EphrinB2 Activation Enhances Vascular Repair Mechanisms and Reduces Brain Swelling After Mild Cerebral Ischemia

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Objective—Cerebral edema caused by the disruption of the blood–brain barrier is a major complication after stroke. Therefore, strategies to accelerate and enhance neurovascular recovery after stroke are of prime interest. Our main aim was to study the role of ephrinB2/EphB4 signaling in mediating the vascular repair and in blood–brain barrier restoration after mild cerebral ischemia occlusion/reperfusion.

Approach and Results—Here, we show that the guidance molecule ephrinB2 plays a key role in neurovascular protection and blood–brain barrier restoration after stroke. In a focal stroke model, we characterize the stroke-induced damage to cerebral blood vessels and their subsequent endogenous repair on a cellular, molecular, and functional level. EphrinB2 and its tyrosine kinase receptor EphB4 are upregulated early after stroke by endothelial cells and perivascular support cells, in parallel to their reassembly during neurovascular recovery. Using both retroviral and pharmacological approaches, we show that the inhibition of ephrinB2/EphB4 signaling suppresses post-middle cerebral artery occlusion neurovascular repair mechanisms resulting in an aggravation of brain swelling. In contrast, the activation of ephrinB2 after brain ischemia leads to an increased pericyte recruitment and increased endothelial–pericyte interaction, resulting in an accelerated neurovascular repair after ischemia.

Conclusions—We show that reducing swelling could result in improved outcome because of reduction in damaged brain tissue. We also identify a novel role for ephrinB2/EphB4 signaling in the maintenance of the neurovascular homeostasis and provide a novel therapeutic approach in reducing brain swelling after stroke.

Visual Overview—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:867–878. DOI: 10.1161/ATVBAHA.116.308620.)

Key Words: blood–brain barrier ■ brain edema ■ brain ischemia ■ endothelial cells ■ homeostasis

Ischemic stroke is the third leading cause of death worldwide and a leading cause of adult disability. Ischemic insult causes rapid cell death and a disruption of functional circuits such as the neurovascular unit (NVU) in the affected areas. The most serious clinical complication of cerebral ischemia is brain swelling due to brain edema. The lethality of brain edema after a malignant middle cerebral artery infarction is ≤80%, and the only therapeutic intervention today is surgical removal of cranial bone to give brain room for swelling.

See cover image

Alterations and disruption of the blood–brain barrier (BBB) play a key role in the development of cerebral edema. Anatomically, the BBB is a part of a NVU. NVU is composed of neurons, astrocytes, endothelial cells (ECs), pericytes, and the tight junctions (TJs) between the ECs. Change to any of these components leads to disruption of the BBB. Little is known about the role of pericytes in the BBB. Recent studies have shown that pericytes migrate away from ECs after hypoxia. In vitro experiments show that pericytes stabilize capillary-like structures. A recent study has also suggested that pericyte constriction may be the cause of the long-lasting decrease of cerebral blood flow, thus damaging the BBB. Pericytes have also been shown to be involved in angiogenesis and EC TJ formation, which is another important constituent of the BBB. The TJ consists of 3 integral membrane proteins: claudin, occludin, and junction adhesion molecules. Decreased expression of TJ proteins (TJPs) is associated with BBB leakage after a focal cerebral ischemia.

Although survival from cerebral ischemia has improved over years, treatments affecting cerebral edema and BBB repair and regeneration are still lacking. Ischemia is one of the strongest stimuli for gene induction in the brain; it activates genes involved in a process of repair, remodeling, and angiogenesis. Recent studies have shown that many of the genes upregulated in brain pathologies are usually active during the developmental
stages and are known as guidance molecules. EphrinB2 and its receptor EphB4 belong to the most important guidance molecules shown to participate both in vascular remodeling and are involved in different components of the NVU formation. The Eph receptors comprise large families of tyrosine kinases receptors with 14 mammalian Ephs subdivided into EphA and EphB. Their ligands ephrin also fall into 2 subclasses; Ephrins tethered to the membrane via glycosylphosphatidylinositol linkage and Ephrins that are transmembrane glycoproteins. In general, EphAs (EphA1–8) preferentially bind to Ephrins (ephrins A1–5) and EphBs (EphB1–6) to Ephrins (ephrinB1–3). During development, the Eph–ephrin signaling system plays a diverse role in tissue patterning, cell migration, and axon guidance. Recently, genetic experiments in mice have shown that ephrinB2 is a critical regulator of vessel survival and pruning in the vasculature of the eye. Most recently, ephrinB has been shown to play a crucial role in pericyte-to-endothelial assembly. Foo et al. have demonstrated that the expression of ephrinB2 in pericytes and mural cells is critical for the assembly of the vessel wall. This feature contributes to blood vessel maturation and stability, which is essential for the intact and functional NVU. Furthermore, different Eph–ephrin molecules have shown to be activated after stroke: EphA2 activation after stroke contributes to BBB damage and neuronal cell death, whereas astrocytic ephrinA5 inhibits axonal sprouting, and ephrinB3 deficiency enhances poststroke neurogenesis. In contrast, the role of ephrinB2/EphB4 signaling in ischemic stroke is still unexplored. In this study, we were able to show that an exogenous pharmacological agent can stimulate neurovascular ephrinB2/EphB4 signaling after stroke, thereby enhancing vascular repair and reducing cerebral brain swelling. Furthermore, our retroviral modulation of ephrinB2/EphB4 signaling after stroke provides a proof of concept that ephrinB2/EphB4 signaling is important in the maintenance of neurovascular homeostasis, neurovascular protection, and in enhancing vascular repair mechanism. Thus, we present a novel therapeutic approach to reduce brain swelling after stroke.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Stroke and Damage to the NVU

We have used a 30-minute middle cerebral artery occlusion (MCAo) and reperfusion model in C57BL/6 mice, which leads to focal, ischemic cell death in striatum but not in the cortex (Figure IA). Ischemic stroke and damage to the different cell types of the NVU was determined by immunostainings at different post-MCAo reperfusion time points (24 hours, 72 hours, 7 days, 14 days, and 28 days). Double staining for TUNEL and cell-type–specific markers was performed to examine the ischemic damage caused to different cell populations. Mature neuron-specific marker NeuN costained with TUNEL marked the neuronal cell loss in the ischemic lesion core on 7 days post-MCAo (Figure IA in the online-only Data Supplement). Fewer EC (CD31) and pericytic cells (PCs; desmin) were also TUNEL positive (Figure IB; Figure IB in the online-only Data Supplement) at 24 hours post-MCAo.

Damage to the different cellular components of the NVU was accompanied by the loss of neurovascular integrity and disruption of the BBB, resulting in the development of brain swelling which peaked 24 hours after reperfusion, as demonstrated by T2-weighted magnetic resonance imaging (Figure IC in the online-only Data Supplement). A temporary lethargy of the animals and 10% loss of body weight was also observed (Figure ID in the online-only Data Supplement). To further examine the neurovascular integrity, we injected Evans Blue dye, which vastly extravasated at 24 hours after reperfusion, confirming the post-MCAo disruption of BBB and loss of neurovascular integrity (Figure IC). Multifluorescence immunostainings for TJPs in the areas of mild ischemia-induced neurovascular damage revealed decreased surface expression of claudin-5 and occludin at 24 hours post-MCAo (Figure IC). As control, TJPs were unaltered, and no extravasation of Evans Blue was observed in the contralateral hemisphere (Figure IC).

Vascular Recovery and Restoration of BBB

The longitudinal analysis of Evans Blue data demonstrated a stepwise reduction of Evans Blue extravasation, which was accompanied by increased surface expression of TJPs on blood vessels (Figure IC). Thus, we sought to better understand this course of BBB recovery and neurovascular repair after MCAo. Brains examined 24 hours to 28 days post-MCAo showed increased cell proliferation in the ipsilateral striatal region. Cell proliferation significantly increased (P<0.001) after 72 hours post-MCAo with a few cells continuing to proliferate ≤28 days (Figure IIA in the online-only Data Supplement). Then, we aimed to identify the proliferating cell types. Both vascular and perivascular cells showed proliferation after mild ischemia. Double labeling of CD31 and K667 showed EC proliferation in perilesion and lesion regions after 72 hours, 7 days (Figure IIB in the online-only Data Supplement), 14 days, and on 28 days post-MCAo. This continued EC proliferation hallmarking the process of post-MCAo angiogenesis, which turned the perilesion and lesion core vascular system into a highly plastic vascular system with multiple angiogenic sprouts (Figure IIA and IIB in the online-only Data Supplement). EC proliferation and the formation of angiogenic sprouts finally resulted in a significantly increased blood vessel density, both in the perilesion and lesion region from 72 hours and 7 days onwards, respectively. Remarkably, the density of this new vascular system exceeded the density of the contralateral, unaffected hemisphere (Figure IIC in the online-only Data Supplement).
Data Supplement). From day 14 post-MCAo, the blood vessel density both in lesion and perilesion regions stabilized. Lectin perfusion on days 7 and 14 showed that the newly formed vessels were perfused, and thus functional in terms of providing nutritive blood supply (Figure IV in the online-only Data Supplement).

Next, we sought to understand the involvement of perivascular supporting cells in the course of BBB postischemic recovery. Desmin staining revealed that pericytic drop-off contributed to a loss of blood vessel integrity and increased vascular permeability within 24 hours post-MCAo (shown in Figure 1D). However, from 72 hours on, double staining of desmin with Ki67 showed pericyte proliferation both in lesion and perilesion regions (Figure VA in the online-only Data Supplement). NG2 another pericyte marker also confirmed the pericyte proliferation (Figure VB in the online-only Data Supplement). The repopulation of the endothelial lining with pericytes led to enhance endothelial–pericyte interactions and thus stabilization of the newly formed vessels (shown in Figure 1D).

Overall, these results suggested that different mechanisms are initiated after mild brain ischemia to restore neurovascular homeostasis and to repair the NVU. Next, we aimed to examine, which molecules are involved in this repair mechanism.

**EphrinB2/EphB4 Expression Time Course Profile After Ischemia**

We focused our study on the relevance of neurovascular guidance molecules, which generally orchestrate the parallel development of the nervous and vascular system during embryological development. As part of a candidate screen, we investigated the mRNA expression of different guidance molecules at different time points after brain ischemia. EphrinB2 and EphB4 showed an early mRNA upregulation after ischemia which gradually normalized at 7 and 28 days post-MCAo, respectively (Figure 2A and 2B). Because the increased mRNA expression of ephrinB2 and EphB4 paralleled the BBB recovery, we focused our further studies on the role of ephrinB2/EphB4 in vascular repair after...
ischemia. Distribution and expression patterns revealed that EC and perivascular cells express ephrinB2 and EphB4 in the adult brain only at low levels under physiological conditions (Figure 2C and 2D, left). However, after mild ischemia, both EC and perivascular cells expressed ephrinB2 and EphB4 at higher levels (Figure 2C and 2D, right). In addition, we were able to show that reactive astrocytes highly express ephrinB2 and EphB4 after mild ischemia (Figure 2C and 2D). Under normal condition, ephrinB2/EphB4 are not expressed by astrocytes (C and D, left); however, after MCAo, reactive astrocytes highly express both ephrinB2 and EphB4 (C and D, right). Representative images for (C) and (D) were taken from 7 d post-MCAo. n=10 animals per experimental groups. Magnification ×200; scale bar, 50 μm.

Overexpression of EphB4 Evokes Changes in Blood Vessel Morphology and Stabilizes BBB Via Recruiting Pericytes

To further examine their potential functional role in repair and recovery after mild ischemia, we manipulated the ephrinB2 signaling using a retroviral approach with the aim to achieve EphB4 overexpression that will result in an ephrinB2 activation phenotype, as previously shown by our group. Therefore, an ecotropic retroviral vector (pLXSN) containing cDNA encoding full-length EphB4 (EphB4wt) was used to manipulate EphB4 expression.

The coordinates for the viral injection were determined using T2-weighted magnetic resonance images (Figure VIA in the online-only Data Supplement). Forty-eight hours post-MCAo, Phoenix E virus-producing cells were stereotactically injected into the ischemic hemisphere. The effect of infection with the virus containing the cDNA for EphB4wt was compared with the effects induced by the empty control vector (pLXSN) after 72 hours and 7 days post-MCAo. Before implantation, the Phoenix E cells were labeled with DiI to track the injected cell, which revealed that the cells were only localized at the site of implantation (Figure VIB and VIC in the online-only Data Supplement). Immunofluorescent staining confirmed a local overexpression of EphB4 at the site of EphB4wt implantation (Figure VID in the online-only Data Supplement). The EphB4 overexpression was accompanied by a significantly increased cell proliferation compared with

Figure 2. Time course of gene expression analysis and expression pattern of neurovascular guidance molecules ephrinB2 and EphB4 after middle cerebral artery occlusion (MCAo). RT-PCR demonstrates an upregulation of mRNA expression of ephrinB2 and EphB4 in ipsilateral hemisphere after middle cerebral artery occlusion (MCAo) compared with the corresponding hemisphere of sham-operated animals. Relative quantification (RQ) value of sham-operated animal is normalized to 1 (A, B; n=10 for MCAo and n=5 for sham, ±SEM). Double fluorescence staining in (C and D, left) shows the baseline expression of ephrinB2 and EphB4 by endothelial cell (CD31), pericytic cells (desmin) and astrocytes (GFAP). After MCAo, an increased expression of ephrinB2 and EphB4 is observed by endothelial and pericytic cells (C and D, right). Representative images for (C) and (D) were taken from 7 d post-MCAo. n=10 animals per experimental groups. Magnification ×200; scale bar, 50 μm.
the normal post-MCAo recovery (Figure 3B). This included enhanced EC proliferation, which hallmarkd vessel growth (Figure 3C and 3E).

EphB4 overexpression also resulted in a remarkable increase in pericyte proliferation (Figure 3D and 3F). The increased pericyte recruitment at the site of EphB4wt injection compared with the pLXSN injection resulted in an enhanced endothelial–pericyte interaction with more cerebral vessels being in contact with perivascular pericytes (Figure 4A and 4B). To further examine, if pericyte recruitment elicited an effect on vessel permeability and BBB characteristics, we injected Evans Blue dye and studied its extravasation kinetics. After EphB4 overexpression, Evans Blue extravasation was significantly reduced at 72 hours post-MCAo (Figure VIIA in the online-only Data Supplement). Quantification of the Evans Blue extravasation area and the number of leaky vessels with extravascular Evans Blue deposits showed a significantly improved vessel permeability at the site of EphB4wt implanted with extravascular Evans Blues deposits revealed a significantly increased cerebrovascular permeability (Figure 4C and 4D). Furthermore, staining for TJP showed increased occludin expression in the EphB4wt group (Figure VIIIB in the online-only Data Supplement). In parallel, the Bederson score, which assesses the neurological function of the animals, was significantly improved in the EphB4wt group (Figure VIIIC in the online-only Data Supplement). There was no difference in the regaining of the body weight (Figure VIIID in the online-only Data Supplement).

These results emphasize the importance of ephrinB2/EphB4 signaling in recovery after ischemic stroke. Activation of ephrinB2 accelerates vascular repair via pericytes recruitment, enhances endothelial–pericyte interaction, and leads to a more rapid blood vessel sealing.

Local Knockdown of EphB4 Blocks EphrinB2-Mediated BBB Repair
We then analyzed whether siRNA-mediated knockdown of EphB4 (and thus inhibition of ephrinB2 signaling) would impair pericyte recruitment and restoration of the BBB. After verification of siRNA-mediated knockdown of EphB4 in NIH 3T3 (Figure VIII A and VIIIIB in the online-only Data Supplement), we injected siRNA-EphB4 cells into the ischemic region 48 hours post-MCAo. Immunofluorescent staining after siRNA-EphB4 showed decreased focal expression of EphB4 after 72 hours (Figure VIIIC in the online-only Data Supplement). Focal reduction of EphB4 had an impact on vascular normalization. It resulted in a reduced pericyte proliferation (Figure 5A) and, hence, reduced pericyte repopulation and coverage of blood vessels (Figure 5B and 5C). The failure of pericyte–EC communication resulted in a severe increase of blood vessel leakiness, as illustrated by vast extravasation of Evans Blue dye on 7 days post-MCAo (Figure 5E). Quantification of the Evans Blue extravasation area and the number of leaky vessels with extravascular Evans Blues deposits revealed a significantly increased cerebrovascular permeability in siRNA-EphB4 animals compared with control animals (Figure 5D; Figure VIIID in the online-only Data Supplement). Furthermore, EphB4 knockdown impaired improvement of neurological function and recovery of the body weight after ischemia (Figure VIIIIE and VIIIF in the online-only Data Supplement).

Pharmacological Stimulation of EphrinB2 Signaling
To study the therapeutic potential of ephrinB2/EphB4 signaling in repairing the BBB disturbances after ischemia, we stereotactically infused EphB4-Fc into the ischemic region 48 hours post-MCAo. The local infusion of EphB4-Fc enhanced the vascular recovery and BBB repair. Endothelial–pericyte association was dramatically improved from 72 hours onwards in the EphB4-Fc infused ischemic hemisphere compared with phosphate-buffered saline infusion (Figure 6A and 6C). Concurrently, Evans Blue extravasation was vastly decreased already at 72 hours post-MCAo (Figure 6B). Quantification of Evans Blue data confirmed the significantly reduced Evans Blue extravasation area and number of leaky vessels with extravascular Evans Blues deposits 72 hours post-MCAo (Figure 6D; Figure IXA in the online-only Data Supplement).

Multifluorescent immunostaining for the TJP occludin further revealed increased expression of these blood vessel sealing molecules (Figure IXB in the online-only Data Supplement). These data are in line with the Phoenix EphB4wt cell implantation data, indicating that activation of ephrinB2 signaling results in pericyte recruitment after mild ischemia and stabilization of the BBB.

EphrinB Phosphorylation During Vascular Remodeling and BBB Repair
Postnatal angiogenesis has been associated with ephrinB phosphorylation. Next, we aimed at investigating the status of ephrinB phosphorylation during the vascular repair phase after ischemia using a Phospho-EphrinB–specific antibody. The overexpression of EphB4 via phoenix cells led to increased ephrinB phosphorylation 72 hours post-MCAo, when compared with the pLXSN implantation. The increase of ephrinB phosphorylation was mainly detected in the vicinity of blood vessel, suggesting that ephrinB phosphorylation is mainly of perivascular nature (Figure XA in the online-only Data Supplement). Similar ephrinB phosphorylation pattern was observed after stereotactic infusion of EphB4-Fc into the ischemic hemisphere (Figure XA in the online-only Data Supplement). This is in line with our data showing the poor BBB repair after the siRNA-mediated knockdown of EphB4. Taken together, our data suggest that ephrinB phosphorylation mediates post-MCAo BBB repair.

EphrinB2 Activation Leads to Reduced Damage in Brain Tissue
To determine the influence of ephrinB2 activation on apoptosis, analysis of apoptotic cells was performed by using TUNEL staining. Total amount of TUNEL-positive cells were counted in the ipsilateral region. Our data demonstrate that the activation of ephrinB2 via EphB4 overexpression leads to reduced numbers of apoptotic cells when compared with pLXSN group. Whereas the knockdown of EphB4 significantly
Figure 3. Local overexpression of EphB4 enhances endothelial and pericytic cell proliferation. Schematic timeline representation of the experimental procedure (A). Modulation of ephrinB2/EphB4 signaling with PhoenixE full-length EphB4 (EphB4wt) cells implanted stereotactically into the ischemia lesion region. Quantification of proliferating cells (Ki67 staining) showed a significant increase 72 h and 7 d post-middle cerebral artery occlusion (MCAo) in EphB4wt compared with pLXSN control (B). This mitotic activation was mainly attributable to enhanced proliferation of endothelial (C) and pericytic cells (D) in EphB4wt compared with pLXSN. Immunofluorescence staining after EphB4wt implants illustrates activation of endothelial and pericyte cell proliferation (E and F, respectively). Arrows indicate some of the proliferating cells, and the dotted line indicates the site of injection. n=8 in all the experimental groups. Values are represented as ±SEM. (***P<0.001, *P<0.05) values were calculated using 2-tailed Student t test. Magnification ×200; scale bar, 50 μm. MRI indicates magnetic resonance imaging.
increases the number of cells undergoing apoptosis on day 7 post-MCAo (Figure 7A, left and Figure 7B). In addition, the number of detectable neurons in the ipsilateral hemisphere was significantly decreased in the knockdown group, whereas the number of neurons is high in the EphB4 overexpression group (Figure 7A, right and Figure 7C). These data suggest a neuroprotective role of the ephrinB2/EphB4 signaling.

Furthermore, the critical role of inflammation after brain ischemia and the contribution of ephrin/Eph signaling in regulating the process of inflammation and inflammatory cells infiltration have been previously discussed.21,22 Our data suggest that the inhibition of ephrinB2/EphB4 does not enhance inflammation after ischemia (Figure XI in the online-only Data Supplement).

**EphB4 Overexpression Involves Angiopoietin-1/Tie2 System at the Endothelium/Pericyte Interface**

As the involvement of angiopoietin-1 (Ang-1)/Tie2 in reducing vascular leakiness has been suggested,20,21 we investigated a link between ephrinB2 activation via EphB4 and Ang-1/Tie2 system. The activation of ephrinB2 via EphB4 leads to an increase in Ang-1 expression in EphB4wt group compared with control group (pLXSN) and siRNA-EphB4 group (Figure 8A). This increased Ang-1 expression in EphB4wt group is mainly observed in PCs (Figure 8B). In addition, EphB4 overexpression also activates receptor Tie2 on endothelium. In EphB4wt group receptor, Tie2 was expressed at a higher level by the ECs (Figure 8C). On the other hand, expression of ligand Ang-2 was also detected in all groups (EphB4wt, pLXSN, and siRNA-EphB4); however, in siRNA-EphB4 group, where ephrinB2/EphB4 signaling was inhibited, Ang-2 was expressed at a higher level (Figure 8D). Taken together, EphrinB2 activation via EphB4 seems to activate Ang-1/Tie2 system, which promotes blood vessel maturation and exerts a vessel sealing effect. Increased Ang-2 in siRNA-EphB4 suggests its involvement in BBB breakdown in the absence of EphB4.

**Discussion**

Stroke triggers a series of events that lead to a drastic loss of different cell types in the affected brain region. Within 24 hours, all principal neurovascular components undergo an orchestrated ischemic-induced damage. The early pericytic drop-off hallmarks the highly permeable vascular system resulting in the development of brain swelling. However, to some extent, brain also demonstrates the ability to recover from brain ischemia. It endeavors to compensate for the cellular loss by increasing cell proliferation and migration of proliferating cells to the damaged brain region.24–26 Proliferation and migration of ECs has been shown to be an essential criterion for angiogenesis.27–29 We demonstrate active EC proliferation that turns the ischemic hemisphere into a highly plastic vascular system with multiple angiogenic sprouts. The continued angiogenic sprouting results in an increased vessel density in both perilesion and lesion region. The increase in vessel density after MCAo correlates with longer survival in patients.30 Both angiogenesis and EC turnover stay constant from 14 days post-MCAo, suggesting that during the first 2 weeks cell recruitment, migration and vascular sprouting occurs, whereas later on the newly formed vascular system stabilizes. These
observations suggest that, in response to brain ischemia, an intrinsic mechanism reorganizes and repairs the damaged vascular system in the lesion and perilesion areas.

Altogether, our current knowledge of post-MCAo recovery suggests an important role of angiogenesis in vascular remodeling and thus improving collateral circulation. However, angiogenesis alone would have less impact on repair, if the BBB is not restabilized. In this study, we present insights into the integral role of pericytes in the normal course of endogenous BBB recovery after cerebral ischemia. Recent studies have shown the importance of pericytes in stabilizing and maintaining the BBB. Pericytes have also been shown to migrate away from blood vessels in response to traumatic brain injury. Gonul et al demonstrated that pericytes of the BBB are the first cells to respond to brain hypoxia in cats. In agreement with this, we show 40% pericyte-negative vessels in the infarct core region 24 hours after MCAo. The pericyte drop-off has a drastic impact on the vascular permeability and in part is responsible for post-MCAo brain swelling. In the context of a repair of this vascular damage, our data demonstrate enhanced pericyte recruitment to the blood vessel wall within 72 hours, which is accompanied by reduced extravasation of Evans Blue. In summary, pericyte recruitment to the nascent blood vessels is pivotal in stabilization and repair of the cerebral vascular system after mild ischemia.

Our data are in agreement with the current state of knowledge that post-MCAo recovery involves vascular remodeling and BBB repair. However, the molecular players behind this functional recovery had not yet been identified. Here, we identify the guidance molecule ephrinB2 as a key player in the context of repair of this vascular damage, our data demonstrate enhanced pericyte recruitment to the blood vessel wall within 72 hours, which is accompanied by reduced extravasation of Evans Blue. In summary, pericyte recruitment to the nascent blood vessels is pivotal in stabilization and repair of the cerebral vascular system after mild ischemia.

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Figure 5. Local knockdown of EphB4 maintains post-middle cerebral artery occlusion (MCAo) cerebral blood vessel damage and hyperpermeability and retards blood–brain barrier repair. Timeline representation of the experimental procedure is same as in Figure 3A. Costaining of Ki67-desmin showed reduced proliferating pericytic cells (A). Endothelial–pericyte interaction was decreased, resulting in blood vessels lacking pericyte coverage on d 7 post-MCAo (B). Focal knockdown of EphB4 leads to a significant decrease in the percentage of desmin-positive vessels (C). Quantification of the extravasation area and immunofluorescence staining reveals a significantly increased cerebrovascular permeability and delay of vascular repair in EphB4-siRNA versus control animals (D and E). n=8 (**P<0.01, *P<0.05) values are represented as ±SEM, and 2-tailed Student t test is conducted. Magnification ×200; scale bar, 50 μm.
by stimulating angiogenesis through ephrinB2. Hayashi et al.\(^39\) show increased expression of ephrinB2 and EphB4 already 1 hour post-MCAo. We found ephrinB2 to be upregulated even at 72 hour post-MCAo, and the expression of its tyrosine kinase receptor EphB4 continues to be elevated ≤14 days post-MCAo. We demonstrated upregulation of these guidance molecules in endothelial and perivascular support cells. Angiogenesis involves the proliferation of ECs, and the recruitment of perivascular cells ensures that the newly formed blood vessels are stable and nonleaky. Therefore, post-MCAo upregulation of ephrinB2/EphB4 in these cell types provides further evidence of their active participation in repair and remodeling. Furthermore, it is well known that ephrinB2/EphB4 possess a unique bidirectional signaling system.\(^40\) EphrinB2 can also function as a receptor, depending in part on tyrosine phosphorylation of the ephrinB2 cytoplasmic domain mediated by the Src family kinase (reverse signaling).\(^41\) Activation of ephrinB2 reverse signaling by

**Figure 6.** Local infusion of EphB4-Fc enhances vascular repair and blood–brain barrier recovery. Highly increased endothelial–pericyte association already after 72 h post-middle cerebral artery occlusion (MCAo) in the ischemic hemisphere (A). Increased endothelial–pericyte interaction led to reduced vascular permeability (B). Local infusion of EphB4-Fc significantly increases the percentage of desmin-positive vessels (C). Measurement of the extravasation area showed a significantly improved vessel permeability \((P<0.001)\) EphB4-Fc versus control (phosphate-buffered saline [PBS]; D). \(n=8\) \((**P<0.001)\) values were determined by 2-tailed Student t test and are represented as ±SEM. Magnification ×200; scale bar, 50 \(\mu m\).

**Figure 7.** EphrinB2 activation reduces apoptosis and neuronal loss. Number of TUNEL-positive cells were significantly decreased in full-length EphB4 (EphB4wt) overexpression, whereas knockdown of EphB4 leads to significantly increased apoptotic cells (A, left). TUNEL Immunofluorescence staining revealed increased apoptotic cells in the EphB4 knockdown group compared with the empty vector (pLXSN) and EphB4wt overexpressing group (B). Counting the neuronal nuclei further showed increased neuronal loss in EphB4 knockdown group and significantly decreased neurons in siRNA animals (A, right and C). Representative images are taken from 7 d post-middle cerebral artery occlusion (MCAo). \(n=5\) \((**P<0.001)\) values are represented as ±SEM and 1-way ANOVA with Tukey test is conducted. Magnification ×200; scale bar, 50 \(\mu m\).
EphB4 has been shown to be proangiogenic and to induce cell attachment and migration. These observations led us to hypothesize that local overexpression of EphB4 should activate ephrinB2 and may accelerate vascular recovery via pericyte recruitment and tightening of endothelium–pericyte interactions. Indeed, we demonstrated that the activation of ephrinB2 via EphB4 overexpression significantly increased EC and perivascular cell proliferation. EphrinB2 activation via EphB4wt led to an enhanced endothelial–pericyte interaction compared with our pLXSN implantation, resulting in a markedly reduced vascular permeability. In parallel, TJPs, such as claudin-5 and occludin, were markedly increased from 72 hours after MCAo. These results suggest that pericytes not just stabilize the blood vessels but also rehabilitate the TJPs by increasing the expression of TJPs, hereby leading to the early BBB repair after mild ischemia. Furthermore, the activation of ephrinB2 was accompanied by accelerated clinical recovery of the animals, thus providing evidence that early BBB repair leads to faster recovery from ischemia and modulating that ephrinB2/EphB4 signaling regulates angiopoietins/Tie2 system under ischemic condition.

Next, we explored the possibility of using ephrinB2/EphB4 signaling system as a therapeutic target by applying EphB4-Fc fragments. EphB4-Fc has been shown to activate ephrinB2 and being involved in angiogenesis. Furthermore, it has been shown that the treatment of human retinal cells with EphB4-Fc results in activating Scr pathway (ephrinB2 reverse signaling). In our experiments, local infusion of EphB4-Fc had a similar effect as the overexpression of EphB4 by the Phoenix E cells. Comparing our results to other Eph receptor such as EphA2, our data show that overexpressing EphB4 improves vascular permeability after ischemia.

**Figure 8.** EPHrinB2 activation via EphB4 involves angiopoietin (Ang)/Tie2 system in reducing vascular permeability. Ang-1 expression was increased in full-length EphB4 (EphB4wt) group compared with pLXSN and siRNA-EphB4 groups (A). Ang-1/desmin costaining showed increased Ang-1 signal in EphB4wt group on pericytic cells, whereas reduced Ang-1 signal is observed in pLXSN and siRNA-EphB4 group (B). Receptor Tie2 expression was increased on endothelial cells in EphB4wt group compared with pLXSN and siRNA-EphB4 group (C). siRNA-EphB4 animals show increased Ang-2 expression compared with pLXSN and EphB4wt group (D). n=4, and representative images are taken from 7 d post-middle cerebral artery occlusion (MCAo). Magnification ×200; scale bar, 50 μm.
EphA2 promotes permeability after lung injury46–50 and has an inhibitory effect on angiogenesis in brain ECs.51 Furthermore, EphA2 directly contributes to BBB damage after ischemic stroke.17 Our data suggest that increase in EphB4 expression directly contributes to BBB damage after ischemic stroke.48–50 and has an inhibitory effect on angiogenesis in brain ECs.51 Furthermore, EphA2 directly contributes to BBB damage after ischemic stroke.17 Our data suggest that increase in EphB4 expression directly contributes to BBB damage after ischemic stroke.48–50 and has an inhibitory effect on angiogenesis in brain ECs.51 Furthermore, EphA2 directly contributes to BBB damage after ischemic stroke.17 Our data suggest that increase in EphB4 expression.

Our study is an exploratory (discovery) study design conducted according to the initial Stroke Therapy Academic Industry Roundtable recommendations, where the initial studies are recommended in young, healthy animals.52 Using 12 to 14 weeks animals, we analyze the reorganization of the neurovascular network in the adult brain after ischemic stroke and the role played by ephrinB2/EphB4 signaling in mediating the poststroke repair. Further studies using aged animals should be conducted as a part of confirmatory study.

One limitation of our study is that we are not able to clarify the contribution of endothelial–endothelial and endothelial–pericyte dysfunction on BBB function when targeting ephrinB2/EphB4. The focus of our study was to analyze the role of ephrinB2/EphB4 in vascular repair and in BBB restoration after mild cerebral ischemia. Our data provide mechanistic insights into how ephrinB2/EphB4 act at the BBB; however, with our experimental approach, we cannot definitively conclude which cell–cell interaction mainly influences the BBB regulation. The ephrinB2/EphB4 effects observed at the BBB might be because of the intercellular communication between EC and PC. However, endothelial–endothelial interaction might also be involved in BBB regulation. BBB is a part of NVU, which is composed of neurons, astrocytes, ECs, pericytes, and TJs between the ECs. Because of this complex composition, both BBB and NVU cannot be broken down to EC–EC contacts. As also shown in our expression pattern of ephrinB2/EphB4 on all cells involved in forming, the BBB and NVU make clear that intercellular communication within this microenvironment cannot be reduced to the level of the endothelial lining (Figure 2C and 2D). Our data point out that ephrinB2/EphB4 are upregulated in both ECs and pericytes after MCAo and also improved our understanding of the role of EC and pericyte dysfunction in BBB. However, for the translation of the mechanism into clinical relevance, it is crucial to conduct further studies in decoding the role of ephrinB2/EphB4 signaling at BBB interface after mild ischemia.

In summary, our findings suggest that the guidance molecule ephrinB2 plays a key role in vascular repair and remodeling after ischemia and may offer an attractive target for novel approaches, which aim at accelerating vascular repair after MCAo and reducing post-MCAo brain swelling. Therapeutic activation of ephrinB2 reverse signaling will enhance EC and PC proliferation, tighten endothelial–pericyte interaction, and repair the BBB.

Sources of Funding

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Disclosures

None.

References


**Highlights**

- The guidance molecules ephrinB2 and EphB4 are involved in neurovascular protection and repair, by mediating direct cell–cell contacts at the vessel wall.
- The activation of ephrinB2 accelerates vascular recovery via pericyte repopulation of the vessel wall, enhances endothelial–pericyte interaction, and leads to a more rapid blood vessel sealing.
- EphrinB2 is an attractive target for novel approaches, which aim at accelerating vascular repair after middle cerebral artery occlusion and reducing post-middle cerebral artery occlusion brain swelling.
EphrinB2 Activation Enhances Vascular Repair Mechanisms and Reduces Brain Swelling After Mild Cerebral Ischemia
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EphrinB2 activation enhances vascular repair mechanisms and reduces brain swelling after mild cerebral ischemia

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Supplemental Material

Materials and Methods

Animals

Animal experiments were conducted in accordance with institutional guidelines and the German Laws for Animal Protection. All animal experiments were approved by
the local ethics committee on animal research (Landesamt für Gesundheit und Soziales Berlin, Germany, LaGeSo No. G 0047/10). 12-14 weeks old male C57BL/6 mice (total body weight: app. 22-25 g) at the start of the experiment were used for the study. The animals were maintained at 22°C room with a 12-hour light/dark cycle and received drinking water ad libitum.

**Model of Cerebral Ischemia**

Animals were anesthetized with a combination of ketamin and rompun injection. Middle Cerebral Artery Occlusion (MCAO) was performed as described 1-4. Cerebral infarcts were produced by 30-minute left MCA occlusion followed by reperfusion. Occlusion was conducted by introducing a 7-0-intraluminal suture (Doccol Corporation) in the internal carotid artery and advanced it. After 30 minutes the filament was withdrawn to allow reperfusion. The temperature was obtained at 37°C with a temperature control table (Medax, Germany). The sham-operated animals (controls) underwent the same surgery except the occlusion of the artery.

**Magnetic Resonance Imaging and Analysis**

MRI was performed using a 7 Tesla rodent Bruker Pharmascan 70/16 scanner (Bruker Biospin, Ettlingen, Germany) with a 16-cm horizontal bore magnet and a 9 cm (inner diameter) shielded gradient with a H-resonance-frequency of 300 MHz and a maximum gradient strength of 300 mT/m. For imaging a 1H-RF quadratur-volume resonator with an inner diameter of 20 mm was used. Data acquisition and image processing were carried out with the Bruker software Paravision 4.0. During the examinations mice were placed on a heated circulating water blanket to ensure constant body temperature of 37°C. Anaesthesia was induced with 2.0% and maintained with 1.3-1.8% isoflurane (Forene, Abbot, Wiesbaden, Germany) delivered in a O2/N2O mixture (30/70%) via a facemask under constant ventilation monitoring (Small Animal Monitoring and Gating System, SA Instruments, Stony Brook, New York, USA). For imaging the mouse brain a T2-weighted 2D turbo spin-echo sequence was used (imaging parameters TR / TE = 4200 / 36 ms, rare factor 8, 4 averages). 20 axial slices with a slice thickness of 0.5 mm, a field of view of (FOV) 2.6 x 2.6 cm and a matrix of 256 x 256 were positioned over the brain from olfactory
bulb to cerebellum. Calculation of lesion volume was carried out with the program Analyze 5.0 (AnalyzeDirect, Inc.; Lenexa USA). The hyperintense ischemic areas in T2-weighted images were assigned with a region of interest tool. This enables a threshold based segmentation by connecting all pixels within a specified threshold range about the selected seed pixel and results in a 3D object map of the whole stroke region. Further the total volume of the whole object map was automatically calculated.

**Real-time PCR**

After sacrifice tissues were quickly harvested, snap frozen in liquid nitrogen and kept at -80°C. Only striatal infarct, peri-infarct and corresponding contralateral striatal region was carefully dissected using mouse brain slicer matrix (Zivic Instruments, Pittsburgh, USA). Total RNA isolation (Invisorb spin tissue RNA kit, Invitrogen Berlin) reverse transcription (QuantiTect reverse transcription kit, Qiagen), real-time PCR (QuantiTect SYBR green PCR, Qiagen) was performed using company's protocols. Real-time PCR was performed with the 7900 fast real-time PCR System, Applied Biosystems. The following primer sequences were used; ephrinB2 forward 5’-AATCAGGTCACAAGACG-3’ and reverse 5’-GTCTCCTGCCTGACTTACGC-3’ and EphB4 forward 5’-GAGCTATGTCCACCGAGACC-3’ and reverse 5’-GAACTTCTCCTGaAGGCAATGG-3’ and GAPDH forward 5’-GGCCTTCCGTGTCCTACC-3’ and reverse 5’ AACCTGGTCCTCAGTGTAGC-3’.

**Immunofluorescence and Confocal Analysis**

The brains were perfused with PBS and immediately shock frozen in chilled isopentane (2-methylbutane, sigma-aldrich). Immunofluorescence studies were performed on frozen sections 20 μm made on cryostat (HM560 microm GmbH). The sections were fixed in methanol for 10 minutes then incubated with blocking solution containing 1% Casein in PBS for 30 minutes followed by the primary antibodies overnight at 4°C or 2 h at room temperature [rat anti CD31 (BD Pharmingen 550274; 1:50), rabbit anti-desmin (abcam ab15200; 1:100), mouse anti-desmin (Thermo Fischer scientific 1:200), mouse anti-NeuN (MAB377 Millipore; 1:200), rabbit anti-
EphB4 (sc-5536 Santa Cruz; 1:100), Goat anti-Ephrin-B2 (GT15026 Neuromics; 1:100), Rabbit anti-Ki-67 (RM-9106-S1 Thermo Fisher scientifics; 1:200), rat anti-KI-67 (Clone TEC-3, Dakocytomation; 1:25), rabbit anti-NG2 (AB5320 Millipore; 1:200), chicken anti-GFAP (abcam ab4674; 1:1000), mouse anti-Claudin-5 (35-2500, invitrogen; 1:100), rabbit anti-ANG-1 (abcam ab8451; 1:100), rabbit anti-ANG-2 (abcam ab8452; 1:100), rabbit anti-Tie2 (C-20) (Santa Cruz sc-324; 1:150), rat anti-CD11b (abcam ab8878; 1:100), rabbit Anti-EphrinB2 (phospho Y316) antibody (abcam ab119323), after three washes with 0.5% casein in PBS, the sections were incubated with secondary antibody for 2hr [Cy3, FITC, Cy5 and AMCA-conjugated antibodies from Dianova were used; 1:200 dilution]. Sections were washed with PBS and they processed for double or triple staining. For TUNEL Staining ApopTag red In situ kit (S7165, Chemicon Inc.) was used. For TUNEL double staining, the sections were first processed for TUNEL staining and then co-stained with CD31, NG2 or NeuN using above mentioned protocol and antibodies. For imaging, we used a Carl Zeiss LSM 5 exciter confocal laser-scanning microscope equipped with an argon/neon laser and a Carl Zeiss Axio observer Z1 inverted immunofluorescence microscope equipped with standard DAPI, FITC, Cy3 and Cy5 filter.

Protein Extraction and Western Blotting

For protein extraction, the cell lysates were prepared by incubation in RIPA lysis buffer complemented with protease inhibitor cocktail (Thermo fischer). The lysates were passed 10 times through pipette and then through a 25G needle. To pellet cell debris, the lysates were centrifuged for 15 minutes at 14000g at 4°C. For the quantification of total protein BCA protein assay (Pierce) was performed using company's protocols. After determination of protein concentration, the samples were prepared in a LDS loading buffer containing β- Mercapethanol and cooked for 5 minutes at 85°C. 20 µg protein/well were loaded in 10% SDS resolving gel. The samples were run in mini protean tetra electrophoresis system (Bio-Rad) at 120V for 60 minutes. Following separation by SDS-PAGE, gels were then transferred to 0.45µm (for EphB4) PVDF membrane paper sandwiches using bio-rad blotter with plate electrodes at 0.4 Ampere for 2 hours. Tris-Buffered saline (TBS) with 0.05% Tween20 (TBST) was used as a diluent and for all wash steps. Membranes were incubating in blocking buffer (5% non-fat dry milk/TBS) for 1 hour at room
temperature, followed by an overnight incubation at 4°C in the presence of antibodies (anti-EphB4, SC-5536 Santa Cruz, 1:500 and anti-actin-HRP, A3854 Sigma Aldrich, 1:10000). Membranes were washed with TBS 0.05% Tween 20 for 10 minutes and incubated in the presence of HRP-conjugated secondary antibody (Jackson immuno research) for 60 minutes. Membranes were washed 3 times with TBS 0.05% Tween 20 for 10 minutes. Band detection was performed by enhanced chemiluminescent substrate (SuperSignal West Pico, Thermo-Fisher Scientific) and visualized by GeneGnome (Syngene).

Evans Blue Administration and Quantification

The integrity of blood brain barrier was assessed by administration of Evans Blue dye. A 2% Evans blue solution in saline was administered intravenously at a volume of 100mg/kg at 24 hr, 72hr and 7 days post-stroke. Evans blue was always administered 4 hr prior to animal sacrifice. Animals were perfused with PBS, brains were frozen and processed for the presence of Evans Blue using fluorescence microscope.

For the quantification of the extravasation area, the Evans Blue accumulation area on a 200x field (i.e., 20x objective lens and 10x ocular; 0.7386 mm² per field) was measured and quantified using ImageJ (NIH) software. For the quantification of the leaky vessel; all the vessels (labelled with CD31) which showed Evans Blue deposition or accumulation around them in the 200x field were considered to be leaky. These vessels were counted and quantified using ImageJ software.

Plasmid Generation and Retroviral Transduction

The Phoenix packaging cell lines are developed by the Nolan laboratory at Stanford University. These cell lines are variants of the 293T cell line and are capable of producing gag-pol, and envelope protein for ecotropic viruses. Thus, the resultant viral particles are capable of infecting dividing murine cells. For our study, full length EphB4 (EphB4WT represents the entire coding region (2992 bp) of the EphB4 receptor) transduced cell line was generated using pLXSN vector, EphB4 siRNA cell line was generated using pSR vector. PhoenixE virus producing cell line was
transfected with pLXSN EphB4WT, pLXSN or pSREphB4siRNA. The supernatant containing viruses were collected and 3T3 cells were infected and selected with G418 (800µg/ml, PAA) in case of pLXSN EphB4WT, pLXSN and with Pyromycin (1µg/ml, Life technologies) in case of pSREphB4siRNA. The scheme and design of retroviral vectors containing cDNA encoding EphB4wt and verification of virus production information are described in Erber et al 5.

**Stereotaxic Injection into Striatal Region**

Phoenix E cells producing EphB4wt viruses were injected (1 ml containing approx. 2.5x10^5 cells) into the ipsilateral striatal region. For the stimulation of ephrinB2, 2 µl (200 µg/ml) EphB4-FC (R&D systems) were injected in the ischemic hemisphere. The injection coordinates were –3.5 mm interior-posterior and ± 2.5 mm mediolateral relative to bregma.

**Cell Culture and Dil Staining**

PhoenixE cells were cultured in DMEM with 4.5 g/l glucose, 10%FCS and Penicillin/Streptomycin (PAA). PhoenixE cells were selected for neomycin resistance with 800 µg/ml G418. Prior implantations transduced cells were stained with Dil (7.5µg/ml) overnight.

**Lectin Perfusion**

Perfusion Lycopersicon esculentum (tomato) lectin, FITC conjugated. The animals were anesthetized with ketamin and rompun and 0.1 ml of 1 mg/ml lectin (in PBS, made on the same day) was slowly injected through a tail vein. After 5 minutes, the animals were perfused with PBS and brain was harvested. We have included this information in method section.

**Quantification of Angiogenesis and Vessel Counting**

Blood vessel counting followed procedures as previously published 6-8. For quantification of angiogenesis, the numbers of branch points were counted, the
number of blood vessel branch points is indicative of the number of new blood vessel sprouts arising from pre-existing blood vessels. Vessel density was determined from infarct core, peri-infarct and contralateral area on a 200x field (i.e., 20x objective lens and 10x ocular; 0.7386 mm² per field). ImageJ (NIH) software was used for quantification.

Cell Counting and Quantification

Both proliferating cells labelled with Ki67 and TUNEL+ cells were counted using ImageJ (NIH) software. Six brain slices that is three slides per brain were used, the sections were between -1.0 mm to +1.4 mm from bregma. The cells were counted on 200x field (i.e., 20x objective lens and 10x ocular; 0.7386 mm² per field).

Neurological score

A global neurological assessment, the Bederson test, was conducted to measure neurological impairments after stroke⁹. The test was conducted after the animals were recovered from anaesthesia and again on the day of sacrifice. Mice were held gently by the tail, suspended couple of meters above the floor, and were tested for forelimb flexion, resistance to lateral push and circling behaviour. A grading scale of 0-3 was used to assess the effects of occlusion. Mice with no neurological deficits extended both forelimbs toward the floor, hence were assigned grade 0. Mice with ischemia flexed the forelimb contralateral to the injured hemisphere; grades were assigned from 1-3 depending upon the severity of resistance to the lateral push and circular behaviour. All the experiments were performed in a blinded manner.

Software

Analyze 10.0, Image J, Matlab, SPSS and Graphpad Prism (6.01) softwares were used for data analysis and figures.

Statistical analysis
Results are presented as mean ± SEM. Data was analysed using statistical program SPSS and Graphpad Prism. Statistical significance was determined by Student’s t-test and ANOVA when indicated. Mann-Whitney test was used for Bederson test. P<0.05 was considered statistically significant.

References

EphrinB2 activation enhances vascular repair mechanisms and reduces brain swelling after mild cerebral ischemia

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Supplementary Figure. I. TUNEL positive cells co-stained with NeuN and DAPI show the cell death and neuronal loss (A). Quantification of TUNEL positive endothelial and pericytic cells 24h post-MCAo (B). n=10 animals per experimental groups. Volume of brain swelling peaked at 24 h post-MCAo indicating loss of vascular and blood-brain barrier integrity. However, compared to 24 h post-MCAo the volume of brain swelling significantly decreased over 14 days post-MCAo (C) (**p<0.001). Animal’s body weight significantly decreased after 24 h and 72 h post-MCAo, but the animals regained the weight (D). n=10 animals per experimental groups. P values were determined by Student’s t-test and ANOVA. Values are represented as ± SEM. Magnification 200x; Scale bar 50 µm.
Supplementary Figure. II. Kinetic of proliferating cells in the ipsilateral striatum over 28 days post-MCAo. Proliferating cells labelled with Ki67 were counted. The number of proliferating cells is significantly increased from 72 h onwards (p<0.001, ± SEM) (A). P values was determined by one-way ANOVA. Double labelling of Ki67 with endothelial cell marker CD31 shows proliferating endothelial cells both in peri-lesion and ischemic lesion from 72 h onwards. Note, under baseline condition proliferating cells are only labelled in subventrical zone (SVZ) (B). Arrows indicate proliferating endothelial cells. Magnification 200x; scale bar 50 µm. N=8 animals per experimental groups.
Supplementary Figure. III. Loss of endothelial cells led to reduced blood vessels 24 h post-MCAo. Increased angiogenic sprouts were observed 7 days post-MCAo and stayed active even after 28 days post-MCAo in the ischemic hemisphere (A). Time courses of angiogenic sprouts (B) and blood vessel density (desmin-positive vessels) (C) indicate post-MCAo angiogenesis in peri-lesional region, ischemic lesion core and contralateral region (***p<0.001,*p<0.05 peri-lesion, lesion core compared to contralateral). Values are represented as ± SEM and n=10 animals per experimental groups. P values were determined using ANOVA followed by Bonferroni’s post hoc test for multiple comparisons. Magnification 200x; Scale bar 50 µm.
Supplementary Figure. IV. Perfusion staining with *L. esculentum* lectin. (A) shows the baseline of perfused vessels. Newly formed blood vessels in the ischemic lesion and the peri-lesional region 7 day and 14 days post-MCAo showed Perfusion staining with *L. esculentum* lectin (B, C). Magnification 200x; scale bar 50 µm. N=5 animals per experimental groups.
Supplementary Figure. V. Double labelling of Ki67 with pericytic cell markers desmin and NG2 show proliferating pericytes both in peri-lesional region and ischemic lesion core (A, B right). On contralateral side proliferating cells are only labelled in SVZ (A, B left). Arrows indicate some of the proliferating cells. N=8 animals per experimental groups. Double labelling of CD31 with ephrinB2 showed ephrinB2 expression prefentially in angiogenic sprouts (C). Magnification 200x; Scale bar 50 µm. N=10 animals per experimental groups.
Supplementary Figure. VI. T2 weighted MRI images 24 h post-MCAo were used to determine the ischemic region and to determine the coordinates for implantation of virus-producing cells (A left). Prior to brain harvesting T2 weighted MRI images were again acquired to determine the infarct volume post injection (A right). Prior to implantation, the Phoenix E cells were labeled with Dil to track the injected cell (B). Dil labelled Phoenix E cells co-stained with DAPI at the implantation site revealed that the cells did not migrate (C). Thus, the changes induced by the Phoenix E cells were only localized at the site of implantation. Staining with EphB4 antibody showed an increased expression of EphB4 in EphB4-wt compared to pLXSN control indicating successful overexpression of EphB4 at the ischemic lesion implantation site (D). Magnification 200 x; scale bar 50 µm.
Supplementary Figure. VII. Decreased extravasation of Evans Blue is observed from 72 h onwards in EphB4-wt sections (A). Tight junction protein occludin showed increased blood vessel coverage in EphB4-wt compared to pLXSN control (B). Bederson neurological score showed significant improvement in EphB4wt (p<0.001) versus pLXSN (C) (***p<0.001, **p<0.01 *P<0.05, Mann-Whitney test). Animals in both EphB4-wt and pLXSN group regain weight over time (D). N=8 in all the experimental groups. Values are represented as ± SEM. Magnification 200x; Scale bar 50 µm.
Supplementary Figure. VIII. Downregulation was verified by infection of NIH 3T3 murine fibroblasts with the supernatants of siRNA-EphB4 cell cultures. Western blot analysis of siRNA-EphB4 in infected NIH 3T3 showed downregulation of EphB4 at the predicted molecular weight of approx. 120kDa, when compared to the PLXSN infected NIH 3T3 (A,B). Staining of the cryofixed sections following implantation of Phoenix E EphB4-siRNA cells showed decreased focal expression of EphB4 (C). Quantification of the number of leaky vessels revealed a significantly increased cerebrovascular permeability EphB4-siRNA versus control animals (D). EphB4 knockdown prevents recovery of neurological function and body weight after MCAo (E,F respectively). ***p<0.001, **p<0.01 *P<0.05 Values are represented as ± SEM and are determined by Mann-Whitney test and student’s t-test. Magnification 200x; Scale bar 50 µm. n=8 for both siRNA and MCAO group.
Supplementary Figure. IX. Measurement of the leaky vessels showed a significantly improved vessel permeability (p<0.001) EphB4-FC versus normal MCAo recovery (PBS group) (A). Tight junction protein occludin showed increased blood vessel coverage (B) N=8 for EphB4-Fc group. P values were determined using Student’s *t*-test Magnification 200x; Scale bar 50 µm.
Supplementary Figure. X. EphrinB phosphorylation during recovery phase. Compared to empty vector (pLXSN), increased ephrinB phosphorylation was detected from 72h after the manipulation of ephrinB2 signalling via EphB4 (A). Pharmacological application of EphB4-Fc in the ischemic hemisphere leads to increased ephrinB phosphorylation from 72 h onwards comparing to the PBS injection (B). SiRNA mediated knockdown of EphB4 leads to much diminished ephrinB phosphorylation (C). The increased ephrinB phosphorylation is mainly in perivascular cells. N=5, Magnification 200x; Scale bar 50 µm.
Supplementary Figure. XI. EphrinB2/EphB4 signaling in inflammation. Compared to empty vector (pLXSN), activation or inhibition of ephrinB2/EphB4 did not enhance inflammation after MCAo. N=4, Magnification 200x; Scale bar 50 µm.