DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Impact of methamphetamine on astrocytes and endothelial cells: role of aquaporin-4

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Ana Paula Silva (Universidade de Coimbra) e do Professor Doutor Carlos B. Duarte (Universidade de Coimbra)

Ricardo Alexandre Gomes Leitão

2012

Agradecimentos

Após um ano de trabalho e muita aprendizagem e, também, de muita diversão porque a vida não é só trabalho. Chegou agora o momento de passar para o papel todo este ano, mas uma tese como esta não podia começar sem os devidos agradecimentos às pessoas que de alguma maneira me ajudaram a passar este último ano.

Não podia começar estes agradecimentos sem reconhecer todo o apoio e ensinamentos da Doutora Ana Paula Silva, já que sem eles este trabalho não tinha sido possível. Por, mais uma vez, me ter aberto o seu laboratório e a minha visão da, e para a, ciência. Queria ainda agradecer-lhe toda a confiança que depositou, e que continua a depositar, em mim.

Quero ainda agradecer à Professora Doutora Emília Duarte pelo apoio e sugestões ao longo destes dois anos do Mestrado.

À Vanessa pelo companheirismo ao longo deste ano. Pelo tempo que passámos e pela conversa que jogámos fora na sala de cultura. Por me aturar a mim e às minhas maluqueiras. Pela ajuda nos estudos de morte das bEnd.3.

Não podia deixar de agradecer também à Joana e à Sofia.

À Joana por tudo o que me ensinou, e continua a ensinar. Pelas dicas, truques e técnicas que me permitiram fazer um bom trabalho. Pelo apoio e conversas, umas sérias e outras nem tanto. Pelos "puxar de orelhas" que me deu, porque é também a eles que devemos o nosso crescimento. Por tão bem me ter acolhido no gabinete, deixando-me ser o seu braço direito. Pelos ensinamentos de vida que me deu.

À Sofia por todo o apoio. Pela ajuda quando tinha dúvidas. Pela confiança que depositou em mim, ao achar que era merecedor de algumas responsabilidades. Pelo fantástico último jantar que tivemos todos juntos. Pela herança que me deixou, a qual tão bem tenho cuidado, e a qual agradeço muito. E, claro, não podia deixar de fazer um agradecimento na sua nova língua

diária: «J'ai eu à faire de vous un petit merci. Merci pour tout lesoutien que j'ai cette année. J'espère que le texte n'a pasd'erreurs, si vous avez une excuse.»

À Andreia e à Rita por tão boa integração e recepção na “salinha”, que tanto me custou a abandonar. Pelos excelentes convívios que fizemos. Pelas conversas tão “profundas”. Pelos momentos de descontração ao seguir ao almoço e os cafés pela manhã. Pelos momentos triviais em que tanto gostávamos de falar em tricódigo.

Ao resto do pessoal do Laboratório de Farmacologia e do grupo Ocular and Brain Pharmacology por toda a ajuda que me deram durante este ano. E também pelos jantares e picnics que fizemos para descontrair, porque nem só de trabalho vive o Homem.

Ao pessoal do mestrado pelos convívios, saídas e momentos de estudo.

Aos meus amigos por todo o apoio que me deram, mesmo que indirecto ou à distância.

E claro não podia acabar estes agradecimentos sem fazer o devido reconhecimento aos meus pais. Sem os quais nada disto seria possível. Agradeço-lhes por todo o apoio financeiro. Pelo apoio incondicional, por me puxarem para cima nos momentos em que estava mais em baixo. Por sempre acreditarem em mim, e incentivarem-me a continuar a lutar pelos meus sonhos e objectivos e a nunca desistir perante qualquer adversidade.

Index

Abbreviations

AB	Arachnoid barrier
ACM	Astrocyte-conditioned medium
AJ	Adherens junction
ANOVA	Analysis of variance
AQP	Aquaporin
ATP	Adenosine-5'-triphosphate
BBB	Blood-brain barrier
BCFB	Blood-cerebrospinal fluid barrier
BEC	Brain endothelial cells
BRB	Blood-retinal barrier
BSCB	Blood-spinal cord barrier
CaMKII	Calcium/calmodulin dependent protein kinase II
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
Cu	Copper
DNA	Deoxyribonucleic acid
dUTP	2’-deoxy-uridine-5’-triphosphate
EC	endothelial cells
ER	endoplasmic reticulum
GABA	gamma-Aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fribillary acidic protein
GLUT-1	Glutamate transporter 1
GUK	Guanyl kinase-like domain
Hg	Mercury
i.p.	intraperitoneal
JAM	Junctional adhesion molecules
JAM	Junctional adhesion molecule
LPS	Lipopolysaccharide
MAGUK	Membrane-associated guanylate kinase-like protein

MDMA	3,4-methylenedioxymethamphetamine
METH	Methamphetamine
MMP	Matrix metalloproteinase
NOS	Nitric Oxide Synthase
OAP	Orthogonal arrays of particles
Pb	Lead
PBS	Phosphate-buffered saline
PKC	Protein Kinase C
PKG	cGMP-dependent protein kinase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S.E.M.	Standard error of the mean
TJ	Tight junction
TNF-α	Tumor necrosis factor alpha
TNF-α	Tumour necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labelling
VEB	Ventricular ependyma barrier
Zn	Zinc
ZO	Zonula occludens

Resumo

A metanfetamina (MET) é uma droga psicoestimulante altamente viciante cujo consumo na Europa tem vindo a aumentar nos últimos anos. Vários estudos têm demonstrado que o *stress* oxidativo, a excitotoxicidade, inflamação e a disfunção mitocondrial são alguns dos efeitos neurotóxicos da MET. Recentemente foi demonstrado que a MET compromete a função da barreira hemato-encefálica (BHE) e causa edema cerebral. A BHE é uma estrutura responsável por proteger o cérebro de compostos tóxicos, mas também permite a passagem de nutrientes e várias moléculas importantes para o parênquima cerebral. A função de barreira é determinada pelas células endoteliais, que em conjunto com os pericitos, astrócitos, lâmina basal, microglia e neurónios formam a unidade neurovascular.

Entre as diferentes regiões cerebrais, bem como entre o parênquima cerebral e a corrente sanguínea, ocorre constantemente um grande fluxo de água e perturbações na homeostasia da água podem ter efeitos prejudiciais na função cerebral. As aquaporinas (AQPs) são canais de água responsáveis pelo transporte de água através da BHE, sendo a AQP4 uma das mais importantes no Sistema Nervoso Central (SNC). A AQP4 é expressa nas extremidades dos astrócitos que estão em contacto com os vasos sanguíneos. Além disso, alterações na AQP4 podem levar à formação de edema cerebral devido a um aumento anormal do conteúdo de água no cérebro e um consequente inchaço cerebral. De facto, o edema cerebral tem sido observado em várias neuropatologias, incluindo em condições de consumo de MET.

Assim, o objetivo do presente trabalho foi avaliar se a MET induz alterações na expressão da AQP4 e qual o papel dos astrócitos na toxicidade induzida por MET em células endoteliais.

Os nossos resultados mostram que a MET leva a um aumento da expressão de AQP4 em culturas primárias de astrócitos, sem interferir com os níveis da proteína glial fibrilar acídica (GFAP - *Glial Fibrillary Acidic Protein*). Além disso, também testámos o efeito desta droga num modelo animal de intoxicação por MET (*binge*). Com este protocolo mostrámos que a MET induz um aumento da expressão da AQP4 no hipocampo e uma diminuição no córtex pré-frontal, mostrando que as diferentes regiões apresentam diferente suscetibilidade para a MET. No entanto, quando avaliámos o conteúdo de água no cérebro total não observámos alterações significativas.

Tendo em consideração o papel dos astrócitos na função da BHE, usámos para os estudos seguintes uma linha celular de células endoteliais de murganho (bEnd.3). Foi possível concluir que os meios condicionados de astrócitos (MCA) protegeram as células endoteliais (CEs) da morte celular induzida por MET (3 mM). No entanto, quando expusemos as CEs aos MCA

recolhidos de células expostas a concentração não tóxicas de MET (1 μM e 50 μM), observámos uma diminuição da expressão das proteínas das junções oclusivas (JOs), ocludina e claudina-5, mas não se verificou nenhum efeito na condição MCA controlo.

Em suma, os nossos resultados mostram que a MET causa alterações na expressão da AQP4 quer em culturas primárias de astrócitos quer no hipocampo de murganhos. Além disso, os astrócitos numa situação controlo parecem proteger as células endoteliais da morte celular induzida por MET, mas quando previamente expostos a MET os astrócitos provavelmente libertam fatores que interferem negativamente com a expressão de proteínas da JOs o que em última análise pode aumentar a permeabilidade da barreira.

Palavras-chave: metanfetamina, barreira hemato-encefálica, astrócitos, aquaporina-4, células endoteliais

Texto escrito conforme o Acordo Ortográfico - convertido pelo Lince.

Abstract

Methamphetamine (METH) is a potent and highly addictive psychostimulant which consumption in Europe has been increased over the last years. Several reports have demonstrated that oxidative stress, excitotoxicity, inflammation and mitochondrial dysfunction are some of the neurotoxic features of METH. More recently, it was shown that METH can also compromise the blood-brain barrier (BBB) function and cause cerebral edema. BBB is a structure responsible for protecting the brain from toxic compounds, but nevertheless allows the passage of nutrients and several important molecules into the brain parenchyma. The barrier function is determined by the endothelial cells, that together with pericytes, astrocytes, basal lamina, microglia and neurons form the neurovascular unit.

Large water fluxes continuously take place between the different compartments of the brain, as well as between the brain parenchyma and the blood. Disturbances in this well-regulated water homeostasis may have deleterious effects on brain function. Aquaporins (AQPs) are water channels that contribute to water transport across BBB, being AQP4 one of the most important at the Central Nervous System (CNS). AQP4 is express on astrocytic end-feet in contact with brain vessels. Moreover, alterations in AQPs can originate cerebral edema due to abnormally increased water content and consequent brain swelling. Indeed, brain edema has been observed in several neuropathologies, including under conditions of METH consumption.

Therefore, the aim of the present work was to investigate if METH induces alterations in the expression of AQP4 and the role of astrocytes against METH-induced toxicity of endothelial cells.

Our results show that METH leads to an increase of AQP4 expression in primary cultures of astrocytes, without interfering with the glial fibrillary acidic protein (GFAP) levels. Furthermore, we also tested the effect of the drug in an animal model of METH intoxication (binge paradigm). With this protocol we showed that METH leads to an increase of AQP4 expression in the hippocampus and to a decrease in the frontal cortex, demonstrating that different brain regions present different susceptibilities to METH. However, when the water content of whole brain was measured we did not observe significant alterations.

Taking into consideration the role of astrocytes in the BBB function, we further used a mouse brain endothelial cell line (bEnd.3). It was possible to conclude that astrocyte-conditioned medium (ACM) was able to protect endothelial cells (ECs) against METH-induced cell death (3 mM). However, when we exposed the ECs to ACM collected from cells exposed to non-toxic METH concentrations (1 μM and 50 μM), we observed that both ACM METH conditions caused

a decreased in the expression of the tight junction proteins (TJs), occludin and claudin-5, without any effect of the ACM control.

Overall, our results show that METH causes an increase in AQP4 expression in both primary cultures of astrocytes and in mice hippocampus using an acute METH administration protocol. Moreover, astrocytes in a control situation seem to protect the endothelial cells from METH-induced cell death, but when previously exposed to METH they probably release some factors that negatively interfere with the expression of TJs proteins that ultimately may increase barrier permeability.

Keywords: methamphetamine, blood-brain barrier, astrocytes, aquaporin-4, endothelial cells

CHAPTER 1

INTRODUCTION

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1.1. METHAMPHETAMINE

1.1.1. METHAMPHETAMINE CONSUMPTION

Methamphetamine (METH) is a psychostimulant compound that belongs to the amphetamine class of drugs, along with amphetamine and ecstasy (or 3,4-methylenedioxymethamphetamine, MDMA), which has great similarity with the neurotransmitter dopamine (figure 1). METH can be consumed in various ways such as smoked, the most commonly, but can also be injected, snorted and taken orally (Winslow 2007; Nakama 2008). Although METH is drug of abuse, it is also approved by the FDA to treat attention deficit hyperactivity disorder and extreme obesity (Kish, 2008).

According to the last United Nations World Drug Report the worldwide amphetamines consumption is between 14 and 52.5 million among people aged 15-64, which represents 0.3% to 1.2% of annual prevalence, making METH the second most widely used drug globally (United Nations Office on Drugs and Crime, 2012). Concerning the consumption in Portugal, the last report is from “Instituto da Droga e da Toxicodepência, 2009” which showed that in 2007, 0.9% of total population or 1.3% of young adult, was amphetamine consumer. However,

this report includes the group of amphetamines, excluding ecstasy, which means that there are no national statistics of detailed consumption of METH.

Immediately after consumption, METH leads to euphoria, alertness, wakefulness, increased activity (Quinton et al., 2006; Kish et al., 2008), hyperthermia, and decrease in appetite (Yamamoto *et al.*, 2010). Moreover, at long-term this drug originates anxiety, confusion, insomnias, mood disturbances, weight loss, among others (Buchanan *et al.*, 2010). Indeed, chronic METH abusers can experience psychotic and violent behavior, impaired verbal learning and memory, visual and auditory hallucinations, delusions, and even seizures (Quinton *et al.*, 2006; Ramirez *et al.*, 2009, Yamamoto *et al.*, 2010; Buttner, 2011).

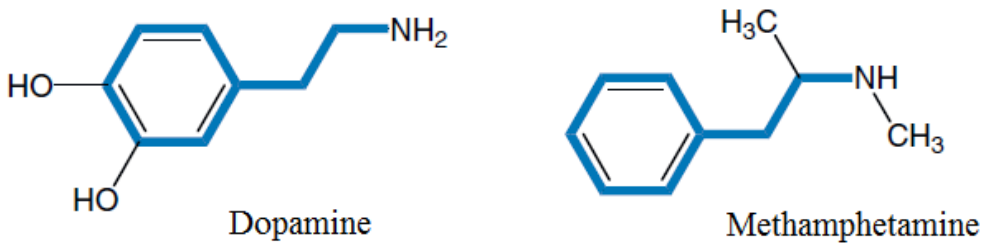

Figure 1. The chemical structure of dopamine and methamphetamine clearly shows the similarity between both (adapted from Fleckenstein et al., 2007).

1.1.2. THE EFFECTS OF METHAMPHETAMINE IN THE CNS: A QUICK OVERVIEW

At the cellular level, when this drug is present in the extracellular space it can enter into the cell by two ways: diffuse transport due to lipophilic characteristics of METH or *via* membranar dopamine transporter. Due to its characteristics, METH can also enter the mitochondria or other cellular compartments. More specifically, it can be stored in vesicles containing dopamine by a mechanism similar to that above mentioned (Yamamoto *et al.*, 2010). With the

increase of METH inside of these vesicles, there will be an increase of dopamine release from those vesicles into the cytoplasm (figure 2), which consequently causes oxidative stress accompanied by production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Moreover, the increase of dopamine in the cytoplasm can reverse the membrane transporters leading to a great release of dopamine into the synaptic cleft, which in the case of neurons can cause an overactivation of postsynaptic neurons (figure 2; Kish, 2008).

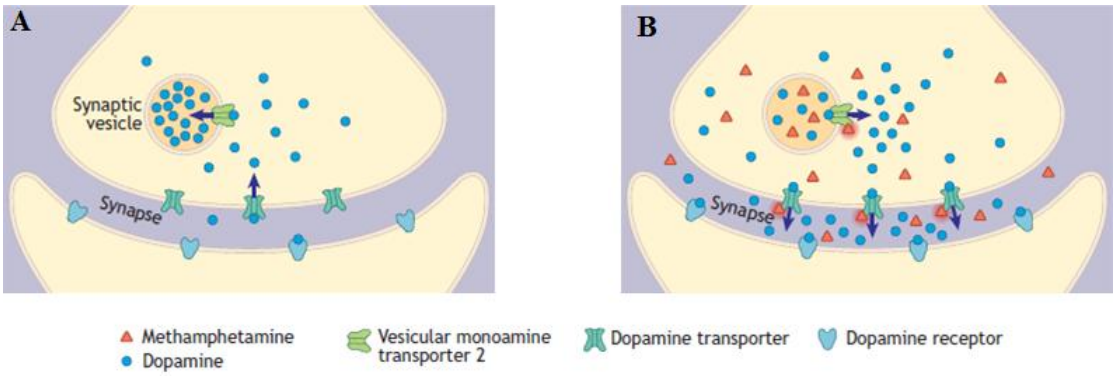

Figure 2. Effect of METH on neuronal dopaminergic system. Methamphetamine is able to cross neuronal membranes, leading to a release of dopamine from storage vesicles, which consequently reverse the membrane transporter and dopamine is released to the synapse. A – control situation; B – after METH exposure (adapted from Kish, 2008).

METH is also capable of inhibit the dopamine uptake (Rothman *et al.*, 2001) leading to an increase in cytoplasmic and extracellular levels of dopamine, serotonin and norepinephrine, which can cause an increase in the production of ROS and RNS (Yamamoto *et al.*, 2008; Kish, 2008; Dietrich, 2009). This increase in oxidative stress has already been described either in human brain microvascular endothelial cells (Ramirez *et al.*, 2009) and *in vivo* accompanied by an increased in lipid peroxidation in rat striatum (Yamamoto *et al.*, 1998). Moreover, METH is able to inhibit glucose uptake in neurons and astrocytes (Muneer *et al.*, 2011), as well as to

inhibit mitochondrial electron transport chain enzyme complexes, with consequent inhibition of mitochondrial function (Quinton *et al.*, 2006; Yamamoto *et al.*, 2010).

Some cellular effects of METH can also be due to an interaction with the glutamatergic system. In fact, METH can lead to an increase of glutamate release (Mark *et al.*, 2004), with a consequent increase in intracellular calcium levels and activation of several kinases and proteases resulting in the disruption of cytoskeleton and formation of ROS (figure 3; Cadet *et al.*, 2007). These observations are supported by some previous works showing that pretreatment with glutamate receptor antagonists can prevent METH neurotoxicity (review in Yamamoto *et al.*, 2008). It was also described by several authors that METH triggers a neuroinflammatory process (Yamamoto *et al.*, 2008; Gonçalves et al., 2010) characterized by astrogliosis in the cortex, hippocampus and striatum (Pubill *et al.*, 2003; Thomas *et al.*, 2004; Sharma *et al.*, 2009), as well as microglial activation in striatum and caudate-putamen (Pubill *et al.*, 2003; Thomas *et al.*, 2004; Bowyer *et al.*, 2008). Similar results were observed in humans (Sekine, *et al.*, 2008; Kitamura *et al.*, 2010). Moreover, neuronal dysfunction or/and death, has already been demonstrated in mice and rats exposed to METH, and in several brain regions such as the hippocampus (Gonçalves *et al.*, 2010) and caudate-putamen (Bowyer *et al.*, 2008) in mice, and neocortex and limbic system in rats (Kuczenski *et al.*, 2007).

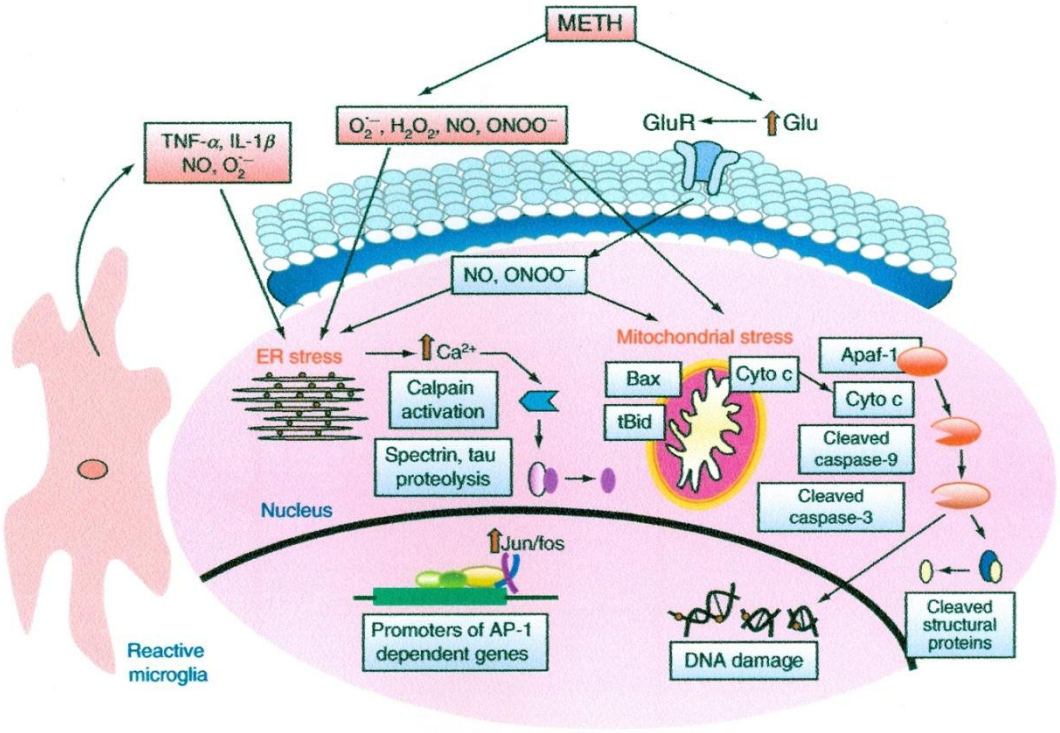

Figure 3. Scheme summarizing the cytotoxicity mechanisms triggered by METH. After drug exposure there is an increase in neuronal glutamate release, as well as an increase in the levels of neuronal and glial reactive oxygen and nitrogen species (ROS and RNS, respectively), and cytokines, such as tumor necrosis factor α (TNF- α) and interleukins. The intracellular effects of a METH exposure include endoplasmic reticulum stress (ER stress) and mitochondrial stress. The ER stress is followed by release of calcium, activation of calpain and proteolysis of cytoskeleton proteins, such as tau and spectrin. The mitochondrial stress is characterized by the involvement of pro-apoptotic proteins, such as Bax and tBid, release of cytochrome C (Cyto c), and activation of caspases. At the nuclear level, METH can damage the DNA and increase the c-Jun and c-fos (Jun/fos) polymerization, which form the activator protein 1 transcription factor (AP-1). In turn, this can lead to the expression of several genes involved in differentiation, proliferation and apoptosis (adapted from Cadet and Krasnova, 2009).

1.2. BLOOD-BRAIN BARRIER: FUNCTION AND STRUCTURE

Blood-brain barrier (BBB), which is one of the blood-neural barriers, is present in almost all brain regions, except in the brain ventricular system, also denominated circumventricular organs, which are responsible for the regulation of autonomic nervous system and endocrine glands (Ballabh *et al.*, 2004; Cardoso *et al.*, 2010). The BBB is formed by endothelial cells, pericytes, basal lamina, astrocytes, and also microglia and neurons (figure 4), which gives raised to the new concept of Neurovascular Unit.

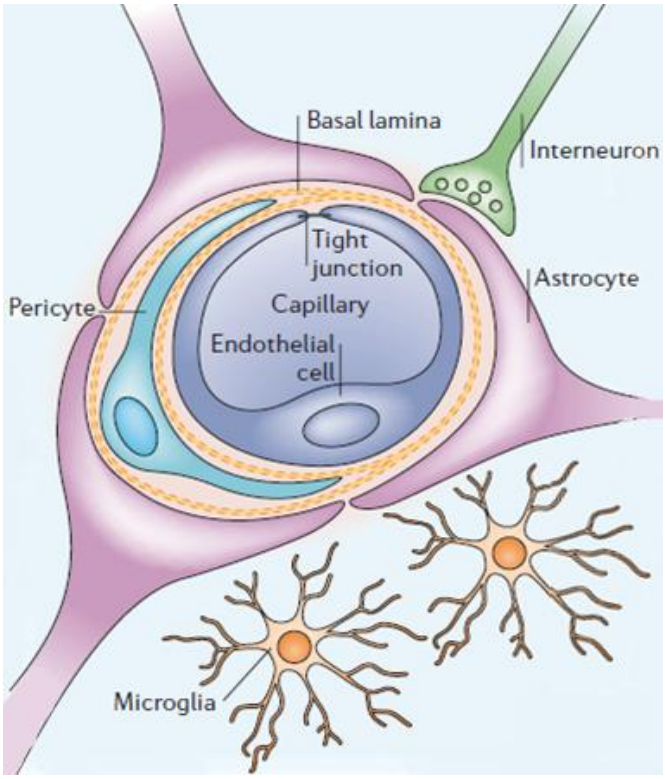

Figure 4. Neurovascular Unit. Endothelial cells and pericytes are surrounded by basal lamina. More recently, it has been described an important role for

astrocytes, microglia and neurons in BBB function (adapted from Abbott *et al.*, 2006)

The BBB maintains the brain homeostasis and protects the brain against toxic compounds and blood fluctuations, but simultaneously provides nutrients essential for the normal brain function (Abbott *et al.*, 2006; Kim *et al.*, 2006; Dietrich, 2009; Cardoso *et al.*, 2010). The transports across the BBB are limited and highly selective and generally we can divide into paracellular and transcellular routes (Figure 5). The first is controlled by tight junctions between adjacent endothelial cells, whereas the second is mainly due to the presence of a complex transport system and selective receptors (Abbott *et al.*, 2006; Cardoso *et al.*, 2010). Water soluble agents go into the brain *via* tight-junctions, and ions, solutes and small lipophilic molecules cross BBB by diffuse transport according to the concentration gradient (Ballabh *et al.*, 2004; Cardoso *et al.*, 2010). The transporters are very important to allow the entrance of small and large hydrophilic molecules and nutrients into the brain, and also to eliminate cellular wastes. This system involves ATP consumption and is composed by a variety of carriers, such as glucose-transporter 1 (GLUT1), amino acids transporters (LAT1), ATP-biding cassettes (ABC) family of transporters, P-glycoprotein (Pgp) and multidrug resistance-related proteins (MRP) (Abbott *et al.*, 2006; Kim *et al.*, 2006; Cardoso *et al.*, 2010). Regarding large hydrophilic molecules, like peptides and proteins, the transport is done by receptor-mediated endocytosis. Proteins, such as transferrin, insulin and leptin, have specific receptors, whereas, positively charged molecules like albumin and histone are transported by adsorptive-mediated endocytosis. However, under normal conditions, brain endothelial cells have less endocytic activity than non-cerebral endothelial cells (Ballabh *et al.*, 2004; Abbott *et al.*, 2006; Kim *et al.*, 2006).

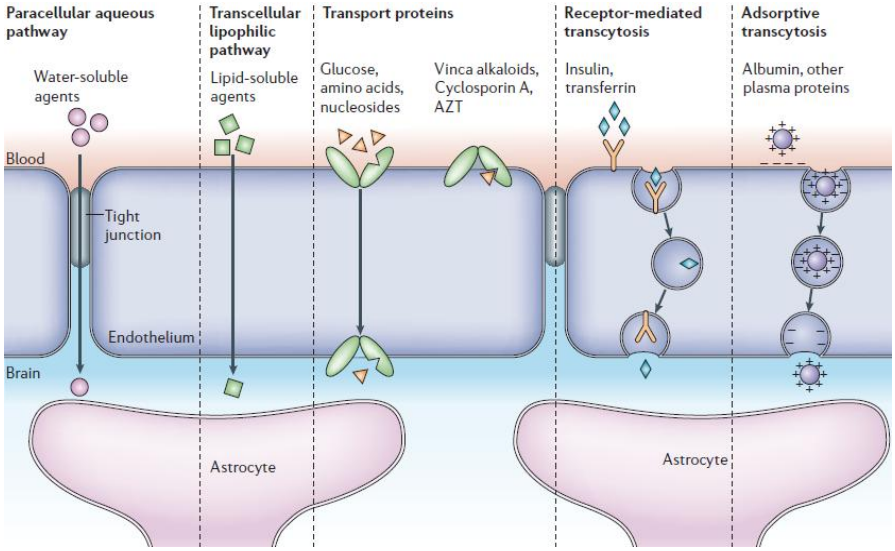

Figure 5. Routes across blood-brain barrier. The transporters of proteins and transcellular pathway are important to suppress the nutritional needs. The paracellular transport is relevant for ionic homeostasis. Transcytosis may also occur under physiological conditions, but at a low level (adapted from Abbott *et al.*, 2006).

1.2.1. COMPONENTS OF THE NEUROVASCULAR UNIT

1.2.1.1. ENDOTHELIAL CELLS

The brain endothelial cells (BECs) are the major cellular component of the BBB, and have unique characteristics that differentiate them from other endothelial cells, such as more active metabolism, more mitochondrial quantity and activity, presence of transporters, lower pinocytosis activity and also absence of fenestrations (Ballabh *et al.*, 2004; Kim *et al.*, 2006; Cardoso *et al.*, 2010). These features provide to BECs the capacity to import and export different types of molecules, such as nutrients and toxic compounds, respectively, and also to provide energy to all transport systems (Cardoso *et al.*, 2010). Between adjacent BECs there

are intercellular complexes - tight and adherens junctions - which seal the brain microvasculature forming the primary structure of BBB.

1.2.1.2. PERICYTES

Pericytes, also known as vascular smooth muscle cells, have contractile proteins which give them the capacity to regulate blood flow providing structural stability to brain microvasculature (Ballabh *et al.*, 2004; Kim *et al.*, 2006; Cardoso *et al.*, 2010). These cells seem to be important to the maintenance of BBB basal lamina since they produce and release components of the basal lamina, such as proteoglycans (Cardoso *et al.*, 2010). Communication of pericytes with BEC occurs *via* gap and tight junctions (Kim *et al.*, 2006, Cardoso *et al.*, 2010), and seems to be important for the maintenance of microvasculature and regulation of blood flow.

1.2.1.3. BASAL LAMINA

The basal lamina is the acellular component of BBB composed predominantly of collagen type IV, laminin, fibronectin and proteoglycans (Kim *et al.*, 2006; Cardoso *et al.*, 2010). This structure is important for anchoring endothelial cells and pericytes, and for connecting these cells with other neighbour cells by cell adhesion molecules (Cardoso *et al.*, 2010).

1.2.1.4. ASTROCYTES

Astrocytes, which are one type of glial cells, play an important role in the formation and maintenance of BBB (Ballabh *et al.*, 2004; Abbott *et al.*, 2006; Kim *et al.*, 2006) and, indeed, one of their functions is to connect BECs with close neurons (Kim *et al.*, 2006). Nevertheless, astrocytes can have a dual role. Regarding the protective effect, they release trophic and soluble factors important for neuronal survival and BBB formation and maintenance (Ballabh

et al., 2004), and are also responsible for the reuptake and metabolism of some neurotransmitters, more precisely glutamate and gamma-Aminobutyric acid (GABA) (figure 6) (Abbott *et al.*, 2006; Allaman *et al.*, 2011). Also, there are some studies clearly showing that astrocytes are important to neurogenesis and synaptogenesis (reviewed in Kim *et al.*, 2006), and to the formation of tube-like structures (Ramsauer *et al.*, 2002; Hawkins *et al.*, 2005). Astrocytes are also involved in brain water homeostasis since they are the major cells that express water channels, denominated aquaporins, in the brain (Abbott *et al.*, 2006; Nag *et al.*, 2009).

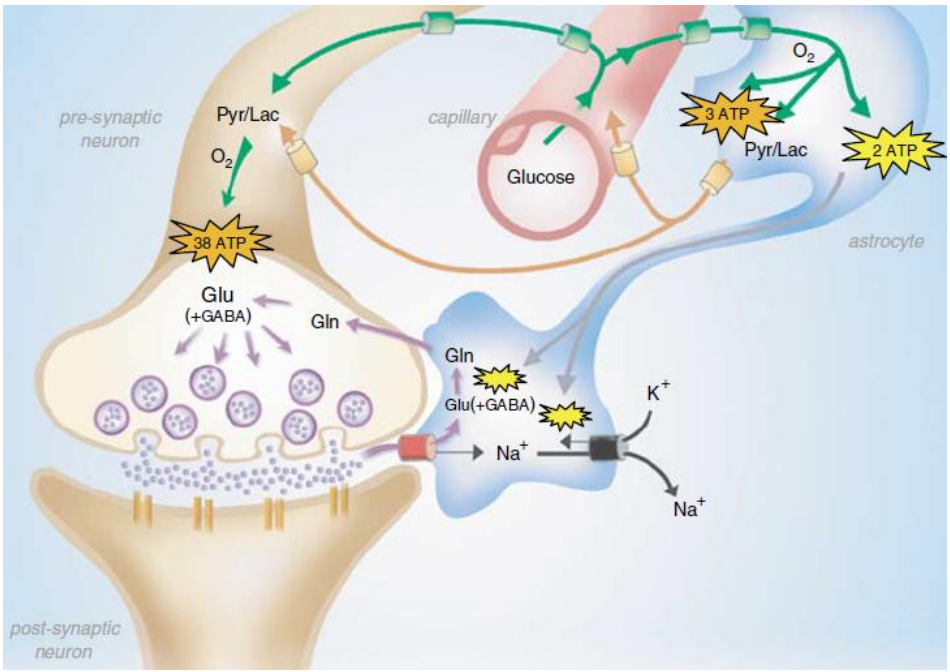

Figure 6. Role of astrocytes in glutamate and GABA metabolism. These cells are responsible for the reuptake of glutamate and GABA, and then metabolize them to glutamine which is released back to neurons (adapted from Hyder *et al.*, 2006). GABA: gamma-Aminobutyric acid

On the other hand, astrocytes can release proinflammatory molecules, such as interleukin-1beta (IL-1β) and tumour necrosis factor-alpha (TNF-α) (Abbott, 2002; Abbott *et al.*, 2006)

(figure 7), as well as high concentrations of calcium to the extracellular space leading to excitotoxicity (Rossi *et al.*, 2009). Additionally, astrocytes can also produce and release matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases capable of extracellular matrix degradation, such as basal lamina and tight junctions in the BBB (Rosenberg *et al.*, 2002).

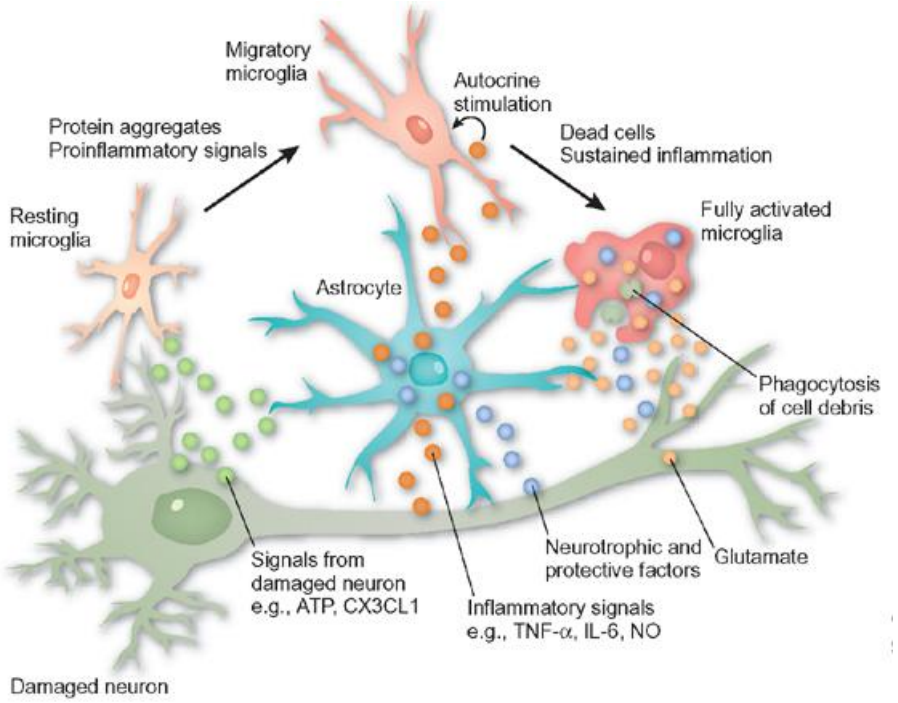

Figure 7. Astrocytic and microglial response to damaged neuron. When there is an insult, astrocytes and microglia are recruited to the lesion area. The recruited astrocytes are able to release both trophic factors and inflammatory molecules (adapted from Monk *et al.*, 2006).

1.2.1.5. MICROGLIA

Microglia is the major component of the brain immune system, and can present itself in three different phenotypes: surveying (resting) microglia, with small bodies; alerted microglia, with longer processes; and reactive microglia with phagocytic activity, (figure 8) (Hanisch and

Kettenmann, 2007). Importantly, resting microglia is responsible for the supervision of the brain microenvironment (Ransohoff *et al.*, 2009). Activation of microglia can be triggered by several stimuli, such as inflammatory molecules release by astrocytes, increase in extracellular glutamate and blood serum molecules which can be a signal of BBB disruption (Ransohoff *et al.*, 2009). Recently, these cells were found in the surroundings of BBB which suggest their participation in the barrier function (Choi *et al.*, 2008). Microglia has been widely related with neuroinflammation, particularly due to their capacity to release inflammatory and cytotoxic molecules, such as TNF-α (Choi *et al.*, 2008).

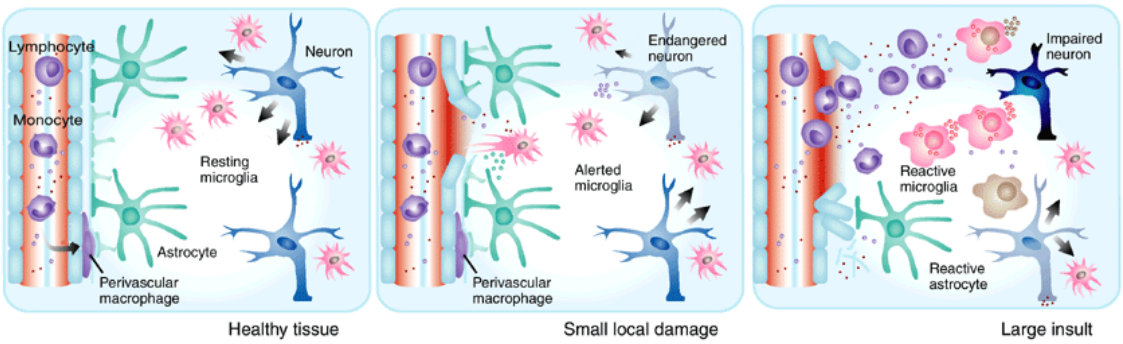

Figure 8. Schematic representation of the microglia phenotypes. In healthy tissue the microglia remains its surveying (resting) state, but when a small damage happens in the brain tissue these cells became alert. In the last situation when the brain is exposed to strong or prolonged damage the microglial cells acquire its reactive phenotype (adapted from Hanisch and Kettenmann, 2007).

1.2.1.6. NEURONS

Neurons are considered the major and more important cells in the brain. Regarding BBB, neurons can regulate blood flow by local neuronal activation (Kim *et al.*, 2006; Cardoso *et al.*, 2010) since BBB can be innervated by noradrenergic, serotonergic, cholinergic, and GABAergic neurons (Hawkins *et al.*, 2005). Although this important role of neurons, they are not directly connected to BECs, and required astrocytes to mediate this link (Kim *et al.*, 2006).

1.2.2. INTERCELLULAR JUNCTIONS

1.2.2.1. TIGHT JUNCTIONS

Tight junctions (TJs) are formed by several proteins present between adjacent BECs that are responsible for the structure and organization of BBB. TJs act as physical barrier by limiting the paracellular transport, and also as membrane domain barrier, dividing apical and basal domains (Abbott *et al.*, 2006; Kim *et al.*, 2006; Cardoso *et al.*, 2010). This polarity can be seen in transporter systems.

The TJ proteins can be transmembranar, such as occludin, claudins and junctional adhesion molecules (JAMs), and cytoplasmic, like zonula occludens (ZO) and cingulin (figure 9). Moreover, transmembranar proteins can be divided into two groups: claudins/occludin, which have four transmembrane domains, and JAM, with one transmembrane domain (Cardoso *et al.*, 2010). The cytoplasmic proteins are important to mediated interactions between transmembrane proteins and actin cytoskeleton (Mahajan *et al.*, 2008).

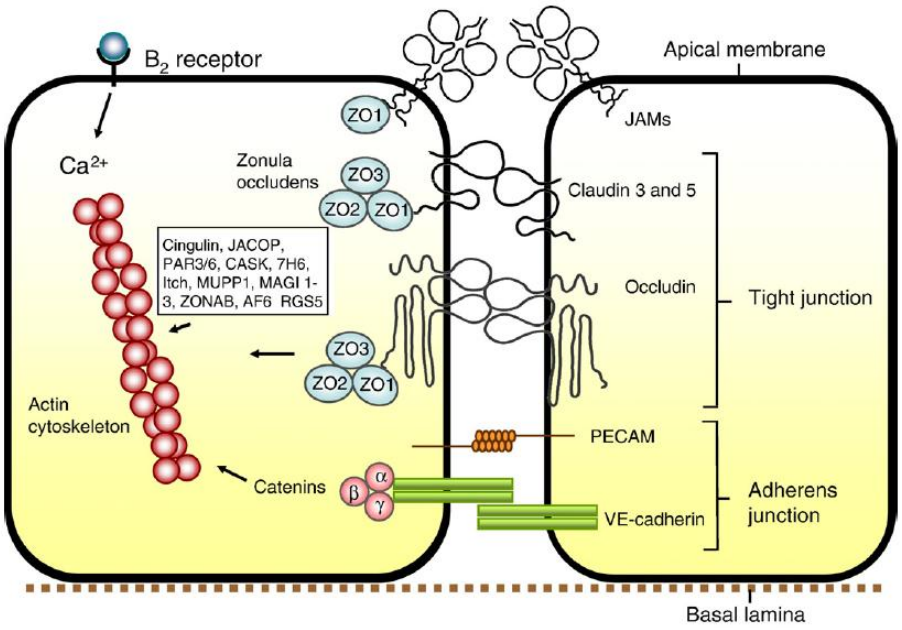

Figure 9. Structure of tight and adherens junctions. The tight junctions (TJs) are formed by occludin and claudins, and by accessory proteins of zonula occludens family. Adherens junctions (AJs) are formed by cadherin and accessory proteins catenins. Accessory proteins of both tight and adherens junctions are able to connected the transmembrane proteins with actin cytoskeleton (adapted from Abbott *et al.*, 2010)

1.2.2.2. OCCLUDIN

Occludin is a phosphoprotein with 65 kDa (Ballabh *et al.*, 2004; Abbott *et al.*, 2006) with four transmembrane domains and two extracellular loops. The first extracellular loop mediates Ca^{2+}-independent adhesion, although ZO-1 presence is required (Cardoso *et al.*, 2010). The cytoplasmic domain is responsible for the association between this protein and cytoskeleton, mediated by accessory proteins, like ZO-1 (Ballabh *et al.*, 2004; Abbott *et al.*, 2006). This protein is responsible for the high electrical resistance of the BECs contributing also for BBB stabilization. Nevertheless, recent data showed that occludin is not fundamental for TJ organization and function (Hawkins *et al.*, 2005; Abbott *et al.*, 2006; Persidsky *et al.*, 2006; Cardoso *et al.*, 2010).

1.2.2.3. CLAUDINS

Claudins are a family of proteins with, at least so far, 24 members. Claudin-1 and -5 are the most important in brain microvascular TJs and these phosphoproteins with 20-22 kDa have four transmembrane domains (Ballabh *et al.*, 2004; Cardoso *et al.*, 2010). The domain responsible for the connection with actin cytoskeleton is the cytoplasmic and involves the ZO proteins (Ballabh *et al.*, 2004). In fact, occludin and claudins show strong structural homology but with no amino acid sequence homology (Ballabh *et al.*, 2004). The literature reports that claudins are the most important TJs protein, and forms the primary seal of TJs by homotypically connection with claudins in the adjacent BEC (Ballabh *et al.*, 2004; Cardoso *et al.*, 2010). Claudin-3 and -5 are associated with BBB integrity, contributing to lower permeability and also high transendothelial electrical resistance (Abbott *et al.*, 2006; Mahajan *et al.*, 2008; Cardoso *et al.*, 2010).

1.2.2.4. JUNCTIONAL ADHESION MOLECULES

Junctional adhesion molecules (JAM-1, -2, and -3) have a molecular weight of 40 kDa, one transmembrane domain and one large extracellular domain with two loops (Hawkins *et al.*, 2005; Cardoso *et al.*, 2010). These proteins belong to the immunoglobulin family and are important in cell-to-cell adhesion contributing to BBB maintenance and stabilization (Abbott *et al.*, 2006). JAM-1 and -3 are expressed mainly in cerebral vessels (Ballabh *et al.*, 2004; Cardoso *et al.*, 2010) and do not need accessory proteins to bind with cytoskeleton but can be linked to ZO-1 protein (Mahajan *et al.*, 2008).

1.2.2.5. CYTOPLASMIC PROTEINS

This group of accessory proteins includes zonula occludens proteins (ZO-1, -2 and -3), cingulin, 7H6 and others (Ballabh *et al.*, 2004). ZO proteins are found in submembranous region and mediate the connection between claudin or occludin with actin cytoskeleton (Kim *et al.*, 2006). These proteins belong to the membrane-associated guanylate kinase-like protein (MAGUK) family (Ballabh *et al.*, 2004, Cardoso *et al.*, 2010), and share between them several characteristics such as three PDZ domains, one SH3 domain and one guanyl kinase-like (GUK) domain (Ballabh *et al.*, 2004). One of the PDZ domains, the PDZ1, is important to link claudin, whereas the GUK domain links occludin (Ballabh *et al.*, 2004).

ZO-1 is a 220 kDa phosphoprotein, and seems to be one of the most important ZO proteins (Kim *et al.*, 2006; Cardoso *et al.*, 2010), because its loss leads to an increase in permeability and decrease in tightness of the BBB (Choi *et al.*, 2008; Cardoso *et al.*, 2010). ZO-2 is a 160 kDa phosphoprotein that shows a great homology in amino acid sequence and structure with ZO-1 (Ballabh *et al.*, 2004; Cardoso *et al.*, 2010). ZO-2 acts also as a transcription factor (Persidsky *et al.*, 2006; Cardoso *et al.*, 2010). Regarding ZO-3, it is a 130 kDa protein with homology to ZO-1 and -2, and some studies have suggested that ZO-3 is capable to directly bind occludin and ZO-1 (Cardoso *et al.*, 2010).

1.2.2.6. ADHERENS JUNCTIONS

These proteins are present in the basal side of BEC, and so they regulate the paracellular transport and BECs adhesion (Hawkins and Davis, 2005). The adherens junctions (AJs) are formed by cadherin and catenin, the membrane and accessory protein, respectively (Perrière *et al.*, 2007) (Figure 9). In fact, cadherin is a transmembranar glycoprotein responsible for Ca^{2+}-dependent cell-cell adhesion (Cardoso *et al.*, 2010). This molecule establishes homophilic interactions with cadherins present in adjacent BECs (Ballabh *et al.*, 2004). Catenin is an

accessory protein that mediates the connection between cytoplasmic domain of cadherins and actin cytoskeleton (Ballabh *et al.*, 2004; Cardoso *et al.*, 2010). However, catenins are not essential to AJ formation but for its stabilization (Cook *et al.*, 2008), and there are four types of catenins: α-, β-, δ-, and γ-catenin (Cook *et al.*, 2008; Cardoso *et al.*, 2010).

1.2.3. OTHER BARRIERS AT THE CNS

In the CNS there are other barriers, such as the blood-cerebrospinal fluid barrier, arachnoid barrier, ventricular ependyma barrier, blood-retinal barrier and blood-spinal cord barrier (figures 10 and 11). Regarding brain barriers, the blood-cerebrospinal fluid barrier (BCFB; figure 10) is present in choroid plexus and it is important for the production and secretion of cerebrospinal fluid (CSF), which are related with protection of brain and spinal cord. The capillaries of this barrier are fenestrated and the barrier forming cells are the epithelial cells. In BCFB, TJs proteins like claudins, occludin and ZO-1 are also present but regarding claudins they differ from those in BBB because whereas claudins 1, 2 and 11 are present in BCFB, in the BBB there are claudins 1, 5 and 11 (Wolburg *et al.*, 2001; Ballabh *et al.*, 2004). The arachnoid barrier (AB; figure 10) is present in the meninges and is the least studied of all brain barriers. The barrier function of AB is formed by cells present in arachnoid membrane since the blood vessels are fenestrated. The AB function is to separate the subarachnoid space from the brain parenchyma (Saunders *et al.*, 2008). The ventricular ependyma barrier (VEB; figure 10), also denominated fetal CSF-brain barrier, is a temporary barrier and only exist during the early developing brain being only permeable to small molecules like sucrose. In adult ependyma the VEB does not exhibit a restriction regarding passage of molecules between CSF and brain tissue (Saunders *et al.*, 2008).

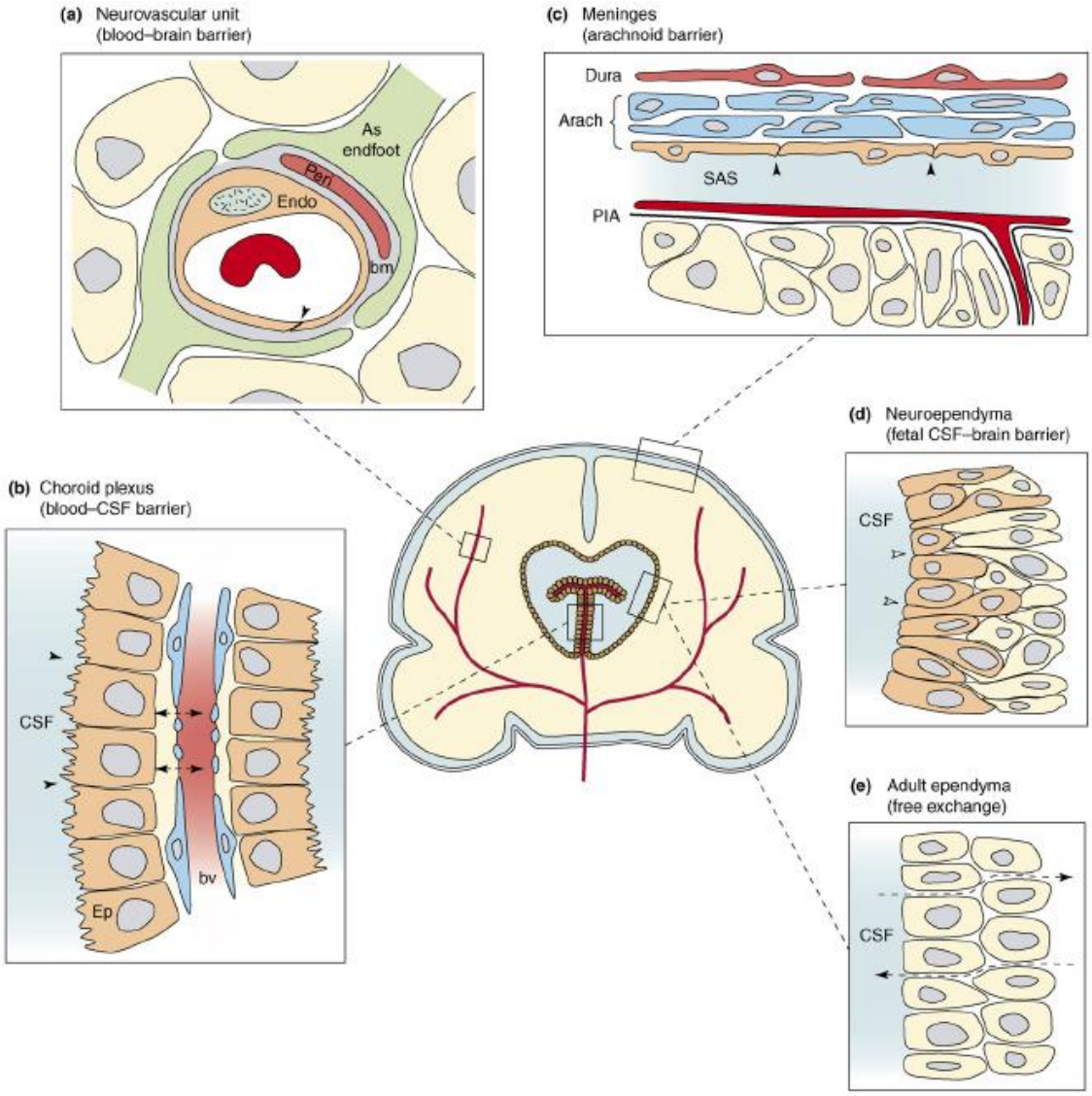

Figure 10. Schematic representation of barriers present in the brain. (a) The blood-brain barrier (BBB) separates the blood from de cerebral parenchyma, and is constituted by endothelial cells (endo), pericytes (peri), basal lamina (bm) that embrace endothelial cells and pericytes, and astrocytes (As endfoot) are also present and surround the brain microvessels. **(b)** The blood-cerebrospinal fluid barrier (blood-CSF barrier), with fenestrated capillaries, is located in the choroid plexus and is responsible for the production of cerebral spinal fluid. **(c)** The arachnoid barrier (AB) is present in the meninges and the blood vessels in arachnoid (Arach) and pial surface (PIA) has tight junctions similar to that found in BBB, however the vessels present in dura are fenestrated. **(d, e)** The ventricular ependyma barrier (VEB) differ in developing brain (d; fetal CSF-brain barrier) with

high tightness compared to adult ependyma (e) which allows a free passage between CSF and brain parenchyma (adapted from Saunders, *et al.*, 2008).

The blood-retinal barrier (BRB; figure 11) is important to deliver oxygen and nutrients to retina, and can be divided in two barriers: the inner and outer blood-retinal barrier. The outer BRB is formed by retinal epithelium with fenestrations, unlike the BBB, and the inner BRB by retina microvessels surrounded by pericytes and astrocytic end-feet (Choi *et al.*, 2008). The blood-spinal cord barrier (BSCB; figure 11) is much similar to BBB, since it is also formed by microvessels surrounded by pericytes and astrocytes end-feet. The only differences between these two barriers are that BSCB has glycogen deposits and an increased permeability when compared to BBB (Choi *et al.*, 2008).

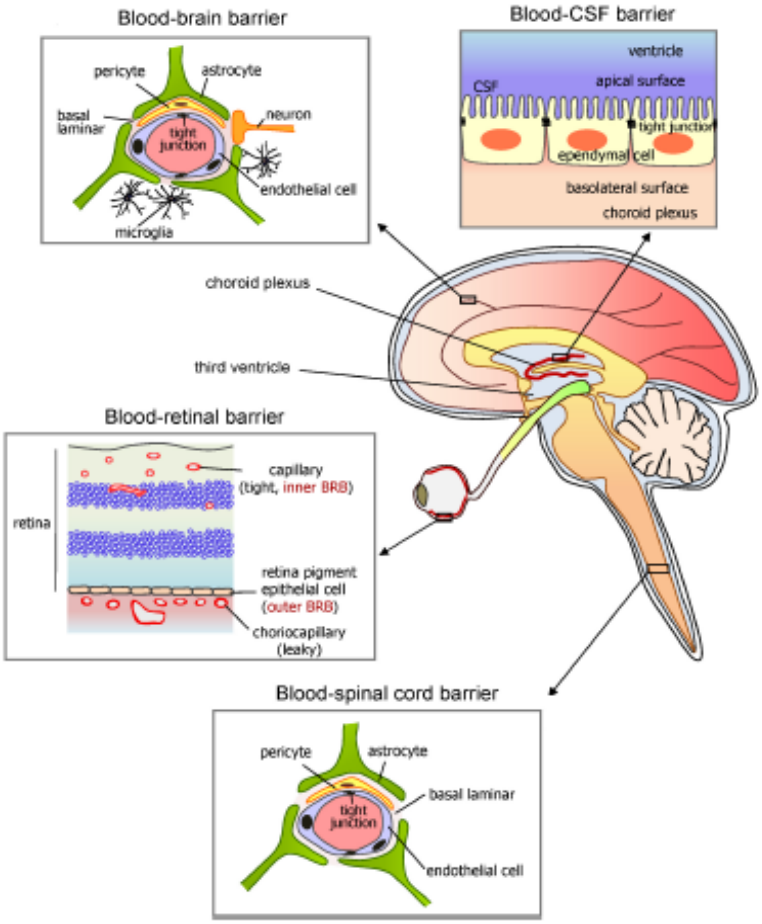

Figure 11. The four blood-neural barriers found in the CNS. There are four different types of barriers. The blood-brain barrier (BBB) separates the blood from de cerebral parenchyma. The ependymal cells present in the blood-cerebrospinal fluid barrier are responsible for production of the cerebral spinal fluid. In this barrier the brain vessels are fenestrated which makes this barrier nonrestrictive however the epithelial cells have apical tight junctions conferring restrict intercellular molecule passage characteristic to the barrier. The blood-retinal barrier (BRB) segregates the retinal space from blood flow in two areas, the inner BRB, with a structure very similar to BBB, formed by microvessels surrounded by pericytes and astrocytes end-feet, and the outer BRB with fenestrated blood vessels and the retinal pigment epithelium layer forming the barrier. The blood-spinal cord barrier protects the spinal cord microenvironment (adapted from Choi *et al.*, 2008).

1.3. IMPACT OF METH ON BBB FUNCTION: WHAT IS KNOWN?

Some groups have explored the effects of multiple or single doses of METH in BBB, and showed that this drug can cause BBB disruption through a decreased in TJ protein expression and/or structural rearrangement of these proteins (Mahajan *et al.*, 2008; Ramirez *et al.*, 2009; Martins *et al.*, 2011). The BBB disruption induced by METH was described in several brain regions, such as cortex, hippocampus, thalamus, hypothalamus, cerebellum, amygdala and striatum (Bowyer *et al.*, 2006; Ramirez *et al.*, 2009; Sharma *et al.*, 2009; Gonçalves *et al.*, 2010). Moreover, it has been extensively studied the effect of METH in astrocytes in both rats and mice exposed to acute or chronic METH administration (Pubill *et al.*, 2003; Thomas *et al.*, 2004; Sharma *et al.*, 2009; Gonçalves *et al.*, 2010). This astrocyte activation can lead to neuroinflammation which potentiates the METH neurotoxicity, BBB dysfunction and can activate MMPs (Rosenberg, 2002; Gonçalves *et al.*, 2010; Martins et al., 2011).

Interestingly, Sharma and collaborators (2009) showed that simultaneously to BBB disruption induced by METH there was also water accumulation in the brain. Moreover, brain edema has been related with a dysfunction in astrocytic aquaporins (AQPs) (Nag *et al.*, 2009, Sharma *et al.*, 2009) and with the BBB breakdown. However, remains to be clarified if brain edema is a cause or a consequence of BBB disruption. Additionally, nothing is known about the effect of METH on AQPs, as well as the precise role of these water channels in BBB dysfunction induced by METH.

1.4. AQUAPORINS

Aquaporins (AQPs) are water channels that can be divided in 13 different proteins (0 to 12; Itoh *et al.*, 2005) as following: type 0, 1, 2, 4 and 5 are water permeable; type 3, 7, 9 and 10 are also known as aquaglyceroporins due to their permeability for water and other small nonpolar solutes like glycerol and urea; type 6 and 8 are also permeable to ions; and type 11 and 12 have lack of structural characteristics related with the other aquaporins (Rojek *et al.*, 2008). The most expressed types in the brain tissue are type 1, 4 and 9 (Venero *et al.*, 2001; Tait *et al.*, 2008; Francesca *et al.*, 2010). AQP1 has a role in CSF formation and is present in choroid plexus epithelium (Zelenina, 2010). AQP9 is important to energetic metabolism being present in neurons, in tanycytes in the wall of the third ventricle, and in endothelial cells of subpial blood vessels (Zelenina *et al.*, 2005). The AQP4 has a role in both formation and resolution of cerebral edema, in K^+ clearance during neuronal activity and can be found in astrocytes and ependymal cells (Zelenina *et al.*, 2005).

The AQPs are small proteins with approximately 30 kDa. Although permeability differences between the different types of aquaporins, all share a similar structure: the water channels forms a tetramer and each monomer has its own functional channel (Smith and Agre, 1991). Moreover, each monomer of AQP4 has six transmembrane domains with both terminals, carboxyl and amino, in the intracellular space (Preston *et al.*, 1994; Nag *et al.*, 2009) (figure 12).

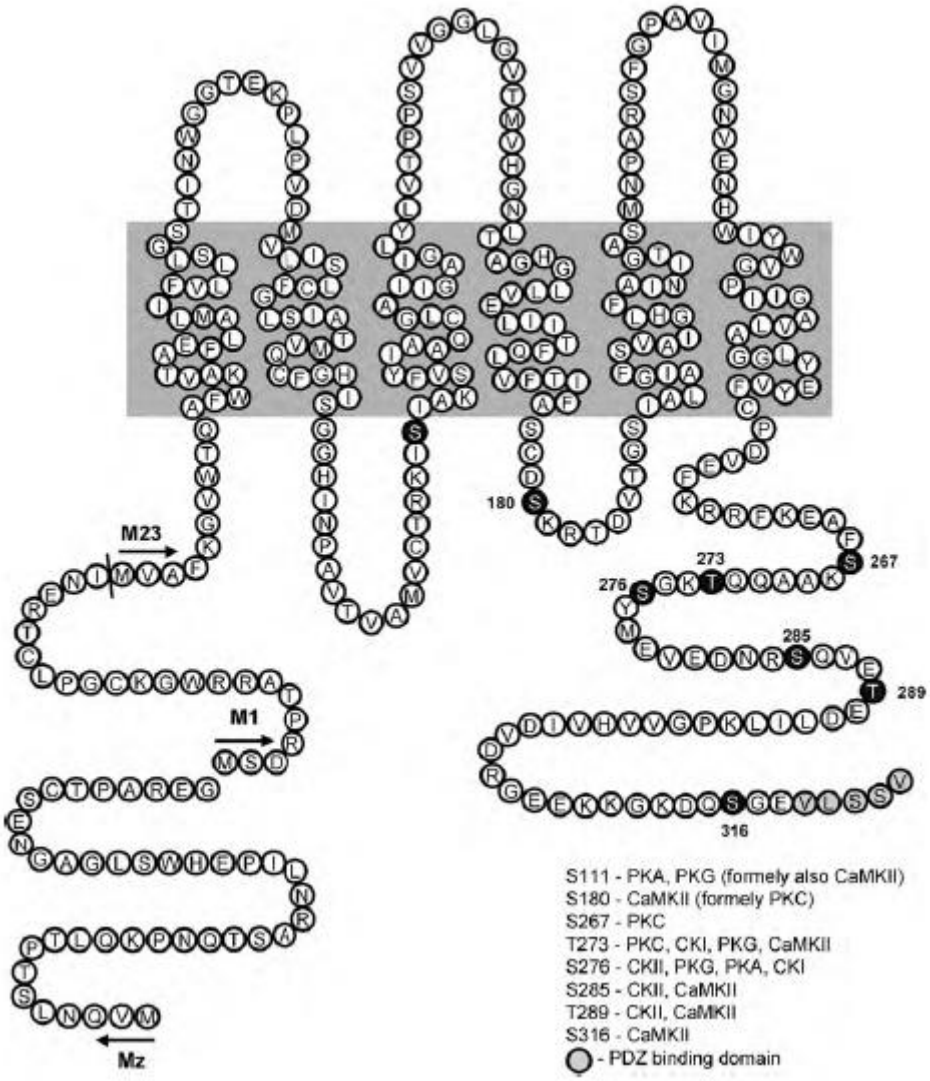

Figure 12. Schematic representation of aquaporin-4. Representation of the 3 AQP4 isoforms, Mz, M1 and M23. The Mz isoform is the longer AQP4 isoform with 364 amino acids. The M1 isoform has 323 amino acids and the M23 isoform is the shorter of the three isoforms with 301 amino acids. In this image is also possible to see residues that can be phosphorilated by different kinases, such as protein kinase C and calcium-calmodulin-dependent protein kinase II (adapted from Zelenina *et al.*, 2010).

AQP4 is expressed in astrocytes endfeet that surround the BECs (Amiry-Moghaddam and Ottersen, 2003). Indeed, AQP4 can be found in 3 different regions: in perivascular astrocyte endfeet where it plays an important role in BBB function (figure 13); in perisynaptic astrocyte

processes where it is involved in neurotransmitter uptake; and in astrocyte processes that are close to nodes of Ranvier and to nonmyelinated axons where AQP4 has a role in the clearance of K^+ (review in Zelenina, 2010). In the brain, AQP4 is related with dystrophin glycoprotein complex, which include dystrophin, β-dystroglycan and syntrophin (Figure 14; Neeley *et al.*, 2001; Connors *et al.*, 2004; Connors and Kofuji, 2006). In fact, Neely *et al.* (2001) showed that in mice lacking α-syntrophin the AQP4 became mislocalized and lose the characteristic distribution in astrocyte endfeet surrounding brain microvasculature. Furthermore, similar effect was observed in null mice for dystrophin (Vadja *et al.*, 2002).

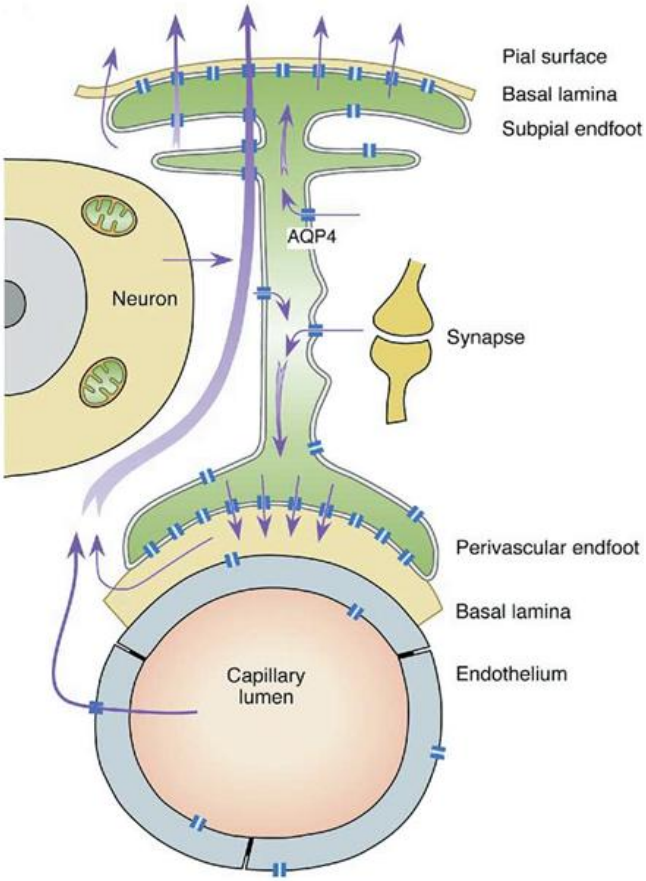

Figure 13. Aquaporin-4 in astrocytes and water movement. This image shows water uptake and release by astrocytic endfeet. The arrows show the water movement (adapted from Amiry-Moghaddam *et al.*, 2004).

AQP4 has two main isoforms (figure 12) originated by alternative splice, known as M1 and M23 (Jung *et al.*, 1994). An additional isoform has been recently identified in rats as Mz and has a Nh2-terminal 41 amino acids longer than the isoform M1 that contain 323 amino acids (Moe *et al.*, 2008). Isoform M23 is the smaller isoform of AQP4 with 301 amino acids but it is the most express isoform in the brain. M23 is also characterized by its ability to form orthogonal arrays of particles (OAPs; Furman *et al.*, 2003; Silberstein *et al.*, 2004), which are supramolecular structures composed of aggregates of M23 and M1 isoforms (Sorlo *et al.*, 2008). However, in Chinese hamster ovary cells transfect with the two AQP4 isoforms, M1 and M23, the presence of M23 alone create large OAPs, whereas the presence of M1 alone creates almost exclusively singlets of AQP4 tetramers. Furthermore, when the two isoforms were expressed, the OAPs formed were more similar to the structures that can be found in astrocytes (Furman *et al.*, 2003). It is already known that AQP4 has a crucial role in the control of water flux in and out the brain, and so on brain edema formation or resolution, respectively. Indeed, in several neuropathologies, such as trauma, ischemia, tumors of astrocytic origin, epilepsy and in neuromyelitis optica, it was demonstrated that the expression of AQP4 is altered (Tait *et al.*, 2008; Nag *et al.*, 2009; Zelenina, 2010). Nevertheless, many questions remain unanswered particularly related with the molecular mechanisms that regulate its function and its possible involvement on BBB dysfunction.

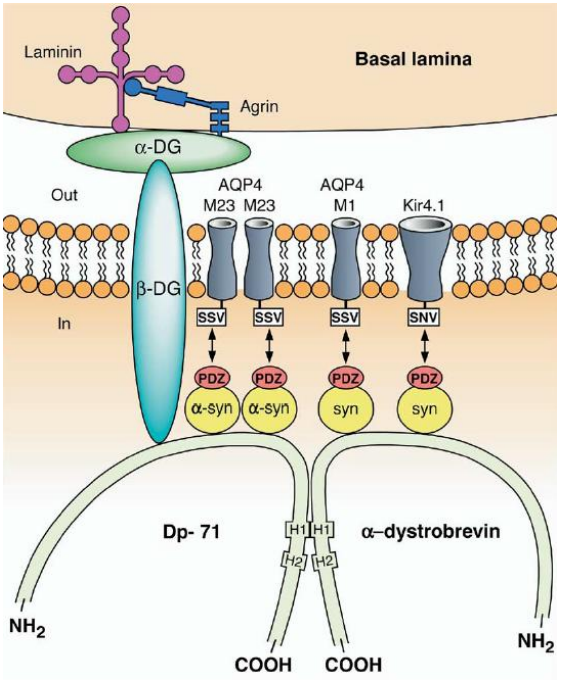

Figure 14. Anchoring of aquaporin-4 and interaction with dystrophin complex. Aquaporin-4 (AQP4) is anchored to cytoplasm and basal lamina through connection with α- and β-dystroglycan (α-DG and β-DG, respectively) and α-syntrophin (α-syn). The association between AQP4 and α-syn occurs *via* PDZ domains. The α-syn links AQP4 to dystrophin-family proteins, such as dystrophin Dp-71 and α-dystrobrevin. Anchorage of AQP4 to basal lamina is mediated by α-DG and β-DG that is associated with laminin and agrin (adapted from Amiry-Moghaddam *et al.*, 2004).

There are two major types of brain edema: vasogenic and cytotoxic edema (figure 15). In vasogenic edema we have an accumulation of water in brain extracellular space, with AQP4 having a role in the clearance of this water excess. In this type of edema, the entry of water is independent of AQPs and seems to be due to BBB opening. However, the exit of water from the brain is AQP-dependent (Venero *et al.*, 2001; Tait *et al.*, 2008; Nag *et al.*, 2009; Francesca *et al.*, 2010). The cytotoxic edema involves the swelling of astrocytes probably due to an increase of AQP4 activity (Tait *et al.*, 2008; Nag *et al.*, 2009; Francesca *et al.*, 2010). Therefore, depending on the type of brain edema, different therapeutic strategies must be adopted. To

treat the vasogenic edema it is necessary to increase the AQP4 activity. However, regarding the cytotoxic edema the treatment necessarily involves the reduction of the activity of this water channel (Nag *et al.*, 2009; Francesca *et al.*, 2010).

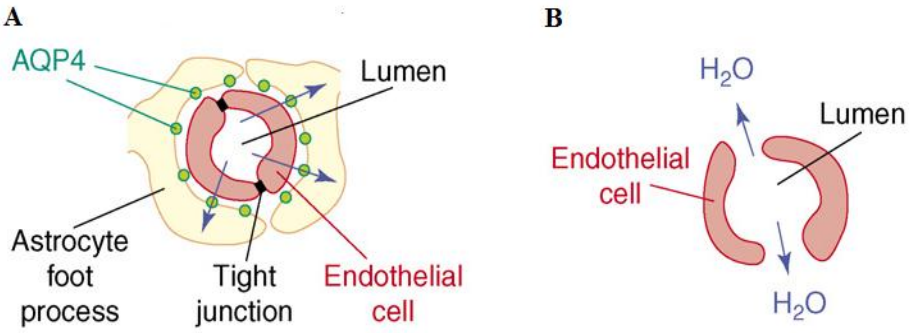

Figure 15. Cytotoxic and Vasogenic Edema. A) Cytotoxic edema, in which the increase in water brain content is due to an increase in astrocytic AQP activity. B) Scheme representing a vasogenic edema, where brain water accumulation is independent of AQP, but AQP4 has a key role in clearance of the excessive water volume (adapted from Tait, 2008).

In an attempt to understand the role of AQP4 in cerebral edema, several studies with knockouts animals were preformed but the results were contradictory. Firstly, in models of cytotoxic brain edema, like hyponatremia (Papadopoulos and Verkman, 2007), bacterial meningitis (Manley *et al.*, 2000) and in early focal cerebral ischemia (Papadopoulos and Verkman, 2005), the knockout animals showed a protection against this type of brain edema. On the other hand, regarding vasogenic edema the knockout animals showed a higher vulnerability to this type of brain edema associated with brain tumors (Papadopoulos *et al.*, 2004). These data demonstrated that in cytotoxic edema AQP4 is responsible for the increase in brain water content. However, in vasogenic edema AQP4 is responsible for removing the excess of water out the brain.

Some studies have shown the involvement of AQP4 under epileptic conditions due to K^+ clearance, and its deletion leads to an increased in seizure threshold and duration (Amiry-

Moghaddam and Ottersen, 2003; Binder *et al.*, 2006). Moreover, AQP4 is up-regulated in astrocyte-derived brain tumors and the level of this up-regulation can be associated with the tumor grade (Warth *et al.*, 2007).

Like other channels and membrane proteins, the regulation of AQPs is very important for normal function. Phosphorilation of two serine residues (Ser111 and Ser180) was demonstrated to be involved in the regulation of AQP4 activity (figure 12 and 16). The serine residue present in position 111 (Ser111) has been reported to be phosphorilated by calcium/calmodulin dependent protein kinase II (CaMKII), although this phosphorilation does not occur directly, but involving the activation of nitric oxide synthase (NOS) and cGMP-dependent protein kinase (PKG; Gunnarson *et al.*, 2008). Some studies suggested that phosphorilation of Ser111 leads to an increase in AQP4 water permeability (Gunnarson *et al.*, 2005; Gunnarson *et al.*, 2008). Moreover, serine residue present in position 180 (Ser180) is phosphorilated by protein kinase C (PKC; Zelenina *et al.*, 2002; McCoy *et al.*, 2009) and this activation by PKC leads to a fast reduction in water permeability of AQP4, being prevented by a PKC inhibitor (Zelenina *et al.*, 2002). On the other hand, in primary cultures of astrocytes there is no evidence of AQP4 phosphorilation by PKC, but the endogenous AQP4 is present in constitutively phosphorilated form (Nicchia *et al.*, 2008; Kadohira *et al.*, 2008). Kadohira and collaborators (2008) have also shown in primary cultures of astrocytes that phosphorilation of other COOH-terminal residues are involved in Golgi transition of AQP4. Moreover, phosphorilation of serine residue in position 276 has been correlated with internalization and degradation of AQP4 in renal epithelial cells (Madrid *et al.*, 2001).

Although the above mentioned evidence, aquaporins are also regulated by metals ions (figure 16) like mercury (Hg) and lead (Pb). Most types of AQPs are inhibited by Hg (Amiry-Moghaddam and Ottersen, 2003) except for AQP6 that is activated by this metal (Hazama *et al.*, 2002). Concerning AQP4, first it was considered mercurial-insensitive (Nicchia *et al.*, 2000;

Shi and Verkman, 1996), but a few years ago Yukutake and collaborators (2008) demonstrated that isoform M23 of AQP4 is inhibited by using Hg involving two cysteine residues in position 178 and 253. The isoform M1 of AQP4 is probably regulated by mercury through NH_2-terminal residues (review in Yukutake and Yasui, 2010). Other metal ions that are able to regulate AQP4 are zinc (Zn) and copper (Cu) (figure 14), which cause an inhibition of isoform M23 of AQP4 (Yukutake and Yasui, 2010).

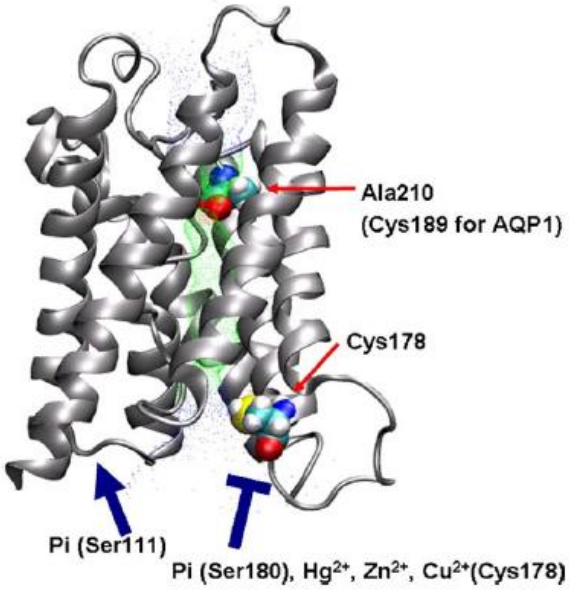

Figure 16. Structural 3D representation of aquaporin-4. Model of AQP4 structure showing the residues involved in AQP4 inhibition by phosphorilation and metal binding (adapted from Yukutake *et al.*, 2010).

CHAPTER 2

MATERIAL AND METHODS

CHAPTER 2

Material and Methods

2.1. Animals and treatments

Male wild-type C57BL/6J mice (3-month-old; 24–26 g body weight; Charles River Laboratories, Barcelona, Spain), were housed under controlled environmental conditions (12 h light:dark cycle, 24 ± 1ºC) with food and water *ad libitum*. All experimental procedures were performed according to the guidelines of the European Community for the use of animals in the laboratory (2010/63/EU) and the Portuguese law for the care and use of experimental animals (DL nº 129/92).

Mice received four intraperitoneal (i.p.) injections, 2 h apart, of 10 mg/kg of METH (Chemistry Department, Faculty of Sciences, University of Porto, Portugal) or 100 µl of 0.9% NaCl (control group), and were sacrificed 24 h after the last injection.

2.2. Primary cultures of mouse cortical astrocytes

Astrocytes were isolated from C57BL/6J mouse pups aged P4-5. After decapitation, brain cortices were isolated and incubated with digestion solution [Hank's Balanced Salt Solution (HBSS; GIBCO, Rockville, MD, USA), 0.25% Trypsin (SAFC, Kansas, USA), 0.001% DNase (SIGMA,

St Louis, MO, USA), 10 µl/ml Gentamicin (SIGMA)] for 20 min at 37ºC with gentle shaking each 2 min. Afterwards, the solution was centrifuged at 700 rpm for 2 min, the supernatant was discarded and the inhibitory digestion solution [HBSS with 10% fetal bovine serum (FBS)] was added and once more centrifuged at 700 rpm for 2 min. After discarded the supernatant, the pellet was resuspended in HBSS in order to dissociate the cells. Then, cell suspension was centrifuged at 1000 rpm for 5 min and the pellet resuspended with astrocyte medium (DMEM high glucose supplemented with 10% FBS and 1% gentamicin).

Cells were plated in T-75 flasks at a density of 40000 cells/cm^2. The medium was changed 6 h after culture and then every 2 days until reach confluence. Then, the flasks were shaking (200 rpm for 4 h at 37ºC), the non-astrocytic cells were discarded (detached cells) and the astrocytes (adherent cells) were washed with dissociation medium [HBSS with 1mM ethylenediamine tetraacetic acid (EDTA, SIGMA)] followed by trypsinization with 0.1% Trypsin in HBSS. This process was stopped by incubation with astrocyte medium followed by centrifugation (1000 rpm for 5 min). The cells were resuspended, counted using Trypan blue dye, and plated at different densities depending on the experiments (Table 1).

Table 1. Astrocyte density according to the experiment

EXPERIMENT	CULTURE MULTIWELL PLATE	FINAL CONCENTRATION (cells/ml)	MEDIUM VOLUME (ml)
Western blot/Astrocyte-conditioned medium	6	240 000	2
Immunocytochemistry	12 with slides	60 000	1
TUNEL assay	24	90 000	0.5

2.3. Astrocyte-conditioned medium (ACM) experiments

Astrocytes were seeded in 6-well plates and after 3 days the medium was removed and the cells were washed with sterile PBS and incubated with two different METH concentrations (1 and 50 µM METH) for 24 h. Afterwards, mediums were collected, centrifuged at 4000 rpm for 10 min at 4ºC and then the supernatant was collected and stored at -80ºC until further use.

2.4. bEnd.3 cell culture

Mouse brain endothelial cells (bEnd.3) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO) supplemented with 10% FBS (GIBCO), 100 U/ml penicillin, and 10 µg/ml streptomycin (GIBCO) in non-coating flasks or multiwells, and maintained at 37ºC in a humidified 5% CO_2/95% air atmosphere. Cells were allowed to reach confluence within 6-8 days. After reaching confluence the cells were trypsinized with 0.25% trypsin (SAFC) in 0.53 mM of EDTA (SIGMA) and then counted using Trypan blue and plated at different densities depending on the experiments (Table 2). Experiments were performed with cells from passages 24 to 33.

Table 2. Cell densities used to plate the endothelial cells depending on the experiments

EXPERIMENT	CULTURE MULTIWELL PLATE	FINAL CONCENTRATION (cells/ml)	MEDIUM VOLUME (ml)
Western blot	6	100 000	2
TUNEL assay	24	10 000	0.5

2.5. Western blot analysis

After appropriate treatment, mice were killed and hippocampi, striata and frontal cortex were dissected on ice. Regarding *in vitro* studies, endothelial cells and astrocytes were seeded in 6-well plates and treated for 24 h with ACM (Control, 1 and 50 µM METH) or different METH concentrations (1 and 50 µM METH), respectively. Total protein of cells or mice brain regions was obtain by lysing the cells/tissue using Radio-Immunoprecipitation Assay lysis buffer (RIPA; 0.150 M NaCl, 0.050 M Tris-base, ethylene glycol tetraacetic acid (EGTA), 0.5% sodium desoxicolate (DAC), 0.1% sodium dodecyl sulfate–polyacrylamide (SDS) and 1% X-Triton, pH 7.5) supplemented with a protease inhibitor cocktail tablets (Roche) and 1 mM dithiothreitol (DTT) (Bioron, Porto, Portugal). Protein quantification was performed using the bicinchoninic acid (BCA) method (Pierce, Rockford, USA) with BSA as a standard, and stored at -20ºC until further use. Protein samples were prepared under reduced conditions by adding sample buffer (0.5 M Tris-HCl, 4% SDS, 30% glycerol, 10% SDS, 0.6 M DTT, bromophenol blue; pH 6.8) and heating at 95ºC for 5 min. Proteins (table 3) were separated by electrophoresis on 10% or 12% polyacrylamide gels at 160 V for 120 min and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Madrid Spain) that were blocked with 5% (w/v) low fat milk in PBS-T (PBS containing 0.1% (v/v) Tween-20 (SIGMA)). Then, membranes were incubated overnight at 4ºC with primary antibodies as described in Table 4. After washing, they were incubated with the respective alkaline phosphatase-conjugated secondary antibody (anti-mouse 1:10000 or anti-rabbit 1:20000) (Amersham GE Healthcare Life Science, USA) for 1 h at RT. The membranes were again washed with PBS-T, and proteins were visualized using the enhanced chemiofluorescence (ECF) reagent (Amersham) assay on the Typhoon FLA 9000 (GE Healthcare Bioscience AB, Uppsala, Sweden). Immunoblots were reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody to ensure equal sample loading. Quantification of band density was performed using ImageQuant 5.0 software.

Table 3. List of proteins identified by western blot analysis

PROTEIN	MOLECULAR WEIGHT (kDa)	AMOUNT OF PROTEIN (µg)
Claudin-5	22	25
Occludin	60-65	25
Aquaporin-4	30	50
GAPDH	37	-

Table 4. List of primary and secondary antibodies used in western blot studies

PRIMARY ANTIBODY	DILUTION	SOURCE	SECONDARY ANTIBODY	DILUTION	SOURCE
Mouse anti-claudin-5	1:100	Zymed laboratories, San Francisco, USA	Anti-mouse IgG alkaline phosphatase conjugated	1:10000	Amersham GE Healthcare Life Science
Rabbit anti-occludin	1:250	Zymed laboratories	Anti-rabbit IgG alkaline phosphatase conjugated	1:20000	Amersham GE Healthcare Life Science
Rabbit anti-aquaporin-4	1:1500	Chemicon, Millipore	Anti-rabbit IgG alkaline phosphatase conjugated	1:20000	Amersham GE Healthcare Life Science
Mouse anti-GAPDH	1:1000	Abcam	Anti-mouse IgG alkaline phosphatase conjugated	1:10000	Amersham GE Healthcare Life Science

2.6. Brain edema evaluation

Brain water accumulation was evaluated by comparing the wet and dry weights, like previously described by Kenne *et al.* (2012). For that, mice were sacrificed and the intact brains were removed and weighed in order to obtain the wet weight (WW). Afterwards, the brains were dried for 72 h at 60ºC, and once again weighed to obtain the dry weight (DW). The percentage of brain water was calculated using the following formula: [(WW – DW)/WW] x 100. As

positive control, mice were administered with 150 ml/kg (i.p.) of distilled water and sacrificed 40 min after the injection (Haj-Yasein *et al.*, 2011).

2.7. TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay permits the quantification of cell death by apoptosis since it will detect DNA fragmentation. This assay is characterized by the detection of specific activity of terminal transferase which attaches labelled biotin-16-2'-deoxy-uridine-5'-triphosphate to the 3'-OH end of DNA generated during apoptotic-induced DNA fragmentation.

Astrocytes and endothelial cells were exposed to different concentrations of METH, and in another set of experiments, endothelial cells were exposed to different ACM for 24 h followed by TUNEL assay. Specifically, cells are fixed with 4% paraformaldehyde (PFA) and rinsed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4). Afterwards, cells were permeabilized with 0.25% Triton X-100 in PBS for 30 min at RT and rinsed with PBS. Cells were then incubated with terminal deoxynucleotidyl transferase buffer (0.25 U/µl terminal transferase, 6 µM biotinylated dUTP, pH 7.5; Roche, Mannheim, Germany) for 1 h at 37ºC in a humidified chamber, and rinsed during 15 min with TB buffer (300 mM NaCl and 30 mM sodium citrate) followed by 5 min in PBS. The incubation with Fluorescein (1:100; Vector Laboratories) was performed for 1 h at RT and the nuclei counterstaining with 4 µg/ml Hoechst 33342 (Sigma) for 5 min at RT. The slides were mounted in Dako Cytomation fluorescent medium (Dako North America, Carpinteria, USA) and stored in the dark. Fluorescent images were recorded using a camera Leica DMIRE2 incorporated on a Leica CTRMIC microscope (Leica, Germany). Six independent microscopy fields were acquired per coverslip with a magnification of x200. The number of apoptotic cells were expressed as percentages of total cells per field stained with Hoechst 33342 (Sigma).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Data was analyzed using the one-way ANOVA analysis of variance followed by Dunnett's post hoc or Bonferroni's post hoc to compare with control or among experimental conditions, respectively. In western blot studies for AQP4 expression in brain regions, statistical significance was determined using an unpaired one tailed Mann-Whitney test. Data were considered to be statistical different at values of $P<0.05$ and were presented as means ± SEM (standard error of the mean). For the quantification of TUNEL-positive cells, six independent microscopy fields per coverslip with 200× magnification were acquired and results are expressed as percentages of total cells stained with Hoechst 33342 per each field (n = number of fields). Every experimental condition was tested in three sets of independent experiments, unless stated otherwise, and performed in duplicates.

CHAPTER 3

RESULTS

CHAPTER 3

Results

3.1. Effect of methamphetamine on astrocytes

Astrocytes are one of the components of neurovascular unit (Kim *et al.*, 2006) and have an important role in tightness and maintenance of the BBB function (Abbott *et al.*, 2006). In the present study, we started by evaluating cell death induced by METH and it was possible to observe an increase in apoptotic cell death at a concentration of 500 µM and 1000 µM (Figure 17; control: 4.03 ± 0.25%; 1 µM METH: 3.44 ± 0.41 %; 50 µM METH: 3.24 ± 0.49%; 250 µM METH: 3.85 ± 0.68%; 500 µM METH: 8.03 ±0.78%; 1000 µM METH: 8.25 ± 0.64%). For further studies in astrocyte cultures the two lower non-toxic concentrations (1 µM and 50 µM) were used.

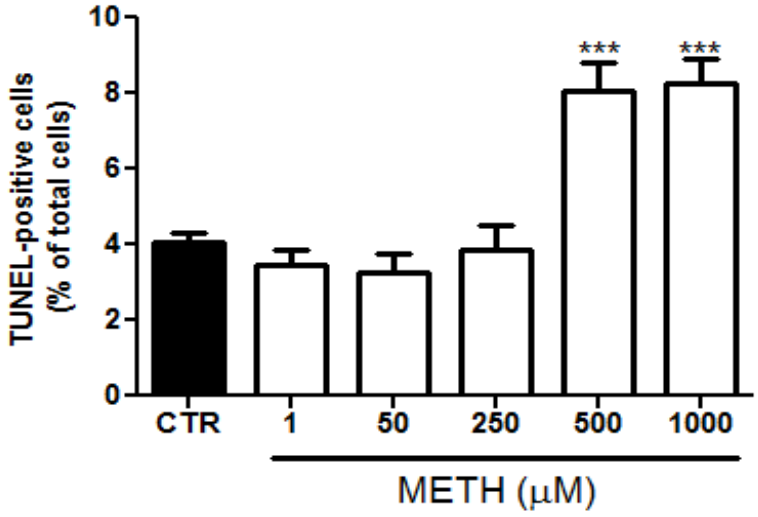

Figure 17. METH-induced astrocytic cell death. Primary cultures of mouse cortical astrocytes were exposed to different METH concentrations (1-1000 μM) for 24 h and apoptotic cell death was evaluated by TUNEL assay. Data are expressed as mean % of total cells ± S.E.M., n = 36. ***$P < 0.001$, significantly different when compared to control using one way ANOVA followed by Dunnett's Multiple comparison test.

Afterwards, possible alterations in AQP4 expression, which is a water channel involved in water movement regulation (Zelenina *et al.*, 2005), was analyzed in the absence and presence of different METH concentrations. As observed in figure 18 A, 50 μM METH leads to a significant increase in the protein levels of AQP4, with no significant effect at the concentration of 1 μM (control: 99.55 ± 1.72%; 1 μM METH: 120.80 ± 5.17%; 50 μM METH: 176.60 ± 10.39%). Furthermore, to ensure that this increase in AQP4 expression is due to a real increase in the protein expression and not due to an increase in the number of astrocytes or astrocyte reactivation, we also evaluated GFAP expression. No differences were observed under various experimental conditions (Figure 18 B; control: 100.70 ± 7.89%; 1 μM METH: 98.45 ± 4.84%; 50 μM METH: 100.90 ± 12.44%).

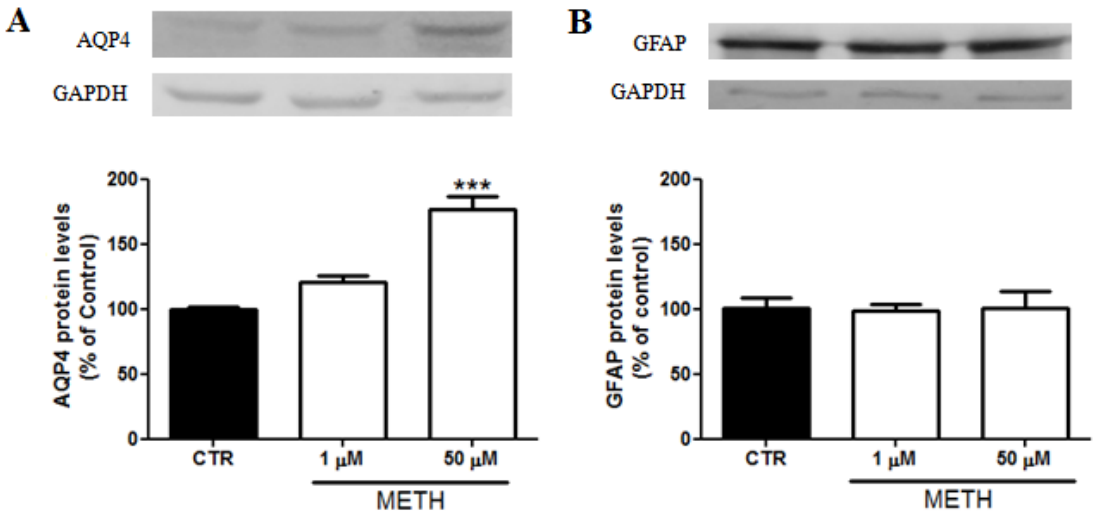

Figure 18. Effect of non-toxic METH concentrations on (A) AQP4 and (B) GFAP expression in astrocytes. (A) Primary cultures of mouse cortical astrocytes were exposed to METH (1 or 50 µM) during 24 h, and protein levels were evaluated by western blot. Above the bars, representative western blot images of AQP4 (32 kDa), GFAP (50 kDa) and GAPDH (37 kDa) are shown. The results are expressed as mean % of control ± S.E.M., n=4-5, ***P < 0.001 significantly different when compared to control using one way ANOVA followed by Dunnett's Multiple comparison test.

3.2. Effect of methamphetamine on brain aquaporin 4 expression and brain edema

Recently, it was shown that acute METH treatment causes brain edema and this increase in brain water content is region-specific, with the hippocampus being more susceptible than the frontal cortex (Sharma and Kiyatkin, 2009; Kiyatkin and Sharma, 2011). Taking into consideration the crucial role of AQP4 water channels in the control of water flux in and out the brain, we further investigated the alteration of its expression in an animal model of METH intoxication (binge administration). It was possible to conclude that in the striatum METH did not induce significant alterations in AQP4 expression (115.50 ± 18.67%; n=3). However, in the hippocampus there was a significant increase in AQP4 expression (156.90 ± 9.56%; n=3). On the other hand, in frontal cortex METH led to a significant decreased in the AQP4 protein levels (58.42 ± 4.17%; n=3).

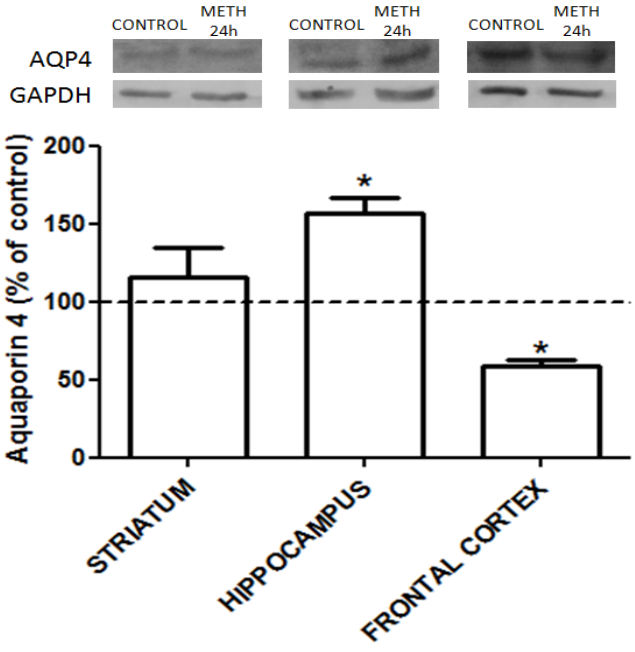

Figure 19. **Effect of acute METH administration on AQP4 expression in different brain regions.** Mice were administered with METH (4x 10 mg/kg, 2h apart) and sacrificed 24 h post-injection. METH increased hippocampal AQP4 protein levels and there was a decrease in the frontal cortex, with no significant effect in the

striatum. Above the bars, representative western blot images of AQP4 (32 kDa) and GAPDH (37 kDa) are shown. The results are expressed as mean % of control ± S.E.M., n=3. *P < 0.05 significantly different when compared to control using Mann-Whitney post-test.

We then hypothesize that the observed alterations in AQP4 expression in the different brain regions could be due astrocyte reactivation (Pubill *et al.*, 2003; Sharma *et al.*, 2009). Thus, GFAP expression was also evaluated, but no significant differences were observed in the three subregions analyzed (Figure 20; striatum: 109.50 ± 24.95%; hippocampus: 104.30 ± 26.12%; frontal cortex: 84.90 ± 19.83%).

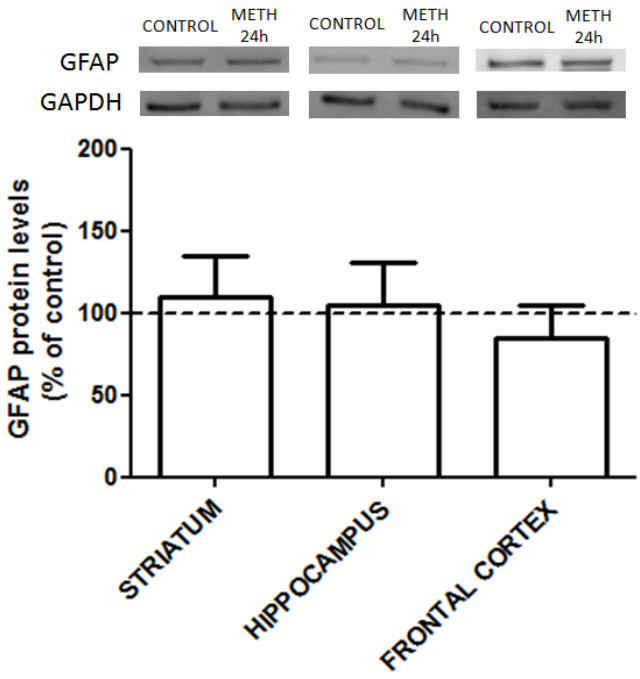

Figure 20. Effect of acute METH administration on GFAP expression in different brain regions. Mice were administered with METH (4x 10 mg/kg, 2h apart) and sacrificed 24 h post-injection. METH did not induce alterations in the GFAP protein levels in the different brain regions analyzed. Above the bars, representative western blot images of GFAP (50 kDa) and GAPDH (37 kDa) are shown. The results are expressed as mean % of control ± S.E.M., n=3.

Afterwards, to confirm if METH also induced brain edema we evaluated the water content as previously described (Kenne *et al.*, 2012). However, no alterations were observed in the brain

water content (control: 78.40 ± 0.09%; METH: 78.60 ± 0.26%; positive control: 80.44 ± 0.22%; figure 21).

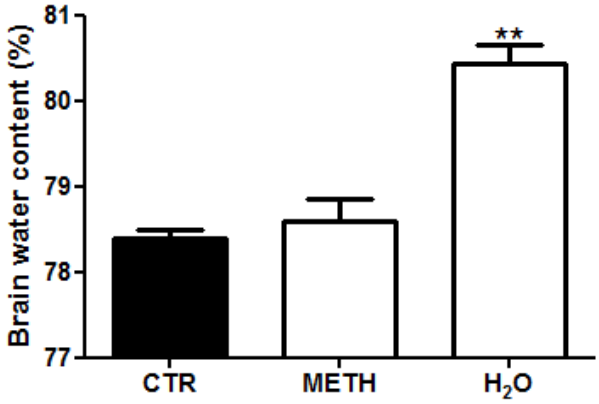

Figure 21. Quantification of brain water content. Mice were administered with METH or water and sacrificed 24 h post-injection. METH did not cause brain edema and the injection of distilled water induced a significant increase in brain water content (positive control). The results are expressed as mean % ± S.E.M., n=3, $^{**}P < 0.01$ significantly different when compared to the control using one way ANOVA followed by Dunnett's Multiple comparison test.

3.3. Effect of astrocyte-conditioned medium on brain endothelial cells

Our group has already demonstrated that METH causes disruption of the BBB and leads to a decreased in the expression of tight junctions proteins (Martins *et al.*, 2011). Moreover, it has been speculated that astrocytes can also have a role in the protection of endothelial cells (Gesuete *et al.*, 2011). So, we evaluated the possible effect of ACM in endothelial cell death, and only 3000 µM METH ACM increased cell death (control: 0.25 ± 0.02%; ACM control: 0.12 ± 0.03%; 1 µM METH ACM: 0.09 ± 0.02%; 50 µM METH ACM: 0.14 ± 0.03%; 250 µM METH ACM: 0.19 ± 0.05%; 500 µM METH ACM: 0.17 ± 0.03%; 1000 µM METH ACM: 0.29 ± 0.07%; 3000 µM METH ACM: 0.54 ± 0.17%). Importantly, the very low number of TUNEL positive cells even in the presence of 3000 µM METH ACM clearly shows the high resistance of these cells.

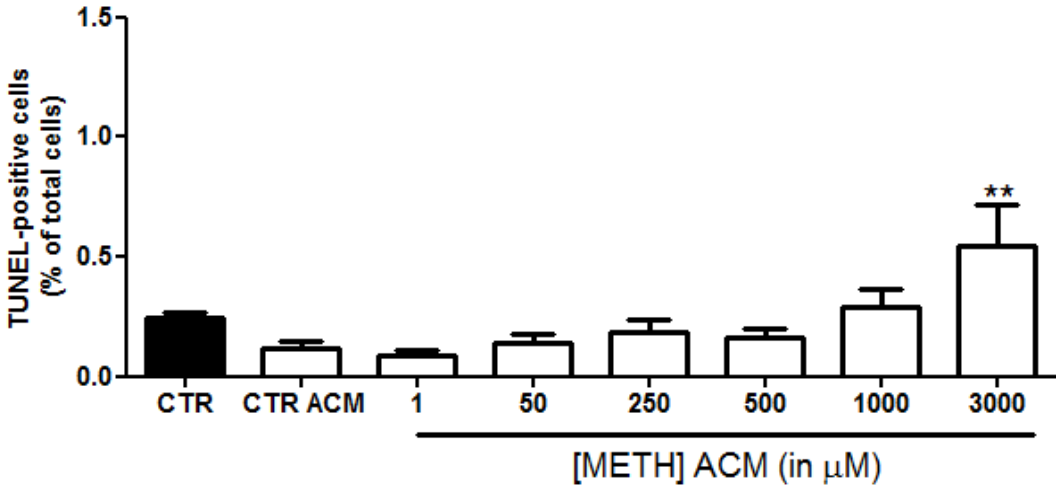

Figure 22. Endothelial cell death induced by ACM. Only 3000 µM METH ACM (24 h exposure) increased cell death and for all the other conditions ACM did not cause a significant alteration in the number of TUNEL positive cells. The results are expressed as mean % of total cells ± S.E.M., n=36, $^{**}P < 0.01$ significantly different when compared to the control using one way ANOVA followed by Dunnett's Multiple comparison test.

In order to study the possible protection of endothelial cells by ACM, we exposed bEnd.3 cells to 3000 µM METH or to the same METH concentration but incubating the cells with CTR ACM medium instead of the normal ECs medium. As observed in figure 23, when we exposed endothelial cells to 3000 µM METH there was a significant increase of cell death. Interestingly, we concluded that ACM was able to protect endothelial cells from METH exposure (control: 0.25 ± 0.02%; ACM control: 0.12 ± 0.03%; 3000 µM METH: 6.12 ± 0.44%; ACM control + 3000 µM METH: 1.45 ± 0.12%).

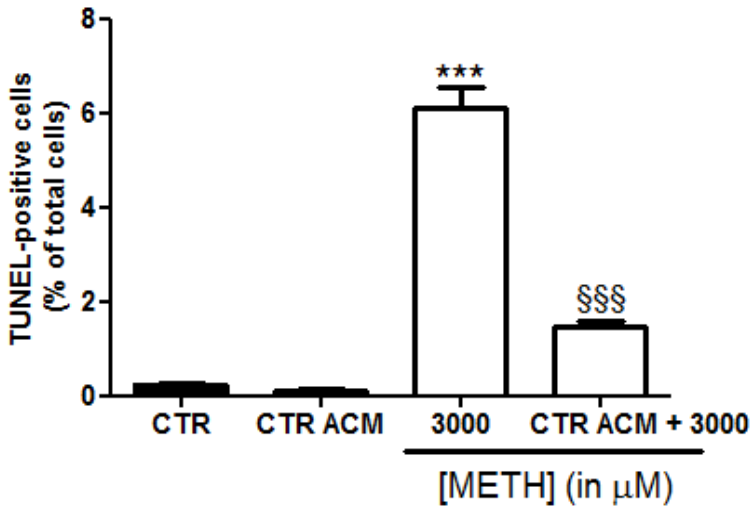

Figure 23. Endothelial cell death induced by METH and the protective role of ACM. The endothelial cells were exposed to 3000 µM METH during 24h or to the same concentration but in the presence of ACM instead of the normal endothelial cell medium. The results are expressed as mean % of total cells ± S.E.M. n = 36-40, ***$P < 0.001$ significantly different when compared to the control (CTR) using one way ANOVA followed by Dunnett's Multiple comparison test; §§§ $P < 0.001$ significantly different when compared to 3000 µM METH, using Bonferroni's Multiple comparison test.

Since METH can also lead to BBB opening, with decrease in tight junctions' proteins expression (Ramirez *et al.*, 2009; Martins *et al.*, 2011), we also evaluated the effect of ACM on the expression of two tight junctions proteins, occludin and claudin-5. Regarding occludin, a very significant decrease in the expression levels can be observed in both ACM METH conditions (1 µM and 50 µM METH ACM) (Figure 24 A; 1 µM METH ACM: 48.44 ± 4.91%; 50 µM METH ACM: 37.17 ± 5.11%). Regarding the protein levels of claudin-5, there was also a significant decrease in its levels (Figure 24 B; 1 µM METH ACM: 56.84 ± 11.89%; 50 µM METH ACM: 57.57 ± 8.21%).

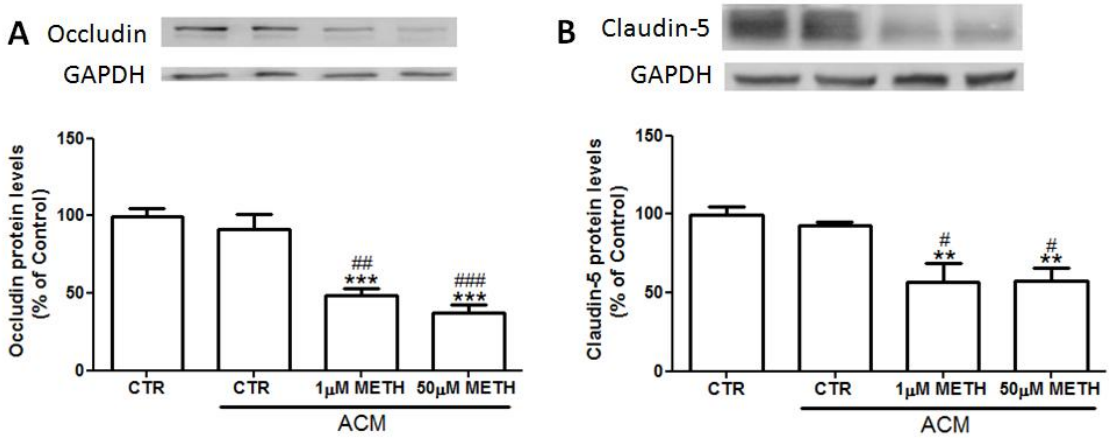

Figure 24. Effect of ACM on the expression of tight junction proteins, (A) occludin and (B) claudin-5. Endothelial cells were exposed to ACM with two different METH concentrations (1 µM and 50 µM) for 24 h. Above the bars, representative western blot images of occludin (65 kDa), claudin-5 (24 kDa) and GAPDH (37 kDa) are shown. The results are expressed as mean % of control ± S.E.M., n=3-4, $^{**}P < 0.01$, $^{***}P < 0.001$ and significantly different when compared to control using one way ANOVA followed by Dunnett's Multiple comparison test; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to control ACM using Bonferroni's Multiple comparison test.

CHAPTER 4

DISCUSSION

CHAPTER 4

Discussion

AQP4 is a water channel widely present in the brain, expressed mostly in astrocytes endfeet that surround brain microvessels (Tait *et al.*, 2008; MacAulay and Zeuthen, 2010). These water channels have a role in controlling water homeostasis (Zelenina *et al.*, 2005) and are involved in the formation (Manley et al., 2000; Papadopoulos and Verkman, 2007) and resolution (Papadopoulos et al., 2004) of brain edema. Moreover, it is already known that METH can cause brain edema in animals (Kiyatkin *et al.*, 2007) and in humans (reviewed in Butner, 2011). So, we hypothesize if METH could have an effect in the expression of AQP4. To clarify this question we used primary cultures of astrocytes and started by evaluating the direct effect of several METH concentrations on cell death. We concluded that only concentrations equal and above 500 µM were toxic to astrocytes. With these results we chose two non-toxic METH concentrations (1 and 50 µM METH) to evaluate the possible alterations in AQP4 expression levels. The results show that METH (50 µM) leads to an increase of AQP4 protein levels. Nevertheless, it was referred by our group and others that METH can cause astrogliosis *in vivo*

followed an acute METH administration (Sharma and Kiyatkin, 2009; Gonçalves *et al.*, 2010), which could mask the real effect of METH on AQP4 expression. In order to clarify this issue, we analysed the levels of GFAP expression in primary cultures of astrocytes after METH treatment, but there were no alterations. This suggests that METH has a direct effect on AQP4 expression. Accordingly, Qi *et al.* (2011) used an ischemia *in vitro* model (oxygen and glucose deprivation, OGD, in primary cultures of rat astrocytes) and showed an increase in the expression levels of AQP4 at 4h after OGD, and this increase was inhibited by the antagonist of cysteinyl leukotriene receptor 2, which is an inflammatory mediator (Qi *et al.*, 2011). Moreover, using an animal model of ischemia it was also observed an increase of AQP4 expression that was prevented by the administration of thrombin, which is a serine protease (Hirt *et al.*, 2009). Regarding the possible link between AQP4 and BBB, Tomás-Camardiel *et al.* (2005) induced BBB disruption by *in* vivo administration of lipopolysaccharide (LPS) and observed an increase in both AQP4 mRNA and protein levels, and this effect was sustained up to 14 days after LPS administration (Tomás-Camardiel *et al.*, 2005). Nevertheless, in our model the functional consequence of this increase remains to be investigated.

It is known that METH causes brain edema with different susceptibilities among brain regions (Sharma and Kiyatkin, 2009). Thus, we also evaluated the levels of AQP4 in the striatum, hippocampus and frontal cortex using an acute protocol of METH administration. The binge treatment is currently the most frequently used model that mimics acute toxic dosing of METH (Krasnova and Cadet, 2009), and provides excellent relevance to intravenous and smoked routes of METH exposure in humans. In addition, it demonstrates the toxic effects of METH in non-tolerant users. Interestingly, we observed different effects of METH in the three regions studied, with an increase of AQP4 expression in the hippocampus and a decrease in frontal cortex. We have also previously observed that METH-induced BBB permeability is dependent on the region, being the hippocampus the most affected brain region (Martins *et al.*, 2011). As

for the *in vitro* studies, we showed that there was no astrogliosis which demonstrated that in the animal model there is also a direct effect on AQP4 expression. An increase in AQP4 levels has already been described in several pathological conditions, such as in inflammation induced by lysolecithin (Tourdias *et al.*, 2011) and by LPS (Alexander *et al.*, 2008), intracerebral hemorrhage stroke that also causes increase in BBB permeability (Tang *et al.*, 2010), and traumatic brain injury with an increase in matrix metalloproteinase 9 expression and BBB permeability (Higashida *et al.*, 2011). In all these studies the increase of AQP4 levels were always accompanied by an increase in the brain water content. Moreover, Yang and collaborators (2008) demonstrated that transgenic mice overexpressing AQP4 presented cytotoxic brain swelling. Interestingly, Jo *et al.* (2011) showed that AQP4 expression is reduced in piriform cortex with vasogenic edema induced by endothelin-1. Thus, since alterations in AQP4 can interfere with brain water content, we further evaluated if METH administration causes brain edema, but we did not observed any differences when compared to the control. One possible explanation for these results can be the fact that we measured the water content in total brain, whereas METH leads to alterations in AQP4 expression in a brain region specific manner. To clarify this question it would be important to evaluate edema formation in the hippocampus, frontal cortex and striatum, together with BBB permeability analysis. Considering that astrocytes are part of the neurovascular unit having a protective role in endothelial cells subject to some type of injury like ischemic (Gesuete *et al.*, 2012), we also studied the effect of astrocyte-conditioned medium in cell death and in expression of tight junctions proteins. We have previously concluded that endothelial cells are quite resistant to METH because only at 3 mM it was possible to observe an increase of cell death (Almeida, C. 2011, Master thesis). Some data shows that METH exposure in endothelial cells cultures leads to a decrease and also disorganization of tight junction proteins, such as occludin and claudin-5 (Mahajan *et al.*, 2008; Ramirez *et al.*, 2009). Moreover, METH causes an impairment of glucose

transport system, with a decrease in the expression of glucose transporter 1 (GLUT-1) and inhibition of its activity (Muneer *et al.*, 2011). Here, for the first time we showed that normal ACM has a protective role against METH-induced endothelial death, suggesting that astrocytes release some molecules that will somehow protect endothelial cells against the toxic effect of the drug. The protective effect has been already demonstrated in human endothelial cells (Siddharthan *et al.*, 2007; Kuo *et al.*, 2011). In these studies, the ACM decreased the permeability and increased transendothelial electric resistance, both used to assess the tightness of BBB. Moreover, Siddharthan *et al.* (2007) demonstrated that permeability decrease and increase of ZO-1 expression are more pronounced using ACM than astrocytes themselves. In future studies it will be important to analyse the composition of this medium in order to identify the protective factor(s).

Astrocytes are very important in the formation and maintenance of BBB (Weiss *et al.*, 2009), having also an important role in the formation of characteristic tube-like structure of BBB (Ramsauer *et al.*, 2002). Additionally, astrocytes induce an upregulation of tight junction proteins, transporters and enzymes (reviewed by Abbott *et al.*, 2006). Thus, we further evaluated the effect of ACM in the expression levels of two tight junctions proteins, claudin-5 and occludin. Claudin-5 is very important to the normal BBB function since the knockout mice for claudin-5 show a disruption of the BBB (Wolburg *et al.*, 2003; Argaw *et al.*, 2009, Cardoso *et al.*, 2010). Regarding occludin, it is a protein important for the BBB stabilization and regulatory properties but not essential for its barrier function because knockouts for this protein show no alterations in tight junctions formation, probably due to compensatory mechanisms (Hawkins and Davis, 2005; Cardoso *et al.*, 2010). Moreover, our group recently showed that METH increases BBB permeability and decrease the protein levels of claudin-5, occludin and ZO-1 (Martins *et al.*, 2011). Here, we concluded that when astrocytes are exposed to non-toxic METH concentrations, they release some molecules that negatively affect the expression of

both TJ proteins. These results regarding ACM strengthens the idea that astrocytes have a dual role in the brain because they can be protective under conditions that trigger cell death, but on the other hand low concentrations of METH can lead to the release of molecules by astrocytes that have a negative impact on barrier functions. Nevertheless, remains to understand if these changes are due to non-metabolized METH that is still present in ACM or to some factor(s) that astrocytes may release, or even due to the increase of AQP4 expression. In fact, some studies have suggested that AQP4 may regulate BBB. In hyperthensive rats it was shown that the increase of AQP4 expression was related with the beginning of hypertension (Ishida *et al.*, 2006). On the other hand, AQP4 knockout mice do not show alterations neither in BBB permeability, TJ proteins, structure of brain microvessels, nor in brain water content (Saadoun *et al.*, 2009; Tait *et al.*, 2010, Haj-Yasein *et al.*, 2011; Eilert-Olsen *et al.*, 2012). However, in pathological situations, the lack of AQP4 leads to severe alterations in brain parenchyma, such as decrease in the expression of glutamate transporter 1 (GLUT1) that interferes with the glutamatergic system (Li *et al.*, 2012), an increase in basal levels of apoptosis, and inhibition of proliferation and neuronal differentiation (Kong *et al.*, 2008). Moreover, regarding the brain edema the lack of AQP4 leads to an increase in brain water content after a subarachnoid hemorrhage due to a disability of removing the excess of water content (Tait *et al.*, 2010). In fact, these observations may explain why we have an increase in AQP4 expression without brain edema, since the system may up-regulate AQP4 expression in order to extrude excess water. However, other studies showed that the AQP4 overexpression aggravates the cytotoxic brain swelling after acute water intoxication demonstrating that in this situation the AQP4 is involved in edema formation (Yang *et al.*, 2008). So, depending on brain edema, AQP4 can have opposite effects. Nevertheless, it is well known that AQP4 is an important protein for the normal function of the brain and BBB, but more studies are

necessary to understand the mechanisms of AQP4 function in normal and pathological situations.

CHAPTER 5

CONCLUSIONS

CHAPTER 5

Conclusions

With this work we showed for the first time that METH interferes with the levels of AQP4. Specifically, METH increased AQP4 expression in both primary cultures of astrocytes and in mouse hippocampus. However, we did not identify brain edema formation. Moreover, we have shown that in a control situation astrocytes release some factors that will protect the endothelial cells from METH toxicity and without any effect on the expression levels of claudin-5 and occludin. However, if astrocytes are directly exposed to non-toxic concentrations of METH (1 μM or 50 μM), they will probably release factor(s) that lead to a downregulation of these two tight junction proteins, suggesting that under such conditions astrocytes may negatively interfere with BBB properties.

CHAPTER 6

REFERENCES

CHAPTER 6

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