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

Robin Lemmens\textsuperscript{1,2,3,*}, Tom Jasper\textsuperscript{1,2}, Wim Robberecht\textsuperscript{1,2,3}, Vincent N. Thijs\textsuperscript{1,2,3}

\textsuperscript{1}Laboratory of Neurobiology, Vesalius Research Center, VIB, Leuven, Belgium

\textsuperscript{2}Experimental Neurology (Department of Neurosciences) and Leuven Research Institute for Neuroscience and Disease (LIND), University of Leuven (KU Leuven), Leuven, Belgium

\textsuperscript{3}Neurology, University Hospitals Leuven, Leuven, Belgium

\textsuperscript{*}Corresponding author: Robin Lemmens MD, PhD, Department of Neurology, University Hospitals Leuven, University of Leuven and VIB, Herestraat 49, B-3000 Leuven, Belgium, Email: robin.lemmens@vib-kuleuven.be, Tel.: ++ 3216344280, Fax: ++3216344285
Abstract

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, 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, while infarct sizes were similar compared to non-transgenic controls. Pharmacological inhibition of the EphA4 signaling cascade using two Rho-associated kinase (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 one 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\(^{-/-}\)) 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\(^{lox/lox}\)). 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\(^{lox/lox}\), heterozygous EphA4\(^{lox/+}\) and non-transgenic (EphA4\(^{+/+}\)) controls (Supplementary Figure 1A 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\(^{lox/lox}\) had reduced accelerating rotarod performance compared to controls, while the performance of heterozygous EphA4\(^{lox/+}\) mice was normal (Supplementary Figure 1C). We therefore excluded the EphA4\(^{lox/lox}\) from the study.

After 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 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 (Figure 1A and B, and Supplementary Figure 2A and B). In contrast, four weeks after stroke, a significant difference in rotarod performance was observed between EphA4\(^{lox/+}\) versus non-transgenic mice (P= 0.02) (Figure 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 less than 25% of baseline performance. Surprisingly, the benefit offered by reduction of EphA4 expression in this group was even more pronounced (P = 0.01) (Figure 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-/- mice (12), we evaluated astrogliosis following ischemic stroke. No difference in GFAP expression was observable between non-transgenic and EphA4lox/lox mice (Figure 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-/- mice cocultured with astrocytes. EphA4-/- neurite outgrowth was significantly increased compared to neurite outgrowth of non-transgenic cultures (P = 0.008) (Figure 2C). This finding supports the hypothesis that reduced expression of EphA4 increases axonal sprouting.

**ROCK-inhibition promotes recovery after experimental stroke**

EphA4 stimulation results in growth cone collapse through activation of GTP-bound RhoA activity (17), which in turn activates ROCK. We investigated whether inhibition of the EphA4 signaling cascade through 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 three days after the induction of photothrombotic stroke, and resulted in clear reduction of coflin 2 phosphorylation in the brain four days later (Figure 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 to placebo-treated animals when evaluated 5 weeks after stroke induction (P = 0.004) (Figure
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 (Figure 3C).

We confirmed these findings by studying a different ROCK inhibitor, fasudil, a drug already approved for clinical use or the treatment of cerebral vasospasm and pulmonary hypertension. Fasudil was administered orally twice daily starting three days after induction of photothrombotic stroke and also resulted in significant reduction of cofilin 2 phosphorylation four days later (Figure 4A). Treatment was prolonged for a period of five weeks after stroke. Fasudil significantly improved recovery from photothrombotic stroke when evaluated six weeks after induction of the photothrombotic lesion (p=0.04) (Figure 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 (Figure 4C). The treatment effect was even more pronounced in the more severely affected animals (P=0.04) (Figure 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 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 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 protects, 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. Vulnerability of neurons to ischemic injury could also vary with reduction of EphA4, but this should be studied by experimental stroke models evaluating neuroprotection.

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 activation of GTP-bound RhoA (17). The direct downstream effector of RhoA is ROCK which is also a downstream target of several other 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 therapy, 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 inhibition of ROCK (24). Moreover a role for 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 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 like 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 phase 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^lox/lox), 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). In these transgenic animals EphA4 expression is reduced. EphA4^-/- 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^-/- 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 KU Leuven. 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 photothrombosis as previously described (29, 30). Mice were anesthetized with 2,5 % isoflurane (Halocarbon, New Jersey, VS) in an oxygen/air mixture, respiration was observed and rectal temperature during the surgical procedure was maintained at 37 ± 0,5 °C with a heating plate (TCAT-2LV Controller, Physitemp instruments inc., New Jersey, VS). 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, VS), 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 a 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 Bengal injection during 5 minutes through the
intact skull.

Functional analysis and pharmacological treatment
Before induction of photothrombosis, animals received training daily for week on an
accelerating rotarod treadmill (Ugo Basile), rotating from 4 r.p.m. 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 one day
after the procedure and animals were excluded if the average and/or maximum performance
over three attempts was more than 75% compared to baseline. We specified a subgroup of
severely affected animals if this percentage was less than 25 for separate additional analysis
post-hoc in the transgenic and prespecified this category in the fasudil study. In the treatment
studies oral gavaging with ROCK-inhibitor Y-27632 and fasudil was initiated three days after
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 four weeks after
stroke and the endpoint was determined one week later. In the fasudil treatment the regimen
was prolonged for one week and the endpoint was determined one 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 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 (1:1,000, Zymed, 37-1600), phosphorylated cofilin 2 (1:500; Millipore), cofilin 2 (1:500; Millipore), and β-actin antibody (1:5,000, Sigma-Aldrich, A5441). We used horseradish peroxidase-conjugated secondary antibodies (1:5,000, Santa Cruz) and enhanced chemiluminescent (ECL) substrate (Pierce) to visualize the protein bands and scanned blots with the 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 immunostaining with GFAP antibody (1:500, Invitrogen, G3893). The sections were incubated with AlexaFluor 555 secondary antibody (Molecular Probes, Eugene, Oregon, USA). Infarct volume was calculated in serial coronal sections and expressed compared to the contralateral side by Adobe Photoshop CS6 software.

Cortical cultures

Glial feederlayers were prepared from 14-d-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-d-old mice was dissociated by trypsinization and trituration. 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, 2% and horse serum. Cultures were kept in a 7% CO2 humidified incubator at 37 °C. Neurons were quantified by direct counting of unfixed cells under phase contrast, as previously described (33), at day-in-vitro (DIV) 2, 4 and five for survival analysis. Co-cultures were fixed 24 hours after seeding and stained for Neuronal Class III β-Tubulin (1:1,000, Covance, MMS-435P) and incubated with either AlexaFluor 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’s 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 four 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.
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Conflict of interest

The authors declare that they have no conflict of interest.
References


**Figure Legends**

**Figure 1**
No difference in rotarod performance 1 day post-stroke, 27.9% ± 6.0 compared to baseline in EphA4\(^{lox/+}\) versus 42.1% ± 4.9 in NTG, was identified (A) and stroke volumes as determined after the functional follow-up were similar: 25.3% of cortical area ± 2.2 (N=6) in EphA4\(^{lox/+}\) and 20.0% ± 3.2 (N=7) in NTG (B). Reduction of EphA4 improved functional recovery as measured by rotarod, compared to baseline performance, at 29 days after induction of stroke: 72.4% ± 4.9 (N=19) in EphA4\(^{lox/+}\) and 89.3 ± 4.3 (N=16) in NTG (P=0.02) (C). Post-hoc analysis of severely affected mice (as specified in methods) showed improved motor performance on rotarod at day 29 after stroke: 88.0% of baseline performance ± 6.3 (N=8) in EphA4\(^{lox/+}\) versus 56.5 ± 8.2 (N=5) in NTG (P=0.01) (D).

Error bars represent means ± s.e.m.

**Figure 2**
Astrogliosis in the peri-infarct area was as pronounced in the NTG (A) compared to EphA4\(^{-/-}\) mice (B). Maximal neurite length of neurons in EphA4\(^{-/-}\) cortical cultures (N=6 dishes, with at least 100 neurons analyzed per dish) on glial feederlayers was increased as compared to NTG (N=6 dishes with at least 100 neurons analyzed per dish) cortical neurons: 162.8% ± 18.8 (P=0.008) (C).

Scale bar: 100µm; GFAP: Glial fibrillary acidic protein; error bars represent means ± s.e.m.

**Figure 3**
Treatment with ROCK inhibitor Y-27632 was initiated at day three post-stroke. Expression of coflin 2 and phosphorylated coflin 2 (p-cofilin 2) was determined in the brain of different
mice after one 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 expression of cofilin 2 was observed (A). Treatment with Y-27632 until four weeks after stroke improved functional recovery as measured by rotarod, compared to baseline performance, at 36 days after induction of stroke: 60.5% ± 3.7 (N=8) in placebo treated versus 78.0% ± 3.6 (N=10) in Y-27632 treated (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 (C).

Error bars represent means ± s.e.m.

**Figure 4**

Treatment with fasudil was initiated at day three post-stroke. Expression of cofilin 2 and p-cofilin 2 was determined in the brain of different mice after treatment duration of one week, which confirmed BBB passage since a reduction of p-cofilin 2 (65% of control, P=0.01) was identified with no difference in expression of cofilin 2 (A). Fasudil treatment until five weeks after stroke improved functional recovery as measured by rotarod, compared to baseline performance, at 43 days after induction of stroke: 74.3% ± 3.0 (N=21) in placebo treated versus 83.5% ± 3.3 (N=19) in fasudil treated (P=0.04) (B). Volumes of infarct were similar: 22.2 ± 0.7 (N=4) in vehicle treated versus 25.9 ± 4.0 (N=5) in fasudil treated mice (C). Pre-specified analysis of severely affected mice showed improved motor performance on rotarod at day 43 after stroke: 67.6% ± 4.7 (N=9) in placebo treated and 83.0% ± 4.9 (N=10) in fasudil treated mice (P=0.04) (D).

Error bars represent means ± s.e.m.