

# G Protein–Coupled Receptor Kinase 6 Acts as a Critical Regulator of Cytokine-Induced Hyperalgesia by Promoting Phosphatidylinositol 3-Kinase and Inhibiting p38 Signaling

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The molecular mechanisms determining magnitude and duration of inflammatory pain are still unclear. We assessed the contribution of G protein–coupled receptor kinase (GRK)-6 to inflammatory hyperalgesia in mice. We showed that GRK6 is a critical regulator of severity and duration of cytokine-induced hyperalgesia. In *GRK6*<sup>-/-</sup> mice, a significantly lower dose (100 times lower) of intraplantar interleukin (IL)-1 $\beta$  was sufficient to induce hyperalgesia compared with wild-type (WT) mice. In addition, IL-1 $\beta$  hyperalgesia lasted much longer in *GRK6*<sup>-/-</sup> mice than in WT mice (8 d in *GRK6*<sup>-/-</sup> versus 6 h in WT mice). Tumor necrosis factor (TNF)- $\alpha$ -induced hyperalgesia was also enhanced and prolonged in *GRK6*<sup>-/-</sup> mice. *In vitro*, IL-1 $\beta$ -induced p38 phosphorylation in *GRK6*<sup>-/-</sup> dorsal root ganglion (DRG) neurons was increased compared with WT neurons. In contrast, IL-1 $\beta$  only induced activation of the phosphatidylinositol (PI) 3-kinase/Akt pathway in WT neurons, but not in *GRK6*<sup>-/-</sup> neurons. *In vivo*, p38 inhibition attenuated IL-1 $\beta$ - and TNF- $\alpha$ -induced hyperalgesia in both genotypes. Notably, however, whereas PI 3-kinase inhibition enhanced and prolonged hyperalgesia in WT mice, it did not have any effect in *GRK6*-deficient mice. The capacity of GRK6 to regulate pain responses was also apparent in carrageenan-induced hyperalgesia, since thermal and mechanical hypersensitivity was significantly prolonged in *GRK6*<sup>-/-</sup> mice. Finally, GRK6 expression was reduced in DRGs of mice with chronic neuropathic or inflammatory pain. Collectively, these findings underline the potential role of GRK6 in pathological pain. We propose the novel concept that GRK6 acts as a kinase that constrains neuronal responsiveness to IL-1 $\beta$  and TNF- $\alpha$  and cytokine-induced hyperalgesia via biased cytokine-induced p38 and PI 3-kinase/Akt activation.

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## INTRODUCTION

Proper functioning of the nociceptive system is essential to protect the body from tissue damage. Inflammation sensitizes the nociceptive system, leading to a lower threshold to painful stimuli (hyperalgesia) (1). This process is thought to

serve adaptive purposes, but becomes maladaptive when hyperalgesia persists after resolution of inflammation. The proinflammatory cytokine interleukin (IL)-1 $\beta$  directly sensitizes nociceptors, leading to transient hyperalgesia (2,3). Peripheral injection of other inflamma-

tory mediators such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) also increases the sensitivity of nociceptors and the response to painful stimuli (4). Moreover, there is evidence that proinflammatory cytokines such as IL-1 $\beta$  and tumor necrosis factor (TNF)- $\alpha$  contribute to the genesis of neuropathic pain (5,6).

Many of the signals involved in inflammatory hyperalgesia are generated via activation of G protein–coupled receptors (GPCRs) expressed in sensory neurons. The activity of GPCRs is regulated by the family of GPCR kinases (G protein–coupled receptor kinase [GRK] 1–7). Agonist-activated GPCRs are phosphorylated by GRKs, inducing rapid uncoupling from the G protein, a process

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called homologous receptor desensitization. GRK-mediated GPCR phosphorylation facilitates binding of arrestin proteins, promoting GPCR internalization (7,8). GRKs are also capable of interacting with a variety of downstream signaling molecules, thereby regulating cellular signaling independently of GPCRs (8,9).

GRK6 plays a crucial role in inflammatory pathologies. For example, GRK6 deficiency increases acute inflammatory arthritis as well as colitis in male mice (10,11). Recently, we showed that post-inflammatory visceral hyperalgesia is enhanced in female *GRK6*<sup>-/-</sup> mice without affecting inflammation (12). This previous study indicated that GRK6 plays a role in regulating visceral post-inflammatory pain, but did not give insight into the mechanisms involved.

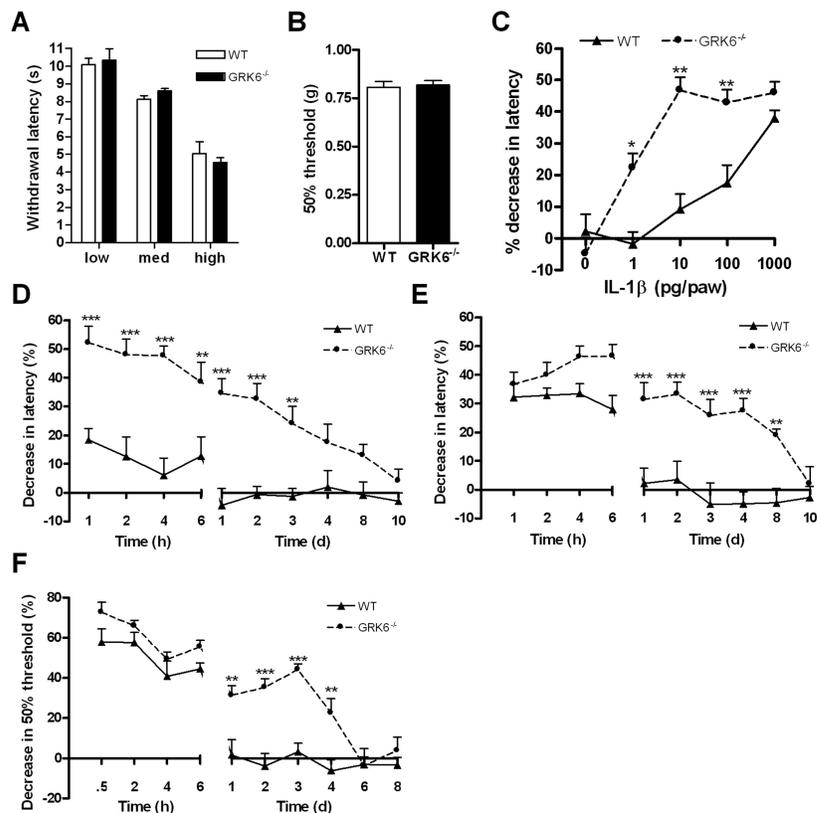
Although most of the substrates of GRK6 are probably still unknown, it has been shown that GRK6 regulates desensitization of the chemokine receptor CXCR4, the BLT1 receptor for the leukotriene B4 (LTB4) and the calcitonin gene-related peptide (CGRP) receptor (13–16). Furthermore, GRK6 binds and phosphorylates PDZ domains in Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor (NHERF) and binds to downstream regulatory element antagonistic modulator (DREAM), both regulators of ion channels, indicating that GRK6 can also regulate cellular signaling via mechanisms independent of GPCR desensitization (17,18).

We aimed to determine the contribution of GRK6 to somatic inflammatory hyperalgesia. As a model, we induced local hyperalgesia by a single injection of carrageenan or the proinflammatory cytokines IL-1 $\beta$  or TNF- $\alpha$  into the paw. In search of the mechanism via which GRK6 regulates hyperalgesia, we analyzed the consequences of GRK6 deficiency for cytokine signaling to p38 and PI 3-kinase.

## MATERIAL AND METHODS

### Animals

Female GRK6-deficient C57BL/6 and wild-type (WT) control littermates were bred in the Utrecht University Central



**Figure 1.** Increased and prolonged IL-1 $\beta$ -induced thermal hyperalgesia and mechanical allodynia in *GRK6*<sup>-/-</sup> mice. (A) Heat withdrawal latencies were determined using the Hargreaves test at three different intensities ( $n = 8$ ). (B) Thresholds to mechanical stimulation were determined using von Frey hairs ( $n = 14$ ). (C) WT and *GRK6*<sup>-/-</sup> mice ( $n = 8$ –12) received an intraplantar injection of IL-1 $\beta$ , and the decrease in heat withdrawal latency was determined 2 h after injection. Two-way ANOVA: genotype:  $P < 0.001$ ; dose:  $P < 0.001$ ; interaction:  $P < 0.001$ . (D, E) Time course of IL-1 $\beta$ -induced thermal hyperalgesia in WT and *GRK6*<sup>-/-</sup> mice (D: 10 pg IL-1 $\beta$ ; E: 1,000 pg IL-1 $\beta$ ;  $n = 8$ –14). (F) Time course of IL-1 $\beta$ -induced mechanical allodynia in WT and *GRK6*<sup>-/-</sup> mice ( $n = 8$ ). Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ ; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Animal Facility (12) and genotyped by polymerase chain reaction (PCR) analysis on genomic DNA. All experiments were performed in accordance with international guidelines and approved by the University Medical Center Utrecht experimental animal committee or were approved under the United Kingdom Home Office Animals (Scientific Procedures) Act 1986.

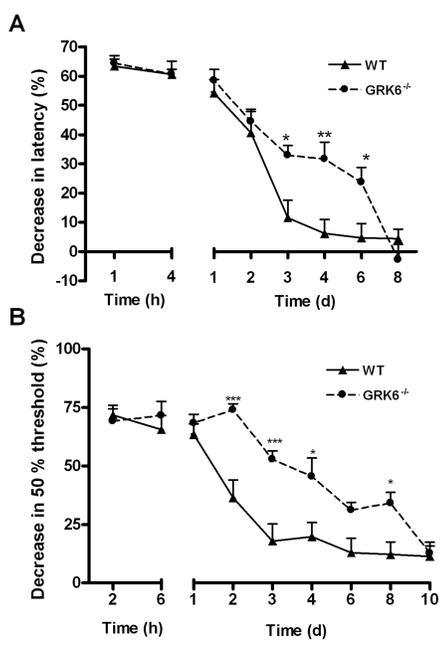
### Induction of Cytokine-Induced Thermal Hyperalgesia

Mice received an intraplantar injection of 5  $\mu$ L 1%  $\lambda$ -carrageenan (Sigma-Aldrich, St. Louis, MO, USA), 5  $\mu$ L re-

combinant mouse IL-1 $\beta$  (0.2–200 ng/mL; Preprotech, Rocky Hill, NC, USA) or TNF- $\alpha$  (20 ng/mL; Preprotech) in saline or 5  $\mu$ L saline as a control (19).

Heat withdrawal latency times were determined using the Hargreaves test (IITC Life Science, Woodland Hills, CA, USA) as described (20). Mechanical allodynia was measured using von Frey hairs (Stoelting, Wood Dale, IL, USA), and the 50% paw withdrawal threshold was calculated using the up-and-down method (21). The observers were blinded to genotype.

The p38 inhibitor SB239063 (5  $\mu$ g/paw; Sigma-Aldrich) or the PI 3-kinase in-



**Figure 2.** Prolonged carrageenan-induced thermal hyperalgesia and mechanical allodynia in *GRK6*<sup>-/-</sup> mice. Time course of carrageenan-induced thermal hyperalgesia (A) and mechanical allodynia (B) in WT and *GRK6*<sup>-/-</sup> mice. *n* = 6 per group. Data are expressed as mean  $\pm$  SEM. \**P* < 0.05; \*\**P* < 0.01, \*\*\**P* < 0.001.

inhibitor LY249002 (10  $\mu$ g/paw; Sigma-Aldrich) was injected intraplantarly, 20 min before cytokine injection (22).

### Chronic Pain Models

**L5 transection (neuropathic).** In isoflurane anesthetized mice, the transverse processes at the L4-S1 levels was accessed, the L5 transverse process was removed and the left L5 spinal nerve was cut. Dorsal root ganglions (DRGs) were isolated 4 wks later.

**Carrageenan (inflammatory).** Carrageenan (2%, 20  $\mu$ L; Sigma-Aldrich) was injected in both hindpaws. DRGs were isolated 6 d later.

### Dorsal Root Ganglia Cell Culture

DRGs 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

phosphate-buffered saline. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Gibco), 2 mmol/L glutamine (Gibco), 10,000 IU/mL penicillin-streptomycin (Gibco) and 100 ng/mL nerve growth factor (NGF) (Sigma-Aldrich) and poly-L-lysine- and laminin-coated wells. Cells were stimulated with IL-1 $\beta$  for 5 min 15–25 h after plating.

### Western Blot Analysis

Cells were homogenized in lysis buffer (200 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.5, 10% glycerol, 1% NP-40, 2 mmol/L sodium orthovanadate, 2 mmol/L phenylmethylsulfonyl fluoride [PMSF], 2  $\mu$ mol/L leupeptin, protease inhibitor mix [p3840, 1:200; Sigma-Aldrich]). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Blots were stained with rabbit-anti-p-p38, rabbit-anti-p38, rabbit-anti-p-Akt and rabbit-anti-Akt (Cell Signaling Technology Inc., Danvers, MA, USA) followed by goat anti-mouse-peroxidase (Jackson Laboratories) or donkey anti-rabbit-peroxidase (Amersham International) and developed by enhanced chemiluminescence plus (Amersham International). Band density was quantified using a GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA, USA).

### mRNA Isolation and Real-Time PCR

Lumbar DRGs were homogenized in Trizol (Invitrogen, Paisley, UK). Total RNA was isolated with RNeasy Mini Kit (Qiagen) and reverse-transcribed using an iScript<sup>TM</sup> Select cDNA Synthesis Kit (Invitrogen). Real-time quantitative PCR was performed with an iQ<sup>TM</sup> SYBR<sup>®</sup> Green Supermix (Invitrogen). Primer pairs used were as follows:

GRK6: CTGG TCTCA TAGGC GTAGG (forward), GCGGA TAAAG AAGCG AAAGG (reverse); GRK2: CGGGACTTC TGCCT GAACC ATCTG (forward), CTCGG CTGCG GACCA CACG (reverse);  $\beta$ -arrestin1: AAGGG

ACACG AGTGT TCAAG A (forward), CCCGC TTTCC CAGGT AGAC (reverse);  $\beta$ -arrestin2: GGCAA GCGCG ACTTT GTAG (forward), GTGAG GGTC ACGAAC ACTTT C (reverse);  $\beta$ -actin: TTCTT TGCAG CTCCT TCGTT (forward), ATGGA GGGGA ATACA GCCC-3' (reverse); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): TGCGA CTTC ACGC AACTC (forward), CTTCG TCAGT GTCCT TGCTG (reverse).

### Data Analysis

Data are expressed as mean  $\pm$  standard error of the mean (SEM) and analyzed using the Student *t* test, one-way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni analysis. A *P* value < 0.05 was considered statistically significant.

All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).

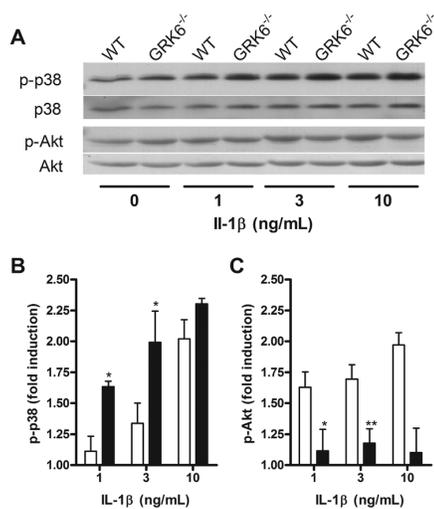
## RESULTS

### Increased IL-1 $\beta$ -Induced Thermal and Mechanical Hyperalgesia in *GRK6*<sup>-/-</sup> Mice

Heat-withdrawal latencies of WT and *GRK6*<sup>-/-</sup> mice at baseline were compared using the Hargreaves test at increasing intensities. Under baseline conditions, there was no genotype-dependent difference in heat sensitivity (Figure 1A). Similarly, sensitivity to mechanical stimuli, as measured with the Von Frey test, was not different between genotypes (Figure 1B).

Intraplantar injection of IL-1 $\beta$  dose-dependently increased thermal hyperalgesia in WT mice, as determined at 2 h after injection (Figure 1C). In *GRK6*<sup>-/-</sup> mice, the dose-response curve was sharply shifted to the left (see Figure 1C). Notably, the lowest dose of IL-1 $\beta$  (100 pg) that induced detectable thermal hyperalgesia in WT mice was 100-fold higher than the lowest dose that induced thermal hyperalgesia in *GRK6*<sup>-/-</sup> mice (1 pg).

To determine whether GRK6 also regulates duration of IL-1 $\beta$ -induced thermal hyperalgesia, we followed intraplantar



**Figure 3.** *In vitro* IL-1 $\beta$ -induced phosphorylation of p38 and Akt in DRG neurons of WT and *GRK6*<sup>-/-</sup> mice. (A) Exemplar dose-response curves for IL-1 $\beta$ -induced p-p38 and p-Akt. Quantification of p-p38 levels (B) and p-Akt levels (C) in DRG neurons after stimulation with increasing concentrations of IL-1 $\beta$  for 5 min (n = 3–7). All data are expressed as mean  $\pm$  SEM. \**P* < 0.05; \*\**P* < 0.01.

IL-1 $\beta$ -induced thermal hyperalgesia over time. Hyperalgesia induced by intraplantar injection of either a low (10 pg/paw) or high (1,000 pg/paw) dose of IL-1 $\beta$  was markedly prolonged in *GRK6*-deficient mice (3 or 8 d after injection of a low or high dose of IL-1 $\beta$  in *GRK6*<sup>-/-</sup> mice versus 6 h or 1 d in WT mice; Figures 1D, E). Mechanical allodynia induced by IL-1 $\beta$  (1,000 pg/paw) was also markedly prolonged in *GRK6*<sup>-/-</sup> mice compared with WT mice (4–6 d after injection in *GRK6*<sup>-/-</sup> mice versus <1 d in WT mice; Figure 1F). At 24 h after intraplantar IL-1 $\beta$  (1,000 pg/paw), there were no differences in expression of COX2, IL-6 and TNF- $\alpha$  mRNA, indicating that the prolongation of hyperalgesia was independent of inflammatory activity (Supplementary Figure 1).

Carrageenan-induced thermal hyperalgesia was also significantly prolonged in *GRK6*<sup>-/-</sup> mice in comparison to WT mice (Figure 2A, recovery within 6–8 d in *GRK6*<sup>-/-</sup> mice versus 2–3 d in WT mice).

Additionally, carrageenan-induced mechanical allodynia in *GRK6*<sup>-/-</sup> mice lasted three times longer than in WT mice (Figure 2B, recovery within 8–10 d in *GRK6*<sup>-/-</sup> versus 2–3 d in WT mice).

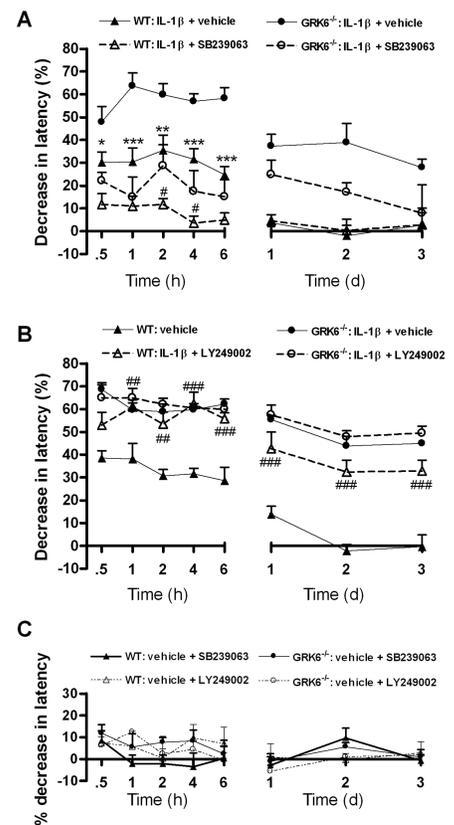
### *In Vitro* Response of *GRK6*<sup>-/-</sup> DRG Neurons to IL-1 $\beta$

Intraplantar IL-1 $\beta$ -induced thermal hypersensitivity is mediated via activation of p38 in primary sensory neurons (2). To determine whether *GRK6* deficiency facilitated IL-1 $\beta$  signaling to p38 in primary sensory neurons, DRG neurons were stimulated *in vitro* for 5 min with increasing doses of IL-1 $\beta$ , and the level of p-p38 was determined as a measure of p38 activation. IL-1 $\beta$ -induced activation of p38 was significantly higher in *GRK6*<sup>-/-</sup> DRG neurons than in WT neurons (Figures 3A, B). The dose-response curve for IL-1 $\beta$ -induced p-p38 *in vitro* was shifted to the left in *GRK6*<sup>-/-</sup> DRG cultures compared with WT DRG neurons (~40-fold more sensitive). Baseline p-p38 did not differ significantly between WT and *GRK6*<sup>-/-</sup> mice (1  $\pm$  0.16 versus 0.95  $\pm$  0.14, n = 7). Next, we determined whether this shift in the dose-response curve of *GRK6*<sup>-/-</sup> DRG neurons to IL-1 $\beta$  was also observed at the level of activation of the PI 3-kinase/Akt pathway, another important signaling cascade that mediates IL-1 $\beta$  responses. WT DRG neurons responded to IL-1 $\beta$  with a clear increase in p-Akt (Figures 3A, C). Interestingly, IL-1 $\beta$  did not induce a significant increase in p-Akt in *GRK6*<sup>-/-</sup> DRG neurons. Baseline p-Akt did not differ significantly between WT and *GRK6*<sup>-/-</sup> mice (1  $\pm$  0.18 versus 0.85  $\pm$  0.08, n = 8).

These *in vitro* data indicate that *GRK6* deficiency prevents activation of PI 3-kinase/Akt while facilitating p38 activation, thereby inducing a switch in IL-1 $\beta$  signaling from activation of both the p38 and PI 3-kinase/Akt pathways toward activation of p38 only.

### *In Vivo* Role of p38 and PI 3-Kinase in the IL-1 $\beta$ -Induced Hyperalgesia

To determine the *in vivo* relevance of the shift in IL-1 $\beta$ -induced activation of the p38 and PI 3-kinase/Akt pathway in



**Figure 4.** Role of p38 and PI 3-kinase in IL-1 $\beta$ -induced hyperalgesia. Mice were injected intraplantarly with the p38 inhibitor SB239063 (A) or the PI 3-kinase inhibitor LY249002 (B) 20 min before intraplantar injection of IL-1 $\beta$  (100 pg/paw; n = 4) or before saline (C). Heat withdrawal latencies were determined over time. All data are expressed as mean  $\pm$  SEM. *GRK6*<sup>-/-</sup>, IL-1 $\beta$  + vehicle, versus *GRK6*<sup>-/-</sup>, IL-1 $\beta$  + inhibitor: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. WT, vehicle, versus WT, treatment: #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001.

DRG neurons of *GRK6*<sup>-/-</sup> mice, we compared the effect of intraplantar administration of the p38 inhibitor SB239063 and the PI 3-kinase inhibitor LY249002 on IL-1 $\beta$ -induced hyperalgesia in WT and *GRK6*<sup>-/-</sup> mice.

In line with previous reports (2), intraplantar administration of SB239063 significantly attenuated IL-1 $\beta$ -induced hyperalgesia in WT mice. The p38 inhibitor SB239063 (5  $\mu$ g/paw) also inhibited the magnitude of acute IL-1 $\beta$ -induced hy-

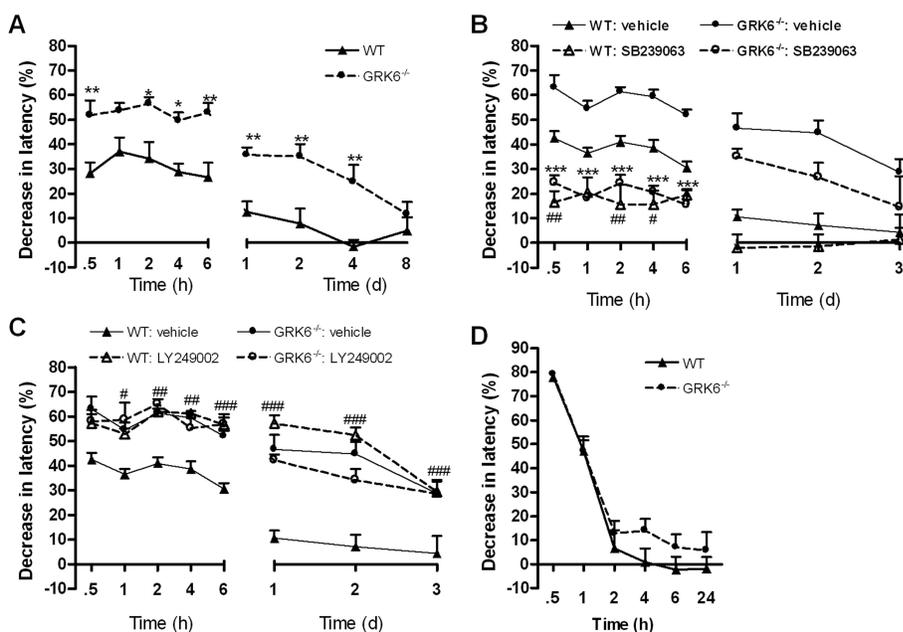
peralgesia in *GRK6*<sup>-/-</sup> mice and partially attenuated the duration of hyperalgesia that develops in these mice (Figure 4A).

In contrast, intraplantar administration of the PI 3-kinase inhibitor LY249002 (10 μg/paw) significantly enhanced the magnitude of acute (0.5–6 h) IL-1β-induced hyperalgesia in WT mice but did not have any effect on acute IL-1β hyperalgesia in *GRK6*<sup>-/-</sup> mice (Figure 4B). Additionally, IL-1β-induced hyperalgesia in WT mice was significantly prolonged after inhibition of PI 3-kinase. LY249002 did not have any effect on the duration of IL-1β-induced hyperalgesia in *GRK6*<sup>-/-</sup> mice. Injection of SB239063 or LY249002 alone did not have any effect on heat withdrawal latencies in WT or *GRK6*<sup>-/-</sup> mice (Figure 4C). These findings indicate that *GRK6*<sup>-/-</sup> mice lack an inhibiting signal that is provided in WT mice by activation of the PI 3-kinase pathway.

#### PGE<sub>2</sub>- and TNF-α-Induced Hyperalgesia in WT and *GRK6*<sup>-/-</sup> Mice

We also determined the effect of *GRK6* deletion on hyperalgesia induced by TNF-α, another proinflammatory cytokine that also signals to p38 and PI 3-kinase/Akt (23). Acute (<1 d) TNF-α-induced hyperalgesia was enhanced in *GRK6*<sup>-/-</sup> mice when compared with WT mice (Figure 5A). In addition, *GRK6*<sup>-/-</sup> mice also developed long-lasting hyperalgesia after a single injection of TNF-α, whereas WT mice recovered within 1 d. Inhibition of p38 with SB239063 significantly attenuated acute TNF-α-induced hyperalgesia in both WT and *GRK6*<sup>-/-</sup> mice (Figure 5B). Moreover, inhibition of PI 3-kinase by intraplantar administration of LY249002 increased and prolonged TNF-α-induced hyperalgesia in WT mice but did not affect TNF-α hyperalgesia in *GRK6*<sup>-/-</sup> mice (Figure 5C). These data indicate that a similar mechanism, a switch from activation of both p38 and PI 3-kinase to p38 only, is operative in *GRK6*<sup>-/-</sup> mice leading to the prolongation of TNF-α-induced hyperalgesia.

To determine whether the effect of *GRK6* deficiency on severity and duration of hyperalgesia was limited to medi-



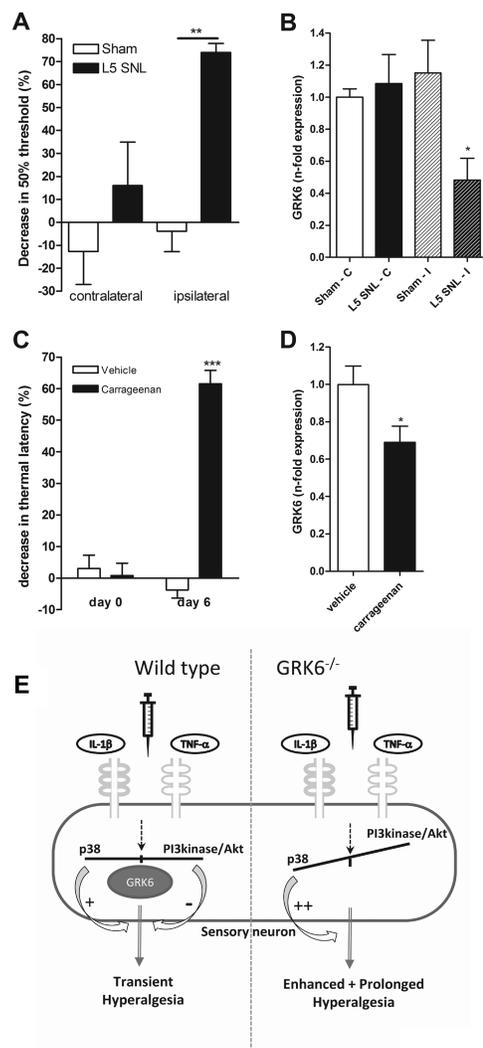
**Figure 5.** Role of p38 and PI 3-kinase in TNF-α-induced thermal hyperalgesia in *GRK6*<sup>-/-</sup> mice. (A) Mice received an intraplantar injection of TNF-α (100 pg/paw; n = 8), and the change in heat withdrawal latency was determined over time. Mice were injected intraplantarly with the p38 inhibitor SB239063 (B) or the PI 3-kinase inhibitor LY249002 (C) 20 min before intraplantar injection of TNF-α (100 pg/paw; n = 4). Heat withdrawal latencies were determined over time. (D) WT and *GRK6*<sup>-/-</sup> mice (n = 8) received an intraplantar injection of PGE<sub>2</sub> (100 ng/2.5 μL), and the decrease in heat withdrawal latency was determined over time. All data are expressed as mean ± SEM. *GRK6*<sup>-/-</sup>, vehicle, versus *GRK6*<sup>-/-</sup>, treatment: \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. WT, vehicle, versus WT, treatment: #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001.

ators signaling via p38, we also analyzed PGE<sub>2</sub>-induced hyperalgesia that is known to be cAMP-dependent protein kinase A (PKA) dependent (24,25). The data in Figure 6C show that PGE<sub>2</sub>-induced thermal hyperalgesia was similar in WT and *GRK6*<sup>-/-</sup> mice (Figure 5D), indicating that *GRK6* deficiency does not affect hyperalgesia induced by an inflammatory mediator that signals via the cAMP/PKA pathway and independently of p38.

#### GRK6 mRNA Expression Levels in DRG of Mice with Neuropathic or Inflammatory Pain

To investigate whether changes in *GRK6* do occur in conditions of chronic pain, we investigated *GRK6* mRNA expression levels in DRGs of mice with chronic neuropathic or inflammatory pain. Four weeks after unilateral L5 nerve transection (L5 SNT), mice were more

sensitive to mechanical stimulation of the ipsilateral paw (Figure 6A). Importantly, at this same time point, *GRK6* mRNA levels were significantly reduced in ipsilateral DRGs compared with contralateral DRGs from sham-operated mice (Figure 6B). L5 SNT did not induce changes in mRNA levels for *GRK2*, β-arrestin1 or β-arrestin2 (Supplementary Figures 2A–C). Chronic inflammatory pain was induced by intraplantar injection of carrageenan. Six days after carrageenan injection, heat-withdrawal latencies were reduced (Figure 6C). At this time, *GRK6* mRNA levels were significantly decreased in the DRGs of carrageenan-treated mice compared with vehicle-treated mice (Figure 6D). mRNA levels for *GRK2* and β-arrestin2 did not differ between carrageenan- and vehicle-treated mice, whereas β-arrestin1 mRNA levels were slightly reduced (Supplementary Figures 2D–F).



**Figure 6.** GRK6 mRNA levels in DRGs during chronic neuropathic and inflammatory pain. (A) The sensitivity to mechanical stimulation was determined in sham-operated mice ( $n = 8$ ) or mice subjected to unilateral L5 SNT 4 wks after surgery ( $n = 10$ ). (B) GRK6 mRNA levels in DRGs innervating the contralateral (-C) or ipsilateral (-I) side of sham-operated ( $n = 4$ ) and L5 SNL ( $n = 5$ ) mice 4 wks after surgery. The sensitivity to thermal stimulation was determined after intraplantar injection of vehicle ( $n = 5$ ) or carrageenan ( $n = 5$ ) (C), and 6 d later, DRG GRK6 mRNA expression levels were determined ( $n = 5$ ) (D). GRK6 mRNA expression levels were corrected for GAPDH and  $\beta$ -actin mRNA expression levels. All data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (E) In WT mice, IL-1 $\beta$  induces activation of the p38 and PI 3-kinase/Akt signaling cascade leading to transient hyperalgesia. The activation of p38 promotes hyperalgesia, whereas activation of the PI 3-kinase/Akt signaling cascade constrains hyperalgesia. Loss of GRK6 enhances p38 activity in sensory neurons, whereas activation of the PI 3-kinase/Akt pathway is attenuated, ultimately leading to enhanced and prolonged cytokine-induced hyperalgesia.

Unfortunately, we were unable to test whether the decrease in GRK6 mRNA was associated with a reduction in GRK6 protein, since no reliable GRK6 antibodies were available.

## DISCUSSION

In this study, we present the novel concept that the kinase GRK6 plays a pivotal role in regulating the duration and intensity of inflammatory hyperalgesia.

GRK6 deficiency strongly enhanced and prolonged thermal hyperalgesia and mechanical allodynia induced by intraplantar injection of either IL-1 $\beta$  or TNF- $\alpha$ . Similarly, hyperalgesia induced by intraplantar injection of carrageenan was markedly prolonged in GRK6<sup>-/-</sup> mice. We also show that GRK6 deficiency promotes activation of p38 while the activation of PI 3-kinase/Akt is dampened. Thus, the novelty and significance of these results is that GRK6 emerges here as a kinase that constrains neuronal responsiveness to IL-1 $\beta$  and TNF- $\alpha$  and ultimately cytokine-induced hyperalgesia via biased cytokine-induced p38 and PI 3-kinase/Akt activation (Figure 6E). The potential pathophysiological significance of these findings is substantiated by our finding that DRG GRK6 expression levels are reduced in a model of chronic neuropathic pain and inflammatory pain.

Recent evidence indicates that hyperalgesia induced by intraplantar administration of IL-1 $\beta$  is mediated via activation of p38 and subsequent modulation of the activity of tetrodotoxin-resistant sodium channels (2). TNF- $\alpha$  is also known to signal to p38 and is capable of inducing hyperalgesia via mechanisms that involve increased transient receptor potential cation channel subfamily V member 1 (TRPV1) expression and modulation of tetrodotoxin-resistant sodium channels (26,27). We show here for the first time that GRK6 constrains IL-1 $\beta$ -induced p38 activation in sensory neurons. This conclusion is on the basis of our *in vitro* finding that IL-1 $\beta$ -induced p38 phosphorylation was enhanced in GRK6<sup>-/-</sup> DRG cultures. Additionally, our *in vivo* studies showed that p38 activation is required for IL-1 $\beta$  and TNF- $\alpha$  hyperalgesia both in WT and GRK6<sup>-/-</sup> mice. Finally, we show that *in vivo* IL-1 $\beta$ - and TNF- $\alpha$ -induced hyperalgesia is increased in GRK6-deficient mice. Collectively, these findings support a central role of p38 in cytokine-induced hyperalgesia and position GRK6 as a pivotal regulator of cytokine-induced hyperalgesia.

Our findings indicate that GRK6 deficiency enhances IL-1 $\beta$  signaling to p38 in

nociceptors. The IL-1 receptor is expressed in virtually all sensory neurons (28). Moreover, IL-1 $\beta$  injection into the rat paw enhances p-p38 in peripherin-positive sensory nerves (2). Finally, in mice in which Nav1.8-positive nociceptors are deleted, peripherin-positive neurons are reduced >85% and inflammatory pain does not develop (29). Therefore, we propose that GRK6 deficiency enhances IL-1 $\beta$ -induced hyperalgesia via promoting p38 signaling in peripherin-positive nociceptors.

Our experiments identified GRK6 as a prerequisite for IL-1 $\beta$ -induced activation of the PI 3-kinase/Akt pathway in primary sensory neurons. This conclusion is on the basis of our finding that IL-1 $\beta$  induced a significant increase in Akt phosphorylation in WT mice, but was unable to do so in *GRK6*<sup>-/-</sup> mice. Second, we show here that IL-1 $\beta$ - and TNF- $\alpha$ -induced PI 3-kinase activity is required to constrain cytokine-induced hyperalgesia in magnitude as well as duration. Inhibition of PI 3-kinase enhanced and prolonged IL-1 $\beta$  and TNF- $\alpha$  hyperalgesia in WT mice, whereas inhibition of PI 3-kinase did not affect hyperalgesia in *GRK6*<sup>-/-</sup> mice. Thus, our studies in GRK6-deficient mice reveal a completely novel role of the PI 3-kinase/Akt signaling cascade in attenuating the severity and duration of cytokine-induced hyperalgesia. Interestingly, recent evidence shows that PI 3-kinase also mediates a negative feedback loop in preventing neuronal hyperexcitability in the *Drosophila* neuromuscular junction (30). In mice, it was shown that PI 3-kinase inhibition blocks capsaicin- and NGF-induced increases in pain sensitivity (hyperalgesia) (31), whereas the morphine-induced reduction in pain sensitivity (analgesia) is mediated via PI 3-kinase (32). Our present data demonstrate that PI 3-kinase inhibition increases and prolongs cytokine-induced hyperalgesia in WT mice. The differential effects of PI 3-kinase activity in determining pain sensitivity may depend on the fact that the isoform of PI 3-kinase that is activated in response to NGF is of a different subtype than the PI 3-kinase

activated by cytokines or morphine, PI3K $\gamma$  (PI 3-kinase  $\gamma$ ) (32). Thus, if GRK6 is only required for activation of the PI3K $\gamma$  isoform of PI 3-kinase activated by TNF- $\alpha$  and IL-1 $\beta$ , one would expect that NGF-induced hyperalgesia would not be affected by GRK6. Indeed, we show that NGF-induced hyperalgesia was similar in *GRK6*<sup>-/-</sup> and WT mice (Supplementary Figures 3A, B).

Opposing effects of the mitogen-activated protein kinase p38 and PI 3-kinases on cellular functioning have been shown previously. Lipopolysaccharide (LPS)-induced IL-6 release is inhibited when p38 is blocked, whereas inhibition of the PI 3-kinase/Akt pathway enhances IL-6 production (33). Additionally, TNF- $\alpha$  and IL-1 $\beta$  can both induce a PI 3-kinase/Akt-dependent decrease in potassium currents (34), whereas p38 activation enhances potassium currents (35). The exact molecular mechanisms that explain how p38 and PI 3-kinase define the balance in the effects on cytokine-induced hyperalgesia remain to be elucidated (Figure 6E).

The question arises through which mechanisms GRK6 is required for the activation of PI 3-kinase and how GRK6 inhibits p38 activation. IL-1 $\beta$ -induced activation of PI 3-kinase requires recruitment of PI 3-kinase to the interleukin-1 receptor (IL-1R) (36). Another member of the GRK family, GRK2, was implicated in facilitating agonist-induced PI3K $\gamma$  recruitment to the  $\beta$ 2-adrenergic receptor (37). Similarly, it is possible that GRK6 regulates recruitment of PI3K $\gamma$  to the IL-1 $\beta$  receptor, enabling IL-1 $\beta$ -induced activation of PI 3-kinase. In addition, GRK2 inhibits p38 activation by phosphorylation of p38 at Thr-123, a residue located at its docking groove (38). Importantly, the central serine/threonine kinase catalytic domain as well as the N-terminal regulator of G protein signaling (RGS)-like domain are highly conserved in all GRKs (39). Thus, it is possible that GRK6 interferes with p38 activation via binding and phosphorylation of p38 similar to what has been described for GRK2. Future studies will have to unravel the precise mechanisms.

We recently described that a partial reduction in GRK2 increases hyperalgesia induced by carrageenan and the GPCR ligands CCL3 and PGE<sub>2</sub> (24,40). However, reduced GRK2 did not affect the magnitude of IL-1 $\beta$ -induced acute hyperalgesia (40,41). Vice versa, we show here that GRK6 deficiency does not affect PGE<sub>2</sub>-induced hyperalgesia. These findings indicate that GRK6 and GRK2 regulate hyperalgesia induced by inflammatory mediators via separate mechanisms. These distinct effects of GRK6 and GRK2 on inflammatory hyperalgesia conform to the notion that the different GRKs have specific and often contrasting effects on signaling pathways (42).

GRK6 and other members of the GRK family have been originally identified because of their capacity to phosphorylate agonist-occupied GPCRs (43). However, to the best of our knowledge, there is no evidence that IL-1 $\beta$  and TNF- $\alpha$  signal directly via a GPCR. One possible explanation for our findings could be that IL-1 $\beta$  induces the local production of GPCR ligands (for example, PGE<sub>2</sub>) and that GRK6 regulates signaling via this receptor with consequences for hyperalgesia. However, inhibition of COX2 did not affect IL-1 $\beta$ -induced hyperalgesia in WT or *GRK6*<sup>-/-</sup> mice (Supplementary Figure 4). Additionally, we showed that the magnitude and duration of PGE<sub>2</sub> hyperalgesia was similar in WT and *GRK6*<sup>-/-</sup> mice. We cannot exclude that other GPCR ligands are produced at the site of injection of the cytokines. However, this explanation seems unlikely in view of our finding that the change in phosphorylation of p38 and Akt in sensory neurons was already observed 5 min after stimulation with IL-1 $\beta$  *in vitro*. Overall, these data point to a role of GRK6 in regulating cytokine-induced events that are independent of GPCR signaling.

The data presented herein indicate that GRK6 deficiency enhances the sensitivity to IL-1 $\beta$ -induced hyperalgesia. It may therefore be that GRK6 deficiency also facilitates other inflammatory processes involving IL-1 $\beta$ . Indeed,

this hypothesis is supported by our earlier studies. For example, we have shown that increased inflammatory visceral hyperalgesia in *GRK6*<sup>-/-</sup> mice is characterized by increased neuronal and behavioral responses to noxious colonic stimulation with capsaicin. We also showed that overexpression of GRK6 attenuated IL-1 $\beta$ -induced TRPV1 sensitization (12). Interestingly, blocking TRPV1 with capsazepine partially inhibited the ongoing IL-1 $\beta$  hyperalgesia 24 h after IL-1 $\beta$  administration (Supplementary Figure 5), suggesting enhanced TRPV1 sensitization may underlie the enhanced intraplantar IL-1 $\beta$ -induced heat hyperalgesia as well. Finally, we demonstrated that *in vivo*, GRK6 deficiency leads to increased severity as well as duration of colitis (11). In these models of visceral hyperalgesia and colon inflammation, IL-1 $\beta$  is produced in the colon, and thus increased sensitivity to IL-1 $\beta$ -induced events may contribute to the increased and prolonged pain and colitis. In addition, Tarrant *et al.* (10) showed that *GRK6*<sup>-/-</sup> mice develop more severe joint inflammation in the K/BxN model of acute inflammatory arthritis (10). These authors showed that serum IL-1 levels are normal, but IL-6 levels are elevated in *GRK6*<sup>-/-</sup> mice in this model of inflammatory arthritis. Because IL-1 is a potent IL-6 inducer, it may be that increased IL-1 signaling also underlies the higher levels of IL-6 and increased joint inflammation in this model.

The pathophysiological relevance of our finding that GRK6 deficiency increases severity and duration of cytokine-induced hyperalgesia is underlined by our findings on GRK6 expression in DRGs in two different chronic pain models. We show that sensory neuron GRK6 expression is reduced in DRGs innervating the sensitized paw of mice with chronic neuropathic or inflammatory pain. Moreover, chronic inflammatory diseases that are accompanied by pain, such as rheumatoid arthritis, are associated with a decrease in intracellular GRK6 levels (44).

## CONCLUSION

In summary, here we have identified for the first time GRK6 as a crucial kinase that is required to constrain cytokine signaling and cytokine-induced hyperalgesia. This contribution of GRK6 to hyperalgesia is likely to be mediated by regulating the balance of cytokine-induced p38 and PI 3-kinase/Akt activation in normal mice. This important relevant function of GRK6 in modulating cytokine-induced signaling events may also play a role in chronic inflammatory conditions.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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