

# Low Nociceptor GRK2 Prolongs Prostaglandin E<sub>2</sub> Hyperalgesia via Biased cAMP Signaling to Epac/Rap1, Protein Kinase C $\epsilon$ , and MEK/ERK

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Hyperexcitability of peripheral nociceptive pathways is often associated with inflammation and is an important mechanism underlying inflammatory pain. Here we describe a completely novel mechanism via which nociceptor G-protein-coupled receptor kinase 2 (GRK2) contributes to regulation of inflammatory hyperalgesia. We show that nociceptor GRK2 is downregulated during inflammation. In addition, we show for the first time that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)-induced hyperalgesia is prolonged from <6 h in wild-type (WT) mice to 3 d in mice with low GRK2 in Na<sub>v</sub>1.8<sup>+</sup> nociceptors (*SNS-GRK2*<sup>+/-</sup> mice). This prolongation of PGE<sub>2</sub> hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice does not depend on changes in the sensitivity of the prostaglandin receptors because prolonged hyperalgesia also developed in response to 8-Br-cAMP. PGE<sub>2</sub> or cAMP-induced hyperalgesia in WT mice is PKA dependent. However, PKA activity is not required for hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice. *SNS-GRK2*<sup>+/-</sup> mice developed prolonged hyperalgesia in response to the Exchange proteins directly activated by cAMP (Epac) activator 8-pCPT-2'-O-Me-cAMP (8-pCPT). Coimmunoprecipitation experiments showed that GRK2 binds to Epac1. *In vitro*, GRK2 deficiency increased 8-pCPT-induced activation of the downstream effector of Epac, Rap1, and extracellular signal-regulated kinase (ERK). *In vivo*, inhibition of MEK1 or PKC $\epsilon$  prevented prolonged PGE<sub>2</sub>, 8-Br-cAMP, and 8-pCPT hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice. In conclusion, we discovered GRK2 as a novel Epac1-interacting protein. A reduction in the cellular level of GRK2 enhances activation of the Epac-Rap1 pathway. *In vivo*, low nociceptor GRK2 leads to prolonged inflammatory hyperalgesia via biased cAMP signaling from PKA to Epac-Rap1, ERK/PKC $\epsilon$  pathways.

## Introduction

Inflammatory mediators sensitize primary sensory neurons, causing increased responsiveness to noxious stimuli (hyperalgesia). The well-known analgesic effect of inhibition of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis by COX-2 inhibitors clearly points toward a major role for this eicosanoid in inflammatory pain (Aley and Levine, 1999; Southall and Vasko, 2001; Moriyama et al., 2005). The action of PGE<sub>2</sub> on primary neuronal afferents can be mediated via various subtypes of PGE<sub>2</sub> receptors (EP1–EP4) that all belong to the class of G-protein-coupled receptors (GPCRs). At the molecular level, it is known that local administration of PGE<sub>2</sub> induces transient hyperalgesia and nociceptor sensitization via activation of the cAMP/PKA-dependent second-messenger system (Minami et al., 2001; Southall and Vasko,

2001; Lin et al., 2006). When animals are primed by a previous transient inflammation, PGE<sub>2</sub> hyperalgesia is prolonged and the signaling pathway switches from a cAMP/PKA-dependent pathway to a PKA, PKC $\epsilon$ , and extracellular signal-regulated kinase (ERK)-mediated route (Aley et al., 2000; Dina et al., 2003).

The kinase GRK2 regulates GPCR signaling via interference at various levels of the signal transduction cascade. GRK2 phosphorylates agonist-occupied GPCR, leading to receptor desensitization and internalization (Zhang et al., 1997; Ribas et al., 2007). GRK2 also limits signaling via direct interaction with specific downstream intracellular kinases such as Akt, MEK1/2, phosphoinositide-3 kinase, and p38, leading to inhibition of their activity (Reiter and Lefkowitz, 2006; Ribas et al., 2007). In addition, GRK2 binds and phosphorylates a variety of cytoskeletal proteins, including tubulin, ezrin, and synuclein (Haga et al., 1998; Pitcher et al., 1998; Pronin et al., 2000; Cant and Pitcher, 2005).

We and others have shown that the intracellular level of GRK2 is tightly regulated. *In vivo*, the level of GRK2 in leukocytes from patients with autoimmune diseases such as rheumatoid arthritis and multiple sclerosis is decreased, and similar changes in GRK2 expression occur in animals with experimental autoimmune en-

Received June 17, 2010; revised July 26, 2010; accepted Aug. 1, 2010.

We thank Dr. Gerald W. Dorn II at Washington University (St. Louis, MO) for providing GRK2-LOX mice.

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DOI:10.1523/JNEUROSCI.3142-10.2010

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cephalomyelitis and adjuvant arthritis (Lombardi et al., 1999; Vroon et al., 2005, 2006). Moreover, neuropathic pain in animals subjected to nerve ligation is associated with decreased spinal cord neuronal and microglial GRK2 expression, suggesting that GRK2 may contribute to regulation of pain signaling under inflammatory conditions (Kleibecker et al., 2007, 2008; Eijkelkamp et al., 2010).

Recently, we described that a 50% reduction of GRK2 in nociceptors (*SNS-GRK2<sup>+/-</sup>* mice) increased and prolonged carrageenan-induced inflammatory hyperalgesia in mice (Eijkelkamp et al., 2010). The aim of this study is to investigate the mechanisms as to how neuronal GRK2 biases signaling responsible for the increased duration of hyperalgesia induced by a single injection of the prototypic inflammatory mediator PGE<sub>2</sub> in conditions of low nociceptor GRK2.

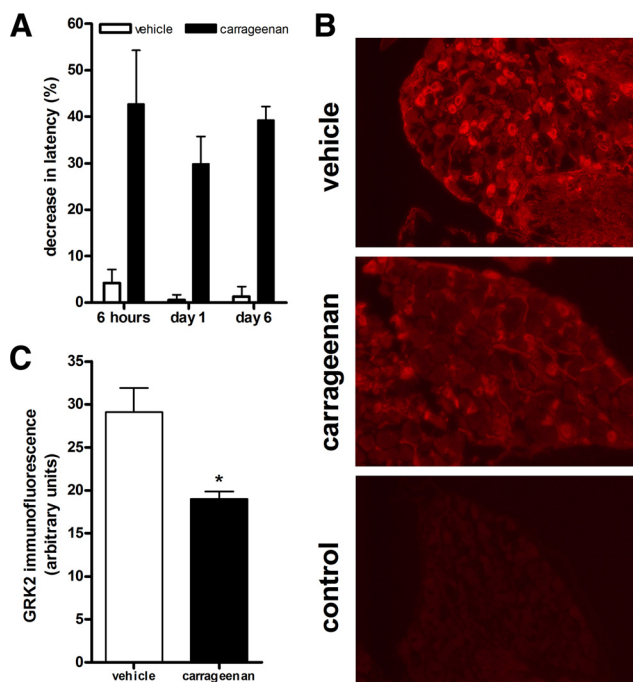
## Materials and Methods

**Animals.** Female C57BL/6 (12–14 weeks) *SNS-GRK2<sup>+/-</sup>*, *SNS-GRK2<sup>-/-</sup>*, and control *SNS-GRK2<sup>+/+</sup>* offspring was generated by breeding homozygous *Nav1.8-CRE* transgenic mice (Stirling et al., 2005; Abrahamson et al., 2008) with heterozygous GRK2-*LOX* mice (Matkovich et al., 2006). Mice heterozygous for targeted deletion of the GRK2 gene (*GRK2<sup>+/-</sup>*) and their wild-type (WT) littermates were used (Jaber et al., 1996). Because *GRK2<sup>-/-</sup>* mice die *in utero*, only *GRK2<sup>+/-</sup>* mice can be used for experiments. Inducible *GRK2<sup>+/-</sup>* mice were generated by crossing transgenic mice overexpressing Cre-recombinase protein fused to two mutant estrogen receptor ligand-binding domains under the control of the  $\alpha$ -myosin heavy chain ( $\alpha$ MHC) promoter ( $\alpha$ MHC-MerCreMer) (Sohal et al., 2001) (The Jackson Laboratory) with heterozygous mice with the floxed GRK2 alleles. To induce Cre expression, adult mice were treated with tamoxifen (2 mg/mouse, i.p., in saline containing 9% ethanol and 51% sunflower oil for 5 consecutive days) (Sigma-Aldrich). GRK2 expression in dorsal root ganglia (DRGs) homogenates was determined 2 weeks after the last injection of tamoxifen. Female C57BL/6 *GRK6<sup>-/-</sup>* and WT littermates were generated by breeding heterozygous *GRK6<sup>+/-</sup>* mice (Eijkelkamp et al., 2007).

Mice were genotyped by PCR analysis on genomic DNA. Mice were bred and maintained in the animal facility of the University of Utrecht (Utrecht, The Netherlands). All experiments were performed in accordance with international guidelines and approved by the University Medical Center Utrecht Experimental Animal Committee.

**Measurement of thermal hyperalgesia.** Heat-withdrawal latency times were determined using the Hargreaves (IITC Life Science) test as described previously (Hargreaves et al., 1988). Intensity of the light beam was chosen to induce a heat-withdrawal latency time at baseline of ~8 s. Baseline withdrawal latencies were determined three times before intraplantar injection of the inflammatory agent.

**Pharmacological agents.** All pharmacological agents were injected intraplantarly in a volume of 2.5  $\mu$ l, except that H89 (*N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-isquinoline-sulfonamide 2HCl) was injected in a volume of 5  $\mu$ l. A solution of 40  $\mu$ g/ml PGE<sub>2</sub> (Sigma-Aldrich) was made in saline containing <1% ethanol. EP receptor agonists were applied at a dose equivalent to the effective dose of PGE<sub>2</sub> for that particular receptor subtype. Dissociation constant of the compounds were obtained from manufactures and <http://www.iuphar-db.org/index.jsp>. The EP4 agonist L-902688 (5-[(1*E*, 3*R*)-4, 4-difluoro-3-hydroxy-4-phenyl-1-buten-1-yl]-1-[6-(2*H*-tetrazol-5*R*-yl)hexyl]-2-pyrrolidinone) (18.85  $\mu$ g/ml; generous gift from Merck Frosst Canada Ltd.), the EP1/3 agonist sulprostone [*N*-(methylsulfonyl)-9-oxo-11 $\alpha$ , 15*R*-dihydroxy-16-phenoxo-17,18,19,20-tetranor-prosta-5*Z*, 13*E*-dien-1-amide] (40  $\mu$ g/ml; Sigma-Aldrich), the EP2 agonist ONO-AE1-259-01 [(*z*)-7-[(1*R*, 2*R*, 3*R*, 5*R*)-5-chloro-3-hydroxy-2-[(*E*, 4*S*)-4-hydroxy-4-(1-prop-2-enylcyclobutyl)but-1-enyl]cyclopentyl]hept-5-enoic acid] (12.23  $\mu$ g/ml; generous gift from Ono Pharmaceutical Co. Ltd.), or the specific EP3 agonist ONO-AE-248 [(*z*)-7-[(1*R*, 2*R*, 3*R*)-3-methoxy-2-[(*E*, 3*S*)-3-methoxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid] (0.41 mg/ml; generous gift from Ono Pharmaceuticals) were dissolved in saline containing 10–20% DMSO. 8-Br-cAMP (4  $\mu$ g/ml; Sigma-Aldrich), the PKA activator 6-Bnz-cAMP (4.46  $\mu$ g/ml; Biolog LifeScience Institute), and the Epac activator 8-pCPT-2'-*O*-Me-cAMP (8-pCPT) (5.04  $\mu$ g/ml; Biolog LifeScience Institute) were dissolved in saline.

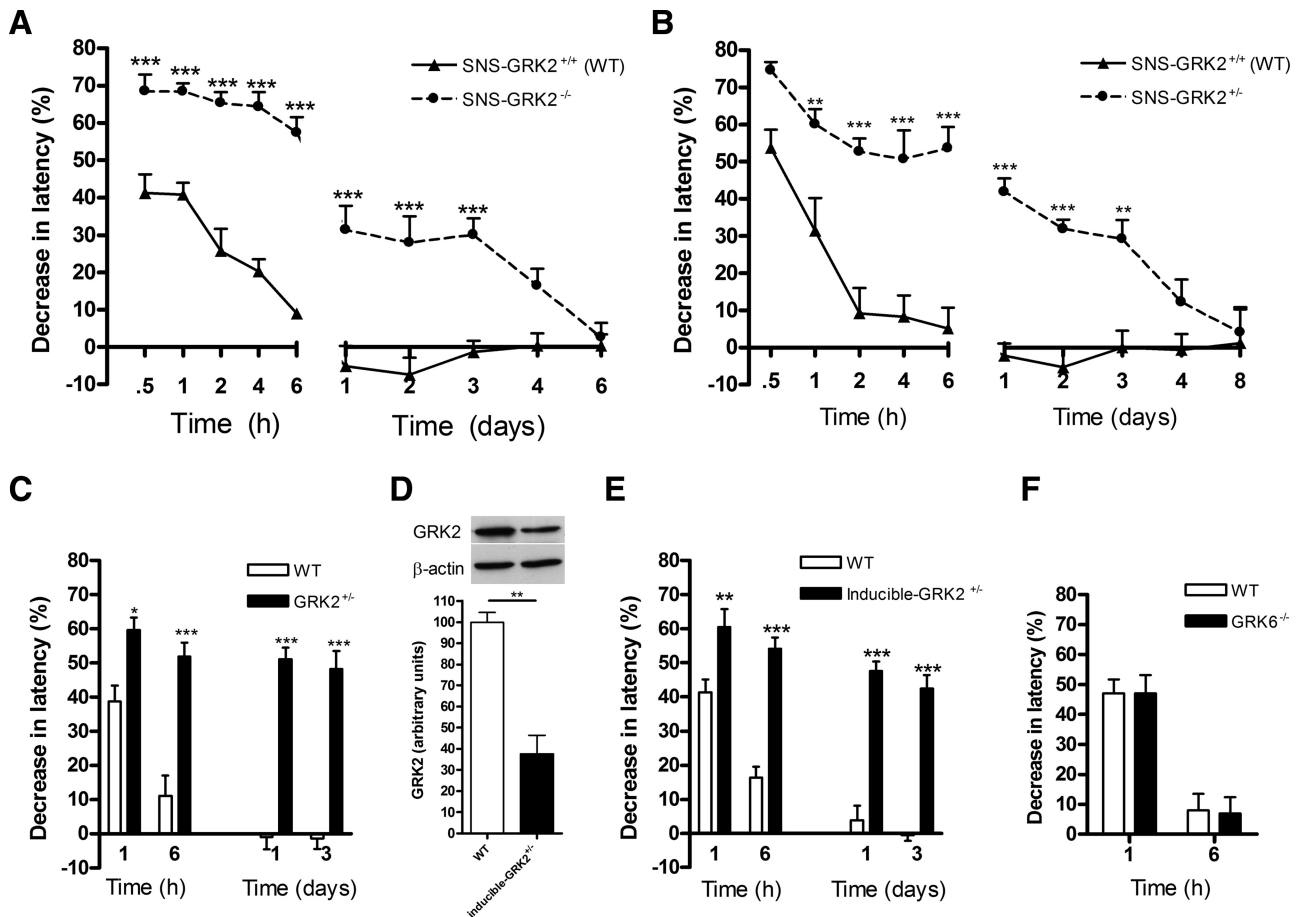


**Figure 1.** Chronic carrageenan hyperalgesia is associated with reduced GRK2 in DRG neurons. **A**, Percentage decrease in heat-withdrawal latency time after intraplantar carrageenan injection (20  $\mu$ l, 2%) in WT mice ( $n = 8–12$  per genotype). **B**, GRK2 expression in dorsal root ganglia isolated 6 d after intraplantar carrageenan or saline administration was compared by immunofluorescence analysis. Representative pictures of GRK2 staining of dorsal root ganglia. **C**, Bar graph represents average GRK2 immunofluorescence intensity of ~40 neurons on two to three different slides per animal ( $n = 4$  animals per group). Data are expressed as mean  $\pm$  SEM fluorescence intensity. \* $p < 0.05$ .

Inhibitors were injected intraplantarly 15 min before the injection of the pharmacological agents that induced hyperalgesia. The MEK1 inhibitor PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one] (1 mg/ml Sigma-Aldrich) (Dina et al., 2003) was dissolved in saline containing 20% DMSO. The PKA inhibitor H89 (5.4 mg/ml; Sigma-Aldrich) (Cunha et al., 1999) or the PKC $\epsilon$  translocation inhibitory peptide (TAT-PKC $\epsilon_{V1-2}$ ; YGRKKRRRQRRRCCEAVSLKPT-COOH) or scrambled control peptide [1 mg/ml (W. M. Keck Facility, Yale University, New Haven, CT); YGRKKRRRQRRRCSETKPAV-COOH] were dissolved in saline.

**Carrageenan paw inflammation and immunohistochemical staining of dorsal root ganglia.** Mice received an intraplantar injection of 20  $\mu$ l of  $\lambda$ -carrageenan [2% (w/v); Sigma-Aldrich] in saline in both hindpaws. At day 6 after carrageenan, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused intracardially with 0.9% saline containing, followed by 4% paraformaldehyde in PBS. DRGs of lumbar level 2–6 (L2–L6) were isolated, postfixed for 4 h at 4°C, and then kept overnight in 20% sucrose in PBS at 4°C, followed by 30% sucrose in PBS for 24–48 h. Dissected tissue was mounted in OCT compound and frozen at  $-20^{\circ}\text{C}$ . DRG sections (10  $\mu$ m) were cut using a cryostat. Sections were washed in PBS and blocked in 5% normal goat serum, 2% bovine serum albumin, and 0.3% Triton X-100. Sections were then incubated overnight at 4°C with rabbit anti-GRK2 (1:200; Santa Cruz Biotechnology). GRK2 staining was controlled by using primary anti-GRK2 antibody blocked with a GRK2 blocking peptide (Santa Cruz Biotechnology). Tissue samples were then washed and incubated with Alexa Fluor 594-conjugated goat anti-rabbit antibody (1:200; Invitrogen). Cells were photographed with an EVOS fl (AMG Inc.), and GRK2 levels in small-diameter DRG neurons were analyzed with NIH ImageJ software.

The average background fluorescence (primary GRK2 antibody plus GRK2 blocking peptide) of 15 small-diameter neurons of DRGs from vehicle- and carrageenan-treated animals was subtracted before calculation of the percentage change in GRK2 staining. Exposure times of photographs were identical for all slices. All stainings were done in parallel for DRGs from vehicle- and carrageenan-treated mice.



**Figure 2.** Reduced nociceptor GRK2 prolongs PGE<sub>2</sub> hyperalgesia. **A**, Percentage decrease in heat-withdrawal latency time after intraplantar PGE<sub>2</sub> injection (100 ng/paw) in *SNS-GRK2*<sup>+/+</sup> control animals and in *SNS-GRK2*<sup>-/-</sup> mice ( $n = 8$  per genotype). **B**, Percentage decrease in heat-withdrawal latency times after intraplantar PGE<sub>2</sub> injection in *SNS-GRK2*<sup>+/+</sup> control animals and *SNS-GRK2*<sup>+/-</sup> mice ( $n = 8$  per genotype). **C**, Percentage decrease in heat-withdrawal latency time after intraplantar PGE<sub>2</sub> injection (100 ng/paw) in WT and *GRK2*<sup>+/-</sup> mice ( $n = 8$  per genotype). **D**, GRK2 levels in dorsal root ganglia of inducible *GRK2*<sup>+/-</sup> and WT mice after tamoxifen treatment ( $n = 3$ ). **E**, Percentage decrease in heat-withdrawal latency time after intraplantar PGE<sub>2</sub> injection in WT mice and inducible *GRK2*<sup>+/-</sup> ( $n = 7–8$  per genotype). **F**, Percentage decrease in heat-withdrawal latency time after intraplantar PGE<sub>2</sub> injection in WT and *GRK6*<sup>-/-</sup> mice ( $n = 7–8$  per genotype). Data are expressed as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

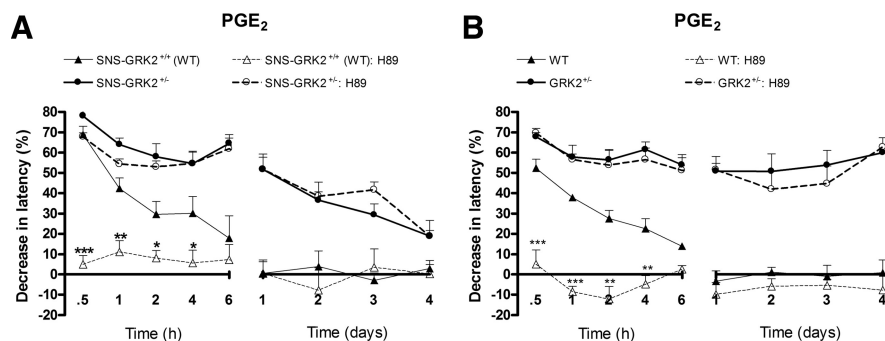
**DRG cell culture.** Dorsal root ganglia were obtained from adult WT and *GRK2*<sup>+/-</sup> mice. Ganglia were digested in collagenase (type XI, 0.6 mg/ml; Sigma-Aldrich), protease (*Streptomyces griseus*, 0.4 mg/ml; Sigma-Aldrich), and glucose (1.8 mg/ml; Sigma-Aldrich) in Ca<sup>2+</sup> and Mg<sup>2+</sup>-free PBS for 40 min before mechanical trituration. Cells were then washed and resuspended in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 2 mM glutamine (Invitrogen), 10,000 IU/ml penicillin–streptomycin (Invitrogen), and 100 ng/ml nerve growth factor (NGF) (Sigma-Aldrich) and plated in a 24-well plate coated with poly-L-lysine and laminin. Cells were stimulated 15–25 h after plating and after 4 h of NGF starvation. Cells were homogenized in RAL lysis buffer [200 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM MgCl<sub>2</sub>, 10% glycerol, 1% NP-40 containing a mixture of protease inhibitors (Sigma) with the addition of 2 mM NaF, 10 mM  $\beta$ -glycerol-phosphate, and 2 mM phenylmethylsulfonyl fluoride].

**Splenocyte isolation.** Single-cell suspensions of spleens from adult WT and *GRK2*<sup>+/-</sup> mice were obtained by dispersing the tissue through a 70  $\mu$ m filter in cold RPMI (Invitrogen). Erythrocytes were removed by lysis in hypotonic buffer for 2 min. Subsequently, splenocytes were washed twice in RPMI supplemented with 5% fetal bovine serum and 50  $\mu$ M  $\beta$ -mercaptoethanol. Cells were stimulated for the indicated time periods with 8-pCPT (100  $\mu$ M; Biolog LifeScience Institute).

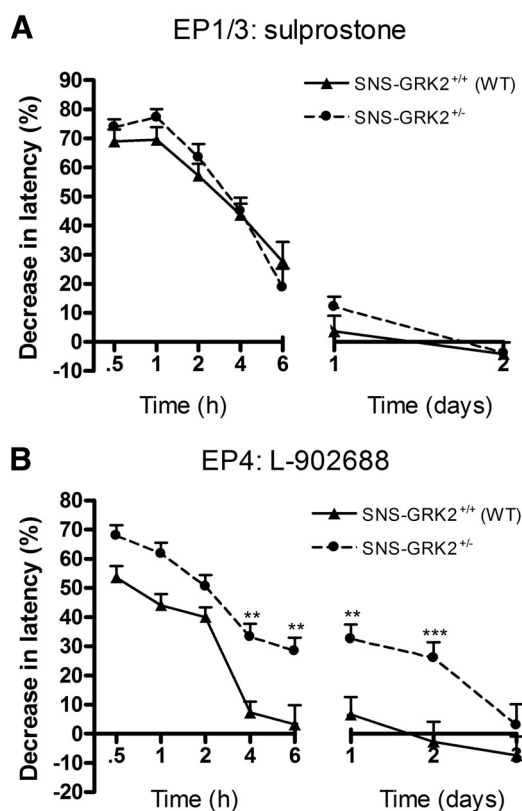
**Rap1 activation assay using RalGDS-RBD.** We used splenocytes as a source of primary cells from WT and *GRK2*<sup>+/-</sup> mice because these cells are easily available in large amounts and do respond to 8-pCPT. After stimulation with 8-pCPT, isolated splenocytes were lysed by addition of 700  $\mu$ l of cold RAL lysis buffer. Lysates were clarified by centrifugation at maximal

speed in an Eppendorf centrifuge for 10 min at 4°C. Rap activation assays were performed as described previously (Franke et al., 1997). Briefly, equal amounts of cell lysates were incubated with glutathione–agarose beads that were precoupled with glutathione S-transferase fused to the minimal Ras-binding domain (RBD) of Ral-guanine nucleotide-dissociation stimulator (GDS) to specifically pulldown-activated GTP-bound forms of Rap1. The amount of Rap1 was quantified by Western blotting. Total Rap1 in cell lysates was determined to verify equal input into the Rap1–GTP pulldown assay.

**Cell transfection and immunoprecipitation.** HEK293a cells were transfected with hemagglutinin (HA)-tagged Epac1 and as control HA–tuberous sclerosis 1 (TSC1) using Lipofectamine (Invitrogen) following the protocol of the manufacturer. Transfected HEK293a cells or spinal cord or dorsal root ganglion tissue were washed in PBS and lysed in ice-cold modified radioimmunoprecipitation assay buffer [20 mM Tris-HCl, pH 8, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, containing a mixture of protease inhibitors (Sigma) with the addition of 2 mM NaF and 10 mM  $\beta$ -glycerol-phosphate]. The lysate was centrifuged at 14,000  $\times$   $g$  for 10 min at 4°C. The cell lysate (200  $\mu$ g) was precleared by adding 50  $\mu$ l of 50% Protein A Sepharose bead slurry per 200  $\mu$ l of cell lysate. Mouse anti-GRK2/3 (Millipore Corporation) or mouse anti-HA (Invitrogen) were coupled to Sepharose A beads overnight at 4°C with gentle tumbling. These beads were added to the lysate for 3 h at 4°C with gentle tumbling. The Sepharose beads were washed three times with ice-cold wash buffer (5 mM Tris-HCl, pH 7.4, 20 mM NaCl, and 0.5% Triton X-100) and resuspended in 50  $\mu$ l of 2 $\times$  sample buffer, followed by elution through boiling.



**Figure 3.** PGE<sub>2</sub> hyperalgesia is PKA independent in *SNS-GRK2*<sup>+/-</sup> and *GRK2*<sup>+/-</sup> mice. Percentage change in heat-withdrawal latencies after intraplantar injection of the PKA inhibitor H89 (27 μg/paw) before PGE<sub>2</sub> injection in *SNS-GRK2*<sup>+/-</sup> (WT) animals and *SNS-GRK2*<sup>+/-</sup> mice (*n* = 4 per group) (**A**) or WT and *GRK2*<sup>+/-</sup> mice (*n* = 4 per group) (**B**). Data are expressed as mean ± SEM. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 *SNS-GRK2*<sup>+/-</sup> (WT) versus *SNS-GRK2*<sup>+/-</sup> (WT) treated with inhibitor.



**Figure 4.** Reduced nociceptor GRK2 only affects hyperalgesia induced by an EP4 agonist. **A**, Percentage decrease in heat-withdrawal latency times after intraplantar sulprostone (EP1/3 agonist) injection in *SNS-GRK2*<sup>+/-</sup> (WT) and *SNS-GRK2*<sup>+/-</sup> mice (*n* = 8 per genotype). **B**, Percentage decrease in heat-withdrawal latency time after intraplantar L-902688 (EP4 agonist) injection in *SNS-GRK2*<sup>+/-</sup> control animals and in *SNS-GRK2*<sup>+/-</sup> (*n* = 8 per genotype). Data are expressed as mean ± SEM. \*\**p* < 0.01, \*\*\**p* < 0.001.

**Western blot analysis.** Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corporation). Blots were stained with rabbit anti-GRK2 (Santa Cruz Biotechnology), mouse anti-p-ERK1/2 (Santa Cruz Biotechnology), rabbit anti-ERK1/2 (Cell Signaling Technology), rabbit anti-Rap1 (Santa Cruz Biotechnology), mouse anti-Epac1 (Price et al., 2004), mouse anti-Epac2 (generated at Department of Physiological Chemistry of Dr. J. L. Bos, University Medical Center Utrecht, Utrecht, The Netherlands), mouse anti-HA-HRP (Miltenyi), mouse anti-α-tubulin (Santa Cruz Biotechnology), and goat anti-β-actin (Santa Cruz Biotechnology). Subsequently, blots were incubated for 1 h with goat

anti-mouse peroxidase IgG plus IgM (heavy and light chain) (Jackson Immuno-Research) or donkey anti-rabbit peroxidase IgG (GE Healthcare) and developed by enhanced chemiluminescence (GE Healthcare). Band density was determined using a GS-700 Imaging Densitometer (Bio-Rad).

**Data analysis.** Data are expressed as mean ± SEM. Measurements were compared using Student's *t* test, one-way ANOVA, or two-way ANOVA followed by Bonferroni's analysis. A *p* value < 0.05 was considered to be statistically significant.

## Results

### GRK2 protein levels in sensory neurons during peripheral paw inflammation

To test whether ongoing inflammation changes GRK2 levels in sensory neurons,

we injected a dose of carrageenan that induces long-lasting (>6 d) paw inflammation (Honore et al., 2006). We quantified GRK2 levels in small-diameter neurons in DRG from mice that had received an intraplantar injection of 20 μl of a 2% carrageenan solution. Intraplantar injection of this high dose of carrageenan induced hyperalgesia that was still present at day 6 (Fig. 1A). At this time point, there was a reduction in GRK2 levels in small-diameter sensory neurons of ~35% compared with vehicle-treated animals (Fig. 1B). The average diameter of measured small-diameter neurons in which GRK2 was quantified was 14.30 ± 0.08 μm in saline-treated and 14.65 ± 0.08 μm in carrageenan-treated animals.

### Contribution of GRK2 to PGE<sub>2</sub>-induced hyperalgesia

To examine the contribution of nociceptor GRK2 to PGE<sub>2</sub> hyperalgesia, we used mice homozygous (*SNS-GRK2*<sup>-/-</sup>) or heterozygous (*SNS-GRK2*<sup>+/-</sup>) for deletion of the GRK2 gene in Na<sub>v</sub>1.8<sup>+</sup> sensory neurons. Mice homozygous for GRK2 deletion in all cells die *in utero*. In *SNS-GRK2*<sup>+/-</sup> mice, GRK2 protein levels are reduced by ~50% in DRGs compared with *SNS-GRK2*<sup>+/-</sup> (WT) controls (Eijkelkamp et al., 2010). This reduction is similar to the reduction in GRK2 observed in small-diameter sensory neurons during chronic carrageenan-induced paw inflammation (Fig. 1).

*SNS-GRK2*<sup>-/-</sup> mice, *SNS-GRK2*<sup>+/-</sup> mice, and *SNS-GRK2*<sup>+/-</sup> (WT) littermates were injected intraplantarly with PGE<sub>2</sub>. PGE<sub>2</sub> hyperalgesia was significantly increased in *SNS-GRK2*<sup>-/-</sup> mice (Fig. 2A) and *SNS-GRK2*<sup>+/-</sup> mice (Fig. 2B) compared with *SNS-GRK2*<sup>+/-</sup> (WT) littermates. Moreover, PGE<sub>2</sub>-induced hyperalgesia was significantly prolonged and lasted 3 d in *SNS-GRK2*<sup>-/-</sup> mice and *SNS-GRK2*<sup>+/-</sup> mice, whereas *SNS-GRK2*<sup>+/-</sup> (WT) littermates recovered within 4–6 h (Fig. 2A,B). The severity and duration of PGE<sub>2</sub> hyperalgesia was similar in *SNS-GRK2*<sup>-/-</sup> and *SNS-GRK2*<sup>+/-</sup> mice. Intraplantar saline injection did not induce a detectable change in thermal sensitivity in any genotype. Baseline latency times of *SNS-GRK2*<sup>-/-</sup> mice were significantly longer than in *SNS-GRK2*<sup>+/-</sup> (WT) mice (8.1 ± 0.2 vs 10.7 ± 0.5 s; *n* = 14, *p* < 0.001). However, in *SNS-GRK2*<sup>+/-</sup> mice, baseline heat-withdrawal latency times were comparable with WT littermates (8.3 ± 0.1 vs 8.3 ± 0.1 s; *n* = 40) (Eijkelkamp et al., 2010). In view of the fact that inflammation reduces GRK2 from 35 to 50% in various cell types including DRG, we decided to use mice heterozygous for deletion of the GRK2 gene in Na<sub>v</sub>1.8<sup>+</sup> nociceptors (*SNS-GRK2*<sup>+/-</sup> mice) and *SNS-GRK2*<sup>+/-</sup> (WT) littermate controls for additional experiments.

To address the question whether the increased and prolonged PGE<sub>2</sub> hyperalgesia in GRK2-deficient mice was caused

by developmental adaptation to GRK2 deficiency, we also used tamoxifen-treated inducible *GRK2*<sup>+/-</sup> mice compared with *GRK2*<sup>+/-</sup> mice that have a 40–60% decrease in GRK2 protein in all cells throughout development (Kleibauer et al., 2008; Eijkelkamp et al., 2010).

PGE<sub>2</sub>-induced thermal hyperalgesia was increased and prolonged in *GRK2*<sup>+/-</sup> mice (Fig. 2C). Baseline heat-withdrawal latencies in *GRK2*<sup>+/-</sup> mice did not differ from WT mice as we described previously (7.8 ± 0.1 vs 8.0 ± 0.1 s; *n* = 30) (Eijkelkamp et al., 2010). Tamoxifen-treated inducible *GRK2*<sup>+/-</sup> mice show an ~50% reduction in GRK2 levels in DRG tissue extracts compared with WT mice (Fig. 2D). Importantly, tamoxifen-inducible *GRK2*<sup>+/-</sup> mice developed increased and prolonged PGE<sub>2</sub> hyperalgesia similar to that observed in *GRK2*<sup>+/-</sup> mice (Fig. 2E). Tamoxifen-treated *GRK2*<sup>+/-</sup> mice responded like WT mice and only developed a transient PGE<sub>2</sub> hyperalgesia (Fig. 2E). Baseline heat-withdrawal latency times did not differ between tamoxifen-treated *GRK2*<sup>+/-</sup> and *GRK2*<sup>+/+</sup> mice (8.5 ± 0.2 vs 8.4 ± 0.3 s; *n* = 8).

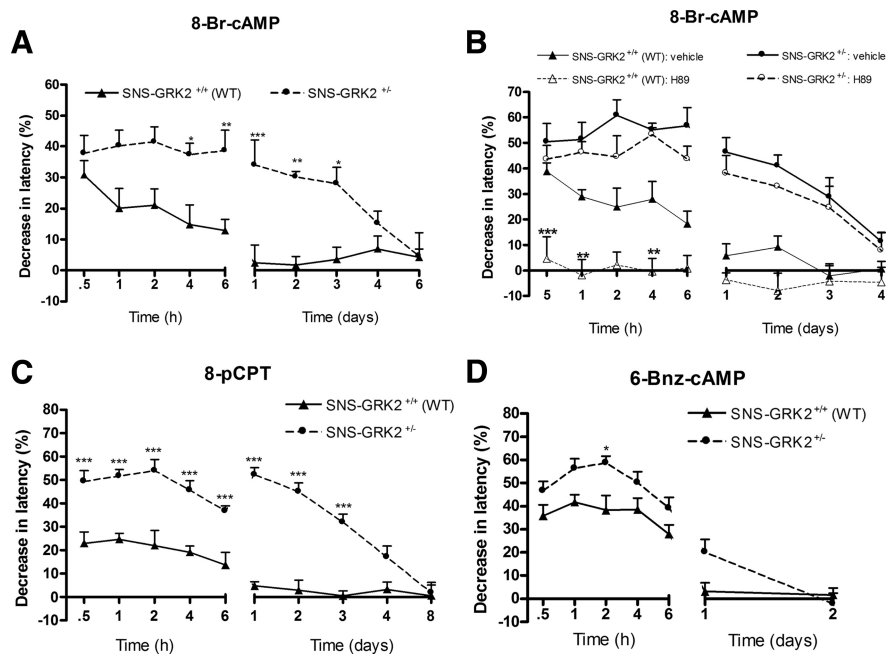
To test whether the effect on the regulation of the hyperalgesic response to PGE<sub>2</sub> is specific for GRK2, we also determined the magnitude and duration of PGE<sub>2</sub> hyperalgesia in *GRK6*<sup>-/-</sup> mice. In Figure 2F, it is shown that deletion of GRK6 does not affect the intensity and duration of PGE<sub>2</sub> hyperalgesia.

### Contribution of PKA in PGE<sub>2</sub> hyperalgesia in WT and *SNS-GRK2*<sup>+/-</sup> mice

In WT rats, PGE<sub>2</sub> hyperalgesia is mediated via a cAMP/PKA-dependent pathway (Dina et al., 2001, 2003). To evaluate whether the increased and prolonged hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice is mediated via (increased) activation of PKA, mice were pre-treated with an intraplantar injection of the PKA inhibitor H89. In line with data in the literature, H89 completely prevented PGE<sub>2</sub> hyperalgesia in *SNS-GRK2*<sup>+/+</sup> (WT) mice (Fig. 3A). In contrast, H89 did not affect PGE<sub>2</sub> hyperalgesia in mice with reduced nociceptor GRK2 (Fig. 3A), indicating that PGE<sub>2</sub> activates signaling cascades independent of PKA, leading to prolonged hyperalgesia in mice with reduced nociceptor GRK2. In *GRK2*<sup>+/-</sup> mice with low GRK2 in all cells, PGE<sub>2</sub> hyperalgesia was also independent of PKA activity (Fig. 3B).

### Role of nociceptor GRK2 in EP receptor subtype agonist-induced hyperalgesia

All four cloned PGE<sub>2</sub> receptor subtypes (EP receptors 1–4) are members of the G-protein-coupled receptor family and are expressed on nerve terminals of nociceptors (Oida et al., 1995). The EP receptors couple to different G proteins, leading to the activation of different signaling cascades. EP1 receptors couple to G<sub>q</sub>, and activation of the receptor lead to an increase in inositol trisphosphate; EP2 and EP4 receptors couple to G<sub>s</sub> and cause an increase in cAMP formation. EP3 receptors couple to G<sub>i</sub>, leading to a decrease in cAMP formation (Coleman et al., 1994). In ro-

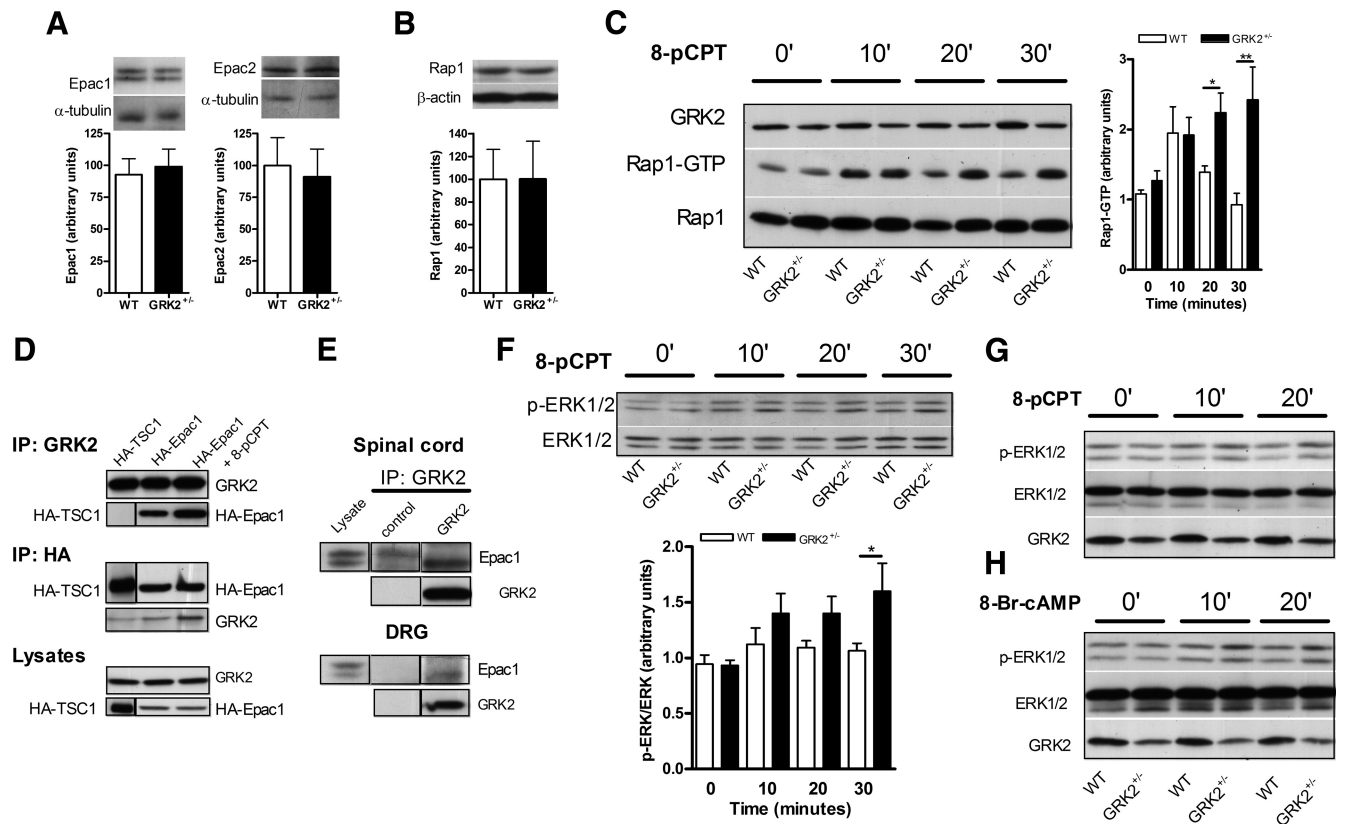


**Figure 5.** Reduced nociceptor GRK2 specifically prolongs cAMP- and Epac-mediated hyperalgesia but not PKA-mediated hyperalgesia. **A**, Percentage change in heat-withdrawal latencies after intraplantar injection of 8-Br-cAMP (10 ng/paw) in *SNS-GRK2*<sup>+/+</sup> (WT) animals and *SNS-GRK2*<sup>+/-</sup> mice (*n* = 8 per genotype). **B**, Percentage change in heat-withdrawal latencies after intraplantar injection of the PKA inhibitor H89 (27 μg/paw) before 8-Br-cAMP injection in *SNS-GRK2*<sup>+/+</sup> (WT) animals and *SNS-GRK2*<sup>+/-</sup> mice (*n* = 4 per group). **C**, Percentage decrease in heat-withdrawal latency time after intraplantar injection of 8-pCPT; 12.6 ng/paw) in *SNS-GRK2*<sup>+/+</sup> control animals and in *SNS-GRK2*<sup>+/-</sup> (*n* = 12 per genotype). **D**, Percentage decrease in heat-withdrawal latency times after intraplantar injection of 6-Bnz-cAMP (cAMP analog that specifically activates PKA; 11.2 ng/paw) in *SNS-GRK2*<sup>+/+</sup> control mice and *SNS-GRK2*<sup>+/-</sup> mice (*n* = 8 per genotype). Data are expressed as mean ± SEM. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

idents, EP4 and to a lesser extent EP1 and EP3 have been shown to mediate PGE<sub>2</sub> hyperalgesia (Minami et al., 2001; Lin et al., 2006). We used EP receptor subtype-specific agonists to determine whether prolonged hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice was caused by a switch in the use of EP receptor subtype(s). The EP1/3 agonist sulprostone induced transient hyperalgesia that did not differ between genotypes (Fig. 4A). Intraplantar injection of the specific EP2 agonist ONO-AE1-259-01 or the specific EP3 agonist ONO-AE-248 did not significantly affect thermal sensitivity in both genotypes (ONO-AE1-259-01: 9.7 ± 4.6 vs 8.0 ± 6.0% maximal decrease in latency, *n* = 8; ONO-AE-248: 6.5 ± 3.7 vs 12.0 ± 5.4% maximal decrease in latency, *n* = 8, *p* = 0.42). However, the EP4 agonist L-902688 completely mimicked the effect of PGE<sub>2</sub>; L-902688-induced transient hyperalgesia in WT mice and L-902688 hyperalgesia was increased and prolonged in *SNS-GRK2*<sup>+/-</sup> mice (Fig. 4B). Because the specific receptor antagonists were not available, we could not test the receptor specificity in more detail. Nonetheless, our current data indicate that the prolonged PGE<sub>2</sub>-induced hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice is likely to rely on EP4 receptor signaling.

### Reduced nociceptor GRK2 regulates hyperalgesia downstream of cAMP

To test whether the prolongation of hyperalgesia in mice with reduced nociceptor GRK2 is cAMP-mediated but PKA independent, we used the cAMP analog 8-Br-cAMP; H89 was applied to block PKA activation. 8-Br-cAMP-induced hyperalgesia was significantly prolonged in *SNS-GRK2*<sup>+/-</sup> mice compared with control *SNS-GRK2*<sup>+/+</sup> littermates; 8-Br-cAMP-induced hyperalgesia lasted for 3 d in *SNS-GRK2*<sup>+/-</sup> mice, whereas *SNS-GRK2*<sup>+/+</sup> (WT) littermates recovered within 6 h (Fig. 5A). Acute 8-Br-cAMP-induced



**Figure 6.** Low primary sensory neuron GRK2 enhances 8-pCPT-induced Rap1 and ERK1/2 activation *in vitro*. **A**, Epac1 and Epac2 expression levels in isolated primary sensory neurons of WT and GRK2<sup>+/-</sup> mice ( $n = 5$  per genotype). **B**, Rap1 expression levels in isolated primary sensory neurons of WT and GRK2<sup>+/-</sup> mice ( $n = 4$  per genotype). **C**, 8-pCPT-induced Rap1 activation in splenocytes from WT and GRK2<sup>+/-</sup> mice. Bar graphs show quantification of Rap1 activation after 8-pCPT (100  $\mu$ M) treatment ( $n = 4-6$  per genotype). **D**, HA-Epac1, HA-TSC1, or GRK2 was immunoprecipitated (IP) from lysates of HEK293 cells that were transfected with HA-Epac or with HA-TSC1 as a control. 8-pCPT (100  $\mu$ M) was added during the precipitation to keep Epac in an activated conformation. **E**, Immunoprecipitates of GRK2 from lysates of either spinal cord or DRG immunoblotted for Epac1. **F**, 8-pCPT-induced ERK1/2 phosphorylation in splenocytes from WT and GRK2<sup>+/-</sup> mice. Bar graphs show quantification of activated p-ERK1/2 after 8-pCPT treatment (100  $\mu$ M;  $n = 3-6$  per genotype). **G**, 8-pCPT-induced (100  $\mu$ M) ERK1/2 phosphorylation in primary sensory neurons of WT and GRK2<sup>+/-</sup> mice. **H**, cAMP-induced (1 mM) ERK1/2 phosphorylation in primary sensory neurons of WT and GRK2<sup>+/-</sup> mice. Data are expressed as mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ .

hyperalgesia was also significantly elevated in SNS-GRK2<sup>+/-</sup> mice compared with control littermates (Fig. 4A).

Pretreatment with H89 totally prevented 8-Br-cAMP hyperalgesia in SNS-GRK2<sup>+/+</sup> (WT) but failed to affect 8-Br-cAMP hyperalgesia in mice with reduced nociceptor GRK2 (Fig. 5B). These findings indicate that GRK2 reduction in nociceptors causes biased signaling toward a PKA-independent pathway downstream of cAMP production, resulting in increased and prolonged hyperalgesia.

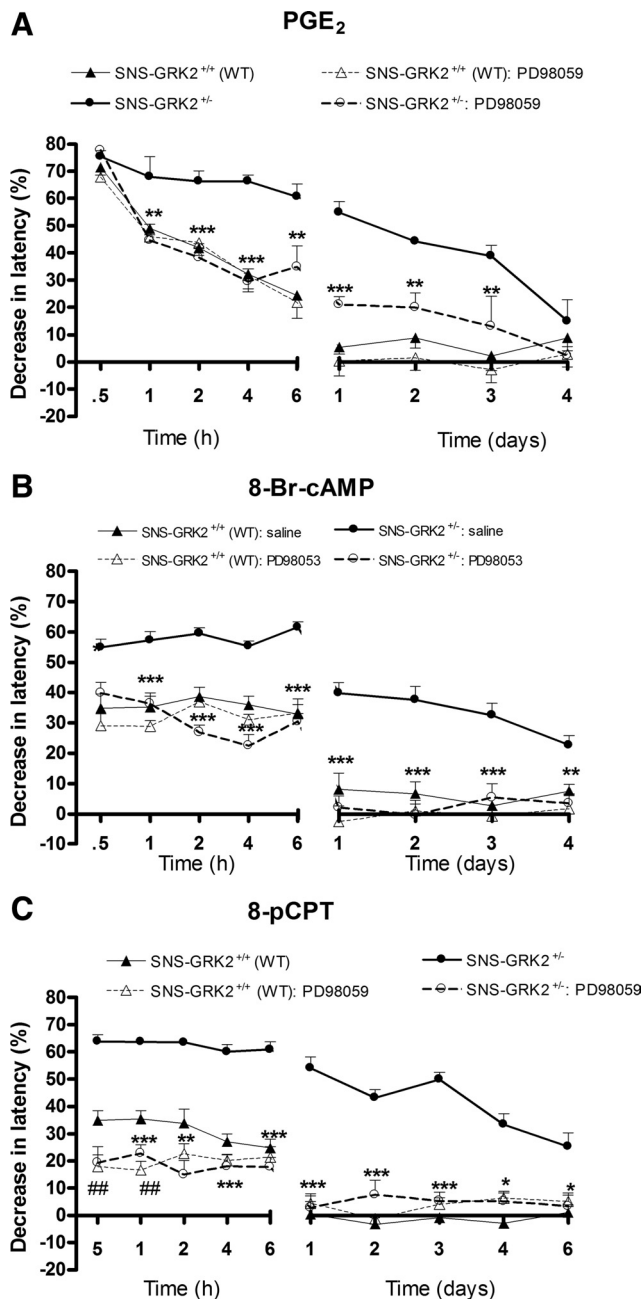
cAMP not only regulates cellular activity via activation of PKA but also of a family of guanine nucleotide exchange factors (GEFs) known as exchange proteins directly activated by cAMP (Epac). Specific inhibitors of Epac are not available. Therefore, we used the specific Epac activator 8-pCPT to determine the contribution of Epac-mediated pathways to the prolonged hyperalgesia in SNS-GRK2<sup>+/-</sup> mice. Hyperalgesia induced by intraplantar injection of the Epac activator 8-pCPT lasted 3 d in SNS-GRK2<sup>+/-</sup>, whereas SNS-GRK2<sup>+/+</sup> (WT) littermates recovered within 6 h (Fig. 5C), similar to what was observed after PGE<sub>2</sub> or 8-Br-cAMP. To verify the contribution of a PKA-mediated pathway in prolonged hyperalgesia in mice with low nociceptor GRK2, we used the specific PKA activator 6-Bnz-cAMP. Although acute hyperalgesia induced by the specific PKA-activator 6-Bnz-cAMP was slightly increased in SNS-GRK2<sup>+/-</sup> mice compared with SNS-GRK2<sup>+/+</sup> (WT) mice, PKA activation was not

sufficient to induce prolonged hyperalgesia in SNS-GRK2<sup>+/-</sup> mice (Fig. 5D).

### GRK2 modulates Rap1 activation via Epac

To test whether reduced GRK2 leads to changes in the level of Epac expression that may affect the duration of PGE<sub>2</sub> hyperalgesia, we determined Epac protein expression in isolated DRG neurons. Western blot analysis of isolated DRG neurons from WT and GRK2<sup>+/-</sup> mice showed that reduced GRK2 did not affect the level of Epac1 or Epac2 protein (Fig. 6A).

The GEFs Epac1 and Epac2 activate Rap1 and Rap2. Rap1 and Rap2 are small GTP binding proteins of the Ras family of GTPases. In its inactive state, Rap is bound to GDP. After binding of cAMP, the conformation of Epac changes so that Rap can bind and this induces the exchange of GDP for GTP, resulting in activation of Rap. Activated Rap induces downstream signaling, including the activation of PKC $\epsilon$  (Wang et al., 2007; Oestreich et al., 2009). Finally, Rap1 activation has been implicated in the modulation of nociceptor functioning (Delcroix et al., 2003; Taniguchi et al., 2008). Western blot analysis of isolated DRG neurons of WT and GRK2<sup>+/-</sup> neurons showed that Rap1 is expressed and that expression levels are similar between WT and GRK2<sup>+/-</sup> neurons (Fig. 6B). We investigated whether GRK2 deficiency affects Epac signaling to Rap1. The data in Figure 6C demonstrate that GRK2 deficiency significantly enhances



**Figure 7.** PGE<sub>2</sub>, 8-Br-cAMP, and 8-pCPT-induced hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice is biased to MEK/ERK. Percentage change in heat-withdrawal latencies after intraplantar injection of the MEK inhibitor PD98059 (2.5 μg/paw) before PGE<sub>2</sub> (A), 8-Br-cAMP (B), or 8-pCPT (C) injection in *SNS-GRK2*<sup>+/+</sup> (WT) animals and *SNS-GRK2*<sup>+/-</sup> mice (*n* = 8 per group). Data are expressed as mean ± SEM. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 *SNS-GRK2*<sup>+/-</sup> versus *SNS-GRK2*<sup>+/+</sup> treated with inhibitor. ##*p* < 0.01 *SNS-GRK2*<sup>+/+</sup> (WT) versus *SNS-GRK2*<sup>+/+</sup> (WT) treated with inhibitor.

8-pCPT-induced Rap1 activation (Fig. 6C). To investigate whether the effect of low GRK2 on Epac-to-Rap1 signaling could be mediated via a direct interaction between GRK2 and Epac, we performed coimmunoprecipitation studies. Cells transfected with HA-tagged Epac1 or with control HA-tagged TSC1 were stimulated with 8-pCPT or medium to get Epac in an active or non-active conformation and coimmunoprecipitated using either anti-GRK2 or anti-HA antibodies. HA-Epac1 was coimmunoprecipitated with GRK2, especially in 8-pCPT-stimulated extracts, whereas the control, HA-TSC1 was not found in the GRK2

immunoprecipitate (Fig. 6D). Conversely, only after stimulation with 8-pCPT, GRK2 was coprecipitated with HA-Epac1 (Fig. 6D). Finally, coimmunoprecipitation studies using a specific GRK2 antibody on tissue extracts of DRG and spinal cord tissue revealed that the low-molecular-weight splice variant of Epac1 binds GRK2 in these tissues (Fig. 6E).

Apart from Rap1 activation, we also show here that 8-pCPT moderately induced ERK1/2 phosphorylation in *GRK2*<sup>+/-</sup> lymphocytes and sensory neurons but not in WT cells (Fig. 6F, G). Similar results were obtained for 8-Br-cAMP-induced ERK1/2 phosphorylation in *GRK2*<sup>+/-</sup> primary sensory neurons (Fig. 6H).

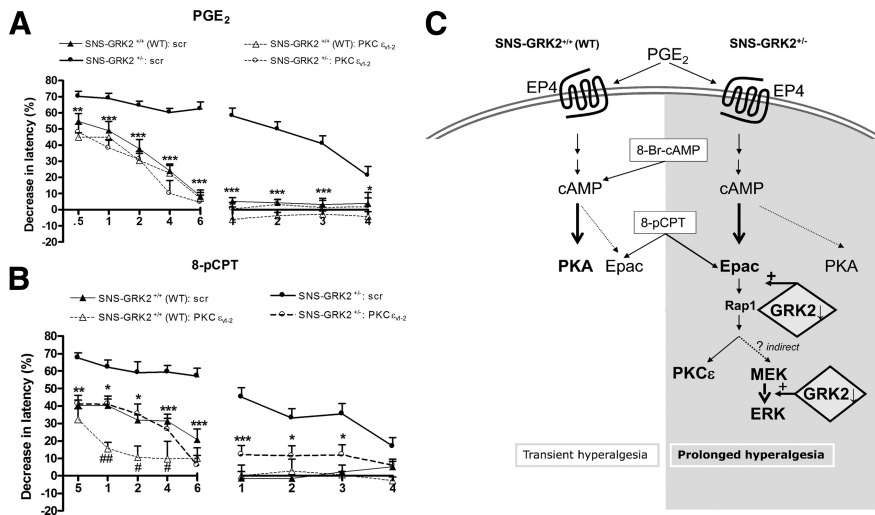
### *In vivo* contribution of the MEK/ERK and PKCε pathway in PGE<sub>2</sub>, 8-Br-cAMP, and 8-pCPT-induced hyperalgesia

Our *in vitro* results indicated that low sensory neuron GRK2 facilitates cAMP signaling toward Epac/Rap1 and ERK. To test whether *in vivo* low nociceptor GRK2 prolongs PGE<sub>2</sub>, 8-Br-cAMP, and 8-pCPT hyperalgesia via biased signaling to the mitogen-activated protein kinase kinase (MEK)/ERK1/2 pathway, we used the MEK1 inhibitor PD98059. In *SNS-GRK2*<sup>+/+</sup> (WT) mice, intraplantar pretreatment with PD98059 did not affect PGE<sub>2</sub> or 8-Br-cAMP-induced hyperalgesia (Fig. 7A, B). In contrast, the MEK1 inhibitor PD98059 inhibited the prolonged PGE<sub>2</sub>, 8-Br-cAMP, and 8-pCPT hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice (Fig. 7A–C). In addition, after PD98059 treatment, we no longer observed the increased acute hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice (Fig. 7A–C). PD98059 slightly reduced acute 8-pCPT hyperalgesia in *SNS-GRK2*<sup>+/+</sup> mice (Fig. 7C). Vehicle treatment did not affect PGE<sub>2</sub>, 8-Br-cAMP, or 8-pCPT hyperalgesia (data not shown).

PKCε is a well-defined target of Epac activation (Hucho et al., 2005; Oestreich et al., 2009). We used the inhibitory peptide TAT-PKCε<sub>v1-2</sub> that prevents translocation of PKCε to the membrane (Cesare et al., 1999). In control *SNS-GRK2*<sup>+/+</sup> mice, intraplantar TAT-PKCε<sub>v1-2</sub> administration 20 min before intraplantar PGE<sub>2</sub> did not have any effect on PGE<sub>2</sub> hyperalgesia (Fig. 8A). In contrast, intraplantar pretreatment with TAT-PKCε<sub>v1-2</sub> completely prevented the prolonged phase of PGE<sub>2</sub> hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice (Fig. 8A). In addition, intraplantar TAT-PKCε<sub>v1-2</sub> attenuated acute PGE<sub>2</sub> hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice (Fig. 8A). Similarly, in *SNS-GRK2*<sup>+/-</sup> mice, the enhanced and prolonged 8-pCPT hyperalgesia was completely prevented by the PKCε inhibitory peptide (Fig. 8B). In control *SNS-GRK2*<sup>+/+</sup> mice, 8-pCPT-induced hyperalgesia was also attenuated by intraplantar pretreatment with TAT-PKCε<sub>v1-2</sub> (Fig. 8B). Pretreatment with a TAT-coupled scrambled peptide that does not affect PKCε translocation did not affect PGE<sub>2</sub> or 8-pCPT-induced hyperalgesia in *SNS-GRK2*<sup>+/+</sup> and *SNS-GRK2*<sup>+/-</sup> mice at any of the time points tested (data not shown).

### Discussion

This study demonstrates that low nociceptor GRK2 protein levels such as those occurring during ongoing inflammatory hyperalgesia can induce biased PGE<sub>2</sub>/cAMP signaling from a PKA to an Epac/PKCε/ERK-dependent pathway, resulting in markedly prolonged PGE<sub>2</sub>/cAMP hyperalgesia. PGE<sub>2</sub> hyperalgesia lasted ~12 times longer in mice with a cell-specific reduction of GRK2 in Na<sub>v</sub>1.8-expressing sensory neurons than in littermate controls. The biased PGE<sub>2</sub> signaling in *SNS-GRK2*<sup>+/-</sup> mice occurred downstream of cAMP, because injection of a cAMP analog that activates both PKA and Epac, or a specific Epac activator, also induced PKA-independent prolonged hyperalgesia in these mice. Moreover, our *in vitro* studies showed that GRK2 physically binds to Epac1. This Epac1-GRK2 interaction may well underlie our finding



**Figure 8.** PGE<sub>2</sub>, 8-pCPT-induced hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice is biased to PKC<sub>ε</sub>. Percentage change in heat-withdrawal latencies after intraplantar injection of the PKC inhibitor PKC<sub>ε</sub>v1-2 (2.5 μg/paw) before PGE<sub>2</sub> injection (**A**) or 8-pCPT (**B**) in *SNS-GRK2*<sup>+/+</sup> (WT) animals and *SNS-GRK2*<sup>+/-</sup> mice (*n* = 4 per group). As a control, mice were treated with scrambled peptide before PGE<sub>2</sub> injection (scr). Data are expressed as mean ± SEM. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 *SNS-GRK2*<sup>+/-</sup> versus *SNS-GRK2*<sup>+/+</sup> treated with inhibitor. #*p* < 0.05, ##*p* < 0.01 *SNS-GRK2*<sup>+/+</sup> (WT) versus *SNS-GRK2*<sup>+/-</sup> (WT) treated with inhibitor. **C**, Model of the role of reduced nociceptor GRK2 in prolonged PGE<sub>2</sub> hyperalgesia. Low levels of nociceptor GRK2 promote cAMP-to-Epac signaling, whereas in nociceptor with “normal” GRK2 levels cAMP signals mainly to PKA. Additionally, low levels of GRK2 enhance ERK1/2 activation at the MEK/ERK1/2 interface. The effect of low nociceptor GRK2 levels ultimately lead to enhanced cAMP-to-Epac and ERK/PKC<sub>ε</sub> signaling resulting in prolonged hyperalgesia.

that reduced GRK2 results in enhanced Rap1 activation in response to stimulation with a specific activator of Epac, leading to prolongation of hyperalgesia.

The maximal effect of low nociceptor GRK2 on PGE<sub>2</sub> hyperalgesia was already obtained in heterozygous *SNS-GRK2*<sup>+/-</sup> mice with a 40–60% reduction in GRK2. Recently, we have observed a similar phenomenon when comparing the effect of partial or total GRK2 deletion in microglia/macrophages on IL-1β-induced hyperalgesia (Willemsen et al., 2010). These findings indicate that there is a threshold level for the effect of reduction of GRK2 in both neurons and microglia. We have described previously a similar threshold level for the effect of GRK6 on colitis; experimental colitis was prolonged to the same extent in mice that were heterozygous or homozygous for deletion of GRK6 (Eijkelkamp et al., 2007).

Using specific EP receptors agonists, we showed that the prolonged hyperalgesia in mice with reduced nociceptor GRK2 does not result from a switch to an EP receptor subtype that signals to second messengers other than cAMP. Only activation of the G<sub>s</sub>-coupled EP4 receptor mimicked the increased and prolonged PGE<sub>2</sub> hyperalgesia in mice with reduced nociceptor GRK2.

GRK2 is known to desensitize signaling via a wide variety of GPCR. This classical model of GRK2 action predicts that GRK2 reduction would enhance EP4-mediated signaling, leading to a stronger cAMP response and increased PKA activation (Ribas et al., 2007). There is indeed evidence that human EP4 receptors can be phosphorylated by GRK2 (Neuschäfer-Rube et al., 1999). Conversely, PGE<sub>2</sub>-induced cAMP production in smooth muscle cells is unaffected in conditions of low GRK2 (Kong et al., 2008). We cannot exclude that low GRK2 in nociceptors has an additional effect upstream of cAMP, e.g., at the level of G-protein coupling to the receptor (Dina et al., 2009). However, we show that the cAMP analog 8-Br-cAMP also induced increased and prolonged hyperalgesia in *SNS-GRK2*<sup>+/-</sup> mice. These findings indicate that the prolongation of hyperalgesia in *SNS-GRK2*<sup>+/-</sup>

mice is not dependent on GRK2 regulation of EP4 receptor desensitization but depends of GRK2 regulation at a level downstream of cAMP.

We recently showed that CCL3-induced hyperalgesia was increased but not prolonged in mice with reduced nociceptor GRK2 (Eijkelkamp et al., 2010). The chemokine CCL3 acts via GPCRs regulated by GRK2 (Oppermann et al., 1999; Vroon et al., 2004). However, CCL3 receptors do not signal to cAMP but to an IP<sub>3</sub>/calcium-dependent pathway. Apparently, regulation of the duration of inflammation-induced hyperalgesia by low nociceptor GRK2 levels occurs only if a cAMP-dependent but not an IP<sub>3</sub>/calcium-dependent signaling cascade is activated.

For many years, the downstream effects of an intracellular rise in cAMP have been attributed to PKA only. However, our present data clearly demonstrate that PGE<sub>2</sub> or cAMP-induced hyperalgesia are no longer PKA dependent in mice with low GRK2 in nociceptors. Nowadays, the contribution of another cAMP target, Epac, has become widely accepted (de Rooij et al., 1998). We show here for the first time using coimmunoprecipitation studies that Epac1 is present in a protein complex with GRK2, especially after stimulation with the specific Epac activator 8-pCPT. Moreover, *in vitro* GRK2 reduction enhanced 8-pCPT-induced activation of the direct downstream target of Epac Rap1. The direct physical interaction between GRK2 and activated Epac may well contribute to this enhanced Epac/Rap1 activation in cells with low GRK2. *In vivo* PGE<sub>2</sub>, cAMP-, and 8-pCPT-induced hyperalgesia in mice with reduced nociceptor GRK2 is prolonged and occurs via a PKA-independent pathway. Based on our *in vivo* and *in vitro* findings, we hypothesize that reduced GRK2 reorganizes the cAMP signaling cascade to favor Epac/Rap1 activation (Fig. 8C). We also propose that this enhanced activation of the Epac/Rap1 pathway underlies the prolongation of PGE<sub>2</sub> hyperalgesia that we observed in *SNS-GRK2*<sup>+/-</sup> mice.

Studies on the functional role of Epac in the neuronal system have shown that Epac enhances neurotransmitter release in glutamatergic synapses (Sakaba and Neher, 2003; Zhong and Zucker, 2005; Gekel and Neher, 2008) and that Epac1 and Epac2 modulate neuronal excitability (Ster et al., 2007). Additionally, PGE<sub>2</sub>-induced sensitization of ATP currents in sensory neurons is enhanced in DRG neurons primed by inflammation in an Epac-dependent manner (Wang et al., 2007). Finally, Epac has been implicated in integrin signaling (Bos, 2006), which is important in the maintenance of inflammatory hyperalgesia (Dina et al., 2004). Thus, if low GRK2 biases cAMP signaling to Epac, as our present findings indicate, such activation of Epac may ultimately result in long-lasting changes in neurotransmitter release, neuronal excitability, and/or integrin signaling and thereby cause prolonged hyperalgesia.

Epac activation has been shown to directly stimulate PKC<sub>ε</sub> activation (Wang et al., 2007; Oestreich et al., 2009). It has also been described that Epac-to-PKC<sub>ε</sub> signaling is critical in P2X<sub>3</sub> sensitization in primed neurons (Wang et al., 2007). Addition-



ally, hyperalgesic priming that significantly prolongs hyperalgesia appears to be highly dependent on PKC $\epsilon$  signaling (for review, see Reichling and Levine, 2009). Finally, PKC $\epsilon$  activation can potentiate Na $_v$ 1.8 and TRPV1 currents (Srinivasan et al., 2008; Cang et al., 2009). We show here that inhibition of PKC $\epsilon$  completely blocked the prolongation of PGE $_2$  or 8-pCPT-induced hyperalgesia in *SNS-GRK2<sup>+/-</sup>* mice. Therefore, we propose that the switch to Epac/Rap1 activation in the nociceptors with low GRK2 leads to PKC $\epsilon$  activation downstream of enhanced Epac/Rap1 activation, finally resulting in prolongation of inflammatory hyperalgesia (Fig. 8C).

Our *in vivo* data showed that not only inhibition of PKC $\epsilon$  but also inhibition of the MEK/ERK pathway completely prevented prolonged PGE $_2$ -, cAMP-, and 8-pCPT-induced hyperalgesia in mice with reduced nociceptor GRK2. In contrast, inhibition of the MEK/ERK pathway did not affect hyperalgesia in WT mice. These findings indicate that PKC $\epsilon$  and MEK/ERK signaling act in parallel, and activation of both is required for the induction of prolonged hyperalgesia in *SNS-GRK2<sup>+/-</sup>* mice. It is also possible, however, that PKC $\epsilon$  and MEK are part of the same pathway and one is downstream of the other. At present, there is no evidence that activation of Epac by cAMP can lead to direct activation of MEK/ERK pathway *in vitro* (Enserink et al., 2002; Lin et al., 2003; Gelinis et al., 2008). Consistently, we observed a small 8-pCPT-induced increase in p-ERK in GRK2-deficient cells but not in WT cells. Nevertheless, *in vivo*, inhibition of the MEK-ERK pathway inhibits 8-pCPT-induced prolonged hyperalgesia in GRK2-deficient mice. These data indicate that, *in vivo* in *SNS-GRK2<sup>+/-</sup>* mice, activation of Epac can lead to MEK-ERK activation. It remains to be determined whether this activation of the MEK-ERK pathway in *SNS-GRK2<sup>+/-</sup>* mice is induced via indirect or direct pathways downstream of Epac and potentially via PKC $\epsilon$ . In this respect, it is of interest that GRK2 binds to MEK1 and inhibits ERK activation at the MEK1-ERK interface (Jiménez-Sainz et al., 2006). Thus, the possibility exists that, in our model, low nociceptor GRK2 prolongs hyperalgesia via enhancing cAMP-induced Epac/Rap1 activation in combination with increased MEK1-to-ERK signaling that results from a direct interaction of GRK2 with MEK1 (Fig. 8C).

Our finding that marked prolongation of PGE $_2$ -induced hyperalgesia occurs in the condition of a 50% reduction in nociceptor GRK2 levels raises the question whether similar changes in GRK2 levels occur in (patho)physiological conditions. Previously, we have shown that GRK2 protein levels in peripheral blood mononuclear cells from patients with rheumatoid arthritis or multiple sclerosis was reduced compared with healthy controls. Similarly, in animal models of rheumatoid arthritis or multiple sclerosis, splenocyte GRK2 protein levels were reduced (Lombardi et al., 1999; Vroon et al., 2005). Our present results show that GRK2 levels are reduced in small-diameter DRG neurons during carrageenan-induced hyperalgesia. In addition, we described that spinal cord neuronal and microglial GRK2 was reduced in rat or mouse models of neuropathic pain (Kleibeuwer et al., 2007, 2008; Eijkelkamp et al., 2010). It is therefore tempting to speculate that an inflammation-induced decrease in GRK2 levels may contribute to the chronic pain that develops in conditions of chronic inflammation or nerve damage.

Overall, this study shows that low nociceptor GRK2 is a novel and important risk factor for the switch from acute to persistent pain by inducing biased intracellular cAMP signaling. Moreover, we propose that GRK2 restrains Epac/Rap1 signaling. Our data strongly indicate that low nociceptor GRK2 favors cAMP signal-

ing to Epac/Rap1, leading to PKC $\epsilon$  and MEK/ERK-dependent signaling and prolonged hyperalgesia.

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