Modifying expression of EphA4 and its downstream targets improves functional recovery after stroke

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Received December 17, 2012; Revised February 4, 2013; Accepted February 9, 2013

Functional recovery after stroke varies greatly between patients, potentially due to differences in gene expression. Several processes like angiogenesis, neurogenesis, axonal reorganization and synaptic plasticity act in concert to restore neurological functions. The ephrin family has known roles in all these processes. EphA4 is the most abundant ephrin receptor in the nervous system. Therefore, we investigated whether EphA4 affects functional recovery from stroke, and evaluated the potential of this receptor as a therapeutic target. Motor recovery after photothrombotic stroke was studied in transgenic mice in which expression of EphA4 was reduced. Furthermore, blocking a downstream target of EphA4, ROCK (Rho-associated kinase), by two different compounds was evaluated in the same model. Motor recovery after photothrombotic stroke was markedly enhanced in transgenic mice with reduced levels of EphA4, whereas infarct sizes were similar compared with non-transgenic controls. Pharmacological inhibition of the EphA4 signaling cascade using two ROCK inhibitors, Y-27632 and fasudil, improved motor function of mice after stroke. Infarct size was comparable in all groups studied, suggesting that the benefit obtained by EphA4 inhibition is not neuroprotective in nature but due to an effect on the mechanisms underlying recovery. Our findings show that reduction of EphA4 improves motor function after experimental stroke and demonstrate that ROCK inhibition is a promising therapeutic strategy to enhance recovery after ischemic stroke.

INTRODUCTION

Stroke causes 1 in 10 deaths worldwide and is the most important cause of disability in adults (1). Traditionally, treatment strategies have tried to limit the initial damage resulting from the ischemic insult. This strategy has for the most part proven unsuccessful (2). The only consistently proven effective treatment, intravenous tissue plasminogen activator, has a narrow time window, which decreases its application to a limited percentage of patients with ischemic stroke (3,4). New treatment paradigms are therefore required. Enhancing the recovery processes after stroke might be a promising therapeutic avenue (5,6). Yet, the often limited recovery seen after stroke is poorly understood.

The brain after stroke responds by reverting to a quasi-developmental state in perilesional areas (7,8). Proteins that promote axonal regeneration, neurogenesis, synaptogenesis and angiogenesis are profusely expressed in the region of tissue adjacent to the ischemic lesion. In the axon, these restorative responses are stunted through myelin-associated proteins, the formation of a perineuronal net and a glial scar surrounding the damaged brain tissue and expression of growth-inhibiting genes, e.g. repellants of the semaphorin and ephrin families (9). A promising therapeutic measure might be to inhibit this growth inhibitory response, which in turn could allow inherent repair mechanisms to exert their full potential (6). Various reports have determined a role for the ephrin family as growth inhibitory proteins following neuronal injury. One particular receptor, EphA4, is predominantly expressed in the central nervous system (10) and is able to interact with all ephrin ligands (11). Interestingly, EphA4−/− mice showed enhanced recovery of spinal cord trauma possibly due to lack of inhibition of regrowth of descending axons (12). We have shown that EphA4 is a disease modifier of amyotrophic lateral sclerosis, supporting a role for ephrin signaling not only in acute neurological diseases.
but also in neurodegeneration (13). After experimental stroke, an upregulation of EphA4 was documented using whole-genome expression analysis of sprouting neurons in peri-infarct cortex, underscoring the inhibitory environment following stroke (14), and blocking of ephrinA5 has recently been shown to influence neural plasticity after stroke (15). Several studies have suggested that EphA4 plays an important role in the inhibition of axonal outgrowth and that targeting EphA4 might be a promising means of neural repair. Here we report on the effect of reducing the expression of EphA4 and blocking the downstream signaling pathway on the functional recovery after stroke.

RESULTS
Reduction of EphA4 results in improved functional outcome
In order to study the role of EphA4 signaling in the functional recovery after stroke, we used transgenic animals with reduced levels of EphA4. EphA4 knockout mice (EphA4<sup>−/−</sup>) have a motor phenotype hindering the applicability of these transgenic mice in the study of functional recovery after stroke (12). However, a mouse has been generated in which the EphA4 gene has been floxed (EphA4<sup>lox/lox</sup>). This mouse was reported to be overall normal, but the process of transgenesis resulted in the reduction of basal EphA4 expression levels to 20% of normal (16). First, we determined the expression of EphA4 in homozygous EphA4<sup>lox/lox</sup>, heterozygous EphA4<sup>lox/+</sup> and non-transgenic (EphA4<sup>+/+</sup>) controls (Supplementary Material, Fig. S1A and B). Expression was 24% of normal in the brain of homozygous mice and 51% of normal in heterozygous mice as described before (16). We then evaluated the rotarod performance of these mice and found that EphA4<sup>lox/lox</sup> had reduced accelerating rotarod performance compared with controls, whereas the performance of heterozygous EphA4<sup>lox/+</sup> mice was normal (Supplementary Material, Fig. S1C). We therefore excluded the EphA4<sup>lox/lox</sup> from the study.

After the induction of a cortical lesion by photothrombosis in the right sensorimotor cortex, there was no difference in motor deficit on the first day after stroke. Similarly, infarct volume determined by the percentage of the involved cortical area when animals were sacrificed at the end of the study was not different, showing that the level of EphA4 expression did not affect the infarct size induced by photothrombosis (Fig. 1A and B, and Supplementary Material, Fig. S2A and B). In contrast, 4 weeks after stroke, a significant difference in rotarod performance was observed between EphA4<sup>lox/+</sup> versus non-transgenic mice (P = 0.02) (Fig. 1C). It can be thought that the most severely affected animals have the smallest chance of recovery. We therefore evaluated the subgroup of mice with a deficit at day 1 of <25% of baseline performance. Surprisingly, the benefit offered by the reduction of EphA4 expression in this group was even more pronounced (P = 0.01) (Fig. 1D).

We next investigated the potential mechanism underlying the improved functional recovery associated with reduced EphA4 expression. As the glial scar induced by spinal cord injury is less pronounced in EphA4<sup>−/−</sup> mice (12), we evaluated astrogliaosis following ischemic stroke. No difference in GFAP expression was observable between non-transgenic and EphA4<sup>lox/+</sup> mice (Fig. 2A and B). EphA4 has been reported to inhibit axonal outgrowth in vitro and in vivo, the latter during normal development as well as in disease models in adulthood (12,13,15). We therefore studied neurite outgrowth in cortical neurons from EphA4<sup>−/−</sup> mice co-cultured with astrocytes. EphA4<sup>−/−</sup> neurite outgrowth was significantly increased compared with neurite outgrowth of non-transgenic cultures (P = 0.008) (Fig. 2C). This finding supports the hypothesis that reduced expression of EphA4 increases axonal sprouting.

Rho-associated kinase-inhibition promotes recovery after experimental stroke
EphA4 stimulation results in growth cone collapse through the activation of GTP-bound RhoA activity (17), which in turn activates Rho-associated kinase (ROCK). We investigated whether inhibition of the EphA4 signaling cascade through the inhibition of ROCK affects outcome after ischemic stroke, by studying the effect of Y-27632, a ROCK inhibitor. Oral administration of Y-27632 was started 3 days after the induction of photothrombotic stroke, and resulted in clear reduction of cofilin 2 phosphorylation in the brain 4 days later (Fig. 3A), confirming that this compound reached the target in the disease model used. Mice treated with Y-27632 had a significantly better outcome after stroke. They recovered to a greater extent compared with placebo-treated animals when evaluated 5 weeks after stroke induction (P = 0.004) (Fig. 3B). The initial deficit evaluated on day 1 was the same for both groups, 25.5 ± 7.1% of baseline performance in placebo-treated versus 26.4 ± 4.9% in Y-27632-treated animals (P = 0.9), and infarct size was equally unaffected by the treatment (Fig. 3C).

We confirmed these findings by studying a different ROCK inhibitor, fasudil, a drug already approved for clinical use for the treatment of cerebral vasospasm and pulmonary hypertension. Fasudil was administered orally twice daily, starting 3 days after the induction of photothrombotic stroke and also resulted in significant reduction of cofilin 2 phosphorylation 4 days later (Fig. 4A). Treatment was prolonged for a period of 5 weeks after stroke. Fasudil significantly improved recovery from photothrombotic stroke when evaluated 6 weeks after the induction of the photothrombotic lesion (P = 0.04) (Fig. 4B). The initial deficit was similar in the two groups, 29.7 ± 3.7% of baseline performance in placebo-treated versus 31.2 ± 4.2% in fasudil-treated animals (P = 0.7), and the infarct size was comparable for both groups (Fig. 4C). The treatment effect was even more pronounced in the more severely affected animals (P = 0.04) (Fig. 4D).

DISCUSSION
In this study, we have identified a role for EphA4 in the recovery after ischemic stroke since reduced expression was associated with improved outcome. Several lines of research indicate that, after central nervous injury, the eph-ephrin system plays a role in recovery (9,12,18). Neural plasticity was shown to be improved in the brain after stroke by blocking...
ephrinA5, establishing a role for ephrins in recovery after ischemic injury (15). We have previously reported on an important role for EphA4 as a modifier of a chronic neurodegenerative disease, ALS, likely by reducing the vulnerability of motor neurons as well as improving the regenerative potential (13).

The mechanism of action could be diverse since axonal outgrowth has previously been shown, by others (12,15) and us (13), to be improved by reducing EphA4. Here we have provided evidence for increased sprouting in cortical neurons in vitro, and in previous work, we have identified reduced levels of EphA4 to be associated with improved regeneration following sciatic nerve injury (13). Additionally, since ephrins have been implicated in synapse formation and in the regulation of long-term synaptic plasticity and memory (11), synaptogenesis post-stroke could be influenced. A broad study of the axonal outgrowth and synapse formation, and this at multiple levels of the neuraxis, is required to fully dissect the mechanism of action after experimental stroke. In addition, it is fairly well possible that the mechanism, through which inhibition of the EphA4 protecns, is related to neuro-inflammation or changes in glutamate-induced toxicity. Indeed, it has been described (16) and confirmed by us that EphA4 inhibits EAAT2 expression. Inhibition of EphA4 may thus increase EAAT2 and decrease glutamate toxicity. Furthermore, ephrins are modulators of angiogenesis and neurogenesis (19–21), processes known to occur in the brain after stroke and found to be correlated with recovery. In our future research, we intend to analyze these various mechanisms in order to elucidate the pathophysiology of EphA4 in neural repair after experimental stroke.

Our study does not fully dissect the mechanism underlying the role of reduced EphA4 levels in stroke recovery; however, we believed that translation of the findings in the transgenic animal model into a potential pharmacological treatment would be of great interest. Downstream signaling following EphA4 stimulation results in the activation of GTP-bound RhoA (17). The direct downstream effector of RhoA is ROCK, which is also a downstream target of several other

Figure 1. No difference in rotarod performance 1 day post-stroke. 27.9 ± 6.0% compared with baseline in EphA4lox/+ versus 42.1 ± 4.9% in NTG, was identified (A), and stroke volumes as determined after the functional follow-up were similar: 25.3 ± 2.2% of cortical area (n = 6) in EphA4lox/+ and 20.0 ± 3.2% (n = 7) in NTG (B). Reduction of EphA4 improved functional recovery as measured by rotarod, compared with baseline performance, at 29 days after the induction of stroke: 72.4 ± 4.9% (n = 19) in NTG and 89.3 ± 4.3% (n = 16) in EphA4lox/+ (P = 0.02) (C). Post hoc analysis of severely affected mice (as specified in Materials and Methods) showed improved motor performance on rotarod at day 29 after stroke: 88.0 ± 6.3% of baseline performance (n = 8) in EphA4lox/+ versus 56.5 ± 8.2% (n = 5) in NTG (P = 0.01) (D). Error bars represent means ± SEM.
proteins, e.g. NogoA and MAG, strongly implicated in neural repair (22,23). Although the therapeutic potential in animal models has been clearly established for anti-NogoA and anti-MAG therapies, clinical translation has only begun to emerge with the intention to study safety and efficacy. However, safety data and even efficacy in stroke patients have already been obtained on the inhibition of ROCK (24). Moreover, a role for the inhibition of ROCK has been established after experimental stroke (25), but the treatment was always initiated during or shortly after the ischemic injury. In treated animals, smaller infarct sizes were reported and increased blood flow (possibly through an effect on endothelial cells) was assumed as a pathophysiological mechanism (25).

Unfortunately, whenever early interventions are required in stroke patients, these treatment strategies will only be applicable to a limited subset of patients as for thrombolysis. Interestingly, in vitro studies have suggested ROCK inhibition to be implicated in neurogenesis (26,27). Here we have identified a yet-unknown role for ROCK inhibition after ischemic stroke by establishing its potential in improving the functional recovery after the lesion has been irreversibly established. We determined that the severely affected animals were (even more) likely to benefit from the therapy. Moreover, ROCK inhibition might have dual benefits: reducing acute infarct size as has been previously shown (25) but also improved functional recovery after stroke, as reported in this study; additionally, it might also augment the therapeutic potential of thrombolysis. Tissue-type plasminogen activator (t-PA) modulates the permeability of the neurovascular unit by the Rho/ROCK pathway, and inhibition of ROCK blocks the increase in permeability and could thereby reduce the incidence of intracranial hemorrhage during thrombolytic therapy in stroke (28).

We report on a novel effect of ROCK inhibition in the subacute and chronic phases after stroke, when the infarct size probably can no longer be modified, in improving functional outcome. Translating these findings into the clinical setting would lead to a novel treatment strategy for patients in the acute phase of stroke regardless of eligibility for tPA as well as in the subacute phase after stroke when rehabilitation occurs. Fasudil can be safely administered to stroke patients (24) and this study supports the potential applicability to a much larger patient population than current treatment strategies in the (sub)acute setting after ischemic stroke. Furthermore, the ROCK pathway is downstream of other promising therapeutic targets as NOGO and MAG. Therefore, it merges the beneficial effects of several promising targets for enhancing neuronal plasticity following ischemic brain injury. Although experimental models have greatly improved our knowledge on the mechanisms of neural plasticity, neurogenesis and angiogenesis, limited translation has been obtained in the study of functional recovery. Hopefully, ROCK inhibition will prove to be a target for the many stroke patients suffering from severe disability.

**MATERIALS AND METHODS**

**Mice**

Two different transgenic mice (C57BL/6 background) with reduced levels of EphA4 were used. A conditional EphA4 knock out mouse (EphA4<sup>lox/lox</sup>), in which two lox sites have been cloned at both sides of exon 3 of the EphA4 gene (16), has been kindly provided by R. Klein (Max Planck Institute, Munich) and K. Kullander (Uppsala University, Uppsala, Sweden). In these transgenic animals, EphA4 expression is reduced. EphA4<sup>−/−</sup> mice have been kindly provided by Dr A. Turnley, University of Melbourne. These transgenic mice develop a hopping gait around week 8, but their life span is normal (12). The EphA4<sup>−/−</sup> mice were used for in vitro experiments only, since functional analysis of recovery is impaired due to the hopping gait. All experiments were in accordance with the Guide of Care and Use of Experimental Animals of the Ethical Committee of the University of Melbourne. The Ethical Committee of KU Leuven approved all animal experiments.

**Photothrombotic cortical stroke**

Focal cortical ischemia was induced in adult male C57BL6J mice aged 3–4 months by photothermolysis as previously described (29,30). Mice were anesthetized with 2.5% isoflurane (Halocarbon, NJ, USA) in an oxygen/air mixture, respiration was observed and rectal temperature during the surgical procedure was maintained at 37 ± 0.5°C with a
was average and/or maximum performance over three attempts after the procedure and animals were excluded if the week after training. Induction of stroke was evaluated 1 day baseline performance was recorded over six attempts the 300 s in three attempts to evaluate motor performance. The (Ugo Basile), rotating from 4 to 40 r.p.m. over the course of training daily for 1 week on an accelerating rotarod treadmill

Before the induction of photothrombosis, animals received Bengal injection for 5 min through the intact skull. The brain was illuminated immediately after Rose bregma. The brain was illuminated immediately after Rose Hamamatsu Photonics, Japan) with an aperture of 2.4 mm

In normal saline, was infused by tail vein injection. For illu- lination, a laser beam of wavelength 565 nm (L4887-13, Louis, MO, USA), 0.1 ml with a concentration of 3 mg/ml in normal saline, was infused by tail vein injection. For for midline incision of the skin. Rose Bengal (Sigma, St Louis, MO, USA), 0.1 ml with a concentration of 3 mg/ml normal saline, was infused by tail vein injection. For illu- lination, a laser beam of wavelength 565 nm (L4887-13, Hamamatsu Photonics, Japan) with an aperture of 2.4 mm was focused 0.5 mm posterior and 1.8 mm right of the bregma. The brain was illuminated immediately after Rose Heating plate (TCAT-2LV Controller, Physitemp Instruments, Inc., NJ, USA). After fixation in a stereotactic frame (David Kopf Instruments, Bilaney, Germany), the skull was exposed by midline incision of the skin. Rose Bengal (Sigma, St Louis, MO, USA), 0.1 ml with a concentration of 3 mg/ml in normal saline, was infused by tail vein injection. For illumination, a laser beam of wavelength 565 nm (L4887-13, Hamamatsu Photonics, Japan) with an aperture of 2.4 mm was focused 0.5 mm posterior and 1.8 mm right of the bregma. The brain was illuminated immediately after Rose

Functional analysis and pharmacological treatment

Before the induction of photothrombosis, animals received training daily for 1 week on an accelerating rotarod treadmill (Ugo Basile), rotating from 4 to 40 r.p.m. over the course of 300 s in three attempts to evaluate motor performance. The baseline performance was recorded over six attempts the week after training. Induction of stroke was evaluated 1 day after the procedure and animals were excluded if the average and/or maximum performance over three attempts was >75% compared with baseline. We specified a subgroup of severely affected animals if this percentage was <25 for separate additional analysis post hoc in the transgenic and pre-specified this category in the fasudil study. In the treatment studies, oral gavaging with ROCK inhibitor Y-27632 and fasudil was initiated 3 days after the induction of experimental stroke. For Y-27632, a dose of 30 mg/kg once daily was chosen as reported in other mice models (31), and fasudil was administered twice daily at a dose of 30 mg/kg (32). Control animals received the same regimen of oral gavaging with placebo (water). Each cage with animals used in the functional studies contained mice in both treatment arms to control for environmental factors. Y-27632 was given for 4 weeks after stroke and the endpoint was determined 1 week later. In the fasudil treatment, the regimen was prolonged for 1 week, and the endpoint was determined 1 week after the end of the treatment as well. Experimental procedures and functional analysis in all studies were done by examiners blinded to genotype and/or pharmacological treatment arm. The survival after the induction of stroke was similar in all studies reported (as analyzed by chi-square test).

Western blotting

We homogenized the mouse brain in RIPA buffer containing 150 mM NaCl, pH 7.5, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS and one tablet Complete-EDTA (Roche). Protein concentration was determined using the Micro BCA Protein Assay Reaction Kit 207 (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded on all blots. Primary antibodies used were EphA4 antibody (1:1000, Zymed, 37–1600), phosphorylated coflin 2 (1:500, Millipore), coflin 2 (1:500, Millipore) and β-actin antibody (1:5000, Sigma-Aldrich, A5441). We used horseradish peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz) and enhanced chemiluminescent substrate (Pierce) to visualize the protein bands and scanned blots with Image Quant LAS 4000.

Immunohistochemistry

After the experimental regimen, mice were perfused and brains fixed (4% paraformaldehyde), dehydrated (30% sucrose) and snap-frozen in Tissue-Tec (Sakura); cryostat sections of 40 μm thickness were made for cresyl violet (Sigma) or 20 μm thickness for immunostaining with GFAP antibody (1:500, Invitrogen, G3893). The sections were incubated with AlexaFluor 555 secondary antibody (Molecular Probes, Eugene, OR, USA). Infarct volume was calculated in serial coronal sections and expressed compared to the contralateral side using the Adobe Photoshop CS6 software.

Cortical cultures

Gliaal feeder layers were prepared from 14-day-old mice embryos as previously described (33) and plated in culture dishes coated with poly-l-ornithine and laminin. For cortical cultures, the cortex of 17-day-old mice was dissociated by

![Image](https://academic.oup.com/hmg/article-abstract/22/11/2214/635125/2218)

**Figure 3.** Treatment with ROCK inhibitor Y-27632 was initiated at day 3 post-stroke. Expression of coflin 2 and phosphorylated coflin 2 (p-cofilin 2) was determined in the brain of different mice after 1 week of treatment, which confirmed BBB passage since a reduction of p-cofilin 2 was clearly present (69% of control, \( P = 0.002 \)) and no difference in the expression of coflin 2 was observed (A). Treatment with Y-27632 until 4 weeks after stroke improved functional recovery as measured by rotarod, compared with baseline performance, at 36 days after the induction of stroke: 60.5 ± 3.7% (n = 8) in placebo-treated versus 78.0 ± 3.6% (n = 10) in Y-27632-treated mice (\( P = 0.004 \)) (B). Volumes of infarct were similar: 29.2 ± 2.0% (n = 6) in placebo-treated versus 30.3 ± 2.2% (n = 6) in Y-27632-treated mice (C). Error bars represent means ± SEM.
The culture medium consisted of L15 supplemented with 0.2% sodium bicarbonate, 3.6 mg/ml glucose, 20 nM progesterone, 5 µg/ml insulin, 0.1 mM putrescine, 0.1 mg/ml conalbumin, 30 nM sodium selenite, 100 U/ml penicillin, 100 µg/ml streptomycin, 5% chick embryo extract and 2% horse serum. Cultures were kept in a 7% CO₂ humidified incubator at 37°C. Co-cultures were fixed 24 h after seeding and stained for neuronal class III β-tubulin (1:1000, Covance, MMS-435P) and incubated with AlexaFluor 555 secondary antibody (Molecular Probes). The longest neurite per neuron of more than 100 neurons per experimental condition was measured. Statistics

All reported data were analyzed by a Student t-test and all P-values reported are two-tailed. The significance level was set at 0.05. Based on previous experiments, we estimated the recovery in NTG mice to be 65 ± 20% after 4 weeks. A sample size of 17 in each group would provide 80% power at α = 0.05 to detect an effect of 20%. In the subgroup of severely affected animals, we aimed to identify a difference of 30% for which a sample size of 8 in each group would be required. A sample size of 11 in each group would provide 80% power at α = 0.05 to detect an effect of 25% after Y-27632 treatment. Based on the observed difference in recovery in that study, the effect size for the fasudil study was readjusted to 20% for all and 30% for the severely affected animals.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.
ACKNOWLEDGEMENTS

We would like to thank Dr R. Klein and Dr A. Turnley for providing the different mouse strains used in this study.

Conflict of Interest statement. None declared.

FUNDING

R.L. and V.N.T. are senior clinical investigators of FWO Flanders. W.R. is supported by the E. von Behring Chair for Neuromuscular and Neurodegenerative Disorders at the University of Leuven. This work is supported by grants from the Flanders Institute for Biotechnology (VIB) and the University of Leuven (GOA 11/014).

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