Published in final edited form as:

Eur J Neurosci. 2014 June ; 39(12): 2129–2138. doi:10.1111/ejn.12556.

Chronic metformin treatment improves post-stroke angiogenesis and recovery after experimental stroke

Venugopal Reddy Venna¹, Jun Li¹, Matthew D. Hammond¹, Nickolas S. Mancini², and Louise D. McCullough^{1,3}

¹Department of Neuroscience, University of Connecticut Health Center, Farmington, CT 06030, USA

²School of Medicine, University of Connecticut Health Center, Farmington, CT 06030, USA

³Department of Neurology, University of Connecticut Health Center, Farmington, CT 06030, USA

Abstract

Metformin is currently the first-line treatment drug for type 2 diabetes. Metformin is a well-known activator of AMP-activated protein kinase (AMPK). In experimental studies, metformin has been shown to exert direct vascular effects by increasing vascular endothelial growth factor expression and improving microvascular density. As stroke is the leading cause of long-term disability and angiogenesis is implicated as an important mechanism in functional recovery, we hypothesized that chronic metformin treatment would improve post-stroke functional recovery by enhancing functional microvascular density. For this study, C57BL/6N male mice were subjected to a 60-min middle cerebral artery occlusion, and were given 50 mg/kg/day metformin beginning 24 h post-stroke for 3 weeks. Behavioral recovery was assessed using adhesive-tape removal and the apomorphine-induced turning test. The role of angiogenesis was assessed by counting vessel branch points from fluorescein-conjugated lectin-perfused brain sections. Importantly even if metformin treatment was initiated 24 h after injury it enhanced recovery and significantly improved stroke-induced behavioral deficits. This recovery occurred in parallel with enhanced angiogenesis and with restoration of endogenous cerebral dopaminergic tone and revascularization of ischemic tissue. We assessed if the effects on recovery and angiogenesis were mediated by AMPK. When tested in AMPK α -2 knockout mice, we found that metformin treatment did not have the same beneficial effects on recovery and angiogenesis, suggesting that metformin-induced angiogenic effects are mediated by AMPK. The results from this study suggest that metformin mediates post-stroke recovery by enhancing angiogenesis, and these effects are mediated by AMPK signaling.

Keywords

AMPK; apomorphine; cerebral ischemia; metformin

Correspondence: Dr L.D. McCullough, Department of Neurology, University of Connecticut Health Center, Farmington, CT 06030, USA, lmcullough@uchc.edu.

Conflicts of interest and disclosure: None.

Introduction

More than 750,000 new strokes occur each year in the USA alone. Worldwide, stroke is responsible for approximately 4.4 million deaths (9% of total deaths) annually (Strong *et al.*, 2007; Meyers *et al.*, 2012). As life expectancy increases, the incidence of stroke will continue to rise (Strong *et al.*, 2007) and improvements in medical care will increase survival rates, leading to increased numbers of stroke survivors in our communities (Towfighi & Saver, 2011; Burke *et al.*, 2012). Due to the fact that stroke survivors often experience profound, life-long deficits, stroke remains the primary cause of adult-onset disability (Towfighi & Saver, 2011). Therefore, novel post-stroke interventions to improve functional recovery and quality of life for stroke patients are urgently needed.

Post-stroke functional recovery is thought to occur by the compensation of lost capabilities by functionally related regions in the intact hemisphere or by the restoration of structural rewiring within the ischemic region (Chopp *et al.*, 2007; Ergul *et al.*, 2012). Clinical imaging studies suggest that lost functions are initially shifted to the spared hemisphere and later, as inflammation subsides and blood flow improves, these functions are taken over by the peri-infarct cortex (Rossini *et al.*, 2003; Johnston *et al.*, 2012). It has been reported that the restoration of blood flow by enhanced angiogenesis and revascularization of ischemic tissue is an important mechanism for long-term functional recovery (Rossini *et al.*, 2003; Chopp *et al.*, 2007; Ergul *et al.*, 2012; Johnston *et al.*, 2012).

AMP-activated protein kinase (AMPK), an evolutionarily conserved serine/threonine protein kinase, is activated by phosphorylation of threonine 172 in the catalytic α -subunit when energy supply is limited, such as occurs in stroke. AMPK signaling is a critical sensor of peripheral energy balance, and may be an important determinant of blood vessel recruitment to tissues subjected to ischemic stress (Nagata *et al.*, 2003). The catalytic subunit alpha (α) of AMPK is expressed in most cell types including endothelial cells, and is a major metabolic regulator at both the cellular and organismal level. AMPK activation has also been shown to significantly promote vascular endothelial growth factor (VEGF) expression (Ouchi *et al.*, 2005) and neurogenesis (Wang *et al.*, 2012). Metformin is a well-recognized AMPK activator both *in vitro* and *in vivo* (Zhou *et al.*, 2001). Acute activation of AMPK by metformin is detrimental in experimental stroke models (Li *et al.*, 2010), secondary to lack of substrates such as glucose in the ischemic brain. However, little is known about the role of metformin in chronic stroke recovery after experimental stroke. Recent research suggests that metformin contributes to energy regulation and anti-inflammatory effects in an AMPK-independent manner (Viollet *et al.*, 2012), and clinical studies suggest that chronic treatment with metformin may also reduce stroke incidence (UKPDS Group, 1998).

Metformin is the first-line treatment for glycemic control as it reduces hepatic glucose production and improves insulin sensitivity in type 2 diabetic patients without causing additional weight gain (Nathan *et al.*, 2009). Interestingly, studies have shown that metformin has other beneficial properties, including favorable cardiovascular and cerebrovascular effects (Zimmet & Collier, 1999; Roussel *et al.*, 2010). Previous studies demonstrate that acute treatment with metformin worsens ischemic injury by enhancing AMPK activity and lactic acidosis. However, chronic metformin treatment prior to stroke

offers potent neuroprotective effects similar to preconditioning (Venna *et al.*, 2012a) by downregulation of AMPK by a negative feedback mechanism, blunting ischemia-induced AMPK activation (Li *et al.*, 2010). To date, the effect of metformin treatment on long-term functional recovery in experimental stroke has not yet been investigated except in diabetic animals (Prakash *et al.*, 2013). In this study, we examined whether post-stroke metformin treatment could improve functional recovery and revascularization after ischemia, and investigated the role of AMPK in chronic metformin treatment.

Materials and methods

Focal cerebral ischemia model

The present study was conducted in accordance with NIH guidelines for the care and use of animals in research, and under protocols approved by the Center for Laboratory Animal Care at University of Connecticut Health Center (UCHC). Focal transient cerebral ischemia was induced in male mice (20–25 g) by right middle cerebral artery occlusion (MCAO) followed by reperfusion as described previously (Li *et al.*, 2010; Venna *et al.*, 2012b). To perform surgery, mice were rapidly sedated with 4% isoflurane anesthesia, and the level of sedation was confirmed by the lack of response to tail pinch. Surgery was performed under 1% continuous isoflurane anesthesia. At the end of ischemia (60 min MCAO), the animal was briefly re-anesthetized and reperfusion was initiated by filament withdrawal. Cortical perfusion (laser Doppler flowmetry) was evaluated throughout MCAO and early reperfusion as described previously (Li *et al.*, 2010; Venna *et al.*, 2012b). One group of mice was given metformin or vehicle treatment beginning at 24 h after stroke, and killed for infarct analysis after 72 h of reperfusion. A second group of mice was given metformin or vehicle treatment for 3 weeks initiated at 24 h after stroke. Mice were subjected to behavioral tests for 30 days prior to death. Wild-type (WT) C57BL/6N mice were purchased from Charles River (Charles River Laboratories, Wilmington, MA, USA) and randomly assigned to treatment groups. An additional cohort of AMPK α -2 knockout (KO) mice and WT littermates (C57BL6 background) was treated with metformin or vehicle, and assessed for long-term behavioral recovery and histological outcomes at 30 days post-stroke. These mice were originally obtained from Dr Benoit Viollet in France, and re-derived and bred in-house at UCHC.

Drug treatment

Metformin was dissolved in saline (vehicle) and given in a dose volume of 0.2 mL/20 g body weight by intraperitoneal route (i.p.). The treatment group was administered metformin once daily (50 mg/kg/day) starting 24 h after the onset of MCAO. The cohort used for infarct analysis received two doses, while the long-term survival cohort was treated for 3 weeks. The control group received an equal volume of saline for the same duration.

Infarct analysis and blood glucose analysis

The infarct analysis cohort was subjected to stroke, and received delayed metformin treatment at 24 and 48 h post-stroke. At 72 h of reperfusion, all animals were killed by cervical dislocation. Brains were removed and cut into five 2-mm coronal sections and stained with 1.5% 2,3,5-triphenyltetrazolium chloride (TTC) for 8 min at 38 °C. Slices were

formalin-fixed (4%), digitalized and infarct volumes were analysed (Sigma Scan Pro) as previously described (Venna *et al.*, 2012b). The final infarct volumes are presented as a percentage (percentage of contralateral structures with correction for edema) $\pm$ SD, as previously described (Li *et al.*, 2010; Venna *et al.*, 2012b; $n = 10$ /group). For atrophy measurement in long-term survival cohorts, mice were killed at 30 days post-stroke and perfused transcardially with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde, and the brains were cyroprotected in 30% sucrose. The brains were cut into 30- μ m sections on a freezing microtome, and every eighth slice from the appearance of corpus callosum was stained with Cresyl violet for evaluation of atrophy. Analysis was performed from digitized images of brain sections. Due to the chronic nature of this study, cerebral atrophy was used as an indirect measure of cell death in long-term survival cohorts. The volume of tissue atrophy was determined by measuring both hemispheres, the cystic cavity and lateral ventricles as previously described with $n = 10$ in each group (Bland *et al.*, 2000; Li *et al.*, 2004, 2010). Blood glucose levels were assessed at day 30 with a VetScan iSTAT analyser ($n = 9$ /group).

Neurological deficit scores (NDS)

NDS were evaluated in both the intra-ischemic period and at 72 h post-stroke ($n = 10$ /group). The scoring system was as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling (Venna *et al.*, 2012b).

Adhesive-tape removal test

The adhesive-tape (sticky-tape) removal test is a measure of somatosensory dysfunction after cerebral ischemia in mice (Bouet *et al.*, 2009). Adhesive-backed tape (30 $\times$ 40 mm) was used as tactile stimuli placed on the distal–radial region of the left wrist, and the mean time to remove the tape was recorded. Animals were trained for 5 days once a day prior to stroke, and the latency to remove adhesive tape was measured on days 7, 14 and 28 after stroke ($n = 9$ /group).

Apomorphine-induced rotational activity

Spontaneous motor asymmetry and increased rotational bias toward the lesion side after injection of the dopamine agonist apomorphine (2 mg/kg i.p.) was quantified using an automated rotometer (RotaCount 8 Rotation Counter; Columbus Instruments) in which mice were harnessed to swivels for 5 min of habituation before being injected with drug. Apomorphine was first dissolved in 0.1% ascorbate (in H₂O) and then buffered with 10 $\times$ PBS to a final concentration of 0.3 mg/mL in 1 $\times$ PBS. I.p. injection volumes were 100 μ L/10 g body weight. After apomorphine injection, animals were monitored for 60 min. Mean turning bias is expressed as the difference between the right and left turns over a 60-min period. This test was performed on days 3 and 30 after stroke ($n = 9$ /group). Data are presented as rotational bias (total right turns – total left turns).

Fluorescein-labeled lectin injections and histological analysis

Mice were anesthetized under isoflurane anesthesia and the femoral artery was exposed by a small incision. Fluorescein-labeled lycopersicon esculentum lectin (Vector Labs, CA, USA) was diluted 1 : 1 with saline, and 200 μ L was injected into the femoral artery 5 min before death. To obtain sections for histology, animals were deeply anesthetized and perfused transcardially with ice-cold PBS followed by 4% paraformaldehyde. Brains were harvested, cryoprotected in 30% sucrose and cut on a freezing microtome into 30- μ m sections. Floating sections were stored at -20°C . For bromodeoxyuridine (BrDU) staining, a cohort of mice ($n = 6/\text{group}$) was injected with 50 mg/kg of BrDU (Sigma) once a day from days 3 to 7. BrDU co-labeling was performed on lectin-perfused brain sections.

Immunohistochemistry

To perform immunohistochemistry, 30-micron sections obtained at 30 days after reperfusion were slide-mounted and incubated in blocking solution followed by microwave irradiation for 5 min in 0.1 M, pH 6 citrate buffer solution. Newborn cells were visualized by co-labeling with BrDU in FITC-conjugated lectin-perfused brains. Co-labeling was done on irradiated lectin brain sections using CD31 (1 : 200; Millipore, Billerica, MA, USA) and were subsequently incubated for 60 min with rhodamine-conjugated anti-mouse Ab, after washes sections were examined. Staining for VEGF and glial fibrillary acidic protein (GFAP) quantification was performed on subsequent sections following an overnight incubation with a rhodamine-conjugated GFAP antibody (1 : 250; DAKO) or VEGF antibody (1 : 200; Abcam), and slices were subsequently incubated in secondary antibody for 60 min before washing and coverslipped using Fluoromount. Sections were visualized under an inverted light Zeiss axiovert fluorescence microscope; $n = 9/\text{group}$.

Histological quantification

Thirty-micrometer brain sections were mounted on Superfrost Plus slides (Fisher). Sections were imaged on a Zeiss Axiovert 200M fluorescence microscope under a $20\times$ objective, and images were processed with a 1.3 gamma setting. Three images were taken from three pre-specified areas in the peri-ischemic region of coronal brain per animal ($n = 9$ animals/group) and stained. Vessel branch points were quantified at ~ 0.7 mm from bregma (lateral ~ 1.2 – 1.8 mm and a depth of ~ 1.5 , 2.5 and 3.5 mm), and GFAP and VEGF expression quantified at ~ 0.2 mm bregma (lateral ~ 1.2 – 1.8 mm and a depth of ~ 1.5 , 2.5 and 3.5 mm) in each brain by a blinded investigator in the peri-infarct area adjacent to the ischemic region (see schematic in Fig. 4). A branch point was defined as a single fluorescein-labeled vessel noticeably separating into two distinct and separate vessel tracks. GFAP-positive cell bodies were quantified from GFAP-stained sections, and numbers were obtained from counting GFAP⁺ cell bodies from each image by a blinded investigator ($n = 9/\text{group}$).

Statistics

Data were expressed as mean $\pm$ SEM except for infarct and NDS, which were presented as mean $\pm$ SD and median $\pm$ interquartile range, respectively. All animals were tail marked and assigned to one of the treatments using a randomized number assigned to one of the treatment conditions by SPSS software. Statistics were performed by either univariate

analysis for two groups or two-way analysis of variance ($_{ANOVA}$) for repeated-measures using SPSS version 17. The Mann–Whitney U -test was used for non-parametric tests (NDS). A P -value < 0.05 was considered to be statistically significant. Investigators performing MCAO, behavioral, histological and infarct size analysis were blinded to the treatment group.

Results

Metformin administration has no effect on infarct size or NDS 72 h after MCAO

In order to investigate whether delayed post-stroke metformin administration could influence ischemic infarct volume, mice were subjected to a 60-min MCAO and reperfusion. Mice were treated 24 and 48 h after stroke with either metformin or a vehicle control. At 72 h post-stroke, animals were killed and infarcts were evaluated with TTC staining (Fig. 1B). Infarct analysis revealed that metformin-treated stroke animals had a total infarct volume of $40.03 \pm 5.3\%$ ($n = 10$), and vehicle-treated animals had an infarct area of $38.5 \pm 4.8\%$ ($n = 10$); power analysis from the using G power ver. 3.1.7 confirmed no statistical difference between the two groups. In addition, no significant differences were seen in the striatum or cortical infarct areas ($P > 0.05$; Fig. 1C). There was no significant effect of delayed metformin treatment on the NDS at 72 h post-stroke (metformin 2.5 ± 1 , vehicle 2.5 ± 0.75 ; $P > 0.05$; $n = 10$ /group; Fig. 1D). One animal from the metformin group was excluded due to subarachnoid hemorrhage, and one from the vehicle group was excluded due to excessive weight loss. No mortality was seen in either group at 72 h.

Metformin treatment reduced latency of adhesive-tape removal

In parallel to the treatment with 50 mg/kg/day metformin or vehicle, pre-trained animals were tested for latency in the adhesive-tape removal test. Repeated-measures $_{ANOVA}$ revealed a significant effect of stroke ($F_{1,26} = 173.7$, $P < 0.001$) and of metformin treatment ($F_{1,26} = 6.0$, $P = 0.02$), and a significant stroke $\times$ treatment interaction ($F_{1,26} = 5.7$, $P = 0.02$). These results suggest that stroke significantly increased the latency in tape removal, and that the stroke-enhanced latency in tape removal was significantly improved by metformin treatment (Fig. 2).

Apomorphine-induced rotation bias is reduced with metformin treatment—We next investigated whether chronic metformin treatment reduced apomorphine-induced rotational behavior in mice subjected to 60-min MCAO. Data are expressed as the difference between the right and left turns over a 60-min period (Fig. 2). Although no significant differences between vehicle- and drug-treated groups were seen in rotational behavior at day 3, repeated-measures $_{ANOVA}$ showed a significant effect of treatment ($F_{1,26} = 5.3$, $P = 0.03$), a significant effect of stroke ($F_{1,26} = 126.5$, $P < 0.01$) and an interaction between treatment and surgery ($F_{1,26} = 6.6$, $P = 0.02$), suggesting that chronic metformin treatment significantly attenuated the stroke-induced rotational behavior at day 30 (Fig. 3).

Blood glucose levels, body weights and mortality at 30 days post-stroke—

Blood glucose levels and body weights were analysed at day 30, 1 week following 3 weeks of treatment with metformin. There were no significant differences in body weight in the metformin-treated stroke group compared with vehicle (metformin 33.9 ± 3 vehicle $30.6 \pm$

5; $P > 0.05$; $n = 9/\text{group}$). Similarly, no significant differences were observed in blood glucose levels (metformin 139 ± 15 mg/dL, vehicle 152 ± 18 mg/dL; $P > 0.05$; $n = 9/\text{group}$). One mouse from each group was excluded due to excessive weight loss $> 20\%$. Three mice from the vehicle group and two mice from the metformin group died in the 30-day survival cohort.

Increased vasculature is observed after long-term metformin treatment

At day 30 there was a significant increase in vessel branch points (nodes) in metformin-treated stroke brains, suggesting increased angiogenesis. Chronic treatment with metformin significantly increased the number of branch points compared with control (metformin 9.7 ± 0.67 , vehicle 5.4 ± 0.61 ; $n = 9$, average of 27 images/group). No significant differences were observed in shams (Fig. 4A and B). Two-way ANOVA revealed a significant effect of surgery ($F_{1,78} = 56.8$, $P < 0.001$) and a significant effect of treatment ($F_{1,78} = 9.06$, $P = 0.004$). Similarly, BrDU co-labeling at day 30 showed BrDU⁺ cells incorporated into blood vessels in metformin-treated stroke brains ($n = 6/\text{group}$; Fig. 4C). Together, these findings suggest that long-term metformin treatment promotes angiogenesis after stroke. To further confirm that the increased number of perfused blood vessels in metformin was not secondary to microthrombosis in the vehicle group, we performed co-labeling with CD31. Significant differences between vehicle and metformin groups were seen, confirming increased angiogenesis in metformin-treated animals (Fig. 5A).

Metformin increased VEGF expression and reduced glial scarring

Metformin-treated animals had increased expression of VEGF, and a significant difference in integrated optical density was observed when compared with vehicle in the ipsilateral striatum ($F_{1,54} = 26.68$, $P < 0.001$). In addition, the glial scar area was significantly larger in the vehicle group compared with metformin-treated animals ($F_{1,18} = 6.05$, $P = 0.026$; $n = 9/\text{group}$). A statistically significant reduction in the number of GFAP-positive cells was seen in the metformin-treated group compared with vehicle controls ($F_{1,54} = 10.5$, $P = 0.002$; $n = 27$ images from nine brains per group and 3 images/brain from the ipsilateral region were used for analysis).

Metformin treatment effects on recovery and angiogenesis are mediated by AMPK

AMPK α -2 KO mice were treated with vehicle or chronic metformin to investigate the role of AMPK in metformin-induced recovery. Mice treated with vehicle or metformin starting 24 h after stroke did not show significant differences between groups either in the adhesive-tape removal test (even at day 28: vehicle 48 ± 5 , metformin 36 ± 4.6 ; $P > 0.1$; Fig. 7A) or in the apomorphine turning test. ANOVA 2×2 repeated-measures revealed a significant effect of treatment ($F_{1,30} = 15.6$, $P = 0.03$), but no significant differences between WT and KO mice ($F_{1,30} = 0.05$, $P = 0.82$), and no interaction between genotype and treatment ($F_{1,30} = 2.2$, $P = 0.15$; Fig. 7B). In addition, no significant differences were seen in the branch points between vehicle and metformin groups (vehicle 8.4 ± 1.4 , metformin 9.1 ± 1.7 ; $P > 0.05$; $n = 10/\text{group}$, 3 images/brain section; Fig. 7C and D). In contrast to the beneficial effects of metformin in WT mice, metformin treatment did not reduce post-stroke atrophy in AMPK

α -2 KO mice when compared with vehicle-treated KO mice (13 ± 3.2 vs metformin 12 ± 4.4 ; $n = 10/\text{group}$). Mortality was 2 in each group.

Discussion

This study demonstrates the benefits of chronic post-stroke metformin treatment on behavioral recovery and post-stroke angiogenesis. Delayed post-stroke metformin treatment improved functional recovery, as measured by the adhesive-tape removal test and the apomorphine-induced turning test. Although mice tested 3 days post-stroke showed no behavioral benefit, chronic metformin treatment significantly reduced rotational bias at day 30 post-stroke, suggesting that metformin treatment ameliorated the abnormal dopaminergic tone that occurs following stroke. We also observed that chronic metformin treatment significantly enhanced angiogenesis, confirmed by an increased number of branch points in functional blood vessels and BrDU co-labeling. This suggests that chronic metformin treatment enhances functional recovery in parallel to the induction of angiogenesis.

The intra-arterial suture occlusion of the MCA used in this study is the most commonly used focal stroke model in the mouse (Liu & McCullough, 2011). This ischemic injury model mimics a clinical MCAO, the most prevalent form of human stroke (Liu & McCullough, 2011). A blockage in this vessel leads to obstruction of collateral blood supply, and produces focal ischemic lesions in the striatum and motor cortex (Liu & McCullough, 2011; Truong *et al.*, 2012). Previous studies have reported that with prolonged reperfusion and survival times, the infarct gradually spreads to the other parts of the MCA territory. In this model, the time-dependent increase in infarct volume peaks after 24 h of reperfusion, and is consistent with several other magnetic resonance imaging experiments of transient MCAO (van Lookeren Campagne *et al.*, 1999; Hoehn *et al.*, 2001; Liu & McCullough, 2011). To evaluate recovery independent of ischemic damage, we initiated metformin treatment after ischemic damage had peaked. As pre-stroke acute metformin treatment has been shown to increase ischemic damage (Li *et al.*, 2010), we used an independent cohort to analyse changes in ischemic volume at 72 h. Former studies have indicated that lesion development slows considerably or ceased altogether by 24 h after the onset of ischemia (Dereski *et al.*, 1993; Garcia *et al.*, 1993). Consistent with this, our histological analysis confirmed that no significant difference in infarct size was seen at 72 h with delayed metformin treatment.

Neurological deficits induced by cerebral ischemia typically improve in the first few days or weeks after a stroke. Previous studies have demonstrated that functional benefits can be achieved independently of reduction in the gross infarct volume (Thiyagarajan *et al.*, 2008). Motor deficit and recovery is greatly variable, even among patients with identical clinical severity in the acute phase. Multiple explanations for these differences have been proposed, including degree of edema reabsorption, rate of intracranial pressure change (which can contribute to secondary ischemic damage), variability in the extent to which collateral vessels from adjacent arteries supply blood to the penumbra region (Rossini *et al.*, 2003; Murphy & Corbett, 2009), differences in organization of neuronal connections in different cortical areas (Newton *et al.*, 2006) and variability of functional appropriation by surviving brain regions (Murphy & Corbett, 2009; Fridriksson, 2010). Although activation of AMPK by metformin has been well described, its effect on the post-stroke recovery is not

understood. Chronic AMPK activation has been shown to promote VEGF, which appears to be an important player in regulating angiogenesis (Nagata *et al.*, 2003; Ouchi *et al.*, 2005). Consistent with this, we found increased VEGF expression in metformin-treated animals, suggesting this is an important underlying mechanism in metformin-induced recovery.

Recovery from post-stroke motor deficits has been associated with cerebral plasticity, the ability of the brain to compensate for loss of function through reorganization and rewiring of neuronal networks. It has been previously reported in both experimental and clinical studies that these restoration processes may take several days or weeks. The adhesive removal task is a sensitive indicator of deficits caused by sensorimotor cortex damage after MCAO-induced focal ischemia (Bouet *et al.*, 2009). Impairment of the contralateral limb contributes to increased latency in removal of the adhesive tape, indicating a deficit in sensorimotor function and a possible impairment in motor control. In this study, metformin-treated mice tested 7 days after stroke showed no significant difference in latency compared with vehicle. But when tested at day 14 and day 28 after stroke, chronic metformin-treated animals were significantly faster at removing the adhesive tape, indicating enhanced recovery compared with vehicle-treated animals despite equivalent initial infarct volumes. Consistent with earlier studies, the delay in the recovery of adhesive removal may be due to the time required for restoration of functional and structural plasticity. Metformin has been shown to promote cell proliferation and improve neuronal plasticity, and new cells take several weeks to mature and incorporate into functional networks (Menendez & Vazquez-Martin, 2012; Potts & Lim, 2012; Wang *et al.*, 2012).

Hemiparesis and unilateral spatial neglect following cerebral ischemia are common and disabling conditions. Stroke disrupts the integrity of the mesolimbic dopaminergic system (Kronenberg *et al.*, 2012), causing affected mice to circle to one side, possibly reflecting a stronger push or pull strength of the unaffected limb. Although this rotation behavior deficit is normalized in the first few days post-stroke, mice with unilateral lesions induced by stroke respond to an acute injection of the dopamine agonist apomorphine by exhibiting a pronounced and continuous rotation behavior to the right side. This is consistent with an ipsilateral rotation, presumably because an increased amount of striatal dopamine interacts with dopaminergic receptors on the intact side of the brain. Beneficial effects of the restoration of dopaminergic tone have been previously reported. Normalization of rotation bias may therefore have an important role in restoration of functional recovery in stroke survivors (Veizovic *et al.*, 2001).

Chronic metformin treatment is associated with a significant improvement in the endogenous dopaminergic tone while simultaneously maintaining glycemic control (Uehara *et al.*, 2001; Ortega-Gonzalez *et al.*, 2005). Chronic metformin treatment consistently reduced rotation behavior in stroke mice compared with vehicle, an effect not observed at the 3 days of treatment. One possible explanation is that metformin-induced increases in angiogenesis may contribute to restoration of dopaminergic tone in the ischemic hemisphere (Potts & Lim, 2012; Sun *et al.*, 2012; Wang *et al.*, 2012). It is also possible that reduced rotation may be caused by non-specific sedative effects of apomorphine (Muller *et al.*, 2004) or a drug interaction with metformin. We did not find any visual evidence of sedation at day 3 or day 30 while testing, and did not observe any differences in spontaneous locomotor

activity between the metformin and vehicle groups when tested in open field following the apomorphine rotation test (data not shown). In addition, the metformin treatment was stopped at day 21 post-stroke and the apomorphine test was performed on day 30. Another possible explanation for reduced rotation behavior is that chronic metformin treatment caused overstimulation of dopamine receptors and sensitized dopaminergic tone, resulting in a downregulation of those receptors (Uehara *et al.*, 2001). Further studies are needed to investigate how chronic treatment with metformin eliminated stroke-induced rotation bias, but restoration of neural networks via angiogenesis is a plausible explanation.

Metformin has been shown to rapidly penetrate the blood–brain barrier and differentially accumulate in the brain, where it helps to attenuate vascular inflammation (Labuzek *et al.*, 2010b; Kim & Choi, 2012). Surprisingly, metformin treatment resulted in functional benefits without a significant change in the initial gross infarct volume or in blood glucose levels. It has been shown previously that metformin treatment increases phagocytosis of dead tissue, which could potentially contribute to a reduced rate of intracranial pressure, increasing the percentage of living cells and potentially contributing to enhanced recovery (Lelekov-Boissard *et al.*, 2009; Labuzek *et al.*, 2010a). We have previously reported that delayed post-stroke metformin treatment consistently reduces atrophy (Li *et al.*, 2010). Angiogenesis and remodeling of vessels in the peri-infarct region after stroke also contributes to stroke recovery (Chopp *et al.*, 2007; Johnston *et al.*, 2012). In general, enhancement of angiogenesis has been reported in the ischemic border zone of human brain autopsy sections, an effect that was decreased in aged patients (Szpak *et al.*, 1999). Evidence from experimental studies supports a role for angiogenesis in functional recovery (Chopp *et al.*, 2007; Menendez & Vazquez-Martin, 2012; Potts & Lim, 2012; Sun *et al.*, 2012; Wang *et al.*, 2012). Angiogenesis and neurogenesis genes are upregulated within minutes of cerebral ischemia onset in rodents; these proteins can remain elevated in the ischemic hemisphere for hours to weeks after the insult (Font *et al.*, 2010; Ergul *et al.*, 2012). VEGF is an important regulator of angiogenesis that has been shown to regulate cell proliferation, migration and differentiation of endothelial cells (Shibuya & Claesson-Welsh, 2006; Stahmann *et al.*, 2010). Metformin is a potent AMPK activator, and the increased VEGF with metformin treatment in our study suggests that AMPK-induced VEGF expression might be an important mediator of angiogenesis that then contributes to functional recovery (Stahmann *et al.*, 2010). Collectively, these findings suggest that AMPK signaling is essential for the pro-angiogenic effects of metformin and that contributes to reduced atrophy seen at 30 days seen in metformin-treated animals. This was confirmed by studies using AMPK KO mice, which showed neither enhancement in angiogenesis nor a reduction in chronic tissue atrophy after metformin treatment.

The correlation between angiogenesis and improved functional outcome after ischemic stroke has been reported in both experimental and clinical stroke studies (Chopp *et al.*, 2007; Font *et al.*, 2010; Ergul *et al.*, 2012). In experimental stroke, angiogenesis occurred transiently in the penumbra of the ischemic hemisphere, implying that the new vessels may play an important role in the clearing debris after stroke (Manoonkitiwongsa *et al.*, 2001). Interestingly, we observed reduced glial scar formation in metformin-treated brains. Glial scarring is associated with inhibition of angiogenesis (Rocamonde *et al.*, 2012). In parallel,

we observed an increased number of reactive astrocytes that were identified by their size and upregulation of GFAP immunoreactivity. The reduction in the glial scar may possibly contribute to enhanced tissue-remodeling processes (Rocamonde *et al.*, 2012). Consistent with our results, a very recent study demonstrated that metformin treatment improved the level of vascularity by increasing non-perfused vessel islands in diabetic rats (Prakash *et al.*, 2013). This study showed that a significant increase in angiogenesis in metformin-treated groups paralleled improved glucose control (Prakash *et al.*, 2013). However, it is unclear if the effects of metformin are simply secondary to an improvement in blood glucose control, and the maintenance of blood glucose by metformin can be achieved independently of activation of AMPK (Viollet *et al.*, 2012). Therefore, we examined the possible mechanistic role of AMPK in metformin-induced recovery in AMPK KO mice. Our findings suggest that the behavioral recovery and angiogenic effects of chronic metformin treatment required AMPK, as these effects were absent in AMPK KO mice.

Collectively, our data show that delayed chronic metformin treatment improved post-stroke functional recovery in mice by enhancing angiogenesis after stroke and restoring dopaminergic tone. This effect appears to be mediated by AMPK signaling. Our findings suggest that chronic metformin treatment improves recovery after focal ischemia in non-diabetic mice and the effect is mediated by AMPK. Therefore, enhancing angiogenesis by metformin may have future translational potential even in patients without diabetes by promoting functional recovery in stroke survivors.

Acknowledgments

This work was supported by NIH R01 NS055301 (L.D.M.), NIH R21NS079137 (J.L.) and an AHA postdoctoral fellowship (V.R.V.). The authors would like to thank Meaghan Roy-O'Reilly for her editing of this manuscript.

References

- Bland ST, Schallert T, Strong R, Aronowski J, Grotta JC, Feeney DM. Early exclusive use of the affected forelimb after moderate transient focal ischemia in rats: functional and anatomic outcome. *Stroke*. 2000; 31:1144–1152. [PubMed: 10797179]
- Bouet V, Boulouard M, Toutain J, Divoux D, Bernaudin M, Schumann-Bard P, Freret T. The adhesive removal test: a sensitive method to assess sensorimotor deficits in mice. *Nat Protoc*. 2009; 4:1560–1564. [PubMed: 19798088]
- Burke JF, Lisabeth LD, Brown DL, Reeves MJ, Morgenstern LB. Determining stroke's rank as a cause of death using multicausal mortality data. *Stroke*. 2012; 43:2207–2211. [PubMed: 22821613]
- Chopp M, Zhang ZG, Jiang Q. Neurogenesis, angiogenesis, and MRI indices of functional recovery from stroke. *Stroke*. 2007; 38:827–831. [PubMed: 17261747]
- Dereski MO, Chopp M, Knight RA, Rodolosi LC, Garcia JH. The heterogeneous temporal evolution of focal ischemic neuronal damage in the rat. *Acta Neuropathol*. 1993; 85:327–333. [PubMed: 8460534]
- Ergul A, Alhusban A, Fagan SC. Angiogenesis: a harmonized target for recovery after stroke. *Stroke*. 2012; 43:2270–2274. [PubMed: 22618382]
- Font MA, Arboix A, Krupinski J. Angiogenesis, neurogenesis and neuroplasticity in ischemic stroke. *Curr Cardiol Rev*. 2010; 6:238–244. [PubMed: 21804783]
- Fridriksson J. Preservation and modulation of specific left hemisphere regions is vital for treated recovery from anomia in stroke. *J Neurosci*. 2010; 30:11558–11564. [PubMed: 20810877]

- Garcia JH, Yoshida Y, Chen H, Li Y, Zhang ZG, Lian J, Chen S, Chopp M. Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat. *Am J Pathol.* 1993; 142:623–635. [PubMed: 8434652]
- Group, U.K.P.D.S.U. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet.* 1998; 352:854–865. [PubMed: 9742977]
- Hoehn M, Nicolay K, Franke C, van der Sanden B. Application of magnetic resonance to animal models of cerebral ischemia. *J Magn Reson Imaging.* 2001; 14:491–509. [PubMed: 11747001]
- Johnston DG, Denizet M, Mostany R, Portera-Cailliau C. Chronic in vivo imaging shows no evidence of dendritic plasticity or functional remapping in the contralesional cortex after stroke. *Cereb Cortex.* 2012; 23:751–762. [PubMed: 22499800]
- Kim SA, Choi HC. Metformin inhibits inflammatory response via AMPK-PTEN pathway in vascular smooth muscle cells. *Biochem Biophys Res Commun.* 2012; 425:866–872. [PubMed: 22898050]
- Kronenberg G, Balkaya M, Prinz V, Gertz K, Ji S, Kirste I, Heuser I, Kampmann B, Hellmann-Regen J, Gass P, Sohr R, Hellweg R, Waeber C, Juckel G, Hortnagl H, Stumm R, Endres M. Exofocal dopaminergic degeneration as antidepressant target in mouse model of poststroke depression. *Biol Psychiatry.* 2012; 72:273–281. [PubMed: 22464799]
- Labuzek K, Liber S, Gabryel B, Adamczyk J, Okopien B. Metformin increases phagocytosis and acidifies lysosomal/endosomal compartments in AMPK-dependent manner in rat primary microglia. *Naunyn Schmiedebergs Arch Pharmacol.* 2010a; 381:171–186. [PubMed: 20012266]
- Labuzek K, Suchy D, Gabryel B, Bielecka A, Liber S, Okopien B. Quantification of metformin by the HPLC method in brain regions, cerebrospinal fluid and plasma of rats treated with lipopolysaccharide. *Pharmacol Rep.* 2010b; 62:956–965. [PubMed: 21098880]
- Lelekov-Boissard T, Chapuisat G, Boissel JP, Grenier E, Dronne MA. Exploration of beneficial and deleterious effects of inflammation in stroke: dynamics of inflammation cells. *Philos Trans A Math Phys Eng Sci.* 2009; 367:4699–4716. [PubMed: 19884176]
- Li J, Benashski SE, Venna VR, McCullough LD. Effects of metformin in experimental stroke. *Stroke.* 2010; 41:2645–2652. [PubMed: 20847317]
- Li X, Blizzard KK, Zeng Z, DeVries AC, Hurn PD, McCullough LD. Chronic behavioral testing after focal ischemia in the mouse: functional recovery and the effects of gender. *Exp Neurol.* 2004; 187(1):94–104. [PubMed: 15081592]
- Liu F, McCullough LD. Middle cerebral artery occlusion model in rodents: methods and potential pitfalls. *J Biomed Biotechnol.* 2011; 2011:464701. [PubMed: 21331357]
- Manoonkitiwongsa PS, Jackson-Friedman C, McMillan PJ, Schultz RL, Lyden PD. Angiogenesis after stroke is correlated with increased numbers of macrophages: the clean-up hypothesis. *J Cereb Blood Flow Metab.* 2001; 21:1223–1231. [PubMed: 11598500]
- Menendez JA, Vazquez-Martin A. Rejuvenating regeneration: metformin activates endogenous adult stem cells. *Cell Cycle.* 2012; 11:3521–3522. [PubMed: 22935702]
- Meyers PM, Schumacher HC, Alexander MJ, Derdeyn CP, Furlan AJ, Higashida RT, Moran CJ, Tarr RW, Heck DV, Hirsch JA, Jensen ME, Linfante I, McDougall CG, Nesbit GM, Rasmussen PA, Tomsick TA, Wechsler LR, Wilson JR, Zaidat OO. Performance and training standards for endovascular acute ischemic stroke treatment. *Neurology.* 2012; 79:S234–238. [PubMed: 23008404]
- Muller T, Benz S, Bornke C, Przuntek H. Differential response in choice reaction time following apomorphine based on prior dopaminergic treatment. *Acta Neurol Scand.* 2004; 109:348–354. [PubMed: 15080862]
- Murphy TH, Corbett D. Plasticity during stroke recovery: from synapse to behaviour. *Nat Rev Neurosci.* 2009; 10:861–872. [PubMed: 19888284]
- Nagata D, Mogi M, Walsh K. AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. *J Biol Chem.* 2003; 278:31000–31006. [PubMed: 12788940]
- Nathan DM, Buse JB, Davidson MB, Ferrannini E, Holman RR, Sherwin R, Zinman B. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the

- European Association for the Study of Diabetes. *Diabetes Care*. 2009; 32:193–203. [PubMed: 18945920]
- Newton JM, Ward NS, Parker GJ, Deichmann R, Alexander DC, Friston KJ, Frackowiak RS. Non-invasive mapping of corticofugal fibres from multiple motor areas--relevance to stroke recovery. *Brain*. 2006; 129:1844–1858. [PubMed: 16702192]
- Ortega-Gonzalez C, Cardoza L, Coutino B, Hidalgo R, Arteaga-Troncoso G, Parra A. Insulin sensitizing drugs increase the endogenous dopaminergic tone in obese insulin-resistant women with polycystic ovary syndrome. *J Endocrinol*. 2005; 184:233–239. [PubMed: 15642799]
- Ouchi N, Shibata R, Walsh K. AMP-activated protein kinase signaling stimulates VEGF expression and angiogenesis in skeletal muscle. *Circ Res*. 2005; 96:838–846. [PubMed: 15790954]
- Potts MB, Lim DA. An old drug for new ideas: metformin promotes adult neurogenesis and spatial memory formation. *Cell Stem Cell*. 2012; 11:5–6. [PubMed: 22770236]
- Prakash R, Li W, Qu Z, Johnson MA, Fagan SC, Ergul A. Vascularization Pattern After Ischemic Stroke is Different in Control Versus Diabetic Rats: Relevance to Stroke Recovery. *Stroke*. 2013
- Rocamonde B, Paradells S, Barcia JM, Barcia C, Garcia Verdugo JM, Miranda M, Romero Gomez FJ, Soria JM. Neuroprotection of lipoic acid treatment promotes angiogenesis and reduces the glial scar formation after brain injury. *Neuroscience*. 2012; 224:102–115. [PubMed: 22917609]
- Rossini PM, Calautti C, Pauri F, Baron JC. Post-stroke plastic reorganisation in the adult brain. *Lancet Neurol*. 2003; 2:493–502. [PubMed: 12878437]
- Roussel R, Travert F, Pasquet B, Wilson PW, Smith SC Jr, Goto S, Ravaud P, Marre M, Porath A, Bhatt DL, Steg PG. Metformin use and mortality among patients with diabetes and atherothrombosis. *Arch Intern Med*. 2010; 170:1892–1899. [PubMed: 21098347]
- Shibuya M, Claesson-Welsh L. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Exp Cell Res*. 2006; 312:549–560. [PubMed: 16336962]
- Stahmann N, Woods A, Spengler K, Heslegrave A, Bauer R, Krause S, Viollet B, Carling D, Heller R. Activation of AMP-activated protein kinase by vascular endothelial growth factor mediates endothelial angiogenesis independently of nitric-oxide synthase. *J Biol Chem*. 2010; 285:10638–10652. [PubMed: 20129920]
- Strong K, Mathers C, Bonita R. Preventing stroke: saving lives around the world. *Lancet Neurol*. 2007; 6:182–187. [PubMed: 17239805]
- Sun X, Zhang QW, Xu M, Guo JJ, Shen SW, Wang YQ, Sun FY. New striatal neurons form projections to substantia nigra in adult rat brain after stroke. *Neurobiol Dis*. 2012; 45:601–609. [PubMed: 22005319]
- Szpak GM, Lechowicz W, Lewandowska E, Bertrand E, Wierzbica-Bobrowicz T, Dymecki J. Border zone neovascularization in cerebral ischemic infarct. *Folia Neuropathol*. 1999; 37:264–268. [PubMed: 10705649]
- Thiyagarajan M, Fernandez JA, Lane SM, Griffin JH, Zlokovic BV. Activated protein C promotes neovascularization and neurogenesis in postischemic brain via protease-activated receptor 1. *J Neurosci*. 2008; 28:12788–12797. [PubMed: 19036971]
- Towfighi A, Saver JL. Stroke declines from third to fourth leading cause of death in the United States: historical perspective and challenges ahead. *Stroke*. 2011; 42:2351–2355. [PubMed: 21778445]
- Truong DT, Venna VR, McCullough LD, Fitch RH. Deficits in auditory, cognitive, and motor processing following reversible middle cerebral artery occlusion in mice. *Exp Neurol*. 2012; 238:114–121. [PubMed: 22921463]
- Uehara MH, Kohlmann NE, Zanella MT, Ferreira SR. Metabolic and haemodynamic effects of metformin in patients with type 2 diabetes mellitus and hypertension. *Diabetes Obes Metab*. 2001; 3:319–325. [PubMed: 11703421]
- van Lookeren Campagne M, Thomas GR, Thibodeaux H, Palmer JT, Williams SP, Lowe DG, van Bruggen N. Secondary reduction in the apparent diffusion coefficient of water, increase in cerebral blood volume, and delayed neuronal death after middle cerebral artery occlusion and early reperfusion in the rat. *J Cereb Blood Flow Metab*. 1999; 19:1354–1364. [PubMed: 10598940]
- Veizovic T, Beech JS, Stroemer RP, Watson WP, Hodges H. Resolution of stroke deficits following contralateral grafts of conditionally immortal neuroepithelial stem cells. *Stroke*. 2001; 32:1012–1019. [PubMed: 11283405]

- Venna VR, Li J, Benashski SE, Tarabishy S, McCullough LD. Preconditioning induces sustained neuroprotection by downregulation of adenosine 5'-monophosphate-activated protein kinase. *Neuroscience*. 2012a; 201:280–287. [PubMed: 22120436]
- Venna VR, Weston G, Benashski SE, Tarabishy S, Liu F, Li J, Conti LH, McCullough LD. NF-kappaB contributes to the detrimental effects of social isolation after experimental stroke. *Acta Neuropathol*. 2012b; 124:425–438. [PubMed: 22562356]
- Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. *Clin Sci (Lond)*. 2012; 122:253–270. [PubMed: 22117616]
- Wang J, Gallagher D, DeVito LM, Cancino GI, Tsui D, He L, Keller GM, Frankland PW, Kaplan DR, Miller FD. Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation. *Cell Stem Cell*. 2012; 11:23–35. [PubMed: 22770240]
- Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doeber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. 2001; 108:1167–1174. [PubMed: 11602624]
- Zimmet P, Collier G. Clinical efficacy of metformin against insulin resistance parameters: sinking the iceberg. *Drugs*. 1999; 58(Suppl 1):21–28. discussion 75-82. [PubMed: 10576521]

Abbreviations

AMPK	AMP-activated protein kinase
BrDU	bromodeoxyuridine
GFAP	glial fibrillary acidic protein
i.p	intraperitoneal
KO	knockout
MCAO	middle cerebral artery occlusion
NDS	neurological deficit scores
PBS	phosphate-buffered saline
TTC	2,3,5-triphenyltetrazolium chloride
VEGF	vascular endothelial growth factor
WT	wild-type

Fig. 1.

Delayed metformin treatment has no effect on histological stroke outcome. Mice were subjected to 60-min MCAO, and treated with either vehicle or metformin beginning 24 h after stroke. (A) Experimental design. (B) Representative TTC-stained brain sections from vehicle- and metformin-treated mice 72 h after stroke. (C) Infarcts were quantified 72 h after stroke. Quantitative analysis revealed no significant differences in infarct volumes between the two groups ($n = 10$ per group). (D) With doses of metformin/vehicle 50 mg/kg given at 24 and 48 h of reperfusion, metformin treatment did not influence NDS. Error bars denote SD for infarct analysis and interquartile range for NDS.

Fig. 2.

Chronic post-stroke metformin treatment significantly reduced the latency of adhesive-tape removal. Mice were treated with either vehicle or metformin (50 mg/kg/day i.p.) starting 24 h after MCAO for 21 days. A two-way ANOVA revealed a significant effect of surgery, significant effect of treatment and a significant treatment-by-day interaction, suggesting that chronic metformin treatment improves later behavioral outcomes ($n = 4$ per sham group; $n = 9$ per stroke group). $*P < 0.05$. Error bars denote SEM.

Fig. 3.

Chronic post-stroke metformin treatment reduced apomorphine-induced circling behavior. Although at day 3 no difference between metformin and vehicle treatments was observed, significantly reduced rotational bias was found 30 days after stroke in metformin-treated mice. Moreover, no significant recovery is observed in the vehicle group even at day 30, suggesting the high sensitivity of this test for long-term recovery assessment. Repeated-measures ANOVA revealed a significant effect of treatment, and a significant interaction between treatment and day ($n = 4$ per sham group; $n = 9$ per stroke group). $*P < 0.05$. Error bars denote SEM.

Fig. 4.

Recovery is observed in parallel to increased blood vessel formation. To quantify functional blood vessels in the brains of animals 30 days after stroke, we injected 200 μ L of fluorescein-conjugated lectin into the femoral artery 5 min before death. (A) TTC-stained image with arrowhead pointing to the region where IHC images are obtained from. Representative images of vehicle- and metformin-treated brain sections. (B) Quantitative analyses for the total number of branch points in the focal area were significantly different between vehicle and drug groups within the penumbra region of the ischemic striatum ($n = 27$ images from nine brains per stroke group). (C) BrDU co-labeling confirmed angiogenesis with post-stroke metformin treatment (lectin in green; BrDU in red). * $P < 0.05$. Error bars denote SEM.

Fig. 5. Metformin treatment increased angiogenesis. Immunohistochemical analysis demonstrated co-labeling of CD31 with FITC-conjugated lectin, confirming the increased blood vessel formation in metformin-treated mice compared with vehicle-treated mice (lectin in green; CD31 in red).

Fig. 6. Metformin treatment increased VEGF expression and reduced glial scar formation. (A) Immunohistology revealed increased VEGF expression in the metformin-treated group compared with the vehicle-treated mice. (B) Quantification data of VEGF by Adobe Photoshop for integrated optical density. (C) Representative images of glial scar in vehicle- and metformin-treated groups. (d) Quantification of glial scar showed a significant difference between vehicle and metformin groups. (E) Representative images of GFAP⁺ cell bodies in vehicle- and metformin-treated groups. (F) Quantitative analysis for the number of GFAP⁺ cell bodies showed a significant difference between vehicle and metformin groups within the penumbra region of the ischemic striatum ($n = 27$ images from nine brains per group). * $P < 0.05$. Error bars denote SEM.

Fig. 7.

No significant differences were seen in recovery with or without metformin treatment in AMPK α -2 KO mice. (A) Chronic post-stroke metformin treatment showed no additional beneficial effects on recovery in the adhesive-tape removal test, or in (B) apomorphine-induced rotation behavior in stroke mice. (C) Representative images of vehicle- and metformin-treated brain sections from the ischemic penumbra of AMPK α -2 KO mice. (D) Quantitative analysis for the total number of branch points in focal area $n = 10$ sections/group. Error bars denote SEM.