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Cell-specific roles of GRK2 in onset and severity of hypoxic-ischemic brain damage in neonatal mice

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Abstract

The ubiquitously expressed kinase GRK2 protects against cellular overstimulation by desensitizing G protein-coupled receptors and regulating intracellular signaling. Recently, we described that hypoxia-ischemia (HI)-induced brain damage was accelerated and increased in GRK2^{+/-} neonatal mice. Using Cre-Lox technology we now investigated the role of decreased GRK2 in only microglia/macrophages or forebrain neurons in development of HI brain injury.

Low GRK2 in microglia/macrophages (LysM-GRK2^{fl/+} mice) was sufficient to accelerate onset of HI damage, without affecting the severity of brain injury at 24h post-HI as compared to LysM-GRK2^{+/-} littermates. Consistently, the ipsilateral hemisphere of GRK2^{+/-} mice contained microglia with a more rounded phenotype compared to WT mice at 3h post-HI. Inhibition of microglial/macrophage activity by minocycline treatment prevented the early onset of HI injury in GRK2^{+/-} mice. *In vitro*, primary GRK2^{+/-} microglia stimulated with LPS produced more TNF- α than WT microglia via a p38-dependent pathway. *In vivo*, HI-induced cerebral p38 activation and TNF- α production were increased in GRK2^{+/-} mice or in LysM-GRK2^{fl/+} mice. Our findings indicate that low GRK2 in microglia/macrophages accelerates brain damage via a GRK2/p38/TNF- α -dependent pathway.

Reduced GRK2 only in forebrain neurons (CamKII α -GRK2^{fl/+} mice) significantly increased severity of HI brain damage without affecting the onset of brain damage.

In conclusion, our data indicate that low GRK2 in microglia/macrophages facilitates activation of these cells which may contribute to the earlier onset of cerebral HI injury associated with increased p38 phosphorylation and TNF- α production. The level of GRK2 in neurons is crucial for determining the ultimate severity of HI damage in the newborn brain.

Keywords

hypoxia-ischemia; G protein-coupled receptor kinase 2; microglia; p38; TNF- α ; minocycline; neonatal; neuron

Introduction

Perinatal hypoxia-ischemia (HI) often leads to serious and permanent brain injury in the human neonate. The underlying mechanisms leading to cell death in the brain after HI are numerous and complex and only partially understood (Ferriero, 2004).

We have shown previously that the level of G protein-coupled kinase 2 (GRK2) in whole brain homogenates is decreased after neonatal HI (Lombardi et al., 2004; Nijboer et al., 2008). Cerebral GRK2 expression is also reduced in animal models of Alzheimer and Parkinson disease (Burgos-Ramos et al., 2008; Suo et al., 2004; Ahmed et al., 2008). Moreover, GRK2^{+/-} mice that express 50% of the protein develop both *earlier* and *more pronounced* brain injury after exposure to neonatal HI (Nijboer et al., 2008). Homozygous GRK2^{-/-} mice die *in utero* (Jaber et al., 1996).

GRKs are key kinases involved in regulation of signaling via G protein-coupled receptors (GPCR) such as chemokine receptors and metabotropic glutamate receptors, via a process called homologous desensitization. More recently, it has been revealed that GRK2 also regulates cellular signaling by direct interaction with several downstream molecules including p38 MAP kinase (Reiter and Lefkowitz, 2006; Ribas et al., 2007). *In vitro* studies have demonstrated that GRK2 binds and phosphorylates p38 MAP kinase, thereby preventing activation of p38 (Peregrin et al., 2006). GRK2 can thus function as an endogenous p38 inhibitor *in vitro*. However, the *in vivo* relevance of changes in GRK2 for p38-dependent processes is unknown. Numerous factors that contribute to brain injury, like reactive oxygen species and cytokines, upregulate the activity of p38 (Cuenda and Rousseau, 2007). In addition, p38 is a major kinase involved in microglial activation and cytokine production in the brain (Koistinaho and Koistinaho, 2002). Activation of cerebral microglia and production of pro-inflammatory cytokines is thought to aggravate brain injury (Silverstein et al., 1997; Hedtjrn et al., 2004). In the literature, the general idea is that microglial activation is induced as a consequence of neuronal injury (Saliba and Henrot, 2001). More recent evidence suggests that in the neonatal HI brain microglia also contribute to an *early* phase of inflammation by increasing early cytokine expression (Dommergues et al., 2003; Fox et al., 2005; Bye et al., 2007; Nijboer et al., 2009).

The aim of this study was to delineate the role of GRK2 in microglia/macrophages or in forebrain neurons in determining the onset and magnitude of neonatal HI brain damage, p38 activation and cytokine production.

Materials and Methods

Animals

Experiments were performed in accordance with national guidelines (WOD, 1996) and were approved by the University Medical Center Utrecht experimental animal care committee.

We used heterozygous GRK2^{+/-} and wildtype (WT) C57BL/6J littermates (Jaber et al., 1996). Animals with cell-specific reduction of GRK2 were obtained using Cre-Lox technology; LysM-Cre^{+/+} males (B6.129P2-*Lyz2^{tm1(cre)}**Ifo*/J) or CamKII-Cre^{+/+} males ((Camk2a-cre)T29-1Stl/J; both Jackson Laboratory) were crossed with GRK2^{f/+} females maintained on a mixed 129/C57BL/6J background (Matkovich et al., 2006). Pups had either normal GRK2 levels (LysM-GRK2^{+/+} or CamKII-GRK2^{+/+}) or reduced GRK2 levels in microglia/monocytes or in neurons respectively (LysM-GRK2^{f/+} or CamKII-GRK2^{f/+}). This approach was chosen to avoid confounding effects due to differences in background; in each litter we always obtained Cre^{+/-}/GRK2^{+/+} control mouse pups (normal GRK2 levels) and Cre^{+/-}/GRK2^{f/+} experimental mouse pups (cell-specific reduction of GRK2).

At postnatal day 9, pups of both genders underwent HI by right common carotid artery occlusion followed by hypoxia (10% O₂/90% N₂) for 45 minutes (Rice III et al., 1981). Sham-treated controls underwent anesthesia and incision only. No differences between genders were observed on any of the parameters.

Mortality rates during HI procedures did not differ between the genotypes of the different strains; WT 13.2% vs. GRK2^{+/-} 17% (Nijboer et al., 2008); LysM-GRK2^{+/-} 15.6% vs. LysM-GRK2^{f/+} 13.6%; CamKII α -GRK2^{+/-} 14.3% vs. CamKII α -GRK2^{f/+} 14.6% (not significant using Fisher exact test).

To deplete animals of granulocytes, pups were treated with 28 μ l/g bodyweight rabbit-anti-mouse PMN antibody or normal rabbit serum (Accurate Antibodies, Westbury, NY) i.p. 20h before and directly after HI. Depletion of circulating neutrophils was confirmed by a differential blood count. To inhibit microglial activity, pups were treated directly prior to hypoxia with 45 mg/kg minocycline i.p. (Sigma-Aldrich).

Histological preparations and immunohistochemistry

At 3 h, 24 h or 3 weeks after HI, mice received an overdose of pentobarbital and were perfused with 4% paraformaldehyde in PBS. Brains were embedded in paraffin; coronal sections were cut at hippocampal level (-1.8 mm from bregma) and stained with hematoxylin-eosin (HE). For immunohistochemistry, sections were incubated with mouse-anti-MAP2 (Sigma-Aldrich, Steinheim, Germany) or rabbit-anti-Iba1 (Wako, Osaka, Japan) followed by biotinylated secondary antibodies (Vector laboratories, Burlingame, CA). Visualization was performed using Vectastain ABC kit (Vector Laboratories) and diaminobenzamidine (DAB).

Full HE- or MAP2-stained section images were captured with a Nikon D1 digital camera (Nikon, Tokyo, Japan). Whole hemisphere areas (HE-stained sections) or MAP2-positive brain areas for both hemispheres were outlined manually using image processing tools in Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA) and ipsilateral volume loss or MAP2 loss was calculated as follows: 1- (ipsilateral/contralateral area) (Nijboer et al., 2007).

Western Blotting

At 1 h or 3 h post-HI mice received an overdose pentobarbital followed by decapitation. The entire ipsi- and contralateral hemispheres were pulverized on liquid nitrogen and divided into 2 fractions; one fraction was used for protein extraction and the other fraction was used for mRNA extraction (see below). Pulverized hemispheres were homogenized in 70 mM sucrose, 210 mM mannitol, 5 mM HEPES, 1 mM EDTA and protease and phosphatase inhibitors using a Potter homogenizer (Heidolph, Schwabach, Germany). Cytosolic fractions were prepared as described (Nijboer et al., 2007). Protein concentration was determined using a protein assay (Bio-Rad, Hercules, CA).

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C, Amersham Biosciences, Roosendaal, the Netherlands). Membranes were stained with rabbit-anti-P-p38 or rabbit-anti-total p38 (both Cell Signaling, Danvers MA), rabbit-anti-GRK2 or goat-anti- β -actin (both Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with donkey-anti-rabbit-HRP (Amersham Biosciences) or donkey-anti-goat-HRP (Santa Cruz). Specific bands were visualised by chemiluminescence (ECL, Amersham Biosciences) with X-ray film exposure. Films were scanned with a GS-700 Imaging Densitometer and analyzed with Quantity One Software (Bio-Rad).

Quantitative Real Time reverse transcriptase (RT)-PCR

Total RNA was isolated from pulverized hemispheres with TRIzol® (Invitrogen, Paisley, UK). cDNA was synthesized with SuperScript Reverse Transcriptase (Invitrogen). The PCR reaction was performed with iQ5 Real-Time PCR Detection System (Bio-Rad) using primers for TNF- α . Sequences: TNF- α : forward: GCGGTGCCTATGTCTCAG, reverse: GCCATTTGGGAAGTCTCTCATC. To confirm appropriate amplification, size of PCR products was verified on gel. Data were normalized to expression of β -actin and GAPDH as described (Nijboer et al., 2008).

Primary microglia

Mixed primary cultures of cortical astrocytes and microglia were obtained from 1-day-old mice. Cortices were dissected after removal of meninges and blood vessels, minced and incubated with 0.25% trypsin for 15 min in Gey's balanced salt solution containing 100 U/ml penicillin, 100 μ g/ml streptomycin and 30 mM D(+)-glucose. Cells were dissociated and cultured in poly-L-ornithine (15 μ g/ml) coated culture flasks in DMEM/Ham's F10 (1:1) supplemented with 10% FCS, 2mM glutamine and antibiotics as stated above.

After 10–12 DIV, flasks were shaken overnight at 37°C to collect microglia. Microglia (>95% CD11b-positive) were cultured for 48h in poly-L-ornithine coated 24-well plates at a density of 0.5×10^6 cells/ml before the experimental procedures.

Plated microglia were pre-incubated for 1 h with p38 inhibitors SB203580 (20 μ M) or SB239063 (5 μ M) (both Sigma-Aldrich) or with 10 μ M or 100 μ M of the c-Jun N-terminal kinase (JNK) inhibitor TAT-JBD peptide (Nijboer et al., 2009) before stimulation with 10 ng/ml LPS from *E. Coli* (serotype 0127:B8; Sigma-Aldrich). Three hours after stimulation with LPS, supernatant was collected to determine TNF- α by ELISA (Ucytech, Utrecht, the Netherlands).

Total cell lysates of LysM-GRK2^{+/+} and LysM-GRK2^{f/+} microglia or astrocytes were prepared by incubating plated cells in ice-cold RIPA buffer (20 mM HEPES pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA and protease inhibitors) for 30 min at 4°C followed by 15 min centrifugation at 13,000 rpm.

Statistical Analysis

All data are presented as mean and SEM. One-way or two-way ANOVA with Bonferroni post-tests was used to analyze the effect of time and genotype.

Results

Role of microglial/macrophage GRK2 in HI brain damage

To determine the contribution of GRK2 in microglia/macrophages to onset and severity of brain damage, we used mice in which GRK2 was only reduced in lysozyme M (LysM)-positive cells (microglia/macrophages/granulocytes). GRK2 protein was ~50% reduced in isolated LysM-GRK2^{f/+} microglia compared to control LysM-GRK2^{+/+} microglia, but not in LysM-GRK2^{f/+} astrocytes (Fig. 1A). LysM is also expressed in granulocytes and therefore, GRK2 will also be reduced in these cells. We have previously shown that depletion of GRK2^{+/-} and WT mice from granulocytes did not interfere with the earlier onset of HI brain damage in GRK2^{+/-} mice. In addition, granulocyte depletion reduced severity of brain damage in both WT and GRK2^{+/-} mice but the relative increase in brain damage in GRK2^{+/-} mice was not affected (Nijboer et al., 2008). However, to ensure that the results of our present experiments are not confounded by effects of granulocytes, all mice used in these experiments were depleted of granulocytes prior to exposure to HI.

At 3 h after HI, MAP2 loss as a marker for neuronal injury, was already significantly apparent in LysM-GRK2^{f/+} mice whereas control LysM-GRK2^{+/+} littermates did not show significant MAP2 loss compared to sham-operated animals (Fig. 1B,C). However, at 24 h after HI, brain damage was *similar* in LysM-GRK2^{f/+} and control LysM-GRK2^{+/+} littermates (Fig. 1B, C). These data indicate that reduced GRK2 in microglia/macrophages is sufficient to accelerate onset of HI-induced neuronal loss without affecting severity of brain damage at a later time point (24 h post-HI).

We also observed that the ipsilateral hemisphere of GRK2^{+/-} mice contained more Iba1-positive cells (microglia/macrophages) with rounded morphology as compared to WT mice at 3 h after HI (Fig. 2A).

To further determine the contribution of microglial/macrophage activity to the onset of HI, we used minocycline, a semisynthetic tetracycline antibiotic and a well-described inhibitor of microglia/macrophage activation (Tikka et al., 2001). Treatment with minocycline prior to hypoxia prevented the earlier onset of MAP2 loss in GRK2^{+/-} mice as observed at 3 h post-HI (Fig. 2B). WT mice did not show MAP2 loss at this early time point as shown previously (Nijboer et al., 2008). Interestingly, minocycline treatment did not reduce the total amount of cerebral damage determined at 24 h after HI in both genotypes (Fig. 2B). We did not observe any significant MAP2 loss in WT or GRK2^{+/-} sham-operated animals with or without minocycline treatment (data not shown).

Effect of low GRK2 on p38 activation and TNF- α production *in vitro* and *in vivo*

Recently, it has been shown that *in vitro* GRK2 directly interacts with p38 MAP kinase resulting in inhibition of p38 activation (Peregrin et al., 2006). These authors also showed that GRK2^{+/-} peritoneal macrophages produced more TNF- α after *in vitro* stimulation with LPS and proposed that low GRK2 facilitates TNF- α production via a p38-dependent pathway (Peregrin et al., 2006). The data in Fig. 3 show that primary cultures of GRK2^{+/-} microglia also produced more TNF- α than WT cells after 3 h of stimulation with LPS *in vitro* (Fig. 3). The p38 MAP kinase inhibitors SB203580 or SB239063 reduced TNF- α production and abolished the difference between WT and GRK2^{+/-} microglia (Fig. 3). To get more insight in the specificity of the GRK2/p38 pathway, we also used an inhibitor of the JNK pathway, another MAP kinase regulating cytokine production. Addition of the JNK inhibitor TAT-JBD reduced TNF- α production in both genotypes (Fig. 3). However, the relative increase in TNF- α production by GRK2^{+/-} microglia was maintained. These data indicate that both p38 and JNK MAP kinases are involved in cytokine production by microglia but that increased p38 MAP kinase activity was responsible for increased TNF- α production by GRK2^{+/-} microglia. Similar results on TNF- α production after LPS stimulation and p38/JNK inhibition in WT and GRK2^{+/-} microglia were obtained after 6 h of stimulation (data not shown).

In vivo, exposure of neonatal mice to HI induced a rapid increase in phosphorylated p38 (P-p38), at 1 and 3 h after the insult that was more pronounced in GRK2^{+/-} mice than in WT littermates (Fig. 4A). Similarly, HI-induced activation of cerebral p38 was significantly increased in LysM-GRK2^{f/+} animals compared to LysM-GRK2^{+/+} littermates (Fig. 4B). These data indicate that a reduction of GRK2 in microglia/macrophages only was sufficient for more pronounced HI-induced brain p38 activation. The HI-induced increase in cerebral TNF- α was also significantly more pronounced in GRK2^{+/-} than in WT animals at 1 h and 3 h post-HI (Fig. 4C). Similarly, in LysM-GRK2^{f/+} animals, HI-induced TNF- α production was significantly higher than in LysM-GRK2^{+/+} littermates at 3 h post-insult (Fig. 4D).

HI brain damage in mice with low GRK2 in forebrain neurons

Our present data show that reduced GRK2 in microglia/macrophages contributes to the onset of HI brain damage, but does not control the extent of cerebral injury. However, in our previous study in mice with reduced GRK2 in all cells, we observed not only an *acceleration of onset*, but also *more severe* brain damage in GRK2^{+/-} mice as compared to WT mice (Nijboer et al., 2008). Therefore, we examined the effect of low neuronal GRK2 on neonatal HI injury. We used mice with Ca²⁺/calmodulin-dependent protein kinase II α (CamKII α)-driven Cre expression. It has been shown that CamKII α -promoter activity is restricted to forebrain neurons with enriched levels in the hippocampus and layers of the cerebral cortex, areas known to be highly vulnerable to HI brain damage (Minichiello et al., 1999). HI-induced damage determined at 3 h post-HI was not affected by reduced GRK2 in CamKII α -positive neurons (Fig. 5A, B). However, at 24 h post-HI, CamKII α -GRK2^{f/+} mice showed significantly more MAP2 loss than control CamKII α -GRK2^{+/+} littermates (Fig. 5A, B). At 3 weeks after the insult, total area loss of the ipsilateral hemisphere was also considerably more pronounced in CamKII α -GRK2^{f/+} mice compared to CamKII α -GRK2^{+/+} controls (Fig. 5C, D).

Discussion

Previously, we showed that neonatal HI brain damage is accelerated and aggravated in mice with reduced GRK2 levels in all cells (GRK2^{+/-} mice) (Nijboer et al., 2008). These findings indicated that the endogenous level of GRK2 is a critical player in HI brain damage. In the present study, we employed Cre-Lox technology to decrease GRK2 in specific cell types. This approach allowed us to identify cell-specific effects of low GRK2 on onset and severity of neonatal HI brain damage.

In LysM-GRK2^{f/+} mice with decreased GRK2 only in microglia/macrophages, an earlier onset of HI brain injury was observed, but brain damage at 24 h after HI was not exacerbated like we observed in GRK2^{+/-} mice with low GRK2 in all cells. These data indicate that low microglial/macrophage GRK2 is sufficient to accelerate the onset of HI brain damage.

We have previously described that the maximal increase in the microglial activation marker CD68 was observed at 24–48 h after neonatal HI in both GRK2^{+/-} and WT mice (Nijboer et al., 2008). These kinetics are in line with the prevailing view that microglia activation is induced as a consequence of neuronal injury and that reactive microglia subsequently aggravate brain injury (Saliba and Henrot, 2001). More recent data, however, suggest that there is also an early increase in pro-inflammatory cytokine levels in the neonatal brain after HI and that microglia contribute to cerebral cytokine expression early after HI (Dommergues et al., 2003; Nijboer et al., 2009). We show here that we already detected microglia (Iba1-positive cells) with rounded morphology in the ipsilateral hemisphere at 3 h after HI and that this response was more prominent in GRK2^{+/-} mice than in WT mice. Furthermore, cerebral TNF- α production early after the insult was significantly increased in mice with low GRK2 only in microglia/macrophages. Moreover, *in vitro* primary cultures of GRK2^{+/-} microglia produced more TNF- α than WT microglia in response to LPS. Finally, in our model, treatment with minocycline, a well-described inhibitor of microglial/macrophage activity, abolished the earlier onset of brain damage in GRK2^{+/-} mice. However, minocycline treatment did not affect the extent of brain damage as determined at 24 h after HI in either WT or GRK2^{+/-} mice.

Although there is evidence that inhibition of microglial activity by minocycline treatment has neuroprotective effects in models of brain injury, there is ongoing debate on the duration of this protective effect (Buller et al., 2009). For example, delayed minocycline treatment

only *transiently* reduced infarct volume after MCAO in neonatal rats; protection by minocycline was only observed early after the insult and disappeared later on (Fox et al., 2005). In addition, Bye et al. (2007) showed that minocycline treatment provided early, but not lasting neuroprotection when given after traumatic brain injury. Tsuji et al. (2004) described a moderate positive effect of i.p. minocycline treatment on neonatal HI brain injury in rats when administered at least 12 h before hypoxia, whereas minocycline pre-treatment in neonatal mice increased brain injury after HI. These studies point out an important role for microglia during the early phases of brain injury. The data we present here are in line with these studies and show that increased early microglial activation and pro-inflammatory cytokine production was observed in mice with low GRK2 in these cells. Our data indicate that the increased early activity of GRK2-deficient microglia might accelerate the onset of neonatal HI brain damage, without affecting the extent of damage at later time points after the insult.

GRK2 was originally described as a regulator of homologous G protein-coupled receptor desensitization, by phosphorylating agonist-occupied GPCR, which enhances binding of arrestins, uncoupling from the G protein and GPCR internalization (Pitcher et al., 1998). More recent evidence suggests that GRK2 can also modulate cellular functioning via direct interaction with downstream signaling molecules, including p38 MAP kinase (Reiter and Lefkowitz, 2006; Vroon et al., 2006; Ribas et al., 2007). *In vitro*, it has been shown that GRK2 phosphorylates Thr123 in the docking groove of p38 thereby preventing binding of the upstream activator of p38, MKK6 (Peregrin et al., 2006). The *in vivo* functional consequences have not been elucidated, but it has been shown that *in vitro* GRK2^{+/-} macrophages produce more TNF- α via a p38-dependent pathway (Peregrin et al., 2006).

Here we demonstrate that GRK2^{+/-} microglia also produced more TNF- α upon stimulation with LPS and that p38 inhibition normalized TNF- α production to the level observed in WT mice. Moreover, we are the first to show that *in vivo*, a reduction in GRK2 in microglia/macrophages is associated with an increase in cerebral p38 activation concomitantly with increased TNF- α production early after HI. We propose that low GRK2 in microglia/macrophages enhances activation of these cells and thereby contributes to acceleration of onset of brain damage via a pathway dependent on direct interaction of GRK2 with p38 MAP kinase. However, it is also possible that low microglial/macrophage GRK2 exacerbates GPCR signaling in these cells leading to increased p38 activation and cytokine production. We can also not exclude that enhanced activation of microglia with low GRK2 might increase cerebral p38 activation indirectly in other cell types of the brain, which might also contribute to neuronal damage (see below). Finally, also increased influx of GRK2^{+/-} macrophages from the periphery to the brain might contribute to earlier onset of brain injury after HI.

We also show here that low GRK2 in CamKII α -positive neurons only is sufficient to increase the ultimate amount of neonatal HI-induced brain damage without accelerating onset. Glutamate excitotoxicity evoked by excessive glutamate release during HI is thought to be key to the induction of neuronal damage in this model. In our previous *in vitro* studies, we showed that GRK2^{+/-} cerebellar granule neurons or hippocampal slices were more sensitive to glutamate-induced cytotoxicity (Nijboer et al., 2008). In addition, there is evidence that overexpression of GRK2 inhibits glutamate-induced signaling via mGluR1 and 5 in HEK293 cells (Dhami et al., 2002; Sorensen et al., 2003). Furthermore, it has been shown that mGluR1 antagonists can reduce NMDA receptor-mediated neuronal death (Strasser et al., 1998). Hence, a reduction in GRK2 levels within neurons may facilitate excitotoxic cell death by increased mGluR signaling leading to potentiation of NMDA receptor-induced neuronal death.

It is also possible that direct interaction of GRK2 with downstream kinases such as p38 contributes to neuronal damage in our model. Indeed there is evidence that p38 MAP kinase plays a critical role during neuronal apoptosis (Harper and LaGrasso, 2001). *In vitro*, p38 has been shown to support glutamate-induced apoptosis in cerebellar granule neurons (Kawasaki et al., 1997). In addition, neuronal survival in PC12 neurons is promoted after inhibition of p38 with selective inhibitors like SB203580 (Xia et al., 1995). Furthermore, direct interactions between GRK2 and the PI3kinase/Akt pathway might add to the role of GRK2 in neuronal survival.

In summary, we demonstrated that even relatively small changes in the expression of GRK2 in a single cell type, i.e. microglia/macrophages, can be sufficient to markedly increase HI-induced cerebral p38 MAP kinase activation and TNF- α production. We propose that as a result of increased early microglial activity in mice with low GRK2 in these cells, the onset of neuronal damage is accelerated after HI. However, our data also indicate that low GRK2 levels in neurons are required to exacerbate the ultimate amount of brain injury after HI indicating that neuronal GRK2 levels can determine the vulnerability of the neonatal brain to HI damage. Since inflammatory conditions *in vivo* and *in vitro* down regulate cellular GRK2 levels (Lombardi et al., 1999, 2001; Ramos-Ruiz et al., 2000; Vroon et al., 2003, 2005), our data may be of crucial importance when HI is preceded by or associated with systemic inflammation in the neonate.

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Figure 1. Effect of reduced GRK2 in microglia/macrophages on HI brain damage

A: GRK2 expression in total lysates of primary cultures of LysM-GRK2^{+/+} and LysM-GRK2^{f/+} microglia (left panel) and astrocytes (right panel) was analyzed by Western blotting. ***p<0.001. Insets show representative Western blot examples; β-actin was used as loading control. n=5 animals per group.

B. LysM-GRK2^{f/+} and control LysM-GRK2^{+/+} littermates were exposed to HI and ipsilateral MAP2 loss was quantified as a measure of neuronal damage at the time points indicated. We did not detect any significant MAP2 loss in sham-operated animals (SHAM) or in contralateral hemispheres. n=5–7 animals per genotype per time point. **p<0.01

C. Representative examples of ipsilateral MAP2 loss at 3 and 24 h post-HI in LysM-GRK2^{+/+} (GRK2^{+/+}) and LysM-GRK2^{f/+} (GRK2^{f/+}) animals.

Figure 2. Microglial activation and the effect of minocycline treatment on HI brain damage

A. Representative examples of Iba1 staining in the hippocampus of WT and GRK2^{+/-} sham-controls (SHAM) and at 3 h post-HI in contra- and ipsilateral hemispheres of WT and GRK2^{+/-} mice.

B. WT and GRK2^{+/-} mouse pups were treated directly prior to hypoxia with minocycline. Neuronal damage was quantified as fraction ipsilateral MAP2 loss at 3 and 24 h post-HI. No MAP2 loss was detected in WT or GRK2^{+/-} sham-operated animals with or without minocycline treatment or in contralateral hemispheres (data not shown). n=6–9 animals per genotype per time point. *p<0.05 and ***p<0.001 WT vs GRK2^{+/-}.

Figure 3. TNF- α production by GRK2^{+/-} microglia *in vitro*

Primary cultures of WT and GRK2^{+/-} microglia were stimulated with LPS (10 ng/ml) and TNF- α production was determined by ELISA after 3 h of culture. TNF- α was not detectable in control cultures that were not stimulated with LPS (not shown). The effect of inhibition of p38 activity was determined using two specific p38 inhibitors: SB203580 (20μM) or SB239063 (5μM). The JNK-inhibitor TAT-JBD (10 or 100 μM) was used as a specificity control. Data are from 4 independent experiments performed in duplicate. *p<0.05, ***p<0.001 WT vs. GRK2^{+/-}.

Figure 4. HI-induced p38 activation and TNF- α expression

A: WT and GRK2^{+/-} littermates were subjected to HI and HI-induced p38 activation (phosphorylation at Thr180/Tyr182) at 1 h and 3 h post-HI was determined by Western Blot on cytosolic brain fractions. Note the difference in scale of the two Y-axes. * $p < 0.05$, *** $p < 0.001$ WT vs. GRK2^{+/-}. Insets show representative Western Blots. Total p38 levels were used as loading control. c indicates contralateral and i ipsilateral. A.U.: arbitrary density units. $n = 8-12$ animals per group per time point.

B: Ipsilateral HI-induced p38 activation at 1 h post-HI in LysM-GRK2^{+/+} and LysM-GRK2^{fl/+} mice (** $p < 0.001$). Insets show representative Western Blots. Total p38 levels were used as loading control. $n = 5$ animals per genotype.

C: WT and GRK2^{+/-} mouse pups were subjected to HI and HI-induced TNF- α mRNA expression at 1 h and 3 h post-HI was determined by quantitative real time RT-PCR. Data are normalized for mean expression of β -actin and GAPDH. Contralateral TNF- α expression was comparable to levels in sham-operated animals. * $p < 0.05$, *** $p < 0.001$ WT vs. GRK2^{+/-}. $n = 8-12$ per group per time point.

D: Ipsilateral TNF- α mRNA expression in LysM-GRK2^{+/+} and LysM-GRK2^{fl/+} littermates at 3 h post-HI (** $p < 0.01$). TNF- α expression in contralateral hemispheres was comparable to levels in sham-operated animals. $n = 7$ per group.

Figure 5. Effects on neuronal GRK2 reduction on HI brain damage

A. CamKII α -GRK2^{+/+} and CamKII α -GRK2^{f/+} littermates were exposed to HI. MAP2 loss was determined in the ipsilateral hemisphere as a measure to quantify neuronal damage at 3 h and 24 h post-HI. We did not detect any significant MAP2 loss in sham-operated animals (SHAM) or in contralateral hemispheres (not shown). * $p < 0.05$ CamKII α -GRK2^{+/+} vs. CamKII α -GRK2^{f/+}. $n = 8-15$ animals per genotype per time point.

B. Representative examples of ipsilateral MAP2 loss at 3 and 24 h post-HI in CamKII α -GRK2^{+/+} (GRK2^{+/+}) vs. CamKII α -GRK2^{f/+} (GRK2^{f/+}) animals. **C.** At 3 weeks after HI, hemispheric area loss in the ipsilateral hemisphere of CamKII α -GRK2^{+/+} and CamKII α -GRK2^{f/+} mice was measured on HE-stained sections. ** $p < 0.01$. $n = 9-16$ animals per group.

D. Representative examples of HE staining at 3 wks post-HI in CamKII α -GRK2^{+/+} (GRK2^{+/+}) vs. CamKII α -GRK2^{f/+} (GRK2^{f/+}) animals.