Vascularization Pattern After Ischemic Stroke Is Different in Control Versus Diabetic Rats
Relevance to Stroke Recovery

Roshini Prakash, PhD; Weiguo Li, PhD; Zhi Qu, MS; Maribeth A. Johnson, MS; Susan C. Fagan, PharmD; Advie Ergul, MD, PhD

Background and Purpose—Pre-existing diabetes mellitus worsens brain functionality in ischemic stroke. We have previously shown that type 2 diabetic rats exhibit enhanced dysfunctional cerebral neovascularization and when these rats are subjected to cerebral ischemic reperfusion injury develop hemorrhagic transformation and greater neurological deficits. However, our knowledge of vascular and functional plasticity during the recovery phase of diabetic stroke is limited. This study tested the hypothesis that vascular repair is impaired in the poststroke period in diabetes mellitus, and this is associated with poor sensorimotor and cognitive function. We further hypothesized that glycemic control prevents impaired vascularization and improves functional outcome in diabetes mellitus.

Methods—Vascularization was assessed in the ipsilateral and contralateral hemispheres in control, diabetes mellitus and diabetes mellitus plus metformin groups 14 days after ischemic reperfusion injury, as well as in respective sham controls. Three-dimensional reconstruction of the fluorescein isothiocyanate (FITC)-stained vasculature was achieved by confocal microscopy, and stereological parameters, including vascular volume and surface area, were measured. Astrogliosis was determined by glial fibrillary acidic protein staining. The relative rates of sensorimotor recovery, cognitive decline, and spontaneous activity were assessed.

Results—Vascular density in the peri-infarct area was significantly reduced in diabetes mellitus, whereas there was reparative neovascularization in control rats. Astroglial swelling and reactivity were more pronounced in diabetic stroke compared with control stroke. Diabetes mellitus blunted sensorimotor recovery and also exacerbated anxiety-like symptoms and cognitive deficits. Glycemic control started after stroke partially prevented these changes.

Conclusions—Diabetes mellitus impairs poststroke reparative neovascularization and impedes the recovery. Glycemic control after stroke can improve neurovascular repair and improve functional outcome. (Stroke. 2013;44:2875-2882.)

Key Words: animal behavior ■ astrocytes ■ diabetes mellitus ■ metformin ■ neovascularization ■ recovery ■ type 2

Stroke or cerebral infarction affects 15 million people globally, with one third of the affected population having permanent disability, impacting quality of life.1,2 Diabetes mellitus, hypertension, hypercholesterolemia, and aging add to the complexity of stroke outcomes. It is estimated that >30% of the patients with stroke have diabetes mellitus, and these patients have a greater risk of hemorrhagic transformation, increased mortality, and slower recovery.3-6 Recent clinical studies suggest that diabetes mellitus also contributes to cognitive decline and dementia and these can occur after stroke further compromising functional outcomes.7-11 There is a great need for better understanding of stroke recovery in diabetes mellitus.

Harmonized regulation of angiogenesis and neurogenesis is very important for brain repair and improvement of functional outcome after cerebral injury.12-14 Indeed, stimulation of angiogenesis is being evaluated as a therapeutic modality in stroke.12,15 We have shown that diabetes mellitus causes dysfunctional cerebral neovascularization that is characterized by increased immature microvasculature, augmented remodeling, and permeability. These vessels also have insufficient pericyte coverage, rendering them more susceptible to reperfusion injury.16-18 However, the impact of ischemia/reperfusion on cerebrovascular repair in diabetes mellitus is unknown. A recent study reported that neuronal plasticity and functional recovery after stroke is blunted in type 1 diabetic rats.20 Understanding the changes associated with the vasculature will provide a better insight into the processes responsible for modulation of reparative angiogenesis and neurogenesis and ultimately improve

Received April 1, 2013; final revision received June 20, 2013; accepted June 26, 2013.
From the Department of Physiology, Charlie Norwood Veterans Administration Medical Center, Augusta, GA (R.P., W.L., S.C.F., A.E.); Program in Clinical and Experimental Therapeutics, University of Georgia College of Pharmacy, Augusta, GA (R.P., S.C.F., A.E.); and Departments of Physiology (W.L., Z.Q., A.E.), and Biostatistics (M.A.J.), Georgia Regents University, Augusta, GA.
The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.113.001660/-/DC1.
Correspondence to Advie Ergul, MD, PhD, Department of Physiology, Georgia Regents University, Augusta, GA 30901. E-mail aergul@gru.edu
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Stroke is available at http://stroke.ahajournals.org

DOI: 10.1161/STROKEAHA.113.001660

2875
of functional outcomes. Building on these studies, the current study tested the hypotheses that (1) vascular repair is impaired in the poststroke period in diabetes mellitus, (2) motor and cognitive recovery is blunted after diabetic stroke, and (3) glycemic control in the poststroke period improves cerebrovascularization and this is associated with better functional outcome.

Methods

Animal Procedures and Experimental Design

All animal surgical and behavioral procedures were carried out in accordance with the National Institutes of Health guidelines under protocols approved by Georgia Regents University and Charlie Norwood VA Medical Center. Wistar rats were purchased from Harlan (Indianapolis, IN) and the Goto-Kakizaki rats were purchased from Taconic (Hudson, NY). We used 5 cohorts of male rats (250–300 g; 10–12 weeks) that were randomized to sham or stroke surgery: sham control and diabetes mellitus, stroked control and diabetes mellitus, and stroked diabetic rats treated with metformin (300 mg/kg per day in drinking water) after stroke until killing. Treatment was started when blood glucose reached >140 mg/dL, which occurred at day 1 in majority of the animals (a small fraction of animals reached that level at day 2). Blood glucose was measured during the light cycle every day and the levels ranged from 86 to 103 mg/dL in control rats, 155 to 217 mg/dL in the diabetic group, and metformin treatment lowered the glucose levels to 81 to 115 mg/dL. The mean body weight was 318±6 g in the control group, 271±7 g in the diabetic group, and 299±12 g in the metformin-treated group.

Method of Ischemic Stroke

Ischemia/reperfusion injury was induced by 90-minute occlusion of the middle cerebral artery as previously published (detailed methodology is given in the online-only Data Supplement).

Measurement of Poststroke Vascularization

Vascularization patterns were assessed in cortex and striatum around the infarct territories, as well as in corresponding regions in the contra-lateral hemisphere using the space-filling model, as reported earlier (detailed methodology is given in the online-only Data Supplement).

Assessing Astrogliosis and Swelling

The fluorescein isothiocyanate (FITC)-stained sections were costained with antiglial fibrillary acidic protein antibody and imaged at ×63 immediately around the infarcts and in the corresponding contralateral hemispheres of the same sections. Astrocytic swelling was evaluated by measuring the somatic volumes using Velocity software. Number of processes projecting from individual astrocytes was counted as the astrocytic reactivity using the Fiji software after skeletonizing the cultures compared with control as indicated by white arrows not only the perfused vessels, but also the nonperfused vasculature compared with diabetes mellitus (diabetes mellitus) and intervention (stroke) interaction. There was a decrease in the surface area only in the ischemic cortex. A comparison made between the ischemic and the contralateral hemisphere reveals that the distant sites display a similar response to that seen in the ischemic hemisphere but to a lesser extent. For example, contralateral region in the control groups had greater vascularization than in sham animals, although the contralateral site in diabetic animals displays attenuated vascularization. Metformin intervention initiated after stroke to achieve euglycemia corrected the decrease in vascular volume and surface area restoring it to control levels. When vascularization was assessed directly at the border of the infarct zone (Figure 2C), there were FITC-perfused vessels feeding into the infarcted area while diabetic rats show decreased vascular density. There was evident astrocyte activation as shown by enhanced glial fibrillary acidic protein staining around the infarct. Metformin treatment improved vascularization in the diabetic group (Figure 2B). Diabetes mellitus impaired not only the perfused vessels, but also the nonperfused vasculature compared with control as indicated by white arrows (Figure 3). Metformin intervention improved the level of vascularity by increasing nonperfused vessel islands.

Evaluation of Neurological Outcomes

All neurological outcomes were measured blindly by assessing sensorimotor functions, anxiety-like symptoms using the elevated plus maze and T-maze, short-term cognitive test using the novel object recognition task (detailed methodology is given in the online-only Data Supplement).

Statistical Analysis

All data points are expressed in mean±SEM. Assumptions of normality were tested and rank transformations were used as needed. Vascularization after stroke was compared using a 2 diseases (control versus diabetes mellitus) by 2 stroke hemisphere (sham versus ischemic or sham versus contralateral) ANOVA within area (cortex or striatum). An interaction was tested and if significant would indicate a differential effect of stroke on vascularity dependent on disease status. The effect of diabetes mellitus and of metformin treatment of diabetes mellitus on vascularization and astrogliosis after stroke was determined using a 1-way ANOVA with 3 groups (control, diabetes mellitus, diabetes mellitus plus metformin) with separate analyses for each hemisphere (ischemic or contralateral) and each area (cortex or striatum). Changes in behavior and function were measured by determining the area under the curve (AUC) for responses across 14 days after stroke. The effect of diabetes mellitus and of metformin treatment of diabetes mellitus on behavior and function after stroke was determined using a 1-way ANOVA with 3 groups (control, diabetes mellitus, diabetes mellitus plus metformin) to analyze AUC, as well as within-day measures of behavior and function. Tukey post hoc tests were used to determine mean differences and to adjust for multiple comparisons for significant ANOVA effects. AUC was calculated using NCSS 2007 software (NCSS, LLC, Kaysville, UT), and all statistical analyses were performed using SAS version 9.3 (SAS Institute, Inc, Cary, NC). Statistical significance was determined at α=0.05.

Results

Vascularization in the Ischemic and Contralateral Hemispheres

As reported earlier, the diabetic sham animals had greater vascularization compared with the control group.14 Fourteen days after stroke, vascular volume and surface area were dramatically enhanced in the ischemic hemisphere both in the cortex and striatum in the control group compared with the respective shams (Figure 1). The greater increase in surface area indicates that a significant portion of this increase comes from the enhancement of microvasculature. However, in diabetic animals, vascular volume was significantly decreased in the ischemic cortex and striatum indicating a strong disease (diabetes mellitus) and intervention (stroke) interaction. There was a decrease in the surface area only in the ischemic cortex. A comparison made between the ischemic and the contralateral hemisphere reveals that the distant sites display a similar response to that seen in the ischemic hemisphere but to a lesser extent. For example, contralateral region in the control groups had greater vascularization than in sham animals, although the contralateral site in diabetic animals displays attenuated vascularization. Metformin intervention initiated after stroke to achieve euglycemia corrected the decrease in vascular volume and surface area restoring it to control levels. When vascularization was assessed directly at the border of the infarct zone (Figure 2C), there were FITC-perfused vessels feeding into the infarcted area while diabetic rats show decreased vascular density. There was evident astrocyte activation as shown by enhanced glial fibrillary acidic protein staining around the infarct. Metformin treatment improved vascularization in the diabetic group (Figure 2B). Diabetes mellitus impaired not only the perfused vessels, but also the nonperfused vasculature compared with control as indicated by white arrows (Figure 3). Metformin intervention improved the level of vascularity by increasing nonperfused vessel islands.

Diabetes Mellitus Aggravates Astrogliosis After Stroke

Astrocytic swelling was evaluated by measuring the somatic volumes. Diabetic animals showed increased swelling and number of astrocytic processes both around the zone of infarction and in the contralateral hemisphere. Metformin treatment prevented this response around the region of infarction (Figure 4A–4C).
Glycemic Intervention After Stroke Improves Sensorimotor Functions in Diabetes Mellitus

Neurological scores were similar at baseline, but decreased significantly in diabetic rats 24 hours after stroke. The control group had gradually improved sensorimotor function. The recovery in the diabetic group was significantly attenuated (Figure 5A). Metformin treatment reduced the sensorimotor deficits after day 5 in the diabetic group, and the composite neurological scores were similar to that of the control group. AUC (day 1–14) was 112±31, 67±14, and 108±21 (P=0.012) for control, diabetes mellitus, and diabetes mellitus plus metformin groups, respectively. Parallel results were obtained with the forelimb grip strength after stroke (Figure 5B). AUC (day 0–14) was 11.4±1.6, 8.2±0.3, and 10.6±0.3 (P=0.0007) for control, diabetes mellitus, and diabetes mellitus plus metformin groups, respectively.

Diabetes Mellitus Induces Anxiety-Like Behaviors and Cognitive Deficits Which Are Further Exacerbated After Stroke

At baseline, diabetic rats demonstrated anxiety-like behaviors as they spent longer time in the closed arm of the elevated plus maze. After stroke, both groups showed a similar pattern (Figure 6A). This response improved in subsequent days in the control group and at day 14, control animals spent significantly less time than diabetics. Percent time spent in the open arm and freezing time or the time in the center of the maze was greater in diabetes mellitus (Figure 6B and 6C).

Spontaneous T-maze alterations scores show a dramatic decrease in alteration scores tested at 48 hours after stroke in both groups (Figure I in the online-only Data Supplement). At baseline, diabetes mellitus causes a reduction in the novel object recognition index, indicating the negative impact of even short-term diabetes mellitus on cognition. All groups
experienced a similar degree of deficits in the first few days after stroke, but rats recovered moderately from cognitive dysfunction that is seen after stroke. Recovery in the diabetic stroked rats was impaired and metformin partially restored the cognitive function (Figure 6D).

Discussion

Diabetes mellitus increases the risk and severity of stroke ultimately resulting in poor outcomes. Clinical evidence suggests that no history of diabetes mellitus is a predictor of recovery implicating that diabetes mellitus hampers recovery after stroke. However, preclinical evidence as to how diabetes mellitus influences stroke recovery is scarce. This study addressed this important gap in our knowledge and investigated the impact of diabetes mellitus on cerebrovascular remodeling and vascularization patterns, and motor and cognitive recovery after stroke.

Studies conducted on human and animal models reveal a critical place for therapeutic angiogenesis in recovery after stroke. Angiogenesis around the infarct boundary in the ipsilateral ischemic hemisphere has been well characterized in animal models. Increased angiogenesis has also been observed around the infarction in stroke patients. Morbidity, survival rates, and neurological recovery are directly correlated to the degree of angiogenesis, microvascular density, and restoration of blood flow after stroke. Few studies have been conducted to assess brain repair in diabetic stroke and these reports suggest impaired vascular restoration after diabetic stroke. Type 2 diabetic mice subjected to ischemia showed decreased number of microvessels after stroke in the ischemic hemisphere. Another study, conducted on diabetic Goto-Kakizaki rats, similar to these studies, also show a reduced rate of angiogenesis 7 days after stroke. Most, if not all, of these studies used conventional 2-dimensional (2D) strategies to assess microvascular density in the ischemic hemisphere, and the contralateral hemisphere was used as control tissue. We now provide a comprehensive report of 3D changes occurring in the brain vasculature in the infarcted zone, as well as in perilesional cortical and subcortical regions in both ischemic and contralateral hemispheres, 14 days after stroke as compared with sham-operated animals. This approach allowed us to not only compare the vascularization in control and diabetic animals, but also assess the impact of stroke on cerebrovascular architecture in the contralateral hemisphere. As recently

Figure 2. Diabetes mellitus impairs poststroke cerebral neovascularization at the infarct border zone. A, Representative image depicting the localization of the infarct border zone. B, Representative images comparing vascular density at infarct border zone taken under ×10 objective. C, Graphical representation of the % vascular density around the area of infarction in all the groups. Mean±SEM; n=4 to 6. GFAP indicates glial fibrillary acidic protein.
normal rats stimulated an angiogenic response in the current study. Although we did not directly study angiogenic markers, greater increase in surface area is indicative of increased microvasculature. In a given volume of tissue, an increase in vascular volume, which measures mainly the vascular lumen space, can be because of either remodeling of the vessel to get larger lumen and because of new vessel formation. In the latter, an increase in surface area, a measure of the area the vessel wall occupies, accompanies increased volume. As such when one looks at the relationship between volume and surface area, a linear association suggests that the increase in these 2 parameters increases in a parallel fashion and there is significant new microvessel formation. In this study, we detected increased vascular volume and even a greater increase in surface area. However, 14 days after stroke the diabetic Goto-Kakizaki rats had severely impaired vasculature around the infarct borders. Decreased vascularization was also observed in the contralateral hemisphere. Greater reduction in vascular volume suggests that there is a significant vascular regression of existing vessels. Potential mechanisms underlying this response merit further investigation.

Glial cells, especially astrocytes, respond to vascular damage by cellular swelling and increased reactivity. Astrogliosis has been reported around the ischemic core and is increased progressively closer to the infarct border associated with glial scarring. This study provides evidence that stroke in association with diabetes mellitus heightens astrogliosis by increasing the swelling and number of astrocytic process densities in the peri-infarct zones. Marked increase in astrogliosis in the brain may alter neurovascular coupling to restore blood flow to the damage regions, cause inflammatory and trophic response.

Clinical and preclinical studies report memory and cognition deficits associated with metabolic alterations and after ischemic reperfusion. Acutely after stroke, motor coordination is weakened that can propagate to permanent paralysis of the extremities. Studies report increased synaptic plasticity and neuronal reorganization with increased motor learning and activities. Animal behavior assessed after stroke also provides evidence of synaptic plasticity and progressive improvement in functional behavior associated with learning. A recent study showed that cortical plasticity in diabetic stroke is challenged compared with the stroke injury on animals without diabetes mellitus. Functional restoration was also compromised long-term after diabetic stroke. The current study evaluated temporal changes in sensorimotor functions, anxiety-like symptoms, spontaneous activity, and cognitive function after stroke with or without diabetes mellitus. Sensorimotor deficits occurred because of ischemic reperfusion injury improved and saturated after 10 days of stroke in the control rats. The extent of recovery was diminished in diabetic rats. Emerging evidence suggests that both type 1 and type 2 patients with diabetes mellitus have poor neuropsychological functions affecting cognition. In our current study, we elaborated on the temporal profile of cognitive functions after stroke in control and diabetic rats. Our results with novel object recognition and spontaneous alteration at T-maze experiments show that even at baseline memory and cognition-related tasks are impaired in diabetes mellitus. When stroke is overlayed on this pathology, recovery of cognitive function is severely affected.
We have earlier provided evidence of chronic glycemic control being an effective preventive strategy that confers vascular protection and reduces the risk of hemorrhagic transformation after ischemic reperfusion injury in diabetes mellitus. Although clinical trials focusing on the impact of glycemic control on cardiovascular outcomes resulted in

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**Figure 4.** Diabetes mellitus exacerbates astrogliosis after stroke. **A.** Representative images showing astrocytic morphology in the infarct border zone and nonlesional hemisphere, as well as in the corresponding regions in sham animals. **B.** Diabetic stroke dramatically increases astrocytic swelling compared with control strokes and metformin treatment decreased swelling. **C.** Number of astrocytic projections is significantly increased even after 14 days in diabetic stroke compared with control and glycemic intervention conserved astrocytic processes. Blue, indicates fluorescein isothiocyanate (FITC)-perfused vasculature; green, glial fibrillary acidic protein (GFAP); and red, neuronal nuclei. Mean±SEM; n=3 to 4.

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**Figure 5.** Glycemic intervention after stroke improves sensorimotor functions in diabetes mellitus. **A** and **B.** Temporal profile of sensorimotor functions represented as composite neurological score. Diabetes mellitus significantly reduced the neurological scores after stroke. Improvement in the neurological scores was slower in the diabetic stroke group compared with control stroke. Glycemic intervention with metformin improved and restored the composite neurological scores comparable with the control group. **B.** Temporal profile of forelimb grip strength shows a similar trend. The drop in the forelimb grip strength is not restored 14 days after stroke in the diabetic group compared with control. Glycemic intervention with metformin improved the forelimb grip strength. *P*<0.05 vs diabetes mellitus, **P**<0.01 vs diabetes mellitus, ***P***<0.001 vs diabetes mellitus, *area under the curve (AUC) P*=0.012 vs diabetes mellitus, 4AUC *P*=0.0007 vs diabetes mellitus. Mean±SEM; n=6 to 8.
Debatable information, tight glycemic control has been an effective treatment for prevention of microvascular complications, such as diabetic nephropathy and retinopathy.\textsuperscript{45-49} Metformin is a first-line choice of drug commonly used to treat patients type 2 diabetes mellitus. Metformin exerts its antihyperglycemic actions by reducing hepatic gluconeogenesis and increasing peripheral glucose uptake, insulin sensitivity, and fatty acid oxidation. Metformin also has protective roles independent of its antihyperglycemic actions. Metformin improved antioxidant capacity in patients with type 2 diabetes mellitus, who display excessive increased oxidation and glycation of albumin in the serum.\textsuperscript{50} Rösen and Wiemersperger\textsuperscript{51} showed improved antioxidant defense with the use of metformin in Goto-Kakizaki rats. We used metformin as an intervention strategy to target hyperglycemic status in diabetic rats in the poststroke period. Clearly, our findings provide compelling evidence that glycemic control by metformin in the poststroke period in rats that had not received any hypoglycemic therapy before effectively promotes vascular repair and improves sensorimotor and cognitive function after stroke.

In conclusion, these studies show that brain responds to repair ischemic injury by increasing vascularization even in remote sites, but in the presence of diabetes mellitus this response is completely reversed and there is vascular regression. These changes are associated with delayed functional recovery after stroke. The mechanisms by which diabetes mellitus impairs vascular repair, as well as mechanisms linking vascular regression to poor functional recovery, are lines of research that we are pursuing further. Our results lend a strong support for glycemic control as an effective strategy in the prevention and improvement of cerebrovascular complications in diabetes mellitus.

Disclosures
This work was supported in part by VA Merit Awards to S. C. Fagan (BX000891) and Dr Ergul (BX000347), National Institutes of Health (NIH) grants to S. C. Fagan (NS063965), Dr Ergul (NS054688), and American Heart Association grants to Dr Ergul (ELA 0740002 N) and Dr Prakash (10PRE4030037). Dr Ergul is a research pharmacist at the Charlie Norwood Veterans Affairs Medical Center in Augusta, Georgia. The contents do not represent the views of the Department of Veterans Affairs or the United States Government. The other authors report no conflicts.

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Stroke. 2013;44:2875-2882; originally published online August 6, 2013;
doi: 10.1161/STROKEAHA.113.001660

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/44/10/2875

Data Supplement (unedited) at:
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SUPPLEMENTAL METHODS AND DATA

Method of ischemic stroke

I/R injury was achieved by occluding the middle cerebral artery (MCA) with a nylon suture under anesthesia maintained at 3% isoflurane mixed with 30% oxygen and 70% nitrogen. The skin on the cervical region was incised to access the common carotid artery. The external carotid artery was separated, ligated, and severed. The nylon suture with rounded-tip was inserted into the internal carotid artery to approach the origin of MCA. The nylon suture occluding the MCA was secured along the external carotid artery at its base and the incision was closed. Ischemia was induced for a period of 90 minutes following which the animals were re-anesthetized, and the occlusion suture was removed to allow reperfusion. Laser Doppler (PIM-3, Perimed, Stockholm, Sweden) was used to confirm reduction in cerebral perfusion. Animals were maintained warm throughout the procedure using a heating pad. Animals were returned to their home cages after MCA occlusion (MCAO) with easy access to food and water. 2 ml of 0.9% saline was injected intraperitoneally daily for the first 2 days after stroke. One cohort of the diabetic rats was treated with metformin (300mg/kg body weight, provided in the drinking water) to achieve euglycemia. 2 ml of 0.9% saline was injected intraperitoneally daily for the first 2 days after stroke. Treatment was started within 1 or 2 days after stroke when blood glucose reached over >140 mg/dl. All the animals were tested for functional outcomes for a period of 14 days at regular intervals. Blood glucose was monitored to maintain euglycemic status.

Measurement of post-stroke vascularization

Regions of interests are depicted in Figure 1. 3 dimensional parameters such as vascular volume and surface areas were assessed by methodologies published earlier. In addition, vascularization at the edge of infarction was measured. For these studies, slides were co-stained with NeuN (neuronal nuclei) and GFAP (glial fibrillary acidic protein) (Chemicon, Temecula, CA) to identify the neurons and surrounding astrocytes, respectively, and regions where the measurements were made are indicated on Fig 2.

Vascular volume representing volume of the vasculature perfusing the brain tissue in the region of interest and surface area representing the area available on the vasculature for the exchange of vascular components in the surrounding tissue were measured using the Volocity Improvision software after thresholding the images and eliminating the background. Non-FITC perfused vasculature was assessed by co-staining the sections with biotinylated isolectin B4 (Vector Laboratories Inc. Burlingame, CA) using the methodology described earlier. Signal intensity was measured to calculate % nonperfused vessels = (total vascular volume-perfused vascular volume)*100/total vascular volume using the volocity software.

Evaluation of neurological outcomes

All the rats were housed in 12h light/dark cycles and all the behavior tests were performed during the day with ambient light of about 30 lux. Animals were handled for 5-7 days prior to behavior testing in rooms where behavior testing was to be carried out. Behavioral tests were recorded and analysis of neurobehavioral tests was done in a blinded fashion. Bederson’s score, beam walk and grip strength tests, which assess sensorimotor function, were performed before and after stroke for a period of 14 days to calculate a composite score.
Bederson’s score for each rat was obtained by using 3 parameters which include (a) observation of spontaneous ipsilateral circling, graded as 2 (no circling), 1 (partial circling), 0 (continuous circling), (b) contralateral hindlimb retraction, (c) forelimb retraction which measures the ability of the animal to replace the limb after it is displaced laterally by 2 to 3 cm, graded 1 (immediate replacement) and 0 (replacement after minutes or no replacement). Maximum score of 5 was allotted to a normal rat. A lower score represents a poor neurological outcome 4, 5.

Beam walking ability graded based on 7-point scale method as described by Feeney et al. 6 A score 7 was assigned for a rat that readily traverses a 2.4-cm-wide, 80-cm-long beam with no more than 2 foot slips, 6 for rat that crosses the beam with the help of the affected paw but slips more than twice, 5 for a rat that crosses the beam with limited use of the affected limb, 4 for a rat that crosses the beam and puts the affected paw on the beam but not use it for movement, 3 for a rat that crosses the beam dragging the feet, 2 for a rat that puts the affected limb on the horizontal surface and maintains balance for 5 sec, and 1 for a rat unable to place the affected hindpaw on the horizontal surface of the beam.

Forelimb grip strength was measured with a standard grip strength meter (Columbus Instrument). 7.

For evaluation of cognition and memory-related tasks, spontaneous novel object recognition (NOR) test was performed using a grey plastic box of (63L x 38W x 42H cm) that was layered with animal bedding. Animals were habituated to the box one day prior to the day of testing with no objects in it. Objects with greater intricacy and details and similar in appearances with equal and unbiased preferences for one over the other were chosen to perform the test. On the day of testing, the rats were allowed to explore two identical objects during the A/A session for a period of 5 minutes. The rats were returned to their home cages for a delay/retention interval of 15 minutes following which the rats were confronted to A/B sessions in the consisting of 5 minutes, during which a novel object was paired with a familiar object used in the A/A session. All objects were cleaned after each session with 30% ethanol and the bedding was ruffled and cleaned to discard cues. The objects were placed equidistant from the walls of the box, in the center and spaced 20 cm apart and the rats were placed in between both the objects at the start of the experiment. The time spent in exploring each object during the A/B session was recorded and recognition indices were calculated by the ratio of time spent in exploring the novel object over the total time spent in exploring both the objects 7.

Anxiety like symptoms were assessed using the elevated plus maze experiments. The apparatus consisted of 2 open arms and 2 closed arms elevated 20” from the ground. Each rat was placed in the central region at the junction of the open and closed arms and its behavior was recorded for about 5 minutes. The time spent in the open and closed arms of the maze arm was recorded. Time spent in the center was also measured as the freezing time. High anxiety states are directly related to the degree to which the rodent avoids the open arms of the maze 8.

Spontaneous arm alterations were also tested using a T-maze made of plexiglass that consisted of 3 arms. This memory test is based on the fact that animals will alternate the arms if they remember which arm was entered last. The end of each arms and the surrounding were marked with different symbols that served as cues for the rats to identify the arena. The rats were placed in the start arm and were allowed to make a choice. The first turn was considered as a choice trial and the sliding door was let down after the first choice that allowed the rat to remain in the choice arm for about 30 seconds. After which the rats were taken out and the three sample
trials/chances were given to alternate from the choice trial. Graded scores were allotted based on the number of trials taken by the rat to make a correct choice or the alternate turn from that of the choice trial. Correct choice in the first sample trial was scored as 3 and third trial was recorded as 1. No correct alternation as scored as 0.9, 10.

Supplementary Figure. Spontaneous T-maze activity and anxiety like symptoms are aggravated in diabetic stroke. Graphical plot of graded scores given to the rats based on the delay in correct choice made on the spontaneous T-maze task. Diabetic group had decreased scored depicting poor outcome in the T-maze test. Stroke further worsened this activity in the diabetic group more so compared to the control. *p<0.05 vs control, Mean ± SEM, n=6-8.

REFERENCES

Supplementary Figure 1

Spontaneous alteration scores vs. Days after stroke for control, diabetes, and diabetes + metformin groups.