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# IMPACT OF METHYLPHENIDATE ON MICROGLIAL CELLS

Dissertação de Mestrado em Biologia Celular e Molecular com especialização em Neurobiologia, orientada pela Doutora Ana Paula Pereira da Silva Martins e co-orientada pela Doutora Emília da Conceição Pedrosa Duarte e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Cover image: Microglia from the prefrontal cortex of Wistar Rats treated with saline or MPH (1.5 mg/kg) solutions once a day during 21 days (blue: nuclei; red: microglia; green: endothelial cells).

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**Teresa Silva Lourenço**

**August 2017**

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Sê todo em cada coisa. Põe quanto és no mínimo que fazes.

Ricardo Reis in “Odes”
(Heterónimo de Fernando Pessoa)

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## ABREVIATIONS

**A**

**ADHD –** Attention-deficit hyperactivity disorder
**ADP –** Adenosine diphosphate
**AMPA –** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
**ASC –** Apoptosis-associated speck-like protein containing a caspase recruitment domain
**ATP –** Adenosine triphosphate

**B**

**BBB –** Blood-brain barrier
**BCA –** Bocinchoninic acid
**BDNF –** Brain derived neurotrophic factor
**BSA –** bovine serum albumin

**C**

**cAMP –** Cyclic adenosine monophosphate
**CARD –** Caspase recruitment domain
**CAV-1 –** Calveolin-1
**$C_{max}$ –** Peak plasma concentration
**CNS –** Central nervous system
**CSF-1 –** Colony-stimulating factor 1
**CSF-1R –** Colony-stimulating factor 1 receptor
**$CX_3CL1$ –** Fractalkine
**$CX_3CR1$ –** Fractalkine receptor

**D**

**DA –** Dopamine
**DAMPS –** Danger-associated molecular patterns
**DARPP-32 –** cAMP-regulated neuronal phosphoprotein
**DAT –** Dopamine transporter
**DGS –** General direction of Health – *Direcção Geral de Saúde*
**DHE –** Dihydroethidium
**DNA –** Deoxyribonucleic acid
**d-MPH –** *d-threo-methylphenidate*

**E**

**ECs –** Endothelial cells
**ER –** Extended-release
**ERK –** Extracellular signal regulated activated kinases

**F**

**FBS –** Fetal bovine serum
**FDA –** Federal drug Administration

**G**

**GAPDH –** Glyceraldehyde-3-phosphate dehydrogenase
**GDNF –** Glial-derived neurotrophic factor

**H**

**$H_2O_2$ –** Hydrogen peroxide

**I**

**IFN-ϒ –** Interferon gamma
**IGF-1 –** Insulin-like growth factor 1
**IL –** Interleukin
**IL-1RI –** Interleukin-1 type I receptor
**IL-1RAcP –** IL-1R accessory protein
**IR –** Immediat- release

**J**

**JNK –** c-jun N-terminal kinase

**K**

**$K^+$ –** Potassium

**L**

**LPS –** Lipopolysaccharide
**l-MPH –** *l-threo-methylphenidate*

**M**

**METH –** Methamphetamine
**MPH –** Methylphenidate
**mRNA –** Messenger ribonucleic acid

**N**

**NADPH –** Nicotinamide adenine dinucleotide phosphate oxidase
**NE –** Norepinephrine
**NET –** Norepinephrine transporter
**NFκB –** Nuclear factor-κB
**NGF –** Nerve growth factor
**NLRs –** NOD-like receptors
**NLRP3 –** NOD-like receptor family pyrin domain containing 3
**NMDA** – N-methyl-D-aspartate
**NO –** Nitric oxide

**NOX –** Nicotinamide adenine dinucleotide phosphate oxidase

**O**

**$O_2^{\cdot}$ –** Superoxide
**$^{-}OH$ –** Hydroxide
**$ONOO^{-}$ –** Peroxynitrite

**P**

**PAMPS –** Pathogen-associated molecular patterns
**PBS –** Phosphate buffer saline
**PFA –** Paraformaldehyde
**PFC –** Prefrontal cortex
**PI –** Propidium iodide
**PKA –** Protein kinase A
**PRR –** Pattern recognition receptor
**pTrKB –** Brain-derived neurothrophic factor receptor phosphorylated
**PYD –** Pyrin domain
**P2X7R –** Ligand-gated P2X7 receptor
**p.o –** Per os (oral administration)

**R**

**Rac1 –** Ras-related C3 botulinum toxin substrate 1
**ROS –** Reactive oxygen species
**RT –** Room temperature

**S**

**SCN –** Suprachiasmatic nucleus
**SNpc –** Substantia nigra pars compacta

**T**

**$T_{1/2}$ –** Plasma half-time
**TLR –** Toll-like receptor
**TNF-α –** Tumor-necrosis factor α

**V**

**VitC –** Vitamin C

## ABSTRACT

Methylphenidate (MPH) is an amphetamine-like psychostimulant widely prescribed for attention-deficit hyperactivity disorder (ADHD). Therapeutic use of MPH is considered safe and produces few side effects. However, MPH has become very popular among healthy young adults in search for cognitive and competitive sports enhancement. At the present moment, little is known about MPH effects in the brain, especially in a non-pathological condition. Taking into account that MPH misuse/abuse is becoming worrisome it is imperative to understand how MPH consumption affects the brain of healthy individuals.

Microglial cells are considered the immune resident macrophages of the central nervous system (CNS) and are responsible for the brain homeostasis and immune defense. Yet, under certain conditions they may have a deleterious effect on the brain. Upon a classical activation, these cells can display a pro-inflammatory phenotype characterized by pro-inflammatory cytokines expression and reactive oxygen species (ROS) production that, according to the levels released, will have a positive effect of signaling/defense or a negative effect leading to neurodegeneration. Nevertheless, an alternatively activation of microglial cells can trigger an anti-inflammatory response by these cells and contribute to tissue repair and regeneration.

There are only a few studies relating MPH consumption and microglial activation. Therefore, the aim of the present work was to evaluate MPH effects in microglial cells and clarify some of the consequences that may result from MPH misuse.

Our results show that MPH (500 μM) does not cause microglial cell death but leads to a fast and transient upregulation of the pro-inflammatory cytokine IL-1β and the inflammasome NLRP3. Moreover, these effects were accompanied by an increase in intracellular ROS levels, which was prevented by vitamin C (200 μM). Furthermore, we investigated the impact of chronic MPH administration (1.5mg/kg/day, p.o, during 21 days) in healthy Wistar Rats. We showed that MPH leads to microglial activation in the prefrontal cortex (PFC), which was accompanied by ROS production without astrocyte activation. Interestingly, MPH also decreased endothelial staining, suggesting a blood-brain barrier dysfunction.

Overall, our results show that MPH causes microglial activation both in microglia N9 cellular line and in rat PFC. Moreover, microglial response is accompanied by several cellular pro-inflammatory hallmarks (IL-1β expression, NLRP3 activation and intracellular ROS production) which can be involved in microglia response and/or adaptation to the surrounding environment.

**Keywords:** Methylphenidate, microglia, IL-1β, NLRP3, reactive oxygen species

## RESUMO

O metilfenidato (MFD) é um psicostimulante da família das anfetaminas que é prescrito como primeira linha de tratamento para a Perturbação de Hiperactividade e Défice de Atenção (PHDA). Em doses terapêuticas o MFD é considerado seguro e manifesta poucos efeitos secundários. Contudo, o uso deste fármaco tem também muito sucesso entre jovens adultos saudáveis que procuram uma melhoria no desempenho cognitivo e em desportos de alta competição. Neste momento ainda se sabe muito pouco sobre os efeitos do MFD no cérebro, essencialmente numa condição não-patológica. Tendo em conta que o seu consumo sem controlo médico atingiu números preocupantes, é extremamente importante esclarecer como o uso de MFD pode afetar o cérebro de indivíduos saudáveis.

As células da microglia são consideradas os macrófagos residentes do sistema nervoso central (SNC) e são responsáveis pela homeostasia e defesa imune do cérebro. No entanto, em determinadas condições têm um efeito deletério. Após uma activação clássica, estas células apresentam um fenótipo pró-inflamatório caracterizado pela expressão de citoquinas pró-inflamatórias e produção de espécies reactivas de oxigénio (ERO) que de acordo com os níveis libertados podem ter um efeito positivo de sinalizações/defesa, ou negativo contribuindo para a neurodegenerescência. Por outro lado, uma ativação alternativa da microglia pode desencadear uma resposta anti-inflamatória por parte destas células e contribuir para a regeneração e recuperação de tecidos.

Existem poucos estudos na literatura que relacionam o consumo de MFD com a activação da microglia. Assim, o objectivo do presente trabalho foi avaliar o papel do metilfenidato nas células da microglia e esclarecer alguns dos efeitos que podem advir do uso incorrecto deste fármaco.

Os nossos resultados mostram que o MFD (500 μM) não induz morte das células da microglia mas leva a um aumento rápido e transitório dos níveis proteicos da citoquina pró-inflamatória interleucina-1β (IL-1β) e do inflamassoma NLRP3. Além disso, estes efeitos fazem-se acompanhar por um aumento na produção intracelular de ERO, o qual foi prevenido pela vitamina C (200 μM). Testámos ainda o efeito deste fármaco em ratos Wistar adultos saudáveis, que foram administrados oralmente com uma dose de 1.5 mg/kg/dia durante 21 dias consecutivos. Concluímos que a administração crónica deste psicoestimulante levou a uma ativação das células da microglia no córtex pré-frontal (CPF), a um aumento da produção intracelular de ERO, mas sem efeito significativo nos astrócitos. Mostrámos ainda uma

diminuição significativa da marcação de células endoteliais sugerindo uma disfunção da barreira hemato-encefálica.

Em conclusão, os nossos resultados mostram que o MFD causa a activação da microglia quer na linha celular N9 quer no córtex pré-frontal de ratos submetidos a um protocolo de administração crónica deste fármaco. Para além disto, a activação da microglia é acompanhada por várias características pró-inflamatórias (expressão de IL-1β, activação do NLRP3 e produção intracelular de ERO), as quais podem estar envolvidas na resposta e/ou adaptação das células da microglia ao ambiente envolvente.

**Palavras-chave:** Metilfenidato, microglia, IL-1β, NLRP3, espécies reativas de oxigénio.

# CHAPTER I

## *Introduction*

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# *Introduction*

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## 1.1 METHYLPHENIDATE

The psychostimulant methylphenidate (MPH; commercially known as Methylin®, Ritalin®, Concerta® and Metadate®)[1] (Fig. 1.1) was synthesized in 1944 and marketed for the first time as Ritalin by Ciba-Geigy Pharmaceutical Company. [2]

Methylphenidate was first prescribed to chronic fatigue, depressed states, depression-associated psychoses and disturbed senile behavior.[2] Additionally, MPH has also been used in the treatment of narcolepsy, giggle incontinence, and to alleviate distress that patients might have related to HIV infection and cancer.[2]

Nowadays, MPH is a FDA-approved drug for use both in children and adults[3], and is considered the primary drug of choice in attention-deficit hyperactivity disorder (ADHD) treatment.[4] In fact, according to the General direction of Health (DGS), the National Program for Mental Health states that children in Portugal under the age of 14 took about 5 million MPH doses in 2015, being more commonly prescribed to children between ages 10 and 14.[5]

Noteworthy, patients should have some cautions when under medication, including alcohol abstinence due to the fact that alcohol may intensify the adverse central nervous system (CNS) effects of this psychostimulant. Furthermore, when MPH is used alongside with alcohol, it can lead to the formation of ethylphenidate, a metabolite that is toxic to human body.[6] Moreover, MPH is contraindicated to patients suffering from agitation, tension and anxiety, becauste it can  exacerbate these symptoms.[3] The use of MPH can also have a few side effects. The most common are insomnia, decreased appetite, abdominal pain, bodyweight loss, irritability and anxiety. Some effects such as increased heart rate and blood pressure, psychosis and hypersensitivity reactions have also been reported.[7]

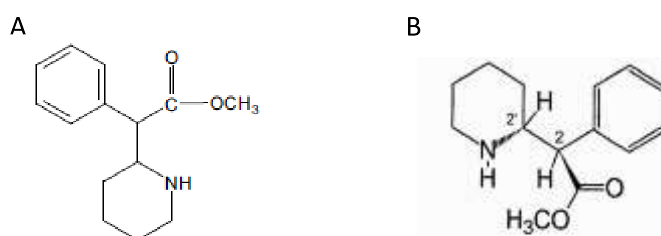


**Figure 1.1| Structure of methylphenidate**. (A) Molecular structure of methylphenidate and (B) its active enantiomer *d-threo-methylphenidate.* Adapted from Challman and colleagues [4] and Kimko and colleagues [8].

MPH, classified as an amphetamine-like stimulant, acts at the CNS through the blockage of dopamine and norepinephrine transporters (DAT and NET, respectively), resulting in a decrease on the uptake of these neurotransmitters by the pre-synaptic terminal and, consequently, an increase of dopamine (DA) and norepinephrine (NE) levels in the synaptic cleft (Fig. 1.2). Thereby, an intensified DA and NE post-synaptic signal is observed.[1] This DAT and NET blockage mechanism occurs by competitive inhibition[8] and can be considered similar to cocaine effect on CNS.[3] Moreover, MPH also binds to other receptors in the brain such as muscarinic and serotonergic receptors but with a lower affinity.[3, 8] Taking into consideration different observations, it is plausible to suggest that MPH effects are due to multiple neurotransmitters influence and not only due to DA and NE.[3]

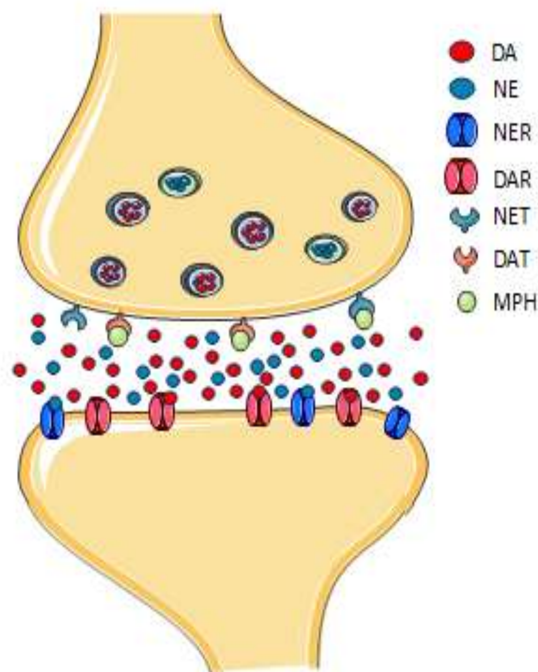


**Figure 1.2| Mechanisms of action of MPH at the synaptic level.** Methylphenidate blocks dopamine and norepinephrine transporters and potentiates dopamine and norepinephrine levels at the synapse. Legend: DA, dopamine; DAR, dopamine receptor; DAT, dopamine transporter; MPH, methylphenidate; NE, norepinephrine; NER, norepinephrine receptor; NET, norepinephrine transporter.

A single-photon emission computed tomography study suggested that nucleus accumbens, prefrontal cortex (PFC) and striatum are the brain areas where MPH has a preferential effect.[3] In addition to these CNS effects, MPH is also able to trigger several peripheral responses, as described below. Nevertheless, its cellular and molecular effects are far from being unravel.

### 1.1.1 Pharmacokinetics

Methylphenidate molecule has two chiral centers and a total of four enantiomers.[3] However, only its *threo* pair is used in therapy[7] since the *erythro* isomers do not have any major stimulant effect on CNS.[3] As a result, therapeutic MPH consists on a racemic (50:50) mixture of only *d,l-threo-methylphenidate*.[3, 9] Despite the fact that both of these isomers (*d-threo-methylphenidate* and *l-threo-methylphenidate*) share a lot of resembles, both pharmacokinetic and pharmacodynamics differences are well stablished and it is suggested that *d-threo-methylphenidate* (Fig. 1.1) is the major pharmacological effective isomer.[9] For therapy, *d,l-threo-methylphenidate* is available both as immediate-release (IR) and extended-release (ER), being the ER the major formulation used.[3] Moreover, a product only with *d-MPH* isomer [d-threo-(R,R)-MPH, usually known as dexmethylphenidate] is also available.[10]

Following oral administration, MPH has a rapid and almost complete absorption, being up to 50% of the dose eliminated from the circulatory system through metabolite products by urine and faecal excretion 8 h after the dose administration.[7] About 60%-86% of MPH is excreted as ritalinic acid (α-phenyl-2-piperidine acetic acid), and the rest is eliminated as minor metabolites. Only less than 1% of unchanged MPH appear in the urine.[2]

Peak plasma concentration ($C_{max}$) happens 1 to 3 h ($T_{max}$) after oral MPH administration[7], with a plasma half-time ($T_{1/2}$) of approximately 1.5 to 2.5 hours for children and 3.5 hours for adults.[3] This brief $T_{1/2}$ might be explained due to low protein binding of MPH (10%-33%).[3] According to the “free hormone hypothesis”, the biological activity of an hormone is defined by its free (unbound) concentration on the plasma.[11] Taking into consideration this hypothesis, and the fact that MPH has a high lipid solubility, this indicates that a significant amount of MPH is available to penetrate into CNS[7] and so to promote its stimulant effect. Moreover, at oral therapeutic doses (0.3-0.6 mg/kg), MPH is estimated to occupy more than half of brain dopamine transporters, showing a high affinity for DAT.[3]

The major metabolic pathway to metabolize MPH is through de-eterification[7] by the carboxylesterase CES1A1[12] to form ritalinic acid, which is pharmacologically inactive.[7] Only few amounts of hydroxylated metabolites, formed by MPH biotransformation, are detectable in plasma, such as hydroxymethylphenidate and hydroxyritalinic acid[3]

The MPH metabolism shows stereoselective clearance, resulting on a gradual shift in the plasma of *d*-MPH and *l*-MPH levels over time. After 1.5 h is possible to notice a significant difference between the plasma concentrations of these isomers[7], being the metabolism of *l*-MPH enhanced, and therefore a decrease in *l*-MPH levels is seen.[3] This can also be translated into a greater bioavailability of *d*-MPH.[7]

When administered intravenously, the total body clearance (CL) of *l*-MPH is significantly higher when compared with *d*-MPH and the $C_{max}$ is detected at 5 to 15 minutes after the injection.[7]

Oral administration can be performed through several forms, such as immediate release tablets, sustained release tablets or sustained release tablets chewed before swallowing.[7] Despite different formulations, the immediate release formulation is the one that has demonstrated more positive results on disruptive behavior, including cognitive function. It is important to note that there is no interconversion between the *threo*-enantiomers when pure *d*- or *l*-MPH are orally administered separately.[7]

### 1.1.2 Clinical uses

Due to its short half-time, MPH is commonly prescribed in multiple daily doses[7], however the total dosage is determined clinically and depends on each individual. Moreover, based on recent meta-analyses reports of heterogeneous results, it is difficult to predict which patients will respond to MPH treatment. Several facts can justify this variability, such as MPH dose, regimen and duration of the treatment.[13] In addition, brain imaging studies suggest that long-term MPH use can lead to an increase tolerance to stimulants, creating the need of patients to get higher doses to exhibit the same medical effects than previously stimulant-naïve patients.[14]

#### *1.1.2.1 Attention-deficit hyperactivity disorder*

MPH is one of the most commonly prescribed psychostimulants for ADHD, being important in reducing impulsivity symptoms and hyperactivity. [2] ADHD is a chronic psychiatric disorder characterized essentially by inattention, and/or impulsivity and hyperactivity, which is translated through an excessive motor activity.[15, 16] ADHD is one of the most common neuropsychiatric diseases in children. Overall, it is estimated that 7.2% of children and adolescents have ADHD[17] and about 60% to 80% of these individuals continue manifesting symptoms in adulthood.[4, 18] In terms of treatment, MPH immediate-release requires three times-daily administration, which can lead to missed doses. To overcome this problem, long-acting formulations with biphasic release were developed, appearing to deliver sustained activity and allowing one daily administration,[19] conferring a more practical treatment approach.

#### *1.1.2.2 Narcolepsy and Cancer*

MPH is also a FDA-approved drug for the management of narcolepsy. MPH (dose range 20-40 mg/day) can improve the sleepiness and sleep attacks that patients suffer, improving the patient's ability to stay awake.[3, 20]

MPH can also have benefits in improving mood, cognition and pain control in cancer patients. Several case reports show that MPH can have a positive role in reduce sedation, improve appetite and depressive symptoms.[3]

### 1.1.3 Methylphenidate misuse

The misuse of MPH has gained attention it the past few years, in a way that it is the second most used illicit substance by college students, just after marijuana[21], and is mostly sold on the black market among adolescents.[22] The term misuse refers to the use of MPH that is not prescribed by a physician or not taken in accordance with physician guidance. Among individuals between ages 18 and 25, MPH misused (nonprescribed) increased significantly from 3.6% in 2000 to 5.4% by 2006.[21]

#### *1.1.3.1 Attention-deficit hyperactivity disorder misdiagnosis*

ADHD diagnosis is a sensitive matter, since a proper diagnosis is critical in terms of health[23] and children's outcomes both in school and social interactions.

Recent reports have shown some areas of subjectivity on this diagnosis. For exemple, there is a relative-age effect since children born closer to the school start age (younger than their colleagues) are more frequently diagnosed with ADHD.[23] Also, a study revealed that boys born in December were 30% more likely to be diagnosed with ADHD than those born in January.[24] Therefore, there is an increasing concern to include relative maturity in this diagnosis to help in a more precise ADHD diagnosis.[23] Moreover, boys are more diagnosed than girls due to the fact that boys often exhibit more stereotypical symptoms than girls.[24] However, girls can also be affected by this subjectivity. Comorbidities, like anxiety and depression, can lead to both ADHD missed or misdiagnosis.[25]

To avoid the high rates of ADHD misdiagnosis, it is important to promote educational sessions about managing differences in child maturity and about factors affecting children's behavior.[23]

#### *1.1.3.2 Abuse potential*

Methylphenidate is a Schedule II drug conferring positive medicinal outcomes but with a significant abuse potential.[21] Thereby, this drug can also be converted into an abused and addictive substance when taken in excessive amounts either by intranasal, intravenous or oral administration.[2]

Physiological effects of MPH can be considered similar to cocaine, since both of these drugs block DA and NE transporters and interfere with both dopaminergic and noradrenergic pathways. This is further confirmed by the fact that, when administered intravenously, these two drugs are indistinguishable.[2] However, some reports have attributed a low abuse potential to MPH, since it has a relative slow clearance rate from the brain when compared to cocaine and hence less likelihood of repeated administrations.[26]

A rapid release of high levels of dopamine produces an instant "high" and euphoria giving a rewarding sensation that can be accompanied by paranoia, delusions, confusion and hallucinations.[2] Besides that, MPH abuse can also lead to psychiatric symptoms of extreme anger, aggressive behavior, repetitive behaviors and toxicity upon overdose.[2]

#### *1.1.3.3 Cognitive enhancement*

The use of psychostimulants to achieve an improvement of intellectual capacity, better working memory and sustained attention has long been reported.[27] MPH is one of the most popular drugs under consideration for this purpose. It has been shown that low doses of MPH (0.5 to 2 mg/kg in normal rats), similar to ADHD therapeutic doses, improve cognitive performance, while higher doses impaired performance.[28] At optimal doses, dopamine and norepinephrine bind with higher affinity to D1-like receptors and $\alpha_2$ noradrenergic receptors, respectively. This results on a strengthening neuronal communication. However, when these levels are higher than optimal, DA and NE activate $D_2$-like receptors and $\alpha_1$ and β noradrenergic receptors.[29]

Stimulation of $\alpha_2$ receptors inhibits cAMP signaling, which results on blockade of potassium channels and strengthen PFC physiological connections.[30] Stimulation of $D_1$ receptors results on a significant ERK 1/2 pathway activation and, together with PKA/DARPP-32 signaling, $D_1$ receptors phosphorylate AMPA and NMDA receptor subunits[31], which are responsible for synaptic plasticity and cognition.[32]

The proposed mechanism by which MPH improves cognition is the stimulation of $D_1$ and $\alpha_2$ receptors due to the increase of DA and NE neurotransmitters levels, respectively.[33] NE improves response inhibition, working memory and decreases distractibility through

interactions with $\alpha_2$ adrenoceptors on the PFC, while DA enhances the working memory through $D_1$ receptors also in PFC.[34] In addition, recent evidence suggest that MPH also improves emotion and motivation-related processes in healthy participants.[27]

#### *1.1.3.4 Performance enhancement in competition sports*

As previously mentioned, MPH improves both concentration and performance and some professional athletes use stimulants like MPH to gain an advantage over others.[35]

MPH can have several boost effects on sports performance of healthy athletes such as improvement of the task perception, increased attention, concentration, balance and enhanced acceleration together with the decrease on fatigue.[35, 36] Due to these effects, MPH is strictly regulated by International Olympic Committee (IOC), only allowing MPH use by athletes with adequate documentation of ADHD diagnosis and continued follow up.[35]

### 1.1.4 Central and peripheral effects of methylphenidate

#### *1.1.4.1 Methylphenidate effects on glial cells and neurons*

One of the main proposed MPH effects is the increased DA levels on the synaptic cleft. Previous studies demonstrate that free dopamine is responsible for inducing an inflammatory response in the brain, leading to microgliosis and an increase in chemokines and cytokines levels.[1]

A recent report showed that chronic treatment of mice with MPH (1 mg/kg and 10 mg/kg/day 5 days a week during 3 months) can sensitize substantia nigra pars compacta (SNpc) dopaminergic neurons to an oxidative stress, due to both increased inflammatory response and reduced levels of trophic factors, such as brain-derived neurotrophc factor (BDNF) and glial cell derived neurotrophic factor (GDNF).[1] The authors also showed that the chronic administration protocol of 10 mg/kg/day MPH could induce a significant loss of dopaminergic neurons also in mice SNpc region.[1] In addition, this study also reported an increase on activated microglial cells in the SNpc area after chronic MPH treatment (10 mg/kg/day 5 days a week during 3 months). However, the exact mechanism underlying this process is not yet fully understood.[1]

More recently, Schmitz and colleagues[37] reported that chronic MPH (2 mg/kg/day for 30 days) consumption is responsible for neuronal and astrocyte loss in the hippocampus of juvenile rats. This cellular loss appears to be apoptotic and is accompanied by a decrease in the levels of BDNF, its active receptor (pTrkB) as well as nerve growth factor (NGF) neurotrophic

levels. In fact, this study showed that the same protocol of chronic MPH treatment can alter the signaling pathways of extracellular signal-regulated kinase (ERK) and calcium/calmodulin-dependent protein kinase II (PKCaMII) by decreasing their levels and that can be, at least in part, due to impairment in the ERK-pTrkB pathway. Moreover, synaptosome-associated protein (SNAP-25) content is decreased in the hippocampus of juvenile rats after chronic MPH treatment. Noteworthy, neurite outgrowth, neuronal development, synaptogenesis, exocytosis, as well as neurotransmitter and hormone release are also compromised by MPH use. Iba-1 immunocontent, a microglial marker, pro-inflammatory cytokines [interleukin (IL)-6 and tumor necrosis factor α (TNF-α)] and cleaved caspase-3 are also increased upon chronic MPH administration (2 mg/kg/day during 30 days) and can have a role on promoting apoptosis, and reducing neurogenesis and proliferation.[37]

Regarding microglia, there are only a two studies[1, 37] showing MPH effect on these cells, as mentioned above, and further investigations are needed to understand and clarify the exact role of this drug and its repercussions.

#### *1.1.4.2 Methylphenidate and endothelial cells*

Our group has recently demonstrated for the first time that MPH also interferes with brain endothelial permeability via caveolae-mediated transcytosis.[38]

Caveolae primarily mediates the vesicular transport across brain endothelial cells (ECs)[39], and its main structural component, calveolin-1 (Cav1)[40], is important to modulate the activity of several signaling molecules. Cav1 phosphorylation on tyrosin 14 by Src family kinases is a crucial step involved on initiation of plasmalemmal vesicle formation, fission and transendothelial vesicular transport.[39, 41] MPH acts by stimulating caveolae-dependent vesicular transport due to activation of Ras-related C3 botulinum toxin substrate 1 (Rac1), which promotes the nicotinamide adenine dinucleotide phosphate oxidasse (NADPH oxidase also known as NOX) activity and an increase of reactive oxygen species (ROS). This NOX-induced ROS generation activates c-Src kinase at the plasma membrane, which phosphorylates Cav1 and promotes the transcytosis of macromolecules in brain ECs.[38]

Since ECs are the main component of the blood-brain barrier (BBB)[42], and caveolae-dependent transcytosis has been related to the transport of macromolecules[43], virus[44] and fungal pathogens[45] across brain ECs, these findings suggest that the BBB permeability might be compromised under MPH use.

In addition, MPH-increased ROS levels can also interfere with brain antioxidant defenses[46] leading to an overpower of the intracellular antioxidant defense systems, creating

an imbalance in redox homeostasis, oxidative stress and resulting on endothelial dysfunction.[47]

#### *1.1.4.3 Other effects*

Shin and colleagues[48] showed that MPH treatment in children and young people with ADHD can increase the risk of heart arrhythmia. Regarding a more delayed outcome, it is hypothesized that MPH can cause myocardial infarction. [48]

MPH treatment can also change the circadian activity profile of healthy mice. A recent report showed that in health adult animals MPH change both sleep and circadian rhythms, as demonstrated by the delay of animal's beginning light period. The change of circadian system can be also explained by the impact of MPH on the central clock's properties, probably due to alterations of monoamines levels.[49] However, in animals with suprachiasmatic nucleus (SCN) lesions, MPH was able to restore circadian rhythms.[49]

In terms of drug dependence, MPH can increase cocaine addiction vulnerability in adolescent ADHD animal models during adulthood, especially after discontinuation of treatment. The exact mechanism is still unclear; however one possibility is that MPH decreases DA clearance in the PFC, exarcebating cocain effects.[50]

## 1.2 MICROGLIAL CELLS

Microglia were first reported by William Ford Robertson and Franz Nissl who, based on the rod-like shape of their nuclei, called them as "Staebchenzellen" and highlightened their phagocytic, proliferative and migratory capacity.[51] Later, the name "microglia" was given by Pio del Rio-Hortega, who distinguished their capacity to differentiate from ramified to amoeboid cells.[52]

It was assumed for decades that adult microglia was originated by infiltration of circulating blood monocytes followed by differentiation.[53] However, after an intence debate, it is now known that microglia arises in yolk sac islands during primitive haematopoiesis as mesodermally-derived mononuclear cells that invade the brain rudiment at eraly stages of fetal development, and these cells persist in the CNS until the adulthood.[54]

Microglial cells are considered the resident mononuclear macrophages of the CNS and belong to the glial system that protects and supports a proper neuronal function.[55] In humans, the microglia population accounts for 0.5% to 16.6% of the total cells in the brain, depending on the region analyzed[56], being the hippocampus, substantia nigra, basal ganglia and telencephalon the regions where microglia has its highest concentration.[57] These cells are one of the most important cells within the brain and spinal cord and theirs adequate function is crucial for the CNS homeostasis in both health and diseased conditions.[58]

Microglia has the functional capacity of immune defense and also CNS maintenance. These cells are active sensors of the disturbances in brain microenvironment, detecting the first signs of pathogenic invasion, and are capable of responding in different ways to restore tissue homeostasis.[59] Microglia react to almost any form of disturbance of CNS homeostasis, such as infection, acute and chronic injury. In addition, these cells are also responsible for control neuronal proliferation and differentiation, the formation of synapses, synaptic pruning[60, 61] and regulation of the growth of dopaminergic axons.[62] In addition to the canonical role of microglia, these cells can also limit the infections of CNS by the release of many effectors molecules that are responsible for the recruitment of other blood immune cells, and can themselves play as phagocytic cells to eliminate material or be antigen presenting cells. Nonetheless, under pathological or inflammatory conditions, microglia can also secrete growth factors and anti-inflammatory molecules that help tissue repair and regeneration.[63]

Due to its high range of action, microglia has gain a lot of attention and become a potential key therapeutic target to recover from brain injury or under different neurological conditions.

### 1.2.1 Microglia functional states

The regional heterogeneity of the brain affects the microglia phenotype and gives a remarkable anatomical diversity to this cellular population[64]. These changes in microglial morphology are necessary and crucial for a healthy brain and for an efficient immune response upon brain injury or damage.

#### *1.2.1.1 Resting/Surveilling microglia*

Under healthy conditions, microglia seems to have a scavenger function for its surrounding area. The morphology of a “resting” microglia is described as a small cell soma with several elongated ramified processes (Fig. 1.3). Usually, the cell soma stays stable, where

the cell's processes are continuously elongating and retracting to scan the neighbor environment.[65, 66] Besides that, the resting microglia avoid contact between their cellular processes to ensure a stable cell size and a stable mosaic distribution.[66] The maintenance of the resting state of microglia can be due to several soluble or membrane-bound molecules, and microglia dynamics can be stimulated by factors like adenosine triphosphate (ATP)[67], and reduced by factors such as fractalkine ($CX_3CL1$)[68].

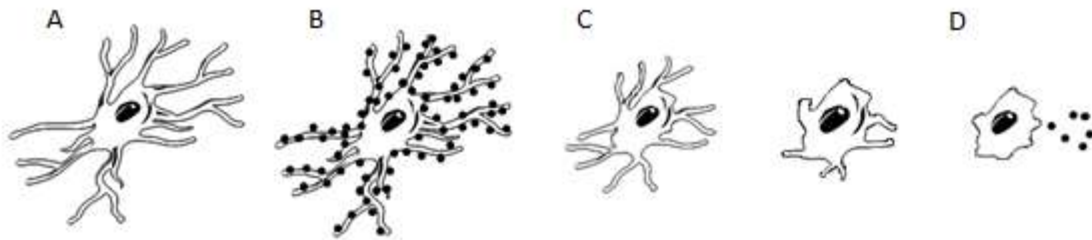


**Figure 1.3| Morphological changes during microglial activation.** (A) Resting/surveilling microglia has a ramified morphology with elongated processes to assure CNS homeostasis. (B) A triggering event, like infection, activates microglia with antigen surface expression and prompt (C) microglia transition to an activated, amoeboid and less ramified morphology. (D) Activated microglia with phagocytic capacity and capable of produce/secrete multiple bioactive molecules, such as cytokines.

#### *1.2.1.2 Pruning and neuromodulatory microglia*

The initial events of postnatal brain development are marked by a process of great brain plasticity characterized by glial cell death and synaptic remodeling, also known as synaptic pruning. Synaptic pruning is important to a healthy adult brain, since it assures the elimination of extranumerary synapses and strength the remaining ones, forming the adult brain connectivity.[69, 70]

Microglia has been associated with synaptic pruning due to evidences showing that these cells engulf some synapses via complement (C3)[71] and $CX_3CR1$-$CX_3CL1$ system[72], directly shaping neuronal connectivity. In addition do synaptic pruning, it was suggested a role for microglia on synaptic stripping, the process of separation of a presynaptic terminal from a damaged or injured postsynaptic terminal[73]. Moreover, microglia has also been implicated in the reorganization of adult circuits during ischemia and following sensory loss.[74, 75]

#### *1.2.1.3 Activated microglia*

Upon brain injury or damage, microglia activation is one of the main occurring events, which is crucial for restore brain homeostasis and a healthy brain function, as previous mentioned. Moreover, microglial activation is a common hallmark of neurodegenerative

diseases, and several conditions such as massive trauma and CNS infections can trigger its activation.[76] This cellular activation comprehends dramatic morphological alterations, changing from a resting/surveilling stage, where microglia is ramified, into an activated state characterized by a cellular amoeboid form that contributes to its mobility and facilitate phagocytosis (Fig. 1.3).[77]

Activated microglia can be divided into classically activated or alternatively activated (Fig. 1.4). The classically activated microglia, (known as M1 phenotype, characterized by a pro-inflammatory response) became activated in response to interferon gamma (IFN-ϒ) and to the proinflammatory agent lipopolysaccharide (LPS). These cells can mediate inflammatory tissue damage since they can produce ROS, and increase the levels of proinflammatory cytokines such as TNF-α and IL-1β.[78] This classical activation is thereby associated with cytotoxicity and inflammatory responses.[79] On the other hand, alternative activation of microglia (known as M2 phenotype, characterized by an anti-inflammatory response) is considered beneficial and can be subdivided into M2a, M2b and M2c phenotypes. M2a microglia became activated in response to IL-4 or IL-13 and can remove cellular debris and promote tissue repair, since these have a phagocytotic activity and produce growth factors such as anti-inflammatory cytokines like IL-10 and insulin-like growth factor 1 (IGF-1), being involved in repair and regeneration.[78, 79] The binding of LPS or IL-1β to immunoglobulin Fc gamma receptors (FcϒRs) (CD16, CD32 or CD64) induces a M2b phenotype which is immunoregulatory, associated with an increased phagocytic activity, increased secretion of IL-10, increased expression of human leukocyte antigen (HLA-DR) and reduced expression of IL-12.[80] M2c phenotype is related to an acquired-deactivaton and can be induced by glucocorticoids or IL-10 and is characterized by an increased expression of transforming growth factor beta (TGF-β).[79, 81]

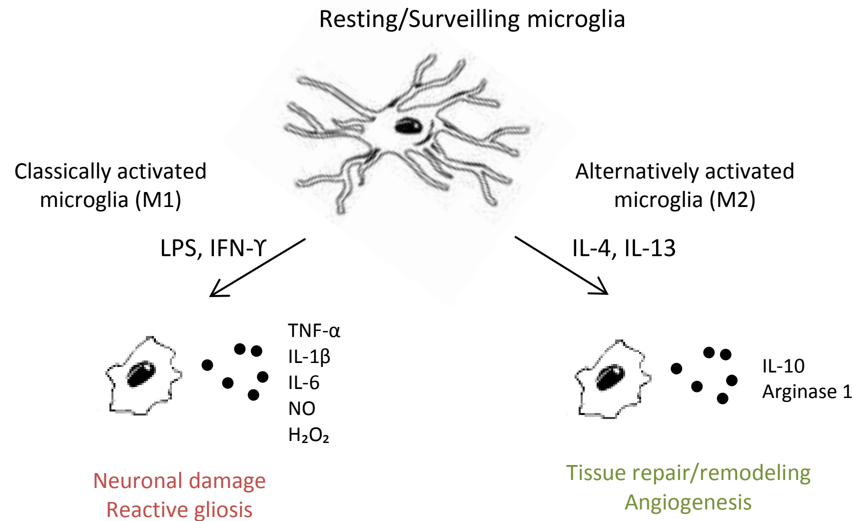


**Figure 1.4| Classically and alternatively activated microglia.** Microglial activation can be subdivided into classical activation (in response to factors such as lipopolysaccharide and interferon gamma), and alternative activation (in response to interleukins such as IL-4 and IL-13). The classical activation is associated with cytotoxicity and inflammatory responses, whereas alternatively activation is correlated with tissue repair and regeneration. Legend: $H_2O_2$, hydrogen peroxide; IFN-ϒ, Interferon gamma; IL-1β, interleukin 1β; IL-4, interleukin-4; IL-6, interleukin-6; IL-13, interleukin-13; LPS, lipopolysaccharide; NO, nitric oxide; TNF-α, tumor necrosis factor alpha.

Recently, microglial M3 phenotype has arisen, since microglial pro- and anti-inflammatory phenotypes do not include microglia undergoing cell division as a response to cytokines such as macrophage colony-stimulating factor 1 (CSF-1) and IL-34. Both cytokines act through the same receptor, CSF-1R, and in addition to induce cell division, they can have an impact on microglial development, maturation and survival.[82]

Although the binary concept of microglial M1/M2 remains a topic for debate[83], the functional classification of microglia as being either neurotoxic (M1) or neuroprotective (M2) is useful for illustrating the pathobiology of inflammatory and neurodegenerative disorders. In fact, the use of functional modulators of microglial phenotypes as potential therapeutic approaches for the treatment of neurodegenerative diseases has garnered considerable attention.

### 1.2.2 Microglial dual role: friend or a foe?

Normally, microglial activation upon injury has the ultimate goal of immune protection and tissue repair, involving inflammatory cytokines and phagocytosis. However, if the inflammatory stimulus persists or if the immune response fails to restore homeostasis, there is

a persistent stage of chronic inflammation that will lead to neurotoxicity and neuronal death.[84]

Microglia is seen as a “double-edge sword” and “friend or a foe”, since these cells can play a benefitial role, as abovementiones, but its over-activation can cause a state of chronic neuroinflammation and contribute to axonal damage and neuronal dysfunction.[84] The deleterious role of microglia has been reported in several conditions such as Alzheimer’s and Parkinson’s diseases, multiple sclerosis, stroke and spinal cord injury (SCI).[57, 58, 85] The microglial responses are not linear, but multifaceted, depending on the nature of stimulus and the prior state of the cell.[53] In sum, these cells can be both “friend or a foe”, depending on the stimulus and surrounding environment.

## 1.3 NEUROINFLAMMATION AND MICROGLIAL CELLS

Neuroinflammation is the inflammation of the CNS, and an immune response to several brain injuries and pathogens having a crucial role for brain protection, elemination of damaged cells and for the extracellular matrix repair.[86] The major contribution for this immune response relies on the action and signaling of microglia and astrocytes.[87] As mentioned above, microglial cells secrete several molecules such as pro- and anti-inflammatory cytokines, oxidative stress-inducing factors and growth factors[78]. Astrocytes can also secrete cytokines, chemokines and growth factors and can contribute to attract periphery immune cells from the blood vessels.[88]

### 1.3.1 Neuroinflammation and microglial activation

Neuroinflammation is characterized by the activation of the resident glial cells that can include different external signals such as “non-self” molecules derived from invading pathogens, designated as pathogen-associated molecular patterns (PAMPS), and molecules derived from endogenous stress named danger-associated molecular patterns (DAMPs) that function as “eat me” signals.[89, 90] These PAMPs and DAMPs are recognized by pattern recognition receptors (PRR), such as toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain-like receptors (NOD-like receptors or NLRs) that includes family members NLRP1, NLRP3 and NLRC4, expressed on the surface of resident brain cells such as microglia.[91, 92] This interaction between ligand and receptor triggers phagocytosis and/or production and release of pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α.[89]

Microglia can also be spontaneously activated by their surrounding cells and by endogenous signals that can trigger microglial activation without any CNS injury. Microglia communicates with several surrounding cells within the brain that can secrete or express on their membranes different molecules that act as exogenous activators signals. One of the main contributions for these events comes from healthy neurons (Fig 1.5)[93]. Neurons can express on their surface glycoproteins and glycolipids that interact with the "triggering receptor expressed on myeloid cells 2" (TREM2) associated with the adaptor protein DNAX-activating protein of 12 kDa (DAP12) complex on microglial membrane and cause an anti-inflammatory response and phagocytosis.[94] Furthermore, neurons secrete adenosine diphosphate (ADP) and ATP molecules that are recognized by microglial purinergic receptors and act as neuronal injury help signals.[67] However, not all molecules contribute to microglial activation. The interaction between CD200 (also expressed by astrocytes and oligodendrocytes) and its receptor CD200R on microglia, as well as the binding of $CX_3CR1$ to its ligand fractalkine ($CX_3CL1$), is needed for maintain the resting stage of these cells in the healthy CNS.[72, 95] Moreover, CD47 is expressed by neurons and is known to help microglia recognizing a non-harmful stimulus[95], and the cytokines IL-34 and Csf1 interact via Csf1R and transmit a survival signal for microglia.[96, 97]

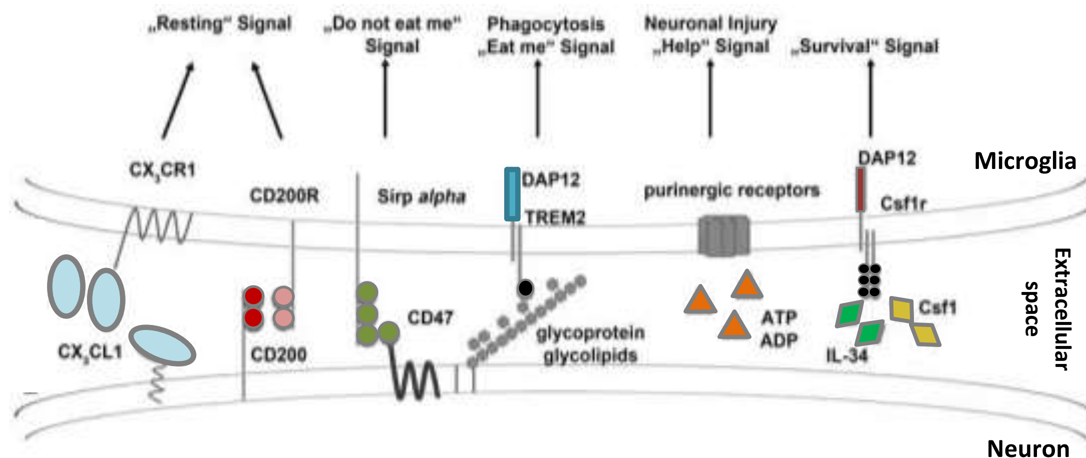


**Figure 1.5| Neuronal factors regulating microglial activation.** Microglia can be regulated by several exogenous signals that are provided by neurons. These crosstalk between microglia and neurons is important for the resting and vigilante stage of microglia, for trigger inflammatory response and for cell survival. Legend: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CD47, cluster of differentiation 47; CD200, cluster of differentiation 200; CD200R, cluster of differentiation 200 receptor; $CX_3CL1$, fractalkine; Csf1, colony stimulating factor 1; Csf1r, colony stimulating factor 1 receptor; $CX_3CR1$, fracralkine receptor; DAP12, adaptor protein DNAX-activating protein of 12 kDa; IL-34, interleukin-34; TREM2, triggering receptor expressed on myeloid cells 2. Adapted from Kierdorf and Prinz [93].

Nevertheless, endogenous signals controlling microglia activation and maturation can be included as well, such as transcription factors like Runt-related transcription factor 1 (Runx1), interferon regulatory factor 8 (Irf8) and the transcription factor Pu.1.[98]

### 1.3.2 Neuroinflammation and Inflammasome

Inflammasomes are protein complexes constituted by several molecules that assemble in the cytosol after recognizing PAMPs or DAMPs.[99] One of the main inflammasome components is PRRs including the NLRs and the absent in melanoma 2 (AIM)-like receptors (ALRs).[100] These complexes have two main effector mechanisms of action: the release of IL-1β and IL-18 cytokines[101] and activation of pyroptosis, which is a cell death pathway characterized by cell swelling, lysis and the release of cytoplasmic content to the extracellular milleu, being considered morphologically different from apoptosis.[102]

Activation of inflammasomes can be involved in inflammatory diseases as a causative or contributer factor, since it can amplify the pathology in response to host-derived factors.[103] Nevertheless, despites the fact that inflammasomes defend the brain against pathogens, they can lead to the development of cancer, autoimmune and neurodegenerative diseases.[104]

#### *1.3.2.1 NLRP3 Inflammasome*

The NLRP3 inflammasome (also known as cryopyrin) is expressed by microglia but not by astrocytes.[105] This inflammasome is activated in response to a wide variety of stimuli such as potassium efflux, increases in intracellular calcium, the production of ROS by mitochondria, uric acid crystals, extracellular ATP, pore-forming toxins, release of mitochondrial DNA or cardiolipin, and by several viral, bacterial and fungal pathogens.[106, 107] It is required at least two signals to activate NLRP3 (Fig. 1.6): priming by extracellular inflammatory stimuli, leading to transcriptional induction of NLRP3 via nuclear factor-κB (NF-κB),[108] and a signal from PAMPs and DAMPs that promote NLRP3 assembly for processing pro-caspase-1.[109]

Once the inflammatory ligand is recognized by the receptor, a signaling cascade leading to oligomerization and recruitment of the adaptor protein Apoptosis-Associated Speck-Like Protein Containing a CARD (ASC) is activated. ASC is constituted by two domains: a pyrin domain (PYD) and a caspase recruitment domain (CARD) that allows ASC to bridge and act as a scavenger molecule, recruiting pro-caspase-1 to the inflammasome.[110] ASC protein connects with NLRP3 through PYD-PYD interactions, and pro-caspase-1 interacts with ASC through CARD-CARD interactions, forming filaments that branch off the complex core.[104] Afterwards, pro-caspase-1 suffers autoproteolytic cleavage into its active form caspase-1 and cleaves the

precursor cytokines pro-IL-1β and pro-IL-18 into mature and active IL-1β and IL-18 cytokines, respectively.[104] Active caspase-1 is also capable of inducing pyroptosis.[111]

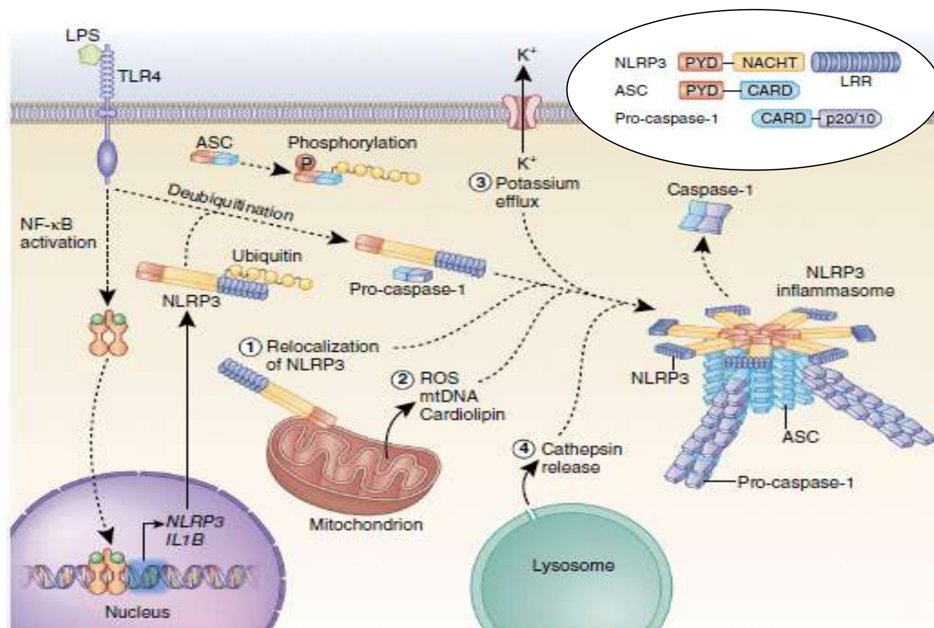


**Figure 1.6| Mechanisms of NLRP3 inflammasome activation.** Priming of NLRP3 inflammasome leads to an increased expression of NLRP3 and pro-IL1β proteins, as well as NLRP3 deubiquitination. ASC protein must be phosphorylated and ubiquitinated to allow inflammasome assembly. After priming, a second signal (such as potassium efflux or mitochondrial ROS) acts as an activating stimuli for NLRP3, which in turn recruits ASC through PYD-PYD interactions. Pro-caspase-1 subsequently binds to ASC through CARD-CARD interactions, allowing autoproteolytic activation of caspase-1 and cleavage of pro-IL1β and pro-IL18. Adapted from Haitao Guo and colleagues [104].

Furthermore, there is a noncanoncal activation of NLRP3 by caspase-11 in mice and by caspase-4 and caspase-5 in humans. These caspases are known to activate caspase-1 and caspase-3[112], and recently were shown to promote NLRP3 activation by indirectly enhancing the maturation of IL-1β and IL-18.[113, 114]

On the other hand, mutations in the NLRP3 are usually translated into cryopyrin-associated periodic syndromes (CAPS), with fever, conjunctivitis and fatigue as hallmarks.[115] NLRP3 dysregulation can also contribute to other diseases such as obesity, type 2 diabetes, atherosclerosis and rheumatoid arthritis.[116-118]

### 1.3.3 Neuroinflammation and Interleukin-1β

IL-1β is one of the most extensive reviewed pro-inflammatory cytokines produced by microglia but it can also be produced and secreted by neurons, oligodendrocytes, brain endothelial cells and astrocytes; yet, this event seems to be secondary to microglial activation.[119] Nevertheless, it is important to notice that IL-1β is expressed at low levels at

healthy brain conditions, and upon brain injury its levels are upregulated.[120] Regarding its biosynthesis, IL-1β is produced as a precursor form named pro-IL-1β and, in order to convert to its mature an active form, it is cleaved by the IL-1 converting enzyme (ICE; also known as caspase-1).[104]

IL-1β binds to interleukin-1 type I receptor (IL-1RI) and causes the recruitment of the IL-1R accessory protein (IL-1RAcP), which is essential to increase IL-1β binding affinity to IL-1RI and allow the signal transduction (Fig. 1.7).[121] In turn, this complex needs the intracellular Toll/IL-1R (TIR) domain-containing adapter protein MyD88 to activate serine/threonine kinases IL-1R-associated kinase (IRAK), which then will interact with tumor necrosis receptor associated factor-6 (TRAF6) and activate several protein kinases that will ultimately lead to activation of NF-κB and c-Jun N-terminal kinase (JNK) signaling pathways, contributing to cellular survival and immune response.[122] In addition to this canonical pathway, IL-1β signaling can also activate p38 and ERK1/2 (extracellular signal-regulated activated kinase 1 and 2) cascades.[122] Ultimately, IL-1β can upregulate the expression of its own levels and several other molecules that mediate an immune response in the brain, such as TNF-α, IL-6 and cyclooxygenase 2 (COX-2). Moreover, IL-1β is responsible for the upregulation of nitric oxide synthase and chemokines, contributing to the pathogenesis of both acute and chronic neuroinflammation.[120, 123]

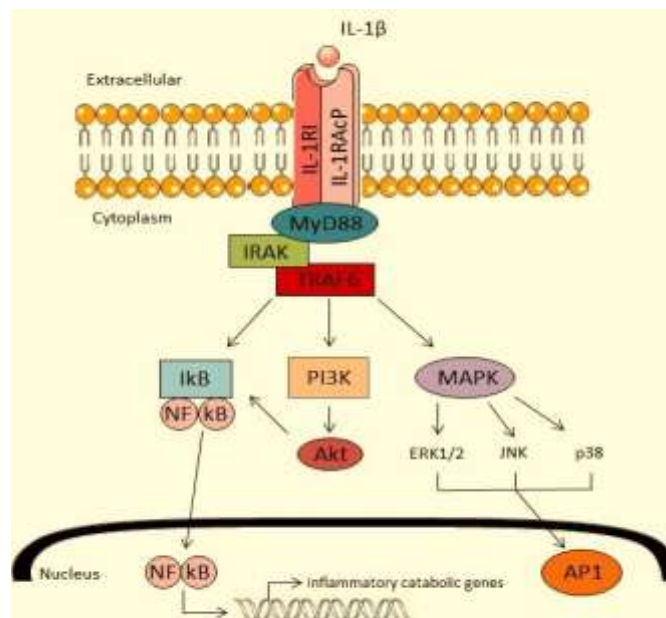


**Figure 1.7| Signaling pathway activated by interleukin-1β.** IL-1β binds to its receptor IL-1RI and recruits IL-1RAcP protein to the complex. A signal transduction cascade is started that ultimately triggers MAP kinases and NFκB activation. Legend: Akt, protein kinase B; AP1, activator protein 1; ERK1/2, extracellular signal-regulated kinases 1/2; IκB, NF-κB inhibitor; IL-1R, interleukin-1 receptor; IL-1RAcP, IL-1R accessory protein; IRAK, IL-1R-associated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-associated protein kinase; MyD88, myeloid differentiation primary response gene 88; NFκB, nuclear factor κB; p38, P38 mitogen-activated protein kinase; PI3K, phosphoinositide-3 kinase; TRAF6, tumor necrosis receptor associated factor-6.

Despite the fact that mature IL-1β acts via IL-1RI, there is also the interleukin-1 type II receptor that does not activate any posterior signaling cascade upon IL-1β binding.[124] Noteworthy, IL-1β has a dual role since it can be involved in chronic brain pathologies such as Alzheimer's and epilepsy, but can be considered beneficial when released in low concentrations, evidenced by its protective role on the survival of neurons and glial cells.[120]

### 1.3.4 Neuroinflammation and Reactive Oxygen Species

Reactive oxygen species are small molecules or ions characterized by the presence of unpaired electrons (radicals), with the exception of hydrogen peroxide ($H_2O_2$) which is not a free radical. Regarding their biosynthesis, they are generated in a controlled manner from specific enzymes, such as NADPH or as end-products of oxidative metabolism.[125]

Microglial cells are known to be a strong source of ROS such as nitric oxide (NO), peroxynitrite, superoxide and $H_2O_2$. Extracellular ROS are able to induce oxidative neuronal damage since they are strong neurotoxic factors. On the other hand, intracellular ROS act as second messengers, being a crucial interplay in the cellular homeostasis and proinflammatory function.[100] Microglia can produce both extra- and intracellular ROS, which make them as a potential and promising therapeutic target for neurotoxicity. [100]

Basal ROS levels are important for a normal cellular function, since these molecules can influence and selectively modify proteins by targeting thiol functional groups on cysteine amino acid residues.[126] These alterations can regulate protein function and control cellular signal transduction.[127] Indeed, ROS regulate several physiological functions such as gene activation, cellular growth, chemical reactions in the cell and prostaglandins production, among others.[128] However, as expected, excessive ROS can damage several biomolecules, impair cellular functions and participate in neurodegenerative diseases.[127]

The production of ROS in microglia can occur through different sources such as peroxidases inside the cell, NADPH oxidase on the membrane surface and oxidative processes in the mitochondria.[129] However, the NADPH oxidase is considered the predominant source of microglial ROS.[127]

#### *1.3.4.1 Microglia and NADPH oxidase*

NADPH oxidase (NOX) is an enzyme that catalyzes the production of superoxide from oxygen and NADPH, being the primary source of microglial-derived extracellular ROS. It is also involved in pro-inflammatory signaling microglia,[130] and in morphology changes of microglia.[131] In fact, NOX can also initiate an intracellular ROS signaling pathway that can

activate microglia and contribute to increase the production of IL-1β and TNF-α, and induce a pro-inflammatory M1 microglia polarization.[127] However, extremely high concentrations of ROS are capable of inhibiting pro-inflammatory signaling and induce lipid peroxidation and oxidative modifications of proteins.[132]

NADPH oxidase is a multi-subunit enzyme that catalyses the production of $O_2^{\bullet -}$ from molecular oxygen, hydroxyl radical ($OH^{\bullet -}$), lipid hydroperoxides, and their byproducts such as $H_2O_2$.[133] The NOX family includes seven enzymes, NOX1-5 and DUOX 1-2. Within this family, NOX1, NOX2 and NOX4 expression has been documented in microglial cells. Yet, NOX2 is highly expressed in microglia and it is considered the major NADPH oxidase enzyme in these cells. [134]

The NADPH oxidase enzyme is composed by cytosolic subunits that are regulatory (p40phox, p47phox, p67phox, and the GTPase Rac1) and are distributed between the cytosol and the membranes of intracellular vesicles and organelles, together with a membrane-bound flavocytochrome $b_{558}$ complex containing a p22phox subunit and a catalytic subunit (gp91phox/NOX2) (Fig 1.8). NOX2 is composed by a cytosolic N-terminal domain, six transmembrane domains and a long C-terminus that has binding sites for both flavin adenine dinucleotide (FAD) and NADPH. Upon microglia activation, the cytosolic subunits translocate to membrane and bind to the catalytic subunit, whereas gp91phox recieves electrons from cytosolic NADPH and shuttles them through the membrane for the reduction of molecular oxygen leading to the production of $O_2^-$.[125, 130] Furthermore, $O_2^-$ and $H_2O_2$ can reduce several other molecules and produce hydroxyl radical ($^{\cdot}OH$) and peroxynitrite ($ONOO^-$), which are highly reactive and destructive.[125]

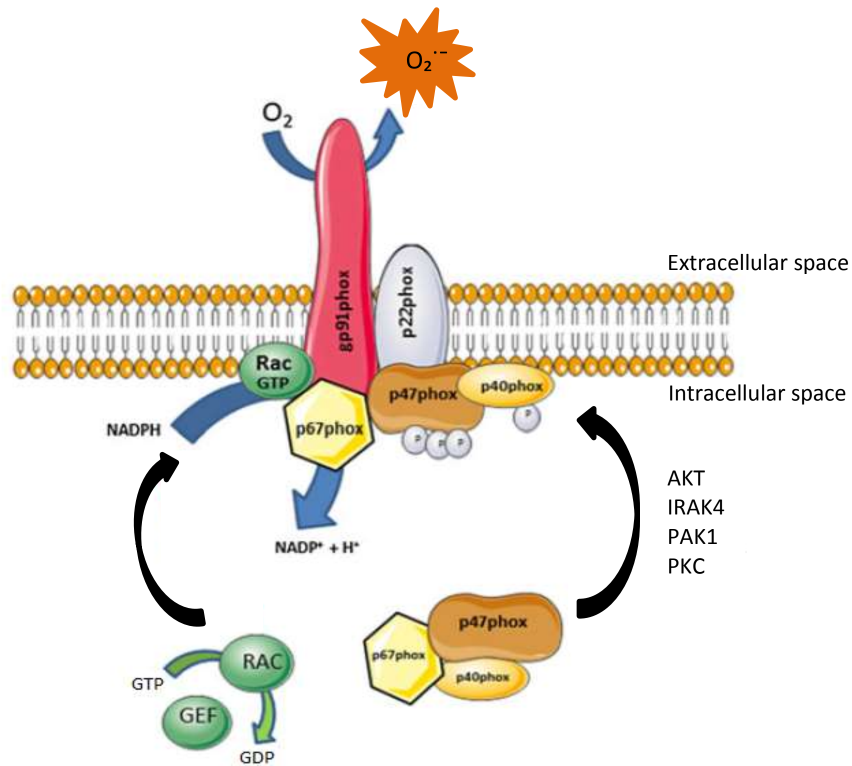


**Figure 1.8| The phagocyte NADPH oxidase.** Assembly of NADPH oxidase consists of the flavocytochrome $b_{558}$ complex [cyt $b_{558}$; composed of gp91phox (NOX2) and p22phox subunits] and cytosolic subunits p67phox and Rac1 (catalytic subunits), p40 and p47 (both serve to guide and retain p67phox interations with cyt $b_{558}$). In resting cells, p22phox is inhibited but, upon multiple phosphorilations on serine and threonine residues by activated kinases, the p22phox inhibition is released and either p47phox or p40phox transports p67phox to cyt $b_{558}$ in the membrane. Rac1 is activated by a GTP/GDP exchange factor (GEF) and, along with p67phox, mediates electron transfer from NADPH to the redox centers of gp91phox (FAD and heme) and to molecular oxygen on the extracellular side of the membrane to form $O_2^{\cdot}$. Adapted from Haslund-Vinding and colleagues [125].

The subcellular localization of the ROS that are generated, as well as the timing and its nature, are essential features for biological functions due to the different membrane permeability to ROS molecules (Fig. 1.9). For example, $H_2O_2$ and $ONOO^-$ molecules generated in the cytosol can diffuse across the plasma membrane through anion channels, while the membrane permeability to both $O_2^{\cdot}$ and $^{\cdot}OH$ is low, and the presence of NOX in the cell surface is required to a substantial release of these molecules to the surroundings.[125]

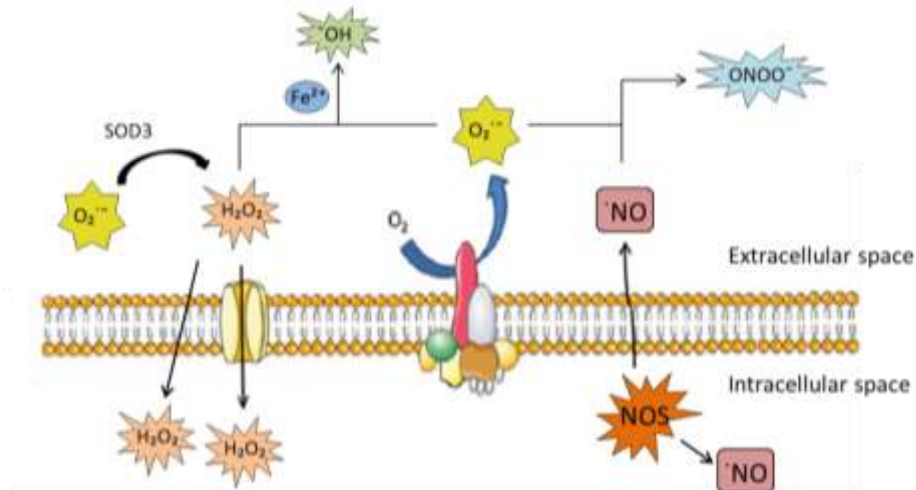


**Figure 1.9| NOX2-derived oxidante reactions.** Released $O_2^-$ quickly dismutates to $H_2O_2$ either spontaneously or through the action of extracellular SOD (SOD3). In the presence of free iron, both $H_2O_2$ and $O_2^-$ can react to form hydroxyl radical $^-OH$, and $O_2^-$ can also react in the presence of NO and form peroxynitrite ($ONOO^-$). A small portion of extracellularly produced $H_2O_2$ can enter into cytosol either by diffusion through the membrane or via aquaporin channels and alter the activity of redox targets. Adapter from Hauslund-Vinding and colleagues [125].

## 1.4 OBJECTIVES

The increasing misuse/abuse of MPH has been reported particularly associated with cognitive improvement among young adults. Recently, our group showed that this psychostimulant is able to promote the blood-brain permeability[38], which contributed to awakened a huge concern about the precise role of MPH in the healthy brain. Some recent reports have contributed to improve our knowledge about the cellular effects of MPH[1, 37, 38]. However, very little is known about its impact in microglia, despite their crucial role in brain immune surveillance and function.

Therefore, the aim of the present work was to characterize the effects of MPH in microglial cells, looking particularly for cell viability and inflammatory features. For that, it was used a microglial cell line and microglial primary cultures for an *in vitro* approach and several studies were carried out, including evaluation of cellular viability, IL-1β, NLRP3 and ROS levels. Complementary animal studies were also performed.

Ultimately, with the present work we aim to contribute to a better understanding of the brain consequences of MPH misuse and provide new insights in the field.

# CHAPTER II

## *Materials and Methods*

# CHAPTER II
# *Materials and methods*

## 2.1 Cell cultures

### 2.1.1 N9 cell line

The murine microglial cell line N9 was kindly provided by Prof. Claudia Verderio from the CNR Institute of Neuroscience, Cellular and Molecular Pharmacology, Milan, Italy. These cells were obtained by immortalization of E13 mouse embryonic brain cultures with the 3RV retrovirus carrying an activated v-myc oncogene.[135]

N9 cells were cultured in Roswell Park Memorial Institute medium (RPMI; Gibco, Paisley, UK) supplemented with 5% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (Gibco), 23.8 mM sodium bicarbonate (Sigma-Aldrich, St. Louis, MO, USA) and 30 mM D-glucose (Milipore, Madrid, Spain). Cells were kept in a humidified incubator (Thermo Scientific Forma Series II, Marietta, USA) at 37ºC in a 95% atmospheric air and 5% $CO_2$ environment and grown in 25 $cm^2$ tissue flasks. Before seeding cells for the experimental procedures, the number of viable cells was accessed by trypan-blue dye with a cell counting chamber, and plated at appropriated densities according with different experiments (Table 2.1).

**Table 2.1|** Microglial N9 cells densities used in different experiments

| Experiment | Nº cells/well | Multiwell culture plate | Volume of medium (µL) | Density (cells/mL) |
|---|---|---|---|---|
| TUNEL assay | $1.6 \times 10^4$ | 24 | 500 | $3.2 \times 10^4$ |
| ROS quantification | $1.2 \times 10^4$ | 96 | 100 | $12 \times 10^4$ |
| Western blot | $50 \times 10^4$ | 6 | 2000 | $25 \times 10^4$ |

### 2.1.2 Primary Cultures of Microglia

Microglia were isolated from C57BL/6J mouse pups aged P3-P5. After decapitation, the whole brain, with the exception of cerebellum, was isolated and incubated on microglial medium [DMEM high glucose (4.5 g/L, Gibco), supplemented with 10% FBS and 1% penicillin/streptomycin] for mechanical dissociation. The suspension was then filtered with a Corning® Cell Strainer 70 µm Nylon mesh (Corning Incorporated, Durham, USA) and centrifuged at 230×*g* during 10 min at room temperature (RT). Afterwards, the supernatant was discarded and the pallet was resuspended in microglial medium. Cells were plated on T-

flasks coated with poly-D-lysine (Sigma-Aldrich) at a density of $1.2 \times 10^5$ cells/$cm^2$ (approximately 3 brains per flask). The medium was changed the day after culture and then every 3 or 4 days until reach confluence. Then, the flasks were shacked (200 rpm for 2 h at 37ºC) and the medium containing the detached cells (microglia) were collected and once again filtrated with a 70 µm nylon mesh. The solution was centrifuged at 230×*g* during 8 min and the supernatant discarded. The cells were resuspendend, and plated at a density of $7 \times 10^4$ cells/mL on 24 MW plates coated with poli-D-lysine. Cells were kept in a humidified incubator (Thermo Scientific Forma Series II) at 37ºC in a 95% atmospheric air and 5% $CO_2$ environment until reach confluence (approximately 14 days). Microglia primary culture purity was stablished as 80%.

Regarding MPH treatments, acute MPH therapeutic doses can be translated in brain concentrations as much as 103 µM[136], and higher concentrations compared to 500 µM MPH can be found in brain upon chronic- and over-consumption. Thus, concentrations used in our study are within MPH doses identified in patients.

## 2.2 Animals and treatments

Five months old male wild-type Wistar rats (total of six animals; 500-600g body weight; Charles River Laboratories, Barcelona, Spain) were housed under controlled conditions (12h light-dark cycle, 24±1 ºC) with food and water *ad libitum*. All experimental procedures were carried out according to the guidelines of the European Community for the use of animals in laboratory (2010/63/EU) and the Portuguese law for the care and use of experimental animals (DL nº 129/92). The present work was part of a broader study approved by the Institutional Animal Care and Use Committee (FMUC/CNC, University of Coimbra, Portugal) and Portuguese National Authority for Animal Health "DGAV". All efforts were made to minimize animal suffering and to reduce the number of animals used.

Animals were subjected to saline (control animals) or chronic 1.5 mg/kg MPH (Sigma-Aldrich) treatment by gavage (oral administration) once a day, always at the same hour, for 21 consecutive days. MPH was dissolved in saline solution and given to animals at a volume of 1 ml/kg of body weight. Control group received equivalent volume of saline solution. This administration protocol (time frame and dose) was chosen to mimic what happens under chronic MPH use, in terms of neurochemical and behavioral effects.[137]

The control group (total of 2 animals) and the MPH group (total of 4 animals) were sacrificed 24 h after the last treatment and the animals were transcardially perfused with 4% paraformaldehyde (PFA; Sigma-Aldrich). Brains were removed, post-fixed in 4% PFA for 24 h at RT and changed to a 30% sucrose solution (Sigma-Aldrich) for another 24 h. After that, coronal sections were cut on a cryostat (Leica CM3050S, Nussloch, Germany) in accordance with Table 2.2.

**Table 2.2|** Storage conditions for the rat brain slices

| Thickness | Storage | Experiment |
|---|---|---|
| 14 µm | -80 ºC | ROS detection |
| 50 µm | -20ºC in cryoprotectant solution (0.1M Phosphate buffer, 30% sucrose and etilenoglicol) | Immunohistochemistry (free-floating) |

## 2.3 Propidium Iodide (PI) and terminal eoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays

The TUNEL and PI assays were used to evaluate cell death by apoptosis (it detects DNA fragmentation) and necrosis, respectively, as previous described by our group.[138] Specifically, microglial cells were incubated with different concentrations of MPH (10 µM – 2000 µM for N9 cells, and only 500 µM for primary cultures) for 24 h. Forty minutes (40 min) before the end of the treatments, PI (3 µg/mL; Sigma-Aldrich) was added to the cells. PI is a polar non-toxic compound that in normal conditions does not cross the plasma membrane. After treatments, the culture medium supernatant was collected (containing dead or dying cells that have detached from the well) and the adherent cells were trypsinized and harvested, followed by a centrifugation for 5 min at 22×*g*. The supernatant was discarded, and the pelleted cells were fixated in 4% PFA for 30 min at RT. After that, cells were washed with phosphate buffered saline (PBS) and centrifuged 10 min at 206×*g*, with supernatant being once again discarded. The cell suspension was centrifuged (Cellspin I, Tharmac GmbH, Waldsolms, Germany) for 5 min at 113×*g*, in order to adhere to SUPERFROST® PLUS slides (Thermo Scientific).

The analysis of apoptotic cell death was performed using the Click-iT™ Plus TUNEL assay (Thermo Fisher Scientific, Life Technologies Corporation, Eugene, Oregon USA) as specified in the datasheet for both N9 cells and primary cultures of microglia. Briefly, cells were permeabilized with 0.25% Triton X-100 (Sigma-Aldrich) for 30 min at RT, washed twice with PBS and incubated with terminal deoxynucleotidyl transferase buffer for 1 h at 37ºC in a humidified chamber. Cells were then washed three times with 3% bovine serum albumin (BSA;

Sigma-Aldrich) and incubated with Alexa Fluor® 488 picolyl azide dye for 30 min at 37ºC, protected from light. Finally, cells were washed for 5 min with 3% BSA, and incubated with 5 µg/mL Hoechst 33342 (Sigma-Aldrich) for 5 min for nuclei counterstaining. The slides were mounted with Dako fluorescent medium (Dako North America Inc., Carpinteria, CA, USA) and cells were observed using an Axiovert 200 M fluorescence microscope (Carl Zeiss, Oberkochen, Germany). For the quantification of TUNEL-positive cells, six independent microscopic fields per coverslip were acquired in duplicates from at least 2 independent cultures.

### 2.4 Immunocytochemistry

Immunocytochemistry was performed after TUNEL assay in primary microglial cells to stain with a microglial marker (rabbit anti-Iba-1, 1:500) overnight at 4ºC and to characterize the purity of cultures together with cell death. Afterwards, cells were washed with PBS, incubated with the secondary antibody Alexa Fluor® 594 (1:200) and Hoechst 33342 (4 µg/mL; Sigma-Aldrich) for 1h30min and mounted with Dako fluorescent medium. Images were then analyzed using an Axiovert 200 M fluorescence microscope (Carl Zeiss, Oberkochen, Germany). For the quantification, six independent microscopic fields per coverslip were acquired from 2 independent cultures.

### 2.5 Western blot analysis

N9 cells were treated for 30 min or for 3 h with 500 µM MPH or 1 µg/mL lipopolysaccharide (LPS). Total protein was obtained by lysing the cells with Radio-Immunoprecipitation Assay lysis buffer (RIPA; 0.15 M NaCl, 0.05 M Tris-base, 0.005 M ethylene glycol tetraacetic acid (EGTA), 0.5% sodium desoxicolate (DOC), 0.1% sodium dodecyl sulfate-polyacrylamide (SDS) and 1% X-Triton, pH 7.5) supplemented with a protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and 1 mM dithiothreitol (DTT) (Sigma-Aldrich). Protein quantification was performed using the bicinchoninic acid (BCA) method (Pierce, Rockford, USA) and stored at -20ºC until further use. Afterwards, protein samples were prepared under reduced conditions by adding sample buffer (0.5M Tris-HCl, 30% glycerol, 10% SDS, 0.6M DTT, 0.01% bromophenol blue; pH 6.8) and heating at 95ºC for 5 min. Proteins were separated by electrophoresis on 10% or 15% polyacrylamide gels at 130V for 90 min and transferred to polyvinylidene fluoride (PVDF) membranes (Milipore). After blocking membranes [5% (w/v) low fat milk in PBS-T [PBS containing 0.1% (v/v) Tween-20 (Sigma-Aldrich) for IL-1β and

GAPDH, or 5% BSA for NLRP3 protein], for 1 h at RT, they were incubated overnight at 4ºC with primary antibodies as follow: IL-1β (1:100, Santa Cruz Biotechnology Inc.), NLRP3 (1:100, Santa Cruz Biotechnology Inc.) and GADPH (1:10000, Thermo Scientific). After washing, membranes were incubated with the respective alkaline phosphatase-conjugated secondary antibodies [anti-mouse (1:5000) or anti-rabbit (1:5000)] (Amersham GE Healthcare Life Science, USA) for 1 h at RT. Membranes were once again washed with PBS-T and proteins were visualized using the enhanced chemiofluorescence (ECF, Amersham) reagent assay on the Typhoon FLA 9000 (GE Healthcare Bioscience AB, Uppsala, Sweden). For the quantification, cell lysates were obtained from six independent cultures and the levels of the proteins of interest were accessed for each independent culture. Quantification of band density was performed using Image Studio$^{TM}$ Lite 5.2 software (LI-COR, Lincoln, Nebraska USA).

### 2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

N9 cells were treated with 500 µM MPH or with 1 µg/mL LPS either during 30 min or 3 h and the IL-1β cytokine levels were assessed using the Mouse IL-1 beta ELISA Ready-SET-Go kit (eBioscience, San Diego, CA, USA), as specified in the datasheet. Briefly, the ELISA plate was coated with 100 µL/well of capture antibody (1:250), sealed and incubated overnight at 4ºC. Then, wells were washed 3 times with Wash Buffer (PBS with 0.05% Tween-20) and blocked for 1 h at RT. Afterwards, the Lyophilized Standard was reconstituted and a 2-fold serial dilutions of the top standard concentration (1000 pg/mL) was performed. After 2 h of incubation at RT, the plate was washed 5 times and incubated with the detection antibody (1:250) for 1 h at RT. After that, the enzyme Avidin-HRP was added for 30 min, before mixing with the substrate 1x TMB Solution. Lastly, a Stop Solution (1 M $H_3PO_4$) was used to end the reaction, and the ELISA plate was read at 450/570 nm in a microplate reader (Biotek, Synergy HT, Winooski, USA). For quantification, IL-1β levels were measured in duplicates from 4-6 independent cultures.

### 2.7 Detection of intracellular levels of reactive oxygen species (ROS)

Intracellular ROS levels were detected using dichlorofluorescein ($H_2DCFDA$) or dihydroethidium (DHE) probes. $H_2DCFDA$ reacts with intracellular esterases loosing its acetate group, and forming the $H_2DCF$ form. This molecule is able to interact with several cytotoxic oxygen species and produce 2´,7´-dichlorodihydrofluorescein (DCF), which can be detected and

used to measure intracellular ROS levels. Regarding DHE, it reacts directly with superoxide anion, and it is oxidized to ethidium form, which can also be detected by fluorescence. Importantly, these probes can detect different types of ROS molecules (Table 2.3).[139]

**Table 2.3|** ROS detection by fluorescent probes

| Fluorescent Probe | ROS detected | Fluorescent product |
| --- | --- | --- |
| $H_2DCFDA$ | Hydrogen peroxide, hydroxyl radical, carbonate radical and nitrogen dioxide | Dichlorofluorescein (DCF) |
| DHE | Cytosolic superoxide, peroxynitrite and hydroxyl radical | 2-Hydroxyethidium and ethidium |

N9 microglial cells were treated with MPH (100 µM, 250 µM or 500 µM) or 250 µM $H_2O_2$ (used as positive control) for 30 min, 1 h, 4 h and 24 h with or without a pre-incubation with 200 µM Vitamin C (VitC; Sigma-Aldrich) for 30 min. Immediately after treatments, the culture medium was removed, cells were washed with warm PBS and incubated with 5 µM $H_2DCFDA$ (Thermo Fisher Scientific,) diluted in Krebs Solution (142 mM NaCl, 4 mM KCl, 1 mM MgCl, 1 mM $CaCl_2$, 10 mM Glucose, 10 mM HEPES) for 1 h in the dark at 37ºC in a 95% atmospheric air and 5% $CO_2$ humidified incubator (Thermo Scientific Forma Series II). The $H_2DCFDA$ fluorescence was determined by a 485/20 nm excitation filter and a 528/35 nm emission filter in a microplate reader (Biotek, Synergy HT). For the quantification, ROS levels were measured in duplicates from 3 or more independent cultures. Each sample was normalized to total protein, quantified by BCA assay.

Prefrontal cortex slices (14 µm) obtained from Wistar rat brains were analyzed to identify signs of oxidative stress by using the DHE probe (Thermo Fisher Scientific). Slices were equilibrated in Krebs solution for 20 min, and incubated with 5 µM DHE and Hoechst 33342 (4 µg/mL) for 30 min at 37ºC, in the dark. Images were acquired on a LSM 710 Meta confocal microscope (Carl Zeiss, Göttingen, Germany). For the quantification, we follow the same method as others described[140, 141]. DHE staining was quantified by counting the number of DHE-positive cells nuclei in 7 random fields for each experimental condition (CTR and MPH animals). The results are expressed as the % of positive nuclei of the total nuclei in the selected field (% of total cells).

## 2.8 Immunohistochemistry

Double-labeling immunofluorescence was performed on 50 µm-thick slices obtained from the prefrontal cortex of both rat groups (saline and MPH-treated group). A microglial

marker (Iba-1), an endothelial marker (platelet endothelial cell adhesion molecule, PECAM1 or CD31), and an astrocyte marker (Glial fibrillary acidic protein, GFAP) were used (Table 2.4).

Briefly, 50 µm thickness free floating slices were incubated in 1% triton for 10 min, blocked in 5% BSA for 1 h 30 min and incubation with primary antibodies for 2 h at RT with agitation, followed by another 2 h without agitation and left overnight at 4ºC. Afterwards, slices were washed every 20 min for 2h with PBS. The slices were then incubated with secondary antibodies and Hoechst 33342 (4 µg/mL) for 2 h 30 min and from this step forward slices were protected from light. Slices were then washed every 20 min for 1 h 30 min with PBS and mounted with Dako fluorescent medium in Superfrost® Plus slides (Thermo Scientific) and stored in the dark at 4ºC until visualization in a LSM 710 Meta confocal microscope (Carl Zeiss, Göttingen, Germany).

**Table 2.4|** List of primary and secondary antibodies used in immunohistochemistry studies.

| **Primary Antibodies** | | | | |
|---|---|---|---|---|
| **Antibody** | **Antigen** | **Company** | **Specificity** | **Dilution** |
| Iba-1 (rabbit) | Ionized calcium binding adaptor molecule-1 | Abcam, Cambridge, MA, USA | Microglial marker | 1:250 |
| CD31 (goat) | Platelet endothelial cell adhesion molecule | R&D Systems Inc, Minneapolis, USA | Endothelial marker | 1:100 |
| GFAP-Cy3 conjugate | Glial fibrillary acidic protein | Sigma-Aldrich, St. Louis, MO, USA | Astrocyte marker | 1:500 |
| **Secondary Antibodies** | | | | |
| **Alexa Fluor 488** | | Invitrogen, Paisley, UK | Anti-goat | 1:200 |
| **Alexa Fluor 594** | | Invitrogen, Paisley, UK | Anti-rabbit | 1:200 |

## 2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Statistical significance was considered relevant for $P<0.05$ using one-way analysis of variance followed by Dunnett's post hoc test for comparison with control condition or followed by Bonferroni's post hoc test for comparison between experimental conditions. Regarding ROS quantification in brain slices, statistical significance was determined by an unpaired two tailed Mann-Whitney test. Data were present as mean+SEM (standard error of the mean) and the "*n*" represents the total number of experiments performed for each condition.

# CHAPTER III

## *Results*

# CHAPTER III
# *Results*

## 3.1 MPH does not cause microglial cell death

The available studies about the direct effects of MPH on brain glial cells, including microglial cells, are very scarce. Nevertheless, Schmitz and colleagues[37] recently showed that MPH can be responsible for neuronal and astrocyte loss in the hippocampus of juvenile rats. Thus, our first aim was to access microglial cell viability when exposed to different MPH concentrations to better understand if this drug could have a deleterious effect on microglia. To do so, we used the N9 microglial cell line and primary cultures of microglia to perform TUNEL and PI assays.

N9 cells were exposed to MPH (10, 100, 1000 and 2000 μM) during 24h. Here, we observed that 24 h after MPH administration (Fig. 3.1) there was no significant cell death for any of the analyzed drug concentrations, showing that at these concentrations and time exposure, MPH is not causing cell death.

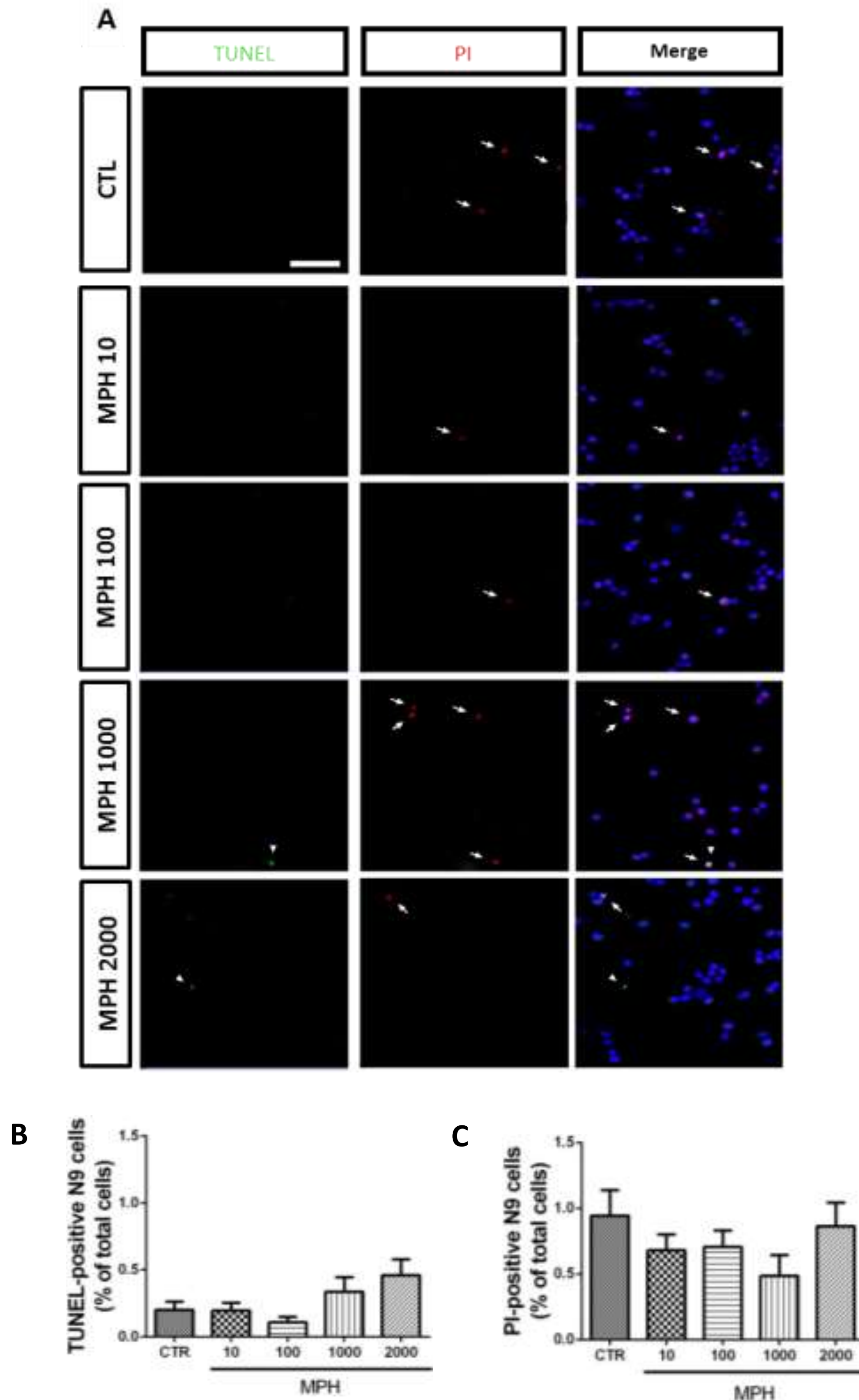


**Figure 3.1| MPH does not cause microglial cell death.** (A) Representative fluorescence images of N9 cells exposed to increasing MPH concentrations (10 µM – 2000 µM) for 24 h and cell viability analyzed by TUNEL (green, arrowheads) and PI (red, arrows) assays. Scale bar=50 µm. (B, C) Quantification of (B) TUNEL and (C) PI positive cells in the absence (CTR) or presence of increasing MPH concentrations. The results are expressed as % of total cells + S.E.M., n=36 from three independent cultures.

Microglial viability was also evaluated in primary cultures to confirm the obtained results with N9 cell line. Accordingly, 500 µM MPH treatment for 24 h did not trigger a significant cellular death when compared to the control (Fig. 3.2) showing once again that MPH does not cause microglial cell death in the analyzed conditions.

Based on the previous results, for the following studies a specific concentration of 500 µM MPH was used to evaluate microglial alterations under drug exposure.

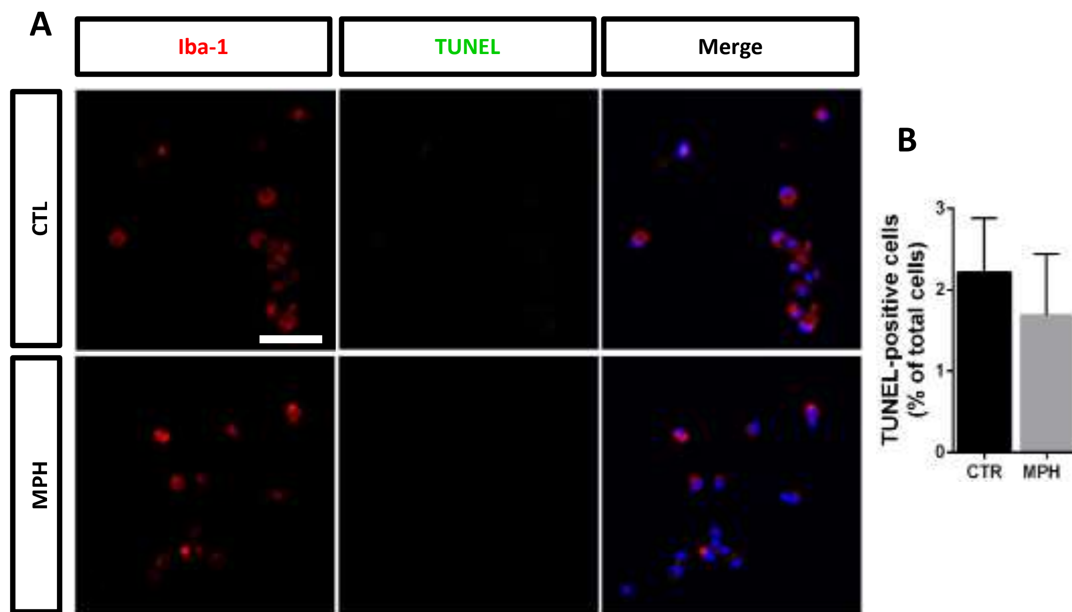


**Figure 3.2| MPH does not cause cell death of primary microglial cells.** (A)Primary cultures of microglia were exposed to 500 µM MPH for 24h and cell viability analyzed by TUNEL (green) and immunocytochemistry with Iba-1 marker (1:500, red) was performed. Scale bar=50µm. (B) Quantification of TUNEL-positive cells in the absence (CTR) or presence of MPH. The results are expressed as % of total cells + S.E.M., n=30-34 obtained from 2 independent cultures.

## 3.2 MPH effects on microglial-mediated inflammation

Microglia is the brain's first line of defense against several types of pathogens, injury or damage. Taking into account their inflammatory features, microglia is considered one of the most important cells that assure a healthy brain function. Upon activation, these cells can trigger inflammasome assembly and secretion of several cytokines, such as IL-1β. NLRP3 inflammasome is known to be activated in these cells, contributing to the immune response and it is a significant source of mature and bioactive IL-1β.[104]

In order to unravel MPH effects on NLRP3 and IL-1β protein levels, N9 cells were exposed to 500 µM MPH or to LPS (1 µg/mL), during 30 min or 3 h and the protein content was evaluated by western blot analysis. LPS, a macromolecule synthesized by gram negative

bacteria, [57] was used as an inflammatory insult. In fact, LPS has been extensively used to activate microglial signaling pathways responsible for production and release of pro-inflammatory cytokines.[142]

Herein, we demonstrate that 500 µM MPH significantly increased IL-1β (140 + 9.6 % of control) and NLRP3 (130.5 + 9.7 % of control) protein levels at 30 min post-treatment. This effect was not observed at 3h after the treatment when compared to the control (Fig. 3.3). Additionally, LPS administration increased IL-1β (151.8 + 15.2 % of control) and NLRP3 (128.8 + 6.1 % of control) protein levels at 30 min but after 3h there was only an upregulation of NLRP3 (136.4 + 8.7 % of control) suggesting a quick and transitory effect on IL-1β.

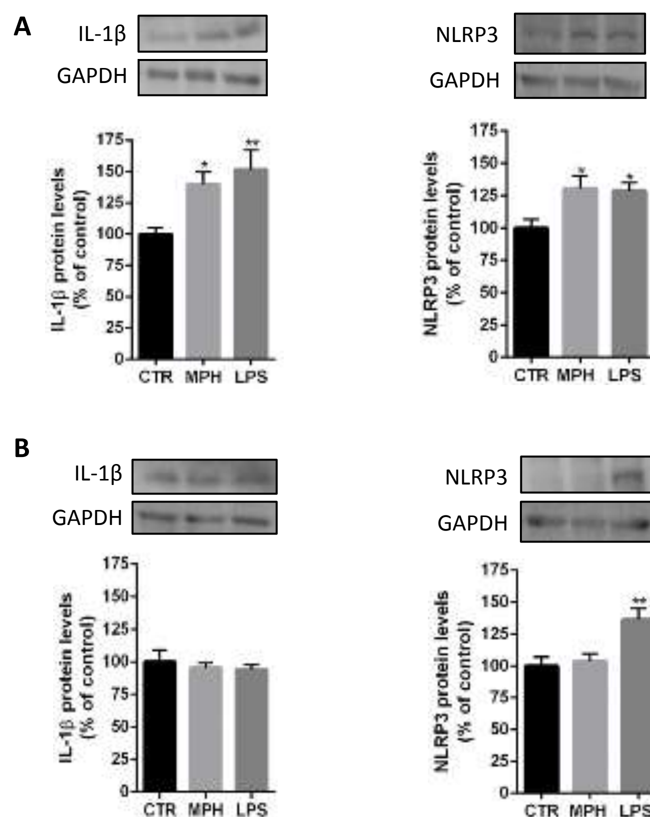


**Figure 3.3| MPH is able to increase microglial IL-1β and NLRP3 protein levels.** N9 cells were treated with 500 µM MPH or with 1 µg/mL LPS during (A, C) 30 min or (B, D) 3 h and protein levels of (A, B) IL-1β and (C, D) NLRP3 were measured by western blot analysis. Above the bars, representative western blot images of IL-1β (17 kDa), NLRP3 (106 kDa) and GAPDH (37 kDa) are shown. MPH upregulates IL-1β and NLRP3 protein levels after 30 min of treatment. The results are expressed as % of control + SEM., n=6 obtained from 6 independent cultures. *P<0.5, **P<0.01 significantly different when compared to control using one way ANOVA followed by Dunnett's Multiple comparison test.

Extracellular IL-1β levels were also assessed by ELISA, however the released levels were below the limit of detection and so it was not possible to identify this pro-inflammatory

cytokine after MPH or LPS treatments under our specific experimental conditions (data not shown).

## 3.3 MPH-induced ROS production in microglial cells

Upon injury, microglial can also produce several ROS molecules that trigger intracellular signaling pathways important for cell survival and/or for immune response. ROS production has been closely related to NLRP3 activation and IL-1β expression, acting as a trigger for inflammatory response. In turn, NLRP3 activation can be responsible for the intracellular ROS increase. [143] Moreover, previous work of our group demonstrated that MPH has the capability of inducing NOX activation and consequent ROS production in brain endothelial cells.[38] Taking into account these observations, we further aimed to clarify if MPH was also able to induce ROS production in microglial cells. For that, we performed a time course study (30 min, 1 h, 4 h and 24 h) to evaluate ROS production using several MPH concentrations (100, 250 and 500 µM). Hydrogen peroxide ($H_2O_2$) is a well-known ROS inductor and was used as a positive control. Here, we show that both 100 and 250 µM of MPH did not interfere with ROS production at all time points analysed (Fig. 3.4). Interestingly, with the higher concentration of MPH (500 µM) it was possible to observe a significant increase of ROS levels after 30 min of treatment (175.6 + 7 % of control). At longer periods, there were no significant alterations compared to the control suggesting that microglial cells present a rapid response to MPH, followed by a recovery. As expected, 250 µM $H_2O_2$ was able to trigger ROS production in all time points anlaysed (142.4 + 5.1, 174.5 + 15.9 %. 208.8 + 25.8 %, 312.5 + 21.5 % of control at 30 min, 1 h, 4h and 24 h, respectively).

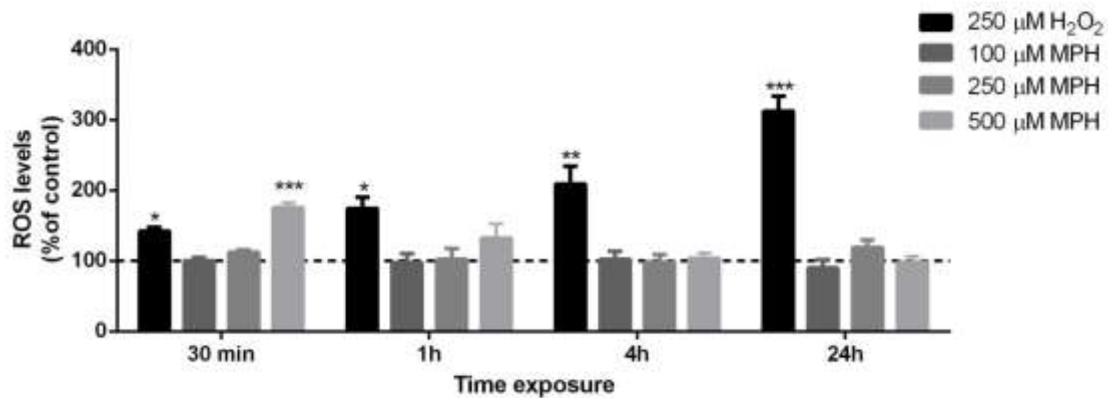


**Figure 3.4| MPH is able to induce ROS production by microglia.** N9 cells were exposed to increasing concentrations of MPH (100 μM – 500 μM) and to 250 μM $H_2O_2$ for 30 min, 1 h, 4 h and 24 h. After the treatments, microglia ROS production was analyzed by $H_2$DCFDA fluorescence. MPH (500μM) induced microglia ROS production only after 30 min. The $H_2$DCFDA was added to the cells in a final concentration of 5 μM during 1 h and the fluorescence was accessed with an excitation spectrum of 485/20 and emission spectrum of 528/20. The results are expressed as % of control + SEM., n=4-16 obtained from at least 4 independent cultures. *P<0.5; **P<0.01; ***P<0.001, significantly different from control using Dunett's post-test.

To further investigate if this alteration on microglial ROS production could be prevented, N9 cells were pre-exposed (30 min prior to MPH or $H_2O_2$ treatments) to 200 μM Vitamin C (VitC), which is a powerful antioxidant. Results show that this antioxidant was capable of keeping the ROS levels similar to the control condition (dashed line), and thereby preventing MPH (or $H_2O_2$)-induced ROS production (Fig 3.5).

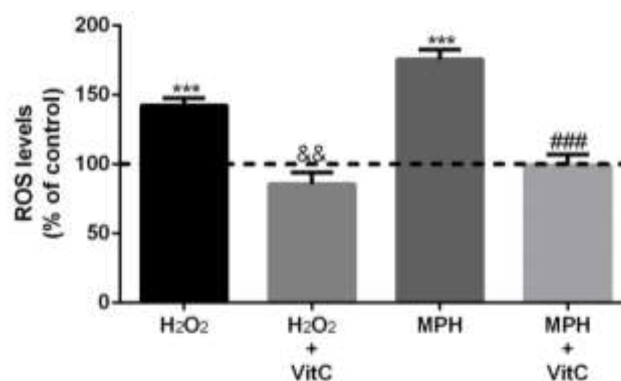


**Figure 3.5| Vitamic C (ascorbic acid) prevents MPH-induced microglial ROS production.** N9 cells were treated during 30 min with 500 μM MPH or 250 μM $H_2O_2$ in the presence/absence of 200 μM Vitamin C (VitC). VitC prevented MPH and $H_2O_2$-induced microglia ROS production. The $H_2$DCFDA probe was added to the cells in a final concentration of 5 μM during 1h (excitation spectrum of 485/20 and emission spectrum of 528/20). The results are expressed as % of control + SEM., n=9-16 obtained from at least 3 independent cultures. ***P<0.001, significantly different from control (dashed line) using Dunett's post-test. $^{\&\&}$P<0.01, significantly different from 250 μM $H_2O_2$ using Bonferroni's post-test. $^{\#\#\#}$P<0.001, significantly differente from 500 μM MPH using Bonferroni's post-test.

## 3.4 MPH promotes microglial activation in the PFC of adult rats

In order to better understand the impact of MPH on microglial cells, we further performed immunohistochemistry analysis of microglia alterations in brain slices obtained from the PFC of adult Wistar rats chronicly treated with MPH (1.5 mg/kg/day for 21 consecutive days). For that, we used a microglial marker (Iba-1) that allowed us to analyse morphological changes in these cells. In fact, we clearly observed microglial activation upon chronic MPH exposure (Fig. 3.6). Specifically, we were able to distinguish between a more ramified and surveilling microglia (in brain slices from control animals; Veh), and an amoeboid activated microglia (MPH-treated rats). Moreover, we observed a decrease in CD31 staining (Figs. 3.6 and 3.7), suggesting that MPH is also able to interfere with blood-brain barrier as previously shown by us *in vitro*[38].

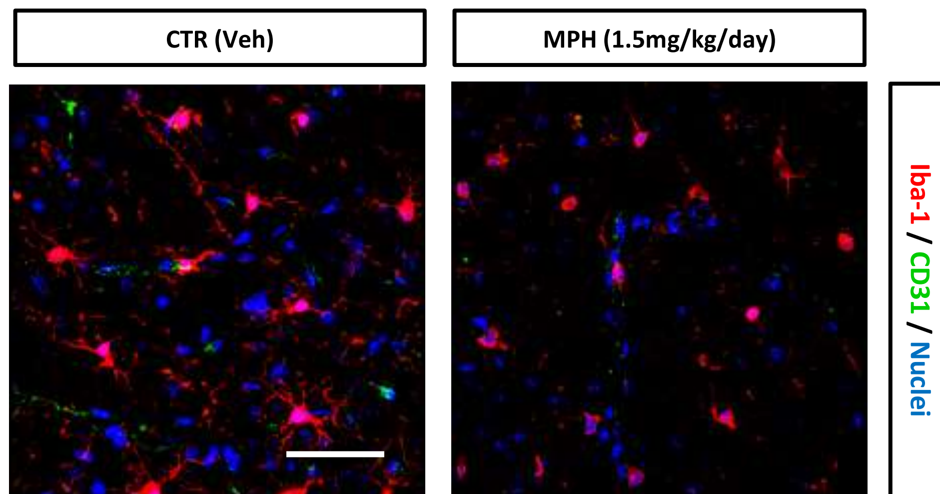


**Figure 3.6| MPH induces microglial activation in the prefrontal cortex of adult Wistar rats.** Animals (5 months old) were treated with saline solution (CTR, Veh) or with MPH (1.5 mg/kg/day) for 21 days. Immunocytochemistry of 50 µm-thick slices to Iba-1 (1:250; Red), CD31 (1:100; Green) and Hoechst 33342 (blue, 4 µg/mL) markers was performed. Based on the short length of microglia extensions it is predicted that MPH induces microglia activation. Scale bar=50µm

Astrocytes are very important for neuronal function, since they can provide energy and substrates for neuron's metabolism and also have a huge contribution for brain homeostasis.[144] Schmitz and colleagues[37] reported that chronic MPH treatment (2.0 mg/kg/day MPH for 30 days) was able to induce astrocytic loss in the hippocampus of juvenile rats. In contrast, we did not observe alterations in astrocytic morphology or even in cell number in the PFC of our animal models (Fig. 3.7).

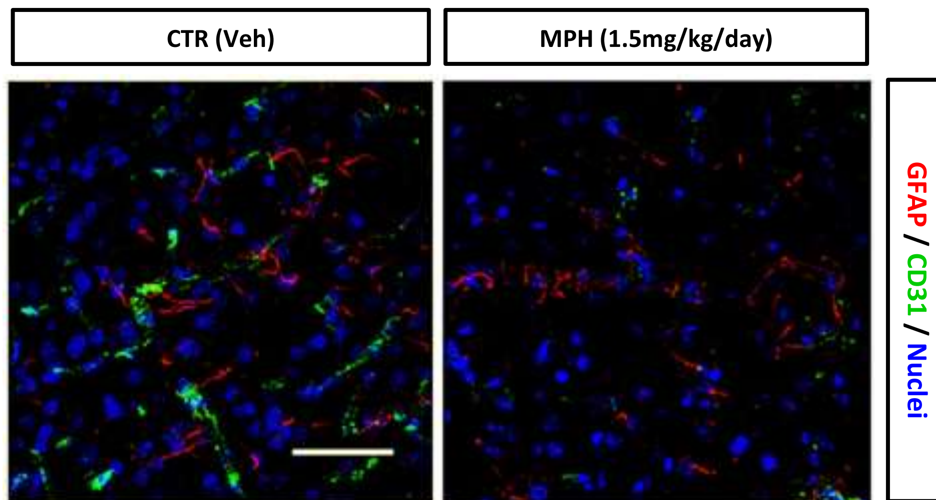


**Figure 3.7| MPH does not induce astrocyte alterations in the prefrontal cortex of adult Wistar rats.** Wistar Rats (5 months old) were treated with saline solution (CTR, Veh) or with MPH (1.5 mg/kg/day) for 21 days. Representative images of immunocytochemistry for GFAP (1:500; Red), CD31 (1:100; Green) and Hoechst 33342 (blue, 4 µg/mL). Scale bar=50µm.

To further corroborate our *in vitro* results, we also evaluated ROS content in PFC slices obtained from rats that underwent MPH chronic administration. For that, we stained 14 µm-thick PFC slices with 5 µM DHE fluorescent probe and once again we observed a significant increase in the ROS levels in MPH-treated animals (22.2 + 2.9 % of control) when compared to the control condition (Fig. 3.8). Despite this promising result, future studies are needed to identify the cellular source(s) of ROS under chronic MPH exposure.

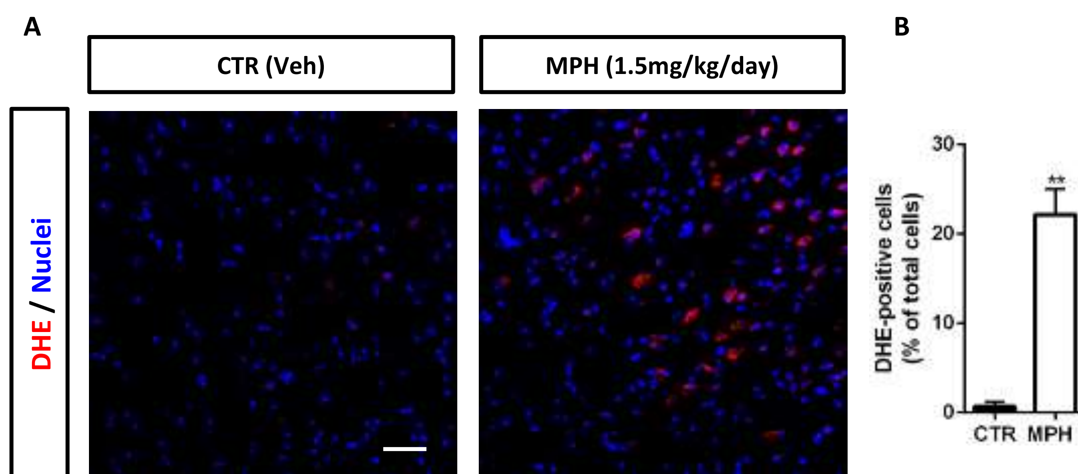


**Figure 3.8| MPH induces the production of reactive oxygen species in the prefrontal cortex of adult Wistar Rats.** Wistar Rats (5 months old) were treated with saline solution or with MPH (1.5 mg/kg/day) for 21 days. (A) Representative images of DHE (5 µM; red) and Hoechst 33342 (blue, 4 µg/mL) double-staining. (B) Quantification of DHE-positive cells. Scale bar=50µm. The results are expressed as % of total cells + S.E.M., n=7 from one animal per condition, **P<0.01, significantly different from control using Mann-Whitneypost-test.

# CHAPTER IV
## *Discussion*

# CHAPTER IV

## *Discussion*

Microglia plays a fundamental role in the brain function and homeostasis. In fact, these cells are responsible for the immune response upon brain injury or pathogen entry, and they can also be involved in tissue repair, formation of new synapses and neuronal proliferation. Methylphenidate (MPH) is the first-line drug for the treatment of attention deficit hyperactivity disorder (ADHD) and is being used by children and adults worldwide to attenuate the hallmarks of this disease. Nevertheless, the misuse/abuse of MPH has become common among healthy young adults for competitive sports and cognitive enhancement. Moreover, the problematic about ADHD misdiagnosis has been extensively discussed because the prescription of this drug reached alarming numbers and the available studies about long-term consequences of MPH use are scarce. In fact, there are only a few studies relating MPH administration and microglia response; yet, without exploring the consequences or identifying key players. Taking into consideration this important gap in the field, we aimed to unravel the direct impact of MPH on microglia.

Herein, we showed that MPH neither induced microglial cell death nor astrocyte loss. In fact, we only assessed cell death in cultures and not in the rat PFC but cell density suggests no significant alterations in the total number but instead a strong activation of only microglial cells. In contrast, Sadasivan and colleagues[1] reported that chronic MPH administration (10 mg/kg/day for 90 days) caused dopamine neuronal loss in the mice basal ganglia. In addition, Schmitz and colleagues[37] showed that MPH (2 mg/kg/day for 30 days) also caused loss of neurons and astrocytes in the hippocampus of juvenile rats by apoptosis since there was an activation of caspase-3. The differences observed between these studies and ours can be explained by the use of different doses of MPH, duration of the treatment, route of administration and the brain region analyzed. Nevertheless, we cannot exclude the hypothesis that MPH could indeed cause cell death with a higher period of drug exposure or in other brain regions.

Despite no alterations on cell viability under our experimental conditions, MPH was able to trigger microglial upregulation of NLRP3, IL-1β and ROS. Interestingly, we observed a significant increase in these proteins after 30 min of drug treatment, returning to basal levels within 3 h. Importantly, cells normally function with a basal level of intracellular ROS. Yet, increasing levels of intracellular ROS act as second messengers to amplify the pro-

inflammatory function of the cell[57]. NADPH oxidase is the major source of microglial ROS production and increased intracellular ROS levels in these cells might result in microglial overactivation and neurotoxic consequences[57]. Moreover, ROS production can act both as signaling mechanism to prime and activate NLRP3 inflammasome, which in turn will produce bioactive cytokines, and as "bonfire" or "effector" resulting in pathological processes[143]. In fact, IL-1β expression and ROS production can up-regulate the levels of other pro-inflammatory molecules and contribute to an inflammatory response. However, whether ROS production observed in the present work is a trigger or a consequence of NLRP3 activation and IL-1β upregulation is still unknown and further studies are needed to clarify this issue. Microglial activation is thought to participate in methamphetamine (METH; another psychostimulant like MPH) neurotoxicity and contribute to neuronal damage through the release of several inflammatory factors[145]. In fact, METH enhances the outward potassium ($K^+$) currents through the voltage-gated Kv1.3 channel, and inhibition of these channels protect against METH-induced cell damage[146]. Moreover, the efflux of $K^+$ from the cells can activate NLRP3 inflammasome and leads to pro-inflammatory cytokines production and contributes to inflammation[105]. Yet, if MPH has the same microglial effects as METH is still unknown. Contradictory to our findings, Sadasivan and colleagues[1] reported that acute administration of 10 mg/kg MPH to mice did not alter IL-1β mRNA levels when compared to control animals. Differences between our study and Sadasivan's can be explained by the fact that they used an animal model, a higher dose of MPH, analyzed mRNA levels and looked to the striatum. We can also hypothesize that MPH is not responsible for the synthesis "*de novo*" of pro-IL-1β but instead be involved in the maturation for its active form. In fact, METH is known to increase the expression of the ligand-gated P2X7 receptor (P2X7R) in microglia[147] and activation of this receptor results in IL-1β maturation and release[148]. In addition to NLRP3 priming, this could underlie the mechanism by which MPH is capable of induce IL-1β production in microglia. Nevertheless, further studies accessing P2X7R activation upon MPH insult are needed to confirm this hypothesis.

Interestingly, the transitory effects that we observed together with the absence of cell death suggest that microglia is reacting to MPH insult with a modulatory and/or protective response instead of a cytotoxic response. In fact, MPH-increased IL-1β levels can activate the NF-κB transcription factor, which is known to translocate to the nucleus and bind to DNA and altering the expression of several target genes. Some of these are pro-survival genes that participate in anti-apoptotic pathways, such as B-cell lymphoma 2 (Bcl-2) and cellular inhibitor of apoptosis (c-IAP)[149]. Besides that, NF-κB can also contribute to the protection of cells from

oxidative stress through the expression of antioxidant genes such as manganese superoxide dismutase[149].

Microglial cells are very dynamic and can be influenced by surrounding cells, such as astrocytes and neurons. Neurons and astrocytes can communicate with microglia and trigger a shift between a resting/surveilling state to an activated state via the release of several molecules[98, 150]. Schmitz and colleagues[37] demonstrated that MPH (2 mg/kg/day for 30 days) induced TNF-α and IL-6 expression in the hippocampus of juvenile rats, yet they did not explore the cellular origin of these molecules. The authors also reported an increase of the Iba-1 (microglial marker) immunocontent. Accordingly, we also showed that chronic MPH treatment (1.5 mg/kg/day for 21 days) activated microglia, suggesting that these cells could indeed be an important source of inflammatory mediators. Moreover, we focused our study in the PFC because this brain region is highly altered in ADHD and MPH is known to have its therapeutic effects by acting primarily in the PFC, which is the center of control of judgment, behavior inhibition, emotion and decision making[151]. Nevertheless, the role of other brain cells such as astrocytes, neurons or even endothelial cells cannot be excluded[152, 153], as well as significant alteration in other brain regions. In fact, our group previously demonstrated that MPH is capable of induce ROS production through NADPH oxidase activation in brain endothelial cells and promote blood-brain barrier disruption[38], which can increase the brain susceptibility to peripheral factors. Regarding astrocytes, Bahcelioglu and colleagues[154] reported that these cells were activated upon MPH treatment (5-20 mg/kg, 5 days a week for 3 months), but in the present study, we did not observe astrogliosis in the PFC of MPH-treated animals. Once again, these differences can be explained by different MPH treatment protocols, since Bahcelioglu and colleagues[154] used a much higher MPH dose and a longer treatment period.

Although MPH has been associated with cognitive enhancement under ADHD conditions, to date no clinical studies have examined the long-term effects of chronic MPH consumption under non-pathological conditions (misuse/abuse). Pardey and colleagues[155] were the firsts to report that chronic MPH treatment (2 mg/kg/twice a day) during 28 days increased impulsive choice in adult control rats. Moreover, chronic treatment with cocaine, a psychostimulant with a similar mechanism of action to MPH, has been associated with impulsive-like behaviors in rats[156] and monkeys[157]. Overall, MPH has a suggestive role in cognitive enhancement when acutely administered. However, taking into consideration the reports above mentioned, when administered chronically, MPH can have unexpected consequences like impulsivity behaviors, not contributing to cognitive enhancement. One key player involved in such effects could be ROS since consequences of excessive ROS production are not limited to neuroinflammation, and are also involved in cognitive impairment[158].

Taking together, our results showed that MPH leads to microglial activation and endothelial alterations, without affecting astrocytes. Moreover, microgliosis was accompanied by a transient increase of ROS, NLRP3 and IL-1β levels. Since no cell death was observed within our experimental window, we can hypothesize that these mediators are probably acting as signaling molecules to counteract the insult induced by MPH. Nevertheless, future studies are needed to better clarify our working hypothesis.

# CHAPTER V

## *Conclusions*

# CHAPTER V

## *Conclusions*

With this work we showed for the first time that MPH displays direct effects in microglial cells. MPH causes microglial activation both in microglia N9 cellular line and in rat prefrontal cortex, without causing microglial cell death. Moreover, microglial activation was accompanied by increased protein levels of IL-1β and NLRP3 inflammasome as well as ROS production, which was prevented by Vit C (200 μM). MPH also increased ROS levels in the PFC without affecting astrocytes. Moreover, we observed that MPH affects the brain endothelium suggesting a possible role on blood-brain barrier breakdown.

# CHAPTER VI

## *References*

# CHAPTER VI

## *References*


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