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Mitogen- and stress-activated kinase 1 in primary sensory neurons contributes to formalin-induced tonic pain

Jahanzaib Irfan[a], Rizki M. Febrianto[a], Paolo La Montanara[a,b], Michelle Y Meng[a], Thomas H. Hutson[b], David Zimmermann[c], Krisztina Deak-Pocsai[d], Deemah Aldossary[a,f], Jianglin Wang[a,e], Simone Di Giovanni[b], Michaela Kress[c], Istvan Nagy[a,d,*]

Abstract

Introduction: The nuclear mitogen- and stress-activated kinases (MSKs) play a critical role in the development and persistence of pain after tissue injury.

Objectives: Here, we ascertained the MSK isoform, the cells and mechanisms, which mediate MSKs' pronociceptive function.

Methods: Nocifensive behaviour evoked by subcutaneous formalin injection into the paw was quantified in wild type (WT), MSK1 and MSK2 global knock out (MSK1$^{-/-}$ and MSK2$^{-/-}$) mice, and a month after injecting adeno-associated viral vector carrying short-hairpin (sh) RNA directed towards the MSK1-encoding gene *Rps6ka5* mRNA or scrambled shRNA into the sciatic nerve of WT mice. *Rps6ka5* expression in nociceptors was ascertained by analysing publicly available single cell and single nucleus RNA sequencing datasets on primary sensory neurons and reverse transcription polymerase chain reaction on dorsal root ganglia (DRG). Mitogen- and stress-activated kinase 1 expression was verified by immunofluorescent staining on DRG sections.

Results: MSK1$^{-/-}$ but not MSK2$^{-/-}$ mice exhibited significantly attenuated evoked nocifensive behaviour specifically in the second but not the first phase of the formalin test. Downregulating *Rps6ka5* in nociceptors by the viral vector tool attenuated formalin-induced pain behaviour to the same extent as observed in MSK1$^{-/-}$ animals. *Rps6ka5* expression was found in DRG and various transcriptionally defined groups of nociceptive primary sensory neurons (nociceptors). Immunofluorescence confirmed the presence of MSK1 predominantly in peptidergic nociceptors.

Conclusion: MSK1 constitutes the principal MSK isoform, which is critically important for regulating cellular components that enable the transient activation of a specific subpopulation of nociceptors by formalin.

Keywords: MSK1, Formalin test, Nociceptors, Calcitonin gene–related peptide, Dorsal root ganglion

1. Introduction

Transcriptional processes in the nociceptive system are critical for the acute and persistent activation of nociceptors enabling them to respond to painful stimuli after tissue injury.[13,16,35,42] Mitogen- and stress-activated kinase (MSK) 1 and 2, via histone 3 (H3) phosphorylation-induced alterations in the epigenetic landscape,

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J. Irfan and R. M. Febrianto contributed equally.

M. Kress and I. Nagy contributed equally.

[a] Nociception Group, APMIC, Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital, London, United Kingdom, [b] Department of Brain Sciences, Imperial College London, Hammersmith Hospital, London, United Kingdom, [c] Austria Division of Neuroscience, Department of Physiology and Biomedical Physics, Institute of Physiology, Medical University of Innsbruck, Innsbruck, Austria, [d] Department of Physiology, University of Debrecen, Debrecen, Hungary, [e] Department of Pain Management, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan Province, PR China, [f] Department of Anaesthesia, Prince Sultan Military College of Health Sceince, Dhahran, Saudi Arabia

**Corresponding author. Address: Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital Campus, Du Cane Rd, London W12 0NN, United Kingdom. Tel.: +44-20-7594-9062. E-mail address: i.nagy@imperial.ac.uk (I. Nagy).*

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and transcription factor activation, regulate gene expression both in neurons and nonneuronal cells and MSKs constitute one of the most recently identified putative nuclear checkpoint node molecules, in the somatosensory system, regulating transcriptional alterations after injury.[2,28,29,34,36,42] Mitogen- and stress-activated kinases' role is evidenced by recent studies reporting that strong nociceptive stimuli including subcutaneous formalin injection induce rapid MSK1/2-dependent upregulation of the permissive epigenetic tag, phosphorylated serine 10 in H3 (p-S10H3) in the spinal cord.[34,36] Intrathecal injection of MSK1/2 inhibitors attenuates pain-related behaviour 35 minutes after the formalin injection.[34] At that time point, the activity of the immediate early gene *Fos* becomes detectable in the spinal cord; *Fos* is regulated by S10H3 phosphorylation and its protein product cFos is part of the transcription factor activator protein 1 (AP-1) complex that regulates a series of nociception-related genes including prodynorphin, chemokine (C-X-C motif) ligand 1 (CXCL1), and chemokine (C-C motif) ligand 2 (CCL2).[3,9,18,24,25,32,40,41]

In contrast to the contribution of nociceptive mechanisms in the spinal cord during late phases after formalin injection, pain-like behaviours during the first hour of the formalin test mostly depend on activation of ion channels particularly the transient receptor potential ion channel ankyrin subfamily member 1 (TRPA1), although in a narrow concentration range, in nociceptive primary afferent neurons (nociceptors).[5,11,15,20–22,26] Therefore, we hypothesised that MSK1 and/or MSK2 regulate molecules in nociceptors needed for fast formalin-induced nocifensive behaviour. Accordingly, we explored the impact of global depletion of MSK1 vs MSK2 or downregulation of MSK1 in nociceptors on pain-like behaviours in the formalin test to elucidate the principal MSK isoform and the site within the somatosensory system where the principal isoform sets responses to formalin.

2. Methods

2.1. Animals

All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986; National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Revised Guidelines), the European Communities Council Directive (86/609/EEC) and the guidelines of the Committee for Research and Ethical Issues of International Association for the Study of Pain, published in Pain, 16 (1983): 109 to 110. Good Lab Practice was followed during the entirety of the project in addition to ARRIVE guidelines for reporting the project. All procedures on animals were approved by Animal Welfare and Ethical Review Body, Imperial College London, UK and conducted under a Homme Office Project Licence.

MSK1/2 double knockout mouse sperm was generous gift from Professor Simon Arthur (University of Dundee, United Kingdom) and used for in vitro fertilisation of eggs collected from wild type (WT) C57BL6 mice at Imperial College London Central Biological Services to breed MSK1, MSK2 single knock out mice (MSK1$^{-/-}$, MSK2$^{-/-}$, respectively), and WT littermates. Genotypes were confirmed with polymerase chain reaction. Mice were held in climate-controlled rooms with 12 hours light/dark cycles with food and water ad libitum. Mice were used, in approximately 50%-50% male and female ratio, at the age of 10 to 35 weeks. Animals were randomly allocated to various studies. Although, MSK1$^{-/-}$ and MSK2$^{-/-}$ mice appeared healthy, the number of offsprings was very low. Therefore, about half of young animals were used as breeders, which then were used for experiment at older age. The number of animals older than 13 weeks, however, was less than 15%, and we did not observe any difference in the behaviour of animals younger or older than 13 weeks. When feasible, samples were processed and analysed in a blinded fashion.

2.2. Formalin test

Mice were gently held in hand and injected with 25 μL of 1% paraformaldehyde (2.5%; 833 mM formaldehyde) intraplantarly in the left hind paw to assess formalin-induced nocifensive behaviours. Injected mice were placed in a plexiglass box positioned on a smooth horizontal surface. The ambient temperature was 22°C. Scoring formalin-induced nocifensive behaviours involved counting licking, biting, flinching, and/or lifting the injected paw commenced immediately after the injection in 1-minute epochs for 60 minutes.

2.3. Sciatic nerve injection

After anaesthesia (Isoflurane) and a skin incision, the sciatic nerve of WT mice was exposed at mid-thigh level, and 2.5 μL of each viral vector (AAV-MSK1-shRNA or AAV-sc-shRNA; Vector Biolabs; Malvern, PA, shAAV-271100) was injected into the sciatic nerve with a Hamilton syringe and Hamilton needle (NDL small RN ga34/15 mm/pst45°). After a month, animals were used for formalin test as described above.

2.4. Reverse transcription polymerase chain reaction

Total RNA from dorsal root ganglia (DRGs), the cortex, and the spinal cord was isolated using the RNeasy Mini Kit (Qiagen; Manchester, United Kingdom) according to the manufacturer's instructions. Contaminating DNA was removed with DNaseI (Invitrogen, Paisley, United Kingdom), and RNA was quantified with a NanoDrop UV Visible Spectrophotometer. The quality of the RNA was assessed through agarose gel electrophoresis. cDNA was synthetised from 200 ng of RNA using the SuperScript First Strand reverse transcriptase kit (Invitrogen) as indicated by the manufacturer. Polymerase chain reaction was performed in 20 μL using 50 ng of cDNA and the following cycling parameters: 30 cycles, which consisted 95°C for 2 minutes, 56°C for 1 minute, and 72°C for 1 minute. Supplementary Table 1, http://links.lww.com/PR9/A341 shows primer sequences.

2.5. In silico study

Single-cell and single nuclei datasets were downloaded (GEO: GSE139088; GSE154659; GSE155622; http://mousebrain.org),[27,30,37,39] preprocessed individually using R (4.2) and Seurat (4.3), and integrated via the RNA slot. Cell type annotation was based on labels from Wang et al.[37] and transferred to the integrated data object. Neuronal subpopulation labels were transferred from Renthal et al.[27] dataset. Feature plots and coexpression analysis were performed on the RNA slot. A cell was considered as *Rps6ka5* or *Trpa1* positive with any detected count without thresholding.

2.6. Western blotting

Lumbar (L) 3 to 5 DRGs were extracted from WT, MSK1$^{-/-}$, and the ipsilateral side of AAV-MSK1-shRNA– or AAV-sc-shRNA–injected mice, snap frozen on dry ice, and stored at −80°C until processing. Tissues were homogenized NP40 lysis buffer (Thermo Fisher Scientific, Altrincham, United Kingdom) supplemented protease inhibitor cocktail (Thermo Fisher Scientific). After centrifugation (14000 rpm at 4°C), the protein concentration was determined (Bio-Rad Protein Assay Dye Reagent Concentrate; Bio-Rad, Hamel Hempstead, United Kingdom) before denaturation in LDS sample buffer (Thermo Fisher Scientific) at 98°C and loading samples to onto

NuPAGE 4% to 12% Bis-Tris Gel (Invitrogen). After electrophoresis, proteins were transferred into iBlot 2 Transfer Stacks nitrocellulose membrane (Invitrogen) and unspecific binding blocked with incubation of the membranes in 5% bovine serum albumin (BSA; Sigma, Gillingham, United Kingdom), diluted in *tris-buffered saline with tween 20* (TBST). Membranes were then incubated in an anti-MSK1 (Cell Signalling, London, United Kingdom; 1:1,000) and antiglyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:20,000, Sigma) antibodies followed by HRP-conjugated secondary antibodies. The reaction was visualised with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Gillingham, United Kingdom).

2.7. Immunohistochemistry

After terminal anaesthesia with sodium pentobarbital, WT mice were perfused transcardially with saline followed by 4% (wt/vol) paraformaldehyde in phosphate buffered saline (PBS) and the L4-5 DRG dissected. Dorsal root ganglia tissues were put into 30% (wt/vol) sucrose in PBS until they sank. Tissue was embedded in Tissue-Tek O.C.T. compound (VWR, Lutterworth, United Kingdom), sliced into 20-μM thick sections on a cryostat, and thaw-mounted onto Superfrost Plus microscope slides (Thermo Fisher Scientific) and stored at −20°C until use.

Slides were washed 3 times with 0.3% Triton X-100 in PBS (PBS-T) and blocked in 10% (vol/vol) donkey serum for 30 minutes at room temperature. Slides were incubated with primary antibodies in a humidified chamber overnight at 25°C then washed 3 times with PBS-T. Secondary antibodies were incubated for 2 hours at room temperature and washed once with PBS-T. For slides where a streptavidin-conjugated secondary antibody was used, the Avidin/Biotin blocking kit (Vector, United Kingdom) was used before application of secondary antibodies according to manufacturer's instructions. Slides were mounted with Vectashield Hard Set mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Peterborough, United Kingdom) and imaged on a Leica DMBL fluorescence microscope (Leica, Wetzlar, Germany). Images were captured on a Retiga 2000R digital camera (QImaging, Darmstadt, Germany) and false coloured (MSK1; red and calcitonin gene-related paptide (CGRP), Griffonia simplicifolia Isolectin B4 (IB4; green) using ImageJ.

2.8. Statistics

Data were checked for normal distribution using skewness and kurtosis z-values test. Normality was assumed if z-value was within ±1.96 and *P*-value>0.05 in Shapiro–Wilk data analysis, and it showed that all data were normally distributed. Thus, statistical analyses were done using 1-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Data are presented as mean ± SEM (standard error of the mean). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Depletion of mitogen- and stress-activated kinase 1 but not mitogen- and stress-activated kinase 2 reduces second phase responses in the formalin test

After unilateral formalin injection into one of the hind paws, WT mice exhibited the characteristic biphasic pattern of nocifensive behaviour (**Figs. 1A and B**)[5,11,15,20–22,26]: A high number of

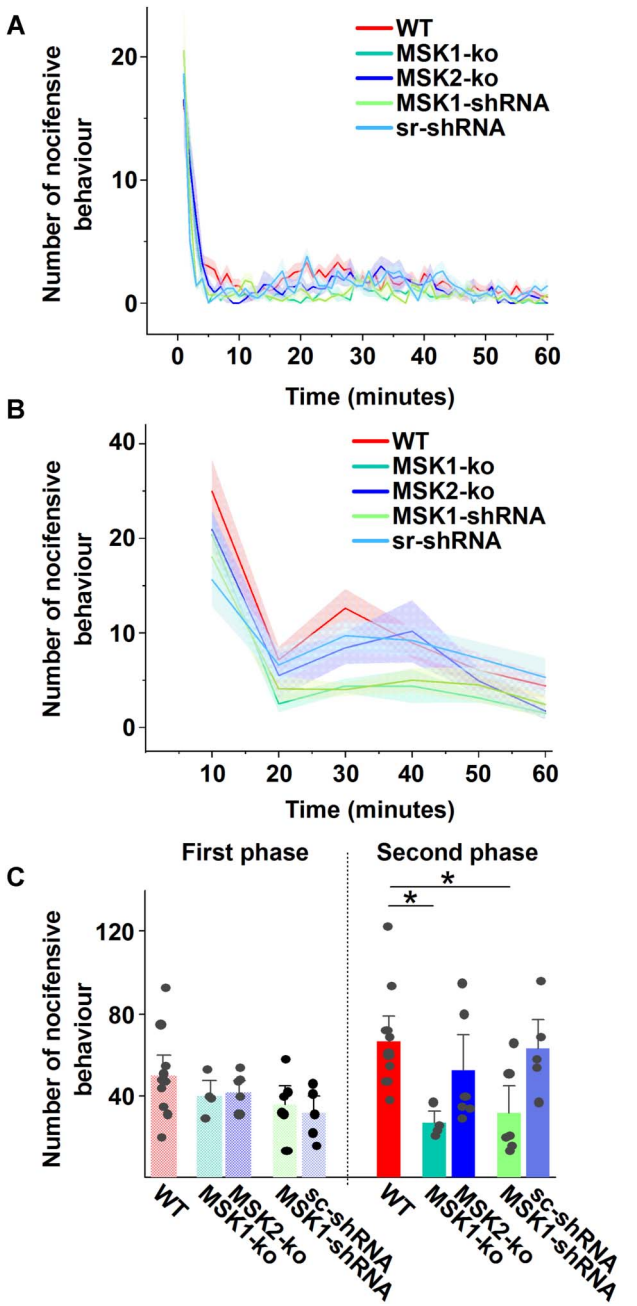

Figure 1. Global depletion, or downregulation in primary sensory neurons, of MSK1 but not MSK2 reduces second phase responses but not first phase responses in the formalin test in mice. (A and B) The number of nocifensive responses counted in 1 minute (A) or 10 minutes (B) bins for 60 minutes after injecting formalin into one of the hind paws of wild type (WT), MSK1$^{-/-}$ (MSK1-ko), MSK2$^{-/-}$ (MSK2-ko) mice or mice injected into the sciatic nerve of shRNA directed towards *Rps6ka5* mRNA (MSK1-shRNA) or sc-shRNA. WT, MSK2$^{-/-}$, and sc-shRNA–injected mice exhibited similar responses both in the first and second phases of the formalin test. However, responses during the second phase in MSK1$^{-/-}$ or MSK1-shRNA–injected mice were inhibited. Solid lines indicate average, and shaded areas indicate SEM. (C) Total number of nocifensive responses in the first phase and second phase responses of WT, MSK1$^{-/-}$ (MSK1-ko), MSK2$^{-/-}$ (MSK2-ko), and MSK1-shRNA– or sc-shRNA–injected mice. No statistical differences were found in first phase responses, whereas second phase responses were significantly inhibited in MSK1-ko ($P < 0.05$) and MSK1-shRNA–injected ($P < 0.05$) mice (1-way ANOVA, Bonferroni post hoc test; n = 4–10). MSK, mitogen- and stress-activated kinase.

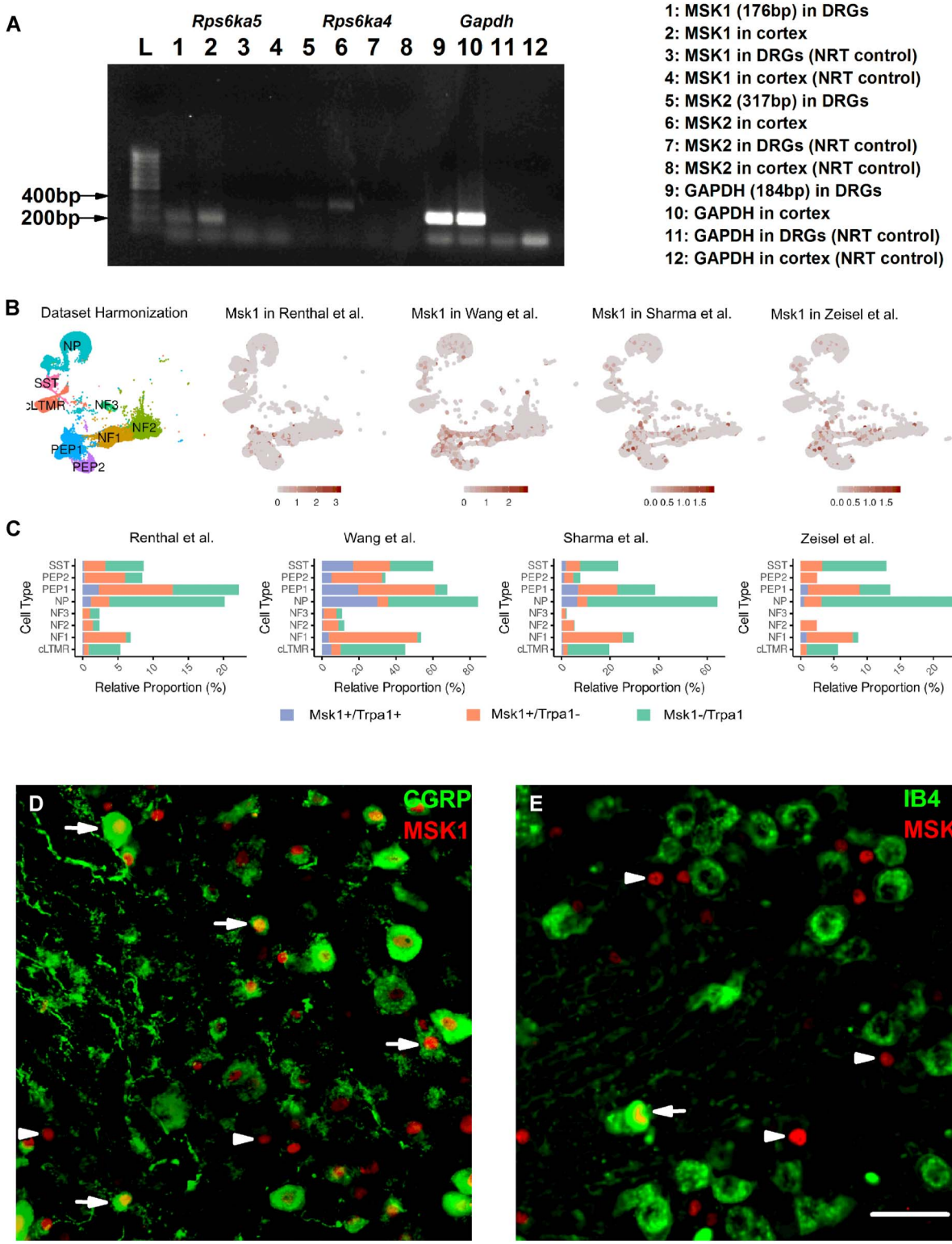

Figure 2. MSK1 is expressed in a group of nociceptors. (A) RT-PCR gel image showing *Rsp6ka6* (lanes 1–4), *Rsp6ka4* (lanes 5–8), and *Gapdh* (lanes 9–12) mRNA expression in DRG (lanes 1, 5, 9) and cortex (lanes 2, 5, 10) and identifies more abundant *Rps6ka5* than *Rps6ka4* mRNA expression in DRG. Lanes 3, 4, 7, 8, 11, 12 are no RT controls. (B) Harmonized single-cell RNA sequencing datasets showing the population of MSK1 expressing cells. (C) Relative proportions of *Msk1* and *Trpa1* coexpressing cells identified in various neuronal subtypes. (D and E) Combined immune staining of mouse DRG sections using anti-MSK1 and anti-CGRP antibodies (D), or anti-MSK1 antibody and biotin-conjugated IB4 (F). Although a major proportion of CGRP-expressing neurons expressed MSK1 in the nucleus, very few IB4-binding cells with MSK1 expression in the nucleus were found. Scale bar indicates 50 μm. DRG, dorsal root ganglia; MSK, mitogen- and stress-activated kinase; RT-PCR, reverse transcription polymerase chain reaction.

nocifensive responses was observed in the first few minutes, which gradually declined by the 10th minute ("first phase"; **Figs. 1A and B**). After a ~10-minute quiescent period, the nocifensive behaviour returned, however, with a significantly lower frequency ("second phase"; **Figs. 1A and B**).

The formalin evoked behaviour in both MSK1$^{-/-}$ and MSK2$^{-/-}$ mice was indistinguishable from that seen in WT mice in the first phase (**Figs. 1A–C**). In contrast, second phase response in MSK1$^{-/-}$ mice was significantly reduced compared to the other 2 genotypes. This finding indicated that MSK1 but not MSK2 was important for the second phase of responses in the formalin test (**Figs. 1A–C**).

3.2. Peripheral downregulation of mitogen- and stress-activated kinase 1 attenuated second phase responses in the formalin test

To find whether MSK1's pain-promoting role in the formalin test was, as we hypothesised, mediated by peripheral effects, we injected adeno-associated virus vector serotype 5 carrying short-hairpin (sh) RNA directed towards the MSK1-encoding gene *Rps6ka5* mRNA (AAV5-MSK1-shRNA) or scrambled shRNA (AAV5-sc-shRNA as negative control) into the sciatic nerve of WT mice 4 weeks before the formalin test. First phase responses in noninjected, AAV5-sc-shRNA–injected and AAV5-MSK1-shRNA–injected mice were undistinguishable (**Fig. 1**). In contrast, second phase responses of AAV5-MSK1-shRNA–injected but not control mice were significantly reduced (**Fig. 1**). The extent of this reduction after AAV5-MSK1-shRNA injection was similar to that seen in MSK1$^{-/-}$ mice indicating that MSK1-mediated mechanism in nociceptors contributed to the development of pain-like behaviour after formalin injection. Western blotting of L3-5 DRG of collected from the injected side confirmed MSK1 downregulation (Supplementary Figure 1, http://links.lww.com/PR9/A341).

3.3. Mitogen- and stress-activated kinase 1 expressed in nociceptors

Since *Rps6ka5* expression was detectable in WT mouse DRG harbouring the cell bodies of primary somatosensory afferents including all nociceptors using reverse transcription polymerase chain reaction (**Fig. 2A**), we reanalysed publicly available single cell and single nuclei RNA sequencing databases of DRG (GEO: GSE139088; GSE154659; GSE155622; http://mousebrain.org).[27,30,37,39] These analyses revealed expression of *Rps6ka5* in various types of nociceptors, predominantly in nonpeptidergic and PEP1 peptidergic neurons conserved throughout all 4 datasets (**Figs. 2B and C**, Supplementary Table 1, http://links.lww.com/PR9/A341). Surprisingly, only a minority of the *Rps6ka5* expressing cells expressed *Trpa1* mRNA (**Fig. 2C**).

Immunostaining further confirmed MSK1 expression in approximately 25% of primary sensory neurons in the DRG. Most of the MSK1-expressing neurons had small diameters and expressed calcitonin gene–related peptide, a marker of peptidergic sensory neurons with nociceptive function (**Figs. 2D and E**).[23]

4. Discussion

We found that global depletion or downregulation of MSK1 but not MSK2 attenuated nocifensive behaviour in the second but not in the first phase of the formalin test. *Rps6ka5* mRNA and MSK1 protein were expressed in nociceptors and together these data support a critical role of MSK1 in regulating nociceptor activity during the second phase of the formalin test.

Formalin induces a characteristic pain-related behaviour, and this behaviour, in the first 60 minutes after the injection, depends on the excitation of primary sensory neurons.[5,11,15,26] Multiple mechanisms are currently discussed: these include direct activation of ion channels in a group of nociceptors, induction of temporal hyperpolarisation, and the development of "spontaneous" discharge activity in nociceptors starting 20 minutes after the injection and indicative of the second phase of the response.[11,20–22] The exact cellular and molecular mechanism generating the spontaneous nociceptor discharge activity is unclear at present; however, activation of TRPA1 by formalin significantly contributes to it, even at gradually decreasing concentrations in the injected tissue.[11,15] Although, MSK1 impacts on transcriptional processes and regulates the expression of a series of genes,[2,28,29] this mode of action unlikely applies to a fast modulation of TRPA1 expression by the second phase of the formalin response since *Rsp6ka5* coexpression with *Trpa1* mRNA is restricted to a small population of neurons. Second, first phase responses also depend largely on TRPA1 activity at the concentrations used in the present study but were neither altered by downregulating the MSK1 expression in primary sensory neurons nor by global depletion of MSK1. This is puzzling and may rather suggest more complex regulatory processes of MSK1 targeting other functional components involved in nociceptor activation by formalin.

Several other ion channels impact on the second phase of the formalin response such as other members of the transient receptor potential channel superfamily, acid sensing ion channels, nicotinic acetyl choline receptors, calcium- or ATP-activated K^+-channels, or even pacemaker channels of the hyperpolarisation-activated cyclic nucleotide-gated (HCN) ion channel family.[8,12,17,19,31,33] The latter are particularly interesting since they can control the membrane potential and initiate discharge activity in nociceptive neurons to induce inflammatory and neuropathic pain, and the HCN blocker ivabradine inhibits the second phase of the formalin response.[1,7,10,38]

Mitogen- and stress-activated kinase 1 not only regulates the transcription of ion channels, but it appears to control cell surface expression of ion channel subunits via the induction of the calcium regulated Arc/Arg3.1 immediate early gene.[4,6] Similar processes may be relevant during the second phase of the formalin response shifting the balance of ionic currents in nociceptors toward depolarization and leading to hyperexcitability through a mechanism resembling inflammation where differentially controlled transport of depolarizing voltage-gated sodium channels vs hyperpolarizing potassium channels drive nociceptor discharge activity.[14]

In summary, our findings support MSK1 as relevant MSK isoform, which regulates the ability of nociceptors to generate discharge activity during the second phase of the formalin test. However, further studies are needed to dissect its expression, activation pattern, and up- and down-stream signalling pathways in nociceptors and particularly the wider role of this nuclear kinase in pathological pain conditions.

Disclosures

The authors have no conflict of interest to declare.

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