Aus dem Bereich Physiologie
Theoretische Medizin und Biowissenschaften
der Medizinischen Fakultät
der Universität des Saarlandes, Homburg/Saar

Neuroprotection following cerebral ischemia: the potential therapeutic role for cannabidiol

Dissertation

zur Erlangung des Grades eines Doktors der Naturwissenschaften

der Medizinischen Fakultät
der UNIVERSITÄT DES SAARLANDES

2022

vorgelegt von: Erika Meyer

geb. am: 24.12.1985 in Apucarana, Brasilien

UNIVERSIDADE ESTADUAL DE MARINGÁ

Departamento de Farmacologia e Terapêutica

Programa de Pós-Graduação em Ciências Farmacêuticas

Neuroprotection following cerebral ischemia: the potential therapeutic role for cannabidiol

ERIKA MEYER

Orientadores:
Prof. Dr. Rúbia Maria Monteiro Weffort de Oliveira
Prof. Dr. Frank Kirchhoff

Tese apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas (Área de concentração – Produtos Naturais e Sintéticos Biologicamente Ativos), da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do Título de Doutor em Ciências Farmacêuticas

Maringá

2022

Date of oral examination: _____

Dean of the faculty (University of Saarland):
Univ.-Prof. Dr. med. Michael D. Menger

1. Referee:
Prof. Dr. Frank Kirchhoff
University of Saarland
Center for Physiology and Molecular Medicine
Department of Molecular Physiology
Building 48
66421 Homburg

2. Referee:
Prof. Dr. Rúbia Maria Monteiro Weffort de Oliveira
State University of Maringá
Department of Pharmacology and Therapeutics
Building K68
87020-900 Maringá

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Weitere Personen waren an der inhaltlich-materiellen Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die entgeltliche Hilfe von Vermittlungs-bzw. Beratungsdiensten (Promotionsberaterinnen/ Promotionsberater oder anderer Personen) in Anspruch genommen. Außer den Angegebenen hat niemand von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder in ähnlicher Form in einem anderen Verfahren zur Erlangung des Doktorgrades einer anderen Prüfungsbehörde vorgelegt.

Ich versichere an Eides statt, dass ich nach bestem Wissen die Wahrheit gesagt und nichts verschwiegen habe.

Die Bedeutung der eidesstattlichen Erklärung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Erklärung sind mir bekannt.

Ort, Datum

Unterschrift der/des Promovierenden

CONTENT

Comments on thesis organization

Chapter 3-7 represent an extended summary of this thesis including results and their discussion which are mainly extracted from four publications (3 original studies, 1 review) and 1 manuscript in preparation. Chapter 9 (Appendix) contains the 4 main publications and the manuscript deposited in bioRxiv in draft form.

The experimental work of this thesis is described in four main studies, called Part I, II, III and IV, each addressing a different animal model of CI in mice or rats and the effect of CBD thereon. Part I, Part II and Part III have already been published in peer-reviewed journals and Part IV is presented in draft form. Since these studies have received collaborative support from several expert colleagues, the specific author contributions are given in chapter 4.2.

List of abbreviations

$5\text{-}HT_{1A}$	5-hydroxytryptamine receptor 1A
BCCAO	bilateral common carotid artery occlusion
BDNF	brain-derived neurotrophic factor
CB1 and CB2	cannabinoid receptors type 1 and type 2
CBD	cannabidiol
CI	cerebral ischemia
FCI	focal cerebral ischemia
GCI	global cerebral ischemia
HI	hypoxic-ischemic
MCAO	middle cerebral artery occlusion
PPAR-γ	peroxisome proliferator-activated receptor-γ
PSD-95	postsynaptic density protein-95
SYN	synaptophysin
TGCI	Transient global cerebral ischemia
tPA	tissue plasminogen activator
VO	vessel occlusion

1. ABSTRACT

Cerebral ischemia is a cerebrovascular condition characterized by the lack of blood flow to the brain, resulting in multiple and complex pathophysiological processes including excitotoxicity, inflammation, oxidative stress and apoptosis. All these processes are potential targets for pharmacotherapy. However, the pharmacological treatment for cerebral ischemia is very limited, despite the efforts in preclinical research. Cannabidiol, a non-psychotomimetic phytocannabinoid present in *Cannabis sativa*, is a pleiotropic compound acting in a variety of molecular targets and may affect many pathophysiological processes resulting in improvement of cerebral ischemia outcomes. Hence, we wanted to investigate the effects of cannabidiol on functional recovery in different animal models of cerebral ischemia in mice and rats, focusing on cellular and pharmacological mechanisms that could be involved in the cannabidiol neuroprotective effects.

The effect of repeated cannabidiol treatment was studied after transient global cerebral ischemia in rats, with a particular focus on ischemia-caused spatial memory impairments and neuroplastic changes in the hippocampus. Cannabidiol treatment attenuated ischemia-induced memory deficits and neurodegeneration while increasing brain-derived neurotrophic factor levels in the hippocampus. Additionally, cannabidiol protected neurons against the deleterious effects of global ischemia on dendritic spine number and dendritic arborization. This study indicated that the beneficial effects of cannabidiol against ischemia-induced memory impairments may involve changes in hippocampal synaptic plasticity.

Next, the effects of cannabidiol on the cognitive and emotional impairments induced by bilateral common carotid artery occlusion, a model of transient global cerebral ischemia in mice, was investigated. Short-term cannabidiol treatment prevented the cognitive and emotional impairments, attenuated hippocampal neurodegeneration and white matter injury, and reduced glial response that were induced by global ischemia. In addition, cannabidiol also stimulated neurogenesis and promoted dendritic restructuring in the hippocampus of ischemic animals. This study demonstrated that short-term cannabidiol treatment results in global functional

recovery in ischemic mice and impacts multiple and distinct targets involved in the pathophysiology of brain ischemic injury.

Additionally, the pharmacological mechanisms of cannabidiol action were explored using bilateral common carotid artery occlusion in mice. Here, cannabidiol prevented ischemia-induced anxiety-like behavior, memory impairments, and despair-like behaviors. The beneficial effects of cannabidiol on anxiety- and despairs-like behaviors were prevented by antagonists of the cannabinoid receptor type 1-, cannabinoid receptor type 2-, 5-hydroxytryptamine receptor 1A- and peroxisome proliferator-activated receptor-γ. These results suggested that cannabidiol produces functional recovery through different neurotransmission systems following global ischemia.

Lastly, the anti-inflammatory potential of short-term treatment with cannabidiol was investigated in transgenic mice with middle cerebral artery occlusion, a model of focal cerebral ischemia. Cannabidiol prevented stroke-induced neurological impairments and neuronal loss and reduced ischemia-caused microglial activation as observed in fixed tissue as well as in in vivo conditions. This study added information indicating that the neuroprotective effects of cannabidiol occur in the subacute phase of stroke and may involve microglial modulation.

Taken together, the results of this work indicate that cannabidiol might be considered as a therapeutic strategy for the treatment and management of cerebral ischemic conditions.

2. ZUSAMMENFASSUNG

Die zerebrale Ischämie ist ein zerebrovaskuläres Geschehen, das durch den Mangel an Blutfluss zum Gehirn gekennzeichnet ist und zu multiplen und komplexen pathophysiologischen Prozessen führt, einschließlich Excitotoxizität, Entzündung, oxidativem Stress und Apoptose. Alle diese Prozesse sind potenzielle Ziele für eine Therapie. Allerdings ist die pharmakologische Behandlung der zerebralen Ischämie trotz der Bemühungen in der präklinischen Forschung sehr begrenzt. Cannabidiol, ein nicht-psychotomimetisches Phytocannabinoid, das in der Pflanze *Cannabis sativa* enthalten ist, ist ein pleiotroper Stoff, der an einer Vielzahl von molekularen Zielen wirkt und viele pathophysiologische Prozesse beeinflussen kann, was zu einer Verbesserung der Folge einer zerebralen Ischämie führen kann. Daher haben wir die Wirkung von Cannabidiol auf die funktionelle Erholung an verschiedenen Tiermodellen mit zerebraler Ischämie bei Mäusen und Ratten untersucht, wobei wir uns auf zelluläre und pharmakologische Mechanismen konzentriert haben, die an den neuroprotektiven Auswirkungen von Cannabidiol beteiligt sein könnten.

Die Wirkung einer wiederholten Behandlung mit Cannabidiol wurde nach transienter, globaler zerebraler Ischämie bei Ratten untersucht, mit besonderem Fokus auf durch Ischämie verursachte Beeinträchtigungen des räumlichen Gedächtnisses und neuroplastischer Veränderungen. Die Cannabidiol- Behandlung schwächte die Ischämie induzierten Gedächtnisdefizite und Neurodegeneration ab, während die Spiegel zerebraler, neurotrophischer Wachstumsfaktoren im Hippocampus angestiegen sind. Zusätzlich schützte Cannabidiol die Neuronen vor den schädlichen Auswirkungen einer globalen Ischämie, die Anzahl der dendritischen Dornen und die dendritische Verzweigung betreffend. Diese Studie weist darauf hin, dass die vorteilhaften Auswirkungen von Cannabidiol auf durch Ischämie induzierte Gedächtnisstörungen mit Veränderungen der synaptischen Plastizität des Hippocampus einhergehen können.

Als nächstes wurden die Wirkungen von Cannabidiol nach bilateralen Okklusion der *Arteria carotis communis* (Halsschlagader) bei Mäusen untersucht, was einem Modell der transienten, globalen zerebralen Ischämie entspricht, mit besonderem Fokus auf Ischämie-induzierte kognitive und emotionale Beeinträchtigungen. Die

Behandlung mit Cannabidiol verhinderte die Beeinträchtigungen, schwächte die Hippocampus-Neurodegeneration und die Verletzung der weißen Substanz ab und reduzierte die Gliareaktion, die durch die globale Ischämie induziert wurden. Darüber hinaus stimulierte Cannabidiol auch die Neurogenese und förderte die dendritische Umstrukturierung im Hippocampus von ischämischen mäusern. Diese Studie zeigte, dass eine kurzzeitige Behandlung mit Cannabidiol zu einer globalen funktionelle Erholung bei ischämischen Mäusen führt und unterschiedliche Ziele beeinflusst, die an der Pathophysiologie der ischämischen Gehirnverletzung beteiligt sind.

Desweiteren wurden die pharmakologischen Mechanismen der Wirkung von Cannabidiol unter Verwendung einer bilateralen Okklusion der *Arteria carotis communis* (Halsschlagader) bei Mäusen untersucht. Hier verhinderte Cannabidiol durch Ischämie verursachtes ängstliches und depressives Verhalten und Gedächtnisstörungen. Die positiven Auswirkungen von Cannabidiol auf ängstliches und depressives Verhalten wurden durch Antagonisten des Cannabinoid Rezeptor Typ 1-, Cannabinoid Rezeptor Typ 2-, Serotonin Rezeptor 1A- und Peroxisom-Proliferator-aktivierten Rezeptors-γ verhindert. Diese Studie legt nahe, dass Cannabidiol eine funktionelle Erholung nach einer globalen Ischämie durch verschiedene Neurotransmissionssysteme bewirkt.

Schließlich wurde das entzündungshemmende Potenzial einer Kurzzeitbehandlung mit Cannabidiol an transgenen Mäusen nach Okklusion der *Arteria cerebri media* (mittlere Gehirnschlagader) das eine fokale zerebrale Ischämie nachahmt, untersucht. Cannabidiol verhinderte durch Schlaganfall induzierte neurologische Beeinträchtigungen, sowie neuronalen Schaden und reduzierte die durch Ischämie verursachte Aktivierung von Mikroglia, welches in fixiertem Gewebe, sowie unter *in vivo* Bedingungen nachgewiesen wurde. Diese Studie liefert Informationen, die darauf hinweisen, dass die neuroprotektiven Wirkungen von Cannabidiol in der subakuten Phase des Schlaganfalls auftreten und eine Anpassung von Mikroglia beinhalten können.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit, dass Cannabidiol als therapeutische Strategie für die Behandlung und Regie von zerebralen ischämischen Zuständen in Betracht gezogen werden sollte.

3. GENERAL INTRODUCTION

Cerebral ischemia (CI) is a common neurological condition that results from impaired blood flow to the brain. It results to local cerebral infarctions or global hypoxic-ischemic (HI) encephalopathy which can lead to death or permanent disabilities (Sommer, 2017; DeSai and Shapshak, 2021). Broadly, CI is classified into two types: global or focal. Focal cerebral ischemia (FCI) or stroke can be described as the neurological deficits that arise after the rapid onset of a local lesion in the brain with vascular origin (Campbell and Khatri, 2020). Transient global cerebral ischemia (TGCI) is a result of systemic processes including cardiac arrest, gas poisoning, perinatal asphyxia, surgical procedures, and shock (Anderson and Arciniegas, 2010).

The lack of blood flow during cerebral ischemic conditions results in multiple and intricate pathophysiological mechanisms including excitotoxicity, oxidative stress, neuroinflammation, white matter injury, and blood-brain barrier impairment. These events are often interconnected and overlapped and may lead to neurodegeneration and neuronal death (Dirnagl et al., 1999; Leker and Shoham, 2002; Mehta et al., 2007). In consequence, patients who survive a cerebral ischemic episode may present long-term dysfunctions, the most prominent of which are cognitive deficits, spatial/temporal disorientation, impaired decision making, anxiety, and depression (Donkor et al., 2018).

Despite numerous experimental studies that have been conducted in preclinical settings, current pharmacological therapies for CI are limited. For acute ischemic stroke the standard of care is the tissue plasminogen activator (tPA) (Cronin, 2010). However, due to various contraindications and the short therapeutic window, only 1-10 % of acute stroke patients can be treated with tPA (Demaerschalk et al., 2016). Besides, no safe and effective pharmacological therapy has yet been discovered that can treat the long-term cognitive and emotional impairments that are caused by global cerebral ischemic insults (Lapchak and Zhang, 2017).

Several animal models of CI have been implemented to explore cellular mechanisms of ischemic injury and evaluate the potential of novel therapeutic strategies and putative neuroprotective compounds. Besides allowing precise

analysis of stroke pathophysiology and drug effects, animal models of CI also reproduce neurological deficits observed in humans after an ischemic episode, including sensorimotor and cognitive deficits (Hermann and Kleinschnitz, 2019). The most common type of animal model of CI is the FCI, which is evoked of transient or permanent middle cerebral artery occlusion (MCAO) in both mice and rats (Traystman, 2003; Macrae, 2011). Not less important but applied less extensively in experimental research are the models of global cerebral ischemia (GCI). These models consist of different variations of vessel occlusion (VO; Povroznik et al., 2018), including the bilateral common carotid artery occlusion (BCCAO) in mice and the 4-VO in rats (León-Moreno et al., 2020).

Several drugs have demonstrated neuroprotective efficacy in preclinical studies of CI with no translational value for clinical practice so far (O'Collins et al., 2006). Indeed, CI is a highly heterogeneous condition, and treatment targeted at a single mechanism in the ischemic cascade is unlikely to be universally effective. Combination therapy or single drugs with multiple targets and actions (pleiotropic drugs) are more likely to confer adequate and successful neuroprotection in cerebral ischemic processes.

Cannabidiol (CBD) is the second most prevalent bioactive compound of *Cannabis sativa* and may represent up to 40% of Cannabis content (Mechoulam et al., 2002). This compound is devoid of euphoric actions which makes it an attractive compound for the treatment of several medical conditions. Preclinical studies point out anxiolytic, antidepressant, antipsychotic, and neuroprotective actions for CBD (Campos et al., 2016; White, 2019; Garcia-Gutierrez et al., 2020). Neuroprotective effects of CBD in animal models of CI have also been demonstrated (Braida et al., 2003; Mishima et al., 2005; Hayakawa et al., 2008). The pleiotropic action of CBD indicates that it can affect many pathophysiological processes resulting in improvement of CI outcomes. However, even though promising results have been already obtained in different animal models, further molecular, cellular, and animal studies are needed to elucidate CBD pharmacological profile to allow a successful translation of preclinical research to clinical settings.

Therefore, our objective was to investigate the effect of CBD on functional recovery in different animal models of CI in mice and rats and explore the molecular and

pharmacological mechanisms involved in its action, thus contributing to increase the translational potential this compound.

Of note: A more comprehensive introduction you will find as published review in the chapter 9.1.

4. AIMS OF THESIS AND AUTHOR CONTRIBUTION

4.1. Aims

Previous studies have highlighted the beneficial effects of cannabidiol (CBD) in animal models of cerebral ischemia (CI). However, the mechanisms by which CBD promotes its effects after CI are still not fully explored.

The central research question of this study was unveiling the impact of CBD on functional recovery after CI and exploring the mechanisms involved in its action.

To address this question, the following aims were defined:

Aim 1 (Part I): To investigate CBD effect on functional impairments and neuronal plasticity after global cerebral ischemia (GCI) in rats

The effect of CBD on memory performance was evaluated in rats submitted to transient global cerebral ischemia using different behavioral paradigms (open-field, eight-arm aversive radial maze, and object location test). The effects of CBD were investigated using immunohistochemistry to access neurodegeneration, Western blot to detect changes in the levels of synaptic proteins, and Golgi-Cox method to study dendritic remodeling.

Aim 2 (Part II): To examine CBD effect on long-term functional impairments after GCI in mice

The effects of CBD on behavioral performance were evaluated in mice submitted to bilateral common carotid artery occlusion (BCCAO), using a multi-tiered behavioral testing battery, that included the open field (locomotor activity), elevated zero maze (anxiety-like behaviors), Y-maze and object location test (memory), and forced swim test (despair-like behaviors). The effects of CBD at cellular levels were investigated using histology, immunohistochemistry, and Western blot to access neurodegeneration, white matter injury, neuroinflammation and neuroplasticity.

Aim 3 (Part III): To explore the pharmacological mechanisms of CBD action and its effect on functional recovery after GCI in mice

The effects of CBD on behavioral performance were evaluated in mice submitted to BCCAO using a multi-task testing battery, that included the open field (locomotor activity), elevated zero maze (anxiety-like behaviors), Y-maze (memory), and forced swim test (despair-like behaviors). The involvement of different receptors in CBD action in ischemic mice was evaluated using cannabinoid receptors type 1 and type 2, 5-hydroxytryptamine 1A receptor, and peroxisome proliferator-activated receptor-γ specific antagonists.

Aim 4 (Part IV): To examine the CBD impact on glial cells reaction in the subacute phase of focal cerebral ischemia (FCI) in mice

The impact of CBD on neuroinflammation was investigated in mice submitted to middle cerebral artery occlusion and evaluated via *in vivo* two-photon imaging in the subacute phase of the injury. Transgenic mice with enhanced green fluorescent protein (EGFP) knocked into the fractalkine receptor (CX_3CR_1) gene locus were used to study the CBD effect on microglial behavior. In addition, the CBD effect on astrocytic calcium signaling was examined using transgenic mice with Cre-recombinase dependent cell-specific expression of the genetically encoded calcium sensor GCaMP3 in astrocytes. The effects of CBD on stroke-induced neurological impairments and neuronal death were also assessed.

4.2. Declaration of author contribution

Part I:

Meyer E, Bonato JM, Mori MA, Mattos BA, Guimarães FS, Milani H, de Campos AC, de Oliveira RMW. (2021). Cannabidiol Confers Neuroprotection in Rats in a Model of Transient Global Cerebral Ischemia: Impact of Hippocampal Synaptic Neuroplasticity. *Mol Neurobiol* **58**, 5338-5355.

Author contribution: EM and RMWO conceived and designed the experiments with inputs from ACC. EM performed behavioral tests, western blot, and Golgi analysis. She wrote the first draft of the manuscript. EM and JMB conducted the

animals' surgeries. BAM performed the immunohistochemistry. MAM performed data analysis. FSG and HM helped with statistical analysis and data interpretation. All authors read and approved the final manuscript.

Part II:

Mori MA, Meyer E, Mori MA, Soares LM, Milani H, Guimarães FS, de Oliveira RMW. (2017). Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia. *Prog Neuropsychopharmacol Biol Psychiatry* **75**, 94-105.

Author contribution: MA and RMWO conceived and designed the experiments with inputs from FSG. MA performed behavioral tests and immunohistochemistry. EM performed the western blot. MA and EM conducted the animals' surgeries. MA performed data analysis. HM and LMS helped with statistical analysis and data interpretation. MA wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Part III:

Mori MA*, Meyer E*, da Silva FF, Milani H, Guimarães FS, Oliveira RMW. (2021). Differential contribution of CB_1, CB_2, 5-HT1A, and PPAR-γ receptors to cannabidiol effects on ischemia-induced emotional and cognitive impairments. *Eur J Neurosci* **53**, 1738-1751. *Contributed equally

Author contribution: MAM and EM conducted the animals' surgeries and behavioral tests. They wrote the first draft of the manuscript. FFS took care of the animals and performed drugs' treatment. HM analyzed the data and helped with statistical analysis. FSG and RMWO conceived the experimental design, planned the experiments, and performed the data work-up. They also contributed to writing the manuscript. All authors read and approved the final manuscript.

Part IV:

Meyer E, Rieder P, Gobbo D, Candido G, Scheller A, Oliveira RMW, Kirchhoff F. (2022). Time-lapse imaging of microglial activity and astrocytic calcium activity

reveals a neuroprotective effect of cannabidiol in the subacute phase of stroke in mice. bioRxiv doi: 10.1101/2022.05.31.494189.

Author contribution: EM and FK conceived and designed the experiments with inputs from RMWO. EM conducted the animals' surgeries and treatment, performed behavioral tests and immunohistochemistry. She wrote the first draft of the manuscript. PR and DG helped with 2P-LSM experiments and data analyses. GC performed the morphological analysis of microglia. AS performed AxioScan imaging and data interpretation. All authors read and approved the draft of the manuscript.

Additional work contributed to the following publications:

Meyer E, Mori MA, Campos AC, Andreatini R, Guimarães FS, Milani H, de Oliveira RM (2017). Myricitrin induces antidepressant-like effects and facilitates adult neurogenesis in mice. *Behav Brain Res* **316**, 59-65.

Bonato JM, **Meyer E**, de Mendonça PSB, Milani H, Prickaerts J, Weffort de Oliveira RM. (2021). Roflumilast protects against spatial memory impairments and exerts anti-inflammatory effects after transient global cerebral ischemia. *Eur J Neurosci* **53**, 1171-1188.

Raupp-Barcaro IFM, da Silva Dias IC, **Meyer E**, Vieira JCF, da Silva Pereira G, Petkowicz AR, de Oliveira RMW, Andreatini R. (2021). Involvement of dopamine D2 and glutamate NMDA receptors in the antidepressant-like effect of amantadine in mice. *Behav Brain Res* **413**, 113443.

Huang W, Bai X, **Meyer E**, Scheller A. (2020). Acute brain injuries trigger microglia as an additional source of the proteoglycan NG2. *Acta Neuropathol Commun* **8**, 146.

Aguiar RP, Soares LM, **Meyer E**, da Silveira FC, Milani H, Newman-Tancredi A, Varney M, Prickaerts J, Oliveira RMW. (2019). Activation of 5-HT(1A) postsynaptic receptors by NLX-101 results in functional recovery and an increase in neuroplasticity in mice with brain ischemia. *Prog Neuropsychopharmacol Biol Psychiatry* **99**, 109832.

Soares LM, **Meyer E**, Milani H, Steinbusch HW, Prickaerts J, de Oliveira RM (2017). The phosphodiesterase type 2 inhibitor BAY 60-7550 reverses functional impairments induced by brain ischemia by decreasing hippocampal neurodegeneration and enhancing hippocampal neuronal plasticity. *Eur J Neurosci* **45**, 510-520.

5. SUMMARIES OF THE RESEARCH RESULTS

5.1. Part I: Cannabidiol confers neuroprotection in rats in a model of transient global cerebral ischemia: impact of hippocampal synaptic neuroplasticity

5.1.1 Abstract

Evidence for the clinical use of neuroprotective drugs for the treatment of cerebral ischemia is still greatly limited. Spatial/temporal disorientation and cognitive dysfunction are among the most prominent long-term sequelae of cerebral ischemia. Cannabidiol, a major phytocannabinoid constituent of cannabis, exerts neuroprotective effects against experimental cerebral ischemia. The present study investigated possible neuroprotective mechanisms of action of cannabidiol on spatial memory impairments that are caused by transient global cerebral ischemia in rats. Hippocampal synaptic plasticity is a fundamental mechanism of learning and memory. Thus, we also evaluated the impact of cannabidiol on neuroplastic changes in the hippocampus after transient global cerebral ischemia. Wistar rats were trained to learn an eight-arm aversive radial maze task and underwent either sham or transient global cerebral ischemia surgery. The animals received intraperitoneal injections of vehicle or 10 mg/kg cannabidiol 30 min before surgery, 3 h after surgery, and then once daily for 14 days. On days 7 and 14, we performed a retention memory test. Another group of rats that received the same pharmacological treatment was tested in the object location test. Brains were removed and processed to assess neuronal degeneration, synaptic protein levels, and dendritic remodeling in the hippocampus. Cannabidiol treatment attenuated ischemia-induced memory deficits. In rats that were subjected to transient global cerebral ischemia, cannabidiol attenuated hippocampal *Cornu Ammonis 1* neurodegeneration and increased brain-derived neurotrophic factor levels. Additionally, cannabidiol protected neurons against the deleterious effects of ischemia on dendritic spine number and the length of dendritic arborization. These results suggest that the neuroprotective effects of cannabidiol against ischemia-induced memory impairments involve changes in synaptic plasticity in the hippocampus.

5.1.2. Highlighted results

In this study, repeated CBD treatment (10 mg/kg, *i.p.* 30 min before surgery, 3 h after reperfusion, and daily for 14 days) prevented spatial memory impairments in a rat model of GCI. CBD also decreased hippocampal neurodegeneration in the *Cornu Ammonis* 1 hippocampal area that were caused by TGCI. Additionally, CBD increased hippocampal brain-derived neurotrophic factor (BDNF) levels, attenuated the TGCI-induced decreases in synaptophysin (SYN) and postsynaptic density protein-95 (PSD-95) levels, and elevated dendritic spine number and arborization in the hippocampus in ischemic animals.

Of note: A detailed information to this part can be found in the chapter 9.2.

5.2. Part II: Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia

5.2.1. Abstract

This study investigated the effects of cannabidiol on the cognitive and emotional impairments induced by bilateral common carotid artery occlusion in mice. Using a multi-tiered behavioral testing battery during 21 days, we found that ischemic mice exhibited long-lasting functional deficits reflected by increase in anxiety-like behavior (day 9), memory impairments (days 12-18) and despair-like behavior (day 21). Short-term cannabidiol 10 mg/kg treatment prevented the cognitive and emotional impairments, attenuated hippocampal neurodegeneration and white matter injury, and reduced glial response that were induced by bilateral common carotid artery occlusion. In addition, ischemic mice treated with cannabidiol exhibited an increase in the hippocampal brain derived neurotrophic factor protein levels. Cannabidiol also stimulated neurogenesis and promoted dendritic restructuring in the hippocampus of ischemic animals. Collectively, the present results demonstrate that short-term cannabidiol treatment results in global functional recovery in ischemic mice and impacts multiple and distinct targets involved in the pathophysiology of brain ischemic injury.

5.2.2. Highlighted results

In this study, short-term CBD treatment (10 mg/kg, *i.p.* 30 min before, 3, 24, and 48h after surgery) prevented cognitive and emotional impairments induced by a mice model of GCI. The neuroprotective effects of CBD were apparent 21 days after BCCAO and may be related to the prevention of hippocampal neuronal loss, white matter protection, a decrease in neuroinflammation, and an increase in hippocampal plasticity.

Of note: Detailed information on this experiment can be found in the chapter 9.3.

5.3. Part III: Differential contribution of CB_1, CB_2, 5-HT_{1A}, and PPAR-γ receptors to cannabidiol effects on ischemia-induced emotional and cognitive impairments

5.3.1. Abstract

An ever-increasing body of preclinical studies has shown the multifaceted neuroprotective profile of cannabidiol against impairments caused by cerebral ischemia. In this study, we have explored the neuropharmacological mechanisms of cannabidiol action and its impact on functional recovery using a model of transient global cerebral ischemia in mice. C57BL/6J mice were subjected to bilateral common carotid artery occlusion and received vehicle or cannabidiol (10 mg/kg) 30 min before and 3, 24 and 48 h after reperfusion. To investigate the neuropharmacological mechanisms of cannabidiol, the animals were injected with cannabinoid receptor type 1 (AM251, 1 mg/kg), cannabinoid receptors type 2 (AM630, 1 mg/kg), 5-hydroxytryptamine receptor 1A (WAY-100635, 10 mg/kg) or peroxisome proliferator-activated receptor-γ (GW9662, 3 mg/kg) antagonists 30 min before each injection of cannabidiol. The animals were evaluated using a multi-task testing battery that included the open field, elevated zero maze, Y-maze and forced swim test. Cannabidiol prevented the anxiety-like behavior, memory impairments and despair-like behaviors induced by bilateral common carotid artery occlusion in mice. The anxiolytic-like effects of cannabidiol in ischemic mice were attenuated by AM251, AM630, WAY-100635 and GW9662. In the Y-maze, both cannabidiol and the cannabinoid receptor type 1 antagonist AM251 increased the exploration of the novel arm in ischemic animals, indicating the beneficial effects of these treatments in spatial memory performance. Together, these findings indicate the involvement of cannabinoid receptor type 1 and 2, 5-hydroxytryptamine receptor 1A and peroxisome proliferator-activated receptor-γ in the functional recovery induced by cannabidiol in ischemic mice.

5.3.2. Highlighted results

In this study, we investigated the effects of CBD on the behavioral changes in a mice model of GCI and whether these effects were sensitive to selective cannabinoid receptor type 1 (CB_1), cannabinoid receptor type 2 (CB_2), 5-

hydroxytryptamine receptor 1A (5-HT_{1A}) or peroxisome proliferator-activated receptor-γ (PPAR-γ) antagonism. CBD (10 mg/kg, *i.p.*) prevented the anxiogenic-like effect, memory impairment, and despair-like behavior induced by BCCAO. The anxiolytic-like effects of CBD were prevented by CB_1, CB_2, 5-HT_{1A}, and PPAR-γ receptor antagonists. CBD did not significantly change the number of crossings of BCCAO mice in the elevated zero maze, pre-treatment with WAY-100635 or GW9662 decreased this parameter, suggesting interference of 5-HT_{1A} and PPAR-γ receptors with general locomotor activity in CI conditions. In the Y-maze, both CBD and the CB_1 receptor antagonist AM251 increased the exploration of the novel arm of the Y-maze in ischemic animals. Finally, CBD and GW9662 when administered alone attenuated despairs-like behaviors in ischemic mice evaluated in the forced swim test, while no effect was detected when CBD target receptors were blocked by antagonists.

Of note: A detailed information to this part can be found in the chapter 9.4.

5.4. Part IV: Time-lapse imaging of microglial activity and astrocytic calcium signaling reveals a neuroprotective effect of cannabidiol in the subacute phase of stroke

5.4.1. Abstract

Neuroprotective agents that limit secondary tissue loss and/or improve functional outcomes after stroke are still limited. Cannabidiol, the major non-psychoactive component of *Cannabis sativa*, has been proposed as a neuroprotective agent against experimental focal cerebral ischemia. The effects of cannabidiol have generally been related to the modulation of neuroinflammation, including the control of glial activation and the toxicity exerted by pro-inflammatory mediators. However, so far, most information concerning cannabidiol neuroprotective effects was obtained from immunohistochemical and biochemical *post-mortem* assays. To test whether the effects of cannabidiol on glial cells could be also detected *in vivo*, we performed time-lapse imaging of microglial activity and astrocytic calcium signaling in the subacute phase of stroke using two-photon laser-scanning microscopy. C57BL/6N mice underwent either sham or transient middle cerebral artery occlusion surgery. The animals received intraperitoneal injection of vehicle or cannabidiol (10 mg/kg) 30 min, 24 h, and 48 h after surgery. One day later the neurological score test was performed. Brain tissue was processed to evaluate the neuronal loss and microglial activation. Transgenic mice with microglial expression of the enhanced green fluorescent protein and astrocyte-specific expression of the calcium sensor GCaMP3 were used to access *in vivo* microglial activity and astrocytic calcium signaling, respectively. The animals were submitted to the same experimental design described above and to imaging sessions before, 30 min, 24 h and, 48 h after surgery. Astrocytic calcium signaling was also assessed in acutely isolated slices 5 h after transient middle cerebral artery occlusion surgery in the presence of perfusion or cannabidiol (100 μM) solution. Cannabidiol prevented ischemia-induced neurological impairments as well as protected against neuronal loss in ischemic wild-type mice. Cannabidiol also reduced ischemia-induced microglial activation in transgenic mice as demonstrated in fixed tissue as well in *in vivo* conditions. No difference in the amplitude and duration of astrocytic calcium signals was detected neither before nor after the middle cerebral artery occlusion *in vivo*.

Similarly, no significant difference was found in the astrocytic calcium signals between contra or ipsilateral sides of acutely isolated brain slices. The present results suggest that the neuroprotective effects of cannabidiol after stroke may occur in the subacute phase of ischemia and reinforces the strong anti-inflammatory property of this compound.

5.3.2. Highlighted results

The effect of CBD treatment given after the onset of ischemia in a mouse model of FCI was investigated. CBD (10 mg/kg, *i.p.* 30 min, 24, and 48 h after ischemia) reverted neurodegeneration and improved functional recovery that were induced by MCAO. Short-term treatment with CBD reduced neuroinflammation, reflected by a decrease in microglial activation, evidentiated in *in situ* and in *vivo* conditions. No difference in the astrocytic calcium signals was observed before and after MCAO *in vivo*.

Of note: A detailed information to this part can be found in the chapter 9.5.

6. GENERAL DISCUSSION

The effects of CBD treatment in ischemic conditions were addressed using different animal models of CI, that lead to motor, cognitive and emotional impairments. In both, mice and rats, we focused on the contribution of CBD to improve functional outcomes after CI. We also investigated the involvement of molecular and pharmacological processes underlying the CBD neuroprotective effects following CI. The GCI models applied in this study led to long-term sequelae, including cognitive and emotional impairments in mice and rats. The FCI model induced neurological deficits and microglial activation, as demonstrated in the subacute phase of the injury. Treatment with CBD given before and/or shortly after the onset of ischemia was revealed to be neuroprotective in the tested animal models, thereby attenuating the ischemia-induced behavioral impairments and neuroinflammation, and impacting neuronal plasticity.

The neuroprotective effects of CBD on neurobehavioral scores have been well documented following FCI (Hayakawa et al., 2008; Ceprián et al., 2017). However, in GCI conditions only a few studies have investigated the effects of CBD on cognitive and emotional impairments (Pazos et al., 2012. Schiavon et al., 2014). In this study, we confirmed the beneficial effects of CBD in mice after focal and global CI and provided evidence for CBD's neuroprotective effects after GCI/reperfusion in rats.

Despite decades of intense clinical and preclinical efforts, few advances have been made in the development of effective pharmacotherapies to prevent or minimize sequelae of CI (Ao et al., 2018; Madhok et al., 2018; Hadley et al., 2019). Despite some pharmacological strategies have provided neuroprotection in experimental animals, several clinical trials have produced negative results when testing many different compounds. Several reasons have been suggested for the current gap between preclinical and clinical results. Most of the brain damage arising after CI occurs within a very short period. The time from symptom onset to treatment in which a substantial reduction of damage is feasible might be as short as 1 h (Chamorro et al., 2016). In this study, short-term CBD treatment, given shortly before and immediately after the onset of GCI, led to sustained functional recovery in mice. Furthermore, when CBD treatment was initiated 30 min after stroke, a

protective effect was also observed. However, MCAO mice treated with CBD from day 5 after the injury did not show any improvement of neurological score or motor performance (Hayakawa et al., 2009). These findings indicate that the neuroprotective actions of CBD might occur in the complex acute/subacute phase of injury.

Another limitation for the success in the development of pharmacotherapy for CI consists of the fact that the neuroprotective agents must present multiple effects at different stages of the complex signaling cascade involved in the pathophysiology of CI (Dirnagl and Endres, 2014; George and Steinberg, 2015). The possible targets of CBD action have been extensively examined in neurodegenerative and neuropsychiatric experimental models (Esposito et al., 2011; Fagherazzi et al., 2012; Campos et al., 2013). Particularly in CI conditions, converging evidence suggests that CBD induces neuroprotective effects through multiple molecular and pharmacological targets, such as decreasing oxidative stress and neuroinflammation and, impacting neuronal plasticity. Such experimental evidence reinforces the broad-spectrum pharmacological profile of CBD (Mishima et al., 2005; Hayakawa et al., 2008; Hayakawa et al., 2009).

An important aspect after CI consists of the functional recovery mediated by neuroplasticity. Indeed, experimental pieces of evidence suggest that a time window of intense synaptic plasticity opens after CI, during which the greatest gains in recovery occur. In this sense, a treatment that provides neuroprotection while stimulating synaptic remodeling may provide more effective avenues for treating CI-related impairments. Our study demonstrated the ability of CBD to promote functional improvement and neuroplastic processes, which can be beneficial in CI conditions.

Given the very substantial large unmet clinical needs, the characterization of CBD as a possible therapeutic agent in CI is of significant interest. Due to its ability to reduce ischemia-related harmful pathophysiological processes, ameliorate functional outcomes, and promote neuroplastic changes, this molecule represents a promising new pharmacological approach to treat CI sequelae.

7. CONCLUSION AND OUTLOOK

Neuroprotective effects of CBD were detected in different animal models of CI, reflected by an improved functional performance in different behavioral paradigms. The beneficial effect of CBD was accompanied by modulation of plastic events and a decrease in neuroinflammation. In addition, the involvement of different neurotransmission systems in CBD's action was also reported.

Although animal models of CI are predictive of human pathophysiology, emphasis should also be placed on factors present when human CI occurs. Key experiments will be to determine whether the neuroprotective effects of CBD can also be demonstrated in aged rodents with comorbidities (such as hypertension, hyperlipidemia, or diabetes) that are often observed in those patients that suffer a cerebral ischemic episode.

Taken together, our results provided evidence for a neuroprotective action of CBD in diverse CI models, highlighting its profile to act on several molecular and pharmacological targets, making this compound a potential neuroprotective pharmacological strategy in CI conditions.

8. REFERENCES

Anderson CA, Arciniegas DB. (2010). Cognitive sequelae of hypoxic-ischemic brain injury: a review. *NeuroRehabilitation* **26**, 47–63.

Ao LY, Yan YY, Zhou L, Li CY, Li WT, Fang WR, Li YM. (2018). Immune Cells After Ischemic Stroke Onset: Roles, Migration, and Target Intervention. *J Mol Neurosci* **66**, 342-355.

Braida D, Pegorini S, Arcidiacono MV, Consalez GG, Croci L, Sala M. (2003). Post-ischemic treatment with cannabidiol prevents electroencephalographic flattening, hyperlocomotion, and neuronal injury in gerbils. *Neurosci Lett* **346**, 61-64.

Campbell BCV, Khatri P. (2020). Stroke. *Lancet* **396**, 129-142.

Campos AC, Fogaça MV, Sonego AB, Guimarães FS. (2016). Cannabidiol, neuroprotection and neuropsychiatric disorders. *Pharmacol Res* **112**, 119-127.

Campos AC, Ortega Z, Palazuelos J, Fogaça MV, Aguiar DC, Díaz-Alonso J, Ortega-Gutiérrez S, Vázquez-Villa H, Moreira FA, Guzmán M, Galve- Roperh I, Guimarães FS. (2013). The anxiolytic effect of cannabidiol on chronically stressed mice depends on hippocampal neurogenesis: involvement of the endocannabinoid system. *Int J Neuropsychopharmacol* **16**, 1407-1419.

Ceprián M, Jiménez-Sánchez L, Vargas C, Barata L, Hind W, Martínez-Orgado J. (2017). Cannabidiol reduces brain damage and improves functional recovery in a neonatal rat model of arterial ischemic stroke. *Neuropharmacology* **116**, 151-159.

Chamorro Á, Dirnagl U, Urra X, Planas AM. (2016). Neuroprotection in acute stroke: targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. *Lancet Neurol* **15**, 869-881.

Cronin CA. (2010). Intravenous tissue plasminogen activator for stroke: a review of the ECASS III results in relation to prior clinical trials. *J Emerg Med* **38**, 99-105.

Demaerschalk BM, Kleindorfer DO, Adeoye OM, Demchuk AM, Fugate JE, Grotta JC, Khalessi AA, Levy EI, Palesch YY, Prabhakaran S, Saposnik G, Saver JL, Smith EE, American heart association stroke council and council on epidemiology and prevention. (2016). Scientific rationale for the inclusion and exclusion criteria for intravenous alteplase in acute ischemic stroke: a statement for healthcare professionals from the American heart association/American stroke association. *Stroke* **47**, 581- 641.

DeSai C, Shapshak AH. (2021). Cerebral ischemia. *Treasure Island (FL): StatPearls Publishing.*

Dirnagl U, Endres M. (2014). Found in translation: preclinical stroke research predicts human pathophysiology, clinical phenotypes, and therapeutic outcomes. *Stroke* **45**, 1510-1518.

Dirnagl U, Iadecola C, Moskowitz MA. (1999). Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* **22**, 391-397.

Donkor ES. (2018). Stroke in the 21st century: a snapshot of the burden, epidemiology, and quality of life. *Stroke Res Treat* **2018**, 3238165.

Esposito G, Scuderi C, Valenza M, Togna GI, Latina V, De Filippis D, Cipriano M, Carratù MR, Iuvone T, Steardo L. (2011). Cannabidiol reduces Aβ- induced neuroinflammation and promotes hippocampal neurogenesis through PPARγ involvement. *PLoS One* **6**, e28668.

Fagherazzi EV, Garcia VA, Maurmann N, Bervanger T, Halmenschlager LH, Busato SB, Hallak JE, Zuardi AW, Crippa JA, Schröder N. (2012). Memory rescuing effects of cannabidiol in an animal model of cognitive impairment relevant to neurodegenerative disorders. *Psychopharmacology (Berl)* **219**, 1133-1140.

García-Gutiérrez MS, Navarrete F, Gasparyan A, Austrich-Olivares A, Sala F, Manzanares J. (2020). Cannabidiol: A potential new alternative for the treatment of anxiety, depression, and psychotic disorders. *Biomolecules* **10**, 1575.

George PM, Steinberg GK. (2015). Novel stroke therapeutics: Unraveling stroke pathophysiology and its impact on clinical treatments. *Neuron* **87**, 297- 309.

Hadley G, Beard DJ, Couch Y, Neuhaus AA, Adriaanse BA, DeLuca GC, Sutherland BA, Buchan AM. (2019). Rapamycin in ischemic stroke: Old drug, new tricks? *J Cereb Blood Flow Metab* **39**, 20-35.

Hayakawa K, Irie K, Sano K, Watanabe T, Higuchi S, Enoki M, Nakano T, Harada K, Ishikane S, Ikeda T, Fujioka M, Orito K, Iwasaki K, Mishima K, Fujiwara M. (2009). Therapeutic time window of cannabidiol treatment on delayed ischemic damage via high-mobility group box1-inhibiting mechanism. *Biol Pharm Bull* **32**, 1538-1544.

Hayakawa K, Mishima K, Irie K, Hazekawa M, Mishima S, Fujioka M, Orito, K, Egashira N, Katsurabayashi S, Takasaki K, Iwasaki K, Fujiwara M. (2008). Cannabidiol prevents a post-ischemic injury progressively induced by cerebral ischemia via a high-mobility group box1- inhibiting mechanism. *Neuropharmacology* **55**, 1280-1286.

Hermann DM, Kleinschnitz C. (2019). Modeling vascular risk factors for the development of ischemic stroke therapies. *Stroke* **50**, 1310-1317.

Lapchak PA, Zhang JH. (2017). The high cost of stroke and stroke cytoprotection research. *Transl Stroke Res* **8**, 307-317.

Leker RR, Shoham E. (2002). Cerebral ischemia and trauma-different etiologies yet similar mechanisms: neuroprotective opportunities. *Brain Res Brain Res Rev* **39**, 55-73.

León-Moreno LC, Castañeda-Arellano R, Rivas-Carrillo JD, Dueñas-Jiménez SH. (2020). Challenges and improvements of developing an ischemia mouse model through bilateral common carotid artery occlusion. *J Stroke Cerebrovasc Dis* **29**, 104773.

Macrae IM. (2011). Preclinical stroke research--advantages and disadvantages of the most common rodent models of focal ischaemia. *Br J Pharmacol* **164**, 1062-1078.

Madhok DY, Vitt JR, Nguyen AT. (2018). Overview of Neurovascular Physiology. *Curr Neurol Neurosci Rep* **18**, 99.

Mechoulam R, Parker LA, Gallily R. (2002). Cannabidiol: an overview of some pharmacological aspects. *J Clin Pharmacol* **42**, 11S-19S.

Mehta SL, Manhas N, Raghubir R. (2007). Molecular targets in cerebral ischemia for developing novel therapeutics. *Brain Res Rev* **54**, 34-66.

Mishima K, Hayakawa K, Abe K, Ikeda T, Egashira N, Iwasaki K, Fujiwara M. (2005). Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine 1A receptor-dependent mechanism. *Stroke* **36**, 1077-1082.

O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW. (2006). 1,026 experimental treatments in acute stroke. *Ann Neurol* **59**, 467-477.

Pazos MR, Cinquina V, Gómez A, Layunta R, Santos M, Fernández-Ruiz J, Martínez-Orgado J. (2012). Cannabidiol administration after hypoxia-ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function. *Neuropharmacology* **63**, 776-783.

Povroznik JM, Ozga JE, Vonder Haar C, Engler-Chiurazzi EB. (2018). Executive (dys)function after stroke: Special considerations for behavioral pharmacology. *Behav Pharmacol* **29**, 638-653.

Schiavon AP, Soares LM, Bonato JM, Milani H, Guimarães FS, Weffort de Oliveira RM. (2014). Protective effects of cannabidiol against hippocampal cell death and cognitive impairment induced by bilateral common carotid artery occlusion in mice. *Neurotox Res* **26**, 307-316.

Sommer CJ. (2017). Ischemic stroke: experimental models and reality. *Acta Neuropathol* **133**, 245-261.

Traystman RJ. (2003). Animal models of focal and global cerebral ischemia. *ILAR J* **44**, 85-95.

White CM. (2019). A review of human studies assessing Cannabidiol's (CBD) therapeutic actions and potential. *J Clin Pharmacol* **59**, 923-934.

9. APPENDIX

9.1 Review: Meyer et al. Rev Bras Farmacogn. 2021

Revista Brasileira de Farmacognosia
https://doi.org/10.1007/s43450-021-00199-6

REVIEW

Neuroprotective Effects of Cannabidiol Under Cerebral Ischemic Conditions

Erika Meyer[1,2] · Bianca Andretto de Mattos[1] · Frank Kirchhoff[2] · Rúbia Maria Weffort de Oliveira[1]

Received: 9 September 2021 / Accepted: 4 October 2021

Abstract
The lack of blood flow during cerebral ischemic conditions results in multiple and intricate pathophysiological mechanisms including excitotoxicity, oxidative stress, neuroinflammation, white matter injury, and blood–brain barrier impairment. Despite numerous experimental studies that have been conducted in preclinical settings, current treatments for cerebral ischemia including mechanical and pharmacological therapies are limited, and often accompanied by significant side effects. Therefore, it is necessary to investigate new strategies for treating these conditions. Cannabidiol, the most abundant non-psychotomimetic compound obtained from *Cannabis sativa* L., Cannabaceae, is a pleiotropic compound acting in a variety of molecular targets and may affect many pathophysiological processes resulting in improvement of cerebral ischemia outcomes. In this review, we summarize the main effects of cannabidiol in different animal models of cerebral ischemia and discuss some of its putative mechanisms of neuroprotection.

Keywords Animal models · Cannabinoids · Cerebral blood flow · Impaired blood flow · Marijuana · Neuroprotection

Introduction

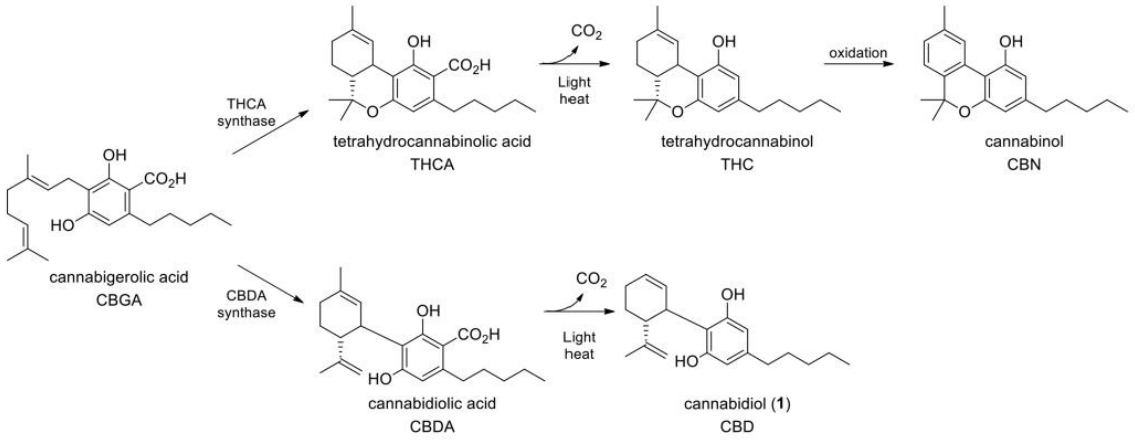
Cerebral ischemia (CI) is a common neurological condition that results from impaired blood flow to the brain. It is a devastating emergency and a paramount cause of mortality and morbidity worldwide (GBD 2021). Many pharmacological agents are promising agents for providing neuroprotection in different animal models of CI. However, despite numerous efforts, only one pharmacological therapy, *i.e.*, the intravenous tissue plasminogen activator (tPA; thrombolytic agent), has been approved for the treatment of the acute phase of CI (Cronin 2010). Besides, the pharmacological treatment for the long-term functional consequences due to cerebral ischemic injury remains limited. Because of the complexity of the ischemia cascade and downstream signaling pathways, finding an agent which acts at multiple targets may provide a promising pharmacological therapy for CI management. Cannabidiol (**1**, CBD) is the most abundant non-psychotomimetic compound from *Cannabis sativa* L., Cannabaceae (Mechoulam et al. 2002), known in English with the common names of marijuana, hemp, and hashish. This is one of the oldest medicinal plants used by humans (Hardy 2020). The first appearance of *Cannabis* was believed to be central Asia about 5000 BC. For millennia, the plant has also been used for fiber and oil production, and simply as additive for food products (Farag and Kayser 2017). It contains several medicinally important compounds, such as cannabinoids, terpennoids, stilbenoids, flavonoids, alkaloids, and fatty acids, among others. Cannabinoids are a unique class of terpenophenolic compounds to *Cannabis* plants, accumulated mainly in the cavity of inflorescence trichomes. More than 80 cannabinoids have been isolated from *C. sativa*, including (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC or simply THC), the one responsible for its main psychological effects. Nowadays, its applications in the clinical world span from multiple sclerosis to epilepsy, neuropathic pain, arthritis, nausea, and vomiting due to chemotherapy, appetite stimulation in HIV/AIDS, depression, anxiety disorders, sleep

This article is part of a Special Issue to celebrate the 35th anniversary of the *Brazilian Journal of Pharmacognosy*

✉ Rúbia Maria Weffort de Oliveira
rmmwoliveira@uem.br

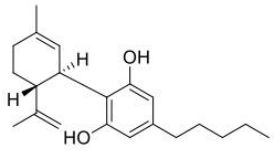
[1] Departamento de Farmacologia e Terapêutica, Universidade Estadual de Maringá, Av. Colombo, 5790, Maringá, PR 87020-900, Brazil

[2] Molecular Physiology, Center for Integrative Physiology and Molecular Medicine (CIPMM), University of Saarland, 66421 Homburg, Germany



Scheme 1 Biosynthetic route of tetrahydrocannabinolicacid (THCA) and cannabidiolic acid (CBDA) from cannabigerolic acid (CBGA), formation of Δ^9-tetrahydrocannabinol (THC) and CBD (**1**) by light and/or heat, and oxidation of THC to cannabinol (CBN). Reproduced from Citti et al. (2018)

disorders, psychosis, glaucoma, and Tourette syndrome (Citti et al. 2018).



1

The therapeutic purposes of *C. sativa* depend on the ratio between THC and CBD, which is known to possess several pharmacological properties but not the psychotropic one of THC. Analgesic, antioxidant, and antiepileptic activities have been attributed to this compound, which seems also to reduce THC side effects (Citti et al. 2018). Three main chemotypes of *Cannabis* inflorescence have been identified based on the cannabinoid constituents for medicinal purposes: THC-dominant chemotype; intermediate chemotype with both THC and CBD; and CBD-dominant chemotype (Sarma et al. 2020). Although CBD and THC have such therapeutical significance, these molecules are biosynthesized in the plant from cannabidiolic acid (CBDA) and tetrahydrocannabinolicacid (THCA). A chemical reaction triggered by heat also leads to the decarboxylation of these compounds to get the corresponding decarboxylated (or neutral) bioactive species CBD and THC. CBDA and THCA are the major components of cannabis inflorescence. Other minor cannabinoids are cannabichromenic acid (CBCA), cannabigerolic acid (CBGA), the parent compounds for other cannabinoid acids, and cannabinolic acid (CBNA). All these compounds upon decarboxylation lead to the neutral derivatives, respectively, cannabichromene (CBC), with anti-inflammatory, antibacterial, and antifungal activity; cannabigerol (CBG) with analgesic, antibacterial, and antifungal activity; and cannabinol (CBN), which derives from the oxidation of THC as a result of prolonged storage and has potent sedative properties (Citti et al. 2018). A schematic representation of the biosynthetic route of THCA and CBDA, their conversion into THC and CBD, and the oxidation of THC to CBN is illustrated in Scheme 1.

CBD (**1**) has been proposed as a pleiotropic compound by acting in a variety of molecular targets that may affect several mechanisms of CI resulting in improvement of functional outcomes and recovery. In this mini-review, the main effects of CBD in different animal models of CI are summarized and its putative mechanisms of neuroprotection are also discussed.

Search Strategy

This study is a narrative review on the neuroprotective effects of CBD, a non-psychotomimetic compound obtained from *Cannabis sativa*, in cerebral ischemic conditions. The review was based on an extensive literature search using PubMed, Google Scholar, ScienceDirect, and Scielo databases. The keywords used as search terms included the following, both individually and in various combinations: cannabidiol, brain, cerebral ischemia, stroke, animal models, neuroinflammation, neuroprotection. Reference lists in the identified papers were also searched, and additional

publications were subsequently traced online. Inclusion criteria were papers reporting the effects of CBD and its biological activities and mechanisms of action under cerebral ischemic conditions.

Discussion

Cerebral Ischemia

Cerebral ischemia can lead to local cerebral infarctions or global hypoxic-ischemic (HI) encephalopathy which can lead to death or permanent disabilities. The extent of neuronal injury resulting from compromised cerebral blood perfusion depends on multiple factors such as the local, extension and duration of the hypoperfusion, and the existence of collateral system etiologies, as well as age, comorbidities with the respective multi-medication, and genetic background (Sommer 2017).

Broadly, CI can be classified into two types: global or focal. Focal ischemia or stroke can be described as the neurological deficits that arise after the rapid onset of a local lesion in the brain with vascular origin (Campbell and Khatri 2020). Focal ischemia can be classified as ischemic when a thrombus or embolus occludes a major cerebral artery, and hemorrhagic stroke when a blood vessel ruptures, and blood accumulates in the adjacent tissue (Lindsay et al. 2019). The symptoms and consequences of focal ischemia will depend upon the specific brain region and extension and duration of hypoperfusion. For example, lesions in cerebral hemispheres may result in contralateral weakness and/or sensory loss. Large artery obstruction, such as the commonly affected middle cerebral artery (MCA), may result in expressive aphasia, visual field defects, acalculia, and hemiagnosia (Stinear and Barber 2020). In 2019, the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD 2021) indicated that stroke is the second leading cause of disability-adjusted life-years worldwide in both 50–74 years and 75 years and older age groups (De Vos et al. 2019). The management of stroke is generally correcting the underlying cause. Mechanical thrombectomy (Catanese et al. 2017; Liaw and Liebeskind 2020) or the tissue plasminogen activator (tPA) has been used for the management of acute ischemic stroke (Cronin 2010). However, due to various contraindications (*e.g.*, diabetes, use of oral anticoagulants, advanced age) and the short therapeutic window (administration within 3–4.5 h of symptom onset), only 1–10% of acute stroke patients can be treated with tPA (Demaerschalk et al. 2016). Besides, many stroke patients, even when treated successfully, suffer from residual long-term neuropsychiatric symptoms which may dramatically impact their quality of life (Karsy et al. 2017; Donkor 2018). Outside of the acute period, the treatment of stroke is focused on secondary prevention and promoting recovery.

Transient global cerebral ischemia (TGCI) is a result of systemic processes including cardiac arrest, gas poisoning, perinatal asphyxia, surgical procedures, and shock (Anderson and Arciniegas 2010). When the effect of global ischemia is transient, the condition manifests acutely as a presyncope or syncope. However, prolonged global ischemia can result in permanent neurological injury and long-term dysfunctions, the most prominent of which are cognitive deficits, spatial/temporal disorientation, impaired decision-making, anxiety, and depression (Li et al. 2020). Currently, no safe and effective pharmacological therapy has yet been discovered that can treat the long-term cognitive and emotional impairments that are caused by global cerebral ischemic insult (Lapchak and Zhang 2017).

Perinatal asphyxia results from a severe global cerebral anoxia during the neonatal period. It is a major cause of neonatal HI encephalopathy and can lead to premature mortality or life-long morbidities including epilepsy, cognitive dysfunction, behavioral and emotional disorders, and hearing, visual, and feeding impairments (Millar et al. 2017; Greco et al. 2020). Perinatal HI has an incidence of 2–6 cases per 1000 live births bringing deleterious consequences for the child and family well-being (Lehtonen et al. 2017; Gale et al. 2018). The current standard of care for HI newborns is hypothermia. However, hypothermia is only partially effective since not more than half of HI neonates achieve an improved outcome (Greco et al. 2020).

Cerebral ischemic conditions remain the leading cause of death, disability, and healthcare expense worldwide (Rajsic et al. 2019; GBD 2021). Complex neural circuits damaged by CI make restoration and recovery difficult. Besides, long-term pharmacological treatment remains limited. Taken together, the large numbers of patients who suffered CI, the devasting long-term consequences, and the lack of viable treatment options highlight a critical need to develop new interventions for the treatment of CI and its secondary and long-term consequences. In this line, several animal models of CI have been implemented to explore cellular mechanisms of ischemic injury and evaluate the potential of novel therapeutic strategies and putative neuroprotective compounds. The use of animal models, therefore, is essential to investigate mechanisms and to predict the value and effect of therapeutic approaches in human subjects (Traystman 2003; Sommer 2017).

Animal Models

Rodents are the first-choice animal species to model CI. They present low costs, the vascular anatomy is similar to humans, and the cascade of pathophysiological mechanisms leading to ischemic cell death are well described (Fig. 1).

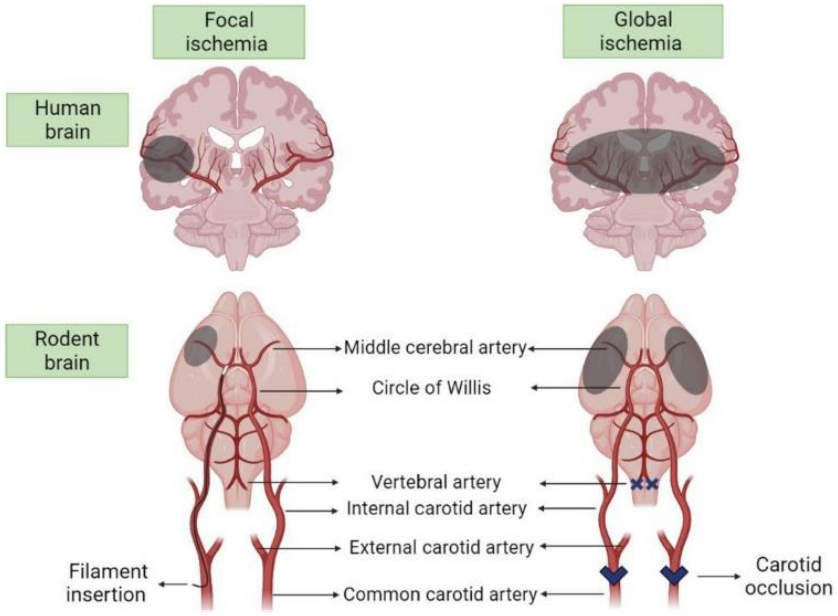


Fig. 1 Representation of animal models of cerebral ischemia. Focal and global cerebral ischemia can be modeled in rodents due to similarities in cerebrovascular anatomy (Circle of Willis) between these species and humans. Focal cerebral ischemia can be achieved through transient or permanent occlusion of one major cerebral blood vessel, *i.e.*, the middle cerebral artery. Global cerebral ischemia can be modeled through different variations of vessel occlusion. In rodents, established methods consist of coagulation of both vertebral arteries, followed by the transient occlusion of both common carotid arteries

Besides, thrombolysis, the one treatment known to be efficacious for stroke in humans, is equally efficacious in rodent focal cerebral ischemia (FCI) models (Back et al. 2007).

The most common type of animal model of CI is the FCI, which can be modeled through transient or permanent occlusion of MCA (MCAo) in both mice and rats with purported relevance to human thromboembolic stroke (Fig. 1) (Traystman 2003; Macrae, 2011). Mechanical (*e.g.*, intraluminal filaments, ligation sutures, cauterization) or chemical (*e.g.*, injection of a clot-forming agent) ways to occlude the MCA can be used. MCAo results in a reduction of cerebral blood flow predominantly in both the striatum and cerebral cortex. However, the degree and distribution of blood flow reduction depend on the duration of MCAo, the site of occlusion along with the MCA, and the amount of collateral blood flow into the MCA territory (Traystman 2003; Gennaro et al. 2019). In general, MCAo in rodents has been associated with robust neurological deficits, including sensorimotor and cognitive deficits (Hermann and Kleinschnitz 2019).

Not less important but applied less extensively in experimental research are the models of global cerebral ischemia. These models consist of different variations of vessel occlusions (VO; Povroznik et al. 2018). The 2-, 3-, or 4-VO are achieved through ligation of different combinations of the carotid, cerebral, and basilar arteries (Traystman 2003; Cechetti et al. 2010). The TGCI can be modeled through bilateral common carotid artery occlusion (BCCAO) in mice and Mongolian gerbils via two vessels (2-VO) or four-vessel occlusion (4-VO) in rats (León-Moreno et al. 2020). Usually, TGCI affects areas of the forebrain, such as the highly vulnerable hippocampal pyramidal neurons (CA1 and CA3 subfields), the medium-sized dorsoventral striatum neurons, and the pyramidal neurons in the prefrontal cortex (PFC) (Raval et al. 2009; Khodanovich and Kisel 2015). Short- and long-term impairments have been associated with neuronal loss in the vulnerable cerebral areas affected by TGCI. For example, in rodents, TGCI provokes lasting cognitive deficits that have been associated with delayed neuronal loss in the hippocampus (Li et al. 2011).

There are numerous animal models in application to study perinatal HI at molecular, cellular, and behavioral levels (Hamdy et al. 2020). HI may occur acutely or chronically during the prenatal, perinatal, or postnatal period resulting in significant brain injury (Yıldız et al. 2017) and the parameters of CI exposure have a significant influence on the outcomes. Nevertheless, HI insults produced damage of the gray and white matter in rodents resulting in long-term functional alterations and impairments comparable to that observed in human infants (Ceprián et al. 2019). In particular, oligodendrocytes (OL) and their precursors (preOL) are sensitive to HI because of the combination of high metabolic demand, an undeveloped antioxidant system, and strong glutamate toxicity (Ceprián et al. 2019).

Pathophysiology

The interruption of the blood–brain flow during a cerebral ischemic episode triggers a complex cascade of cellular and molecular events that may lead to neurodegeneration and neuronal death. These events are often interconnected and

overlapped (Dirnagl et al. 1999; Leker and Shoham 2002; Mehta et al. 2007). At first, lack of oxygen and glucose by the reduction of cerebral blood flow results in insufficient production of energy. When the energy supply is interrupted, neurons can no longer maintain their transmembrane gradient, leading to dysfunctions of ion pumps and impairment of neuronal signaling. Anoxic depolarization at presynaptic terminals leads to excessive glutamate release and calcium influx. The intracellular increase of calcium leads to activation of several calcium-dependent processes such as activation of inducible nitric oxide synthase with consequent free radical production (Iadecola and Ross 1997) and initiation of cell death processes including oxidative stress, apoptosis, necrosis, and autophagy (Fig. 2). After glutamate receptor, overactivation, sodium and potassium ions enter neurons through monovalent cation channels resulting in brain edema and apoptosis (George and Steinberg 2015; Campbell et al. 2019).

Almost immediately after the onset of cerebral ischemia, resident microglia are activated and cytokines are extensively produced, which leads to an influx of macrophages, neurophil polymorphs, and lymphocytes to the brain parenchyma (Campbell et al. 2019). The activated microglia are highly plastic and can manifest predominantly as the classic pro-inflammatory (M1) or the alternative anti-inflammatory (M2) phenotype depending on the different stages of cerebral ischemia (Gulyás et al. 2012; Hu et al. 2012). Endothelial cells, astrocytes, and oligodendrocytes are damaged by similar injury pathways to neurons including glutamate toxicity (Sánchez-Gómez et al. 2011). Astrocytes also became reactive and release pro-inflammatory cytokines leading to further neuronal damage (Fig. 2). During the post-ischemic reperfusion phase, oxidative stress and neuroinflammation aggravate and amplify such responses, increasing and spreading neuron and glial cell damage (Martínez-Orgado et al. 2007).

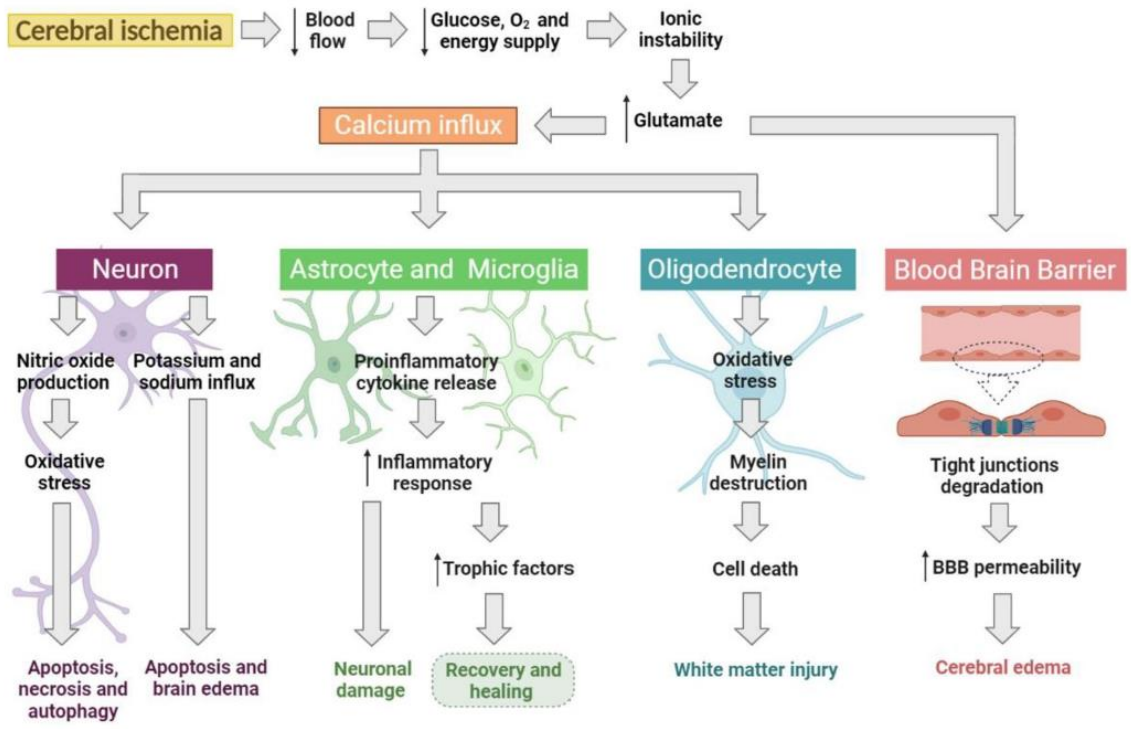


Fig. 2 Overview of pathophysiological mechanisms involved in cerebral ischemia. The reduction of cerebral blood flow leads to energy failure and ionic instability. Subsequent activation of ionotropic glutamate receptors dramatically increases the level of intracellular messenger calcium. Generation of nitric oxide and free radicals, which damage membranes, mitochondria, and DNA, triggers different mechanisms of cell death including autophagy, activation of glial response (astrocytes and microglia), and formation and release of inflammatory mediators. Increased oxidative stress and neuroinflammation lead to blood–brain barrier (BBB) disruption and oligodendrocyte damage with consequent white matter injury. However, the ischemic tissue may be spared by restorative mechanisms such as the release of trophic factors, collateral, and spontaneous reperfusion, vasculogenesis, and neurogenesis

The blood–brain barrier (BBB) disruption may occur within minutes to hours after CI. It is characterized by proteolytic degradation of tight junctions and basement membranes, endothelial tied junctions, and increased permeability of chemicals from blood-borne cells across the BBB leading to cerebral edema and further progression of cerebral damage (Fig. 2) (Prakash and Carmichael 2015; Liu et al. 2018). The underlying events of CI may affect all components of BBB including loss of endothelial cells, astrocytes, pericytes, and the extracellular matrix (Knowland et al. 2014; Haley and Lawrence 2017).

The white matter is also highly vulnerable to reduced blood supply due to cerebral ischemic injuries because proportionally it receives less circulation than gray matter. The OL present in the white matter is the primary target of glutamate excitotoxicity. CI triggers P2X7 receptors in OL, which contributes to calcium overload and depolarization (Wang et al. 2009). Overactivation of glutamate receptors leads to mitochondrial alterations, oxidative stress, cell death, and myelin destruction (Sánchez-Gómez et al. 2003). These effects result in white matter damage with a consequent increase in morbidity and mortality.

The complex immune response induced by CI induces not only deleterious consequences but also beneficial effects on the surviving cerebral tissue (George and Steinberg 2015). The timing and extension of injury contribute to the balance between degenerative and restorative processes after a cerebral insult (Dirnagl 2012). Degeneration overwhelms protection while the ischemic tissue may be spared by restorative mechanisms including collateral and spontaneous reperfusion, vasculogenesis, and neurogenesis (Dirnagl 2012). In addition, reactive microglia and astrocytes can modulate the release of trophic factors which collaborate with recovery and healing (Rolls et al. 2009; Wang et al. 2013). Yet, neurons that survive an ischemic event can respond with ultrastructural changes, including dendritic restructuring, synaptogenesis, and growth-promoting processes that, in turn, can result in functional recovery (García-Chávez et al. 2008).

Several drugs have demonstrated neuroprotective efficacy in preclinical studies of CI with no translational value for clinical practice so far (O'Collins et al. 2006). Indeed, CI is a highly heterogeneous condition, and treatment targeted at a single mechanism in the ischemic cascade is unlikely to be universally effective. Combination therapy or single drugs with multiple targets and actions (pleiotropic drugs) are more likely to confer adequate and successful neuroprotection in cerebral ischemic processes.

Cannabidiol

Cannabidiol (**1**, CBD) is the second most prevalent bioactive compound of *Cannabis sativa* and may represent up to 40% of *Cannabis* content (Mechoulam et al. 2002). Unlike Δ^9-tetrahydrocannabinol (Δ^9-THC), CBD is devoid of euphoric actions which makes it an attractive compound for the treatment of several medical conditions. The wide range of putative pharmacological properties of CBD includes antimicrobial, immunosuppressive, antiemetic, spasmolytic, antitumor, anti-pain, and anti-inflammatory actions (Mechoulam et al. 2002; White 2019; Larsen and Shahinas 2020). Some of these properties rendered CBD its clinical approval for treating spasticity in multiple sclerosis (Syed et al. 2014) and seizures associated with Lennox-Gastaut or Dravet syndromes in children (Devisnky et al. 2018). Currently, preclinical studies point out anxiolytic, antidepressant, antipsychotic, and neuroprotective actions for CBD (Campos et al. 2016; White 2019; García-Gutiérrez et al. 2020). However, only a few controlled clinical trials investigating CBD pharmacological actions have been conducted to define its therapeutic potential (Britch et al. 2021).

CBD belongs to the group of terpenophenols. The hydroxyl groups and double bonds contribute to increasing its highest occupied orbital (HOMO) value, indicating the CBD's ability to donate an electron, as an active redox agent, making this compound a powerful antioxidant molecule (Borges et al. 2013; Atalay et al. 2020). Besides its potent antioxidant actions, CBD may operate throughout components of the endocannabinoid system (ECS) as well as in more than 50 different molecular targets. The majority of CBD effects, however, occur in an ECS-independent manner (Pertwee 2008).

The ECS is composed of two G-protein-coupled receptors (GPCR) cannabinoid receptors, named CB_1 and CB_2, their endogenous ligands (*i.e.*, anandamide (AEA) and 2-aracdonylglicerol (2-AG)), the enzymes of endocannabinoid syntheses (*N*-acylphosphatidylethanolamine (NAPE-PLD), diacylglycerol lipase α (DAGLα) and β (DAGLβ)), and the enzymes of endocannabinoids degradation, such as fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) (Di Marzo et al. 2004; Cristino et al. 2020). However, CBD presents only low affinities for the endocannabinoid CB_1 and CB_2 receptors (Pertwee 2008). CBD actions at CB_1 receptors are primarily due to indirect effects such as inhibiting the enzymatic hydrolysis and the uptake of AEA, resulting in increased levels of this endocannabinoid (Fig. 3) (Bisogno et al. 2001). CBD can act as a negative allosteric modulator of CB_1 receptors (Chung et al. 2019; Laprairie et al. 2016; Tham et al. 2019). Besides, CBD has been suggested to act as a weak agonist (Muller et al. 2019) or as an inverse agonist of the CB_2 receptor (Fig. 3) (Pertwee 2008).

Some of the CBD effects are mediated by direct activation of serotonin receptors including the 5-hydroxytryptamine 1A (5-HT_{1A}) and 5-HT_{2A} receptors (Russo et al. 2005; Peres et al. 2018; Sales et al. 2018). CBD is also an agonist of the peroxisome proliferator-activated receptor γ (PPAR-γ)

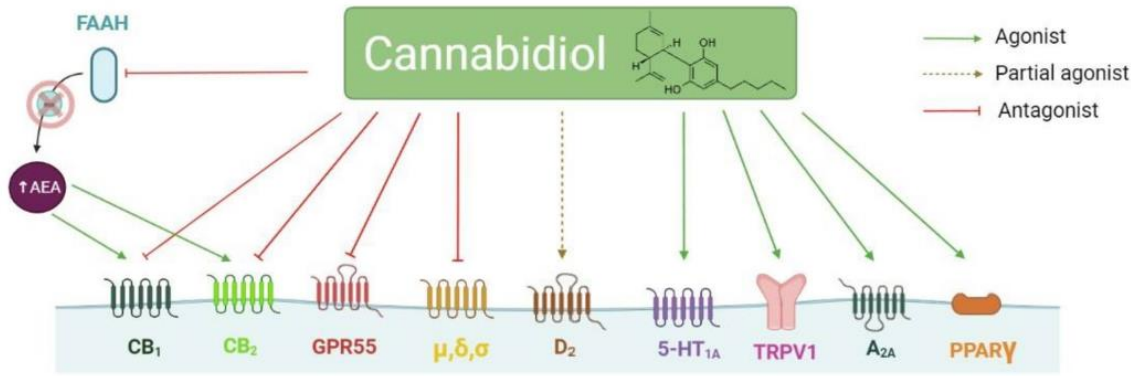


Fig. 3 Multiple molecular targets of cannabidiol (CBD). CBD has low affinity to cannabinoid receptors (CB_1 and CB_2) and may act as a negative allosteric modulator of CB_1 receptors as well as an inverse agonist of the CB_2 receptors. CBD actions at CB receptors are primarily due to its indirect effects through inhibition of fatty acid amide hydrolase (FAAH), which leads to an increase of anandamide levels such as inhibiting the enzymatic hydrolysis and the uptake of anadamide (AEA). Anandamide, in turn, activates CB_1 and CB_2 receptors. CBD acts as opioid receptors (μ, δ, and σ) and G-protein-coupled receptors (GPR55) antagonist. A partial agonist action of CBD on dopamine 2 receptor (D2) has been also proposed. Several pharmacological effects of CBD are related to the activation of 5-hydroxytryptamine 1 A receptor ($5\text{-}HT_{1A}$), transient receptor potential (TRPV1), peroxisome proliferator-activated receptor γ (PPAR-γ), and adenosine receptor (A_{2A})

receptor which participated in the modulation of inflammation by inhibiting pro-inflammatory pathways including the nuclear factor kappa B (NFκB)-mediated inflammatory signaling (Vallée et al. 2017). In addition, CBD activity can be enhanced by the action of AEA and 2-AG, which are $5\text{-}HT_{1A}$ (Haj-Dahmane and Shen 2011) and PPAR-γ (O'Sullivan 2016) direct agonists and whose levels are elevated by CBD. Other proposed molecular targets for CBD are the transient receptor potential (TRP) including the TRPV1, TRPV2, and TRPA1 (De Petrocellis et al. 2011; Morelli et al. 2014). These receptors belong to a family of ion channels implicated in the modulation of neuronal hyperactivity. The opioid system is also a target for CBD actions. At micromolar concentrations, CBD inhibits μ, δ, and σ opioid receptors (Rodríguez-Muñoz et al. 2018). A partial agonist action on the dopamine 2 (D_2) receptor has also been proposed to CBD (Seeman 2016). Besides, CBD stimulates adenosine A_{2A} receptor signaling (Ribeiro et al. 2012) and modulates energy metabolism by increasing mitochondrial complex and creatine kinase activity (Valvassori et al. 2013). Finally, CBD may act towards the orphan G-protein-coupled receptors (GPR55), a class of receptors involved in synaptic transmission (Ross 2009).

Neuroprotective Mechanisms

Studies on the effects of CBD in cerebral ischemic conditions from the 2000s are listed in Table S1. CBD (3 mg/kg, *i.p.*) when given either immediately before or 3 h after MCAo reduced the infarct size, ameliorated the survival rate, and increased the cerebral blood flow in ddY mice (Hayakawa et al. 2004; 2007a, b, 2008; Mishima et al. 2005). The effects of CBD were independent of CB_1 or CB_2 receptor activation but inhibited by the $5\text{-}HT_{1A}$ receptor antagonist, WAY100135. Other studies demonstrated that CBD (3 mg/kg, *i.p.*) repeated treatment (12–14 days) from 1 day or 3 days after CI improved motor coordination and survival rates in mice with MCAo. In these studies, CBD prevented the increase of the inflammation mediator, the high-mobility group box1 (HMGB1), levels in plasma and decreased the number of microglia, and reduced apoptosis in the brain (Hayakawa et al. 2008; 2009). However, CBD (3 mg/kg) administered from day 5 did not change functional outcomes on day 14 after MCAo in mice (Hayakawa et al. 2009). Similar neuroprotective effects of CBD were detected in rats subjected to MCAo. Ceprián et al. (2017) observed that only a CBD dose (5 mg/kg, *i.p.*) administered 15 min after ischemic stroke could prevent neurological and sensory impairments, as well as decrease microglial reactivity in the brain of ischemic rats.

Neuroprotective effects of CBD on cerebral ischemic injury have also been demonstrated when it is directly injected into the brain. Infusion of CBD (100 and 200 ng, *i.c.v.*) into the lateral ventricles 5 days before ischemic injury resulted in a significant reduction in the neurological deficits, brain edema, and BBB permeability provoked by MCAo in rats. Concomitantly, downregulation of TNF-α, TNFR1, and NF-κB expression was observed by CBD in the core and penumbra areas of MCAo rats (Khaksar and Bigdeli 2017a, b). In the MCA electrocoagulation model, CBD (10 nmol, *i.c.v.*) decreased the infarct size in mice. In this study, the CBD effects were reduced by σ1 receptor

agonists and absent in σ1 receptor knockout mice. These findings suggest that CBD displays an antagonist-like activity towards the σ1 receptor against neuronal injury caused by CI (Rodríguez-Muñoz et al. 2018).

Concerning animal models of global CI, Braida et al. (2003) have demonstrated that gerbils with BCCAO and treated with only a dose of CBD (15 mg/kg, *i.p.*), 5 min after injury, presented a reduction in the hippocampal cell loss and hyperlocomotion induced by ischemia (Table S1). In mice, 17–20 min of BCCAO results in hippocampal neurodegeneration, neuroinflammation, and functional deficits characterized by cognitive impairments and increased expression of anxiety- and despair-like behaviors. Short-term CBD treatment (10 mg/kg, *i.p.*, 30 min before, 3, 24, and 48 h after ischemia) ameliorated the functional impairments, attenuated hippocampal neurodegeneration and inflammation (microglia and astrocyte reactivity), and reduced white matter injury in ischemic mice (Schiavon et al. 2014; Mori et al. 2017). Moreover, CBD stimulated neurogenesis and promoted dendritic restructuring in the hippocampus of BCCAO mice. In a recent study, Mori et al. (2021) have demonstrated the differential contribution of CB_1, CB_2, PPAR-γ, and 5-HT_{1A} receptors in the functional recovery of mice subjected to BCCAO. For example, the anxiolytic-like effects of CBD (10 mg/kg, *i.p.*) were attenuated by CB_1, CB_2, PPAR-γ, and 5-HT_{1A} receptor antagonists while the cognitive performance was mediated by CB_1 receptor activation.

Interested in the possible effects of CBD on neural plasticity, Meyer et al. (2021) have demonstrated that CBD (10 mg/kg, *i.p.*), administered 30 min before and then once daily for 14 days, increased the brain-derived neurotrophic factor (BDNF) levels and the dendritic spine number and the length of dendritic arborization in the hippocampus of rats subjected to 4-VO/ICA occlusion model of TGCI. In addition, CBD prevented memory impairments and attenuated hippocampal neurodegeneration in those ischemic rats.

As shown in Table S1, the effects of CBD were also studied in animal models of HI. In newborn piglets subjected to BCCAO plus hypoxia, CBD (1 mg/kg, *i.v.*) administered following the insult (15–240 min after) reduced seizures frequency, prevented brain damage and edema, and decreased cerebral hemodynamic impairments (Alvarez et al. 2008; Lafuente et al. 2011; Pazos et al. 2012). Moreover, CBD decreased neuroinflammation and TNF-α levels and reduced oxidative stress (Pazos et al. 2012; 2013; Lafuente et al. 2016). These findings agreed with previous studies in vitro showing that CBD reduced the release of glutamate, decreased oxidative stress and inflammation, and normalizes the release of cytokines as well as the induction of inducible NOS and COX-2 in forebrain slices of newborn mice exposed to oxygen-glucose (OG) deprivation (Castillo et al. 2010). Co-incubation of CBD with WAY635100 (5-HT_{1A} receptor antagonist), AM630 (CB_2 receptor antagonist), or SCH58261 (A_{2A} receptor antagonist) abolished those CBD protective effects, indicating the involvement of 5-HT_{1A}, CB_2, and A_{2A} receptors in the CBD protective effects also in immature brain cells of piglets (Castillo et al. 2010).

Lafuente et al. (2016) demonstrated that a combination of CBD treatment and therapeutic hypothermia produced an accumulative effect on excitotoxicity, oxidative stress, neuronal death, and enhanced levels of caspase-3 and TNF-α in HI newborn piglets. However, CBD (1–50 mg/kg, *i.v.*) 15–20 min after HI did not present significant protection in newborn piglets as measured by the neurological score, levels of astrocytic marker S100B in cerebrospinal fluid, and magnetic resonance spectroscopy (Garberg et al. 2016; 2017). In addition, high doses of CBD (25–50 mg/kg) induced severe hypotension in HI newborn piglets (Garberg et al. 2017).

Similar to newborn piglets, HI injury in newborn rats and mice results in long-lasting myelinization disturbance characterized by OL and myelin basic protein (MBP) loss which are directly related to long-term functional impairments. Newborn rats received CBD (1 mg/kg, *s.c.*) after brain damage induced by left carotid artery electrocoagulation plus 10% oxygen for 112 min (electrocoagulation plus hypoxia). CBD administration resulted in normal function associated with normal OL and MBP expression, as well as normal axon density and myelin thickness in the cerebral cortex and white matter in HI rats (Ceprián et al. 2019). These results add information to CBD neuroprotective effects on white matter injury induced by CI.

Perspectives and Future Directions

Current pharmacological treatments for cerebral ischemia are limited, and often accompanied by significant side effects. The pleiotropic action of CBD indicates that it can affect many pathophysiological processes resulting in improvement of cerebral ischemia outcomes. Even though promising results have been already obtained in different animal models, further molecular, cellular, and animal studies are needed to elucidate CBD pharmacological profile to allow a successful translation of preclinical research to clinical settings.

Conclusions

It has become clear that CBD (**1**) represents a valuable compound for the treatment and management of cerebral ischemic conditions. The complex pathogenesis of CI associated with the multi-target effects of CBD indicates a potential and promising future of CBD as a neuroprotective

agent. Antioxidant, anti-inflammatory, antiapoptotic, and neuroplastic effects are believed to be involved in the CBD neuroprotective effects. Besides, activation of 5-HT_{1A}, PPAR-γ, and CB_2 receptors, among others, may also mediate the beneficial CBD effects in CI.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s43450-021-00199-6.

Author Contribution Both EM and RMWO wrote the article. BAM prepared the supplementary material and figures. FK made a critical revision of the article before submission to the Journal. All authors discussed and commented on the manuscript.

Funding This work is based on a collaboration supported by Serviço Alemão de Intercâmbio Acadêmico (DAAD)/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)—PROBRAL, University of Maringá, Maringá, Brazil, and University of Saarland, Homburg, Germany. EM benefits from a PhD scholarship provided by CAPES and BAM benefits from a PhD scholarship provided by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Declarations

Conflict of Interest The authors declare no competing interests.

References

Alvarez FJ, Lafuente H, Rey-Santano MC, Mielgo VE, Gastiasoro E, Rueda M, Pertwee RG, Castillo AI, Romero J, Martínez-Orgado J (2008) Neuroprotective effects of the nonpsychoactive cannabinoid cannabidiol in hypoxic-ischemic newborn piglets. Pediatr Res 64:653–658. https://doi.org/10.1203/PDR.0b013e318186e5dd

Anderson CA, Arciniegas DB (2010) Cognitive sequelae of hypoxic-ischemic brain injury: a review. NeuroRehabilitation 26:47–63. https://doi.org/10.3233/NRE-2010-0535

Atalay S, Dobrzyńska I, Gęgotek A, Skrzydlewska E (2020) Cannabidiol protects keratinocyte cell membranes following exposure to UVB and hydrogen peroxide. Redox Biol 36:101613. https://doi.org/10.1016/j.redox.2020.101613

Back T, Otto D, Kittner D, Schüler OG, Hennerici MG, Mennel HD (2007) Failure to improve the effect of thrombolysis by memantine in a rat embolic stroke model. Neurol Res 29:264–269. https://doi.org/10.1179/174313206X154012

Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, Moriello AS, Davis JB, Mechoulam R, Di Marzo V (2001) Molecular targets for cannabidiol and its synthetic analogs: effect on vanilloid VR1 receptors and one cellular uptake and enzymatic hydrolysis of anandamide. Br J Pharmacol 134:845–852. https://doi.org/10.1038/sj.bjp.0704327

Borges RS, Batista J Jr, Viana RB, Baetas AC, Orestes E, Andrade MA, Honório KM, da Silva AB (2013) Understanding the molecular aspects of tetrahydrocannabinol and cannabidiol as antioxidants. Molecules 18:12663–12674. https://doi.org/10.3390/molecules181012663

Braida D, Pegorini S, Arcidiacono MV, Consalez GG, Croci L, Sala M (2003) Post-ischemic treatment with cannabidiol prevents electroencephalographic flattening, hyperlocomotion and neuronal injury in gerbils. Neurosci Lett 346:61–64. https://doi.org/10.1016/s0304-3940(03)00569-x

Britch SC, Babalonis S, Walsh SL (2021) Cannabidiol: pharmacology and therapeutic targets. Psychopharmacology 238:9–28. https://doi.org/10.1007/s00213-020-05712-8

Campbell BCV, Khatri P (2020) Stroke. Lancet 396:129–142. https://doi.org/10.1016/S0140-6736(20)31179-X

Campbell B, De Silva DA, Macleod MR, Coutts SB, Schwamm LH, Davis SM, Donnan GA (2019) Ischaemic stroke. Nat Ver Dis Primers 5:70. https://doi.org/10.1038/s41572-019-0118-8

Campos AC, Fogaça MV, Sonego AB, Guimarães FS (2016) Cannabidiol, neuroprotection and neuropsychiatric disorders. Pharmacol Res 112:119–127. https://doi.org/10.1016/j.phrs.2016.01.033

Castillo A, Tolón MR, Fernández-Ruiz J, Romero J, Martinez-Orgado J (2010) The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB(2) and adenosine receptors. Neurobiol Dis 37:434–440. https://doi.org/10.1016/j.nbd.2009.10.023

Catanese L, Tarsia J, Fisher M (2017) Acute ischemic stroke therapy overview. Circ Res 120:541–558. https://doi.org/10.1161/CIRCRESAHA.116.309278

Cechetti F, Worm PV, Pereira LO, Siqueira IR, Netto CA (2010) The modified 2VO ischemia protocol causes cognitive impairment similar to that induced by the standard method, but with a better survival rate. Braz J Med Biol Res 43:1178–1183. https://doi.org/10.1590/s0100-879x2010007500124

Ceprián M, Jiménez-Sánchez L, Vargas C, Barata L, Hind W, Martínez-Orgado J (2017) Cannabidiol reduces brain damage and improves functional recovery in a neonatal rat model of arterial ischemic stroke. Neuropharmacology 116:151–159. https://doi.org/10.1016/j.neuropharm.2016.12.017

Ceprián M, Vargas C, García-Toscano L, Penna F, Jiménez-Sánchez L, Achicallende S, Elezgarai I, Grandes P, Hind W, Pazos MR, Martínez-Orgado J (2019) Cannabidiol administration prevents hypoxia-ischemia-induced hypomyelination in newborn rats. Front Pharmacol 10:1131. https://doi.org/10.3389/fphar.2019.01131

Chung H, Fierro A, Pessoa-Mahana CD (2019) Cannabidiol binding and negative allosteric modulation at the cannabinoid type 1 receptor in the presence of delta-9-tetrahydrocannabinol: an *in silico* study. PLoS ONE 14:e0220025. https://doi.org/10.1371/journal.pone.0220025

Citti C, Braghiroli D, Vandelli MA, Cannazza G (2018) Pharmaceutical and biomedical analysis of cannabinoids: a critical review. J Pharm Biomed Anal 147:565–579. https://doi.org/10.1016/j.jpba.2017.06.003

Cristino L, Bisogno T, Di Marzo V (2020) Cannabinoids and the expanded endocannabinoid system in neurological disorders. Nat Ver Neurol 16:9–29. https://doi.org/10.1038/s41582-019-0284-z

Cronin CA (2010) Intravenous tissue plasminogen activator for stroke: a review of the ECASS III results in relation to prior clinical trials. J Emerg Med 38:99–105. https://doi.org/10.1016/j.jemermed.2009.08.004

De Petrocellis L, Ligresti A, Moriello AS, Allarà M, Bisogno T, Petrosino S, Stott CG, Di Marzo V (2011) Effects of cannabinoids and cannabinoid-enriched *Cannabis* extracts on TRP channels and endocannabinoid metabolic enzymes. Br J Pharmacol 163:1479–1494. https://doi.org/10.1111/j.1476-5381.2010.01166.x

De Vos L, De Herdt V, Timmermans F (2019) Misdiagnosis or missed diagnosis: digging out the "Near-Field Clutter" artifact in a patient with stroke. CASE (Phila) 4:2–6. https://doi.org/10.1016/j.case.2019.10.007

Demaerschalk BM, Kleindorfer DO, Adeoye OM, Demchuk AM, Fugate JE, Grotta JC, Khalessi AA, Levy EI, Palesch YY, Prabhakaran S, Saposnik G, Saver JL, Smith EE, American Heart Association Stroke Council and Council on Epidemiology and

Prevention (2016) Scientific rationale for the inclusion and exclusion criteria for intravenous alteplase in acute ischemic stroke: a statement for healthcare professionals from the American heart association/American stroke association. Stroke 47:581–641. https://doi.org/10.1161/STR.0000000000000086

Devinsky O, Patel AD, Cross JH, Villanueva V, Wirrell EC, Privitera M, Greenwood SM, Roberts C, Checketts D, VanLandingham KE, Zuberi SM, GWPCARE3 Study Group (2018) Effect of cannabidiol on drop seizures in the lennox-gastaut syndrome. N Engl J Med 378:1888–1897. https://doi.org/10.1056/NEJMoa1714631

Di Marzo V, Bifulco M, De Petrocellis L (2004) The endocannabinoid system and its therapeutic exploitation. Nat Ver Drug Discov 3:771–784. https://doi.org/10.1038/nrd1495

Dirnagl U (2012) Pathobiology of injury after stroke: the neurovascular unit and beyond. Ann N Y Acad Sci 1268:21–25. https://doi.org/10.1111/j.1749-6632.2012.06691.x

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci 22:391–397. https://doi.org/10.1016/s0166-2236(99)01401-0

Donkor ES (2018) Stroke in the 21st century: a snapshot of the burden, epidemiology, and quality of life. Stroke Res Treat 2018:3238165. https://doi.org/10.1155/2018/3238165

Farag S, Kayser O (2017) The cannabis plant: botanical aspects. In: Preedy VR (ed) Handbook of Cannabis and Related Pathologies. Academic Press, London, pp 3–12. https://doi.org/10.1016/B978-0-12-800756-3.00001-6

Gale C, Statnikov Y, Jawad S, Uthaya SN, Modi N, Injuries B, expert working group, (2018) Neonatal brain injuries in England: population-based incidence derived from routinely recorded clinical data held in the National Neonatal Research Database. Arch Dis Child Fetal Neonatal Ed 103:F301–F306. https://doi.org/10.1136/archdischild-2017-313707

Garberg HT, Huun MU, Escobar J, Martinez-Orgado J, Løberg EM, Solberg R, Didrik Saugstad O (2016) Short-term effects of cannabidiol after global hypoxia-ischemia in newborn piglets. Pediatr Res 80:710–718. https://doi.org/10.1038/pr.2016.149

Garberg HT, Solberg R, Barlinn J, Martinez-Orgado J, Løberg EM, Saugstad OD (2017) High-dose cannabidiol induced hypotension after global hypoxia-ischemia in piglets. Neonatology 112:143–149. https://doi.org/10.1159/000471786

García-Chávez D, González-Burgos I, Letechipía-Vallejo G, López-Loeza E, Moralí G, Cervantes M (2008) Long-term evaluation of cytoarchitectonic characteristics of prefrontal cortex pyramidal neurons, following global cerebral ischemia and neuroprotective melatonin treatment, in rats. Neurosci Lett 448:148–152. https://doi.org/10.1016/j.neulet.2008.10.043

García-Gutiérrez MS, Navarrete F, Gasparyan A, Austrich-Olivares A, Sala F, Manzanares J (2020) Cannabidiol: a potential new alternative for the treatment of anxiety, depression, and psychotic disorders. Biomolecules 10:1575. https://doi.org/10.3390/biom10111575

GBD 2017 US Neurological Disorders Collaborators (2021) Burden of neurological disorders across the us from 1990–2017: a global burden of disease study. JAMA Neurol 78:165–176. https://doi.org/10.1001/jamaneurol.2020.4152

Gennaro M, Mattiello A, Pizzorusso T (2019) Rodent models of developmental ischemic stroke for translational research: strengths and weaknesses. Neural Plast 2019:5089321. https://doi.org/10.1155/2019/5089321

George PM, Steinberg GK (2015) Novel stroke therapeutics: unraveling stroke pathophysiology and its impact on clinical treatments. Neuron 87:297–309. https://doi.org/10.1016/j.neuron.2015.05.041

Greco P, Nencini G, Piva I, Scioscia M, Volta CA, Spadaro S, Neri M, Bonaccorsi G, Greco F, Cocco I, Sorrentino F, D'Antonio F, Nappi L (2020) Pathophysiology of hypoxic-ischemic encephalopathy: a review of the past and a view on the future. Acta Neurol Belg 120:277–288. https://doi.org/10.1007/s13760-020-01308-3

Gulyás B, Tóth M, Schain M, Airaksinen A, Vas A, Kostulas K, Lindström P, Hillert J, Halldin C (2012) Evolution of microglial activation in ischaemic core and peri-infarct regions after stroke: a PET study with the TSPO molecular imaging biomarker [((11))C]vinpocetine. J Neurol Sci 320:110–117. https://doi.org/10.1016/j.jns.2012.06.026

Haj-Dahmane S, Shen RY (2011) Modulation of the serotonin system by endocannabinoid signaling. Neuropharmacology 61:414–420. https://doi.org/10.1016/j.neuropharm.2011.02.016

Haley MJ, Lawrence CB (2017) The blood-brain barrier after stroke: structural studies and the role of transcytotic vesicles. J Cereb Blood Flow Metab 37:456–470. https://doi.org/10.1177/0271678X16629976

Hamdy N, Eide S, Sun HS, Feng ZP (2020) Animal models for neonatal brain injury induced by hypoxic ischemic conditions in rodents. Exp Neurol 334:113457. https://doi.org/10.1016/j.expneurol.2020.113457

Hardy K (2020) Paleomedicine and the evolutionary context of medicinal plant use. Rev Bras Farmacogn 31:1–15. https://doi.org/10.1007/s43450-020-00107-4

Hayakawa K, Mishima K, Abe K, Hasebe N, Takamatsu F, Yasuda H, Ikeda T, Inui K, Egashira N, Iwasaki K, Fujiwara M (2004) Cannabidiol prevents infarction via the non-CB1 cannabinoid receptor mechanism. NeuroReport 15:2381–2385. https://doi.org/10.1097/00001756-200410250-00016

Hayakawa K, Mishima K, Nozako M, Hazekawa M, Irie K, Fujioka M, Orito K, Abe K, Hasebe N, Egashira N, Iwasaki K, Fujiwara M (2007a) Delayed treatment with cannabidiol has a cerebroprotective action via a cannabinoid receptor-independent myeloperoxidase-inhibiting mechanism. J Neurochem 102:1488–1496. https://doi.org/10.1111/j.1471-4159.2007.04565.x

Hayakawa K, Mishima K, Nozako M, Ogata A, Hazekawa M, Liu AX, Fujioka M, Abe K, Hasebe N, Egashira N, Iwasaki K, Fujiwara M (2007b) Repeated treatment with cannabidiol but not delta9-tetrahydrocannabinol has a neuroprotective effect without the development of tolerance. Neuropharmacology 52:1079–1087. https://doi.org/10.1016/j.neuropharm.2006.11.005

Hayakawa K, Mishima K, Irie K, Hazekawa M, Mishima S, Fujioka M, Orito K, Egashira N, Katsurabayashi S, Takasaki K, Iwasaki K, Fujiwara M (2008) Cannabidiol prevents a post-ischemic injury progressively induced by cerebral ischemia via a high-mobility group box1-inhibiting mechanism. Neuropharmacology 55:1280–1286. https://doi.org/10.1016/j.neuropharm.2008.06.040

Hayakawa K, Irie K, Sano K, Watanabe T, Higuchi S, Enoki M, Nakano T, Harada K, Ishikane S, Ikeda T, Fujioka M, Orito K, Iwasaki K, Mishima K, Fujiwara M (2009) Therapeutic time window of cannabidiol treatment on delayed ischemic damage via high-mobility group box1-inhibiting mechanism. Biol Pharm Bull 32:1538–1544. https://doi.org/10.1248/bpb.32.1538

Hermann DM, Kleinschnitz C (2019) Modeling vascular risk factors for the development of ischemic stroke therapies. Stroke 50:1310–1317. https://doi.org/10.1161/STROKEAHA.118.024673

Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, Gao Y, Chen J (2012) Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke 43:3063–3070. https://doi.org/10.1161/STROKEAHA.112.659656

Iadecola C, Ross ME (1997) Molecular pathology of cerebral ischemia: delayed gene expression and strategies for neuroprotection. Ann

NY Acad Sci 835:203–217. https://doi.org/10.1111/j.1749-6632.1997.tb48631.x

Karsy M, Brock A, Guan J, Taussky P, Kalani MY, Park MS (2017) Neuroprotective strategies and the underlying molecular basis of cerebrovascular stroke. Neurosurg Focus 42:E3. https://doi.org/10.3171/2017.1.FOCUS16522

Khaksar S, Bigdeli MR (2017a) Intra-cerebral cannabidiol infusion-induced neuroprotection is partly associated with the TNF-α/TNFR1/NF-κB pathway in transient focal cerebral ischaemia. Brain Inj 31:1932–1943. https://doi.org/10.1080/02699052.2017.1358397

Khaksar S, Bigdeli MR (2017b) Anti-excitotoxic effects of cannabidiol are partly mediated by enhancement of NCX2 and NCX3 expression in animal model of cerebral ischemia. Eur J Pharmacol 794:270–279. https://doi.org/10.1016/j.ejphar.2016.11.011

Khodanovich MYu, Kisel AA (2015) Animal models of cerebral ischemia. AIP Conf Proc 1688https://doi.org/10.1063/1.4936032

Knowland D, Arac A, Sekiguchi KJ, Hsu M, Lutz SE, Perrino J, Steinberg GK, Barres BA, Nimmerjahn A, Agalliu D (2014) Stepwise recruitment of transcellular and paracellular pathways underlies blood-brain barrier breakdown in stroke. Neuron 82:603–617. https://doi.org/10.1016/j.neuron.2014.03.003

Lafuente H, Alvarez FJ, Pazos MR, Alvarez A, Rey-Santano MC, Mielgo V, Murgia-Esteve X, Hilario E, Martinez-Orgado J (2011) Cannabidiol reduces brain damage and improves functional recovery after acute hypoxia-ischemia in newborn pigs. Pediatr Res 70:272–277. https://doi.org/10.1203/PDR.0b013e3182276b11

Lafuente H, Pazos MR, Alvarez A, Mohammed N, Santos M, Arizti M, Alvarez FJ, Martinez-Orgado JA (2016) Effects of Cannabidiol and hypothermia on short-term brain damage in new-born piglets after acute hypoxia-ischemia. Front Neurosci 10:323. https://doi.org/10.3389/fnins.2016.00323

Lapchak PA, Zhang JH (2017) The high cost of stroke and stroke cytoprotection research. Transl Stroke Res 8:307–317. https://doi.org/10.1007/s12975-016-0518-y

Laprairie RB, Bagher AM, Kelly ME, Denovan-Wright EM (2016) Biased type 1 Cannabinoid receptor signaling influences neuronal viability in a cell culture model of Huntington disease. Mol Pharmacol 89:364–375. https://doi.org/10.1124/mol.115.101980

Larsen C, Shahinas J (2020) Dosage, efficacy and safety of Cannabidiol administration in adults: a systematic review of human trials. J Clin Med Res 12:129–141. https://doi.org/10.14740/jocmr4090

Lehtonen L, Gimeno A, Parra-Llorca A, Vento M (2017) Early neonatal death: a challenge worldwide. Semin Fetal Neonatal Med 22:153–160. https://doi.org/10.1016/j.siny.2017.02.006

Leker RR, Shoham E (2002) Cerebral ischemia and trauma-different etiologies yet similar mechanisms: neuroprotective opportunities. Brain Res Rev 39:55–73. https://doi.org/10.1016/s0165-0173(02)00157-1

León-Moreno LC, Castañeda-Arellano R, Rivas-Carrillo JD, Dueñas-Jiménez SH (2020) Challenges and improvements of developing an ischemia mouse model through bilateral common carotid artery occlusion. J Stroke Cerebrovasc Dis 29:104773. https://doi.org/10.1016/j.jstrokecerebrovasdis.2020.104773

Li LX, Cheng YF, Lin HB, Wang C, Xu JP, Zhang HT (2011) Prevention of cerebral ischemia-induced memory deficits by inhibition of phosphodiesterase-4 in rats. Metan Brain Dis 26:37–47. https://doi.org/10.1007/s11011-011-9235-0

Li Y, Cao Y, Xiao J, Shang J, Tan Q, Ping F, Huang W, Wu F, Zhang H, Zhang X (2020) Inhibitor of apoptosis-stimulating protein of p53 inhibits ferroptosis and alleviates intestinal ischemia/reperfusion-induced acute lung injury. Cell Death Differ 27:2635–2650. https://doi.org/10.1038/s41418-020-0528-x

Liaw N, Liebeskind D (2020) Emerging therapies in acute ischemic stroke. F1000Res 9:F1000 Faculty Rev-546. https://doi.org/10.12688/f1000research.21100.1

Lindsay MP, Norrving B, Sacco RL, Brainin M, Hacke W, Martins S, Pandian J, Feigin V (2019) World Stroke Organization (WSO): global stroke fact sheet. Int J Stroke 14:806–817. https://doi.org/10.1177/1747493019881353

Liu YC, Tsai YH, Tang SC, Liou HC, Kang KH, Liou HH, Jeng JS, Fu WM (2018) Cytokine MFI enhances blood-brain barrier permeability: impact for therapy in ischemic stroke. Sci Rep 8:743. https://doi.org/10.1038/s41598-017-16927-9

Macrae IM (2011) Preclinical stroke research–advantages and disadvantages of the most common rodent models of focal ischaemia. Br J Pharmacol 164:1062–1078. https://doi.org/10.1111/j.1476-5381.2011.01398.x

Martínez-Orgado J, Fernández-López D, Lizasoain I, Romero J (2007) The seek of neuroprotection: introducing cannabinoids. Recent Pat CNS Drug Discov 2:131–139. https://doi.org/10.2174/157488907780832724

Mechoulam R, Parker LA, Gallily R (2002) Cannabidiol: an overview of some pharmacological aspects. J Clin Pharmacol 42:11S-19S. https://doi.org/10.1002/j.1552-4604.2002.tb05998.x

Mehta SL, Manhas N, Raghubir R (2007) Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res Rev 54:34–66. https://doi.org/10.1016/j.brainresrev.2006.11.003

Meyer E, Bonato JM, Mori MA, Mattos BA, Guimarães FS, Milani H, de Campos AC, de Oliveira R (2021) Cannabidiol confers neuroprotection in rats in a model of transient global cerebral ischemia: impact of hippocampal synaptic neuroplasticity. Mol Neurobiol [Epub ahead of print]. https://doi.org/10.1007/s12035-021-02479-7

Millar LJ, Shi L, Hoerder-Suabedissen A, Molnár Z (2017) Neonatal hypoxia ischaemia: mechanisms, models, and therapeutic challenges. Front Cell Neurosci 11:78. https://doi.org/10.3389/fncel.2017.00078

Mishima K, Hayakawa K, Abe K, Ikeda T, Egashira N, Iwasaki K, Fujiwara M (2005) Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine1A receptor-dependent mechanism. Stroke 36:1077–1082. https://doi.org/10.1161/01.STR.0000163083.59201.34

Mohammed N, Ceprian M, Jimenez L, Pazos MR, Martínez-Orgado J (2017) Neuroprotective effects of Cannabidiol in hypoxic ischemic insult. The therapeutic window in newborn mice. CNS Neurol Disord Targets 16:102–108. https://doi.org/10.2174/1871527315666160927110305

Morelli MB, Offidani M, Alesiani F, Discepoli G, Liberati S, Olivieri A, Santoni M, Santoni G, Leoni P, Nabissi M (2014) The effects of cannabidiol and its synergism with bortezomib in multiple myeloma cell lines. A role for transient receptor potential vanilloid type-2. Int J Cancer 134:2534–2546. https://doi.org/10.1002/ijc.28591

Mori MA, Meyer E, Soares LM, Milani H, Guimarães FS, de Oliveira R (2017) Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia. Prog Neuropsychopharmacol Biol Psychiatry 75:94–105. https://doi.org/10.1016/j.pnpbp.2016.11.005

Mori MA, Meyer E, da Silva FF, Milani H, Guimarães FS, Oliveira R (2021) Differential contribution of CB1, CB2, 5-HT1A, and PPAR-γ receptors to cannabidiol effects on ischemia-induced emotional and cognitive impairments. Eur J Neurosci 53:1738–1751. https://doi.org/10.1111/ejn.15134

Muller C, Morales P, Reggio PH (2019) Cannabinoid ligands targeting TRP channels. Front Mol Neurosci 15:487–518. https://doi.org/10.3389/fnmol.2018.00487

O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW (2006) 1,026 experimental treatments in acute

stroke. Ann Neurol 59:467–477. https://doi.org/10.1002/ana.20741

O'Sullivan SE (2016) An update on PPAR activation by cannabinoids. Br J Pharmacol 173:1899–1910. https://doi.org/10.1111/bph.13497

Pazos MR, Cinquina V, Gómez A, Layunta R, Santos M, Fernández-Ruiz J, Martínez-Orgado J (2012) Cannabidiol administration after hypoxia-ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function. Neuropharmacology 63:776–783. https://doi.org/10.1016/j.neuropharm.2012.05.034

Pazos MR, Mohammed N, Lafuente H, Santos M, Martínez-Pinilla E, Moreno E, Valdizan E, Romero J, Pazos A, Franco R, Hillard CJ, Alvarez FJ, Martínez-Orgado J (2013) Mechanisms of Cannabidiol neuroprotection in hypoxic-ischemic newborn pigs: role of 5HT(1A) and CB2 receptors. Neuropharmacology 71:282–291. https://doi.org/10.1016/j.neuropharm.2013.03.027

Peres FF, Diana MC, Levin R, Suiama MA, Almeida V, Vendramini AM, Santos CM, Zuardi AW, Hallak J, Crippa JÁ, Abílio VC (2018) Cannabidiol administered during peri-adolescence prevents behavioral abnormalities in an animal model of schizophrenia. Front Pharmacol 9:901. https://doi.org/10.3389/fphar.2018.00901

Pertwee RG (2008) The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, Cannabidiol and delta9-tetrahydrocannabivarin. Br J Pharmacol 153:199–215. https://doi.org/10.1038/sj.bjp.0707442

Povroznik JM, Ozga JE, Vonder Haar C, Engler-Chiurazzi EB (2018) Executive (dys)function after stroke: special considerations for behavioral pharmacology. Behav Pharmacol 29:638–653. https://doi.org/10.1097/FBP.0000000000000432

Prakash R, Carmichael ST (2015) Blood-brain barrier breakdown and neovascularization processes after stroke and traumatic brain injury. Curr Opin Neurol 28:556–564. https://doi.org/10.1097/WCO.0000000000000248

Rajsic S, Gothe H, Borba HH, Sroczynski G, Vujicic J, Toell T, Siebert U (2019) Economic burden of stroke: a systematic review on post-stroke care. Eur J Health Econ 20:107–134. https://doi.org/10.1007/s10198-018-0984-0

Raval AP, Bhatt A, Saul I (2009) Chronic nicotine exposure inhibits 17beta-estradiol-mediated protection of the hippocampal CA1 region against cerebral ischemia in female rats. Neurosci Lett 458:65–69. https://doi.org/10.1016/j.neulet.2009.04.021

Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Vitoretti LB, Mariano-Souza DP, Quinteiro-Filho WM, Akamine AT, Almeida VI, Quevedo J, Dal-Pizzol F, Hallak JE, Zuardi AW, Crippa JA, Palermo-Neto J (2012) Cannabidiol, a non-psychotropic plant-derived cannabinoid, decreases inflammation in a murine model of acute lung injury: role for the adenosine A(2A) receptor. Eur J Pharmacol 678:78–85. https://doi.org/10.1016/j.ejphar.2011.12.043

Rodríguez-Muñoz M, Onetti Y, Cortés-Montero E, Garzón J, Sánchez-Blázquez P (2018) Cannabidiol enhances morphine antinociception, diminishes NMDA-mediated seizures and reduces stroke damage via the sigma 1 receptor. Mol Brain 11:51. https://doi.org/10.1186/s13041-018-0395-2

Rolls A, Shechter R, Schwartz M (2009) The bright side of the glial scar in CNS repair. Net Rev Neurosci 10:235–241. https://doi.org/10.1038/nrn2591

Ross RA (2009) The enigmatic pharmacology of GPR55. Trends Pharmacol Sci 30:156–163. https://doi.org/10.1016/j.tips.2008.12.004

Russo EB, Burnett A, Hall B, Parker KK (2005) Agonistic properties of cannabidiol at 5-HT1a receptors. Neurochem Res 30:1037–1043. https://doi.org/10.1007/s11064-005-6978-1

Sales AJ, Crestani CC, Guimarães FS, Joca S (2018) Antidepressant-like effect induced by cannabidiol is dependent on brain serotonin levels. Prog Neuropsychotopharmacol Biol Psychiatry 86:255–261. https://doi.org/10.1016/j.pnpbp.2018.06.002

Sánchez-Gómez MV, Alberdi E, Ibarretxe G, Torre I, Matute C (2003) Caspase-dependent and caspase-independent oligodendrocyte death mediated by AMPA and kainate receptors. J Neurosci 23:9519–9528. https://doi.org/10.1523/JNEUROSCI.23-29-09519.2003

Sánchez-Gómez MV, Alberdi E, Pérez-Navarro E, Alberch J, Matute C (2011) Bax and calpain mediate excitotoxic oligodendrocyte death induced by activation of both AMPA and kainate receptors. J Neurosci 31:2996–3006. https://doi.org/10.1523/JNEUROSCI.5578-10.2011

Sarma ND, Waye A, ElSohly MA, Brown PN, Elzinga S, Johnson HE, Marles RJ, Melanson JE, Russo E, Deyton L, Hudalla C, Vrdoljak GA, Wurzer JH, Khan IA, Kim NC, Giancaspro GI (2020) Cannabis inflorescence for medical purposes: USP considerations for quality attributes. J Nat Prod 83:1334–1351. https://doi.org/10.1021/acs.jnatprod.9b01200

Schiavon AP, Soares LM, Bonato JM, Milani H, Guimarães FS, Weffort de Oliveira RM (2014) Protective effects of cannabidiol against hippocampal cell death and cognitive impairment induced by bilateral common carotid artery occlusion in mice. Neurotox Res 26:307–316. https://doi.org/10.1007/s12640-014-9457-0

Seeman P (2016) Cannabidiol is a partial agonist at dopamine D2High receptors, predicting its antipsychotic clinical dose. Transl Psychiatry 6:e920. https://doi.org/10.1038/tp.2016.195

Sommer CJ (2017) Ischemic stroke: experimental models and reality. Acta Neuropathol 133:245–261. https://doi.org/10.1007/s00401-017-1667-0

Stinear CM, Barber PA (2020) Neurophysiology to guide acute stroke treatment. Clin Neurophysiol 131:2284–2285. https://doi.org/10.1016/j.clinph.2020.06.014

Syed YY, McKeage K, Scott LJ (2014) Delta-9-tetrahydrocannabinol/cannabidiol (Sativex®): a review of its use in patients with moderate to severe spasticity due to multiple sclerosis. Drugs 74:563–578. https://doi.org/10.1007/s40265-014-0197-5

Tham M, Yilmaz O, Alaverdashvili M, Kelly M, Denovan-Wright EM, Laprairie RB (2019) Allosteric and orthosteric pharmacology of cannabidiol and cannabidiol-dimethylheptyl at the type 1 and type 2 cannabinoid receptors. Br J Pharmacol 176:1455–1469. https://doi.org/10.1111/bph.14440

Traystman RJ (2003) Animal models of focal and global cerebral ischemia. ILAR J 44:85–95. https://doi.org/10.1093/ilar.44.2.85

Vallée A, Lecarpentier Y, Guillevin R, Vallée JN (2017) Effects of cannabidiol interactions with Wnt/β-catenin pathway and PPARγ on oxidative stress and neuroinflammation in Alzheimer's disease. Acta Biocheim Biophys Sin 49:853–866. https://doi.org/10.1093/abbs/gmx073

Valvassori SS, Bavaresco DV, Scaini G, Varela RB, Streck EL, Chagas MH, Hallak JE, Zuardi AW, Crippa JA, Quevedo J (2013) Acute and chronic administration of cannabidiol increases mitochondrial complex and creatine kinase activity in the rat brain. Braz J Psychiatry 35:380–386. https://doi.org/10.1590/1516-4446-2012-0886

Wang G, Guo Q, Hossain M, Fazio V, Zeynalov E, Janigro D, Mayberg MR, Namura S (2009) Bone marrow-derived cells are the major source of MMP-9 contributing to blood-brain barrier dysfunction and infarct formation after ischemic stroke in mice. Brain Res 1294:183–192. https://doi.org/10.1016/j.brainres.2009.07.070

Wang Y, Zhao Z, Chow N, Rajput PS, Griffin JH, Lyden PD, Zlokovic BV (2013) Activated protein C analog protects from ischemic stroke and extends the therapeutic window of tissue-type plasminogen activator in aged female mice and hypertensive rats.

Stroke 44:3529–3536. https://doi.org/10.1161/STROKEAHA.113.003350

White CM (2019) A review of human studies assessing cannabidiol's (CBD) therapeutic actions and potential. J Clin Pharmacol 59:923–934. https://doi.org/10.1002/jcph.1387

Yıldız EP, Ekici B, Tatlı B (2017) Neonatal hypoxic ischemic encephalopathy: an update on disease pathogenesis and treatment. Expert Rev Neurother 17:449–459. https://doi.org/10.1080/14737175.2017.1259567

SUPPLEMENTARY MATERIAL

Neuroprotective Effects of Cannabidiol Under Cerebral Ischemic Conditions

Table 1: Effects of Cannabidiol on *in vivo* models of cerebral ischemia

FOCAL CEREBRAL ISCHEMIA						
Reference	**Model**	**Animal species**	**CBD dose, route**	**Treatment schedule**	**CBD effects**	**Molecular mechanism**
Hayakawa et al., 2004	MCAO for 4 h	ddY mice	3 mg/kg, *i.p.*	Immediately before and 3 h after occlusion	↓ Infarct size	Effects not mediated by CB_1 R
Mishima et al., 2005	MCAO for 4 h	ddY mice	1 and 3 mg/kg, *i.p.*	Immediately before and 3 h after occlusion	↓ Infarct size ↑ Cerebral blood flow	$5HT_{1A}$ R Effects not mediated by CB_1 or TRPV1 R
Hayakawa et al., 2007a	MCAO for 4 h	ddY mice	3 mg/kg, *i.p.*	Immediately before and 3 h after occlusion + inject 14 days	↓ Infarct size ↑ Cerebral blood flow	$5HT_{1A}$ R Effects not mediated by CB_1 R
Hayakawa et al., 2007b	MCAO for 4 h	ddY mice	3 mg/kg, *i.p.*	Immediately before and 3 h after occlusion	↓ Motor impairment ↓ Infarct size ↑ Cerebral blood flow ↓ Neutrophil accumulation	Effects not mediated by CB_1 or CB_2 R
Hayakawa et al., 2008	MCAO for 4 h	ddY mice	3 mg/kg, *i.p.*	Immediately before and 3 h after occlusion	↓ Neurological/motor impairment ↓ Infarct size ↓ Neuronal death ↓ Number of microglia ↓ Neutrophil accumulation	Effects not mediated by CB1 or CB2 R
Hayakawa et al., 2009	MCAO for 4 h	ddY mice	3 mg/kg, *i.p.*	1 day after occlusion and daily for 14 days or 3 days after occlusion and daily for 12 days	↓ Neurological/motor impairment ↑ Survival rate ↓ HMGB1 levels ↓ Iba-1 expression	-

continue

Reference	Model	Animal species	CBD dose, route	Treatment schedule	CBD effects	Molecular mechanism
Ceprián et al., 2017	MCAo for 3 h	Wistar rats, 7-9 days	5 mg/kg, *i.p.*	15 min after reperfusion	↓ Neurological/motor/sensory impairment ↓ Excitotoxicity ↓ Microglial reactivity	-
Khaksar and Bigdeli, 2017a	MCAO for 60 min	Wistar rats	100 and 200 ng/rat, *i.c.v.*	During 5 days before occlusion	↓ Neurological impairment ↓ Infarct size ↓ Cerebral edema ↑ BBB integrity ↑ NCX expression	-
Khaksar and Bigdeli, 2017b	MCAO for 60 min	Wistar rats	100 and 200 ng/rat, *i.c.v.*	During 5 days before occlusion	↓ Neurological impairment ↓ Infarct size ↓ Cerebral edema ↓TNF-α, TNFR1 and NF-κB	-
Rodríguez-Munoz et al., 2018	MCA electro-coagulation	CD1 WT and KO σ1R$^{-/-}$ mice	10 nmol, *i.c.v.*	60 min after surgery	↓ Infarct size	σ1 R antagonism
GLOBAL CEREBRAL ISCHEMIA						
Reference	**Model**	**Animal species**	**CBD dose, route**	**Treatment schedule**	**CBD effects**	**Molecular mechanism**
Braida et al., 2003	BCCAO for 10 min	Gerbils	15 mg/kg, *i.p.*	5 min after BCCAO	↓ Hyperlocomotion ↓ Neuronal death in CA_1 subfield ↓ Electroencephalographic flattening	-
Schiavon et al., 2014	BCCAO for 17 min	Swiss mice	3, 10 and 30 mg/kg, *i.p.*	30 min before, 3, 24 e 48 h after BCCAO	↓ Cognitive impairments ↓ Neuronal death ↓ Astrocyte reactivity	-
Mori et al., 2017	BCCAO for 20 min	C57BL/6 mice	10 mg/kg, *i.p.*	30 min before, 3, 24 e 48 h after BCCAO	↓ Cognitive/emotional deficits ↓ Neuronal death ↓ Caspase-9 ↓ Astrocyte/microglia reactivity ↓ White matter injury ↑ BDNF/ DCX/ MAP2	-

continue

Reference	Model	Animal species	CBD dose, route	Treatment schedule	CBD effects	Molecular mechanism
Mori et al., 2021	BCCAO for 20 min	C57BL/6 mice	10 mg/kg, *i.p.*	30 min before, 3, 24 e 48 h after BCCAO	↓ Cognitive/ emotional impairments	$5HT_{1A}$, CB_1, CB_2 and PPAR-γ R
Meyer et al., 2021	4-VO for 15 min	Wistar rats	10 mg/kg, *i.p.*	30 min before, 3 h after reperfusion and daily for 14 days	↓ Memory impairments ↓ Neuronal death ↑ BDNF/ SYN and PSD-95 ↑ Dendritic spine number and arborization	-
GLOBAL CEREBRAL ISCHEMIA + HYPOXIA						
Reference	**Model**	**Animal species**	**CBD dose, route**	**Treatment schedule**	**CBD effects**	**Molecular mechanism**
Alvarez et al., 2008	BCCAO + hypoxia for 20 min	Newborn piglets, 3-5 days	0.1 mg/kg, *i.v.*	15 and 240 min after ischemia	↓ Seizures ↓ Neuronal death ↓ Cerebral hemodynamic/metabolic impairments	-
Lafuente et al., 2011	BCCAO + hypoxia for 20 min	Newborn piglets, 1-3 days	0.1 mg/kg, *i.v.*	15 and 240 min after ischemia	↓ Neurological deficits ↑ Neuronal/astrocytic protection ↑ tissue oxygenation ↓ TNF-α	-
Pazos et al., 2012	CCA electrocoa-gulation + hypoxia for 120 min	Wistar rats, 7-10 days	1 mg/kg, *s.c.*	10 min after ischemia	↓ Motor/cognitive impairment ↓ Infarct size ↓ Excitotoxicity ↓ Oxidative stress ↓ Neuroinflammation ↓TNF-α	-
Pazos et al., 2013	BCCAO + hypoxia for 30 min	Newborn piglets, 1-2 days	1 mg/kg, *i.v.*	30 min after ischemia	↓ Viable neurons ↓ Excitotoxicity ↓ Oxidative stress ↓ Neuroinflammation	CB_2, $5HT_{1A}$ and A_{2A} R

continue

Reference	Model	Animal species	CBD dose, route	Treatment schedule	CBD effects	Molecular mechanism
Lafuente et al., 2016	BCCAO + hypoxia for 30 min	Newborn piglets, 1-2 days	1 mg/kg, *i.v.*	30 min after ischemia + 10 min hypothermia	↓ Neuronal death ↓ Excitotoxicity ↓ Oxidative stress ↓ Neuroinflammation ↓ Caspase-3 and TNF-α	-
Mohammed et al., 2017	CCA electrocoa-gulation + hypoxia for 90 min	C57BL/6 mice, 9-10 days	1 mg/kg, *s.c.*	15 min or 1, 3, 6, 12 or 18 h after ischemia	↓ Neuronal death ↓ Astrogliosis ↓ Microglial population	-
Ceprián et al., 2019	CCA electrocoa-gulation + hypoxia for 112 min	Wistar rats, 7-10 days	1 mg/kg, *s.c.*	10 min after ischemia	↓ Oligodendrocyte loss ↓ Myelination loss	-
			HYPOXIA			
Reference	**Model**	**Animal species**	**CBD dose, route**	**Treatment schedule**	**CBD effects**	**Molecular mechanism**
Garberg et al., 2016	Hypoxia-ischemia	Newborn piglets 12-36 h	1mg/kg, *i.v.*	30 min after ischemia + hypothermia	No histological protection	-
Garberg et al., 2017	Hypoxia-ischemia	Newborn piglets	50, 25, 10 and 5 mg/kg, *i.v.*	30 min after ischemia	No histological protection	-

5-HT_{1A}: 5-hydroxytryptamine; A2A: adenosine A2A receptor; BCCAO: bilateral common carotid artery occlusion; BDNF: brain-derived neurotrophic factor; CA_1: Cornu Ammonis 1; CB_1: cannabinoid receptor type 1; CB_2: cannabinoid receptor type 2; CBD: cannabidiol; CCA: common carotid artery; DCX: doublecortin; HMGB1: high mobility group box 1; *i.c.v.*: intracerebroventricular; *i.p.*: intraperitoneal; *i.v.*: intravenous; Iba-1: ionized calcium-binding adaptor molecule 1; KO: knockout; MAP-2: microtubule associated protein 2; MCAO: middle cerebral artery occlusion; NCX: Na/Ca^{2+} exchanger; NF-κB: nuclear factor kappa B; R: receptor; *s.c.*: subcutaneous; TNFR1: tumor necrosis factor receptor 1; TNF-α: tumor necrosis factor- α; TRPV1: transient receptor potential vanilloid 1; WT: wild type.

9.2 Part I: Meyer et al. Mol Neurobiol. 2021

Molecular Neurobiology
https://doi.org/10.1007/s12035-021-02479-7

Cannabidiol Confers Neuroprotection in Rats in a Model of Transient Global Cerebral Ischemia: Impact of Hippocampal Synaptic Neuroplasticity

Erika Meyer[1] · Jéssica Mendes Bonato[1] · Marco Aurélio Mori[1] · Bianca Andretto Mattos[1] · Francisco Silveira Guimarães[2] · Humberto Milani[1] · Alline Cristina de Campos[2] · Rúbia Maria Weffort de Oliveira[1]

Received: 22 April 2021 / Accepted: 28 June 2021

Abstract
Evidence for the clinical use of neuroprotective drugs for the treatment of cerebral ischemia (CI) is still greatly limited. Spatial/temporal disorientation and cognitive dysfunction are among the most prominent long-term sequelae of CI. Cannabidiol (CBD) is a non-psychotomimetic constituent of *Cannabis sativa* that exerts neuroprotective effects against experimental CI. The present study investigated possible neuroprotective mechanisms of action of CBD on spatial memory impairments that are caused by transient global cerebral ischemia (TGCI) in rats. Hippocampal synaptic plasticity is a fundamental mechanism of learning and memory. Thus, we also evaluated the impact of CBD on neuroplastic changes in the hippocampus after TGCI. Wistar rats were trained to learn an eight-arm aversive radial maze (AvRM) task and underwent either sham or TGCI surgery. The animals received vehicle or 10 mg/kg CBD (i.p.) 30 min before surgery, 3 h after surgery, and then once daily for 14 days. On days 7 and 14, we performed a retention memory test. Another group of rats that received the same pharmacological treatment was tested in the object location test (OLT). Brains were removed and processed to assess neuronal degeneration, synaptic protein levels, and dendritic remodeling in the hippocampus. Cannabidiol treatment attenuated ischemia-induced memory deficits. In rats that were subjected to TGCI, CBD attenuated hippocampal CA1 neurodegeneration and increased brain-derived neurotrophic factor levels. Additionally, CBD protected neurons against the deleterious effects of TGCI on dendritic spine number and the length of dendritic arborization. These results suggest that the neuroprotective effects of CBD against TGCI-induced memory impairments involve changes in synaptic plasticity in the hippocampus.

Keywords Cannabidiol · Transient global cerebral ischemia · Memory · Hippocampus · Neuroplasticity

Introduction

Transient global cerebral ischemia (TGCI) is a devastating outcome of reversible cardiac arrest and other clinical conditions, such as severe cardiac arrhythmias, respiratory arrest, gas poisoning, hypotensive shock, and perinatal asphyxia, which may result in hypoxic/ischemic brain damage [1, 2]. A few minutes of global cerebral ischemia (GCI) can produce extensive neuronal damage and impact synaptic plasticity in vulnerable areas of the brain [3, 4]. Hippocampal pyramidal neurons in the cornu ammonis (CA), especially in the CA1 subfield, are dramatically affected by TGCI in both humans [5, 6] and experimental animals [7, 8]. Hippocampal damage is associated with long-term sequelae of GCI, such as cognitive impairments, spatial/temporal disorientation, and deficits in learning, memory, and attention [9]. Despite intense research efforts, no safe and effective pharmacological therapy has yet been discovered that can treat cognitive impairments that are caused by cerebral ischemic insult [10, 11].

Cannabidiol (CBD) is a major non-psychotomimetic phytochemical that is present in the *Cannabis sativa* plant and has emerged as a potential treatment for several clinical conditions,

✉ Rúbia Maria Weffort de Oliveira
rubiaweffort@gmail.com

[1] Department of Pharmacology and Therapeutics, State University of Maringá, Av. Colombo, Maringá, Paraná 5790, 87020-900, Brazil

[2] Department of Pharmacology, School of Medicine, USP, Av. Bandeirantes, Ribeirão Preto, São Paulo 14015-000, Brazil

Published online: 24 July 2021

many of which are characterized by alterations of memory processing. For example, CBD treatment improved memory deficits in patients with treatment-resistant epilepsy [12–14]. In preclinical models, CBD improved cognition in animal models of schizophrenia, Alzheimer's disease, meningitis, cerebral malaria, and hepatic encephalopathy (for review, see [15]). However, only a few studies have investigated the effects of CBD on cognitive impairments in GCI. Pazos et al. (2012) investigated the effects of CBD on cognition in newborn rats that were subjected to hypoxia/ischemia (HI)-induced brain injury. A single injection of CBD (1 mg/kg, s.c.) 10 min after the HI insult led to long-lasting neuroprotection, reflected by better performance in the novel object recognition test 30 days after the insult [16]. In mice with global GCI that was induced by bilateral common carotid artery occlusion (BCCAO), daily CBD treatment (3–30 mg/kg) for 14 days improved spatial memory in the Morris water maze [17]. Mice that were subjected to BCCAO and received short-term CBD treatment (10 mg/kg, 30 min before and 24, 48, and 72 h after BCCAO) performed better than sham controls in spatial memory tests (i.e., Y-maze test and object location test (OLT)) [18].

The beneficial effects of CBD on spatial memory performance in ischemic mice have been associated with decreases in hippocampal neuronal death and markers of neuroinflammation and increases in hippocampal levels of trophic factors and proteins that are involved in synaptic plasticity [17, 18]. Fogaça et al. (2018) reported that CBD induced anxiolytic-like responses in chronic stressed mice by stimulating hippocampal neurogenesis and dendritic remodeling [19]. Remaining unknown, however, is whether CBD promotes such plastic changes in the hippocampus in rodents with GCI.

In the present study, we used a four-vessel occlusion (4-VO) model of TGCI in rats to investigate the impact of long-term treatment with CBD on ischemia-induced memory impairments and neuroplastic changes in the hippocampus. We first evaluated the effects of CBD treatment on spatial memory performance in ischemic rats in the aversive radial maze (AvRM) and OLT. We then evaluated the effects of CBD on the expression of key proteins that are involved in synaptic plasticity, including brain-derived neurotrophic factor (BDNF), synaptophysin (SYN), and postsynaptic density protein-95 (PSD-95). Dendritic remodeling in the hippocampus was investigated in CBD-treated TGCI rats using Golgi-Cox staining.

Materials and Methods

Ethics Statement

This study was performed at the State University of Maringá in strict accordance with Brazilian College of Animal Experimentation (COBEA) recommendations. The animal experiments were approved by the local Ethics Committee on Animal Experimentation of the State University of Maringá (animal license No. CEUA 1,555,230,316).

Animals

A total of 145 young adult male Wistar rats (3–4 months old) were acquired from the local vivarium of the State University of Maringá (Paraná, Brazil). The animals were housed in groups of three animals per cage in a temperature-controlled room (22 C ± 1 °C) with a 12-h/12-h light/dark cycle (lights on at 7:00 a.m., lights off at 7:00 p.m.). The animals had free access to tap water and a standard commercial chow diet (Nutrilab-CR1; Nuvital Nutrients, Curitiba, PR, Brazil) during the experiments. The local Ethics Committee on Animal Experimentation of the State University of Maringá approved the experimental procedures in accordance with the guidelines of the U.S. National Institutes of Health and Brazilian College for Animal Experimentation (CEUA No. 1555230316). All efforts were made to minimize the number of animals used and reduce their suffering.

Drugs

Cannabidiol (THC Pharma, Frankfurt, Germany) was dissolved in 2% Tween 80 (Synth, Maringá, Brazil) in sterile isotonic saline (vehicle). The 10-mg/kg dose of CBD was based on previous studies that reported a neuroprotective effect of CBD against CI in rodents [18, 20].

Transient Global Cerebral Ischemia

Transient global cerebral ischemia was induced using the 4-VO model [21] with modifications [22]. The animals were fist anesthetized in a chamber that contained halothane (Isoforine, Cristália, SP, Brazil), and the dorsal and ventral regions of the neck were trichotomized. The rats were then fixed in a stereotaxic frame, and a halothane/oxygen mixture was delivered (0.5 L/min) through a face mask that was affixed to the rat's snout. After bilateral exposure of the alar foramen of the first cervical vertebrae, the vertebral arteries were permanently electrocoagulated (unipolar current, 3–4 mA). The common carotid arteries were exposed through an incision on the ventral neck and loosely tied with silk thread. Four to 5 h later, when the animals completely recovered from anesthesia, the silk thread was carefully tightened for 15 min. During this time, such signs as loss of the righting reflex, mydriasis, tonic stretching of the paws, and the absence of responses to touch were considered indicative of effective ischemia. After reperfusion, the animals were maintained in a warming box (37 °C ± 1 °C) for 1 h to avoid ischemia-induced cerebral hypothermia [23].

Sham-operated animals were subjected to the same surgical procedures without occlusion of the vertebral or carotid arteries.

Experimental Design

Experiment 1

In Experiment 1 (Fig. 1), naïve rats were trained to learn the eight-arm AvRM task (see details below). After 10 days of training, the rats underwent sham or TGCI surgery. Vehicle or 10 mg/kg CBD was administered (i.p.) 30 min before reperfusion, 3 h after reperfusion, and then daily for 14 days. On days 7 and 14 of reperfusion, retention memory trials (RMTs) were conducted to assess retrograde memory performance. The animals were then euthanized under deep anesthesia, and their brains were processed for immunohistochemistry and Western blot. Neuronal nuclei (NeuN), SYN, PSD-95, and BDNF protein levels were measured in the hippocampus.

Experiment 2

In Experiment 2 (Fig. 1), TGCI rats were treated with vehicle or 10 mg/kg CBD following the same administration protocol as in Experiment 1. On days 7, 13, and 14 postischemia, the animals were evaluated for locomotor behavior in the open-field (OF) test and memory in the OLT (see below). One day after the OLT, the animals were euthanized under deep anesthesia, and brains were collected and assayed for Golgi-Cox staining.

Behavioral Tests

Eight-Arm Aversive Radial Maze

The AvRM consisted of a central, polygonal platform with eight arms that radiated outward from alternate sides (Fig. 2A). Aversive illumination was provided by spotlights that were positioned 1 m above the maze. An opening at the end of each arm provided access to a dark wooden box, which served as a shelter for the animals. Only one arm contained the true goal box (closed-end box). In the remaining arms, the boxes were open-ended (i.e., false goal box). Additional details about the AvRM apparatus can be found elsewhere [24].

The test was divided into two phases. In the training phase, naive rats were placed in the apparatus for 10 days to learn the goal box's spatial location, with one session/day and three trials/session. The animals were placed

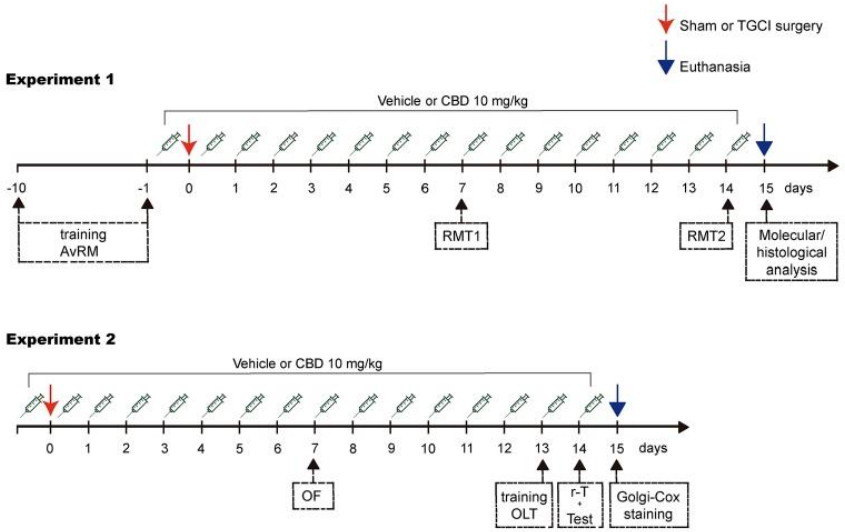
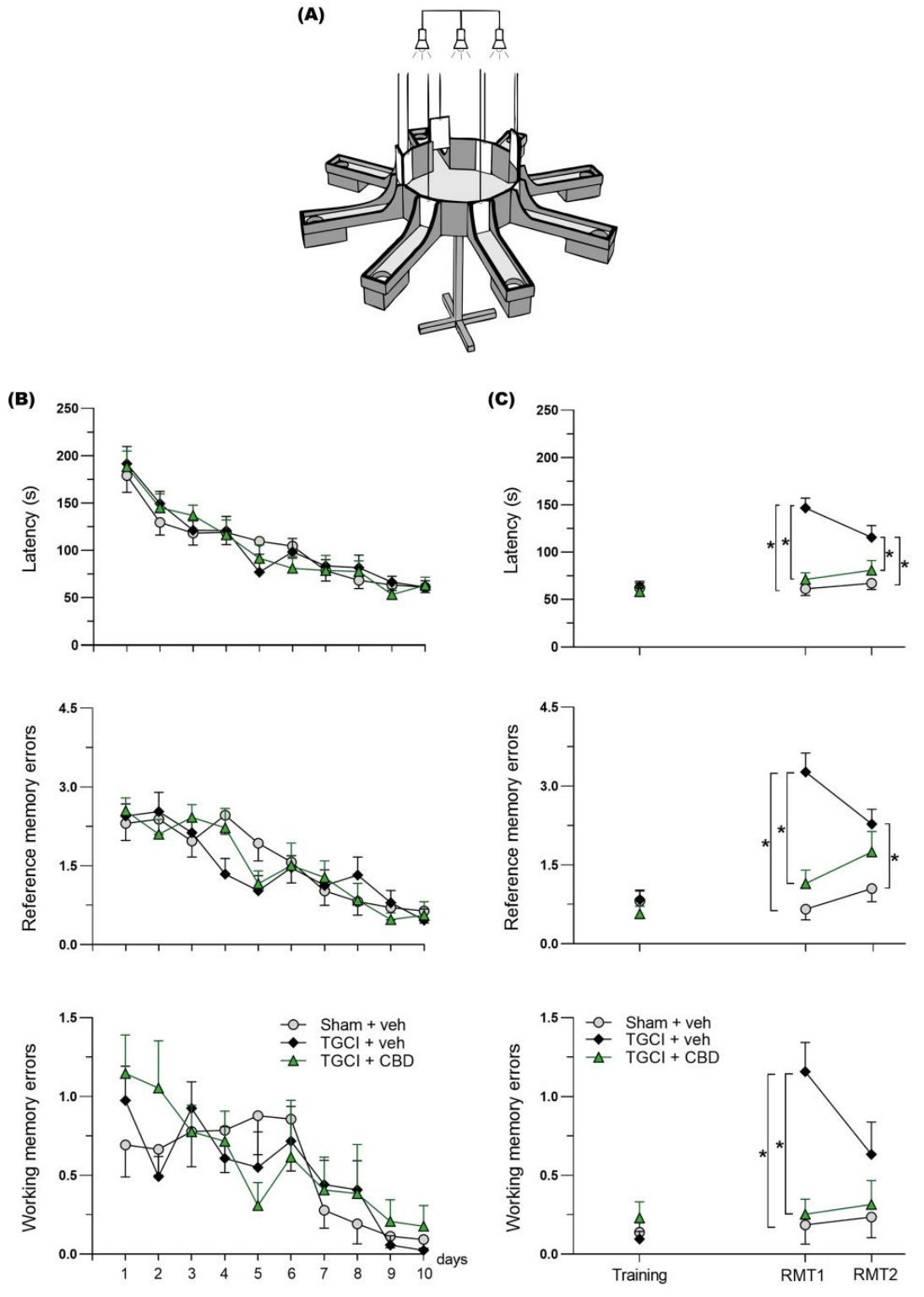


Fig. 1 Experimental design. In Experiment 1, intact rats were trained for 10 days in the 8-arm aversive radial maze (AvRM) to find the goal box location, and then they were subjected to sham or transient global cerebral ischemia (TGCI) procedure. Vehicle or CBD 10 mg/kg (i.p.) were administered 30 min before, 3 h after surgery, and daily for 14 days once a day. On the 7th and 14th days after reperfusion, the retention memory trials (RMT) in the AvRM were carried out. One day after behavioral testing (day 15), the animals were euthanized and their brains were freshly collected for molecular and histological analysis. In Experiment 2, rats underwent TGCI and received vehicle or CBD treatment as described above. Seven days after TGCI, the animals were evaluated in the open-field test (OF), and 13 and 14 days after brain ischemia, they were subject to the object location test (OLT). One day after behavioral testing, the animals had their brains removed and assayed for Golgi-Cox staining


(A)
(B)
(C)
Latency (s)
Reference memory errors
Working memory errors
Sham + veh
TGCI + veh
TGCI + CBD
days
Training
RMT1
RMT2

◂**Fig. 2** Cannabidiol prevented the amnestic effect of TGCI. **A** Schematic representation of the 8-arm aversive radial maze (AvRM). **B** Preoperative learning (acquisition) performance over 10 days of training in the AvRM, as expressed by the parameter latency (s), reference memory errors (number), and working memory errors (number). **C** Temporal distribution of memory performance measured in each retention trial (RMT1 and RMT2). Preoperative performance (training) is expressed as the mean of the last 2 days of training. The bars represent the means ± SEM of the experimental groups: Sham + veh ($n = 14$), TGCI + veh ($n = 12$), and TGCI + CBD ($n = 13$). *$p < 0.05$

individually in the center of the arena. Thirty seconds later, the guillotine doors were opened simultaneously, thereby allowing the animal to explore the entire maze. When the animal entered an arm that contained the false goal box, the remaining arm doors were closed. After returning to the central arena, the animal was again confined there for another 10 s and then released to explore the arms. A trial ended when the rat found the true goal box or a cut-off time of 4 min elapsed. If the goal box was not found within that time, then the rat was led by the experimenter to the arm that contained the true goal box and gently guided to enter it. The rat remained in the goal box for 1 min and then was returned to its home cage. After each trial, the maze was cleaned and randomly rotated on its central axis, and the goal box was moved randomly to any other arm, but its spatial position remained unchanged relative to extra-maze cues. Preoperative learning performance was estimated by the following parameters: (*i*) latency to find the goal box, (*ii*) number of reference memory errors, and (*iii*) number of working memory errors. During each trial, a reference memory error was counted every time the rat first visited an arm that contained a false goal box. If it returned to an arm that had been previously visited, then a working memory error was recorded. After 10 days of training, the rats were subjected to TGCI and allowed to recover from surgery for 1 week. Retrograde memory performance was then assessed on day 7 (RMT1) and day 14 (RMT2) post-ischemia according to the same procedure that was used during the learning phase. All of the behavioral tests were conducted in a temperature-controlled (23 °C) and sound-attenuated room.

Open-Field Test

Locomotor activity was evaluated in the OF, which consisted of a wooden square box (70 cm × 70 cm) with 40 cm high walls. The rats were individually placed in the central area of the OF and allowed to freely explore the arena for 10 min. After each session, the OF was cleaned with 70% ethanol and water and then dried. The distance traveled (in meters) was evaluated using a contrast-sensitive video tracking system (ANYmaze, Stoelting, Wood Dale, IL, USA).

Object Location Test

The OLT is a one-trial test that measures the recognition memory of an object's location [25]. The apparatus consisted of a circular arena (83 cm diameter, 40 cm high wall) where the rats were exposed to two identical objects (i.e., glass bottles; 9 cm maximum diameter, 22 cm height) that were filled with water and sand so that the rat could not move them. On day 1 (i.e., day 13 post-ischemia), both objects were positioned equidistant (10 cm) from the arena wall. The animal was placed in the apparatus and allowed to explore it for 3 min. This process, considered the training session, was repeated five times at 15 min intervals. On day 2 (i.e., day 14 post-ischemia), a retraining (r-T) session was performed, in which the animal was placed in the arena for 3 min with the objects in the same positions as the previous day. After the r-T session, the animal was returned to its home cage, and the location of one of the objects was moved diagonally relative to the other object. After 15 min, the rat was placed again in the OF arena for the test session. The time spent exploring the two objects during the r-T and test sessions was manually recorded.

Another parameter, the discrimination index (D2), was also analyzed and calculated as the following: D2 = (exploration time in the novel location – exploration time in the familiar location) / (exploration time in the novel location + exploration time in the familiar location). Exploratory behavior was considered when the rat directed its nose toward the object at a distance of ≤ 1 cm and/or touched the object with its nose. Animals that explored the objects for less than 5 s were excluded from further analysis since sufficient exploratory behavior is required to produce a reliable memory performance [26].

Between sessions, the objects and arena were cleaned with 70% ethanol and water.

Biochemical and Histological Analyses

On day 15 post-surgeries, 6 animals of each experimental group were randomly chosen and destined for immunohistochemistry (Experiment 1), Western blot (Experiment 1), and Golgi-Cox staining (experiment 2).

Immunohistochemistry

The animals were deeply anesthetized with sodium thiopental (75 mg/kg, Thiopentax, Cristália, SP, Brazil) and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were removed and postfixed with the same fixative solution for 24 h and then cryoprotected by immersion in 30% sucrose

for 72 h. The brains were frozen and sliced in a cryostat (Leica CM1860 UV, Wetzlar, HE, Germany) into 30 µm coronal sections (− 2.30 to − 4.52 mm from Bregma) that encompassed dorsal portions of the hippocampus [26]. The brain slices were collected in replicates in tubes that contained antifreeze solution (15% sucrose and 30% ethylene glycol in PBS) and stored at − 24 °C until further processing.

NeuN immunohistochemistry was used to identify mature neurons. Free-floating sections were first washed with PBST (0.1 M PBS [pH 7.4] plus 0.3% Triton X-100) and then incubated with citrate solution at 50 °C for 30 min. Endogenous peroxidase activity was blocked by incubating the slices in 1% H_2O_2 in PBS for 30 min. After rinsing in PBST, the sections were blocked with 2% bovine serum albumin in PBST for 60 min. After three washes in PBST, the sections were incubated with rabbit polyclonal anti-NeuN antibody (1:500, catalog No. ab177487, Abcam, Cambridge, MA, USA) in PBST overnight at 4 °C. The sections were incubated with specific biotinylated secondary antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 90 min and then in ABC solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The colorimetric peroxidase reaction was performed using 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) and 0.05% H_2O_2. $NiCl_2$ was added to the DAB solution to increase staining contrast. After the immunolabeling procedures, the sections were mounted on gelatin-coated slides and coverslipped with Permount mounting medium.

Quantification was performed by an experimenter who was blind to the experimental groups. The analysis was performed using an Olympus BX41 microscope (Olympus, Tokyo, Japan) coupled to a color high-performance device camera (QColor3, Ontario, Canada) with $10^{\times}$, $20^{\times}$, and $40^{\times}$objectives for the dentate gyrus (DG) and CA3/CA1 areas of the hippocampus, respectively. The lighting conditions and magnifications were maintained constant during image capture to avoid signal saturation. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to calculate the integrated optical density (IOD) when appropriate. Immunoreactive cells were quantified in prefixed digital microscopic areas of the CA1 and CA3, encompassing the entire region of interest. The DG, including the subgranular zone and granular cell layer, was measured in each section. Ten to 12 brain sections were evaluated per animal. For the IOD measurements, the selected images were converted to 16-bit image grayscale, and the background was subtracted. The threshold for a positive signal was predefined, and the IOD was calculated. The results are expressed as the mean IOD/area ± standard error of the mean (SEM).

Western Blot

BNDF, SYN, and PSD-95 protein levels were determined in the hippocampus of 6 randomly chosen rats of each experimental group (Experiment 1). The animals were deeply anesthetized with isoflurane/oxygen (Isoforine, Cristália, SP, Brazil) and decapitated. Brains were removed, and the whole hippocampus was carefully isolated. Tissues were macerated for protein extraction with lysis buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.5], 5 mM NaF, 1 mM Na_3VO_4, 2.5 mM $Na_2H_2O_7P_2$, and 1% Triton-X-100) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The samples were centrifuged at 12,000 rotations per minute (rpm) for 15 min at 4 °C, and the supernatant was preserved. For the experiments that were performed with the synaptosome fraction (synaptic proteins), a group of animals had their hippocampus homogenized in a solution that contained 0.32 M sucrose, 20 mM HEPES, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM NaF, 1 mM Na_3VO_4, and protease inhibitor cocktail. The homogenate was centrifuged at 2800 rpm for 10 min at 4ºC, and the supernatant was discarded. Pellets that contained synaptosomes were resuspended in lysis buffer (150 mM NaCl, 50 mM Tris–HCl [pH 7.4], 1% Triton-X-100, 0.1% sodium dodecyl sulfate, 2 mM EDTA, 1 mM Na_3VO_4, and 5 mM NaF). Total proteins were quantified using the Bradford method. Homogenates were exposed to 4–12% NuPAGE Bis–Tris gel (Invitrogen, Carlsbad, CA, USA) for protein separation and transferred to a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA). The membranes were blocked with 5% nonfat milk in TBST (Tris-buffered saline [pH 7.6] plus 0.1% Triton X-100) and incubated overnight at 4ºC with primary antibodies at the following dilutions: anti-PSD-95 (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-synaptophysin (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-BDNF (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAPDH (1:5000; Abcam), and anti-β-actin (1:10,000; Cell Signaling Technology, Danvers, MA, USA). The membranes were then incubated for 2 h with specific secondary antibodies (anti-rabbit or anti-mouse; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and revealed using Novex ECL chemiluminescent reagent (Invitrogen, Carlsbad, CA, USA). The density of preserved and unsaturated bands was quantified using Image Studio Lite 5.2 software (LI-COR Biosciences, Lincoln, NE, USA). The results are expressed as percentages (± SEM) relative to sham-operated rats that were treated with vehicle.

Golgi-Cox Staining

Six animals of each experimental group from experiment 2 were randomly chosen and had their brains destined to Golgi-Cox staining. Golgi-Cox staining was based on

previous studies [22, 27] with some modifications. Under deep anesthesia (75 mg/kg, Thiopentax, Cristália, SP, Brazil), the rats were transcardiacally perfused with PBS. The brains were removed and maintained in Golgi solution for 24 h at 37ºC and then transferred to a new Golgi solution where they remained for 19 days in the dark. After this period, the brains were immersed in a 30% sucrose/saline solution for at least 6 days. Afterward, the brains were rapidly frozen in isopentane and dry ice and cut into 100 µm thick sections in a cryostat (Leica CM1860 UV, Wetzlar, HE, Germany) to obtain slices of the hippocampus. The sections were then transferred to gelatinized slides. After drying for 2 days at room temperature, the sections were stained with the FD Rapid GolgiStain kit (FD NeuroTechnologies, Columbia, MD, USA). The mounted tissue was dehydrated in successive alcohol baths, followed by xylene baths. Finally, the sections were covered with Permount and coverslipped.

Neurons that were impregnated with Golgi-Cox in the DG, CA1, and CA3 of the hippocampus were chosen based on the following criteria: (*i*) relatively isolated neurons, (*ii*) defined soma, (*iii*) whole-cell impregnation, and (*iv*) well distinguished dendritic arborization. Five to six neurons/animal were analyzed using a DM2500 M polarizing microscope (Leica Microsystem, Wetzlar, HE, Germany) with 20ˣand 100ˣobjectives. The length of apical and basal dendrites, number of branches, and number of dendritic spines were recorded. Neuron images were captured, and dendritic arborization was traced using 3D Simple Neurite Tracer FIJI 3.1.3. software (National Institutes of Health, Bethesda, MD, USA). The number of dendritic spines was counted manually in a 10 µm linear length of an apical and basal tertiary branch [19]. The data are expressed as mean ± SEM.

Statistical analysis

Statistica 8.0 software (StatSoft, Palo Alto, CA, USA) and Prism 8 software (GraphPad, San Diego, CA, USA) were used for the statistical analysis. Data were examined for assumptions of a normal distribution using the D'Agostino and Pearson omnibus test and tested for homoscedasticity using Levene's test. The data met the normality and homoscedasticity criteria. The behavioral data were analyzed using Student's *t*-test and one- or two-way analysis of variance (ANOVA) as appropriate, followed by the Duncan multiple-comparison post hoc test. In the two-way repeated-measures ANOVA, group was the between-subject factor, and trial (test day) was the within-subject factor. In the OLT, functional spatial memory within groups (i.e., a D2 value that differed significantly from zero) was analyzed with a two-way one-sample *t*-test [28].

For the molecular data, the generalized linear model with a gamma distribution was used for continuous data (i.e., IOD for NeuN immunoreactivity, Western blots, and dendritic length based on Golgi-Cox staining). One-way ANOVA followed by Duncan's post hoc test was used for the number of branches and number of dendritic spines based on Golgi-Cox staining. Data are expressed as mean ± SEM of biological replicates (rats). Values of $p \leq 0.05$ were considered statistically significant.

Results

In Experiment 1 (AvRM task), 69 naïve animals were used. All of them were trained for 10 days to learn the task in the AvRM. Because 5 animals did not learn the task, they did not undergo sham or TGCI surgery and were excluded from the experiment. The left 64 animals were randomly distributed in the sham ($n = 21$) or ischemic ($n = 43$) groups. During or immediately after the TGCI procedure, 18 rats died, reflecting a severe effect of TGCI procedure. The remaining 46 animals were randomly divided into the following experimental groups: Sham + vehicle ($n = 14$); Sham + CBD ($n = 7$); TGCI + vehicle ($n = 12$) and TGCI + CBD ($n = 13$).

For Experiment 2 (OF and OLT), 76 naïve rats entered the experiment. They were randomly distributed in sham ($n = 21$) or TGCI ($n = 55$) groups. Nineteen animals died during or after TGCI, remaining 36 ischemic animals. The following experimental groups were generated: Sham + vehicle ($n = 21$); TGCI + vehicle ($n = 20$) and TGCI + CBD ($n = 16$). Because 8 animals (sham + vehicle, $n = 4$; TGCI + vehicle, $n = 2$; TGCI + CBD, $n = 2$) did not explored the objects for 5 s or more, they were excluded from statistical analysis.

Behavioral Tests

Cannabidiol Prevented the Amnestic Effect of Ischemia

Figure 2B shows learning performance curves in the AvRM before ischemia. Intact rats that were allocated to each treatment condition learned the task very well and similarly, indicated by a highly significant main effect of time for all three parameters ($F_{9, 324} = 7.58–35.34$, $p < 0.0001$) but no between-group differences ($F_{2, 36} = 0.03–0.12$, $p > 0.05$).

The postoperative retrograde memory performance in each group is shown in Fig. 2C. The two-way ANOVA revealed a main effect of group for all parameters (latency: $F_{2, 36} = 22.91$, $p < 0.0001$; reference memory errors: $F_{2,36} = 16.60$, $p < 0.0001$; working memory errors: $F_{2, 36} = 10.10$, $p < 0.001$; Fig. 2C). A main effect of trial was not detected for any of the three parameters ($F_{1, 36} = 0.74–1.62$, $p > 0.05$), although memory performance appeared to improve from RMT1 to RMT2 in the TGCI + vehicle group. A significant group × trial interaction was found for latency ($F_{2, 36} = 4.58$, $p < 0.05$),

reference memory errors ($F_{2, 36} = 6.04$, $p < 0.01$), and working memory errors ($F_{2, 36} = 3.08$, $p = 0.05$). Compared with the sham + vehicle group, the TGCI + vehicle group had a longer latency and committed more reference and working memory errors during RMT1 (Duncan's test, $p < 0.0001$; Fig. 2C). Similar outcomes for the sham + vehicle *vs*. TGCI + vehicle groups were found for latency and reference memory errors in RMT2 (Duncan's test, $p < 0.001$–0.01; Fig. 2C). These results indicate that TGCI caused persistent retrograde amnesia, despite some degree of improvement. The amnestic effect of ischemia was prevented by CBD treatment. The longitudinal analysis (Fig. 2C) indicated that both the latency and number of errors significantly decreased in the TGCI + CDB group compared with the TGCI + vehicle group ($p < 0.0001$–0.01). In the sham-operated group, memory performance did not differ between the pre- and postischemic phases, indicating the endurance of retrograde memory in the AvRM task.

Cannabidiol Alleviated TGCI-Induced Spatial Memory Impairments Without Affecting General Motor Activity

No differences were found in the distance traveled in the OF ($F_{2, 46} = 0.31$, $p > 0.05$), demonstrating that CBD treatment and the TGCI procedure did not affect general locomotor activity (Fig. 3B).

Figure 3C, D shows the effects of ischemia on memory in the OLT. During r-T, the objects were in a familiar location, and all groups similarly explored both objects (data not shown). During the test session (Fig. 3C), the sham + vehicle group exhibited greater exploration of the object in the new location (O2; Student's *t*-test, $t_{32} = 3.43$, $p < 0.01$) compared with the object in the familiar location (O1), indicating the rats' ability to discriminate between the familiar and unfamiliar locations (i.e., spatial memory performance). This discrimination ability was lost in the TGCI + vehicle group (Student's *t*-test, $t_{34} = 0.35$, $p > 0.05$), indicating that they did not retain memory of the familiar location that was learned

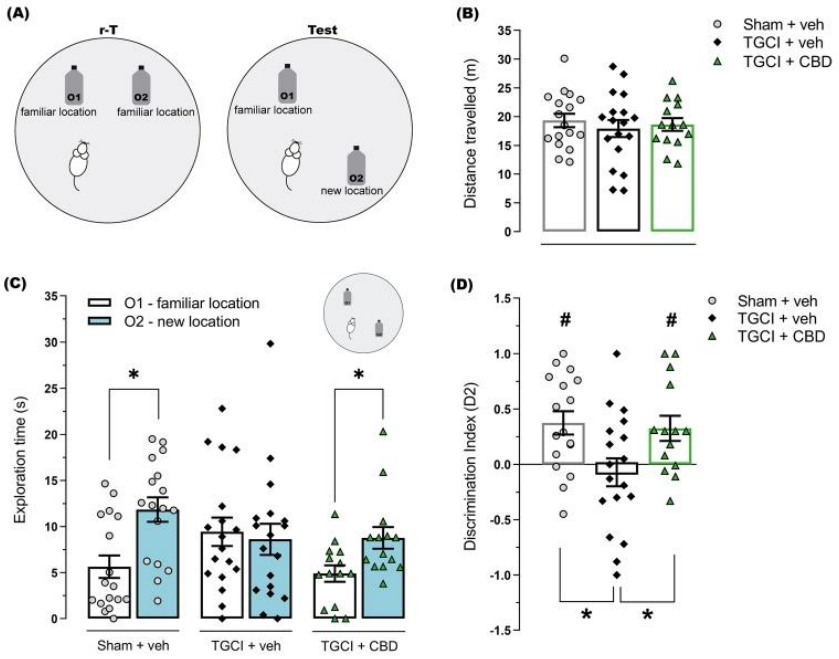


Fig. 3 Cannabidiol alleviated TGCI-induced spatial memory impairments without affecting general locomotor activity. **A** Schematic representation of the identical objects (O1 and O2) located in the open field during retraining session (r-T) in a familiar position and during the test session in which the O2 was moved diagonally relative to O1. **B** Locomotor activity was evaluated 7 days post-ischemia by measuring the distance traveled (m) in the open field (OF). **C** On the 13th day after ischemia, the animals were trained in the OLT and afterward (day 14) re-exposed to the arena for the r-T and test. Time spent exploring the two identical-object zone during the test session was recorded. **D** The discrimination index in the OLT D2 was calculated as follows: D2 = (exploration time in the novel location − exploration time in the familiar location) / (exploration time in the novel location + exploration time in the familiar location). Data are shown as individual values (dots) and the means ± SEM (columns and bars) of the experimental groups: Sham + veh ($n = 17$), TGCI + veh ($n = 18$), and TGCI + CBD ($n = 14$). $^{*}p < 0.05$ and $^{\#}p < 0.05$ compared to zero (i.e., no memory in the OLT)

during training. This effect of TGCI was alleviated by CBD treatment (Student's *t*-test, $t_{26}=2.60$, $p<0.05$).

The effects of ischemia and CBD on spatial memory in the OLT were also indicated by the discrimination index (D2; Fig. 3D). The one-way ANOVA revealed significant differences in D2 among groups ($F_{2,46}=4.67$, $p<0.05$). Duncan's post hoc analyses revealed that the TGCI + vehicle group had a lower, negative D2 score than the sham + vehicle group ($p<0.05$). The TGCI + CBD group exhibited a significant decrease in spatial memory impairment to sham levels compared with the TGCI + vehicle group ($p=0.05$). No difference in spatial memory was observed between sham + vehicle and TGCI + CBD groups ($p > 0.05$). Object location memory is also reflected by D2 relative to zero, where zero indicates no memory (Fig. 3D). The sham + vehicle group had a positive D2 score relative to zero (Student's *t*-test, $t_{29}=3.21$, $p<0.01$), indicating the presence of spatial memory capacity. The TGCI + vehicle group did not present a statistically significant D2 score relative to zero (Student's *t*-test, $t_{30}=0.50$, $p>0.05$), indicating memory impairment that was caused by ischemia. The TGCI + CBD group had a D2 score that was significantly different from zero (Student's *t*-test, $t_{26}=2.87$, $p<0.01$), indicating that CBD prevented TGCI-induced spatial memory deficits when the animals were treated with CBD before ischemia.

Biochemical and Histological Analyses

Cannabidiol Attenuated TGCI-Induced Neurodegeneration

TGCI-induced neurodegeneration was assessed by analyzing NeuN immunoreactivity in the DG, CA1, and CA3 of the hippocampus. As shown in Fig. 4B, a significant difference in NeuN immunoreactivity in the CA1 was found among groups ($\chi^2=7.07$, $p<0.05$). The loss of hippocampal pyramidal neurons was detected in the TGCI + vehicle group compared with the sham + vehicle group ($p<0.01$), and CBD treatment attenuated this neurodegeneration ($p=0.07$). No differences in NeuN immunoreactivity were found in the CA3 ($\chi^2=4.09$, $p>0.05$). Significant differences in NeuN immunoreactivity were found in the DG among groups ($\chi^2=7.33$, $p<0.05$). Cannabidiol increased NeuN immunoreactivity in the TGCI + CBD group compared with the sham + vehicle and TGCI + vehicle groups ($p<0.01$–0.05).

Cannabidiol Changed Neuroplasticity Markers in the Hippocampus of Rats that Were Subjected to TGCI

Figure 5 shows the influence of CBD on the impact of ischemia on synaptic protein levels in the hippocampus. Significant differences in BDNF protein levels were observed among groups ($\chi^2=11.93$, $p<0.01$; Fig. 5A). No differences in BDNF levels were found between the TGCI + vehicle and sham + vehicle groups ($p>0.05$). BDNF expression increased in the TGCI + CBD group compared with the sham + vehicle and TGCI + vehicle groups ($p<0.01$–0.05).

Significant differences in SYN protein levels were found among groups ($\chi^2=5.61$, $p=0.05$; Fig. 5B). A decrease in SYN levels was detected in the TGCI + vehicle group compared with the sham + vehicle group ($p=0.05$). No differences in SYN levels were found between the TGCI + CBD and sham + vehicle groups ($p>0.05$).

Significant differences in PSD-95 levels were found among groups ($\chi^2=7.60$, $p<0.05$; Fig. 5C). A decrease in PSD-95 levels was found in the TGCI + vehicle group compared with the sham + vehicle group ($p<0.001$). No difference in PSD-95 levels was found between TGCI + CBD and sham + vehicle groups ($p>0.05$).

Cannabidiol Prevented Ischemia-Induced Dendritic Spine Loss

As shown in Fig. 6B, ischemia affected the length of dendrites of granular neurons in the DG ($\chi^2=10.45$, $p<0.01$; Fig. 6B). Interestingly, however, the total dendritic length increased in the TGCI + vehicle group compared with the sham + vehicle group ($p<0.05$) but significantly decreased in the TGCI + CBD group compared with the TGCI + vehicle group ($p<0.001$). No difference in the number of branches was found among groups ($F_{2,12}=0.38$, $p>0.05$; Fig. 6B). The one-way ANOVA revealed significant differences in the number of dendritic spines in the DG among groups ($F_{2,14}=20.12$, $p<0.0001$). Duncan's post hoc test showed that the TGCI + vehicle group exhibited a decrease in the number of dendritic spines compared with the sham + vehicle group ($p<0.001$). Cannabidiol treatment alleviated this effect compared with the TGCI + vehicle group ($p<0.05$).

The one-way ANOVA also revealed significant differences in apical, basal, and the total number of dendritic spines in the CA1 subfield among groups ($F_{2,14}=5.84$–18.02, $p<0.001$–0.01; Fig. 7A). Further analysis revealed that the number of dendritic spines decreased in the TGCI + vehicle group compared with the sham + vehicle group ($p<0.001$–0.05). Cannabidiol reversed this ischemia-induced dendritic spine loss in both apical and basal dendritic branches compared with the TGCI + vehicle group ($p<0.001$–0.05). No differences in dendritic length ($\chi^2=0.44$–0.93, $p>0.05$) or the number of branches ($F_{2,12}=0.12$–0.31, $p>0.05$) were found among groups (Fig. 7A).

As shown in Fig. 7C, ischemia did not alter the dendritic length of pyramidal neurons in the CA3 subfield. However, ischemic rats that were treated with CBD exhibited a significant increase in apical dendritic length compared with the sham + vehicle and TGCI + vehicle groups ($p<0.01$–0.05). The one-way ANOVA revealed

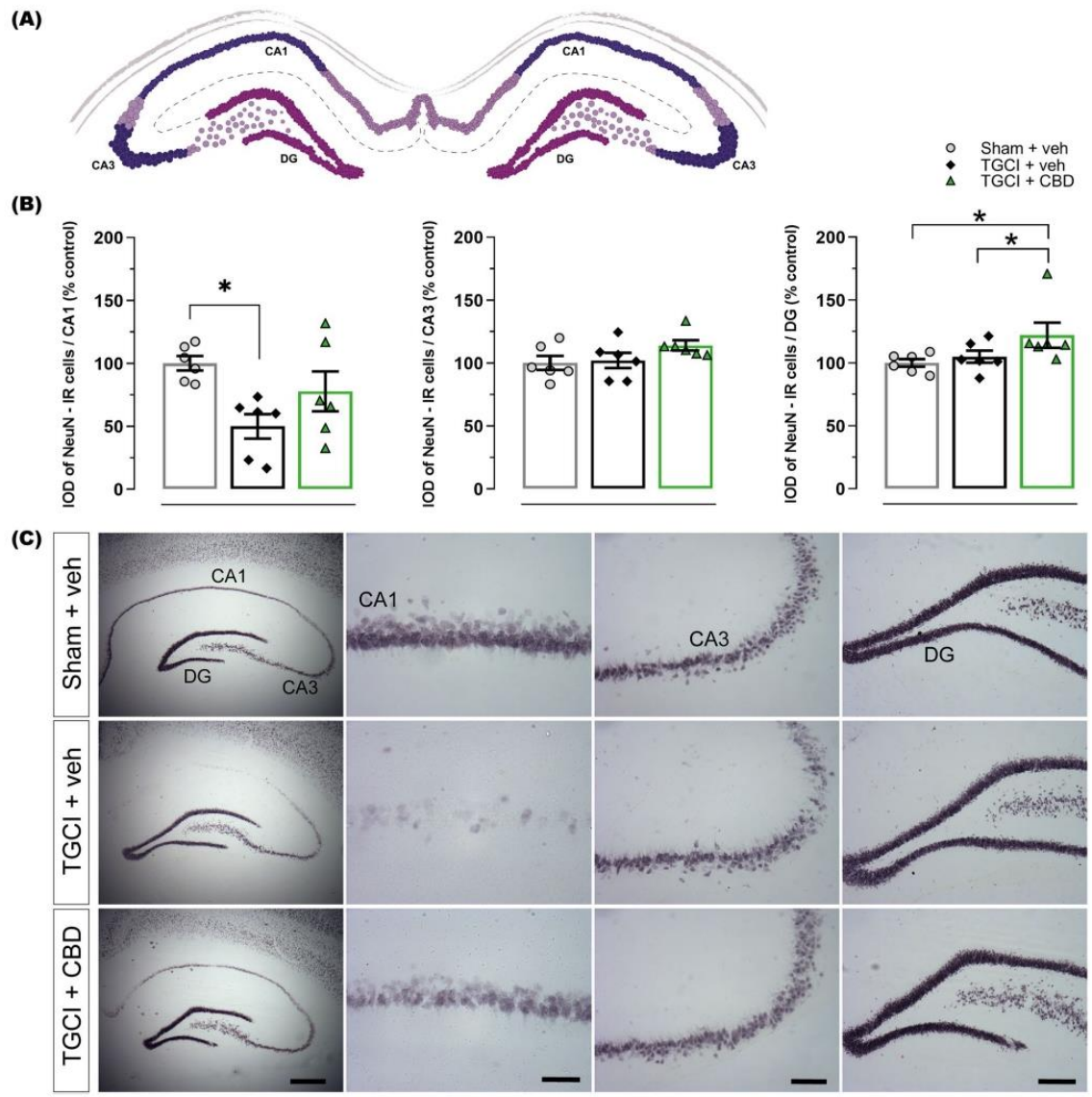


Fig. 4 Cannabidiol attenuated TGCI-induced hippocampal neurodegeneration. **A** Representative diagram illustrating a coronal brain section at the intermediate level of the hippocampus showing the DG, CA1, and CA3 subfields where the analysis for NeuN-IR was performed. **B** Integrated optical density (IOD) of NeuN-positive neurons in the different hippocampal areas. **C** Representative photomicrographs of NeuN-IR cells in the CA1 and CA3 subfields and DG in the different experimental groups, using 40×, 20×, and 10×objectives, respectively. Data are shown as individual values (dots) and the means ± SEM (columns and bars) of the experimental groups ($n = 6$/group). *$p < 0.05$. Scale bars: 500 µm (hippocampal formation); 50 µm (CA1, CA3, and DG)

significant differences in apical, basal, and the total number of branches ($F_{2, 12} = 2.81–3.67$, $p < 0.05$). Ischemic mice that were treated with CBD exhibited a significant increase in the number of apical branches compared with the TGCI + vehicle group ($p = 0.05$). A significant decrease in the number of basal branches was found in the TGCI + vehicle group compared with the sham + vehicle group ($p < 0.05$).

Ischemia-induced dendritic spine loss in the CA1 subfield was also observed in apical branches of pyramidal neurons in the CA3 subfield ($F_{2, 12} = 4.05$, $p < 0.05$; Fig. 7C). The TGCI + vehicle group exhibited a significant decrease in

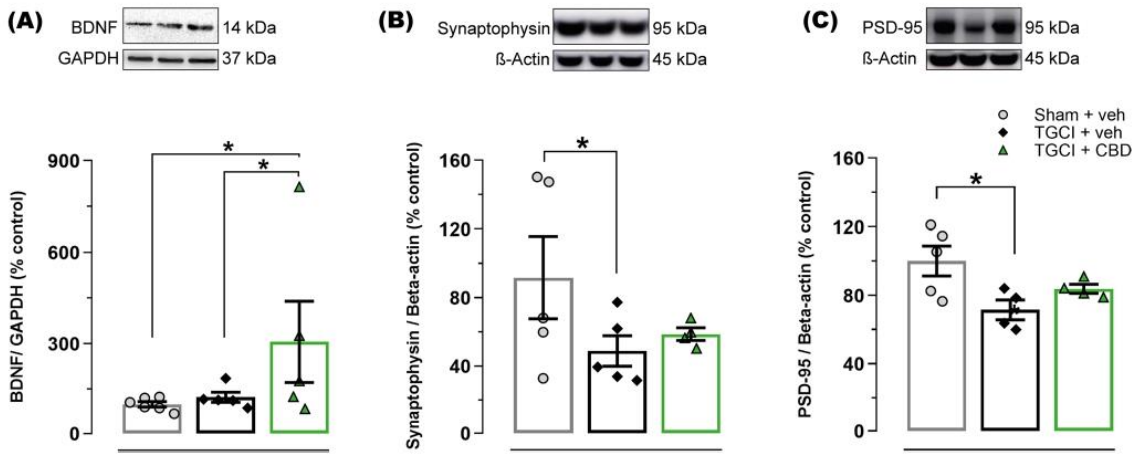


Fig. 5 Cannabidiol changed neuroplasticity markers in the hippocampus of rats that were subjected to TGCI. **A**–**C** Protein levels of BDNF, synaptophysin, and PSD-95 in the hippocampus of sham or ischemic rats evaluated by Western blot. Data are shown as individual values (dots) and the means ± SEM (columns and bars) of the experimental groups ($n = 4$–6/group). *$p < 0.05$

the number of apical dendritic spines compared with the sham + vehicle group ($p < 0.05$). The one-way ANOVA revealed differences in the number of basal dendritic spines among groups ($F_{2, 14} = 4.32, p < 0.05$). No significant difference in the number of basal dendritic spines was observed between the TGCI + vehicle and sham + vehicle groups ($p < 0.05$), whereas the TGCI + CBD group exhibited an increase in the number of basal dendritic spines compared with the other groups ($p < 0.05$).

Discussion

In the present study, we found that repeated CBD treatment prevented memory impairments and decreased hippocampal CA1 neurodegeneration that were caused by ischemia. Cannabidiol also increased hippocampal BDNF levels, attenuated the TGCI-induced decreases in SYN and PSD-95 levels, and elevated dendritic spine number and arborization in the hippocampus in ischemic animals.

The effects of TGCI on spatial memory performance have been well documented [29–32]. Studies from our group showed deleterious effects of TGCI on retrograde memory in rats in the AvRM up to 39 days after the ischemic insult [33–35]. In the present study, memory impairments were detected up to 14 days after TGCI in rats in the AvRM and OLT. Cannabidiol attenuated these effects of TGCI, reflected by decreases in latency and the number of errors in the AvRM, indicating improvements in spatial memory performance. Cannabidiol also ameliorated memory deficits in the AvRM paradigm in middle-aged diabetic rats that underwent chronic cerebral hypoperfusion [36]. Spatial memory recovery in the Y-maze, OLT, and Morris water maze was also observed in BCCAO mice that were treated with CBD [17, 18]. The beneficial effects of CBD on memory function may extend to other conditions that are associated with deficits in memory processing. For example, single or repeated (14 days) injections of CBD (10 mg/kg, i.p.) ameliorated memory function in the object recognition test in rats that were subjected to iron overload [37]. Moreover, accumulating evidence indicates that CBD reduces learned fear in paradigms that are translationally relevant to phobias and posttraumatic stress disorder [38].

Selective hippocampal CA1 damage is known to impair hippocampus-dependent memory, such as spatial learning and memory performance [29, 39–41]. In the present study, we found the significant loss of CA1 neurons (detected by NeuN immunohistochemistry) in TGCI animals, which paralleled memory impairments in those animals in the AvRM. Cannabidiol treatment attenuated hippocampal CA1 neuronal loss that was induced by TGCI in rats. These results extend previous studies that reported histological neuroprotection in the CA1 subfield after CBD administration in mice [18] and gerbils [42] that were subjected to TGCI.

The extent to which the modest reduction of CA1 pyramidal loss that was elicited by CBD treatment contributes to memory preservation (or recovery) is uncertain. Fish oil treatment was reported to restore memory loss that was caused by TGCI without rescuing hippocampal CA1 pyramidal cells [24, 43–45]. Alterations at the electrophysiological, synaptic, and subcellular levels and morphological changes that extend beyond certain structures can result in the dysfunction of complex behaviors and their recovery [46]. Hippocampal damage contributes

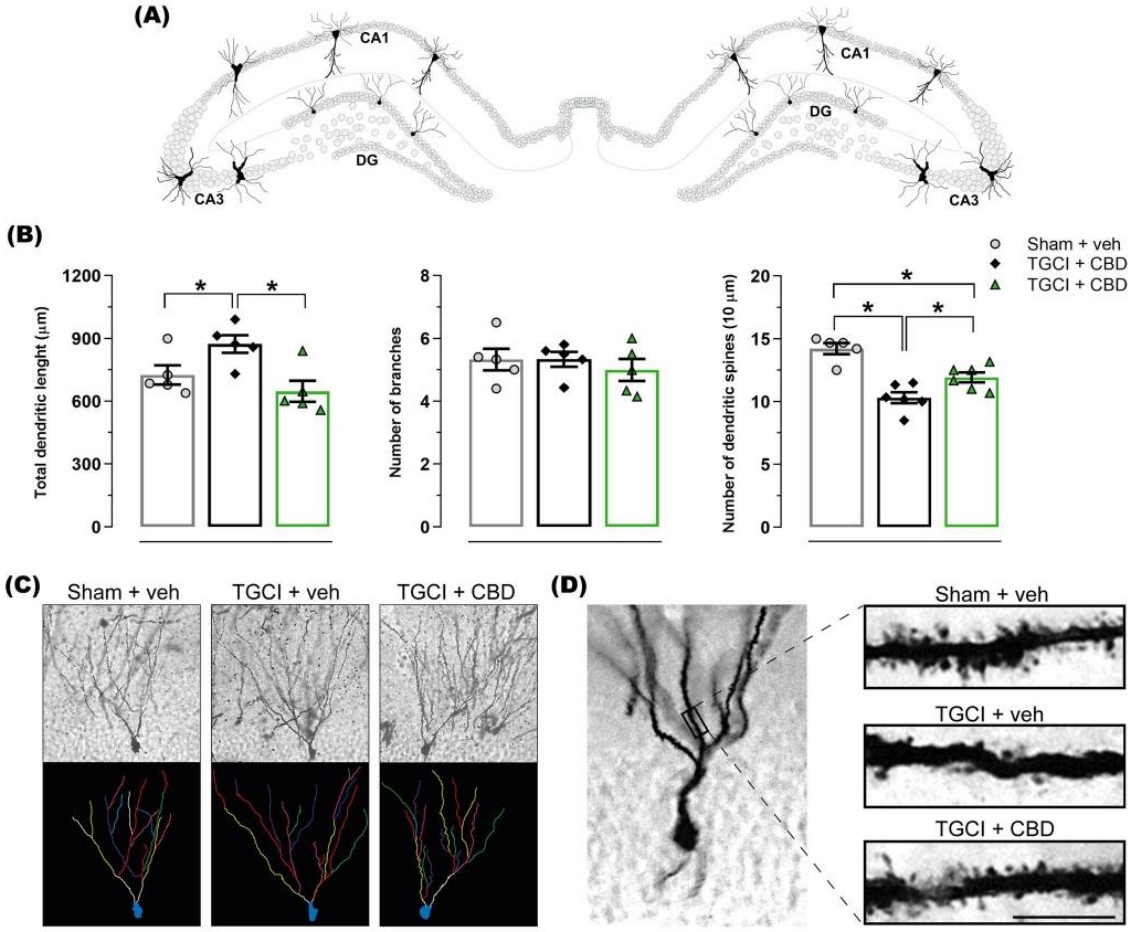


Fig. 6 Cannabidiol ischemia-induced dendritic spine loss in hippocampal granular neurons. **A** Representative diagram illustrating a coronal brain section at the intermediate level of the hippocampus showing the DG, CA1, and CA3 subfields where the analysis for Golgi-Cox was performed. **B** Total dendritic length, number of branches, and dendritic spines of granular neurons among the groups. **C** Representative photomicrography of stained neurons in the hippocampal DG and respective reconstructions. **D** Representative photomicrography of dendritic spines in the different experimental groups. Data are shown as individual values (dots) and the means ± SEM (columns and bars) of the experimental groups ($n = 5–6$ mice/group). *$p < 0.05$

to ischemia-induced cognitive deficits, and the extent of such deficits depends on whether such damage is intra- or extra-hippocampal, the task that is applied to assess such deficits, and the specific memory process that is measured [47]. Interestingly, TGCI rats that were treated with CBD exhibited an increase in NeuN immunoreactivity in the DG of the hippocampus compared with respective controls. However, the implications of this finding are unclear. Neurogenesis in the DG has been shown to reflect a compensatory mechanism that is triggered by TGCI in rats [48] and mice [49]. Moreover, CBD increases doublecortin expression, a marker of newborn neurons, in the DG in ischemic mice. The increase in NeuN immunoreactivity in the DG may reflect hippocampal neurogenesis, but further studies are necessary to confirm this possibility.

Several possible mechanisms may underlie the neuroprotective effects of CBD in GCI, such as CBD-induced neurogenesis, the rescue of CA1 neuronal death, and improvements in synaptic plasticity [18, 44, 50, 51]. BDNF is a neurotrophin that regulates activity-dependent synaptic plasticity and contributes to learning and memory processes [52]. Favorable effects of BDNF on functional recovery and neuroplasticity after CI have been reported [27, 53–55]. However, the levels of BDNF seem to vary temporally and regionally in response to TGCI. For example, a decrease in the hippocampal BDNF levels was detected at 7 [56], 14

[57] or 30 [58] days after TGCI in rats. Otherwise, Kapoor et al. have observed a decrease in the hippocampal BDNF levels at 7 days while hippocampal BDNF levels were found elevated at 14 and 21 days after the ischemic insult [59]. The authors interpreted this fluctuation in the hippocampal BDNF levels as a compensatory and repair mechanism induced by the ischemic injury. Differences in the age and animal species might have contributed to the contradictory findings above mentioned. In the present study, hippocampal BDNF levels did not significantly decrease 14 days after TGCI. Nevertheless, CBD treatment increased BDNF in the hippocampus, suggesting that this effect may be related to functional recovery in ischemic rats.

Evidence indicates that CBD interacts with serotonin 5-hydroxytryptamine-1A (5-HT_{1A}) receptors, which may positively regulate BDNF levels. Mishima et al. [60] reported that CBD reduced the infarct size in rats with middle cerebral artery occlusion, a model of focal cerebral ischemia. The effect of CBD was inhibited by the 5-HT_{1A} receptor antagonist WAY100135 [60]. Notably, the direct activation of postsynaptic 5-HT_{1A} receptors in the hippocampus resulted in higher BDNF levels and an increase in neuroplasticity in mice with BCCAO [55]. Moreover, the antidepressant-like effects of CBD have been associated with higher levels of SYN and PSD-95 in the medial prefrontal cortex and higher levels of BDNF in the prefrontal cortex and hippocampus. An intracerebroventricular injection of the TrkB receptor antagonist K252a and mammalian/mechanistic target of rapamycin inhibitor rapamycin abolished the antidepressant-like effects of CBD. The positive behavioral effects of CBD were related to plastic changes through activation of the BDNF-TrkB signaling pathway [61].

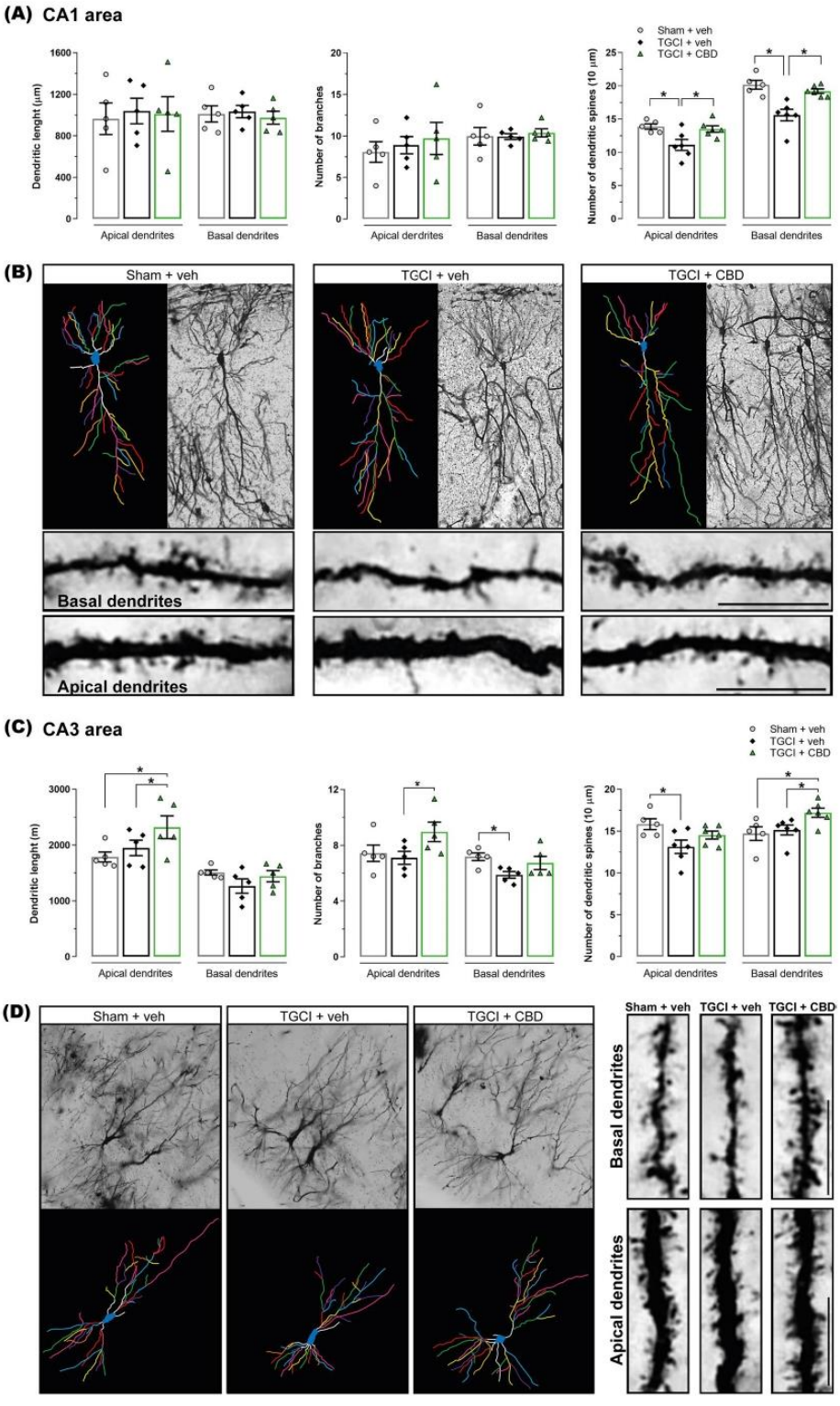
Both SYN and PSD-95 are the main proteins that participate in structural synaptic plasticity [62]. Synapsin is a marker of presynapse development and activity [63], and PSD-95 is an essential factor for synaptic plasticity and postsynaptic membrane stabilization [64]. Experimental evidence indicates that SYN and PSD-95 levels markedly decrease in the hippocampus after CI [65–68]. In the present study, TGCI significantly decreased hippocampal levels of SYN and PSD-95. However, CBD at the tested dose (10 mg/kg) only slightly attenuated these effects of TGCI. Supporting our data, Sales et al. did not observe any difference in SYN or PSD-95 levels in the hippocampus in healthy mice that were treated with 10 mg/kg CBD for 7 days [61]. In contrast, treatment with 10 mg/kg CBD for 14 consecutive days reversed iron-induced reductions of hippocampal SYN levels in rats [63]. Moreover, CBD reversed the decrease in hippocampal PSD-95 levels in mice that were exposed to a model of chronic unpredictable stress [19].

Under some conditions, modifications of the structure of dendritic spines are strongly associated with synaptic plasticity, which is critical for cognitive flexibility [69, 70]. Cerebral ischemia can cause poor spatial memory performance as a result of hippocampal damage and a decrease in synaptic function [71, 72]. In the present study, TGCI significantly decreased the number of dendritic spines in neurons that survived the ischemic insult in the DG (granular neurons), CA1, and CA3. These effects of TGCI on dendritic deterioration were prevented by CBD treatment. Various pharmacological interventions can stimulate neuroplastic changes. For example, acute melatonin administration attenuated dendritic spine loss in the hippocampus in TGCI rats [50]. Treatment with progesterone resulted in a similar recovery of dendritic spines and mitigated ischemia-induced learning and memory deficits [72]. Acetyl-L-carnitine treatment before and after 2-vessel occlusion (i.e., a model of ischemia) prevented ischemia-induced dendritic spine loss in the hippocampus, paralleled by the normalization of long-term potentiation in the hippocampus [73]. The biased 5-HT_{1A} receptor agonist NLX-101 was recently reported to reverse dendritic spine loss in mice that were subjected to BCCAO [55]. The ability of CBD to increase hippocampal BDNF levels and reverse or prevent dendritic spine loss in the hippocampus suggests its influence on neuroplasticity and consequently cognitive recovery (or preservation) under conditions of GCI.

One limitation of the present study was that we did not investigate the pharmacological mechanism of action of CBD in rats with TGCI. We recently demonstrated the involvement of cannabinoid CB_1, CB_2, 5-HT_{1A}, and peroxisome proliferator-activated receptor-γ (PPAR-γ) receptors in functional recovery that is elicited by CBD in BCCAO mice [74]. Whether similar mechanisms are engaged in TGCI rats remains to be determined. The pharmacological profile of CBD is complex. It has potent anti-inflammatory and antioxidant properties [75, 76] and exerts its actions throughout both the endocannabinoid system and other neurotransmitter systems. Treatment with CBD increases anandamide levels, in turn further activating cannabinoid CB_1 receptors [77, 78]. Cannabidiol has been reported to act as a CB_1/CB_2 receptor inverse agonist [79]. Other effects of CBD have been reported to be mediated by PPAR- γ [80] and G-protein-coupled receptors [81]. Moreover, CBD was reported to increase adenosine A_{2A} receptor signaling [82] and modulate energy metabolism through elevations of the mitochondrial complex and creatine kinase activity [83].

Overall, the present findings suggest that the beneficial effects of CBD on spatial memory recovery in TGCI rats at least partially occur through molecular mechanisms that underlie synaptic plasticity and dendritic remodeling, suggesting that CBD may be useful for functional improvement after CI.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12035-021-02479-7.


(A) CA1 area
Dendritic lenght (µm)
Number of branches
Number of dendritic spines (10 µm)
Sham + veh
TGCI + veh
TGCI + CBD
Apical dendrites
Basal dendrites
(B)
Sham + veh
TGCI + veh
TGCI + CBD
Basal dendrites
Apical dendrites
(C) CA3 area
Dendritic lenght (m)
Number of branches
Number of dendritic spines (10 µm)
(D)
Sham + veh
TGCI + veh
TGCI + CBD
Basal dendrites
Apical dendrites

◂**Fig. 7** Cannabidiol protected pyramidal neurons against dendritic spine loss caused by TGCI. **A** Total dendritic length, number of branches, and dendritic spines of pyramidal neurons of CA1 subfield among the groups. **B** Representative photomicrography of stained neurons in the hippocampal CA1 area and basal/apical dendritic spines among the groups. **C** Total dendritic length, number of branches, and dendritic spines of pyramidal neurons of CA3 subfield. **D** Representative photomicrography of stained neurons in the hippocampal CA3 area and basal/apical dendritic spines in the different experimental groups. Data are shown as individual values (dots) and the means ± SEM (columns and bars) of the experimental groups ($n = 5$–6 mice/group). *$p < 0.05$

Acknowledgements This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Universidade Estadual de Maringá and FAPESP (2017/24304-0), São Paulo, Brazil. The authors thank ADCA – Indústria e Comércio de Material Cirúrgico for kindly donating the aneurysm clips and Marco Alberto Trombelli for his technical support.

Author Contribution EM and RMWO conceived and designed the experiments with inputs from ACC. EM performed behavioral tests, western blot, and Golgi analysis. She wrote the first draft of the manuscript. EM and JMB conducted the animals' surgeries. BAM performed the immunohistochemistry. MAM performed data analysis. FSG and HM helped with statistical analysis and data interpretation. All authors read and approved the final manuscript.

Funding This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Universidade Estadual de Maringá, Paraná, Brazil and FAPESP (2017/24304–0), São Paulo, Brazil.

Data Availability Experimental data will be made available under reasonable request.

Declarations

Research Involving Human Participants and/and or Animals The local Ethics Committee on Animal Experimentation of the State University of Maringá approved the experimental procedures in accordance with the guidelines of the U.S. National Institutes of Health and Brazilian College for Animal Experimentation (animal license number: CEUA 1555230316).

Ethics Approval and Consent to Participate This study was carried out at the State University of Maringá in strict accordance with the Brazilian College of Animal Experimentation (COBEA) recommendations. Animal experiments were approved by the local Ethics Committee on Animal Experimentation of the State University of Maringá (animal license number: CEUA 1555230316).

Consent for Publication All of the co-authors approved the final version of the manuscript and agreed to submit it to Molecular Neurobiology.

Competing Interests FSG is a co-inventor (Mechoulam R, JC, Guimaraes FS, AZ, JH, Breuer A) of the patent "Fluorinated CBD compounds, compositions, and uses thereof. Pub. No.: WO/2014/108899. International Application No.: PCT/IL2014/050023" Def. US No. Reg. 62193296; 29/07/2015; INPI on 19/08/2015 (BR1120150164927). The University of São Paulo has licensed the patent to Phytecs Pharm (USP Resolution No. 15.1.130002.1.1). The University of São Paulo has an agreement with Prati-Donaduzzi (Toledo, Brazil) to "develop a pharmaceutical product containing synthetic cannabidiol and prove its safety and therapeutic efficacy in the treatment of epilepsy, schizophrenia, Parkinson's disease, and anxiety disorders."

References

1. Horstmann A, Frisch S, Jentzsch RT, Müller K, Villringer A, Schroeter ML (2010) Resuscitating the heart but losing the brain: brain atrophy in the aftermath of cardiac arrest. Neurology 74(4):306–312. https://doi.org/10.1212/WNL.0b013e3181cbcd6f
2. Sekhon MS, Ainslie PN, Griesdale DE (2017) Clinical pathophysiology of hypoxic ischemic brain injury after cardiac arrest: a "two-hit" model. Crit Care 21(1):90. https://doi.org/10.1186/s13054-017-1670-9
3. Canese R, Podo F, Fortuna S, Lorenzini P, Michalek H (1997) Transient global brain ischemia in the rat: spatial distribution, extension, and evolution of lesions evaluated by magnetic resonance imaging. MAGMA 5(2):139–149. https://doi.org/10.1007/BF02592245
4. Wahul AB, Joshi PC, Kumar A, Chakravarty S (2018) Transient global cerebral ischemia differentially affects cortex, striatum and hippocampus in Bilateral Common Carotid Arterial occlusion (BCCAo) mouse model. J Chem Neuroanat 92:1–15. https://doi.org/10.1016/j.jchemneu.2018.04.006
5. Petito CK, Feldmann E, Pulsinelli WA, Plum F (1987) Delayed hippocampal damage in humans following cardiorespiratory arrest. Neurology 37(8):1281–1286. https://doi.org/10.1212/wnl.37.8.1281
6. Ng T, Graham DI, Adams JH, Ford I (1989) Changes in the hippocampus and the cerebellum resulting from hypoxic insults: frequency and distribution. Acta Neuropathol 78(4):438–443. https://doi.org/10.1007/BF00688181
7. Pulsinelli WA, Brierley JB, Plum F (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 11(5):491–498. https://doi.org/10.1002/ana.410110509
8. Kirino T (2000) Delayed neuronal death. Neuropathology 20:S95–S97. https://doi.org/10.1046/j.1440-1789.2000.00306.x
9. Anderson CA, Arciniegas DB (2010) Cognitive sequelae of hypoxic-ischemic brain injury: a review. NeuroRehabilitation 26(1):47–63. https://doi.org/10.3233/NRE-2010-0535
10. Dirnagl U, Endres M (2014) Found in translation: preclinical stroke research predicts human pathophysiology, clinical phenotypes, and therapeutic outcomes. Stroke 45(5):1510–1518. https://doi.org/10.1161/STROKEAHA.113.004075
11. Lapchak PA, Zhang JH (2017) The high cost of stroke and stroke cytoprotection research. Transl Stroke Res 8(4):307–317. https://doi.org/10.1007/s12975-016-0518-y
12. Martin RC, Gaston TE, Thompson M, Ampah SB, Cutter G, Bebin EM, Szaflarski JP (2019) Cognitive functioning following long-term cannabidiol use in adults with treatment-resistant epilepsy. Epilepsy Behav 97:105–110. https://doi.org/10.1016/j.yebeh.2019.04.044
13. Gaston TE, Allendorfer JB, Nair S, Bebin EM, Grayson LP, Martin RC, Szaflarski JP, Program UABCBD (2020) Effects of highly purified cannabidiol (CBD) on fMRI of working memory in treatment-resistant epilepsy. Epilepsy Behav 112:107358. https://doi.org/10.1016/j.yebeh.2020.107358
14. Metternich B, Wagner K, Geiger MJ, Hirsch M, Schulze-Bonhage A, Klotz KA (2021) Cognitive and behavioral effects of cannabidiol in patients with treatment-resistant epilepsy. Epilepsy Behav 114(Pt A):107558. https://doi.org/10.1016/j.yebeh.2020.107558

15. Osborne AL, Solowij N, Weston-Green K (2017) A systematic review of the effect of cannabidiol on cognitive function: relevance to schizophrenia. Neurosci Biobehav Rev 72:310–324. https://doi.org/10.1016/j.neubiorev.2016.11.012
16. Pazos MR, Cinquina V, Gómez A, Layunta R, Santos M, Fernández-Ruiz J, Martínez Orgado J (2012) Cannabidiol administration after hypoxia-ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function. Neuropharmacology 63(5):776–783. https://doi.org/10.1016/j.neuropharm.2012.05.034
17. Schiavon AP, Soares LM, Bonato JM, Milani H, Guimarães FS, Weffort de Oliveira RM (2014) Protective effects of cannabidiol against hippocampal cell death and cognitive impairment induced by bilateral common carotid artery occlusion in mice. Neurotox Res 26(4):307–316. https://doi.org/10.1007/s12640-014-9457-0
18. Mori MA, Meyer E, Soares LM, Milani H, Guimarães FS, de Oliveira RMW (2017) Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia. Prog Neuropsychopharmacol Biol Psychiatry 75:94–105. https://doi.org/10.1016/j.pnpbp.2016.11.005
19. Fogaça MV, Campos AC, Coelho LD, Duman RS, Guimarães FS (2018) The anxiolytic effects of cannabidiol in chronically stressed mice are mediated by the endocannabinoid system: Role of neurogenesis and dendritic remodeling. Neuropharmacology 135:22–33. https://doi.org/10.1016/j.neuropharm.2018.03.001
20. Hayakawa K, Mishima K, Irie K, Hazekawa M, Mishima S, Fujioka M, Orito K, Egashira N, Katsurabayashi S, Takasaki K, Iwasaki K, Fujiwara M (2008) Cannabidiol prevents a post-ischemic injury progressively induced by cerebral ischemia via a high-mobility group box1-inhibiting mechanism. Neuropharmacology 55(8):1280–1286. https://doi.org/10.1016/j.neuropharm.2008.06.040
21. Pulsinelli WA, Brierley JB (1979) A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 10(3):267–272. https://doi.org/10.1161/01.str.10.3.267
22. de Oliveira DV, Bernardi TC, de Melo SR, Godinho J, de Oliveira RMW, Milani H (2019) Postischemic fish oil treatment restores dendritic integrity and synaptic proteins levels after transient, global cerebral ischemia in rats. J Chem Neuroanat 101:101683. https://doi.org/10.1016/j.jchemneu.2019.101683
23. Seif el Nasr M, Nuglisch J, Krieglstein J (1992) Prevention of ischemia-induced cerebral hypothermia by controlling the environmental temperature. J Pharmacol Toxicol Methods 27(1):23–26. https://doi.org/10.1016/1056-8719(92)90016-t
24. Fernandes JS, Mori MA, Ekuni R, Oliveira RM, Milani H (2008) Long-term treatment with fish oil prevents memory impairments but not hippocampal damage in rats subjected to transient, global cerebral ischemia. Nutr Res 28(11):798–808. https://doi.org/10.1016/j.nutres.2008.09.004
25. Ennaceur A, Michalikova S, Bradford A, Ahmed S (2005) Detailed analysis of the behavior of Lister and Wistar rats in anxiety, object recognition and object location tasks. Behav Brain Res 159(2):247–266. https://doi.org/10.1016/j.bbr.2004.11.006
26. Akkerman S, Blokland A, Reneerkens O, van Goethem NP, Bollen E, Gijselaers HJ, Lieben CK, Steinbusch HW, Prickaerts J (2012) Object recognition testing: methodological considerations on exploration and discrimination measures. Behav Brain Res 232:335–347. https://doi.org/10.1016/j.bbr.2012.03.022
27. Gull S, Ingrisch I, Tausch S, Witte OW, Schmidt S (2015) Consistent and reproducible staining of glia by a modified Golgi-Cox method. J Neurosci Methods 256:141–150. https://doi.org/10.1016/j.jneumeth.2015.08.029
28. Soares LM, De Vry J, Steinbusch HWM, Milani H, Prickaerts J, Weffort de Oliveira RM (2016) Rolipram improves cognition, reduces anxiety- and despair-like behaviors and impacts hippocampal neuroplasticity after transient global cerebral ischemia. Neuroscience 326:69–83. https://doi.org/10.1016/j.neuroscience.2016.03.062
29. Block F (1999) Global ischemia and behavioural deficits. Prog Neurobiol 58(3):279–295. https://doi.org/10.1016/s0301-0082(98)00085-9
30. Erfani S, Moghimi A, Aboutaleb N, Khaksari M (2018) Nesfatin-1 Improve Spatial Memory Impairment Following Transient Global Cerebral Ischemia/Reperfusion via Inhibiting Microglial and Caspase-3 Activation. J Mol Neurosci 65(3):377–384. https://doi.org/10.1007/s12031-018-1105-3
31. Montes P, Vigueras-Villaseñor RM, Rojas-Castañeda JC, Monfil T, Cervantes M, Moralí G (2019) Progesterone treatment in rats after severe global cerebral ischemia promotes hippocampal dentate gyrus neurogenesis and functional recovery. Neurol Res 41(5):429–436. https://doi.org/10.1080/01616412.2019.1576356
32. Zhang B, Zhong Q, Chen X, Wu X, Sha R, Song G, Zhang C, Chen X (2020) Neuroprotective effects of celastrol on transient global cerebral ischemia rats via regulating HMGB1/NF-κB signaling pathway. Front Neurosci 14:847. https://doi.org/10.3389/fnins.2020.00847
33. Paganelli RA, Benetoli A, Milani H (2006) Sustained neuroprotection and facilitation of behavioral recovery by the Ginkgo biloba extract, EGb 761, after transient forebrain ischemia in rats. Behav Brain Res 174(1):70–77. https://doi.org/10.1016/j.bbr.2006.07.005
34. Baccarin CC, Godinho J, de Oliveira RMW, Matsushita M, Gohara AK, Cardozo-Filho L, Lima JC, Previdelli IS, Melo SR, Ribeiro MHDM, Milani H (2016) Postischemic fish oil treatment restores long-term retrograde memory and dendritic density: an analysis of the time window of efficacy. Behav Brain Res 311:425–439. https://doi.org/10.1016/j.bbr.2016.05.047
35. Bonato JM, Meyer E, de Mendonça PSB, Milani H, Prickaerts J, Weffort de Oliveira RM (2021) Roflumilast protects against spatial memory impairments and exerts anti-inflammatory effects after transient global cerebral ischemia. Eur J Neurosci 53(4):1171–1188. https://doi.org/10.1111/ejn.15089
36. Santiago AN, Mori MA, Guimarães FS, Milani H, Weffort de Oliveira RM (2019) Effects of cannabidiol on diabetes outcomes and chronic cerebral hypoperfusion comorbidities in middle-aged rats. Neurotox Res 35(2):463–474. https://doi.org/10.1007/s12640-018-9972-5
37. Fagherazzi EV, Garcia VA, Maurmann N, Bervanger T, Halmenschlager LH, Busato SB, Hallak JE, Zuardi AW, Crippa JA, Schröder N (2012) Memory rescuing effects of cannabidiol in an animal model of cognitive impairment relevant to neurodegenerative disorders. Psychopharmacology (Berl) 219(4):1133–1140. https://doi.org/10.1007/s00213-011-2449-3
38. Lee JLC, Bertoglio LJ, Guimarães FS, Stevenson CW (2017) Cannabidiol regulation of emotion and emotional memory processing: relevance for treating anxiety-related and substance abuse disorders. Br J Pharmacol 174(19):3242–3256. https://doi.org/10.1111/bph.13724
39. Morris RG, Garrud P, Rawlins JN, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297(5868):681–683. https://doi.org/10.1038/297681a0
40. Nunn JA, LePeillet E, Netto CA, Hodges H, Gray JA, Meldrum BS (1994) Global ischaemia: hippocampal pathology and spatial deficits in the water maze. Behav Brain Res 62(1):41–54. https://doi.org/10.1016/0166-4328(94)90036-1
41. Hartman RE, Lee JM, Zipfel GJ, Wozniak DF (2005) Characterizing learning deficits and hippocampal neuron loss following transient global cerebral ischemia in rats. Brain Res 1043(1–2):48–56. https://doi.org/10.1016/j.brainres.2005.02.030
42. Braida D, Pegorini S, Arcidiacono MV, Consalez GG, Croci L, Sala M (2003) Post-ischemic treatment with cannabidiol prevents electroencephalographic flattening, hyperlocomotion, and

neuronal injury in gerbils. Neurosci Lett 346(1–2):61–64. https://doi.org/10.1016/s0304-3940(03)00569-x
43. Bacarin CC, Mori MA, Ferreira EDF, Romanini CV, de Oliveira RMW, Milani H (2013) Fish oil provides robust and sustained memory recovery after cerebral ischemia: influence of treatment regimen. Physiol Behav 119:61–71. https://doi.org/10.1016/j.physbeh.2013.06.001
44. Bacarin CC, Godinho J, de Oliveira RMW, Matsushita M, Gohara AK, Cardozo-Filho L, Lima JC, Previdelli IS, Melo SR, Ribeiro MHDM, Milani H (2016) Postischemic fish oil treatment restores long-term retrograde memory and dendritic density: An analysis of the time window of efficacy. Behav Brain Res 311:425–439. https://doi.org/10.1016/j.bbr.2016.05.047
45. de Oliveira JN, Reis LO, Ferreira EDF, Godinho J, Bacarin CC, Soares LM, de Oliveira RMW, Milani H (2017) Postischemic fish oil treatment confers task-dependent memory recovery. Physiol Behav 177:196–207. https://doi.org/10.1016/j.physbeh.2017.05.009
46. Aronowski J, Samways E, Strong R, Rhoades HM, Grotta JC (1996) An alternative method for the quantitation of neuronal damage after experimental middle cerebral artery occlusion in rats: analysis of behavioral deficits. J Cereb Blood Flow Metab 16(4):705–713. https://doi.org/10.1097/00004647-199607000-00022
47. Bachevalier J, Meunier M (1996) Cerebral ischemia: are the memory deficits associated with hippocampal cell loss? Hippocampus 6(5):553–560. https://doi.org/10.1002/(SICI)1098-1063(1996)6:5%3c553::AID-HIPO8%3e3.0.CO;2-J
48. Kawai T, Takagi N, Miyake-Takagi K, Okuyama N, Mochizuki N, Takeo S (2004) Characterization of BrdU-positive neurons induced by transient global ischemia in adult hippocampus. J Cereb Blood Flow Metab 24(5):548–555. https://doi.org/10.1097/00004647-200405000-00009
49. Soares LM, Schiavon AP, Milani H, de Oliveira RM (2013) Cognitive impairment and persistent anxiety-related responses following bilateral common carotid artery occlusion in mice. Behav Brain Res 249:28–37. https://doi.org/10.1016/j.bbr.2013.04.010
50. García-Chávez D, González-Burgos I, Letechipía-Vallejo G, López-Loeza E, Moralí G, Cervantes M (2008) Long-term evaluation of cytoarchitectonic characteristics of prefrontal cortex pyramidal neurons, following global cerebral ischemia and neuroprotective melatonin treatment, in rats. Neurosci Lett 448(1):148–152. https://doi.org/10.1016/j.neulet.2008.10.043
51. Murphy TH, Corbett D (2009) Plasticity during stroke recovery: from synapse to behaviour. Nat Rev Neurosci 10(12):861–872. https://doi.org/10.1038/nrn2735
52. Begni V, Riva MA, Cattaneo A (2017) Cellular and molecular mechanisms of the brain-derived neurotrophic factor in physiological and pathological conditions. Clin Sci (Lond) 131(2):123–138. https://doi.org/10.1042/CS20160009
53. Schäbitz WR, Steigleder T, Cooper-Kuhn CM, Schwab S, Sommer C, Schneider A, Kuhn HG (2007) Intravenous brain-derived neurotrophic factor enhances poststroke sensorimotor recovery and stimulates neurogenesis. Stroke 38(7):2165–2172. https://doi.org/10.1161/STROKEAHA.106.477331
54. Espinera AR, Ogle ME, Gu X, Wei L (2013) Citalopram enhances neurovascular regeneration and sensorimotor functional recovery after ischemic stroke in mice. Neuroscience 247:1–11. https://doi.org/10.1016/j.neuroscience.2013.04.011
55. Aguiar RP, Soares LM, Meyer E, da Silveira FC, Milani H, Newman-Tancredi A, Varney M, Prickaerts J, Oliveira RMW (2020) Activation of 5-HT1A postsynaptic receptors by NLX-101 results in functional recovery and an increase in neuroplasticity in mice with brain ischemia. Prog Neuropsychopharmacol Biol Psychiatry 99:109832. https://doi.org/10.1016/j.pnpbp.2019.109832
56. Cheng CY, Kao ST (2020) Lee YC (2020) *Angelica sinensis* extract protects against ischemia-reperfusion injury in the hippocampus by activating p38 MAPK-mediated p90RSK/p-Bad and p90RSK/CREB/BDNF signaling after transient global cerebral ischemia in rats. J Ethnopharmacol 24(252):112612. https://doi.org/10.1016/j.jep.2020.112612
57. Wang W, Liu X, Yang Z, Shen H, Liu L, Yu Y, Zhang T (2020) Levodopa improves cognitive function and the deficits of structural synaptic plasticity in hippocampus induced by global cerebral ischemia/reperfusion injury in rats. Front Neurosci. 30(4):586321. https://doi.org/10.3389/fnins.2020.586321
58. de la Tremblaye PB, Benoit SM, Schock S, Plamondon H (2017) CRHR1 exacerbates the glial inflammatory response and alters BDNF/TrkB/pCREB signaling in a rat model of global cerebral ischemia: implications for neuroprotection and cognitive recovery. Prog Neuropsychopharmacol Biol Psychiatry 3:234–248. https://doi.org/10.1016/j.pnpbp.2017.06.021
59. Kapoor M, Sharma S, Sandhir R, Nehru B (2019) Temporal changes in physiological and molecular markers in various brain regions following transient global ischemia in rats. Mol Biol Rep 46(6):6215–6230. https://doi.org/10.1007/s11033-019-05060-7
60. Mishima K, Hayakawa K, Abe K, Ikeda T, Egashira N, Iwasaki K, Fujiwara M (2005) Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine1A receptor-dependent mechanism. Stroke 36(5):1077–1082. https://doi.org/10.1161/01.STR.0000163083.59201.34
61. Sales AJ, Fogaça MV, Sartim AG, Pereira VS, Wegener G, Guimarães FS, Joca SRL (2019) Cannabidiol induces rapid and sustained antidepressant-like effects through increased BDNF signaling and synaptogenesis in the prefrontal cortex. Mol Neurobiol 56(2):1070–1081. https://doi.org/10.1007/s12035-018-1143-4
62. Sadigh-Eteghad S, Geranmayeh MH, Majdi A, Salehpour F, Mahmoudi J, Farhoudi M (2018) Intranasal cerebrolysin improves cognitive function and structural synaptic plasticity in photothrombotic mouse model of medial prefrontal cortex ischemia. Neuropeptides 71:61–69. https://doi.org/10.1016/j.npep.2018.07.002
63. Tarsa L, Goda Y (2002) Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. Proc Natl Acad Sci U S A 99(2):1012–1016. https://doi.org/10.1073/pnas.022575999
64. Luo J, Zhang L, Ning N, Jiang H, Yu SY (2013) Neotrofin reverses the effects of chronic unpredictable mild stress on behavior via regulating BDNF, PSD-95 and synaptophysin expression in rat. Behav Brain Res 253:48–53. https://doi.org/10.1016/j.bbr.2013.07.014
65. Ishimaru H, Casamenti F, Uéda K, Maruyama Y, Pepeu G (2001) Changes in presynaptic proteins, SNAP-25 and synaptophysin, in the hippocampal CA1 area in ischemic gerbils. Brain Res 903(1–2):94–101. https://doi.org/10.1016/s0006-8993(01)02439-8
66. Zhao Y, Wang J, Liu C, Jiang C, Zhao C, Zhu Z (2011) Progesterone influences postischemic synaptogenesis in the CA1 region of the hippocampus in rats. Synapse 65(9):880–891. https://doi.org/10.1002/syn.20915
67. Yan BC, Park JH, Ahn JH, Lee JC, Won MH, Kang IJ (2013) Postsynaptic density protein (PSD)-95 expression is markedly decreased in the hippocampal CA1 region after experimental ischemia-reperfusion injury. J Neurol Sci 330(1–2):111–116. https://doi.org/10.1016/j.jns.2013.04.023
68. da Silva VK, de Freitas BS, da Silva DA, Nery LR, Falavigna L, Ferreira RD, Bogo MR, Hallak JE, Zuardi AW, Crippa JA, Schröder N (2014) Cannabidiol normalizes caspase 3, synaptophysin, and mitochondrial fission protein DNM1L expression levels in rats with brain iron overload: implications for neuroprotection. Mol Neurobiol 49(1):222–233. https://doi.org/10.1007/s12035-013-8514-7

69. González-Burgos I, Letechipía-Vallejo G, López-Loeza E, Moralí G, Cervantes M (2007) Long-term study of dendritic spines from hippocampal CA1 pyramidal cells, after neuroprotective melatonin treatment following global cerebral ischemia in rats. Neurosci Lett 423(2):162–166. https://doi.org/10.1016/j.neulet.2007.06.050
70. Hasbani MJ, Schlief ML, Fisher DA, Goldberg MP (2001) Dendritic spines lost during glutamate receptor activation reemerge at original sites of synaptic contact. J Neurosci 21(7):2393–2403. https://doi.org/10.1523/JNEUROSCI.21-07-02393.2001
71. Shih PC, Yang YR, Wang RY (2013) Effects of exercise intensity on spatial memory performance and hippocampal synaptic plasticity in transient brain ischemic rats. PLoS One 8(10):e78163. https://doi.org/10.1371/journal.pone.0078163
72. Moralí G, Montes P, González-Burgos I, Velázquez-Zamora DA, Cervantes M (2012) Cytoarchitectural characteristics of hippocampal CA1 pyramidal neurons of rats, four months after global cerebral ischemia and progesterone treatment. Restor Neurol Neurosci 30(1):1–8. https://doi.org/10.3233/RNN-2011-0605
73. Kocsis K, Knapp L, Gellért L, Oláh G, Kis Z, Takakuwa H, Iwamori N, Ono E, Toldi J, Farkas T (2014) Acetyl-L-carnitine normalizes the impaired long-term potentiation and spine density in a rat model of global ischemia. Neuroscience 269:265–672. https://doi.org/10.1016/j.neuroscience.2014.03.055
74. Mori MA, Meyer E, da Silva FF, Milani H, Guimarães FS, Oliveira RMW (2021) Differential contribution of CB1, CB2, 5-HT1A, and PPAR-γ receptors to cannabidiol effects on ischemia-induced emotional and cognitive impairments. Eur J Neurosci. https://doi.org/10.1111/ejn.15134
75. Fernández-Ruiz J, Sagredo O, Pazos MR, García C, Pertwee R, Mechoulam R, Martínez-Orgado J (2013) Cannabidiol for neurodegenerative disorders: important new clinical applications for this phytocannabinoid? Br J Clin Pharmacol 75(2):323–333. https://doi.org/10.1111/j.1365-2125.2012.04341.x
76. Campos AC, Fogaça MV, Sonego AB, Guimarães FS (2016) Cannabidiol, neuroprotection and neuropsychiatric disorders. Pharmacol Res 112:119–127. https://doi.org/10.1016/j.phrs.2016.01.033
77. Devane WA, Dysarz FA 3rd, Johnson MR, Melvin LS, Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 34(5):605–613
78. Bisogno T, Hanus L, De Petrocellis L, Tchilibon S, Ponde DE, Brandi I, Moriello AS, Davis JB, Mechoulam R, Di Marzo V (2001) Molecular targets for cannabidiol and its synthetic analogues: effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. Br J Pharmacol 134(4):845–852. https://doi.org/10.1038/sj.bjp.0704327
79. Thomas A, Baillie GL, Phillips AM, Razdan RK, Ross RA, Pertwee RG (2007) Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. Br J Pharmacol 150(5):613–623. https://doi.org/10.1038/sj.bjp.0707133
80. Esposito G, Scuderi C, Valenza M, Togna GI, Latina V, De Filippis D, Cipriano M, Carratù MR, Iuvone T, Steardo L (2011) Cannabidiol reduces Aβ-induced neuroinflammation and promotes hippocampal neurogenesis through PPARγ involvement. PLoS One 6(12):e28668. https://doi.org/10.1371/journal.pone.0028668
81. Brown KJ, Laun AS, Song ZH (2017) Cannabidiol, a novel inverse agonist for GPR12. Biochem Biophys Res Commun 493(1):451–454. https://doi.org/10.1016/j.bbrc.2017.09.001
82. Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Vitoretti LB, Mariano-Souza DP, Quinteiro-Filho WM, Akamine AT, Almeida VI, Quevedo J, Dal-Pizzol F, Hallak JE, Zuardi AW, Crippa JA, Palermo-Neto J (2012) Cannabidiol, a non-psychotropic plant-derived cannabinoid, decreases inflammation in a murine model of acute lung injury: role for the adenosine A(2A) receptor. Eur J Pharmacol 678(1–3):78–85. https://doi.org/10.1016/j.ejphar.2011.12.043
83. Valvassori SS, Bavaresco DV, Scaini G, Varela RB, Streck EL, Chagas MH, Hallak JE, Zuardi AW, Crippa JA, Quevedo J (2013) Acute and chronic administration of cannabidiol increases mitochondrial complex and creatine kinase activity in the rat brain. Braz J Psychiatry 35(4):380–386. https://doi.org/10.1590/1516-4446-2012-0886

9.3 Part II: Mori et al. Prog Neuropsychopharmacol Biol Psychiatry. 2017

Progress in Neuro-Psychopharmacology & Biological Psychiatry 75 (2017) 94–105

Contents lists available at ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia

Marco Aurélio Mori [a], Erika Meyer [a], Ligia Mendes Soares [a], Humberto Milani [a], Francisco Silveira Guimarães [b], Rúbia Maria Weffort de Oliveira [a,*]

[a] Department of Pharmacology and Therapeutics, State University of Maringá, Av. Colombo, 5790, 87020-900 Maringá, Paraná, Brazil
[b] Department of Pharmacology, School of Medicine, USP, Av. Bandeirantes, 14015-000 Ribeirão Preto, São Paulo, Brazil

ARTICLE INFO

Article history:
Received 29 September 2016
Accepted 22 November 2016
Available online 23 November 2016

Keywords:
Cannabidiol
Bilateral common carotid arteries occlusion
White matter
Neuroplasticity
Neuroprotection

ABSTRACT

This study investigated the effects of cannabidiol (CBD), a non-psychotomimetic phytochemical present in *Cannabis sativa*, on the cognitive and emotional impairments induced by bilateral common carotid artery occlusion (BCCAO) in mice. Using a multi-tiered behavioral testing battery during 21 days, we found that BCCAO mice exhibited long-lasting functional deficits reflected by increase in anxiety-like behavior (day 9), memory impairments (days 12–18) and despair-like behavior (day 21). Short-term CBD 10 mg/kg treatment prevented the cognitive and emotional impairments, attenuated hippocampal neurodegeneration and white matter (WM) injury, and reduced glial response that were induced by BCCAO. In addition, ischemic mice treated with CBD exhibited an increase in the hippocampal brain derived neurotrophic factor (BDNF) protein levels. CBD also stimulated neurogenesis and promoted dendritic restructuring in the hippocampus of BCCAO animals. Collectively, the present results demonstrate that short-term CBD treatment results in global functional recovery in ischemic mice and impacts multiple and distinct targets involved in the pathophysiology of brain ischemic injury.

1. Introduction

Brain ischemia can result from stroke or cardiac arrest and is one of the leading causes of death and disability worldwide, presenting a significant global burden to patients, their relatives, and entire economies (Flynn et al., 2008; Kim and Johnston, 2011). Patients who survive an ischemic brain insult are particularly vulnerable to the development of cognitive impairment, depression, and anxiety disorders (Geri et al., 2014; Moulaert et al., 2010). Different experimental models have been used to induce neuronal damage and behavioral impairments that recapitulate conditions of brain ischemia (Hall and Traystman, 2009). These models have allowed investigations of the pathophysiology of brain ischemia and the identification of potential targets for neuroprotective compounds. Despite intense preclinical efforts, however, only limited advances have been made to develop effective therapies to the deleterious effects of brain ischemia (Dirnagl and Endres, 2014; Ginsberg, 2009). The reasons that such translational studies have failed include the fact that the majority of neuroprotective agents have focused on specific neuronal targets, and functional outcomes were investigated using single short-term endpoints (Dirnagl and Endres, 2014).

Cannabidiol (CBD) is the major non-psychotomimetic phytochemical that is present in the *Cannabis sativa* plant (Mechoulam and Gaoni, 1965; Pertwee et al., 2005). In the last decade, CBD administration has emerged as a potential therapeutic strategy for the treatment of several neuropsychiatric conditions (Campos et al., 2012a; Campos et al., 2016; Fernandez-Ruiz et al., 2013). Moreover, CBD has been proposed to exert neuroprotective effects in neurodegenerative conditions, including Alzheimer's (Martin-Moreno et al., 2011) and Parkinson's disease (Garcia-Arencibia et al., 2007), epilepsy (Leo et al., 2016), and multiple sclerosis (Leo et al., 2016). Regarding ischemic disorders, CBD reduces neuronal damage both in vitro and in vivo in models of hypoxia/ischemia (H/I) (Alvarez et al., 2008; Castillo et al., 2010; Lafuente et al., 2011; Pazos et al., 2013). Cannabidiol also increased survival rates, decreased infarct volume, improved neurological scores and motor coordination in a model of stroke induced by middle cerebral artery occlusion (MCAo) in mice (Hayakawa et al., 2009; Hayakawa et al., 2008; Mishima et al., 2005). In gerbils subjected to transient global cerebral ischemia, CBD prevented electroencephalographic flattening, hyperlocomotion, and neurodegeneration (Braida et al., 2003). We have recently reported that CBD protects against memory impairments and hippocampal cell

Abbreviations: 5-HT1A, 5-hydroxytryptamine 1A; BCCAO, bilateral common carotid artery occlusion; BDNF, brain derived neurotrophic factor; CB1 and CB2, types 1 and 2 cannabinoid receptors; CBD, cannabidiol; DCX, doublecortin; DG, dentate gyrus; EZM, elevated zero maze; FST, forced swim test; GCL, granular cell layer; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule 1; IOD, integrated optical density; MAP-2, microtubule-associated protein 2; MCAo, middle cerebral artery occlusion; OFT, open field test; OLT, object location test; SGZ, subgranular zone; WM, white matter; YM, Y maze.

* Corresponding author.
E-mail address: rmmwoliveira@uem.br (R.M.W. de Oliveira).

http://dx.doi.org/10.1016/j.pnpbp.2016.11.005
0278-5846/

loss in mice subjected to bilateral common carotid artery occlusion (BCCAO) (Schiavon et al., 2014). However, in most of these studies, the histological and/or behavioral effects of CBD were measured within a short period (i.e., from hours to several days) after the ischemic insult. They have also commonly used only a single behavioral test.

Multiple targets have been proposed to mediate the pharmacological effects of CBD. Cannabidiol has negligible activity on cannabinoid receptors (i.e., CB_1 and CB_2) but may interfere with the endocannabinoid system and directly or indirectly stimulate 5-hydroxytryptamine 1A (5-HT_{1A}) receptors, adenosine receptors, transient receptor potential vanilloid subtype 1 (TRPV1), and nuclear receptors of the peroxisome proliferator-activated receptor family (for review, see Fernandez-Ruiz et al., 2013). Experimental evidence indicates that CBD exerts a combination of antioxidant, anti-inflammatory, and neuroprotective effects against ischemic insult. For example, CBD reduced glutamate excitotoxicity, oxidative stress, and inflammation in immature brains (Pazos et al., 2012; Pazos et al., 2013). In adult MCAo mice, CBD increased cerebral blood flow (Hayakawa et al., 2008) and reduced microglia activation (Hayakawa et al., 2008), resulting in a decrease of the infarct size. Moreover, a reduction of astrogliosis and attenuation of hippocampal cell loss were also observed in BCCAO mice that were treated with CBD (Schiavon et al., 2014). However, the neuroprotective effects of CBD were detected when the treatment was initiated before or up to 3 days after the insult (Hayakawa et al., 2009). This observation indicates that the neuroprotective effects of CBD might be significant at early stages after the ischemic insult. However, concerning functional recovery after ischemic brain damage, a relevant issue is the ability of the brain to reorganize neuronal circuits around or distant from the primary site of the ischemic injury. Neurons that survive an ischemic event can respond with plastic changes, including dendritic restructuring, reactive synaptogenesis, and growth-promoting processes that, in turn, can promote functional recovery (Garcia-Chavez et al., 2008). However, whether CBD promotes such plastic changes in response to an ischemic insult remains unknown.

The evaluation of global functional recovery over the course of several days or weeks after brain ischemia and the use of a multi-tiered battery of behavioral tests have been proposed as key elements in improving the clinical validity of experimental studies of brain ischemia (Balkaya et al., 2013; Kronenberg et al., 2014). To our knowledge, no study has evaluated the effects of CBD on long-term cognitive and emotional responses that are induced by transient global brain ischemia. Therefore, the present study sought to answer the following questions: (*i*) does CBD treatment alleviate both cognitive and emotional deficits in a multi-tiered battery of behavioral tasks after ischemia? (*ii*) Does CBD provide sustained hippocampal neuronal rescue and white matter (WM) protection in ischemic mice? (*iii*) Does CBD counteract ischemia-induced neuroinflammation and stimulate the expression of hippocampal plasticity markers, such as doublecortin (DCX), microtubule-associated protein 2 (MAP-2), and brain-derived neurotrophic factor (BDNF)?

2. Materials and methods

2.1. Animals

Experiments were conducted with 2- to 3-month-old male C57BL/6 mice, weighing 25–30 g. The animals were housed in groups ($n = 3$–8) under conditions of controlled temperature (22 ± 1 °C) and a 12-h/12-h alternating light/dark cycle (lights on at 7:00 AM) for two weeks prior to the experiments. A standard commercial chow diet (Nutrilab-CR1; Nuvital Nutrients, Curitiba, Brazil) and water were provided ad libitum. All efforts were made to minimize the number of animals used and reduce their suffering. The experimental procedures conformed to the ethical principles of the Brazilian College of Animal Experimentation (COBEA) and were approved by the local Ethics Committee on Animal Experimentation of the State University of Maringá (CEEA 073/2013).

2.2. Drugs and injections

Cannabidiol (THC Pharma, Frankfurt, Germany) was diluted in 1% Tween 80 in sterile saline (vehicle). The injection regimen and the doses of CBD were based on Hayakawa et al. (2009). The animals were randomly assigned to receive intraperitoneal injections of vehicle or 10 mg/kg CBD 30 min before and 3, 24, and 48 h after surgery.

2.3. Surgery

Transient cerebral ischemia was induced by BCCAO as previously described (Soares et al., 2013). Briefly, the mice were initially anesthetized with a mixture of isoflurane/oxygen (Isoforine®, Cristália, São Paulo, Brazil) using a small-animal anesthesia delivery system that consisted of a mask adapted to the nose. The animals were fixed in a stereotaxic frame, and anesthesia was maintained with 1.3–1.5% isoflurane in 100% oxygen for approximately 6 min. An incision was made in the ventral neck to expose the common carotid arteries. Brain ischemia was induced by 20 min of BCCAO using aneurysm clips (ADCA, Barbacena, Brazil). Throughout the occlusion procedures, the mice were maintained in a warming box (inner temperature, 30 ± 1 °C) to avoid ischemia-induced cerebral hypothermia (Seif el Nasr et al., 1992). At the end of the occlusions, the aneurism clips were removed, and the carotid arteries were visually inspected for reperfusion. The animals were again anesthetized for 2 min, and the incision was closed with sutures. For 3 h after reperfusion, the mice were maintained in a warming box at 30 °C. Sham-operated animals were subjected to the same surgical interventions, with the exception that the carotid arteries were not occluded.

2.4. Experimental design

Behavioral testing began 7 days after sham or BCCAO surgery and was conducted from 7:00 AM to 1:00 PM (Fig. 1). The animals were divided into three experimental groups: Sham + Veh ($n = 12$), BCCAO + Veh ($n = 13$), and BCCAO + CBD ($n = 13$). Over 21 days, the animals were consecutively evaluated in the open field test (OFT), elevated zero maze (EZM), Y-maze (YM), object location test (OLT), and forced swim test (FST). To eliminate possible bias that may be caused by odors left by previous animals, the apparatus were cleaned with 70% ethanol and water and then dried before another mouse was tested. Behaviors were recorded using a contrast-sensitive video tracking system (ANYmaze, Stoelting, Wood Dale, IL, USA). At the end of the drug treatment and behavioral testing, the animals were sacrificed, and the brains were processed for immunohistochemistry and neurochemistry. Additional groups of matched mice ($n = 6$) were used for histopathological evaluation of the hippocampus using Nissl and Kluver Barrera staining.

2.5. Behavioral tests

2.5.1. Open field test

Locomotor activity was evaluated in the OFT, which consisted of a circular arena (43 cm diameter) with a 40 cm high wall. The apparatus was made of transparent polyvinyl chloride. Each animal was individually placed in the central area and allowed to freely explore the arena for 10 min. The distance traveled (in meters) was recorded.

2.5.2. Elevated zero maze

The EZM was employed to analyze anxiety-like behavior (Carola et al., 2002). The apparatus consisted of a circular runway (46 cm diameter, 5.5 cm width) that was made from gray plastic material and elevated 20 cm above the floor. The runway was divided into four quadrants: two opposing open quadrants with a low border (3 mm height) to prevent the mouse from stepping down and two opposite closed quadrants with 11 cm high sidewalls. Each mouse was individually placed in one of

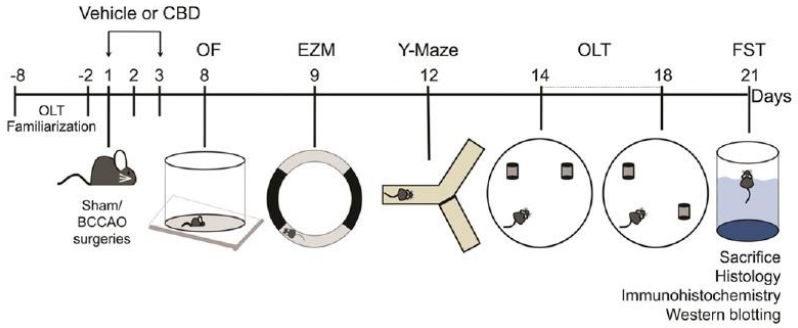


Fig. 1. Experimental design. Vehicle or 10 mg/kg CBD was administered intraperitoneally 30 min before and 3, 24, and 48 h after Sham or BCCAO surgery. Behavioral testing was performed from day 8 to day 21 after BCCAO. Immediately after the last behavioral test, the animals were sacrificed, and their brains were processed for histological, immunohistochemical, and Western blot analysis. OF, open field; EZM, elevated zero maze; OLT, object location test; FST, forced swim test.

the open arms and allowed to explore the maze for 6 min. The time spent in the open quadrants and number of crossings between the open and closed quadrants of the maze were recorded.

2.5.3. Y-maze

The YM is a simple two-trial test that measures spatial recognition memory (Dellu et al., 2000). The YM was made of gray wood and consisted of three identical arms (12 cm width, 41 cm length, and 15 cm height) with a central triangular area. The three arms were randomly designated into two familiar arms (always open) and a novel arm (blocked during the first trial but open during the second trial). In the first training trial, the mouse was allowed to freely explore the open arms for 6 min. After 1 h, the second trial was conducted with all arms accessible for exploration during a cut-off period of 5 min. Discrimination was analyzed as the time spent in the novel arm.

2.5.4. Object location test

The OLT was conducted to measure spatial memory performance (Rutten et al., 2007). The apparatus consisted of a circular arena, similar to the OF above. The mouse was tested in this arena with three different sets of objects. Each object was available in triplicate and could not be moved by the mouse. The objects were the following: (*i*) a small glass bottle (200 ml, 5.5 cm diameter, 15.0 cm height) that was filled with water and sand, (*ii*) a large porcelain cube (9.5 × 6.5 × 6.5 cm), and (*iii*) a large aluminum cube with a tapered top (4.5 × 4.5 × 8.5 cm). The OLT consisted of two sessions: training and testing. Initially, the animals were familiarized with the OLT apparatus for 1 week before the surgical procedures (Fig. 1). They were allowed to explore the arena (without any objects) on 2 consecutive days (3 min/day). On the following 4 days, the mice were adapted to the test until they showed stable discrimination performance (i.e., good object discrimination at a 1 h interval; habituation). The OLT was conducted 14 and 18 days after BCCAO at 1, 4, and 24 h intervals, respectively. The test session consisted of two trials, with each trial lasting 3 min. During the first trial (T1), the arena contained two identical objects. The mouse was introduced to the arena. After the first exploration period had elapsed, the mouse was returned to its homecage. After the predetermined time interval, the mouse was put back into the arena with the same objects for the second trial (T2). However, during T2, one of the objects was relocated. The time spent exploring the two objects during T1 and an experienced observer recorded T2 manually. The discrimination index (D2; i.e., an indication of spatial memory) indicated whether the mouse spent more time exploring the relocated object, while correcting for the total exploration time in T2. $D2 = (\textit{exploration time novel location} - \textit{exploration time familiar location}) / (\textit{exploration time novel location} + \textit{exploration time familiar location})$. Exploratory behavior was defined as when the mouse directed its nose toward the object at a distance of ≤1 cm and/or touched the object with its nose. Sitting on the object was not considered exploration. Animals that explored the objects for <5 s were excluded from further analysis. To avoid the presence of olfactory cues, all of the objects and the arena were thoroughly cleaned with 70% ethanol and water between sessions. The order of object movement and object positions was balanced throughout the experiment and between groups to reduce potential bias toward particular objects, sides, or locations.

2.5.5. Forced swim test

The FST was used to assess behavioral despair as a rodent test for screening the antidepressant activity of drugs (Porsolt et al., 1978). Each mouse was individually placed in an acrylic cylinder (20 cm diameter, 32 cm height) that contained water (24 ± 1 °C) to a depth of 20 cm. The latency to the first episode of immobility and total immobility time, during which the mouse did not struggle and made only movements necessary to keep its head above the water, were recorded over 6 min. Immobility time was registered during the last 4 min of test. After each session, the water was changed, and the animals were dried and returned to their homecage.

2.6. Histology and immunohistochemistry

For Nissl and Kluver Barrera staining, the mice were transcardially perfused with saline followed by Bouin's fixative. After decapitation, the head was immersed in crushed ice (1–2 °C) for 2 h. The brains were then carefully removed and postfixed in Bouin's solution for 3 days and then embedded in paraffin. Coronal brain sections (7 μm) were obtained at the medial level of the hippocampus from −1.06 mm to −2.70 mm posterior to bregma (Franklin and Paxinos, 1997) using a rotating microtome (RM2445; Leica, Goettingen, Germany). The sections were distributed into three sets of slides that contained three coronal sections each, 126 μm apart. After standard dehydration and diaphanization procedures, a set of slides was immersed in distilled water and submerged in 0.2% Cresyl violet (Sigma, St. Louis, MO, USA) for 5 min (Nissl staining). Other set of slides destined for Kluver Barrera was soaked in 0.1% solvent Blue 38 (Sigma, St. Louis, MO, USA) solution at 60 °C overnight. The dye was removed using lithium carbonate solution, distilled water, and 70% ethanol. The slides were then soaked in 0.1% Cresyl violet solution for 10 min and then sequentially in 95% ethanol, 100% ethanol, and xylene for dehydration and clearing.

For immunohistochemistry, the animals were deeply anesthetized with sodium thiopental i.p. (Thiopentax; Cristália, São Paulo, Brazil) and transcardially perfused with 0.1 M phosphate-buffered saline

(PBS) followed by 4% paraformaldehyde in 0.2 M phosphate buffer (PB). The brains were postfixed in the same fixative solution for 24 h and then cryoprotected by immersion in a sucrose gradient (10%, 20%, and 30%) for 24 h in each concentration. Finally, the brains were embedded in tissue freezing medium (Tissue-Tek® OCT, Sakura Finetek, Torrance, USA), quickly frozen in liquid nitrogen, and kept at −80 °C. The frozen brains were sectioned into 30 μm serial coronal sections between bregma coordinates −1.06 mm and −2.70 mm (Franklin and Paxinos, 1997) using a cryostat (Criocut 1800, Reichert-Jung, Heidelberg, Germany). The sections were collected into six alternating Eppendorf tubes that contained antifreeze solution (15% sucrose and 30% ethylene glycol in PBS) and stored at −24 °C until further processing.

Free-floating sections were washed three times with PBST (0.1 M PBS, pH 7.4, plus 3% Triton X-100) to remove the antifreeze solution. Endogenous peroxidase activity was blocked in 1% H_2O_2 in PBS for 30 min. After rinsing in PBST, the sections were incubated with 2% bovine serum albumin (BSA) in PBS for 60 min at room temperature. After three washes in PBST, the sections were incubated overnight in the same medium with the following polyclonal antibodies: goat anti-DCX (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-MAP-2 (1:250; Sigma-Aldrich, Saint Louis, MO, USA), rabbit anti-glial fibrillary acidic protein (GFAP; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1; 1:1000; Wako Chemicals, Cambridge, MA, USA). The sections were then incubated with the respective biotinylated secondary antibodies (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h and then in ABC solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature. The colorimetric peroxidase reaction was performed using 3,3′-diaminobenzidine (DAB; Sigma) and 0.05% H_2O_2. $NiCl_2$ was added to the DAB solution to increase the staining contrast.

2.6.1. Quantitative analysis

Histological and immunohistochemical analyses were performed using an Olympus BX41 microscope (Tokyo, Japan) coupled to a color high-performance device camera (QColor3, Ontario, Canada). The camera settings and microscope parameters were accurately kept constant to avoid signal saturation. ImageJ software (NIH, Bethesda, MD, USA) was used to calculate the number of cells and integrated optical density (IOD) when indicated. For IOD measurements, selected images were converted to 32-bit image gray scale, and the background was subtracted. The threshold for a positive signal was predefined, and the IOD was calculated. All of the analyses were conducted under blind conditions.

2.6.2. Nissl and Kluver Barrera staining

With the aid of the ImageJ cell counter plug-in, the number of intact-appearing pyramidal neurons along the Ammon horn CA1, CA2/CA3, and CA4 subfields of the hippocampus in both cerebral hemispheres was estimated. Hippocampal neurodegeneration was deduced from the reduction of the number of normal-looking (viable) pyramidal neurons relative to the sham-operated groups, and the effect of CBD thereon was assessed to indicate the presence or absence of neuronal rescue. Cells that presented a well delimited, spherical form with a distinct nucleus and nucleolus were counted as viable neurons. Cells that had shrunken cell bodies or surrounding empty spaces were considered neurons that were destined to die and were excluded from the counting. The values were averaged and used to represent the data (mean ± SEM) for each experimental group. The data are expressed as a percentage of the sham-operated group.

Kluver Barrera staining enables the evaluation of white matter (WM), focusing on the disarrangement of nerve fibers, formation of marked vacuoles, and disappearance of myelinated fibers (Wakita et al., 2002). For the semiquantitative analysis of WM, digital microscopic images of the corpus callosum located immediately above the stratum oriens of the CA1 hippocampal subfield were obtained (Fig. 3a). The IOD measurements were determined in prefixed areas (0.04 mm^2) located in the corpus callosum. The data are expressed as mean ± SEM.

2.6.3. Immunohistochemistry

The number of DCX-immunoreactive (DCX-IR) neurons was manually quantified in the subgranular zone (SGZ) and inner granular cell layer (GCL) of the dentate gyrus (DG) in both the right and left brain hemispheres. The results are expressed as the mean ± SEM of 6–8 sections per animal. For the Iba-1 analysis, prefixed digital microscopic areas were captured bilaterally for the CA1 (0.12 mm^2), CA2/CA3 (0.15 mm^2), and CA4 (0.07 mm^2) hippocampal subfields. All Iba-1-IR cells in the selected areas were manually counted and classified as being in a resting or reactive microglial state according to their morphological aspects (Gomes et al., 2015). Cellular processes (≤2) or cells that had 3–5 short branches were considered resting microglia. Cells with numerous and longer cell processes, a large soma, and retracted and thicker processes and cells with an amoeboid cell body, numerous short processes, and intense Iba-1 immunoreactivity were considered reactive microglia. The results are expressed as the mean ± SEM of the number of reactive microglia/area (mm^2). The GFAP immunoreactivity data were obtained by measuring the IOD in prefixed digital microscopic areas captured bilaterally for the CA1 (0.12 mm^2), CA2/CA3 (0.15 mm^2), and CA4 (0.07 mm^2) hippocampal subfields. The results are expressed as mean ± SEM/area (mm^2).

MAP-2 is known as a marker of structural integrity and plasticity related to the morphological stabilization of dendrite processes (Di Stefano et al., 2001). Images were captured bilaterally in a prefixed area (0.09 mm^2) in the stratum radiatum of the CA1 and the stratum lucidum of CA2/CA3 hippocampal subfields. The data are expressed as mean ± SEM/area (mm^2).

2.7. Western blot

Protein levels were assessed for NeuN, caspase-9, GFAP, and BDNF. Hippocampal homogenates (30 μg protein each) in sample buffer were separated on a 15% (BDNF) or 12% (NeuN, caspase-9, and GFAP) sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel using a total of four different blots to measure all of the different proteins. All of the blots were stripped to protein control with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After protein transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), the membranes were blocked with 2% BSA in Tris-buffered saline (TBS) and incubated with the primary antibody at 4 °C overnight at the following dilutions: rabbit anti-NeuN (1:500; Abcam, Cambridge, MA, USA), mouse anti-caspase-9 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-GFAP (1:2.000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-BDNF (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-GAPDH (1:5.000; Abcam, Cambridge, MA, USA). After a washing step with TBS, the membranes were incubated for 2 h with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000; Abcam, Cambridge, MA, USA) or donkey anti-mouse IgG (1:5000; Abcam, Cambridge, MA, USA) and developed using ECLplus® (Invitrogen, Carlsbad, CA, USA). The bands were visualized using the ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA). The IODs of the specific bands were quantified using ImageJ software and normalized to GAPDH levels.

2.8. Statistical analysis

Statistical analyses were performed using SAS 9.3 software. The behavioral and histological data were examined for assumptions of a normal distribution (D'Agostino and Pearson omnibus test) and homoscedasticity (Levene's test). Because the behavioral data fit the assumptions of a normal distribution and homoscedasticity, one-way analysis of variance (ANOVA) was used for between-group comparisons. Bonferroni's post hoc test was used to determine significant

differences among groups. Functional spatial memory within groups (i.e., D2 value in the OLT that differs significantly from 0) was analyzed using a two-way one-sample *t*-test. A generalized linear model for data with a Poisson distribution was used for the count data (i.e., the number of Nissl-stained cells, DCX-IR cells, and Iba-1-IR cells). A generalized linear model for data with a gamma distribution was used for continuous data (i.e., the IOD for Kluver Barrera-stained cells, MAP-2-IR, and GFAP-IR cells and Western blot data). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Cannabidiol decreases BCCAO-induced anxiety-like behavior

As shown in Fig. 2a, no differences were found in the distance traveled in the OFT ($F_{2,37} = 0.58$, $p = 0.56$), indicating that short-term CBD treatment and the BCCAO procedure did not affect general motor activity. The ANOVA indicated significant effects on the time spent in the open quadrants ($F_{2,37} = 11.50$, $p = 0.0001$) and number of crossings ($F_{2,37} = 7.68$, $p = 0.001$) in the EZM. The BCCAO + Veh group spent less time in the open quadrants compared with the Sham + Veh group ($p < 0.01$), likely reflecting an anxiogenic-like effect of BCCAO (Fig. 2b). The BCCAO + Veh group also exhibited a decrease in the number of crossings compared with the Sham + Veh group ($F_{2,37} = 7.68$, $p = 0.001$). This BCCAO-induced anxiogenic-like effect was reversed by CBD, reflected by an increase in the time spent in the open quadrants of the EZM ($p < 0.001$) and the number of crossings ($p = 0.002$) compared with the BCCAO + Veh group.

3.2. Cannabidiol improves cognitive performance in BCCAO mice

Fig. 2c and d show the effects of CBD in BCCAO mice in the YM and OLT. The ANOVA detected a significant difference in the total time spent in the novel arm of the YM ($F_{2,37} = 11.62$, $p = 0.0001$; Fig. 2c). The BCCAO + Veh group spent less time in the novel arm compared with the Sham + Veh group ($p < 0.001$), indicating that they failed to discriminate (or recognize) the novel arm relative to the familiar arms. This spatial memory deficit was significantly reduced in the CBD-treated group ($p < 0.05$, vs. vehicle).

In the OLT, a significant main effect of group was found at 1 h ($F_{2,37} = 19.86$, $p = 0.0001$) but not at 4 h ($F_{2,37} = 2.76$, $p = 0.07$) or 24 h ($F_{2,37} = 0.85$, $p = 0.43$; Fig. 2d). At 1 h, the BCCAO + Veh group had lower, negative D2 scores compared with the Sham + Veh group ($p < 0.001$), reflecting location memory impairment. This memory deficit was reversed by CBD ($p < 0.001$, vs. vehicle). When examining the D2 indices at 1 h intervals in more detail, the Sham + Veh and BCCAO + CBD groups could distinguish between the familiar and novel locations ($t = 4.72$–6.81, $p = 0.0001$–0.0005), whereas the BCCAO + Veh group could not ($t = 2.46$, $p = 0.03$).

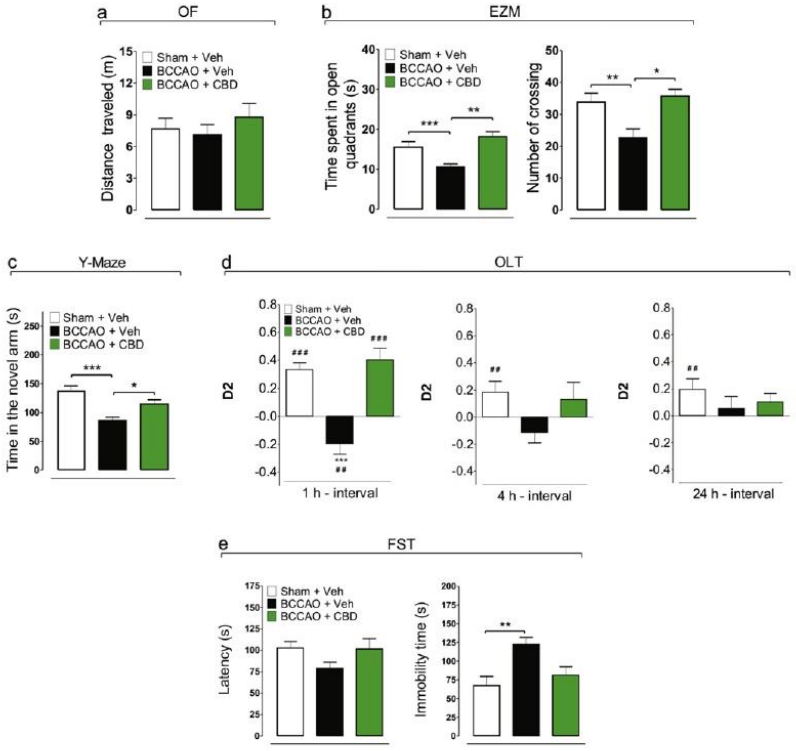


Fig. 2. Effects of 10 mg/kg CBD on motor, cognitive, and emotional behaviors in BCCAO mice. (a) Motor activity was evaluated in the open field (OF) by measuring the distance traveled. (b) Anxiety-like behavior was evaluated in the elevated zero maze (EZM) by measuring the time spent in the open quadrants and number of crossings between quadrants. (c) Cognitive performance was evaluated in the Y-maze by measuring the time spent in the novel arm. (d) Hippocampus-dependent memory was analyzed in the object location test (OLT) using the D2 exploration index ($D2 = [exploration\ time\ novel\ location - exploration\ time\ familiar\ location] / [exploration\ time\ novel\ location + exploration\ time\ familiar\ location]$) at 1, 4, and 24 h intervals. (e) The latency and immobility time were recorded in the forced swim test (FST). The bars represent the mean ± SEM in the different groups ($n = 12$–13/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA followed by Bonferroni's test); $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared with zero (i.e., chance level or no memory in the OLT; one-sample *t*-test).

3.3. Cannabidiol induces antidepressant-like effects in BCCAO mice

Fig. 2e shows the results of the FST. No differences were detected in the latency to the first episode of immobility among the experimental groups ($F_{2,37} = 2.15$, $p = 0.13$). However, immobility time was affected by BCCAO ($F_{2,37} = 4.31$, $p = 0.02$). The BCCAO + Veh group exhibited an increase in immobility time compared with the Sham + Veh group ($p < 0.01$). This effect was prevented by CBD.

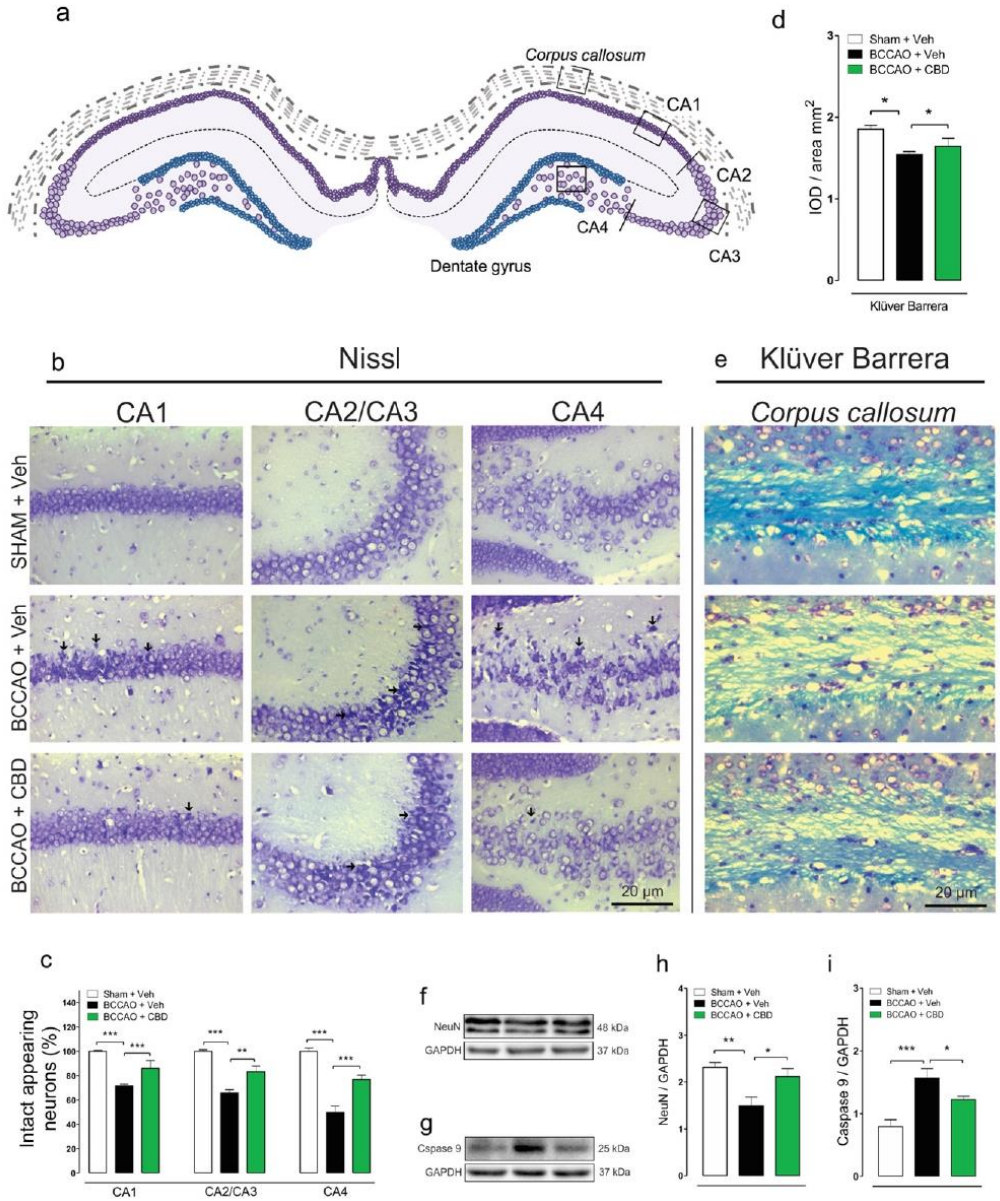


Fig. 3. Cannabidiol reduces hippocampal neurodegeneration and white matter injury in BCCAO mice. (a) Representative diagram illustrating a coronal brain section at the intermediate level of the hippocampus showing the CA1–CA4 subfields and a selected area in the corpus callosum where the analysis was performed. (b) Representative photomicrographs of the CA1, CA2/CA3, and CA4 hippocampal subfields (Nissl staining), indicating intact-appearing neurons and shrunken and dark-stained neurons (arrows) that indicate neurodegeneration. (c) Intact-appearing neurons were counted along the CA1, CA2/CA3, and CA4 hippocampal subfields 21 days after BCCAO. The results were normalized to the mean of the Sham + Veh group (100%). (d) The IOD of the corpus callosum (Kluver-Barrera staining). (e) Representative photomicrographs showing the corpus callosum. Notice the presence of vacuolization and fiber disarrangement in the BCCAO + Veh group compared with the Sham + Veh group. (f–i) Western blot results showing NeuN and caspase-9 protein levels in the hippocampus 21 days after BCCAO. The bars represent the mean ± SEM in the different groups ($n = 6$/group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.4. Cannabidiol decreases hippocampal neurodegeneration and WM injury induced by BCCAO

Hippocampal damage was evaluated by the reduction in the number of intact-appearing pyramidal neurons assessed by Nissl staining 21 days after sham or BCCAO surgery (Fig. 3b, c). A main group effect was detected in all hippocampal subfields ($\chi^2 = 28.37–102.60$, $p < 0.0001$). Between-group comparisons revealed that BCCAO reduced the number of intact neurons in the CA1, CA2/CA3, and CA4 subfields ($p < 0.001$) compared with the Sham + Veh group. Cannabidiol treatment decreased this neurodegenerative effect of BCCAO in the CA1 ($p = 0.004$), CA2/CA3 ($p = 0.005$), and CA4 ($p < 0.0001$) subfields.

As shown in Fig. 3d and e, Kluver-Barrera staining revealed changes in the structural arrangement of WM in the *corpus callosum* (above *stratum oriens*) in BCCAO mice. The BCCAO + Veh group exhibited more vacuolization and fiber disarrangement, reflected by a significant decrease in the IOD compared with the Sham + Veh group ($\chi^2 = 10.18$, $p < 0.006$). Cannabidiol treatment significantly recovered the IOD measurements ($p = 0.05$), indicating a protective effect against WM injury.

To quantitatively confirm injury in the hippocampus, the expression of NeuN protein levels was assessed using immunoblotting (Fig. 3f, h). The statistical analysis revealed a significant effect of BCCAO on NeuN hippocampal protein levels compared with the Sham + Veh group ($\chi^2 = 10.45$, $p = 0.005$). Cannabidiol treatment attenuated this effect in BCCAO mice compared with vehicle ($p = 0.005$). BCCAO also altered caspase-9 expression in hippocampal tissue ($\chi^2 = 16.27$, $p = 0.0003$). The BCCAO + Veh group exhibited higher expression of caspase-9 than the Sham + Veh group ($p = 0.0001$). Compared with vehicle, CBD treatment decreased caspase-9 levels in BCCAO mice ($p < 0.05$).

3.5. Cannabidiol decreases hippocampal neuroinflammation caused by BCCAO in mice

Neuroinflammation was assessed by analyzing the expression of Iba-1 (microglia) and GFAP (astrocytes) in the hippocampus (Fig. 4). The analysis of Iba-1 immunoreactivity indicated no differences in the total number of microglial cells in the CA1 ($\chi^2 = 3.95$, $p = 0.13$),

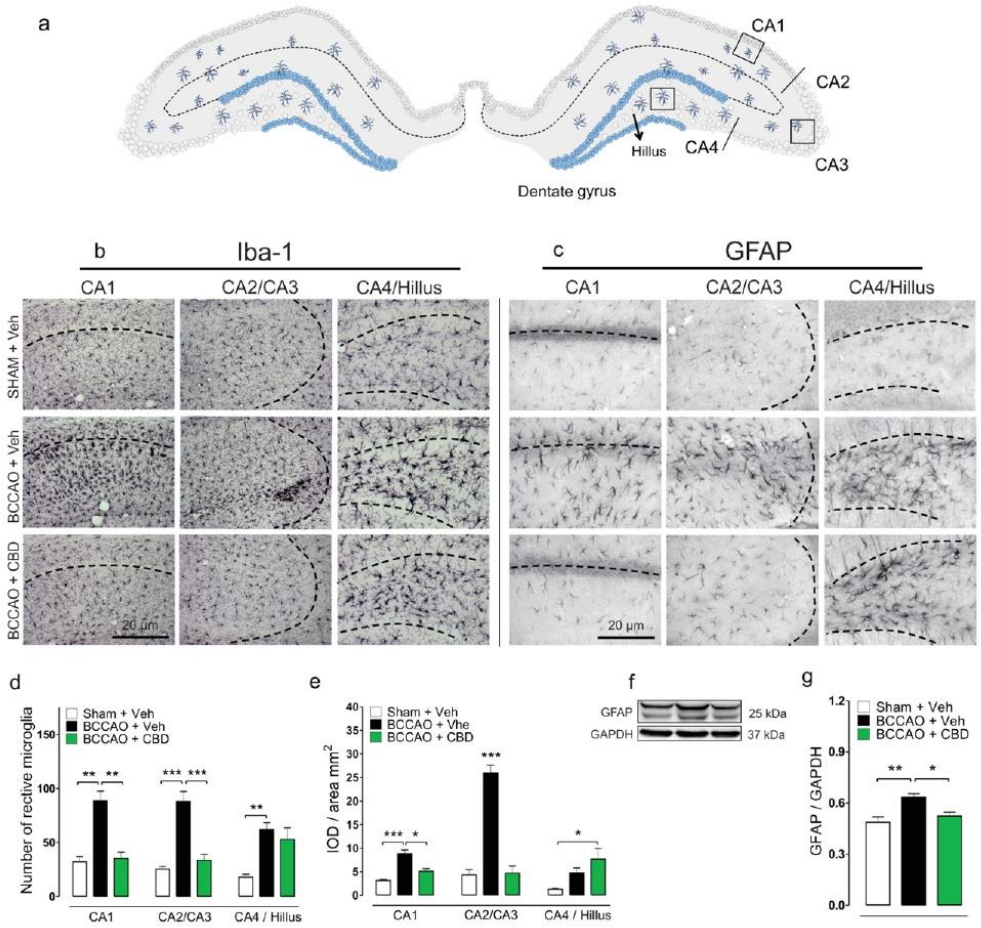


Fig. 4. Effects of CBD on the glial response 21 days after BCCAO. (a) Representative diagram illustrating a coronal brain section at the intermediate level of the hippocampus. (b, c) Representative photomicrographs of Iba-1-IR and GFAP-IR cells in the hippocampus. (d) Number of reactive microglia in the hippocampus. (e) The IOD of GFAP immunoreactivity in the hippocampus induced by BCCAO. (f, g) GFAP protein levels in the hippocampus. The bars represent the mean ± SEM in the different groups ($n = 6$/group). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

CA2/CA3 ($\chi^2 = 3.63$, $p = 0.16$), or CA4/hillus ($\chi^2 = 4.87$, $p = 0.09$). However, a significant effect on the number of reactive microglia was observed in the CA1 ($\chi^2 = 215.09$, $p < 0.0001$), CA2/CA3 ($\chi^2 = 269.88$, $p < 0.0001$), and CA4 ($\chi^2 = 171.68$, $p = 0.0001$) hippocampal subfields (Fig. 4b, d). A significant increase in the number of reactive microglia was found in all hippocampal subfields in the BCCAO + Veh group compared with the Sham + Veh group ($p < 0.0001$). Cannabidiol treatment reversed this effect in the CA1 and CA2/CA3 subfields in BCCAO mice ($p < 0.001$) but not in the CA4 subfield ($p > 0.04$).

As shown in Fig. 4c and e, BCCAO altered GFAP immunoreactivity in the CA1 ($\chi^2 = 23.10$, $p < 0.0001$), CA2/CA3 ($\chi^2 = 24.51$, $p < 0.0001$), and CA4 ($\chi^2 = 16.14$, $p = 0.0003$) hippocampal subfields. Increases in the IOD of GFAP immunoreactivity were observed in the CA1 ($p < 0.001$), CA2/CA3 ($p < 0.001$), and CA4/hillus ($p < 0.0003$) in the BCCAO + Veh group compared with the Sham + Veh group. Compared with vehicle, CBD treatment attenuated this effect of BCCAO in the CA1 ($p < 0.001$) and CA2/CA3 ($p < 0.0002$) subfields but not in the CA4/hillus subfield ($p < 0.2$).

GFAP protein levels were also altered in the hippocampus in BCCAO mice ($\chi^2 = 10.64$, $p = 0.005$) compared with the Sham + Veh group ($p = 0.0002$). Cannabidiol treatment prevented the increase in the IOD of GFAP-IR cells compared with the BCCAO + Veh group ($p = 0.03$).

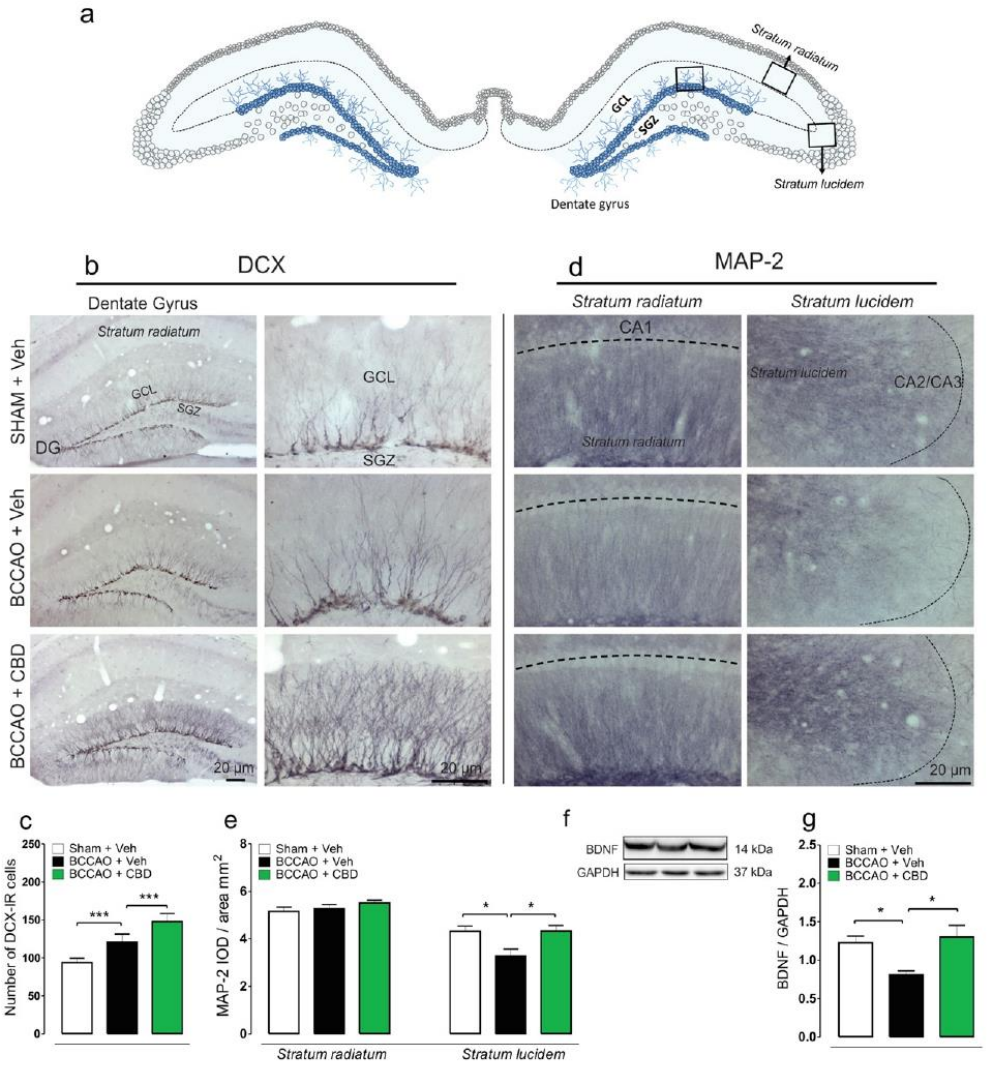


Fig. 5. Cannabidiol increases the number of DCX-IR neurons, MAP-2-IR cells, and BDNF protein levels in the hippocampus in BCCAO mice. (a) Representative diagram illustrating a coronal brain section at the intermediate level of the hippocampus showing the granular cell layer and selected areas in the CA1/stratum radiatum and CA3/stratum lucidem where the analyses were performed. (b) Representative photomicrographs of DCX-IR neurons in the granular cell layer (GCL) of the hippocampal dentate gyrus. (c) Number of DCX-IR neurons in the SGZ and GCL of the dentate gyrus. (d) Representative photomicrographs of MAP-2-IR cells in the stratum radiatum and stratum lucidem. (e) The IOD of MAP-2 immunoreactivity in the CA1/stratum radiatum and CA3/stratum lucidem. (f, g) BDNF protein levels in the hippocampus. The bars represents the mean ± SEM in the different groups ($n = 6$/group). $^*p < 0.05$, $^*p < 0.01$, $^{**}p < 0.001$.

3.6. Cannabidiol facilitates hippocampal neuroplasticity in BCCAO mice

The number of newborn neurons (DCX-IR), MAP-2 expression, and BDNF protein levels were used to evaluate neuronal plasticity in the hippocampus. A highly significant main effect of group was found on the number of DCX-IR neurons in the SGZ and GCL of the DG ($\chi^2 = 73.02$, $p < 0.0001$; Fig. 5b, c). BCCAO alone increased the number of DCX-IR neurons compared with the sham control group ($p > 0.0001$). This neurogenic response was further increased in the BCCAO + CBD group compared with the vehicle group ($p < 0.0001$).

As shown in Fig. 5d, no significant effect on the IOD of MAP-2 immunoreactivity was found in the stratum radiatum ($\chi^2 = 2.79$, $p = 0.25$), in contrast to the significant effect that was observed in the stratum lucidem ($\chi^2 = 8.95$, $p = 0.01$). In this region, BCCAO reduced the IOD of MAP-2 immunoreactivity ($p = 0.003$, BCCAO + Veh vs. Sham + Veh), indicating dendritic degeneration. This effect was attenuated in CBD-treated BCCAO animals compared with vehicle-treated BCCAO animals ($p = 0.003$).

The protein levels of BDNF were also investigated as an indicator of neuroplasticity. BCCAO mice exhibited a decrease in hippocampal BDNF levels compared with controls ($\chi^2 = 14.01$, $p = 0.0003$). Cannabidiol treatment prevented the decrease in BDNF protein levels in the BCCAO + Veh group compared with the BCCAO + CBD group ($p < 0.0001$).

4. Discussion

Cognitive and emotional dysfunctions are among the most impactful and enduring consequences of brain ischemic events. In the present study, using a multi-tiered behavioral testing battery, we found that BCCAO mice exhibited long-lasting functional deficits, reflected by increases in anxiety-like behavior in the EZM (day 9), memory impairments in the YM (day 12) and OLT (days 14–18), and despair-like behavior in the FST (day 21). Short-term CBD treatment prevented these cognitive and emotional impairments and attenuated hippocampal neurodegeneration and WM injury that were induced by BCCAO. Additionally, CBD reduced hippocampal neuroinflammation in BCCAO mice, reflected by decreases in reactive microglia and astrocytes. After 21 days of BCCAO, ischemic mice that were treated with CBD exhibited an increase in BDNF protein levels and DCX and MAP-2 expression in the hippocampus. These results show that short-term CBD treatment led to long-term functional and structural protective effects in mice after BCCAO.

Anxiety-like behavior was previously observed in mice 2–7 days after BCCAO (Nakashima et al., 2003, Neigh et al., 2009), an outcome that was later found to persist for at least 28 days after reperfusion (Soares et al., 2016, Soares et al., 2013). In the present study, BCCAO mice exhibited anxiogenic-like behavior, reflected by a decrease in open quadrant exploration in the EZM 9 days after reperfusion. These effects were prevented by CBD treatment. Indeed, CBD has been shown to have anxiolytic properties after acute administration in non-stressed rodents (Campos and Guimaraes, 2008, Guimaraes et al., 1990, Moreira et al., 2006, Resstel et al., 2006) and after chronic administration in stressed mice (Campos et al., 2013) as well as in a murine model of cerebral malaria (Campos et al., 2015).

Poor cognitive performance is a usual feature in rodents after global brain ischemia and is associated with hippocampal neurodegeneration (Kiryk et al., 2011, Soares et al., 2013). Our results indicated that BCCAO mice exhibited cognitive impairments in the hippocampus-dependent YM and OLT, associated with significant cell loss in the hippocampus. In the OLT, sham mice could distinguish the familiar from the novel object locations at 1, 4, and 24 h intervals, whereas BCCAO mice could not. Cannabidiol treatment improved working memory performance at 1 h in mice in the OLT. A similar positive effect of CBD was also detected in BCCAO mice at 1 h in the YM. These findings are consistent with previous studies that reported beneficial effects of CBD in BCCAO mice that were exposed to the Morris water maze task (Schiavon et al., 2014). Furthermore, positive effects of CBD on cognition were recently demonstrated in Alzheimer's disease transgenic mice, reflected by improvements in social recognition memory deficits (Cheng et al., 2014), and in a murine model of cerebral malaria (Campos et al., 2015). In both of these studies, the authors noted the impact of CBD treatment on neuroinflammation that was induced by ischemia. Accordingly, persistent hippocampal inflammation contributed to cognitive disruptions after lipopolysaccharide challenge in mice (Cibelli et al., 2010). One possibility is that the effects of CBD on reducing neuroinflammation contribute to its facilitative effect on memory recovery after BCCAO.

The present study confirmed that BCCAO mice exhibited significant hippocampal cell loss, reflected by Nissl staining and NeuN protein levels, compared with sham animals. In a previous study, we demonstrated that CBD prevented hippocampal cell loss and decreased the number of cells that underwent degeneration 7 days after BCCAO (Schiavon et al., 2014). Our results indicate that BCCAO in C57BL/6 mice may induce hippocampal neurodegeneration up to 21 days, reflected by an increase in caspase-9 levels, a key marker that is involved in triggering and promoting the activation of the apoptosis cascade. Cannabidiol treatment appears to act in a sustained way on the mechanisms that lead to long-term cell death (e.g., over 21 days), reflected by a reduction of caspase-9 protein levels and the maintenance of NeuN protein levels at control levels during this period. Cannabidiol prevented hippocampal and cortical neurodegeneration by normalizing caspase-3 levels in rats with brain iron overload (da Silva et al., 2014) and decreased caspase-9 concentrations in forebrain slices from newborn mice that underwent oxygen and glucose deprivation (Castillo et al., 2010).

White matter has been shown to be vulnerable to ischemia because of the low levels of intrinsic antioxidants and abundance of lipids that are present in the myelin sheath, which can be easily peroxidized after ischemia (Ueno et al., 2009). Using neurofilament protein (SMI-32) immunostaining to identify axonal injury, researchers found that the corpus callosum was consistently injured 3 days after 22 min of BCCAO in C57BL/6 mice (Yoshioka et al., 2011). In the present study, 20 min of BCCAO resulted in nerve fiber disarrangement, vacuolization, and the disappearance of myelinated fibers, reflected by a decrease in the IOD in the corpus callosum (i.e., Kluver-Barrera staining). Although the behavioral consequences of WM injury in BCCAO mice are still unclear, a link between corpus callosum injury and impairments in cognitive performance has been reported in models of focal brain ischemia (Blasi et al., 2014) and chronic cerebral hypoperfusion (Shibata et al., 2007). In rats, recognition memory is compromised after lesions of the corpus callosum (Kouhsar et al., 2011). Nevertheless, WM injury along with hippocampal neurodegeneration might contribute to memory deficits after BCCAO, in which WM tracts connect broadly distributed neuronal networks that coordinate several aspects of cognitive function. This possibility is consistent with the observation that WM lesions negatively impact a broad range of cognitive functions, such as memory, processing speed, attention, and executive function, in older humans, in whom cognitive impairments and cerebrovascular deficiency are common occurrences (Bolandzadeh et al., 2012). Recently, the development of delayed dementia after intracerebral hemorrhage was found to be associated with the severity of WM lesions, assessed by computerized tomography (Biffi et al., 2016). Importantly, in the present study, WM injury in BCCAO mice was attenuated by CBD treatment. To the best of our knowledge, these are the first findings indicating a protective effect of CBD against WM injury following brain ischemia.

Neuroinflammation is a central aspect of the brain ischemia process that includes the activation of astrocytes and rapid synthesis of cytokines and chemokines, which are closely associated with ischemia-induced neurodegeneration (Gehrmann et al., 1992, Stoll et al., 2006). Mice that were subjected to global brain ischemia exhibited an increase

in cytokine expression and glial activation in the hippocampus from days 2 to 7 after the insult (Schiavon et al., 2014, Taguchi et al., 2016). In an extensive study in rats, was reported an intense microglial and astroglial response in the hippocampus 2–3 days after global brain ischemia, which was accentuated on day 21 after the insult. With the progression of neuronal death, the authors suggested that the impact of dead cells on glial cells and cytokine expression is even stronger than the influence of ischemic stress per se (Yasuda et al., 2011). We observed a persistent glial response following BCCAO in mice. An increase in the expression of Iba-1 and GFAP immunoreactivity was observed in the whole hippocampus 21 days after BCCAO. Moreover, a large proportion of microglial cells undergo phenotypic transformation and present a reactive phenotype, indicating that these cells actively produce inflammatory cytokines, which may lead to neuronal apoptosis (Graeber et al., 2011). Cannabidiol treatment prevented both astroglial and microglial responses in the CA1 and CA2/CA3 hippocampal subfields in BCCAO mice. Because CBD was administered on the first 3 days after BCCAO, the present findings indicate that the neuroprotective effect of CBD occurred in the acute/early phase of ischemia. We previously found that CBD decreased GFAP expression 7 days after BCCAO (Schiavon et al., 2014). Using the same CBD treatment regimen in a model of focal brain ischemia, Hayakawa et al. (2009) showed that CBD inhibited the expression of high-mobility group box 1 (HMGB1), a proinflammatory cytokine that is massively released during the acute phase of ischemic processes and stimulates microglia activation (Kim et al., 2006). Castillo et al. (2010) showed neuroprotective effects of CBD in vitro, reflected by an increase in adenosine levels in forebrain slices from newborn mice under conditions of oxygen and glucose deprivation. However, these proposed mechanisms of action of CBD have been investigated in models of brain ischemia other than BCCAO. Whether CBD impacts HMGB1 expression or increases adenosine levels after BCCAO in mice needs further investigation.

Extending our previous studies (Soares et al., 2016), the present study found that BCCAO resulted in an increase in immobility time in the FST 21 days after BCCAO. We also detected a decrease in hippocampal BDNF protein levels in BCCAO mice compared with sham animals. A decrease in BDNF levels in the hippocampus has been associated with depressive-like behavior in ischemic mice (Kim et al., 2016, Moriyama et al., 2011, Pang et al., 2015). BDNF signaling has been shown to be necessary for antidepressant drug action (Castren et al., 2007), neuronal survival, and maintenance of the structural integrity of neurons (Moriyama et al., 2011). Under ischemic conditions, intraventricular administration of BDNF attenuated hippocampal damage after global forebrain ischemia (Beck et al., 1994, Wu and Pardridge, 1999) and reduced infarct size after MCAo (Schabitz et al., 1997). Moreover, fluoxetine (Kim et al., 2007) and escitalopram (Lee et al., 2011) protected against neuronal damage after transient global brain ischemia, an effect that was related to the upregulation of BDNF expression. In the present study, BCCAO mice that were treated with CBD exhibited an increase in hippocampal BDNF protein levels. The increase in BDNF levels 21 days after BCCAO coincided with the behavioral recovery in the FST. These findings suggest that prior treatment with CBD triggered protective mechanisms that might be involved in long-term improvements in emotional behavior in ischemic mice.

Compensatory hippocampal neurogenesis is believed to contribute to brain repair and functional recovery following experimental transient global cerebral ischemia. The survival of new neurons after an ischemic episode is small and transient (Lei et al., 2014, Zhang et al., 2011, Zhang et al., 2004). For example, DCX, a microtubule-associated protein that is expressed in newborn neurons (Brown et al., 2003), peaks 7–10 days after BCCAO but returns to baseline levels within 2–4 weeks (Soares et al., 2013). Accordingly, we observed an increase in the number of DCX-IR neurons 21 days after BCCAO, which may indicate compensatory neurogenesis that is induced by brain ischemia. Moreover, CBD treatment enhanced hippocampal neurogenesis in BCCAO mice compared with ischemic animals that received vehicle. BDNF is a potent stimulator of neurogenesis in intact and ischemic brains (Blondeau et al., 2009), and one possibility is that the neurogenic effect of CBD that was observed in BCCAO mice is related to an enhancement of hippocampal BDNF protein levels.

MAP-2 is a microtubule protein that is linked to the structural integrity of the cytoskeleton, dendrite growth, and synapse formation (Jones et al., 1996, Zepeda et al., 2004). The loss of MAP-2 in the CA1 and CA3 hippocampal subfields has been considered one of the earliest pathogenic events that indicate dendrite breakdown following global cerebral ischemia in rats (Johansen et al., 1984, Yan et al., 2013). MAP-2 expression days or weeks after ischemic injury may reflect a degree of dendritic restructuring (Briones et al., 2006). BCCAO mice exhibited a decrease in MAP-2 expression in the stratum lucidem in the CA3 hippocampal subfield. No changes in MAP-2 expression were detected in the CA1 subfield 21 days following BCCAO. Our findings indicate regional differences in hippocampal MAP-2 expression that was induced by BCCAO in mice. Cannabidiol treatment prevented the loss of MAP-2 immunoreactivity in the CA3 subfield, pointing that CBD may have interrupted dendritic degeneration and/or stimulated dendritic regeneration. The reason for the selective changes in MAP-2 immunoreactivity in the CA3 subfield is unclear. Rats that were subjected to a global model of brain ischemia (Bacarin et al., 2016) and were treated with fish oil presented recovery of MAP-2 immunoreactivity in the CA3 subfield, suggesting that the number of viable neurons and their processes in the stratum radiatum of the CA3 subfield served as a primary site for the neuroplastic response by axons and dendrites following brain ischemia. This interpretation is supported by another study that subjected rats to stroke, in which MAP-2 immunoreactivity was greatly reduced in the ischemic core but selectively increased in the boundary, penumbral zone of the infarct, reflecting a neuroplastic response of the axons and dendrites that survived ischemia (Li et al., 1998).

The mechanisms of CBD effects have not been investigated in the present paper but are likely to involve multiple pharmacological targets. For example, facilitation of serotonin 5-HT_{1A} receptor-mediated neurotransmission have been reported to be involved not only in the anxiolytic and antidepressive-like effects of CBD (Campos et al., 2012b) but also in its neuroprotective effects in mice subjected to MCAo (Mishima et al., 2005) and in an H/I model in newborn pigs (Pazos et al., 2013). Under ischemic conditions, CBD seems to exert neuroprotective effects by modulating excitotoxicity, oxidative stress, and inflammation (Castillo et al., 2010, Hayakawa et al., 2009). By inhibiting anandamide metabolism, CBD could indirectly activate CB1 and CB2 receptors and modulate these process (Campos et al., 2012a, Campos et al., 2016). Other mechanisms, such as activation of nuclear receptors of the peroxisome proliferator-activated receptor family and blockade of adenosine uptake, are also likely to be involved (for review, see Campos et al., 2012a, 2012b, Campos et al., 2016). This multitarget effect of CBD may represent a novel approach to providing neuroprotection in brain ischemia processes and perhaps other neuropsychiatric disorders.

5. Conclusion

The success of a future neuroprotective agent in brain ischemia may depend on targeting multiple mechanisms to elicit global functional recovery. The present study found that short-term CBD treatment promoted sustained neuroprotective effects in mice that were subjected to the BCCAO model of brain ischemia. The benefits of CBD may be related to the prevention of hippocampal neuronal loss, WM protection, a decrease in neuroinflammation, and an increase in hippocampal plasticity, reflected by increases in neurogenesis, MAP-2 immunoreactivity, and BDNF protein levels. The fact that short-term CBD treatment has protective effects that are apparent 21 days after BCCAO implies a promising therapeutic action of this compound against the long-term consequences of BCCAO.

Conflict of interest

There is no conflict of interest regarding the information of this manuscript.

Acknowledgments

The authors thank Marco Alberto Trombelli for his technical support. This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (4598/2012), Universidade Estadual de Maringá and FAPESP, São Paulo, Brazil.

References

Alvarez, F.J., Lafuente, H., Rey-Santano, M.C., Mielgo, V.E., Gastiasoro, E., Rueda, M., et al., 2008. Neuroprotective effects of the nonpsychoactive cannabinoid cannabidiol in hypoxic-ischemic newborn piglets. Pediatr. Res. 64, 653–658.

Bacarin, C.C., Godinho, J., de Oliveira, R.M., Matsushita, M., Gohara, A.K., Cardozo-Filho, L., et al., 2016. Postischemic fish oil treatment restores long-term retrograde memory and dendritic density: an analysis of the time window of efficacy. Behav. Brain Res. 311, 425–439.

Balkaya, M., Krober, J., Gertz, K., Peruzzaro, S., Endres, M., 2013. Characterization of long-term functional outcome in a murine model of mild brain ischemia. J. Neurosci. Methods 213, 179–187.

Beck, T., Lindholm, D., Castren, E., Wree, A., 1994. Brain-derived neurotrophic factor protects against ischemic cell damage in rat hippocampus. Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism 14, 689–692.

Biffi, A., Bailey, D., Anderson, C.D., Ayres, A.M., Gurol, E.M., Greenberg, S.M., et al., 2016. Risk factors associated with early vs delayed dementia after intracerebral hemorrhage. JAMA Neurology 73, 969–976.

Blasi, F., Wei, Y., Balkaya, M., Tikka, S., Mandeville, J.B., Waeber, C., et al., 2014. Recognition memory impairments after subcortical white matter stroke in mice. Stroke; A Journal of Cerebral Circulation 45, 1468–1473.

Blondeau, N., Nguemeni, C., Debruyne, D.N., Piens, M., Wu, X., Pan, H., et al., 2009. Subchronic alpha-linolenic acid treatment enhances brain plasticity and exerts an antidepressant effect: a versatile potential therapy for stroke. Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology 34, 2548–2559.

Bolandzadeh, N., Davis, J.C., Tam, R., Handy, T.C., Liu-Ambrose, T., 2012. The association between cognitive function and white matter lesion location in older adults: a systematic review. BMC Neurol. 12, 126.

Braida, D., Pegorini, S., Arcidiacono, M.V., Consalez, G.G., Croci, L., Sala, M., 2003. Post-ischemic treatment with cannabidiol prevents electroencephalographic flattening, hyperlocomotion and neuronal injury in gerbils. Neurosci. Lett. 346, 61–64.

Briones, T.L., Suh, E., Jozsa, L., Woods, J., 2006. Behaviorally induced synaptogenesis and dendritic growth in the hippocampal region following transient global cerebral ischemia are accompanied by improvement in spatial learning. Exp. Neurol. 198, 530–538.

Brown, J.P., Couillard-Despres, S., Cooper-Kuhn, C.M., Winkler, J., Aigner, L., Kuhn, H.G., 2003. Transient expression of doublecortin during adult neurogenesis. J. Comp. Neurol. 467, 1–10.

Campos, A.C., Guimaraes, F.S., 2008. Involvement of 5HT1A receptors in the anxiolytic-like effects of cannabidiol injected into the dorsolateral periaqueductal gray of rats. Psychopharmacology 199, 223–230.

Campos, A.C., Ortega, Z., Palazuelos, J., Fogaça, M.V., Aguiar, D.C., Díaz-Alonso, J., Ortega-Gutiérrez, S., Vázquez-Villa, H., Moreira, F.A., Guzmán, M., Galve-Roperh, I., Guimarães, F.S., 2013. The anxiolytic effect of cannabidiol on chronically stressed mice depends on hippocampal neurogenesis: involvement of the endocannabinoid system. Int. J. Neuropsychopharmacol 16, 1407–1419.

Campos, A.C., Brant, F., Miranda, A.S., Machado, F.S., Teixeira, A.L., 2015. Cannabidiol increases survival and promotes rescue of cognitive function in a murine model of cerebral malaria. Neuroscience 289, 166–180.

Campos, A.C., Ferreira, F.R., Guimaraes, F.S., 2012a. Cannabidiol blocks long-lasting behavioral consequences of predator threat stress: possible involvement of 5HT1A receptors. J. Psychiatr. Res. 46, 1501–1510.

Campos, A.C., Fogaca, M.V., Sonego, A.B., Guimaraes, F.S., 2016. Cannabidiol, neuroprotection and neuropsychiatric disorders. Pharmacol. Res.

Campos, A.C., Moreira, F.A., Gomes, F.V., Del Bel, E.A., Guimaraes, F.S., 2012b. Multiple mechanisms involved in the large-spectrum therapeutic potential of cannabidiol in psychiatric disorders. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 367, 3364–3378.

Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F., Renzi, P., 2002. Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. Behav. Brain Res. 134, 49–57.

Castillo, A., Tolon, M.R., Fernandez-Ruiz, J., Romero, J., Martinez-Orgado, J., 2010. The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB(2) and adenosine receptors. Neurobiol. Dis. 37, 434–440.

Castren, E., Voikar, V., Rantamaki, T., 2007. Role of neurotrophic factors in depression. Curr. Opin. Pharmacol. 7, 18–21.

Cheng, D., Spiro, A.S., Jenner, A.M., Garner, B., Karl, T., 2014. Long-term cannabidiol treatment prevents the development of social recognition memory deficits in Alzheimer's disease transgenic mice. Journal of Alzheimer's Disease: JAD 42, 1383–1396.

Cibelli, M., Fidalgo, A.R., Terrando, N., Ma, D., Monaco, C., Feldmann, M., et al., 2010. Role of interleukin-1beta in postoperative cognitive dysfunction. Ann. Neurol. 68, 360–368.

da Silva, V.K., de Freitas, B.S., da Silva, D.A., Nery, L.R., Falavigna, L., Ferreira, R.D., et al., 2014. Cannabidiol normalizes caspase 3, synaptophysin, and mitochondrial fission protein DNM1L expression levels in rats with brain iron overload: implications for neuroprotection. Mol. Neurobiol. 49, 222–233.

Dellu, F., Contarino, A., Simon, H., Koob, G.F., Gold, L.H., 2000. Genetic differences in response to novelty and spatial memory using a two-trial recognition task in mice. Neurobiol. Learn. Mem. 73, 31–48.

Di Stefano, G., Casoli, T., Fattoretti, P., Gracciotti, N., Solazzi, M., Bertoni-Freddari, C., 2001. Distribution of MAP2 in hippocampus and cerebellum of young and old rats by quantitative immunohistochemistry. J. Histochem. Cytochem. 49, 1065–1066.

Dirnagl, U., Endres, M., 2014. Found in translation: preclinical stroke research predicts human pathophysiology, clinical phenotypes, and therapeutic outcomes. Stroke; A Journal of Cerebral Circulation 45, 1510–1518.

Fernandez-Ruiz, J., Sagredo, O., Pazos, M.R., Garcia, C., Pertwee, R., Mechoulam, R., et al., 2013. Cannabidiol for neurodegenerative disorders: important new clinical applications for this phytocannabinoid? Br. J. Clin. Pharmacol. 75, 323–333.

Flynn, R.W., MacWalter, R.S., Doney, A.S., 2008. The cost of cerebral ischaemia. Neuropharmacology 55, 250–256.

Franklin, K.B.J., Paxinos, G., 1997. The Mouse Brain in Stereotaxic Coordinates. 2th Edn. Academic Press, San Diego.

Garcia-Arencibia, M., Gonzalez, S., de Lago, E., Ramos, J.A., Mechoulam, R., Fernandez-Ruiz, J., 2007. Evaluation of the neuroprotective effect of cannabinoids in a rat model of Parkinson's disease: importance of antioxidant and cannabinoid receptor-independent properties. Brain Res. 1134, 162–170.

Garcia-Chavez, D., Gonzalez-Burgos, I., Letechipia-Vallejo, G., Lopez-Loeza, E., Morali, G., Cervantes, M., 2008. Long-term evaluation of cytoarchitectonic characteristics of prefrontal cortex pyramidal neurons, following global cerebral ischemia and neuroprotective melatonin treatment, in rats. Neurosci. Lett. 448, 148–152.

Gehrmann, J., Bonnekoh, P., Miyazawa, T., Hossmann, K.A., Kreutzberg, G.W., 1992. Immunocytochemical study of an early microglial activation in ischemia. Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism 12, 257–269.

Geri, G., Mongardon, N., Daviaud, F., Empana, J.P., Dumas, F., Cariou, A., 2014. Neurological consequences of cardiac arrest: where do we stand? Annales francaises d'anesthesie et de reanimation 33, 98–101.

Ginsberg, M.D., 2009. Current status of neuroprotection for cerebral ischemia: synoptic overview. Stroke; A Journal of Cerebral Circulation 40, S111–S114.

Gomes, F.V., Llorente, R., Del Bel, E.A., Viveros, M.P., Lopez-Gallardo, M., Guimaraes, F.S., 2015. Decreased glial reactivity could be involved in the antipsychotic-like effect of cannabidiol. Schizophr. Res. 164, 155–163.

Graeber, M.B., Li, W., Rodriguez, M.L., 2011. Role of microglia in CNS inflammation. FEBS Lett. 585, 3798–3805.

Guimaraes, F.S., Chiaretti, T.M., Graeff, F.G., Zuardi, A.W., 1990. Antianxiety effect of cannabidiol in the elevated plus-maze. Psychopharmacology 100, 558–559.

Hall, E.D., Traystman, R.J., 2009. Role of animal studies in the design of clinical trials. Frontiers of Neurology and Neuroscience 25, 10–33.

Hayakawa, K., Irie, K., Sano, K., Watanabe, T., Higuchi, S., Enoki, M., et al., 2009. Therapeutic time window of cannabidiol treatment on delayed ischemic damage via high-mobility group box1-inhibiting mechanism. Biol. Pharm. Bull. 32, 1538–1544.

Hayakawa, K., Mishima, K., Irie, K., Hazekawa, M., Mishima, S., Fujioka, M., et al., 2008. Cannabidiol prevents a post-ischemic injury progressively induced by cerebral ischemia via a high-mobility group box1-inhibiting mechanism. Neuropharmacology 55, 1280–1286.

Johansen, F.F., Jorgensen, M.B., Ekstrom von Lubitz, D.K., Diemer, N.H., 1984. Selective dendrite damage in hippocampal CA1 stratum radiatum with unchanged axon ultrastructure and glutamate uptake after transient cerebral ischaemia in the rat. Brain Res. (291), 373–377.

Jones, L., Fischer, I., Levitt, P., 1996. Nonuniform alteration of dendritic development in the cerebral cortex following prenatal cocaine exposure. Cereb. Cortex 6, 431–445.

Kim, A.S., Johnston, S.C., 2011. Global variation in the relative burden of stroke and ischemic heart disease. Circulation 124, 314–323.

Kim, Y.R., Kim, H.N., Hong, K.W., Shin, H.K., Choi, B.T., 2016. Anti-depressant effects of phosphodiesterase 3 inhibitor cilostazol in chronic mild stress-treated mice after ischemic stroke. Psychopharmacology 233, 1055–1066.

Kim, D.H., Li, H., Yoo, K.Y., Lee, B.H., Hwang, I.K., Won, M.H., 2007. Effects of fluoxetine on ischemic cells and expressions in BDNF and some antioxidants in the gerbil hippocampal CA1 region induced by transient ischemia. Exp. Neurol. 204, 748–758.

Kim, J.B., Sig Choi, J., Yu, Y.M., Nam, K., Piao, C.S., Kim, S.W., et al., 2006. HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. J. Neurosci. Off. J. Soc. Neurosci. 26, 6413–6421.

Kiryk, A., Pluta, R., Figiel, I., Mikosz, M., Ulamek, M., Niewiadomska, G., et al., 2011. Transient brain ischemia due to cardiac arrest causes irreversible long-lasting cognitive injury. Behav. Brain Res. 219, 1–7.

Kouhsar, S.S., Karami, M., Tafreshi, A.P., Roghani, M., Nadoushan, M.R., 2011. Microinjection of l-arginine into corpus callosum cause reduction in myelin concentration and neuroinflammation. Brain Res. 1392, 93–100.

Kronenberg, G., Gertz, K., Heinz, A., Endres, M., 2014. Of mice and men: modelling post-stroke depression experimentally. Br. J. Pharmacol. 171, 4673–4689.

Lafuente, H., Alvarez, F.J., Pazos, M.R., Alvarez, A., Rey-Santano, M.C., Mielgo, V., et al., 2011. Cannabidiol reduces brain damage and improves functional recovery after acute hypoxia-ischemia in newborn pigs. Pediatr. Res. 70, 272–277.

Lee, C.H., Park, J.H., Yoo, K.Y., Choi, J.H., Hwang, I.K., Ryu, P.D., et al., 2011. Pre- and post-treatments with escitalopram protect against experimental ischemic neuronal

damage via regulation of BDNF expression and oxidative stress. Exp. Neurol. 229, 450–459.
Lei, S., Zhang, P., Li, W., Gao, M., He, X., Zheng, J., et al., 2014. Pre- and posttreatment with edaravone protects CA1 hippocampus and enhances neurogenesis in the subgranular zone of dentate gyrus after transient global cerebral ischemia in rats. ASN Neuro. 6.
Leo, A., Russo, E., Elia, M., 2016. Cannabidiol and epilepsy: rationale and therapeutic potential. Pharmacol. Res. 107, 85–92.
Li, Y., Jiang, N., Powers, C., Chopp, M., 1998. Neuronal damage and plasticity identified by microtubule-associated protein 2, growth-associated protein 43, and cyclin D1 immunoreactivity after focal cerebral ischemia in rats. Stroke; A Journal of Cerebral Circulation 29, 1972–1980 (discussion 80-1).
Martin-Moreno, A.M., Reigada, D., Ramirez, B.G., Mechoulam, R., Innamorato, N., Cuadrado, A., et al., 2011. Cannabidiol and other cannabinoids reduce microglial activation in vitro and in vivo: relevance to Alzheimer's disease. Mol. Pharmacol. 79, 964–973.
Mechoulam, R., Gaoni, Y., 1965. A total synthesis of DL-delta-1-tetrahydrocannabinol, the active constituent of hashish. J. Am. Chem. Soc. 87, 3273–3275.
Mishima, K., Hayakawa, K., Abe, K., Ikeda, T., Egashira, N., Iwasaki, K., et al., 2005. Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine1A receptor-dependent mechanism. Stroke; A Journal of Cerebral Circulation 36, 1077–1082.
Moreira, F.A., Aguiar, D.C., Guimaraes, F.S., 2006. Anxiolytic-like effect of cannabidiol in the rat vogel conflict test. Prog. Neuro-Psychopharmacol. Biol. Psychiatry 30, 1466–1471.
Moriyama, Y., Takagi, N., Tanonaka, K., 2011. Intravenous injection of neural progenitor cells improved depression-like behavior after cerebral ischemia. Transl. Psychiatry 1, e29.
Moulaert, V.R., Wachelder, E.M., Verbunt, J.A., Wade, D.T., van Heugten, C.M., 2010. Determinants of quality of life in survivors of cardiac arrest. J. Rehabil. Med. 42, 553–558.
Nakashima, M.N., Ajiki, K., Nakashima, K., Takahashi, M., 2003. Possible role of nitric oxide in anxiety following transient cerebral ischemia in mice. J. Pharmacol. Sci. 91, 47–52.
Neigh, G.N., Karelina, K., Glasper, E.R., Bowers, S.L., Zhang, N., Popovich, P.G., et al., 2009. Anxiety after cardiac arrest/cardiopulmonary resuscitation: Exacerbated by stress and prevented by minocycline. Stroke; A Journal of Cerebral Circulation 40, 3601–3607.
Pang, C., Cao, L., Wu, F., Wang, L., Wang, G., Yu, Y., et al., 2015. The effect of trans-resveratrol on post-stroke depression via regulation of hypothalamus-pituitary-adrenal axis. Neuropharmacology 97, 447–456.
Pazos, M.R., Cinquina, V., Gomez, A., Layunta, R., Santos, M., Fernandez-Ruiz, J., et al., 2012. Cannabidiol administration after hypoxia-ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function. Neuropharmacology 63, 776–783.
Pazos, M.R., Mohammed, N., Lafuente, H., Santos, M., Martinez-Pinilla, E., Moreno, E., et al., 2013. Mechanisms of cannabidiol neuroprotection in hypoxic-ischemic newborn pigs: role of 5HT(1A) and CB2 receptors. Neuropharmacology 71, 282–291.
Pertwee, R.G., Thomas, A., Stevenson, L.A., Maor, Y., Mechoulam, R., 2005. Evidence that (−)-7-hydroxy-4′-dimethylheptyl-cannabidiol activates a non-CB(1), non-CB(2), non-TRPV1 target in the mouse vas deferens. Neuropharmacology 48, 1139–1146.
Porsolt, R.D., Bertin, A., Jalfre, M., 1978. Behavioural despair in rats and mice: strain differences and the effects of imipramine. Eur. J. Pharmacol. 51, 291–294.
Resstel, L.B., Joca, S.R., Moreira, F.A., Correa, F.M., Guimaraes, F.S., 2006. Effects of cannabidiol and diazepam on behavioral and cardiovascular responses induced by contextual conditioned fear in rats. Behav. Brain Res. 172, 294–298.
Rutten, K., Lieben, C., Smits, L., Blokland, A., 2007. The PDE4 inhibitor rolipram reverses object memory impairment induced by acute tryptophan depletion in the rat. Psychopharmacology 192, 275–282.
Schabitz, W.R., Schwab, S., Spranger, M., Hacke, W., 1997. Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats. Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism 17, 500–506.
Schiavon, A.P., Soares, L.M., Bonato, J.M., Milani, H., Guimaraes, F.S., Weffort de Oliveira, R.M., 2014. Protective effects of cannabidiol against hippocampal cell death and cognitive impairment induced by bilateral common carotid artery occlusion in mice. Neurotox. Res. 26, 307–316.
Seif el Nasr, M., Nuglisch, J., Krieglstein, J., 1992. Prevention of ischemia-induced cerebral hypothermia by controlling the environmental temperature. J. Pharmacol. Toxicol. Methods 27, 23–26.
Shibata, M., Yamasaki, N., Miyakawa, T., Kalaria, R.N., Fujita, Y., Ohtani, R., et al., 2007. Selective impairment of working memory in a mouse model of chronic cerebral hypoperfusion. Stroke; A Journal of Cerebral Circulation 38, 2826–2832.
Soares, L.M., De Vry, J., Steinbusch, H.W., Milani, H., Prickaerts, J., Weffort de Oliveira, R.M., 2016. Rolipram improves cognition, reduces anxiety- and despair-like behaviors and impacts hippocampal neuroplasticity after transient global cerebral ischemia. Neuroscience 326, 69–83.
Soares, L.M., Schiavon, A.P., Milani, H., de Oliveira, R.M., 2013. Cognitive impairment and persistent anxiety-related responses following bilateral common carotid artery occlusion in mice. Behav. Brain Res. 249, 28–37.
Stoll, M., Capper, D., Dietz, K., Warth, A., Schleich, A., Schlaszus, H., et al., 2006. Differential microglial regulation in the human spinal cord under normal and pathological conditions. Neuropathol. Appl. Neurobiol. 32, 650–661.
Taguchi, N., Nakayama, S., Tanaka, M., 2016. Single administration of soluble epoxide hydrolase inhibitor suppresses neuroinflammation and improves neuronal damage after cardiac arrest in mice. Neurosci. Res.
Ueno, Y., Zhang, N., Miyamoto, N., Tanaka, R., Hattori, N., Urabe, T., 2009. Edaravone attenuates white matter lesions through endothelial protection in a rat chronic hypoperfusion model. Neuroscience 162, 317–327.
Wakita, H., Tomimoto, H., Akiguchi, I., Matsuo, A., Lin, J.X., Ihara, M., McGeer, P.L., 2002. Axonal damage and demyelination in the white matter after chronic cerebral hypoperfusion in the rat. Brain Res. 924, 63–70.
Wu, D., Pardridge, W.M., 1999. Neuroprotection with noninvasive neurotrophin delivery to the brain. Proc. Natl. Acad. Sci. U. S. A. 96, 254–259.
Yan, B.C., Park, J.H., Ahn, J.H., Lee, J.C., Won, M.H., Kang, I.J., 2013. Postsynaptic density protein (PSD)-95 expression is markedly decreased in the hippocampal CA1 region after experimental ischemia-reperfusion injury. J. Neurol. Sci. 330, 111–116.
Yasuda, Y., Shimoda, T., Uno, K., Tateishi, N., Furuya, S., Tsuchihashi, Y., et al., 2011. Temporal and sequential changes of glial cells and cytokine expression during neuronal degeneration after transient global ischemia in rats. J. Neuroinflammation 8, 70.
Yoshioka, H., Niizuma, K., Katsu, M., Sakata, H., Okami, N., Chan, P.H., 2011. Consistent injury to medium spiny neurons and white matter in the mouse striatum after prolonged transient global cerebral ischemia. J. Neurotrauma 28, 649–660.
Zepeda, A., Sengpiel, F., Guagnelli, M.A., Vaca, L., Arias, C., 2004. Functional reorganization of visual cortex maps after ischemic lesions is accompanied by changes in expression of cytoskeletal proteins and NMDA and GABA(A) receptor subunits. J. Neurosci. Off. J. Soc. Neurosci. 24, 1812–1821.
Zhang, C., Wu, H., Zhu, X., Wang, Y., Guo, J., 2011. Role of transcription factors in neurogenesis after cerebral ischemia. Rev. Neurosci. 22, 457–465.
Zhang, R., Zhang, Z., Zhang, C., Zhang, L., Robin, A., Wang, Y., et al., 2004. Stroke transiently increases subventricular zone cell division from asymmetric to symmetric and increases neuronal differentiation in the adult rat. J. Neurosci. Off. J. Soc. Neurosci. 24, 5810–5815.

9.4 Part III: Mori et al. Eur J Neurosci. 2021

Received: 16 September 2020 | Revised: 3 December 2020 | Accepted: 17 January 2021

DOI: 10.1111/ejn.15134

RESEARCH REPORT

EJN European Journal of Neuroscience FENS WILEY

Differential contribution of CB1, CB2, 5-HT1A, and PPAR-γ receptors to cannabidiol effects on ischemia-induced emotional and cognitive impairments

Marco Aurélio Mori[1] | **Erika Meyer**[1] | **Francielly F. da Silva**[1] | **Humberto Milani**[1] | **Francisco Silveira Guimarães**[2] | **Rúbia Maria Weffort Oliveira**[1]

[1]Department of Pharmacology and Therapeutics, State University of Maringá, Maringá, Brazil

[2]Department of Pharmacology, School of Medicine, USP, Ribeirão Preto, Brazil

Correspondence
Rúbia Maria Weffort Oliveira, Department of Pharmacology and Therapeutics, State University of Maringá, Av. Colombo, 5790, 87020-900 Maringá, Paraná, Brazil.
E-mail: rubiaweffort@gmail.com

Funding information
Coordenação de Aperfeiçoamento de Pessoal de Nível Superior; Conselho Nacional de Desenvolvimento Científico e Tecnológico; Instituto Nacional de Ciência e Tecnologia Translacional em Medicina; Universidade Estadual de Maringá; FAPESP, Grant/Award Number: 2017/24304-0

Abstract

An ever-increasing body of preclinical studies has shown the multifaceted neuroprotective profile of cannabidiol (CBD) against impairments caused by cerebral ischemia. In this study, we have explored the neuropharmacological mechanisms of CBD action and its impact on functional recovery using a model of transient global cerebral ischemia in mice. C57BL/6J mice were subjected to bilateral common carotid artery occlusion (BCCAO) for 20 min and received vehicle or CBD (10 mg/Kg) 0.5 hr before and 3, 24, and 48 hr after reperfusion. To investigate the neuropharmacological mechanisms of CBD, the animals were injected with CB_1 (AM251, 1 mg/kg), CB_2 (AM630, 1 mg/kg), 5-HT_{1A} (WAY-100635, 10 mg/kg), or PPAR-γ (GW9662, 3 mg/kg) receptor antagonists 0.5 hr prior to each injection of CBD. The animals were evaluated using a multi-task testing battery that included the open field, elevated zero maze, Y-maze (YM), and forced swim test. CBD prevented anxiety-like behavior, memory impairments, and despair-like behaviors induced by BCCAO in mice. The anxiolytic-like effects of CBD in BCCAO mice were attenuated by CB_1, CB_2, 5-HT_{1A}, and PPAR-γ receptor antagonists. In the YM, both CBD and the CB_1 receptor antagonist AM251 increased the exploration of the novel arm in ischemic animals, indicating beneficial effects of these treatments in the spatial memory performance. Together, these findings indicate the involvement of CB_1, CB_2, 5-HT_{1A}, and PPAR-γ receptors in the functional recovery induced by CBD in BCCAO mice.

KEYWORDS

5-HT_{1A}, behavior, brain ischemia, cannabidiol, CB_1, CB_2, PPAR-γ

Abbreviations: 5-HT_{1A}, 5-hydroxytryptamine 1A; BBB, blood-brain barrier; BCCAO, bilateral common carotid artery occlusion; BDNF, brain-derived neurotrophic factor; CB1 and CB2, cannabinoid receptors type 1 and type 2; CBD, cannabidiol; EZM, elevated zero maze; FST, forced swim test; GCI, global cerebral ischemia; HI, hypoxic-ischemic; MCAo, middle cerebral artery occlusion; mTOR, mammalian target of rapamycin; OF, open field; PPAR-γ, peroxisome proliferator-activated receptor γ; TRK, tyrosine receptor kinase; YM, Y-maze.

Marco Aurélio Mori and Erika Meyer contributed equally to this study.

Edited by Dr. Bita Moghaddam

1 | INTRODUCTION

Global cerebral ischemia (GCI) occurs as a result of an abrupt reduction of cerebral blood flow which leads to a significant decrease in oxygen and nutrient supply to the brain causing neuronal loss and neurodegeneration. Several pathologies such as cardiac arrest, cardiomyopathies, ventricular fibrillation, and aortic rupture are among the main causes of GCI (Anderson & Arciniegas, 2010; Neumann et al., 2013). Most of the affected surviving patients develop long-lasting disabilities including motor, cognitive and emotional impairments with deleterious consequences for the patient's well-being and relatives (Alexander et al., 2012; Jorgensen et al., 2014; Lim et al., 2004). Despite decades of intense clinical and preclinical efforts, few advances have been made in the development of effective pharmacotherapies to prevent or minimize complications of cerebral ischemia (Ao et al., 2018; Hadley et al., 2019; Madhok et al., 2018). One of the limitations in the search for an ideal pharmacological treatment is the difficulty for a particular agent to act on complex pathophysiological events occurring during and after GCI and reperfusion (Chamorro et al., 2016).

Several studies have shown that cannabidiol (CBD), the second most abundant phytocannabinoid of the *Cannabis sativa* plant (Pertwee, 2006), might play neuroprotective effects in cerebral ischemia by modulating excitotoxicity, oxidative stress, and neuroinflammation, processes that play key roles in the pathophysiology and progression of ischemic injury (Cassano et al., 2020; Hayakawa, Mishima, Nozako, Hazekawa, et al., 2007; Izzo et al., 2009; Mori et al., 2017). From a functional point of view, CBD normalized the neurobehavioral scores in both hypoxic-ischemic (HI) newborn pigs (Lafuente et al., 2011, 2016; Pazos et al., 2012, 2013) and rodents subjected to middle cerebral artery occlusion (MCAo; Ceprian et al., 2017; Hayakawa et al., 2008; Hayakawa, Mishima, Nozako, Hazekawa, et al., 2007). In gerbils subjected to transient global brain ischemia, CBD decreased hyperlocomotion and electroencephalographic flattening (Braida et al., 2003). We have recently demonstrated that CBD prevents memory deficits and anxiety- and despair-like behaviors in mice subjected to bilateral common carotid artery occlusion (BCCAO; Mori et al., 2017; Schiavon et al., 2014), an experimental model of GCI/reperfusion.

Besides its potent anti-inflammatory and antioxidant actions (Campos et al., 2016; Fernandez-Ruiz et al., 2013), CBD may operate throughout components of the endocannabinoid system as well as in different neurotransmitter systems and pathways. CBD enhances enzymatic hydrolysis and the uptake of anandamide (Bisogno et al., 2001), resulting in increased levels of this endocannabinoid, which in turn further activates the cannabinoid receptor type 1 (CB_1; Devane et al., 1988). However, CBD presents a low affinity for the endocannabinoid CB_1 and CB_2 receptors (Pertwee, 2008) and may act as CB_1/CB_2 receptors inverse agonist (Thomas et al., 2007). Other CBD effects may be mediated by direct activation of 5-hydroxytryptamine 1A (5-HT_{1A}) receptors (Peres et al., 2018; Sales et al., 2018) and peroxisome proliferator-activated receptor γ (PPAR-γ; Esposito et al., 2011; O'Sullivan & Kendall, 2010). CBD also increases adenosine A_{2A} receptor signaling (Ribeiro et al., 2012) and modulates energy metabolism by increasing mitochondrial complex and creatine kinase activity (Valvassori et al., 2013). Recently, it has been shown that CBD presents antagonist activity on G-protein-coupled receptors (ex. GPR3, GPR6, GPR12, GPR55). The discovery that these receptors are novel targets for CBD provides a new perspective on CBD potential therapeutic, due to the important role played by these receptors in many normal physiological functions and a variety of pathological conditions (Brown et al., 2017; Laun et al., 2018; Laun et al., 2018; Laun & Song, 2017; Ryberg et al., 2007).

The direct or indirect interference with molecular mechanisms through CBD may represent a promising pharmacological strategy for reducing the damage caused by cerebral ischemia. Indeed, rats subjected to MCAo showed long-lasting reductions of cortical lesion sizes and improvements in motor deficits when treated with the CB_1 and CB_2 agonist, KN38-72717 (Schmidt et al., 2012). The 5-HT_{1A} receptor antagonist WAY-100635 prevented the effects of CBD on the reduction of cortical infarct size in MCAo mice (Hayakawa, Mishima, Nozako, Ogata, et al., 2007; Mishima et al., 2005). In HI newborn pigs, CBD attenuated glutamate excitotoxicity, oxidative stress, and neuroinflammation, effects that were prevented by the CB_2 and 5-HT_{1A} receptor antagonists AM630 and WAY-100635 respectively (Pazos et al., 2013). CBD also reduced cell damage and decreased blood-brain barrier (BBB) permeability in endothelial and astrocyte human cocultures subjected to oxygen-glucose deprivation through 5-HT_{1A} and PPAR-γ but not CB_1, CB_2, or adenosine A_2 receptor activation (Hind et al., 2016). It is unclear, however, whether and how the activation of CBD target receptors would implicate in functional recovery promoted by CBD after CGI.

In the present study, we evaluated if CBD would attenuate behavioral impairments induced by BCCAO in mice using a multitask behavioral battery including both cognitive and emotional tests. To explore the putative neuropharmacological mechanisms, we further investigated the involvement of CB_1, CB_2, 5-HT_{1A}, and PPAR-γ receptors in the behavioral effects of CBD in ischemic mice.

2 | MATERIALS AND METHODS

2.1 | Ethics statements

This study was carried out at the State University of Maringá in strict accordance with the recommendations

of the Brazilian College of Animal Experimentation (COBEA). Animal experiments were approved by the local Ethics Committee on Animal Experimentation of the State University of Maringá (animal license numbers: CEEA 073/2013).

2.2 | Animals

Inbred, male C57BL/6J mice (2- to 3-month-old) weighing 25–30 g were housed in polyethylene cages (39 × 32 × 12,7 cm) in groups of 4–8 mice per cage, under conditions of controlled temperature (22 ± 1°C), and a 12 hr/12 hr alternating light/dark cycle. A standard commercial chow diet (Nutrilab-CR1; Nuvital Nutrients) and water were provided ad libitum.

2.3 | Drugs

Cannabidiol (CBD, THC Pharma), AM251 (Abcam), AM630 (Sigma-Aldrich), WAY-100635 (Sigma-Aldrich), and GW9662 (Sigma-Aldrich) were dissolved in 1% Tween 80 in sterile saline (vehicle). The animals were randomly assigned to receive intraperitoneal injections of vehicle or CBD 10 mg/kg 0.5 hr before and 3, 24, and 48 hr after surgeries. The CBD doses and route of administration were based on previous studies (Hayakawa, Mishima, Nozako, Hazekawa, et al., 2007; Hayakawa, Mishima, Nozako, Ogata, et al., 2007; Mori et al., 2017). The receptor antagonists were intraperitoneally (*i.p.*) injected 0.5 hr before each CBD injection. The dosage regimens and route of administration doses were based on previously published studies as following: CB_1 receptor antagonist AM251 1 mg/kg (Caltana et al., 2015), CB_2 receptor antagonist AM630 1 mg/kg (Hayakawa, Mishima, Nozako, Hazekawa, et al., 2007; Pazos et al., 2013), 5-HT_{1A} receptor antagonist WAY-100635 10 mg/kg (Hayakawa, Mishima, Nozako, Ogata, et al., 2007), and PPAR-γ antagonist GW9662 3 mg/kg (Sun et al., 2012).

2.4 | Bilateral common carotid artery occlusion

Transient global brain ischemia was induced by BCCAO as described previously (Mori et al., 2017). Briefly, animals were placed under anesthesia with a mixture of isoflurane/oxygen (1.3%–1.5% isoflurane in 100% oxygen, Isoforine®, Cristália) delivered through a universal vaporizer (Oxigel). First, an incision was made in the ventral neck to expose the common carotid arteries. Next, the arteries were occluded for 20 min using aneurysm clips (ADCA). Rectal temperature was monitored during surgery and maintained at approximately 37.5°C using a heat blanket. After the 20 min occlusion time, the aneurism clips were carefully removed, and the arteries were visually inspected for reperfusion. The incision was then closed with sutures. During the 3 hr period after reperfusion, the animals were kept in a warming box (inner temperature 30 ± 1°C) to avoid ischemia-induced cerebral hypothermia. Then, they returned to their cages with free access to water and food. Mice in which the carotid arteries were exposed but not occluded were used as sham-operated animals and designated as controls. All efforts were made to minimize animal suffering. The animals were sacrificed 21 days after the surgeries.

TABLE 1 Experimental groups

Experimental groups	*"n"*
Sham+vehicle+vehicle (Sham)	16
BCCAO+vehicle+vehicle (BCCAO)	15
BCCAO+vehicle+CBD (Veh+CBD)	11
BCCAO+AM251+vehicle (251+veh)	12
BCCAO+AM251+CBD (251+CBD)	13
BCCAO+AM630+vehicle (630+veh)	14
BCCAO+AM630+CBD (630+CBD)	15
BCCAO+WAY−100635+vehicle (WAY+veh)	11
BCCAO+WAY−100635+CBD (WAY+CBD)	11
BCCAO+GW9662+vehicle (GW+veh)	12
BCCAO+GW9662+CBD (GW+CBD)	12

2.5 | Experimental design

The behavioral tests were conducted during the light phase (between 7:00 a.m. and 1:00 p.m.) in a sound-attenuated experimental room kept at 22 ± 2°C. The mice were acclimatized to the experimental room for 30 min before each test. C57BL/6Jmice were randomly distributed in 11 experimental groups as shown in Table 1. The order of tests within the battery was adapted from McIlwain et al. (2001). Mice were evaluated on what were thought to be the least aversive tests before being tested on more aversive assays. The sequence took into account the sensitivity of specific tests that could significantly influence the outcome of subsequent tests. The timeline and the sequence of behavioral tests, and the days of testing were based on previous studies with BCCAO mice (Mori et al., 2017; Schiavon et al., 2014; Soares et al., 2013, 2016). As seen in Figure 1, over 14 days after the surgery, the animals were evaluated in the open field (OF; day 8), elevated zero maze (EZM; day 9), Y-maze (YM; day 14), and forced swim test (FST; day 21). Each apparatus was cleaned with 70% ethanol and water before testing another mouse. The water in the FST cylinder was changed after every session to avoid any influence on the next mouse. The test sessions were recorded

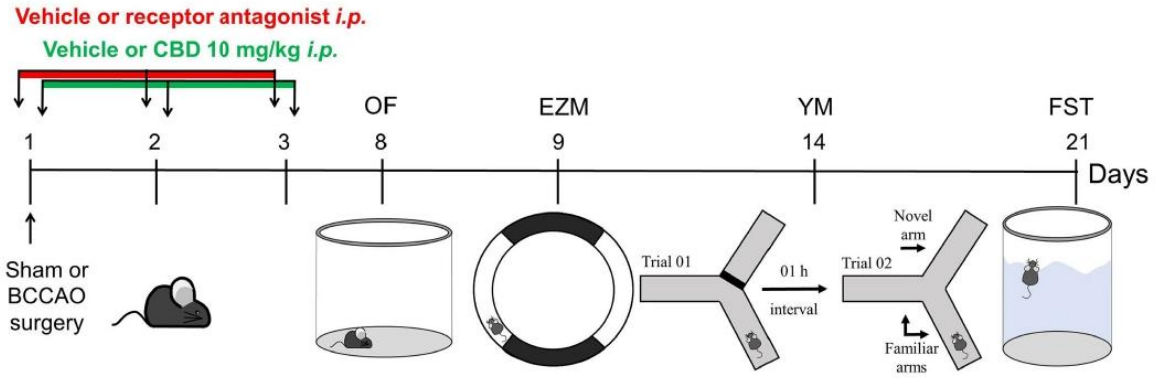


FIGURE 1 Experimental design. Vehicle or cannabidiol (CBD; 10 mg/kg) was intraperitoneally (*i.p.*) injected 0.5 hr before and 3, 24, and 48 hr after Sham or BCCAO surgeries. Vehicle or CB_1 (AM251, 1 mg/kg), CB_2 (AM630, 1 mg/kg), 5-HT_{1A} (WAY-100635, 10 mg/kg), and PPAR-γ (GW9662, 3 mg/kg) receptor antagonists were *i.p.* injected 30 min prior to each CBD injection. Behavioral testing was performed from day 8 to day 21 after the surgery. BCCAO, bilateral common carotid artery occlusion; EZM, elevated zero maze; FST, forced swim test; OF, open field; YM, Y-maze test

and analyzed using a contrast-sensitive video tracking system (ANYmaze, Stoelting). All behavioral assessments were conducted by a blinded investigator.

2.6 | Behavioral tests

2.6.1 | The open field

The OF test was carried out to measure locomotor activity. The apparatus consisted of a circular arena made of transparent polyvinyl chloride (43 cm diameter, 40 cm high wall). Each animal was gently placed in the central area and allowed to freely explore the OF for 10 min. The distance traveled (in meters) during this period was recorded.

2.6.2 | The elevated zero maze

The EZM maze was constructed of gray plastic material and consisted of a circular runway (46 cm diameter, 5.5 cm width) elevated 20 cm above the floor. The maze was divided into four quadrants of equal lengths with two opposing open quadrants surrounded by a low border (3 mm height) to prevent falls and two opposite closed quadrants with 11 cm high sidewalls. Each mouse was allowed to explore the apparatus for 6 min. The % time spent in the open quadrants (time spent in the open quadrant/total time × 100) and the number of crossings between the open and closed quadrants were recorded and express anxiety-related behaviors and locomotor activity respectively (Nebuka et al., 2020; Tucker et al., 2017).

2.6.3 | The Y-maze

Spatial memory was examined by the 2-trial YM test (Dellu et al., 1992). The apparatus was made of gray wood and consisted of three arms (12 cm width, 41 cm length, 15 cm height) with an angle of 120° between adjacent arms and a central triangular area. The three identical arms were randomly designated as (a) two familiar arms (open arms) or (b) a novel arm (blocked during the first trial but open during the second trial). In the first trial, mice were allowed to freely explore two open arms for 6 min. The animal was returned to its home cage for a 1-hr delay. In the second trial, mice were positioned in one of the open arms, previously designated as the starting arm, and allowed to explore all 3 arms of the YM apparatus freely for 5 min. The percentage of time spent in the novel arm (time in the novel arm divided by total time in all arms × 100) was calculated.

2.6.4 | The forced swim test

This test is commonly used to screen for the antidepressant-like activity of compounds (Porsolt et al., 1977). Each mouse was individually placed in an acrylic cylinder (20 cm diameter, 32 cm height) that contained 20 cm of water at 24 ± 1°C. The animals were left in the cylinder for 6 min. The latency (seconds) for the first episode of immobility and the duration (seconds) of the immobility (during which the mouse did not struggle and only made movements that were necessary to keep its head above the water) were recorded during the last 4 min of the 6 min testing period. After each session, the animals were dried and returned to their home cages.

2.7 | Statistical analysis

GraphPad Prism 8 software (2020 GraphPad Software) was used for statistical analysis. The Student's *t*-test was used to identify significant differences between Sham+vehicle+vehicle (Sham) and BCCAO+vehicle+vehicle (BCCAO) groups. To evaluate the involvement of CB_1, CB_2, 5-HT_{1A}, and PPAR-γ receptors on CBD effects, the data were analyzed by two-way ANOVA using the first (vehicle, AM251, AM630, WAY-100635, or GW9662) and the second (vehicle or CBD) injections as factors. When interactions between factors were observed, one-way ANOVA was performed. Post-hoc analysis was conducted using the Newman-Keuls multiple comparisons test. The normal distribution of the data was confirmed by the Shapiro–Wilk test. Data are presented as means ± *SEM* of biological replicates (mice). The levels of statistical significance were set as $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.00$; $^{t}p < 0.05$; $^{tt}p < 0.01$.

3 | RESULTS

3.1 | Open field

Seven days after BCCAO, mice treated only with vehicle (BCCAO group) presented an increase in the distance traveled when compared with Sham animals (Figure 2, Student *t*-test, $t = 2.23$, $df = 29$, $p = 0.03$). These data suggest an increase in overall exploratory/locomotor activity in mice under cerebral ischemic conditions.

Although a significant effect of the first injection was observed ($F_{4,116} = 4.61$, $p = 0.002$, two-way ANOVA), no significant interactions between the first and the second injections were detected for the distance traveled in the OF ($F_{4,116} = 1.32$, $p = 0.56$, two-way ANOVA).

3.2 | Elevated zero maze

In the EZM, BCCAO mice spent significantly less time in the open quadrants than Sham mice (Figure 3a, Student *t*-test, $t = 4.1$, $df = 29$, $p = 0.0003$), indicating an anxiogenic-like effect induced by cerebral ischemia. However, no significant effect was detected on the number of crossings between BCCAO and Sham groups (Figure 3b, Student *t*-test, $t = 0.99$, $df = 29$, $p = 0.32$).

Two-way ANOVA showed significant interactions between the first and the second injections for the % of time spent in the open area of the EZM (Figure 3a, $F_{4,116} = 4.94$, $p = 0.001$). Further analysis indicated significant differences among the experimental groups for this parameter ($F_{9,116} = 5.33$, $p < 0.0001$, one-way ANOVA). CBD treatment increased the % of time spent by BCCAO mice in the open area of the EZM when compared to the BCCAO group treated only with vehicle ($p < 0.01$, Neumann-Keuls). The effect of CBD was attenuated by pretreatment with AM251, AM630, WAY-100635 and GW9662 (Figure 3a, $p < 0.05$ to 0.0001, Neumann-Keuls).

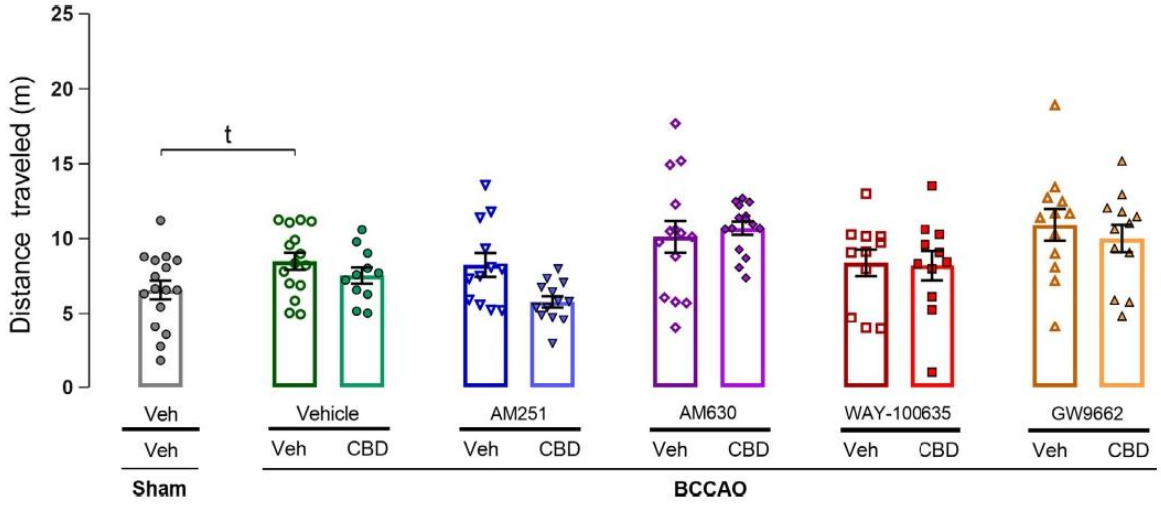


FIGURE 2 Effects of cannabidiol (CBD) and CB1, CB2, 5-HT1A, and PPAR-γ receptor antagonists on motor behavior in ischemic mice. Locomotor activity was evaluated by measuring the distance traveled (m) in the open field 7 days after bilateral common carotid artery occlusion (BCCAO). CBD (10 mg/kg) was intraperitoneally (*i.p.*) injected 0.5 hr before and 3, 24, and 48 hr after Sham or BCCAO surgeries. AM251, AM630, WAY-100635, or GW9662 (CB1, CB2, 5-HT1A, and PPAR-γ receptor antagonists, respectively) were *i.p.* injected 30 min prior to each CBD injection. All values represent the means ± *SEM* of number of the animals indicated in the figure ($n = 11$–16 mice/group). $^{t}p < 0.05$ (Student's *t*-test)

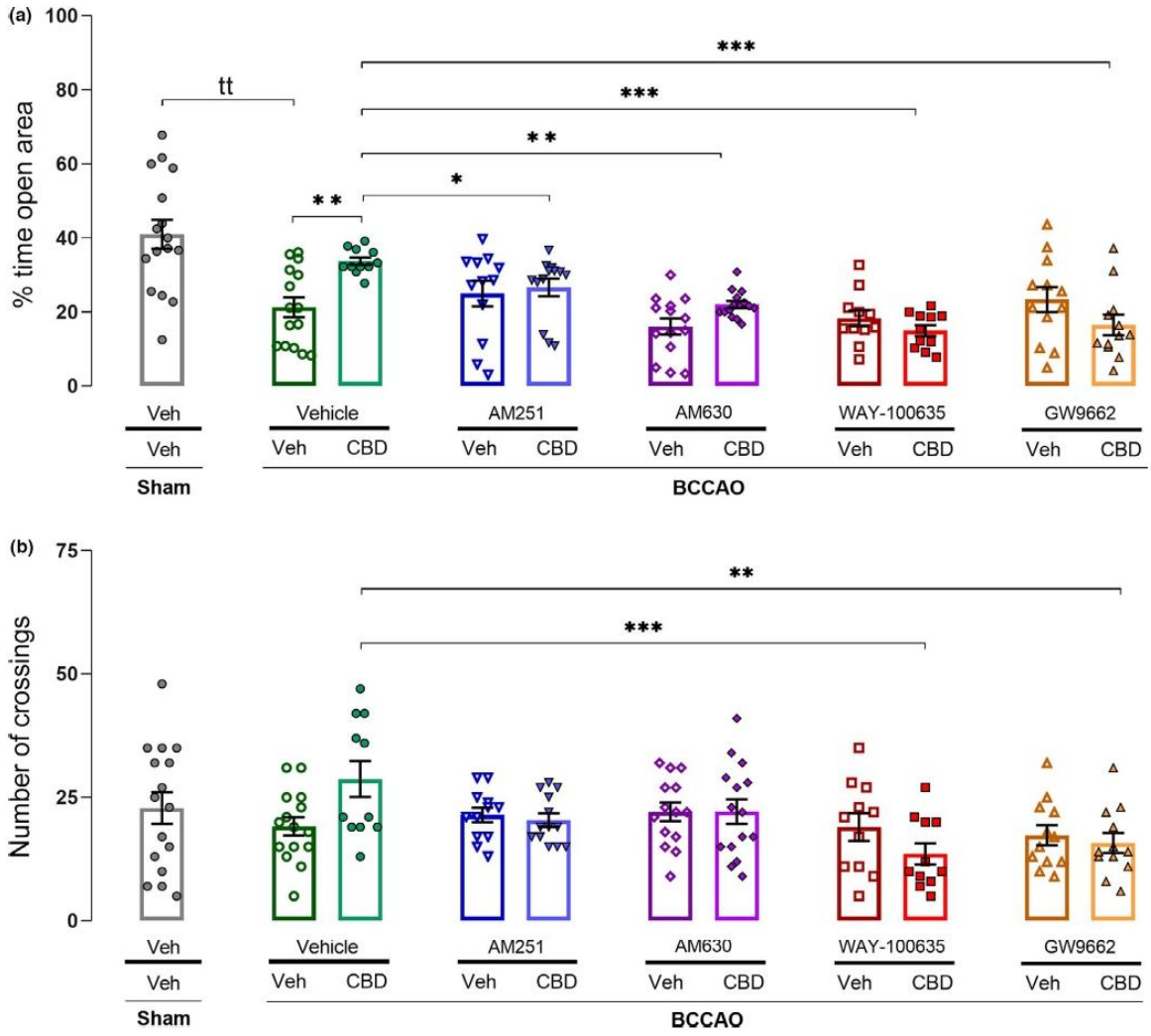


FIGURE 3 Effects of cannabidiol (CBD) and CB1, CB2, 5-HT1A, and PPAR-γ receptor antagonists on the anxiogenic-like effect induced by BCCAO in mice. Anxiety-like behavior and locomotor activity were evaluated in the EZM by measuring the time spent in the open quadrants (a) and the number of crossings between quadrants (b) respectively. See detailed information in Figure 2. BCCAO, bilateral common carotid artery occlusion; EZM, elevated zero maze. All values represent the means ± *SEM* of number of the animals indicated in the figure (n = 11–16 mice/group). $^{tt}p < 0.01$ (Student's *t*-test); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-way ANOVA followed by Neumann-Keuls test)

A significant interaction was also detected for the number of crossings in the EZM ($F_{4,116} = 3.12, p = 0.01$, two-way ANOVA). Pre-treatment with WAY-100635 or GW9662 attenuated the effects of CBD in BCCAO mice ($F_{9,116} = 3.22$, $p = 0.001$, one-way ANOVA, $p < 0.01$ to 0.001, Neuman-Keuls).

3.3 | Y-Maze

Bilateral common carotid artery occlusion mice treated with vehicle presented a significant reduction in the % of time spent in the novel arm of the YM when compared to Sham animals (Figure 4, Student *t*-test, $t = 3.83$, $df = 29$, $p = 0.0006$), indicating that cerebral ischemia interfered with spatial memory. There were significant interactions between the first and second injections ($F_{4,116} = 7.95, p < 0.0001$, two-way ANOVA). One-way ANOVA ($F_{9,116} = 3.85$, $p = 0.0003$) revealed that BCCAO animals that received CBD presented an increase in the % of the time in the novel arm of the YM when compared to BCCAO animals treated only with vehicle (Figure 4, $p < 0.01$, Neuman-Keuls). However, AM251 treatment, per se, also led to an increased exploration time in the novel arm

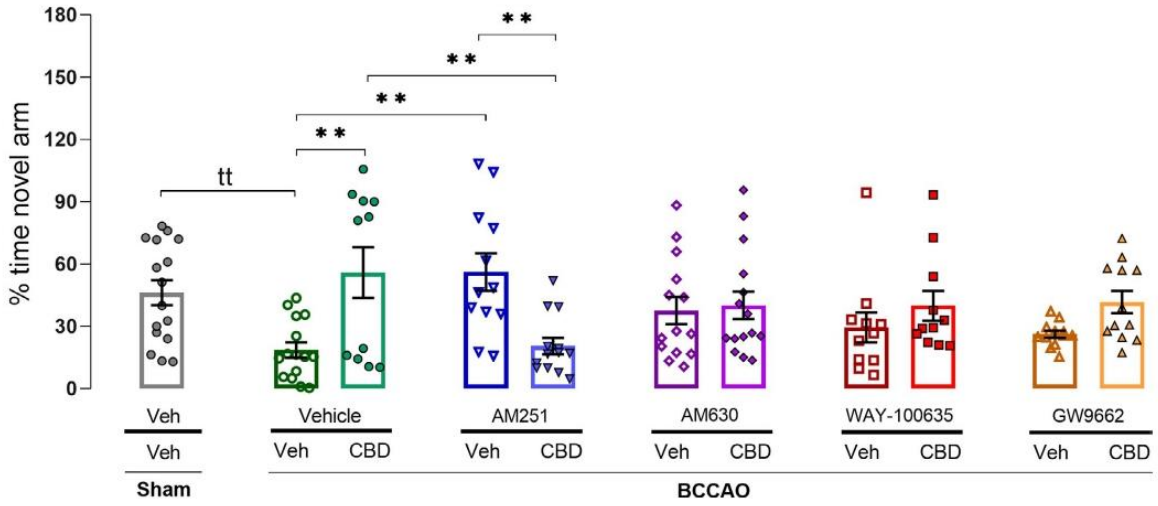


FIGURE 4 Effects of cannabidiol (CBD) and CB1, CB2, 5-HT1A, and PPAR-γ receptor antagonists in BCCAO mice evaluated in the Y-maze (YM) test. Hippocampus-dependent memory was evaluated by measuring the time spent in the novel arm of the YM. See detailed information in Figure 2. BCCAO, bilateral common carotid artery occlusion. All values represent means ± *SEM* of the number of the animals indicated in the figure ($n = 11–16$ mice/group). $^{tt}p < 0.01$ (Student's *t*-test); $^{**}p < 0.01$ (two-way ANOVA followed by Neumann-Keuls test)

of the YM compared with the BCCAO group ($p < 0.01$, Neuman-Keuls). Jointed administration of AM251 + CBD resulted in a decreased % of time spent in the novel arm of the YM compared with the groups that received only CBD and AM251 ($p < 0.01$, Neumann-Keuls).

3.4 | Forced swim test

In the FST, BCCAO provoked a decrease in the latency for the first episode of immobility when compared to the Sham group (Figure 5a, Student *t-test*, $t = 3.29$, $df = 29$, $p = 0.003$). Additionally, BCCAO mice treated with vehicle showed an increase in the immobility time when compared to Sham animals (Figure 5b, Student *t-test*, $t = 2.73$, $df = 29$, $p = 0.01$). Concerning latency, significant interaction between the injections were observed ($F_{4,116} = 4.85$, $p = 0.001$, two-way ANOVA). Further analysis with one-way ANOVA ($F_{9,116} = 5.80$, $p < 0.0001$) showed that CBD increased the latency for the first episode of immobility in BCCAO mice as compared to BCCAO mice that received only vehicle ($p < 0.01$, Neumann-Keuls). Pre-treatment with GW-9662 increased the latency when compared to BCCAO animals that received vehicle ($p < 0.001$, Neuman-Keuls).

For the immobility time parameter, there was no interaction between the first and second injections ($F_{4,116} = 1.19$, $p = 0.32$, two-way ANOVA). However, there was a trend effect for the second injection, i.e., CBD injection ($F_{1,116} = 3.52$, $p = 0.06$).

4 | DISCUSSION

The present work investigated the effects of CBD on the behavioral changes induced by BCCAO and whether these effects were sensitive to selective CB_1, CB_2, 5-HT_{1A}, and PPAR-γ receptor antagonism. CBD (10 mg/kg) prevented the anxiogenic-like effect, memory impairment, and despair-like behavior induced by BCCAO in mice. The anxiolytic-like effects of CBD were attenuated by CB_1, CB_2, 5-HT_{1A}, and PPAR-γ receptor antagonists, indicating the involvement of these receptors in the modulation of anxiety in ischemic mice. Although CBD did not significantly change the number of crossings of BCCAO mice in the EZM, pre-treatment with WAY-100635 or GW9662 decreased this parameter, suggesting interference of 5-HT_{1A} and PPAR-γ receptors with general locomotor activity in cerebral ischemia conditions. In the YM, both CBD and the CB_1 receptor antagonist AM251 increased the exploration of the novel arm of the YM in ischemic animals. Finally, CBD and GW9662 when administered alone attenuated despair-like behaviors in ischemic mice evaluated in the FST, while no effect was detected when CBD target receptors were blocked by antagonists.

Bilateral common carotid artery occlusion mice presented an increase in the distance traveled in the OF, suggesting an increase in the general locomotor activity induced by cerebral ischemia. However, BCCAO mice recovered over time, since there was no change in the number of crossings in the EZM recorded 8 days post-insult. Indeed, transient motor changes have been described after GCI in gerbils (Ramos-Zúñiga *et al*., 2008), rats (Milot & Plamondon, 2009), and

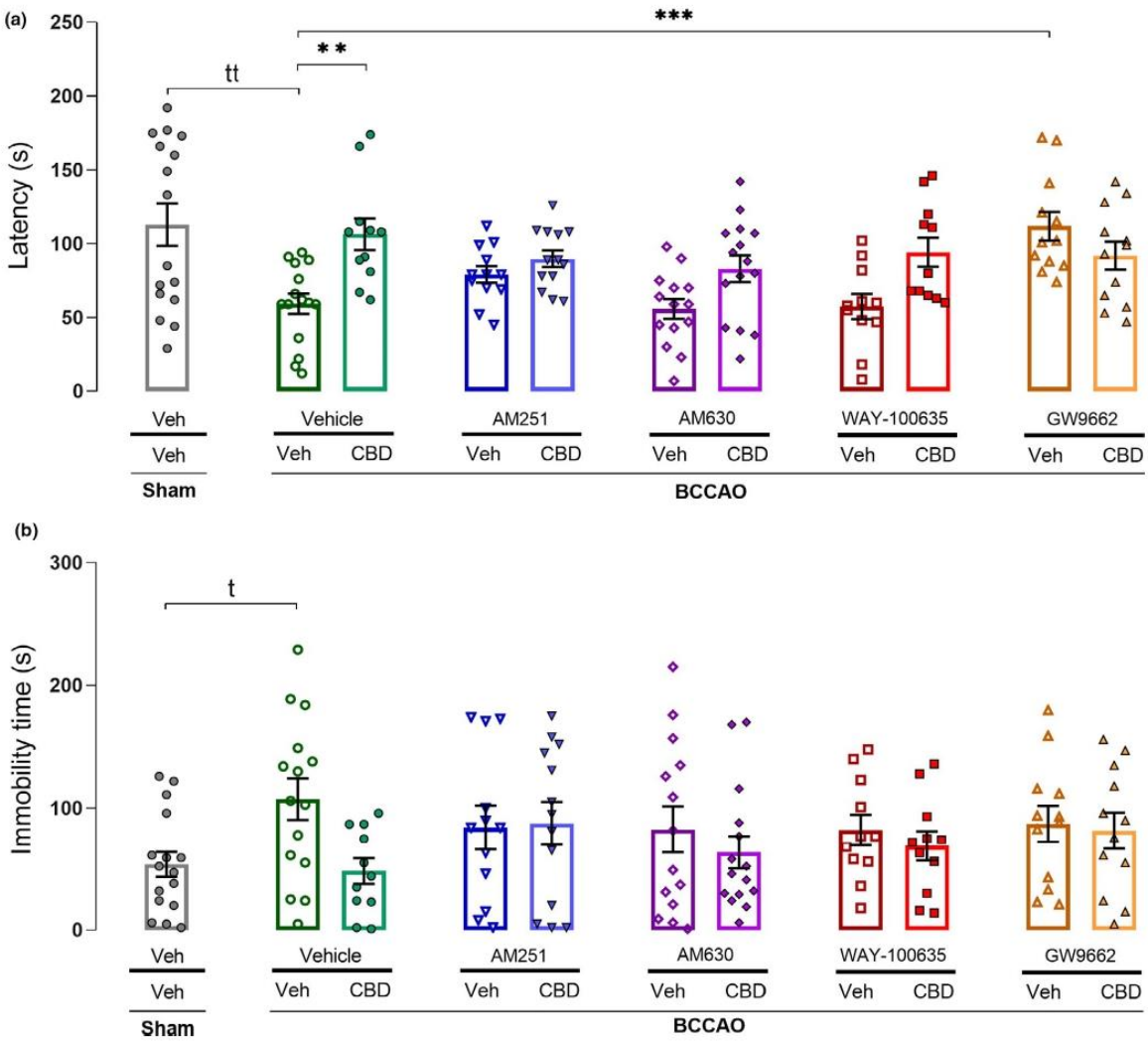


FIGURE 5 Effects of cannabidiol (CBD) and CB1, CB2, 5-HT1A, and PPAR-γ receptor antagonists in BCCAO mice tested in the forced swim test (FST). Latency to the first episode of immobility (a) and the immobility time during the last 4 min of the FST (b) were recorded. See detailed information in the Figure 2. BCCAO, bilateral common carotid artery occlusion. All values represent the means ± *SEM* of the number of the animals indicated in the figure ($n = 11–16$ mice/group). $^{t}p < 0.05$, $^{tt}p < 0.01$ (Student's *t*-test); $^{**}p < 0.01$, $^{***}p < 0.001$ (two-way ANOVA followed by Neumann-Keuls test)

mice (Soares et al., 2013). Therefore, adaptive changes or compensatory mechanisms may enable the restoration of motor impairments following BCCAO over time. Otherwise, 20 min of BCCAO increased anxiety-like behaviors, seen as a decreased time spent in the open quadrants of the EZM. Others have already described anxiogenic-like effects resultant of cerebral ischemia in mice (Nakashima et al., 2003; Neigh et al., 2009; Soares et al., 2013, 2016) which were attenuated by CBD administration (Mori et al., 2017).

The CB_1, CB_2, 5-$HT_{1A,}$ and PPAR-γ receptor antagonists prevented the anxiolytic-like effect produced by CBD in BCCAO animals. Fogaca et al. (2018) proposed that CBD reduced the anxiety-like behavior in stressed mice by enhancing endocannabinoid neurotransmission, leading to the consequent activation of both CB_1 and CB_2 receptors (Fogaca et al., 2018). Similar mechanisms might have been involved in this study with BCCAO mice since AM251 and AM630 receptor antagonists blocked the anxiolytic-like effect of CBD. In agreement, Knowles et al. (2016) have shown that AM251, attenuated emotional changes induced by global ischemia in rats, indicating a prominent role of CB_1 receptors on the modulation of anxiety-like behaviors induced by cerebral ischemia in rodents (Knowles et al., 2016). The mechanisms by which CB_2 receptors are involved in BCCAO-induced anxiety is still

unclear. In gerbils submitted to GCI, the N-Linoleytyrosine, an anandamide analog, improved hippocampal neurodegeneration and inflammation via CB_2 receptor involvement of PI3K/Akt signaling pathway. This mechanism was related to the amelioration of animal behavior after the brain insult (Cheng et al., 2019). However, it remains to be determined the role of CB_2 receptors in emotional impairments, like anxiety induced by BCCAO.

Cannabidiol has been proposed to act as a 5-HT_{1A} receptor positive allosteric modulator leading to enhanced serotonergic signaling (Rock et al., 2012, 2017). In a model of neuropathic pain in rats, the anxiolytic-like effect of CBD was blocked by WAY100365 administration, demonstrating a possible involvement of 5-HT_{1A} receptors on the CBD effect (De Gregorio et al., 2019). In line with these findings, microinjection of CBD into the dorsolateral periaqueductal gray matter (Campos & Guimaraes, 2008) and the bed nucleus of stria terminallis (Gomes et al., 2011) increased open arms exploration in the elevated plus maze (EPM), suggesting an anxiolytic-like effect of CBD. In both studies, pretreatment with the 5-HT_{1A} receptor antagonist WAY100635 blocked the observed anxiolytic-like effect of CBD. However, in the prelimbic medial prefrontal cortex, CBD produced opposite effects in rats evaluated in the contextual fear conditioning paradigm and the EPM test, promoting anxiolytic- and anxiogenic-like effects respectively (Fogaca et al., 2014). Such effects were also prevented by WAY100635, indicating that both anxiolytic- and anxiogenic-like effects of CBD depended on local 5-HT_{1A}-mediated neurotransmission. Interestingly, when rats were previously submitted to restraint stress (2 hr), the anxiogenic-like effect of CBD turned into an anxiolytic-like effect in the EPM. These findings demonstrate the complex modulation of anxiety responses by CBD through 5-HT_{1A} mediated neurotransmission in the prelimbic medial prefrontal cortex and highlight the influence of a previous stressful experience on CBD effects (Fogaca et al., 2014). Considering that cerebral ischemia is an acute stressor per se (De La Tremblaye et al., 2014) the effects of CBD on anxiety-induced by BCCAO must be interpreted carefully.

Cannabidiol also acts as a partial agonist of PPAR-γ receptors (O'Sullivan & Kendall, 2010). In an animal model of Alzheimer's disease, the blockade of PPAR-γ receptors was able to significantly blunt CBD effects on reactive gliosis and subsequently on neuronal damage. Moreover, due to its interaction at PPAR-γ receptors, CBD was observed to stimulate hippocampal neurogenesis (Esposito et al., 2011). CBD also decreased the permeability of the BBB following oxygen-glucose deprivation-reperfusion, in an in vitro model of the BBB disruption, through interaction with PPAR-γ receptors (Hind et al., 2016). These findings indicate a role of this receptor in mediating CBD actions. In the present study, prior administration of GW9662, a PPAR-γ receptor antagonist, prevented the anxiolytic-like effect of CBD in BCCAO mice. In this way, it has been reported that genetic deletion of neuronal PPAR-γ receptors or administration of GW9663 elicited a marked anxiogenic response in mice (Domi et al., 2016), indicating that dampened transmission may contribute to exacerbate anxiety.

Cannabidiol prevented the memory impairment evaluated in the YM 13 days after brain ischemia. The CB_1 antagonist AM251 had per se a neuroprotective effect. Similar findings were described by others, showing that CB_1 blockage resulted in neuroprotective effects after brain ischemia in rats (Berger et al., 2004; Knowles et al., 2016). Curiously, when AM251 was combined with CBD administration, the neuroprotective effect of CBD and AM251 disappeared. The outcomes of CBD interaction with CB_1 receptors are still controversial. CBD has been proposed as a negative allosteric modulator of CB_1-mediated signaling (Laprairie et al., 2015; Pertwee et al., 2002; Straiker et al., 2018). However, CBD could indirectly increase anandamide levels through inhibition of its metabolism/uptake (Bisogno et al., 2001; Murillo-Rodriguez et al., 2006; Pertwee, 2008). CBD and anandamide, therefore, could activate TRPV1 receptors, facilitating glutamate release. CB_1 blockade might influence anandamide effects toward this latter receptor (Moreira et al., 2012) resulting in opposing effects on cognition evaluated in the YM.

Antidepressant-like effects of CBD are dependent on 5-HT_{1A}, CB_1, tyrosine receptor kinase, and mammalian target of rapamycin activation in the central nervous system (Sales, Crestani, et al., 2018; Sales, Fogaca, et al., 2018; Sartim et al., 2016; Zanelati et al., 2010). CBD has been shown to induce antidepressant-like effects by increasing endocannabinoid signaling (Campos et al., 2013). Recently, we have shown that CBD increased the levels of brain-derived neurotrophic factor and stimulated hippocampal neurogenesis, effects that contributed to its antidepressant-like action in BCCAO mice (Mori et al., 2017). Here, the antidepressant-like effects of CBD in ischemic mice were attenuated but not blocked by all of the antagonists tested (i.e., AM251, AM630, WAY-100635, and GW9662). These effects indicate that CBD, at least partially, induces antidepressant-like effects through CB_1, CB_2, 5-HT_{1A}, and PPAR-γ receptors. Unexpectedly, the PPAR-γ receptor antagonist GW9662, when administered alone, increased the latency for the first immobility episode in BCCAO mice, indicating an antidepressant-like effect. In several clinical and preclinical experiments, PPAR-γ receptor agonists (not antagonists) exerted antidepressant-like activities, which have been related to their anti-inflammatory actions (Colle et al., 2017; Sadaghiani et al., 2011; Shahsavarian et al., 2014). The behavioral effect observed with the PPAR-γ receptor antagonist GW9662 on FST is intriguing. However, we cannot rule out the possibility of a false positive finding due to the behavioral variability showed by BCCAO mice in the FST.

One limitation of the present study is the use of single doses of the receptor antagonists and CBD, although all doses used have been based on previous studies. As discussed above, CBD has complex pharmacology and it is conceivable that different molecular targets are preferentially engaged by distinct behavioral responses (Campos et al., 2012). Another feature of the present study that deserves attention refers to the interindividual variability in all behavioral responses measured. Notably, this interindividual variability was also present in the sham-operated animals, which could be interpreted as a low-quality experimental protocol. This cannot be the case, however, since the animals used in the present study were inbred, the experimental procedures (tests) are well-standardized (validated), and well-trained experimenters conducted the surgery and the behavioral testing. Instead, it is well-known that inbred C57BL/6 mice display a remarkably high interindividual variability in their trait anxiety, i.e., the intrinsic basal anxiety characteristic of an individual, which can predict the behavioral and other physiological responses to an acute stressor (Jakovcevski et al., 2008). Highly interindividual variability has also been observed in the Swiss male mice for their behavioral reactions to the elevated plus-maze test, a phenomenon that seems to correlate with animal´s innate emotional profile, and the influence of social factors (Ferrari et al., 1998). Accordingly, individual variability in the basal level of emotion alone or in combination with other BCCAO-related factors may have contributed to the high variability of the behavioral response that was measured after BCCAO (acute stressor per se). The impact of BCCAO on brain damage and functions may vary according to individual differences in the vascular anatomy, and/or neuroanatomical and neurochemical properties (Guadalupe et al., 2013). For instance, a large inter-individual variation in the severity of neuronal degeneration and cellular inflammatory responses was observed in the retina of C57/Bl6J mice after chronic BCCAO, which degree ranged from highly severe lesion to no pathological changes at all (Crespo-Garcia et al., 2018). This variable pattern of neuronal damage triggered by BCCAO in the retina may extend to the brain and impact differently onto the behavioral responses that are measured in different behavioral tests. Therefore, while the interindividual variability of behavioral responses after brain ischemia may represent an inherent characteristic of the model, care should be taken to use, as much as possible, a sufficient sample size associated with careful statistical analysis to avoid misinterpretation of data. Even so, taken together the present study indicated that CBD could produce functional recovery in BCCAO mice through different neurotransmission systems including CB_1, CB_2, $5\text{-}HT_{1A,}$ and PPAR-γ receptors.

ACKNOWLEDGEMENTS

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Universidade Estadual de Maringá and FAPESP (2017/24304-0), São Paulo, Brazil. The authors thank ADCA—Indústria e Comércio de Material Cirúrgico for kindly donating the aneurysm clips and Marco Alberto Trombelli for his technical support.

CONFLICT OF INTEREST

There is no conflict of interest in this study.

AUTHOR CONTRIBUTIONS

MA Mori and E Meyer conducted the animals' surgeries and behavioral tests. They wrote the first draft of the manuscript. FF Silva took care of the animals and performed drugs' treatment. H Milani analyzed the data and helped with statistical analysis. FS Guimarães and RMW Oliveira conceived the experimental design, planned the experiments, and performed the data work-up. They also contributed to writing the manuscript. All authors discussed the results and commented on the manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ejn.15134.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from corresponding author, RMWO, upon request.

ORCID

Marco Aurélio Mori https://orcid.org/0000-0003-1231-8756
Erika Meyer https://orcid.org/0000-0002-8425-6358
Humberto Milani https://orcid.org/0000-0002-2157-8317
Francisco Silveira Guimarães https://orcid.org/0000-0003-4092-7778
Rúbia Maria Weffort Oliveira https://orcid.org/0000-0002-6181-1881

REFERENCES

Alexander, L. D., Pettersen, J. A., Hopyan, J. J., Sahlas, D. J., & Black, S. E. (2012). Long-term prediction of functional outcome after stroke using the Alberta Stroke Program Early Computed Tomography Score in the subacute stage. *Journal of Stroke and Cerebrovascular Diseases: the Official Journal of National Stroke Association*, *21*, 737–744. https://doi.org/10.1016/j.jstrokecerebrovasdis.2011.03.010

Anderson, C. A., & Arciniegas, D. B. (2010). Cognitive sequelae of hypoxic-ischemic brain injury: A review. *NeuroRehabilitation*, *26*, 47–63. https://doi.org/10.3233/NRE-2010-0535

Ao, L. Y., Yan, Y. Y., Zhou, L., Li, C. Y., Li, W. T., Fang, W. R., & Li, Y. M. (2018). Immune cells after ischemic stroke onset: Roles, migration, and target intervention. *Journal of Molecular Neuroscience*, *66*, 342–355. https://doi.org/10.1007/s12031-018-1173-4

Berger, C., Schmid, P. C., Schabitz, W. R., Wolf, M., Schwab, S., & Schmid, H. H. (2004). Massive accumulation of N-acylethanolamines after stroke. Cell signalling in acute cerebral ischemia? *Journal of Neurochemistry*, *88*, 1159–1167.

Bisogno, T., Hanus, L., De Petrocellis, L., Tchilibon, S., Ponde, D. E., Brandi, I., Moriello, A. S., Davis, J. B., Mechoulam, R., & Di Marzo, V. (2001). Molecular targets for cannabidiol and its synthetic analogues: Effect on vanilloid VR1 receptors and on the cellular uptake and enzymatic hydrolysis of anandamide. *British Journal of Pharmacology*, *134*, 845–852. https://doi.org/10.1038/sj.bjp.0704327

Braida, D., Pegorini, S., Arcidiacono, M. V., Consalez, G. G., Croci, L., & Sala, M. (2003). Post-ischemic treatment with cannabidiol prevents electroencephalographic flattening, hyperlocomotion and neuronal injury in gerbils. *Neuroscience Letters*, *346*, 61–64. https://doi.org/10.1016/S0304-3940(03)00569-X

Brown, K. J., Laun, A. S., & Song, Z. H. (2017). Cannabidiol, a novel inverse agonist for GPR12. *Biochemical and Biophysical Research Communications*, *493*, 451–454. https://doi.org/10.1016/j.bbrc.2017.09.001

Caltana, L., Saez, T. M., Aronne, M. P., & Brusco, A. (2015). Cannabinoid receptor type 1 agonist ACEA improves motor recovery and protects neurons in ischemic stroke in mice. *Journal of Neurochemistry*, *135*, 616–629.

Campos, A. C., Fogaca, M. V., Sonego, A. B., & Guimaraes, F. S. (2016). Cannabidiol, neuroprotection and neuropsychiatric disorders. *Pharmacological Research*, *112*, 119–127. https://doi.org/10.1016/j.phrs.2016.01.033

Campos, A. C., & Guimaraes, F. S. (2008). Involvement of 5HT1A receptors in the anxiolytic-like effects of cannabidiol injected into the dorsolateral periaqueductal gray of rats. *Psychopharmacology (Berl)*, *199*, 223–230. https://doi.org/10.1007/s00213-008-1168-x

Campos, A. C., Moreira, F. A., Gomes, F. V., Del Bel, E. A., & Guimaraes, F. S. (2012). Multiple mechanisms involved in the large-spectrum therapeutic potential of cannabidiol in psychiatric disorders. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *367*, 3364–3378. https://doi.org/10.1098/rstb.2011.0389

Campos, A. C., Ortega, Z., Palazuelos, J., Fogaca, M. V., Aguiar, D. C., Diaz-Alonso, J., Ortega-Gutierrez, S., Vazquez-Villa, H., Moreira, F. A., Guzman, M., Galve-Roperh, I., & Guimaraes, F. S. (2013). The anxiolytic effect of cannabidiol on chronically stressed mice depends on hippocampal neurogenesis: Involvement of the endocannabinoid system. *International Journal of Neuropsychopharmacology*, *16*, 1407–1419. https://doi.org/10.1017/S1461145712001502

Cassano, T., Villani, R., Pace, L., Carbone, A., Bukke, V. N., Orkisz, S., Avolio, C., & Serviddio, G. (2020). From *Cannabis sativa* to cannabidiol: Promising therapeutic candidate for the treatment of neurodegenerative diseases. *Frontiers in Pharmacology*, *11*, 124. https://doi.org/10.3389/fphar.2020.00124

Ceprian, M., Jimenez-Sanchez, L., Vargas, C., Barata, L., Hind, W., & Martinez-Orgado, J. (2017). Cannabidiol reduces brain damage and improves functional recovery in a neonatal rat model of arterial ischemic stroke. *Neuropharmacology*, *116*, 151–159. https://doi.org/10.1016/j.neuropharm.2016.12.017

Chamorro, A., Dirnagl, U., Urra, X., & Planas, A. M. (2016). Neuroprotection in acute stroke: Targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. *The Lancet Neurology*, *15*, 869–881. https://doi.org/10.1016/S1474-4422(16)00114-9

Cheng, L., Li, J., Zhou, Y., Zheng, Q., Ming, X., & Liu, S. (2019). N-Linoleyltyrosine protects against transient cerebral ischemia in gerbil via CB2 receptor involvement in PI3K/Akt signaling pathway. *Biological and Pharmaceutical Bulletin*, *42*, 1867–1876.

Colle, R., de Larminat, D., Rotenberg, S., Hozer, F., Hardy, P., Verstuyft, C., Feve, B., & Corruble, E. (2017). PPAR-gamma agonists for the treatment of major depression: A review. *Pharmacopsychiatry*, *50*, 49–55.

Crespo-Garcia, S., Reichhart, N., Skosyrski, S., Foddis, M., Wu, J., Figura, A., Herrspiegel, C., Füchtemeier, M., Sassi, C., Dirnagl, U., Joussen, A. M., & Strauss, O. (2018). Individual and temporal variability of the retina after chronic bilateral common carotid artery occlusion (BCCAO). *PLoS One*, *13*(3), e0193961. https://doi.org/10.1371/journal.pone.0193961

De Gregorio, D., McLaughlin, R. J., Posa, L., Ochoa-Sanchez, R., Enns, J., Lopez-Canul, M., Aboud, M., Maione, S., Comai, S., & Gobbi, G. (2019). Cannabidiol modulates serotonergic transmission and reverses both allodynia and anxiety-like behavior in a model of neuropathic pain. *Pain*, *160*, 136–150. https://doi.org/10.1097/j.pain.0000000000001386

de la Tremblaye, P. B., Raymond, J., Milot, M. R., Merali, Z., & Plamondon, H. (2014). Evidence of lasting dysregulation of neuroendocrine and HPA axis function following global cerebral ischemia in male rats and the effect of Antalarmin on plasma corticosterone level. *Hormones and Behavior*, *65*(3), 273–284. https://doi.org/10.1016/j.yhbeh.2014.01.003

Dellu, F., Mayo, W., Cherkaoui, J., Le Moal, M., & Simon, H. (1992). A two-trial memory task with automated recording: Study in young and aged rats. *Brain Research*, *588*, 132–139. https://doi.org/10.1016/0006-8993(92)91352-F

Devane, W. A., Dysarz, F. A. 3rd, Johnson, M. R., Melvin, L. S., & Howlett, A. C. (1988). Determination and characterization of a cannabinoid receptor in rat brain. *Molecular Pharmacology*, *34*, 605–613.

Domi, E., Uhrig, S., Soverchia, L., Spanagel, R., Hansson, A. C., Barbier, E., Heilig, M., Ciccocioppo, R., & Ubaldi, M. (2016). Genetic deletion of neuronal PPARgamma enhances the emotional response to acute stress and exacerbates anxiety: An effect reversed by rescue of amygdala PPARgamma function. *Journal of Neuroscience*, *36*, 12611–12623.

Esposito, G., Scuderi, C., Valenza, M., Togna, G. I., Latina, V., De Filippis, D., Cipriano, M., Carratu, M. R., Iuvone, T., & Steardo, L. (2011). Cannabidiol reduces Abeta-induced neuroinflammation and promotes hippocampal neurogenesis through PPARgamma involvement. *PLoS One*, *6*, e28668.

Fernandez-Ruiz, J., Sagredo, O., Pazos, M. R., Garcia, C., Pertwee, R., Mechoulam, R., & Martinez-Orgado, J. (2013). Cannabidiol for neurodegenerative disorders: Important new clinical applications for this phytocannabinoid? *British Journal of Clinical Pharmacology*, *75*, 323–333. https://doi.org/10.1111/j.1365-2125.2012.04341.x

Ferrari, P. F., Palanza, P., Parmigiani, S., & Rodgers, R. J. (1998). Interindividual variability in Swiss male mice: Relationship between social factors, aggression, and anxiety. *Physiology & Behavior*, *63*(5), 821–827. https://doi.org/10.1016/S0031-9384(97)00544-1

Fogaca, M. V., Campos, A. C., Coelho, L. D., Duman, R. S., & Guimaraes, F. S. (2018). The anxiolytic effects of cannabidiol in chronically stressed mice are mediated by the endocannabinoid system: Role of neurogenesis and dendritic remodeling. *Neuropharmacology*, *135*, 22–33. https://doi.org/10.1016/j.neuropharm.2018.03.001

Fogaca, M. V., Reis, F. M., Campos, A. C., & Guimaraes, F. S. (2014). Effects of intra-prelimbic prefrontal cortex injection of cannabidiol on anxiety-like behavior: Involvement of 5HT1A receptors and previous stressful experience. *European Neuropsychopharmacology*, *24*, 410–419. https://doi.org/10.1016/j.euroneuro.2013.10.012

Gomes, F. V., Resstel, L. B., & Guimaraes, F. S. (2011). The anxiolytic-like effects of cannabidiol injected into the bed nucleus of the stria terminalis are mediated by 5-HT1A receptors. *Psychopharmacology (Berl)*, *213*, 465–473. https://doi.org/10.1007/s00213-010-2036-z

Guadalupe, S., Raúl, T., Ana, M.-M., Lluïsa, C., Dafnis, B., Emma, M.-M., Elisenda, E., Josep, P., Salvador, P., Elisabet, V., Alberto, P. G., & Anna, M. P. (2013). The ins and outs of the BCCAO model for chronic hypoperfusion: A multimodal and longitudinal MRI approach. *PLoS One*, *8*(9), e74631.

Hadley, G., Beard, D. J., Couch, Y., Neuhaus, A. A., Adriaanse, B. A., DeLuca, G. C., Sutherland, B. A., & Buchan, A. M. (2019). Rapamycin in ischemic stroke: Old drug, new tricks? *Journal of Cerebral Blood Flow & Metabolism*, *39*(1), 20–35. https://doi.org/10.1177/0271678X18807309

Hayakawa, K., Mishima, K., Irie, K., Hazekawa, M., Mishima, S., Fujioka, M., Orito, K., Egashira, N., Katsurabayashi, S., Takasaki, K., Iwasaki, K., & Fujiwara, M. (2008). Cannabidiol prevents a post-ischemic injury progressively induced by cerebral ischemia via a high-mobility group box1-inhibiting mechanism. *Neuropharmacology*, *55*, 1280–1286. https://doi.org/10.1016/j.neuropharm.2008.06.040

Hayakawa, K., Mishima, K., Nozako, M., Hazekawa, M., Irie, K., Fujioka, M., Orito, K., Abe, K., Hasebe, N., Egashira, N., Iwasaki, K., & Fujiwara, M. (2007). Delayed treatment with cannabidiol has a cerebroprotective action via a cannabinoid receptor-independent myeloperoxidase-inhibiting mechanism. *Journal of Neurochemistry*, *102*, 1488–1496. https://doi.org/10.1111/j.1471-4159.2007.04565.x

Hayakawa, K., Mishima, K., Nozako, M., Ogata, A., Hazekawa, M., Liu, A. X., Fujioka, M., Abe, K., Hasebe, N., Egashira, N., Iwasaki, K., & Fujiwara, M. (2007). Repeated treatment with cannabidiol but not Delta9-tetrahydrocannabinol has a neuroprotective effect without the development of tolerance. *Neuropharmacology*, *52*, 1079–1087.

Hind, W. H., England, T. J., & O'Sullivan, S. E. (2016). Cannabidiol protects an in vitro model of the blood-brain barrier from oxygen-glucose deprivation via PPARgamma and 5-HT1A receptors. *British Journal of Pharmacology*, *173*, 815–825.

Izzo, A. A., Borrelli, F., Capasso, R., Di Marzo, V., & Mechoulam, R. (2009). Non-psychotropic plant cannabinoids: New therapeutic opportunities from an ancient herb. *Trends in Pharmacological Sciences*, *30*, 515–527. https://doi.org/10.1016/j.tips.2009.07.006

Jakovcevski, M., Schachner, M., & Morellini, F. (2008). Individual variability in the stress response of C57BL/6J male mice correlates with trait anxiety. *Genes, Brain, and Behavior*, *7*(2), 235–243. https://doi.org/10.1111/j.1601-183X.2007.00345.x

Jorgensen, M. E., Torp-Pedersen, C., Gislason, G. H., Jensen, P. F., Berger, S. M., Christiansen, C. B., Overgaard, C., Schmiegelow, M. D., & Andersson, C. (2014). Time elapsed after ischemic stroke and risk of adverse cardiovascular events and mortality following elective noncardiac surgery. *The Journal of the American Medical Association*, *312*, 269–277. https://doi.org/10.1001/jama.2014.8165

Knowles, M. D., de la Tremblaye, P. B., Azogu, I., & Plamondon, H. (2016). Endocannabinoid CB1 receptor activation upon global ischemia adversely impact recovery of reward and stress signaling molecules, neuronal survival and behavioral impulsivity. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *66*, 8–21. https://doi.org/10.1016/j.pnpbp.2015.10.010

Lafuente, H., Alvarez, F. J., Pazos, M. R., Alvarez, A., Rey-Santano, M. C., Mielgo, V., Murgia-Esteve, X., Hilario, E., & Martinez-Orgado, J. (2011). Cannabidiol reduces brain damage and improves functional recovery after acute hypoxia-ischemia in newborn pigs. *Pediatric Research*, *70*, 272–277. https://doi.org/10.1203/PDR.0b013e3182276b11

Lafuente, H., Pazos, M. R., Alvarez, A., Mohammed, N., Santos, M., Arizti, M., Alvarez, F. J., & Martinez-Orgado, J. A. (2016). Effects of cannabidiol and hypothermia on short-term brain damage in new-born piglets after acute hypoxia-ischemia. *Frontiers in Neuroscience*, *10*, 323. https://doi.org/10.3389/fnins.2016.00323

Laprairie, R. B., Bagher, A. M., Kelly, M. E., & Denovan-Wright, E. M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *British Journal of Pharmacology*, *172*, 4790–4805.

Laun, A. S., Shrader, S. H., Brown, K. J., & Song, Z. H. (2018). GPR3, GPR6, and GPR12 as novel molecular targets: Their biological functions and interaction with cannabidiol. *Acta Pharmacologica Sinica*. https://doi.org/10.1038/s41401-018-0031-9

Laun, A. S., Shrader, S. H., & Song, Z. H. (2018). Novel inverse agonists for the orphan G protein-coupled receptor 6. *Heliyon*, *4*, e00933. https://doi.org/10.1016/j.heliyon.2018.e00933

Laun, A. S., & Song, Z. H. (2017). GPR3 and GPR6, novel molecular targets for cannabidiol. *Biochemical and Biophysical Research Communications*, *490*, 17–21. https://doi.org/10.1016/j.bbrc.2017.05.165

Lim, C., Alexander, M. P., LaFleche, G., Schnyer, D. M., & Verfaellie, M. (2004). The neurological and cognitive sequelae of cardiac arrest. *Neurology*, *63*, 1774–1778. https://doi.org/10.1212/01.WNL.0000144189.83077.8E

Madhok, D. Y., Vitt, J. R., & Nguyen, A. T. (2018). Overview of neurovascular physiology. *Current Neurology and Neuroscience Reports*, *18*, 99. https://doi.org/10.1007/s11910-018-0905-8

McIlwain, K. L., Merriweather, M. Y., Yuva-Paylor, L. A., & Paylor, R. (2001). The use of behavioral test batteries: Effects of training history. *Physiology & Behavior*, *73*(5), 705–717. https://doi.org/10.1016/S0031-9384(01)00528-5

Milot, M. R., & Plamondon, H. (2009). Time-dependent effects of global cerebral ischemia on anxiety, locomotion, and habituation in rats. *Behavioral Brain Research*, *200*, 173–180. https://doi.org/10.1016/j.bbr.2009.01.009

Mishima, K., Hayakawa, K., Abe, K., Ikeda, T., Egashira, N., Iwasaki, K., & Fujiwara, M. (2005). Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine1A receptor-dependent mechanism. *Stroke*, *36*, 1077–1082.

Moreira, F. A., Aguiar, D. C., Terzian, A. L., Guimaraes, F. S., & Wotjak, C. T. (2012). Cannabinoid type 1 receptors and transient receptor potential vanilloid type 1 channels in fear and anxiety-two sides of one coin? *Neuroscience*, *204*, 186–192. https://doi.org/10.1016/j.neuroscience.2011.08.046

Mori, M. A., Meyer, E., Soares, L. M., Milani, H., Guimaraes, F. S., & de Oliveira, R. M. W. (2017). Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *75*, 94–105. https://doi.org/10.1016/j.pnpbp.2016.11.005

Murillo-Rodriguez, E., Millan-Aldaco, D., Palomero-Rivero, M., Mechoulam, R., & Drucker-Colin, R. (2006). Cannabidiol, a

constituent of *Cannabis sativa*, modulates sleep in rats. *FEBS Letters*, *580*, 4337–4345.

Nakashima, M. N., Ajiki, K., Nakashima, K., & Takahashi, M. (2003). Possible role of nitric oxide in anxiety following transient cerebral ischemia in mice. *Journal of Pharmacological Sciences*, *91*, 47–52. https://doi.org/10.1254/jphs.91.47

Nebuka, M., Ohmura, Y., Izawa, S., Bouchekioua, Y., Nishitani, N., Yoshida, T., & Yoshioka, M. (2020). Behavioral characteristics of 5-HT2C receptor knockout mice: Locomotor activity, anxiety-, and fear memory-related behaviors. *Behavioral Brain Research*, *379*, 112394. https://doi.org/10.1016/j.bbr.2019.112394

Neigh, G. N., Karelina, K., Glasper, E. R., Bowers, S. L., Zhang, N., Popovich, P. G., & DeVries, A. C. (2009). Anxiety after cardiac arrest/cardiopulmonary resuscitation: Exacerbated by stress and prevented by minocycline. *Stroke*, *40*, 3601–3607. https://doi.org/10.1161/STROKEAHA.109.564146

Neumann, J. T., Cohan, C. H., Dave, K. R., Wright, C. B., & Perez-Pinzon, M. A. (2013). Global cerebral ischemia: Synaptic and cognitive dysfunction. *Current Drug Targets*, *14*, 20–35. https://doi.org/10.2174/138945013804806514

O'Sullivan, S. E., & Kendall, D. A. (2010). Cannabinoid activation of peroxisome proliferator-activated receptors: Potential for modulation of inflammatory disease. *Immunobiology*, *215*, 611–616. https://doi.org/10.1016/j.imbio.2009.09.007

Pazos, M. R., Cinquina, V., Gomez, A., Layunta, R., Santos, M., Fernandez-Ruiz, J., & Martinez-Orgado, J. (2012). Cannabidiol administration after hypoxia-ischemia to newborn rats reduces long-term brain injury and restores neurobehavioral function. *Neuropharmacology*, *63*, 776–783. https://doi.org/10.1016/j.neuropharm.2012.05.034

Pazos, M. R., Mohammed, N., Lafuente, H., Santos, M., Martinez-Pinilla, E., Moreno, E., Valdizan, E., Romero, J., Pazos, A., Franco, R., Hillard, C. J., Alvarez, F. J., & Martinez-Orgado, J. (2013). Mechanisms of cannabidiol neuroprotection in hypoxic-ischemic newborn pigs: Role of 5HT(1A) and CB2 receptors. *Neuropharmacology*, *71*, 282–291. https://doi.org/10.1016/j.neuropharm.2013.03.027

Peres, F. F., Diana, M. C., Levin, R., Suiama, M. A., Almeida, V., Vendramini, A. M., Santos, C. M., Zuardi, A. W., Hallak, J. E. C., Crippa, J. A., & Abilio, V. C. (2018). Cannabidiol administered during peri-adolescence prevents behavioral abnormalities in an animal model of schizophrenia. *Frontiers in Pharmacology*, *9*, 901. https://doi.org/10.3389/fphar.2018.00901

Pertwee, R. G. (2006). Cannabinoid pharmacology: The first 66 years. *British Journal of Pharmacology*, *147*(Suppl. 1), S163–171. https://doi.org/10.1038/sj.bjp.0706406

Pertwee, R. G. (2008). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. *British Journal of Pharmacology*, *153*, 199–215.

Pertwee, R. G., Ross, R. A., Craib, S. J., & Thomas, A. (2002). (-)-Cannabidiol antagonizes cannabinoid receptor agonists and noradrenaline in the mouse vas deferens. *European Journal of Pharmacology*, *456*, 99–106. https://doi.org/10.1016/S0014-2999(02)02624-9

Porsolt, R. D., Bertin, A., & Jalfre, M. (1977). Behavioral despair in mice: A primary screening test for antidepressants. *Archives Internationales de Pharmacodynamie et de Therapie*, *229*, 327–336.

Ramos-Zúñiga, R., Gómez, P. U., Navarro Ruiz, A., de Luquín, A. S., & García-Estrada, J. (2008). Locomotor activity is a predictive test after global ischemia-reperfusion in Mongolian gerbils. *Minim Invasive Neurosurg*, *51*(2), 87–90. https://doi.org/10.1055/s-2007-1022531

Ribeiro, A., Ferraz-de-Paula, V., Pinheiro, M. L., Vitoretti, L. B., Mariano-Souza, D. P., Quinteiro-Filho, W. M., Akamine, A. T., Almeida, V. I., Quevedo, J., Dal-Pizzol, F., Hallak, J. E., Zuardi, A. W., Crippa, J. A., & Palermo-Neto, J. (2012). Cannabidiol, a non-psychotropic plant-derived cannabinoid, decreases inflammation in a murine model of acute lung injury: Role for the adenosine A(2A) receptor. *European Journal of Pharmacology*, *678*, 78–85. https://doi.org/10.1016/j.ejphar.2011.12.043

Rock, E. M., Bolognini, D., Limebeer, C. L., Cascio, M. G., Anavi-Goffer, S., Fletcher, P. J., Mechoulam, R., Pertwee, R. G., & Parker, L. A. (2012). Cannabidiol, a non-psychotropic component of cannabis, attenuates vomiting and nausea-like behaviour via indirect agonism of 5-HT(1A) somatodendritic autoreceptors in the dorsal raphe nucleus. *British Journal of Pharmacology*, *165*, 2620–2634. https://doi.org/10.1111/j.1476-5381.2011.01621.x

Rock, E. M., Limebeer, C. L., Petrie, G. N., Williams, L. A., Mechoulam, R., & Parker, L. A. (2017). Effect of prior foot shock stress and delta(9)-tetrahydrocannabinol, cannabidiolic acid, and cannabidiol on anxiety-like responding in the light-dark emergence test in rats. *Psychopharmacology (Berl)*, *234*, 2207–2217.

Ryberg, E., Larsson, N., Sjogren, S., Hjorth, S., Hermansson, N. O., Leonova, J., Elebring, T., Nilsson, K., Drmota, T., & Greasley, P. J. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. *British Journal of Pharmacology*, *152*, 1092–1101. https://doi.org/10.1038/sj.bjp.0707460

Sadaghiani, M. S., Javadi-Paydar, M., Gharedaghi, M. H., Fard, Y. Y., & Dehpour, A. R. (2011). Antidepressant-like effect of pioglitazone in the forced swimming test in mice: The role of PPAR-gamma receptor and nitric oxide pathway. *Behavioral Brain Research*, *224*, 336–343. https://doi.org/10.1016/j.bbr.2011.06.011

Sales, A. J., Crestani, C. C., Guimaraes, F. S., & Joca, S. R. L. (2018). Antidepressant-like effect induced by Cannabidiol is dependent on brain serotonin levels. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *86*, 255–261. https://doi.org/10.1016/j.pnpbp.2018.06.002

Sales, A. J., Fogaca, M. V., Sartim, A. G., Pereira, V. S., Wegener, G., Guimaraes, F. S., & Joca, S. R. L. (2018). Cannabidiol induces rapid and sustained antidepressant-like effects through increased BDNF signaling and synaptogenesis in the prefrontal cortex. *Molecular Neurobiology*. https://doi.org/10.1007/s12035-018-1143-4

Sartim, A. G., Guimaraes, F. S., & Joca, S. R. (2016). Antidepressant-like effect of cannabidiol injection into the ventral medial prefrontal cortex-Possible involvement of 5-HT1A and CB1 receptors. *Behavioural Brain Research*, *303*, 218–227. https://doi.org/10.1016/j.bbr.2016.01.033

Schiavon, A. P., Soares, L. M., Bonato, J. M., Milani, H., Guimaraes, F. S., & Weffort de Oliveira, R. M. (2014). Protective effects of cannabidiol against hippocampal cell death and cognitive impairment induced by bilateral common carotid artery occlusion in mice. *Neurotoxicity Research*, *26*, 307–316. https://doi.org/10.1007/s12640-014-9457-0

Schmidt, W., Schafer, F., Striggow, V., Frohlich, K., & Striggow, F. (2012). Cannabinoid receptor subtypes 1 and 2 mediate long-lasting neuroprotection and improve motor behavior deficits after transient focal cerebral ischemia. *Neuroscience*, *227*, 313–326. https://doi.org/10.1016/j.neuroscience.2012.09.080

Shahsavarian, A., Javadi, S., Jahanabadi, S., Khoshnoodi, M., Shamsaee, J., Shafaroodi, H., Mehr, S. E., & Dehpour, A. (2014).

Antidepressant-like effect of atorvastatin in the forced swimming test in mice: The role of PPAR-gamma receptor and nitric oxide pathway. *European Journal of Pharmacology*, *745*, 52–58. https://doi.org/10.1016/j.ejphar.2014.10.004

Soares, L. M., De Vry, J., Steinbusch, H. W. M., Milani, H., Prickaerts, J., & Weffort de Oliveira, R. M. (2016). Rolipram improves cognition, reduces anxiety- and despair-like behaviors and impacts hippocampal neuroplasticity after transient global cerebral ischemia. *Neuroscience*, *326*, 69–83. https://doi.org/10.1016/j.neuroscience.2016.03.062

Soares, L. M., Schiavon, A. P., Milani, H., & de Oliveira, R. M. (2013). Cognitive impairment and persistent anxiety-related responses following bilateral common carotid artery occlusion in mice. *Behavioral Brain Research*, *249*, 28–37. https://doi.org/10.1016/j.bbr.2013.04.010

Straiker, A., Dvorakova, M., Zimmowitch, A., & Mackie, K. (2018). Cannabidiol inhibits endocannabinoid signaling in autaptic hippocampal neurons. *Molecular Pharmacology*, *94*, 743–748. https://doi.org/10.1124/mol.118.111864

Sun, H., Xiong, W., Arrick, D. M., & Mayhan, W. G. (2012). Low-dose alcohol consumption protects against transient focal cerebral ischemia in mice: Possible role of PPARgamma. *PLoS One*, *7*, e41716.

Thomas, A., Baillie, G. L., Phillips, A. M., Razdan, R. K., Ross, R. A., & Pertwee, R. G. (2007). Cannabidiol displays unexpectedly high potency as an antagonist of CB1 and CB2 receptor agonists in vitro. *British Journal of Pharmacology*, *150*, 613–623.

Tucker, L. B., Burke, J. F., Fu, A. H., & McCabe, J. T. (2017). Neuropsychiatric symptom modeling in male and female C57BL/6J mice after experimental traumatic brain injury. *Journal of Neurotrauma*, *34*, 890–905. https://doi.org/10.1089/neu.2016.4508

Valvassori, S. S., Bavaresco, D. V., Scaini, G., Varela, R. B., Streck, E. L., Chagas, M. H., Hallak, J. E., Zuardi, A. W., Crippa, J. A., & Quevedo, J. (2013). Acute and chronic administration of cannabidiol increases mitochondrial complex and creatine kinase activity in the rat brain. *Brazilian Journal of Psychiatry*, *35*, 380–386. https://doi.org/10.1590/1516-4446-2012-0886

Zanelati, T. V., Biojone, C., Moreira, F. A., Guimaraes, F. S., & Joca, S. R. (2010). Antidepressant-like effects of cannabidiol in mice: Possible involvement of 5-HT1A receptors. *British Journal of Pharmacology*, *159*, 122–128. https://doi.org/10.1111/j.1476-5381.2009.00521.x

How to cite this article: Mori MA, Meyer E, da Silva FF, Milani H, Guimarães FS, Oliveira RMW. Differential contribution of CB1, CB2, 5-HT1A, and PPAR-γ receptors to cannabidiol effects on ischemia-induced emotional and cognitive impairments. *Eur J Neurosci*. 2021;53:1738–1751. https://doi.org/10.1111/ejn.15134

9.5 Part IV: Meyer et al. bioRxiv [preprint]. 2022

Time-lapse Imaging of Microglial Activity and Astrocytic Calcium Signaling Reveals a Neuroprotective Effect of Cannabidiol in the Subacute Phase of Stroke

Erika Meyer[1,2,*], Phillip Rieder[1], Davide Gobbo[1], Gabriela Cândido[2], Anja Scheller[1], Rúbia Maria Weffort de Oliveira[2], Frank Kirchhoff[1]

1. Molecular Physiology, Center for Integrative Physiology and Molecular Medicine, University of Saarland, D-66421 Homburg, Germany

2. Laboratory of Brain Ischemia and Neuroprotection, Department of Pharmacology and Therapeutics, State University of Maringá, CEP-87020900, Maringá, Brazil

(*) To whom correspondence should be addressed.

The authors have declared no competing interest.

Abstract

Pharmacological agents that limit secondary tissue loss and/or improve functional outcomes after stroke are still limited. Cannabidiol, the major non-psychoactive component of *Cannabis sativa*, has been proposed as a neuroprotective agent against experimental focal cerebral ischemia. The effects of cannabidiol have generally been related to the modulation of neuroinflammation, including the control of glial activation and the toxicity exerted by pro-inflammatory mediators. However, so far, most information concerning cannabidiol neuroprotective effects was obtained from histological and biochemical *post-mortem* assays. To test whether the effects of cannabidiol on glial cells could be also detected *in vivo*, we performed time-lapse imaging of microglial activity and astrocytic calcium signaling in the subacute phase of stroke using two-photon laser-scanning microscopy. First, C57BL/6N wild-type mice underwent either sham or transient middle cerebral artery occlusion surgery. The animals received intraperitoneal injection of vehicle or cannabidiol (10 mg/kg) 30 min, 24 h, and 48 h after surgery. One day later the neurological score test was performed. Brain tissue was processed to evaluate the neuronal loss and microglial activation. Transgenic mice with microglial expression of the enhanced green fluorescent protein and astrocyte-specific expression of the calcium sensor GCaMP3 were used to access *in vivo* microglial activity and astrocytic calcium signaling, respectively. The animals were submitted to the same experimental design described above and to imaging sessions before, 30 min, 24 h and, 48 h after surgery. Astrocytic calcium signaling was also assessed in acutely isolated slices 5 h after transient middle cerebral artery occlusion surgery in the presence of perfusion or cannabidiol solution. Cannabidiol prevented ischemia-induced neurological impairments as well as protected against neuronal loss in ischemic mice. Cannabidiol also reduced ischemia-induced microglial activation, as demonstrated in fixed tissue as well in *in vivo* conditions. No difference in the amplitude and duration of astrocytic calcium signals was detected before and after the middle cerebral artery occlusion *in vivo*. Similarly, no significant difference was found in the astrocytic calcium signals between contra and ipsilateral side of acutely isolated brain slices. The present results suggest that the neuroprotective effects of cannabidiol after stroke may occur in the subacute phase of ischemia and reinforce the strong anti-inflammatory property of this compound.

INTRODUCTION

Stroke is one of the most important causes of morbidity and mortality worldwide. Globally, from 1990 to 2019, the number of incident strokes and deaths due to stroke increased by 70% and 43% (GBD, 2021). Stroke survivors are particularly vulnerable to the stroke-secondary consequences that include sensory, motor and cognitive impairments, as well as mood dysfunction. Despite some recent advances in ischemic stroke caused by large-vessel occlusions, such as mechanical thrombectomy, a neuroprotective pharmacological agent that reduces the consequences of stroke remains needed in clinical practice.

Resident microglia are the first cell of the brain to respond to the pathophysiological changes induced by an ischemic stroke. Several studies have demonstrated the involvement of microglia in cerebral ischemic conditions, but their contribution to the progression of stroke remains under debate, despite the research effort in this field (Kitamura et al., 2004, 2005; Lalancette-Hébert et al., 2007; Jolivel et al., 2015; Szalay et al., 2016). During the acute phase of cerebral ischemia (CI), astrocytes undergo also important morphological modifications, such as hyperplasia and hypertrophy, followed by the production of neurotoxic substances, possibly aggravating the ischemic lesion (Buskila et al., 2005; Basic Kes et al., 2008).

The complex pathophysiology of ischemic stroke may be the reason for the ineffectiveness of treatments that act only on some mechanism of the ischemic cascade. Thus, therapies acting on multiple pathophysiological processes can offer promising results in stroke research. Due to its pleiotropic action and good safety profile cannabidiol (CBD) has been emerging as a candidate for the multifactorial treatment of stroke and its consequences.

Current evidence for the neuroprotective effect of CBD in stroke is predicated on preclinical settings. CBD decreased infarct size, increased cerebral blood flow and improved neurological scores and motor coordination in a model of stroke induced by middle cerebral artery occlusion (MCAO) in mice (Hayakawa et al., 2004; Mishima et al., 2005; Hayakawa et al., 2008). CBD also protected against neuronal death and reduced microglial activation resulting in a decrease of the infarct size in MCAO mice (Hayakawa et al., 2007; Hayakawa et al., 2008; Ceprián et al., 2017). A reduction of microglia and astrocytic reactivity were observed in mice after bilateral common carotid artery occlusion (Schiavon et al., 2014; Mori et al., 2017), a model of transient global CI. Although these studies indicate an effect of CBD in decreasing microglial and astrocytic activation after an ischemic insult, so far these observations were only obtained from immunohistochemical assays.

In the present study we were particularly interested in utilizing *in vivo* imaging to examine how microglia behave after CBD treatment during the early phase of CI induced by MCAO. Using genetically encoded Ca^{2+} indicator and two-photon imaging we also asked if CBD treatment would impact astrocytic Ca^{2+} signaling in the penumbra area of ischemic mice.

MATERIALS AND METHODS

Ethics statement

All animal procedures were carried out at the University of Saarland in strict accordance with the recommendations to European and German guidelines for the welfare of experimental animals and approved by the Saarland state's "Landesamt für Gesundheit und Verbraucherschutz" in Saarbrücken/Germany (animal license number: 36/2016).

Animals

Mice were housed at the animal facility of the Center for Integrative Physiology and Molecular Medicine (CIPMM) in Homburg under conditions of controlled temperature (22 ± 1 °C) and a 12 h light-dark cycle, with food and water *ad libitum*. Experiments were conducted with 12- to 15-week-old male and female C57BL/6N wild-type (WT) and transgenic mice. To image microglia *in vivo,* the knock-in mouse line TgH(CX_3CR_1-EGFP) was used. To visualize astrocytic calcium signals, inducible CreERT2 DNA recombinase knock-in mice TgH(GLAST-CreERT2) were crossed to the floxed reporter mice TgH(Rosa26-CAG-fl-stop-fl-GCaMP3-WPRE). To simplify, in this study the mouse lines are termed $CXCR^{EGFP}$ and $GLAST^{GCAMP3}$, respectively. Further mouse line information and respective constructs are listed below (Table 1).

TgH (CX_3CR_1-EGFP)

The mouse line was maintained in the C57BL/6N background and only heterozygous mice were used. In this mouse line, microglia express the enhanced green fluorescent protein (EGFP). EGFP expression is achieved through placement of the EGFP reporter gene into the *Cx3cr1* locus encoding the fractalkine receptor CX_3CR_1 (Jung et al., 2000).

TgH (Glast-CreERT2)

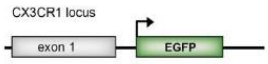
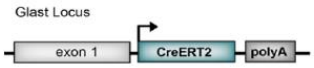
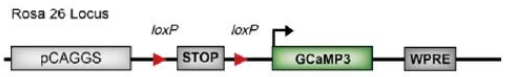
In this transgenic mouse line, CreERT2 is knocked into the locus of the L- Glutamate/L-aspartate transporter (GLAST; Mori et al., 2006). The Cre DNA recombinase is bound to the ligand-binding domain of the modified estrogen receptor (ERT2) and remains in the

cytoplasm. After tamoxifen administration, this fusion protein translocates into the nucleus and mediates recombination. For this study, only heterozygous animals were used.

TgH (Rosa 26-CAG-fl-stop-fl-GCaMP3-WPRE)

GCaMP3 is a Ca^{2+} indicator, consisting of the M13 fragment from myosin light chain kinase (M13), EGFP, and calmodulin. The M13 domain is bound at the N-terminus of the EGFP and is target sequence for calmodulin. After binding of Ca^{2+} to calmodulin, the conformation of the protein is changed and the fluorescence intensity of EGFP is thereby enhanced (Nakai et al., 2001). The expression of GCaMP3 is controlled by the CAGGS promoter (Niwa et al., 1991) and the construct is inserted into the Rosa26 locus. Upon tamoxifen administration, the CreERT2 recombinase leads to the deletion of a floxed STOP cassette ahead of the GCaMP3 sequence allowing the inducible expression. The WPRE (woodchuck hepatitis virus posttranscriptional regulatory element) leads to the early exit of mRNA from the nucleus and enhances mRNA stability (Paukert et al., 2014). For this study, only homozygous animals were used.

Table 1 - Mouse constructs

Mouse line	Construct	Reference
TgH(CX_3CR_1-EGFP)		Jung et al., 2000
TgH(Glast-CreERT2)		Mori et al., 2006
TgH(Rosa 26-CAG-lsl-GCAMP3-WPRE)		Paukert et al., 2014

Genotyping

Genomic DNA extraction was performed on tail biopsy or ear punch samples. Tissue digestion for DNA extraction and subsequent polymerase chain reaction (PCR) was performed with the prepared sample buffer from REDextract-N-Amp PCR KIT (Sigma-Aldrich, St. Louis, United States) or DreamTaq™ Hot Start Green DNA Polymerase (Thermo Fischer Scientific, Walthan, United States) and different oligonucleotide primers (Table 2). First, the samples were incubated with 62.5 µl of extraction solution for 10 min by shaking. Next, the solution was incubated for 20 min at 95 °C. Following cooling down to RT, 50 µl of neutralization buffer was given to the samples. The reactions were run in 96-well PCR plates in Thermocyclers (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Gel

electrophoresis was run on 1.5 - 2 % agarose gels with ethidium bromide (*f.c.* 0.015 %) and, lastly, exposed and documented with the Quantum Gel documentation system (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

Table 2 - Genotyping primers

Line	Primer		Sequence	(bp)
CX3CR1	5927 5928	KI	5'-TCAGTGTTTTCTCCCGCTTGC-3' 5'-GTAGTGGTTGTCGGGCAGCAG-3'	825
CX3CR1	5929	WT	5'-CAGTGATGCTCTTGGGCTTCC -3'	407
GLAST	11984 11986	KI	5'-GAGGCACTTGGCTAGGCTCTGAGGA-3' 5'-GGTGTACGGTCAGTAAATTGGACAT-3'	400
GLAST	11985 11986	WT	5'-GAGGAGATCCTGACCGATCAGTTGG-3' 5'-GGTGTACGGTCAGTAAATTGGACAT-3'	700
GCaMP3	27490 27632	KI	5'-GGCATTAAAGCAGCGTATCC-3' 5'-CACGTGATGACAAACCTTGG-3'	245
GCaMP3	14025 14026	WT	5'-CTCTGCTGCCTCCTGGCTTCT-3' 5'-CGAGGCGGATCACAAGCAATA-3'	327

Drugs

Tamoxifen induced gene recombination

Tamoxifen (Sigma-Aldrich, St. Louis, United States) was dissolved in Miglyol (Sigma-Aldrich, St. Louis, United States) at a concentration of 10 mg/ml, aliquoted and stored at 4 °C. The mice received injections of tamoxifen (100 mg/kg, *i.p.*), once per day, for five consecutive days (Jahn et al., 2018). Animals were treated at 7 weeks of age, resulting in 5 weeks intervals before the experiment start (Fig. 1C).

Cannabidiol treatment

Cannabidiol (CBD; THC Pharma, Frankfurt, Germany) was diluted in 1 % Tween 80 in sterile isotonic saline (vehicle). The animals were randomly assigned to receive injections (*i.p.*) of CBD 10 mg/kg or vehicle 30 min, 24, and 48 h after surgery (Fig. 1A, B, D).

Surgeries

For all described surgeries, the procedures were performed under conditions as sterile as possible. First, the animals were anesthetized with a mixture of isoflurane (5 % for induction and 2 % for maintenance), and O_2 (0.6 l/min) and N_2O (0.4 l/min) using Harvard Apparatus equipment (Holliston, United States). A temperature probe soaked in vaseline was inserted

rectally to control the body temperature between 36.6 - 37.5 °C. The eyes were covered with Bepanthen® (Bayer, Leverkusen, Germany) to prevent the cornea from drying out. After the surgeries, the mice received 10 % glucose (0.5 ml/30 g body weight, *s.c.*) as a fluid replacement, buprenorphine *i.p.* (0.01 µg/30 g body weight; Temgesic, Essex Pharma, Munich, Germany) and tramadol hydrochloride (Grünenthal GmbH, Aachen, Germany) to the drinking water (100 mg/200 ml) for three days, including the surgery day.

Cortical craniotomy

The cranial window procedure was performed as previously described with few modifications (Cupido et al., 2014). Briefly, after disinfecting the area with 70 % ethanol and removing the tissue, a 3-4 mm-diameter craniotomy was made over the somatosensory cortex (3.4 mm posterior to bregma and mediolateral 1.5 mm) using a driller. The drilling procedure was stopped at short intervals to remove bone particles and cool down the area with cortex buffer (in mM) 125 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 $CaCl_2$, 2 $MgSO_4$ [pH ~ 7.4] to prevent overheating of the underlying cortex. Next, using forceps (Fine Science Tools, Foster City, United States) the remaining bone was carefully removed to avoid damaging the meninges. Small bleeding was stopped with sponges (Gelastypt, Sanofi Aventis, Paris, France). A coverslip was placed on the exposed brain and the edge was sealed with dental cement (RelyX®, 3M ESPE, Saint Paul, United States). Finally, a custom-made metal holder (5 mm diameter) was put over the coverslip and glued with dental cement onto the bone.

Middle cerebral artery occlusion

Focal cerebral ischemia (FCI) was induced according to the Koizumi method of MCAO (Koizumi et al., 1986). In short, the left common carotid artery (CCA) and the external carotid artery were permanently ligated with silk sutures. A silicone-coated filament (Doccol Corp, Sharon, United States) was introduced through an arteriotomy and advanced into the right internal carotid artery until mild resistance was felt, indicating the filament reached the origin of the MCA to occlude the blood flow. After 15 min occlusion, the filament was gently withdrawn and a suture was made around the CCA, to prevent backflow through the arteriotomy. After recovery from anesthesia, the mice were kept in their cages with free access to food and water.

Neurological Score

Mice were evaluated for neurological deficits using the modified Bederson Score System

(Bederson et al., 1986; Bieber et al., 2019). The animals were examined for motor impairments 24 h after the MCAO as listed below (Table 3; Fig. 1A). Animals presenting a score of 5 were euthanized and excluded from the experiment.

Table 3 - Bederson neurological score (0-5)

Score	Mouse behavior
0	no observable deficit
1	forelimb flexion
2	forelimb flexion and decreased resistance to lateral push
3	circling
4	circling and spinning around the cranial-caudal axis
5	no spontaneous movement

Immunohistochemical analysis

Preparation of vibratome slices

After experimental procedures, mice were deeply anesthetized with Ketamine (Ketavet®, Pfizer, New York City, United States) / Xylazine (Rumpon®, Bayer Healthcare, Leverkusen, Germany) in 0.9 % NaCl (100 µl/10 µg body weight; *i.p.*) and dissected by performing a bilateral axillary thoracotomy to expose the heart. A butterfly needle was inserted into the left ventricle and perfusion was started with 1x phosphate buffered saline (PBS) followed by 4 % paraformaldehyde (PFA) in phosphate buffer. Simultaneously, an incision of the superior vena cava allowed the blood to drain off. The brains were carefully removed and postfixed in the same fixative solution overnight at 4 °C.

The fixed brains were sliced in PBS into coronal sections (40 µm) at a Leica VT1000S vibratome (Leica Biosystems, Wetzlar, Germany). The coronal sections at the hippocampal level (Bregma -1.34 mm to -2.70 mm according to Franklin and Paxinos, 1997) were collected in 48-well culture plates containing PBS and used for free-floating immunohistochemistry.

Antibody incubation

Vibratome sections were incubated in blocking solution (0.3 % Triton X-100 and 5 % horse serum in PBS) for 1 h at room temperature (RT). Next, the sections were incubated with polyclonal rabbit anti-Iba1 antibody (1:1000, Wako Chemicals USA, Richmond, United States), diluted in blocking solution, overnight at 4 °C. On the next day, the sections were washed 3x for 10 min with PBS and incubated with the anti-rabbit secondary antibody

(1:1000; Alexa Invitrogen, Walthan, United States), diluted in blocking solution, for 2 h at RT. Additionally, 4′,6-Diamidin-2-phenylindol (DAPI, *f.c.* 1 µg/ml, AppliChem, Darmstadt, Germany), a specific dye for nucleic acids, was added to the secondary antibody incubation. After washing with PBS, the slices were placed in a water bath and mounted with Immu-Mount™ (Thermo Fisher Scientific, Walthan, United States).

Fluoro-Jade C staining

To monitor MCAO-induced cell death, Fluoro-Jade C (FJC) staining was used, which is an established detection technique for degenerating neurons (Schmued and Hopkins, 2000). Vibratome sections, collected in 0.1 M PBS, were mounted on gelatin-coated slides and then dried on a slide warmer at 50 °C for 30 min. The slides containing the sections were immersed in a basic alcohol solution consisting of 1 % sodium hydroxide in 80 % ethanol for 5 min. Next, they were rinsed for 2 min in 70 % EtOH, for 2 min in distilled water, and then incubated in 0.06 % potassium permanganate solution for 15 min. Following a 1 min water rinse, the slides were transferred for 20 min to a 0.0001 % solution of FJC (Millipore, Burlington, United States) dissolved in 0.1 % acetic acid vehicle and kept in the dark. Thereafter, slides were washed in distilled water three times for 1 min, air dried, and coverslipped with DPX (Sigma-Aldrich, St. Louis, United States). FJC staining images were taken with the automated slide scanner microscope (Axio Scan.Z1, Zeiss, Jena, Germany).

Microscopy

Automated epifluorescence microscopy on fixed brain slices

Epifluorescent images were taken by the automated slide scanner AxioScan.Z1 (Zeiss, Jena, Germany) equipped with an LED Light Source Colibri 7 (Zeiss, Jena, Germany). A Plan-Apochromat 10×/0.45 objective for pre-focusing and a Plan-Apochromat 20×/0.8 objective for fine focus image acquisition were applied with appropriate emission and excitation filters. Offline image stitching (8 µm stacks, variance projection) for overviews of brain slices and further analysis was performed using ZEN 1 Software Black Edition (Zeiss, Oberkochen, Germany).

Confocal laser-scanning microscopy

Confocal images were taken using a laser-scanning microscope (LSM-710, Zeiss, Jena, Germany) with a Plan-Apochromat 40×/1.4 Oil DIC (UV) VISIR M27 objective. Z-stacks of images taken at 1 µm intervals were processed with Fiji/ImageJ software (Schindelin et al.,

2012) and displayed as maximum intensity projections for analyses with the MIA Software (provided by Prof. Bart Eggen, University of Groningen, Netherlands). The images were taken in the cortex, hippocampus, and thalamus in both brain hemispheres.

2-Photon laser-scanning microscopy

High-resolution *in vivo* imaging was performed using a custom-made two photon laser-scanning microscope (2P-LSM) equipped with a mode-locked Ti:Sapphire laser (Vision II, Coherent, Santa Clara, United States). Scanning and image acquisition were controlled using custom-written software ScanImage (Pologruto et al., 2003). Additionally, the setup was equipped with XY-galvanometer-based scanning mirrors (Cambridge Technology). The excitation wavelength of the laser was set at 890 nm and a 20x/1.0 water-immersion objective (W Plan-Apochromat, Carl Zeiss, Jena, Germany) was used. To minimize photodamage, the average excitation laser intensity was kept at a minimum for a sufficient signal-to-noise ratio, ranging from 30 to 50 mW depending on depth. The emitted light was detected by photomultiplier tubes (H10770PB-40, Hamamatsu Photonics, Hamamatsu, Japan). The imaging settings were selected every time equally (256 x 256 px, Zoom 2, frame rate 1.9 Hz).

Microglia imaging *in vivo*

For microglia imaging *in vivo*, isoflurane anesthesia was used as described. After sedation, the animals were fixed with a metal holder on a custom-made head restrainer. Before the imaging session was started, animals were injected with circa 50 µl Texas Red-dextran (50 mg/ml, 70 kDa; Invitrogen, Waltham, United States), via tail vein injection, for visualization of brain blood vessels. The marked blood vessels served as a map to image the same region of interest (ROI) over several sessions. Time-lapse imaging of cortical microglia was performed by the repeated acquisition of fluorescence image stacks (15 focal planes with 2 µm axial spacing) recorded 50 - 70 µm below the dura mater. Subsequent image stacks were recorded every 60 s, totalizing 20 stacks acquisition from a single ROI. The mice were imaged before (baseline), 30 min, 24, and 48 h after the MCAO (Fig. 1B).

Ca^{2+} imaging *in vivo* in slightly anesthetized mice

First, the animals were anesthetized as described. After fixing the animal's head, the isoflurane was reduced to 0.5 % until the end of the imaging session. Astrocytic GCaMP3 signals were recorded on a single focal plane located in the somatosensory cortex at a

depth of 50 - 70 µm inferior to the dura mater. Three to four ROIs were recorded for each mouse. The same experimental design was used as described above (Fig. 1D). All images and movies were further processed using Fiji/ImageJ software.

Ca^{2+} imaging in acute brain slices

For acute brain slice imaging, the animals were sacrificed by decapitation 3 h after MCAO. First, the brain was dissected and placed in an ice-cooled, carbogen-saturated (5 % CO_2 - 95 % O_2, pH 7.4) cutting solution, containing (in mM) 87 NaCl, 3 KCl, 1.25 $Na_2H_2PO_4$, 25 $NaHCO_3$, 25 Glucose, 75 Sucrose, 3 $MgCl_2$, and 0.5 $CaCl_2$. Next, the brain tissue was coronally cut (300 µm slice thickness) using a vibratome (Leica VT 1200S, Wetzlar, Germany) and transferred into a nylon basket as a slice holder with incubation solution, containing (in mM) 12.6 NaCl, 3 KCl, 1.2 $Na_2H_2PO_4$, 25 $NaHCO_3$, 15 Glucose, 2 $MgCl_2$, and 1 $CaCl_2$ 35 °C for 30 min. No more than 5 slices were collected from each brain. After incubation, the slices were let to recover with continuous oxygenation for at least 30 min at RT before recording. Subsequently, slices were transferred to an imaging chamber under the 2P-LSM and kept submerged by a custom-made platinum grid with nylon threads for mechanical stabilization. The imaging chamber was continuously perfused with oxygenated perfusion solution, containing (in mM) 12.6 NaCl, 3 KCl, 1.2 $Na_2H_2PO_4$, 25 $NaHCO_3$, 15 Glucose, 2 $MgCl_2$, and 1 $CaCl_2$ at a flow rate of 2-5 ml/min. The imaging sessions were done in both hemispheres (contra- and ipsilateral) and the chosen ROIs were located in the somatosensory cortex at a depth of 50 µm. For the investigation of the CBD effect over time, the slice incubated in oxygenated perfusion solution was imaged at 0, 10, 20 and 30 min. At the end of the period of 30 min the perfusion solution was switched to perfusion solution containing CBD 100 µM and the ROI was recorded again using the same schedule described above (Fig. 1E). Afterwards each application, the slice was removed and the chamber was washed completely with perfusion solution. CBD was first dissolved in a stock solution of 10 mM in dimethyl sulfoxide (DMSO), which was then added to the perfusion solution to obtain the desired 100 µM final concentration. CBD concentration was based on previous studies (Castillo et al., 2010).

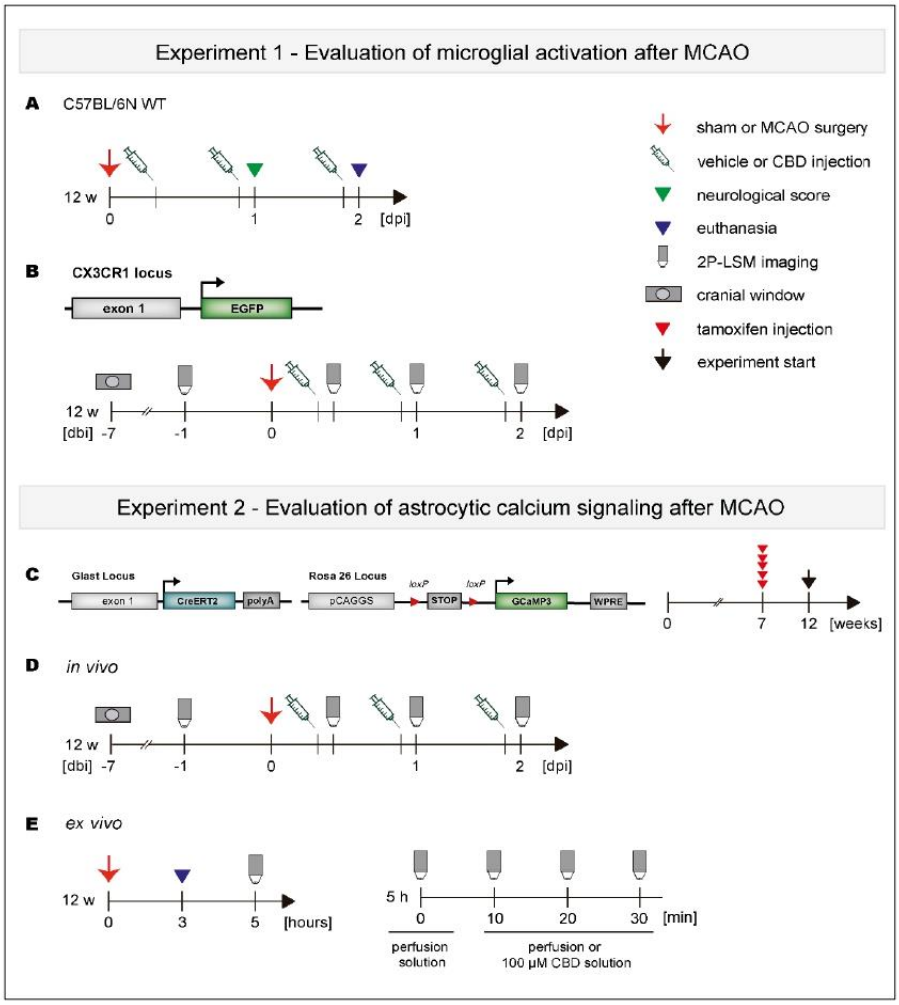


Figure 1 - Experimental design

A-B In Experiment 1, C57BL/6N WT mice were subjected to sham or MCAO surgery. Vehicle or 10 mg/kg CBD was administered (*i.p.*) 30 min, 24, and 48 h after reperfusion. One day after reperfusion the neurological score was carried out. One day after behavioral testing the animals were euthanized and their brains were collected for analysis. In another protocol, $CXCR^{EGFP}$ mice underwent cortical craniotomy. After one week of recovering, the mice underwent the MCAO surgery. The MCAO mice were treated with vehicle or 10 mg/kg CBD following the same administration protocol cited above. 2P-LSM imaging was performed before MCAO (baseline), 30 min, 24, and 48 h after reperfusion. **C** In experiment 2, the tamoxifen-inducible GLAST-CreERT2 x GCaMP3 mouse line was employed for expression of the Ca^{2+} sensor GCaMP3 in astrocytes. GCaMP3 expression in astrocytes was induced five weeks prior to the experimental start. **D** Mice underwent, first, the cortical craniotomy, and six days later, the MCAO surgery. The mice were submitted to the same treatment- and *in vivo* imaging protocol as described in B. **E** For the *ex vivo* imaging procedure, the mice underwent MCAO, and 3 h later were euthanized and had their brains processed. The brain slices (contra- and ipsilateral site) were incubated in perfusion- or 100 µM CBD solution and imaged over time. CBD: cannabidiol; MCAO: middle cerebral artery occlusion.

Data analysis

Staining data

FJC staining was analyzed, first, via measurement of fluorescence intensity on the total area of the brain slice, to obtain the percentage (%) of affected area per slice (Fig. 2C). Next, the number of FJC-positive cells was counted in the hippocampal dentate gyrus (GD) and CA1 area, thalamus and striatum (Fig. 2D, F, G). The ROIs were determined by drawing the respective structures on the slice. All slices were analyzed at the same coronal level and using the same ROI position and size.

MIA Software

MIA is a semi-automatic morphological parameter extractor for single-microglia images. Sholl profiles detailing about 23 parameters of microglia morphology can be obtained after analyses with MIA. This software was developed by Prof. Bart Eggen and his team (University of Groningen, Groningen, Netherlands). The cortex, hippocampus and thalamus in both brain hemispheres (contra- and ipsilateral side) were investigated. For each mouse, three microglia cells per brain region were analyzed.

Analyses of Ca^{2+} imaging data

Ca^{2+} imaging data were analyzed, using the custom-made MATLAB application *MSparkles* (Stopper et al., in preparation). MSparkles was specifically designed for the visualization and analysis of cellular events, visualized using fluorescence indicator dyes. A novel algorithm to estimate fluorescence fluctuations at basal concentrations of signaling molecules (F_0), such as Ca^{2+} or Na^+, removes fluorescence background and enables the detection of microdomain events. F_0 was computed by fitting a polynomial curve along the temporal axes of each pixel. Before polynomial fitting, statistically large values were removed. Subsequent event detection and analysis were based on the range projection of the normalized and detrended image stack ($\Delta F/F_0$). Fluorescence events were detected as temporally correlated, local brightness peaks, and segmented into individual ROIs. ROI traces were integrated by computing the mean fluorescence ($\Delta F/F_0$) per ROI per recorded time point. These ROI traces were then subjected to peak analysis for transient extraction and classification, based on a transient's peak amplitude.

Before analysis, Ca^{2+} imaging data were run through a pre-processing pipeline performing de-noising using the PURE-LET algorithm, image registration as well as a temporal median filter of size 3.

Statistical analyses

Prism 8 software (GraphPad, San Diego, CA, USA) was used for the statistical analysis. Data were examined for assumptions of a normal distribution using the Shapiro-Wilk normality test. In case of a normal distribution, the data were analyzed using Student's *t*-test and one- or two-way analysis of variance (ANOVA) as appropriate, followed by the Tukey multiple-comparison post hoc test. In the two-way repeated measures ANOVA, the group was the between-subject factor, and time (test day) was the within-subject factor. For data sets with a non-normal distribution, the non-parametric Wilcoxon matched-pairs signed rank and Mann-Whitney tests were applied, as appropriate. For calcium-signals data, outlier detection with GraphPad Prisms Rout method (Q = 1 %) was performed. The data are expressed as mean ± SEM or median with interquartile range (IQR). Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Ischemia-induced neurodegeneration and neurological deficits are reduced after cannabidiol treatment

The effects of ischemia and CBD on neurological function were evaluated 24 h after reperfusion by the neurological deficit score, as shown in Fig. 2A. The one-way ANOVA revealed significant differences in the scores among groups ($F_{2,21} = 7.92$, $p < 0.05$). Tukey's post hoc analyses revealed that the sham+vehicle group had a lower score compared with the MCAO+vehicle group ($p < 0.05$). The MCAO+CBD group exhibited a significantly lower score compared with the MCAO+vehicle group ($p < 0.05$).

Neurodegeneration was evaluated by the increase in the number of dying neurons assessed by FJC staining two days after sham or MCAO surgery in the hippocampal CA1 subfield, DG, thalamus, and striatum, as illustrated in Fig. 2B. An increased percentage of FJC-positive cells per slice was found in the MCAO+vehicle compared with the MCAO+CBD group (Student's *t*-test, $t_4 = 3.10$, $p < 0.05$), reflecting the beneficial effect of CBD on MCAO-induced neurodegeneration (Fig. 2C). As shown in Fig. 2D, there were no statistical differences in FJC staining of hippocampal CA1 area and DG between the groups (Student's *t*-test, $t_4 = 1.13$ - 1.16, $p > 0.05$). However, a significant loss of neurons in the MCAO+vehicle group was detected when compared to the MCAO+CBD group (Student's *t*-test, $t_4 = 2.87$, $p < 0.05$; Fig. 2F). There was no statistical difference in the FJC-positive cell number in the striatum between the groups (Student's *t*-test, $t_4 = 2.87$, $p > 0.05$; Fig. 12G).

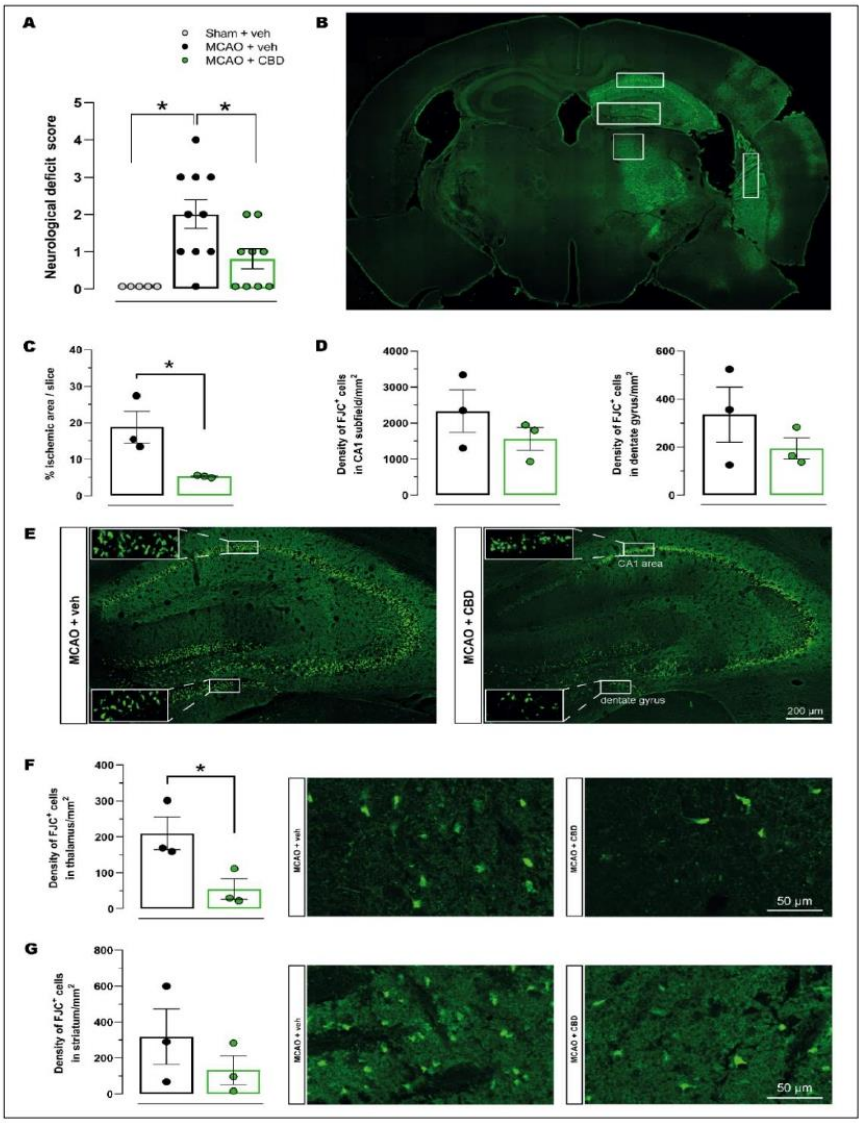


Figure 2 - Cannabidiol protected against neurological impairments and neuronal death induced by MCAO

A Neurological impairment was evaluated 24 h post-ischemia using the neurological deficit score. **B** Representative figure illustrating a coronal brain section at the intermediate level of the hippocampus showing the CA1 subfield, DG, thalamus, and striatum where the analysis for FJC was performed. **C** Percentage (%) of FJC-positive neurons per slice. **D** The density of FJC-positive neurons in the hippocampal CA1 area and DG. **E** Representative photomicrographs of FJC-positive cells in the CA1 and DG in the different experimental groups. **F** Density of FJC-positive neurons in the thalamus and respective representative photomicrographs. **G** The density of FJC-positive neurons in the striatum and respective representative photomicrographs among the groups. Stats: data are shown as individual values (dots) and the means ± SEM (columns and bars) of the experimental groups (n= 3-10/group). *$p \leq 0.05$. DG: dentate gyrus; FJC: Fluoro-Jade C; MCAO: middle cerebral artery occlusion.

Reduction of ischemia-caused microglia activation by cannabidiol treatment

MCAO-induced microglia activation was assessed by analyzing Iba-1 immunoreactivity in the cortex, hippocampus and thalamus comparing contra- and ipsilateral sides from animals' brains (Fig. 3A). As shown in Fig. 3C, ischemia did not affect the soma area, total branch length, and number of nodes of microglia in the cortex ($F_{2,9}$ = 1.08 - 2.45, $p > 0.05$) and thalamus ($F_{2,9}$ = 5.85 - 10.85, $p > 0.05$) from the contralateral side of the lesion. However, the one-way ANOVA revealed significant differences in microglial soma area, total branch length, and the number of nodes in the hippocampus from the contralateral side among groups ($F_{2,9}$ = 5.53 - 27.62, $p < 0.05$). Further analysis revealed that these three parameters were increased in the MCAO+vehicle group when compared with the sham+vehicle group ($p < 0.05$). Cannabidiol reversed this ischemia-induced effect in the hippocampus compared with the MCAO+vehicle group ($p < 0.05$).

Ischemia-induced microglial activation was also observed on the ipsilateral side of the brain (Fig. 3C). The one-way ANOVA revealed significant differences in the soma area of microglia in the cortex, hippocampus, and thalamus among groups ($F_{2,9}$ = 104.0 - 111.2, $p < 0.05$). Tukey's post hoc test showed that the MCAO+vehicle group exhibited an increase in these parameters in all three brain regions compared with the sham+vehicle group ($p < 0.05$). CBD treatment alleviated this effect compared with the MCAO+vehicle group ($p < 0.05$).

Significant statistical differences were also found in the total branch length in the cortex, hippocampus, and thalamus on the ipsilateral side among groups ($F_{2,9}$ = 16.97 - 39.41, $p < 0.05$; Fig. 3C). A significant increase in the total branch length in the cortex was found in the MCAO+vehicle group compared with the sham+vehicle group ($p < 0.05$). In the hippocampus and thalamus, the opposite ischemia-induced effect was observed, comparing the MCAO+vehicle with sham+vehicle group ($p < 0.05$). Ischemic mice that were treated with CBD exhibited a significant decrease in the total branch length in the cortex, and an increase in this parameter in the hippocampus and thalamus compared with the MCAO+vehicle group ($p < 0.05$). The one-way ANOVA also revealed significant differences in the number of nodes in the cortex and thalamus of the ipsilateral side of the lesion among groups ($F_{2,9}$ = 4.72 - 33.14, $p < 0.05$). Tukey's post hoc test showed that the MCAO+vehicle group exhibited an increase in the number of nodes in the cortex and a decrease in the thalamus compared with the sham+vehicle group ($p < 0.05$). CBD treatment alleviated the ischemia-induced increase in the number of nodes of microglia in the cortex compared with the MCAO+vehicle group ($p < 0.05$).

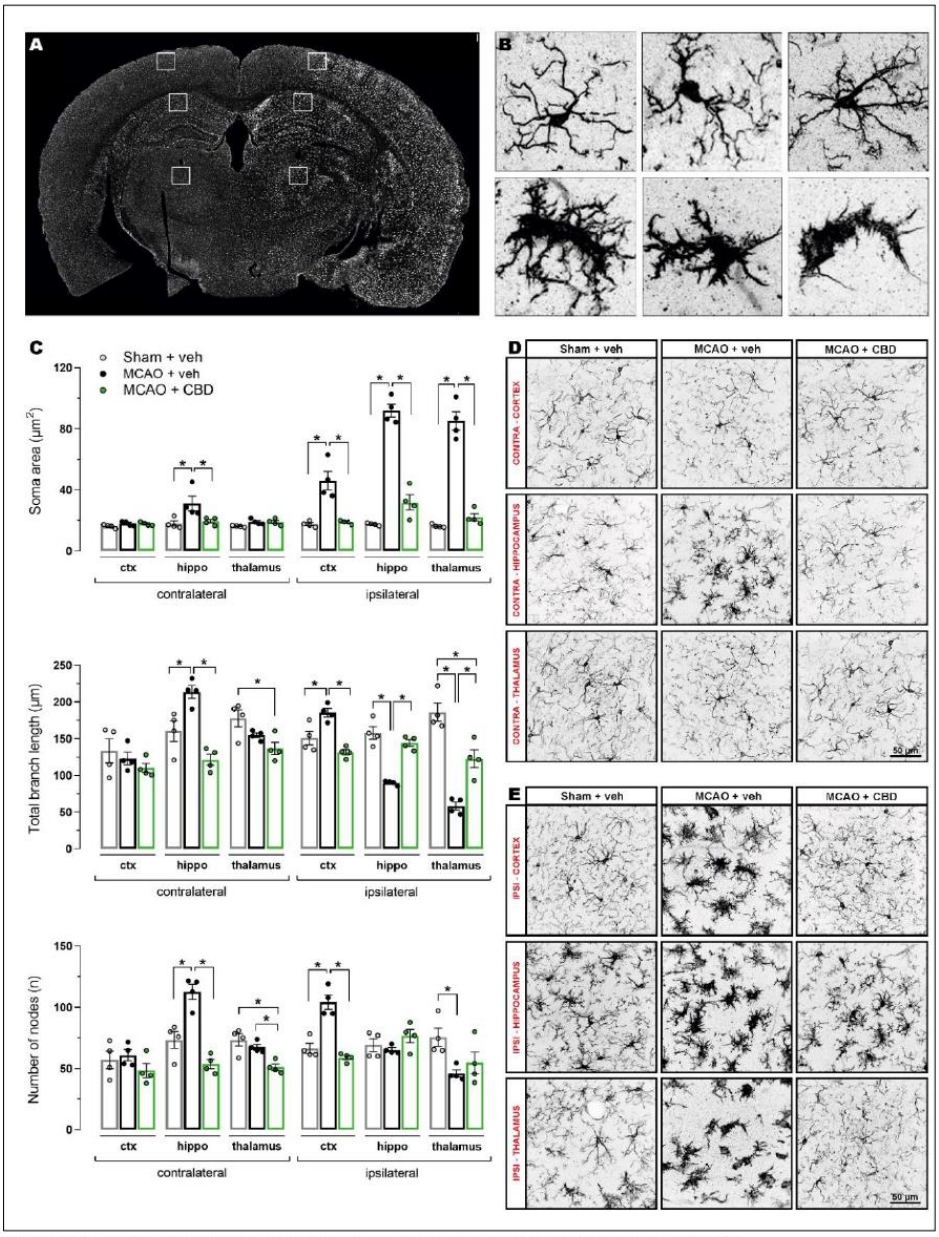


Figure 3 - Cannabidiol reduced microglial activation induced by MCAO
A Representative figure illustrating a coronal brain section showing the cortex, hippocampal CA1 subfield and thalamus where the analysis for microglial morphology was performed. **B** Heterogeneity in the morphology of microglial cells was found among the groups. **C** Soma area, total branch length and number of nodes in the cortex, hippocampus and thalamus on the contra- and ipsilateral side among the groups. **D** Representative photomicrographs of Iba-1-IR cells on the contralateral side among the experimental groups. **E** Representative photomicrographs of Iba-1-IR cells on the ipsilateral side among the experimental groups. Stats: data are shown as individual values (dots) and the means ± SEM (columns and bars) of the experimental groups (n= 4/group). *$p \leq 0.05$. MCAO: middle cerebral artery occlusion.

I*n vivo* 2P-LSM demonstrates a reduced microglial activation in ischemic animals treated with cannabidiol

The microglial reaction during *in vivo* 2P-LSM recording in each group is shown in Fig. 4B. The two-way ANOVA revealed a main effect of group for the number of cells: $F_{2,5} = 6.60$, $p < 0.05$) and soma area ($F_{2,5} = 6.81$, $p < 0.05$). A main effect of time ($F_{3,15} = 7.65$, $p < 0.05$) and a significant group × time interaction ($F_{6,15} = 8.01$, $p < 0.05$) was found for the soma area. At baseline conditions, no between-group differences were found for the number of cells and soma area, indicating no microglial activation before the injury for all experimental groups (Tukey's post hoc test, $p > 0.05$). Comparing the MCAO+vehicle with sham+vehicle group at 30' after MCAO, the ischemic animals presented more cells and increased soma area (Tukey's post hoc test, $p < 0.05$). The ischemia-induced microglial activation was prevented by CBD treatment. The longitudinal analysis indicated that both the number of cells and the soma area significantly decreased at 30' after MCAO in the MCAO+CDB group compared with the MCAO+vehicle group ($p < 0.05$). Similar outcomes for the MCAO+CBD *vs.* MCAO+vehicle groups were found for both parameters at 24 h after MCAO ($p < 0.05$). In the sham-operated and ischemic treated with CBD groups, the number of cells and soma area did not differ between the recorded time points, indicating no microglial activation along time ($p > 0.05$). However, the MCAO+vehicle group presented more cells and increased microglial soma area at 30' and 24 h after MCAO compared to baseline conditions ($p < 0.05$).

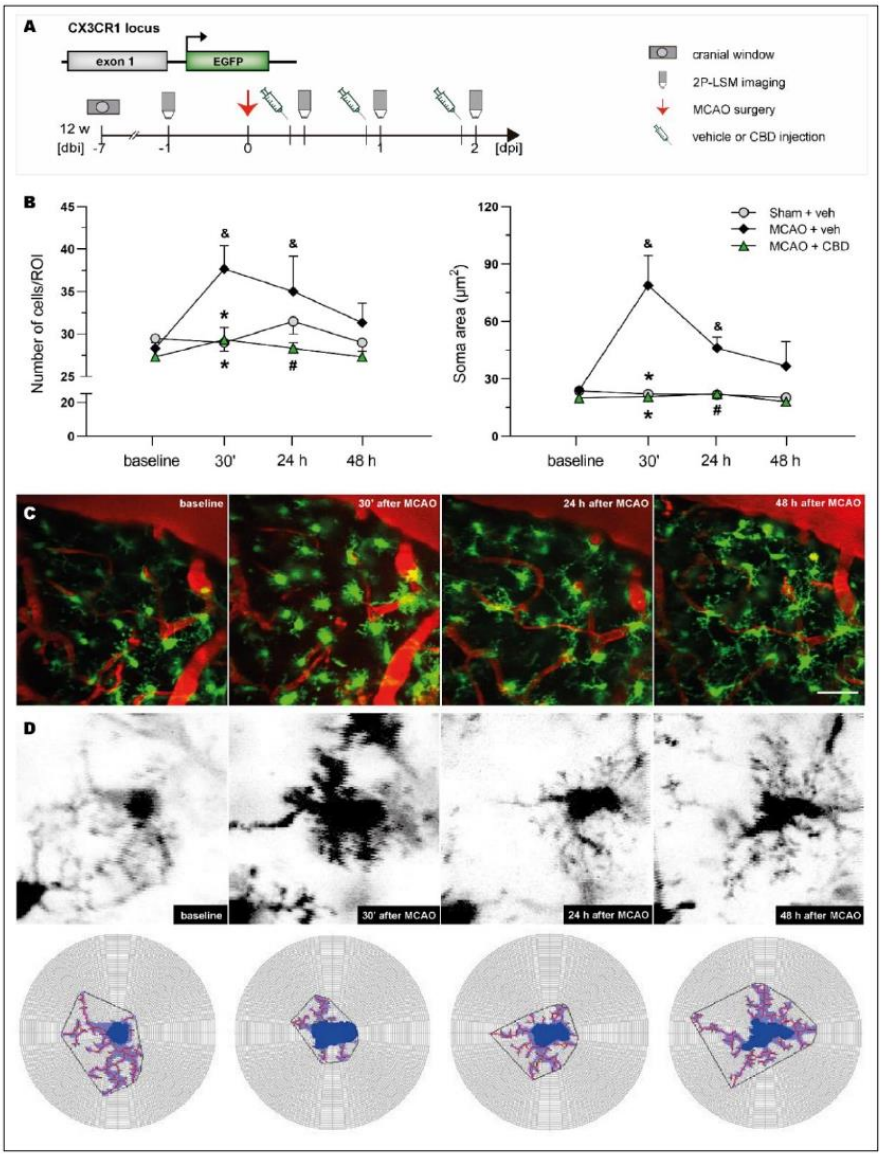


Figure 4 - Cannabidiol prevented microglia activation in the somatosensory cortex of MCAO animals

A *In vivo* microglia imaging was performed after vehicle or cannabidiol injection during baseline, 30 min, 24, and 48 h after reperfusion. **B** Temporal distribution of number and soma area of microglia in the somatosensory cortex on the ipsilateral side among the groups. **C** Representative maximum-intensity projection of one stack of one field of view at recorded time points from MCAO + veh group. **D** Representative photomicrographs of microglia from an ischemic mouse at recorded time points and respective Sholl analysis with MIA software. Stats: the bars represent the means ± SEM of the experimental groups (n= 3/group). $^{*}p \leq 0.05$ compared to MCAO + veh group at 30', $^{\#}p \leq 0.05$ compared to MCAO + veh group at 24 h and $^{\&}p \leq 0.05$ compared to MCAO + veh group at baseline. Scale bar = 50 µm. MCAO: middle cerebral artery occlusion.

Cannabidiol treatment did not change astrocytic calcium signaling *in vivo* after ischemia

Astrocytic calcium signaling was evaluated *in vivo* by visualization of the genetically encoded Ca^{2+} indicator GCaMP3. This experiment aimed, first, to determine whether MCAO changes the amplitude and duration of astrocytic Ca^{2+} signals. Secondly, we examined whether treatment with CBD modulates Ca^{2+} signals in the somatosensory cortex on the ipsilateral side of the brain of ischemic mice.

As shown in Fig. 5A and B, neither amplitude nor duration of Ca^{2+} signals was changed over the days analyzed in the group treated with vehicle (within group comparison, W = -6, W = -6 – -4, $p > 0.05$, respectively). Similarly, no difference in the signal amplitude (within group comparison, W = -6 – 0, $p > 0.05$) and duration (within group comparison, W = -6 – -4, $p > 0.05$) was found over time in the group of ischemic mice treated with CBD. Comparing the signals obtained in the MCAO+vehicle group with those obtained in the MCAO+CBD group, in each time point, no difference in the signal amplitude and duration were found (U = 1 – 4, U = 2 – 4, $p > 0.05$, respectively).

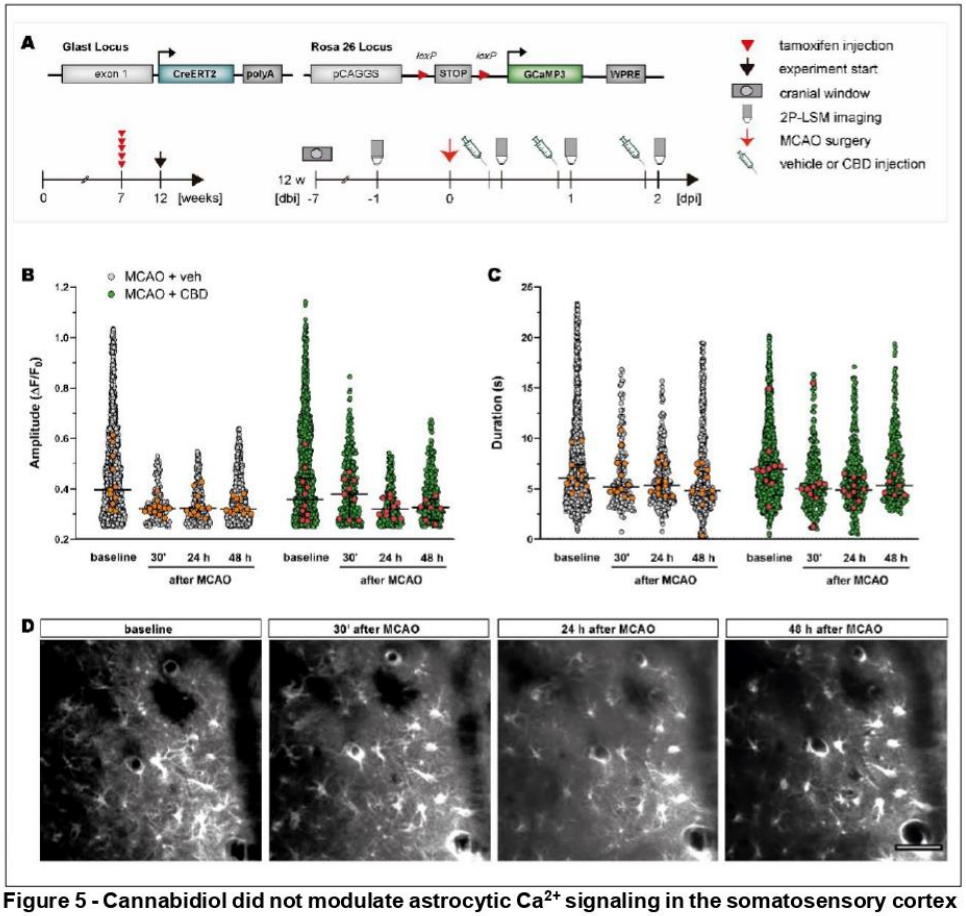


Figure 5 - Cannabidiol did not modulate astrocytic Ca^{2+} signaling in the somatosensory cortex after MCAO

A GCaMP3 expression in astrocytes was induced five weeks before the experiment started. *In vivo* two-photon Ca^{2+} imaging was performed after vehicle or CBD injection during baseline, 30 min, 24, and 48 h after MCAO. **B-C** Amplitude and duration of astrocytic Ca^{2+} signals on the ipsilateral side of the brain after MCAO among groups. **D** Representative single frames of *in vivo* two-photon recordings of one FOV at recorded time points. Scale bars = 50 µm. Stats: data points represent FOV of the experimental groups (*n*= 3/group) and are displayed as median ± IQR. Grey and green data points in the background display single Ca^{2+} signals. CBD: cannabidiol; FOV: field of view; MCAO: middle cerebral artery occlusion.

Astrocytic calcium signals are not affected in the early phase of focal ischemia

To confirm the results obtained in the experiment with GLAST^{GCaMP3} mice *in vivo*, we performed imaging of Ca^{2+} calcium signals *ex vivo*. Firstly, Ca^{2+} signals in the somatosensory cortex from both brain hemispheres were compared. Contra and ipsilateral side did not display differences in the amplitude (U = 12 , p > 0.05) and duration (U = 15, p > 0.05) of astrocytic Ca^{2+} signals (Fig. 6B).

To investigate if Ca^{2+} signals could be modulated by the presence of CBD, the acutely isolated brain slices were incubated, firstly, with perfusion solution and then CBD solution. As shown in Fig. 6E, on the contralateral side, the amplitude and duration of Ca^{2+} signals were not changed after incubation with perfusion solution comparing the time points analyzed (within group comparison, W = 2 – 4, W = 10, p > 0.05, respectively). Similarly, no difference in the signal amplitude and duration was found when the slices were incubated with CBD solution (within group comparison, W = -8 – -6, W = 8 – 10, p > 0.05, respectively). Finally, no difference in the signal amplitude and duration was found over time comparing the signals obtained after incubation with perfusion solution and CBD solution (W = -6 – 8, W = 4 – 8, p > 0.05, respectively).

Fig. 6F shows that, on the ipsilateral side, there was no change in the Ca^{2+} signal's amplitude over time in the presence of perfusion solution (within group comparison, W = -2 – 2, p > 0.05) or CBD solution (within group comparison, W = -8 – -4, p > 0.05). Similarly, no significant difference was found for the duration of signal overtime when the slices were incubated with perfusion solution (within group comparison, W = 10, p > 0.05). Comparing the signal duration after incubation with CBD solution no difference was found among the time points recorded (within group comparison, W = 4 - 10, p > 0.05). No significant difference in the amplitude nor duration of Ca^{2+} signals was found comparing the incubation with perfusion solution and CBD solution (W = -8 – 0, W = -6 – -2, p > 0.05, respectively).

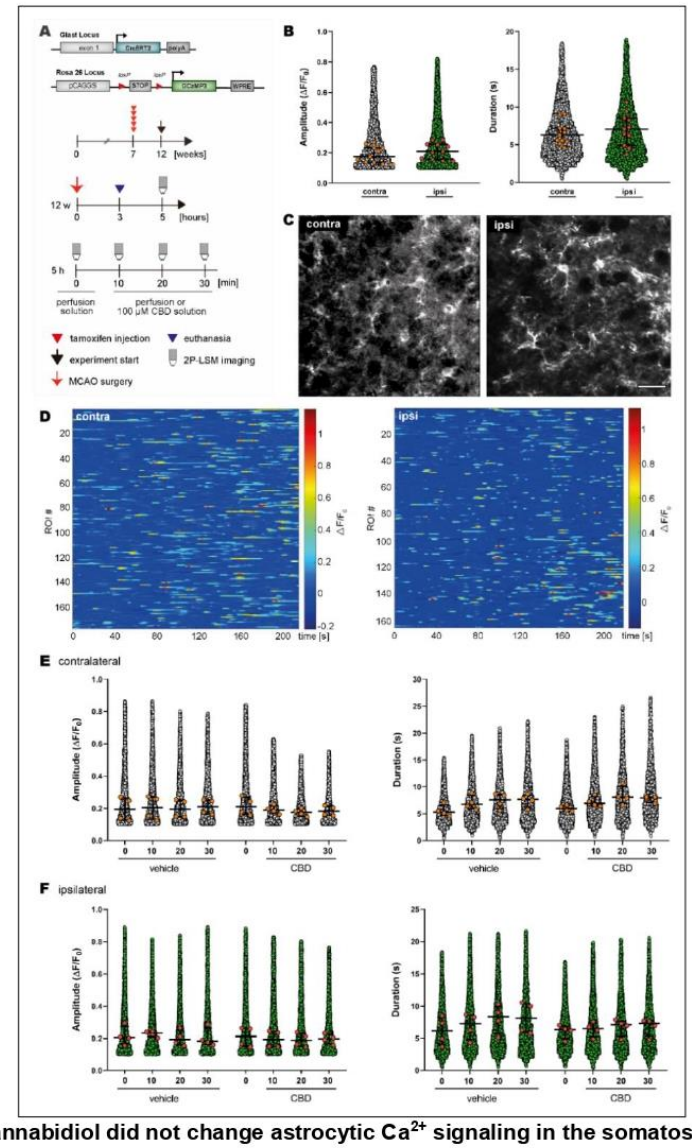


Figure 6 - Cannabidiol did not change astrocytic Ca^{2+} signaling in the somatosensory cortex of acutely isolated slices of ischemic mice

A GCaMP3 expression in astrocytes was induced five weeks before the experiment started. *Ex vivo* two-photon Ca^{2+} imaging was performed 5 h after MCAO at different time points in the presence of perfusion or CBD solution. **B** Amplitude and duration of Ca^{2+} signals in astrocytes on the brain's contra- and ipsilateral side after MCAO. **C** Representative single frames of ex *vivo* two-photon recordings of one FOV on the contra- and ipsilateral side. **D** Representative heatmaps of fluorescence amplitude ($\Delta F/F_0$) from the contra- and ipsilateral side of the brain. **E-F** Amplitude and duration of astrocytic Ca^{2+} signals over time in the presence of perfusion or CBD solution on the brain's contra- and ipsilateral side, respectively. Stats: data points represent FOV of the experimental groups (*n*= 4-5/group) and are displayed as median ± IQR. Grey and green data points in the background display single Ca^{2+} signals. Scale bar = 25 µm. CBD: cannabidiol; FOV: field of view; MCAO: middle cerebral artery occlusion.

DISCUSSION

In the present study, we found that CBD (10 mg/kg, *i.p.* 30 min, 24, and 48 h after ischemia) reverted neurological deficits that was induced by FCI in mice. CBD treatment reduced by 75 % the ischemia-induced neurodegeneration area. CBD also reduced neuroinflammation in ischemic mice, reflected by *in situ* and in *vivo* decreases in reactive microglia.

Impairment of sensorimotor and cognitive performance is a common outcome in rodents with MCAO (Hattori et al., 2000; Truong et al., 2012; Linden et al., 2014). In the present study, the neurological impairments were detected in mice in the subacute phase of injury, *i.e.*, one day after MCAO, using the Bederson score system. CBD attenuated the effects of MCAO, reflected by a decrease in the neurological score, indicating an improvement in neurological function. Consistent with our results, CBD led to an improvement of motor and neurological deficits in mice submitted to 4 h MCAO and treated immediately before and 3 h after the occlusion. These effects of CBD were accompanied by a reduction in infarct size and an increase in cerebral blood flow (Hayakawa et al., 2004; Mishima et al., 2005; Hayakawa et al., 2007). Another study demonstrated that CBD (5 mg/kg, *i.p.*) given once 15 minutes after reperfusion led to the functional and sensorimotor recovery in neonatal rats submitted to MCAO (Ceprián et al., 2017). Similar beneficial effects of CBD on neurological function were also observed in MCAO rats treated with CBD, *i.c.v.*, for 5 days before surgery (Khaksar and Bigdeli, 2017). On the other hand, mice submitted to 4 h MCAO and treated with CBD (3 mg/kg) from day 5 did not show improved neurological score and motor coordination on day 14 after reperfusion. These results indicate that the therapeutic time window is an important feature of CBD treatment in CI, with neuroprotective actions occurring in the subacute phase of ischemia.

MCAO is known to cause a robust reduction in cerebral blood flow and consequent massive neuronal death. Accordingly, we found significant neurodegeneration (detected by FJC staining) in the hippocampus, thalamus and striatum of MCAO animals, which paralleled neurological impairments in those animals. The short-term CBD treatment reduced the extension of neuronal loss induced by MCAO, especially in the thalamus. Other studies have shown reduced neuronal death in the striatum and hippocampus after CBD treatment in mice (Hayakawa et al., 2008) and gerbils (Braida et al., 2003) submitted to focal and global CI, respectively. Moreover, a reduction of necrotic neurons in the cortex was observed in newborn pigs submitted to a hypoxic-ischemic brain injury and treated with CBD (1 mg/kg, *i.v.*) 30 min after the ischemic insult (Pazos et al., 2013). Notably, CBD (10 mg/kg, *i.p.* 30 min before and 3, 24, and 48 h after ischemia) decreased neurodegeneration and normalized

caspase-9 protein levels 21 days after GCI in mice, demonstrating that the neuroprotective action of CBD led to sustained beneficial effects after the injury (Mori et al., 2017).

Neuroinflammation is a critical aspect of stroke, which includes the early activation of microglia and production of cytokines and chemokines (Iadecola and Anrather, 2011; Kim *et al*., 2016; Jayaraj et al., 2019). Depending on injury severity, microglia may present distinct functional and spatiotemporal-dependent profiles in CI, which may protect or contribute to the ischemic injury evolution (Yasuda et al., 2011; Benakis et al., 2015; Fumagalli et al., 2015). We have observed an extensive microglial activation 2 days after MCAO, which extended from the ipsilateral side of the brain, including large areas in the cortex, hippocampus, striatum, and thalamus, to the contralateral hippocampus. In line with our results, activated microglia were abundant in the cortex and thalamus 2 days after MCAO in rats. In the same study, early microglial activation was also observed in regions outside of the MCA territory, such as the contralateral cortex and hippocampus (Morioka et al., 1993). In this sense, microglial reactivity not only indicates imminent ischemic neuronal damage but possibly may reflect subtle changes in neuronal activity outside the MCA territory. Selective neuronal loss, which refers to the death of single neurons with preserved extracellular matrix, *i.e.*, in the ischemic penumbra zone, is consistently associated with microglial activation in the first few days after injury (Yasuda et al., 2011; Emmrich et al., 2015; Park et al., 2018). As both these processes, *i.e.*, selective neuronal death and activation of microglia, affect the salvageable penumbra, hindering functional recovery after reperfusion, they represent potential therapeutic targets in ischemic stroke (Hughes et al., 2010; Baron et al., 2014).

CBD mediated-neuroprotection after experimental CI has generally been related to the modulation of inflammation, including the control of microglia activation, and the toxicity exerted by these cells by producing pro-inflammatory mediators (Pazos et al. 2013; Mohammed et al., 2017; Mori et al., 2017). In mice submitted to 4 h MCAO, repeated CBD treatment from day 1 after ischemia reduced the number of Iba1-positive cells expressing HMGB1 a proinflammatory cytokine that is massively released during the acute phase of ischemic processes (Hayakawa et al., 2009). A reduction in the number of Iba1-positive cells was also reported in neonatal rats submitted to a model of ischemic stroke and treated once with CBD (5 mg/kg, *i.p.*) 15 min after the injury (Ceprián et al., 2017). Our results also demonstrated the anti-inflammatory potential of CBD, as shown by a potent reduction in microglial activation in several cerebral areas 2 days after the onset of ischemia.

To test whether the effects of CBD on microglia could be also detected *in vivo*, we performed time-lapse imaging of microglial activity using the 2P-LSM. Soon after reperfusion, we have

observed that microglia become activated and increased in number in the ischemic penumbra. Supporting our findings, *in vivo* imaging of mice submitted to a cortical microhemorrhage has shown a coordinated pattern of microglia migration, where microglia within 200 µm of the injury migrated toward the lesion, leading to an increased microglia local density (Ahn et al., 2018). Moreover, in mice submitted to MCAO, it was demonstrated that many round-shaped microglia migrated to the peri-infarct area 24 h after the insult (Tanaka et al., 2003). According to our results and also using an *in vivo* approach in mice, Jolivel and coworkers (2015) have shown that all microglia in the penumbra were found associated with blood vessels within 24 h post reperfusion. The authors have also demonstrated that these perivascular microglia started to phagocytose endothelial cells, leading to an activation of the local endothelium and contributing to the degradation of blood vessels with an eventual breakdown of the BBB. Considering these findings, the inhibition of microglial activation within the first day after stroke could stabilize blood vessels in the penumbra, improving the outcomes after ischemia through an increase in the blood flow (Jolivel et al., 2015). Our results show that CBD treatment decreased microglia activation after MCAO, which is consistent with other findings and reinforces the strong anti-inflammatory profile of CBD in ischemic conditions.

Besides microglia, astrocytes play an active role in the neuroinflammation process as well, producing complex and not yet completely understood responses after CI (Liu and Chopp, 2016). In newborn piglets submitted to hypoxia-ischemia, treatment with CBD attenuated the loss of cortical GFAP-positive cells and decreased the levels of S100β in the cerebrospinal fluid (Lafuente et al., 2011). In mice submitted to GCI, CBD (10 mg/kg, *i.p.*) decreased the hippocampal reactivity of astrocytes (GFAP-positive) and total levels of GFAP 21 days after the insult (Mori et al., 2017). In cultured astrocytes, CBD treatment decreased the ß-amyloid-induced release of proinflammatory mediators such as nitric oxide, TNF-α, S100B, and IL-1β. In the same study, CBD treatment (10 mg/kg, *i.p.*) for 15 days diminished the pro-inflammatory response and gliosis triggered by the intrahippocampal injection of ß-amyloid (Esposito et al., 2011).

We tested whether focal ischemia would impact astrocytic Ca^{2+} signaling, a characteristic form of excitability in this cell type, and whether CBD treatment could modulate these signals. Unexpectedly, ischemic mice did not display significant differences in cortical astrocytic Ca^{2+} signals up to 2 days after MCAO. Additionally, no difference in astrocytic Ca^{2+} signals was observed in the somatosensory cortex between contra- and ipsilateral brain sides. Our results are not in line with others showing an increase in astrocytic Ca^{2+} signals after CI. For example, in mice submitted to photothrombotic-induced FCI for 20 min,

in vivo imaging showed an increase in frequency and amplitude of transient Ca^{2+} signals in astrocytes. The authors reported, an increased astrocytic Ca^{2+} signal in the penumbra although it was smaller than that in the core region (Ding et al., 2009). Moreover, permanent MCAO led to an increased astrocytic Ca^{2+} activity in the penumbra of aged mice, while a reduction of astrocytic Ca^{2+} activity was observed in adult mice (Fordsmann et al., 2019). A strong increase of intracellular Ca^{2+} in astrocytes associated with detrimental peri-infarct depolarizations was also reported by Rakers and Petzold (2017) in mice after permanent MCAO. The reason we did not detect a significant difference in astrocytic Ca^{2+} signals after MCAO is unknown. Considering that neurons are more sensitive to ischemia than astrocytes, it is possible that the short occlusion time applied in our study was insufficient to elicit astrocytic responses in the somatosensory cortex after the ischemia. Yet, the cited studies have examined astrocytic Ca^{2+} signaling *in vivo* using a permanent model of FCI or photothrombotic-induced FCI, which display significant differences with the transient FCI model used in this study, mostly for not allowing cerebral reperfusion. Our results involving the absence of astrocytic activation in the somatosensory cortex after MCAO remain elusive and factors other than those mentioned above could be implicated in the absence of astrocytic response found in our study.

One limitation of the present study was that we did not distinguish between resident brain microglia and eventually infiltrating peripheral macrophages. Whether treatment with CBD might differently impact the microglial subpopulations after MCAO remains to be determined.

Overall, the present findings suggest that the functional and structural protective effects of CBD are closely associated with anti-inflammatory action in the subacute phase of ischemia.

REFERENCES

Ahn SJ, Anrather J, Nishimura N, Schaffer CB. (2018). Diverse Inflammatory Response After Cerebral Microbleeds Includes Coordinated Microglial Migration and Proliferation. *Stroke* **49**, 1719-1726.

Baron JC, Yamauchi H, Fujioka M, Endres M. (2014). Selective neuronal loss in ischemic stroke and cerebrovascular disease. *J Cereb Blood Flow Metab* **34**, 2-18.

Basic Kes V, Simundic AM, Nikolac N, Topic E, Demarin V. (2008). Pro-inflammatory and anti-inflammatory cytokines in acute ischemic stroke and their relation to early neurological deficit and stroke outcome. *Clinical biochemistry* **41,** 1330-1334.

Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. (1986). Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke* **17**, 472-476.

Benakis C, Garcia-Bonilla L, Iadecola C, Anrather J. (2015). The role of microglia and myeloid immune cells in acute cerebral ischemia. *Front Cell Neurosci* **8**, 461.

Bieber M, Gronewold J, Scharf AC, Schuhmann MK, Langhauser F, Hopp S, Mencl S, Geuss E, Leinweber J, Guthmann J, Doeppner TR, Kleinschnitz C, Stoll G, Kraft P, Hermann DM. (2019). Validity and Reliability of Neurological Scores in Mice Exposed to Middle Cerebral Artery Occlusion. *Stroke* **50**, 2875-2882.

Braida D, Pegorini S, Arcidiacono MV, Consalez GG, Croci L, Sala M. (2003). Post-ischemic treatment with cannabidiol prevents electroencephalographic flattening, hyperlocomotion, and neuronal injury in gerbils. *Neurosci Lett* **346**, 61-64.

Buskila Y, Farkash S, Hershfinkel M, Amitai Y. (2005). Rapid and reactive nitric oxide production by astrocytes in mouse neocortical slices. *Glia* **52**, 169-176.

Castillo A, Tolón MR, Fernández-Ruiz J, Romero J, Martinez-Orgado J. (2010). The neuroprotective effect of cannabidiol in an in vitro model of newborn hypoxic-ischemic brain damage in mice is mediated by CB(2) and adenosine receptors. *Neurobiol Dis* **37**, 434-440.

Ceprián M, Jiménez-Sánchez L, Vargas C, Barata L, Hind W, Martínez-Orgado J. (2017). Cannabidiol reduces brain damage and improves functional recovery in a neonatal rat model of arterial ischemic stroke. *Neuropharmacology* **116**, 151-159.

Cupido A, Catalin B, Steffens H & Kirchhoff F. (2014). Surgical procedures to study microglial motility in the brain and in the spinal cord by in vivo two-photon laser-scanning microcopy. Book: Laser Scanning Microscopy and Quantitative Image Analysis of Neuronal Tissue, *Springer Protocols*, 37-50.

Ding S, Wang T, Cui W, Haydon PG. (2009). Photothrombosis ischemia stimulates a sustained astrocytic Ca2+ signaling in vivo. *Glia* **57**, 767-776.

Emmrich JV, Ejaz S, Neher JJ, Williamson DJ, Baron JC. (2015). Regional distribution of selective neuronal loss and microglial activation across the MCA territory after transient focal ischemia: quantitative versus semiquantitative systematic immunohistochemical assessment. *J Cereb Blood Flow Metab* **35**, 20-27.

Esposito G, Scuderi C, Valenza M, Togna GI, Latina V, De Filippis D, Cipriano M, Carratù MR, Iuvone T, Steardo L. (2011). Cannabidiol reduces Aβ-induced neuroinflammation and promotes hippocampal neurogenesis through PPARγ involvement. *PLoS One* **6**, e28668.

Fordsmann JC, Murmu RP, Cai C, Brazhe A, Thomsen KJ, Zambach SA, Lønstrup M, Lind BL, Lauritzen M. (2019). Spontaneous astrocytic Ca^{2+} activity abounds in electrically suppressed ischemic penumbra of aged mice. *Glia* **67**, 37-52.

Franklin KBJ, Paxinos C. (1997). The mouse brain in stereotaxic coordinates. 6th Edition, Academic Press, San Diego.

Fumagalli S, Perego C, Pischiutta F, Zanier ER, De Simoni MG. (2015). The ischemic environment drives microglia and macrophage function. *Front Neurol* **6**, 81.

GBD 2019 Stroke Collaborators. (2021). Global, regional, and national burden of stroke and its risk factors, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet Neurol* **2**0, 795-820.

Hattori K, Lee H, Hurn PD, Crain BJ, Traystman RJ, DeVries AC. (2000). Cognitive deficits after focal cerebral ischemia in mice. *Stroke* **31**, 1939-1944.

Hayakawa K, Irie K, Sano K, Watanabe T, Higuchi S, Enoki M, Nakano T, Harada K, Ishikane S, Ikeda T, Fujioka M, Orito K, Iwasaki K, Mishima K, Fujiwara M. (2009). Therapeutic time window of cannabidiol treatment on delayed ischemic damage via high-mobility group box1-inhibiting mechanism. *Biol Pharm Bull* **32**, 1538-1544.

Hayakawa K, Mishima K, Abe K, Hasebe N, Takamatsu F, Yasuda H, Ikeda T, Inui K, Egashira N, Iwasaki K, Fujiwara M. (2004). Cannabidiol prevents infarction via the non-CB1 cannabinoid receptor mechanism. *Neuroreport* **15**, 2381-2385.

Hayakawa K, Mishima K, Irie K, Hazekawa M, Mishima S, Fujioka M, Orito, K, Egashira N, Katsurabayashi S, Takasaki K, Iwasaki K, Fujiwara M. (2008). Cannabidiol prevents a post-ischemic injury progressively induced by cerebral ischemia via a high-mobility group box1-inhibiting mechanism. *Neuropharmacology* **55**, 1280-1286.

Hayakawa K, Mishima K, Nozako M, Hazekawa M, Irie K, Fujioka M, Orito K, Abe K, Hasebe N, Egashira N, Iwasaki K, & Fujiwara M. (2007). Delayed treatment with cannabidiol has a cerebroprotective action via a cannabinoid receptor-independent myeloperoxidase-inhibiting mechanism. *J Neurochem* **102**, 1488-1496.

Hughes JL, Beech JS, Jones PS, Wang D, Menon DK, Baron JC. (2010). Mapping selective neuronal loss and microglial activation in the salvaged neocortical penumbra in the rat. *Neuroimage* **49**, 19-31.

Iadecola C, Anrather J. (2011). The immunology of stroke: from mechanisms to translation. *Nat Med* **17**, 796-808.

Jahn HM, Kasakow CV, Helfer A, Michely J, Verkhratsky A, Maurer HH, Scheller A & Kirchhoff F. (2018). Refined protocols of tamoxifen injection for inducible DNA recombination in mouse astroglia. *Sci Rep* **8**, 5913.

Jayaraj RL, Azimullah S, Beiram R, Jalal FY, Rosenberg GA. (2019). Neuroinflammation: friend and foe for ischemic stroke. *J Neuroinflammation* **16**, 142.

Jolivel V, Bicker F, Binamé F, Ploen R, Keller S, Gollan R, Jurek B, Birkenstock J, Poisa-Beiro L, Bruttger J, Opitz V, Thal SC, Waisman A, Bäuerle T, Schäfer MK, Zipp F, Schmidt MHH. (2015). Perivascular microglia promote blood vessel disintegration in the ischemic penumbra. *Acta Neuropathol* **129**, 279-295.

Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, Littman DR. (2000). Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol* **20**, 4106-4114.

Khaksar S, Bigdeli MR. (2017). Anti-excitotoxic effects of cannabidiol are partly mediated by enhancement of NCX2 and NCX3 expression in animal model of cerebral ischemia. *Eur J Pharmacol* **794**, 270-279.

Kim JY, Park J, Chang JY, Kim SH, Lee JE. (2016). Inflammation after Ischemic Stroke: The Role of Leukocytes and Glial Cells. *Exp Neurobiol* **25**, 241-251.

Kitamura Y, Takata K, Inden M, Tsuchiya D, Yanagisawa D, Nakata J, Taniguchi T. (2004). Intracerebroventricular injection of microglia protects against focal brain ischemia. *J Pharmacol Sci* **94**, 203-206.

Kitamura Y, Yanagisawa D, Inden M, Takata K, Tsuchiya D, Kawasaki T, Taniguchi T, Shimohama S. (2005). Recovery of focal brain ischemia-induced behavioral dysfunction by intracerebroventricular injection of microglia. *J Pharmacol Sci* **97**, 289-293.

Koizumi J-i, Yoshida Y, Nakazawa T, Ooneda G. (1986). Experimental studies of ischemic brain edema. A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. *Jpn J Stroke* **8**, 1-8.

Lafuente H, Alvarez FJ, Pazos MR, Alvarez A, Rey-Santano MC, Mielgo V, Murgia-Esteve X, Hilario E, Martinez-Orgado J. (2011). Cannabidiol reduces brain damage and improves functional recovery after acute hypoxia-ischemia in newborn pigs. *Pediatr Res* **70**, 272-277.

Lalancette-Hébert M, Gowing G, Simard A, Weng YC, Kriz J. (2007). Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. *J Neurosci* **27**, 2596-2605.

Linden J, Fassotte L, Tirelli E, Plumier JC, Ferrara A. (2014). Assessment of behavioral flexibility after middle cerebral artery occlusion in mice. *Behav Brain Res* **258**, 127-137.

Liu Z, Chopp M. (2016). Astrocytes, therapeutic targets for neuroprotection and neurorestoration in ischemic stroke. *Prog Neurobiol* **144**, 103-120.

Mishima K, Hayakawa K, Abe K, Ikeda T, Egashira N, Iwasaki K, Fujiwara M. (2005). Cannabidiol prevents cerebral infarction via a serotonergic 5-hydroxytryptamine 1A receptor-dependent mechanism. *Stroke* **36**, 1077-1082.

Mohammed N, Ceprián M, Jimenez L, Pazos MR, Martínez-Orgado J. (2017). Neuroprotective effects of Cannabidiol in hypoxic ischemic insult. The therapeutic window in newborn mice. *CNS Neurol Disord Targets* **16**, 102-108.

Mori MA, Meyer E, Soares LM, Milani H, Guimarães FS, de Oliveira RMW. (2017). Cannabidiol reduces neuroinflammation and promotes neuroplasticity and functional recovery after brain ischemia. *Prog Neuropsychopharmacol Biol Psychiatry* **75**, 94-105.

Mori T, Tanaka K, Buffo A, Wurst W, Kühn R & Götz M. (2006). Inducible gene deletion in astroglia and radial glia – a valuable tool for functional and lineage analysis. *Glia* **54**, 21-34.

Morioka T, Kalehua AN, Streit WJ. (1993). Characterization of microglial reaction after middle cerebral artery occlusion in rat brain. *J Comp Neurol* **327**, 123-132.

Nakai J, Ohkura M, Imoto K. (2001). A high signal-to-noise Ca(2+) probe composed of a single green fluorescent protein. *Nat Biotechnol* **19**, 137-141.

Niwa H, Yamamura K, Miyazaki J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-199.

Park JH, Cho JH, Ahn JH, Choi SY, Lee TK, Lee JC, Shin BN, Hong S, Jeon YH, Kim YM, Hwang IK, Lee YJ, Won MH, Kang IJ. (2018). Neuronal loss and gliosis in the rat striatum subjected to 15 and 30 minutes of middle cerebral artery occlusion. *Metab Brain Dis* **33**, 775-784.

Paukert M, Agarwal A, Cha J, Doze VA, Kang JU, Bergles DE. (2014). Norepinephrine controls astroglial responsiveness to local circuit activity. *Neuron* **82**, 1263-1270.

Pazos MR, Mohammed N, Lafuente H, Santos M, Martínez-Pinilla E, Moreno E, Valdizan E, Romero J, Pazos A, Franco R, Hillard CJ, Alvarez FJ, Martínez-Orgado J. (2013). Mechanisms of Cannabidiol neuroprotection in hypoxic-ischemic newborn pigs: role of 5HT(1A) and CB2 receptors. *Neuropharmacology* **71**, 282-291.

Pologruto TA, Sabatini BL, Svoboda K. (2003). ScanImage: flexible software for operating laser scanning microscopes. *Biomedical engineering online* **2**, 13.

Rakers C, Petzold GC. (2017). Astrocytic calcium release mediates peri-infarct depolarizations in a rodent stroke model. *J Clin Invest* **127**, 511-516.

Schiavon AP, Soares LM, Bonato JM, Milani H, Guimarães FS, Weffort de Oliveira RM. (2014). Protective effects of cannabidiol against hippocampal cell death and cognitive impairment induced by bilateral common carotid artery occlusion in mice. *Neurotox Res* **26**, 307-316.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch, S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682.

Schmued LC, Hopkins KJ. (2000). Fluoro-Jade: novel fluorochromes for detecting toxicant-induced neuronal degeneration. *Toxicol Pathol* **28**, 91-99.

Szalay G, Martinecz B, Lénárt N, Környei Z, Orsolits B, Judák L, Császár E, Fekete R, West BL, Katona G, Rózsa B, Dénes Á. (2016). Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. *Nat Commun* **7**, 11499.

Tanaka R, Komine-Kobayashi M, Mochizuki H, Yamada M, Furuya T, Migita M, Shimada T, Mizuno Y, Urabe T. (2003). Migration of enhanced green fluorescent protein expressing bone marrow-derived microglia/macrophage into the mouse brain following permanent focal ischemia. *Neuroscience* **117**, 531-539.

Truong DT, Venna VR, McCullough LD, Fitch RH. (2012). Deficits in auditory, cognitive, and motor processing following reversible middle cerebral artery occlusion in mice. *Exp Neurol* **238**, 114-121.

Yasuda Y, Shimoda T, Uno K, Tateishi N, Furuya S, Tsuchihashi Y, Kawai Y, Naruse S, Fujita S. (2011). Temporal and sequential changes of glial cells and cytokine expression during neuronal degeneration after transient global ischemia in rats. *J Neuroinflammation* **8**, 70.

10. ACKNOWLEDGMENTS

Firstly, I would like to express my gratitude to my supervisor Prof. Rúbia Monteiro Weffort de Oliveira for her supervision and for always being supportive to my plans and let me experience science from Ribeirão Preto to Homburg. Thank you for the calmness and patience in the moments I lost it.

My deep appreciation goes to Dr. Frank Kirchhoff, my second supervisor, for giving me the opportunity to study in his fantastic lab. I really appreciated being part of the Glia lab.

I owe my gratitude to Prof. Alline Cristina de Campos who took over the supervision during my stays at the University of São Paulo. Thank you for your help and for sharing your expertise and time with me.

In addition, I want to thank Prof. Humberto Milani. Thank you for being an example of integrity and for your insightful comments on my projects.

Furthermore, I am deeply thankful to Dr. Anja Scheller. Thank you for answering my naïve questions and for your friendship beyond the lab work. Thank you for explaining me how the vibratome works Oo. I enjoyed our time in Ribeirão Preto a lot, specially our never ending churrasco by Gui's place.

My special thanks go to all current and former members of the Cerebral Ischemia lab in Brazil. Lígia Mendes Soares, Emilene Fiuza Ferreira, Jacqueline Godinho, Bianca Andretto de Mattos, Daniela Velasquez, and Gabriella Cândido thank you for the partnership and for accompanying me through the ups and downs of PhD life.

My exceptional gratitude goes to my special friend Jéssica Mendes Bonato, for her overwhelming support and efforts. Thank you for your always positive and encouraging vibrations. It was a great pleasure working with you!

I owe an extra big hug to my friends Marco Aurélio Mori and Rafael Pazinatto Aguiar. Thank you so much for our time together in the lab and for sharing pain and joy with me throughout these years.

My special thanks go to all current and former members of the Kirchhoff lab in Germany. A special thank you goes to Fei (Wenhui Huang), Mariza Bortolanza, Laura Stopper, Gebhard Stopper, Laura Caudal, Elisa Damo, Cai (Xianshu Bai),

Lipao Fang, Qilin Guo, Qing Liu, Na Zhao, Michael Schweigmann, Emeline Buttigieg and Daniel Schauenburg. Thank you all for the support and the great working atmosphere.

A special thank goes to Frank Rhode. Thank you so much for your help in the lab, for picking me up when I just arrived in Homburg and for offering me a warm meal after the long trip.

I am particularly deeply thankful to my Jungs Phillip Rieder and Davide Gobbo. We had hilarious moments together, we traveled, cried, laughed, partied, loved and hated science together. Thank you so much for taking care of me after my surgery and for your friendship. It means a lot to me.

My deepest gratitude goes to my partner Guilherme, for always standing by my side. Thank you for always accepting and supporting my plans. You give me the strength to achieve my dreams.

My most special thanks go to my family. Thank you for your unconditional support, and for being proud of me. Without you absolutely none of this would be possible.

Meus agradecimentos mais especiais vão para minha família. Obrigada pelo seu suporte incondicional e por se orgulharem de mim. Sem vocês absolutamente nada disso seria possível.