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A selective cannabinoid CB2 agonist attenuates damage and improves memory retention following stroke in mice

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Abstract

Aims—We have recently demonstrated that treatment with a cannabinoid CB2 agonist was protective in a mouse middle cerebral artery occlusion model of cerebral ischemia/reperfusion injury. The present study aimed to determine whether these protective effects of CB2 agonism would extend to a mouse photoinjury model of permanent ischemia and determine associated alterations in cognition and infarct size.

Main Methods—Mice received three injections of the CB2 selective agonist O-1966 or vehicle 1 h prior to and 2 and 5 days following induction of stroke. Infarct size was assessed at 1, 3, or 7 days post injury and learning and memory effects of injury and O-1966 treatment were assessed on days 6 and 7 using a novel object recognition task and an operant acquisition and retention procedure.

Key Findings—O-1966 treated mice had significantly smaller infarct volumes compared with vehicle treated mice. Photoinjury was also associated with a significant memory impairment on day 7 post-injury, and this deficit was reversed with O-1966 treatment. Surprisingly, sham-operated mice receiving O-1966 treatment showed a significant learning *deficit* in both the recognition and operant tasks compared with vehicle treated sham mice.

Significance—We conclude that CB2 activation is protective against cognitive deficits and tissue damage following permanent ischemia, but may dysregulate glial or neuronal function of learning and memory circuits in the absence of injury and/or inflammation.

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Keywords

Cannabinoid CB2; stroke; permanent ischemia; photoinjury; learning and memory

Introduction

Although stroke is the leading cause of morbidity and the third leading cause of death in the United States, therapeutic options other than preventive measures, such as anticoagulants, remain extremely limited (Lakhan, Kirchgeessner et al. 2009). In addition, approximately 30 – 50% of stroke survivors demonstrate cognitive impairments (Whyte et al 2011). Therefore there is a pressing need to develop effective treatment strategies not only to reduce morbidity, but also improve quality of life functions such as cognition following stroke.

Ischemic stroke models can be either transient or permanent. In the case of transient ischemia, blood flow to the ischemic area is reinstated after a fixed period of time. Numerous studies have demonstrated that the damage that occurs following transient ischemia is the result of both the ischemia and of reperfusion injury, which exacerbates the amount of damage. Previous results from our laboratory and from others have demonstrated that activation of the CB2 receptor provides protection from the secondary injury associated with inflammation. For example, we have previously demonstrated that administration of a selective cannabinoid CB2 agonist can attenuate injury in a mouse model of transient middle cerebral artery occlusion (MCAO) (Zhang, Martin et al. 2007; Zhang, Martin et al. 2008; Zhang, Adler et al. 2009; Zhang, Mahadevan et al. 2012). The protective effect of the selective CB2 agonist was found to be associated with a reduction in inflammatory responses following injury, including a reduction in the production of proinflammatory markers and a decrease in invasion by circulating inflammatory cells. The progression of injury is somewhat different in permanent models of ischemia, where the inflammation associated with reperfusion injury does not play a prominent role. We were therefore interested in determining whether modification of the endocannabinoid system via activation of the CB2 receptor would influence infarct size following permanent occlusion using the photoinjury model.

Cognitive impairment following stroke in humans is most often observed as deficits in executive function, attention, processing speed, and spatial memory. Cognitive deficits have also been reported in rodent models of transient ischemia, such as MCAO. Deficits most widely reported include those of spatial learning (radial arm maze task; Sakai et al 1996) and acquisition and retention (step-down passive avoidance; Bouet et al 2007, Hattori et al 1002). A challenge of this model, however, is that rodents that have undergone MCAO exhibit severe sensorimotor deficits which confound the evaluation of cognitive function (Bingham et al 2012). A recent study by Deiderich et al (2014) investigated the effect of the photoinjury model of permanent occlusion on sensorimotor and cognitive performance, and reported selective deficits in retention, but not acquisition, of a spatial learning task (Morris water maze). This deficit was seen in the absence of motor impairment in the spatial learning task.

The purpose of the present study was to investigate whether CB2 agonist treatment would be protective in a photoinjury model of a permanent ischemia, with a focus on characterizing cognitive function. In the present study, two cognitive learning and memory assays were employed. The novel object recognition task is a model of visual attention and short or long term memory (depending on the design) that does not require training and is not dependent on reward or punishment. The test involves both cortical and hippocampal processing. The operant learning and memory task is a model of acquisition and retention of conditioned reinforcement and involves activation of corticolimbic circuits. Based on our previous work coupled with the findings of Deiderich et al 2014, we hypothesized that photoinjury would produce cognitive deficits in the novel object recognition and operant learning and memory tasks in conjunction with a focal infarct without reperfusion. We also hypothesized that treatment with the selective CB2 agonist O-1966 would reduce infarct size while attenuating cognitive impairment in these assays.

Methods

All experiments were conducted in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at Temple University. Male C57B/6 mice (9-12 wk old males) were anesthetized by an intraperitoneal injection of a Ketamine (100 mg/ml) - Xylazine (20 mg/kg) mixture (1:1) in a volume of 1ml/kg. Body temperature was maintained at $37\pm 5^{\circ}\text{C}$ by using a heating lamp and pad. The scalp was excised over the skull and the periosteum removed. A marker was used to identify the sensorimotor cortex 2 mm posterior and 2 mm lateral to bregma. Rose Bengal (0.1 ml of 10 mg/ml) dissolved in saline was administered i.p. and five min later a cold light source was placed on the skull at the sensorimotor cortex marker and was left in place for 20 min (Watson, Dietrich et al. 1985; Lee, Park et al. 2007). A subset of mice was subjected to sham stroke in which they underwent each step of the described procedure but with the light source turned off. The CB2 agonist O-1966 (Organix, Inc. Woburn MA) was dissolved in a 1:1:18 ethanol: cremophor: saline solution. Animals were treated with the CB2 agonist O-1966 (5 mg/kg IP) or vehicle one hour prior to ischemia and again 2 and 5 days post ischemia. This dosing regimen was based on previous work with the MCAO model (Zhang et al 2009) and our pilot studies with the photoinjury model showing that this combined prophylactic/protective approach was the optimal dosing regimen to significantly decrease infarct size following ischemia.

A total of 62 mice were exposed to sham or photoinjury to determine whether these protective effects of CB2 agonism would extend to a mouse photoinjury model of permanent ischemia by determining alterations in cognition and infarct volume.

Infarct size

Thirty six mice (n=6/group) were treated with the O-1966 or vehicle regimen and euthanized on Day 1, 3 or 7 following photoinjury for assessment of infarct volume. Mice were perfused with PBS and the brains were removed and placed in PBS on ice for 20 min. The brain was sliced into 2mm coronal sections using a mouse brain matrix (Zivic lab). The brain slices were then stained with 2% Triphenyltetrazolium Chloride (TTC) (Sigma, Inc), a

redox indicator and marker of cellular respiration, dissolved in saline for 20 minutes at 37 degrees Celsius in the dark. Slices were then imaged for quantification using Image J software as described in Zhang et al 2007.

Cognitive testing

An additional twenty six mice underwent sham (n=13) or photoinjury (n=13), and mice in each group received either the CB2 agonist dosing regimen or cremophor vehicle injections in a randomized design for final n's of 6 for Vehicle/Sham, 7 for Vehicle/Stroke, 7 for O-1966/Sham, and 6 for O-1966/Stroke. On day 5 following induction of photoinjury, mice were singly housed and food restricted to increase their motivation for a palatable food reward. Novel object testing was performed in the morning on day 6 following induction of photoinjury. Acquisition and retention of a food-motivated operant task was tested in the afternoon on days 6 (acquisition) and 7 (retention). Mice were euthanized immediately following behavioral testing on day 7. Figure 1 shows a schematic of the experimental design.

Cognitive performance was assessed using a novel object recognition test and a food-motivated operant procedure. All behavioral testing was done by an experimenter blinded to the treatment of the individual animals. The novel object recognition task was executed as described previously by our laboratory (Bisen-Hersh et al 2013). On the morning of day 6, mice were individually placed into the testing cage for a 20-min acclimation period and then returned to their home cage for 30 min. Each mouse was then placed in the same testing cage for trial 1 in the presence of two novel objects. The amount of time spent exploring each object is recorded over a 5-min period. At the end of trial 1, mice were placed back into the home cage for the duration of the 1-h delay period. Following the 1-h delay, each mouse was returned to the same testing cage for trial 2, which contained one of the previous objects (a familiar object) and a novel object, placed in the same locations. The amount of time spent exploring each object was recorded over a 5-min period. A preference score for each mouse is calculated by dividing the total amount of time the animal spends exploring both objects in trial 2 by the amount of time the animal spends exploring the novel object, expressed as a percentage. Total exploration time is also used to impose an inclusion criterion of 10 s for an animal to be included in the analysis of preference for the novel object.

The operant procedure used was modified by our laboratory from the method previously described for mice by (Vanover and Barrett 1998) by removing the non-contingent dipper presentation (Walker, Foley et al. 2011). On day 6 following the novel object recognition task, mice were placed in standard mouse operant testing chambers (MedAssociates, VT) to test for performance in a food motivated operant task. The vanilla-flavored liquid nutritional drink Ensure is available under a variable-time schedule signaled by the presence of 6 s tone. A nose-poke in the center dipper receptacle is only reinforced in the presence of the tone. Learning of the task is measured by the acquisition latency, or the amount of time it takes the mouse to learn the contingency to earn 10 reinforcers. The session lasts for 2 h or until 20 reinforced nose-pokes are recorded. Two other variables are measured, non-reinforced head entries into the center dipper hole, and non-reinforced responses into two

additional nose-poke holes located to the left and right of the center hole. Analysis of these variables allows for determination of overall exploratory behavior within the testing chamber and helps to rule out potential locomotor deficits, sedative effects, or amotivational state of animals as a source of poor performance in the test. During the following afternoon of day 7, recall of the task is measured by the retention latency, which is calculated identically to the acquisition latency. The session again lasts for 2 h and non-reinforced responses are also recorded in order to assess recall of the learned task as well as overall exploratory behavior.

All data were analyzed using Two-way Analyses of Variance (ANOVAs) using GraphPad Prism 6 software. Post-tests were performed using multiple comparisons tests.

Results

Infarct size

Treatment with O-1966 led to a significant decrease in infarct volume. Two-way ANOVA revealed significant main effects of treatment [$F_{(1, 30)} = 31.5$, $p < 0.05$] and time [$F_{(2, 30)} = 28.6$, $p < 0.05$]. Tukey's multiple comparison revealed significant differences between vehicle and O-1966 treated mice at the 1 day and 7 days post stroke time points ($p < 0.05$; Figure 2a). Figure 2b shows the location of the infarct in coronal section and its attenuation following O-1966 treatment in two representative mice. The center of the lesion is located in parietal cortex area 1 (primary somatosensory cortex) at approximately -2.0 mm from Bregma, and the extent of the infarct can be observed in the coronal slices from approximately 0.20 mm rostral to Bregma to approximately -2.80 mm caudal to Bregma. The infarct spreads to the border of the corpus callosum, but is not observed in deeper tissue such as the CA1-3 fields of the hippocampus or striatum as measured with the TTC staining.

Novel object recognition

Sham animals treated with vehicle showed an average preference for exploring the novel object of 69.8% during Trial 2 (test trial, Day 6 post stroke) (Figure 3a). Interestingly, sham mice treated with O-1966 failed to show a preference and explored the familiar and novel object for nearly equal amounts of time (average 47.1%). Following stroke, mice treated with vehicle also did not show a preference for the novel object (average 55.5%), while exploration time of the novel object in stroked mice treated with O-1966 was very similar to control levels (average 72.4%). Two-way ANOVA showed a significant interaction between stroke treatment and drug treatment [$F_{(1, 15)} = 23.3$, $p < 0.05$], demonstrating that exploration of the novel object was increased in stroked mice treated with O-1966 while novel object exploration was decreased in sham mice treated with O-1966. Multiple comparisons posttests revealed a significant difference between O-1966/Sham mice vs Vehicle/Sham mice and O-1966/Sham mice vs O-1966 mice/Stroke mice. There was no main effect of stroke treatment [$F_{(1, 15)} < 1.0$, n.s.] or main effect of drug treatment [$F_{(1, 15)} = 1.63$, n.s.]. Only mice that explored the objects for greater than 10 s during Trial 2 were included in these analyses (4/6 Vehicle/Sham mice; 6/7 Vehicle/Stroke mice; 4/7 O-1966/Sham mice; 5/6 O-1966/Stroke mice). Two-way ANOVA also revealed no significant effects of stroke or drug treatment on total exploration time (Figure 3b).

Food motivated operant task

Vehicle-treated sham animals took on average approximately 1000 seconds to acquire the operant task as measured by latency to earn 10 food reinforcers. Stroked animals treated with vehicle took nearly twice as long to acquire the task (approx. 1900 seconds), and this deficit was attenuated in stroked mice treated with O-1966 (approx. 1300 seconds). Sham animals treated with O-1966 took three times as long to acquire the operant task as the sham controls (approx. 2900 seconds). Two-way ANOVA showed significant interaction between stroke treatment and drug treatment [$F_{(1, 22)} = 6.82, p < 0.05$], again demonstrating that acquisition learning was impaired in sham mice treated with O-1966 but improved in stroked mice treated with O-1966 (Figure 4a). There was no main effect of stroke treatment [$F_{(1, 22)} = 0.76, n.s.$] or main effect of drug treatment [$F_{(1, 22)} = 1.76, n.s.$]. Total head entries into the dipper hole is depicted in Figure 4b. There was a significant main effect of increased non-reinforced head entries in stroked mice versus shams [$F_{(1, 22)} = 5.20, p < 0.05$]. Head entries into two inactive nose poke holes located on either side of the center dipper hole are depicted in Figure 4c. There was also a significant main effect of increased non-reinforced responding in these side holes in stroked mice versus shams [$F_{(1, 22)} = 5.12, p < 0.05$], revealing that while stroked animals showed no motor or motivational impairment in their ability to respond in the operant chambers, their accuracy in responses directed toward the reinforced manipulandum during the reinforced time period is impaired.

On Day 7, sham animals treated with vehicle earned 10 reinforcers in approximately half the time as it took them on Day 6, showing retention of the food-motivated operant task, and this time the performance was not altered in the sham mice treated with O-1966 (Figure 4d). Stroked animals treated with vehicle retained the task, but their Day 7 latency was double that of the sham controls. Stroke mice treated with O-1966 improved performance from Day 6 to Day 7 and took approximately the same amount of time to earn 10 reinforcers on Day 7 as the sham controls. Two-way ANOVA showed significant main effect of stroke [$F_{(1, 21)} = 17.13, p < 0.05$] and significant interaction between stroke treatment and drug treatment [$F_{(1, 21)} = 12.81, p < 0.05$], demonstrating that stroke impaired retention of the operant task and that this impairment was attenuated by O-1966 treatment. Multiple comparisons posttest revealed that Vehicle/Stroke treated mice performed significantly poorer on the retention task compared with Vehicle/Sham controls, while no other group was statistically different. On Day 7 there were no significant effects of stroke or drug treatment on food seeking or non-reinforced behaviors in the nose poke holes (Figures 4e, f).

Discussion

Despite the high incidence of cognitive impairment in stroke survivors, there is no specific treatment for this complication. Evaluation of post-stroke cognitive deficits in animal models and associated preclinical evaluation of candidate drugs has been limited, but in general impairments are seen in executive function, working memory, spatial memory, and information processing speed. The present set of experiments used the photoinjury model of permanent ischemia to test the efficacy of CB2 agonist treatment in attenuating tissue damage following stroke as well as associated cognitive deficits. Treatment with the CB2 selective agonist O-1966 resulted in a reduction in infarct size at each time point observed,

supporting our previous findings with the MCAO model and demonstrating that activation of the CB2 receptor is protective from tissue damage even in the absence of reperfusion injury. The present results also demonstrated that the photoinjury model was associated with impaired retention of a food-motivated operant learning task, and that O-1966 was protective against deficits in retention. In addition, intriguing effects of CB2 receptor agonism on decreasing novel object recognition and acquisition learning in non-stroked animals were observed.

We did not observe a significant effect of photoinjury on novel object recognition. Others have reported deficits in this task following other stroke models but we are unaware of any reports on this task following photoinjury. Our novel object recognition study may also have been underpowered due to the fact that some animals in each treatment group had to be excluded because they did not reach the exploration criterion on the test session. A significant interaction was observed however, meaning that performance in stroked animals was increased following O-1966 treatment while performance decreased in sham animals treated with O-1966.

The mouse photoinjury model of permanent ischemia was associated with deficits in retention of a food-motivated operant task and in accuracy in responding in this paradigm. We observed a trend toward impaired acquisition of the task but the effect was not statistically significant. Similarly, a recent report by Diederich et al 2014 demonstrated that photoinjury was selectively deleterious to recall, and not acquisition learning, in the Morris water maze in rats. Importantly, as was also reported in the Diederich study, our observed deficits were not associated with an overall motor impairment. We observed that exploratory behavior and motivation to perform in the operant chamber were not impacted by photoinjury, in that stroked mice explored the center dipper receptacle and non-reinforced adjacent nose poke holes as frequently as sham controls during both the acquisition and retention session. In fact, during the acquisition session there was a significant main effect of stroke on increasing head entries into all holes, suggesting that while the stroked mice responded as much as sham controls, they were not associating reinforcer delivery to the correct response criterion (center receptacle entries in the presence of the tone), an effect selective to learning deficits and not motor or motivational impairment.

Furthermore, we observed that treatment with O-1966 beginning 1 h prior to stroke induction and again on days 2 and 5 following stroke significantly reversed the retention deficit in the operant responding task. The operant responding task is an example of instrumental/reinforcement learning and involves frontal cortical and many subcortical brain regions, including the hippocampus, ventral tegmental area, nucleus accumbens, and amygdala (Cardinal, Parkinson et al. 2002; Bisen-Hersh, Hineine et al. 2013). These brain regions were not primary sites of damage induced by the stroke, as seen by their ability to take up the TTC staining. However, the fact that the stroked animals still showed significant impairment of retention of the operant task suggests that secondary damage caused by inflammatory sequelae impact the integrity of the circuits involved in this cognitive task. Future exploration of the extent of inflammation stemming from the primary infarct into neighboring regions relevant to learning and memory, as well as the impact of infarct on neurobiological processes such as long term potentiation are warranted.

These results supported our hypothesis that a permanent ischemic insult to the mouse brain would lead to measurable deficits in cognitive performance. Furthermore, the selective CB2 agonist O-1966 with already demonstrated anti-inflammatory and neuroprotective properties was capable of attenuating the insult and consequent memory impairment. What was unexpected was the effect of O-1966 administration on learning and memory performance in the sham-operated mice. In the novel object recognition task and to an even greater extent in the acquisition of the food motivated task, O-1966 treatment significantly impaired performance in sham mice. However, the same treatment regimen reversed cognitive deficits observed in the stroked animals, especially evident during retention of the food motivated task. These findings bring up two areas of current interest: 1) what is the localization and function (if any) of neuronal CB2 receptors (for review on this topic see (Atwood and Mackie 2010); 2) what is the function of glia (which do express CB2 receptors) in learning and memory processes (for review see (Marin and Kipnis 2013)). The present data suggest that if neurons do express the CB2 receptor within brain regions involved in learning and memory, that activation of such receptors may be detrimental to normal neuronal function during these processes. Alternately, it may be that glial CB2 receptor activation suppresses normal functioning of microglia and/or astrocytes, which are increasingly being recognized for playing important roles in synaptic events critical to learning and memory, such as synaptic remodeling and neurogenesis (for review see Paolicelli et al 2014, Wake et al 2013.)

Conclusion

In conclusion, although we and others have previously demonstrated that administration of a selective CB2 agonist decreases infarct size following ischemia/reperfusion injury, the results of the current investigation demonstrates that administration of a selective CB2 agonist is also protective not only from tissue damage but from cognitive impairment as well as a consequence of permanent ischemia. The results of this study suggest that while activation of CB2 receptors is protective against CNS injury and associated behavioral deficits, CB2 receptor activation in the uninjured brain also appears to influence neuronal and/or glial activity that may negatively impact normal function such as cognition.

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Figure 1. Experimental Design

Arrows above the timeline show the treatment regimen for both the infarct size and cognition studies. Mice are treated with 5.0 mg/kg O-1966 or vehicle IP 1 hr, 2 days, and 5 days post photoinjury or sham surgery. Separate groups of mice in the infarct study were sacrificed at three timepoints for TTC staining and quantification of infarct size. Mice in the cognitive study were tested on day 6 post infarct in the novel object recognition task and on days 6 and 7 in the operant learning and memory task.

Figure 2. Effect of O-1966 treatment on infarct size in mice

A) O-1966 administration significantly decreased infarct volume. Mice sacrificed at 24 h received one dose of O-1966 1 h prior to photoinjury. Those sacrificed on day 3 received an additional dose on day 2 and those sacrificed at day 7 received another dose on day 5. Two-way ANOVA revealed significant effect of treatment and time, and multiple comparisons showed significance at the 1 day and 7 day post-injury timepoints. **(B).** In representative vehicle- and O-1966 treated subjects sacrificed on Day 1 or Day 7 post stroke, the extent of the infarct at approx. -2.80 mm caudal to Bregma is shown. In the vehicle-treated mice, the infarct spreads to the border of the corpus callosum but is not observed in deeper tissue such as the CA1-3 fields of the hippocampus or striatum. The infarcts of representative O-1966 treated subjects are smaller by comparison. When TTC is taken up by living cells its metabolite turns the cell red. As a result, viable tissue is stained red while dead tissue is left unstained.

Figure 3. Effect of stroke and/or the CB2 agonist O-1966 on novel object recognition in mice O-1966 or vehicle was administered 1 h prior to stroke or sham surgery, and again 2 and 5 days post stroke. **A)** The # symbol indicates a significant interaction between stroke treatment and drug treatment, $p < 0.05$. The * symbol indicates that multiple comparisons posttest revealed a significant difference between O-1966/Sham mice vs Vehicle/Sham mice and O-1966/Sham mice vs O-1966 mice/Stroke mice. **B)** There was no effect of stroke treatment or drug treatment on the total exploration time on the test day. Mice were excluded from the data analysis if they spent less than 10 s exploring both objects.

Figure 4. Effect of stroke and/or CB2 agonist O-1966 on acquisition and retention of a food-motivated operant task

The # symbol indicates significant main effects of stroke or of O-1966 treatment or significant interactions between the two using two-way ANOVA. The * symbol indicates that a multiple comparisons posttest revealed significance between individual groups. **A, D**) There was a significant interaction between stroke and O-1966 treatment on acquisition and retention of the task ($\#p < 0.05$). There was also a significant main effect of stroke on retention, $p < 0.05$. Multiple comparisons posttest revealed that the Vehicle/Stroke group was the only group statistically different from Vehicle/Sham. **B, E**) There was a significant effect of stroke on head entries into the dipper receptacle on Day 6 (**B**) but not on Day 7 (**E**). **C, F**) Stroke treatment caused a significant increase in responding in the non-reinforced nose poke holes on Day 6 (**C**) but not on Day 7 (**F**).