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Mariana Carvalho Muga

METHYLPHENIDATE-INDUCED ALTERATIONS IN ASTROCYTES:
A COMPREHENSIVE CHARACTERIZATION

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular, ramo de Neurobiologia, orientada pela Professora Doutora Ana Paula Pereira da Silva Martins e pela Professora Doutora Emília da Conceição Pedrosa Duarte e apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra, Departamento de Ciências da Vida.

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Resumo

O metilfenidato (MTF) é o fármaco mais prescrito no tratamento de primeira linha da perturbação de hiperatividade/défice de atenção (PHDA), condição com grande prevalência nas crianças e que pode também persistir na idade adulta. O diagnóstico de PHDA, feito apenas com base no historial clínico do paciente, está a aumentar, a par com a prescrição de MTF. O uso não-terapêutico de MTF é uma prática comum principalmente entre estudantes que procuram melhorar a sua performance cognitiva. Isto representa um problema, que é intensificado pelo escasso conhecimento atual sobre os efeitos crónicos e a longo prazo do tratamento com MTF, em particular no cérebro, tanto em condições de PHDA como fisiológicas. Por outro lado, os efeitos neurobiológicos do MTF também não são bem conhecidos, em especial nas células da glia, como os astrócitos, cuja função cerebral é fundamental em condições saudáveis e de doença. Assim, este trabalho teve como principais objetivos estudar os efeitos diretos do MTF nos astrócitos, e o impacto do seu consumo crónico num modelo animal de PHDA e controlo, simulando desta forma o uso terapêutico e não-terapêutico do MTF, respetivamente. Para atingir estes objetivos, foram usadas culturas primárias de astrócitos corticais de rato como modelo *in vitro*, bem como dois modelos animais, Wistar-Kyoto (*WKY*) e ratos espontaneamente hipertensos (*SHR*), tratados com uma dose clinicamente relevante de MTF.

Este estudo demonstrou que o tratamento direto dos astrócitos com MTF não induziu astrogliose, mas provocou morte celular por apoptose de forma dependente da concentração. Ainda, esta droga demonstrou ser capaz de induzir a produção intracelular de espécies reativas de oxigénio (*ROS*) e de óxido nítrico (*NO*) nos astrócitos, bem como um aumento nos níveis de proteína da sintase do óxido nítrico induzível (*iNOS*). Para além disso, a via de sinalização do fator nuclear kappa B (*NF-κB*) foi ativada pelo MTF. Relativamente aos estudos em animais, os ratos *SHR* não tratados apresentaram uma tendência para níveis aumentados da proteína ácida fibrilar glial (*GFAP*) e de *iNOS* no hipocampo, comparando com os respetivos animais controlo, o que sugere que este modelo de PHDA apresenta um estado de inflamação basal. Curiosamente, o tratamento com MTF levou a um aumento significativo dos níveis proteicos de *GFAP* e *iNOS* em animais controlo, enquanto nos animais *SHR* foi verificado um leve efeito benéfico. Relativamente à peroxidação lipídica, esta foi diminuída pelo tratamento com MTF, apenas no modelo animal de PHDA, tanto no hipocampo como no córtex pré-frontal. O modelo animal de PHDA apresentou ainda uma capacidade antioxidante basal reduzida no hipocampo, a qual sofreu uma tendência para ser aumentada pelo tratamento com MTF. Também no hipocampo, o tratamento com MTF diminuiu a capacidade antioxidante dos ratos *WKY*.

Em conclusão, o MTF induziu um efeito direto moderado nos astrócitos, especialmente relacionado com stress oxidativo e nitrosativo. Não obstante, num contexto mais complexo como os estudos em animais, as respostas observadas, associadas à astrogliose, stress oxidativo/nitrosativo e capacidade antioxidante, evidenciam que o uso não-terapêutico do MTF tem um efeito prejudicial, enquanto o seu uso terapêutico, em condições de PHDA, é benéfico.

Este trabalho descreve pela primeira vez alguns dos efeitos diretos do MTF nos astrócitos, e enriquece o nosso conhecimento sobre os efeitos cerebrais tanto do uso terapêutico como do uso não terapêutico do MTF.

Palavras-chave: Metilfenidato, Astrócitos, Stress oxidativo/nitrosativo, PHDA, Hipocampo.

Abstract

Methylphenidate (MPH) is the first-line drug of choice for the treatment of attention deficit hyperactivity disorder (ADHD), a highly prevalent condition in children that may also persist into adulthood. ADHD diagnosis, which is only based on the clinical history of the patient, is rapidly increasing, along with MPH prescription. MPH non-medical use is also a common practice mainly among students with intent to improve their cognitive performance. This represents a critical problem, intensified by the fact that there is poor knowledge regarding the chronic and long-term effects of MPH treatment on the brain, both under ADHD and healthy conditions. In addition, the neurobiological effects of MPH have not yet been fully characterized, particularly in glial cells, such as astrocytes, that play essential roles in healthy and diseased brain. Thus, the present work aimed to characterize the direct effects of MPH on astrocytes, and also to investigate the impact of MPH chronic exposure in control and ADHD animal models, simulating MPH non-therapeutic and therapeutic use, respectively. To achieve these goals, primary cultures of rat cortical astrocytes and both Wistar-Kyoto (WKY) and Spontaneously Hypertensive (SHR) rats treated with a clinically relevant dose of MPH were used as *in vitro* and animal models, respectively.

The present study showed that direct MPH treatment did not induce astrogliosis, but promoted apoptotic cell death in a concentration-dependent manner. Moreover, MPH was able to induce reactive oxygen species (ROS) and nitric oxide (NO) intracellular production by astrocytes, as well as an increase of inducible nitric oxide synthase (iNOS) protein levels. In addition, the nuclear factor-κB (NF-κB) signaling pathway was activated by MPH. Regarding animal studies, SHR animals showed a tendency to an increment of glial fibrillary acidic protein (GFAP) and iNOS levels in the hippocampus comparing to control animals, suggesting that this ADHD model presents a basal inflammatory status. Curiously, MPH treatment led to a significant increase of both GFAP and iNOS protein levels in control animals, whereas in SHR rats there was a slight beneficial effect. Regarding lipid peroxidation, MPH treatment decreased such levels only in ADHD animals both in the prefrontal cortex (PFC) and hippocampus. Interestingly, the ADHD animal model presented, particularly in the hippocampus, a basal lower antioxidant capacity compared to control rats, which MPH treatment tended to increase. Still in the hippocampus, MPH chronic treatment decreased the antioxidant capacity of controls (WKY rats).

In conclusion, MPH induced a moderate direct effect in astrocytes particularly related with oxidative and nitrosative stress. Nevertheless, in a more complex context using animal studies, the observed responses related to astrogliosis, oxidative/nitrosative stress and antioxidant capacity, demonstrate that MPH non-therapeutic use has detrimental outcomes, while MPH therapeutic use is beneficial under ADHD conditions.

This work unveils for the first time some of the direct effects of MPH on astrocytes, and improved our knowledge about the central effects of both therapeutic and misuse of MPH.

Keywords: Methylphenidate, Astrocytes, Oxidative/Nitrosative stress, ADHD, Hippocampus.

List of abbreviations

AA	Arachidonic Acid
aCSF	artificial Cerebrospinal Fluid
ADHD	Attention Deficit Hyperactivity Disorder
Aldh1L1	Aldehyde dehydrogenase 1 family member L1
AM	Acetoxymethyl
ANOVA	Analysis of Variance
APA	American Psychiatric Association
AQ4	Aquaporin 4
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BCA	Bicinchoninic Acid
BDNF	Brain-Derived Neurotrophic Factor
BHT	Butylhydroxytoluene
BSA	Bovine Serum Albumin
C1q	Complement component 1, q subcomponent
Ca^{2+}	Calcium
Cd11b	Cluster of differentiation 11b
CNS	Central Nervous System
CRs	Complement Receptors
CTCF	Corrected Total Cells Fluorescence
Cx	Connexin
DA	Dopamine
DAF-FM	amino-5-methylamino-2',7'-difluorofluorescein diacetate
DAN	Dorsal Attention Network
DAT-1	Dopamine Transporter-1
DEA	Drug Enforcement Administration
DMEM	Dulbecco's Modified Eagle's Medium
DMN	Default Mode Network
DSM-5	Diagostic and Statistical Manual of Disorders-5
DTT	Dithiothreitol

EAAT1	Excitatory Amino Acid Transporter-1
EAAT2	Excitatory Amino Acid Transporter-2
ECL	Enhanced Chemiluminescence
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
fMRI	functional Magnetic Resonance Imaging
FRAP	Ferric Reducing Antioxidant Power
GABA	Gamma-Aminobutyric Acid
GAD	Glutamate Decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
GFRα1	GDNF Family Receptor Alpha 1
GLAST	Glutamate Aspartate Transporter
GLT-1	Glutamate Transporter-1
GS	Glutamine Synthetase
GSH	Glutathione
HRP	Horseradish Peroxidase
Iba1	Ionized calcium-binding adaptor molecule 1
IL-1α	Interleukin 1 alpha
iNOS	inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
MAO	Monoamine oxidase
MAP2	Microtubule-Associated Protein 2
MAPK	Mitogen-Activated Protein Kinase
MDA	Malondialdehyde
METH	Methamphetamine
MPH	Methylphenidate
NAc	Nucleus Accumbens
NADPH	Nicotinamide-Adenine Dinucleotide Phosphate
NCAM	Neural Cell Adhesion Molecule

NE	Norepinephrine
NET	Norepinephrine Transporter
NeuN	Neuronal Nuclei
NF-kB	Nuclear Factor-kB
NGF	Nerve Growth Factor
NO	Nitric Oxide
NOX	Nicotinamide-Adenine Dinucleotide Phosphate Oxidase
Nrfr2	Nuclear factor erythroid 2-related factor 2
NTFs	Neurotrophic Factors
NVU	Neurovascular Unit
OPCs	Oligodendrocyte Precursor Cells
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
PFC	Prefrontal Cortex
PI3K	Phosphoinositide 3-kinase
PNS	Peripheral Nervous System
PRRs	Pattern-Recognition Receptors
PVDF	Polyvinylidene Fluoride
RIPA	Radio-Immunoprecipitation Assay
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Room Temperature
SEM	Standard Error of the Mean
SDS	Sodium Dodecyl Sulfate
SHR	Spontaneously Hypertensive
SOD	Superoxide Dismutase
SRs	Scavenger Receptors
SVZ	Subventricular Zone
TAS	Total Antioxidant Status
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances

TdT	Deoxynucleotidyl Transferase
TGF-β	Transforming Growth Factor Beta
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor alpha
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
VMAT-2	Vesicular Monoamine Transporter-2
VRAC	Volume-Regulated Anion Channel
VZ	Ventricular Zone
WKY	Wistar-Kyoto

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CHAPTER 1
INTRODUCTION

Chapter 1 – Introduction

1.1 Methylphenidate

1.1.1 Psychostimulants

Psychostimulants include drugs that exert their activity within the central and peripheral nervous systems (CNS and PNS, respectively). In general, they increase monoaminergic neurotransmission, increasing psychomotor and sympathetic nervous system activity. At the behavioral level, they are known to afford for positive effects, such as increased energy, alertness, attention, arousal and enthusiasm, enhanced self-esteem, or even the feeling of euphoria, reason why they are colloquially called as "uppers" (Meyer & Quenzer, 2005; Prus, 2013). Additionally, some of the physiological effects of psychostimulants use are increased heart rate, constriction of blood vessels, airways' relaxation, dilation of pupils, inhibition of salivation and digestion, among others. There are also evidences that they may be able to suppress appetite, through actions at the hypothalamic level (Prus, 2013). Cardiovascular and pulmonary dysfunction, and psychotic behavior are some of the possible adverse effects. The above-mentioned behavioral outcomes account for the therapeutic utility of psychostimulants, but also for their misuse or even abuse. A chronic use of these substances might induce tolerance, sensitization and/or dependence, and its cessation may provoke withdrawal symptoms. However, it is important to notice that among these substances, there are controlled and illicit drugs highly addictive, such as amphetamine, methamphetamine (METH) and cocaine (Prus, 2013), and others that are widely prescribed specially for the treatment of Attention Deficit Hyperactivity Disorder (ADHD), such as methylphenidate (MPH). Also, in some countries, controlled substances can be prescribed under specific conditions.

1.1.2 Pharmacodynamics and Pharmacokinetics

Methylphenidate was first synthetized in 1944 by the chemist Leandro Panizzon as a treatment for chronic fatigue and depression (Leonard et al., 2004; Prus, 2013). Nowadays, it is the first-line drug of choice for the treatment of ADHD and is also used to control the symptoms of narcolepsy, a sleep disorder (Dinis-Oliveira, 2017).

Concerning the pharmacodynamics of MPH and particularly its locals of action, this drug facilitates and increases catecholaminergic transmission primarily by inhibiting

catecholamines, dopamine (DA) and norepinephrine (NE), reuptake via their transporters (dopamine transporter-1 - DAT-1 and norepinephrine transporter - NET, respectively) into the cytoplasm of the presynaptic nerve terminal, which is the main mechanism to terminate the action of these neurotransmitters after release (Ebenezer, 2015; Faraone, 2018; Meyer & Quenzer, 2005; Sharma & Couture, 2014). Furthermore, it has been reported to have negligible effect on serotonergic transmission (Dinis-Oliveira, 2017; Suzuki et al., 2007). Beyond the described classic mechanism of action, there is also evidence that MPH indirectly affects the vesicular monoamine transporter-2 (VMAT-2), leading to the redistribution of VMAT-2 containing vesicles into the cytoplasm and to an increase of DA transport into the vesicles, which results in an enhanced exocytotic DA release (Volz, Farnsworth, Hanson, et al., 2008; Volz, Farnsworth, Rowley, et al., 2008). Additionally, MPH may also inhibit monoamine oxidase (MAO), preventing the catabolic action of this enzyme, that is responsible for the metabolization of monoamines (Sharma & Couture, 2014). However, further details on these mechanisms remain to be elucidated. The combination of these molecular mechanisms results in an augmented and sustained effect of DA and NE on their postsynaptic receptors (Ebenezer, 2015) (Figure 1.1). MPH is structurally related to the neurotransmitter DA, which explains why it presents most effectiveness at the dopaminergic system level.

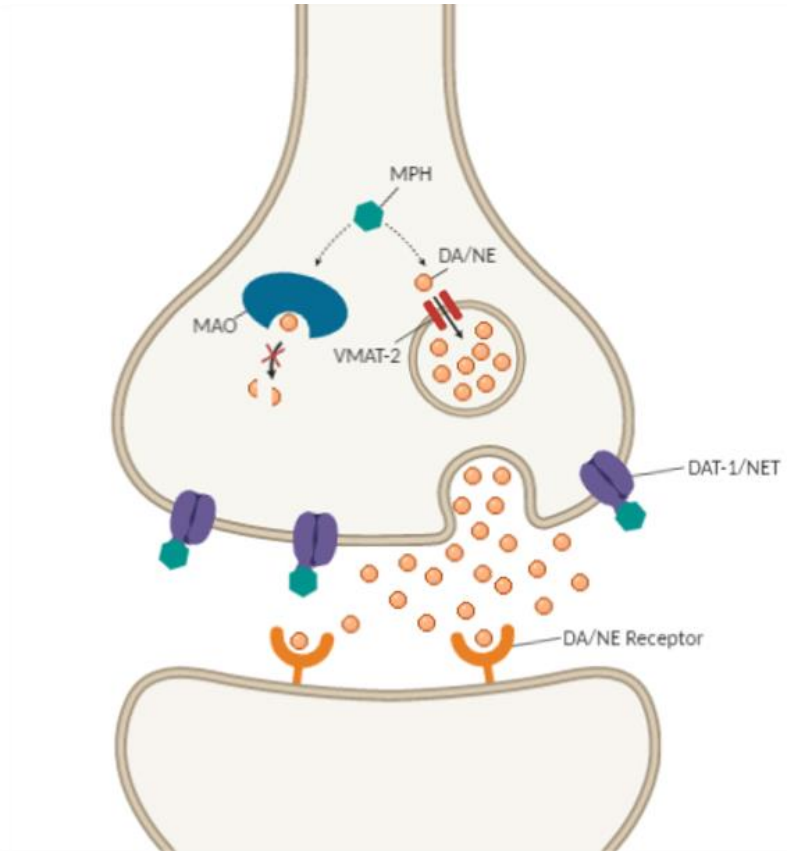

Figure 1.1. **Mechanism(s) of action of methylphenidate.** MPH interacts with DAT-1 and NET, blocking their activity and inhibiting the reuptake of DA and NE at the presynaptic level. This is the established and widely accepted mechanism of action of MPH. Two additional mechanisms have been described as follows: 1) the indirect redistribution of VMAT-2 containing vesicles and consequent increase of DA transport into the vesicles; 2) the inhibition of MAO and thus of its catabolic activity on DA and NE. Even though the last two molecular mechanisms are missing a better understanding, all together contribute to an increased synaptic availability of DA and NE and, consequently, to the strength of their interaction with the respective receptor at the postsynaptic level, reinforcing neuronal communication. Legend: DA, Dopamine; DAT-1, Dopamine Transporter-1; MAO, Monoamine Oxidase; NE, Norepinephrine; NET, Norepinephrine Transporter; VMAT-2, Vesicular Monoamine Transporter-2.

Regarding pharmacokinetics, MPH is orally administrated for its therapeutic purposes, and rapidly absorbed by the gastrointestinal tract, being a short-acting stimulant (its half-life is short, 2 to 3 hours, averagely) (Arnold, 2000; Freese et al., 2012). It is highly lipophilic, easily crossing the blood-brain barrier (BBB) (Dinis-Oliveira, 2017) and spreading to brain tissue, where it reaches its specific target sites. Metabolization of MPH occurs in the liver, where it is converted to ritalinic acid, an inactive metabolite. MPH is classified, by the Drug Enforcement Administration (DEA), as a schedule II substance, evidencing its potential of abuse. Still, its therapeutic use has been demonstrated to be safe and effective (Pereira et al., 2018). Different formulations of

MPH are clinically used according to the therapeutic purpose (Prus, 2013). It is only available in prescription form (in pill form to be orally administered), in immediate (like Rubifen and Ritalin, brand name) and extended-release pharmaceutical formulations (like Concerta, brand name) (Meyer & Quenzer, 2005; Prus, 2013; Sharma & Couture, 2014). The formulations of immediate release are effective for 3 to 5 hours after administration, working quickly and rapidly reaching its peak performance, which represents a disadvantage for children during the school day. On the other hand, Concerta provides relief during all day, staying longer in the system (Meyer & Quenzer, 2005). Besides these two key features, pharmacogenetics needs to be considered as well, since genetic differences between individuals may lead to variations in methylphenidate's response or even to its absence.

The effects of MPH can be explained by exploring its action throughout the brain. Studies have mainly focused on cortical and striatal regions, such as the prefrontal cortex (PFC), striatum and nucleus accumbens (NAc), which are related to executive and attentional functions, cognitive performance, reward and addiction mechanisms (Ebenezer, 2015; Faraone, 2018; Freese et al., 2012; Sharma & Couture, 2014). Nevertheless, more recently its widespread activity on different brain regions has been focus of interest. Catecholamines tune the action of glutamatergic and gabaergic transmission, and MPH, by increasing DA in the striatum, enhances glutamatergic responses, activating this area. Increased alertness and arousal are related to augments of catecholamines in the PFC and the feeling of euphoria is due to their increase in the limbic system (Ebenezer, 2015). The action within the NAc, center of the reward circuitry, explains the potentiality of MPH to be abused (Carlezon et al., 2003). It was also demonstrated that MPH increases the activation of dorsal attention network (DAN) and deactivation of default mode network (DMN), a resting-state condition that engages when someone is not focused on any specific task, which may contribute to the effects of this stimulant on improving cognitive performance (Faraone, 2018; Tomasi et al., 2011). It is important to take into consideration that almost all of these effects are described in task-based functional magnetic resonance imaging (fMRI) studies, so they depend on the context and also on the subject's baseline level of performance and arousal (Ebenezer, 2015; Faraone, 2018; Mueller et al., 2014; Tomasi et al., 2011).

1.1.3 Therapeutic use – Attention Deficit Hyperactivity Disorder (ADHD)

ADHD is one of the most common neurodevelopmental disorders, having its onset during childhood, normally apparent before 12 years old, and often persisting into

adulthood (in about 40 to 50 % of the cases) (Ebenezer, 2015; Zigmond et al., 2015). Recent data estimates that it affects among 3 to 7 % of children and 2 to 5 % of adults (Faraone, 2018; Pereira et al., 2018; Sayal et al., 2017). The Diagnostic and Statistical Manual of Disorders (DSM)-5 by the American Psychiatric Association (APA), widely used by clinicians for classification and diagnosis of mental disorders, identifies three different presentations of ADHD (that can change throughout time): predominantly inattentive, predominantly hyperactive-impulsive and combined presentation, each with different levels of severity, proving, in advance, the heterogeneity of this condition (Ebenezer, 2015; Epstein & Loren, 2013). Thus, inattention, hyperactivity and impulsivity are the main symptoms of ADHD, which greatly impact the daily life of patients (Sharma & Couture, 2014). Children with ADHD exhibit poor social skills and academic performance; generally, they display deficits in executive functions, which are the cognitive abilities required to plan, monitor and execute actions towards a specific goal, and that encompass inhibition (or inhibitory control, *i.e.* the ability to inhibit context-inappropriate automatic/impulsive responses), working memory and sustained attention (Diamond, 2013; Zigmond et al., 2015). Bipolar disorder, depression, anxiety and autism are common psychiatric comorbidities of ADHD (Ebenezer, 2015; Sharma & Couture, 2014).

Regarding the etiology of ADHD, the causes remain elusive, nonetheless there is consistent evidence for the role of genetic and environmental factors on the development of the disorder. In respect of the genetic factors, ADHD is highly inheritable, and multiple genes have been found to be altered and to play a role, almost all related to catecholaminergic transmission, such as genes coding for dopamine receptors, dopamine transporter, monoamine oxidase, among others (Ebenezer, 2015). Concerning the environmental factors, several have been proposed to increase the risk of ADHD, and most are prenatal risk factors. Among them are maternal risky behaviors during pregnancy, such as smoking, alcohol abuse, substance use and stress, and pregnancy, delivery and infancy complications (Banerjee et al., 2007; Kim et al., 2020; Sciberras et al., 2017). These factors may induce fetal deprivation of nutrients and oxygen, brain structures anomalies and behavioral and cognitive problems, impacting the normal neurodevelopment (Banerjee et al., 2007; Sciberras et al., 2017). Yet, these are identified associations that still lack sufficient evidence to imply causality (Kim et al., 2020; Sciberras et al., 2017).

Symptoms arise from a dysfunctional neurotransmission at important brain structures. These brain regions typically present structural and functional alterations, including in the white matter integrity, volume, and connectivity (Ebenezer, 2015;

Faraone, 2018; Zigmond et al., 2015). There are several regions, in a broad and complex interaction, contributing for the pathophysiology of ADHD (Faraone, 2018; Zigmond et al., 2015). For instance, the PFC, basal ganglia, thalamus and cerebellum are highly affected in ADHD. Indeed, they account for the control of movement, and cognitive, emotional and social behaviors, usually impaired in this disorder (Ebenezer, 2015; Leonard et al., 2004; Mueller et al., 2014; Sharma & Couture, 2014). Different studies disclose an ADHD-related hypoactivation of networks involved in executive and attentional functions (frontoparietal and ventral attentional networks) and hyperactivation of the DMN (which disturbs ongoing cognitive performance) (Zigmond et al., 2015). It is accepted that there is an imbalance of the catecholamines levels, DA and NE, related to a dysfunctional reuptake and release, which alters the communication between the previously mentioned brain regions, leading to ADHD symptoms (Ebenezer, 2015).

Some studies have also suggested that ADHD is not a perceptual issue, but an inhibition problem. Individuals with ADHD tend to respond to distracting factors much more than healthy individuals, an impulsive behavior that hinders goals' accomplishment. It is considered a performance disorder and not a knowledge disorder, and MPH exerts its effect by acting at the performance level. In fact, the brain areas affected in ADHD and those where MPH exerts its activity, by restoring the neurotransmitters' balance, are largely coincident (Faraone, 2018). Even so, different approaches are available for the treatment of ADHD, from medication to behavioral therapy. Medication is the most effective approach; it includes stimulants as the leading option, such as MPH and amphetamines, and nonstimulants (atomoxetine) (Meyer & Quenzer, 2005; Sharma & Couture, 2014). Behavioral therapy is beneficial as a co-treatment along with medication.

Diverging views have culminated in an intense debate around ADHD prevalence and overdiagnosis. Several specialists claim that ADHD diagnosis is rapidly increasing, mainly due to a greater awareness and attention from the parents and society in general. Notwithstanding, an accurate diagnosis remains unattainable, since it is still based only on clinical history, demonstrated behavioral symptoms and reports from parents and schoolteachers (Ebenezer, 2015). Also, different physicians, from distinct backgrounds, may apply the DSM-5 criteria differently, which may result in misdiagnosis. As a consequence, MPH prescription is increasing as well (Bruggisser et al., 2011; Freese et al., 2012; Pereira et al., 2018), and it is likely that many children are being subjected to drug exposure unnecessarily. On the other hand, the lack of diagnosis and intervention can lead to devastating consequences to the undiagnosed patient.

Thus, regardless of the point of view, all recognize that an accurate ADHD diagnosis is crucial for the patients and their families. Keeping on exploring the neurobiology of ADHD is fundamental to overcome the subjectivity inherent to ADHD diagnosis by complementing it with objective measures.

1.1.4 Non-medical use

In the last few years, there was a significant increase in MPH non-medical use, which means that people are taking it without prescription for non-medical purposes, including as a recreational drug. The motivations for this behavior are diverse, since stimulant effects gather increased wakefulness, focus and attention, and suppressed appetite, among others. The most frequent intent of MPH misuse is to enhance concentration, alertness and focus, and to decrease tiredness (Pereira et al., 2018; Thier & Gresser, 2017), which is very common among students that want to improve their performance (Freese et al., 2012). Recreational purposes are also common, since MPH can induce pleasant feelings like euphoria and increased self-esteem (Bruggisser et al., 2011; Freese et al., 2012; Meyer & Quenzer, 2005; Pereira et al., 2018).

Evidence show that one of the factors that contribute to the increasing of MPH misuse is the easy access to this drug through relatives or friends with a clinical prescription (Freese et al., 2012; Pereira et al., 2018). Moreover, ADHD patients are a group of risk for MPH abuse, since these patients can decide to consume higher doses than those prescribed (Bruggisser et al., 2011; Thier & Gresser, 2017). Although therapeutic use of MPH has shown to be safe (Pereira et al., 2018), there are adverse effects associated to its non-therapeutic use such as abdominal pain, tachycardia, insomnia, lack of appetite and anorexia, nausea, headache, anxiety and hypertension. Other studies have also suggested a correlation between its misuse and the abuse of other drugs particular by non-ADHD patients (Bruggisser et al., 2011; Pereira et al., 2018; Thier & Gresser, 2017). Through intravenous injection, the effects of MPH are amplified comparing to oral administration, since this route of administration provides a faster drug absorption and bioavailability (Meyer & Quenzer, 2005; Pereira et al., 2018; Prus, 2013). Indeed, Bruggisser et al (2011) demonstrated a severe toxicity of MPH misuse through its injection, whereas oral and nasal administrations led only to mild to moderate toxicity. This toxicity may be associated to paranoia, delirium and hallucinations (Bruggisser et al., 2011; Freese et al., 2012).

MPH has been ranked as the 12th substance to cause physical harm and the 13th to cause dependence (Sharma & Couture, 2014). Despite this, the rates of MPH illicit production are irrelevant, since it is considered a weak drug, even though its abuse potential. *Kiddie coke* and *the smart drug* are common street names for MPH (Pereira et al., 2018; Prus, 2013). Although the frequent and increasing use of MPH, there is an overall lack of information about complications derived from non-therapeutic use of MPH (Bruggisser et al., 2011), as well as about brain alterations and underlying mechanisms. Since it is consumed mainly by children at key phases of development, it would be crucial to understand the implications of the early exposure to MPH. There are no studies regarding its misuse in Portugal. Nonetheless, a study by Lopes et al. (2015) with focus on young people (aged 18-29 years) revealed that most respondents had already consumed medication for enhanced performance purposes and that there is a wide acceptance and disposition for its use for non-therapeutic purposes, especially for improvements of cognitive performance. Despite the importance of this topic, not much is known about the effect of MPH, including its impact in non-neuronal cells and the long-term consequences of its use.

1.2 Astrocytes

1.2.1 Brief note on glial cells

The present concept of neuroglia was built over more than one century, and nowadays the subdivision of glial cells into microglia, astrocytes, oligodendrocytes and their progenitors, NG2 cells (or oligodendrocyte precursor cells - OPCs), is well established (Jäkel & Dimou, 2017). Virchow, in 1856, was the first to use the term neuroglia (or Nervenkitt) as “nerve-glue”, a sort of connective tissue of the nervous system. Without knowing, he opened doors for the discovery and study of glial cells. But was in the beginnings of the 20th century that a determinant step in the field was given, fairly driven by the findings of Santiago Ramón y Cajal and Pío del Río-Hortega, considered the basis of modern glial biology research (Sierra et al., 2016; Somjen, 1988). In the last few years, the way of looking at these cells from a functional point of view has completely changed. Today, glial cells are well-known as highly active and dynamic cells, playing countless key roles in the nervous system, and should be set at the same level of relevance and importance as neurons. Nevertheless, many of the primordial questions remain to be answered.

1.2.2 Origin and astrocytic features

Astrocytes, also collectively known as astroglia, are the most abundant glial cell type (Jäkel & Dimou, 2017) in the human brain, tiling the entire CNS. These cells were, for many years, understood mainly as neuron supportive cells. In fact, the term "astrocyte" was coined in 1895 by Michael von Lenhossek, to refer to the cellular element of neuroglia (Sierra et al., 2016). Nowadays, the variety and complexity of functions carried out by astrocytes are well stablished, in both healthy and diseased CNS. This potential to play a broad range of key roles, unveils their great morphologic, molecular and genetic heterogeneity.

To understand the dynamism and heterogeneity of astrocytes, one must start by looking into their origin. Glial cells have two different origins: astrocytes, oligodendrocytes and NG2 cells originate from the ectodermal tissue, while microglia, the primary immune cells of the brain, originate from mesodermal tissue (Jäkel & Dimou, 2017). Astrogliogenesis, i.e. astrocytes' formation, starts during embryonic development and persists throughout the neonatal and postnatal period, reaching its peak during late prenatal to early postnatal stages (Yang et al., 2013). Astrocytes develop from different pools of progenitor cells (that lead to distinct astrocyte lineages) within the ventricular zone (VZ), like radial glial cells, and the subventricular zone (SVZ), such as glial progenitor cells (Oberheim et al., 2012; Wang & Bordey, 2008).

Regarding morphology and spatial location, this cell population is generally distinguished into two main categories: protoplasmic astrocytes, found in gray matter and highly branched, and fibrous (or fibrillary) astrocytes, which are found in white matter and have a narrower less complex shape (Barres, 2008; Oberheim et al., 2012; Sofroniew & Vinters, 2010; Somjen, 1988). Still, this nomenclature might be reductive considering the great heterogeneity of these cells. Specialized astrocytic cells include Müller cells in the retina and Bergmann glial cells in the cerebellum, both similar to protoplasmic astrocytes (Barres, 2008; Oberheim et al., 2012). Studies have demonstrated that hippocampal and cortical astrocytes are organized in nonoverlapping domains, each astrocyte, within the respective domain, contacting thousands of synapses, which makes this architecture favorable for the role of these cells on synaptic activity (Halassa et al., 2007; Oberheim et al., 2012; Wang & Bordey, 2008). Although not well understood, this organization and its preservation, may account for this astrocytic role (Oberheim et al., 2008 and 2012).

There is evidence that astrocytes' variety is related to their domain environment. Transcriptomic and proteomic analysis, as well as transgenic studies, are crucial to understand the differential regulation of transcription factors and gene expression, and to reveal the heterogenous expression of several proteins in different brain regions and during development (Oberheim et al., 2012). The genetic and molecular profile of astrocytes is very broad, thereby only a few of the expressed proteins considered to be the most relevant, from a functional point of view, will be referred. Glial fibrillary acidic protein (GFAP), which stains intermediate filaments, is the classically used molecular marker for astrocytes' identification. GFAP expression is highly regulated both in physiologic and pathological conditions, unveiling its importance. Glutamine synthetase (GS), an enzyme that catalyzes the conversion of ammonia and glutamate into glutamine, and S100β, that belongs to the S100 family of calcium binding proteins, are also widely used. They all have limitations, for instance, none is entire exclusive for astrocytes and their detection varies per region, within the cell, and with the CNS environment (Sofroniew & Vinters, 2010; Wang & Bordey, 2008). GFAP, in particular, stains only the major astrocytic processes, not revealing the entire cellular structure (Nag, 2011; Zhou et al., 2019). In addition, the aldehyde dehydrogenase 1 family member L1 (Aldh1L1) gene was found to be a highly specific and very useful marker for astrocytic cells, with a more robust staining (Cahoy et al., 2008).

The main feature that confers astrocytes a key position in the CNS is the expression of a wide variety of receptors, through which they respond to different neurotransmitters, such as glutamate, adenosine triphosphate (ATP), gamma-aminobutyric acid (GABA), adenosine, norepinephrine, acetylcholine, endocannabinoids and serotonin (Nag, 2011; Oberheim et al., 2012; Poskanzer & Molofsky, 2018). Moreover, astrocytic cells express several pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs), particularly TLR3, scavenger receptors (SRs) and complement receptors (CRs), like CR1, CR2, C3aR and C5aR. This feature enables astrocytes to regulate innate immune responses in the CNS (Farina et al., 2007). These cells also express glutamate transporters, predominantly glutamate aspartate transporter (GLAST; human excitatory amino acid transporter 1 - EAAT1) and glutamate transporter 1 (GLT-1; human excitatory amino acid transporter 2 - EAAT2). Astrocytic response varies among brain regions and may be coupled to intracellular calcium signaling (Barres, 2008), which has been shown to trigger the release of neuroactive substances, like glutamate, ATP, adenosine, D-serine, cytokines, eicosanoids, neuropeptides and

trophic factors. Nevertheless, gliotransmission is still a matter of debate (Nag, 2011; Oberheim et al., 2012).

At the cell membrane, different channels can be found. One of them, which is also a relevant astrocytic marker, is the aquaporin 4 (AQP4) channel, through which water molecules diffuse (Nag, 2011). Potassium channels, mainly Kir 4.1, are densely expressed on these glial cells as well, contributing to their very low resting potential (-85 to -90 mV). Indeed, astrocytes are not electrically excitable, do not conduce action potentials, thus being electrophysiologically silent. Despite that, they exhibit intracellular Ca^{2+} activity, as it will be depicted afterwards (Oberheim et al., 2012; Poskanzer & Molofsky, 2018; Wang & Bordey, 2008).

Other interesting feature of astrocytes is their connection through gap junctions, where connexin (Cx) 30 and Cx43 colocalize. Connexins may enable the diffusion of signaling molecules between astrocytes (Nag, 2011), and Cx30 seems to regulate astrocytes' morphology as well as the orientation of their motile protrusions. This regulation also involves members of the Rho GTPase family needed to astrocytes structural plasticity (Zhou et al., 2019).

The depicted expression profile of astrocytes is adapted according to the surrounding environment and, along with their morphology and organization, constitutes the basis for their numerous functions in the CNS.

1.2.3 A myriad of functions in (patho)physiology

Astrocytes have a very strategic position at the CNS. Astrocytic processes envelop the pre- and postsynaptic compartments of synapses. Once they present countless branching processes, a single astrocyte can closely contact thousands of synapses, sensing their activity and regulating their function (Barres, 2008).

Astrocytic cells are part of the neurovascular unit (NVU). With their endfeet, astrocytes ensheath blood vessels, which makes them the ideal agents for regulate cerebral microcirculation and blood flow, that suffer constant changes due to neural activity (Iadecola & Nedergaard, 2007). Indeed, they produce and release molecular mediators, such as nitric oxide (NO) and arachidonic acid (AA), that play an important role on those functions (Attwell et al., 2010; Sofroniew & Vinters, 2010). These cells promote the integrity of the BBB, via astrocytic signals like transforming growth factor beta (TGF-β) and glial cell line-derived neurotrophic factor (GDNF), and control its permeability (Wang & Bordey, 2008).

Astrocytes play also a critical role in the maintenance of ionic homeostasis, by buffering excess extracellular ions, like potassium and calcium, and water homeostasis, thus promoting the preservation of a viable CNS environment (Barres, 2008; Matias et al., 2019; Wang & Bordey, 2008). These glial cells are sensitive to the ionic extracellular environment, undergoing volume changes in response to alterations of the extracellular osmolarity. Cell swelling occurs after exposition to hypoosmotic solutions and can be paired to oscillations of Ca^{2+} waves. This process is an early change that occurs in most types of acute cell injury, that may be repaired by restoration of normal volume or lead up to further damage. Different types of specialized channels may be involved, like AQP4 and volume-regulated anion channel (VRAC) (Lafrenaye & Simard, 2019; Nag, 2011).

Calcium imaging techniques have provided evidence of astrocytic communication by Ca^{2+} signaling and it is currently known that astrocyte calcium activity is involved in many functions of these cells (Semyanov, 2019). This signaling may be limited to a single cell, in an oscillatory form, or may be propagated in the form of calcium waves along the astrocytic network mediated by gap-junctions (Semyanov, 2019). Calcium signaling can be also transmitted to neuronal cells (Nag, 2011), eliciting neuronal responses. Increases in intracellular calcium are part of astrocytes' response to neurotransmitters' stimulation and may trigger the release of gliotransmitters.

The third element of synapses

As above-mentioned, the intimate contact of astrocytes with neuronal cells enables a strong astrocyte-neuron bidirectional communication that led to the concept of tripartite synapse, proposed by Araque and colleagues (1999). The traditional picture of synapse comprised two elements, the pre- and postsynaptic neuronal terminals, and the tripartite synapse concept proposes a more accurate picture of the synapse, by including a third element, the astrocyte (Araque et al., 1999). Indeed, astrocytes exert an active regulation of synaptic features and neuronal activity in general. These glial cells promote synaptogenesis and induce neuronal maturation, by providing growth-promoting molecules like laminin and neural cell adhesion molecule (NCAM) and trophic factors (Wang & Bordey, 2008). The release of glial-derived factors can also modulate neurotransmission (Wang & Bordey, 2008).

Astrocytes contribute for neurons' survival and activity by providing metabolic support. Actually, they are in the right position to be the ones capturing nutrients from blood and to furnish them to neurons (Sofroniew & Vinters, 2010; Wang & Bordey,

2008). Furthermore, one of their major functions is the metabolism of neurotransmitters, since they express several transporters for glutamate, GABA and glycine, and others, being able to reuptake them from the synaptic cleft. The metabolism of glutamate by astrocytes is well-known: after glutamate uptake, it is converted into glutamine, through the enzyme glutamine synthetase, recycling it back into the neurons, for de-novo synthesis of glutamate catalyzed by the enzyme glutaminase (Figure 1.2). Astrocytic-released glutamine is also utilized by neurons as precursor for the synthesis of GABA by the enzyme glutamate decarboxylase (GAD). This influences neurotransmission and prevents excitotoxicity (Mahmoud et al., 2019; Oberheim et al., 2012; Schousboe, 2019).

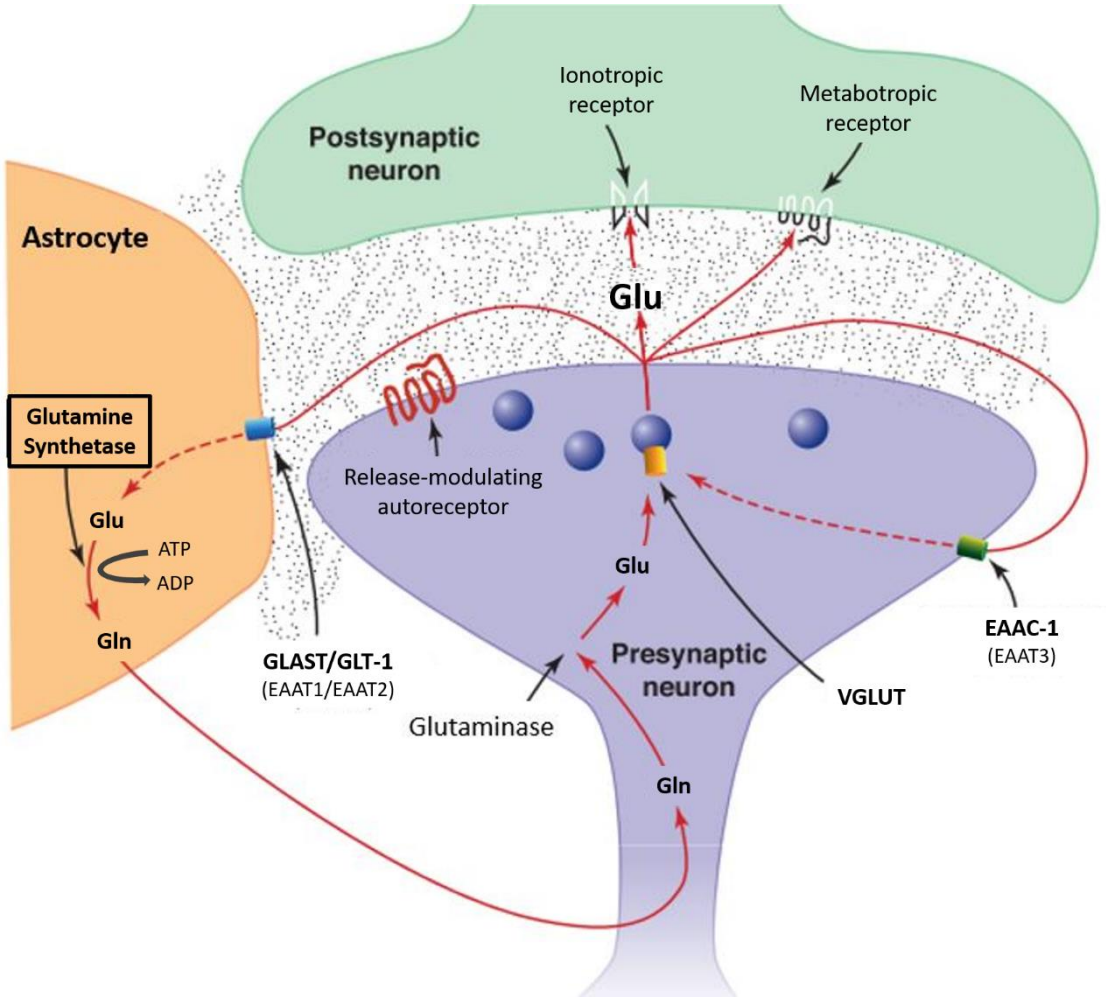

Figure 1.2. **Representative image of glutamate-glutamine cycle.** In the presynaptic neuron, glutamate is stored in synaptic vesicles (blue spheres in the figure) through transport by VGLUT. When released into the synaptic cleft, glutamate can activate its receptors at the post synaptic terminal (ionotropic and metabotropic glutamate receptors), and interact with its reuptake transporters expressed by the presynaptic neurons (EAAC-1; human EAAT3) or by astrocytes (GLAST and GL-T1; human EAAT1 and EAAT2, respectively), being cleared from the synaptic cleft. In the presynaptic terminal, there are receptors controlling glutamate release (the release-modulating autoreceptor). Astrocytes uptake most of the glutamate released by neurons, only a low percentage is taken up back into the presynaptic neurons. At the astrocytic level, glutamate is converted into glutamine through action of glutamine synthetase, an ATP-dependent reaction. Astrocytes are responsible for the synthesis of glutamine that will be provided to glutamatergic neurons, for conversion into glutamate by glutaminase, and further maintenance of glutamate reservoirs. Legend: ADP, Adenosine Diphosphate; ATP, Adenosine Triphosphate; EAAC-1, Excitatory Amino Acid Carrier-1; EAAT1, Excitatory Amino Acid Transporter 1; EAAT2, Excitatory Amino Acid Transporter 2; EAAT3, Excitatory Amino Acid Transporter 3; GLAST, Glutamate Aspartate Transporter; Gln, Glutamine; GLT-1, Glutamate Transporter 1; Glu, Glutamate; VGLUT, Vesicular Glutamate Transporter. Adapted from (Byrne et al., 2014).

Neurotrophic factors

Astrocytes are highly secretory cells and an important source of neurotrophic factors (NTFs) in the CNS. Their basal levels are usually very low at normal conditions, but can increase under stress conditions (Pöyhönen et al., 2019). The impact of astrocyte-released neurotrophic factors on neurons has been long known (Lin et al., 1993). NTFs, such as GDNF, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), stimulate synaptogenesis and have neuroprotective effects, promoting neuronal survival. Also, astrocyte-released NTFs may modulate microglial activity (Rocha et al., 2012). Looking particularly to GDNF, this neurotrophic factor belongs to the GDNF ligand family, and exerts its role through binding to GDNF family receptor alpha 1 (GFRα1) followed by association with RET receptor tyrosine kinase or the NCAM (Ibáñez & Andressoo, 2017; Rocha et al., 2012). The downstream signaling activated by this receptor complex may include the activation of different signaling pathways such as mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K)/AKT pathways (Ibáñez & Andressoo, 2017; Pöyhönen et al., 2019) (Figure 1.3).

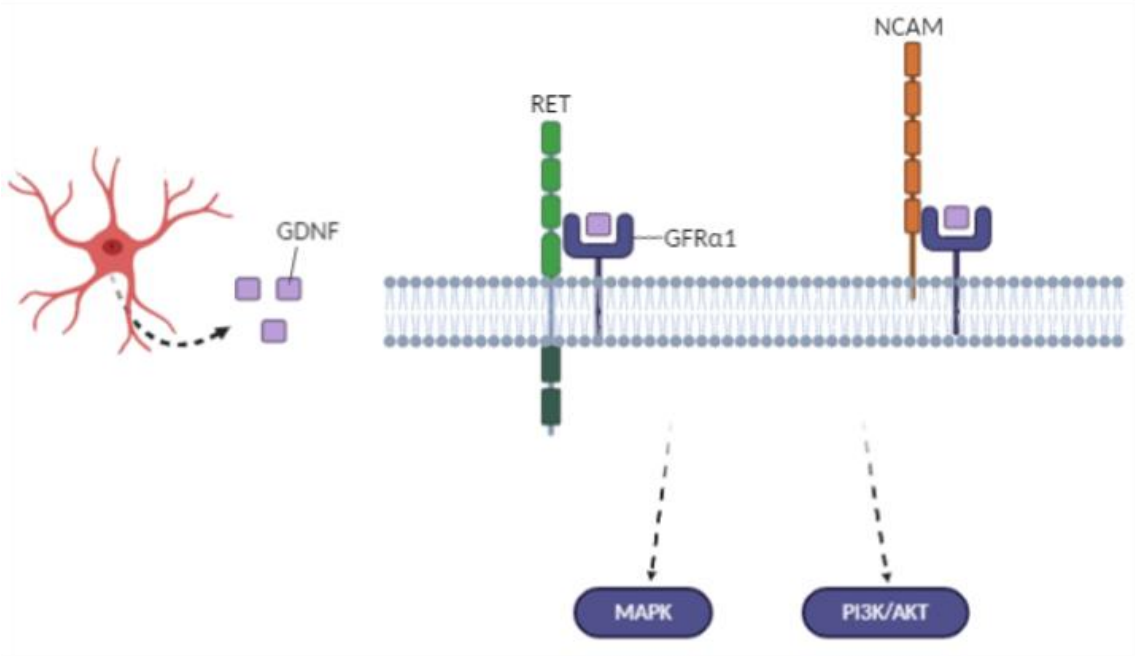

Figure 1.3. **Simplified mechanism of GDNF action.** Astrocytes release GDNF that will bind to one of the two protein complexes: GFRα1/RET-receptor complex or GFRα1/NCAM complex. Binding just to GFRα1 does not allow GDNF to activate downstream signaling pathways. After protein complexes are formed, PI3K/AKT and MAPK signaling pathways are activated. Legend: AKT, protein kinase B; GDNF, glial cell line-derived neurotrophic factor; GFRα1, GDNF family receptor alpha 1; MAPK, mitogen-activated protein kinase; NCAM, neural cell adhesion molecule; PI3K, phosphoinositide 3-kinase; RET, receptor with tyrosine kinase activity.

Astrogliosis

Astrocytes are influenced at many levels by the surrounding environment (Poskanzer & Molofsky, 2018) and so, under several CNS conditions, such as infection, mechanical injury and neurodegenerative disorders, astrocytes suffer changes at different levels, becoming reactive, a process called astrogliosis. Astrocytes exhibit cellular plasticity, which can be observed when they turn reactive, through the upregulated expression of GFAP (Oberheim et al., 2012; Wang & Bordey, 2008) (Figure 1.4). Indeed, the classical hallmarks of astrogliosis comprise overexpression of GFAP (whose level correlates with the degree of reactivity), hypertrophy of cell body and increase in the number of astrocytic processes (Matias et al., 2019). Besides GFAP, other proteins can be also upregulated in the astrogliosis process, such as S100β, iNOS and NF-kB as well (Nag, 2011).

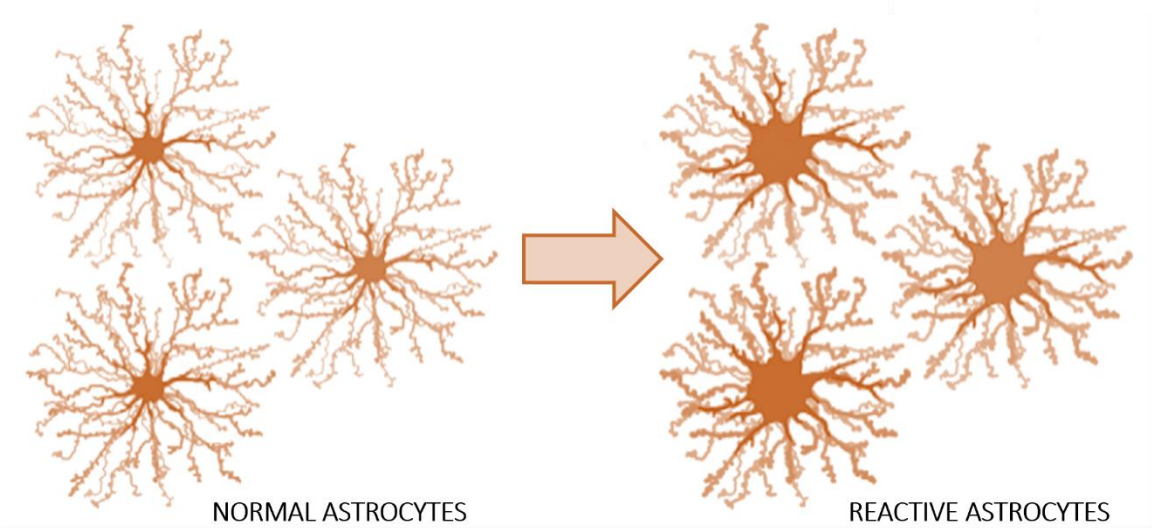

Figure 1.4. **Morphologic alterations of astrocytes induced by an insult.** Astrocytes respond to stress conditions on their surrounding environment by turning reactive, a process identified as astrogliosis. Major characteristics of this change are the hypertrophy of astrocytes' cell body and the increase and enhanced size of astrocytic processes, mediated primarily by the regulation of glial fibrillary acidic protein (GFAP) expression. Adapted from (Sofroniew, 2009).

This process occurs in a spectrum, from mild to moderate forms to highly severe ones, in a progressive way, and triggered by different signaling molecular mechanisms having different possible consequences. Some of the molecules that will affect astrocytes are growth factors, cytokines, lipopolysaccharide (LPS), neurotransmitters

and ROS. Highly severe forms of astrogliosis are followed by scar formation. This process may have protective and repairing functions, but it may also contribute to the onset and progression of CNS disorders. Although astrogliosis is considered a hallmark of diseased CNS, and unconsciously associated to a negative outcome, it is important to notice that it exerts both beneficial and detrimental effects. As above-mentioned, an optimal marker for astrocytes is still lacking, preventing a complete comprehension and characterization of astrogliosis (Sofroniew, 2009; Sofroniew & Vinters, 2010).

Zamanian and colleagues have described two different subtypes of reactive astrocytes (Zamanian et al., 2012), “A1” and “A2”, the first being induced by LPS-induced neuroinflammation and the second through ischemia. The A1 phenotype, characterized by the upregulation of complement cascade genes, was found to be neurotoxic and destructive to synapses, losing normal functions and gaining cytotoxic ones. On the contrary, A2 is neuroprotective, promoting homeostasis and repair of CNS tissue. LPS induces microglia activation, which in turn leads to astrocytic reactivity. Recently, the same group demonstrated that activated microglia, , by secreting a mix of pro-inflammatory factors [interleukin 1 alpha (IL-1α), tumor necrosis factor alpha (TNF-α), and the complement component 1, q subcomponent (C1q)] can induce the A1 neurotoxic astrocyte phenotype, *in vivo* and *in vitro* (Liddelow et al., 2017) (Figure 1.5). Moreover, even though both injury models induced microglia activation, these authors revealed that this feature is mandatory for LPS-induced A1 phenotype *in vivo*. Also, they showed that this mix of cytokines is sufficient for induction of A1 phenotype *in vitro* (Liddelow et al., 2017).

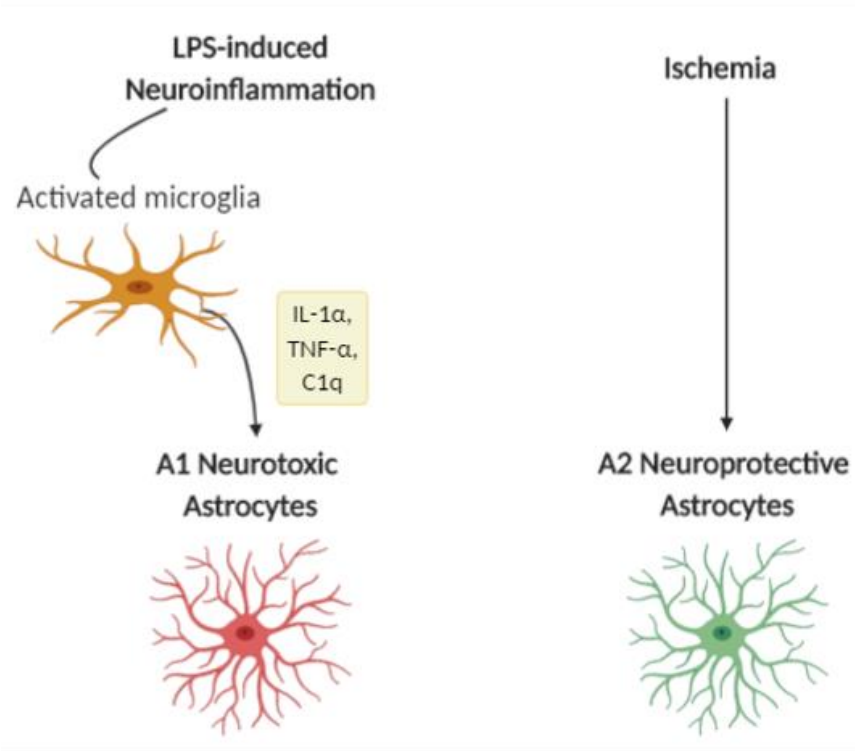

Figure 1.5. **Two different subtypes of reactive astrocytes are induced by different injury models.** LPS-induced neuroinflammation triggers microglia activation, which secretes a mix of three inflammatory mediators, IL-1α, TNF-α and C1q, finally inducing the A1 astrocytic phenotype. The A2 phenotype is induced directly by ischemia. This happens both *in vivo* and *in vitro*. A1 astrocytes were demonstrated to be neurotoxic, widely expressing complement cascade genes, in contrast to A2 astrocytes, which revealed neuroprotective functions, expressing high levels of neurotrophic factors. Legend: IL-1α, Interleukin 1 alpha; TNF-α, Tumor Necrosis Factor alpha; C1q, Complement Component 1, q subcomponent; LPS, Lipopolysaccharide.

The description of these two subtypes of reactive astrocytes was based only on expression profiling analysis. Very recently, Sofroniew (2020) questioned the accuracy of identifying and defining reactive astrocytic subtypes merely on differences in molecular expression patterns. Indeed, the author stressed the need of information about cell morphology, proliferation, molecular expression, functions, and cellular interactions for that purpose. Sofroniew proposes a more complete and accurate type of categorization of reactive astrocytes that should be further applied on future studies. This proposition relies on the heterogeneity of astrogliosis, across disorders and tissue regions, and over time during disorders, and collides with the previously described findings, unveiling them as possibly speculative and misleading.

Astrogliosis and oxidative stress

In the CNS there is an uninterrupted metabolic activity due to the great consumption of oxygen and glucose. This metabolic activity relies on reduction-oxidation (redox) reactions that lead to the production of reactive oxygen and nitrogen species (ROS and RNS, respectively) (Rizor et al., 2019). In the brain, ROS have diverse sources, like mitochondrial and the nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase (NOX) pathway (Adibhatla & Hatcher, 2010; Chen et al., 2020; Ebenezer, 2015). For RNS, a typical source is the nitric oxide synthase (NOS) pathway (Rizor et al., 2019). Indeed, astrocytes may act as main sources of ROS and RNS (Chen et al., 2020), since they express NOX (Chen et al., 2020; Zhu et al., 2009) and the inducible nitric oxide synthase (iNOS) (Brahmachari et al., 2006).

Astrocytes play a major antioxidant activity, contributing for the redox balance in the CNS (Chen et al., 2020). Compared to astrocytes, neurons have weak antioxidant defenses, revealing the importance of these glial cells, which is further evident given the fact that the brain is extremely vulnerable to oxidative stress (Adibhatla & Hatcher, 2010). By producing and releasing reducing agents such as glutathione (GSH) and the enzyme superoxide dismutase (SOD), astrocytes lead to the conversion of ROS into non-reactive products (Rizor et al., 2019). The expression of these antioxidants is regulated by the nuclear factor erythroid 2-related factor 2 (Nrfr2) (Chen et al., 2020). The process of astrogliosis, as already mentioned, may have protective functions, restricting the damage, by, for instance, maintaining this balance, but can also lead to an increased production of ROS and RNS, disrupting the oxidative balance and aggravating neural injury (Figure 1.6).

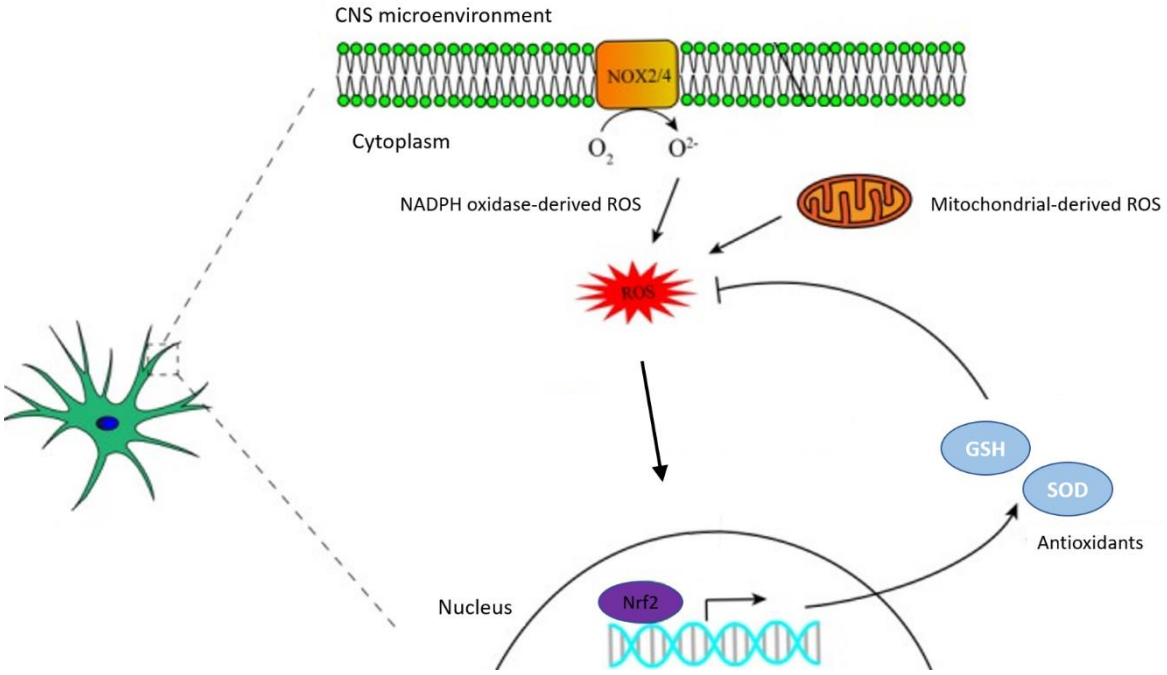

Figure 1.6. **Astrocytes are a source of both antioxidants and ROS.** Under physiologic conditions, astrocytes are major players on the CNS antioxidant defenses, producing and releasing antioxidants like GSH and SOD, which process is actively regulated by Nrfr2 signaling. However, under an insult or disease conditions that may lead to astrogliosis, they can function as main sources of ROS. In astrocytes, ROS may arise from the action of NOX or from mitochondrial activity and can prevent the degradation of Nrf2 in the cytoplasm (mechanism not depicted), allowing its entry into the nucleus and regulation of production of antioxidants. Legend: CNS, central nervous system; NOX, nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase; ROS, reactive oxygen species; GSH, glutathione; SOD, superoxide dismutase; Nrfr2, nuclear factor erythroid 2-related factor 2. Adapted from Chen et al., 2020.

There is no doubt that astrocytes are active pivotal players in the CNS, in both physiological and pathological conditions, providing an overall microenvironment homeostasis or contributing to (putatively all) brain diseases, respectively. These roles rely on intrinsic characteristics of astrocytes and on intercellular crosstalk with other cells. Many examples of the bidirectional conversation between astrocytes and other cells, such as neurons and microglia, have been reported. This communication is crucial for CNS development and function. The normal neuronal functioning is not possible without the normal glial functioning, and vice-versa; they are intrinsically related.

1.3 Outline of the (unexplored) methylphenidate-induced effects in CNS

Several studies exploring the action of methylphenidate (MPH) within the brain have determined the regions most affected by this psychostimulant, being the prefrontal cortex and striatum. In agreement, these are widely implicated areas in ADHD. Therefore, cortical and striatal regions have been receiving almost all attention with regard the impact of MPH consumption in the CNS. Nonetheless, some efforts have been done to counter this tendency, and despite the undoubtedly importance of these areas, there is now multiple evidence that other brain regions deserve the same attention. The problematic of the non-therapeutic use of MPH along with ADHD overdiagnosis discloses another issue on this research field, that is the poor comprehension of the implications (and the underlying mechanisms) of MPH consumption at such critical phase of brain development.

Hippocampus is another brain region that needs more studies, since it is critical for learning, memory, and drug use processes and affective behaviors. Moreover, it can influence brain's reward system (Schmitz et al., 2017). Interestingly, some studies have already raised the importance of hippocampus in this area of knowledge by showing that MPH increases catecholamines' levels (Kuczenski & Segal, 2002), affects cell survival (Schmitz et al., 2017), induces astrogliosis, neuroinflammation and oxidative stress (Faraone, 2018; Martins et al., 2006; Meftahi et al., 2020; Motaghinejad et al., 2016) and alters BBB permeability (Coelho-Santos et al., 2019) in this region.

Another existent gap regarding research concerning the impact of MPH in the brain is the lack of knowledge on its effects in glial cells. Such information is almost absent in the available literature; yet, there are a few studies that have started to explore this issue, on both astrocytes and microglia, and have already gave some insights namely into morphological alterations of glial cells (Bahcelioglu et al., 2009; Carias et al., 2018; Cavaliere et al., 2012; Coelho-Santos et al., 2018; Sadasivan et al., 2012). Thus, based on the importance of glial cells in the brain properties and function, together with the widely use of MPH, it is urgent to investigate in detail the consequences of MPH consumption.

CHAPTER 2
AIMS

Chapter 2 – Aims

The main goal of this project was to unveil and characterize the direct effects of MPH in astrocytes. To achieve this purpose, relevant astrocytic features were analyzed on primary cultures of rat cortical astrocytes as follows: GFAP protein levels, cell volume and death, ROS and NO production, NF-κB signaling pathway activation, and release of GDNF.

Additionally, this project aimed at exploring some effects of MPH chronic treatment, comparing its misuse and therapeutic use. This was performed on the hippocampus, PFC and serum of control and ADHD animal model (WKY and SHR rats, respectively), by looking into GFAP protein levels, oxidative and nitrosative stress, and antioxidant capacity.

In sum, this work aimed to fulfill some of the gaps concerning MPH neurobiological effects particularly in glial cells, and also the consequences of its chronic use in the brain.

CHAPTER 3
METHODS

Chapter 3 – Methods

3.1 Primary Cultures of Rat Cortical Astrocytes

Astrocytes were isolated from newborn (postnatal day 0-2) Wistar rats (Figure 3.1). Under sterile conditions, rat pups were decapitated, the cerebral cortices dissected, and meningeal layers and superficial blood vessels carefully removed. The tissue was finely minced and further mechanically dissociated by pipetting with pierced tips and by filtration through a cell strainer (70 µm nylon mesh). The cell suspension was centrifuged at 300 ×g for 10 min at room temperature (RT), the supernatant discarded and the pellet resuspended in astrocytic culture medium [Dulbecco's Modified Eagle's Medium high glucose (DMEM; Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1 % gentamicin (Gibco)]. Afterwards, cells were plated in 75 cm^2 cell culture flasks (at a density of 1.2×10^5 cells/cm^2) and maintained at 37˚C in a humidified 5 % CO_2/95 % air atmosphere (Series II CO_2 incubator; Thermo Fisher, Waltham, USA). The medium was changed on the day after the isolation and then on every 2-3 days, until cells reached confluency. At that point, flasks, containing mixed glial cells, were shaken at 250 rpm for 4 h at 37˚C, on an orbital shaker incubator (Stuart, Cole-Parmer, UK). Next, the non-astrocytic cells (detached cells) were discarded, and astrocytes (adherent cells) were washed with phosphate-buffered saline (PBS) and then detached with 0.25 % trypsin-EDTA (Gibco) at 37˚C. Culture medium was then added, ceasing trypsinization, and the suspension was centrifuged at 375 ×g for 5 min. The pellet was resuspended in medium, viable cells were counted in a Neubauer chamber using Trypan blue dye (Sigma-Aldrich) and seeded at different densities according to the experimental procedures (Table 3.1).

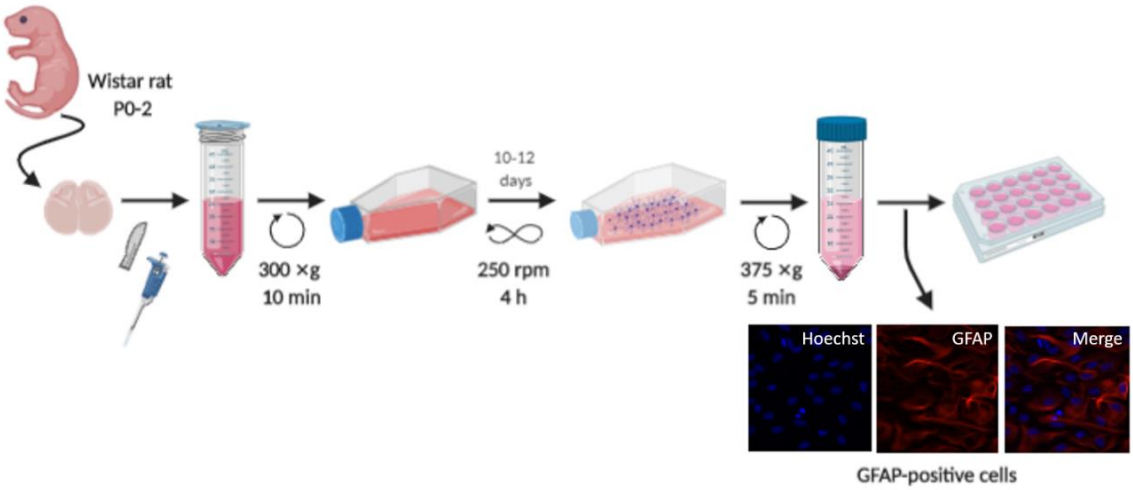

Figure 3.1. **Schematic representation of the isolation and culture of primary rat cortical astrocytic cells.** Astrocytes were isolated from the cortices of newborn Wistar rats (P0-2). The cortical tissue was mechanically digested: firstly, tissue was minced with a bistoury, then it suffered pipetting with pierced tips and finally, for further dissociation of the cells, it was filtered through a cell strainer. Cell suspension was centrifuged and cells were plated in T75 flasks. When cells reached confluency, after 10-12 days, the flasks were shaken at 250 rpm for 4 h, in order to detach contaminating cells, which were discarded. The adherent cells, astrocytes, were detached by trypsinization, and cell suspension was centrifuged. The pellet was resuspended, viable cells were counted and plated at appropriate density, depending on the experimental procedure. Each isolated primary culture was assessed for its purity in astrocytic cells (GFAP-positive cells) by immunofluorescence. Legend: GFAP, Glial Fibrillary Acidic Protein.

Table 3.1. Astrocytic cell densities used according to the experimental procedure.

EXPERIMENTAL ASSAY	MULTIWELL CULTURE PLATE	CONCENTRATION (CELLS/ML)	VOLUME OF MEDIUM/WELL (ML)
TUNEL assay	24	20×10^4	500
Immunocytochemistry	24 with coverslips	45×10^4	500
Calcein-AM	96	10×10^4	150
ELISA	24	20×10^4	500
Western Blot	6	25×10^4	2000

3.1.1 Evaluation of astrocytic culture purity

The purity of the primary astrocytic culture was evaluated by immunofluorescence, using the following cell markers: mouse anti-Glial Fibrillary Acidic Protein (GFAP)-Cy3 conjugated (1:500, Sigma-Aldrich) and mouse anti-S100β-subunit (S100β) (1:200, Sigma-Aldrich) for astrocytes; rat anti-Cluster of differentiation 11b (Cd11b) (1:500, Bio-Rad AbD Serotec, CA, USA) and rabbit anti-Ionized calcium-binding adaptor molecule 1 (Iba1) (1:500, Wako, Japan) for microglia; mouse anti-Neuronal Nuclei (NeuN) (1:500, Millipore, Darmstadt, Germany) and mouse anti-Microtubule-Associated Protein 2 (MAP2) (1:500, Santa Cruz Biotechnology, Dallas, TX, USA) for neurons. Anti-rabbit Alexa Fluor 488, anti-mouse Alexa Fluor 488 and anti-rat Alexa Fluor 488 (1:500, Invitrogen, Inchinnan Business Park, UK) were used as secondary antibodies and nuclei were counterstained with Hoechst 33342 (2 µg/mL, Sigma-Aldrich) (Figure 3.2). Purity was assessed by the percentage of cells positive for GFAP, which was found to be 94 ± 0.4 % of the total cells.

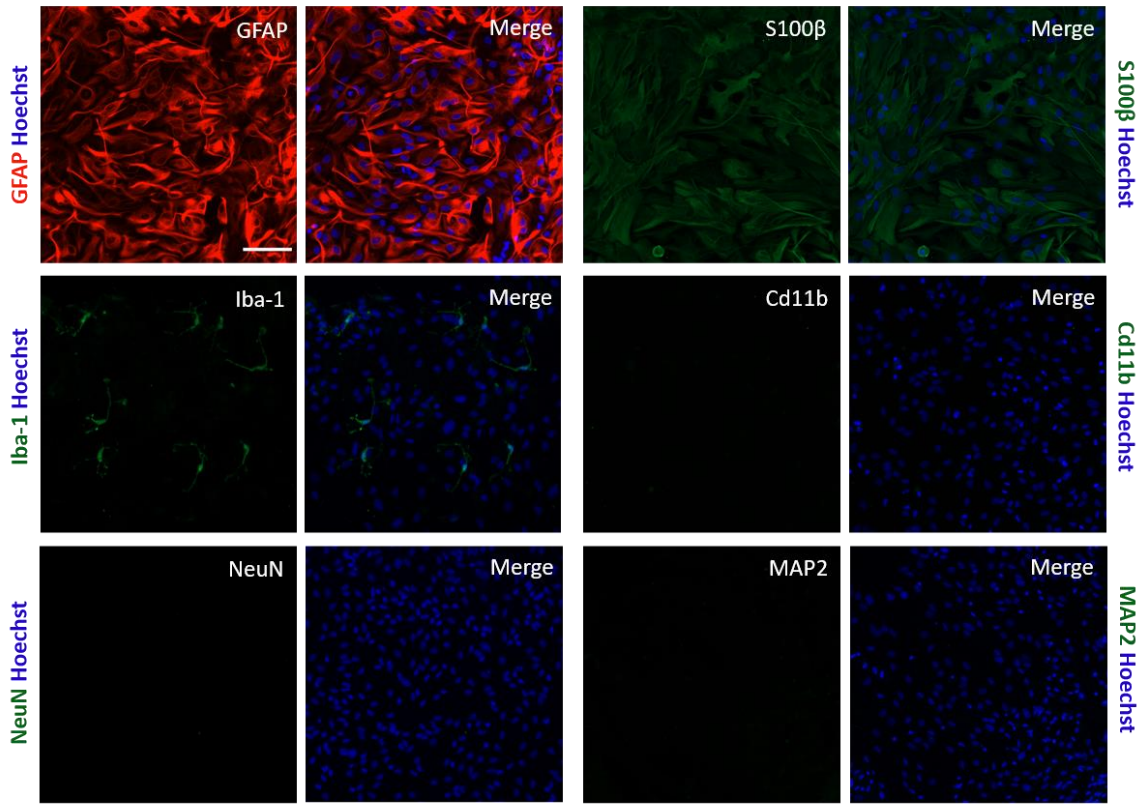

Figure 3.2. **Characterization of primary cortical rat astrocytes culture purity.** In order to validate the astrocytic culture purity, an immunofluorescence was performed, using different cell markers for astrocytes (GFAP and S100β, red and green, respectively), microglia (Cd11b and Iba1, both green) and neurons (NeuN and MAP2, both green). Once GFAP is the classic astrocytic marker, purity was assessed specifically by calculating the percentage of GFAP-positive cells, which was 94 ± 0.4 %. Legend: GFAP, glial fibrillary acidic protein; S100β, anti-S100β-subunit; Cd11b, cluster of differentiation 11b; Iba1, ionized calcium-binding adaptor molecule 1; NeuN, neuronal nuclei; MAP2, microtubule-associated protein 2. Scale bar = 100 µm.

3.2 Animals

To study the effects of chronic exposure to MPH under control and ADHD conditions, Spontaneously Hypertensive (SHR) rats, the most used ADHD model (Sagvolden & Johansen, 2012), and Wistar-Kyoto (WKY) rats, the strain from which SHR model was developed thus being used as its control, were used. This is a genetic model that displays hyperactive and impulsive behaviors, as well as learning and memory deficits. Moreover, abnormalities on the catecholaminergic system at cortical and striatal areas were observed, making it the best-validated animal model of ADHD.

WKY and SHR rats (Charles River Laboratories, France) were housed under controlled environmental conditions (12h light/dark cycle, 24 ± 1 ˚C) with food and water *ad*

libitum. Animals were uninterruptedly and orally administrated with MPH between P28-P55 at a clinically relevant dose. Two animal groups for each animal strain were formed, as follows: WKY and SHR control groups (vehicle, tap water), and WKY and SHR MPH groups (1.5 mg/kg/day; Sigma-Aldrich) as previously described (Coelho-Santos et al., 2018 and 2019). After animal sacrifice, the hippocampi and prefrontal cortices were isolated, and blood serum collected, and stored at -80°C. All the described procedures were done by certified researchers (Federation for Laboratory Animal Science Association) with approval by the Institutional Animal Care and Use Committee (FMUC/CNC, University of Coimbra, Coimbra, Portugal) and Portuguese National Authority for Animal Health "DGAV".

Hippocampal and cortical tissue samples were homogenized with a solution containing saline phosphate buffer and the butylhydroxytoluene (BHT) antioxidant (Sigma-Aldrich), for lipid peroxidation and antioxidant capacity assays (3.9). Samples were vortexed, sonicated and centrifuged at 9425 ×g for 10 min at 4°C. The supernatant was collected, and went for another two steps of sonication and centrifugation at 1508 ×g for 15 min at 4°C. The resulting supernatant was collected, and total protein concentration quantified by the bicinchoninic acid (BCA) method. For western blot assay (3.3), hippocampal tissue samples were homogenized by adding Radio-Immunoprecipitation Assay (RIPA) lysis buffer to the samples. Then, samples were vortexed, sonicated and centrifuged at 24127 ×g for 20 min at 4°C, and supernatants were stored until further use.

3.3 Western Blot analysis

Astrocytes were treated with MPH at the concentrations of 0.5 mM or 1 mM during 24 h at 37°C. Then, the cells were lysed with RIPA lysis buffer supplemented with a protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany), phosphatase inhibitor (PhosSTOPTM, Roche Applied Science) and 1 mM dithiothreitol (DTT) (Bioron, Porto, Portugal). Cell lysates were centrifuged at 1300 ×g for 15 min at 4°C, supernatants (containing the extracted protein) were collected and sonicated for 2-3 min.

Protein concentration of cell lysates and hippocampal tissue (previously processed as described in 3.2) was determined using the BCA method (Pierce, Rockford, USA) with bovine serum albumin (BSA) as standard, and protein samples were prepared for electrophoresis taking into account the defined final protein concentration. Sample loading buffer and 5 % sodium dodecyl sulfate (SDS) were added to the samples, and

proteins were denatured at 95°C for 5 min. Thereafter, proteins (50 µg) were separated through electrophoresis on 10 or 15 % polyacrylamide gels (depending on the target protein) at 100 V for 15 min and at 130 V until the end of the run. Afterwards, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) in Tris-glycine buffer at 0.25 A for 2 h. Membranes were blocked with 5 % nonfat milk in PBS-T (PBS containing 0.1 % (v/v) Tween-20) for 2 h at RT. Then, membranes were incubated overnight at 4˚C with the primary antibodies as follow: rabbit anti-inducible nitric oxide synthase (iNOS; 1:500, Novus Biotechne, CO, USA) or rabbit anti-GFAP (1:750, Sigma-Aldrich). After washing with PBS-T, membranes were incubated with horseradish peroxidase conjugated anti-rabbit secondary antibody (1:10000, GE Healthcare Life Science, USA) for 1 h at RT. Proteins were visualized using the enhanced chemiluminescence (ECL) method on ImageQuant LAS 500 (GE Healthcare). Immunoblots were reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:5000, Millipore) to ensure equal sample loading. Analysis and quantification were performed using Image Studio Lite Software (LI-COR, NE, USA) and results were expressed in percentage of control.

3.4 Cell Volume Measurement

Changes in astrocytes' volume due to water movements were investigated using the calcein fluorescence quenching method as previously described (Leitão et al., 2018). Calcein-acetoxymethyl ester (Calcein-AM) is a nonfluorescent cell-permeable dye which easily diffuses across cell membranes into the cells. Once inside the cell, cytoplasmic esterases cleave the acetoxymethyl (AM) group, resulting on its retention inside the cell and green fluorescence. Since fluorescence is proportional to cell volume, it is through its quantification that changes in cellular volume was determined.

Confluent astrocytic cells, seeded on sterile black 96-well plates coated with laminin (10 µg/mL in PBS; Invitrogen), were treated with MPH at the concentrations of 0.1 mM, 0.5 mM or 1 mM for 2 and 24 h at 37°C. Afterwards, cells were rinsed in PBS and incubated with a 5 µM Calcein-AM (Invitrogen) solution in artificial cerebrospinal fluid (aCSF; in mM: 120 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1 NaH_2PO_4, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 10 glucose) during 1 h at 37°C. Then, the solution was removed and replaced by an equal volume of aCSF, and fluorescence was immediately read at 485/530 nm (excitation/emission wavelengths, respectively) using a microplate reader (Biotek Synergy HT, Winooski, USA). Results were expressed as fold change from control condition.

3.5 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay

The TUNEL assay was used to evaluate cell death by apoptosis. This experiment is based on the incorporation of modified dUTPs, by the action of the enzyme terminal deoxynucleotidyl transferase (TdT), at the 3'-OH ends of fragmented DNA, a hallmark of programmed cell death. These dUTPs undergo a copper-catalyzed reaction, through which they acquire fluorescence, allowing the accurately detection and quantification of apoptotic cells.

The Click-iT Plus TUNEL Assay (Invitrogen) was used according to the manufacturer's recommendations. Briefly, confluent astrocytic cells were treated with methylphenidate (MPH; Sigma-Aldrich) at concentrations of 0.1 mM, 0.5 mM or 1 mM, or with 250 µM Hydrogen Peroxide (H_2O_2; Sigma-Aldrich) for 24 h at 37°C. Then, the whole population of cells (suspended and attached cells) was collected by trypsinization and rinsed in PBS. Cell suspension was centrifuged at 43 ×g for 5 min at 4˚C, supernatant was discarded, and cells were fixed with 4 % paraformaldehyde (PFA; Sigma-Aldrich) for 20 min at RT, followed by further centrifugation at 390 ×g for 10 min at 4˚C. The pellet was resuspended, and cells were adhered to superfrost microscope slides (Thermo Fisher) by centrifugation in the cellspin (Tharmac, Wiesbaden, Germany) at 704 ×g for 5 min. Afterwards, cells were washed with PBS, permeabilized with 0.25 % triton X-100 (Sigma-Aldrich) for 30 min and washed again. The TdT reaction buffer was added to the cells for 10 min at 37 °C, followed by an incubation with the TdT reaction mixture for 1 h at 37 °C, which enabled the nucleotides' incorporation by TdT. Cells were rinsed in 3 % bovine serum albumin (Sigma-Aldrich), followed by an incubation with the Click-iT Plus TUNEL Reaction Cocktail for 30 min at 37 °C in the dark. At this step, the click-reaction, catalyzed by copper, occurred between the nucleotides and the fluorescent dye. Finally, after washing with 3 % BSA, nuclei were counterstained with Hoechst 33342 (4 µg/mL, Sigma-Aldrich) for 5 min at RT and slides were mounted in Dako mounting medium (Dako North America, Carpinteria, USA). Images were acquired using the Axio observer inverted widefield microscope (Carl Zeiss, Oberkochen, Germany). Six different microscopy fields were acquired per coverslip and images were analyzed using Fiji (ImageJ software by the US National Institutes of Health). The number of TUNEL-positive cells was expressed as percentage of total cells.

3.6 Detection of oxidative and nitrosative stress in cell cultures

Intracellular ROS production was evaluated using the CellROX Green Reagent probe (Thermo Fisher). This is a cell-permeable probe that acquires bright green fluorescence once oxidized by ROS and subsequent binding to DNA. It is detectable by fluorescence microscopy with absorption/emission maxima of 485/520 nm. Intracellular production of NO was assessed by using the 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) probe (Thermo Fisher). This nonfluorescent probe is cell-permeable and once inside the cells, it is converted into a cell-impermeant form that when exposed to NO becomes fluorescent and detectable (Kojima et al., 1999; Namin et al., 2013). Fluorescence intensity of DAF-FM diacetate is measured by fluorescence microscopy with excitation/emission at 495/515 nm.

Astrocytes were treated with 0.1, 0.5 or 1 mM MPH, or 250 µM H_2O_2 for 2 h at 37°C. Then, cells were rinsed in PBS and incubated with 10 µM CellROX or 10 µM DAF-FM diacetate, which were freshly prepared in KREBS solution containing 0.02 % Pluronic F-127 (Sigma-Aldrich), for 45 min at 37°C. Following incubation, the solution was removed, and culture medium added for 15 min. Cells were rinsed in PBS and fixed with 4 % PFA (Sigma-Aldrich) for 15 min at RT. After cell fixation, cells were permeabilized with 0.1 % Triton X-100 (Sigma-Aldrich) for 5 min at RT and blocked with 3 % BSA (Sigma-Aldrich) during 1 h at RT. Thereafter, cells were incubated with a primary antibody against GFAP (mouse anti-GFAP-Cy3 conjugated; 1:500, Sigma-Aldrich) overnight at 4°C, washed with PBS and nuclei were counterstained with Hoechst 33342 (2 µg/mL, Sigma-Aldrich) for 5min at RT protected from light. Cells were mounted in Dako fluorescence medium (Dako North America) and six different microscopy fields per coverslip were acquired using the Axio observer inverted widefield microscope (Carl Zeiss) and analyzed using Fiji (ImageJ software by the US National Institutes of Health). Total fluorescence (considering the total area of the image) was quantified as well as three different areas corresponding to background, and the corrected total cells fluorescence (CTCF) was calculated using the following formula:

CTCF = integrated intensity – (area of the picture × mean background)

Data was represented as the mean of fluorescence intensity per total cell number.

3.7 Detection of NF-κB pathway activation by immunofluorescence

The activity of the nuclear factor-kB (NF-kB) signaling pathway in astrocytes was investigated through the detection of the cellular localization of the NF-kB p65 subunit, which is translocated from the cytoplasm into the nucleus when the pathway is activated.

Astrocytic cells were treated with 0.5 mM MPH for 30 min or 2 h, or with tumor necrosis factor α (TNF-α) (10 ng/mL, Sigma-Aldrich) for 2 h at 37°C. After treatment, cells were rinsed in PBS and fixed with 4 % PFA (Sigma-Aldrich) for 15 min at RT. Next, fixed cells were permeabilized with 0.1 % Triton X-100 (Sigma-Aldrich) for 5 min at RT and blocked with 3 % BSA (Sigma-Aldrich) for 1 h at RT. Afterwards, cells were incubated with a primary antibody against nuclear factor-kB (NF-kB) p65 (rabbit anti-NF-kB p65; 1:400, Cell Signaling Technology, MA, USA) overnight at 4°C, washed with PBS and co-incubated for 1 h at RT with the secondary antibody anti-rabbit Alexa Fluor 488 (1:200, Invitrogen), mouse anti-GFAP-Cy3 conjugated antibody (1:500, Sigma Aldrich) and Hoechst 33342 (2 µg/mL, Sigma-Aldrich). Then, cells were mounted in Dako mounting medium (Dako North America) and images were acquired using the Axio observer inverted widefield microscope (Carl Zeiss). In general, six different microscopy fields were acquired per coverslip and images were analyzed by counting the nuclear NF-kB-p65-positive cells, using Fiji (ImageJ software). The number of nuclear-NF-kB-p65-positve cells was expressed as percentage of total cells.

3.8 Measurement of Glial cell line-Derived Neurotrophic Factor (GDNF) levels by Enzyme-Linked Immunosorbent Assay (ELISA)

The release levels of GDNF were measured using the Rat GDNF ELISA-kit (Sigma-Aldrich) in accordance with the manufacturer's instructions.

After challenging astrocytes with 0.1 mM, 0.5 mM or 1 mM MPH for 24 or 48 h at 37°C, the cell media was collected. Cell samples and standards were added, in duplicates, to the plate, coated with the capture antibody, and incubated overnight at 4°C. Then, the plate wells were carefully washed with wash solution kit. The biotinylated detection antibody was incubated for 1 h at RT, followed by incubation with the horseradish peroxidase (HRP)-streptavidin solution for 45 min at RT. After another washing step, the colorimetric 3,3',5,5'-tetramethylbenzidine (TMB) reagent was added for a maximum of 30 min at RT, in the dark. At this point, color develops in proportion

to the amount of target protein bound. Finally, a stop solution kit was added, changing the color from blue to yellow, and the plate was immediately read at 450 nm in a microplate reader (Biotek Synergy HT). For the results' analysis, the mean absorbance of each set of duplicates was calculated and subtracted by the average zero standard optical density. A standard curve was used to calculate the respective protein levels, expressed as pg/mL.

3.9 Lipid peroxidation and antioxidant capacity assays

Products of lipid peroxidation from hippocampus, prefrontal cortex and serum of WKY and SHR rats were assessed using the thiobarbituric acid reactive substances (TBARS) assay. Specifically, TBARS measures lipid peroxidation (the oxidative degradation of lipids), through the assessment of its end products (like malondialdehyde – MDA). Under acidic conditions (conferred by hydrochloric acid), MDA reacts with thiobarbituric acid (TBA), giving rise to a pink-colored compound (that develops over time, usually at 95°C), which absorbs light and ultimately can be measured and quantified (Ohkawa et al., 1979; Tsikas, 2017). Briefly, samples were incubated in an acid solution (pH 3.5) containing TBA, BHT (an antioxidant, added to prevent further sample peroxidation during the process, and to minimize artificial formation of MDA; Sigma-Aldrich) and a catalyzer (Iron III chloride; Sigma-Aldrich) for 1 h at RT in the dark, followed by 1 h incubation in a 95°C water-bath. Samples were then cooled to RT and butanol extraction was performed (to extract/precipitate the pink pigment produced after the reaction of TBA with MDA). Samples were vigorously vortex and centrifuged at 2773 ×g for 10 min at 4°C. Supernatant was collected and absorbance read at 532 nm (Biotek Synergy HT). Concentration of MDA was calculated based on a calibration curve using the precursor 1,1,3,3-tetramethoxypropane (TMB; Sigma-Aldrich) as the external standard (range: 0.1-83.5 µM). Results were expressed as µM of lipid peroxides formed per milligram of protein.

The total antioxidant status of the hippocampus, prefrontal cortex and serum of WKY and SHR rats was measured using the ferric reducing antioxidant power (FRAP) assay. This assay is based on a redox reaction, in which, at low pH, a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to a ferrous (F^{2+}) blue-colored form, in the presence of antioxidants, due to their action of electron donation (Benzie & Strain, 1996). For the experimental procedure, FRAP solution (the source of the F^{3+}-TPTZ complex) was added to the samples and, after 15 min of incubation at 37˚C, absorbance was measured at 593 nm (Biotek Synergy HT). For the calibration curve (range from 250

µM to 2000 µM), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), an analog of vitamin E (with antioxidant activity), was used as standard, and the total antioxidant status of each sample was defined as the concentration of Trolox equivalent antioxidant activity expressed as µM/mg of protein.

3.10 Statistical Analysis

GraphPad Prism 8 (GraphPad Software, San Diego, CA) was used to perform statistical analysis. For the assays concerning astrocytic primary cultures data were analyzed using the Kruskal-Wallis nonparametric test followed by Dunn's multiple comparison test. Regarding the animal material assays, data were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All data were expressed as the mean ± standard error of the mean (S.E.M.). Differences were considered statistically significant at $P<0.05$. The "n" represents the number of experiments obtained from independent cell cultures or animals, as specified in the respective figure legends.

CHAPTER 4
RESULTS

Chapter 4 – Results

4.1 Direct effect of methylphenidate on astrocytes

Astrocytes present heterogeneity and dynamism, which confer them the ability to play pivotal roles in both healthy and diseased brain. Different roles rely on different and specific intrinsic features of these cells. The first part of this work aimed to unravel the astrocytic response to a direct exposure to methylphenidate (MPH).

4.1.1 MPH does not induce astrogliosis

Astrogliosis consists of several changes in astrocytes' gene expression and morphology, being the upregulation of GFAP and hypertrophy of cell body and astrocytic processes the classical hallmarks of this process (Matias et al., 2019; Sofroniew & Vinters, 2010). Thus, in attempt to characterize astrocytic responses elicited by MPH, we analyzed protein levels of GFAP, which is the most widely marker of astrogliosis. Curiously, there was no alterations induced by MPH (Figure 4.1). After, astrocytic volume was also measured through the calcein fluorescence quenching method. MPH exposure during 2 and 24 h did not exert any alteration in astrocytic cell volume, at any of the three concentrations used (0.1, 0.5 or 1 mM) (Figure 4.2). The present data suggest that MPH does not lead to significant astrocytic reactivity, including alterations in cell volume.

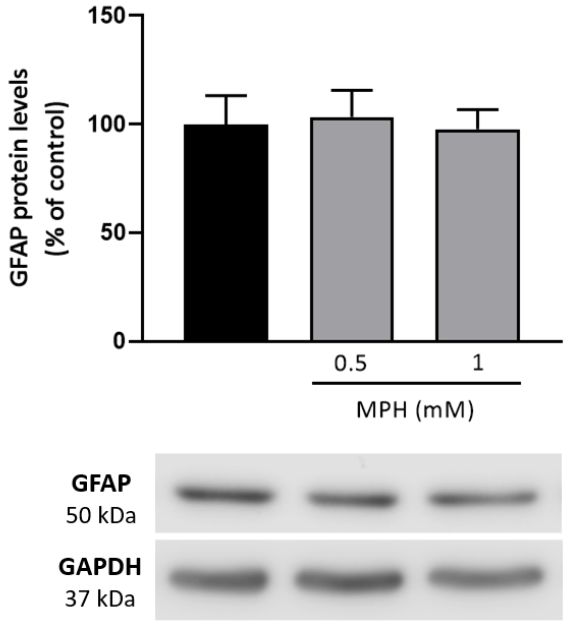

Figure 4.1. **MPH does not alter GFAP protein levels in astrocytes.** Upon a 24 h exposure to 0.5 or 1 mM MPH, protein levels of astrocytic GFAP were analyzed through western blot. MPH did not induce any alteration on GFAP expression. Under the respective bars, representative western blot images of GFAP (50 kDa) and GAPDH (37 kDa) are shown. Results are expressed as mean % of control ± S.E.M., n=5-12 from 5 independent cultures.

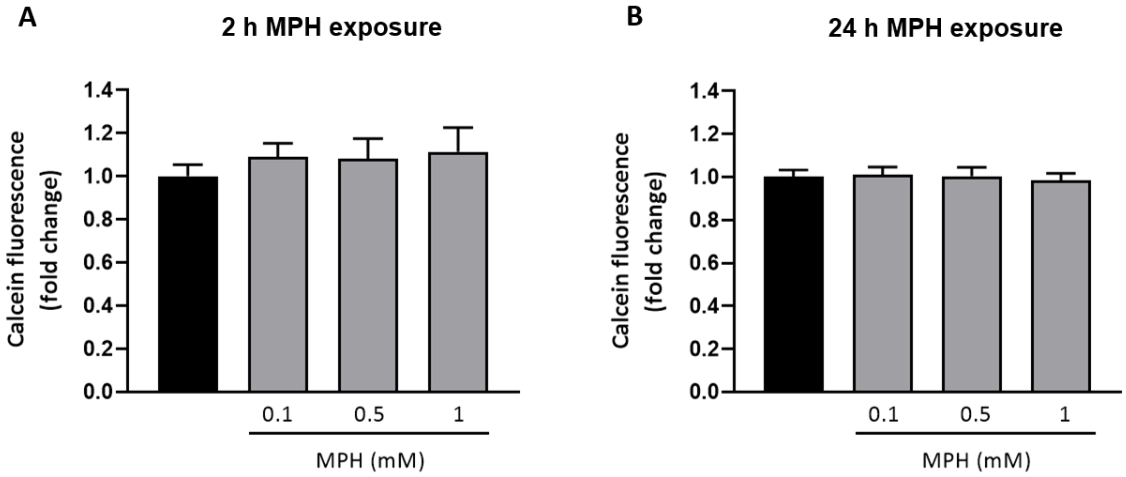

Figure 4.2. **MPH does not induce volume changes in astrocytes.** Quantification of calcein fluorescence was used to measure cell volume changes in astrocytes. Upon exposure to 0.1, 0.5 or 1 mM MPH for **(A)** 2 h and **(B)** 24 h, astrocytes did not present cell volume alteration, in comparison to control conditions (black bar). Results are expressed as mean fold change ± S.E.M. **(A)** n=5-11 from 2-4 independent cultures; **(B)** n=11-25 from 4-7 independent cultures.

4.1.2 Methylphenidate induces astrocytic cell death in a concentration-dependent manner

Despite no evident effects of MPH on astrocytic reactivity or cell volume, toxicity was further evaluated by specifically investigating cell death. Thus, astrocytic death through apoptosis was assessed after treatment with MPH for 24 h and hydrogen peroxide (250 µM H_2O_2), a reactive oxygen species known to promote cell death in astrocytes (Hamdi et al., 2011) that was used as positive control. Incubation of astrocytes with increasing concentrations of MPH (0.1, 0.5 or 1 mM) induced a significant concentration-dependent increase in the number of TUNEL positive cells as follows: 0.1 mM MPH, 1.5 ± 0.2 %; 0.5 mM MPH, 2.4 ± 0.4 %; 1 mM, 3.5 ± 0.3 % of total cells. As expected, H_2O_2 led to very prominent increase of astrocytic death (94.5 ± 0.6 %) (Figure 4.3). These results demonstrate that MPH promotes astrocytic cell death through apoptosis in a concentration-dependent manner.

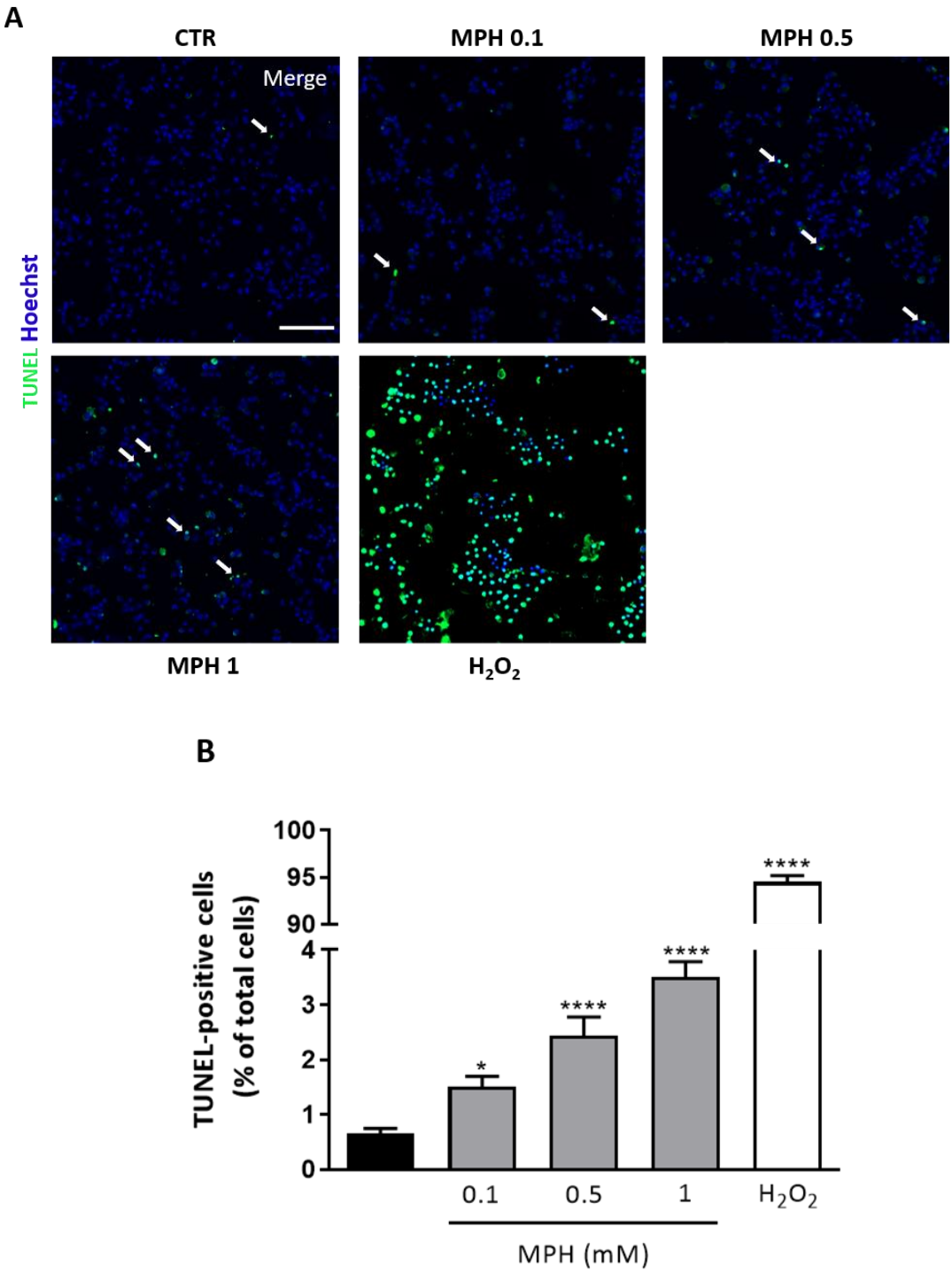

Figure 4.3. **MPH promotes astrocytic apoptosis in a concentration-dependent manner. (A)** Representative fluorescence images of MPH-induced cell death assessed through the TUNEL assay, which confers green fluorescence to the nucleus of apoptotic cells. White arrows indicate TUNEL-positive cells (green). Nuclei were stained with Hoechst (blue). **(B)** Quantification of TUNEL-positive cells after astrocytic treatment with 0.1, 0.5 or 1 mM of MPH, or 250 µM H_2O_2. All three concentrations led to a significant concentration-dependent increase in cell death. The results are expressed as the mean % of total cells ± S.E.M., n=42-83, from 4 independent cell cultures. *P<0.05, ****P<0.0001 significantly different when compared to the control (black bar), using Kruskal Wallis-test followed by Dunn's multiple comparisons test. Scale bar = 100 µm.

4.1.3 Methylphenidate promotes ROS and NO production by astrocytes

Insults may lead to a variety of cell responses that can be associated with oxidative and nitrosative stress. Thus, the intracellular production of ROS by astrocytes was measured following exposure to MPH. Astrocytic treatment with H_2O_2 (250 µM) was used as positive control. Challenging astrocytes with 0.1 or 1 mM of MPH, for 2 h, did not induce significant alterations in ROS production, in contrast to 0.5 mM MPH, which significantly increased it ($1.69\times10^5 \pm 1.25\times10^4$ a.u.), in comparison to the control. H_2O_2 also triggered a significant increase in astrocytic ROS production ($3.95\times10^5 \pm 3.91\times10^4$ a.u.) (Figure 4.4). The observed results demonstrate that MPH, at 0.5 mM, promotes intracellular ROS production by astrocytes.

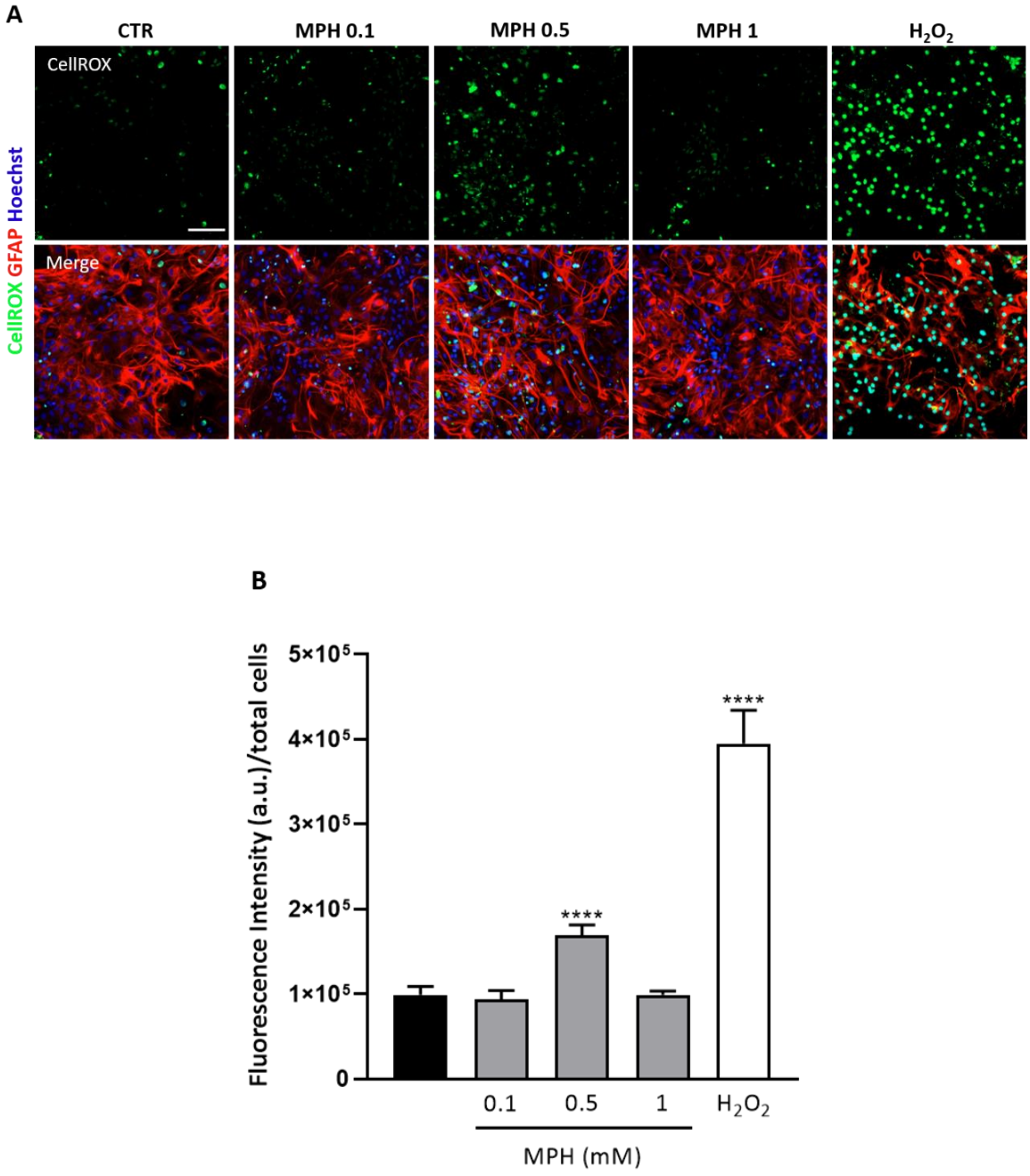

Figure 4.4. **MPH is able to induce astrocytic ROS production. (A)** Representative fluorescence images of ROS production, by MPH-treated astrocytes, through CellROX staining (green). Astrocytes were also stained with anti-GFAP (red) and nuclei with Hoechst (blue). **(B)** CellROX immunostaining analysis was performed by quantifying fluorescence intensity, after astrocytic exposure to different concentrations of MPH (0.1, 0.5 or 1 mM) or to H_2O_2. Only the concentration of 0.5 mM led to a significant increase of intracellular ROS production by astrocytes. Data are expressed as the mean of fluorescence intensity (a.u.) per total cells ± S.E.M, n=14-32, from 4-6 independent cell cultures. ****P<0.0001 significantly different from the control (black bar), using Kruskal Wallis-test followed by Dunn's multiple comparisons test. Scale bar = 100 µm; Legend: a.u., arbitrary units.

Afterwards, intracellular NO production by astrocytes exposed to MPH was also evaluated. As in the previous experiments, H_2O_2 (250 µM) was used as positive control. All three concentrations of MPH to which astrocytes were exposed to, during 2 h, led to a significant increase in the NO production (0.1 mM MPH, $1.78\times10^5 \pm 1.13\times10^4$ a.u.; 0.5 mM MPH, $1.88\times10^5 \pm 2.03\times10^4$ a.u.; 1 mM MPH, $1.79\times10^5 \pm 1.41\times10^4$ a.u.). Likewise, H_2O_2 significantly increased intracellular NO production ($3.42\times10^5 \pm 2.77\times10^4$ a.u)(Figure 4.5) These results show that MPH increases astrocytic intracellular production of NO.

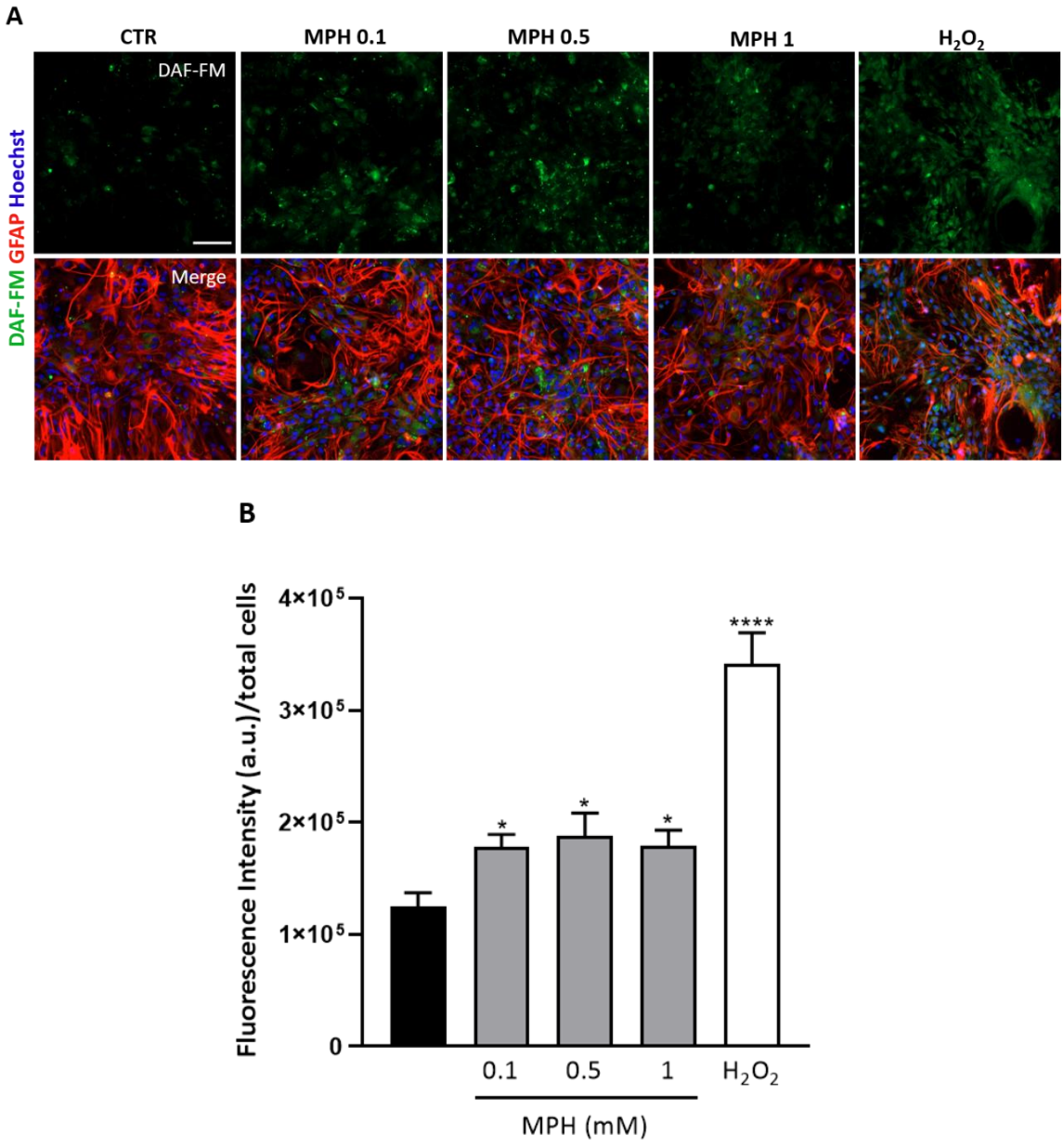

Figure 4.5. MPH promotes astrocytic production of NO. (A) Representative fluorescence images of NO production by astrocytes upon MPH exposure, assessed by DAF-FM staining (green). Astrocytes were also stained with anti-GFAP (red) and nuclei with Hoechst (blue). **(B)** Upon astrocytic exposure to 0.1, 0.5 or 1 mM MPH, or to H_2O_2, DAF-FM immunoreactivity staining was quantified through the calculation of fluorescence intensity. All three MPH concentrations similarly triggered intracellular NO production by astrocytes. Results are expressed as the mean of fluorescence intensity (a.u.) per total cells ± S.E.M., n=12-18, from 4 independent cell cultures. *P<0.05, ****P<0.0001 significantly different from the control (black bar), using Kruskal Wallis-test followed by Dunn's multiple comparisons test. Scale bar = 100 µm; Legend: a.u., arbitrary units.

Then, a specific enzyme of nitric oxide metabolism, iNOS, was also investigated, and it was possible to conclude that 1 mM MPH (24 h of exposure) triggered a significant increase of iNOS protein levels by astrocytes (325.5 ± 76.8 % of control) (Figure 4.6). Despite not being statistically significant, 0.5 mM MPH showed a tendency to also upregulate iNOS (P=0.16).

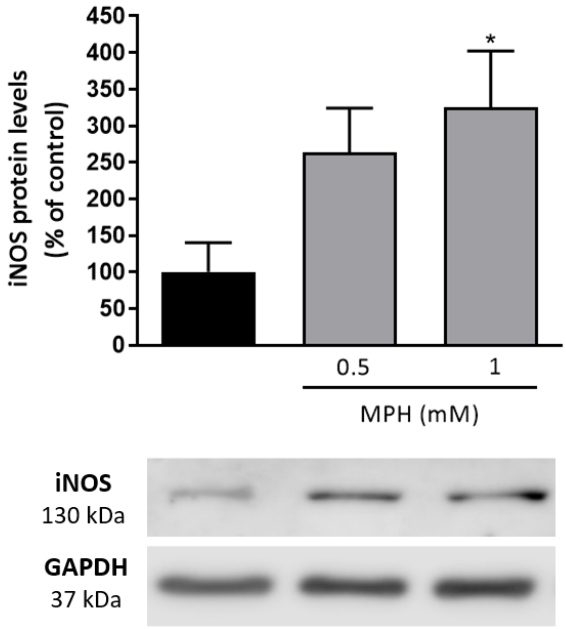

Figure 4.6. **MPH increases astrocytic iNOS protein levels.** Upon a 24 h exposure to 0.5 or 1 mM MPH, protein levels of astrocytic iNOS were analyzed through western blot. MPH significantly increased iNOS protein levels at 1 mM. Under the respective bars, representative western blot images of iNOS (130 kDa) and GAPDH (37 kDa) are shown. Data are shown as mean % of control ± S.E.M., n=3-6, from 3 independent cultures. *P<0.05 significantly different when comparing to the control (black bar), using Kruskal Wallis-test followed by Dunn's multiple comparisons test.

4.1.4 MPH activates the NF-κB signaling pathway

The nuclear factor kappa B (NF-κB) pathway, which regulates many cellular processes including proliferation, apoptosis, and survival, has emerged as an important therapeutic target. Activation of the NF-κB transcription factor is associated with nuclear translocation of the p65 component of the complex (Giridharan & Srinivasan, 2018), and can initiate ROS and reactive nitrogen species (RNS) production by astrocytes (Rizor et al., 2019). So, in order to investigate the NF-κB signaling pathway activation in astrocytes upon MPH exposure, the translocation of the NF-κB p65 subunit to the nucleus was analyzed. The pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) was used as positive control (10 ng/mL for 2 h; 64.8 ± 7.2 % of total cells). MPH (0.5 mM) significantly increased the number of nuclear NF-κB p65-positive cells following 30 min and 2 h of astrocytic treatment, in a very similar way (30 min, 1.1 ± 0.3 %; 2h, 1.1 ± 0.3 %; Figure 4.7). Summing up, data show that MPH is able to promote nuclear translocation of NF-kB p65 subunit in astrocytes, and therefore to affect the activity of this signaling pathway.

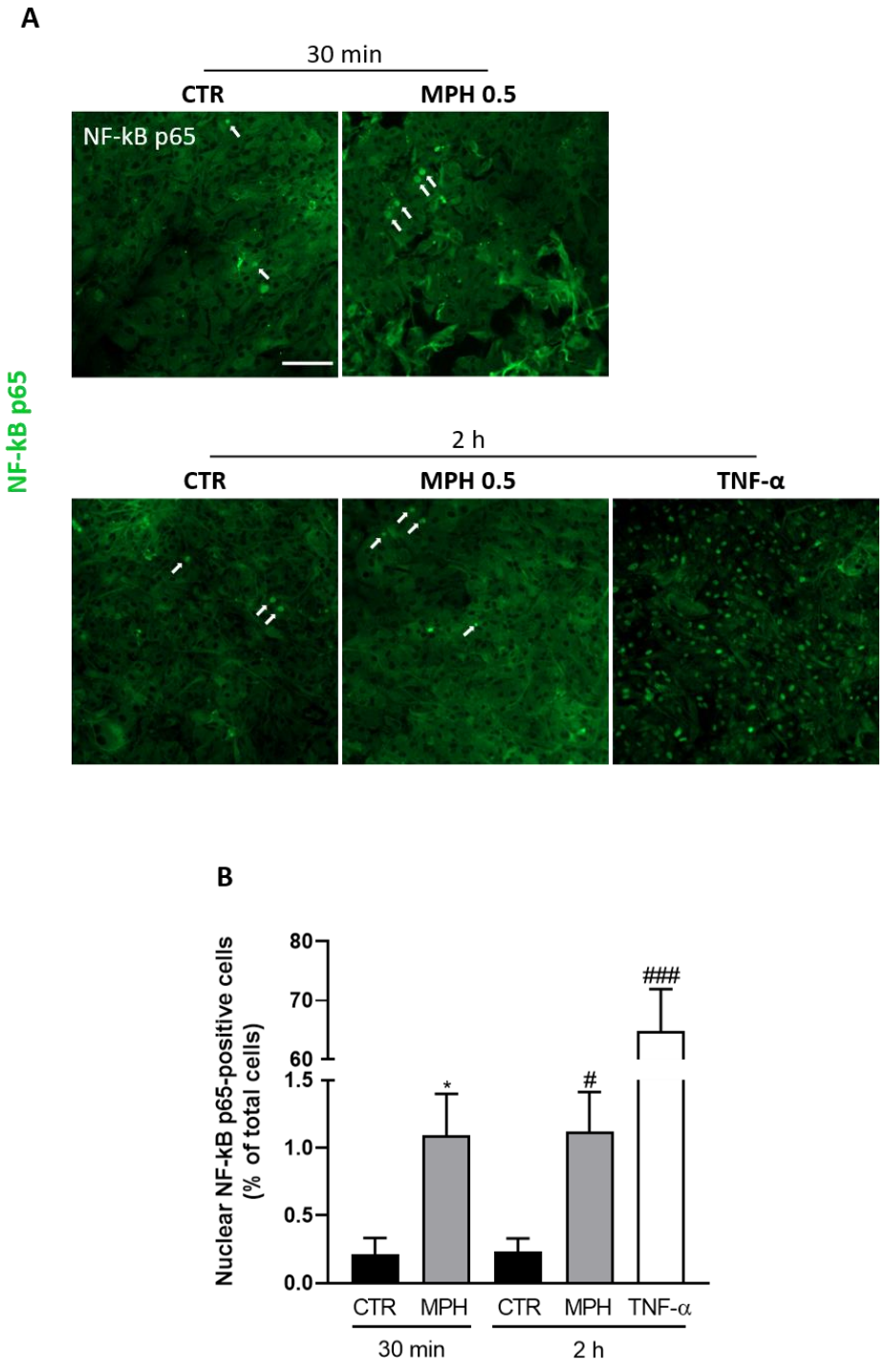

Figure 4.7. **MPH promotes the activation of NF-κB pathway in astrocytes. (A)** Representative fluorescence images of NF-κB p65 localization within astrocytes detected by immunofluorescence. Green fluorescence dots indicate the presence of the p65 subunit in the nucleus, which under resting conditions remains in the cytoplasm, translocating into the nucleus only in response to some stimulus. White arrows indicate nuclear NF-kB p65-positive cells. **(B)** MPH (0.5 mM) induced a significant increase in nuclear NF-κB p65-positive cells at both timepoints (30 min and 2 h). Results are expressed as mean % of total cells ± S.E.M, n=5-12, from 2 independent cultures. *P<0.05 significantly different comparing to 30 min control (CTR – 30 min), $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 significantly different comparing to 2 h control (CTR – 2h), using Kruskal Wallis-test followed by Dunn's multiple comparisons test. Scale bar = 100 µm.

4.1.5 MPH effect on astrocytic GDNF release

Astrocytes are an important source of neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF), known for its neuroprotective functions (Tanabe et al., 2009). Thereby, the release of GDNF by astrocytes upon MPH treatment was measured. After challenging astrocytes with different concentrations of MPH for 24 and 48 h, no statistically significant alterations were observed, despite a tendency to increase with 1 mM of MPH after 24 h (P=0.13) or both 0.5 and 1 mM after 48 h (P=0.24 and P=0.09, respectively). More experiments are needed to confirm this tendency (Figure 4.8). At this point, our results suggest that MPH may promote the release of GDNF by astrocytes.

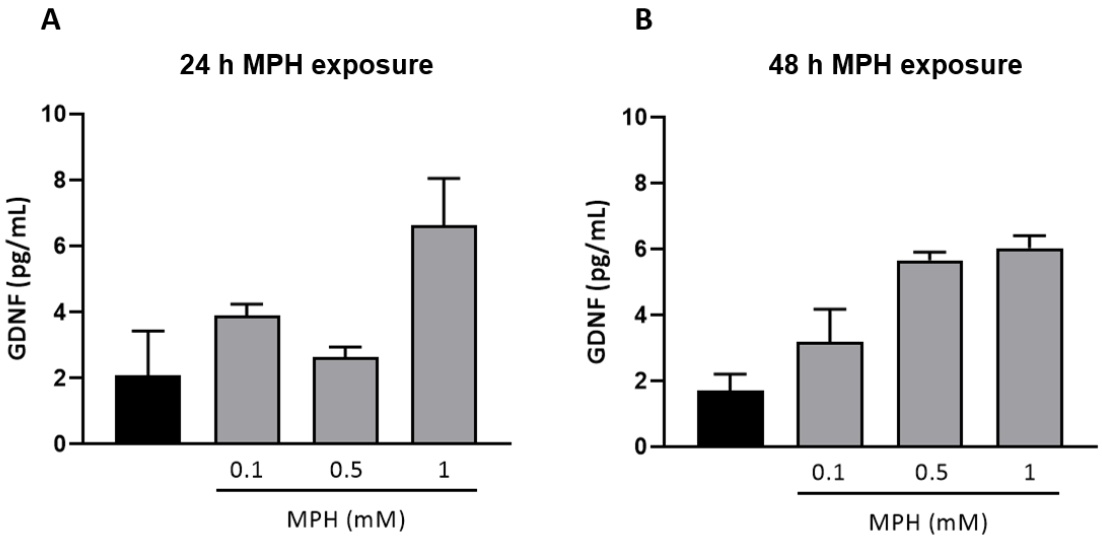

Figure 4.8. **MPH does not induce significant alterations in astrocytic GDNF release.** After treating astrocytes with 0.1, 0.5 or 1 mM of MPH, the levels of GDNF, present in the cell culture media, were measured through ELISA after **(A)** 24 h and **(B)** and 48 h of MPH exposure. None of the conditions resulted in MPH-induced significant alterations of GDNF levels. Results are expressed as mean GDNF levels (pg/mL) ± S.E.M. **(A)** n=2-3 from 2-4 independent cultures; **(B)** n=2 from 2 independent cultures.

4.2 Effect of methylphenidate administration in control *versus* ADHD animal model: focus on oxidative and nitrosative stress

In the second part of the work, some effects of chronic exposure to a clinically relevant dose of MPH were investigated in an animal model of ADHD (Spontaneously Hypertensive Rats, SHR), comparing to controls (Wistar Kyoto, WKY) to simulate MPH therapeutic and non-therapeutic use, respectively. We started by evaluating possible alterations in GFAP and iNOS protein levels in the hippocampus, a brain region intrinsically related to drug consumption and poorly studied in this field. This could give information about possible astrocytic responses and nitrosative stress induced by chronic MPH consumption.

Protein levels of GFAP and iNOS were evaluated through western blot analysis, in the hippocampus of WKY and SHR rats chronically administrated with MPH. Between the two rat strains, under basal conditions (Veh), no statistically differences were observed, despite a tendency to an increase of GFAP and iNOS levels in ADHD model (Figure 4.9). Moreover, chronic MPH significantly increased both GFAP and iNOS protein levels in WKY rats. In SHR animals (ADHD model), despite no statistical differences observed, there was a tendency (P=0.09) to a decrease of GFAP protein levels, comparing to the respective control. Comparing only the effect of MPH between strains, treated ADHD animals present significantly lower GFAP and iNOS levels comparing to treated control rats suggesting a different effect of MPH on both strains.

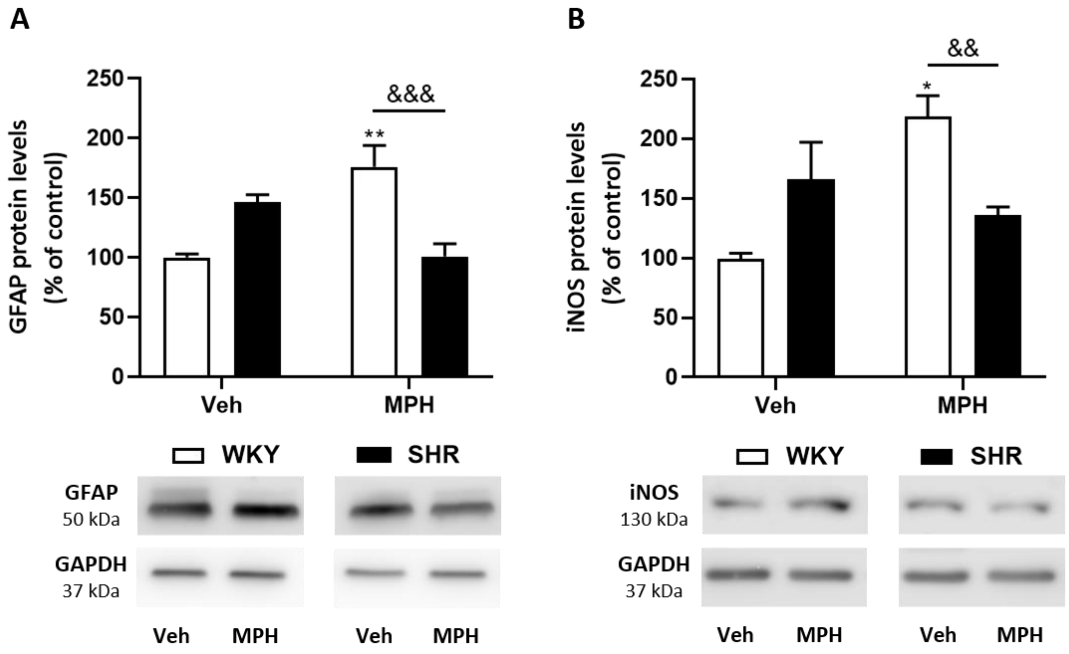

Figure 4.9. **Chronic treatment with MPH leads to a significant increase of GFAP and iNOS protein levels in the hippocampus of control animals. (A)** GFAP and **(B)** iNOS protein levels in the hippocampus of WKY and SHR rats, under basal or MPH treatment conditions, were measured through western blot analysis. Chronic MPH induced a significant increase of GFAP and iNOS protein levels in WKY rats. Also, under MPH-treatment conditions, it is observed a significant difference between control and ADHD animals regarding both protein levels, which are significantly lower in treated ADHD rats. Data are expressed as the mean % of control ± S.E.M., **(A)** n=6-10, from 5-6 animals per group; **(B)** n=6-13, from 5-6 animals per group.*P < 0.05, **P < 0.01 significantly different from the respective vehicle (Veh)-treated strain; $^{\&\&}$P < 0.01, $^{\&\&\&}$P < 0.001 significant difference between WKY and SHR within the same treatment, using two-way ANOVA followed by Tukey's test. Below the respective bars, representative western blot images of GFAP (50 kDa), iNOS (130 kDa), and GAPDH (37 kDa) are presented.

Astrogliosis, here detectable by evaluation of GFAP protein levels, may have both protective and negative outcomes, the latter being associated with oxidative and nitrosative stress and an overall neuroinflammation. Once different alterations in GFAP and iNOS levels were observed dependent on the animal strain and treatment, possibly indicating different responses regarding astrogliosis and nitrosative stress, to better clarify if chronic MPH administration leads to oxidative stress, analysis was performed not only in the hippocampus, but also in the prefrontal cortex and serum of both control and ADHD animals.

Thus, lipid peroxidation was measured through the assessment of its products, via malondialdehyde (MDA), in the hippocampus, prefrontal cortex and serum of WKY and SHR rats upon chronic exposure to MPH or vehicle. Under basal conditions (Veh), no significant differences were observed between rat strains neither in brain regions nor

in serum. Curiously, MPH treatment in ADHD animals both in hippocampus and PFC showed a tendency to decrease MDA levels that reached static significance in the PFC. Nevertheless, the treatment with MPH had a different impact in both control (WKY) and ADHD (SHR) animals, being the levels of lipid peroxidation (MDA) significantly higher in control rats. In serum, no differences were observed (Figure 4.10).

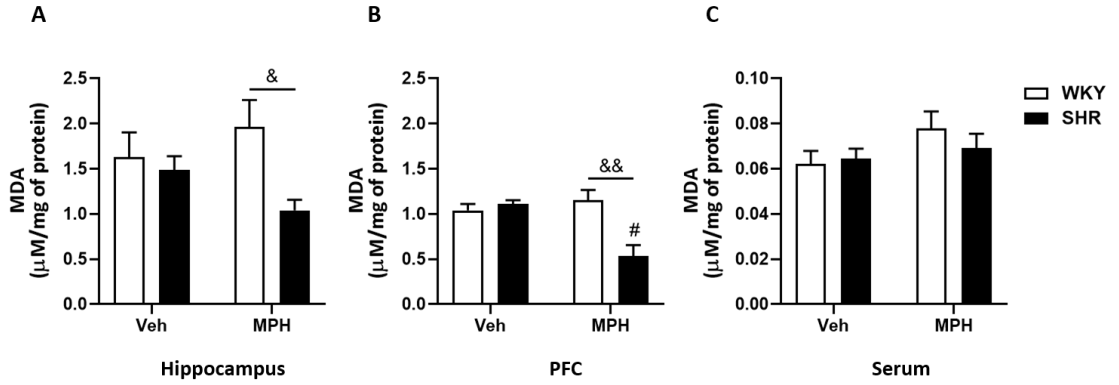

Figure 4.10. **Chronic administration of MPH leads to different MDA levels between WKY and SHR rats.** Malondialdehyde (MDA) levels in the **(A)** hippocampus, **(B)** prefrontal cortex and **(C)** serum of WKY and SHR rats upon chronic MPH treatment or vehicle (Veh) was measured. In the PFC, SHR treated rats demonstrate a significant decrease in MDA levels. A comparison between both rat strains under MPH treatment reveals that MDA levels are significantly different in the hippocampus and PFC, being higher in WKY. Regarding rats' serum, no significant changes are observed. Also, under basal conditions, no differences are observed between both strains. Results are expressed as mean µM/mg of protein ± S.E.M., **(A)** n=3-5, **(B)** n=3-5, **(C)** n=5-6, from 5-6 animals per group. $^{\#}P<0.05$ significantly different from the respective vehicle (Veh)-treated strain; $^{\&}P<0.05$, $^{\&\&}P < 0.01$ significant difference between WKY and SHR within the same treatment, using two-way ANOVA followed by Tukey's test.

The total antioxidant status (TAS) was further investigated under the same conditions. It was concluded that the hippocampus of ADHD animal model presents a basal lower antioxidant capacity comparing to control animals. Additionally, MPH treatment decreased TAS levels in control rats. Under ADHD conditions, even though no statistical differences were observed, there was a tendency (P=0.13) for the increase of TAS levels under MPH treatment. These results suggest that the antioxidant capacity of hippocampus, under control conditions, is impaired by chronic MPH exposure, in contrast to the PFC and serum, in which no alterations were observed (Figure 4.11).

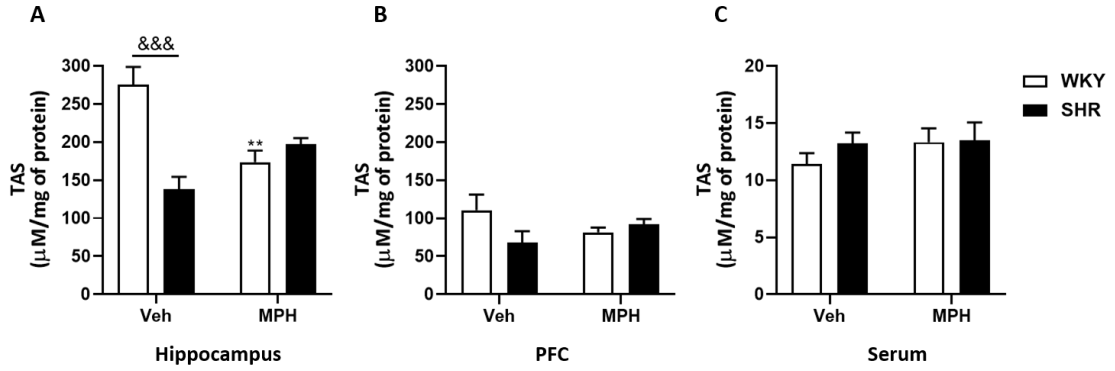

Figure 4.11. **Chronic exposure to MPH decreases total antioxidant status in the hippocampus of WKY rats.** Antioxidant capacity of the **(A)** hippocampus, **(B)** PFC and **(C)** serum of WKY and SHR rats was evaluated following chronic exposure to MPH or vehicle (Veh). Regarding the PFC and serum, no significant differences were observed. However, the hippocampus showed to be differently affected depending on the condition. Different rat strains (WKY and SHR) present, in this brain region and under basal conditions, significantly different levels of antioxidant capacity that is lower in ADHD animals (SHR). Also, MPH significantly decreases the total antioxidant status in control (WKY) rats. Results are expressed as mean µM/mg of protein ± S.E.M, **(A)** n=4-6, **(B)** n=2.5, **(C)** n=6, from 4-6 animals per group. **P<0.01 significantly different from the respective vehicle (Veh)-treated strain, $^{\&\&\&}$P<0.001 significant difference between WKY and SHR within the same treatment, using two-way ANOVA followed by Tukey's test.

CHAPTER 5
DISCUSSION

Chapter 5 – Discussion

Methylphenidate is the first-line pharmacological treatment for ADHD, which is highly prevalent among children and can also persist into adulthood (Ebenezer, 2015; Faraone, 2018). ADHD diagnosis is rapidly increasing, along with MPH prescription. Besides the therapeutic use, MPH is also misused, mostly to reach an overall enhanced performance, to lose weight, and as a recreational drug to achieve pleasant sensations, which is nowadays a critical problem particularly among adolescents and young adults (Freese et al., 2012; Pereira et al., 2018). Despite the importance of this topic and the intense debate and controversy around it, not much is known about the chronic and long-term effects of MPH treatment in the brain, as well as the impact of its non-medical use. Particularly, little is known about how MPH use affects glial cells, such as astrocytes, which play crucial roles on both physiologic and pathological CNS conditions. Thereby, the present work aimed not only to characterize the direct effects of MPH on astrocytes, but also to explore some effects of chronic exposure to MPH in control and ADHD conditions, thus simulating non-therapeutic and therapeutic use, respectively.

In response to some stimulus or alteration on the surrounding environment, astrocytes, as dynamic and adjustable cells, can undergo several changes typically identified as astrogliosis. This response of astrocytes may at first have protective functions but, when prolonged, it leads to negative effects usually associated with neurodegeneration and neuroinflammation. The upregulation of GFAP expression (Oberheim et al., 2012; Wang & Bordey, 2008), a classic marker for the identification of astrocytes, and the hypertrophy of cell body are hallmarks of this reactive state. The present results revealed that MPH does not induce astrogliosis since GFAP protein levels did not alter upon direct exposure to the drug. In contrast to this result, an *in vitro* study by Suzuki et al. (2007) demonstrated that 0.01 and 0.1 mM of MPH induced astrocytic activation, which was found to be reversible. They also exposed cells to methamphetamine (METH), a more powerful psychostimulant than MPH, and observed a more pronounced and long-lasting astrogliosis than that induced by MPH. These results were observed after both 1 and 3 days of drug incubation. The differences observed can be explained by the fact that the authors used limbic neuron/glia co-cultures from mice and a different experimental approach, which consisted in the measurement of the intensity of GFAP-like immunoreactivity. Interestingly, our animal studies indicate evidence of astrogliosis upon MPH treatment, as it will be depicted later, in accordance with Coelho-Santos et al. (2018) that demonstrated astrocytic hypertrophy in control animals treated with MPH.

Astrocytic cells are known to support the maintenance of brain ionic homeostasis, by sensing and adapting to the ionic extracellular environment, which can be observed by volume changes (Barres, 2008; Matias et al., 2019). Herein, it was demonstrated that MPH does not alter astrocytic volume, suggesting that this psychostimulant does not directly induce astrocytes' morphologic changes, which is consistent with the previous results demonstrating absence of astrocytic reactivity. Leitão and colleagues (2018) showed that METH triggers astrocytic swelling, and also that AQP4, mainly expressed by astrocytes (Lafrenaye & Simard, 2019), plays a crucial role in the process. Therefore, it may be suggested that MPH, when compared to other psychostimulants such as METH, does not represent a severe stimulus to astrocytes or that a pronounced response of astrocytes to MPH may be dependent on other neural cells being mainly due to an indirect effect.

Despite no significant astrogliosis triggered by MPH, once the present work is the first to explore the direct effect of MPH on astrocytes, we found it relevant to investigate in more detail other possible consequences. Looking specifically to cell death upon drug exposure, it was demonstrated that this psychostimulant induces astrocytic cell death through apoptosis in a concentration-dependent manner and, even though the differences were significant, they were low, comparing to positive control, indicating a relative low toxicity of MPH. Indeed, the used range of concentrations had already been employed in other studies where different concentrations were tested from clinically relevant to extreme concentrations (Gopal et al., 2007; Ludolph et al., 2006; Suzuki et al., 2007), so a big increase in cell death was not expected. In fact, Ludolph et al. (2006) tested the toxicity of MPH in a well-established cell line (human embryonic kidney cells – HEK-293) and in primary cultures of mesencephalic neurons, under the same range of concentrations, for 24 and 72 h, and did not found any cytotoxic potential of MPH. This conclusion was based on viability measurement and morphologic analysis, which revealed no MPH-induced effects. Nonetheless, to fully characterize astrocytic death, cell death through necrosis and necroptosis need to be also dissected.

Astrocytes are an important source of ROS and RNS (Chen et al., 2020), being involved in oxidative and nitrosative stress responses. Thus, keeping this in mind and to further characterize the prompt astrocytic response to MPH, it was demonstrated that MPH promotes both ROS and NO intracellular production by astrocytes. Curiously, an increase in ROS was observed only with 0.5 mM MPH. Lau et al. (2000), using astrocytic cultures from the cortex, striatum and mesencephalon of mice treated with 4 mM METH for 4 to 48 h, demonstrated an increase in ROS production, a decrease in ATP levels and mitochondrial depolarization mainly in striatal astrocytes, showing that astrocytes from

different brain regions are differently affected by METH treatment. Also, the authors verified a correspondence between the decrease in mitochondrial membrane potential and the increase in ROS formation in all three regions. Even though METH is a more severe drug than MPH, this work demonstrates that mitochondria are a possible source of ROS in response to psychostimulants. Indeed, unstable mitochondrial membrane potential, resulting from diverse insults, has been described to lead to augmented ROS (Zorov et al., 2006), and there is evidence that mitochondria are distributed not only in the astrocytic cell body, but also in its processes, consistent to an important function of mitochondrial metabolism in the astrocytic oxidative system (Chen et al., 2020). Additionally, it is known that astrocytes express the enzyme NOX (mainly NOX2 and NOX4) (Chen et al., 2020; Zhu et al., 2009). In fact, in another study, using a human fetal astrocytic cell line (SVGA) and primary human fetal astrocytes (HFAs), it was showed that METH (500 μM, 24 h) induced astrocytic apoptosis by inducing oxidative stress through the NOX pathway (Shah et al., 2013). Based on these findings, an interesting approach would be to depict the source(s) of ROS production upon MPH exposure. For instance, mitochondrial membrane potential could be analyzed to detect possible alterations, mitochondrial-specific ROS could be suppressed, and a NOX specific inhibitor could be used as performed previously by Coelho-Santos et al. (2016) in brain endothelial cells to perceive which is the origin of MPH-induced ROS. In agreement with the increased levels of NO, MPH also increased the protein levels of iNOS, suggesting that this enzyme may be involved in the synthesis of NO triggered by MPH. Furthermore, the present work demonstrated that MPH promoted the nuclear translocation of the NF-κB p65 subunit, suggesting that MPH may also modulate the activity of the NF-κB signaling pathway. The activation of this pro-inflammatory transcription factor can contribute for ROS and RNS production by astrocytes and an overall inflammatory response, typical of glial cells that are main sources of inflammatory mediators (Rizor et al., 2019). Thus, the observed astrocytic oxidative and nitrosative stress may be related to the activation of this pathway; yet further experiments are necessary to dissect this hypothesis.

Astrocytes also represent an important source of neurotrophic factors in the CNS, whose basal levels are low under normal and healthy conditions but may increase under stressful conditions. GDNF, one of these factors, is known for its neuroprotective and therapeutic potential (Tanabe et al., 2009). The present results revealed that, even though not significant, there is a tendency for the increase of release levels of GDNF by astrocytes upon MPH treatment. There is evidence of astrocytic GDNF-release in

response to brain insults (Yamagata et al., 2007), thus this may be an astrocytic protective defense against the MPH-induced oxidative and nitrosative stress.

In sum, MPH induces a response in astrocytes particularly related with ROS and NO production that can be somehow involved in the observed cell death. Nevertheless, these effects are tenuous comparing to other psychostimulants and can be considered of low severity. Given the therapeutic context in which MPH is used, these are valuable findings, since they may account for the classification of its safety. Despite these interesting observations, this *in vitro* study included a unique exposure to MPH and was done under control conditions, representing only MPH misuse conditions. It would be important not only to investigate other possible responses of these cells such as calcium signals, which have been described as crucial to intracellular processes and intercellular communication (Shigetomi et al., 2019), but also the underlying mechanisms through which MPH induces the described effects in astrocytes. The classic and more established mechanism of action of this drug consists in the interaction and blocking of the activity of DAT and NET (Faraone, 2018). Even though it was not explored in the present work, there is evidence that cultured astrocytes express functionally NET (Inazu et al., 2003), but regarding DAT there are still no consistent data (Jennings et al., 2016). Also, MAO and VMAT-2, which are involved in other MPH mechanisms of action (inhibition of MAO and redistribution of VMAT-2 containing vesicles, respectively), are present in astrocytes (Inazu et al., 2003; Petrelli et al., 2018), suggesting that MPH may have different possible targets in astrocytic cells. Interestingly, a study on pheochromocytoma cells (PC12) cells, that do not express DAT but express NET (Inazu et al., 2003), demonstrated a diversity of MPH-induced effects, which were independent of DAT inhibition, including alterations in catecholamines and synaptic genes' expression levels and enhanced cell proliferation (Bartl et al., 2010; Grünblatt et al., 2013).

In vitro studies are important to dissect the cellular and molecular mechanisms triggered by several insults, including drugs, but they also present several limitations that can be overcome, at least in part, with animal models. Thus, in the present study WKY and SHR rats were used to investigate the impact of chronic treatment with MPH, simulating a misuse and therapeutic use, respectively. Animals received a MPH dose that ensures therapeutically relevant plasma concentrations found in ADHD patients, with a duration that mimics chronic use in humans and that occurred in a period of time equivalent to late-childhood through late-adolescence/early adulthood in humans (Coelho-Santos et al., 2018 and 2019; Schmitz et al., 2017; Somkuwar et al., 2013). Some effects of chronic exposure to MPH were explored, with focus on oxidative and nitrosative stress. Here, it was revealed that, in the hippocampus, under basal

conditions, GFAP and iNOS levels tend to be higher in SHR than in WKY rats, suggesting that this ADHD model presents a basal inflammatory status, which appears to be reversed by MPH. Also, MPH induced astrogliosis under control conditions (in WKY rats), identified by the increased GFAP protein levels. This contrasts to what was found in the *in vitro* study previously described, probably by the fact that in the brain environment all cells and circuits contribute to the outcomes of MPH treatment. Curiously, Coelho-Santos and colleagues (2019) reported no alterations of hippocampal GFAP levels in WKY rats, but their treatment protocol (orally treatment with 1.5 mg/kg/day MPH for 4 weeks) included only week days (Monday to Friday), whereas ours was continuous without interruptions. Nevertheless, the same group demonstrated similar results as in the present work, but in the PFC (orally treatment with 1.5 mg/kg/day MPH for 4 weeks) (Coelho-Santos et al., 2018). In the cerebellum, GFAP expression was also seen to be increased in untreated ADHD rats, and decreased upon MPH treatment (orally treatment with 1 mg/kg/day MPH for 28 consecutive days) (Yun et al., 2014). Additionally, Bahcelioglu et al. (2009) demonstrated an increased number of GFAP-positive cells with increased reactivity in non-ADHD rats' cortical region (orally treatment with 5, 10 and 20 mg/kg MPH for 3 months, 5 days per week). Astrocytic activation in rat striatum and PFC after MPH treatment (intraperitoneally treatment with 2 mg/kg/day MPH for 14 days) was also reported by Cavaliere et al. (2012), in non-ADHD conditions. Also in the hippocampus, MPH was revealed to increase iNOS protein levels in non-ADHD conditions, which suggests that MPH treatment induces NO production and consequent nitrosative stress under healthy conditions, in accordance with what was found in astrocytes reactivity. The cell source of iNOS was not explored in the present study and it would be also interesting to unravel the specific involvement of hippocampal astrocytes on such increment.

Additionally, present data demonstrated no differences in lipid peroxidation levels between rat strains under basal conditions, neither in brain regions nor in serum. MPH chronic treatment leads to significantly higher levels of MDA in the hippocampus and PFC of WKY rats, comparing to SHR rats. Regarding the impact of MPH in ADHD animals, in the PFC there was a decrease of MDA levels comparing to vehicle-treated SHR animals, showing the same tendency in the hippocampus, although not statically different, suggesting a protective function of MPH against oxidative stress under ADHD conditions. Coelho-Santos et al. (2018) demonstrated that chronic treatment with MPH upregulates ROS and MDA levels in the PFC and serum of WKY rats, and downregulates them in ADHD animals. However, in contrast to the present work, in which no alterations

were observed in serum, this group showed a correspondence between brain and serum alterations. Motaghinejad et al. (2016) also demonstrated that MPH chronic treatment (intraperitoneally treatment with 2, 5, 10 and 20 mg/kg MPH for 21 days) induced oxidative stress in the hippocampus of Wistar rats in a dose-dependent manner. Regarding antioxidant capacity, basal hippocampal TAS levels in SHR rats are reduced when compared to WKY, indicating that ADHD may be related to a decreased antioxidant capacity. Curiously, MPH had a negative impact in WKY rats since it decreased the levels of TAS to levels similar to those observed in the ADHD model. Under ADHD conditions, MPH tends to increase TAS levels. Coelho-Santos et al. (2019) presented evidence that MPH treatment (orally treatment with 5 mg/kg/day MPH for 4 weeks) decreased TAS levels in the hippocampus of WKY rats, in accordance with the present results. Concerning PFC and serum, no alterations were observed.

Overall, the results obtained with animal studies, particularly in the hippocampus support the idea that MPH non-medical use induces oxidative and nitrosative stress, as well as astrogliosis, and also a decrease in the antioxidant capacity. In opposition, MPH therapeutic use decreases oxidative and nitrosative stress along with astrogliosis and increases antioxidant capacity. Therefore, there is strong evidence of the injurious outcomes of a non-medical consume of MPH and the benefits and ameliorative effects of MPH consumption under ADHD condition, in the brain, accordingly to what has been described in the literature. This direct comparison between the two types of MPH consumptions, representing both healthy and ADHD conditions, is extremely relevant, since most of the studies focus on only one of the conditions (usually control, non-ADHD conditions). Additionally, the results from animal studies include the whole system with the involvement of a variety of different cells that may be differently contributing for the observed effects.

In a transversal interpretation from *in vitro* to animal studies, it can be hypothesized that astrocytes change into a reactive state upon MPH exposure through the involvement of other(s) cell type(s). Also, the increased levels of iNOS in astrocytes can contribute to the observed nitrosative stress in the hippocampus. Moreover, astrocytes are known to present a more prominent antioxidant activity than neurons, containing a large amount of antioxidants, like glutathione and superoxide dismutase (Adibhatla & Hatcher, 2010; Chen et al., 2020). Thus, this antioxidant capacity may be compromised by MPH, which could contribute to the observed decreased total antioxidant status in the brain. Additional investigation of the impact of MPH on other

astrocytic features, such as astrocytic genes involved in the control of cellular redox state, would be of major importance.

CHAPTER 6
CONCLUSIONS AND FUTURE PERSPECTIVES

Chapter 6 – Conclusions and Future Perspectives

This study investigated not only the direct effects of MPH on astrocytes, but also some effects of MPH chronic treatment in both physiological and ADHD conditions, simulating misuse and therapeutic use, respectively.

Direct exposure of astrocytes to MPH did not induce astrogliosis, including cell volume alterations. Nevertheless MPH did promote an astrocytic response as follows: apoptotic cell death, increased ROS and NO intracellular production along with increased iNOS protein levels, activation of the NF-kB signaling pathway and a tendency for increased GDNF release levels (Figure 6.1).

MPH chronic use under healthy conditions, *i.e.* MPH non-medical use, was revealed to induce harmful outcomes, in opposition to MPH therapeutic use in ADHD conditions, which exhibited beneficial and ameliorative effects. These effects focused on oxidative and nitrosative stress, astrocytic reactivity and antioxidant capacity, and were especially notorious in the hippocampus (Figure 6.2). The direct comparison between the two types of MPH consumption is an unusual approach, and so this study results provides accurate data on MPH impact depending on baseline conditions.

This work discloses for the first time some of the MPH-induced effects in astrocytes, along with interesting data on the two types of MPH consumption, overall contributing to the improvement of the knowledge about the impact of MPH in the brain and also to the classification of MPH safety.

Future studies are still necessary to deeply characterize the effect of MPH on astrocytes. Many other interesting astrocytic features can be explored, and of particular interest is the study of the effects of MPH on the crosstalk between astrocytes and other cells, including microglia. Given the problematic around increased MPH misuse and ADHD diagnosis, along with major gaps on this research field, the path must be lead in direction to the full characterization of MPH-induced responses in the brain of both healthy individuals and ADHD patients.

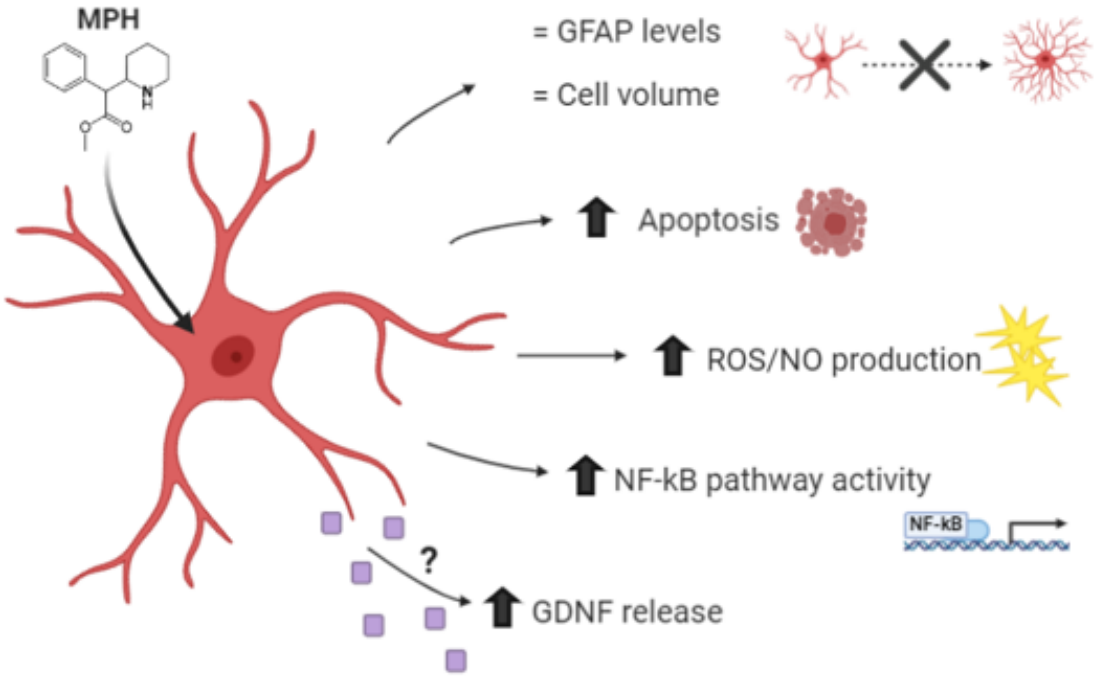

Figure 6.1. **MPH-induced alterations in astrocytes.** This image is representative of the astrocytic response induced by MPH that comprises increased cell death through apoptosis, increased intracellular production of both ROS and NO (consistent with the observed increase of iNOS protein levels – not shown), increased activity of the NF-kB signaling pathway and a tendency for increased release of GDNF. Overall, MPH induced an astrocytic response of moderate severity, since astrogliosis was not detected, but yet an oxidative and nitrosative response was identified. Legend: MPH, Methylphenidate; GFAP, Glial Fibrillary Acidic Protein; ROS, Reactive Oxygen Species; NO, Nitric Oxide; NF-kB, Nuclear Factor kappa B; GDNF, Glial Cell Line-Derived Neurotrophic Factor.

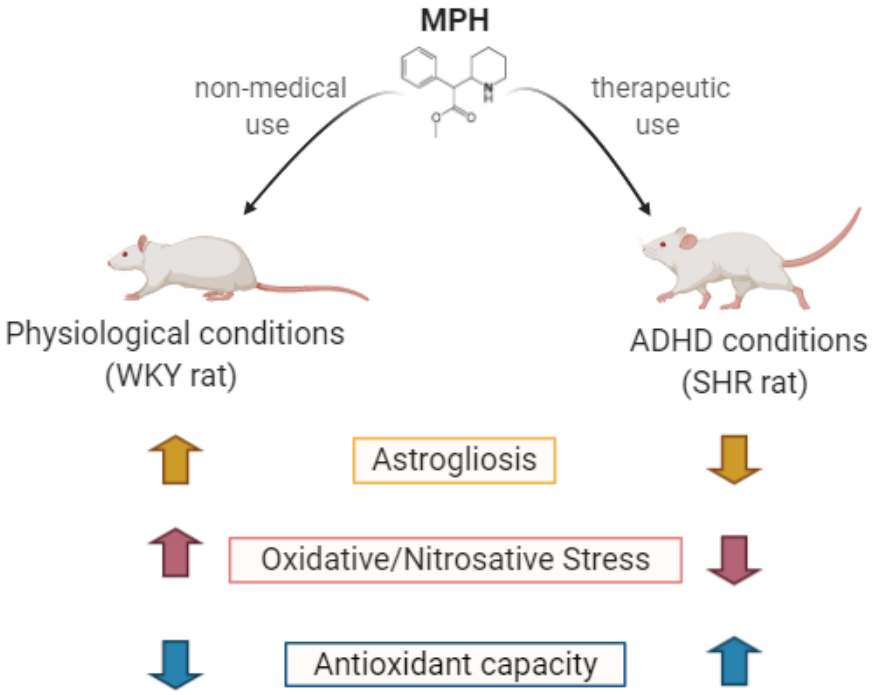

Figure 6.2. **Overall outcomes of non-medical and therapeutic use of MPH.** Taking together the observations from the animal studies, the overall and generalized outcomes of non-medical use and therapeutic use of MPH are opposite: the non-therapeutic use of MPH, under physiological conditions, promoted astrogliosis and oxidative/nitrosative stress, and decreased antioxidant capacity; and the therapeutic use of MPH, under ADHD conditions, decreased basal astrogliosis and oxidative/nitrosative stress and increased antioxidant capacity. In sum, non-medical use of MPH was demonstrated to be injurious, whereas its therapeutic use was showed to be beneficial, in accordance with what has been described in the literature. Legend: ADHD, Attention Deficit Hyperactivity Disorder; MPH, Methylphenidate; WKY, Wistar-Kyoto; SHR, Spontaneously Hypertensive.

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