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G Protein-Coupled Receptor Kinase 2 in Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis

Anne Vroon,* Annemieke Kavelaars,* Volker Limmroth,† Maria Stella Lombardi,* Marion U. Goebel,‡ Anne-Marie Van Dam,§ Marc G. Caron,¶ Manfred Schedlowski,** and Cobi J. Heijnen†*†

Many modulators of inflammation, including chemokines, neuropeptides, and neurotransmitters, signal via G protein-coupled receptors (GPCR). GPCR kinases (GRK) can phosphorylate agonist-activated GPCR thereby promoting receptor desensitization. Here we describe that in leukocytes from patients with active relapsing-remitting multiple sclerosis (MS) or with secondary progressive MS, GRK2 levels are significantly reduced. Unexpectedly, cells from patients during remission express even lower levels of GRK2. The level of GRK2 in leukocytes of patients after stroke, a neurological disorder with paralysis but without an autoimmune component, was similar to GRK2 levels in cells from healthy individuals. In addition, we demonstrate that the course of recombinant myelin oligodendrocyte glycoprotein (1–125)-induced experimental autoimmune encephalomyelitis (EAE), an animal model for MS, is markedly different in GRK2−/− mice that express 50% of the GRK2 protein in comparison with wild-type mice. Onset of EAE was significantly advanced by 5 days in GRK2−/− mice. The earlier onset of EAE was associated with increased early infiltration of the CNS by T cells and macrophages. Although disease scores in the first phase of EAE were similar in both groups, GRK2−/− animals did not develop relapses, whereas wild-type animals did. The absence of relapses in GRK2−/− mice was associated with a marked reduction in inflammatory infiltrates in the CNS. Recombinant myelin oligodendrocyte glycoprotein-induced T cell proliferation and cytokine production were normal in GRK2−/− mice. We conclude that down-regulation of GRK2 expression may have important consequences for the onset and progression of MS. The Journal of Immunology, 2005, 174: 4400–4406.

Multiple sclerosis (MS) is a demyelinating chronic inflammatory disease of the CNS. Infiltration of activated T lymphocytes and macrophages into the brain and spinal cord is an important factor in the pathogenesis of this disease. The family of G protein-coupled receptors (GPCR) plays an important role in regulation of inflammation by a variety of ligands, including chemokines, leukotrienes, prostaglandins, neurotransmitters, and adrenergic agonists (1). GPCR activate intracellular effector enzymes via coupling to heterotrimeric G proteins. The signaling event produced by agonist-induced activation of these receptors is rapidly attenuated by a phenomenon called homologous receptor desensitization. This phenomenon occurs as a consequence of receptor phosphorylation by GPCR kinases (GRK) (2, 3). GRK-mediated phosphorylation uncouples the receptor from the G protein, which abrogates receptor signaling. In addition, GRK-mediated receptor phosphorylation facilitates subsequent binding of β-arr teins, which promotes GPCR internalization (3, 4).

Cells of the immune system express particularly high levels of GRK2 protein (5, 6) and its expression level is precisely regulated. For example, proinflammatory cytokines and oxygen radicals induce a down-regulation of GRK2 expression in PBMC (7, 8). Moreover, in vivo, the expression of GRK2 protein in cells of the immune system is altered in a number of disease states, including hypertension, cardiac failure, and the chronic inflammatory disease rheumatoid arthritis (7, 9–11). The intracellular level of GRK2 is an important factor in determining the response of multiple GPCR to agonist stimulation. For example, overexpression of GRK2 in cell lines inhibits signaling of a number of receptors including the chemokine receptors CCR5 and CCR2b (12, 13), the β2-adrenergic receptor (14), and the metabotropic glutamate receptor (15). Conversely, we have recently described that the 50% decrease in GRK2 expression in T cells from mice that are heterozygous for deletion of the GRK2 gene (GRK2−/+ mice) results in an increased chemotactic response to the chemokines MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) that signal via CCR5 (16). Reduced cellular levels of GRK2 can increase not only the response to chemokines, but also of multiple other GPCR, including β2-adrenergic receptors. We have shown that leukocytes from patients with rheumatoid arthritis express a reduced level of GRK2. The reduced level of GRK2 in cells from patients with rheumatoid arthritis was associated with an increased inhibition of TNF-α production by a β2-adrenergic agonist (7). Similarly, the cAMP response of splenocytes from GRK2−/+ mice to β-adrenergic stimulation was significantly higher than that of wild-type (WT) control cells (M. S. Lombardi, unpublished observations).
Knowing that GRK2 can modulate the activity of multiple GPCR involved in regulation of inflammation and that chronic inflammation can reduce GRK2 levels, we hypothesized that changes in GRK2 expression will contribute to the pathogenesis of MS. We analyzed expression of GRK2 in PBMC from patients with active relapsing-remitting MS (RR-MS), patients with secondary progressive MS, and patients with RR-MS during remission. To get more insight in the clinical significance of low GRK2 levels for the course of MS, we followed the clinical course of relapsing-remitting experimental autoimmune encephalomyelitis (EAE) in heterozygous GRK2+/− mice, which have a 50% reduction in GRK2 protein expression.

**Materials and Methods**

**Patients**

Fourteen patients suffering from an acute exacerbation of RR-MS (active RR-MS: 8 females, 6 males, mean age 35.7 ± 9.8 years; expanded disability status scale (EDSS): 3.3 ± 1.4, and disease duration 4.7 ± 5.8 years), 9 patients with secondary progressive MS (4 females, 5 males; EDSS 5.6 ± 1.2 and disease duration 13.3 ± 5.5 years, 7 patients (6 male, 1 female) with RR-MS in remission (no exacerbations during the past 3 months) and median age 45 ± 4.5, EDSS 2.5 ± 1.4 and disease duration 5.4 ± 3.3 years), and 15 healthy gender- and age-matched controls were included in the study. As an additional neurological disease control we included 6 male patients after stroke (age 71 ± 7.2 years, time since stroke 3–6 days, treatment with low dose aspirin). Subjects were recruited from the Department of Neurology, University Hospital Essen, Germany and the Department of Neurology of the VU Medical Center (Amsterdam, The Netherlands). The study was approved by the medical ethical committee of the Medical Faculty of the University of Essen, Germany and participants signed an informed consent. Seven blood samples from active RR-MS patients were collected before any treatment, and seven blood samples were from MS patients on steroid treatment (1000–5000 mg methylprednisolone). Samples from patients with secondary progressive MS were collected following treatment with steroids (n = 4), mitoxantrone (10 mg/m² ± 2) or steroids/mitoxantrone (n = 3). PBMC were isolated from heparinized whole blood by Ficoll-Isopaque (Pharmacia) density gradient centrifugation. We included samples from a separate group of patients with RR-MS during the remission phase of RR-MS, GRK2 levels in PBMC were incubated in 10% sucrose for at least 24 h. Immunohistochemical analyses were performed on 10-μm cryostat sections of spinal cord, cerebellum, and brain stem. Endogenous peroxidase activity was inactivated with 0.3% H₂O₂ in PBS for 20 min. Sections were incubated for 48 h at 4°C in TBS/0.5% Triton X-100 containing 2% BSA (Sigma-Aldrich) with rat-anti-mouse Abs against F4/80 (Serotec), CD45 (clone MP33), CD3 (clone KT3), CD4 (clone GK1.5), or CD8 (clone 33.6-67). (Abs were a generous gift of Dr. R. Mebius, Department of Cell Biology, VU Medical Center, Amsterdam, The Netherlands). Subsequently, sections were incubated for 2 h at room temperature with mouse anti-rat biotinylated IgGs (Jackson ImmunoResearchLaboratories, followed by Vectastain ABC kit (Vector Laboratories). The specificity of the immunoreactivity was determined by incubating sections without primary Ab, which yielded no staining (data not shown).

The frequency of inflammatory infiltrates in anti-CD45 stained sections of spinal cord or cerebellum from 3 to 4 mice per group was quantified using a morphometric lattice placed in the microscope ocular lens. At least 20 random fields (ocular ×10, objective ×10) from at least 4 sections per animal were scored. The number of infiltrating CD3-positive T cells, the number of activated microglia/macrophages (F4/80-positive cells) and the ratio of CD4- and CD8-positive cells was determined by analyzing at least 100 random high power fields (ocular ×10, objective ×100) per animal.

**Splenocyte proliferation and cytokine production**

Spleens were collected from naïve mice or from mice at day 48 after immunization with rMOG. Splenocytes were obtained by dispersion through filter chambers (NBP). Subsequently, RBC were lysed and splenocytes were resuspended in culture medium (RPMI 1640; Invitrogen Life Technologies) supplemented with 5% FCS (Invitrogen Life Technologies), 2 mM l-glutamine, 100 μM penicillin, 100 μg/ml streptomycin, and 50 μg/ml 2-ME. Splenocytes were cultured at 1.5 × 10⁴ cells per well with Con A or at 5 × 10⁴ cells per well with Con A or at 5 × 10⁴ cells per well with rMOG. After 48–72 h of culture, supernatants were collected and cytokines were determined by ELISA (IFN-γ, U-Cytech; IL-10, OptEIA kits, BD Pharmingen), Incorporation of [3H]thymidine (1 μCi/well; Amersham) was determined by pulsing cultures for 15–20 h.

**Anti-MOG Abs**

The level of MOG-specific IgG was determined by ELISA in serum obtained at 48 days after immunization as described (18).

**Statistical analysis**

Data represent mean ± SEM and were analyzed using Student’s t test. Results obtained in animal experiments were confirmed in at least two independent experiments, using animals from different litters.

**Results**

**GRK2 protein levels in PBMC from MS patients and controls**

We analyzed the expression of GRK2 in PBMC from patients with RR-MS, suffering from an acute exacerbation of the disease. GRK2 expression in PBMC from healthy gender and age-matched individuals served as a control. Levels of immunodetectable GRK2 in PBMC from patients with active RR-MS were significantly decreased by ~40% compared with GRK2 levels in PBMC from healthy individuals (Fig. 1). Similarly, we observed an ~40% decrease in the level of GRK2 in PBMC from patients with secondary progressive MS (Fig. 1). The decrease in GRK2 levels does not reflect an overall down-regulation in protein expression, because expression of the GRK cofactor β-arrestin as well as β-actin levels were similar in patients and controls (Fig. 1).

There was no significant correlation between the level of GRK2 and gender, age, disability status (EDSS) or disease duration. In addition, GRK2 expression did not differ between active RR-MS patients receiving steroid treatment (n = 7) or no treatment (n = 7; p = 0.912). These data suggest that treatment did not have a major effect on GRK2 expression.

To investigate whether the decrease in GRK2 during an acute exacerbation of RR-MS was related to disease activity, we also included samples from a separate group of patients with RR-MS during the remission phase of the disease. Interestingly, also during the remission phase of RR-MS, GRK2 levels in PBMC were
PBMC, we included a group of patients after stroke. GRK2 levels out an autoimmune component also affects expression of GRK2 in

\[ \text{Expression of GRK2 in PBMC from RR-MS patients in } \]

decreased as compared with healthy controls (Fig. 2). More importantly, the level of GRK2 in PBMC from patients with RR-MS in remission was even lower than in patients with active RR-MS (active RR-MS: 65.2 ± 10% of GRK2 in healthy individuals; RR-MS in remission: 29 ± 9.8% of healthy individuals; \( p < 0.05 \)).

To test whether a neurological disorder with paralysis, but without an autoimmune component also affects expression of GRK2 in PBMC, we included a group of patients after stroke. GRK2 levels after stroke were similar to GRK2 levels in healthy individuals (87 ± 11% of healthy individuals).

Because we studied a mixed population of cells (PBMC) it is possible that the decrease in GRK2 in PBMC from MS patients is limited to certain subsets of cells and/or that altered composition of the PBMC population is responsible for the observed decrease in GRK2. To address this possibility, we analyzed the expression of GRK2 in CD4\(^+\) cells, CD8\(^+\) cells, and a T cell-depleted fraction of PBMC from healthy individuals and active RR-MS patients. Expression levels of GRK2 were similar in all subsets tested. Moreover, the decrease in GRK2 was observed in all cell fractions tested, and the extent of the decrease was similar in all fractions (Fig. 3). Similarly, the more pronounced reduction in GRK2 in cells from RR-MS patients in remission was observed in all subsets (data not shown). Therefore, we conclude that the observed decrease in GRK2 cannot be attributed to subset-specific effects.

**Course of EAE in GRK2\(^{-/-}\) mice**

To determine whether the reduction in GRK2 expression as observed in patients with MS can contribute to the pathophysiology of the disease, we induced EAE in heterozygous GRK2\(^{-/-}\) mice and in WT littermate controls. GRK2\(^{-/-}\) mice show a 50% reduction in GRK2 protein levels in various cells and organs, including lymphocytes (16, 19), and therefore provide an excellent model to analyze the contribution of reduced GRK2 to the course of EAE. Due to embryonic lethality, GRK2\(^{-/-}\) animals cannot be used. Relapsing-remitting EAE was induced by s.c. administration of rMOG in Freund’s complete adjuvant. In WT mice, onset of clinical signs occurred on day 13 after immunization (mean day of onset 13.3 ± 0.8; Fig. 4A). Interestingly, disease onset in the GRK2\(^{-/-}\) mice was significantly advanced by 5 days (mean day of onset 8.6 ± 0.4; \( p < 0.001 \), Fig. 4A).

Maximal disease scores during the acute phase (determined as maximal score between days 7 and 20 postimmunization) did not differ between GRK2\(^{-/-}\) mice (mean score 2.1 ± 0.3) and WT mice (mean score 2.7 ± 0.5). The incidence of EAE was similar in both groups (>83% in all experiments).

When we followed progression of EAE over the course of 48 days, we observed a marked difference between WT and GRK2\(^{-/-}\) mice. Immunization of C57BL/6 mice with rMOG in Freund’s complete adjuvant (Fig. 4A). Surprisingly, GRK2\(^{-/-}\) mice only developed an acute phase of the disease without relapses. In GRK2\(^{-/-}\) animals, disease scores gradually decreased over time (Fig. 4A). As mentioned before, it should be noted that GRK2\(^{-/-}\) and WT animals developed similar maximal disease scores during the acute phase.

**Early cellular infiltration into the CNS**

CCR5-induced infiltration of inflammatory cells into the CNS has been implicated in the pathogenesis of EAE and MS (20, 21). We have recently described that the chemotactic response of GRK2\(^{-/-}\) T cells toward CCR5-binding chemokines is significantly increased, as compared with the chemotactic response of T cells from WT animals (16). Therefore, we hypothesized that the earlier onset of EAE in GRK2\(^{-/-}\) mice is associated with increased infiltration of inflammatory cells into the CNS. To test this
hypothesis, we determined leukocyte infiltration into the CNS on day 13 after immunization. The data in Fig. 4 show that earlier onset of EAE in GRK2/H11001/H11002 mice was associated with markedly higher leukocyte-specific CD45 immunoreactivity in spinal cord as determined on day 13 after immunization. The quantitative data shown in Table I confirm these observations and show that the number of cellular infiltrates in the spinal cord on day 13 was significantly higher in GRK2/H11001/H11002 animals than in WT animals.

More detailed investigation of the infiltrating cells showed that on day 13, the number of activated macrophages/microglia (F4/80-positive cells) and the number of T cells (CD3-positive cells) in the spinal cord of GRK2/H11001/H11002 mice was markedly elevated compared with WT mice (Fig. 5 and Table I). Increased immunoreactivity for CD45, CD3, and F4/80 was also observed in the brain stem and cerebellum of GRK2/H11001/H11002 animals compared with WT animals (data not shown). To further characterize the infiltrating T cells, we also analyzed CD4 and CD8 immunoreactivity. In the spinal cord of both WT and GRK2/H11001/H11002 mice on day 13, all infiltrating T cells were CD4 positive. We could not detect CD8-positive cells in the infiltrates on day 13 (data not shown).

Late cellular infiltration in the CNS

To investigate whether the lack of relapses in GRK2/H11001/H11002 mice was associated with changes in the number of inflammatory infiltrates or in the number of T cells and activated microglia/macrophages in the CNS, we analyzed the cerebellum on day 45 after immunization. At day 45 after immunization, when GRK2/H11001/H11002 mice no longer show signs of disease whereas WT animals still develop relapses, we observed a significantly lower number of inflammatory infiltrates in the cerebellum of GRK2/H11001/H11002 animals (Fig. 6 and Table II).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>GRK2+/−</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of infiltrates/mm²</td>
<td>0.5 ± 0.2</td>
<td>2.8 ± 0.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Number of infiltrating T cells</td>
<td>5.1 ± 0.7</td>
<td>27.2 ± 7.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Number of activated microglia/macrophages</td>
<td>8.3 ± 2.7</td>
<td>52.1 ± 10.8</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

The number of inflammatory infiltrates after staining of spinal cord slices with anti-CD45 Ab was determined as described in Materials and Methods.
The number of inflammatory infiltrates after staining of spinal cord slices with anti-CD45 Ab, the number of infiltrating T cells (CD3-positive cells), and the number of activated microglia/macrophages (F4/80-positive cells) were determined as described in Materials and Methods.

Table II. The number of T cells (CD3+ and CD4+/CD8+) and the number of F4/80-positive activated microglia/macrophages was significantly lower in GRK2+/− mice than in WT animals (Fig. 6 and Table II). The difference between GRK2+/− and WT animals was more pronounced for T cells (GRK2+/−: 16% of WT) than for activated microglia/macrophages (GRK2+/−: 36% of WT). The ratio of CD4- and CD8-positive cells in the cerebellum of WT and GRK2+/− mice was similar (WT: 3.8 ± 0.5; GRK2+/−: 3.2 ± 0.5).

Anti-MOG Abs
Ab-mediated demyelination may contribute to the pathogenesis and severity of MOG-EAE (22, 23). Therefore, we determined the level of anti-MOG IgG in serum obtained from WT and GRK2+/− mice at day 48 after immunization.

WT and GRK2+/− mice had similar levels of MOG-specific IgG in serum (WT: 110 ± 26 U/ml; GRK2+/−: 135 ± 24 U/ml, NS), suggesting that the differences in the clinical course are not dependent on differences in the anti-MOG Ab response.

T cell proliferation and cytokine production
We examined whether the differences in the clinical course of EAE between WT and GRK2+/− mice could be explained by differences in the ability to respond to the specific immunizing Ag, rMOG. Therefore, we determined the in vitro rMOG-induced proliferative responses of splenocytes from GRK2+/− and WT mice at day 48 postimmunization. The MOG-induced proliferative response of GRK2+/− and WT splenocyte was similar (Table III).

Table III. Proliferative response and cytokine production of splenocytes from WT and GRK2+/− mice

<table>
<thead>
<tr>
<th>MOG (µg/ml)</th>
<th>WT</th>
<th>GRK2+/−</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAE animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferation (S.I.)</td>
<td>100</td>
<td>3.23 ± 0.52</td>
<td>3.58 ± 0.37</td>
</tr>
<tr>
<td>IFN-γ (ng/ml)</td>
<td>10</td>
<td>170.0 ± 15.5</td>
<td>144.5 ± 23.1</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>10</td>
<td>236.7 ± 22.0</td>
<td>225.7 ± 34.6</td>
</tr>
<tr>
<td>Naive animals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferation (S.I.)</td>
<td>Con A</td>
<td>12.8 ± 2.2</td>
<td>15.9 ± 3.6</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>Con A</td>
<td>3590 ± 751</td>
<td>2987 ± 426</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>Con A</td>
<td>98.5 ± 29</td>
<td>92.9 ± 18.1</td>
</tr>
</tbody>
</table>

The proliferative response of naive splenocytes to the T cell mitogen Con A was not different between WT and GRK2+/− cells either (Table III). Similar results were obtained after stimulation with 0.25 and 0.5 µg/ml Con A (data not shown).

Because Th1-type cytokines are thought to play an important role in the pathogenesis of EAE, it is possible that differences in the capacity of lymphocytes from GRK2+/− and WT animals to produce Th1- vs Th2-type cytokines contribute to changes in the course of the disease. Therefore, we examined rMOG- as well as mitogen-induced IL-10 and IFN-γ production by splenocytes from WT and GRK2+/− animals. Data in Table III show that there was no difference between WT and GRK2+/− cells with respect to IL-10 or IFN-γ production after stimulation with rMOG or after stimulation with the mitogen Con A. Finally, splenocytes from untreated mice were phenotypically analyzed by flow cytometry. No significant difference in percentages of CD4+ and CD8+ cells, B cells, macrophages, and neutrophils were observed in GRK2+/− mice vs WT mice thereby excluding a possible effect of decreased GRK2 on the cellular composition of the spleen (data not shown).

Discussion
In the present study, we describe a significant reduction of GRK2 protein expression in PBMC from MS patients compared with healthy donors. The data suggest that down-regulation of GRK2 is at least a peripheral marker for MS, even though the inflammatory process occurs predominantly beyond the blood-brain barrier. More importantly, however, we show that there is a clear relation between the clinical phase of MS and the extent of GRK2 down-regulation. In patients with active RR-MS and in patients with secondary progressive MS, GRK2 is reduced by ~40%. Unexpectedly, the decrease in GRK2 was even more pronounced during the remission phase in patients with RR-MS; during this phase GRK2 levels were decreased by ~70%. The data we present here were obtained in a cross-sectional study and careful longitudinal studies are now needed to further delineate the relation between changes in GRK2 and the course of MS.

In an earlier study, we have shown that also during an active phase of the inflammatory autoimmune disease, rheumatoid arthritis, GRK2 levels in PBMC are decreased (7). In vitro, it has been shown that the proinflammatory cytokines IL-1β, TNF-α, IL-6, and IFN-γ, as well as oxidative stress can down-regulate GRK2 protein levels (7, 8, 24). In addition, activation of chemokine receptors can stimulate the degradation of GRK2 (25). Although MS is an inflammatory disease of the CNS, increased levels of cytokines and chemokines can be detected in the circulation of both acute RR-MS and secondary progressive MS patients (20, 26). The systemic effects of these inflammatory mediators might thus be involved in the observed down-regulation of GRK2 expression in PBMC of active RR-MS patients. During the remission phase of RR-MS the balance between pro- and anti-inflammatory cytokines changes (27, 28). It remains to be determined whether anti-inflammatory cytokines that are thought to play a role in MS remission, e.g., TGF-β, IL-4, and IL-10, are more potent in reducing GRK2 levels than the proinflammatory cytokines.

Previous studies have already suggested that changes in GRK2 can contribute to the induction and/or severity of diseases in which GPCR play a pivotal role. Hypertension and chronic heart failure are associated with increased expression and activity of GRK2 in PBMC (9, 10). Interestingly, vascular targeted overexpression of GRK2 in mice was shown to induce hypertension and cardiac hypertrophy (29). Moreover, targeted cardiac overexpression of a GRK2 dominant-negative mutant prevented the development of cardiomyopathy in a murine model of heart failure (30). These data clearly implicate the functional importance of “normal” GRK2.
levels for the cardiovascular system. Our present data point to an important novel role for GRK2 expression levels in onset and progression of the inflammatory autoimmune disease MS. The earlier onset of the acute phase and absence of relapses of EAE in GRK2-/-/- animals and the more pronounced reduction in GRK2 levels during the remission phase of RR-MS suggests that low GRK2 levels may have both pathogenic and protective effects.

The most investigated GPCR in EAE are receptors for chemokines and chemotaxtractants and a number of these molecules are thought to be involved in the onset and regulation of EAE. We have recently shown that activated T cells from GRK2-/-/- mice have an increased chemotactic response toward the chemokines RANTES (CCL5), MIP-1β (CCL4), and MIP-1β (CCL3). Moreover, signaling toward ERK, protein kinase B, and calcium is increased in GRK2-/-/- T cells stimulated with these chemokines (16). In addition, studies in overexpression systems demonstrated that GRK2 is involved in regulation of the activity of the chemokine receptors CCR5, CCR2b, CXCR1 as well as of the chemotaxtractant receptors C5aR, C3aR, and FMLP (1). In these studies, it has been shown that increased levels of GRK2 reduced the response of cells to stimulation with their ligand. It is known that activation of chemokine receptors and increased production of chemokines in the CNS play an important role in regulating migration of inflammatory cells into the brain. For example, treatment of animals with anti-MIP-1α Abs or MIP-1α naked DNA vaccine inhibits EAE and is associated with impaired infiltration of inflammatory cells in the CNS (31, 32). Similarly, treatment with an Ab against the CCR2-binding chemokine MCP-1 or MCP-1 naked DNA vaccine inhibits the development of EAE (31, 32). Our data showing that infiltration of cells into the CNS is increased in GRK2-/-/- mice in the early phase of the disease are compatible with the concept of increased responsiveness of cells to chemokines and chemotaxtractants in GRK2-/-/- animals. Together, these findings suggest a role for reduced GRK2 in the acute phase of EAE, via increased chemokine-induced inflammatory cell infiltration into the CNS.

The mechanism of the apparent protective effect of low GRK2 in GRK2-/-/- mice during the relapsing phase of EAE is more difficult to explain on the basis of existing literature. We show that the absence of relapses in the GRK2-/-/- mice is associated with a marked reduction in the number of inflammatory lesions in the CNS. Moreover, the number of infiltrating T cells (CD3+, CD4+, and CD8+ cells) as well as the number of macrophages/activated microglia in the CNS of GRK2-/-/- mice was reduced. However, the infiltrates in the CNS of WT and GRK2-/-/- did not differ with respect to the relative contribution of the various cell types.

Chemokines and chemokine receptors are thought to play a central role in the regulation of relapses in EAE. In general, however, data in the literature suggest that increased responsiveness to chemokines would lead to more infiltration and more severe relapses. However, there are also chemokine receptors involved in the attraction of regulatory T cells and Th2 T cells to the CNS. Increased intracerebral expression of the chemokine receptors CCR3 and CCR4, that are predominantly expressed on Th2 and on regulatory T cells (33), has been shown to correlate with protection from EAE (34). Moreover, regulatory T cells are involved in inhibition of disease activity during the relapsing-remitting phase of EAE. Prevention of the activity of regulatory T cells by in vivo treatment with anti-CD25 results in marked exacerbation of the disease (35). Although it is not known whether CCR3 and CCR4 are also regulated by GRK2, it might be possible that increased responsiveness of these chemokine receptors contributes to the absence of relapses in GRK2-/-/- mice.

Changes in the balance of Th1 and Th2 cytokines have been implicated in the regulation of remission and relapses during EAE. For example, intrathecal delivery of IFN-γ results in a clinical course of EAE similar to the EAE course in GRK2-/-/- animals: an earlier onset of EAE but a milder course of the disease during the relapsing-remitting phase (36). However, our data demonstrate that the capacity of MOG-specific T cells to produce IFN-γ is not increased in GRK2-/-/- mice, suggesting that this mechanism is not responsible for the observed absence of relapses. Moreover, the balance between IFN-γ and IL-10 production by Ag-specific T cells is not changed either, suggesting that changes in the activity of peripheral Th1/Th2 cells or regulatory T cells is not involved in the lack of relapses in GRK2-/-/- mice. Finally, Ab-mediated demyelination is thought to contribute to the pathogenesis and severity of MOG-EAE (22, 23). However, we do not have evidence for differences in anti-MOG Ab levels between GRK2-/-/- and WT mice.

Another, perhaps more likely, explanation for the lack of relapses in GRK2-/-/- mice may be that down-regulation of GRK2 increases the responsiveness of GPCR that can directly suppress the inflammatory activity in the CNS. Based on the current knowledge of receptors that are regulated by GRK2 and whose ligands are potential inhibitors of EAE, we can identify a number of candidates. For example, administration of a β2-adrenergic receptor agonist can decrease the number of relapses in rats with EAE (37). Interestingly, we have evidence that the sensitivity of the β2-adrenergic receptor on splenocytes from GRK2-/-/- mice is significantly increased; stimulation of the β-adrenergic receptor results in a larger increase in cAMP that is associated with a marked reduction in agonist-induced receptor internalization (M. S. Lombardi, A. Kavelaars, A. Vroon, and C. J. Heijne, unpublished observations). Moreover, we have shown previously that reduced GRK2 levels in patients with the inflammatory autoimmune disease rheumatoid arthritis are associated with enhanced β2-adrenergic agonist-induced cAMP formation and increased β2-adrenergic agonist-mediated inhibition of TNF-α production (7). Because cells from GRK2-/-/- mice are more sensitive to stimulation with β-adrenergic receptor agonists it is possible that the increased effect of the endogenous ligands (noradrenaline and adrenaline) may contribute to the absence of relapses in the GRK2-/-/- mice.

In conclusion, the more benign course of EAE in GRK2-/-/- mice and the fact that GRK2 expression is reduced the most during the remission phase in patients with MS, suggest that reduction of GRK2 activity during MS may be a novel target for therapy.

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Disclosures
The authors have no financial conflict of interest.

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