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Evaluation of the role of C-terminal binding proteins (CtBP) in the neuroimmune response

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Resumo

A neuroinflamação é uma resposta inflamatória que ocorre ao nível do sistema nervoso central (SNC). Vários estímulos ou lesões podem desencadear esta resposta, como por exemplo isquémia, trauma, infeções ou exposição a toxinas. As células da microglia, consideradas células imunitárias inatas residentes no SNC, contribuem para a resposta neuroinflamatória. Estas células detetam alterações à homeostasia cerebral, migram para local lesado e libertam mediadores inflamatórios capazes de impactar na sobrevivência neuronal. O lipopolissacarídio (LPS), um dos componentes da membrana exterior de bactérias gram-negativas, é amplamente utilizado como modelo para compreender os mecanismos patológicos envolvidos na neurodegeneração induzida pela neuroinflamação, bem como para identificar potenciais moléculas terapêuticas. As proteínas de ligação C-terminal (CtBPs) são correguladores transcricionais que interagem com fatores de transcrição e reprimem a transcrição. Estas proteínas são altamente expressas no SNC durante o desenvolvimento embrionário e regulam o desenvolvimento, a sobrevivência e a função neuronal. Contudo, a compreensão da regulação da expressão e função das CtBPs em condições de neuroinflamação induzida pelo LPS, ainda é escassa.

O primeiro objetivo deste trabalho foi avaliar a expressão das CtBPs em modelos experimentais *in vivo* e *ex vivo* de neuroinflamação induzidos por LPS e, posteriormente, avaliar a sua função num modelo de fatias organotípicas de hipocampo *ex vivo*. A expressão das CtBPs foi avaliada por análise Western-Blot no hipocampo extraído de cérebros de murganhos adultos C57BL/6J e em fatias organotípicas de hipocampo de murganhos C57BL/6J com 6 a 9 dias de idade. Concluiu-se que a concentração de 500 µg/kg de LPS foi a mais eficaz a induzir o aumento de expressão de CtBP1 e CtBP2 quando os animais foram expostos durante 4 dias. Em murganhos expostos à mesma concentração de LPS, durante 7 dias, verificou-se o aumento de expressão de CtBP2. Por outro lado, a exposição com 2 mg/kg de LPS durante 24h induziu um aumento da expressão de CtBP1. O LPS, a diferentes concentrações, também induziu o aumento da expressão das CtBPs em culturas organotípicas de hipocampo. De seguida, avaliamos a resposta glial em fatias organotípicas de hipocampo, através da deteção da expressão da proteína glial fibrilar ácida (GFAP) e do fator citosólico 1 de neutrófilos (P47phox) por Western-Blot. Para avaliar o envolvimento das CtBPs na resposta neuroinflamatória, as culturas foram expostas ao ácido 4-metiltio-2-oxobutírico (MTOB), um modulador da atividade das CtBPs, podendo atuar como inibidor a altas concentrações e como agonista

a baixas concentrações. Em algumas experiências foi também adicionado o ácido retinóico (RA), um potente agente anti-inflamatório. Observámos que a expressão de P47phox se encontrava diminuída em fatias expostas a LPS e/ou MTOB e que o AR foi capaz de contrariar este efeito. A diminuição da expressão deste marcador de células fagocíticas sugere implicações na resposta inflamatória dessas células. Por outro lado, o aumento da expressão deste marcador para níveis semelhante ao controlo induzido pelo AR, pode indicar uma tentativa de atenuar a resposta inflamatória. Relativamente à análise da expressão de GFAP, observou-se uma diminuição da sua expressão em todos os grupos experimentais em comparação com o grupo controlo, sugerindo que estes compostos podem estar a afetar a sobrevivência dos astrócitos. Em suma, podemos concluir que a neuroinflamação induzida por LPS desencadeia a expressão de CtBPs no hipocampo consoante a dose e o tempo de exposição ao agente inflamatório e que o AR consegue atenuar os efeitos induzidos pelos LPS+MTOB, podendo ser considerado um potencial agente anti-inflamatório.

Palavras-chave

Neuroinflamação; Lipopolissacarídeo; Proteínas de ligação c-terminal; MTOB; Ácido Retinóico

Resumo Alargado

As doenças neurológicas têm vindo a revelar-se uma preocupação crescente em todo o mundo, uma vez que têm sido consideradas umas das principais causas de mortalidade e morbidade. Subjacente à grande maioria destas doenças está a neuroinflamação que pode ser definida como uma resposta inflamatória ao nível do sistema nervoso central. Vários estímulos podem desencadear esta resposta, entre eles a isquémia, trauma, infeções e toxinas. A neuroinflamação pode desempenhar dois papéis distintos. Em condições fisiológicas, permite a homeostase do sistema nervoso central ajudando-o a responder e a reparar danos neuronais. Por outro lado, em condições crónicas, pode induzir danos neuronais podendo desencadear a etiologia e/ou progressão de doenças neurodegenerativas. Desta forma, é fundamental compreender os mecanismos e efeitos da resposta inflamatória em cada uma destas condições. O perfil prejudicial ou benéfico da neuroinflamação é determinado pelas alterações fenotípicas e funcionais das células da microglia e dos astrócitos.

O lipopolissacarídeo (LPS), presente na membrana externa de bactérias gram-negativas, tem demonstrado ser um potente ativador da neuroinflamação. O principal recetor do LPS é o recetor toll-like 4 (TLR4) que é expresso pelas células da microglia e pelos astrócitos. O LPS é utilizado como modelo experimental de neuroinflamação, podendo ser administrado em roedores *in vivo*, através de várias vias de administração e utilizando protocolos variados de frequência única ou múltipla. Desta forma, consoante o protocolo utilizado, o LPS pode induzir imunidade inata ou pode levar à disfunção neuronal e consequente neurodegeneração.

As proteínas de ligação C -terminal (CtBP) são proteínas que têm como principal função atuar como co-repressores transcricionais. Existem dois membros desta família, CtBP1 e CtBP2, que são altamente expressas em vertebrados e compartilham 76% de homologia. Há evidências que mostram que as CtBPs exercem um papel na resposta neuroinflamatória. Contudo, desconhece-se o efeito das CtBP na resposta neuroimune, em particular na resposta das células da microglia, em resposta ao LPS.

Desta forma, o primeiro objetivo deste trabalho foi caracterizar a expressão das CtBPs em modelos de neuroinflamação induzidos por LPS. Inicialmente, começou-se por analisar a expressão das CtBPs no hipocampo de murganhos adultos expostos ao LPS, através da via intraperitoneal, com diferentes tempos e doses de exposição. Os nossos resultados mostraram uma maior expressão de CtBP1 em resposta a LPS com maior

dosagem (2 mg/kg, 24h) , um aumento da expressão de CtBP2 em resposta a 500 μg/kg LPS durante 7 dias e um aumento da expressão de ambas as CtBPs em resposta a uma exposição com 500 μg/kg LPS durante 4 dias.

Posteriormente, analisou-se a expressão destas proteínas em fatias organotípicas de hipocampo, para corroborar os resultados obtidos nos estudos *in vivo*. Os resultados demonstraram uma maior expressão de CtBP1 em resposta a doses mais baixas de LPS (100 ng/mL) e maior expressão de ambas as CtBPs a doses mais altas (1 μg/mL).

Como observámos um aumento da expressão das CtBPs em culturas organotípicas e nos estudos *in vivo,* na segunda parte do trabalho modulou-se a atividade das CtBPs através da exposição das fatias a MTOB (25 μM e 250 μM). O MTOB tem um duplo efeito nas CtBPs, a elevadas concentrações atua como um inibidor enquanto a baixas concentrações atua como um ativador. Algumas fatias foram também expostas ao ácido retinóico (AR, 10 μM), um potente agente anti-inflamatório. A reatividade glial foi avaliada através de Western-Blot para P47phox, um marcador de células fagocíticas, e GFAP, um marcador astrocitário. Observamos que os grupo expostos apenas ao LPS e ao LPS+MTOB foram os que apresentaram menor expressão de P47phox, ou seja, a capacidade fagocítica das células expostas a estes compostos está diminuída, o que pode ter implicações na resposta inflamatória dessas células. Por outro lado, constatámos que houve um aumento da expressão de células fagocíticas nos grupos expostos ao AR próximos dos valores do controlo o que pode indicar uma tentativa de restaurar a função normal das células fagocíticas ou atenuar a resposta inflamatória, sugerindo o seu papel anti-inflamatório. Em relação ao GFAP, os resultados mostraram uma diminuição da sua expressão em todos os grupos o que pode sugerir morte dos astrócitos como consequência da inflamação. Apesar da necessidade de experiências adicionais para esclarecer essas respostas gliais, no geral, os nossos resultados sugerem que as CtBPs desempenham um papel na neuroinflamação induzida por LPS.

Abstract

Neuroinflammation is an inflammatory response that occurs at the level of the central nervous system (CNS). Various stimuli or injuries can trigger this response, such as ischemia, trauma, infections or exposure to toxins. Microglial cells, considered innate immune cells residing in the CNS, contribute to the neuroinflammatory response. These cells detect changes in brain homeostasis, migrate to the injured site and release inflammatory mediators capable of impacting neuronal survival. Lipopolysaccharide (LPS), one of the components of the outer membrane of gram-negative bacteria, is widely used as a model to understand the pathological mechanisms involved in neurodegeneration induced by neuroinflammation, as well as to identify potential therapeutic molecules. C-terminal binding proteins (CtBPs) are transcriptional coregulators that interact with transcription factors and repress transcription. These proteins are highly expressed in the CNS during embryonic development and regulate neuronal development, survival and function. However, knowledge about the regulation of the expression and function of CtBPs in conditions of neuroinflammation induced by LPS is still scarce.

The first objective of this work was to evaluate the expression of CtBPs in *in vivo* and *ex vivo* experimental models of neuroinflammation induced by LPS and, subsequently, to evaluate their function in an ex vivo model. The expression of CtBPs was evaluated by Western-Blot analysis of hippocampi from adult C57BL/6J mice and in organotypic hippocampal slice cultures from 6- to 9-day-old C57BL/6J mice. It was concluded that the concentration of 500 μg/kg of LPS was the most effective in inducing an increase in the expression of CtBP1 and CtBP2 when the animals were exposed for 4 days. In mice exposed to the same concentration of LPS for 7 days, there was an increase in CtBP2 expression. On the other hand, exposure to 2 mg/kg of LPS for 24h induced as increase in CtBP1 expression. LPS, at different concentrations, also induced increased expression of CtBPs in organotypic hippocampal cultures. Next, we evaluated the glial response, in organotypic hippocampal slices obtained from C57BL/6J mice by Western-Blot analysis against glial fibrillary acidic protein (GFAP) and neutrophil cytosolic factor 1 (P47phox). To evaluate the involvement of CtBPs in the neuroinflammatory response, cultures were exposed to 4-methylthio-2-oxobutyric acid (MTOB), a modulator of CtBP activity that can act either as an inhibitor at high concentrations or an agonist at low concentrations. In some experiments, retinoic acid (RA) was also added as a potent anti-inflammatory agent. We observed that P47phox expression was reduced in slices exposed to LPS

and/or MTOB and that RA was able to counteract this effect. The decreased expression of this phagocytic cell marker suggests implications for the inflammatory response of these cells. On the other hand, the increase in expression of this marker to levels similar to the control induced by RA may indicate an attempt to attenuate the inflammatory response. Regarding the analysis of GFAP expression, a decrease in its expression was observed in all experimental groups, suggesting that these compounds may be affecting the survival of astrocytes. In short, we can conclude that neuroinflammation induced by LPS triggers the expression of CtBPs in the hippocampus depending on the dose and time of exposure to the inflammatory agent and that RA can attenuate the effects induced by LPS+MTOB and can be considered a potential anti-inflammatory agent.

Keywords

Neuroinflammation; Lipopolysaccharide; C-terminal binding proteins; MTOB; Retinoic acid

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List of Abbreviations

AD	Alzheimer's disease
BBB	Blood-Brain Barrier
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CtBP	C-terminal Binding Protein
DAMPS	Damage associated molecular patterns
GBSS	Gey's Balanced Salt Solution
GFAP	Glial Fibrillary Acid Protein
HDAC	Histone Deacetylase
HMT	Histone Methyltransferases
icv	Intracerebroventricular
ip	Intraperitoneal
IL	Interleukin
LIF	Hypoxia induced factor
LPS	Lipopolysaccharide
MD-2	Myeloid Differentiantion protein-2 co-receptor
MTOB	4-methylthio-2-oxobutyric acid
NAD+	Oxidized Nicotinamide Adenine
NADH	Reduced Nicotinamide Adenine Dinucleotide
NO	Nitric Oxide
NSC	Neural Stem Cell
PAMPS	Pathogen-associated molecular patterns
PD	Parkinson's Disease
PRR	Pattern recognition receptor
PXDLS	Pro-X-Asp-Leu-Ser
RA	Retinoic Acid
ROS	Reactive Oxygen Species
RT	Room temperature
TGF	Transforming growth factor
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor

Chapter 1

Introduction

1. Neuroinflammation

1.1. Definition

All over the world, due to the aging of the population, the incidence of neurological disorders has been increasing. The main reason for this global burden is the lack of a clear understanding of the etiopathology of these neurological conditions. Studies indicate that the neuroinflammatory cascade has been identified as one of the common etiopathology factors in different neurological disorders (1).

Neuroinflammation can be defined as an inflammatory response that occurs at the level of the central nervous system (CNS), that acts in response to distinct stimuli and conditions, including ischemia, trauma, infection, and toxins. During these processes, the innate immune cells of the CNS, mainly microglia, produce pro-inflammatory cytokines, including IL-1β, IL-6, IL-18 and tumor necrosis factor (TNF)-alpha, chemokines such as C-C motif chemokine ligand (CCL1), CCL5 and C-X-C motif chemokine ligand 1 (CXCL1), small-molecule messengers, including prostaglandins and nitric oxide (NO), and further reactive oxygen species (ROS) (2).

This condition may play two distinct roles. On the one hand, under physiological conditions, it allows CNS homeostasis by helping the CNS respond to neuronal damage or dysfunction. On the other hand, in chronic conditions, neuroinflammation exacerbates neuronal damage and glial reactivity, ultimately leading to acute or chronic degenerative conditions (3). It is therefore crucial to understand when inflammation is protective and when it is harmful to devise novel therapeutic approaches for brain diseases (4).

1.2. The role of microglia and astrocytes in neuroinflammation

Microglia and astrocytes are the main glial players in neuroinflammatory responses.
In 1919, Pío del Río-Hortega classified a new type of phagocytic brain cells, of mesodermal origin, which he called microglia. Microglia originate in the yolk sac and migrate to the CNS during embryogenesis, they are the most abundant immune cells in the brain, representing more than 80% of all immune cells (5). These cells are

ubiquitously distributed in the brain, considered resident brain macrophages, and are important for regulating homeostasis and neuronal behavior. Recent studies indicate that these can modulate neuronal activity, facilitate learning, and shape social behavior. These studies further indicated that microglia have a dual role: driving neuroinflammation and subsequent pathology, as well as ameliorating neuroinflammation and repairing the nervous system. Indeed, there is evidence that inflammation simultaneously leads to tissue damage and repair (6).

Depending on the microenvironment in which it is found, physiological or pathological, microglia undergo morphological changes from a ramified (resting) form to an amoeboid (active) form. These cells patrol the brain providing immune surveillance and neuronal survival (7). The neuroinflammatory response begins when microglia recognize specific stimuli, namely molecules expressed by specific pathogens or that signal the presence of cellular damage or necrosis, designated pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). These compounds bind to pattern recognition receptors (PRRs) and to Toll-like receptors (TLR), which will initiate signaling pathways that will subsequently activate the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), ultimately enhancing the expression of pro-inflammatory mediators, such as IL-1β, IL-6 and TNF-α (8).

Several stimuli like lipopolysaccharide (LPS) or high-mobility group protein box-1 (HMGB1), particularly the ones that activate TLR4 on the microglia cell's membrane, lead to this particular neuroinflammatory cascade. On the other hand, other stimuli like IL-4, IL-13, IL-10 or transforming growth factor beta (TGF-β), can develop a neuroprotective phenotype, characterized by the release of anti-inflammatory mediators, such as interleukin IL-10, transforming growth factor beta (TGF-β) and glucocorticoids (9) (Figure 1).

Astrocytes are glial cells that derive from neural stem cells (NSCs) during embryonic development, with an important role in synaptic formation and plasticity, and free radical removal. Furthermore, they play a crucial function in the maintenance of homeostasis and providing metabolites and growth factors to neurons (7). Under physiological conditions, they also restrict the entry of peripheral immune cells that cross the Blood-Brain Barrier (BBB) (9).

Like microglia, astrocytes also undergo morphological and functional changes in response to the microenvironment, leading to the activation of astrocytes, which in the

initial stages aim to eliminate damage to the brain but in more chronic conditions can be harmful (10). Reactive astrocytes can be of two types: pro-inflammatory which elevate the levels of many genes of the classical complement cascade, such as C1r, C1s, C3 and C4, or anti-inflammatory that release neurotrophic factor and cytokines such as LIF (hypoxia inducing factor), IL-6, IL-10 (9) (Figure 1).

Traditionally, microglia and astrocytes were classified into two opposing phenotypes: neurotoxic and neuroprotective. It was considered that there were A1 and A2 astrocytes and M1 and M2 microglia. On the one hand, A1 and M1 were activated cells, with neuroinflammatory characteristics that positively regulate many genes and increase the expression of pro-inflammatory cytokines. On the contrary, A2 and M2 were non-reactive cells with neuroprotective properties promoting neuron survival and increasing the secretion of anti-inflammatory cytokines. However, this simple dichotomized classification does not reflect the various phenotypes of microglia and astrocytes. A more recent hypothesis suggests that glia exist physiologically as heterogeneous and mixed populations and that they differ from a morphological and functional point of view. Microglia and astrocytes are considered to have multiple reactive phenotypes related to the type of insult, the stage of the disease, the type of cell, and the area of the CNS in which the cells are located (11,12).

The activation of innate immune system cells, such as microglia and astrocytes, can also disrupt the BBB and allow and chemotactically attract several peripheral immune cells, such as monocytes, macrophages, neutrophils and T cells, aggravating inflammation (12).

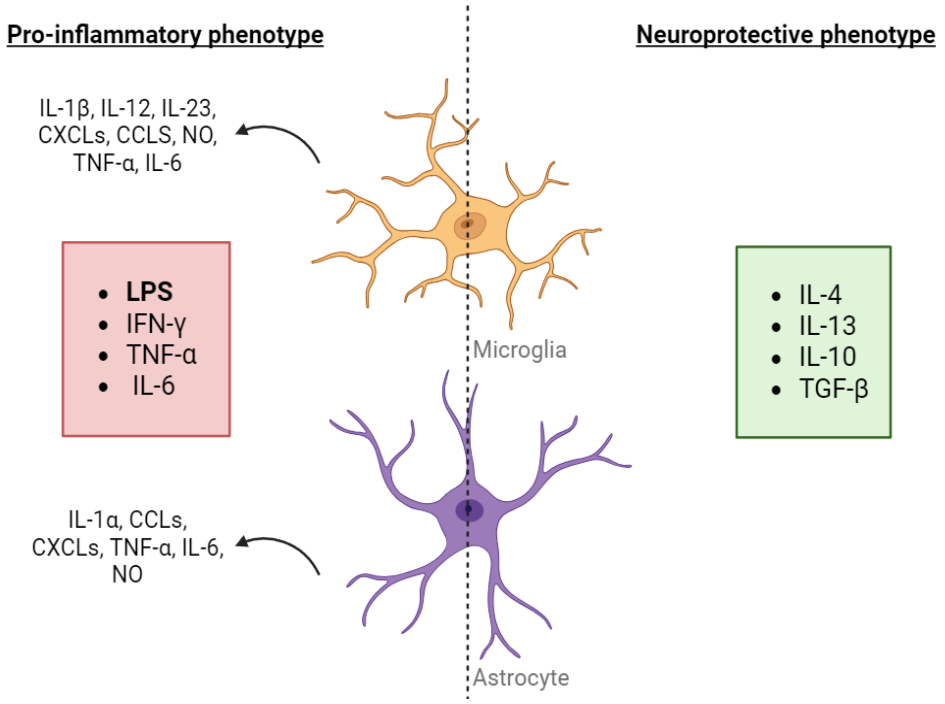

Figure 1 – Schematic representation of signals released by microglia (yellow) and astrocytes (purple) in pro-inflammatory and neuroprotective phenotypes. For example, LPS activates the pro-inflammatory phenotype of microglia through the NF-κB and STAT1 pathway, which lead to the release of IL-1β, IL-12, IL-23, CXCLs, CCLs, NO, TNF-α and IL-6. On the other hand, the neuroprotective phenotype of microglia is promoted by IL-4, IL-13, IL-10 and TGF-β through activation of STAT3 and STAT6 pathway. The pro-inflammatory phenotype of astrocytes is induced by LPS, INF-γ, TNF-α and IL-6 and produces IL-1α, CCLs, CXCLs, TNF-α, IL-6 and NO. Neuroprotective astrocytes interact with anti-inflammatory cytokines such as IL-13, IL-10, TGF-β and IL-4; IL-4 suppresses TNF-α, IL-6 and NO (12). Created by Biorender.

1.3. Role of neuroinflammation in neurodegeneration

Neuroinflammation frequently precedes the development of neurodegenerative disease (13). Brains from patients with neurodegenerative diseases are characterized by marked astrocytosis, microglial activation and elevated levels of pro-inflammatory cytokines. This set of factors associated with neuroinflammation indicates that this is an important feature in the pathogenesis and progression of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia, and amyotrophic lateral sclerosis (14).

AD is characterized by the aggregation of extracellular beta-amyloid (Aβ) plaques and neurofibrillary tangles of hyperphosphorylated tau proteins which leads to memory loss and cognitive dysfunction. These accumulated proteins can be known as DAMPs and activate TLRs present in different cells residing in the CNS. Overactivation of these TLR-dependent pathways can generate a harmful neuroinflammation response contributing to disease progression (15).

PD is characterized by the loss of dopaminergic neurons in the substantia nigra which leads to the development of various symptoms such as resting tremors, rigidity, shuffling gait and bradykinesia. Neurodegeneration is driven by the abnormal accumulation of misfolded α-synuclein. Abnormally shaped aggregation of α-synuclein is recognized as a DAMP and activates TLR-mediated signaling pathways, which subsequently leads to activation of the MyD88 and NF-κB response resulting in the production of TNF-α and IL-1β (16).

McGeer et al. were the first to describe evidence of neuroinflammation in PD in 1988, reporting the presence of activated microglia and infiltrating lymphocytes in the substantia nigra of a patient with PD. Additional studies carried out in patients with PD observed a significant increase in TNF-α, IL-1β, IL-2 and IL-10, which demonstrated that elevated levels of pro-inflammatory cytokines and lower levels of anti-inflammatory cytokines were associated with a faster progression of symptoms. Furthermore, TLRs can be stimulated through various molecules, such as α-synuclein and heat shock proteins (HSPs), serving as immunological receptors in PD, through which neuroinflammation can be triggered (15).

2. Lipopolysaccharide

Lipopolysaccharide (LPS) is a polysaccharide present in the outer membrane of gram-negative bacteria. Substantial studies have shown that LPS is a potent activator of neuroinflammation that, depending on the exposure protocol, can lead to neuronal dysfunction and neurodegeneration or neuronal protection. The main receptor of LPS is the toll-like receptor 4 (TLR4) which is expressed in microglia and astrocytes. Activation and dimerization of this receptor lead to the recruitment of intracellular adapter proteins that trigger NFκB signaling pathway and cytokine production, expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in the brain (14,17,18).

LPS can be used both in *in vitro* protocols, and *in vivo*, injected into the CNS or in the periphery through single or multiple injections, leading to a variety of effects that may vary according to the protocol (13).

2.1. Mechanism of action

LPS is made up of three parts: antigen O, central oligosaccharide, and lipid A, which is the main PAMP of LPS (19). The main receptor of LPS is TLR4, which belongs to a subfamily of pattern recognition receptors that recognize invading pathogens and

endogenous harmful stimuli to induce innate and adaptive immune responses. When this receptor is expressed on the cell membrane, LPS binds and causes TLR4 dimerization with the myeloid differentiation protein-2 co-receptor (MD-2) forming a complex. The formation of this complex results in the activation of downstream mediators, including the nuclear factor of the transcription factor nuclear factor NF-κB, which increases the production of pro-inflammatory molecules such as chemokines, enzymes, reactive oxygen and nitrogen species and cytokines, namely factor of tumor necrosis (TNF-α), interleukin IL-1β and IL-6 (20) (Figure 2).

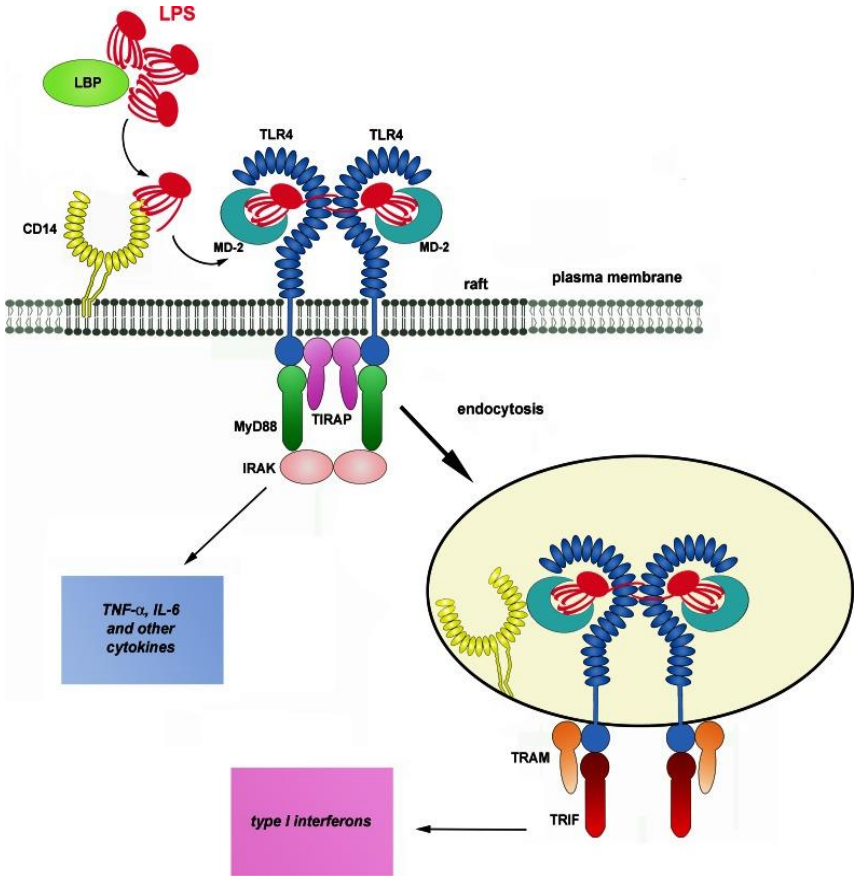

Figure 2 – Schematic representation of TLR4 activation by LPS. LPS-binding protein (LBP) binds to LPS and transfers its monomers to cluster of differentiation 14 (CD14). It subsequently transfers the endotoxin to the TLR4/MD-2 complex. After heterodimer formation, the intracellular signal can follow two distinct directions: the formation of a myddosome in the TIR domain of TLR4, TIRAP/MyD88/IRAK, inducing a signaling pathway that leads to the production of pro-inflammatory cytokines. Or after endocytosis, TRAM and TRIF associate with TLR4 activating a signaling pathway that controls the production of type I interferons. IRAK (interleukin-1 receptor associated kinase); MyD88 (myeloid differentiation primary response gene); TIRAP (TIR domain-containing adaptor protein); TRAM (TRIF-related adaptor molecule); and TRIF (TIR-domain-containing adapter-inducing interferon-β) Adapted from (21).

2.2. The role of LPS in Neuroinflammation

Understanding the effects of neuroinflammation on cellular and behavioral processes has been a growing concern. To this end, different experimental strategies have been used to promote a state of inflammation such as the administration of different elements or compounds, namely heavy metals, pro-inflammatory mediators, and bacterial components, such as LPS (8).

How animals respond to LPS stimuli can vary depending on the species, age, stimulus source, dose, route, and duration of administration used in each study. For example, single intracerebroventricular (icv) injections of LPS resulted in increased levels of interleukin-1β (IL-1β) in the brainstem and diencephalon of rats 2 hours after injection. Furthermore, single intrahippocampal injections of LPS produced elevations of glial fibrillary acidic protein (GFAP) 24 hours and 28 days after the injection and increased CR3 and CD45 expression indicating chronic microglial activation (13).

In general, after systemic administration of LPS in rodents, microglia display elongated somata and retraction of processes and astrocytes exhibit hypertrophy, in addition, both cells exhibit a pro-inflammatory secretory profile. Furthermore, the hippocampus is a region of the brain that has a high content of PRRs and cytokine receptors found mainly in astrocytes, microglia and neurons, which makes cells in this structure especially vulnerable to the harmful effects of neuroinflammation (8). The BBB is a highly selective structural and functional barrier that separates the CNS from the rest of the organism, creating a unique microenvironment necessary for normal brain function and homeostasis (22). A common feature of neuroinflammation-mediated neurodegenerative diseases is disruption of the BBB. This membrane restricts the transport of certain plasma proteins and immune cells from the blood to the brain parenchyma and its rupture allows lymphocytes, macrophages and plasma proteins to enter the brain parenchyma. The infiltration of peripheral immune cells induces activation of microglia and consequent neurodegeneration (23). Vascular endothelial cells are the main cellular constituents of the BBB, and structural damage or a decrease in the number of these cells culminates in an increase in the permeability of the BBB. There is evidence that LPS disrupts the functional state of the BBB by directly affecting processes such as the proliferation or apoptosis of endothelial cells. Studies indicate that LPS can damage the BBB by promoting apoptosis of endothelial cells through the inhibition of endothelial nitric oxide synthase (eNOS) and guanosine triphosphate cyclohydrolase 1 (GTPCH1). In fact, studies showed that increasing the expression of eNOS and GTPCH1 managed to reverse the effects of LPS in human microvascular endothelial cells (24).

If, on the one hand, the administration of high doses of LPS is one of the main models for inducing chronic neuroinflammation, several studies indicate that preconditioning with a lower dose of LPS could induce a neuroprotective effect against higher doses of LPS. This exposure to lower doses induces tolerance against further administration with

higher doses, suggesting a modulation of the inflammatory response and consequent release of anti-inflammatory cytokines such as IL-10 (25).

3. C-terminal Binding Proteins

C-terminal binding proteins are 48 kDa cellular phosphoproteins whose main function is to link DNA/histone modifying proteins to sequence-specific DNA binding proteins to be a transcriptional co-repressor (26,27). The first member of the CtBP family to be identified was CtBP1 based on its interaction with the C-terminal region of human adenovirus E1A proteins. Posteriorly, a second CtBP was discovered and named CtBP2 (27). Both proteins (CtBP1 and CtBP2) are highly expressed in different tissues of vertebrate species and share 76% homology (26).

It is known that CtBPs interact with a wide range of transcription factors. Some *in situ* hybridization studies have shown ubiquitous embryonic expression of CtBP1 and CtBP2, with high expression of both in the nervous system (28). On the other hand, double mutant mice for CtBP1 and CtBP2 showed severe developmental defects in the nervous system (29). In the adult brain, Hubler et al., observed the expression of CtBP1 in the polymorphic layer of the dentate gyrus and in the stratum lucidum of the CA3 region of the hippocampus, in the granular cell layer of the dentate gyrus, in the pyramidal cell layer of CA1 and weaker in CA2. Concerning CtBP2, they observed greater immunoreactivity in the cell body layers of the dentate gyrus, CA3 and especially in CA2. Furthermore, they observed diffuse staining evident in the strati lucidum of CA3 and in the polymorphic cell layer of the dentate gyrus pointing to the synaptic location of CtBP2 in the hippocampus (30).

3.1. Structure and function of CtBPs

In vertebrates, CtBPs have multiple isoforms that exhibit functional and structural similarities, although each also has many distinct functions. Some of these isoforms, in addition to their nuclear functions, also play important roles in the cytoplasm that are not related to the activity of CtBP co-repressor (31).

CtBP1 has 2 splice isoforms, CtBP1-L (long isoform) and CtBP1-S (short isoform) better known as CtBP1/BARS and which is located predominantly in the cytoplasm, participates in the process of membrane trafficking and Golgi partitioning during mitosis. CtBP1 is known to localize to the synapses of the strands in sensory neurons, where it is believed to provide structure to the synaptic strands. On the other hand, CtBP2 has 3 splice isoforms, CtBP2-L, CtBP2-S and RIBEYE. The latter is located

predominantly in the cytoplasm and is an important structural component of the synaptic strands that are important for the precise and accurate transmission of sensory signals (31,32). However, their preferential location is in the nucleus where they act as transcriptional corepressors and alter the chromatin structure to repress gene expression. They bind DNA-binding proteins to chromatin-modifying enzymes such as histone deacetylases (HDAC1/2) and histone methyl transferases, which alter the pattern of post-translational modifications of histones to form a repressive structure of chromatin in the promoters of target genes (33,34).

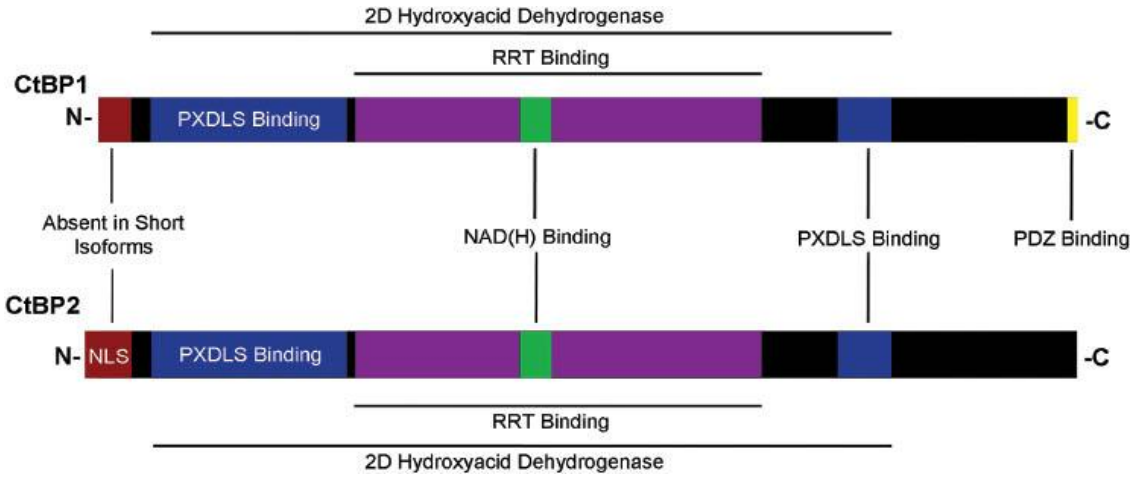

Figure 3 – Schematic representation of structure of CtBP1 and CtBP2. Both CtBPs have a PXDLS-binding cleft, an RRT-binding cleft, and the dehydrogenase domain. However, there are some slight differences between the two proteins: CtBP2 possesses an NLS-domain in its N-terminal while CtBP1 have a PDZ-binding domain in its C terminal. Adapted from (31).

CtBPs are made up of three main domains: (1) the substrate-binding domains, which contain the Pro-X-Asp-Leu-Ser (PXDLS; X being a hydrophobic amino acid) binding sequence, this domain is essential for the formation of the CtBP co-repressor complex as it recruits histone deacetylases (HDACs), histone methyltransferases (HMTases) and transcriptional repressors that are essential components for CtBPs carry out their co-repressor functions; (2) the central domain Arg-Arg-Thr (RRT), where NAD(H) binds and is responsible for dimerization, like the previous domain, it is predominantly used to bind and recruit members of the co-repressor complex and CtBP-interacting proteins (CtIPs); and (3) a C-terminal domain (26,31) (Figure 3).

3.2. Regulation of CtBPs

The regulation of CtBPs is done mainly at two levels: metabolic and redox sensitivity, and post-translational modifications. In terms of metabolic and redox sensitivity of CtBPs, it is known that the corepressor activity of CtBPs is dependent on the NADH/NAD^+ ratio

in the cell. Furthermore, CtBPs act as sensors of the oxygen level in the cell's microenvironment, which is proportional to the concentration of intracellular NADH. CtBPs have at least 100 times greater affinity for NADH than for NAD^+. In short, under hypoxic conditions, when oxygen levels are low, there is an increase in the concentration of NADH within the cell resulting in the dimerization of CtBPs (27). On the other hand, when the NADH/NAD^+ ratio is low, CtBP is in its monomeric form, which suppresses the activation of the P300/CBP acetyltransferase, leading to the acetylation of histones and NF-κB (35).

At the level of post-translational modifications, phosphorylation of CtBPs often targets these proteins for ubiquitination and subsequent proteasomal degradation (31). The serine/threonine kinase HIPK2 (Homeodomain Interacting Protein Kinase 2) phosphorylates CtBP1 and 2, leading to their degradation and cell death. In turn, c-Jun NH_2-terminal kinase (JNK1) plays an analogous role by phosphorylating CtBP1 in a p53-independent manner. These two pathways together play roles in the regulation of CtBP-mediated apoptosis (32).

In addition to these, CtBP1 can also be phosphorylated by AMP-activated protein kinase (AMPK) in response to metabolic stress that results in the inhibition of its repressive function. SUMOylation of CtBP1 leads to its retention in the nucleus and opposes its interaction with proteins such as nNOS, which improves its cytoplasmic localization (31) (Figure 4).

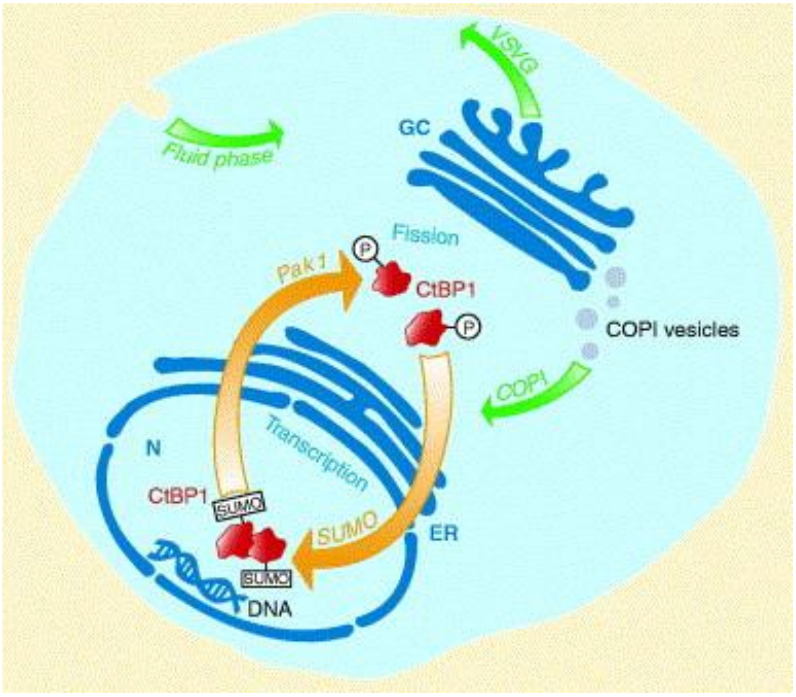

Figure 4 – Schematic representation of the mechanisms that mediate the location and functions of CtBP in the cell. Depending on its location in the cell, CtBP1 can have different functions. In the nucleus, SUMOylated CtBP1 acts as a transcriptional corepressor, regulating the assembly of multiprotein complexes. In the cytoplasm, CtBP1, phosphorylated by Pak1, acts in membrane fission, and is involved in several dynamin-independent membrane fission steps such as: fragmentation of Golgi complex membranes during mitosis, transport of the VSVG cargo reporter from the trans network-Golgi to the basolateral plasma membrane in epithelial cells, fluid phase endocytosis and retrograde transport mediated by COPI vesicles. ER, endoplasmic reticulum; N, nucleus; P, phosphorylation; SUMO, sumoylation. Adapted from (36).

3.3. Role of CTBPs in neuroinflammation and neurological disorders

In recent years, several studies have shown that CtBPs are highly expressed in inflammatory diseases. Its overexpression results in the upregulation of pro-inflammatory cytokine genes through direct or indirect mechanisms (37).

CtBPs play a role in regulating the inflammatory response of microglia and astrocytes. On the one hand, it can inhibit the inflammatory response in these cells by exerting transcriptional repression of pro-inflammatory genes. Saijo et al., found that the endogenous estrogen receptor β ligand, 5-androsten-3β,17β-diol (ADIOL), mediated the recruitment of CtBP1 and CtBP2 to the promoter region of c-Jun/c-Fos AP1-dependent promoters, leading to the transcriptional repression of genes that are implicated in the activation of the inflammatory response in Th17 T cells (38). On the other hand, there is evidence that shows that they can also induce the inflammatory response through TLRs. A study using an LPS-induced neuroinflammation model in rats observed an increase in CtBP2 expression in activated microglia and astrocytes. The knockdown of CtBP2 in cultured microglia prevented the release of TNF-α (31).

For example, traumatic brain injury (TBI) induces an acute inflammatory response in the CNS. One study demonstrated that depending on severity and time in a mouse model of TBI, both CtBPs are highly induced. In this study, the induction of a set of NF-κB target genes, such as IL-1β, IL-6 and TNF-α, was also observed (37).

On the other hand, transcriptional repression by CtBPs regulates gene expression in epileptogenesis, to generate a repressive chromatin environment around the BDNF promoter suggesting the participation of CtBPs in activity-dependent gene expression, indispensable for higher brain functions, including memory and learning (30).

Most studies on CtBPs are focused on cancer due to their participation as a transcription factor in several functions associated with cell proliferation and apoptosis (26). CtBPs act as oncogenes in several types of cancer, including osteosarcoma, melanoma, pancreatic ductal adenocarcinoma, colon, prostate breast, ovarian cancer, and leukemia. These proteins regulate several genes that control different aspects of tumorigenesis and progression, including apoptosis, cell proliferation, migration, invasion, metastasis, and multidrug resistance (37). In addition to their overexpression, they can also be hyperactivated in cancer cells since these cells normally have higher levels of NADH due to hypoxia. In this situation, NADH binds to CtBPs with high affinity promoting a conformational change that favors its binding to transcriptional repressors and leading to homo and heterodimerization of CtBPs (32).

Taking into account that overexpression of CtBPs mediates aggressive and metastatic neoplastic behavior, it is expected that pharmacological inhibition of CtBP may be beneficial in the treatment of cancer. Several approaches have been proposed in recent times where these agents act by inhibiting enzymatic activity, interfering with dimerization, or inhibiting the interaction of CtBP with other co-regulators (32).

Our research group was a pioneer in characterizing the effect of CtBPs, where it was observed that they play an important role in neuronal function and survival. It has been demonstrated that both CtBPs are expressed in the subventricular zone where they promote neurogenesis (39). Furthermore, their expression has also been demonstrated in neurons, dopaminergic neurons, astrocytes and microglial cells in the substantia nigra and striatum of adult wild-type mice where they are involved in dopaminergic survival (40).

Considering the abovementioned evidence on the participation of CtBPs in neuroinflammation and neurological diseases, future studies should be carried out on the role of these proteins in neuroinflammation and subsequently consider them as potential therapeutic targets for some neurodegenerative diseases. This work aims to decipher the expression and function of CtBPs in experimental models of LPS-induced neuroinflammation.

Chapter 2

Objectives

CtBPs are highly expressed in the hippocampus and can regulate neuronal survival and function (39). However, the role CtBPs in neuroinflammation, particularly how their expression and function are altered under these conditions, is not well known.

The first objective of this work was to evaluate the expression of CtBP1 and 2 in *in vivo* and *ex vivo* experimental models of neuroinflammation induced by LPS.

After this characterization, the second objective of this project was to evaluate the function of CtBP1 and 2 in the LPS-induced neuroinflammation model *ex vivo*, using organotypic hippocampal slice cultures.

Chapter 3

Materials and Methods

All animal experiments were conducted following protocols approved by the Direção-Geral de Alimentação e Veterinária (DGAV) and Órgão de Bem-Estar e Ética Animal (ORBEA) from the Health Sciences Research Centre (CICS-UBI). All efforts were made to minimize the number of animals used in this study and potential suffering, according to the 3R's principles (replacement, reduction, and refinement).

1. Organotypic Hippocampal Slice Cultures

Hippocampal slice cultures were obtained from 7-day-old C57BL/6 mice, as described by Ferreira et al. (2012) (41). Animals were sacrificed by puncturing the back of the neck to cut the spinal cord and brains were removed to isolate both hippocampi, under sterile conditions. Each hippocampus is cut into 350 μm-thick slices using a tissue chopper (Mcllwain), slices were selected in Geiss medium (composed of Gey's Balanced Salt Solution (GBSS) (Sigma-Aldrich) and 10 μL/mL glucose 50%) and displayed in groups of 6 in 0.4 μm porous insert membranes (Millipore), which were placed in six-well plates containing culture medium (composed of 25% heat-inactivated horse serum (Pan Biotech), 50% OPTI-MEM (Gibco), 25% Hank's Balanced Salt Solution (HBSS) (Gibco), 10 μL/mL glucose 50%, 10,000 U/mL penicillin and 10,000 μg/mL streptomycin (Gibco)) kept at 37ºC, 5% CO_2. Cultures were kept in a humidified atmosphere (5% CO_2) at 37º C and media were renewed every 2 days for 2 weeks.

After two weeks, the culture medium was switched to Neurobasal medium (composed of 98% Neurobasal Medium (Gibco), 2% B27 supplement (Gibco), 10 μL/mL glutamine (12,5 mM) (Gibco), 10,000 U/mL penicillin and 10,000 μg/mL streptomycin) for 24 hours. To induce neuroinflammation, slice cultures were exposed to 100 ng/mL or 1 μg/mL LPS (from *Escherichia coli* L2880, Sigma-Aldrich), at 37º C, 5% CO_2 for 24 hours. Some hippocampal slices were exposed to a CtBPs antagonist, α-Keto-γ-(methhylthio) butyric acid sodium salt (MTOB) (Aldrich) at 25 μM and 250 μM, and retinoic acid (RA) (Sigma) at 10 μM, for 24 hours. Non-treated controls were included in all experiments (Figure 5).

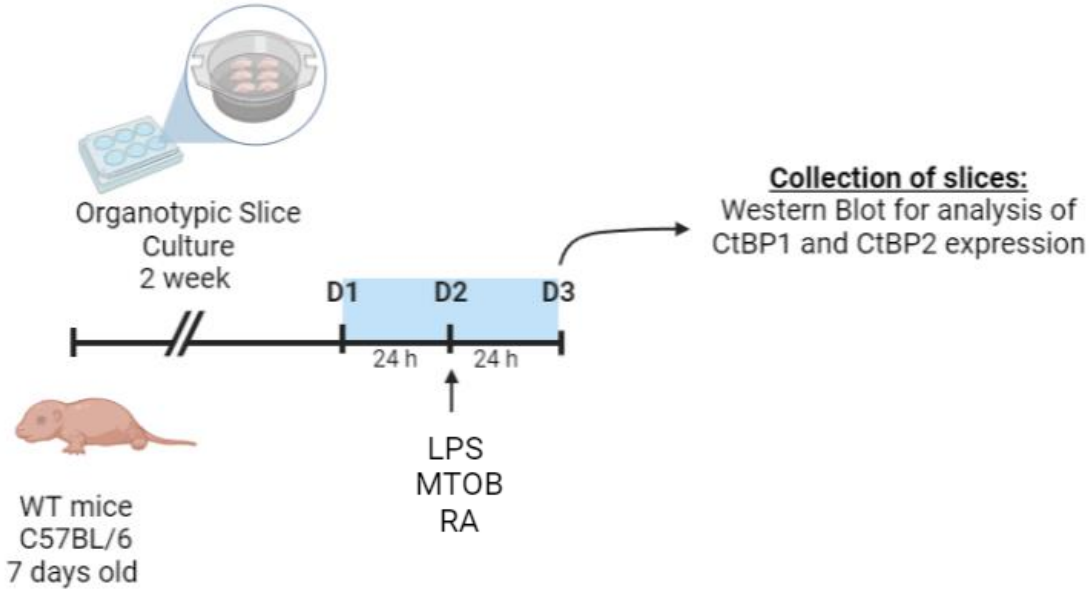

Figure 5 – Schematic representation of the experimental protocol for culturing organotypic hippocampal slice cultures. Organotypic hippocampal slice cultures obtained from 7-day-old C57BL/6 mice were treated for 24 hours with 100 ng/mL or 1 µg/mL LPS to analyze CtBP expression or LPS 100ng/mL, LPS 100ng/mL more MTOB (250µM or 25 µM), or LPS 100ng/mL more MTOB (250µM or 25µM) and RA 10 µM to analyze the modulation of CtBPs functions. The control group was treated with Neurobasal medium only.

2. *In vivo* Studies

Wild-type C57BL/6 mice (three to five months old) were used for the *in vivo* experiments. Mice were maintained under controlled conditions (12 hours of light/dark cycle at a temperature of 22°C with free access to food and water).

In these studies, three protocols with different concentrations of LPS (from *Escherichia coli* L2880, Sigma-Aldrich) and different time points were performed to mimic distinct inflammatory reactions. In all experiments, mice injected intraperitoneally with PBS were considered the control group (saline).

In the first protocol (Group 1), mice were injected intraperitoneally for four consecutive days with LPS at 300 µg/kg or 500 µg/kg. This protocol has previously been used by Wendeln et al., 2018 to assess immune memory induced in the brain by peripheral stimulation, originating a mild sickness behavior and temporary weight loss (42). Mice were euthanized 3 hours after the last injection (Figure 6). The second group of animals (Group 2) received an intraperitoneal injection *per* day for seven consecutive days with LPS at 500 µg/kg (Figure 7). Previous studies have shown that repeated exposure to LPS is capable of triggering prolonged neuroinflammation resulting in the progressive loss of dopaminergic neurons (43). In the last group (Group 3), mice were injected intraperitoneally with LPS at 2 mg/kg and euthanized four days after (Figure 8). As

previously described by Machado-Pereira et al. (2022), this protocol induces neuroinflammation and neuronal dysfunction (44).

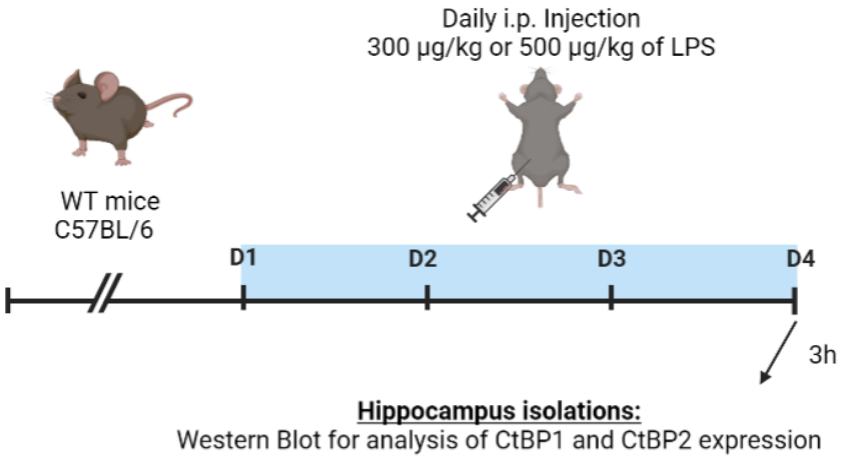

Figure 6 – Schematic representation of the experimental protocol for Group 1. Adult wild-type (WT) mice C57BL/6 received daily intraperitoneal (i.p.) injections of LPS 300 µg/kg or 500 µg/kg for 4 days. The control group received injections with the same volume of sterile PBS.

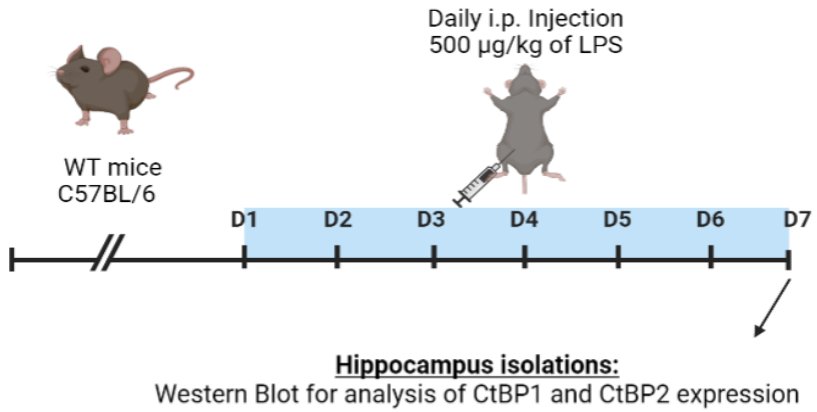

Figure 7 – Schematic representation of the experimental protocol for Group 2. Adult wild-type (WT) mice C57BL/6 received daily intraperitoneal (i.p.) injections of LPS 500 µg/kg for 7 days. The control group received injections with the same volume of sterile PBS.

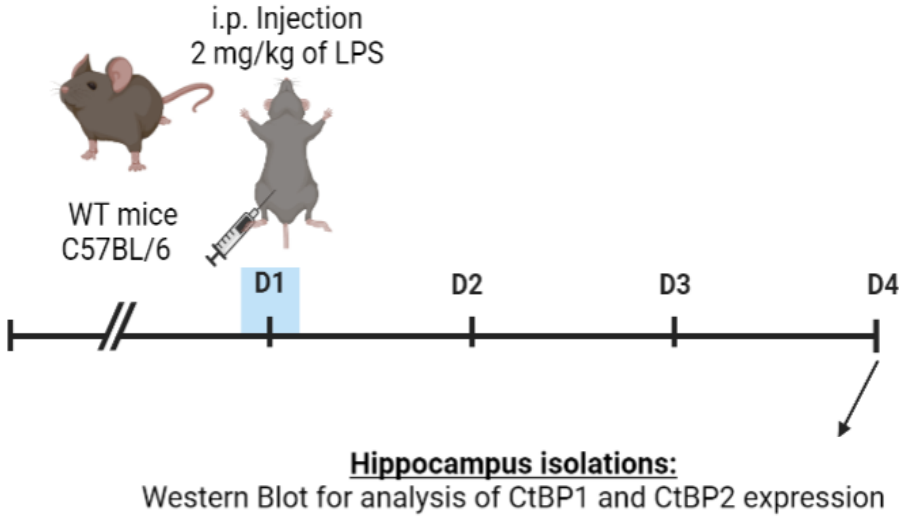

Figure 8 – Schematic representation of the experimental protocol for Group 3. Adult wild- type (WT) mice C57BL/6 received an intraperitoneal (i.p.) injection of LPS 2 mg/kg and hippocampi were collected 3 days after the injection. The control group received an injection with the same volume of sterile PBS.

3. Protein Quantification

After treatments, organotypic hippocampal slices and one hippocampus *per* animal were mechanically dissociated, lysed on ice in RIPA Buffer (50 mM Tris-Base, pH=8.0; 150 mM NaCl; 1% Triton X-100; 0,5% Sodium Deoxycholate; 0.1% Sodium dodecyl sulfate (SDS)), containing a cocktail of protease inhibitors (Sigma-Aldrich) and sonicated for 10 minutes. Then, a centrifugation at 12000 rpm, for 20 minutes, at 4°C was made and the supernatant was collected. The total protein was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher). The absorbance was measured at 570 nm and protein concentration was calculated.

4. Western Blot

A total of 30 µg of protein was loaded into the 10% SDS polyacrylamide gels and proteins were separated using a running buffer solution (25 mM Tris, 190 mM glycine, 0,1% SDS; pH=8.3) by SDS-PAGE electrophoresis at 120V, 90 minutes, at room temperature (RT). After, the proteins were transferred to a polyvinylidene fluoride membrane (Millipore) through a semidry transfer for 90 minutes at 0.75 A on ice using transfer buffer (25 mM Tris, 190 mM glycine, 20% Methanol; pH=8.3). Then, membranes were blocked in Tris Buffer Saline (TBS) containing 0.1% Tween 20 and 0.05% BSA for 1 hour at RT. Membranes were incubated with primary antibody mouse anti-CtBP1 (1:2500, 48 kDa;

BD Bioscience), mouse anti-CtBP2 (1:2500, 48 kDa; BD Bioscience), mouse anti-GFAP (1:1000, 50 kDa; Cell Signaling) and rabbit anti-P47-phox (1:1000, 47 kDa; St John's Laboratory) overnight at 4ºC. After washing three times with TBS-T, they were incubated with the respective secondary antibody m-IgGκ BP (1:10000; Santa Cruz Biotechnology) and anti-rabbit antibody (1:10000; Santa Cruz Biotechnology), diluted in washing buffer, at RT for 90 minutes. After, the membranes were incubated with the housekeeping antibody mouse anti-GAPDH (1:5000, 37 kDa, Millipore) for 90 minutes and the corresponding secondary antibody m-IgGκ BP (1:5000; Santa Cruz Biotechnology) for another 90 minutes, both at RT, to normalize the expression of the target protein. Finally, the membranes were incubated with Pierce™ ECL Western Blotting Substrate and protein levels were detected by ChemiDocTM MP and quantified using the software ImageJ.

5. Data Analysis

Experimental conditions were performed at least in two sets of independent experiments (n). Statistical analysis was made using GraphPad Prism 8.0 Software, using unpaired two-tailed Student's t-test or one-way ANOVA followed by Dunnett's multiple comparisons test. Values of $P<0.05$ were considered significant and data are shown as the mean ± standard error of the mean (SEM).

Chapter 4

Results

1. Expression of CtBP1 and CtBP2 *in vivo*

The first task of this work was to characterize the protein expression of CtBPs in the hippocampi of adult mice exposed to one or several consecutive ip injections with different concentrations of LPS to mimic distinct inflammatory conditions. The control group received ip injections of the same volume of sterile PBS. Protein expression of CtBPs was analyzed by Western-Blot.

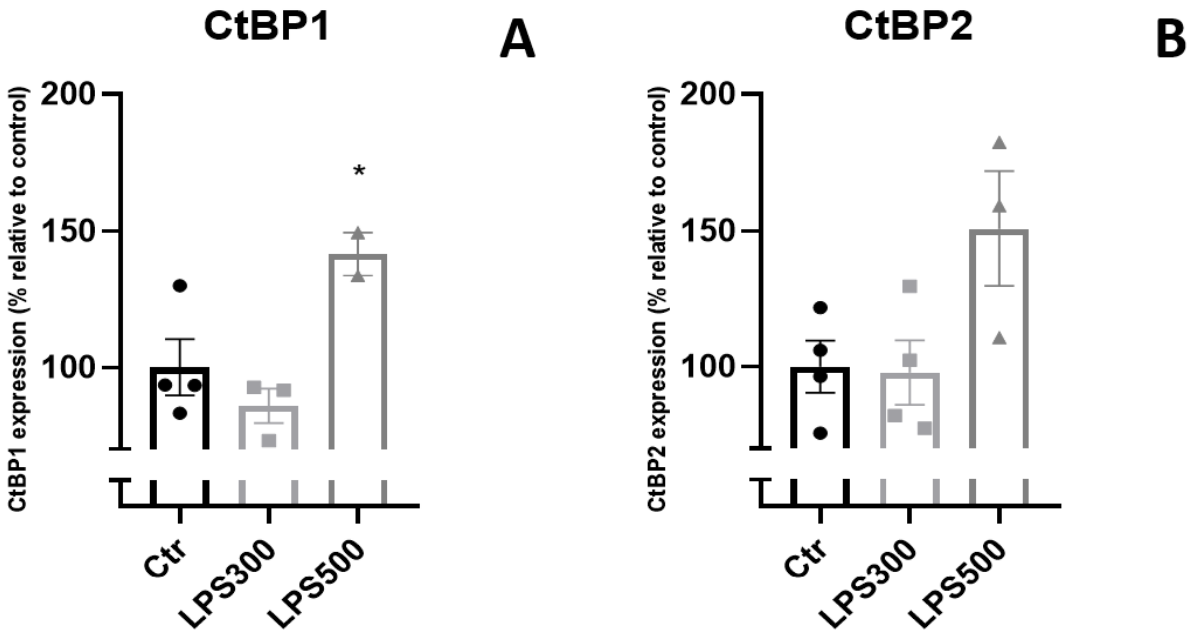

Figure 9 - The effects of LPS on the expression levels of CtBP1 and CtBP2 in the hippocampus (Group 1), *in vivo*. Mice were injected with daily intraperitoneal injections of LPS 300 µg/kg (LPS300) or 500 µg/kg (LPS500) for 4 days. The control group received PBS. Graphs depict the percentage of (A) CtBP1 and (B) CtBP2 expression in the hippocampus. Protein expression was normalized to GAPDH. Data are expressed as a percentage of mean ± SEM (n=2-4) and the controls were set to 100%. Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. *p<0.05 when compared with control.

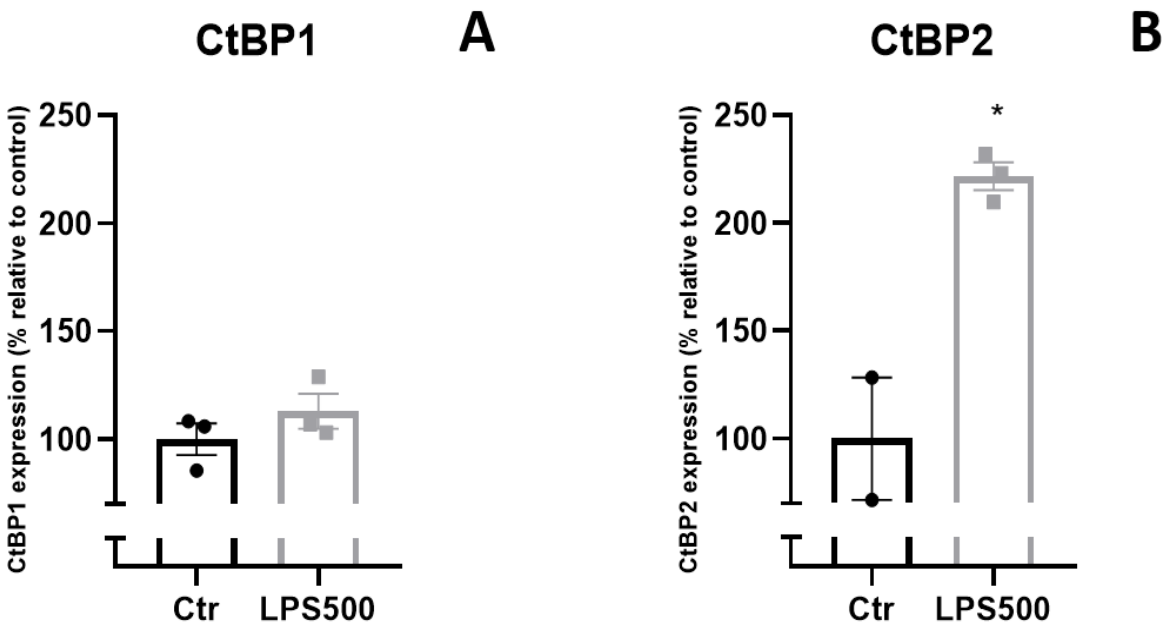

Figure 10 – The effects of LPS on the expression levels of CtBP1 and CtBP2 in the hippocampus (Group 2), *in vivo*. Mice were injected with daily intraperitoneal injections of LPS 500 µg/kg (LPS500) for 7 days. The control group received PBS. Graphs depict the percentage of (A) CtBP1 and (B) CtBP2 expression in the hippocampus. Protein expression was normalized to GAPDH. Data are expressed as a percentage of mean ± SEM (n=2-3) and the controls were set to 100%. Statistical analysis was performed using unpaired two-tailed Student's t-test. *p<0.05 when compared with control.

In Group 1 (Figure 9), a significant increase in the expression levels of CtBP1 was found in animals exposed to a dose of LPS 500 µg/kg for 4 consecutive days as compared to the control. The same trend was found for CtBP2 but without reaching statistical significance. On the contrary, no significant differences were found in animals exposed to a dose of LPS 300 µg/kg as compared to the control (Figure 9A, $mean_{Ctr}$ =100.0 ± 10.2, n=4; $mean_{LPS300}$ =85.9 ± 6.3, n=3; $mean_{LPS500}$ =141.5 ± 7.9, n=2; *p<0.05; Figure9B, $mean_{Ctr}$ =100.0 ± 9.6, n=4; $mean_{LPS300}$ =97.9 ± 11.9, n=4; $mean_{LPS500}$ =150.7 ± 21.2, n=3).

Similar results were obtained for the second protocol (Group 2) (Figure 10), in which animals were exposed to a dose of LPS 500 µg/kg for 7 consecutive days. The expression of both CtBP1 and CtBP2 was increased as compared to controls, while only CtBP2 reached a statistically significant result (Figure 10A, $mean_{Ctr}$ =100.0 ± 7.2, n=3; $mean_{LPS500}$ =113.0 ± 8.0, n=3; Figure 10B, $mean_{Ctr}$ =100.0 ± 28.3, n=2; $mean_{LPS500}$ =221.6 ± 6.4, n=3; *p<0.05).

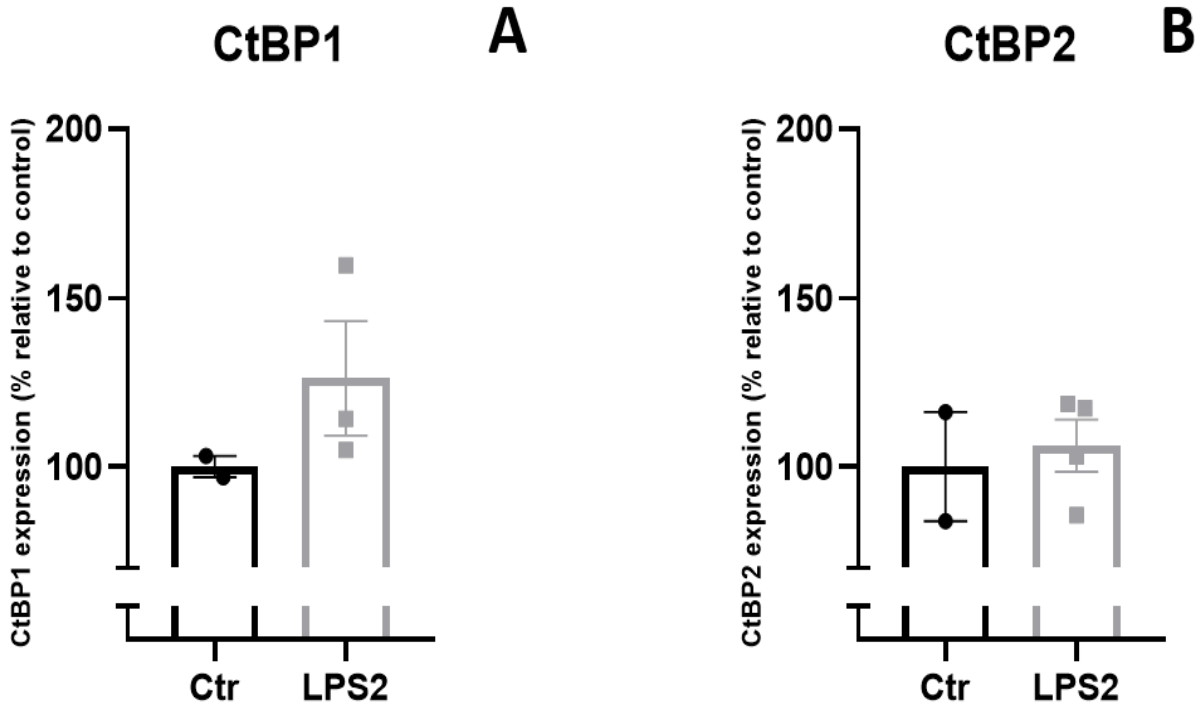

Figure 11 – The effects of LPS on the expression levels of CtBP1 and CtBP2 in the hippocampus (Group 3), *in vivo*. Mice were injected with an intraperitoneal injection of LPS 2 mg/kg (LPS2) and hippocampi were collected 3 days after the injection. The control group received PBS. Graphs depict the percentage of (A) CtBP1 and (B) CtBP2 expression in hippocampus. Protein expression was normalized to GAPDH. Data are expressed as a percentage of mean ± SEM (n=2-4) and the controls were set to 100%. Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test.

In animals exposed to a higher concentration of LPS (2 mg/kg; Group 3) (Figure 11), we found an increasein the expression of CtBP1 still not reaching statistical significance as compared to controls. The expression of CtBP2 was similar to controls (Figure 11A, $mean_{Ctr}$ =100.0 ± 3.1, n=2; $mean_{LPS2}$ =126.2 ± 17.0, n=3; Figure 11B, $mean_{Ctr}$ =100.0 ± 16.1, n=2; $mean_{LPS2}$ =106.1 ± 7.7, n=4).

Our results showed that distinct inflammatory environments induced by ip injections with several doses and timings of LPS induced different responses in terms of the expression of both CtBPs. Overall, there is a tendency for increased CtBPs expression, particularly when the dosage of 500 μg/mL is used and mice are exposed for 4 days.

2. Expression of CtBP1 and CtBP2 *ex vivo*

Then, we evaluated the expression of CtBPs in organotypic hippocampal slice cultures to assess whether we could mimic the *in vivo* data. Hippocampal slices were treated with 100 ng/mL or 1 μg/mL LPS for 24 hours (45,46). An increase in the expression of both CtBPs was observed in cultures treated with both LPS concentrations similarly to *in vivo*

data (Figure 12). The highest increase was observed with CtBP1 expression at the concentration of LPS 100 ng/mL, where statistical significance was obtained in relation to the control (Figure 12A, $mean_{Ctr}$ =100.0 ± 7.8, n=5; $mean_{LPS100}$ =131.4 ± 5.5, n=5; $mean_{LPS1}$ =112.9 ± 4.8, n=6; **p<0.01; Figure 12B, $mean_{Ctr}$ =100.0 ± 14.1, n=6; $mean_{LPS100}$ =102.9 ± 7.4, n=6; $mean_{LPS1}$ =111.1 ± 13.6, n=4).

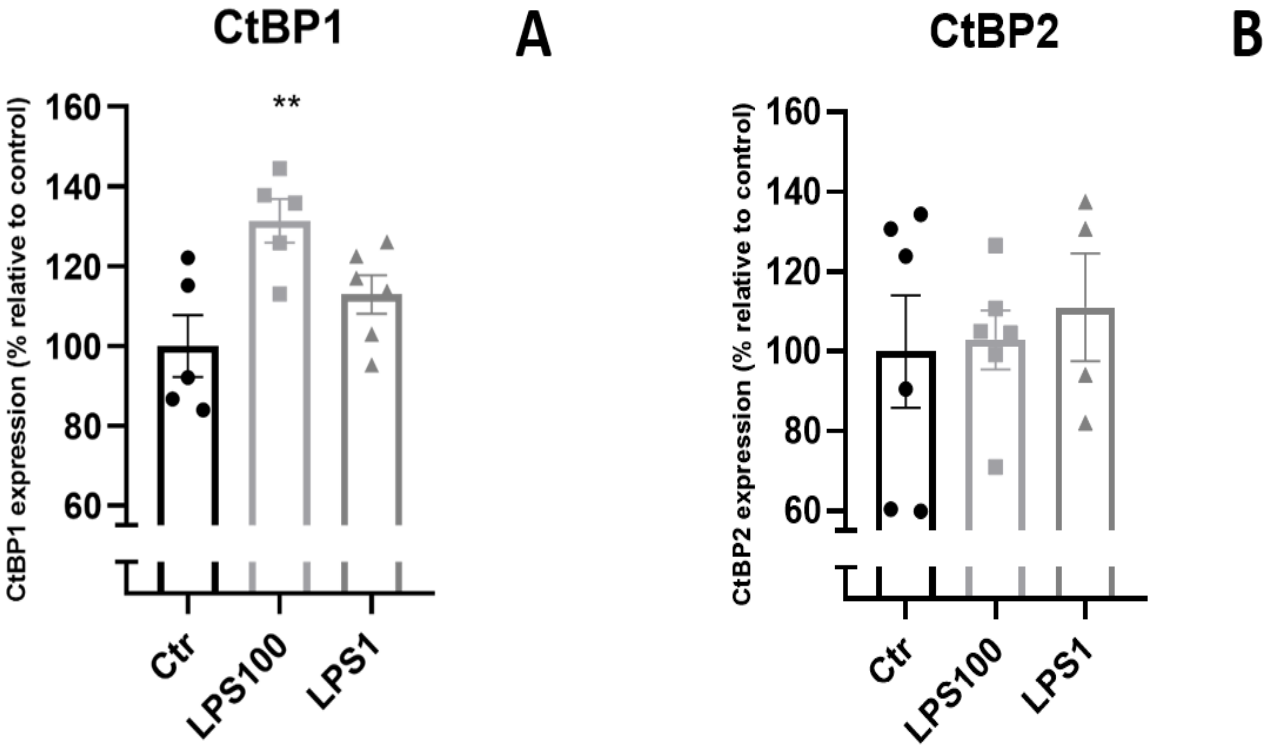

Figure 12 – The effects of LPS on the expression levels of CtBP1 and CtBP2 in hippocamp*al* organotypic slice cultures. Hippocampal slices were treated with LPS 100 ng/mL (LPS100) or 1 µg/mL (LPS1) for 24 hours. The control group (Ctr) was treated with Neurobasal medium only. Graphs depict the percentage of (A) CtBP1 and (B) CtBP2 protein expression relative to control conditions (set to 100%). Protein expression was normalized to GAPDH. Data are expressed as a percentage of mean ± SEM (n= 4-6). Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test. **p<0.01 when compared with control.

3. Modulation of CtBP1 and CtBP2 functions *ex vivo*

Finally, we evaluated the function of CtBPs in organotypic hippocampal slice cultures by using MTOB, a well-known CtBP antagonist that depending on concentrations may also act as an agonist (39,40). Moreover, we also used retinoic acid (RA) a well-known anti-inflammatory and neuroprotective agent (47,48). Protein expression of P47phox and GFAP was analyzed by Western-Blot, as markers of phagocytic cells and astrocytes, respectively (Figure 13). Hippocampal slices were treated with LPS 100ng/mL (LPS100) alone, LPS 100ng/mL more MTOB 250µM or 25 µM (MTOB250; MTOB25), or LPS 100ng/mL more MTOB 250µM or 25µM and retinoic acid 10 µM (MTOB250+RA10, MTOB25+RA10) for 24 hours. Regarding the expression of P47phox, a decreased expression was found in slices treated with LPS 100ng/mL and LPS with MTOB (both concentrations) compared to controls, while not reaching statistical significance. Interestingly, RA was able to counteracy this decrease to levels similar to control.

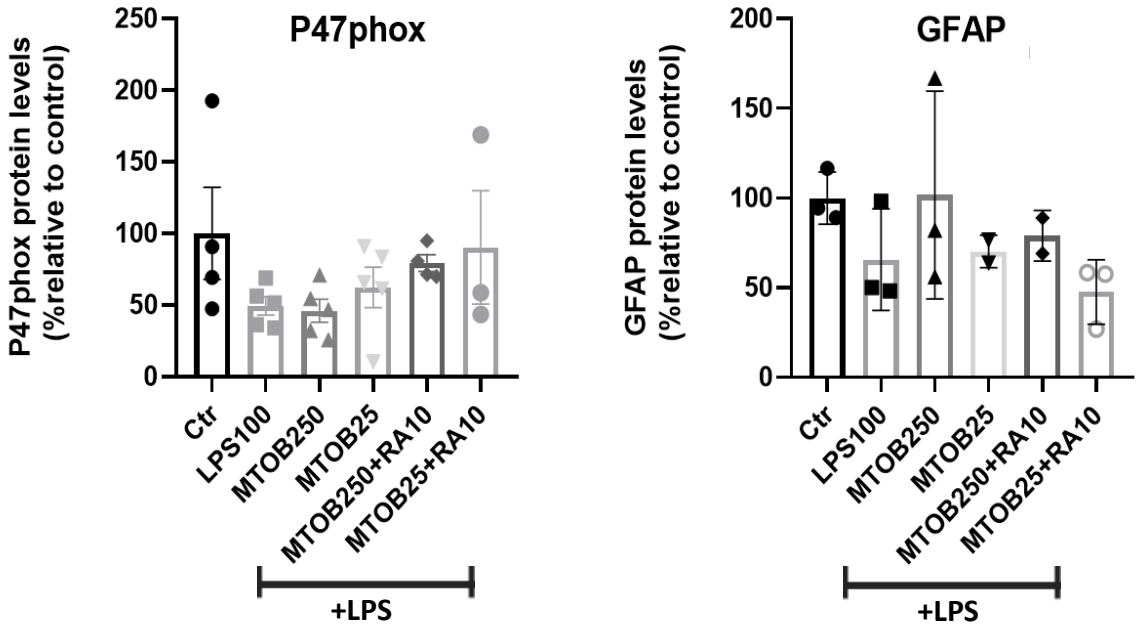

Figure 13 – The effects of MTOB on the expression levels of P47phox and GFAP in hippocamp*al* organotypic slice cultures. Hippocampal slices were treated with LPS 100ng/mL (LPS100), LPS 100ng/mL more MTOB 250µM (MTOB250), LPS 100 ng/mL more MTOB 25 µM (MTOB25), LPS 100ng/mL more MTOB 250µM and retinoic acid 10 µM (MTOB250+RA10) or LPS 100 ng/mL more MTOB 25µM and retinoic acid 10 µM (MTOB25+RA10) for 24 hours. The control group (Ctr) was treated with Neurobasal medium only. Graphs depict the percentage of (A) P47phox and (B) GFAP protein expression relative to control conditions (set to 100%). Protein expression was normalized to GAPDH. Data are expressed as a percentage of mean ± SEM (n= 2-5). Statistical analysis was performed using one-way ANOVA, followed by the Dunnett's multiple comparison test.

Regarding the astrocyte marker, a more pronounced decrease was observed in the group exposed to LPS and in the group treated with LPS plus MTOB 25 µM and RA 10 µM. The remaining groups presented similar results to each other, still showing a decrease as compared to controls. (Figure 13A, $mean_{Ctr}$ =100.0 ± 32.1, n=4; $mean_{LPS100}$ =49.4 ± 6.5, n=5; $mean_{MTOB250}$ =50.0 ± 8.0, n=5; $mean_{MTOB25}$ =75.2 ± 7.1, n=4; $mean_{MTOB250+RA10}$ =79.2

± 5.8, n=4; $mean_{MTOB25+RA10}$ =90.4 ± 39.6, n=3; Figure 13B, $mean_{Ctr}$ =100.0 ± 8.4, n=3; $mean_{LPS100}$ =65.6 ± 16.4, n=3; $mean_{MTOB250}$ =101.7 ± 33.5, n=3; $mean_{MTOB25}$ =70.2 ± 6.4, n=2; $mean_{MTOB250+RA10}$ =79.0 ± 10.0, n=2; $mean_{MTOB25+RA10}$ =47.6 ± 10.4, n=3).

Chapter 5

Discussion

It is known that inflammatory responses are influenced at the transcriptional level by factors that affect the cellular bioenergetic state. A potential mechanism that alters this cellular state is the cytosolic NADH:NAD^+ ratio, which is thermodynamically coupled to glycolysis and directly related to inflammation. Only a very limited number of NADH-sensitive proteins affect genetic transcription, among them are CtBPs that function as transcriptional corepressors (49). However, there is scarce information about the role of CtBPs in neuroinflammation. The first objective of this study was to evaluate how CtBP1 and 2 are expressed in LPS-induced neuroinflammation *in vivo* and *ex vivo* models.

Several studies have demonstrated that LPS administration in mice induces the activation of astrocytes and microglia, COX-2, iNOS and expression of pro-inflammatory cytokines in the brain such as IL-1β, IL-6 and TNF-α. However, the route of administration, dose and duration of the stimulus can vary the type of inflammatory, neurotoxic, and neuroprotective response (14).

We started this project by using two concentrations of LPS, 300μg/kg and 500μg/kg, that are widely described in the literature to induce an activation of the innate immune system (42). A third dose of 2 mg/kg was also used to induce acute neuroinflammation. However, because is such a high dose, it can only be administered in a single dose to avoid the mortality of the animals (44). In addition to distinct doses, we also used different administration paradigms to mimic mild and chronic neuroinflammation responses.

In this study, we found an upregulation in the expression of CtBPs in various models of neuroinflammation. Through western-blot analysis, we observed that in mild and chronic stimuli (4 and 7 days) there is a significant expression of CtBPs, namely CtBP1 and and CtBP2, the latest especially in the chronic stimuli (7 days). Furthermore, we observed that the dosage of 500 μg/kg for 4 days induces the expression of both CtBPs. However, when we changed the protocol to 7 days with the same concentration, we only observed an increase in CtBP2 expression. These results may suggest that CtBP2 expression is involved in more chronic situations, such as neurodegenerative diseases. In fact, this chronic protocol has been described to induce dopaminergic neurodegeneration therefore mimicking PD pathogenesis (43). Previous studies by

others suggested that CtBP2 could play a functional role in the glial cell-mediated immune response. Confocal laser scanning microscopy revealed that CtBP2 expression was co-localized with neurons, astrocytes and microglia and its expression significantly increased in astrocytes and microglia after exposure to LPS. Therefore, CtBP2 may play an important role in immune responses mediated by glial cells in the CNS (50). A pre-incubation with the Pep1-E1A peptide of primary microglia and mouse astrocytes before LPS stimulation increased the expression of nine pro-inflammatory genes regulated by CtBP. On the other hand, the simultaneous knockdown of CtBP1 and CtBP2 suppressed the mRNA expression of pro-inflammatory genes in LPS-activated microglia and macrophages (51).

A careful imaging analysis of the expression patterns for both CtBP1 and CtBP2 in the mouse brain demonstrated an almost complementary expression of the CtBP family members. For example, the hippocampal CA1 reaction showed greater immunoreactivity for CtBP1 while CtBP2 showed greater immunoreactivity in the CA2 and CA3. However, the cellular nuclei of the granule cells of the dentate gyrus exhibited a special labeling of both CtBPs. Furthermore, staining for both CtBPs in glutamatergic and GABAergic cells in dissociated hippocampal cultures revealed greater nuclear expression of CtBP2 in excitatory cells while CtBP1 showed slightly higher levels in inhibitory cells (30). In a study carried out in mice with AD, an increase in memory and learning was found after overexpression of CtBP1. Furthermore, it improved the morphological changes of hippocampal and cortical neurons, increased neuronal activity, and inhibited neuronal apoptosis (52). Altogether, these studies suggest that CtBPs are expressed in the hippocampus and that the expression of these proteins may vary depending on the subregion. Furthermore, it appears that modulating the expression of these proteins may be beneficial in neurodegenerative diseases.

To reduce the number of animals used for *in vivo* experiments, we then evaluated the expression of CtBPs in organotypic hippocampal slice cultures. These cultures have a cellular and tissue organization that mimics the *in vivo* conditions (53). Similarly, to *in vivo* experiments, we observed an increase in the expression of CtBPs in the group exposed to the LPS concentration of 100 ng/mL, particularly CtBP1. Therefore, we concluded that organotypic hippocampal slice cultures are capable of mimicking what happens *in vivo*.

For the second part of this work, we analyzed de role of CtBPs in neuroinflammation in the presence of MTOB (a CtBP antagonist) and RA. MTOB acts as a substrate for both

CtBPs, 80 to 5,000 times more specific than other similar α-keto acids due to its interaction in the tryptophan active site, which is unique among other dehydrogenases. The catalysis of this compound has biphasic kinetics. At high concentrations (millimolar) it inhibits the function of CtBPs (antagonist) by preventing NADH-dependent dimerization of CtBPs and suppressing their downstream activity while at lower concentrations it activates the functions of CtBPs (agonist). Most studies focus on testing high concentrations of MTOB in neurons resulting in cell apoptosis. In this work, low concentrations of MTOB were used because were already validated by us in previous studies and because organotypic slices are sensitive structures that could suffer degradation with higher concentrations (39,40).

RA is the main biologically active derivative of vitamin A (retinol) and plays an important role in neuronal differentiation and axon growth(48). RA has been considered as a therapeutic option for some neurodegenerative diseases. The signal from this compound is translated by specific nuclear receptors: retinoic acid receptors (RAR) and retinoid X receptors (RXR), which are members of the nuclear receptor superfamily. They heterodimerize and bind to a DNA sequence called retinoic acid response element (RARE) and induce genetic transcription (47). Studies demonstrated that de 9-cis-RA isoform inhibited the production of NO by microglia stimulated with cytokines or LPS and suppressed the production of NO by astrocytes stimulated with LPS. Furthermore, this isoform potently inhibited the production of TNF-α and IL-1β by microglia (54). Previously, our group developed nanoparticles loaded with retinoic acid capable of inducing a neuroprotective phenotype of microglia. RA-loaded nanoparticles inhibited an M1 microglial phenotype while inducing the M2 (neuroprotective) stage in LPS-induced injuries (48).

We thus incubated organotypic hippocampal cultures with LPS, MTOB and RA and evaluated the expression of the glial markers P47phox and GFAP. p47phox is a marker of phagocytic cells (44). In response to LPS stimulation, in the cytoplasm, P47phox aggregates with gq91 phox to form NOX enzymatic cells that are subject to several levels of regulation, including epigenetic modifications and modulation by SUMO (55). In a study carried out in mice injected intraperitoneally with LPS (2 mg/kg), a decrease in P47phox in the cortex was observed with the administration of LPS (44). We found a decrease in its expression in LPS-treated cultures and levels close to control in groups treated with LPS+MTOB+RA. These results may indicate that RA has an anti-inflammatory response, as already described by us (47,48). On the other hand, MTOB was not able to modulate LPS response.

GFAP is considered a marker of astrocytic cells (44). Studies done to understand LPS-induced neuroinflammation measured GFAP expression in the hippocampus and prefrontal cortex where immunofluorescence results demonstrated that LPS treatment significantly increased GFAP expression in the DG and CA3 regions of the hippocampus as well as in the prefrontal cortex (56). A decrease of GFAP was found in all groups in relation to the control, which may indicate the death of astrocytes or deregulation expression induced by an unknown mechanism. In this set of data, RA was not able to counteract the decreased GFAP expression induced by LPS and/or MTOB. Besides the need for additional experiments to clarify these glial responses, our data suggest that CtBPs play an active role in LPS-induced neuroinflammation.

Chapter 6

Conclusions and Perspectives

Our results suggest that LPS-induced neuroinflammation triggers CTBPs expression with a certain dose and time of exposure pattern. In general, it can be concluded that the lower dose tested (300 ug/kg) was not able to modify CtBPs expression, while the higher dose (500 ug/kg) at a shorter time exposure (4 days) induced the expression of both CtBP1 and CtBP2. Interestingly, the same dose (500 ug/kg) but subject to longer exposure (7 days), only increased the expression of CtBP2, suggesting a possible involvement of this isoform in pathologies with a more chronic duration, such as neurodegenerative diseases. On the other hand, acute stimuli at a higher dose (2 mg/kg) were able to increase the expression of CtBP1, which may indicate the participation of this isoform in more acute phases of neuroinflammation. The pattern observed in organotypic hippocampal slices was similar to *in vivo* data, in which 100 ng/mL LPS induced increased expression of CtBP1 only while 1 μg/mL LPS induced the expression of both CtBPs (while not statistically significant).

Unexpectedly, we observed that LPS induced a decrease of P47phox and GFAP expression in organotypic hippocampal cultures, which is similar when MTOB is added together with LPS. On the other hand, RA was able to counteract reduced expression of P47phox only, suggesting an anti-inflammatory role.

However, it is important to note that the number of animals *per* group is very low and more studies are needed to corroborate these preliminary conclusions, namely: fluorescent immunostaining analysis to evaluate the cellular expression of CtBPs in the hippocampus, LDH assays to evaluate cellular viability, and studies to find out which types of cytokines are being released to better elucidate the neuroprotective or neurotoxic effect. For example, immunoenzymatic assays using ELISA kits ((Enzyme-Linked Immunosorbent Assay) could be done to measure specific cytokines such as IL-1β, IL-6, TNF-α, relevant to neuroprotection or neurotoxicity. In addition, it would also be interesting to carry out real-time PCR (qPCR) to identify which cytokines are being produced locally in brain tissue in response to these stimuli. Furthermore, the effect of CtBPs on neuroinflammation can be investigated using a set of siRNAs specific for each isoform.

Chapter 7

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