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THE ROLE OF MICROGLIA IN METHAMPHETAMINE EXPOSURE

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The Role of Microglia in Methamphetamine Exposure

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"A menos que modifiquemos a nossa maneira de pensar, não seremos capazes de resolver os problemas causados pela forma como nos acostumamos a ver o mundo".

(Albert Einstein)

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Resumo

A metanfetamina (Meth) é um psicostimulante altamente viciante que causa graves consequências à saúde e que produz efeitos neurotóxicos a longo prazo. As células da microglia, macrófagos residentes do sistema nervoso central (SNC), desempenham um papel crucial nos processos inflamatórios. Uma vez activada, a microglia segrega uma variedade de moléculas, incluindo citocinas pró-inflamatórias, óxido nítrico e superóxido, que podem causar danos oxidativos no tecido neuronal. Recentemente, a microglia tem vindo a ser considerada como mediador da neurotoxicidade induzida pela Meth. Além disso, tem sido descrito que Meth induz activação das células da microglia no cérebro de consumidores humanos.

Neste estudo, investigámos os efeitos da Meth nas células da microglia e nos astrócitos, tal como na intercomunicação entre estes dois tipos de células. De forma a obter maior conhecimento sobre a activação da micróglia, analisamos os efeitos deste psicostimulante em culturas primárias de microglia através de marcadores pró- e anti-inflamatórios utilizando imunocitoquímica, microscopia de fluorescência quantitativa e RT-PCR quantitativo. Mostramos que a Meth não induz uma assinatura nem pró- nem anti-inflamatória na microglia. Estudámos também os efeitos da Meth em culturas primárias de astrócitos através de marcadores de reactividade (F-actina, GFAP e iNOS). Os nossos resultados demonstram que Meth não induz astrogliose *in vitro*. Na tentativa de esclarecer de que forma a Meth poderia afectar a *intercomunicação* entre as células da microglia e os astrócitos, avaliámos o efeito do meio condicionado de astrócitos (ACM) tratados com Meth na microglia. Verificamos que este ACM altera o perfil da microglia promovendo uma assinatura pró-inflamatória, verificando-se um aumento significativo da actividade fagocitária, produção de espécies reactivas de oxigénio e expressão da iNOS comparativamente com células incubadas com meio condicionado de astrócitos não tratados. Em suma, aqui demonstramos que Meth, por si só não induz nem um perfil pró- nem anti-inflamatório em células da microglia cortical. A Meth também não aumenta a reactividade dos astrócitos em cultura. No entanto, a activação da microglia induzida pela Meth aparece ser mediada por factores solúveis libertados pelos astrócitos tratados com Meth, sugerindo que Meth induz a activação da microglia de uma forma dependente dos astrócitos.

Keywords: Assinatura pró-inflamatória; Astrócitos; Meio condicionado de astrócitos; Metanfetamina; Microglia.

Abstract

Methamphetamine (Meth) is a highly addictive psychostimulant posing severe health consequences and long-term neurotoxic effects. Microglial cells, the resident macrophages of the central nervous system (CNS), play crucial roles in inflammatory processes. Upon activation, microglia secrete an array of molecules including pro-inflammatory cytokines, nitric oxide and superoxide, which can cause oxidative damage to the neuronal tissue. Recently, microglia have been implicated as mediators of Meth-induced neurotoxicity. Besides, it has been described that Meth induces microglial cell activation in the brain of human abusers.

In this study, we investigated the effects of Meth in microglial cells and astrocytes, as well as the crosstalk between these two cell types. To gain further insight into microglia activation we analyzed the effects of this psychostimulant in pro- and anti-inflammatory markers in primary cortical microglial cell cultures using immunocytochemistry, quantitative fluorescence microscopy and quantitative RT-PCR. We show that Meth triggered neither a pro-inflammatory nor an anti-inflammatory signature in microglia. We also studied the effects of Meth on primary astrocyte cultures using reactivity markers (f-actin, GFAP and iNOS). Our results demonstrate that Meth did not trigger astrogliosis in vitro. Moreover, in an attempt to clarify how Meth could affect the crosstalk between microglial cells and astrocytes, the effect of conditioned media from astrocytes (ACM) treated with Meth was evaluated on microglia. We found that ACM treated with Meth shifted microglia profile to a pro-inflammatory signature significantly increasing in their phagocytic activity, reactive oxygen species production and iNOS expression when compared to cells incubated with conditioned media from naïve astrocytes. Overall, here we describe that Meth per se does not trigger neither a pro nor an anti-inflammatory signature in cortical microglial cells. Likewise, Meth does not increase the reactivity of cultured astrocytes as well. Nonetheless, Meth-induced microglia activation appears to be mediated by soluble factors released from Meth-sensitized astrocytes, suggesting that Meth induces microglial activation in an astrocyte-dependent fashion.

Keywords: Astrocyte conditioned medium; Astrocyte; Methamphetamine; Microglia; Pro-inflammatory signature.

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Abbreviations

ACM Astrocytes conditioned medium
ADP Adenosine diphosphate
AIF Apoptosis inducing factor
ANOVA Analysis of variance
ATP Adenosine triphosphate
BBB Blood-brain barrier
BDNF brain-derived neurotrophic factor
BSA Bovine serum albumin
Ca^{2+} Calcium
CCL2 chemokine (C-C motif) ligand 2
CM Conditioned medium
CNS Central Nervous System
CO_2 Carbon dioxide
Csf-1 Colony stimulating factor 1
CX3CL Chemokine (C-X3-C motif) ligand 1
CXCL10 C-X-C motif chemokine 10
DA Dopamine
DAT Dopamine transporter
DMEM Dulbecco's Modified Eagle's Medium
FBS Fetal bovine serum
GFAP Glial fibrillary acidic protein
GM-CSF Granulocyte macrophage colony-stimulating factor
H_2O_2 Hydrogen peroxide
HBSS Hank's balanced salt solution
Iba-1 Ionized calcium binding adaptor molecule 1
IGF1 insulin-like growth factor 1
IL-10 Interleukin 10
IL-13 Interleukin 13
IL-1β Interleukin 1β
IL-23 Interleukin 23
IL-34 Interleukin 34
IL-4 Interleukin 4
IL-6 Interleukin 6

INF-Ɣ Interferon-ϒ

MAO Monoamine oxidase

Meth Methamphetamine

MHC-II Major histocompatibility complex class II

MP-PFA Paraformaldehyde microtubule protection buffer

NGF nerve growth factor

NLRs nucleotide-binding oligomerization domain receptors

NMDA N-Methyl-D-aspartate

NOS Nitric oxide synthase

PAMPs Pattern recognition receptors

PBS Phosphate-buffered saline

PRRs Pathogen-associated molecular patterns

qRT-PCR Quantitative reverse transcriptase polymerase chain reaction

RNS Reactive nitrogen species

ROS Reactive oxygen species

RT Room temperature

SEM Standard error of the mean

TGFβ Transforming growth factor-β

TH Tyrosine hydroxylase

TLRs Toll-like receptors

TNF- α Tumor necrosis factor α

VMAT Vesicular monoamine transporter

YWHAZ Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

cDNA Complementary deoxyribonucleic acid

mRNA Messenger ribonucleic acid

1 Overview

1.1 Methamphetamine

Methamphetamine (Meth) is an illegal psychostimulant drug that is widely abused all over the world. It belongs to the group of amphetamines and it is estimated that there are about 14 to 52 million users worldwide (Granado, Ares-Santos et al. 2013). Meth is the second most consumed drug of abuse after cannabis and presents a higher consumption rate in young-aged groups, (Krasnova and Cadet 2009) which establishes Meth consumption to be considered a significant public health problem in several countries (Karila, Weinstein et al. 2010).

The Japanese scientist Nagai Nagayoshi first synthesized Meth from ephedrine in 1893, six years after the synthesis of amphetamine (Anglin, Burke et al. 2000, Panenka, Procyshyn et al. 2013). The chemical structure of Meth (N-methyl-O-phenylisopropylamine) comprehends a phenyl ring connected to an amino group by two-carbon side chain with a methyl group on carbon-1 of the side chain (Fleckenstein, Volz et al. 2007, Huang, Wu et al. 2009, Krasnova and Cadet 2009). There are two enantiomers of this compound, D-Methamphetamine and L-Methamphetamine (Fig.1). The D-Methamphetamine is the most powerful and is the form manufactured for illicit use (Petit, Karila et al. 2012).

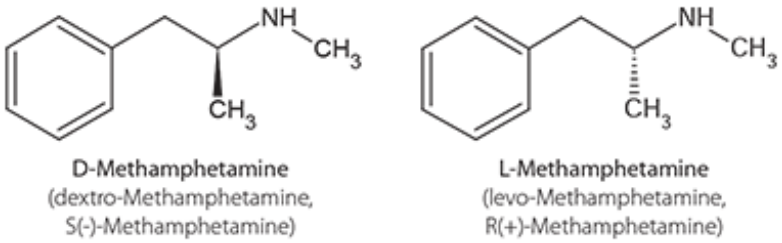


Figure 1. Chemical structure of Meth isomers: D- Methamphetamine and L-Methamphetamine (adapted from http://www.sigmaaldrich.com/technical-documents/articles/analytical/bioanalytical/chiral-lcms-methamphetamine.html).

This illicit drug, also called ice, Meth, crank, jib, speed or crystal, is available in different forms such as a pure crystalline hydrochloride salt or as formulated tablets. Meth is soluble in water, and may be administered by intranasal sniffing, pulmonary inhalation, injection and oral ingestion (Karila, Weinstein et al. 2010, Granado, Ares-Santos et al. 2013, Panenka, Procyshyn et al. 2013). Acute use of Meth leads to euphoria, increased vigilance, cardiovascular changes, hyperactivity, hypersexuality, decreased anxiety and increased energy. Chronic abuse of this psychostimulant can, however, result in adverse physiological and neurological disorders, including weight and muscular loss, accelerated aging, brain injury, cognitive impairment, mood disturbances and psychomotor dysfunction. Meth symptoms may persist for several hours due to its half-life, which is about 10 to 12 hours (Krasnova and Cadet 2009).

1.1.1 Neurotoxicity

Meth consumption leads to neuropsychiatric and neurotoxic impairment in humans. Meth is lipophilic, which allows the compound to rapidly cross the blood-brain barrier (BBB). In the brain, Meth leads to an impairment of the monoaminergic system (dopamine, serotonin and norepinephrine), BBB breakdown, glutamate release, overproduction of free radicals, mitochondrial dysfunction and neuroimflammation that all-together contribute for neuronal cell death and neurotoxicity (Huang, Wu et al. 2009).

1.1.1.1 Blood Brain Barrier dysfunctions

Several studies examining the effects of Meth on the BBB function have shown that it leads to the breakdown of the BBB. Such effect has been established as one of the most prominent events of Meth toxicity (Sharma and Ali 2006). In rodents, it has been shown that Meth leads to BBB damage in the cortex, hippocampus, thalamus, hypothalamus, cerebellum and amygdala (Bowyer and Ali 2006, Kiyatkin, Brown et al. 2007, Krasnova and Cadet 2009, Sharma and Kiyatkin 2009).

In line with this, some studies suggested that neurotoxic effects induced by Meth such as hyperthermia, ROS production and neuroimflammation lead to an increase in BBB permeability (Ramirez, Potula et al. 2009). Oxidative stress and

cytokines production seem to be a possible mediator of BBB breakdown, since these molecules may modify tight junction function and extracellular matrix remodelling, which leads to the impairment of endothelial cells form the BBB (Yamamoto, Moszczynska et al. 2010).

1.1.1.2 Dopaminergic system dysfunction

Dopamine (DA) is an important neurotransmitter that modulates locomotion, motivation, cognition and reward-associated functions. Dopaminergic dysfunction has been implicated in several pathologies such as Parkinson, Schizophrenia, bipolar disorder, attention deficits, hyperactivity and drug addiction (Saha, Sambo et al. 2014). Under normal conditions, DA is released into the synaptic cleft in response to neuronal activity. When stimuli cease, DA is taken up through dopamine transporters (DAT), which are located at the plasma membrane of the pre-synaptic neuron. Once inside the neuron, DA is stored into vesicles by vesicular monoamine transporter (VMAT), which protects DA from oxidation (Krasnova and Cadet 2009).

Due to the structural similarity between Meth and DA (Fig.2), Meth can enter dopaminergic neurons via DAT, by acting as a competitive inhibitor for DA re-uptake, leading to DA accumulation in the synaptic cleft. Such mechanistic route seems to be important for Meth toxicology as DAT-knockout mice are resistant to the persistent dopaminergic deficits produced by Meth administration (Volz, Hanson et al. 2007).

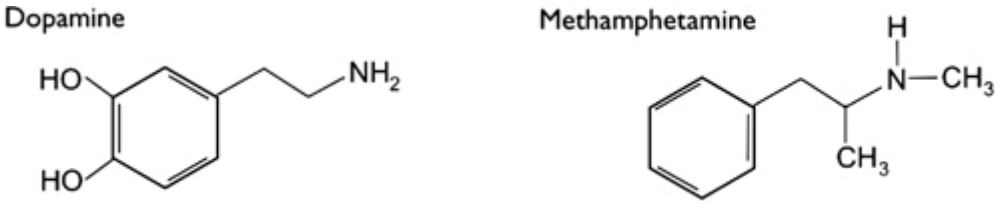


Figure 2. Chemical structure of DA (Dopamine) and Meth (Methamphetamine). Adapted from (Hart, Marvin et al. 2011).

Meth lipophilic diffusion across neuronal membranes as also been described (Saha, Sambo et al. 2014). Once inside the cell, Meth promotes cytoplasmatic accumulation of monoamines, which would be stored in synaptic vesicles, via the inhibition of VMAT. While such effect causes a rapid depletion of DA from synaptic vesicles (Volz, Hanson et al. 2007, Krasnova and Cadet 2009), this accumulation in the cytosol leads to an increased metabolism of DA by monoamine oxidase A (MAO-A), resulting in the production of high amounts of H_2O_2 and other reactive oxygen species (ROS; Fig.3). Moreover, DA is also oxidized forming reactive quinones, which modifies lipids and proteins at neuronal membranes, further increasing the formation of ROS (Brown and Yamamoto 2003). Besides, Meth inhibits the expression of tyrosine hydroxylase (TH), an essential enzyme for dopamine biosynthesis (Thomas and Kuhn 2005). Taken together, these studies show that Meth leads to a complete disruption of cellular DA homeostasis in the dopaminergic neuron.

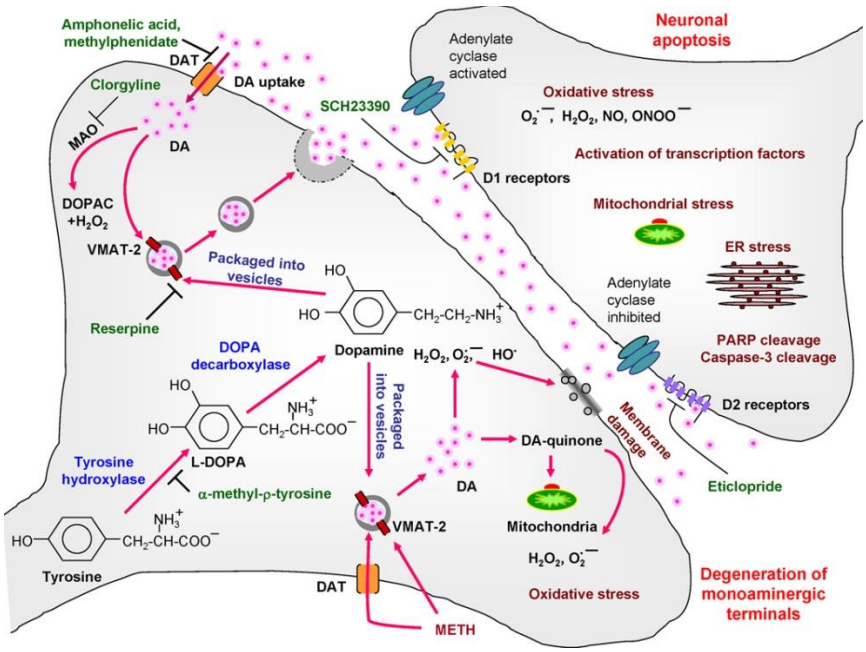


Figure 3. Mechanisms whereby Meth causes dopaminergic system dysfunction: DA, dopamine; DAT, dopamine transporters; MAO, monoamine oxidase; VMAT-2, vesicular monoamine transporter; METH, methamphetamine; DOPAC, 3,4-Dihydroxyphenylacetic acid; RE, endoplasmic reticulum; L-DOPA, L-3,4-dihydroxyphenylalanine. Adapted from (Krasnova and Cadet 2009).

1.1.1.3 Mitochondrial dysfunction

Due to its lipophilic properties, Meth penetrates and accumulates in the mitochondria. Such accumulation of positively charged molecules disrupt the electrochemical gradient established by the oxidative phosphorylation, impairing ATP synthesis, which leads to energy deficits and increases ROS generation. This loss in mitochondrial membrane potential, induced by Meth, also leads to the release of mitochondrial proteins such as cytochrome C and apoptosis inducing factor (AIF) into the cytosol. Likewise, it has been shown that cytochrome C is implicated in cytoskeleton rearrangements through proteolysis of cytoskeletal proteins in a caspase-3-dependent manner (Fig.4) (Krasnova and Cadet 2009).

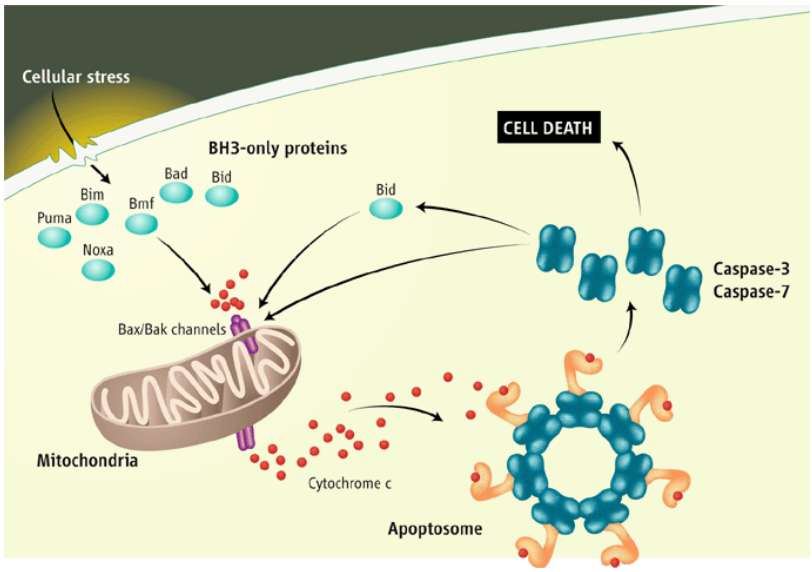


Figure 4. The role of caspase activation in the apoptotic pathway. Cellular stress promotes the expression of BH3 proteins, which binds Bax/Bak channels in the mitochondria, leading to the release of cytochrome c. Cytochrome c activates the apoptosome, which activates both caspases 3 and 7 and triggers apoptosis. Adapted from (Adrain and Martin 2006).

1.1.1.4 Excitotoxicity

Excitotoxicity is mediated by exacerbated glutamate release and activation of ionotropic glutamate receptors, mainly the NMDA sub-type. It has already been shown that Meth induces an exacerbated release of glutamate causing neurotoxicity (Krasnova and Cadet 2009). Increased nitric oxide (NO) and reactive

nitrogen species (RNS) induced by Meth administration, inhibits oxidative phosphorylation, causing ATP depletion in neurons. Such effect is followed by a disruption in the sodium gradient across the neuronal plasma membrane and alters the membrane potential, resulting in further glutamate release and neuronal damage (Brown 2010). This increase in glutamate release induces hyperactivation of the NMDA receptors, increasing Ca^{2+} influx and activating several intracellular enzymes, such as Nitric Oxide Synthase (NOS). The calcium influx and the activation of NOS can lead to the establishment oxidative stress in neurons by the formation of ROS and RNS, further contributing for neuronal damage and neurodegeneration (Dawson, Beal et al. 1995, Lipton and Nicotera 1998).

1.1.1.5 Reactive species and hyperthermia

The production of ROS and RNS induced by Meth arises mainly from the disruption of dopaminergic system and excessive DA oxidation and the subsequently formation of toxic substances such as the radical superoxide (Yamamoto and Zhu 1998). ROS react with and oxidize many cellular constituents such as proteins, nucleic acids and lipids. While the brain is particularly vulnerable to the excess of such reactive species, oxidative stress can significantly disrupt neuronal signalling and synaptic homeostasis (Slemmer, Shacka et al. 2008).

Hyperthermia appears as an important factor in neurotoxic responses induced by Meth (Krasnova and Cadet 2009). Meth increases body temperature by enhancing heat production and retention. This psychostimulant increases metabolic activation and augment vasoconstriction directly contributing for hyperthermia induction (Matsumoto, Seminerio et al. 2014). Even though it is not clear how Meth induces hyperthermia, it seems that the disruption of the dopaminergic system is a critical event. Studies have demonstrated that the prevention of hyperthermia by low ambient temperature blocked the Meth-induced increase in DA oxidation, preventing its toxic effects. Likewise, it has been shown that hyperthermia facilitates Meth-induced ROS production further potentiating the Meth toxic effects (Krasnova and Cadet 2009).

1.2 Neuroinflammation

Neuroinflammation is a term used to designate immune responses at the level of central nervous system (CNS). This inflammatory response differs from peripheral inflammation mainly by the cells involved. In inflammatory responses of the brain parenchyma, the cells involved are mainly, microglia, astrocytes, neurons and circulating leukocytes. BBB that physically separates the CNS from others tissues can be stimulated and become more permeable, enabling the entry of leukocytes into the brain. TNF-α, IL-6, IL-1β and others pro-inflammatory cytokines modulate the permeability of BBB (Merrill and Murphy 1997). Exacerbation of neuroinflammatory responses can result in synaptic impairment, neuronal cell death and several neurodegenerative diseases (Lyman, Lloyd et al. 2014).

1.2.1 Microglia

Nissl was the first to recognize microglial cells, characterizing them as reactive neuroglia and suggesting that such cells had the capacity of migrating and phagocyting (Nissl 1899). Many years later, in 1932, Pio del Rio-Hortega, for the first time, distinguished microglia from macroglia and suggested that the formers were of mesodermal origin. Hortega also proposed that microglia would enter the brain early during development and would be found dispersed throughout the CNS (del Río Hortega 1932). He additionally recognized that microglia experience cell shape transformation after a pathologic condition, acquiring an amoeboid morphology (Kettenmann, Hanisch et al. 2011).

Nowadays, all of these statements are not only perfectly plausible but also well characterized. Microglia are a small and distinct population of glial cells that differs from macroglia in origin, function, morphology and gene expression profile. Glial cells represent more than 90% of the human brain, and 10% of the adult glial cell population in the normal brain is composed of microglia. These cells can support and protect neuronal cells, being distributed throughout the brain and the spinal cord parenchyma, although there are differences between their density in different CNS regions. The hippocampus, basal ganglia and *substantia nigra* are the most microglia-enriched areas of the CNS (Kim and de Vellis 2005, Walter and Neumann 2009, Greter and Merad 2013).

1.2.1.1 Origin of Microglia

Unlike the other cells in the CNS, that are derived from the neuroectoderm, microglia are derived from the mesoderm. Before the BBB formation, microglial cells derive from embryonic hematopoietic precursors that colonize the CNS during early development. Primitive macrophages derived from the yolk sac migrate through the blood vessels to the neuroepithelium to originate microglia. After that, the BBB formation segregates the developing brain from fetal liver haematopoiesis, isolating the primitive macrophages poll, which segregates these populations. Microglial cells expand and colonize the whole CNS and maintain themselves until adulthood, proliferating during all development as well as in the challenged adult brain during inflammatory processes (Ling and Wong 1993, Ginhoux, Lim et al. 2013).

However, this does not mean that in special cases, as inflammatory conditions, circulating monocytes or any other bone marrow-derived progenitors cannot migrate into the CNS, to support the resident microglia population (Fig.5). Nevertheless, it is not clear whether this foreign population persist to become permanently incorporated in the neuronal parenchyma, or are a momentary addiction to the endogenous pool. It also remains unknown if new microglia population are functionally and morphologically equivalent to the resident microglia (Saijo and Glass 2011, Ginhoux, Lim et al. 2013, Greter and Merad 2013).

Microglial cells display a plethora of phenotypic markers of the macrophage lineage and several of them are surface antigens/receptors with important functional roles. These small cells express the Csf-1 receptor (CD115), fraktalkine receptor (Cx3cr1), glycoproteins (F4/80; CD68), integrin (CD11b), Iba-1 and low levels of CD45 (Greter and Merad 2013).

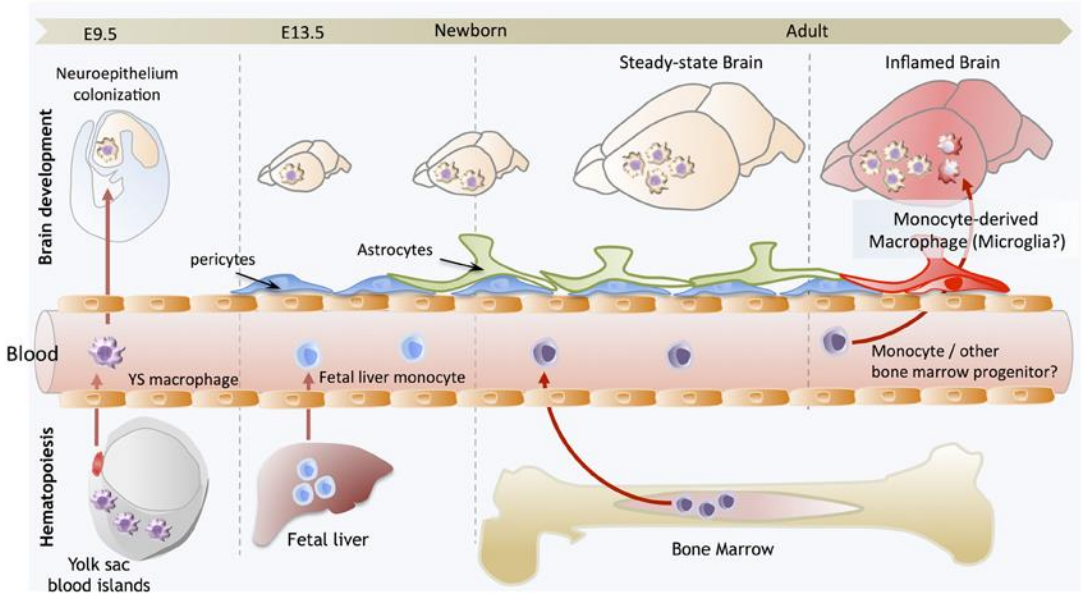


Figure 5. Primitive macrophages existing in the yolk sac migrate and colonize the neuroepithelium to give rise to microglia. These cells colonize the CNS by proliferating during brain development. However, in the inflamed brain, circulating monocytes can also enter the CNS for supplementing further the microglia population. Adapted from (Ginhoux, Lim et al. 2013).

1.2.1.2 Steady-state microglia and homeostasis in the CNS

Adequate microglia functioning is essential for the CNS homeostasis in health and disease. Non-appropriate microglia activation is observed in brain pathologies, contributing for disease progression and neuronal damage. Microglia are not only the first cell type that respond to a CNS challenge, such as infiltrating pathogens but also execute important roles in CNS homeostasis, such as regulation of synapse function, neurogenesis, production of trophic factors and clearance of apoptotic cells and debris (Cronk and Kipnis 2013).

In normal conditions microglia are in the steady state, exhibiting an extensively branched morphology. In the surveillance state, these cells surround the healthy CNS parenchyma continuously extending and retracting their processes, effectively surveilling the CNS parenchyma (Boche, Perry et al. 2013). This surveillance state can be modulated by neuron-derived signals. The CX3C-chemokine ligand 1, which is expressed by neurons, binds to CX3C receptor (CX3CR1) in microglia, playing an important role in inhibiting microglia-induced neurotoxicity. Also, in this branching phenotype, microglia secrete neurotrophic factors such as insulin-like growth factor 1 (IGF1), nerve growth factor (NGF),

transforming growth factor-β (TGF-β) and brain-derived neurotrophic factor (BDNF) that are important for neuronal function and synaptic activity. Still, the interaction between neuronal proteins such as CD47, CD200 and CD22 with their respective receptors in microglia (CD172, CD200R, CD45) has also been reported to block pro-inflammatory activation of microglia (Fig.6) (Hanisch 2002, Saijo and Glass 2011, Chavarría and Cárdenas 2013, Suzumura 2013). However, this ramified microglia stage rapidly modifies when these cells detect some environmental change and, in response, microglia migrate to the area of this misbalance and rapidly change their morphology and function (Cronk and Kipnis 2013).

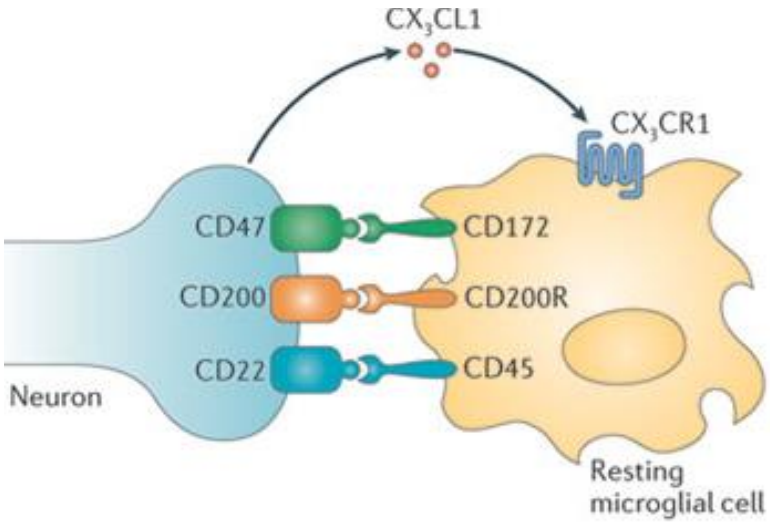


Figure 6. Under normal conditions, microglia exhibit a branched morphology. This phenotype is maintained in part through interactions between neuronal signals and the correspondent microglia receptors. Adapted from (Saijo and Glass 2011).

1.2.1.3 Modulation of microglial cell phenotypes

According to macrophages classification, microglia are classified into M1 and M2 phenotypes. The M1 phenotype or classical activation is cytotoxic, with increased phagocytosis and release of pro-inflammatory cytokines, such as, IL-1β, TNF, IL-6 and IL-23 in addition to ROS and RNS. Interferon, lipopolysaccharide (LPS) and TNF stimulus are associated with triggering the M1 polarization, a response associated with host defence to pathogens. However, this classical activation may cause damage to others cells, inclusive neurons.

M2 polarization or alternative activation is characterized by a neuroprotective profile through the release of anti-inflammatory cytokines and growth factors. There are two types of M2 polarization, M2 wound healing and M2 regulatory. The M2 wound healing is stimulated by IL-4 and IL-13 and this function is related to tissue repair, phagocytosis and increased production/ remodelling of the extracellular matrix. In this phenotype, microglia produce extracellular matrix complements, arginase 1 and chitinase. On the other hand, the M2 regulatory phenotype is stimulated by IL-10, displays high phagocytic activity and inhibits inflammatory processes. This regulatory/alternative activation also includes the production of TGFβ1 and IL-10 (Raivich, Bohatschek et al. 1999, Saijo and Glass 2011, Boche, Perry et al. 2013).

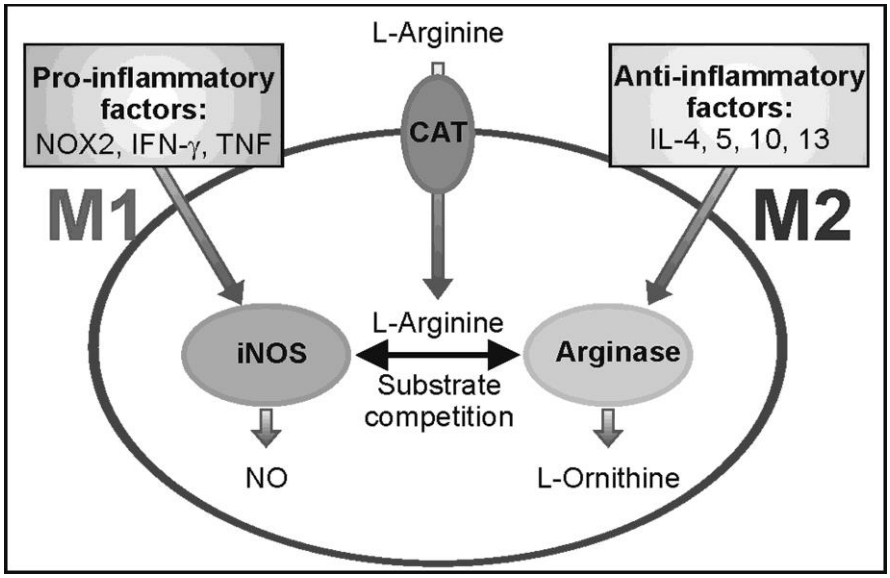


Figure 7. Microglial cells polarization: M1 and M2. Pro-inflammatory factors such as INFϒ and TNF induce microglial M1 phenotype and anti-inflammatory factors such IL-4, IL-5 and IL-10 polarize microglia for M2 phenotype. NOX2:; INFϒ: Interferon-ϒ; TNF: Tumor necrosis factor; IL-4:Interleukin 4; IL-5:Interleukin 5; IL-10:Interleukin 10; IL-13:Interleukin 13. Adapted from (Rojo, McBean et al. 2014).

1.2.1.3.1 Classical microglia activation

Activated microglia are identified by an "amoeboid" morphology in response of process retraction, becoming highly motile, migrating to the misbalanced area, proliferating and phagocyting cell debris and/or damaged

neurons. Once activated, microglia secrete a variety of factors such as inflammatory cytokines, ROS and RNS, in addition to an enhance in the expression of MHCII, which is required for activation of naïve T lymphocytes (Fig.8) (Hanisch 2002, Saijo and Glass 2011).

Microglia cells express pattern recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs). The recognition of PAMPs by microglia through PRRs normally induces the classical microglial activation (Saijo and Glass 2011).

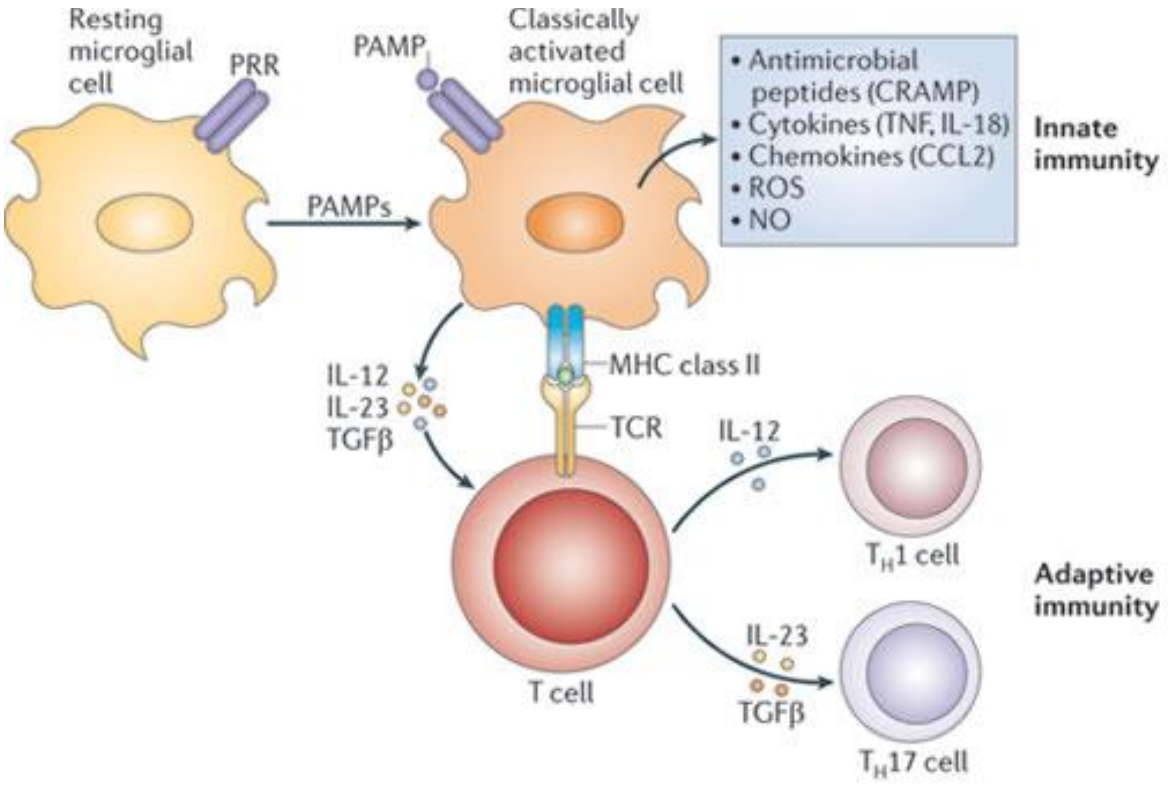


Figure 8. Classical activation microglial cells triggered by PAMPs participate in both innate and adaptive responses. Microglia express pattern recognition receptors (PRRs) that identify various pathogen-associated molecular patterns (PAMPS). Following the recognition of PAMPs microglia induces the production of cathelicidin-related antimicrobial peptide (CRAMP), TNF, IL-18, CCL2, ROS and NO. Activated microglia also express MHC class II molecules to enable them to present antigens to T cells. In addition, activated microglia produce IL-12, IL-23 and TGF β that activates T cells. CCL2: CC-chemokine ligand 2; ROS: reactive oxygen species; NO: nitric oxide; TCR: T cell receptor; TNF: tumour necrosis factor; IL-18: Interleukin 18; IL-23: Interleukin 23; IL-12: Interleukin 12; TGF β: Transforming growth factor β; MHC class II: Major histocompatibility class II. Adapted from (Saijo and Glass 2011).

1.2.1.3.2 Alternative microglial activation

Alternative microglial activation is associated with damage resolution, such as tissue repair and extracellular matrix reconstitution. This microglial activation is induced by anti-inflammatory cytokines such as IL-4, IL-13 and IL-10. Once activated, microglial cells produce IL-10, IL-4, IGF-1 and TGF-β, which contribute for inflammatory resolution. This phenotype is characterized by the modification of inflammatory signals, promoting debris clearance and neuroprotective proprieties (Fig.9) (Saijo and Glass 2011, Zhou, Spittau et al. 2012, Wang, Zhang et al. 2013).

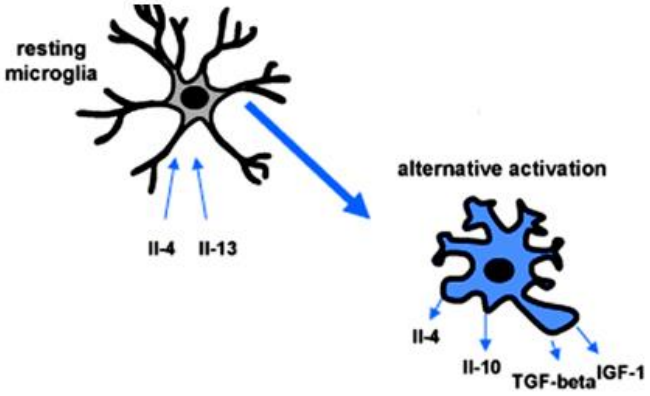


Figure 9. Alternative microglial activation induced by IL-4 and IL-13. This activation induces production of IL-4, IL-10, TGF-β and IGF-1. IL-4: interleukin 4; IL-13: interleukin 13; IL-10: interleukin 10; TGF- β: transforming growth factor 1 and IGF-1: insulin-like growth factor 1. Adapted from (http://jonlieffmd.com/blog/are-microglia-the-most-intelligent-brain-cells).

1.2.1.4 Microglial migration and proliferation

In a trauma response, microglia become amoeboid (round and disbranched) and migrate to the injury site. Damage in the CNS leads to a release of chemotactic factors such as CXCL10, which induces microglial migration. Studies have demonstrated *in vivo* and *in vitro* that microglia migrate along chemotactic gradients including morphine, epidermal growth factor, cannabinoids and bradykinin (Walter, Franklin et al. 2003, Garden and Möller 2006, Noda and Suzumura 2012, Shi, Yuan et al. 2013). Extracellular ATP and ADP release from traumatic brain also stimulate microglia migration. Besides,

NOS play an important role in microglial migration. A significant reduction in microglial accumulation was observed in lesion sites with decreased NOS production (Chen, Kumar et al. 2000, Garden and Möller 2006).

Microglia proliferation is essential for an adequate immune response. Cytokines such as IL-1β, IL-4 and INFϒ have been described as proliferation inducers; however, the most potent factor that induces microglia proliferation is GM-CSF. Neurotrophic factors, such as BDNF and NT-3, released by activated microglia, also induce the proliferation of these cells (Garden and Möller 2006).

1.2.1.5 Microglia phagocytosis

Microglia are the predominant phagocytes in the CNS. Phagocytosis is the processes that comprehend recognition, internalization and digestion of the antigen by a phagocytic cell (Lampron, Pimentel-Coelho et al. 2013). Recognition mechanisms can be mediated by cellular receptors of the pathogen by antibodies or complement proteins, which are recognized by specific receptors in the phagocyting cell.

There are two distinct functional types of phagocytic receptors in microglia: (1) receptors that recognise pathogens and simultaneously stimulate pro-inflammatory responses, for example, in the case of Toll-like receptors (TLRs) or Fc- receptors' which interaction with antigens, and (2) receptors that identify apoptotic material and stimulate an anti-inflammatory response, such as phosphatidylserine (PSRs) or receptor expressed on myeloid cells-2 (TREM-2) (Fig.8). The removal of apoptotic cells by phagocytosis is defined as a silent process, which is essential during brain development, for example (Neumann, Kotter et al. 2009).

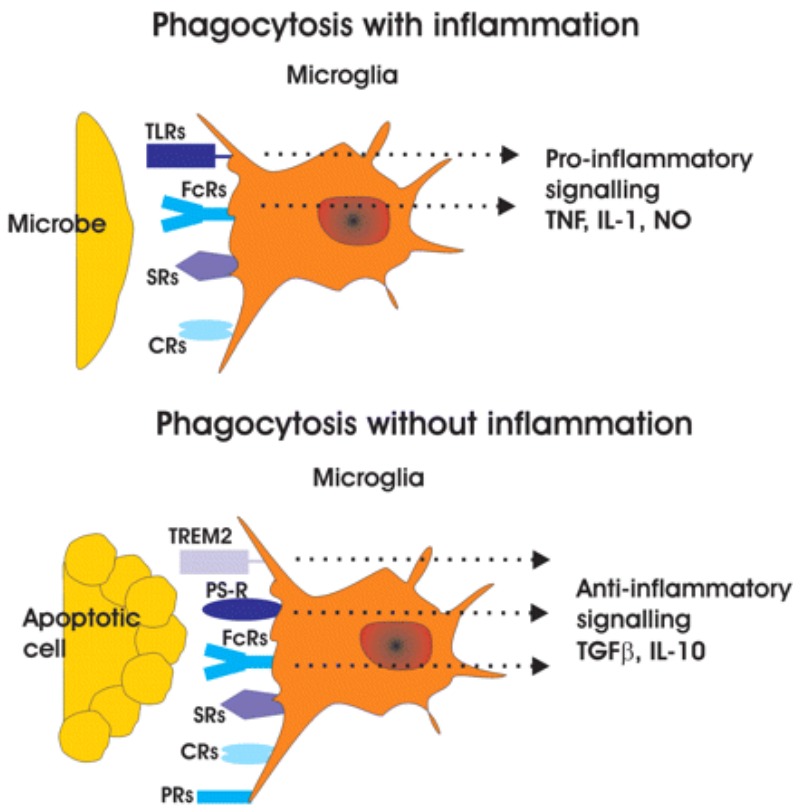


Figure 10. Microglia phagocytic response. In inflammatory response microglia detect the pathogen, which induces the phagocytic response and leads to the release pro-inflammatory mediators by microglia. Without inflammation microglial cells detect apoptotic cells, phagocyte and release anti-inflammatory signals. Phagocytic receptors: purine receptors (PRs); phosphatidylserine receptors (PSRs); complement receptors (CRs); toll like receptors (TLRs); Fc-receptors (FcR); scavenger receptors (SRs) and triggering receptor expressed on myeloid cells-2 (TREM2). Adapted from (Neumann, Kotter et al. 2009).

1.2.2 Astrocytes

Astrocytes are the major and most abundant glial cell type in the CNS (Mahesh, Dhandapani et al. 2006). These specialized cells outnumber neurons by over five-fold and are ubiquitous throughout all CNS regions (Hamby and Sofroniew 2010). Astrocytes, as the name suggests, are star-shaped cells distributed throughout the brain and the spinal cord (Montgomery 1994). These glial cells are derived from the neuroectoderm during embryonic development and are essential for brain homeostasis in health and disease (Farina, Aloisi et al. 2007). Traditionally, these cells are divided into two main subtypes, protoplasmic and fibrous based on their morphologic appearance and anatomical localization in the CNS (Montgomery 1994). Protoplasmic astrocytes are found in the grey matter and exhibit many branching processes. On the other hand, fibrous

astrocytes are most common in the white matter bearing many long processes, which intersect blood capillaries through their end-feet (Gee and Keller 2005). Functionally, protoplasmic astrocytes are associated with neuronal cell bodies and synapses while fibrous astrocytes are associated with axons (Allen and Barres 2009, Chaboub and Deneen 2012).

Astrocytes play important physiologic roles in the brain, including the regulation of blood flow, which supplies neurons with energy and other metabolites, participation in synaptic dynamics, maintenance of extracellular ionic homeostasis and recycling of neurotransmitters (Figure.11) (Allen and Barres 2009, Hamby and Sofroniew 2010).

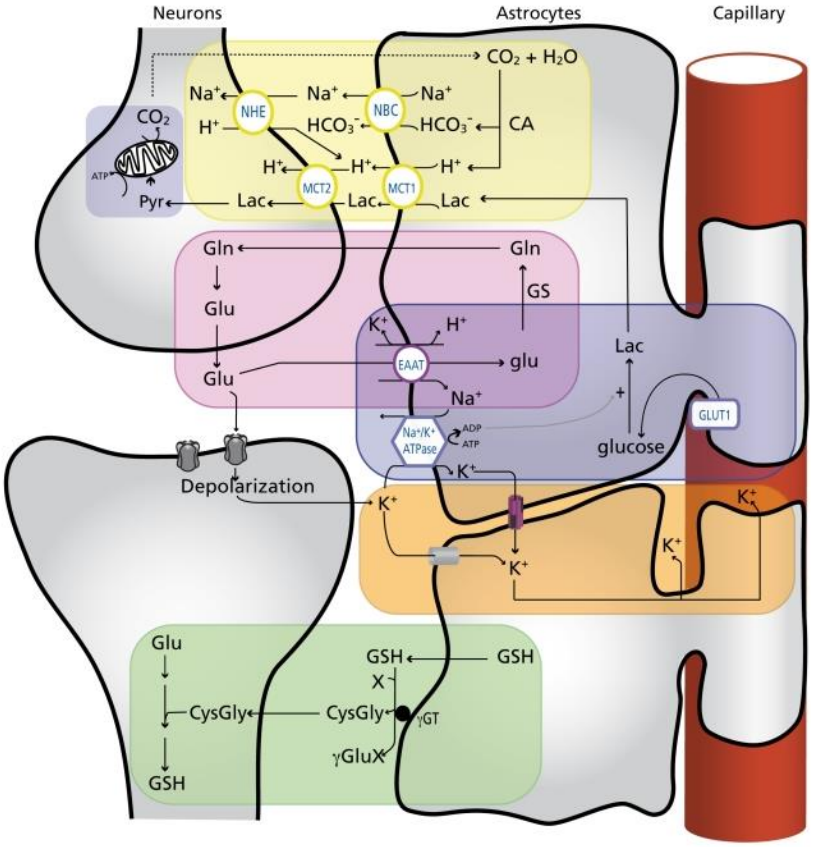


Figure 11. Representation of the main roles of astrocytes in brain homeostasis. Astrocytic excitatory amino acid transporters (EAATs) are responsible for the uptake of a large fraction of glutamate (Glu) at the synapse. Glu is converted into glutamine (Gln) by glutamine synthetase (GS) and shuttled back to neurons for glutamate resynthesis. Glutamate uptake by astrocytes is accompanied by Na^+ entry which is counteracted by the action of the Na^+/K^+ ATPase. The resulting increase in ADP/ATP ratio triggers anaerobic glucose utilization in astrocytes and glucose uptake from the circulation through the glucose transporter GLUT1. The lactate (Lac) produced is shuttled to neurons through monocarboxylate transporters MCTs, where it can be used as an energy substrate after its conversion to pyruvate (Pyr). Abundant carbonic anhydrase (CA) in astrocytes

converts CO^2 into H^+ and HCO_3^-. Two HCO_3^- are transported into the extracellular space along vyith one Na^+ via the Na^+-HCO_3^- co-transporter (NBC), thereby increasing the extracellular buffering power. Protons left in the glial compartment may drive the transport of lactate outside of astrocytes and into neurons through MCTs. Excess H^+ in neurons is extruded via sodium-hydrogen exchange (NHE). Astrocytes buffer excess potassium ions (K^+) released into the extracellular space as a result of neuronal activity. K^+ travel through the astrocytic syncitium down their concentration gradient and are released in sites of lower concentration. Astrocytes release glutathione (GSH) in the extracellular space where it is cleaved by the astrocytic ectoenzyme γ-glutamyl transpeptidase (γGT). The resulting CysGly selves as a precursor for neuronal GSH synthesis. X represents an acceptor for the γ-glutamyl moiety in the reaction catalyzed by γGT. Adapted from (Bélanger and Magistretti 2009).

Recent evidence also implicates astrocytes as important immune regulators in the CNS. Their strategic location in close association with other glial cells, neurons and blood vessels allows them to efficiently respond to changes in the CNS environment (Fig.12).

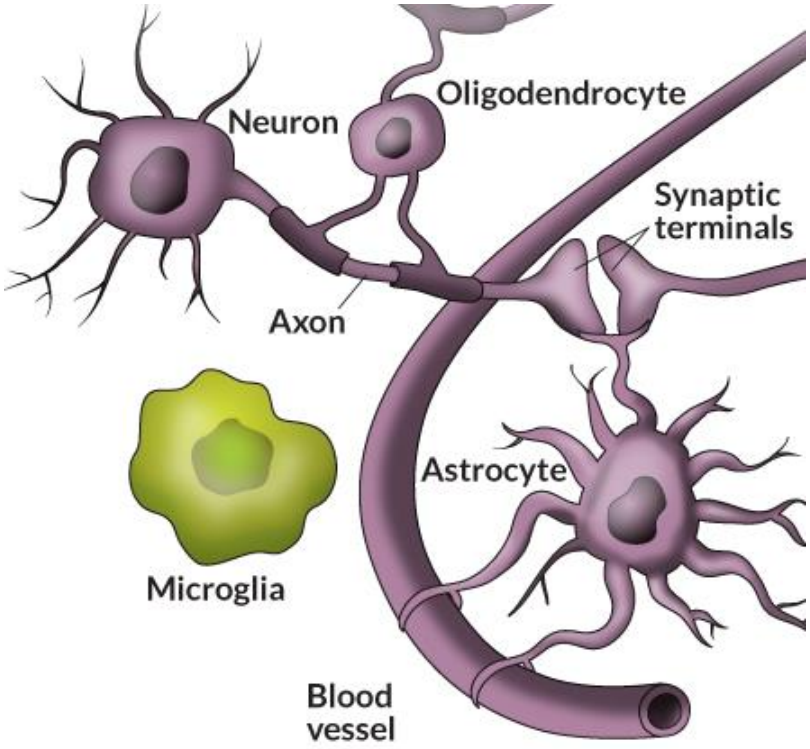


Figure 12. The strategic location of astrocytes, close microglia, neurons and blood vessels, make these cells play an important role in both innate and adaptive immunity. Adapted from (Allen and Barres 2009)

Astrocytes also respond to many forms of CNS injury, such as infection, trauma and ischemia. Similarly to microglia, astrocytes may become reactive, a process known as astrogliosis, which is characterized by changes in gene expression profile, proliferation, and migration. In this activated stage, astrocytes release a plethora of inflammatory mediators such as cytokines, chemokines and growth factors (Ridet, Privat et al. 1997, Sofroniew 2009).

These inflammatory mediators produced by astrocytes, participate in tissue repair and remodelling both in the brain and spinal cord following an acute injury to the neuronal parenchyma. These cells also modulate neuronal viability by restricting the spread of inflammatory cells and infection. However, reactive astrocytes can also be harmful to neurons by producing and releasing neurotoxic amounts of glutamate and ROS and by further exacerbating neuroinflammation via the increased release of pro-inflammatory cytokines (Sofroniew and Vinters 2010).

Astrocytes express major histocompatibility complex class II (MHC II) molecules indicating their functional capability as antigen presenting cells, contributing for adaptative immunity (Dong and Benveniste 2001). Astrocytes also display direct effects on endothelial cells, for example, regulating the permeability of the BBB, which indirectly contributes for the recruitment of T lymphocytes to the brain (Bush, Puvanachandra et al. 1999, Argaw, Zhang et al. 2006, Argaw, Gurfein et al. 2009). Once within the brain, these T cells may affect the immune response and modulate brain homeostasis by the secretion of a variety of both pro and anti-inflammatory cytokines (Dong and Benveniste 2001). Therefore, immune activation of astrocytes can either be beneficial or harmful depending on the conditions under which activation occurs.

1.2.2.1 Interplay between Microglia and Astrocytes

Astrocytes produce and secrete immune mediators such as INF-ϒ, TNF, IL-1β and IL-6, which may lead to pro-inflammatory microglia activation. On the other hand, the release of TGF-β and IL-10 may shift microglia activation toward an anti-inflammatory signature (Hanisch 2002, Lu, Ma et al. 2010, Norden, Fenn et al. 2014). Chemokines such as CCL2 and CXCL10 also modulate microglia functions such as migration (Flynn, Maru et al. 2003, Farina, Aloisi et al. 2007).

Studies have demonstrated that fractalkine (CX3CL1) expressed by astrocytes leads to inhibition of neurotoxicity by suppressing the production of pro-inflammatory cytokines and NO by activated microglia (Mizuno, Kawanokuchi et al. 2003). Several studies have also demonstrated that microglia treated with astrocyte conditioned medium, reduce iNOS expression and ROS production induced by H_2O_2 and INF-Υ through an increased expression of microglial antioxidant enzymes (Min, Yang et al. 2006, Shih, Fernandes et al. 2006).

1.3 Neuroinflammation and Methamphetamine

Microglia have recently been implicated as mediator of Meth-induced neurotoxicity. Some studies have shown that Meth consumption might cause both microglial and astrocytes activation. Regarding microglia activation, it has already been shown that Meth induces a substantial microglial response in areas of the brain showing neuronal degeneration (Krasnova and Cadet 2009).

It was also demonstrated that Meth promotes neuroinflammation by damaging the dopaminergic system, inducing hyperthermia, increasing ROS generation and glutamate release. Furthermore, this psychostimulant also increases the release of pro-inflammatory cytokines by microglia (Gonçalves, Martins et al. 2008, Tocharus, Khonthun et al. 2010). All of these elements are recognized either to cause or aggravate neuronal damage, and some studies have suggested that microglial cell activation seems to precede the appearance of damage produced by Meth consumption (LaVoie, Card et al. 2004). This has been confirmed by studies demonstrating that anti-inflammatory drugs protect against Meth-induced neurotoxicity (Asanuma, Tsuji et al. 2003, Tsuji, Asanuma et al. 2009).

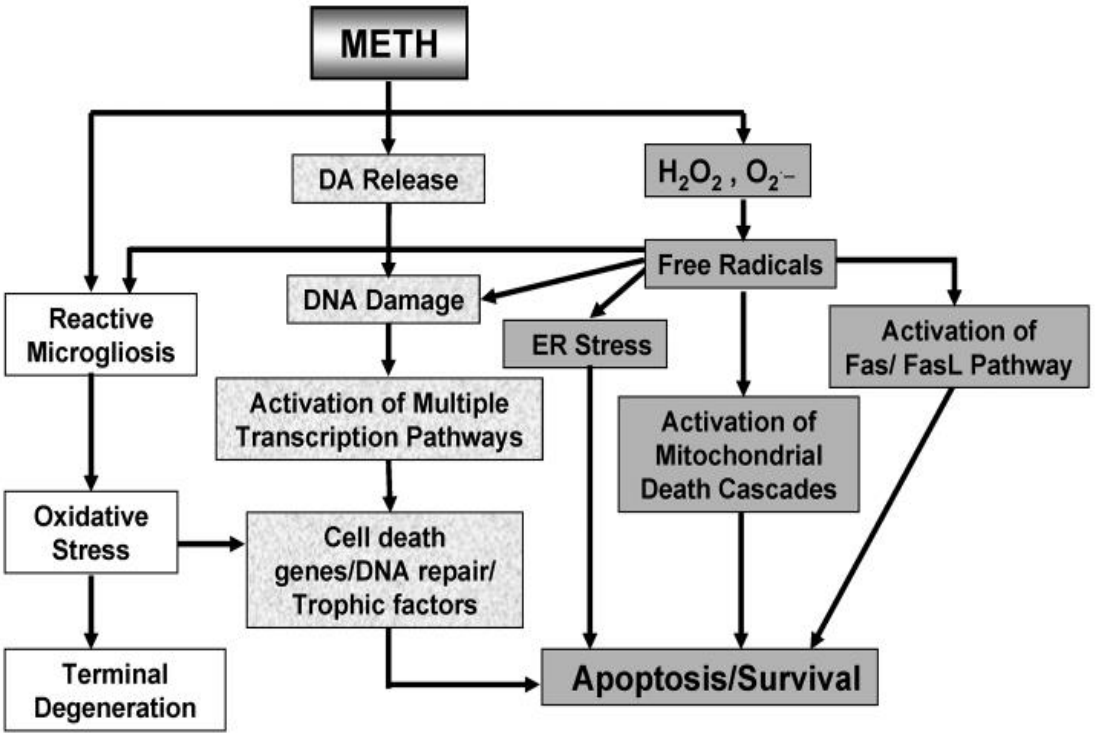


Figure 11. Signalling pathways of Meth-induced neurodegeneration and loss of dopaminergic terminals. Adapted from (Cadet, Krasnova et al. 2007).

2 Aim

Our main goal was to comprehend how isolated microglia (assessed using primary cultures) would respond to Meth exposure. For that we will analyse pro-inflammatory and anti-inflammatory markers in cortical purified microglial cells exposed to Meth. We will also study activation markers in cultured cortical astrocytes challenged with Meth. Finally, we will observe the effect of conditioned media from Meth-treated astrocytes on cultured microglial cells analysing different set of classical activation markers.

3 Experimental Procedures

3.1 Reagents

DMEM/F-12 GlutaMAX™-I, DMEM GlutaMAX™-I, Fetal Bovine Serum (FBS), Penicillin-Streptomycin (10.000U/ml) 0.25% trypsin-EDTA, HBSS and CellROX® were acquired from Life Technologies. Granulocyte macrophage colony-stimulant factor (GM-CSF) was obtained from Peprotech®, latex beads and poly-D-lysine were obtained from Sigma-Aldrich.

3.2 Mixed glial cell cultures

Primary mixed glial cell cultures were obtained from cerebral cortices of newborn Wistar rats (P1-P3). The cortices were separated from other tissues; the meninges were removed in HBSS and then dissociated with 0.25% trypsin-EDTA. Glial cells were plated into T75 flasks coated with poly-D-lysine in DMEM/F-12 GlutaMAX™-I supplemented with 10% FBS and 1% penicillin/streptomycin. These cells were cultured for 10 days at 37ºC in a humidified incubator in an atmosphere containing 5% CO_2 and 95% O_2 ratio. The culture media was totally replenished twice a week. Each mixed glial cell culture batch was shaked three times, once a week, for collecting microglial cells (3.2.1) and, after that, it was used for purified astrocyte cultures (3.2.2).

3.2.1 Purified microglial cell cultures

Mixed glial cell cultures, 10 days after plating, were submitted to orbital shaking three times, once a week, at 200 rpm for 2 hours to detach microglia cells. After that, culture medium containing the microglial cells in suspension was centrifuged at 290g for 10min. The pellet was re-suspended in DMEM/F-12 GlutaMAX™-I supplemented with 10% FBS, 1% penicillin/streptomycin and 1ng/ml granulocyte macrophages colony-stimulating factor (GM-CSF). Microglial cells were plated in non-coated coverslips on the bottom of a 12-well tissue culture plate (1×10^5 cells/ml) or in non-coated culture dishes (2×10^5 cells/ml), and the

media was totally replenished on the day after plating. The cells were cultured for 3 to 4 days at 37ºC in a humidified incubator in an atmosphere containing 5% CO_2 and 95% O_2 ratio.

3.2.2 Purified astrocyte cell cultures

After the third shake of the mixed glial cell culture, the adherent cells were washed with PBS (phosphate buffer saline solution), trypsinized (0.25% trypsin-EDTA), and splited to non-coated 100mm culture dishes. Splited cultures were re-splited, at least, three more times, in order to obtain purified astrocyte cultures. These cultures were then frozen (10% DMSO; 20% FBS in DMEM GlutaMAX™-I) until use. For experimental procedures, these cells were thaw and plated.

3.3 Drug treatment

The drug used was (+)- Methamphetamine hydrochloride obtained from Sigma under an INFARMED license. In order to be used for cell treatment, the compound was dissolved in sterile PBS and incubated in microglial or astrocyte cell culture media. Microglial cells and astrocyte were exposed to 10 or 100 μM of Meth for 24 hours.

The concentrations of Meth used were based on previous *in vitro* studies published (Lee, Hennig et al. 2001) and (Narita, Miyatake et al. 2006). These concentrations are similar to levels found in blood, urine or tissue samples of Meth users that range from ≤ 2 μM to 600 μM (Takayasu, Ohshima et al. 1995, Kalasinsky, Bosy et al. 2001, Schepers, Oyler et al. 2003).

3.4 Phagocytic assay

Primary cultures of microglial cells were incubated with fluorescent latex beads with 0.5 μm diameter in order to analyse the changes in phagocytosis capacity induced by Meth. Latex beads (green) were diluted in the culture medium (1:1000 (v/v)) and incubated 24 hours after Meth treatment for 1 hour at 37ºC. After beads incubation, cells were washed three times with PBS and fixed for 12 minutes in 4% MP-PFA (Paraformaldehyde microtubule protection buffer) : 65mM

pipes, 25mM HEPES, 10mM EGTA, 3mM $MgCl_2$, pH 6.9). The number of beads per cell was counted and the phagocytic efficiency was calculated based on a weighted arithmetic mean of phagocyted beads per cell (Liu, Hao et al. 2006). In order to establish a score, beads *per* cell were classified according to the number of phagocyted beads as described in the following table:

Range of phagocyted Beads	Score
1 to 7	1
8 to 14	2
15 to 21	3
22 to 28	4
28 to 35	5
Up to 36 beads	6

Table 1. Score of beads per cells.

The number of cells classified in each score was multiplied by the respective score number, as a weight value for the weighted arithmetic mean, as described by the following equation:

$$\bar{X} = \frac{(nº\ cells\ in\ score\ 1 \times 1) + (nº\ cells\ in\ score\ 2 \times 2) + \cdots + (nº\ cells\ in\ score\ 6 \times 6)}{Total\ number\ of\ cells}$$

3.5 Oxidative Stress assay

Primary microglial cultures were incubated with CellROX® Oxidative Stress Reagents in order to analyse the production of ROS induced by Meth. CellROX® Green Reagent was added to primary cultures 24 hours after drug treatment at a final concentration of 5 μM, for 30 min at 37ºC. Then, cells were washed three times with PBS and fixed for 12 minutes in 4% MP-PFA.

3.6 Astrocyte-conditioned medium assay

Astrocytes were thaw and plated. After 2 days, cells were treated for 24 hours with different Meth concentrations (10 µM and 100 µM). Afterwards, the medium was collected and stored at -80ºC until use. The conditioned medium was added to microglial cell cultures and, 24 hours later, cells were washed three times with PBS and fixed for 12 minutes in 4% MP-PFA. Control microglia conditions received astrocyte conditioned medium from non Meth exposed cultures.

3.7 Immunocytochemistry

After MP-PFA fixation, cultures were permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature (RT), washed with PBS three times for 10 minutes and then the coverslips were blocked with 3% bovine serum albumin (BSA) in PBS for 60 min at RT. Afterwards, cells were incubated with primary antibody (Table 2) or Alexa conjugated 647 Phalloidin (1:50) overnight at RT in blocking solution. Cells were then washed three times for 10 min and incubated with a secondary antibody Alexa Fluor® 488, 568 or 647 in blocking solution for one hour at RT. Finally, cells were incubate with DAPI, a nuclear staining, for 15 minutes, and washed in PBS for 10 minutes with PBS. Coverslips were mounted using Immu-Mount™ mounting media and observed using DIM6000B inverted microscope (Leica Microsystem) with an HCX Plan Apo 63x/1.4-0.6 NA oil immersion objective.

Primary antibody	Dilution	Reference	Company
Anti-Arginase 1	1:100	Sc-18354	Santa Cruz Biotechnology
Anti-CD11b	1:200	Ab8879	Abcam
Anti-GFAP	1:500	G9269	Sigma-Aldrich
Anti-iba-1	1:500	016-20001	Wako

Anti-NOS2	1:200	Sc-650	Santa Cruz Biotechnology

Table 2. List of primary antibodies used in immunocytochemistry analysis.

3.8 Intensity Quantification

For the intensity quantification, images were exported using the Leica LAS AF program in TIF format (16-bit). In ImageJ software these micrographs had their background subtracted using 50%-off pixels radius by the rollerball algorithm. Next, cells were segmented using the OTSU algorithm with automatic complementation for both the bottom and upper threshold ramps with background values set to black and foreground values set to red. Individual segmented cells were transposed to ImageJ's ROI manager using the analyse particles tool. A range between 100-to-infinity of non-calibrated pixels2 defined each segmented cell. Mean gray values for the intensities were returned for each cell individually using the multi-measure function on ImageJ`s ROI manager.

3.9 Morphologic assessment of microglia

Images were processed in ImageJ software to assess microglial cell morphology. Microglia were immunostained for CD11b and images were acquired in a DIM6000B inverted microscope (Leica Microsystem). Micrographs were exported as 16 bit tiff images, segmented, converted to binary 8 bit gray scale and the background subtracted 25% by the rollerball algorithm in ImageJ. FracLac plugin was implemented to assess the complexity of microglia morphology on a cell-by-cell basis. Regions of interest were traced using the particle analyzer tool in ImageJ to contour microglial cells and complexity (D_B) was extracted by box counting with FracLac plugin as previously described (Karperien, Ahammer et al. 2013).

3.10 Quantitative Real-Time RT-PCR

3.10.1 RNA isolation from microglial and Astrocyte cells.

The total mRNA was isolated using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Briefly, samples were lysed in a denaturating buffer and homogenized. Ethanol was added into the lysate to provide ideal binding conditions. The lysate was loaded onto RNeasy Mini spin column and, after washes, concentrated RNA was eluted in 20µl RNase free water as schematically explained in Fig 12. The total amount of RNA yields was measured using NanoDrop® 1000 Spectrophotometer (Thermo scientific) at 260nm and the purity of the RNA was evaluated through the ratio 260/280nm.

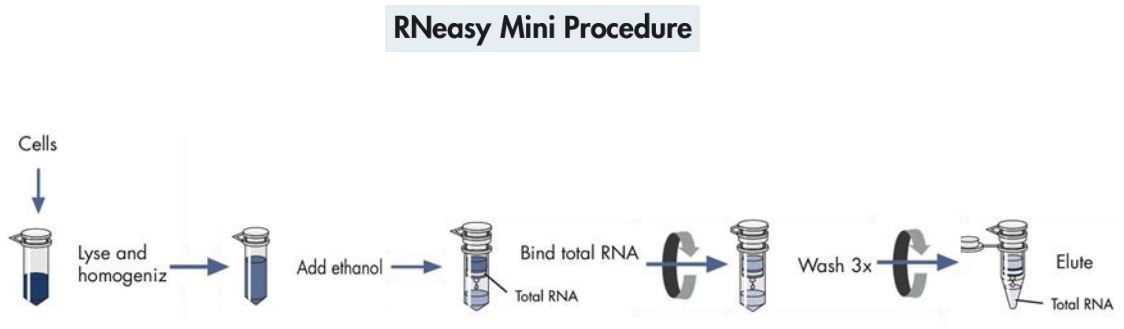


Figure 12. Procedure of RNA extraction by RNeasy Mini kit (Qiagen). Adapted from (http://www.qiagen.com/pt/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en).

3.10.2 Reverse transcription for cDNA synthesis

cDNA was synthesized using 1µg of total RNA of samples through RT² Easy First Strand (Qiagen) according to manufacturer's instructions. Briefly, to 1µg of RNA was added buffer GE for each sample in order to eliminate genomic DNA and then, the reverse transcriptase mix was added, reaching a final volume of 20µl. On a thermocycler the samples were heated until 42°C for 15 min and then heated until 95°C for 5 minutes, finishing the reaction. These samples were stored at -20°C until used.

3.10.3 Quantitative Real-time PCR and Analysis

Gene expression of classical inflammatory markers was evaluated using quantitative real time PCR (qRT-PCR). The PCR was performed using iQ™5 multicolor real time PCR detection system (Bio Rad). The reaction was performed following the iQ™ SYBR® Green Supermix manufacturer's protocol. The reaction components are summarized in table 3.

Component	Volume per 20uL reaction
iQ™ SYBR® Green Supermix (2×)	10µl
Primers Forward	Variable
Primer reverse	Variable
cDNA diluted (1:10)	2µl
H_2O	Variable
Total	20µl

Table 3. Real Time PCR reaction components.

For TNF, iNOS, IL-10 and TGF-β detection, the primers concentration used was 200nM, for YWHAZ (reference gene) 250nM and for IL-1β and IL-6 300nM. The primers (all obtained for Sigma-Aldrich) used in PCR reactions were described in table 4.

Primers	Sequences (5'⟶3')	
	Forward	**Reverse**
IL-10	ATCCGGGGTGACAATAACTG	TGTCCAGCTGGTCCTTCTTT
IL-1β	TAAGCCAACAAGTGGTATTC	AGGTATAGATTCTTCCCCTTG
IL-6	ACTCATCTTGAAAGCACTTG	GTCCACAAACTGATATGCTTAG

iNOS	AGCCGTAACAAAGGAAATAG	ATGCTGGAACATTTCTGATG
TGF-β	TGAGTGGCTGTCTTTTGACG	GTTTGGGACTGATCCCATTG
TNF-α	CTCACACTCAGATCATCTTC	GAGAACCTGGGAGTAGATAAG
Ywhaz	GATGAAGCCATTGCTGAACTTG	GTCTCCTTGGGTATCCGATGTC

Table 4 . List of primers used in qPCR.

Each reaction was performed in duplicated and the PCR cycling profile for IL-1β, IL-6, iNOS, TNF-α and Ywhaz was 3 min at 94ºC for polymerase activation and DNA denaturation, followed by 40 cycles of 15 sec at 94ºC (denaturation), 20 sec at 58ºC (annealing), and 15 sec at 72ºC (extension) and 81 cycles of 30 sec between 55ºC and 95ºC (final extension period). For IL-10 and TGF-β the cycling profile was 3 min at 94ºC for polymerase activation, followed by 40 cycles of 15 sec at 94ºC (denaturation), 20 sec at 62ºC (annealing) and 81 cycles of 30 sec between 55ºC and 95ºC (final extension period).

Melting point analyses were performed for each reaction to confirm single amplified products. Gene expression was extrapolated from standard curves generated concurrently for each gene using a control cDNA dilution series. Results were analysed using iQ™5 Optical System software version 2.1(BioRad). All values were normalized to levels of the reference gene, Ywhaz, and expressed compared to unstimulated control samples. Results were calculated using relative quantification method, Pffalf (Pfaffl 2001) by following formula:

$$RATIO = \frac{(E_{target})^{\Delta Ct,target(calibrator-test)}}{(E_{ref})^{\Delta Ct,ref(calibrator-test)}}$$

3.11 Statistical analyses

Data are shown as mean ± SEM (standard error of the mean). Statistical analysis of data was performed either using one-way ANOVA followed by Dunnett's post-test or two-sample equal variance Student's t test. Differences were considered significant for *$p<0.05$. Every experimental condition was tested, at least, in three sets of separate and independent experiments. Statistical analysis was performed using the GraphPad Prism® software version 6.02.

4 Results

4.1 Methamphetamine does not induce a pro-inflammatory signature in microglial cells

It was previously described that microglia activation represents an early step in Meth-induced neurotoxicity (Thomas, Dowgiert et al. 2004). In this section, primary cultures of microglial cells were used as a model to better understand the role of this cell type on Meth-induced neuroinflammation. For that, microglial cells were challenged with Meth 10 µM or 100 µM for 24 hours and, in order to examine the activation state of microglial cells, classical activation markers were observed such as cell morphology, phagocytosis efficiency, ROS production, iNOS expression and pro-inflammatory cytokines profile.

Firstly, we analysed microglial cell morphology using a microglial marker, CD11b, and observed that cell complexity significantly increased when cells were incubated with Meth 100 µM (Fig.12), suggesting that Meth doesn't induce an amoeboid morphology. We also evaluated the phagocytic capacity of microglial cells using inert fluorescent microbeads. For that, microglia were treated with Meth 10 µM or 100 µM and, as shown in Fig.13, the number of cells containing beads, the number of beads *per* cell and the phagocytic activity of microglial cells significantly decreased when these cells were treated with Meth 100 µM but not in cells treated with Meth 10 µM when compared to cells not treated with Meth. We concluded that Meth reduces the phagocytosis of microglial cells.

Regarding ROS production, we observed that Meth at neither 10 µM nor 100 µM was capable of increasing ROS production, as can be observed in figure 14. Moreover, we also analysed iNOS expression and observed that Meth could not induce changes in iNOS expression when compared with untreated microglial cells (Fig.15). Pro-inflammatory cytokines and iNOS mRNA expression levels were also evaluated by qRT-PCR. Our results suggest that Meth did not induce any significant change in the mRNA levels of IL-1β, IL-6 or TNF. Microglial cells were incubated with both Meth 10 µM and 100µM in two different time points (3 and

24 hours; Fig 16 A-C). Regarding the mRNA expression of iNOS, it could not be detected any significant change at 24 hours of Meth exposure (Fig 16 D).

The results described herein indicate that Meth does not induce a pro-inflammatory activation of microglial cells. In order to better understand the Meth effect in primary microglial cultures, we hypothesized whether Meth could trigger an anti-inflammatory signature in primary microglial cell cultures. To answer this question we analysed anti-inflammatory hallmarks of microglia activation.

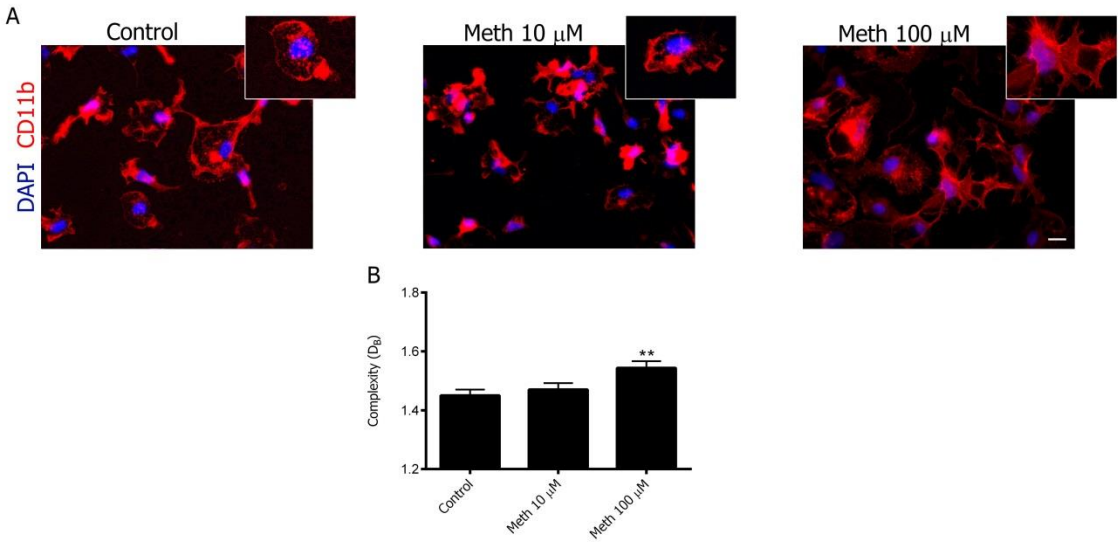


Figure 12. Meth increased the complexity in primary cortical microglial cells. (A) Representative photomicrographs of purified microglial cells cultures treated with 10 μM or 100 μM of Meth for 24 hours stained for CD11b (red) and Dapi (blue). (B) Quantification of complexity show a significant increase of complexity on cells treated with Meth 100 μM compared with untreated cells (control). Results are expressed as mean ± SEM of three independent experiments. Statistical significant differences are signed as **p< 0.01 compared with control. Calibration bar: 10 μm

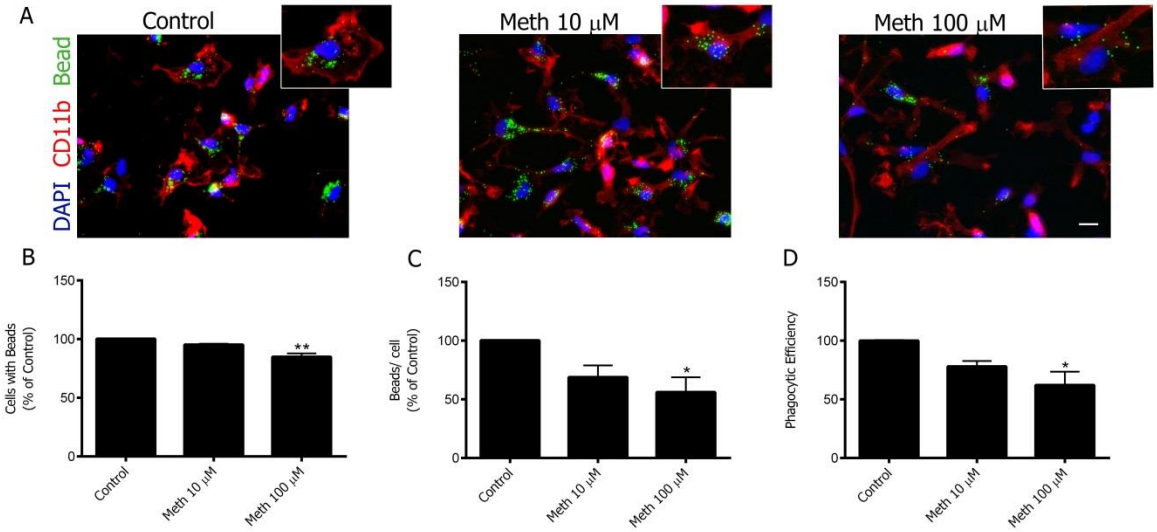


Figure 13. Meth reduced the phagocytosis in primary cortical microglial cells. (A) Representative photomicrographs of purified microglial cells cultures treated with 10 μM or 100 μM of Meth for 24 hours stained for CD11b (red) and Dapi (blue). (B-D) Quantification of phagocytosis activity show a significant decrease of phagocytosis on cells treated with Meth 100 μM compared with untreated cells (control). The graphs represent: percentage of cells with beads (B), number of beads *per* cell (C) and phagocytic efficiency (D). Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group. Statistical significant differences are signed as *p< 0.05, **p< 0.01 compared with control. Calibration bar: 10 μm.

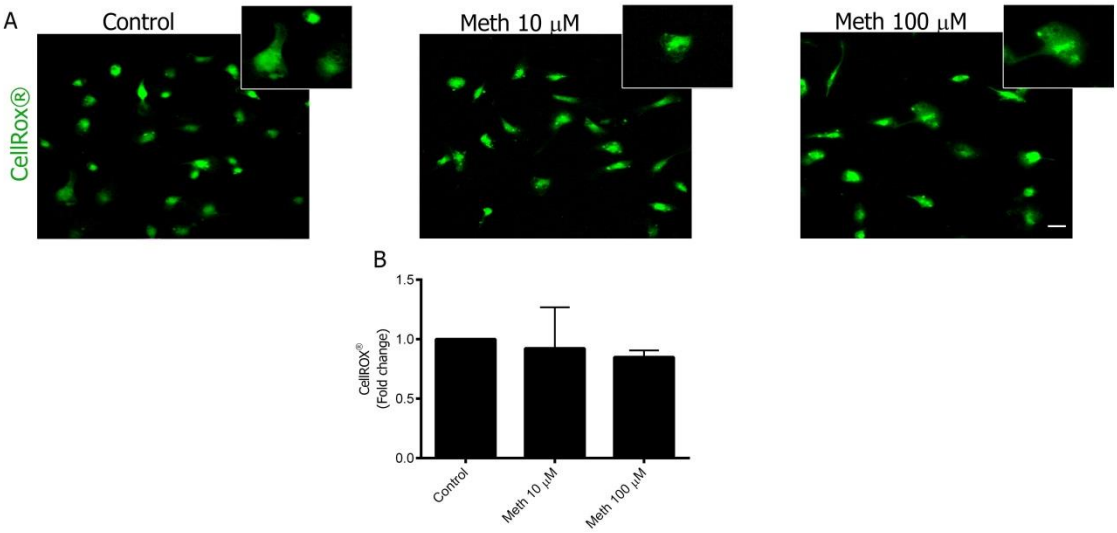


Figure 14. Effects of Meth in reactive oxygen species production by primary cortical microglial cells. (A) Representative photomicrographs of purified microglial cells cultures treated with 10 μM or 100 μM of Meth for 24 hours. (B) Quantification of CellRox® (green) shows no significant changes when compared to control. Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group. Calibration bar: 10 μm.

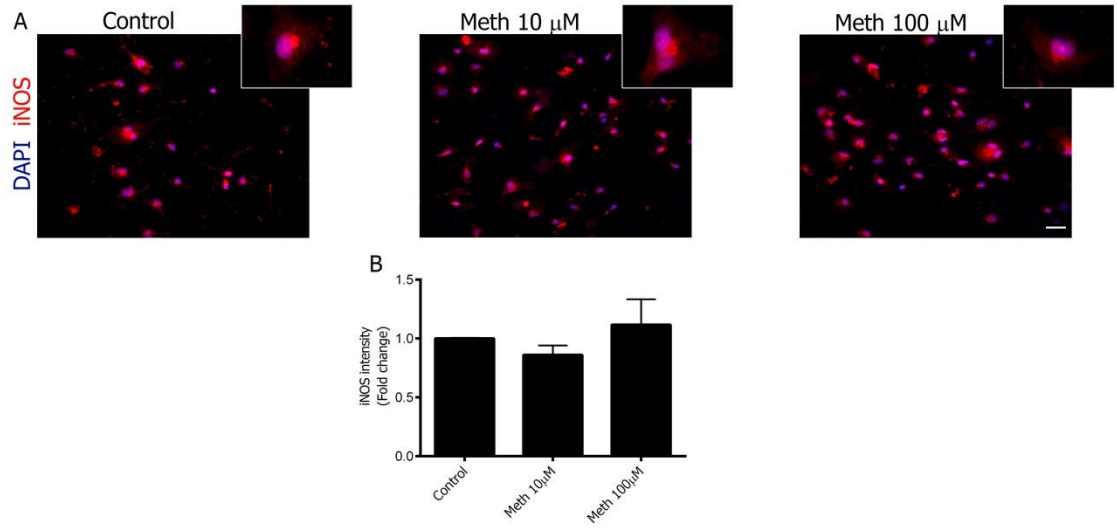
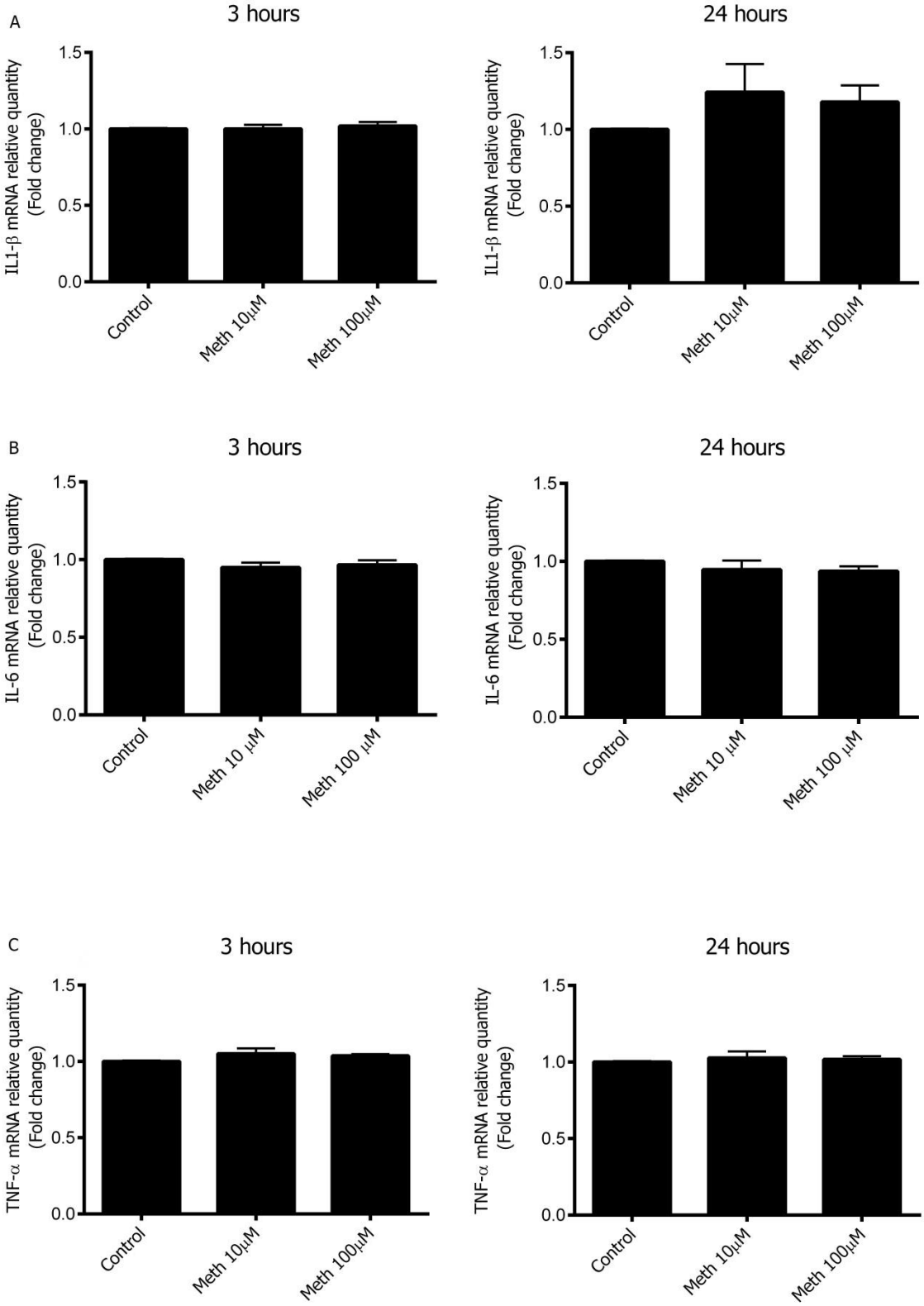


Figure 15. Effects of Meth in nitric oxide production by primary cortical microglial cells. (A) Representative photomicrographs of purified microglial cells cultures treated with 10 μM or 100 μM of Meth for 24 hours stained for iNOS (red) and Dapi (blue). (B) Quantification of inducible nitric oxide synthase show not significant changes when compared to control. Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group. Calibration bar: 10 μm


A
3 hours
24 hours
IL1-β mRNA relative quantity (Fold change)
1.5
1.0
0.5
0.0
Control
Meth 10μM
Meth 100μM
B
3 hours
24 hours
IL-6 mRNA relative quantity (Fold change)
Control
Meth 10 μM
Meth 100 μM
C
3 hours
24 hours
TNF-α mRNA relative quantity (Fold change)
Control
Meth 10μM
Meth 100μM

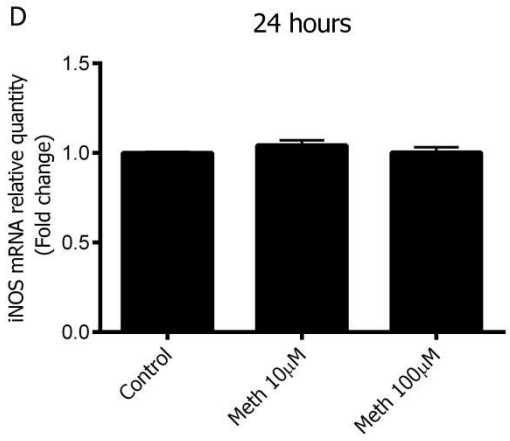


Figure 16. Levels of classical pro-inflammatory markers mRNA in primary microglial cell cultures after Meth exposure. The mRNA levels of (A) IL-1β, (B) IL-6, (C) TNF-α and (D) iNOS were analysed 3and 24 hours after Meth treatment, by RT-PCR. Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group.

4.2 Methamphetamine does not trigger an anti-inflammatory profile in microglial cells

In order to evaluate whether Meth induces an anti-inflammatory signature in microglial cells we first evaluated Arginase 1 expression as an anti-inflammatory marker. We found that Meth did not induce any significant changes in the expression of this anti-inflammatory marker for both doses (Fig. 17). Then, we evaluated the mRNA expression levels of anti-inflammatory markers by qRT-PCR. Our results demonstrate that Meth induced no significative change in IL-10 or TGF-β mRNA expression levels. The cells were tested for both Meth doses (10 μM and 100μM) and, at two different time points (3 and 24 hours) after Meth exposure (Fig. 18). Taken together, these data suggest that Meth does not trigger an anti-inflammatory profile in cultured microglial cells.

It was previously reported that Meth may induce microglial cell activation *in vivo* (LaVoie, Card et al. 2004, Thomas, Dowgiert et al. 2004, Sekine, Ouchi et al. 2008). In order to better comprehend how Meth could induce this pro-inflammatory activation we hypothesized that other CNS cell type could contribute for his Meth-induced microglia activation *in vivo*. Likewise, we searched for cell types that could be influenced by Meth and found that Meth also regulates astrocytes reactivity in both *in vivo* and *in vitro* paradigms (Narita, Miyatake et al. 2005, Friend and Keefe 2013). Based on these facts, we hypothesized that Meth might modify astrocytes basal activation state, which could promote the release of pro-inflammatory mediators to indirectly influence microglial cell function.

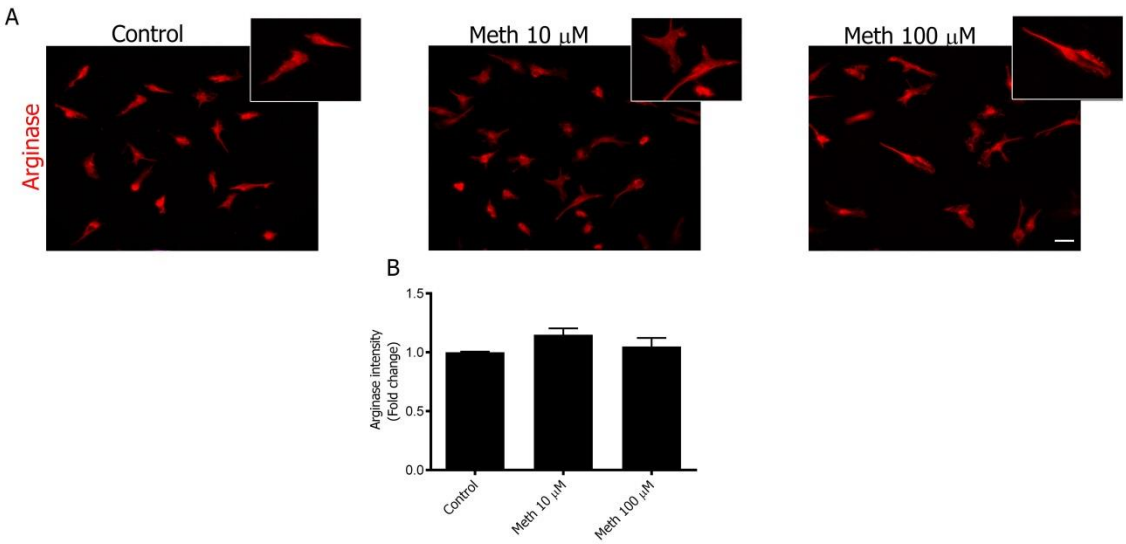


Figure 17. Effects of Meth on Arginase 1 expression, in primary cortical microglial cells. (A) Representative photomicrographs of purified microglial cells cultures treated with 10 μM or 100 μM of Meth for 24 hours. (B) Quantification of Arginase 1 (red) intensity shows no statistically significant changes compared to untreated cells (control). Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group. Calibration bar: 10 μm.

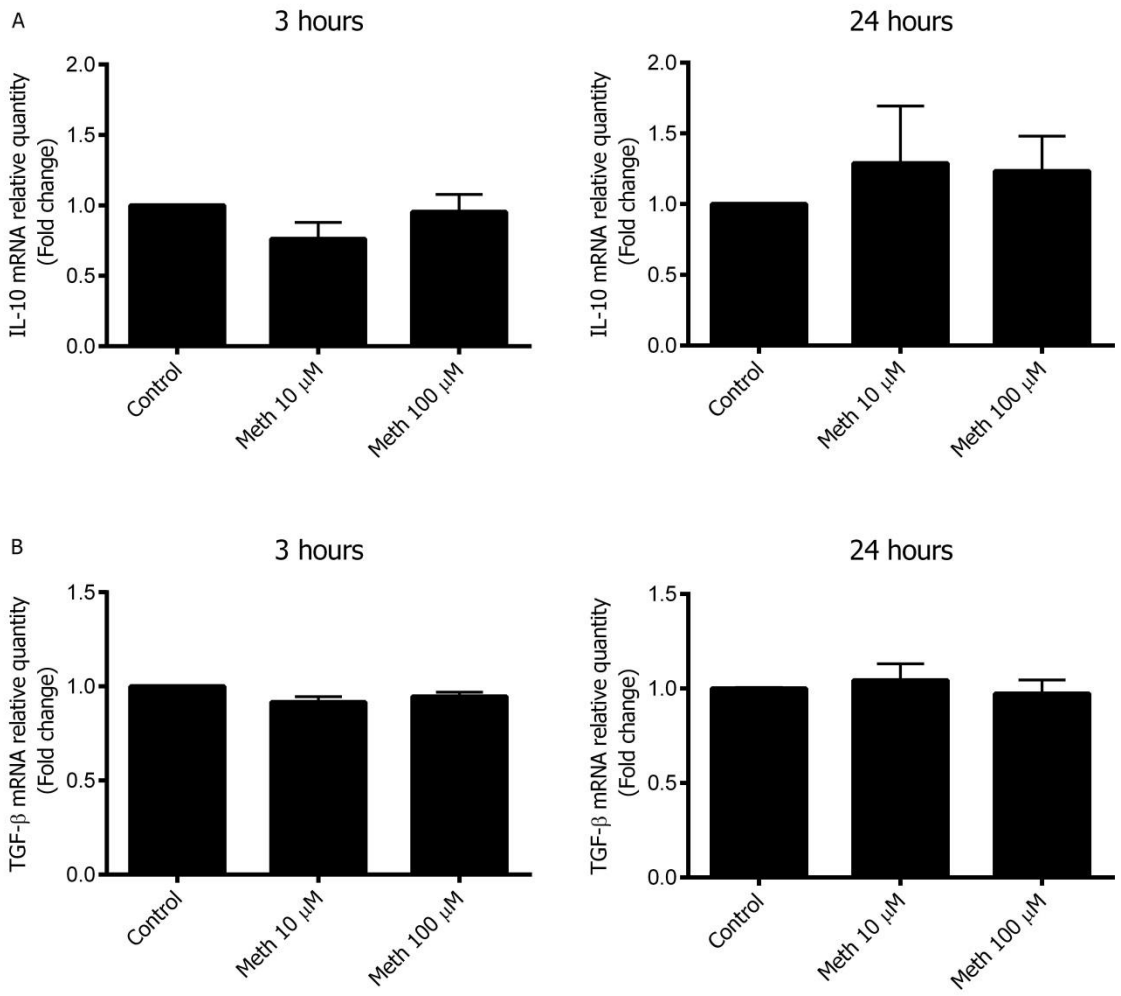


Figure 18. Levels of (A) IL-10 and (B) TGF-β mRNA in primary microglial cell cultures after Meth exposure. The mRNA levels were analysed 3and 24 hours after Meth treatment, by RT-PCR. Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group.

4.3 Methamphetamine does not regulate astrocytes reactivity

In order to analyse Meth effects on astrocytes, we analysed astrocyte reactivity markers such as the expression of iNOS and GFAP, as well as the formation of actin stress fibers. As can be observed in figure 19, we found that Meth did not induce a significant change in the expression of iNOS in astrocytes 24 hours after treatment. Then, we evaluated the Meth effect on the formation of actin stress fibers. For that we stained astrocytes F-actin with Phalloidin-conjugated fluorochrome and observed that Meth could not increase stress fibers formation (Fig. 20). We also evaluated GFAP expression as a marker for *in vitro* astrogliosis. Surprisingly, we observed that Meth treatment significantly decreased GFAP expression in astrocytes primary cultures (Fig.21).

Our results strongly suggest that, in presence of Meth, astrocytes do not become activated. Notwithstanding, we evaluated the effects of conditioned media (CM) of astrocytes treated with Meth in microglial cells.

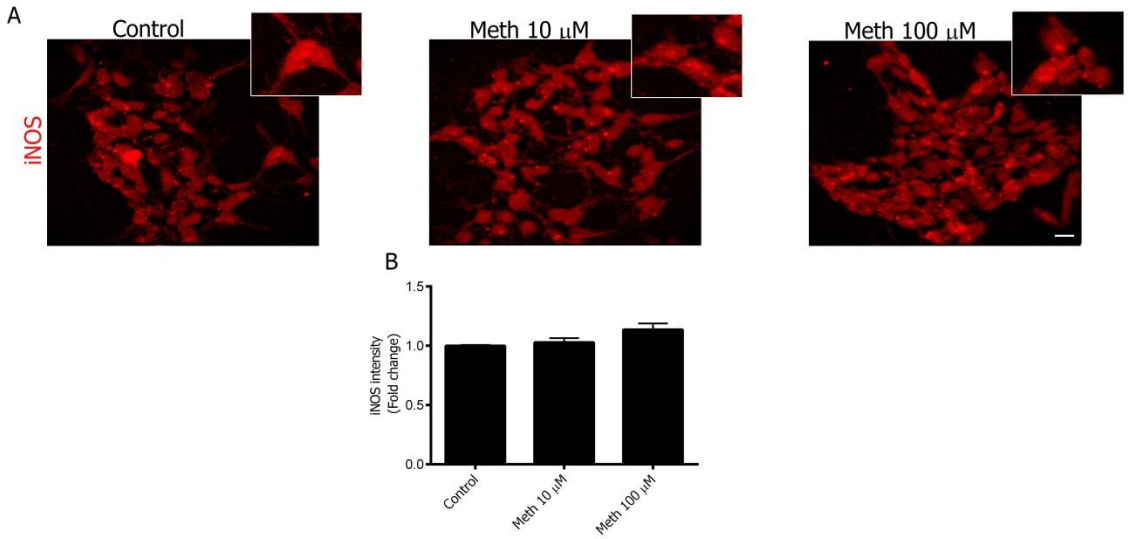


Figure 19. Effects of Meth on iNOS expression, in primary cortical astrocytes cultures. (A) Representative photomicrographs of purified astrocytes cells cultures treated with 10 μM or 100 μM of Meth for 24 hours. (B) Quantification of iNOS (red) intensity shows no statistically significant changes compared to untreated cells (control). Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group. Calibration bar: 10 μm.

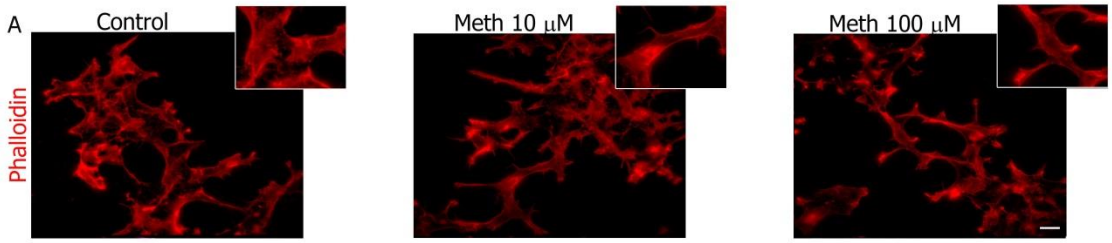


Figure 20. Effects of Meth, on stress fibers formation, in primary cortical astrocytes cultures. (A) Representative photomicrographs of purified astrocytes cells cultures treated with 10 μM or 100 μM of Meth for 24 hours. Calibration bar: 10 μm.

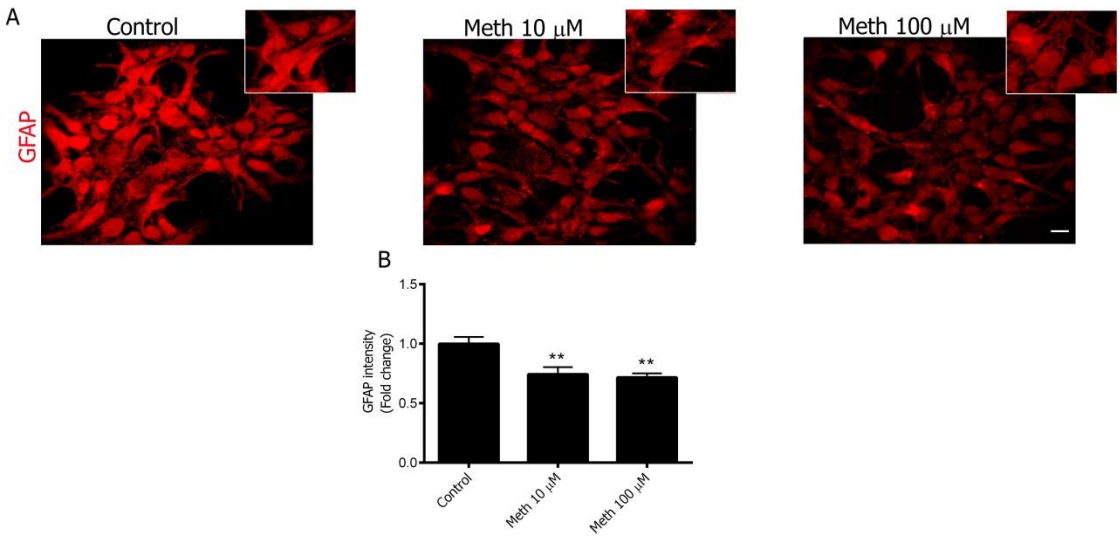


Figure 21. Effects of Meth on GFAP expression in primary cortical astrocytes cultures. (A) Representative photomicrographs of purified astrocytes cells cultures treated with 10 μM or 100 μM of Meth for 24 hours. (B) Quantification of GFAP (red) intensity shows statistically significant decrease of GFAP expression on astrocytes treated with Meth 10 μM and 100 μM compared with untreated cells (control). Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group. Statistical significant differences are signed as **p< 0.01 compared with control. Calibration bar: 10 μm.

4.4 Conditioned medium (CM) of Meth-treated astrocytes promotes microglial cell activation

In order to determine whether CM of astrocytes (ACM) treated with Meth can activate microglial cells, we analysed activation hallmarks, such as, phagocytic efficiency, iNOS expression and ROS production.

As shown in figure 22, ACM significantly increased phagocytic efficiency suggesting an activated profile of microglial cells. Then, we also evaluated iNOS expression and our results show that CM of astrocytes treated with Meth 100 μM significantly increased iNOS expression in microglial cells (Fig. 23). Moreover, we also observed that CM of astrocytes treated with Meth significantly enhanced ROS production by cultured microglial (Fig. 24). Taken together, these results clearly show that Meth induces indirect microglial cell activation, mediated in this case by astrocytes. Here, one can clearly advocate that soluble factors released by astrocytes, in presence of Meth, would play an essential role in microglial cell activation.

In order to have a hint of which factor would regulate this ACM-mediated microglial cell activation, we evaluated the expression of pro-inflammatory cytokines, such as TNF, IL-1β and IL-6 that were previously reported to be released from astrocytes (Lau and Yu 2001) and could potentially lead to the microglia activation we observed. Therefore, we analysed the mRNA expression levels of TNF, IL-1β and IL-6 in astrocytes treated with 100μM Meth for 24 hours. However, the mRNA levels of these cytokines were so low in cultured primary astrocytes that they could not detect by quantitative PCR, even in the presence of Meth. These data indicate that these cytokines were not directly responsible for microglial cell activation.

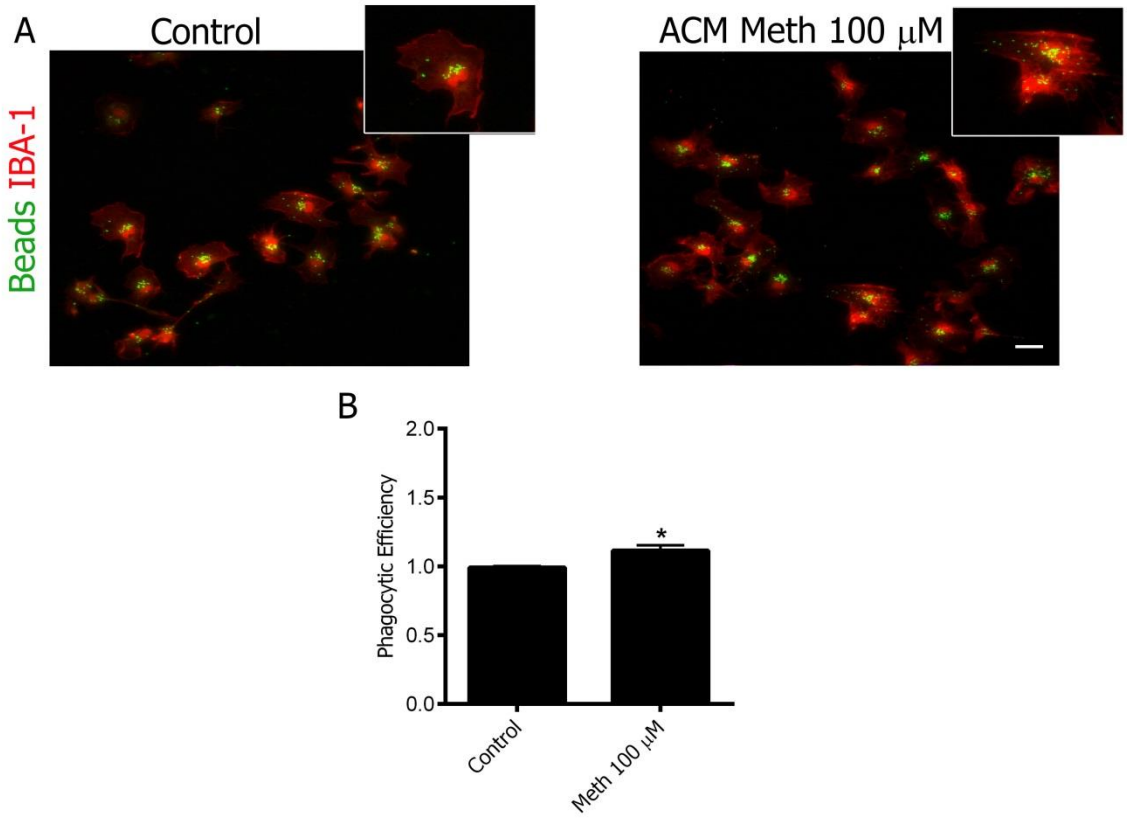


Figure 22. CM of astrocytes exposed to Meth increases the phagocytosis in microglial cells. (A) Representative photomicrographs of purified microglial cells cultures treated with CM of astrocytes treated with 100μM of Meth for 24 hours stained for IBA-1 (red) (B) Quantification of phagocytic efficiency shows a significant increase of phagocytosis on cells treated with CM of astrocytes incubated with 100μM of Meth compared with control. Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group. Statistical significant differences are signed as *p<0.05, compared with control. Calibration bar: 10 μm.

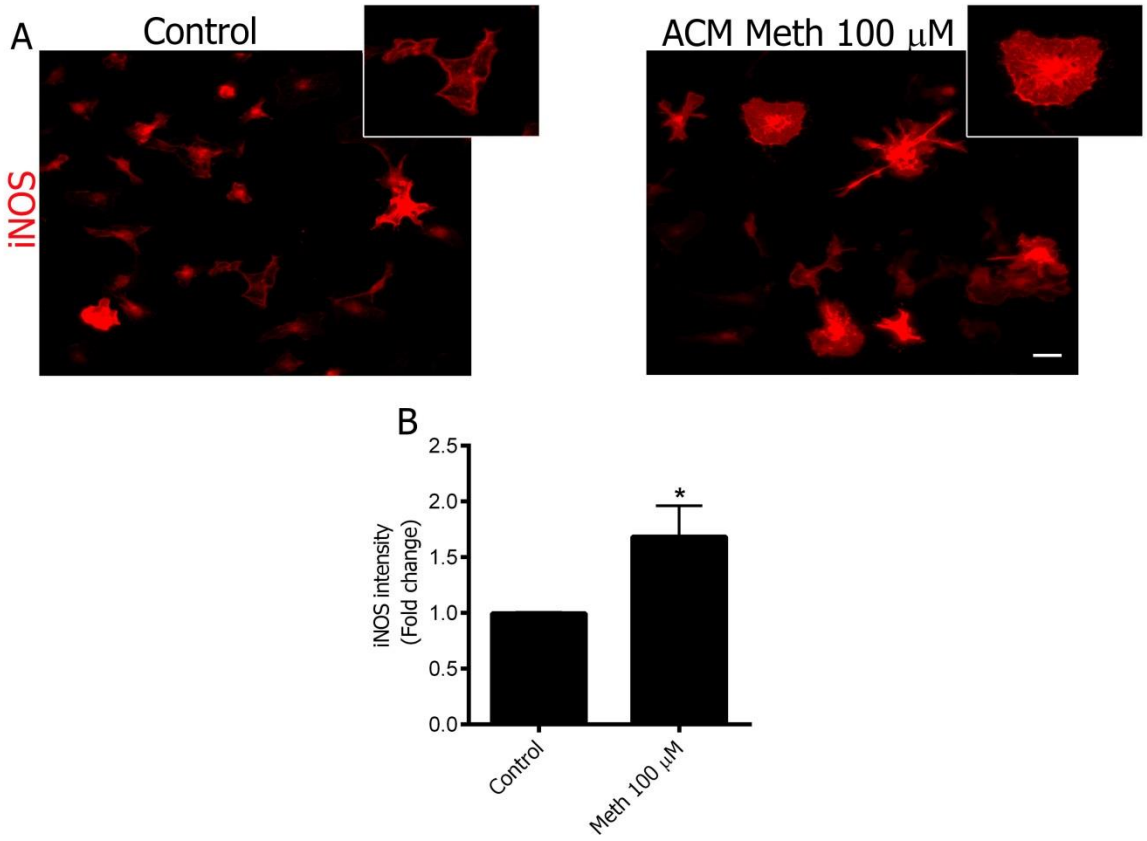


Figure 23. CM of astrocytes exposed to Meth increases iNOS expression in primary cortical microglial cells. (A) Representative photomicrographs of purified microglial cells cultures treated with CM of astrocytes treated with 100μM of Meth for 24 hours (B) Quantification of iNOS (red) expression shows a significant increase of iNOS on cells treated with CM of astrocytes incubated with 100μM of Meth compared with control. Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group. Statistical significant differences are signed as *p<0.05, compared with control. Calibration bar: 10 μm.

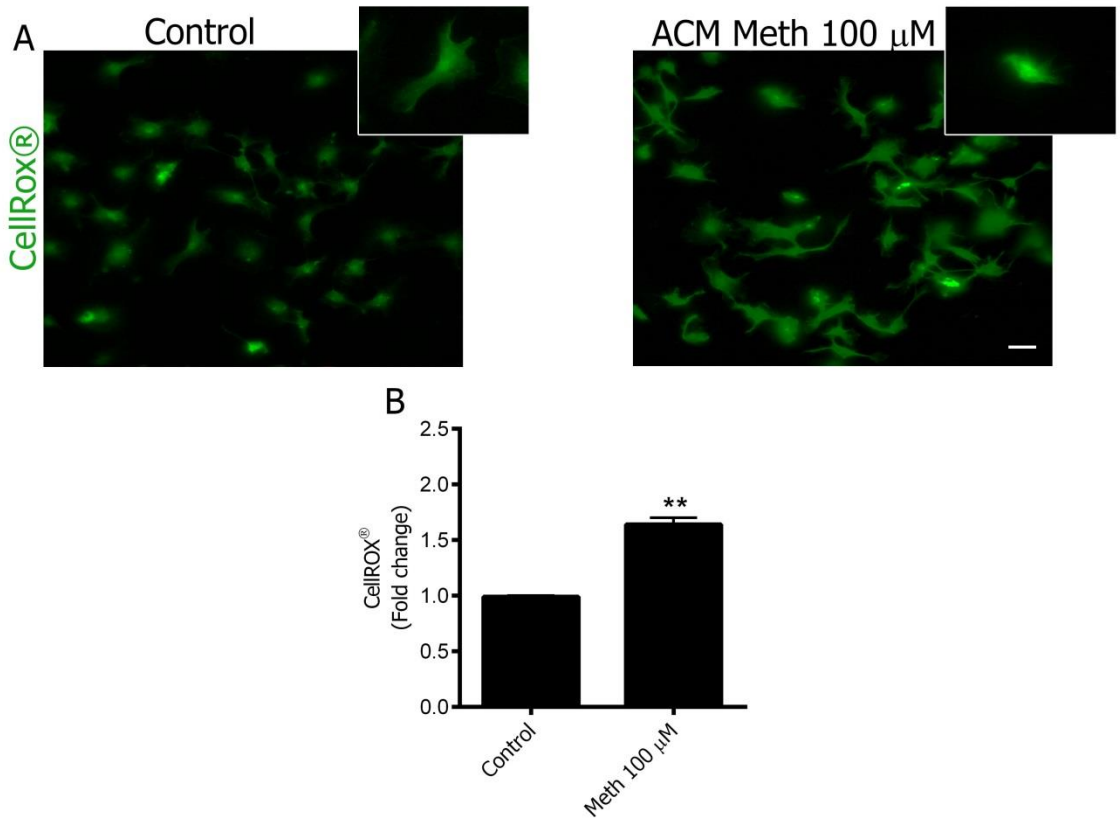


Figure 24. CM of astrocytes exposed to Meth increases ROS production in microglial cells. (A) Representative photomicrographs of purified cortical microglial cells cultures treated with CM of astrocytes treated with Meth for 24 hours stained with CellRox® (green) to assess ROS production. (B) Quantification of CellRox® expression shows a significant increase of ROS production on microglia treated with CM of astrocytes incubated with Meth 100 μM compared with control. Results are expressed as mean ± SEM of three independent experiments. Values displayed were normalized in relation to the control group. Statistical significant differences are signed as **p<0.01, compared with control. Calibration bar: 10 μm

5 Discussion

In this work we show that Meth did not induce, directly, neither to a pro-inflammatory nor to an anti-inflammatory signature in primary purified microglial cells. We also observed that Meth can only induce microglial cells activation indirectly, when astrocytes were challenge with this drug and the conditioned medium was administrated to microglial cells. Thus, we can accomplish that astrocytes act as inductors of microglia activation.

5.1 Methamphetamine and Microglia activation:

It has been previously established that Meth induces microglia activation in *in vivo* models (LaVoie, Card et al. 2004, Thomas, Walker et al. 2004, Thomas and Kuhn 2005) and also in brain of human addicts (Sekine, Ouchi et al. 2008). Regarding *in vitro* models, there is much less evidence of Meth-induced microglia activation, but it was demonstrated in HAPI microglial cell lines that high doses of Meth induce pro-inflammatory cytokines mRNA expression (Tocharus, Khonthun et al. 2010). However, this result is not directly comparable to our work, since they worked with immortalyzed cells exposed to high amounts of Meth. Overall, at the best of our knowledge, this was the first time that the Meth effects on microglial activation was evaluated in primary purified microglial cells with physiologic concentrations of Meth (Fornai, Lenzi et al. 2007) And, in these conditions, we verified that Meth did not directly activate microglial cells towards a pro-inflammatory profile. We analysed the phagocytic activity, iNOS expression, reactive oxygen species and pro-inflammatory cytokines mRNAs levels as hallmarks of pro-inflammatory activation. Surprisingly, we observed that Meth did not increase any of these parameters, which strongly suggest that Meth did not induce activation of primary purified microglia.

Accumulated evidence has involved overproduction of nitric oxide (NO) in methamphetamine (Meth)-induced neurotoxicity (Sheng, Cerruti et al. 1996). Several studies have demonstrated that the use of NO synthase blockers can provide significant attenuation of the toxic effects of Meth in both *in vitro* and *in vivo* (Di Monte, Royland et al. 1996, Sheng, Cerruti et al. 1996, Ali and Itzhak

1998). However, in the present study, exposure of microglial cells to Meth did not result in increased production of NO (accessed indirectly through expression of iNOS) which corroborates our findings showing that, in microglia cells, Meth is not able to increase iNOS expression in a direct way.

Furthermore, it has also been proposed that pro-inflammatory cytokines are involved in the toxic effects of Meth in CNS. Studies demonstrated that Meth increased levels of IL-1β mRNA in the hypothalamus (Yamaguchi, Kuraishi et al. 1991, Halladay, Kusnecov et al. 2003), and TNF mRNA in the mouse brain (Flora, Lee et al. 2002). Moreover, it was described that TNF-α and IL-6 mRNA levels rapidly increase in the hippocampus of Meth-exposed mice (Gonçalves, Baptista et al. 2010). Furthermore, Meth–induced neurotoxic effects were attenuated in mice with a null mutation for IL-6, suggesting that this cytokine might be an important component of the toxic cascade triggered by this psychostimulant (Ladenheim, Krasnova et al. 2000). In our work, we evaluated Meth effect in pro-inflammatory cytokines through mRNA levels in primary microglial cell cultures at 3 and 24 hours post treatment. Our results showed that Meth does not induce significant changes in the mRNA levels in none of the analysed pro-inflammatory markers (IL-1β, IL-6 and TNF). Based on these results it is possible to conclude that in the presence of Meth isolated microglial cells do not acquire an activated signature, which goes against previous results described by other authors (Tocharus, Chongthammakun et al. 2008, Tocharus, Khonthun et al. 2010). As already mentioned, these differences can be related to the use of microglial immortalized cell lines, and may also be attributed to the higher Meth doses used in those studies, which do not seem to transcribe a physiologic microglia response.

Another relevant aspect is that many of the existing studies reporting Meth-induced activation of microglia were conducted *in vivo* (LaVoie, Card et al. 2004, Sekine, Ouchi et al. 2008, Robson, Turner et al. 2014) where many other CNS cells are also affected by Meth exposure, which may obviously influence or exacerbate the microglial response. Importantly, from these studies in yet not possible to difference between the acute and chronic effects of Meth at the microglial level. Therefore, further studies will be needed to clarify this profile.

Based on the results presented above it is possible to conclude that Meth does not induce a pro-inflammatory signature in microglial cells, so, we decided to evaluate the hypothesis that, in presence of Meth, microglia could acquire an anti-inflammatory profile. To clarify this we evaluated the expression of anti-

inflammatory markers in microglial cells such as Arginase 1, TGF-β and IL-10. Arginase 1 increased expressed in microglial cells is associated to an anti-inflammatory state (Michelucci, Heurtaux et al. 2009, Goldmann and Prinz 2013). It is known that arginase 1 competes with iNOS to the use of L-arginine as a substrate leading to reduced inhibit NO production (Chang, Liao et al. 1998). In the present study, we did not observe any significant changes in Arginase 1 expression in presence of Meth, which does not favor the hypothesis that Meth could induce an anti-inflammatory state in microglial cells.

IL-10 and TGF-β are key anti-inflammatory markers that modulate astrocytes and microglia activation (Ridet, Privat et al. 1997, Hanisch 2002, Kremlev and Palmer 2005, Norden, Fenn et al. 2014). Studies have demonstrate that administration of IL-10 suppresses LPS-induced IL-1β expression in the hippocampus (Lynch, Walsh et al. 2004), while in IL-10 deficient mice, LPS challenge lead to increased and prolonged neuroinflammation (Richwine, Sparkman et al. 2009). TGF-β is known to inhibit pro-inflammatory cytokines and nitric oxide (NO) production by activated microglia (Suzumura, Sawada et al. 1993, Orellana, Montero et al. 2013). Our results do not show increased mRNA levels of any of these anti-inflammatory markers in microglial cells exposed to Meth. Together with the Arginase 1 results, these findings indicate that microglia cells do not acquire an anti-inflammatory signature in presence of Meth.

As Meth was not able to act on the microglial activation state directly, and in vivo studies clearly show an activation of these cells after Meth administration, it seems reasonable to suppose that this in vivo activation may result from the interplay with other cellular players such as the astrocytes or different neuronal types.

5.2 Methamphetamine and Astrocyte activation:

Both *in vivo* and *in vitro* studies reported astrogliosis in the presence of Meth (Narita, Miyatake et al. 2006, Robson, Turner et al. 2014). In this activated stage, astrocytes can release many inflammatory factors that may influence microglial cells. It was already demonstrated that repeated *in vivo* Meth treatment induced a robust activation of astrocytes as shown by significant increase in glial fibrillary acidic protein (GFAP) levels in several mice brain regions such as the

cingulate cortex, nucleus accumbens, hippocampus (Gonçalves, Baptista et al. 2010) and the striatum (Narita, Miyatake et al. 2005, Zhu, Xu et al. 2005). GFAP was also shown to be increased in the brain astrocytes of human Meth-addicts (Kitamura, Takeichi et al. 2010).

Thus, we accessed the activation of astrocytes after Meth exposure, by analyzing the stress fibers, GFAP and iNOS expression in these cells. As reported, we did not observe any Meth-induce significant increase in the expression of iNOS, GFAP or stress fibers when compared to the control. Therefore, as observed for microglial cells, Meth exposure does not seem to be able to directly activate primary astrocytes. Other authors however, have reported increased GFAP expression in astrocytes exposed to similar concentrations of Meth for a period of 3 days (Narita, Miyatake et al. 2005). Another in vitro study also reported Meth-increased expression of GFAP when using co-cultures of neuron-glia, which does not allow to evaluate Meth effects on isolate astrocytes (Narita, Miyatake et al. 2005, Narita, Miyatake et al. 2006). Therefore, although we did not observe an activation of astrocytes at 24h, it is possible that longer Meth exposure periods could lead to a different result.

5.3 Astrocytes and microglia cross-talk under Meth exposure:

The bidirectional communication between neurons and astrocytes through the so-called gliotransmitters (such as glutamate, ATP, and D-serine) has been shown to modulate synaptic transmission and plasticity through several mechanisms (Araque, Carmignoto et al. 2014). Glutamatergic signalling in particular has been implicated in the modulation of drug-seeking behaviour (Reissner, Gipson et al. 2014). Increased levels of glutamate are well known to lead to sustained Ca^{2+} influx and initiating a cascade-like effect that leads to cell death (Dong, Wang et al. 2009). In the process, there is substantial ROS formation and consequently increased oxidative damage. Increased glutamate release is often associated to neuroinflammation (Tzschentke and Schmidt 2003).

In vitro studies showed that astrocytes could cooperate with microglia to prevent excessive inflammatory responses in the brain, as it was demonstrated that microglia treated with astrocyte conditioned medium (ACM) reduce iNOS and ROS production by an increased expression of microglial antioxidant enzymes

(Min, Yang et al. 2006). Furthermore, it was also reported that in microglia treated with ACM suppressed interferon-gamma (IFN-ϒ)-induced ROS production, leading to reduced iNOS expression and NO release (Min, Yang et al. 2006, Shih, Fernandes et al. 2006). However, it was also reported that microglial release of TNF reduces the activity of glutamate transporters in astrocytes, further contributing to a hyperglutamate state (Zou and Crews 2005). Although activation of microglia and astrocytes are normal homeostatic mechanisms in brain injury, excessive neuroinflammation may result in further damage.

As such, although Meth did not induce astrocyte activation, we used the conditioned medium of Meth treated astrocytes to incubate microglial cells. We observed that ACM treated with Meth significantly increase phagocytic activity, iNOS expression and ROS production in primary microglia cell cultures. These results strongly suggest that, in presence of CM of astrocytes treated with Meth, microglial cells acquire an activated signature. Although Meth did not directly activate astrocytes, this compound induced astrocytes to release soluble factors that directly modulate microglia activation.

In order to isolate possible astrocyte-released factors involved in triggering microglial activation under Meth exposure, we analyzed the production, in astrocytes, of pro-inflammatory cytokines such as IL-6, TNF and IL-1β (Lau and Yu 2001, Hanisch 2002). However the mRNA levels of such cytokines were too low to be detected through quantitative PCR, indicating that further studies are needed to reliably understand the concrete role of these cytokines (IL-6, TNF and IL-1β) in microglia activation induced by ACM.

Another possible mediator in the astrocyte-microglia cross-talk is the excitotoxic release of glutamate. Further studies will be necessary to confirm this hypothesis.

In conclusion, Meth *per se* does not induce a pro-inflammatory or an anti-inflammatory signature in microglial cells nor increase reactivity in astrocytes. Under Meth exposure, microglia activation appears to be mediated by astrocyte-released factors, however at this point is not possible to exclude a possible role for other cellular players, as the different neuronal types. Mainly, dopaminergic and glutamatergic neurons, classic targets of Meth, may also contribute to the activation of microglia.

6. Conclusion

In this work we investigated the effects of Meth on primary microglial cells cultures. We observed that Meth *per se* did not shift microglial cells neither to a pro-inflammatory nor anti-inflammatory signature.

Also, we found that Meth *per se* has not induced an activated state on astrocytes in culture. However, microglia activation appears to be mediated by conditioned medium of astrocytes treated with Meth.

The results described above clearly show that Meth induces indirectly microglial activation and astrocytes appear to be a vehicle for this activation, however it is important to not rule out the hypothesis that another CNS cell can also influence microglial activation induced by Meth.

7. References

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