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Surprising outcomes in cannabinoid CB1/CB2 receptor double knockout mice in two models of ischemia

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Abstract

Aims—We tested the hypothesis that CB1/CB2 receptor double knockout would produce significant increases in infarct size and volume and significant worsening in clinical score, using two mouse models, one of permanent ischemia and one of ischemia/reperfusion.

Main methods—Focal cerebral infarcts were created using either photo induced permanent injury or transient middle cerebral artery occlusion. Infarct volume and motor function were evaluated in cannabinoid receptor 1/ cannabinoid receptor 2 double knockout mice.

Key Findings—The results surprisingly revealed that CB1/CB2 double knockout mice showed improved outcomes, with the most improvements in the mouse model of permanent ischemia.

Significance—Although the number of individuals suffering from stroke in the United States and worldwide will continue to grow, therapeutic intervention for treatment following stroke remains frustratingly limited. Both the cannabinoid 1 receptor (CB1R) and the cannabinoid 2 receptor (CB2R) have been studied in relationship to stroke. Deletion of the CB2R has been shown to worsen outcome, while selective CB2R agonists have been demonstrated to be neuroprotective following stroke. Although initial studies of CB1R knockout mice demonstrated increased injury following stroke, indicating that activation of the CB1R was neuroprotective, later studies of selective antagonists of the CB1R also demonstrated a protective effect. Surprisingly the double knockout animals had improved outcome. Since the phenotype of the double knockout is not dramatically changed, significant changes in the contribution of other homeostatic pathways in compensation for the loss of these two important receptors may explain these apparently contradictory results.

Keywords

Stroke; cannabinoid1 receptor; cannabinoid 2 receptor; double knockouts; photo injury; transient middle artery occlusion

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INTRODUCTION

Although the number of individuals suffering from stroke in the United States and worldwide will continue to grow, therapeutic intervention for treatment following stroke remains frustratingly limited. Stroke is the third leading cause of death and the primary cause of adult disability in the industrial world[1,2]. The development of novel therapies to treat the secondary expansion of damage following stroke are desperately needed. It has been clearly demonstrated that inflammation following stroke is both a component of secondary injury and of healing[3,4]. Therefore modulation of inflammatory changes resulting from stroke represents an attractive target for treatment.

Treatment with cannabinoids has been proposed as a potential way to beneficially modify inflammatory changes following stroke[5–7]. The endocannabinoid system, comprised of the enzymes responsible for the production of endogenous cannabinoids, cannabinoid receptors, and the enzymes responsible for the degradation of the endogenous cannabinoids have all been viewed as potential therapeutic targets for the treatment of stroke[8,9]. Both the cannabinoid 1 receptor (CB1R) and the cannabinoid 2 receptor (CB2R) have been studied in relationship to stroke. The CB1R, primarily located on neurons, inhibits synaptic transmission. Endogenous cannabinoids released from the post-synaptic terminal serve as retrograde neurotransmitters for this receptor. The CB2 receptor is primarily located on cells involved in inflammatory responses[10–12]. While it is clear that there are alterations of the endocannabinoid system following stroke, and that selective agonists and antagonists of cannabinoid receptors can influence outcome following stroke, our understanding of these changes and possibilities is far from complete. Deletion of the CB2R has been shown to worsen outcome, while selective CB2R agonists have been demonstrated to be neuroprotective following stroke[5,7].

Although initial studies of CB1R knockout mice demonstrated increased injury following stroke, indicating that activation of the CB1R was neuroprotective, later studies of selective antagonists of the CB1R also demonstrated a protective effect[13–16]. As a result of conflicting reports, a better understanding of the effects of deletion of the cannabinoid receptors is needed. A complication to interpretation of results from knockout studies is the ever present problem that permanent deletion of a receptor almost invariably results in other compensatory changes, especially when involving receptors that are as important for homeostasis as the cannabinoid receptors. However, based on the current literature, it would be predicted that the effect of double CB1/CB2 receptor knockout would be as or more deleterious than either knockout alone.

In the present set of experiments, we tested the hypothesis that CB1/CB2 receptor double knockout would produce significant increases in infarct size and volume and significant decrease in clinical score. We tested this hypothesis using two mouse models of stroke, the middle cerebral artery occlusion model (MCAO) of transient ischemia/reperfusion, and the photothrombosis model of permanent ischemia.

METHODS

Animals

Male wild type and CB1 receptor/CB2 double knockout mice (CB_1 -/- CB_2 -/-) generated on a full C57Bl/6 background were used. The mice used in the photothrombotic studies were 11 weeks old and weighed 27–30 grams. The cerebral ischemia studies were conducted on 7 to 8-week-old mice weighing 18–23 grams. Wild type and CB1/CB2 receptor knockout mice were age matched littermates obtained from the Center for Substance Abuse Research (CSAR) Breeding Core at Temple University. Studies were conducted in accordance with the guidelines approved by the Institute for Animal Care and Use Committee at Temple University as well as the National Institute of Health Guidelines. Animals were maintained under a 12-h light/dark cycle on a regular chow diet and had access to food and water ad libitum before and after the procedure. The experimenters were blinded to the treatment groups throughout the experiments.

Prior to inducing stroke, the animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) (1:1) at a volume of 1 mL/kg. Prior to any intervention, an appropriate anesthetic plane was confirmed by regular respiration, lack of response to toe pinch, and lack of response to corneal touch, and anesthetized body temperature was maintained at 37°C ± 0.5°C using heating lamps and a heating pad.

Photothrombotic cerebral ischemia

The technique was carried out as described by Kleinshnitz et al [17]. Briefly, 0.1 mL of Rose Bengal (Sigma Aldrich) 10 mg/mL dissolved in 0.9% saline was injected intraperitoneally. The fur on the head was clipped and the skin and periosteum covering the right parietal bone were removed and the head secured in position. A cold light source was positioned over the exposed area, and 7 minutes after the injection of dye, the light was activated. After 20 minutes of illumination at approximately 28,000 lux, the mouse was returned to its home cage.

Middle Cerebral Artery Occlusion and Reperfusion (MCAO/R) and Cerebral Blood Flow Monitoring (CBF)

After ensuring each animal was brought to an appropriate anesthetic plane, the skin on the dorsal aspect of the head was retracted and the periosteum removed. An ink mark 2 mm poster and 4 mm lateral to Bregma was placed over the right parietal bone. Body temperature was maintained at 37°C ± 0.5°C with heating lamps and a heating pad during the period of occlusion. Middle cerebral artery occlusion (MCAO) was induced with the intraluminal filament method and slight modifications[7,14,18]. Briefly, after securing the animal in place, a midline incision was made on the ventral aspect of the neck. Using an operating microscope, the submaxillary glands were located and retracted to allow a clear visualization of the trachea and surrounding anatomy. The right external carotid artery (ECA) was identified, ligated with 6-0 silk suture, and cauterized distal to the bifurcation of the common carotid artery (CCA) into the ECA and internal carotid artery (ICA). Another 6-0 silk suture was tied around the right ICA loosely. A third 6-0 silk suture was tied around

the right ECA proximal to the point of cauterization. The vagus nerve was separated from the CCA with care taken to not damage it.

A laserPro Blood Perfusion Monitor (TSI, Inc., Shoreview, MN, USA) was employed to monitor regional cerebral blood flow (rCBF) before ischemia and during MCAO. A 1-mm diameter microfiber laser-Doppler probe was aligned so that it covered the mark previously indicated on the parietal bone. Baseline rCBF readings were collected. The second suture placed around the ICA was tightened. With a microvascular clamp, the CCA was clamped. A 30-gauge needle made a small incision in the ECA. A blunted 5-0 monofilament nylon suture coated with poly-L-lysine (0.1% in deionized water, Sigma Inc., St. Louis, MO USA) was inserted into the ECA, advanced into the Circle of Willis, and finally, to the origin of the middle cerebral artery (MCA) [19]. Slight resistance upon advancing indicated it was in the proper position. The third suture was secured around the ECA to prevent the suture from dislodging and preclude backflow. The MCAO was considered adequate if rCBF showed a sharp decrease to 25% of baseline levels [20]. After 50 minutes, the nylon suture was withdrawn and the ECA permanently tied and cauterized. Reperfusion was confirmed when pulsations were again observed in the ICA.

Neurological Evaluation

The severity of neurological deficits was evaluated 24 hours after the ischemic insult using a five point deficit score. The scale utilized the following criteria adapted from Hata: 0 = normal motor function, 1 = flexion of torso and of contralateral forelimb on lifting of the animal by the tail, 2 = circling but normal posture at rest, 3 = leaning while at rest, 4 = no spontaneous motor activity or lateral rolling[18].

Infarct Volume Assessment

Animals were euthanized with an overdose of pentobarbital (200 mg/kg intraperitoneal) 24hr after cerebral ischemia. Brains were submerged in cold PBS briefly and then cut into 6 2 mm coronal sections using a mouse brain matrix (Zivic Lab, Pittsburgh, PA, USA). The brain sections were placed in 2% triphenyltetrazolium chloride (Sigma, Inc) dissolved in saline and stained for 5 minutes at 37°C in the dark. The brain sections were fixed in 4% paraformaldehyde at 4°C for 24hr. Next, the anterior and posterior face of each section was scanned by a flatbed color scanner (Microtek Inc., Carson, CA USA). Images were saved as JPEG files and analyzed with Image-J Software (NIH). The infarct volumes were expressed as mm^3as well as the percentage of overall brain tissue after correcting for edema using the following formulas

$$\text{Infarct Fraction} = \left(\frac{\text{Infarct Volume} + \text{Contralateral Volume} - \text{Ipsilateral Volume}}{\text{Contralateral Volume}}\right) \times 100$$

$$\text{Edema} = \left(\frac{\text{Infarct Volume} - \text{Contralateral Volume}}{\text{Contralateral Volume}}\right) \times 100$$

as described in the literature [7,14,21–24].

Statistical analyses

Data were analyzed using either Student's unpaired t-tests or two-way ANOVA using Graph Pad Prism version 6.

Results

Student's unpaired t-test showed a significant decrease in infarct volume ($p = 0.0002$) and infarct fraction ($p = 0.001$), as well as a lower clinical score ($p = 0.003$) in CB1/CB2 DKOs as compared with wild type controls, indicative of improved outcome in the DKOs following photoinjury (Figure 1).

Student's unpaired t-test showed no significant decrease in infarct volume or infarct fraction, but a significant improvement of clinical score ($p = 0.008$) in CB1/CB2 DKOs as compared with wild type controls (Figures 2A-C). Furthermore, two-way ANOVA with repeated measures showed there was a significant main effect of genotype on rCBF, as flow was significantly improved in the DKOs as compared to the wild type controls [$F_{(10, 88)} = 36.25$, $p < 0.0001$]. Sidak's multiple comparisons test showed a significant different within the second 5 minutes of occlusion between wildtype and CB1/CB2 DKO mice. There was no significant main effect of time and no significant interaction (Figure 2D).

DISCUSSION

Previous reports have indicated that deletion of either the CB1R or the CB2R increased damage following stroke[7,13] The surprising finding from the current investigation was that combined deletion of both the CB1R and the CB2R in a single animal resulted in a decrease in infarct size in a model of permanent ischemia and improved recovery following stroke following both permanent and transient ischemia.

The CB1R provides for a negative feedback mechanism to inhibit synaptic transmission. Depolarization of the postsynaptic terminal results in the formation of the endogenous cannabinoid 2-AG, which then diffuses through the membrane to serve as a retrograde neurotransmitter, inhibiting the anterograde release of either excitatory or inhibitory neurotransmitters from the presynaptic terminal[8,9]. This provides an explanation for the consistent finding that antagonism or deletion of the CB1R increases brain excitotoxic injury[25–28]. However, early studies demonstrated that deletion of the CB1R in mice exacerbated injury following stroke. As the CB1R has been found to play an important role in normal neuronal development, it is therefore possible that chronic deletion of the CB1R causes neurons to be much more susceptible to injury, and that acute or conditional deletion would not have the same effect. This hypothesis is supported by the finding that administration of the selective CB1R antagonist SR141716 (rimonabant) was neuroprotective following ischemia reperfusion injury. Interpretation of these results is complicated by the fact that SR141716 may interact with other non- CB1Rs, including 5-HT_{1A} and Vanilloid VR1 receptor[6,17,29–31]. The contribution of the CB1R to secondary injury following stroke has therefore remained unclear.

The results obtained from modulation of activity of the CB2R have been more consistent than the CB1R results. The CB2R is present on numerous cells that are involved in inflammatory responses following stroke, including microglia, monocytes/macrophages, dendritic cells, neutrophils, lymphocytes and endothelial cells[32–35]. The CB2R contributes to downregulation of proinflammatory responses by all of these cells. Numerous studies have provided evidence that activation of the CB2R on endothelial cells decreases expression of adhesion molecules, reduces leukocyte rolling and adhesion, and causes tightening of the blood brain barrier[5,36,37]. These actions contribute to decreasing inflammatory cell invasion of the brain and reduce their contribution of secondary injury following stroke. We have also demonstrated that activation of the CB2R on both microglia and dendritic cells, resident inflammatory cells within the central nervous system, attenuates their production of pro-inflammatory cytokines and their contribution to the promotion of inflammation. In addition, there is significant evidence that direct activation of CB2R on peripheral inflammatory cells also decreases their proinflammatory phenotype[34]. Reports of CB2R on neurons also indicate the possibility that the CB2R is present in very restricted areas[38,39]and therefore could also play a role in neuroprotection. Interestingly, a group of investigators have reported that administration of what they describe as a CB2 inverse agonist is protective following traumatic brain injury[40,41]. While these studies demonstrated that the agent investigated (SMM-189) functions as a CB2 inverse agonist in HEK-CNG cells, the possibility remains that this agent could actually function as a selective CB2 agonist for microglial cells activated following traumatic brain injury. This, if true, would provide an explanation for the apparent conflicting results. Clearly, additional work is required to elucidate the precise mechanism(s) through which selective modulators of CB2 receptor activation provide neuroprotection.

As mentioned in the introduction, reports of single knockouts for either CB1 or CB2 receptors would lead one to predict an increase in infarct size and poorer motor performance in the double knockout animals. Surprisingly, we found that following transient ischemia, damage was not greater in the double knockout as compared with wild type controls. Furthermore, neurological function was significantly improved based on the clinical score. This may be the result of the finding that the magnitude of blood flow reduction was less during the occlusion period in the double knockout animals as compared with the wild type controls, demonstrating improved autoregulatory ability in the double KO mice following occlusion. One possible explanation of this improved flow would be vascular remodeling in the double receptor knockout mice. Unfortunately it is not possible to evaluate this using the laser Doppler method because it does not measure absolte values of flow but instead a change in flow from baseline.

Interestingly, the results of this investigation demonstrated that the double knockout mice are less susceptible to damage in the model of permanent ischemia. The photothrombosis model of permanent ischemia was chosen because of its high reproducibility of lesion size[42]. Damage is thought to be the result of the generation of free radicals following exposure of the Rose Bengal in the cerebral vasculature to light. Although platelet aggregation and coagulation may contribute to the injury with this model, it has been shown that the injury can occur even without the presence of functionally active platelets[17]. While it was not possible to use the laser Doppler technique to evaluate flow with the photothrombosis

model, flow has been found to be almost completely eliminated in the area of illumination. Therefore the smaller reduction in blood flow in the area of illumination is unlikely to be an explanation for the greater protective effect of the CB2 agonist observed with this model. However, it is possible that flow was improved in the ischemic penumbra, contributing to less expansion of the infarct. It is also possible that the smaller magnitude of injury in the permanent ischemia model afforded the possibility for greater recovery in the double knockout animals.

One concern we had with using the photothrombosis model in these experiments was the possibility that changes in bone density could interfere with light transmission to the brain[43,44]. Cannabinoids have been shown to influence bone growth in a number of studies. We therefore examined whether there was a decrease in light transmission in a small number of knockout animals (data not shown). In fact, light transmission was actually enhanced in the double knockout animals and therefore an increase in bone density cannot explain the results obtained.

Conclusion

The reason the double knockouts do not show a similar susceptibility to injury following stroke to the single knockouts of these receptors remains unclear. Both the CB1 and CB2 receptor are important contributors to homeostasis. Since the phenotype of the double knockout is not dramatically changed, there must be significant changes in the contribution of other homeostatic pathways in compensation for the loss of these two important receptors. One pathway intimately related to the endocannabinoid system is the eicosanoid system. Future investigations will explore alterations in the eicosanoid system following deletion of the cannabinoid receptors.

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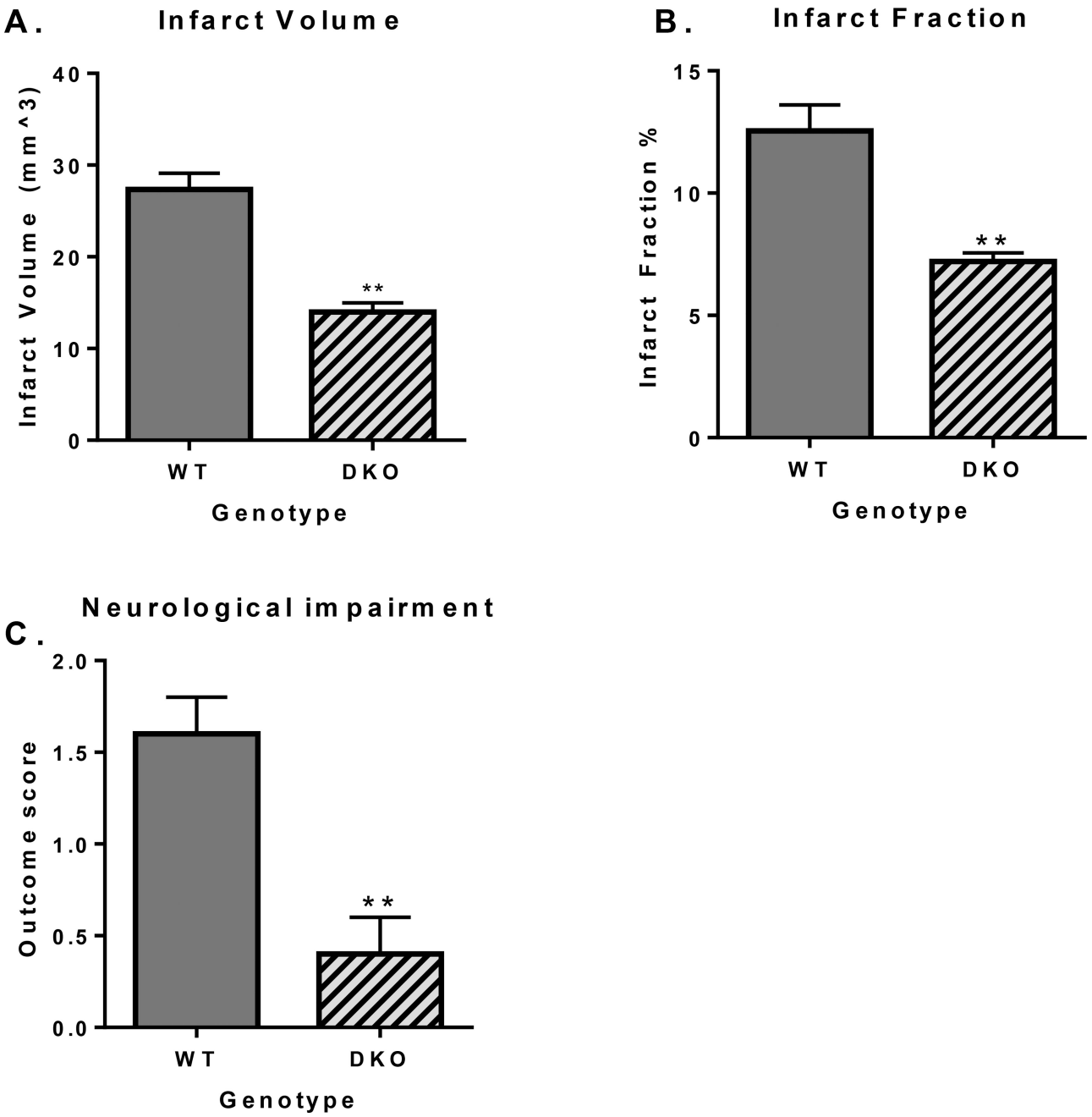

Figure 1.

Effect of CB1/CB2 double knockout on infarct volume, infarct fraction, and clinical score in a mouse model of permanent ischemia (photoinjury). A: X axis: genotype, Y axis, infarct volume in mm^3. Double knockout (DKO) mice show significantly smaller infarct volume as compared to wild type controls ($p = 0.0002$). B: X axis: genotype, Y axis, infarct fraction as a percent of the whole ipsilateral hemisphere. DKO mice show significantly smaller infarct fraction as compared to wild type controls ($p = 0.0014$). C: X axis: genotype, Y axis, outcome of the neurological assessment (a higher score indicates more severe impairment).

DKO mice show significantly improved neurological performance compared to wild type controls ($p = 0.003$).

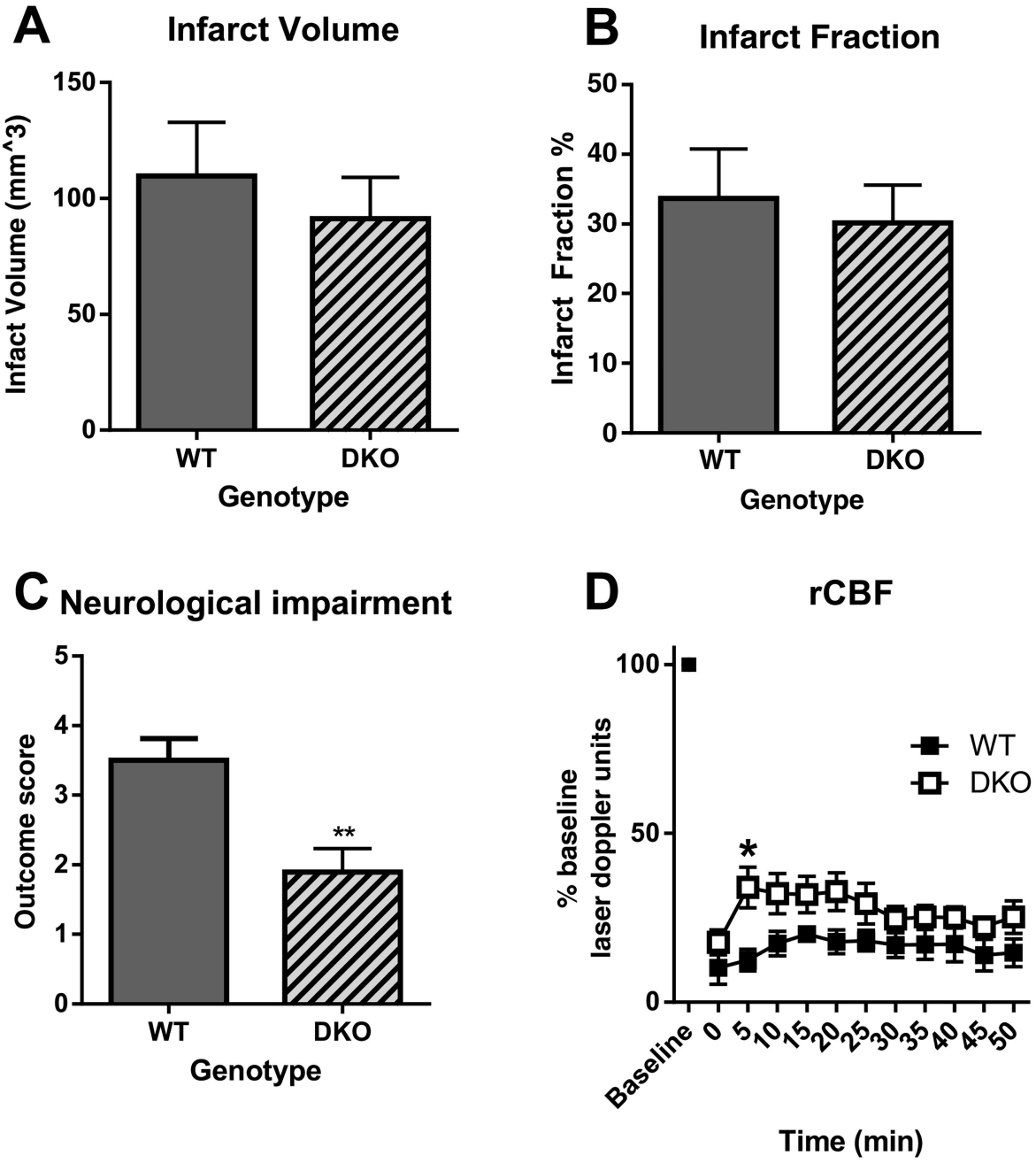

Figure 2.

Effect of CB1/CB2 double knockout on infarct volume, infarct fraction, clinical score, and regional cerebral blood flow in a mouse model of transient ischemia/reperfusion (MCAO). A: X axis: genotype, Y axis, infarct volume in mm^3. Double knockout (DKO) are not significantly different compared to wild type controls. B: X axis: genotype, Y axis, infarct fraction as a percent of the whole ipsilateral hemisphere. DKO mice are not significantly different as compared to wild type controls ($p = 0.0014$). C: X axis: genotype, Y axis, outcome of the neurological assessment (a higher score indicates more severe impairment). DKO mice show significantly improved neurological performance compared to wild type

controls ($p = 0.008$). D: X axis, time following middle cerebral artery occlusion, Y axis, % baseline flow measured in laser Doppler units. Double knockout (DKO) mice show significantly improved neurological performance compared to wild type controls (two way ANOVA significant main effect of genotype $F= 36.25$, $p < 0.0001$; Sidak's multiple comparison test shows significance within the second 5 minutes of occlusion * $p<0.05$)).