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 earlier1, 2. In addition, vascularization at the edge of infarction was measured1, 2. 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 interest1, 2 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 background1. Non-FITC perfused vasculature was assessed by co-staining the sections with biotinylated isolectin B4 (Vector Laboratories Inc. Burlingame, CA) using the methodology described earlier2. Signal intensity was measured to calculate % nonperfused vessels = (total vascular volume-perfused vascular volume)*100/total vascular volume using the volocity software2, 3.

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. 9\(^p\leq0.05\) vs control, Mean ± SEM, n=6-8.

**REFERENCES**

Supplementary Figure 1

- Control
- Diabetes
- Diabetes + Metformin

Spontaneous alteration scores

Days after stroke

metformin intervention