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NLRP3 inflammasome inhibition with MCC950 improves diabetes-mediated cognitive impairment and vasoneuronal remodeling after ischemia

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Abstract

Diabetes increases the risk and worsens the progression of cognitive impairment via the greater occurrence of small vessel disease and stroke. Yet, the underlying mechanisms are not fully understood. It is now accepted that cardiovascular health is critical for brain health and any neurorestorative approaches to prevent/delay cognitive deficits should target the conceptual neurovascular unit (NVU) rather than neurons alone. We have recently shown that there is augmented hippocampal NVU remodeling after a remote ischemic injury in diabetes. NLRP3 inflammasome signaling has been implicated in the development of diabetes and neurodegenerative diseases, but little is known about the impact of NLRP3 activation on functional and structural interaction within the NVU of hippocampus, a critical part of the brain that is involved in forming, organizing, and storing memories. Endothelial cells are at the center of the NVU and produce trophic factors such as brain derived neurotrophic factor (BDNF) contributing to neuronal survival, known as vasotrophic coupling. Therefore, the aims of this study focused on two hypotheses: 1) diabetes negatively impacts hippocampal NVU remodeling and worsens cognitive outcome after stroke, and 2) NLRP3 inhibition with MCC950 will improve NVU remodeling and cognitive outcome following stroke via vasotrophic (un)coupling between

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Conflicts of interest

R Ward declares she has no conflict of interest. W Li declares he has no conflict of interest. Y Abdul declares he has no conflict of interest. L Jackson declares she has no conflict of interest. G Dong declares he has no conflict of interest. S Jamil declares she has no conflict of interest. J Filosa declares she has no conflict of interest.SC Fagan declares she has no conflict of interest. A Ergul declares she has no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2019.01.035.

endothelial cells and hippocampal neurons. Stroke was induced through a 90-min transient middle cerebral artery occlusion (MCAO) in control and high-fat diet/streptozotocin-induced (HFD/STZ) diabetic male Wistar rats. Saline or MCC950 (3 mg/kg), an inhibitor of NLRP3, was injected at 1 and 3 h after reperfusion. Cognition was assessed over time and neuronal density, blood-brain barrier (BBB) permeability as well as NVU remodeling (aquaporin-4 [AQP4] polarity) was measured on day 14 after stroke. BDNF was measured in endothelial and hippocampal neuronal cultures under hypoxic and diabetes-mimicking condition with and without NLRP3 inhibition. Diabetes increased neuronal degeneration and BBB permeability, disrupted AQP4 polarity, impaired cognitive function and amplified NLRP3 activation after ischemia. Inhibition with MCC950 improved cognitive function and vascular integrity after stroke in diabetic animals and prevented hypoxia-mediated decrease in BDNF secretion. These results are the first to provide essential data showing MCC950 has the potential to become a therapeutic to prevent neurovascular remodeling and worsened cognitive decline in diabetic patients following stroke.

Keywords

Diabetes; Vascular cognitive impairment and dementia (VCID); Hippocampus; NLRP3 inflammasome; Stroke; Neurovascular unit

1. Introduction

Diabetes affects more than 422 million people worldwide and doubles the risk and worsens the progression of vascular cognitive impairment/dementia via the greater occurrence of small vessel disease and stroke [1,2]. Although stroke and cognitive impairment are considered aging diseases, the number of younger patients being diagnosed with diabetes is dramatically increasing and accordingly, the risk of stroke and cognitive impairment occurring at earlier ages is elevated [1,3–5]. The underlying reasons for how diabetes contributes to worsened cognition and stroke outcome are not fully understood [6–8]. It is now accepted that cardiovascular health is critical for brain health and any approaches to prevent and/or delay cognitive impairment should target the conceptual neurovascular unit (NVU) rather than neurons alone [9,10]. The NVU, which is composed of neurons, endothelial cells, astrocytes and the basement membrane, emphasizes the unique interaction between cerebrovasculature and the surrounding brain cells [11]. Vascular contributions to cognition and dementia (VCID) have been long neglected. Indeed, two major statements from the AHA emphasized the disconnection between “neuroscience” and “vascular physiology” [9,10]. Historically, there have been rigid distinctions made between diseases considered neurodegenerative (such as Alzheimer’s) compared to those seen as cerebrovascular (such as stroke). Therefore, there is a need to bridge the gap between these two fields. As a highly metabolic organ lacking its own energy reserves, the brain relies on a constant blood flow to provide nutrients and remove waste products to remain properly functional [12]. The brain has tight regulation of constant blood flow and provides delivery of blood where it is needed in the brain through NVU coupling (functional hyperemia) [13]. We have recently shown worse post-stroke outcomes in diabetic animals that are associated with augmented NVU remodeling and chronic inflammation in the hippocampus, an

important domain in learning and memory [14]. However, the underlying mechanism remains unknown.

Activation of the immune system and inflammation is a common factor in diabetes, stroke and cognitive impairment [15–19]. Induction of cytokines and their downstream effectors causes astrocytic reactivity and breakdown of blood brain barrier (BBB) integrity due to endothelial injury [20]. The innate immune system is activated through pattern recognition receptors such as membrane-bound toll-like receptors (TLRs) and cytosolic NOD-like receptors (NLRs) [21]. Activation of NLRs enables the formation and activation of inflammasome complexes [22,23]. NLRP3 is the most widely studied inflammasome and has been implicated in the development of diabetes [15,16,24–26] and neuro-degenerative diseases [27,28], including stroke [29,30]. Elevated levels of NLRP3 are observed in diabetic patients [16] and its activation leads to diabetic retinopathy, a microvascular complication of diabetes [24,31]. Mice deficient in NLRP3 had ameliorated neurovascular damage and improved outcome after stroke [30], suggesting that in-flammasomes may play an important role in worsened stroke outcome. A recent study implicates inhibition of NLRP3 in diabetic mice with improved functional outcome 24 h after ischemia and better long-term survival than vehicle treated animals [32]. Yet, little is known about the impact of NLRP3 activation on functional and structural interaction within the NVU of the hippocampus. Therefore, our first goal of this study was to determine the role of NLRP3 activation on hippocampal NVU remodeling and cognitive outcome after stroke in diabetes. Our working hypothesis is that NLRP3 inflammasome activation contributes to NVU remodeling and associated cognitive decline after stroke in diabetes.

Beyond the function of providing cerebral blood flow (CBF) and nutrients, endothelial cells can provide neurotrophic and angiogenic trophic factors to neurons and oligodendrocytes, a termed coined as vasotrophic coupling [33–36]. Neurotrophins are vital to the central nervous system and play a role in neuronal survival and differentiation [37]. Brain-derived neurotrophic factor (BDNF), an important neurotrophic factor, contributes to learning and memory and has both neuroprotective and angiogenic effects [38–41]. Guo and colleagues elegantly reported the importance of BDNF-mediated vasotrophic coupling between endothelial and neuronal cells [33]. Reduced BDNF after oxidative stress in endothelial cells suggests that under pathophysio-logical conditions, vasotrophic neuroprotection may be compromised [33]. Multiple studies have shown the beneficial impact of BDNF signaling after ischemic injury [34,34,35,36]. While BDNF has been implicated in stroke, little is known about impact of BDNF secretion in the hippocampus after stroke in diabetes. Therefore, our second goal of this study was to determine the role of NLRP3 activation on vasotrophic (un)coupling between endothelial cells and neurons after hypoxic injury under diabetic conditions. We hypothesize that NLRP3-mediated decrease in neurotrophic mBDNF secretion from endothelial cells contributes to reduced survival ability of hippocampal neurons under diabetic and/or hypoxic conditions.

2. Materials and methods

2.1. Animal model

Male Wistar rats (Envigo RMS, Inc., Indianapolis, IN) were housed in the animal care facility at Augusta University, which is approved by the American Association for Accreditation of Laboratory Animal Care. All experiments were conducted in accordance with the National Institute of Health guidelines for the care and use of animals in research. Furthermore, all protocols were approved by the institutional animal care and use committee.

Diabetes was induced using a high-fat diet/low-dose streptozotocin (HFD/STZ) in male Wistar rats. At 4 weeks of age, rats in the diabetic group were started on a 45% kcal fat diet (Research Diets Inc., New Brunswick, NJ). A single dose STZ injection (30 mg/kg; Cayman Chemical, Ann Arbor, MI) was administered intraperitoneally at 6 weeks of age. If blood glucose was not above 150 mg/dl 5 days post-injection, a second small dose (20 mg/kg) was administered. Control rats received regular chow with 4% kcal fat. Body weight and blood glucose were measured twice a week until euthanasia. Mechanical suture occlusion was induced in 12-week-old animals. After stroke induction, diabetic animals remained on HFD until euthanasia. In the post-operative period (first 5 days) blood glucose was monitored daily. 2 sets of experiments were conducted. Number of animals included, and mortality rates are given in Table 1.

Experiment 1: Control and diabetic male rats were subjected to sham or 90-minute mechanical middle cerebral artery occlusion (MCAO) surgery and followed for 14 days. A battery of sensorimotor and cognitive/memory tests were performed as described below. Detailed methodology for MCAO surgery, sensorimotor and cognitive tests are described in the supplementary materials.

Experiment 2: Control and diabetic male rats were subjected to 90-min MCAO, treated with vehicle or NLRP3 inhibitor MCC950 as described below and followed for 14 days for outcomes. Greater numbers of animals were used in the diabetic group due to an expected higher mortality. Animals which lost greater than 20% of their weight were sacrificed. 3 of the 9 diabetic animals that died before day 14 were sacrificed due to weight loss. The animals that died before Day 14 were not included in analyses.

2.2. MCC950 treatment

MCC950 treatments (3 mg/kg) were given at 1 h and 3 h after reperfusion through tail vein injection [42–44]. The MCC950 solution was prepared by dissolving the MCC950 powder (Cayman Chemical, Ann Arbor, MI) in DMSO. Sterile saline was then mixed with this solution at a 1:1 ratio. Animals were placed in a restraining device while their tail was soaked in warm water for 3 min to dilate the vein. After dampening with alcohol to increase vein visibility, the prepared MCC950 solution was injected into the lateral tail veins using a BD 1 ml TB syringe. Saline treatments were injected with equal volume of sterile saline at 1 h and 3 h post-reperfusion.

2.3. Evaluation of sensorimotor and neurobehavioral outcomes

Sensorimotor and cognitive tests were recorded and scored in a blinded fashion. For 5–7 days prior to surgery, animals were handled and trained in the room where tests were to be administered. Sensorimotor function was examined by composite neurological score as previously described [45]. Neurobehavioral outcomes were assessed by the two-trial Y-maze, novel object recognition (NOR), and novel social recognition (NSR). Tests were administered, in that order, after sensorimotor assessment was completed. Details on the experimental design can be found in the Supplemental Methods.

2.4. Assessment of vascular, neuronal and glial indices

Hippocampal vascular networks were visualized with the space-filling FITC-dextran injection method, as we previously described [46,47]. Volocity software was used to measure vascular volume and surface area, while Fiji was used to measure branch density. Immunohistochemistry for IgG with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) on 20 μm sections was used to measure BBB permeability. Images were analyzed using Metamorph Image Analysis Software (Molecular Devices, LLC, San Jose, CA). Immuno-fluorescence microscopy was used to investigate the number of NeuN-positive neurons. AQP4 polarity was used as a measurement of astrocytic dysfunction. Number of microglia process endpoints/cell, total processes length, number of branches and cell body size was measure for microglia stained with Iba-1. Details on the experimental design can be found in the Supplemental Methods.

2.5. Cell culture

Immortalized HT22 hippocampal cells and rat brain microvascular endothelial cells (BMVECs) were studied in an in vitro system. BMVECs were isolated as described previously from 10 to 12-week old male Wistar rats [48]. BMVECs were grown in MCBD complete media (VEC Technologies Inc., Rensselaer, NY) and HT22 cells in DMEM media supplemented with 10% FBS and 1% penicillin streptomycin cocktail in a humidified incubator at 37°C with 5% CO_2. Cells were not passaged more than 5 times. To mimic the in vivo model of diabetes, cells were cultured in regular or high glucose (HG)/palmitate (Pal)-containing medium. Due to the high metabolic demands, HT22 cells require higher glucose than endothelial cells therefore control conditions contained 25 mM glucose. Cells were grown in control (5.5 mM glucose in BMVECs) or HG plus Pal (25 mM and 50 mM in BMVECs and HT22 cells, respectively). Pal was chosen because it is the most abundant saturated fatty acid and has been used in in vitro models of diabetes [24,49,50]. For each experiment, cells were either grown in control or HG/Pal for 24 h. Cells were placed in serum-free media for 1 h prior to oxygen glucose deprivation (OGD) for 6 h. Glucose free DMEM medium was used for OGD conditions and cells were placed in a 37°C incubated hypoxia chamber (94% N_2, 5% CO_2, < 1% O_2). During the reoxygenation stage (24 h), medium was changed to control or diabetic conditions and either no treatment or MCC950 (100 nM).

A second set of in vitro studies used endothelial conditioned media (CM) from BMVECs on HT22 hippocampal cells. Cells were cultured in a 96-well plate under control or HG + Pal conditions as described above. BMVECs were cultured for 24 h in control/vehicle, control/

MCC950, HG + Pal/vehicle or HG + Pal/MCC950 conditions. HT22 neurons were grown concurrently under normoxic or OGD conditions for 6 hs. After 6 h in normal or low glucose levels, HT22 cells were put into one of 8 groups: 1) control/no treatment (NT), 2) control/CM (control glucose), 3) control/MCC950, 4) control/CM + MCC950 (control glucose), 5) HG + Pal/NT, 6) HG + Pal/CM (HG + Pal), 7) HG + Pal/MCC950, and 8) HG + Pal/CM + MCC950 (HG + Pal). Western blot analysis was used to examine protein expression and cell viability was measured using a RealTime Glo-MT viability assay kit (Promega, USA) (Supplemental methods).

2.6. Data analysis

All data points are expressed by mean ± SEM. Prism7 was used for all analyses. The interaction between diabetes and ischemia on functional (composite score) and cognitive outcome (NOR, NSR, Y-maze) in control versus diabetic animals was analyzed by repeated measures and regular ANOVA, respectively (Fig. 1). The interaction of disease (control, diabetes) and surgery (sham, MCAO) on neurovascular injury was analyzed by two-way ANOVA (Figs. 2–4). NLRP3 expression and activation was analyzed in vitro by two-way ANOVA to compare glucose (control vs high) and presence/absence of palmitate in Fig. 5. The interaction of diabetes and treatment on functional (composite score) and cognitive outcome (NOR, NSR, Y-maze) in control versus diabetic animals was analyzed by repeated measures and regular ANOVA [(control vs diabetes) × (saline vs MCC950)], respectively (Fig. 6). For Figs. 7–9, interaction of disease (control, diabetes) and treatment (saline, MCC950) on neurovascular injury was analyzed by two-way ANOVA. If an interaction was identified, only interaction was marked on graphs rather than marking main effects, which are given in figure legends. Bonferroni's post-hoc test was used to compare means from significant ANOVAs. Statistical significance was determined at alpha < 0.05.

3. Results

3.1. Diabetes induces hippocampal-dependent cognitive function

While stroke caused sensorimotor deficits at days 3 and 7, both control and diabetic rats returned to baseline by day 14 in composite score measurements (Fig. 1A). Cognition was assessed by the NOR task (Fig. 1B), two-trial Y-maze (Fig. 1C) and NSR task (Fig. 1D). All recognition tasks revealed impaired hippocampal-dependent cognition in diabetic groups as early as 6 weeks after diabetes induction. While spatial learning and social recognition did not further decline, the hippocampal cognitive deficit was exacerbated in diabetic rats 14 days after 90-min MCAO as determined by measurement of discrimination index with NOR. In all tasks, control animals recovered to baseline (BL) 2 weeks after ischemia. Together, these data suggest that diabetes worsens cognitive outcome after stroke.

3.2. Diabetes induces neurovascular remodeling in the hippocampus after stroke

Since the hippocampus is essential in learning and memory, we next examined changes to the NVU in diabetes after stroke. Neuronal changes were investigated in sham and stroke animals in the CA1 and DG regions of the hippocampus. These two regions were examined because the CA1 region has been shown to be a target after MCAO and diabetes has been correlated with reduced volume of the DG in patients [51,52]. Although neither stroke nor

diabetes impacted neuronal density in the CA1 region (Fig. 2A), diabetes did reduce number of neurons in the DG (Fig. 2B). Ischemic stroke did not cause a further decrease in neuron numbers. This finding suggests that deterioration of cognitive function after diabetic stroke is not associated with exacerbated neuronal loss.

Next, vascular changes were assessed by measuring BBB permeability and vascular architecture. Diabetes alone increased permeability in the CA1 and DG. This was further elevated in the DG after ischemic injury (Fig. 3A–D). In the CA1 and DG regions, diabetes alone increased branch density but reduced vascular volume. Ischemic injury in control rats induced increased branch density along with a decrease in volume. Diabetes increased branch density, vascular volume, and surface in sham but not stroked animals (CA1 disease effect: $p = 0.0214$, ischemia effect: $p = 0.0022$; DG disease effect: $p = 0.0515$, ischemia effect $p = 0.0004$). A similar interaction was noted in vascular volume (disease effect: $p = 0.0721$ in CA1 and $p = 0.0314$ in DG) and surface area (ischemia effect: $p = 0.0024$ in CA1 and $p = 0.0329$ in DG). While diabetes reduced vasular volume and surface area in shams, there was no difference after stroke. The combination of diabetes and stroke increased vascular volume and surface area (Fig. 3E–H). Significant interactions between disease (control vs diabetes) and surgery (sham vs MCAO) were found in branch density, vascular volume and surface area of the CA1 and branch density as well as vascular volume in the DG region of the hippocampus. Together, this suggests diabetes promotes vascular remodeling resulting in compromised BBB integrity in the hippocampus following transient MCAO.

Since astrocytes are an integral part of the NVU, we next characterized astrocytic changes in the hippocampus in diabetes and stroke by two approaches. Diabetes alone disrupted AQP4 polarity, i.e. increased un-polarized AQP4, which was then further increased after 90 min MCAO in both the CA1 and DG (Fig. 4B–D; CA1 disease and ischemia effect $p < 0.001$). Next, we examined changes in astrogliosis by measuring surface area of GFAP positive astrocytes. Both the CA1 and DG had elevated astrocytic surface area 14 days after stroke in diabetic animals, suggesting increased astrogliosis in these animals (Fig. 4E–F).

3.3. NLRP3 inflammasome is elevated in the hippocampus in diabetes and stroke

Since NLRP3 inflammasome has been implicated in both diabetes and stroke [15,16,30,53,54], we investigated the role of NLRP3 in the hippocampus of diabetic animals after stroke. NLRP3 and IL-1β expression was greater in the hippocampus of diabetic animals after 90-min MCAO (Fig. 5A–B). Since the expression seemed to be located in neurons, we further confirmed NLRP3 expression and activation in an in vitro system. Immortalized HT22 hippocampal cells were grown in control or HG conditions in the presence or absence of palmitate to mimic our in vivo HFD/STZ diabetic model. Both glucose levels and treatment had a significant effect ($p < 0.0001$) such that Pal reduced expression of the NLRP3 scaffold protein compared to no treatment and HG increased expression (Fig. 5C). While Pal had no impact of IL-1β expression in control conditions, HG elevated IL-1β which was further exacerbated by Pal (interaction $p = 0.0096$) (Fig. 5D). These data suggest diabetes increases NLRP3 inflammasome in hippocampus of diabetic animals.

3.4. NLRP3 inhibition with MCC950 reduces mortality, improves cognitive function and neurovascular remodeling in diabetic rats

Since NLRP3 upregulation after transient MCAO was amplified in diabetic rats, we next investigated the impact of NLRP inhibition (using MCC950) of stroke outcomes. In control-saline and control-MCC950 groups, 100 (8/8) and 89 (1/9) %, respectively, of animals survived until day 14. In diabetes-saline and diabetes-MCC950 groups, 40 (6/15) and 83 (5/6) %, respectively, completed the study. These results suggest early inhibition of NLRP3 with MCC950 improves long-term survival after stroke in diabetes. In a limited number of animals (n = 3–4), infarct analysis was examined on TTC-stained Sections 3 days after MCAO in diabetic rats treated with vehicle (saline) and MCC950. No difference in infarct size was seen between treatment groups (data not shown).

Recovery of sensorimotor function in control and diabetic animals was similar in saline-treated and MCC950-treated groups. At day 3 post-MCAO lower composite scores were observed compared to BL (Fig. 6A). By D14, all groups recovered back to BL. The effect of NLRP3 inhibition on cognition was measured by NOR, two-trial Y-maze and NSR. At BL, there was a disease effect ($p = 0.0126$) in which diabetes reduced recognition index. After ischemia, MCC950 had no impact in control animals but there was a trend in diabetic animals treated with MCC950 14 days after stroke compared to saline treatment (Fig. 6B). When D14 results were compared to BL, only the diabetic group treated with saline was further reduced after stroke ($p = 0.0249$). Diabetes reduced the time spent in the novel arm of the two-trial Y-maze ($p = 0.0013$; Fig. 6C). Although not significant, there was a trend towards an interaction effect between disease and treatment at day 14 ($p = 0.0976$). NLRP3 inhibition in diabetic animals significantly increased time spent in novel arm compared to saline treated animals at day 14 post-MCAO ($p = 0.0141$). Furthermore, these animals had improved function compared to BL ($p = 0.0538$). To investigate long-term hippocampal memory, NSR was used (Fig. 6D). When disease (control and diabetes) and treatment (saline and MCC950) was compared by two-way ANOVA, diabetes had a significant effect at BL ($p = 0.0002$) such that the recognition index was higher in control than diabetes in both saline ($p = 0.0048$) and MCC950 ($p = 0.0156$) groups. Recognition index at day 14 after stroke had a treatment effect in which MCC950 treatment had higher index than saline treated animals in both control ($p = 0.0232$) and diabetic animals ($p = 0.0111$). Stroke and treatment effect in control animals had a significant interaction ($p = 0.0440$) in which the saline treatment groups has a lower recognition index at D14 compared to BL ($9 = 0.0657$) but MCC950 had no change. In diabetic rats, inhibition of NLRP3 with MCC950 increased recognition index after ischemia ($p = 0.0198$). These data show MCC950 treatments ameliorate diabetes-mediated cognitive deficits after stroke.

To confirm that NLRP3 activation was inhibited, immuno-fluorescence was used to determine the intensity and localization of NLRP3 inflammasome and IL-1β in the hippocampus (Fig. 7). IL-1β has a higher intensity of expression in diabetes compared to the control group in both the CA1 and DG hippocampal regions. Furthermore, NLRP3 inhibition with MCC950 reduced levels of IL-1β 14 days after MCAO, suggesting less activation of the inflammasome. IL-1β co-localized highly in neurons in the CA1 (Fig. 7A) and DG (not shown). Expression of IL-1β was increased in diabetes compared to controls in

the CA1 ($p = 0.0219$) and a trend was seen in the DG ($p = 0.0765$). MCC950 treatment significantly decreased CA1 IL-1β in diabetic animals ($p = 0.0265$), but not the DG, although a trend was observed ($p = 0.0514$). NLRP3 was similarly increased in diabetic rats treated with saline compared to controls, while inhibition decreased expression 14 days after ischemic injury (Fig. 7B). Comparable to IL-1β expression, NLRP3 scaffold protein was co-localized more in neurons than astrocytes. Diabetes increased NLRP3 staining threshold area compared to control rats in the CA1 ($p = 0.0262$) and DG (0.0069) of the hippo-campus. NLRP3 inhibition with MCC950 reduced expression of NLRP3 in both control and diabetic groups in the CA1 ($p = 0.0120$ and $p < 0.0001$ for control and diabetes, respectively) and DG ($p = 0.0051$; $p < 0.0001$ for control and diabetes, respectively). Together, this shows chronic NLRP3 expression and activation is blunted by MCC950 treatments in diabetic animals following stroke.

Next, changes to the NVU in MCC950 treated animals were investigated. Diabetes increased BBB permeability as seen by higher % threshold area for IgG staining (Fig. 8). A significant interaction between disease (control and diabetes) and treatment (saline and MCC950) was observed in the DG such that MCC950 had no effect after stroke in control animals, but NLRP3 inhibition improved BBB integrity in diabetic rats after stroke (interaction $p = 0.0028$) (Fig. 8B). To determine if MCC950 treatments reduced neurodegeneration in the hippocampus, apoptotic cells were counted by TUNEL fluorescein staining (Fig. 8C–D). Diabetes increased the number of TUNEL-positive neurons in the CA1 (C) and DG (D) after 90-min MCAO. Treatment with MCC950 significantly reduced cell death in both control and diabetic animals. Un-polarized AQP4, an indicator of astrocytic disruption, was increased in diabetic animals compared to controls (CA1: $p = 0.0029$; DG: $p = 0.0012$). An interaction effect was observed such that treatment with MCC950 had no impact of AQP4 polarity in controls after stroke, while un-polarized AQP4 was drastically reduced in diabetic animals compared to saline treatments in the CA1 ($p < 0.001$) and DG ($p = 0.0009$).

Since NLRP3 activation amplifies immune responses, microglia reactivity was next investigated after ischemic injury in MCC950 treated animals by measuring cell swelling, number of protrusions from the cell body, summed number of endpoints per cell and summed branch length per cell. Diabetes trended towards an increase in cell swelling as seen by higher cell body size (Bonferroni's post-hoc: $p = 0.0992$). A significant treatment effect was observed in which MCC950 reduced cell swelling in diabetes compared to saline treatment (Fig. 9C). NLRP3 inhibition with MCC950 increased the number of endpoints per microglia compared to saline groups in both control and diabetes ($p = 0.0136$) (Fig. 9D). The number of protrusions per cell was lower in saline treated diabetic animals ($p = 0.0125$) and was increased to control numbers with MCC950 treatments (Fig. 9E). While no significance between control and diabetic saline treated groups was observed, there was a trend towards greater total processes length in diabetes after MCC950 injections ($p = 0.0591$) (Fig. 9F). Together, these data show improved integrity of the NVU in MCC950-treated diabetic animals after MCAO.

3.5. Diabetic conditions reduce BDNF levels in endothelial cells and HT22 hippocampal neurons

To evaluate the vasotrophic coupling between endothelial cells and hippocampal neurons, multiple comparisons were made. First, the effect of MCC950 on endothelial or HT22 neuronal BDNF levels in control and diabetes-mimicking conditions under normoxia was assessed [(control vs HG + Pal) X (vehicle vs MCC950)]. In HT22 cells, diabetes-mimicking conditions reduced mBDNF levels and MCC950 had no effect ($p = 0.0485$, Fig. 10A). On the other hand, after OGD MCC950 tended to increase BDNF (Fig. 10A, $p = 0.0758$). When the interaction of hypoxia and MCC950 treatment on BDNF levels under control or diabetic conditions [(Normoxia vs OGD) × (vehicle vs MCC950)] was assessed, NLRP3 inhibition was effective in preventing the decrease in BDNF levels in cells grown in diabetic (Fig. 10A, b:$p = 0.0125$) but not control conditions.

In BMVECs, under normoxic settings, diabetes effect was greater ($p = 0.0047$) and a trend for increased BDNF with MCC950 treatment ($p = 0.0623$) was observed. Under OGD conditions, there was a strong diabetes effect (Fig. 10B, $p = 0.009$). There was an interaction such that MCC950 lowered BDNF level in control with no effect on diabetic conditions (Fig. 10B, $p = 0.0102$). When normoxic and OGD settings were compared for control or diabetes-mimicking conditions, an interaction was observed for control cells such that OGD increased BDNF level in vehicle treated cells and lowered it in MCC950 treated cells (Fig. 10B, $p = 0.0082$). Overall, we show that in hypoxia NLRP3 inhibition does not improve decreased *endothelial* BDNF levels in diabetic conditions but increases BDNF in hippocampal neurons.

Next, cell viability was investigated in HT22 cells 6 h after hypoxia in the presence and absence of MCC950 (100 nM). The role of vasotrophic coupling was examined by adding endothelial conditioned media (CM) collected from BMVECs grown under control or HG/Pal conditions in the presence or absence of MCC950 (Fig. 10C). A significant interaction between growth conditions (diabetes/hypoxia) and treatment was observed ($p = 0.0286$). Post-hoc tests showed HT22 cells grown under HG/Pal conditions had lower survival than control conditions under normoxia ($p = 0.0014$). Survival of CM ($p = 0.0050$) or MCC950 ($p = 0.0020$) groups remained lower in HG/Pal than controls under normoxic conditions. Hypoxia reduced cell viability, regardless of treatment of CM or MCC950 ($p < 0.0001$). While hypoxia further reduced survival in diabetic conditions ($p = 0.0095$), treatment with CM, MCC950 or in combination did not have an additional decline in cell viability after hypoxia (Fig. 10D). These experiments show treatment with MCC950 improves hippocampal cell and BMVEC viability after hypoxia.

4. Discussion

The experiments in the current study were conducted to determine the extent and mechanism by which NLRP3 activation contributes to poor cognitive function after stroke in diabetes. The results provide evidence that 1) diabetes upregulates NLRP3 inflammasome expression which is further amplified by stroke, 2) inhibition with MCC950 improves survival, cognitive function and vascular integrity after stroke in diabetic animals, and 3) while NLRP3 inhibition does not improve decreased endothelial BDNF levels in diabetic

conditions, it increases BDNF in hippocampal neurons, an effect more pronounced after a hypoxic injury. These results are the first to provide essential data showing MCC950 has the potential to become a therapeutic to prevent neurovascular remodeling and worsened cognitive decline in diabetic patients following stroke.

Cognitive impairment is an increasingly recognized complication of diabetes that is further amplified by greater incidence of stroke in the U.S. population [1,51]. As the global incidence of diabetes continues to rise, the burden of cognitive impairment and dementia will continue to amplify due to small vessel disease and stroke. Unfortunately, there are no current therapeutics that target cognitive impairment after stroke. In our study, MCC950 administration in the acute phase of stroke provided sustained benefits 14 days after stroke such as decreased BBB permeability, neuronal degeneration, AQP4 polarity disruption and microglial activation, all of which were associated with improved cognitive outcomes in diabetes. These findings support the notion that this small molecule inhibitor of inflammasome may provide a new therapeutic target.

A range of diseases fall under the umbrella of the term vascular contributions to cognitive impairment and dementia (VCID) [55]. The two main settings in which VCID occur are 1) post-stroke, in which cognitive impairment results due to ischemic injury, and 2) without recent stroke, in which injury manifests through neuropathology rather than stroke [56]. We have previously reported vascular contributions to cognitive impairment and worsened outcome after ischemia in the cortex and striatum [45,47,48,57–59]. In the present study, we further support these findings in the hippocampus, an area remote to the site of injury. Indeed, treatment with MCC950 improved both cognitive function and vascular integrity in diabetic rats. These results suggest a role for the NLRP3 inflammasome in VCID and warrant further investigation.

Unlike many of the other therapeutics targeting inflammation after stroke, MCC950 specifically inhibits NLRP3-induced ASC oligomerization and thereby preventing IL-1β cleavage into its active form. Furthermore, this small-molecule inhibitor only targets NLRP3 and no other inflammasome, such as NLRP1, AIM2 or NLRC4 [43]. This allows for IL-1β processing to still occur through other inflammasome complexes, but potentially preventing exacerbation of inflammatory cascades in diseased states such as diabetes. A report comparing pharmacokinetics of a variety of inflammasome inhibitors commented on the high stability of the compound as well as great pharmacokinetic properties of MCC950 [60]. Furthermore, the half-life of MCC950 was reported to be 3.27 h. Some published reports have used higher doses such as 10 mg/kg [61] or 50 mg/kg [62] rather than 3 mg/kg used in the current study. This dose was selected based on the dosing used by Coll et al [43] and due to preliminary studies in diabetic rats showing improved mortality rates with MCC950 treatments after 60-min MCAO (data not shown). The notable improvement in cognition by day 14 after a 90-min MCAO was associated with better BBB integrity, less neurovascular remodeling and reduced inflammation as seen by lower IL-1β expression and reactive microglia. Interestingly, this dose did not reduce infarct size suggesting that even small doses of MCC950 given shortly after reperfusion can improve stroke outcomes by providing resolution of neuroinflammation in the repair/recovery phase of stroke. Improved

neurovascular coupling with MCC950 treatment in diabetic rats can be further studied by measuring functional hyperemia.

In the current study, a qualitative reduction in NLRP3 expression and subsequent IL-1β activation was observed in diabetic animals treated with MCC950. NLRP3 expression was low 14 days after stroke, which suggests that it may be upregulated and activated earlier during the acute phase of stroke. Higher levels of IL-1β in the hippocampus at day 14, especially in diabetic animals, further corroborate this. Future studies should examine NLRP3 activation at earlier time points after stroke in diabetic animals. Treatment with MCC950 blunted IL-1β expression. Some of the IL-1β expressed after ischemia may be attributed to NLRP1 activation, which has been associated with neuronal in-flammasome activation [63].

Diabetes is a chronic inflammatory state [17]. Microglia are the resident immune cells of the brain and upon activation are responsible for the initiation of an immune response. A secondary insult, such as a stroke, exacerbates inflammation within the brain. Microglia are essential to detecting signals from damaged tissue after ischemic injury. Ramified microglia monitor the healthy brain and are characterized as having a small cell body with extensive branching off the soma [64,65]. On the contrary, activated microglia have enlarged somas and retraction of processes [66–68]. Additionally, patients with cerebral infarction and diabetes have higher levels of microglial proliferation and activation as seen by ferritin-stained post-mortem sections [69]. These results suggest that diabetes amplifies the immune response to stroke, even in remote areas of the brain, and may be a contributing factor to worsened stroke outcome. The presence of greater activated microglia suggests that diabetes results in sustained inflammation after ischemic injury. Intriguingly, it has been shown that hippocampal microglial activation mediates post-operative cognitive decline and this effect is more pronounced in diabetes [70]. Based on these previous studies, we measured cell body swelling, number of endpoints/cell and total length of processes of microglia in the hippocampus of control and diabetic animals. Early treatment with MCC950 reduced activated microglia in the hippocampus 14 days after stroke. This suggests that restricting the acute neuroinflammatory response, in this specific case microglia, can potentially provide improvement after stroke. Developing inhibitors for treatment toward of stroke through blockade of a specific inflammatory complex, such as the inflammasome, may be more beneficial by targeting over exaggerated inflammation, rather than inhibiting beneficial immune cell response.

While improved functional and neurovascular outcome was reported here, the trigger for NLRP3 elevation in diabetes and amplified activation after stroke remains elusive. The NVU is essential to proper brain health and this concept emphasizes the interaction between the cerebrovasculature and the surrounding brain cells. Vasotrophic coupling, the idea that endothelial cells provide neurotrophic as well as angiogenic factors to neurons and oligodendrocytes, provides insight to how the NVU may be targeted in ischemic injury. Guo et al reported that primary cortical neurons cultured in a transwell system with primary mouse endothelial cells survived better than neurons alone after hypoxia/reoxygenation [33]. Since this suggests a role for secreted factors from endothelial cells, they then used conditioned media from endothelial cells to determine if trophic factors secreted from

vasculature provide neuroprotection. Using this paradigm, they showed improved neuronal survival after numerous insults (amyloid Aβ, oxygen glucose deprivation, oxidative stress and endoplasmic reticulum stress)in the presence of endothelial conditioned media. This study elegantly shows the importance of vasotrophic coupling after injury. This led us to investigate the impact of NLRP3 inhibition on BDNF signaling in both neurons and endothelial cells.

In the in vitro studies presented, we expected to observe reduced cell death and higher expression of the neuroprotective mBDNF after treatment with MCC950 in diabetic conditions exposed to normoxic or OGD/reoxygenation. mBDNF expression was not different with these treatments after ODG in HG + Pal grown endothelial cells. It is possible that mBDNF is being secreted into the media, which was not measured in the present study. Indeed, it has been noted that neurons secrete roughly 1 pg per 10^6 cells whereas endothelial cells secrete 50 pg per 10^6 cells over 24 h [33]. Therefore, BDNF secretion may be altered rather than protein expression within the cell lysate. Diabetes alters the ratio between mBDNF and proBDNF in which pro-apoptotic proBDNF was greater and neurotrophic mBDNF was reduced in neurons and endothelial cells in vitro [71]. This imbalance may contribute to the neurovascular dysfunction observed in diabetes. Bands on the western blot for proBDNF in MCC950 treated cells were faint in BMVECs, therefore we were unable to measure this ratio in these treatments. Alternatively, improved cognition and neurovascular function after inhibition of NLRP3 may not be mediated through the BDNF system. Further studies should be pursued to investigate the mechanism in which the NLRP3 inflammasome activation becomes amplified in diabetes and stroke. Understanding the mechanism behind this in-flammatory pathway may provide critical insight to diabetes-induced cognitive impairment.

While this study shows promising use of MCC950 as a therapeutic for stroke, there are several limitations that should be noted. First, intravenous injection of MCC950 results in systemic inhibition of NLRP3. By lowering systemic inflammation, improvements in cognition could also be a result of improved neurovascular coupling in other brain regions essential for cognition, such as the prefrontal cortex. Administration of MCC950 at a later time point after reperfusion should be investigated. While we had improved stroke survival after MCC950 treatment in diabetic rats, later administration may provide evidence for chronic inflammation contributing to worsened outcome. Conversely, as documented low grade chronic inflammation contribute to the repair process and this response may differ in diabetic animals [72,73]. Additionally, only one method, immunohistochemistry, was used to investigate NLRP3 expression and activation in vivo. This method was selected to be able to co-localize NLRP3 and its components with either vascular cells, neurons or glial cells within the CA1 and DG hippocampal regions. Total expression within the hippocampus could be studied at the gene and protein level by rt-PCR and western blotting, respectively. Additionally, we did not quantify co-localization of NLRP3 with neurons or astrocytes. Another caveat of the present study is that MCC950 treatment was not tested in aged animals or females. Stroke is considered an aging disease and although diabetes does increase stroke incidence in patients, little is known about neurovascular changes in aged animals. Diabetic females are more likely to have worsened functional outcome following a stroke and often suffer from stroke at younger ages [74–78]. Future studies are needed to see

if MCC950 treatment would be beneficial in these groups as well. Lastly, animals were only followed up to day 14 after stroke due to increased mortality in the diabetic groups and longer-term studies with multiple cognitive/memory tasks are needed. Nevertheless, NLRP3 inhibition by MCC950 remains a promising therapeutic to treat cognitive impairment in diabetes and stroke.

5. Conclusions

In summary, the present study provides evidence that diabetes impairs the integrity of the NVU in the hippocampus, a critical area in learning and memory, as seen by increased BBB permeability, vascular remodeling, and higher levels of hippocampal cell death, greater astrocyte reactivity and disruption of the AQP4 polarity within the astrocytes. These indices were further exacerbated by ischemia in diabetic animals. Furthermore, low dose treatments with the specific NLRP3 inflammasome inhibitor MCC950 ameliorated hippocampal-dependent memory deficits after ischemia in diabetic rats through 1) resolution of inflammation as seen through greater incidence of resting microglia and lower IL-1β expression, 2) improve BBB integrity, and 3) lower cell death of the neurons in the CA1 and DG regions of the hippocampus.

Collectively, this work may provide a piece of the puzzle explaining how diabetes leads to cognitive impairment and worsens outcome following acute ischemic injury, and it provides a potential therapeutic target to treat cognitive impairment after stroke, especially in diabetic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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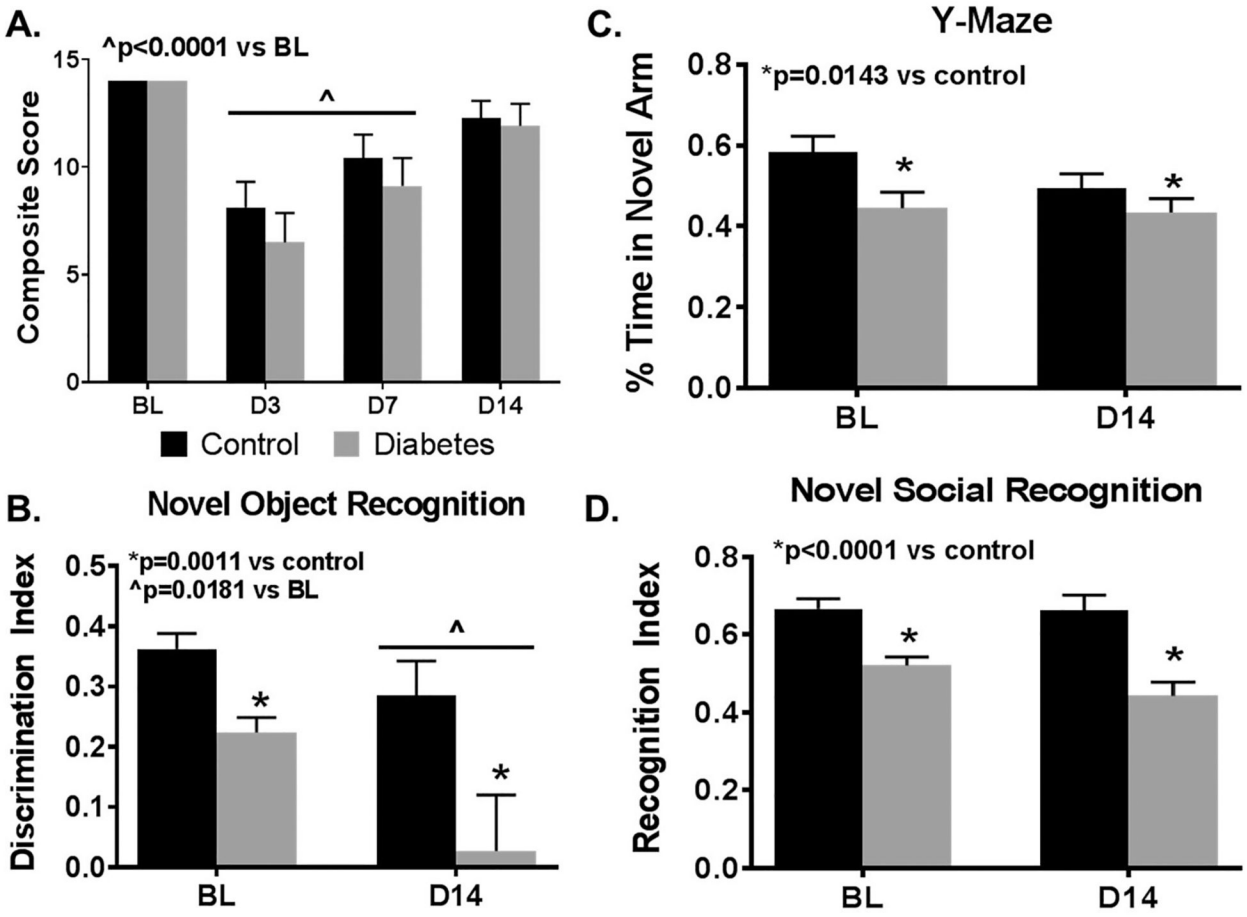

Fig. 1.

Stroke exacerbates diabetes-mediated cognitive deficits. (A) Sensorimotor deficits were investigated by composite score. Diabetes had no impact on functional outcomes 2 weeks after MCAO. Novel object recognition (B), two-trial Y-maze (C) and novel social recognition (D) were used to examine cognitive function in control and diabetic rats. Diabetes impaired cognition even before stroke (B–D). Short-term learning and memory deficits were further exacerbated by MCAO as seen by NOR (n = 8; BL = baseline, 3–5 days prior to MCAO induction).

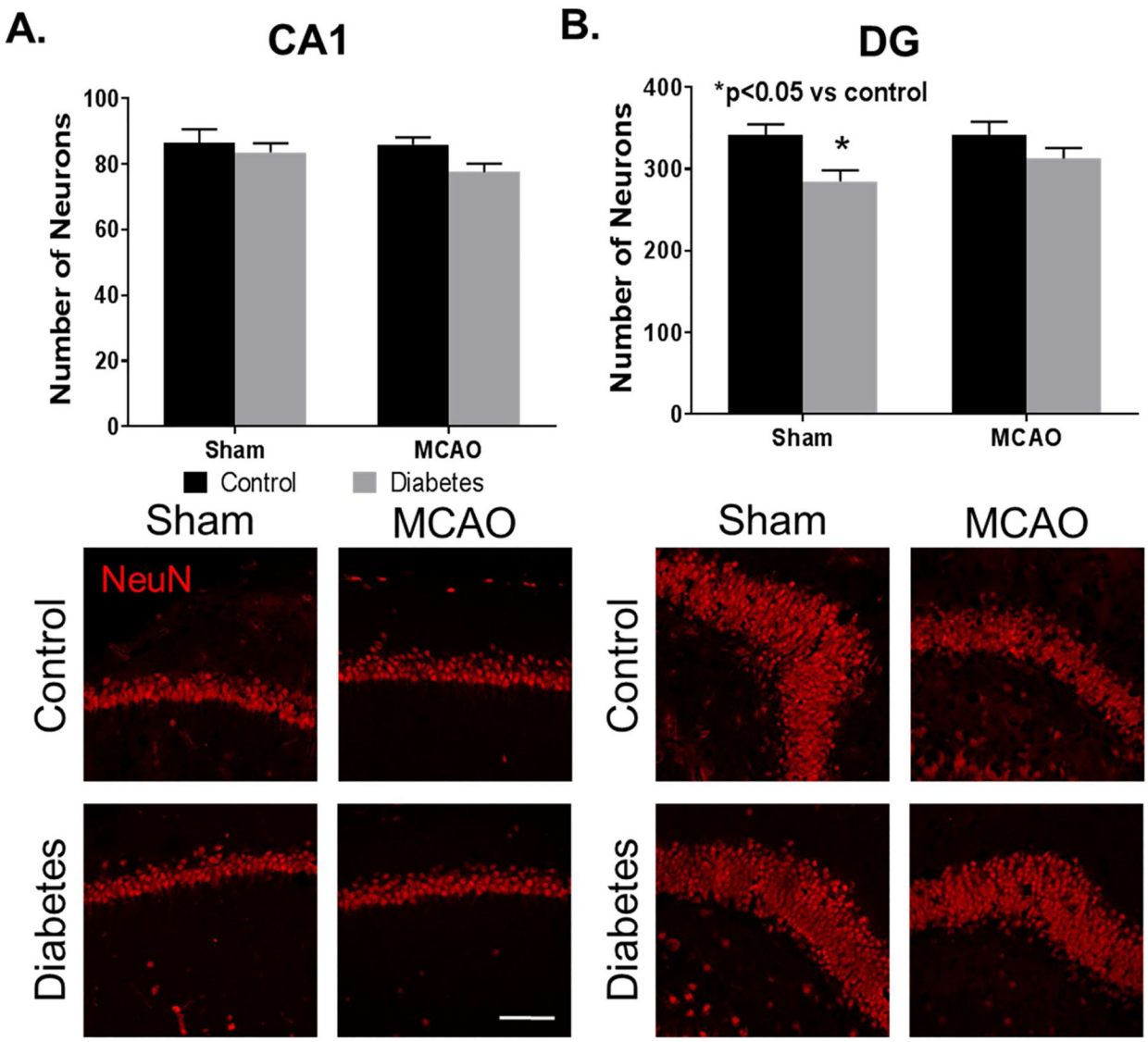

Fig. 2.

Diabetes reduced neuronal density in the DG region of hippocampus. Neuronal density was unchanged in the CA1 region after induction of diabetes and/or middle cerebral artery occlusion (MCAO) (A). Diabetes alone reduced number of NeuN-positive neurons in the DG, but stroke had no effect (B) (n = 6–8; scale bar = 100 μm).

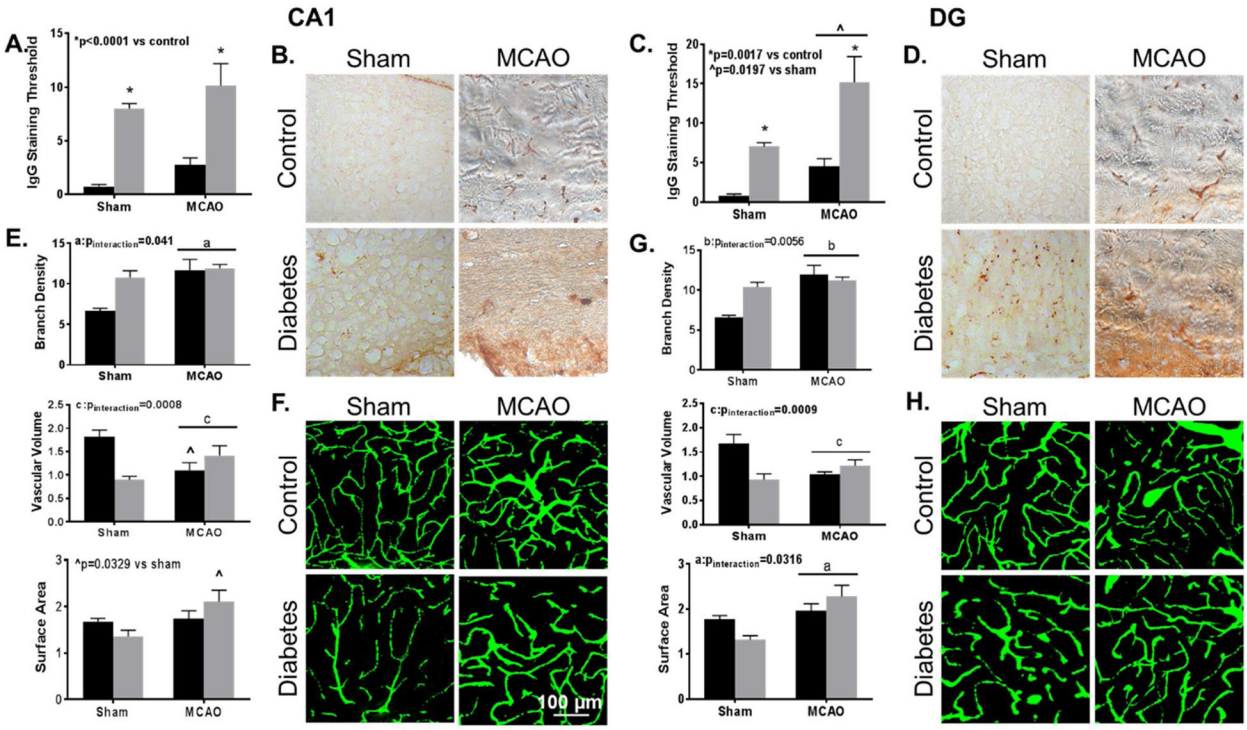

Fig. 3.

Transient MCAO exacerbates hippocampal vascular injury in diabetic rats. Diabetes disrupted blood brain barrier integrity in the hippocampus as seen by increased IgG staining (A and C; $p < 0.0001$). Representative images from the CA1 and the dentate gyrus are shown in Panels B and D. Stroke further elevated IgG staining in the DG of diabetic rats. Representative images of FITC-filled blood vessels in the CA1 and DG are shown in Panels F and H, respectively. Diabetes increased branch density, vascular volume, and surface in sham but not stroked animals (E, G) (n = 6–8; scale bar = 100 μm).

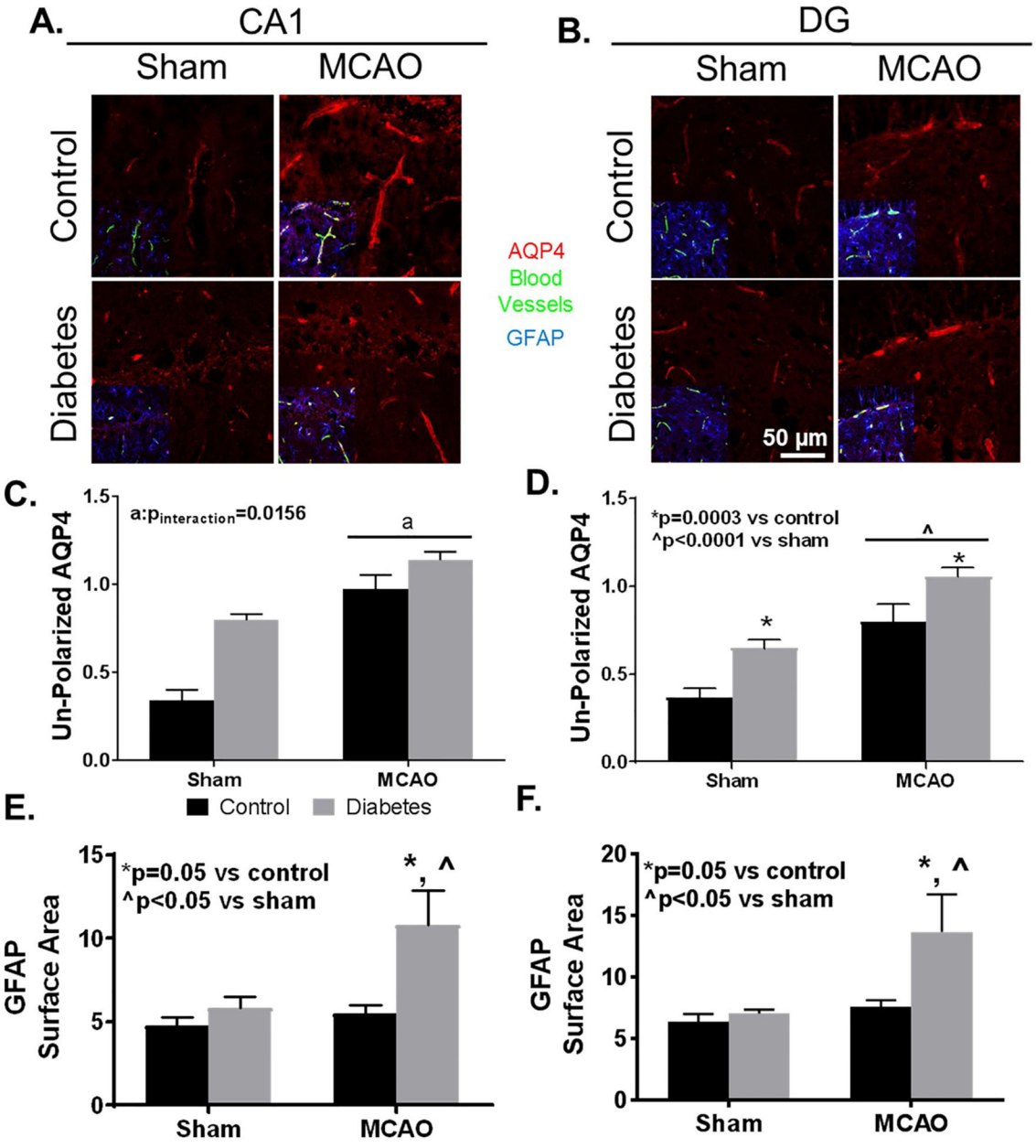

Fig. 4.

Diabetes disrupts AQP4 polarity and is exacerbated after ischemia. Representative images of aquaporin 4 (AQP4; red), GFAP (blue) and FITC-filled vessels (green) in the CA1 and dentate gyrus (DG) of the hippocampus (A–B). Diabetes increases un-polarized AQP4 in sham animals which is exacerbated after stroke in the CA1 (C) and DG. Astrogliosis was increased after stroke in diabetic rats as seen by elevated surface area in the CA1 (E) and DG (F) of the hippocampus. (n = 8; scale bar = 50 µm) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

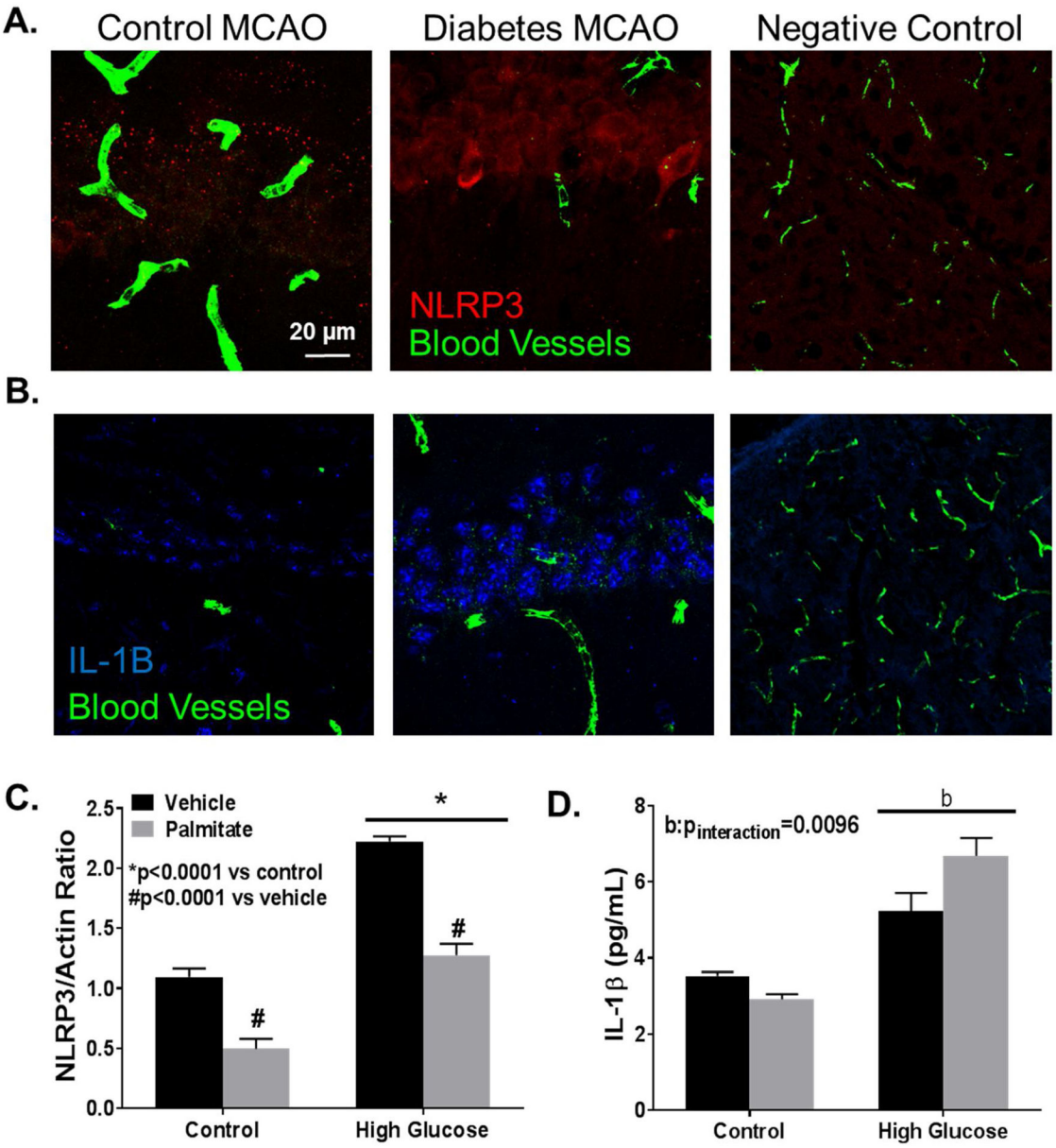

Fig. 5.

Diabetes elevated NLRP3 expression and activation in the hippocampus after ischemic injury. NLRP3 inflammasome (A) and activated IL-1β (B) was up-regulated in the hippocampus after ischemia in diabetic rats. HT22 hippocampal neurons had increased NLRP3 activation as seen by elevated protein levels or NLRP3 scaffold protein (C) and IL-1β (D). (n = 3–4; magnification 63x; scale bar = 20 μm).

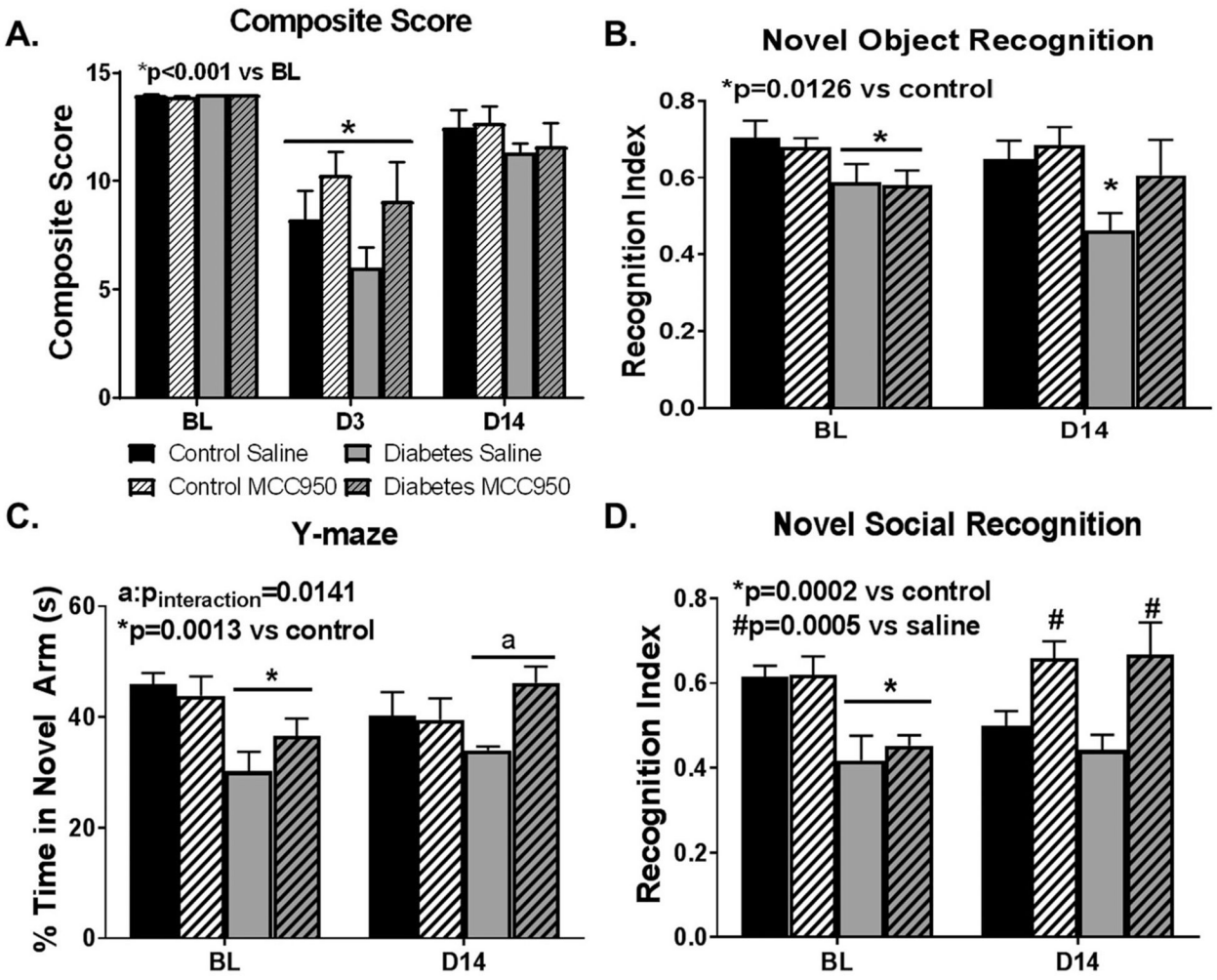

Fig. 6.

MCC950 improves post-stroke cognition in diabetic rats. All groups had lower composite scores 3 days after stroke but recovered by day 14 (D14) (A). Cognitive function was measured by novel object recognition (NOR; B), two-trial Y-maze (C), and novel social recognition (NSR; D). Diabetic animals had lower baseline (BL) scores compared to controls. Treatment with MCC950 prevented further decline observed in saline-treated diabetic animals. (Control n = 8; diabetes n = 5).

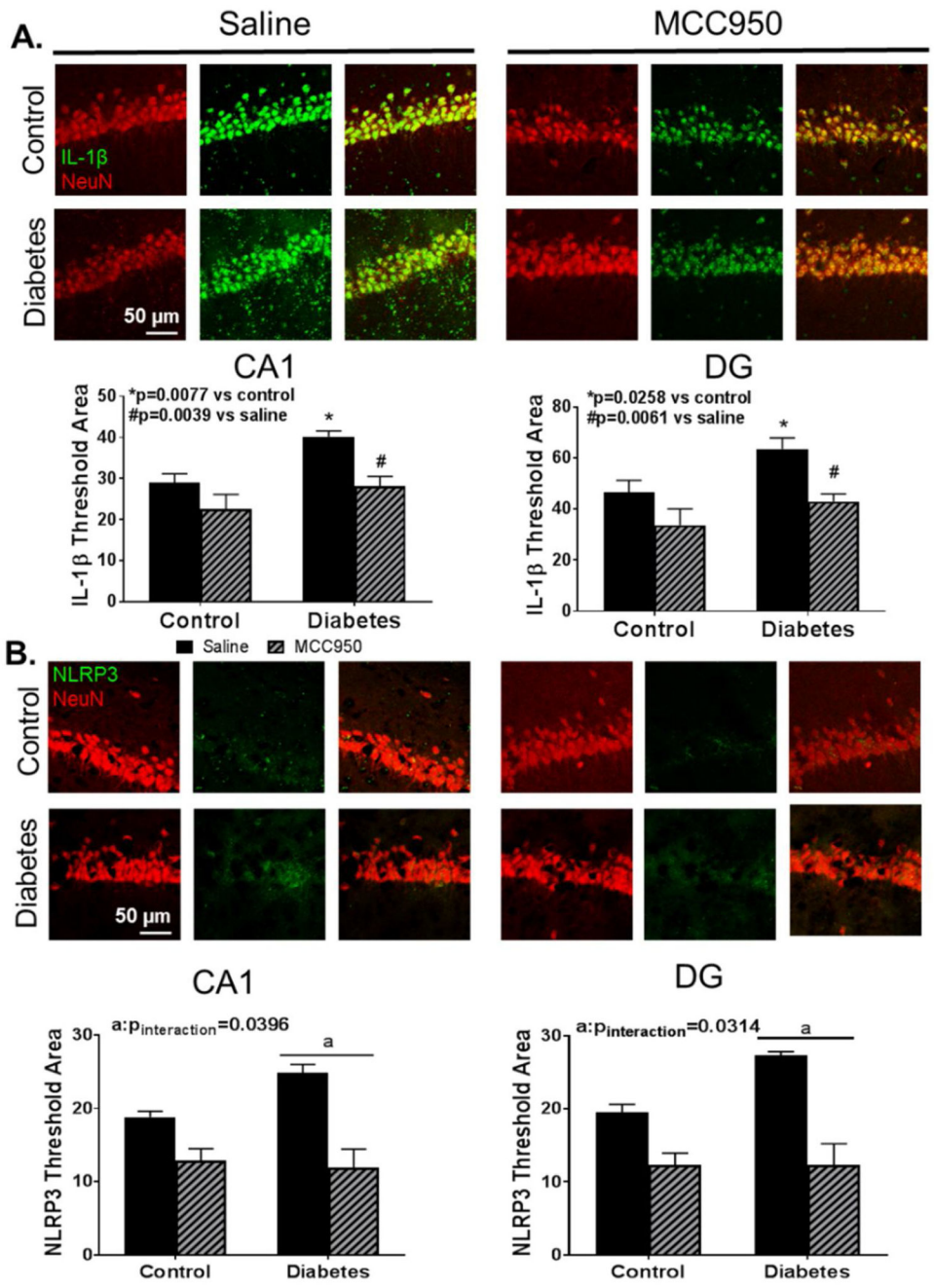

Fig. 7.

IL-1β and NLRP3 stained sections in the hippocampus. (A) Diabetes amplified IL-1β expression in the CA1 and DG of the hippocampus. IL-1β was mainly co-localized in the neurons as seen by NeuN staining. Treatment with MCC950 appeared to reduce IL-1β staining in both hippocampal regions (identical parameters were used). (B) Sections were co-stained for the scaffold protein in the NLRP3 inflammasome and NeuN. (Magnification 40x; scale bar = 50 μm).

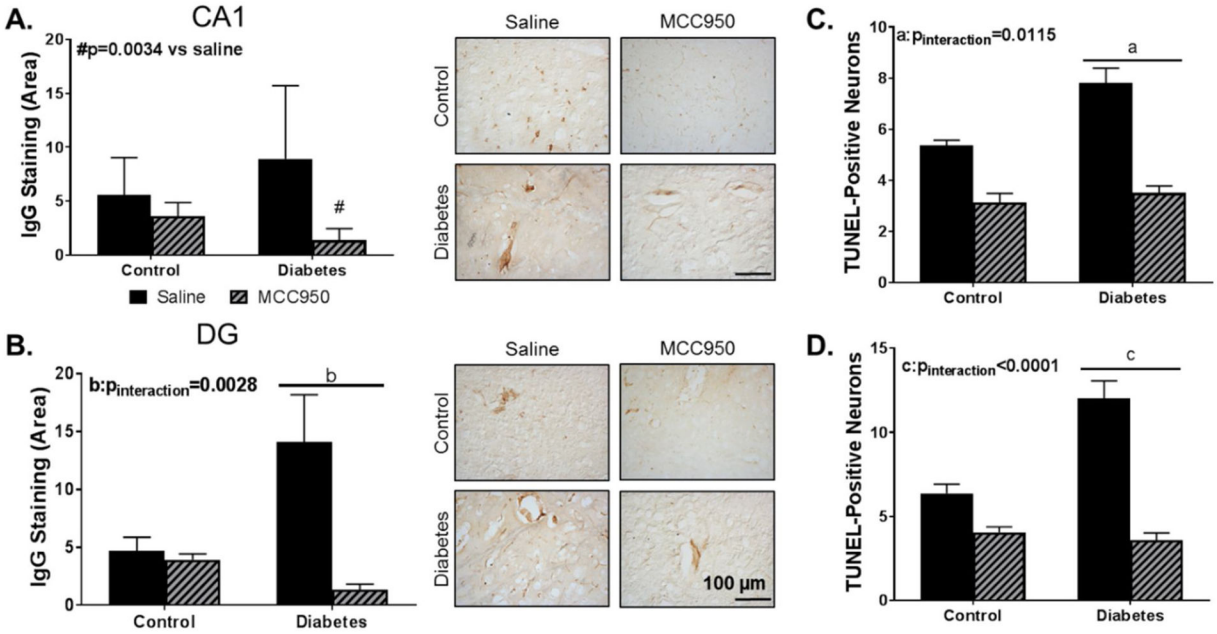

Fig. 8.

MCC950 treatments improved blood brain barrier (BBB) integrity and reduced neurodegeneration in diabetic animals after stroke. MCC950 had no effect on control rats, but improved BBB integrity by reducing percent IgG area threshold (A–B.) Diabetes increased the number of TUNEL-positive neurons in the CA1 (C) and DG (D) after 90-min middle cerebral artery occlusion (MCAO). Treatment with MCC950 significantly reduced cell death in both control and diabetic animals. (n = 8 in control groups and 5 in diabetic groups; scale bar = 100 μm).

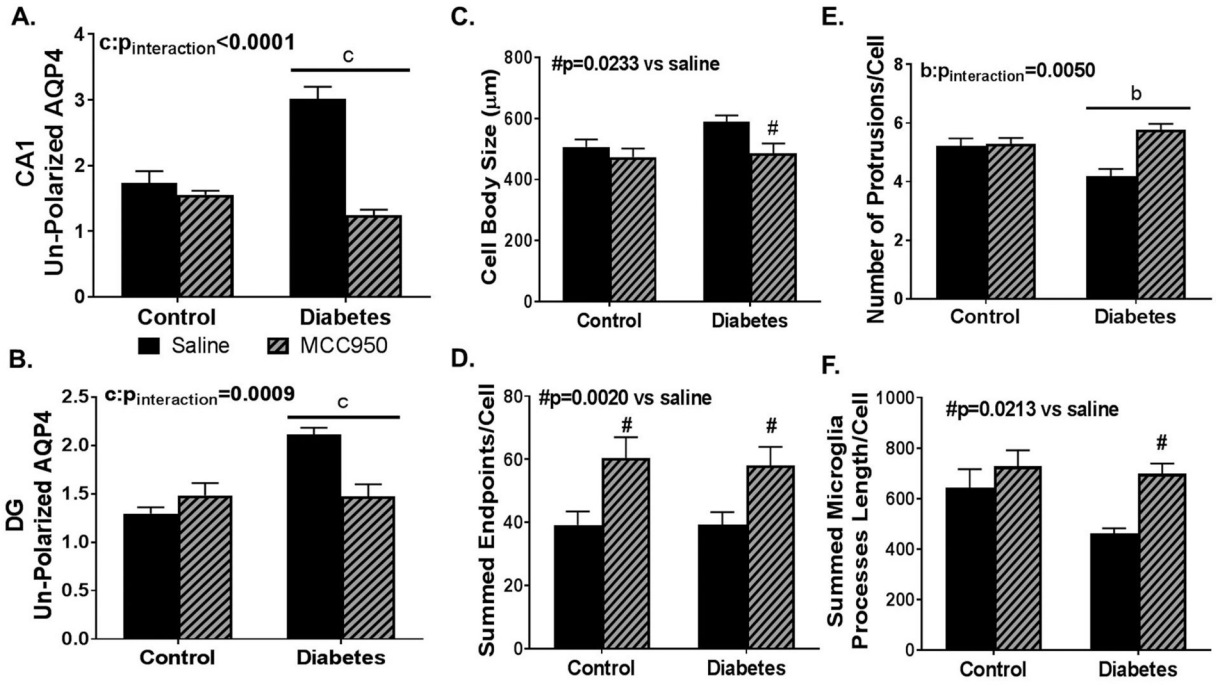

Fig. 9.

MCC950 reduced un-polarized aquaporin 4 (AQP4) and reactive microglia phenotype after stroke in diabetes. 90-min middle cerebral artery occlusion (MCAO) induces greater un-polarization of AQP4 in the CA1 (A) and DG (B) in diabetic rats which was ameliorated with MCC950 treatments. NLRP3 inhibition reduced markers of reactive microglia as seen by reduced cell body size (C), elevated number of endpoints (D), protrusions per cell (E) and total processes length (F) in Iba-1 positive microglia of the CA1 region after ischemia. (n = 8 for control; n = 5 for diabetes).

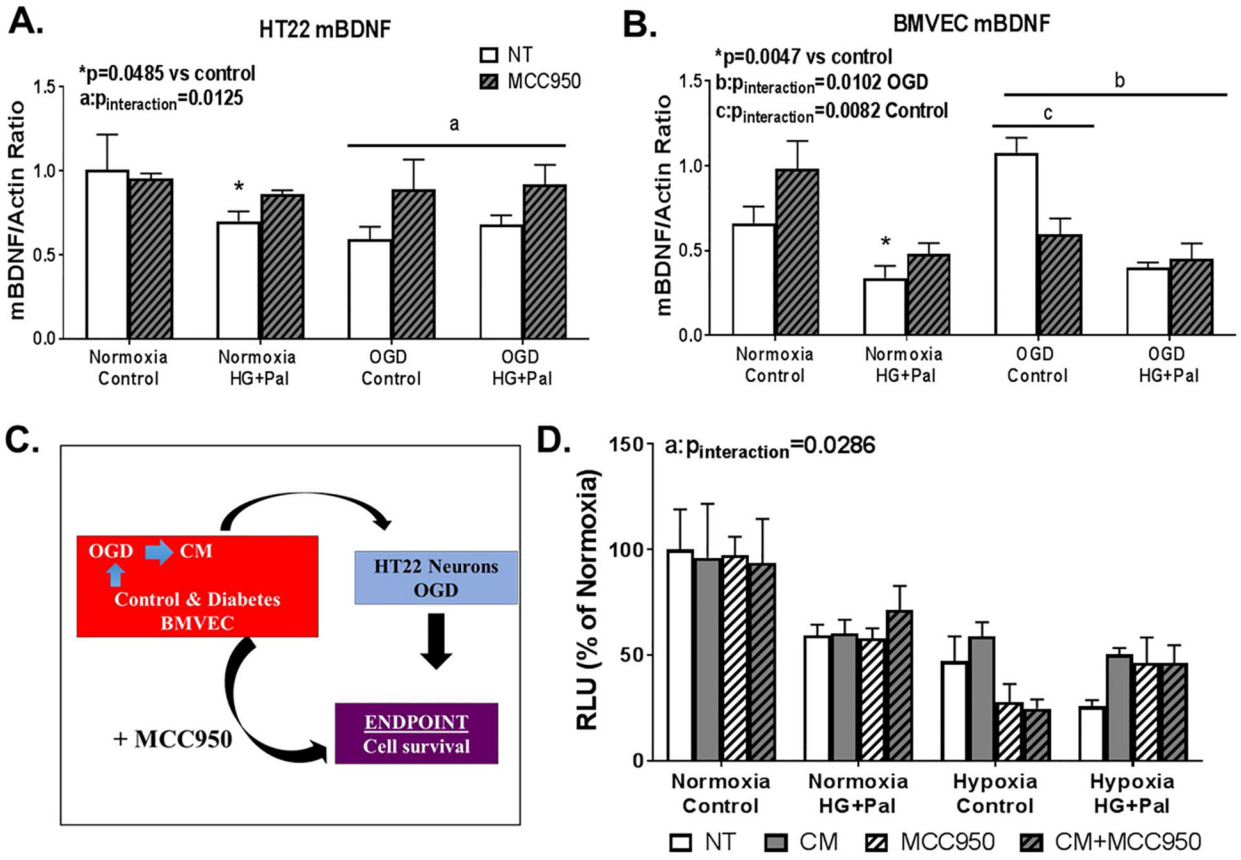

Fig. 10.

MCC950 treatment improved cell viability in HT22 hippocampal cells and brain microvascular endothelial cells (BMVECs) after hypoxia in control and high glucose/palmitate (HG/Pal) conditions. Neurotrophic mature brain derived neurotrophic factor (mBDNF) was measured by western blot in HT22 cells grown in control or HG + Pal in normoxic or oxygen glucose deprivation (OGD) conditions. HG + Pal reduced mBDNF expression in normoxic condition (A). In endothelial cells, BDNF expression was reduced in HG + Pal conditions during normoxia. mBDNF was increased after OGD, but MCC950 reduced expression after hypoxia (B). HT22 were treated with endothelial conditioned media (CM) as shown by the schematic (C). Diabetes reduced cell survival of HT22 cells under normal conditions. Hypoxia reduced cell viability in untreated cells grown in control or HG/Pal diabetic conditions compared to normoxia CM and/or MCC950 seemed to slightly improve cell viability after hypoxia in diabetic conditions (D). (n =. 3–4).

Table 1

Number (n) and mortality rates for experiment 1 and experiment 2.

		Number of animals at the start	Number of animals that died	Number of animals at the end	Morality (%)
Experiment 1	Control Sham	8	0	8	0%
	Control MCAO	9	1	8	11%
	Diabetes Sham	8	0	8	0%
	Diabetes MCAO	12	4	8	33%
Experiment 2	Control Vehicle	8	0	8	0%
	Control MCC950	9	1	8	11%
	Diabetes Vehicle	11	5	6	45%
	Diabetes MCC950	6	1	5	17%