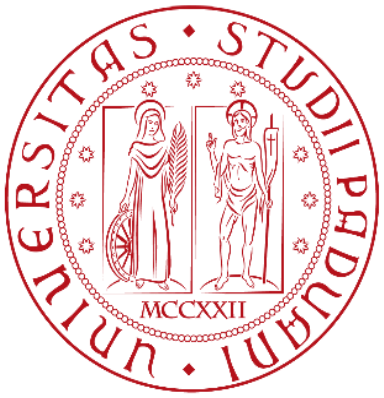


UNIVERSITY OF PADOVA

Department of Pharmaceutical

and Pharmacological Sciences

Doctoral Course in Pharmacological Sciences

***Curriculum* Pharmacology, Toxicology and Therapy**

Cycle XXIX

Characterization of an *in vivo* model of neuroinflammation and evaluation of the anti-inflammatory and neuroprotective effects of curcumin as a potential lead compound for the development of new agents useful to treat neuroinflammatory disorders

PhD student: **Vincenzo Sorrenti**

Supervisor: **Dr Morena Zusso**

Coordinator: **Prof Piero Maestrelli**

I. General index

II. List of abbreviations

AA arachidonic acid

AD Alzheimer's disease

APOE apolipoprotein E

BBB blood brain barrier

CCR2 C-C chemokine receptor type 2

CLR C-type lectin receptor

CNS central nervous system

CSF cerebrospinal fluid

DAMPs Damage-associated molecular patterns

GFAP glial-fibrillary acidic protein

GLT-1 glial glutamate transporter 1

HMGB1 High mobility group box 1

HSP heat shock protein

Iba-1 ionized calcium-binding adapter molecule 1

IFN interferon

IKK-β inhibitor of κB kinase

IL interleukin

iNOS inducible nitric oxide synthase

JNK c-jun N-terminal kinase

KO knockout

LPS lipopolysaccharide

MHC major histocompatibility complex

MS multiple sclerosis

NADPH nicotinamide adenine dinucleotide phosphate

NO nitric oxide

NOD nucleotide-binding-protein oligomerization domain

NLR NOD-like receptor

PAMPs pathogen-associated molecular patterns

PD Parkinson's disease

PGE prostaglandin

PGE2 prostaglandin E2

POMC pro-opiomelaocortin

PRRs pattern recognition receptors

RAGE advanced glycation end-product-specific receptor

RLR RIG-like receptor

ROS reactive oxygen species

SOCS suppressor of cytokine signalling

TGF-β transforming growth factor-β

TH tyrosine hydroxylase

TLR toll-like receptor

TLR4 toll-like receptor 4

TNF-α tumor necrosis factor-α

VTA ventral tegmental area

WT wild-type

III. Abstract

Neuroinflammation is a complex and multifactorial response of the central nervous system (CNS) to trauma, infection and neurodegenerative diseases orchestrated by specialized immune cells (microglial and astrocytes). In particular, microglia, the main resident immune cells of the CNS, undergo rapid "activation" in response to noxious stimuli, releasing a plethora of inflammatory and potentially neurotoxic soluble factors, such as cytokines [*e.g.*, interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α], chemokines and reactive oxygen and nitrogen species (*e.g.*, nitric oxide). Although an efficient microglial immune response is necessary and critical for proper resolution of pathological events, it is clear that an excessive activation of these cells contributes to neuronal cell damage in neurodegenerative and psychiatric disorders. However, the molecular mechanism(s) by which these cells exert their deleterious effects on neurons remain poorly understood. Recent evidence suggests that microglial cells can be "activated" in response to a systemic inflammatory stimulus. For example, a single peripheral administration of the bacterial endotoxin lipopolysaccharide (LPS), the main component of Gram-negative bacteria walls, in adult mice, can induce microglial activation and an inflammatory state in the CNS that persists long after peripheral stimulus has decline.

Identification of molecules which prevent or down-regulate microglial inflammatory responses or direct microglia towards a protective anti-inflammatory phenotype could prove efficacious in neurodegenerative diseases in which inflammation is implicated. Recently, increasing interest has focused on identifying natural compounds with potential inhibitory effects on microglial activation and subsequent inflammatory processes. Among these compounds, curcumin (diferuloylmethane), the main bioactive component isolated from the rhizome of the turmeric plant (*Curcuma longa*) with multiple pharmacological effects, including anti-inflammatory activities, possesses neuroprotective properties against many neurodegenerative conditions.

The principal focus of this doctoral project has been the study of the possible anti-inflammatory effect of curcumin in an *in vivo* model of neuroinflammation based

on a single systemic LPS injection. Young adult mice were intraperitoneally injected with a single dose of LPS (0.5 or 5 mg/kg) or vehicle and then tested for "sickness behavior" (*e.g.*, changes in body weight and food intake), mRNA (real-time RT-PCR) and protein (enzyme-linked immunosorbent assay, ELISA) expression of pro-inflammatory mediators and microglia morphological changes (immune-staining with the microglial marker ionized calcium binding adaptor molecule 1) in different brain areas. Both LPS doses induced a significant decrease in food intake and body weight within the first 4 days, followed by a gradual recovery to control values; however, only 5 mg/kg LPS significantly increased TNF-α, IL-1β, IL-6, COX-2 and iNOS gene expression 2 h post-injection. Pre-treatment with 50 mg/kg curcumin (orally administered by *gavage* for 2 consecutive days before LPS injection) facilitated the recovery from sickness behavior (anorexia and weight loss), suppressed LPS-induced microglial morphological changes and increased of mRNA levels of TNF-α, IL-1β and COX-2 in all brain areas, while limiting expression of IL-6 and iNOS to more selected brain regions.

Possible neuroprotective properties of curcumin were also investigated. In particular, mRNA expression of brain-derived neurotrophic factor (BDNF), known to play a key role in the regulation of neuronal function, as well as in learning and memory processes. Its reduced expression, described in numerous neurodegenerative disorders, contributes to the onset of structural abnormalities and functional damage in the CNS. LPS treatment reduced mRNA levels of BDNF in all brain areas analyzed up to 7 days after treatment. Pre-treatment with 50 mg/kg curcumin restored BDNF gene expression 24 hours after LPS injection, while a higher dose of curcumin (100 mg/kg) completely restored the expression of BDNF 2 hours after the inflammatory stimulus.

Finally, behavioral tests demonstrated that LPS induced motor and memory impairments, that persisted even when the inflammatory process had been solved. Moreover, curcumin reversed motor deficits induced by LPS.

Taken together, these data show that curcumin can prevent neuroinflammation by modulating the expression of brain pro-inflammatory mediators *in vivo* and also suggest a potential role of curcumin as a neuroprotective molecule able to restore

BDNF levels and behavioral impairments under inflammatory conditions. In conclusion, curcumin represents a promising lead compound to discover new drug candidates, with improved therapeutic efficacy in the treatment of neurodegenerative and age-related diseases with an inflammatory etiology.

1. Introduction

1.1 Inflammation and neuroinflammation

Inflammation is a complex cascade of self-defensive response to infectious or sterile tissue damage and has the physiological purpose of restoring tissue homeostasis [Medzhitov, 2010]. However, uncontrolled or unresolved inflammation can drive tissue damage, giving rise to a plethora of chronic inflammatory diseases, including neurodegenerative and autoimmune diseases with eventual loss of organ function [Nathan and Ding, 2010]. In fact, signs of persistent and unresolved inflammation are not merely typical of classical inflammatory diseases but they represent also intrinsic features of a wide spectrum of human conditions not previously thought to have an inflammatory component [Serhan *et al*., 2007]. This, justifies the increasing interest in studying inflammatory processes. In this context, an important milestone has been achieved with the knowledge that the resolution of an acute inflammatory condition is crucial to avoid chronic persistent inflammation and ensure proper return to homeostasis [Lawrence and Gilroy, 2007]. Inflammation underlies a wide variety of physiological and pathological processes. Although the pathological aspects of many types of inflammation are well characterized, their physiological functions are mostly elusive [Medzhitov, 2008].

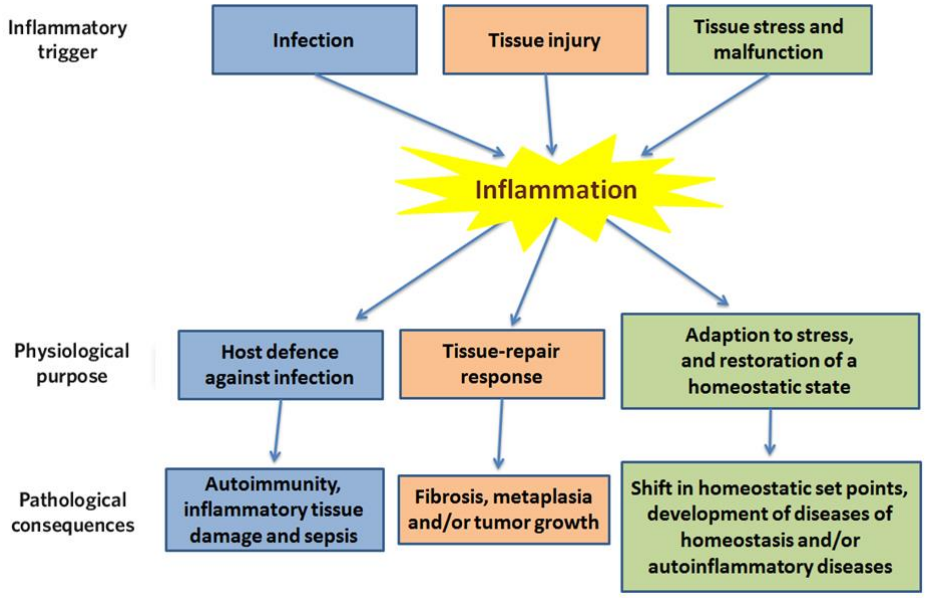


Figure 1. Causes and physiological and pathological outcomes of inflammation. Depending on the trigger, the inflammatory response has a different physiological purpose and pathological consequences. Of the three possible initiating stimuli, only infection induced inflammation is coupled with the induction of an immune response (modified from Medzhitov, 2008).

Typically, the acute inflammatory response, triggered by infection or tissue damage, implies the coordinated migration of the blood components (plasma and leukocytes) to the site of infection or tissue damage (Fig.1) [Majno and Joris, 2004; Kumar *et al*., 2003]. This response has been well characterized for microbial infections, especially bacterial, in which, the innate immune system is activated by binding to receptors known as Toll-like (TLR) and NOD (nucleotide-binding-protein oligomerization domain)-like receptors(NLRs), among others [Barton, 2008]. Tissue resident macrophages and mast cells became activated and release a plethora of inflammatory mediators, including chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades which elicit an inflammatory exudate locally with selective extravasation of neutrophils afforded by the inducible ligation of endothelial-cell selectins with integrins and chemokine receptors on leukocytes [Poeber and Sessa, 2007]. Once reached damaged tissue, neutrophils become activated, either by direct contact with pathogens or through the actions of cytokines secreted by tissue resident cells, and

begin to fight the invading agents by releasing the toxic contents of their granules, which contain reactive oxygen species (ROS) and reactive nitrogen species, proteinase 3, cathepsin G and other molecules [Nathan, 2006]. These highly potent effectors do not discriminate between microbial and host targets, so collateral damage to host tissues is unavoidable [Nathan, 2002]. The successful resolution of the acute inflammatory response is mainly mediated by resident and recruited macrophages producing transforming growth factor-β and growth factors as well as anti-inflammatory lipid mediators (lipoxins, resolvins and protectins) which inhibits the recruitment of neutrophils and promote the recruitment of monocytes and results in the elimination of the infectious agents followed by tissue remodelling and repair [Serhan and Savill, 2005; Serhan, 2007].

If the acute inflammatory response fails to eliminate the pathogen, the inflammatory process persists and acquires new characteristics, recruiting macrophages, and in case of infection, T cells. If the combined effect of these cells is still inadequate, a chronic inflammatory state takes place. Persistent injury or infection, prolonged exposure to a toxic agent, undegradable foreign bodies and autoimmune diseases can induce a chronic inflammatory state characterized by long duration, lymphocyte, plasma cell and macrophage infiltration, tissue destruction (mediated by inflammatory cells)and unsuccessful repair with fibrosis and angiogenesis [Lawrence and Gilroy, 2007]. The complex architecture of systemic chronic inflammation plays a central role in the development of chronic diseases, such as neurodegenerative and autoimmune diseases, although the exact mechanism is still poorly understood.

In the central nervous system (CNS), the inflammatory response to various stimuli (trauma, infection, etc.) represents a highly regulated biological program that enables the innate and adaptive immune systems to effectively fight pathogens, restores cerebral damages and preserves tissue homeostasis. In this context, cellular and molecular immune components such as specialized glial cells (microglia and astrocytes) are the main actors [Glass *et al*., 2010; Hirsh and Hunot, 2009].In particular, microglia, the resident innate immune cells of the CNS, undergo rapid “activation” in response to injurious stimuli, producing a plethora of inflammatory and potentially neurotoxic soluble factors, such as

cytokines [*e.g.*, interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α], chemokines and reactive oxygen and nitrogen species (*e.g.*, nitric oxide) (Fig. 2).

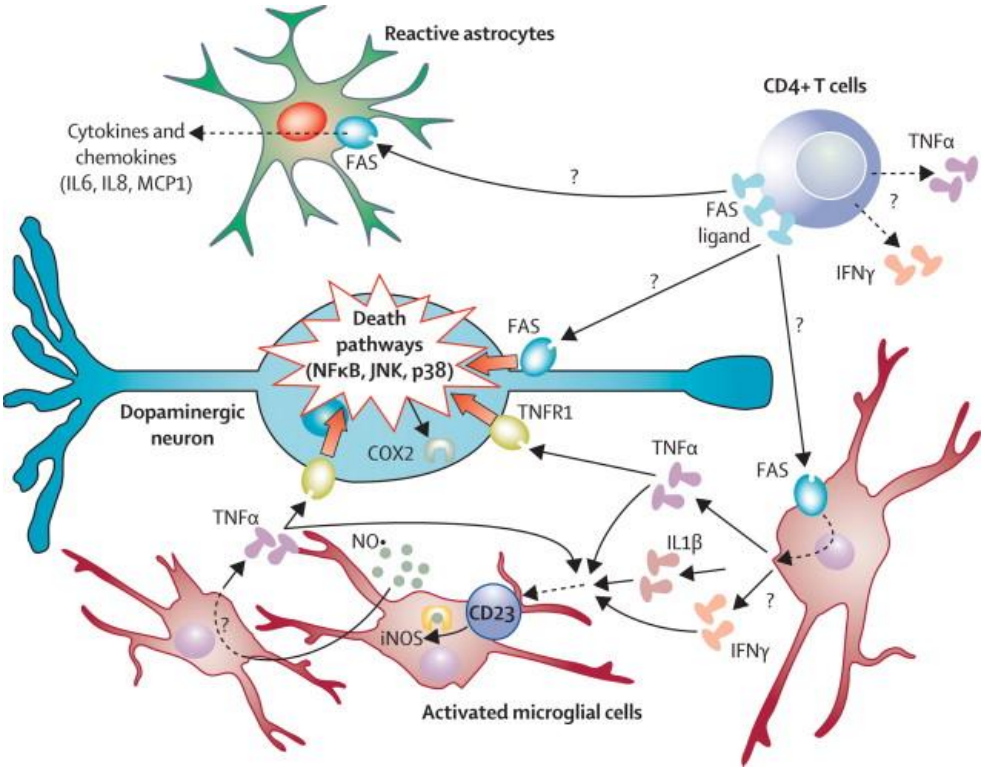


Figure 2. Cells involved in neuroinflammation and neurodegeneration. Under pathological conditions, microglial cells become activated and express iNOS, leading to the production and release of NO free radicals. These cells also have upregulated expression and activation of NADPH oxidase, leading to the production of high amounts of O^{2-} free radicals. In turn, O^{2-} and NO free radicals might react to generate the highly reactive $ONOO^{-}$, which can cause oxidative damage to several proteins within dopaminergic neurons. Reactive astrocytes express increased concentrations of myeloperoxidase, might directly inflict oxidative damage to dopaminergic neurons through, for example, amine conversion into chloramine. Apart from these non-cell-autonomous mechanisms of neuronal cell death, inflammatory-associated oxidative stress might also originate from the damaged dopaminergic neurons through COX2 expression.(from Hirsch and Hunot, 2009)

Although an efficient microglial immune response is necessary and critical for host defence, it is clear that over-activation of microglia contribute to neuronal cell damage [Norden *et al*., 2015]. In response to a peripheral infection, microglial cells produce pro-inflammatory cytokines that act on the brain to cause what is called the "acute phase response (APR)" and "sickness behavior". The latter is characterized by endocrine, autonomic and behavioral changes and includes fever, reductions in activity, food intake and social interactions [Dantzer *et al*., 2008]. When activation of the peripheral immune system continues unabated, such as during systemic infections, cancer or autoimmune diseases, the ensuing immune signalling to the brain can lead to an exacerbation of sickness and the development of a chronic state of neuroinflammation. During a normal infection, neuroinflammation and the resulting adaptive sickness behaviour persists only for several days. However, if these responses become exaggerated or prolonged, the outcomes may become established, leading to neurodegeneration, cognitive impairment and depression [Dantzer *et al*., 2008]. Recent evidence suggests that microglia can be "activated" in response to a systemic inflammatory stimulus. One important and well-known model exhibiting a unique gene expression profile in the brain and an exaggerated neuroinflammatory response is the activation of the peripheral innate immune system by a single peripheral injection of lipopolysaccharide (LPS), the major component of Gram-negative bacterial wall. In adult mice LPS administration can lead to microglial activation and CNS inflammation that persists long after peripheral events have declined [Qin *et al*., 2007; Noh *et al*., 2014].

1.2 Inducers and mediators of neuroinflammation

In the brain, the inflammatory pathway is regulated by a wide range of inducers and mediators that form complex regulatory networks. Inducers initiate the inflammatory response and activate specialized sensors, which then elicit the production of specific sets of mediators. The mediators, in turn, alter the functional states of tissues and organs, like the brain, in a way that allows them to adapt to the conditions indicated by the particular inducer of inflammation. Inducers of neuroinflammation can be exogenous or endogenous agents.

1.2.1 Endogenous inducers

Endogenous inducers are signals produced by stressed, damaged or otherwise malfunctioning of brain tissue. The identity and characteristics of these signals are poorly defined but they probably belong to various functional classes according to the nature and the degree of tissue anomalies on which they report. During necrotic neuronal death, for example, the integrity of the plasma membrane is disrupted, resulting in the release of certain cellular constituents, including ATP, K^+ ions, uric acid, HMGB1 (high-mobility group box 1 protein) and several members of the S100 calcium-binding protein family (S100A8, S100A9 and S100A12) [Rock and Kono, 2008; Bianchi, 2007]. ATP binds to purinoceptors (including P2X7) at the surface of microglia and macrophages, resulting in K^+ ion efflux, and can cooperate with other signals to activate the NALP3 inflammasome [Halle *et al*., 2008]. ATP also activates nociceptors (which are sensory receptors), thereby reporting tissue injury to the nervous system [Keller *et al*., 2008].

HMGB1 and S100A12 engage the receptor RAGE (advanced glycation end-product-specific receptor; also known as AGER), which (at least in the case of HMGB1) cooperates with TLRs to induce an inflammatory response. S100A8 and S100A9 signal through TLR4 [Hofmann *et al*., 1999; Vogl *et al*., 2007]. It should be noted that, although intracellular proteins are thought to be passively released when the plasma membrane of necrotic cells is disrupted, numerous intracellular proteins can be secreted by a non-canonical (endoplasmic-reticulum–Golgi-independent) pathway. In light of this finding, it will be necessary to examine whether inflammatory intracellular proteins are passively released from necrotic cells or secreted by a dependent mechanism. Finally, damage to the cerebral vascular endothelium allows plasma proteins and platelets to gain access to extravascular cerebral spaces [Poeber and Sessa, 2007].

1.2.2 Exogenous inducers

Exogenous inducers consist of two groups: non-microbial and microbial .Non-microbial exogenous inducers include allergens, irritants, foreign bodies and toxic compounds [Kumar *et al*., 2003]. Microbial inducer can be classified into two groups: virulence factors and pathogen-associated molecular patterns

(PAMPs).Virulence factors are a class of microbial inducers not linked directly by dedicated receptors. Instead, the effects of their activity, particularly their adverse effects on host tissues, are responsible for triggering the inflammatory response. Typical activities of virulence factors can be detected by specialized sensors. For example, the pore-forming exotoxins produced by Gram-positive bacteria are detected by the NALP3 (NACHT-, leucine-richrepeat- and pyrin-domain-containing protein) inflammasome, which is sensitive to the efflux of K^+ ions that results from pore formation [Mariathasan *et al*., 2006].

The main class of microbial inducer, PAMPs, is a limited and defined set of conserved molecular patterns that is carried by all microorganisms of a given class (Gram-positive and -negative bacteria, DNA and RNA viruses, fungi and protozoa) [Medzhitov and Janeway, 1997]. PAMPs are specifically recognized by pattern recognition receptors (PRRs), a crucial parts of the innate immune reaction. The intracellular signalling cascades triggered by these PRRs lead to transcriptional expression of inflammatory mediators that coordinate the elimination of pathogens and infected cells. However, aberrant activation of this system leads to immunodeficiency, septic shock, and seems to be related with the onset of neuroinflammatory diseases [Lehnardt, 2010].A variety of intracellular and extra-cellular PRRs are known up to date. Among others, the growing family includes TLRs, NLRs, RIG-like receptors (RLRs) and C-type lectin receptors (CLRs) [Takeuchi and Akira, 2010] (Table 1).

Table 1. PRRs and their ligands.

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

Germline-encoded pattern recognition receptors (PRRs) are responsible for sensing the presence of microorganisms. They do this by recognizing structures conserved among microbial species, which are called pathogen-associated molecular patterns (PAMPs). Recent evidence indicates that PRRs are also responsible for recognizing endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs). Currently, four different classes of PRR families have been identified. These families include transmembrane proteins such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (from Takeuchi and Akira, 2010).

The RLR family is composed of RIG-I, melanoma differentiation-associated gene 5 (MDA5) and LGP2[Takeuchi and Akira, 2009]. RLRs are composed of two N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain and a C-terminal regulatory domain. They are localized in the cytoplasm and recognize the genomic RNA of dsRNA viruses and dsRNA generated as the replication intermediate of ssRNA viruses. The expression of RLRs is greatly enhanced in response to type I IFN stimulation or virus infection [Yoneyama and Fujita, 2008]. RLRs play a major role in pathogen sensing of RNA virus infection to initiate and modulate antiviral immunity. The RLRs detect viral RNA ligands or processed self RNA in the cytoplasm to triggers innate

immunity and inflammation and to impart gene expression that serves to control infection. Importantly, RLRs cooperate in signalling crosstalk networks with TLRs and other factors to impart innate immunity and to modulate the adaptive immune response [Loo and Gale, 2011].

The NLR family consists of cytoplasmic pathogen sensors that are composed of a central nucleotide-binding domain and C-terminal leucine-rich repeats. The N-terminal portions of most NLRs harbor protein-binding motifs, such as CARDs, a pyrin domain, and a baculovirus inhibitor of apoptosis protein repeat (BIR) domain. NLRs harboring a pyrin domain or a BIR domain in their N terminus are not involved in the transcriptional activation of inflammatory mediators and are components of the inflammasome that regulates caspase1 activation [Inohara *et al*., 2005]. NOD1 and NOD2, which harbor CARDs in addition to NOD and LRR domains, activate nuclear factor-kappa B (NF-κB) via an adaptor, RIP2/ RICK. NOD1 and NOD2 induce transcriptional upregulation of pro-inflammatory cytokine genes. NOD1 and NOD2 recognize the structures of bacterial peptidoglycans, g-D-glutamyl-mesodiamino pimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively. As TLRs also recognize bacterial peptidoglycan components, TLRs and NODs synergistically activate pro-inflammatory cytokine production [Franchi *et al*., 2009].

CLRs comprise a transmembrane receptor family characterized by the presence of a carbohydrate-binding domain. CLRs recognize carbohydrates on microorganisms such as viruses, bacteria, and fungi. CLRs either stimulate the production of pro-inflammatory cytokines or inhibit TLR-mediated immune complexes [Geijtenbeek and Gringhuis, 2009].

Out of this intricate system of innate immune receptors, the TLR family is one of the best characterized PRR families and is responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes [Glass *et al*., 2010; Akira *et al.*, 2006].Mammalian TLRs were initially discovered because of their sequence similarities to *Toll* involved in *Drosophila* dorso-ventral embryonic development and antifungal immunity [Nüsslein-Volhard and Wieschaus, 1980; Lemaitre *et al.*, 1996]. In 1997, Medzhitov and colleagues cloned a human homolog of the *Drosophila* Toll protein, now known as TLR4,

and showed that Toll signalling was able to stimulate adaptive immune responses [Medzhitov *et al*., 1997]. Shortly after, the Toll gene was discovered to be an important component for the detection of microbes in *Drosophila melanogaster*, as well as increasing studies demonstrated that TLR4 mediated the inflammatory response to LPS in mice [Poltorak *et al.*, 1998; Poltorak *et al.*, 2000]. This led to the identification of the target molecule of LPS on the cellular surface of macrophages. These discoveries substantially extended the knowledge of pathogen-mediated intra-cellular signal transduction, and were crucial for understanding the mechanisms that govern innate immunity [Bode *et al.*, 2012]. To date, ten different TLRs have been discovered in humans and 12 in mice; for most of them their specific ligands are known. These receptors have the same basic structure of a type 1 transmembrane glycoprotein receptor. However, whereas some TLRs are localized at the cell membrane (TLR1, 2, 4, 5, 6 and 10), others are anchored in the endosome (TLR3, 7, 8 and 9) and therefore lie intracellularly [Kumar *et al.*, 2009].TLRs are characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain. Different TLRs recognize the different molecular patterns of microorganisms and self-components (Table 1).In recent years it has become clear that activation of TLRs is not restricted to the initiation of innate and adaptive immune reactions. In the gastrointestinal tract for instance, TLRs and their interaction with commensal microflora seem to be required for the maintenance of normal homeostasis [Rakoff-Nahoum *et al.*, 2004]. On the other hand, aberrant activation of TLR pathways has been implicated in various chronic and autoimmune diseases affecting the CNS, the gastrointestinal tract, kidneys, skin, lungs and joints. Thereby, not only exogenous but rather endogenous ligands have been suggested to act as TLR activators. Evidence that intracellular proteins or products of protein cleavage can act as endogenous ligands for TLRs supported the hypothesis that TLRs are not only of importance in mediating a response to infections but to stress, damage and death of cells in general [Gallucci *et al.*, 1999; Shi *et al.*, 2000]

1.2.3 Mediators of neuroinflammation

Inducers of inflammation trigger the production of numerous inflammatory mediators, which in turn alter the functionality of many tissues and organs. Many of the inflammatory mediators have effects in common on the vasculature and on the recruitment of leukocytes. These mediators can be derived from plasma proteins or secreted by cells [Majno and Joris, 2004; Kumar *et al.*, 2003]. The cellular mediators can be produced by specialized leukocytes (particularly tissue-resident macrophages and mast cells) or by cells present in local tissues. Some mediators (such as histamine and serotonin) are preformed and stored in the granules of mast cells, basophils and platelets. Others are preformed and circulate as inactive precursors in the plasma. The plasma concentration of these mediators can increase markedly as a result of increased secretion of the precursors by hepatocytes during the acute-phase response. Other mediators are produced directly in response to appropriate stimulation by inducers of inflammation. Inflammatory mediators can be classified into seven groups according to their biochemical properties [Majno and Joris, 2004; Kumar *et al.*, 2003]: vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes.

In particular, lipid mediators (eicosanoids and platelet-activating factors) are derived from phospholipids, such as phosphatidylcholine, that are present in the inner leaflet of cellular membranes. After activation by intracellular Ca^{2+} ions, cytosolic phospholipase A2 generates arachidonic acid and lysophosphatidic acid, the precursors of the two classes of lipid mediator, from phosphatidylcholine. Arachidonic acid is metabolized to form eicosanoids either by cyclooxygenase (COX-1 andCOX-2), or by lipoxygenases, which generate leukotrienes and lipoxins2.Lipoxins (and dietary ω3-fatty-acid-derived resolvins and protectins) inhibit inflammation and promote resolution of inflammation, and tissue repair [Serhan and Savill, 2005]. COX catalyses the first committed step in the prostanoids synthesis, a large family of arachidonic acid metabolites comprising PGs, prostaclyclin and tromboxanes. The prostaglandins PGE2 and PGI2, cause vasodilation, and PGE2 is also hyperalgesic and a potent inducer of fever [Higgs *et al.*, 1984]. COX exists as constitutive and inducible isoforms. Normally,

COX-2 is the inducible isoform which elicits an inflammatory reactions in peripheral tissues.

In the CNS, COX-2 plays a major role in a complex and interlocking metabolic pathway, converting a structural membrane lipid into a plethora of biologically active eicosanoids, with opposite physiological activity. Further, other biomolecules such as endocannabinoids or docosanoids are COX-2 substrates, expanding the important role of COX-2 in the brain [Strauss, 2008]. In physiological condition, significant levels of COX-2 mRNA were found in rodent and mammalian brain [Feng *et al.*, 1993; Seibert *et al.*, 1994]. High levels of COX-2 mRNA were detected in the hippocampus, piriform cortex, neocortex and amigdala, and low levels in striatum, thalamus, cerebellum and hypothalamus [Fujimi *et al.*, 2007; Minghetti, 2004] The constitutive COX-2 expression in the brain seems to be related to neuronal synaptic plasticity [Minghetti, 2004; Kaufmann *et al.*, 1996] and COX-2 inhibitors in rodent Morris Water Maze Test have been shown to impair spatial memory [Teather *et al.*, 2002]. Moreover, steady-state COX-2 appears to be regulated by normal glutamatergic synaptic activity in the adult brain, supporting the role of COX-2 in synaptic plasticity, memory consolidation [Strauss, 2008]. In many neurodegenerative diseases, COX-2 becomes up-regulated and can promote neuroinflammatory response in concert with cytokines, chemokines and other pro-inflammatory molecules [Strauss, 2008; Minghetti, 2004]. Glutamatergic excitotoxicity, for instance, strongly induces neuronal COX-2 overexpression, as seen in ALS [Strauss, 2008; Minghetti, 2004]. In a mouse model of PD, COX-2 overexpression were seen in the dopaminergic neurons of the *substantia nigra* [Teismann *et al.*, 2003] and in microglial cells [Knott *et al.*, 2000]. Despite numerous evidence underlies COX-2 involvement in neurodegenerative diseases, the direct role of COX-2 in brain pathology is still controversial and requires further experimental and clinical studies to explore, more deeply, neurodegenerative diseases pathology and to understand the beneficial or detrimental effect of COX-2 inhibitors in neuroinflammation and neurodegeneration [Minghetti, 2004].

Another important class of mediators and effectors in inflammation are the inflammatory cytokines (TNF-α, IL-1, IL-6 and many others). These cytokines are

produced by many cell types, most importantly by macrophages or microglia and mast cells. They have several roles in the inflammatory response, including activation of the endothelium, leukocytes, the induction of the acute-phase response and activation of microglial cells during neuroinflammation.

The potent pro-inflammatory cytokine TNF is a member of the TNF superfamily of ligands, many of which promote inflammatory signalling. TNF signalling through TNFR1 and TNFR2 can elicit a variety of cellular responses depending on many factors, including the metabolic state of the cell and the adaptor proteins present in the cell. These differences then influence the activation of a number of intracellular signalling pathways including NF-κB, p38, c-jun N-terminal kinase (JNK), and the ceramide/sphingomyelinase signalling pathway, resulting in a number of responses including inflammation, proliferation, cell migration, apoptosis, and necrosis [Eissner *et al.*, 2000; Wajant *et al.*, 2003]. TNF signalling has been shown to have several important functions within the CNS including injury-mediated microglial and astrocyte activation, and regulation of blood brain barrier permeability, febrile responses, glutamatergic transmission, and synaptic plasticity and scaling [McCoy and Tansey, 2008; McCoy *et al.*, 2006]. A number of pre-clinical and clinical studies in various disease models and in chronic neurodegenerative conditions suggest that targeting TNF action in the brain may be an attractive disease-modifying strategy to slow progression or attenuate severity of the disease [McCoy *et al.*, 2006; Perry *et al.*, 2001].

IL-1 was the first interleukin to be identified [Garlanda *et al.*, 2013]. IL-1 is a central mediator of innate immunity and inflammation and exerts a number of diverse actions in the brain [Patel *et al.*, 2003]. Studies using the IL-1 receptor antagonist, which inhibits cell death caused by ischemia or brain injury contribute to understand the role of IL-1 in neurodegeneration [Allan *et al.*, 2005; Vezzani *et al.*, 2000]. Members of the IL-1 family are expressed at low or undetectable levels in healthy brain but their expression become rapidly up-regulated by various experimental brain insults including ischaemia, trauma, hypoxia and neurotoxic or inflammatory stimuli (LPS) [Lucas *et al.*, 2006; McColl *et al.*, 2007]. Central or peripheral administration of IL-1 dramatically increases neuronal death following acute brain injury [Yamasaki *et al.*, 1995]. Pro-IL-1β must be cleaved by the

enzyme caspase 1, to produce the active form and to allow cellular release [Garlanda *et al.*, 2013]. Deletion, inhibition or inactivation of caspase 1 inhibits experimentally induced neuronal cell death [Hara *et al.*, 1997]. Increasing evidence suggests that IL-1 might also be involved in chronic neurodegenerative diseases. In experimental models of AD in mice, β-amyloid-activated microglia produce IL-1, which in turn promotes production and deposition of neurotoxic β-amyloid peptides [Griffin *et al.*, 1998; Ranaivo *et al.*, 2006]. Chronic expression of IL-1 in rat brain results in extensive demyelinating lesions, mimicking multiple sclerosis, and IL-1RA slows disease progression in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis [Brough *et al.*, 2002; Ferrari *et al.*, 2004].

IL-6 is an important cytokine originally identified almost 30 years ago as a B-cell differentiation factor with novel many critical roles in major physiological systems including the nervous system [Erta *et al.*, 2012]. In the brain, IL-6 influences neurogenesis and it was shown to be involved in multiple physiological CNS processes such as neuron homeostasis, astrogliogenesis and neuronal differentiation. In many respects, IL-6 behaves in a neurotrophin-like fashion [Spooren *et al.*, 2011]. However, the first evidence implicating IL-6 in brain-related processes originated from its dysregulated expression in several neurological disorders such as multiple sclerosis, Alzheimer's disease and Parkinson's disease [Spittau *et al.*, 2012; Ramesh *et al.*, 2013]. Its expression is affected in several of the main brain diseases, and animal models strongly suggest that IL-6 could have a role in the observed neuropathology. In addition, the molecular mechanisms underlying IL-6 functions in the brain have only recently started to emerge. Further, IL-6 plays a central role in neuroinflammation, which can evolve as a beneficial process, aimed at maintaining tissue homeostasis, but which can become malignant when exaggerated [Allan and Rothwell, 2001]. In this perspective, it is not surprising that 'well-meant' actions of IL-6 are often causing harm instead of leading to recovery and therefore it could be a clear target of strategic therapies in neurodegenerative diseases in which persistent inflammation is the main feature [Erta *et al.*, 2012; Spooren *et al.*, 2011].

It should be noted that it is unclear to what extent the nature of an inflammatory trigger dictates the type of mediator induced. In addition, many (but not all) mediators not only have direct effects on target tissues but also themselves induce the production of additional mediators. It will be important to understand the logic underlying this hierarchy of mediators. The effectors of an inflammatory response are the tissues and cells, the functional states of which are specifically affected by the inflammatory mediators. Responsiveness to certain inflammatory mediators (such as TNF-α and IL-1) is almost ubiquitous, although these mediators have distinct effects in different tissue and cell types. Although the most obvious effect of inflammatory mediators is to induce the formation of an exudate (through their effects on the vasculature and on leukocyte migration), many inflammatory mediators have other, equally important, effects on neuroendocrine and metabolic functions and on the maintenance of tissue homeostasis in general [Turnbull and Rivier, 1999]. These functions of inflammatory mediators reflect a more general role for inflammation in the control of tissue homeostasis and in adaptation to noxious conditions.

1.3 TLRs in the brain: focus on TLR4

TLRs are expressed in a wide variety of immune, as well as on non-immune cells. Whereas dendritic cells (DCs), neutrophils or macrophages express an almost complete panel of the different TLRs, other cells only express a restricted repertoire. Given the crucial role TLRs, but also other innate immune receptors, play in the initiation of innate as well as adaptive immune responses, their involvement in the pathogenesis of chronic inflammatory or autoimmune diseases is not surprising. In fact, functionally active TLRs were found to be expressed in different cells of the CNS, and interestingly also seem to be involved in neurodegenerative diseases [Kielan, 2006; Konat*et al.*, 2006].To date, there is accumulating evidence that TLR4 and TLR2 activation play a crucial role in the development and progression of a number of neurodegenerative diseases [Schröder and Schumann, 2005; Ziegler *et al.*, 2011].

TLR4 was first identified, in 1998, as the signalling receptor for LPS or endotoxin from the outer membrane of Gram-negative bacteria [Poltorak *et al.*, 1998]. In

CNS, constitutive expression of TLR4 transcripts has been described in distinct anatomical areas of the brain such as circumventricular organs, choroid plexus and leptomeninges [Lacroix *et al.*, 1998; Laflamme and Rivest, 2001]. TLR4 has also been reported to be expressed in microglia and astrocytes [Lehnardt *et al.*, 2003; Lehnardt *et al.*, 2002; Gorina *et al.*, 2011].In addition, the expression of TLR4 has recently been documented, despite controversial, in mammalian neurons and appears to be implicated in several processes such as neurogenesis and brain development [Wadachi and Hargreaves, 2006; Rolls *et al.*, 2007]. TLRs have been shown to be implicated in several CNS diseases, and accumulating evidence demonstrates that TLR4 contributes to neuronal death, blood brain barrier damage, cerebral edema, and inflammatory responses in the brain injury induced by ischemia [Caso *et al.*, 2007; Hua *et al.*, 2007]. TLR4-mediated activation of NF-κB signalling plays a vital role in the initiation of cerebral inflammation in CNS diseases [Hua *et al.*, 2007; Kerfoot *et al.*, 2004], leading to the transcription of many pro-inflammatory genes encoding cytokines, chemokines, and enzymes such as COX-2 and MMP-9, that are involved in the development of secondary brain injury following traumatic brain injury (TBI) [Wang *et al.*, 2009; Lucas *et al.*, 2006]. The upregulation of cytokines and chemokines could activate microglia, thus initiating infiltration of inflammatory cells into the brain which may cause neuronal loss [Allan and Rothwell, 2001; Morganti-Kossmann *et al.*, 2002]. Considering the critical role of TLR4 in neuroinflammation and brain injury, recent insights into the role and activation mechanisms of TLR4 in the brain should be better characterized, not only in pathological events but also in physiological conditions, as well as the therapeutic benefit that could derive from TLR4 modulation.

1.3.1 LPS and TLR4 pathways

The constitutive expression of TLR4 may explain the innate immune response in the brain, which originates from the structures devoid of the blood–brain barrier in the presence of circulating LPS, thus suggesting a role for TLR4 acting as a sensor for engaging the cerebral innate immune response in the case of invasion during systemic bacterial infections, which may have detrimental consequences for the

neuronal structures [Laflamme and Rivest, 2001]. In this respect, in an *in vivo* model of neurodegeneration, LPS administration was reported to be able to stimulate innate immunity, causing extensive neuronal and axonal loss in the cortex. In contrast, animals bearing a loss-of-function mutation in the TLR4 gene are resistant to neuronal injury, thus demonstrating a mechanistic link among innate immunity, TLRs, and neurodegeneration [Lehnardt *et al.*, 2003].

In the host system, LPS capture is facilitated by the LPS binding protein (LBP) which transfers it to the receptor complex composed of CD14, MD-2 (or LY96) and TLR4. Upon LPS binding, TLR4 recruits, through its short intracellular TIR domain, adaptor molecules and kinases, thus initiating a downstream signalling cascade that culminates in the secretion of pro-inflammatory cytokines and chemokines [Takeda and Akira,2005;Takeuchi and Akira, 2002]. Activation of TLR4 by LPS induces two signalling pathways known as the myeloid differentiation primary response gene 88 (MyD88) dependent and independent pathways (Fig.3) [Akira *et al.*, 2006]. The MyD88 dependent pathway in TLR4 signalling requires the adaptor proteins TIRAP (TIR domain containing adaptor protein) and MyD88 to initiate a downstream cascade leading to nuclear translocation of the NF-κB and mitogen associated protein (MAP) kinase signalling pathways (such as ERK-CREB, JNK-AP1and p38 pathways), resulting in the production of pro-inflammatory cytokines [Kargan and Medzhitov, 2006]. This leads to the rapid expression of inducible nitric oxide synthase (iNOS) and a wide variety of pro-inflammatory cytokines, chemokines and their receptors, including TNF-α, IL-1α, IL-1β, IL-1ra, IL-6, IL-8, IL-10, IL-12p40, IL-23, macrophage inflammatory protein (MIP)-1α, and MIP-1β [Lee and Kim, 2007]. These factors initiate the inflammatory response, increase vascular permeability, direct DC and macrophage migration from the periphery to the central lymphoid organs, activate glial cells and regulate various aspects of adaptive immunity development. On the other hand, the independent signalling pathway is controlled by the adaptors TICAM (Toll-like receptor adaptor molecule) 1 or TRIF (TIR-domain-containing adaptor inducing interferon-β) and TICAM 2 or TRAM (TRIF-related adaptor molecule), which activate the transcription factor IRF3 (IFN regulatory factor 3) and the production of IFN-β and chemokine RANTES

(regulated on activation normal T cell expressed and secreted) [Yamamoto *et al.*, 2003a].TLR4 engagement leads to the production of neurotoxic molecules such as pro-inflammatory cytokines, NO, ROS and peroxynitrite [Xie *et al.*, 2002].

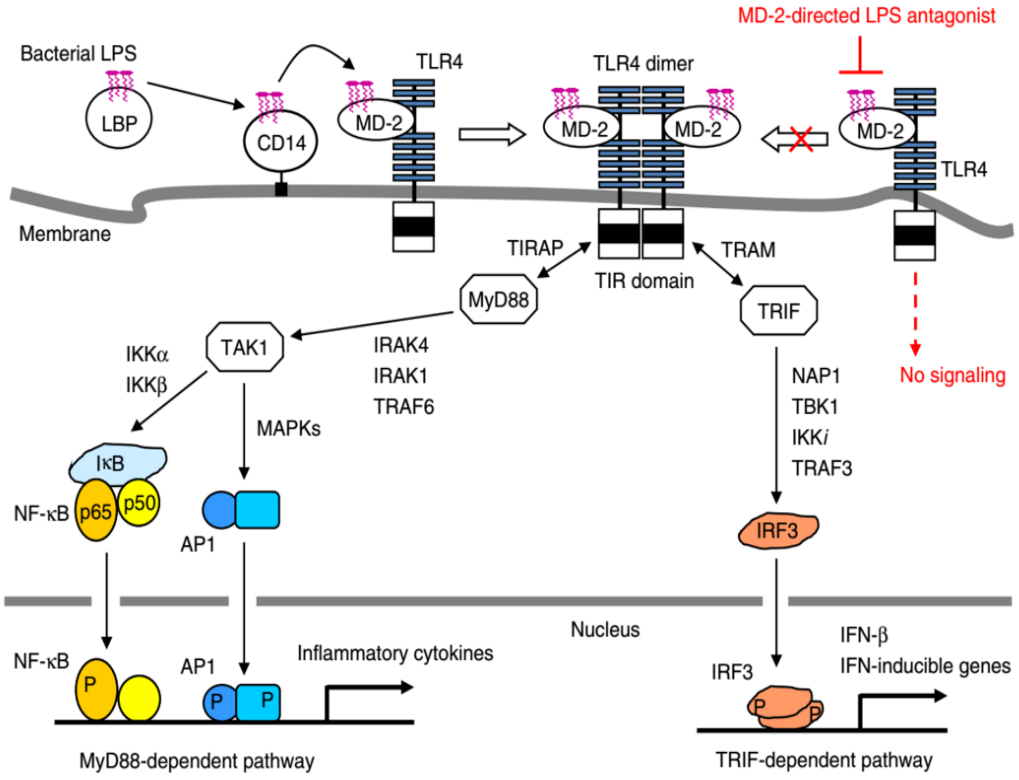


Figure 3 :Overview of LPS/TLR4 signalling pathway. Stimulation of TLR4 by LPS involves the participation of several molecules (LBP, CD14 and MD-2). Activation of TLR4 induces two downstream signalling pathways. First, the MyD88-dependent pathway is initiated by recruitment of TIRAP and MyD88 to the TLR4 complex which leads to early-phase activation of NF-κB and subsequent induction of the expression of NF-κB-controlled genes including pro-inflammatory cytokines (TNF-α). The MyD88-dependent pathway can also activate JNK, leading to transcription of several genes including TNF-α, via activation of AP-1. Second, the MyD88-independent pathway is initiated by recruitment of TRAM and TRIF to the TLR4 complex, followed by late activation of NF-κB complex and activation of IRF3 which leads to the transcription of IFN-β as well as other interferon-induced genes. (from Lehnardt et al., 2002)

Moreover, LPS-activated microglia produce a large amount of glutamate, an important neurotransmitter which in some circumstances acts as a potent

neurotoxin [Takeuchi *et al.*, 2006]. LPS challenge may also activate TLR4 on the microglia surface, leading to oligodendrocyte injury [Lehnardt *et al.*, 2002]. Recently, CNS-relevant *in vitro* and *in vivo* studies have highlighted the function of suppressor of cytokine signalling (SOCS) proteins under various neuroinflammatory or neuropathological conditions. SOCS1 and SOCS3 have been described as having a short half-life (1–2 h) and their expression levels are reported to increase rapidly following macrophage exposure to inflammatory cytokines and TLR ligands. Expression of SOCS1 and SOCS3 is regulated primarily by activation of STAT1 and STAT3, respectively, although their expression can be mediated through other signalling cascades, including MAPK and NF-κB pathways [Wang and Campbell, 2002; Blach-Olszewska and Leszek, 2007]. Moreover, SOCSs not only influence cytokine and growth hormone signalling, but also the signalling pathway initiated by the reaction of a TLR with PAMP or an endogenous molecule. SOCS-1 negatively regulates TLR signalling by mediating the degradation of the adapter protein Mal. This protein is involved in signalling via TLR2 and TLR4. Because of their obvious biological importance, the SOCS proteins have been the subject of intense investigation, including the development of strategies to utilize these proteins to control cytokine induced JAK/STAT signal transduction for therapeutic purposes [Blach-Olszewska and Leszek, 2007]. Although it is undisputed that TLR4 recognizes LPS, an important role has been attributed to molecules of host origin that have lately emerged as potential endogenous ligands of TLR4, including different components of the extracellular matrix, intracellular proteins, or modified lipids or lipoproteins (Table 1). Interestingly, many of them are able to activate both TLR4 and TLR2 without having any substantial structural similarity to their natural ligands (endotoxin or lipopeptides, respectively) [Oblak and Jerala, 2011]. However, as a potent immune activator LPS can activate TLR4 at smaller concentrations than other activators (*e.g.*, heat shock proteins, fibrinonectins, oligosaccharides, heparin sulphate, and fibrinogen) [60]. The emerging evidence is that many of these molecules may be more accurately described as PAMP-binding molecules or PAMP sensitizing molecules, rather than genuine ligands of TLR2 or TLR4. Consequently, these molecules may serve a beneficial purpose initially by

enhancing the sensitivity of compromised tissues to a potential microbial challenge, or, alternatively, it is proposed that they may also play a role in the maintenance of chronic inflammatory diseases [Erridge, 2010]. Paradoxically, TLR activation by endogenous ligands following ischemia worsens stroke damage, therefore these molecules, recently termed "alarmins", have been suggested to serve as mediators of inflammation that may be expressed or released in response to tissue damage and, therefore, have also been described as DAMPs [Bianchi, 2007]. In the brain the following molecules have been reported as DAMPs: heat shock proteins (HSPs), β-amyloid (Aβ), hyaluronan, heparin sulphate, DNA or RNA immune complex, oxidized low-density lipoproteins (oxLDL), and others, all able to stimulate TLRs [Marsh *et al*., 2009; Rivest, 2009; Yanai *et al*., 2009; Stewart *et al*., 2010; Zhang *et al*., 2010]. Among DAMPs, high-mobility group box-1 (HMGB1), a non-histone nuclear protein, has been reported to exert its biological effects through the activation of signalling pathways coupled to TLRs, including TLR4 and RAGE, both of which are involved in inflammatory responses [Park *et al*., 2004; Yang *et al*., 2010; Rauvala and Rouhiainen, 2010]. Recent studies show that elevated brain levels of HMGB1 induce memory abnormalities which may be mediated by either TLR4, or RAGE. This mechanism may contribute to memory deficits under various neurological and psychiatric conditions associated with increased HMGB1 levels, such as epilepsy, Alzheimer's disease and stroke [Mazarati *et al*., 2011].

1.4 Microglia: the key regulator of brain microenvironment

Microglia are bone marrow-derived cells of the brain displaying multiple roles in regulatory processes such as maintaining homeostasis and repairing from injury. This heterogeneous group of monocyte-derived cells orchestrate innate immune response in the brain and show different phenotypes depending on functional connotation [Kettenmann *et al.*, 2011]. In 1932, Pio del Rio Hortega provided an early description of microglia. He postulated that morphological classification of microglia fell into three different phenotypes: ramified, intermediate forms and amoeboid [Tremblay *et al.*, 2011]. Scientists have shown that during postnatal development, progenitors from mesodermal/mesenchymal origin migrate through

the peripheral blood into the brain where they become tissue specific macrophages. The colonization of the brain by microglia occurs parallel to the vascularization of the CNS. Microglial cells initially show amoeboid shape and once migrated into the brain, they transform into a branched, ramified morphological phenotype termed "resting" microglia [Kettenmann *et al.*, 2011; Tremblay *et al.*, 2011; Ginhoux, 2013]. The switch from an amoeboid state to the resting state seems to be linked to the release of molecules such as purines (*e.g.*, ATP or adenosine), cytokines from astrocytes [transforming growth factor-β (TGF-β), macrophage colony-stimulating factor (M-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF)] and chloride channels [Kettenmann *et al.*, 2011; Dheen *et al.*, 2007]. Microglia present different inwardly rectifying K^+ channels, receptors for purines (*e.g.*, P2X7R) and neurotransmitters (GABA, NMDA, D2 etc.) [Kettenmann *et al.*, 2011]. The resting microglial cells tight regulate CNS microenvironment, surveying brain parenchyma via dynamic movement of the ramified processes. They control the extracellular environment and perceive even small changes in the ionic composition of the extracellular fluid surrounding neurons, without disturbing the fragile neuronal circuitry [Kettenmann *et al.*, 2011; Tremblay *et al.*, 2011]. To protect vulnerable nervous tissue from a potential harmful immune response, the brain is under a tight regulated control termed "immune-privilege" and microglial cells participate in this complex regulation [Carson *et al.*, 2006].

Microglial immune function is regulated by two different types of signals known as "Off" and "On" signals. Neuronal Off signals are found in healthy neurons and can maintain microglia in a resting state by secreting neurotransmitters (*e.g.*, GABA), the immunoglobulin CD200, the chemokine CX3CL1 and neurotrophins, such as neuronal growth factor (NGF), brain derived neutrophic factor (BDNF), neurotrophin-3 (NT3) and cytokines with neurotrophic activity. By contrast, On signals start to be produced when a defined microglial activation is required. In response to impaired neurons On signals are released. These signals include neurotransmitters (*e.g.*, ATP, UTP and glutamate), matrix metalloproteinases (MMPs), chemokines (*e.g.*, CCL21, CXCL10), and membrane On signals such as TREM2 ligand in the membrane of apoptotic neurons to drive microglia

phagocytosis [Biber *et al.*, 2007; Ransohoff and Perry, 2009]. This represent the "proof of principle" of a bidirectional microglia-neuron communication in the brain and provides novel insights in understanding microglial function in both the healthy and diseased brain [Eyo and Wu, 2013].

1.4.1 "Activation" of microglia during neuroinflammation

During alterations in brain activity or invading microbes, microglia can be activated to perform the first line of defence. They initiate rapid changes in gene expression, cell shape and functional behaviour becoming "activated" (Fig.4) [Kettenmann *et al.*, 2011; Saijo and Glass, 2011]. Activated microglia moved to the lesion following chemotactic gradients by reverting their resting state to an amoeboid state. They undergo complex changes in intracellular enzymes activity and start to release a plethora of pro-inflammatory and immunoregulatory factors such as cytokines, reactive nitrogen species, chemokines, neurotrophic factors and chemoattractive factors [Kettenmann *et al.*, 2011; Saijo and Glass, 2011; Hoogland *et al.*, 2015]. Microglia physically associate with damaged neurons, presenting the antigen to T cell and lead to neuronal apoptosis. In acute pathological condition microglia are not inevitably neurotoxic. In fact, it has been demonstrated that microglial neurotoxicity can occur after excessive, chronic, uncontrolled stimulation of microglia or when microglia function is impaired [Hoogland *et al.*, 2015¸ Lull and Block, 2010]. One physiological event that could induce microglial exaggerated response and neurotoxicity is normal aging. Healthy adult brain shows an higher microglial expression of the major histocompatibility complex(MHC) class II and IL-6, also accompanied with an increased oxidative stress, decreased IL-10 and age-related decline in cognitive and motor function [Norden and Godbout, 2013; Von Bernhardi *et al.*, 2015].This evidence could explain why in aged individuals a systemic infection can induce an exaggerated inflammatory response in the brain due to an over-activation of microglia and neuronal cell damages. Finally, microglia, in a close crosstalk with astrocytes, neurons and other brain cells, play crucial functions as the scavenger system of the CNS, providing beneficial functions as tissue repair in the CNS. However, chronic, dysregulated activation of microglia appears to lead to deleterious effects inducing malfunction and damage of brain cells. What drives

this dysregulation is not fully understood, but age-related impairment of regulatory mechanisms represent a promising hypothesis for understanding cytotoxic activation in aged individuals [Von Bernhardi *et al.*, 2015].

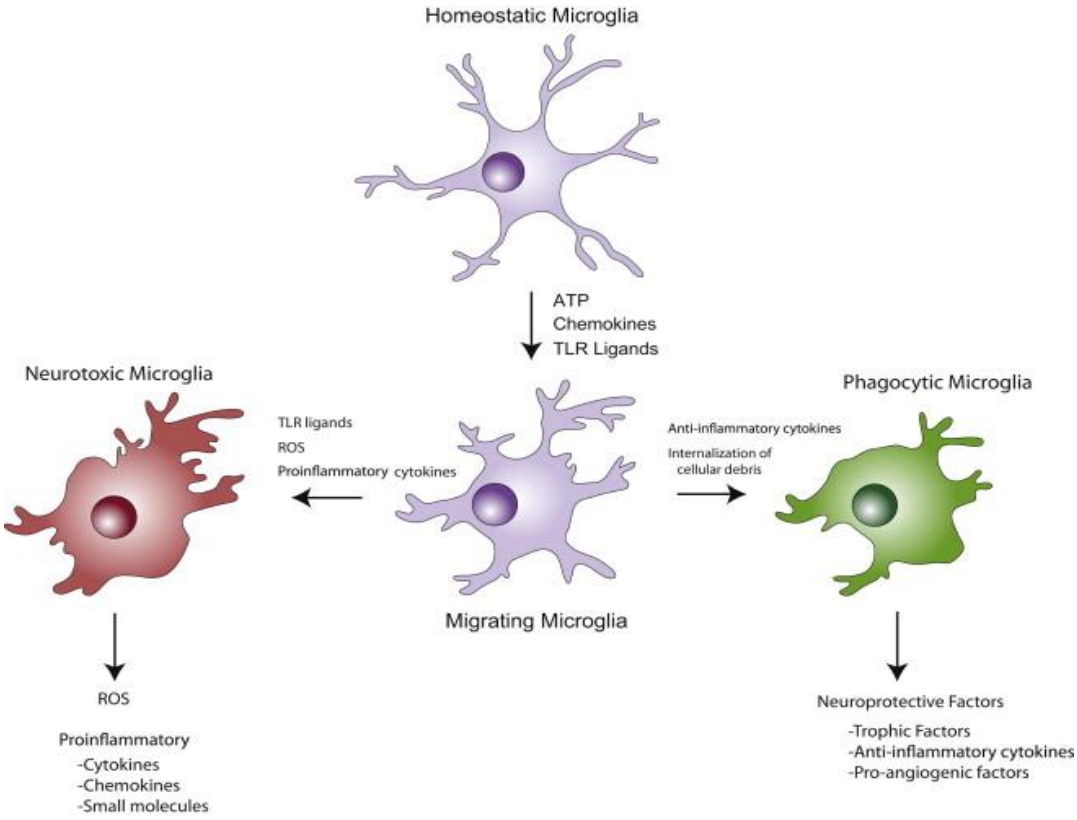


Figure 4. Microglial activation states. In the healthy CNS, microglia survey their immediate environment, and in this "resting state", do not express inflammatory mediators. However, after exposure to a number of chemical signals from damaged neurons, microglia respond rapidly and physically migrate to the site of injury. Responding microglia may then adopt a pattern of behavior similar to pro-inflammatory macrophages (shown in red), as they release molecules intended to protect against pathogens, including neurotoxic cytokines, reactive oxygen species (ROS), and small molecules, such as quinolinic acid, that promote excitotoxicity. The release of cytokines and chemokines can lead to the recruitment of additional inflammatory cells from adjacent blood vessels, and may also engage astrocytes in the pro-inflammatory response. Alternatively, activated microglia may exhibit behaviors associated with anti-inflammatory macrophages (shown in green), secreting molecules that promote tissue repair, and internalizing cellular debris including aggregated, misfolded proteins such as β-amyloid, through phagocytosis. Whether two distinct populations of microglia exist that are committed to either of these response patterns, or all microglia can be induced to exhibit either response behavior when exposed to the correct combination of signals, remains to be determined (from Garden and La Spada, 2012).

Nonetheless, despite the undeniable potential of activated microglia to become deleterious, microglia have a profound immune-modulatory and reparative potential in the CNS. Thus, instead of abolishing microglia activation as it is most often proposed, strategies to potentiate those beneficial functions while inhibiting cytotoxic activation should be developed. Such strategy may well constitute the way to treat neurodegenerative disorders, but demands a better understanding of the protective and modulatory pathways of immune activation. Additional research is needed for the identification of new pathways that may decrease the impact of microglial cell dysfunction, in order of breaking the vicious circle leading to neurotoxicity. In that perspective, multi-target pharmacological approaches, like natural compounds and their phytocomplexes, aimed to reestablish normal regulation of microglia could prove highly effective, especially as preventive treatments. Furthermore, non-pharmacological strategies, like exercise, life style changes and dietary restriction could promote a healthy aging through their effects on promoting microglial physiological functions, while reducing inflammation and ROS production.

1.5 Astrocytes in neurodegeneration

Astrocytes are characteristic star-shaped glial cells located in CNS and enteric nervous system and play a critical role in the viability and function of the CNS, including biochemical support of endothelial cells that form the blood–brain barrier, provision of nutrients to the nervous tissue and involved in the maintenance of extracellular ion balance and in the repair and scarring process of the brain and spinal cord following traumatic injuries [Sofroniew and Vinters, 2010]. Astrocytes become reactive in response to virtually all pathological conditions in the CNS, both following acute injuries (stroke, trauma) and during progressive diseases [Sofroniew, 2005]. Thus, it is not surprising that these multifunctional cells have been implicated in the onset and progression of several neurodegenerative diseases like Parkinson (PD), Alzheimer(AD), Amyotrophic lateral sclerosis (ALS); and multiple sclerosis (MS) among others [Clement *et al.*, 2003; Wisniewski and Wegiel, 1991; Haughey and Mattson, 2003; Forman *et al.*,

2005; Shin *et al.*, 2005; Forno *et al.*, 1992; Teismann and Schulz, 2004; Heales *et al.*, 2004].

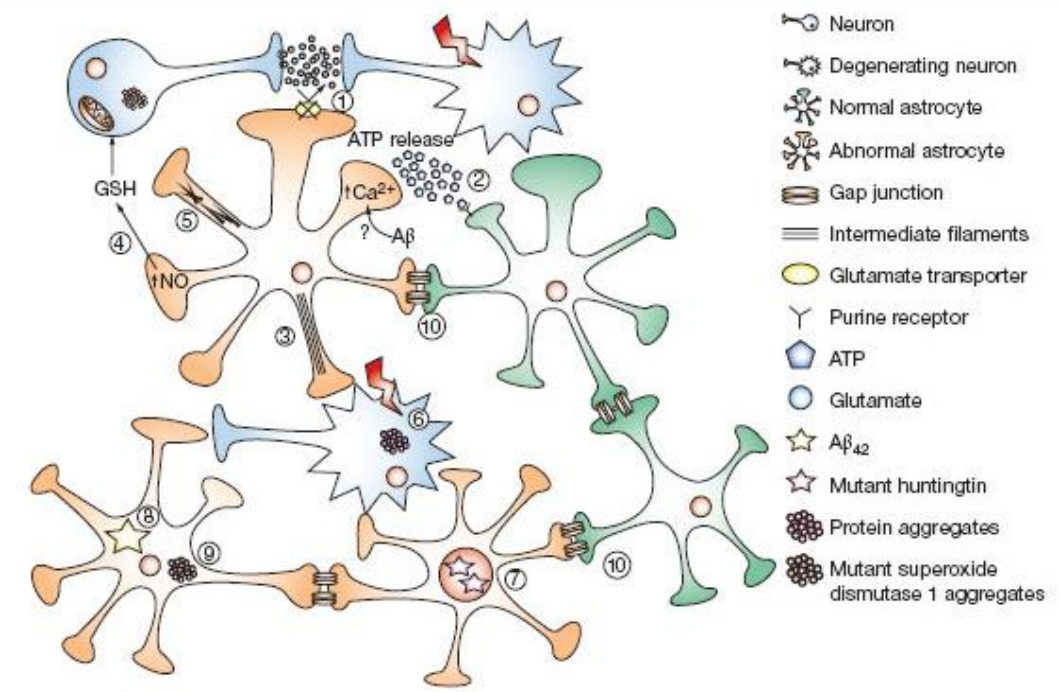


Figure 5. Potential astrocyte dysfunction in neurodegenerative diseases. (1) Impairment of glutamate transporters, through reduced expression, aberrant RNA synthesis or altered function, results in increased synaptic glutamate and excitotoxicity [Heales *et al.*, 2004]. (2) Amyloid-β could potentially increase the amount of ATP released by astrocytes, as well as interacting with gap junctions to alter calcium signalling and glial communication [Haughey and Mattson, 2003]. (3) Upregulation of glial fibrillary acidic protein is a consistent pathological feature in neurodegenerative diseases, although the significance of this observation is not completely understood [Luo *et al.*, 2010; Brenner *et al.*, 2001]. (4) Nitric oxide stimulates the release of glutathione from astrocytes to neurons, thereby increasing neuronal antioxidant reserves and limiting oxidative damage to neuronal mitochondria [Sofroniew, 2005]. (5) Mutations in glial fibrillary acidic protein associated with Alexander disease result in the development of Rosenthal fibers, and lead to disordered intermediate neurofilament organization [Brenner *et al.*, 2001]. (6) Neurons surrounded by mutant astrocytes develop protein aggregates and axonal pathology, and are more susceptible to cell death in several neurodegenerative disease models, including amyotrophic lateral sclerosis, Alzheimer's disease and Huntington's disease [Wakabayashi *et al.*, 2000; Teismann and Schulz, 2004; Watanabe *et al.*, 2001]. (7) Mutant huntingtin expressed in astrocytes forms intranuclear aggregates and also influences neuronal cell death in vitro [Shin *et al.*, 2005]. (8) Amyloid-β42-positive material is seen within activated astrocytes in the tissues of individuals with Alzheimer's disease, and is abundant in regions with the most active Alzheimer's disease pathology [Haughey and Mattson, 2003]. (9) Accumulation of mutant superoxide dismutase 1 is also observed in astrocytes [Clement *et al.*, 2003]. (10) Finally, communication between abnormal or injured astrocytes might affect the biology and function of surrounding normal astrocytes, through either hemichannels or soluble mediators [Sofroniew *et al.*, 2005]. Abbreviations: Aβ, amyloid-β; GFAP, glial fibrillary acidic protein; GSH, glutathione (from Maragakis and Rothstein, 2006).

The involvement of astrocytes in the neuropathology of these diseases is likely a consequence of both the loss of normal homeostatic functions and gain of toxic functions [Sofroniew and Vinters, 2010]. Intracellular aggregates in astrocytes are a common feature of various neurodegenerative diseases, and these aggregates perturb normal astrocytic functions in ways that can be harmful to neuronal viability [Wakabayashi *et al.*, 2000].It has been very difficult to distinguish the contribution of astrocytes from that of microglia because they usually become reactive in concert and both are involved in neuroinflammation. However, they have quite different functions in the brain in normal conditions; therefore, they may also play different roles during neurodegenerative diseases [Sofroniew and Vinters, 2010].In response to insults or injuries in the CNS, astrocytes respond through the so called "reactive astrogliosis", which consist of a graded response that encompasses a spectrum of changes that range from hypertrophy to proliferation and migration [Sofroniew and Vinters, 2010].Astrocyte hallmark of reactivity is a strong Glial fibrillary acidic protein(GFAP) expression and upregulation of vimentin and nestin. Another cardinal feature of astrocyte reactivity is hypertrophy, which was reported by early neuropathologists. Reactive astrocytes display an enlarged cell body and processes [Sofroniew, 2005; Brenner *et al.*, 2001; Sofroniew and Vinters, 2010; Sun *et al.*, 2010]. In addition, astrocyte arborization is reorganized with reactivity: the number of primary processes changes or they polarize toward the site of injury or toward amyloid plaques in AD (Fig.5) [Sun *et al.*, 2010; Bardehle *et al.*, 2013].The nature and extent of the astrocytic response is determined by the context in which it occurs and by the duration and nature of the instigating stimulus. For example, LPS, stroke, and other insults can induce very different kinds of reactive gliosis [Zamanian *et al.*, 2012; Gorina *et al.*, 2011]. Reactive astrocytes can release a wide variety of extracellular molecules, including inflammatory modulators, chemokines and cytokines, and various neurotrophic factors. These factors can be either neuroprotective (*e.g.*, cytokines, such as IL-6 in some condition and TGF-β) or neurotoxic (such as IL-1β and TNF-α) [Sofroniew *et al.*, 2010].In light of their many actions on neurons, strategies targeting reactive astrocytes may effectively sustain neuronal function and hence survival during neurodegenerative diseases.

However, given the complex changes that occur in reactive astrocytes during neurodegenerative diseases, complete ablation of astrocyte reactivity may be counterproductive because these cells also display beneficial adaptative changes during disease. Identifying the complex interplay between shared intracellular pathways mediating reactivity and disease specific signals may enable the design of selective therapeutic cocktails to engage reactive astrocytes in protective actions. Huge progress has been made recently as a result of the heightened interest in glial cells, and the development of innovative and cell type-specific approaches. However, these cells remain enigmatic, and many aspects of their physiology need to be clarified. Although the molecular pathways leading to astrocyte reactivity during ND have been described, it is crucial to elucidate what disease-, region- and environmental-specific mechanisms control the functional outcomes associated with astrocyte reactivity. In any case, considering reactive astrocytes as key partners in neuronal dialog during neurodegenerative diseases opens new avenues for neuroscience and biomedical research [Dantzer *et al.*, 2008].

1.6 Oligodendrocytes and neurodegeneration

Nowadays, an important component of neuroglia, oligodendrocyte, has received more attention in the field of neurodegenerative disease research. Traditionally, oligodendrocytes were recognized as myelin sheath producers by which saltatory conduction of action potential is guaranteed. However, emerging evidence has suggested that oligodendrocytes exert supportive functions for neurons and their axons in a myelin sheath-independent manner [Bradl and Lassmann, 2010], and thus, impairment in oligodendrocyte functions has been reported in several neurodegenerative diseases, such as ALS and AD. These diseases are considered to be not associated with myelin-forming cells [Lee *et al.*, 2012; Roth *et al.*, 2005; Nonneman *et al.*, 2014]. The interesting observations prompt us to rethink our traditional views on oligodendrocytes and their possible roles in the pathogenesis of neurodegenerative disorders. A clear experimental evidence, showing the supportive function of oligodendrocytes in axon health independent of myelin sheath, came from the study using 2',3′-cyclic nucleotide 3′ phosphodiesterase

(CNP1) mutant mice in which the CNP1 gene was disrupted by Cre insertion [Lappe-Siefke *et al.*, 2003]. The overall pattern of myelin proteins, amount and ultrastructure of the central myelin were almost the same as littermate controls in the absence of CNP1. However, by four months of age, progressive motor deficits in motor performance, axon pathology and reactive gliosis became more visible. In this mutant mouse line, the supportive role of oligodendrocytes in axon integrity was completely uncoupled from its role of myelin sheath maintenance, suggesting that dysfunction of oligodendrocytes is sufficient to cause secondary axonal neurodegeneration. Further evidence supports this hypothesis. For example, absence of functional peroxisomes in oligodendrocytes resulted from inactivation of perioxisomal biogenesis factor-5 (PEX5), a factor known to be essential for peroxisomal protein import, caused wide-spread axonal degeneration, progressive demyelination and pronounced neuroinflammation [Kassmann *et al.*, 2007]. These data highlight the importance of oligodendrocytes on neuroprotection. More recently, two very interesting studies revealed that metabolite coupling between oligodendrocytes and neurons is important for neuronal survival and its deregulation contributes to neurodegeneration. In these studies, cytochrome c oxidase assembly protein 10 (Cox10) gene, a haem A farnesyl transferase essential for assembly of mitochondria complex IV, and monocarboxylate transporter 1 (MCT1) were depleted specifically in oligodendrocytes [Fünfshilling *et al.*, 2012; Lee *et al.*, 2012]. More importantly, Lee and colleagues found that monocarboxylate transporter 1 (MCT1) was highly enriched in oligodendrocytes and its levels were reduced in ALS mouse models and neural samples of ALS patients, suggesting the involvement of oligodendrocytes in ALS pathogenesis [Lee *et al.*, 2012].

In summary, oligodendrocytes are multi-functional glial cells in the CNS and they actively play important roles under both physiologic and pathological conditions. Their dysfunction can contribute to several neurodegenerative diseases including ALS, MSA and AD, although the precise molecular and cellular mechanisms are still unknown. More detailed investigations will significantly broaden our understanding of disease pathogenesis and oligodendrocyte pathophysiology and could help to identify new therapeutic targets in neurodegenerative diseases.

1.7 Neuroinflammatory basis of neurodegenerative diseases

Nowadays, neurodegenerative diseases are chronic and incurable conditions whose disabling effects may continue for years or even decades, representing an enormous disease load, regarding human suffering and economic cost. The disproportionate impact on countries with longer life expectancies make neurodegenerative diseases the fourth highest source of overall disease burden in the high-income countries. According to the World Health Organization, 37 million people currently have dementia worldwide, and about 50% of them are being affected by AD and this number is expected to grow up to 115.4 million people by 2050 [Brown *et al.*, 2005].

Neurodegenerative diseases are characterized by chronic and progressive degeneration of selective neuronal populations. Inflammation is a common hallmark in many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis. [Glass *et al.*, 2010]. Extensive research so far has identified many molecular and cellular factors that contribute to the initiation and progression of inflammatory response in neurodegenerative diseases. Among them, glial activation, mainly astrocytes and microglia, takes an important place in the pathology of neurodegenerative disorders. This may be due to their widespread involvement in the maintenance and regulation of brain homeostasis [Glass *et al.*, 2010; Mena and Garcìa de Yébenes, 2008].Sustained inflammation resulting in tissue pathology implies persistence of an inflammatory stimulus or a failure in normal resolution mechanisms. A persistent stimulus may result from environmental factors or the formation of endogenous factors (*e.g.*, protein aggregates) that are perceived by the immune system as “stranger” or “danger” signals. Inflammatory responses that establish feed-forward loops may overwhelm normal resolution mechanisms. Although some inflammatory stimuli induce beneficial effects (*e.g.*, phagocytosis of debris and apoptotic cells), and inflammation is linked to tissue repair processes, uncontrolled inflammation may result in production of neurotoxic factors that amplify underlying disease states [Glass *et al.*, 2010].

1.7.1 Alzheimer's Disease

Initially described almost 100 years ago by Alois Alzheimer, AD is one of the most common age-related neurodegenerative diseases, affecting approximately 30% of people older than 70 years worldwide. The symptoms of AD are characterized by loss of memory, progressive impairment of cognition, and various behavioral and neuropsychiatric disturbances. The pathological hallmarks of AD in the brain include extracellular amyloid plaques comprising aggregated, cleaved products of the amyloid precursor protein (APP) and intracellular neurofibrillary tangles (NFTs) generated by hyperphosphorylated forms of the microtubule-binding protein tau [Glass *et al.*, 2010; Parihar and Hemnani, 2004]. The role of neuroinflammation in AD has been assumed to be merely a response to pathophysiological events. However, novel insights from preclinical and clinical studies have determined that immune system-mediated actions, in fact, contribute to and drive AD pathogenesis [Heppner *et al.*, 2015]. Inflammatory response in AD includes activation of microglial cells, from ramified (resting) to amoeboid (active), and astrogliosis (increase in the number, size, and motility of astrocytes) surrounding the senile plaques. Moreover, activated microglia releases pro-inflammatory mediators, including COX-2, TNF-α, IL-1β, IL-6 and MCP-1 [Glass *et al.*, 2010; Heppner *et al.*, 2015]. The latter is known to induce astrocytes chemotaxis and recruitment around senile plaques [Wyss-Coray*et al.*, 2003]. In addition, elevated levels of chemokines and cytokines and their receptors, including IL-1α, CXCR2, CCR3, CCR5, and TGF-β, have been reported in post-mortem AD brains [Khandelwal *et al.*, 2011].

1.7.2 Parkinson's disease

Parkinson's disease is the second most common neurodegenerative disease after AD, characterized by motor dysfunction (bradykinesia, tremor, rigidity, and postural instability) and non-motor-related symptoms (olfactory deficits, autonomic dysfunction, depression, cognitive deficits, and sleep disorders). Pathological hallmarks in PD are accumulation and aggregation of misfolded α-synuclein in the so called Lewy bodies and Lewy neuritis, and the loss of dopaminergic neurons in the *pars compacta* of *substantia nigra* of the midbrain

and in other brain regions primarily involved in the control of motor functions [Braak *et al.*, 2003]. Dopaminergic neurons are very sensitive to the surrounding environment and inflammatory processes can easily cause them degeneration. In fact, activation of microglia seems to precedes DA neuronal cell loss and neurons undergoing degeneration may be phagocytosed prematurely by phagocytic microglia (Fig.6) [Marinova-Mutafchieva *et al.*, 2009]. An increase in astroglial cells in postmortem tissue from the brains of PD patients and an increased number of dystrophic astrocytes have also been reported [Braak *et al.*, 2007]. Although these inflammatory components are not specific for PD, they might provide useful biomarkers for monitoring disease progression.

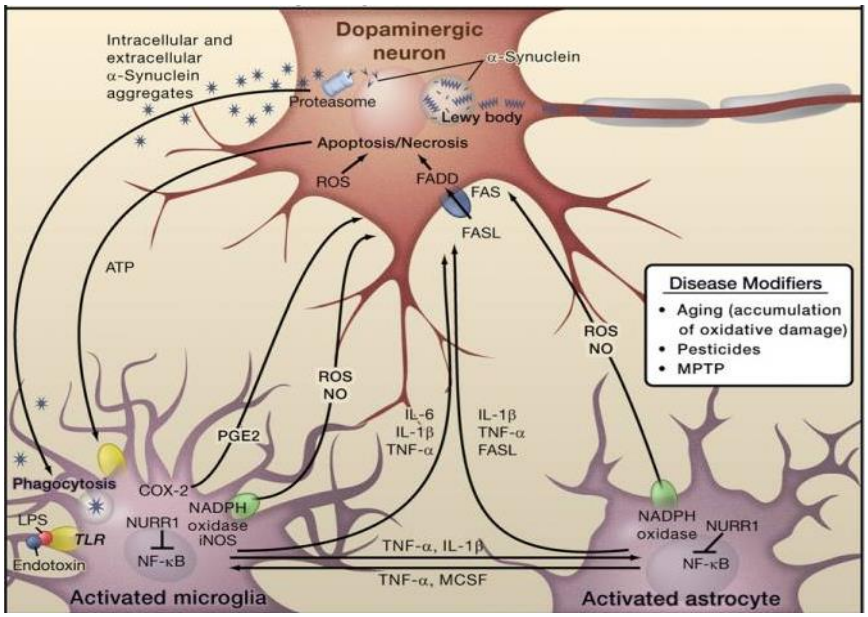


Figure 6. Inflammation in Parkinson's Disease. Prominent neuropathological hallmarks of Parkinson's disease are the loss of dopaminergic neurons in the *substantia nigra* of the midbrain and the presence of intracellular inclusions containing aggregates of the α-synuclein protein, called Lewy bodies. Besides forming Lewy bodies, aggregates of α-synuclein form intermediate-state oligomers that when released from neurons activate microglia through Toll-like receptor (TLR)-independent mechanisms. This leads to activation of NF-κB and production of reactive oxygen species (ROS) and pro-inflammatory mediators. These factors act directly on dopaminergic neurons of the *substantia nigra*, which are the principal (although not the only) neurons that die in PD. These factors also activate microglia, which amplify the inflammatory response in a positive feedback loop, leading to further activation of microglia. Products derived from microglia and astrocytes act in a combinatorial manner to promote neurotoxicity (from Glass *et al.*, 2010)

1.7.3 Multiple sclerosis

Multiple sclerosis is a chronic autoimmune inflammatory neurodegenerating disorder affecting movement, sensation, and body functions with considerable social impact and economic consequences. Onset of MS typically occurs during adulthood and patients experience an early loss in productivity, making MS the major cause of serious physical disability in young adult, especially women [Compston and Coles, 2008; Dendrou *et al.*, 2015].The main pathological features of MS are inflammatory infiltrates, destruction of the myelin sheath and oligodendrocytes, axonal and neuronal damage as well as glia proliferation [Brück, 2005]. These hallmarks vary over time both quantitatively and qualitatively between MS subclinical types and among individuals with the same form [Lassmann, 2014]. MS lesions are characterized by infiltration of lymphocytes and antibody-producing plasma cells into the perivascular region of the brain and spinal cord white matter, an increase in microglia and astrocytes, and demyelination [Compston and Coles, 2008; Brück, 2005]. The deposition of antibodies and complement around demyelinated lesions and axonal degeneration in the progression phase of MS have also been observed [Brück, 2005;Lassmann, 2014]. When damage and the ensuing inflammatory response are transient, remyelination of nerves can take place as part of normal repair. However, in the presence of chronic inflammation, such as in MS, remyelination is severely impaired and leads to neuron degeneration.

As mentioned above, neurodegenerative diseases can be distinguished by disease-specific mechanism for induction of inflammatory response. However, once inducers are generated, effector mechanisms that lead to amplification of inflammatory responses, neurotoxicity, and neuronal death are convergent. In fact, activation of innate immune cells in the CNS, such as microglia and astrocytes, is one of the universal components of neuroinflammation. In particular, activation of TLRs and other pattern recognition receptors expressed on microglia initiate the inflammatory responses that are further amplified by astrocytes. This, lead to signal transduction pathways downstream which activate transcription factors such as NF-κb and AP-1 and the production of quite the same amplifiers and

effector molecules, such as cytokines , ROS, and NO found in all the neurodegenerative diseases discussed before [Glass *et al.*, 2010; Gorina *et al.*, 2011].

It is likely that sustained inflammatory responses that contribute to neurodegeneration are driven, at least in part, by positive feedback loops. Crosstalk between microglia and astrocytes is predicted to lead to amplification of inflammation and release of ATP by necrotic neurons that would be expected to activate microglia in each of the disease. Such feedback loops could, in principle, become independent of the original inducing molecules that are required to initiate inflammatory responses. In addition, inflammation may itself influence the production of disease-specific inducers. It is important to understand how activated immune cells affects specific types of neurons. For example, many of the same cytokines are suggested to play pathological roles in AD, PD, and ALS, but the patterns of neuronal loss are distinct. It will therefore be important to determine whether this difference reflects different sensitivities of specific neurons to generic neurotoxic factors or the production of neurotoxic factors with neuron-specific activities [Glass *et al.*, 2010].

In this context, therapeutic approaches logically would aim to modulate the sensor/transducer/effector functions of the innate immune system. In terms of timing, efficacy, and safety targeting inflammatory pathways might be more effective in preventing disease progression than in reversing existing pathology. To be clinically effective, anti-inflammatory therapies will have to gain access to the CNS and target specific cells and pathways that are quantitatively important in disease pathogenesis in humans. As AD, PD, and ALS are chronic degenerative diseases, it is likely that their prevention and treatment will require long-term therapy, imposing a corresponding requirement for a high level of safety. Among multi-targeting therapies, phytocomplexes can successfully and rationally being used together with both already available drugs and those in process of development. Phytocomplexes contain combinations of bioactive molecules and thus provide synergistic effects and have attracted extensive attention worldwide. These complex systems combine many active compounds that may strike multiple

biological targets involved in neurodegenerative diseases [Glass *et al.*,, 2010; Lehàr *et al.*, 2009].

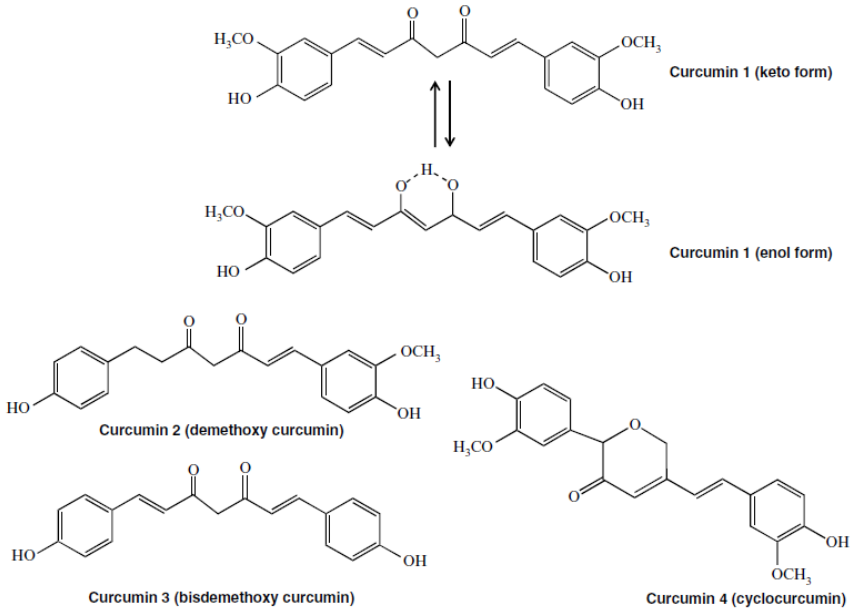
1.8 Herbal remedies in neurodegenerative diseases

Recently, a great number of natural medicinal plants have been tested for their therapeutic properties, showing that the raw extracts or the whole plants could be used as complementary agents in many neurodegenerative diseases. These properties are due mainly to the presence in the phytocomplexe of polyphenols, alkaloids and terpenes, among others, that are micronutrients produced by plants as secondary metabolites[Li *et al.*, 2014]. Several evidences (epidemiological studies, animal studies, and human clinical trials) suggest the use of polyphenols in a wide range of pathologies associated with inflammation [Habauzit and Morand, 2012; Wright, 2013]. Polyphenols may act to protect the brain in a number of ways, including by protection of vulnerable neurons, the enhancement of existing neuronal function or by stimulating neuronal regeneration [Youdim and Joseph, 2001]. For example, polyphenols have been shown to protect neurons against oxidative stress [Li *et al.*, 2005] and Aβ-induced- neuronal injury [Luo *et al.*, 2002] and polyphenol-rich *Ginkgo biloba* extracts have been shown to be neuroprotective by protecting hippocampal neurons from nitric oxide- and Aβ-induced neurotoxicity [Tchantchou *et al.*, 2007].Among various polyphenols, curcumin, a polyphenolic antioxidant derived from the root of *Curcuma longa* has undergone extensive preclinical development, showing remarkable efficacy in neuroinflammatory disorders, particularly in AD [Hu *et al.*, 2015].

1.9 Curcumin: metabolism, bioavailability and biochemical effects in the brain

Curcumin or diferuloylmethane (bis-α,β-unsaturated β-diketone) is a hydrophobic polyphenolic compound (mol mass of 368.38) present in the Indian spice turmeric (curry powder). It is the principal curcuminoid of turmeric (*Curcuma longa*), a member of the ginger family (Zingiberaceae). It has been used for centuries in Chinese traditional medicine and Indian medicine (Ayurvedic medicine) as a nociceptive, anti-inflammatory, and anti-shock drug to relieve pain and

inflammation in muscles and for the treatment of many pathological conditions, such as rheumatism, digestive and inflammatory disorders, intermittent fevers, urinary discharges, leukoderma and amenorrhoea as part of traditional medicine [Anand *et al.*, 2008]. Turmeric products have been declared as safe not only by the FDA in the USA, and the Natural Health Products Directorate of Canada, but also by the Joint Expert Committee of the Food and Agriculture Organization/World Health Organization (FAO/WHO) (National Cancer Institute, 1996). Thus, curcumin is a safe and non-toxic compound, which exhibits a wide range of pharmacological activities, such as anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antifertility, antidiabetic, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic, antivenom, antiulcer, hypotensive and hypocholesteremic activities [Amand *et al.*, 2008; Prasad *et al.*, 2014]. The chemical structure of curcumin consists of two methoxyl groups, two phenolic hydroxyl groups, and three double conjugated bonds. The two aryl rings containing ortho-methoxy phenolic OH– groups are symmetrically linked to a β-diketone moiety. The presence of intramolecular hydrogen atoms transfer at the β-diketone chain of curcumin results in the existence of keto- and enol-tautomeric conformations in equilibrium (Figure7). Keto-enoltautomers of curcumin also exist in several cis and trans forms. The relative concentrations of cis and trans forms vary according to temperature, polarity of solvent, pH and substitution of the aromatic rings [Cornago *et al.*, 2008]. Thus, a predominant keto form occurs in acidic and neutral solutions and as a stable enol form, which occurs in alkaline media. The amount of keto-enol-enolate of the heptadienone moiety in equilibrium plays acrucial role in the physicochemical properties of curcumin. The particular chemical structure of curcumin is predictive of the multi biological effects in human and animals. The o-methoxyphenol group and methylenic hydrogen are responsible for the antioxidant activity of curcumin, and curcumin donates an electron/hydrogen atom to reactive oxygen species.



7. Chemical structures of tautomers of curcumin along with curcumin 2 and curcumin 3.

Curcumin interacts with a number of biomolecules through non-covalent and covalent binding. As stated above, the hydrogen bonding and hydrophobicity of curcumin, arising from the aromatic and tautomeric structures along with the flexibility of the linker group are responsible for the non-covalent interactions. The α,β-unsaturated β-diketone moiety covalently binds with thiols-residues in protein, through Michael reaction [Priyadarsini, 2013]. The β-diketo group is capable of chelating transition metals, thereby reducing the metal induced toxicity and some of the metal complexes exhibit improved antioxidant activity as enzyme mimics [Priyadarsini, 2013]. The alcoholic extract of turmeric contains three curcuminoids: curcumin (also referred as curcumin I, 77%), desmethoxycurcumin (curcumin II, 17 %), and bisdesmethoxycurcumin (curcumin III, 3%) (see Fig.7). In addition, turmeric also contains volatile oils (natlantone, tumerone and zingiberone), proteins, sugar and resins. Curcumin is insoluble in water. It is readily soluble in organic solvents, such as dimethylsulfoxide, acetone, and ethanol.

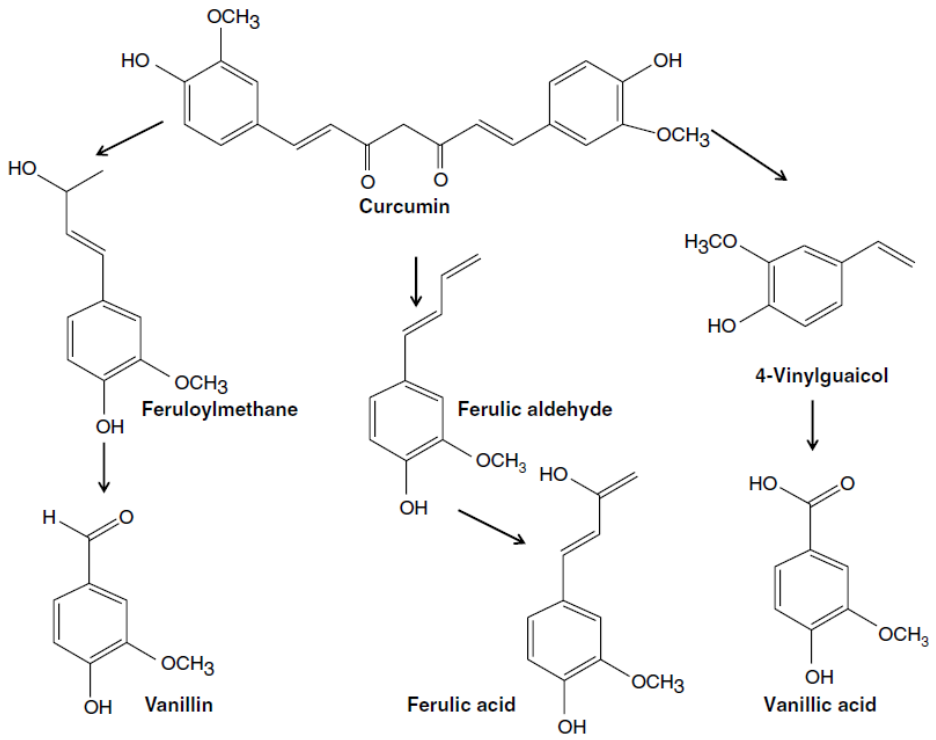


Figure 8. Degradation of curcumin under physiological conditions

More than 90% decomposition of curcumin occurs in a serum free 0.1 M phosphate buffer (pH 7.2) at 37 °C. The degraded products have been identified as trans-6-(4′-hydroxy-3′-methoxyphenyl)- 2,4-dioxo-5-hexenal (major products), vanillin, vanillic acid, ferulic acid (4-hydroxy-3- methoxycinnamic acid) and feruloylmethane using high performance liquid chromatography (HPLC) and gas chromatography mass spectrophotometry (GC-MS) analyses [Wang *et al*., 1997] (see Fig.8). Among degradation products of curcumin, ferulic acid possesses three distinctive structural motifs, which contribute to its free radical scavenging activity. The presence of electron donating groups on the benzene ring (3 methoxy and more importantly 4-hydroxyl) of ferulic acid gives the additional property of terminating free radical chain reactions. Furthermore, carboxylic acid group in ferulic acid with an adjacent unsaturated C–C double bond provides additional attack sites for free radicals and thus preventing them from attacking the membrane. In addition, the carboxylic acid group in ferulic acid also acts as an

anchor for binding with the lipid bilayer and providing some protection against lipid peroxidation [Srinivasan *et al*., 2007; Kanski *et al*., 2002]. The addition of ascorbic acid, N-acetylcysteine and glutathione slow the decomposition of curcumin in both cell culture medium and 0.1 M phosphate buffer under alkaline conditions [Wang *et al*., 1997].

In the brain, curcumin acts by modulating multiple pathways. The analgesic effects of curcumin are mediated by the suppression of nitrite, TNF-α, and capsaicin-induced TRPV1 activity, and through the descending noradrenergic and serotonergic systems [Yeon *et al*., 2010; Zhao *et al*., 2012]. Anti-inflammatory and antioxidant activities of curcumin are mediated by the inhibition of cytosolic phospholipase A2 (cPLA2), COX-2, 5-lipoxygenase (5-LOX), glutathione S-transferases, down-regulation of inflammatory transcription factors, and upregulation of heme oxygenase-1 [Aggarwal *et al*., 2007]. Curcumin also interacts and modulates activities of DNA (cytosine-5)-methyltransferase-1, thioredoxin reductase, and protein kinases (such as protein kinase C, mammalian target of rapamycin, MAPK and Akt). Many of above mentioned enzymes contribute not only to its ability to interfere with multiple signalling cascades, such as cell cycle regulators, apoptotic proteins, pro-inflammatory cytokines, but also to its modulatory effects on proliferative regulators and transcription factors such as (NF-κB, Nrf2, Stat3, TNF-α, forkhead box O3a, AP-1 and CRAC [Shishodia, 2013]. It also inhibits secretion of interleukins and cytokines as well as the expression of human epidermal growth factor receptor [Chen *et al*., 2006]. Curcumin also inhibits the development of cancer cells, tumor growth and blocks angiogenesis and inflammation [Shehzad and Lee, 2013].

1.9.1 Bioavailability of curcumin and its analogs

Delivery of curcumin to the body can be performed through oral, nasal, intraperitoneal, and intravenous injections. Despite its safety, curcumin presents a very low bioavailability, which is the greatest challenge for its therapeutic use in human [Prasad *et al*., 2014]. There are three major reasons for the low bioavailability:(a) its poor absorption, (b) its rapid metabolism, and (c) its rapid systemic elimination. In addition, curcumin has a short biological half-life in

circulation and visceral organs [Prasad *et al*., 2014]. In rodents, curcumin undergoes rapid metabolism by conjugation and reduction, and its disposition after oral dosing. However, information about comprehensive pharmacokinetic data is not available. It is reported that 10 mg/kg of curcumin given intravenously to rats yielded a maximum serum level of 0.36 ± 0.05 μg/mL, whereas 500 mg/kg of curcumin administered orally only yielded a 0.06 ± 0.01 μg/mL maximum serum level [Yang *et al*., 2007]. Furthermore, oral administration of curcumin at a dose of 2 g/kg results in a maximum serum concentration of 1.35 ± 0.23 μg/mL at time 0.83 h, while in humans the dose of 2 g of curcumin results in either undetectable or very low (0.006 ± 0.005 μg/mL at 1 h) serum levels [Shoba *et al*., 1998].

Very little information is available on pharmacokinetic of curcumin in humans. First phase I and II clinical trials of curcumin have been performed in patients with advanced colorectal cancer for up to 4 months at several doses (500, 1000, 2000, 4000, 8000and 12,000 mg/day) without any toxicity [Sharma *et al*., 2001; Cheng *et al*., 2001].The serum concentration of curcumin usually peaks at 1–2 h after oral intake of curcumin and gradually declines within 12 h. The average peak serum concentrations after taking 4000 mg, 6000 mg and 8000 mg of curcumin were 0.51 ± 0.11 μM,0.63 ± 0.06 μM and 1.77 ± 1.87 μM, respectively. In this study, serum levels of curcumin peak after one hour, and two hours post-administration they declined rapidly. So far, an upper level of toxicity has not been established for curcumin. Studies have shown that a dosage as high as 12 g/day is safe and tolerable to humans with a few reporting mild side-effects [Goel *et al*., 2008; Jiao *et al*., 2009]. Studies on curcumin metabolism in animals indicate that it is rapidly metabolized through glucuronidation (Fig. 9) and sulfation or it is reduced to hexahydrocurcuminin liver, and intestine [Metzler *et al*., 2013]. Glucuronidation and sulfation of curcumin is catalyzed by UDP-Glucuronosyltransferases(UGTs) and sulfotransferases in liver and intestine [Metzler *et al*., 2013].

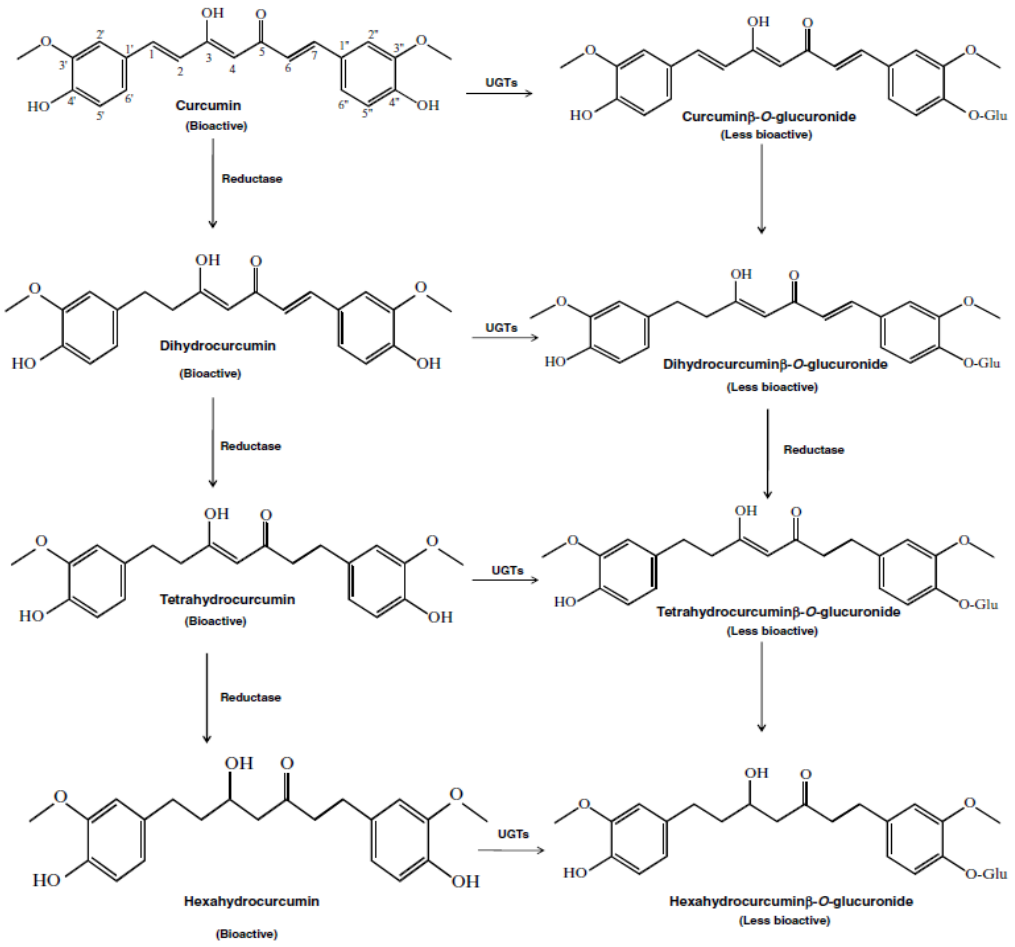


Figure 9. Curcumin metabolites after biotransformation.

1.9.2 Different approaches to delivery curcumin to the brain

It is well known that the brain and spinal cord are protected by the blood–brain barrier (BBB), a protective mechanism that controls cerebral homeostasis and provides the CNS with unique protection against all foreign matter [Roney *et al.*, 2005]. The BBB retards the entry of 98% of small molecules and 100% of large molecules in the brain. It is located at the level of the capillaries between the blood and cerebral tissue, and is characterized by the presence of tight intracellular junctions and polarized expression of many transport systems [Abbott *et al.*, 2010]. BBB reduces the penetration of curcumin. In this case, the use of certain delivery systems (liposome, nanoparticles etc. [Ghalandarlaki *et al.*, 2014;

Mourtas *et al*., 2014]) may prove particularly effective. These approaches have not only resulted in longer circulation and increase in the cellular permeability, but also resistance against metabolic processing leading to efficient delivery to tissues and higher half-life in the circulation. Some of these approaches have also allowed curcumin to penetrate the BBB effectively [Shoba *et al*., 1998; Ghalandarlaki *et al*., 2014]. The delivery of curcumin to the brain can enhance the adult hippocampus neurogenesis in the dentate gyrus region of hippocampus [Kim *et al*., 2008].As stated above, curcumin has a poor absorption rate and undergoes rapid metabolism which severely curtails its bioavailability. Piperine, a major alkaloidal constituent of black pepper (Piper nigrum), is a powerful inhibitor of hepatic and intestinal glucuronidation. Several studies have indicated that piperine increases the bioavailability and bioefficacy of curcumin by inhibiting glucuronidation [Shoba *et al*., 1998; Suresh and Srinivasan, 2010].

1.9.3 Biochemical activities and targets of curcumin action

Curcumin mediates its effects through the modulation of transcription factors (NF-κB,AP-1, STAT, and Nrf2), inflammatory cytokines (TNF-α, IL-1β, and IL-6),enzymes (COX-2, LOX, MMP9, MAPK, mTOR, Akt), growth factors (VEGF,EGF, and FGF), growth factor receptors (EGFR, HER-2, and AR), adhesion molecules(ELAM-1, ICAM-1, VCAM-1), and apoptosis-related proteins (Bcl-2, caspases, DR, Fas) (Fig. 10) [Goel *et al*., 2008; Zhou *et al*., 2011]. These targets are associated with regulation of signal transduction processes involved in antioxidant, anti-inflammatory, antidepressant, antidiabetic, anti-hyperalgesic, anticarcinogenic, antimicrobial, hepatoprotective, thrombosuppressive, and antinociceptive effects of curcumin [Anand *et al*., 2008; Prasad *et al*., 2014]. It should be noted that most above mentioned activities of curcumin have been assigned to methoxy, hydroxyl, α,β-unsaturated carbonyl moiety or to diketone groups present in the curcumin structure. A major metabolite of curcumin is called as tetrahydrocurcumin(THC), which lacks α,β-unsaturated carbonyl moiety and is white in colour. It differs from curcumin in its chemical and biochemical activities [Aggarwal *et al*., 2014].Some investigators have indicated that curcumin exhibits both pro-oxidant and antioxidant properties, while THC mediates superior

antioxidant activities than curcumin. Curcumin increases the expression of HO-1 through the activation of the Nrf2. In contrast, THC has no effect on HO-1 expression [Aggarwal *et al*., 2014]. Other investigators have reported that THC mediates higher antioxidant activity due to its ability to better activate GSH peroxidase, glutathione-S-transferase, NADPH: quinine reductase as well as superior free radical quenching activities than curcumin [Aggarwal *et al*., 2014].

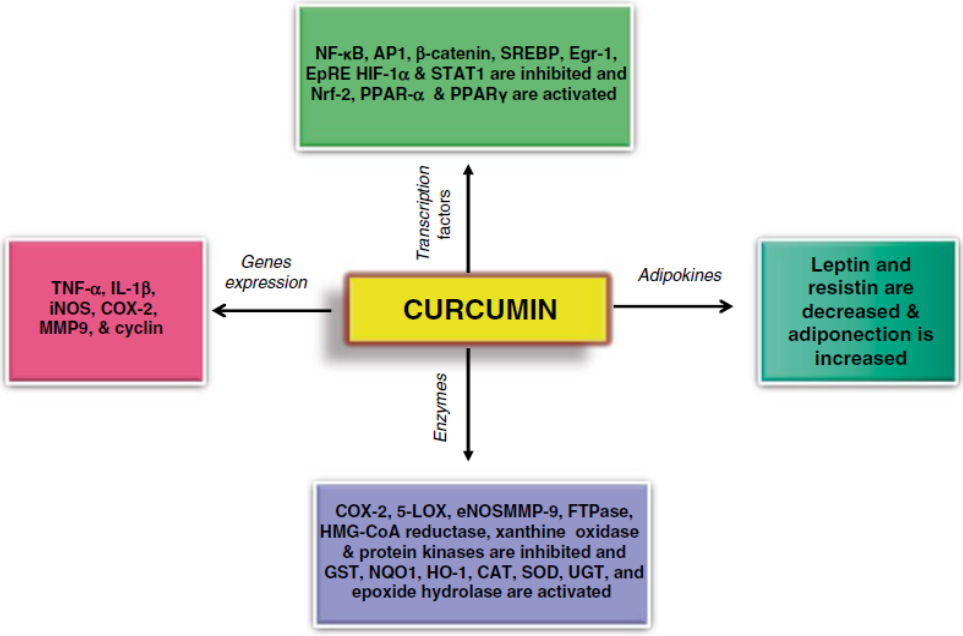


Figure 10. Effect of curcumin on gene expression, adipokines, transcription factors and enzymes. Curcumin mediates its effects through the modulation of transcription factors (NF-κB,AP-1, STAT, and Nrf2), inflammatory cytokines (TNF-α, IL-1β, and IL-6),enzymes (COX-2, LOX, MMP9, MAPK, mTOR, Akt), growth factors (VEGF,EGF, and FGF), growth factor receptors (EGFR, HER-2, and AR), adhesion molecules(ELAM-1, ICAM-1, VCAM-1), adipokines (resistin and leptin) and apoptosis-related proteins (Bcl-2, caspases, DR, Fas) (from Farooqi, 2016).

1.9.4 Neuroprotective activities of curcumin

It is well known that the brain hippocampus plays an important role in memory formation and spatial navigation. Adult hippocampal neurogenesis is closely related to memory formation [Snyder *et al*., 2005]. Progenitor cells located in the subgranular zone of the hippocampal dentate gyrus divide, proliferate,

differentiate, and give rise to new neurons [Jin *et al*., 2001]. Overall decline in cellular proliferation has been reported to occur during brain aging and stress[Cameron and McKay, 1999]. In the dentate gyrus of aged mice neurogenesis is promoted by low levels of ROS, expression of BDNF, caloric restriction, exercise, and physiological activation. Wnt-catenin signalling pathway contributes to neurogenesis through the maintenance of synaptic plasticity, survival, proliferation, and differentiation in embryonic and adult brains [Van Praag*et al*., 2002]. Curcumin mediates its neuroprotective effects and memory restoring effects through the prevention of oxidative stress [Cole *et al*., 2007]. In the okadaic acid-mediated neural impairment model, oral administration of curcumin significantly improves the memory function as assessed by both Morris water maze and passive avoidance tests [Rajasekar *et al*., 2013]. Curcumin not only reverses Aβ-mediated cognitive deficits and neuropathological alterations [Lim *et al*., 2001], but also promotes hippocampal neurogenesis through activation of the canonical Wnt pathway [Tiwari *et al*., 2014].

1.9.5 Anti-inflammatory properties of curcumin

Oxidative stress and neuroinflammation are interlinked with neurodegenerative processes in neurological disorders although it is difficult to establish the temporal sequence of their relationship. Pro-inflammatory transcription factor, NF-κB is redox sensitive. ROS stimulates NF-κB, which exists in the cytoplasm of neural cells as a heteromeric p50/p65 complexed with an inhibitory subunit, IκB. High oxidative stress promotes the dissociation NF-κB from NF-κB-IκB complex. This frees NF-κB and allows it to translocate to the nucleus, where it binds to genome through NF-κB response element, facilitating the expression of more than 200 target genes including genes for inflammatory cytokines resulting in the onset of neuroinflammation. Many of genes contribute to cell proliferation, invasion, metastasis, and chemoresistance. Therefore, ROS trigger the release of inflammatory cytokines, which through the activation of phospholipasesA2, COX-I2, and LOXs in turn enhance ROS production, thus establishing a vicious circle [Farooqui, 2014]. Curcumin retards neuroinflammation not only by inhibiting p65 translocation to the nucleus and suppressing IκBα degradation in numerous cell

types, but also by blocking COXs and LOXs and the uptake of arachidonic acid by macrophages[Singh and Aggarwal, 1995]. By inhibiting NF-κB activation, curcumin suppresses the expression of various cell survival and proliferative genes, including Bcl-2, Bcl-xL, cyclin D1, interleukin (IL)-6,cyclooxygenase 2 (COX-2) and matrix metallopeptidase (MMP)-9, and subsequently arrests cell cycle, inhibits proliferation, and induces apoptosis [Zhou *et al*., 2011]. In addition, Curcumin may also act by increasing the level and activity of proteins involved in antioxidative defense. Thus, curcumin enhances activities of protein kinases, and GST, CAT, SOD, UDP-glucuronosyltransferase (UGT), HO-1,andsirtuins. Activation of these enzymes not only contributes to neuroprotection, but is also essential for homeostasis in the vascular system [Zhang *et al*., 2015].

Finally, numerous evidence demonstrates that curcumin can prevent neuroinflammation by modulating microglial activation [Lee et al., 2007]. Microglia mediated neuroinflammation is an important contributor to the inflammatory injury in various brain pathologies such as intracerebral hemorrhage (ICH).Curcumin could suppress ICH induced inflammatory injury and represent a novel herbal sources for ICH therapeutical strategy [Yang et al., 2014].Morover, the HIV-1 gp120 model of neuroinflammation increases ROS, TNF-α and MCP-1 production in microglia, and induces cortical neuron apoptosis by affecting the delayed rectification and transient outward K+ channel current. Curcumin reduces production of ROS and inflammatory mediators in HIV-1-gp120-stimulated microglia, and protects cortical neurons against HIV-1-mediated apoptosis, most likely through inhibition of HIV-1 gp120-induced elevation of the delayed rectification and transient outward K+ current [Guo et al., 2013].

2. Aim of the thesis

The aim of this research project was the characterization of an *in vivo* model of neuroinflammation suitable for studying potential anti-inflammatory compounds, useful in the prevention and/or treatment of neurodegenerative (*e.g.*, Parkinson's and Alzheimer's diseases, multiple sclerosis and amyotrophic lateral sclerosis) and psychiatric (psychosis, schizophrenia, depression) disorders, where inflammation is a main actor.

Our attention was focused on curcumin, the main bioactive component in the rhizome of the turmeric plant (*Curcuma longa*). Curcumin is a safe and a highly pleiotropic molecule endowed with a wide range of beneficial activities, including anti-inflammatory, antitumor, anti-oxidative and cardiovascular protective effects, interacting with diverse molecular targets.

The research activity of this project was focused on:

- Characterization of the *in vivo* model of neuroinflammation, based on a single intraperitoneal injection of LPS. This model was characterized by molecular, biochemical and behavioral studies, that allowed us to identify a CNS inflammatory state.

- Study of curcumin effects on the *in vivo* model of neuroinflammation. First, a dose-response study of curcumin was performed to choose the first efficacy dose able to prevent LPS-induced pro-inflammatory gene expression increase. Then, following an oral administration of curcumin (by *gavage* of a single dose for 2 consecutive days), molecular, biochemical and behavioral studies were performed to test the possible anti-inflammatory and neuroprotective role of curcumin.

3. Materials and Methods

3.1 Animals and treatments

Animal-related procedures were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and those of the Italian Ministry of Health (D.L. 26/2014). The study was approved by the OPBA of the University of Padova and the Italian Ministry of Health (Protocol number 722/2015-PR).

Three-month-old male C57BL/6J mice were housed under standard laboratory conditions with food and water *ad libitum* on a 12/12 h dark/light cycle (light on between 07:00 and 19:00 h). Before each experiment mice were handled during 3 min daily for 6 days. The use of proper restraint and handling techniques reduces novelty-induced stress to animals and ensure greater results reliability. Animals were randomly divided into different experimental groups and intraperitoneally (i.p.) injected with either a single dose of LPS (0.5 or 5 mg/kg; *E. Coli*, 026:B6; Sigma, Italy) or vehicle (sterile endotoxin-free PBS; Life Technologies, Italy).

For curcumin treatment, mice were orally treated with a single daily dose of either curcumin (5, 10 or 50 mg/Kg *via gavage*, in a 1% methylcellulose solution; Sigma-Aldrich, Italy) or vehicle following periodic fasting for two consecutive days. On the second day, one hour after curcumin treatment, mice were i.p. injected with a single dose of 5 mg/Kg LPS and sacrificed 2 h, 24h and 7 days post-injection for the analysis of animal behavior, cytokine profile, microglial morphology and neuronal cell loss (Fig. 11).

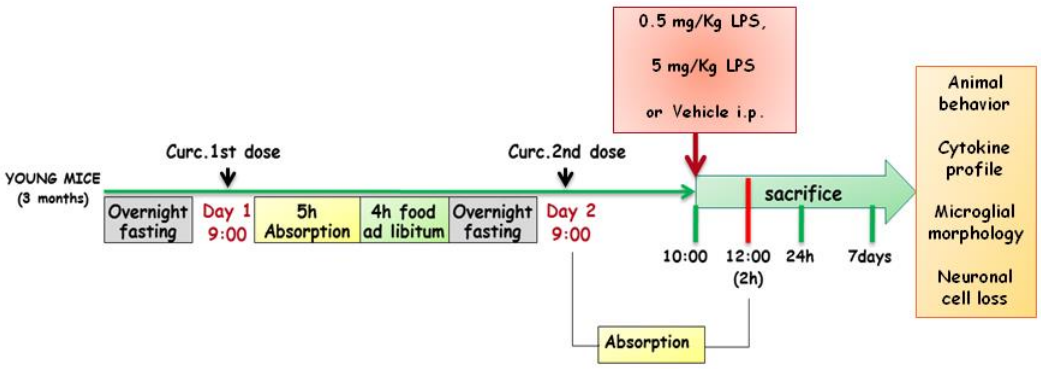


Figure 11: Schematic paradigm of mouse treatment. Mice were orally treated with either a single daily dose of curcumin or vehicle following periodic fasting for two consecutive days. On the second day, one hour after curcumin treatment, mice were i.p. injected with a single dose of 5 mg/Kg LPS and sacrificed 2 h, 24h and 7 days post-injection.

3.2 Measurements of body weight and food intake

Body weight and food intake were measured every morning (9-10 am) for 7 days to minimize any variation due to the circadian rhythm. Food intake was obtained by subtracting remaining food, including any spilled food in cages, from a weighed aliquot of food

3.3 Locomotor Activity: Open field test

Open field test (OFT) is used for measuring anxiety and exploration as well as general locomotor activity levels [Tosini G, 2007]. The apparatus consists of four gray arenas (open field) delimited by a 30-cm-high wall (Fig. 12) exposed to an adjusted amount of light, which is measured with a Luxmeter. Arena was cleaned with 70% ethanol one hour before and at the end of each behavioral evaluation. Each mouse was allowed to freely move in the empty open field and locomotor activity was video-recorded during the one-hour session, using an overhead camera with a videotracking system. Total distance traveled in the arena was analyzed using ANY-maze™ Video tracking (Stoelting Co.)

Figure 12: Gray arenas disposition during LMA test

3.4 Novel Object Recognition (NOR) test

The NOR test is used to evaluate recognition memory in murine models of CNS disorders. This test is based on the spontaneous tendency of rodents to spend more time exploring a new object with respect to a familiar one. The choice of exploring the new object reflects the capacity for learning and recognition.

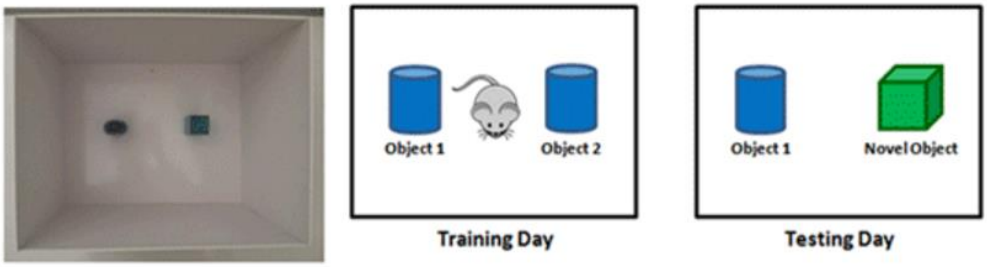


Figure 13: Novel object recognition test apparatus and schematic rappresentation.

The apparatus consists of the same arena where LMA test was previously performed. Arena and object were cleaned with 70% ethanol one hour before and

at the end of each behavioral evaluation. The test was composed of two different sessions each lasting 10 min:

- *habituation phase*: during this phase, two identical objects in shape and color were placed at the front of the arena and at equal distance from both sides, in order to allow the mouse to freely explore the objects Mice, previously habituated to the apparatus, were placed inside the arena for a 10-min period. Behavior was videotaped and the duration engaged in object investigation was determined from the video records by an observed who was blind to experimental treatments. Exploration was recorded when the animal's nose or mouth was in direct contact with the object. Climbing or sitting on the object was not considered exploration [Frühauf *et al*., 2015].
- *retention phase*: this test session was carried out 2 hours after habituation phase. Mice were placed back in the arena and one of the familiar object (object 2, Fig.13) was replaced by a novel object, different in shape and color. The times spent exploring the familiar and the novel object were videotaped for 10 min.

The *preference index* was the calculated, considering the difference of time spent exploring the new and familiar objects, using the formula:

$(T_{novel}) / (T_{novel}+T_{familiar})$

This index was used as a memory parameter.

3.5 Brain area extraction

Mice were rapidly sacrificed by cervical dislocation 2 hours, 24 hours and 7 days after treatments, limiting possible manipulation induced-stress. Brains were gently removed from the skull and immediately placed on a glass plate on ice. Frontal cortex, hippocampus, striatum, cerebellum and hypothalamus were dissected, placed into sterile tubes (Eppendorf®) and stored at -80°C for subsequent analysis.

3.6 RNA extraction

Total RNA was extracted from frontal cortex, hippocampus, striatum, cerebellum and hypothalamus tissues by TRIzol® Reagent (Life Technologies), according to the manufacturer's instructions. One mL TRIzol® Reagent was added per 30-50 mg of brain tissue. Tissues were homogenized using a power homogenizer, immediately placed on ice for 15 min and then incubated for 5 min at room temperature (RT) to permit complete dissociation of the nucleoprotein complex. 0.2 mL of chloroform per 1 mL of TRIzol® Reagent were subsequently added for homogenization. Tubes were capped and shook vigorously by hand for 15 s and incubated for 15 min at 4°C. Samples were centrifuged at 12,000 × g for 15 min at 4°C, to allow the mixture to separate into a lower red phenol/chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remained exclusively in the aqueous phase. The upper aqueous phase was ~50% of the total volume. Aqueous phase was removed by angling the tube at 45° and pipetting the solution out into a new labelled tube, paying attention to avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase. 0.5 mL of 100% isopropanol/mL of TRIzol® Reagent used for homogenization were added to the aqueous phase and incubated at RT for 10 min before centrifuging at 12,000 × g for 10 min at 4°C. RNA is often invisible prior to centrifugation and forms a gel-like pellet on the side and bottom of the tube. After centrifugation supernatant was removed from the tube, leaving only the RNA pellet. One mL of 75% ethanol/mL of TRIzol® Reagent used in the initial homogenization was used to wash the pellet. Samples were centrifuged at 7,500 × g for 5 min at 4°C, supernatant was discarded and RNA pellet was vacuumed or air dried for 5–10 min, avoiding the RNA to dry completely. Finally, pellet was dissolved in diethylpyrocarbonate (DEPC) water and stored at -80°C. RNA integrity and quantity were determined by spectrophotometric analysis (NanoDrop 2000, Thermo Fisher Scientific Inc.). One microgram of RNA was reverse transcribed with Superscript IV Reverse Transcriptase using random oligonucleotides (Life Technologies).

3.7 Real-time RT-PCR analysis

The real-time RT-PCR reaction was performed in a MX 3000P thermal cycler in a final volume of 10 µl, containing 100 nM of each primer pair (Table 2) and 1X SYBR green JumpStart Taq ReadyMix (Sigma). The PCR cycling conditions comprised an initial denaturation step at 94°C for 4 min, followed by 45 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 15 s and extension phase at 72°C for 30 s, followed by a dissociation thermal profile at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Amounts of each gene product were calculated using linear regression analysis from standard curves, demonstrating amplification efficiencies ranging from 90 to 100%. Dissociation curves were generated for each primer pair, showing single product amplification. Relative mRNA levels were obtained after normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels.

Table 2: Gene names and primer used for Real-time PCR analysis

TNF-α	F	CAAGTGGAGGAGCAGCTGGA
	R	CATCGGCTGGCACCACTAGT
IL-1β	F	CTGGTGTGTGACGTTCCCATTA
	R	CCGACAGCACGAGGCTTT
IL-6	F	GAGGATACCACTCCCAACAGACC
	R	AAGTGCATCATCGTTGTTCATACA
IL-18	F	AGGTGGGGAGGGTTTGTGTT
	R	TGCAGCCTCGGGTATTCTGT
COX-2	F	GCTGGCCTGGTACTCAGTAGGTT
	R	CGAGGCCACTGATACCTATTGC
iNOS	F	TGTACCCTCAGTTCTGCGCC
	R	TGTTGGGGCAAGCTGAGAGG
POMC	F	GCCCAAGGACAAGCGTTACG
	R	TGCGCGTTCTTGATGATGGC

3.8 Protein extraction

Proteins were extracted from brain regions of interest (frontal cortex, striatum, hippocampus, cerebellum). 50 mL of lysis solution [890 µL NP-40 cell lysis buffer (Invitrogen), 100 µL protease inhibitor cocktail (Sigma Aldrich) and 10 µL of 0.1 M Pefabloc SC (Sigma Aldrich)] was added per mg of brain tissue. Tissues were homogenized using a power homogenizer and immediately placed on ice for 45 min. Samples were then cleared by centrifugation at 14,000 × g for 20 min at 4°C. The supernatants were transferred into new labelled tubes and stored at -20°C until analysis (by avoiding repeated cycles of freezing/thawing).

3.9 Bicinchoninic acid (BCA) protein assay

Protein amount was determined by the BCA method, using the BCA Protein Assay Reagent Kit (Pierce) and bovine serum albumin (BSA, Pierce) as standard, following to the manufacturer's protocol. In brief, 100 µL of working reagent, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, cupric sulphate, sodium tartrate were mixed with tissue lysate. The mixture was incubated for 30 min at 37°C. All samples were then measured for their absorbance at 570 nm, using a Microplate Reader (1420 Victor2 Multilabel Counter, Perkin Elmer Wallac). Total amount of protein was calculated from the BSA calibration curve and expressed in µg/ml of tissue.

3.10 Enzyme-linked immunosorbent assay (ELISA)

The TNF-α levels were analyzed by ELISA, using a commercially available ELISA kit (Antigenix America, Huntington Station, NY, USA), according to the manufacturer's instructions. Cytokine concentration was determined with reference to a standard curve obtained with known amounts of TNF-α. A 96-well plate was incubated ON at 4°C with a Coating Antibody Working Solution (1 µg/mL). After solution removal and four washings with Washing Solution (0.05% Tween-20 in 1X PBS), 100 µL of Coating Stabilizer/Blocking Reagent were placed in each well for 1 hour. Solution was then removed and standard and samples were transferred in the plate for 2 hours. At the end of incubation, plate was extensive washed (4 times) with washing solution and incubated with

Detection Antibody Working Solution for 1 hour at RT. After extensive rinsing (4 times) with Washing Solution, plate was incubated with an Avidin-HRP Working Solution for 30 min at RT, washed with Washing Solution and incubated with a Colour Development Solution for 10 min at RT. Finally, Stop Solution (2M sulfuric acid) was added and the plate was read at 450 nm, using a Microplate Reader (Victor2 Multilabel Counter). TNF-α concentrations (pg/mg protein) were determined by reference to standard curves obtained with known amounts of TNF-α.

3.11 Immunohistochemistry

Brains were gently extracted from the skull, washed in PBS (3 times), fixed in 4% paraformaldehyde for 2 hours at 4°C, cryopreserved in a sucrose gradient (12%, 16%, 18%, 30%) for 4 consecutive days and then embedded in Tissue-Tek OCT compound (Sakura Finetek), as previously described [Majno and Joris, 2004]. Frozen tissues were cryostat sectioned (12 μm), mounted onto SuperFrost glass slides (Fisher), and stored at -20°C until use.

Immunofluorescence experiments were performed by removing OCT excess, surrounding slides with a PAP-PENto avoid fluid loss, and rehydrating slides with PBS for 10 min at RT. Immunofluorescence staining involving mouse primary antibodies was performed using a "Mouse on Mouse" Kit (Vector Laboratories). All other immunofluorescence experiments were performed by first blocking non-specific staining with a blocking solution containing 5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS for 1 h. Sections were then washed 3 times with PBS for 10 min at RT and sequentially incubated with primary (2 hours) and secondary (1 hours) antibodies diluted in blocking solution. The following primary antibodies were used: rabbit polyclonal anti-ionized calcium binding adaptor molecule 1 (Iba1) (1:800, Wako Chemicals USA Inc., Richmond, VA, USA) and mouse monoclonal anti-tyrosine hydroxylase (TH, (1:800, Sigma Aldrich). The fluorescent-conjugated secondary antibodies used included the Alexa Fluor 488 and 555 series (1:1000; Invitrogen). Sections were counterstained (2 min at RT) with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Sigma) to visualize nuclei, and mounted with Fluoromount-G mounting medium (Fisher

Scientific, Milan, Italy). All images were captured with a confocal laser-scanning microscope (Zeiss LSM 800; Carl Zeiss AG, Oberkochen, Germany).

3.12 Plasma and tissue sample preparation for HPLC-MS analysis

Fifty µL of analytical standard (137 µg/mL benzanilide) and 200 µL of methanol were added to 100 µL of plasma samples. Samples were stirred, centrifugated for 10 min at 13, 000 rpm, then supernatants were collected and placed in vials.

Brain were homogenized using a power homogenizer and added of 50 µL of analytical standard (137 µg/mL benzanilide) and 500 µL/brain of methanol. After stirring, samples were sonicated in an ultrasonic bath for 10 min, centrifuged at 13, 000 rpm for 10 min, then supernatants were collected and placed in vials.

3.13 HPLC-MS analysis

An Agilent series 1260 HPLC chromatograph equipped with a Prostar 410 auto-sampler (Varian, Cernusco Sul Naviglio, Milan, Italy) coupled with a Varian 320 TQD MS spectrometer was used as HPLC-MS system. The mass spectrometer was equipped with electrospray ionization (ESI) source as the interface and analysis was conducted in positive ion mode. Analyses were performed on Phenomenex Kinetex EVO C-18 100A, 100 x 3 mm 5 µm column. The mobile phase was composed of solvent A (water-formic acid, 100:1.0 v/v) and solvent B (acetonitrile). A gradient program was used as follows: 0 → 9th min: A:B (80:20) → A:B (5:95) 9 → 15th min: A:B (5:95) → A:B (5:95) 15 → 15.5th min: A:B (5:95) → A:B (80:20) 15.5→20th min: A:B (80:20) → A:B (80:20). The mobile phase flow rate was 200 µL/min and the injection volume was 10 µL. The ESI source was set in positive ionization mode. Quantification was performed using multiple reaction monitoring (MRM) with m/z 369 → 177 transition for curcumin and m/z 198 → 105 transition for benzanilide standard.

Analyte	Mass (M+H)	Fragment
Curcumin	369	177
Benzanilide	198	105

3.14 Statistical Analysis

Statistical comparisons were made using Student's t test or one-way analysis of variance (ANOVA) followed by *post-hoc* tests for either selected or multiple comparisons. Values are expressed as mean ± standard error of the mean (SEM). All statistical tests were performed with GraphPad Prism software version 3.03. Significance level was set at $p < 0.05$. Additional details are provided in the figure legends, where appropriate.

4. Results

4.1 Effect of systemic LPS administration on body weight and food intake.

Mice were i.p. injected with either vehicle (sterile PBS) or two doses of LPS (0.5 and 5 mg/Kg) and body weight and food intake were monitored daily for 7 days after injection. Both doses of LPS induced a significant decrease in body weight 24 hours after LPS injection (- 3.20 ± 0.34 g and - 2.90 ± 0.27 g, with 0.5 and 5 mg/Kg LPS, respectively), compared with control group (0.02 ± 0.009 g) . On day 2 after treatment, body weight was significantly reduced only in 5 mg/Kg LPS-treated mice (-0.58 ± 0.22). From the third day, mice completely recovered LPS-induced body weight loss (Fig. 14).

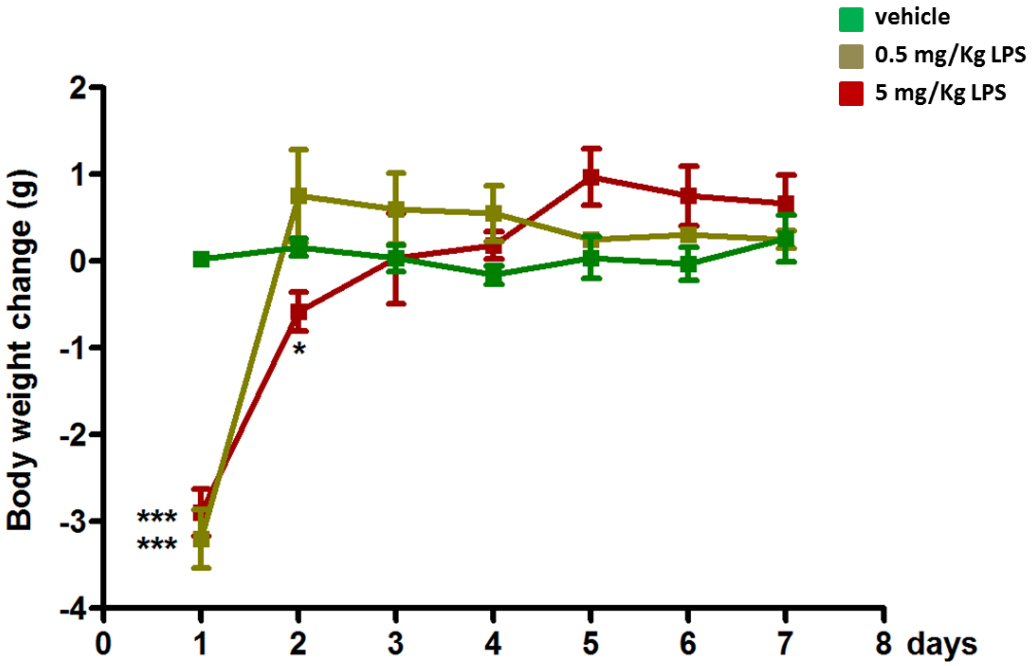


Figure 14: Effect of LPS on body weight changes in mice. Mice were treated with a single intraperitoneal injection of vehicle (sterile PBS; green line) or two doses of LPS [0.5 mg/Kg (ochre line) or 5 mg/Kg (red line)] and body weight change was monitored daily for 7 days after injection. Data are means ± SEM (n=10 animals/group). *p<0.05 and ***p<0.001 *vs* vehicle. Student's t-test.

Food intake was also monitored daily for 7 days after LPS treatment. Both LPS doses induced a significant decrease of total food intake up to 4 days after injection. Starting from day 5 total food intake gradually returned to control values (Fig. 15).

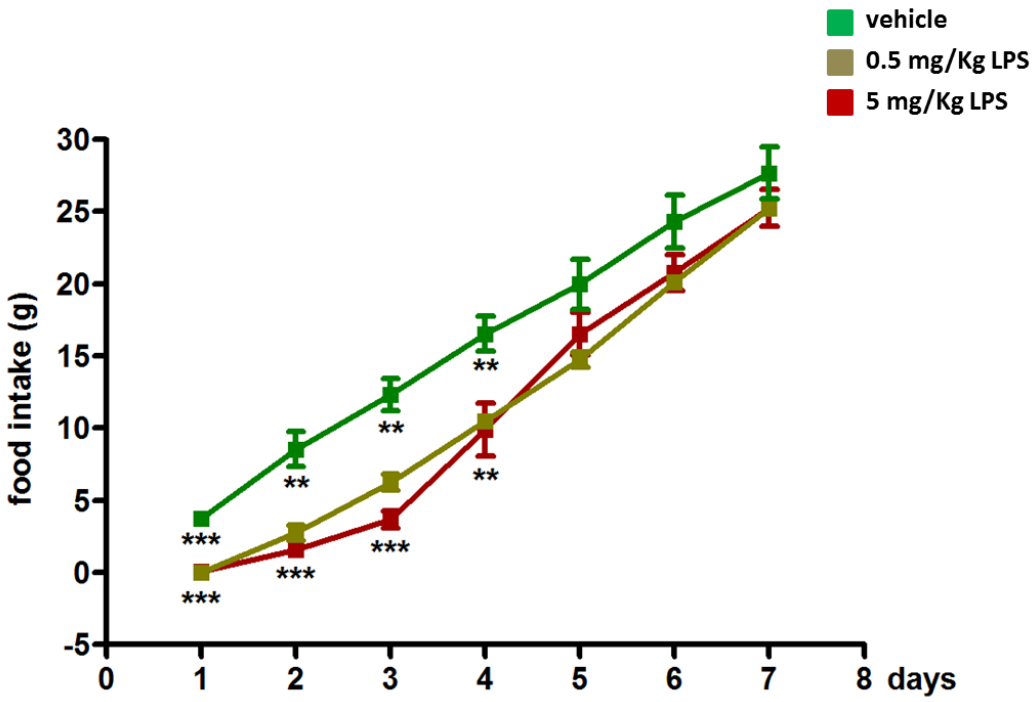


Figure 15: Effect of LPS on food intake in mice. Mice were treated with a single intraperitoneal injection of vehicle (sterile PBS; green line) or two doses of LPS [0.5 mg/Kg (ochre line) or 5 mg/Kg (red line)] and total food intake was monitored daily for 7 days after injection. Data are means ± SEM (n=10 animals/group). **p<0.05 and ***p<0.001 *vs* vehicle. Student's t-test.

4.2 Effect of systemic LPS administration on gene expression profile of major pro-inflammatory molecules in different brain areas.

In order to evaluate the effect of a systemic injection of 0.5 and 5 mg/Kg LPS on brain inflammation, mRNA levels of different inflammation-related cytokines and enzymes, known to be involved in neuroinflammation, were measured. In particular, gene expression of TNF-α, IL-1β, IL-6, IL-18, COX-2 and iNOS were analyzed in frontal cortex, striatum, hippocampus and cerebellum, by real-time RT-PCR (see "Materials and Methods" section for more details). With regard to TNF-α , its mRNA levels were significantly increased in 5 mg/Kg LPS (red lines) *versus* control (green lines) animals within the first 24 hours after LPS treatment in all brain areas analyzed, with the exception of cerebellum, where a significant increase was observed only 2 hours post-treatment. No significant differences in TNF-α mRNA levels were found between 0.5 mg/Kg LPS (ochre lines) and control (green lines) mice (Fig. 16).

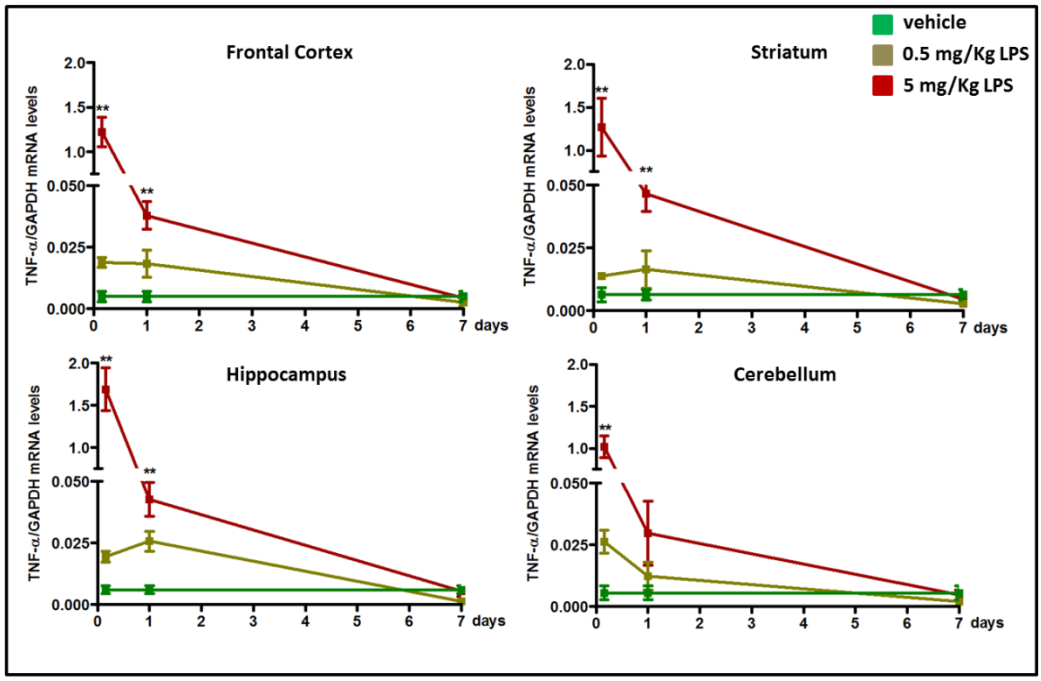


Figure 16: Effect of LPS on TNF-α mRNA levels in different brain areas. Mice were treated with a single i.p. injection of vehicle (sterile PBS; green lines) or two doses of LPS [0.5 mg/Kg (ochre lines) or 5 mg/Kg (red lines)] and mRNA levels of TNF-α were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours, 24 hours and 7 days post-treatment by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). **p<0.01 *vs* vehicle. ANOVA followed by *post-hoc* Dunnett's test.

IL-1β, IL-6 and iNOS mRNA levels (Figs. 17,18,19) increased in all brain areas 2 hours after 5 mg/Kg LPS treatment (red lines), declining to control levels by 24 hours post-treatment. No significant gene expression changes with low dose of LPS (0.5 mg/Kg, ochre lines) were observed.

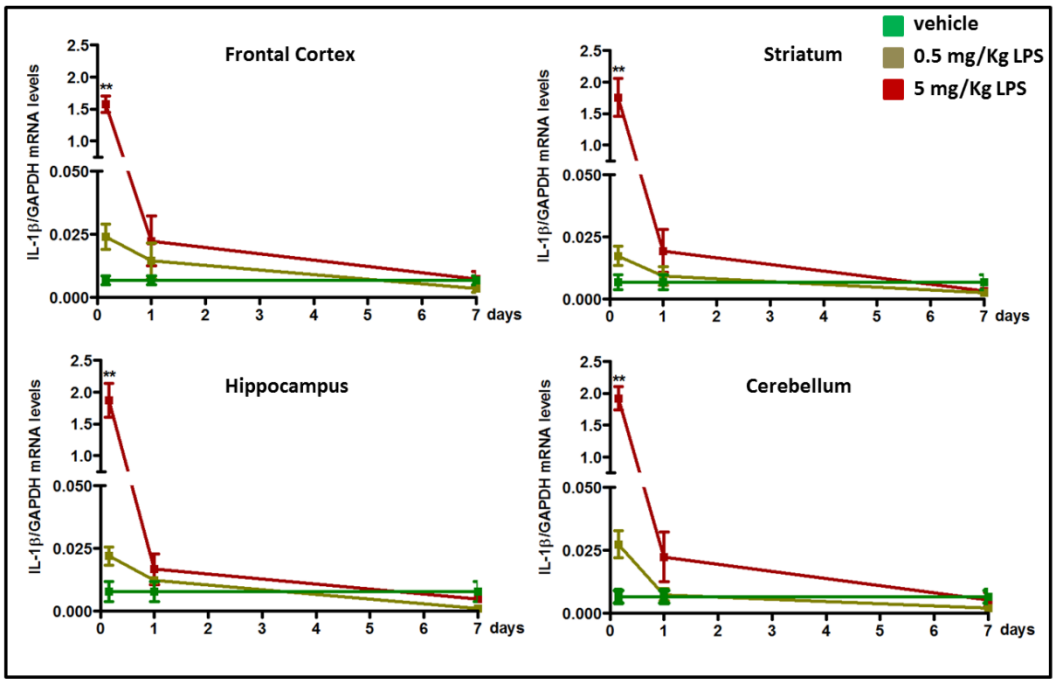


Figure 17: Effect of LPS on IL-1β mRNA levels in different brain areas. Mice were treated with a single i.p. injection of vehicle (sterile PBS; green lines) or two doses of LPS [0.5 mg/Kg (ochre lines) or 5 mg/Kg (red lines)] and mRNA levels of IL-1β were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours, 24 hours and 7 days post-treatment by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). **p<0.01 *vs* vehicle. ANOVA followed by *post-hoc* Dunnett's test.

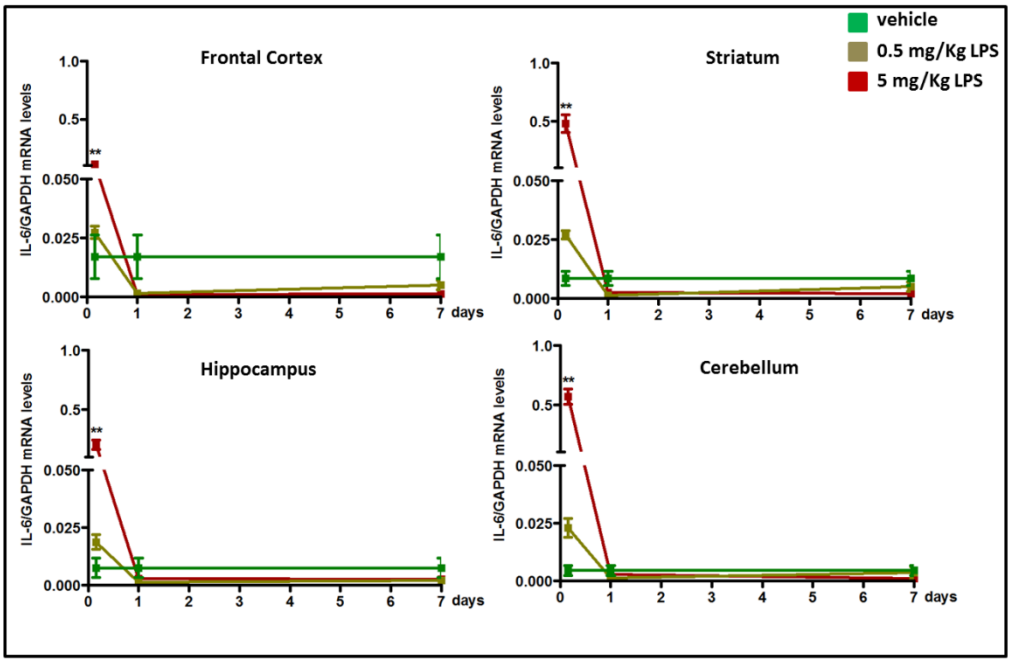


Figure 18: Effect of LPS on IL-6 mRNA levels in different brain areas. Mice were treated with a single i.p. injection of vehicle (sterile PBS; green lines) or two doses of LPS [0.5 mg/Kg (ochre lines) or 5 mg/Kg (red lines)] and mRNA levels of IL-6 were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours, 24 hours and 7 days post-treatment by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). **p<0.01 *vs* vehicle. ANOVA followed by *post-hoc* Dunnett's test.

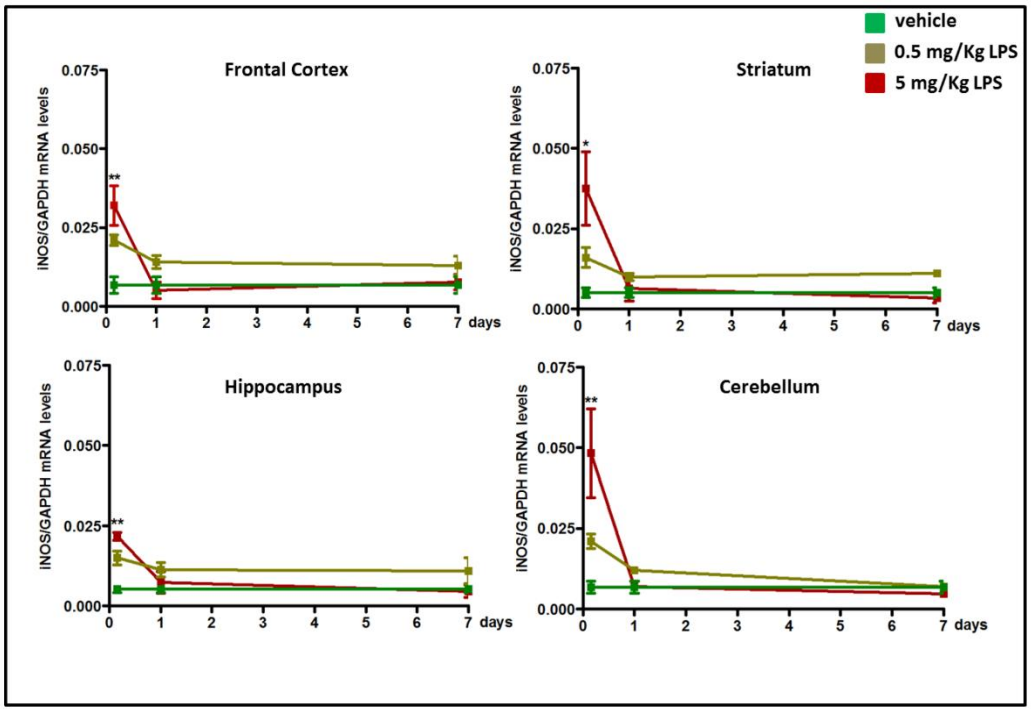


Figure 19: Effect of LPS on iNOS mRNA levels in different brain areas. Mice were treated with a single i.p. injection of vehicle (sterile PBS; green lines) or two doses of LPS [0.5 mg/Kg (ochre lines) or 5 mg/Kg (red lines)] and mRNA levels of iNOS were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours, 24 hours and 7 days post-treatment by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). **p<0.01 *vs* vehicle. ANOVA followed by *post-hoc* Dunnett's test.

Interestingly, unlike the previously analyzed pro-inflammatory molecules, treatment with both LPS doses did not induce any significant change in IL-18 gene expression levels in the brain regions analyzed (Fig. 20).

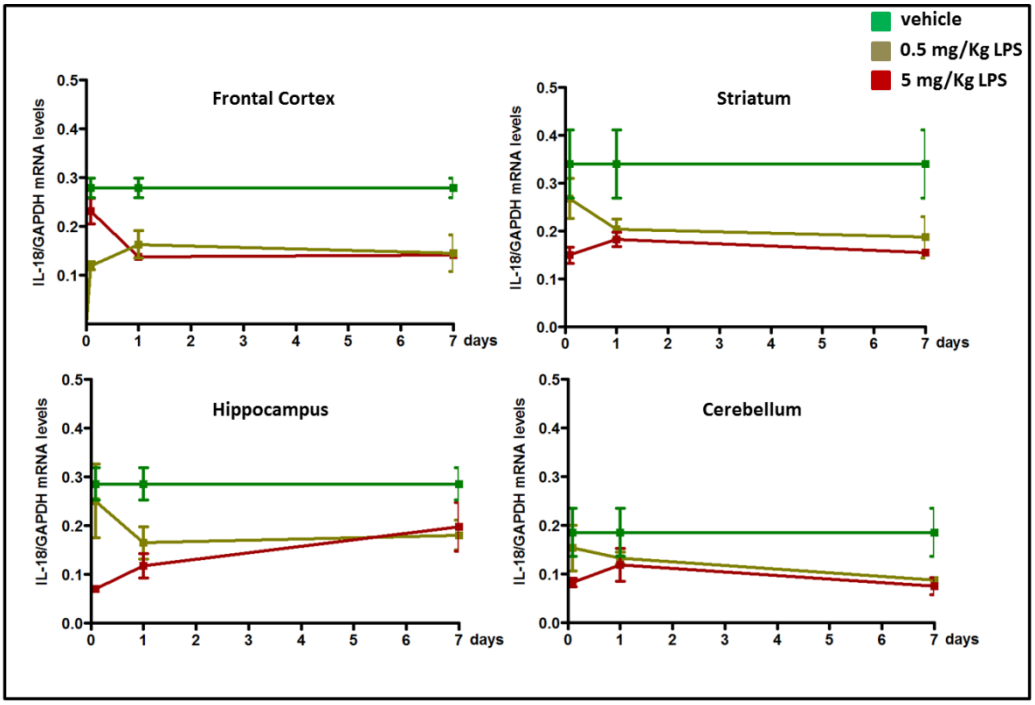


Figure 20: Effect of LPS on IL-18 mRNA levels in different brain areas. Mice were treated with a single i.p. injection of vehicle (sterile PBS; green lines) or two doses of LPS [0.5 mg/Kg (ochre lines) or 5 mg/Kg (red lines)] and mRNA levels of IL-18 were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours, 24 hours and 7 days post-treatment by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). ANOVA followed by *post-hoc* Dunnett's test.

Finally, mRNA levels of COX-2 were evaluated. Both doses of LPS significantly increased COX-2 mRNA levels in frontal cortex, striatum, hippocampus and cerebellum 2 hours after LPS injection. Furthermore, highest LPS dose (5 mg/kg) maintained these levels elevated in striatum and cerebellum up to 24 hours. To note that basal mRNA levels of COX-2 (green lines) were higher in frontal cortex and hippocampus compared to striatum and cerebellum, where LPS effect was evident also 24 hours post-treatment.

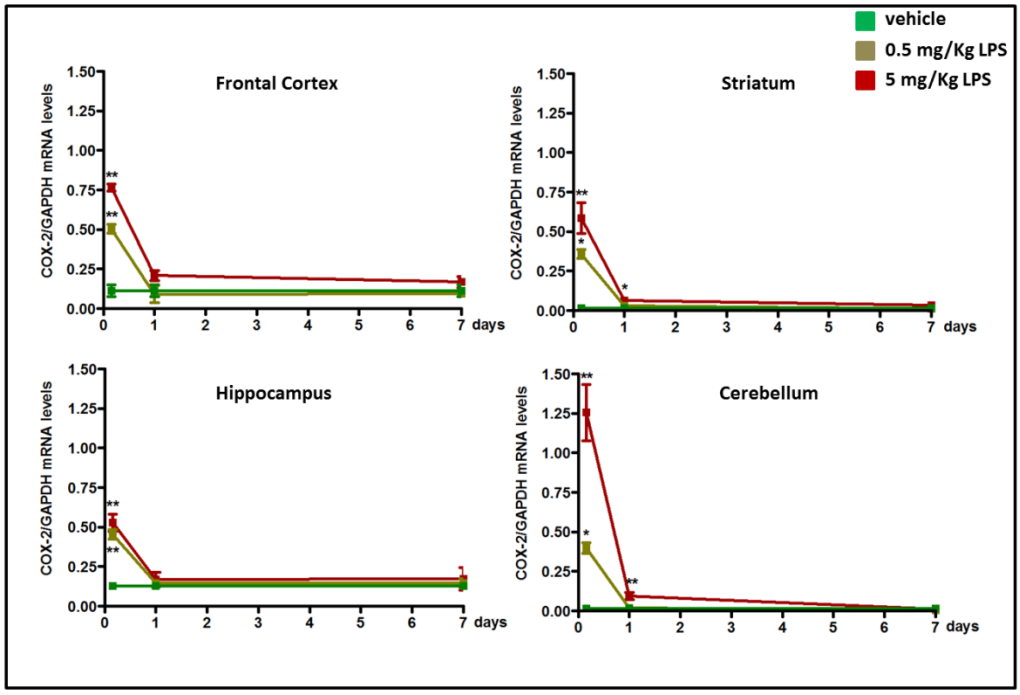


Figure 21: Effect of LPS on COX-2 mRNA levels in different brain areas. Mice were treated with a single i.p. injection of vehicle (sterile PBS; green lines) or two doses of LPS [0.5 mg/Kg (ochre lines) or 5 mg/Kg (red lines)] and mRNA levels of COX-2 were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours, 24 hours and 7 days post-treatment by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). **p<0.01 *vs* vehicle. ANOVA followed by *post-hoc* Dunnett's test.

4.3 Concentration of curcumin in brain and plasma after oral administration.

To verify whether curcumin could reach the CNS, mice were orally treated with 50 mg/Kg curcumin for 2 consecutive days and then i.p. injected with 5 mg/Kg LPS. Two hours after LPS injection, mice were sacrificed and plasma and brains were analyzed by HPLC. Brain levels of curcumin were 1.80 ± 0.50 µg/g and this amount did not differ after LPS stimulation (2.71 ± 1.40 µg/g) (Table 3). Moreover, no metabolites in the brain tissue samples were detected, although some glucuronide metabolites were found in plasma (data not shown).

Table 3. Concentration of curcumin in brain and plasma after oral administration.

	Brain (µg/g)	Plasma (ng/ml)
curcumin	1.80 ± 0.50	1.32 ± 0.17
curcumin + LPS	2.71 ± 1.40	1.26 ± 0.18

Curcumin (50mg/Kg) was administered one hour before LPS (5 mg/Kg) injection. Concentration of curcumin in the brain and plasma was evaluated 2h after LPS treatment. Data are means ± SEM (n=5 animals/group).

4.4 Effect of curcumin on gene expression profile in LPS-induced inflammatory state.

To test possible anti-inflammatory properties of curcumin in the CNS, mice were orally pre-treated with increasing doses (5, 10 and 50 mg/kg) of curcumin before LPS treatment. As shown in Fig. 11, curcumin was administered *via gavage*, with a single daily dose, for 2 consecutive days, before LPS injection to induce an inflammatory response in the CNS. Two hours post-treatment with 5 mg/kg LPS mice were sacrificed and brain areas collected for real-time RT-PCR analysis, as previously described (see "Materials and Methods" section). Low basal levels of TNF-α, IL-1β, IL-6, iNOS and COX-2 gene expression remained unchanged after treatment with increasing doses of curcumin (Figs. 22,23,24,25,26). Conversely, pre-treatment with curcumin, dose-dependently, suppressed LPS-induced increase of mRNA levels of TNF-α, IL-1β and COX-2 in all brain areas (Figs.22,23,26), while limiting expression of IL-6 and iNOS to striatum and cerebellum (Figs.24,25).

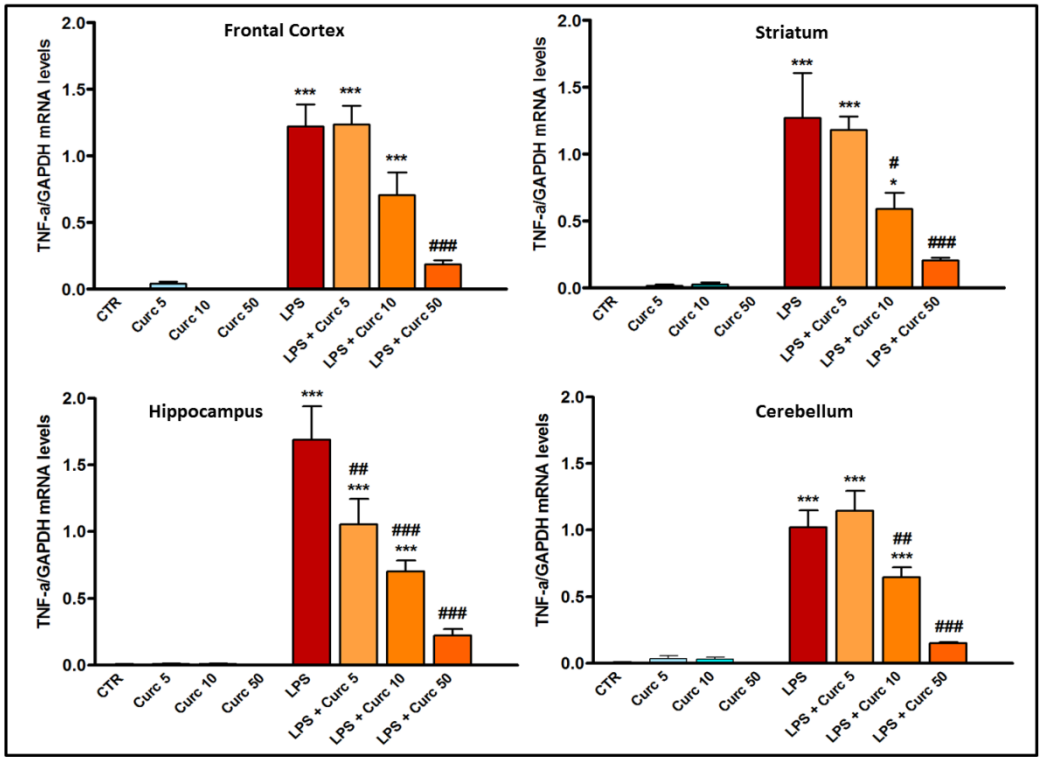


Figure 22: Effect of curcumin on TNF-α mRNA levels in LPS-treated mice. Mice were treated for 2 consecutive days with a single daily dose of vehicle, 5, 10 or 50 mg/kg curcumin before LPS treatment. mRNA levels of TNF-α were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours after treatment with LPS, by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). *p<0.05, ***p<0.001 *vs* vehicle; #p<0.05, ##p<0.01 and ###p<0.001 *vs* LPS-treatment. ANOVA followed by *post-hoc* Tukey's test.

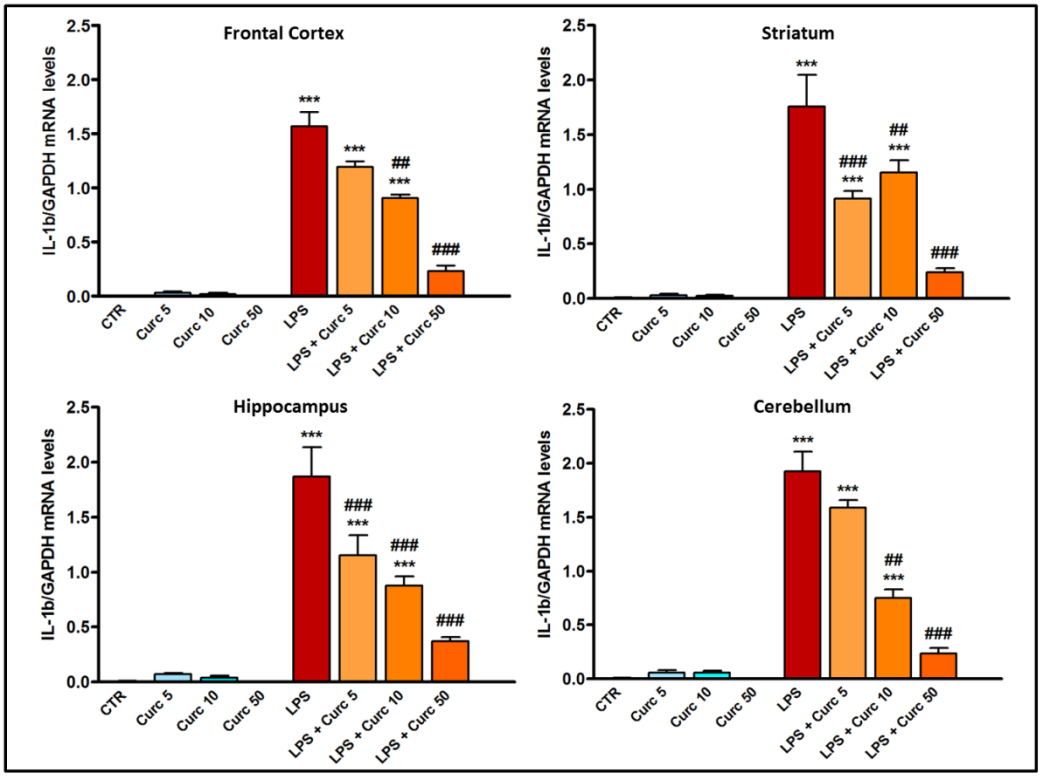


Figure 23: Effect of curcumin on IL-1β mRNA levels in LPS-treated mice. Mice were treated for 2 consecutive days with a single daily dose of vehicle, 5, 10 or 50 mg/kg curcumin before LPS treatment. mRNA levels of IL-1β were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours after treatment with LPS, by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). ***p<0.001 *vs* vehicle; ##p<0.01 and ###p<0.001 *vs* LPS-treatment. ANOVA followed by *post-hoc* Tukey's test.

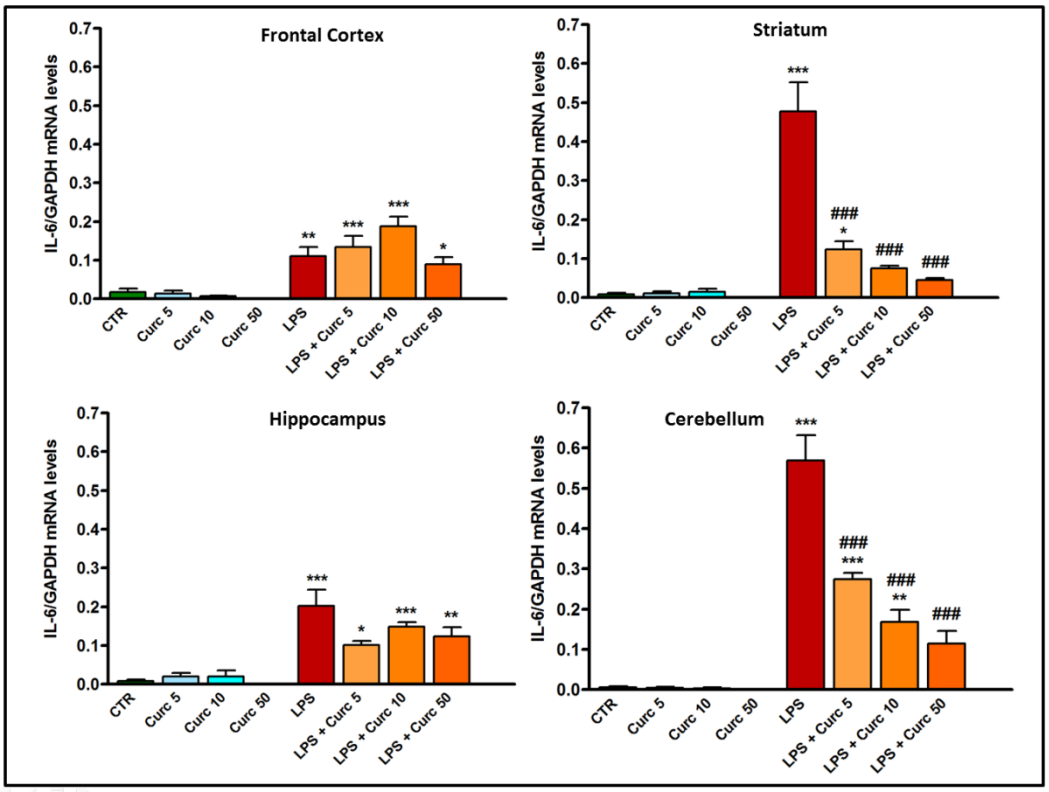


Figure 24: Effect of curcumin on IL-6 mRNA levels in LPS-treated mice. Mice were treated for 2 consecutive days with a single daily dose of vehicle, 5, 10 or 50 mg/kg curcumin before LPS treatment. mRNA levels of IL-6 were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours after treatment with LPS, by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). *p<0.05, **p<0.01 and ***p<0.001 *vs* vehicle; ###p<0.001 *vs* LPS-treatment. ANOVA followed by *post-hoc* Tukey's test.

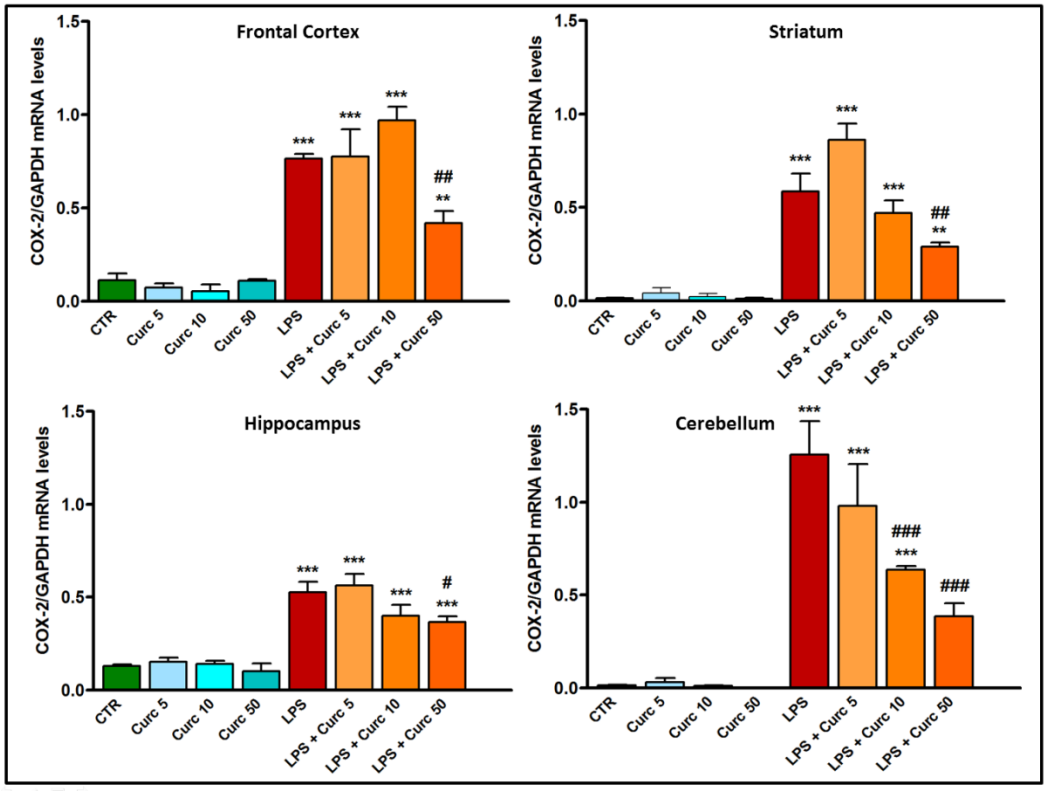


Figure 25: Effect of curcumin on COX-2 mRNA levels in LPS-treated mice. Mice were treated for 2 consecutive days with a single daily dose of vehicle, 5, 10 or 50 mg/kg curcumin before LPS treatment. mRNA levels of COX-2 were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours after treatment with LPS, by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). ***p<0.001 *vs* vehicle; #p<0.05, ##p<0.01 and ###p<0.001 *vs* LPS-treatment. ANOVA followed by *post-hoc* Tukey's test.

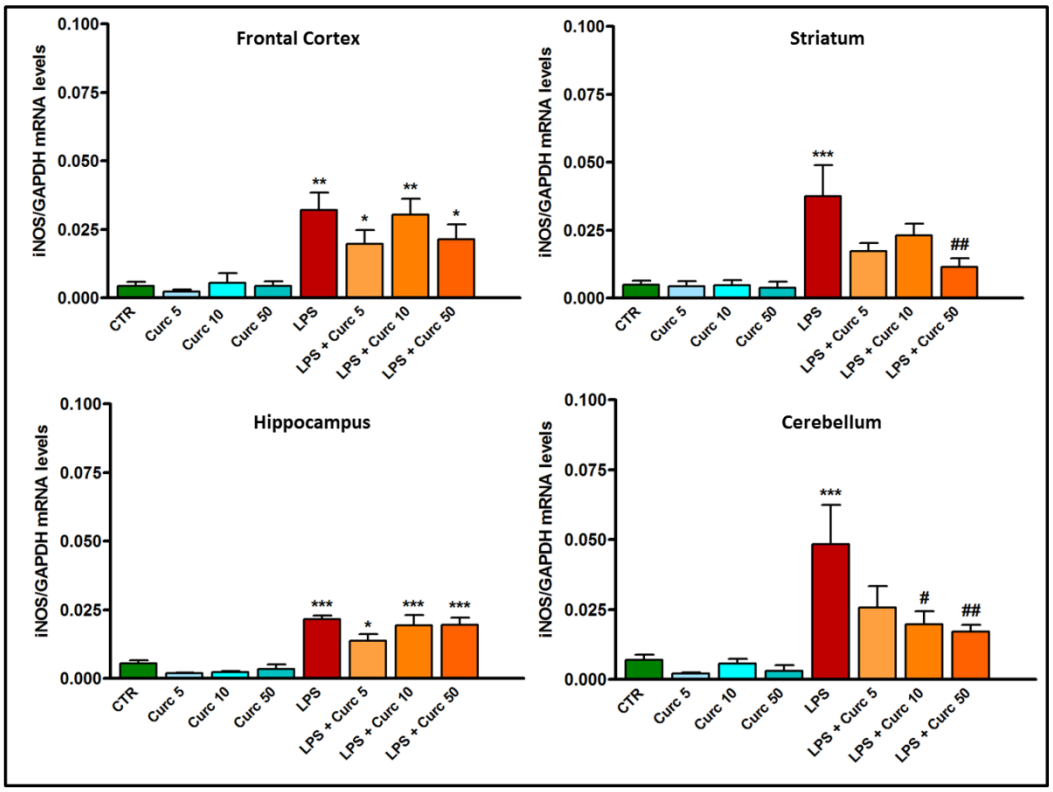


Figure 26: Effect of curcumin on iNOS mRNA levels in LPS-treated mice. Mice were treated for 2 consecutive days with a single daily dose of vehicle, 5, 10 or 50 mg/kg curcumin before LPS treatment. mRNA levels of iNOS were evaluated in frontal cortex, striatum, hippocampus and cerebellum, 2 hours after treatment with LPS, by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). *p<0.05, **p<0.01 and ***p<0.001 *vs* vehicle; #p<0.05 and ##p<0.01 *vs* LPS-treatment. ANOVA followed by *post-hoc* Tukey's test.

4.5 Effect of curcumin on LPS-induced body weight and food intake changes.

Mice were orally pre-treated with 50 mg/Kg curcumin before LPS treatment and body weight and food intake were monitored daily for 7 days after LPS injection. Curcumin completely prevented LPS-induced body weight loss during the first 2 days after treatment. Interestingly, treatment with curcumin alone induced a significant increase in mouse body weight during the 24 hours after treatment (+ 1.63 g ± 0.15 g) (Fig. 27).

Food intake was also monitored daily for 7 days after LPS treatment. Pre-treatment with curcumin completely prevented LPS-induced food intake reduction during the 4 days after treatment (orange line). Starting from day 5 curcumin treatment induced a significant increase in food intake in mice treated with LPS (orange line) compared to control (green line) and LPS-treated group (red line) (Fig. 28).

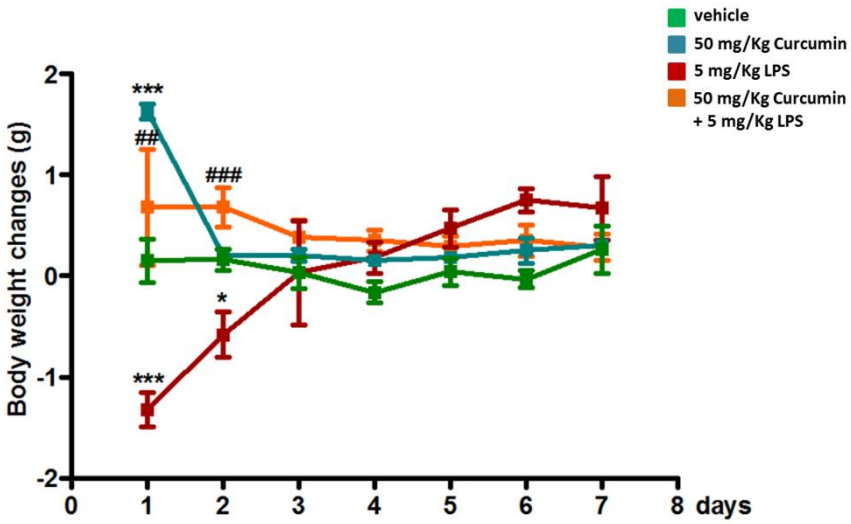


Figure 27: Effect of curcumin on LPS-induced body weight changes. Mice were orally pre-treated with 50 mg/Kg curcumin before treatment with vehicle or 5 mg/Kg LPS and body weight change was monitored daily for 7 days after LPS injection. Data are means ± SEM (n=10 animals/group). *p<0.05, ***p<0.001 *vs* vehicle. $^{\#\#}$p<0.01 and $^{\#\#\#}$p<0.001 *vs* LPS. Student's t-test.

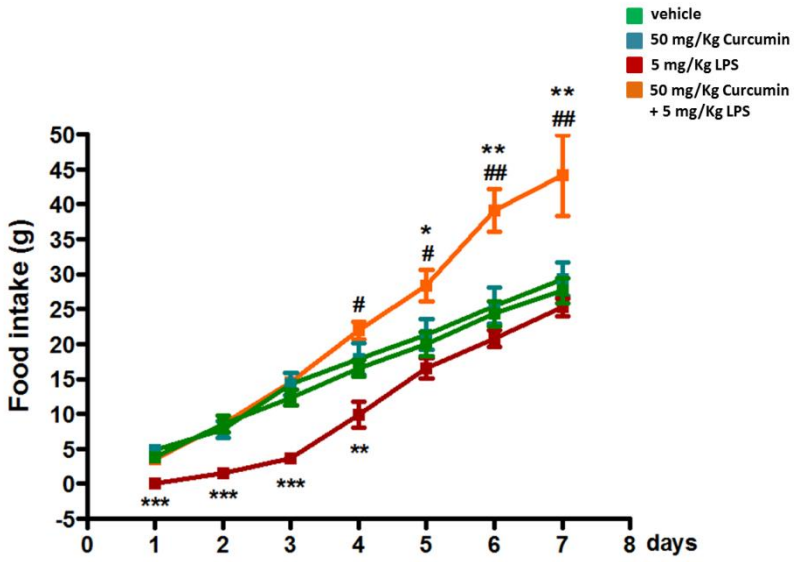


Figure 28: Effect of curcumin on LPS-induced food intake changes. Mice were orally pre-treated with 50 mg/Kg curcumin before treatment with vehicle or 5 mg/Kg LPS and food intake was monitored daily for 7 days after LPS injection. Data are means ± SEM (n=10 animals/group). *p<0.05, **p<0.01 *vs* vehicle. $^{\#}$p<0.05 and $^{\#\#}$p<0.01 *vs* LPS. Student's t-test.

4.6 Effect of LPS and curcumin on hypothalamic proopiomelanocortin (POMC) gene expression.

Based on the observed effects of LPS and curcumin pre-treatment on body weight and food intake changes, a preliminary analysis on their effects on the hypothalamic expression of the feeding-related neuropeptide POMC [Millington, 2007] was performed. As shown in Fig. 29, the mRNA expression of the anorexigenic gene POMC was significantly increased by LPS 2 hours after injection, while pre-treatment with curcumin decreased LPS-induced increase of gene expression. However, a direct effect of curcumin on POMC gene expression was not observed. Furthermore, no significant differences in POMC mRNA levels were found 24 hours and 7 days after LPS-treatment, both in the absence and presence of curcumin pre-treatment (data not shown).

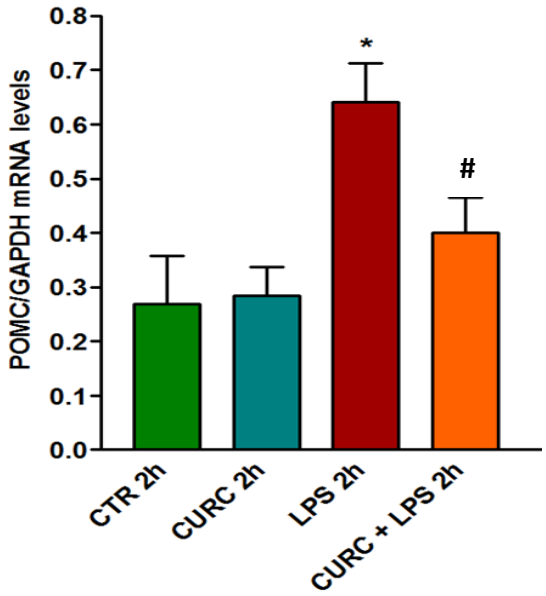


Figure 29: Effect of LPS and curcumin on POMC mRNA levels in the hypothalamus. Mice were orally pre-treated 50 mg/Kg curcumin before treatment with vehicle or a single dose of LPS And mRNA levels of POMC were evaluated in the hypothalamus 2 hours post-treatment, by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). *p<0.05 *vs* vehicle; #p<0.05 *vs* LPS treatment. ANOVA followed by *post-hoc* Tukey's test.

4.7 Effect of curcumin on TNF-α protein expression in LPS-induced inflammatory state.

To further support the anti-inflammatory effect of curcumin on LPS-induced neuroinflammation, TNF-α protein levels were measured in different brain areas. Mice were treated with vehicle or 50 mg/kg LPS before LPS injection (5 mg/kg), brain areas were collected 2 hours, 24 hours and 7 days after systemic LPS administration and TNF-α content was determined by ELISA. Basal protein expression of TNF-α was not modified by curcumin treatment in all brain areas analyzed (orange bars in Fig. 30). Two hours post-treatment, LPS induced a significant increase in TNF-α protein expression in all areas analyzed with the exception of striatum. Moreover, TNF-α levels remained significantly higher up to 7 days in frontal cortex. Pre-treatment with curcumin decreased to control values the LPS-induced increase of TNF-α protein expression (Fig. 30).

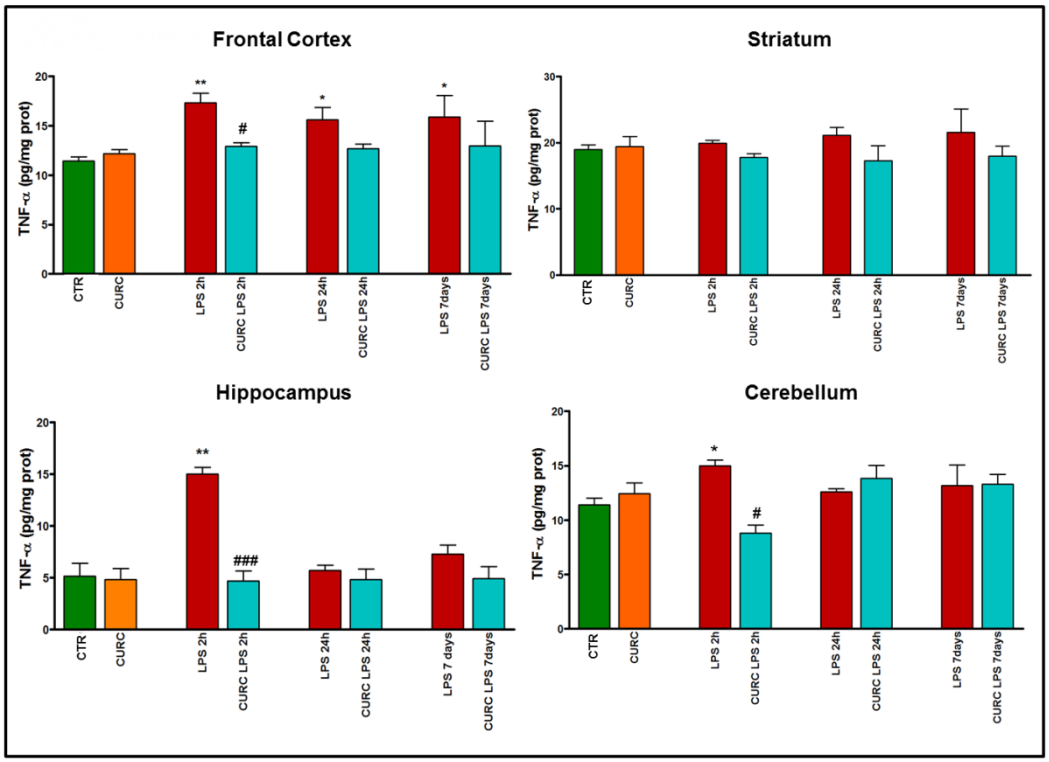


Figure 30: Effect of curcumin on TNF-α protein levels in in LPS-treated mice. Mice were treated for 2 consecutive days with a single daily dose of vehicle or 50 mg/kg curcumin before LPS treatment. Protein expression of TNF-α was evaluated in frontal cortex, striatum, hippocampus and cerebellum 2 hour, 24 hours and 7 days after treatment with LPS, by ELISA. Data are means ± SEM (n=6 animals/group). *p<0.05, **p<0.01 *vs* vehicle; $^{\#}$p<0.05 and $^{\#\#\#}$p<0.001 *vs* LPS-treatment. ANOVA followed by *post-hoc* Tukey's test.

4.8 Effect of curcumin on LPS-induced microglia morphological changes.

Since activation of microglia is accompanied by morphological changes [Nimmerjahn *et al.*, 2005], immunohistochemical analysis was performed to evaluate the possible effect of curcumin on microglial morphology. Two hours after LPS treatment, Iba1 staining in frontal cortex showed an increased cellular density and cells characterized by a morphology typical of primed and reactive microglia: presence of enlarged and less round cell body and thicker and more ramified ramifications (middle panel in Fig. 31). Curcumin pre-treatment completely prevented LPS-induced morphological changes in microglia and Iba1 staining showed cells characterized by a small cell body and many thin

ramifications (lower panel in Fig. 31), similar to that of the control mice (upper panel in Fig. 31)

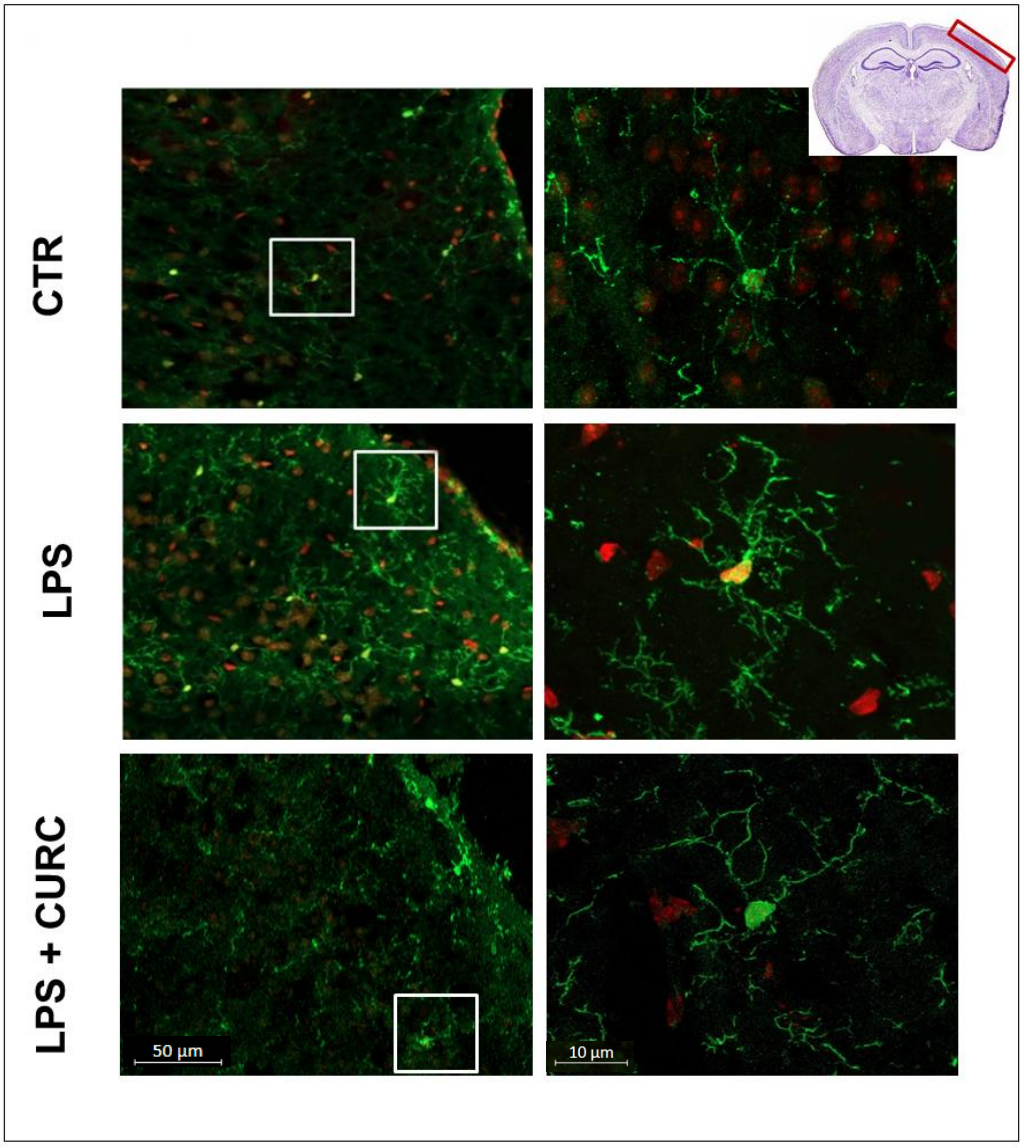


Figure 31: Effect of curcumin on microglial cell morphology in cerebral cortex. Mice were treated for 2 consecutive days with a single daily dose of vehicle or 50 mg/kg curcumin before LPS treatment (5 mg/Kg) and sacrificed 2 hours after LPS injection. Brain sections were immunostained with Iba1 specific microglial marker and counterstained with DAPI to visualize cell nuclei. Experiments were performed 4 times and representative confocal images are shown.

4.9 Effect of curcumin on locomotor activity in LPS-treated mice.

To evaluate the effects of LPS and curcumin on spontaneous behavior activity, mice pre-treated with curcumin were i.p. injected with vehicle or LPS and locomotor activity was assessed by LMA test as described in "Materials and Methods" 24 hours and 7 days after LPS injection. Twenty-four hours after LPS treatment, mice showed a marked decrease of total distance travelled in the arena (38.55 ± 12.01 m), compared to control mice (124.26 ± 22.30 m) (Fig. 32A). This effect was evident until 7 days post-treatment (163.32 ± 12.94 m, control mice and 104.63 ± 16.34 m, LPS-treated mice) (Fig. 32B). Pre-treatment with curcumin (50 mg/Kg) abolished the inhibitory effect of LPS on locomotor activity (112.19 ± 22.91 m after 24 hours and 127.99 ± 25.49 m 7 days after LPS injection) (Fig. 32A and B).

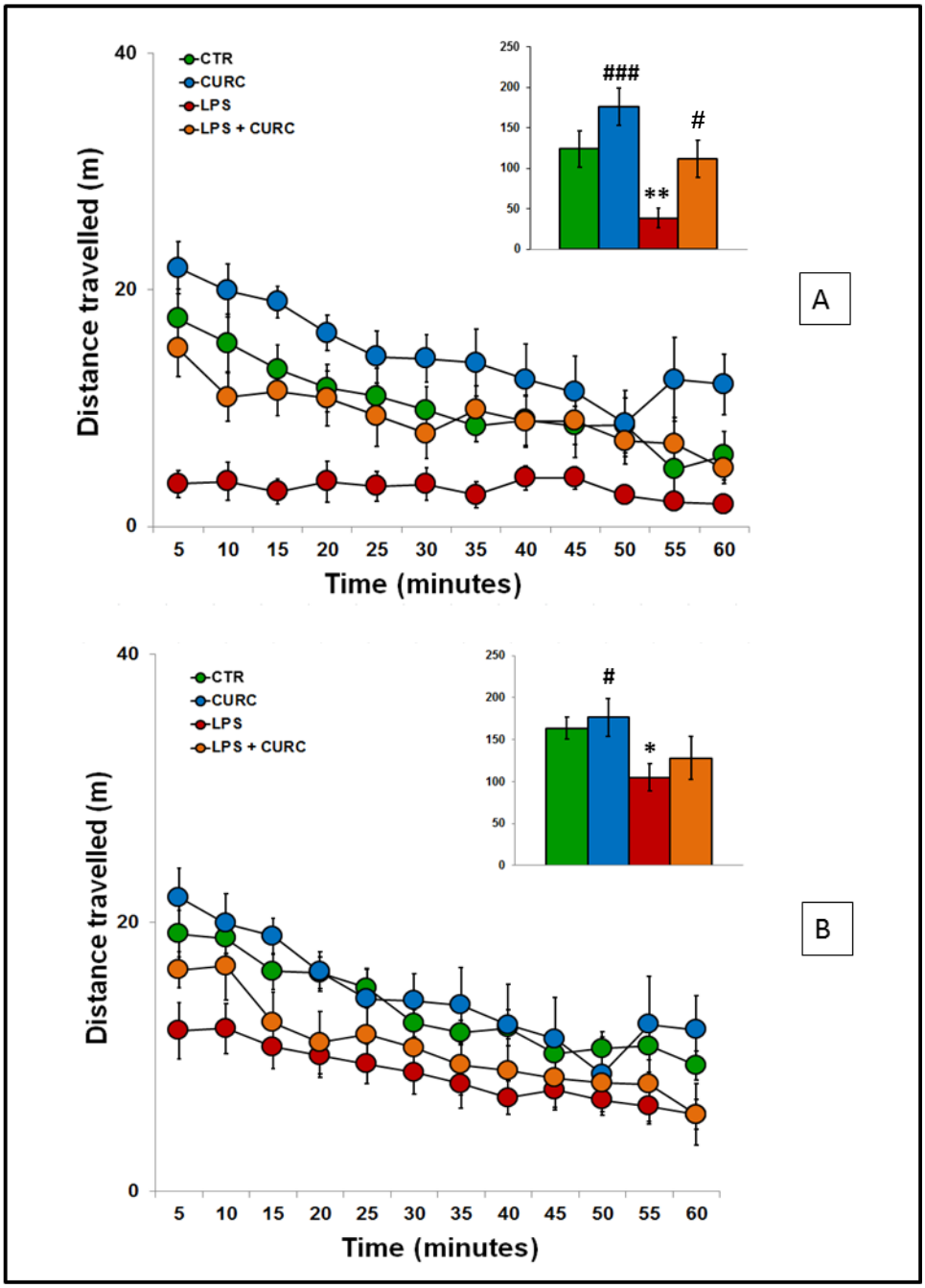


Figure 32: Effect of curcumin on locomotor activity in LPS-treated mice. Mice were orally pre-treated with 50 mg/Kg curcumin before treatment with vehicle or 5 mg/Kg LPS and spontaneous activity was assessed by LMA test 24 hours (**A**) and 7 days (**B**) after LPS injection. Data are means ± SEM (n = 12 animals/group). *p <0.05, **p <0.01 *vs* vehicle; $^{\#}$p <0.05, $^{\#\#\#}$p <0.001 *vs* LPS treatment. Student's t test.

4.10 Effect of curcumin on dopaminergic neurons in the *substantia nigra* and ventral tegmental area (VTA) of LPS-treated mice.

To investigate possible neuroprotective effect of curcumin on the dopaminergic neurons of *substantia nigra* and ventral tegmental area, mice were treated as described and damage of the dopaminergic neurons were observed by using tyrosine-hydroxylase (TH) staining 7 days after LPS treatment. Fig. 33 showed that 7 days after injection, LPS induced a slight reduction in the number of TH-labeled neurons (middle panel), while pre-treatment with curcumin protected from dopaminergic neuronal cell loss (lower panel).

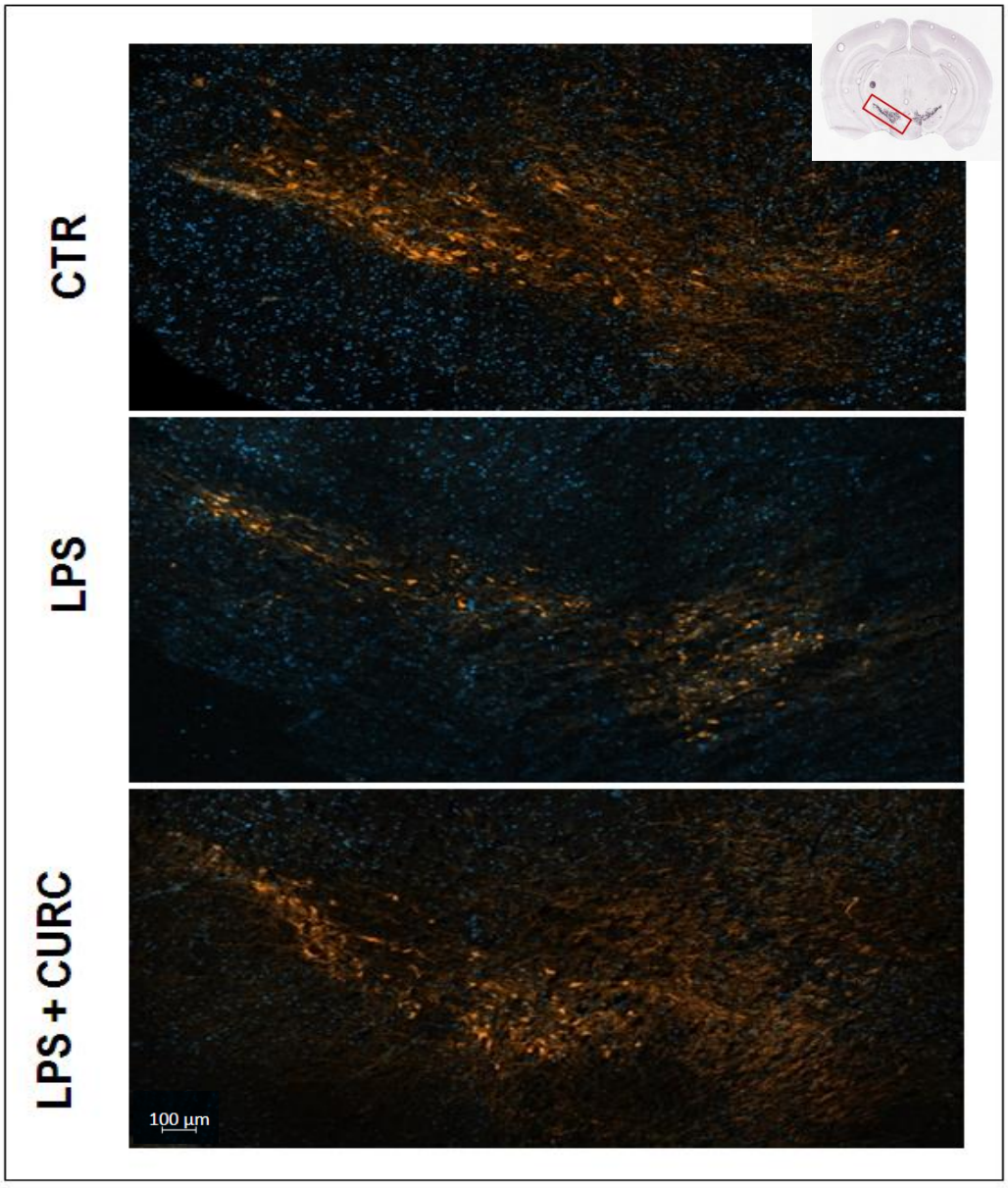


Figure 33: Effect of curcumin on dopaminergic neurons in the *substantia nigra* and VTA of LPS-treated mice. Mice were treated for 2 consecutive days with a single daily dose of vehicle or 50 mg/kg curcumin before LPS treatment (5 mg/Kg) and sacrificed 7 days after LPS injection. Brain sections were immunostained with anti-TH antibody and counterstained with DAPI to visualize cell nuclei. Experiments were performed 3 times and representative confocal images are shown.

4.11 Effect of systemic LPS administration on memory function.

To evaluate the effect of LPS on recognition memory, mice systemically treated with LPS were subjected to NOR test as described in "Materials and Methods" 7 days and 1 month after LPS injection. LPS decreased the preference index compared to the control group at both time points (Fig. 34), indicating a memory impairment in LPS-treated animals.

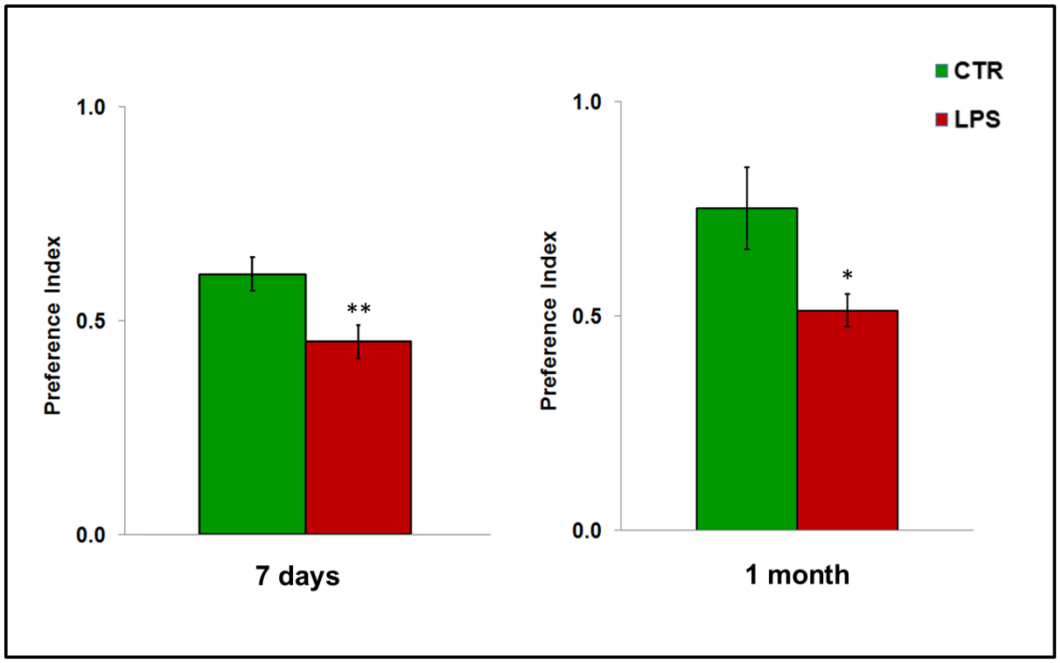


Figure 34: Effect of systemic LPS administration on memory. Mice were treated with 5mg/Kg LPS and recognition memory was assessed 7 days and 1 month post-treatment. Data are means ± SEM (n=12 animals/group). *p<0.05, **p<0.01*vs* vehicle; Student's *t* test.

4.12 Effect of curcumin on BDNF mRNA levels in LPS-treated mice.

To investigate possible effect of curcumin on BDNF mRNA levels in the LPS-induced inflammatory state, mice were treated with curcumin and LPS, sacrificed 2 hours, 24 hours and 7 days post-treatment and brain areas collected for real-time RT-PCR analysis, as previously described (see "Materials and Methods" section). Basal levels of BDNF gene expression was not affected by curcumin treatment. Conversely, LPS treatment significantly reduced BDNF gene expression starting from 2 hours after treatment in all brain areas analysed, with the exception of striatum, where low levels of BDNF mRNA were found. Pre-treatment with 50

mg/kg curcumin restored BDNF mRNA levels to control values 24 hours after LPS injection (Fig. 35). Interestingly, a higher dose of curcumin (100 mg/Kg) significantly recovered LPS-induced decrease of BDNF gene expression to control values 2 hours after LPS injection, showing a dose- and time-dependent effect of curcumin (data not shown).

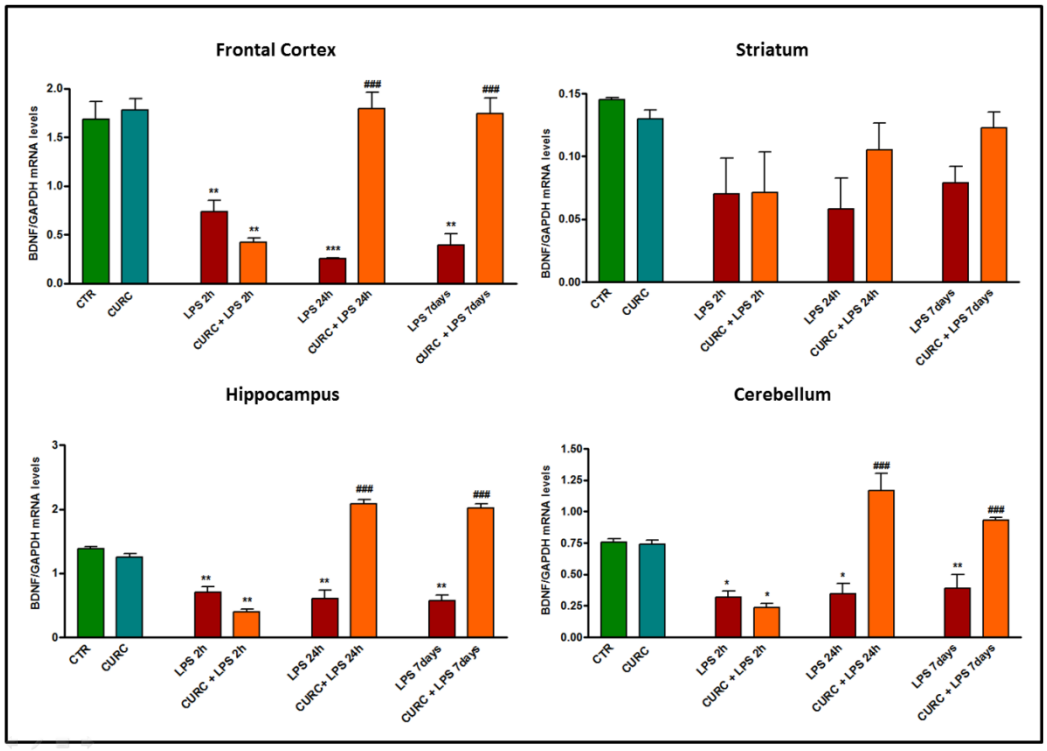


Figure 35: Effect of curcumin on BDNF mRNA levels in different brain areas. Mice were treated for 2 consecutive days with a single daily dose of 50 mg/Kg curcumin before LPS treatment. mRNA levels of BDNF were evaluated 2 hours, 24 hours and 7 days after treatment with LPS in frontal cortex, striatum, hippocampus and cerebellum, by real-time RT-PCR. Data are means ± SEM (n=6 animals/group). *p<0.05, **p<0.01, ***p<0.001 *vs* vehicle; $^{###}$p<0.001 *vs* LPS treatment. ANOVA followed by *post-hoc* Tukey's test.

5. Discussion

Innate immune response is emerging as a crucial component of major neurodegenerative diseases. Microglia are resident immune cells in the CNS that can be activated in response to injurious stimuli, infection, misfolded proteins or aberrantly localized nucleic acids, contributing to neuroinflammation. Activated microglia secrete pro-inflammatory mediators, that aggravate and propagate neuroinflammation contributing to neuronal cell damage and loss and impaired brain functions in both animal models and human patients [Heneka *et al*., 2014, Réus *et al*., 2015]. Therefore, inhibition of neuroinflammation and microglial activation may represent valuable strategic targets for therapeutic intervention in neurological disorders associated with inflammation. In recent years, research has focused on the study of anti-inflammatory agents in *in vivo* experimental models of neuroinflammation.

The *in vivo* mouse model used in this study has been realized through a single intraperitoneal injection of LPS (5 mg/kg) in adult male mice. This model is widely used for studying neuroinflammatory processes in rodents and its use is supported by the knowledge that an acute inflammatory condition is essential for the occurrence of a state of chronic inflammation [Medzhitov, 2010]. Moreover, the 5 mg/Kg dose of LPS is capable of inducing neurodegeneration, without causing animals death [Liya *et al*., 2007]. A comparison between two doses of LPS (0.5 and 5 mg/Kg), in terms of gene expression changes of pro-inflammatory molecules (TNF-α, IL-1β, IL-6, IL-18, COX-2 and iNOS) was performed to verify if the lower dose also induced neuroinflammation and subsequent neurodegeneration. High dose of LPS induced a significantly increase in gene expression of almost all the inflammatory molecules analyzed, 2 hours after injection. IL-18 mRNA levels were not modified after LPS treatment, probably because this cytokine follows different kinetics of activation that other members of IL-1-family [Felderhoff-Mueser *et al*., 2005]. Conversely, low dose of LPS (0.5 mg/kg) did not induce any significantly increase in gene expression of inflammatory molecules, although it was able to induce a state of sickness behavior, characterized by a reduction in body weight and food intake, comparable to the higher dose of LPS.

After molecular characterization of the *in vivo* model of neuroinflammation, the anti-inflammatory and possible neuroprotective effect of curcumin was tested. Extensive research and clinical trials over the past half century have shown that curcumin can modulate multiple cell signalling pathways involved in neuroinflammation. Anti-inflammatory properties of curcumin are linked to numerous molecular mechanisms [He *et al*., 2015], involving also its ability to antagonize the binding of LPS with its binding site in the TLR4. Binding of LPS activates the inflammatory intracellular signalling, leading to activation of transcription factor NF-κB, which controls the final immune response through the synthesis of inflammatory molecules [Lu *et al*., 2008].

A treatment protocol was first developed in order to optimize the effects of curcumin in mice. Since this molecule is poorly water soluble, it was administered using a daily administration, for 2 consecutive days, *via gavage* in a 1% methylcellulose suspension, leaving the animals fasted overnight in order to increase curcumin absorption [Begum *et al*., 2008]. Given the low bioavailability of curcumin, initially we verified whether this molecule could cross the BBB to enter brain tissue. HPLC-MS analysis showed that curcumin entered unmodified brain tissue 3 hours after oral administration, reaching clinically relevant concentrations (1.80 ± 0.50 µg/g). Conversely, 3 hours after administration, plasma concentration of curcumin was very low (1.32 ± 0.17 ng/ml); furthermore, some glucuronide metabolites were detected as biotransformation products in plasma samples of curcumin, confirming previously published studies [Anand *et al*., 2007].Then, a dose-response study was performed administering three doses of curcumin (5, 10 and 50 mg/kg) that were tested for their anti-inflammatory properties, in terms of prevention of pro-inflammatory gene expression increase induced by LPS. Only 50 mg/Kg curcumin significantly reduced the LPS-induced increase of mRNA levels of several pro-inflammatory molecules (TNF-α, IL-1β, IL-6 and COX-2) in different brain areas (cortex, striatum, hippocampus and cerebellum). This dose of curcumin is widely used in the scientific literature; in particular, a mouse daily dose of 50 mg/Kg curcumin corresponds to 3.5 g/day for a man weighing 70 Kg, which is considered a safe dose, because dose-escalating

studies have indicated the safety of curcumin at dose as high as 12 g/day over 3 months [Chainani-Wu , 2003].

An important aspect of neuroinflammatory response is the activation of microglial cells that can assume different phenotypes depending on the type of stimulation. Microglial activation consists in morphological changes, followed by functional changes, characterized by increased expression of membrane receptors and by the release of pro-inflammatory molecules, which support the inflammatory process [Melinda *et al.*, 2010]. Curcumin pre-treatment completely prevented LPS-induced microglial morphological changes, as demonstrated by immunohistochemical analysis, showing cells characterized by a small cell body and many thin ramifications, similar to that of the control mice.

An excessive neuroinflammatory response can alter brain functions and lead to brain pathologies. Many studies have suggested the onset of motor and memory deficits induced by a neuroinflammatory state [Robert *et al*., 2008]. In this study, a locomotor activity test was carried out to assess the effect of curcumin on LPS-induced motor deficit. LPS-treated mice showed a significant decrease in total distance traveled 24 hours and 7 days after injection, while pre-treatment with curcumin significantly prevented LPS-induced motor deficits. Several studies suggest that motor deficits induced by LPS can be related to an altered functionality of voluntary movement control centers, such as basal ganglia, which use dopaminergic and glutamatergic neurons as principal connections [Martyn, 2009]. Moreover, dopaminergic neurons are very sensitive to cerebral microenvironment and easily degenerate in response to traumas or inflammatory processes [Drui, 2014]. To test the effect of neuroinflammation on neuronal survival, a TH-immunohistochemistry of dopaminergic neurons within the *substantia nigra pars compacta* and ventral tegmental area 7 days after LPS injection was performed. LPS injection decreased the number of dopaminergic neurons in the *substantia nigra pars compacta* and ventral tegmental area, which could be related to the observed motor dysfunction in LMA paradigm. Further studies evaluating the effect of neuroinflammation in other neuronal cell types involved in motor control are needed to confirm this relationship. Interestingly, curcumin treatment prevented dopaminergic neuronal cell loss, suggesting a

possible neuroprotective effect of this molecule that can be used in the treatment of brain pathologies associated with motor deficits, such as Parkinson disease.

Molecular mechanisms underlying neuroprotective effect of curcumin are multiple and may involve several neurotrophic factors, including BDNF, especially in terms of synaptic plasticity [Calabrese *et al.,* 2014]. The BDNF synthesis occurs ubiquitously in the brain, with the exception of some areas, such as striatum where BDNF is anterogradely transported from cell bodies located in the cerebral cortex, *substantia nigra*, amygdala and thalamus [Baydyuk and Xu, 2014]. LPS induced a significant reduction of BDNF gene expression levels up to 7 days after treatment, in cerebral cortex, hippocampus and cerebellum, whereas in striatum mRNA of BDNF appeared to be absent. Pre-treatment with curcumin restored BDNF mRNA expression to control values, in a dose- and time-dependent manner, suggesting a possible neuroprotective role of curcumin. To further confirm these results BDNF protein levels should be measured.

Finally, based on previous observations showing that "sickness behavior" induced by LPS includes alterations in central processes involved in learning and memory [Song *et al.*, 2013], the presence of possible memory deficits in the LPS-induced inflammatory state was evaluated. To this aim, a behavioral test (novel object recognition) which discriminates the differences in the exploration time of novel and familiar objects [Antunes and Biala, 2012] was used. Our preliminary observations showed a reduction in the ability to discriminate novel objects, induced by LPS, suggesting a link between neuroinflammation and the onset of mnesic deficits. Further studies will be needed to study the possible protective effect of curcumin.

6. Conclusions

The results reported in this thesis show the importance of curcumin as an *in vivo* anti-inflammatory agent, able to modulate microglial activation and CNS inflammatory processes.

In particular, curcumin protected against LPS-induced:

- gene expression increase of TNF-α, IL-1β, IL-6 and COX-2 in different brain areas;
- increase of TNF-α protein levels;
- microglial morphological "activation";
- behavioral symptoms of sickness, such as reduction of body weight and food intake;
- mouse motor deficits;
- dopaminergic neuron loss in *substantia nigra pars compacta* and ventral tegmental area;
- gene expression changes of BDNF in different brain areas.

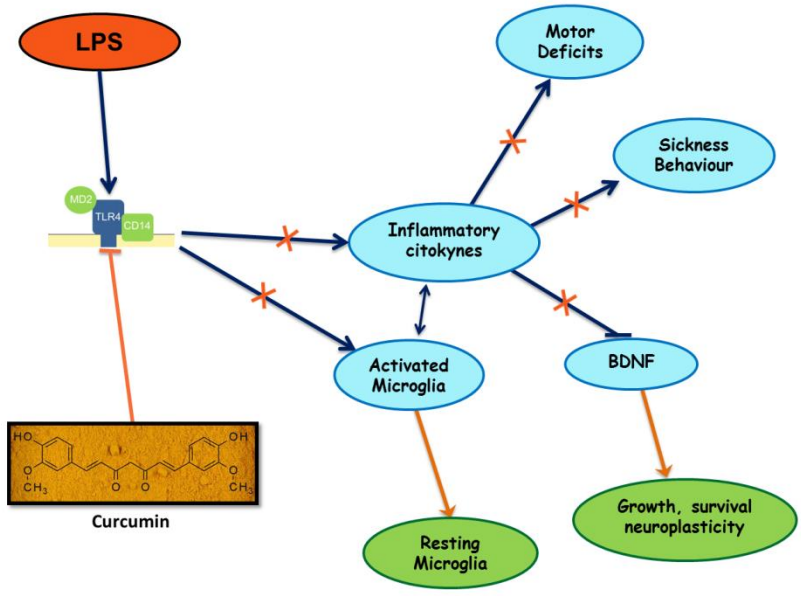


Figure 36: Anti-inflammatory and neuroprotective properties of curcumin.

Taken together, these data support the idea that pharmacological strategies aimed at decreasing neuroinflammation associated with microglial activation are important for improving recovery from sickness and reducing neurobehavioral deficits.

These results may also provide useful information to develop novel therapeutic approaches for neurodegenerative diseases, that represent a growing unmet medical need, given the increasing shift in population dynamics and longevity. Current treatment at best represent a symptomatic approach, while disease-modifying therapeutics remain to be successfully developed. More work will be needed to establish the relevance of curcumin anti-inflammatory effects observed in animal models to its clinical effects and to elucidate the precise molecular mechanisms underlying the anti-inflammatory and neuroprotective effects of this natural molecule. A more complete understanding of these issues would promote the design of novel curcumin derivatives with improved availability and therapeutic efficacy, for the management of neurological diseases associated with inflammation.

7. References

Abbott, N.J., Patabendige, A.A.K., Dolman, D.E.M., Yusof, S.R., Begley, D.J., 2010. Structure and function of the blood-brain barrier. Neurobiol. Dis. 37, 13–25.

Aggarwal, B.B., Deb, L., Prasad, S., 2014. Curcumin differs from tetrahydrocurcumin for molecular targets, signalling pathways and cellular responses. Molecules 20, 185–205.

Aggarwal, B.B., Sundaram, C., Malani, N., Ichikawa, H., 2007. Curcumin: the Indian solid gold. Adv. Exp. Med. Biol. 595, 1–75.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.

Allan, S.M., Rothwell, N.J., 2001. Cytokines and acute neurodegeneration. Nat. Rev. Neurosci. 2, 734–744.

Allan, S.M., Tyrrell, P.J., Rothwell, N.J., 2005. Interleukin-1 and neuronal injury. Nat. Rev. Immunol. 5, 629–640.

Anand, P., Kunnumakkara, A.B., Newman, R.A., Aggarwal, B.B., 2007. Bioavailability of curcumin: problems and promises. Mol. Pharm. 4, 807–818.

Anand, P., Thomas, S.G., Kunnumakkara, A.B., Sundaram, C., Harikumar, K.B., Sung, B., Tharakan, S.T., Misra, K., Priyadarsini, I.K., Rajasekharan, K.N., Aggarwal, B.B., 2008. Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. Biochem. Pharmacol. 76, 1590–1611.

Antunes, M., Biala, G., 2012. The novel object recognition memory: neurobiology, test procedure, and its modifications. Cogn Process 13, 93–110.

Bardehle, S., Krüger, M., Buggenthin, F., Schwausch, J., Ninkovic, J., Clevers, H., Snippert, H.J., Theis, F.J., Meyer-Luehmann, M., Bechmann, I., Dimou, L., Götz, M., 2013. Live imaging of astrocyte responses to acute injury reveals selective juxtavascular proliferation. Nat. Neurosci. 16, 580–586.

Barton, G.M., 2008. A calculated response: control of inflammation by the innate immune system. J. Clin. Invest. 118, 413–420.

Baydyuk, M., Xu, B., 2014. BDNF signalling and survival of striatal neurons. Front Cell Neurosci. 8, 254.

Begum, A.N., Jones, M.R., Lim, G.P., Morihara, T., Kim, P., Heath, D.D., Rock, C.L., Pruitt, M.A., Yang, F., Hudspeth, B., Hu, S., Faull, K.F., Teter, B., Cole, G.M., Frautschy, S.A., 2008. Curcumin structure-function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease. J Pharmacol Exp Ther. 326(1),196-208.

Bianchi, M.E., 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. J. Leukoc. Biol. 81, 1–5.

Biber, K., Neumann, H., Inoue, K., Boddeke, H.W.G.M., 2007. Neuronal “On” and “Off” signals control microglia. Trends Neurosci. 30, 596–602.

Blach-Olszewska, Z., Leszek, J., 2007. Mechanisms of over-activated innate immune system regulation in autoimmune and neurodegenerative disorders. Neuropsychiatr. Dis. Treat. 3, 365–372.

Bode, J.G., Ehlting, C., Häussinger, D., 2012. The macrophage response towards LPS and its control through the p38(MAPK)-STAT3 axis. Cell. Signal. 24, 1185–1194.

Braak, H., Del Tredici, K., Rüb, U., de Vos, R.A.I., Jansen Steur, E.N.H., Braak, E., 2003. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging 24, 197–211.

Braak, H., Sastre, M., Del Tredici, K., 2007. Development of alpha-synuclein immunoreactive astrocytes in the forebrain parallels stages of intraneuronal pathology in sporadic Parkinson's disease. Acta Neuropathol. 114, 231–241.

Bradl, M., Lassmann, H., 2010. Oligodendrocytes: biology and pathology. Acta Neuropathol 119, 37–53.

Brenner, M., Johnson, A.B., Boespflug-Tanguy, O., Rodriguez, D., Goldman, J.E., Messing, A., 2001. Mutations in GFAP, encoding glial fibrillary acidic protein, are associated with Alexander disease. Nat. Genet. 27, 117–120.

Brough, D., Le Feuvre, R.A., Iwakura, Y., Rothwell, N.J., 2002. Purinergic (P2X7) Receptor Activation of Microglia Induces Cell Death via an Interleukin-1-Independent Mechanism. Molecular and Cellular Neuroscience 19, 272–280.

Brown, R.C., Lockwood, A.H., Sonawane, B.R., 2005. Neurodegenerative Diseases: An Overview of Environmental Risk Factors. Environ. Health Perspect. 113, 1250–1256.

Brück, W., 2005. The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage. J. Neurol. 252 Suppl. 5, v3-9.

Calabrese, F., Rossetti, A.C., Racagni, A., Gass, P., Riva, M.A., Molteni, R., 2014. Brain-derived neurotrophic factor: a bridge between inflammation and neuroplasticity. Front. Cell .Neurosci. 8, 430.

Cameron, H.A., McKay, R.D., 1999. Restoring production of hippocampal neurons in old age. Nat. Neurosci. 2, 894–897.

Carson, M.J., Doose, J.M., Melchior, B., Schmid, C.D., Ploix, C.C., 2006. CNS immune privilege: hiding in plain sight. Immunol. Rev. 213, 48–65.

Caso, J.R., Pradillo, J.M., Hurtado, O., Lorenzo, P., Moro, M.A., Lizasoain, I., 2007. Toll-like receptor 4 is involved in brain damage and inflammation after experimental stroke. Circulation 115, 1599–1608.

Chainani-Wu, N., 2003. Safety and anti-inflammatory activity of curcumin: a component of tumeric (Curcuma longa). J. Altern. Complement. Med. 9, 161–168.

Chen, A., Xu, J., Johnson, A.C., 2006. Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1. Oncogene 25, 278–287.

Cheng, A.L., Hsu, C.H., Lin, J.K., Hsu, M.M., Ho, Y.F., Shen, T.S., Ko, J.Y., Lin, J.T., Lin, B.R., Ming-Shiang, W., Yu, H.S., Jee, S.H., Chen, G.S., Chen, T.M., Chen, C.A., Lai, M.K., Pu, Y.S., Pan, M.H., Wang, Y.J., Tsai, C.C., Hsieh, C.Y., 2001. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res. 21, 2895–2900.

Clement, A.M., Nguyen, M.D., Roberts, E.A., Garcia, M.L., Boillée, S., Rule, M., McMahon, A.P., Doucette, W., Siwek, D., Ferrante, R.J., Brown, R.H., Julien, J.-P., Goldstein, L.S.B., Cleveland, D.W., 2003. Wild-type nonneuronal cells extend survival of SOD1 mutant motor neurons in ALS mice. Science 302, 113–117.

Cole, G.M., Teter, B., Frautschy, S.A., 2007. Neuroprotective effects of curcumin. Adv. Exp. Med. Biol. 595, 197–212.

Compston, A., Coles, A., 2008. Multiple sclerosis. Lancet 372, 1502–1517.

Cornago, P., Claramunt, R.M., Bouissane, L., Alkorta, I., Elguero, J., 2008. A study of the tautomerism of β-dicarbonyl compounds with special emphasis on curcuminoids. Tetrahedron 64, 8089–8094.

Dantzer, R., O'Connor, J.C., Freund, G.G., Johnson, R.W., Kelley, K.W., 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat. Rev. Neurosci. 9, 46–56.

Dendrou, C.A., Fugger, L., Friese, M.A., 2015. Immunopathology of multiple sclerosis. Nat. Rev. Immunol. 15, 545–558.

Dheen, S.T., Kaur, C., Ling, E.-A., 2007. Microglial activation and its implications in the brain diseases. Curr. Med. Chem. 14, 1189–1197.

Drui, G., Carnicella, S., Carcenac, C., Favier, M., Bertrand, A., Boulet, S., Savasta, M., 2014. Loss of dopaminergic nigrostriatal neurons accounts for the motivational and affective deficits in Parkinson's disease. Mol. Psychiatry. 19(3), 358-67.

Eissner, G., Kirchner, S., Lindner, H., Kolch, W., Janosch, P., Grell, M., Scheurich, P., Andreesen, R., Holler, E., 2000. Reverse signalling through transmembrane TNF confers resistance to lipopolysaccharide in human monocytes and macrophages. J. Immunol. 164, 6193–6198.

Erridge, C., 2010. Endogenous ligands of TLR2 and TLR4: agonists or assistants? J. Leukoc. Biol. 87, 989–999.

Erta, M., Quintana, A., Hidalgo, J., 2012. Interleukin-6, a Major Cytokine in the Central Nervous System. International Journal of Biological Sciences 8, 1254–1266.

Eyo, U.B., Wu, L.-J., 2013. Bidirectional microglia-neuron communication in the healthy brain. Neural Plast. 2013, 456857.

Farooqui, A.A., 2014. Inflammation and Oxidative Stress in Neurological Disorders: Effect of Lifestyle, Genes, and Age. Springer Science & Business Media.

Farooqui A.A. Therapeutic Potentials of Curcumin for Alzheimer Disease (Springer, 2016).

Felderhoff-Mueser, U., Schmidt, O.I., Oberholzer, A., Bührer, C., Stahel, P.F., 2005. IL-18: a key player in neuroinflammation and neurodegeneration? Trends in Neurosciences 28, 487–493.

Feng, L., Sun, W., Xia, Y., Tang, W.W., Chanmugam, P., Soyoola, E., Wilson, C.B., Hwang, D., 1993. Cloning two isoforms of rat cyclooxygenase: differential regulation of their expression. Arch. Biochem. Biophys. 307, 361–368.

Ferrari, C.C., Depino, A.M., Prada, F., Muraro, N., Campbell, S., Podhajcer, O., Perry, V.H., Anthony, D.C., Pitossi, F.J., 2004. Reversible Demyelination, Blood-Brain Barrier Breakdown, and Pronounced Neutrophil Recruitment Induced by Chronic IL-1 Expression in the Brain. The American Journal of Pathology 165, 1827–1837.

Forman, M.S., Lal, D., Zhang, B., Dabir, D.V., Swanson, E., Lee, V.M.-Y., Trojanowski, J.Q., 2005. Transgenic mouse model of tau pathology in astrocytes leading to nervous system degeneration. J. Neurosci. 25, 3539–3550.

Forno, L.S., DeLanney, L.E., Irwin, I., Monte, D.D., Langston, J.W. (1992) Astrocytes and Parkinson's disease. Prog. Brain Res. 94, 429–436.

Franchi, L., Warner, N., Viani, K., Nuñez, G., 2009. Function of Nod-like receptors in microbial recognition and host defense. Immunol. Rev. 227, 106–128.

Frühauf, P.K., Ineu, R.P., Tomazi, L., Duarte, T., Mello, C.F., Rubin, M.A., 2015. Spermine reverses lipopolysaccharide-induced memory deficit in mice. J. Neuroinflammation 12, 3.

Fujimi, K., Noda, K., Sasaki, K., Wakisaka, Y., Tanizaki, Y., Iida, M., Kiyohara, Y., Kanba, S., Iwaki, T., 2007. Altered expression of COX-2 in subdivisions of the hippocampus during aging and in Alzheimer's disease: the Hisayama Study. Dement. Geriatr. Cogn. Disord. 23, 423–431.

Fünfschilling, U., Supplie, L.M., Mahad, D., Boretius, S., Saab, A.S., Edgar, J., Brinkmann, B.G., Kassmann, C.M., Tzvetanova, I.D., Möbius, W., Diaz, F., Meijer, D., Suter, U., Hamprecht, B., Gallucci, S., Lolkema, M., Matzinger, P., 1999. Natural adjuvants: endogenous activators of dendritic cells. Nat. Med. 5, 1249–1255.

Garden, G.A., La Spada, A.R., 2012. Intercellular (Mis)communication in Neurodegenerative Disease. Neuron 73, 886–901.

Garlanda, C., Dinarello, C.A., Mantovani, A., 2013. The interleukin-1 family: back to the future. Immunity 39, 1003–1018.

Geijtenbeek, T.B., and Gringhuis, S.I., 2009. Signalling through C-type lectin receptors: shaping immune responses. Nat. Rev. Immunol. 9, 465–479.

Ghalandarlaki, N., Alizadeh, A.M., Ashkani-Esfahani, S., 2014. Nanotechnology-Applied Curcumin for Different Diseases Therapy. BioMed Research International, e394264.

Ginhoux, F., Lim, S., Hoeffel, G., Low, D., Huber, T., 2013. Origin and differentiation of microglia. Front Cell Neurosci. 7, 45.

Glass, C.K., Saijo, K., Winner, B., Marchetto, M.C., Gage, F.H., 2010. Mechanisms underlying inflammation in neurodegeneration. Cell 140, 918–934.

Goel, A., Kunnumakkara, A.B., Aggarwal, B.B., 2008. Curcumin as "Curecumin": from kitchen to clinic. Biochem. Pharmacol. 75, 787–809.

Gorina, R., Font-Nieves, M., Márquez-Kisinousky, L., Santalucia, T., Planas, A.M., 2011. Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFκB signalling, MAPK, and Jak1/Stat1 pathways. Glia 59, 242–255.

Griffin, W.S., Sheng, J.G., Royston, M.C., Gentleman, S.M., McKenzie, J.E., Graham, D.I., Roberts, G.W., Mrak, R.E., 1998. Glial-neuronal interactions in Alzheimer's disease: the potential role of a "cytokine cycle" in disease progression. Brain Pathol. 8, 65–72.

Guo, L., Xing, Y., Pan, R., Jiang, M., Gong, Z., Lin, L., Wang, J., Xiong, G., Dong, J., 2013. Curcumin protects microglia and primary rat cortical neurons against HIV-1 gp120-mediated inflammation and apoptosis. PLoS ONE 8, e70565.

Habauzit, V., Morand, C., 2012. Evidence for a protective effect of polyphenols-containing foods on cardiovascular health: an update for clinicians. Ther. Adv. Chronic Dis. 3, 87–106.

Halle, A. et al., 2008. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol. 9(8), 857-65.

Hara, H., Fink, K., Endres, M., Friedlander, R.M., Gagliardini, V., Yuan, J., Moskowitz, M.A., 1997. Attenuation of Transient Focal Cerebral Ischemic Injury in Transgenic Mice Expressing a Mutant ICE Inhibitory Protein. J. Cereb. Blood Flow Metab. 17, 370–375.

Haughey, N.J., Mattson, M.P., 2003. Alzheimer's amyloid beta-peptide enhances ATP/gap junction-mediated calcium-wave propagation in astrocytes. Neuromolecular Med. 3, 173–180.

He, Y., Yue, Y., Zheng, X., Zhang, K., Chen, S., Du, Z., 2015. Curcumin, inflammation, and chronic diseases: how are they linked? Molecules 20, 9183-9213.

Heales, S.J. et al., 2004. Neurodegeneration or neuroprotection: the pivotal role of astrocytes. Neurochem. Res. 29, 513–519.

Heneka, M.T., Kummer, M.P., Latz, E., 2014. Innate immune activation in neurodegenerative disease, Nat. Rev. Immunol. 14, 463-477.

Heppner, F.L., Ransohoff, R.M., Becher, B., 2015. Immune attack: the role of inflammation in Alzheimer disease. Nat. Rev. Neurosci. 16, 358–372.

Higgs, G.A., Moncada, S., Vane, J.R., 1984. Eicosanoids in inflammation. Ann Clin. Res. 16, 287–299.

Hirsch, E.C., Hunot, S., 2009. Neuroinflammation in Parkinson's disease: a target for neuroprotection? The Lancet Neurology 8, 382–397.

Hofmann, M.A., Drury, S., Fu, C., Qu, W., Taguchi, A., Lu, Y., Avila, C., Kambham, N., Bierhaus, A., Nawroth, P., Neurath, M.F., Slattery, T., Beach, D., McClary, J., Nagashima, M., Morser, J., Stern, D., Schmidt, A.M., 1999. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell 97, 889–901.

Hoogland, I.C.M., Houbolt, C., van Westerloo, D.J., van Gool, W.A., van de Beek, D., 2015. Systemic inflammation and microglial activation: systematic review of animal experiments. Journal of Neuroinflammation 12, 114.

Hu, S., Maiti, P., Ma, Q., Zuo, X., Jones, M.R., Cole, G.M., Frautschy, S.A., 2015. Clinical development of curcumin in neurodegenerative disease. Expert Review of Neurotherapeutics 15, 629–637.

Hua, F., Ma, J., Ha, T., Xia, Y., Kelley, J., Williams, D.L., Kao, R.L., Browder, I.W., Schweitzer, J.B., Kalbfleisch, J.H., Li, C., 2007. Activation of Toll-like Receptor 4 Signalling Contributes to Hippocampal Neuronal Death Following Global Cerebral Ischemia/Reperfusion. J, Neuroimmunol, 190, 101–111.

Inohara, Chamaillard, McDonald, C., Nuñez, G., 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu. Rev. Biochem. 74, 355–383.

Jiao, Y., Wilkinson, J., Di, X., Wang, W., Hatcher, H., Kock, N.D., D'Agostino, R., Knovich, M.A., Torti, F.M., Torti, S.V., 2009. Curcumin, a cancer chemopreventive and chemotherapeutic agent, is a biologically active iron chelator. Blood 113, 462–469.

Jin, K., Minami, M., Lan, J.Q., Mao, X.O., Batteur, S., Simon, R.P., Greenberg, D.A., 2001. Neurogenesis in Dentate Subgranular Zone and Rostral Subventricular Zone after Focal Cerebral Ischemia in the Rat. Proceedings of the National Academy of Sciences of the United States of America 98, 4710–4715.

Kagan, J.C., Medzhitovd, R., 2006. Phosphoinositide mediated adaptor recruitment controls Toll-like receptor signalling. Cell 125, 943–955.

Kanski, J., Aksenova, M., Stoyanova, A., Butterfield, D.A., 2002. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems in vitro: structure-activity studies. J. Nutr. Biochem. 13, 273–281.

Kassmann, C.M., Lappe-Siefke, C., Baes, M., Brügger, B., Mildner, A., Werner, H.B., Natt, O., Michaelis, T., Prinz, M., Frahm, J., Nave, K.-A., 2007. Axonal loss and neuroinflammation caused by peroxisome-deficient oligodendrocytes. Nat. Genet. 39, 969–976.

Kaufmann, W.E., Worley, P.F., Pegg, J., Bremer, M., Isakson, P., 1996. COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. Proc. Natl. Acad. Sci. U.S.A. 93, 2317–2321.

Keller, M., Rüegg, A., Werner, S., Beer, H.-D., 2008. Active caspase-1 is a regulator of unconventional protein secretion. Cell 132, 818–831.

Kerfoot, S.M., Long, E.M., Hickey, M.J., Andonegui, G., Lapointe, B.M., Zanardo, R.C.O., Bonder, C., James,W.G., Robbins, S.M., Kubes, P., 2004. TLR4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease. J. Immunol. 173, 7070–7077.

Kettenmann, H., Hanisch, U.-K., Noda, M., Verkhratsky, A., 2011. Physiology of microglia. Physiol. Rev. 91, 461–553.

Khandelwal, P.J., Herman, A.M., Moussa, C.E.-H., 2011. Inflammation in the early stages of neurodegenerative pathology. J. Neuroimmunol. 238, 1–11.

Kielian, T., 2006. Toll-Like Receptors in Central Nervous System Glial Inflammation and Homeostasis. J. Neurosci. Res. 83, 711–730.

Kim, S.J., Son, T.G., Park, H.R., Park, M., Kim, M.-S., Kim, H.S., Chung, H.Y., Mattson, M.P., Lee, J., 2008. Curcumin Stimulates Proliferation of Embryonic Neural Progenitor Cells and Neurogenesis in the Adult Hippocampus. J. Biol. Chem. 283, 14497–14505.

Konat, G.W., Kielian, T., Marriott, I., 2006. The role of Toll-like receptors in CNS response to microbial challenge. J. Neurochem. 99, 1–12.

Knott, C., Stern, G., Wilkin, G.P., 2000. Inflammatory regulators in Parkinson's disease: iNOS, lipocortin-1, and cyclooxygenases-1 and -2. Mol. Cell. Neurosci. 16, 724–739.

Kumar, H., Kawai, T., Akira, S., 2009. Toll-like receptors and innate immunity. Biochem. Biophys. Res. Commun. 388, 621–625.

Kumar, V., Cotran, R. S. & Robbins, S. L. Robbins Basic Pathology (Saunders, 2003).

Lacroix, S., Feinstein, D., Rivest, S., 1998. The bacterial endotoxin lipopolysaccharide has the ability to target the brain in upregulating its membrane CD14 receptor within specific cellular populations. Brain Pathol. 8, 625–640.

Laflamme, N., Rivest, S., 2001. Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. FASEB J. 15, 155–163.

Lappe-Siefke, C., Goebbels, S., Gravel, M., Nicksch, E., Lee, J., Braun, P.E., Griffiths, I.R., Nave, K.-A., 2003. Disruption of Cnp1 uncouples oligodendroglial functions in axonal support and myelination. Nat. Genet. 33, 366–374.

Lassmann, H., 2014. Multiple sclerosis: lessons from molecular neuropathology. Exp. Neurol. 262 Pt A, 2–7.

Lawrence, T., Gilroy, D.W., 2007. Chronic inflammation: a failure of resolution? Int. J. Exp. Pathol. 88, 85–94.

Lee, H.S., Jung, K.K., Cho, J.Y., Rhee, M.H., Hong, S., Kwon, M., Kim, S.H., Kang, S.Y., 2007. Neuroprotective effect of curcumin is mainly mediated by blockade of microglial cell activation. Pharmazie 62, 937–942.

Lee, M.S., Kim, Y.J., 2007. Signalling pathways downstream of pattern-recognition receptors and their cross talk. Ann. Rev. Biochem. 76, 447–480.

Lee, Y., Morrison, B.M., Li, Y., Lengacher, S., Farah, M.H., Hoffman, P.N., Liu, Y., Tsingalia, A., Jin, L., Zhang, P.-W., Pellerin, L., Magistretti, P.J., Rothstein, J.D., 2012. Oligodendroglia metabolically support axons and contribute to neurodegeneration. Nature 487, 443–448.

Lehár, J., Krueger, A.S., Avery, W., Heilbut, A.M., Johansen, L.M., Price, E.R., Rickles, R.J., Short, G.F., Staunton, J.E., Jin, X., Lee, M.S., Zimmermann, G.R., Borisy, A.A., 2009. Synergistic drug combinations tend to improve therapeutically relevant selectivity. Nat. Biotechnol. 27, 659–666.

Lehnardt, S., 2010. Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. Glia 58, 253–263.

Lehnardt, S., Lachance, C., Patrizi, S., Lefebvre, S., Follett, P.L., Jensen, F.E., Rosenberg, P.A., Volpe, J.J., Vartanian, T., 2002. The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. J. Neurosci. 22, 2478–2486.

Lehnardt, S., Massillon, L., Follett, P., Jensen, F.E., Ratan, R., Rosenberg, P.A., Volpe, J.J., Vartanian, T., 2003. Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. Proc. Natl. Acad. Sci. U.S.A. 100, 8514–8519.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., Hoffmann, J.A., 1996. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86, 973–983.

Li, G., Min, B.-S., Zheng, C., Lee, J., Oh, S.-R., Ahn, K.-S., Lee, H.-K., 2005 Neuroprotective and free radical scavenging activities of phenolic compounds from Hovenia dulcis. Arch. Pharm. Res. 28, 804–809.

Li, N., Ma, Z., Li, M., Xing, Y., Hou, Y., 2014. Natural potential therapeutic agents of neurodegenerative diseases from the traditional herbal medicine Chinese dragon's blood. J. Ethnopharmacol. 152, 508–521.

Lim, G.P., Chu, T., Yang, F., Beech, W., Frautschy, S.A., Cole, G.M., 2001. The Curry Spice Curcumin Reduces Oxidative Damage and Amyloid Pathology in an Alzheimer Transgenic Mouse. J. Neurosci. 21, 8370–8377.

Liya, Q., Xuefei, W., Michelle, L.B., Yuxin, L., George, R.B., Jau-Shyong, H., Darin, J.K., and Fulton, T.C., 2007. Systemic LPS Causes Chronic Neuroinflammation and Progressive Neurodegeneration Glia. 55(5), 453–462.

Loo, Y.-M., Gale, M., 2011. Immune signalling by RIG-I-like receptors. Immunity 34, 680–692.

Lu, Y.C., Yeh, W.C., Ohashi, P.S., 2008. LPS/TLR4 signal transduction pathway. Cytokine 42, 145–151.

Lucas, S.-M., Rothwell, N.J., Gibson, R.M., 2006. The role of inflammation in CNS injury and disease. British Journal of Pharmacology 147, S232–S240.

Lull, M.E., Block, M.L., 2010. Microglial activation and chronic neurodegeneration. Neurotherapeutics 7, 354–365.

Luo, X.-G., Ding, J.-Q., Chen, S.-D., 2010. Microglia in the aging brain: relevance to neurodegeneration. Molecular Neurodegeneration 5, 12.

Luo, Y., Smith, J.V., Paramasivam, V., Burdick, A., Curry, K.J., Buford, J.P., Khan, I., Netzer, W.J., Xu, H., Butko, P., 2002. Inhibition of amyloid-beta aggregation and caspase-3 activation by the Ginkgo biloba extract EGb761. Proc. Natl. Acad. Sci. U.S.A. 99, 12197–12202.

Majno G. & Joris I. Cells, Tissues and Disease (Oxford Univ. Press, 2004).

Maragakis, N.J., Rothstein, J.D., 2006. Mechanisms of Disease: astrocytes in neurodegenerative disease. Nature Clinical Practice 2, 679-689.

Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W.P., Weinrauch, Y., Monack, D.M., Dixit, V.M., 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440, 228–232.

Marinova-Mutafchieva, L., Sadeghian, M., Broom, L., Davis, J.B., Medhurst, A.D., Dexter, D.T., 2009. Relationship between microglial activation and

dopaminergic neuronal loss in the substantia nigra: a time course study in a 6-hydroxydopamine model of Parkinson's disease. J. Neurochem. 110, 966–975.

Marsh, B., Stevens, S.L., Packard, A.E., Gopalan, B., Hunter, B., Leung, P.Y., Harrington, C.A., Stenzel-Poore, M.P., 2009. Systemic lipopolysaccharide protects the brain from ischemic injury by reprogramming the response of the brain to stroke: a critical role for IRF3. J. Neurosci. 29, 9839–9849.

Martyn, G., 2009. Circuits controlling vertebrate locomotion: moving in a new direction. Nat. Rev. Neurosci. 10(7), 507–518.

Mazarati, A., Maroso, M., Iori, V., Vezzani, A., Carli, M., 2011. High-mobility group box-1 impairs memory in mice through both Toll-like receptor 4 and receptor for advanced glycation end products. Exp. Neurol. 232, 143–148.

McColl, B.W., Rothwell, N.J., Allan, S.M., 2007. Systemic inflammatory stimulus potentiates the acute phase and CXC chemokine responses to experimental stroke and exacerbates brain damage via interleukin-1- and neutrophil-dependent mechanisms. J. Neurosci. 27, 4403–4412.

McCoy, M.K., Martinez, T.N., Ruhn, K.A., Szymkowski, D.E., Smith, C.G., Botterman, B.R., Tansey, K.E., Tansey, M.G., 2006. Blocking soluble tumor necrosis factor signalling with dominant-negative tumor necrosis factor inhibitor attenuates loss of dopaminergic neurons in models of Parkinson's disease. J. Neurosci. 26, 9365–9375.

McCoy, M.K., Tansey, M.G., 2008. TNF signalling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J. Neuroinflammation 5, 45.

Medzhitov R. 2008. Origin and physiological roles of inflammation. Nature 454, 428–435.

Medzhitov R. 2010. Inflammation 2010: new adventures of an old flame. Cell 140, 771–776.

Medzhitov, R., Janeway, C.A., 1997. Innate immunity: the virtues of a nonclonal system of recognition. Cell 91, 295–298.

Medzhitov, R., Preston-Hurlburt, P., Janeway, C.A., 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388, 394–397.

Melinda, E.L, Michelle, L.B., 2010. Microglial Activation & Chronic Neurodegeneration. Neurotherapeutics 7(4), 354–365.

Mena, M.A., García de Yébenes, J., 2008. Glial cells as players in parkinsonism: the "good," the "bad," and the "mysterious" glia. Neuroscientist 14, 544–560.

Metzler, M., Pfeiffer, E., Schulz, S.I., Dempe, J.S., 2013. Curcumin uptake and metabolism. Biofactors 39, 14–20.

Millington, G.W., 2007. The role of proopiomelanocortin (POMC) neurones in feeding behaviour. Nutr. Metab. (Lond). 4, 18.

Minghetti, L., 2004. Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. J. Neuropathol. Exp. Neurol. 63, 901–910.

Morganti-Kossmann, M.C., Rancan, M., Stahel, P.F., Kossmann, T., 2002. Inflammatory response in acute traumatic brain injury: a double-edged sword. Curr. Opin. Crit. Care 8, 101–105.

Mourtas, S., Lazar, A.N., Markoutsa, E., Duyckaerts, C., Antimisiaris, S.G., 2014. Multifunctional nanoliposomes with curcumin-lipid derivative and brain targeting functionality with potential applications for Alzheimer disease. Eur. J. Med. Chem. 80, 175–183.

Nathan C, Ding A. 2010. Nonresolving inflammation. Cell 140, 871–882.

Nathan C. 2002. Points of control in inflammation. Nature 420, 846–852.

Nathan C. 2006. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 6, 173–182.

Nimmerjahn, A., Kirchhoff, F., Helmchen, F., 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308, 1314–1318.

Noh, H., Jeon, J., Seo, H., 2014. Systemic injection of LPS induces region-specific neuroinflammation and mitochondrial dysfunction in normal mouse brain. Neurochem. Int. 69, 35–40.

Nonneman, A., Robberecht, W., Van Den Bosch, L., 2014. The role of oligodendroglial dysfunction in amyotrophic lateral sclerosis. Neurodegener. Dis. Manag. 4, 223–239.

Norden, D.M., Godbout, J.P., 2013. Review: microglia of the aged brain: primed to be activated and resistant to regulation. Neuropathol. Appl. Neurobiol. 39, 19–34.

Norden, D.M., Muccigrosso, M.M., Godbout, J.P., 2015. Microglial priming and enhanced reactivity to secondary insult in aging, and traumatic CNS injury, and neurodegenerative disease. Neuropharmacology 96, 29–41.

Nüsslein-Volhard, C., Wieschaus, E., 1980. Mutations affecting segment number and polarity in Drosophila. Nature 287, 795–801.

Oblak, A., Jerala, R., 2011. Toll-like receptor 4 activation in cancer progression and therapy. Clin. Dev. Immunol. 2011, 609579.

Parihar, M.S., Hemnani, T., 2004. Alzheimer's disease pathogenesis and therapeutic interventions. Journal of Clinical Neuroscience 11, 456–467.

Park, J.S., Svetkauskaite, D., He, Q., Kim, J.Y., Strassheim, D., Ishizaka, A., Abraham, E., 2004. Involvement of toll like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J. Biol. Chem. 279, 7370–7377.

Patel, H.C., Boutin, H., Allan, S.M., 2003. Interleukin-1 in the Brain. Annals of the New York Academy of Sciences 992, 39–47.

Perry, R.T., Collins, J.S., Wiener, H., Acton, R., Go, R.C., 2001. The role of TNF and its receptors in Alzheimer's disease. Neurobiol. Aging 22, 873–883.

Pober, J.S., Sessa, W.C., 2007. Evolving functions of endothelial cells in inflammation. Nat. Rev. Immunol. 7, 803–815.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., Beutler, B., 1998. Defective LPS signalling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282, 2085–2088.

Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S., Beutler, B., 2000. Physical contact between lipopolysaccharide and Toll-like receptor 4 revealed by genetic complementation. PNAS 97, 2163–2167.

Prasad, S., Gupta, S.C., Tyagi, A.K., Aggarwal, B.B., 2014. Curcumin, a component of golden spice: from bedside to bench and back. Biotechnol. Adv. 32, 1053–1064.

Prasad, S., Tyagi, A.K., Aggarwal, B.B., 2014. Recent Developments in Delivery, Bioavailability, Absorption and Metabolism of Curcumin: the Golden Pigment from Golden Spice. Cancer Res Treat 46, 2–18.

Priyadarsini, K.I., 2013. Chemical and structural features influencing the biological activity of curcumin. Curr. Pharm. Des. 19, 2093–2100.

Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J.-S., Knapp, D.J., Crews, F.T., 2007. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55, 453–462.

Rajasekar, N., Dwivedi, S., Tota, S.K., Kamat, P.K., Hanif, K., Nath, C., Shukla, R., 2013. Neuroprotective effect of curcumin on okadaic acid induced memory impairment in mice. Eur. J. Pharmacol. 715, 381–394.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., Medzhitov, R., 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118, 229–241.

Ramesh, G., MacLean, A.G., Philipp, M.T., 2013. Cytokines and Chemokines at the Crossroads of Neuroinflammation, Neurodegeneration, and Neuropathic Pain. Mediators of Inflammation 2013, e480739.

Ranaivo, H.R., Craft, J.M., Hu, W., Guo, L., Wing, L.K., Eldik, L.J.V., Watterson, D.M., 2006. Glia as a Therapeutic Target: Selective Suppression of Human Amyloid-β-Induced Upregulation of Brain Proinflammatory Cytokine Production Attenuates Neurodegeneration. J. Neurosci. 26, 662–670.

Ransohoff, R.M., Perry, V.H., 2009. Microglial Physiology: Unique Stimuli, Specialized Responses. Annual Review of Immunology 27, 119–145.

Rauvala, H., Rouhiainen, A., 2010. Physiological and pathophysiological outcomes of the interactions of HMGB1 with cell surface receptors. Biochim. Biophys. Acta 1799, 164–170.

Réus, G.Z., Fries, G.R., Stertz, L., Badawy, M., Passos, I.C., Barichello, T., *et al*., 2015. The role of inflammation and microglial activation in the pathophysiology of psychiatric disorders, Neuroscience 300, 141-154.

Rivest, S., 2009. Regulation of innate immuneresponses in the brain. Nat. Rev. Immunol. 9, 429–439.

Robert, D.J.C., Gregory, G.F., Rodney, W.J., Kate, W.K., 2008. From inflammation to sickness and depression: when the immune system subjugates the brain. Nat. Rev. Neurosci. 9(1), 46–56.

Rock, K.L., Kono, H., 2008. The inflammatory response to cell death. Annu. Rev. Pathol. 3, 99–126.

Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R., Schwartz, M., 2007. Toll-like receptors modulate adult hippocampal neurogenesis. Nat. Cell Biol. 9, 1081–1088.

Roney, C., Kulkarni, P., Arora, V., Antich, P., Bonte, F., Wu, A., Mallikarjuana, N.N., Manohar, S., Liang, H.-F., Kulkarni, A.R., Sung, H.-W., Sairam, M., Aminabhavi, T.M., 2005. Targeted nanoparticles for drug delivery through the blood-brain barrier for Alzheimer's disease. J. Control. Release 108, 193–214.

Roth, A.D., Ramírez, G., Alarcón, R., Von Bernhardi, R., 2005. Oligodendrocytes damage in Alzheimer's disease: beta amyloid toxicity and inflammation. Biol. Res. 38, 381–387.

Saijo, K., Glass, C.K., 2011. Microglial cell origin and phenotypes in health and disease. Nat. Rev. Immunol. 11, 775–787.

Schröder, N.W.J., Schumann, R.R., 2005. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect. Dis. 5, 156–164.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Lee, L., Isakson, P., 1994. Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. Proc. Natl. Acad. Sci. U.S.A. 91, 12013–12017.

Sereda, M.W., Moraes, C.T., Frahm, J., Goebbels, S., Nave, K.-A., 2012. Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. Nature 485, 517–521.

Serhan, C.N., 2007. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. Annu. Rev. Immunol. 25, 101–137.

Serhan, C.N., Brain, S.D., Buckley, C.D., Gilroy, D.W., Haslett, C., O'Neill, L.A.J., Perretti, M., Rossi, A.G., Wallace, J.L., 2007. Resolution of inflammation: state of the art, definitions and terms. FASEB J. 21, 325–332.

Serhan, C.N., Savill, J., 2005. Resolution of inflammation: the beginning programs the end. Nat. Immunol. 6, 1191–1197.

Sharma, R.A., Ireson, C.R., Verschoyle, R.D., Hill, K.A., Williams, M.L., Leuratti, C., Manson, M.M., Marnett, L.J., Steward, W.P., Gescher, A., 2001. Effects of dietary curcumin on glutathione S-transferase and malondialdehyde-DNA adducts in rat liver and colon mucosa: relationship with drug levels. Clin. Cancer Res. 7, 1452–1458.

Shehzad, A., Lee, Y.S., 2013. Molecular mechanisms of curcumin action: signal transduction. Biofactors 39, 27–36.

Shi, Y., Zheng, W., Rock, K.L., 2000. Cell injury releases endogenous adjuvants that stimulate cytotoxic T cell responses. PNAS 97, 14590–14595.

Shin, J.-Y., Fang, Z.-H., Yu, Z.-X., Wang, C.-E., Li, S.-H., Li, X.-J., 2005. Expression of mutant huntingtin in glial cells contributes to neuronal excitotoxicity. J. Cell Biol. 171, 1001–1012.

Shishodia, S., 2013. Molecular mechanisms of curcumin action: gene expression. Biofactors 39, 37–55.

Shoba, G., Joy, D., Joseph, T., Majeed, M., Rajendran, R., Srinivas, P.S., 1998. Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Med. 64, 353–356.

Singh, S., Aggarwal, B.B., 1995. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane). J. Biol. Chem. 270, 24995–25000.

Snyder, J.S., Hong, N.S., McDonald, R.J., Wojtowicz, J.M., 2005. A role for adult neurogenesis in spatial long-term memory. Neuroscience 130, 843–852.

Sofroniew, M.V., 2005. Reactive astrocytes in neural repair and protection. Neuroscientist 11, 400–407.

Sofroniew, M.V., Vinters, H.V., 2010. Astrocytes: biology and pathology. Acta Neuropathol. 119, 7–35.

Song, J.H., Lee, J.W., Shim, B., Lee, C.Y., Choi, S., Kang, C., Sohn, N.W., Shin, J.W., 2013. Glycyrrhizin alleviates neuroinflammation and memory deficit induced by systemic lipopolysaccharide treatment in mice. Molecules 18, 15788-15803.

Spittau, B., Zhou, X., Ming, M., Krieglstein, K., 2012. IL6 Protects MN9D Cells and Midbrain Dopaminergic Neurons from MPP+-Induced Neurodegeneration. Neuromol. Med. 14, 317–327.

Spooren, A., Kolmus, K., Laureys, G., Clinckers, R., De Keyser, J., Haegeman, G., Gerlo, S., 2011. Interleukin-6, a mental cytokine. Brain Res. Rev. 67, 157–183.

Srinivasan, M., Sudheer, A.R., Menon, V.P., 2007. Ferulic Acid: therapeutic potential through its antioxidant property. J Clin Biochem Nutr 40, 92–100.

Stewart, C.R., Stuart, L.M., Wilkinson, K., van Gils, J.M., Deng, J., Halle, A., Rayner, K.J., Boyer, L., Zhong, R., Frazier, W.A., Lacy-Hulbert, A., El Khoury, J., Golenbock, D.T., Moore, K.J., 2010. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. Nat. Immunol. 11, 155–161.

Strauss, K.I., 2008. Antiinflammatory and neuroprotective actions of COX2 inhibitors in the injured brain. Brain Behav. Immun. 22, 285–298.

Sun, D., Lye-Barthel, M., Masland, R.H., Jakobs, T.C., 2010. Structural Remodeling of Fibrous Astrocytes after Axonal Injury. J. Neurosci. 30, 14008–14019.

Suresh, D., Srinivasan, K., 2010. Tissue distribution & elimination of capsaicin, piperine & curcumin following oral intake in rats. Indian J. Med. Res. 131, 682–691.

Takeda, K., Akira, S., 2005. Toll-like receptors in innate immunity. Int. Immunol. 17, 1–14.

Takeuchi, H., Jin, S., Wang, J., Zhang, G., Kawanokuchi, J., Kuno, R., Sonobe, Y., Mizuno, T., Suzumura, A., 2006. Tumor necrosis factor-alpha induces neurotoxicity via glutamate release from hemichannels of activated microglia in an autocrine manner. J. Biol. Chem. 281, 21362–21368.

Takeuchi, O., Akira, S., 2002. MyD88 as a bottle neck in Toll/IL-1 signalling. Curr. Top. Microbiol. Immunol. 270, 155–167.

Takeuchi, O., Akira, S., 2009. Innate immunity to virus infection. Immunol. Rev. 227, 75–86.

Takeuchi, O., Akira, S., 2010. Pattern recognition receptors and inflammation. Cell 140, 805–820.

Tchantchou, F., Xu, Y., Wu, Y., Christen, Y., Luo, Y., 2007. EGb 761 enhances adult hippocampal neurogenesis and phosphorylation of CREB in transgenic mouse model of Alzheimer's disease. FASEB J. 21, 2400–2408.

Teather, L.A., Packard, M.G., Bazan, N.G., 2002. Post-training cyclooxygenase-2 (COX-2) inhibition impairs memory consolidation. Learn. Mem. 9, 41–47.

Teismann, P., Schulz, J.B., 2004. Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation. Cell Tissue Res. 318, 149–161.

Teismann, P., Tieu, K., Choi, D.-K., Wu, D.-C., Naini, A., Hunot, S., Vila, M., Jackson-Lewis, V., Przedborski, S., 2003. Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration. Proc. Natl. Acad. Sci. U.S.A. 100, 5473–5478.

Tiwari, S.K., Agarwal, S., Seth, B., Yadav, A., Nair, S., Bhatnagar, P., Karmakar, M., Kumari, M., Chauhan, L.K.S., Patel, D.K., Srivastava, V., Singh, D., Gupta, S.K., Tripathi, A., Chaturvedi, R.K., Gupta, K.C., 2014. Curcumin-loaded nanoparticles potently induce adult neurogenesis and reverse cognitive deficits in Alzheimer's disease model via canonical Wnt/β-catenin pathway. ACS Nano 8, 76–103.

Tosini, G., 2007. Locomotor activity in rodents. Methods Mol. Biol. 362, 95-101.

Tremblay, M.-È., Stevens, B., Sierra, A., Wake, H., Bessis, A., Nimmerjahn, A., 2011. The Role of Microglia in the Healthy Brain. J. Neurosci. 31, 16064–16069.

Turnbull, A.V., Rivier, C.L., 1999. Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. Physiol. Rev. 79, 1–71.

Van Praag, H., Schinder, A.F., Christie, B.R., Toni, N., Palmer, T.D., Gage, F.H., 2002. Functional neurogenesis in the adult hippocampus. Nature 415, 1030–1034.

Vezzani, A., Moneta, D., Conti, M., Richichi, C., Ravizza, T., De Luigi, A., De Simoni, M.G., Sperk, G., Andell-Jonsson, S., Lundkvist, J., Iverfeldt, K., Bartfai, T., 2000. Powerful anticonvulsant action of IL-1 receptor antagonist on

intracerebral injection and astrocytic overexpression in mice. Proc. Natl. Acad. Sci. U.S.A. 97, 11534–11539.

Vogl, T., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., van Zoelen, M.A.D., Nacken, W., Foell, D., van der Poll, T., Sorg, C., Roth, J., 2007. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat. Med. 13, 1042–1049.

Von Bernhardi, R., Eugenín-von Bernhardi, L., Eugenín, J., 2015. Microglial cell dysregulation in brain aging and neurodegeneration. Front. Aging Neurosci. 7.

Wadachi, R., Hargreaves, K.M., 2006. Trigeminal nociceptors express TLR-4 and CD14: a mechanism for pain due to infection. J. Dent. Res. 85, 49–53.

Wakabayashi, K., Hayashi, S., Yoshimoto, M., Kudo, H., Takahashi, H., 2000. NACP/alpha-synuclein-positive filamentous inclusions in astrocytes and oligodendrocytes of Parkinson's disease brains. Acta Neuropathol. 99, 14–20.

Wajant, H., Pfizenmaier, K., Scheurich, P., 2003. Tumor necrosis factor signalling. Cell Death Differ. 10, 45–65.

Wang, L., Harrington, L., Trebicka, E., Shi, H.N., Kagan, J.C., Hong, C.C., Lin, H.Y., Babitt, J.L., Cherayil, B.J., 2009. Selective modulation of TLR4-activated inflammatory responses by altered iron homeostasis in mice. J. Clin. Invest. 119, 3322–3328.

Wang, J., Campbell, I.L., 2002. Cytokine signalling in the brain: putting a SOCS in it? J. Neurosci. Res. 67, 423–427.

Wang, Y.J., Pan, M.H., Cheng, A.L., Lin, L.I., Ho, Y.S., Hsieh, C.Y., Lin, J.K., 1997. Stability of curcumin in buffer solutions and characterization of its degradation products. J. Pharm. Biomed. Anal. 15, 1867–1876.

Watanabe, M., Dykes-Hoberg, M., Culotta, V.C., Price, D.L., Wong, P.C., Rothstein, J.D., 2001. Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic lateral sclerosis neural tissues. Neurobiol. Dis. 8, 933–941.

Wisniewski, H.M., Wegiel, J., 1991. Spatial relationships between astrocytes and classical plaque components. Neurobiol. Aging 12, 593–600.

Wright, B., 2013. Forging a modern generation of polyphenol-based therapeutics. Br. J. Pharmacol. 169, 844–847.

Wyss-Coray, T., Loike, J.D., Brionne, T.C., Lu, E., Anankov, R., Yan, F., Silverstein, S.C., Husemann, J., 2003. Adult mouse astrocytes degrade amyloid-β in vitro and in situ. Nat. Med. 9, 453–457.

Xie, Z.,Wei, M., Morgan, T.E., Fabrizio, P., Han, D., Finch, C.E., Longo, V.D., 2002. Peroxynitrite mediates neurotoxicity of amyloid beta-peptide 1–42- and lipopolysaccharideactivated microglia. J. Neurosci. 22, 3484–3492.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., Akira, S., 2003a. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signalling pathway. Science 301, 640–643.

Yamasaki, Y., Matsuura, N., Shozuhara, H., Onodera, H., Itoyama, Y., Kogure, K., 1995. Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. Stroke 26, 676–680; discussion 681.

Yanai, H., Ban, T., Wang, Z., Choi, M.K., Kawamura, T., Negishi, H., Nakasato, M., Lu, Y., Hangai, S., Koshiba, R., Savitsky, D., Ronfani, L., Akira, S., Bianchi, M.E., Honda, K., Tamura, T., Kodama, T., Taniguchi, T., 2009. HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. Nature 462, 99–103.

Yang, K.-Y., Lin, L.-C., Tseng, T.-Y., Wang, S.-C., Tsai, T.-H., 2007. Oral bioavailability of curcumin in rat and the herbal analysis from Curcuma longa by LC-MS/MS. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 853, 183–189.

Yang, Q.W., Xiang, J., Zhou, Y., Zhong, Q., Li, J.C., 2010. Targeting HMGB1/TLR4 signalling as a novel approach to treatment of cerebral ischemia. Front. Biosci. 2, 1081–1091.

Yang, Z., Zhao, T., Zou, Y., Zhang, J.H., Feng, H., 2014. Curcumin inhibits microglia inflammation and confers neuroprotection in intracerebral hemorrhage. Immunol. Lett. 160, 89–95.

Yeon, K.Y., Kim, S.A., Kim, Y.H., Lee, M.K., Ahn, D.K., Kim, H.J., Kim, J.S., Jung, S.J., Oh, S.B., 2010. Curcumin produces an antihyperalgesic effect via antagonism of TRPV1. J. Dent. Res. 89, 170–174.

Yoneyama, M., Fujita, T., 2008. Structural mechanism of RNA recognition by the RIG-I-like receptors. Immunity 29, 178–181.

Youdim, K.A., Joseph, J.A., 2001. A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects. Free Radic. Biol. Med. 30, 583–594.

Zamanian, J.L., Xu, L., Foo, L.C., Nouri, N., Zhou, L., Giffard, R.G., Barres, B.A., 2012. Genomic analysis of reactive astrogliosis. J. Neurosci. 32, 6391–6410.

Zhang, J., Hu, Z., Lu, C., Bai, K., Zhang, L., Wang, T., 2015. Effect of Various Levels of Dietary Curcumin on Meat Quality and Antioxidant Profile of Breast Muscle in Broilers. J. Agric. Food Chem. 63, 3880–3886.

Zhang, Q., Raoof, M., Chen, Y., Sumi, Y., Sursal, T., Junger, W., Brohi, K., Itagaki, K., Hauser, C.J., 2010. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature 464, 104–107.

Zhao, X., Xu,Y., Zhao, Q., Chen, C.-R., Liu A.-M., Huang, Z.-L., 2012. Curcumin exerts antinociceptive effects in a mouse model of neuropathic pain: descending monoamine system and opioid receptors are differentially involved. Neuropharmacology 62, 843–854.

Zhou, H., Beevers, C.S., Huang, S., 2011. The targets of curcumin. Curr. Drug Targets 12, 332–347.

Ziegler, G., Freyer, D., Harhausen, D., Khojasteh, U., Nietfeld, W., Trendelenburg, G., 2011. Blocking TLR2 in vivo protects against accumulation of

inflammatory cells and neuronal injury in experimental stroke. J. Cereb. Blood Flow Metab. 31, 757–766.

Don't get stuck in the "comparison trap".
Just challenge yourself everyday
and keep getting better and better…

Anonymous