GRK2 DEFICIENCY IS A PAIN

Wendy Kleibeuker

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Promotor: Prof. dr. C.J. Heijnen

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Voor mijn zusje

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CHAPTER

General introduction

PAIN PERCEPTION

Pain has been defined as "an unpleasant sensory and emotional experience that is associated with actual or potential tissue damage, or both" (IASP, 1979). Acute pain functions as a protective warning mechanism and is sensed during an event, which is or may become potentially harmful. However, chronic pain may develop when noxious stimuli cause tissue damage, nerve trauma, and/or inflammation. Chronic pain is often characterized by:

1. spontaneous pain: pain in the absence of stimulation,
2. hyperalgesia: an increased response to a painful stimulus,
3. allodynia: pain due to a stimulus which does normally not provoke pain[1].

Chronic pain can be subdivided into inflammatory and neuropathic pain. Neuropathic pain is a consequence of nerve trauma due to disease, surgery, or injury. Neuropathic pain often emerges in patients with spinal cord injury, diabetic mellitus, herpes zoster, carpal tunnel syndrome, phantom limb pain, or multiple sclerosis[2]. Inflammatory pain is initiated by tissue damage or inflammation and is present in many patients with inflammatory diseases like rheumatoid arthritis, infections, and inflammatory bowel diseases (e.g. ulcerative colitis and Crohn's disease), and so-called "pain syndromes" such as fibromyalgia[3]. However, from a theoretical point of view, it is hard to distinguish between inflammatory and neuropathic pain, as inflammation is also a feature of neuropathic disorders. In this respect, it is of interest that signs of inflammation (i.e. the production of inflammatory mediators and recruitment of leukocytes) often take place during neuropathic pain. On the other hand, inflammation can result in damage to terminal nerve endings, such as is operative during bone cancer[4].

Several mechanisms contributing to the development of increased pain perception have been described, however, the exact mechanisms that lead to chronic pain have not been unraveled yet[2]. Table 1 depicts peripheral and central mechanisms contributing to increased processing of nociceptive signals. The most important mechanisms are discussed in the following paragraphs.

PERIPHERAL MECHANISMS CONTRIBUTING TO INCREASED PAIN PERCEPTION

When peripheral nerve endings are triggered by innoxious or noxious stimuli, sensory nerve fibers become activated and transmit peripheral, sensory information to the spinal cord. Sensory nerve fibers are grouped into Aβ-, Aδ-, and C-fibers. Innoxious stimuli are mainly transmitted by Aβ-fibers, which are myelinated, large-diameter fibers. The thinly-myelinated, medium-diameter Aδ-fibers are involved in transmitting signals from thermal as well as mechanical noxious stimuli. The slow-conducting C-fibers are unmyelinated, small-diameter fibers that transmit mechanical, chemical, as well as thermal information. In the spinal cord, sensory nerves mainly project to pain-transmission neurons, which send the message to the

Mechanisms contributing to the development chronic pain
Peripheral mechanisms:
- Recruitment of leukocytes.
- Release of inflammatory mediators (e.g. pro-inflammatory cytokines, bradykinin, prostaglandins, and NGF).
- Increased expression or sensitivity of ion channels:
o Lowered threshold for depolarization,
o Ectopic and spontaneous discharges,
o Increased responsiveness to pain stimuli.
- Collateral sprouting of sensory nerves fibers.
- Recruitment of "silent" receptors.
Central mechanisms:
- Early phase long-term potentiation:
o Increased neurotransmitter release from C-fibers induces the opening of NMDA receptor channels or T-type voltage-gated Ca^{2+} channels,
o Increased release of intracellular Ca^{2+}.
- Late phase long-term potentiation:
o Phosphorylation of ion channels,
o Increased insertion of receptors into the plasma membrane,
o Increased protein synthesis (e.g. BDNF, COX-2, NMDA receptor).
- Activation of glial cells.
- Activation of MAPKs.
- Release of inflammatory mediators.
- Cross-excitation between neurons.
- Neuronal reorganization (sprouting of spinal cord neurons to new locations).
- Central disinhibition.

Table 1: Peripheral and central mechanisms contributing to the development of chronic pain. Table is adapted from Pasero *et al.* (2004)[2].

brain and are located in laminae I, II, IV, V, and X[5]. The excitability of peripheral nerve endings is regulated by a number of processes (Table 1) including:

1. recruitment of leukocytes,
2. expression of inflammatory mediators,
3. changes in ion channels and signal transduction pathways.

1. Recruitment of leukocytes

Leukocytes have an important function in the modulation of pain perception during chronic pain. These cells are not involved in the perception of acute pain. Leukocytes infiltrate inflamed or damaged tissue and nerve fibers. Invasion of T-lymphocytes has been demonstrated at the site of nerve lesion[6-8]. The induction of mechanical allodynia and thermal hyperalgesia by chronic constriction injury (CCI) of the sciatic nerve was reduced in athymic nude rats. Passive transfer of type 1 T-lymphocytes, that produce pro-inflammatory cytokines, into nude rats restored the development of neuropathic pain[6]. However, the role of T- and B-lymphocytes during inflammatory pain has been less well-studied and is still unclear[9].

On the other hand, infiltrating mast cells, macrophages, as well as neutrophils have been reported to contribute to inflammatory as well as neuropathic pain[9]. A reduction in the mast

cell population or macrophage depletion results in a decrease in inflammatory pain evoked by injection of zymosan or acetic acid[10]. In addition, sciatic nerve CCI-induced hyperalgesia is reduced by inhibition of mast cell degranulation or by depletion of macrophages or neutrophils[11-13]. However, the function of neutrophils during inflammatory pain is controversial, since neutrophil migration into the inflamed area can have multiple consequences. For example, nerve growth factor (NGF)-induced inflammatory hyperalgesia is dependent on the recruitment of neutrophils[14]. In contrast, infiltrating neutrophils mediate anti-nociception during inflammatory pain via the release of endogenous opioids induced by stimulation with corticotropin-releasing factor or swim-stress[15,16]. However, selective neutrophil recruitment by C-X-C chemokine ligand (CXCL)1 or CXCL2/3 injection does not contribute to inflammatory pain at all[17].

2. Expression of inflammatory mediators

During chronic pain, a variety of substances that are known to contribute to the increase in pain sensitivity are produced. These mediators include factors related to tissue damage (e.g. reactive oxygen species (ROS) and ATP), factors related to inflammatory processes (e.g. pro-inflammatory cytokines, histamine, chemokines, NGF, and prostaglandins), and factors released by terminal nerve endings (e.g. substance P (SP) and calcitonin gene-related peptide (CGRP)). These inflammatory mediators contribute to chronic pain via direct excitation of peripheral nerves, via sensitization of neurons, or via the induction of changes in gene expression in peripheral nerves[3].

Production of inflammatory mediators and their contribution to chronic pain has already been demonstrated in a number of animal models. For instance, during λ-carrageenan-induced inflammatory pain, a cascade of pro-inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, prostaglandin E (PGE)2, and several chemokines (C-C chemokine ligand 2 (CCL2), CCL3, CXCL8, and CXCL10), is produced in the paw[18,19]. Blocking the effects of CXCL8, IL-1β, or TNF-α by antagonist treatment reduces inflammatory hyperalgesia in this model[18]. Neuropathic pain induced by chronic compression of dorsal root ganglia (DRG) or CCI of the sciatic nerve is associated with an increased production of CCL2 in DRG[20,21]. Furthermore, production of IL-6, TNF-α, and IL-1β is observed in the sciatic nerve during CCI[22]. Intraneural injections of IL-1β or TNF-α into the sciatic nerve resulted in thermal hyperalgesia[23]. Moreover, the observation that intraplantar injection of CCL3, bradykinin, SP, PGE2, NGF, or IL-1β induces hyperalgesia demonstrates that these mediators can induce an increase in pain sensitivity[18,24-27].

3. Changes in ion channels and signal transduction pathways

An important mechanism that enhances the excitability of peripheral nerve endings involves the increase in the sensitivity or expression of ion channels. Transient receptor potential vanilloid type 1 (TRPV1) and sodium channels belong to the group of ion channels that are

expressed on peripheral nerve endings. Sodium channels open rapidly and transiently in response to membrane depolarization, whereas TRPV1 is activated by noxious heat (> 43°C), acid, and capsaicin[28]. Sensitization of TRPV1 occurs through protein kinase C (PKC)ε-mediated phosphorylation, downregulation of phosphatidylinositol 4,5-bisphosphate (PIP2)-mediated inhibition, or the increased insertion of receptors in the plasma membrane via c-Src-dependent phosphorylation[29]. This event can take place in response to prostaglandins, bradykinin, NGF, and the chemokine CCL3[24,30,31]. In addition, an increase in TRPV1 mRNA expression, in anterograde transport of TRPV1, and in expression of TRPV1 in peripheral nerve endings has been observed during inflammatory as well as neuropathic pain[32]. Moreover, an increase in expression of the TTX-sensitive sodium channels $Na_v1.3$, $Na_v1.7$, and $Na_v1.8$ has been reported in DRG following λ-carrageenan injection[33].

Changes in sensory nerve fibers are often reflected in DRG, which contain the cell bodies of these fibers. Inflammation and nerve trauma leads to mitogen-activated protein kinase (MAPK) activation, altered gene transcription, and changes in protein synthesis in DRG[34]. For instance, phosphorylation of p38 MAPK has been demonstrated during inflammation evoked by injection of complete Freund's adjuvant (CFA) or λ-carrageenan as well as during CCI- and spinal nerve ligation (SNL)-induced neuropathic pain[35-38]. In addition, phosphorylation of ERK1/2 in DRG has been observed during CCI-, SNL-, and CFA-evoked pain hypersensitivity[37,39,40]. Recently, Katsura *et al.* (2007) showed increased phospho-ERK5 levels in DRG during CFA-induced hyperalgesia[41].

Activated MAPKs translocate to the nucleus where they phosphorylate transcription factors (e.g. CREB, Elk-1). In this way, transcription of a variety of genes including c-Fos, brain-derived neurotrophic factor (BDNF), cyclooxygenase (COX)-2, and pro-inflammatory cytokines is increased[34]. During CFA-induced inflammatory thermal hyperalgesia, the upregulation of phospho-p38 MAPK is associated with an increase in TRPV1 protein expression and inhibition of p38 MAPK phosphorylation prevents development of hyperalgesia as well as the upregulation of TRPV1[35]. In addition, neuropeptide Y, NGF, and BDNF production in DRG during CCI of the sciatic nerve is prevented by MAPK inhibitors[37].

CENTRAL MECHANISMS CONTRIBUTING TO INCREASED PAIN PERCEPTION

The development of chronic pain is not wholly dependent on peripheral mechanisms, but is also a result of several central mechanisms (Table 1). As a consequence of changes in central pain processing, chronic pain is not only characterized by an increase in pain sensitivity in the affected tissue, but may also comprise the development of secondary hyperalgesia. Secondary hyperalgesia occurs in the uninjured tissue surrounding the site of injury or contralateral to the site of injury (mirror-image pain)[2]. The mechanisms contributing to facilitation of central pain transmission include among others:

1. activation of glial cells,
2. production of inflammatory mediators in the spinal cord,
3. activation of MAPKs in the spinal cord,
4. long-term potentiation.

1. Activation of glial cells

Glial cells (astrocytes and microglia) are non-neuronal cells present in the central nervous system. The main functions of glial cells are the transport of nutrients to neurons, removal of cell debris, and maintenance of the blood-brain-barrier. Microglia also participate in the immune defense, a function in which they show great resemblance to macrophages. Additionally, activated glial cells have a key role in the control of synaptic transmission via the regulation of clearance of neurotransmitters from the synaptic cleft and the release of substances which modulate pre- as well as post-synaptic function[42]. A number of mediators, which are produced in the spinal cord, are responsible for glial activation during chronic pain including neuronal chemokines (e.g. C-X_3-C chemokine ligand (CX_3CL)1, CCL2), pro-inflammatory cytokines, ATP, glutamate, and neuropeptides[43].

Activation of glial cells is associated with increased pain sensitivity in a variety of animal models including spinal nerve trauma, CCI of the sciatic nerve, and inflammation induced by λ-carrageenan or CFA injection[44-47]. Application of glial inhibitors has demonstrated that glial activation is indeed critical for the upregulation of pain sensitivity. Fluorocitrate, which blocks activity of microglia as well as astrocytes, inhibits and/or reverses pain states induced by sciatic inflammatory neuritis (SIN), viral components, or formalin[48-50]. In addition, minocycline, a specific microglia inhibitor, prevents the development of chronic pain induced by SIN, viral components, λ-carrageenan, and L5 spinal nerve transection (SNT), but fails to reverse established chronic pain[46,51,52]. These data suggest that microglia activation is involved in initiating the increase in pain sensitivity, whereas astrocytes have a more prominent role in its maintenance. Finally, the essential role for microglia activation in the development of chronic pain comes from the observation that intrathecal injection of ATP-activated microglia leads to an increase in mechanical allodynia[53].

2. Production of inflammatory mediators in the spinal cord

Activated glial cells contribute to chronic pain via the production of inflammatory mediators (i.e. pro-inflammatory cytokines, ATP, neurotransmitters, nitric oxide (NO), and prostaglandins). These inflammatory mediators facilitate pain processing via increasing neurotransmitter release, upregulating ion channel expression and/or conductivity, and increasing neuron excitability[54,55].

The production of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 is elevated in the spinal cord following SNI, CCI of the sciatic nerve, and CFA-induced inflammation[47,52,56]. The contribution of these pro-inflammatory cytokines to pain hypersensitivity becomes apparent

from the observations that they increase nociception after intrathecal administration and that intrathecal administration of cytokine antagonists can block and/or reverse established mechanical allodynia and thermal hyperalgesia[52,56-59]. Moreover, the development of mechanical allodynia during L5 SNT is abrogated in mice with a deficiency in IL-1β signaling[60]. In addition to pro-inflammatory cytokines, λ-carrageenan-induced inflammatory pain and L5 SNT-induced neuropathic pain is associated with the production of PGE2 in the spinal cord, whereas CCL2 production has been observed in the spinal cord during sciatic nerve CCI-induced neuropathy[21,61,62]. Furthermore, the production of ROS plays a role in the development of neuropathic pain behaviors, since non-selective inhibition of nitric oxide synthase (NOS) inhibits CCI-induced hyperalgesia[63].

3. Activation of MAPKs in the spinal cord

As a consequence of receptor stimulation, multiple intracellular signaling cascades, including the MAPK pathways, are turned on (for detailed description see below). The activation of MAPKs (p38 MAPK, ERK1/2, and ERK5) has been reported during inflammatory as well as neuropathic pain. ERK1/2 phosphorylation was detected in glial cells as well as in neurons, whereas p38 MAPK phosphorylation was mainly detected in microglia. Contribution of these MAPKs to the development of chronic pain was confirmed by blockade of MAPK activation by specific inhibitors. ERK1/2 phosphorylation in the spinal cord was demonstrated during CFA- and λ-carrageenan-induced inflammatory hyperalgesia as well as during neuropathic pain (e.g. CCI of the sciatic nerve, L5 SNT) and inhibition of ERK1/2 activation blocked mechanical allodynia and/or thermal hyperalgesia in these models[39,64-67]. Increased pain sensitivity induced by CCI of the sciatic nerve, L5 SNT, and injection of formalin or λ-carrageenan is associated with an upregulation of p38 MAPK phosphorylation. Like ERK1/2, specific inhibition of p38 MAPK phosphorylation prevented the development of pain hypersensitivity[46,68-70]. Recently, Obata *et al.* (2007) reported phosphorylation of ERK5 in spinal cord microglia during L5 SNL-induced neuropathic pain. ERK5 siRNA knock-down prevented the nerve-injury neuropathic pain as well as microglia activation in this model[71].

As described above, activation of MAPKs is responsible for phosphorylation of transcription factors and the subsequent increase in gene expression. For example, inhibition of p38 MAPK activation prevented the upregulation of COX-2 and c-Fos expression in the formalin model[70]. In addition, inhibition of ERK1/2 activation prevents the CFA-induced increase in neurokinin-1 receptor (NK-1R) and prodynorphin levels[64]. Moreover, formalin-induced hyperalgesia is associated with an increase in phosphorylation of the transcription factor CREB and c-Fos expression in the spinal cord[72].

4. Long-term potentiation

An important mechanism underlying hyperalgesia is long-term potentiation, which has been defined as facilitation of synaptic transmission as a result of an increase in neurotransmitter

release from C-fibers and/or an increase in the post-synaptic effects of neurotransmitters. In early phase long-term potentiation, an exaggerated C-fiber activity induces the opening of NMDA receptor channels (release of Mg^{+} block) or T-type voltage-gated Ca^{2+} channels and an increase in intracellular Ca^{2+} release. Late phase long-term potentiation involves the activation of protein kinases and the upregulation of transcription, which facilitates pain transmission through phosphorylation of ion channels (e.g. AMPA receptor, NMDA receptor), alterations in trafficking of synaptic proteins (e.g. increased insertion of receptors into the plasma membrane), and increased protein synthesis (e.g. COX-2, TRPV1, NMDA receptor)[5,73].

GPCRS AND PAIN

G protein-coupled receptors (GPCRs) constitute a large family of proteins with seven transmembrane-spanning domains and are involved in signal transduction across the cell membrane. Numerous substances (i.e. neurotransmitters, neuropeptides, and chemokines) involved in the regulation of nociceptive transmission during chronic pain signal through GPCRs. GPCR signaling plays an essential role in the regulation of pain perception, which is supported by several lines of evidence. First of all, peripheral or central stimulation of GPCRs initiates an increase in pain perception. For example, intrathecal administration of metabotropic glutamate receptor (mGluR) group I agonists, CCL2, or CX_3CL1 induces an increase in pain sensitivity[74-76]. Furthermore, an array of GPCR agonists, including CCL2, CCL3, CCL5, CXCL12, PGE2, epinephrine, and SP, have been reported to induce hyperalgesia and/or mechanical allodynia when injected into the hind paw of rodents[18,24-26,77,78]. In addition, abrogation of GPCR signaling blocks the development of pain hypersensitivity. Inhibition of mGluR group I, PGE2, or α_2-adrenergic receptors (ARs) by intrathecal administration of antagonists inhibits chronic pain associated with CCI of the sciatic nerve[79-81]. Inflammatory pain is inhibited by intrathecal injection of anti-CX_3CL1 or mGluR group I antagonists[75,76]. Moreover, mechanical allodynia caused by CCI of the sciatic nerve or intraplantar CFA injection is impaired in C-C chemokine receptor 2 $(CCR2)^{-/-}$ mice[77]. Several studies also demonstrated an increase in sensitivity of GPCRs during various pain models. During sciatic nerve CCI-induced neuropathic pain, pain-related behaviors induced by intrathecal injection SP are increased[82]. Similar results were obtained by intrathecal injection of PGE2 during L5 SNT[62]. Furthermore, pain-related behaviors in response to intrathecal injection of mGluR group I agonists or intradermal injection of PGE2, 5-hydroxytryptamine (HT) or A2 adenosine agonists were increased during inflammatory pain[83,84].

Stimulation of GPCRs leads to the activation of G proteins and intracellular second messengers, which in turn activate specific protein kinases (for detailed description see below). These kinases phosphorylate several targets and in this way induce changes in cellular proteins and ion channels. Indeed, intradermal injection of PGE2 or epinephrine produces

hyperalgesia which is dependent on protein kinase A (PKA), PKC, and ERK1/2 activation[26]. Furthermore, the increase in PGE2-, 5-HT-, and A2-adenosine agonist-mediated pain behaviors during inflammatory pain is reduced by a PKCε inhibitor[84]. Moreover, signaling induced by GPCR agonists (e.g. CCL3 and PGE2) can increase neuronal excitability via the upregulation of TRPV1 sensitivity[24,30]. In addition to TRPV1, mGluR group I receptor signaling initiates neuronal sensitization through phosphorylation of the NMDA receptor NR2B subunit in the spinal cord during CFA-induced inflammatory hyperalgesia[85].

GPCR SIGNALING

G protein signaling

Signaling through GPCRs is mediated by heterotrimeric G proteins, which are composed of a Gα, Gβ, and Gγ subunit. In the absence of receptor stimulation, the Gα subunit is GDP-bound and tightly associated with the Gβγ subunit. An agonist-activated receptor functions as guanine-nucleotide exchange factor, which activates the G protein by catalyzing the exchange of the Gα-bound GDP for GTP. The succeeding conformational change of Gα leads to the dissociation of Gα from the Gβγ subunits. The Gα as well as the Gβγ subunits are responsible for the activation of multiple intracellular signaling routes via the activation of several downstream effector proteins. The Gα subunit possesses intrinsic GTPase activity, which hydrolysis GTP to GDP. This process is facilitated by regulators of G protein signaling (RGS) proteins, which function as GTPase-activating proteins. The GDP-bound form of Gα reassociates with Gβγ, which terminates activation of downstream effectors[86,87].

As yet, 17 different Gα, 5 Gβ, and 14 Gγ subunits have been discovered. The Gα subunits can be grouped into 4 subgroups: (1) $G\alpha_s$ ($G\alpha_s$, $G\alpha_{olf}$), (2) $G\alpha_q$ ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, $G\alpha_{16}$), (3) $G\alpha_i$ ($G\alpha_{i1\text{-}3}$, $G\alpha_z$, $G\alpha_{oA\text{-}B}$, $G\alpha_{t1\text{-}2}$), and (4) $G\alpha_{12}$ ($G\alpha_{12}$, $G\alpha_{13}$). Most Gα subunits are ubiquitously expressed, except for $G\alpha_{15/16}$, $G\alpha_{olf}$, and $G\alpha_{t1\text{-}2}$, which are mainly present in hematopoietic cells, olfactory nerves, and the retina, respectively[86]. $G\alpha_s$ is able to activate adenylyl cyclases that convert ATP to cAMP, which initiates activation of PKA. Conversely, $G\alpha_i$ inhibits adenylyl cyclases. $G\alpha_q$ subunits activates phospholipase C (PLC)β that hydrolyses PIP2 into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which are responsible for the activation of PKC and the release of intracellular calcium, respectively. The $G\alpha_{12}$ subunits regulate the activation of Rho and Rho guanine-nucleotide exchange factors. In addition, activated Gβγ subunits control the activation of several effectors, including K^+ channels, small G proteins, phosphoinositide-3 kinases (PI3 kinases) and PLCβ[87,88].

MAPK cascades

The MAPK cascades are typically composed of a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates a MAPK. The 4 members

of MAPKs present in cells are ERK1/2, JNK1-3, p38 MAPKα/β/γ, and the more recent identified ERK5[89,90]. All Gα and Gβγ subunits have been described to trigger activation of the different MAPK pathways. Activation of MAPKKK by G proteins is mainly mediated via PKC, PKA and PLCβ. Conversely, some Gα subunits have been described to exert inhibitory effects on MAPKs[88,91]. Activated MAPKs translocate from the cytosol to the nucleus, where they adjust the activity of a variety of transcription factors (e.g. CREB, Elk-1, and ATF-2) and in this way regulate the expression of multiple genes. For instance, the amount of c-Fos transcription in the spinal cord was illustrated to depend on the level of ERK1/2 phosphorylation[92]. During hyperalgesia, activated MAPKs in the spinal cord or DRG initiate increased transcription of several proteins (e.g. c-Fos, dynorphin, COX-2, and IL-1β), which contributes to the upregulation of neuronal excitability during hyperalgesia[34].

MAPK signaling is regulated by scaffold proteins such as MEK-partner 1, β-arrestins and several JNK interacting proteins. These scaffolding proteins promote kinase-substrate interactions and regulate the activity and localization of MAPKs[93]. For example, binding of MEK-partner 1 to MEK1 and ERK1 has been shown to enhance ERK1-mediated phosphorylation of the transcription factor Elk-1[94].

PI3 kinase-Akt pathway

PI3 kinases are composed of a catalytic and regulatory subunit and based on the composition of these subunits they are subdivided into class I-III. Class I PI3 kinases generate the lipid products that are responsible for Akt activation. Class IA PI3 kinases are composed of the regulatory p85 subunit, that is associated with a p100α, p110β, or p110δ catalytic subunit, whereas class IB PI3 kinases contains one member (PI3 kinase-γ), which is composed of the regulatory p101 subunit and the catalytic p110γ subunit. Upon GPCR stimulation, Gβγ subunits directly bind to p110γ resulting in the activation of PI3 kinase-γ[95]. Although GPCRs mainly stimulate PI3 kinase-γ, for some receptors alternative pathways have been identified. CCR2 signaling leads to $G\alpha_i$-mediated activation of class IA PI3 kinases as well as class II PI3 kinase-C2α in THP-1 cells[96]. In addition, upon LPA stimulation liberated Gβγ subunits mediate activation of p110γ and p110β through Gβγ-mediated transactivation of tyrosine kinases[97].

Class I PI3 kinases generate phosphatidylinositol 3,4,5-triphosphate (PIP3) via the phosphorylation of PIP2. Production of PIP3 is responsible for the recruitment of PIP3-binding proteins, such as PDK-1 and Akt, to the plasma membrane. These proteins selectively bind PIP3 through their pleckstrin homology (PH) domain. Membrane translocation and PIP3 binding triggers the succeeding activation of Akt. Complete Akt activation requires phosphorylation at Thr308 by PDK-1 as well as the subsequent phosphorylation of Ser473 by an unknown protein PDK-2. Active Akt translocates back to the cytosol or nucleus, where it phosphorylates multiple targets involved in transcription (e.g. IκB kinase), cell survival and apoptosis (e.g. GSK-3 and caspase-9)[98]. Interestingly, activation of Akt signaling pathways in spinal cord or peripheral neurons has been shown to contribute to pain hypersensitivity[99,100].

TERMINATION OF GPCR SIGNALING

Receptor desensitization (the loss of receptor responsiveness to an agonist) is an essential feedback mechanism that prevents a cell from overstimulation. Receptor desensitization is regulated by different mechanisms, which are initiated upon GPCR activation. The receptor is first uncoupled from heterotrimeric G proteins in response to receptor phosphorylation. Further desensitization is achieved by subsequent internalization of agonist-activated receptors. When stimulation is chronically present, the number of receptors is downregulated via receptor degradation or a reduction in receptor mRNA expression. Whereas uncoupling of the G protein (seconds) and receptor internalization (minutes) are relatively rapid events, downregulation of receptor expression takes several hours to days to occur. Two forms of receptor desensitization have been identified and phosphorylation is an essential step for both forms of desensitization. Homologous desensitization is restricted to receptors that are activated by the agonist and has no effect on other receptors present in the same cell. On the other hand, during heterologous desensitization the stimulation by one agonist attenuates the responsiveness to a variety of unrelated agonists. GPCR kinases (GRKs) and arrestins play a major role in homologous desensitization, whereas second-messenger protein kinases are involved in homologous as well as heterologous desensitization[101,102].

Second-messenger protein kinases

GPCR stimulation leads to the activation of several intracellular signaling cascades, which results in the activation of second-messenger proteins kinases (PKA and PKC). PKA and PKC mainly phosphorylate G_i- and G_q-coupled GPCRs, which results in a conformational change of the receptor. In this way, further coupling of heterotrimeric G proteins to the receptor is inhibited. PKA and PKC not only phosphorylate agonist-occupied receptors, but also receptors which have not been exposed to an agonist and thus are involved in homologous as well as heterologous receptor desensitization[101].

GRKs and arrestins

The GRK family consists of serine-threonine protein kinases of which, until now, 7 members have been discovered: GRK1-7. Based on structural and functional resemblance, the GRK family can be subdivided into 3 subgroups: (1) the visual GRKs consisting of GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase), (2) the GRK2-like subfamily containing GRK2 (β-ARK1) and GRK3 (β-ARK2), and (3) the GRK4-like subfamily consisting of GRK4, GRK5, and GRK6. GRK1/7 and GRK4 show tissue-specific distribution to the retina and the testis respectively, whereas other GRKs are more or less ubiquitously expressed[102,103]. GRK2 levels are predominantly high in cells of the immune and nervous system[104,105]. In the nervous system, GRK2 is mainly detected in neurons with specific localization in post-synaptic densities and axon

terminals[106]. This indicates that GRK2 is particularly localized to cell compartments containing GPCRs that are substrate of GRK2.

Upon ligand binding, GRK2 exclusively associates with agonist-occupied GPCRs and phosphorylates threonine-serine residues within the intracellular loops and the C-terminal domain of these receptors (Figure 1). This event initiates homologous receptor desensitization[107]. Complete desensitization, however, requires the subsequent binding of a member of the arrestin family. Thus far, the arrestins family constitutes 4 members: visual arrestin (arrestin-1), β-arrestin-1 (arrestin-2), β-arrestin-2 (arrestin-3), and cone arrestin (arrestin-4). Visual and cone arrestin are expressed in the retina, whereas β-arrestins are widely distributed. Binding of β-arrestins to GRK-phosphorylated, agonist-activated receptors leads to uncoupling of heterotrimeric G proteins by preventing receptor-G protein interactions. Subsequently, β-arrestins initiate clathrin-mediated internalization of GPCRs resulting in receptor degradation or resensitization inside endosomal compartments[102]. Recent studies demonstrated that β-arrestins can also promote the activation of intracellular signaling pathways. β-arrestin can

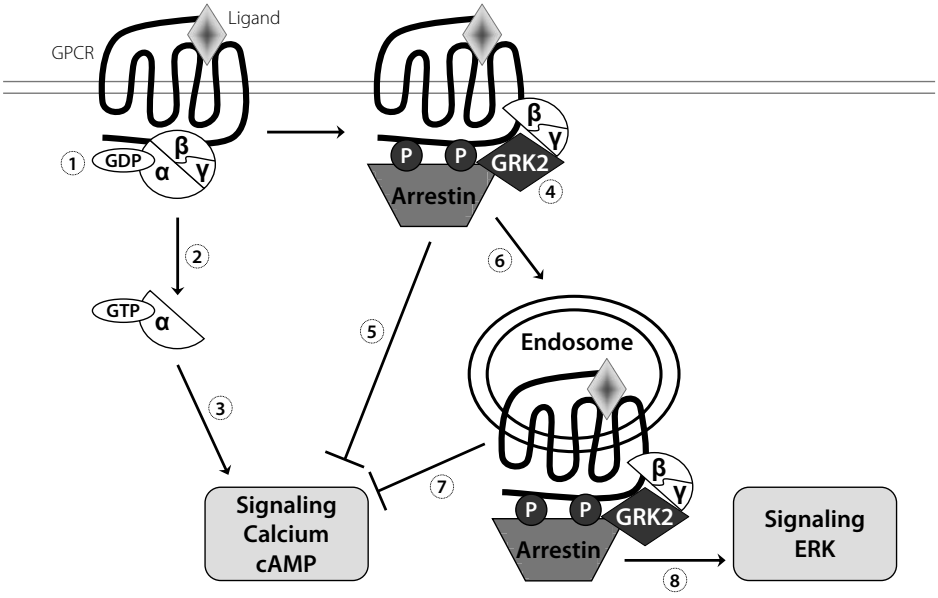

Figure 1: Schematic model of GRK2-mediated desensitization of GPCRs. (1) In the absence of receptor stimulation, the Gα subunit is GDP-bound and tightly associated with the Gβγ subunits. (2) Upon ligand binding, Gα-bound GDP is exchanged for GTP. A subsequent conformational change of the Gα subunit leads to dissociation of Gα from the Gβγ subunits. (3) The Gα and Gβγ subunits activate several downstream effector proteins, which initiate the activation of multiple intracellular signaling cascades. (4) Subsequently, GRK2 is recruited to agonist-occupied GPCRs and phosphorylates threonine-serine residues within the intracellular domains of GPCRs, which results in the subsequent recruitment of β-arrestins. (5) β-Arrestin binding leads to uncoupling of heterotrimeric Gα proteins, which inhibits further signaling through GPCRs. (6) Subsequently, β-arrestins target GPCRs to clathrin-mediated internalization, (7) which results in further inhibition of GPCR signaling. (8) In addition, β-arrestins can also promote the activation of intracellular signaling pathways (e.g. ERK1/2).

serve as a scaffold for specific components of the MAPK cascades (e.g. ERK1/2, JNK, c-Src, and Raf-1) and PI3 kinase-Akt pathway, which facilitates signaling. For example, β-arrestin1 can recruit c-Src to activated β_2-ARs resulting in ERK1/2 activation[108,109]. In addition, β-arrestin binding to ERK1/2 facilitates ERK1/2 phosphorylation following angiotensin AT1a receptor stimulation, but prevents translocation of ERK1/2 to the nucleus and thus inhibits ERK1/2-induced activation of transcription factors[110].

REGULATION OF GRK2 ACTIVITY AND EXPRESSION

Subcellular localization

Most of the cellular GRK2 content is located in the cytosol and GRK2 needs to be recruited to the plasma membrane in order to phosphorylate an agonist-occupied receptor. Membrane translocation of GRK2 requires the specific interaction of the PH domain with phospholipids (i.e. PIP2) present in the plasma membrane[111]. In addition, membrane targeting of GRK2 occurs through binding of the PH domain to Gβγ subunits, which are liberated upon receptor-mediated G protein activation[112]. The association of GRK2 with Gβγ subunits was reported to increase GRK2 kinase activity[113]. Moreover, translocation of GRK2 is promoted by agonist-induced GRK2 phosphorylation at Ser685 by PKA, which increases GRK2 binding to Gβγ subunits[114].

Regulation of GRK2 activity

Activation of GPCRs by agonist stimulation leads to the production of intracellular messengers which can regulate activity of GRK2 (Table 2). GRK2 activity is under control of calmodulin binding, which inhibits GPCR phosphorylation by GRK2 in a Ca^{2+}-dependent manner[115]. Receptor phosphorylation by GRK2 is also modulated by the action of several protein kinases that directly phosphorylate GRK2. Activation of β_2-ARs lead to a rapid and transient increase in c-Src-mediated phosphorylation of tyrosine residues resulting in an upregulation of GRK2 kinase activity[116]. In contrast, GRK2 phosphorylation at Ser670 by ERK1/2 inhibits receptor phosphorylation by GRK2 and impairs Gβγ-mediated GRK2 activation[117,118]. However, as a long-term effect of c-Src- and ERK1/2-mediated GRK2 phosphorylation, GRK2 protein is degraded via a proteasome-dependent mechanism[119,120]. In addition, GRK2 activity is promoted by PKC-mediated phosphorylation at Ser29, which relieves the inhibition of GRK2 by calmodulin and enhances membrane-targeting of GRK2[115,121]. RKIP is a physiological inhibitor of GRK2. Upon agonist stimulation, PKC-mediated phosphorylation of RKIP leads to the dissociation of RKIP from Raf-1. Subsequent binding of RKIP to GRK2 inhibits GRK2 activity[122]. Furthermore, GRK2 activity is inhibited by α-actinin and caveolin[123,124].

An additional mechanism to regulate GRK2 catalytic properties is the modulation of its conformation. Intramolecular interactions between the N-terminal, C-terminal, and catalytic

Interaction Partner	Functional consequences
GPCRs	GRK2 phosphorylates serine-threonine residues within the intracellular loops and C-terminal domain of GPCRs resulting in receptor desensitization.
α-Actinin	α-Actinin inhibits GRK2 activity.
Akt	GRK2 inhibits kinase activity of Akt.
Calmodulin	Ca^{2+}-dependent binding of calmodulin to GRK2 inhibits GRK2-mediated receptor phosphorylation.
Caveolin	Caveolin inhibits GRK2 activity.
Clathrin	Association of clathrin with GRK2 is required for GPCR internalization.
c-Src	c-Src phosphorylates GRK2, which increases GRK2 kinase activity and promotes GRK2 degradation on the long-term.
DREAM	GRK2 phosphorylates DREAM, which blocks DREAM-mediated membrane expression of Kv4.2 potassium channels.
Epithelial sodium channels	GRK2 interacts with epithelial sodium channels.
ERK1/2	ERK1/2-mediated GRK2 phosphorylation decreases GRK2-induced receptor phosphorylation, whereas it enhances GRK2 degradation on the long-term.
Ezrin	GRK2 phosphorylates ezrin, which maintains ezrin in its active conformation.
Gα	The RH-domain of GRK2 binds to $G\alpha_{q/11}$ and inhibits $G\alpha_{q/11}$-mediated signaling via a phosphorylation-independent mechanism.
Gβγ	Activated Gβγ subunits bind and recruit GRK2 to the plasma membrane, thereby increasing GRK2 kinase activity.
GIT1	Translocation of GIT to the membrane requires GRK2.
HSP90	An interaction of GRK2 with HSP90 is involved in proper folding and maturation of GRK2.
MEK1/2	A MEK1/2-GRK2 interaction inhibits agonist-induced ERK1/2 activation.
p38 MAPK	Phosphorylation of p38 MAPK by GRK2 impairs p38 MAPK activation.
Phosducin	GRK2 phosphorylates phosducin, which disrupts its interaction with Gβγ.
PI3 kinase	GRK2-mediated recruitment of PI3 kinase increases receptor endocytosis.
PIP2	PIP2 targets GRK2 to the plasma membrane.
PKA	Phosphorylation of GRK2 by PKA promotes GRK2 binding to Gβγ subunits.
PKC	GRK2 is phosphorylated by PKC, which increases GRK2 activity by relieving the inhibition of GRK2 by calmodulin.
Ribosomal protein P2	GRK2-mediated P2 phosphorylation increases its activity.
RKIP	Upon phosphorylation by PKC, RKIP uncouples from Raf-1 and binds GRK2 to inhibit GRK2 activity.
Synucleins	GRK2 phosphorylates synucleins.
Tubulin	GRK2 phosphorylates tubulin, which may affect the interaction of microtubules with microtubule-associated proteins.

Table 2: Proteins that interact with GRK2. Table is adapted from Hansen *et al.* (2004)[166].

domains of GRK2 are involved in keeping GRK2 in a forced inactive, basal conformation. Disruption of intramolecular interactions by binding to regulatory proteins promotes conformational changes within GRK2, which are required for GRK2 translocation and activation[125,126]. In addition, HSP90 interacts with GRK2 to assure proper folding and maturation of the GRK2 protein. *In vitro*, inhibition of HSP90 lead to an increase in degradation of newly synthesized GRK2[127].

Alterations in GRK2 expression during pathologies

Changes in GRK2 expression in cells of the immune system, the heart, and the central nervous system has been reported during various pathologies. Peripheral blood leukocytes from patients with rheumatoid arthritis or multiple sclerosis show a decrease in GRK2 protein levels, whereas no changes in GRK2 mRNA expression were observed[128,129]. In line with these findings, the amount of GRK2 present in lymphocytes was decreased during an animal model for adjuvant arthritis or relapsing-progressive experimental autoimmune encephalomyelitis (EAE)[130,131]. Furthermore, several studies described an enhanced GRK2 protein expression and GRK2 activity in lymphocytes and cardiac tissue during heart failure and hypertension in humans and in related animal models[132-135]. Interestingly, the amount of leukocyte GRK2 correlated with blood pressure and inversely correlated with β-AR responsiveness[132,134]. In addition, portal hypertensive rats display an increase in GRK2 protein levels in sinusendothelial cells[136]. In the brain, GRK2 levels are downregulated during a neonatal hypoxia-ischemia model, whereas an upregulation of GRK2 was observed in rats during chronic morphine treatment and in patients with Alzheimer's disease or major depression[137-140].

Obviously, above mentioned studies do not exclude the possibility that the observed changes in GRK2 are just an adaptation mechanism of cells as a consequence of the disease state. Proof for a functional relationship between GRK2 levels and pathology comes from multiple animal studies. GRK2$^{+/-}$ mice, that have a 50% decrease in GRK2 expression, show an advanced onset of EAE[129]. In addition, targeted overexpression of vascular GRK2 in mice resulted in cardiac hypertension and hypertrophy and attenuated β_2-AR signaling[141,142]. Moreover, inhibition of GRK2 by overexpression of its C-terminal domain (GRK2ct) prevented the development of cardiomyopathy in a murine model of heart failure[143].

Mechanisms that regulate GRK2 levels

A number of possible mechanisms that can adjust GRK2 levels during disease processes have been identified. One important mechanism is the regulation of GRK2 expression by pro-inflammatory mediators. IL-6 and interferon (IFN)-γ induce a decrease in GRK2 protein levels in peripheral blood leukocytes from healthy human donors[128]. In line with these findings, IL-1β, TNF-α, and IFN-γ reduced GRK2 promoter activity in an aortic smooth muscle cell line[144]. In addition to pro-inflammatory mediators, GRK2 levels can be altered upon treatment with factors that induce mitotis. PMA increases GRK2 protein expression in the promyelocytic HL-60 cell line and increases GRK2 promoter activity in an aortic smooth muscle cell line[144,145]. PHA treatment of human lymphocytes leads to enhancement of GRK2 protein and mRNA levels, as well as GRK2 activity[104,145]. Less is known about the regulation of GRK2 expression in neurons. Thakker *et al.* (2002) demonstrated that prolonged activation of the opioid receptor-like 1 ORL1 induces an upregulation of GRK2 protein levels in two human neuroblastoma cell lines[146].

Changes in GRK2 protein expression are not always reflected by changes in mRNA expression, therefore post-transcriptional mechanisms appear to play an important role in

the regulation of GRK2 protein levels. In fact, exposure of lymphocytes to oxygen radicals facilitates GRK2 degradation through a calpain-dependent mechanism, which requires phosphorylation by tyrosine kinases[147]. However, oxygen radical treatment of the C6 glioma cell line led to reduced GRK2 protein via downregulation of its translation[148]. In addition, GRK2 can also be targeted to proteasome-dependent degradation and this process is stimulated by GPCR stimulation with β-AR agonists or CXCL12[120,149]. Proteasome-dependent degradation requires phosphorylation of GRK2 by c-Src as well as by ERK1/2 and is dependent on β-arrestin function[119,120].

CONSEQUENCES OF ALTERED GRK2 FOR GPCR FUNCTIONING

As mentioned above, GRK2 expression is modulated during various pathologies, which may influence GPCR responsiveness. In this way, the level of GRK2 could have its impact on the induction and/or course of the disease state. Many of the receptors which are substrate of GRK2 are activated by chemokines, neuropeptides, and neurotransmitters that play a role in the development of hyperalgesia and allodynia. GRK2-mediated phosphorylation of agonist-stimulated GPCRS has been reported for several receptors including the NK-1R, mGluR1a, A1/3 adenosine receptor, CCR2, CCR5, α_2- and β_2-ARs[150-155].

The level of cellular GRK2 expression modulates the amount of signaling and desensitization of these receptors. In general, a reduction in GRK2 expression correlates with enhanced receptor signaling, whereas an increase in GRK2 associates with decreased receptor function. Using overexpression systems for GRK2, the impact of increased GRK2 levels on GPCR responsiveness was investigated. GRK2 overexpression promoted agonist-induced internalization of CCR5[155]. The CCL2-induced release of intracellular calcium was shown to be decreased in Mono Mac1 cells overexpressing GRK2[154]. Vascular smooth muscle cells, that overexpressed GRK2, displayed attenuated β_2-AR-induced adenylyl cyclase inhibition and MAPK activation[141]. In addition, phosphatidylinositol (PI) hydrolysis upon α_2-AR stimulation was decreased in COS-7 cells overexpressing GRK2[152]. In HEK293 cells, overexpression of GRK2 inhibited the mGluR1a-mediated IP3 formation[156].

To study the effects of decreased GRK2 levels, cells derived from GRK2$^{+/-}$ mice, which show a 50% decrease in GRK2 expression, were used[157]. Increased migration and release of intracellular calcium in response to CCL3, CCL4, and CCL5 was reported for T-lymphocytes derived from GRK2$^{+/-}$ mice[158]. In addition, β_2-AR internalization was impaired in splenocytes from GRK2$^{+/-}$ mice[159]. Moreover, knock-down of GRK2 by siRNA treatment demonstrated that low levels of GRK2 resulted in a decrease in opioid receptor-like 1, μ-opioid receptor, and β_2-AR desensitization[160,161].

NOVEL ROLE OF GRK2 IN THE REGULATION OF GPCR SIGNALING

Recent studies using kinase-deficient mutants of GRK2 revealed that phosphorylation is not absolutely required for GPCR desensitization[154,162-164]. In addition, it was reported that GRK2 phosphorylates non-receptor substrates (e.g. tubulin, synuclein, ezrin) and binds to several proteins involved in intracellular signaling and receptor trafficking such as PI3 kinase, $G\alpha_q$ subunit, Gβγ subunits, Akt, caveolin, MEK1/2, clathrin, p38 MAPK, and RKIP (Table 2)[165,166]. These studies have uncovered novel mechanisms for GRK2 in the regulation of GPCR signaling.

One additional mechanism for GRK2 to regulate receptor signaling is at the G protein level. GRK2 interacts with $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{14}$, but not with $G\alpha_s$, $G\alpha_i$, $G\alpha_{12/13}$, and $G\alpha_{16}$[167,168]. Binding of the N-terminal domain of GRK2 to $G\alpha_{q/11}$ is sufficient to attenuate $G\alpha_{q/11}$-mediated signaling of several receptors including the angiotensin II receptor, endothelin receptor, thromboxane A2 receptor, 5-HT_{2c} receptor, m2 muscarinic acetylcholine receptor, thyrotropin receptor, and mGluR1a/5. This process is phosphorylation-independent, requires the RGS homology (RH) domain of GRK2, and depends on the interaction of GRK2 with $G\alpha_{q/11}$ as well as with a GPCR[162,164,167,169,170].

In addition, GRK2 interacts with components of the PI3 kinase-Akt and MAPK signaling cascades with consequences for receptor signaling and desensitization. GRK2 binding to PI3 kinase promotes recruitment of PI3 kinase to the plasma membrane resulting in an increase in receptor endocytosis in HEK293 cells overexpressing GRK2[171]. It has also been reported that an interaction between GRK2 and Akt inhibits the kinase activity of Akt in sinusoidal endothelial cells from portal hypertensive rats[136]. Additionally, p38 MAPK associates with endogenous GRK2 and is phosphorylated by GRK2, which attenuates binding and activation by its upstream activator MKK6 and inhibits the ability of p38 MAPK to activate downstream substrates. The activation of p38 MAPK is reduced or enhanced, when GRK2 expression is increased or decreased, respectively. Moreover, altered GRK2 expression modifies p38 MAPK-dependent processes such as LPS-induced cytokine production in macrophages[172]. Furthermore, a GRK2-MEK1/2 interaction reduces CCL2-induced ERK1/2 activation in HEK293 cells overexpressing GRK2. Moreover, the GRK2-mediated inhibition of CCL2-induced ERK1/2 activation did not require GRK2 kinase activity[173].

AIM AND OUTLINE OF THE THESIS

GRK2 regulates the responsiveness of many GPCRS, e.g. CCR1, CCR2, CCR5, NK-1R, and mGluRs, which are involved in the development of inflammatory and neuropathic pain. During several inflammatory processes, GRK2 levels are downregulated in leukocytes. In addition, the amount of cellular GRK2 determines the course and outcome of the disease.

To investigate whether GRK2 levels are regulated in neuronal tissue during neuropathic and inflammatory pain, we analyzed the levels of spinal cord dorsal horn GRK2 expression during two models for nerve injury-induced mechanical allodynia: chronic constriction injury (CCI) of the sciatic nerve in rats (**chapter 2**) and L5 spinal nerve transection (SNT) in mice (**chapter 3**).

Since pro-inflammatory cytokines are known to regulate GRK2 expression in leukocytes, we hypothesized that also in neurons, cytokines may mediate the downregulation of GRK2 during mechanical allodynia. Therefore, the role of IL-1β signaling in downregulating GRK2 was investigated by examining GRK2 expression in IL-1R$^{-/-}$ mice, which do not develop mechanical allodynia after L5 SNT (**chapter 3**). In addition, the effects of IL-1β treatment on GRK2 levels in *ex vivo* cultured spinal cord slices were determined (**chapter 2**).

To investigate whether low GRK2 levels contribute to increased pain sensitivity, we compared λ-carrageenan-induced mechanical allodynia (**chapter 2**) and thermal hyperalgesia (**chapter 4**) between wild type (WT) and GRK2$^{+/-}$ mice. In **chapter 4**, the underlying mechanisms of the increased thermal hyperalgesia in GRK2$^{+/-}$ mice were explored.

Next to neurons, we determined in **chapter 5** whether changes in endogenous GRK2 levels could be induced by IL-1β treatment of primary astrocyte and microglia cultures. Since IL-1β only induced changes in endogenous GRK2 expression of astrocytes, we used these cells to study the effects of physiological changes in endogenous GRK2 levels on chemokine receptor signaling. Moreover, we also investigated in depth the consequences of low GRK2 for signaling pathways in astrocytes from GRK2$^{+/-}$ mice.

A summary of the findings, concluding remarks, and implications for future research are presented in **chapter 6**.

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2

CHAPTER

A role for G protein-coupled receptor kinase 2 in mechanical allodynia

Wendy Kleibeuker[1]
Annemarie Ledeboer[2,3]
Niels Eijkelkamp[1]
Linda R. Watkins[2]
Steven F. Maier[2]
Jitske Zijlstra[1]
Cobi J. Heijnen[1]
Annemieke Kavelaars[1]

[1] University Medical Center Utrecht, Laboratory of Psychoneuroimmunology, 3584 EA Utrecht, the Netherlands
[2] University of Colorado at Boulder, Department of Psychology & Center for Neuroscience, Boulder, CO 80309-0345, USA
[3] Present address: Avigen, Inc., Alameda, CA 94502-1301, USA

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ABSTRACT

Inflammation and nerve injury can both induce mechanical allodynia via mechanisms involving production of pro-inflammatory cytokines and increased neuronal activity. Many neurotransmitters involved in pain signal via G protein-coupled receptors (GPCRs). GPCR kinase (GRK)2 is a member of the GRK family that regulates agonist-induced desensitization and signaling of GPCRs. Low intracellular GRK2 levels are associated with increased receptor signaling. The aim of this study was to investigate whether mechanical allodynia is associated with decreased spinal cord GRK2 expression and whether reduced GRK2 increases inflammation-induced mechanical allodynia. Mechanical allodynia was induced in rats by chronic constriction injury (CCI) of the sciatic nerve. After two weeks, neuronal GRK2 expression was decreased bilaterally in the superficial layers of the lumbar spinal cord dorsal horn. Moreover, interleukin (IL)-1β significantly reduced GRK2 expression *ex vivo* in spinal cord slices. To investigate whether reduced GRK2 potentiates inflammation-induced mechanical allodynia, we used $GRK2^{+/-}$ animals expressing decreased GRK2. At baseline, the threshold for mechanical stimulation did not differ between $GRK2^{+/-}$ and wild type (WT) mice. However, $GRK2^{+/-}$ animals were more sensitive to mechanical stimulation than WT animals after intraplantar λ-carrageenan injection. We propose cytokine-induced down-regulation of spinal cord neuronal GRK2 expression as a novel mechanism that contributes to increased neuronal signaling in mechanical allodynia.

INTRODUCTION

Peripheral nerve injury and inflammation can both induce hyperalgesia and mechanical allodynia. Increased excitability of neurons in the spinal cord dorsal horn plays a pivotal role in increased pain responses[1]. The intracellular mechanisms of increased neuronal excitability are thought to include changes in expression and function of receptors, activation levels of several kinases, levels of neuropeptides and neurotrophins, and inflammatory mediators[2-7].

Many mediators involved in pain signal via G protein-coupled receptors (GPCRs) and there is evidence that increased sensitivity of GPCRs may contribute to hyperalgesia and mechanical allodynia. For example, during chronic constriction injury (CCI) of the sciatic nerve, pain-related behaviors induced by intrathecal substance P (SP) administration are increased, suggesting that sensitivity of the GPCR for SP (neurokinin-1 receptor (NK-1R)) is increased[8]. In addition, intrathecal administration of antagonists for metabotropic glutamate group I receptors inhibits mechanical allodynia during CCI[4]. Chronic compression of dorsal root ganglia (DRG) also facilitates pain and is associated with increased responses to the GPCR agonist C-C chemokine ligand (CCL)2[9]. During λ-carrageenan-induced inflammatory hyperalgesia, increased mechanical allodynia occurs in response to intradermal administration of the GPCR agonists prostaglandin E2, epinephrine, 5-hydroxytryptamine (HT), and an A2 adenosine receptor agonist[10,11].

The responsiveness of GPCRs can be regulated by GPCR kinase (GRK)2. GRK2 phosphorylates agonist-occupied GPCRs, which facilitates binding of arrestins, uncoupling from the Gα protein and receptor internalization[12]. Changes in GRK2 levels can modulate the responsiveness of GPCRs involved in mechanical allodynia including C-C chemokine receptor (CCR)2, NK-1R, adrenergic receptor (AR), and adenosine receptor[13,14]. In general, low cellular GRK2 levels are associated with increased receptor signaling[15,16], whereas over-expression of GRK2 is related to decreased receptor signaling[13,17].

GRK2 expression is reduced in inflammatory conditions including rheumatoid arthritis[18] and multiple sclerosis[19], and in response to cerebral hypoxia-ischemia[20]. *In vitro*, inflammatory mediators (e.g. interleukin (IL)-1β, IL-6, interferon (IFN)-γ) reduce GRK2 levels[18,21].

Since pro-inflammatory cytokines are thought to be important in mechanical allodynia[6,7], we hypothesized that mechanical allodynia is associated with decreased neuronal GRK2 and that reduced GRK2 contributes to increased pain responses. We analyzed spinal cord dorsal horn GRK2 expression during sciatic nerve CCI-induced mechanical allodynia. In addition, we examined the influence of IL-1β on GRK2 expression in cultured spinal cord slices. To determine whether decreased GRK2 levels contribute to increased behavioral pain responses, we analyzed inflammation-induced mechanical allodynia in GRK2 heterozygous mice that express a decreased level of GRK2.

MATERIALS AND METHODS

Animals

Pathogen-free adult male Sprague-Dawley rats (300-450 g; Harlan Laboratories, Madison, WI, USA) were used for CCI experiments. Rats were housed in temperature (23±3°C) and light (12/12 light/dark; lights on at 07:00 h) controlled rooms with standard rodent food and water available *ad libitum*. All procedures for the CCI experiments were approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder.

In addition, mice heterozygous for the targeted deletion of the catalytic subdomain I of the GRK2 gene ($GRK2^{+/-}$) and their wild type (WT) littermates were used[22]. Offspring were genotyped by PCR analysis on genomic DNA extracted from the tail. As $GRK2^{-/-}$ mice die *in utero*, only $GRK2^{+/-}$ mice were used for experiments. Mice used for the λ-carrageenan-induced mechanical allodynia were 8-12 weeks old, whereas for the *ex vivo* cultured spinal cord slices WT mice of postnatal day 7 were used. All mice were bred and maintained in the animal facility of the University of Utrecht (the Netherlands). All experiments involving mice were performed in accordance with international guidelines and approved by the University Medical Center Utrecht experimental committee.

CCI

CCI was created at mid-thigh level of the left hind leg as described previously[23]. Four sterile chromic gut sutures (cuticular 4-0, chromic gut, 27 inches, cutting FS-2; Ethicon, Somerville, NJ, US) were loosely tied around the gently isolated sciatic nerve under isoflurane anesthesia (Phoenix Pharm., St. Joseph, MO, USA). The sciatic nerves of sham animals were identically exposed and manipulated, but not ligated. Thresholds for behavioral responses of the hind legs to calibrated mechanical stimuli (von Frey test) were assessed using blinded procedures as described previously[6]. At different time points post CCI surgery, rats were anaesthetized with sodium pentobarbital (60 mg/kg) and transcardially perfused with 4% paraformaldehyde after which spinal cords were dissected and post-fixed in the same fixative at 4°C during 24-48 h. Subsequently, the tissue was cryo-protected in 30% sucrose in PBS (pH 7.5) and frozen in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, the Netherlands) using cooled isopentane, and stored at -80°C until further use.

Immunohistochemistry

Transverse sections of the spinal cord (6-8 µm) were cut in a cryostat. For immunohistochemistry, endogenous peroxidase was inactivated by 1.5% H_2O_2 (Merck, Darmstadt, Germany) in acetone. Subsequently, spinal cord sections were blocked with 10% normal goat serum at 37°C for 30 min. Sections were stained with rabbit anti-GRK2 1:600 (sc-562; Santa Cruz Biotechnology Inc., Santa Cruz, United Kingdom) overnight at room temperature (21°C), biotinylated goat anti-rabbit IgG 1:100 (Vector laboratories, Burlingame, CA, USA), and subsequently

with Vectastain ABC (Vector laboratories). Finally, the reaction product was visualized with DAB. Sections were counter-stained with hematoxylin, dehydrated, and mounted in DePex (VWR international ltd., Poole, England). GRK2 expression was scored by an observer blinded for the experimental group in lamina I-II and in lamina III-IV of the spinal cord dorsal horn in segments T1-T2, L1-L2, and L4-L6 on a 5-point scale, ranging from 4 for the highest GRK2 expression and 0 for no GRK2 expression.

For immunofluorescent staining, sections were treated with acetone and washed with TBS and TBS containing 0.05% Triton X-100 (TBS-T). Spinal cord sections were blocked with 5% normal goat serum at 37°C for 30 min and incubated at 4°C with rabbit anti-GRK2 1:400 combined with mouse anti-MAP2 1:1000, mouse anti-NeuN 1:50 (both Chemicon international, Temecula, CA, USA), or mouse anti- glial fibrillary acidic protein (GFAP) 1:50 (Cymbus Biotechnology, Southampton, United Kingdom) during 48 h. Subsequently, sections were incubated with biotinylated goat anti-rabbit IgG 1:100 and Alexa Fluor-488 goat anti-mouse IgG (H+L) 1:200 (Molecular Probes, Eugene, OR, USA) followed by ExtrAvidin-Cy3 conjugate 1:100 (Sigma-Aldrich, St. Louis, MO, USA). Sections were mounted in DABCO.

IL-1β treatment of *ex vivo* cultured spinal cord slices

Organotypic spinal cord cultures were prepared from the spinal cord of mouse pups (postnatal day 7) as described previously[24]. In brief, pups were decapitated, spinal cords were dissected, and 350 µm thick sections were cut with a McIlwain tissue chopper (Laméris laboratorium B.V., Breukelen, the Netherlands). The slices were cultured on 30 mm Millicell culture plate inserts (Millipore, Billerica, MA, USA) in medium containing 25% Hank's balanced salt solution, 25% OPTI-MEM, 50% horse serum, 35 mM glucose, 2 mM glutamine, 100 U/ml Penicillin, and 100 µg/ml streptomycin (all from Gibco, Invitrogen, Breda, The Netherlands). After 7 days, spinal cord slices were treated chronically with IL-1β by adding IL-1β (Peprotech, Rocky Hill, NJ, USA) to the media during 2 weeks. Medium with or without IL-1β was replaced twice weekly.

Western blotting

Lysates from cultured spinal cord slices and spinal cords from WT and GRK2$^{+/-}$ mice were obtained by sonication in ice-cold RIPA buffer (20 mM Hepes pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 2 mM 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF), 20 µg/ml leupeptin, and 200 µg/ml benzamidine) and subsequent incubation for 30 min at 4°C. 20-30 µg of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C, Amersham Int., Buckinghamshire, UK) by electroblotting. Blots were stained with rabbit anti-GRK2 1:400 and mouse anti-ERK2 1:1000 (Santa Cruz Biotechnology Inc.) as described previously[18]. Immunoreactivity was detected by enhanced chemiluminescence (Amersham Int.). Band density was determined using a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

λ-Carrageenan induced mechanical allodynia

Female GRK2$^{+/-}$ mice and their WT littermates received an intraplantar injection of 20 µl of 2% λ-carrageenan (Sigma-Aldrich) in saline in the right paw. As a control, 20 µl of saline was injected in the contralateral paw. Thresholds for behavioral responses of the hind paw to calibrated mechanical stimuli (von Frey test) were assessed before and 6 h after λ-carrageenan injection using a blinded procedure as described previously[6]. As a measure for development of inflammation, the paw thickness was measured.

Data analysis

All data shown were confirmed in at least 2 independent experiments. Statistical comparisons for the von Frey test were computed using Statview 5.0.1 for the Macintosh. Data from the von Frey test were analyzed as the interpolated 50% threshold (absolute threshold) in log base 10 of stimulus intensity (monofilament stiffness in mg x 10). Baseline measures for the von Frey test were analyzed by one-way ANOVA. Time-course measures were analyzed by repeated measures ANOVA followed by Fisher's protected least significant difference posthoc comparisons, where appropriate.

Western blot data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. Immunohistochemical data were analyzed using a Mann-Whitney test. A P-value < 0.05 was considered to be statistically significant.

RESULTS

GRK2 expression in the spinal cord

We investigated the anatomical and cellular localization of GRK2 expression in the spinal cord of control rats using immunohistochemistry. Previously, we have shown that the GRK2 antibody used in this study recognizes a single protein co-migrating with GRK2 using Western blot analysis of rat brain homogenates and that data obtained by Western blot analysis correlated with data obtained by immunohistochemical analyses[20]. When spinal cord sections were stained, GRK2 expression was detected in cells throughout the entire grey matter, although regional expression differences were observed. The level of expression was particularly high in the superficial layers of the dorsal horn. However, GRK2 expression was hardly detectable in the white matter (Figure 1A). The observed GRK2 expression was specific, since staining without the first antibody was negative (Figure 1B).

The sensitivity of this method for detecting differences in GRK2 expression levels was tested by staining spinal cord sections from WT and GRK2$^{+/-}$ C57BL/6 mice. As shown in Figure 2A-B, GRK2 staining was lower in the spinal cord dorsal horn from GRK2$^{+/-}$ mice, which express 28% of GRK2 protein compared to WT littermates as shown by Western blotting (P < 0.0001, Figure 2C).

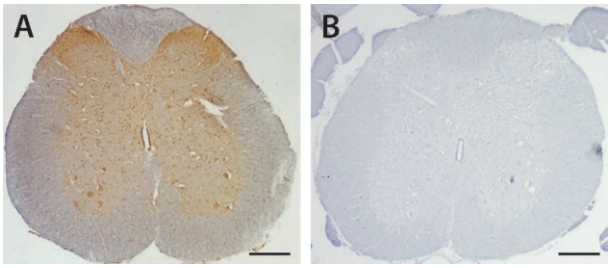

Figure 1: GRK2 expression in the lumbar spinal cord of control rats. Immunohistochemical staining of rat lumbar spinal cord (L4-L6) with rabbit anti-GRK2 followed by biotinylated goat anti-rabbit IgG. Slices were stained using avidin-biotin-peroxidase complex and diaminobenzidine (A). Staining of rat spinal cord as in A but without rabbit anti-GRK2 (B). Scale bar, 250 µm.

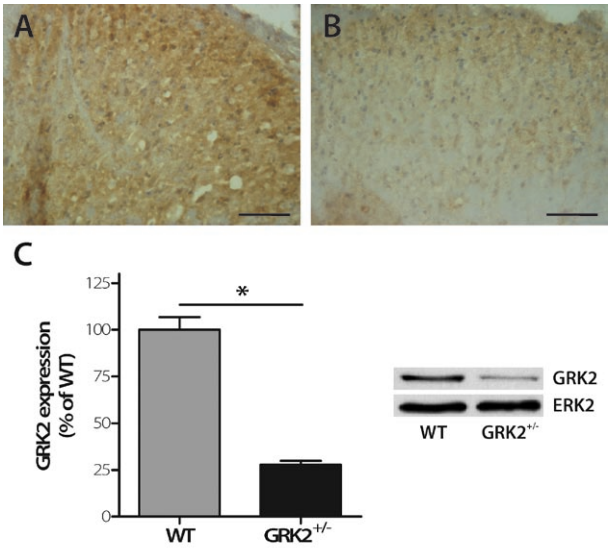

Figure 2: GRK2 expression in the lumbar spinal cord of WT and GRK$^{2+/-}$ mice. GRK2 expression was determined in the lumbar spinal cord dorsal horn (L5-L6) from WT (A) and from GRK2$^{+/-}$ (B) C57BL/6 mice using immunohistochemistry as described in the legend to Figure 1. GRK2 protein expression in spinal cords from WT and GRK2$^{+/-}$ mice determined by Western blotting (C). Data are represented as the mean ± SEM of 6 spinal cords and GRK2 expression was normalized for the amount of ERK2 present (*$P < 0.0001$, WT vs. GRK2$^{+/-}$ spinal cords). Scale bar, 62.5 µm.

To determine cellular localization of GRK2 in the rat spinal cord, sections were stained for GRK2 and markers for neurons (MAP2 and NeuN) or astrocytes (GFAP). Double-staining for GRK2 with the cytosolic neuronal protein MAP2 shows that MAP2 and GRK2 are co-localized in neurons (Figure 3A-C). Double-staining for the nuclear marker NeuN and GRK2 showed that GRK2 staining surrounded the NeuN staining indicating that the nucleus is not a major source of GRK2 (Figure 3D-F). Co-localization of GRK2 and GFAP was not detectable using immunofluorescence (Figure 3G-I). These results indicate that in the spinal cord, GRK2 is mainly expressed in neurons.

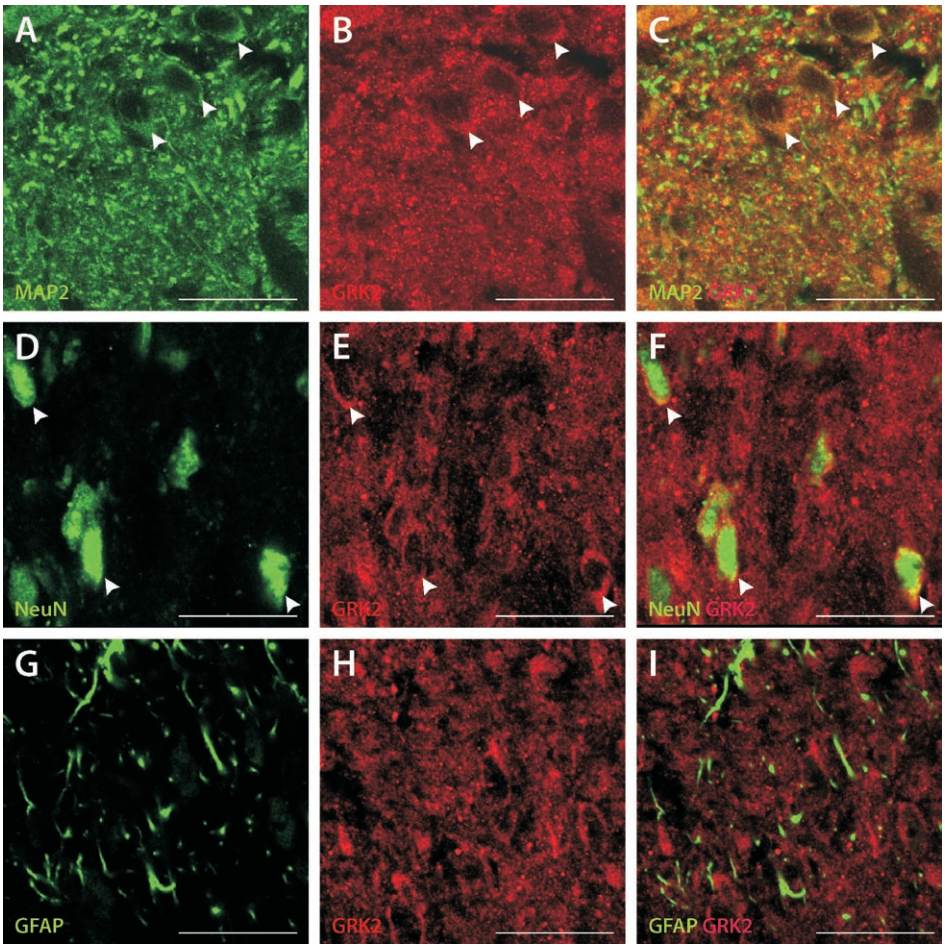

Figure 3: Cellular localization of GRK2 expression in the superficial layers of the dorsal horn in control rats. Immunofluorescent double-staining for rabbit anti-GRK2 combined with mouse anti-MAP2 (A-C), mouse anti-NeuN (D-F), or mouse anti-GFAP (G-I). Slices were stained with Alexa Fluor-488 goat anti-mouse IgG (green) and biotinylated goat anti-rabbit IgG followed by ExtrAvidin-Cy3 conjugate (red). ➤, MAP2 and GRK2 co-localization in A-C; GRK2 expression surrounding NeuN expression in D-F. Scale bar, 25 µm.

CCI induces bilateral mechanical allodynia

In this study, GRK2 expression in the spinal cord dorsal horn was investigated after induction of mechanical allodynia by CCI of the left sciatic nerve. Development of mechanical allodynia was assessed using the von Frey test. As shown in Figure 4, baseline measurements were similar in the various groups. After induction of CCI, mechanical allodynia was evident in the ipsilateral as well as in the contralateral paw of CCI animals ($P < 0.0001$, sham controls vs. CCI animals) but not in sham controls. No significant differences in sensitivity to mechanical stimulation were observed between the ipsi- and contralateral paw of CCI animals. Maximal mechanical allodynia was already observed at day 4 and lasted at least until day 14 after CCI surgery.

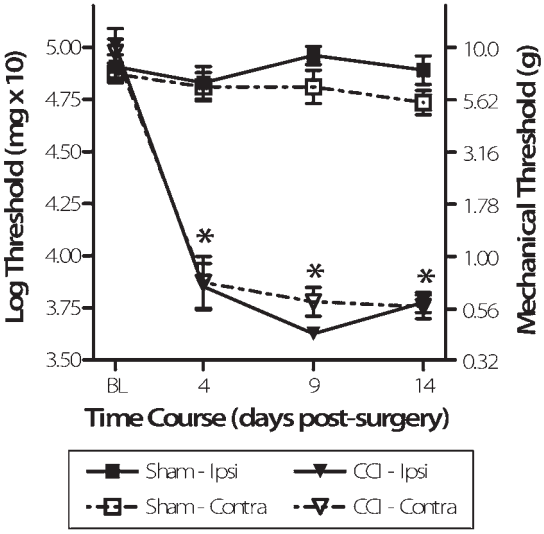

Figure 4: Time course for development of mechanical allodynia following CCI. Mechanical withdrawal thresholds were determined using the von Frey test. Data are represented as the mean ± SEM of 6 rats (*$P < 0.0001$, sham controls vs. CCI animals).

CCI decreases neuronal GRK2 expression in the lumbar dorsal horn

The dorsal horn in the lumbar segment L4-L6 receives sensory input from the sciatic nerve. GRK2 expression in this part of the spinal cord was compared between sham control and CCI animals (n=6 per group). Two weeks after CCI, a clear reduction in GRK2 expression was observed in segments L4-L6 of the spinal cord from CCI animals. Down-regulation of GRK2 was particularly pronounced in lamina I-II of the dorsal horn at L4-L6 (Figure 5I-J). Analysis of GRK2 expression scores confirms that GRK2 was significantly and markedly down-regulated in lamina I-II and to a lesser extent in lamina III-IV (Figure 5K-L). To investigate whether the decrease in GRK2 only took place in the segments where the sciatic nerve enters the spinal cord (L4-L6), we also determined GRK2 expression in other spinal cord segments. Our data show that GRK2 was also significantly decreased in lamina I-II of segment L1-L2, whereas the decrease in lamina III-IV did not reach statistical significance ($P = 0.065$; Figure 5E-H). CCI did not induce a change in GRK2 expression in the thoracic segments T1-T2 of the spinal cord (Figure 5A-D).

Although CCI was only created in the left hind leg, GRK2 expression was decreased to the same extent in the ipsi- and contralateral dorsal horn of the spinal cord (compare Figure 6C and F). This bilateral change mirrors the bilateral pain changes induced in these animals (Figure 4). The surgical procedure itself did not induce any changes in GRK2, because GRK2 expression did not differ between the ipsi- and contralateral side of sham controls (compare Figure 6A and D) and between sham control and untreated control animals (data not shown).

We also analyzed the expression of GRK2 in the lumbar spinal cord dorsal horn at earlier time points after CCI surgery. A decreased GRK2 was found in 1 out of 3 animals two days after CCI, whereas one week after CCI, all animals had decreased GRK2 levels compared to the

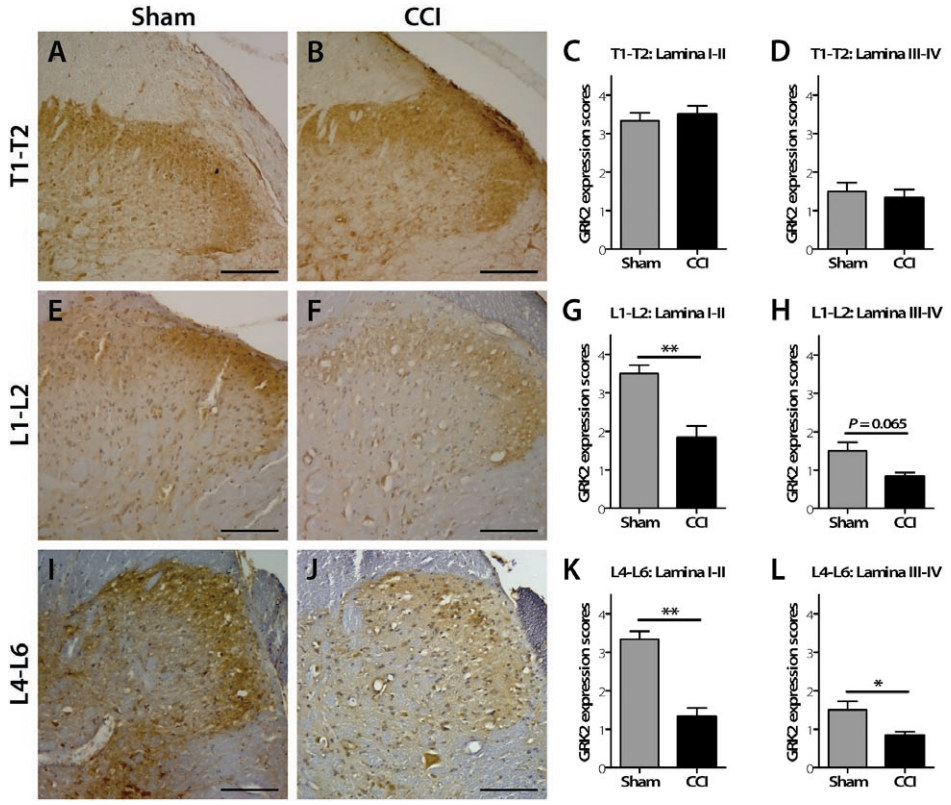

Figure 5: GRK2 expression in the spinal cord dorsal horn 2 weeks after CCI. A representative example of GRK2 expression in the ipsilateral dorsal horn of the spinal cord of sham controls (A, E, I) and CCI-operated animals (B, F, J) two weeks after CCI (n=6 per group). Slices were stained as indicated in the legend to Figure 1. Photographs were taken from segment T1-T2 (A, B), L1-L2 (E, F) and L4-L6 (I, J) and GRK2 expression scores were determined by an observer blinded to the experimental condition on a scale from 0-4 in lamina I-II and in lamina III-IV of segment T1-T2 (C, D), L1-L2 (G, H) and L4-L6 (K, L). Data are represented as the mean ± SEM of 6 rats. A Mann-Whitney test was used to analyze the difference in GRK2 expression scores between sham and CCI animals (*$P < 0.01$, **$P < 0.005$, sham controls vs. CCI animals). Scale bar, 125 µm.

sham controls (n=3 per group). However, at this earlier time point the decrease in GRK2 was less pronounced than two weeks after nerve constriction (Figure 6B, E).

As shown in Figure 3, in spinal cord GRK2 is expressed by neurons. To determine whether CCI results in decreased GRK2 expression in neurons, spinal cord sections were double-stained with GRK2 (red dye) as well as the neuronal marker MAP2 (green dye). In spinal cords from sham-operated animals, a clear co-localization of both proteins is present in the spinal cord dorsal horn. In the CCI-operated animals, GRK2 expression decreases which is accompanied by a decrease in the co-localization of GRK2 and MAP2. This decrease in co-localization of GRK2 and MAP2 does not result from a decrease in MAP2 staining, since MAP2 expression is similar in CCI and sham-operated animals (Figure 7). These results suggest that CCI induces a decrease in neuronal GRK2 expression.

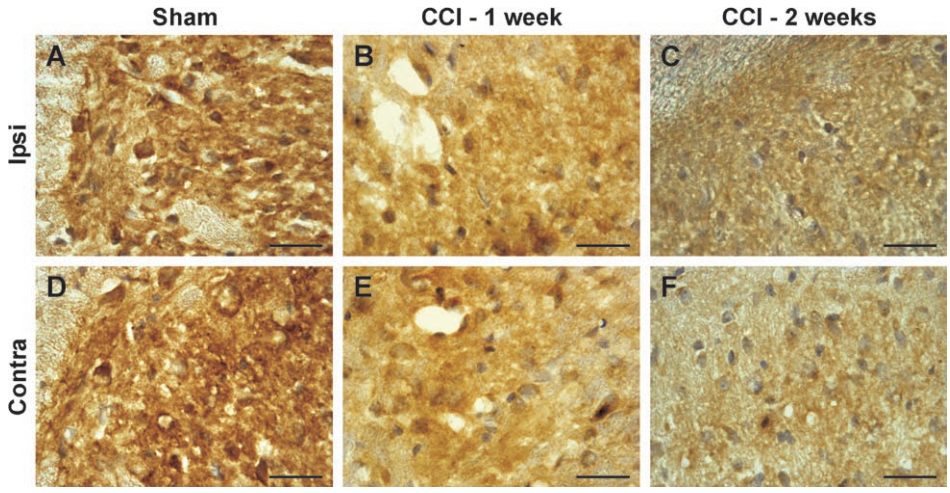

Figure 6: GRK2 expression in the dorsal horn after CCI. GRK2 expression was determined by immunohistochemistry as described in the legend to Figure 1 in the superficial layers of the dorsal horn in segment L4-L6 ipsilateral (A-C) and contralateral (D-E) to surgery from sham controls (A, D), animals 1 week (B, E) and 2 weeks (C, F) after CCI operation. Representative example out of 6 animals is shown. Scale bar, 25 µm.

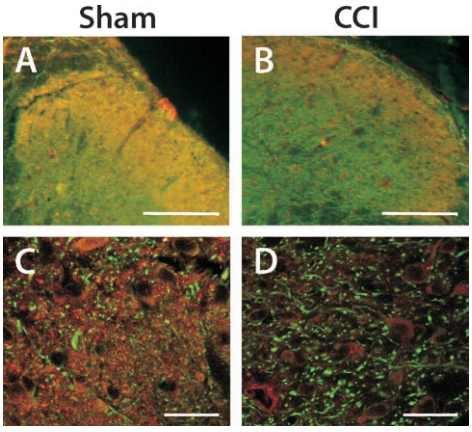

Figure 7: GRK2 expression in MAP2-positive cells in the dorsal horn. Slices from sham operated animals (A, C) and CCI animals (B, D) were stained with anti-GRK2 (red) and anti-MAP2 (green) as described in the legend to Figure 3. Photographs were taken from the spinal cord dorsal horn (scale bar, 200 µm) (A, B) and the superficial layers of the dorsal horn (scale bar, 25 µm) (C, D).

IL-1β treatment decreases GRK2 expression in *ex vivo* cultured spinal cord slices

It has been shown that production of cytokines, including IL-1β in the spinal cord is important in the development of mechanical allodynia. To explore the possibility that a chronic increase in IL-1β may contribute to the observed reduction in GRK2 expression, we used an *ex vivo* spinal cord culture system. Spinal cord slices were cultured with IL-1β and changes in GRK2 levels were determined by Western blotting. Two weeks of IL-1β treatment resulted indeed in a dose-dependent decrease in GRK2 expression. Culture with 0.01 or 0.1 ng/ml

IL-1β significantly decreased GRK2 expression by 43% and 34%, respectively ($P < 0.05$, Figure 8). Although GRK2 expression was decreased by 22% after stimulation with 1 ng/ml IL-1β, this effect was not statistically significant. When spinal cords slices were treated with 0.001 ng/ml IL-1β no significant decrease in GRK2 was detected (data not shown). Bell-shaped dose-response curves for the effect of cytokines in *in vitro* systems have also been described by others[25].

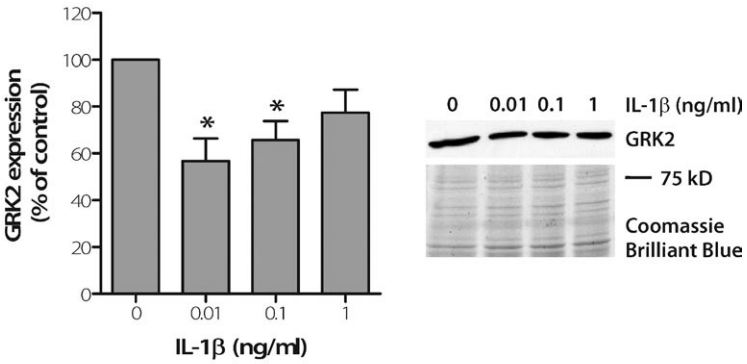

Figure 8: GRK2 expression in spinal cord slices treated with IL-1β in *ex vivo* cultures. Spinal cord slices were cultured *ex vivo* for two weeks with IL-1β and GRK2 protein expression was determined using Western blotting. Data are represented as mean ± SEM and normalized for the total amount of protein present using Coomassie Brilliant Blue staining (*$P < 0.05$; n=4).

Reduced GRK2 potentiates inflammation-induced mechanical allodynia

To address the question whether reduced GRK2 levels can indeed contribute to mechanical allodynia, we used GRK2$^{+/-}$ and WT mice. As mentioned earlier, expression levels of GRK2 protein in various organs of these animals including spinal cord are reduced by approximately 50%. To induce mechanical allodynia, λ-carrageenan was injected into the hind paw of GRK2$^{+/-}$ animals and WT littermates and the behavioral responses to stimulation with von Frey hairs were determined 6 hours later. An acute inflammatory challenge was used rather than CCI as it provides the strongest test of whether alterations in GRK2 levels immediately alter pain responses. The data in Figure 9 show that λ-carrageenan induces a significant decrease in mechanical thresholds compared to vehicle injection. Moreover, GRK2$^{+/-}$ mice were significantly more sensitive to mechanical stimulation than WT littermates. We did not observe differences in paw thickness between GRK2$^{+/-}$ and WT littermates, indicating that the λ-carrageenan-induced inflammatory response was identical between the GRK2$^{+/-}$ and WT animals (data not shown).

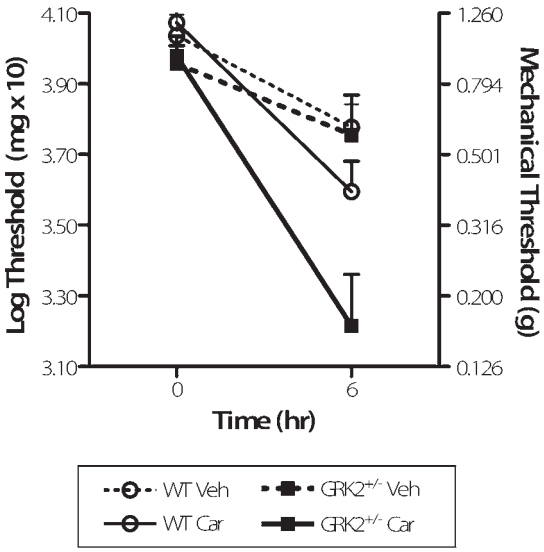

Figure 9: Development of mechanical allodynia following intraplantar injection of λ-carrageenan. Mechanical withdrawal thresholds were determined using the von Frey test. Data are represented as the mean ± SEM of 6 mice. Data were analyzed by repeated-measures ANOVA. There was a significant effect of λ-carrageenan injection ($P < 0.05$). Moreover, GRK2$^{+/-}$ mice were significantly different from WT littermates ($P < 0.05$).

DISCUSSION

The aim of this study was to investigate the possible role of changes in GRK2 expression in mechanical allodynia. Our data demonstrate that the mechanical allodynia induced by CCI in rats is associated with a reduction in neuronal GRK2 levels in the dorsal horn of the lumbar spinal cord. Moreover, we show that treatment of rat spinal cord slices with IL-1β reduces the expression of GRK2. Finally, GRK2$^{+/-}$ mice that express a reduced level of GRK2 display an increase in inflammation-induced mechanical allodynia.

GRK2 in the spinal cord is mainly localized in neurons and is co-localized with MAP2. It has been suggested that CCI can induce death of a limited percentage of spinal cord neurons[26,27]. However, the decrease in GRK2 is much more pronounced than the amount of cell death that has been reported and therefore cannot explain the large decrease in GRK2 expression we observed after CCI. Moreover, we show that after CCI, GRK2 still co-localizes with MAP2, but is present at a lower level. GRK2 expression was below detection levels in GFAP-positive cells. The finding that GRK2 is expressed mainly by spinal cord neurons is consistent with the observation that in the brain GRK2 is also found predominantly in neurons[20,28]. It should be noted, however, that cell lines of glial origin as well as primary cultures of astrocytes express significant levels of GRK2 that can be modulated by exposure of these cells to the cytokine

IL-1β or to oxygen radicals (Kleibeuker *et al.*, non-published observations)[29]. Therefore, we cannot exclude that GRK2 is also present in glial cells and can be regulated during CCI.

In line with the bilateral mechanical allodynia that occurs after CCI, the CCI-induced decrease in neuronal GRK2 occurred bilaterally in the superficial layers of the spinal cord dorsal horn. The observed down-regulation of spinal cord GRK2 expression was restricted to the area receiving sensory input from the constricted nerve (L4-L6) and the segments immediately adjacent to it (L1-L2). GRK2 was not altered in the thoracic segment T1-T2. These results suggest that the reduction in GRK2 expression is anatomically limited to the segments of the spinal cord that process signals from the part of the body that is hypersensitive to pain.

The down-regulation of GRK2 in the spinal cord during CCI was time-dependent. Two days after CCI, GRK2 was decreased in 1 out of 3 animals, whereas one and two weeks after CCI, the expression was decreased in all animals examined. In addition, the reduction in GRK2 levels was more pronounced two weeks after CCI than one week after CCI. Although the maximal induced mechanical allodynia was already reached 4 days after CCI (Figure 4), the maximal reduction in GRK2 staining was not even reached 7 days after CCI. This might indicate that reduced GRK2 is not required for the induction of mechanical allodynia, but that it may contribute to its maintenance.

To investigate whether decreased GRK2 levels are causally related to a decrease in pain threshold, inflammatory mechanical allodynia was induced in WT and GRK2$^{+/-}$ mice by intraplantar injection of λ-carrageenan. Indeed, λ-carrageenan induced mechanical allodynia was more pronounced in GRK2$^{+/-}$ mice than in WT littermates. At baseline, however, there was no difference in pain threshold, These data show that reduced GRK2 levels potentiate inflammation-induced mechanical allodynia, without affecting the normal pain threshold. During λ-carrageenan-induced inflammation, several GPCR agonists (e.g. noradrenalin, CCL2, and CCL3) are produced that are not are not present under basal conditions[30,31]. From previous studies, we know that cells from GRK2$^{+/-}$ mice have an increased response to the GPCR agonists CCL2 and CCL3, suggesting that increased reactivity to these (inflammatory) mediators may contribute to the increased mechanical allodynia in the GRK2$^{+/-}$ animals[13,16]. In addition, several overexpression studies showed that increased GRK2 levels were associated with decreased signaling of α-ARs[32,33].

During CCI, production of reactive oxygen species (ROS) plays a role in the development of neuropathic pain behaviors, since inhibition of nitric oxide synthase inhibits CCI-induced hyperalgesia[34]. It has been shown that GRK2 expression can be down-regulated in lymphocytes by ROS[35]. In addition to ROS, pro-inflammatory cytokines (e.g. IL-1β, tumor necrosis factor (TNF)-α) are produced and released in the spinal cord during CCI[6,7]. These pro-inflammatory cytokines can increase nociception after intrathecal administration[36], whereas intrathecal administration of cytokine antagonists (e.g. IL-1RA) can block and/or reverse established mechanical allodynia and thermal hyperalgesia[6]. In addition, CCI-induced neuropathic pain is decreased in IL-1α/β knock-out mice[37]. It has been described that CCI induces bilateral me-

chanical allodynia and that treatment with minocycline (a microglia inhibitor) or with IL-1RA prevents the allodynia at both sides to the same extent[6]. These data suggest that bilateral IL-1β-dependent mechanisms are responsible for the bilateral allodynia that occurs in this model and lead us to the hypothesis that the contribution of IL-1β to bilateral allodynia also includes a bilateral decrease in GRK2. Indeed our data show that *ex vivo* IL-1β treatment of cultured spinal cord slices leads to a reduction in GRK2 expression. In line with these findings, it was shown in previous studies that stimulation of peripheral blood leukocytes with pro-inflammatory cytokines (e.g. IFN-γ, IL-6) leads to a down-regulation of GRK2 expression[18]. Moreover, IFN-γ, TNF-α, and IL-1β have been shown to decrease the activity of the GRK2 promoter in an aortic smooth muscle cell line[21].

In conclusion, the data presented in this paper lead us to propose that inflammation or neuronal injury induce a cytokine-mediated decrease in neuronal GRK2 expression that contributes to increased neuronal excitability and thereby to mechanical allodynia.

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3

CHAPTER

IL-1β signaling is required for mechanical allodynia induced by nerve injury and for the ensuing reduction in spinal cord neuronal GRK2

Wendy Kleibeuker[1]
Eran Gabay[2]
Annemieke Kavelaars[1]
Jitske Zijlstra[1]
Gilly Wolf[3]
Nadav Ziv[2,3]
Raz Yirmiya[3]
Yehuda Shavit[3]
Michael Tal[2]
Cobi J. Heijnen[1]

[1] University Medical Center Utrecht, Laboratory of Psychoneuroimmunology, 3584 EA Utrecht, the Netherlands
[2] Department of Anatomy and Cell Biology, Faculty of Medicine, The Hebrew University, Jerusalem 91010, Israel
[3] Department of Psychology, The Hebrew University, Mount Scopus, Jerusalem 91905, Israel

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ABSTRACT

Many neurotransmitters involved in pain perception transmit signals via G protein-coupled receptors (GPCRs). GPCR kinase (GRK)2 regulates agonist-induced desensitization and signaling of multiple GPCRs and interacts with downstream molecules with consequences for signaling. In general, low GRK2 levels are associated with increased responses to agonist stimulation of GPCRs. Recently, we reported that in mice with reduced GRK2 levels, inflammation-induced mechanical allodynia was increased. In addition, mice with impaired interleukin (IL)-1β signaling did not develop mechanical allodynia after L5 spinal nerve transection (SNT). We hypothesized that in the L5 SNT model mechanical allodynia would be associated with reduced neuronal GRK2 levels in the spinal cord dorsal horn and that IL-1β signaling would be required to induce both the decrease in GRK2 and mechanical allodynia. We show here that in wild type (WT) mice L5 SNT induces a bilateral decrease in neuronal GRK2 expression in the lumbar spinal cord dorsal horn, 1 and 2 weeks after L5 SNT. No changes in GRK2 were observed in the thoracic segments. Moreover, spinal cord GRK2 expression was not decreased in IL-1R$^{-/-}$ mice after L5 SNT. These data show that IL-1β signaling is not only required for the development of mechanical allodynia, but also to reduce neuronal GRK2 expression. These results suggest a functional relation between the L5 SNT-induced IL-1β-mediated decrease in GRK2 and development of mechanical allodynia.

INTRODUCTION

Nerve injury caused by trauma, surgery, or inflammation often results in the development of mechanical allodynia and/or hyperalgesia. The increased pain responsiveness during mechanical allodynia is mediated at least in part by increased excitability of neurons in the spinal cord[1]. The intracellular mechanisms of increased neuronal excitability are thought to include changes in expression and responsiveness of receptors, activation of several kinases, release of neuropeptides and neurotrophins, and local production of inflammatory mediators[2-6].

Many mediators involved in pain perception and allodynia (e.g. substance P, glutamate, chemokines, prostaglandins) signal via G protein-coupled receptors (GPCRs). GPCR kinase (GRK)2 regulates the responsiveness of multiple GPCRs. GRK2 phosphorylates agonist-occupied GPCRs, which facilitates binding of arrestins, uncoupling from the Gα protein, and receptor internalization[7]. More recent studies described an important additional mechanism via which GRK2 can regulate signaling, i.e. via a direct interaction with intracellular signaling molecules (e.g. MEK1/2, Akt, RKIP, p38 mitogen-activated protein kinase (MAPK))[8,9]. Changes in GRK2 levels modulate the responsiveness of GPCRs including C-C chemokine receptor (CCR)1/5, CCR2, β_2-adrenergic receptors, and metabotropic glutamate receptors[10-13]. In general, low cellular GRK2 expression is associated with increased receptor signaling[10,12,14], whereas GRK2 overexpression is related to decreased receptor signaling[15].

Based on the role of GRK2 in the regulation of GPCR signaling and the fact that GPCR signaling plays an important role in mechanical allodynia, we have suggested that changes in GRK2 would contribute to inflammation-associated mechanical allodynia. Indeed, we recently demonstrated that λ-carrageenan-induced acute mechanical allodynia was increased in GRK2$^{+/-}$ mice compared to WT mice, indicating that low GRK2 levels are associated with increased sensitivity for inflammatory allodynia[16]. Conversely, we showed that neuronal GRK2 expression is diminished in the lumbar spinal cord dorsal horn of rats during chronic constriction injury (CCI) of the sciatic nerve, a model of mechanical allodynia[16]. During CCI and other models of mechanical allodynia several pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α are produced in the spinal cord that contribute to increased pain sensitivity[6,17,18]. Intrathecal administration of IL-1β can increase nociception, whereas intrathecal administration of IL-1 receptor antagonist (IL-1RA) can prevent the development of and/or reverse established mechanical allodynia[6,19]. Additional evidence for the role of IL-1β in the development of mechanical allodynia comes from studies using mice with deficiencies in IL-1β signaling. Mechanical allodynia induced by L5-L6 spinal nerve ligation or CCI is decreased in IL-1α/β$^{-/-}$ mice[20]. Moreover, Wolf *et al.* (2006) showed that mechanical allodynia is abrogated in mice with a targeted deletion of the IL-1 type I receptor (IL-1R$^{-/-}$) and in mice overexpressing IL-1RA, indicating that IL-1β is crucial for the development of mechanical allodynia after L5 spinal nerve transection (SNT).

GRK2 expression is downregulated during various inflammatory diseases including rheumatoid arthritis, multiple sclerosis, adjuvant arthritis, and experimental allergic encephalomyelitis[21-24], but also after pro-inflammatory cytokine treatment. *In vitro*, chronic IL-1β treatment of cultured spinal cord slices decreases GRK2 levels[16]. In addition, IL-1β and several other pro-inflammatory cytokines (e.g. interferon (IFN)-γ, IL-6, TNF-α) downregulated GRK2 expression in human peripheral blood lymphocytes or in a smooth muscle cell line[21,25].

In the present study, we investigated whether L5 SNT-induced mechanical allodynia in mice is associated with a reduced neuronal GRK2 expression in the spinal cord dorsal horn. This may answer the question whether downregulation of neuronal GRK2 is a general mechanism, which may contribute to the development of mechanical allodynia. In addition, we hypothesized that IL-1β signaling is required to induce mechanical allodynia as well as a decrease in GRK2 levels. To test this hypothesis, mechanical sensitivity and expression of GRK2 in the spinal cord dorsal horn were determined in the L5 SNT model in mice with a deficiency of IL-1β signaling (IL-1R$^{-/-}$) and in wild type (WT) mice.

MATERIALS AND METHODS

Animals

IL-1R$^{-/-}$ mice[26] and their C57BL/6 X 129/Sv WT control male mice, 10-14 weeks old, were employed in this study (Jackson Laboratory, Bar Harbor, ME, USA). All mice were housed in groups of 4-5 in 26.5 x 20 x 13.5 cm cages or groups of 7-10 in 37 x 30 x 15 cm cages. Food and water were available *ad libitum*. All measurements were performed during the dark phase of a reversed 12 h light-dark cycle (lights off at 08:00). The experiments were performed according to international guidelines and approved by the Hebrew University Committee on Animal Care and Use.

Mice heterozygous for the targeted deletion of the catalytic subdomain I of the GRK2 gene (GRK2$^{+/-}$) and their WT littermates were used to test the specificity of the GRK2 antibody[27]. Offspring were genotyped by PCR analysis on genomic DNA extracted from the tail. As GRK2$^{-/-}$ mice die *in utero*, only GRK2$^{+/-}$ mice could be used.

L5 SNT

L5 SNT (modification of Kim & Chung model[28]) was performed on mice. Mice were anesthetized with 4% chloral hydrate (400 mg/kg in 10 ml, i.p.), the lower back was shaved and the skin was disinfected with 70% ethanol. A 2-cm long incision was made along the back, at the level of the posterior iliac crest. The transverse process of L6 was exposed and carefully removed by No. 5 forceps, revealing the left L5 spinal nerve. With delicate scissors, the L5 was cut and the distal stump was excised forming a gap of a few millimeters between the stumps to avoid nerve regeneration. The incisions were closed in layers, using silk sutures (5-0) and Michel clips.

Mechanical pain sensitivity test

Withdrawal responses of the hind paws to calibrated mechanical stimuli (von Frey filament test) were assessed using blinded procedures. Mice were placed on a metal mesh floor under a transparent plastic box. Five successive measures for each filament were taken from each hind paw, starting with 1.4 g. Mechanical sensitivity was measured before as well as during 7 successive days following L5 SNT, determining the lightest filament which induced hind paw withdrawal for at least 3 of the 5 measures.

Tissue sample collection

One and two weeks after L5 SNT, mice were anaesthetized with an overdose of chloral hydrate solution and transcardially perfused with buffered saline and subsequently with 4% paraformaldehyde, after which spinal cords were dissected and post-fixed in the same fixative at 4°C during 24-48 h. Subsequently, the tissue was cryo-protected in 20% sucrose in PBS (pH 7.5) containing 0.01% sodium azide, frozen in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, the Netherlands) using cooled isopentane, and stored at -80°C until further use.

Immunohistochemistry

Transverse sections of the spinal cord (6 µm) were cut in a cryostat. For immunofluorescent staining, sections were treated with acetone and washed with TBS and TBS containing 0.05% Triton X-100 (TBS-T). Spinal cord sections were blocked with 5% normal goat serum at 37°C for 30 min and incubated at 4°C with rabbit anti-GRK2 1:400 (Santa Cruz Biotechnology Inc., Santa Cruz, United Kingdom; sc-562) combined with mouse anti-MAP2 1:1000 (Chemicon international, Temecula, CA, USA) or mouse anti-glial fibrillary acidic protein (GFAP) 1:50 (Cymbus Biotechnology, Southampton, United Kingdom) during 48 h. Subsequently, sections were incubated with biotinylated goat anti-rabbit IgG 1:100 (Vector laboratories, Burlingame, CA, USA) and Alexa Fluor-488 goat anti-mouse IgG (H+L) 1:200 (Molecular Probes, Eugene, OR, USA) followed by ExtrAvidin-Cy3 conjugate 1:100 (Sigma-Aldrich, St. Louis, MO, USA). Sections were mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingham, CA, USA).

Statistical analysis

Data of the pain assessment (von Frey filament test) were analyzed using a 3-way ANOVA with repeated measures, consisting of the following factors: strain (WT vs. IL-1R$^{-/-}$), hind paw (ipsilateral vs. contralateral), and the repeated measure time post L5 SNT (days). A P-value < 0.05 was considered to be statistically significant.

RESULTS

GRK2 expression in the mouse spinal cord

We have previously shown that the GRK2 antibody used in this study can be used to detect differences in GRK2 expression in the rat spinal cord using immunohistochemistry. Moreover, the antibody recognizes a single band on Western blots of mouse spinal cord and GRK2 expression, as determined by Western blotting, is significantly reduced in the spinal cord of GRK2$^{+/-}$ mice[16]. As shown in Figure 1A-B, the difference in GRK2 expression between WT and GRK2$^{+/-}$ mice can also clearly be detected by immunofluorescence. The staining for GRK2 expression was specific, since staining without the primary antibody was negative (Figure 1C).

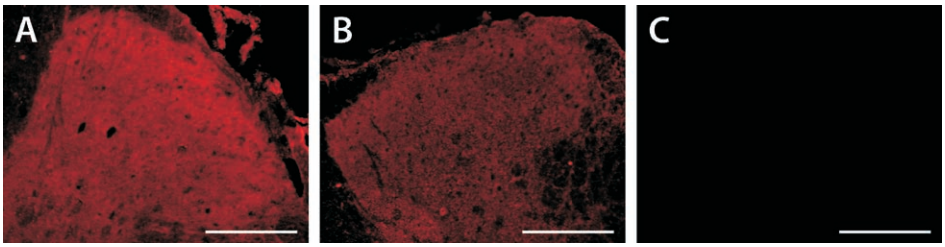


Figure 1: GRK2 expression in the lumbar spinal cord dorsal horn of WT and GRK2$^{+/-}$ mice. Using immunofluorescence, spinal cord sections were stained with rabbit anti-GRK2 followed by biotinylated goat anti-rabbit IgG and ExtrAvidin-Cy3 conjugate. GRK2 expression was determined in the lumbar spinal cord dorsal horn from WT (A) and GRK2$^{+/-}$ (B) C57BL/6 mice. (C) Staining of the spinal cord dorsal horn as in A-B but without rabbit anti-GRK2. Scale bar, 200 µm.

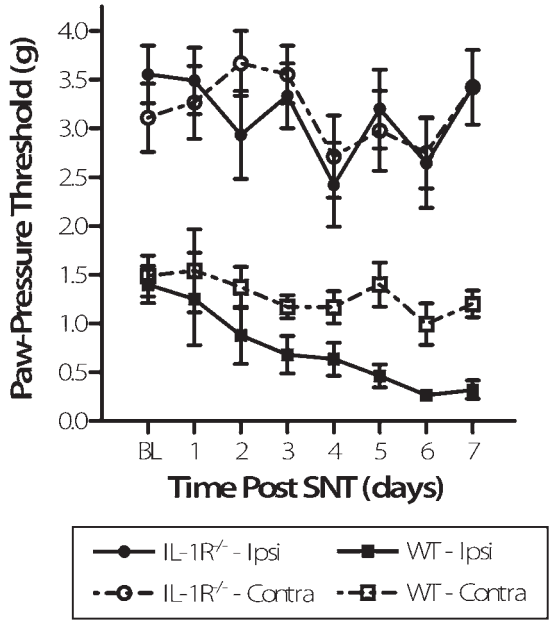
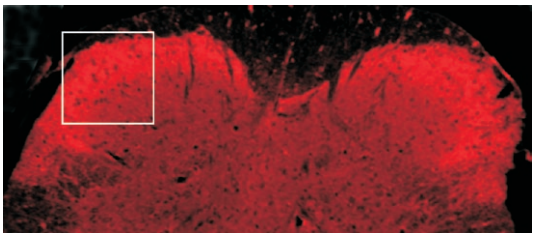


Figure 2: Time course for development of mechanical allodynia following L5 SNT in WT en IL-1R$^{-/-}$ mice. Mechanical withdrawal thresholds were determined using the von Frey test. Data are represented as the mean ± SEM (n=8 WT mice; n=9 IL-1R$^{-/-}$ mice). Data were analyzed by a 3-way ANOVA with repeated-measures. WT mice developed increased mechanosensitivity in the hind paw ipsilateral to L5 SNT compared to the contralateral hind paw, but there was no significant effect of L5 SNT in both hind paws of IL-1R$^{-/-}$ mice (strain by paw by time interaction; $F(7,147) = 2.122$, $^{*}P < 0.05$).



Supplementary Figure 1: Representation of the area of the spinal cord dorsal that is shown in Figure 3, 4 and 6. The photomicrograph shows the dorsal horns of the lumbar spinal cord. The square that is drawn represents the location in the superficial layers of the spinal cord dorsal horn depicted in Figure 3, 4, and 6.

L5 SNT induces mechanical allodynia in WT, but not in IL-1R$^{-/-}$ mice

A 3-way ANOVA with repeated measures revealed that C57BL/6 X 129/Sv WT mice developed mechanical allodynia in the hind paw ipsilateral to the injury compared to the contralateral hind paw, whereas IL-1R$^{-/-}$ mice did not display increased mechanosensitivity in either hind

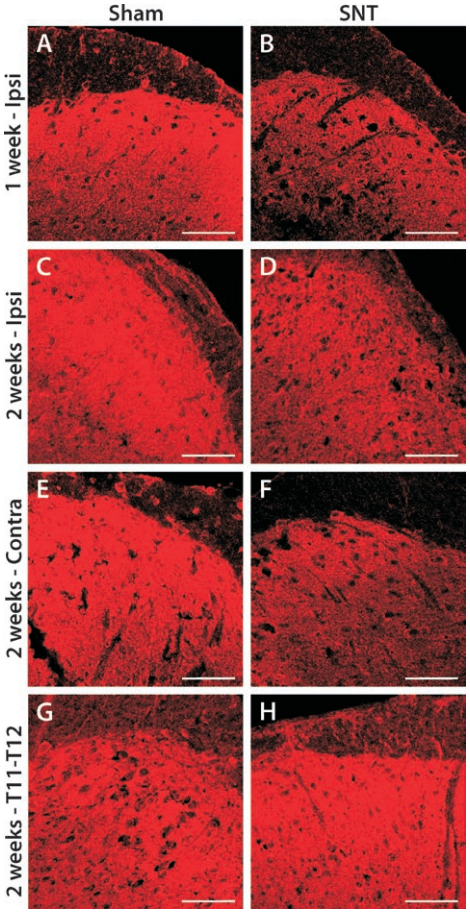


Figure 3: GRK2 expression in lamina I-II of the spinal cord dorsal horn of WT mice 1 and 2 weeks after L5 SNT. GRK2 expression after L5 SNT was determined in lamina I-II of the lumbar spinal cord dorsal horn (L4-L6) in sham-operated mice (A,C) and mice with L5 SNT (B, D) at 1 (A, B) and 2 weeks (C, D) after surgery. In addition, 2 weeks after surgery GRK2 expression was determined at the contralateral side in sham-operated controls (E) and mice with L5 SNT (F). To investigate whether the effect of L5 SNT was spread throughout the spinal cord, GRK2 expression was also determined in lamina I-II of spinal cord segments T11-L2 in sham-operated (G) and nerve transected (H) mice. Slices were stained as indicated in the legend to Figure 1 and a representative example of GRK2 expression is shown. Scale bar, 50 µm.

paw, as reflected by a significant triple interaction (strain by paw by time interaction; $F(7,147) = 2.122$, $P < 0.05$)(Figure 2). These data confirm previous published results[29]. The maximal reduction in mechanical threshold was reached after 7 days, and as previously reported, this effect was still present 14 days after L5 SNT[29].

L5 SNT decreases neuronal GRK2 expression in the lumbar dorsal horn in WT mice

To determine whether L5 SNT induced a reduction in GRK2 expression in the superficial layers of the spinal cord dorsal horn in WT mice, sections from spinal cord, obtained at 1 and 2 weeks after L5 SNT, were analyzed for GRK2 expression using immunofluorescence. Supplementary Figure 1 shows the area of the spinal cord dorsal horn that was analyzed for changes in GRK2 expression. One week after L5 SNT, we observed a decrease in GRK2 levels in lamina I-II of the lumbar spinal cord dorsal horn (L4-L6) in 4 out of 4 mice (Figure 3A-B). The reduction in GRK2 expression was still detectable 2 weeks after nerve transection (n=5; Figure 3C-D). In 2 out of 5 mice the decrease in GRK2 was even more pronounced 2 weeks compared to 1 week after L5 SNT. Although the left L5 spinal nerve was transected, GRK2 expression was reduced to the same extent in both the ipsilateral and contralateral sides of the lumbar spinal cord (compare Figure 3C-D with E-F).

To investigate whether the decrease in GRK2 was restricted to lumbar segments L4-L6 or whether it was a more generalized effect throughout the spinal cord, GRK2 expression was also determined at T11-L2. As shown in Figure 3G-H, no change in GRK2 expression was seen in these segments two weeks after L5 SNT. Similarly, at higher thoracic segments, there was no change in GRK2 expression after L5 SNT (data not shown).

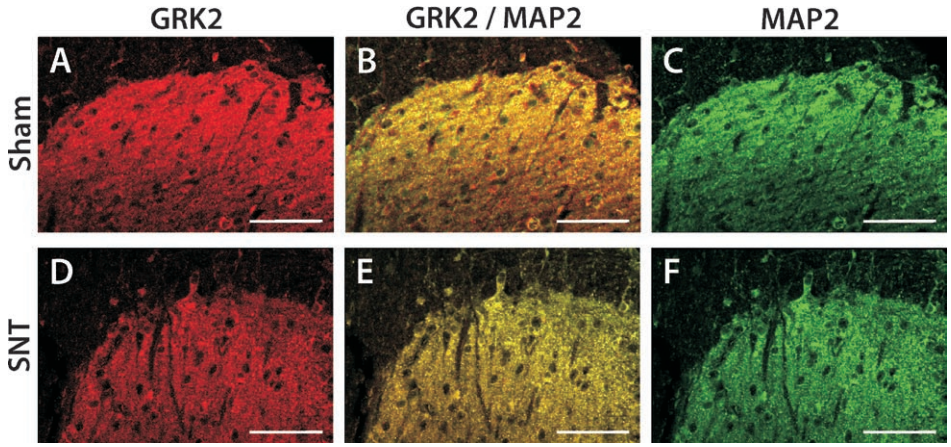


Figure 4: GRK2 expression 2 weeks after L5 SNT in MAP2-positive cells in lamina I-II of the lumbar dorsal horn. Slices from sham-operated mice (A-C) and mice with L5 SNT (D-F) were double-stained with rabbit anti-GRK2 and mouse anti-MAP2 2 weeks after surgery. Slices were stained with Alexa Fluor-488 goat anti-mouse IgG (green) and biotinylated goat anti-rabbit IgG followed by ExtrAvidin-Cy3 conjugate (red). Photographs were taken from lamina I-II of the lumbar spinal cord dorsal horn (L4-L6). Scale bar, 50 µm.

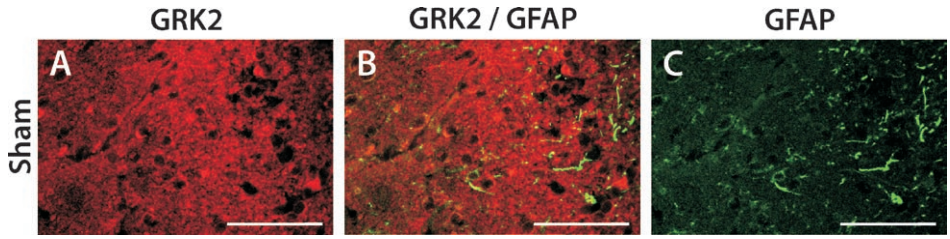


Figure 5: Lack of GRK2 expression in GFAP-positive cells in lamina I-II of the lumbar dorsal horn. Slices from sham-operated animals were double-stained with rabbit anti-GRK2 and mouse anti-GFAP. Slices were stained with Alexa Fluor-488 goat anti-mouse IgG (green) and biotinylated goat anti-rabbit IgG followed by ExtrAvidin-Cy3 conjugate (red). Photographs were taken from lamina I-II of the lumbar spinal cord dorsal horn (L4-L6). Scale bar, 50 µm.

To determine in which cell types L5 SNT induced a decrease in GRK2 expression, spinal cord sections were double-stained with GRK2 (red) and the neuronal marker MAP2 (green) or GFAP (green), which is present in astrocytes. A clear co-localization of MAP2 and GRK2 (yellow) was observed in the spinal cords from sham-operated controls, showing that GRK2 is highly expressed in neuronal cells (Figure 4A-C). Expression of GRK2 was below detection limit in astrocytes (Figure 5). Two weeks after L5 SNT, the reduction in GRK2 expression at

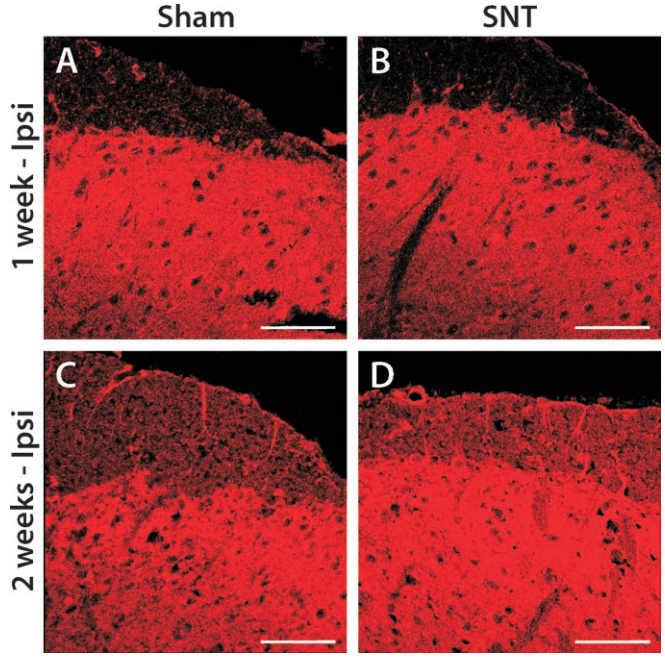


Figure 6: GRK2 expression in lamina I-II of the lumbar spinal cord dorsal horn of IL-1R$^{-/-}$ mice 1 and 2 weeks after L5 SNT. GRK2 expression after L5 SNT was determined in lamina I-II of the lumbar spinal cord dorsal horn (L4-L6) in sham-operated mice (A, C) and mice with L5 SNT (B, D), at 1 (A, B) and 2 weeks (C, D) after surgery. Slices were stained as indicated in the legend to Figure 1 and a representative example of GRK2 expression is shown. Scale bar, 50 µm.

L4-6 was accompanied by a decrease in co-localization of GRK2 and MAP2. This decrease was not associated with a decrease in MAP2 staining (Figure 4) indicating that the decrease in staining is not caused by loss of neurons but rather that L5 SNT is associated with a decrease in neuronal GRK2 expression.

L5 SNT does not alter spinal cord GRK2 levels in IL-1R$^{-/-}$ mice

To investigate whether IL-1β signaling is required for this phenomenon, we determined GRK2 expression in the spinal cord dorsal horn after L5 SNT in IL-1R$^{-/-}$ mice. In contrast to WT mice, no change in GRK2 expression was observed in IL-1R$^{-/-}$ mice 1 and 2 weeks after L5 SNT compared to sham-operated knockout mice (n=3; Figure 6).

DISCUSSION

This study demonstrates that neuronal GRK2 levels in the lumbar spinal cord dorsal horn were downregulated in a murine model of mechanical allodynia (L5 SNT). Interestingly, in IL-1R$^{-/-}$ mice, which did not develop mechanical allodynia after L5 SNT, no such reduction was observed. These results indicate that IL-1β signaling is required for the decrease in neuronal GRK2 levels, as well as for the development of L5 SNT-induced mechanical allodynia.

We previously demonstrated that CCI of the sciatic nerve in rats is associated with a downregulation of neuronal GRK2 expression in the superficial layers of the lumbar spinal cord dorsal horn[16]. The present data show that in the murine L5 SNT model mechanical allodynia is also associated with a decrease in GRK2 levels in lamina I-II of the lumbar spinal cord. There are, however, some major differences between the present study and our recently published data, which concern the use of different species (mice vs. rats), the affected nerve (L5 spinal nerve vs. sciatic nerve), the type of injury (transection vs. ligation), and the spreading of the GRK2 decrease in the spinal cord. In contrast to the CCI model in rats, which shows spreading of the reduction in GRK2 expression to other sections of the spinal cord (L1-L6)[16], downregulation of GRK2 after L5 SNT was restricted to the segment directly receiving sensory input from the transected nerve (L5) and the segments immediately adjacent to it (L4-L6). GRK2 was not altered in segments T11-L2. The observation that neuronal GRK2 is decreased in two completely different models for mechanical allodynia indicates that downregulation of GRK2 may be an important general mechanism for the development of mechanical allodynia.

We demonstrated that in the spinal cord GRK2 was mainly expressed in neurons and below detection levels in GFAP-positive cells (Figure 4, 5). Furthermore, we showed that the observed decrease in GRK2 after L5 SNT represents mainly a decrease in neuronal GRK2 as shown by double-staining for GRK2 and the neuronal marker MAP2.

Based on *in vitro* experiments, we previously suggested that IL-1β is involved in reducing spinal cord GRK2[16]. In the present study, we showed the completely novel finding that *in*

vivo IL-1β signaling is required not only to induce mechanical allodynia after L5 SNT, but also to downregulate GRK2 expression in the spinal cord dorsal horn. The observed reduction in GRK2 might be due to a direct effect of IL-1β signaling. Indeed, IL-1β has been shown to downregulate GRK2 promoter activity[25]. Moreover, we have recently shown that chronic IL-1β treatment of *ex vivo* cultured spinal cord slices resulted in downregulation of GRK2 expression, indicating that IL-1β is able to directly diminish spinal cord GRK2 protein levels[16]. On the other hand, IL-1β might also indirectly cause a reduction in GRK2 via downstream processes of mechanical allodynia, e.g. alterations in production of other inflammatory mediators or in neurotransmitter output. *In vitro*, IL-1β treatment of primary astrocyte cultures leads to the production of TNF-α, as well as IL-6[30,31]. These two cytokines are produced in the spinal cord after L5 SNT and are involved in the development of mechanical allodynia[17,18,32,33]. In addition, TNF-α and IL-6 are also capable of downregulating GRK2[21,25]. Thus, IL-1β-induced production of IL-6 or TNF-α could also be responsible for the reduction of GRK2 levels during L5 SNT.

Several studies illustrated that mechanical allodynia is associated with increased sensitivity of multiple GPCRs (e.g. neurokinin-1 receptor, CCR2, and adrenergic receptors)[34-36]. The reduction in GRK2 protein levels could be responsible for the enhancement of GPCR sensitivity during mechanical allodynia. Indeed, agonist-induced signaling of several GPCRs, that show enhanced sensitivity during mechanical allodynia, is increased during low levels of GRK2[11,12]. Therefore, we propose that the downregulation of GRK2 may contribute to the mechanical allodynia observed after L5 SNT. However, it should be noted that downregulation of GRK2 expression after L5 SNT was observed at both sides of the spinal cord dorsal horn, whereas mechanical allodynia was observed solely in the ipsilateral paw. This could imply that the two processes are independent. A more likely explanation, however, is that a cytokine-mediated reduction of GRK2 expression is necessary but by itself not sufficient to produce mechanical allodynia. This conclusion is supported by our previous findings showing that under basal conditions, $GRK2^{+/-}$ mice do not display increased sensitivity to mechanical stimulation, despite the marked reduction in spinal cord GRK2 expression in these animals[16]. In other words, low GRK2 levels per se and the associated increased sensitivity of certain GPCRs is not sufficient to induce a decrease in pain threshold. We therefore hypothesize that a reduction in GRK2 facilitates the development of mechanical allodynia solely in the presence of an additional peripheral stimulus, i.e. the spinal nerve damage in our present experiments. In line with this hypothesis, we have previously reported that $GRK2^{+/-}$ mice develop more pronounced mechanical allodynia and/or thermal hyperalgesia during acute paw inflammation induced by either intraplantar injection of λ-carrageenan or the GPCR agonist C-C chemokine ligand 3 (CCL3)(Kleibeuker *et al.*, unpublished results)[16]. In both models of acute peripheral paw inflammation, $GRK2^{+/-}$ animals develop more pronounced inflammatory pain in the paw that was exposed to the peripheral inflammatory stimulus. There was no change in pain threshold in the contralateral paw. In the case of L5 SNT, it may well be that as the peripheral stimulus from the spinal nerve trauma is solely present at the ipsilateral side, the mechanical

allodynia is also present in the ipsilateral paw, despite bilateral reductions in GRK2 levels. The peripheral damage induced by L5 SNT might result in activation of astrocytes, microglia, p38 MAPK, and ERK1/2 and thereby contribute to the decrease in pain threshold in conditions of low neuronal GRK2[37-39]. In addition, it should be noted that mechanical allodynia may involve activation of other sets of GPCRs, e.g. chemokine receptors, which are not involved in normal pain processing, but induced or upregulated by the peripheral stimulus[40]. Such an event will exclusively occur at the ipsilateral site as well, thereby facilitating the development of ipsilateral mechanical allodynia in association with bilateral low GRK2 levels.

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4

CHAPTER

Endogenous GRK2 regulates inflammatory thermal hyperalgesia

Wendy Kleibeuker
Niels Eijkelkamp
Jitske Zijlstra
Ilona J.M. den Hartog
Annemieke Kavelaars
Cobi J. Heijnen

University Medical Center Utrecht, Laboratory of Psychoneuroimmunology, 3584 EA Utrecht, the Netherlands

ABSTRACT

G protein-coupled receptor (GPCR) kinase (GRK)2 controls agonist-induced GPCR signaling and desensitization. Recently, we described downregulation of spinal cord neuronal GRK2 in two models of nerve damage-induced mechanical allodynia. Here, we investigated whether a reduction in GRK2 is sufficient to alter thermal sensitivity and/or inflammatory hyperalgesia. Using the Hargreaves test, we observed equal thermal sensitivity in naive wild type (WT) and GRK2$^{+/-}$ mice, which have a 50% lower GRK2 expression. Subsequently, inflammatory hyperalgesia was induced by intraplantar injection of λ-carrageenan. The inflammatory response, determined as paw swelling, influx of neutrophils, and cytokine expression 6 h after injection, was similar in both genotypes. Since inflammatory responses of the two genotypes were comparable, the λ-carrageenan model was used to analyze the effect of reduced GRK2 on inflammatory hyperalgesia. At 6 h after induction of inflammation, mice showed thermal hyperalgesia. Interestingly, this inflammation-induced thermal hyperalgesia was significantly more pronounced in GRK2$^{+/-}$ mice. The increased sensitivity of GRK2$^{+/-}$ mice was not restricted to λ-carrageenan-induced inflammation, since intraplantar injection of the chemokine CCL3 also provoked increased thermal hyperalgesia in GRK2$^{+/-}$ mice compared to WT mice. To investigate the pathways responsible for increased inflammatory hyperalgesia in GRK2$^{+/-}$ mice, we analyzed TRPV1 expression and phosphorylation, as well as MAPK activation. However, no effects of genotype on these parameters were observed at 1 and 6 h after λ-carrageenan injection.

In conclusion, GRK2$^{+/-}$ mice showed increased inflammatory thermal hyperalgesia compared to WT mice, whereas baseline thermal sensitivity and inflammatory activity were identical. The mechanism for increased inflammatory thermal hyperalgesia in GRK2$^{+/-}$ mice requires further investigation, but may involve increased signaling in response to GPCR agonists. Moreover, the increase in inflammatory thermal hyperalgesia is probably not the result of an increase in central sensitization.

INTRODUCTION

Pain is a common complaint in patients with inflammatory diseases (e.g. rheumatoid arthritis, ulcerative colitis, and Crohn's disease). Inflammatory pain is characterized by hyperalgesia (an increased response to a painful stimulus) and/or allodynia (pain due to a stimulus which does normally not provoke pain). An increase in the excitability of peripheral nerves and spinal cord neurons is a critical event in the development of hyperalgesia[1,2]. Several phenomena which contribute to inflammatory hyperalgesia have been reported, including increased activation of mitogen-activated protein kinases (MAPK) in dorsal root ganglia (DRG) and spinal cord, the activation of glial cells and neuronal c-Fos in the spinal cord, the production of pro-inflammatory mediators, and an altered expression and sensitivity of ion channels present on sensory nerve fibers (e.g. transient receptor potential vanilloid subtype 1 (TRPV1))[3-9]. Although the exact mechanisms that lead to inflammatory hyperalgesia are not completely understood, it is known that inflammatory mediators that signal via G protein-coupled receptors (GPCRs) are important in the induction of inflammatory hyperalgesia. The GPCR agonists and inflammatory mediators C-C chemokine ligand (CCL)2, CCL3, Keratinocyte-Derived Chemokine (KC), prostaglandin E (PGE)2, and CCL5 have been described to provoke hyperalgesia and/or mechanical allodynia when injected into the hind paw[7,10-13]. In addition, during inflammatory hyperalgesia induced by intraplantar injection of λ-carrageenan, a number of GPCR agonists (e.g. CCL2, CCL3, C-X-C chemokine ligand (CXCL)10, KC, and PGE2) are locally produced[7,14,15].

GPCR kinase (GRK)2 is a serine-threonine kinase that regulates GPCR function. In the classic model, GRK2 regulates signaling and desensitization of GPCRs via a phosphorylation-dependent mechanism. Upon ligand binding, GPCRs become phosphorylated by GRK2 leading to the binding of arrestins. Subsequently, GPCR signaling is abrogated via inactivation of the G protein and receptor internalization[16]. In addition, more recent studies have shown that GRK2 can also regulate signaling via an interaction with several intracellular signaling molecules (e.g. $G\alpha_{q/11}$, Akt, MEK1/2, and phosphoinositide-3 kinase (PI3 kinase))(Kleibeuker *et al.*, submitted)[17,18]. Multiple studies reported that a reduction in GRK2 expression correlated with enhanced receptor signaling[19-21]. Interestingly, we recently described a decrease in neuronal GRK2 expression in the spinal cord dorsal horn during mechanical allodynia provoked by chronic constriction injury (CCI) of the sciatic nerve or L5 spinal nerve transection (SNT), suggesting that a reduced level of GRK2 may contribute to increased sensitivity to pain[22,23].

In the present study, we investigated whether low endogenous GRK2 levels in fact result in an increase in thermal sensitivity and/or hyperalgesia. For this purpose, we tested the sensitivity for thermal stimulation of the hind paw in wild type (WT) mice and $GRK2^{+/-}$ mice, which express approximately 50% of the GRK2 protein[24,25], under baseline and under inflammatory conditions. In addition, we tried to unravel the underlying mechanisms for the increased inflammatory thermal hyperalgesia in $GRK2^{+/-}$ mice.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (12-14 weeks old) heterozygous for targeted deletion of the GRK2 gene ($GRK2^{+/-}$) and their WT littermates were used[24]. Offspring were genotyped by PCR analysis on genomic DNA. As $GRK2^{-/-}$ mice die *in utero*, only $GRK2^{+/-}$ mice could be used for experiments. Mice were bred and maintained in the animal facility of the University of Utrecht (the Netherlands). All experiments were performed in accordance with international guidelines and approved by the University Medical Center Utrecht experimental animal committee.

Induction of thermal hyperalgesia

$GRK2^{+/-}$ mice and their WT littermates received an intraplantar injection of 20 µl 2% λ-carrageenan (Sigma-Aldrich, St. Louis, MO, USA) in saline or 2.5 µl 100 µg/ml recombinant murine CCL3 (R&D systems, Minneapolis, MN, USA) in phosphate-buffered saline (PBS; pH 7.5) in the hind paw. As a control, a similar amount of vehicle was injected in the contralateral hind paw. Thresholds for behavioral responses to heat stimuli applied to the hind paw were determined using the Hargreaves test as described previously[26,27]. Thresholds were determined prior to (baseline) and at the indicated time points after intraplantar injection. In brief, baseline paw withdrawal latency (PWL) was calculated from an average of 2 successive PWLs of each hind paw. The intensity of the heat source was adjusted to yield mean baseline PWLs ranging of 7-8 sec. A cut-off time of 20 sec was used to avoid tissue damage. As a measure for development of paw edema, the paw thickness was measured using a Digimatic Micrometer (Mitutoyo, Veenendaal, the Netherlands).

Tissue collection

For tissue collection, mice were sacrificed by an overdose of pentobarbital. Paw biopsies were taken using a 4-mm biopsy punch (Stiefel, Offenbach am Mein, Germany) and lumbar spinal cords (L1-S1) were isolated. All samples were frozen in liquid nitrogen. For immunohistochemistry, animals were transcardially perfused with 4% paraformaldehyde in PBS (pH 7.5) after which spinal cords were dissected. Tissue was post-fixed in the same fixative at 4°C during 24-48 h. Subsequently, tissue was cryo-protected in 30% sucrose in PBS (pH 7.5) and frozen in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, the Netherlands) using cooled isopentane, and stored at -80°C until further use.

Frozen paw biopsies were homogenized in 50 mM HEPES buffer (pH 8.0) using a Potter homogenizer. Homogenates were centrifuged for 30 min at 10.000 g at 4°C, supernatants were collected for ELISA and pellets were used to determine myeloperoxidase (MPO) activity.

Neutrophil influx

MPO activity in biopsies from the paw was determined as a measure of infiltrated neutrophils. The protocol for measuring MPO activity was adapted from Schneider *et al.* (1996)[28]. Pellets from homogenized paw biopsies were sonicated in 0.5% cetyltrimethylammonium chloride (CTAC; Merck, Darmstadt, Germany) and centrifuged for 30 min at 10.000 g at 4°C. Supernatants were taken for analysis of MPO activity. Samples and MPO standard (Sigma-Aldrich) were diluted in 10 mM sodium-citrate buffer (pH 5.0) containing 0.22% CTAC. Subsequently, substrate solution containing 3 mM 3',5,5'-tetramethylbenzidine dihydrocloride (TMB; Sigma-Aldrich), 120 µM resorcinol (Sigma-Aldrich), and 2.2 mM H_2O_2 was added 1:1. Reaction mixtures were incubated for 30 min at 37°C protected from light. The reaction was stopped by adding 4 N H_2SO_4, followed by determination of OD at 450 nm in an ELISA-plate reader.

Cytokines/chemokines

Mouse KC and interleukin (IL)-1β levels were measured in paw homogenates (see tissue collection) using ELISA according to manufacturer's protocol (R&D systems).

Chemotactic assay

Femurs and tibias from WT and GRK2$^{+/-}$ mice were cleaned and crushed using a mortar. A total bone marrow suspension was obtained and resuspended in RPMI containing 5% FCS (Invitrogen, Breda, the Netherlands). The cell suspension was filtered and centrifuged. Subsequently, the cells were resuspended in RPMI containing 5% FCS and counted. Cell migration was performed using a transwell system with 5 µm pore filters (Costar, Cambridge, MA, USA). Before use, the filters were incubated with RPMI containing 5% FCS at 37°C. Subsequently, cells were placed in the upper well in a total volume of 100 µl and 600 µl of recombinant murine KC (Peprotech, London, United Kingdom) or medium (RPMI containing 5% FCS) was added to the bottom chamber. After 2 h incubation at 37°C, cells in the lower well were collected and the number of migrated neutrophils was determined using flow cytometry, based on forward-sideward scatter characteristics (FACS Calibur, Becton Dickinson, San Jose, CA, USA).

Western blot analysis

For the preparation of membrane fractions, paw biopsies or spinal cord tissue were lysed in ice-cold cell lysis buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA) by homogenization using an Ultra-Turrax and subsequent sonication. Unbroken cells and nuclei were pelleted by centrifugation (800 x g for 5 min) and discarded. The supernatant was subsequently centrifuged (48.000 x g for 20 min at 4°C) and the membrane pellet was resuspended in ice-cold RIPA buffer (20 mM Hepes pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA) and sonicated. For determination of pTRPV1 (Ser800) expression, 1 mM DTT was added to the cell lysis buffer and membrane fractions were resuspended in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA

and 1% Triton. All buffers used contained the following protease and phosphatase inhibitors: 2 mM 4-(2-Aminoethyl) benzenesulphonyl fluoride (AEBSF), 20 µg/ml leupeptin, 200 µg/ml benzamidine, 10 mM β-glycerolphosphate, 1 mM NaVO3, and 20 mM NaF.

Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C; Amersham Int.) or PVDF membranes (Millipore, Bedford, MA, USA) for pTRPV1 (Ser800) expression by electroblotting. Blots were stained with the following antibodies: mouse anti-pERK1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; sc-7383), rabbit anti-ERK1/2 (Cell Signaling Technology, Beverly, MA, USA; #9102), rabbit anti-p-p38 MAPK (Cell Signaling; #4631), rabbit anti-p38 MAPK (Cell signaling; #9212), rabbit anti-pTRPV1 (Ser800) (Transgenic Inc., Kobe, Japan; KM112), rabbit anti-TRPV1 (Abcam, Cambridge, MA, USA; ab10296), goat anti-actin (Santa Cruz Biotechnology Inc.; sc-1616). Subsequently, blots were incubated for 1 h with goat anti-mouse-peroxidase IgG + IgM (H+L)(Jackson Laboratories, Bar Harbor, ME, USA), donkey anti-rabbit-peroxidase IgG (Amersham Int.), or donkey anti-goat-peroxidase IgG (Santa Cruz Biotechnology Inc.; sc-2020) and developed by enhanced chemiluminescence (Amersham Int.). Band density was determined using a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

c-Fos staining

Transverse free-floating sections of the spinal cord (30 µm) were cut in a cryostat (Leica CM3050 S, Rijswijk, Netherlands). Sections were incubated with rabbit anti-c-Fos (1:20.000; Vector laboratories, Burlingame, CA, USA) followed by biotinylated donkey anti-rabbit IgG (1:1500; Jackson ImmunoResearch Laboratories; West Grove, PA, USA) and stained using the ABC nickel-enhanced diaminobenzidine method (Vector laboratories). The mean number of c-Fos positive cells was determined in the superficial laminae (I-II) of the dorsal horn of at least 3 slices per spinal segment per animal (L1-L6).

Data analysis

Data are expressed as a mean value ± 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

Thermal sensitivity in naive WT and GRK2$^{+/-}$ mice

Using the Hargreaves test, thermal sensitivity was compared between naive WT mice and naive GRK2$^{+/-}$ mice, which express approximately 50% of the GRK2 protein in various tissues compared to WT mice[24,25]. The intensity of the heat source was adjusted to the level at which naive WT mice withdrew their paw 7-8 sec after heat application onto the hind paw. Using

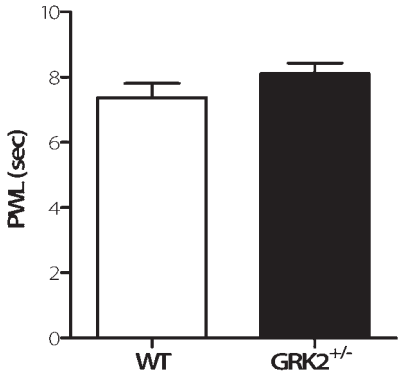

Figure 1: Thermal sensitivity in naive GRK2$^{+/-}$ mice and their WT littermates. Using the Hargreaves test, PWLs were determined in naive WT mice and naive GRK2$^{+/-}$ mice. Data are presented as mean ± SEM of 10 animals.

this setting, paw withdrawal latencies (PWLs) of GRK2$^{+/-}$ mice did not significantly differ from WT mice (Figure 1). These results suggest that decreased GRK2 does not have consequences for thermal sensitivity in naive mice.

λ-Carrageenan-induced inflammation of the hind paw

To induce inflammatory hyperalgesia, paw inflammation was induced by intraplantar injection of λ-carrageenan. In a pilot experiment we observed that the optimal time point for induction of inflammatory hyperalgesia was 6 h post injection. For this reason, this time point was used for further experiments. First, the magnitude of the λ-carrageenan-induced inflammatory response was compared between both genotypes. As a measure of paw edema formation, which is a characteristic feature of λ-carrageenan-induced inflammation, paw thickness was determined. At 6 h after λ-carrageenan injection, paw thickness was increased to the same extent in WT and GRK2$^{+/-}$ mice (Figure 2A). Thickness of vehicle-injected paws was not significantly different from paw thickness of naive mice. The amount of IL-1β and KC present in the inflamed tissue of the paw was determined using ELISA. As shown in Figure 2B-C, a comparable increase in the amount of IL-1β and KC was found in the paws of WT and GRK2$^{+/-}$ mice 6 h after λ-carrageenan injection. Since neutrophils are attracted in response to the production of pro-inflammatory mediators (e.g. KC), the MPO activity in paw biopsies 6 h after λ-carrageenan injection was determined as a measure for infiltrated neutrophils. The influx of neutrophils in WT and GRK2$^{+/-}$ mice provoked by λ-carrageenan injection was not different (Figure 2D). Since it has been shown before that low GRK2 is associated with an increased capacity of T-lymphocytes to migrate in response to certain chemokines (i.e. CCL3, CCL4, and CCL5)[29], we determined the *in vitro* chemotactic activity of neutrophils isolated from WT and GRK2$^{+/-}$ mice towards KC and to a number of other chemokines (CXCL12, CCL2, and CCL3). However, neutrophils of GRK2$^{+/-}$ mice showed the same capacity to migrate towards chemokines as neutrophils of WT mice did (Figure 3; data not shown).

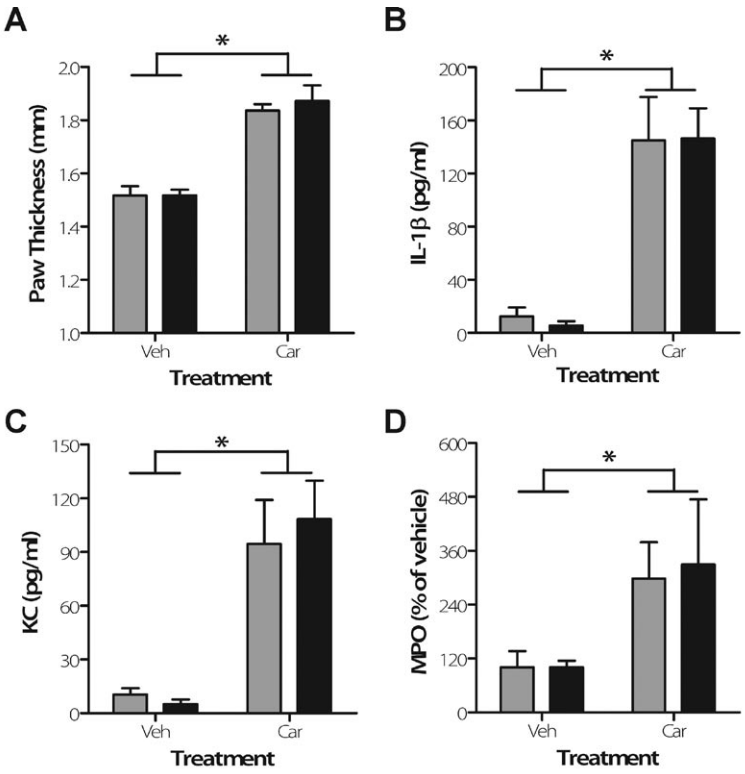

Figure 2: Inflammatory response in the paws of WT and GRK2$^{+/-}$ mice in response to intraplantar λ-carrageenan injection. (A) Paw thickness was determined using a Digimatic Micrometer (n=10). ELISA was employed to determine the amount of (B) IL-1β (n=4-6) and (C) KC (n=3) in paw biopsies. (D) MPO activity was determined in paw biopsies as a measure for infiltrated neutrophils (n=4-6). Measurements were performed 6 h after intraplantar injection of vehicle or λ-carrageenan in WT mice and GRK2$^{+/-}$ mice. Data are presented as mean ± SEM (gray bars, WT mice; black bars, GRK2$^{+/-}$ mice; *$P < 0.05$).

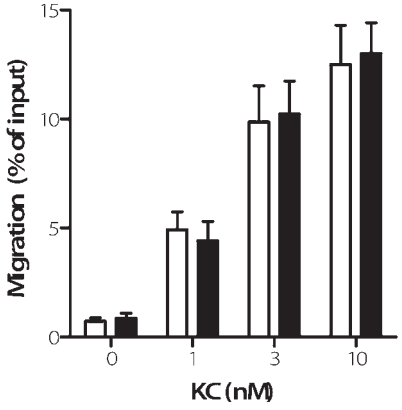

Figure 3: Migration of neutrophils derived from WT and GRK2$^{+/-}$ mice towards KC. Neutrophils were isolated from bone marrows from WT mice and GRK2$^{+/-}$ mice. Migration towards recombinant murine KC was determined using a chemotactic assay. Data are presented as mean ± SEM of 3 animals (gray bars, WT mice; black bars, GRK2$^{+/-}$ mice).

Inflammatory thermal hyperalgesia is increased in GRK2$^{+/-}$ mice

Since λ-carrageenan induced a similar inflammatory response in GRK2$^{+/-}$ mice and their WT littermates, we used the λ-carrageenan model to compare the development of inflammatory thermal hyperalgesia between both genotypes. At 6 h after λ-carrageenan injection, WT mice showed an increased sensitivity for thermal stimulation of the hind paw characterized by a significant decrease in PWL. Interestingly, the inflammation-induced decrease in PWL was significantly more pronounced in GRK2$^{+/-}$ mice compared to WT mice at 6 h after λ-carrageenan injection (Figure 4). The PWL of the contralateral vehicle-injected hind paw did not significantly change in both genotypes (data not shown). Thus, inflammation-induced thermal hyperalgesia was more pronounced in GRK2$^{+/-}$ mice compared to WT mice.

To investigate whether stimulation of a GPCR could also result in an upregulation of thermal hyperalgesia in GRK2$^{+/-}$ mice, we compared the effect of intraplantar injection of the GPCR agonist CCL3 on thermal sensitivity between both genotypes. Zhang *et al.* (2005) described thermal hyperalgesia in mice 30 min after intraplantar injection of CCL3[13]. For this reason, we determined sensitivity for thermal stimulation prior to and 30 min after intraplantar injection of CCL3. In WT mice as well as in GRK2$^{+/-}$ mice, CCL3 indeed evoked an increase in thermal sensitivity compared to baseline levels. Moreover, the CCL3-induced thermal hyperalgesia was significantly higher in GRK2$^{+/-}$ mice compared to WT mice (Figure 5). The PWL of the contralateral vehicle control paw did not significantly change in both genotypes over time (data not shown). In addition, intraplantar injection of CCL3 did not induce an increase in paw thickness.

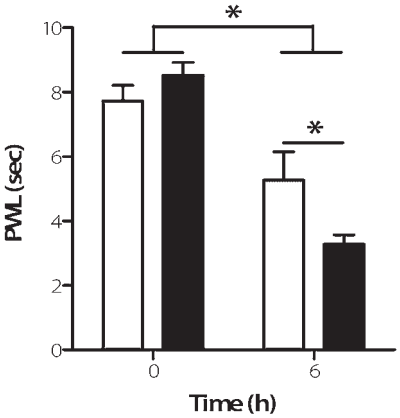

Figure 4: Thermal sensitivity in WT and GRK2$^{+/-}$ mice 6 h after λ-carrageenan-induced paw inflammation. Using the Hargreaves test, PWLs were determined in WT and GRK2$^{+/-}$ mice prior to and 6 h after induction of inflammation by intraplantar injection of λ-carrageenan. Data are presented as mean ± SEM of 13 animals (gray bars, WT mice; black bars, GRK2$^{+/-}$ mice; *$P < 0.05$).

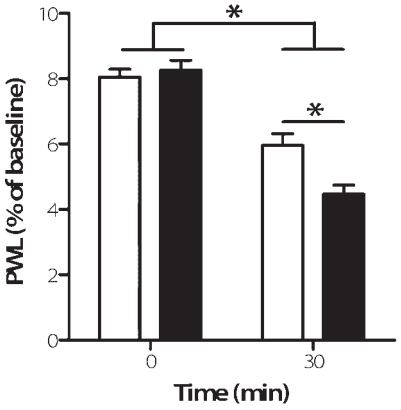

Figure 5: CCL3-induced thermal hyperalgesia in WT and GRK2$^{+/-}$ mice. Using the Hargreaves test, PWLs were determined in WT and GRK2$^{+/-}$ mice prior to and 30 min after intraplantar injection of CCL3. Data are shown as mean ± SEM of 30 animals (gray bars, WT mice; black bars, GRK2$^{+/-}$ mice; *$P < 0.05$).

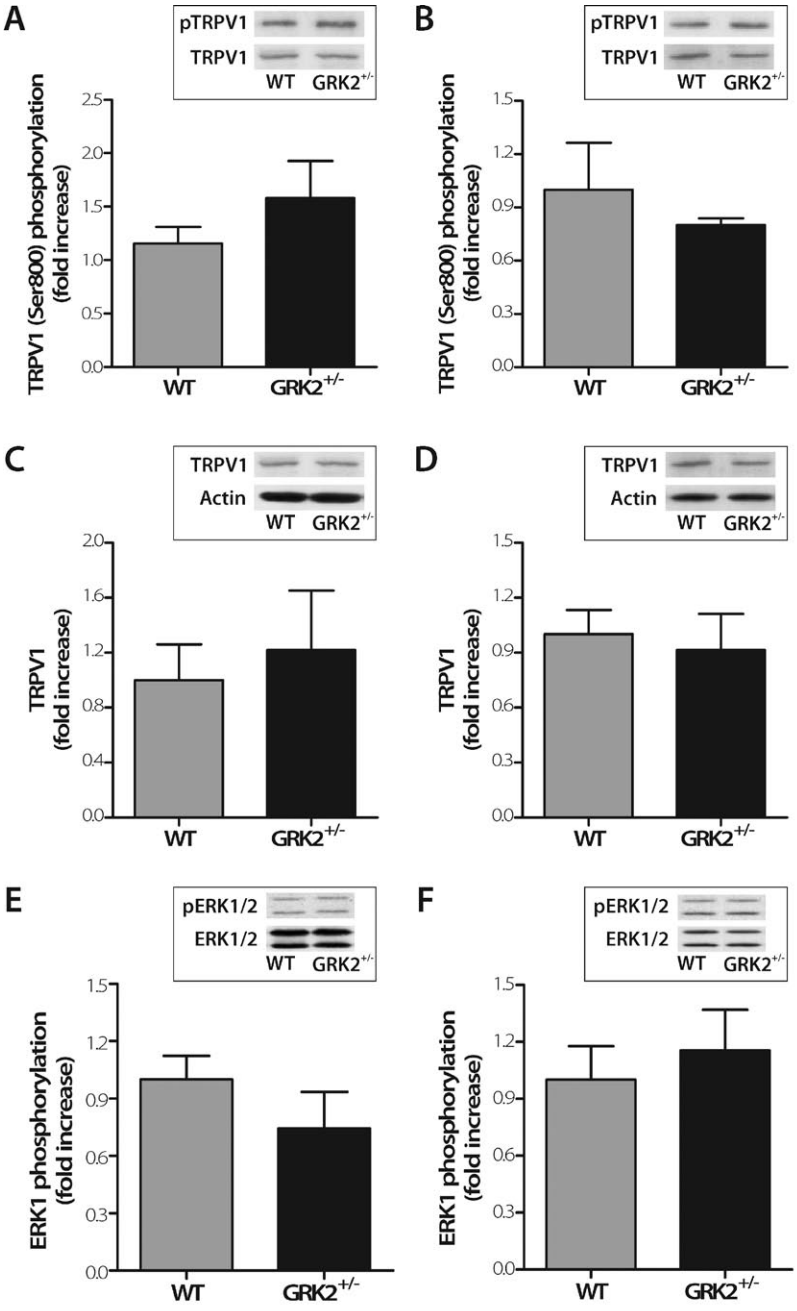

Figure 6: pTRPV (Ser800), TRPV1 and pERK1/2 levels in the paws of WT and GRK2$^{+/-}$ mice during inflammatory hyperalgesia. Paw biopsies from WT and GRK2$^{+/-}$ mice were taken 1 h (A, C, E) and 6 h (B, D, F) after induction of inflammatory thermal hyperalgesia by intraplantar λ-carrageenan injection. Western blotting analysis was used to determine pTRPV1 (Ser800) (A-B), TRPV (C-D), and pERK1/2 (E-F) levels. Insets show representative Western blots. Data are presented as mean ± SEM of 8 mice and normalized for the total amount TRPV1 (A, B), actin (C, D) or ERK1/2 (E, F) present.

Possible mechanisms contributing to increased inflammatory thermal hyperalgesia in GRK2$^{+/-}$ mice

To identify a possible mechanism that leads to the increase in inflammatory thermal hyperalgesia in GRK2$^{+/-}$ mice, we investigated several peripheral mechanisms involved in the upregulation of thermal sensitivity. One important mechanism that could contribute to increased heat sensitivity is an increase in expression or sensitivity of TRPV1. An increase in TRPV1 sensitivity occurs via various mechanisms including protein kinase C (PKC)ε-mediated phosphorylation at Ser800 as well as increased expression of TRPV1[30]. TRPV1 and pTRPV1 (Ser800) levels were determined in the paws of WT and GRK2$^{+/-}$ mice after λ-carrageenan injection. Western blot analysis showed that TRPV1 as well as pTRPV1 (Ser800) levels were similar in WT and GRK2$^{+/-}$ mice 1 and 6 h after λ-carrageenan injection (Figure 6A-D). In addition, pERK1/2 levels were measured in the paw using Western blotting. As shown in Figure

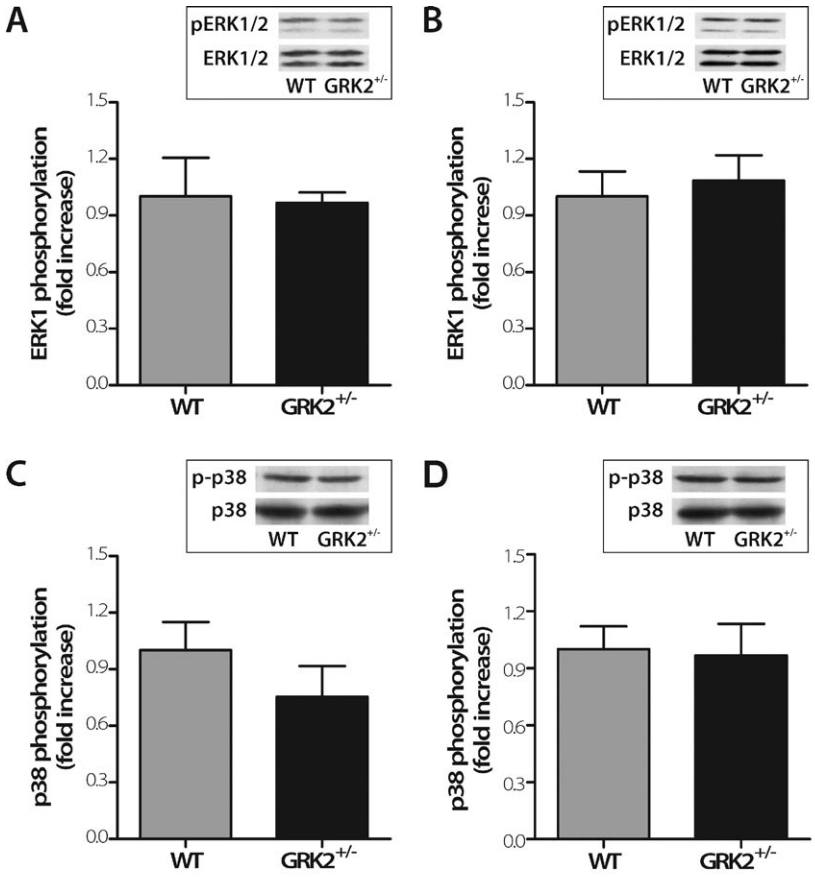

Figure 7: ERK1/2 and p38 MAPK phosphorylation in the lumbar spinal cord during inflammatory hyperalgesia. Lumbar spinal cord tissue (L1-S1) was isolated 1 h (A, C) and 6 h (B, D) after intraplantar injection of λ-carrageenan. pERK1/2 (A-B) and p-p38 MAPK (C-D) levels were determined using Western blotting. Data are presented as mean ± SEM of 4 mice and normalized for the total amount ERK1/2 or p38 present.

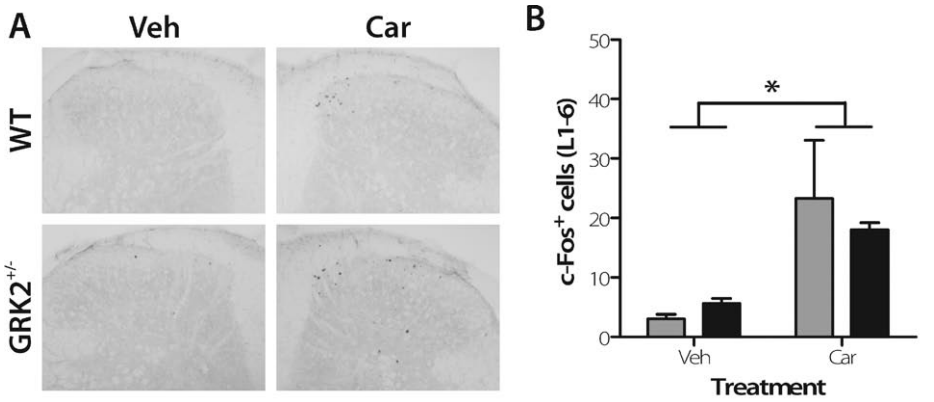

Figure 8: λ-Carrageenan-evoked c-Fos expression in the superficial layers of the lumbar spinal cord in WT and GRK2$^{+/-}$ mice. Immunohistochemical staining of lumbar spinal cord (L1-L6) with rabbit anti-c-Fos followed by biotinylated donkey anti-rabbit IgG. Slices were stained using the ABC nickel-enhanced diaminobenzidine method. (A) Representative photomicrographs of vehicle- and λ-carrageenan-induced c-Fos expression in the lumbar spinal cord dorsal horn. (B) Quantified data for the number of c-Fos positive cells in the superficial layers of the lumbar spinal cord dorsal horn 6 h following intraplantar injection of vehicle or λ-carrageenan. The mean number of c-Fos positive cells was determined of at least 3 slices per spinal segment. Results are presented as the sum of c-Fos positive cells in segments L1-L6 and are expressed as mean ± SEM of 3 animals (gray bars, WT mice; black bars, GRK2$^{+/-}$ mice; $*P < 0.05$).

6E-F, the level of pERK1/2 was similar in the paws of WT and GRK2$^{+/-}$ mice at 1 and 6 h after injection of λ-carrageenan.

Since no differences in peripheral mechanisms were observed between both genotypes we also investigated the possible contribution of a number of central mechanisms involved in sensitization. For that purpose, p-p38 MAPK and pERK1/2 levels were determined in the lumbar spinal cord (L1-S1) from WT and GRK2$^{+/-}$ mice during inflammatory hyperalgesia. As demonstrated in Figure 7A-D, there was no difference in the level of pERK1/2 and p-p38 MAPK in the lumbar spinal cord of WT and GRK2$^{+/-}$ mice 1 and 6 h after λ-carrageenan injection. Furthermore, the data in Figure 8A show that λ-carrageenan injection into the hind paw induced an increase in c-Fos expression at 6 h in the superficial laminae of the spinal cord dorsal horn compared to vehicle treatment. However, when c-Fos-positive cells were counted in this area, no difference in the number of c-Fos-positive cells was observed between WT and GRK2$^{+/-}$ mice (Figure 8B). Finally, no glial activation, as measured by CD68 and glial fibrillary acidic protein (GFAP) staining of the lumbar spinal cord, was observed 6 h after λ-carrageenan injection in WT as well as GRK2$^{+/-}$ mice (data not shown).

DISCUSSION

The data presented in this study demonstrate that low endogenous GRK2 levels resulted in an increase in λ-carrageenan- and CCL3-induced thermal hyperalgesia. However, naive GRK2$^{+/-}$ and WT mice responded in a similar way to thermal stimulation of the hind paw. These results

indicate that GRK2 is involved in regulating thermal sensitivity during inflammation but not under baseline conditions. The more pronounced inflammation-induced thermal hyperalgesia in GRK2$^{+/-}$ animals was not dependent on increased λ-carrageenan-induced inflammatory activity in these animals. In addition, no difference in central sensitization was observed between both genotypes during λ-carrageenan-induced inflammation. Finally, during inflammatory hyperalgesia we could not detect differences between WT and GRK2$^{+/-}$ mice in a number of factors known to be involved in peripheral sensitization.

Our data demonstrate that reduced GRK2 levels are not sufficient for a change in the normal sensitivity to heat, since thermal stimulation provoked similar PWLs in WT and GRK2$^{+/-}$ mice under basal conditions. However, inflammation-induced thermal hyperalgesia was significantly elevated in GRK2$^{+/-}$ mice. These data are in line with our recently published data which showed that naive WT and GRK2$^{+/-}$ mice did not differ in their sensitivity for *mechanical* stimuli, whereas GRK2$^{+/-}$ mice displayed increased λ-carrageenan-evoked *mechanical allodynia* compared to WT mice[22]. Thus, naive GRK2$^{+/-}$ mice display normal pain sensitivity, but during λ-carrageenan-induced inflammation the upregulation of sensitivity for mechanical as well as thermal stimuli is increased in GRK2$^{+/-}$ mice compared to WT mice.

The difference in inflammatory thermal hyperalgesia between the two genotypes is probably not related to a difference in the inflammatory response, since intraplantar injection of λ-carrageenan into the hind paw provoked a comparable inflammatory response in GRK2$^{+/-}$ mice and their WT littermates as measured by paw edema formation, the amount of cytokines present in the paw, and the influx of neutrophils. These results seem to be counterintuitive, since we previously showed an advanced onset of experimental autoimmune encephalomyelitis in GRK2$^{+/-}$ mice, which was characterized by an increased infiltration of T-lymphocytes in the spinal cord[25]. It should be noted that λ-carrageenan-induced inflammation is mainly characterized by infiltration of neutrophils in the inflamed area. Interestingly, T-lymphocytes isolated from GRK2$^{+/-}$ mice show an increased migration towards several chemokines (CCL3, CCL4, and CCL5)[29]. However, as is shown here, neutrophils derived from GRK2$^{+/-}$ animals did not show increased migration towards a number of chemokines. These observations suggest that migration of T-lymphocytes is regulated by GRK2, but that influx of neutrophils is not dependent on the level of cellular GRK2.

The question remains why there is an increase in thermal sensitivity during λ-carrageenan-induced inflammation in GRK2$^{+/-}$ mice, but not under baseline conditions. Several studies have reported the production of GPCR agonists, which are hardly present in naive animals, in the paw during λ-carrageenan-induced inflammation e.g. PGE2 and several chemokines (CCL2, CCL3, CCL5, and KC)[14,15,31,32]. Moreover, injection of CCL2, CCL3, KC, PGE2, and CCL5 into the hind paw has been shown to induce hyperalgesia and/or mechanical allodynia[7,10-13]. In addition, intracellular signaling induced by a number of these GPCR ligands is regulated by GRK2[20,29,33]. Increased GPCR signaling in GRK2$^{+/-}$ mice might therefore contribute to the increased inflammatory thermal hyperalgesia in these mice. In line with this hypothesis, we

showed an increase in thermal hyperalgesia in GRK2$^{+/-}$ mice compared to WT mice after intraplantar injection of the GPCR agonist CCL3. These results demonstrate that GPCR signaling is indeed able to induce a more pronounced increase in thermal sensitivity in GRK2$^{+/-}$ mice. However, the exact mechanism contributing to the increase in CCL3- and λ-carrageenan-induced inflammatory thermal hyperalgesia in GRK2$^{+/-}$ mice still remains to be determined.

An important mechanism to enhance the sensitivity for thermal stimuli is TRPV1 sensitization, which occurs via multiple mechanisms including phosphorylation at Ser800 by PKCε and the reduction of phosphatidylinositol 4,5-biphosphate (PIP2)-mediated inhibition of TRPV1[30,34]. A study by Zhang *et al.* (2005) showed that CCL3 treatment of small-diameter neurons increased the release of intracellular calcium evoked by capsaicin (a TRPV1 agonist) via a PKC-dependent mechanism[13]. TRPV1 sensitization can also be initiated by multiple other mediators, including GPCR agonists such as PGE2 and bradykinin[35,36]. Here, we reported that TRPV1 levels in the paw were similar in WT and GRK2$^{+/-}$ mice during λ-carrageenan-induced inflammation. Furthermore, phosphorylation of TRPV1 at Ser800 was equal in both genotypes. However, we can not exclude the possibility that TRPV1 sensitization is increased in GRK2$^{+/-}$ mice, since sensitization might still occur through the reduction of PIP2-mediated inhibition, PKCε-mediated phosphorylation of Ser502 or phosphorylation on other residues by other PKC isoforms. Besides TRPV1, a role for TRPV4 during λ-carrageenan-induced hyperalgesia has been demonstrated[37]. In addition, increased expression of the sodium channels $Na_v1.3$, $Na_v1.7$, and $Na_v1.8$ has been reported to contribute to inflammatory hyperalgesia[8]. Moreover, an association between GRK2 and epithelial sodium channels has been demonstrated[38]. Based on these findings, it might be worthwhile to investigate sensitivity and expression of these sodium channels and TRPV4 to clarify the mechanism involved in the increase in inflammatory hyperalgesia in GRK2$^{+/-}$ mice.

To test the hypothesis that peripheral signaling is increased in GRK2$^{+/-}$ mice during inflammatory thermal hyperalgesia, pERK1/2 levels in the paws of GRK2$^{-/-}$ and WT mice were compared. However, no difference in pERK1/2 levels was detected between the paws of WT and GRK2$^{+/--}$ mice 1 and 6 h after λ-carrageenan injection. One might therefore conclude that signaling to ERK1/2 is not increased in GRK2$^{+/-}$ mice. However, numerous cell types are present in the paw and it may still be possible that sensory neurons showed an increased signaling to ERK1/2, but that this response was underestimated due to the contribution of various other cell types. Therefore, the increase in sensory neuronal pERK1/2 could be 'diluted' in total paw lysates and for this reason impossible to demonstrate by Western blotting in a paw homogenate. Using immunofluorescence, Zhuang *et al.* reported pERK1/2 activation in sensory nerve endings in the skin during capsaicin-induced hyperalgesia[39]. Thus, immunofluorescent staining for pERK1/2 might provide a better insight than the use of Western blotting. In addition to pERK1/2, it might be worthwhile to investigate the activation of other protein kinases. Good candidate molecules are p38 MAPK, whose activation has been demonstrated to be under control of GRK2[40], and PKC isoforms, which regulate sensitivity of TRPV1.

In the spinal cord, activation of MAPK, c-Fos expression, and glial activation have been reported to occur in response to λ-carrageenan-induced paw inflammation[3-5]. Inhibition of microglia activation prevented the development of λ-carrageenan-induced thermal hyperalgesia[4]. In our model, c-Fos expression in the lumbar segments was upregulated to the same extent in WT and GRK2$^{+/-}$ mice in response to paw inflammation. Likewise, equal amounts of p-p38 MAPK and pERK1/2 were found in the lumbar spinal cord. These results suggest that differences in the activation of these pathways at the spinal cord level does not play a role in increasing the thermal sensitivity in GRK2$^{+/-}$ mice. Although prior administration of the microglia inhibitor minocycline has been demonstrated to prevent λ-carrageenan-induced hyperalgesia[4], we did not detect glia activation in the spinal cord in both WT and GRK2$^{+/-}$ mice. A possible explanation for this observation might be the use of different species, since Hua *et al.* (2005) used rats, whereas mice were used in the present study. In addition, microglia activation might occur earlier than 6 h and for this reason, we did not detect glial activation at 6 h after λ-carrageenan injection. We suggest that the regulatory effect of low GRK2 might not reside at the central level, but will have its effect peripherally. This conclusion is also supported by the fact that we observe already an increase in CCL3-induced thermal hyperalgesia after 30 min, which makes it rather unlikely that central sensitization mechanisms will signify the difference in thermal hyperalgesia between GRK2$^{+/-}$ and WT mice.

In conclusion, the data presented in this study demonstrated that GRK2$^{+/-}$ mice developed a more pronounced inflammatory thermal hyperalgesia compared to WT mice. In contrast, baseline thermal sensitivity was not different between both genotypes. Additional research is required to elucidate the underlying mechanism for increased inflammatory thermal hyperalgesia in GRK2$^{+/-}$ mice. We suggest, however, that increased GPCR signaling in GRK2$^{+/-}$ mice induced by the production of GPCR agonists (e.g. CCL3) plays an important role.

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5

CHAPTER

Physiological changes in GRK2 regulate CCL2-induced signaling to ERK1/2 and Akt but not to MEK1/2 and calcium

Wendy Kleibeuker[1]
Maria Jurado-Pueyo[2]
Cristina Murga[2]
Niels Eijkelkamp[1]
Federico Mayor Jr.[2]
Cobi J. Heijnen[1]
Annemieke Kavelaars[1]

[1] University Medical Center Utrecht, Laboratory of Psychoneuroimmunology, 3584 EA, Utrecht, the Netherlands
[2] Universidad Autónoma de Madrid, Departamento de Biología Molecular and Centro de Biología "Severo Ochoa", 28049 Madrid, Spain

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ABSTRACT

G protein-coupled receptor (GPCR) kinase (GRK)2 regulates GPCR signaling via agonist-induced receptor phosphorylation and desensitization. GRK2 can also modulate cellular activation by interacting with downstream signaling molecules. The intracellular GRK2 level changes during inflammatory conditions. We investigated how interleukin (IL)-1β-induced changes in endogenous GRK2 expression influence chemokine receptor signaling in primary astrocytes. Culturing astrocytes with IL-1β for 24 h induced a 2-3-fold increase in GRK2 and decreased CCL2-induced ERK1/2 activation. Conversely, the 45% decrease in GRK2 expression in astrocytes from $GRK2^{+/-}$ animals resulted in a more pronounced CCL2-induced ERK1/2 phosphorylation. Increased GRK2 inhibited CCL2-induced Akt phosphorylation at Thr308 and Ser473 as well as pPDK-1 translocation. In contrast, altered GRK2 levels did not change the CCL2-induced increase in intracellular calcium or MEK1/2 phosphorylation. These data suggest that altered GRK2 expression modulates chemokine signaling downstream of the receptor. We found that GRK2 kinase activity was not required to decrease chemokine-induced ERK1/2 phosphorylation, whereas regulation of CCL2-induced Akt phosphorylation did require an active GRK2 kinase domain. Collectively, these data suggest that changes in endogenous GRK2 expression in primary astrocytes regulate chemokine receptor signaling to ERK1/2 and to PDK-1-Akt downstream of receptor coupling via kinase-dependent and kinase-independent mechanisms, respectively.

INTRODUCTION

Chemokines play a very important role in the pathophysiology of various neuro-inflammatory processes such as multiple sclerosis, Alzheimer disease, ischemic cerebral damage and the development of inflammatory pain[1,2]. Astrocytes express several functional chemokine receptors including C-C chemokine receptor (CCR)2[1]. Stimulation of astrocytes with the CCR2 ligand C-C chemokine ligand (CCL)2 leads to CCL2-directed migration, a rise in intracellular calcium ($[Ca^{2+}]_i$), and CCR2 internalization via clathrin- and caveolin-coated vesicles[3,4]. Chemokine receptors belong to the family of G protein-coupled receptors (GPCRs). Upon GPCR binding, chemokines induce the activation of heterotrimeric G-proteins and several intracellular signaling pathways (e.g. calcium signaling, mitogen-activated protein kinase (MAPK) cascades, and the phosphoinositide-3 kinase (PI3 kinase)-Akt pathway).

GPCR kinase (GRK)2 is a serine-threonine kinase that regulates the intracellular signaling of various GPCRs[5,6]. GRK2 phosphorylates serine or threonine residues of agonist-occupied GPCRs, thus downregulating receptor signaling by initiating the binding of the co-factors β-arrestin1/2, which induces Gα uncoupling and facilitates receptor internalization[7]. Changes in the intracellular level of GRK2 lead to changes in agonist-induced, phosphorylation-dependent receptor desensitization with consequences for signaling through specific GPCRs. For example, GRK2 is capable of phosphorylating agonist-stimulated CCR5 and β_2-adrenergic receptors (ARs)[8,9]. Low levels of GRK2 are associated with decreased agonist-induced phosphorylation of CCR5 and increased signaling to calcium and Akt after triggering of this receptor[10]. In addition, decreased expression of GRK2 increases β_2-AR signaling, whereas increased expression of GRK2 results in decreased receptor signaling through the β_2-AR and CCR2[11-13].

Next to its classical role in phosphorylation-dependent receptor desensitization, GRK2 can phosphorylate non-receptor substrates (e.g. tubulin, synuclein) and can bind to several proteins involved in signaling and receptor trafficking such as PI3 kinase, $G\alpha_q$, Gβγ, Akt, caveolin, MEK1/2, clathrin, p38 MAPK, and RKIP[6,14-17]. Recruitment of PI3 kinase to the plasma membrane via binding to GRK2 leads to an increase in receptor endocytosis in cells overexpressing GRK2[18]. In addition, evidence has been presented that an interaction between GRK2 and Akt inhibits the kinase activity of Akt[15]. Recently, we demonstrated that a GRK2-MEK1/2 interaction reduced CCL2-induced ERK1/2 activation in HEK293 cells overexpressing GRK2. Moreover, we showed that the GRK2-mediated inhibition of CCL2-induced ERK1/2 activation did not require GRK2 kinase activity[16]. Thus, several studies have uncovered a novel role for GRK2 in the regulation of GPCR signaling, which does not necessarily require kinase activity of GRK2 and takes place downstream of the receptor. Since a lot of these studies have been performed in transfected immortalized cells, we wanted to investigate the consequences of changes in GRK2 in primary cells, using cortical astrocytes.

Changes in the intracellular level of GRK2 have been described during various inflammatory diseases such as rheumatoid arthritis and multiple sclerosis, but also after pro-inflamma-

tory cytokine treatment[19,20]. Interleukin (IL)-1β treatment of rat lungs increases GRK2 expression[21]. *In vitro*, we recently demonstrated that chronic treatment of cultured spinal cord slices with IL-1β decreases GRK2 levels[22]. In addition, several other pro-inflammatory cytokines (e.g. interferon (IFN)-γ, IL-6, tumor necrosis factor (TNF)-α) downregulate GRK2 in different cell types[19,23]. These results suggest that pro-inflammatory mediators modulate endogenous GRK2 expression.

During neuro-inflammatory processes, several pro-inflammatory cytokines like IL-1β are produced in the central nervous system. In this study, we investigated whether IL-1β modulates the endogenous GRK2 level in primary cortical astrocytes. Subsequently, we studied the consequences of the IL-1β-induced physiological changes in endogenous GRK2 for signaling in response to the GPCR agonist CCL2 in astrocytes. In addition, the effect of reducing GRK2 expression on CCL2-induced signaling was determined using astrocytes from GRK2$^{+/-}$ mice. In particular, we investigated whether changes in endogenous GRK2 level have consequences for chemokine signaling to the PI3 kinase-PDK-1-Akt pathway, the agonist-induced increase in $[Ca^{2+}]_i$, and activation of the MEK1/2-ERK1/2 pathway.

MATERIALS AND METHODS

Animals

C57BL/6 mice (Harlan Laboratories, Horst, the Netherlands) were used to study the effects of IL-1β on CCL2-induced signaling, whereas C57BL/6 mice heterozygous for the targeted deletion of the GRK2 gene (GRK2$^{+/-}$) and their wild type (WT) littermates were used to study the effects of decreased GRK2 on CCL2-induced signaling[24]. The offspring was genotyped by PCR analysis on genomic DNA. Experiments were performed in accordance with international guidelines and approved by the University Medical Center Utrecht experimental animal committee.

Primary cultures of cortical astrocytes

Primary cultures of cortical astrocytes were obtained from one-day old mice as described previously[25]. In brief, meninges and visible blood vessels were removed from the brain and cortices were dissected. Cortices were minced and incubated with 0.25% trypsin for 15 min in Gey's balanced salt solution containing 100 U/ml Penicillin, 100 µg/ml streptomycin (Invitrogen, Breda, The Netherlands), and 30 mM D(+)-glucose (Brunschwig Chemie, Amsterdam, the Netherlands). Cells were washed, dissociated by pipetting, and cultured in poly-L-ornithine- (15 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) coated culture flasks in culture medium: Dulbecco's modified Eagle's medium (DMEM)/Ham's F10 (1:1) supplemented with 10% FCS, 2 mM Glutamine, 100 U/ml Penicillin, and 100 µg/ml streptomycin (Invitrogen). After 10 days, flasks were placed overnight on a rotary shaker at 37°C to remove adherent and loosely attached

microglia. This procedure was repeated twice in the following week. The remaining astrocytes were trypsinized, and replated in poly-L-ornithine-coated 24-well plates at a density of 500.000 cells per well and grown for 48 h before use. The obtained cultures of astrocytes were > 94% pure as determined by staining for glial fibrillary acidic protein (GFAP).

Treatment of primary cortical astrocyte cultures

At the beginning of the experiment, culture medium was replaced with medium containing recombinant murine IL-1β (Peprotech, London, United Kingdom) at the indicated concentrations and cells were incubated during 24 h. To determine changes in GRK2 expression, cell lysates were prepared or RNA was extracted. For intracellular signaling experiments, cells were serum-deprived for 4 h at 37°C and subsequently stimulated with 10 nM recombinant murine CCL2 (R&D systems, Minneapolis, MN, USA) or 100 ng/ml epidermal growth factor (EGF; Sigma-Aldrich) during the indicated time periods after which cell lysates were prepared.

U87 cell culture and transfection

U87 human astrocytoma cells (ATCC number HTB-14) were seeded one day before transfection in 24-well plates at a density of 200.000 cells per well. Transfection was performed using the Lipofectamine Plus method (Invitrogen) following the manufacturer's guidelines[13]. After 24 h, cells were serum-deprived for 4 h, stimulated with 10 nM recombinant human CCL2 (R&D systems) for 0, 3 or 10 min, and subsequently cell lysates were made.

Western blotting

Total cell lysates were prepared by incubation in ice-cold RIPA buffer (20 mM Hepes pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 2 mM 4-(2-Aminoethyl) benzenesulphonyl fluoride (AEBSF), 20 μg/ml leupeptin, 200 μg/ml benzamidine, 10 mM β-glycerolphosphate, 1 mM $NaVO_3$, 20 mM NaF) for 30 min at 4°C followed by 15 min centrifugation at 13.000 x g.

CCR2 expression and agonist-induced pPDK-1 translocation to the membrane was quantified by Western blot analysis of pPDK-1 and CCR2 in membrane fractions. For the preparation of membrane fractions, cells were lysed in ice-cold cell lysis buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 2 mM AEBSF, 20 μg/ml leupeptin, 200 μg/ml benzamidine, 10 mM β-glycerolphosphate, 1 mM $NaVO_3$, 20 mM NaF) using sonication. Unbroken cells and nuclei were pelleted by centrifugation (800 x g for 5 min) and discarded. The supernatant was then centrifuged (48.000 x g for 20 min at 4°C) and the membrane pellet was resuspended in ice-cold RIPA buffer and sonicated.

20-30 μg of protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C; Amersham Int., Buckinghamshire, United Kingdom) by electroblotting. Blots were stained with the following antibodies: rabbit anti-GRK2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; sc-562), mouse anti-β-arrestin1 (Transduction Laboratories, Lexington, KY, USA), goat anti-actin (Santa Cruz Biotechnology Inc.; sc-1616), mouse anti-

phospho-ERK1/2 (Santa Cruz Biotechnology Inc.; sc-7383), rabbit anti-ERK1/2 (Cell Signaling Technology, Beverly, MA, USA; #9102), rabbit anti-phospho-MEK1/2 (Ser217/221; Cell Signaling Technology; #9121), rabbit anti-MEK1/2 (Cell Signaling Technology; #9122), goat anti-CCR2 (Santa Cruz Biotechnology Inc.; sc-6228), rabbit anti-phospho-Akt (Ser473; Cell Signaling Technology; #9271), rabbit anti-phospho-Akt (Thr308; Cell Signaling Technology; #9275), rabbit anti-Akt1/PKBα (Sigma-Aldrich; P-1601), or anti-phospho-PDK-1 (Ser241; Cell Signaling Technology; #3061). Blots were then incubated for 1 h with goat anti-mouse-peroxidase IgG + IgM (H+L) (Jackson Laboratories, Bar Harbor, ME, USA), donkey anti-rabbit-peroxidase IgG (Amersham Int.), or donkey anti-goat-peroxidase IgG (Santa Cruz Biotechnology Inc.; sc-2020) and developed by enhanced chemiluminescence (Amersham Int.). Band density was determined using a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

Real-time RT-PCR

Total RNA was extracted using Trizol (Invitrogen). cDNA was synthesized from 0.5 µg total RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real-time RT-PCR was performed with use of DNA Master SYBR-green kits (Roche, Basel, Switzerland) and a LightCycler apparatus (Roche) using the forward primer 5'-AAGACGAGGCTCCGCAGA-3' and reverse primer 5'-GCACCAGCTCAGGGTCACTAT-3' for GRK2 mRNA. Melting curve analysis and agarose gel electrophoresis confirmed amplification specificity. 18S rRNA was amplified using the forward primer 5'-GTAACCCGTTGAACCCCATT-3' and the reverse primer 5'-CCATCCAATCGGTAGTAGCG-3' and used for data normalization. All oligonucleotide primers were from TibMolbiol (Berlin, Germany).

Immunofluorescence

To determine CCR2 expression, cells were fixed in 100% methanol and incubated overnight with goat anti-CCR2 at 4°C and subsequently with Fluorescein (FITC) rabbit anti-goat IgG (H+L)-FITC (Jackson Laboratories) during 1 h at room temperature.

Analysis of $[Ca^{2+}]_i$ flux analysis by microscope-based ratiometric imaging

For calcium measurements, astrocytes were plated on poly-L-ornithine-coated glass coverslips 2 days before the experiment. After 24 h, medium was replaced by medium containing 1 ng/ml IL-1β and cells were incubated for an additional 24 h. Cells were loaded for 30 min at 37°C with 10 µM FURA-2-acetomethoxy ester (Molecular Probes, Paisley, United Kingdom) in 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.4. Subsequently, the coverslips were put into DMEM without phenol red (Invitrogen) for 30 min and afterwards mounted in a perfusion chamber on a Zeiss UltraVIEW Live Cell Imager (PerkinElmer, Milan, Italy). Fluorimetric measurements were done using a CCD camera supported by MetaFluor imaging software. Digital images of the cells were obtained at an emission wavelength of 510 nm using paired exposures to 340 and 380 nm excitation wavelength sampled every 4 sec.

Fluorescence values representing spatial averages from a defined pixel area were recorded online. Changes in $[Ca^{2+}]_i$ concentrations upon stimulation with 10 nM recombinant murine CCL2 are expressed as the ratio of the 510 nm emission at 340/380 nm excitation.

Statistical analysis

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

RESULTS

IL-1β increases GRK2 protein expression in astrocytes

To analyze potential changes in endogenous GRK2 expression, primary cultures of murine astrocytes were incubated with the pro-inflammatory cytokine IL-1β for 24 h. Stimulation with 0.01-10 ng/ml IL-1β dose-dependently increased GRK2 expression (Figure 1A). No changes in GRK2 expression were detected when astrocytes were treated with 1 ng/ml IL-1β for shorter periods of time (4-8 h; data not shown). IL-1β treatment did not induce changes in the expression of related proteins, such as β-arrestin1 (Figure 1A). Treatment with heat-inactivated IL-1β (1 ng/ml) did not lead to a significant change in GRK2 expression compared to untreated cells. Moreover, addition of the IL-1 receptor antagonist (IL-1RA; 100 ng/ml) completely prevented the effect of IL-1β and no significance difference compared to untreated astrocytes

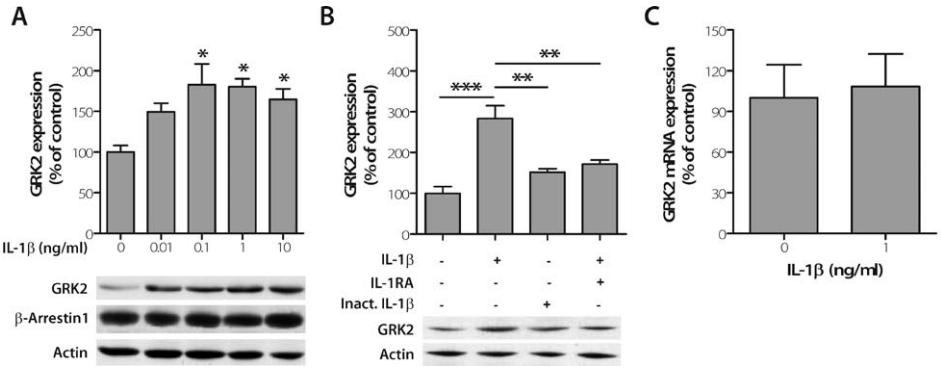

Figure 1: Effect of IL-1β treatment on GRK2 expression in primary cultures of murine astrocytes. A) Astrocytes were treated with 0-10 ng/ml IL-1β during 24 h and GRK2 and β-arrestin1 protein expression in total cell lysates was determined by Western blotting. B) Effect of treatment with 1 ng/ml IL-1β in combination with 100 ng/ml IL-1RA or treatment with 1 ng/ml IL-1β, that was heat-inactivated at 90°C for 20 min, on GRK2 protein levels. C) GRK2 mRNA expression after treatment with 1 ng/ml IL-1β during 24 h was determined by real-time RT-PCR. Data are represented as the mean ± SEM of 4 independent experiments and analyzed using 1-way ANOVA followed by Bonferroni analysis (A, B) or Student's t-test (C). GRK2 expression was normalized for the amount of actin (protein) or 18S rRNA (mRNA) present. Insets show a representative experiment (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

was found (Figure 1B). These data suggest that the effect of IL-1β is specific and involves the IL-1 receptor, which is known to be expressed on astrocytes[26]. GRK2 mRNA expression was not altered 24 h after treatment with IL-1β (1 ng/ml; Figure 1C) suggesting that the effect of IL-1β on GRK2 protein expression is not caused by increased transcription of the GRK2 gene.

Altered GRK2 levels are associated with modulation of CCL2-induced ERK1/2 but not MEK1/2 activation

To analyze the effect of the change in GRK2 expression on chemokine signaling in primary cells, we stimulated IL-1β-treated astrocytes and untreated astrocytes with the chemokine CCL2. Basal phosphorylation levels of ERK1/2 were not significantly different between untreated and IL-1β-treated astrocytes. Stimulation with CCL2 leads to phosphorylation of ERK1/2, peaking around 3 min. CCL2-induced phosphorylation of ERK1/2 was significantly lower in IL-1β-treated astrocytes compared to untreated astrocytes (Figure 2A, C). However, we did not observe any effect of IL-1β on CCL2 signaling to MEK1/2 at any of the time points analyzed (Figure 2B-C). ERK1/2 phosphorylation upon stimulation with EGF (100 ng/ml) was similar in untreated and IL-1β-treated astrocytes (data not shown), thus indicating that the effect of increased GRK2 on ERK1/2 activation is not a general phenomenon. No differences in CCR2 expression, as assessed by immunofluorescence and Western blotting of membrane fractions were found between untreated and IL-1β-treated astrocytes (data not shown).

To investigate whether a change in the intracellular level of GRK2 is sufficient to alter CCL2-induced ERK1/2 phosphorylation, we used astrocytes from GRK2$^{+/-}$ mice. As shown in Figure 3A, astrocytes from GRK2$^{+/-}$ mice have a 45% lower GRK2 protein level compared to WT astrocytes. Phosphorylation of ERK1/2 in response to CCL2 stimulation was significantly higher in GRK2$^{+/-}$ astrocytes (Figure 3B, D). Again, no differences in CCL2-induced MEK1/2 phosphorylation were observed between WT and GRK2$^{+/-}$ astrocytes at any of the time points analyzed (Figure 3C-D). ERK1/2 phosphorylation upon EGF (100 ng/ml) stimulation did not

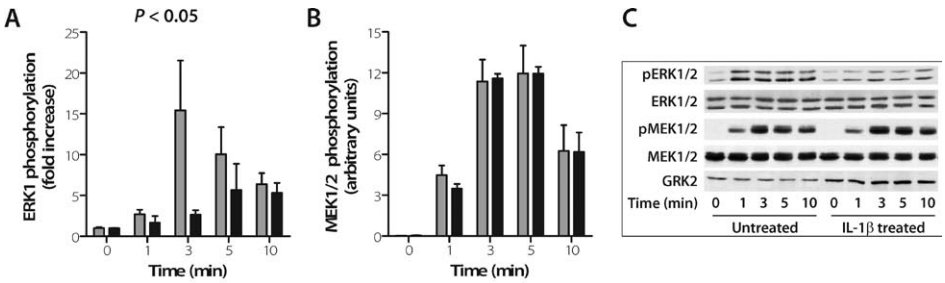

Figure 2: CCL2-induced ERK1/2 and MEK1/2 phosphorylation in untreated and IL-1β-treated astrocytes. Astrocytes were treated with IL-1β for 24 h and subsequently stimulated with 10 nM CCL2 for 0-10 min. pERK1/2 (A) and pMEK1/2 (B) were determined in total cell lysates using Western blotting. Data are represented as mean ± SEM of 4 independent samples (gray bars, untreated; black bars, IL-1β-treated) and analyzed using 2-way ANOVA with the factors treatment and time followed by Bonferroni analysis. pERK1/2 and pMEK1/2 levels were normalized for the amount of total ERK1/2 or MEK1/2 present. A representative Western blot is shown in C.

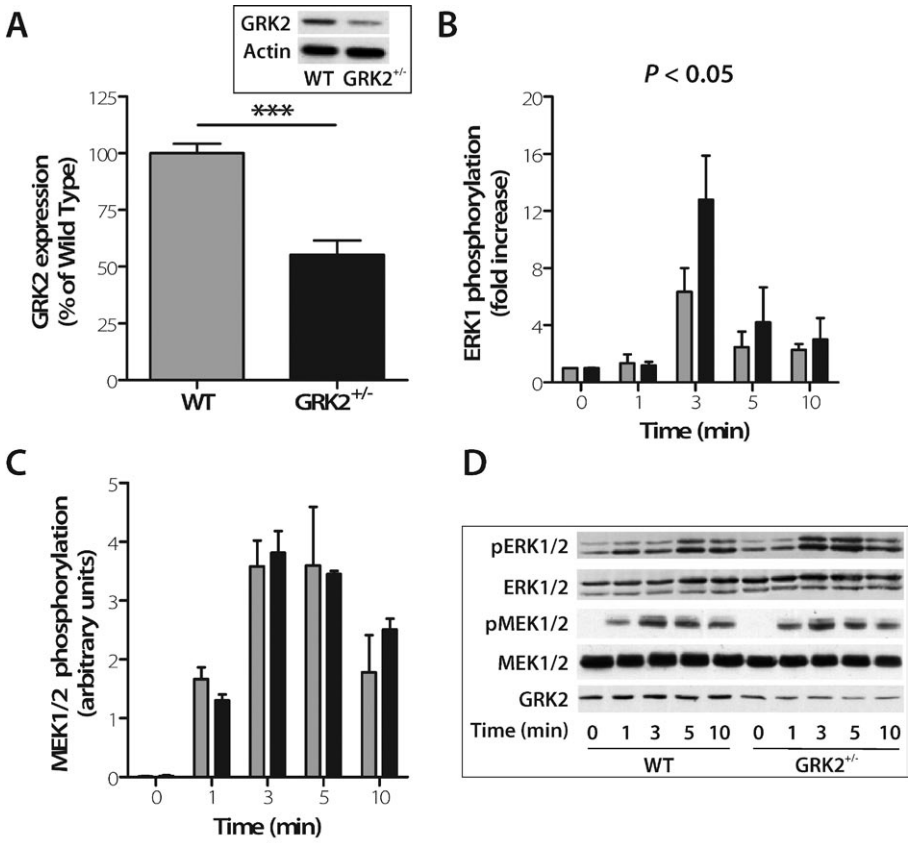

Figure 3: CCL2-induced ERK1/2 and MEK1/2 phosphorylation in WT and GRK2$^{+/-}$ astrocytes. GRK2 protein expression in total cell lysates of astrocytes from WT and GRK2$^{+/-}$ mice was compared using Western blotting (A). GRK2 levels were normalized for the amount of actin present. WT and GRK2$^{+/-}$ astrocytes were stimulated with 10 nM CCL2 for 0-10 min and subsequently pERK1/2 (B) and pMEK1/2 (C) levels were detected using Western blotting. A representative Western blot is shown in D. Data are represented as mean ± SEM of 3 independent samples (gray bars, WT; black bars, GRK2$^{+/-}$) and analyzed using Student's t-test (A) or 2-way ANOVA with the factors genotype and time followed by Bonferroni analysis (B, C). pERK1/2 and pMEK1/2 levels were normalized for the amount of total ERK1/2 or MEK1/2 present (***$P < 0.001$).

differ between GRK2$^{+/-}$ and WT cells (data not shown). No differences in CCR2 expression were found between WT and GRK2$^{+/-}$ astrocytes (data not shown).

$[Ca^{2+}]_i$ mobilization in astrocytes with altered GRK2 expression

Since CCL2-induced ERK1/2, but not MEK1/2 activation was altered in cells with changes in GRK2 expression, the question arose whether CCL2-induced signaling upstream of MEK1/2 was influenced by changes in GRK2. Therefore, astrocytes were stimulated with CCL2 (10 nM) and changes in $[Ca^{2+}]_i$ were determined. CCL2 induced a rise in $[Ca^{2+}]_i$ with a peak 15 sec after application of CCL2 which was followed by a gradual decrease to baseline levels (Figure 4A, C). The effect of CCL2 on $[Ca^{2+}]_i$ was not different between WT and GRK2$^{+/-}$ astrocytes (Figure

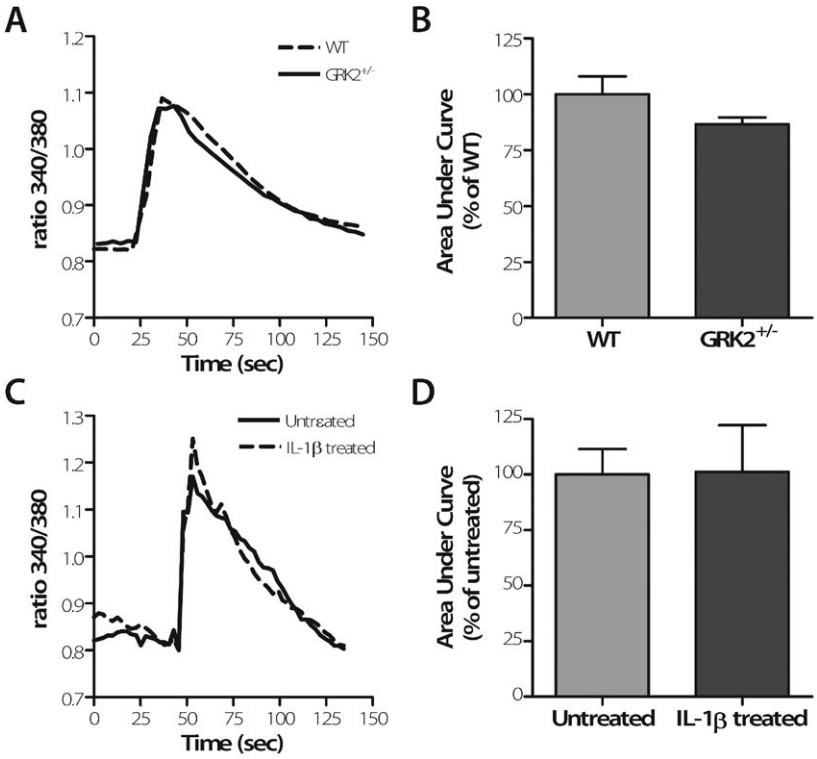

Figure 4: CCL2-induced increase in $[Ca^{2+}]_i$ in astrocytes. Astrocytes were loaded by incubation with 10 μM FURA-2-acetomethoxy ester, and the change in $[Ca^{2+}]_i$ induced by 10 nM CCL2 was monitored by fluorescence ratiometric imaging as described in "Materials and Methods". Representative examples of the CCL2-induced increase in $[Ca^{2+}]_i$ in WT and GRK2$^{+/-}$ astrocytes (A) and untreated and IL-1β-treated astrocytes (C) . Cumulative effects of CCL2 on $[Ca^{2+}]_i$ were calculated as the Area Under Curve and compared between WT and GRK2$^{+/-}$ astrocytes (B) and untreated and IL-1β-treated astrocytes (D). Data are represented as mean ± SEM (n=7/8) and analyzed using Student's t-test.

4A-B). In addition, the CCL2-induced increase in $[Ca^{2+}]_i$ was similar in untreated and IL-1β-treated cells (Figure 4C-D).

Regulation of Akt activation in astrocytes with altered GRK2 levels

CCL2 stimulation of CCR2 leads to the activation of several parallel pathways including the MEK1/2-ERK1/2 pathway and the PI3 kinase-Akt pathway[16,27,28]. To determine whether changes in GRK2 could also affect signaling to the PI3 kinase pathway, we analyzed CCL2-induced Akt phosphorylation at Ser473. The basal phosphorylation level of Akt was similar in untreated and IL-1β-treated astrocytes. The CCL2-induced upregulation in the level of pAkt (Ser473) was reduced in IL-1β-treated astrocytes compared to untreated astrocytes (Figure 5A). Conversely, in astrocytes from GRK2$^{+/-}$ mice the CCL2-induced formation of pAkt (Ser473) was increased compared to WT cells (Figure 5B). In contrast, EGF-induced pAkt (Ser473) was similar in untreated and IL-1β-treated cells (Figure 5C) as well as in WT and GRK2$^{+/-}$ cells (Figure 5D).

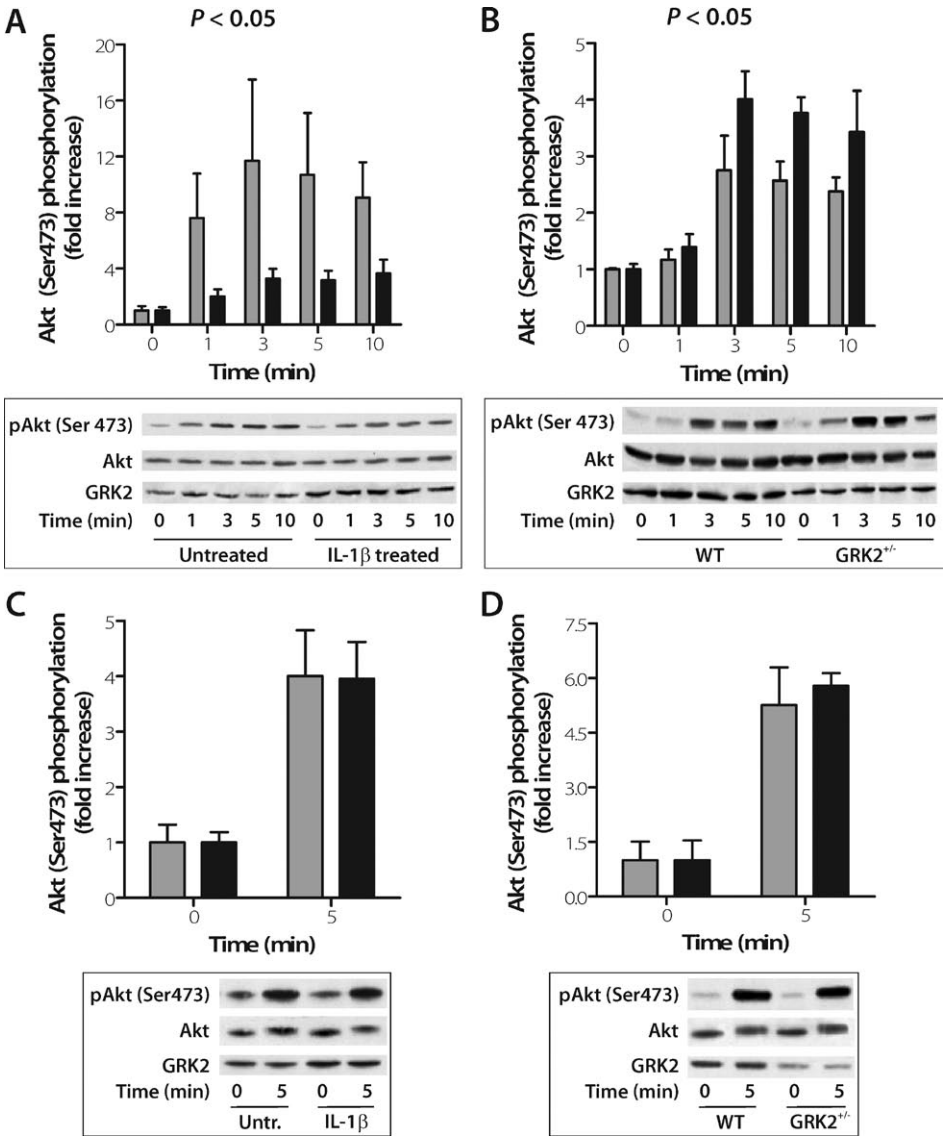

Figure 5: CCL2- and EGF-induced Akt (Ser473) phosphorylation. Astrocytes were treated with IL-1β for 24 h and subsequently stimulated with 10 nM CCL2 for 0-10 min (A) or 100 ng/ml EGF for 0-5 min (C). pAkt (Ser473) levels were determined in total cell lysates by Western blotting. In addition, CCL2- (B) and EGF- (D) induced changes in pAkt (Ser473) levels in WT and GRK2$^{+/-}$ astrocytes were determined. Data are represented as the mean ± SEM of 4 independent samples ((A, C) gray bars, untreated; black bars, IL-1β-treated; (B, D) gray bars, WT; black bars, GRK2$^{+/-}$) and analyzed using 2-way ANOVA with the factors treatment and time followed by Bonferroni analysis. pAkt (Ser473) expression was normalized for the amount of total Akt present. Insets show representative Western blots.

We previously showed that the inhibition of chemokine-induced ERK1/2 phosphorylation by overexpression of GRK2 did not require GRK2 kinase activity[16]. To address the question whether the GRK2-induced change in chemokine-induced Akt phosphorylation requires

GRK2 kinase activity, GRK2 or the kinase-deficient mutant GRK2-K220R were overexpressed in the astrocytoma cell line U87. Transfection of U87 cells with GRK2 or GRK2-K220R did not result in changes in CCR2 expression (data not shown). CCL2-induced Akt (Ser473) phosphorylation was decreased in cells overexpressing GRK2, but not in cells overexpressing GRK2-K220R. In contrast, however, this mutant was effective at reducing CCL2-mediated ERK phosphorylation (Figure 6). These data indicate that GRK2-mediated effective inhibition of chemokine-induced Akt phosphorylation requires the kinase activity of GRK2, whereas the inhibition of pERK1/2 formation does not depend on kinase activity.

We have shown that changes in the intracellular level of GRK2 lead to an alteration in CCL2-induced phosphorylation of ERK1/2 as well as of Akt, but not in MEK1/2 activation or in signaling to calcium. To investigate the possibility that this effect on the MEK1/2-ERK1/2

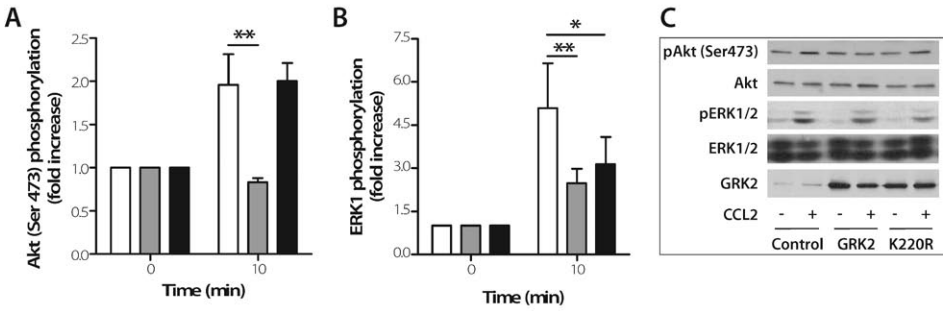

Figure 6: Effect of GRK2 or GRK2-K220R overexpression on CCL2-induced Akt (Ser473) and ERK1/2 phosphorylation in U87 cells. U87 cells were transfected with GRK2 or the GRK2 kinase-deficient mutant GRK2-K220R. pAkt (Ser473) (A) and pERK1/2 (B) levels in total cell lysates were determined 0-10 min after stimulation with 10 nM CCL2 using Western Blotting. Insets show representative Western blots (C). Data are represented as the mean ± SEM of 4 independent experiments and analyzed using repeated-measures ANOVA followed by Bonferroni analysis (white bars, control; gray bars, GRK2; black bars, GRK2-K220R). pAkt (Ser473) or pERK1/2 expression was normalized for the amount of total Akt or ERK1/2 present (*$P < 0.05$, **$P < 0.01$).

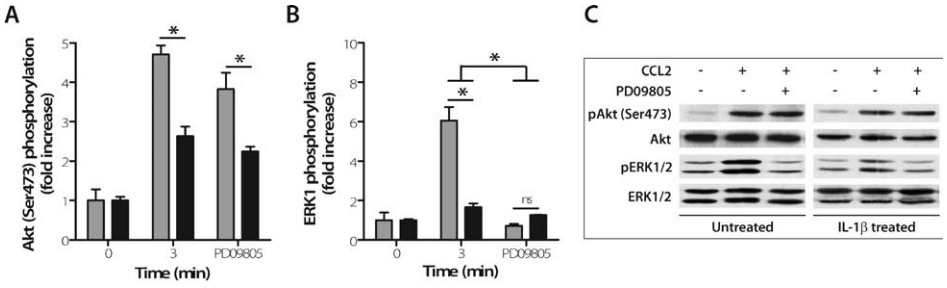

Figure 7: Effect of PD09805 on the CCL2-induced pAkt (Ser473) and pERK1/2 formation in untreated and IL-1β-treated astrocytes. Astrocytes were pretreated with 50 μM PD09805, a well established MEK1/2 inhibitor, for 1 h and subsequently stimulated with 10 nM CCL2 for 3 min. pAkt (Ser473)(A) and pERK1/2 (B) expression was determined in total cell lysates using Western Blotting in untreated and IL-1β-treated astrocytes. Insets show representative Western blots (C). Data are represented as mean ± SEM of 4 independent samples and analyzed using ANOVA followed by Bonferroni analysis (gray bars, untreated; black bars, IL-1β-treated). pAkt (Ser473) expression was normalized for the amount of total Akt present (*$P < 0.05$).

pathway is functionally related to the effect on the PI3 kinase-Akt pathway, we tested whether MEK1/2 was capable of activating the PI3 kinase-Akt signaling pathway with the aid of a specific pharmacological inhibitor of MEK1/2. As shown in Figure 7, PD09805, a well established MEK1/2 inhibitor[29], significantly reduced the CCL2-induced phosphorylation of ERK1/2 but not that of Akt. Moreover, phosphorylation of ERK1/2, but not of Akt, was inhibited by PD098059 in IL-1β-treated astrocytes, indicating that IL-1β treatment also is not associated with MEK1/2-dependent changes in Akt phosphorylation (Figure 7). These results indicate that MEK1/2 does not signal to the PI3 kinase-Akt pathway.

Upon chemokine stimulation, PI3 kinase becomes activated via the stimulation of heterotrimeric G proteins[30]. PI3-phosphate subsequently activates PDK-1, which in turn phosphorylates Akt at Thr308. Complete activation of Akt requires subsequent phosphorylation at Ser473 by an additional kinase (also referred to as PDK-2)[31]. As is shown in Figure 8A-B,

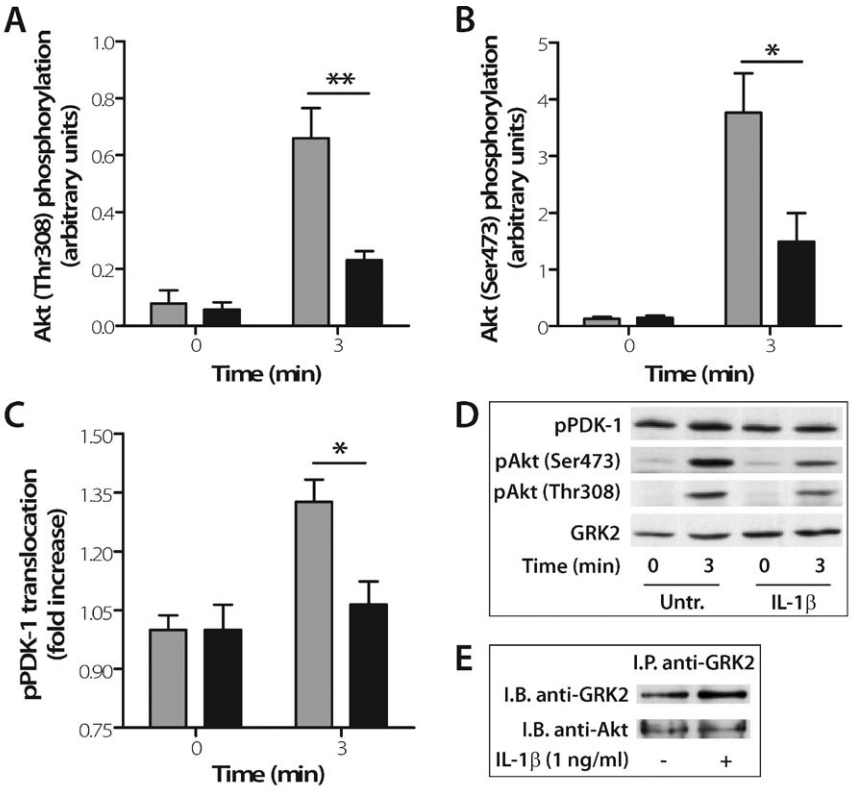

Figure 8: CCL2-induced Akt phosphorylation and pPDK-1 translocation in untreated and IL-1β-treated astrocytes. Astrocytes were treated with IL-1β for 24 h and subsequently stimulated with 10 nM CCL2 for 3 min. pAkt (Thr308) (A), pAkt (Ser473) (B), pPDK-1 (C) levels were determined in membrane fractions using Western blotting (gray bars, untreated; black bars, IL-1β-treated). A representative Western blot is shown in D. Total cell lysates (150 µg total protein) from untreated and IL-1β-treated were subjected to immunoprecipitation with anti-GRK2. Immunoprecipitates were blotted with anti-GRK2 and anti-Akt (E). Data are represented as mean ± SEM of 4 independent samples and analyzed using 2-way ANOVA followed by Bonferroni analysis (*$P < 0.05$, **$P < 0.01$).

CCL2-induced Akt phosphorylation at both Thr308 and Ser473 is diminished in cells that have increased GRK2 levels after IL-1β treatment. These results indicate that GRK2 inhibits the phosphorylation of Akt at Thr308 as well as Ser473, which may represent sequential events.

To investigate whether high levels of GRK2 prevented Akt phosphorylation by inhibiting the activation of the upstream activator PDK-1, we measured translocation of pPDK-1 from cytosol to membrane as a measure of pPDK-1 activation[32,33]. Figure 8C-D shows that pPDK-1 levels are increased in the membrane fraction upon CCL2 stimulation. Moreover, in IL-1β-treated astrocytes, that have an increased level of GRK2, CCL2-induced membrane translocation of pPDK-1 is reduced (Figure 8C-D). These data suggest that GRK2 inhibits Akt phosphorylation at the level of pPDK-1 activation or upstream of this event.

To determine whether a direct interaction between GRK2 and Akt contributes to the inhibition of Akt activation, we performed co-immunoprecipitation analysis. Although we do detect co-immunoprecipitation of Akt with GRK2, the amount of co-immunoprecipitated Akt was not increased by IL-1β treatment (Figure 8E). These results suggest that the IL-1β-induced alterations in CCR2 signaling to Akt are not associated with increased interactions between GRK2 and Akt.

DISCUSSION

The interaction between IL-1β and chemokine signaling in astrocytes might be important during the pathophysiology of various neuro-inflammatory processes such as multiple sclerosis, Alzheimer disease, and cerebral ischemia[1]. During these neuro-inflammatory processes various pro-inflammatory mediators (e.g. IL-1β) are produced in the central nervous system. These mediators can regulate GRK2 expression and in this way indirectly modulate chemokine receptor signaling, since GRK2 is well known for its capacity to regulate signaling of GPCRs directly via agonist-induced phosphorylation-mediated receptor desensitization. In the present study, we show that physiological changes in endogenous GRK2 expression induced by IL-1β treatment or as present in GRK2$^{+/-}$ cells modulate CCL2 signaling to pPDK-1, Akt and ERK1/2, but not to calcium and MEK1/2, suggesting that regulation takes place at a level downstream of receptor coupling.

We report that IL-1β treatment of primary astrocytes resulted in an increase in GRK2 protein expression that was not associated with an increase in GRK2 mRNA, suggesting that the IL-1β-induced upregulation of GRK2 occurs at the post-transcriptional or post-translational level. However, we can not exclude the possibility that at earlier time points GRK2 mRNA levels may have been affected by stimulation of cells with IL-1β. Earlier studies showed that IL-1β can decrease rather than increase GRK2 promoter activity in a model system of aortic smooth muscle cells[23]. Moreover, accumulating evidence suggests that GRK2 protein expression can indeed be regulated independently of changes in mRNA synthesis or in mRNA sta-

bilization, and that this post-transcriptional regulation may have a more profound impact on total GRK2 levels than control at the mRNA level. For example, we have previously shown that in peripheral blood cells from patients with chronic inflammatory diseases like multiple sclerosis or rheumatoid arthritis, GRK2 protein levels are decreased without changes in mRNA level[19,20]. In addition, it has been reported that GRK2 protein expression was increased in lymphocytes from patients with hypertension, whereas mRNA levels remained constant[34]. The rate of degradation of GRK2 can be regulated in various ways. GRK2 can be targeted to proteasome-dependent degradation and this process is stimulated by treatment with β-AR agonists or CXCL12[35]. In addition, in response to exposure to hydrogen peroxide, GRK2 degradation through a calpain-dependent process is facilitated in T-lymphocytes[36]. Finally, we have previously shown that reduced translation downregulated GRK2 protein expression in the C6 glioma cell line in response to treatment with oxygen radicals[37].

The role of GRK2 in cellular signaling has often been investigated in model cell systems. The focus of this paper, however, is on the effect of physiological changes in GRK2 protein levels in primary cells that naturally express the kinase. In primary cultures of astrocytes, we demonstrated that the IL-1β-induced increase in endogenous GRK2 lead to a decrease in the CCL2-induced phosphorylation of Akt and of ERK1/2 as well as to a decrease in translocation of pPDK-1 to the membrane. Conversely, CCL2-induced pAkt and pERK1/2 formation was increased in astrocytes from GRK2$^{+/-}$ mice, which have a 45% decrease in GRK2 expression. Overall, these results indicate that physiological changes in GRK2 level dose-dependently regulate CCL2 signaling. Moreover, these data suggest that the effect of IL-1β on CCL2-induced signaling is mediated via the increase in GRK2. However, we can not exclude completely the possibility that IL-1β influences signaling via another way which is independent of the regulation of GRK2 expression. To address this issue we have performed experiments using siRNA to prevent the IL-1β-induced change in GRK2 protein. Treatment with siRNA reduced basal GRK2 protein. However, under these conditions IL-1β could still upregulate GRK2 protein which is in line with our conclusion that IL-1β regulates GRK2 protein at a level downstream of mRNA. Therefore, this approach could not be used to confirm the hypothesis that the IL-1β-induced upregulation of GRK2 protein is indeed responsible for changes in CCL2 signaling.

In the classic model, GPCR desensitization requires GRK2-mediated phosphorylation of an agonist-occupied GPCR. The subsequent recruitment of β-arrestin leads to inactivation of the G protein, which inhibits intracellular signaling, and to receptor internalization. However, GRK2 can also regulate GPCR signaling at the level of the G protein in a phosphorylation-independent manner. An interaction of the N-terminal RGS homology (RH) domain of GRK2 with $G\alpha_{q/11}$ is sufficient to attenuate $G\alpha_{q/11}$-mediated signaling[38]. Moreover, recent studies have demonstrated that GRK2 as well as β-arrestin can interact with a number of intracellular signaling molecules and thereby regulate GPCR signaling. β-Arrestins can serve as scaffolds for the activation of e.g. ERK, JNK, p38 MAPK and Akt, whereas GRK2 has been shown to

regulate GPCR signaling via the interaction with components of the PI3 kinase-Akt and MAPK signaling cascades[39].

Interestingly, we show that in primary astrocytes the CCL2-induced increase in $[Ca^{2+}]_i$ and in phosphorylation of MEK1/2, the upstream ERK1/2 activator, was not affected by changes in endogenous GRK2 expression. It seems to be counterintuitive that calcium signaling in response to CCL2 stimulation was not affected in $GRK2^{+/-}$ and IL-1β-treated astrocytes, since earlier studies have clearly demonstrated that overexpression of GRK2 can inhibit calcium signaling in response to a number of GPCR ligands including CCL2[13]. It should be noted, however, that we are not dealing with overexpression of GRK2, but rather with relatively small physiological changes in endogenous GRK2 expression. It may well be possible that these physiological changes in GRK2 expression do not affect GRK2 functioning at the receptor level, but do have consequences for the interaction with other signaling molecules. In this respect it is of interest that previous studies have shown that overexpression of the kinase-deficient mutant GRK2-K220R does not affect calcium signaling induced by CCL2 in HEK293 cells. However, overexpression of WT GRK2 did reduce calcium signaling in this system[13]. In addition, it has also been shown that reducing endogenous GRK2 levels does not necessarily affect signaling to inositol 1,4,5-triphosphate (IP3), the second messenger upstream of calcium. For example, a 50-60% decrease in GRK2 expression did not affect M1 muscarinic acetylcholine receptor signaling to IP3 in HEK293 cells and in hippocampal neurons[40]. Another possible explanation for the lack of effect of reduced GRK2 expression on calcium signaling in response to CCL2 could be that other GRK subtypes compensate for reduced GRK2 expression at the level of receptor-G protein interaction, but not at more downstream levels. Indeed, primary rat astrocytes do also express GRK3 and it has been shown that this member of the GRK family can also regulate CCL2-induced calcium signaling[13,41].

Since no changes in CCL2-induced calcium signaling and MEK1/2 phosphorylation were found, it is very unlikely that in our system GRK2 regulates CCL2-induced intracellular signaling via the classic model of phosphorylation-dependent GPCR desensitization. This is supported by our observation that in U87 cells the GRK2 kinase domain is not required for inhibition of CCL2 signaling to ERK1/2. In addition, a mechanism in which GRK2 regulates signaling at the level of Gα can also be excluded, since this would affect the CCL2-mediated increase in $[Ca^{2+}]_i$. Based on the data presented here, we suggest that physiological changes in GRK2 alter CCL2-induced signaling in primary astrocytes downstream of the receptor via an interaction of GRK2 with components of intracellular signaling cascades.

In a previous study, we have shown that overexpression of GRK2 in HEK293 cells also inhibits CCL2 signaling to ERK1/2 but not to MEK1/2. Our present data suggest that even a modest increase in GRK2, such as that induced by treatment of cells with IL-1β, is sufficient to downregulate chemokine signaling to ERK1/2 at a level downstream of MEK1/2. Earlier, we presented evidence that the effect of GRK2 overexpression is mediated by a direct interaction between GRK2 and MEK1/2 and does not require GRK2 kinase activity[16]. In line with these

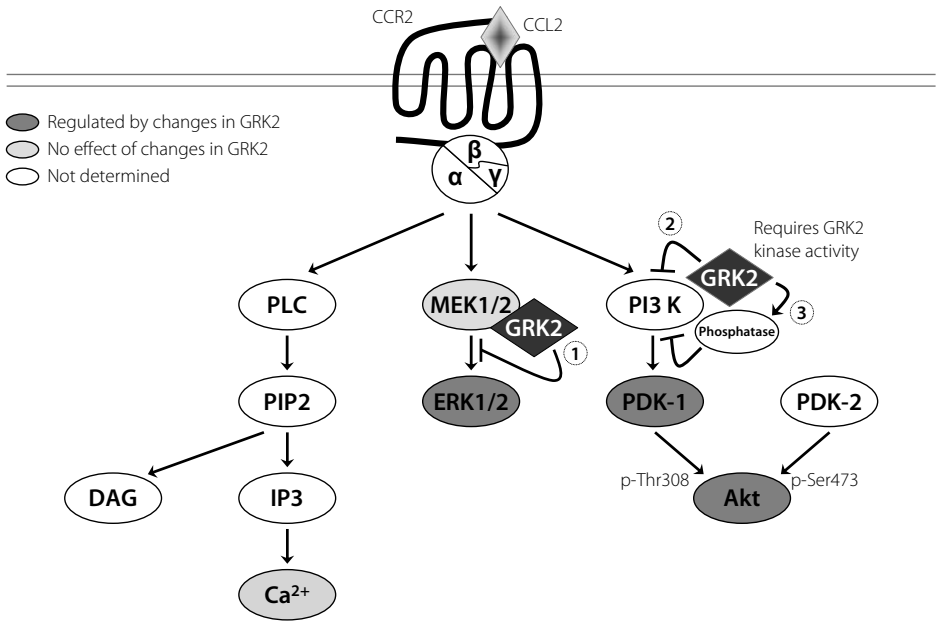

Figure 9: Hypothetical model for the regulation of CCL2-induced signaling by GRK2. A physiological change in GRK2 expression does not influence CCL2-induced activation of MEK1/2 and the release of $[Ca^{2+}]_i$. GRK2, however, regulates CCL2-mediated activation of ERK1/2, Akt, and PDK-1. (1) Association of GRK2 with MEK1/2 prevents downstream ERK1/2 activation. (2) An interaction of GRK2 with PI3 kinase inhibits PI3 kinase-mediated activation of PDK-1 and Akt via a mechanism that requires GRK2 kinase activity. (3) As an alternative, GRK2 might upregulate the activity of phosphatases that downregulate PI3 kinase activity via a GRK2 kinase activity-dependent mechanism.

data, we show here that also in U87 cells the kinase domain is not required for inhibition of CCL2 signaling to ERK1/2. We hypothesize that also in astrocytes an MEK1/2-GRK2 interaction interferes with downstream ERK1/2 activation (see hypothetical model in Figure 9). The finding that GRK2 and MEK1/2 interact does not necessarily mean that GRK2 inhibits MEK1/2 activation, but rather that GRK2 impedes proper MEK1/2 activity or MEK1/2 interaction with substrates, and in this way reduces downstream signaling of MEK1/2.

In the present study we show that not only chemokine signaling to ERK1/2, but also signaling to Akt was altered by changes in the intracellular level of GRK2. The IL-1β-induced increase in GRK2 expression inhibited CCL2-induced signaling to Akt, and the decrease in GRK2 levels in $GRK2^{+/-}$ cells enhanced CCL2-induced signaling to Akt. These effects of changes in GRK2 levels were independent of their effect on CCL2-induced signaling to the MEK1/2-ERK1/2 pathway, since they were maintained in the presence of the MEK1/2 inhibitor PD098059.

The exact mechanism via which changes in intracellular GRK2 level alters chemokine-induced pAkt formation remains to be determined. Previous studies by Liu *et al.* (2006) have described that GRK2 can interfere with the PI3 kinase-Akt pathway at a level downstream of Akt activation[15]. In a liver portal hypertension model, these authors showed that the increase

in intracellular GRK2 level in liver endothelial cells resulted in decreased phosphorylation of eNOS by activated Akt via a mechanism that involves direct interaction of GRK2 with Akt. Our data, however, do not suggest that GRK2 regulates the CCL2-induced activation of the Akt pathway via a direct interaction with Akt. First, we showed that the amount of Akt that co-immunoprecipitated with GRK2 was not increased by IL-1β treatment. Second, we demonstrated that PDK-1, the upstream activator of Akt, is influenced by changes in GRK2; agonist-induced translocation of pPDK-1 to the plasma membrane, a critical step in PDK-1 activation, was altered by changes in GRK2[32,33]. However, it is unlikely that GRK2 regulates PDK-1-Akt activation through an interaction of GRK2 with pPDK-1 since we detected no co-immunoprecipitation of pPDK-1 and GRK2 in primary astrocytes or in U87 cells transfected with GRK2 (own unpublished results). pPDK-1 translocation to the plasma membrane is known to be mainly dependent on the formation of phosphatidylinositol 3,4,5-triphosphate (PIP3), a major product of PI3 kinase activation[42]. Furthermore, pPDK-1 translocation is sensitive to wortmannin, an inhibitor of PI3 kinase[32]. Finally, studies by Naga Prasad *et al.* (2001) showed that GRK2 can directly interact with PI3kinase thereby regulating isoproterenol-induced PI3 kinase recruitment to the plasma membrane[18]. Taken together, we propose that the effect of changes in GRK2 level on CCL2 signaling to PDK-1-Akt result from an interaction of GRK2 with PI3 kinase (Figure 9). However, we can not provide direct evidence that GRK2 affects PI3 kinase activity in our system, since PI3 kinase activity was not detectable in phospho-tyrosine immunoprecipitates from CCL2-stimulated cells (data not shown).

In contrast to the effect of changes in intracellular GRK2 level on chemokine-induced ERK1/2 phosphorylation, its effect on Akt phosphorylation does require an intact GRK2 kinase domain; overexpression of the kinase-deficient mutant GRK2-K220R in the U87 astrocytoma cell line did not alter chemokine signaling to Akt, whereas overexpression of WT GRK2 did. Several studies have shown that GRK2 can phosphorylate a number of non-receptor substrates. These substrates include synuclein, phosducin, and tubulin, and phosphorylation of these proteins by GRK2 might play a role in regulation of receptor endocytosis or cellular migration[39]. Moreover, it has been recently demonstrated that phosphorylation of p38 MAPK by GRK2 leads to inactivation of p38 MAPK[17]. Kinase activity of GRK2 might play a similar role in the inhibition of the PI3 kinase-PDK-1-Akt pathway i.e. through phosphorylation-mediated inhibition of PI3 kinase activity (Figure 9). However, it has not been demonstrated that regulation of PI3 kinase requires GRK2 kinase activity. Moreover, there is no evidence available that phosphorylation of PI3 kinase at serine-threonine residues, the target residues of GRK2, regulates PI3 kinase activity. It is known, however, that dephosphorylation of PI3 kinase by phosphatases (e.g. PTEN and SHIP2) downregulates PI3 kinase signaling[43]. Therefore, an alternative possibility might be that GRK2 indirectly influences PI3 kinase activity by regulating the rate of PI3 kinase dephosphorylation via a mechanism that requires GRK2 kinase activity (Figure 9).

In conclusion, our current data show that in primary astrocytes, the activity of GRK2 is not restricted to phosphorylation of GPCR but that GRK2 can also have important regulatory effects downstream of receptor coupling. Moreover, we demonstrate that mediators like IL-1β, have the potential to regulate neuro-inflammation by changing the net effect of GPCR activation.

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6

CHAPTER

General discussion and summary of the results

G protein-coupled receptor (GPCR) kinase (GRK)2 regulates the responsiveness of many GPCRs involved in inflammatory and neuropathic pain. During several inflammatory disease states, the amount of cellular GRK2 is modulated in various organs. The experiments performed in this thesis focused on:

1. the expression of spinal cord neuronal GRK2 during neuropathic pain,
2. the consequences of low GRK2 on pain perception,
3. the mechanism of GRK2-mediated regulation of GPCR signaling.

We demonstrated a reduction of neuronal GRK2 in the spinal cord dorsal horn during two models of nerve injury-induced mechanical allodynia: chronic constriction injury (CCI) of the sciatic nerve in rats and L5 spinal nerve transection (SNT) in mice. In addition, we reported that interleukin (IL)-1 type 1 receptor knock-out (IL-1R$^{-/-}$) mice did not show a reduction in spinal cord GRK2 during L5 SNT and also did not develop mechanical allodynia in this model. Furthermore, chronic IL-1β treatment of *ex vivo* cultured spinal cord slices resulted in a decrease in GRK2. These data demonstrate a role for IL-1β signaling in downregulating spinal cord GRK2 during mechanical allodynia.

Moreover, we showed that low GRK2 lead to increased mechanical allodynia and thermal hyperalgesia in animals with paw inflammation. No changes in pain perception were observed in naive GRK2$^{+/-}$ mice compared to wild type (WT) mice.

Finally, we reported that GRK2 not only plays a role in neurons, but also in astrocytes by regulating GPCR (chemokine) signaling in primary cultures of astrocytes via interaction with intracellular signaling molecules.

NOVEL CONCEPTS OF GRK2-MEDIATED REGULATION OF GPCR SIGNALING

In the classic model, GRK2-mediated phosphorylation of ligand-occupied GPCRs induces desensitization of the GPCRs. Receptor phosphorylation by GRK2 leads to subsequent binding of β-arrestins, which results in inactivation of the G protein and in receptor internalization. Although the classical concept of phosphorylation-dependent GPCR desensitization is of great importance for many GPCRs, we found that GRK2 also modulates cellular signaling in a receptor-independent way after triggering the C-C chemokine receptor (CCR)2 expressed by astrocytes. We propose that GRK2 regulates C-C chemokine ligand (CCL)2-induced signaling via an *interaction with downstream signaling molecules*. In **chapter 5**, we demonstrated that an increase in endogenous GRK2, via IL-1β treatment, leads to a decrease in the CCL2-induced pAkt and pERK1/2 formation as well as to a decrease in activation of PDK-1. Conversely, CCL2-induced phosphorylation of Akt and ERK1/2 was increased in astrocytes from GRK2$^{+/-}$ mice, which have a 45% decrease in GRK2 expression. In contrast, changes in GRK2 did not alter the intracellular calcium release and MEK1/2 activation. Based on this observation, we assume that GRK2 regulates CCL2-induced signaling in primary astrocytes via the interaction with

components of the mitogen-activated protein kinase (MAPK) cascade and phosphoinositide-3 kinase (PI3 kinase)-Akt pathway.

We proposed that an interaction of GRK2 with MEK1/2 prevents downstream ERK1/2 phosphorylation via a mechanism that does not require GRK2 kinase activity. In addition, we hypothesized that an interaction of GRK2 with PI3 kinase directly or indirectly inhibits PI3 kinase activity via a GRK2 kinase-dependent mechanism, which results in reduced PDK-1 translocation and Akt activation. However, we were not able to provide direct evidence that GRK2 affects PI3 kinase activity in our system, since CCL2-induced PI3 kinase activity was not detectable in phospho-tyrosine immunoprecipitates from CCL2-stimulated astrocytes. The reason might be that the PI3 kinase activity in these endogenous systems is below the detection level or that other PI3 kinase isoforms become activated by CCL2.

In contrast to our study, Aragay *et al.* (1998) showed that in Mono-Mac-1 cells overexpressing GRK2, GRK2 attenuated the CCL2-induced release of intracellular calcium via a phosphorylation-dependent mechanism[1]. A possible explanation for this discrepancy might be the use of an overexpression system instead of the use of primary cell cultures with physiological changes in the amount of GRK2 protein, like we used in **chapter 5**. Overexpression of proteins might alter the compartmentalization of receptors and intracellular signaling molecules in clusters and/or lipid rafts. For this reason, overexpression systems might not always provide the same outcome as physiological systems when studying the mechanism of GRK2-mediated regulation of GPCR signaling and one should be careful when translating results of overexpression studies to studies using physiological systems.

If one receptor is regulated via phosphorylation-dependent GPCR desensitization, are other GPCRs in the same cell regulated in a similar fashion by GRK2 or could signaling through these GPCRs still be regulated via the interaction of GRK2 with intracellular signaling molecules? It is possible that GRK2-mediated regulation of GPCR function depends on the compartmentalization of the receptor in question. If receptors are compartmentalized within different clusters and/or lipid rafts, which vary in the composition of intracellular signaling molecules, it might be that for example β_2-adrenergic receptor (AR) function is regulated by GRK2 via phosphorylation-dependent receptor desensitization, whereas CCR2 signaling in the same cell is regulated via the interaction of GRK2 with intracellular signaling molecules.

The latter phenomenon is not restricted to the CCR2. For instance, A1 adenosine receptor-induced ERK1/2 phosphorylation was efficiently inhibited by overexpression of GRK2. Similar to our study, ERK1/2 activation was not affected by overexpression of a GRK2 kinase deficient mutant. Conversely, the A1 adenosine receptor-mediated inhibition of cAMP production was not influenced by changes in GRK2[2]. In addition, reducing GRK2 levels by GRK2 siRNA knock-down in primary cultures of hippocampal neurons or HEK293 cells did not modulate agonist-induced phospholipase C (PLC) activity[3]. These data suggest that GRK2 can indeed regulate cellular signaling through other GPCRs at a level downstream of the receptor (i.e. via the interaction with intracellular signaling molecules such as ERK1/2).

CYTOKINE-INDUCED DOWNREGULATION OF NEURONAL GRK2 DURING CHRONIC PAIN

In vitro studies have demonstrated that treatment with pro-inflammatory cytokines or reactive oxygen species (ROS) induces changes in GRK2 expression in various cell types[4-6]. Many organ-specific diseases are often associated with (low-grade) systemic inflammation. Therefore, we suggest that GRK2 is decreased in peripheral organs, because of peripheral production of cytokines or the formation of ROS. Indeed, a number of studies have demonstrated changes in the amount of cellular GRK2 in the periphery of patients with diverse pathologies. For instance, we showed in previous studies that patients with multiple sclerosis and rheumatoid arthritis have a reduction of GRK2 content in peripheral blood lymphocytes[4,7]. In addition, in a study performed in collaboration with the Department of Medical Psychology from the University of Duisburg-Essen, we demonstrated that GRK2 was decreased in peripheral blood leukocytes isolated from patients with inflammatory bowel disease (IBD) (unpublished data).

In this thesis, we hypothesized that mediators produced in spinal cord during inflammation or nerve injury cause a reduction in GRK2, which subsequently leads to an increased processing of nociceptive signals and in this way contributes to neuropathic pain. Although above mentioned studies already demonstrated downregulation of GRK2 in peripheral organs of patients with inflammatory diseases, we wondered whether the downregulation of GRK2 is also reflected on the spinal cord. Preliminary results suggest that we do not see changes in the GRK2 expression in the spinal cord during the chronic phase of collagen-induced arthritis in mice (unpublished data).

In **chapter 2** and **3** of this thesis, we investigated GRK2 expression in the spinal cord during two animal models of neuropathic pain: CCI of the sciatic nerve and L5 SNT. Some major differences exist between these two animal models: the affected nerve (sciatic nerve vs. L5 spinal nerve), the type of nerve damage (ligation vs. transection), and use of different species (rats vs. mice). Even though these major differences are present between the two models, we demonstrated that nerve injury-induced mechanical allodynia was associated with a reduction in GRK2 expression in the superficial layers of the spinal cord dorsal horn in both models. These results imply that reducing GRK2 may be a mechanism of general importance involved in the development of neuropathic pain.

In contrast to L5 SNT, the decrease in GRK2 during CCI of the sciatic nerve shows spreading throughout the lumbar spinal cord. With respect to this finding, one has to assume that the activation of glial cells and subsequent production of inflammatory cytokines caused by CCI will spread through the lumbar spinal cord and in this way will lead to a decrease in GRK2, up- and downstream of the site where the affected nerve enters the spinal cord. The latter effect may contribute to a more general increase in sensitivity for pain after nerve damage and may also contribute to mirror-image pain syndromes.

The reduction in GRK2 in the spinal cord during nerve injury-induced mechanical allodynia was mainly observed in neurons. In the spinal cord, GRK2 was below detection limits in glial cells and was mainly present in neuronal cells as shown by immunohistochemistry (**chapter 2** and **3**). Since glial cells outnumber neurons by 10 to 1 and make up about 50% of the nervous system[8], this suggests that GRK2 expression will be much higher in spinal cord neurons than in glial cells. Surprisingly, in primary cultures of astrocytes or microglia as well as in the C6 glioma cell line, GRK2 can easily be detected by Western blotting. Interestingly, in isolated peritoneal macrophages, which show functional resemblance to microglia, GRK2 content is very low as well. Also in the case of peritoneal macrophages, GRK2 could be detected in *in vitro* cultures (unpublished data). These observations imply that during *in vitro* activation, glial cells and macrophages increase their amount of cellular GRK2. Alternatively, although less likely, *in vivo* expression of GRK2 might be actively suppressed in these cells. It still remains to be determined which role GRK2 plays in macrophages and cells of the glial lineage. At the moment, this problem is attacked by applying cell type-specific deletions of GRK2 using the CRE-Lox system.

Which mechanism is responsible for the decrease in spinal cord neuronal GRK2? The amount of GRK2 present in a cell is regulated by a number of mediators that control the balance between transcription, translation, and protein degradation of GRK2. Some of these mediators may also be involved in reducing neuronal GRK2 in the spinal cord during neuropathic pain. As discussed above for inflammatory diseases, we propose that pro-inflammatory cytokines (IL-1β, IL-6, and tumor necrosis factor (TNF)-α) are candidates for the downregulation of neuronal GRK2 during neuropathic pain. Pro-inflammatory cytokines play a significant role in upregulating pain sensitivity during chronic pain; production of IL-1β, TNF-α, and IL-6 in the spinal cord contribute to increased pain perception during inflammatory and neuropathic pain[9-11]. Several lines of evidence presented in this thesis favor a pivotal role for IL-1β signaling in downregulating neuronal GRK2 in the spinal cord dorsal horn during neuropathic pain. We showed that chronic IL-1β treatment of *ex vivo* cultured spinal cord slices reduced GRK2 expression (**chapter 2**). Furthermore, chronic LPS treatment, which induces production of multiple cytokines (e.g. IL-1β), also downregulated the amount of GRK2 in cultured spinal cord slices (own unpublished data). Moreover, we demonstrated that L5 SNT did not induce a decrease in GRK2 expression in the spinal cord dorsal horn in IL-1R$^{-/-}$ mice, which also do not develop mechanical allodynia in this model (**chapter 3**). The latter suggests a functional relationship between IL-1β signaling and the development of mechanical allodynia as well as downregulation of spinal cord GRK2. The contribution to nerve injury-induced mechanical allodynia of other pro-inflammatory cytokines (e.g. IL-6 and TNF-α), which have been shown to regulate GRK2 expression and the development of neuropathic pain, should still be investigated. However, IL-1R activation is probably a prerequisite for regulating GRK2 and in this respect, it is of interest that IL-1β is probably at the top of the cytokine hierarchy.

Pro-inflammatory cytokines might have multiple functions in the regulation of neuropathic pain. Next to their ability of reducing GRK2, cytokines may modify GPCR function. It has, for example, been reported that IL-1β upregulates expression of GPCRs (e.g. neurokinin-1 receptor (NK-1R) and metabotropic glutamate receptor (mGluR)5) in astrocytes[12,13]. In addition, Khoa *et al.* (2006) recently demonstrated a cross-link between TNF-α signaling and GPCR function. These authors demonstrated that TNF-α treatment downregulated the recruitment of GRK2 to the plasma membrane in response to GPCR stimulation. The reduction in membrane-bound GRK2 resulted in a decrease in GPCR desensitization[14]. These observations indicate that pro-inflammatory cytokines are able to regulate GPCR functions in other ways than only regulation of GRK2 levels.

Next to pro-inflammatory cytokines, it may be possible that ROS are engaged in downregulating GRK2 during neuropathic pain. Hydrogen peroxide treatment of peripheral blood leukocytes as well the C6 glioma cell line reduced GRK2 levels[5,15]. In addition, ROS have an important function in the regulation of pain sensitivity during neuropathic pain. O'rielly *et al.* (2006) demonstrated an increase expression of inducible and neuronal nitric oxide synthase in the rat spinal cord after L5-L6 spinal nerve ligation (SNL)[16]. Furthermore, inhibition of oxygen radical production prevented the development of CCI-induced neuropathic pain behaviors[17]. Thus, production of ROS could be an additional way to regulate GRK2 expression during chronic pain. However, based on our IL-1R$^{-/-}$ studies, we would like to underline that IL-1β is absolutely required for the modulation of pain sensitivity and that ROS production may act downstream of IL-1R signaling in regulating GRK2 expression.

Could it be that additional mechanisms than the production of a number of mediators (i.e. pro-inflammatory cytokines and ROS) play a role in the regulation of neuronal GRK2 during nerve injury-induced mechanical allodynia? It has been demonstrated that increased stimulation of GPCRs, such as the β-ARs or C-X-C chemokine receptor (CXCR)4, leads to an increase in GRK2 protein degradation via the proteasome pathway[18,19]. During chronic pain, increased noxious stimuli-evoked neurotransmitter release from sensory primary afferents may lead to increased GPCR stimulation. For example, capsaicin stimulation induced an increase in the release of calcitonin gene-related peptide (CGRP) and substance P (SP) from spinal cord slices isolated from rats with λ-carrageenan-induced thermal hyperalgesia[20]. Also, spinal cords, that were isolated from rats with L5 SNT, released significantly more SP upon electrical stimulation of Aβ-fibers[21]. Furthermore, noxious stimulation of the hind paw produced significantly higher release of glutamate in the spinal cord during CCI[22]. Thus, the possibility that an enhancement of degradation GRK2 via an increase in GPCR stimulation is involved in the modulation of GRK2 protein levels during chronic pain as well should also be addressed.

DOES A DOWNREGULATION OF GRK2 HAVE IMPLICATIONS FOR PAIN PERCEPTION?

Two studies presented in this thesis demonstrated a decrease in neuronal GRK2 spinal cord expression during nerve injury-induced mechanical allodynia. We hypothesized that a reduction in the amount of neuronal GRK2 contributes to an increase in pain perception (**chapter 2** and **4).** For this purpose, we compared sensitivity for thermal and mechanical stimuli between WT mice and GRK2$^{+/-}$ mice, which have 50% reduction in GRK2[7,23]. Naive GRK2$^{+/-}$ mice and naive WT mice exhibited equal sensitivity for thermal and mechanical stimuli indicating that during baseline conditions a reduction in GRK2 as such does not lead to changes in pain perception. These data seem to be counterintuitive since a decrease in GRK2 in general leads to an increase in GPCR signaling and therefore one would expect to find an increase in pain perception.

Next, we considered the possibility that low GRK2 will only play a role in the induction of increased pain perception in the context of inflammation. For instance, pain-related behaviors in response to intrathecal injection of mGluR group I agonists or intradermal injection of prostaglandin (PGE)2, 5-hydroxytryptamine (HT), or A2 adenosine agonists are increased during animal models of inflammatory pain[24,25]. These data imply that sensitivity of GPCRs is increased during inflammatory pain. To further study the interaction between inflammation and low GRK2, we induced paw inflammation by injecting λ-carrageenan. We clearly showed that inflammation of the paw evoked a more pronounced mechanical allodynia (**chapter 2**) and thermal hyperalgesia (**chapter 4**) in GRK2$^{+/-}$ mice compared to WT mice. Apparently, the effects of low GRK2 are only operative in the context of the production of inflammatory mediators in the GRK2$^{+/-}$ mice. So apart from regulation of GRK2 levels, inflammatory mediators give a second signal in a condition of low GRK2 to evoke inflammatory pain. The molecular interactions of signal 1 (low GRK2) and signal 2 (unknown) leading to the establishment of increased hyperalgesia is at the moment a subject of intense investigation.

A number of studied have reported production of GPCR ligands in affected tissue as well as in the spinal cord during animal models for inflammatory pain (e.g. PGE2, CCL2, CCL3, and CCL5) and blocking the effects of these mediators inhibited the development of increased pain sensitivity in these models[16,26-30]. In addition, an increased neurotransmitter release (e.g. SP, and glutamate) by primary sensory afferents takes place in the dorsal horn of the spinal cord during neuropathic pain[21,22]. The nature of signal 2 might also be the production of inflammatory GPCR ligands, such as the chemokine CCL3, which does not occur under naive conditions, but only during neuropathic or inflammatory pain. As an experimental approach for this model, we demonstrated that intraplantar injection of the chemokine CCL3 as such is already sufficient to induce an increased thermal hyperalgesia in GRK2$^{+/-}$ mice compared to WT mice (**chapter 4**). For this reason, we propose that IL-1β can function as signal 1, whereas the inflammatory GPCR ligands such as CCL3, can function as signal 2. Furthermore, it is un-

likely that increased neurotransmitter release functions as signal 2, since naive GRK2$^{+/-}$ mice do not display increased pain sensitivity during activation of peripheral nerves with thermal or mechanical stimuli.

Another interesting possibility to be studied is that an inflammatory mediator like IL-1β not only decreases GRK2, but is also involved in the arrangement of the cytoskeleton leading to changes in raft formation and subsequently to changes in receptor/signal transduction molecule clustering in the plasma membrane. It could be that these conformational changes (signal 2), together with low GRK2, will be a prerequisite for activating signaling pathways by GPCR ligands that result in an increased sensitivity for pain. For that reason, we would like to hypothesize that only the production of those ligands through which changes occur in the compartmentalization of the receptor/signal transduction molecules located inside clusters or lipid rafts will be capable of changing the sensitivity for pain in conditions of low GRK2.

How could an increase in GPCR signaling, due to low GRK2, result in inflammatory pain? Under normal conditions, painful stimuli (e.g. heat or capsaicin) activate transient receptor potential vanilloid receptor subtype 1 (TRPV1), which is expressed on sensory nerve terminals. A possible mechanism that might be involved in increasing pain sensitivity during chronic pain is TRPV1 sensitization in response to GPCR signaling. For instance, TRPV1 sensitization occurs in response to stimulation of neuronal cells with the GPCR agonists CCL3, PGE2, and bradykinin[31-33]. The mechanism through which GPCR stimulation increase the sensitivity of TRPV1 includes phosphorylation by protein kinase C (PKC)ε or a reduction in the phosphatidylinositol 4,5-biphosphate (PIP2)-mediated inhibition[34]. Thus, GPCR signaling, in response to the production of GPCR agonists, may lead to an increase in the sensitivity for painful stimuli via TRPV1 sensitization during inflammatory pain. In addition, during chronic pain several pro-inflammatory cytokines (e.g. IL-1β), which may reduce GRK2 levels, are produced. The reduction in GRK2 would lead to increased GPCR signaling and in this way to even higher TRPV1 sensitization (Figure 1).

In addition to TRPV1, GPCR signaling may also induce phosphorylation of other ion channels, which might lead to an increase in neuronal sensitivity. Indeed, mGluR group I signaling initiates neuronal sensitization via phosphorylation of the NMDA receptor NR2B subunit in the spinal cord during complete Freund's adjuvant (CFA)-induced inflammatory hyperalgesia[35]. Moreover, SP and bradykinin-induced activation of protein kinases contributes to potentiation of currents mediated by ATP-gated ion channels $P2X_2$ and $P2X_3$[36]. The sensitivity of a number of ion channels is thus under regulation by GPCR-mediated signaling and thereby possibly under the control of GRK2.

As discussed in the beginning of this chapter, GRK2 does not only regulate cellular activity by phosphorylation of GPCR, but also via interaction with downstream signaling molecules. In **chapter 5**, we show that GRK2 regulates CCL2 signaling via a mechanism downstream of receptor phosphorylation via the interaction with intracellular signaling molecules MEK1/2 and PI3 kinase. We hypothesize that the interactions of GRK2 with intracellular proteins may

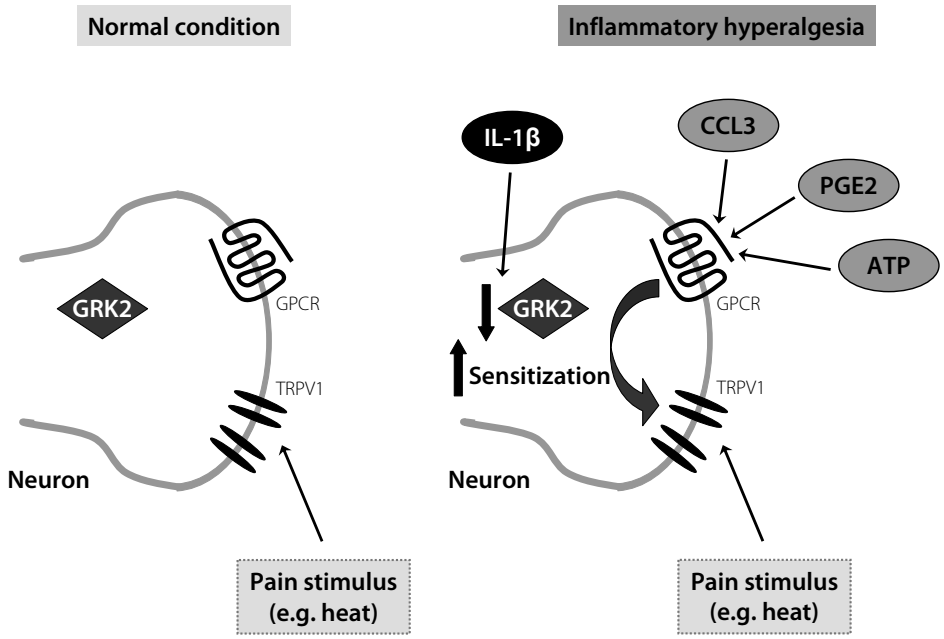

Figure 1: Hypothetical model for GRK2-mediated TRPV1 sensitization during inflammatory hyperalgesia. During normal conditions, painful stimuli (e.g. heat, capsaicin) activate TRPV1, which is present on peripheral nerve terminals. During inflammation, several GPCR ligands (e.g. CCL3 and PGE2) are produced in the surrounding of peripheral nerve terminals. These mediators bind to several GPCRs, which are present on sensory nerve endings. GPCR signaling is responsible for TRPV1 sensitization via PKCε-mediated phosphorylation and a reduction in PIP2-mediated inhibition of TRPV1. TRPV1 sensitization results in an increased sensitivity for painful stimuli and thus leads to hyperalgesia. During inflammation, however, several pro-inflammatory cytokines (e.g. IL-1β) are produced, which reduce neuronal GRK2 expression. As a consequence of lower GRK2 expression, signaling through GPCRs will be increased resulting in an upregulation of TRPV1 sensitization and therefore in an increased hyperalgesia.

also occur in lipid rafts, which imply that these downstream interactions will play a role as well in the establishment of hyperalgesia. Evidence for this hypothesis comes from a study by Peregrin *et al.* (2006). These authors showed that phosphorylation of p38 MAPK by GRK2 resulted in a decrease in activation of p38 MAPK by its upstream activator MKK6. The LPS-induced cytokine production, which is a p38 MAPK dependent process, was significantly increased in macrophages derived from GRK2$^{+/-}$ mice[37]. If such an event also takes place in the spinal cord, an increase in cytokine production might take place independent of GPCR signaling during low levels of GRK2.

Clustering of GRK2 with ion channels may also indirectly regulate pain perception. For instance, GRK2 is able to regulate the function of DREAM, which is a transcriptional repressor of the dynorphin gene, but also regulates trafficking of Kv4.2 potassium channels to the plasma membrane. It has been shown that GRK2-mediated phosphorylation of DREAM inhibits DREAM-mediated trafficking of Kv4.2 potassium channels to the plasma membrane, but does not affect the function of DREAM as a repressor of dynorphin transcription[38]. In addition to DREAM, an association between GRK2 and epithelial sodium channels has been

demonstrated[39]. However, functional consequences for the interaction of GRK2 with sodium channels have not been demonstrated yet.

Taken together, changes in GRK2 can modulate pain perception during inflammatory pain by regulating GPCR signaling via its classical way of phosphorylation-mediated receptor desensitization. However, the more recently identified role of GRK2 (i.e. the regulation of activation of intracellular molecules by GRK2) could also be of great importance during chronic pain.

DOES GRK2 HAVE A UNIQUE ROLE IN THE DEVELOPMENT OF CHRONIC PAIN?

GRK2 is not the only protein involved in regulating the desensitization and signaling of GPCRs. GRK2 belongs to a larger family of protein kinases, of which until now 7 members have been identified. The studies presented in this thesis on the use of the GRK2$^{+/-}$ mice provide evidence that other GRK family members and β-arrestins do not (completely) compensate for the loss of GRK2.

In this thesis, we did not investigate expression of other GRKs and β-arrestins during chronic pain. However, it may well be possible that the expression of these proteins also changes during chronic pain. It has been reported that next to a decrease in GRK2, a reduction of GRK6 is observed in lymphocytes isolated from patients with multiple sclerosis or rheumatoid arthritis[4,7]. Therefore, it could theoretically be possible that when neurons are confronted with inflammatory mediators such as during CCI, these cells do not only downregulate GRK2, but also GRK6, which could have consequences for pain perception. In naive animals, it has been demonstrated that β-arrestin2$^{-/-}$, GRK3$^{-/-}$, GRK5$^{-/-}$, or GRK6$^{-/-}$ mice do not differ in thermal sensitivity during baseline conditions (Eijkelkamp *et al.*, unpublished results)[40-42].

In this respect, the results of Eijkelkamp *et al.* on GRK6 and (post-)inflammatory pain are of interest (unpublished data). Upon intracolonic instillation of capsaicin, an increase in behavioral pain responses was observed in GRK6$^{-/-}$ mice post-colitis compared to WT mice post-colitis, indicating that reduced GRK6 levels resulted in increased post-inflammatory visceral hyperalgesia. In addition, inflammatory thermal hyperalgesia induced by intraplantar injection of IL-1β was more pronounced in GRK6$^{-/-}$ mice compared to WT mice. These results indicate that similar to GRK2, low GRK6 levels result in an increased inflammatory pain as well. Next to GRK6, changes in β-arrestin2 expression may result in changes in pain perception during chronic pain. Indeed, siRNA knock-down of β-arrestin2 prevented or reversed CCI-induced thermal cold allodynia and tended to inhibit mechanical allodynia in rats. Furthermore, downregulation of β-arrestin2 levels reduced morphine tolerance[43]. In addition, β-arrestin2$^{-/-}$ mice showed a delay and decrease in development of morphine tolerance[42]. In conclusion, GRKs and β-arrestins contribute to changes in pain sensitivity during conditions of inflammation or nerve trauma, but do not play a role during normal pain perception. In-

terestingly, genetic deletion of one specific GRK has already consequences for inflammatory hyperalgesia, indicating that there is no complete functional overlap.

THE THERAPEUTIC IMPORTANCE OF DECREASED GRK2

Chronic pain is a significant problem, which affects daily life to a large extent. Chronic pain is frequently observed in patients with spinal cord injury, carpal tunnel syndrome, phantom limb pain, and inflammatory bowel diseases (e.g. ulcerative colitis and Crohn's disease)[44,45]. It might be possible that spinal elevations in cytokines or oxygen radicals may also influence pain perception in patients with systemic inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, and in some cases IBD. Even a low-grade inflammatory response as observed in patients with so-called "pain syndromes" (e.g. fibromyalgia and sympathetic reflex dystrophy)[46,47] might be sufficient to induce a decrease in neuronal GRK2. In this respect, it is interesting to investigate whether the GRK expression levels in peripheral blood leukocytes may be a marker for GRK levels in the spinal cord in patients with pain syndromes.

Could the prevention or reversal of the reduction in GRK2 in the spinal cord become a possible treatment strategy for chronic pain? Indeed, we showed that a deficiency in IL-1β signaling prevented the downregulation of neuronal GRK2 in the spinal cord as well as the development of mechanical allodynia (**chapter 3**). As a proof of principle, we would like to develop a treatment strategy that prevents degradation of GRK2. We already know that GRK2 is ubiquitinated and subsequently degraded via the proteasome pathway. The development of a peptide that will block the ubiquitination site of GRK2 might be of great significance for the treatment of chronic pain. However, the design of this peptide will be difficult for several reasons; one should be able to block a common structure involved in ubiquitination, because multiple sites within GRK2 can be ubiquitinated.

Next to a specific blocking GRK2 peptide, treatment with cytokine antagonists may also prevent GRK2 downregulation. It may be worthwhile to investigate in animal models whether e.g. inhibition of IL-6, TNF-α or GPCR signaling has the same effect on GRK2 as abrogation of IL-1β signaling. In addition, it would also be interesting to investigate whether GRK2 levels return to baseline levels in patients with chronic pain that are treated with IL-1RA or anti-TNF-α.

We demonstrated in **chapter 2** and **4** of this thesis that low GRK2 results in an upregulation of mechanical allodynia and thermal hyperalgesia during inflammation. However, the amount of GRK2 is decreased in all cell types in GRK2$^{+/-}$ mice. Therefore we would like to test whether a specific reduction in only neuronal GRK2 leads to an increase in inflammatory or neuropathic pain. For this purpose, CRE-Lox mice, in which we specifically knock-out neuronal GRK2, can be used. If these mice indeed show more pronounced inflammatory pain

than their WT littermates, this would indicate that a specific reduction in neuronal GRK2 contributes to the development of inflammatory and/or neuropathic pain.

In conclusion, the studies performed in this thesis revealed an important function for GRK2 in the regulation of pain sensitivity during inflammation and nerve trauma. GRK2 inhibits the development of inflammatory thermal hyperalgesia and mechanical allodynia. Furthermore, neuropathic pain is associated with a decrease in neuronal GRK2 in the spinal cord in which IL-1β signaling has a crucial regulatory function. Future research might focus on the underlying mechanisms for the contribution of GPCR signaling and its regulation by GRK2 to increased pain perception. In particular, the identification of signal 2 that induces an increase in pain perception during a condition of low GRK2 and the contribution of GRK2 to regulate cellular function via the interaction with intracellular molecules might be of great importance. In addition, the contribution of other components of the GPCR desensitization machinery in chronic pain might be subject of future research. Elucidating the function of GRK2 during states of increased pain perception might uncover new opportunities for the treatment of patients with chronic pain.

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7

CHAPTER

Samenvatting in het Nederlands

Pijn is een onplezierige sensorische en/of emotionele ervaring, die wordt veroorzaakt door een weefselbeschadiging ergens in het lichaam of door de dreiging voor mogelijke weefselbeschadiging (IASP, 1979). Pijnlijke prikkels activeren perifere zenuwcellen, die het signaal doorgeven aan zenuwcellen in het ruggenmerg, die wederom in verbinding staan met de hersenen. Op deze manier wordt de informatie over pijnlijke prikkels doorgestuurd naar de hersenen. Weefselbeschadigingen of ontstekingen kunnen de oorzaak zijn van het ontstaan van ontstekingsgerelateerde pijn, zoals die bijvoorbeeld optreedt bij patiënten met reumatoide artritis en prikkelbare darm syndroom. Bij patiënten met fantoompijn of ruggenmergbeschadigingen hebben zenuwbeschadigingen geleid tot de ontwikkeling van neuropathische pijn. Het onderscheid tussen neuropathische en ontstekingsgerelateerde pijn is echter niet altijd goed te maken. Beide worden gekenmerkt door spontane pijn (pijn zonder de aanwezigheid van pijnlijke prikkels), hyperalgesie (een verhoogde gevoeligheid voor pijnlijke prikkels) en/of allodynie (pijn ten gevolge van een prikkel die normaalgesproken geen pijn zou veroorzaken).

Een verhoogde gevoeligheid voor pijnlijke prikkels ontstaat door een verhoogde gevoeligheid van zenuwcellen voor prikkels, waardoor ze eerder of sneller prikkels gaan doorgeven. Deze verhoogde gevoeligheid kan optreden in zenuwcellen aanwezig in de periferie en/of in het ruggenmerg.

Er zijn al enkele mechanismen beschreven die een rol spelen bij het ontstaan van de verhoogde gevoeligheid van zenuwcellen. In de periferie speelt de migratie van immuuncellen naar de plek van ontsteking, weefsel- of zenuwbeschadiging een belangrijke rol. De activering van twee type gliacellen, astrocyten en microgliacellen (gliacellen zijn cellen die in het ruggenmerg voorkomen en zenuwcellen "verzorgen") is in het ruggenmerg zeer belangrijk. Daarnaast treedt zowel in de periferie als in het ruggenmerg productie op van mediatoren die bijdragen aan het ontstaan van een verhoogde gevoeligheid voor pijnlijke prikkels. Tot deze mediatoren behoren onder andere mediatoren die vrijkomen tijdens weefselschade (zuurstofradicalen en ATP), ontstekingsmediatoren (cytokinen en chemokinen) en mediatoren die worden uitgescheiden door zenuwcellen (neuropeptiden en neurotransmitters). Deze mediatoren dragen bij aan het ontstaan van een verhoogde pijngevoeligheid, doordat zij boodschappereiwitten in cellen activeren (bijv. MAPK), een verandering in de hoeveelheid of de gevoeligheid van receptoren aanwezig op het oppervlak van zenuwcellen veroorzaken (bijv. TRPV1 en NMDA receptoren) en/of de aanmaak van bepaalde eiwitten verhogen (bijv. COX-2 en cytokinen).

Veel van deze mediatoren (chemokinen, neuropeptiden en neurotransmitters) binden aan een lid van de familie van receptoren genaamd G-eiwit gekoppelde receptoren (GPCR). GPCR zijn aanwezig op het oppervlak van cellen en spelen een rol bij het doorgeven van signalen tussen cellen. Wanneer een mediator bindt aan de receptor, worden via de activering van G-eiwitten boodschappereiwitten in de cel aangezet. Deze boodschappereiwitten zijn

verantwoordelijk voor het aan- en/of uitschakelen van allerlei processen in de cel. De rol van GPCR bij het ontstaan van een verhoogde pijngevoeligheid is in een groot aantal studies aangetoond. Wanneer activatoren van GPCR in de poot of het ruggenmerg worden gespoten, kan hyperalgesie en/of allodynie ontstaan. Verder leidt blokkade van de activering van GPCR tot een remming van ontstekingsgerelateerde en neuropathische pijn.

De functie van GPCR kan gereguleerd worden door een proces genaamd desensitisatie, dat wil zeggen een afname in de mogelijkheid van receptoren om boodschappereiwitten te activeren. Desensitisatie van GPCR wordt gereguleerd door twee belangrijke groepen van eiwitten: GPCR kinases (GRKs) en arrestines. Nadat een mediator aan een receptor bindt en boodschappereiwitten zijn aangezet kan een GRK aan de geactiveerde receptor binden. GRK fosforyleert de receptor met als gevolg de binding van arrestines. Arrestines zijn verantwoordelijk voor het uitzetten van geactiveerde G-eiwitten, zodat boodschappereiwitten niet meer aangezet kunnen worden. Tevens zorgen arrestines ervoor dat GPCR worden opgenomen in de cel (geïnternaliseerd), zodat het aantal receptoren aanwezig op het oppervlak van de cel afneemt.

GRKs behoren tot een familie van eiwitten, waarvan er tot nu toe zeven zijn ontdekt: GRK1-7. Het onderzoek in dit proefschrift is voornamelijk gericht op GRK2, omdat dit eiwit in grote hoeveelheid voorkomt in het zenuwstelsel. Het is tevens bekend dat het gehalte van GRK2 in een cel de signalering door GPCR kan beïnvloeden. Een laag gehalte van GRK2 leidt tot een verhoogde activering van boodschappereiwitten, omdat minder desensitisatie optreedt, terwijl een hoog gehalte van GRK2 juist het aanzetten van boodschappereiwitten remt. Op deze manier kan GRK2 dus indirect allerlei processen, die worden geactiveerd of geremd door GPCR, in de cel reguleren.

VERLAGING VAN HET GRK2 GEHALTE TIJDENS NEUROPATHISCHE PIJN

Er zijn verschillende studies beschreven, die onderzoek hebben gedaan naar het gehalte van GRK2 in cellen van het immuunsysteem, het zenuwstelsel en het hart. Zo is bijvoorbeeld bekend dat witte bloedcellen in patiënten met reumatoïde artritis of multiple sclerose (MS) een verlaagd GRK2 gehalte hebben. In patiënten met hartfalen of een verhoogde bloeddruk treedt echter een verhoging op van de hoeveelheid GRK2 in het hart en witte bloedcellen. In de hersenen treedt tijdens een diermodel voor neonatale hersenschade een verlaging op van het GRK2 gehalte. Het is echter nog niet onderzocht of de hoeveelheid GRK2 ook van belang is in situaties waarin de pijngevoeligheid is verhoogd. Wij denken dat een verlaging van GRK2 zou kunnen bijdragen aan een verhoogde gevoeligheid van GPCR (aanwezig op het oppervlak van zenuwcellen) die betrokken zijn bij het ontstaan van verhoogde pijngevoeligheid.

In dit proefschrift (**hoofdstuk 2** en **3**) onderzoeken wij in twee diermodellen voor neuropathische pijn of het gehalte GRK2 in het ruggenmerg is veranderd. In het eerste model

wordt de zenuwschade veroorzaakt door chronische constrictie van de nervus sciaticus (CCI). De nervus sciaticus is een zenuw die het gehele onderbeen innerveert. In het andere model wordt een spinale zenuw ter hoogte van de lumbale verdikking (niveau L5) doorgesneden (L5 SNT). Spinale zenuwen zijn de zenuwen die tussen twee wervels van de wervelkolom vanuit het ruggenmerg naar buiten komen. De spinale zenuw ter hoogte van L5 is verantwoordelijk voor de innervatie van een deel van het onderbeen. In beide modellen is de gevoeligheid voor mechanische prikkels getest met de "von Frey test". In deze test worden plastic naalden van oplopende diktes tegen de poot aangedrukt en wordt bepaald bij welke haardikte het dier reageert. Hoe eerder het dier reageert, hoe gevoeliger het dier is voor mechanische prikkels. In beide modellen veroorzaakt de zenuwschade een toename in de gevoeligheid voor mechanische prikkels (mechanische allodynie). Opvallend is dat in het CCI-model deze mechanische allodynie ook optreedt in de poot waar de zenuw niet is beschadigd. We zien in beide modellen een vermindering van de hoeveelheid GRK2 in de zenuwcellen van het ruggenmerg optreden. Deze verlaging in GRK2 is alleen terug te vinden in het deel van het ruggenmerg dat verantwoordelijk is voor de innervatie van de poten (lumbale deel) en niet op andere hoogtes in het ruggenmerg.

DE ROL VAN IL-1β BIJ HET VERLAGEN VAN DE HOEVEELHEID GRK2

Er zijn verschillende manieren beschreven om het gehalte van GRK2 in cellen te reguleren. Het gehalte van GRK2 in cellen kan worden verlaagd door de cellen te behandelen met bepaalde ontstekingsmediatoren (cytokinen), waaronder interleukine-1β (IL-1β). Het is tevens bekend dat IL-1β een zeer belangrijke rol speelt bij het verhogen van de pijngevoeligheid tijdens neuropathische pijn. In dit proefschrift onderzoeken wij of IL-1β ook betrokken zou kunnen zijn bij het verlagen van het GRK2 gehalte in het ruggenmerg tijdens neuropathische pijn. In een eerste experiment hebben we aangetoond dat chronische behandeling van gekweekte ruggenmergplakjes met IL-1β leidt tot een vermindering van het GRK2 gehalte (**hoofdstuk 2**). Daarnaast hebben we de rol van IL-1β onderzocht in een diermodel voor neuropathische pijn (**hoofdstuk 3**). Hierbij hebben we gebruikt gemaakt van muizen die geen IL-1 receptor hebben (IL-1 receptor knock-out muizen). In deze muizen kan IL-1β geen signalen tussen cellen meer doorgeven, omdat de cellen geen receptor hebben voor IL-1β. In IL-1 receptor knock-out muizen veroorzaakt L5 SNT geen verandering in de gevoeligheid voor mechanische prikkels; er treedt dus geen mechanische allodynie op. Tevens treedt in deze muizen geen verlaging van het GRK2 gehalte op in het ruggenmerg. Signalering door IL-1β lijkt dus niet alleen betrokken te zijn bij het ontstaan van een verhoogde pijngevoeligheid, maar ook bij het verlagen van GRK2 in het ruggenmerg.

LAAG GRK2: GEVOLGEN VOOR PIJNGEVOELIGHEID

In twee diermodellen, waarin mechanische allodynie optreedt, hebben we aangetoond dat een verlaging van GRK2 in zenuwcellen optreedt. Deze observatie wil nog niet zeggen dat verlaagd GRK2 inderdaad bijdraagt aan een verhoging van de pijngevoeligheid. In **hoofdstuk 2** en **4** hebben we onderzocht of laag GRK2 leidt tot een verhoging in de gevoeligheid voor pijnlijke prikkels. Hierbij hebben we gebruik gemaakt van GRK2 heterozygote muizen. In deze muizen is slechts 50% van het normale gehalte aan GRK2 in cellen aanwezig. De gevoeligheid voor mechanische prikkels en hitte is vergeleken tussen GRK2 heterozygote muizen en wild type (normale) muizen. De gevoeligheid voor mechanische prikkels is wederom bepaald met de "von Frey test" (hierboven beschreven). Voor de bepaling van de hittegevoeligheid hebben we gebruik gemaakt van de "Hargreaves test". In de Hargreaves test wordt de onderkant van de poot belicht met een warme laserstraal en vervolgens wordt bepaald na hoeveel tijd het dier zijn poot wegtrekt. Hoe korter deze tijd is, hoe gevoeliger het dier is voor hitte. Gezonde GRK2 heterozygote muizen blijken niet te verschillen van wild type muizen in de gevoeligheid voor zowel hitte als mechanische prikkels. In de muizen is vervolgens een pootontsteking veroorzaakt door in de voetzool carrageenan (een polysaccharide geisoleerd uit rood zeewier, dat een ontstekingsreactie opwekt) te injecteren. Deze ontsteking veroorzaakt een verhoging in de gevoeligheid voor zowel hitte als mechanische prikkels. In GRK2 heterozygote muizen is de pijngevoeligheid nog meer verhoogd in vergelijking met wild type muizen. Omdat beide muizensoorten eenzelfde ontstekingsreactie vertonen in de poot is een verhoogde ontstekingsreactie in GRK2 heterozygote muizen waarschijnlijk niet de oorzaak van deze verhoging in de gevoeligheid voor pijnlijke prikkels.

Uit deze resultaten blijkt dus dat een verlaging van GRK2 alleen niet genoeg is om een verhoogde pijngevoeligheid te veroorzaken, maar dat hiernaast dus nog een tweede, onbekend signaal, dat optreedt tijdens een ontstekingsreactie, nodig is. In **hoofdstuk 4** hebben we verschillende onderliggende mechanismen onderzocht om de verhoogde pijngevoeligheid in GRK2 heterozygote muizen tijdens ontsteking te verklaren. Wat het tweede signaal is om de verhoogde pijngevoeligheid tijdens laag GRK2 te veroorzaken, hebben we nog niet helemaal opgehelderd. Wij suggereren dat tijdens ontsteking, weefsel- of zenuwbeschadigingen, de productie van ontstekingsstoffen die de GPCR activeren op zenuwcellen nodig is om de pijngevoeligheid te verhogen. Veel studies hebben inderdaad aangetoond, dat tijdens ontstekingsgerelateerde pijn in de periferie en in het ruggenmerg productie optreedt van meerdere GPCR activatoren: chemokinen (CCL2, CCL3, CCL5), prostaglandines, ATP en noradrenaline. Deze mediatoren zijn niet aanwezig onder normale omstandigheden. Wanneer één van deze mediatoren (CCL3) in de poot wordt ingespoten veroorzaakt dit een verhoogde gevoeligheid voor mechanische prikkels (mechanische allodynie), die inderdaad hoger is in GRK2 heterozygote muizen in vergelijking met wild type muizen (**hoofdstuk 4**). Pijnlijke prikkels leiden tot de activering van de receptor TRPV1, die aanwezig is op zenuwcellen. Active-

ring van GPCR door CCL3 en prostaglandine E2 leidt tot het aanzetten van boodschappereiwitten, welke een proces in gang zetten, wat leidt tot een verhoogde gevoeligheid van TRPV1 en dus een verhoogde gevoeligheid voor pijnlijke prikkels. Bij laag GRK2 zou een verhoogde activering van deze boodschappereiwitten kunnen optreden, die dus gepaard gaat met een nog hogere gevoeligheid van deze receptor en dus een verhoogde pijngevoeligheid.

REGULATIE VAN GPCR FUNCTIE DOOR GRK2: NIEUWE MECHANISMEN

Naast de hierboven beschreven klassieke manier van GPCR desensitisatie kan GRK2 de functie van GPCR ook op twee andere manieren reguleren. GRK2 kan aan een bepaald onderdeel van het G-eiwit (Gα-eiwit) binden. Door de binding van GRK2 aan het Gα-eiwit remt GRK2 het aanzetten van boodschappereiwitten door het G-eiwit. Voor deze manier van regulatie is het deel van GRK2 dat betrokken is bij fosforylering van de eiwitten niet noodzakelijk. Meer recente studies hebben aangetoond dat GRK2 ook zelf kan binden aan bepaalde boodschappereiwitten (bijv. MEK1/2, PI3 kinase en Akt). Op deze manier reguleert GRK2 het aanzetten van boodschappereiwitten.

In **hoofdstuk 5** is de regulatie door GRK2 van de receptor, die wordt geactiveerd door het chemokine CCL2, onderzocht. Wij hebben gekozen voor CCL2, omdat is beschreven dat deze chemokine een belangrijke rol speelt bij het ontstaan van ontstekingsgerelateerde en neuropathische pijn. Deze receptor zou dus een belangrijk doelwit kunnen zijn voor de regulatie van GRK2. Om te onderzoeken of GRK2 het aanzetten van boodschappereiwitten door CCL2 reguleert, hebben wij gebruik gemaakt van gekweekte astrocyten, die we hebben geïsoleerd uit de hersenen (cortex) van muizenpups. Vervolgens hebben wij bepaald of een verandering in de hoeveelheid GRK2, aanwezig in de cel, invloed heeft op het aanzetten van boodschappereiwitten door CCL2. Het gehalte van GRK2 in gekweekte astrocyten is verhoogd door de cellen gedurende 24 uur te behandelen met IL-1β. Daarnaast hebben we astrocyten gebruikt van GRK2 heterozygote muizen. In deze muizen is slechts 50% van het normale gehalte aan GRK2 in de cellen aanwezig.

Wij laten zien dat zowel de activering van het boodschappereiwit MEK1/2 als de verhoging van calcium in de cel door CCL2 niet wordt beïnvloedt door de hoeveelheid GRK2. Activering van MEK1/2 is vervolgens verantwoordelijk voor activering van het boodschappereiwit ERK1/2. De mate van ERK1/2 activering is echter wel aan verandering onderhevig wanneer meer of minder GRK2 in de cel aanwezig is. Een hogere hoeveelheid GRK2 remt ERK1/2 activering, terwijl bij een lagere hoeveelheid GRK2 juist meer ERK1/2 wordt geactiveerd. Voor de regulatie van ERK1/2 activering door GRK2 blijkt het deel van GRK2, dat betrokken is bij fosforylering van eiwitten, niet noodzakelijk te zijn.

Omdat zowel MEK1/2 als calcium niet worden beïnvloed door de hoeveelheid GRK2, is het niet logisch dat GRK2 de functie van de receptor voor CCL2 reguleert via de klassieke manier

van desensitisatie of via de binding van GRK2 aan het Gα-eiwit. Beide manieren van regulatie zouden namelijk juist wel een verandering in MEK1/2 en calcium teweeg moeten brengen. Wij veronderstellen dat binding van GRK2 aan MEK1/2 de activering van ERK1/2 reguleert, zoals al in een eerdere studie is aangetoond.

Naast de MEK1/2-ERK1/2 route hebben we ook een tweede route van boodschappereiwitten onderzocht, namelijk de route PI3 kinase-PDK-1-Akt. We hebben laten zien dat de activering van zowel PDK-1 als Akt wordt gereguleerd door GRK2. Voor deze regulatie is echter juist wel weer het deel van GRK2 nodig dat betrokken is bij fosforylering van eiwitten. Wij suggereren dat GRK2 direct of indirect de activering van PI3 kinase remt, wat vervolgens verantwoordelijk is voor de remming van de activering van PDK-1 en Akt.

CONCLUSIES

De studies, die staan beschreven in dit proefschrift, tonen een belangrijke en nieuwe rol aan voor GRK2 bij de regulatie van gevoeligheid voor pijnlijke prikkels tijdens ontsteking en zenuwbeschadiging. Laag GRK2 leidt tot een verhoging van de gevoeligheid voor zowel hitte als mechanische prikkels tijdens ontstekingsprocessen. Verder is neuropathische pijn geassocieerd met een verlaging van het GRK2 gehalte in zenuwcellen in het ruggenmerg. Hiermee samenhangend is aangetoond dat het cytokine IL-1β een cruciale rol speelt bij zowel de ontwikkeling van mechanische allodynie als het verlagen van GRK2 tijdens zenuwbeschadigingen. In de toekomst is het van belang om het onderliggende mechanisme van regulatie van pijngevoeligheid door GRK2 verder te onderzoeken. Ook zou de betrokkenheid van andere GRKs en arrestines bij de ontwikkeling van ontstekingsgerelateerde pijn een belangrijk onderwerp kunnen zijn van toekomstig onderzoek. Wanneer de rol van GRK2 bij het ontstaan van een verhoogde gevoeligheid voor pijnlijke prikkels is opgehelderd, zou farmacologische modulatie van GRK2 expressie een belangrijk doelwit kunnen zijn voor behandeling van patiënten met ontstekingsgerelateerde en neuropathische pijnklachten.

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 12 oktober 1979 geboren te Gouda en groeide op in Ouderkerk aan den IJssel. Na het behalen van het VWO-diploma in 1998 aan het Krimpenerwaard College te Krimpen aan den IJssel, begon zij in datzelfde jaar met de studie Medische Biologie aan de Universiteit Utrecht. Als onderdeel van deze studie volgde zij een tweetal stages. De eerste stage werd uitgevoerd bij de afdeling Medische Microbiologie van het Universitair Medisch Centrum Utrecht onder begeleiding van prof. dr. Jos van Strijp en dr. Carla de Haas. Als vervolg op deze stage heeft zij vier maanden als research analist gewerkt bij de afdeling Medische Microbiologie. De andere stage werd doorlopen bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht, alwaar zij werd begeleid door prof. dr. Marc Timmers en dr. Chris van Oevelen. Het doctoraalexamen werd in februari 2003 cum laude behaald. Vanaf mei 2003 was zij werkzaam als promovendus bij het laboratorium Psychoneuroimmunologie van het Wilhelmina Kinderziekenhuis, onderdeel van het Universitair Medisch Centrum Utrecht. Tijdens deze periode stond zij onder begeleiding van prof. dr. Cobi Heijnen en dr. Annemieke Kavelaars. De resultaten van dit onderzoek zijn weergegeven in dit proefschrift.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

AR	adrenergic receptor
BDNF	brain-derived neurotrophic factor
CCI	chronic constriction injury
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
COX	cyclooxygenase
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
CX_3CL1	$C\text{-}X_3\text{-}C$ chemokine ligand
DAG	diacylglycerol
DRG	dorsal root ganglia
EAE	experimental autoimmune encephalomyelitis
GFAP	glial fibrillary acidic protein
GPCR	G protein-coupled receptor
GRK	GPCR kinase
HT	hydroxytryptamine
IBD	inflammatory bowel disease
IFN	interferon
IL	interleukin
IP3	inositol 1,4,5-trisphosphate
KC	keratinocyte-derived chemokine
MAPK	mitogen-activated protein kinase
mGluR	metabotropic glutamate receptor
NGF	nerve growth factor
NK-1R	neurokinin-1 receptor
NO	nitric oxide
NOS	nitric oxide synthase
PGE	prostaglandin E
PH	pleckstrin homology
PI	phosphatidylinositol
PI3 kinase	phosphoinositide-3 kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PIP3	phosphatidylinositol 3,4,5-trisphosphate

PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
RGS	regulators of G protein signaling
RH	RGS homology
ROS	reactive oxygen species
SIN	sciatic inflammatory neuritis
SNI	spinal nerve injury
SNL	spinal nerve ligation
SNT	spinal nerve transection
SP	substance P
TNF	tumor necrosis factor
TRPV1	transient receptor potential vanilloid subtype 1
WT	wild type