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The effect of methamphetamine on the crosstalk between endothelial cells and astrocytes.

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 supervisão da FCTUC pela Professora Doutora Emília Duarte (Universidade de Coimbra).

Catarina Oliveira Praça Almeida

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Contents

Abbreviations

5-HT	Serotonin
ACM	Astrocytes conditioned medium
AJ	Adherent junction
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
AMPH	Amphetamine
ATS	Amphetamine-type stimulants
BBB	Blood-brain barrier
BMVEC	Brain microvascular endothelial cells
BSA	Bovine serum albumin
CAV	Caveolin
CNS	Central nervous system
CSF	Cerebrospinal fluid
DA	Dopamine
DAT	Dopamine transporter
DMEM	Dulbecco's Modified Eagle's Medium
EC	Endothelial cell
E	Epinephrine
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GLU	Glutamate

HBSS	Hank's balanced saline solution
IL	Interleukine
ISF	Interstitial fluid
JAM	Junctional adhesion molecules
MMPs	Matrix metalloproteinases
METH	Methamphetamine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NE	Norepinephrine
NO	Nitric oxide
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PKC	Protein kinase C
PLP	Proteolipid protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SEM	Standard error of the mean
TJ	Tight junction
TNF- α	Tumor necrosis factor α
TUNEL	Terminal deoxynucleotidyl transferased UTP nick-end labelling
UCP	Uncoupling mitochondrial protein
VMAT-2	Vesicular monoamine transporter-2
ZO	Zonula occluden

Resumo

A metanfetamina (MET) é uma droga de abuso psicoestimulante que causa alterações severas no sistema nervoso central. De forma a explicar a neurotoxicidade induzida pela MET vários mecanismos celulares e moleculares têm vindo a ser propostos, sendo que a maioria deles estão associados ao stresse oxidativo, disfunção mitocondrial, excitotoxicidade e neuroinflamação. Recentemente, alguns estudos têm sugerido que o dano associado ao consumo de MET pode resultar da sua capacidade em comprometer a função da barreira hemato-encefálica (BHE). Devido ao papel crucial desta barreira na manutenção da homeostasia cerebral e na protecção contra moléculas tóxicas, é importante compreender o mecanismo subjacente à disfunção da BHE induzida pela MET.

Neste estudo, investigámos o efeito da MET nas células endoteliais (CEs) e nos astrócitos, tal como no *crosstalk* entre estes dois tipos de células. Os nossos resultados demonstraram que a MET não induz alterações na viabilidade celular das CEs quando expostas durante 24 h a concentrações baixas da droga, assim como também não se verifica morte celular por apoptose. Mais ainda, de forma a tentar esclarecer se a MET poderia afectar a expressão de proteínas das junções oclusivas, avaliámos os níveis das seguintes proteínas: claudina-5, ocludina e *zonula occludens* (ZO) -1 e -2. Os níveis de caveolina (cav) -1 foram também analisados. Verificámos que a MET induz uma diminuição significativa dos níveis proteicos da claudina-5, ocludina e ZO-1. Relativamente ao efeito da MET nos astrócitos, verificámos que estas células são mais susceptíveis à MET já que observámos morte por apoptose a concentrações mais baixas (500 μM MET) comparativamente aos resultados obtidos com as CEs. Numa tentativa de também esclarecer como a MET poderia afectar o *crosstalk* entre as CEs e os astrócitos, avaliámos o efeito da exposição das CEs a meio-condicionado de astrócitos (MCA) durante 24 h. Curiosamente observou-se que o MCA induz alterações na viabilidade das CEs. Deste modo, por si só o MCA induz um aumento na viabilidade das CEs, contrariamente ao observado com o MCA de astrócitos tratados com MET em que se verificou uma diminuição da viabilidade das CEs. Também demonstrámos uma diminuição da expressão da claudina-5 e cav-1 após a exposição a MCA sugerindo que os astrócitos libertam determinados factores para o meio após exposição a MET, que induzem alterações significativas nas CEs.

No geral, o presente trabalho demonstra que a MET diminui a viabilidade das CEs e dos astrócitos, embora seja mais tóxica para os astrócitos. Verificámos também que esta droga de abuso afecta a expressão de diversas proteínas das CEs, tal como o *crosstalk* entre as CEs e os astrócitos.

Palavras-chave: Metanfetamina; Barreira hemato-encefálica; Células endoteliais; Astrócitos; Junções oclusivas; Caveolina-1

Abstract

Methamphetamine (METH) is a powerful psychostimulant drug of abuse that causes severe alterations in the central nervous system (CNS). Several cellular and molecular mechanisms have been proposed in order to explain the METH-induced neurotoxicity, being most of them associated with oxidative stress, mitochondrial dysfunction, excitotoxicity and neuroinflammation. Recently, some studies have suggested that the damage associated with METH might result from its ability to compromise the blood-brain barrier (BBB) function. Duo to the crucial role of the BBB in the maintenance of brain homeostasis and protection against toxic molecules, it is important to understand the mechanisms underlying METH-induced BBB dysfunction.

In this study, we investigated the effect of METH on the endothelial cells (ECs) and astrocytes, as well as on the crosstalk between these two cell types. Our results demonstrate that METH does not induce alterations in ECs cell viability and apoptosis when exposed for 24 h to low concentrations of the drug. Moreover, in an attempt to clarify if METH could affect the expression of the tight junction (TJ) proteins, we evaluated the possible changes of claudin-5, occludin, zonula occludens (ZO)-1 and -2 protein levels, as well as caveolin (cav)-1 expression. We verified that METH induces a significant decrease of claudin-5, occludin and ZO-1 protein levels. Regarding astrocytes, we concluded that they are more susceptible to METH since it was possible to observe cell death by apoptosis with lower concentrations (500 μM METH). Moreover, in attempt to clarify how METH could affect the crosstalk between the ECs and astrocytes, the effect of astrocytes-conditioned medium (ACM) on ECs was evaluated after 24 h of ACM exposure. Interestingly we observed that ACM induces alterations on the viability of the ECs. In fact, ACM *per se* induces an increase in the ECs viability, whereas the ACM obtained from astrocytes treated with METH induces a decrease in ECs viability. Also, we observed a decrease in the expression of claudin-5 and cav-1 after ACM exposure, suggesting that astrocytes can release factors after METH exposure that can induce significant alterations on the ECs.

Overall, the present work demonstrates that METH decreases ECs and astrocytes viability, being, however, more toxic to astrocytes. Also, this drug of abuse induces alterations on important proteins of the ECs, and can also affect the crosstalk between the ECs and astrocytes.

Keywords: Methamphetamine; Blood-brain barrier; Endothelial cells; Astrocytes; Tight junctions; Caveolin-1.

CHAPTER 1

Introduction

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Introduction

1.1. Methamphetamine

1.1.1.Methamphetamine consumption

Methamphetamine (METH) belongs to amphetamine-type stimulants (ATS) group, and is a popular synthetic drug of abuse with psychostimulant effects. All the substances that belongs to ATS group, are structural similar to endogenous neurotransmitters, the monoamines [dopamine (DA), epinephrine (E), norepinephrine (NE) and serotonin (5-HT)], and so, simulate their biological actions and effects.

METH is commonly used by teenagers and young adults at bars, nightclubs, concerts and parties. Some of the street names of METH are speed, meth, chalk, ice, crystal, glass, among others, and it can be taken orally, snorted, injected or smoked (National Institute on Drug Abuse). This drug was first synthesized in Japan in 1983 (Kast and Focosi, 2010) and despite its addiction effect, METH is approved by Food and Drug Administration (FDA)(Kast, 2007; Kast, 2009) being prescribe in cases of narcolepsy, attention deficit hyperactivity disorder in children and also for weight loss (Thrash et al., 2009; Kast and Focosi, 2010). It has a circulating half-life of 9 to 15h with C_{max} of 1h route, and when given in FDA approved doses (maximum daily dose of 25 mg) (Cruickshank and Dyer, 2009), is well-tolerated even in individuals medically ill and frail (Kast, 2007; Kast, 2009).

Regarding METH synthesis, since 1990 ATS-related manufacture has been reported in over 60 countries worldwide, with illicit laboratories increasing in size, sophistication and production, and the majority is reported from North America. However, METH synthesis has increased in East and South-East Asia, specifically in China, Myanmar and Philippines. Moreover, an increase was also reported in Oceania, Europe and Southern Africa (United Nations Office on Drugs and Crime, 2009, World Drug Report). In Europe, METH manufacture is limited to a small number of countries in Central Europe and East Europe, mainly in Czech Republic and Slovakia, but with increasing evidence in Baltic countries,

like Lithuania and Poland (United Nations Office on Drugs and Crime, 2010, World Drug Report; European Monitoring Center for Drugs and Drug Addiction, 2010).

Data regarding METH consumption suggest that in 2009 between 13.7 and 56.4 million people aged 16-64 have consumed amphetamine (AMPH)-group substances (annual prevalence 0.3%-1.3%), at least once in their lives. Moreover, ATS is the second most commonly used drug group after cannabis, with AMPH being the most prominent of the group. A significant number of METH users are present in East and South-East Asia, North America, South Africa and Oceania. In Europe, AMPH is the most used, with few exceptions, like in Czech Republic where METH is used predominantly (United Nations Office on Drugs and Crime, 2010, World Drug Report).

In Portugal, cannabis, cocaine and ecstasy are the most consumed drugs of abuse, with cannabis at the top of the list (Annual Report of the Portuguese Institute for Drugs and Dependence, 2008). However, Portugal is still one of the European country with less drug consume, apart of heroin consumption. Moreover, according with this report, during 2001 and 2007 the consumption of drug throughout life increased, with AMPH increasing 0.4% in total population and 0.7% in young adult population (Fig. 1) (Annual Report of the Portuguese Institute for Drugs and Dependence, 2008).

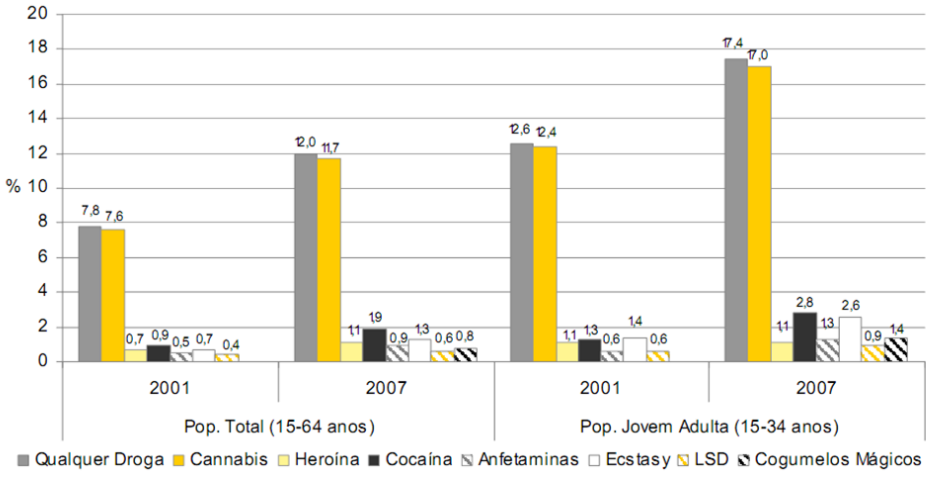


Figure 1. Prevalence of drug use in total and young adult population aged 15-34 years and throughout life (Annual Report of the Portuguese Institute for Drugs and Dependence, 2008).

1.1.2. Methamphetamine toxicity in the CNS

Psychostimulant drugs have several acute effects, which include well-being sensation, euphoria, alertness, hyperthermia, hypersexuality, increases in energy and locomotor

activity and decreases in appetite (Yamamoto et al., 2010). In addition, these drugs have cardiovascular effects, such as increased heart beet and blood pressure, and effects on metabolism with an increase in the levels of blood glucose and mobilization of fat. The chronic effects include extreme weight loss, dental problems, anxiety, confusion, mood disturbances, insomnia and violent behaviors, as well as the development of psychosis (Yamamoto et al., 2010) and addiction (Cunha-Oliveira et al., 2008).

Studies with human subjects have shown that METH chronic users demonstrate structural abnormalities in the brains, namely loss of gray matter, white matter hypertrophy and altered glucose metabolism in specific regions like hippocampus, prefrontal cortex, cingulated gyrus and amygdala. These findings could explain some of the problems identified in METH users, such as behavioral alterations, recall capacity, memory and performance deficits observed on verbal memory tests and executive functions like problem solving (Thompson et al., 2004). McCann et al (2008) also made neuropsychiatric tests to abstinent METH users and demonstrated modest deficits in memory, executive and motor function which are known to be modulated, in part, by the neurotransmitter DA (McCann et al., 2008).

It is known that many of the toxic effects of METH are due to the similarity of its chemical structure with the monoaminergic neurotransmitters, in particular with DA, which allows the entry of METH to the DA axons and also to mimic DA effects in the central nervous system (CNS) (Fig. 2) (Fleckenstein et al., 2007; Krasnova and Cadet, 2009).

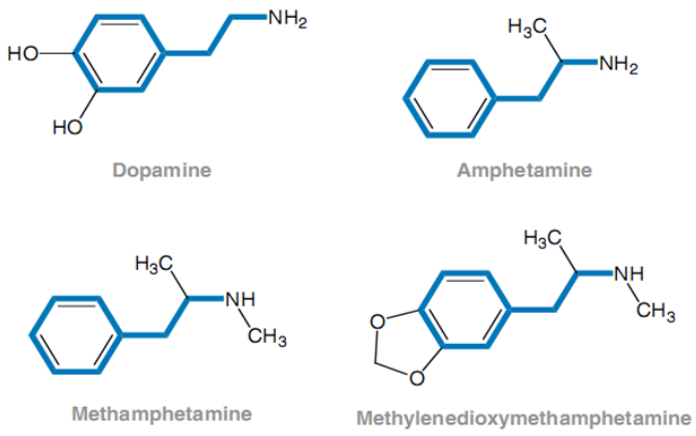


Figure 2. Chemical structure of dopamine and amphetamine related drugs. The common structure is represented in blue (Fleckenstein et al., 2007).

Under normal conditions, DA is released into the synaptic cleft due to neuronal activation, which then binds to DA receptors (D_1 and D_2 receptors). When the stimulus is ceased, the DA is removed from the synaptic cleft by the dopamine transporters (DAT) to the

cytoplasm, and then to vesicles by the vesicular monoamine transporter-2 (VMAT-2). METH can affect this normal mechanism in various forms, leading to neurotoxicity (Fig. 3)(Krasnova and Cadet, 2009).

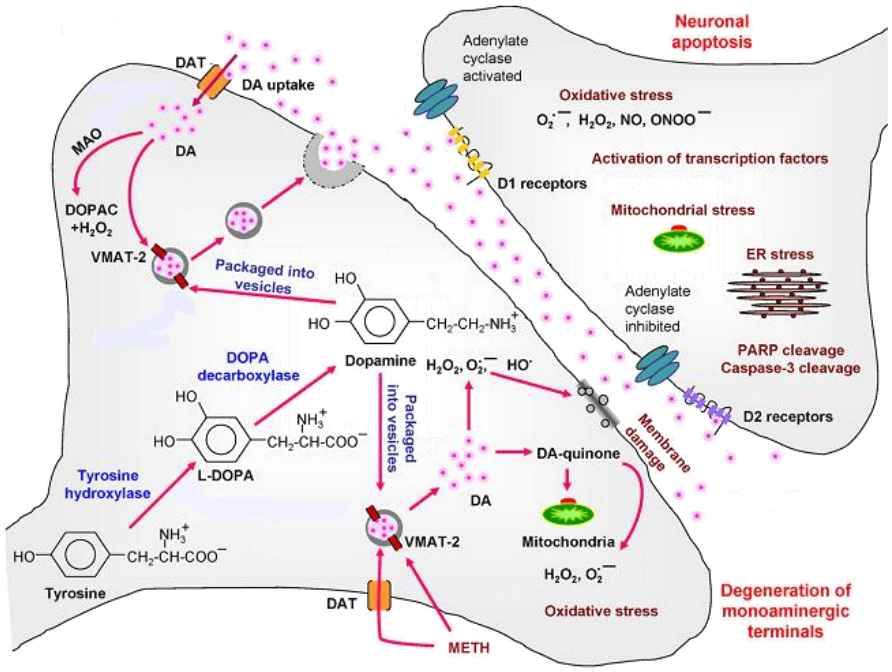


Figure 3. Schematic representation of cellular and molecular events involved in METH-induced DA terminal degeneration and neuronal apoptosis within the striatum. Legend: DA, dopamine; DAT, dopamine transporters; DOPAC, 2,4-dihydroxyphenylacetic acid; ER, endoplasmic reticulum; L-DOPA, L-3,4-dihydroxyphenylalanine; MAO, monoamine oxidase; METH, methamphetamine; VMAT-2, vesicular monoamine transporter 2 (adapted from Krasnova and Cadet, 2009).

Several studies suggest that METH toxicity involves the occurrence of three main events: an increase in extracellular and intracellular DA, an increase in extracellular glutamate (GLU) and hyperthermia (Yamamoto et al., 2010). The main cellular mechanisms by which METH induces long-term effects include: oxidative stress, excitotoxicity, mitochondrial dysfunction, hyperthermia, neuroinflammation and blood-brain barrier (BBB) dysfunction, among others (Quinton and Yamamoto, 2006; Krasnova and Cadet, 2009). These mechanisms can lead to neural damage or even to cell death.

1.1.2.1. Oxidative stress

METH can induce the production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and lipid peroxidation products, as well as the decrease in the levels of antioxidants in DAergic terminals (Yamamoto et al., 2010). The production of these molecules results from the entry of METH to the DAergic terminal and from its weak base

properties. After entry into the cell, METH diffuses into the vesicles where it will cause the disruption of vesicular proton gradient and VMAT-2 function. This leads to the exchange of DA by METH in the vesicle, causing the accumulation of DA in the cytoplasm, which is followed by the production of toxic metabolites due to DA auto-oxidation (Fleckenstein et al., 2007; Yamamoto et al., 2010). Moreover, the elevated levels of DA in the cytoplasm leads to the release of DA by DAT due to its inversion (Fleckenstein et al., 2007).

1.1.2.2. Mitochondrial dysfunction

Due to cationic lipophilic properties, METH can diffuse into mitochondria and be retained there, inducing an inhibition of mitochondrial electron transport chain enzyme complexes, leading to a decrease in the adenosine 5'-triphosphate (ATP) stores in the brain (Quinton and Yamamoto, 2006; Krasnova and Cadet, 2009). Additionally, mitochondrial dysfunction can also lead to the production of ROS (Wu *et al.*, 2007). Some studies showed that METH can lead to an increase in pro-apoptotic proteins, like Bax, Bad and Bid and a decrease in anti-apoptotic proteins, such as Bcl-2 and Bcl-$X_{L,}$ suggesting that the alteration of mitochondrial function induced by this drug of abuse can alter the mitochondria-dependent death pathway (Krasnova and Cadet, 2009).

1.1.2.3. Excitotoxicity

The excitotoxic damage results from GLU release and hyperactivation of GLU receptors, which induces an increase in intracellular Ca^{2+} levels, generation of free radicals, activation of dependent-Ca^{2+} enzymes, like kinases, lipases and proteases, and results in the activation of apoptotic pathways. Together, these events eventually cause the breakdown of cytoskeletal proteins, DNA damage and failure of cellular organelles (Quinton and Yamamoto, 2006; Krasnova and Cadet, 2009; Yamamoto et al., 2010). Also, our group has shown that METH alters the protein levels of the ionotropic glutamate receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and N-methyl-D-aspartate (NMDA) in the rat hippocampus (Simões et al., 2007), frontal cortex and striatum (Simões et al., 2008).

Excitotoxicity produced by METH can also occur by the production of nitric oxide (NO) due to the activation of nitric oxide synthase in a Ca^{2+}-mediated event. NO can react with superoxide to form peroxynitrite, and is possible that both can induce toxicity throughout their ability to oxidize tyrosine and cysteine residues on proteins (Yamamoto and Raudensky, 2008).

1.1.2.4. Hyperthermia

Hyperthermia has been pointed out as one of the most important events in the development of METH-induced neurotoxicity (Riddle et al., 2006; Yamamoto et al., 2010). Even though is not clear by which mechanisms METH induces hyperthermia, it seems that DA release and activation of DA receptors is a critical event. Moreover, it has been shown that hyperthermia facilitates METH-induced ROS production (Krasnova and Cadet, 2009). Other possible mechanism by which METH induces hyperthermia can be related with the skeletal muscle uncoupling mitochondrial protein 3 (UCP-3), protein responsible for the energy dissipation as heat. The use of UCP-3 $^{-/-}$ KO mice revealed that this strain does not sustain the elevation of temperature METH-induced, being protected against the lethal effect of METH caused by hyperthermia (Sprague et al., 2004).

Currently, there are new mechanisms that attempt to explain the neurotoxicity induced by METH, such as neuroinflammation and BBB dysfunction (Yamamoto et al., 2010).

1.1.2.5. Neuroinflammation

Neuroinflammation is the inflammatory response in the CNS after an injury in order to repair the damage produced. The neural cells participate in this inflammatory response, with neurons changing their gene expression and glial cells producing and releasing inflammatory mediators. Microgliosis and astrogliosis are beneficial in response to an injury, but in some cases microglial and astrocytes activation can be harmful, exacerbating the damage (Silva et al., 2010). Some studies have shown, that certain drugs of abuse, like METH can cause microglial and astrocytes activation (Hanisch, 2002; Thomas et al., 2004). The astrocytes respond to several forms of tissue damage, throughout proliferation, up-regulation of cell-type specific glial fibrillary acidic protein (GFAP) and in several cases can form a glial scar (Sofroniew, 2009). Indeed, several studies have shown that METH induces marked astrogliosis in striatum (O'Callaghan and Miller, 1994; Guillot et al., 2008; O'Callaghan et al., 2008), cortex (O'Callaghan et al., 2008), hippocampus (Simões et al., 2007; Gonçalves et al., 2010) and in different cortical regions (Stadlin et al., 1998; Sharma and Kiyatkin, 2009).

METH treatment can induce neuroinflammation as well by the release of GLU, which can act as an inflammatory intermediary of METH-induced toxicity to monoaminergic and nonmonoaminergic neurons (Yamamoto et al., 2010). The GLU released can activate the microglia, which in turn leads to an increase in the production and release of proinflammatory cytokines, like interleukine-1β (IL-1β), interleukine-6 (IL-6) and tumor

necrosis factor α (TNF-α)(Gonçalves et al., 2008), as well as to an increase in ROS, NOS, prostaglandins (Krasnova and Cadet, 2009; Yamamoto et al., 2010), NO and superoxide (Yamamoto and Raudensky, 2008). All of these elements are known to cause neuronal damage, and some studies have suggested that microglial activation seems to precede the appearance of damage produced by METH (Krasnova and Cadet, 2009; Yamamoto et al., 2010). However, despite these pieces of evidence, it remains unclear if the glia activation is a consequence or a cause of METH-induced neurotoxicity.

1.1.2.6. Blood-brain barrier disruption

Several studies have shown that METH can lead to BBB dysfunction and this can contribute to METH-induced neurotoxicity (Sharma and Ali, 2006). METH can have a more potent action in the CNS when compared to other related drugs, due to the methyl group in its chemical structure which induces high lipid solubility, allowing the rapid transport of the drug across the BBB (Martins et al., 2011).

Increased of the BBB permeability has been shown in rodents. However, at the present moment it is still not clear how METH leads to BBB damage, but some studies suggested that, at least in part, it is related with hyperthermia and ROS production in a METH-dependent way. Also, matrix metalloproteinases (MMPs), which are likely to be activated by oxidative stress and cytokine production, seems to be a possible mediator of BBB breakdown, because they can degrade the tight junction (TJ) proteins and the extracellular matrix, which supports the endothelial cells (ECs) that form the BBB (Yamamoto et al., 2010).

1.2. Blood-Brain Barrier

The brain is a fragile structure that needs to be protected from injury, and so it is important to maintain the homeostasis of the neuronal microenvironment (Boron and Boulpaep, 2004; Abbott et al., 2010). There are three principal barriers between blood and brain responsible for this regulation: the blood-cerebrospinal fluid barrier, the arachnoid barrier and the BBB (Fig. 4) (Abbott et al., 2006; Abbott et al., 2010).

The blood-cerebrospinal fluid barrier is formed by the epithelial cells of the choroid plexus, which lies in the four ventricles of the brain (Abbott et al., 2010). The choroid plexus is the major site of the cerebrospinal fluid (CSF) that fills the ventricular system (Boron and Boulpaep, 2004). This barrier is formed by fenestrated and leaky ECs, and TJs are present in epithelial cells of the plexus (Zheng, 2004; Abbott et al., 2010). On the other hand, the arachnoid barrier is a multilayer formed by closely adhering cells with TJs in the inner layer that lies under the dura mater involving the brain. Finally, the BBB is formed by the ECs that line the walls of the capillaries, leading to a large interface of blood-brain exchange (Abbott et al., 2006). BBB is present in almost all brain regions, with exception in those that control autonomic nervous system and endocrine glands of the body (Cardoso et al., 2010).

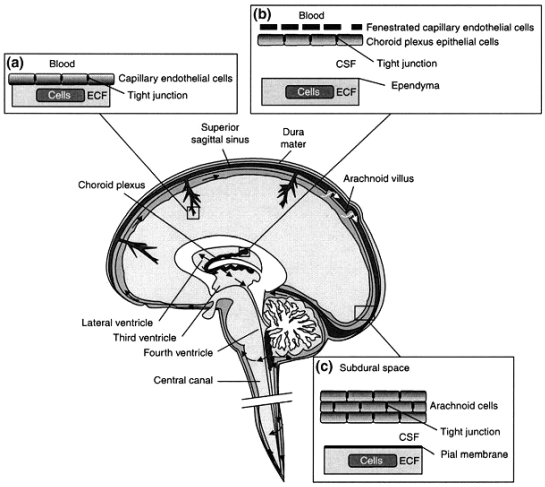


Figure 4. Barriers of the brain, (a) Blood-brain barrier, (b) blood-cerebrospinal fluid barrier and (c) arachnoid barrier. Legend: CSF, cerebrospinal fluid; ECF, extracellular fluid (Abbott et al., 2010).

The BBB should be thought as a physical and dynamic barrier, responsible for the maintenance of brain and spinal cord homeostasis and protection, because it controls the

exchange of organic solutes between the blood and brain, prevents the entry of toxic molecules and pathogenic organisms (Boron and Boulpaep, 2004; Dietrich, 2009), and supply the brain with metabolites, nutrients and hormones (Cardoso et al., 2010). It is also considered a "metabolic barrier", due to the presence of intracellular and extracellular enzymes, such as ecto-enzymes, monoamino oxidase and cytochrome P450 (Abbott et al., 2006).

1.2.1. Blood-brain barrier functions

Ion Regulation

The BBB has specific ion channels (Na^+, K^+, Cl^-, among others) and transporters (organic anion transporting polypeptides and organic anion transporters) (Fig. 13) (Bernacki et al., 2008; Abbott et al., 2010) which provide an optimal ionic composition for synaptic signaling that in turn allows maintenance of solute concentrations in the brain even when there are changes in plasma concentrations. BBB is also responsible for the pH control, and levels of Ca^{2+} and Mg^{2+} (Abbott et al., 2010).

Neurotransmitters

The BBB is important to maintain the equilibrium of GLU levels between the blood plasma and the brain, because if this neurotransmitter is released in an uncontrolled way it can lead to permanent damage in the neural tissue (Abbott et al., 2006). The BBB also expresses efflux transporters for GABA, and transporters for NE at the abluminal membrane and for 5-HT in both luminal and abluminal membranes (Bernacki et al., 2008).

Macromolecules

Many of the plasma proteins, like albumin, pro-trombin and plasminogen, are prevented to enter in the brain by the BBB, since they can damage the neural tissue, leading to cellular activation, which can culminate in apoptosis (Abbott et al., 2010).

Neurotoxins

Neurotoxins, like endogenous metabolites, or proteins and xenobiotics ingested in the diet or acquired from the environment, are kept out of the brain by the BBB. Some of the components that are important in this function are the ATP-binding cassette transporters, which pump actively these agents out of the brain (Fig. 5)(Abbott et al., 2010; Cardoso et al., 2010).

Brain nutrition

There are specific transport systems expressed in the BBB that allow an adequate supply of the brain with essential water-soluble nutrients and metabolites necessary to the nervous tissue (Fig. 5)(Abbott et al., 2010; Cardoso et al., 2010).

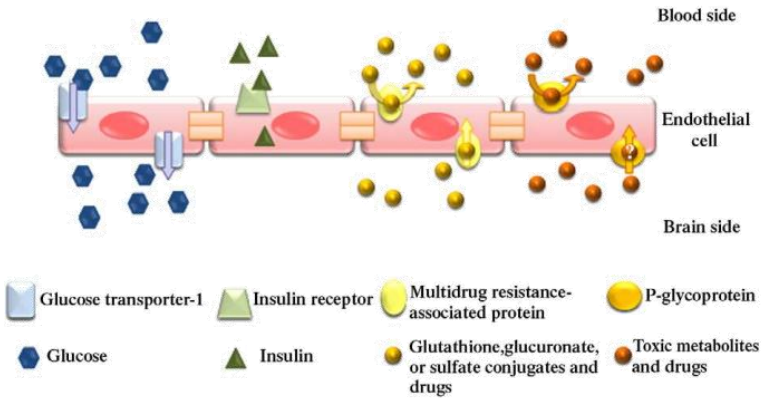


Figure 5. Some of the specific transporters and receptors present in the endothelial cells of the blood-brain barrier (Cardoso et al., 2010).

1.2.2. Blood-brain barrier structural components

The BBB is a specialized system of brain microvascular endothelial cells (BMVEC) and other components, such as astrocytes, basal lamina, pericytes and neurons, forming the functional neurovascular unit (Fig. 6) (Persidsky et al., 2006; Abbott et al., 2010). This specific and stable structure allows BBB to protect the brain. However, BBB also requires plasticity for adaptation to changing conditions. These different properties are due to many structural and functional aspects of the BBB components (Bernacki et al., 2008).

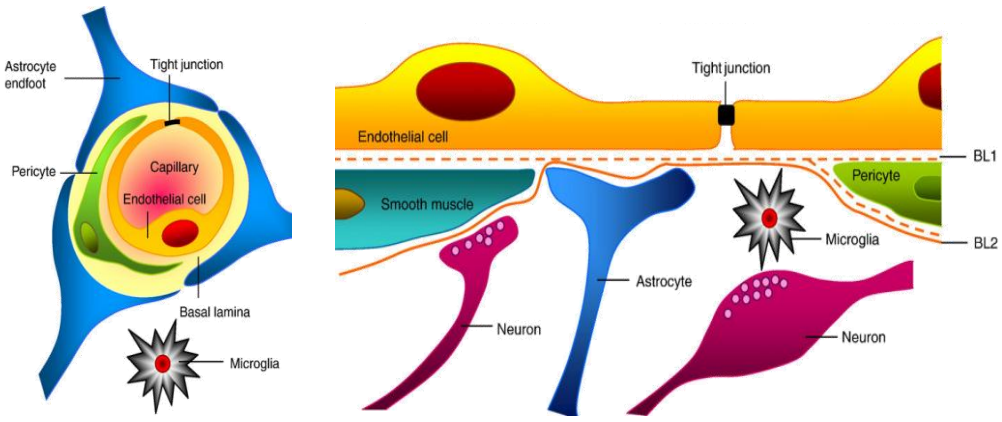


Figure 6. Schematic representation of the neurovascular unit. Legend: BL 1, basal lamina 1; BL 2, brain parenchyma (Abbott et al., 2010).

1.2.2.1. Brain Endothelial Cells

Brain ECs are situated at the interface between the blood and the brain, performing essential functions, such as, barrier, transport of micronutrients and macronutrients, receptor-mediated signaling, leukocyte trafficking and osmoregulation (Persidsky et al., 2006). There are structural features of the BMVEC which allow them to perform these functions: the presence of tight (TJs) and adherens junctions (AJs); a polarized surface between the apical and basal laminas (Fig. 7) (Boron and Boulpaep, 2004; Persidsky et al., 2006; Cecchelli et al., 2007); the negative surface charge that repulse negatively charged compounds (Cardoso et al., 2010). These cells are linked to the basal lamina by transmembrane proteins (selectins, immunoglobulin superfamily and integrins) that also participate in the intercellular adhesion (Cardoso et al., 2010).

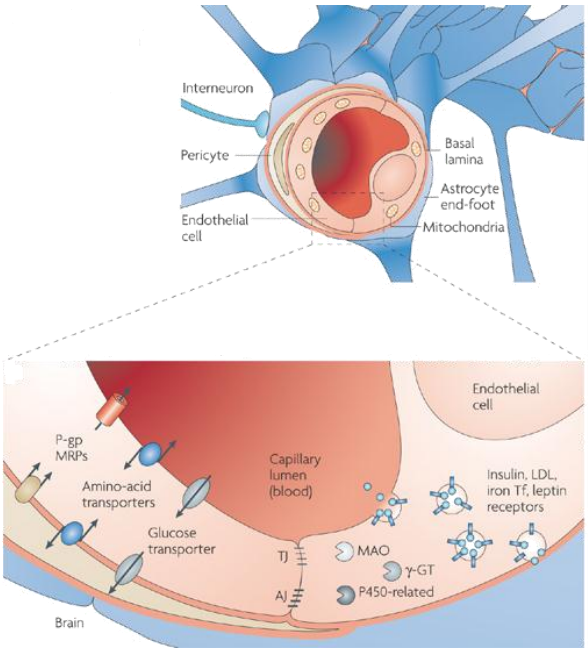


Figure 7. Neurovascular unit and molecules expressed by cerebral endothelial cells (ECs). Cerebral ECs express tight junctions that seal the paracellular pathway. There are also adherent junctions which stabilize cell–cell interactions. The presence of transporters and enzymes allow the blood-brain barrier to perform their functions. Large molecules can also be transferred to the brain by adsorptive-mediated transcytosis or receptor-mediated transcytosis which include receptors to insulin, low-density lipoprotein (LDL), iron transferrin (Tf) and leptin. Legend: MAO, monoamine oxidase; ϒ-GT, ϒ-glutamyltranspeptidase; P-gp, P-glycoprotein; MRP, multidrug resistance-associated protein family (Adapted from Cecchelli et at., 2007).

Brain ECs also express a high number of vesicles (caveolae), which are important in the transport of fluids and other substances from the blood to the brain (Simionescu and Antohe, 2006).

Other structural characteristics are the high number and volume of mitochondria, which increases the energy potential to break down molecules and to selective transport systems of nutrients and other compounds that function actively from into and out of the brain (Cardoso et al., 2010), as well as the lack of fenestration and uniform thickness (Persidsky et al., 2006; Bernacki et al., 2008).

a) Tight junctions

The TJs (Fig. 7), located on the apical region of ECs, are responsible for BBB paracellular permeability of water-soluble molecules between the ECs from the blood plasma to the brain extracellular fluid (Persidsky et al., 2006; Bernacki et al., 2008; Abbott et al., 2010). They are responsible for ECs polarity, which is revealed by the non-uniform distribution of proteins (e.g. transporters) between the luminal and abluminal membranes (Fig. 7). TJs confer the high electrical resistance of the ECs, and their integrity depends on extracellular Ca^{2+} ion concentration (Bernacki et al., 2008). Other functions have been recently proposed for TJs regarding to cell signaling. This signaling is bi-directional with the TJs receiving signals from the cell interior in order to regulate is assembly and function, or transmitting information to the cell interior in order to regulate gene expression and subsequent cell responses. So, it has been proposed that these junctions are involved in control of gene expression, cell proliferation and differentiation (Cardoso et al., 2010).

This group of proteins includes transmembrane proteins, like claudins, occludin and junctional adhesion molecules (JAMs), and intracellular proteins such as zonula occludens (ZO)-1, -2 and -3 (Fig. 8). The ZO proteins are accessory proteins that link the TJ transmembrane proteins to the actin cytoskeleton (Ballabh et al., 2004; Bernacki et al., 2008; Abbott et al., 2010), which is the key cytoskeleton protein for maintenance of structural and functional integrity of the endothelium (Huber et al., 2001; Bernacki et al., 2008).

Claudins

Until now, twenty four members of this protein family have been identified. Claudins are 22 kDa phosphoproteins consisting of four transmembrane domains and are the most important component of TJs. The claudins from one EC bind to the claudins of the adjacent EC, creating the primary seal of TJs, and the carboxyl terminal of each claudin binds to ZO proteins (Ballabh et al., 2004; Bernacki et al., 2008). Claudins appear to be express in tissue-specific manner with most of the cells type possessing more than two isoforms (Cardoso et al., 2010). Claudin-1, -3 and -5 are mainly express in BMVEC (Ballabh et al., 2004; Persidsky et al., 2006; Bernacki et al., 2008), and some studies have revealed that the loss of claudin-3 or -5 compromised the BBB function (Abbott et al., 2010). Moreover, claudin-3 and -5 appears to contribute to the high resistance of the TJ (Abbott et al., 2006) and claudin-1 and -5 beside the important role in the maintenance of normal BBB function, seem to be important in angiogenesis and in disease processes with increase in vessel permeability (Cardoso et al., 2010).

Occludin

Occludin is a regulatory protein responsible for the increased electrical resistance and decreased paracellular permeability across the barrier (Huber et al., 2001; Bernacki et al., 2008). It is a phosphoprotein of 60-65 kDa with four transmembrane domains. The two extracellular loops of claudin and occludin bind to form the paracellular component of the TJ, and, as described for claudins, its cytoplasmic domain connects with ZO proteins allowing the connection with the cytoskeleton (Ballabh et al., 2004; Persidsky et al., 2006; Bernacki et al., 2008).

Expression of occludin has been identified in rodents and adult human brain, but not in human newborns or fetuses (Ballabh et al., 2004; Bernacki et al., 2008), and its expression is much higher in brain ECs when compared to nonneural tissues (Ballabh et al., 2004).

It appears that occludin expression is not essential for TJ formation, contrary to claudins that seems to be the primary seal of TJ, but is crucial to enhance the TJ tightness (Hawkins and Davis, 2005; Persidsky et al., 2006; Yamamoto et al., 2008), since it has been shown that occludin guarantee decreased paracellular permeability (Huber et al., 2001) and high electrical resistance of the BMVEC monolayers (Persidsky et al., 2006). Together, the presence of claudins and occludin seems to be essential for the correct function of the TJs and, consequently, the proper function of the BBB (Bernacki et al., 2008).

Junctional adhesion molecules (JAMs)

JAM and the endothelial selective adhesion molecule are TJ-associated proteins and belong to the immunoglobulin superfamily (Abbott et al., 2006; Cardoso et al., 2010). The JAM proteins have a molecular weight around 40 kDa, and have a single transmembrane domain with a loop in its extracellular portion. Three members of this superfamily were identified in rodent brains: JAM-1, -2 and -3 but only JAM-1 and -3 were found in brain vessels (Ballabh et al., 2004; Bernacki et al., 2008). JAM are involved in cell adhesion, with JAM-1 being important on the TJ organization and formation (Cardoso et al., 2010), monocyte migration through the BBB (Ballabh et al., 2004; Bernacki et al., 2008) and in development processes (Persidsky et al., 2006).

Cytoplasmic accessory proteins

The cytoplasmic proteins associated with TJs are ZO proteins, cingulin and 7H6, among others (Ballabh et al., 2004).

ZO-1, -2 and -3 belong to a family of proteins known as membrane-associated guanylate kinase-like protein which acts as recognition proteins for TJ location and as support structure for signal transduction proteins (Huber et al., 2001). They contain three PDZ domains (PDZ1, PDZ2 and PDZ3 – postsynaptic density-95, disc large and zonula occludens-1 protein), one SH3 domain (SRC homology 3 domain) and one guanyl kinase-like domain. These domains function as protein binding molecules promoting the plasma membrane integrity (Ballabh et al., 2004; Bernacki et al., 2008), which is important for protein arrangements. It was demonstrated that ZO-1 is mainly expressed in endothelial and epithelial cells, which normally form the TJ assembly and connects the transmembrane TJ proteins with the actin cytoskeleton. These studies also showed that ZO-2 has a significant homology with ZO-1 and binds to transmembrane TJ proteins, whereas ZO-3 is concentrated in TJs of epithelial cells (Persidsky et al., 2006). All of the membrane proteins bind to ZO proteins, and actin binds to the carboxyl terminal of ZO-1 and ZO-2, and so this cross-link of elements provides structural support to the ECs (Ballabh et al., 2004).

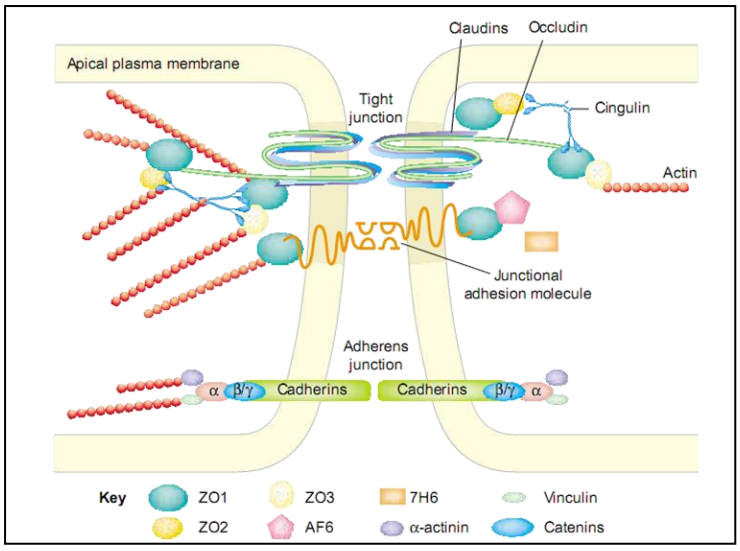


Figure 8. Schematic representation of tight and adherent junctions present in the endothelial cells. Legend: ZO, Zonula-Ocludens (Huber et al., 2001).

b) Adherens junctions

Bellow the TJ, in the basal region of lateral plasma membrane, there are the AJs which are responsible for holding the cells together giving the tissue structural support (Abbott et al., 2010). Thus, these proteins are another example of tightening structures between ECs, which are formed by a large family of membrane proteins, cadherins (Fig. 9). They are also responsible for the initiation of cell polarity and regulation, in part, of cell paracellular permeability and for the contact inhibition (natural process of cell growth arrest) during vascular growth and remodeling being, therefore, important for the correct organization

of new vessels in angiogenesis (Cardoso et al., 2010). The cadherins interact with each other on the surface of adherent cells in the presence of Ca^{2+}, and are anchored to the cytoplasm by the catenins. Intracellular Ca^{2+} binds to beta or gamma catenin which links to actin cytoskeleton *via* alpha catenin (Ballabh et al., 2004; Bernacki et al., 2008).

These junctions are fundamental for TJ formation, and disruption of AJs leads to barrier disruption (Abbott et al., 2010).

It is possible that AJ and TJ proteins contribute to the human BBB due to their proximal spatial organization, and also some studies revealed that ZO-1 and catenins interaction influence TJ assembly (Ballabh et al., 2004; Bernacki et al., 2008).

c) Caveolae

Caveolae (Fig. 9) are invaginations of the plasma membrane with a diameter between 50 - 100 nm (Parton and Simons, 2007; Hansen and Nichols, 2010), and exist as single, chain or as clusters. Morphologically, caveolae are characterized as spherical or flask-shape (Bauer et al., 2005; Stan, 2005; Parton and Simons, 2007) and with no clear coat (Parton and Simons, 2007). They are present in most of the mammalian tissue and cells, and are abundant in muscle, endothelia, adipocytes (Razani et al., 2002; Deurs et al., 2003; Hansen and Nichols, 2010), and type I pneumocytes (Razani et al., 2002).

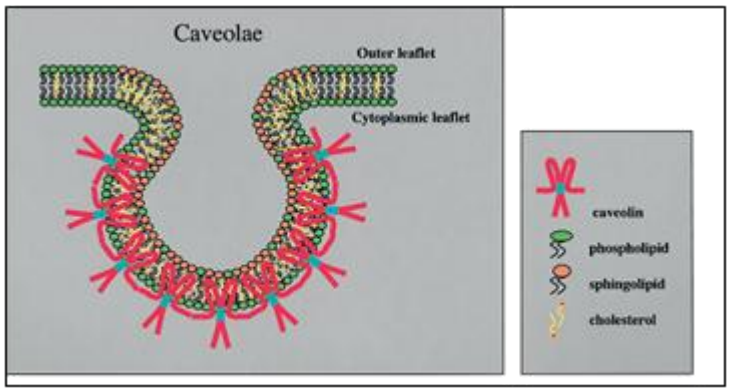


Figure 9. Organization of caveolae membranes (Razani et al., 2002).

Caveolae have been implicated in many functions, like endocytosis, transcytosis, Ca^{2+} signaling, cholesterol transport and homeostasis, and signaling transduction events (Deurs et al., 2003; Parton and Simons, 2007). Caveolin (Cav)-1 interacts and modulates directly some signaling molecules present on the caveolae, such as endothelial nitric-oxide synthase (eNos), H-Ras, α subunit of G-proteins, among others, leading to an inhibition of downstream signaling (Razani et al., 2002). Several studies with cav-$1^{-/-}$ KO mice show an increased permeability through the paracellular pathway, which could be explain by the excess of NO production due to the non-existing inhibition of eNOS (Deurs et al., 2003; Mehta and Malik, 2006). Moreover, others have demonstrated that the same mice show a

defect in the morphology of TJs in lung capillaries. Together, these studies suggest that cav-1 is not only involved in caveolae mediated transcytosis, but can also modulate negatively the paracellular permeability (Mehta and Malik, 2006).

Cav is the major structural protein component of the caveolae and is necessary for the formation of invaginated caveolae (Deurs et al., 2003). Gene family of cav has three members: Cav-1, -2 and -3. Cav-1 and -2 are expressed in most cell types, whereas cav-3 is muscle specific (skeletal-, cardic- and smooth-muscle cells). Several studies demonstrated the important role of cav proteins in the caveolae structure, but only cav-1 and -3 are sufficient to induce the formation of caveolae, whereas cav-2 alone is not able to exert this function (Deurs et al., 2003; Parton and Simons, 2007).

Caveolins are multiply acylated 22-24 kDa proteins (Hansen and Nichols, 2010), inserted in the cytoplasm membrane with both N-terminus and C-terminus (attached by palmitoyl groups) in the cytoplasm. N-terminal is a highly conserved region and contains the scaffolding domain, which is responsible for the interaction with signaling proteins and might be important in cav oligomers formation and interaction with cholesterol (Fig. 10) (Deurs et al., 2003; Parton and Simons, 2007).

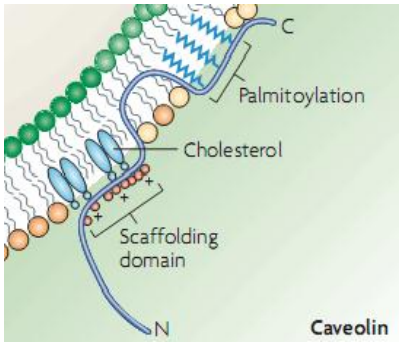


Figure 10. Caveolin structure (Adapted from Parton and Simons, 2007).

Caveolae are immobilized in the plasma membrane due to actin cytoskeleton, which anchorage is mediated by filamin (ligand of cav). Caveolae can be internalized due to specific stimulus (Deurs et al., 2003; Parton and Simons, 2007) and their fission process is possible associated with dynamin activity (Deurs et al., 2003; Mehta and Malik, 2006; Parton and Simons, 2007), which is a GTPases responsible for the scission of a vesicle formed in the membrane in the endocytosis phenomena.

1.2.2.2. Astrocytes

The close anatomical association of astrocytes with the ECs (Fig. 6) allows them to influence each other's structure, being important on the development and/or maintenance of the BBB characteristics (Haseloff et al., 2005; Hawkins and Davis, 2005; Persidsky et al., 2006). Several *in vitro* studies showed that brain ECs co-cultured with astrocytes or cultured with astrocytes-conditioned medium (ACM) display better barrier properties, like increased in tight formation, increased in electrical resistance and decrease in

permeability, among others (Nag, 2011). Some *in vivo* studies in adult mice suggested that astrocytes can regulate BBB properties through specific bone morphogenetic proteins (BMP) signaling mechanisms on astrocytes end-feet and when disrupted cause BBB leaks (Sofroniew and Vinters, 2010).

Confocal images, shows that astrocytes form a rosette-like structure on the vessel wall due to the termination of the end-feet of astrocytes into multiple looped processes. The vessels can be covered with multiple end-feet originated from different astrocytes, once each astrocyte can contact with different EC through different end-feet (Nag, 2011).

Astrocytes present a molecular and structural heterogeneity which is responsible for the astrocytes polarity. In their end-feet luminal surface is expressed a variety of specialized proteins, like glucose transporter-1, P-glycoprotein, purinergic receptors P2Y, α_1 and β adrenergic receptors (Nag, 2011). The perivascular end-feet of astrocytes due to their localization near the ECs, show others specific characteristics, namely high density of water channel aquaporin 4 and Kir4.1 potassium channel, which are important in ion and volume regulation (Abbott et al., 2006).

This cell interaction also enhances endothelial cell TJs and reduces gap junctional area, which in turn increases the number of astrocytic membrane particle assemblies and astrocyte density (Persidsky et al., 2006). Astrocytes can also upregulate many BBB features like expression and polarized localization of transporters and specialized enzyme systems (Abbott et al., 2006).

Many studies have confirmed the importance of astrocytes in the BBB formation and function at a physical, transporter and metabolic levels. Also connections ECs - astrocytes are essential for the morphogenesis and development of vessel wall, and both can originate essential factors that can affect each other (Fig. 11) (Abbott et al., 2006; Bernacki et al., 2008).

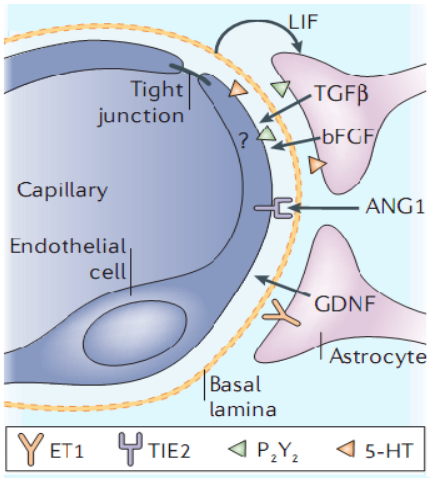


Figure 11. Bidirectional astroglial-endothelial interaction. Legend: ANG1, angiopoetin 1; bTGF, basic fibroblast growth factor; GDNF, glial cell line-devived neurotrophic factor; LIF, leukemia inhibitory factor; TGFβ, transforming growth factor-β (Adapated from Abbott et al., 2006).

The continuing induction of the ECs by the astrocytes during adult life is crucial for normal function, and in many neurological diseases the BBB dysfunction is caused by alterations in the normal endothelial-glial interaction (Abbott et al., 2006), such as in multiple sclerosis, epilepsy, brain tumors, Parkinson and Alzheimer´s disease and in AIDS-related dementia (AIDS, acquired immune deficiency syndrome) (Silva et al., 2010; Sofroniew and Vinters, 2010; Nag, 2011). Thus, it is crucial to understand the crosstalk between ECs and astrocytes.

1.2.2.3. Basal lamina – The extracellular matrix

The extracellular matrix of the basal lamina also interacts with the ECs (Fig. 6). This matrix is formed by three opposed laminas, composed by different classes of molecules, playing as an "anchor" to the BMVEC via interaction of elastin and collagen type IV which are structural proteins. Moreover, the specialized proteins, like fibronectin and laminin, and the proteoglicans also compose the matrix (Cardoso et al., 2010). The interaction between the ECs and the proteins from the matrix is responsible for stimulation of many intracellular signaling pathways (Hawkins and Davis, 2005; Persidsky et al., 2006).

The matrix proteins stimulate the expression of endothelial TJs proteins, and so it is possible that these proteins are likely involved in TJ maintenance (Hawkins and Davis, 2005; Persidsky et al., 2006).

1.2.2.4. Pericytes

Pericytes are flat, undifferentiated and contractile connective tissue cells that develop around the capillary walls, being embedded within the basal lamina of microvessels (Fig. 6). Gap junctions provide direct connections between the cytoplasm of pericytes and ECs, enabling the exchange of ions and small molecules. They also exhibit long cytoplasmic processes, which can contact with ECs and also penetrate in basal membrane (Ballabh et al., 2004; Bergers and Song, 2005; Persidsky et al., 2006).

Pericytes can migrate away from brain microvessels in response to hypoxia (Gonul et al., 2002) or brain trauma (Dore-Duffy et al., 2000), which are situations associated with increased BBB permeability. In fact, these cells are important in the induction and/or maintenance of barrier properties, acting in a similar way as astrocytes (Hawkins and Davis, 2005; Persidsky et al., 2006). Moreover, the association between pericytes and blood vessels is important in the regulation of ECs proliferation, survival, migration, differentiation and vascular branching (Persidsky et al., 2006).

1.2.2.5. Neurons

A close relationship between the blood flow and neurons is requiring due to the high levels of brain activity and high metabolic needs. It has also been suggested that the interaction between the neurons and the vasculature is important for the modulation of the BBB function by inducing the expression of unique enzymes in the ECs (Hawkins and Davis, 2005; Persidsky et al., 2006; Cardoso et al., 2010).

Different types of neurons, such as noradrenergic, serotonergic, cholinergic and GABAergic, can innervate the BMVEC and/or be associated with astrocytic processes (Fig. 6) (Hawkins and Davis, 2005; Persidsky et al., 2006).

In conclusion, all the previous described components of the neurovascular unit and their interactions are responsible for the dynamic regulation of the cerebral microvascular permeability (Fig. 12)(Hawkins and Davis, 2005).

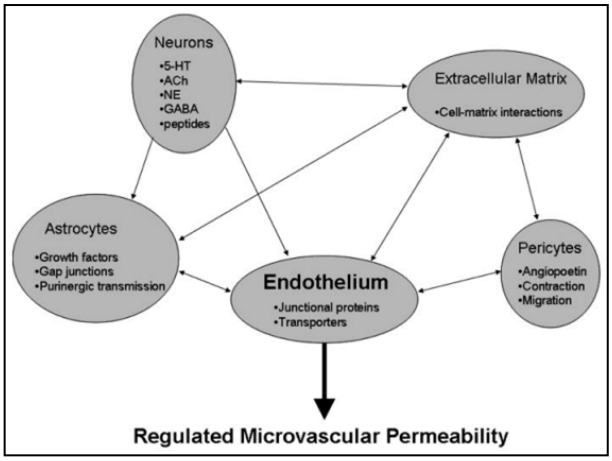


Figure 12. Contribution of each component of the neurovascular unit to the dynamic regulation of microvascular permeability (Hawkins and Davis, 2005).

1.2.3. Blood-brain barrier transport systems

The BBB acts also as a dynamic barrier because it offers several potential routes of transport, making a selection of the molecules that can enter the brain tissue, and so maintaining the brain homeostasis (Fig. 13 and 14).

The paracellular pathway (Fig. 14) is limited by the TJs, forcing most of the molecules to take another route (Abbott et al., 2006). In turn, the transcellular pathway allows the entry of lipophilic substances, including drugs like barbiturates and ethanol, and also the diffusion of small molecules such as O_2 and CO_2 (Fig. 13) (Huber et al., 2001; Abbott et al., 2006).

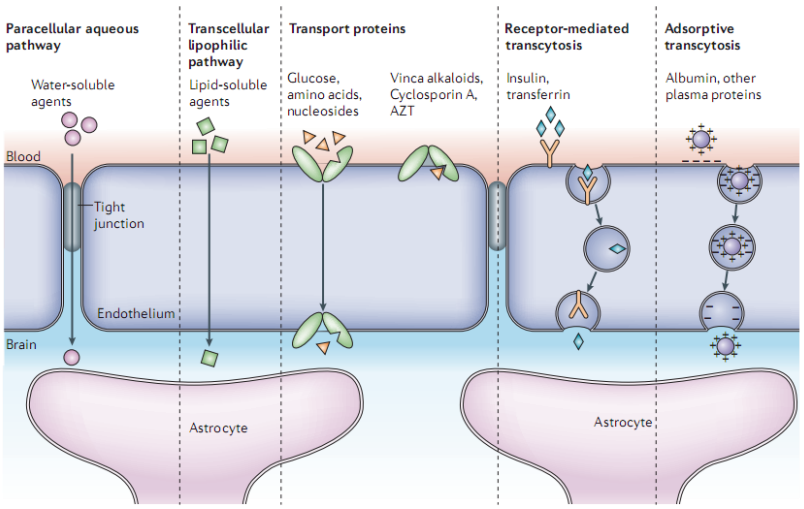


Figure 13. Pathways across the blood-brain barrier (Abbott et al., 2006).

BBB endothelium also express a variety of specific transporters to supply the brain with several nutrients like glucose, amino acids, organic anions and cations, among others (Huber et al., 2001; Abbott et al., 2006). The expression of these transporters is polarized and so they can be expressed in the luminal and/or in the basal membrane, and also their orientation controls the direction of the molecules transport (Fig. 14)(Abbott et al., 2010).

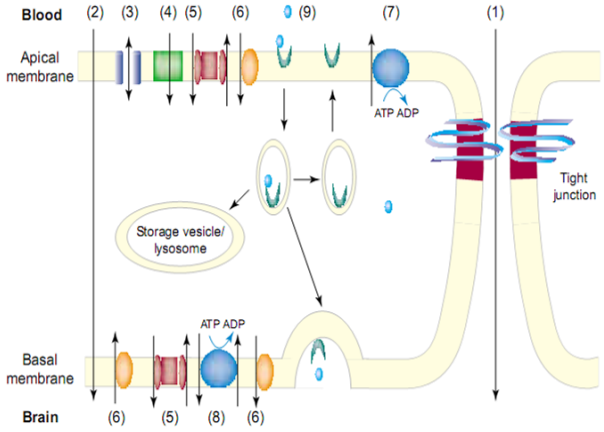


Figure 14. Blood-brain barrier transport mechanisms: 1. Paracellular diffusion; 2. Transcellular diffusion; 3. Cation channels; 4. Ion symports; 5. Ion antiports; 6. Facilitated diffusion active transport; 7. Active transport; 8. Active antiport transport; 9. Endocytosis (receptor or adsorptive mediated) (Huber et al., 2001).

The large hydrophilic molecules normally are excluded, but they can enter by a mechanism of endocytosis, which can be specific (receptor-mediated transcytosis) or less specific (adsorptive-mediated transcytosis) (Huber et al., 2001; Abbott et al., 2006; Abbott et al., 2010). The receptor-mediated transcytosis mechanism relies on the binding of the macromolecular ligand to the specific receptors on the surface, leading to an endocytic event. On the other hand, the less specific transcytosis needs an excess of positive charge on the molecule, which will allow it to interact with binding sites on the cell surface (Fig. 13 and 14) (Abbott et al., 2010)

1.3. Alteration of blood-brain barrier by methamphetamine: Underlying mechanisms

The most common cellular mechanisms involved in METH-induced neurotoxicity have been previously discussed in section 1.1.2. , but more recently another toxic aspect of this drug of abuse was raised. In fact, we have now new and strong evidence pointing to a deleterious effect of METH on BBB function.

Under several pathological conditions, such as METH consumption, BBB function can be altered (Dietrich, 2009). However, the mechanisms underlying the effect of this drug of abuse on BBB are still poorly understood and many hypothesis have been proposed, mainly related with oxidative stress, hyperthermia and MMPs (Yamamoto et al., 2010).

Mahajan and co-authors (2008) firstly suggested that modulation of TJ proteins expression induces alteration in BBB permeability. They observed that METH induced a decrease in the resistance of the BMVEC in a dose dependent manner with maximal decrease at a concentration of 50 nM during 48 h. Moreover with a concentration of 10 nM METH, they observed a decrease in claudin-5, occludin and ZO-1 expression. Also an increase of Rho-A (important in regulation of actin cytoskeleton) expression were observed after METH exposure (Mahajan et al., 2008).

It is known that oxidative stress and ROS production are increased by METH in different brain areas, which can also contribute to alterations in the BBB function (Dietrich, 2009). In fact, Ramirez and collaborators (2009) proposed that ROS production induced by METH could occur in the brain endothelium leading to BBB compromise due to TJs and cytoskeleton modifications via activation of myosin light chain kinase. They observed that mice exposure to METH induces an increase in BBB permeability, and when a primary human BMVEC were exposed to METH there was ROS production, decrease in TJ proteins levels (claudin-5 and occludin) and decrease in BBB resistance. Moreover, the same authors observed that antioxidants prevent ROS formation *in vivo* and *in vitro* and restore monolayer integrity (Ramirez et al., 2009). Therefore, these results suggest that oxidative stress contributes to BBB dysfunction. Both studies showed that METH interferes with BBB functionality by affecting the TJs, and also, that this injury induces an increase in monocyte transendothelial migration, which can initiate many pathological processes (Aghajanian et al., 2008).

Other studies have suggested that BBB disruption is the result of METH-induced hyperthermia (Bowyer and Ali, 2006; Sharma and Kiyatkin, 2009), which is an important symptom of acute METH consume as well as an important factor implicated in neurotoxicity. These authors demonstrated that hyperthermia affects BBB permeability allowing the entrance of substances into the brain inducing neuronal damage. Sharma and Kiyatkin (2009) demonstrated that Evans blue content and albumin staining in the brain of rats treated with METH raised significantly with temperature increase (significant differences between rats injected at 23^{o}C and 29^{o}C), reveling a relationship between temperature and BBB disruption.

Another possible mediator of the BBB damage is the MMPs, which cleave the majority of extracellular matrix components, basal lamina and TJ proteins in ECs resulting in the increase of BBB permeability (Candelario-Jalil et al., 2009; Rosenberg, 2009). These endopeptidases are produced by all cell-types of the neurovascular unit, but the

expression is different, according with the cell and the stimuli that induce the MMPs production (Rosenberg, 2002; Candelario-Jalil et al., 2009). Few studies have been performed in order to understand the role of MMPs in the disruption of the BBB. Yang and co-authors (2007) proposed that the BBB damage was due to TJ proteins degradation mediated by MMPs. To test this hypothesis they used a model of ischemic/reperfusion, where they demonstrated that the increase in MMP-2 activity and in less extend of MMP-9, correspond with early opening of the BBB. A second opening of the BBB was seen at 24 h associated with increase of MMP-9. Moreover, it was verified that BB-1101, a MMPs inhibitor, reduced the BBB opening. They concluded that the changes in TJ proteins, namely claudin-5 and occludin, are due to MMP-2 in an early reversible disruption of the BBB, and that MMP-9 is important in the delayed damage (Yang et al., 2007).

It was shown that METH increases the release of MMP-1 in neuron-astrocyte co-cultures (Yamamoto et al., 2010), and repeated METH treatment is accompanied by an increase in MMP-2 and -9 in the rat brain, which are the MMPs mainly involved in BBB damage (Gurney et al., 2006). Moreover, METH has been shown to alter the expression of TJ proteins and to increase the permeability of brain-derives primary microvascular endothelial cells (Yamamoto et al., 2010). Thus, knowing that MMPs can be activated by oxidative stress or inflammation, is plausible to suggest that METH can activate MMPs in a process mediated by oxidative stress or inflammation, which in turns leads to the breakdown of TJ proteins resulting in BBB disruption (Yamamoto and Raudensky, 2008; Yamamoto et al., 2010). Indeed, recently in our group it was demonstrated *in vivo* that METH treatment for 24 h induces in the hippocampus an increase in the active form of MMP-9 and also an increase in immunoreactivity. Moreover, this increase in MMP-9 immunoreactivity was prevented by the pre-incubation with an inhibitor of the MMP-9 (Martins et al., 2011).

More recently, Park and his collaborators (2011) showed in an immortalized human brain microvascular endothelial cells (hCMEC/D3) that METH (10 μM) treatment can activate the NAD(P)H- oxidase (NOX) complex, which is an important source of ROS in different cells, namely in the ECs. This activation leads to activation of ERK1/2 signaling and phosphorylation of cav-1. These events were followed by ROS production, alterations in the occludin protein levels, increase in the transendothelial migration of monocytes and compromise of the ECs function. Using inhibitors of NOX the authors realized that METH-induced alterations of occludin levels and transendothelial migration are prevented. Also, the silencing or disruption of functional caveolae phosphorylation and blockage of ERK1/2 activation protects against METH-induced production of ROS and disruption of occludin

levels. This work demonstrates the importance of NOX complex and functional caveolae in METH-induced oxidative stress in ECs (Park et al., 2011).

Based on the available results, it is clear that BBB breakdown can be mediated by different toxic mechanisms, like oxidative stress, hyperthermia and MMPs, but more studies are needed in order to full field the existing gaps, particularly related with the mechanisms that cause the BBB dysfunction induced by METH.

1.4. Objectives

BBB disruption is a critical event in the development and progression of several diseases that affect the CNS, being a consequence of the pathology or in other cases being a precipitating event. Regarding drugs of abuse, there are increasing evidences that psychoactive drugs, like METH, induce BBB dysfunction.

The work done so far in order to elucidate the effect of METH on the BBB is focused on the ECs, the most important cell type of the neurovascular unit. However, it is known that astrocytes play an important role in the BBB function. Thus, the present study aimed to evaluate the effect of METH on the ECs and astrocytes regarding cell viability and death, as well as the expression of important endothelial proteins. Moreover, we aimed to disclose how METH can affect the crosstalk between these two cell types. Our main goal was to contribute to a better understanding of the mechanism(s) involved in BBB dysfunction induced by METH.

CHAPTER 2

Material and Methods

Chapter 2

Material and Methods

2.1. Cell culture

2.1.1. bEnd.3 Cell line

A mouse brain endothelial cell line, bEnd.3 (Fig. 15) originally generated in 1990 (Montesano et al., 1990), was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GIBCO, Rockville, MD, USA), 100 U/ml penicillin, and 10 μg/ml streptomycin (GIBCO) in non-coating flask or in fibronectin (from bovine plasma, Sigma, St Louis, MO, USA) coating for the different studies. The cells were maintained in a humidified cell culture incubator (Thermo Scientific Forma Series II, Marietta, USA) at 37^{0}C and 5% CO_2/95% room air. Cells were allowed to reach confluence within 5-7 days and were used 3 days after reaching confluence. For all experiments, cells were in passage 24-35 and plated at a density of 1.0 x 10^4 cells/cm^2.

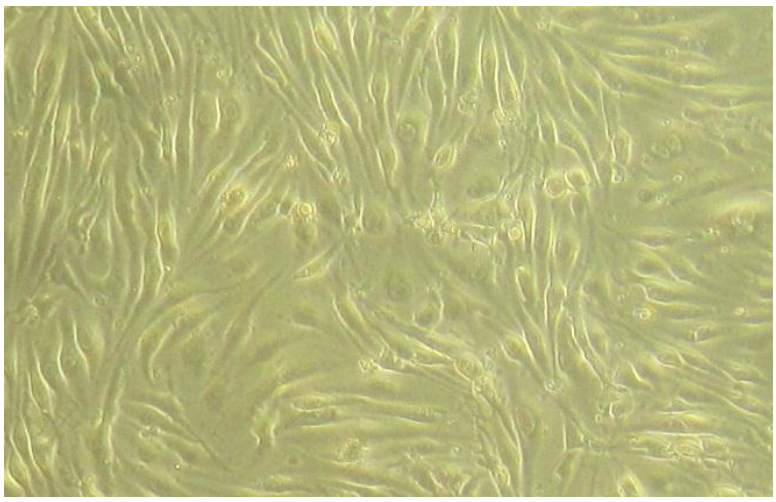


Figure 15. Representative image of bEnd.3 cell line (Magnification x100).

2.1.2. Primary Culture of Mice Cortical Astrocytes

All experiments were performed in accordance with European Community Guidelines (86/609/EEC) and the Portuguese law for the care and use of experimental animals (DL nº129/92). All efforts were made to minimize animal suffering and to reduce the number of animals used. The procedure used was adapted from others protocol (McCarthy and Vellis, 1980; Miranda et al., 2009).

Astrocytes were isolated from newborn (P3-P6) C57BL/6J mice. After decapitation, the cortices were isolated and treated with digestion solution [Hank's balanced saline solution (HBSS), 10 µl/ml gentamicin, 0.25% trypsin, 0.001% DNase] (GIBCO; GIBCO; Sigma and SAFC, Kansas, USA, respectively) during 20 min at 37^{o}C, with gentle agitation every 2 min on the first 10-15 min. After digestion, the cells were centrifuged at 700 rpm during 1 min, followed by other centrifugation with inhibitory digestion solution (HBSS supplemented with 10% FBS). Then, the pellet was dissociated in HBSS followed by centrifugation at 1000 rpm during 5 min, and finally the pellet was homogenized in astrocyte medium [DMEM (GIBCO) supplemented with 10% FBS and 10 ml/L gentamicin]. The astrocytes were cultured in a humidified cell culture incubator (Nuaire™ IR Autoflowat, Plymouth, USA) at 37^{o}C and 5% CO_2/95% room air for 8-14 days until the mixed glia culture reached confluence. The medium was changed 6 h after culture and then every 2 days. After reaching confluence, cultures were shaking for 4 h at 37^{o}C at 200 rpm on an orbital shaker. Suspended non-astrocytic cells were discarded, and the adherent astrocytes were washed with dissociation medium [HBSS supplemented with 1 mM Ethylenediaminetetraacetic acid (EDTA)] and trypsinized (0.1% trypsin in HBSS). Then the cells were plated in non-coating dishes, except in studies of cell death in which the dishes were coated with poly-D-lysine (Sigma), at different densities according with the experiments (Table 1) and were allowed to stabilize for 2 days.

Table 1. Cell densities used to plate the astrocytes according to the experiment.

Experiment	Nº cells/well	Multiwell culture plate	Medium volume	Final concentration
ACM	240 000 cells/well	6	2 ml	120 000 cells/ml
Immunocytochemistry	60 000 cells/well	12	100 µl + 900 µl	500 000 cells/ml
TUNEL assay	90 000 cells/well	12	1 ml	90 000 cells/ml

2.2. Viability and Cell death assays

2.2.1. MTT assay

The reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to tetrazole was use to evaluate cell viability.

The cells bEnd.3 were seeded on a 96–multiwell plate and were allowed to grown until reach confluence and then incubated with a wide range of METH [(+)-Methamphetamine hydrochloride, Sigma, USA] concentrations (in mM: 0.001, 0.050, 0.100, 0.250, 1 and 3) or ACM (in µM METH: 1, 50 and 250) for 24 h (Fig. 16A). After 24 h, the medium was removed and the cells were washed with Krebs solution [142 mM NaCl, 4 mM KCl, 1 mM $MgCl_2.6H_2O$, 1 mM $CaCl_2.2H_2O$, 10 mM Glucose, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4] and 0.5 mg/ml MTT solution was added to each well and incubated for 2-3 h. The crystals were then dissolved in dimethyl sulfoxide (DMSO) with vigorous resuspension. Absorbance was measured at 570 nm (reference 620 nm) on the Synergy™ HT multi-detection microplate reader (BioTek, Windoski, USA).

2.2.2. TUNEL assay

Cell death by apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay which detects DNA fragmentation. This method is based on the 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.

ECs were exposed to different METH concentrations (in mM: 0.001, 0.050, 0.250, 3) or to ACM (in µM METH: 1, 50 and 250) for 24 h (Fig. 16A). Regarding astrocytes, they were exposed to different METH concentrations (in µM: 1, 50, 250 and 500) for 24 h (Fig. 16B),

followed by TUNEL assay. Cells were fixed with 4% paraformaldehyde (PFA) and rinsed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 nM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Afterwards, cells were permeabilized with 0.25% Triton X-100 (Sigma) for 30 min at room temperature (RT) washed in PBS and 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 37^{0}C in a humidified chamber. Then, the cells were rinsed with TB buffer (300 mM NaCl and 30 mM sodium citrate) for 15 min followed by 5 min in PBS. Incubation with Fluorescein (1:100; Vector Laboratories) was performed for 1 h at RT and nuclei were counterstaining with 4 µg/ml Hoechst 33342 (Sigma) for 5 min. The lamellas were mounted in DakoCytomation fluorescent medium (Dako North America, Carpinteria, USA) and stored in dark at 4^{0}C. Fluorescent images were recorded using the camera Leica DMIRE2 incorporate on Leica fluorescence microscope Leica CTRMIC (Leica, Germany).

For astrocytes TUNEL analysis twelve independent microscopy fields were acquired per coverslip (n=3) with a magnification of x400 and for the ECs TUNEL analysis six independent microscopy fields were acquired per coverslip (n=3) with a magnification of x200. In order to evaluate cell death, the number of apoptotic cells were expressed in the percentages of total cells stained with Hoechst 33342.

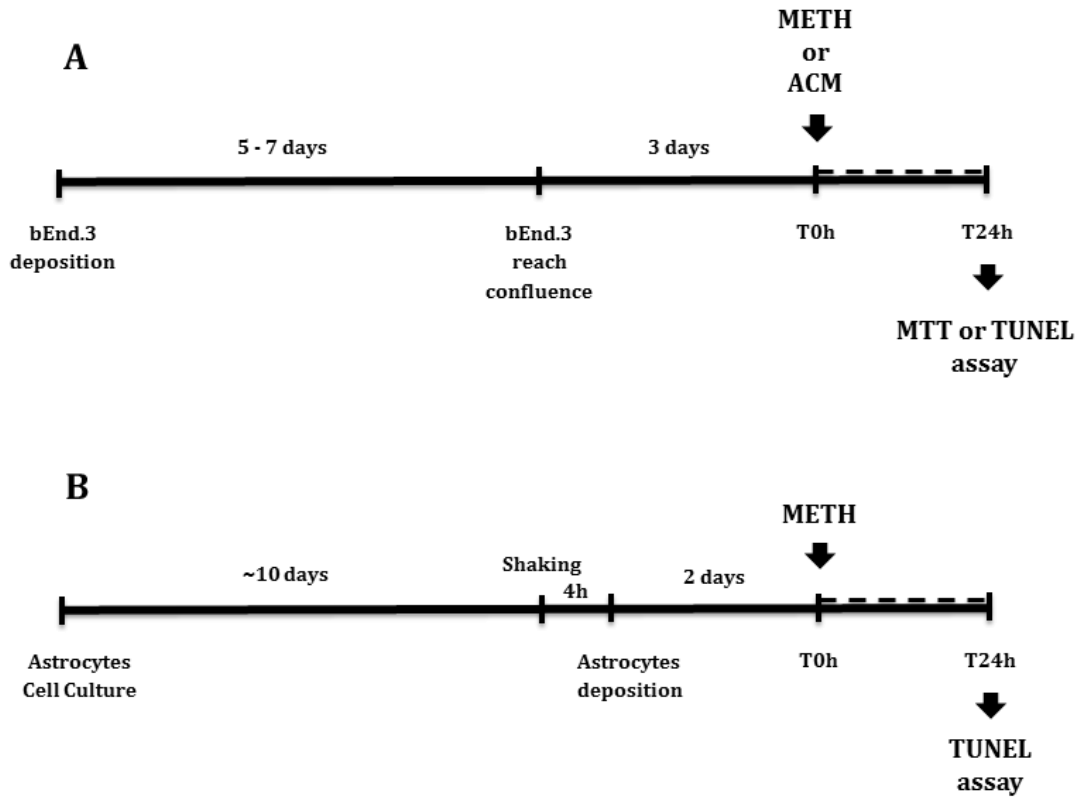


Figure 16. Schematic protocol used to performed MTT and TUNEL assay on bEnd.3 cells and TUNEL assay on the primary culture of mice astrocytes, in order to understand how methamphetamine (METH) induces toxicity in the two different models. (A) The bEnd.3 cell line was exposed to METH (in mM: 0.001, 0.050, 0.100, 0.250, 1 and 3) and to ACM (in µM METH: 1, 50 and 250) and the (B) astrocytes were incubated with METH (in µM: 1, 50, 250 and 500).

2.3. Astrocyte-conditioned medium assays

Astrocytes were platted and after 2 days, the medium was removed and the cells were washed with sterile PBS and incubated with different METH concentrations (in µM: 1, 50 and 250) for 24 h. Afterwards, the medium was collected, centrifuged at 3900 rpm for 10 min and supernatant was collected to a falcon tube and store at -80^{0}C until use.

2.4. Immunocytochemistry

2.4.1. Primary Cultures of Mice Cortical Astrocytes

Immunocytochemistry was performed to assess the purity of primary astrocyte cultures. For that, double immunocytochemistry was performed, for A) astrocytes/microglia, B) astrocytes/oligodendrocytes.

A) Cells were fixed in 4% PFA for 30 min at RT. After permeabilization with 1% Triton X-100 during 5 min at RT, the cells were blocked with 1% Triton X-100 and 3% bovine serum albumin (BSA) for 30 min. Then, cells were incubated with primary antibody rat anti-mouse CD11b (1:500, Serotec, Oxford, UK) in PBS with 0.1% Triton and 0.3% BSA overnight at 4ºC. After rinsed with PBS, cells were incubated with secondary antibody Alexa Fluor ® 488 donkey anti-rat IgG (1:200, Invitrogen) in PBS with 0.1% Triton and 0.3% BSA for 1 h at RT at dark. Cells were again washed and blocked with 1% Triton X-100 and 3% BSA for 30 min and incubated with mouse anti-GFAP conjugated with Cy3 (1:1000, Sigma) (Table 2) in PBS with 0.1% Triton and 0.3% BSA overnight at 4ºC. The cells were then washed and incubated with 4 μg/ml Hoechst 33342 for 5 min at RT in dark. Finally the cells were washed and mounted in DakoCytomation fluorescent medium and stored in dark at 4ºC.

B) For the double immunocytochemistry of astrocytes and oligodendrocytes, the protocol performed was identical, however the primary antibodies used were rabbit anti-GFAP (1:1000, Sigma) and mouse anti-proteolipid protein (PLP; 1:700, Sigma), respectively. The secondary antibodies used were Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 594 donkey anti-rabbit IgG (1:200, Invitrogen), respectively (Table 2). All the images were obtained using an Axioskop 2 Plus fluorescent microscope (Carl Zeiss, Göttinger, Germany).

To evaluate the purity of cell cultures, six independent microscopy fields were acquired per coverslip (n=3). The number of astrocytes was expressed in percentage of total cells stained with Hoechst 33342. With this protocol we were able to conclude that in our cultures, 99% of the cells were astrocytes (Fig. 17).

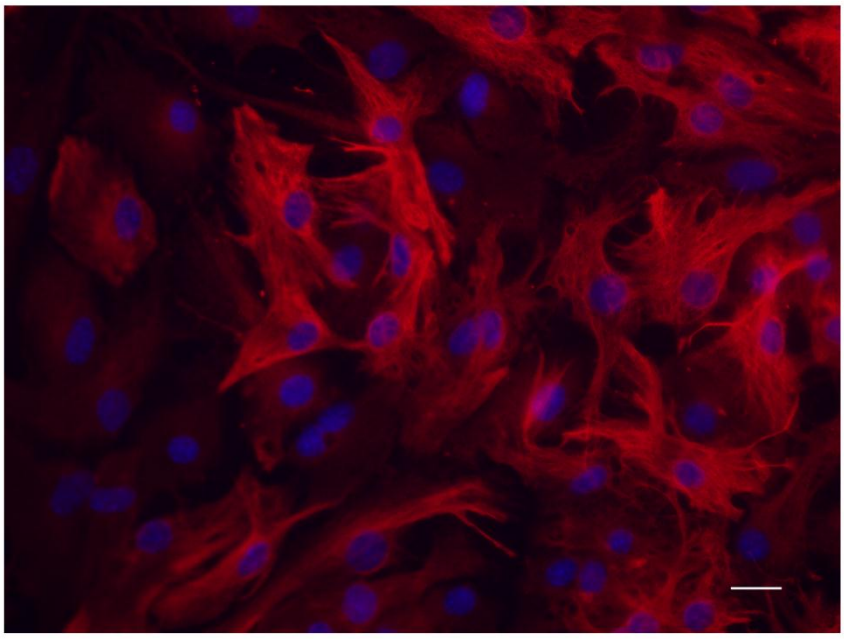


Figure 17. Representative fluorescent image of primary culture of mice astrocytes. Scale bar: 50 µm.

2.4.2. bEnd.3 cell line

Immunocytochemistry on the ECs were performed in order to visualize the TJs proteins: claudin-5 and occludin.

After treating the cells with METH (in µM: 1, 50 and 250) for 24 h, the cells were fixed with 4% PFA for 30 min at RT, washed with PBS followed by permeabilization with 0.2% Triton-X 100 during 10 min and blocked with a blocking solution of 3% BSA and 0.1% Triton-X 100 for 1 h. Then, the cells were incubated with primary antibodies mouse anti-claudin-5 and rabbit anti-occludin (1:100, Zymed laboratories) (Table 2) in blocking solution for 1 h at 37^{0}C. Afterwards, cells were washed and followed by incubation with the secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG (H+L) and Alexa Fluor® 594 donkey anti-rabbit IgG (1:200, Invitrogen) (Table 2), respectively, for 1 h at RT in dark. The cells were again washed and incubated with 4 µg/ml Hoechst 33342 for 5 min at RT in the dark. Finally, the cells were washed and mounted in DakoCytomation fluorescent medium and stored in dark at 4^{0}C. The fluorescent images were recorded using the LSM 710 confocal microscope (Carl Zeiss).

Table 2. List of primary and secondary antibodies used to characterize the primary astrocyte cell cultures and to identified claudin-5 and occludin on the bEnd.3 cell line.

Primary antibody	Dilution	Source	Secondary antibody	Dilution	Source
Rat anti-CD11b	1:500	Serotec	Alexa Fluor ® 488 donkey anti-rat IgG	1:200	Invitrogen
Anti-GFAP conjugated with Cy3	1:1000	Sigma	-	-	-
Rabbit anti-GFAP	1:1000	Sigma	Alexa Fluor® 594 donkey anti-rabbit IgG	1:200	Invitrogen
Mouse anti-PLP	1:700	Sigma	Alexa Fluor® 488 goat anti-mouse IgG	1:200	Invitrogen
Mouse anti-Claudin-5	1:100	Zymed laboratories	Alexa Fluor® 488 goat anti-mouse IgG	1:200	Invitrogen
Rabbit anti-Occludin	1:100	Zymed laboratories	Alexa Fluor® 594 donkey anti-rabbit IgG	1:200	Invitrogen

2.5. Western Blot

Total protein from confluent bEnd.3 treated for 24 h with different METH concentrations (in μM: 1, 50 and 250) or with ACM (in μM METH: 1, 50 and 250) (Fig. 21), were isolated with 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) . The protein concentration was measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, USA) with BSA as a standard and stored at -20^{o}C until further use. Protein samples were prepared according to the final protein concentration (μg/μl) wanted (Table 3), under reduced conditions by adding sample buffer (0.5 M Tris-HCl, 4% SDS pH 6.8, 30% glycerol, 10% SDS, 0.6 M DTT, bromophenol blue) and heating at 95^{o}C for 5 min. Proteins were separated by electrophoresis on 6%–14% polyacrylamide gels at 160 V for 60-120 min. and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Madrid Spain). After blocking the nonspecific reactions with PBS-0.1% Tween® 20 (Sigma)-5% low fat milk, the membranes were incubated overnight at 4^{o}C with primary antibodies as described in Table 4. After washing the membranes with PBS-0.1% Tween® 20, they were incubated with the respective alkaline phosphatase-conjugated secondary antibody (anti-mouse 1:10000 and anti-rabbit 1:20000) (Amersham GE Healthcare Life Science, USA) (Table 4) for 1 h at RT. The membranes were again washed with PBS-0.1% Tween® 20, and proteins were visualized using the enhanced chemiofluorescence (ECF) reagent (Amersham) assay on the Typhoon FLA 9000 (GE Healthcare Bioscience AB, Uppsala, Sweden), and quantification of band density was performed using ImageQuant 5.0 software. Immunoblots were reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody to ensure equal sample loading,

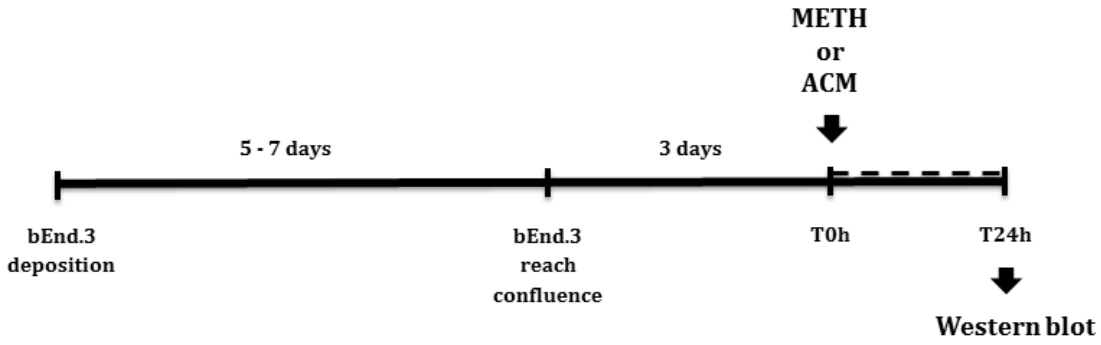


Figure 18. Schematic representation of the protocol used to evaluate the levels of tight junction proteins and caveolin-1. The cells were exposed to methamphetamine (METH) (in μM: 1, 50 and 250) or astrocytes-conditioned medium (ACM) (in μM METH: 1, 50 and 250) for 24 h.

Table 3. List of immunoreactive proteins identified by Western blot.

Protein	Molecular weight (kDa)	Concentration of protein (μg/μl)
Cav-1	22	5
Claudin-5	22	5
Occludin	60-65	15
ZO-1	225	25
ZO-2	160	25
GAPDH	37	-

Table 4. List of antibodies used in Western blot analysis.

Primary antibody	Dilution	Source	Secondary antibody	Dilution	Source
Rabbit anti-Cav-1	1:200	Santa Cruz Biotechonology	Anti-rabbit IgG alkaline phosphatase conjugated	1:20000	Amersham GE Healthcare Life Science
Mouse anti-Claudin-5	1:500	Zymed laboratories	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-ZO-1	1:250	Invitrogen	Anti-rabbit IgG alkaline phosphatase conjugated	1:20000	Amersham GE Healthcare Life Science
Rabbit anti-ZO-2	1:250	Zymed laboratories	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. Statistical analysis

The results obtained were treated statistically with the software GraphPad Prism, Version 5.0. The statistical analysis used was 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. Data were considered to be statistical different at values of $P<0.05$ and were presented as means ± SEM (standard error of the mean). Every experimental condition was tested in, at least, three sets of independent experiments.

CHAPTER 3

Results

Chapter 3

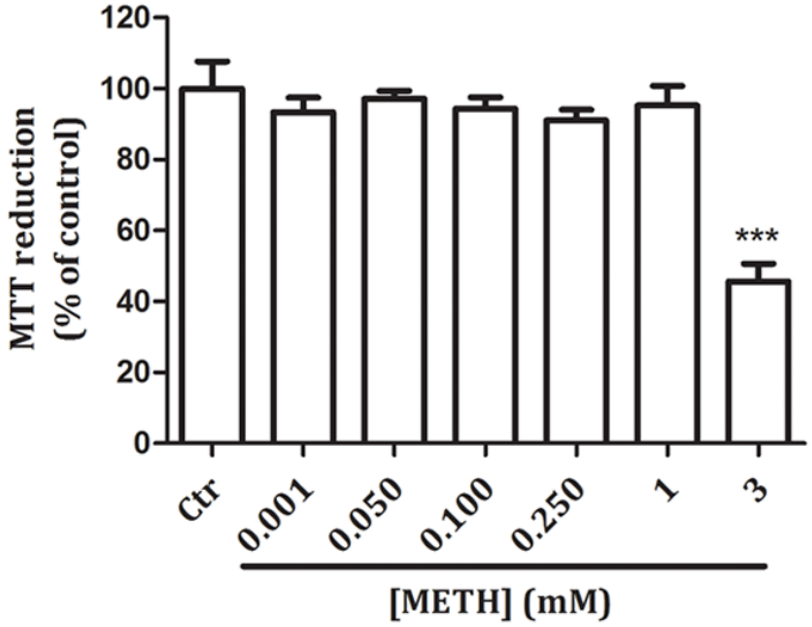
Results

3.1. Effect of methamphetamine on endothelial cell viability

It is known that METH induces deleterious effects on the BBB function. In fact, several studies have shown that METH leads to BBB disruption on several brain regions, such as cortex (Sharma and Kiyatkin, 2009), hippocampus (Kiyatkin and Sharma, 2009) and caudate-putamen (Bowyer and Ali, 2006). Recently, some authors demonstrated that METH can directly affect ECs, since it was able to decrease the BMVEC tightness (Mahajan et al., 2008; Ramirez et al., 2009).

In the present study, bEnd.3 cell line was used as a biological model to investigate the effect of METH on brain ECs viability. For that, cells were treated with a wide range of METH concentrations for 24 h. As described in Fig. 19A, METH decreased bEnd.3 cell viability only at 3 mM, with no significant effect at lower concentrations (control: 100.00±7.62; METH: 0.001 mM, 93.39±4.06%; 0.05 mM, 97.38±2.21%; 0.1 mM, 94.38±3.17%; 0.25 mM, 91.01±3.06%; 1 mM, 95.29±5.39%; 3 mM, 45.69±4.91% of control; ***P<0.001; n=3). In order to verify if this reduction of viability was due to cell death by apoptosis, TUNEL assay was also performed. However, we concluded that METH did not induce EC death by apoptosis (Fig. 19B).

A


MTT reduction
(% of control)
120
100
80
60
40
20
0
***
Ctr
0.001
0.050
0.100
0.250
1
3
[METH] (mM)

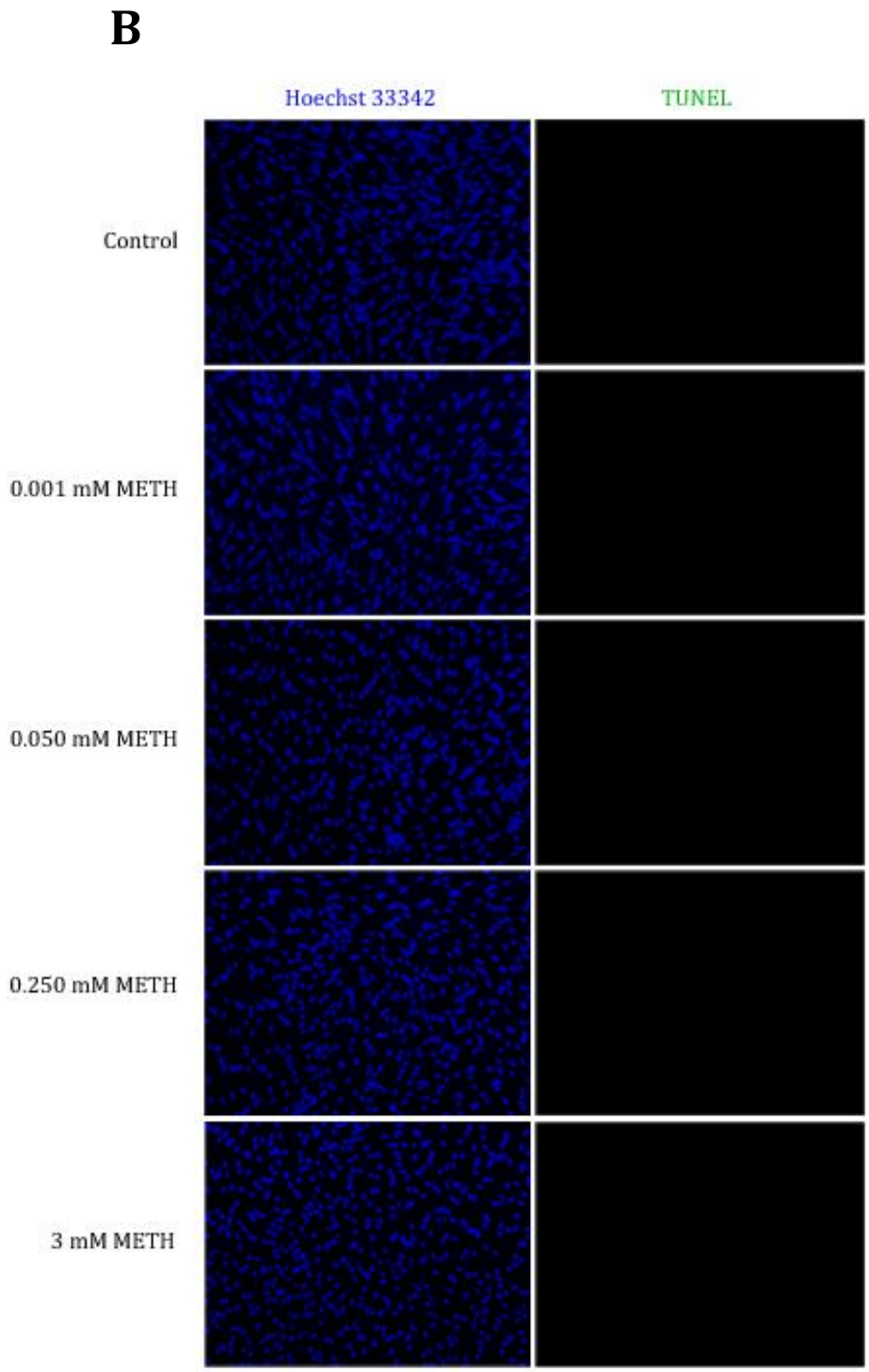


Figure 19. Concentration-dependent effect of methamphetamine (METH) on bEnd.3 cells viability. (A) Cells were exposed to increasing METH concentrations (0.001 mM-3 mM) for 24 h, and only 3 mM METH decreased cell viability. The results are expressed as mean % of control ± SEM, n=3. ****P*<0.001, significantly different when compared to control using Dunnett's Multiple comparison test. (B) No significant apoptotic cell death was observed in these cultures after METH exposure (Magnification x200).

3.2. Tight junction and caveolin-1 protein levels: Alterations induced by methamphetamine

Recent *in vitro* studies revealed that METH induces alterations on the expression of the TJ proteins, namely claudin-5 (Ramirez et al., 2009) and occludin (Ramirez et al., 2009; Park et al., 2011). Also, in our group it was demonstrated that an acute administration of METH induced a decrease on claudin-5, occludin and ZO-1 protein levels in the mice hippocampus after 24 h drug-injection (Martins et al., 2011). Since these alterations are normally linked with increased BBB permeability (Zlokovic, 2008), we further evaluated the changes of claudin-5, occludin, ZO-1 and ZO-2 protein levels induced by METH in ECs. It was observed a significant decrease on claudin-5 levels with 50 and 250 μM METH (58.74±6.87% and 70.42±7.72% of control, respectively; **P*<0.05 and ***P*<0.01; n=6; Fig. 20A), with no alterations at 1 μM METH (97.94±8.07% of control; n=6). As observed for claudin-5, there was also a decrease in occludin protein levels, but in this case at all METH concentrations analyzed, as follows: 1 μM, 71.36±2.93%; 50 μM, 72.40±4.39%; 250 μM, 72.19±19.16% of control; **P*<0.05; n=3-5 (Fig. 20B). Regarding ZO-1 there was a significant decrease of protein levels in the presence of 50 and 250 μM METH (63.73±10.27% and 60.27±8.782% of control, respectively; **P*<0.05; n=3; Fig. 20C). No significant differences were observed in the presence of 1 μM METH, however there was a tendency to decrease (67.71±9.63% of control; n=3; Fig. 20C). Concerning ZO-2 protein levels, no alterations were observed when compared to the control (1 μM, 88.13±8.52%; 50 μM, 78.59±1.21%; 250 μM, 75.48±8.13 % of control; n=3; Fig. 20D).

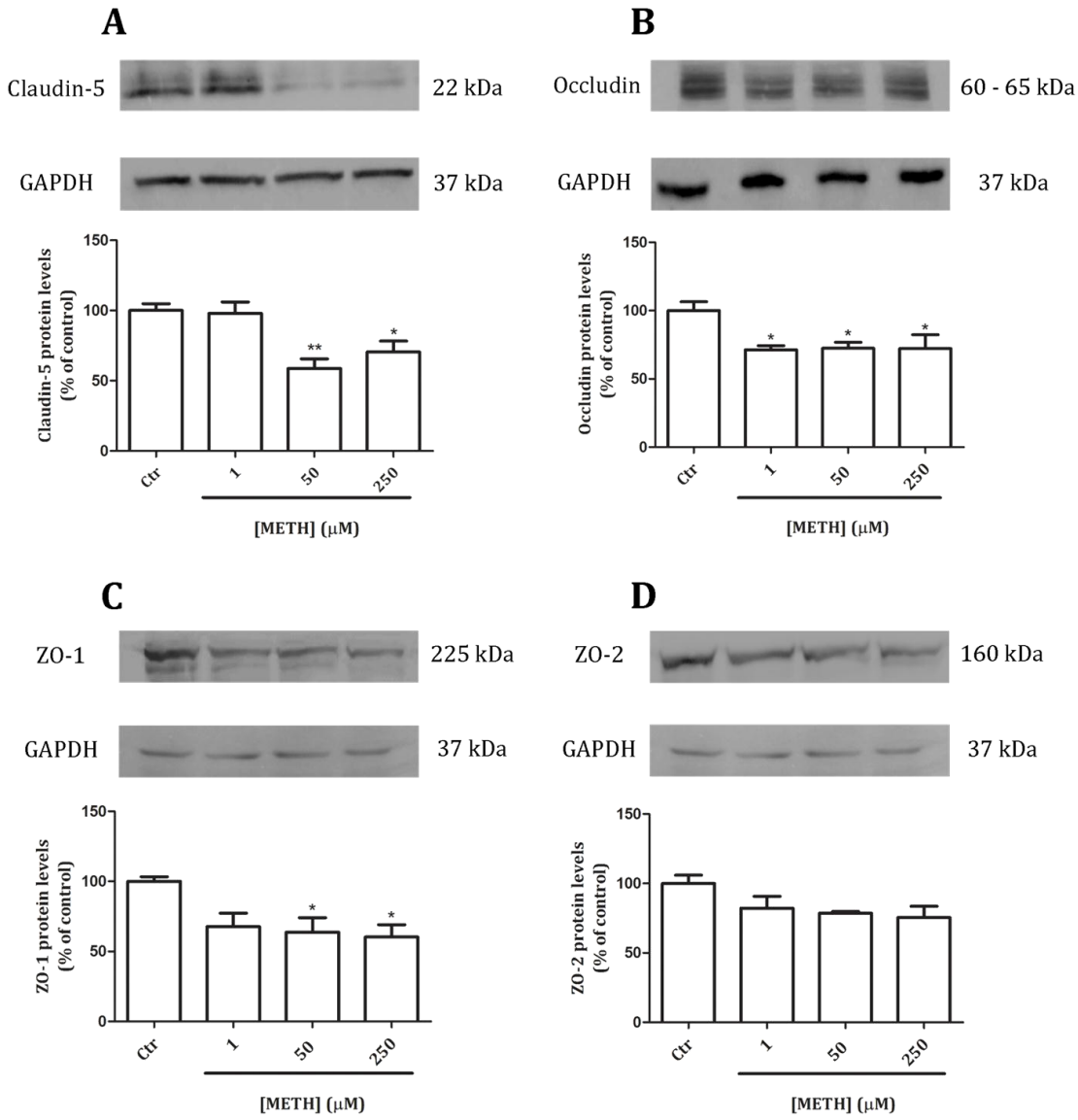


Figure 20. Methamphetamine (METH) induced a decrease of (A) claudin-5, (B) occludin, (C) zonula occludens (ZO)-1 and had no effect on (D) ZO-2 protein levels, at 24 h after treatment. Above the bars, representative Western blot images of claudin-5 (22 kDa), occludin (60-65 kDa), ZO-1(225 kDa), ZO-2 (160 kDa) and GAPDH (37 kDa) are shown. The results are shown as mean % of control ± SEM, n=3-6. **P*<0.05 and ***P*<0.01, significantly different when compared to the control using Dunnett's Multiple comparison test.

The ECs are rich in caveolae (Stan, 2005) which have been implicated in different cell events such as endocytosis, transcytosis and signal transductions (Parton and Simons, 2007). Cav-1 is one of the most important proteins present in caveolae and recently it has been demonstrated to play an important role in conditions of BBB disruption (Nag et al., 2007; Park et al., 2011). Thus, we were also interested in understanding how cav-1 was being affected in our model after METH treatment. As observed in Fig. 21, METH did not induce significant alterations on cav-1 expression, as follows: 1 μM, 91.65±4.73%; 50 μM, 86.81±4.26; 250 μM, 86.21±5.01% of control; n=3.

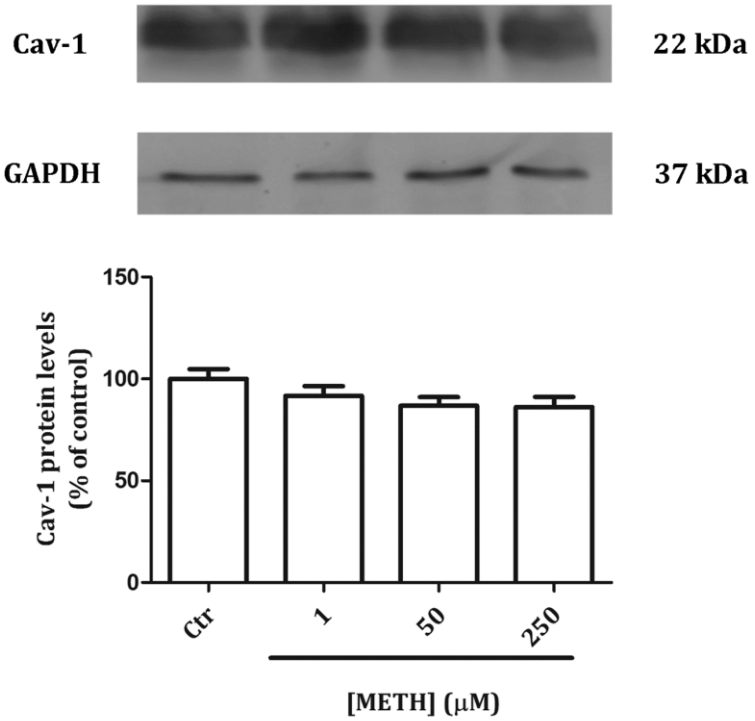
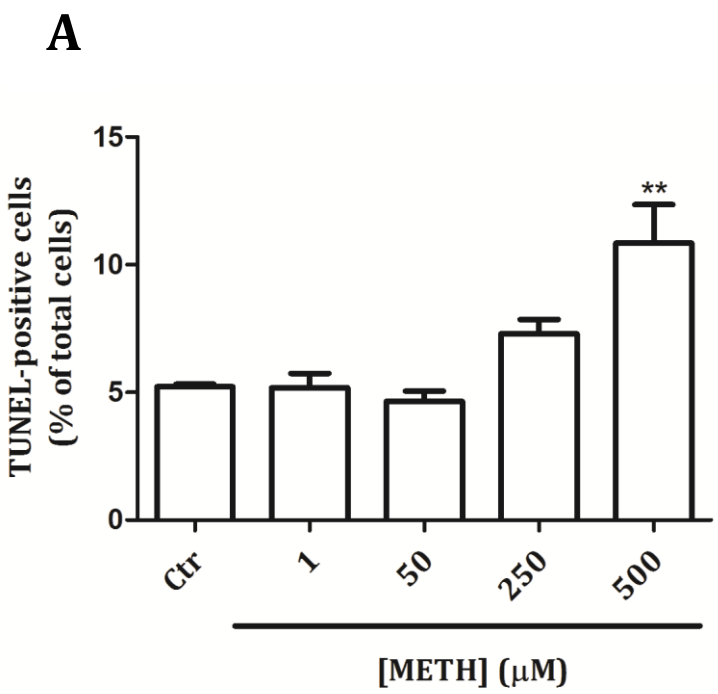


Figure 21. Methamphetamine (METH) had no effect on caveolin (cav)-1 protein levels. Above the bars, representative Western blot images of cav-1 (22 kDa) and GAPDH (37 kDa) are shown.

3.3. Evaluation of astrocytic cell death induced by methamphetamine

Astrocytes play an important role in physiological CNS functions (Hamby and Sofroniew, 2010) being also crucial in the maintenance of BBB properties (Nag, 2011). Most of the studies that aimed to understand the effect of METH on astrocytes showed that this drug of abuse induces astrogliosis (Stadlin et al., 1998), but very little is known about its impact on cell death. Thus, primary cultures of mice cortical astrocytes were exposed to different METH concentrations for 24 h and cell death by apoptosis was evaluated. We observed that 500 μM METH induced a significant increase in the number of TUNEL-positive cells (10.84±1.51% of total cells; ***P*<0.01; n=4; Figs. 22A, B) when comparing to the control (5.22±0.09% of total cells; n=3; Figs. 22A, B). At lower concentrations, METH did not induce significant changes comparing to the control (1 μM, 5.18±0.56%; 50 μM, 4.64±0.41%; 250 μM, 7.29±0.56% of total cells; n=3; Figs. 22A, B).



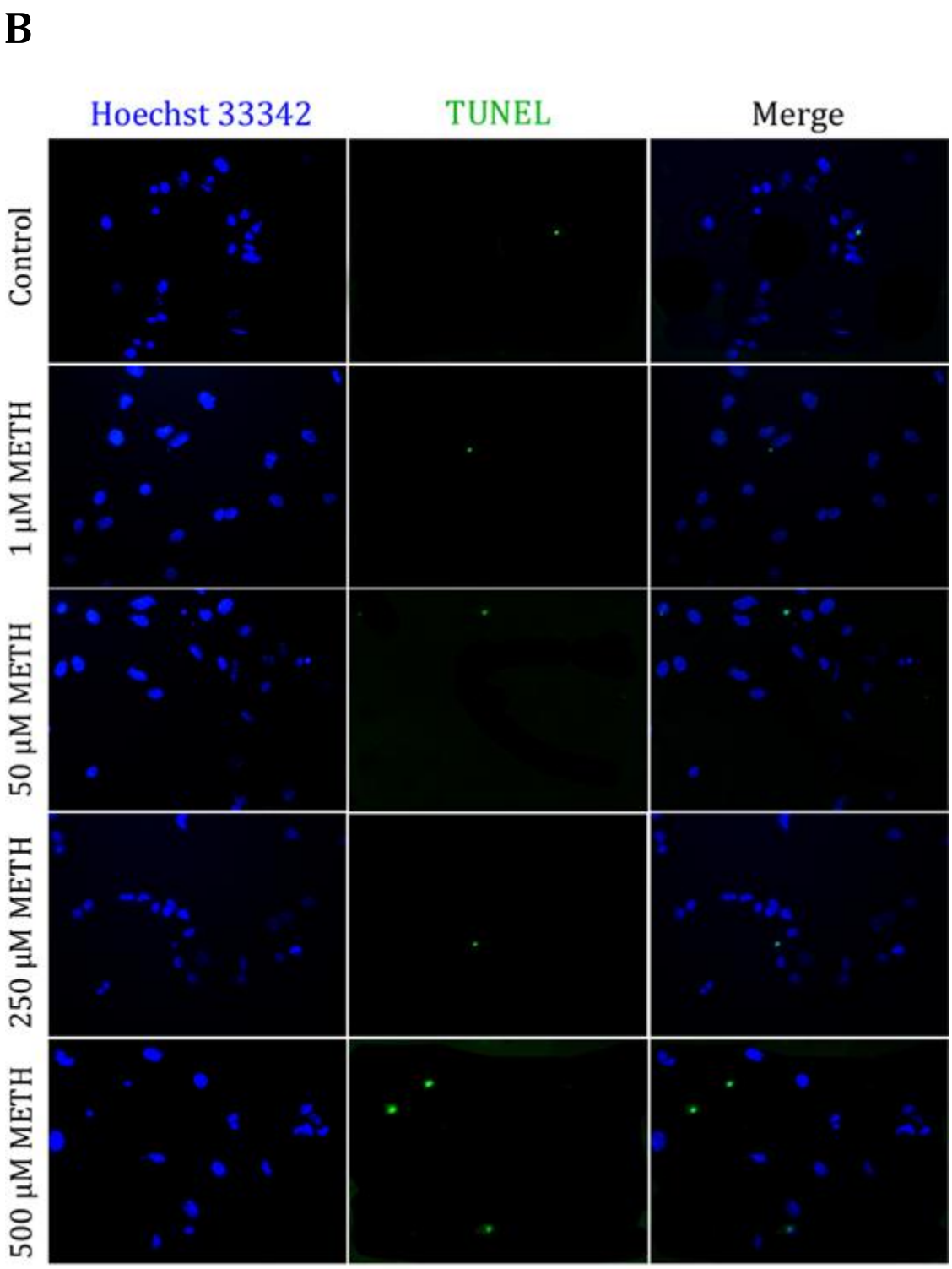


Figure 22. Cell death evaluation in astrocyte cultures exposed to different concentrations of methamphetamine (METH) (1-500 µM) for 24 h. (A) Only 500 µM METH increased the number of TUNEL-positive cells. The results are expressed as mean % of total cells ± SEM, n=3. ***P*<0.01, significantly different when compared to control using Dunnett's Multiple comparison test. (B) Representative fluorescent images of cultures treated with different METH concentrations (1-500µM) for 24 h, and only the 500 µM increases the number of TUNEL-positive cells (TUNEL-positive cells, green; Hoechst positive cells, blue) (Magnification x400).

3.4. Effect of astrocyte-conditioned medium on endothelial cell viability

Several studies have shown that ECs treated with ACM display better barrier properties (Nag, 2011). So, we also aimed to investigate whether ACM affects the viability of the ECs. For that, bEnd.3 cells were treated for 24 h with ACM obtained from astrocyte cultures non-exposed (ACM control) or exposed to different concentrations of METH, as follows: ACM 1 μM METH, ACM 50 μM METH and ACM 250 μM METH. We concluded that EC viability was altered when exposed to ACM since it increased when exposed to control ACM (111.10±3.94% of control; **P*<0.05; n=3; Fig. 23) but when ECs were exposed to ACM obtained from astrocytes incubated with different METH concentrations, cell viability slightly decreased, as follows: ACM 1 μM METH, 96.75±2.73%; ACM 50 μM, 91.84±1.77%; ACM 250 μM, 89.24±3.30% of control; **P*<0.05,$^{\#}$*P*<0.05, $^{\#\#\#}$*P*<0.001; n=3; Fig. 23).

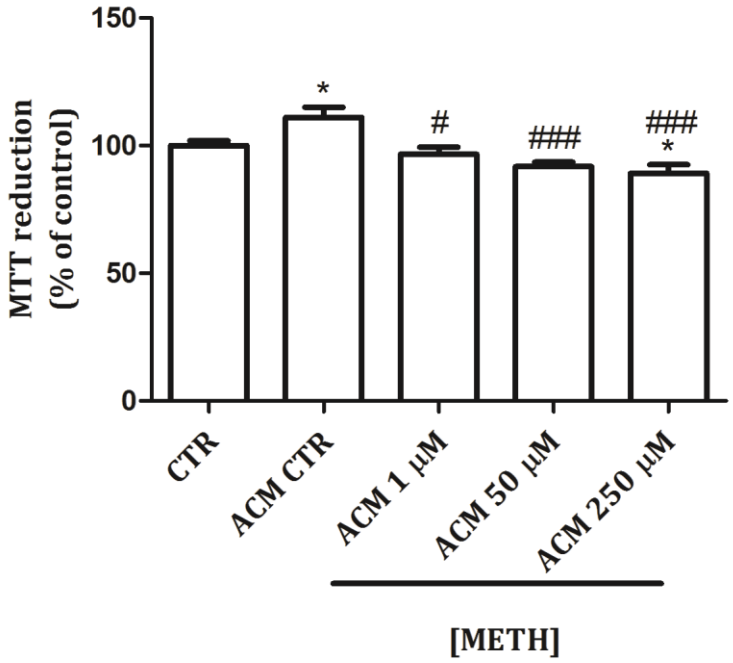


Figure 23. Effect of astrocyte-conditioned medium (ACM) obtained from astrocytes exposed to methamphetamine (METH) on bEnd.3 cell viability. The ACM control increases cell viability when compared to the control and ACM 1 μM METH, ACM 50 μM METH or ACM 250 μM METH decreases cell viability when compared to the ACM control. Also a significant decrease is seen with ACM 250 μM METH when compared to the control. The results are expressed as mean % of control ± SEM, n=3, **P*<0.05 significantly different when compared to control using Dunnett's Multiple comparison; $^{\#}$*P*<0.05 and $^{\#\#\#}$*P*<0.001 significantly different when compared to ACM control using Bonferroni's Multiple comparison test.

3.5. The effect of astrocyte-conditioned medium on claudin-5 and caveolin-1 protein levels

After evaluation of the effect of ACM on cell viability, we further investigated how this treatment affects protein expression. As observed in Fig. 24A, ACM METH induces a decrease on claudin-5 protein levels when compared to the control (ACM 1 μM METH, 66.27±6.70%; ACM 50 μM METH, 60.00±15.76%; ACM 250 μM METH, 56.94±5.37% of control; **P*<0.05 and ***P*<0.005; n=3). However, no differences are observed when compared to the ACM control. Concerning cav-1 expression, all ACM METH treatments induced a decrease in the expression of this protein comparing to both control and ACM control (ACM 1 μM METH, 36.16±2.94%; ACM 50 μM METH, 24.71±5.90%; ACM 250 μM METH, 38.40±2.44 % of control; ****P*<0.001 and ###*P*<0.001; n=3; Fig. 25B), in opposite to what we observed for METH *per se* (see Fig. 21). No differences are observed between the control and the ACM control for both proteins (91.64±6.15% and 103.70±5.83% of control for claudin-5 and cav-1, respectively; n=3).

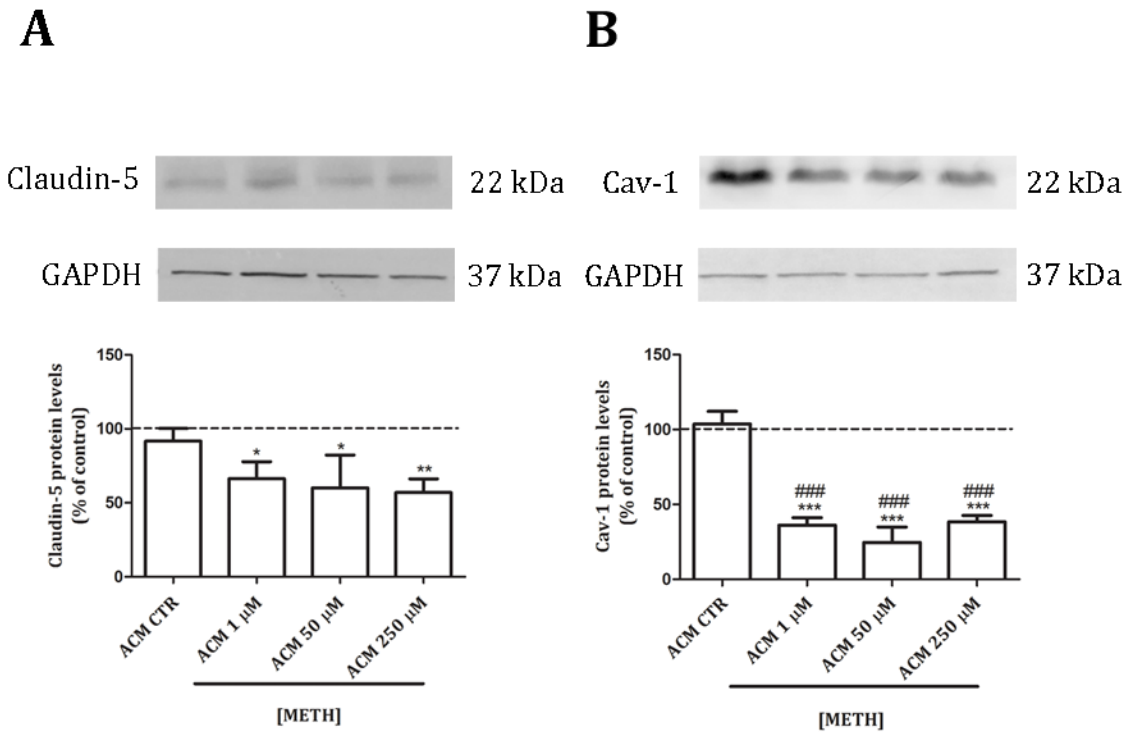


Figure 24. Astrocyte-conditioned medium (ACM) induces a decrease of (A) claudin-5 and (B) caveolin (cav)-1 protein levels after 24 h of ACM exposure. Above the bars, representative Western blot images of claudin-5 (22 kDa), cav-1 (22 kDa) and GAPDH (37 kDa) are shown. The results are shown as mean % of control ± SEM, n=3. **P*<0.05, ***P*<0.01 and ****P*<0.001, significantly different when compared to the control using Dunnett's Multiple comparison test; ###*P*<0.001 significantly different when compared to ACM control using Bonferroni's Multiple comparison test.

CHAPTER 4

Discussion

Chapter 4

Discussion

METH is a powerful drug of abuse with more potent effects on the CNS comparing to others in part due to its lipophilic and weak base properties, low molecular weight and low protein binding capacity, allowing a rapid diffusion across the plasma membranes and lipid layers, such as the BBB (Torre et al., 2004; Fleckenstein et al., 2007). Indeed, recently our group demonstrated in an acute intoxication model of METH , that this drug reach to brain tissue within 1 h post-administration whereas after 8 h no differences were observed comparing to the control (Martins et al., 2011).

This drug of abuse causes severe alterations in the CNS (Thomas and Kuhn, 2005; Scott et al., 2007), and indeed studies performed with METH abusers showed severe brain abnormalities in specific brain regions, such as hippocampus, prefrontal cortex and amygdala, which could be associated with some of the psychiatric problems identified in these individuals (Thompson et al., 2004; Tobias et al., 2010). Several molecular and cellular mechanisms associated with oxidative stress, mitochondrial dysfunction, excitotoxicity and neuroinflammation (Yamamoto and Raudensky, 2008; Krasnova and Cadet, 2009; Yamamoto et al., 2010) have been proposed to explain the severe brain abnormalities induced by METH. However, many questions remain to be answered. Currently, it was proposed that BBB dysfunction can also be responsible for the neurotoxicity induced by METH. In fact, some studies have revealed that METH induces alterations in BBB, most of them associated with hyperthermia (Bowyer and Ali, 2006; Sharma and Kiyatkin, 2009), oxidative stress (Ramirez et al., 2009; Park et al., 2011) and MMPs (Martins et al., 2011). It is well documented that METH can lead to neuronal cell death in several brain regions, such as striatum, cortex and hippocampus (Deng et al., 2001), however the effect of METH in BBB, and more precisely on the ECs, has been overlooked. Our present results show that METH has a direct effect on ECs viability, but only at high concentrations (3 mM METH). However, this decrease in cell viability was not accompanied by an increase in cell death by apoptosis. In fact, the MTT assay is used to evaluate the metabolic state of the cells, and is known that METH alters mitochondrial

function affecting the electron transport chain enzyme complexes (Tata and Yamamoto, 2007; Yamamoto et al., 2010) leading to alterations in the energy state of the cell. So, it is possible that this effect on cell viability with 3 mM METH could be due to alterations in the normal ability of the cell to maintain and provide energy for the normal metabolic cell function. Moreover, another possible explanation for the absence of cell death by apoptosis is the exposure period to the drug. Previous *in vivo* studies with male CD-1 mice showed an increase of neural cell death in striatum, cortex and hippocampus three days after the drug administration (Deng et al., 2001; Jayanthi et al., 2004). However, *in vitro* studies with an immortalized rat striatal cell line (Deng et al., 2002) and oligodendroglial cultures (Genc et al., 2003) showed an increase in cell death by apoptosis in 24 h after 2 mM and 1 mM METH, respectively. This suggests that the ECs are more resistant to METH-induced cell death. Also, it was demonstrated previously that METH can induce cell death by necrosis (Bento et al., 2011), but we did not evaluated necrosis in the present study.

It has been shown that METH can induce an increase in BBB permeability and some studies have suggested that changes on the TJ proteins could be responsible for this event (Mahajan et al., 2008; Ramirez et al., 2009; Martins et al., 2011). The TJ complex is the main responsible of paracellular permeability across the BBB (Persidsky et al., 2006; Bernacki et al., 2008), and it is composed by transmembrane proteins, namely, claudin-5, occludin and JAM that are linked to the actin cytoskeleton through the cytoplasmic accessory proteins ZO-1, -2 and -3 (Ballabh et al., 2004; Persidsky et al., 2006; Bernacki et al., 2008). It is well establish that alterations in TJ proteins are associated with BBB disruption in several CNS pathologies, like Alzheimer's and Parkinson's disease, multiple sclerosis and AIDS-related dementia (Persidsky et al., 2006; Forster, 2008; Zlokovic, 2008). However, alterations of BBB through modifications on TJ proteins by drugs of abuse are not well characterized. Thus, the ECs were exposure for 24 h to METH and several TJ proteins were analyzed. Accordingly with previous studies (Ramirez et al., 2009; Martins et al., 2011), we observed a significant decrease in claudin-5, occludin and ZO-1 protein levels, but no differences were observed regarding ZO-2. In this study we demonstrated that the levels of claudin-5 decrease only with 50 μM METH and 250 μM METH, whereas all concentrations of METH decreased occludin levels. These observations suggest that claudin-5 appear to be more stable than occludin. In contrast to what was observed by others (Ramirez et al., 2009), we concluded that 50 μM and 250 μM METH induced a decrease in ZO-1 protein levels. The apparent contradiction could be explained by the use of different *in vitro* models, since we used bEnd.3 mouse cell line and Ramirez and collaborators (2009) used a human BMVEC. Immunocytochemistry for claudin-5 and occludin after METH exposure was also performed (data not shown). However, due to the

fact that an endothelial cell line do not retain several BBB properties, as discussed previously in several studies (Nicolazzo et al., 2006), it was not possible to verify properly the alterations in proteins distribution and expression. Nevertheless, our results clearly show that METH is able to decrease the protein levels of several TJs, which corroborate the results previously shown by others (Ramirez et al., 2009; Martins et al., 2011).

Another protein that has been related with BBB disruption is the cav-1, which is one of the resident proteins on caveolae and has been associated with important functions such as cell signaling transduction events (Razani et al., 2002; Deurs et al., 2003). Some studies have demonstrated that within minutes after brain-injury there is an increase in endothelial caveolae on the vessel segments followed by TJs alterations. This suggests that changes on cav-1 expression precede alterations on the TJs proteins during BBB breakdown after an injury (Nag et al., 2007). In fact, Zhong and co-authors (Zhong et al., 2008) demonstrated that cav-1 upregulation may constitute an early and critical modulator that controls signaling pathways leading to the disruption of TJ proteins. More recently, Park and collaborators (2011) demonstrated that 30 min after METH exposure there was an increase in the phosphorylation of cav-1, and when cav-1 was silenced the changes in occludin levels induced by METH were prevented. This suggests an important role for the functional caveolae in the regulation of TJ protein levels. In the present work we evaluate the effect of METH on the total levels of cav-1 after 24 h and no differences were observed when compared to the control. The apparent inconsistency between our and previous results can be explain by the different time point that was used to evaluate the levels of cav-1, since most of the alterations on cav-1 expression seems to occur as an early event, as was previously described with a model of cold injury in male Wistar rats (Nag et al., 2007) and with a human BMVECs in studies of BBB dysfunction induced by Tat protein (Zhong et al., 2008).

Glial cells include microglia, oligodendrocytes and astrocytes, which have important roles in the CNS. Indeed, astrocytes are the most numerous glial cell type present in the CNS and play different roles that are crucial for the maintenance of neuronal homeostasis and function, such as modulation of synaptic activity, removal of excitatory neurotransmitters from the synaptic cleft and can also influence the neurite outgrowth (Chen and Swanson, 2003). Moreover, astrocytes are important for the development and maintenance of the BBB properties (Abbott, 2002; Abbott et al., 2006). In several pathological conditions where alterations on the BBB occur, such as stroke, trauma, brain tumors, epilepsy and Alzheimer's disease (Abbott et al., 2006), it is possible to observe an abnormal endothelial-glial interaction. So, it appears very important to understand how drugs of

abuse can alter this interaction. Indeed, some studies have demonstrated that drugs of abuse, such as METH, can induce microglial and astrocytes activation by a process known as gliosis (Hanisch, 2002; Thomas et al., 2004). Astrogliosis has been suggested has a marker of neurotoxicity and has been demonstrated in several brain regions like in striatum, cortex (O'Callaghan et al., 2008), and hippocampus (Simões et al., 2007; Gonçalves et al., 2010). In our study, we were interested in understand how METH affects cultured mouse astrocytes, and we concluded that astrocytes are more susceptible to METH than ECs, since we observed a significant increase in cell death with 500 μM METH, showing that METH can have direct and toxic effects on astrocytes.

Knowing that brain ECs cocultured with astrocytes or with ACM demonstrate a significant increase in the BBB features, like increase in the TJ formation, increase in electrical resistance and decrease in permeability (Abbott, 2002; Nag, 2011), we were also interested in understand how METH could affect the crosstalk between astrocytes and ECs. In this work we clearly show that ACM induces alterations on the viability of ECs. Interestingly, we observed that ACM *per se* induces an increase in cell viability showing that, indeed, astrocytes may produce and release important soluble molecules that can increase the metabolic status of the ECs. On the other hand, when the ECs were treated with ACM obtained from astrocytes exposed to METH, the cells displayed a significant decrease in cell viability, suggesting that astrocytes when exposed to METH release critical products that decrease the viability of ECs. In fact, Lau and co-authors (2000) demonstrated that METH increases the production of ROS in cultured astrocytes, which could affect the ECs by altering their metabolic state. It is also known that under pathological conditions astrocytes can produce and release several pro-inflammatory cytokines like IL-1 β, IL-6 and TNF-α that are associated with BBB opening (Abbott et al., 2006). This increase in the levels of pro-inflammatory cytokines is also observed in cases of METH exposure (Gonçalves et al., 2008; Gonçalves et al., 2010). So, it is plausible to suggest that this inflammatory response could lead to alterations on the ECs viability. In an attempt to clarify if these alterations in cell viability were also accompanied by modifications in protein expression, we further evaluated the levels of claudin-5 and cav-1 in the presence of ACM. Our results demonstrate that ACM led to a slight decrease of claudin-5 when compared to the control; however no differences are observed when compared to the ACM control. In fact, the alterations of claudin-5 levels are quite similar to those observed with 50 μM and 250 μM METH alone, so is possible to suggest that, at these conditions the alterations are mainly due to METH *per se* and not to the products released by the astrocytes when exposed to METH. However, we also observed that ACM 1 μM METH, besides the fact that 1 μM METH by itself did not affect claudin-5 levels, it

triggered the release of astrocytic factors that decreased the expression of claudin-5. Interestingly, ACM significantly decreased the protein levels of cav-1, an effect that was not observed with METH by itself. The alteration on the levels of cav-1 can lead to alterations in cell permeability due to the fact that this protein is responsible for the negative regulation of several molecules, namely eNOS. The non-inhibition of eNOS originates an increase in NO production (Razani et al., 2002; Deurs et al., 2003; Mehta and Malik, 2006) that has been associated with the opening of the BBB (Thiel and Audus, 2001). Moreover, it was verified that METH can induce an increase in the NO levels in several *in vivo* models, that could be correlated with METH-induced neural damage in the mouse striatum (Zhu et al., 2009; Wang and Angulo, 2011). Despite these evidences, it will be important to analyze the molecular content of the ACM in order to understand which soluble molecules are released by the astrocytes, as well as to verify the concentration of METH present in the medium.

In this work we demonstrated that METH has a direct effect on ECs decreasing their viability and the levels of TJ proteins, which can induce important alterations in the BBB. Moreover, we also demonstrated that METH is toxic to astrocytes and triggers the release of molecules that can alter the ECs viability, and decreases the protein levels of claudin-5 and cav-1. In future work it will be important to understand how this crosstalk between endothelial cells-astrocytes can affect other TJ proteins and to verify if these alterations can also occur in a more complex *in vitro* model, such as co-cultures. Nevertheless, the present work demonstrates for the first time that METH can also induce indirect alterations on the ECs through modifications on astrocytes.

CHAPTER 5

Conclusion

Chapter 5

Conclusion

BBB disruption is a critical event in the development and progression of several diseases that affect the CNS, being a consequence of the pathology or in other cases being a precipitating event. Regarding drugs of abuse, there is increasing evidence that psychoactive drugs, like METH, induce BBB dysfunction. Although these noxious effects, is possible that in the future, METH could be used as a therapeutic tool in order to facilitate drug delivery to CNS to treat several brain disorders, such as chronic myelogenous leukemia and glioblastoma (Kast, 2009; Kast and Focosi, 2010).

Until that day comes, many studies need to be performed in order to clarify the mechanisms by which METH leads to BBB disruption. According with our results METH does not alter the ECs viability at low doses and also does not induce cell death by apoptosis. Moreover, we demonstrate that this drug induces direct effects on the ECs, through a decrease on the expression of TJs proteins. We also demonstrate that astrocytes are more susceptible to METH, and that METH can induce alterations on the ECs by affecting directly the astrocytes.

In conclusion, the present work allows us to better understand how METH can affect directly the ECs and astrocytes, as well as the impact on the crosstalk between the endothelial cells-astrocytes.

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