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CB₂ receptor activation attenuates microcirculatory dysfunction during cerebral ischemic/reperfusion injury

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Abstract

Previous studies from our laboratory indicated that selective cannabinoid CB₂ agonists were able to attenuate cerebral ischemia/reperfusion (I/R) injury. The goal of current study is to further test whether this attenuation involves cerebral microcirculatory function during I/R injury. Middle cerebral artery occlusion with reperfusion (MCAO/R) was performed in male mice. A selective CB₂ agonist was administered at different dosages and different times. Cerebral infarction volume, neurological function and cerebral microcirculatory function (leukocyte/endothelial interactions,

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cell adhesion molecule expression and blood-brain barrier disruption) were examined *in vivo* and *in vitro*. CB₂ knockout mice were subjected to MCAO/R following same procedures. Administration of the CB₂ agonist at middle dosage exerted optimal effects in reducing cerebral infarction and improving neurological function compared with other dosage groups and control group. Treatment with the CB₂ agonist at the optimal dose was still effective when given 3 h after MCAO. Transient ischemia significantly increased leukocyte/endothelial interactions, adhesion molecules expression and blood-brain barrier disruption which were all attenuated by pre-treatment with a CB₂ agonist. CB₂ knockout mice showed larger cerebral infarction and worse neurological function compared to wide type. In conclusion, CB₂ activation contributed to protecting the brain through the attenuation of cerebral microcirculatory dysfunction during cerebral I/R injury.

Keywords

Cerebral ischemia; Leukocyte/endothelial interactions; Adhesion molecules; Blood-brain barrier disruption; Cannabinoids; CB₂ receptor

Introduction

Cannabinoids, including the synthetic analogs of natural cannabis, have been found to have neuro-modulatory and immuno-modulatory properties in both *in vivo* and *in vitro* studies (Klein et al., 2001). There are two cloned cannabinoid receptors, designated CB₁ and CB₂. The CB₁ receptor is primarily expressed in the central nervous system (CNS), exhibiting a presynaptic location and playing a prominent role in synaptic neurotransmission and glia differentiation. The CB₂ receptor is expressed predominantly by cells of the immune system, such as lymphocytes, neutrophils and macrophages (Pazos et al., 2005; Rodriguez de Fonseca et al., 2005). Recently, it has been shown that CB₂ is also found in residing inflammatory cells within the CNS such as microglia (Maresz et al., 2005). CB₂ stimulation has been shown to have immuno-modulatory properties, such as decreasing antigen presenting cell (APC) activity and down-regulation of inflammatory cytokine (IFN- γ and TNF- α) production during inflammatory responses (Klein and Gabral, 2006; Lombard et al., 2007). A number of investigations have shown that CB₂ receptor activation has anti-inflammatory therapeutic potential in CNS diseases, such as multiple sclerosis, traumatic brain injury and Alzheimer's disease (Mauler et al., 2003; Ni et al., 2004; Ramirez et al., 2005).

Because of the involvement of synaptic neurotransmission and neuroinflammation, the cannabinoid receptors have been tested as targets for the modulation of the cerebral ischemic injury (Hayakawa et al., 2004; Muthian et al., 2004; Nagayama et al., 1999; Parmentier-Batteur et al. 2002; Zhang et al., 2008). Our laboratory first reported that selective activation of the CB₂ receptor attenuated cerebral ischemia/reperfusion injury (Zhang et al., 2007). In the current investigation, further studies were performed to delineate the dose and time dependent responses of CB₂ activation during cerebral ischemia and explore the mechanisms responsible for their neuroprotective effects. Furthermore, CB₂ receptor knockout mice were utilized to determine the role of the CB₂ receptor in cerebral ischemic injury.

It has been clearly demonstrated that microcirculatory dysfunction, including leukocyte rolling and adhesion on cerebral micro-vasculature, blood-brain barrier disruption and cerebral edema play important roles in secondary injury following ischemia and eventually exacerbate neurological deficits (Heinel et al., 1994; Ritter et al., 2000). In the current

investigation we examined the hypothesis that CB₂ activation attenuates microcirculatory dysfunction following cerebral ischemia/reperfusion injury.

Materials and methods

Animals

The cerebral ischemia/reperfusion studies were carried out on 8 week old male C57BL/6 mice from Taconic Farms (Hudson, NY). CB₂ receptor knockout mice (strain name: B6.129P2-Cnr^{2tm1Dgen}/J) and wide type mice were obtained from the Jackson lab (Bar Harbor, MI). All experiments were conducted in accordance with the guidelines approved by Institutional Animal Care and Use Committee at Temple University.

Middle cerebral artery occlusion and reperfusion (MCAO/R)

The animals were anesthetized with an intraperitoneal injection of a Ketamine (100 mg/ml)–Xylazine (20 mg/kg) mixture (1:1) at a dose of 1 ml/kg. Body temperature was maintained at 37±5 °C by a heating lamp and heating pad. Middle cerebral artery occlusion was achieved by the intraluminal filament methods (Hata et al., 1998). Briefly, a midline neck incision was made using an operation microscope; the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were isolated. The ECA was ligated with 6–0 silk suture distal from the ICA-ECA branch and then cut distal from ligated point. Another 6–0 silk suture was tied loosely around ECA close to the origin at the CCA. A blunted 5–0 monofilament nylon suture coated with poly-L-lysine (0.1% in deionized water, Sigma Inc, St Louis MO) was introduced from a small incision on ECA and then advanced into the circle of Willis, and finally to the origin of the middle cerebral artery. The silk suture around the ECA stump was tied tightly to prevent bleeding and secure the nylon suture. The nylon suture was removed after 60 min of occlusion and ECA was permanently tied. Reperfusion was confirmed when pulsations were again observed in the ICA. The same surgical procedures were performed on sham animals without occlusions of the middle cerebral artery.

A laserPro Blood Perfusion Monitor (TSI Inc, Shoreview, MN, USA) was used to monitor regional cerebral blood flow (rCBF) prior to ischemia, during MCAO and during reperfusion. A 1 mm diameter microfiber laser-Doppler probe was attached to the skull 4 mm lateral and 2 mm posterior of bregma. The MCAO was considered adequate if rCBF showed a sharp drop to 25% of baseline (pre-ischemia) level, otherwise, animals were excluded.

Cranial windows

On the day of cranial window implantation, the animals were anesthetized with an intraperitoneal injection of Ketamine (100 mg/ml)–Xylazine (20 mg/kg) mixture (1:1) at a dose of 1 ml/kg. The head was shaved and positioned in a stereotactic head holder. A 1 cm area of skin on the dorsal surface of the skull over the right cortical hemisphere was excised and the periosteum was removed. A 4 mm diameter circular craniotomy was performed using a high speed drill (Champ-Air Dental Drill Benco Dental) over the right parietal cortex extending from attachment of the temporal muscle to the midpoint of the sagittal suture in the coronal direction and aligned to the middle of the sagittal suture, so that the window contained terminal branches of the middle cerebral artery. Normal saline was dripped over the cranium to avoid thermal injury to the cortex. A 5 mm diameter coverglass was then placed over the exposed brain, and an airtight seal was produced using Nexaband Quick Gel. The coverglass provided adequate mechanical protection from infection or contamination. A recovery period of four days was allowed between implantation of the cranial window and the induction of transient focal ischemia (Ni et al., 2004).

Injection of the CB₂ agonist

The CB₂ agonist O-1966 (Wiley et al., 2002; Zhang et al., 2007) was obtained from BTG Inc (London, UK) and was dissolved in a pure ethanol: emulphor: saline mixed solution at 1:1:18. In dose response experiments, the CB₂ agonist was given at doses of 1 mg/kg, 5 mg/kg, and 10 mg/kg as intraperitoneal injections 1 h before MCAO; an equal volume of vehicle was administered at same time in control animals. In the time of administration experiments, the CB₂ agonist was administered 1 h before MCAO, 1 h after MCAO or 3 h after MCAO using the optimal dosage obtained from the dose response experiment (5 mg/kg).

Intravital microscopy and measurement of leukocyte/endothelial interactions

The animals were anesthetized and immobilized on a plexiglass stage at 24 h after MCAO. Intravital microscopy was performed with an epi-illuminescence microscope (BX10, Olympus, Japan) which was equipped with a Digital Camera (Cooke 1600, Cooke Corporation, Romulus, Michigan). Leukocytes were stained *in vivo* by a bolus injection of 0.05 ml of a 0.01% solution of the fluorescent dye Rhodamine 6G (Sigma Inc, St Louis, MO) into the facial vein (Levene et al., 2007). The light leaving the lamp housing was filtered to allow light with a peak wavelength of 605 nm to be transmitted to tissue. Excitation of fluorescent dyes in the leukocytes caused a shift in the wavelength of the emitted light. Selective filtering allowed visualization of the fluorescent cells on a dark background. The image from the camera was then displayed on the computer monitor, captured and recorded by Camfire software at a video frame rate of 25 frames/s. The interactions between leukocytes and endothelium were investigated offline.

Three venules (with diameter 30–40 μ m) in each animal were assessed. The number of rolling leukocytes was considered to be the total number of leukocytes moving along the endothelial cells at substantially slower velocity compared with the midstream blood cell velocity. They were counted when they passed an arbitrary line perpendicular to the longitudinal axis of the vessel over a period of 30 s. Adhering leukocytes were defined as the total number of the leukocytes firmly attached to the microvascular endothelium that did not change their location during the entire 30 s of observation period. Adhering leukocytes were scored as the number of cells per mm² of the vascular surface area, calculated from the diameter and standardized length (100 μ m) of the vessel segment under investigation.

Infarct volume and cerebral edema assessment

The animals were euthanized with an overdose of pentobarbital (200 mg/kg *i.p.*) 24 h after MCAO and the brains were removed, and chilled on ice for 10 min to slightly harden the tissue. Five 2 mm coronal sections were cut using a mouse brain matrix (Zivic lab, Pittsburgh, PA, USA). The brain sections were placed in 2% triphenyltetrazolium chloride (TTC) (Sigma Inc, St Louis, MO, USA) dissolved in saline and stained for 20 min at 37 °C in the dark. The brain sections were then fixed in 4% paraformaldehyde at 4 °C for 24 h and the anterior and caudal face of each section was scanned by a flatbed color scanner (Microtek Inc, Carson, CA, USA). The resulting images were captured as JPEG files and analyzed with NIH image software. The infarct volumes were corrected for brain edema/swelling: the hemispheric infarct volume in each section was calculated by subtracting the area of normal TTC stained tissue in the hemisphere ipsilateral to the ligation, from the contralateral non-ischemic area to generate the infarct fraction (%) as described by Swanson et al. (1990) and Lin et al. (1993); cerebral edema was determined by the percent increase of the ipsilateral/contralateral area (Vannucci et al., 2001).

Neurological deficit score and Rota rod test

The severity of neurological deficits was evaluated 24 h after ischemic insult using a five-point deficit score (0 = normal motor function; 1 = flexion of torso and of contralateral forelimb upon lifting of the animal by tail; 2 = circling to the contralateral side but normal posture at rest; 3 = leaning to contralateral side at rest; and 4 = no spontaneous motor activity) (Hata et al., 1998). Rota rod was used to evaluate the motor skills 24 h after MCAO (Gupta et al., 2002): animals were trained on Rota rod at speed of 10 rpm before MCAO and tested 24 h after MCAO at same speed. The running time on Rota rod was recorded automatically.

Immunofluorescent staining

Brain tissues from mice subjected to 1 h MCAO and 23 h reperfusion were stored in 30% sucrose in 4 °C. Tissues were then placed in the center of a chuck covered with O.C.T and quickly frozen in -20 °C then cut into 10 mm thickness slide by an IEC Cryotomy Microtome. Brain tissue sections from MCAO model were air-dried, blocked with 5% BSA for 30 min, washed twice by Tris Buffered Saline (TBS) and then incubated with FITC-conjugated anti mouse Mac-1 or FITC-conjugated anti mouse ICAM-1 in the dark. After incubation for 45 min, the slides were washed twice in TBS and gently washed by tap water for 15 s (Gonzalez-Rey et al., 2006). The slides were dried and mounted in permount. An Olympus BX10 fluorescent microscope with a FITC filter (excitation: 488 nm; emission: 520 nm) equipped with a Cooke 1600 monochrome digital camera was used to visualize and record the slide images. The mean fluorescent intensity (M.F.I) in the ischemic areas and the same sites in the non-ischemic hemisphere were measured by Image J software. The M.F.I in non-ischemic area was used as background to eliminate non specific binding and the results were shown as mean fluorescent intensity ratio changes of ischemic areas to the non-ischemic hemisphere.

Quantitative evaluation of Evans blue extravasation

Blood-brain barrier disruption was assessed by quantitatively measuring Evans blue extravasation using the technique described by Belayev et al. (1996) with some modifications. Briefly, 24 h after MCAO, mice were intravenously injected with 0.1 ml 2% Evans blue and allowed to circulate for 60 min. Animals were perfused transcardially with 0.9% saline to remove intravascular EB dye. The ischemic and non-ischemic hemispheres were dissected and weighed. The hemispheres were homogenized in 500 µl 0.1 M PBS and 500 µl of TCA (trichloroacetic acid 100%). The samples were incubated at 4 °C for at least 1 h and centrifuged at 10,000 g for 30 min. The resulting supernatants were measured for absorbance of EB at 610 nm using a spectrophotometer.

Statistical analysis

Bonferroni's test after one-way analysis of variance (ANOVA) was used for analyzing differences in infarct fraction, cerebral edema, neurological score, Rota rod running time and Evans blue extravasation. Leukocyte/endothelial interactions and adhesion molecules expression data were analyzed by one-way ANOVA followed by Bonferroni's test. Student's *t*-test was used to evaluate the difference of cerebral infarction and neurological score in CB₂^{-/-} and wild type mice. Data were presented as means±SEM. A statistically significant difference was assumed at *p*<0.05.

Results

Dose response experiments

One hour pre-MCAO administration of the CB₂ agonist (O-1966) at a dosage of 1 mg/kg significantly decreased infarct fraction ($14.3 \pm 1.8\%$) compared to control group ($25.1 \pm 2.4\%$); the dose of 5 mg/kg exerted much more protection, decreasing infarct fraction to ($5.4 \pm 1.5\%$); while the dose of 10 mg/kg did not have a significant neuroprotective effect ($21.4 \pm 3.9\%$). Only the dose of 5 mg/kg of CB₂ agonist significantly decreased cerebral edema ($5.7 \pm 1.8\%$) compared to the vehicle treated control group ($10.6 \pm 1.5\%$). The neurological deficit score and Rota rod test results showed the same trend as the cerebral infarction data: both the doses of 1 mg/kg and 5 mg/kg improved neurological and motor function, and 5 mg/kg showed the best neuroprotective effects (Fig. 1).

Time of administration experiments

The CB₂ agonist (O-1966) was administered at 5 mg/kg (optimal dosage obtained from above experiments) 1 h before MCAO (1 h pre-treatment), 1 h after MCAO (1 h post-treatment) or 3 h after MCAO (3 h post-treatment). The results indicated that all treatment groups showed significantly decreased cerebral infarction compared to vehicle treated control group. Both 1 h pre-treatment and 1 h post-treatment decreased cerebral edema and improved neurological and motor function, but there was no statistical significance between 3 h post-treatment group and control in edema formation, although there was a trend for improvement (Fig. 2).

Effects of the CB₂ agonist on leukocyte/endothelial interactions during MCAO/R

Treatment with CB₂ agonist (O-1966) at 5 mg/kg 1 h prior to MCAO attenuated leukocyte/endothelial interactions during cerebral ischemia/reperfusion injury. Mice subjected to MCAO/R shown tremendously increased numbers of leukocytes rolling (9.3 ± 0.9) and adhering (144 ± 40) on venules compared to sham mice (3.2 ± 0.33) and (32 ± 12), while CB₂ agonist treatment significantly decreased leukocyte rolling (6.2 ± 0.4) and adhesion (72 ± 20) 24 h after cerebral ischemia (Fig. 3).

Effects of the CB₂ agonist on adhesion molecule expression after MCAO/R

In concurrence with the leukocyte/endothelial interaction results, treatment with the CB₂ agonist (O-1966) at 5 mg/kg 1 h prior to MCAO attenuated both Mac-1 and ICAM-1 expression after cerebral ischemia/reperfusion injury. Mice subjected to MCAO/R showed tremendously increased expression of both Mac-1 and ICAM-1 compared to sham mice, while the CB₂ agonist significantly decreased the Mac-1 M.F.I ratio change (1.45 ± 0.1 vs 2.04 ± 0.15) and ICAM-1 M.F.I ratio change (1.6 ± 0.13 vs 2.4 ± 0.14) compared to vehicle the treated control group at 24 h after cerebral ischemia (Fig. 4).

Effects of the CB₂ agonist on blood-brain barrier disruption after MCAO/R

Focal cerebral ischemia significantly increased Evans blue extravasation in the ischemic hemisphere of vehicle treated control mice (3956 ± 331 ng/g brain) compared to sham animals (1407 ± 147 ng/g brain). Treatment with CB₂ agonist O-1966 at 5 mg/kg 1 h prior to MCAO significantly attenuated Evans blue extravasation in ischemic hemisphere (1834 ± 309 ng/g brain) compared to the vehicle treated control group (3956 ± 331 ng/g brain). The results also indicated that the blood-brain barrier was not altered in the non-ischemic hemisphere in either vehicle treated control mice (1403 ± 338 ng/g brain) or O-1966 treated mice (1151 ± 173 ng/g brain) when compared to sham mice (1407 ± 147 ng/g brain) (Fig. 5).

Effects of CB₂ receptor deficiency in MCAO/R

As would be expected from the data demonstrating a protective effect of CB₂ receptor activation, CB₂ receptor knockout mice showed a larger cerebral infarct fraction (32.6% $\pm$ 2.9%) compared to wild type mice (23.4% $\pm$ 2.6%) as well as poorer neurological function after MCAO (Fig. 6).

Discussion

The primary goal of this study was to further elucidate the mechanisms through which selective CB₂ cannabinoid receptor agonists provide protection for the brain following cerebral ischemia/reperfusion injury in a mouse MCAO/R model.

We had previously demonstrated that O-1966, a selective CB₂ agonist, administered at a dose of 1 mg/kg reduced infarct size following ischemia/reperfusion injury. In the current study we examined the dose response relationship for the neuroprotective effect of O-1966 following stroke. The results showed a bell shaped dose dependent response effects following cerebral ischemia. The low dose of CB₂ agonist (1 mg/kg) decreased cerebral infarction and improved neurological function while the optimal effects were achieved by using middle dose (5 mg/kg). However, the high dose (10 mg/kg) lost its neuroprotective effects after cerebral ischemia. The bell shaped dose response effects of cannabinoids have been commonly described in various studies (Darmani et al., 2003; Steffens et al., 2003). This phenomenon could be due to the cross talk with other receptors in the brain with high doses of the cannabinoids (Ferraro et al., 2001; Pegorini et al., 2006). However, the exact mechanism for bell shaped dose response curves is still unclear, seems to depend on the models used, and needs to be elucidated.

By using the optimal dosage chosen from the above experiments, mice subjected to MCAO were treated with O-1966 at different times to evaluate the therapeutic window following cerebral ischemia. We found that O-1966 given 1 h before ischemia, 1 h after ischemia or even as late as 3 h after ischemia was still effective in reducing cerebral infarction. Since as hypothesized, CB₂ activation exerts its neuroprotection through anti-inflammatory responses by inhibition of inflammatory cell invasion after stroke, it is not surprising that the effects of 3 h post-ischemia treatment were not as strong as pre-ischemia treatment. It is very likely that the CB₂ agonist lost some of its protective effect when administered after inflammatory cells had already invaded brain parenchyma and caused the secondary neuronal damage. Since the inflammatory cells invading the brain during this period of time are almost exclusively neutrophils, these results indicate a significant effect of the CB₂ agonist on neutrophil invasion of the brain. This is, to the best of our knowledge, the first *in vivo* demonstration of a CB₂ agonist influencing neutrophil function.

In parallel with cerebral infarction data, the CB₂ agonist demonstrated a bell shaped dose response effect on neurological function: mice receiving a dose at 5 mg/kg showed the least neurological deficits after stroke. Although the neurological deficit score scale is widely used in the mouse model of cerebral ischemic injury research, other more specific evaluations of motor function are also useful. Therefore, a Rota rod evaluation was introduced as a more accurate and objective test to evaluate the muscle strength, balance and coordination skills (Gupta et al., 2002). The running time on Rota rod correlated with the neurological function data but demonstrated a much clearer bell shaped dose dependent trend and reflected more significant difference.

The possible mechanisms through which CB₂ agonist might modulate cerebral I/R injury have been investigated in the current study. We have previously reported that administration of a CB₂ agonist reduced leukocyte/endothelial interactions in the pial circulation during

cerebral I/R injury (Zhang et al., 2007). To further evaluate the mechanisms responsible for this effect we examined how CB₂ agonist administration influenced adhesion molecule expression. After cerebral ischemia, microvascular endothelium is activated and endothelial cell adhesion molecules such as ICAM-1 are induced by pro-inflammatory cytokines such as TNF- α (Yang et al., 1998). Activated leukocytes also express integrins (such as Mac-1) which bind to ICAM-1 on endothelial cells and mediate leukocyte firm adhesion. Subsequent to adhesion, leukocytes transmigrate through endothelial cells into brain parenchyma (Kataoka et al., 2004). Leukocytes activation and migration have been implicated as primary contributors to ischemia/reperfusion injury (Heinel et al., 1994; Ritter et al., 2000). In addition to their role in physical obstruction of capillaries, they participate in inflammatory responses and cause brain tissue damage. Pro-inflammatory cytokines (TNF- α and IL-1 β) secreted by leukocytes not only activate vascular endothelial cells and amplify inflammatory response but also directly induce neuronal injury (Wood, 2003). Matrix metalloproteases (MMPs), secreted by macrophages, can degrade constituents of basal lamina thus contribute to blood-brain-barrier disruption and cerebral edema during stroke (Gasche et al., 2001). All of these studies highlight the involvement of leukocytes in exacerbating ischemic injury, and the protection offered by interfering with the ability of leukocytes adhesion to endothelial cells and diapedesis into the brain. By using the optimal dosage, we confirmed our previous finding that CB₂ activation decreased leukocyte/endothelial interactions in post-capillary venules *in vivo*. Consistent with the results from other laboratories (Kishimoto and Rothlein, 1994; Lindsberg et al., 1996), we found that cerebral ischemia tremendously increased adhesion molecules expression on both leukocytes (Mac-1) and endothelial cells (ICAM-1), and this increase was attenuated by treatment with the CB₂ agonist. It has also been shown in previous studies that selective CB₂ stimulation could modulate leukocyte trafficking in various disease models and *in vitro* studies (Rajesh et al., 2007a). CB₂ activation could have inhibited this cell trafficking through a direct effect on endothelial cells (Golech et al., 2004), leukocytes and/or inhibition of chemokines/cytokines (Ghosh et al., 2006; Rajesh et al., 2007b) and/or endothelial cells. As discussed previously, invading leukocytes could damage blood-brain-barrier by secreting MMPs leading to degradation of the basement membrane and further exacerbate microcirculatory dysfunction (Gasche et al., 2001). The break down of BBB allows additional influx of neuro-toxic mediators into the brain parenchyma and induces vasogenic cerebral edema which further deteriorates stroke outcomes. In this study, we found that selective CB₂ agonist significantly attenuated blood-brain barrier disruption after cerebral I/R injury which is accompanied with reduction of cerebral edema and improved neurological function. Therefore, our results strongly imply that CB₂ stimulation protect brain against cerebral ischemic injury through improving microcirculatory function.

In addition to the exogenous CB₂ agonists, the effects of endogenous cannabinoids, which are produced inside body and serve as natural ligands for the cannabinoid receptors, have also been explored in cerebral ischemic research. CB₁ knockout mice have been utilized in previous investigations of cerebral ischemic injury, with some studies implicating a protective effect for the CB₁ receptor (Parmentier-Batteur et al., 2002). However, there are also conflicting results in the literature (Muthian et al., 2004; Zhang et al., 2008), which indicated CB₁ blockade was neuroprotective. When interpreting these results it must be recognized that embryonic deletion of CB₁ receptor may lead to abnormal CNS development and differentiation, making these animals more susceptible to ischemia and may not reflect the actual contributions of the CB₁ receptor in ischemic damage. To the best of our knowledge we are the first to carry out the cerebral ischemia/reperfusion studies on CB₂ receptor deficient animals. Our results indicted that the CB₂ knockout mouse had larger cerebral infarction and worse neurological function compared to wild type mice. These findings provide evidence for a role of endogenous cannabinoids in protection of the brain following ischemia/reperfusion injury through the CB₂ receptor. Since the CB₂ receptor is

predominantly located outside the CNS, this is not a confounding factor in our study. Thus the actual role of CB₂ receptor in cerebral ischemic injury could be fully reflected by CB₂ knockout mice.

In conclusion, CB₂ receptor activation demonstrated a bell shaped dose dependent response in reducing cerebral infarction and improving neurological function. This neuroprotective effect occurred when the CB₂ agonist was administered prior to and up to 3 h following ischemia. Administration of the CB₂ agonist attenuated numerous aspects of ischemia/reperfusion induced microcirculatory dysfunction including increased leukocyte/endothelial interactions, adhesion molecule expression and blood-brain barrier disruption. Finally, mice lacking the CB₂ receptor showed larger cerebral infarction, which confirmed the neuroprotective properties of the CB₂ receptor and a potential neuroprotective effect of endogenous cannabinoids.

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Fig. 1.

Dose dependent effects of O-1966 administration after MCAO/R. 1 h pre-treatment with O-1966 at 1 mg/kg significantly decreased infarct fraction compared with the vehicle treated control group ($p < 0.05$). The 5 mg/kg dose exerted much more protective effect ($p < 0.001$), while protection was no longer apparent after administration of the higher dose (10 mg/kg) (A). Only the 5 mg/kg dose caused a significant decrease of cerebral edema compared to control group (B). The neurological deficit score and Rota rod test results demonstrated the same trend as cerebral infraction data in a bell shaped dose response curve: both the dosage of 1 mg/kg and 5 mg/kg improved neurological and motor function and 5 mg/kg demonstrated the best neuroprotective effects (C, D). (Data were expressed as Mean $\pm$ SEM, $n=6-7$ in each groups, * $p < 0.05$ vs vehicle treated group, ** $p < 0.01$ vs vehicle treated group, *** $p < 0.001$ vs vehicle treated group).

Fig. 2.

Effects of administration of the CB₂ agonist O-1966 after MCAO/R. O-1966 (5 mg/kg) was given 1 h before MCAO, 1 h after MCAO or 3 h after MCAO. All three treatment groups had significantly decreased cerebral infarction compared to the vehicle treated control group. The 1 h pre-treatment group exerted highest protection ($p < 0.001$). Both 1 h pre-treatment and 1 h post-treatment significantly decreased cerebral edema compared to the vehicle group. (A, B) Treatment with O-1966 at either 1 h before MCAO or 1 h after MCAO significantly improved neurological function compared to control ($p < 0.05$) (C). The Rota rod test indicated an improved motor skills in the 1 h pre-ischemic treatment group ($p < 0.01$) and the 1 h post-ischemic group ($p < 0.05$) (D). (Data were expressed as Mean $\pm$ SEM, $n = 6-8$ in each groups, * $p < 0.05$ vs vehicle treated group, ** $p < 0.01$ vs vehicle treated group, *** $p < 0.001$ vs vehicle treated group).

Fig. 3.

Representative video images of leukocytes labeled with Rhodamine 6G in the ischemic area from a closed cranial window. The arrow points to a Rhodamine 6G-labeled leukocyte flowing through venules. Scale bar=40 μ m (A). Cerebral ischemia significantly enhanced leukocyte rolling and adhesion on venules compared to sham ($p<0.001$) this enhancement was attenuated by pre-treatment with O-1966 at 5 mg/kg ($p<0.05$) (B, C). (Data were expressed as Mean $\pm$ SEM, $n=4$ in each groups, * $p<0.05$ vs vehicle treated group, *** $p<0.001$ vs vehicle treated group).

Fig. 4.

Representative slides showing, ICAM-1 expression and Mac-1 expression in vehicle treated or O-1966 treated groups after MCAO. Scale bar=2.5 mm (A). Cerebral ischemia significantly enhanced both ICAM-1 and Mac-1 expression in the ischemic area compared to sham ($p<0.01$). This enhancement was attenuated by pre-treatment with O-1966 at 5 mg/kg ($p<0.05$) (B, C). (Data were expressed as ratio change of mean fluorescent intensity in ischemic areas with regard to the same sites in non-ischemic hemisphere. $n=4$ in each groups, * $p<0.05$ vs vehicle treated group, ** $p<0.01$ vs vehicle treated group).

Fig. 5.

Representative pictures of Evans Blue extravasation in mouse brains from the sham group, the vehicle treated control group or the O-1966 treated group after MCAO (A). Cerebral ischemia significantly enhanced Evans Blue extravasations in ischemic hemisphere of vehicle treated mice compared to sham mice ($p < 0.01$). Treatment with the CB₂ agonist (O-1966) at 5 mg/kg significantly attenuated Evans Blue extravasations in ischemic hemisphere compared to vehicle treated control group. ($p < 0.05$) (B). (Data were expressed as Mean $\pm$ SEM, $n = 5-6$ in each groups, * $p < 0.05$ vs ischemic hemisphere in vehicle treated group, ** $p < 0.001$ vs ischemic hemisphere in vehicle treated group).

Fig. 6.

CB₂ knockout mice and wild type mice were subjected to 1 h MCAO followed by 23 h reperfusion. CB₂ knockout mice showed larger cerebral infarction (A) and worse neurological function (B) compared to the wide type ($p < 0.05$). (Data were expressed as Mean $\pm$ SEM, $n=5$ in each groups, * $p < 0.05$ vs wide type).