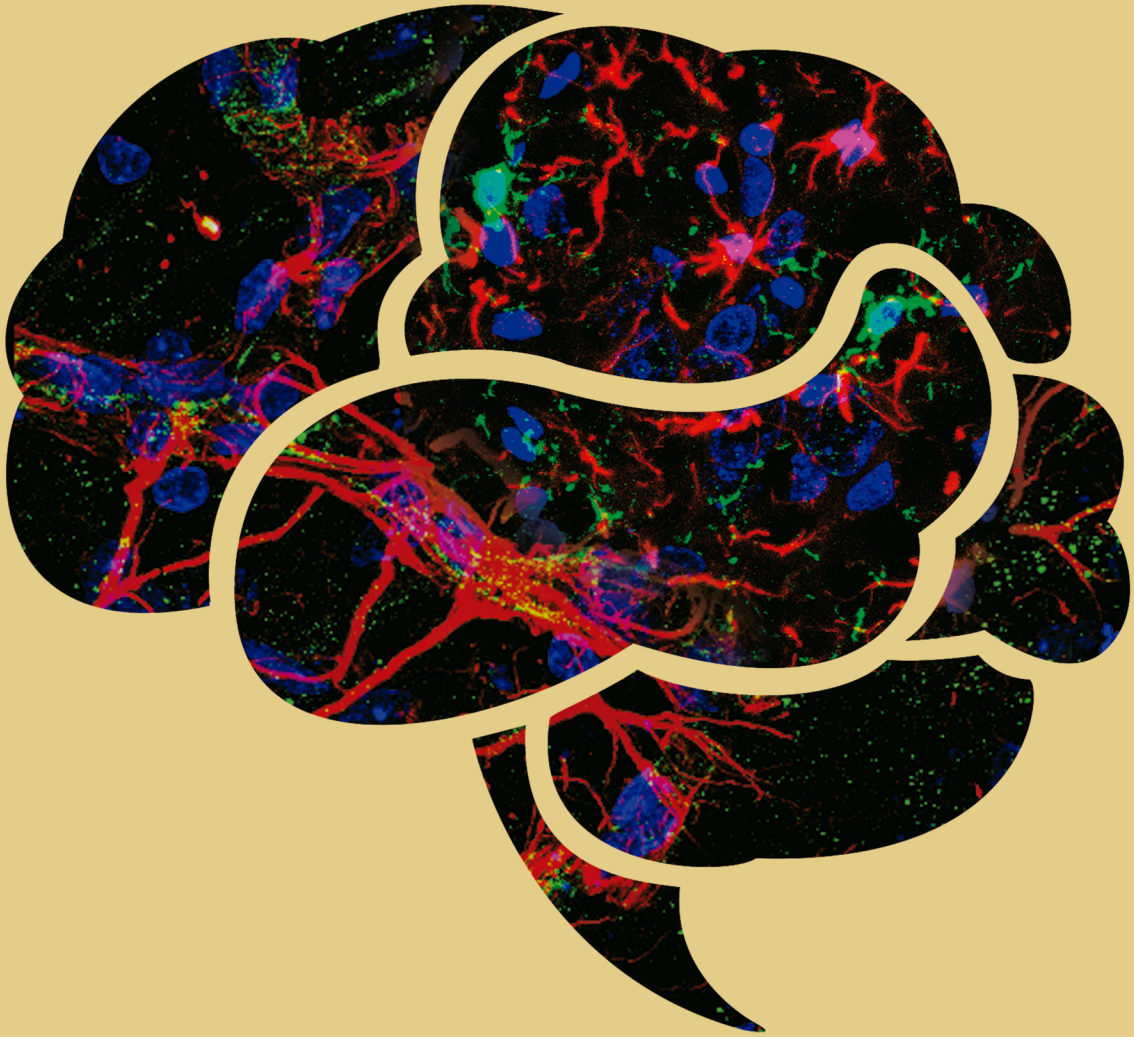

Vanessa Filipa Coelho Santos

Effect of methylphenidate on blood-brain barrier function in health and attention deficit hyperactivity disorder

Doctoral Thesis of the Inter-University Doctoral Program in Aging and Chronic Diseases, supervised by Doctor Ana Paula Pereira da Silva Martins, and presented to the Faculty of Medicine of the University of Coimbra

August 2017

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UNIVERSIDADE DE COIMBRA

Vanessa Filipa Coelho Santos

O metilfenidato e as alterações na barreira hemato-encefálica numa situação fisiológica e na perturbação de hiperatividade e défice de atenção

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Cover: Overlay of two images. One image of glial cells, microglia (green; stained to Iba-1) and astrocytes (red; stained for GFAP) in hippocampus from ADHD model. The other belongs to a healthy rat treated with 5 mg/kg of methylphenidate. This one, presents a brain microvessel (green; stained for CD-31) covered by astrocytes (red). Cell nuclei in blue.

Para os meus pais

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"O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis." — Fernando Pessoa

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“Não sou nada.
Nunca serei nada.
Não posso querer ser nada.
À parte isso, tenho em mim todos os sonhos do mundo.”

Tabacaria - poesias de Álvaro de Campos

Table of contents

Abbreviations

ADHD	Attention-deficit hyperactivity disorder
AJs	Adherens junctions
AM	Adhesion molecules
AMPH	Amphetamine
ANOVA	Analysis of variance
AP-1	Activator protein-1
AKT	Protein kinase B
BBB	Blood-brain barrier
BMVECs	Brain microvascular endothelial cells
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
Cav1	Caveolin1
CCL2	C-C motif chemokine ligand 2
CD	Cluster of differentiation
CBF	Cerebral blood flow
CNS	Central nervous system
COX-2	Cyclooxygenase-*2*
CREB	cAMP-response element binding protein
CSF	Cerebrospinal fluid
CX3CR1	CX3C chemokine receptor 1
DA	Dopamine
DAergic	Dopaminergic
DAT	Dopamine transporter
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DSM	Statistical manual of mental disorders
DTT	Dithiothreitol
ECs	Endothelial Cells
ECF	Enhanced chemifluorescence
ECM	Extracellular matrix
ERK	Extracellular signal-regulated activated kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GAP-43	Growth associated protein 43
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
GLUT-1	Glucose transporter-1
HBMVECs	Human brain microvascular endothelial cells
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase

HUVEC	Human umbilical vein endothelial cells
IBA1	Ionized calcium-binding adapter molecule 1
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
i.p.	Intraperitoneal
JAM	Junctional adhesion molecule
LPA	Lysophosphatidic acid
MDA	Malondialdehyde
MAPK	Mitogen-activated protein kinase
M-β-C	Methyl-β-cyclodextrin
MCP-1	Monocyte chemotactic protein 1
MMP	Matrix metalloproteinases
MPH	Methylphenidate
3-NT	Neurotrophin-3
NAC	N-acetylcystein
NAc	Nucleus accumbens
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluorescein
NMDA	N-methyl-D-aspartate
NE	Norepinephrine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NLR Family Pyrin Domain Containing 3
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NAPDH oxidase
NVU	Neurogliovascular unit
PBS	Phosphate-buffered saline
PECAM	Platelet endothelial cell adhesion molecule
PFC	Prefrontal cortex
PFA	Paraformaldehyde
PKC	Protein kinase C
PSD-95	Postsynaptic density-95
RNS	Reactive nitrogen species
ROS	Reactive species of oxygen
RT	Room temperature
SDS	Sodium dodecyl sulfate
S.E.M.	Standard error of the mean
SHR	Spontaneously hypertensive rat
siRNA	Small interference ribonucleic acid
SNAP-25	Synaptosomal-associated protein 25
SUD	Substance use disorder

TBARS	Thiobarbituric acid reactive substances
TBI	Traumatic brain injury
TEER	Transendothelial electrical resistance
TEM	Transmission electron microscopy
TJs	Tight junctions
TIMPs	Tissue inhibitor of metalloproteinases
TNF-α	Tumor necrosis factor-alpha
Tx-100	Triton X-100
VCAM-1	Vascular adhesion molecule-1
VE-cadherin	Vascular endothelial cadherin
VitC	Vitamin C
WKY	Wistar Kyoto
ZO	Zonula occludens

Publications

The results presented in this dissertation have been published or submitted for publication in international peer-reviewed scientific journal:

Vanessa Coelho-Santos, Renato Socodato, Camila Portugal, Ricardo A. Leitão, Manuel Rito, Marcos Barbosa, Pierre-Olivier Couraud, Ignacio Romero, Babette Weksler, Robert Minshall, Carlos Fontes-Ribeiro, Teresa Summavielle, João B Relvas, Ana Paula Silva. (2016) Methylphenidate-triggered ROS generation promotes caveolae-mediated transcytosis via Rac1 signaling and c-Src-dependent caveolin-1 phosphorylation in human brain endothelial cells. Cell Mol Life Sci. Dec;73(24):4701-4716. Epub 2016 Jul 4. doi: 10.1007/s00018-016-2301-3

Vanessa Coelho-Santos, Filipa L. Cardoso, Ana Magalhães, Ricardo A. Leitão, Manuel Rito, Marcos Barbosa, Carlos A. Fontes-Ribeiro, Ana Paula Silva. Unraveling the aftermath of methylphenidate use on hippocampal neurogliovascular unit and memory performance. (Submmited for publication)

Vanessa Coelho-Santos, Filipa L. Cardoso, Ricardo A. Leitão, Carlos A. Fontes-Ribeiro, Ana Paula Silva. Impact of developmental exposure to methylphenidate on rat brain's immune privilege and behavior: control versus ADHD. (Under revision in Brain, Behavior and Immunity)

The following publications were under the scope of the present dissertation:

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Ricardo A. Leitão, **Vanessa Coelho-Santos,** Ana Paula Silva. (2016) Methamphetamine and the blood-brain barrier. In Victor R Preedy (ed) Neuropathology of Drug Addictions

and Substance Misuse Volume 2: Part I Stimulants, 1st edn Academic Press-Elsevier, London, pp 155-168.

Note: In this dissertation, the results presented in Chapter 2, 3 and 4 are formatted according to the style of the journal where the papers were published or submitted for publication, with minor modifications, and respective licenses for reproduction of the material.

Resumo

O metilfenidato (MFD) é um psicoestimulante do grupo das anfetaminas usado como primeira linha de tratamento da perturbação de hiperatividade e défice de atenção (PHDA). PHDA é uma perturbação neuropsiquiátrica altamente prevalente com inicio na infância e que inclui sintomas comportamentais e cognitivos, nomeadamente falta de atenção e/ou impulsividade/hiperatividade. Normalmente, em mais de 50% das crianças com PHDA os sintomas persistem até à adolescência e fase adulta. Além disso, o diagnóstico desta patologia não é consensual com vários relatórios a afirmar que os médicos tendem a estar mais preocupados com a redução dos sintomas através da medicação e muitas vezes não conseguem reconhecer o contexto em que o comportamento é exibido, o que sugere que a PHDA está a ser sobrediagnosticada em crianças. Apesar de ser benéfico em condições de PHDA, o uso indevido de MFD é hoje em dia um problema com alto impacto na sociedade. Os mecanismos celulares/moleculares desencadeados pelo uso de MFD ainda são largamente desconhecidos. Até à data, a maioria dos estudos que explicam os efeitos no sistema nervoso central subjacentes ao uso do MFD têm-se focado nos eventos intraneuronais, particularmente nas alterações dopaminérgicas. Neste sentido, o impacto do MFD na função de barreira hematoencefálica (BHE) nunca foi abordado antes, quer em condições não patológicas quer em situações de PHDA. A BHE é uma barreira altamente seletiva responsável pela regulação e manutenção da homeostasia cerebral, criando um microambiente adequado para a função neuronal. Visto que a disfunção na BHE é considerada um evento precoce em muitas patologias do cérebro, não é surpreendente que haja um interesse crescente na BHE como um alvo terapêutico.

Desta forma, o principal objetivo da presente tese foi esclarecer os efeitos do MFD na função da BHE em condições fisiológicas e de PHDA. Especificamente, pretendeu-se investigar o efeito direto do MFD nas células endoteliais da microvasculatura do cérebro humano (CEs) e avaliar o efeito do uso crónico de MFD durante o desenvolvimento neurológico normal e numa condição de PDHA, tendo como principais focos a unidade neurogliovascular, a resposta imunitária e neuroinflamatória, bem como as alterações comportamentais.

Esta tese encontra-se organizada em 5 capítulos. No capítulo 1, é apresentada uma revisão da literatura onde se exploram tópicos essenciais à compreensão deste trabalho dando ênfase à PHDA, MFD e BHE.

No capítulo 2, avaliamos o efeito direto do MFD nas CEs. Verificou-se que o MFD aumenta a permeabilidade das CEs promovendo o transporte vesicular. Especificamente, identificámos a via de sinalização intracelular responsável pela transcitose induzida por MFD, mostrando que a c-Src é ativada por espécies reativas de oxigénio intracelular gerados pelo complexo Rac1/NOX que por sua vez interage e fosforila a caveolina 1, resultando na formação de cavéolas e consequente permeabilidade vesicular.

Em seguida, no capítulo 3, desvendámos os efeitos resultantes do consumo crónico do MFD no cérebro saudável, bem como as consequências cognitivas que podem surgir do seu uso indevido. Tendo em consideração que o hipocampo está amplamente envolvido em processos de memória/aprendizagem, focámos o nosso estudo nesta região cerebral. Concluímos que doses mais altas de MFD causam disfunção e ativação endotelial com o consequente aumento da infiltração de leucócitos por transcitose. Além da disfunção da vasculatura do hipocampo, também foram observadas alterações na morfologia dos astrocítos e na maquinaria sináptica. Estas alterações neurogliovasculares culminaram em défice de memória. Por outro lado, uma menor dose de MFD melhorou a memória em simultâneo com um aumento dos prolongamentos astrocíticos, mas sem efeito significativo na permeabilidade da BHE. Adicionalmente, mostrámos que o uso indevido do MFD é capaz de modular a via de sinalização da AKT/CREB, o que pode explicar as alterações no desempenho cognitivo.

Posteriormente, no capítulo 4, avaliámos o impacto do consumo crónico de MFD no período correspondente desde a infância até à adolescência tardia na vigilância imunológica do cérebro e na neuroinflamação em condições fisiológicas e de PHDA. Este trabalho foi focado no córtex pré-frontal, região cerebral com um papel proeminente na fisiopatologia da PHDA. Os nossos resultados mostram que a exposição crónica a uma dose mais elevada de MFD causou um aumento da permeabilidade da BHE e incitou um comportamento do tipo ansioso em ambas as condições estudadas, saudável e PHDA. No entanto, a disfunção da BHE foi mais proeminente nos animais sem patologia, o que foi evidenciado por uma diminuição dos níveis e rutura das junções intercelulares, um aumento de vesículas nos microvasos e ativação do endotélio, concomitante com a infiltração de células do sistema imunitário periférico para o cérebro. Para além disso, independentemente da dose utilizada, o MFD desencadeou uma resposta oxidativa e neuroinflamatória robusta nos ratos saudáveis comprovado pela ativação dos astrócitos e das células da microglia, assim como pelo aumento de citocinas pró-inflamatórias. Pelo contrário, no modelo animal de PHDA, o MFD na dose mais baixa restaurou a homeostasia cerebral, diminuindo o estado

inflamatório e oxidativo. Além disso, os efeitos imunomoduladores do MFD em ambos os modelos animais parecem ser mediados pela via de sinalização do NF-κB/NLRP3.
Finalmente, no capítulo 5, uma discussão geral com considerações finais é apresentada.
Em conclusão, esta tese dá-nos evidências de que o MFD atua diretamente nas CEs prejudicando a integridade da BHE, o que pode favorecer o acesso das células periféricas ao cérebro. De realçar que o MFD tem também um efeito nas células da glia, desencadeando uma resposta neuroinflamatória e causando stress oxidativo. Alterações comportamentais também foram observadas, incluindo défice de memória de trabalho e comportamento do tipo ansioso. Desta forma, contribuímos para uma melhor compreensão das alterações que ocorrem na unidade neurogliovascular devido a um tratamento crónico com MFD durante o desenvolvimento numa condição fisiológica *versus* PHDA, e como essas alterações dependem ainda da dose de MFD. Estas observações evidenciam a importância da dose terapêutica de MFD que é prescrita e de um diagnóstico mais objetivo para evitar o uso indevido de MFD.

Palavras-chave: astrócitos, barreira hemato-encefálica, células endoteliais, comportamento, metilfenidato, microglia, neuroinflamação, perturbação de hiperatividade com défice de atenção, stress oxidativo.

Summary

Methylphenidate (MPH) is an amphetamine-like psychostimulant that has become the primary drug of choice for treating attention-deficit hyperactivity disorder (ADHD), a highly prevalent neuropsychiatric disorder with an onset in early childhood. ADHD includes behavioral and cognitive symptoms, such as inattention and/or impulsivity/hyperactivity. In more than 50% of children with ADHD, the symptoms persist into adolescence and adulthood. Moreover, the diagnosis of ADHD itself is non-consensual and several reports claim that physicians tend to be preoccupied with reducing symptoms via medication but often fail to recognize the context in which the behavior exhibits, which suggest that ADHD is being overdiagnosed in children. Despite being beneficial under ADHD conditions, MPH misuse is nowadays a problem with high impact on society.

The full comprehension of the cellular/molecular mechanisms trigger by MPH use is still elusive. To date, most of the studies that explain the underlying MPH effects on central nervous system have focused on intra-neuronal events, particularly on dopaminergic alterations. The impact of MPH on blood brain-barrier (BBB) function has never been addressed before neither in non-pathological conditions nor in ADHD. The BBB is a barrier highly selective responsible for the regulation and maintenance of brain homeostasis creating a proper microenvironment for neural function. Given the evidence of BBB damage as an early event in many neurological conditions, it is not surprising that there is a growing interest in the BBB as a therapeutic target.

Thus, the major goal of the present thesis was to clarify the effects of MPH on the BBB function in both physiological and ADHD conditions. Specifically, we aimed to investigate the direct impact of MPH on human brain microvascular endothelial cells (HBMVECs), and to evaluate the effect of chronic MPH use during normal and ADHD rat neurodevelopmental on the neurogliovascular unit, brain immune surveillance neuroinflammation, and behavior.

This thesis is organized in 5 chapters. In chapter 1 is presented a review of the literature about the topics explored in this work with emphasis in ADHD, MPH and BBB.

In chapter 2, we evaluated the direct effect of MPH on HBMVEC. It was found that MPH increases brain endothelial cell permeability by promoting the vesicular transport. Specifically, we identified the molecular event critical to MPH-induced transcytosis, showing that c-Src is activated in result of intracellular reactive oxygen species generated

by Rac1/NOX that interacts and phosphorylates Caveolin1, resulting in caveolae formation and consequent vesicular permeability.

Next in chapter 3, we unraveled MPH chronic outcome in the healthy brain as well as cognitive concerns that might arise from its misuse. Since hippocampus is highly involved in memory/learning processes we focused on this brain region. Our data revealed that higher doses of MPH cause endothelial dysfunction and activation with a consequent increase of leukocyte infiltration by transcytosis. In addition to disruption of hippocampal vasculature, alterations in astrocytic morphology and synaptic machinery were also observed. These neurogliovascular disturbances culminated in memory deficit. Contrariwise, a lower dose of MPH improved cognition in parallel with an increase of astrocytic processes but with no major effect on BBB permeability. Furthermore, we showed that MPH misuse modulated AKT/CREB signaling pathway, which can explain alterations on cognitive performance.

Afterwards, in chapter 4, we dissected the impact of early-life chronic consumption of MPH on brain immune surveillance and neuroinflammation in both physiological and ADHD conditions. This work was focused on the prefrontal cortex that is a brain region with a prominent role in the pathophysiology of ADHD. Our results show that chronic exposure to a higher dose of MPH caused BBB leakage and elicited an anxious-like behavior in both healthy and ADHD conditions. Nevertheless, BBB dysfunction was more prominent in control animals, which was proved by a downregulation and disruption of intercellular junctions, an increase of microvessels vesicles, and endothelium activation concomitant with infiltration of peripheral immune cells. Moreover, independently of the dose used, MPH triggered a robust oxidative and neuroinflammatory response in healthy rats shown by astrocytes and microglial cells activation and upregulation of pro-inflammatory cytokines. On the contrary, in ADHD animal model, MPH at the lower dose restored brain homeostasis by decreasing the inflammatory and oxidative status. Moreover, the MPH immune-modulatory effects in both animal conditions seem to be mediated through NF-κB/NLRP3 signaling pathways.

Finally, in chapter 5, a general discussion with final remarks is presented.

Overall, this thesis provides evidence that MPH acts directly on endothelial cells impairing BBB integrity, which may favor the access of peripheral cells into the brain. Additionally, MPH has an effect on glial cells leading to a neuroinflammatory response and oxidative status. Behavior alterations were also observed, including spatial working memory deficit and anxiety-like behavior. Herein, we improved our understanding about neurogliovascular

unit alterations associated with chronic MPH treatment during development, and how these changes depend on the dose of MPH and (non)-pathological conditions, which highlights the importance of an appropriate MPH dose regimen for ADHD and an adequate diagnosis to avoid MPH misuse.

Keywords: astrocytes, attention deficit hyperactivity disorder, behavior, blood-brain barrier, endothelial cells, methylphenidate, microglia, neuroinflammation, neurogliovascular unit, oxidative stress.

Chapter 1

General Introduction

1.1 Attention deficit hyperactivity disorder

1.1.1 Pathophysiology and diagnosis

First called hyperkinetic impulse disorder, the American Psychiatric Association formally recognized attention-deficit hyperactivity disorder (ADHD) as a mental disorder in second Diagnostic and Statistical Manual of Mental Disorders (DSM), in 1968 (Lange et al., 2010). This disorder is classically characterized by hyperactivity, inattentiveness and impulsiveness. Nevertheless, there are three subtypes of ADHD: predominantly inattentive, predominantly hyperactive, and combined (American Psychiatric et al., 2013). ADHD is among the most common neurobehavioral disorder of childhood and is now recognized as a chronic condition. In fact, the persistence of ADHD symptoms into adulthood was first recognized in the 1970s (Wood et al., 1976) and was formally included in the nomenclature of the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) in 1994.

ADHD is typically manifests by age 7, although the symptoms can be identified as early as 2 years old (Egger and Angold, 2006). About 60-80% of these children have symptoms into adulthood (Wilens et al., 2004). Its prevalence is approximately 5-7.2% in the school age population (Polanczyk et al., 2007; Thomas et al., 2015) and 2.5%-4% in the adult population (Faraone and Biederman, 2005; Fayyad et al., 2007; Simon et al., 2009). The US Census Bureau has estimated 1,795,734,009 ADHD people worldwide aged 5-19, in 2013. Moreover, ADHD seems to occur two to four times more often in boys than girls. Although there is no national epidemiologic study, the Portuguese Association of hyperactive children estimates that 3-7% school-age children have this disorder, and the boys are 2-9 times more affected than the girls (Azevedo et al., 2012).

Currently, all clinical criteria are behavioral and there is no biomarker or laboratory test to confirm the diagnosis of ADHD. The diagnosis is based mainly on the International Classification of Mental and Behavioral Disorders 10th revision (ICD-10) and DSM-V. ICD-10 uses a limited diagnostic category, which includes people with more severe symptoms and impairment. DSM-V has a broader, more inclusive definition, which

includes a number of different ADHD subtypes and many European clinicians prefer to use this classification. Despite the fact that DSM-V (Table 1) has improved over DSM-IV-TR, the criteria still misses gender differences in ADHD (Sharma and Couture, 2014). Since there are three subtypes of ADHD, children need to present at least six symptoms from either (or both) the inattention, hyperactivity and impulsivity criteria, while older adolescents and adults (over the age of 17) must present five symptoms. The symptoms of inattention and/or hyperactivity-impulsivity should be present for at least 6 months to an extent that is disruptive and inappropriate for the person's developmental level and should be observed at two or more setting (e.g., at home, school or work; with friends or relatives; in other activities). Moreover, several inattentive or hyperactive-impulsive symptoms should be present before age 12, with significant impact on quality of social life, school, or work functioning. Additionally, the symptoms must not happen only during the course of schizophrenia or another psychotic disorder. Diagnosing ADHD is also complicated because it is often present with one or more comorbidities, like major depressive disorder, bipolar, anxiety disorders, oppositional defiant disorder. Children with ADHD can present significant functional problems, as learning difficulties with poor performance, social and emotional adjustment problems. These children are usually disorganized, clumsy for motor tasks, including sports, limited social skills and with an inconsistent academic performance. These behavioral characteristics promote failure, and generating a low self-esteem, social isolation and sometimes are associated with symptoms of depression, anxiety and tics. In adults, ADHD interferes with psychosocial functioning and quality of life including educational and professional performance. Manifestations in adolescents and also observed in adults include relationship problems, parenting difficulties, lower socioeconomic status, reduced self-esteem (Wehmeier et al., 2010; Knapp et al., 2011), tendency toward risk such as dangerous driving habits and risky sexual behavior, substance use disorder (SUD) and criminality (Barkley et al., 2004; Flory et al., 2006; Fletcher and Wolfe, 2009; Odell et al., 2017).

The early diagnosis and appropriate treatment is fundamental to prevent the development of more serious psychopathology in adolescence and adulthood.

Table 1 – DSM-V Diagnostic Criteria for Attention Deficit/Hyperactivity Disorder (American Psychiatric et al., 2013).

ADHD consists of a pattern of behavior that is present in multiple settings where it gives rise to social, educational, or work performance difficulties.	
A. Either (A1) and/or (A2).	
A1. Inattention: Six (or more) of the following symptoms have persisted for at least 6 months to a degree that is inconsistent with developmental level and that impact directly on social and academic/occupational activities.	**A2. Hyperactivity and impulsivity: Six (or more) of the following symptoms have persisted for at least 6 months to a degree that is inconsistent with developmental level and that impact directly on social and academic/occupational activities.**
a. Often fails to give close attention to details or makes careless mistakes in schoolwork, at work, or during other activities (e.g., overlooks or misses details, work is inaccurate). b. Often has difficulty sustaining attention in tasks or play activities (e.g., has difficulty remaining focused during lectures, conversations, or reading lengthy writings). c. Often does not seem to listen when speaking directly (e.g., mind seems elsewhere, even in the absence of any obvious distraction). d. Often does not follow through on instructions and fails to finish schoolwork, chores, or duties in the workplace (e.g., starts tasks but quickly loses focus and is easily sidetracked; fails to finish schoolwork, household chores, or tasks in the workplace). e. Often has difficulty organizing tasks and activities (e.g., difficulty in managing sequential tasks; difficulty in keeping the materials and belongings in order; messy, disorganized, and work; poor time management; tends to fail to meet deadlines). f. Often avoids, dislikes, or is reluctant to engage in tasks that require sustained mental effort (e.g., schoolwork or homework; for older adolescents and adults, preparing reports, completing forms, or reviewing lengthy papers). g. Often loses things necessary for tasks or activities (e.g., school materials, pencils, books, tools, wallets, keys, paperwork, eyeglasses, or mobile telephones). h. Is often easily distracted by extraneous stimuli (for older adolescents and adults, may include unrelated thoughts). i. Is often forgetful in daily activities (e.g., chores, running errands; for older adolescents and adults, returning calls, paying bills, and keeping appointments).	a. Often fidgets with or taps hands or feet or squirms in seat. b. Often leaves seat in situations when remaining seated is expected (e.g., leaves his/her place in the classroom, office or other workplace, or in other situations that require remaining seated). c. Often runs about or climbs in situations where it is inappropriate (in adolescents or adults, may be limited to feeling restless). d. Often unable to play or engage in leisure activities quietly. e. Is often ‘‘on the go,’’ acting as if ‘‘driven by a motor’’ (e.g., is unable or uncomfortable being still for an extended time, as in restaurants, meetings, etc.; may be experienced by others as being restless and difficult to keep up with). f. Often talks excessively. g. Often blurts out an answer before a question has been completed (e.g., completes people’s sentences and ‘‘jumps the gun’’ in conversations, cannot wait for next turn in conversation). h. Often has difficulty in waiting for his/her turn (e.g., while waiting in line). i. Often interrupts or intrudes on others (e.g., butts into conversations, games, or activities; may start using other people’s things without asking or receiving permission, adolescents or adults may intrude into or take over what others are doing).

B. Several inattentive or hyperactive-impulsive symptoms were present prior to age 12.
C. Criteria for the disorder are met in two or more settings (e.g., at home, school, or work, with friends or relatives, or in other activities).
D. There must be clear evidence that the symptoms interfere with or reduce the quality of social, academic, or occupational functioning.
E. The symptoms do not occur exclusively during the course of schizophrenia or other psychotic disorder and are not better accounted for by other mental disorder (e.g., mood disorder, anxiety disorder, dissociative disorder, or a personality disorder).
Specific based on current presentation
Combined presentation: If both criterion A1 (inattention) and Criterion A2 (hyperactivity-impulsivity) are met for the past 6 months.
Predominantly inattentive presentation: If criterion A1 (inattention) is met, but criterion A2 (hyperactivity-impulsivity) is not met, and three or more symptoms from criterion A2 have been present for the past 6 months.
Inattentive presentation (restrictive): If criterion A1 (inattention) is met, but no more than two symptoms from criterion A2 (hyperactivity- impulsivity) have been present for the past 6 months.
Predominantly hyperactive/impulsive presentation: If criterion A2 (hyperactivity-impulsivity) is met, and criterion A1 (inattention) is not met for the past 6 months.
Coding note: For individuals (especially adolescents and adults) who currently have symptoms with impairment that no longer meet full criteria, ‘‘in partial remission’’ should be specified.

1.1.2 Etiology

The exact etiology of ADHD is still unknown but neurocognitive, neurophysiological and neuroimaging studies propose that brain dysfunction resultant from genetic or environmental factors act together to create a spectrum of neurobiological liability in children and adults (Figure 1.1).

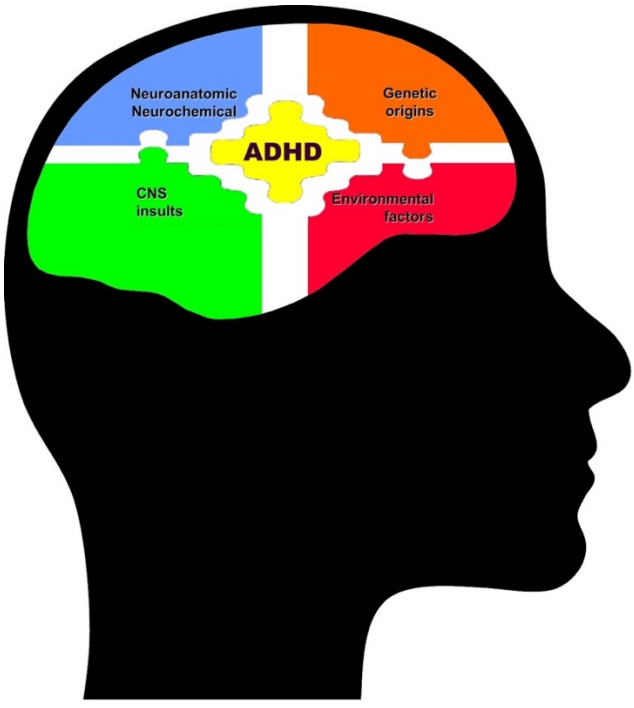


Figure 1.1. Attention-deficit hyperactivity disorder (ADHD) is a heterogeneous behavioral disorder with multiple possible etiologies, such as neuroanatomic and neurochemical, central nervous system insults, genetic origins and environmental factor.

Several reports have considered the prefrontal cortex (PFC), caudate and cerebellum as the primary areas that present alterations in ADHD (Emond et al., 2009), specifically a reduced volume and activity. These impaired areas are interconnected by a network of neurons and together regulate attention, emotions, thoughts, behavior and actions. Developmental trajectory studies in ADHD patients showed a delay in cortical maturation, most prominent in prefrontal regions important in the control of cognitive processes including attention and motor planning (Shaw et al., 2006; Shaw et al., 2007), which result in different clinical outcomes associated with different developmental trajectories in adolescence and beyond (Shaw and Rabin, 2009). The PFC, basal ganglia and cerebellum are differentially affected and evidence indicating reduced connectivity in white matter tracts in key brain areas is emerging. Additionally, diffusion tensor imaging studies have revealed developmental changes in white matter pathways in prefrontal regions and in pathways surrounding the basal ganglia and cerebellum in patients with ADHD (D'Agati et al., 2010), which presumably reflect a decreased myelination of axons and consequently a decrease in the speed of neuronal communication. The fronto-subcortical circuits that comprise lateral PFC, dorsal anterior cingulate cortex, caudate, and putamen, are rich in catecholamines. In fact, neuropharmacological studies have provided evidence that ADHD involves dysregulation of both dopamine (DA) and norepinephrine (NE) neurotransmitter systems (Pliszka, 2005). The theory of hypodopaminergic suggests alteration in the mesolimbic DA signaling and is the most studied and the most scientific accepted (Sagvolden et al., 2005a). It was proposed an altered reinforcement of novel behavior and deficient extinction of this reinforced behavior. Both processes are associated with dopaminergic (DAergic) system, since dopamine D1 receptor activity is associated with strengthening of the behavior reinforcement and D2 receptor with weakening of this reinforced behavior. Likewise, it is suggested that hypofunction of DAergic system results on a deficient extinction of processes, causing excessive behavior that is usually translated and seen as hyperactivity (Sagvolden et al., 2005a). Genetic defects in DA metabolism, resulting in a hypo-dopaminergic state in the limbic system and frontal lobes, result in a compensatory increase in DAergic activity in the basal ganglia (Blum et al., 2008). Compensatory networks to overcome response inhibition deficits, including basal ganglia, insula and cerebellum, seem to be implicated in relative low cognitive load tasks in ADHD patients (Castellanos et al., 2002).

This disorder is highly heritable, with a genetic basis in about 80% of the cases, involving a number of different genes (Banaschewski et al., 2010). Data from numerous families and twin studies indicate a high heritability and have been identified a number of genes that convey risk for ADHD. However, it is important to note that the effects of each gene marker are too small to be of clinical utility and explain only a small fraction of the overall genetic influence. Moreover, all the candidate genes identified account for less than 5% of genetic variation (Neale et al., 2010). Yet, several genetic studies and meta-analyses have stated polymorphisms of genes that reduced functionality of DAergic system in ADHD (Gizer et al., 2009), such as DA D4 receptors (DRD4) (Holmes et al., 2000; Faraone et al., 2001), DA D5 receptors (DRD5) (Manor et al., 2004), and DA transporter (DAT-1) (Cook et al., 1995; Gill et al., 1997). More recently, mutations (A559V and R615C) in DAT-1 were identified in some ADHD patients. Furthermore, a gene important in synaptic vesicle docking fusion, the synaptosomal-associated protein 25 (SNAP25), seems also to be linked with ADHD etiology (Barr et al., 2000; Mill et al., 2004). Additionally, genes coding for enzymes that catalyze the biosynthesis of DA have been also suggested as candidates for ADHD (Ernst et al., 1998; Kopeckova et al., 2006). Equally important, it has been also suggested a role for noradrenergic (NEergic) genes, particularly the alpha-2 receptor (α2) (Rivero et al., 2013). Even though the meaning of decreased receptor density or genetic polymorphisms of NE in ADHD has not yet been unraveled, the α2 receptor was identified as leading to impaired attention and impulsive control, as well as hyperactivity (Arnsten and Pliszka, 2011). Genetic research and *in vivo* imaging observations have put the focus on DA dysfunction in ADHD by documenting increased dopa-decarboxylase activity in the midbrain (Ernst et al., 1999), decreased sensitivity of the DA D4 receptor and increased density of the DAT in the striatum/nucleus accumbens (NAc) (Dougherty et al., 1999; Castellanos et al., 2002; Fisher et al., 2002). In the PFC, there is a reduced DA storage in ADHD patients (Ernst et al., 1998), and neither DAT nor D2 receptors are detected (Meador-Woodruff et al., 1994).

In addition, monoamine oxidase (MAO-A) that is important to degrade biogenic amines such as DA and serotonin, providing control of the level of these neurotransmitters in the CNS, has been proposed as a potential susceptibility gene for ADHD (Manor et al., 2002; Lawson et al., 2003). MAO-A polymorphisms, such the 30-bp VNTR in the promoter and the Fnu4HI 941T⟶G, were reported to be associated with aggression and

impulsivity (Lawson et al., 2003). Other studies showed that the gene coding catechol-*O*-methyltransferase (COMT), an important enzyme involved in the inactivation of cathecolamines, is linked with ADHD neuropsychiatric condition (Eisenberg et al., 1999; Tunbridge et al., 2006). Additional genes from other neurotransmitters systems that can affect DA/NE regulation, as serotonin-transporter-linked polymorphic region (Ehli et al., 2012) and 5-hydroxytryptamine serotonin receptor 1B (Guimarães et al., 2009), as well as the ionotropic glutamate receptor N-methyl-d-aspartate 2A (Turic et al., 2004) are markers associated with ADHD (Gizer et al., 2009).

Environmental factors are also associated with ADHD, particularly prenatal risk factors such as exposure to alcohol (Bhatara et al., 2006), nicotine (Agrawal et al., 2010), illicit drugs (Bandstra et al., 2001; Ornoy, 2003), high blood pressure and maternal stress during pregnancy (Grizenko et al., 2012), as well as preterm birth and low birth weight (Polanska et al., 2012; Silva et al., 2014). In fact, perinatal factors have also been implicated, with a two-fold increase in ADHD in very low-birthweight children and an increased rate of pregnancy and birth complications in mothers of children later diagnosed with ADHD (Taylor and Rogers, 2005). Furthermore, children who experienced ischemic-hypoxic conditions (lack of oxygen and blood to the brain) prior or during birth have more rick to develop ADHD (Lou, 1996; Zhu et al., 2016).

As abovementioned, the etiology of ADHD is thought to include abnormal regulation of neurotransmitter systems particularly DA and NE. In fact, the accumulation of catecholamines in the synaptic cleft undergo enzymatic oxidation by MAO or oxygen mediated non-enzymatic degradation that culminate in the increase of reactive oxygen species (ROS). Recent evidence strongly suggests an association between ADHD and changes in serum levels of nitric oxide synthase (NOS), xanthine oxidase, glutathione S-transferase and paraoxonase-1 activities, which are important markers of oxidative stress, and adenosine deaminase activity, marker of cellular immunity (Ceylan et al., 2012). Additionally, several peripheral indicators of oxidative stress namely markers of lipid peroxidation like the ubiquitous lipid peroxidation product, malondialdehyde (MDA), have been found to be significantly increased in ADHD patients (Bulut et al., 2007; Selek et al., 2008; Ceylan et al., 2010; Archana et al., 2012; Ceylan et al., 2012; Selek et al., 2012; Sezen et al., 2016). These bioenergetics crisis and/or phenotypes resulting from mitochondrial defects could be a contributory factor for ADHD pathology (Verma et al., 2016). It was also suggested that fatty acid deficiencies or imbalances may contribute to ADHD (Joshi et al., 2006). Noteworthy, the treatment with antioxidant compounds such

as omega-3 fatty acids, vitamin C, pycnogenol, and N-acetylcysteine (NAC) significantly reduced symptoms of ADHD (Joshi et al., 2006; Bloch and Qawasmi, 2011; Garcia et al., 2013). Some patients with ADHD have normal levels of antioxidant production but their response to oxidative stress is insufficient, leading to oxidative damage (Joseph et al., 2015), whereas others have decreased levels of total antioxidant status (Sezen et al., 2016).

Beside the molecular findings, nutritional and dietary also seems to influences the behavior and learning of children with ADHD. About 90% of total brain growth occurs in the first three years of life and so nutrients required for its development must be supplied to support optimal brain health in these early years. Individuals with ADHD have a brain volume with 3 to 4 percent smaller, which can be related with nutritional deficits. Interestingly, a number of studies point to an association between zinc deficiency and ADHD (Arnold et al., 2000). Zinc is an important co-factor for metabolism relevant to neurotransmitters, which has a direct action on serotonin, NE, and DA (Arnold et al., 2000). Zinc deficiency can also increase oxidative stress and decrease the antioxidant defense system as well (Taysi et al., 2008).

Interestingly, inflammation has also been related with ADHD (Mitchell and Goldstein, 2014). Cytokine values, as interleukin (IL)-6 and IL-13, tend to be higher in ADHD patients serum than in the control group (Oades et al., 2010b) and return to normal values with medication (Oades et al., 2010a). Likewise, in cerebrospinal fluid from ADHD childhood was detected intermediate amounts of several cytokines (Mittleman et al., 1997).

Finally, about 20% of the ADHD cases are a result of an acquired brain insult (Voeller, 2004). Any injury to the brain that affects the prefrontal-subcortical circuits can result in an ADHD-like picture. Approximately 30 to 50% of children are reported to develop symptoms of ADHD soon after traumatic brain injury (TBI) (Max et al., 2004; Li and Liu, 2013). However, roughly 20% of children hospitalized for TBI meeting criteria for pre-injury ADHD. Attention problems are often present before TBI and may be predictive for sustaining head and other injuries (Li and Liu, 2013).

A demand for an ADHD biomarker is a hot topic in the field, however is very complex due the heterogeneous nature of this psychiatric disorder. A collection set of biomarkers could be useful for a most effective diagnosis and treatment selection. It will be important to take into consideration the deep integration of “omics” sciences such as “pharmacogenomics”, “phenomics”, “epigenomics,” “proteomics”, “transcriptomics”,

and "metabolomics" (Faraone et al., 2014). Although important data have been provided to improve our knowledge about brain and behavior interactions in ADHD, the underlying pathaetiological basis of ADHD remains largely unknown. Up to now, there is no biomarker for ADHD that could be useful to eliminate subjective diagnoses based on clinical interviews. Some clinicians are starting to use brain imaging in ADHD diagnosis but this is a rare practice. It thereby creates a challenge to medical and scientific communities to unravel the etiology of this disorder and consequently to improve the diagnostic process.

1.1.3 Treatment

ADHD treatment may consist on drug therapy and/or behavioral therapy. There are also a few scientific supports that highlight the importance of formal guidelines on diet and adopt nutritional approaches (Nigg et al., 2012; Heilskov Rytter et al., 2014; Rucklidge et al., 2014), but this is not consensual among physicians. Regardless the treatment used, it is recommended for all children an early and effective treatment after the diagnosis. Due to behavioral characteristics of ADHD children, it is usually suggested extracurricular activities to escape to the accumulated tensions during day school. These activities can be sports, hobbies or others that allow them to get a feeling of success.

Drug therapy was shown to be more effective than behavioral therapy (Sharma and Couture, 2014). Medication is crucial to restore the balance of neurotransmitters, DA/NE, and required for optimal brain function, particularly in PFC that is the main brain area affected in ADHD. Undoubtedly, pharmacotherapy is recognized as the most effective component of ADHD treatment, but some roles are also fundamental for proper educational placement, parent management training and social skills development. Drugs approved by the Food and Drug Administration (FDA) for treating ADHD include stimulants (considered first-line agents) such as methylphenidate (MPH) and amphetamines, and nonstimulants (considered alternative agents), such as atomoxetine and extended-released α-2 agonists (clonidine and guanfacine) (Sharma and Couture, 2014). Immediate-released α-2 agonist, tricyclic antidepressants and bupropion have been used off-label to treat ADHD only if the above agents fail to show benefit or cannot be used (Sharma and Couture, 2014). MPH is indeed the most widely used pharmacological agent for the treatment of ADHD, 1/3 of all cases, and is considered a first line treatment for children with this disorder (Goldman et al., 1998). MPH has a positive effect on 60–

80% of the children with ADHD, reducing behavioral adjustment problems and improving attention (Tannock et al., 1995; Swanson et al., 1999). Studies have shown a huge increase in the use of ADHD medications during the last years. It was published that one year of treatment with MPH may be beneficial to endure normalization of neural correlates of attention.

Several authors have been explored the association between ADHD and SUD. Despite the controversy around this topic, there are strong indications that psychostimulant treatment, when initiated in childhood, reduces or delays the onset of SUD in individuals with ADHD (Wilens et al., 2008; Frodl, 2010). In line with this, undiagnosed ADHD and consequently non-medicated individuals present a higher prevalence of SUD in adolescence or adulthood (McCabe et al., 2017). Interestingly, this phenomenon has been identified as a self-medication hypothesis that explain the illicit use of drugs to ameliorate psychological suffering (Odell et al., 2017).

However, little is known about the long-term effects of stimulants on the functional organization of the developing brain (Konrad et al., 2007). One of the myths of ADHD is that ADHD children show a paradoxical effect of being calmed by stimulants, while "normal" individuals are stimulated by them.

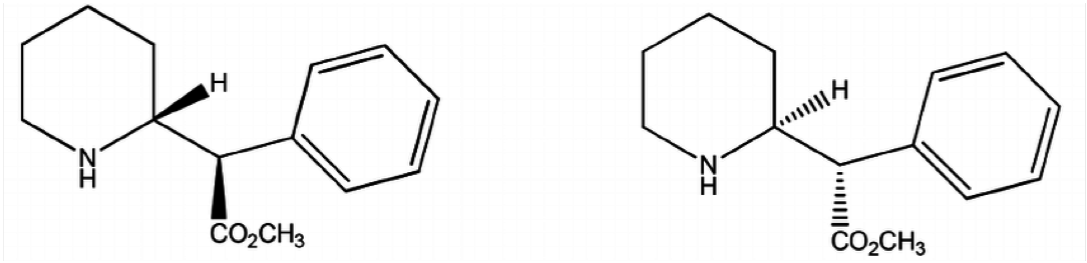
Patients receiving pharmacological treatment for ADHD should always be closely monitored for both common and unusual potentially severe adverse effects.

1.2 Methylphenidate

MPH [dl-threo-methyl-2(or a)-phenyl-2(or a)-(2-piperidyl) acetate; commercially known as Ritalin®, Concerta®, Rubifen®, Methylin® and Metadate®] is a piperazine-substituted phenylisopropylamine psychomotor stimulant that is traditionally related to amphetamine (Figure 1.2). This psychostimulant drug was synthesized for the first time in 1944 and sold by Ciba-Geigy Pharmaceutical Company as Ritalin and patented in 1954. In the first years, MPH was used for a number of conditions such as obesity, chronic fatigue, depressed states, depression-associated psychoses and disturbed senile behavior (Ferguson and Funderburk, 1956; Jacobson, 1956; Kovitz and Madi, 1956; Fitzgerald and Mc, 1957).

Currently, MPH is an U.S. FDA-approved drug considered a first line ADHD treatment for children, adolescents and adults (Goldman et al., 1998; Greenhill et al., 2002;

Subcommittee on Attention-Deficit/Hyperactivity et al., 2011). It can also be used as treatment complement in other conditions such as narcolepsy, depression, brain injury, pain, cancer, cognitive disorders and in patients with human immunodeficiency virus infection (Challman and Lipsky, 2000).



(*d*)-*threo*-Methylphenidate **(*l*)-*threo*-Methylphenidate**

Figure 1.2. Chemical structure of the stereoisomers of methylphenidate, d-threo-methylphenidate and l-threo-methylphenidate.

As shown in Figure 1.2, MPH contains two stereogenic centers and therefore can exist as four possible stereoisomers (i.e., *dl*-erythro and *dl*-threo-terms used for diastereomers with two adjacent chiral carbons with two similar groups on the same or opposite sides of the carbon chain, respectively). The structure has been altered over the years to improve therapeutic index (Markowitz and Patrick, 2008). Nowadays, only the racemic mixture of *d*-threo (R, R)-MPH (or dexmethylphenidate) and *l*-threo (S, S)-MPH is used (Ding et al., 2004; Zhang et al., 2016a). However, since the *d*-threo-enantiomer is much more active than *l*-threo-enantiomer, some countries as Switzerland and United States use pure formulations of *d*-threo-enantiomer (i.e. Focalin ®) (Patrick et al., 1987; Ding et al., 2004; Markowitz and Patrick, 2008).

1.2.1 Pharmacokinetics, metabolism and elimination

There are three pharmaceutical formulations of MPH as follows: (1) immediate release or short-acting formulation; (2) sustained release or intermediate-acting MPH and (3) extended release or long-acting MPH. The release mechanism of the intermediate-acting MPH produces variable results making this preparation less useful. Due to the short duration of action, MPH-immediate release needs to be administered repeatedly during the day to maintain effectiveness, 2-3 daily doses being required for most children (Ferguson, 2000). On the other hand, MPH-extended release formulations provide a rapid onset of therapeutic effect, while having a sufficient duration to eliminate the need for

additional doses. In fact, extended release MPH is the major formulation used (Challman and Lipsky, 2000), although the immediate release formulation is the one that has demonstrated more positive results regarding disruptive behavior (Durand-Rivera et al., 2015). Furthermore, brain imaging studies suggest that the MPH use for long periods can increase the tolerance to stimulants, creating the need of patients to get higher doses to exhibit the same medical effects than previously stimulant-naïve patients (Konstenius et al., 2014).

Current clinical practice recommends the administration of MPH 30 to 45 minutes before meals since the absorption and consequently its potency may be altered by the presence of food (Kimko et al., 1999; Midha et al., 2001). Nonetheless, some studies showed no significant difference in the behavioral effect of MPH neither serum concentrations when the drug is administered with food (Swanson et al., 1983; Midha et al., 2001; Sallee et al., 2017).

After oral administration, MPH is quickly and totally absorbed at gastrointestinal tract (Kimko et al., 1999). The therapeutic action is rapid and short-lived, with onset within 30 min until 1-4 h of duration of immediate-release MPH and 3 to 9 hours after sustained-release (Adjei et al., 2014). However, extended release MPH continued action throughout a 10–12-hour period. The half-life is age-dependent with ranges from 2 to 7 h, although in children is about 2.5 h and 3.5 h in adults (Gualtieri et al., 1982). MPH has a low degree of protein binding and it is very lipophilic (Ding et al., 1994), so it is rapidly distributed and relatively large concentrations of MPH reach the brain crossing blood-brain barrier (BBB) with a brain:plasma ratio in rats of 3:4 (Ding et al., 1994). Remarkably, some findings have demonstrated sex differences in the responsiveness to MPH, specifically an increase of females' sensitivity to its stimulatory effects due to the achieved higher brain concentrations, particularly regarding the active *d*-enantiomer (Bentley et al., 2015). Depending on the route of administration, the pharmacokinetics of MPH can change. In fact, when administered intravenously its pharmacokinetics is quite similar to cocaine (Fowler and Volkow, 1998). Moreover, with this route of administration MPH reaches a peak concentration in the basal ganglia within 8 to 15 minutes, while the associated "high" feeling peaked in 1 to 3 min (Volkow et al., 1996).

MPH is metabolized *via* endoplasmic reticulum carboxylesterase 1 (CES1A1 in human, *Ces1a in* mouse and rat; a serine esterase) through de-esterification to pharmacologically inactive metabolite *d*- or *l*-threo-ritalinic acid [2(or a)-phenyl-2(or a)-(2-piperidyl) acetic acid], which has a half-life of 3–4 h (Frolich et al., 2014) (Figure 1.3). Additionally, minor

pathways (less than 2%) involving microsomal oxidation, aromatic hydroxylation, and conjugation have been reported to form the *6*-oxo-MPH (also called Lactam), hydroxyl-MPH, and conjugated metabolites (Wargin et al., 1983; Kimko et al., 1999; Wada et al., 2011). Though, there is no evidence that any of these MPH metabolites contribute significantly to the pharmacological activity. Still, MPH may undergo transesterification forming a pharmacologically active metabolite, ethylphenidate, which can occur when MPH is used concurrently with ethanol and may be a contributing factor in MPH-induced toxicity (Markowitz et al., 1999).

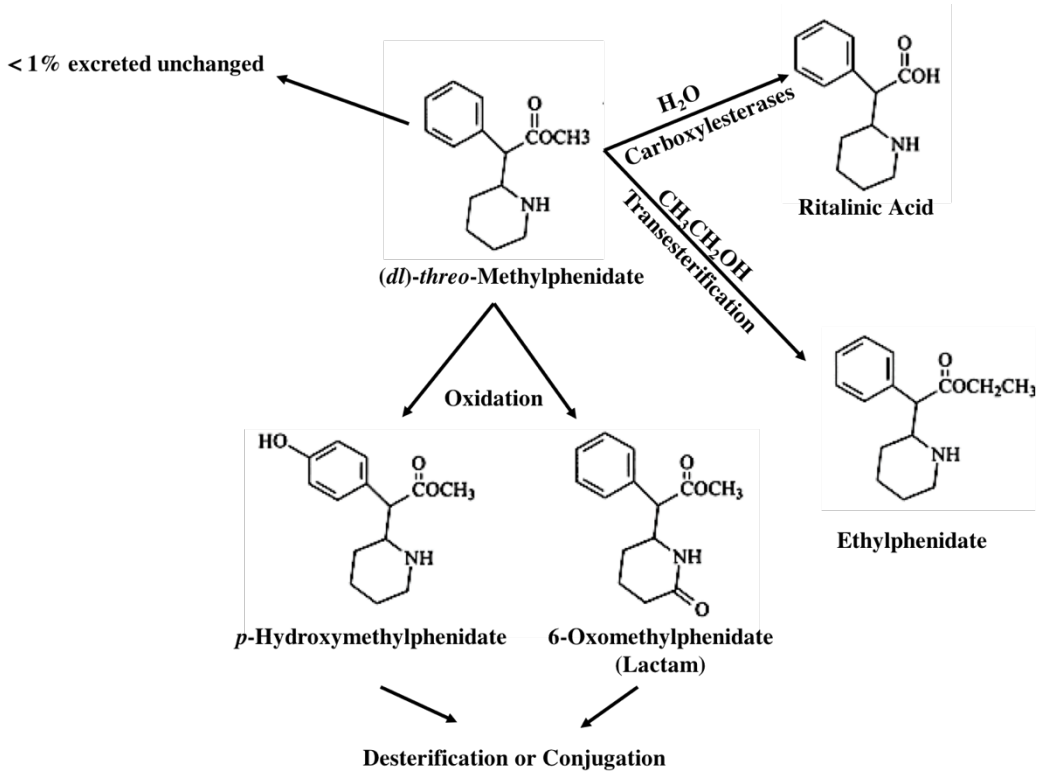


Figure 1.3. Metabolic pathways of methylphenidate (MPH). *MPH is metabolized predominantly by hydrolysis (de-esterification) to the pharmacologically inactive ritalinic acid. Carboxylesterase (CES) 1A1 has been shown to be the major enzyme responsible for the stereoselective hydrolysis of MPH. The major metabolite of MPH identified in urine is the hydrolytic metabolite ritalinic acid, accounting for 80% of the total urinary excretion, following both oral and i.v. administration, while unmetabolized MPH accounted for less than 1%. Co-administration of dl-MPH with ethanol results in CES1-mediated enantioselective transesterification of l-MPH to l-Ethylphenidate. In addition, other minor metabolites are produced through oxidation and subsequent conjugation or hydrolysis, including the pharmacologically active metabolite p-hydroxymethylphenidate.*

About 60%-86% of MPH is excreted as ritalinic acid, less that 1% of MPH is excreted unchanged in the urine and the rest is eliminated as minor metabolites (Patrick et al., 1987). Regarding the MPH bioavailability there are differences between enantiomers. After oral administration of *dl*-threo-MPH, the bioavailability of *d*-threo is 23% and of *l*-

threo-MPH 5% (Srinivas et al., 1993). These low values suggest that MPH is highly metabolized presystemically.

Besides the widely use and misuse of MPH further investigation is needed to improve our knowledge about the pharmacological profiles of its enantiomers, as well as the contribution of its metabolites for MPH pharmacodynamics (Dinis-Oliveira, 2016).

1.2.2 Consumption and dosage

According to the report of International Narcotics Control Board for 2014, MPH worldwide consumption in 2013 was around 718 tons. In Portugal, IMS Health communicate state that Portuguese pharmacies sold 270 492 drugs with MPH in 2016, and between 2011 and 2017 the sales of MPH increased 77%. Moreover, according to the General direction of Health (DGS), the National Program for Mental Health states that children in Portugal under the age of 14 years old take about 5 million MPH doses, being more prescribed to children between ages 10 and 14 (de Carvalho et al., 2015).

The optimal therapeutic oral MPH dosage is variable and marked individual variability. Thus, dosage must be titrated for optimal effects in each person, avoiding possible adverse effects. Additionally, the daily regimen for ADHD treatment as well as the gradual increment depends on MPH formulation. In children, the effective dose range is 0.25–1.0 mg/kg MPH, avoiding the daily dose above 2 mg/kg that usually causes adverse effects, such as insomnia, irritability and anxiety. The average daily dose is 20 to 30 mg and most of the reports recommend that the daily dose should not exceed 60 mg, although some individuals may require higher doses (Morton and Stockton, 2000). In adults, MPH is administered orally with a starting dose of 10 mg. The recommended dose is 0.3–1.5 mg/kg/day (Kolar et al., 2008) and daily maximum dose for adults ranges from 80 to 108 mg.

Concerning MPH abuse, intranasal doses have been reported as high as 200 mg (Jaffe, 1991), and intravenous dose ranges from 40 to 1000 mg (Levine et al., 1986).

Since the knowledge about the biology of ADHD in humans is limited, as well the effect of medication on the brain, the research using animal models of ADHD can contribute to better understand the effects of MPH treatment in diagnosed children and adults. Nevertheless, the pharmacokinetics of MPH must be taken into account when comparing the dosages between humans and rodents, since rodents require a higher drug dose to produce the same effect. This difference is thought to result from the increased metabolic

rate in rodents (Gatley et al., 1999). In rodents, a dose smaller than 5.0 mg/kg is considered low and is comparable to dosages being used in the clinical setting (Gerasimov et al., 2000; Brandon et al., 2001; Somkuwar et al., 2013). Dosages between 5.0 and 10.0 mg/kg of MPH are considered moderate (Solanto, 2000; Brandon et al., 2001; Kollins et al., 2001). In addition to obvious difficulties inherent in any interspecies comparison, interpretation of preclinical studies with MPH are even more complicated due to different routes of administration in animals (i.v. and i.p.) compared with humans (oral) (Gerasimov et al., 2000). Also, the response to psychostimulants has been reported to be age-dependent (Brandon et al., 2001). Rats exposed to MPH at an early age experienced behavioral changes that endured into adulthood, suggesting that MPH has a long-term effect on normal neurodevelopment (Brandon et al., 2001; Andersen et al., 2002). The majority of studies are with adolescence and/or adult rats. There are only few studies using MPH in rats until 4-5 weeks that mimics human childhood, and the administrations is variable. Moreover, the majority of these studies use healthy/control rats instead of an adequate ADHD model.

Criteria for assessing models for ADHD were proposed (Sagvolden, 2000). An ADHD model must conform to three validation criteria: face, construct, and predictive validity. Face validity is the ability to fundamentally mimic the behavioral clinical characteristics of the disorder; construct validity corresponds to a theoretical rationale for the disorder; and predictive validity is the ability to forecast previously unknown aspects of behavior, genetics, and neurobiology of the disorder from the model. The spontaneously hypertensive rat (SHR) is the most well characterized and frequently used animal model of ADHD (Sagvolden, 2000; Adriani et al., 2003). It is a genetic model bred from progenitor Wistar Kyoto rats (WKY) (Okamoto and Aoki, 1963), which is used as a control to the SHR strain (Johnson et al., 1992; Sagvolden et al., 2009). Of all the animal models that have been proposed, the SHR best fits the criteria for ADHD especially in children (Sagvolden, 2000; Adriani et al., 2003; Sagvolden et al., 2005b). When SHRs are tested with correspondent behavioral schedule used in children, they exhibit all the behavioral characteristics of ADHD as operationalized: sustained attention deficit without obvious sensory problems, motor impulsiveness, and hyperactivity that develops over time in novel situations with few reinforcers (Sagvolden et al., 2005b). The deficits observed seem to be related to dysfunction in the fronto-striatal system. In fact, the SHR has an impaired release of DA in the PFC, NAc and caudate-putamen (Myers et al., 1981; Jones et al., 1995; Russell et al., 1995; Watanabe et al., 1997). Moreover, young male

SHR have an increased density of D1 and D5 receptors in the neostriatum and NAc (Carey et al., 1998), and reduced expression of the D4 receptor gene in the PFC (Li et al., 2007). The limitation of this model is the fact that it is hypertensive; however, hypertension develops only in adults and so prepubertal SHR (4-5 weeks old) and pubertal/young SHR (up to 10 weeks of age) show hyperactivity without hypertension symptoms (Sagvolden, 2000).

1.2.3 Mechanism of action – molecular pharmacology

Although the mechanism of action is not completely understood, MPH is considered an indirect adrenomimetic since it does not act directly on the adrenoreceptors (Figure 1.4).

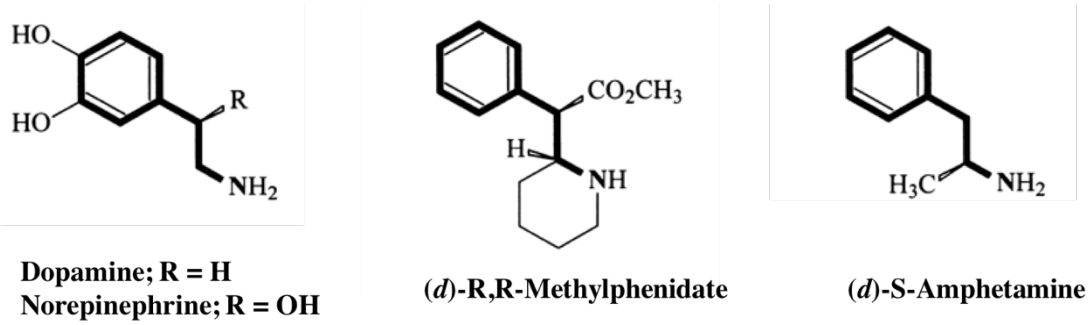


Figure 1.4. Chemical structures of the neurotransmitter dopamine and norepinephrine, methylphenidate and amphetamine.

By competing for the uptake binding sites (DAT and NET), MPH inhibits the reuptake of DA and NE. Thus, the therapeutic effects of MPH are postulated to be mediated by the increase of DA and NE levels in the synaptic cleft (Figure 1.5), which will increase cellular signaling promoting prolonged neuronal activity.

Major CNS DAergic circuits comprise the mesolimbic, mesocortical, and nigrostriatal pathways, which are responsible for the attention and executive functions, emotional motivation responses, reward systems, and motor control (Wise, 2004). Regarding NEergic regions, of particular interest is the hippocampus, important in memory consolidation, the PFC, which processes cognitive functions, and the medial basal forebrain that mediates arousal (Samuels and Szabadi, 2008). In addition to DA, NE has also a key role on providing the needed stimulation and proposed activation of the motor inhibitory system in the orbital-frontal-limbic axis.

As abovementioned, converging evidence has demonstrated that ADHD symptoms arise from dysregulation of PFC/striatal and cerebellar circuits. Genetic defects in DA

metabolism cause a hypodopaminergic state in the limbic system and frontal lobes, resulting in a compensatory increase in DAergic activity in the basal ganglia. Deficits in PFC function lead to poor impulse control, distractibility, hyperactivity, forgetfulness and poor organization and planning. There is a general agreement that ADHD involves weakened PFC function, and that medication might strengthen PFC abilities. There is evidence that the action of MPH on the PFC would be related primarily with NEergic modulation promoting cognitive enhancement in working memory and associative learning (Mehta et al., 2000). Moreover, in the PFC there are a few D2 receptors, while D1 receptors are the more abundant ones (Kirouac and Ganguly, 1993; Watanabe et al., 1997).

In the NAc, essential for acute drug reward, pleasure and motivation, the increase in DA is related with increased motivation and consequently an improved task performance (Volkow and Swanson, 2003). In the basal ganglia, ADHD hyperactivity is associated to excessive DA activity. MPH decreases the DAergic stimulation in this area, due to the high density of D2 receptors (Volkow et al., 2002). Furthermore, MPH decreases the functional connectivity by direct stimulation of GABAergic inhibitory projections and indirect pathways of the basal ganglia (Ramaekers et al., 2013). The increase on this inhibition results in a decrease of impulsiveness, helping the patients focus their attention. The striatum contains very few NET, and thus the actions of MPH on the NE system have received far less attention. Consequently, the action of MPH in the striatum seems mediated by DAT and are clinically related with improved attention and decreased distraction (Volkow et al., 2001).

Overall, the current data are consistent with the hypothesis that at clinical doses, MPH improves performance by increasing the availability of DA and NE, which in turn stimulate D1-like and α2 receptors, preferentially within the PFC (Arnsten and Dudley, 2005). Importantly, these data indicate that the effect of MPH on NEergic system is as important as the drug's effects on DAergic system

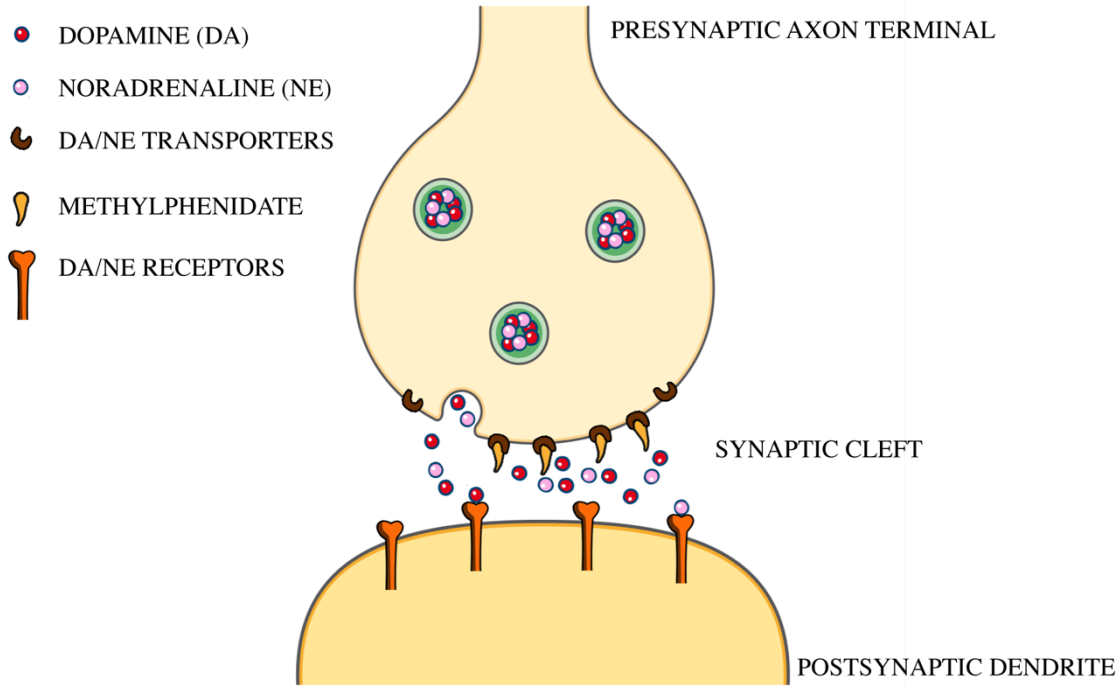


Figure 1.5. Mechanisms through which methylphenidate (MPH) increases the synaptic levels of dopamine (DA) and norepinephrine (NE). *MPH is capable of directly bind to DA/NE transporters, inhibiting DA/NE influx into the cell and leading to the accumulation of these neurotransmitters in the synaptic cleft. The difference with amphetamine is that MPH does not promote dopamine release from synaptic vesicles.*

1.2.4 Methylphenidate side effects and misuse

MPH is generally well tolerated by patients and side effects are usually mild. The most common are insomnia, decreased appetite, abdominal pain, bodyweight loss, irritability and anxiety. Less frequent side effects such as increased heart rate and blood pressure have also been reported. In fact, MPH treatment can lead to a significant higher heart arrhythmia risk in children and young people with ADHD (Shin et al., 2016), and some patients can also exhibit psychosis and hypersensitivity reactions (Man et al., 2016). MPH is contraindicated to patients suffering from agitation, tension, anxiety (once again due to the possibility of exacerbate these symptoms), glaucoma, motor tics and during treatment with monoamine oxidase inhibitors (Challman and Lipsky, 2000).

Besides therapeutic use of MPH, its misuse is currently a significant problem being the second most abused substance just after marijuana (Lakhan and Kirchgessner, 2012), and the most sold on the black market among adolescents (Urban and Gao, 2014). Misuse refers when the use of MPH was not prescribed by a physician or is not taken according with clinical dosing schedule. MPH nontherapeutic use has increased significantly from

3.6% in 2000 to 5.4% by 2006 in individuals from 18 to 25 years (Lakhan and Kirchgessner, 2012). Importantly, MPH abuse can also lead to psychiatric symptoms of extreme anger, aggressive behavior, repetitive behaviors and toxicity upon overdose (Morton and Stockton, 2000). The rapid release of synaptic DA produces an immediate "high" and euphoria contributing to a pleasing sensation that can be accompanied by paranoia, delusions, confusion and hallucinations (Morton and Stockton, 2000).

MPH has also been used to achieve an enhancement of intellectual capacity, better working memory and sustained attention (Ilieva and Farah, 2013). This improvement of cognitive performance seems to occur particularly at low doses of MPH, 0.5 to 2 mg/kg in normal rats comparable to ADHD relevant therapeutic doses, while higher doses impaired performance (Arnsten and Dudley, 2005; Urban and Gao, 2014). At optimal doses, DA and NE bind with higher affinity to D1-like receptors and α2 receptors, respectively. There is a co-work between both neurotransmitters where NE improves response inhibition, working memory and decreases distractibility through interactions with α2 adrenoceptors on the PFC, DA enhances the working memory through D1 receptors also in PFC. This results on a strengthening neuronal communication and consequently cognitive improvement. Particularly, the stimulation of D1 receptors results on a significant extracellular signal-regulated activated kinase 1 and 2 (ERK1/2) pathway activation and, together with PKA/DARPP-32 signaling, D1 receptors phosphorylate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptor subunits (Sarantis et al., 2009), which are responsible for synaptic plasticity and involved in cognition. Moreover, dopamine D1/D5 receptors activate the cyclic adenosine monophosphate/phosphokinase A/cAMP response element binding protein pathway (cAMP/PKA/CREB) improving synaptic plasticity and memory (Otmakhova and Lisman, 1998). Nevertheless, when these levels are higher than optimal, DA and NE activate D2-like receptors and NEergic receptors α1 and β, causing weakening of the signal-to-noise ratio via activation of neurons that may not be involved in the current task (Arnsten, 2009). This nonspecific activation impairs attentional selectivity and results in a manifestation of locomotor hyperactivity, distractibility and poor impulse control.

Other studies suggest that MPH also improve emotion and motivation-related processes in healthy participants (Ilieva and Farah, 2013). In addition, this stimulant is used by healthy professional athletes since it can have several boost effects improving task perception, attention, concentration, balance, and enhancing acceleration together with

the decrease of fatigue (Svetlov et al., 2007; Nazeer et al., 2014). MPH is indeed strictly regulated by International Olympic Commitee (IOC), being only allowed to athletes with adequate documentation of ADHD diagnosis and continued follow up (Nazeer et al., 2014).

It is important to keep in mind that according to FDA drug classifications or drug schedules, MPH is considered a drug schedule II, which means that is medically accepted but has high potential for abuse which may lead to severe psychological or physical dependence. In fact, the brain areas that comprise the clinical effects of MPH are the same that are involved in the different mechanisms of chemical dependence, and the increased levels of DA found in areas of the limbic system are similar to the reinforcing effects observed for drugs of abuse. As abovementioned, physiological and pharmacological effects of MPH are similar to cocaine or amphetamine, since they all block DA and NE transporters. Moreover, studies have shown that MPH has similar drug-seeking reinforcing effects as cocaine (Volkow et al., 1997; Kollins, 2003). Likewise, when both drugs are intravenously administrated are indistinguishable. However, since MPH has a slow clearance rate from brain compared to cocaine, it presents less abuse potential (Kollins, 2003). MPH abuse by intravenous or inhalation routes can provoke intoxication effects as tachycardia, hypertension, paranoia, delirium, agitation, and hyperactivity. As a consequence of the long-term use of MPH, symptoms such as tolerance, compulsive drug use, anorexia, personality changes, depression, and abstinence may be observed (Parran and Jasinski, 1991). In MPH-naive subjects, the toxic dose may be very close to the therapeutic dose when compared with patients under long-term treatment. Cases of intoxication have been described after the administration of chewed or crushed tablets at doses ranging from 2 mg/kg to 60 mg for MPH- Immediate Release and 4 mg/kg to 120 mg for MPH-extended release (Scharman et al., 2007). Conversely, several studies on the clinical use of MPH attest its effectiveness and low toxicity (Mehta et al., 2000) and recommend its use with a personalized medical prescription.

1.2.5 Neurotoxicity of MPH

MPH exhibits low plasma protein binding (approximately 15%) and has a small molecular weight of 269.8 Da. Moreover, it can efficiently traverse cell plasma membranes including the BBB by passive diffusion.

As abovementioned, this Schedule II CNS stimulant exerts its pharmacological effects

via preferential blockade of the DAT and NET. It is recognized that MPH results in an increase of DA/NE levels on the synaptic cleft. In fact, the excess of DA has been shown to be toxic both *in vitro* and *in vivo* due to the production of superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the dopamine quinone (Graham, 1978; Filloux and Townsend, 1993; Miyazaki and Asanuma, 2008). Both acute and chronic treatment with MPH have been shown to result in superoxide production in the brain (Gomes et al., 2009). DA can also induce an inflammatory response in the brain characterized by an increase in cytokines and chemokines (Le et al., 2001; Gomez-Santos et al., 2007) that lead to microgliosis. In line with this, it was observed a robust glial activation in primary cultures (Suzuki et al., 2007) and in rodent brains after MPH exposure (Bahcelioglu et al., 2009; Sadasivan et al., 2012; Schmitz et al., 2016b).

Furthermore, MPH can lead to neuronal dysfunction or even death (Brandon et al., 2003; Gopal et al., 2007; Sadasivan et al., 2012; Urban et al., 2012; Schmitz et al., 2016b). Bart and co-works showed that low doses of MPH downregulated several synaptic proteins in PC12 cells (Bartl et al., 2010). In accordance, a recent study demonstrating that a chronic misuse of MPH results in a decrease on synaptic machinery, including downregulation of ERK and PKCaMII signaling pathways that are associated with neuronal loss, which culminated in exploratory activity and object recognition memory impairment (Schmitz et al., 2016b). DNA damage was also described in the striatum of young and adult rats chronically administrated with MPH (Andreazza et al., 2007).

Alterations in energy metabolism via mitochondrial function impairment (Fagundes et al., 2007; Fagundes et al., 2010a; Fagundes et al., 2010b) have been also suggested to mediate MPH-induced neurotoxicity. In fact, young healthy rats chronically treated with MPH present a dose-dependent increase of oxidative damage, protein carbonyls formations, as well as lipid peroxidation (Martins et al., 2006; Schmitz et al., 2012). In adult rats, there are several evidences that repeated MPH treatment in both healthy (Motaghinejad et al., 2016) and ADHD conditions (Comim et al., 2014) increases brain oxidative stress levels.

Despite the extensive use of MPH in school age and adult populations with ADHD, there are only a few studies that have investigated the neuropathological consequences of long-term MPH exposure (Schmitz et al., 2016b). Consequently, many questions remain answered regarding signaling mechanisms together with the downstream effector and molecular endpoints adjacent to MPH use. Thus, the effect of MPH on CNS and the long-term neurochemical and neurobehavioral outcomes remain unknown.

1.3 Blood-brain barrier

More than 100 years ago, Paul Ehrlich demonstrated that a blue dye injected into the circulatory systems stained tissues of the whole body except the brain and spinal cord (Ehrlich, 1885, 1904). This effect was firstly attributed to a low affinity of nervous tissue to the dye. Lately, an Ehrlich's student, Edwin Goldman, performed the opposite experiment and noticed that injecting trypan blue directly into cerebro-spinal fluid was able to stain the brain but none of the peripheral organs (Goldmann, 1913). Thus, for the first time was suggested the presence of a barrier between the peripheral circulation and brain. However, the term *bluthirnschranke* (german for blood-brain barrier), was only used by Lewandowsky while studying the limited permeation of potassium ferrocyanate into brain (Lewandowsky, 1900).

The brain homeostasis is skillful and well-maintained through barrier layers localized at three main interfaces between blood and neural tissue or its fluid spaces (Figure 1.6). These three barriers that exist between the blood and the brain are the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (BCSFB), and the arachnoid barrier. The BBB is formed by microvascular endothelial cells (ECs) that constitute the primary interface for the exchanges between the blood and the brain. Brain endothelium present a large surface area (between 12 and 18 m^2 in human adult) and short diffusion distance between neurons and capillaries (Abbott et al., 2010).

The BCSFB is formed by epithelial cells of the choroid plexus facing the cerebrospinal fluid (CSF), protecting the brain and the spinal cord. The BCSFB fenestrated cells are leaky but between epithelial cells at the apical surface (the CSF-facing surface) exist intercellular tight junctions. The avascular arachnoid epithelium lies under the dura and constitutes an effective seal between the CNS extracellular fluids and the rest of the body. Although a relatively small surface area, the arachnoid barrier does not constitute a significant interface for blood-brain exchange (Abbott et al., 2010). Thus, the BCSFB and the arachnoid barrier contain epithelial tight junctions (TJs), rather than endothelial TJs (Engelhardt and Sorokin, 2009; Abbott, 2013).

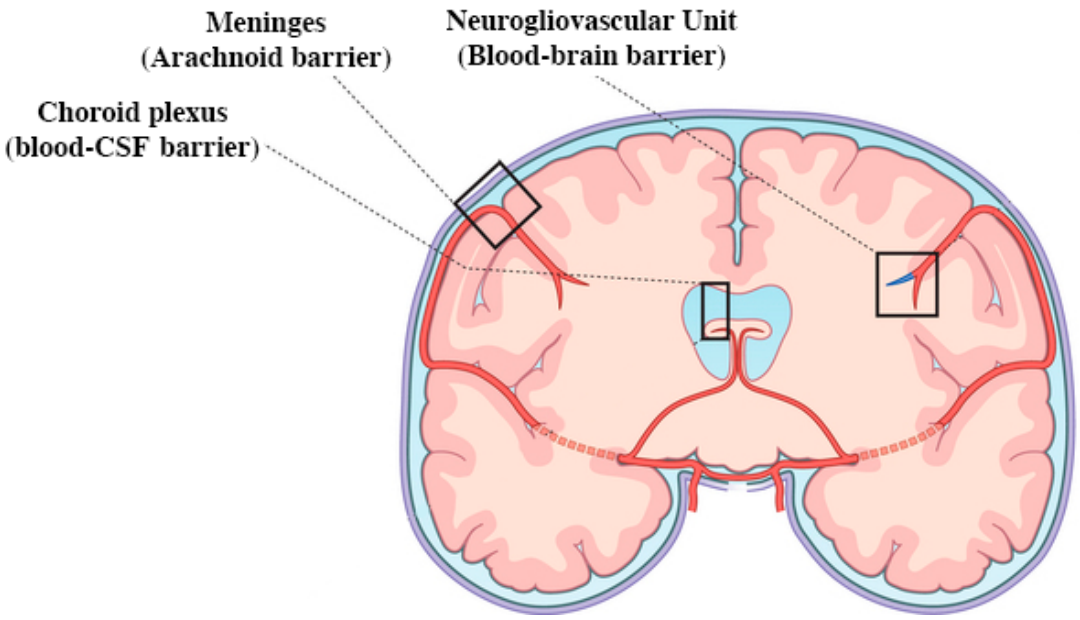


***Figure 1.6. Localization of the blood-neural barriers present in the central nervous system**. The barriers in the brain are localized at three main sites: the choroid plexus epithelium, which secretes cerebrospinal fluid (CSF) and forms the blood-CSF barrier; the arachnoid epithelium that forms the middle layer of the meninges; the brain endothelium forming the blood-brain barrier. At each interface, the tight junctions between cells provide a physical barrier, reducing flux via the paracellular pathway.*

BBB is the bodyguard of the CNS being responsible for protecting the brain from extracellular environment by limiting the entry of toxins, pathogens, and immune cells into the neural tissue (de Vries et al., 1997; Abbott et al., 2010; Cardoso et al., 2010). On the other hand, the intact structure of BBB is a major obstacle for the pharmacological treatment of CNS disorders, since about 98% of small molecules and 100% of large molecules are not able to cross the BBB and reach the CNS (Zlokovic, 2008; Cardoso et al., 2010).

BBB has also an important role to maintain the CNS homeostasis, thought the regulation of ion balance and compounds influx/efflux avoiding changes in normal blood ionic composition. The other main function of BBB is nutrition providing the essential nutrients to all cerebral population by specific and complex transport systems (Abbott et al., 2010; Cardoso et al., 2010). BBB also interact directly with inflammatory cells to act in response to changes in local environment (Persidsky et al., 2006b).

1.3.1 Components of the Neurogliovascular Unit

During brain development, capillaries are differentiated and matured into BBB. This results from multiple interactions between microvasculature and neighboring cells. BBB

is not composed of only one type of cells but rather by different cells such as ECs, pericytes, astrocytes, microglia and also neurons that all together form the neurogliovascular unit (NVU) (Figure 1.7). The complex interactions within this unit promote, regulate and maintain the specialized and important functions of the endothelium.

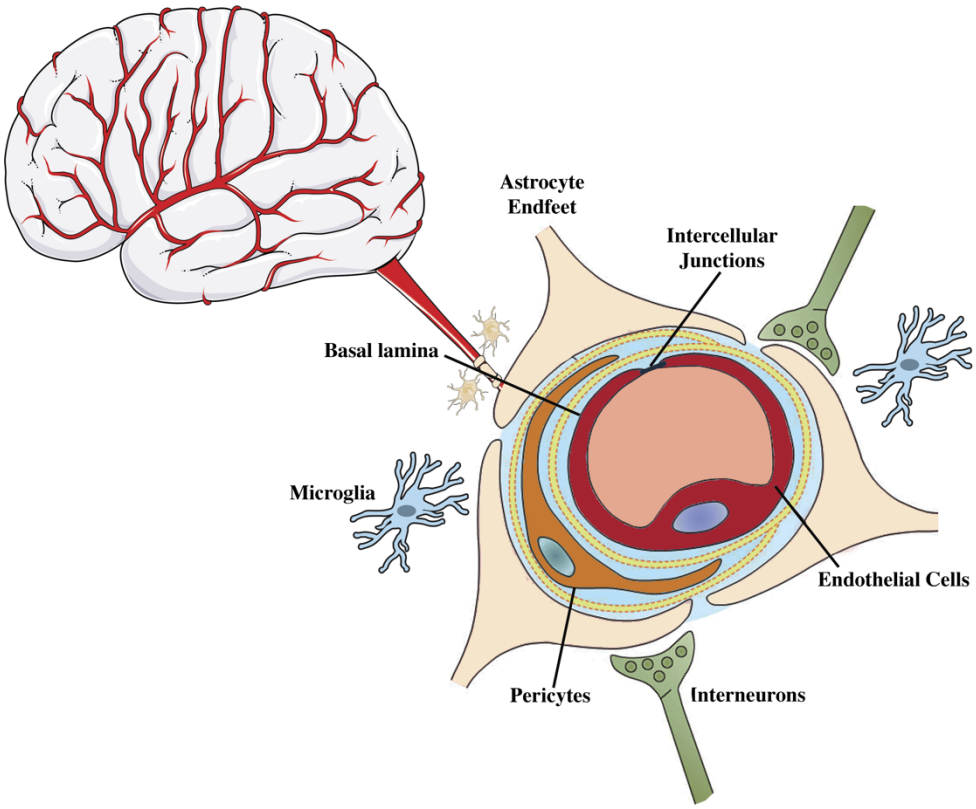


Figure 1.7. Schematic representation of the neuro(glial)vascular unit. The cerebral endothelial cells comprise intercellular junctions, which confer the barrier properties of the blood-brain barrier. *Pericytes are distributed discontinuously along the length of the cerebral capillaries. Both the cerebral endothelial cells and the pericytes are enclosed by basement membrane. Astrocyte foot processes form a complex network surrounding the capillaries. Axonal projections from neurons contain vasoactive neurotransmitters that regulate local cerebral blood flow. Microglial cells have an immune surveillance function in the barrier.*

I. Endothelial cells

The ECs are the most important cells in BBB since they delimitate the brain microvasculature and provide the transport of micro and macronutrients, receptor-mediated signaling, leukocyte trafficking and osmoregulation (Persidsky et al., 2006b). The endothelium represents an efficient physical, biochemical, and metabolic barrier between the blood and the brain due to the presence of dynamic structures, intercellular junctions, between adjacent ECs. There are two types of intercellular junctions in BBB, the TJs and the adherens junctions (AJs) (Tietz and Engelhardt, 2015)(Figure 1.8). The

TJs are formed by transmembranar proteins, claudins and occludin and junctional adhesion molecules (JAM), and by a family of cytoplasmic proteins, *zonulla occludens* (ZO). The AJs are composed by cadherins, the transmembranar proteins, and by catenins, the cytoplasmic component of these junctions. Both TJs and AJs are essential for barrier properties and alterations of these proteins may lead to BBB dysfunction (Persidsky et al., 2006b; Tietz and Engelhardt, 2015). Other structure that normally is less mentioned are the gap junctions. The gap junctions are transmembrane hydrophilic channels that allow the direct exchange of ions and small molecules between adjacent ECs. These junctions can establish homotypic (endothelial-endothelial) or heterotypic (endothelial-pericytes, endothelial-macrophages) communications (Dejana et al., 1995) important to barrier functions of TJs (Nagasawa et al., 2006). The hallmark of BBB endothelium is its highly restricted and regulated permeability to plasmatic compounds and ions, characterized by a very high transendothelial electrical resistance (TEER) (Butt et al., 1990; Petty and Lo, 2002).

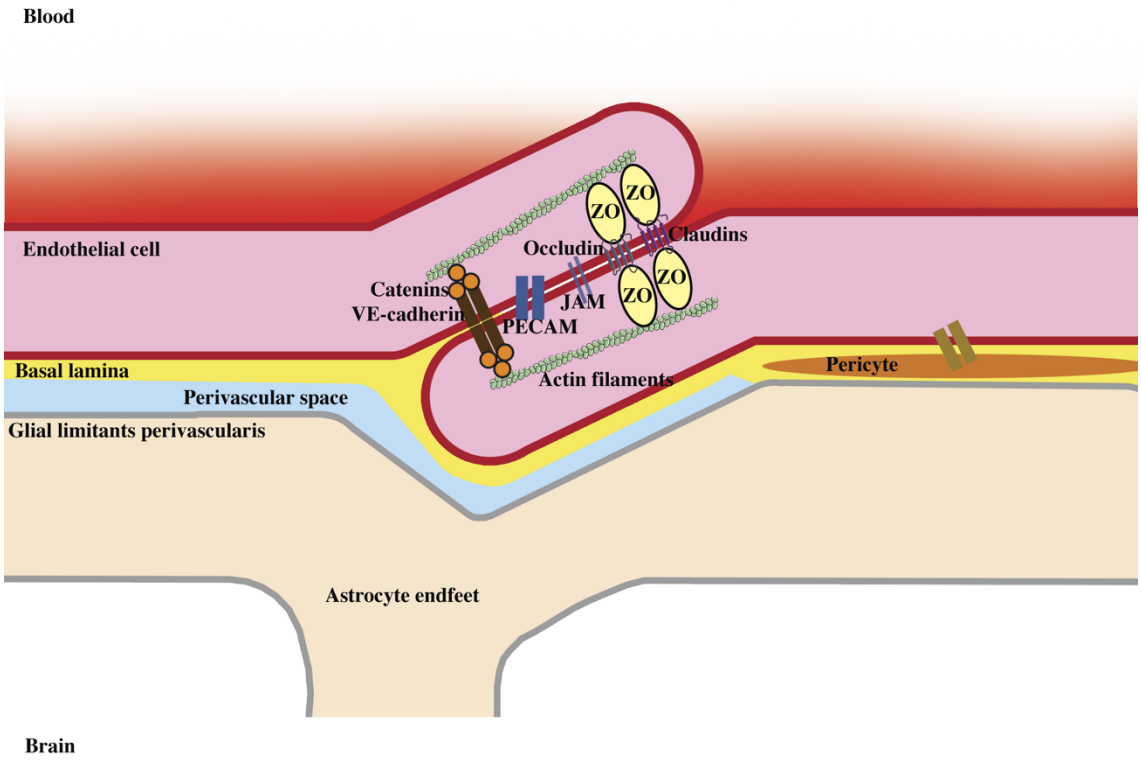


Figure 1.8. Structure of blood-brain barrier complex junctions. *The intercellular complex between two adjacent endothelial cells is composed by tight and adherens junctions. The tight junctional (TJs) complex comprises claudins, occludin and junctional adhesion molecules (JAMs). The claudins and occludin are linked to the scaffolding proteins zonula occludens (ZO) which in turn are linked via cingulin dimers to the actin/myosin cytoskeleton within the cell. In the adherens junctions (AJs), vascular endothelial cadherin (VE)-cadherin proteins are linked to the actin cytoskeleton by the scaffolding proteins catenin. Abbreviations: PECAM, platelet/endothelial cell adhesion molecule.*

Brain ECs differ from those of non-neuronal tissues since they are highly polarized, show absence of fenestrations that limit the movement of molecules and ions between cells (paracellular flux), and also have low rates of pinocytosis/transcytosis, which limits the movement of molecules through the cell (transcellular flux), and are surrounded by a continuous basement membrane (Ballabh et al., 2004; Daneman, 2012). Another structural characteristic of these cells is the great number of mitochondria, providing energy for enzymes to break down compounds and allowing diverse selective transport systems to actively transport nutrients and other compounds into and out of the brain (Cardoso et al., 2010). This distribution of several transporters contributes to ECs polarity.

Tight Junctions

Tight junctions are the ‘zip-locked' structures responsible for barrier properties of ECs regulating the permeability to plasmatic compounds and ions, and characterized by a very high TEER. These proteins are found and concentrated in the luminal side of lateral plasma membrane and act as seal that regulates lateral diffusion between the luminal and abluminal plasma membrane, limiting the paracellular permeability. TJs include members of the claudin family, occludin, and JAMs (Ballabh et al., 2004).

Claudins

Claudins are small transmembrane proteins with a molecular weight around 20-27 kDa, and claudin protein family comprises, until now, 27 members (Gonçalves et al., 2013). Among these, types 3 and 5 are the most expressed in the brain endothelium. Claudins are not only important for barrier formation but are also responsible for the selective permeability of the paracellular pathway (Gonçalves et al., 2013). Claudin-5 seems to be of vital importance for normal function and structure of BBB since knockout animals showed an abnormal BBB function and a high post-natal mortality (Nitta et al., 2003). Accordingly, down-regulation of claudin-5 expression correlates with breakdown of the BBB (Argaw et al., 2009). On the other hand, the overexpression of claudin-5 can improve the barrier function in cultured brain ECs (Ohtsuki et al., 2007). Also, claudin-3 seems to have a role in maintaining the BBB structure since its expression is lost during experimental autoimmune encephalomyelitis and human glioblastoma (Wolburg et al., 2003). Furthermore, new functions of claudin family proteins in response to cellular stress

(Romanitan et al., 2010), as well as in the regulation of embryonic morphogenesis (Gupta and Ryan, 2010) were suggested.

Occludin

Occludin is a 60-65 kDa integral membrane phosphoprotein with four transmembrane domains and two extracellular loops (Ballabh et al., 2004; Cardoso et al., 2010). Its expression is much higher in brain ECs, showing a continuous distribution, when compared to nonneural tissues that display a discontinuous pattern (Hirase et al., 1997). Interestingly, this protein is not essential for BBB formation and structure. Saitou and colleagues (2000) demonstrated that occludin knockout animals did not show any morphologic changes in TJs but have an impairment in development and procreation. However, it was also demonstrated that occludin is responsible for the high electrical resistance of the brain ECs contributing to BBB stabilization and a decrease in its protein levels impairs the BBB function (Huber et al., 2002; Brown and Davis, 2005). Nevertheless, the expression and localization of other junctional proteins can compensate occludin loss (Zlokovic, 2008). In fact, occludin seems to act more in a regulatory context than as a major structural protein in the development of the BBB properties since it is responsible for the association with the cytoskeleton through the accessory protein ZO-1.

JAM

The junctional adhesion molecule (JAM) family is constitute by 3 members (JAM-1, -2 and -3) with the JAM-1 expressed almost exclusively in the brain, whereas JAM-2 is highly expressed in lymphatic endothelial cells, and JAM-3 is found in most endothelial contacts ranging from brain vasculature to high endothelium venules (Aurrand-Lions et al., 2001). These are 40 kDa proteins involved in cell-cell adhesion and in the regulation of leukocytes migration through BBB (Persidsky et al., 2006b). Moreover, JAM are also implicated in the organization and formation of TJs (Vorbrodt and Dobrogowska, 2003) since arbitrate homophilic and probably also heterophilic interactions in the TJs (Vorbrodt and Dobrogowska, 2003; Stamatovic et al., 2008). Although the functions of JAM are still largely unknown in mature BBB, it has been suggested that altered expression of JAM-1, in addition to affecting the junctional tightness, may also disturb leukocyte trafficking, with implications for immune status within the diseased CNS (Zlokovic, 2008).

Cytoplasmic accessory proteins

Cytoplasmic proteins involved in TJs formation include ZO protein family, cingulin, 7H6, and AF-6, among others. Regarding the cytoplasmic proteins, the ZO proteins belong to the family of membrane-associated guanylate kinase (MAGUK) proteins, being the most expressed in ECs and compromising three members, ZO-1, ZO-2 and ZO-3 (Ballabh et al., 2004). The ZO proteins have as main role the connection between the transmembrane proteins, such as claudins and occludin, with the actin cytoskeleton. The ZO-1, a 220 kDa phosphoprotein, is the most expressed in brain ECs and it is essential for normal localization of occludin and also TJs formation (Kniesel and Wolburg, 2000). Therefore, the loss of ZO-1 expression can be associated with the increase in BBB permeability. In addition, ZO-1 may also act as a signaling molecule by communicating the state of the TJs to the interior of the cell, or vice-versa (Gottardi et al., 1996). For instance, under conditions of proliferation and injury, ZO-1 confines to the nucleus interfering with transcription factors (Gottardi et al., 1996). The ZO-2 protein (160 kDa) in addition to structural features is also related with gene expression, as a nuclear transcription factor, and cell cycle progression (Gonzalez-Mariscal et al., 2009; Wolburg et al., 2009). ZO-3 (130 kDa) share structural homology with ZO-1 and ZO-2; however the function of this ZO family member is not well understood but it is capable of binding to both occludin and ZO-1 (Haskins et al., 1998). Additionally, while KO mice for ZO-1 and ZO-2 exhibited embryonic lethality, KO mice for ZO-3 are viable, suggesting that ZO-3 is not essential *in vivo* (Katsuno et al., 2008; Xu et al., 2008).

Phosphoprotein cingulin (140 – 160 kDa) is localized at the cytoplasmic surface of TJs of both endothelial and epithelial connected with ZO and JAM proteins, AF-6 and myosin, implying a role as a scaffold between TJs cytoplasmic face and the cytoskeleton (Cordenonsi et al., 1999). Further, this protein has a role in the regulation of TJs permeability (Cordenonsi et al., 1999). The phosphoprotein 7H6 (155 kDa), also found at both epithelial and endothelial, seems to be correlated with TJs impermeability to ions and large molecules playing a role in the maintenance of paracellular barrier function (Satoh et al., 1996). There is evidence that this protein may detach from TJs when ATP levels decrease, resulting in increased paracellular permeability (Mitic and Anderson, 1998). AF-6, a 180 kDa protein, contains two Ras-associating domains, a PDZ domain, and a myosin V-like domain, and has been reported either at the TJs or at the AJs (Bazzoni and Dejana, 2004; Hawkins and Davis, 2005), and the disruption of the ZO-1/AF-6

complex seems be critical in the modulation of TJs by pathways that involve Ras activation (Yamamoto et al., 1997).

Adherens Junctions

The AJs are localized below TJs in the basal region of lateral plasma membrane and more close to the abluminal side of ECs, and are responsible for cell-cell adhesion, for cell polarity, and regulation of paracellular permeability (Hawkins and Davis, 2005). AJs act as a gate between luminal and abluminal sides giving the tissue a structural support, mediating the cell–cell adhesion through actin filaments linking. In the transmembrane domain of this junctions the glycoproteins cadherins can be found. In addition to contributing to the barrier function during vascular growth and remodeling, AJs also modulate the contact inhibition (Carvey et al., 2009).

The vascular endothelial (VE)-cadherin is expressed only in cells of vascular epithelial origin (Navarro et al., 1998) mediating cell-cell adhesion via homophilic interactions between the extracellular domains of proteins expressed in adjacent cells (Vincent et al., 2004), and being important for BBB integrity but not formation (Vorbrodt and Dobrogowska, 2003). Nevertheless, ECs also express neuronal cadherin (N-cadherin), which is present in other cell types such as neural cells and smooth muscle cells (Bazzoni and Dejana, 2004). VE-cadherin mediates cell adhesion in a Ca^{2+}-dependent manner, inhibits cell proliferation, and when are overexpressed decreases cell permeability and migration (Tiruppathi et al., 2002; Xu et al., 2016a). However, this protein alone is insufficient to promote junction formation, and must be linked to a group of proteins identified as catenins (Vincent et al., 2004). The catenins are responsible for the connection between cadherins and actin cytoskeleton but also interact with several actin-binding proteins, including ZO-1. There are four different types (α-, β-, δ- and γ-catenin) of catenins with β-catenin being essential in vascular patterning (Vincent et al., 2004). Moreover, β-catenin is linked to the cell membrane in a complex with VE-cadherin and platelet–endothelial cell adhesion molecule (PECAM-1 or CD31), which mediates homophilic adhesion. Despite the role of β-catenin in the anchorage of cadherins in cellular cytoskeleton this protein also plays an important role as transcription factor, regulating several genes and participating in signaling pathways after moving to the nucleus (Petty and Lo, 2002; Vorbrodt et al., 2008). Further, δ-catenin has been implicated as a regulator of the nuclear factor kappa-light-chain-enhancer of activated B cells pathway (NF-κB) transcription factor (Perez-Moreno et al., 2006).

Overall, the cadherin family and their intracellular associated catenin proteins form complexes of central importance to the sorting and morphogenic processes of developing animal tissues and in maintaining the integrity and identity of adult tissues (Vincent et al., 2004; Stamatovic et al., 2008).

II. Basal Membrane

Basement membrane surround ECs and engulfs pericytes, anchoring and supporting cells and establishing the connection with surrounding brain resident cells. This structure is an essential part of BBB being generate and maintain by ECs, pericytes and astrocytes (Cardoso et al., 2010). Basal lamina is composed by different structural proteins (collagen and elastin), specialized proteins (fibronectin and laminin) and proteoglycans (Cardoso et al., 2010). Basal membrane also includes cell adhesion molecules (CAM), as signaling proteins. Alterations and disruption of basement membrane can lead to changes in the ECs cytoskeleton, which in turn affects TJ proteins and barrier integrity. Accordingly, several neuropathologies have been related with BBB permeability mediated by ECM disruption (Hawkins and Davis, 2005; Zlokovic, 2008; Carvey et al., 2009).

Dynamic regulation of basal membrane in both physiological and inflammatory conditions are mediated by matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) (Lu et al., 2011). While some MMPs are secreted into the extracellular space, others are expressed on the cell surface, the membrane-type MMPs (Milward et al., 2007). The major contributor to basal downregulation and degradation are MMPs, a family of zinc-dependent endopeptidases that catalyse the proteolysis of a number of extracellular matrix and basement membrane proteins, including collagens, laminin, glycoproteins and proteoglycans (Milward et al., 2007). They are also capable to cleave TJs (Rosenberg, 2002b). MMP-2 and MMP-9 (also called gelatinases A and B, respectively) are capable of cleaving collagen IV and V, laminin, and chondroitin sulfate proteoglycan, which are associated with cell adhesion (Lu et al., 2011). MMP-9 is probably the best characterized MMP in the CNS. MMP-9 expression has been detected in both limbic and non-limbic structures in adult rat brain, with preferential expression within the hippocampus (Szklarczyk et al., 2002), might because is required for hippocampal long-term potentiation and memory (Nagy et al., 2006).

III. Pericytes

Pericytes, also known as mural cells or even myofibroblast, are found directly abluminal to the endothelium, sharing basement membrane with ECs (Bagley et al., 2005). They have ovoid cell bodies, thin and elongated processes that run for hundreds of micrometers along brain microvessels, covering 22-32% of the capillaries. Pericytes are critical in regulating vessel stabilization and BBB integrity (Sweeney et al., 2016). In fact, the location of pericytes and the coverage of microvessels depend on the type of microvasculature (Allt and Lawrenson, 2001) and seem to be correlated with the degree of tightness of intercellular junctions. In addition, these cells synthetize and release laminal proteins, as proteoglycans, that are thought to be a critical step in BBB differentiation and maturation (Armulik et al., 2010). Furthermore, pericyte-derived angiopoietin can stimulate endothelial expression of occludin (Hori et al., 2004), showing once again the involvement of these cells in the induction and/or maintenance of barrier properties in the cerebral endothelium. In accordance, severe defects in the integrity of the BBB arise in mutant mice that are deficient in pericytes (Armulik et al., 2010; Daneman et al., 2010).

Capillary pericytes have important contractile proteins that allow the regulation of blood flow, presenting rich contents of α-smooth muscle actin (Peppiatt et al., 2006). Pericytes and ECs communicate using innumerous cellular apparatuses such as gap junctions, TJs, adhesion plates and soluble factors (Allt and Lawrenson, 2001; Bagley et al., 2005). There are also evidence showing that pericytes induced and up-regulation of endothelium P-glycoprotein functional activity (Dohgu et al., 2005). Moreover, pericytes are involved in regulatory adjustments in response to stress stimuli, as during severe and prolonged hypoxia situations (Al Ahmad et al., 2009).

Several functions of pericytes relevant to angiogenesis and proliferation have been also proposed. Pericytes may sense the physiological needs of the tissue and the presence of angiogenic stimuli, sense the hemodynamic forces within the vessel, deposit or degrade extracellular matrix, act in paracrine and cell-cell contact-dependent control of endothelial proliferation and differentiation, and contact numerous ECs and thus integrate the signals along the vessel length (Gerhardt and Betsholtz, 2003; Bergers and Song, 2005; Hall et al., 2014). Moreover, pericytic coverage has been suggested to be a key to vascular maturation during the remodeling process that follows new growth of vessels (Benjamin et al., 1998).

IV. Astrocytes

Astrocytes form an endfeet lacework of fine lamellae closely opposed to the outer surface of the BBB endothelium and respective basement membrane (Abbott, 2002; Abbott et al., 2006). Their perivascular astrocytic endfeet are important and highly specialized cellular compartments that are enriched in astrocyte-specific proteins such as water channels aquaporin-4, connexin-43, purinergic receptors, and potassium channels (Simard et al., 2003). Astrocytes control brain water and ion homeostasis, and also connect ECs with close neurons by its processes (Abbott et al., 2006; Belanger et al., 2011).

Astrocytes promote the synthesis of proteoglycan, important protein of basal membrane, which results in an increase in the charge selectivity of brain microvascular ECs (Cardoso et al., 2010). Several studies demonstrated that astrocytes can have a dual role. Astrocytes can release trophic and soluble factors upregulating TJs proteins and decreasing the transendothelial permeability across ECs, important for barrier properties formation and maintenance (Ballabh et al., 2004; Siddharthan et al., 2007; Colgan et al., 2008). On the other hand, astrocytes can release MMPs (Rosenberg, 2002a), proinflammatory cytokines, such as interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) (Abbott, 2002; Abbott et al., 2006; Coelho-Santos et al., 2015), and high concentrations of Ca^{2+} to the extracellular space leading to BBB hyperpermeability (Abbott, 2002). These findings showed that astrocytes may modulate the BBB phenotype without being directly involved in the physical BBB properties. In accordance, in some areas of the CNS the microvessels lack astrocytic ensheathment but still exhibit some BBB features, which are likely due to soluble factors acting from the glia limitans or the subarachnoid CSF (Abbott, 2002). Subsequent studies also showed a transient loss of barrier integrity *in vivo* following a temporary focal loss of astrocytes (Willis et al., 2004; Persidsky et al., 2006b).

V. Microglia

Microglia are the resident immune cells that play a vital role in the immune response. However, these cells are also an important component of the NVU particularly because they survey local microenvironment and change the phenotype according to disturbances of the CNS (da Fonseca et al., 2014). In fact, microglia can be found in the perivascular space and contribute to the BBB properties (Choi and Kim, 2008). On the other hand, microglia are a source of cytotoxic mediators that can induce disruption of TJs (Poritz et al., 2004; da Fonseca et al., 2014) and so having a negative impact on BBB.

VI. Neurons

Neurons are the most widely studied cells in the CNS but little is known about them on BBB proprieties. However, pieces of evidence show that neurons can regulate the blood vessels function in response to metabolic necessities through inducing expression of enzymes unique for ECs (Persidsky et al., 2006b; Cardoso et al., 2010). Particularly due the dynamic nature of neural activity and the considerable metabolic needs of nervous tissue, the microcirculation of the brain must be highly responsive to tissue necessities. In fact, "metabolic coupling" of regional brain activity to blood flow is the basis of functional neuroimaging (Buxton and Frank, 1997). Gliovascular cells are innervated by NEergic, cholinergic, serotonergic and GABAergic neurons, among others (Hawkins and Davis, 2005). Actually, the astrocytes are required to mediate the communication between neurons and ECs. Neurons regulate blood flow by local stimulation and mature endothelium has a reciprocal function in inducing a stable brain environment that allows neuronal activity.

1.3.2 Transport across the BBB

ECs have also a key role in controlling the transport through the brain endothelium (Figure 1.9). The endothelium has an unique pattern of receptors and specific transport systems that facilitate the uptake of important nutrients and hormones, in addition to active pumps that help to regulate the concentrations of ions, metabolites and xenobiotics in the brain (Zlokovic, 2008). Furthermore, the transport across the BBB is limited and highly selective, and can occur by two main routes, the paracellular pathway that take place between adjacent ECs, and the transcellular pathway that occurs through the ECs.

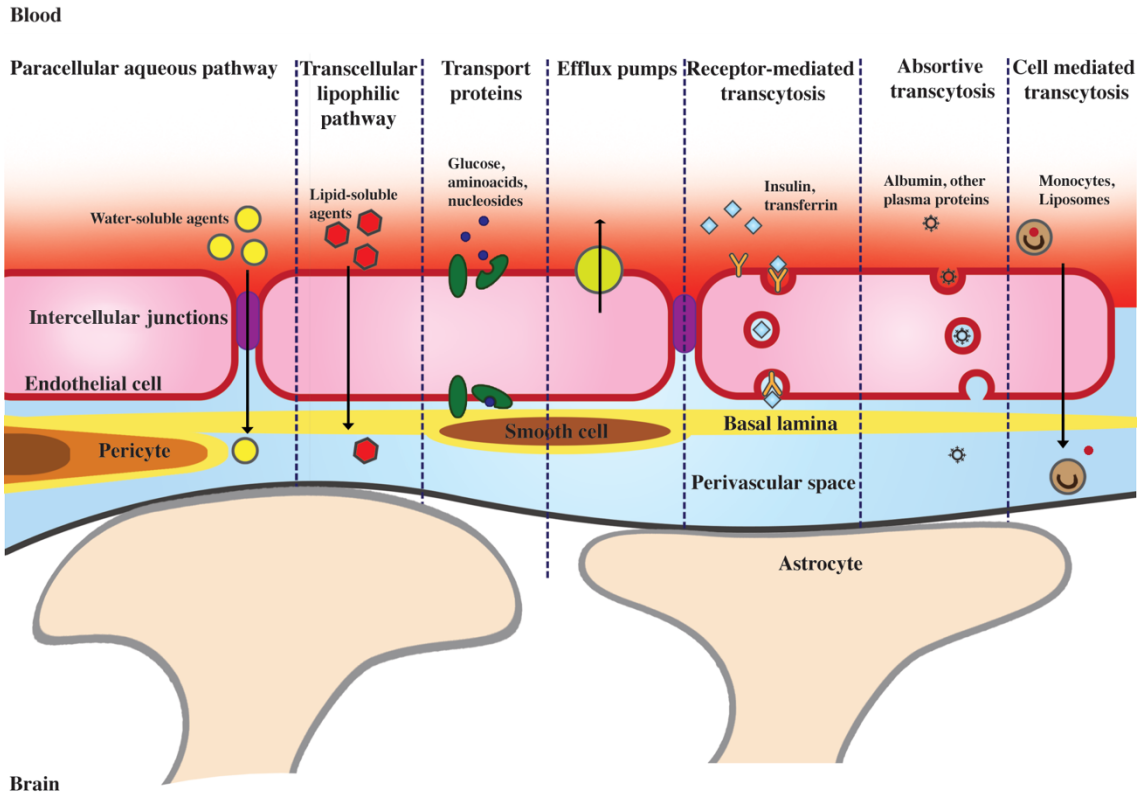


Figure 1.9. Transport mechanisms at the blood-brain barrier (BBB). *The paracellular transport is important for ionic homeostasis. The presence of specific transporters and transcytosis are essential to suppress the nutritional needs of the brain parenchyma. Nevertheless, transcytosis occur at a low level under physiological conditions. The intercellular junctions (Tight and adheren junctions) restrict the passage of water-soluble compounds, including polar drugs. Lipid-soluble molecules can diffuse through the lipid membranes of the endothelium. Active efflux carriers (ABC transporters) may intercept some of these lipophilic solutes and pump them out of the endothelial cells. P-glycoprotein and Breast Cancer Resistance Protein are strategically placed in the luminal membrane of the endothelium, while Multidrug Resistance-associated Proteins are inserted into either luminal or abluminal membranes. Carrier-mediated influx via solute carriers can transport many essential polar molecules such as glucose, amino acids and nucleosides. Receptor-mediated transcytosis requires receptor binding of ligand and transport a variety of macromolecules such as peptides and proteins across the BBB endothelium, while adsorptive-mediated transcytosis is induced in a non- specific manner by positively charged molecules.*

I. Paracellular Pathway - Passive diffusion

The paracellular pathway is used by ions and solutes that cross the BBB accordingly to their concentration gradient (Petty and Lo, 2002). Nevertheless, this type of transport is regulated by TJs and AJs, so impairment of these ECs junctions will increase the permeability by this type of route. Passive transcellular transport of a substance across the BBB depends mostly on physico-chemical properties of compounds, such as molecular weight, hydrophilicity/lipophilicity, electrical charge and hydrogen bond potential. In fact, in health conditions only small and lipophilic molecules can cross the BBB. Small lipophilic molecules, such as oxygen, CO_2 and ethanol, can pass the BBB freely by diffusion (Cardoso et al., 2010). However, some small and large hydrophilic

molecules (e.g. essential nutrients, such as glucose and certain amino acids) with molecular weight no greater than 450 Da and/or 80 Å can enter in brain by active transport and specific transporter mechanisms (Rubin and Staddon, 1999; Daneman, 2012).

II. Transporters: focus on carrier mediated transport and ATP-binding cassette transporters (ABC transporters)

The transcellular pathway can be mediated by membranar transporters or vesicles and can also occur by diffuse transport exclusively for lipophilic compounds (Abbott et al., 2006). Some of the solute carriers are involved in active transcellular transport of hydrophilic and polar nutrients that cannot diffuse through cell membranes. Carrier-mediated transport systems are specific and facilitate the transport of nutrients as follows: hexoses (glucose, galactose); neutral, basic, and acidic amino acids and monocarboxylic acids (lactate, pyruvate, ketone bodies); nucleosides (adenosine, guanosine, uridine); purines (adenine, guanine); amines (choline); and vitamins (Hawkins and Davis, 2005; Zlokovic, 2008). Most of these systems are polarized and are inserted into the abluminal membrane. Likewise, they are active transporters, where energy may be harnessed directly by the hydrolysis of ATP or indirectly by coupling to the cotransport of a countering down its electrochemical gradient (e.g., Na^+, H^+, Cl^-) (Zhang et al., 2002).

Regarding glucose transporter-1 (GLUT1), it is expressed at higher concentration in the abluminal membrane when compared with the luminal side (Cardoso et al., 2010). This asymmetric expression is particular relevant to prevent the accumulation of glucose in the brain in concentrations higher than those present in the blood stream. GLUT1 ensures nutrient delivery, supplying the brain with glucose (Persidsky et al., 2006b), the main energy source. GLUT1 is expressed by brain EC very early during brain angiogenesis and is considered a feature of the early BBB (Zheng et al., 2010). In fact, expression is also required for the formation of tight cell-cell junctions between EC during brain angiogenesis. Mutations in GLUT1 gene cause a deficiency syndrome that leads to mental retardation accompanied by a variety of neurological symptoms (Brockmann, 2009).

Other group of transporters present in ECs are the adenosine triphosphate-binding cassette transporters (ABC transporters), which includes the P-glycoprotein 1 (P-gp), the multidrug resistance-associated proteins (MDR1, 2, 4, 5) and breast cancer-related protein (Bcrp). The ABC transporters acts as efflux pumps of a diversified range of lipid-soluble compounds, removing potentially neurotoxic endogenous, xenobiotic molecules and anionic compounds from the brain, having therefore a vital neuroprotective and

detoxifying function (Abbott et al., 2010). P-gp transporter is three times more expressed in luminal membrane than in the abluminal cell membrane, which prevents the passage of blood-borne molecules into the brain and facilitates their transport out of the brain tissue. Further, P-gp can be found on the cell membrane of astrocytes and pericytes (Bendayan et al., 2006). Noteworthy, ABC transporters although play a crucial role in protecting the brain from neurotoxic compounds, they are also responsible for the limited entry of potential therapeutic drugs to treat CNS diseases.

III. Transcytosis

Macromolecules transcytosis across BBB can be divided in receptor-mediated (RMT) or the adsorptive-mediated (AMT) transcytosis. The RMT involves the binding of a ligand to its specific receptor that will trigger the internalization of the newly formed complex and ultimately leads to its exocytosis on the opposite side of the cell (Herve et al., 2008). BBB has several receptors that can carry ligands into and across the ECs via RMT, as the insulin receptor, the low-density lipoprotein receptor, and the transferrin receptor, the latest being the best characterized receptor system at the BBB (Herve et al., 2008). Otherwise, in AMT an excessively charged molecule interacts with cell surface binding sites inducing the endocytic process (Herve et al., 2008). Solutes that undergo this form of transcytosis include wheat germ agglutinin, basic and cationized peptides, glycoproteins and glycopeptides, and virus (Herve et al., 2008). Thus, a variety of large lipophilic molecules and complexes can cross the BBB by non-specific transcytotic mechanisms. Fluid-phase transcytosis is a constitutive and non-specific process in which solutes together with extracellular fluid are caught in the lumen of the vesicle that are formed at the surface and then enters the cell (Predescu et al., 2007; Hawkins and Egleton, 2008). This transport process is independent of any interaction between the transported molecule and the vesicle membrane. Solutes that undergo fluid-phase endocytosis at the BBB include horseradish peroxidase (HRP) (Defazio et al., 1997) and Lucifer yellow (Guillot and Audus, 1990). Noteworthy, brain ECs have a low pinocytic activity (Claudio et al., 1989), which highly increase under pathological conditions. Indeed, endocytotic vesicles have an important role in transport into and out the brain, and the most studied are the caveolae-derived vesicles.

Caveolae

Caveolae or "small caves" were first identified in 1953 by George Emil Palade and were described and named 'caveola intracellularis' by Yamada in 1955 (Yamada, 1955; Palade and Bruns, 1968). Caveolae are dynamic pieces of membrane with a defined omega (Ω) shape and a diameter of 60-80 nm, which can only be unambiguously identified by electron microscopy (Yamada, 1955; Palade and Bruns, 1968). These vesicles are either opened to receive and release material or closed to process, store, and deliver (Anderson, 1993).

Caveolins (18–24 kDa) are integral membrane proteins that constitute the major protein component of caveolae vesicles. Three mammalian caveolin-resident genes [cav; firstly, described as VIP-21 (Glenney and Zokas, 1989)] were identified: caveolin-1 (Cav1), cav2 (Cav2) and cav3 (Cav3). Smooth muscle expresses all three isoforms, while skeletal and cardiac muscle express only cav3. Cav1 and 2 are also expressed in non-muscle cells. Caveolae formation is strictly dependent on Cav1 or Cav3, depending on the tissue (Le Lay and Kurzchalia, 2005). In BBB endothelium, the major structural protein of caveolae is Cav1 (Virgintino et al., 2002) suggesting that may be involved in BBB function. Other cells of NVU also express Cav1, like pericytes and vascular astrocytes (Virgintino et al., 2002). Approximately 144 caveolin molecules have been suggested to be incorporated in a single caveolar structure (Pelkmans and Zerial, 2005). Cav1 oligomerizes in the endoplasmic reticulum after synthesis and is transported to the Golgi apparatus where oligomerized Cav1 interacts with cholesterol. Caveolae formation is highly dependent of cholesterol and glycosphingolipids, as its depletion flattens caveolae. Microtubules are involved in the trafficking of cav1-positive vesicles to different organelles and may also regulate the density of caveolae. There is a functional and biochemical interaction between actin-crosslinking filamin proteins and caveolin where integrins have a role helping caveolae trafficking (Head et al., 2006). Intracellular caveolar vesicles are concomitantly recycled to the plasma membrane (Pelkmans and Zerial, 2005). A second family of proteins, the cavins (a family of four proteins), also known as polymerase I and transcript release factor has been shown to be important in caveolae formation (Hill et al., 2008). Still, more studies are needed to define the role of each cavin and their interactions with caveolins. It was recently identified the membrane curvature regulator pacsin2, which has been implicated in sculpting and caveolae formation (Hansen et al., 2011; Senju et al., 2011).

The signaling mechanism that mediate the release of caveolae from the plasma membrane is not fully understood. Phosphorylation events are the most accepted since caveolar fission is increased by phosphatase inhibition and decreased by kinase inhibition (Parton et al., 1994). Cav1 is phosphorylated by Src family kinases on tyrosine residue 14 (Li et al., 1996), suggesting a relationship between tyrosine kinase activity and release of caveolae from the membrane (Place et al., 2011).

Caveolae are sites of endothelial transcytosis, endocytosis (Schnitzer and Oh, 1996), lipid and cholesterol regulation (Fielding and Fielding, 1995), signal transduction, and act as docking sites (Schlegel and Lisanti, 2001) (Figure 1.10). The transport of albumin occurs through a caveolae-dependent transcytosis (Predescu et al., 2004) involving the gp60 receptor that is localized in caveolae (Tiruppathi et al., 1997; Schubert et al., 2001). Importantly, in contrast to WT, cav1 KO ECs are not able to transport albumin by transcytosis (Schubert et al., 2001).

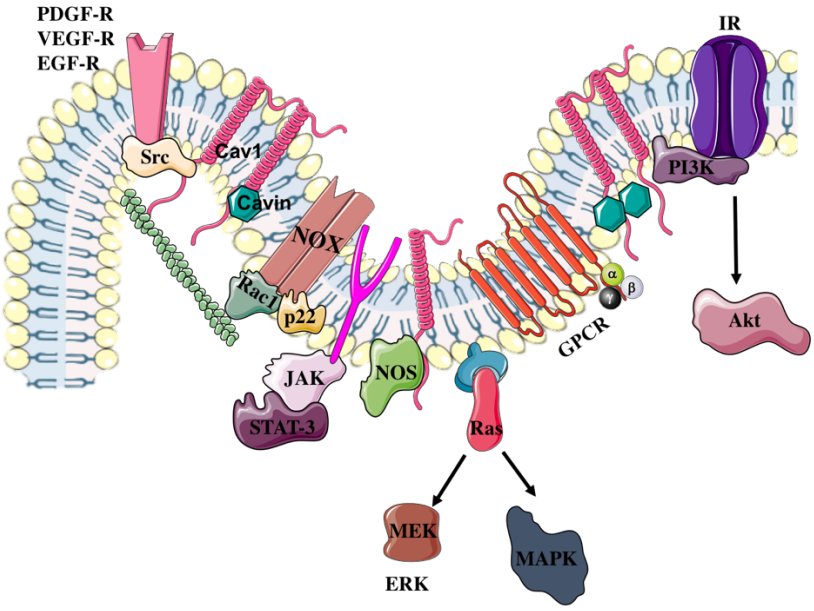


Figure 1.10. Participation of Caveolin-1 in signal transduction. *Caveolin-1 (Cav1) functions as a broad-spectrum kinase inhibitor with a relevant role in cytokine signaling. G-protein–coupled receptors (GPCR), G-proteins (α and βγ), and receptor tyrosine kinases can reside in or translocate in and out of caveolae. Cav1, the primary coat protein of caveolae (typically oligomeric in cells, but shown graphically as half hairpin) may directly regulate protein function or indirectly by regulating Ras-Stat, extracellular signal-regulated kinases (Erk), and Janus kinases (Jak)-Stat signaling pathways. Lipid-modified proteins such as endothelial nitric oxide synthase (eNOS) and the Src family of kinases can target to caveolae and interact with Cav1.*

The broad spectrum of signaling pathways sensitive to caveolae is remarkable, with impacts on gene expression, cell proliferation, cell growth, directional cell migration, and

extracellular matrix remodeling (Minshall et al., 2003) (Figure 1.10). Caveolae is a platform for many different signaling pathways namely because Cav1 acts as a scaffold to interact with and to regulate signaling molecules that are located in this structure (Patel et al., 2008). Cav proteins serve as both positive and negative regulators of intracellular signaling. For instance, receptor tyrosine kinases [eg. mitogen-activated protein kinase (MAPK)], epidermal growth factor receptor, verve growth factor receptor, nitric oxide synthase (eNOS), G proteins, protein kinase C and Src family protein are negatively regulated by cav, whereas insulin receptor, estrogen receptor and Ephrins Receptors are positively regulated (Patel et al., 2008).

Among the binding partners of cav, the first non-receptor proteins found to be localized to plasma membrane caveolae was endothelial eNOS (Feron et al., 1996). Under basal conditions, Cav1 inhibit eNOS activity by binding to eNOS in brain ECs (Chen et al., 2012). Besides regulate nitrite oxide signaling, Cav1 is also important for redox signaling and function. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) complex may be preassembled and functional in caveolae, and its enzymatic activity enhanced by recruitment of additional components (Yang and Rizzo, 2007). Cav1 can serve as a sensor of stress stimuli in ECs and thereby regulate ROS-mediated signaling via NOX.

Is well know that the subcellular localization of ion channels and pumps is critical for their regulation and impact on cell function. Ca^{2+}-ATPase, inositol 1, 4, 5-trisphosphate receptors, Ca^{2+} pumps, L-type Ca^{2+} channels, large-conductance Ca^{2+}-activated K^+ channels, calmodulin and transient receptor potential channels, Na^+ and Cl^- channels are targeted to caveolae and associate with caveolins. Some findings stated that alterations in caveolin expression may shift the localization of channels, thereby altering cellular excitability and functional activity (Patel et al., 2008).

Several neuropathologies have been associated to caveolar dysfunction. Head and colleagues (2010) demonstrated that loss of cav1 in a transgenic mouse model produces neuropathology similar to that exhibited with Alzheimer, i.e., Aβ production, elevated astrogliosis, reduced cerebrovasculature and neuronal loss in the hippocampus, accelerating neurodegeneration and aging. At BBB, cav1 seems to be a critical determinant of its permeability by regulating TJs protein expression and MMPs activity (Zhong et al., 2008a; Stamatovic et al., 2009). Actually, both occludin and ZO-1 are organized within the TJs by association with cav1 in detergent-insoluble glycolipid rafts, membrane specializations closely related to caveolae. Besides, in the rat cortical cold

injury model, increased expression of Cav1 precedes the decreased expression of occludin and claudin-5 (Nag et al., 2007), and cav1 signaling mainly exists in the form of phosphorylation in early BBB breakdown (Nag et al., 2009). The deletion of the Cav1 gene increased BBB disruption, degradation of TJs proteins, and proteolytic activity of MMP-9 and MMP-2 in infarcted mice subjected to photothrombosis. Interestingly, Cav1 re-expression ameliorated vasogenic cerebral edema, TJs protein degradation, and MMP activity during cerebral ischemia (Choi et al., 2016).

It is clear that the study of the mechanisms underlying caveolae biogenesis and function, as well as the individual proteins involved in caveolae assembly and function, is of crucial interest in different fields of research.

1.3.3 BBB in pathology

Chronic BBB dysfunction can contribute to persistent neurological deficits and exacerbate the overall brain pathology, as it leads to extravasation of immune cells, poorly regulated flux of molecules and ions, as well as impaired transport processes. In fact, the dysfunction of the BBB is a characteristic feature in several inherited CNS disorders and pathologies (de Vries et al., 1997; Zlokovic, 2008; Carvey et al., 2009; Rosenberg, 2012), including Alzheimer's disease (Algotsson and Winblad, 2007), Parkinson's disease (Kortekaas et al., 2005), Huntington disease (Drouin-Ouellet et al., 2015), infectious or inflammatory processes (Leppert et al., 2001; Argaw et al., 2012; Chai et al., 2014), hypoxia and ischemia (Latour et al., 2004; Zhu et al., 2010), multiple sclerosis (Shinohara et al., 2012), amyotrophic lateral sclerosis (Zhong et al., 2008b), epilepsy (van Vliet et al., 2007), brain tumors (Wolburg et al., 2003), pain (McCaffrey et al., 2008), and lysosomal storage diseases (Begley et al., 2008).

Multitude of factors can cause BBB disruption, which include secreted elements by immune cells and pathogens (Wardill et al., 2016), ROS (Haorah et al., 2007; Schreibelt et al., 2007), activation of MMPs (Rosenberg et al., 1998), and chronic up-regulation of angiogenic factors and pro-inflammatory cytokines (Argaw et al., 2006; Argaw et al., 2012; Coelho-Santos et al., 2015). When BBB integrity is compromised, it normally manifest initially as increased barrier permeability with reduced expression of TJs/AJs proteins and/or increased of vesicular transport. However, depending upon the severity, it may show other features such as pericyte detachment, astrocyte end-feet swelling or loss, and disrupted basal lamina (Obermeier et al., 2013). Disruption of the BBB

eventually culminates in neuronal dysfunction, neurodegeneration and a neuroinflammatory cascade of events (Taheri et al., 2011).

Despite the fact that BBB disturbance is usually associated with CNS diseases, in most cases is challenging to define whether barrier impairment is itself responsible for the disease onset. In line with this, is essential to improve our knowledge about the mechanisms underlying BBB dysfunction for targeting the brain endothelium to aid recovery from injury and to help in the formulation of new therapeutic strategies to prevent or/and treat many neurological disorders.

1.4 Neurogliovascular crosstalk

An innumerous integrated, interdependence and fundamental crosstalk between neurons, glia and brain blood vessels contribute to an optimal environment of CNS (Figure 1.11). A growing body of evidence indicates that neurons, glia (astrocytes, microglia, oligodendrocytes), and vascular cells (endothelium, smooth muscle cells or pericytes, adventitial cells) are closely related developmentally, structurally, and functionally.

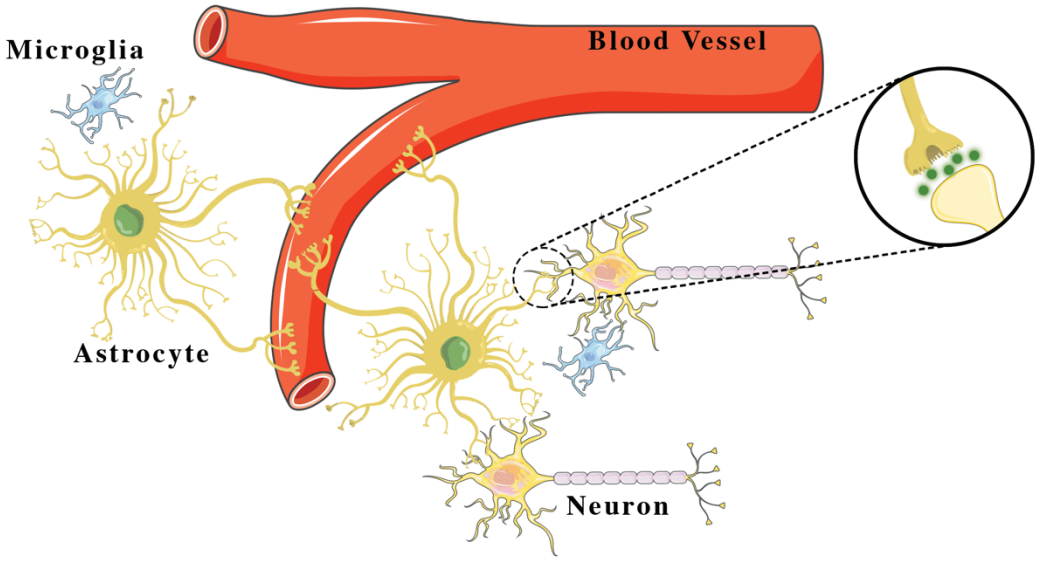


Figure 1.11. Neurogliovascular crosstalk. *Penetrating capillaries are covered by astrocytes. Interneurons targeted onto astrocytes at the perivascular zone. Astrocytes also detect synaptic activity at neuronal synapses. Microglia patrolling the perivascular space and astrocyte-neurons communications.*

1.4.1 The neurovascular coupling

The brain function, namely neural activity, is intimately tied and dependent on a continuous supply of oxygen and glucose through cerebral blood flow (CBF). Normally, CBF correlates precisely with the regional energy utilization in the brain matter (Klein et al., 1986). Neuronal activity is dependent on the hemodynamic response in the brain which is vital in supplying energy nutrients (glucose and oxygen) and residues removal from neuronal activity (CO_2, lactate excess, as well as other metabolites and heat) (Iadecola, 2004). Neurovascular coupling reflects this close temporal and regional linkage between neural activity and cerebral blood flow, involving the coordinated interaction of neurons, glia, and vascular cells.

For many years, the mechanisms underlying neurovascular coupling have been the subject of enquiry, and numerous vasoactive factors have been implicated in such phenomenon (Petzold and Murthy, 2011). Nevertheless, the molecular nature of these highly complex processes has not been elucidated in sufficient detail.

Astrocytes are anatomical intermediaries between neurons and blood vessels mainly because the vascular external surface is almost completely covered by astrocytic endfeet. At the ultrastructural level, the processes of many vasoactive interneurons, in particular those expressing noradrenaline, synapse onto astrocytes rather than directly onto blood vessels (Hamel, 2006). Thus, all signaling molecules, with the possible exception of gaseous transmitters, targeted to the vasculature must first act on or pass through astrocytes in order to reach the endothelial cells (Petzold and Murthy, 2011). Briefly, glutamate is released from presynaptic terminals acting on post-synaptic NMDA receptors leading to the production of nitric oxide (NO) by neuronal NO synthase (nNOS) (Fergus and Lee, 1997). NO is involved in the communication between the endothelium and vascular smooth muscle cells surrounding small brain arteries and arterioles, and also with surrounding capillary pericytes to relax, resulting in vasodilation and increasing blood flow (Fernandez-Klett et al., 2010; Hill et al., 2015; Sweeney et al., 2016; Kisler et al., 2017). In addition, glutamate released by neurons can act on astrocyte metabotropic glutamate receptors, increasing the intracellular levels of Ca^{2+} in astrocytes where it will activate phospholipase A2 leading to the release of arachidonic acid from phosphatidylinositol signal cascade, and converted by cyclooxygenase into vasoactive prostanoids. These vasoactive messengers (particularly prostaglandin E2) release from astrocytic endfeet play an important role in regulating contraction and relaxation allowing

simultaneous changes in vessel diameter, (Porter and McCarthy, 1996; Takano et al., 2006; Kisler et al., 2017). On the other hand, low levels of glutamate lead to vasoconstriction.

Neurovascular coupling is disrupted in pathological conditions, such as hypertension, Alzheimer´s disease, and ischemic stroke, perturbing the delivery of substrates to active brain cells and impairing the removal of potentially deleterious by-products resultant from cerebral metabolism.

1.4.2 Glia-neuron crosstalk

Neurons and glia are intimately packed and closely associated, working as an integrative functional unit and independently. The importance of glia in regulating neuronal survival has already been shown to be particularly important in development, differentiation and physiological processes.

During CNS development astrocytes participate in axonal guidance (Marchetti, 1997). Astrocytes when in close contact with both axons and oligodendrocytes, form membranous blebs that ensheath axons and synapses through extending thousands of thin processes (Molofsky et al., 2012; Ioannidou et al., 2014). The percentage of synapses ensheathed by astrocytic processes as well as the absolute number of synapses ensheathed by one astrocyte varies greatly with synapse type and brain region. Astrocytes could stimulate the formation of inhibitory or excitatory synapses by secreting molecules that regulate presynaptic and postsynaptic differentiation (Elmariah et al., 2005; Hughes et al., 2010; Allen et al., 2012; Chung et al., 2015). It is known that astrocytes convert glucose into lactate. Lactate is then exported to neurons, where it is converted to pyruvate to produce ATP. Astrocytes are also responsible for terminating the action of neurotransmitters secreted by neurons and for mediating their recycling back to neurons in a process known as the glutamate–glutamine cycle (Belanger et al., 2011).

Beside astrocytes, microglia are also in close communication with neurons. These immune cells not only provide immune-brain interactions and response to several pathologies as play important roles for normal brain physiology, both in development and in the mature nervous system (Wake et al., 2013). The microglia-neuron interaction is a complex process that occurs via several processes such as glutamate uptake, removal of cell debris and production of neurotrophic factors, among others. However, it can also be mediated by physical association with endangered neurons in the case of detrimental

conditions. In fact, the physical connection of fractalkine CX3CL1 (expressed by neurons) and CX3CR1 (expressed by microglia), not only keeps microglia in a resting surveillance state but it is also involved in synaptic pruning (Neiva et al., 2014). Recent studies have suggested that microglia have effects on synapses and neuronal circuits by promoting synapse formation (Ueno and Yamashita, 2014; Miyamoto et al., 2016).

In sum, these interactions demonstrate the need of a mutual control between neuronal and glial cells supporting a role for glia in synaptic development and guidance of neuronal migration and process outgrowth in order to maintain the integrity of CNS circuits. Interestingly, glial cells have been shown to play and important role in neuropsychiatric disorders (Kato et al., 2013).

1.4.3 Synaptic pruning

Synaptic pruning is the process by which extra synapses are eliminated thereby increasing the efficiency of the neural network. During embryogenesis and in the postnatal brain the removal of excess excitatory synapses is fundamental for the normal neuronal synaptic activity (Tremblay, 2011). Not less important, this process also occurs in mature brain in order to prevent epileptic activity and in the adaptive remodeling of neural circuits. Both microglia and astrocytes are important in synaptic pruning through their phagocytic activities. Microglia cells supervise synaptic activity, eliminate less active synapse structures and remodel specific regions of neuronal dendrites by synaptic stripping and consequently limiting secondary neurodegeneration after brain injury (Wake et al., 2009; Paolicelli et al., 2011; Schafer et al., 2012). Regarding astrocytes, they can control synapse elimination by releasing signals that activate the complement cascade, but also express phagocytic proteins (al-Ali and al-Hussain, 1996; Sokolowski et al., 2011; Clarke and Barres, 2013; Chung et al., 2015).

Glial cells promote synaptic elimination in response to inflammatory events, developmental axon death, and to brain injury engulfing whole dead cells and peptides. Nevertheless, this process also occur in healthy CNS conditions, sculpting neural circuits, and regulating neuronal function from development to degeneration.

1.5 Neuroinflammation

Neuroinflammation (Figure 1.12) comprises a complex and integrated interplay between different cellular types of the peripheral immune system (macrophages, T and B lymphocytes, dendritic cells), ECs and resident cells of the CNS (microglia, astrocytes, oligodendrocytes, neurons) as well as a complex orchestra of adhesion molecules, free radicals, chemokines, cytokines and their receptors (Farooqui et al., 2007; Sprague and Khalil, 2009). Importantly, in the healthy developing brain inflammatory mediators play an important role in neurogenesis, apoptosis and pruning of neuronal processes (Yirmiya and Goshen, 2011; de Miranda et al., 2017). However, during pro-inflammatory conditions the developing neural circuits may be negatively affected (Yirmiya and Goshen, 2011). The balance between suppressive and pro-inflammatory signals determines the localization, intensity and course of immune responses in the brain.

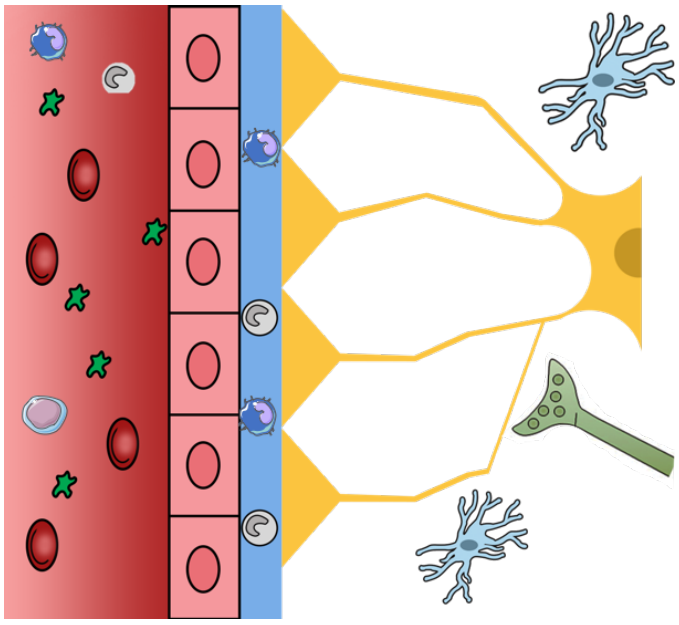


Figure 1.12. Immune surveillance in the central nervous system*. Intact blood-brain barrier (BBB) is formed by tightly sealed endothelial cells (ECs) and the basal lamina containing extracellular matrix components and astrocytes endfeet. The BBB restricts the entrance of pathogens, toxins and blood-borne molecules into the brain parenchyma. Perivascular patrolling macrophages and T-cells ensure continuous surveillance and contribute to the maintenance of the perivascular space homeostasis, mainly via its phagocytic activity. When the brain experiences a major insult, peripheral immune cells join microglia in the parenchyma. Quiescent microglia maintain a healthy brain microenvironment suitable for neuronal function, by continuously sensing any changes via their thin and long ramifications, secreting neurotrophic factors, and promoting neuronal remodeling and synaptic plasticity.*

1.5.1 Is brain an "immune privilege" organ?

For a long time it was assumed that BBB should be completely impermeable to the entrance of peripheral immune system cells into the brain parenchyma. Because of this there was a traditional view of the brain as an immune privileged organ. The immune privilege of the brain is certainly not absolute but is relative to other organs (Galea et al., 2007). In fact, there are several distinctive features in brain inflammatory responses compared with other organs (Lucas et al., 2006; Galea et al., 2007): (i) the brain parenchyma is separated from the blood circulation by the BBB; (ii) the brain lacks lymphatic drainage; and (iii) the brain displays low major histocompatibility complex class II (MHCII) expression.

There is an apparent lack of communication between the CNS parenchyma and the peripheral immune system. Monocyte recruitment was delayed to the third day after injection of LPS in mice brain and only occurred with higher doses (Andersson et al., 1992). Thus, inflammatory responses seem to be more limited and delayed in the brain than other organs. Although systemic inflammation often does not induce evident CNS lesions, it contributes to cerebral vulnerability and exacerbate cell damage (Perry, 2004). However, the opposite seems also to occur since administration of *Bacille Calmatte-Guerin* and influenza virus intracerebroventricularly resulted in tissue rejections, delayed-type hypersensitivity lesions in choroid plexus and humoral and cytotoxic T-cell responses (Matyszak and Perry, 1996; Stevenson et al., 1997).

The equilibrium between immune privilege in health and effective responses in disease or injury result in an efficient regulation of the inflammatory cascade.

I. Innate and adaptive immunity in CNS

The essence of innate immunity is the detection of pathogens-associated molecular patterns (PAMPS) that are unique to infectious organisms and noxious substances. This will induce clearance of the intruders and it also dictates the conduct of the subsequent immune response either to promote a strong inflammatory response to engage acquired immunity or to favor tissue repair (Elward and Gasque, 2003). To understand immune responses in CNS in the absence of pathogens it was formulated the "danger theory" (Matzinger, 2002). This theory stated in the ability of immune system to detect danger molecules or distressed cells within the organism itself. Thus, damage-associated

molecular patterns (DAMPS) can be either endogenously derived "alarmins" or exogenous "pathogens".

Immune system uses an elaborate network of immune sensors, the pattern recognition receptor (PRR'S) that are expressed by most cells including neurons and also immune cells (Czirr and Wyss-Coray, 2012). These DAMPs/PAMPs are recognized by the toll-like receptors (TLRs), the first element of contact between pathogens and the host (Akira, 2001) and play a pivotal role between the innate immune response and the adaptive immunity.

Innate immunity is usually sufficient to counteract a simple infection, and the adaptive response is not required. The acquired immunity has a specific response to the pathogen and development of memory signals. In fact, it appears to be more difficult to CNS locally initiate adaptive immune reaction. However, when innate process failed to clear the infectious organism following TLRs/PAMPs interaction, glial cells and neurons begin to produce inflammatory cytokines, chemokines and adhesion molecules. This way activates and stimulates the traffic of immune cells with a role in adaptive immune system, such as T and B lymphocytes, to the sites of lesion in the brain (Kielian et al., 2002; Simard and Rivest, 2005). B lymphocytes have the major role in acquired immunity, responsible for the humoral defense. Despite increasing evidence showing that B lymphocytes can enter the CNS under pathological conditions, differentiate into plasma cells and secrete antibodies (Knopf et al., 1998), the ability of B lymphocytes to patrol the neural parenchyma is less understood. There is a specific group of highly specialized immune cells, antigen presenting cells (APCs), that process and expose the antigens bound to the MHC molecules on their cell surface, making these antigens recognizable by specific T lymphocytes, $CD4^+$ (helper) or $CD8^+$ (cytotoxic), which become activated (Nguyen et al., 2002).

Overall, one hallmark of neuroinflammation is the infiltration of peripheral immune cells into the multiple compartments of the CNS.

II. Immune surveillance: BBB is the gatekeeper of CNS

Immune surveillance at the BBB is ensured by ECs of brain capillaries (Engelhardt and Coisne, 2011) and by perivascular cells surrounding cerebral vessels (Thomas, 1999). CNS-specific T cell present at the brain limitans coordinate CNS communication with periphery, modulate immune response in inflammatory scenarios and maintain the functional plasticity of healthy brain (Ransohoff and Engelhardt, 2012). The endothelium

controls the flow of immune cells from the blood to the CNS and vice-versa and can respond rapidly to antigens (Huber et al., 2001). In fact, brain ECs are the first line of defense of CNS by contacting with toxic and pathogens circulating in the systemic circulation, and this way might be the first player in the signaling mechanisms that triggers neuroinflammation. During damage, brain ECs are both target and sources of inflammatory mediators such as cytokines, chemokines and ROS that are secreted into the CNS or the blood. Thus, ECs in response to inflammatory stimuli can induce the activation of microglia and astrocytes by itself. The expression of adhesion molecules like vascular and intercellular adhesion molecule-1 (VCAM-1 and ICAM-1, respectively) will facilitate the passage of lymphocytes from blood to CNS (Couraud, 1994; Wong et al., 1999). As abovementioned, ECs also express PAMP and TLR that are able to recognize and respond to pathogen infection (Mallard et al., 2009).

When occurring systemic inflammation blood-born cytokines cross BBB and affect directly brain tissue or can bind to ECs receptor and alter intracellular functions. Perivascular cells localized in the perivascular space (between the vascular ECs and CNS parenchyma) show constitutive and inflammatory-induced expression of MHCII, cytokines, chemokines and adhesion molecules (Thomas, 1999; Rucker et al., 2000; Williams et al., 2001).

Vascular system acts as signaling interface playing a key role in immune-CNS communication, transferring information between both sites.

III. Recruitment and extravasation of leukocytes

The response to inflammatory stimulus urge the need to recruit additional immune cell from blood-circulation into the brain to help and restore homeostasis, a process that is called leukocyte transendothelial migration (TEM), or diapedesis. This type of migration is rare in normal conditions. Immune cells can gain access to the CNS at several sites, including the BBB present along the capillaries in the brain parenchyma, the choroid plexus, meningeal vessels that extend into the brain parenchyma, and postcapillary venules (Ransohoff et al., 2003; Engelhardt and Ransohoff, 2005; Man et al., 2007). While in the periphery migrating cells directly enter the tissue parenchyma, in the CNS, migrating cells have only access to perivascular spaces. To access the CNS parenchyma, they need to reach beyond the glia limitans, which is unique to the architecture of the BBB. In fact, the leukocyte recruitment occurs only under certain defined host conditions

and it is highly controlled by BBB. Additionally, the resting T cells or monocyte-derived macrophages are usually excluded from health brain given that endothelium limits their passage, and are only recruit to resolve an CNS injury (Engelhardt, 2006; Shechter and Schwartz, 2013). Moreover, some findings highlight that T cell activation alone is not sufficient for entry into the brain parenchyma and that additional events outside the CNS are required to make these cells responsive to the adhesion molecules and chemokine signals that facilitate access to the brain (Engelhardt and Ransohoff, 2012; Odoardi et al., 2012; Sallusto et al., 2012). However, it is important to notice that BBB disruption may enhance the infiltration of peripheral circulating lymphocytes, monocytes/macrophages or even plasma components as albumin or fibrinogen (Davalos et al., 2012).

Leukocytes can cross into CNS through the BBB via a multistep process that involves four consecutive steps: rolling, tethering, adhesion and para-cellular or trans-endothelial migration through ECs (Engelhardt and Ransohoff, 2005; Engelhardt, 2006; Muller, 2011). The steps are as follows (Figure 1.13): (I) rolling: fragile adhesion of leukocytes to ECs that is mainly mediated by interactions between selectins and their carbohydrate counter-receptors; (II) activation: leukocyte activation through chemokine stimulation of G-protein-linked receptors, resulting in functional activation of adhesion molecules along their surface; (III) arrest: leukocyte attachment to ECs through connections between integrins associated with leukocytes and cell adhesion molecules on ECs; (IV) crawling: leukocytes looking for preferred sites of transmigration across the endothelium; (V) transmigration: migration of leukocytes across CNS endothelia into the perivascular space and progression across the glia limitans into the brain parenchyma, a process driven in part by chemokine–chemokine receptor interactions.

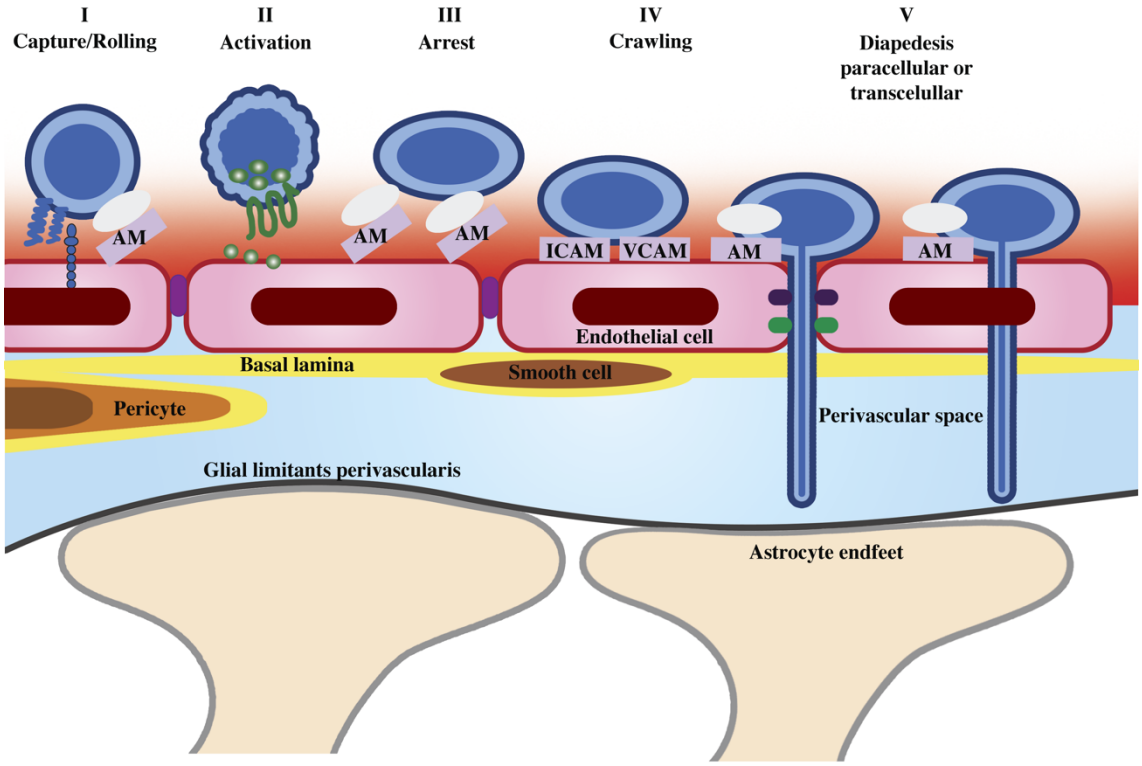


Figure 1.13. Key transmigratory steps of leukocytes across endothelium into brain parenchyma. *Upon inflammatory activation of the endothelium, leukocytes are attracted to and captured on the endothelial surface. Capture and subsequent rolling over the endothelium is mediated by selectins and their ligands on either cell surface. During rolling, leukocytes become partially activated by chemokines/cytokines leading to firm adhesion that is mediated by the leukocyte integrin and endothelial adhesion molecules. Firm adhesion initiates signaling cascades leading to the loosening of junctions and increased endothelial permeability. Along with PECAM-1 and CD99, JAM-A contributes to the migration of leukocytes in-between endothelial cells by diapedesis. In some cases where leukocytes cannot pass through the basal membrane, the leukocyte can exit the abluminal compartment by reverse transmigration. Leukocytes are able also to pass across the endothelium using a transcellular route.*

In this the last stage of this multi-step process is not clear whether the leukocytes cross the ECs through tight junctions or via a large pore or vacuole in the EC. At the paracellular level, the binding of leukocyte integrin to EC CAMs will stimulate signaling cascades in the ECs that further support adhesion and the subsequent process of TEM. Particularly, clustering of ICAM-1 stimulates phosphorylation of cortactin, enhancing further actin-induced clustering of ICAM-1. This process instigates the enrichment of ICAM-1 around tightly adherent leukocytes, and ICAM-1 multimerization increases the content of cytosolic free Ca^{2+} and RhoA activation (Muller, 2011). Clustering of VCAM-1 also stimulates an increase in cytosolic free Ca^{2+}, activation of Rac-1, and production of ROS in ECs (Muller, 2011). As consequence of these events the junctional structures are loosed. Also, both ICAM-1 and VCAM-1 lead to the phosphorylation of VE-cadherin, causing VE-cadherin dissociation from its links to the actin cytoskeleton, which promote weakness of the endothelial junctions. Other important structure involved in TEM is the

lateral border recycling compartment (LBRC), which is a complex vesicular-tubule invagination of the plasma membrane of the EC borders (Mamdouh et al., 2009). Usually, membrane traffics constitutively between the lateral cell border and this compartment, recycling uniformly along the lateral border with a life time of roughly 10 min. However, when a leukocyte transmigrates, membrane from the LBRC is targeted rapidly and extensively to the site of diapedesis to surround the leukocyte (Mamdouh et al., 2003). This structure contains important molecules required for TEM as platelet/endothelial CAM (PECAM), CD99 and JAM-1 proteins (Mamdouh et al., 2003; Mamdouh et al., 2009).

Regarding transcellular route, leukocytes are able to penetrate directly through the cytoplasm of brain ECs by diapedesis, which does not disrupt TJs (Engelhardt and Wolburg, 2004), but in a process that requires the involvement of MMP to digest the basement membrane (Man et al., 2007). Even though JAM-1 is located at TJs, this protein can redistribute to the apical surface of the endothelium during inflammation or monocyte transmigration to bind to the monocytes' integrins (Nourshargh et al., 2006). After the leukocytes enter the EC, the luminal membrane closes before the opening of the abluminal membrane in order not to create a fluid-filled channel through the cell (Carman and Springer, 2008; Wallez and Huber, 2008). ICAM-1 and VCAM-1 concentrates at the site of diapedesis and is enriched in the membrane channel that surrounds the crossing leukocyte as it goes through the EC body (van Buul et al., 2007). Although VCAM-1-mediated signaling in EC has received little attention, it results in the activation of Rac1 that in turn will induce the generation of ROS in ECs, important also for activation of MMP, promoting EC retraction (van Wetering et al., 2003; Deem and Cook-Mills, 2004). In parallel, VCAM-1-driven ROS production activates PKC, which was also found to be required for efficient TEM (Abdala-Valencia and Cook-Mills, 2006).

Other molecules normally considered restricted to the cell borders, such as PECAM, CD99, and JAM-1, are also observed around leukocytes migrating transcellularly (Carman et al., 2007; Mamdouh et al., 2009) and appear to be functional. In fact, by blocking PECAM and CD99 it is possible to prevent transcellular migration but not adhesion (Mamdouh et al., 2009). Curiously, after leukocyte interaction, PECAM-1 is translocated to the cell surface. Moreover, endothelial vesicles and vesiculo-vacuolar organelles (VVOs) were observed immediately adjacent to the region of transcellular migration (Carman et al., 2007). As previously mentioned, cav1 was shown to accumulate in the site of transcellular diapedesis, showing that caveolae is also involved in

transcellular migration (Millan et al., 2006). Additionally, Rho GTPases as RhoA and Rac1 have been reported to localize to lipid rafts and caveolae in ECs (Gingras et al., 1998), and this is important not only for control both actin assembly and actomyosin-based contractility as for their ability to signal to some of their downstream targets.

Other mechanism propose for transcytosis, is phagocytosis since endothelium has been shown to possess a remarkable phagocytic capacity (Howland et al., 2015; Rengarajan et al., 2016). Thus, ECs may use a similar machinery to phagocytose cells and to allow transcellular passage of leukocytes (Faille et al., 2012).

The factors that regulate the preferred route for TEM are not well understood. Nevertheless, if the endothelial cell junctions are particularly tight, or when leukocytes are strongly activated or have difficulty reaching the cell junctions, transcellular migration tends to occur (Mamdouh et al., 2009). However, during pathological conditions that can lead to the opening of the TJs, leukocytes may enter by both paracellular and transcellular routes.

1.5.2 Parenchyma resident cells in CNS

I. Neurons

The neurons' function in immune reactions in brain parenchyma is restrict. Although neurons are the most passive cells in the inflammatory response, they have the ability to express class I MHC molecules (Foster et al., 2002; Ribic et al., 2010) to induce apoptosis or suppress the activation of T cell (Flugel et al., 2000), to produce several cytokines (Neumann et al., 1997) and express their receptors (Friedman, 2001). Thus, neurons can modulate the outcome of neuronal injury and neuroinflammation (Orellana et al., 2005; Lai et al., 2006).

II. Glial response to inflammation

Glia cells play an active role in normal physiology and pathology and occupy the majority of the brain volume (Raivich et al., 1999; Giaume et al., 2007). These cells support and sustain proper neuronal function (Giaume et al., 2007). Glial cells appear to play a dual role, amplifying the effects of inflammation and maintaining neuronal survival.

Microglia

Microglia are the main cellular regulators of brain's innate immune response in both physiological and pathological conditions. These are cells of the monocyte/macrophage lineage, its progenitors arise from pial macrophages and mesenchymal tissue, and colonize the CNS during embryogenic and fetal periods before closure of BBB (Chan et al., 2007; Czeh et al., 2011).

Under physiological situations, microglia display a down-regulated immune phenotype, resting/surveillance stage, which is characterized by a small soma and numerous thin and highly branched processes in constant moving showing a ramified surveillant-like morphology, and unable to phagocytize cells (Aloisi, 2001).

Microglia are activated rapidly in response to insults, infection and inflammation, and it cell-surface receptor expression is modified and the cells change from a monitoring role to a protective and repair role, acquiring an amoeboid morphology and phagocytic capacity (Streit, 2002). As part of the innate immunologic system resident in the brain, microglia recognize the injury or pathogen and quickly initiates the production of inflammatory molecules like cytokines, free radicals, chemokines, fatty acid metabolites, and quinolinic acid that promote the inflammatory state (Hanisch, 2002; Liu and Hong, 2003). Circulating pro-inflammatory cytokines like IL-1, IL-6 and TNF-α are major activators of microglial cells, via a cyclooxygenase (COX)2-dependent mechanism that subsequently produces prostaglandin E2 and may induce fever. Blood-borne proteins such as albumin, thrombin, plasminogen activator, and the complement system have also been shown to activate microglia (Moller et al., 2000; Siao and Tsirka, 2002; Ralay Ranaivo and Wainwright, 2010; Ramaglia et al., 2012).

Although microglial cells have been implicated in lesion progression following various CNS injuries and neurodegeneration, their activation is a key factor in the defense of the brain parenchyma against infectious diseases, inflammation, trauma, ischemia, tumors and neurodegeneration (Kreutzberg, 1996). Actually, activation of microglia can be beneficial or harmful depending on the type of stress and damage signals, duration, microenvironment, interaction and the age of the organism (Walter and Neumann, 2009). In course of recovery from injury, normally microglia overactivation is followed by microglial apoptosis in order to reestablish the normal brain functionality. Microglia are mainly scavenger cells, eliminate invading pathogens, cell debris and several neurotoxins (Streit, 2002), but have also various functions related to tissue repair and neuronal

regeneration (Jin and Yamashita, 2016). Subsequent defense activation, microglia may assume reparation functions and eventually reverse to a resting state or remain primed. During this response, microglia produce trophic factors and anti-inflammatory cytokines (Kerschensteiner et al., 2003; Neumann et al., 2009; Jin and Yamashita, 2016).
The signaling pathways within microglia that direct their responses toward neuronal protection or damage are not fully understood.

Astrocytes

Astrocytes constitute nearly 40% of the total CNS cell population in the adult human brain. These cells are targeted by pro-inflammatory cytokines or directly activated by several pathogens, once they express receptors to recognize various types of pathogens, like TLR2 (Combes et al., 2012). Following stress and/or injury astrocytes proliferate, redistribute around inflammatory cells and release inflammatory/potentially neurotoxic molecules like pro-inflammatory cytokines (Piehl and Lidman, 2001; Moynagh, 2005). Their activation is morphologically identified by the hypertrophy of cellular process and the upregulation of the cytoskeleton known as intermediate filaments, which are composed of nestin, vimentin, and glial fribillar acid protein (GFAP) (Pekny et al., 2007). Astrocytes stabilize and maintain homeostatic repair of tissues, supports neuronal metabolism, control BBB function, regulate intercellular Ca^{2+} signaling, and contribute to early wound repair (Mucke and Eddleston, 1993). Reactive astrocytes may play a pivotal role in maintaining neuronal survival under pathological situations through the delivery of specific neurotrophic factors (Pekny et al., 2007). As abovementioned, they can also be involved in more complex functions such as synaptic integration, plasticity and processing of neuronal information (Perea and Araque, 2006; Pekny et al., 2007). Astrocytes express numerous receptors that enable them to respond to virtually all known neuroactive compounds, including neurotransmitters, neuropeptides, growth factors, cytokines, small molecules and toxins, which allow not only participate in signal processing, but to function as sentinels (Liberto et al., 2004). In fact, astrocytes may decrease brain inflammation by inhibiting microglia through production of IL-12, blocking microglia from presenting antigens (Acevedo et al., 2013).
Reactive gliosis ranges in a scale from mild to prominent. The latter is connected with a damage-associated molecular-patter that induces neurovascular remodeling by endothelial progenitor cells (Hayakawa et al., 2013), where BBB permeability could occur and ultimately promote lymphocyte trafficking and further damage CNS (Ogier et

al., 2005). Latter astrogliosis is a key event in the formation of glial scar, where reactive astrocytes form a physical barrier around a lesion separating it from the surrounding tissue which, depending on the context, can be beneficial for repair and neuronal survival, by producing neurotrophins, or can be detrimental by inhibiting reinnervation (Hatten et al., 1991; Sofroniew, 2005; Raposo and Schwartz, 2014).

Overall, the activation of astrocytes is normally a neuroprotective attempt to support CNS regeneration.

1.5.3 Inflammatory mediators in the brain

After an injury, infection or disease, resident CNS cells generate inflammatory mediators, namely ROS, chemokines and cytokines, which can trigger, exacerbate or inhibit cellular injury and promote tissue repair (Allan and Rothwell, 2003; Lucas et al., 2006).

I. Reactive oxygen species

ROS are a group of small, diffusible radical and non-radical molecules with one or more unpaired electrons such as H_2O_2, superoxide ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), and the hydroxyl radical (•OH), sub-products resultant of the normal cellular metabolism of living organisms (Valko et al., 2007; Birben et al., 2012; Pisoschi and Pop, 2015). The $O_2^{\bullet-}$ is considered the primary ROS, since it interacts with other molecules inducing the formation of different free radicals, namely H_2O_2, which can be converted into •OH or hypochlorous acid (Valko et al., 2007).

There are multiple sources of free radical molecules, both endogenous and exogenous. The endogenous sources include oxidative phosphorylation, P450 metabolism, peroxisomes, and inflammatory cell activation. Cellular ROS are mainly generated at three sites: mitochondria, endoplasmic reticulum, and NOX complex (Figure 1.14). Exogenous sources of ROS include the action of pollutants/toxins such as cigarette smoke, alcohol, ionizing and UV radiations, pesticides, hyperoxia, as well as ozone and heavy metal ions exposure (Birben et al., 2012; Pisoschi and Pop, 2015).

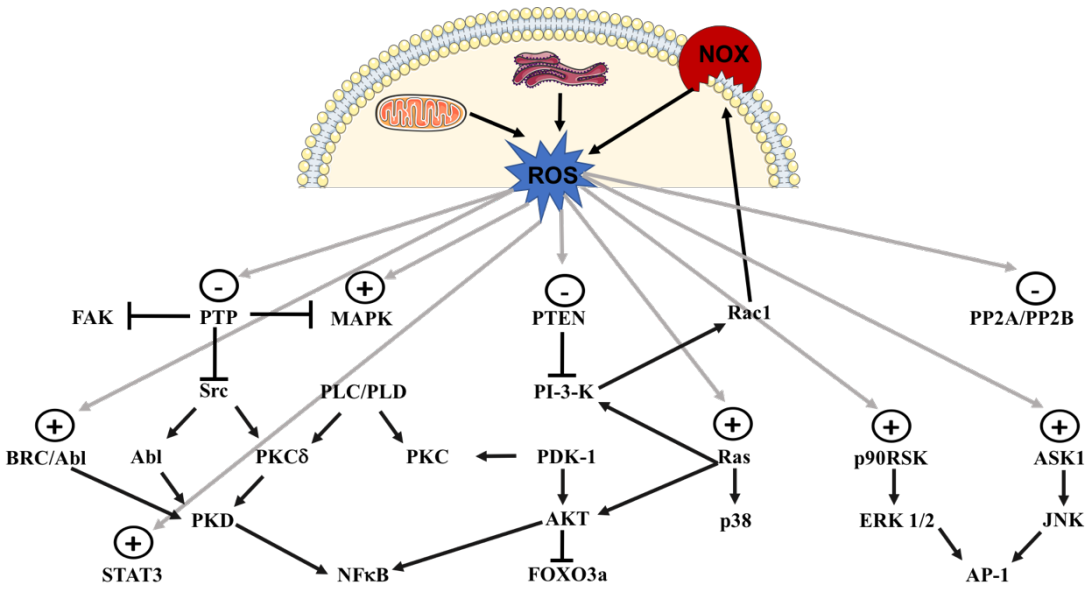


Figure 1.14. Redox signaling cascades. *Reactive oxygen species (ROS) inhibit phospho-serine/threonine-, phosphotyrosine- and phospholipid-phosphatases such as PP, PTP and PTEN, probably by directly regulating their active site cysteines. On the contrary, ROS activates other signaling cascades such as PI 3-Kinase-dependent, MAPK-dependent and Src/Abl kinase-dependent signaling pathways. These signaling cascades, as well as dimer formation due to inter-chain disulphide bridging mediated by oxidation (e.g. STAT3) lead to the activation of several redox-regulated transcription factors such as AP-1, FOXO3a, STAT3 and NF-κB.*

During aerobic process, which employs oxygen to produce energy and heat, molecular oxygen is stepwise reduced to a series of intermediate species producing ROS (Pisoschi and Pop, 2015). Mitochondria are the primary cellular organelle consumer of oxygen, where the oxidoreduction energy of mitochondrial electron transport is converted to the high-energy phosphate bond of ATP. Consequently, mitochondria also contain numerous redox enzymes capable of transferring single electrons to oxygen, generating superoxide anion through the tricarboxylic acid cycle enzymes, electron-transport chain complexes I, II and III, among others enzymes (Lin and Beal, 2006). In fact, approximately 5% of molecular oxygen is converted to ROS, primarily superoxide anion (Lin and Beal, 2006). Under normal physiological conditions, cells are capable of counterbalancing ROS production with scavengers. The antioxidant defenses can be divided in enzymatic and non-enzymatic (Birben et al., 2012; Pisoschi and Pop, 2015). The major antioxidant enzymes directly involved in the neutralization of ROS are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (Valko et al., 2007; Birben et al., 2012; Pisoschi and Pop, 2015). As the first line of defense against free radicals, SOD catalyzes the superoxide anion radical into hydrogen peroxide. Then, CAT or GPX convert the hydrogen peroxide into water and oxygen. The GPX removes the hydrogen peroxide through the oxidation of reduced glutathione (GSH) into oxidized

glutathione (GSSG). Additionally, glutathione reductase regenerates GSH from GSSG, using NADPH as reducing power (Valko et al., 2007; Birben et al., 2012; Pisoschi and Pop, 2015). The GSH/GSSG ratio reflects the oxidative state and is responsible for the maintenance of appropriate redox balance in the cell (Jones, 2006; Handy and Loscalzo, 2012). Cells can also defend itself through non-enzymatic antioxidants, including vitamins (vitamins C and E), i-carotene, uric acid, melatonin, polyphenols (flavonoids and nonflavonoids), and NAC. Some nutrients such as minerals, fibers, fatty acids or amino acids, are also source of exogenous antioxidants (Valko et al., 2007; Birben et al., 2012; Pisoschi and Pop, 2015).

ROS has been recognized for playing a dual role in living organisms, since they can be either harmful or beneficial (Valko et al., 2007). Normally, the beneficial effects of ROS occur at low/moderate concentrations and involve physiological cell processes, for example in defense against infectious agents and maintenance of signal transduction pathways involved in cell growth, proliferation and differentiation (Valko et al., 2007). Conversely, at high concentrations, ROS have harmful effect causing potential biological damage to cell components (such as lipids, proteins, and DNA) and inhibiting their normal function.

Cells constitutively produce ROS, which act as second messengers to regulate the activity of redox-sensitive enzymes including phosphatases and kinases. These signal transduction pathways rely on ROS as signaling molecules acting on different levels in the signaling cascade. Most of the growth factor receptors, namely epidermal growth factor receptor, protein tyrosine phosphatases, as well as tyrosine kinase receptors and serine/threonine kinases are targeted and regulated by ROS. ROS are involved in the inhibition of tyrosine phosphatases by the oxidation of their cysteine residues and also contribute to the activation and/or maintenance of signaling pathways driven by kinases such as extracellular signal-regulated kinases. Particularly, ROS inhibit phospho-serine/threonine-, phosphotyrosine- and phospholipid-phosphatases such as PP, PTP and PTEN (Figure 1.14). This leads to the upregulation of several signaling cascades, such as PI 3-Kinase-dependent, MAPK-dependent and Src/Abl kinase-dependent signaling pathways. These signaling cascades, as well as dimer formation due to inter-chain disulphide bridging mediated by oxidation, lead to the activation of several redox-regulated transcription factors such as activator protein-1(AP-1), FOXO3a, signal transducer and activator of transcription (STAT3) and NF-κB (Thannickal and Fanburg, 2000; Storz, 2005; Valko et al., 2007; Birben et al., 2012).

These redox-responsive signaling pathways regulate several physiological functions, namely gene expression, cell survival, growth and migration (Thannickal and Fanburg, 2000; Storz, 2005; Valko et al., 2007; Birben et al., 2012). Redox signaling is also required for cell expansion in their niches of proliferation (Chaudhari et al., 2014). Moreover, it has been proposed a relationship between ROS and neuroplasticity/cognitive functions (Massaad and Klann, 2011).

The establishment of an imbalance between oxidant/antioxidant, in favor of the former, is termed "oxidative stress" (Birben et al., 2012). This condition occurs when an overproduction of ROS and/or a deficiency of enzymatic and non-enzymatic antioxidants are established (Valko et al., 2007; Birben et al., 2012). Oxidative stress has been implicated in ageing and in various pathological conditions including cardiovascular disease, cancer, neurological disorders, and diabetes (Valko et al., 2007).

Vascular Oxidative Stress: a Final Common Pathway to Cerebrovascular Dysfunction

Although brain accounts for ~2% of total body mass, it requires ~20% of the total blood flow from the heart in order to supply the brain cells with continuous fresh oxygen and energy for the maintenance of homeostasis (Shulman et al., 2004). The BBB and its exquisite vascular network within the brain accomplishes this delivery of oxygen and nutrients, as well as the removal of numerous potentially toxic molecules. Therefore, a number of features that have evolved for the high-level functioning of the BBB and cerebral ECs themselves due the high number of mitochondria also come at a price, with the prospect for producing oxidative stress. Many function of the endothelium are affect by ROS as vasodilatation, apoptosis and angiogenesis (Taniyama and Griendling, 2003). Expression of several adhesion molecules, including VCAM-1 and ICAM-1, is also ROS-dependent (Marui et al., 1993). These cells must be equipped with a selective and unique repertoire of redox and metabolic mechanisms that play a crucial role to preserve redox balance, and adjust metabolic conditions in both normal and pathological conditions.

There is accumulating evidence that vascular oxidative stress leads to deep alterations in cerebrovascular function (Faraci, 2005). Specifically, oxidative stress can increase BBB permeability by inducing alterations on TJs (Schreibelt et al., 2007; Lochhead et al., 2010), activating endothelium (Pueyo et al., 2000; Haorah et al., 2007) and mediating vesicular transcytosis (Peterson et al., 1999; Bian et al., 2017) that occurs by disruption

of endothelium-dependent NO signaling.

Inability of the BBB to perform its normal physiologic functions is thought to contribute to numerous disease states affecting the CNS (Rosenberg, 2012). In fact, several neurological diseases as hypertension (Capone et al., 2012), Alzheimer disease (Hamel et al., 2008), and cerebral ischemia (Kleinschnitz et al., 2010) have been associated with oxidative stress in cerebral blood vessels (Girouard and Iadecola, 2006). ROS are produced by several enzymatic systems, as above mentioned, but was identified NOX as a major source of ROS at the vascular level (Cai et al., 2003). Namely, ROS-derived from NOX may serve as important signaling molecules for the regulation of vascular tone in BBB (Paravicini et al., 2006). NOX has seven isoforms, with Nox-1, -2, and -4 the most well-described in the vasculature (Ray and Shah, 2005). NOX is a membrane-bound enzyme that catalyzes the production of superoxide from oxygen, which is constituted by several cytosolic subunits (p47, p67, p40 and Rac2) distributed between the cytosol and the membranes of intracellular vesicles and organelles (Babior, 1999). In line with this, NOX blocking attenuates the ROS production in models of hypertension and AD, whereas mice lacking the catalytic subunit of the enzyme (gp91phox) are protected from the deleterious cerebrovascular effects of hypertension or Aβ (Kazama et al., 2004; Park et al., 2005). Moreover, mice lacking gp91phox showed reduce brain damage after middle cerebral artery occlusion (Walder et al., 1997). Therefore, NOX-produced ROS could also play a role in post-ischemic cerebrovascular dysregulation, once intrathecal administration of the NADPH oxidase inhibitor, VAS2870, was also shown to reduce brain injury after focal ischemia (Kleinschnitz et al., 2010).

The current awareness that oxidative stress plays a pivotal role in the pathophysiologic processes of vascular dysfunction resulted in several treatment strategies to alter ROS levels by decreasing production and/or increasing radical scavenging (Freeman and Keller, 2012).

II. Chemokines

Chemokines are a family of functionally related small secreted molecules named “chemo-kine” because of leukocyte chemoattractant and cytokine-like activities (Asensio and Campbell, 1999). According to cysteine's number and spacing, four chemokine subfamilies have been defined: CC, CXC, XC, and CX3C subfamilies (Bajetto et al., 2002). Chemokines exert their biological activity by activating surface seven-

transmembrane domain receptors (GPCR) that signal through coupled heterodimeric G-proteins (Gao and Ji, 2010). In the CNS, different types of cells have been identified as sources of chemokines, including microglia, astrocytes, neurons and ECs (Mennicken et al., 1999; Bajetto et al., 2002). As representative arrest chemokines, CXCL12, CCL11, and CCL21 can trigger integrin-dependent adhesion of leukocytes, preceding crawling towards interendothelial junctions (Campbell et al., 1998; Cinamon et al., 2001). Their expression can be regulated by different pro- and anti-inflammatory and seem to be associated with several acute and chronic inflammatory conditions in the CNS (Biber et al., 2002; Belmadani et al., 2006).

III. Cytokines

Cytokines are low weight proteins that can be produced by infiltrating peripheral immune cells lymphocytes and macrophages or by CNS resident cells (glial cells, neurons and ECs) (Ramesh et al., 2013). The functional role of cytokines in CNS inflammation can shift from beneficial to detrimental being anti-inflammatory (e.g. IL-10) or pro-inflammatory (e.g. IL- β and TNF-α), respectively. Nevertheless, cytokines can also exhibit pleiotropic and redundancy since an individual cytokine can have different functions according to the cell type and different cytokines can act on the same cell population to induce similar effects.

IL-1β

The cytokine IL-1β belong to the IL-1 family, like IL-α (agonist) and IL-1 receptor agonist (IL-1ra) (Luheshi et al., 2009). IL-1β is a pleiotropic cytokine that plays a major role in coordinating the inflammatory response being rapidly synthesized and released, primarily by microglia (Pearson et al., 1999; Luheshi et al., 2009) but also by circulating immune cells invading the CNS upon injury. Neurons, astrocytes, oligodendrocytes, and ECs may also produce IL-1, but evidence suggest that their production is subsequent to the microglial response (Basu et al., 2004). IL-1β is released as a pro-form (31 kDa), due to the presence of a pathogen or an inflammatory response, and is activated by its cleavage through the caspase-1 or caspase-8. Caspase-1 is also released as procaspase-1 and then is activated by large multimolecular signaling platforms, the inflammasomes. This complex is composed by a sensor molecule from the NOD-like receptor (NLR) family or the pyrin and HIN domain-containing protein family, a caspase 1, and the apoptosis-

associated speck-like protein containing a caspase recruitment domain (ASC). The NLR-family pyrin domain-containing 3 inflammasome (NLRP3, also known as NALP3 or cryopyrin) is the most important inflammasome in the inflammatory response. NLRP3 can be stimulated by a large number of factors, namely bacteria and viruses, endogenous danger signals from damaged cells, and others. Afterwards, NLRP3 stimulate caspase-1 to cleave the pro-form of IL-1β (18 kDa), raising the levels of IL-1β secreted. When activated, NLRP3 recruits the ASC to form the inflammasome because this protein is responsible for the connection between the NALP and the caspase-1 (Haneklaus and O'Neill, 2015). Many autoinflammatory disorders result from mutations in the genes that codify for NALP3, because of the resulting exacerbated production of IL-1β (Kummer et al., 2007). Similarly to other pro-inflammatory cytokines, in healthy brain IL-1β is expressed at low levels that are up-regulated under pathological conditions. This cytokine has been implicated in a number of neurodegenerative/neurotoxic conditions (Bernardino et al., 2005; Simi et al., 2007) although it has been also reported that IL-1β can play a neuroprotective role (Rothwell and Luheshi, 2000; Friedman, 2005). The biological actions of IL-1β are exerted by the membrane-bound IL-1 Receptor I (IL-1RI), which then associates with the IL-1R accessory protein (IL-1RAcP) and activates intracellular signaling pathways involved in survival and inflammation including the NF-κB, MAPKs, p38 and ERK1/2, and c-Jun N-terminal kinases (JNK) (O'Neill and Greene, 1998). In fact, this cytokine upregulates several inflammatory mediators, such IL-1β itself, TNF-α, COX-2, NO and chemokines.

TNF-α

TNF-α is a multifunctional inflammatory cytokine produced as a 26 kDa membrane-associated precursor (tmTNF), which is cleaved by TNF-α converting enzyme (TACE; a matrix metalloproteinase family member) releasing the soluble form (sTNF) with 17 kDa as a homotrimer (Montgomery and Bowers, 2012). This pleiotropic cytokine can be produced by immune cells as activated microglia and astrocytes, but under pathological conditions it can also be produced by CNS-infiltrating lymphocytes and macrophages (Vassalli, 1992). However, non-immune cells like ECs, muscle cells, fibroblasts and neurons can also produce TNF-α. In fact, we have demonstrated that ECs have a pro-active role by triggering a stronger and faster release of TNF-α than astrocytes (Coelho-Santos et al., 2015).

TNF-α binds to two distinct, but structurally related, receptors, TNFR1 and TNFR2 (Tartaglia and Goeddel, 1992; Tartaglia et al., 1993) and can be activated by a membrane binding or a soluble receptors form (sTNFR) (Kriegler et al., 1988; Idriss and Naismith, 2000). However, sTNF has high affinity to both receptors, whereas tmTNF has a greater affinity to TNFR2 than to TNFR1 (Pozniak et al., 2014). Both receptors can be found in membrane-bound or in proteolytic cleaved form, however the soluble fragments have a role as a neutralizing molecule against TNF-α, protecting the tissue from its deleterious effects (Montgomery and Bowers, 2012). TNF-α receptors are activated by distinct signaling pathways, such as NF-κB, MAPK, AP-1, JNK, and sphingosine-base ceramide, contributing to a variety of different biological responses mediated by TNF-α (Grell et al., 1994; Sriram and O'Callaghan, 2007; Montgomery and Bowers, 2012; Pozniak et al., 2014).

TNF-α has been suggested to be toxic to neurons (Gelbard et al., 1993) and found overexpressed and correlated with neurological conditions as Alzheimer's and Parkinson's disease and traumatic brain injury. Indeed, the use of antibodies against TNF-α had a protective role to improve the neurological outcome of these disorder conditions. However, studies have also demonstrated neuroprotective effects of this cytokine. For instance, TNF-α prevented cell death *in vitro* after exposure of neurons to β-amyloid (Barger et al., 1995), and in brain cells of TNFR-KO mice oxidative stress was increased and levels of an antioxidant enzyme reduced (Bruce et al., 1996). Additionally, this cytokine has also a role in synaptic transmission by inducing expression of both glutamate receptors, AMPA and NMDA (Stellwagen and Malenka, 2006; Jara et al., 2007).

IL-10

IL-10 is a 18 kDa cytokine and a member of the so-called IL-10 family (including IL-10, IL-19, IL-20, IL-22, IL-24 and IL-26) (Volk et al., 2001). IL-10 is an anti-inflammatory cytokine produced by nearly all leukocytes, microglia and astrocytes. Cerebral EC in culture produce and release IL-10 in a polarized manner, mostly at the apical cell surface (Verma et al., 2006).

IL-10 receptor (IL-10R) is composed of at least two subunits, IL-10R1 and ILR2 (Moore et al., 2001). The best characterized IL-10 downstream signaling pathway is the Janus kinase (JAK) signal transducer and activator of transcription (STAT) (JAK/STAT) system, particularly STAT3 (Murray, 2006). The IL-10/IL-10R interaction engages several steps of the immune response, from decreasing cytokine gene expression to down-

regulating the expression of MHC-II and thus antigen presentation to T cells that have been shown to prevent apoptosis. Conversely, while the absence of IL-10 is often initially beneficial to the host, prolonged IL-10 deficiency can often be detrimental. Because inflammatory molecules can often be potent activators of cell death, increasing levels of IL-10 can moderate the extent of apoptosis that is induced in response to infection.

The generation of an effective immune response to an infection while also limiting tissue damage requires a delicate balance between pro- and anti-inflammatory responses. IL-10 is to date the most studied suppressive molecule of the immune system and is essential for immune responses regulation. IL-10 is able to inhibit the production of pro-inflammatory cytokines by microglia and potentiate astrocytes to produce transforming growth factor (TGF)-β. In neurons, IL-10R signaling has been associated with increased cell survival (Zhou et al., 2009) and the regulation of adult neurogenesis (Pereira et al., 2015). At BBB, IL-10 production and biological function is poorly understood, but it appears to have a protective function in bacterial infections (Londono et al., 2011), inhibiting the monocyte and lymphocyte adhesion (Krakauer, 1995).

Based on its immunomodulating functions, IL-10 has been considered an attractive candidate for therapeutic applications, including the treatment of acute and chronic inflammation, autoimmunity, cancer and infectious disease.

1.6 Objectives

MPH is the most frequently prescribed drug for the symptomatic treatment of ADHD. During the last years, the number of children diagnosed with ADHD, and consequently the consumption of MPH, has greatly increased. However, the effects of stimulant medication are still controversial and some reports state a nonconsensual diagnosis among physicians claiming that many children are misdiagnosed with ADHD. Taking into consideration that MPH is an amphetamine-like CNS stimulant highly used among children for long periods, it is urgent to clarify its impact at the cellular/molecular level. Despite the importance of the problem very little is known about this issue. In fact, the effect of MPH on BBB function has never been addressed before.

Given the limited understanding of the impact of MPH on the BBB, the purpose of the present thesis was to clarify the cellular mechanisms underlying the BBB dysfunction associated with MPH consumption. To do so, we have defined the following steps:

Chapter 2

Rationale: Barrier function is mainly provided by ECs that respond to physical and chemical stimuli regulating hemostasis, vasomotor tone, and both immune and inflammatory responses.

Aim: Explore the direct effect of MPH on the integrity of human brain microvascular EC by evaluating both paracellular and vesicular transport and, unraveling the underlying intracellular pathway.

Chapter 3

Rationale: MPH is widely misuse to improve cognitive performance and the hippocampus is highly involved in learning and memory processes. Nevertheless, alterations of the hippocampal NVU under such conditions had never been investigated. Importantly, changes in the structure and organization of BBB may affect its permeability and subsequently the movement of leukocytes and immune mediators into the brain, which in turn can interfere with the vessel-astrocyte-neurons crosstalk culminating in neural damage/dysfunction.

Aim: Uncover the impact of chronic MPH use in non-pathological conditions by specifically investigate BBB alterations, leukocyte transmigration, astrocytic morphology, changes in synaptic protein content and memory performance.

Chapter 4

Rationale: Concerns have been raised about ADHD overdiagnosis and the unknown chronic neurological consequences of MPH treatment. Thus, it is crucial to simultaneously clarify the misuse versus therapeutic use of this psychostimulant.

Aim: Clarify the effect of chronic MPH treatment on ADHD versus control conditions in the main region affected by this pathology, the prefrontal cortex, with particular focus on neuroinflammatory and oxidative responses both in BBB and brain parenchyma, as well as in anxiety-like behavior.

In sum, we aim to contribute to the clarification of cellular and behavioral consequences of therapeutic use and misuse of MPH. The outcome of this field may be very important taking into consideration that MPH is prescribed essentially to children and adolescents for long periods.

Chapter 2

Methylphenidate-triggered ROS generation promotes caveolae-mediated transcytosis via Rac1 signaling and c-Src-dependent caveolin-1 phosphorylation in human brain endothelial cells

Methylphenidate-triggered ROS generation promotes caveolae-mediated transcytosis via Rac1 signaling and c-Src-dependent caveolin-1 phosphorylation in human brain endothelial cells

2.1 Abstract

Methylphenidate (MPH) is an amphetamine-like stimulant commonly prescribed for attention deficit hyperactivity disorder (ADHD). Despite its widespread use, the cellular/molecular effects of MPH remain elusive. Here, we report a novel direct role of MPH on the regulation of macromolecular flux through human brain endothelial cells (ECs). MPH significantly increased caveolae-mediated transcytosis of horseradish peroxidase through ECs without affecting paracellular permeability. Using FRET-based live cell imaging, together with pharmacological inhibitors and lentiviral-mediated shRNA knockdown, we demonstrate that MPH promoted ROS generation via activation of Rac1-dependent NADPH oxidase (NOX) and c-Src activation at the plasma membrane. c-Src in turn was shown to mediate the phosphorylation of caveolin-1 (Cav1) on Tyr^{14} leading to enhanced caveolae formation and transendothelial transport. Accordingly, inhibition of Cav1 phosphorylation by overexpression of a phospho-defective $Cav1^{Y14F}$ mutant or knocking down Cav1 expression abrogated MPH-induced transcytosis. Also, both vitamin C and inhibition of NOX blocked MPH-triggered vesicular transport. This study therefore identifies Rac1/NOX/c-Src dependent signaling in MPH-induced increase in transendothelial permeability of brain endothelial cell monolayers via caveolae-mediated transcytosis.

2.2 Introduction

Methylphenidate (MPH) is the drug of choice for treatment of attention deficit hyperactivity disorder (ADHD), a common neuropsychiatric disorder in children that can persist into adulthood (Sharma and Couture, 2014). Despite the well-known therapeutic effects of MPH on ADHD, several reports state a nonconsensual diagnosis among

physicians claiming that many children are being misdiagnosed with this disorder (Elder, 2010). Additionally, the consumption of stimulants to sustain attention, augment memory, and enhance intellectual capacity is increasing globally. In fact, the use of MPH for cognitive enhancement is a subject that has received much attention in recent years (Sharma and Couture, 2014; Sahakian et al., 2015). Thus, it is crucial to better understand the overall impact of MPH on the central nervous system.

Endothelial cells (ECs) are the primary cellular component of the blood-brain barrier (BBB), a dynamic and highly specialized structure that plays a key role in brain homeostasis and protection (Cardoso et al., 2010). Continuous non-fenestrated ECs with well-developed tight junctions (TJs) are responsible for maintaining high electrical resistance and thereby preventing the diffusion of small molecules between adjacent ECs of the BBB. In addition, the existence of selective transporters and a low rate of fluid-phase endocytosis, or (macro)pinocytosis, greatly limits the transport of large molecules through the brain endothelium (Cardoso et al., 2010). An increase in transcellular transport is usually associated with brain injuries, such as traumatic brain injury and stroke (Cipolla et al., 2004). Vesicular transport across brain ECs is primarily mediated by caveolae, which represent >95% of ECs vesicles (Sverdlov et al., 2007). Despite the deleterious role of caveolae in vascular and neurological diseases (Gaudreault et al., 2004; Chang et al., 2011), these vesicles can also be used for drug delivery (Sverdlov et al., 2007; Head et al., 2014). Caveolin-1 (Cav1) is the main structural component of caveolae which is thought to form an oligomeric coating on the cytoplasmic surface (Fernandez et al., 2002). Cav1 also acts as an important scaffold protein that interacts with and modulates the activity of numerous signaling molecules (Sverdlov et al., 2007). Cav1 phosphorylation on tyrosine 14 by the Src family kinases (SFKs) is essential to initiate plasmalemmal vesicle formation, fission and transendothelial vesicular transport (Sverdlov et al., 2007; Zimnicka et al., 2016).

Vascular endothelial cell dysfunction has been described as a critical event in the development and progression of brain pathologies, such as Alzheimer's disease (Rosenberg, 2012). Also, the use of MPH seems to be associated with cardiovascular risks (Sharma and Couture, 2014), increased brain blood flow (Marquand et al., 2012), and capillary wall structural changes such as thickening of basement membrane and increased density of pinocytotic vesicles (Bahcelioglu et al., 2009). Nevertheless, the impact of MPH on brain ECs is not known. Herein, we report for the first time that MPH increases the permeability of human brain endothelial cell monolayers by stimulating caveolae-

dependent vesicular transport. We reveal that MPH activates the GTPase Rac1 which promotes the assembly of NADPH oxidase (NOX) and increased production of reactive oxygen species (ROS). NOX-induced ROS generation activates c-Src which then phosphorylates Cav1, promoting transcytosis of macromolecules via caveolae-mediated transcellular transport in brain ECs.

2.3 Material and methods

2.3.1 Cell cultures

Human samples were obtained from discarded temporal lobe tissue during operative treatment of epilepsy (outside epileptogenic foci) after informed consent and institutional review board ethical approval at the Neurosurgery Service, Coimbra Hospital and University Centre, Portugal. Primary cultures of human brain microvascular endothelial cells (HBMVECs) were isolated as previously described (Bernas et al., 2010). HBMVECs were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12, Biochrom AG, Berlin, Germany) media containing 10% fetal bovine serum (FBS, GIBCO, Rockville, MD, USA), endothelial cell growth supplement (ECGS, BD Biosciences, Franklin Lakes, NJ, USA), heparin (1 mg/mL, Biochrom AG), and 1% antibiotic-antimycotic (Sigma-Aldrich, St. Louis, MO, USA). After reaching confluence, ECGS and heparin were removed.

The human cerebral microvascular endothelial cell line (hCMEC/D3) shows similar properties to primary BMVEC and are routinely used as an *in vitro* model of the BBB (Weksler et al., 2005). This cell line was cultured in EBM-2 medium (Lonza, Walkersville, MD, USA) supplemented with 1 ng/mL basic fibroblast growth factor (bFGF, Sigma-Aldrich), chemically defined lipid concentrate (1:100; Invitrogen), 1.4 µM hydrocortisone (Sigma-Aldrich), 5 µg/mL acid ascorbic (Sigma-Aldrich), 1% Penicillin-Streptomycin (Gibco, Paisley, UK), 5% FBS (Invitrogen, Inchinnan Business Park, UK), and 10 mM HEPES (Lonza).

Medium was changed every 2 days until the cells reached confluence. HBMVECs and hCMEC/D3 (passage 26-35) were seeded on culture plates coated with collagen type I and maintained at 37°C with 5% CO_2. ECs were either left untreated (control) or treated for different time-points, as specified in the figure legends.

2.3.2 Plasmids

pUSE-Src-YF-UniRapR-mCerulean-myc (hereafter termed c-Src CFP; Plasmid 45381) and pFRET-HSP33 (plasmid 12260) were obtained from Addgene. Cells transfection was performed using 1-2 µg of each plasmid with jetPRIME® (Polyplus transfection, Illkirch-Graffenstaden, France) according to the manufacturer's protocol. c-Src Mission shRNA clone TRCN0000023597, Rac1 Mission shRNA clone TRCN0000055188, Cdc42 Mission shRNA clone TRCN0000071686, RhoA Mission shRNA clone TRCN0000068198 and Cav1 Mission shRNA clone TRCN0000008002 were from Sigma-Aldrich.

2.3.3 Lentivirus Production

The production was similar to that described previously (Socodato et al., 2015). Briefly, HEK293T cells were seeded in 90 mm culture dishes and at 80% confluence were co-transfected overnight with virus-producing plasmids using jetPRIME® (Polyplus transfection) according to the manufacturer's instructions. Transfection ratios were as follows: 6 µg of shRNA plasmids to 3 µg of psPAX2 to 3 µg of VSVG (2:1:1). The next day, normal growth media were replaced by transfection media, and cells were cultivated for an additional 48 h. Then, media with viral particles were collected, centrifuged at 1500 rpm for 5 min, and the supernatant was collected into new tubes.

2.3.4 TUNEL assay

The protocol was performed as previously described (Coelho-Santos et al., 2012). Briefly, HBMVECs and hCMEC/D3 were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich), permeabilized in 0.25% Triton X-100 for 30 min at room temperature (RT), and incubated with terminal deoxynucleotidyl transferase buffer for 1 h at 37ºC in a humidified chamber. Incubation with fluorescein (1:100; Vector Laboratories, Burlingame, CA, USA) was performed for 1 h, followed by nuclei counterstaining with 5 µg/mL Hoechst 33342 (Sigma-Aldrich) for 5 min. The slides were mounted in Dako fluorescent medium (Dako North America Inc., Carpinteria, CA, USA) and fluorescent images for cell counts were recorded using an Axiovert 200 M fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2.3.5 Evaluation of endothelial cell monolayer integrity

Cell monolayer integrity was determined as previously described (Martins et al., 2013). Briefly, HBMVECs were grown on collagen type I-coated 12-mm Transwell filters (Costar, Corning, NY, USA) and sodium fluorescein (376 Da Na-F; 10 μg/mL), fluorescein isothiocyanate (4 kDa FITC; 1 mg/mL), or rhodamine B isothiocyanate dextran (70 kDa RITC; 1 mg/mL; all from Sigma-Aldrich) were added to the apical side. Samples (50 μL) were collected from the basal chamber at 30 min intervals for 120 min and 240 min before and after treatments, respectively. Fluorescence was measured and plotted against time. Permeability was determined from linear slope changes before and after addition of the compounds.

Transendothelial electrical resistance (TEER) of monocultures was measured using a STX-2 electrode coupled to an EVOM resistance meter (World Precision Instruments, Hertfordshire, United Kingdom). TEER readings of cell-free inserts were subtracted from the values obtained with cells and results were expressed as % of control.

2.3.6 Immunocytochemistry

For β-catenin and ZO-1 identification, HBMVECs were fixed with 4% PFA (Sigma-Aldrich) for 20 min at RT, whereas for claudin-5 staining, cells were fixed with methanol for 15 min at -20ºC. Afterwards, cells were permeabilized with 0.2% or 0.1% Triton X-100 (Sigma-Aldrich) and blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at RT or with 5% BSA (Sigma-Aldrich) for 45 min at RT for β-catenin and ZO-1 or claudin-5, respectively. Incubation with primary antibodies was performed as follows: rabbit ZO-1 (1:200, Invitrogen) and rabbit β-catenin (1:100, Invitrogen) for 1 h at 37°C; and mouse claudin-5 (1:100, Invitrogen) overnight at 4ºC. Cells were incubated with secondary antibodies (Alexa Fluor 488 or Alexa Fluor 594, 1:200, Invitrogen) for 1 h at RT in the dark and nuclei were stained with 4 μg/mL Hoechst 33342 (Sigma-Aldrich) for 5 min at RT. Finally, cells were mounted in Dako fluorescence medium (Dako North America) and images were recorded using a LSM 710 Meta Confocal microscope (Carl Zeiss, Oberkochen, Germany).

Analysis of claudin-5 immunofluorescence images was performed using the FIJI Software version 2.0 as previously described (Fernandes et al., 2016). A total of three independent cultures were used, and from each coverslip, six images were blindly captured and analyzed.

2.3.7 Horseradish peroxide transport

HBMVEC and hCMEC/D3 were seeded on collagen type I-coated collagen 12-mm Transwell filters (Costar) and grown to confluence. Horseradish peroxide (10 mg/mL HRP, Sigma-Aldrich) was added to the apical chamber, and after 3 h of incubation, the top and the bottom of the Transwells were washed and new medium was added. Samples were taken for 2 h at 30 min intervals. Absorbance was measured in a microplate reader (Biotek, Synergy HT, Winooski, USA), and HRP activity was plotted against time, and transport rates determined by linear regression.

2.3.8 Transmission electron microscopy analysis

HBMVECs were manipulated as mentioned for HRP transport assay and then washed with 0.01 M phosphate-buffered saline (PBS - 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM disodium hydrogen phosphate, 1.47 mM monopotassium dihydrogen phosphate, pH 7.4) followed by fixation in 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M cacodylate buffer for 30 min. For the diaminobenzide (DAB) reaction, cells were incubated with 0.05% DAB and 0.015% hydrogen peroxide (H_2O_2) in the dark for 10 min at RT, washed with PBS, and incubated with 1.5% potassium ferricyanide and 1% osmium tetroxide (all from Sigma-Aldrich) for 1 h in the dark at 4°C. Afterwards, cells were dehydrated in a graded ethanol series (50-100%), and impregnated using an epoxy embedding kit (Fluka Analytical, USA). Ultrathin sections (70 nm) were mounted on copper grids with no post-staining and observations carried out on a FEI-Tecnai G2 Spirit Bio Twin at 100kV.

2.3.9 Western blot analysis

Experiments were performed as previously described (Coelho-Santos et al., 2012). Primary antibodies were as follows: rabbit anti-Cav1 (1:200, Santa Cruz Biotechnology Inc.), rabbit anti-p-Cav1 Tyr^{14} (1:50, Santa Cruz Biotechnology Inc.), mouse anti-Rac1 (1:500, Cytoskeleton, Inc., Denver, CO, USA), mouse anti-Cdc42 (1:500, Cytoskeleton, Inc), mouse anti-RhoA (1:500, Cytoskeleton, Inc.), rabbit anti-p-c-Src Tyr^{416} (1:500, Cell Signaling Technology, USA), and rabbit anti-c-Src (1:500, Abcam, Cambridge, UK). Then, membranes were then probed with alkaline phosphatase-conjugated secondary

antibodies, anti-rabbit (1:20000) and anti-mouse (1:10000) (Amersham GE Healthcare Life Science, USA). Immunoblots were stripped and reprobed with an antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:500; Abcam, Cambridge, UK) to ensure equal sample loading. Bands were visualized using the enhanced chemiofluorescence (ECF) reagent assay (Amersham) on the Typhoon FLA 9000 (GE Healthcare Bioscience AB, Uppsala, Sweden), and quantification was performed using the ImageJ 1.44o software.

2.3.10 Fluorescence resonance energy transfer (FRET) and image analysis

The immortalized hCMEC/D3 cell line was plated on glass-bottom culture dishes (µ-Dish 35 mm, iBidi). As previously described (Socodato et al., 2015), imaging was performed using an inverted epifluorescence microscope (DMI6000B, Leica Microsystems). The excitation light source was a mercury metal halide bulb with an integrated light attenuator (EL6000, Leica Microsystems). High-speed low vibration external excitation and emission filter wheels (equipped with CFP/YFP excitation and emission filters) were mounted on the microscope (Fast Filter Wheels, Leica Microsystems). A 440-520 nm dichroic mirror (CG1, Leica Microsystems) and a PlanApo 63X 1.3N.A glycerol immersion objective were used. Images were acquired with 4x4 binning using a digital CMOS camera (ORCA-Flash4.0, Hamamatsu Photonics). All modules were controlled by LAS AF software (Leica Microsystems). At each time-point, CFP and FRET images were sequentially acquired using different combination of filters (YFP excitation plus CFP emission, and CFP excitation plus YFP emission, respectively). Images were exported as 32 bit tiff files and processed using FIJI software. Background was dynamically subtracted from all slices from both channels using a routine macro, and images were filtered using the second momentum of a mean filter in FIJI. Segmentation was achieved on a pixel-by-pixel basis. After background subtraction and filtering, ratiometric images (CFP/FRET or FRET/CFP) were generated in intensity modulated display mode using the FRET images as intensity modulators.

2.3.11 RhoA/Rac1/Cdc42 pull-down assay

Rho GTPase activation was assessed by a pull-down assay kit (Cytoskeleton, Inc.) according to the manufacturer's protocol. In brief, hCMEC/D3 lysates were collected and

GTP-bound RhoA or Rac1/Cdc42 was captured using pull-down assays with immobilized Rhotekin-RBD or PAK-PBD, respectively. The levels of activated small GTPases, as well as total amount of GTPase pulled down were evaluated by western blot analysis using specific antibodies (1:500).

2.3.12 Reactive oxygen species detection

HBMVECs were cultured in coated black 96-well plates and after treatments, 2',7'-dichlorodihydrofluorescein diacetate (5 μM H_2DCFDA; Molecular Probes, Eugene, Oregon, USA) or dihydroethidium (2 μM DHE; Molecular Probes) was added to the cells during 1 h at 37^{o}C in the dark. The fluorescence intensity was measured (DHE, ex/em: 485/590; H_2DCFDA, ex/em: 485/528) and divided by the amount of protein.

2.3.13 Statistical analysis

Evaluation of EC monolayer integrity, HRP transport, claudin-5 immunofluorescence images, and western blot analysis were performed by a person blinded to treatments. Results are expressed as mean + standard error of the mean (S.E.M.). Data were analyzed using one-way ANOVA followed by Dunnett's or Bonferroni's post hoc test, or Mann-Whitney test using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The level of significance was $P<0.05$ and the "n" represents the total number of experiments obtained from at least three independent cell cultures.

2.4 Results

2.4.1 MPH increases human brain endothelial cell permeability via caveolae-dependent transcytosis

To investigate the impact of MPH on the integrity of the brain endothelial barrier, we measured the macromolecular flux of fluorescent dyes across confluent HBMVECs. For these studies, 100 μM of MPH was used since this concentration is within the range of the recommended daily dosage prescribed for ADHD (Gopal et al., 2007). Also, studies with animal models have suggested that due to an active accumulation process, brain concentrations of MPH are substantially higher than those found in the plasma (Balcioglu

et al., 2009) and are usually between 23.3-242 μM (Huff and Davies, 2002; Phan et al., 2013).

In the present study, we first demonstrated that MPH (100 μM) did not cause EC death when applied to either the primary cultures (Figure 2.1 A, B) or cell line (Figure 2.1 C).

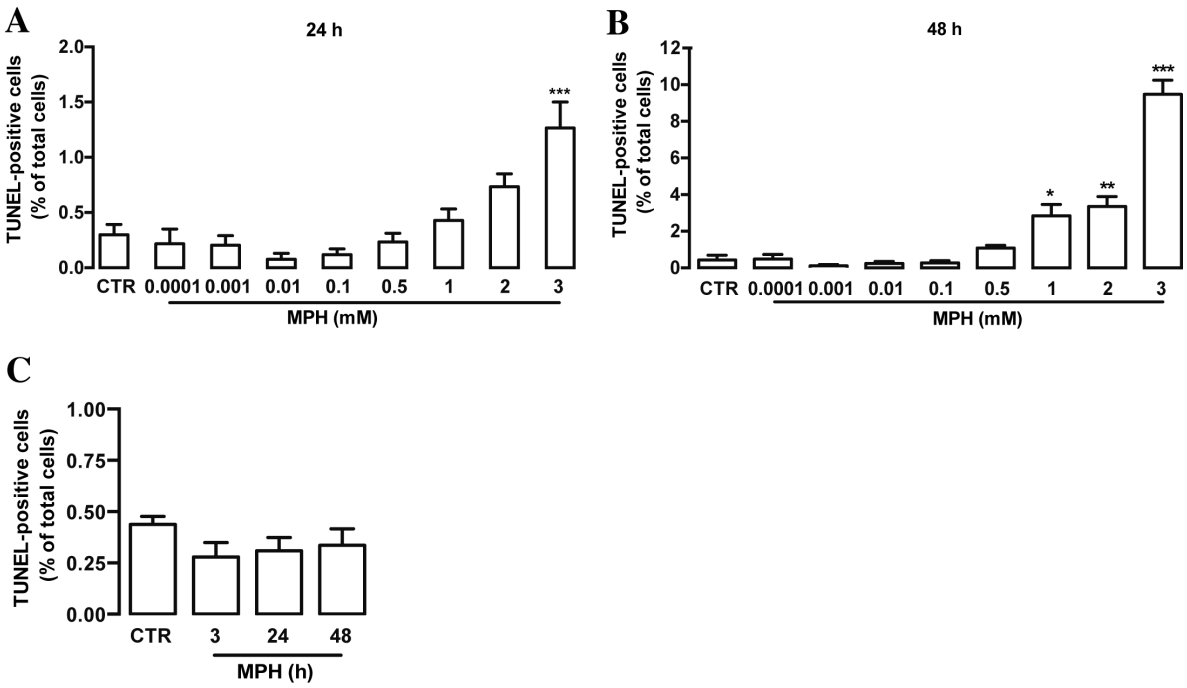


Figure 2.1. Effect of MPH on ECs viability. *HBMVECs were exposed to increasing MPH concentrations (0.001-3 mM) for* ***(A)*** *24 h or* ***(B)*** *48 h to evaluate cell death. MPH increased the number of TUNEL-positive cells to concentration above 3 or 1 mM for 24 or 48 h of exposure, respectively.* ***(C)*** *hCMEC/D3 cells were incubated with MPH (100 μM) during 3, 24, and 48 h, and no cell death was observed at any time-point analyzed. The results are expressed as mean % of control ± S.E.M., n=11-24.* $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, *significantly different when compared to the control.*

Nevertheless, it increased 376 Da Na-F and 4 kDa FITC-dextran flux across HBMVECs (Figure 2.2 A, B) without changing the permeability to 70 kDa rhodamine B (Figure 2.2 C). It should be noted that larger tracers are excluded from the paracellular pathway if intercellular junctions are intact (Matter and Balda, 2003; Hasebe et al., 2010). Additionally, despite the MPH-induced increase in endothelial permeability to small molecular weight tracers, there were no alterations in TEER values (Figure 2.2 D) suggesting that hyperpermeability promoted by MPH was not due to alterations in the paracellular permeability pathway.

Accordingly, no changes were observed in the expression and organization of inter-endothelial junctions, specifically in both tight and adherens junction proteins claudin-5 (Figure 2.2 E), zonula occludens (ZO)-1, and β-catenin, respectively (Figure 2.2 F). To

support our hypothesis, we further investigated the impact of MPH on tight junction organization by morphometric analysis of claudin-5 staining. Our results show that MPH did not alter the membrane/cytoplasm ratio of claudin-5 localization compared to control (Figure 2.2 E).

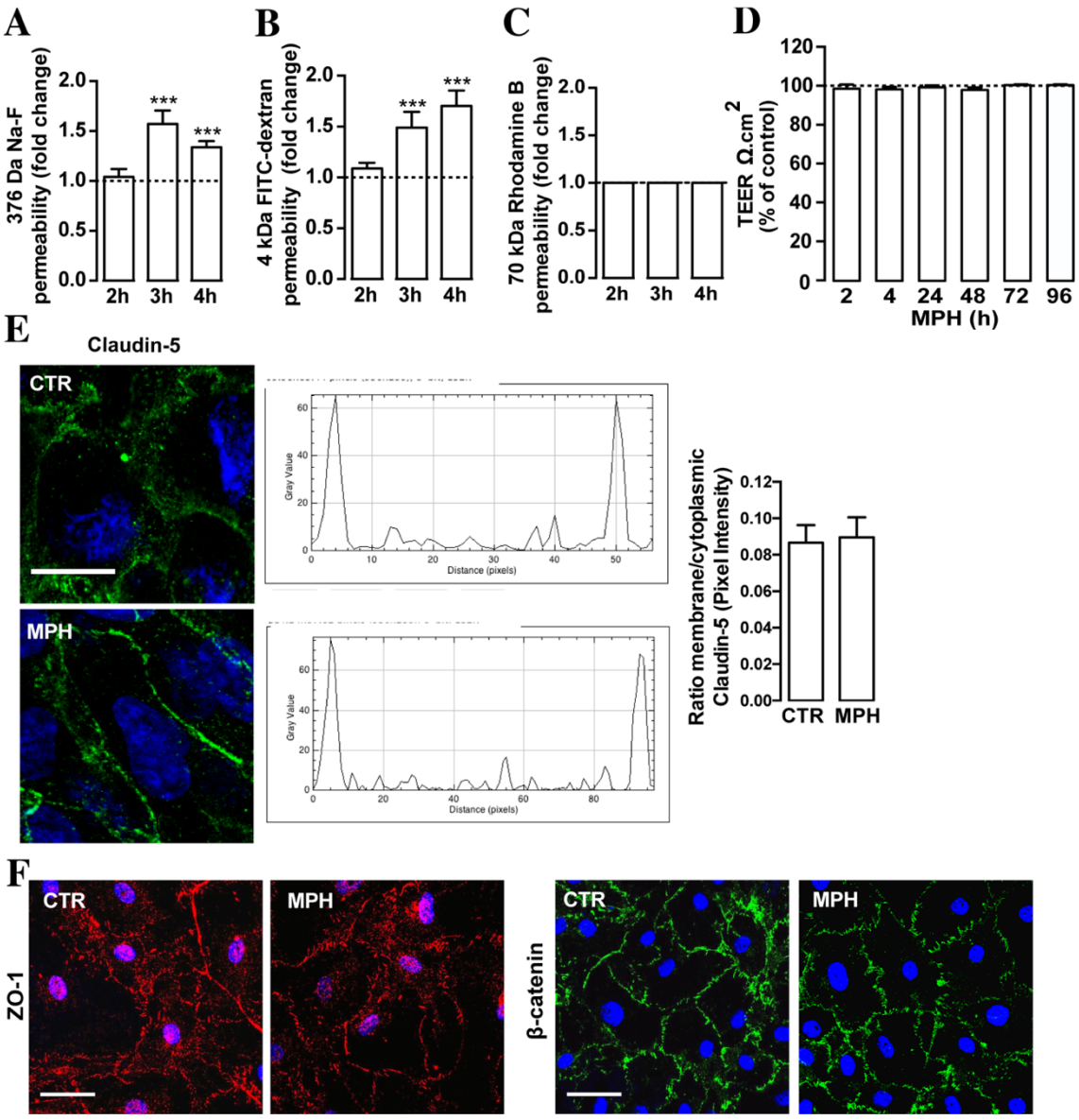


Figure 2.2. MPH increases the permeability of human brain ECs. *Macromolecular flux across HBMVECs was assessed using* ***(A)*** *376 Da Na-F,* ***(B)*** *4 kDa FITC-dextran or (C) 70 kDa rhodamine B-dextran at different time points after MPH exposure (100 µM).* ***(D-F)*** *MPH altered neither* ***(D)*** *transendothelial electrical resistance (TEER) nor* ***(E, F)*** *tight and adherens junction protein expression/organization.* ***(E)*** *Representative confocal images of claudin-5 immunofluorescence and plot intensity profile that enables calculation of the ratio between membrane and cytoplasm pixel intensity. Scale bar: 20 µm.* ***(F)*** *Representative confocal images of ZO-1 and β-catenin immunoreactivity. Nuclei were stained with Hoechst 33342. Scale bar: 50 µm. The results are expressed as mean + S.E.M., n=4-12.* $^{***}P<0.001$ *vs control (dashed line).*

We next explored whether MPH-induced increase in macromolecular flux was due to transcytosis. We observed endothelial membrane-invaginations by electron microscopy (Figure 2.3 A) suggesting MPH may have increased unidirectional and non-junctional HRP flux across HBMVECs via a vesicular pathway (Figure 2.3 B). To further identify the type of vesicles formed upon MPH exposure, HBMVECs were treated with either caveolae or clathrin disrupting agents. We used methyl-β-cyclodextrin (5 mM M-β-C) or hypertonic sucrose (0.4 M) to inhibit caveloae- or clathrin-mediated endocytosis, respectively (Stamatovic et al., 2009). Our results showed that only the depletion of caveolar plasmalemmal vesicles blocked MPH-induced transcytosis in HBMVECs (Figure 2.3 C). Given that Cav1 is the major protein constituent of caveolae, we then demonstrated that Cav1 knockdown in hCMEC/D3 cells (validated in Figure 2.3 D) abolished MPH-induced HRP transport (Figure 2.3 E). Also, MPH increased Cav1 phosphorylation at Tyr^{14} (p-$Cav1^{Y14}$), which is required for caveolae-mediated endocytosis (Sun et al., 2009; Zimnicka et al., 2016) in a time-dependent manner in both HBMVECs (Figure 2.3 F) and hCMEC/D3 cells (Figure 2.3 G). The phosphorylation kinetics triggered by MPH has never been shown, and our results are in accordance with other studies that investigated different stimuli, including the psychostimulant methamphetamine (Vihanto et al., 2006; Maniatis et al., 2012; Park et al., 2012). Additionally, MPH was not able to induce Cav1 phosphorylation in ECs expressing the phosphodefective Cav1 mutant ($Cav1^{Y14F}$; Figure 2.3 G).

To specifically address the role of p-Cav1 in MPH-induced vesicular transport of HRP, we used hCMEC/D3 cells overexpressing either phosphomimicking ($Cav1^{Y14D}$) or phosphodefective ($Cav1^{Y14F}$) Cav1 mutants (Figure 2.2 H). While MPH-induced increase in HRP transport was blocked by $Cav1^{Y14F}$ overexpression (Figure 2.2 H), $Cav1^{Y14D}$ by itself was sufficient to increase HRP transport, mimicking the MPH effect. Taken together, our data show that MPH promotes transcytosis across human ECs through a caveolae-dependent process.

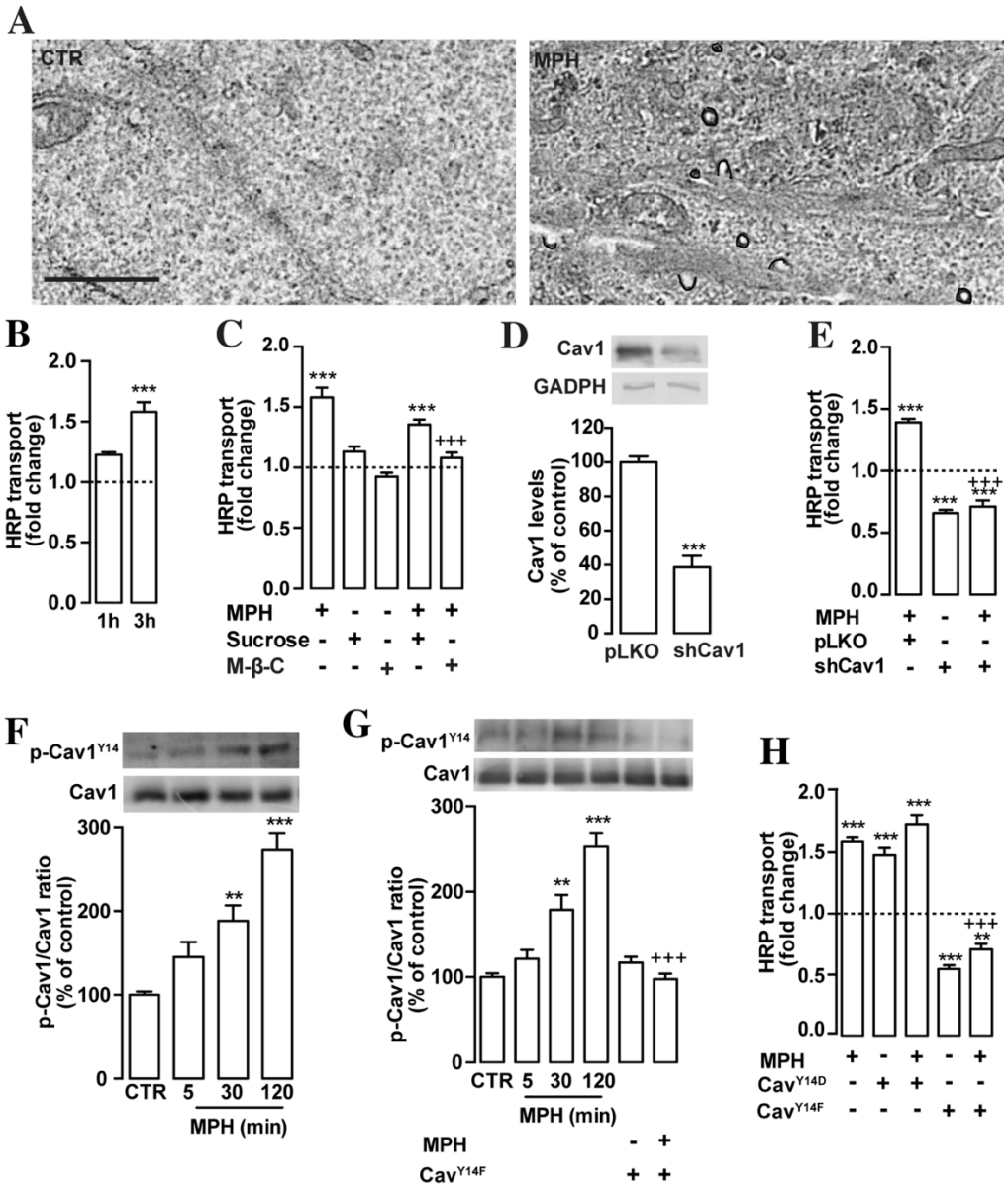


Figure 2.3. MPH promotes caveolae-mediated transcytosis in human brain ECs. ***(A)*** *Representative transmission electron micrographs of HBMVECs under control conditions (CTR) or 3 h after MPH exposure (100 µM) showing the formation of caveolar structures (outlined in black). Scale bar: 2 µm.* ***(B)*** *MPH enhanced vesicular transport of HRP (44 kDa) in HBMVECs, n=16-18.* ***(C)*** *Disruption of caveolae with methyl-β-cyclodextrin (5 mM M-β-C) blocked the effect of MPH in HBMVECs, whereas blockade of clathrin-coated vesicles formation with sucrose (0.4 M) had no effect, n=7-18.* ***(D)*** *Quantification of Cav1 protein levels in hCMEC/D3 cells after shRNA-mediated knockdown or infection with empty vector (pLKO). Top: representative western blot image of Cav1 (22 kDa) and GAPDH (37 kDa) are shown, n=4-6.* ***(E)*** *In the absence of Cav1 (shCav1), MPH was not able to promote HRP transport in hCMEC/D3 cells, n=3-12. (f-g) MPH increased the phosphorylation of Cav1 Tyr^{14} (p-Cav1^{Y14}) in both* ***(F)*** *HBMVECs and* ***(G)*** *hCMEC/D3 cells. Expression of phosphodefective Cav1^{Y14F} abolished MPH-induced phosphorylation (analyzed after 120 min). Top: representative western blot images of p-Cav1^{Y14} and Cav1 (22 kDa) are shown, n=12-14.* ***(H)*** *Phosphomimicking Cav1^{Y14D} mutant is sufficient to increase HRP vesicular transport, similar to that observed with MPH, whereas phosphodefective Cav1^{Y14F} mutant blocked the effect of MPH, n=11-33. The results are expressed as mean + S.E.M. $^{**}P<0.01$, $^{***}P<0.001$ vs control (CTR or dashed line) or pLKO; $^{+++}P<0.001$ vs MPH.*

2.4.2 ROS production via Rac1/NOX activation are key mediators of MPH-induced vesicular transport

The Rho family of small GTPases regulates many aspects of actin cytoskeletal dynamics, including vesicle trafficking (Long et al., 2012; Lim et al., 2014). Thus, we further focused on the impact of MPH on Rho GTPase activity in brain ECs by studying the dynamic activity of Rho family members (RhoA, Rac1 and Cdc42) using live cell imaging with FRET-based Rho biosensors in hCMEC/D3 transfected cells. MPH induced robust Rac1 activation at membrane ruffles (Figure 2.4 A), as detected by a significant increase in the FRET/CFP ratio of the Raichu Rac1 FRET probe (Ouyang et al., 2008) (Figure 2.4 A, B, red circles). On the contrary, there was a decrease in RhoA activity over time (Figure 2.4 A, B, green circles) and no alteration of Cdc42 activity (Figure 2.4 A, B, blue circles). These observations were confirmed by pull-down assay in hCMEC/D3 cells (Figure 2.5 A-C). Moreover, a causal link between Rac1 activation and MPH-induced EC permeability was further demonstrated in that knockdown of Rac1 in hCMEC/D3 cells with shRNA (validated in Figure 2.4 C) prevented the increase in MPH-triggered vesicular transport of HRP (Figure 2.4 F). However, knockdown of RhoA or Cdc42 (validated in Figure 2.4 D, E) had no effect on the MPH-induced response (Figure 2.4 G, H).

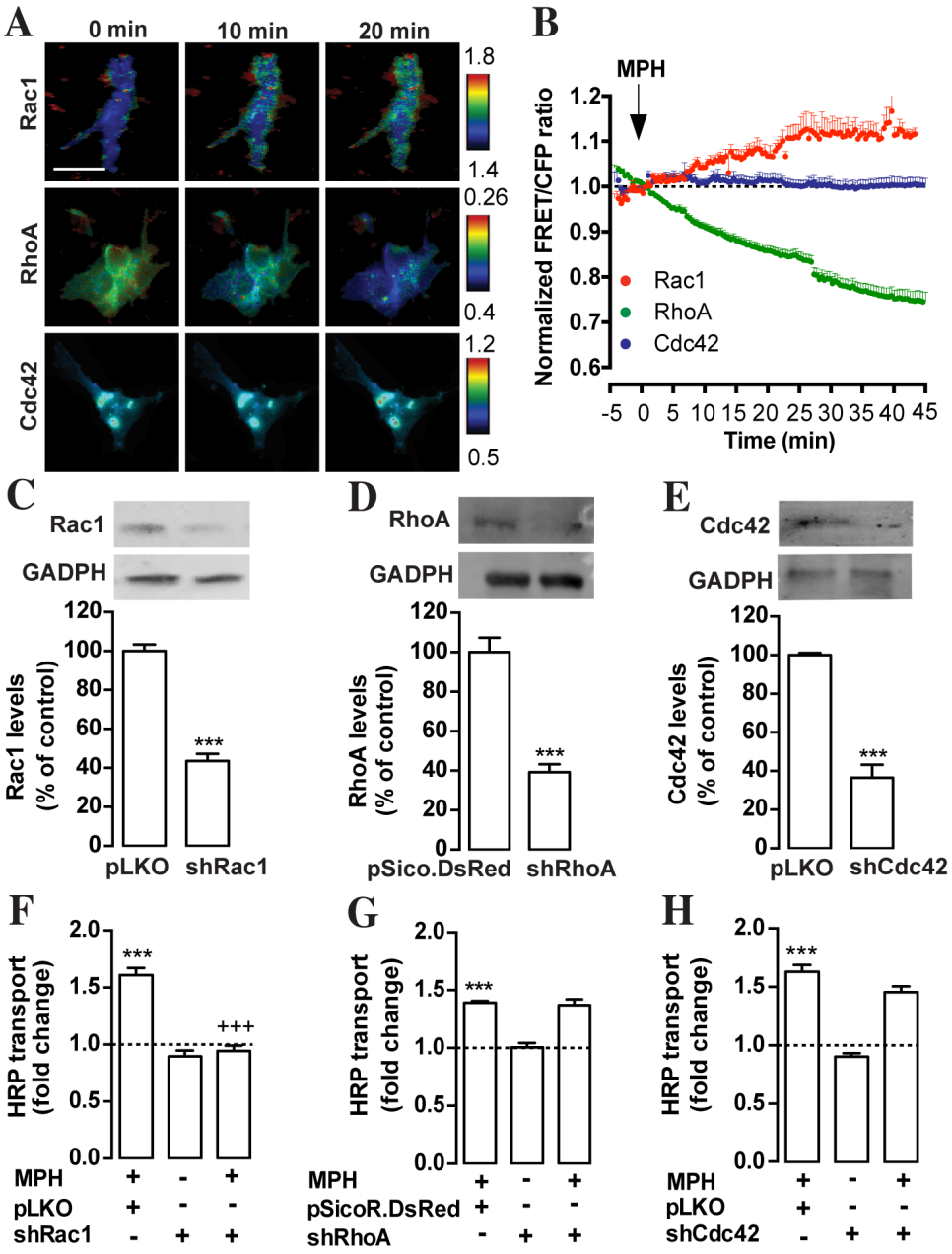


Figure 2.4. Impact of MPH on Rac1, RhoA and Cdc42 GTPase activity and their involvement in vesicular transport. ***(A)*** *Representative FRET/CFP ratio images of hCMEC/D3 cells expressing Rac1, RhoA and Cdc42 biosensors after MPH (100 µM) exposure at the indicated time points and coded according to a pseudocolor scale. Scale bar: 40 µm.* ***(B)*** *MPH increased Rac1 activity (red circles), decreased RhoA (green circles), and had no effect on Cdc42 (blue circles) activity. The mean FRET/CFP emission ratios were normalized with a reference value acquired from unstimulated cells. Symbols represent the mean + S.E.M., n=4-7 cells.* ***(C-E)*** *Quantification of* ***(C)*** *Rac1,* ***(D)*** *RhoA, and* ***(E)*** *Cdc42 protein levels in hCMEC/D3 cells after shRNA-mediated knockdown or infection with empty vector (pLKO or pSicoR-DsRed). Top: representative western blot images of the Rho GTPases (22 kDa) and GAPDH (37 kDa), n=4-6.* ***(F-H)*** *hCMEC/D3 cells infected with lentiviral shRNA clone to* ***(F)*** *Rac1,* ***(G)*** *RhoA and (H) Cdc42 indicate that Rac1 is required for MPH-increased caveolar transport. pLKO and pScicoR.DsRed were the respective empty vectors. The results are expressed as mean + S.E.M, n=6-8.* $^{***}P<0.001$ *vs pLKO, pSicoR.DsRed, or control (dashed line);* $^{+++}P<0.001$ *vs MPH+pLKO.*

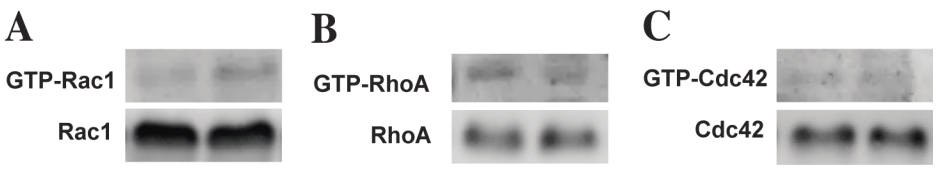


Figure 2.5. Impact of MPH on Rac1, RhoA, and Cdc42 GTPases activity analyzed by a pull-down assay. *hCMEC/D3 cells were exposed to MPH (100 µM) during 30 min.*

Previous reports have demonstrated that MPH increases oxidative stress in the brain (Martins et al., 2006; Gomes et al., 2009), and oxidant signaling in ECs has been linked to caveolae-mediated transcytosis (Hu et al., 2008b; Sun et al., 2009). Thus, we assessed cellular redox responses to MPH in live ECs using a redox-sensitive FRET biosensor [heat shock protein 33 fluorescence resonance energy transfer (HSP-FRET)] (Socodato et al., 2015). We observed that MPH increased ROS generation in hCMEC/D3 cells, as detected by an increase in the CFP/FRET ratio using the HSP sensor (Figure 2.6 A, B, green circle). Interestingly, the antioxidant vitamin C (80 µM VitC) attenuated ROS production triggered by MPH (Figure 2.6 A, B, blue circles). Since it is known that the NOX complex plays a key role in vascular ROS production (Frey et al., 2009), we further showed that a specific NOX inhibitor, VAS2870 (5µM VAS) (Stielow et al., 2006) blocked ROS generation triggered by MPH (Figure 2.6 A, B, black circles). Also, taking into consideration that the small GTPase Rac1 mediates ligand-dependent ROS generation by NOX assembly in ECs (Chen et al., 2008), we further evaluated the potential involvement of Rac1 activation in oxidative events associated with MPH exposure by conducting FRET studies with the HSP sensor in ECs after knocking down Rac1. We observed that MPH-induced ROS production was largely attenuated upon shRNA-mediated Rac1 knockdown (Figure 2.6 A, B, orange circles). As proof of concept, we also showed that ROS production was reestablished in cells transfected with the empty vector (transduction control) of Rac1 (pLKO; Figure 2.6 A, B, yellow circles), and hydrogen peroxide (H_2O_2) was used as a positive control (Figure 2.6 A, B, red circles). Taken together, these data indicate that MPH increases ROS generation in brain ECs via the Rac1/NOX signaling pathway.

Similar to what was demonstrated with the hCMEC/D3 cell line (Figure 2.6 A, B), we observed that VitC also prevented MPH-induced ROS formation in HBMVECs (Figure 2.6 C). The use of DHE, which is specifically oxidized by superoxide anion, also allowed us to identify an increase in superoxide anion content after MPH exposure (Figure 2.6 D).

As expected, H_2O_2 (positive control) robustly increased ROS generation (Figure 2.6 C, D). We then explored the possibility that ROS participated in MPH-induced increase of vesicular transport. In fact, both VitC and the NOX inhibitor (5 μM VAS) blocked HRP transport promoted by MPH (Figure 2.6 E). These results demonstrate that MPH-induced Rac1/NOX-dependent ROS generation culminates in vesicular transport in human brain ECs.

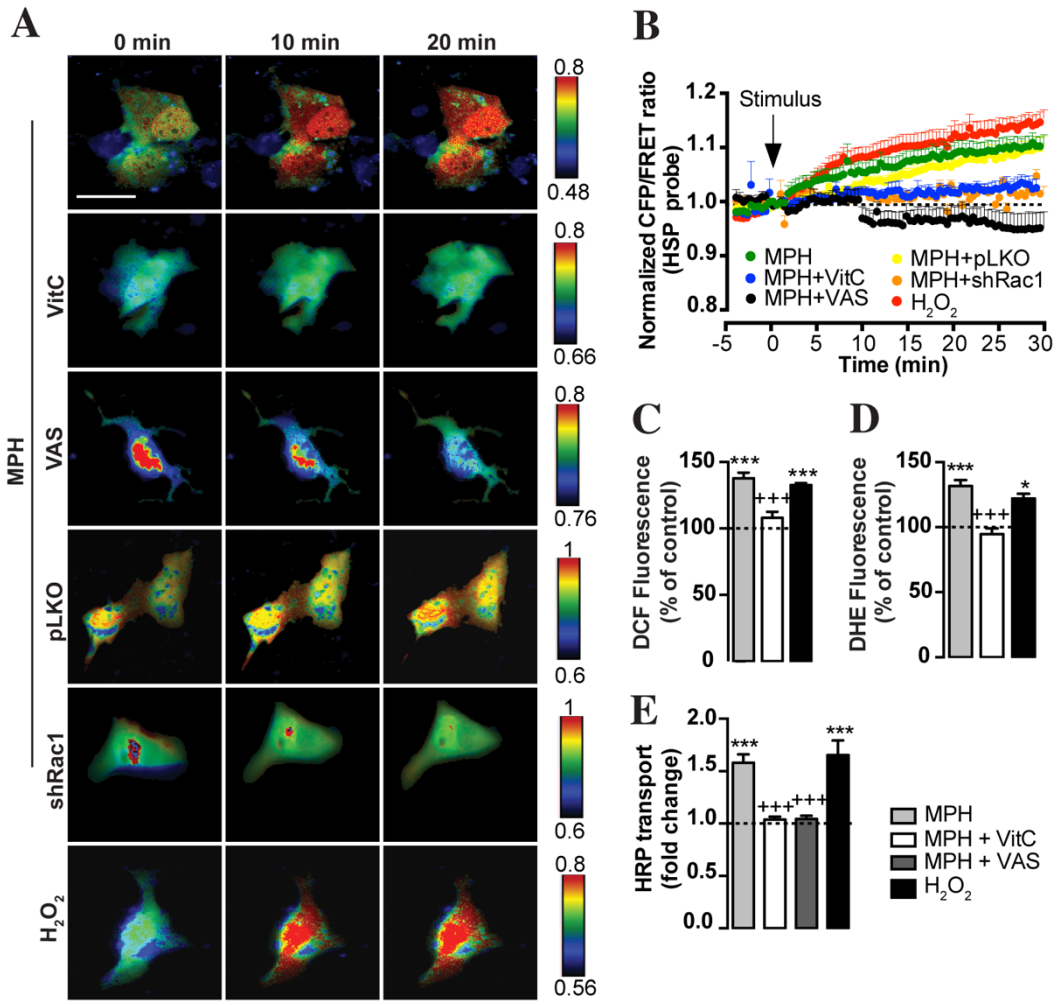


Figure 2.6. MPH increases the production of intracellular ROS by activation of Rac1/NOX complex culminating in enhanced caveolae-mediated transcytosis in brain ECs. (A, B) *hCMEC/D3 cells expressing ROS FRET probe (HSP) were treated with MPH (100 μM; green circles) alone and in the presence of the antioxidant vitamin C (80 μM VitC; blue circles) or NOX inhibitor VAS2870 (5 μM VAS; black circles). To specifically identify the role of Rac1 on MPH-induced ROS production, hCMEC/D3 cells were infected with lentivirus carrying pLKO (empty vector; yellow circles) or Rac1 shRNA (orange circles, and then transfected with HSP probe. H_2O_2 (500 μM; red circles) was used as a positive control.* ***(A)*** *Representative ratio images of hCMEC/D3 cells show time-lapse CFP/FRET color-coded according to the pseudocolor ramp. Scale bar: 40 μm.* ***(B)*** *CFP/FRET emission ratios of the chimera over time were normalized with a reference value acquired from unstimulated cells. Symbols represent the mean + S.E.M., n=5-18 cells.* ***(C)*** *MPH increased the production of ROS (DCF dye), namely* ***(D)*** *superoxide anion (DHE dye), n=16-48.* ***(E)*** *The increase in HRP transport mediated by MPH in HBMVECs was prevented by the antioxidant VitC or NOX inhibitor VAS. H_2O_2 (500 μM) was used as a positive control, n=5-18. Results are expressed as mean + S.E.M. $^{*}P<0.05$, $^{***}P<0.001$ vs control (dashed line), $^{+++}P<0.001$ vs MPH.*

2.4.3 Oxidant signaling triggered by MPH activates c-Src

ROS have been described as regulators of c-Src activity via oxidization (Giannoni et al., 2005). Thus, we asked whether MPH-mediated oxidant signaling could activate c-Src in human brain ECs. We first showed that MPH significantly increased c-Src phosphorylation (p-c-Src^{Y416}) in both HBMVECs (Figure 2.7 A) and hCMEC/D3 cells (Figure 2.7 B). Then, tyrosine kinase activity of c-Src was also evaluated at the plasma membrane using a specific c-Src FRET-based biosensor (KRas Src YPet) which does not recognize the activity of other SFKs (Ouyang et al., 2008). From these studies, we conclude that MPH induced a rapid and sustained increase in c-Src activity in live hCMEC/D3 cells, as demonstrated by an increase in CFP/FRET emission ratio of the KRas Src YPet probe (Figure 2.7 C, d, green circles). This effect was blocked by SKI-1 (100 nM SKI, 1 h pretreatment; Figure 2.7 C, D, cyan circles). To further confirm that MPH specifically activates c-Src, we used the negative control KRas Src (RV) construct (Ouyang et al., 2008) and showed that MPH had no effect on FRET in ECs under the same conditions (Figure 2.7 C, D purple circles).

The antioxidant VitC (Figure 2.7 C, D, blue circles) or NOX inhibition with VAS (Figure 2.7 A, B, black circles) blocked MPH-mediated increase of c-Src activation. Since it was previously shown that Rac1 plays a key role in redox-sensitive signal transduction in ECs (Chen et al., 2008; Frey et al., 2009), we assessed MPH-induced activation of c-Src following Rac1 knock-down in hCMEC/D3 cells. As shown in Figure 2.7, Rac1 shRNA decreased Src activity (Figure 2.7 B, C, orange circles) compared to control cells (pLKO empty vector; Figure 2.7 B, C, yellow circles). ROS mediated c-Src activation was confirmed by stimulating hCMEC/D3 cells with H_2O_2 (Figure 2.7 C, B, red circles). We conclude that MPH activates c-Src via Rac1/NOX-dependent ROS generation in human brain ECs.

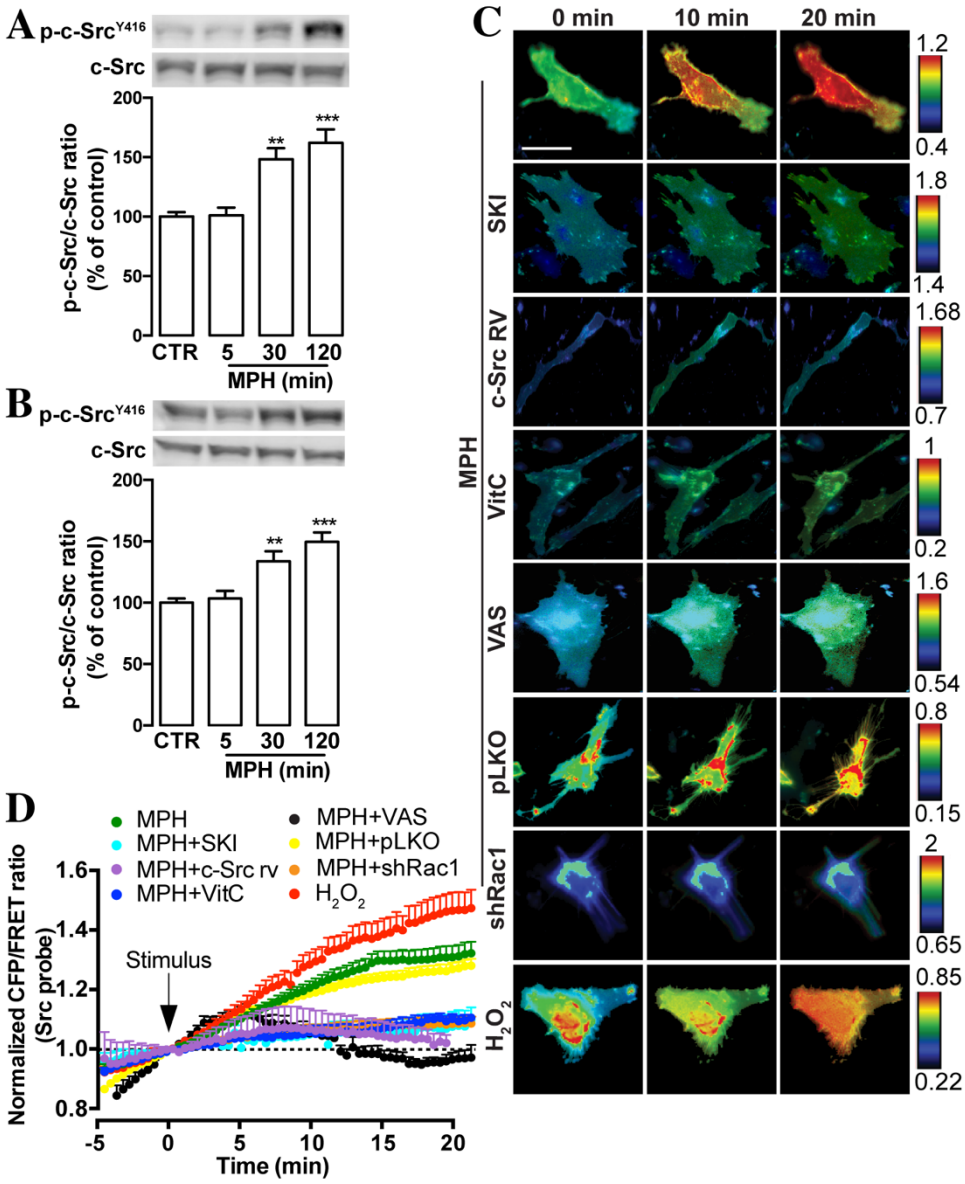


Figure 2.7. ROS generation by Rac1/NOX activation is required for MPH-mediated c-Src activation in human brain ECs. (A, B) *MPH induced c-Src activation, as shown by the increased phosphorylation at Tyr^{416} (p-c-Src) in both* ***(A)*** *HBMVECs and* ***(B)*** *hCMEC/D3 cells. Top: representative western blot images of c-Src Tyr^{416} (60 kDa) and total c-Src are shown. Results are expressed as mean + S.E.M. $^{**}P<0.01$, $^{***}P<0.001$ vs control (CTR), n=10-14.* ***(C)*** *hCMEC/D3 cells expressing c-Src FRET probe (KRas Src YPet) or its negative control (Kras Src (RV) YPet) were exposed to MPH (100 µM; green circles) alone or in the presence of vitamin C (80 µM VitC; blue circles) or VAS2870 (5 µM VAS; black circles). Other cells were infected with lentivirus carrying pLKO (control empty vector; yellow circles) or Rac1 shRNA (shRac1; orange circles). H_2O_2 (500 µM; red circles) was used as positive stimulus to trigger ROS production. Representative ratio images show time-lapse CFP/FRET color-coded according to the pseudocolor ramp. Scale bar: 40 µm.* ***(D)*** *Time course of CFP/FRET emission ratios of c-Src activity in hCMEC/D3 cells under the same experimental conditions as mentioned previously. The results were normalized with a reference value acquired from unstimulated cells. Symbols represent the mean + S.E.M., n=5-18 cells.*

2.4.4 MPH-mediated c-Src activation leads to Cav1 Tyr^{14} phosphorylation and transendothelial hyperpermeability

Cav1 phosphorylation has been implicated in the mechanism of oxidative stress-induced transcellular transport, and it is a well-known substrate for c-Src (Sverdlov et al., 2007). Thus, the role of c-Src in MPH-induced vesicular transport was investigated. We observed that MPH-triggered HRP hyperpermeability of HBMVECs was prevented by c-Src inhibition by SKI (100 nM; Figure 2.8 A), and that shRNA-mediated c-Src knockdown (validated in Figure 2.8 B) had the same effect in hCMEC/D3 cells (Figure 2.8 C). Also, pharmacological inhibition or c-Src knockdown blocked MPH-induced Cav1 phosphorylation (at 120 min) in both HBMVECs (Figure 2.8 D) and hCMEC/D3 cells (Figure 2.8 E), respectively. To further clarify whether MPH-induced transcytosis was dependent on Cav1 phosphorylation by c-Src, hCMEC/D3 cells were co-transfected with a rapamycin (Rap)-inducible c-Src heterodimerization chimera (RapR-Src) (Socodato et al., 2015), and/or with Cav1^{Y14F}. We observed that c-Src activation with Rap in RapR-Src-transfected ECs was sufficient to trigger HRP transport, whereas the overexpression of Cav1^{Y14F} prevented RapR-Src-induced HRP transport (Figure 2.8 F). c-Src and Cav1 interaction assessed by FRET in hCMEC/D3 cells transfected with Cav1-YFP and RapR-Src-CF further demonstrate that RapR-Src-CFP binding to Cav1-YFP was significantly increased by Rap activation of RapR-Src (Figure 2.8 G, H). The same FRET result was obtained with MPH co-administration, further indicating MPH-triggered Cav1 Tyr^{14} phosphorylation was mediated by activated c-Src. Overall, we conclude that MPH-induced vesicular transport in human ECs requires Cav1 Tyr^{14} phosphorylation by c-Src downstream of Rac1/NOX-mediated ROS generation.

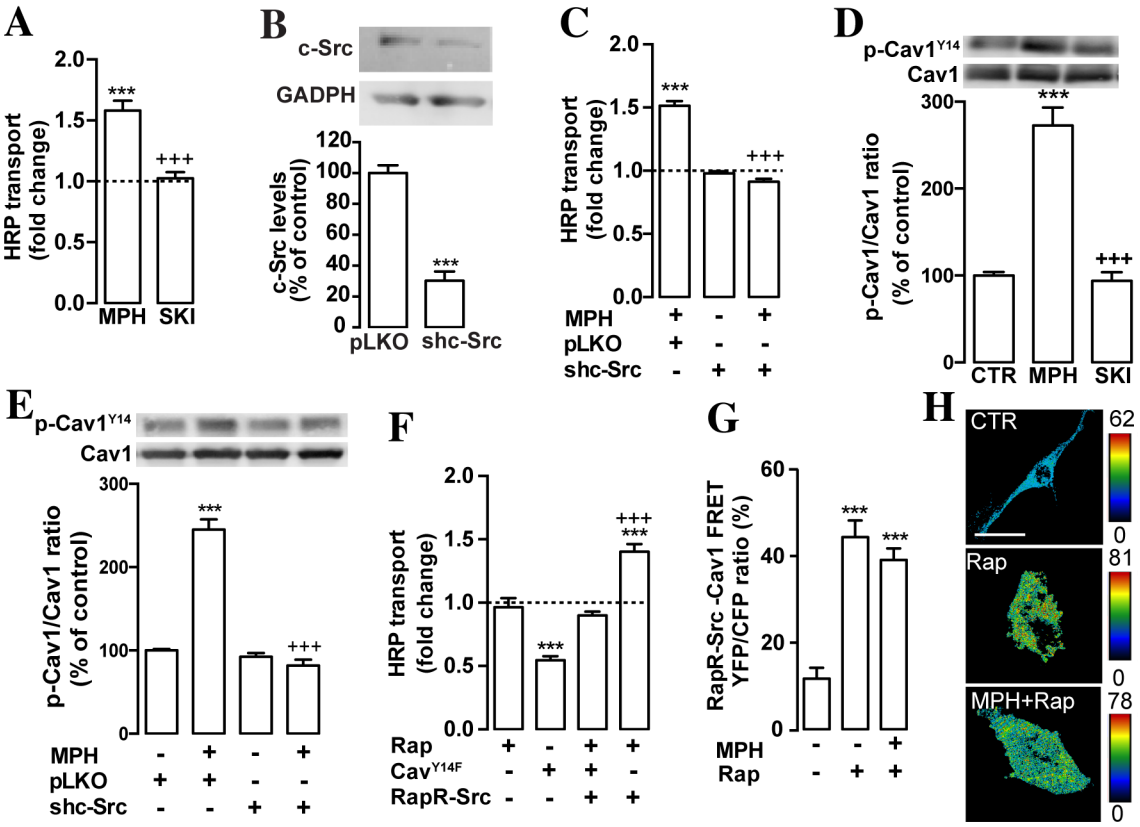


Figure 2.8. MPH-induced HRP vesicular transport is dependent on c-Src activation. ***(A)*** *The inhibition of Src pathway using SKI-1 (100 nM SKI) prevented MPH-induced increase in transcytosis in HBMVECs, n=10-18.* ***(B)*** *Quantification of c-Src protein levels in hCMEC/D3 cells after shRNA-mediated knockdown or infection with empty vector (pLKO). Top: representative western blot images of c-Src (60 kDa), and GAPDH (37 kDa) are shown, n=5-9.* ***(C)*** *Knockdown of c-Src (shc-Src) prevented MPH-induced vesicular transport compared with cells infected with pLKO (empty vector), n=4-5.* ***(D, E)*** *Increase in Cav1 Tyr^{14} phosphorylation (p-Cav1) triggered by MPH (100 µM for 120 min) was prevented by* ***(D)*** *SKI (100 nM) in HBMVECs and by* ***(E)*** *c-Src knockdown in hCMEC/D3 cells. Top: representative western blot images of p-Cav1 (22 kDa) and Cav-1 (22 kDa) are shown, n=10-15.* ***(F)*** *hCMEC/D3 cells were transfected with Cav1^{Y14F} and/or RapR-Src, and vesicular transport was assessed. Rap (200 nM) used to activate c-Src construct increased HRP transport, n=9-12.* ***(G)*** *Quantification and* ***(H)*** *representative ratio images of Cav1-YFP and RapR-Src-CFP interaction assessed by FRET in hCMEC/D3 cells, n=12-15. Results are expressed as mean + S.E.M. $^{***}P<0.001$ vs control (CTR or dashed line) or pLKO; $^{+++}P<0.001$ vs MPH, pLKO+MPH or Rap.*

2.5 Discussion

MPH is the drug of choice for the treatment of ADHD and its use has increased significantly over the last few years. Several studies have shown beneficial effects of MPH in ADHD (Sharma and Couture, 2014), but nevertheless its use among children is non-consensual (Sharma and Couture, 2014; Sahakian et al., 2015). In addition, there is currently a concern regarding MPH misuse for cognitive enhancement. Thus, it crucial to better clarify the central effects of MPH. In the present study, we investigated whether

MPH affects human brain ECs, the main component of the BBB, using a representative therapeutic dose range of MPH (Gopal et al., 2007). We demonstrate, for the first time, that MPH increases brain EC permeability by stimulating vesicular transport and unveil the intracellular signaling pathway responsible for this effect (Figure 2.9).

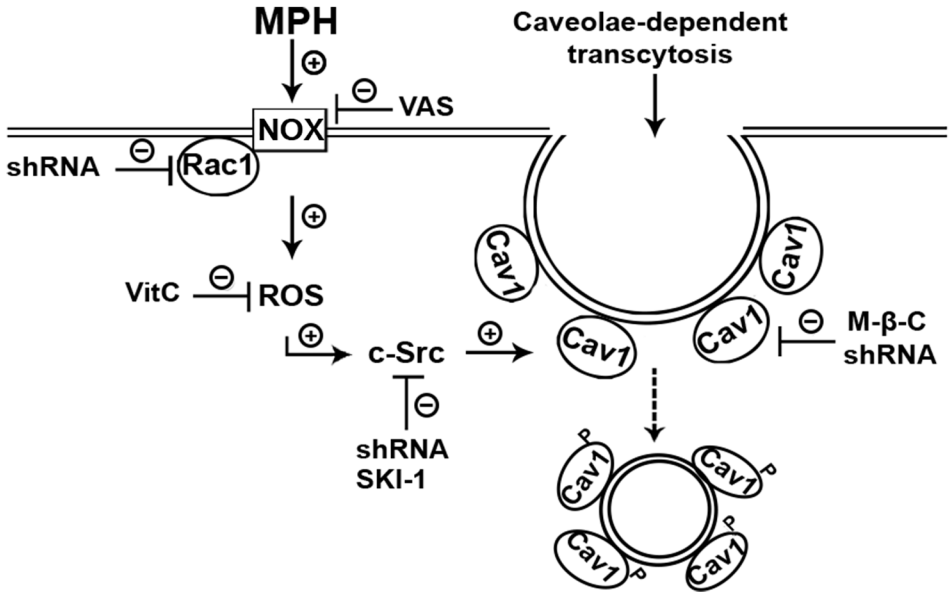


Figure 2.9. Schematic illustrating the mechanism by which MPH promotes caveolae-mediated transcellular transport in human brain endothelial cells. *Plus and minus signs represent stimulation and inhibition, respectively. Cav1, caveolin-1; MPH, methylphenidate; M-β-C, methyl-β-cyclodextrin; NOX, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase; ROS, reactive oxygen species; VitC, vitamin C.*

It was previously demonstrated that MPH augments the number of vesicles in ECs (Balcioglu et al., 2009) and that methamphetamine, a powerful psychostimulant drug of abuse, increases both paracellular and transcellular transport in ECs (Coelho-Santos et al., 2012; Martins et al., 2013). Paralleling these findings, we showed that MPH increased transendothelial flux without altering monolayer electrical resistance or the expression/distribution of intercellular junctional proteins in human brain ECs. Instead, MPH promoted vesicular transport across ECs via a caveolae-dependent mechanism. We also revealed that this psychostimulant increased Cav1 phosphorylation. Induction of Cav1 expression has been identified in a number of brain disorders, such as Alzheimer's disease (Gaudreault et al., 2004) and intracerebral hemorrhage (Chang et al., 2011). Moreover, caveolae-dependent processes are involved in the transport of macromolecules (Hu et al., 2008b), virus (Coyne et al., 2007), and fungal pathogens across ECs (Long et al., 2012). Caveolae have also been implicated in the internalization of TJs

transmembrane proteins leading to brain endothelial barrier disruption during CNS inflammation (Stamatovic et al., 2009), although recycling of TJs proteins also allows for faster re-establishment of barrier function without new protein synthesis (Hopkins et al., 2003). Additionally, Cav1 can regulate the expression of junction-associated proteins since its reduction was associated with deletion of tight junction-associated proteins and a consequent increase of barrier permeability (Song et al., 2007; Li et al., 2015). In the present study, we identified neither alterations in the expression/organization of inter-endothelial junctions nor in the expression of Cav1 protein. Nevertheless, several studies have suggested that Cav1 phosphorylation plays a crucial role in the regulation of caveolae formation and transcytosis in endothelial cells (Sverdlov et al., 2007; Hu et al., 2008b; Sun et al., 2009). Very recently, it was clearly shown that endocytosis and trafficking of caveolae are associated with a Cav1 Tyr^{14} phosphorylation-dependent conformational change in rat lung microvascular endothelial cells (Zimnicka et al., 2016). Accordingly, in the present study we demonstrated for the first time that shRNA-induced depletion of Cav1 or the use of $Cav1^{Y14F}$ mutant prevented increase in brain endothelial transcytosis induced by MPH. On the contrary, overexpression of $Cav1^{Y14D}$ by itself enhanced vesicular transport, supporting the hypothesis that phosphorylation of Cav1 at Tyr^{14} promotes caveolae formation upon MPH stimulation. These data were further corroborated by immunoelectron microscopy, wherein numerous caveolae-like invaginations on the plasma membrane and in the cytosol of ECs were observed after exposure to MPH.

We also investigated the mechanism by which MPH increased caveolae vesicular transport. It has been shown that young rats treated with MPH exhibit oxidative damage as evidenced by an increase in both lipid peroxidation and protein carbonyl adducts in the brain (Martins et al., 2006). In addition, MPH seems to interfere with important brain antioxidant defenses (Gomes et al., 2008). Whereas ROS generation by ECs at low levels signal important physiological activities such as cell growth and differentiation (Frey et al., 2009), excessive generation of ROS overwhelms the intracellular antioxidant defense systems leading to an imbalance in redox homeostasis, oxidative stress, and endothelial dysfunction (Frey et al., 2009). In fact, we found that intracellular ROS generation in human ECs, namely superoxide anion, increased upon exposure to MPH. Likewise, acute MPH administration in young rats increased the amount of brain superoxide (Gomes et al., 2009). We also identified NOX as the source of ROS production triggered by MPH. Our findings are consistent with previous studies showing a crucial role for NOX-

generated ROS in BBB disruption (Kahles et al., 2007). Collectively, these observations led us to hypothesize that an antioxidant strategy could have a beneficial effect on ECs exposed to MPH. Indeed, the antioxidant VitC was able to prevent both MPH-induced ROS generation and transcytosis. Accordingly, others have reported that VitC could prevent microvascular endothelial barrier dysfunction during septic insult by blocking NOX-dependent ROS generation (Han et al., 2010).

ROS signaling plays an important role in the control of endothelial permeability (Frey et al., 2009) by interfering with the dynamics of the actin cytoskeleton via Rho GTPases (Stielow et al., 2006). It is known that Rho-regulated cytoskeletal remodeling is essential for targeting vesicles to their correct location, enabling exocytosis (Long et al., 2012; Lim et al., 2014). However, the mechanisms by which Rho GTPases exert their effects on intracellular trafficking are still largely not known. Herein, we observed that MPH had different effects on the activities of the endothelial Rho GTPases Rac1, RhoA and Cdc42. In particular, while MPH induced the rapid activation of Rac1, it decreased the activity of RhoA and had no effect on Cdc42. Likewise, Rac-mediated ROS production in HeLa cells results in the downregulation of Rho activity, which is required for Rac-induced formation of membrane ruffles and integrin-mediated cell spreading (Nimnual et al., 2003). Interestingly, others have demonstrated the involvement of Rac1in the recruitment and assembly of the endothelial NOX complex (Chen et al., 2008). Here, we showed that MPH activated Rac1 and that shRNA-mediated Rac1 knockdown prevented ROS generation and consequently the effect of MPH. Although the exact role of Rac1 in EC barrier function is not fully understood, Rac-dependent generation of ROS is known to cause barrier dysfunction (Chen et al., 2008). Chen *et al.* (2008) reported that Rac1 inhibition in human pulmonary artery ECs contributes to barrier protection via the inhibition of NOX and superoxide generation. Similarly, we demonstrated that Rac1 knockdown inhibits MPH-induced caveolae-mediated transcytosis. Furthermore, Rac1 activation was reported to be required for bacterial entry into human ECs (Lim et al., 2014). Several pathogens use caveolae as a carrier vacuole to hijack endosomal trafficking in host cells and escape lysosomal degradation (Zaas et al., 2005). In light of these observations, it is plausible to speculate that MPH-induced caveolae formation and trafficking might ultimately promote the entrance of pathogens into the brain.

RhoA activation is known to be involved in functional changes of tight junction proteins, such as claudin-5, and also reorganization of the actin cytoskeleton leading to disruption of endothelial cell-cell contacts and paracellular hyperpermeability (Hopkins et al., 2003;

Persidsky et al., 2006a). RhoA inhibition and dominant negative RhoA mutant prevented the loss of tight and adherens junctions, the decrease of transendothelial resistance, and stress fiber formation in human umbilical vein endothelial cells (Wojciak-Stothard et al., 2001). Moreover, RhoA inactivation caused the disassembly of actomyosin stress fibers and reorganized F-actin and phosphotyrosine-containing proteins to β-catenin-containing cell-cell junctions, a process that increased the size-selective permeability of endothelial monolayers (Carbajal and Schaeffer, 1999). Also, reduction of RhoA activity and elevation of Rac1 signaling are important steps in the control of endothelial permeability due to their involvement in the re-annealing of the intercellular junctions (Birukova et al., 2011). In our study, we did not observe alterations in the intercellular junctions, which may be justified by data indicating inactivation of RhoA in response to MPH.

The activity of c-Src has been implicated in endothelial hyperpermeability (Hu et al., 2008a), and its inhibition ameliorates vascular leakage and inflammation in rodent brains (Liang et al., 2009). Here, we demonstrated that oxidant signaling is a key feature of c-Src activation in response to MPH in brain ECs, since the antioxidant VitC or NOX inhibition abrogated MPH-induced c-Src activation. Intracellular ROS may regulate the activity of c-Src via oxidation of two cysteine residues that control c-Src conformational changes necessary for its activation (Giannoni et al., 2005). The c-Src and Rac1 hierarchy is generally context and stimuli specific in different cells. Our results show that MPH-mediated Rac1 activation is upstream of c-Src in human brain ECs. In response to oxidative stress, Tyr^{14} on Cav1 is the principal c-Src target (Hu et al., 2008b; Sun et al., 2009). We showed that MPH leads to Cav1 phosphorylation by c-Src, and consequently to HRP transcytosis. Direct activation of c-Src via rapamycin increased vesicular transport and promoted the interaction between c-Src and Cav1 at the plasma membrane. In this respect, our results are consistent with other reports showing that Src-Cav1 interaction requires c-Src activity to promote caveolae-mediated pulmonary endothelial hyperpermeability and edema formation (Hu et al., 2008b). Additionally, overexpression of $Cav1^{Y14F}$ in brain ECs abrogated RapR-Src-mediated transcytosis, supporting the premise that c-Src-dependent phosphorylation of Cav1 on Tyr^{14} is necessary and sufficient to promote caveolae trafficking dynamics and endothelial hyperpermeability. Accordingly, Zimnicka and collaborators (2016) have recently demonstrated a key role of Src-dependent Cav1 phosphorylation in promoting caveolae release from the plasma membrane via phosphorylation-dependent destabilization of Cav1 oligomers. Overall, our data indicate that MPH induces Rac1/NOX-dependent ROS generation and

subsequent c-Src activation-dependent Cav1 Tyr^{14} phosphorylation promotes transcellular transport (transcytosis) in human brain ECs.

The present study shows for the first time that MPH has a direct effect on human brain ECs and provides new insights into the mechanism underlying MPH-induced BBB hyperpermeability. In this context, our data raise the question of whether MPH use enhances brain susceptibility to peripheral factors and the risk of neurological disease. However, while the cerebral vasculature provides a crucial protective role in maintaining brain homeostasis, the BBB also represents a substantial obstacle to the delivery of many neurotherapeutic drugs. Thus, our results also suggest that transient and controlled MPH exposure might constitute a potential strategy for enhancing drug delivery into the brain.

Chapter 3

Unravelling the aftermath of methylphenidate use on hippocampal neurogliovascular unit and memory performance

Unraveling the aftermath of methylphenidate use on hippocampal neurogliovascular unit and memory performance

3.1 Abstract

Methylphenidate (MPH) is the classic treatment for attention deficit hyperactivity disorder (ADHD). Some reports state a nonconsensual diagnosis and claim that many children are being misdiagnosed with ADHD. Due to the limited knowledge about the consequences of this psychostimulant use, the purpose of the current study was to uncover the neurogliovascular and cognitive effects of chronic misuse of MPH during a critical period of development.

To achieve our goal, healthy rats were treated with MPH (1.5 or 5 mg/kg/day at weekdays, per os) from P28-P55. Our results showed that the higher dose of MPH caused hippocampal blood-brain barrier (BBB) hyperpermeability by specifically promoting vesicular transport (transcytosis) concomitantly with the presence of peripheral immune cells in the brain parenchyma. These observations were confirmed by *in vitro* studies, in which the knockdown of caveolin-1 in human brain microvascular endothelial cells prevented the increased permeability and leukocytes transmigration triggered by MPH (100 μM for 24h).

In addition to endothelial alterations, MPH highly interfered with astrocytic morphology, namely the length and degree of ramification. Interestingly, we verified that a low-dose of MPH increased astrocytic processes, as well as the protein levels of several neuronal proteins and signaling pathways involved in synaptic plasticity culminating in the improvement of working memory. On the contrary, the higher dose of MPH led to astrocytic atrophy and to a decrease in the levels of several synaptic proteins and impairment of AKT/CREB signaling together with working memory deficit.

In sum, our results allow us to conclude that the central effect of MPH is dose-dependent being harmful at higher doses, which highlights the problematic of MPH misuse among non-ADHD population.

3.2 Introduction

Methylphenidate (MPH) is the first line treatment for the symptomatic treatment of attention deficit hyperactivity disorder (ADHD), one of the most common neuropsychiatric disorders of childhood (Sharma and Couture, 2014). Despite evidence for improvement of ADHD behavioral and neurocognitive impairment with MPH, its prescription among children is still non-consensual. Additionally, there is currently a concern about MPH misuse for cognitive enhancement (Urban and Gao, 2014) that together with the ADHD misdiagnosis problematic have raised an intense debate. The underlying mechanisms contributing to the effectiveness of MPH have also received little attention besides its role on dopaminergic system. In this context, chronic MPH use during development can modulate genes and transcription factors associated with long-term potentiation, synaptogenesis and synaptic plasticity (Andersen et al., 2002; Chase et al., 2003; Adriani et al., 2006a). In fact, the hippocampus is an important region to memory (Bird and Burgess, 2008; Urban and Gao, 2014), and seems to be very susceptible to psychostimulants use (Gonçalves et al., 2010; Martins et al., 2011). The administration of MPH to pre-pubertal and adolescent rats dramatically attenuates c-fos expression (Chase et al., 2003) and cyclic adenosine monophosphate (cAMP) response element-binding (CREB) with behavioral and neurobiological adaptations that endure into adulthood (Andersen et al., 2002). Moreover, rats treated during adolescence with MPH exhibited an increase of *dynorphin* expression at the end of adolescent (Brandon and Steiner, 2003). Importantly, the use of MPH under non-pathological conditions may have abuse potential since it will interact with the same brain pathways activated by other psychostimulants, such as amphetamine, methamphetamine and cocaine (Volkow et al., 2002). Therefore, MPH misuse is nowadays a major public health concern.

Blood-brain barrier (BBB) is a selective and dynamic structure that separates the central nervous system (CNS) from the peripheral blood circulation. BBB sustains a stable environment for the CNS function through highly specialized transport mechanisms on both the luminal and abluminal membrane surfaces, avoiding ions and molecules out of the CNS and allowing oxygen and nutrients into the CNS according to neural cell´s needs (Cardoso et al., 2010). The core anatomical element of the BBB is the microvascular endothelium formed by endothelial cells (ECs) that are associated with pericytes, basal lamina, astrocytes, microglia and neurons forming a neurogliovascular unit (NVU). Brain ECs present unique properties as lacking fenestrations and undergoing extremely low

rates of transcytosis (Cardoso et al., 2010). The adherens (AJs) and tight junctions (TJs) between adjacent ECs regulate the paracellular permeability, while the transcellular route can occur through caveolae vesicles (Cardoso et al., 2010) that are dynamic pieces of membrane composed by the structural protein caveolin-1 (Cav1) and enriched in cholesterol and sphingolipids (Fernandez et al., 2002).

Alterations at the BBB are likely involved in most of all neurodegenerative diseases (Rosenberg, 2012). Interestingly, MPH has been demonstrated to affect blood flow in different brain regions (Marquand et al., 2012). Also, a study in rats showed that MPH causes neuronal degeneration and capillary wall structural changes such as basal membrane thickness and augmentation of the pinocytic vesicles in ECs (Bahcelioglu et al., 2009). Accordingly, our recent results show that an acute exposure of MPH promotes human brain endothelial permeability by interfering specifically with the caveolae-dependent vesicular transport (Coelho-Santos et al., 2016), Chapter 2. Despite this first attempt to understand the effect of MPH on endothelial signaling, many questions remain unanswered regarding the BBB function together with the downstream effectors and molecular endpoints that ultimately modulate MPH-induced BBB dysfunction.

As previously mentioned, BBB is a complex structure and astrocytes play here an important role since they ensheath blood vessels at the same time that communicate with neurons. Regarding the effect of MPH on astrocytes, it was previously observed a robust astrocytic activation in primary cultures (Suzuki et al., 2007) and in rodent brains (Bahcelioglu et al., 2009; Sadasivan et al., 2012; Schmitz et al., 2016b). Therefore, it is of high interest to determine if MPH modulates astrocyte morphology, vessels coverage and how it may reflect in behavior.

Thus, we investigated the effect of chronic MPH use on hippocampal NVU and memory performance using a clinical dosing schedule for ADHD treatment (Marco et al., 2011) in healthy rats (Wistar Kyoto, WKY) to simulate a misuse condition, as well as *in vitro* BBB model to better explore some cellular effects.

3.3 Material and methods

3.3.1 Animals and treatments

Male WKY rats (arrived at 24 days-old; ~55 g body weight; Charles River Laboratories, Lyon, France) were housed under controlled environmental conditions (12 hours light:dark cycle, 24±1°C) with food and water ad libitum. Animals were divided into three different groups as follows: control group (vehicle, tap water), 1.5 mg/kg/day MPH group (MPH 1.5) and 5 mg/kg/day MPH group (MPH 5) (Sigma-Aldrich, Si. Louis, MO, USA). Drug doses and the pathway for drug administration are important factors to be taken into account when trying to understand physiological mechanisms of treatments used in human therapies studying animal models (Clark et al., 2007). Normally, given the existing differences in metabolism between rodents and humans, higher doses of different drugs (approx. 3-fold) are required to achieve blood levels in rats within the range found in humans (Gerasimov et al., 2000). The clinical use of MPH for the treatment of ADHD in children typically involves oral administration of doses between 0.25 and 1.0 mg/kg, which result in peak plasma levels in the range of 8–40 ng/ml (Swanson and Volkow, 2002). An oral dose of 1.5 mg/kg/day of MPH or 5 mg/kg/day produces peak plasma concentrations between 9–36 ng/ml (Kuczenski and Segal, 2002) or 36-80 ng/ml in rats (Wheeler et al., 2007; Balcioglu et al., 2009). Based on these observations, the use of both 1.5 and 5 mg/kg/day oral dose of MPH ensures that therapeutic relevant plasma drug levels are achieved and mimic the dosage range in humans (Harvey et al., 2011; Somkuwar et al., 2013). Individual administration was performed by gavage from Monday to Friday between postnatal days (P)28-P55 (4 weeks), equivalent to late-childhood through late-adolescence in humans (Marco et al., 2011). Behavioral tests were performed at P56, and animals were sacrificed after 24h (P57). All experiments were performed by certified researchers in accordance with European Community Council Directives (2010/63/EU) and Portuguese law for care and use of experimental animals (DL no 113/2013). The present study was approved by the Institutional Animal Care and Use Committee (FMUC/CNC, University of Coimbra, Coimbra, Portugal) and Portuguese National Authority for Animal Health "DGAV" and Federation for Laboratory Animal Science Associations, FELASA. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.3.2 Immunohistochemistry

For immunohistochemical analysis, after anesthesia with intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg), rats were transcardially perfused with 4% paraformaldehyde (PFA; Sigma-Aldrich) in 0.01 M phosphate buffered saline (PBS), pH 7.4. Then, brains were removed, post fixed in 4% PFA for 24h at RT and transferred to 30% sucrose in 0.01 M PBS, pH 7.4, for at least 24h at 4°C. Coronal sections were cut on a cryostat (Thermo Sahndon Inc.), slices with 14 µm were mounted directly onto superfrost microscope slides and 50 µm slices were collected in crioprotector solution (0.1 M phosphate buffer, 30% sucrose and 30% ethylene glycol) and stored at -80°C until further use.

Immunolabelling for albumin and collagen IV was performed as previously published by us (Coelho-Santos et al., 2015) using slices with 14 µm. Antibodies used were as follows: goat anti-albumin (1:2000; Bethyl Laboratories, Inc, Montogomery, TX, USA), rabbit anti-collagen IV (1:200; Abcam, Cambridge, UK), rabbit anti-matrix metalloproteinase-9 (MMP-9; 1:200; Abcam) and rabbit anti-3-nitrotyrosine (3-NT; 1:200; Millipore, Madrid, Spain). Moreover, free-floating slices with 50 µm were permeabilized with 10% Triton X-100 (Sigma-Aldrich) for 10 min, blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS for 1h30 min at room temperature (RT), and incubated overnight at 4ºC with anti-GPAP-Cy3 conjugated (1:500; Sigma-Aldrich) and CD31 antibodies (microvessels marker, 1:100; R&D system, Minneapolis, USA).

Afterwards, slices were washed with 0.01 M PBS and incubated with Alexa Fluor 488 or 594 secondary antibodies (1:200; Invitrogen), followed by nuclei staining with 5 µg/mL Hoechst dye 33342 (Sigma-Aldrich). Slices were then mounted with Dako fluorescence medium (Dako North America) and images recorded using a LSM 710 Meta Confocal microscope (Carl Zeiss, Göttingen, Germany).

Quantification of fluorescence and co-localization were determined using the FIJI J software (NIH, USA) based on a previous study (Cardoso et al., 2015).

3.3.3 Western blot analysis

As above mentioned, after anesthesia rats were transcardially perfused with 0.01 M PBS, pH 7.4. Then, hippocampi were dissected on ice, and lysed in RIPA buffer supplemented with protease inhibitor cocktail tablets (Roche Applied Science, Basel, Switzerland) and anti-phosphatases (PhosSTOP^{TM}, Roche Applied Science, Mannheim, Germany).

Supernatants were quantified using the BCA method and stored at -20ºC. Samples of total protein (Table 3.1) were separated by electrophoresis, transferred onto polyvinylidine difluoride membrane (Millipore) and blocked with 5% non-fat milk or 4% BSA.

Table 3.1. *List of primary antibodies used in western blot analysis*

Primary antibody	MW (kDa)	Dilution	Reference	Company
Goat anti-albumin	67	1:2000	A80-129	Bethyl Laboratories, Montogomery, TX, USA
Mouse anti-claudin-5	25	1:100	35-2500	Invitrogen Inchinnan Business Park, UK
Goat anti-VE-cadherin	130	1:100	sc-6458	Santa Cruz Biotechnology, Inc., Dallas, TX, USA
Rabbit anti-caveolin-1	20	1:200	sc-894	Santa Cruz Biotechnology
Rabbit anti-VCAM-1	110	1:200	sc-8304	Santa Cruz Biotechnology
Rabbit anti-ICAM-1	110	1:100	sc-366318	Santa Cruz Biotechnology
Rabbit anti-MMP-9	98-95 kDa pro 82 or 63 kDa active form	1:1000	ab38898	Abcam, Cambridge, UK
Rabbit anti-GFAP	55	1:2000	G9269	Sigma-Aldrich, St. Louis, MO, USA
Mouse anti-SNAP-25	25	1:1000	S5187	Sigma-Aldrich
Mouse anti-syntaxin-1	39	1:5000	110011	Synaptic Systems
Mouse anti-synaptophysin	37	1:5000	S5768	Sigma-Aldrich
Mouse anti-PSD-95	110	1:1000	Ab2723	Abcam
Rabbit anti-calbindin D28K	28	1:1000	#2136	Cell Signaling Technology, Danvers, MA, USA
Rabbit anti-GAP-43	43	1:1000	Ab7462	Abcam

Abbreviations: MW, molecular weight; VE-cadherin, vascular endothelial cadherin; VCAM-1, vascular cell adhesion protein-1; ICAM-1, intercellular adhesion molecule-1; MMP-9, matrix metallopeptidase-9 or gelatinase B; GFAP, glial fibrillary acidic protein; SNAP-25, synaptosome-associated protein of 25kDa; PSD-95, postsynaptic density protein 95; GAP-43, growth associated protein 43 kD.

The membranes were incubated overnight at 4ºC with primary antibodies (Table 3.1) followed by incubation with respective alkaline phosphatase-conjugated secondary antibody for 1h at RT and visualized using ECF reagent on Typhoon FLA 9000 (GE Healthcare Bioscience AB, Uppsala, Sweden). Immunoblots were reprobed with glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH, 1:5000; Life Technologies, Cambridge, UK) to ensure equal sample loading. Densitometric analyses were performed using the Fiji J software (NIH, USA).

3.3.4 Transmission electron microscopy

Rats were anesthetized and transcardially perfused with 4% PFA. Hippocampi (1-mm pieces) were immersed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2h. Following rinsing in the same buffer, post-fixation was performed using 1% osmium tetroxide for 1h 30 min. Then, hippocampi pieces were again rinsed in buffer and in distilled water, followed by the addition of 1% aqueous uranyl acetate for 1h in the dark, for contrast enhancement. After rinsing once again in distilled water, samples were dehydrated in a graded ethanol series (30-100%), and impregnated and embedded in epoxy resin (Fluka Analytical). Ultrathin sections (70 nm) were mounted on copper grids and stained with 0.2% lead citrate, for 7 min. Observations were carried out on a FEI-Tecnai G2 Spirit Bio Twin transmission electron microscope at 100kV.

3.3.5 Reactive oxygen species (ROS) assay

The ROS assay was performed by N,N-Diethyl-p-phenylenediamine (DEPPD) staining adapted from (Tiwari et al., 2011). In brief, 5 µL of serum or hippocampi lysates were added to 140 µL of 0.1 M sodium acetate buffer (pH 4.8) at 37°C in a 96-well plate. Samples were taken in triplicate and 100 µL of the mixed DEPPD solution (DEPPD was dissolved in 0.1 M sodium acetate buffer [pH 4.8]) and ferrous sulfate (4.37 µM ferrous sulfate dissolved in 0.1 M sodium acetate buffer, pH 4.8) at a ratio of 1:25 was added to each well to initiate reaction. Thereafter, the microtiter plate was incubated at 37°C for 5 min. Absorbance was measured by a spectrophotometer plate reader (Biotek, Synergy HT) at 505 nm. Serum levels of ROS were calculated from a calibration curve of H_2O_2 and expressed as H_2O_2 equivalent (1 unit = 1.0 mg H_2O_2/L). The calibration curve for standard solution was obtained by calculating slopes from an optical density graph.

3.3.6 Antioxidant status

The total antioxidant status of the hippocampi supernatants were measured using a colorimetric ferric-reducing antioxidant potential (FRAP) assay (Benzie and Strain, 1996). Briefly, 10 µL of samples was added to 300 µL of the freshly prepared working solution of FRAP that consists of 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine; Sigma-Aldrich) and 20 mM $FeCl_3.6H_2O$ (Iron III chloride; Sigma-Aldrich). The reduction of a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to a ferrous form was monitored by measuring the absorbance at 593 nm after 15 min incubation at 37 °C. Results were expressed as Trolox equivalent µM/mg proteins (Sigma-Aldrich), calculated from the calibration curve.

3.3.7 Lipid Peroxidation

The thiobarbituric acid reactive-species (TBARs) assay was used to assess hippocampi products of lipid peroxidation, via malondialdehyde (MDA) adapted from (Ohkawa et al., 1979). Briefly, 100 µL of hippocampi tissue supernatant were incubated at RT in the dark for 1 h in a TBA solution together with butylhydroxytoluene (BHT; Sigma-Aldrich) and a catalyzer (Iron III chloride; Sigma-Aldrich). Afterwards, samples were incubated at 95-100°C for 60 min followed by butanol extraction. The supernatants were read spectrophotometrically at 532 nm (Biotek, Synergy HT) and the concentration of MDA was calculated with respect to a calibration curve using 1,1,3,3-tetramethoxypropane (Sigma-Aldrich) as the external standard (range: 0.1–83.5 µM). Results were expressed as µmol/mg of hippocampi tissue concentration of lipid peroxides.

3.3.8 Y-maze spontaneous alternation

The Y-maze spontaneous alternation paradigm is based on the natural tendency of rodents to explore a novel environment (Dudchenko, 2004). When placed in the Y-maze, rats will explore the least recently visited arm, and thus tend to alternate visits between the three arms. The Y-maze consisted of three arms made of black plastic (50 cm long, 20 cm high, 10 cm wide) extending from a central platform at an angle of 120°. Percent of spontaneous alternation was defined as the ratio of actual (= total alternations) to possible (= total arm entries-2) number of alternations × 100. Total entries were scored as an index of

ambulatory activity in the Y-maze, and rats showing scores below six entries were excluded.

3.3.9 Cell cultures

Human samples were obtained from discarded temporal lobe tissue during operative treatment of epilepsy (outside epileptogenic foci) after informed consent and institutional review board ethical approval at the Neurosurgery Service, Coimbra Hospital and University Centre, Portugal. Primary cultures of human brain microvascular endothelial cells (HBMVECs) were isolated as previously described (Bernas et al., 2010; Coelho-Santos et al., 2016). HBMVECs were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 media (DMEM/F-12, Biochrom AG, Berlin, Germany) containing 10% fetal bovine serum (FBS, GIBCO, Rockville, MD, USA), ECGS (endothelial cell growth supplement, BD Biosciences, Franklin Lakes, NJ, USA), heparin (1 mg/mL, Biochrom AG) and 1% antibiotic-antimycotic (Sigma-Aldrich). After reaching confluence, ECGS and heparin were removed.

3.3.10 Caveolin-1 silencing by small-interfering RNA-based knockdown

HBMVECs at about 70% confluence were transiently transfected with Cav1 specific short interfering RNA (siRNAs) (Hs CAV1 13 FlexiTube siRNA, Qiagen, Venlo, Netherlands) at a final concentration of 10 nM using ScreenFect (InCellA, Eggenstein-Leopolshafen, Germany) diluted in Opti-MEM (1:50; Invitrogen, Inchinnan Business Park, UK) according with the manufacturer's protocol. AllStars Negative Control siRNA (Qiagen) were used as controls. Cells were incubated with the transfection complexes for 48 h under normal growth conditions before use.

3.3.11 Horseradish peroxidase transport

HBMVECs were seeded in collagen type I-coated collagen 12-mm transwell filters (Costar, Corning, NY, USA) and grown to confluence. Experiments were performed as previously described (Coelho-Santos et al., 2016). Briefly, horseradish peroxide (10 mg/mL HRP, Sigma-Aldrich) was added to the apical chamber, and after 24 h of incubation with 100 μM MPH, the top and the bottom of the transwells were washed and new medium was added. Samples were taken during 2 h at 30 min intervals. Absorbance

was measured in a microplate reader (Biotek, Synergy HT, Winooski, USA), and HRP transport was plotted against time and rates determined by linear regression.

3.3.12 Transendothelial migration of peripheral blood mononuclear cells

Approval was obtained from the Ethics Review Board for the use of peripheral blood mononuclear cells from healthy donors buffy coats, provided by the Portuguese Blood Institute. Briefly, peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation (Ficoll-Hypaque; GE Healthcare, Uppsala, Sweden), and the fraction of leukocytes was stored at -80ºC. The transmigration assays were performed with Transwell TM inserts (Costar) containing confluent HBVMEC culture. For quantification of transmigrated cells, PBMCs were labelled with calcein-AM (5 μM; Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions and resuspended in 100 μL TEM buffer (RPMI 1640 without phenol red + 1% bovine serum albumin) prior to transmigration assays. PBMCs (10^6) from the same donor suspended in TEM buffer were added to the upper side of the insert (luminal side of the endothelium) and allowed to transmigrate at 37ºC in a 100% humid atmosphere with 5% CO_2 for 24h. For chemokine-driven migration we used as positive control 600 μL of TEM buffer containing 100 ng/mL CXCL12 (SDF1-α; Proteintech Group Inc, Manchester, UK) that was placed in the lower chamber (abluminal side of the endothelium). Transmigrated PBMCs were counted in samples from the bottom chamber by measuring fluorescence at 530 nm using microplate spectroflurometer. Fluorescence values of migrated PBMCs were compared to a standard curve of known cell numbers for quantitation.

3.3.13 Statistical Analysis

Results are expressed as mean + standard error of the mean (S.E.M.). Data were analyzed using Student's t-test or one-way ANOVA followed by Dunnett's or Bonferroni's post hoc test, as indicated in figure legends. The level of significance was $P<0.05$ and the "n" represents the total number of animals or the total number of experiments obtained from at least three independent human donors cell cultures used in each experiment. Statistical analysis was calculated using Prism 6.0 (GraphPad Software, San Diego, CA, USA).

3.4 Results

3.4.1 MPH increases hippocampal BBB permeability by promoting vesicular transport

We recently demonstrated that acute MPH exposure causes endothelial hyperpermeability through caveolae-mediated transcytosis (Coelho-Santos et al., 2016). However, nothing is known about the impact of chronic MPH exposure on BBB. Herein, for the first time we concluded that chronic exposure of MPH (5 mg/kg/day) promoted hippocampal BBB permeability, which was proved by the presence of albumin extravasation in the hippocampal parenchyma (Figure 3.1 A) and by its quantification (Figure 3.1 B; $F_{(2, 56)} = 3.318$, $p = 0.0435$). Albumin is a blood serum protein with a high molecular weight that under normal conditions does not cross BBB.

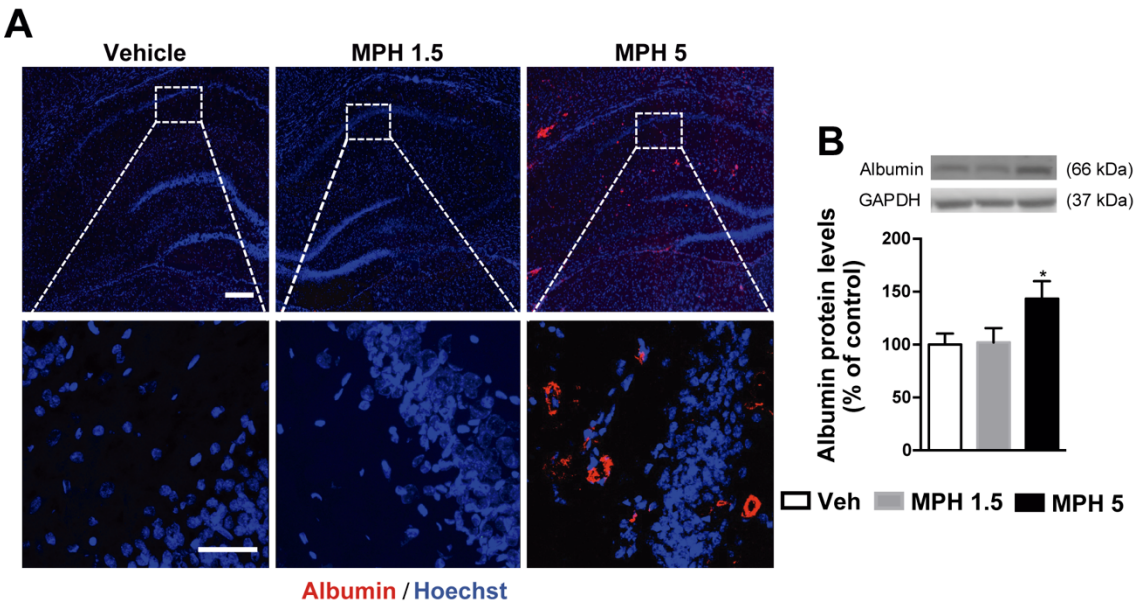


Figure 3.1. Hippocampal BBB permeability triggered by MPH chronic administration. *(**A**) Representative confocal images of albumin immunoreactivity (red) in rat hippocampus (CA1 subregion) after MPH treatment (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively). Brain sections were also stained with Hoechst 33342 (blue; nuclear marker). Scale bars: 50 and 500 µm. (**B**) Quantification of albumin protein levels in the hippocampus. Above the bars, representative western blot images of albumin (66 kDa) and GAPDH (37 kDa) are shown. MPH compromised BBB permeability and allowed the access of circulating factors, namely serum albumin. The results are shown as mean % of control + S.E.M., n=6-9 animals of each condition. *P<0.05, compared to the vehicle (Veh), using one-way ANOVA followed by Bonferroni's test.*

Endothelial intercellular junction seal is critical for the BBB function (Cardoso et al., 2010). Thus, we also evaluated the protein levels of the TJs protein claudin-5 (Figure 3.2 A; $F_{(2, 54)} = 0.3236$, $p = 0.7249$) and the AJ protein vascular-endothelial cadherin (VE-cadherin; Figure 3.2 B; $F_{(2, 18)} = 0{,}7409$, $p = 0.4907$). No significant alterations were observed with both MPH doses. In contrast, cav1, the major protein constituent of caveolae vesicles, was upregulated with MPH 5 (Figure 3.2 C; $F_{(2, 66)} = 5{,}076$; $p = 0.0089$) together with an increase in the number of vesicles observed by electron microscopy (highlighted in black). Interestingly, there were no changes at the intercellular junctions (arrow head; Figure 3.2 D) pointing to an intact paracellular pathway.

Brain microvascular ECs constitute the anatomic basis of BBB and their dysfunction will contribute to BBB disruption. Thus, to unravel the specific mechanisms that contribute to BBB permeability, we also evaluated the impact of MPH on HBMVECs obtained from discarded temporal lobe tissues removed from patients with intractable epilepsy undergoing neurosurgical operative procedures (removal of hippocampal outside epileptogenic foci). We showed that MPH (100 μM for 24h) increased Cav1 phosphorylation at Tyr^{14} (p-$Cav1^{y14}$, Figure 3.2 E; $F_{(2, 44)} = 0.2585$, $p < 0.0001$), which is necessary to caveolae-mediated endocytosis (Fernandez et al., 2002). To further validate the involvement of Cav1 in MPH-induced vesicular unidirectional transport of horseradish peroxidase (HRP) across HBMVECs, we knockdown cav1 by transfecting HBMVECs with Cav1 siRNA, as shown in Figure 3.2 F ($F_{(2, 44)} = 16.60$, $p< 0.0001$). In addition, Cav1 siRNA also significantly abrogated MPH-mediated transcellular transport (Figure 3.2 G; $F_{(2, 28)} = 0.4675$, $p< 0.0001$). These findings underscored the role of Cav1 in promoting MPH-induced vesicular endothelial transport.

Our data demonstrate that MPH interferes with hippocampal BBB function and directly with ECs properties by increasing vesicular transport. Moreover, we identified cav1 signaling pathway as a key mechanism in MPH-induced transcytosis.

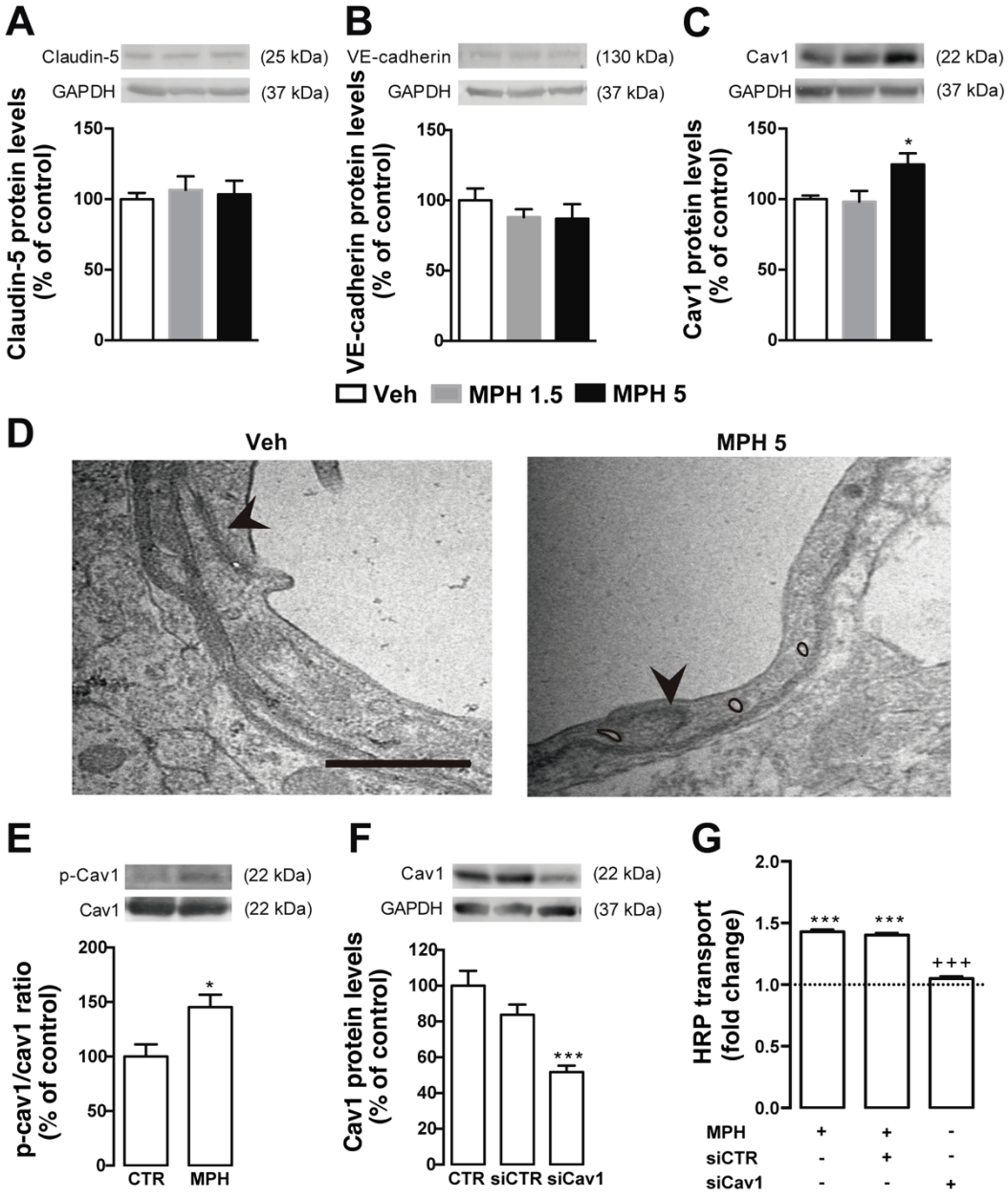


Figure 3.2. MPH increases endothelial vesicular transport without affecting intercellular junctions. *MPH chronic treatment (1.5 or 5 mg/kg/day, os; P28-P55) did not alter the protein levels of (**A**) claudin-5 and (**B**) VE-cadherin, but increased (**C**) caveolin-1 (Cav1) at 5 mg/kg/day. Above the bars, representative western blot images of claudin-5 (25 kDa), VE-cadherin (130 kDa), cav1 (22 kDa) and GAPDH (37 kDa) are shown. (**D**) Effect of MPH on intercellular junctions and formation of pinocytotic vesicles was analyzed by transmission electron microscopy. MPH had no effect on intercellular junctions (head arrows), but triggered the formation of pinocytotic vesicles (outlined in black). Scale bar: 1 µm. (**E**) MPH (100 µM during 24h) increased the phosphorylation of cav1 Tyr14 (p-Cav1^{Y14}) in HBMVECs. (**F**) Quantification of cav1 protein levels in HBMVECs cells after siRNA-mediated knockdown. Above the bars, representative western blot images of p-Cav1^{Y14} and Cav1 (22 kDa) are shown. (**G**) In the absence of Cav1 (siCav1), MPH was not able to promote HRP transport in HBMVECs. The results are shown as mean + S.E.M., n=6-9 animals of each condition or n=4 brain donors cultures. $^{*}P<0.05$, $^{***}P<0.001$, compared to the vehicle (Veh) or untreated cells (CTR), $^{+++}P<0.001$ compared to MPH using Student's t-test one-way ANOVA followed by Bonferroni's test or by Dunnett's.*

3.4.2 MPH upregulates the expression of adhesion molecules and promotes leukocyte infiltration

Alterations in the structure and organization of BBB may affect its permeability and subsequently the movement of leukocytes and immune mediators into the brain, which can cause neural damage/dysfunction. ECs actively participate in inflammatory events by regulating leukocyte recruitment *via* the expression of inflammation-related genes, including cell adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). Thus, we hypothesized that MPH could upregulate the expression of cell adhesion molecules in ECs, and therefore promoting infiltration of leukocytes. We concluded that MPH 5 increased the hippocampal protein levels of VCAM-1 (Figure 3.3 A; $F_{(2, 44)} = 4.709$, $p = 0.0140$) and ICAM-1 (Figure 3.3 B; $F_{(2, 28)} = 3.375$, $p = 0.0486$). Additionally, we observed the presence of $CD45^+$ leukocytes and $CD169^+$ macrophages in the hippocampus of WKY rats treated with MPH 5 (Figure 3.3 C).

In accordance with animal studies we also found that MPH (100 μM, 24h) upregulated VCAM-1 protein levels in HBMVECs. Cav1 is also involved in regulating endothelial permeability and leukocyte diapedesis (Choi et al., 2016; Wu et al., 2016). In fact, when we knowdown Cav1 the levels of VCAM-1 decreased to levels similar to the control (Figure 3.3 D; $F_{(3, 42)} = 27.29$, $p < 0.0001$). Furthermore, to unravel the involvement of cav1 in MPH-induced diapedesis we investigated transendothelial migration of leukocytes. Specifically, T, B, and natural killer cells, monocytes, and neutrophils-labeled with calcein-AM were added to HMBMECs in the presence or absence of MPH. We concluded that MPH promoted the transmigration of leukocytes (Figure 3.3 E), and also that cav1 is involved in such process because the effect of MPH was abolished in the presence of siCav1 (Figure 3.3 E; $F_{(4, 18)} = 30.82$, $p < 0.0001$). As positive control, we used CXCL12 (SDF-1, 100 ng/mL) in the abluminal side of the transwell system (Rossi et al., 2013). CXCL12 induced a significant increase in PBMCs transmigration across HBMECs (Figure 3.3 E).

We concluded that MPH promotes the infiltration of leukocytes into the brain through a caveolae-dependent transcytosis pathway.

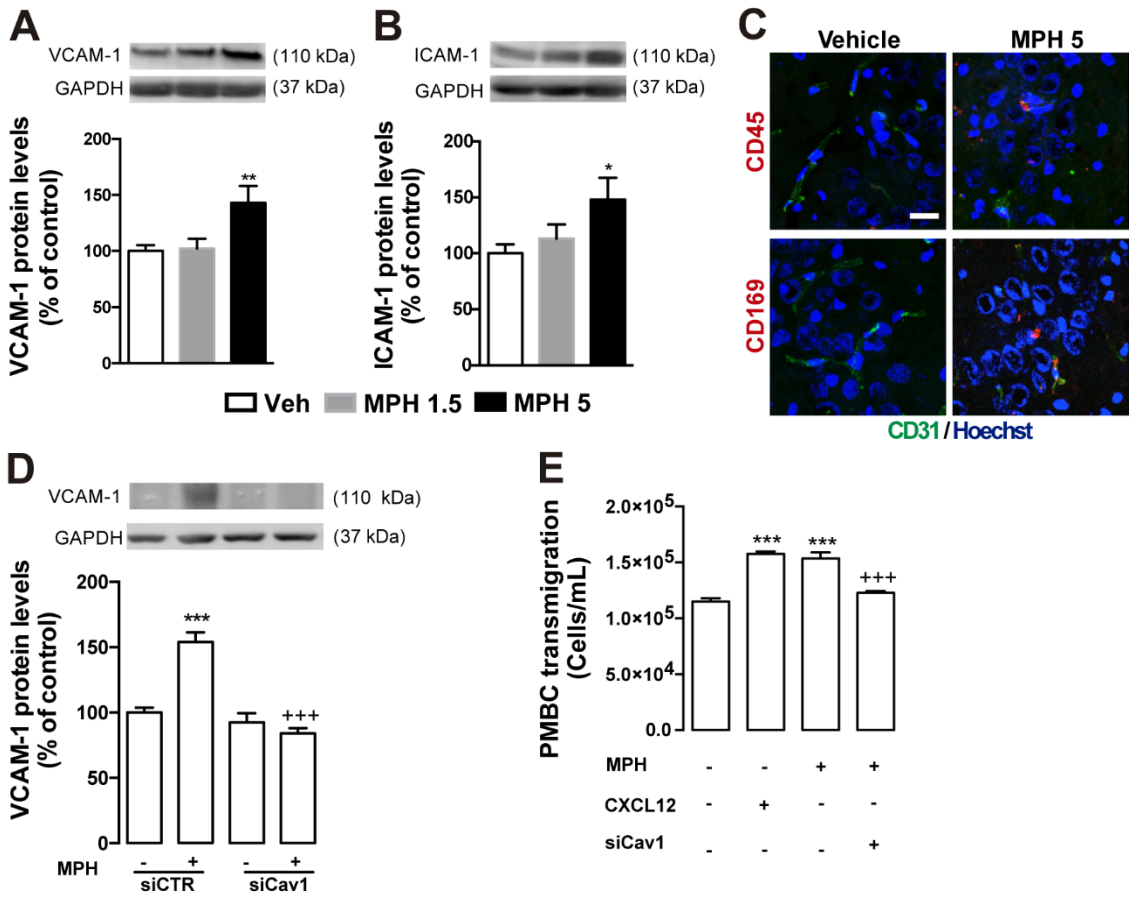


Figure 3.3. Impact of MPH on brain immune quiescence. *Protein levels of (**A**) vascular cell adhesion molecule-1 (VCAM-1) and (**B**) intercellular adhesion molecule-1 (ICAM-1) after MPH administration (1.5 or 5 mg/kg/day, os; P28-P55). There was an increase in VCAM-1 and ICAM-1 protein levels triggered by MPH 5. (**C**) Representative images of positive cells for CD31 (green, protein of brain endothelial cells), CD45 (red, lymphocyte common antigen), and CD169 (red, Sialoadhesin - a macrophage-restricted cell surface receptor). Brain sections were also stained with Hoechst 33342 (blue; nuclear marker). Scale bar: 20 µm. (**D**) HBMVECs without cav1 (siCav1) prevented MPH-increased levels of VCAM-1. (**E**) Leukocytes (Peripheral Blood Mononuclear Cells; PBMC) transmigration triggered by MPH (100 µM for 24h) was abolished by downregulation of cav1 (siCav1). CXCL12 (100 ng/mL) was used as positive stimulus to promote PBMCs transmigration. The results are shown as mean + S.E.M., n=6-9 animals or n=4 brain donors cultures. $^{*}P<0.05$, $^{***}P<0.001$, compared to the vehicle (veh) or untreated cells; $^{+++}P < 0.001$ compared to MPH, using one-way ANOVA followed by Bonferroni's test or by Dunnett's.*

3.4.3 MPH induces extracellular matrix degradation concomitantly with increased matrix metalloproteinase-9 expression and oxidative stress

The process of leukocyte transmigration involves breaching of the basal lamina, where collagen IV is one of the most expressed extracellular matrix protein responsible for the mechanical support of ECs (Hamann et al., 2002; Zhang et al., 2013). As we observed a transcellular migration, we further searched for the effect of this psychostimulant on basal

lamina. In fact, a decrease of collagen IV staining was observed with MPH 5 (Figure 3.4 A, B), which is coincident with the presence of albumin in the brain parenchyma (Figure 3.1 A, B). Additionally, leukocyte extravasation requires metalloproteinase (MMPs) secretion that will degrade the extracellular matrix allowing leukocyte to penetrate through the basement membrane into the brain parenchyma (Leppert et al., 1995; Lakhan et al., 2013). Since MMP-9 has been associated with BBB leakage via degradation of basement membrane components such as collagen IV, special attention was paid to this MMP (Asahi et al., 2001; Svedin et al., 2007). In fact, the increase of MMP-9 immunoreactivity on the perivascular zone induced by MPH 5 was concomitant with the decrease of collagen IV (Figure 3.4 C; $F_{(2, 22)} = 57.49$, $p < 0.0001$). In accordance, MPH administration resulted in a significant increase of MMP-9 protein levels (Figure 3.4D; $F_{(2, 44)} = 13{,}26$, $p < 0.0001$), specifically the 63-67 kDa bands that correspond to the active form of MMP-9 (Piedagnel et al., 1999).

Oxidative stress is closely related to pathological alterations of BBB involving the activation of MMPs (Gu et al., 2002). Our previous study demonstrated that acute treatment with MPH triggered an oxidative signaling that culminated in endothelial transcytosis (Coelho-Santos et al., 2016). Herein, we evaluated if chronic MPH treatment interferes with rat hippocampal ROS levels, concluding that MPH 5 significantly increased ROS levels (Figure 3.4E; $F_{(2, 13)} = 76.78$, $p < 0.0001$). Additionally, the non-enzymatic antioxidant status (Figure 3.4 F; $F_{(2, 15)} = 4.305$, $p < 0.0333$) decreased with MPH 5 exposure. MDA, the breakdown product of polyunsaturated fatty acids oxidation that is a reliable oxidant marker of oxidative stress-mediated lipid peroxidation (Uzar et al., 2006), was upregulated under the same experimental condition (Figure 3.4 G; $F_{(2, 13)} = 16.73$, $p < 0.0022$). Interestingly, 3-nitrotyrosine (3-NT) immunoreactivity, a maker of oxidative/nitrosative stress, was identified in the cerebral microvessels (Figure 3.4 H) suggesting a perivascular production of ROS.

Overall, we concluded that the administration of MPH promoted the degradation of the basal lamina, which was concomitant with the generation of MMP-9 and an oxidative response at the perivascular zone that can explain the previously observed BBB permeability

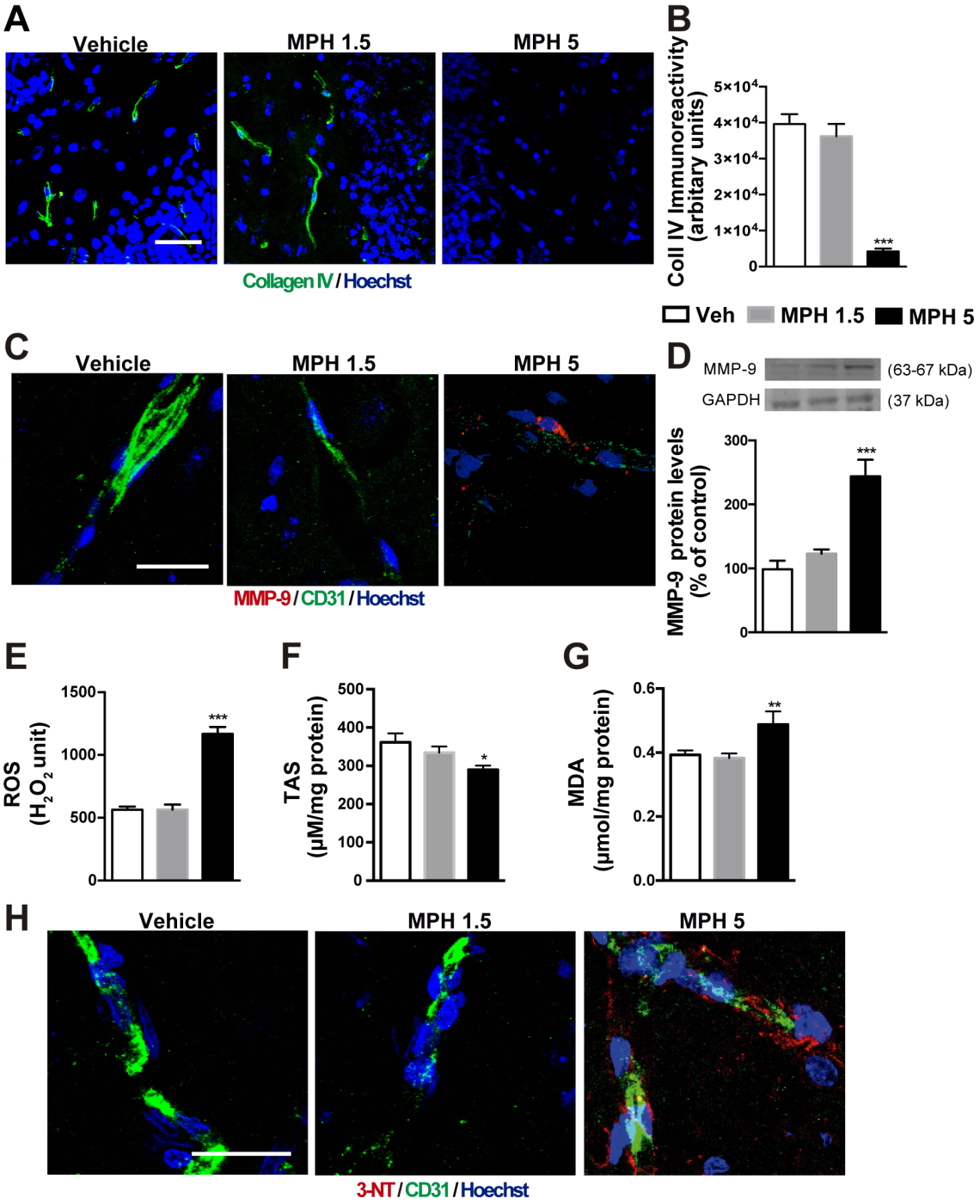


Figure 3.4. MPH promotes the degradation of lamina basal concomitantly with an upregulation of MMP-9 and an oxidative stress response. *(**A**) Representative images of collagen IV (Coll IV; green) after MPH chronic exposure (1.5 or 5 mg/kg/day, os; P28-P55). Brain sections were also stained with Hoechst 33342 (blue; nuclear marker). Scale bar: 50 µm. (**B**) Quantification of Coll IV immunofluorescence showing its down-regulation induced by MPH. (**C**) Representative images of matrix metalloproteinase-9 (MMP-9; red) at the perivascular zone (CD31, vessels marker; green). Brain sections were also stained with Hoechst 33342 (blue; nuclear marker). Scale bar: 50 µm. (**D**) Quantification of MMP-9 protein levels. Above the bars, representative western blot images of MMP-9 (63-67 kDa) and GAPDH (37 kDa) are shown. MPH promoted the degradation of basal lamina concomitantly with the up-regulation of MMP-9. (**E**) Reactive oxygen species (ROS) production, (**F**) non-enzymatic antioxidant activity levels (TAS) and (**G**) malondialdehyde (MDA) formation in the rat hippocampi. (**H**) Representative images of immunofluorescent staining of 3-nitrotirosyne (3NT; red) in hippocampal microvessels stained with CD31 (green) in control and MPH-treated animals (1.5 or 5 mg/kg/day). The results are shown as mean + S.E.M., n=6-9 animals of each condition. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the vehicle (Veh), using one-way ANOVA followed by Bonferroni's test.*

3.4.4 Astrocytic morphological changes and interaction with vasculature after MPH administration

Brain capillaries are separated from circumferential astrocyte end-feet by the intervening extracellular membrane (ECM) of the basal lamina. Likewise, BBB integrity and function is dependent on the continuous crosstalk between the cellular elements that compose the NVU (Cardoso et al., 2010). Since EC-astrocyte stability requires matrix adhesion, we further investigated if MPH-induced ECM degradation could affect astrocytic morphology.

Glial fibrillary acidic protein (GFAP) expression in the hippocampus was determined by western blotting and no statistical significances were observed in the MPH 1.5 group (Figure 3.5 A, B). Curiously, with the higher dose of MPH it was possible to observe a decrease of GFAP immunoreactivity and protein levels compared to the vehicle group (Figure 3.5 A, B; $F_{(2, 94)} = 4{,}189$, $p = 0.0181$), as well as a significant reduction in their cytoskeletal surface area. Looking in more detail to cell morphology, we observed thinner and shorter astroglial stained processes (Figure 3.5 A, D-F; $F_{(2, 22)} = 16.75$, $p < 0.0001$), whereas in vehicle rats was possible to identify well-defined GFAP-positive astrocytes processes. Also, the blood vessels of rats treated with MPH 5 showed less GFAP expression in the perivascular zone than vehicle rats, which was translated by the decrease of astrocytic-coverage vessels (Figure 3.5 C; $F_{(2, 22)} = 16.75$, $p < 0.0001$). Interestingly, the lower dose of MPH (1.5 mg/kg) increased the number of astrocytic processes although the length was not altered (Figure 3. 5 E, F; $F_{(2, 22)} = 16.75$, $p < 0.0001$).

These observations suggest that low doses of MPH increase the number of astrocytic processes, whereas a higher dose impairs astrocytic cytoskeletal branching and decreases the vessel coverage, which is coincident with the degradation of collagen IV, the entrance of immune cells into the brain parenchyma, and in sum, with BBB hyperpermeability.

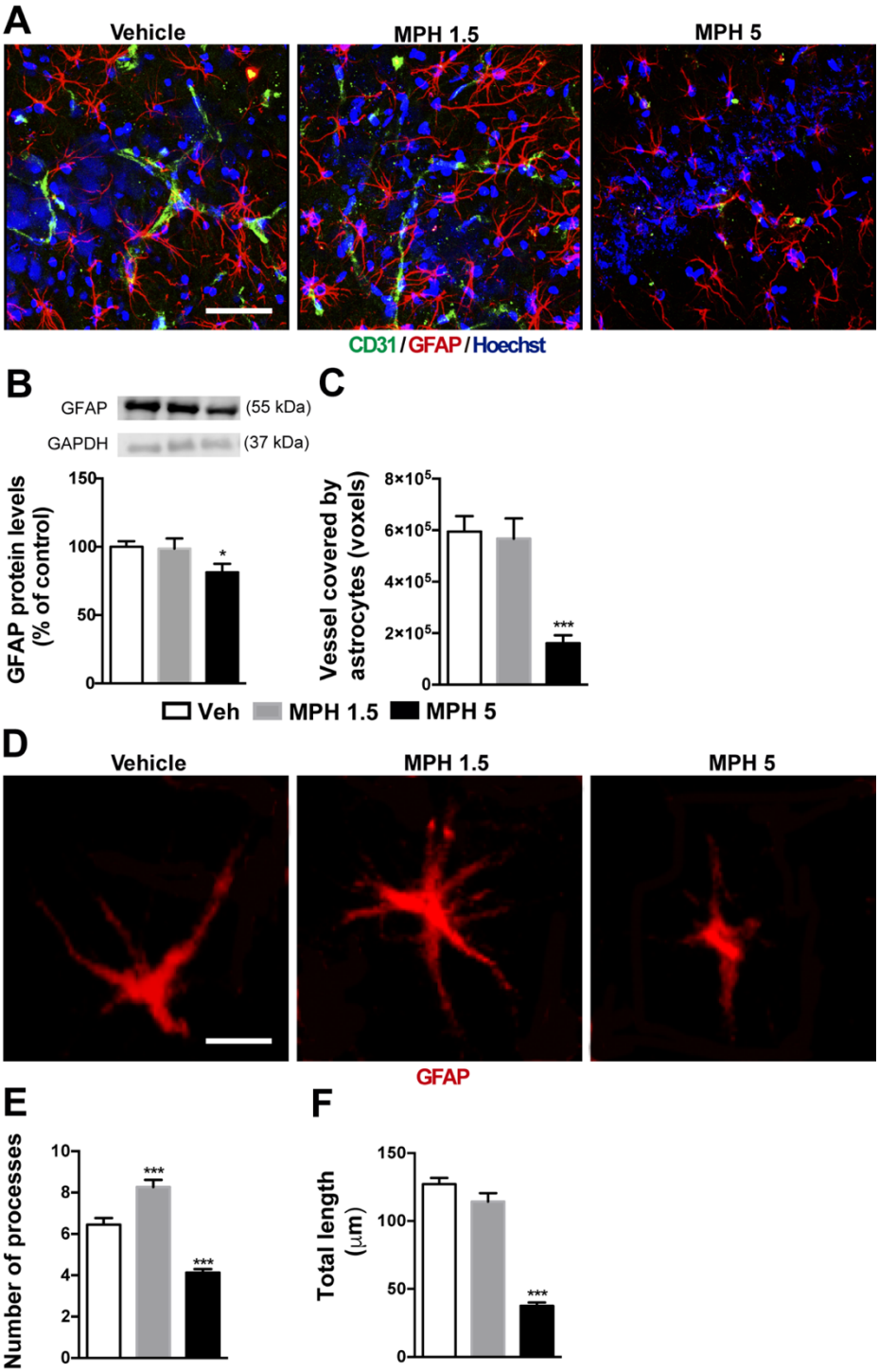


Figure 3.5. MPH disrupts astrocytic-vascular coverage and induces astrocytic morphological alterations. *(**A**) Representative confocal images of hippocampal sections from WKY rats after MPH administration (1.5 or 5 mg/kg/day, os; P28-P55) co-labelled with the brain microvascular endothelial cells cluster differentiation (CD31; green) and the astrocyte-specific GFAP antibody (red). Sections were counterstained with Hoechst 33342 (blue) to detect nuclei. Scale bar: 20 µm. (**B**) GFAP protein levels in the hippocampus after a chronic MPH treatment. Above the bars, representative western blot images of GFAP (55 kDa) and GAPDH (37 kDa) are shown, n=6-9 animals of each condition. (**C**) Area fraction per field of CD31 and GFAP positive staining, along with astrocytes colocalization with vessels, were determined using Fiji J software (NIH, USA). n=3 animals of each condition. (**D**) Representative confocal images of astrocytes morphology (anti-GFAP). Scale bar: 20 µm. (**E, F**) Morphological alterations in astrocytes were analyzed in detailed by the quantification of (**E**) total number and (**F**) total length of cell processes. The results are shown as mean + S.E.M. $^{*}P<0.05$, $^{***}P<0.001$ compared to the vehicle, using one-way ANOVA followed by Bonferroni's test.*

3.4.5 Neuronal alterations triggered by MPH

Astrocytes provide a link between neurons and blood vessels allowing a bidirectional communication. Moreover, in the hippocampus ~60% of synapses are ensheathed by astrocyte processes (Ostroff et al., 2014). To understand if astrocytic morphological alterations could reflect neuronal synaptic alterations we evaluated the effect of chronic MPH administration on several pre- and post-synaptic proteins that constitute the exocytotic machinery, namely synaptosomal-associated protein-25 (SNAP-25), syntaxin-1, synaptophysin and postsynaptic density-95 (PSD-95). It is well know that the regulation of intracellular calcium concentration is critical for synaptic function (Catterall and Few, 2008), and so we also looked to calbindin D28k, a calcium-binding protein that plays an important role in neuronal survival, spatial learning paradigms and some forms of synaptic plasticity (Sloviter, 1989). Other important protein that we explored was the neuronal growth-associated protein 43 kD (GAP-43) a marker for axonal sprouting (Aigner et al., 1995). GAP-43 and calbindin D28k are important proteins in synaptic plasticity, essential for the formation of new synapses and their control of synapses.

MPH 1.5 increased the protein levels of the pre-synaptic proteins SNAP-25 (Figure 3.6 A; $F_{(2, 46)} = 71.55$, $p < 0.0001$), synaptophysin (Figure 3.6 B; $F_{(2, 37)} = 12.24$, $p < 0.0001$), but with no effect on syntaxin-1 (Figure 3.6 C; $F_{(2, 44)} = 6.955$, $p = 0.0024$). Moreover, PSD-95 (Figure 3.6 D; $F_{(2, 36)} = 64.99$, $p < 0.0001$), calbindin D28K (Figure 3.6 E; $F_{(2, 72)} = 1.148$, $p < 0.0001$) and GAP-43 (Figure 3.6 F; $F_{(2, 36)} = 1.148$, $p < 0.0001$) were upregulated at this dose. On the contrary, MPH 5 chronic exposure decreased both pre- and post-synaptic proteins, as well the GAP-43 and calbindin D28k suggesting that at this dose MPH can impair neuronal communication.

In sum, our results indicate that low doses of MPH promote the expression of synaptic proteins and proteins related with neurite outgrowth and axonal sprouting, which is coincident with increased astrocytic processes. On the contrary, a higher dose of MPH leads to a down-regulation of neuronal synaptic proteins suggesting neuronal dysfunction, in parallel with astrocytic atrophy morphology.

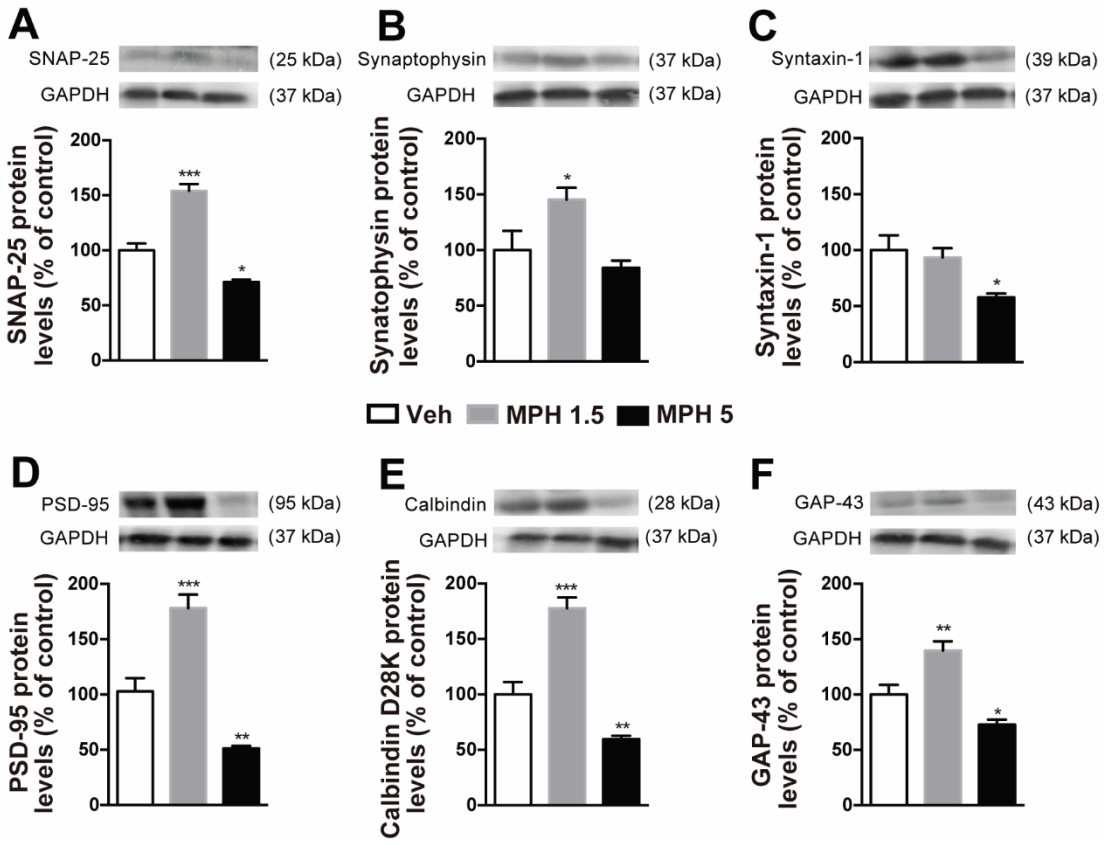


Figure 3.6. MPH treatment influences the network of synaptic proteins. *The chronic MPH treatment (1.5 or 5 mg/kg/day, os; P28-P55) regulates several synaptic proteins. The protein levels of (**A**) SNAP-25, (**B**) synaptophysin, (**C**) syntaxin-1, (**D**) PSD-95, (**E**) calbindin D28K, and (**F**) GAP-43 were analyzed. Above the bars, representative western blot images of the respective proteins are shown. The results are shown as mean % of control + S.E.M, n=6-9 animals of each condition. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the vehicle (Veh), using one-way ANOVA followed by Bonferroni's test.*

3.4.6 Impact of MPH chronic treatment on cognitive performance and signaling pathways

The ability of synapses to undergo lasting biochemical and morphological changes in response to stimuli and neuromodulators is known as synaptic plasticity, which likely forms the cellular basis of learning and memory. Also, brain vasculature dysfunction has been associated with cognitive performance (Gorelick et al., 2011). Thus, based on our observations regarding BBB leakage and neuronal machinery alterations, we further analyzed the rat spatial working memory performance using the Y-maze test. The number of total entries were not altered by MPH (Figure 3.7 A; $F_{(2, 27)} = 1.148$, $p = 0.3324$). However, MPH at a lower dose (1.5 mg/Kg) increased the number of spontaneous alternations (Figure 3.7 B; $F_{(2, 26)} = 10.40$, $p = 0.0005$), whereas 5 mg/kg of MPH

decreased the number of spontaneous alternations compared to vehicle (Figure 3.7 B), pointing to an impairment of working memory.

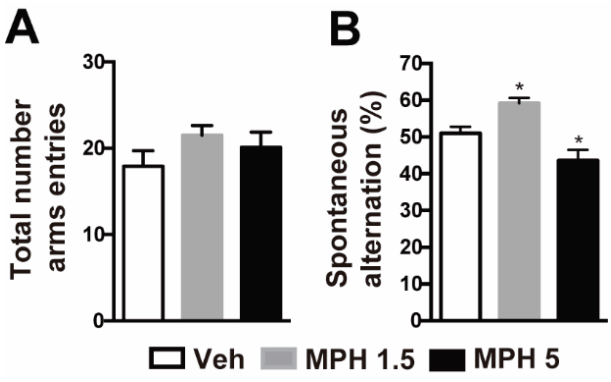


Figure 3.7. Effect of MPH on spatial working memory. *(**A**) The chronic MPH treatment (1.5 or 5 mg/kg/day, os; P28-P55) did not alter the total number of arm entries. However, it modulated the (**B**) percentage of spontaneous alternation in a dose dependent-manner. The results are shown as mean + S.E.M, n=9-13 animals of each condition. *P<0.05, compared to the vehicle (Veh), using one-way ANOVA followed by Bonferroni's test.*

To unravel the signaling pathways involved on the modulation of cognitive effects observed after MPH chronic administration, we also investigated possible alterations of key signaling proteins known to be involved on both synaptic plasticity and learning/memory, such as the protein kinase B (AKT) and CREB transcription factor (Cunha et al., 2010).

Rats treated with 1.5 mg/kg MPH showed a significant increase in the phosphorylation of AKT (Figure 3.8 A; $F_{(2, 16)} = 49.97$, $p < 0.0001$) and CREB (Figure 3.8 B; $F_{(2, 26)} = 80.74$, $p < 0.0001$) in comparison to vehicle rat. In accordance with the memory impairment induced by MPH 5, we observed that this dose significantly decreased phosphorylation of AKT (Figure 3.8 C; $F_{(2, 16)} = 49.97$, $p < 0.05$) and CREB (Figure 3.8 B; $F_{(2, 26)} = 80.74$, $p < 0.01$). However, p38-mitogen-activated protein kinase (MAPK) signaling was activated, here shown by the increase of p38 phosphorylation (Figure 3.8 C; $F_{(2, 21)} = 78.42$, $p < 0.001$).

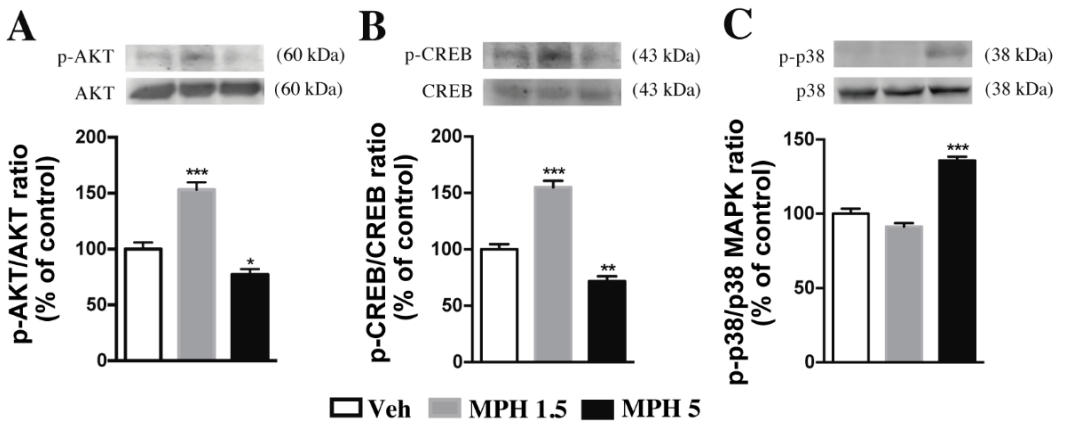


Figure 3.8. MPH modulates AKT, CREB and p-38 MAPK pathways in the rat hippocampus. *The levels of phosphorylated levels of (**A**) AKT, (**B**) CREB and (**C**) p38 MPAK after MPH chronic exposure (1.5 or 5 mg/kg/day, os; P28-P55) were analyzed. MPH 1.5 increased phosphorylation levels of AKT and CREB, whereas MPH 5 has the opposite effect; however, increasing the phosphorylation of p38 MAPK. Above the bars, representative western blot images of phosphorylated AKT (60 kDa), CREB (43 kDa), p38 MAPK (38 kDa) and respective total proteins are shown. The results are shown as mean % of control + S.E.M., n=6-9 animals of each condition. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared to the vehicle, using one-way ANOVA followed by Bonferroni's test.*

Altogether, these findings show that MPH can induce a beneficial or detrimental effect on working memory dependent on the dose probably by modulating AKT and CREB signaling pathways.

3.5 Discussion

Despite the widespread use of MPH in school aged and adult populations with ADHD, as well as its misused to cognitive enhancement and recreation in non-ADHD individuals (Sahakian et al., 2015), only few studies have explored the neuropathological impact of chronic MPH consumption. Herein, we investigated the effect produced by long-lasting MPH exposure during development in healthy individuals using for that control WKY rats (Drolet et al., 2002). Overall, our results provide direct evidence that chronic administration of MPH at a dose of 5 mg/kg/day causes hippocampal BBB disruption together with peripheral immune cells infiltration, and significant changes in astroglial cells morphology and neuronal synaptic proteins culminating in memory impairment. On the contrary, low doses of MPH did not affect brain vasculature but enhanced memory in normal rats probably by promoting an increase in synaptic proteins.

BBB is a highly selective barrier responsible for the regulation and maintenance of brain microenvironment proper for neuronal function (Cardoso et al., 2010). Previously, we

demonstrated that acute MPH exposure affects the vesicular transport across human brain ECs (Coelho-Santos et al., 2016). Thus, we decided to explore the chronic impact of MPH. Curiously, we found that MPH increased BBB permeability without affecting the levels of claudin-5 and VE-cadherin, but with an upregulation of Cav1 protein and the number of cell vesicles. In fact, caveolae is implicated in transcytosis, a process that is involved in the transcellular transport of albumin (Schubert et al., 2001). In line with this, we proved that the depletion of Cav1 prevented the increase in brain endothelial transcytosis induced by chronic MPH exposure. Moreover, endothelial dysfunction was accompanied by the upregulation of adhesion molecules increase, VCAM-1 and ICAM-1. It is well known that adhesion molecules have a decisive role on leukocyte migration across microvasculature into brain parenchyma (Ley et al., 2007). Noteworthy, we detected a significant increase of leukocytes, namely macrophages, in the hippocampus. By using an *in vitro* approach, we further concluded that Cav1 plays a crucial role on the transcytotic process since Cav1 knockdown decreased MPH-induced PMBCs infiltration. Thus, our findings offer a new insight to the clarification of MPH-induced caveolae endorsing hyperpermeability and provides a new perspective for designing endothelial-targeted interventions to modulate vascular permeability.

A disruption in the molecular framework of the cerebrovascular basal lamina, which serves as the structural basis of the BBB (Gidday et al., 2005), is thought to underlie the transmigration of leukocytes. Basal membrane is mostly made of structural proteins such as collagen type IV, fibronectin and laminin, including also cell adhesion molecules and immobilized signaling proteins (Baeten and Akassoglou, 2011). Herein we showed that MPH 5 decreased collagen IV-positive microvessels and increased of MMP-9 expression. MMP-9 has been involved in the loss of BBB integrity and consequent diapedesis (Gidday et al., 2005) because many of the molecular constituents comprising the cerebrovascular basal lamina are substrates for active MMP-9 (Van den Steen et al., 2002). In fact, peripheral immune cells are producers and releasers of MMPs (Haorah et al., 2007).

Besides MMP-9, it is well known that oxidative stress play a pivotal role in damage brain endothelium and so affecting BBB permeability by disrupting the paracellular and vesicular transport (Haorah et al., 2007; Coelho-Santos et al., 2016). ROS can indeed activate redox signaling pathways triggering an inflammatory response and expression of adhesion molecules in brain ECs (Kim et al., 2008). Our study shows that 5 mg/kg of MPH shifts the balance in favor of free-radical generation leading to oxidative tissue

damage observed by increased lipid peroxidation that can cause tissue damage by react with polyunsaturated fatty acids in cellular membranes. In accordance, previous studies have documented that long-term administration of MPH induces oxidative stress by a decrease in the antioxidant defenses through TBARS content and protein carbonyls formation that culminated in brain cells damage in young rats (Martins et al., 2006; Motaghinejad et al., 2016). Likewise, others have demonstrated that ROS promote permeability and monocyte migration across BBB, namely by activating MMP-1, -2, and -9 and decreasing tissue inhibitors of MMPs (Gidday et al., 2005). The production of ROS by migrating leukocytes, and particularly by monocytes, is thought to result from their activation following interaction with ECs (Van der Goes et al., 2001). Herein, the increase of 3-NT staining at the perivascular zone suggest that oxidative stress is involved in MPH-induced endothelial permeability.

Cerebral microvasculature is separated from astrocyte endfeet by the extracellular matrix of the basal lamina. Moreover, the astrocytic endfeet embrace brain endothelial cells covering almost entirely abluminal vascular surface forming glial basal lamina or glial limiting membrane, with an important role in the structure and function of cerebral endothelium (Abbott, 2002). Thus, the loss of basal lamina results in disruption of BBB, probably due to the loss of a physical barrier at the EC-astrocyte interface and/or lack of signaling from extracellular matrix molecules (Wang and Shuaib, 2007). The decrease of astrocytic vessels coverage is frequently associated with psychiatric disorders as a result of alterations in water homeostasis, blood flow, glucose transport, metabolism and BBB (Rajkowska et al., 2013; Watkins et al., 2014). Here, we also observed that MPH decreased the coverage of vessels by astrocytes and downregulated GFAP expression concomitantly with BBB leakage. Since GFAP seems to play a key role in astrocytic and neuronal glutamate transporter trafficking and function (Hughes et al., 2004), changes in the distribution of this critical and normally stable protein suggest that the glutamate-glutamine cycle could be impaired by MPH. Recently, it was shown that chronic treatment with MPH at an early age causes excitotoxicity associated to the inhibition of glutamate uptake and Na^+, K^+-ATPase in the prefrontal cortex (Schmitz et al., 2016a). Decrease in GFAP expression has also been associated with detrimental conditions in the CNS, such as schizophrenia, bipolar disorder and depression (Johnston-Wilson et al., 2000). Moreover, low levels of GFAP make astrocytes less efficient in dealing with the acute stage of various brain injuries. (Pekny and Pekna, 2004). Actually, deficiency of glial intermediate fibers in astrocytes causes increased permeability of the BBB (Nico et

al., 2004) and neural microenvironment damage, as demonstrated in GFAP knockout mice (Liedtke et al., 1996). Here, chronic exposure to higher doses of MPH promoted BBB dysfunction coincident with the decrease of GFAP and astrocytic vessel coverage. Additionally, we identified astrocytic atrophic with less and thinner processes when compared with the vehicle rats. This same pattern of decreased astrocytic branching was observed in Alzheimer disease and associated with cognitive deficits (Kulijewicz-Nawrot et al., 2012). On the contrary, lower doses of MPH increased the number of total ramifications. A recent study has also showed that enhanced performance on cognitive tasks is coincident with astrocytic plasticity (Brockett et al., 2015).

The disruption of vessels interaction with astrocytes may lead to neurovascular alterations and can also result in neuronal dysfunction. In rodents, one astrocyte ensheath thousands of synapses (Heller and Rusakov, 2015), and structural assembly of excitatory or inhibitory synapses can be mediated by physical contact with astrocytes as well as by different astrocyte-secreted proteins that regulate presynaptic and postsynaptic differentiation (Elmariah et al., 2005). This way, astroglial remodeling can impact the number and functional status of synapses, leading to an increase or decrease connectivity and neurotransmission imbalance (Allaman et al., 2011). Normally, an increase of astrocytic processes around neurons potentiates neuronal plasticity in the hippocampus and increases cognitive function (Wenzel et al., 1991; Heller and Rusakov, 2015). In fact, we found that 1.5 mg/kg of MPH increases the protein levels of PSD-95, SNAP-25, synaptophysin, GAP-43 and calbindin D28k. Synaptophysin and SNAP-25 dysfunction is linked to ADHD (Brophy et al., 2002; Brookes et al., 2006), so this increase could explain MPH effect to improve ADHD symptoms (Turner et al., 2005). It is known that the augment of synapatophysin expression suggest an increase in the number of synaptic vesicles and, consequently, the number of synapses (Calhoun et al., 1996). Regarding calbindin D28K, this protein is downregulated in ADHD animal model and the treatment with 1 mg/kg MPH, was able to increase calbindin D28K (Yun et al., 2014). Although there is no evidence that PSD-95 and GAP-43 are associated with ADHD, both proteins are important to synaptic plasticity and formation of new synapses (Aigner et al., 1995; El-Husseini et al., 2000). Moreover, GAP-43 is also expressed by astrocytes mediating plastic changes and attenuating astrogliosis after inflammatory stimulus. In fact, its upregulation seems to be beneficial in immune modulation and neuronal survival after CNS injury (Hung et al., 2016). This way, the increase of GAP43 could explain our increase of astrocytic processes after a low dose of MPH. By contrast, we found that MPH

induced atrophy of astrocytes expressing less and thinner processes, as well as reduced gliovascular interaction were coincident with the downregulation of the synaptic proteins. In accordance, Lima et al. (2014) demonstrated that a reduction in the number of astrocytes alters cognitive performance. Based on these results, we may conclude that higher doses of MPH can compromise neuronal events culminating in detriment of functional connectivity. Actually, it was already shown that MPH chronic treatment causes loss of neurons in the rat hippocampus (Schmitz et al., 2016b). Additionally, early chronic administration of 5 mg/kg (daily for 21 days) induced hippocampal shape deformations and affected topological features of ventral hippocampal functional networks (van der Marel et al., 2015).

It is well established the involvement of hippocampus in learning and memory processes in humans and animals (Bird and Burgess, 2008). We focus our work on working memory, which is generally defined as cognitive entities related to temporary storage and operation of information. Thus, besides cellular alterations triggered by MPH, we also aimed to understand the effects of chronic consumption of this psychostimulant on memory and decipher the signaling pathways underlying its cognitive effects. MPH is also misused to enhance cognitive skills, and in the past two decades there was an increased nonmedical use of MPH for pharmacological cognitive enhancement in healthy individuals, particularly among college students (Bogle and Smith, 2009; McCabe et al., 2014). Truthfully, MPH modulates neurotransmission interfering with executive functions and working memory in healthy individuals (Camp-Bruno and Herting, 1994; Dommett et al., 2008; Repantis et al., 2010). Moreover, MPH treatment seems to potentiate synaptic plasticity, in an age-dependent manner. Potential benefits and risks of cognitive enhancement highly depends on drug dose and task requirements, but nevertheless its chronic misuse will certainly have negative consequences. Pre-clinical studies showed that rats (30- to 44-days old) administered with MPH (2 mg/kg, i.p once daily) presented an upregulation of striatal genes involved in synaptic plasticity, namely the formation, maturation, and stabilization of new neural connections (Adriani et al., 2006b). Also, it is thought that the administration of MPH to those with “normal” catecholamine function alters cognitive function, with low doses enhancing performance and higher doses increasing catecholamine levels above optimal values leading to glutamate receptors blockade and consequently to neuronal network impairment (Berridge et al., 2006; Cheng et al., 2014; Linssen et al., 2014). Furthermore, several observations converged on the conclusion that the dose-response curve has an asymptotic

U shape, such that there are diminishing therapeutic gains at progressively higher doses of MPH (Smith et al., 1998; Devilbiss and Berridge, 2008).

In the present study, we observed that 1.5 mg/kg MPH increased short-term memory performance and activated AKT/CREB cascade. Accordingly, Andersen et al. (2002) demonstrated that treatment with a similar dose of MPH (2.0 mg/kg, i.p.) during the same developmental period caused a sustained increase in CREB levels in the NAc. On the contrary, a higher dose of MPH caused NVU dysfunction and downregulated AKT/CREB pathways, but caused an activation of p38 MAPK. Noteworthy, it was already demonstrated that BBB opening and subsequent infiltration of serum components into brain parenchyma triggers a sequence of processes that lead to neuronal dysfunction, which can culminate in cognitive impairment (Serlin et al., 2011; Chen et al., 2017). Moreover, at this same dose of MPH an increase of ROS was found in the hippocampus, namely at the perivascular zone. ROS may act as a second messenger arbitrating the cellular pathways (Zhang et al., 2016b), which can explain the inhibition of AKT/CREB. Noteworthy, ROS also induce the activation of p38-MAPK signaling that is involved in long-term depression, a persistent activity-dependent decrease of synaptic efficacy (Huang et al., 2004). In addition, lipid peroxidation affects the membrane biophysical properties and integrity that leads to impairments in long-term potentiation (Ojo et al., 2015). The synaptic modulation and memory effects triggered by chronic MPH treatment in the present study are consistent with the hypothesis that psychostimulants produce a persistent reorganization of patterns of synaptic connectivity in brain regions including the hippocampus, which may impair cognitive behavior in “normal” rats (Robinson and Kolb, 2004).

In conclusion, the present study reveals that low doses of MPH in normal rats improves memory performance associated with modulation of astrocytic morphology and synaptic machinery. However, higher doses of MPH lead to hippocampal NVU alterations and memory impairment. The present findings emphasize the negative impact that chronic use of higher doses of MPH under non-pathological conditions can have in brain function and behavior.

Chapter 4

Impact of developmental exposure to methylphenidate on rat brain's immune privilege: control versus ADHD

Impact of developmental exposure to methylphenidate on rat brain's immune privilege: control versus ADHD

4.1 Abstract

Attention deficit hyperactivity disorder (ADHD) is the most prevalent childhood mental disorders that often persists into adulthood. Moreover, methylphenidate (MPH) is the mainstay of medical treatment for this disorder. Nevertheless, not much is known about the neurobiological impact of MPH on control versus ADHD conditions, which is crucial to simultaneously clarify the misuse/abuse versus therapeutic use of this psychostimulant.

Herein, we applied biochemical and behavioral approaches to broadly explore the early-life chronic exposure of two different doses of MPH (1.5 and 5 mg/kg/day) on control (Wistar Kyoto) and ADHD rats (Spontaneously Hypertensive rats). We concluded that the higher dose of MPH increased blood-brain barrier (BBB) permeability and elicited anxious-like behavior in both control and ADHD animals. Notwithstanding, BBB dysfunction triggered by MPH was particularly prominent in control rats, which was translated by a marked disruption of intercellular junctions, an increase of microvessels vesicles, and an upregulation of adhesion molecules concomitant with infiltration of peripheral immune cells into the prefrontal cortex. Moreover, both doses of MPH induced a robust neuroinflammatory and oxidative response in control control rats. Curiously, in the ADHD model, the lower dose of MPH (1.5 mg/kg/day) had a beneficial effect since it balanced both glial response and pro-/anti-inflammatory mediators.

Overall, the contrasting effects of MPH observed between control and ADHD models support the importance of an appropriate MPH dose regimen for ADHD, and also suggest that MPH misuse has a negative effect.

4.2 Introduction

Attention deficit hyperactivity disorder (ADHD) is the most prevalent neuropsychiatric disorder with onset in childhood that can persist into adulthood (Sharma and Couture, 2014). It is a complex brain condition, which etiology is far from being fully understood. Nevertheless, it is currently accepted that ADHD has a neurobiological and genetic basis (Matthews et al., 2014; Sharma and Couture, 2014). Interestingly, structural and functional imaging studies, particularly in the prefrontal cortex (PFC), provided evidence of ADHD brain alterations, such as lower activity and smaller volumes associated with abnormal developmental brain networks related to cognition, attention, emotion and sensorimotor functions (Seidman et al., 2005).

Methylphenidate (MPH) is the ADHD first-line psychotropic medication (Sharma and Couture, 2014). Still, possible enduring neuroadaptational consequences of chronic MPH treatment are poorly understood. Despite its benefits in ADHD treatment, MPH is an amphetamine-like CNS stimulant highly used among children for long periods, which highlights the importance of clarifying its neurobiological effects. Most of the preclinical studies have used control animals and focused on dopaminergic alterations (Harvey et al., 2011; Somkuwar et al., 2013). The few available papers show that early developmental chronic exposure to MPH promotes the expression of inflammatory mediators (Schmitz et al., 2016b) and oxidative stress (Martins et al., 2006) in the hippocampus affecting recognition memory, neurogenesis (van der Marel et al., 2015), and long-lasting changes in neuronal excitability and synaptic transmission in the PFC (Urban et al., 2012) of control animals.

Peripheral immune cell trafficking into the brain is strictly controlled and limited by blood-brain barrier (BBB) (Russo and McGavern, 2015). This dynamic barrier comprises endothelial cells (ECs) that form brain capillaries and maintain a brain microenvironment proper for neural function (Campos-Bedolla et al., 2014). Cerebral ECs have no fenestrations and are linked by a junctional complex formed by tight (TJs) and adherens junctions (AJs) responsible for BBB low paracellular permeability and high electrical resistance (Campos-Bedolla et al., 2014). Brain endothelium together with pericytes, basal lamina, astrocytes, microglia and neurons form a functional neurogliovascular unit (NVU) (Cardoso et al., 2010). Notwithstanding, the unique brain immune quiescence can be altered by various pathological processes, including psychostimulants use (Kousik et al., 2012), through BBB disturbance allowing diapedesis into brain parenchyma. Consecutively, this can induce or worsen neuroinflammation culminating in neuronal damage.

Concerns have been raised about ADHD overdiagnoses, particularly because this condition overlaps symptoms and comorbidity with other common mental illnesses (Matthews et al., 2014), and also about MPH misuse (Sahakian et al., 2015). Actually, we have recently shown, through *in vitro* approach, that MPH increased endothelial permeability (Coelho-Santos et al., 2016). Nevertheless, it is highly important to further dissect the central impact of MPH on both ADHD and non-ADHD conditions.

With the present work we aimed to simultaneously investigate the effect of chronic MPH treatment on both control and ADHD animal model to simulate its misuse and therapeutic use. Focus was given to BBB function, neuroimmune responses and anxiety-like behavior.

4.3 Methods and Materials

4.3.1 Animals and Treatments

WKY and SHR (arrived at 24 days-old; ~55 g body weight; Charles River Laboratories, Lyon, France) were housed under controlled environmental conditions (12 hours light:dark cycle, 24±1°C) with food and water ad libitum. Animals were divided into three different groups: vehicle group (Veh; tap water), 1.5 mg/kg MPH group (MPH 1.5) and 5 mg/kg MPH (MPH 5) (Sigma-Aldrich, Si. Louis, MO, USA) to mimic the dosage range in humans (Harvey et al., 2011; Somkuwar et al., 2013). Individual administration was performed by gavage from Monday to Friday between P28-P55. Behavioral tests were performed at P56, and animals were sacrificed at P57. All experiments were performed by certified researchers (Federation for Laboratory Animal Science Associations) in accordance with European Community Council Directives (2010/63/EU) and Portuguese law for care and use of experimental animals (DL no 113/2013). The present study was approved by the Institutional Animal Care and Use Committee (FMUC/CNC, University of Coimbra, Portugal) and Portuguese National Authority for Animal Health "DGAV". All efforts were made to minimize animal suffering and to reduce the number of animals used.

Western Blots

Rats were anaesthetized with intraperitoneal injection of ketamine (50 mg/kg), xylazine (10 mg/kg) and transcardially perfused with 0.01 M phosphate buffered saline (PBS), pH 7.4. Western blots were performed as previously described (Coelho-Santos et al., 2015). Primary

antibodies are specified in Table 4.1. Densitometric analyses were performed using the Fiji J software (NIH, USA).

Table 4.1. *List of primary antibodies used in Western blot (WB) and immunohistochemistry (IHC) analysis*

Primary antibody	µg	MW (kDa)	Dilution WB	Dilution IHC	Reference	Company
Goat anti-albumin	50	67	1:20000	1:20000	A80-129	Bethyl Laboratories, Montogomery, TX, USA
Rabbit anti-collagen IV	--	--	---	1:200	ab6586	Abcam, Cambridge, UK
Mouse anti-claudin-5	100	25	1:100	---	35-2500	Invitrogen Inchinnan Business Park, UK
Mouse anti-occludin	100	66	1:200	---	71-500	Invitrogen
Goat anti-VE-cadherin	100	130	1:100	---	sc-6458	Santa Cruz Biotechnology, Inc., Dallas, TX, USA
Rabbit anti-caveolin-1	50	20	1:200	---	sc-53564	Santa Cruz Biotechnology
Rabbit anti-VCAM-1	150	110	1:200	---	sc-8304	Santa Cruz Biotechnology
Rabbit anti-ICAM-1	150	110	1:100	---	sc-366318	Santa Cruz Biotechnology
Rabbit anti-CD4	50	55	1:100	---	sc-7219	Santa Cruz Biotechnology
Mouse anti-CD169	--	--	---	1:200	MAB5610	R&D systems, Inc., Minneapolis, USA
Goat anti-CD-31	--	--	---	1:100	AF3628	R&D systems, Inc.
rabbit anti-GFAP	5	55	1:2000	---	G9269	Sigma-Aldrich, St. Louis, MO, USA
anti-GPAP-Cy3 conjugated	--	--	---	1:500	C9205	Sigma-Aldrich
Rabbit anti-Iba1	--	--	---	1:250	019-19741	Wako Chemical Pure Industries Ltd., Japan
Rabbit anti-CX3CR1	50	50	1:500	---	14-6093	eBioscience, San Diego, CA, USA

Rabbit anti-TNF-α	150	19	1:250	---	P350	Thermo Scientific, Waltham, MA, USA
Rabbit anti-IL-1β	150	15	1:100	---	sc-7884	Santa Cruz Biotechnology
Mouse anti-IL-10	150	20	1:200	---	sc-365858	Santa Cruz Biotechnology
Rabbit anti-p-p65 NF-kB	100	65	1:500	---	#3031	Cell Signaling Technology, Inc., Danvers, MA, USA
Rabbit anti-p65 NF-kB	100	65	1:1000	---	#4764	Cell Signaling Technology
Rabbit anti-NLRP3	100	130	1:200	---	sc-66846	Santa Cruz Biotechnology

Abbreviations: MW, molecular weight; CD4, cluster of differentiation 4; CD31, cluster of differentiation 31; CD169, cluster of differentiation 169; CX3CR1, fractalkine receptor; GFAP, glial fibrillary acidic protein; Iba-1, calcium-binding protein; ICAM-1, intercellular adhesion molecule-1; IL-1β, interleukine-1beta; IL-10, interleukine-10; NLRP3, NACHT, LRR and PYD domains-containing protein 3; NF-kB, factor nuclear kappa B; TNF-α, tumor necrosis factor-alpha; VE-cadherin, vascular endothelial cadherin; VCAM-1, vascular cell adhesion protein 1.

4.3.2 Immunohistochemistry

After anesthesia, rats were transcardially perfused with 4% paraformaldehyde (PFA; Sigma-Aldrich) in 0.01 M PBS, pH 7.4. Brains were post fixed as described previously (Coelho-Santos et al., 2015). Immunolabelling for albumin, collagen IV, CD169 and CD45 was performed as previously (Coelho-Santos et al., 2015) using slices with 14 µm. Free-floating slices with 50 µm were stained against glial fibrillary acidic protein (GFAP) or for ionized calcium-binding protein (Iba)1, both co-stained with CD31. Primary antibodies used are indicated in Supplemental Table S1.

Images were recorded using LSM 710 Meta Confocal microscope (Carl Zeiss). Quantification of fluorescence intensities, co-localization and microglia morphological analysis were determined using FIJI J software (Cardoso et al., 2015).

4.3.3 Transmission Electron Microscopy (TEM)

Rats were transcardially perfused with 4% PFA. The PFC pieces (1 mm) were immersed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 2h. Post-fixation was

performed using 1% osmium tetroxide for 1.5h. Afterwards, 1% aqueous uranyl acetate was added for 1h in the dark, for contrast enhancement. Samples were dehydrated in a graded ethanol series (30–100%), impregnated and embedded in Epoxy resin (Fluka Analytical, USA). Ultrathin sections (70 nm) were mounted on copper grids and stained with 0.2% lead citrate for 7min. Observations were carried out on a FEI-Tecnai G2 Spirit Bio Twin at 100kV.

4.3.4 Blood Collection

Blood samples were withdrawn by cardiac puncture into Tubo BD Vacutainer SST (BD Bioscience, Franklin Lakes, NJ, USA). Serum was separated by centrifugation at 1100×*g* for 10 min, and stored at -80°C until analysis.

4.3.5 Determination of Oxidative Stress Markers

Reactive oxygen species (ROS) in serum and PFC were measured by N, N-diethyl-pera-phenylenediamine assay adapted from (Tiwari et al., 2011). In brief, 5 µL serum or PFC lysates were added to 140 µL of 0.1 M sodium acetate buffer (pH 4.8) at 37°C in a 96-well plate. Samples were taken in triplicate and 100 µL of the mixed DEPPD solution (DEPPD was dissolved in 0.1 M sodium acetate buffer [pH 4.8]) and ferrous sulfate (4.37 µm ferrous sulfate dissolved in 0.1 M sodium acetate buffer, pH 4.8) at a ratio of 1:25 was added to each well to initiate reaction. The microtiter plate was then incubated at 37°C for 5 minutes. Absorbance was measured by a spectrophotometer plate reader (Biotek, Synergy HT) at 505 nm. ROS levels from serum were calculated from a calibration curve of H_2O_2 and expressed as hydrogen peroxide equivalent (1 unit = 1.0 mg H_2O_2/L). The calibration curve for standard solution was obtained by calculating slopes from an optical density graph.

The thiobarbituric acid reactive-species (TBARs) assay was used to assess hippocampi products of lipid peroxidation, via malondialdehyde (MDA) adapted from (Ohkawa et al., 1979). Briefly, 100 µL of PFC tissue supernatant were incubated at RT in the dark for 1 h in a TBA solution together with butylhydroxytoluene (BHT; Sigma-Aldrich) and a catalyzer (Iron III chloride; Sigma-Aldrich). Afterwards, samples were incubated at 95-100°C for 60 min and followed by butanol extraction. The supernatants were read spectrophotometrically at 532 nm (Biotek, Synergy HT) and the concentration of MDA was calculated with respect to a calibration curve using 1,1,3,3-tetramethoxypropane (Sigma-Aldrich) as the external standard (range: 0.1–83.5 µM). Results were expressed as µM/g of PFC tissue and µM of serum concentration of lipid peroxides

4.3.6 Animal Behavior Studies

To evaluate animal locomotor activity and anxiety-like behavior, open field test (Prut and Belzung, 2003) was performed accordingly with (Leitão et al., 2017). Animal behavior was recorded and analyzed by Anymaze Video Tracking Software (Stoelting, Wood Dale, IL, USA).

4.3.7 Statistical Analysis

Results are expressed as mean + standard error of the mean (S.E.M.). Data were analyzed using one-way ANOVA followed by Dunnett's or Bonferroni's post hoc test, as indicated in figure legends. The level of significance was $p < .05$ and the "n" represents the total number of animals used in each experimental group/condition. Statistical analysis was calculated using Prism 6.0 (GraphPad Software, San Diego, CA, USA).

4.4 Results

4.4.1 Impact of MPH on BBB function

The role of brain ECs goes beyond barrier function being also actively involved in brain (dys)function. Herein, no significant differences were detected in BBB permeability of both rat strains using MPH 1.5, with no albumin staining in PFC parenchyma (Figure 4.1 A) and no changes in proteins levels (Figure 4.1 B). However, BBB hyperpermeability was observed in both strains using MPH 5, being slightly higher in WKY rats (Figure 4.1 A, B). Also, basement membrane degradation was observed in MPH-treated rats translated by a decrease in collagen IV staining, and again more evident at MPH 5 (Figure 4.1 A). These observations indicate an altered and weakened structure of brain microvessels after MPH 5 administration.

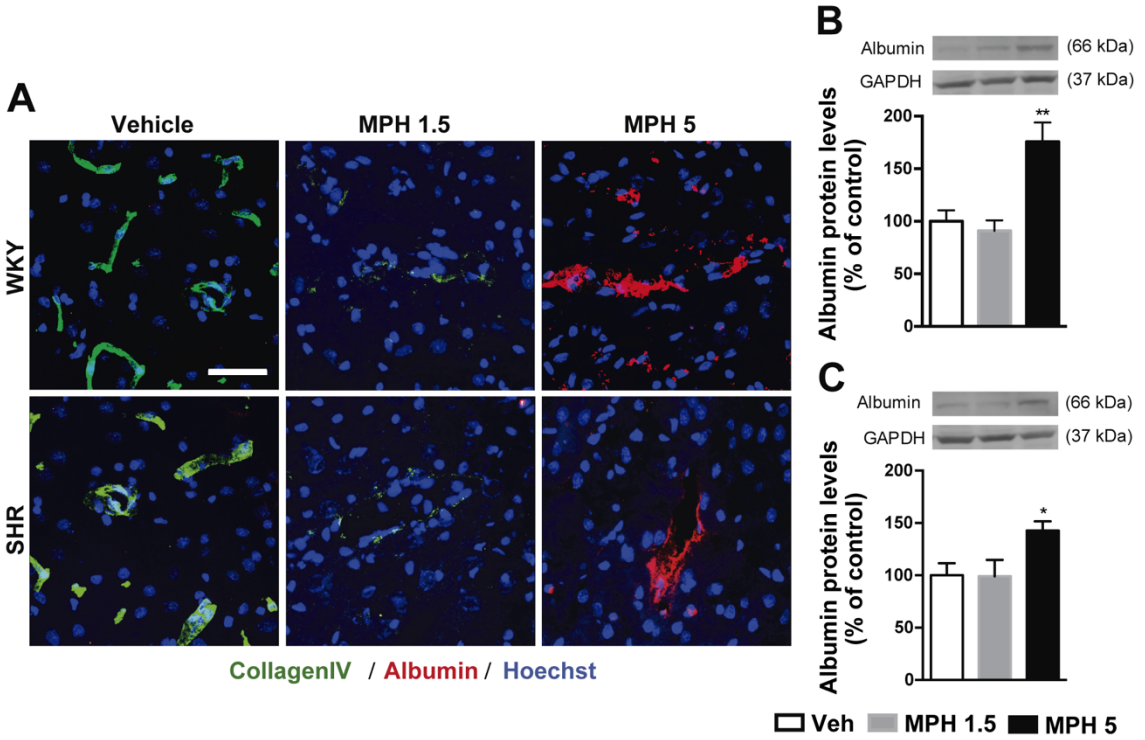


Figure 4.1. MPH increases cortical blood-brain barrier (BBB) permeability. *Wistar-Kyoto (WKY; control) and spontaneously hypertensive rats (SHR; ADHD model) were administered with MPH (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively).* ***(A)*** *Representative images of collagen IV (green), a protein of the basement membrane that surrounds the brain vessels, and albumin (red), that is an indicator of BBB leakage. Sections were counterstained with Hoechst 33342 (blue) for nuclei detection. Scale bar: 50 µm.* ***(B, C)*** *Albumin protein levels in the prefrontal cortex of* ***(B)*** *WKY and* ***(C)*** *SHR rats. MPH 5 compromised BBB permeability by degradation of basal lamina allowing the access of circulating factors to the brain parenchyma, namely serum albumin. BBB dysfunction was more significant in control rats. Data are expressed as the mean + S.E.M., n=6-9 animals of each experimental group. $^{*}P<0.05$, $^{**}P<0.01$ compared to the vehicle rats (Veh), using one-way ANOVA followed by Bonferroni's test.*

To clarify the mechanisms by which MPH induces BBB hyperpermeability, intercellular junctions were investigated. As abovementioned, MPH 1.5 did not alter TJs (claudin-5, occludin) and AJs (VE-cadherin) proteins levels in WKY rats, whereas MPH 5 induced a markedly decreased, concomitant with BBB leakage (Figure 4.2 A, B, C). In SHR, we only observed a significant decrease of claudin-5 levels with MPH 5 (Figure 4.2 D), with no alterations of occludin or VE-cadherin (Figure 4.2 E, F). We also examined ultrastructural changes in microvessels morphology by TEM in WKY and SHR after MPH 5 treatment. In WKY animals, some blood vessels had an abnormal appearance since adjacent EC membranes lost contact forming gaps that lack electron dense material (arrowheads), which suggest significant alterations of TJs and AJs protein (Figure 4.2 G). These structural aberrations were not found in vehicle rats. Concerning SHR treated with MPH 5, we also detected some alteration on intercellular junctions, but less pronounced than in WKY MPH 5 (Figure 4.2 G).

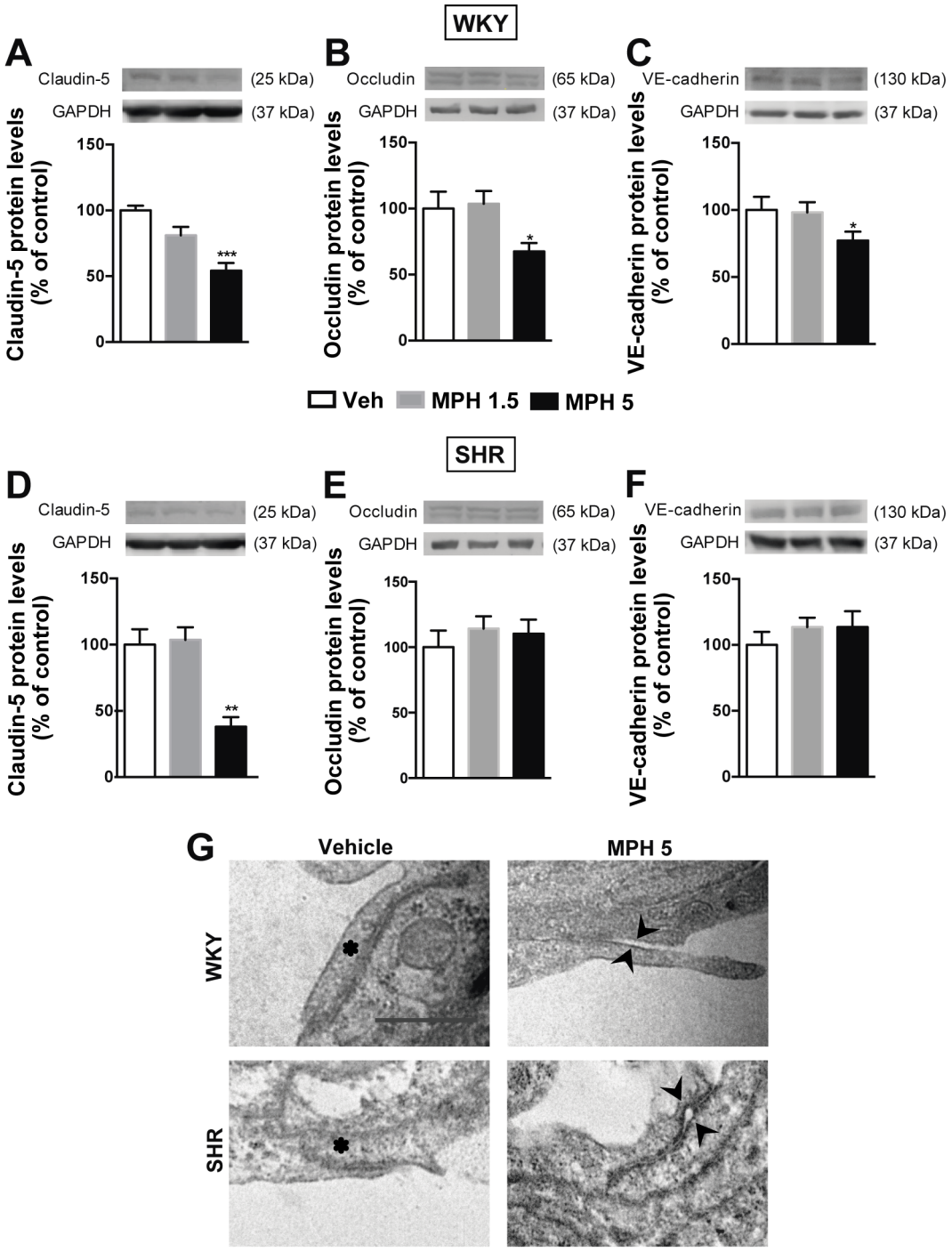


Figure 4.2. MPH interferes with endothelial junctional proteins. *Protein levels of claudin-5, occludin and VE-cadherin were analyzed in the prefrontal cortex after a chronic MPH treatment (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively) in* ***(A, B, C)*** *Wistar-Kyoto (WKY; control) and* ***(D, E, F)*** *spontaneously hypertensive rats (SHR; ADHD model). In WKY animals, MPH induced a downregulation of all intercellular proteins analyzed. Above the bars, representative western blot images of claudin-5 (25 kDa), occludin (65 kDa), VE-cadherin (130 kDa) and GAPDH (37 kDa) are shown. The results are shown as mean % of control + S.E.M., n=6-9 animals of each experimental group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the vehicle rats (Veh), using one-way ANOVA followed by Bonferroni's test.* ***(G)*** *The impact of MPH on intercellular junctional structure was also investigated by transmission electron microscopy. In vehicle conditions there were no alterations (asterisk), whereas MPH 5 induced structural alteration of intercellular junctions (head arrows). Scale bar: 1 µm.*

Besides paracellular transport, we examined if MPH could promoted pinocytosis. Using both immunoblotting for caveolin-1 (Cav1) and TEM, we concluded that in WKY rats, MPH upregulated cav1 protein levels in a dose-dependent manner (Figure 4.3 A) and the number of caveolae in the microvessels (Figure 4.3 C). Neither Cav1 protein levels nor the number of vesicles were altered in SHR after MPH administration (Figure 4.3 B, C).

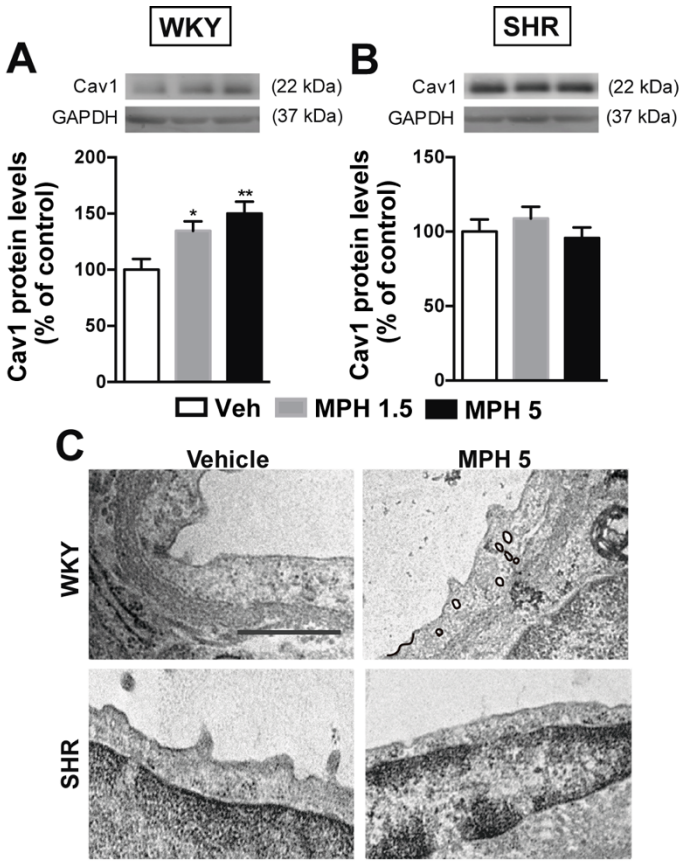


Figure 4.3. MPH promotes vesicular transport in control rats. *Caveolin-1 (Cav1) protein levels in the prefrontal cortex after a chronic MPH administration (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively) in* ***(A)*** *Wistar-Kyoto (WKY; control) and* ***(B)*** *spontaneously hypertensive rats (SHR; ADHD model). MPH upregulated Cav1 in control rats with no effect in the ADHD model. Above the bars, representative western blot images of Cav1 (22 kDa) and GAPDH (37 kDa) are shown. The results are shown as mean % of control + S.E.M., n=6-9 animals of each experimental group. $^{*}P<0.05$, $^{**}P<0.01$ compared to the vehicle (Veh), using one-way ANOVA followed by Bonferroni's test.* ***(C)*** *Representative images of TEM showing that MPH triggers the formation of pinocytotic vesicles in control animals (WKY) (outlined in black). Scale bar: 1 μm.*

Overall, our results show that chronic exposure to MPH impairs barrier properties particularly under physiological conditions.

4.4.2 MPH promotes leukocyte recruitment into the brain of control rats

Although there is a baseline trafficking across BBB, this structure excludes most of the circulating cells (Russo and McGavern, 2015). Thus, we hypothesized that MPH-induced BBB hyperpermeability could culminate on peripheral cell immune infiltration. Interestingly, we identified that MPH promoted different patterns of vascular cell and intercellular adhesion molecules expression (VCAM-1 and ICAM-1) between WKY and SHR rats (Figure 4.4 A, B).

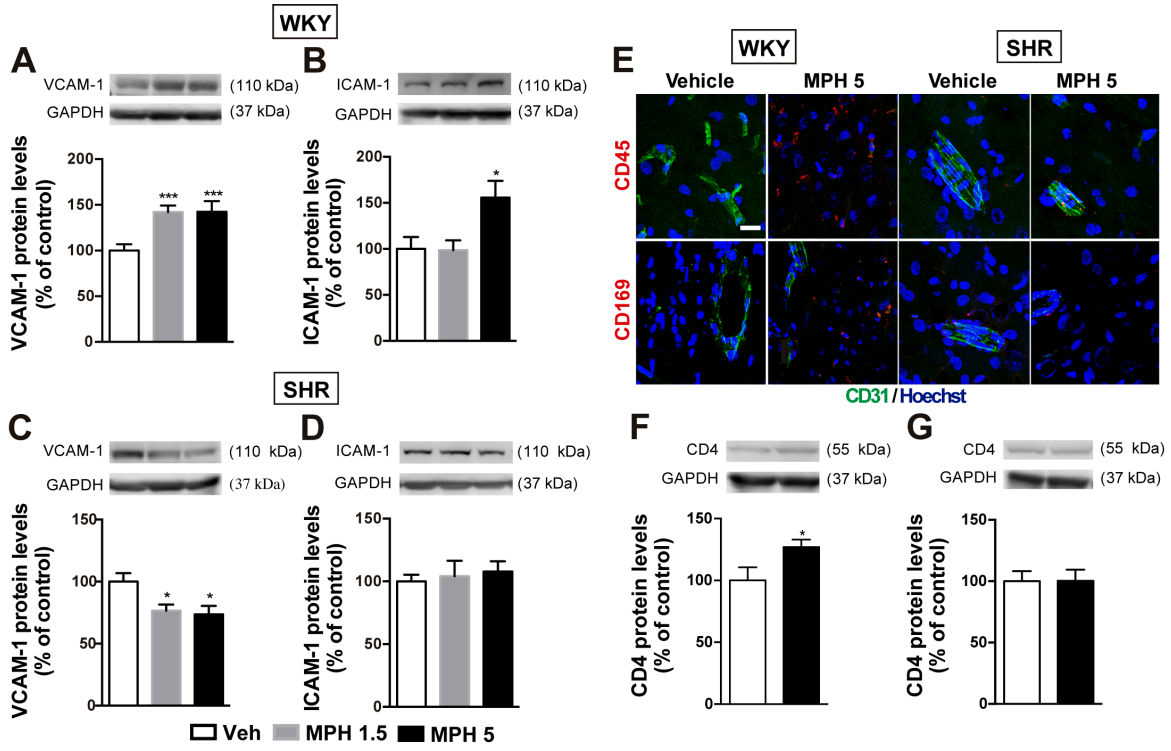


Figure 4.4. Effect of MPH on endothelial adhesion molecules and patrolling leukocytes. *Quantification of vascular cell and intercellular adhesion molecules (VCAM-1 and ICAM-1) protein levels in the prefrontal cortex after a chronic MPH treatment (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively) in (**A**, **B**) Wistar-Kyoto (WKY) and (**C**, **D**) spontaneously hypertensive rats (SHR). WKY animals showed an increase in the protein levels of adhesion molecules triggered by MPH. In SHR rats, there was an opposite effect with a downregulation of VCAM-1, and no alterations of ICAM-1. Above the bars, representative western blot images of VCAM-1 (110 kDa), ICAM-1 (110 kDa) and GAPDH (37 kDa) are shown. (**E**) Representative images of CD31 (green), a protein of brain endothelial cells, lymphocyte common antigen (CD45; red) and sialoadhesin (CD169; red), a macrophage-restricted cell surface receptor. Sections were counterstained with Hoechst 33342 (blue) for nuclei detection. Scale bar: 20 µm. (**F**, **G**) $CD4^+$ T-cells protein levels in the prefrontal cortex were increased in (**F**) WKY rats with no effect on (**G**) SHR after MPH 5 treatment. Above the bars, representative western blot images of CD4 (55 kDa) and GAPDH (37 kDa) are shown. The results are shown as mean % of control + S.E.M., n=6-9 animals of each experimental group. $^{*}P<0.05$, $^{***}P<0.001$, compared to the vehicle rats (Veh), using one-way ANOVA followed by Bonferroni's test.*

Specifically, WKY animals showed an increase of VCAM-1 protein levels with both MPH doses (Figure 4.4 A) and ICAM-1 only with MPH 5 (Figure 4.4 B). Contrary, in ADHD model both doses of MPH decreased VCAM-1 levels (Figure 4.4 C) with no effect on ICAM-1 (Figure 4.4 D). Following the abovementioned results, we demonstrated a dramatic infiltration of $CD45^+$ leukocytes (Figure 4.4 E) and presence of $CD169^+$ macrophages (Figure 4.4 E) in brain parenchyma of WKY rats, simultaneously with an increase of and $CD4^+$ protein levels (Figure 4.4 F). Curiously, no leukocytes were identified in MPH-treated SHR animals (Figure 4.4 E, G).

Our data prove that MPH chronic treatment triggers brain vascular alterations in control conditions interfering with brain immune privilege.

4.4.3 Differential gliovascular response in control versus ADHD following MPH treatment

Astrocytes play a crucial role in NVU, specifically in the maintenance, function and repair of BBB (Cardoso et al., 2010). Herein, we demonstrated that MPH induced astrogliosis in WKY rats since it increased GFAP protein levels in a dose-dependent manner (Figure 4.5 A). Using immunohistochemistry to identify brain vessels (CD31, green) and astrocytes (GFAP, red), we showed that MPH caused morphological changes in astrocytes, with hypertrophy of cell bodies and processes in WKY rats (Figure 4.5 C). Moreover, MPH 5 promoted astrogliosis not only in brain parenchyma but also at microvasculature interface pointing to a perivascular astrogliosis (Figure 4.5 C), as specifically showed by the coverage of vessels by astrocytes (Figure 4.5 D). On the contrary, MPH 1.5 downregulated GFAP levels in SHR animals (Figure 4.5 B), which is in agreement with cell morphology and decrease in the number of vessels coverage by astrocytes (Figure 4.5 E). These observations suggest that in ADHD model there are basal alterations that are counteracted by MPH.

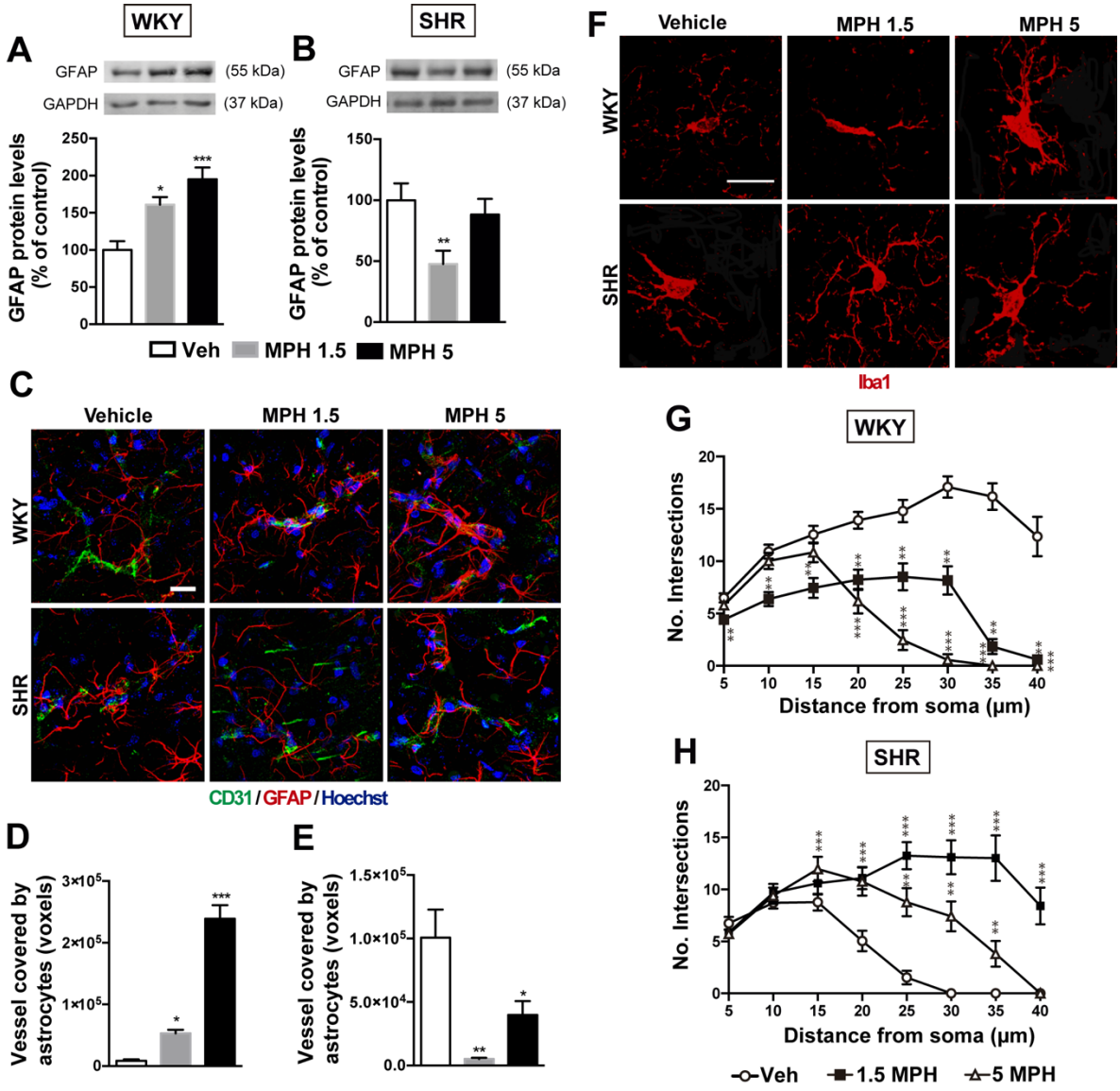


Figure 4.5. Effect of MPH on astrocytic and microglia morphology. *(**A**, **B**) GFAP protein levels in the prefrontal cortex after a chronic MPH treatment (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively) in (**A**) Wistar-Kyoto (WKY) and (**B**) spontaneously hypertensive rats (SHR). Above the bars, representative western blot images of GFAP (55 kDa) and GAPDH (37 kDa) are shown, n=6-9 animals of each experimental group. (**C**) Representative confocal images of prefrontal cortex sections obtain from WKY and SHR rats after MPH administration that were co-labelled with the brain microvascular endothelial cells cluster differentiation (CD31; green) and the astrocyte-specific Glial fibrillary acidic protein antibody (GFAP; red). Sections were counterstained with Hoechst 33342 (blue) for nuclei detection. Scale bar: 20 µm (**D**, **E**) Area fraction per field of CD31 and GFAP positive staining, along with their respective co-localization with vessels, were determined using Fiji J software (NIH, USA). In WKY rats, both MPH doses increased GFAP levels in brain parenchyma and around the capillaries highlighting a clear perivascular astrogliosis. The number of GFAP/CD31 co-localized voxels was up-regulated in the WKY proving that more vessels are covered by astrocytes. In SHR rats, MPH had an opposite effect, reducing both GFAP levels and vessels coverage by astrocytes. (**F**) Representative confocal images of microglial cells morphology (anti-Iba1) in the prefrontal cortex of Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) rats after MPH administration (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively). Scale bar: 20 µm. (**G**, **H**) Microglia Sholl analysis (> 40 cells per animal) by using Fiji J software (NIH, USA) in (**G**) WKY and (**H**) SHR rats. In WKY rats, MPH resulted in hypertrophied/activated microglial cells, whereas in SHR it changed microglia density and morphology from activated to a resting state. The results are shown as mean % of control + S.E.M., n=3 for immunohistochemistry and n=6-9 for western blots animals of each experimental group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the vehicle rats (Veh), using one-way ANOVA followed by Bonferroni's test.*

Microglia are the CNS resident immune cells, being also an important component of the NVU (da Fonseca et al., 2014). Therefore, we decided to examine microglial morphology following chronic MPH treatment.

Analysis of Iba1 staining revealed highly ramified microglia with long processes in WKY vehicle (Figure 4.5 F). With MPH 1.5, microglia presented few and elongated primary branches typical of bipolar/rod morphology (Taylor et al., 2014), and MPH 5 elicited an amoeboid phenotype with enlarged and short processes (Figure 4.5 F, G). This microglia activation by MPH in WKY rats is supported by quantitative data showing that maximum branch length and distance from the soma where microglia process interactions occurred (critical value) decreased with MPH in a dose dependent manner (Figure 4.5 G; Figure 4.6). On the contrary, in ADHD model we observed that MPH increased the process maximum and branches length (Figure 4.5 F, H), suggesting that under ADHD conditions MPH elicited a resting state in microglial cells. Details of microglial Sholl analysis are available in Figure 4.6.

Microglial Sholl analysis						
	WKY			SHR		
	Vehicle	MPH 1.5	MPH 5	Vehicle	MPH 1.5	MPH 5
Process Maximum (NM)	22.46±1.19	14.87±1.42***	15.89±0.69***	14.14±0.87	23.02±2.38**	15.89±0.69
Critical Value (µm)	34.66±1.24	25.64±1.72***	16.32+0.95***	18.20±1.77	35.33±1.52***	21.32±1.59
Maximum branch length (µm)	33.29±1.12	22.92±1.26***	16.01±0.85***	14.07±1.27	29.10±2.36***	19.45±1.28*
Number of primary branches (Np)	6.49±0.45	4.58±0.34***	5.74±0.30	7.042±0.29	6.10±0.60	5.67±0.38
Schoenen ramification index (Nm/Np)	3.20±0.27	3.10±0.32	2.76±0.17**	2.31±0.26	3.65±0.37**	2.92±0.27

Figure 4.6. Summary of microglial Sholl analysis. *Quantitative data of microglial Soll analysis (> 40 cells per animal) in WKY and SHR rats by using Using Fiji J software (NIH, USA). In WKY rats exposure to 5 mg/kg/day of MPH resulted in decrease Schoenen ramification index, which correspond to ameboid microglia. However, in SHR the treatment with 1.5 mg/kg/day MPH 1.5 changed the morphology of microglial cells from activated to a resting state by increasing the number of processes and length which resulted in an increase of Schoenen ramification index. The results are shown as mean % of control + S.E.M., n=3 animals of each experimental group, *P<0.05, **P<0.01, ***P<0.001 compared to the vehicle (control rats), using one-way ANOVA followed by Bonferroni's test.*

Afterwards, we investigated the impact of MPH chronic exposure on CX3CR1, a microglia-specific CX3CL1 (Fractalkine) receptor, important in microglia activation (Cardona et al., 2006) and phagocytosis modulation (Zabel et al., 2016). Moreover, CX3CL1 expression has been detected on activated ECs (Bazan et al., 1997).

Herein, we observed that CX3CR1 protein levels were decreased in control rats with MPH 5 (Figure 4.7 A). Concomitantly there was an increase of microglia (Iba1, red) recruitment to the vessels (CD31, green) (Figure 4.7 C) and perivascular microglia displayed intracellular vesicles containing CD31-positive particles, suggesting a partial or full uptake of blood vessel (Figure 4.7 F).

Instead, both doses of MPH upregulated CX3CR1 in SHR (Figure 4.7 B) and MPH 1.5 decreased microglia around the vessels (Figure 4.7 E).

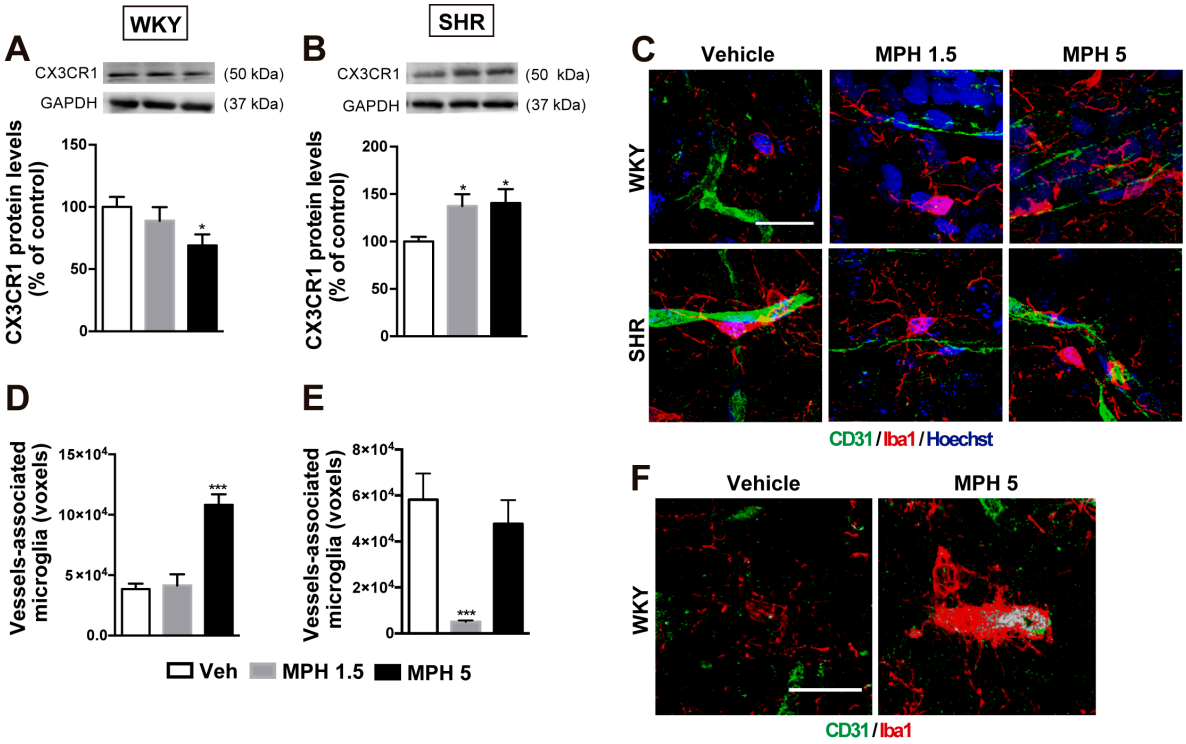


Figure 4.7. Microglia CX3CR1 and cell association with the vasculature *(**A**, **B**) CX3CR1 protein levels in the prefrontal cortex after in **(A)** WKY and **(B)** SHR post-MPH treatment (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively). Above the bars, representative western blot images of CX3CR1 (50 kDa) and GAPDH (37 kDa) are shown. **(C)** Representative confocal images of prefrontal cortex sections obtained from WKY and SHR rats after MPH administration were co-labelled with the brain microvascular endothelial cells cluster differentiation (CD31; green) and the microglia (Iba1; red). Sections were counterstained with Hoechst 33342 (blue) for nuclei detection. Scale bar: 20 µm. (**D**, **E**) Quantification of blood vessel-associated microglia by identifying the area fraction per field of both CD31 and Iba1 positive staining (Fiji J software, NIH, USA). The number of Iba1/CD31 co-localized voxels was up-regulated in WKY by MPH 5 showing that more vessels are associated with microglia. (**E**) Double-staining of microglia (Iba1) and endothelial cells (CD31) revealed an increase of CD31-positive intracellular vesicles inside microglial cells. Scale bar: 20 µm. In control rats, MPH 5 downregulated CX3CR1 which was concomitant with an increase of perivascular microglia and vessels phagocytosis. Yet, in ADHD rats, MPH 1.5 upregulated CX3CR1 and decrease the number of microglia associated to the vessels. No phagocytosis was observed in ADHD condition in any MPH dose. The results are shown as mean % of control + S.E.M., n=6-9 for western blots and n=3 animals for immunohistochemistry of each experimental group. $^{*}P<0.05$, $^{***}P<0.001$ compared to the vehicle rats (Veh), using one-way ANOVA followed by Bonferroni's test.*

Overall, we observed that glial cells respond differently to MPH according with ADHD or non-ADHD condition.

4.4.4 Distinctive modulation of neuroinflammatory mediators by MPH on control versus ADHD rats

It has been identified an oxidant–antioxidant imbalance in ADHD patients (Bulut et al., 2007; Ceylan et al., 2010). Additionally, several studies have reported that MPH induces oxidative stress and metabolic alteration (Martins et al., 2006; Coelho-Santos et al., 2016). Based on this information, and considering the observed glial cells morphological changes, we hypothesized that MPH could also modulate inflammatory and oxidative stress mediators. Indeed, we observed that the protein levels of both proinflammatory cytokines tumor necrosis factor (TNF)-α (Figure 4.8 A) and interleukine (IL)-1β (Figure 4.8 B) were significantly higher in WKY rats after MPH chronic treatment. On the contrary, MPH 5 reduced anti-inflammatory cytokine IL-10 protein levels (Figure 4.8 C). Regarding oxidative stress, both doses of MPH upregulated the levels of ROS (Figure 4.8 D) and MDA (Figure 4.8 E) in rat serum and PFC. Interestingly, in ADHD animal model, the production of TNF-α (Figure 4.8 F), IL-1β (Figure 4.8 G), ROS (Figure 4.8 I) and MDA (Figure 4.8 J) was decreased by MPH 1.5, whereas IL-10 levels were upregulated with both doses of MPH (Figure 4.8 H). However, with MPH 5 there was an increase in PFC and no alterations in serum suggesting once again that different consequences can be triggered according to MPH dose. Overall, our results suggest that a lower dose of MPH has a beneficial effect in the disease model whereas a higher dose seems to have a negative impact independently of the condition. Importantly, we were also able to show a parallelism between brain (PFC) and serum alterations which can be useful to predict central changes through peripheral indicators.

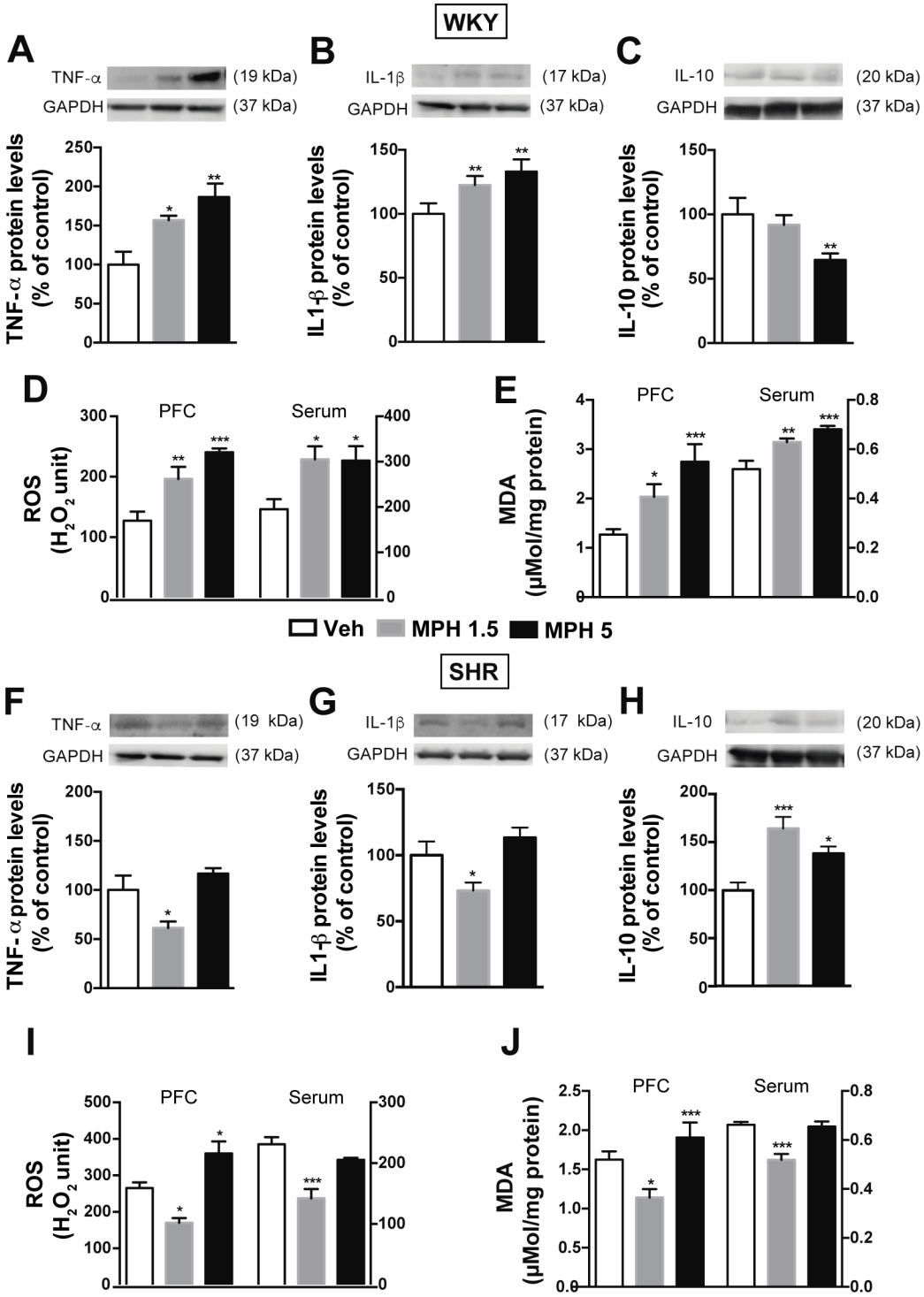


Figure 4.8. Analysis of MPH impact on neuroinflammatory and oxidative stress status. *Tumor necrosis factor-alpha (TNF-α), interleukine-1 beta (IL-1β), IL-10 protein levels, reactive oxygen species (ROS) production and malondialdehyde (MDA) formation in serum and the prefrontal cortex (PFC) homogenates were differently modulated by MPH exposure (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively) in (**A**, **B**, **C**, **D**, **E**) Wistar-Kyoto (WKY) and (**F**, **G**, **H**, **I**, **J**) spontaneously hypertensive rats (SHR). MPH had a distinctive immunomodulatory effect depending on the condition, since in control rats promoted a neuroinflamamtory environment whereas in ADHD endorsed an anti-inflammatory status. Moreover, under control conditions, ROS levels significantly increased with MPH in both serum and PFC culminating in increased lipid peroxidation. However, in ADHD rats MPH was able to attenuate oxidative stress-induced lipid peroxidation. Above the bars, representative western blot images of TNF-α (19 kDa), IL-1β (17 kDa), IL-10 (20 kDa) and GAPDH (37 kDa) are shown. The results are shown as mean % of control + S.E.M., n=10-23 for serum and n=6-9 for PFC supernatants of each experimental group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the vehicle rats (Veh), using one-way ANOVA followed by Bonferroni's test.*

To explore in more detail the signaling cascades underlying MPH-induced immunomodulatory effects, we investigated key inflammatory response pathways (Lawrence, 2009; Pan et al., 2014). NF-κB is a transcription factor and p65-NF-kB activation classically occurs via phosphorylation. Moreover, NLRP3 inflammasome assembly mediates the processing and release of IL-1β, having a central role in inflammatory responses.

MPH promoted p65 phosphorylation (Figure 4.9 A) and increased NLRP3 protein levels (Figure 4.9 B) in WKY rats. However, in SHR both pathways were downregulated after MPH 1.5 treatment and MPH5 only decreased p-p65/p65 ratio (Figure 4.9 C, D).

These results suggest that MPH under a control situation promotes a pro-inflammatory and oxidative status whereas in ADHD situation has an anti-inflammatory effect. Additionally, the MPH immunoregulation seems to involve NF-κB/NLRP3 signaling pathways.

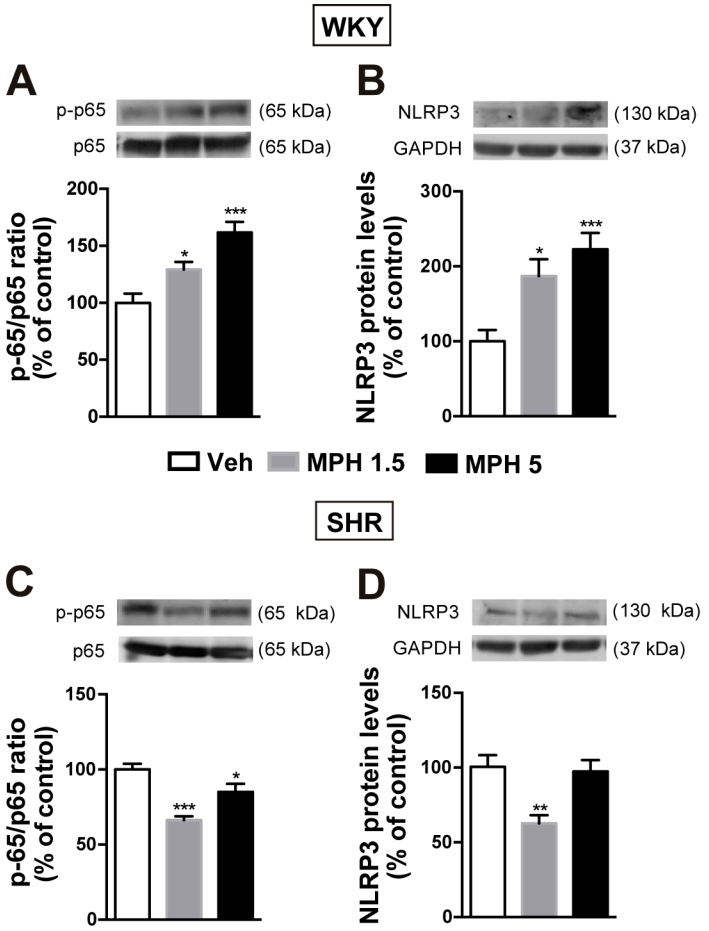


Figure 4.9. Modulation of inflammatory signalling pathways by MPH. *The levels of phosphorylated factor nuclear kappa B (NF-κB) p-65 subunit and NACHT, LRR and PYD domains-containing protein 3 (NLRP3) protein levels were analyzed in* ***(A, B)*** *Wistar-Kyoto (WKY) and* ***(C, D)*** *Spontaneously hypertensive rats (SHR) after chronic MPH exposure (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively). Inflammatory signaling pathways were differently modulated by chronic MPH exposure. MPH increased the ratio of p-p65/p65 and the levels of NLRP3 inflammassome in a dose-dependent manner in WKY rats. In SHR, MPH 1.5 decreased both phosphorylation of p65 as well as the protein levels of NLRP3. Above the bars, representative western blot images of p-p65 and p65 (65 kDa), NLRP3 (130 kDa) and GAPDH (37 kDa) are shown. The results are shown as mean % of control + S.E.M., n=6-9 animals of each experimental group. $^{*}P < 0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the vehicle rats (Veh), using one-way ANOVA followed by Bonferroni's test.*

4.4.5 Chronic MPH treatment increases anxiety-like behavior

Since we observed significant brain alterations, we also investigated whether animals could present behavioral impairment. Throughout the study, no unusual labored breathing, difficulties in moving, hunching, or unusual interactions with cage mates were observed. Also, body weight increased gradually with age during the experimental period (Figure 4.10) and no differences were observed among groups.

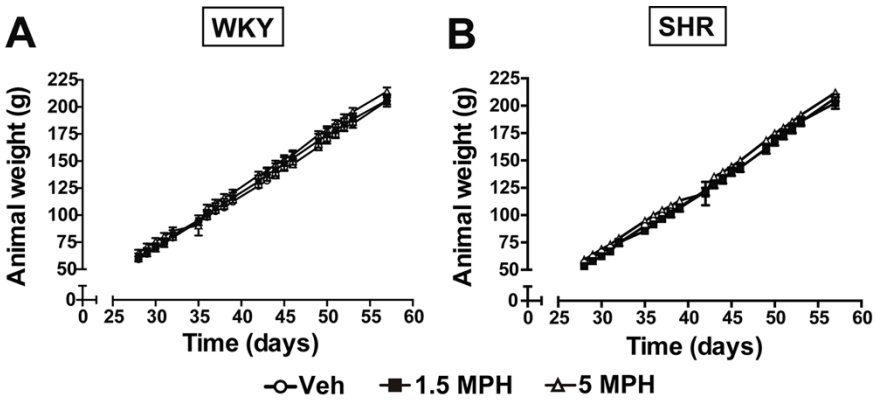


Figure 4.10. Effect of MPH in body weight of (A) WKY and (B) SHR rats over treatment time. *No significant alterations were observed. Each point represents the mean ± S.E.M. n=14-26 animals of each experimental group.*

Our results demonstrated that MPH 1.5 significantly increased both locomotor (Figure 4.11 A) and exploratory (Figure 4.11 B) activity in WKY rats without changing the percentage of time spent in the arena center (Figure 4.11 C). Instead, MPH 1.5 decrease ambulation distance (Figure 4.11 E) in ADHD rats with no alteration on exploratory activity (Figure 4.11 F) and spent in the arena center (Figure 4.11 G). Regarding MPH 5, this dose decreased all the parameters analyzed in both animal models.

In sum, MPH at a lower dose leads to hyperactivity in control rats whereas balances the locomotor activity in ADHD animals. At a higher dose, MPH seems to be anxiogenic inducing anxiety-like behavior in control and ADHD rats.

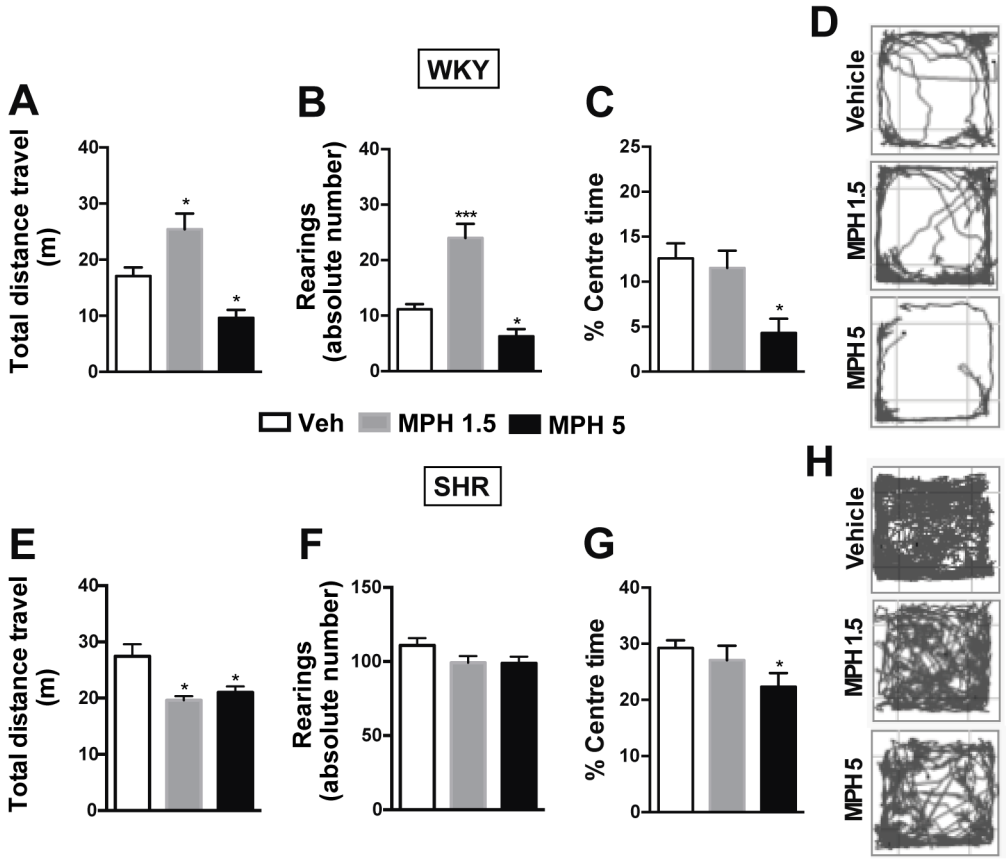


Figure 4.11. Chronic MPH treatment promotes anxiety-like behavior. (A, E) *Total distance travel,* ***(B, F)*** *number of rearings,* ***(C, G)*** *% central time and* ***(D, H)*** *representative track plots of path travelled by in* ***(A, B, C, D)*** *Wistar-Kyoto (WKY) and* ***(E, F, G, H)*** *spontaneously hypertensive rats (SHR) after chronic exposure of MPH (1.5 or 5 mg/kg/day, os; P28-P55; MPH 1.5 and MPH 5, respectively). MPH 5 induced anxiety-like behavior in both strains, non-ADHD and ADHD conditions. Data are expressed as the mean + S.E.M., n=10-23 animals of each experimental group. $^{*}P<0.05$, $^{***}P<0.001$ compared to the vehicle rats (Veh), using one-way ANOVA followed by Dunnett's test.*

4.5 Discussion

Given the prevalence of MPH use in pediatric populations, it is surprising that only a few studies have analyzed the neurobiological and behavioral effects of early developmental MPH exposure. Moreover, most of the studies use healthy rats and intraperitoneal MPH administration, so significance of these findings for ADHD is ambiguous. ADHD is a brain disorder with structural, chemical and functional abnormalities (Seidman et al., 2005; Matthews et al., 2014). Therefore, to better simulate ADHD treatment schedule, we used the most validated ADHD rat model (Sagvolden et al., 2009) and the inbred comparator strain treated orally with clinical relevant doses of MPH from equivalent late-childhood through late-adolescence in humans (Harvey et al., 2011; Somkuwar et al., 2013). Here, we report for the first time that MPH early development treatment of control animals alters BBB function and

triggers a neuroimmune response concomitantly with anxiety-like behavior. Conversely, in ADHD animal model MPH has an ameliorative effect. Also, we show that the central effects are highly dependent on MPH dose.

Brain neuronal activity requires a stable environment maintained by functional BBB (Cardoso et al., 2010; Campos-Bedolla et al., 2014), which dysfunction has been often associated with neurological diseases (Rosenberg, 2012) and cognitive impairment (Shih et al., 2013). Previously, Bahcelioglu et al. (2009) showed capillary wall structural changes and increased endothelial vesicles in rats after MPH chronic treatment (5-20 mg/kg, weekdays, 3 months orally), suggesting that MPH could induce BBB alterations. In fact, we recently proved that MPH promotes human brain endothelial permeability by specifically increasing caveolae-dependent vesicular transport (Coelho-Santos et al., 2016). Still nothing more is known about the impact of MPH on the NVU. Herein, we show that higher dose of MPH increased BBB permeability detected by serum albumin extravasation and basal lamina protein collagen IV degradation, which is in accordance with the previous study showing increased thickness of the basal membrane in brain vessels triggered by MPH (Bahcelioglu et al., 2009). BBB disruption is known to be involved in locomotor impairment (Leitão et al., 2017) and anxiety-like behavior (Yang et al., 2016), which can justify the observed anxiety-like behavior. Furthermore, we showed a significant decrease of both TJs and AJs proteins as well as an increase of microvessels vesicles, proving that endothelial paracellular and transcellular permeability were compromised by MPH particularly in control conditions.

BBB plays an important role in limiting the infiltration of leukocytes mainly due ECs low basal expression of adhesion molecules and inflammatory mediators (Alvarez et al., 2011). Concomitantly with BBB disruption, we concluded that MPH compromised brain immune quiescence in control rats, since it upregulated adhesion molecules and promoted leukocytes recruitment. On the contrary, adhesion molecules were unaltered or even decreased in MPH-treated SHR, which can explain leukocyte absence in the PFC and demonstrates that MPH-induced BBB dysfunction without inducing endothelial activation is insufficient for diapedesis. Diapedesis into brain parenchyma is a highly-regulated process involving integrins and adhesion molecules, chemokines/cytokines, and metalloproteinases to promote vascular basement membrane degradation or structural alteration (Muller, 2013). Likewise, leukocytes can migrate through the ECs involving caveolae (Millan et al., 2006). In the present study, we showed that MPH increased Cav1 levels and ECs vesicles in control rats, which can be responsible for transcellular leukocyte migration.

Besides ECs, astrocytes have also an important role at the NVU. These cells project their endfeed around vessels and secrete factors that provide a barrier-promoting effect and an endogenous anti-inflammatory balance to brain-directed immune attacks (Alvarez et al., 2011). Here, astrocytes and their interactions with brain vasculature were analyzed, and we concluded that, in WKY rats, both MPH doses increased GFAP levels not only in brain parenchyma but also around the capillaries highlighting a clear perivascular astrogliosis. Accordingly, MPH was shown to activate astrocytes in limbic neuron/glia co-cultures (Suzuki et al., 2007), and to induce PFC astrocytes hypertrophy (Bahcelioglu et al., 2009). Also, astroglial swelling and perivascular reactivity may alter neurovascular and neurometabolic coupling causing inflammatory and trophic response (Leybaert, 2005) that could be responsible for BBB disruption and consequently leukocyte infiltration observed in these animals. Curiously, MPH 1.5 in ADHD animal model decreased astrocytic reactivity in brain parenchyma and around vessels, which is in agreement with a previous study showing that orally treatment with MPH (1 mg/kg) for 28 consecutive days reduced astrocytic response in the cerebellar vermis of adult SHR male (Yun et al., 2014). Moreover, we concluded that MPH 5 did not alter GFAP levels but decreased astrocytic microvessels coverage. Interestingly, reduction in the coverage of blood vessels by astrocytic endfeet was shown to be associated with anxiety (Di Benedetto et al., 2016), which could explain our results regarding MPH-induced anxiety-like behavior.

Microglia are the main innate immune cells and their dysregulation is associated with psychiatric disorders (Frick et al., 2013). Likewise, latest studies suggest that microglia activation can be related to BBB disruption (da Fonseca et al., 2014). Specifically, MPH long-term administration (10 mg/kg i.p., 90 days) promoted microglia activation in mice basal ganglia (Sadasivan et al., 2012). Herein, in control animals microglia presented a bipolar/rod morphology after MPH 1.5 chronic exposure, which was also found in the cerebral cortex of patients with neural disorders as Alzheimer's disease and viral encephalitis (Wierzba-Bobrowicz et al., 2002). MPH 5 had even a more pronounced effect and recruited microglial cells to perivascular zones, as verified by the presence of CD31 marker inside microglia suggesting phagocytosis on ECs (Jolivel et al., 2015). These observations were concomitant with CX3CR1 downregulation. Accordingly, it was reported that CX3CR1 deficiency dysregulates microglial responses, resulting in neurotoxicity (Cardona et al., 2006) and increase of phagocytosis (Zabel et al., 2016). Additionally, CX3CR1 signaling can protect from injury and death induced by dysregulated microglia, acting as anti-inflammatory molecule downregulating pro-inflammatory cytokines (Neiva et al., 2014). Curiously, in ADHD animals

MPH had the opposite effect and changed microglia morphology from activated to resting state coincident with increased CX3CR1 levels.

Since we observed differential glial response in control versus ADHD animals following chronic MPH treatment, and knowing that glial cells are the main source of inflammatory mediators, we further dissected this paradoxical effect. In non-ADHD conditions, MPH initiated an inflammatory cascade by increasing proinflammatory cytokine levels and decreasing anti-inflammatory cytokines, probably through NF-κB and NLRP3 activation. Undeniably, both pathways are involved in neuroimmune responses by inducing inflammatory mediators (Lawrence, 2009; Pan et al., 2014). Moreover, considering that CNS inflammation has been reported to be involved in the anxiety-like symptoms (Xu et al., 2016b), MPH-induced neuroinflammation can also explain control rat behavior observed at higher doses. However, in ADHD model, MPH treatment at a clinically more relevant dose decreased inflammatory processes by suppressing the activation of the NF-κB and inflammasome signaling pathway. Accordingly, Aga-Mizrachi et al. (2014) showed that serum levels of IL-1β and IL-6 were decreased by MPH, in a rat model of posttraumatic stress disorder.

Furthermore, studies have shown that MPH chronic exposure alters the energetic metabolism, facilitates ROS formation, and causes both lipid peroxidation and protein damage in PFC of young Wistar rats (Martins et al., 2006). Nonetheless, a comparison between peripheral and central systems in control and ADHD conditions has never been addressed before. Herein, we proved that MPH in control rats increased oxidative stress in a dose-dependent manner in both peripheral and central systems. Importantly, it was previously demonstrated that ADHD children exhibit higher peripheral oxidant levels (Ceylan et al., 2010), and MPH is able to repair the oxidative imbalance by increasing antioxidant defense mechanisms (Guney et al., 2015). Here, we showed that, in ADHD rat, the lower dose of MPH prevented the oxidative stress in serum and PFC. Also, numerous studies have highlighted that anxiety-like behavior may be a consequence of oxidative stress mechanisms (Bouayed et al., 2009). Moreover, it has been proposed that oxidative stress plays a role in psychostimulants-induced BBB dysfunction (Kousik et al., 2012), and we have previously revealed that indeed oxidative stress is involved in MPH-induced endothelial hyperpermeability (Coelho-Santos et al., 2016).

Regarding behavior studies, MPH has a paradoxical effect increasing locomotor and exploratory activity in control rodents with an opposite effect in ADHD animal model. Truthfully, psychostimulant sensitization is commonly demonstrated as increased locomotor activity and was previously described that low doses of MPH induces sensitization in WKY but not in SHR (Yang et al., 2003).

Overall, our findings prove that a clinically relevant dose of MPH has beneficial effects under ADHD conditions, but promotes neurovascular dysfunction, neuroinflammation and oxidative stress in non-disease conditions. Additionally, higher doses have a detrimental impact in both conditions. These observations highlight the importance of an appropriate MPH dose regimen for ADHD and also call our attention for MPH misuse consequences.

Chapter 5

General Discussion and Conclusions

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Methylphenidate (MPH) is an amphetamine-like central nervous system (CNS) psychostimulant widely prescribed for attention deficit hyperactivity disorder (ADHD). ADHD affects 129 million worldwide children aged 4-17 years (Thomas et al., 2015) and generates substantial costs to society at individual and community levels (Pelham et al., 2007). Several studies have stated a huge increase in ADHD medications (Lakhan and Kirchgessner, 2012; Beau-Lejdstrom et al., 2016) and according to the report of the International Narcotics Control Board for 2014, worldwide MPH consumption in 2013 was estimated over to 71.8 tons. In fact, the use of MPH is controversial and concerns have been raised about ADHD overdiagnosed.

Due to the limited knowledge about the consequences of MPH use, the purpose of the current study was to uncover the effect of MPH on the CNS and behavioral alterations in both ADHD and healthy conditions. Special attention was given to the impact of MPH on blood-brain barrier (BBB) since nothing was known about vascular alterations under conditions of MPH use. To achieve our goals, *in vitro* and animal models were used, and in the end we were able to prove that MPH can have a negative impact in BBB proprieties with repercussions on behavior.

We started by investigating how MPH directly affects the BBB (Chapter 2). For that, we used two simplified *in vitro* models of BBB, primary cultures and a cell line of human brain microvascular endothelial cells, which are the BBB principal components and the first line of brain defense against pathogens and peripheral signals (Cardoso et al., 2010). We concluded that acute exposure to a clinically relevant MPH concentration increased endothelial permeability. The intracellular signaling responsible for this effect was dissected by proving that brain endothelial cells (ECs) have a quick response to MPH producing intracellular reactive oxygen species (ROS) after the assembly of NADPH oxidase (NOX) complex. ROS signaling then activated c-Src kinase, which in turn phosphorylated Caveolin-1 (Cav1) at Tyr^{14} and promoted caveolae formation inducing transcytosis. Noteworthy, brain ECs are indeed an important source of ROS which supports their proactive role in brain (dys)function. Additionally, we also concluded that MPH induced the expression of adhesion molecules that culminated in leukocytes migration across cell monolayer (Chapter 3). Downregulation of Cav1 ameliorated both BBB permeability (Chapter 2 and 3) and leukocyte transmigration (Chapter 3).

After understanding the direct impact of MPH on ECs, we performed animal studies to clarify how this psychostimulant interferes with neurogliovascular (NVU) functions under non-pathological conditions. Taking into account that MPH is widely misused for cognitive enhancement, we also investigated its influence on memory performance. Healthy rats were treated with clinical dosing schedule of MPH (1.5 or 5 mg/kg/day, per os, Monday-Friday) from P28-P55 (equivalent to late-childhood through late-adolescence in humans). Chronic administration of the higher dose of MPH enhanced hippocampal BBB permeability. Interestingly, there were no alterations on the expression of intercellular junctions, but Cav1 was significantly upregulated as well the number of vesicles present on microvessels. Additionally, MPH activated endothelium through the increase of adhesion molecules promoting the entrance of peripheral immune cells into the brain parenchyma.

BBB dysfunction was also coincident with the degradation of lamina basal, matrix metalloproteinases-9 (MMP-9) upregulation and oxidative stress. MMPs are important in normal development and in ECs migration given their ability to degrade the extracellular matrix (ECM) surrounding BBB capillaries. However, MMPs may also weaken the barrier properties by impairing the basement membrane integrity (Asahi et al., 2001; Svedin et al., 2007), which might then favor the access of peripheral cells to the brain. Regarding ROS, they can have both beneficial and deleterious effects. In fact, several biological processes are dependent upon appropriate intracellular ROS levels, namely those involved in the activation of signaling pathways. Conversely, high levels of ROS (oxidative stress) can trigger an inflammatory response and expression of adhesion molecules in brain ECs (Kim et al., 2008). The results obtained in animal studies corroborate those found *in vitro*. Moreover, it was possible to conclude that the effect of MPH on BBB properties depends on the dose used, since the lower dose did not affect the function of hippocampal vasculature.

Despite the crucial role of ECs in barrier properties, other cells like astrocytes have also a critical role on the NVU. Astrocytes are the mediators between vessels and neurons, providing glucose from bloodstream and mediating neurovascular coupling. Alterations in these cells may lead to neuropathological conditions (Lima et al., 2014). It was previously shown that MPH decreased GFAP and NeuN content (Schmitz et al., 2016b), but the consequences of such alterations were overlooked. Our data provide evidence that MPH has a dose-dependent effect on glial cells and synaptic plasticity signaling pathways. Low doses of MPH altered astrocytic morphology as shown by the increased

in the degree of ramification and activation of synaptic plasticity signaling pathways. On the contrary, chronic early-life exposure to high doses of MPH led to astrocyte atrophy and abnormal cell distribution around the vessels simultaneously with alterations on synaptic plasticity proteins.

Together, our data (Chapter 2 and Chapter 3; summarized in Figure 5.1) showed that MPH use under non-pathological conditions (misuse) negatively interferes with BBB properties and we identified Cav1 signaling pathway as a key mechanism in MPH-induced endothelial transcytosis. Likewise, these results demonstrate that this psychostimulant can lead to an impairment in vascular-astrocytic-neuronal communication, which has important implications for children misdiagnosed with ADHD and for those who deliberately misuse MPH.

Apart from providing important mechanistic insight into MPH-induced NUV dysfunction, our work also identifies a potentially novel strategy for drug delivery into the brain in order to treat several CNS diseases, which the access is limited by BBB. In fact, other psychostimulants have been suggested for such purpose (Kast, 2009; Focosi and Kast, 2010). The non-specific fluid-phase transcytosis at the BBB is rare under physiological conditions, so a transient increase in BBB opening raises the potential use of MPH for this purpose. Still, it is of high importance to first fully characterize the effects of this psychostimulant in the BBB since there are many unanswered questions. This could be a crucial step toward the development of therapies with fewer adverse effects while preserving the useful properties of the drug to penetrate into the brain. Curiously, MPH is already used in cancer patients to improve the depressive mood (Hardy, 2009), but it was never tested as a facilitator of drug delivery into the brain.

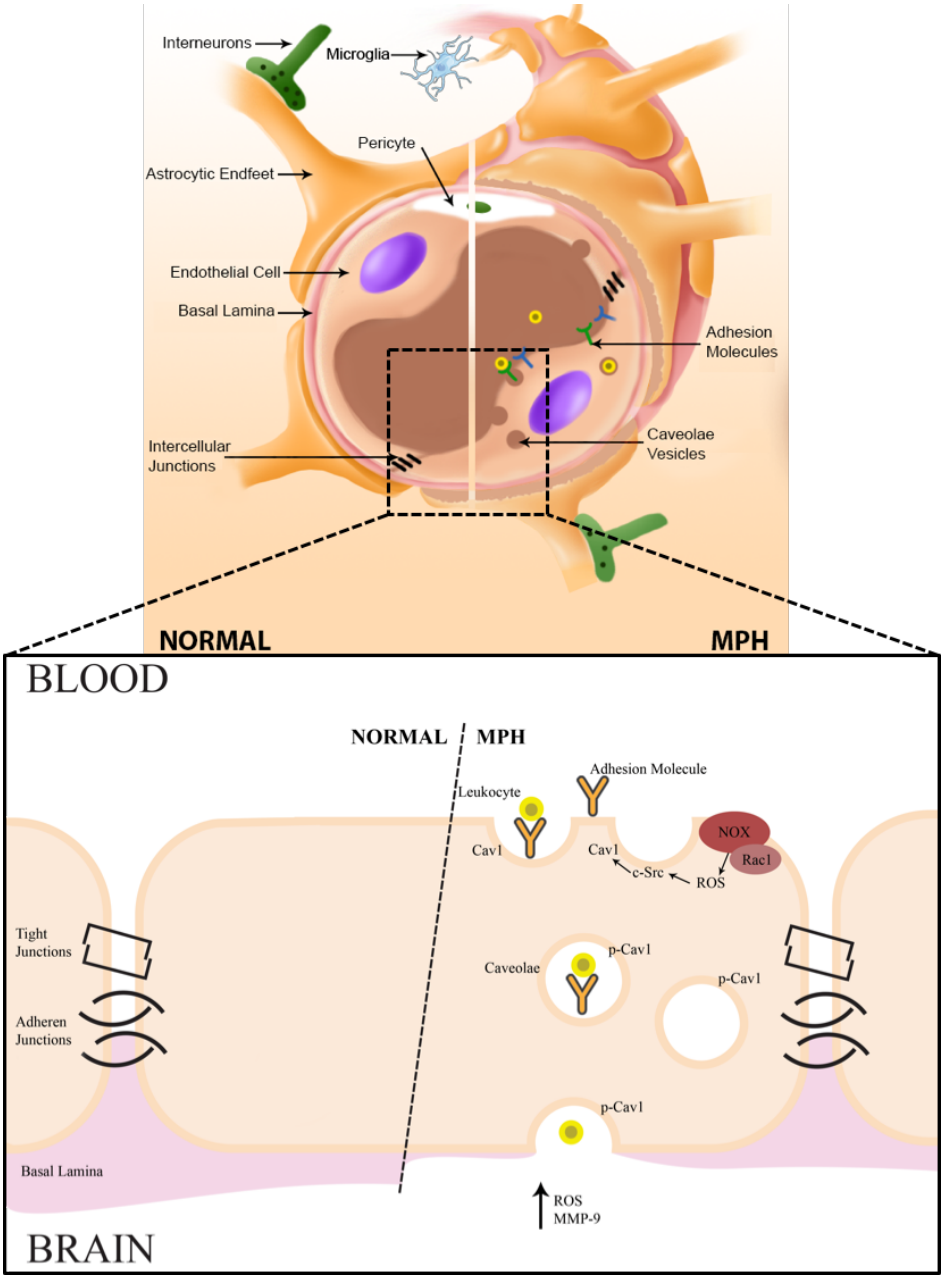

Figure 5.1. Schematic representation of the main alterations that occur at the neurogliovascular unit during MPH exposure. *The BBB is mainly composed of vascular endothelial cells, highly connected by adherens (AJs) and tight junctions (TJs), a sparse layer of pericytes, a basal lamina, and a layer of astrocytic foot processes. Neurons and microglia are also important mediators of BBB integrity in physiological conditions. After MPH, several BBB alterations occur culminating in increased permeability by transcytosis. MPH-induced BBB dysfunction involves increased levels of caveolin-1 (Cav1) and number of caveolae by reactive oxygen species (ROS) signaling. Moreover, MPH response includes the increased matrix metalloproteinase (MMP) activity and oxidative stress (both derived from endothelial cells or from macrophage/peripheral immune cells) lead to basement membrane degradation, an inflammatory response by increasing adhesion molecules at the endothelium. Also, the decrease of astrocytic processes as the endfeet vessel coverage were observed. Altogether these events culminate in albumin extravasation, leukocyte recruitment into brain parenchyma, and neuronal dysfunction. Cav1, caveolin-1; MPH, methylphenidate; NOX, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase; p-Cav1, Cav1 phosphorylated; ROS, reactive oxygen species.*

In chapter 2 and 3 we were able to improve our knowledge about the impact of MPH under physiological conditions. However, it is crucial to understand the differences

between MPH use in ADHD versus non-ADHD conditions. In fact, this approach has been rarely used conducting ourselves to only highlight the "dark side" of MPH. Having this in mind, we determined the chronic impact of MPH treatment during a period of neurodevelopment in physiological and hyperactive situation. The outcome of this field may be very important taking in consideration that MPH is prescribed essentially to children and adolescents, and for long periods. To address this issue at the preclinical level (Chapter 4), experiments were conducted using simultaneously a rat model of ADHD and its control, which were administered as in Chapter 3. This study, show that chronic MPH at higher doses induced prefrontal cortex (PFC) BBB disruption and anxiety-like behavior in both ADHD and non-ADHD conditions. Notwithstanding, BBB dysfunction was more prominent in control rats showed by a disruption of both transcellular and paracellular transport and increase of recruitment and infiltration of immune cells. Astonishingly, in ADHD animal model low doses of MPH had an anti-inflammatory and anti-oxidative effect, and decreased the locomotor activity. MPH was able to adjust glial activation allowing the reestablishment of basal conditions. This is consistent with previous studies showing that MPH can be beneficial in other pathologic conditions decreasing neuroinflammatory status in a rat model of Post-Traumatic Stress Disorder (Aga-Mizrachi et al., 2014) or improving cognitive and behavioral impairments in Jacobsen Syndrome rat model (Huang and Huang, 2012). In fact, it seems unquestionable that MPH improves some of the core symptoms of ADHD by reducing hyperactivity and impulsivity, and helping children to concentrate. On the contrary, at higher doses MPH is harmful in both physiological (Chapters 3 and 4) and ADHD conditions (Chapter 4) leading to neuroinflammation, oxidative stress and vascular alterations.

Interestingly, when we compare the impact of MPH in both hippocampus (Chapter 3) and PFC (Chapter 4) of healthy rats, we conclude that MPH at the higher dose promoted BBB permeability in both regions. The difference was that in the PFC both paracellular and transcellular transport were altered whereas in the hippocampus only the transcellular pathway was affected by MPH. Moreover, there were significant differences regarding astrocytic responses. One important aspect is that the density and proliferation of astrocytes is significantly higher in the rodent cortex that in the hippocampus (Emsley and Macklis, 2006), which may explain the increase intensity of GFAP and edematous astrocytic processes observed around the vessels in the PFC of control rats administered with MPH. Additionally, there are also differences in vascular supply, distribution and

density of brain capillaries in different CNS regions, which is thought to be related to the higher synaptic activity and metabolic demand of neurons (Cavaglia et al., 2001; Wilhelm et al., 2016). Particularly, the blood vessels per mm^2 brain tissue is higher in PFC than in the hippocampus (Miyamoto et al., 2005). Additionally, the lower dose of MPH induced neuroinflammation, oxidative status, and increased the exploratory activity, with no major BBB dysfunction in the PFC (Chapter 4). On the contrary, in the hippocampus (Chapter 3) MPH was beneficial since it promoted astrocytic and neuronal plasticity, as well as memory enhancement. As abovementioned, there is a well-known regional heterogeneity of astrocytic morphology and function that can explain the neuroinflammation observed in the PFC, but not in the hippocampus. Noteworthy, mild inflammation and oxidative stress can be beneficial. Actually, reactive astrocytes play critical roles in neuroprotection, BBB repair, and regulation of inflammation (Burda and Sofroniew, 2014). In line with this, it was already stated that inflammation, oxidative and nitrogen stress mediate the beneficial effect of exercise on anxiety disorder symptoms and behaviors (Moylan et al., 2013).

The work present in this thesis is in accordance with the inverted U dose response triggered by MPH whereby low doses significantly improve cognition, while higher doses produce perseverative errors in many animals (Arnsten and Dudley, 2005). Cheng and collaborator (2014) provided a potential mechanism to explain the improvement of cognition by a low-dose of MPH, and the psychosis-inducing effects of a high-dose of MPH. The aforementioned work revealed that administration of a low-dose of MPH potentiates glutamate receptor NMDAR trafficking and function, enhances PFC-mediated cognition, whereas administration of a high-dose of MPH suppresses PFC glutamatergic transmission.

Collectively, this thesis provides a better understanding of the cellular effects of MPH. Results enlightened that MPH treatment may cause BBB dysfunction and NVU miscommunication under physiological conditions with behavioral repercussions. Moreover, chronic early-life exposure to high doses of MPH seems to be more risky than low doses. One of the most striking findings in this thesis was the opposite effect of MPH between physiological and pathological conditions, which demonstrate that a proper diagnosis and low doses are fundamental to promote brain homeostasis in ADHD. Clarifying CNS mechanisms of MPH action may contribute to prevent its misuse/abuse and to help in a more successful therapeutic accompaniment.

Conclusions

The results presented in this dissertation allowed drawing the following main conclusions:

- ✓ Acute low concentration of MPH increases the permeability of human brain microvascular endothelial cells by promoting caveolae transcytosis. This mechanism triggered by MPH involves the activation of GTPase Rac1 to assemble the NOX complex producing ROS. In turn, NOX-induced ROS generation activates c-Src, which then phosphorylates Cav1 promoting transcytosis via the caveolae-mediated transcellular permeability pathway in brain endothelial cells.

- ✓ Endothelium dysfunction and activation caused by MPH also promotes leukocytes transendothelial migration by vesicular transport.

- ✓ Chronic early-life exposure to higher doses of MPH in physiological conditions leads to BBB dysfunction by increasing vesicular transport and allowing peripheral immune cells to enter into CNS. This BBB hyperpermeability was coincident with astrocytic pathology, synaptic machinery aberration and memory impairment.

- ✓ On the contrary, low doses of MPH modulate hippocampal astrocytic and neuronal plasticity, which seems to be related with the activation of AKT/CREB pathways culminating in memory improvement.

- ✓ Under ADHD conditions, a low dose of MPH has a beneficial effect since it balances both glial response and inflammatory mediators, whereas promotes neurovascular alterations, neuroinflammation and oxidative stress under physiological conditions.

- ✓ A higher dose of MPH promotes BBB permeability and elicits anxious-like behavior in both control and ADHD animals.

- ✓ A parallelism between the impact of MPH regarding oxidative stress status periphery (serum) and brain (PFC) which can be useful to predict some central changes through peripheral indicators.

In summary, these results show that MPH can led to BBB alterations and alter brain immune quiescence particularly under physiological conditions. This highlights the importance of an appropriate MPH dose regimen for ADHD, and also that MPH misuse can have a negative impact in the brain.

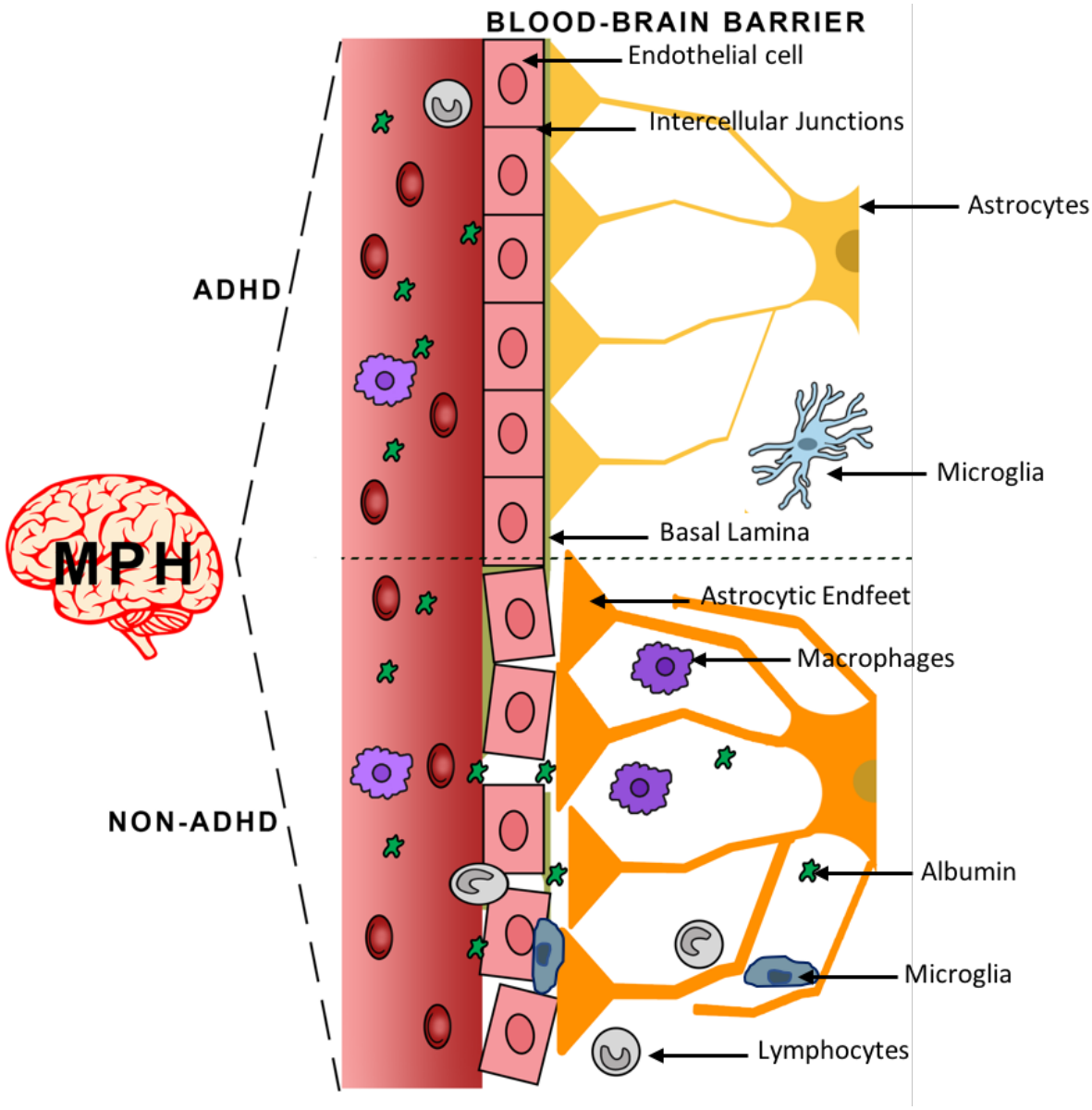

Figure 5.2. Schematic representation of the major findings achieved in the present thesis. *Under ADHD conditions, MPH has a beneficial effect since it balances both glial response and inflammatory mediators, whereas under physiological conditions MPH promotes neurovascular alterations, interfering with brain's privilege immunity and causing neuroinflammation. Legend: ADHD, attention deficit hyperactive disorder; MPH, methylphenidate.*

Chapter 6

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Pedra Filosofal

Eles não sabem que o sonho
é uma constante da vida
tão concreta e definida
como outra coisa qualquer,
como esta pedra cinzenta
em que me sento e descanso,
como este ribeiro manso
em serenos sobressaltos,
como estes pinheiros altos
que em verde e oiro se agitam,
como estas aves que gritam
em bebedeiras de azul.

eles não sabem que o sonho
é vinho, é espuma, é fermento,
bichinho álacre e sedento,
de focinho pontiagudo,
que fossa através de tudo
num perpétuo movimento.

Eles não sabem que o sonho
é tela, é cor, é pincel,
base, fuste, capitel,
arco em ogiva, vitral,
pináculo de catedral,
contraponto, sinfonia,
máscara grega, magia,
que é retorta de alquimista,
mapa do mundo distante,
rosa-dos-ventos, Infante,
caravela quinhentista,
que é cabo da Boa Esperança,
ouro, canela, marfim,
florete de espadachim,
bastidor, passo de dança,
Colombina e Arlequim,
passarola voadora,
pára-raios, locomotiva,
barco de proa festiva,
alto-forno, geradora,
cisão do átomo, radar,
ultra-som, televisão,
desembarque em foguetão
na superfície lunar.

Eles não sabem, nem sonham,
que o sonho comanda a vida,
que sempre que um homem sonha
o mundo pula e avança
como bola colorida
entre as mãos de uma criança.

António Gedeão, In *Movimento Perpétuo*, 1956