Neuroprotective mechanisms of CB2 cannabinoid receptors and PPAR-α in hypoxia/ischemia-induced brain damage

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"What is real? How do you define real? If you're talking about what you can feel, what you can smell, what you can taste and see, then real is simply electrical signals interpreted by your brain"

Morpheus from Matrix (Film)

"Every man can be if he tries, sculptor of his own brain"

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Abstract

Cerebral ischemia is a significant cause of mortality and severe neurologic disability, affecting millions of persons each year. Therefore, great efforts are being made to identify the mechanisms involved in the pathophysiology of this condition, and to develop new therapies to counteract its deleterious consequences. In this thesis, we have developed a hypoxia-ischemia (HI) model in adult mice to study the neuroprotective mechanisms of CB2 cannabinoid receptors (CB2R), and the potential therapeutic effects of the new PPAR-α agonist, octadecylpropyl sulfamide (SUL). First, we determined the behavioural and cognitive alterations induced by HI in CB2R knockout (KO) mice and wild-type (WT) littermates, as well as, the cellular and molecular alterations associated with brain injury. Second, we evaluated the effects of SUL on the behavioural and cognitive alterations induced by HI in C57BL/6J adult mice, and studied the associated neurodegeneration processes, and changes in gene expression related to the neuroinflammation/endocannabinoid signalling systems in the brain. Our findings revealed that the loss of CB2R produces more extensive brain lesions, persistent behavioural and motor coordination deficits and exacerbates gliosis in the hippocampus. Moreover, the expression of the microglial pro-inflammatory factors HIF-1α and TIM-3 was increased in the brain of CB2R KO as compared to WT mice. These findings suggest that CB2R confer neuroprotection following HI insult through the modulation of microglial pro-inflammatory factors acting as a defensive mechanism to reduce subsequent behavioural alterations. In addition, we demonstrated that SUL administered immediately after HI reduced neuronal loss, countered the overexpression of microglia/astroglia, as well as, the associated motor and memory deficits by acting on PPAR-α. Furthermore, SUL normalized the overexpression of genes related to neuroinflammation/endocannabinoid signalling systems in the brain of lesioned mice. These results revealed that the potent and stable PPAR-α agonist, SUL exhibits neuroprotective properties, and could be a potential pharmacological treatment to prevent the impact of hypoxia on brain function in adults.

Resumen

La isquemia cerebral es una causa importante de mortalidad y discapacidad neurológica grave, que afecta a millones de personas cada año. Por lo tanto, se están realizando grandes esfuerzos para identificar los mecanismos implicados en la fisiopatología de esta enfermedad y desarrollar nuevas terapias para evitar sus consecuencias perjudiciales. En la presente tesis, hemos desarrollado un modelo de hipoxia-isquemia (HI) en ratones adultos para estudiar los mecanismos neuroprotectores de los receptores cannabinoides CB2 (CB2R) y los posibles efectos terapéuticos del nuevo agonista de PPAR-α, octadecilpropil sulfamida (SUL). En primer lugar, determinamos las alteraciones conductuales y cognitivas inducidas por HI en ratones knock-out CB2R (KO) y controles (WT), así como las alteraciones celulares y moleculares asociadas con la lesión cerebral. En segundo lugar, evaluamos los efectos de SUL sobre las alteraciones conductuales y cognitivas inducidas por HI y estudiamos los procesos de neurodegeneración y los cambios en la expresión de genes relacionados con los sistemas de neuroinflamación/endocannabinoides en el cerebro en ratones adultos C57BL/6J. Nuestros hallazgos revelaron que la pérdida de CB2R produce lesiones cerebrales más extensas, déficits conductuales y de coordinación motora persistentes y agravamiento de la gliosis en el hipocampo. Además, la expresión de los factores proinflamatorios microgliales, HIF-1α y TIM-3 se incrementó en el cerebro de CB2R KO en comparación con los ratones WT. Estos hallazgos sugieren que CB2R confiere neuroprotección después de HI a través de la modulación de factores proinflamatorios microgliales, actuando como un mecanismo de defensa para reducir las alteraciones conductuales posteriores. Por otra parte, demostramos que la administración de SUL inmediatamente después de HI redujo la pérdida neuronal, contrarrestó la sobreexpresión de microglia/astroglia, así como los déficits motores y de memoria actuando a través de los receptores PPAR-α. Además, SUL normalizó la sobreexpresión de genes relacionados con los sistemas de señalización de neuroinflamación/endocannabinoides en el cerebro de ratones lesionados. Estos resultados revelan que el potente y estable agonista de PPAR-α, SUL exhibe propiedades neuroprotectoras, y podría ser un potencial tratamiento farmacológico para prevenir el impacto de la hipoxia en la función cerebral en adultos.

List of abbreviations

2-AG	2-arachidonoylglycerol
2VO	two-vessel occlusion
4VO	four-vessel occlusion model
ACEA	arachidonyl-2-chloroethylamide
AEA	N-arachidonoylethanolamide (anandamide)
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCCAO	bilateral common carotid artery occlusion
BDNF	brain-derived neurotrophic factor
CB1R	cannabinoid receptors type 1
CB1R KO	cannabinoid receptors type 1 knockout
CB2R	the cannabinoid receptors type 2
CB2R KO	cannabinoid receptors type 2 knockout
CBD	cannabidiol
CBF	cerebral blood flow
CNS	central nervous system
COX-2	cyclooxygenase 2
DAG	diacylglycerol
DAGL	diacylglycerol lipase enzyme
ECS	endocannabinoid system
ER	endoplasmic reticulum
ES	elaidylsulfamide
FAAH	fatty acid amine hydrolase
GFAP	glial fibrillary acidic protein
GPR119	G-protein-coupled receptor 119
GPR55	G-protein-coupled receptor 55
HI	hypoxia-ischemia
HIE	hypoxic-ischemic encephalopathy
HIF	hypoxia-inducible factor
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IGF-1	insulin growth factor
iGluR	ionotropic glutamate receptors
IL-	Interleukin-
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
MAGL	monoacylglycerol lipase

MAGs	monoacylglycerols
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MHC-II	major histocompatibility complex II
NADA	N-arachidonoyldopamine
NAEs	N-acylethanolamides
NAGly	N-arachidonoylglycine
NAPE	N-arachidonoyl-phosphatidylethanolamine
NAPE-PLD	N-arachidonoyl-phosphatidylethanolamine-phospholipase D
NAT	N-acyltransferase
NF-κB	nuclear factor-κB
NMDA	N-methyl-D-aspartic acid
NO	nitric oxide
NOS	nitric oxide synthase
ODA	Cis-9,10-octadecanoamide (oleamide)
OEA	oleoylethanolamide
OGD	oxygen-glucose deprivation
OLHHA	N-[1 -(3,4-dihydroxyphenyl)propan-2-yl]oleamide
PcomA	posterior communicating artery
PEA	palmitoylethanolamide
PLC	phospholipase C
PPAR	peroxisome proliferator-activated receptor
PPAR-α	peroxisome proliferator-activated receptor alpha
ROS	reactive oxygen species
SEA	stearoylethanolamide
SODs	superoxide dismutases
SUL	octadecylpropyl sulfamide
TC	trans-caryophyllene
TGFβ	transforming growth factor β
THC	Δ9- tetrahydrocannabinol
TIM-3	t-cell immunoglobulin and mucin-domain containing-3
TNF	tumour necrosis factor
TRP	transient receptor potential
TRPV1	transient receptor potential cation channel subfamily V member 1
VCAM-1	vascular cell adhesion molecule-1
VDCC	voltage-dependent calcium channels
WT	wild-type

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INTRODUCTION

1. Overview of cerebral ischemia

The human brain is an essential organ in the body with high energy requirements mainly due to the necessity for active maintenance of ion gradients (i.e., Na^+/K^+ ATPase) in excitable neurons. Neuronal discharge and release of neurotransmitters and neuropeptides require large amounts of energy (Attwell and Laughlin, 2001). These characteristics make the brain intolerant to any reduction in cerebral blood flow (CBF), even for brief periods. Therefore, any interruption of blood flow to support this energy demand will lead to a lack of blood oxygenation or cerebral hypoxia and decreased supply of glucose, which will result in a cerebral ischemia, a condition that represents an 85% of all strokes (Durukan and Tatlisumak, 2007).

Cerebral ischemia is a significant cause of severe neurologic disabilities in children and adults, and the resulting consequences will depend on the depth and duration of ischemia, its location within the brain and the size and distribution of the blood vessels involved (Flynn et al., 2008). It is usually categorized into focal and global ischemia. Focal ischemia is caused by a highly localized reduction in CBF in a specific area of the brain and can be caused by a thrombosis or embolism. In contrast, global ischemia is caused by an extensive reduction in CBF to all or most parts of the brain.

Global ischemia is associated with hypoxic-ischemic encephalopathy (HIE), especially in newborn due to birth asphyxia. However, in adults and children it can be caused by cardiac arrest and by severe cerebral hypoperfusion. In this sense, HIE is a neurovascular and neurometabolic syndrome, caused by a deficiency of supply of oxygen and glucose in brain, a situation that can be designated as hypoxia-ischemia (HI). The consequences of HI in brain are variable, mild or moderate HI insults usually result in watershed zone infarcts (Arbelaez et al., 1999). In addition, in mild or moderate hypoxia of short duration the prognosis is favourable with recovery of function. However, in severe insult, when the circulatory arrest is not restored quickly, this circumstance could lead to an unfavourable prognosis since brain injury could be irreversible (Vintila et al., 2010).

1.1. Epidemiology

Cerebral ischemia is a frequent neurologic disease that presents an elevated risk of mortality, with few available treatments. Moreover, the strong association between cerebral ischemia and cardiac arrest underpins the importance of the study of this condition since the incidence of both diseases is very high. Cardiovascular disease is the leading cause of death worldwide, with a mortality rate of 30%, and in most cases, it is caused by cardiac arrest. Meanwhile, stroke is the second leading cause, representing 11% of all deaths, and 5% is caused only by ischemic stroke (World Health Organization, 2016). In Spain, the incidence of death after cardiovascular disease is

around 105 cases per 100.000 inhabitants, whereas the incidence of death after stroke represents 87 cases per 100.000 inhabitants (Instituto Nacional de Estadistica, 2015). Importantly, the burden of morbidity and healthcare costs of stroke are very high due to the neurological consequences, where motor and cognitive impairments are frequent. In this context, stroke is among the top three causes of healthcare costs for the aging adults (Eurostat - European Commission, 2009).

1.2. Cerebral ischemia following cardiac arrest

Despite advances in therapeutic approaches over the past several decades, brain injury continues to be the leading cause of morbidity and mortality after cardiac arrest. Interestingly, the aetiology of cardiac arrest is different between adults and children. In children, the most common cause of cardiac arrest is cardiorespiratory failure or asphyxia. In contrast, in adults, the causes are attributed to ventricular fibrillation or ventricular tachycardia secondary to coronary artery disease (Vintila et al., 2010). The first action, in terms of treatment to recover the ischemic tissue and to improve functional outcome after cardiac arrest, is to restore the blood flow through reperfusion treatment. Reperfusion is characterized by initial reduction of blood supply followed by subsequent restoration of blood flow, providing oxygenation to the tissue. Paradoxically, while quick reperfusion of ischemic brain tissue is critical for restoring normal function, often it may also exacerbate cerebral injury (Nour et al., 2012). In this context, the reperfusion injury

may be caused by extended reactions from the primary insult and often deteriorates the brain metabolism by increasing oxidative stress damage.

Others cardiovascular abnormalities are also the cause of brain injury since they lead to significant periods of low blood pressure (hypotension) and consequently to insufficient oxygenation of tissues. Moreover, cardiovascular abnormalities may slow blood flow to a point where blood begins to clot, leading to a blood clot or embolus formation that can travel to the brain triggering a cerebral ischemia. At the present, the only approved treatment for this condition is the use of thrombolytic agents that targets the thrombus within the blood vessel such as recombinant tissue plasminogen activator (rtPA). This agent, however, is only effective during the first 3-4.5 hours and it is imperative that the patient is correctly diagnosed with having a thrombotic and not a haemorrhagic stroke, since in the latter case thrombolytic treatment would be deleterious (Hacke et al., 2008; Wahlgren et al., 2008).

2. Pathophysiology of hypoxic-ischemic injury

In last years, significant progress has been made in the knowledge of the initiating factors associated with HI. The pathophysiology of cerebral ischemia is very complex, and a wide variety of cellular processes have been shown to be integral to neuronal injury. Under normal conditions, CBF is maintained at a constant rate of 50 mL/100 g/min. If CBF drops below 10 mL/100 g/min, as occurs during HI episodes, energy failure occurs, neuronal depolarization (efflux of K^+ and influx of Ca^{2+}) follows rapidly and neurons undergo death within a short time unless CBF is restored (Patel, 2008). It is well known that HI does not affect all brain structures in the same way. Certain tissues in the brain are more likely to be injured and to be injured earlier than others, a concept known as selective vulnerability. Thus, neurons are more vulnerable than glia and vascular cells, and when exposed to HI quickly become dysfunctional and die (Lipton, 1999). In this context, a higher vulnerability is found in neurons of the hippocampus, cortex and striatum, as well the Purkinje cells of the cerebellum (Patel, 2008; Vintila et al., 2010).

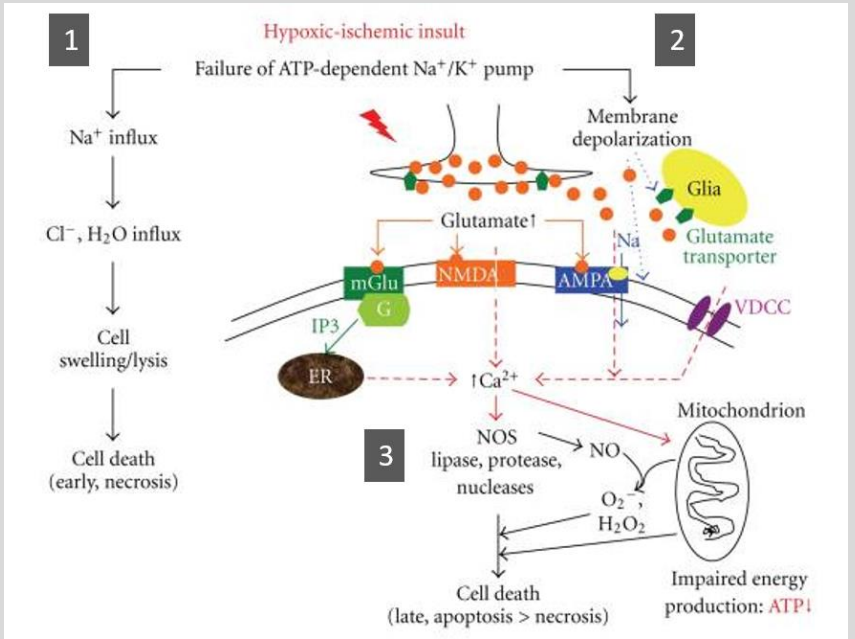


Figure 1. Pathophysiology of hypoxic-ischemic injury. Primary ischemic brain injury: (1) In severe insult, the depletion of energy compounds (ATP) result in an influx of Na^+, Cl^-, and water with consequent cell death (necrosis). (2) In less severe insult, it causes membrane depolarization, that if persistent, results in excessive presynaptic glutamate release, reversal of glutamate transport in glia and neural terminals, and activation of NMDA and Ca^{2+} permeable AMPA receptor channels, and other voltage-dependent Ca^{2+} channels, which leads to high intracellular Ca^{2+}. Secondary ischemic brain injury: (3) After reperfusion, cytosolic Ca^{2+} initiates a cascade of events that include the enhancement of generation of free radicals and NOS, disruption of mitochondrial function, degradation of cellular lipids by activation of phospholipases and proteases, and deterioration of DNA by activation of nucleases leading to a delayed cell death, mainly apoptosis. AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; ATP: adenosine triphosphate; ER: endoplasmic reticulum; mGlu: metabotropic glutamate; NMDA: N-methyl-D-aspartic acid; NOS: nitric oxide synthase; VDCC: voltage-dependent calcium channels (Lai and Yang, 2011).

HI and reperfusion trigger a complex cascade of pathological events, comprising many different pathways that lead to irreversible tissue injury (Lo et al., 2003). HI pathophysiology is characterized by two distinct phases. The primary injury caused by immediate cessation of cerebral blood flow during cardiac arrest and the

secondary injury that takes place in the hours and days following the initial cardiac arrest and reperfusion (Sekhon et al., 2017) (Fig. 1). It is evident that clarifying the pathophysiology of HI is imperative to identify new therapeutic targets.

2.1. Primary ischemic brain injury

The primary or acute injury phase begins immediately after the ischemic insult, during which time the decrease in blood flow disrupts ionic homeostasis. In this phase, the critical damaging processes include cellular energy failure, excitotoxicity and intracellular Ca^{2+} accumulation (Huang and Castillo, 2008). Reduced oxygen supply and decreased energy caused by HI leads to a halt in oxidative phosphorylation in the brain. Although this condition triggers anaerobic metabolism to compensate for lack of oxygen, it is inefficient in maintaining cellular energy demand. This situation results in further depletion of energy compounds, such as phosphocreatine and adenosine triphosphate (ATP), and leads to the production of lactic acid and subsequent tissue acidosis. The depletion of these energy stores leads to the failure of the ATP-dependent Na^+/K^+ pump in the outer cell membrane. In a severe HI insult, this situation leads to a rapid influx and accumulation of intracellular Na^+ with subsequent influx of Cl^-, producing an osmotic gradient which drives water into the cell. The consequence is a rapid cell swelling and lysis with membrane degeneration (necrotic cell death) (Dirnagl et al., 1999). In a less severe insult, the failure of the ATP-dependent Na^+/K^+ pump leads to a cell

depolarization with an increase in intracellular Ca^{2+}. In neurons, the increase in intracellular Ca^{2+} leads to the release of glutamate from axon terminals into the synaptic clef. Subsequently, glutamate binds to receptors in the dendritic cell membrane of adjacent neurons causing influx of Ca^{2+} into the cell. This situation results in a series of successive depolarizations and release of glutamate from neuron to neuron. Synaptic function is rapidly disrupted resulting in a failure by glia to take up glutamate, an energy-dependent process (Lai and Yang, 2011), which contributes to an extracellular accumulation of glutamate to excitotoxic levels. The excess of glutamate sustains the continuous excitatory depolarization with influx of Ca^{2+} into the cell due to activation of NMDA and Ca^{2+} permeable AMPA receptor channels and other voltage-dependent Ca^{2+} channels. The excessive stimulation of glutamate receptor/ion channel which increases the intracellular Ca^{2+} is termed excitotoxicity. This process induces cascades of intracellular events that lead to secondary energy failure and delayed cell death (Fig. 2) (Lai and Yang, 2011).

2.2. The delayed or secondary ischemic brain injury

After reperfusion, the excitotoxic process may lead to delayed cell death, apoptosis, by several routes. This phase starts 6 to 15 hours post-insult and can extend for days (Pulsinelli et al., 1982; Lipton, 1999). In this secondary phase, the high intracellular Ca^{2+} leads to an enhancement of generation of free radicals and nitric oxide (NO) by increase of nitric oxide synthase (NOS), disruption of

mitochondrial function, degradation of cellular lipids by activation of phospholipases and proteases, and deterioration of DNA by activation of nucleases (Lai and Yang, 2011). During this phase, inflammatory process also occurs with the activation of pro-inflammatory cytokines and resident macrophages, such as interleukin-1 (IL-1), and tumour necrosis factor-α (TNF-α) (Allan and Rothwell, 2001) (Fig. 2). Importantly, microglia plays an important role in neuroinflammation since it is the main factor responsible for cytokines activation in response to HI injury (Ashton and Glass, 2007).

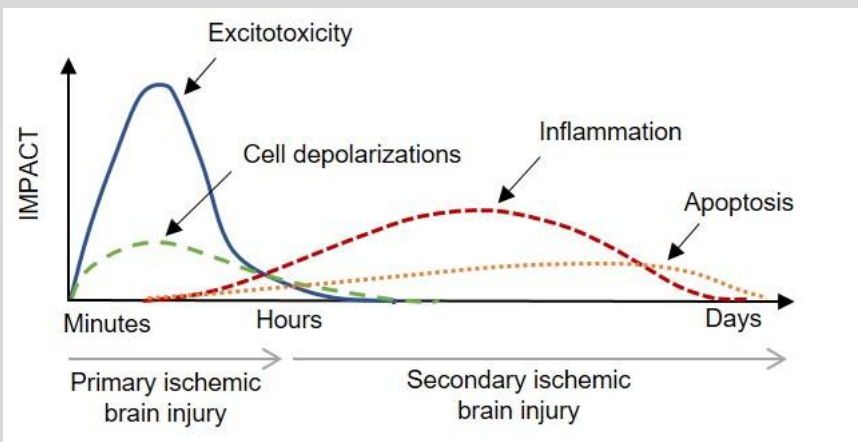


Figure 2. Putative cascade of damaging events following hypoxia-ischemia. Following HI, excitotoxic mechanisms can damage neurons and glia. Excitotoxicity induces many events that can further contribute to the tissue death such as repetitive depolarizations (primary ischemic brain injury), and the more-delayed mechanisms of inflammation and programmed cell death, apoptosis (secondary ischemic brain injury). X-axis: evolution of the cascade over time; Y-axis: impact of each damaging event on outcome. (Adapted from Dirnagl et al., 1999).

As reviewed above, HI induces a cascade of molecular and cellular events in the brain that may result in temporary or permanent changes in both the anatomy as well as the physiology of the

affected structures. Many of these changes are pathological consequences of the injury that leads to a damaging outcome. Traditionally, the primary phase represents a therapeutic time window, where any intervention would be most effective. However, this time window is of a short duration for therapeutic action what has impeded effective treatment for most patients. On the other hand, adaptive processes may occur in the delayed injury stage, resulting in some restoration of function due to the reduction of pathophysiological events, an issue that represents a possible window of opportunity for therapeutic intervention (Nudo, 2013). Thus, current research is also focusing on this phase with the aim to develop new therapeutic treatments to reduce or prevent the pathophysiological effects of HI that lead to cell death.

2.3. Mechanisms of cell death following hypoxic-ischemic injury

Cell death is a fundamental biological process highly relevant to normal histogenesis, to the maintaining of healthy tissues, and to the pathogenesis of tissue damage and disease (Martin et al., 1998). There are two essential mechanisms of cell death after exposure to cerebral ischemia: necrosis and apoptosis. Both types of cellular death differ in their morphological and biochemical markers, since the mechanisms that produce them are different (Majno and Joris, 1995). However, it is not clear why a particular type of cellular death occurs in specific cells. It may be associated with the nature of the insult, the cell type, age, and probably the state of the cell at

the time of the insult (Martin et al., 1998). In this context, neurons can display both necrosis and apoptosis in response to an ischemic insult (Lipton, 1999).

Necrosis is the predominant type of cell death in the primary injury phase following a severe HI, affecting large groups of neighbouring neurons as well as glia (Dirnagl et al., 1999). It is a passive phenomenon that depends on the biochemical alterations produced by the energy deficit, and by the activation of different enzymes which destroy cellular components. Because of the energy depletion, a failure of the membrane function occurs, that leads to the loss of the ionic gradient and passage of water to the cytoplasm and to the organelles interior, producing the consequent cellular oedema. Finally, the lysis of the membranes takes place, and the loss of the differentiation of the cellular compartments. Cell lysis and release of cytoplasmic content cause an inflammatory response reaction with infiltration of neutrophil leukocytes and macrophages, aggravating tissue damage (Alonso de Leciñana et al., 1995; Martin et al., 1998; Elmore, 2007). Interestingly, some regions of the central nervous system (CNS) are more vulnerable to this process than others. For instance, the so-called *selective necrosis* occurs in layers III-V of the neocortex, the CA1 and DG hippocampal regions, and the striatum (Martin et al., 1998; Lawrence and Inder, 2008). This circumstance is associated with the higher expression levels in these areas of receptors to excitotoxic neurotransmitters, such as glutamate and aspartate.

In contrast to necrosis, apoptosis is the predominant type of cell death in a less severe HI, and occurs in the secondary injury phase (Dirnagl et al., 1999). Apoptosis is a highly controlled and organized programmed cell death which is managed by cysteine proteases called caspases, and requires active protein synthesis as well as intact mitochondria (Martin et al., 1998). Apoptosis occurs in a sequence of morphological events characterized by nuclear and cytoplasmic condensation, DNA fragmentation, blebbing of the plasma membrane and subsequent formation of apoptotic bodies and phagocytosis of these elements (Rich et al., 1999; Elmore, 2007). This process plays a critical role in healthy tissues, acting as a defence mechanism to remove damaged, unwanted or potentially harmful cells, while it can induce further damage following cerebral ischemia. In this context, many of the conditions that develop during cerebral ischemia can induce apoptosis in one or more cell types. These conditions comprise increased intracellular Ca^{2+} concentrations, free radical generation, NOS production, reduced mitochondrial activity, degradation of cellular lipids by activation of phospholipases and proteases, and deterioration of DNA by activation of nucleases (Lipton, 1999; Lai and Yang, 2011). Notably, the inflammatory processes resulting in further cell damage as seen following DNA fragmentation through necrosis is not seen following apoptotic cell death (Elmore, 2007). Moreover, apoptosis is considered to be responsible for a considerable proportion of cell loss in the brain after global ischemia (Zhu et al., 2005). It also occurs in selectively vulnerable areas of CNS such as the

hippocampus, the basal ganglia, and the cortical watershed areas (Martin et al., 1998).

2.4. Neuroinflammation following hypoxic-ischemic insult

The inflammation that is triggered in the CNS, also called neuroinflammation, is mainly a protective mechanism for isolating the damaged area, destroying the affected cells and repairing the extracellular matrix (Correale and Villa, 2004). However, there is an increasing amount of evidence suggesting that inflammation induced by cerebral ischemia is a significant contributing factor to the pathogenic process. As previously reviewed, a cascade of cellular and biochemical responses to the initial HI insult can lead to secondary neuronal injury after reoxygenation. One of the crucial mechanisms of secondary neuronal injury after global HI is inflammation.

Although almost all the attention of the ischemic process has been focused on neurons, it is well known that cerebral ischemia induces inflammation processes through the activation of glial cells, not only in the ischemic core but also in places far from it. Glial cells in the CNS are divided into macroglia (astrocytes and oligodendrocytes) and microglia (Kaas, 2016). Brain injury leads to glial activation, also named gliosis, where structural and physiological changes of astrocytes and microglia occur in response to the traumatic lesions in the nervous system.

In general terms, cerebral ischemia leads to inflammatory cell infiltrates from nonspecific immunologic reactions, migration of peripheral leukocytes into the brain, and activation of microglia. The release of inflammatory cytokines such as IL-1 and TNF-α by ischemic neurons and glia leads to the generation of adhesion molecules (intercellular adhesion molecule-1: ICAM-1; selectins; integrins) in the cerebral vasculature. This process results in breakdown of the blood-brain barrier (BBB), and consequently in oedema formation (Wang et al., 2007; Bhalala et al., 2015).

2.4.1. The role of microglia

Microglia, the resident immune cells in the brain, play a vital role in both physiological and pathological conditions. They are differentially distributed along the brain parenchyma. In the normal state, microglia represent 5% of the cells in the cortex and corpus callosum, and 12% of the cells in the substantia nigra of the rodents (Lawson et al., 1990). In the human brain, microglia represent only 0.5% in grey matter areas of cerebellum and cerebral cortex, and 16.6% in the pons and medulla (Mittelbronn et al., 2001). Moreover, they appear to be more abundant in white matter than grey matter (Mittelbronn et al., 2001). Microglia have an important role in many regulatory processes in the brain that are essential for tissue development, maintenance of the neural environment, response to injury, and for promoting repair (Subramaniam and Federoff, 2017). Moreover, the interactions with neurons, astrocytes, migrating T-cells, and the BBB can alter microglial

function (González et al., 2014). Studies have suggested that the faculty of microglia to prevent or aggravate neuronal damage is highly dependent on balanced crosstalk between microglia and neurons. For instance, the addition of apoptotic neuronal cells to microglial cultures decrease the secretion of pro-inflammatory cytokines and promote the release of other molecules such as transforming growth factor β (TGFβ), prostaglandin-E2, and IL-10, suggesting that phagocytosis of apoptotic neurons specifically stimulates microglial cells to acquire an anti-inflammatory phenotype (De Simone et al., 2003). Interestingly, healthy neurons control the amplitude and duration of microglial activation, whereas following brain injury, the lack of specific communication between damaged neurons and microglia could lead to a shift of microglia to a hyperactivated state. This state allows microglia to escape neuronal control that results in a persistent inflammation, and consequently, in an exacerbation of the neuropathology (Polazzi and Contestabile, 2002). In addition, several studies have also suggested that activated microglia may contribute to injury in models of cerebral ischemia. In spontaneously hypertensive rats subjected to ischemia, repetitive hyperbaric oxygen treatment reduced the infarct volume by suppressing microglia activation (Günther et al., 2005). Another study showed that minocycline, a tetracycline family antibiotic, provided significant protection against brain ischemia in gerbil by inhibiting microglial activation and proliferation (Yrjänheikki et al., 1998).

Under physiological conditions, microglia remain in a downregulated state, carrying out their role of sampling and inspection of the local environment. In this state, resting microglia (also called M0 phenotype) adopt the characteristic ramified morphological appearance (small soma and highly ramified and elongated branches), with little or no movement, and serve the role of immune surveillance and host defence (Liu and Hong, 2003). This resting state is partly maintained by signals conveyed by neuronal and astrocyte-derived factors. In order to maintain this state, microglia, secrete several cytokines and neurotrophic factors like insulin growth factor (IGF-1), brain-derived neurotrophic factor (BDNF), colony stimulating factor 1 receptor (CSF1R), signal regulatory protein CD172 (SIRP1A), chemokine CX3CL1 and CD200R (Franco and Fernández-Suárez, 2015; Subramaniam and Federoff, 2017) (Fig. 3).

Microglia are sensitive to even minor disturbances in CNS homeostasis and become readily activated during most neuropathological conditions, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, AIDS, dementia, trauma and stroke (Liu and Hong, 2003; Franco and Fernández-Suárez, 2015). With the activation of microglia, their phenotype changes from a resting state (M0) to two different activation pathways, the classical phenotype known as M1, or the alternative known as M2 (Kofler and Wiley, 2011; Franco and Fernández-Suárez, 2015). Fundamentally, the two states of microglia, the M1 and M2 phenotypes are associated with injury and homeostasis, respectively.

When classically activated, M1 microglia are characterized by pro-inflammatory functions that serve as the first line of defence. The M1 state can be induced by TNF and by the endotoxin lipopolysaccharide (LPS), an endotoxin present in the cell membranes of Gram-negative bacteria, or by other factors related to infections such as interferon-γ (IFN-γ) (Franco and Fernández-Suárez, 2015). Similarly, an inflammatory response also occurs after exposure to trauma, ischemia-reperfusion injury or chemical injury. In this state, microglia adopt an activated amoeboid appearance, and release different pro-inflammatory cytokines such as IL-1β, 6, 12, 17, 18, 23, TNF-α, IFN-γ and NO, and chemokines such as CCL2. Also, the M1 microglial activation state presents phenotypic markers such as inducible NO synthase (iNOS), cyclooxygenase 2 (COX-2), major histocompatibility complex II (MHC-II), CD86, and other substances including reactive oxygen species (ROS) and reactive nitrogen species (Woodroofe et al., 1991; Franco and Fernández-Suárez, 2015; Waisman et al., 2015; Wang et al., 2015; Subramaniam and Federoff, 2017). All these processes will contribute to ischemic brain injury (Jin et al., 2010) (Fig. 3).

On the other hand, the alternative M2 microglial activation state is associated with immunoregulation, inhibition of inflammation, and repair and injury resolution (Kofler and Wiley, 2011; Franco and Fernández-Suárez, 2015). In this state, microglia is morphologically characterized by enlarged cell bodies (Subramaniam and Federoff, 2017). Among the M2 microglial phenotypes, the M2a state can be induced by IL-4 or IL-13 and contributes to tissue repair and

regeneration by expressing anti-inflammatory and immune-regulatory molecules such as Fizz1 and IGF-1. In contrast, the M2b phenotype can be induced by LPS or immune complexes and is involved in the recruitment of regulatory T cells also expressing anti-inflammatory molecules such as MHC-II and IL-10. And finally, the M2c phenotype can be induced by IL-10 or glucocorticoid hormones (GlcH), and it is associated with anti-inflammatory and healing effects, expressing anti-inflammatory molecules such as CXCL13 (Franco and Fernández-Suárez, 2015) (Fig. 3).

Currently, studies have been focused on investigating molecules with the capacity to polarize microglia from an M1 towards an M2 phenotype for the treatment of neuropathological conditions such as cerebral ischemia, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and traumatic brain injury (Franco and Fernández-Suárez, 2015).

In conclusion, microglia are considered the key innate immunity cells of the CNS that are specifically adapted to detect any danger in the brain and react differently with a classical or alternative reparative response. Understanding the role of microglia in the neuroinflammatory process has open new vistas in the development of neurological therapies, where the modulation of microglia can be proposed as a means of modulating neurological disease.

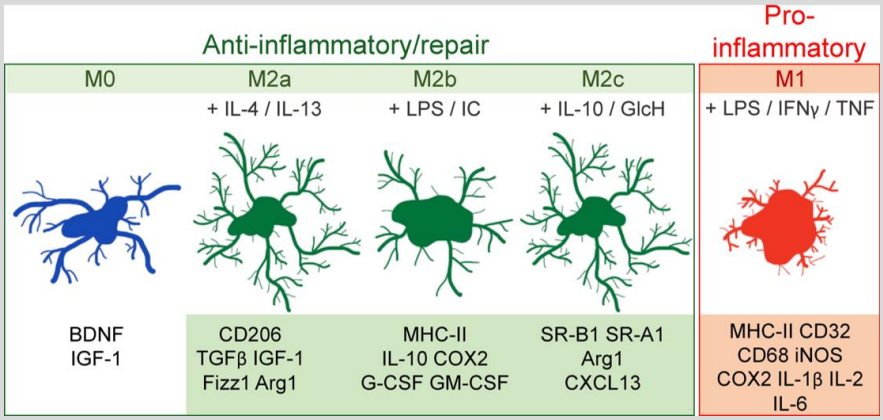


Figure 3. Activated states of microglia. The molecules that induce the classical (M1) or the alternative (M2) activation pathways are indicated for each phenotype. Molecules expressed by the different polarized cells, M1, M2a, M2b and M2c, are indicated in the coloured boxes. In normal physiological conditions microglia acquire the surveillance phenotype (M0) to maintain all CNS cell types including neurons. To maintain this surveillance state, microglia secrete several neurotrophic factors like IGF-1 and BDNF. Upon classical activation when triggered by LPS, IFN-γ, or TNF, microglia acquire M1 pro-inflammatory phenotype leading to neurotoxicity by secreting several pro-inflammatory cytokines and markers such as IL-1β, IL-2, IL-6, MHC-II and COX-2. When activated alternatively by IL-4, 13, 10, IC, microglia attain M2 anti-inflammatory state prompting neuroprotection through secretion of variety of anti-inflammatory molecules such as Fizz1, IGF-1, IL-10 and CXCL13. Arg1, arginase 1; BDNF, brain-derived neurotrophic factor; COX-2, cyclooxygenase 2; CXCL13, chemokine (C-X-C motif) ligand 13; G-CSF, granulocyte colony-stimulating factor; GlcH, glucocorticoid hormones; GM-CSF, granulocyte-macrophage colony-stimulating factor; IC, immune complexes; IFN-γ, interferon gamma;, IGF-1, insulin growth factor; IL, interleukin; iOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MHC-II, major histocompatibility complex type II receptor; SR-A1, scavenger receptor class A1; SR-B1, scavenger receptor class B1; TGFβ, transforming growth factor beta; TNF, tumor necrosis factor (Adapted from Franco and Fernández-Suárez, 2015).

2.4.2. The role of astrocytes

Increasing evidence shows that astrocytes also play an important role in brain inflammation following HI, both in lesion

establishment and tissue repair. Astrocytes are the most abundant glial cells in the CNS, their proportion varies by region and ranges from 20% to 40% of all glia (Verkhratsky and Butt, 2013). Astrocytes are divided into two main subtypes: protoplasmic or fibrous, according to the differences in their cellular morphologies and anatomical locations. Protoplasmic astrocytes are found throughout all grey matter and, display a morphology characterized by stem branches that result in many finely branching processes in a uniform globoid distribution. In contrast, fibrous astrocytes are found throughout all white matter and display a morphology consisting of many long fiber-like processes (Sofroniew and Vinters, 2010).

In physiological condition, astrocytes develop multiple functions that are essential for the normal functioning of neurons, such as glutamate uptake, K^+ and H^+ buffering, water transport and metabolic and trophic support (Swanson et al., 2004). Astrocytes serve as the primary glycogen storage site in the CNS, and they perform metabolite cleansing. Together with endothelial cells, they form the BBB providing selective protection to the brain. Moreover, they can promote neuronal survival during ischemic insult, and in the post-injury period, they can promote neurite outgrowth and regeneration. These factors underline the cross-talk that exists between astrocyte and neurons.

Like microglia, astrocytes can become activated in response to all forms of CNS injury or disease through a process known as

astrogliosis, which is characterized by altered gene expression, hypertrophy, and proliferation (Ridet et al., 1997). In this state, proliferating astrocytes adopt a gemistocytic appearance consisting of a swollen cytoplasm, eccentric nucleus, and increased intracellular content of intermediate filament proteins, especially glial fibrillary acidic protein (GFAP). Increased GFAP is considered a characteristic of astrogliosis and found to be a sensitive biomarker for CNS injury (Sofroniew and Vinters, 2010; Zhang et al., 2010).

An important component of brain oedema after cerebral ischemia is astrocyte swelling. It occurs within 30 minutes of the onset of ischaemia. This swelling is significant since water redistribution into the astrocytes may reduce the total extracellular volume by 50 to 75%. The initiation of astrocyte swelling after ischaemia may be due to the increased levels of glutamate and K^+, which activate astrocyte-mediated uptake and trigger cellular osmotic overload. All this process activates water influx through aquaporins that are highly expressed in astrocyte membranes, and lead to the dramatic increase in their volume (Verkhratsky and Butt, 2013).

Astrogliosis may contribute to the neuroinflammatory process by limiting the area of lesions through glial scar formation, and releasing local mediators (Minghetti, 2005; Sofroniew, 2009). In this context, activated astrocytes release a variety of immune mediators such as cytokines, chemokines, and growth factors (TNF-α, IL-1β, IL-1, 6, 10, 15, IFN-γ, and TGFβ), that may exert either neuroprotective or neurotoxic effects (Farina et al., 2007). The

neurotoxic effects of activated astrocytes may lead to several consequences such as demyelination, leukocyte infiltration and rupture of BBB (Sofroniew and Vinters, 2010). On the other hand, in cases of severe lesions, the formation of scars induced by activated astrocytes is an indicator of many brain injuries and pathologies. Glial scars act as cell migration barriers around the areas with intense inflammation to restrict the spread of inflammatory cells into adjacent healthy tissue. This process contributes to protecting neuronal function and promote tissue repair (Sofroniew and Vinters, 2010). An increase in astrocyte activity, characterized by increased cell proliferation (cell hypertrophy) and upregulation of the astrocyte intermediate filaments (GFAP and vimentin), was shown to accompany different types of brain insult, such as focal and global ischemia (Kindy et al., 1992; Cheung et al., 1999; Olson and McKeon, 2004; Robel et al., 2011; Koh et al., 2015). Moreover, increased GFAP immunoreactivity in the CA1 hippocampal region after brain ischemia has been associated with the extent and maturation of neuronal necrosis (Petito and Halaby, 1993; Stoll et al., 1998). Therefore, decreasing astrogliosis may rescue neuronal damage and may serve as a valuable therapeutic strategy.

Interestingly, astrocytes appear to participate in the suppression of microglial activation. As previously reviewed, activated microglial cells release high levels of pro-inflammatory cytokines and toxic ROS which may negatively impact neuronal survival. In this context, many *in vitro* studies have demonstrated that the presence

of astrocytes attenuates microglial activation in response to various pro-inflammatory stimuli (Hailer et al., 2001; Min et al., 2006). The mechanisms by which astrocyte-derived factors participate in this process have not yet been fully elucidated, but studies suggest that TGFβ may be a contributing element (Vincent et al., 1997). In fact, several studies revealed the neuroprotective effect of TGFβ in experimental models of ischemia or excitotoxicity (Prehn et al., 1993; Henrich-Noack et al., 1996; Ruocco et al., 1999).

In brief, understanding the different roles of activated astrocytes, and determining the molecular signalling events that mediate these specific actions, may lead to new therapeutic strategies that could improve the outcome after HI insult, by promoting certain astrocyte activities and inhibiting others.

3. Animal models of cerebral ischemia

Cerebral ischemia is a significant cause of mortality and severe neurologic disability, affecting millions of persons each year. Thus, it is crucial to make every effort to identify the mechanisms that cause tissue damage, and to develop new therapies to counteract the deleterious consequences of this disease. Several animal models of cerebral ischemia have been designed specifically to identify risk factors, to determine neural repair processes, and to assess novel drugs as neuroprotective, neuroregenerative and anti-inflammatory therapies (Carmichael, 2005). Today, reliable animal models for cerebral ischemia are available in a variety of species, with rats being the most commonly used animals. However, since the mouse is the best-characterized animal in genetics and molecular biology, an increasing number of studies are carried out in this species. Moreover, there are many relevant transgenic mouse models available that can be used to search for new targets to treat cerebral ischemia.

The numerous existing models of cerebral ischemia in rodents reflect the complexity of the disease, and the need for different paradigms to optimize the study of therapeutic approaches. In general, the different models can be classified into global and focal ischemia models (Table 1). As previously reviewed, global ischemia

is characterized by a reduction of CBF throughout most or all of the brain, whereas in focal ischemia the reduction in blood flow occurs in a specific brain region (Traystman, 2003). In this section, some of these rodent models will be briefly summarized.

Table 1. Animal models of ischemia

Focal cerebral ischemia	Proximal MCA occlusion Distal MCA occlusion Thromboembolic MCA occlusion Photothrombosis model
Global cerebral ischemia	Four-vessel occlusion Two-vessel occlusion BCCAO and controlled ventilation Cardiac arrest and resuscitation Hypoxia-ischemia

MCA: middle cerebral artery; BCCAO: bilateral common carotid artery

3.1. Focal cerebral ischemia models

Most models of focal cerebral ischemia comprise the occlusion of one major cerebral blood vessel, the middle cerebral artery (MCA). Middle cerebral artery occlusion (MCAO) leads to a reduction of CBF in the striatum and cortex. However the degree and distribution of blood flow reduction varies as a function of the duration and the site of occlusion along the MCA, and the volume of collateral blood flow into the MCA territory (Traystman, 2003). The MCAO models have been often used because of their relevance to human thromboembolic stroke (Hossmann, 1998). In this sense, there are several techniques in rodents that reproduce the human stroke caused by the occlusion of the MCA: mechanical

occlusion of either the proximal MCA (large vessel occlusion) or distal MCA (small vessel occlusion), thrombotic occlusion either via injection of synthetic spheres, blood clots or thrombin into the MCA or occlusion by photo-thrombosis after intravenous injection of Rose Bengal (Bacigaluppi et al., 2010). Moreover, the occlusion of MCA could be permanent or transient followed by tissue reperfusion. It is important to point out that these variations in the MCAO models could lead to different pathophysiological outcomes (Hossmann, 2012). For instance, in the permanent MCAO, primary core injury expands in peripheral brain regions and reaches its maximum at around 3 hours after occlusion. In contrast, in the transient MCAO model, primary core injury may recover, and a secondary delayed injury develops after an interval of up to 12 hours, a very long therapeutic window without clinical relevance, since it is not observed in human stroke (Hossmann, 2012; Fluri et al., 2015).

Proximal MCAO was originally described by Kozuimi and colleagues (Koizumi et al., 1986), and subsequently modified to reduce subarachnoid haemorrhage and premature reperfusion (Belayev et al., 1996). This model is usually induced by the insertion of a silicon-coated nylon suture into the internal carotid artery up to the circle of Willis to occlude the MCA at its origin. The filament suture can be left in place transiently for a variable duration of time (between 30-120 min) before its removal and subsequent reperfusion. Or, in the case of permanent MCAO, the

filament suture is placed permanently and no reperfusion is allowed (Bacigaluppi et al., 2010).

On the other hand, the distal MCAO, originally developed by Tamura and colleagues (1981), is performed through a craniotomy to directly expose and manipulate superficial MCA branches (Tamura et al., 1981). In this case, the MCA can be also occluded either transiently or permanently distal, using a clip or by electrocoagulation, in the origin of the lenticulostriate branches, which is a group of small arteries that arise at the commencement of the MCA (Iadecola et al., 1997). Compared to proximal MCAO, the damage with distal occlusions is more restricted because the occluded vessel is superficial.

In the thromboembolic models, embolic strokes can be induced through injection into the internal carotid artery of synthetic spheres of different sizes: macrospheres (300-400 μm diameter), which produce large infarcts like those produced by the permanent MCAO, but avoiding hypothalamic damage and hyperthermia (Gerriets et al., 2003); or microspheres (less than 50 μm), which produce smaller and multifocal infarcts restrict to the brain regions of the microsphere-injected hemisphere (Miyake et al., 1993). There are models where autologous blood clots are injected directly into the internal carotid artery (Kilic et al., 1998); or where the generation of an autologous thrombus is induced by a direct *in situ* microinjection of purified murine thrombin into the MCA, causing a very reproducible ischemic injury (Orset et al., 2007).

The photothrombosis model involves the use of a photochemical reaction *in vivo*. In this model, the injection of Rose Bengal, a potent photosensitive dye, followed by irradiation of several branches of the distal MCA with beams from an argon dye laser, induces a very localized stroke (Watson et al., 1985). The advantages of this model are that only a small craniotomy is necessary, and that the dura stays intact. The disadvantage is that a microvascular injury can be induced by the photochemical reaction (Traystman, 2003).

3.2. Global cerebral ischemia models

Global ischemia can be induced by different approaches, and many of them have been carried out in gerbils. These small mammals of the order Rodentia, have been often used for global ischemia studies due to species-specific incomplete circle of Willis (Levine and Payan, 1966). Most of gerbil strains lack the posterior communicating artery (PcomA) that connects the anterior and posterior circulation of the brain. Thus, global ischemia may be induced in these rodents by simple bilateral common carotid artery occlusion (BCCAO) (Murakami et al., 1998), usually resulting in death of the hippocampal CA1 neurons (Giuliani et al., 2006). In contrast, the use of gerbils in BCCAO might present an inconvenient in strains where the circle of Willis is not totally incomplete, allowing the communicating arteries give collateral supply to the forebrain (Seal et al., 2006). An alternative would be to abstain from using these strains known to have a high prevalence

of posterior communicating arteries and use the ones in which the posterior communicating arteries are absent.

In rats or mice, global ischemia cannot be induced by BCCAO alone because of the existence of the PcomA (Levine and Sohn, 1969). Consequently, many rats and mice models of global ischemia have been developed to counteract this problem.

In rats, the four-vessel occlusion model (4VO) is used involving a reversible BCCAO occlusion, and a permanent interruption of the vertebral arteries' blood flow via electrocoagulation (Pulsinelli and Brierley, 1979; Pulsinelli and Buchan, 1988). This model results in bilateral forebrain and brainstem ischemia with a highly predictable brain damage. In this model, striatal neurons are damaged after 30 min of ischemia, hippocampal damage occurs after 3 to 6 h, and neocortex after 1 to 3 days (Pulsinelli and Brierley, 1979). The major disadvantages of this model are the need for a two-step procedure, and the variability in susceptibility found in different strains or within a single strain (McBean and Kelly, 1998). Unsuccessful outcomes occur in approximately 25% to 50% of the animals mainly due to the differences in collateral blood supply (Pulsinelli and Buchan, 1988; Traystman, 2003). Moreover, it is important to note that a precision electrocoagulation of the vertebral arteries is essential, although it is impossible to directly view these vessels (Ginsberg and Busto, 1989). Thus, technical skills and finesse are required during the first-step procedure to avoid injury of the brainstem while coagulating the vertebral

arteries. However, controlling all the experimental conditions and using a short occlusion time, it is possible to obtain a reproducible level of damage.

As an alternative to the 4VO method, global ischemia in rats can also be induced by the two-vessel occlusion model (2VO), where BCCAO is followed by the induction of hypotension for a limited period of time through bleeding (Eklöf and Siesjö, 1972) or by haemorrhage caused by pharmacological agents, such as methaphan or phentolamine (Smith et al., 1984). In this ischemia model, selective injury is observed in the hippocampal area CA1, the caudate putamen and neocortex (Smith et al., 1984). In general, the histopathology outcomes are comparable to those in the 4VO model. The major disadvantage of this model is the use of anaesthesia and hypotension drugs that can confound the interpretation of the results. To counteract this problem, some modifications of the 2VO model have been developed to induce hypotension, such as the use of hypobaric hypotension or the manipulation of the already present halothane anaesthetic (Dirnagl et al., 1993; McBean et al., 1995). Another disadvantage is that the variability in results may be caused by variation in temperature, like most ischemic models (Traystman, 2003). On the other hand, this model has advantages over the 4VO model since it requires less surgical training, and the reperfusion can be easily achieved (Traystman, 2003).

Although many efforts have been made to apply these models of global ischemia to mice, it has led to limited results since high levels of mortality and other complications have been noted (Traystman, 2003). However, the development of relevant transgenic mouse models has motivated the establishment of more reliable global ischemia methods in mice. One of these models consists in applying a BCCAO in association with controlled ventilation (Murakami et al., 1998). Nonetheless, the patency of the PcomA was strongly correlated with the ischemic injury in the striatum, cortex and especially in the hippocampus, where the injury appeared to be more sensitive to the PcomA plasticity (Murakami et al., 1998) .

Another model in mice consists in the induction of cardiac arrest by an injection of potassium chloride, followed by cardio-pulmonary resuscitation (Kofler et al., 2004). In this model, a selective injury of neurons in the hippocampus and striatum is observed (Kofler et al., 2004).

Finally, the HI model consists of a permanent unilateral occlusion of the common carotid artery followed by a systemic hypoxia. HI model was initially used in rats (Levine, 1960), and later applied to adult mice (Vannucci et al., 2001). Although this model is very extended to study perinatal and neonatal hypoxia in newborn and immature rodents (Rice et al., 1981; Vannucci et al., 1999) due to its clinical application, in adult rodents it is relevant in the situations where the brain is deprived of both oxygen tension and blood flow,

such as cardiac arrest (Olson and McKeon, 2004). This procedure induces injury in the ipsilateral cortex and hippocampus that is consistent with functional deficits (Olson and McKeon, 2004). The main advantage of this model is the minimal surgical invasiveness. Moreover, the hypoxic episode can be conducted without anaesthesia, avoiding its influence on post-ischemic outcome. The main disadvantages of the HI model, as for other models of ischemia, are the variability in results and mortality (Olson and McKeon, 2004; El-Hayek et al., 2011).

Although animal models do not entirely reproduce human cerebral ischemia, where the underlying risk factors are heterogeneous and complex, rodent models of both global and focal cerebral ischemia continue being a useful resource for trying to elucidate the mechanisms, prevention, and treatment of ischemic brain injury.

4. The endocannabinoid system

The endocannabinoid system (ECS) includes the cannabinoid type 1 (CB1R) and type 2 (CB2R) receptors, their endogenous ligands called endocannabinoids (anandamide, 2-arachidonoylglycerol, among others), and the enzymes involved in their synthesis and degradation (fatty acid amine hydrolase: FAAH; and monoacylglycerol lipase: MAGL). The ECS acts as a retrograde modulator of several brain neurotransmitters and is widely present throughout the entire brain. The ECS is known to be involved in a large variety of biological functions, including brain development, control of energy expenditure, motivation, pain perception, and stress coping, among others (Chen, 2015). Moreover, the ECS has been studied as a possible target for neuroprotective strategies in ischemia-induced brain damage.

4.1. Cannabinoid receptors

Cannabinoids exert their pharmacological actions mainly through the activation of CB1R and CB2R. The CB1R were the first to be cloned and characterized (Matsuda et al., 1990), while the CB2R were identified three years later (Munro et al., 1993). Both belong to the G-protein-coupled receptor family and are mainly associated to Gi/o proteins, but they display distinct expression patterns in

different tissues. The CB1R are abundantly expressed throughout the CNS, whereas the CB2R are scarcely expressed in the brain and they are mainly present in the immune system. Moreover, diverse studies also point to the existence of other receptors that bind cannabinoid ligands. For instance, the G-protein-coupled receptor 55 (GPR55) (Pertwee, 2007; Ryberg et al., 2007), the peroxisome proliferator-activated receptor (PPAR) (O'Sullivan, 2007) and the transient receptor potential cation channel subfamily V member 1 (TRPV1) (Di Marzo and De Petrocellis, 2010).

4.1.1. Cannabinoid receptors type 1

The CB1R are one of the most abundant seven-transmembrane domain receptors in the CNS and are widely distributed through the CNS. Its distribution has been well characterized both in rodents (Tsou et al., 1998) (Fig. 4) and in humans (Westlake et al., 1994; Burns et al., 2007). They are greatly expressed in the basal ganglia, cerebellum, hippocampus, cortex, amygdala, thalamus and hypothalamus, among other brain regions (Herkenham et al., 1991). Due to their central distribution, the CB1R are involved in the psychotropic effects of Δ9-tetrahydrocannabinol (THC), the principal psychoactive component in cannabis. With regards to peripheral tissues, the CB1R are also expressed in the retina, gonads, peripheral neurons, adipocytes, heart, lung, liver, adrenal gland, and immune and vascular system (Pertwee et al., 2010). At the cellular level, they are mainly expressed at presynaptic terminals of central and peripheral neurons, where they regulate the release of

multiple excitatory and inhibitory neurotransmitters, usually by promoting the inhibition of their release (Tsou et al., 1998; Wilson and Nicoll, 2002).

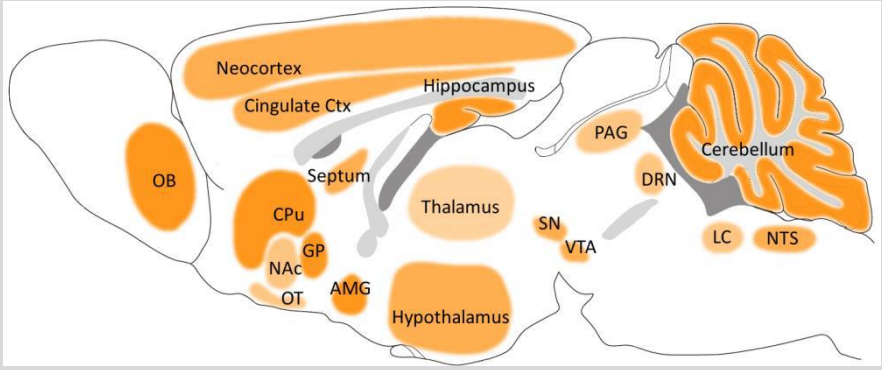


Figure 4. Distribution of CB1R in brain. Sagittal section of mouse brain showing schematic CB1R location (different shading density indicates expression level). AMG, amygdala; ctx, cortex; Cpu, caudate-putamen; DRN, dorsal raphe; GP, globus pallidus; LC, locus coeruleus; NAc, nucleus accumbens; NTS, nucleus of the solitary tract; OB, olfactory bulb; OT, olfactory tubercle; PAG, periaqueductal grey; SN, substantia nigra; VTA, ventral tegmental area (Flores et al., 2013).

The expression of these receptors at presynaptic terminals may vary according to the brain region and synapse type. Moreover, their distribution is also heterogeneous among cellular populations. In this regard, weak but relevant levels are present in glial cells. In microglia, CB1R expression is very low, making it difficult to study how these receptors regulate microglial cell function (Stella, 2010). On the other hand, in astrocytes, they control the release of inflammatory mediators (Molina-Holgado et al., 2002), regulate the energy supply of the brain (Sánchez et al., 1998; Blázquez et al., 1999), and mediate some of the neuron/astrocyte interactions (Navarrete and Araque, 2008). This data indicate that CB1R

expressed by astrocytes might be involved in many fundamental regulatory functions, and might have a possible pathophysiological role in the brain (Stella, 2010).

4.1.2. Cannabinoid receptors type 2

The CB2R are mostly located in the immune system including the spleen, thymus and immune cells, being profoundly involved in inflammatory processes (Walter and Stella, 2004). The presence of these receptors in neurons is still a controversial matter. In fact, early studies showed that the CB2R were absent in CNS neurons, and that healthy brain tissue did not express CB2R (Munro et al., 1993). However, several recent studies have suggested their localization in neurons in the brainstem, dorsal root ganglia, lumbar spinal cord, and possibly in the cerebellum (Van Sickle et al., 2005; Gong et al., 2006; Onaivi et al., 2006). In the immune tissues, the CB2R are present in macrophages, monocytes, neutrophils, among others (Atwood and Mackie, 2010). The presence of CB2R in microglia and astrocytes (Kearn and Hilliard, 1997; Sheng et al., 2005) is particularly relevant since they can modulate glia-mediated inflammation processes in ischemia models (Maresz et al., 2005; Ashton and Glass, 2007; Sagredo et al., 2009). In healthy brain tissue, only weak levels of CB2R are detectable, suggesting that resting microglia do not express CB2R (Munro et al., 1993; Griffin et al., 1999; Carlisle et al., 2002). However, high levels of CB2R are expressed by activated microglia as a result of specific neuropathologies and neuroinflammatory responses (Stella, 2010).

4.2. Cannabinoid-like receptors

Recent studies indicate that some cannabinoid responses are mediated by receptors other than CB1R and CB2R, suggesting the possible existence of additional cannabinoid ligand biding sites that exert activities in numerous physiological processes. In this context, the GPR55 has been suggested as a new cannabinoid receptor (Baker et al., 2006; Pertwee, 2007; Ryberg et al., 2007). This receptor is expressed in some brain areas, including the frontal cortex and striatum in much lower levels than the CB1R (Ryberg et al., 2007). Moreover its expression is modified depending on the microglial activation state (Pietr et al., 2009). Additionally, recent data has suggested the role of GPR55 in neuroprotection (Kallendrusch et al., 2013). Finally, the TRPV1 and PPAR have also been involved in the pharmacological responses induced by cannabinoids compounds, as well in regulating microglial activation and neuroprotection in response to ischemia and neuroinflammation (Di Marzo and De Petrocellis, 2010; O'Sullivan and Kendall, 2010).

The TRPV1 is part of the transient receptor potential (TRP) family, which is a diverse group of channels that control cation entry and participate in several physiological conditions. Located in the plasma membrane, TRP channels act as polymodal integrators due to their activation by several stimuli including temperature, osmolality, mechanical force, chemoattractant and ischemia (Ho et al., 2012). The TRPV1 is widely distributed in the brain suggesting its importance in CNS function. This is supported by recent

evidence of TRPV1-mediated activities in several regions of the rat brain, including the hippocampus, cortex, cerebellum, olfactory bulb, mesencephalon and hindbrain (Tóth et al., 2005).

PPARs belong to a family of nuclear receptors responsible for transducing the effects of an extensive variety of signalling molecules that modify the transcription of target genes. The PPAR family consist of three isoforms: α, δ and γ, encoded by different genes, but displaying considerable amino acid similarity. Ligand binding to PPARs causes the recruitment of further regulator proteins also implicated in the modulation of transactivation (O'Sullivan and Kendall, 2010; Abood et al., 2012). All three isoforms are expressed in the brain and peripheral nervous system (Moreno et al., 2004; Cimini et al., 2005), and they are primarily involved in the regulation of metabolism and energy homeostasis, cell differentiation and inflammation (Ferre, 2004; Moraes et al., 2006; Stienstra et al., 2007).

Although studies suggest the involvement of the TRPV1 and the different PPARs isoforms in neuroprotective processes, in this thesis we will focus in the role of PPAR-α.

4.2.1. Peroxisome proliferator-activated receptor-alpha

PPAR-α is found in metabolically active tissues including the liver, heart and muscle (Stienstra et al., 2007). In the CNS, PPAR-α is widely distributed, exhibiting patterns of expression in various areas and at different developmental stages. It has been predominantly

detected in the cortex, hippocampus, septal complex, thalamus, and in the basal ganglia (Moreno et al., 2004). In addition, studies demonstrate the presence of PPAR-α in neurons, and moderately in astrocytes under normal conditions in both mice and human cells (Moreno et al., 2004; Warden et al., 2016). Moreover, it has been reported that PPAR-α expression in microglia was equally weak under both normal and neuroinflammatory conditions, since the administration of the endotoxin LPS, to induce a strong neuroimmune response, did not increase the expression of PPAR-α (Warden et al., 2016). This finding was unexpected since evidence shows that PPAR-α participates in different inflammatory responses, such as modulating the expression of chemokines, chemokine receptors and adhesion molecules in endothelial cells, smooth muscle cells, monocytes/macrophages and T cells (Duval et al., 2002; Blanquart et al., 2003). Thus, it is possible that the low expression of PPAR-α observed under inflammatory conditions may be due to the short time course for LPS treatment. PPAR-α is also involved in other processes including modulation of cellular differentiation, metabolism of carbohydrates, lipids and proteins, and tumorigenesis (Lee et al., 2003; Marx et al., 2004). In addition, studies also reported its involvement in the cell response to ROS (Rusyn et al., 2000; Inoue et al., 2001).

4.3. Endocannabinoids

Endocannabinoids are a family of lipid molecules that are involved in numerous functions, spanning from neuromodulation to

neuroprotection processes (Lutz, 2004; Mechoulam et al., 2002; Mechoulam and Lichtman, 2003; Piomelli, 2003; van der Stelt et al., 2002; van der Stelt and Di Marzo, 2005). These molecules are divided into two groups that present specific structures, N-acylethanolamides (NAEs) and monoacylglycerols (MAGs).

N-arachidonoylethanol-amide, also named anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized members of the main families of endocannabinoids, NAEs and MAGs, respectively (Fig. 5). AEA was identified first in 1988 (Devane et al., 1988), and behaves as a partial agonist at both CB1R and CB2R (Pertwee et al., 2010). In addition, it is now accepted that AEA possesses the ability to interact with other receptors, such as the TRPV1 (Zygmunt et al., 1999) and PPAR family (O'Sullivan, 2007). The second endocannabinoid identified was 2-AG (Mechoulam et al., 1995; Sugiura et al., 1995). Its concentration in the brain is much higher than AEA, acting as a full agonist for both CB1R and CB2R with higher potency than AEA (Reggio, 2010), and it also activates PPAR. Both lipid signalling molecules are synthetized and metabolized by different enzymes and display distinct pharmacological profiles, as well as regional diversity (De Petrocellis et al., 2004). Contrasting with most neurotransmitters, endocannabinoids are not stored in presynaptic vesicles, but are synthetized and released on demand in the postsynaptic terminals in response to elevations of intracellular calcium (Pertwee, 2010). These compounds act as retrograde synaptic messengers travelling across synapses to activate cannabinoid receptors located on

presynaptic terminals, where they generate a transient decrease in the release of other neurotransmitters (Wilson and Nicoll, 2002) (Fig. 6). Thus, endocannabinoid availability in the synaptic cleft needs to be finely regulated through balancing its biosynthesis and degradation.

Moreover, other putative endogenous compounds that may bind to cannabinoid receptors have also been identified including, 2-arachidonylglycerolether (noladin ether) (Hanus et al., 2001), O-arachidonoylethanolamine (virodhamine) (Porter et al., 2002), N-arachidonoyldopamine (NADA) (Huang et al., 2002), N-arachidonoylglycine (NAGly) (Huang et al., 2001) and Cis-9,10-octadecanoamide (oleamide or ODA) (Leggett et al., 2004). These compounds are amides, esters or ethers of long-chain polyunsaturated fatty acids. Their chemical structures are represented in Fig. 5. Despite the ability of these endogenous lipids to bind to cannabinoid receptors, their functional relevance remains to be elucidated.

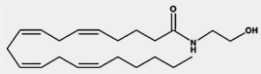
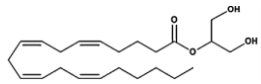
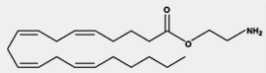
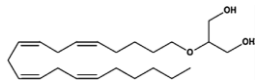
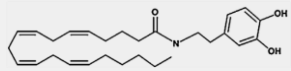
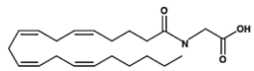
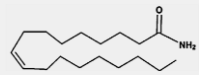
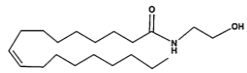
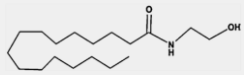
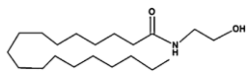
Endocannabinoids	Chemical structure	Receptors affinity
Anandamide (AEA)		CB1R > CB2R TRPV1 PPAR-α GPR55
2-Arachidonoylglycerol (2-AG)		CB1R = CB2R PPAR-γ/δ TRPV1 GPR55
Virodhamine		CB2R > CB1R PPAR-α GPR55
Noladin (2-AGE)		CB1R >> CB2R TRPV1 PPAR-α/γ/δ GPR55
N-Arachidonoyldopamine (NADA)		CB1R >> CB2R TRPV1 PPAR-γ
N-arachidonoylglycine (NAGly)		GPR18 GPR92
Oleamida (ODA)		CB1R CB2R
Oleoylethanolamide (OEA)		TRPV1 PPAR-α GPR55 GPR119
Palmitoylethanolamide (PEA)		TRPV1 PPAR-α GPR55 GPR119
Stearoylethanolamide (SEA)		PPAR-α

Figure 5. Chemical structure and receptor affinity of some endocannabinoids and endocannabinoid-like compounds (Di Marzo et al., 2004; Brown, 2009; Fonseca et al., 2013).

4.4. Endocannabinoid-like compounds

In addition to endocannabinoids, our body synthesizes other compounds that may modulate cannabinoid signalling, although they are not able to activate cannabinoid receptors directly. These compounds are called endocannabinoid-like compounds and share with endocannabinoids some metabolizing enzymes (Lambert and Di Marzo, 1999). The main endocannabinoid-like compounds are: palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and stearoylethanolamide (SEA) (Fig. 5).

PEA was identified in 1965 in the brain, the liver, and in muscle of rat and guinea pig (Bachur et al., 1965). Later, it was discovered in other organs in higher levels than AEA (Schmid et al., 1995). Evidence shows that PEA may take part in several processes, such as regulation of the immune response, pain and neuroprotection (Skaper et al., 1996; Berdyshev et al., 1997; Calignano et al., 1998; Gulaya et al., 1998). Other findings indicate that PEA exhibits an anti-inflammatory effect through PPAR-α mediation (LoVerme et al., 2005), and may be involved in immune cell function and cell migration through GPR55 (Ryberg et al., 2007).

OEA is also a bioactive NAE and appears naturally in many mammalian tissues and cells. However, its biological effects and molecular mechanisms of action are not yet completely elucidated. OEA acts as a lipid mediator of satiety and exerts anorexic effects through peripheral mechanisms (Rodríguez de Fonseca et al., 2001), and it is mediated by PPAR-α (Fu et al., 2003). Moreover, OEA has

also been implicated in neuroprotection and pain processes through its action on PPAR-α (Suardíaz et al., 2007; Sun et al., 2007; Zhou et al., 2012, 2017; Yang et al., 2015). On the other hand, evidence shows that OEA may exert some effects in a PPAR-α-independent manner (Cluny et al., 2009), and could be acting instead through the G-protein-coupled receptor 119 (GPR119), GPR55 and TRPV1 (Wang et al., 2005; Overton et al., 2006; Brown, 2009).

Finally, SEA is one of the least studied compounds in the family of NAEs. Reports indicate that SEA exhibits anti-inflammatory properties *in vivo* (Dalle Carbonare et al., 2008), pro-apoptotic activity *in vitro* (Maccarrone et al., 2002), and exerts anorexic effects that were not associated with PPAR-α expression (Terrazzino et al., 2004). Interestingly, the anti-inflammatory effects of SEA do not seem to be mediated by cannabinoid receptors, since neither CB1R nor CB2R antagonists reversed these effects. In contrast, the competitive antagonist of the TRPV1, capsazepine, fully reversed the anti-inflammatory effects of SEA (Dalle Carbonare et al., 2008). Moreover, a report has shown that SEA appears to be an activator of PPAR-α (Artmann et al., 2008). This finding supports the hypothesis that SEA is a bioactive signalling lipid capable of downregulating inflammation processes; however, further studies are required to understand its potential interest.

Notably, PEA, OEA and SEA have been shown to influence AEA and 2-AG metabolism, potentiating their action by an "entourage effect" (Fonseca et al., 2013). For instance, there is evidence

showing that they can inhibit AEA degradation by competing for fatty acid amine hydrolase (FAAH) activity (Bisogno et al., 1997; Jonsson et al., 2001; Maccarrone et al., 2002). Similarly, there are data revealing that PEA and OEA potentiate the vasorelaxation and hypotensive effects of AEA through CB1R and TRPV1 (Ho et al., 2008). Moreover, it has been shown that PEA may decrease FAAH expression (Di Marzo et al., 2001), and may enhance the affinity of AEA for TRPV1 (De Petrocellis et al., 2001). Thus, all these findings show that it might be more relevant to investigate these lipids as a whole, instead of in isolation.

4.5. Enzymes involved in the biosynthesis and degradation of endocannabinoids signalling

Different enzymes are involved in the synthesis and degradation of endocannabinoids. AEA biosynthesis mainly occurs from enzymatic cleavage of a phospholipid precursor, N-arachidonoyl-phosphatidylethanolamine (NAPE). NAPE is synthesized by the calcium-independent N-acyl-transferase and hydrolysed to AEA by a specific phospholipase D (NAPE-PLD) (Di Marzo et al., 2005, 2014) (Fig. 6). Additionally, AEA can also be synthetized from ethanolamine and arachidonic acid (Herrera et al., 2016). The other main endocannabinoid, 2-AG, is also synthetized in two steps. First, the enzyme phospholipase C (PLC) produces the 2-AG precursor diacylglycerol (DAG) from enzymatic cleavage of membrane phospholipid precursors. Second, DAGs are hydrolysed by the enzyme diacylglycerol lipase (DAGL) to generate 2-AG (Di

Marzo et al., 2005, 2014) (Fig. 6). After their biosynthesis, AEA and 2-AG are immediately released into the extracellular medium acting mostly, and with varying selectivity, on CB1R and CB2R. Endocannabinoids may also act, prior to their release, on intracellular sites on ion channels, such as TRPV1 and T-type $Ca2^{+}$ channels. Thus, in this case, release would be a possible way to inactivate, rather than facilitate the action of endocannabinoids (Di Marzo et al., 2005). Endocannabinoids are degraded by specific hydrolases. AEA is hydrolysed by FAAH into arachidonic acid and ethanolamine (Cravatt et al., 1996), while 2-AG is mainly hydrolysed by MAGL into arachidonic acid and glycerol (Dinh et al., 2002a, 2002b). Both are intracellular enzymes, but FAAH is primarily expressed in the soma and dendrites of postsynaptic neurons (Egertová et al., 2003), whereas MAGL is expressed in presynaptic terminals (Gulyas et al., 2004). Interestingly, in the presence of high concentrations of ethanolamines and unesterified fatty acids, which occurs during cerebral ischemia, FAAH can also catalyse the reverse reaction, producing NAEs (Ueda et al., 1995; Katayama et al., 1999). However, this process does not occur under normal physiological conditions (Kurahashi et al., 1997; Ueda et al., 2000).

Other NAEs, such as PEA and OEA, share the same mechanisms of biosynthesis and degradation with AEA. They are synthetized on demand through NAPE-PDL and degraded by FAAH, but like AEA, PEA and OEA can also be synthetized from ethanolamine and fatty acids, particularly palmitic acid and oleic acid, respectively

(Herrera et al., 2016). Although PEA and OEA do not act through CB1R and CB2R, they may participate in the endocannabinoid signalling pathway, acting as "entourage compounds", increasing AEA activity (see section 4.4). Both NAEs exert their actions mainly through PPAR-α, but they may act through TRPV1, GPR55 and GPR119 as well, besides other PPAR isoforms.

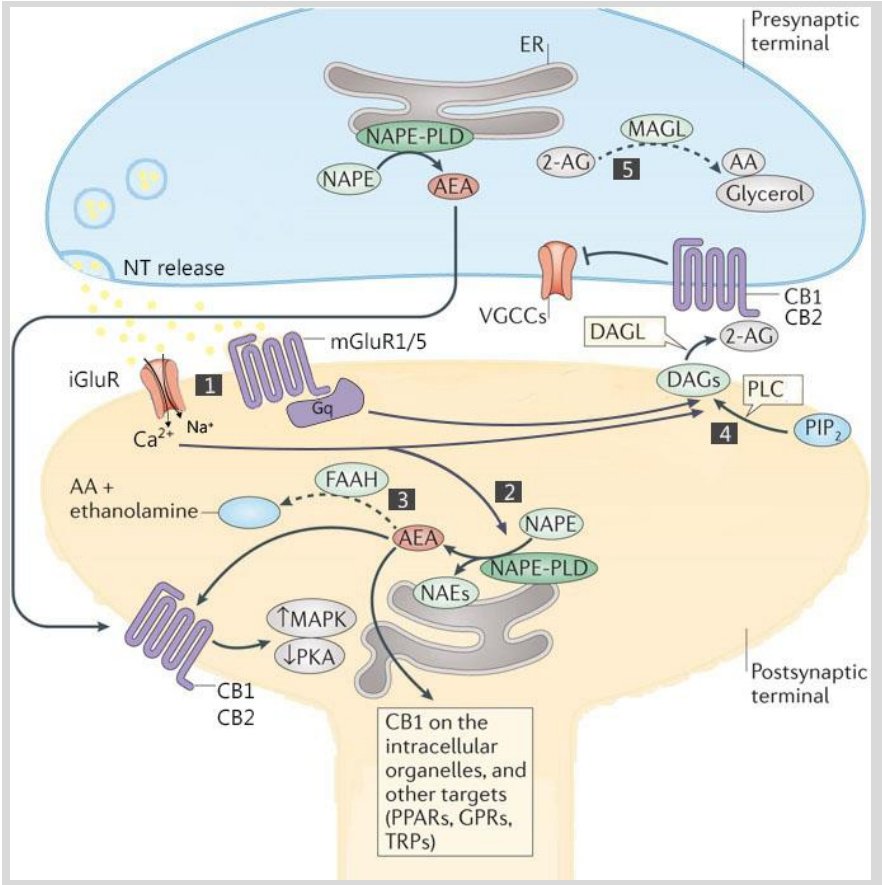


Figure 6. Main enzymes involved in the biosynthesis and degradation of endocannabinoids signalling. (1) Once glutamate is released from presynaptic terminals, stimulates both ionotropic and metabotropic glutamate receptors (iGluR and mGluR), leading to postsynaptic depolarization through $Ca2^{+}$ entrance and Gq-protein activation. (2) High Ca^{2+} concentration promote the biosynthesis of AEA and other NAEs mainly through the action of NAPE-PLD, which is located in intracellular membranes both pre- and

postsynaptically. (3) NAEs are degraded by FAAH, which is located postsynaptically. This distribution of the enzymes responsible for synthesis and degradation of AEA enables this and other NAEs to function as anterograde signals acting at postsynaptic targets, or as intracellular mediators. (4) The biosynthesis of 2-AG occurs mainly through the action of DAGL, which is located postsynaptically. 2-AG synthesis is also mediated by Gq-protein activation. (5) 2-AG is degraded by MAGL, which is located presynaptically, thus accounting for the retrograde signalling action suggested for this endocannabinoid. Solid arrows: transformation into active metabolites or activation; dashed arrows: transformation into metabolites inactive at cannabinoid receptors; blunt arrow: inhibition. AA, arachidonic acid; DAGs, diacylglycerols; DAGL, diacylglycerol lipase enzyme; ER, endoplasmic reticulum; FAAH, fatty acid amine hydrolase; GPRs, orphan G-protein-coupled receptors; MAGL, monoacylglycerol lipase; MAPK, mitogen-activated protein kinases; NAEs, N-acylethanolamides; NAPE-PLD, N-arachidonoyl-phosphatidylethanolamine-phospholipase D; NAPE, N-arachidonoyl-phosphatidylethanolamine; PIP2, phosphoinositide bisphosphate; PKA, protein kinase A; PLC, phospholipase C; PPARs, peroxisome proliferator-activated receptors; TRPs, transient receptor potential channels; VGCCs, voltage-gated calcium channels (Adapted from Di Marzo et al., 2014).

5. Role of the endocannabinoid system in cerebral ischemia

There are several lines of evidence both in humans and experimental animals that show the important impact of cerebral ischemia on the components of the ECS. In first place, the expression of cannabinoid CB1R and CB2R are upregulated in the brain after cerebral ischemia (chapter 5.1). Second, the deletion of cannabinoids receptors in mice seems to greatly affect the outcome of brain injury (chapter 5.2). Third, studies in animal models of cerebral ischemia have shown the implication of cannabinoids in neuroprotection (chapter 5.3). Finally, there is evidence demonstrating that endocannabinoids accumulate in ischemic tissues, supporting the hypothesis that they are activated during ischemia (chapter 5.4). All these data indicate that the ECS may have an important role in the endogenous response to stroke. However, there is contradicting data showing that the activation of the ECS during ischemia could also aggravate injury (chapter 5.3).

In this chapter, the effects of cerebral ischemia on the various components of the ECS, and the role of the ECS in the consequences of cerebral ischemia will be reviewed.

5.1. Changes in the expression of cannabinoid and cannabinoid-like receptors

There are several reports suggesting that ischemia alters the amount of both CB1R and CB2R in the brain. The first evidence came from a study in rats subjected to transient MCAO (Jin et al., 2000). In this study, CB1R-like immunoreactivity was upregulated in neuronal cells in the ischemic boundary zone 2 hours after reperfusion. However, no changes in CB1R-like immunoreactivity in the ischemic core of the infarct was observed (Jin et al., 2000). Similar results were obtained by Zhang and colleagues (2008) in a study using quantitative PCR to measure CB1R mRNA content following transient MCAO in mice. The mRNA for the CB1R in the brain ipsilateral to the occlusion was significantly elevated at 1 hour, and maximally elevated at 6 hours, after the ischemia (Zhang et al., 2008). In contrast to these findings, transient MCAO models revealed that CB1R mRNA expression was slightly reduced in adult rats 1, 5 and 10 days following reperfusion (Yu et al., 2015) or did not change at 24 hours in neonatal rats (Fernández-López et al., 2012). Interestingly, in permanent MCAO models, the levels of CB1R mRNA in mice were decreased 5 hours post-ischemia (Zarruk et al., 2012), while no change was observed in CB1R binding site density in the cortex of rats (Sommer et al., 2006). These findings suggest (i) that CB1R expression may increase only early in the time course of the ischemia, and (ii) that the increase in CB1R expression is associated with reperfusion injury.

In global ischemia, an increase in CB1R mRNA expression was observed in the frontal cortex, associated to an increase in endocannabinoids concentration in rats subjected to 30 min of BCCAO followed by 60 min of reperfusion (Quartu et al., 2017). In contrast, no changes were observed in CB1R protein or binding site density in the hippocampus of gerbils subjected to 5 min of BCCAO followed by 24 or 48 hours of reperfusion (Schomacher et al., 2006). Interestingly, in an ischemic preconditioning model, gerbils were exposed to short ischemic stimulus of 2.5 min duration producing a transient reduction in hippocampal CB1R protein expression, while receptor binding density was permanently decreased at 24, 48 and 96 hours after the ischemia (Schomacher et al., 2006). In sum, these findings suggest the both up and downregulation of CB1R expression can appear in the area at risk of infarction. Therefore, more studies are needed to determine the exact role of CB1R mediated signalling in ischemic injury.

Regarding the effect of ischemia in CB2R expression, there are reports of increases in the expression of these receptors in two different models of ischemia (Ashton et al., 2007). In the HI model, CB2R-positive cells were observed in the ipsilateral hemisphere of the brain following 3 days in 26 days old rats that were colocalized with areas of reactive astrocytosis and had a macrophage-like phenotype (Ashton et al., 2007). In the transient MCAO model in adult rats, 3 days following ischemia, CB2R-positive macrophage or microglial cells were seen in the ischemic penumbra of the cortex (Ashton et al., 2007). In another study, the levels of CB2R mRNA

were significantly elevated in the ischemic cortex of rats, 2 and 5 days following transient MCAO, but normalized on day 10 post-stroke (Yu et al., 2015). Interestingly, in the same model, CB2R mRNA levels decreased in the ischemic hemisphere of mice during first 3 hours after the ischemia, but significantly increased following 24 hours (Zhang et al., 2008). In addition, mice subjected to permanent MCAO, CB2R mRNA expression also increased in reactive microglia in the peri-infarct area 18 hours after injury (Zarruk et al., 2012). These findings suggest that CB2R expression may increase later in the time course of the ischemia, and this is consistent with its presence on macrophages or leukocytes recruited into the injured brain in the secondary phase following ischemia (Heinel et al., 1994; Maresz et al., 2005).

Increases in CB2R expression have also been reported in the model of global ischemia BCCAO. In this context, increases in CB2R expression in the frontal cortex of rats subjected to 30 min of BCCAO followed by 60 min of reperfusion was associated to an increase in the endocannabinoids concentration (Quartu et al., 2017). Although there are few studies regarding the effects of ischemic injury on CB2R expression in brain, these findings indicate the link between CB2R and glia-mediated inflammation processes. This issue will be reviewed in the next chapter.

Regarding the effect of ischemia in PPAR-α expression, different outcomes in models of cerebral ischemia have been observed. For instance, in rats subjected to 20 min of global cerebral ischemia/reperfusion injury, an increase in PPAR-α mRNA

expression in the hippocampus was observed, and this finding was associated to spatial memory and learning function deficits (Luo et al., 2014). Similarly, another study using the same model has shown that the expression of PPAR-α mRNA in rat hippocampus increased following ischemia, reaching the peak level at 24 h (Wang et al., 2012). In contrast, no changes were detectable for the relative levels of PPAR-α in the frontal cortex of rats subjected to 30 min of BCCAO followed by 60 min of reperfusion (Quartu et al., 2017) or in brain of rats subjected to transient MCAO during 120 min (Xiong et al., 2016). The discrepancies found in the levels of PPAR-α mRNA may be due the different models of ischemia used, or to the various brain areas analysed.

5.1.1. Cannabinoid and cannabinoid-like receptors in glia cells

It has been shown that microglial and astrocytic cells express CB1R and CB2R, as well as other cannabinoid-like receptors, and that their activation regulates different cell functions, but also control cell viability (Stella, 2010). Moreover, microglia and astrocytes produce endocannabinoids, a response that probably contributes to the long-lasting increase in endocannabinoid levels observed under neuroinflammatory conditions, such as cerebral ischemia (Franklin et al., 2003). These prolonged increases in endocannabinoid levels represent a mechanism of defence that prevents the propagation of neuroinflammation and the associated cell damage.

In microglia, the expression of CB1R and CB2R depends on the microglial activation profile, becoming activated sequentially following several activation signals from resting to reactive phenotype. A very low expression of CB1R and CB2R is present in resting microglia (M0 phenotype), which can only be found in intact healthy brain tissue (Stella, 2010). However, in activated microglia, CB2R are rapidly upregulated "on demand" being highly detectable in more reactive states (Carlisle et al., 2002; Walter et al., 2003; Maresz et al., 2005; Stella, 2010).

In culture, microglia are found to be intrinsically activated, probably induced by the procedures involved in setting up the cultures (Becher and Antel, 1996). Several studies have demonstrated that such intrinsically activated microglia cultures from human, rat, or mouse tissues express high levels of CB2R. Moreover, CB2R are expressed in the rodent microglial cell line BV-2, which innately exhibit high rates of cell proliferation (Walter et al., 2003; Carrier et al., 2004). CB2R upregulation in activated microglial cells depends on the combination of different signals (such as toxins, cytokines or molecules released by neurons like ATP) after neuroinflammatory events or during neurodegenerative diseases. In this regard, microglial CB2R expression is upregulated in some neurodegenerative disorders such as stroke, Alzheimer's and Huntington's diseases (Ashton et al., 2007; Palazuelos et al., 2009; Sagredo et al., 2009; Zarruk et al., 2012). Concerning stroke, CB2R are upregulated during the activation of microglia following brain injury. Specifically, CB2R-positive cells appear in the brain of rats

and mice following both HI and MCAO (Ashton et al., 2007; Zarruk et al., 2012). Moreover, in a MCAO model induced by endothelin, CB2R were identified 3 and 7 days post-ischemia in activated microglia/macrophages (Schmidt et al., 2012). Another study also demonstrated microglia CB2R upregulation *in vivo* in response to an inflammatory challenge (Maresz et al., 2005). In contrast, a recent study found that CB2R are not expressed on microglial cells in the postmortem human Huntington's disease brain (Dowie et al., 2014).

There are some studies regarding the expression of CB2R in astrocytes with controversial results. For instance, CB2R appears to be poorly expressed by astrocytes in healthy conditions. However, in a rat model of Huntington's disease, malonate-induced striatum injury, a marked elevation of CB2R levels was observed, as revealed by immunocolocalization with GFAP (Sagredo et al., 2009). In a MCAO model induced by endothelin, CB2R was identified in astrocyte 3 and 7 days post-ischemia (Schmidt et al., 2012). In addition, some isolated astrocytes ($GFAP^+$ cells) present in the corpus callosum showed CB2R expression in mice subjected to a permanent MCAO (Zarruk et al., 2012). On the other hand, some studies have shown the lack of expression of this receptor in astrocyte cultures under physiological conditions (Walter and Stella, 2003), in postmortem human Huntington's disease brain (Dowie et al., 2014) or in rat tissue after stroke (Vandeputte et al., 2012).

Interestingly, several studies have tried to elucidate the therapeutic potential of cannabinoids in some of these diseases acting on CB2R. In this context, studies have reported that activation of CB2R increases microglial and astrocytic cell proliferation and migration, while reducing the release of detrimental factors, such as TNF-α and free radicals (Walter et al., 2003; Carrier et al., 2004; Eljaschewitsch et al., 2006; Sagredo et al., 2009). In neurogenerative diseases, cannabinoids have shown to protect the striatum against malonate toxicity, likely through a mechanism involving glial cells (Sagredo et al., 2009), and improve motor symptoms (Palazuelos et al., 2009) in models of Huntington's disease. Moreover, cannabinoids have shown to reduce infarct volume and improve neurological outcome by preventing microglial reactivity in models of MCAO (Zarruk et al., 2012).

CB1R are expressed by cultured microglial cells from different species, such as mollusk, mouse and rat (Stefano et al., 1996; Waksman et al., 1999; Carlisle et al., 2002; Walter et al., 2003). These receptors regulate NO production, an inflammatory mediator released from activated microglial cells. However, how these receptors regulate microglial cell function is controversial. A study carried out in mollusk microglia has shown that the cannabinoid agonist CP 55,940 acting at CB1R increases NO production (Stefano et al., 1996). Though, NO production induced by LPS and IFN-γ was partially inhibited by CP 55,940 administration in rat microglia (Waksman et al., 1999). Moreover, in these studies, the effect of CP 55,940 on NO production was only

partially antagonized by micromolar concentrations of rimonabant, thus the involvement of CB1R in this response is uncertain (Stefano et al., 1996; Waksman et al., 1999). Similarly to CB2R, microglial CB1R expression is also upregulated in stroke. In this context, a study carried out in rats subjected to MCAO induced by endothelin showed that CB1R expression increased in activated microglia 3 days following ischemia (Schmidt et al., 2012).

On the other hand, the existence and the role of CB1R in astrocytes has been a controversial issue (Stella, 2004). For instance, cultured astrocytes from CD1 mice express CB1R (Molina-Holgado et al., 2002), while cultured astrocytes from C57BL/6 do not (Walter and Stella, 2003). These conflicting results may be due discrepancies in culture conditions that may affect the state of differentiation of astrocytes in culture, and CB1R expression changes, depending on the differentiation state of cells (Stella, 2004). On the other hand, the presence of CB1R on astrocytes *in situ* is well documented. CB1R are expressed by astrocytes in the nucleus accumbens of Sprague-Dawley rats (Rodriguez et al., 2001), and in the cingulate cortex, the medial forebrain bundle, and the amygdala of Wistar rats (Moldrich and Wenger, 2000). Moreover, recent data has demonstrated that CB1R are expressed by hippocampal and cortical astrocytes *in situ*, and mediate relevant effects on astrocyte–neuron communication and synaptic modulation (Navarrete and Araque, 2008). Because astroglial CB1R regulate energy metabolism and mediate neuron-glia interactions, they may have a possible pathophysiological role (Stella, 2010). In this context, a recent study

has shown in a rodent BCCAO model that long-term depression induced by endocannabinoids produces neuroprotection through astroglial CB1R (Wang et al., 2018). Moreover, in a MCAO model induced by endothelin, CB1R was identified in astrocyte 3 and 7 days post-ischemia (Schmidt et al., 2012).

The knowledge about the presence of the different PPAR isotypes in glia is limited. There are only few studies investigating the localization of these receptors in microglia and astrocytes despite the well-documented anti-inflammatory effects of PPAR agonists. The PPAR-γ isotype is the dominant isoform in microglia. Astrocytes possess all three PPAR isotypes, although to different degrees depending on the brain area and animal age (Cristiano et al., 2001; Cullingford et al., 2002).

Bernardo and colleagues (2000) have shown that PPAR-γ are constitutively expressed in rat primary microglial cultures and that such expression was downregulated during microglial activation by LPS. They demonstrated that the PPAR-γ natural ligand 15-deoxy-Delta12,14-prostaglandin J2 (15d-PGJ2) inhibited the production of NO, TNF-α and MHC class II. The administration of PPAR-γ agonist, ciglitazone suppressed the production of these pro-inflammatory molecules in a similar manner, thus 15d-PGJ2 was interpreted to act through a PPAR-γ dependent mechanism (Bernardo et al., 2000). Similarly, 15d-PGJ2 was demonstrated to inhibit NO, IL-1β, and TNF-α production in the BV-2 mouse microglial cell line (Petrova et al., 1999; Koppal et al., 2000). In

contrast, the PPAR-γ agonist troglitazone did not suppress the expression of these pro-inflammatory molecules by BV-2 cells, suggesting that 15d-PGJ2 may act through a PPAR-γ-independent mechanism (Petrova et al., 1999). These studies suggest that 15d-PGJ2 likely inhibits microglial cell activation by PPAR-γ-dependent as well as PPAR-γ-independent mechanisms. Warden and colleagues (2016) have shown that PPAR-α, but not PPAR-β/δ or PPAR-γ are expressed in microglia in both adult mouse and adult human brain, under normal conditions. Moreover, LPS administration did not alter PPAR expression in brain tissue (Warden et al., 2016). Kim and colleagues (2002) have shown that PPAR-γ agonist reduced LPS-induced cortical neuronal cell death through NO and iNOS expression from microglia. Moreover, in mixed neurons with glial cells culture, PPAR-α was rarely detectable, while PPAR-γ was strongly expressed (Kim, 2005). In addition, Dentesano and colleagues (2014) demonstrated that PPAR-γ modulates CD200 (mainly expressed in neurons and astrocytes) and CD200R1 (present in microglial cells) gene expression, and that the CD200/CD200R1 interaction is involved in the anti-inflammatory and neuroprotective action of PPAR-γ agonists (Dentesano et al., 2014).

PPAR-α, PPAR-β and PPAR-γ are expressed in rat cortical and cerebellar astrocytes, and in primary cultures of adult cortical astrocytes (Cristiano et al., 2001; Cullingford et al., 2002). Accumulating evidence indicates that PPAR agonists modulate astrocyte functions. For instance, in rat cortical slices and cultured

astrocytes, the TZD pioglitazone (diabetes mellitus type 2 treatment drug) was found to significantly increase glucose consumption in time- and dose-dependent manners, through a mechanism independent of PPAR-γ and involving cAMP/PKA signalling (Dello Russo et al., 2003).

Interestingly, some studies have also demonstrated the role of these receptors in the regulation of microglial and astrocytic activation processes following ischemia (Kim et al., 2002; Zhao et al., 2005, 2006; Lee et al., 2011; Lee and Won, 2014; Yuan et al., 2016). Most of these studies have been focused in PPAR-γ. Lee and Won (2014) have shown lack of PPAR-γ expression in microglial cells in the gerbil dentate gyrus and CA1 of the hippocampus under normal conditions. However, an increase was observed in microglial PPAR-γ expression after BCCAO, indicating that PPAR-γ may be related to the ischemia-induced microglial activation and neuronal damage/death in these brain areas after transient global cerebral ischemia (Lee et al., 2011; Lee and Won, 2014). Similarly, microglial PPAR-γ was observed in peri-infarct cortical areas after MCAO (Zhao et al., 2006). Another study carried out in MCAO has demonstrated an extensive accumulation of activated microglia and macrophages at the periphery of, and to a minor extent, in the ischaemic core, and this accumulation was significantly reduced by the PPAR-γ ligand pioglitazone (Zhao et al., 2005). Regarding astrocytes, immunohistochemical studies showed the presence of PPAR-γ/GFAP double-positive cells in the ischaemic penumbra region in mice following MCAO, and the activation of PPAR-γ

signal pathway in astrocytes could be associated with higher levels of BDNF under ischaemia conditions (Yuan et al., 2016).

In summary, these studies demonstrate the relevance of the ECS as a possible therapeutic target for the modulation of neuroinflammation. In this sense, the stimulation of cannabinoid and cannabinoid-like receptors may reduce microglial and astrocytic activation and neurotoxicity (Stella, 2010).

5.2. Impact of genetic deletion of cannabinoid and cannabinoid-like receptors

It is clear that with the development of transgenic mice, doors opened to the research of receptors and their ligands. In this sense, studies on cerebral ischemia have been carried out in mice with genetic deletions of CB1R, CB2R and other cannabinoid-like receptors such as PPAR-α. The purpose of these studies was to elucidate the effects of these deletions in this neuropathology as well as the mechanisms involved in these effects.

5.2.1. Deletion of cannabinoid receptors

Only a single study using CB1R knockout (CB1R KO) mice was performed. The study showed increased infarct size and neurological deficits following transient MCAO, which were correlated with a reduction in cerebral flow and NMDA excitotoxicity (Parmentier-Batteur et al., 2002).

With respect to CB2R, some authors have reported that the infarct volume and neurological deficits in CB2R knockout (CB2R KO) mice did not differ from that of wild-type (WT) mice in models of MCAO (Murikinati et al., 2010; Zarruk et al., 2012), while others have found significant alterations in CB2R KO mice. The study carried out by Zhang and colleagues (2009) investigated the effects of CB2R deletion in the transient MCAO model in mice. These experiments confirmed the protective role of CB2R, since CB2R KO mice had larger cerebral infarction, and worse neurological function compared to WT mice (Zhang et al., 2009). A similar result was found in a study performed in our laboratory using adult CB2R KO mice following HI. In this study, the absence of CB2R induced more behavioural deficits, and more extensive brain injury. These alterations were associated with the modulation of the inflammatory-related HIF-1α/TIM-3 signalling pathway in microglia (Kossatz et al., 2016). This study is part of the results of this thesis (Article 1).

5.2.2. Deletion of peroxisome proliferator-activated receptor-alpha

To our knowledge, only few studies have been conducted specifically to determine the role of PPAR-α on cerebral ischemia-induced brain damage. In this sense, a study performed on PPAR-α KO and WT mice reported no differences between groups on the lesion volume after permanent MCAO (Arsenijevic et al., 2006). Contrasting with these findings, only a single study revelated that

the deletion of PPAR-α increases the deleterious effect of cerebral ischemia. In this study, using MRI technology, a modest but significant increase in the extent of the lesion was observed in PPAR-α KO mice over the first 14 days after permanent MCAO. Albeit the differences found in the extent of the lesion, no differences in overall brain oedema were observed between groups (Pialat et al., 2007). The reasons for the discrepancy in the different studies carried out in PPAR-α KO may be due to differences in the model used (transient versus permanent occlusion), and in the methodology applied (single time point versus longitudinal study).

Interestingly, gender differences have been observed with respect to cerebral ischemia in PPAR-α KO mice. Thus, larger infarct volume, greater neurological deficits, and an increase in peripheral pro-inflammatory and adhesion molecule gene expression were found in PPAR-α KO males than in females after transient MCAO, indicating that PPAR-α also plays an important role in sex differences in the immune response to cerebral ischemia (Dotson et al., 2016b).

5.3. Effects of cannabinoid and cannabinoid-like receptor ligands

The neuroprotective potential of compounds targeting the ECS has been extensively investigated over the last years. Their potential is due to the capability of these compounds to restrict the influence of multiple deleterious stimuli such as excitotoxicity, oxidative stress, and inflammation on neuronal homeostasis and survival. These

noxious stimuli collaborate in deteriorating neurons in cerebral ischemia. Thus, synthetic compounds have been developed, some of which exhibit high potency at CB1R (arachidonyl-2-chloroethylamide: ACEA; CP 55,940; rimonabant; AM251; SR141716), CB2R (trans-caryophyllene: TC; JWH-133; O-1966; O-3853; AM1241) or at both CB1R/CB2R (HU-210; TAK-937; BAY 38-7271; WIN 55,212-2), and at PPAR-α (fenofibrate; gemfibrozil; Wy-14643; clofibrate).

5.3.1. CB1 receptor ligands

As previously reviewed, studies show that CB1R are abundantly localized in neurons mostly at presynaptically level (Tsou et al., 1998), and in astrocytes (Navarrete and Araque, 2008). Moreover, CB1R expression increases in activated microglia following ischemia (Schmidt et al., 2012), as well as in cortical neurons after MCAO (Jin et al., 2000). These data suggest that CB1R may be involved in cell death and/or inflammatory processes following brain damage, and that endogenous or exogenous CB1R ligands could be crucial as neuroprotective agents against brain injury. In this sense, studies performed in different rodent models of ischemia showed beneficial effects in the use of selective CB1R agonists. For instance, the pre-treatment with the synthetic CB1R agonist, CP 55,940, administered in gerbils with BCCAO, reversed the decrease in electroencephalography (EEG) power and the hyperlocomotion induced by an ischemic episode, and this effect was reverted by rimonabant (Braida et al., 2000). Similarly, the post-

treatment with THC in the same model, reduced hippocampal neuronal damage, and memory deficits, which were blocked by naloxone, suggesting that the interaction between CB1R and opioid receptors may be involved in this neuroprotective mechanism (Zani et al., 2007). In addition, studies carried out in an *in vitro* model of neonatal HI, the neuroprotective effects of the selective CB1R agonist, ACEA were reversed by rimonabant when brain slices from new born rats are exposed to oxygen-glucose deprivation (OGD) (Fernández-López et al., 2006).

Interestingly, it was reported that CB1R are also located in mitochondria, participating in the control of cellular respiration and energy production (Bénard et al., 2012). The expression of CB1 mitochondrial receptors appears to be upregulated in hippocampal neurons following BCCAO and reperfusion, and this effect was potentiated by ACEA (Ma et al., 2015). These data suggest that the overexpression of CB1R could be an adaptive mechanism to counteract neuronal injury following ischemic insult. In the same study, ACEA improved the neurological deficits, and reduced apoptosis. Moreover, this neuroprotective effect was reversed by the CB1R antagonist AM251, while the selective cell-impermeant CB1R antagonist, hemopressin only showed a partial effect (Ma et al., 2015).

Studies using the non-selective CB1R/CB2R agonist, WIN 55,212-2 also support the neuroprotective role of CB1R in ischemia models. Thus, pre-treatment with WIN 55,212-2 showed

neuroprotection in both transient and permanent MCAO, and this effect was abolished by the selective CB1R antagonist, rimonabant (Nagayama et al., 1999). Moreover, in adult rats exposed to transient MCAO, repeated post-ischemia treatment with WIN 55,212-2 for 14 days increased the remyelination in peri-infarcted areas through regulation of the level of the p-ERK 1/2 via CB1R (Sun et al., 2013). Similarly, pre-treatment with WIN 55,212-2 for 3 or 5 days showed neuroprotective effects that were partially reversed by the potent and specific mitogen-activated protein kinase inhibitor, U0126 (Hu et al., 2010). In line with these findings, post-ischemia treatment with WIN 55,212-2 showed neuroprotective effects in *in vitro* and *in vivo* models of HI in new born rats, and these effects were reversed by both CB1R and CB2R antagonists, rimonabant and SR141588, respectively (Fernández-López et al., 2006, 2007).

Most of the studies reported here suggest the neuroprotective role of CB1R activation. However, a very limited number of studies have investigated the mechanisms involved in these neuroprotective effects of CB1R activation, and there is evidence that they may be due to a non-specific hypothermic effect. Consequently, the decreased infarct volume observed in a 4 h MCAO mouse model by the primary psychoactive constituent of cannabis, THC, administered twice (before and 3 h after MCAO) was mediated through a temperature-dependent mechanism via the CB1R (Hayakawa et al., 2007). Likewise, the protective effects of the synthetic CB1R agonist, HU-210, administered in rats 1-6 h

after permanent MCAO, were partially blocked by pre-treatment with rimonabant, but were completely abolished by warming of the animals (Leker et al., 2003). Another study showed that the post-administration of CB1R/CB2R agonist, TAK-937, decreased the brain damage induced by MCAO, an effect that was mediated by CB1R activation, but also in part by the induction of hypothermia (Suzuki et al., 2012). In contrast to these findings, another CB1R/CB2R agonist; BAY 38-7271, induced moderate neuroprotective effects in rats after MCAO, and this effect was attributed to a CB1R mediated mechanism, and not to its hypothermic effects (Mauler et al., 2002).

All these findings support the beneficial effects of both pre- and post-ischemia CB1R activation on brain damage. Despite this, there are data available in different animal models showing that CB1R activation may be detrimental in the ischemic process. In a gerbil model of transient global ischemia, it has been shown that BCCAO induced a marked degeneration of pyramidal cells in the CA1 region of the hippocampus, and that post-administration of WIN 55,212-2 did not display neuroprotective effects, while the CB1R antagonist, AM251, reduced CA1 pyramidal cell loss (Landucci et al., 2011). Accordingly, rimonabant reduced brain damage and prevented the hyperlocomotion and memory impairments observed after BCCAO in gerbils through activation of TRPV1 receptors (Pegorini et al., 2006). Consistent with these findings, in rat stroke models it has been shown that the CB1R antagonist, SR141716,

reduces infarct volume following MCAO (Berger et al., 2004; Muthian et al., 2004; Sommer et al., 2006; Amantea et al., 2007).

At present, the controversial pharmacological data obtained, and the lack of studies using CB1R KO mice, make these results very difficult to interpret. Thus, additional research is needed to understand these conflicting findings in terms of neuroprotection from brain injury.

5.3.2. CB2 receptor ligands

As previously reviewed, several studies have demonstrated that CB2R present in human astrocytes (Sagredo et al., 2009) and microglia (Maresz et al., 2005; Ashton et al., 2007) increase their expression following HI (Fernández-Ruiz et al., 2008; Zhang et al., 2008). Since both microglia and astrocytes have been associated with inflammation processes, and may contribute to cell death and increased infarct size (Jin et al., 2013), research has been focused on the study of the neuroprotective role of CB2R activation in ischemia-induced brain damage.

For this purpose, *in vitro* models of OGD in brain slices and cultured cells have been used to investigate the role of CB2R activation in neuroprotection following cerebral ischemia, as well as the mechanisms involved in this process. For instance, a study performed with the CB2R agonist TC, hypoxia-induced cytotoxicity of cultured BV2 microglia cells was suppressed through inhibition of nuclear factor-κB (NF-κB) activation (Guo et al., 2014).

Similarly, in another study, TC diminished neuronal damage following OGD/re-oxygenation in rat cortical neurons/glia mixed cultures, and this effect was reversed by the selective CB2R antagonist, AM630, but not by the CB1R antagonist, AM251 (Choi et al., 2013). In a study preformed in an *in vitro* model of neonatal hypoxic-ischemic encephalopathy, where brain slices from new born rats are exposed to OGD, the use of the CB2R agonist, JWH-133, was neuroprotective, and its beneficial effects were reversed by the CB2R antagonist, SR141588 (Fernández-López et al., 2006). In this same model, the agonist WIN 55,212 was also neuroprotective, and this effect was mediated through a decrease in glutamate and cytokine release, as well as toxic nitric oxide production. Moreover, this beneficial effect was reversed by both rimonabant and the CB2R antagonist, SR141588 (Fernández-López et al., 2006).

Various *in vivo* models have been used to evaluate the neuroprotective role of pre- and post-treatment with CB2R agonists on ischemia-induced brain damage. In a mouse model of MCAO, the continuous administration of the CB2R agonist, JWH-133 using micro osmotic pumps 4 h before the ischemic insult and during the subsequent 3 days (Murikinati et al., 2010), or a single administration 10 min after the ischemic insult (Zarruk et al., 2012), decreased the infarct volume. Other CB2R agonists such as O-3853 and O-1966 also decreased cerebral infarction, and improved motor function in mice when administered by intravenous injection 1 h before transient MCAO (Zhang et al., 2007, 2009). The neuroprotective effects of these agonists were associated to the

inhibition by CB2R of either neutrophil recruitment (Murikinati et al., 2010), inhibition of leukocyte/endothelial adhesion (Zhang et al., 2007, 2009), or to a decrease in microglia/macrophage activation (Zarruk et al., 2012). In line with these findings, a study performed in rats exposed to MCAO showed that the treatment with TC 3 h post-ischaemia, decreased the cerebral infarct volume and oedema and increased phosphorylated CREB and brain-derived neurotrophic factor expression in neurons. These effects were reversed by co-administration of AM630 (Choi et al., 2013). Another study showed that pre-treatment with the selective CB2R agonist, AM1241 reduced brain infarction and neurological deficits in rats exposed to MCAO, but not when it was administered from 2 to 5 days after the procedure (Yu et al., 2015). In contrast to these studies, one report showed that the CB2R agonist GW405833, administered to adolescent rats either immediately before HI, 30 min after HI or by repeated treatment every 24 h for 6 days post-HI did not reduce brain damage (Rivers-Auty et al., 2014). A possible explanation for these conflicting results may be the different compounds or animal models used in these studies. Interestingly, a study performed in MCAO showed that the most effective neuroprotective treatment against brain damage was the combination of the CB1R antagonist rimonabant, with the CB2R agonist O-1966, indicating a synergistic effect derived from combining both agents (Zhang et al., 2008).

On the other hand, CB2R have also been implicated in the neuroprotective effects of the phytocannabinoid, cannabidiol

(CBD). In a study, after OGD in new born mouse brain slices, it was observed that CBD reduced cell death, glutamate release, cytokine production, COX-2 and iNOS expression and these effects were reversed by the CB2R antagonist AM630, and by the adenosine A2A antagonist SCH58261 (Castillo et al., 2010). Moreover, CBD also reduced excitotoxicity, oxidative stress and inflammation in the HI model using new born piglets, and these effects were blocked by the CB2R antagonist AM630, and by the serotonin 1A receptor antagonist, WAY100635 (Pazos et al., 2013).

In contrast to the controversial results obtained for CB1R, most of the data for CB2R provide clear evidence that its activation induces neuroprotective effects when administered either before or after the onset of ischemia. In addition, the possible mechanisms proposed for these beneficial effects are mostly related to the modulation of inflammatory processes, although it is still not known which downstream mediators participate in these neuroprotective effects of CB2R activation.

5.3.3. PPAR-α ligands

A growing amount of evidence suggests that activation of this receptor by natural (polyunsaturated fatty acids and eicosanoids) or synthetic ligands (hypolipidemic fibrates) regulates inflammation and repair in the brain (Bordet et al., 2006; Collino et al., 2006; Moraes et al., 2006). Several studies have been performed regarding the use of PPAR-α agonists as treatment in neuroinflammation-

related diseases, especially in experimental stroke. In this sense, MCAO and 4VO models have been extensively used in last years to evaluate the effects of synthetics and endogenous PPAR-α agonists on cerebral ischemia-induced brain injury. Among the PPAR-α synthetic agonists, the fibrates, lipids used in clinical practice to decrease triglycerides, have been widely tested (Derosa et al., 2017). Fibrates include various types of drugs, such as fenofibrate, gemfibrozil, Wy-14643 and clofibrate.

A study performed in mice subjected to transient MCAO has shown that 14 days of pre-treatment with fenofibrate reduced the infarct volume through the inhibition of inflammation pathways, and upregulation of brain antioxidant enzymes (Deplanque et al., 2003). Specifically, the neuroprotective effect was associated with a reduction in cerebral oxidative stress due to the increase in copper/zinc superoxide dismutase, an enzyme involved in glutathione metabolism. Moreover, in the same study, chronic treatment with fenofibrate decreased the brain expression of vascular cell adhesion molecule-1 (VCAM-1), and ICAM-1 (Deplanque et al., 2003). Thus, the increase in antioxidant enzyme activities in the brain and the decrease in adhesion protein expression are probably two of the mechanisms by which this agent protects against cerebral injury. Another study has reported that pre-treatment with fenofibrate during 7 days also protected the brain against ischemia in mice subject to transient MCAO (Wang et al., 2010). In this study, fenofibrate attenuated oxidative stress after cerebral ischemia through an increase in mRNAs and activities of

superoxide dismutases (SODs) levels in brain microvessels. Moreover, fenofibrate reduced de infarct size in the cortex when treated 1 hour before MCAO (Wang et al., 2010). Similarly, fenofibrate were found to reduce infarct volume significantly following 3 days of pre-treatment in mice subjected to permanent MCAO (Sun et al., 2007). In addition, Guo and colleagues (2010) have studied the temporal changes of cortical CBF in the ischemic hemisphere in the absence and presence of fenofibrate. Laser speckle imaging showed CBF reductions in the ischemic hemisphere after transient MCAO. In the animals treated with vehicle, the cortical CBF continued to decrease up to 2 h after MCAO, indicating the expansion of ischemic area, while 7 days of pre-treatment with fenofibrate improved CBF in the ischemic brain and reduced the infarct volume. Fenofibrate treatment also enhanced CBF recovery after reperfusion, suggesting that it reduced the no-reflow phenomenon (Guo et al., 2010). Ouk and colleagues (2014a) have investigated the effects of fenofibrate, administered during the acute phase of ischemia, on brain damage, functional impairment, and ischemia-induced memory alterations. In this study, rats were subjected to transient MCAO followed by early fenofibrate administration (1 h after MCAO) for 72h or followed by delayed administration (8 h after MCAO) for 7 days. Early and delayed administration of fenofibrate induced a significant decrease in the infarct size and reduced the functional impairments observed. Moreover, fenofibrate decreased the post-ischemic inflammation resulting from microglial activation and parenchymatous infiltration by polynuclear neutrophils. Other

interesting findings of this study were that fenofibrate increased post-ischemic neurogenesis, prevented the appearance of post-ischemic amyloid deposition that contributes to the occurrence of post-stroke dementia, and induced a delayed protective effect on memory recovery. Finally, in mice subjected to MCAO, fenofibrate induced an acute protective effect on leukocyte rolling and adhesion (Ouk et al., 2014a). In another study, 7 days of post-treatment with fenofibrate significantly reduced hippocampal neuronal death, and improved memory impairment and hippocampal neurogenesis in rats subjected to 4VO (Xuan et al., 2015). Moreover, fenofibrate also inhibited overactivation of microglia induced by 4VO and prevented upregulations of pro-inflammatory mediators in hippocampus. Interestingly, this anti-inflammatory effect was mediated by inhibiting activation of P65, NF-κB and P38 MAPK in the hippocampus (Xuan et al., 2015).

Interestingly, fenofibrate pre-treatment affects male and female mice differently in the MCAO model. Mice were treated with a low-dose of fenofibrate 30 min prior to transient MCAO and once a day for 3 additional days post-ischemia. A reduction in infarct volume in male mice was observed due to an increase in M2 macrophage gene expression in ischemic tissue, while females exhibited an increase in pro-inflammatory cytokine gene expression. Moreover, fenofibrate induced an increase in regulatory cells in the periphery in male mice, but a decrease in female mice. The differences between male and female mice is likely due to the lower PPAR-α expression in cells and tissues in females vs males,

suggesting that males and females respond to PPAR-α activation differently (Dotson et al., 2016a).

Inoue and colleagues (2003) have investigated the effects of two synthetic PPAR-α agonists: fenofibrate and Wy-14,643. In this study, 3 days of pre-treatment with fenofibrate or 7 days of pre-treatment with Wy-14,643 reduced the infarct volume in WT mice subjected to permanent MCAO. They also investigated the effect of resveratrol, a polyphenol that is abundantly found in the skin and seeds of grapes and share a great similarity in their biological actions with the PPAR-α agonists. Three days of pre-treatment with resveratrol also reduced the infarct volume in WT mice subjected to the same model, but not in PPAR-α KO mice, suggesting that resveratrol, as well as fenofibrate, requires the expression of PPAR-α to exert neuroprotection against permanent focal cerebral ischemia (Inoue et al., 2003).

Another study investigated the effects of gemfibrozil, using two different mouse models of cerebral ischemia: permanent MCAO and distal MCAO (Guo et al., 2009). Seven days of pre-treatment with gemfibrozil in mice subjected to permanent MCAO induced a significant reduction in infarct volume at 24h after MCAO. Similar results were obtained in mice subjected to distal MCAO, where 7 days of pre-treatment combined with 7 days of post-treatment with gemfibrozil has also reduced the infarct volume at 7 days after MCAO. However, the results of the current study should be interpreted with caution since neither behavioural nor neurological

deficits were observed following MCAO (Guo et al., 2009). Mohagheghi and colleagues (2013) have studied the effects of pre-treatment with gemfibrozil during 7 days in ischemic injury using the 4VO global model in male and female rats. In female rats, gemfibrozil was neuroprotective through the modulation of inflammatory factors and induction of antioxidant defense system including SOD, catalase, and glutathione level. In contrast, pre-treatment with gemfibrozil was detrimental in male rats, since this agonist increased the expression of inflammatory factors, such as TNF-α, NF-κB, and COX-2, and decreased Nrf-2 expression and SOD activity, leading to hippocampal neurodegeneration (Mohagheghi et al., 2013b). This gender-dependent effect of gembifrozil associated to global cerebral ischemia was also observed in another study from the same group (Mohagheghi et al., 2013a). Following the same treatment protocol, in a 4VO model in rats, gembifrozil confered protection to metestrous females against global cerebral insult, while detrimental within male rats resulting in hippocampal CA1 neurodegeneration. This neuroprotective effect was associated with the induction of mitochondrial pro-survival factors involved in the mitochondrial biogenesis-signaling pathway, and inhibition of caspase-dependent apoptosis (Mohagheghi et al., 2013a).

Collino and collegues (2006) investigated the effect in hippocampus of an acute dose of Wy-14,643, 30 and 60 min before the induction of transient 4VO. In that study, the levels of S100B protein, a marker of cerebral injury, were high in the hippocampus of rats,

and this effect was blunted by Wy-14,643. Moreover, it reversed oxidative stress, decreased the expression of heme oxygenase-1, iNOS and ICAM-1, and suppressed the activation of p38 mitogen-activated protein kinase and NF-κB. The PPAR-α antagonist, MK886 abolished the beneficial effects of Wy-14,643, indicating that this compound protects the brain against excessive oxidative stress and inflammation through the activation of PPAR-α (Collino et al., 2006).

Atorvastatin is a member of the statins that inhibits HMG-CoA reductase, which is used primarily as a lipid-lowering agent. This compound has also demonstrated neuroprotective effects against experimental stroke (Ouk et al., 2014b). In mice subjected to transient MCAO, 14 days of pre-treatment with atorvastatin reduced the infarct volume through PPAR-α. Also, the anti-inflammatory action of atorvastatin was mediated, at least partly, by PPAR-α. Treated animals presented a decrease in IL-6 plasmatic levels, as well as, a reduction in the expression of the adhesion molecule ICAM-1 and VCAM-1 in the cortex. Moreover, atorvastatin also diminished the cerebral expression of iNOS in the cortex, suggesting that PPAR-α is a key mediator of the multitargeted neuroprotective effects of statins in stroke (Ouk et al., 2014b).

Interestingly, endogenous PPAR-α agonists such as OEA and its analogues have also demonstrated a potential neuroprotective role

against cerebral ischemia. This issue is reviewed in depth in the chapter 5.4.

In sum, all these data suggest that PPAR-α expression exert brain protection against neuroinflammation, and particularly cerebral ischemia, indicating that this receptor may be a potential target for developing drugs to counteract the deleterious effect of this disease.

5.4. Neuroprotective role of N-acylethanolamides and their analogues

NAEs are lipid molecules present in animal and plant tissues. Among the NAEs, AEA and OEA have attracted great attention due to their endocannabinoid activity with anti-inflammatory, analgesic, and anorexic properties. Furthermore, the potential neuroprotective actions of NAEs have been widely studied in the last years owing to the increasing amount of evidence showing that these molecules accumulate in the brain during cerebral ischemia (Hillard, 2008).

5.4.1. Anandamide

AEA binds to cannabinoid CB1R (Mackie et al., 1993; Showalter et al., 1996) and CB2R (Felder et al., 1996; Showalter et al., 1996), as well as to the vanilloid TRPV1 (Zygmunt et al., 1999). The content of AEA in the brain varies depending on the structure, with high levels present in hippocampus, thalamus, striatum and brainstem,

while lower expression is observed in the cerebral cortex and cerebellum (Felder et al., 1996; Bisogno et al., 1999).

Additionally, several studies have reported that AEA accumulates following ischaemic events as part of an on-demand compensatory response. This fact has lead several laboratories to hypothesize that this increase is related to an endogenous protective pathway. In a study carried out in a human subject using *in vivo* microdialysis, release of AEA was reported following an ischaemic stroke in the MCA territory. The increase in AEA levels occurred in penumbral tissue, and was significantly associated with elevations in extracellular lactate, an early hypoxic insult marker (Schabitz et al., 2002). In addition, an early increase in AEA levels was also detected in stroke patients, and a positive correlation with stroke volume and neurological deficits was reported (Naccarato et al., 2010).

In animal models, accumulation of AEA following permanent MCAO has been observed in the cortex of mice, which was correlated with an increase in microglial cell motility (Franklin et al., 2003). In rats, permanent MCAO increases AEA levels in ipsilateral striatal and cortical tissues (Berger et al., 2004). Similarly, increases in brain AEA content in the ischaemic hemisphere were reported in a transient MCAO model induced in rats (Muthian et al., 2004). There are evidences that global ischemia also increases AEA levels. Thus, rats subject to 30 min of BCCAO followed by 60 min of reperfusion induced increases of AEA in the frontal cortex and plasma (Quartu et al., 2017). Interestingly, these studies show that

cerebral ischemia increases the levels of other NAEs as well (Schabitz et al., 2002; Berger et al., 2004; Quartu et al., 2017), which could play a relevant role in neuroprotection from ischemic insult.

These studies contrast with other data showing no accumulation of AEA after permanent MCAO induced in adult mice (Degn et al., 2007), or in adult rats after transient MCAO (Kilaru et al., 2011), and the 4VO model (Melis et al., 2006). Thus, it may be possible that the endogenous levels of NAEs attained in some pathological conditions of brain injury are inadequate to provide neuroprotection. In this sense, the administration of exogenous NAEs may be useful to enhance the neuroprotective effect. Indeed, exogenous AEA has shown neuroprotective effects in *in vitro* models of neurodegenerative diseases (Benito et al., 2012), and in ischaemic (Nagayama et al., 1999; Sinor et al., 2000; Hawkins and Butt, 2013), and non-ischaemic brain injury models (Eljaschewitsch et al., 2006). In a model of OGD, neuroprotective effects of AEA and methanandamide (a non-hydrolysable analogue of AEA) were observed on cell survival in cerebral cortical neuron cultures (Nagayama et al., 1999; Sinor et al., 2000), and this protective effect was not mediated by CB1R or CB2R (Sinor et al., 2000). In isolated intact optic nerves subject to OGD, methanandamide significantly protected oligodendrocytes against hypoxic disruption and death (Hawkins and Butt, 2013).

In line with these findings, AEA has also shown neuroprotective effects in *in vivo* models of cerebral ischemia. Post-treatment with

AEA significantly reduced the size of infarcted tissue in rats following transient MCAO, by acting as an agonist on cerebral CB1R (Schomacher et al., 2008). Similarly, AEA administered after perinatal HI brain injury in a rat model, led to a reduction in brain injury, reducing apoptotic cell death, maintaining mitochondrial functionality and improving cellular parameters, including influx of calcium into cells and the production of ROS (Lara-Celador et al., 2012).

Albeit these data, there is existing *in vitro* and *in vivo* data reporting no neuroprotective effects of exogenous AEA. In human brain microvascular endothelial cell and astrocyte co-cultures subjected to OGD, AEA was found to reduce the permeability of the BBB mediated through CB2R, TRPV1 and calcitonin gene-regulated peptide receptors. In addition, AEA and methanandamide did not prevent cerebral infarction in mice when administered immediately before or 3 hours after permanent MCAO (Mishima et al., 2005). In line with these data, it was reported that higher concentrations of AEA may induce neuronal toxicity in *in vitro* and *in vivo* models of neurotoxicity (Cernak et al., 2004; Movsesyan et al., 2004; Landucci et al., 2011). In organotypic hippocampal slices exposed to OGD, AEA increased CA1 injury, and this neurotoxic effect was exacerbated by the FAAH inhibitor, URB597. Moreover these effects appeared to be mediated by CB1R and TRPV1 (Landucci et al., 2011).

In summary, despite the discrepancies noted above, there is evidence suggesting an important contribution of AEA in neuroprotection after cerebral ischemia in different experimental conditions. However, the use of other NAEs as neuroprotective agents has also been proposed due to their potential for enhancing AEA activity as part of what is called an "entourage effect".

5.4.2. Oleoylethanolamide

Despite its structural similarity with AEA, OEA is not a ligand at classical CB1R and CB2R. OEA binds to PPAR-α (Fu et al., 2003, 2005), TRPV1 (Wang et al., 2005), GPR55 (Brown, 2009), and GPR119 (Overton et al., 2006).

Studies that have analysed the content of OEA in the brains of mice and pigs immediately after death (Patel et al., 2004; Schmid et al., 1995) show that the content of OEA is much higher than AEA, and that they both accumulate in a time-dependent manner after death, with different distribution throughout brain structures (Schmid et al., 1995; Patel et al., 2005). Similar results were obtained when evaluating the content of OEA after brain injury in various models of cerebral ischemia. For instance, a study carried out in mice has demonstrated an increased in the content of OEA under CO_2-induced hypercapnia/ischemia (Lin et al., 2017). These data in animal models are consistent with a study performed in a patient with a hemispheric stroke showing the release of OEA in the penumbral tissue surrounding the primary ischemic lesion,

measured by *in vivo* microdialysis (Schabitz et al., 2002). The increase in OEA was significantly associated with elevations in extracellular lactate, an early hypoxic insult marker, suggesting the involvement of the NAEs signalling system within the ischemic cascade (Schabitz et al., 2002).

OEA has shown neuroprotective effects in *in vitro* models of ischaemic brain injury. A study has been carried out to explore the role of OEA in modulating BBB permeability in normal conditions and in an OGD model using human brain microvascular endothelial cells culture (Hind et al., 2015). Interestingly, OEA decreased BBB permeability in normal conditions, conferring resistance to this barrier through PPAR-α. Moreover, the pre-treatment with OEA reduced the OGD-induced increase in permeability during reperfusion, suggesting that OEA provides protection to the BBB during ischaemic stroke (Hind et al., 2015).

These data in human cells are consistent with *in vivo* mouse studies evaluating the neuroprotective role of PPAR-α activation by a 3-day preventive of OEA intraperitoneal administration. In this study, OEA was able to reduce the extent of the lesion in WT mice subject to permanent MCAO, but not in PPAR-α KO mice (Sun et al., 2007). Moreover, OEA increased CNS levels of the anti-inflammatory mediator IκBα, while decreasing the pro-inflammatory enzyme COX-2. In contrast, a single post-treatment with OAE had no effect on lesion size (Sun et al., 2007). In addition, Zhou and colleagues (2012) found that oral pre-treatment

with OEA, during 3 days before transient MCAO in mice, improved neurological dysfunction, reduced infarct volume and alleviated brain oedema in a dose-dependent manner, and that these protective effects were abolished by the PPAR-α antagonist MK886 (Zhou et al., 2012). OEA also exerted therapeutic effects on ischemia brain injury with the administration of a single dose of OEA, immediately or shortly after reperfusion. The protective effects of OEA were complete only when administered at 1 h after reperfusion. With this administration protocol, OEA reduced the oedema and improved neurological score, but did not affect the infarct volume, suggesting that the optimal timing of post-ischemic treatment was within 1 h after reperfusion (Zhou et al., 2012). In the same line, a single intraperitoneal administration with OEA during reperfusion reduced cell apoptosis through the suppression of Bax, and the upregulation of Bcl-2 levels in WT, but not in PPAR-α KO mice subjected to transient MCAO. Moreover, OEA inhibited MCAO-induced TLR4 expression, NF-ϰB activation, IϰBα degradation, and ERK1/2 phosphorylation, suggesting that the anti-apoptosis effects of OEA were mediated, at least in part, by PPAR-α signalling and inhibition of both TLR4/NF-ϰB and ERK1/2 signalling pathways (Zhou et al., 2017).

Another study investigated the effects of chronic OEA treatment on ischemia-induced cognitive impairments, electrophysiology, glial alterations and hippocampal neurogenesis in rats. Treatment with OEA (i.p.) for 28 days following transient MCAO significantly reversed spatial cognitive deficits and attenuated the inhibition of

long-term potentiation (LTP). Interestingly, the recovery of cognitive function was associated with enhanced neurogenesis in the hippocampus, suggesting that chronic OEA treatment can exert neuroprotective effects against cerebral ischemic insult in rats via triggering of neurogenesis in the hippocampus (Yang et al., 2015).

These data indicate that both intraperitoneal and oral administration of OEA protect the brain against acute ischemic injury in mice by activating PPAR-α. Although the outcomes are clinically relevant, the therapeutic window of OEA seems to be too short for clinical use in treating ischemic stroke. Moreover, the rapid degradation of the OEA by FAAH is known to be a problem for the development of an effective therapeutic approach. Therefore, modelling OEA analogue drugs with high affinity for the PPAR-α might solve this issue, and facilitate the development of more stable neuroprotective drugs.

5.4.3. Sulfamoyl derivatives of OEA: Octadecylpropyl Sulfamide

Given the pharmacological profile of OEA as an anti-obesity and neuroprotective agent, novel OEA-modelled PPAR-α agonists based in a sulfamoyl moiety were synthesized (Cano et al., 2007). Recent studies have demonstrated the potential utility of these novel compounds in animal models of obesity. Among them is the trans-analogue of OEA, elaidylsulfamide (ES), a lipid mediator of satiety that interacts with the PPAR-α as an agonist (Decara et al., 2012). Decara and colleagues (2012) have shown that the

administration of ES in obese rats was able to improve hepatic function, reducing feeding and weight gain, lowering plasma cholesterol, and reducing the plasmatic activity of transaminases through the modulation of both cholesterol and lipid metabolism regulatory genes. However, the chronic administration of ES induces insulin resistance, limiting its development as an anti-obesity drug (Decara et al., 2012). Similarly, N-[1 -(3,4-dihydroxyphenyl)propan-2-yl]oleamide (OLHHA), a conjugation of oleic acid with an amphetamine derivative that presents affinity for both CB1 and PPAR-α, has also shown anti-obesity properties (Decara et al., 2015). The study indicated that chronic administration of OLHHA to obese Zucker rats reduced both hepatic lipid accumulation and circulating triglyceride levels. Moreover, a general improvement in plasma parameters related to liver damage was observed in these animals. These findings were associated with anti-apoptotic activity and with a downregulation of the expression of enzymes involved in the biosynthesis of lipids in the liver, suggesting its potential therapeutic application for the treatment of non-alcoholic fatty liver disease (Decara et al., 2015).

Octadecylpropyl sulfamide (SUL) is the most potent and stable compound from the new sulfamide derivatives of OEA. It is characterized as an effective concentration-dependent activator of PPAR-α, with potent hypolipidemic properties, and feeding suppressant effects (Cano et al., 2007). Cano and colleagues (2007) have shown that 8 days of SUL treatment in rats mimics the effects of OEA (Rodríguez de Fonseca et al., 2001; Fu et al., 2003),

reducing body weight and plasma triglycerides. Similar effects were observed in genetically obese Zucker rats, where 11 days of treatment reduced body weight gain and food intake (Cano et al., 2007). Interestingly the potency of SUL was greater than OEA, since the hypolipemiant effects of OEA are present only at doses greater than 5 mg/kg (Fu et al., 2003, 2005), whereas SUL was found to be effective at a dose of 1 mg/kg (Cano et al., 2007).

On the other hand, data obtained in *in vivo* assays in rodents indicated a differential action for OEA as compared to SUL. As previously exposed, both compounds have anorectic effects in rats as appetite-suppressing agents, weight gain inhibitors and hypolipidemic agents (Cano et al., 2007; Moreno-Santos et al., 2014). However, on the writhing test in mice an antinociceptive effect was observed for OEA, but not for SUL. These findings suggest that OEA activity in PPAR-α may be dissociated from others actions at alternative targets, since other non-cannabimimetic ligands that interact with PPAR-α, such as SUL, do not reproduce the full spectrum of the pharmacological activity of OEA (Moreno-Santos et al., 2014).

With regards to neuroprotection, SUL has shown protective effects in neuron cultures exposed to the neurotoxin 6-OHDA, reducing cell death and increasing the cell viability (Rodríguez de Fonseca et al., 2012), indicating a possible efficacy of SUL in neurodegenerative diseases. In this context, our laboratory has evaluated the neuroprotective effects of SUL in global ischemia-

induced brain damage, using an adult mice model of HI (Kossatz et al. 2018, in press). Interestingly, an acute administration of SUL after HI reversed the cognitive and motor coordination impairment induced by HI through the activation of PPAR-α receptors, enhancing CB2R, decreasing FAAH and downregulating CB1R mRNA expression. This treatment also reduced inflammatory parameters and neuronal cell death in the hippocampus and motor cortex. These findings suggest that the potent and stable PPAR-α agonist SUL could be a good candidate to counteract the harmful effect of HI brain damage, especially in restoring the cognitive and motor function. This study is part of the results of this thesis (Article 2).

OBJECTIVES

Specific objectives

Article 1: CB2 Cannabinoid Receptors Modulate HIF-1α and TIM-3 Expression in a Hypoxia-Ischemia Mouse Model

1. To evaluate the neuroprotective role of CB2R and to determine the progression of the cognitive and behavioural alterations associated with HI-induced brain injury in adult mice.

2. To evaluate the role of CB2R in the activation of microglia and astrocyte expression following HI-induced brain damage in the hippocampus, the striatum, and the motor cortex of adult mice.

3. To study the involvement of CB2R in the expression of microglial pro-inflammatory factors HIF-1α and TIM-3 following HI-induced brain damage in different brain areas of adult mice.

Article 2: Octadecylpropyl Sulfamide Reduces Neurodegeneration and Restores the Memory Deficits Induced by Hypoxia-Ischemia in Mice

1. To assess the effects of SUL on the cognitive and behavioural alterations associated with HI-induced brain damage in adult mice.

2. To evaluate the effects of SUL on neurodegeneration processes, microglial and astrocyte expression in the hippocampus and cortex following HI-induced brain damage in adult mice.

3. To evaluate changes in gene expression related to the neuroinflammation/endocannabinoid signalling systems in the hippocampus and cortex following HI-induced brain damage in adult mice.

4. To determine whether the effects of SUL on the cognitive, behavioural, and molecular alterations associated with HI-induced brain damage are mediated by the PPAR-α.

RESULTS

Article 1: CB2 Cannabinoid Receptors Modulate HIF-1α and TIM-3 Expression in a Hypoxia-Ischemia Mouse Model

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European Neuropsychopharmacology. 2016; 26 (12):1972–1988

Kossatz E, Maldonado R, Robledo P. CB2 cannabinoid receptors modulate HIF-1α and TIM-3 expression in a hypoxia-ischemia mouse model. Eur Neuropsychopharmacol. 2016 Dec;26(12):1972–88. DOI: 10.1016/j.euroneuro.2016.10.003

Article 2: Octadecylpropyl Sulfamide Reduces Neurodegeneration and Restores the Memory Deficits Induced by Hypoxia-Ischemia in Mice

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DISCUSSION

1. Cerebral Ischemia

Global cerebral ischemia is caused by a deficiency of oxygen and glucose supply in the brain, resulting often from a cardiac arrest and severe cerebral hypoperfusion in both adults and children. Its consequences in the brain are variable; in mild or moderate hypoxia the prognosis is favourable with the recovery of function, while in severe insult, when the circulatory arrest is not restored quickly the brain injury could be irreversible, leading to severe and permanent neurologic disabilities. Likewise, cerebral ischemia has an elevated risk of mortality, with few available treatments, being the first action to restore the blood flow through a reperfusion treatment.

To reduce the devastating impact of cerebral ischemia on society, research continues to seek ways to achieve better functional recovery in patients suffering from this condition. In this context, neuroprotective agents that can limit the ischemic damage have generated long-term interest. Investigations in stroke have been focused on the search for agents that may modulate receptors to reduce the release of excitatory neurotransmitters and prevent neuroinflammation. In addition, the possibility to expand the therapeutic time window to improve the treatment against this condition is also under investigation. In this context, the ECS has been put forward as a key modulator of excitotoxicity, oxidative stress, and inflammation (*reviewed in* section 5.2), and could thus have an important neuroprotective potential in the treatment of stroke.

Considering these factors, the main purpose of this thesis was to study the neuroprotective function of CB2R and the neuroprotective effects of the potent and stable PPAR-α agonist, SUL in HI-induced brain damage and associated cognitive, behavioural and molecular alterations in mice, as well as the mechanisms involved. The general results presented in this thesis have shown a crucial molecular mechanism involved in the neuroprotective role of CB2R following HI through the modulation of the inflammatory-related protein HIF-1α and TIM-3 in microglia, decreasing the extent of brain damage, preventing inflammation, and reducing the associated behavioural alterations (Article 1). Secondly, the neuroprotective properties of the new OEA analogue, SUL in restoring the HI-induced deficits in cognitive and motor function were shown to be due to a normalization of changes in neuroinflammation/immune system mediators (Article 2).

2. Hypoxia-ischemia model

The HI model has been widely used to study perinatal and neonatal hypoxia in newborn and immature rodents (Rice et al., 1981; Vannucci et al., 1999). In adults, this model is relevant in the study of global ischemia associated with cardiac arrest (Olson and McKeon, 2004). Moreover, it has been suggested that the pattern and course of brain injury following HI resembles the clinical manifestations of large hemispheric stroke (Adhami et al., 2006). The HI is a two-step model that combines permanent unilateral

ligation of the common carotid artery under isoflurane anaesthesia and subsequent respiratory hypoxia. It is well documented that the unilateral ligation alone does not result in appreciable brain lesions in adult mice and rats, although some studies describe white matter lesions to the corpus callosum (Olson and McKeon, 2004). Furthermore, respiratory hypoxia alone also does not produce brain lesions in rodents (Vannucci et al., 2001; Olson and McKeon, 2004; Adhami et al., 2006; Wais et al., 2009).

Importantly, this model presents numerous advantages that makes it suitable for investigating cerebral ischemia in small rodents: the minimal surgical invasiveness, sparing use of anaesthetics, and suitability in age-advanced and various transgenic mice (Vannucci et al., 2001; Olson and McKeon, 2004). Additionally, one of the strong points of the HI model is that the magnitude of the hypoxic insult can be regulated by the duration of the hypoxia. This question has generated certain criticism because it produces inconsistent brain damage in adult animals. However, some studies indicate that controlling the body temperature and/or adjusting the duration of hypoxia improves the consistency of brain damage in adult rodents (Vannucci et al., 2001; Olson and McKeon, 2004; Zhu et al., 2005; Adhami et al., 2006). Other experimental conditions such as O_2 concentration, strain and laboratory environment may also influence the lesion size. Thus, it is important to control all these parameters to diminish the variability. For this thesis, the duration of hypoxia, the concentration of O_2 and the temperature during the process were improved during a

preliminary "set-up" phase. In this first phase, we evaluated different experimental conditions of HI to determine the optimal parameters to use in the following pharmacological and genetic studies. Our goal was to obtain an adult mice model of brain damage with reproducible and quantifiable lesions, low inter-individual variability and decrease mortality. For this purpose, we carried out a series of experimental conditions: (1) 8% oxygen for 20 min, (2) 8% oxygen for 45 min, (3) 10% oxygen for 45 min, (4) 10% oxygen for 60 min, (5) 10% oxygen for 90 min. In behavioural tests we observed that the time of hypoxia and the amount of oxygen affected the performance of the animals. Taking this into consideration, we selected the experimental conditions of 10% oxygen for 60 min with the temperature maintained in 37 ºC inside the chamber, since it produced the greatest deficits in behavioural tests, as well as a higher number of lesioned animals with less mortality (unpublished data). These experimental conditions were used in all experiments.

3. Effects of HI on mortality and pattern of brain injury

The mortality observed following HI in mice was variable in both articles. In Article 1, we used CB2 KO mice and WT littermates on a C57BL/6J background, and in Article 2, we used inbred C57BL/6J mice. Despite the similar background, in Article 1 WT showed 30% and CB2 KO mice showed 17% mortality following HI, while C57Bl/6J mice in Article 2 presented only 4% mortality. Most of these mice died during the HI procedure, suggesting that

the differences in mortality ratio observed in both studies were not due to greater brain damage in WT mice, but were probably related to technical issues.

With respect to the ratio of lesion/non-lesioned mice, in Article 1 we observed a proportion of mice without lesions that ranged from 45.8% to 72.3%, and from 52.6% to 76.4% in Article 2. It is important to highlight that the evaluations were based on qualitative observations of Cresyl violet stained sections, and such assessment would not detect subtle brain injury. Therefore, one explanation for the high number of mice without lesions may be that smaller lesions were not detected with this methodology, which would necessitate a more detailed molecular analysis. Another explanation may be related to the widely reported intra-strain variability in collateral flow through the circle of Willis that could affect the incidence of brain injury (Murakami et al., 1998).

With regards to the extent of brain damage in mice showing overt lesions following HI, WT mice in Article 1 showed injury in the hippocampus, sensory, entorhinal and piriform cortices, while CB2R KO mice presented more extensive brain injury including central structures like the dorsal striatum, the globus pallidus and the amygdala. Thus, CB2R may play a general protective role in adult mice subjected to cerebral ischemia by preventing a more widespread lesion in the brain. In Article 2, C57BL/6J mice showed similar lesions in the hippocampus, the sensory, entorhinal and

piriform cortices than WT mice in Article 1, but also in the striatum.

4. Behavioural consequences of HI in adult mice

To examine the behavioural consequences of HI, several behavioural paradigms were applied including the Irwin test, the rotarod, the beam walking and the open field tests. Moreover, to evaluate the time course of brain injury following HI, behaviour was analysed at different time-points. In Article 1, the evaluation was conducted at 24 h, 72 h and 7 days, while in Article 2 it was performed at 24 h and 7 days. The Irwin test is a systematic observational procedure developed for assessing and scoring the effects of drugs on the behavioural and physiological state of rodents (Irwin, 1968). In our studies, the test was modified to adapt to ischemic mice conditions and detect behavioural dysfunction produced by HI. Thus, the symptoms evaluated were loss of balance, loss of traction, motor incoordination, abnormal gait, low reactivity to touch, piloerection and ptosis. Simultaneously, the neurological deficits were also evaluated and quantified in the same test.

In the Irwin test, a significant increase in symptoms was observed 24 h after HI in WT mice with respect to the control group. Interestingly, WT mice progressively recovered functionality, and at 7 days only minor signs of dysfunction were detected (Articles 1 and 2). In both studies, the locomotor activity evaluated in the open

field test showed slight non-significant decreases in mice 24 h following HI, and a progressive recovery of function was observed with time. To evaluate impairments in motor learning, motor coordination and balance we used the rotarod and the beam walking tests. In Article 1, HI did not induce deficits in these tests in WT mice on either testing day, while in Article 2, motor coordination and balance were significantly and persistently affected by HI in C57BL/6J mice. This discrepancy could be due to the fact that only C57BL/6J mice showed lesions in the motor cortex and striatum, two brain structures known to be involved in motor learning processes.

In order to evaluate the short-term memory impairments induced by HI, the novel object recognition test (NOR) was performed. This test is a behavioural model designed to evaluate whether an object is novel or familiar. This kind of memory is mainly controlled by structures in the medial temporal lobe, including the hippocampus and the adjacent entorhinal, perirhinal, and parahippocampal cortices (Zola-Morgan and Squire, 1993; Zola et al., 2000). To evaluate cognitive deficits, the test was conducted at 72 h and 7 days in Article 1, and at 7 days in Article 2. In both studies, severe cognitive impairments following HI were observed, consistent with the extensive lesions observed in the hippocampus and the entorhinal cortex, structures closely associated with learning and memory processes (Wang and Morris, 2010; Sasaki et al., 2015).

5. Involvement of CB2R in the behavioural deficits induced by HI

The neurophysiological deficits observed 24 h after HI were similar in WT and CB2R KO mice. However, these alterations disappeared 7 days post-insult in WT, but persisted in CB2R KO mice. Additionally, motor learning, motor coordination and balance were impaired selectively in CB2R KO mice on both testing days, consistent with the extensive injury in the motor cortex and the striatum observed in these mice. The results in CB2R KO mice in these motor deficits cannot be due to changes in locomotor activity since no differential effects were observed between WT and KO mice in the open field test. These data support the involvement of CB2R in the recovery of neurophysiological and motor functions following HI.

CB2R KO mice also exhibited severe memory deficits on both testing days that were similar in magnitude to those observed in WT mice. These findings contrast with the greater extent of lesions observed in CB2R KO mice with respect to WT mice in areas such as the hippocampus and entorhinal cortex. This apparent divergence may be due to a possible ceiling effect in the NOR test that could have prevented further deficits to be observed in CB2R KO mice.

6. Involvement of CB2R in glial activation following HI

HI induced an overexpression of reactive astrocytes in the ipsilateral motor cortex, the striatum and the hippocampus. These findings are in agreement with other studies using the HI mice model (Olson and McKeon, 2004; Koh et al., 2015). However, under our experimental conditions, the observed increase in GFAP expression was not modulated in CB2R KO mice, indicating that CB2R may not play a major role in HI-induced astrogliosis. These findings were surprising since several studies have suggested that CB2-mediated regulation of astroglial reactivity can contribute to cannabinoid neuroprotection (Docagne et al., 2007; Garcia-Ovejero et al., 2009). In contrast, microglia expression was increased in the hippocampus of CB2R KO mice with respect to WT littermates, consistent with the larger lesions observed in KO mice. Microglia produce several inflammatory mediators (Woodroofe et al., 1991) that contribute to cell death and increased infarct size. However, it has been demonstrated that microglia can adopt an alternative phenotype changing from the classical pro-inflammatory M1 to an M2 anti-inflammatory phenotype (Franco and Fernández-Suárez, 2015). When we evaluated the involvement of CB2R in modifying microglial phenotypes following HI, we found that in areas with extensive brain injury such as the hippocampus, no differences were observed in the number of M1 or M2 phenotypes present between genotypes. In contrast, in areas where brain damage was sparse, such as the motor cortex and the striatum, more anti-inflammatory microglia was found in both genotypes. These

findings agree with the dual role (pro- and anti-inflammatory) of microglia following HI conditions (Franco and Fernández-Suárez, 2015) by showing that in salvageable tissue the protective microglial phenotype is more abundant. Although CB2R have been implicated in the transformation of microglia to a more protective phenotype (Mecha et al., 2015), our findings using the HI model do not support this contention.

7. Involvement of CB2R in the expression of microglial pro-inflammatory factors following HI

HIF-1 is a heterodimeric transcriptional regulator of various genes that facilitates cellular adaptation to low O_2 conditions (Scholz and Taylor, 2013). HIF is composed of an oxygen sensitive alpha sub-unit (HIF-1α, HIF-2α or HIF-3α) and an oxygen insensitive beta sub-unit (HIF-1β) (Scholz and Taylor, 2013). Importantly, its activation is mainly dependent on HIF-1α protein levels and on low O_2 conditions (Shi, 2009). It is believed to be a key component of the cellular response under, not only hypoxia, but also inflammatory conditions and plays an important role in the pathogenesis of several inflammation-associated diseases such as stroke. In response to ischemic stroke, cells activate HIFs to enhance the transcription of genes involved in glycolysis, angiogenesis, and cell survival, promoting adaptation to hypoxic/ischemic stress (Kaelin and Ratcliffe, 2008). However, also anti-survival factors have been identified as HIF-target genes (Kim et al., 2004; Althaus et al., 2006). Therefore, HIF-1 is considered as

a key regulator responsible for controlling inflammation-associated signalling events.

TIM-3, a member of T cell immunoglobulin and mucin domain family, was first identified as a specific cell surface marker of Th1 cells (Zhao et al., 2011). It has double roles in regulating the inflammatory response (Anderson et al., 2007). When expressed on $CD4^+$T help 1 (Th1) cells, TIM-3 is activated by its ligand galectin-9, which causes calcium influx and cell aggregation, resulting in Th1 cell death. TIM-3 therefore inhibits the inflammatory response by eliminating Th1 cells (Su et al., 2008). However, when TIM-3 is expressed on macrophage, microglia and dendritic cells, it promotes the inflammatory response (Anderson et al., 2007). Interestingly, recent findings have revealed that TIM-3 is upregulated in ischemic neurons, astrocytes and microglia in rodents (Wei et al., 2012; Koh et al., 2015), and this effect is mediated by HIF-1α (Koh et al., 2015). Moreover, the expression of TIM-3 promotes the secretion of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-17, which can aggravate inflammation and secondary brain damage (Anderson et al., 2007; Zhao et al., 2011; Xu et al., 2013).

Considering that: (i) microglia are the key cells for inducing brain inflammation, (ii) CB2R are localized in these cells and are upregulated following HI, (iii) microglial TIM-3 is also upregulated under hypoxia, (iv) HIF-1 mediate the oxygen-dependent expression of TIM-3, we investigated whether CB2R modulated the microglial HIF-1α/TIM-3 axis following HI.

We found that microglial HIF-1α expression was higher in ipsilateral lesioned areas in mice deficient in CB2R than in WT mice, including the hippocampus, amygdala, motor and entorhinal cortices and striatum, but also in several non-lesioned structures such as the olfactory tubercle, the nucleus accumbens, septum and thalamus. Significantly, in the ischemic brain HIF-1α appears to be mostly induced outside the ischaemic infarct that is considered as a salvageable tissue (Bergeron et al., 1999), suggesting a neuroprotective effect. Both neuroprotective and detrimental effects of HIF-1 have been extensively reported in various cerebral ischemic models (*reviewed in* Shi, 2009), but most observations support a concept that HIF-1α may induce cell death in severe and prolonged ischemia, whereas promote cell survival following mild ischemic insults, indicating that its effects possibly depend on the degree of severity of the insults. Our results showing that CB2R KO mice had larger lesions, increased expression of HIF-1α and exacerbated behavioural deficits are consistent with a damaging effect of HIF-α. Thus, our data provide evidence that CB2R is necessary to limit the detrimental action of HIF-1α and to prevent brain damage.

Similarly, we found that microglial TIM-3 expression was present in several brain structures of WT mice following HI injury (striatum, hippocampus, sensory and entorhinal cortices). Our findings are consistent with another study using the HI mice model and primary glial cell culture showing that TIM-3 expression is elevated in hypoxic penumbra, predominantly in activated microglia in a HIF-

1-dependent manner (Koh et al., 2015). Microglial TIM-3 expression was exacerbated in CB2R KO mice in lesioned areas, but also in non-damaged areas (olfactory tubercle and lateral septum). Importantly, the increase in HIF-1α and TIM-3 in CB2KO mice was also observed when we compared animals with similar lesions, especially in entorhinal cortex, hippocampus and septum. These results suggest that HIF-1α and TIM-3 expression is not a consequence of a more extensive lesion size in KO mice, but it is probably due to the lack of CB2R. Thus, the modulation of the pro-inflammatory factors HIF-1α and TIM-3 by CB2R may underlie their neuroprotective role. Accordingly, previous reports have shown that pharmacological activation of CB2R reduces brain damage in the MCAO ischemia model by inhibiting neutrophil recruitment (Murikinati et al., 2010) or by decreasing microglia/macrophage activation (Zarruk et al., 2012). These data, together with recent evidence showing that blockade of TIM-3 reduces neutrophil recruitment by glial cells and diminishes IL-1β and CXCL1 (Koh et al., 2015), supports our hypothesis that CB2R play a crucial role in modulating the HIF-1α/TIM-3 axis in microglia to prevent widespread inflammation and global brain damage following HI insults.

8. Behavioural alterations are associated with higher brain damage and increased expression of TIM-3 in microglia.

We tested the hypothesis that specific behavioural alterations would be associated with the lesion size and with inflammatory processes in particular brain structures. Thus, greater neurophysiological

alterations were observed in mice showing larger lesions, and higher microglial TIM-3 expression in the thalamus and in the whole brain. Motor learning deficits were correlated with the extent of the lesion, and with higher microglial TIM-3 expression in areas involved in motor learning, such as the motor cortex and dorsal striatum, but also in limbic structures like the nucleus accumbens and amygdala. Motor coordination and balance deficits were correlated with the extent of lesioned area, and with microglial TIM-3 expression in another limbic structure, the olfactory tubercle. The associations found between microglial TIM-3 expression in limbic brain structures with motor learning, motor coordination and balance impairments suggest that these tasks are sensitive to inflammatory damage in brain networks related to emotional processing. On the other hand, we did not find correlations between memory impairments and lesion size in the hippocampus or any other structure, or with inflammatory mediators. Again, possibly due to the fact that cognitive deficits in the NOR task reached a ceiling effect that precluded these observations.

9. Effects of SUL on brain damage induced by HI

As previously discussed, in Article 2 HI induced brain injury in several structures including the ipsilateral hippocampus, the sensory, entorhinal, piriform and motor cortices, the striatum and the amygdala. The administration of SUL following HI did not significantly modify the extent of these lesions as compared to

vehicle treatment. This result was unexpected since SUL is a potent OEA analogue, which has been shown to have neuroprotective effects (Moreno-Santos et al., 2014). The lack of protective effect of SUL on this parameter may be due to the fact that it was administered in a single post-treatment dose. In accordance, a study carried out in mice showed that a single administration of OAE immediately or shortly after permanent MCAO had no effect on lesion size (Sun et al., 2007). Despite this finding, SUL showed beneficial effects on various behavioural deficits induced by HI.

10. Effects of SUL on behavioural deficits induced by HI

In the Irwin test, neurophysiological dysfunction was observed 24 h following HI, but SUL did not reduce the deficits in this test. These results contrast with previous studies where the pre- and post-treatment with PPAR-α agonists, OEA (Zhou et al., 2012), and fenofibrates (Ouk et al., 2014a) reversed neurological deficits in models of transient MCAO in rodents. Likewise, SUL showed no effects in the rotarod or in the open field tests. In contrast, SUL was able to reverse the motor coordination and balance deficits in the beam walking test at 7 days after HI, and the PPRA-α antagonist, GW6471 prevented this action. Very few studies have evaluated the effects of PPRA-α agonists on motor impairments induced by cerebral ischemia. One study shows that activation of PPRA-α by fenofibrates improved grip strength alterations in rats and mice subjected to MCAO (Ouk et al., 2014a). Our findings suggest that in terms of motor functions, the PPAR-α agonist SUL

may be preferentially involved in the restoration of coordination and balance following HI.

With respect to the cognitive alterations induced by HI injury, we found that a single dose of SUL (10 mg/kg) totally reversed the short-term memory deficits observed following HI by acting on PPRA-α. Several studies have suggested the important role played by PPAR-α in learning and memory processes, consistent with their abundant localization in different areas involved in cognitive processing, such as the cortex, hippocampus and amygdala (Moreno et al., 2004). Moreover, immunolocalization studies suggested that this nuclear receptor might have a role in regulating the expression of genes involved in cholinergic neurotransmission, where it participates in cognitive processing under normal and pathological conditions (Moreno et al., 2004). Our findings are consistent with recent studies performed in rodent models of ischemia, where two PPAR-α agonist, OEA (Yang et al., 2015), and fenofibrates (Xuan et al., 2015), reduced cognitive deficits and promoted neurogenesis and neuroplasticity in the hippocampus.

To investigate the mechanisms involved in these beneficial effects, we evaluated the consequences of SUL administration on neuronal death, on the levels of microglia and reactive astrocytes, and on the changes in the expression of genes related to the neuroinflammation/endocannabinoid signalling systems.

11. Effects of SUL on neurodegeneration and neuroinflammatory process following HI

The severe cognitive and motor impairments observed in HI mice were associated with brain damage in several different structures of the ipsilateral hemisphere. A more specific immunohistochemical investigation revealed a decrease in NeuN expression in the ipsilateral hippocampus and somatosensory cortex, indicating that HI induced severe neurodegeneration in these structures. Remarkably, SUL significantly reduced this effect in a PPAR-α-dependent manner. In line with these findings, previous studies have shown that SUL (Rodríguez de Fonseca et al., 2012) and OEA (Galan-Rodriguez et al., 2009) reduce neuronal cell death and increase cellular viability in an *in vitro* model of 6-OHDA-induced degeneration of substantia nigra dopamine neurons.

In addition, a higher expression of microglia was observed in the somatosensory, entorhinal and piriform cortices, and in the hippocampus of mice subject to HI, and SUL was able to reduce its expression by acting on PPAR-α. The overexpression of reactive astrocytes in these mice was also decreased by SUL in the hippocampus and in the somatosensory and entorhinal cortices. Significantly, SUL also reduced the expression of several genes involved in excitotoxicity, neuroinflammation and apoptosis in the hippocampus and the motor cortex, such as *Gfap*, *Iba-1*, *Fcgr2b*, *Cox2*, and *Mrc1*. Several studies have been performed regarding the use of endogenous or synthetic PPAR-α agonists for treating neuroinflammation-related diseases such as experimental stroke. In

this context, synthetic PPAR-α agonists, such as fibrates reduce infarct volume through (i) the inhibition of inflammation pathways (Deplanque et al., 2003; Collino et al., 2006), specifically decreasing microglia activation (Ouk et al., 2014a; Xuan et al., 2015), (ii) reducing oxidative stress (Collino et al., 2006; Wang et al., 2010), and (iii) inhibiting cell apoptosis (Mohagheghi et al., 2013a). Similarly, the endogenous PPAR-α agonist OEA also appears to reduce brain damage in stroke models through the inhibition of pro-inflammation enzymes (Sun et al., 2007), and inhibiting cell apoptosis (Zhou et al., 2017). In agreement, our results suggest that the beneficial effects of SUL in preventing cognitive and motor impairment following HI are associated with the neuroprotective and anti-inflammatory actions of PPAR-α activation.

12. Effects of SUL on the expression of the N-acylethanolamides/endocannabinoid – related genes

To investigate the effect of SUL on the components of the NAEs/endocannabinoids signalling systems, we analysed changes in the expression of several related genes in the hippocampus and the motor cortex. The administration of SUL induced contrasting effects in the expression of genes coding for cannabinoid receptors. Thus, SUL upregulated *Cnr2* mRNA, but downregulated *Cnr1* mRNA expression in the hippocampus and motor cortex of both sham and HI mice, indicating a general modulation of these receptors by PPAR-α mechanisms. The increased expression of *Cnr2* mRNA under HI conditions may be promoting protection against neuroinflammation. Indeed, there is evidence showing that

CB2R protein levels can be upregulated by PPAR-α activation in vascular models of inflammation, facilitating endocannabinoid-mediated vascular protection (Xu et al., 2016). In addition, in models of ischemia, such as MCAO and HI, CB2R expression is increased in microglia (Maresz et al., 2005; Ashton et al., 2007; Schmidt et al., 2012; Zarruk et al., 2012), possibly to prevent neuroinflammation mediated by microglial HIF-1α and TIM-3 (Article 1). The decreased expression of *Cnr1* mRNA levels induced by SUL is more difficult to interpret since the role of these receptors in the consequences of ischemic injury is controversial (*reviewed in* chapters 5.2 and 5.3), and there are no studies investigating the effects of other PPAR-α agonists on neurodegeneration or neuroinflammation induced by HI.

In addition, SUL downregulated the expression of *Napepld* and *Faah* mRNA, enzymes that catalyse the release and degradation of NAEs, respectively. Although these effects were observed in the motor cortex and hippocampus of both sham and HI mice, SUL further decreased the expression of *Faah* mRNA specifically in the hippocampus of HI-lesioned mice. This finding suggests that during the HI insult an increase in anandamide and other NAEs meditated by a decrease in FAAH levels may be a mechanism to counteract inflammation and brain damage. Importantly, sulfamoyl derivatives of OEA are not substrates of FAAH, nor inhibitors of its activity (Cano et al., 2007). Thus, the consistent down regulatory effects of SUL on *Faah* mRNA are pharmacologically dependent on SUL-induced activation of PPAR-α receptors.

Finally, the administration of SUL modulated the expression of *Ppar-a* mRNA in a distinctive manner in the hippocampus and the motor cortex of HI-lesioned mice. Thus, in the motor cortex an upregulation was observed, while in the hippocampus a downregulation was found. This area-dependent action of SUL on *Ppar-a* expression might reflect a differential distribution of existing PPAR-α receptors, or of additional modulators of its expression. Upon activation, this receptor is translocated to the nucleus, where it can also induce its own expression. Therefore, an activation or feedback suppression of its mRNA expression could be detected depending on the moment of the analysis. Further investigation is needed to understand this differential regulation regarding the brain areas studied.

In conclusion, an acute administration of SUL may reverse the cognitive and motor coordination impairments induced by HI through the activation of PPAR-α, leading to changes in *Cnr2, Cnr1* and *Faah* gene expression that may contribute to a reduction of inflammatory parameters and neuronal cell death in the hippocampus and cortex.

13. Closing remarks

It is evident that inflammation has an important role in cerebral ischemia. Therefore, great efforts are being made to target these inflammatory pathways and reduce their negative effects on the brain after the ischemic injury. At present, many studies have been carried out to investigate the effects of different anti-inflammatory

treatments after ischemic stroke, most of them reporting worse outcomes or no differences between treatment and placebo (*reviewed in* Bhalala et al., 2015; Tobin et al., 2014).

In this thesis, we demonstrated for the first time that CB2R may act as a defensive mechanism to prevent inflammation mediated by microglial HIF-1α and TIM-3, and reduce subsequent behavioural alterations. We also demonstrated the neuroprotective properties of SUL when administered immediately after HI, and its beneficial effects on preventing the concomitant memory alterations.

Together, the results obtained in this thesis provide evidence for new therapeutic avenues to prevent the enormous negative outcome associated with hypoxic stroke.

CONCLUSIONS

The results obtained allow us to conclude that:

1. Following hypoxia-ischemia, CB2R KO mice exhibited more extensive brain injury, associated with longer behavioural, motor learning, motor coordination and balance deficits than WT mice.
2. Microglia expression was increased in the hippocampus of CB2R KO with respect to WT mice, while no differences were observed in reactive astrocytes between genotypes.
3. In lesioned mice of both genotypes, a predominant anti-inflammatory microglia phenotype was found in brain areas showing moderate lesions such as the motor cortex and the striatum. In the hippocampus, where lesions were larger, no differences were observed in the number of pro- or anti-inflammatory microglial phenotypes.
4. The expression of microglial pro-inflammatory factors HIF-1α and TIM-3 was exacerbated in several lesioned and non-lesioned areas in CB2R KO with respect to WT mice.
5. The overexpression of microglial TIM-3 in specific brain structures significantly correlated with behavioural, motor learning, motor coordination and balance deficits induced by hypoxia-ischemia.
6. The acute administration of octadecylpropyl sulfamide did not modify the extent of the lesion induced by hypoxia-ischemia, suggesting that a single post-injury administration is insufficient to prevent overt neuronal damage.

7. The acute administration of octadecylpropyl sulfamide prevented the cognitive and motor coordination impairments induced by hypoxia-ischemia, reduced neurodegeneration and downregulated the overexpression of microglia and astrocytes in the hippocampus and cortex.
8. The acute administration of octadecylpropyl sulfamide downregulated the expression of genes related to neuroinflammation in the hippocampus and cortex, such as *Gfap*, *Iba-1*, *Fcgr2b*, *Cox2*, and *Mrc1*.
9. The acute administration of octadecylpropyl sulfamide modulated the expression of genes related to the endocannabinoid signalling system, downregulating *Cnr1, Faah* and *Napepld,* and upregulating *Cnr2* expression.
10. The PPAR-α antagonist, GW6471 blocked the beneficial effects of octadecylpropyl sulfamide following hypoxia-ischemia, supporting the involvement of these receptors in the neuroprotective effects of this sulfamide.

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