NEUROSCIENCE
RESEARCH ARTICLE
D. Maftei et al. / Neuroscience 427 (2020) 16–28

Abnormal Pain Sensation in Mice Lacking the Prokineticin Receptor PKR2: Interaction of PKR2 with Transient Receptor Potential TRPV1 and TRPA1

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Abstract—The amphibian Bv8 and the mammalian prokineticin 1 (PROK1) and 2 (PROK2) are new chemokine-like protein ligands acting on two G protein-coupled receptors, prokineticin receptor 1 (PKR1) and 2 (PKR2), participating to the mediation of diverse physiological and pathological processes. Prokineticins (PKs), specifically activating the prokineticin receptors (PKRs) located in several areas of the central and peripheral nervous system associated with pain, play a fundamental role in nociception. In this paper, to improve the understanding of the prokineticin system in the neurobiology of pain, we investigated the role of PKR2 in pain perception using pkr2 gene-deficient mice. We observed that, compared to wildtype, pkr2-null mice were more resistant to nociceptive sensitization to temperatures ranging from 46 to 48 °C, to capsaicin and to protons, highlighting a positive interaction between PKR2 and the non-selective cation channels TRPV1. Moreover, PKR2 knock-out mice showed reduced nociceptive response to cold temperature (4 °C) and to mustard oil-induced inflammatory hyperalgesia, suggesting a functional interaction between PKR2 and transient receptor potential ankyrin 1 ion (TRPA1) channels. This notion was supported by experiments in dorsal root ganglia (DRG) cultures from pkr1 and–pkr2-null mice, demonstrating that the percentage of Bv8-responsive DRG neurons which were also responsive to mustard oil was much higher in PKR1−/− than in PKR2−/− mice. Taken together, these findings suggest a functional interaction between PKR2 and TRP channels in the development of hyperalgesia. Drugs able to directly or indirectly block these targets and/or their interactions may represent potential analgesics.

Key words: pain, nociception, prokineticin system, prokineticin receptor 2, inflammation.

INTRODUCTION

Prokineticins (PKs) are secreted regulatory proteins of 80–90 amino acids that display structural and functional characteristics similar to those of chemokines. The mammalian prokineticin 1 (PROK1 or endocrine gland-derived vascular endothelial growth factor, EG-VEGF) and prokineticin 2 (PROK2 or mammalian Bv8), the amphibian Bv8 and other homologue proteins of snake (Mamba Intestinal Toxin-1, MIT-1), lizard and fishes belong to the prokineticin family. PROK1 and PROK2 are both widely distributed in several organs of the mammalian body. While PROK1 is predominantly expressed in non-nervous tissues, especially in steroidogenic endocrine organs, cardiac and intestinal tissues, PROK2 is mainly expressed in both the central (CNS) and peripheral nervous system (PNS), as well as in the skin, in macrophages, granulocytes or dendritic cells (Negri and Ferrara, 2018).

PKs bind to two G-protein coupled receptors (GPCR), termed prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2), which are co-expressed in the CNS and PNS, as well as in granulocytes, macrophages and

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Abbreviations: CGRP, calcitonin gene related peptide; DRG, dorsal root ganglia; EG-VEGF, endocrine gland-derived vascular endothelial growth factor; PKRs, prokineticin receptors; PNS, peripheral nervous system; TRPA1, transient receptor potential ankyrin 1 ion channel; WT, wild type; ANOVA, analysis of variance; CFA, Complete Freund's Adjuvant; CNS, central nervous system; GPCR, G-protein coupled receptors; MIT-1, Mamba Intestinal Toxin-1; MO, mustard oil; PKs, prokineticins; PROK1, prokineticin 1; PROK2, prokineticin 2; PKR1, prokineticin receptor 1; PKR2, prokineticin receptor 2; PWT, paw withdrawal threshold; TRPV1, transient receptor potential vanilloid 1 channel.

https://doi.org/10.1016/j.neuroscience.2019.12.003
0306-4522/

endothelial cells (Koyama et al., 2006; Negri et al., 2007, 2009). PKR1 is mainly expressed in peripheral tissues (Soga et al., 2002; Battersby et al., 2004), while PKR2 is more abundantly expressed in several brain regions (Cheng et al., 2002; Lin et al., 2002), especially in those associated with pain (Maftei et al., 2014; Franchi et al., 2017). The activation of PKRs mediates multiple intracellular signalling pathways promoting the mobilization of intracellular calcium, MAPK, STAT3 and Akt phosphorylation or cAMP accumulation (Lattanzi et al., 2018; Negri and Ferrara, 2018).

The wide tissue distribution of PKs and of their receptors confirms their involvement in several biological and pathological functions, in particular in nociception: Bv8 causes significant nociceptive sensitization to thermal and tactile stimuli at doses in the femtomolar range (Negri et al., 2002). When injected by systemic route, Bv8 induces hyperalgesia and allodynia with a biphasic time course. The first phase is due to the activity of PKRs expressed in nociceptors and to the peripheral release of neuropeptides involved in pain perception, such as calcitonin gene related peptide (CGRP) and substance P (Mollay et al., 1999; De Felice et al., 2012). Conversely, the second phase has a central origin (Negri et al. 2006; De Felice et al., 2012, Negri and Maftei, 2018). Of note, PKR1 is mainly expressed in small nociceptors also expressing the transient receptor potential vanilloid receptor channel TRPV1, while PKR2 is mainly expressed in medium/large-sized sensory neurons, co-localized with the transient receptor potential ankyrin 1 ion channel (TRPA1) (Negri et al., 2006; Vellani et al., 2006).

The involvement of the PROK2/PKRs system in the regulation of nociceptive thresholds to acute and chronic noxious stimuli was demonstrated by studies performed in prok2- or pkr1-null mutant mice (Hu et al., 2006; Negri et al., 2006). As demonstrated by Hu et al. (2006), the lack of the prok2 gene induces a strong reduction in nociception when the mutant animals are exposed to thermal and chemical stimuli, in comparison to wild type (WT) mice. Among the noxious stimuli used, the impaired nociception exerted by capsaicin treatment was in agreement with the results that demonstrate a potentiation of capsaicin-evoked pain behaviour by peripheral injection of PROK2 and Bv8 (Vellani et al., 2006). In addition, the reduced thermal nociception induced by high noxious temperatures (46–48 °C) in prok2-null mutant mice suggests that PROK2 likely acts through a pathway involving TRPV1, which is activated in that range of temperatures (Le Bars et al., 2001; Tominaga and Tominaga, 2005). However, no difference in local inflammatory response to capsaicin was observed in prok2 null mutant mice, which suggests that the function of the chemonociceptive terminals mediating the acute phase of capsaicin-induced neurogenic inflammation is unaffected. On the other hand, a reduced inflammatory pain response to formalin treatment was observed in prok2-null mutant mice, in agreement with the literature data reporting central sensitization (likely in the spinal cord) in the formalin model of persistent inflammatory pain (Abbadie et al., 1997; Pitcher and Henry, 2002).

Studies from pkr1-null mutant mice explored the role of PKR1 receptors in nociceptive signalling and in nociceptor sensitization, demonstrating that, when compared with WT littermates, the lack of pkr1 gene was able to significantly impair nociception following exposure to noxious heat, mechanical stimuli, capsaicin, and protons (Negri et al., 2006). In the same work it was demonstrated that interaction between PKR1 and TRPV1, both co-expressed in a significant proportion of small-diameter neurons in dorsal root ganglia (DRG), represents a new example of GPCR and TRPV1 interaction mediating hyperalgesia and allodynia (Tominaga and Tominaga, 2005). Indeed, following both acute and chronic inflammatory stimuli, pkr1-null mice exhibited reduced development of hyperalgesia, with a decrease in PROK2 upregulation due to the inflammatory process, as previously demonstrated (Giannini et al, 2009), confirming the involvement of the PKR1 receptor in initiating and maintaining inflammatory pain.

In view of the role of the prokineticin system in pain and hyperalgesia, the present study aims to further explore the role of PKR2 receptors in pain perception following noxious chemical, thermal and inflammatory stimulation using pkr2-deficient mice.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6J WT, PKR1- and PKR2−/− male mice, generated by Lexicon Genetics Incorporated (The Woodlands, TX, USA) as previously described (Negri et al., 2006) were used to perform the experiments. Since PKR2−/− mice are not fertile, it was necessary to cross heterozygous males and females and analyse the genotype of littermate pups at weaning, that is, 21 days after birth (authorization number for mice colony breeding: 116/215-PR). After genotyping, animals were housed five per cage and maintained under controlled conditions of light, temperature and humidity (12:12 h light/dark cycles, 21 ± 2 °C, 50–60% humidity) with *ad libitum* access to food and water.

All experiments were performed on 25–30 g mice (12–14 weeks old) following protocols approved by the Animal Care and Use Committee of the Italian Ministry of Health (authorization number: 116/2015-PR) according to European Community directives. A total number of 60 WT, 30 PKR1−/− and 70 PKR2−/− mice was used. For each experiment different groups of mice were used: 5 mice for each genotype for RNA extraction and genotyping analysis; five mice for each genotype for histological and immunofluorescence assays; five mice for each genotype for intracellular calcium imaging and immunocytochemistry experiments. The basal sensitivity to different noxious stimuli was performed on 20 mice for each genotype. For all the behavioural experiments, involving pharmacological treatments, groups of five mice for each genotype and dose were used.

Mice genotyping

Genotyping of mouse tails was performed using PCR, which allowed the amplification of the wild type pkr2 gene (primers: 5′-AGCTGCGCAACCTTACCAA-3′, 5′-GGGCACGGGCTACCTGT-3′) and of the neomycin-resistant gene cassette, inserted to disrupt the PKR2 coding region (primers: 5′-GCAGCGCATCGCCTTCTATC-3′, 5′-GGGCACGGGCTACCTGT-3′). Genomic DNA was extracted from mouse tail samples by overnight digestion with proteinase K (Sigma, Milan, Italy), precipitated with ethanol, dried and dissolved in sterile water. PCR amplification was obtained using 200 ng of DNA in 25 μl of total reaction volume, using HotStarTaq DNA Polymerase (Qiagen, Milan, Italy) in the presence of the specific primers listed above. The amplification temperature profile used was: 1 cycle at 95 °C for 3 min (denaturation); followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min (annealing), 72 °C for 1 min; and 1 cycle 72 °C for 10 min (extension). Amplified products were resolved on 2% agarose gel, stained with ethidium bromide, and visualized with Versa Doc 3000 imaging system (Bio-Rad, Milan, Italy).

RNA extraction and real-time PCR

Total mRNA from DRG of wild type and pkr2-null mice (n = 5 animals per genotype) was extracted using RNeasy columns (Qiagen) following the manufacturer's protocol. The total amount of mRNA was spectrophotometrically quantified, and 1 μg of mRNA was reverse transcribed to obtain cDNA, using the reverse transcriptase from (Promega, Milan, Italy). The cDNA aliquots were then amplified in real-time PCR (iCycler; Bio-Rad) using the iQ SYBR Green Supermix (Bio-Rad) in the presence of the specific primers (0.3 μM of each primer) for the target genes: PKR1, PKR2, TPRV1 and TRPA1, in a total reaction volume of 25 μl. The real time PCR was carried out using the following thermal protocol: 1 cycle at 95 °C for 15 min; followed by 40 cycles at 95 °C for 30 s, 52 °C for 30 s, and at 72 °C for 30 s. For each PCR product a melting curve analysis from 55 to 95 °C was performed. The specific sense and antisense mouse primers (Biogen, Rome, Italy) used for PCR are reported below:

PKR1, 5′-ATTCTCGGACTTTCTTTGC-3′ and 5′-GCGGCTGTTTGACACTTC-3′
PKR2, 5′-ACTACCTTCGTACGGTCTCC-3′ and 5′-TAACGAGGATGGTTTCTGTG- 3′
TRPV1, 5′-TGTACTTCAGCCATCGCAAG-3′ and 5′-ACCGACACAGGTCTCTGAGG-3′
TRPA1, 5′-CTACTGGCTTTTGGCCTCAG-3′ and 5′-CCAAAGGTCAGGACTGGGTA-3′

To quantify the results, using the same primers as described above, cDNA standards for each individual gene were generated from total RNA extracted from mouse DRG. Once produced, the cDNA fragments were purified using silica columns (QIAquick PCR purification; Qiagen), cloned into a pGEM-T easy vector (Promega) and ligated fragments were transformed using JM109-competent cells (Promega). The cloned plasmid cDNA was further purified with silica cartridges (Nucleobond AX plasmid purification; Macherey-Nagel, Duren, Germany) and the cloned amplicons sequences were analysed using cycle sequencing (PRIMM, Milan, Italy). The concentration of plasmid cDNA was determined by spectrophotometer (OD_{260nm}) and the corresponding copy number was calculated with the following equation: 1 μg of 1000 bpDNA = 9.1×10^{11} molecules. Plasmid cDNA was serially diluted, from 10^2 to 10^7 input copies, to generate a standard curve, which was amplified in each real time PCR run. The expression level of the gene of interest was expressed as input copy number per nanogram of input total RNA.

Immunofluorescence

Immunofluorescence was performed as previously described (Maftei et al., 2014). Briefly, the L4-L5 DRG were dissected from WT, PKR1−/− and PKR2−/− mice (n = 5 animals per genotype), previously transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). The collected tissue was further post-fixed in 4% PFA for 24 h, cryoprotected in 30% sucrose solution and cut in 20 μm thick slices. The DRG slices were blocked for 30 min at room temperature using 3% normal donkey serum dissolved in PBS/0.3% triton X-100 and then incubated overnight at 4 °C with anti-PKR2 antibody (rabbit, 1:200, Alomone labs, Jerusalem, Israel) diluted in PBS/0.3% triton X-100. Section were rinsed in PBS and then incubated with the proper secondary antibody (anti-rabbit IgG coupled to Alexa Fluor®-488, Immunological Sciences) for 2 h at room temperature. The staining of nuclei was performed with DAPI (1:500, Sigma Aldrich). Images were visualized and acquired with a laser-scanning confocal microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany) connected to a digital camera diagnostic instruments operated by I.A.S. software of Delta Systems Italia (Milan, Italy).

Immunohistochemistry

The assay was performed on L4-L5 DRG dissected from WT and PKR2−/− mice (n = 5 animals per genotype) as previously described (Artico et al., 2010). Briefly, DRG were fixed in 10% formaldehyde solution, embedded in paraffin and serially cut (7 μm thickness). Sections (n = 12–17 from each DRG) were deparaffinized, rehydrated and blocked for endogenous peroxidases (incubation in H_2O_2 0.3% diluted in methanol) for 30 min. After antigen retrieval (heated in 10 mM citrate buffer pH 6.0 for 10 min) sections were incubated overnight at 4 °C, with the primary antibody mouse anti-NeuN (1:200, Sigma, Milan, Italy) rinsed and then incubated for 1 h at room temperature with the appropriate secondary antibody (1:100) conjugated to horseradish peroxidase (HRP, Cell Signalling). After a further wash with PBS, slides were treated with 3,3-diaminobenzidine (0.1 mg/ml, Dako) for 10 min. Finally, sections were counterstained with 0.1% o-toluidine blue solution at pH 4.5.

Measurement of acute nociceptive thresholds

Nociceptive behaviour differences to noxious stimuli such as heat, punctate filaments, acetic acid, capsaicin and formalin were measured in WT and PKR2−/− mice. A different group of mice from each genotype was used for each nociceptive test.

– *Thermal nociception* was evaluated by hot plate, tail and paw immersion tests (Negri et al., 2006).

Hot plate test was performed by placing the animal on a hot plate (Ugo Basile, Italy) with temperature ranging from 46 to 52 °C. The time to the first sign of nociception such as paw licking, flinching, jumping and escaping from the hot plate was recorded and the animal was immediately removed.

Tail and paw immersion tests were carried out by briefly immersing the tail or one hindpaw of the mouse into a hot (46, 48 and 52 °C) or cold (4 °C) water bath. The time, in seconds, for tail/paw withdrawal from the water was recorded. To reduce variability in nociceptive threshold measurements, three days before the experiments mice were trained in tail-or paw withdrawal tests at 30 min intervals for 3 h daily. Mice showing non-homogeneous baseline values were discarded.

– *Mechanical nociception:* tactile allodynia was performed using von Frey filaments with logarithmically incrementing stiffness (0.04–2.0 g, 2 Biological Instruments, Besozzo, Varese, Italy). Mice were placed on a mesh grid and after a 30 minute period of habituation the filaments were perpendicularly applied to the plantar surface of mouse paw for 7–8 s. The 50% paw withdrawal threshold (PWT) was determined using Dixon's up-down paradigm (Chaplan et al., 1994).

– *Chemical nociception*: acetic acid-induced writhing test was used to asses visceral pain. Briefly, mice were intraperitoneally injected with 0.8% acetic acid solution (5 ml/kg body weight) and the number of abdominal constrictions were counted for 20 min. Capsaicin test: capsaicin (1.5 μg in 20 μl) was injected into the plantar surface of the mouse hindpaw and the time (seconds, s) spent licking, flinching or biting the paw was measured for 15 min after injection. Formalin test: 5% of formalin solution (20 μl) was injected into dorsal surface of mouse hindpaw and the time (s) spent licking, flinching or biting the paw was measured for each 5 min interval during the early phase (0–10 min) and the late phase (10–60 min).

Measurement of nociceptive sensitization

The nociceptive sensitization produced by the pro-algesic agents such as Bv8 and capsaicin or that produced by acute and chronic paw inflammation were measured in WT, pkr1- and pkr2-null mutant mice, as changes in nociceptive threshold to noxious or non-noxious sensory stimuli. Thermal hyperalgesia was assessed using hot-plate and paw immersion test (48 °C); while tactile allodynia was assessed using von Frey filaments.

Bv8-induced hyperalgesia: was elicited by subcutaneously (s.c., 5 ml/kg b.w.), in doses ranging from 1 to 30 μg/kg, and intraplantar (i.pl., 20 μl), in doses ranging from 0.5 to 5 ng/paw, injection of Bv8. Capsaicin-induced thermal hyperalgesia: was elicited by intraplantar injection of capsaicin (12 μg).

Paw inflammation was elicited by plantar painting with mustard oil (MO) (10% in mineral oil; Sigma-Aldrich, Milan, Italy) or intraplantar injection of Complete Freund's Adjuvant (20 μl CFA, Sigma-Aldrich, Milan, Italy).

Changes of nociceptive thermal and mechanical thresholds after the pro-algesic and pro-inflammatory agents injection were calculated and expressed as percentage change in nociceptive threshold from baseline threshold $\%\Delta NT = 100 \times (NTts - NTb)/NTb$ where NTb is the baseline nociceptive threshold and NTts is the nociceptive threshold in the presence of the pro-algesic/ pro-inflammatory agent.

Body temperature

Mice body temperature was assessed using implantable thermal probes (G2 E-Mitter probe; ER-4000 Energizer receiver; Vital View software; Mini Mitter, Sunriver, OR). To introduce the probe into the peritoneal cavity, mice were, firstly, anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Subsequently, an incision at the linea alba was made, probe was inserted, sutured to the inner musculature and finally the incision was closed. This procedure, showing a very low risk of internal displacement of the sensor, permitted us a continuous and accurate monitoring of mice body temperature. Prior experiment the core body temperature in wild type and pkr2-null mutant mice was continuously monitoring for 1 week. On the day of the experiment capsaicin (1 mg/kg) was s.c. administrated in both WT and PKR2−/− mice and the body temperature was recorded for 3 h.

Intracellular calcium imaging and immunocytochemistry in DRG cultures

Sensory neurons were isolated from mice and cultured as previously described (Vellani et al., 2004, 2006). Animals were killed by cervical dislocation and decapitation under CO_2 anaesthesia. DRGs were incubated for 60 min at 37 °C with 0.125% type IV collagenase (Worthington, Freehold, NJ), mechanically dissociated and plated onto coverslips pre-treated with 10 μg/ml poly-L-lysine (Sigma, St. Louis, MO) and 20 μg/ml laminin (Sigma). The medium was DMEM with 1% penicillin/streptomycin, 10% fetal bovine serum, 1% l-glutamine (Invitrogen, San Diego, CA), 1.5 μg/ml cytosine 1-β-D-arabinofuranoside (Sigma), and 100 ng/ml NGF (2.5S NGF; Invitrogen). After 3 days *in vitro*, cells were loaded with the calcium-sensitive fluorescent dye Fluo-4 AM (10 μm for 10 min, Invitrogen), and experiments performed with an imaging setup equipped with a Cairn monochromator and an electron-multiplying CCD camera with proprietary acquisition software (Andor Technology). During the experiments cells were continuously superfused with HBSS (140 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM KCl, 10 mM HEPES, 4 mM glucose, pH 7.4). Treatments were applied with an automated fast solution changer based on a stepping motor

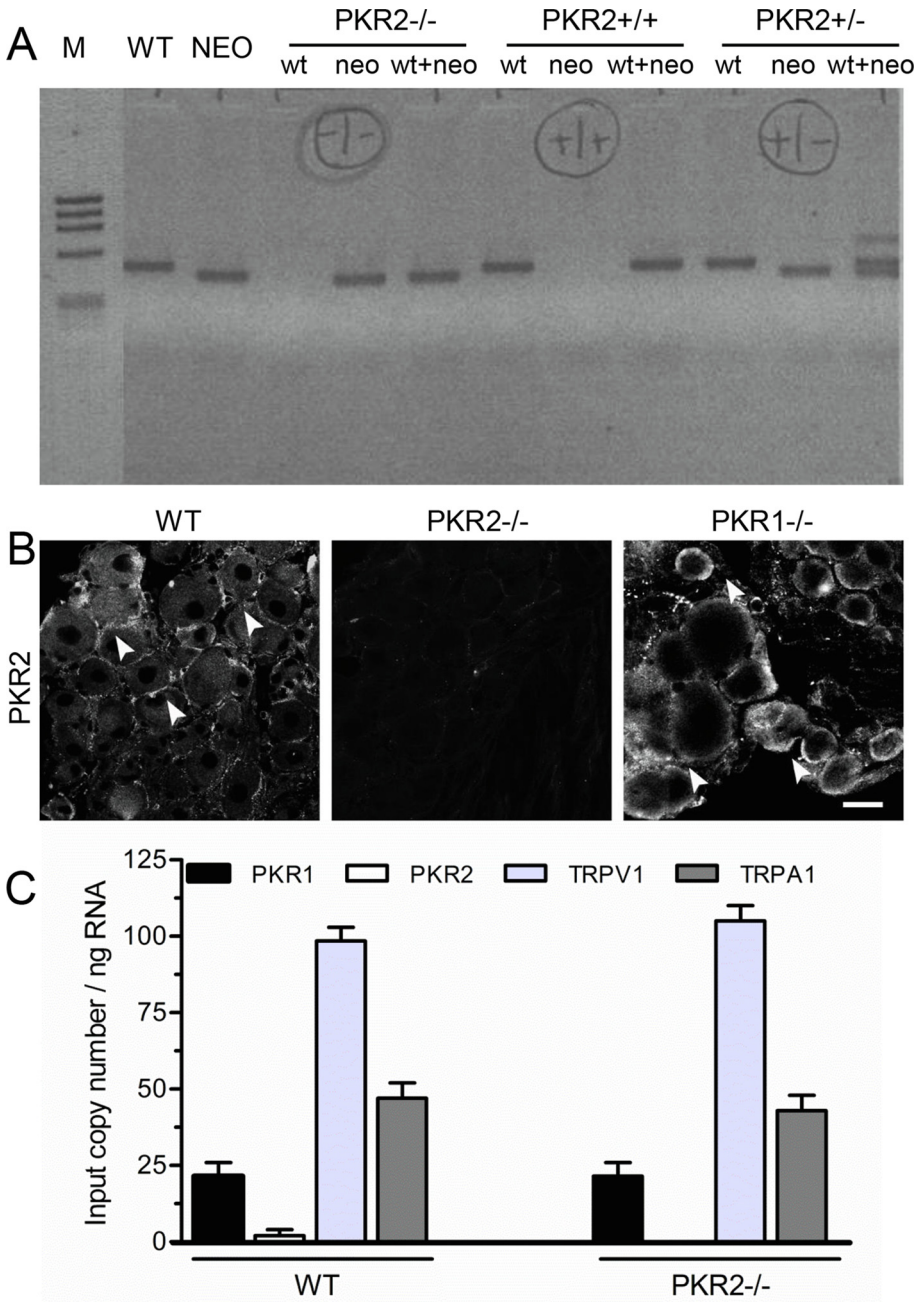

Fig. 1. Mouse tail DNA genotyping, DRG PKR2 protein localization and DRG mRNA expression of PKR1, PKR2, TRPV1 and TRPA1 genes. (**A**) Representative example of WT (PKR2+/+), heterozygous mutant (PKR2+/−) and homozygous mutant (PKR2−/−) mouse tail DNA genotyped by PCR. Lane WT contains PCR amplification products of the wild-type PKR2 gene; lane NEO contains the PCR amplification products of the mutant gene; lanes WT contain the PCR amplification products obtained with primers for wild-type PKR2 gene; lanes NEO contain the PCR amplification products obtained with primers for the mutant gene; lanes WT + NEO indicate multiplex PCR for both the WT and the mutant gene; lane M contains DNA molecular weight markers. (**B**) Immunofluorescence staining showing the presence of PKR2 (arrows) in DRG neurons from WT and PKR1−/− mice but not in DRG neurons from PKR2−/− mice. Scale bar = 20 μm. (**C**) Expression of PKR1, PKR2, TRPV1 and TRPA1 genes in DRG from WT and PKR2−/− mice (n = 5 mice/genotype for DNA/RNA extraction; n = 5 mice/genotype for immunofluorescence).

and a multibarrel microperfusion head (CV Scientific, Modena, Italy). Neuronal cells were routinely distinguished from satellite/glial cells present in DRG cultures by morphology and by their fluorescence response to a brief puff of HBS containing an increased extracellular potassium concentration (25 mM) which causes depolarization and activation of the voltage-sensitive calcium channels expressed in neurons (not shown). At the end of the experiment, the maximal fluorescence (Fmax) was obtained with the application of the calcium ionophore ionomycin (10 μm; Calbiochem, La Jolla, CA) in the presence of high calcium (30 mM) and high potassium (125 mM). The fluorescence individual neurons was carefully analysed offline with ImageJ (EMBL, Heidelberg, DE) (Vellani et al., 2006). Sensitisation of the capsaicin response was investigated with an experimental approach similar to the one used in Bonnington and McNaughton (2004), as described in Results. Fluorescence is expressed as F/Fmax. All experiments were performed at room temperature (RT) (20–22 °C).

Statistical analysis

Statistical analysis and graphing were performed using GraphPad Prism software (Version 6) or Sigmaplot 10.0. Unpaired Student's *t*-test (two-tailed distribution and equal variance) was used to perform the statistical analysis between groups. Two-way ANOVA test was used to analyse the behavioural time-course data; the significant interactions were further subjected to Bonferroni's post-hoc test for multiple comparisons (data normally distributed, equal variance). All data were expressed as mean ± SEM values. Statistical analysis at $p < 0.05$ (*) were considered significant.

RESULTS

Characterization of PKR2−/− mice

As shown in Fig. 1A, mouse tail DNA genotyping confirmed the complete absence of the pkr2 gene sequence. Immunofluorescence studies (Fig. 1B) confirmed the total absence of PKR2 protein in DRG neurons, where normally both PKRs are expressed and produce hyperalgesia by sensitizing TRPV1 and TRPA1 (Vellani

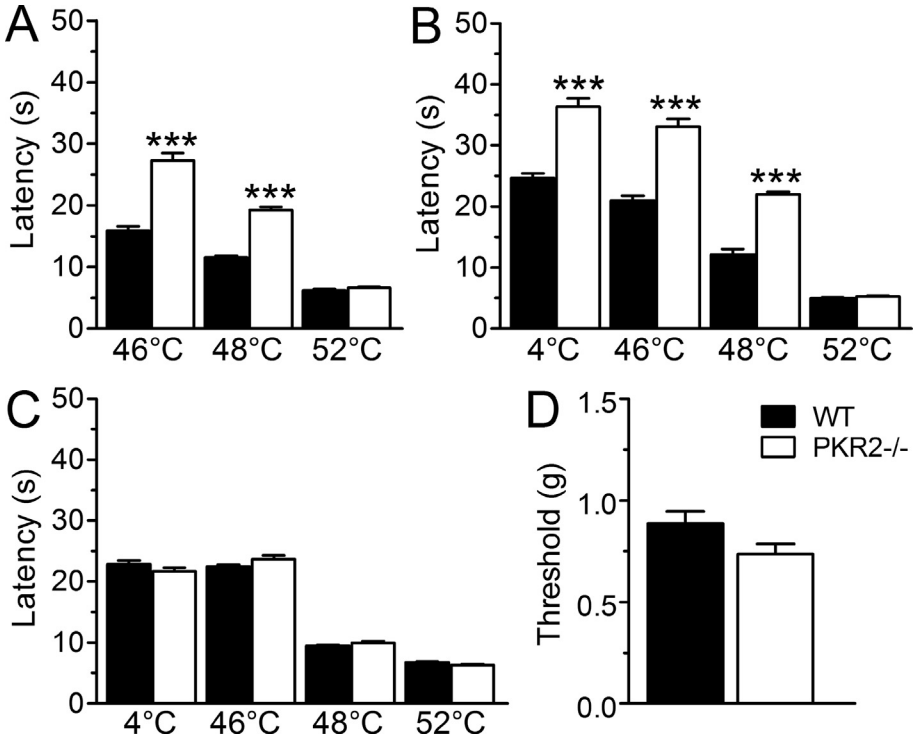

Fig. 2. Nociceptive responses of PKR2−/− mice to thermal and tactile stimuli. (**A**) PKR2−/− mice exhibited significantly increased paw withdrawal latencies from hot plate and (**B**) increased tail withdrawal latencies from cold and hot water in comparison to WT mice (tail immersion test) (**C**) Paw withdrawal latency from both hot and cold water and (**D**) tactile threshold by von Frey filaments did not reveal any difference between the two genotypes. Data are shown as mean ± SEM, $^{***}p < 0.001$ vs WT unpaired Student's *t*-test. For all behavioural test 20 WT and 20 PKR2−/− mice were used.

et al., 2006; Franchi et al., 2017). By qRT-PCR analysis of DRG-extracted RNA, we determined that in WT animalsPKR1 mRNA is 8 times higher than PKR2, and about 4 and 2 times lower than TRPV1 and TRPA1 mRNA. In PKR2−/− mice instead, PKR2 mRNA was completely absent while PKR1, TRPV1 and TRPA1 transcripts were present at the same WT levels (Fig. 1C).

Impaired thermal and mechanical nociception in PKR2−/− mice

The sensitivity of WT and PKR2−/− mice to noxious, thermal and mechanical stimuli were assessed with behavioural experiments.

When exposed to noxious heat and cold, pkr2-null mutant mice showed a reduced nociceptive response. In the hot plate test at 52 °C there were no significant differences in the response latencies of the two genotypes (WT and PKR2−/− mice) ($t(37) = 1.93$, $p = 0.061$), while at lower temperature (< 50 °C) PKR2−/− mice displayed significantly longer (about 2-fold) response latencies (Fig. 2A, $t(38) = 8.17$, $p < 0.0001$ at 46 °C and $t(38) = 12.12$, $p < 0.0001$ at 48 °C). A comparable reduced response was observed in the test using hot water (46–48 °C) applied to the mouse tail. Also in this case the response latencies at 46 and 48 °C were ~ 2-fold longer in pkr2-null mutant compared with the WT littermates ($t(38) = 7.95$, $p < 0.0001$ and $t(38) = 9.67$, $p < 0.0001$, respectively), while no differences were observed at 52 °C ($t(38) = 1.58$, $p = 0.12$). Analogously, pkr2 deletion produced a significantly impaired response to cold stimuli (immersion of the mouse tail in cold water at 4 °C) ($t(36) = 7.29$, $p < 0.0001$), as revealed by the increased latencies in pkr2-null mutant mice (Fig. 2B). On the contrary, no significant differences were detected in the withdrawal latencies between PKR2−/− and WT mice when both cold (4 °C) and hot (46–52 °C) water tests were applied to the mouse paw (Fig. 2C, $t(40) = 1.45$, $p = 0.15$ at 4 °C; $t(38) = 1.84$, $p = 0.07$ at 46 °C; $t(38) = 1.6$, $p = 0.11$ at 48 °C; $t(37) = 1.76$, $p = 0.085$ at 52 °C). Similarly, no significant differences in paw withdrawal threshold to tactile stimuli, assessed with von Frey filaments, was observed between PKR2−/− and WT mice (Fig. 2D, $t(38) = 1.99$, $p = 0.053$).

Overall, these data indicate that pkr2 deletion alters baseline sensitivity to thermal stimuli, leaving unaltered tactile nociception in the tested conditions.

Reduced nociceptive response to noxious chemical stimuli in PKR2−/− mice

The same reduced nocifensive behaviour was observed in the PKR2−/− genotype following noxious chemical stimuli such as intra-peritoneum (i.p.) acetic acid and intra-plantar (i.pl.) capsaicin injection. As shown in Fig. 3A, compared to WT, in PKR2−/− mice the writhing episodes after acetic acid treatment were significantly less intense and shorter-lasting ($t(8) = 4.89$, $p = 0.0012$). Also paw shaking, licking and biting after capsaicin treatment were significantly reduced (Fig. 3B, $t(8) = 17.84$, $p < 0.0001$).

Subcutaneous (s.c.) hindpaw inoculations of formalin in mice is characterized by a biphasic behavioural response, a first short-lived phase of 0–10 min and a subsequent sustained phase of 10–50 min of duration (Hunskaar et al., 1986; Coderre et al., 1990).

In PKR2−/− mice both the early and late phase responses to subcutaneous formalin administration were significantly smaller when compared to the ones observed in WT mice ($t(8) = 5.12$, $p = 0.0009$ early phase and $t(8) = 3.503$, $p < 0.008$ late phase). Conversely, PKR1−/− mice showed a significant reduction only in the early phase response, compared to WT ($t(8) = 3.37$, $p = 0.009$ early phase and $t(8) = 0.79$, $p = 0.44$ late phase), thus demonstrating a significant difference in the nociceptive response between the two null genotypes, pkr1 and pkr2, only in

the late phase ($t(8) = 0.87$, $p = 0.409$ early phase and $t(8) = 2.51$, $p = 0.035$ late phase).

Impaired nociceptive sensitization by proalgesic agents in PKR2−/− mice

As previously demonstrated, the s.c (1 μg/kg) and i.pl (0.5 ng) injection of Bv8 induced powerful and long-lasting sensitization to thermal stimuli (Negri et al., 2006). We demonstrated that pkr2-null mice were also responsive to Bv8 treatment, however a 30 times higher s.c. (Fig. 4A; Time: $F(13,209) = 66.92$, $p < 0.0001$; Group: $F(2,209) = 84,17$, $p < 0.0001$; Interaction: $F(26,209) = 17.08$, $p < 0.0001$; post-hoc $p < 0.001$ PKR2−/−: 1 vs WT:1) and a 10 times higher i.pl. Bv8 dose were necessary to obtain a similar level of hyperalgesia (Fig. 4B; Time: $F(7,119) = 61.79$, $p < 0.0001$; Group: $F(2,119) = 56.47$, $p < 0.0001$; Interaction: $F(14,119) = 10.97$, $p < 0.0001$; post-hoc $p < 0.001$ PKR2−/−: 0.5 vs WT:0.5).

Deletion of pkr2 was also involved in allodynia induced by Bv8 i.pl. injection. Whereas 0.5 ng of Bv8 evoked tactile allodynia in WT mice, a 10 times higher Bv8 dose was necessary to obtain the same effect in pkr2-null mice (Time: $F(7,119) = 87.44$, $p < 0.0001$; Group: $F(2,119) = 157.27$, $p < 0.0001$; Interaction: $F(14,119) = 20.71$, $p < 0.0001$; post-hoc $p < 0.001$ PKR2−/−: 0.5 vs WT:0.5). Interestingly, the Bv8 dose effective in WT animals (0.5 ng) was also able to evoke tactile allodynia in pkr1-null mice (Time: $F(7,79) = 72.75$, $p < 0.0001$; Group: $F(1,79) = 11.61$, $p = 0.001$; Interaction: $F(7,79) = 3.23$, $p = 0.0053$; $p < 0.01$ PKR1−/−: 0.5 vs WT:0.5) (Fig. 4C).

In addition, nociceptive sensitization to thermal stimuli (48 °C, paw immersion) induced by intrapaw injection of capsaicin (12 μg) in WT mice, was absent in pkr2-null mice (Fig. 4D) (Time: $F(6,69) = 41.88$, $p < 0.0001$; Group: $F(1,69) = 53.39$, $p < 0.0001$; Interaction: $F(6,69) = 13.82$, $p < 0.0001$; post-hoc $p < 0.001$ PKR2−/− vs WT).

On the whole, these results show that only pkr2 deletion, but not pkr1 deletion, impaired tactile allodynia.

Lacking of inflammatory hyperalgesia in PKR2−/− mice

We tested the sensibility of pkr2-null mice to both acute and chronic inflammatory hyperalgesia.

Acute inflammatory hyperalgesia was obtained painting mustard oil solution on both hindpaws, and noxious response measured with the hot-plate test. As shown in Fig. 5A, mustard oil-induced thermal hyperalgesia was evident within 15 min after application in WT mice, reaching its maximum after 30 min (40 ± 3% of the baseline paw withdrawal latency) and lasted up to 80–90 min. In pkr2-null mice the thermal nociceptive threshold remained at the baseline values (Time: $F(5,59) = 16.92$, $p < 0.0001$; Group: $F(1,59) = 17.74$, $p = 0.0029$; Interaction: $F(5,59) = 11.21$, $p < 0.0001$; post-hoc $p < 0.001$ PKR2−/− vs WT), while in PKR1−/− mice a significant reduction (24 ± 3%) of the baseline paw withdrawal latency was observed (Time: $F(5,59) = 34.99$, $p < 0.0001$; Group: $F(1,59) = 5.52$, $p = 0.046$; Interaction: $F(5,59) = 4.38$, $p = 0.0028$; post-hoc $p < 0.01$ PKR1−/− vs WT).

Chronic inflammatory hyperalgesia was induced by i.pl. injection of CFA, and tested analysing paw-withdrawal latency to noxious thermal (paw immersion test at 48 °C) and tactile stimuli (Von-Frey filaments) 6, 24, 36, 48, 60 and 72 h after CFA administration. The results showed that the development of CFA-induced thermal hyperalgesia was impeded by the disruption of pkr1 as well as pkr2 genes (Fig. 5B; Time: $F(5,89) = 47.79$, $p < 0.0001$; Group: $F(2,89) = 91.85$, $p < 0.0001$; Interaction: $F(10,89) = 28.54$, $p < 0.0001$; post-hoc $p < 0.001$ PKR1−/− vs WT and PKR2−/− vs WT), while only the pkr2 disruption blocked the development of CFA-induced tactile allodynia (Fig. 5C; Time: $F(8,134) = 150.22$, $p < 0.0001$; Group: $F(2,134) = 107.23$, $p < 0.0001$; Interaction: $F(16,134) = 21.56$, $p < 0.0001$; post-hoc $p < 0.001$ PKR2−/− vs WT).

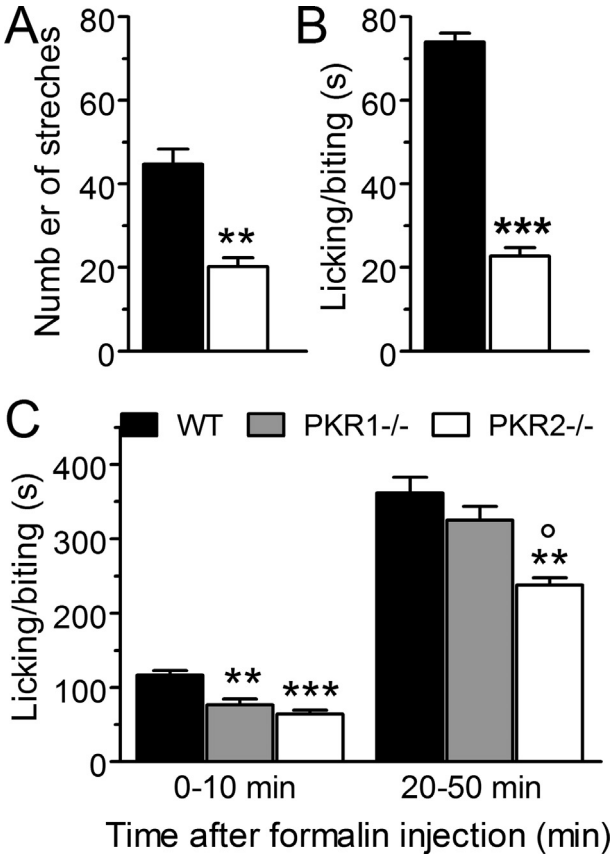

Fig. 3. Nociceptive responses of PKR2−/− mice to noxious chemical stimuli. (**A**) PKR2−/− mice showed a reduced number of writhes in response to intraperitoneal 0.8 % acetic acid injection compared with WT mice during the 20 min observation period ($n = 5$ mice/genotype). (**B**) Paw licking recorded for 10 min after 1.5 μg intraplantar capsaicin injection was shorter in pkr2-null mice than in WT mice ($n = 5$ mice/genotype). (**C**) PKR2−/− mice exhibited significantly increased early phase response to subcutaneous administration of 5% formalin; the late phase response did not show significant difference between WT and PKR2−/− mice. Data are shown as mean ± SEM ($n = 5$ mice/genotype). Unpaired Student's *t*-test: $^{**}p < 0.01$, $^{***}p < 0.001$ vs WT; $^{\circ}p < 0.05$ vs PKR1−/−.

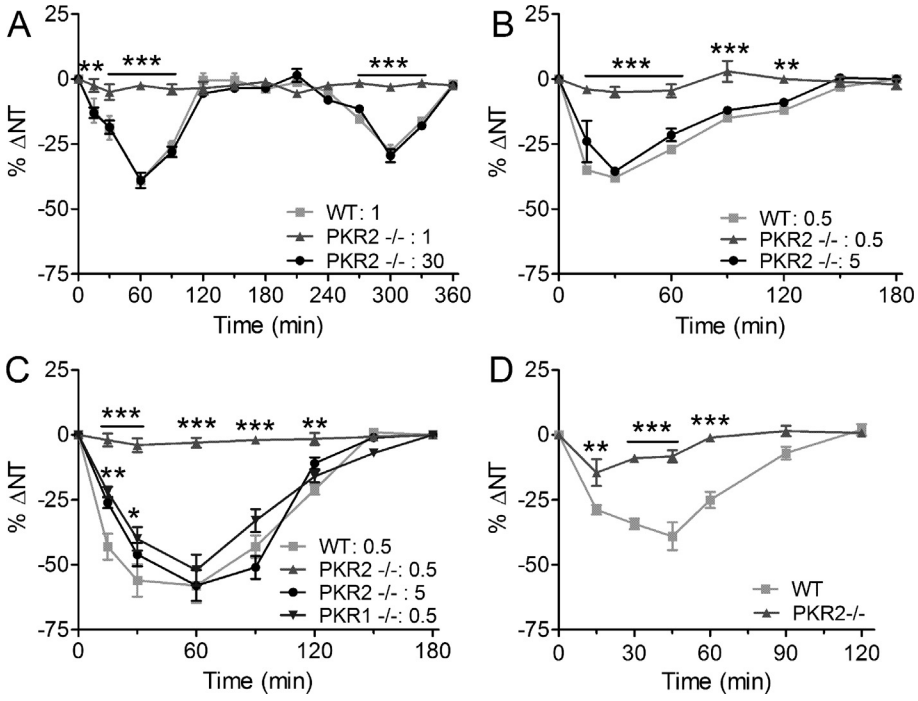

Fig. 4. Nociceptive responses of PKR2−/− mice to pro-algesic agents. (**A**) In WT mice, subcutaneous injection of Bv8 (1 μg/kg) produced a long-lasting sensitization to thermal stimuli (hot-plate test). A 30-fold higher dose was necessary to obtain the same effect in PKR2−/− mice (n = 5 mice/genotype/dose). (**B**) In WT mice, intraplantar (0.5 ng) administration of Bv8 induced thermal hyperalgesia in the injected paw without affecting the contralateral paw (paw-immersion test, 48 °C). A 10-fold higher dose of Bv8 was required to elicit hyperalgesic effects comparable to those obtained in WT mice. (**C**) In WT mice the intraplantar injection of 0.5 ng Bv8 evoked tactile allodynia (Von-Frey filaments); the same dose was effective in PKR1−/− mice but not in PKR2−/− mice, where a 10 times higher Bv8 dose was necessary to obtain the same effect (n = 5 mice/genotype/dose). (**D**) Intraplantar injection of capsaicin (12 μg) induced sensitization to thermal stimuli in WT but not in PKR2-null mice (paw immersion test, 48 °C). Data are shown as mean ± SEM (n = 5 mice/genotype/dose). Two-way ANOVA for repeated measures followed by Bonferroni's post hoc test for multiple comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs WT.

Diminished body temperature reduction in PKR2−/− mice

Systemic capsaicin, by selectively activating chemo- and heat-sensitive TRPV1 receptors, exerts significant effect on thermoregulation, reducing body temperature. By s.c. injection, capsaicin (1 mg/kg) lowered body temperature by about 6 °C in WT animals, while in PKR2−/− mice reduction was about 1 °C (Fig. 6; Time: $F(11,119) = 58.31$, $p < 0.0001$; Group: $F(1,119) = 4.23$, $p < 0.073$; Interaction: $F(11,119) = 20.2$, $p < 0.0001$; post-hoc $p < 0.001$ PKR2−/− vs WT).

Morphology of DRG neurons from PKR2−/− mice

We tested the influence of pkr2 gene deletion in DRGs, analysing their morphology. As shown in Fig. 7A, B, immunostaining for neuronal cells showed no significant differences in morphology of DRGs from PKR2−/− mice, compared to WT mice.

Calcium response in cultured mouse DRG neurons

The functional expression of TRPV1 and TRPA1, and their co-expression with functional PKR receptors was studied with calcium imaging in PKR2−/−, PKR1−/− and WT cultured DRG neurons. The specific agonists capsaicin (600 nM) and mustard oil (MO, 1 μM) were used to elicit specific TRPV1- and TRPA1-mediated calcium signals.

The percentage of TRPV1- and TRPA1-positive neurons in the three genotypes tested is summarised in Fig. 9A. The fraction of the capsaicin-responsive neurons was reduced by more than 50% in both PKR2−/− and PKR1−/− in comparison with WT. Similarly, MO-responsive neurons were reduced in both transgenics, although to a lesser extent in PKR1−/− (Fig. 9A). This indicates a significant role of PKRs in functional expression of both TRPV1 and TRPA1 in sensory neurons.

The Bv8-responsive subpopulation of cultured DRG neurons was detected as the subpopulation that responded to Bv8 (100 nM, 1 min), either with a direct calcium signal (not shown), or with the sensitization of their response to capsaicin (Vellani et al., 2006). When present, the calcium response to Bv8 treatment was typically delayed (3–29 s from the beginning of Bv8 treatment) and large (ΔF > 0.12 Fmax), similar to the response to Bv8 shown previously in the rat (Vellani et al., 2006).

The sensitization of the capsaicin response following Bv8 application was studied with an experimental protocol similar to the one described in Bonnington and McNaughton (2003). A brief puff of capsaicin (500 nM, 600 ms duration) was repeatedly applied at 2 min intervals with an automated multibarrel fast solution changer during continuous superfusion with vehicle solution. After some initial adaptation, the capsaicin responses became more reproducible. The test solution containing Bv8 (100 nM for 1 min) was applied between the 5th and 6th capsaicin pulse. In control experiments, the vehicle solution was applied with the same perfusion protocol used for the test solution containing Bv8. The vehicle treatment *per se* did not produce any response, ruling out mechanical artefacts due to solution turbulence. The response of individual capsaicin-sensitive neurons was carefully quantified offline. The calculated ratios between the 6th and the 5th capsaicin response amplitudes measured in experiments with vehicle were normally distributed, and from the mean and standard deviation of the control distribution a threshold level for classifying a neuron as exhibit-

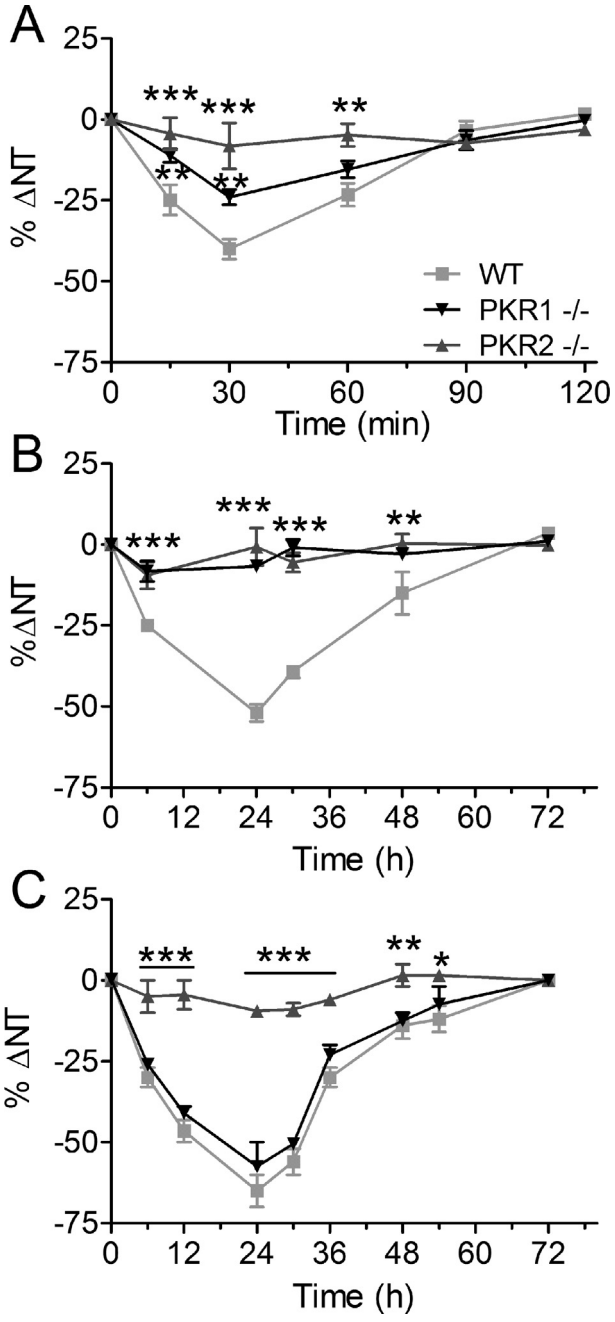

Fig. 5. Inflammatory hyperalgesia in PKR2−/− mice. (**A**) Acute inflammatory hyperalgesia (hot-plate test), induced by painting both hindpaws with mustard oil was intense in WT mice and was impaired in PKR1−/− mice, while PKR2−/− mice were completely unaffected (n = 5 mice/genotype/dose). (**B**, **C**) Chronic inflammatory hyperalgesia elicited by intrapaw injection of CFA was impaired in PKR2−/− mice when both thermal (paw immersion, 48 °C, (**B**)) and tactile (Von-Frey filaments, (**C**)) were applied to the paw. Pkr1-null mice exhibited reduced inflammatory thermal but not tactile CFA-induced hyperalgesia. Data are shown as mean ± SEM (n = 5 mice/genotype/dose). Two-way ANOVA for repeated measures followed by Bonferroni's post hoc test for multiple comparisons: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs WT.

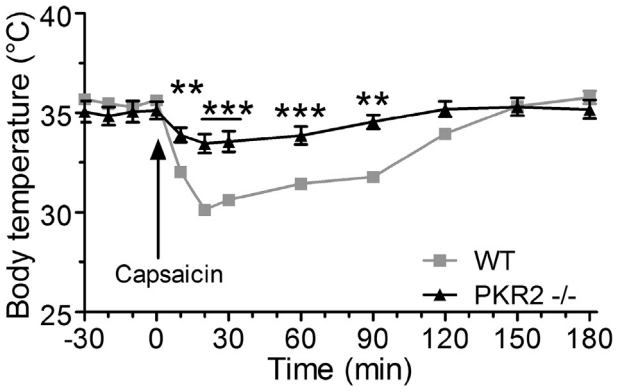

Fig. 6. Impaired capsaicin-induced hypothermia in PKR2−/− mutant mice. Subcutaneous capsaicin (1 mg/kg) induced hypothermic response in WT but not in PKR2−/− mice. Data are shown as mean ± SEM (n = 5 mice/genotype/dose). Two-way ANOVA for repeated measures followed by Bonferroni's post hoc test for multiple comparisons: $^{**}p < 0.01$, $^{***}p < 0.001$ vs WT.

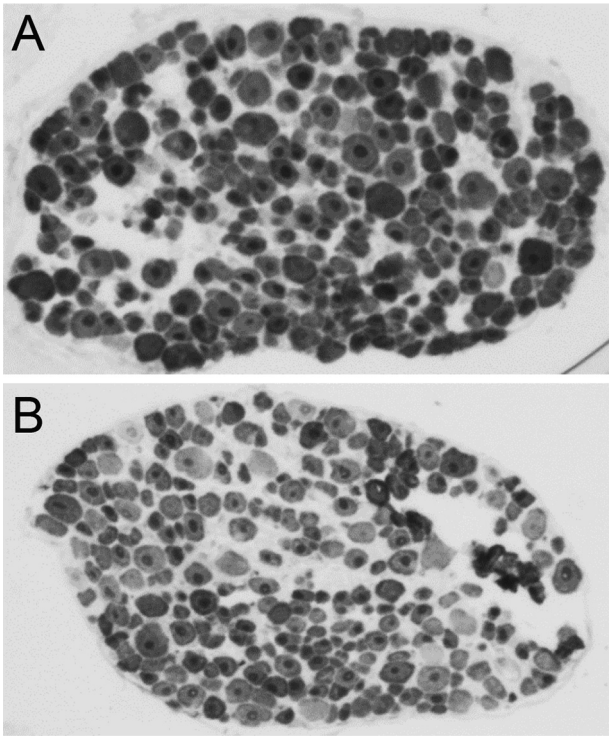

Fig. 7. Morphology of DRG neurons in PKR2−/− mice. Microphotographs of DRG sections from PKR2−/− (**A**) and WT mice (**B**), immunostained with an antibody specific for the neuronal marker NeuN and stained with 0.1% o-toluidine blue. The images demonstrate no differences in the morphology of DRG from WT and PKR2−/− mice (n = 5 mice/genotype/dose).

ing sensitisation in response to Bv8 was set at the calculated two-tailed 99.9% confidence value of 1.62 (Fig. 8B, left panel). As expected, in Bv8-treated cells a normally distributed population of $6^{th}/5^{th}$ ratio values similar to the one in control experiments was observed, together with a subpopulation of values above 1.62 (range: 1.85–8), indicating sensitization of the capsaicin response by Bv8 (Fig. 8B, right panel).

At the end of capsaicin pulse protocol, the TRPA1 agonist allyl isothiocyanate (mustard oil, 10 μM, 30 s), was applied to test for co-localization of Bv8 response

(calcium response or TRPV1 sensitization) with TRPV1 and TRPA1, demonstrating the co-localization of Bv8 response effect only with TRPA1 from neurons of PKR1-null mice, where PKR2s are expressed (Fig. 8C). The total percentage of Bv8-responsive DRG neurons (either with a calcium signal and/or with sensitization to capsaicin in response to Bv8) was 11.6% in PKR2−/−, 5.3% in PKR1−/−, and 18.3% in WT cultures (Fig. 9). The percentage of Bv8-responsive neurons which were also responsive to capsaicin was identical in PKR1−/− and PKR2−/− mice (Fig. 9B) but significantly smaller compared to WT neurons. Conversely, the percentage of Bv8-responsive DRG neurons which were also responsive to MO was much higher in PKR1−/− than in PKR2−/− mice (Fig. 9B). All differences mentioned were statistically significant, as assessed by χ^2 analysis.

DISCUSSION

In this study, to better evaluate the involvement of the prokineticin system in the neurobiology of pain, we investigated the role of the prokineticin receptor 2 (PKR2) in pain perception, using pkr2- gene deficient mice.

As previously established for pkr1-null mice (Negri et al., 2006), we observed that mice lacking the pkr2 gene exhibited several nociceptive deficits in response to thermal noxious stimuli, to pro-algesic agents, and in inflammatory hyperalgesia when compared to wild type littermates.

We found that pkr2-null mice exhibited reduced nociceptive sensitivity to the noxious hot temperatures of 46 °C and 48 °C, in the working range of C-polymodal nociceptors and of TRPV1 channels (Le Bars et al., 2001; Tominaga and Tominaga, 2005). Pkr2-null mice were also less sensitive to the noxious cold temperature of 4 °C, in the working range of TRPA1 channels (Story et al., 2003; del Camino et al., 2010; Knowlton et al., 2010; Moran et al., 2011). Despite PKR2−/− and WT mice showed substantial differences in the thermal nociceptive thresholds in the hot plate and in the tail-immersion test, in the paw immersion test they showed similar results, therefore suggesting that the response to the different tests used may involve separate subpopulations of nociceptors (Negri et al., 2006).

Conversely, as already observed in PKR1−/− mice (Negri et al., 2006), PKR2−/− mice did not show impaired responsiveness to mechanical stimuli in basal conditions.

Moreover, we established that pkr2-null mice were more resistant to nociceptive sensitization induced by chemical and pro-algesic agents, and that PKR2−/− mice exhibited impaired hyperalgesic responses to Bv8. In line with RT-PCR studies on DRG neurons from PKR2−/− mice confirming the absence of PKR2 receptors, we deduce that the residual hyperalgesic effect of Bv8 is explained by the presence of PKR1 receptors expressed in neurons able to induce the typical biphasic hyperalgesia to thermal stimuli, but only at doses 30 times higher compared to those sufficient in WT mice. Interestingly, deletion of pkr2, but not of pkr1, impaired Bv8-induced tactile allodynia, indicating the

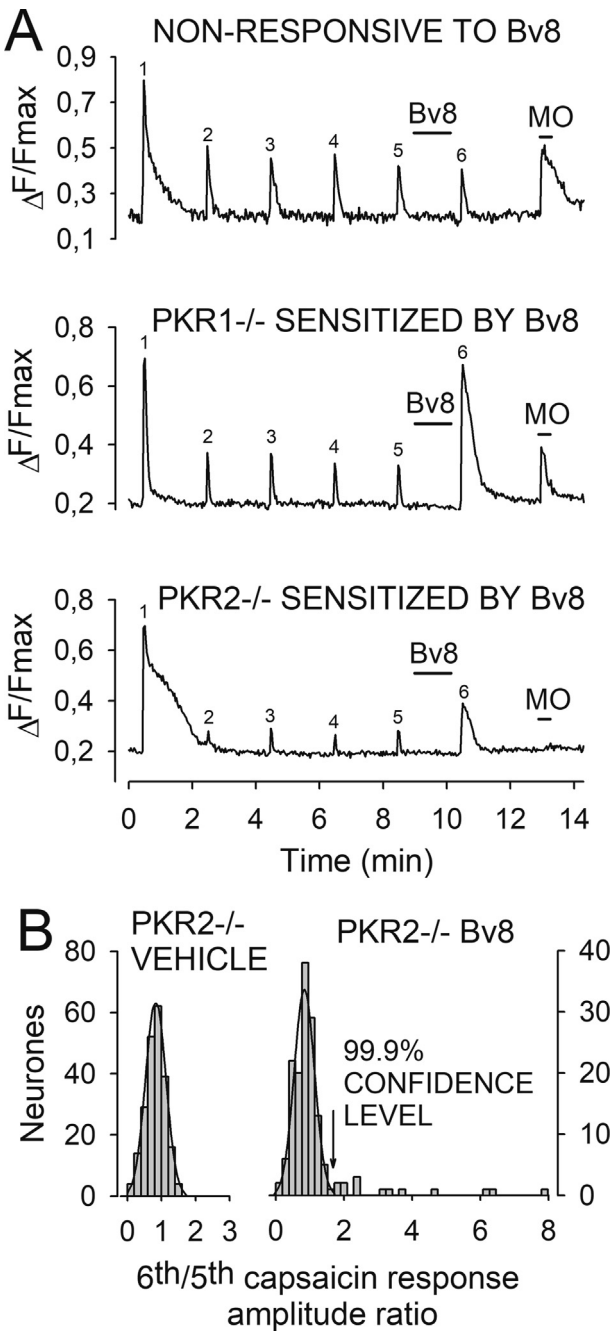

Fig. 8. Sensitization of the capsaicin response by Bv8. (**A**) Calcium responses of cultured sensory neurons during pulsed applications of capsaicin (500 nM, 600 ms), Bv8 (50 nM, 1 min) and mustard oil (MO, 1 μM, applied for 20–40 s). In neurons insensitive to Bv8 (upper panel) the capsaicin response was unaffected by Bv8. Examples of neuronal calcium responses to capsaicin sensitized by Bv8 are shown in the middle (PKR1−/− neuron also responsive to MO) and lower panel (PKR2−/− neuron unresponsive to MO). Individual responses of Bv8-sensitive neurons from WT mice displayed a largely similar behaviour (not shown). (**B**) Distribution of the calculated ratios between the 6th and the 5th capsaicin response amplitude in PKR2−/− neurons treated with vehicle (left panel) or with Bv8 (right panel, experiments as in (**A**)). The Bv8-responsive neurons displayed a 6th/5th ratio above the 99.9% two-tailed confidence limit (1.62 ratio value) determined analysing the response amplitudes to control treatment with vehicle (further explanation in the text). 6th/5th ratio distributions in control experiments on PKR1−/− and WT cultures had an identical distribution and 99.9% two-tailed confidence limit (not shown) (n = 5 mice/genotype/dose).

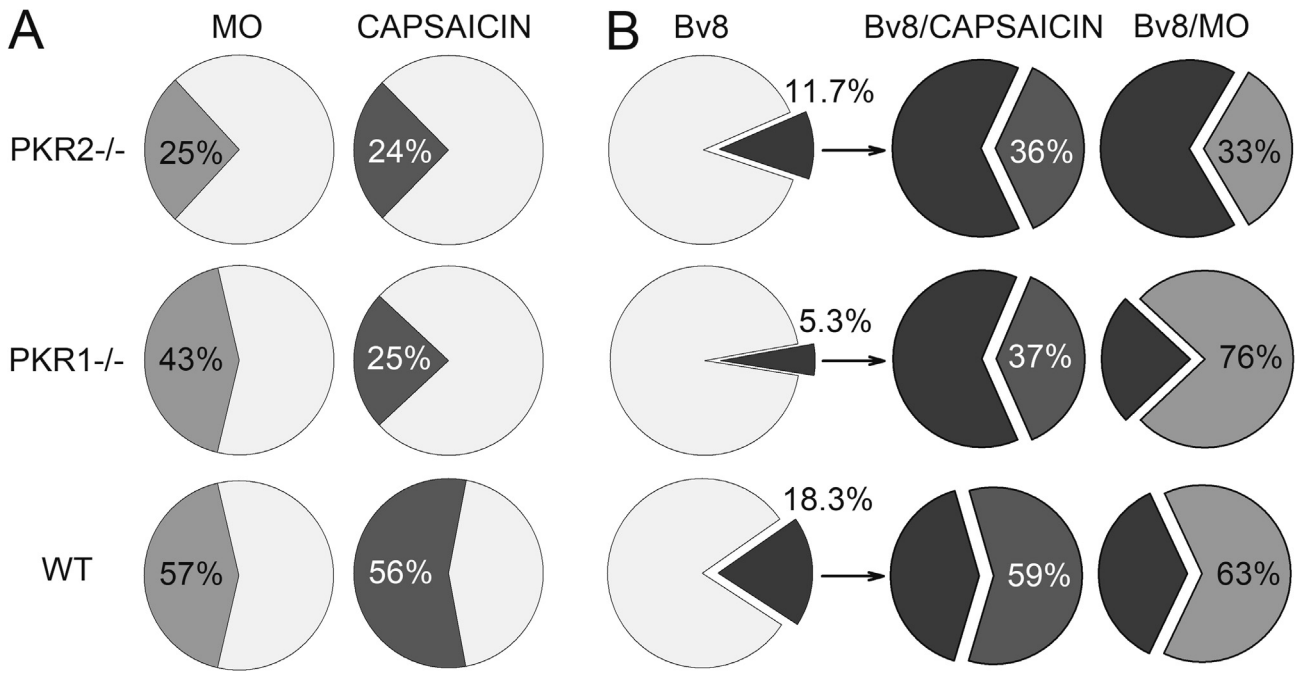

Fig. 9. Summary of calcium imaging data in WT, PKR2−/− and PKR1−/− mice. (**A**) Percentage of cultured DRG neurons responsive to capsaicin and mustard oil (MO). Pooled data from six cultures (WT, overall population of 3240 neurons) and five cultures from PKR2−/− and PKR1−/− (2288 and 2209 neurons, respectively). Percentages of capsaicin- and MO-responsive neurons in comparison to WT cultures were decreased by more than 50% in PKR2−/− cells, while in PKR1−/− the decrease of MO-responsive neurons was limited to about a quarter. (**B**) Neuronal subpopulations responsive to Bv8 either with a calcium signal or with sensitization to capsaicin. The percentages of Bv8-sensitive neurons and their sub-fractions responsive to capsaicin were reduced in both PKR2−/− and PKR1−/− cultures by a fraction similar to the one of the overall population (**A**). Differently, MO-responsive neurons were decreased in PKR2−/− cultures (by almost 50%) while they were fractionally increased in PKR1−/− neurons, where they represented 76% of neurons expressing functional PKR2.

specific role of PKR2 in nociceptor sensitization to tactile stimuli. Disruption of pkr2 gene induced a strong reduction of the capsaicin-induced paw licking response, hyperalgesia and hypothermia, and reduced the acetic acid-induced writhing responses. These observations highlight a positive interaction between the G-protein-coupled receptors PKR2 and the non-selective cation channels TRPV1, which are activated by noxious heat as well as by capsaicin and protons (Caterina et al., 1997; Hu et al., 2006; Negri et al., 2006).

These data are in agreement with those already acquired in pkr1-null mice (Negri et al., 2006) demonstrating an interaction between PKR1 and TRPV1, which are co-expressed in a significant proportion of DRG small-diameter neurons (Tominaga and Tominaga, 2005; Lumpkin and Caterina, 2007). Results obtained in pkr2-null mice suggest a similar positive functional cooperation between PKR2 and TRPV1 in DRG small-sized neurons. Nevertheless, PKR2 receptors are mainly present in medium/large-sized neurons (Negri et al., 2006), and are co-localized with TRPA1 (Dhaka et al., 2006; Vellani et al., 2006).

Accordingly, in this paper we demonstrated that the percentage of DRG neurons, from both pkr1- and pkr2-null mice, that respond to Bv8 respond also to capsaicin. Conversely, the percentage of DRG neurons responsive to Bv8, which responded also to allyl isothiocyanate, the pungent agent also termed mustard oil (a specific TRPA1 activator), was much higher in PKR1−/− than in PKR2−/− mice, proving the anatomical and functional colocalization between PKR2 and TRPA1. These data are supported also by *in vivo* studies, which demonstrated that the PKR2−/− mice are far less sensitive to mustard oil-induced hyperalgesia than WT mice.

The PKR2/TRPA1 functional co-localization is also suggested by the substantially different formalin-induced nocifensive behaviours between pkr2- and pkr1-null mice. While in PKR2−/− mice we observed a significant reduction of both the first and the late phase of the response to subcutaneous formalin administration, in PKR1−/− mice the reduction was detected only in the first phase of the response. The two phases are caused by two different mechanisms of action: the first phase is predominantly due to the direct stimulation of peripheral nociceptors, while the late phase involves both peripheral inflammatory reactions and central functional changes in the spinal cord (Tjølsen et al., 1992). TRPA1 channels localized in the periphery and in the spinal cord participate to both the first and the secondary allodynia and hyperalgesia: studies with TRPA1 antagonists and TRPA1−/− mice show impaired responses to formalin (Colleen et al., 2007; McNamara et al., 2007; Rojas et al., 2018). The lack of TRPA1, consequent to the lack of PKR2 (with which it is colocalized), causes a significant decrease in the early- and late-phase responses to forma-

lin in PKR2−/− mice. Conversely, the lack of TRPV1, consequent to the lack of PKR1 (with which it is colocalized), causes a significant reduction only in the early phase responses to formalin in PKR1−/− mice. These data suggest that PKR2 receptors act at both peripheral and spinal levels, while PKR1 preferentially act in the periphery.

The notion of differential PKR1/2 sites of action is supported also by experiments on inflammatory hyperalgesia. We observed that both pkr2- and pkr1-null mice show a reduced CFA-induced hyperalgesia, thus indicating that both PKR2 and PKR1 receptors are involved in chronic inflammatory pain. Indeed, chronic inflammation is related to a robust upregulation of PROK2, which, once released, interacts with both PKRs on monocytes, macrophages and dendritic cells secreting inflammatory mediators, as previously demonstrated (Giannini et al., 2009).

However, only the disruption of PKR2 impaired the development of CFA-induced tactile allodynia, because tactile noxious stimuli are transmitted by Aδ larger diameter fibres, and the response involves processing by central circuits (Klede et al., 2003), in accordance with the prevalent distribution of PKR2 in the CNS (Chen et al., 2002; Lin et al., 2002; Negri et al., 2007), and its putative colocalization and cooperation with TRPA1 on medium/large size sensory neurons.

These data are in line with recent data from our group which demonstrate that the peripheral nerve damage was able to upregulate PROK2 and PKR2 in the periphery as well as in the spinal cord, and with the evidence that the therapeutic administration of PC1, a prokineticin receptor antagonist, alleviated thermal hyperalgesia and delayed the development of tactile allodynia (Maftei et al., 2014; Guida et al., 2015; Lattanzi et al., 2015), supporting the notion that PKR2 is an inducible receptor, as previously proposed (Kisliouk et al., 2005). PKR2 is normally expressed in spinal cord neurons and in some medium-large DRG neurons, which also express the vanilloid receptor TRPA1, an ion channel which is considered to play an important role in tactile allodynia (Nassini et al., 2011). We therefore hypothesize that the increased expression of PKR2 in nociceptors and in spinal cord neurons associated with the increased expression of its agonist PROK2, may play a crucial role in the induction and maintenance of allodynia.

In conclusion, we demonstrated that PKR2 plays an important role in setting the nociceptive threshold to diverse noxious stimuli and that it is a critical mediator of acute and chronic inflammatory hyperalgesia.

Given the interactions that we propose between PKR2 and TRP channels signalling in the development of pain, it may be important to establish whether these interactions are direct or indirect, with the aim of developing drugs that, by targeting them, may have the potential to achieve broad-spectrum analgesia.

DECLARATIONS OF INTEREST

None.

AUTHOR CONTRIBUTIONS

DM performed PCR and immunofluorescence experiments; VV, CG performed calcium imaging assays; MA performed immunohistochemistry; CS contributed to the manuscript writing and analysed the data; RL designed research, performed behavioural experiments, analysed the data and wrote the manuscript. All authors reviewed the results, read and approved the final version of the manuscript.

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(Received 16 July 2019, Accepted 2 December 2019)
(Available online 26 December 2019)