70KDa believed to be a cleaved form of this chaperone (rarely reported). Therefore, the density of the two bands was quantified separately. Values were corrected against the loading control (γ-actin) and ratios with the control+saline group were calculated afterwards (defined as 1). One-way ANOVA followed by Newman-Keuls post-hoc test allowed to assess differences between the experimental groups for each of the HSP90 forms (N=6 for control+saline; N=5 for Control+17DMAG; N=6 for MA+saline and N=11 for MA+17DMAG). By following a protocol of double extraction of RNA and protein we were able to use the exact same animals for the WB and RT-qPCR analysis, with

the exception of a control+saline animal whose sample was not suitable for WB.

For all the statistical analyses, a level of significance of $P < 0.05$ was assumed.

3. Results

MA animals show movement-induced allodynia

Monoarthritis was successfully induced in all animals injected with CFA. The ipsilateral paws of MA animals showed demarked inflammatory signs (as described in [37,41-43]). The contralateral paws of both MA and control animals have never shown signs of inflammation or hypersensitivity (0.0±0.0 AB scores; data not shown). The AB test confirmed that all inflamed animals at 4d of MA (N=17) showed significantly increased movement-induced nociception in their ipsilateral paws (19.1±0.3 mean AB scores) when comparing to day 0 (prior to MA induction; 0.0±0.0) ($p<0.0001$; Fig. 1B). Since control animals did not show any significant pain-like behavior (2.9±0.5 AB scores, probably due to tissue trauma inherent to the injection procedure, in accordance with previous studies [34-37]; data not shown), the behavioral responses to the AB test after 17-DMAG or saline i.t administration were only scored in inflamed animals to check the drug effects in nociceptive behavior during MA.

17-DMAG alleviates mechanical allodynia

We have previously observed that ATF3 silencing in DRG cell cultures resulted in reduced HSP90 expression [13]. Since ATF3 is highly induced in the DRG of MA animals, we hypothesized about a role of HSP90 in this condition.

To evaluate this, 4dMA animals were randomly separated into two different groups for intrathecal (i.t.) administrations of saline (**MA+saline**; N=6) or of the HSP90 inhibitor 17-DMAG (**MA+17DMAG;** N=11), followed by AB behavioral tests (schematized in 1A). The statistical analyses revealed that 1 hour after the 17-DMAG treatment MA animals show a significant reduction in the AB scores (14.6±1.5; Fig. 1B), comparatively to the animals injected with saline (20.0±0.0; Fig. 1B). Differences were still significant 3h after the drug administration (15.7±1.3 for MA+17DMAG vs 19.5±0.5 for MA+saline; Fig. 1B). Data indicate that MA animals treated with this HSP90 inhibitor show less movement-induced allodynia (Fig. 1B). Interestingly, the statistical differences between the animals receiving saline (19.3±0.7) or HSP90 inhibitor (17.6±0.8) were no longer found at 4hours post-injection, suggesting that after this timepoint there is a reversion of the drug effect (Fig. 1B).

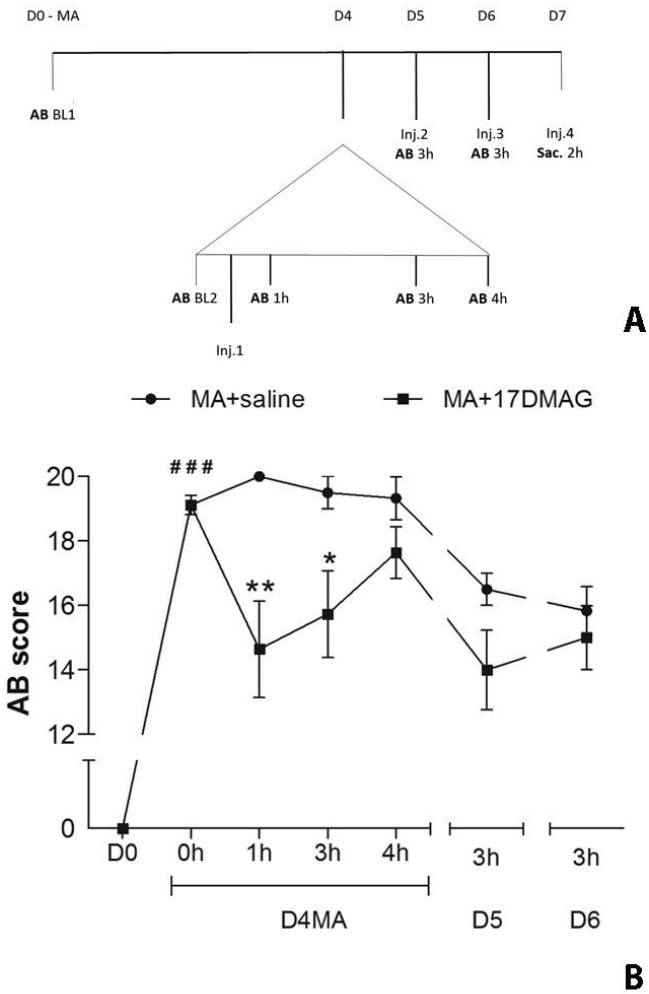


Fig. 1 – 17-DMAG treatment alleviated MA-induced mechanical allodynia. A) Schematic representation of the experiment. After 4d of MA induction, animals were divided into two groups receiving either saline or the drug intrathecally. AB was performed 1, 3 and 4h after the administration. To assess a possible accumulative effect of the drug, animals were again injected in the following days (5 and 6), and tested 3h after. Animals were sacrificed at day 7, 2h after the last injection. **B) 17-DMAG significantly**

reduced AB scores of MA animals. All ipsilateral paws of inflamed rats showed high AB scores at day 4 of MA (near 20, the maximum value) when comparing to day 0 (prior to MA induction). Contralateral paws or control animals never showed any signs of inflammation or pain (data not shown). Values are shown as Mean±SEM (N=17). Differences between D0 and D4 MA were assessed using two-way ANOVA, followed by Bonferroni post-hoc tests (### represents p<0.001). To evaluate the effect of HSP90 inhibition, 4d MA animals were divided in two groups receiving either saline or 17-DMAG. The drug significantly attenuated mechanical allodynia in inflamed rats, mainly 1h-post injection. Values were still significantly different 3h after the injection but at 4h the effect could no longer be observed, suggesting an acute action of the drug. Behavioral analysis at the following days showed that repeated injections do not increase the response to 17-DMAG, which excludes a putative accumulative effect of the drug. Values are shown as Mean±SEM (N=6 for the MA+saline and N=11 for the MA+17-DMAG group). Two-way ANOVA repeated measures analysis of variance was used followed by the LSD post-hoc test. * represents p<0.05 and ** represents p<0.01 between the two groups for each timepoint.

The AB tests performed at 3h after the injection on days 5 and 6 showed no significant differences between the MA animals injected with the drug or saline, excluding the hypothesis of an accumulative effect of the drug. Here, the AB scores of MA+17DMAG animals were very similar to those found on day 4 also 3h after drug injection (14.00±1.23 for day 5 and 15.00± 0.99 for day 6, 3h post-injection), however the AB scores of MA+saline animals considerably decreased (16.50±0.50 for day 5 and 15.83±0.75 for day 6, 3h-post injection), which might explain the lack of differences (Fig. 1B). Therefore, it is shown for the first time in this study that 17-DMAG induces an acute pain alleviation in MA animals.

HSP90 is up-regulated in DRG of MA animals and 17-DMAG treatment reverses it

The RT-qPCR performed in the DRG of MA animals showed that both the inducible (α-HSP90) and the constitutive (β-HSP90) HSP90 isoforms were significantly increased (1.92±0.08 for α-HSP90, Fig. 2A; 1.93±0.07 for β-HSP90, Fig. 2B), comparing to controls (control+saline; normalized to 1). This is the first time that HSP90 isoforms levels are shown to be increased in primary afferents during MA, once again supporting the role of these chaperones in inflammatory chronic conditions. Moreover, treatment with 17-DMAG, shown above to reduce pain-like behavior in these animals, was capable of significantly reversing these increases. Indeed, the HS90 levels of both isoforms decreased considerably after the treatment (1.48±0.16 for α-HSP90 and 1.42±0.13 for β-HSP90; Fig. 2A and B). The drug induced no changes in the HSP90 expression of non-inflamed animals (control+17DMAG; 0.99±0.12 and 0.97±0.05 for α and β isoforms, respectively; Fig. 2A and B).

17-DMAG reverses the increases in GFAP and P2X3 found in MA animals while ATF3 remains up-regulated

The RT-qPCR analyses of the DRG homogenates from MA animals (injected with saline) showed increased mRNA levels of ATF3 (MA+saline; 3.50±0.90) in comparison to non-inflamed controls (control+saline; normalized to 1; Fig 2C), although these changes were not statistically significant (p=0.0609). This is in accordance with our previous work showing that at day 7 the number of ATF3-positive DRG neurons was no longer significantly increased, although still higher than controls [14]. Interestingly, the i.t. administration of the HSP90 inhibitor during 4 consecutive days induced an even higher increase in the ATF3 mRNA levels in ipsilateral DRG of MA animals (4.43±1.00; MA+17DMAG) than that found in non-treated animals (3.50±0.90; MA+saline), which was statistically significant when comparing to controls (control+saline; Fig. 2C). The drug had no effect on ATF3 expression in control animals (controls+17DMAG; 1.00±0.12; Fig. 2C).

The DRG of MA animals also presented significant increases in the mRNA levels of GFAP (MA+saline; 9.13±2.82) when comparing to control+saline (normalized to 1; Fig. 2D), which again corroborates with a previous work where we demonstrated a dramatic increase in GFAP protein levels, by WB in the DRG of MA animals [16]. The MA animals treated with 17-DMAG showed a significant decrease in GFAP gene expression (MA+17DMAG; 4.69±0.70), even though it was still 4 to 5 times higher than in non-inflamed controls (Fig. 2D). Again, the drug induced no

significant changes in control non-inflamed animals (control+17DMAG; 2.07±0.64; Fig. 2D).

The mRNA expression of both the P2X3 and P2X7 purinergic receptors was also significantly increased in MA animals (MA+saline; 1.92±0.11 and 2.11±0.18, respectively) when comparing to non-inflamed control+saline (normalized to 1; Fig. 2E and F) which is also partially in agreement with preliminary data from a previous study [13]. Similarly to GFAP, we observed that 17-DMAG administration to MA animals diminished the expression of the neuronal P2X3R (MA+17DMAG; 1.42±0.13; Fig. 2E). On the other hand, the drug had no effect in the expression of the glial P2X7R in MA animals, which remained high (MA+17DMAG; 2.00±0.22; Fig. 2F). The drug also had no effect on the expression of any of the purinergic receptors in control animals (control+17DMAG; Fig. 2E and F). Altogether data demonstrate that HSP90 inhibition is capable of inducing molecular changes at the DRG level that might be associated with distinct signaling pathways and mechanism

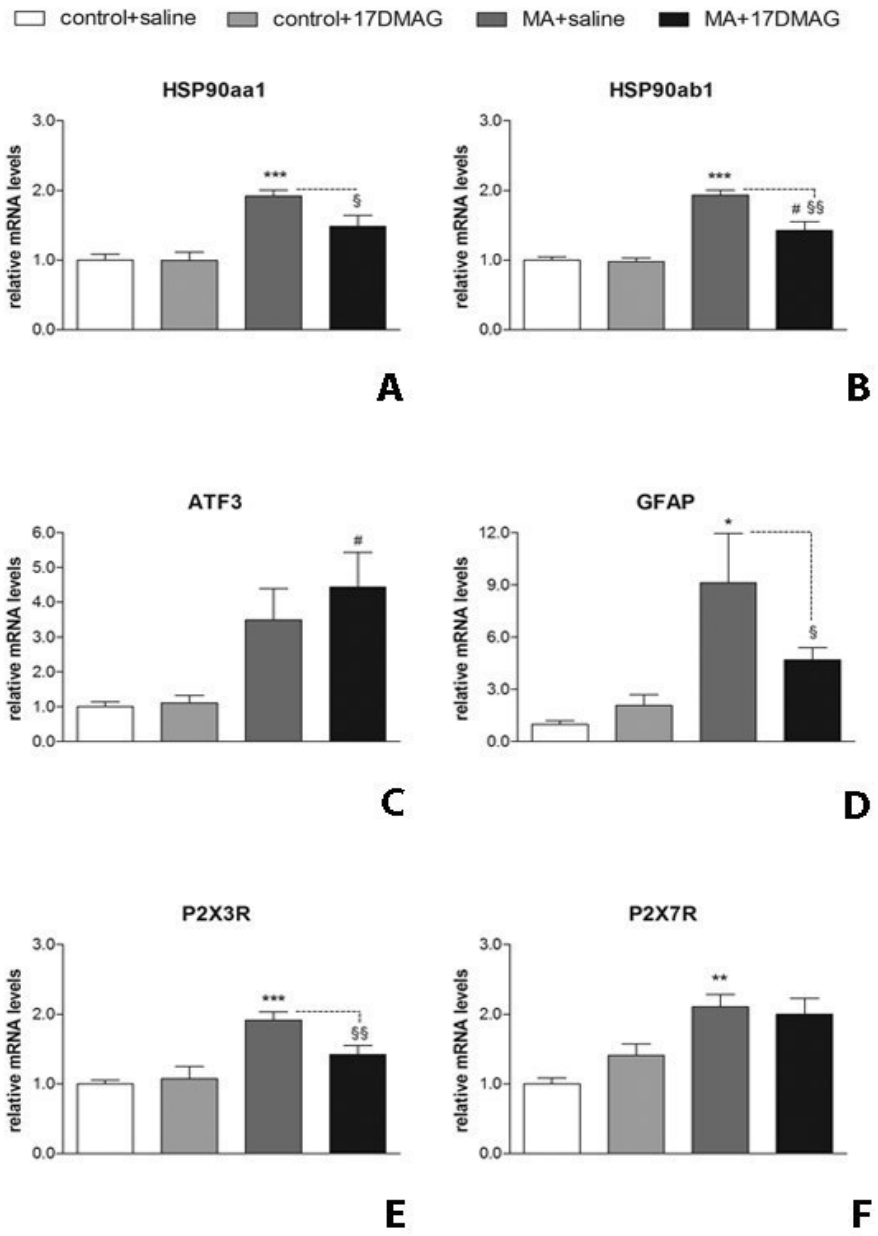


Fig. 2 - HSP90 is up-regulated in MA and its inhibition by 17-DMAG reverses it and attenuates other pathomechanisms in the DRG. A, F) Both inducible **(A)** and constitutive **(B)** isoforms of HSP90 were increased in DRG of MA rats (MA+saline in comparison to control+saline), and these increases were reversed by treatment with 17-DMAG (MA+17DMAG). The decrease in HSP90 expression in treated animals was accompanied by a significant increase in ATF3 expression **(C)** and a decrease in both GFAP **(D**; suggesting less activation of SGCs) and P2X3R (**E**; inferring lower neuronal sensitization). No differences were found for the P2X7R mRNA levels suggesting that the changes observed in SGCs are unlikely to be mediated by this receptor **(F)**. TData suggest that HSP90 inhibitors attenuate MA inflammatory signaling and pathomechanisms which may also explain pain alleviation. Values are shown as Mean±SEM (N=6 for control+saline; N=5 for Control+17DMAG; N=6 for MA+saline and N=11 for MA+17DMAG). One-way ANOVA followed by Newman-Keuls post-hoc test was used to assess differences between the experimental groups. * represents $p<0.05$, ** represents $p<0.01$ and *** represents $p<0.001$ to the control+saline group. # represents $p<0.05$ to the control-17DMAG group. § represents $p<0.05$ and §§ represents $p<0.01$ to the MA-saline group.

HSP90 is cleaved during MA and 17-DMAG treatments seems to prevent it

Two distinct bands were observed in the WB detection of HSP90 using a polyclonal antibody that detects both α and β isoforms of the protein. In the DRG homogenates one band around 90KDa (the predicted weight for HSP90) and another one around 70KDa (Fig. 3A) were detected. Based on a few other reports [3] we hypothesized that the 70KDa band is a cleaved form of the HSP90 chaperone. This was confirmed by a simultaneous detection (Odyssey® CLx infrared system) of the polyclonal antibody for HSP90 with a monoclonal antibody specific for an N-terminal region, showing that the70KDa band lacks the N-terminal (Fig. 3B; left column).We also discarded the possibility of this being a nonspecific detection of the HSP70 chaperone (since these two proteins share some conserved domains) as the signals from these two chaperones were not coincident (even though they have a very similar molecular weight; Fig. 3B right column). Altogether, these data indicate that a full length form (90KDa) and a cleaved form (lacking the N-terminal - 70KDa) of HSP90 has been detected in the DRG homogenates.

Quantification of WB data from ipsilateral ganglia showed a significant increase in the HSP90 cleaved form levels for the MA animals (5.02±0.57 for MA+saline; Fig 3A and C), when comparing to the control non-inflamed group (control+saline; normalized to 1; Fig. 3A and C). Interestingly, 17-DMAG administration to MA animals was capable of reversing the high values of cleaved protein found in the disease, to values that are very similar to those detected in controls (2.28±0.44 for MA+17DMAG, Fig. 3 A and C). In the MA animals the levels of full length protein were slightly decreased, most likely due to the remarkable and immediate cleavage of the protein, but no significant differences were found for this form when comparing to any of the experimental groups (Fig. 3C). Similarly, both forms of the protein remained unaltered in control animals treated with the drug (1.94±0.84 for the full length and 2.27±0.64 for the cleaved form; Fig. 3A and C). These results highly suggest that HSP90 cleavage is a relevant event occurring during MA. Moreover they suggest that 17-DMAG might protect HSP90 from cleavage, therefore reversing the high levels of cleaved protein found in these animals.

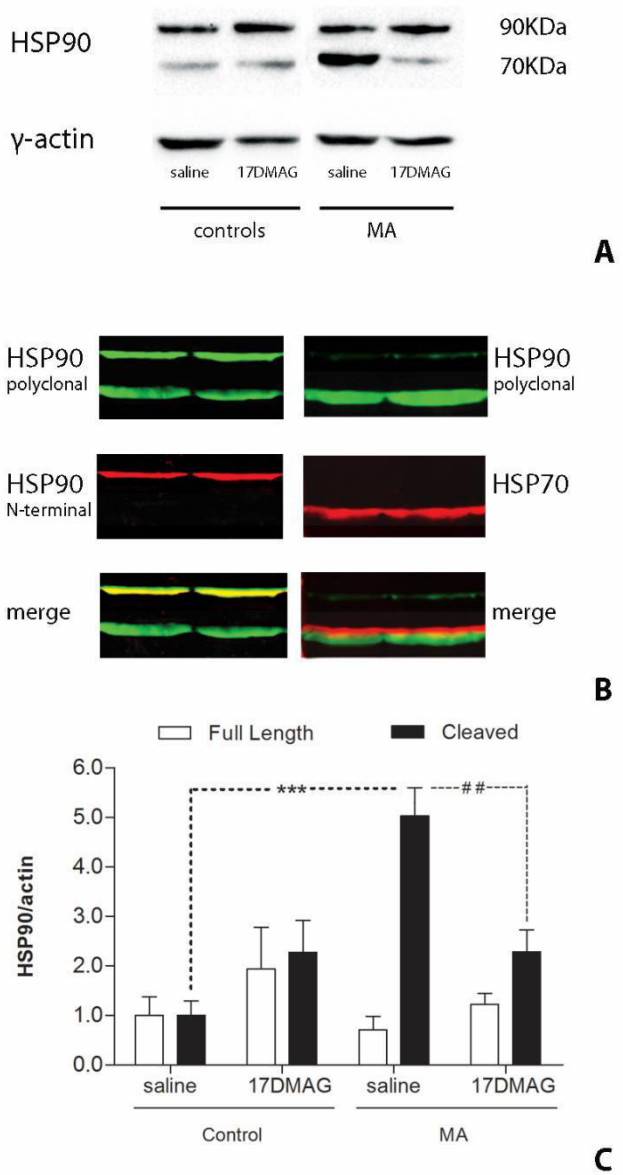


Fig. 3 – HSP90 is highly cleaved in MA animals while 17-DMAG seems to prevent it. A) WB analyses of the ipsilateral DRG homogenates demonstrate the existence of two HSP90 forms of distinct size, one at 90KDa which is the expected size of this chaperone and another one at 70KDa proposed to be a cleaved form. **B)** Odyssey® CLx infrared detection allowed the double detection of the HSP90 polyclonal antibody (green, left column) with a specific HSP90 N-terminal antibody (red, left column), confirming that the lightest form lacks the N-terminal region. The signal of this smaller fragment (green, right column) did not overlap with the signal obtained for the HSP70 detection (red, right column), excluding the hypothesis of an unspecific labeling. **C)** Quantification of the 90KDa ("full length form") and 70KD ("cleaved form") bands separately showed a significant increase in the protein levels of the cleaved form in MA animals. Data further showed that the

administration of 17-DMAG for 4 days protects HSP90 from being cleaved resulting in the lower amounts of cleaved protein (similar to controls). Values were normalized against the control+saline group and are shown as Mean±SEM (N=7 for control+saline; N=5 for Control+17DMAG; N=6 for MA+saline and N=11 for MA+17DMAG). One-way ANOVA followed by Newman-Keuls post-hoc test was used to discriminate statistical significances. *** represents $p<0.001$ differences to the control+saline group and ## represents $p<0.01$ differences to the MA+saline group

4. Discussion

In this study, we show for the first time that the HSP90 mRNA levels are significantly increased in DRG of MA animals, which is accompanied by an augmented cleavage of the N-terminal of the protein. Treatment with an HSP90 inhibitor (17-DMAG) alleviated movement-induced allodynia (ankle bend test) and resulted in a decreased expression of HSP90, mostly observed in the cleaved protein levels. This suggests that HSP90 inhibition most likely protects HSP90 from cleavage. 17-DMAG administration also resulted in a significant up-regulation of ATF3 and decrease in SGCs activation (evaluated by GFAP expression). Consistently, the inhibitor led to a decrease in the expression of the neuronal P2X3 purinergic receptor (but not of P2X7R). Here, we suggest that HSP90 plays a role in the MA pathomechanisms and that HSP90 cleavage might be a central event that needs to be better explored.

The mRNA levels of both the constitutive and inducible forms of HSP90 were significantly increased in the ipsilateral DRG of MA animals. The observed HSP90 up-regulation in MA is in accordance with several studies showing that HSPs are induced in response to many stressful conditions that can go from heat shock, to hypoxia and inflammation [44]. Evidence suggests a role for HSP90 in inflammatory diseases such as rheumatoid arthritis [6], mainly through activation of innate immunity responses via nuclear factor ƙβ (NF-ƙβ) and toll-like receptor 4 (TLR4) signaling [10]. Interestingly, we found out that this protein is considerably cleaved in DRG of MA animals. Even though these mechanisms are not yet well explored, the cleavage of HSP90 by reactive oxygen species (ROS) at a highly conserved N-terminal amino acid motif has already been reported [3]. The exacerbated production of ROS is characteristic of inflammatory conditions [45], like MA, and therefore this is very likely to be the reason for the massive cleavage observed in this disease. As a result of this cleavage, HSP90 was shown to become less functional which leads to the disruption of its chaperoning/stabilization properties and degradation of its client proteins instead [3,46]. Thus, although HSP90 expression is augmented in MA condition, this protein is most likely dysfunctional which might result in the impaired folding of the client proteins [5,28]. We propose that the compromised activity of the chaperone may, *per se*, trigger the increases in HSP90 expression found in the MA animals, as a compensatory mechanism.

Here, and in accordance with previous data, we also demonstrate that the up-regulation of HSP90 in the DRG of MA rats is accompanied by increases in the mRNA levels of GFAP [16] and P2X7R [13], suggesting a possible association of these signaling pathways. Additionally, ATF3 expression was also higher in MA animals than in controls, but these differences were not significant mostly because ATF3 expression is transient and at day 7 its levels are already retuning to physiological values, in accordance with our previous work [14]. Interestingly, in a previous study, we show that knockdown of ATF3 in DRG primary cell cultures decreases HSP90 expression, therefore suggesting a positive ATF3-HSP90 regulation that is also consistent with the present data [13]. Additionally, increased levels of P2X3R mRNA were observed in MA+saline animals. In a previous work, we described a decrease in the number of P2X3R immunoreactive neurons, at the more prolonged timepoints of disease [13] that is likely to be a consequence of the activation of a P2X7R/P2X3R negative feedback control [24-26]. Taking into account this new evidence, it is highly possible that up-regulation of P2X3R (largely associated with pain-like behaviors [47]) occurs within the first week of MA development. Only after, when P2X7R levels re significantly increased, the P2X7R/P2X3R negative control might be triggered and down-regulation of P2X3R is then observed. It is also important to note that the quantification methodologies are distinct and animals from this study were manipulated daily for the AB behavioral tests. Nevertheless, even though all these markers were consistently up-regulated in

MA DRG, more studies are needed to prove a real association of these phenomena.

Thus, in order to better understand the biological significance of HSP90 in the MA condition, the HSP90 inhibitor 17-DMAG was intrathecally (i.t.) administered to MA animals. A reduction in the movement-induced nociception of MA rats was observed at 1 and 3 hours after drug administration in the first day (day 4 of MA). After 4hours the effect was reversed and differences were no longer evident between the MA animals receiving either the inhibitor or saline. These findings show that a single injection of 17-DMAG induces an effective but transient anti-nociceptive effect in MA animals. This is in accordance with another study showing decreased mechanical allodynia (Von Frey test) in chronic constriction injured (CCI) rats following 17-DMAG i.t. injection, in the same temporal window [10]. No differences between the nociceptive behavior of the two groups could be observed on the following days (5 and 6, 3h-post injection), suggesting that repeated (daily) injections do not necessarily increase the response to 17-DMAG, therefore excluding a possible accumulative effect of the drug. In fact, even though differences were not found, the AB values of MA+17DMAG animals on days 5 and 6 were very similar to those on day 4 for the 3h timepoint, suggesting that the drug is similarly effective. On the other hand, MA+saline animals showed considerably reduced AB scores. A possible habituation of the animals to the AB test, together with a slight amelioration of the MA condition (already observed in our previous studies [34]), might have contributed to these events which were obviously more evident in the untreated group where AB scores had been extremely high in the beginning of 17-DMAG treatment. It is also plausible that HSP90 cleavage in MA animals limits the efficacy of the drug as 17-DMAG might not be able to bind to the fragments. Starting the treatment simultaneously to MA induction (prior to ROS production) would perhaps show more dramatic effects. Nevertheless, the strong effects observed in the initial timepoints should further encourage the study of HSP90 inhibitors in pain management.

This anti-nociceptive effect of 17-DMAG was accompanied by considerable changes on the expression of specific molecular targets at the DRG. Indeed, HSP90 expression decreased significantly comparatively to non-treated MA animals. Moreover, it is highly possible that, by binding to HSP90 instead of ROS [3], 17-DMAG prevented HSP90 cleavage, reversing the high levels of cleaved protein detected in MA animals. As above discussed, HSP90 is probably highly dysfunctional in the MA condition due to cleavage, which could trigger increases in its expression in a sort of compensatory mechanism. Likewise, the fact that 17-DMAG prevents HSP90 cleavage could explain why the treatment lowers HSP90 expression in MA animals. However, it is unlikely that the HSP90 functionalities and chaperone properties had been restored since 17-DMAG, by binding to the ATP site, also disrupts the functional activity of these chaperones [5,31]. Therefore, other mechanisms should explain the effects observed with 17-DMAG treatment. In fact, HSP90 is assumed as the major repressor of the heat shock factor 1 (HSF1), forming with it a complex in unstressed cells [31]. In stressful conditions, HSF1 dissociates being therefore able to translocate to the nucleus and activate other molecules [48,49]. Similarly, 17-DMAG (and other related compounds) binds to the HSP90 N-terminal freeing HSF1, which promotes the expression of stress-responsive genes [50]. This mechanism could possibly explain the effects of 17-DMAG even though HSP90 is not functional as a chaperone when the drug is bound to it.

Interestingly, 17-DMAG treatment also reduced GFAP expression in DRG of MA animals suggesting that HSP90 is somehow associated with SGCs activation. In fact it has been previously shown that the treatment with another HSP90 inhibitor (PU-H71) reduced astrocyte activation in an experimental autoimmune encephalomyelitis (EAE) model [18], while 17-DMAG was shown to suppress microglia activation in experimental stroke [51]. Although little is known about the underlying mechanisms, HSP90 involvement with SGCs activation in the MA condition is unlikely to be mediated by P2X7R since their levels remained unchanged with 17-DMAG treatment. We also observed a significant reduction in the mRNA levels of the neuronal P2X3R in 17-DMAG-treated animals, suggesting that HSP90 inhibition attenuates neuronal sensitization in MA. Accordingly, others

have shown that HSP90 inhibitors alter ATP-induced currents in DRG neurons, inferring not only the involvement of HSP90 with purinergic receptors but also a possible role in nociception [52]. Indeed, both SGCs activation [19,21,53] and P2X3R up-regulation [47,54,55] are highly associated with the generation of pain states and likewise, the suppression of these events directly contributes to pain alleviation [56,57]. Therefore, it is likely that the decreased SGCs activation and expression of P2X3R induced by the treatment with 17-DMAG, are implicated in the anti-nociceptive effects of the drug observed in the MA animals. Overall, HSP90 inhibition may activate some mechanisms and suppress others, which ultimately will attenuate typical inflammatory events at the DRG.

The expression of ATF3 was also up-regulated after HSP90 inhibition in MA animals. Others have previously shown that ATF3 expression is regulated by HSP90 at the mRNA level [58] and, in fact, in cancer derived cell lines 17-DMAG was shown to increase ATF3 expression [11]. Moreover, since ATF3 is also a stress-inducible gene, it is likely to be up-regulated by the activated/free HSF1, which further supports that this cascade is part of 17-DMAG's mechanism of action. Recently, consistent and increasing data in the nervous system have been demonstrating the role of ATF3 in neuroregeneration, survival and tissue repair [59-61]. ATF3 is also known to be involved in the resolution of the inflammatory response by negatively regulating the toll-like receptor 4 (TLR4) pro-inflammatory signalling pathway [62]. Interestingly, HSP90 is capable of inducing the production of proinflammatory cytokines via TLR4 signal transduction pathways [63], while HSP90 inhibitors are known to attenuate these responses [10,51,64]. These findings suggest that HSP90 inhibition attenuates the MA inflammatory pathomechanisms at the DRG level (namely SCGs activation and P2X3R expression), revealing a possible protective role of ATF3.

In conclusion, we propose a role for HSP90 in MA pathophysiology. A remarkable cleavage of HSP90 is demonstrated suggesting that the chaperone is most likely non-functional in MA. We show evidence for an anti-nociceptive effect of 17-DMAG (HSP90 inhibitor) in MA animals, possibly due to diminished SGCs activation, decreased purinergic activity and a protective role of ATF3. Data suggest that HSP90 inhibition by 17-DMAG attenuates the inflammatory signaling at the DRG level with implications in the pain behavior of MA animals. 17-DMAG also seems to protect HSP90 from cleavage. We hypothesize that HSP90 cleavage is a central event in the mechanisms underlying the role of this chaperone in DRG neurons, which might also determine the efficacy of HSP90 inhibitors. Even though HSP90 chaperoning properties might not be restored, this can be somehow associated with the molecular changes observed and the nociceptive effects of the drug. Further investigation is needed to clarify this.

5. Acknowledgements

The study was supported by the Chair on Pain Medicine of the Faculty of Medicine, University of Porto and by the Grünenthal Foundation – Portugal. The first author received a doctoral grant **(SFRH/BD/79497/2011)** by *Fundação para a Ciência e a Tecnologia* (FCT), Portugal. The authors would also like to thank José Pedro Castro (from Deutsches Institut für Ernährungsforschung Potsdam-Rehbrücke; Germany) and Francisco Nóvoa Faria (from Dept. Fisiologia e Cirugia Cardiotorácica, CIM-FMUP) for their help and expertize concerning HSP90 fluorescent detection using LI-COR system.

6. Conflict of interest

The authors certify that there are not any personal or financial conflicts of interests related with the presented data.

7. References

1. Osterloh A, Breloer M (2008) Heat shock proteins: linking danger and pathogen recognition. Med Microbiol Immunol 197: 1-8.
2. Ohtsuka K, Suzuki T (2000) Roles of molecular chaperones in the nervous system. Brain Res Bull 53: 141-146.
3. Beck R, Dejeans N, Glorieux C, Creton M, Delaive E, et al. (2012) Hsp90 is cleaved by reactive oxygen species at a highly conserved

N-terminal amino acid motif. PLoS One 7: e40795.
4. Neckers L (2007) Heat shock protein 90: the cancer chaperone. J Biosci 32: 517-530.
5. Neckers L, Neckers K (2002) Heat-shock protein 90 inhibitors as novel cancer chemotherapeutic agents. Expert Opin Emerg Drugs 7: 277-288.
6. Rice JW, Veal JM, Fadden RP, Barabasz AF, Partridge JM, et al. (2008) Small molecule inhibitors of Hsp90 potently affect inflammatory disease pathways and exhibit activity in models of rheumatoid arthritis. Arthritis Rheum 58: 3765-3775.
7. Madrigal-Matute J, Lopez-Franco O, Blanco-Colio LM, Munoz-Garcia B, Ramos-Mozo P, et al. (2010) Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis. Cardiovasc Res 86: 330-337.
8. Waza M, Adachi H, Katsuno M, Minamiyama M, Tanaka F, et al. (2006) Alleviating neurodegeneration by an anticancer agent: an Hsp90 inhibitor (17-AAG). Ann N Y Acad Sci 1086: 21-34.
9. Lu Y, Ansar S, Michaelis ML, Blagg BS (2009) Neuroprotective activity and evaluation of Hsp90 inhibitors in an immortalized neuronal cell line. Bioorg Med Chem 17: 1709-1715.
10. Hutchinson MR, Ramos KM, Loram LC, Wieseler J, Sholar PW, et al. (2009) Evidence for a role of heat shock protein-90 in toll like receptor 4 mediated pain enhancement in rats. Neuroscience 164: 1821-1832.
11. Hackl C, Lang SA, Moser C, Mori A, Fichtner-Feigl S, et al. (2010) Activating transcription factor-3 (ATF3) functions as a tumor suppressor in colon cancer and is up-regulated upon heat-shock protein 90 (Hsp90) inhibition. BMC Cancer 10: 668.
12. Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, et al. (2000) Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. Mol Cell Neurosci 15: 170-182.
13. Nascimento DS (2016) The expression of P2X7 and P2X3 receptors is altered in sensory ganglia of monoarthritic rats (submitted).
14. Nascimento D, Pozza DH, Castro-Lopes JM, Neto FL (2011) Neuronal injury marker ATF-3 is induced in primary afferent neurons of monoarthritic rats. Neurosignals 19: 210-221.
15. Butler SH, Godefroy F, Besson JM, Weil-Fugazza J (1992) A limited arthritic model for chronic pain studies in the rat. Pain 48: 73-81.
16. Nascimento DS, Castro-Lopes JM, Moreira Neto FL (2014) Satellite glial cells surrounding primary afferent neurons are activated and proliferate during monoarthritis in rats: is there a role for ATF3? PLoS One 9: e108152.
17. Kakimura J, Kitamura Y, Takata K, Umeki M, Suzuki S, et al. (2002) Microglial activation and amyloid-beta clearance induced by exogenous heat-shock proteins. FASEB J 16: 601-603.
18. Lisi L, McGuire S, Sharp A, Chiosis G, Navarra P, et al. (2013) The novel HSP90 inhibitor, PU-H71, suppresses glial cell activation but weakly affects clinical signs of EAE. J Neuroimmunol 255: 1-7.
19. Hanani M (2005) Satellite glial cells in sensory ganglia: from form to function. Brain Res Brain Res Rev 48: 457-476.
20. Haydon PG (2001) GLIA: listening and talking to the synapse. Nat Rev Neurosci 2: 185-193.
21. Jasmin L, Vit JP, Bhargava A, Ohara PT (2010) Can satellite glial cells be therapeutic targets for pain control? Neuron Glia Biol 6: 63-71.
22. Ohara PT, Vit JP, Bhargava A, Romero M, Sundberg C, et al. (2009) Gliopathic pain: when satellite glial cells go bad. Neuroscientist 15: 450-463.
23. Chizh BA, Illes P (2001) P2X receptors and nociception. Pharmacol Rev 53: 553-568.
24. Chen Y, Li G, Huang LY (2012) P2X7 receptors in satellite glial cells mediate high functional expression of P2X3 receptors in immature dorsal root ganglion neurons. Mol Pain 8: 9.
25. Chen Y, Li G, Huang LY (2015) p38 MAPK mediates glial P2X7R-neuronal P2Y1R inhibitory control of P2X3R expression in dorsal root ganglion neurons. Mol Pain 11: 68.
26. Chen Y, Zhang X, Wang C, Li G, Gu Y, et al. (2008) Activation of P2X7 receptors in glial satellite cells reduces pain through downregulation of P2X3 receptors in nociceptive neurons. Proc Natl Acad Sci U S A 105: 16773-16778.

27. Adinolfi E, Kim M, Young MT, Di Virgilio F, Surprenant A (2003) Tyrosine phosphorylation of HSP90 within the P2X7 receptor complex negatively regulates P2X7 receptors. J Biol Chem 278: 37344-37351.
28. Nollen EA, Morimoto RI (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins. J Cell Sci 115: 2809-2816.
29. Zimmermann M (1983) Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16: 109-110.
30. Lourenco Neto F, Schadrack J, Platzer S, Zieglgansberger W, Tolle TR, et al. (2000) Expression of metabotropic glutamate receptors mRNA in the thalamus and brainstem of monoarthritic rats. Brain Res Mol Brain Res 81: 140-154.
31. Gorska M, Popowska U, Sielicka-Dudzin A, Kuban-Jankowska A, Sawczuk W, et al. (2012) Geldanamycin and its derivatives as Hsp90 inhibitors. Front Biosci (Landmark Ed) 17: 2269-2277.
32. Ossipov MH, Suarez LJ, Spaulding TC (1988) A comparison of the antinociceptive and behavioral effects of intrathecally administered opiates, alpha-2-adrenergic agonists, and local anesthetics in mice and rats. Anesth Analg 67: 616-624.
33. De la Calle JL, Paino CL (2002) A procedure for direct lumbar puncture in rats. Brain Res Bull 59: 245-250.
34. Borges G, Neto F, Mico JA, Berrocoso E (2014) Reversal of monoarthritis-induced affective disorders by diclofenac in rats. Anesthesiology in press.
35. Cruz CD, Neto FL, Castro-Lopes J, McMahon SB, Cruz F (2005) Inhibition of ERK phosphorylation decreases nociceptive behaviour in monoarthritic rats. Pain 116: 411-419.
36. Neto FL, Castro-Lopes JM (2000) Antinociceptive effect of a group II metabotropic glutamate receptor antagonist in the thalamus of monoarthritic rats. Neurosci Lett 296: 25-28.
37. Pozza DH, Potes CS, Barroso PA, Azevedo L, Castro-Lopes JM, et al. (2010) Nociceptive behaviour upon modulation of mu-opioid receptors in the ventrobasal complex of the thalamus of rats. Pain 148: 492-502.
38. Egorin MJ, Lagattuta TF, Hamburger DR, Covey JM, White KD, et al. (2002) Pharmacokinetics, tissue distribution, and metabolism of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (NSC 707545) in CD2F1 mice and Fischer 344 rats. Cancer Chemother Pharmacol 49: 7-19.
39. Eiseman JL, Lan J, Lagattuta TF, Hamburger DR, Joseph E, et al. (2005) Pharmacokinetics and pharmacodynamics of 17-demethoxy 17-[[(2-dimethylamino)ethyl]amino]geldanamycin (17DMAG, NSC 707545) in C.B-17 SCID mice bearing MDA-MB-231 human breast cancer xenografts. Cancer Chemother Pharmacol 55: 21-32.
40. Butler S, Weil-Fugazza, J (1994) The foot-bend procedure as test of nociception for chronic studies in a model of monoarthritis in the rat. Pharmacol Commun 4: 327-334.
41. Borges G, Neto F, Mico JA, Berrocoso E (2014) Reversal of monoarthritis-induced affective disorders by diclofenac in rats. Anesthesiology 120: 1476-1490.
42. Ferreira-Gomes J, Neto FL, Castro-Lopes JM (2004) Differential expression of GABA(B(1b)) receptor mRNA in the thalamus of normal and monoarthritic animals. Biochem Pharmacol 68: 1603-1611.
43. Potes CS, Neto FL, Castro-Lopes JM (2006) Administration of baclofen, a gamma-aminobutyric acid type B agonist in the thalamic ventrobasal complex, attenuates allodynia in monoarthritic rats subjected to the ankle-bend test. J Neurosci Res 83: 515-523.
44. Sevin M, Girodon F, Garrido C, de Thonel A (2015) HSP90 and HSP70: Implication in Inflammation Processes and Therapeutic Approaches for Myeloproliferative Neoplasms. Mediators Inflamm 2015: 970242.
45. Poulet B, Beier F (2016) Targeting oxidative stress to reduce osteoarthritis. Arthritis Res Ther 18: 32.
46. Castro JP, Reeg S, Botelho V, Almeida H, Grune T (2014) HSP90 cleavage associates with oxidized proteins accumulation after oxidative stress. Free Radic Biol Med 75 Suppl 1: S24-25.

47. Wirkner K, Sperlagh B, Illes P (2007) P2X3 receptor involvement in pain states. Mol Neurobiol 36: 165-183.
48. Wolfgang CD, Liang G, Okamoto Y, Allen AE, Hai T (2000) Transcriptional autorepression of the stress-inducible gene ATF3. J Biol Chem 275: 16865-16870.
49. Zou J, Guo Y, Guettouche T, Smith DF, Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell 94: 471-480.
50. Neef DW, Jaeger AM, Thiele DJ (2011) Heat shock transcription factor 1 as a therapeutic target in neurodegenerative diseases. Nat Rev Drug Discov 10: 930-944.
51. Qi J, Han X, Liu HT, Chen T, Zhang JL, et al. (2014) 17-Dimethylaminoethylamino-17-demethoxygeldanamycin attenuates inflammatory responses in experimental stroke. Biol Pharm Bull 37: 1713-1718.
52. McDowell TS, Yukhananov RY (2002) HSP90 inhibitors alter capsaicin- and ATP-induced currents in rat dorsal root ganglion neurons. Neuroreport 13: 437-441.
53. Dublin P, Hanani M (2007) Satellite glial cells in sensory ganglia: their possible contribution to inflammatory pain. Brain Behav Immun 21: 592-598.
54. Prado FC, Araldi D, Vieira AS, Oliveira-Fusaro MC, Tambeli CH, et al. (2013) Neuronal P2X3 receptor activation is essential to the hyperalgesia induced by prostaglandins and sympathomimetic amines released during inflammation. Neuropharmacology 67: 252-258.
55. Xu GY, Huang LY (2002) Peripheral inflammation sensitizes P2X receptor-mediated responses in rat dorsal root ganglion neurons. J Neurosci 22: 93-102.
56. Barclay J, Patel S, Dorn G, Wotherspoon G, Moffatt S, et al. (2002) Functional downregulation of P2X3 receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. J Neurosci 22: 8139-8147.
57. Cao J, Li Z, Zhang Z, Ren X, Zhao Q, et al. (2014) Intrathecal injection of fluorocitric acid inhibits the activation of glial cells causing reduced mirror pain in rats. BMC Anesthesiol 14: 119.
58. Sato A, Nakama K, Watanabe H, Satake A, Yamamoto A, et al. (2014) Role of activating transcription factor 3 protein ATF3 in necrosis and apoptosis induced by 5-fluoro-2'-deoxyuridine. FEBS J 281: 1892-1900.
59. Hunt D, Raivich G, Anderson PN (2012) Activating transcription factor 3 and the nervous system. Front Mol Neurosci 5: 7.
60. Seijffers R, Mills CD, Woolf CJ (2007) ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration. J Neurosci 27: 7911-7920.
61. Seijffers R, Zhang J, Matthews JC, Chen A, Tamrazian E, et al. (2014) ATF3 expression improves motor function in the ALS mouse model by promoting motor neuron survival and retaining muscle innervation. Proc Natl Acad Sci U S A 111: 1622-1627.
62. Gilchrist M, Henderson WR, Jr., Clark AE, Simmons RM, Ye X, et al. (2008) Activating transcription factor 3 is a negative regulator of allergic pulmonary inflammation. J Exp Med 205: 2349-2357.
63. Tsan MF, Gao B (2004) Cytokine function of heat shock proteins. Am J Physiol Cell Physiol 286: C739-744.
64. Yun TJ, Harning EK, Giza K, Rabah D, Li P, et al. (2011) EC144, a synthetic inhibitor of heat shock protein 90, blocks innate and adaptive immune responses in models of inflammation and autoimmunity. J Immunol 186: 563-575.

Discussion

5. Discussion

The present study focused on the molecular and cellular mechanisms occurring in the sensory ganglia, during the MA joint inflammatory condition. Indeed, chronic pain is one the main causes for disability and loss of joint function which has serious impact in the patient's quality of life and in the governmental finances. Moreover, these conditions are often poorly diagnosed which results in a high percentage of unsuccessful treatments (McDougall, JJ 2006). With this work, we hope we contributed to the better understanding of the pathophysiology mechanisms of MA occurring at the DRG level, namely of the involvement of ATF3 and SGCs.

The intra-articular injection of complete Freund's adjuvant (CFA) produces a prominent, stable and anatomically limited inflammatory condition in the joint. We demonstrated that MA significantly induced ATF3 expression in L3, L4 and L5 ipsilateral DRG **(Publication I)** (Nascimento, D *et al.* 2011). This up-regulation was transient, with the most significant increases being found after 4 days of disease which was followed by a decrease and return to control values around day 14. Since ATF3 is assumed as a neuronal injury marker (Tsujino, H *et al.* 2000), this evidence suggests that the MA inflammatory condition might lead to some degree of neuronal damage **(Publication I)** (Nascimento, D *et al.* 2011). Indeed, others have also observed the induction of ATF3 in models of osteoarthritis induced by MIA injection in the knee joint, suggesting a possible neuropathic component in this condition (Ivanavicius, SP *et al.* 2007). In addition, other studies in OA, either induced by MIA or collagenase injection, support that the initial inflammatory state shifts to a "neuropathic pain phenotype" with the progression of the disease (Ferreira-Gomes, J *et al.* 2012, Adaes, S *et al.* 2015, Su, J *et al.* 2015). Also the injection of capsaicin, formalin, mustard oil or menthol injected into the plantar surface of

mice induced the expression of ATF3 in distinct subpopulations of sensory neurons (Braz, JM and Basbaum, AI 2010).

Despite being expressed during these particular joint painful conditions (like in OA and MA), ATF3 is not often induced in DRG neurons upon peripheral inflammation. For example, in the antigen-induced arthritis (AIA) model none ATF3 expression was observed, is spite of the profound inflammatory state (Segond von Banchet, G *et al.* 2009). Moreover, intraplantar injection of CFA also failed to evoke ATF3 induction (Braz, JM and Basbaum, AI 2010). Indeed, contrarily to our experimental model that is induced by intra-articular injection of CFA (**Publication I**, (Nascimento, D *et al.* 2011)), no ATF3 expression or neuronal damage seems to occur when the same inflammatory agent is injected in the soft tissue (Braz, JM and Basbaum, AI 2010). Therefore, ATF3 expression seems to be somehow selective for the inflammatory agent but also dependent on the severity of the model. Probably, during MA and other similar pathologies in the joint, the increase in the intra-articular pressure activates mechanoreceptors in the terminals of primary afferents that result in higher neuronal excitability and activation of the so-called "neuronal-damage programs" (Su, J *et al.* 2015). Unfortunately, most studies focus on cutaneous nociception, while chronic pain arising from deeper tissues and joints are less explored. Thus, it is likely that ATF3 is selectively induced in neurons marked as damaged but actual nerve lesion/injury is not necessarily required.

The hypothesis of a neuropathic component during MA was further supported by another set of experiments where we demonstrated that the administration of Ketoprofen, a COX inhibitor NSAID, to MA animals failed to reverse ATF3 expression, either when given prior to disease induction or after 2 days **(Publication I)** (Nascimento, D *et al.* 2011). Ketoprofen-treated MA animals showed reduced paw diameters suggesting an attenuation of the inflammatory component, although this exerted no effect on ATF3 expression. Thus, we conclude that production of prostanoids (among these, prostaglandin E2 - PGE2) is

unlikely to be the trigger of ATF3 expression, and these molecules do not seem to be associated with the presumed activation of neuronal damage programs occurring in the MA model. Although the production of prostanoids and up-regulation of COX-2 are intimately associated with thermal and mechanical hyperalgesia in peripheral inflammation (Schaible, HG *et al.* 2002), some authors failed to show pain relief after administration of a non-selective COX-inhibitor in the OA model (Ivanavicius, SP *et al.* 2007). Once again, this evidence supports that pain arising from chronic joint pathology, after a certain timepoint of disease progression, no longer seems to be dependent on the initial inflammatory mechanisms. In fact, even though neuropathic and inflammatory conditions have different etiologies, their mechanisms are nowadays believed to converge over time (Xu, Q and Yaksh, TL 2011). This might also explain why resolution of the original injury (such as joint repair) does not revert persistent pain in arthritic patients and why anti-inflammatory drugs are so often inefficient in those cases (Christianson, CA *et al.* 2010, Xu, Q and Yaksh, TL 2011). Therefore, investigating what triggers neuronal damage programs in these painful conditions is crucial to understand the pathophysiology of these diseases, and ATF3 expression seems to hold the key for some of the answers.

On the other hand, ATF3 was shown to negatively regulate the expression of prostaglandins and COX-2 in acute inflammation (Hellmann, J *et al.* 2015) suggesting that ATF3 itself might down-regulate the expression of these pro-inflammatory mediators and not necessarily the other way around. Indeed, ATF3 is known to be involved in the resolution of the inflammatory response, as it is also a negative regulator of the TLR4 pro-inflammatory signalling pathway (Gilchrist, M *et al.* 2008). Contrarily to its detrimental functions in other tissues, ATF3 actions are frequently protective in the nervous system (Hunt, D *et al.* 2012), where it has been mainly correlated to neuronal regeneration and protection. This is not necessarily contradictory to its use as a neuronal injury marker, since some damage is needed for the activation of regeneration/protective mechanisms

(Xu, Q and Yaksh, TL 2011). ATF3 promotes, for example, neurite outgrowth (Seijffers, R *et al.* 2006), increases the intrinsic growth state of injured neurons (Seijffers, R *et al.* 2007), and improves motor function promoting motor neuron survival (Seijffers, R *et al.* 2014). Additionally, overexpression of ATF3 was shown to induce neurite elongation and promote survival (by inhibiting apoptosis) via Akt activation (Nakagomi, S *et al.* 2003). Therefore, we questioned whether ATF3 was being induced during MA in order to drive cells into a survival/regeneration pathway. However, contrarily to other chronic pain models (like capsaicin injection and axotomy) where pAkt was increased (Nakagomi, S *et al.* 2003, Pezet, S *et al.* 2005, Shi, TJ *et al.* 2009), in the MA animals we observed a reduction in the number of pAkt IR cells (**Publication I**, (Nascimento, D *et al.* 2011)). Although the number of pAkt-positive neurons in the DRG were similar to what is reported (Shi, TJ *et al.* 2009), data suggest that this survival factor and the respective signalling pathway are not activated during MA, and therefore are not associated with MA pathophysiology. This might also justify why pAkt and ATF3 colocalization did not change during MA progression, suggesting that, in this condition, ATF3 is not likely triggered via a pAkt-mediated signaling pathway (**Publication I**, (Nascimento, D *et al.* 2011)).

In order to better understand the biological significance of ATF3 at the DRG level, we also tried to identify the ATF3-expressing neuronal population. Cell size measurement revealed that ATF3 is mainly expressed in small-to-medium populations in L_5 DRG from MA rats. Moreover, expression of ATF3 and CGRP in the same neurons was significantly increased at 2 and 4 days of MA (**Publication I**, (Nascimento, D *et al.* 2011)) which did not occur with IB4. Therefore, data suggest that ATF3 is mainly induced in peptidergic primary afferents at the early time points of MA. Indeed, CGRP is a key mediator of neurogenic inflammation at the periphery, but it also greatly contributes to nociceptive central mechanisms. Primary afferents are the major source of CGRP in the rat dorsal horn and this is known to have major repercussions in central pain processing, namely in the

development of hyperalgesia and allodynia (Galeazza, MT *et al.* 1995, Calza, L *et al.* 1998, Greco, R *et al.* 2008, Seybold, VS 2009). Therefore, the fact that ATF3 is mainly expressed in CGRP-positive neurons (double labelled cells significantly increased in MA) might suggest the involvement of this transcriptional factor in pain signalling (**Publication I**, (Nascimento, D *et al.* 2011)).

Contrarily to some reported increases of CGRP expression in DRG during neuropathic and inflammatory pain states (Tie-Jun, SS *et al.* 2001, Nakanishi, M *et al.* 2010, Wang, Z *et al.* 2013, Quartu, M *et al.* 2014), in MA we observed instead a non-significant decrease on CGRP overall expression, along the disease progression (**Publication I,** (Nascimento, D *et al.* 2011)). Indeed, in a model of colonic inflammation, the initial decreases in CGRP content were suggested to be caused by a greater peripheral and central CGRP demand (Li, XQ *et al.* 2004). The release of this neuropeptide in the dorsal horn of the spinal cord (Ryu, PD *et al.* 1988, Seybold, VS 2009) is proposed to result in a decreased immunoreactivity for CGRP in primary afferents neurons after 2d of CFA subcutaneous injection, an event that might be happening in MA as well. This "compensatory balance" between release and *de novo* production might justify the lack of more significant differences in CGRP expression, during MA. In addition, Ketoprofen-treated animals showed higher CGRP expression in comparison to non-treated MA animals **(Publication I,** (Nascimento, D *et al.* 2011)). Indeed, in neurons from the trigeminal ganglia (TG), CGRP release was shown to be mediated by a COX-2 dependent pathway. Therefore, it is highly possible that COX-2 inhibition by ketoprofen impairs CGRP release from primary afferents, which probably contributes to its accumulation in the DRG (Neeb, L *et al.* 2011). Overall, data concerning the expression of CGRP in the DRG and spinal cord are difficult to interpret since in pathological conditions many factors may contribute to peptides plasticity. The contradictory data found in the literature may result from changes in peptide synthesis, degradation or release (Galeazza, MT *et al.* 1995). In addition, the use

of different experimental models and timepoints contribute to some of this discrepancy, leading to a still incomplete *scenario* regarding the functional plasticity of neuropeptides.

Interestingly, it has been shown that stimulation of primary afferents evokes release of CGRP at the sensory ganglia which results in the activation of neighboring SGCs and release of pro-inflammatory cytokines (Ceruti, S *et al.* 2011). Indeed, the release of neuronal CGRP promotes the expression of several pro-inflammatory mediators such as IL-1β, which is suggested to have major repercussions in the activation of SGCs (Capuano, A *et al.* 2009), in what seems to be a cycle of glial-neuronal continued excitation (Takeda, M *et al.* 2009). SGCs are active modulators of neuronal activity, amplifying the inflammatory responses and sustaining the sensory transmission within the ganglia, therefore being greatly involved in the development of hyperalgesia and allodynia (Hanani, M 2005, Takeda, M *et al.* 2007, Takeda, M *et al.* 2008, 2009, Hanani, M 2012). These findings support the notion of an important bidirectional crosstalk between neurons and glial cells, during pathological painful conditions, where peptides like CGRP may play a role (Capuano, A *et al.* 2009, Takeda, M *et al.* 2009, Neeb, L *et al.* 2011). Moreover, some authors suggest that it is the expression of neuronal injury factors, just like ATF3, that might trigger the activation of SGCs and therefore the initiation of neuron-glia interactions (Elson, K *et al.* 2004). Indeed, we observed a selective increase of ATF3 expression in CGRP-containing peptidergic neurons in the DRG of MA rats (Publication I, (Nascimento, D *et al.* 2011)). Considering all these evidences, we then decided to evaluate the involvement of SGCs in the MA pathophysiological mechanisms, and hypothesized about a possible role of ATF3 in the regulation of those mechanisms.

Thus, we then demonstrated that SGCs are activated mainly after day 7 of MA, as inferred by the significant increases of GFAP protein levels detected by WB analysis. Quantification of GFAP-positive neuronal profiles (neurons surrounded in 50% or more of their perimeter by GFAP labeling) in L5 DRG also showed significantly increased levels at

7 days of MA (**Publication II**, (Nascimento, DS *et al.* 2014)). Our data corroborate with previous studies indicating that the 1st week of disease progression seems to be crucial for the events associated with SGCs activation. In fact, GFAP expression was increased in inflamed DRG, 7 days after chromic gut suture application onto the DRG (Siemionow, K *et al.* 2009), as well as in the TG of rats with orofacial inflammatory pain (Stephenson, JL and Byers, MR 1995). Additionally, CFA injection into the rat whisker pad area induced significant increases in GFAP and IL-1b co-labeling, after 2 days (Takeda, M *et al.* 2007).

However, data show that nerve damage provokes a more demarked and prolonged effect on SGCs activation. Indeed, in neuropathic pain models, such as in chemically-induced neuropathy, GFAP levels were still increased 1 month later (Warwick, RA and Hanani, M 2012). In the spinal nerve ligation (SNL) neuropathic model, GFAP expression increased immediately after 4 hours, gradually increasing up to 7 days and staying high until the end of the experiment at day 56 post-model induction (Liu, FY *et al.* 2012). We observed that GFAP expression (and therefore the activation of SGCs) remains significantly higher than controls at least until 14 days of MA **(Publication II)**, while in other inflammatory models GFAP levels have already returned to control values at this timepoint, or at least started decreasing. The fact that neuronal damage programs are possibly being triggered during MA (as suggested by ATF3 expression) might account for the significantly increased GFAP levels at day 14. Interestingly, we detected significant increases in the GFAP labeling around ATF3-positive neurons (**Publication II**, (Nascimento, DS *et al.* 2014)), suggesting that ATF3 could be one of the injury factors regulating these mechanisms, as suggested by others (Elson, K *et al.* 2004). Indeed, other studies have shown that the number of ATF3-positive TG neurons enclosed by GFAP-immunoreactive SGCs increased in a time-dependent manner in a model of molar extraction in the rat (Gunjigake, KK *et al.* 2009). Although ATF3 is significantly induced in MA, it is only expressed in a small percentage of neurons with a temporal profile that is distinct from

SGCs activation. Therefore, the fact that we observed significant increases in ATF3-GFAP positive profiles is highly suggestive of a relation between these two events.

Additionally, the proliferation of SGCs preferentially occurring around ATF3-positive TG neurons was also observed after chronic constriction injury of the infraorbital nerve (Donegan, M *et al.* 2013). In MA animals, the number of proliferating SGCs (measured by incorporation of BrdU) in the whole DRG was also significantly higher after 7d of disease. Not only the overall number of BrdU-positive SGCs in the DRG increased but also the number of SGCs proliferating around a specific neuron. Moreover, we found significantly more GFAP-positive neuronal profiles in 7d MA animals (**Publication II**, (Nascimento, DS *et al.* 2014). Our data are in accordance with other studies where SGCs proliferation was observed mostly 1 week after L5 nerve transection (Lu, X and Richardson, PM 1991). Additionally, BrdU incorporation was increased up to 5 days after Herpes Simplex virus infection (Elson, K *et al.* 2003). The same research group then showed that proliferation of SGCs also occurs in an animal model of scarification of the skin, considered to be a model of minor tissue trauma (Elson, K *et al.* 2004). Moreover, SGCs' proliferation was equally reported at 4 days after chronic constriction injury of the infraorbital nerve (Donegan, M *et al.* 2013).

Altogether our studies indicate that SGCs are not bystanders in MA pathophysyiology, but instead that they are crucial mediators in the mechanisms underlying articular inflammation. Indeed, many studies demonstrate that administration of fluorocitrate (FC), a metabolic inhibitor of SGCs, not only abolishes GFAP labeling in the DRG but also alleviates pain (Souza, GR *et al.* , Liu, FY *et al.* 2012). Parallel studies in our group showed that in the OA model induced by collagenase injection in the knee, the intrathecal injection of FC alleviated the movement- and loading-induced mechanical allodynia in the first couple of hours after administration (1h for the knee bend and 2h for the CatWalk tests) (Adães, *et al* manuscript submitted). Unfortunately, the exact functional

implications of SGCs activation/proliferation in nociception were not explored in MA. Nevertheless, our findings reinforce that the classical analgesic approaches mainly focusing on neurons should be substituted by broader strategies targeting SGCs and their communication with neurons instead (Ceruti, S *et al.* 2011).

Even though it is currently assumed that activation of SGCs and neuron-glia interactions within the sensory ganglia are crucial for the subsistence of the inflammatory response and modulation of sensory transmission (including nociception) (Hanani, M 2005, Dublin, P and Hanani, M 2007, Ohara, PT *et al.* 2009, Jasmin, L *et al.* 2010), the mediators and signaling pathways involved in these mechanisms remain still poorly elucidated (in comparison with the CNS, for example). However, in the last years, the purinergic system has emerged as one of the most relevant players in those events. Indeed, ATP is one of the major neurotransmitters important for the communication between neurons and glia. Moreover, in the sensory ganglia, P2X7R is exclusively expressed in SGCs while P2X3R can only be found in neurons, which implies that these receptors exert their effects almost exclusively through glia–neuron interactions (Chen, Y *et al.* 2008). Their inhibition/suppression also contributes to pain relief which demonstrates they also play a role in nociception (Dell'Antonio, G *et al.* 2002, Arulkumaran, N *et al.* 2011, Alves, LA *et al.* 2013, Antonioli, L *et al.* 2014).

Therefore, in another study, we have shown that the expression of P2X7R increases in the DRG of rats after 1 week of MA induction (**Publication III**, submitted). Western blot analysis revealed three distinct bands for P2X7R detection, corresponding most probably to different post-translational modifications. Indeed, in order to be addressed to the cell surface and become functional these receptors need to be glycosylated in two or more sites (Dunn, PM *et al.* 2001, North, RA 2002). In MA, we observed no changes in the protein levels of the heavier band, proposed to be the glycosylated form of P2X7R. Controversially, the non-glycosylated forms at 65-69KDa (possibly cytosol stored protein)

were significantly increased in ipsilateral DRG of MA animals (**Publication III**, submitted). Unfortunately, the biological significance of these post-translational modifications is still poorly elucidated. Moreover, with the antibodies available it is still difficult to distinguish homomeric from heteromeric receptors which altogether might result in the divergence that is often found between functional and molecular studies. For that reason, we later demonstrated that P2X7R mRNA levels are also significantly increased in the DRG of 7d MA (**Publication IV,** under submission). Data are in accordance with other studies showing that P2X7R is up-regulated in DRG of animals inflamed by CFA intraplantar injection (Chen, Y *et al.* 2008) or in a model of inflammatory bowel disease (Liu, S *et al.* 2015). Interestingly, P2X7R was also increased in DRG of neuropathic pain patients (Chessell, IP *et al.* 2005). Moreover, these results are in agreement with our previous studies showing that SGCs are activated and proliferate also around 7 days of MA (**Publication II**, (Nascimento, DS *et al.* 2014)). Indeed, since P2X7R is so intimately associated with SGCs activation (Takeda, M *et al.* 2009, Arulkumaran, N *et al.* 2011, Alves, LA *et al.* 2013), the finding of a similar temporal profile between P2X7R up-regulation and SGCs activation during MA development reinforces that these events are interconnected and that P2X7R-mediated signaling is relevant in MA pathophysiology.

Interestingly, others have shown that P2X7R up-regulation exerts a negative feedback control mechanism over neuronal P2X3R expression (Chen, Y *et al.* 2008, Chen, Y *et al.* 2012, 2015). By reducing P2X3R neuronal expression, P2X7R-negative control was proposed to act as a protective mechanism shown to effectively prevent allodynia in inflamed rats. In the MA condition, we did find a significant decrease in the number of P2X3R-positive neurons (**Publication III**, submitted). Therefore, it is likely that the P2X7R/P2X3R negative control mechanism suggested by Chen and colleagues (Chen, Y *et al.* 2008, Chen, Y *et al.* 2012, 2015) is also occurring in MA. Accordingly, others have

detected a more than 50% drop in P2X3R expression in L4 and L5 DRG neurons after sciatic nerve axotomy (Bradbury, EJ *et al.* 1998).

Curiously, we observed that the P2X3R mRNA levels are increased in the DRG of 7d MA animals (**Publication IV**, under submission). Although apparently this is in contradiction with the immunohistochemistry data, this is not necessarily the case. Besides the two methodological approaches, namely immunohistochemistry and RT-qPCR, being totally distinct, it is also known that P2X receptors are highly dependent on post-translational modifications and therefore changes at the mRNA level might not always correlate with data from the protein form or with a functional effect (North, RA 2002). Indeed, P2X3R is increased at the DRG level in both inflammatory (Prado, FC *et al.* 2013, Su, J *et al.* 2015) and neuropathic pain (Jarvis, MF *et al.* 2002, Taylor, AM and Ribeiro-da-Silva, A 2011) models, and these increases are proposed to be critical for the development of hyperalgesia in the peripheral tissue. It is possible that P2X3R expression is initially increased in response to the exacerbated neuronal excitation at the DRG (as suggested by increases in mRNA levels), and only after significant activation of P2X7R occurs, likely around day 7 of MA, the known P2X7R-P2X3R negative feedback control (Chen, Y *et al.* 2008, Chen, Y *et al.* 2012, 2015) is triggered, as suggested by the decreases in the number P2X3R-expressing neurons. Due to their involvement in pain processing, many studies now evaluate these receptors as targets for novel analgesic therapies (Chessell, IP *et al.* 2005, Arulkumaran, N *et al.* 2011). Our data not only provide evidence of the involvement of the purinergic system in MA, but they also demonstrate that P2X7R is most probably controlling an excessive activation of P2X3R, and consequently the extent of neuronal excitation, as part of a mechanism that can be assumed as protective (Tsuzuki, K *et al.* 2001). Therefore, a possible antinociceptive therapeutic approach based on targeting the inhibition of P2X7R should be carefully studied, since disrupting the P2X7R-P2X3R negative feedback might exacerbate the painful condition.

Interestingly, some studies had indicated that the co-expression of P2X3R and ATF3 in the same neurons was linearly correlated with increased mechanical thresholds (Hsieh, YL *et al.* 2012). In the MA model we have not evaluated the co-expression of both molecules. In fact we were not expecting a meaningful induction of ATF3 in P2X3R-positive neurons (which are mainly IB4 non-peptidergic) in the DRG of MA animals, since we had previously shown that this transcriptional factor is preferentially expressed in peptidergic neurons. Moreover, as P2X3R immunoreactivity decreased along MA it would be difficult to find a correlation between ATF3 and P2X3R expression, based solely on immunohistochemistry co-localization assays. Some studies have already shown that P2X3 mRNA decreased in ATF3-expressing neurons, indicating that P2X3R was increased specifically in non-stressed neurons, supposedly as part of a protective mechanism (Tsuzuki, K *et al.* 2001). Indeed, ATF3 is also assumed to have a protective role in neurons. Thus the down-regulation of P2X3R, as observed in MA, might be part of this ATF3-mediated protective mechanism, therefore justifying a lack of co-localization between these two proteins. In addition, some authors proposed that it is the expression of injury factors (just like ATF3) that signalizes and initiates mechanisms of neuron-glia crosstalk (Elson, K *et al.* 2004) which, as previously discussed, are strongly associated with P2X receptors. Therefore, even though they might not co-localize, it is still plausible to hypothesize a relation between ATF3 and P2X receptors.

Thus, in order to evaluate if ATF3 could be regulating both SGCs activation and the expression of purinergic receptors, we silenced ATF3 by using siRNAs in primary cell cultures of DRG. ATF3 knock-down did not induce any changes in the expression of P2X7R and/or P2X3R or even in the activation of SGCs, as inferred by no changes in the levels of GFAP. This suggests that, at least in vitro, ATF3 expression is not implicated in these events (**Publication III**, submitted). Nevertheless, it must be taken in consideration that although in most cases *in vitro* systems are excellent tools, they often fail in reproducing

biological phenomena. Indeed, when culturing DRG cells we disrupted the SGC-neuron structure. Since both cell types constitute functional units, strongly dependent on this morphological organization, the communication between the neuronal cell bodies and their satellite cells in the cultured DRG might have been impaired. Additionally, a knock-down of 60% in the expression of the ATF3 gene might not have been enough to induce significant and quantifiable changes in the purinergic system. However, due to their relevance in pathological conditions like MA, it is still worthy to continue investigating these targets and their association, *in vivo*.

Quite surprisingly, we found that ATF3 knock-down in the DRG cell cultures induced significant decreases in the gene expression of both the inducible and constitutive isoforms of the HSP90 chaperone (**Publication III**, submitted). Indeed, others have shown that ATF3 mRNA expression is regulated by HSP90 (Sato, A *et al.* 2014) but not much is known about the regulation of ATF3 over HSP90. Additionally, the HSP90 role in the nervous system, namely in the DRG, is still poorly elucidated. Despite the lack of more knowledge, it has recently been demonstrated that HSP90 inhibitors can reduce neurodegeneration (Waza, M *et al.* 2006) and promote neuroprotection (Lu, Y *et al.* 2009). Moreover, HSP90 was found to induce the production of proinflammatory cytokines through TLR4 signal transduction pathways (Tsan, MF and Gao, B 2004) while HSP90 blockers were shown to attenuate these responses (Yun, TJ *et al.* 2011, Qi, J *et al.* 2014). Even more remarkably, HSP90 was shown to be necessary (as part of theTLR4 signaling cascade) for the pain enhancement observed in CCI rats, while HSP90 inhibition proved to be effective in pain relief (Hutchinson, MR *et al.* 2009).

Taking all this in consideration, we hypothesized that HSP90 could have a role in MA pathophysiology, and that ATF3 and HSP90 could be possibly associated, in similarity to what had been observed in the *in vitro* studies. In accordance, we then demonstrated that the mRNA levels of both the constitutive and inducible forms of HSP90 are significantly

increased in the ipsilateral DRG of MA animals (**Publication IV**, under submission). These data are in agreement with several studies showing that HSPs are induced in response to many stressful conditions, namely during inflammation (Sevin, M *et al.* 2015). Indeed, some studies suggest HSP90 plays a role in inflammatory diseases such as rheumatoid arthritis (Rice, JW *et al.* 2008). HSP90 up-regulation in MA is also consistent with ATF3 induction, suggesting that the ATF3-HSP90 positive regulation found in DRG cell cultures might also occur *in vivo*.

Curiously, we also detected a significant increase in the cleavage of HSP90 during MA (**Publication IV**, under submission). Although the underlying mechanisms are not fully explored, HSP90 cleavage has been mainly attributed to the binding of ROS at a highly conserved N-terminal amino acid motif (Beck, R *et al.* 2012). Interestingly, inflammatory conditions (like MA) are greatly associated with ROS production (Poulet, B and Beier, F 2016), and therefore this is likely to be the reason for the massive cleavage observed in this disease. As a result, HSP90 chaperoning functions are compromised and degradation of its client proteins might occur instead (Beck, R *et al.* 2012, Castro, JP *et al.* 2014). Thus, although HSP90 expression is augmented in MA condition, we believe that it is most likely dysfunctional. We further propose that it is actually the disruption of HSP90 activity sensed by the cell that may trigger the up-regulation of HSP90 in MA animals, in a sort of compensatory mechanism (**Publication IV**, under submission).

Although some of the HSP90 signaling cascades are known, the studies have been mostly conducted in macrophages, so that the role of this chaperone in sensory neurons is still not clear. Encouraged by our previous findings in DRG cell cultures (**Publication III,** submitted)**,** and other studies suggesting a possible role of HSP90 in pain processing (Hutchinson, MR *et al.* 2009), we then intrathecally administered 17-Dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), a HSP90 inhibitor, to MA animals. Indeed, movement-induced nociception of 4d MA rats was significantly

attenuated with the drug in the first 3h post-injection. After 4h the effect was reverted and differences were no longer evident (**Publication IV**, under submission). Accordingly, others have shown decreased mechanical allodynia (Von Frey test) in chronic constriction injured (CCI) rats following 17-DMAG i.t. injection, in the same temporal window (Hutchinson, MR *et al.* 2009). With this, we show for the first time that a single injection of 17-DMAG induces an acute anti-nociceptive effect in MA animals which further encourages the study of HSP90 inhibitors in pain management (**Publication IV**, under submission).

Moreover, we also observed that 17-DMAG treatment reversed the high levels of cleaved HSP90 observed in MA animals (**Publication IV**, under submission). We propose that, by binding to HSP90 instead, 17-DMAG prevents HSP90 cleavage by ROS (Beck, R *et al.* 2012). According to the compensatory mechanisms mentioned above, it is likely that the decreased expression of HSP90 in 17-DMAG-treated animals is associated with the lower levels of cleaved protein. However, the down-regulation of HSP90 cannot be attributed to the restitution of its chaperone properties, since 17-DMAG, by binding to the ATP site, also disrupts the HSP90 functional activity (Neckers, L and Neckers, K 2002, Gorska, M *et al.* 2012). Thus it is possible that 17-DMAG effects might be mediated by heat shock factor 1 (HSF1), a major repressor of HSP90. Indeed, in unstressed cells HSF1 can be found in its inactive form consisting of a complex with HSP90 (Gorska, M *et al.* 2012). Similarly to what happens in stressful conditions, 17-DMAG binds to HSP90, resulting in HSF1 dissociation (Zou, J *et al.* 1998, Wolfgang, CD *et al.* 2000). This free HSF1 then translocates to the nucleus and promotes the expression of stress-responsive genes (Neef, DW *et al.* 2011). Therefore, this mechanism could possibly explain the molecular changes observed following 17-DMAG treatment, even though HSP90 is probably not functional.

Interestingly, the expression of ATF3 was also up-regulated after HSP90 inhibition in MA animals (**Publication IV**, under submission). In fact, since ATF3 is also a stress-inducible gene, it is likely to be up-regulated by the activated/free HSF1, which further

supports that this cascade is part of 17-DMAG mechanism of action. In fact, in cancer derived cell lines 17-DMAG was shown to increase ATF3 expression (Hackl, C *et al.* 2010), although the mechanisms for this association are still unknown. Many studies have recently demonstrated that ATF3 might have a protective role in the nervous system (Seijffers, R *et al.* 2007, Hunt, D *et al.* 2012, Seijffers, R *et al.* 2014), rather than a detrimental function as suggested for other tissues. Additionally, ATF3 appears to be also involved in the resolution of the inflammatory response by negatively regulating the toll-like receptor 4 (TLR4) pro-inflammatory signalling pathway (Gilchrist, M *et al.* 2008). Interestingly, HSP90 is capable of inducing the production of proinflammatory cytokines via TLR4 signal transduction pathways (Tsan, MF and Gao, B 2004), while HSP90 inhibitors are known to attenuate these responses (Hutchinson, MR *et al.* 2009, Yun, TJ *et al.* 2011, Qi, J *et al.* 2014). Thus, it is plausible that HSP90 inhibition attenuates the pro-inflammatory cascades at the DRG level, possibly through ATF3.

Accordingly, 17-DMAG treatment also reduced GFAP expression in the DRG of MA animals suggesting a suppression of SGCs activation (**Publication IV**, under submission). Similar effects were observed in the CNS, where the HSP90 inhibitor PU-H71 reduced astrocyte activation in an experimental autoimmune encephalomyelitis (EAE) model (Lisi, L *et al.* 2013) while 17-DMAG suppressed microglia activation in experimental stroke (Qi, J *et al.* 2014). Although our studies do not allow greater conclusions regarding the underlying mechanisms, this diminished SGCs activation is unlikely to be mediated by P2X7R as their expression remained unchanged in 17-DMAG treated animals (**Publication IV**, under submission). On the other hand, the expression of the neuronal P2X3R significantly decreased in 17-DMAG-treated animals, suggesting that HSP90 inhibition also attenuates neuronal sensitization in MA (**Publication IV**, under submission). Accordingly, others have shown that HSP90 inhibitors alter ATP-induced currents in DRG neurons, which suggests not only the involvement of HSP90 with purinergic receptors but also a

possible role in nociception (McDowell, TS and Yukhananov, RY 2002). Indeed, both SGCs activation (Hanani, M 2005, Dublin, P and Hanani, M 2007, Jasmin, L *et al.* 2010) and P2X3R-mediated neuronal sensitization (Xu, GY and Huang, LY 2002, Wirkner, K *et al.* 2007, Prado, FC *et al.* 2013) constitute pathomechanisms involved in the MA painful condition. Thus, also these findings indicate that HSP90 inhibition suppresses the inflammatory signaling at the DRG level. Likewise, the decreased SGCs activation and expression of P2X3R may explain the anti-nociceptive effects of 17-DMAG observed in the MA animals.

In summary, with these studies, we unraveled several mechanisms occurring at the DRG (the first place for the processing of information arising from the periphery) upon the induction of the MA inflammatory condition (as schematized in Fig. 11 A). In most of the cases, we were also able to find temporal correlations between the events under study (as schematized in Fig. 11 B). We hope we contributed to a better understanding of the pathophysiological mechanisms underlying the establishment and progression of MA and that, by further elucidating these signaling cascades, we shed light into novel potential therapeutic approaches.

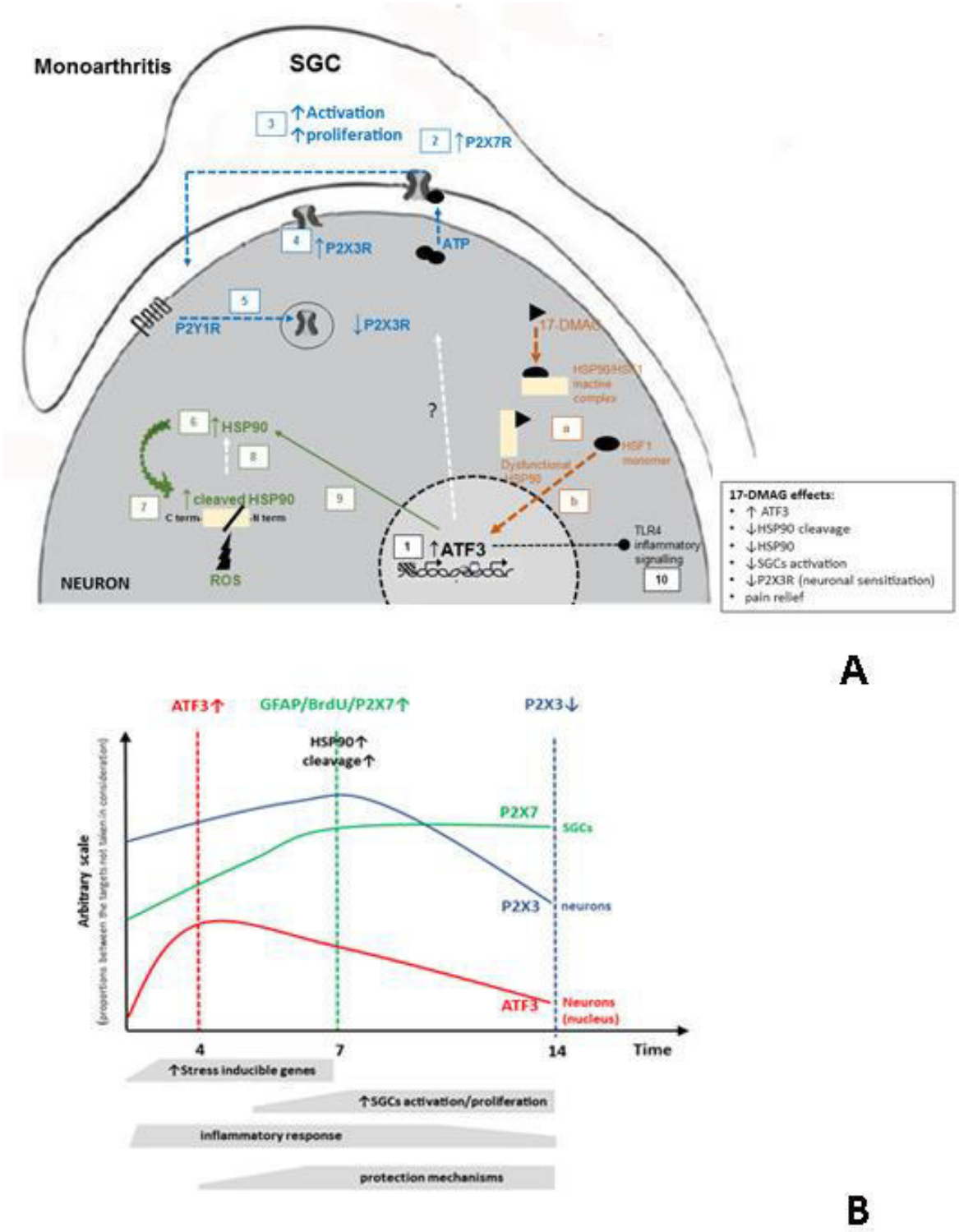


Fig. 11 – Summary of the major findings observed at the DRG during MA (A) and the temporal profile of some of these events (B).

The stress inducible gene ATF3 was induced mainly in small peptidergic DRG neurons (most likely representing C-fiber nociceptors) at the initial timepoints of MA (Publication I), suggesting its possible role in pain processing **(A, 1)**. Being a transcriptional factor that is rapidly and transiently expressed in MA (around day 2 and 4), its peak of expression occurs prior to all the other molecular and cellular changes observed in these studies, therefore suggesting it is upstream the signaling pathways investigated (scheme B). P2X7R, expressed exclusively in SGCs, was also up-regulated around 1 week of MA (Publication III) **(A, 2)** which is proposed as the most relevant mechanism for SCGs activation. Indeed, SGCs activation and proliferation were also observed around the same timepoint of disease progression (Publication II) **(A, 3)**, demonstrating a temporal correlation with P2X7 up-regulation (represented in **B**). Interestingly, SGCs activation was detected preferentially around ATF3-expressing neurons. Indeed, the activation of glial receptors/cells suggests the occurrence of neuron-glia communication events which are proposed by some authors to be initiated by injury markers like ATF3. Moreover, the mRNA levels of the neuronal P2X3R were also increased in the DRG of 7d MA animals (Publication III) **(A, 4;** also represented in **B),** while IHC assays showed that after this timepoint P2X3R immunoreactivity in the ganglia significantly decreased (mostly at 14d MA; Publication III) **(A, 5;** also represented in **B).** This suggests that, as previously reported, a P2X7R-P2X3R negative feedback control might be activated during MA. Finally, we demonstrate that HSP90 is another stress inducible gene up-regulated during MA **(A, 6)**, although it is most likely dysfunctional due to massive cleavage by ROS, as the levels of the cleaved form of HSP90 are also increased in MA animals **(A, 7),** (Publication IV). We propose that, besides being expressed as a stress gene in MA, the high cleavage of HSP90 into non-functional fragments might trigger its own up-regulation in a sort of compensatory mechanism **(A, 8).** Our experiments in primary cell cultures of DRG demonstrated a positive regulation of ATF3 over HSP90 **(A, 9)**, and in fact both genes seem

to be both augmented in the MA condition (Publication III). However, the exact correlation between these two events is still not clear and lacks further investigation. Also supportive of a role of HSP90 in MA were the effects observed with the administration of 17-DMAG (HSP90 inhibitor) to inflamed animals (Publication IV). Indeed, by preventing ROS binding, 17-DMAG seems to protect HSP90 from cleavage **(A, a).** Probably due to the release of HSF1, ATF3 was up-regulated after 17-DMAG treatment in what we believe to be a protective mechanism **(A, b).** HSP90 inhibition was shown before to exert anti-inflammatory effects by the suppression of a HSP90-TLR4-mediated signaling pathway. In fact, also ATF3 is known to be a negative regulator of this cascade and to be involved in the resolution of the inflammatory response **(A, 10).** Moreover, in 17-DMAG treated animals, SGCs activation and P2X3R expression were decreased, which further supports a role of these drugs in the attenuation of the inflammatory signaling. These findings might also explain the pain alleviation observed in MA animals injected with the inhibitor. Hopefully, these studies will allow a better understanding of MA pathophysiology and open a door for new possible targets for pain management in joint inflammatory conditions.(Note: dashed lines in **A** represent documented findings used to explain our data but that were not investigated in the present work. White dashed lines represent possible mechanisms, here proposed, that were still not described, at least directly).

Conclusions

6. Conclusions

In summary, the main conclusions of these studies are:

1. ATF3 is significantly and transiently induced in DRG neurons in early stages of a MA condition induced by intra-articular CFA injection. Ketoprofen (NSAID) administration ameliorated the MA typical inflammatory signs but did not reverse ATF3 expression.

   This suggests some degree of neuronal damage occurs in this inflammatory condition, or at least the activation of "neuronal damage programs" along disease progression.

2. SGCs are activated and proliferate during MA, particularly at the first week of disease.

   SGCs are not bystanders to MA pathophysiology but they are active players in this condition.

3. P2X7R is up-regulated in sensory ganglia of MA rats mainly after day 7 of disease, which is in agreement with the activation of SGCs, while P2X3R is down-regulated after the same timepoint (more significantly at 14d MA).

   The P2X7R/P2X3R purinergic system is activated during MA. Glial P2X7R is most likely down-regulating neuronal P2X3R in a feedback negative control.

SGCs activation in MA is probably mediated by P2X7R, as this receptor is assumed to be the major responsible for these events.

The activation of glial cells and receptors supports the hypothesis of neuron-glia communication events taking place during MA, which are highly correlated with pain states.

4. Silencing of ATF3 expression in DRG cultures significantly reduced the expression of both the inducible and constitutive isoforms of HSP90.

ATF3 seems to positively regulate HSP90 expression in DRG neurons.

5. HSP90 mRNA was up-regulated in DRG of MA animals but protein analysis showed it was massively cleaved in this condition.

HSP90 is most likely dysfunctional in MA due to cleavage by ROS.

6. 17-DMAG administration attenuated MA-induced allodynia and reversed the cleavage of the HSP90 protein. Additionally, HSP90 inhibition decreased GFAP and P2X3R expression while ATF3 was significantly increased.

17-DMAG exerts an antinociceptive effect in MA animals possibly due to the suppression of MA inflammatory cascades at DRG, namely SGCs activation and purinergic signaling. We also propose a protective role for ATF3 as a consequence of HSP90 inhibition.

17-DMAG disrupts HSP90 chaperoning functions but it prevents it from being cleaved. This event seems to be correlated with the molecular changes at the DRG and pain alleviation observed in MA animals following administration of 17-DMAG.

References

7. References

Adaes, S., J. Ferreira-Gomes, M. Mendonca, L. Almeida, J. M. Castro-Lopes and F. L. Neto (2015). "Injury of primary afferent neurons may contribute to osteoarthritis induced pain: an experimental study using the collagenase model in rats." Osteoarthritis Cartilage **23**(6): 914-924.

Adinolfi, E., M. Kim, M. T. Young, F. Di Virgilio and A. Surprenant (2003). "Tyrosine phosphorylation of HSP90 within the P2X7 receptor complex negatively regulates P2X7 receptors." J Biol Chem **278**(39): 37344-37351.

Alvarez, F. J. and R. E. Fyffe (2000). "Nociceptors for the 21st century." Curr Rev Pain **4**(6): 451-458.

Alves, L. A., R. J. Bezerra, R. X. Faria, L. G. Ferreira and V. da Silva Frutuoso (2013). "Physiological roles and potential therapeutic applications of the P2X7 receptor in inflammation and pain." Molecules **18**(9): 10953-10972.

Antonioli, L., M. C. Giron, R. Colucci, C. Pellegrini, D. Sacco, V. Caputi, G. Orso, M. Tuccori, C. Scarpignato, C. Blandizzi and M. Fornai (2014). "Involvement of the P2X7 purinergic receptor in colonic motor dysfunction associated with bowel inflammation in rats." PLoS One **9**(12): e116253.

Arulkumaran, N., R. J. Unwin and F. W. Tam (2011). "A potential therapeutic role for P2X7 receptor (P2X7R) antagonists in the treatment of inflammatory diseases." Expert Opin Investig Drugs **20**(7): 897-915.

Averill, S., D. R. Davis, P. J. Shortland, J. V. Priestley and S. P. Hunt (2002). "Dynamic pattern of reg-2 expression in rat sensory neurons after peripheral nerve injury." J Neurosci **22**(17): 7493-7501.

Azevedo, L. F., A. Costa-Pereira, L. Mendonca, C. C. Dias and J. M. Castro-Lopes (2012). "Epidemiology of chronic pain: a population-based nationwide study on its prevalence, characteristics and associated disability in Portugal." J Pain **13**(8): 773-783.

Azevedo, L. F., A. Costa-Pereira, L. Mendonca, C. C. Dias and J. M. Castro-Lopes (2014). "The economic impact of chronic pain: a nationwide population-based cost-of-illness study in Portugal." Eur J Health Econ.

Barajon, I., G. Serrao, F. Arnaboldi, E. Opizzi, G. Ripamonti, A. Balsari and C. Rumio (2009). "Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia." J Histochem Cytochem **57**(11): 1013-1023.

Baron, R. (2000). "Peripheral neuropathic pain: from mechanisms to symptoms." Clin J Pain **16**(2 Suppl): S12-20.

Basbaum, A. I., D. M. Bautista, G. Scherrer and D. Julius (2009). "Cellular and molecular mechanisms of pain." Cell **139**(2): 267-284.

Beamer, E., F. Goloncser, G. Horvath, K. Beko, L. Otrokocsi, B. Kovanyi and B. Sperlagh (2015). "Purinergic mechanisms in neuroinflammation: An update from molecules to behavior." Neuropharmacology **104**: 94-104.

Beck, R., N. Dejeans, C. Glorieux, M. Creton, E. Delaive, M. Dieu, M. Raes, P. Leveque, B. Gallez, M. Depuydt, J. F. Collet, P. B. Calderon and J. Verrax (2012). "Hsp90 is cleaved by reactive oxygen species at a highly conserved N-terminal amino acid motif." PLoS One **7**(7): e40795.

Bleehen, T. and C. A. Keele (1977). "Observations on the algogenic actions of adenosine compounds on the human blister base preparation." Pain **3**(4): 367-377.

Borges, G., F. Neto, J. A. Mico and E. Berrocoso (2014). "Reversal of monoarthritis-induced affective disorders by diclofenac in rats." Anesthesiology **in press**.

Bradbury, E. J., G. Burnstock and S. B. McMahon (1998). "The expression of P2X3 purinoreceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor." Mol Cell Neurosci **12**(4-5): 256-268.

Braz, J. M. and A. I. Basbaum (2010). "Differential ATF3 expression in dorsal root ganglion neurons reveals the profile of primary afferents engaged by diverse noxious chemical stimuli." Pain.

Breivik, H., B. Collett, V. Ventafridda, R. Cohen and D. Gallacher (2006). "Survey of chronic pain in Europe: prevalence, impact on daily life, and treatment." Eur J Pain **10**(4): 287-333.

Breivik, H., E. Eisenberg, T. O'Brien and Openminds (2013). "The individual and societal burden of chronic pain in Europe: the case for strategic prioritisation and action to improve knowledge and availability of appropriate care." BMC Public Health **13**: 1229.

Butler, S. H., F. Godefroy, J. M. Besson and J. Weil-Fugazza (1992). "A limited arthritic model for chronic pain studies in the rat." Pain **48**(1): 73-81.

Byng-Maddick, R., L. Jeyalingam and A. Keat (2012). "Management of persistent inflammatory large joint monoarthritis." Clin Rheumatol **31**(12): 1657-1662.

Cachemaille, M., C. J. Laedermann, M. Pertin, H. Abriel, R. D. Gosselin and I. Decosterd (2012). "Neuronal expression of the ubiquitin ligase Nedd4-2 in rat dorsal root ganglia: modulation in the spared nerve injury model of neuropathic pain." Neuroscience **227**: 370-380.

Calza, L., M. Pozza, M. Zanni, C. U. Manzini, E. Manzini and T. Hokfelt (1998). "Peptide plasticity in primary sensory neurons and spinal cord during adjuvant-induced

arthritis in the rat: an immunocytochemical and in situ hybridization study." Neuroscience **82**(2): 575-589.

Cao, J., Z. Li, Z. Zhang, X. Ren, Q. Zhao, J. Shao, M. Li, J. Wang, P. Huang and W. Zang (2014). "Intrathecal injection of fluorocitric acid inhibits the activation of glial cells causing reduced mirror pain in rats." BMC Anesthesiol **14**: 119.

Capuano, A., A. De Corato, L. Lisi, G. Tringali, P. Navarra and C. Dello Russo (2009). "Proinflammatory-activated trigeminal satellite cells promote neuronal sensitization: relevance for migraine pathology." Mol Pain **5**: 43.

Carlton, S. M. (2014). "Nociceptive primary afferents: they have a mind of their own." J Physiol **592**(Pt 16): 3403-3411.

Castro, J. P., S. Reeg, V. Botelho, H. Almeida and T. Grune (2014). "HSP90 cleavage associates with oxidized proteins accumulation after oxidative stress." Free Radic Biol Med **75 Suppl 1**: S24-25.

Ceruti, S., G. Villa, M. Fumagalli, L. Colombo, G. Magni, M. Zanardelli, E. Fabbretti, C. Verderio, A. M. van den Maagdenberg, A. Nistri and M. P. Abbracchio (2011). "Calcitonin gene-related peptide-mediated enhancement of purinergic neuron/glia communication by the algogenic factor bradykinin in mouse trigeminal ganglia from wild-type and R192Q Cav2.1 Knock-in mice: implications for basic mechanisms of migraine pain." J Neurosci **31**(10): 3638-3649.

Cervero, F. (2008). Pain Theories. The senses - a comprehensive reference. M. C. B. a. A. I. Basbaum, Elsevier. **Volume 5 - Pain**.

Cervero, F. and J. M. Laird (1996). "Mechanisms of allodynia: interactions between sensitive mechanoreceptors and nociceptors." Neuroreport **7**(2): 526-528.

Chen, B. P., G. Liang, J. Whelan and T. Hai (1994). "ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms." J Biol Chem **269**(22): 15819-15826.

Chen, Y., G. Li and L. Y. Huang (2012). "P2X7 receptors in satellite glial cells mediate high functional expression of P2X3 receptors in immature dorsal root ganglion neurons." Mol Pain **8**: 9.

Chen, Y., G. Li and L. Y. Huang (2015). "p38 MAPK mediates glial P2X7R-neuronal P2Y1R inhibitory control of P2X3R expression in dorsal root ganglion neurons." Mol Pain **11**(1): 68.

Chen, Y., X. Zhang, C. Wang, G. Li, Y. Gu and L. Y. Huang (2008). "Activation of P2X7 receptors in glial satellite cells reduces pain through downregulation of P2X3 receptors in nociceptive neurons." Proc Natl Acad Sci U S A **105**(43): 16773-16778.

Chessell, I. P., J. P. Hatcher, C. Bountra, A. D. Michel, J. P. Hughes, P. Green, J. Egerton, M. Murfin, J. Richardson, W. L. Peck, C. B. Grahames, M. A. Casula, Y. Yiangou, R. Birch, P. Anand and G. N. Buell (2005). "Disruption of the P2X7 purinoceptor gene abolishes chronic inflammatory and neuropathic pain." Pain **114**(3): 386-396.

Chizh, B. A. and P. Illes (2001). "P2X receptors and nociception." Pharmacol Rev **53**(4): 553-568.

Christianson, C. A., M. Corr, G. S. Firestein, A. Mobargha, T. L. Yaksh and C. I. Svensson (2010). "Characterization of the acute and persistent pain state present in K/BxN serum transfer arthritis." Pain **151**(2): 394-403.

Clark, A. K., A. A. Staniland, F. Marchand, T. K. Kaan, S. B. McMahon and M. Malcangio "P2X7-dependent release of interleukin-1beta and nociception in the spinal cord following lipopolysaccharide." J Neurosci **30**(2): 573-582.

Cockayne, D. A., P. M. Dunn, Y. Zhong, W. Rong, S. G. Hamilton, G. E. Knight, H. Z. Ruan, B. Ma, P. Yip, P. Nunn, S. B. McMahon, G. Burnstock and A. P. Ford (2005). "P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effects of ATP." J Physiol **567**(Pt 2): 621-639.

Cockayne, D. A., S. G. Hamilton, Q. M. Zhu, P. M. Dunn, Y. Zhong, S. Novakovic, A. B. Malmberg, G. Cain, A. Berson, L. Kassotakis, L. Hedley, W. G. Lachnit, G. Burnstock, S. B. McMahon and A. P. Ford (2000). "Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice." Nature **407**(6807): 1011-1015.

Costa, F. A. and F. L. Moreira Neto (2015). "[Satellite glial cells in sensory ganglia: its role in pain]." Rev Bras Anestesiol **65**(1): 73-81.

Cruz, C. D., F. L. Neto, J. Castro-Lopes, S. B. McMahon and F. Cruz (2005). "Inhibition of ERK phosphorylation decreases nociceptive behaviour in monoarthritic rats." Pain **116**(3): 411-419.

Dell'Antonio, G., A. Quattrini, E. Dal Cin, A. Fulgenzi and M. E. Ferrero (2002). "Antinociceptive effect of a new P(2Z)/P2X7 antagonist, oxidized ATP, in arthritic rats." Neurosci Lett **327**(2): 87-90.

Donegan, M., M. Kernisant, C. Cua, L. Jasmin and P. T. Ohara (2013). "Satellite glial cell proliferation in the trigeminal ganglia after chronic constriction injury of the infraorbital nerve." Glia **61**(12): 2000-2008.

Dublin, P. and M. Hanani (2007). "Satellite glial cells in sensory ganglia: their possible contribution to inflammatory pain." Brain Behav Immun **21**(5): 592-598.

Dunn, P. M., Y. Zhong and G. Burnstock (2001). "P2X receptors in peripheral neurons." Prog Neurobiol **65**(2): 107-134.

Ebersberger, H.-G. S. a. A. (2009). Pain from the arthritic joint. Synaptic Plasticity in pain. M. Malcangio, Springer Science: 271-288.

Ellis, A. and D. L. Bennett (2013). "Neuroinflammation and the generation of neuropathic pain." Br J Anaesth **111**(1): 26-37.

Elson, K., R. M. Ribeiro, A. S. Perelson, A. Simmons and P. Speck (2004). "The life span of ganglionic glia in murine sensory ganglia estimated by uptake of bromodeoxyuridine." Exp Neurol **186**(1): 99-103.

Elson, K., A. Simmons and P. Speck (2004). "Satellite cell proliferation in murine sensory ganglia in response to scarification of the skin." Glia **45**(1): 105-109.

Elson, K., P. Speck and A. Simmons (2003). "Herpes simplex virus infection of murine sensory ganglia induces proliferation of neuronal satellite cells." J Gen Virol **84**(Pt 5): 1079-1084.

Ferreira-Gomes, J., S. Adaes, R. M. Sousa, M. Mendonca and J. M. Castro-Lopes (2012). "Dose-dependent expression of neuronal injury markers during experimental osteoarthritis induced by monoiodoacetate in the rat." Mol Pain **8**: 50.

Ferreira-Gomes, J., F. L. Neto and J. M. Castro-Lopes (2004). "Differential expression of GABA(B(1b)) receptor mRNA in the thalamus of normal and monoarthritic animals." Biochem Pharmacol **68**(8): 1603-1611.

Ferreira-Gomes, J., F. L. Neto and J. M. Castro-Lopes (2006). "GABA(B2) receptor subunit mRNA decreases in the thalamus of monoarthritic animals." Brain Res Bull **71**(1-3): 252-258.

Fukuoka, T., H. Yamanaka, K. Kobayashi, M. Okubo, K. Miyoshi, Y. Dai and K. Noguchi (2012). "Re-evaluation of the phenotypic changes in L4 dorsal root ganglion neurons after L5 spinal nerve ligation." Pain **153**(1): 68-79.

Galeazza, M. T., M. G. Garry, H. J. Yost, K. A. Strait, K. M. Hargreaves and V. S. Seybold (1995). "Plasticity in the synthesis and storage of substance P and calcitonin gene-related peptide in primary afferent neurons during peripheral inflammation." Neuroscience **66**(2): 443-458.

Gascon, E. and A. Moqrich (2010). "Heterogeneity in primary nociceptive neurons: from molecules to pathology." Arch Pharm Res **33**(10): 1489-1507.

Gerevich, Z. and P. Illes (2004). "P2Y receptors and pain transmission." Purinergic Signal **1**(1): 3-10.

Gilchrist, M., W. R. Henderson, Jr., A. E. Clark, R. M. Simmons, X. Ye, K. D. Smith and A. Aderem (2008). "Activating transcription factor 3 is a negative regulator of allergic pulmonary inflammation." J Exp Med **205**(10): 2349-2357.

Gold, M. and M. Caterina (2008). Molecular Biology of the nociceptor/Transduction. The senses - a comprehensive reference. M. C. B. a. A. I. Basbaum, Elsevier Inc. **volume 5 - Pain:** 43-73.

Gorczyca, R., R. Filip and E. Walczak (2013). "Psychological aspects of pain." Ann Agric Environ Med **Spec no. 1**: 23-27.

Gorska, M., U. Popowska, A. Sielicka-Dudzin, A. Kuban-Jankowska, W. Sawczuk, N. Knap, G. Cicero and F. Wozniak (2012). "Geldanamycin and its derivatives as Hsp90 inhibitors." Front Biosci (Landmark Ed) **17**: 2269-2277.

Greco, R., C. Tassorelli, G. Sandrini, P. Di Bella, S. Buscone and G. Nappi (2008). "Role of calcitonin gene-related peptide and substance P in different models of pain." Cephalalgia **28**(2): 114-126.

Gregory, N. S., A. L. Harris, C. R. Robinson, P. M. Dougherty, P. N. Fuchs and K. A. Sluka (2013). "An overview of animal models of pain: disease models and outcome measures." J Pain **14**(11): 1255-1269.

Grubb, B. D. (2009). Activation in sensory neurons in arthritic joints. Pain in Osteoarthritis. H.-G. S. David T. Felson, Wiley-Blackwell.

Gu, Y., Y. Chen, X. Zhang, G. W. Li, C. Wang and L. Y. Huang (2010). "Neuronal soma-satellite glial cell interactions in sensory ganglia and the participation of purinergic receptors." Neuron Glia Biol **6**(1): 53-62.

Gunjigake, K. K., T. Goto, K. Nakao, S. Kobayashi and K. Yamaguchi (2009). "Activation of satellite glial cells in rat trigeminal ganglion after upper molar extraction." Acta Histochem Cytochem **42**(5): 143-149.

Habermacher, C., K. Dunning, T. Chataigneau and T. Grutter (2015). "Molecular structure and function of P2X receptors." Neuropharmacology **104**: 18-30.

Hackl, C., S. A. Lang, C. Moser, A. Mori, S. Fichtner-Feigl, C. Hellerbrand, W. Dietmeier, H. J. Schlitt, E. K. Geissler and O. Stoeltzing (2010). "Activating transcription factor-3 (ATF3) functions as a tumor suppressor in colon cancer and is up-regulated upon heat-shock protein 90 (Hsp90) inhibition." BMC Cancer **10**: 668.

Hai, T. and M. G. Hartman (2001). "The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis." Gene **273**(1): 1-11.

Hai, T., C. D. Wolfgang, D. K. Marsee, A. E. Allen and U. Sivaprasad (1999). "ATF3 and stress responses." Gene Expr **7**(4-6): 321-335.

Hai, T., C. C. Wolford and Y. S. Chang (2010). "ATF3, a hub of the cellular adaptive-response network, in the pathogenesis of diseases: is modulation of inflammation a unifying component?" Gene Expr **15**(1): 1-11.

Hanani, M. (2005). "Satellite glial cells in sensory ganglia: from form to function." Brain Res Brain Res Rev **48**(3): 457-476.

Hanani, M. (2012). "Intercellular communication in sensory ganglia by purinergic receptors and gap junctions: implications for chronic pain." Brain Res **1487**: 183-191.

Hanani, M. (2015). "Role of satellite glial cells in gastrointestinal pain." Front Cell Neurosci **9**: 412.

Hellmann, J., Y. Tang, M. J. Zhang, T. Hai, A. Bhatnagar, S. Srivastava and M. Spite (2015). "Atf3 negatively regulates Ptgs2/Cox2 expression during acute inflammation." Prostaglandins Other Lipid Mediat **116-117**: 49-56.

Hsieh, Y. L., H. Chiang, J. H. Lue and S. T. Hsieh (2012). "P2X3-mediated peripheral sensitization of neuropathic pain in resiniferatoxin-induced neuropathy." Exp Neurol **235**(1): 316-325.

Huang, L. Y., Y. Gu and Y. Chen (2013). "Communication between neuronal somata and satellite glial cells in sensory ganglia." Glia **61**(10): 1571-1581.

Hunt, D., G. Raivich and P. N. Anderson (2012). "Activating transcription factor 3 and the nervous system." Front Mol Neurosci **5**: 7.

Hutchinson, M. R., K. M. Ramos, L. C. Loram, J. Wieseler, P. W. Sholar, J. J. Kearney, M. T. Lewis, N. Y. Crysdale, Y. Zhang, J. A. Harrison, S. F. Maier, K. C. Rice and L. R. Watkins (2009). "Evidence for a role of heat shock protein-90 in toll like receptor 4 mediated pain enhancement in rats." Neuroscience **164**(4): 1821-1832.

Isacsson, A., M. Kanje and L. B. Dahlin (2005). "Induction of activating transcription factor 3 (ATF3) by peripheral nerve compression." Scand J Plast Reconstr Surg Hand Surg **39**(2): 65-72.

Ivanavicius, S. P., A. D. Ball, C. G. Heapy, F. R. Westwood, F. Murray and S. J. Read (2007). "Structural pathology in a rodent model of osteoarthritis is associated with neuropathic pain: increased expression of ATF-3 and pharmacological characterisation." Pain **128**(3): 272-282.

Jang, M. K. and M. H. Jung (2015). "ATF3 inhibits PPARgamma-stimulated transactivation in adipocyte cells." Biochem Biophys Res Commun **456**(1): 80-85.

Jarvis, M. F., E. C. Burgard, S. McGaraughty, P. Honore, K. Lynch, T. J. Brennan, A. Subieta, T. Van Biesen, J. Cartmell, B. Bianchi, W. Niforatos, K. Kage, H. Yu, J. Mikusa, C. T. Wismer, C. Z. Zhu, K. Chu, C. H. Lee, A. O. Stewart, J. Polakowski, B. F. Cox, E.

Kowaluk, M. Williams, J. Sullivan and C. Faltynek (2002). "A-317491, a novel potent and selective non-nucleotide antagonist of P2X3 and P2X2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat." Proc Natl Acad Sci U S A **99**(26): 17179-17184.

Jasmin, L., J. P. Vit, A. Bhargava and P. T. Ohara (2010). "Can satellite glial cells be therapeutic targets for pain control?" Neuron Glia Biol **6**(1): 63-71.

Ji, R. R., R. W. t. Gereau, M. Malcangio and G. R. Strichartz (2009). "MAP kinase and pain." Brain Res Rev **60**(1): 135-148.

Jimenez-Andrade, J. M., C. M. Peters, N. A. Mejia, J. R. Ghilardi, M. A. Kuskowski and P. W. Mantyh (2006). "Sensory neurons and their supporting cells located in the trigeminal, thoracic and lumbar ganglia differentially express markers of injury following intravenous administration of paclitaxel in the rat." Neurosci Lett **405**(1-2): 62-67.

Kakimura, J., Y. Kitamura, K. Takata, M. Umeki, S. Suzuki, K. Shibagaki, T. Taniguchi, Y. Nomura, P. J. Gebicke-Haerter, M. A. Smith, G. Perry and S. Shimohama (2002). "Microglial activation and amyloid-beta clearance induced by exogenous heat-shock proteins." FASEB J **16**(6): 601-603.

Khakh, B. S. and R. A. North (2006). "P2X receptors as cell-surface ATP sensors in health and disease." Nature **442**(7102): 527-532.

Kidd, B. L., V. H. Morris and L. Urban (1996). "Pathophysiology of joint pain." Ann Rheum Dis **55**(5): 276-283.

Kivitz, A., M. Fairfax, E. A. Sheldon, Q. Xiang, B. A. Jones, A. R. Gammaitoni and E. M. Gould (2008). "Comparison of the effectiveness and tolerability of lidocaine patch 5% versus celecoxib for osteoarthritis-related knee pain: post hoc analysis of a 12 week, prospective, randomized, active-controlled, open-label, parallel-group trial in adults." Clin Ther **30**(12): 2366-2377.

Kleggetveit, I. P., B. Namer, R. Schmidt, T. Helas, M. Ruckel, K. Orstavik, M. Schmelz and E. Jorum (2012). "High spontaneous activity of C-nociceptors in painful polyneuropathy." Pain **153**(10): 2040-2047.

Kobayashi, K., T. Fukuoka, H. Yamanaka, Y. Dai, K. Obata, A. Tokunaga and K. Noguchi (2005). "Differential expression patterns of mRNAs for P2X receptor subunits in neurochemically characterized dorsal root ganglion neurons in the rat." J Comp Neurol **481**(4): 377-390.

Labasi, J. M., N. Petrushova, C. Donovan, S. McCurdy, P. Lira, M. M. Payette, W. Brissette, J. R. Wicks, L. Audoly and C. A. Gabel (2002). "Absence of the P2X7 receptor alters leukocyte function and attenuates an inflammatory response." J Immunol **168**(12): 6436-6445.

Lai, P. F., C. F. Cheng, H. Lin, T. L. Tseng, H. H. Chen and S. H. Chen (2013). "ATF3 Protects against LPS-Induced Inflammation in Mice via Inhibiting HMGB1 Expression." Evid Based Complement Alternat Med **2013**: 716481.

Laveti, D., M. Kumar, R. Hemalatha, R. Sistla, V. G. Naidu, V. Talla, V. Verma, N. Kaur and R. Nagpal (2013). "Anti-inflammatory treatments for chronic diseases: a review." Inflamm Allergy Drug Targets **12**(5): 349-361.

Lee, J. R., M. H. Lee, H. J. Eo, G. H. Park, H. M. Song, M. K. Kim, J. W. Lee and J. B. Jeong (2014). "The contribution of activating transcription factor 3 to apoptosis of human colorectal cancer cells by protocatechualdehyde, a naturally occurring phenolic compound." Arch Biochem Biophys **564**: 203-210.

Li, J., X. Wang, F. Zhang and H. Yin (2013). "Toll-like receptors as therapeutic targets for autoimmune connective tissue diseases." Pharmacol Ther **138**(3): 441-451.

Li, X. Q., V. M. Verge, J. M. Johnston and D. W. Zochodne (2004). "CGRP peptide and regenerating sensory axons." J Neuropathol Exp Neurol **63**(10): 1092-1103.

Liang, L., Z. Wang, N. Lu, J. Yang, Y. Zhang and Z. Zhao (2010). "Involvement of nerve injury and activation of peripheral glial cells in tetanic sciatic stimulation-induced persistent pain in rats." J Neurosci Res **88**(13): 2899-2910.

Lisi, L., S. McGuire, A. Sharp, G. Chiosis, P. Navarra, D. L. Feinstein and C. Dello Russo (2013). "The novel HSP90 inhibitor, PU-H71, suppresses glial cell activation but weakly affects clinical signs of EAE." J Neuroimmunol **255**(1-2): 1-7.

Liu, F. Y., Y. N. Sun, F. T. Wang, Q. Li, L. Su, Z. F. Zhao, X. L. Meng, H. Zhao, X. Wu, Q. Sun, G. G. Xing and Y. Wan (2012). "Activation of satellite glial cells in lumbar dorsal root ganglia contributes to neuropathic pain after spinal nerve ligation." Brain Res **1427**: 65-77.

Liu, S., Q. Shi, Q. Zhu, T. Zou, G. Li, A. Huang, B. Wu, L. Peng, M. Song, Q. Wu, Q. Xie, W. Lin, W. Xie, S. Wen, Z. Zhang, Q. Lv, L. Zou, X. Zhang, M. Ying, G. Li and S. Liang (2015). "P2X(7) receptor of rat dorsal root ganglia is involved in the effect of moxibustion on visceral hyperalgesia." Purinergic Signal **11**(2): 161-169.

Lourenco Neto, F., J. Schadrack, S. Platzer, W. Zieglgansberger, T. R. Tolle and J. M. Castro-Lopes (2000). "Expression of metabotropic glutamate receptors mRNA in the thalamus and brainstem of monoarthritic rats." Brain Res Mol Brain Res **81**(1-2): 140-154.

Lu, D., J. Chen and T. Hai (2007). "The regulation of ATF3 gene expression by mitogen-activated protein kinases." Biochem J **401**(2): 559-567.

Lu, X. and P. M. Richardson (1991). "Inflammation near the nerve cell body enhances axonal regeneration." J Neurosci **11**(4): 972-978.

Lu, Y., S. Ansar, M. L. Michaelis and B. S. Blagg (2009). "Neuroprotective activity and evaluation of Hsp90 inhibitors in an immortalized neuronal cell line." Bioorg Med Chem **17**(4): 1709-1715.

Madrigal-Matute, J., O. Lopez-Franco, L. M. Blanco-Colio, B. Munoz-Garcia, P. Ramos-Mozo, L. Ortega, J. Egido and J. L. Martin-Ventura (2010). "Heat shock protein 90 inhibitors attenuate inflammatory responses in atherosclerosis." Cardiovasc Res **86**(2): 330-337.

Mason, M. R., A. R. Lieberman and P. N. Anderson (2003). "Corticospinal neurons up-regulate a range of growth-associated genes following intracortical, but not spinal, axotomy." Eur J Neurosci **18**(4): 789-802.

McDougall, J. J. (2006). "Arthritis and pain. Neurogenic origin of joint pain." Arthritis Res Ther **8**(6): 220.

McDowell, T. S. and R. Y. Yukhananov (2002). "HSP90 inhibitors alter capsaicin- and ATP-induced currents in rat dorsal root ganglion neurons." Neuroreport **13**(4): 437-441.

Merskey, H. and N. Bogduk (1994). Part III: Pain terms, A current list with definitions and notes on usage. . Classification of chronic pain, second edition. Seattle: IASP Press, Merskey H, Bogduk N, editors**:** 209-214.

Messlinger, K. (1997). "[What is a nociceptor?]." Anaesthesist **46**(2): 142-153.

Milligan, E. D. and L. R. Watkins (2009). "Pathological and protective roles of glia in chronic pain." Nat Rev Neurosci **10**(1): 23-36.

Moalem, G. and D. J. Tracey (2006). "Immune and inflammatory mechanisms in neuropathic pain." Brain Res Rev **51**(2): 240-264.

Nakagomi, S., Y. Suzuki, K. Namikawa, S. Kiryu-Seo and H. Kiyama (2003). "Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation." J Neurosci **23**(12): 5187-5196.

Nakanishi, M., K. Hata, T. Nagayama, T. Sakurai, T. Nishisho, H. Wakabayashi, T. Hiraga, S. Ebisu and T. Yoneda (2010). "Acid activation of Trpv1 leads to an up-regulation of calcitonin gene-related peptide expression in dorsal root ganglion neurons via the CaMK-CREB cascade: a potential mechanism of inflammatory pain." Mol Biol Cell **21**(15): 2568-2577.

Nascimento, D., D. H. Pozza, J. M. Castro-Lopes and F. L. Neto (2011). "Neuronal injury marker ATF-3 is induced in primary afferent neurons of monoarthritic rats." Neurosignals **19**(4): 210-221.

Nascimento, D. S., J. M. Castro-Lopes and F. L. Moreira Neto (2014). "Satellite glial cells surrounding primary afferent neurons are activated and proliferate during monoarthritis in rats: is there a role for ATF3?" PLoS One **9**(9): e108152.

Neckers, L. (2007). "Heat shock protein 90: the cancer chaperone." J Biosci **32**(3): 517-530.

Neckers, L. and K. Neckers (2002). "Heat-shock protein 90 inhibitors as novel cancer chemotherapeutic agents." Expert Opin Emerg Drugs **7**(2): 277-288.

Neeb, L., P. Hellen, C. Boehnke, J. Hoffmann, S. Schuh-Hofer, U. Dirnagl and U. Reuter (2011). "IL-1beta stimulates COX-2 dependent PGE(2) synthesis and CGRP release in rat trigeminal ganglia cells." PLoS One **6**(3): e17360.

Neef, D. W., A. M. Jaeger and D. J. Thiele (2011). "Heat shock transcription factor 1 as a therapeutic target in neurodegenerative diseases." Nat Rev Drug Discov **10**(12): 930-944.

Neto, F. L., A. R. Carvalhosa, J. Ferreira-Gomes, C. Reguenga and J. M. Castro-Lopes (2008). "Delta opioid receptor mRNA expression is changed in the thalamus and brainstem of monoarthritic rats." J Chem Neuroanat **36**(2): 122-127.

Neto, F. L. and J. M. Castro-Lopes (2000). "Antinociceptive effect of a group II metabotropic glutamate receptor antagonist in the thalamus of monoarthritic rats." Neurosci Lett **296**(1): 25-28.

Neto, F. L., J. Schadrack, A. Ableitner, J. M. Castro-Lopes, P. Bartenstein, W. Zieglgansberger and T. R. Tolle (1999). "Supraspinal metabolic activity changes in the rat during adjuvant monoarthritis." Neuroscience **94**(2): 607-621.

Neto, F. L., J. Schadrack, S. Platzer, W. Zieglgansberger, T. R. Tolle and J. M. Castro-Lopes (2001). "Up-regulation of metabotropic glutamate receptor 3 mRNA expression in the cerebral cortex of monoarthritic rats." J Neurosci Res **63**(4): 356-367.

Nobori, K., H. Ito, M. Tamamori-Adachi, S. Adachi, Y. Ono, J. Kawauchi, S. Kitajima, F. Marumo and M. Isobe (2002). "ATF3 inhibits doxorubicin-induced apoptosis in cardiac myocytes: a novel cardioprotective role of ATF3." J Mol Cell Cardiol **34**(10): 1387-1397.

Nollen, E. A. and R. I. Morimoto (2002). "Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins." J Cell Sci **115**(Pt 14): 2809-2816.

North, R. A. (2002). "Molecular physiology of P2X receptors." Physiol Rev **82**(4): 1013-1067.

Obata, K., H. Yamanaka, T. Fukuoka, D. Yi, A. Tokunaga, N. Hashimoto, H. Yoshikawa and K. Noguchi (2003). "Contribution of injured and uninjured dorsal root ganglion

neurons to pain behavior and the changes in gene expression following chronic constriction injury of the sciatic nerve in rats." Pain **101**(1-2): 65-77.

Ohara, P. T., J. P. Vit, A. Bhargava, M. Romero, C. Sundberg, A. C. Charles and L. Jasmin (2009). "Gliopathic pain: when satellite glial cells go bad." Neuroscientist **15**(5): 450-463.

Ohba, N., M. Maeda, S. Nakagomi, M. Muraoka and H. Kiyama (2003). "Biphasic expression of activating transcription factor-3 in neurons after cerebral infarction." Brain Res Mol Brain Res **115**(2): 147-156.

Osterloh, A. and M. Breloer (2008). "Heat shock proteins: linking danger and pathogen recognition." Med Microbiol Immunol **197**(1): 1-8.

Park, H. J., J. A. Stokes, M. Corr and T. L. Yaksh (2014). "Toll-like receptor signaling regulates cisplatin-induced mechanical allodynia in mice." Cancer Chemother Pharmacol **73**(1): 25-34.

Pavel, J., Z. Oroszova, L. Hricova and N. Lukacova (2013). "Effect of subpressor dose of angiotensin II on pain-related behavior in relation with neuronal injury and activation of satellite glial cells in the rat dorsal root ganglia." Cell Mol Neurobiol **33**(5): 681-688.

Peters, C. M., J. M. Jimenez-Andrade, B. M. Jonas, M. A. Sevcik, N. J. Koewler, J. R. Ghilardi, G. Y. Wong and P. W. Mantyh (2007). "Intravenous paclitaxel administration in the rat induces a peripheral sensory neuropathy characterized by macrophage infiltration and injury to sensory neurons and their supporting cells." Exp Neurol **203**(1): 42-54.

Pezet, S., A. Spyropoulos, R. J. Williams and S. B. McMahon (2005). "Activity-dependent phosphorylation of Akt/PKB in adult DRG neurons." Eur J Neurosci **21**(7): 1785-1797.

Potes, C. S., F. L. Neto and J. M. Castro-Lopes (2006). "Administration of baclofen, a gamma-aminobutyric acid type B agonist in the thalamic ventrobasal complex, attenuates allodynia in monoarthritic rats subjected to the ankle-bend test." J Neurosci Res **83**(3): 515-523.

Poulet, B. and F. Beier (2016). "Targeting oxidative stress to reduce osteoarthritis." Arthritis Res Ther **18**(1): 32.

Pozza, D. H., C. S. Potes, P. A. Barroso, L. Azevedo, J. M. Castro-Lopes and F. L. Neto (2010). "Nociceptive behaviour upon modulation of mu-opioid receptors in the ventrobasal complex of the thalamus of rats." Pain **148**(3): 492-502.

Prado, F. C., D. Araldi, A. S. Vieira, M. C. Oliveira-Fusaro, C. H. Tambeli and C. A. Parada (2013). "Neuronal P2X3 receptor activation is essential to the hyperalgesia

induced by prostaglandins and sympathomimetic amines released during inflammation." Neuropharmacology **67**: 252-258.

Priestley, J. V. (2009). Trophic factors and their receptors in pain pathways. Synaptic Plasticity in Pain. M. Malcangio, Springer Science.

Puchalowicz, K., M. Tarnowski, I. Baranowska-Bosiacka, D. Chlubek and V. Dziedziejko (2014). "P2X and P2Y receptors-role in the pathophysiology of the nervous system." Int J Mol Sci **15**(12): 23672-23704.

Qi, J., X. Han, H. T. Liu, T. Chen, J. L. Zhang, P. Yang, S. H. Bo, X. T. Lu and J. Zhang (2014). "17-Dimethylaminoethylamino-17-demethoxygeldanamycin attenuates inflammatory responses in experimental stroke." Biol Pharm Bull **37**(11): 1713-1718.

Quartu, M., V. A. Carozzi, S. G. Dorsey, M. P. Serra, L. Poddighe, C. Picci, M. Boi, T. Melis, M. Del Fiacco, C. Meregalli, A. Chiorazzi, C. L. Renn, G. Cavaletti and P. Marmiroli (2014). "Bortezomib treatment produces nocifensive behavior and changes in the expression of TRPV1, CGRP, and substance P in the rat DRG, spinal cord, and sciatic nerve." Biomed Res Int **2014**: 180428.

Reichling, D. B. and J. D. Levine (1999). "The primary afferent nociceptor as pattern generator." Pain **Suppl 6**: S103-109.

Reid, K. J., J. Harker, M. M. Bala, C. Truyers, E. Kellen, G. E. Bekkering and J. Kleijnen (2011). "Epidemiology of chronic non-cancer pain in Europe: narrative review of prevalence, pain treatments and pain impact." Curr Med Res Opin **27**(2): 449-462.

Rice, J. W., J. M. Veal, R. P. Fadden, A. F. Barabasz, J. M. Partridge, T. E. Barta, L. G. Dubois, K. H. Huang, S. R. Mabbett, M. A. Silinski, P. M. Steed and S. E. Hall (2008). "Small molecule inhibitors of Hsp90 potently affect inflammatory disease pathways and exhibit activity in models of rheumatoid arthritis." Arthritis Rheum **58**(12): 3765-3775.

Rozanski, G. M., Q. Li, H. Kim and E. F. Stanley (2013). "Purinergic transmission and transglial signaling between neuron somata in the dorsal root ganglion." Eur J Neurosci **37**(3): 359-365.

Rozanski, G. M., Q. Li and E. F. Stanley (2013). "Transglial transmission at the dorsal root ganglion sandwich synapse: glial cell to postsynaptic neuron communication." Eur J Neurosci.

Ruan, H. Z., L. A. Birder, W. C. de Groat, C. Tai, J. Roppolo, C. A. Buffington and G. Burnstock (2005). "Localization of P2X and P2Y receptors in dorsal root ganglia of the cat." J Histochem Cytochem **53**(10): 1273-1282.

Ruan, H. Z., E. Moules and G. Burnstock (2004). "Changes in P2X3 purinoceptors in sensory ganglia of the mouse during embryonic and postnatal development." Histochem Cell Biol **122**(6): 539-551.

Ryu, P. D., G. Gerber, K. Murase and M. Randic (1988). "Actions of calcitonin gene-related peptide on rat spinal dorsal horn neurons." Brain Res **441**(1-2): 357-361.

Sandkuhler, J. (2009). "Models and mechanisms of hyperalgesia and allodynia." Physiol Rev **89**(2): 707-758.

Sato, A., K. Nakama, H. Watanabe, A. Satake, A. Yamamoto, T. Omi, A. Hiramoto, M. Masutani, Y. Wataya and H. S. Kim (2014). "Role of activating transcription factor 3 protein ATF3 in necrosis and apoptosis induced by 5-fluoro-2'-deoxyuridine." FEBS J **281**(7): 1892-1900.

Saul, K. E., J. R. Koke and D. M. Garcia (2010). "Activating transcription factor 3 (ATF3) expression in the neural retina and optic nerve of zebrafish during optic nerve regeneration." Comp Biochem Physiol A Mol Integr Physiol **155**(2): 172-182.

Schadrack, J., F. L. Neto, A. Ableitner, J. M. Castro-Lopes, F. Willoch, P. Bartenstein, W. Zieglgansberger and T. R. Tolle (1999). "Metabolic activity changes in the rat spinal cord during adjuvant monoarthritis." Neuroscience **94**(2): 595-605.

Schafers, M., D. H. Lee, D. Brors, T. L. Yaksh and L. S. Sorkin (2003). "Increased sensitivity of injured and adjacent uninjured rat primary sensory neurons to exogenous tumor necrosis factor-alpha after spinal nerve ligation." J Neurosci **23**(7): 3028-3038.

Schaible, H. G. (2007). "[Pathophysiology of pain]." Orthopade **36**(1): 8, 10-12, 14-16.

Schaible, H. G., A. Ebersberger and G. S. Von Banchet (2002). "Mechanisms of pain in arthritis." Ann N Y Acad Sci **966**: 343-354.

Schaible, H. G., F. Richter, A. Ebersberger, M. K. Boettger, H. Vanegas, G. Natura, E. Vazquez and G. Segond von Banchet (2009). "Joint pain." Exp Brain Res **196**(1): 153-162.

Segond von Banchet, G., M. K. Boettger, N. Fischer, M. Gajda, R. Brauer and H. G. Schaible (2009). "Experimental arthritis causes tumor necrosis factor-alpha-dependent infiltration of macrophages into rat dorsal root ganglia which correlates with pain-related behavior." Pain **145**(1-2): 151-159.

Seijffers, R., A. J. Allchorne and C. J. Woolf (2006). "The transcription factor ATF-3 promotes neurite outgrowth." Mol Cell Neurosci **32**(1-2): 143-154.

Seijffers, R., C. D. Mills and C. J. Woolf (2007). "ATF3 increases the intrinsic growth state of DRG neurons to enhance peripheral nerve regeneration." J Neurosci **27**(30): 7911-7920.

Seijffers, R., J. Zhang, J. C. Matthews, A. Chen, E. Tamrazian, O. Babaniyi, M. Selig, M. Hynynen, C. J. Woolf and R. H. Brown, Jr. (2014). "ATF3 expression improves motor function in the ALS mouse model by promoting motor neuron survival and retaining muscle innervation." Proc Natl Acad Sci U S A **111**(4): 1622-1627.

Sevin, M., F. Girodon, C. Garrido and A. de Thonel (2015). "HSP90 and HSP70: Implication in Inflammation Processes and Therapeutic Approaches for Myeloproliferative Neoplasms." Mediators Inflamm **2015**: 970242.

Seybold, V. S. (2009). "The role of peptides in central sensitization." Handb Exp Pharmacol(194): 451-491.

Shan, Y., A. Akram, H. Amatullah, D. Y. Zhou, P. L. Gali, T. Maron-Gutierrez, A. Gonzalez-Lopez, L. Zhou, P. R. Rocco, D. Hwang, G. M. Albaiceta, J. J. Haitsma and C. C. Dos Santos (2015). "ATF3 Protects Pulmonary Resident Cells from Acute and Ventilator-Induced Lung Injury by Preventing Nrf2 Degradation." Antioxid Redox Signal **22**(8): 651-668.

Shi, T. J., P. Huang, J. Mulder, S. Ceccatelli and T. Hokfelt (2009). "Expression of p-Akt in sensory neurons and spinal cord after peripheral nerve injury." Neurosignals **17**(3): 203-212.

Siemionow, K., A. Klimczak, G. Brzezicki, M. Siemionow and R. F. McLain (2009). "The effects of inflammation on glial fibrillary acidic protein expression in satellite cells of the dorsal root ganglion." Spine (Phila Pa 1976) **34**(16): 1631-1637.

Solle, M., J. Labasi, D. G. Perregaux, E. Stam, N. Petrushova, B. H. Koller, R. J. Griffiths and C. A. Gabel (2001). "Altered cytokine production in mice lacking P2X(7) receptors." J Biol Chem **276**(1): 125-132.

Soti, C., E. Nagy, Z. Giricz, L. Vigh, P. Csermely and P. Ferdinandy (2005). "Heat shock proteins as emerging therapeutic targets." Br J Pharmacol **146**(6): 769-780.

Souza, G. R., J. Talbot, C. M. Lotufo, F. Q. Cunha, T. M. Cunha and S. H. Ferreira "Fractalkine mediates inflammatory pain through activation of satellite glial cells." Proc Natl Acad Sci U S A **110**(27): 11193-11198.

Stenberg, L., M. Kanje, K. Dolezal and L. B. Dahlin (2012). "Expression of activating transcription factor 3 (ATF 3) and caspase 3 in Schwann cells and axonal outgrowth after sciatic nerve repair in diabetic BB rats." Neurosci Lett **515**(1): 34-38.

Stephenson, J. L. and M. R. Byers (1995). "GFAP immunoreactivity in trigeminal ganglion satellite cells after tooth injury in rats." Exp Neurol **131**(1): 11-22.

Su, J., T. Gao, T. Shi, Q. Xiang, X. Xu, Z. Wiesenfeld-Hallin, T. Hokfelt and C. I. Svensson (2015). "Phenotypic changes in dorsal root ganglion and spinal cord in the

collagen antibody-induced arthritis mouse model." J Comp Neurol **523**(10): 1505-1528.

Suganami, T., X. Yuan, Y. Shimoda, K. Uchio-Yamada, N. Nakagawa, I. Shirakawa, T. Usami, T. Tsukahara, K. Nakayama, Y. Miyamoto, K. Yasuda, J. Matsuda, Y. Kamei, S. Kitajima and Y. Ogawa (2009). "Activating transcription factor 3 constitutes a negative feedback mechanism that attenuates saturated Fatty acid/toll-like receptor 4 signaling and macrophage activation in obese adipose tissue." Circ Res **105**(1): 25-32.

Takeda, M., M. Takahashi and S. Matsumoto (2008). "Contribution of activated interleukin receptors in trigeminal ganglion neurons to hyperalgesia via satellite glial interleukin-1beta paracrine mechanism." Brain Behav Immun **22**(7): 1016-1023.

Takeda, M., M. Takahashi and S. Matsumoto (2009). "Contribution of the activation of satellite glia in sensory ganglia to pathological pain." Neurosci Biobehav Rev **33**(6): 784-792.

Takeda, M., T. Tanimoto, J. Kadoi, M. Nasu, M. Takahashi, J. Kitagawa and S. Matsumoto (2007). "Enhanced excitability of nociceptive trigeminal ganglion neurons by satellite glial cytokine following peripheral inflammation." Pain **129**(1-2): 155-166.

Takii, R., S. Inouye, M. Fujimoto, T. Nakamura, T. Shinkawa, R. Prakasam, K. Tan, N. Hayashida, H. Ichikawa, T. Hai and A. Nakai (2010). "Heat shock transcription factor 1 inhibits expression of IL-6 through activating transcription factor 3." J Immunol **184**(2): 1041-1048.

Taylor, A. M. and A. Ribeiro-da-Silva (2011). "GDNF levels in the lower lip skin in a rat model of trigeminal neuropathic pain: implications for nonpeptidergic fiber reinnervation and parasympathetic sprouting." Pain **152**(7): 1502-1510.

Taylor, P. C. and M. Feldmann (2009). "Anti-TNF biologic agents: still the therapy of choice for rheumatoid arthritis." Nat Rev Rheumatol **5**(10): 578-582.

Thompson, M. R., D. Xu and B. R. Williams (2009). "ATF3 transcription factor and its emerging roles in immunity and cancer." J Mol Med (Berl) **87**(11): 1053-1060.

Tie-Jun, S. S., Z. Xu and T. Hokfelt (2001). "The expression of calcitonin gene-related peptide in dorsal horn neurons of the mouse lumbar spinal cord." Neuroreport **12**(4): 739-743.

Tracey, I. and M. C. Bushnell (2009). "How neuroimaging studies have challenged us to rethink: is chronic pain a disease?" J Pain **10**(11): 1113-1120.

Treede, R. D. (1999). "Transduction and transmission properties of primary nociceptive afferents." Ross Fiziol Zh Im I M Sechenova **85**(1): 205-211.

Tsan, M. F. and B. Gao (2004). "Cytokine function of heat shock proteins." Am J Physiol Cell Physiol **286**(4): C739-744.

Tsan, M. F. and B. Gao (2009). "Heat shock proteins and immune system." J Leukoc Biol **85**(6): 905-910.

Tsujino, H., E. Kondo, T. Fukuoka, Y. Dai, A. Tokunaga, K. Miki, K. Yonenobu, T. Ochi and K. Noguchi (2000). "Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury." Mol Cell Neurosci **15**(2): 170-182.

Tsuzuki, K., E. Kondo, T. Fukuoka, D. Yi, H. Tsujino, M. Sakagami and K. Noguchi (2001). "Differential regulation of P2X(3) mRNA expression by peripheral nerve injury in intact and injured neurons in the rat sensory ganglia." Pain **91**(3): 351-360.

Vallejo, R., D. M. Tilley, L. Vogel and R. Benyamin (2010). "The role of glia and the immune system in the development and maintenance of neuropathic pain." Pain Pract **10**(3): 167-184.

Vihervaara, A. and L. Sistonen (2014). "HSF1 at a glance." J Cell Sci **127**(Pt 2): 261-266.

Villa, G., M. Fumagalli, C. Verderio, M. P. Abbracchio and S. Ceruti (2010). "Expression and contribution of satellite glial cells purinoceptors to pain transmission in sensory ganglia: an update." Neuron Glia Biol **6**(1): 31-42.

Wang, Z., D. Chen, Z. Zhang, R. Zhang, S. An and L. Yu (2013). "Protease-activated receptor 4 activation increases the expression of calcitonin gene-related peptide mRNA and protein in dorsal root ganglion neurons." J Neurosci Res **91**(12): 1551-1562.

Warwick, R. A. and M. Hanani (2012). "The contribution of satellite glial cells to chemotherapy-induced neuropathic pain." Eur J Pain.

Watkins, L. R. and S. F. Maier (2003). "Glia: a novel drug discovery target for clinical pain." Nat Rev Drug Discov **2**(12): 973-985.

Waza, M., H. Adachi, M. Katsuno, M. Minamiyama, F. Tanaka and G. Sobue (2006). "Alleviating neurodegeneration by an anticancer agent: an Hsp90 inhibitor (17-AAG)." Ann N Y Acad Sci **1086**: 21-34.

Weng, X., T. Smith, J. Sathish and L. Djouhri (2012). "Chronic inflammatory pain is associated with increased excitability and hyperpolarization-activated current (Ih) in C- but not Adelta-nociceptors." Pain **153**(4): 900-914.

Whitmore, M. M., A. Iparraguirre, L. Kubelka, W. Weninger, T. Hai and B. R. Williams (2007). "Negative regulation of TLR-signaling pathways by activating transcription factor-3." J Immunol **179**(6): 3622-3630.

Wirkner, K., B. Sperlagh and P. Illes (2007). "P2X3 receptor involvement in pain states." Mol Neurobiol **36**(2): 165-183.

Wolfgang, C. D., G. Liang, Y. Okamoto, A. E. Allen and T. Hai (2000). "Transcriptional autorepression of the stress-inducible gene ATF3." J Biol Chem **275**(22): 16865-16870.

Wright, D. E., J. M. Ryals, K. E. McCarson and J. A. Christianson (2004). "Diabetes-induced expression of activating transcription factor 3 in mouse primary sensory neurons." J Peripher Nerv Syst **9**(4): 242-254.

Xie, J. J., Y. M. Xie, B. Chen, F. Pan, J. C. Guo, Q. Zhao, J. H. Shen, Z. Y. Wu, J. Y. Wu, L. Y. Xu and E. M. Li (2014). "ATF3 functions as a novel tumor suppressor with prognostic significance in esophageal squamous cell carcinoma." Oncotarget **5**(18): 8569-8582.

Xu, G. Y. and L. Y. Huang (2002). "Peripheral inflammation sensitizes P2X receptor-mediated responses in rat dorsal root ganglion neurons." J Neurosci **22**(1): 93-102.

Xu, Q. and T. L. Yaksh (2011). "A brief comparison of the pathophysiology of inflammatory versus neuropathic pain." Curr Opin Anaesthesiol **24**(4): 400-407.

Yun, T. J., E. K. Harning, K. Giza, D. Rabah, P. Li, J. W. Arndt, D. Luchetti, M. A. Biamonte, J. Shi, K. Lundgren, A. Manning and M. R. Kehry (2011). "EC144, a synthetic inhibitor of heat shock protein 90, blocks innate and adaptive immune responses in models of inflammation and autoimmunity." J Immunol **186**(1): 563-575.

Zhang, X., Y. Chen, C. Wang and L. Y. Huang (2007). "Neuronal somatic ATP release triggers neuron-satellite glial cell communication in dorsal root ganglia." Proc Natl Acad Sci U S A **104**(23): 9864-9869.

Zhou, H., H. Guo, J. Zong, J. Dai, Y. Yuan, Z. Y. Bian and Q. Z. Tang (2014). "ATF3 regulates multiple targets and may play a dual role in cardiac hypertrophy and injury." Int J Cardiol **174**(3): 838-839.

Zou, J., Y. Guo, T. Guettouche, D. F. Smith and R. Voellmy (1998). "Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1." Cell **94**(4): 471-480.