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IMPACT OF METHAMPHETAMINE ON BLOOD-BRAIN BARRIER: ROLE OF EXERCISE

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TRABALHO COM VISTA À ATRIBUIÇÃO DO GRAU DE MESTRE NO ÂMBITO DO ENSINO PÓS-GRADUADO EM MEDICINA DO DESPORTO

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IMPACTO DA METANFETAMINA NA BARREIRA HEMATO-ENCEFÁLICA: O PAPEL DO EXERCÍCIO

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RESUMO

O exercício físico é reconhecido e aceite como uma estratégia comportamental para melhorar os parâmetros de saúde. No entanto, o uso de substâncias dopantes ganhou grande popularidade em desportos de competição, apesar do facto de poderem ser tóxicas para vários órgãos, incluindo o cérebro. Algumas substâncias, como as anfetaminas, podem facilmente atingir o cérebro atravessando a barreira hemato-encefálica (BHE), tendo também um efeito direto nesta estrutura celular. Esta barreira protege o cérebro contra moléculas nocivas e organismos patológicos, e é também responsável por manter a sua homeostasia. A disfunção da BHE pode ter consequências graves, tais como o dano irreversível de células cerebrais, o que por sua vez pode originar alterações neurológicas e psiquiátricas. Os principais responsáveis pela integridade desta barreira são as junções intercelulares oclusivas e aderentes existentes entre células endoteliais adjacentes que controlam a via paracelular através da BHE. As células endoteliais em conjunto com os astrócitos, pericitos, neurónios e a matriz extracelular, constituem a "unidade neurovascular" que é essencial para o normal funcionamento do sistema nervoso central. Atualmente, já é do nosso conhecimento que a metanfetamina (MET) pode promover a disrupção da BHE através de alterações na expressão e conformação das junções intercelulares, promoção da astrogliose, remodelação do citoesqueleto, e através da indução de processos neuroinflamatórios.

Várias estratégias têm sido apontadas como protetoras em condições de uso de substâncias ilícitas. Neste âmbito, o exercício físico parece promissor pelos seus efeitos benéficos na função cognitiva (aprendizagem e memória), assim como pelo seu potencial efeito não farmacológico de manutenção da homeostasia cerebral e de

melhoria dos sintomas em condições de doenças neurodegenerativas e/ou doenças psiquiátricas. Ainda assim, pouco se sabe sobre o papel do exercício físico na disrupção da BHE causada pela MET, assim como sobre os seus mecanismos celulares. Deste modo, o objetivo deste trabalho foi avaliar o papel do exercício físico moderado na disrupção da BHE induzida pela MET, através da análise de proteínas constituintes da unidade neurovascular e assim inferir sobre a integridade da BHE.

Neste trabalho foram utilizados ratinhos jovens machos com 3 meses e submetidos a um protocolo de exercício físico moderado com ou sem administração concomitante de MET. Em seguida, as possíveis alterações nos níveis proteicos de albumina, claudina-5, VCAM-1 (molécula de adesão celular vascular) e GFAP (proteína ácida fibrilar glial) no córtex pré-frontal e hipocampo foram analisados pela técnica de *western blot*.

Os efeitos foram dependentes da região cerebral. No hipocampo observámos um maior aumento da permeabilidade da BHE induzida pela MET, assim como pelo exercício físico em si. No entanto, quando combinados foi possível observar um efeito protetor do exercício. Por outro lado, no córtex pré-frontal os efeitos foram mais moderados, e não se verificou um efeito protetor do exercício físico.

Podemos assim dizer que o exercício físico pode ter um papel importante no hipocampo aquando uma condição de consumo de MET.

PALAVRAS-CHAVE: Astrócitos, barreira hemato-encefálica, exercício físico, claudina-5, junções intercelulares, molécula de adesão.

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IMPACT OF METHAMPHETAMINE ON BLOOD-BRAIN BARRIER: ROLE OF EXERCISE

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ABSTRACT

Nowadays, physical exercise is widely accepted as a behavioral strategy to improve health outcomes. Nevertheless, performance-enhancing drugs, such as methamphetamine (METH) have gained great popularity in competition sports, despite of their potential neurotoxicity. METH is a powerful psychostimulant drug of abuse that causes severe alterations in the central nervous system (CNS). Its neurotoxicity has been studied over the last years, and is now known that METH can easily reach the brain by crossing the blood-brain barrier (BBB), with a direct effect on its function. This barrier has an important role on the brain homeostasis and protection against toxic molecules and pathogenic organisms and its dysfunction can lead to neurological and psychiatric abnormalities. The intercellular junctions, tight (TJ) and adherens junctions (AJ), present between adjacent endothelial cells, are the main responsible for the barrier properties since they control the paracellular pathway across BBB. Endothelial cells together with pericytes, astrocytes, the extracellular matrix and neurons, constitute the "neurovascular unit" that is essential for the normal function of the CNS. METH can promote BBB dysfunction through alterations in tight junction protein

expression and conformation, increased glial activation, and neuroinflammatory pathways.

Physical exercise has been studied by its positive effects on cognitive functions (learning and memory), being a non-pharmacological approach to improve health, including under conditions of neurodegenerative and/or psychiatric conditions. Still, little is known about the role of physical exercise on METH-induced BBB disruption and its underlying mechanisms. Therefore, the aim of this work was to evaluate the role of moderate exercise on BBB disruption METH-induced, by analyzing neurovascular unit proteins and consequently the BBB integrity.

Male C57BL/6J wild-type mice at 3 months of age were submitted to a moderate exercise protocol with or without concomitant administration of METH. Possible alterations in the protein levels of albumin, claudin-5, VCAM-1 (vascular cell adhesion molecule), and GFAP (glial fibrillary acidic protein) in the pre-frontal cortex and hippocampus were analyzed by *western blot*.

Effects were region-dependent. BBB leakage was significant in the hippocampus when triggered by METH or exercise by themselves although in this region when combined, exercise showed a protective effect against METH alterations. In the cortical region we did observe neither relevant effects nor protective role of exercise. In conclusion, exercise can have an important role by preventing METH-induced neuroinflammatory response, namely in the hippocampus.

KEYWORDS: Astrocytes, blood-brain barrier, physical exercise, claudin-5, intercellular junctions, adhesion molecules.

ABBREVIATIONS:

AJ: Adherens Junctions

BBB: Blood-Brain Barrier

BCA: Bicinchoninic Acid Assay

CNS: Central Nervous System

DL: Law by Decree

ECs: Endothelial Cells

GFAP: Glial Fibrillary Acidic Protein

MMP-9: Matrix Metalloproteinase-9

METH: Methamphetamine

PBS: Phosphate Buffered Saline

PFA: Paraformaldehyde

PFC: Pre-Frontal Cortex

PVDF: Polyvinylidene Difluoride

RIPA: Radio Immunoprecipitation Assay Buffer

RT: Room Temperature

SDS-PAGE: Sodium Dodecyl Sulfate – Polyacrilamide Gel Electroforesis

SEM: Standard Error of Mean

TJ: Tight Junctions

VCAM-1: Vascular Cellular Adhesion Molecule -1

ZO: Zonula Occludens

INTRODUCTION

Nowadays, physical activity is widely accepted as a behavioral strategy to promote health. However, in competition sports, performance-enhancing drugs such as methamphetamine (METH) are being used by a significant number of athletes in order to improve performance (Clarkson and Thompson, 1997; De Hon *et al.*, 2015). METH is a powerful stimulant drug of abuse highly addictive and toxic to the brain, leading to neurological and psychiatric abnormalities (Weiss *et al.*, 2009; Silva *et al.*, 2010). It is used for recreational purposes and also as a performance-enhancing drug by athletes, as it is a sympatico-mimetic drug (Angoorani *et al.*, 2012). Furthermore, METH is included in the list of substances prohibited in-competition (World Anti-Doping Agency list of Prohibited Substances and Methods; http://list.wada-ama.org/). The abuse/misuse of METH induces several adverse effects compromising the capacity of the brain to generate new neurons, and thus decreasing the endogenous brain repair resources (Silva *et al.*, 2010; Gonçalves *et al.*, 2014). Besides the well-known alteration of monoaminergic system, blood-brain barrier (BBB) dysfunction triggered by METH has been recently pointed as a crucial effect.

BBB has an important role on the brain homeostasis and protection against toxic molecules and pathogenic organisms by regulating the flux of components between the blood and the brain (Abbott *et al.*, 2010). This barrier can be disrupted under different conditions involving injury and/or inflammation of the central nervous system (CNS), like multiple sclerosis, ischemic stroke, encephalitis, tumors, Parkinson´s and Alzheimer´s diseases, epilepsy and AIDS-related dementia (Zlokovic, 2008; Martins *et al.*, 2011; Zhao *et al.*, 2015). The BBB is a selective diffusion barrier that is composed

of specialized endothelial cells (ECs) that are linked by tight (TJ) and adherens junctions (AJ) (Tietz and Engelhardt, 2015). ECs together with pericytes, astrocytes, extracellular matrix and neurons constitute a "neurovascular unit" that is essential for the normal function of the CNS (Hawkins and Davis, 2005; Muoio *et al.*, 2014). TJ are constituted by transmembrane proteins, such as claudin-5 and occludin, and by intracellular proteins, like zonula occludens (ZO). Claudin-5, the smallest protein (24 kDa), is responsible for the primary seal of the BBB, and occludin (64 kDa) is responsible for additional support. TJ are primarily responsible for the extremely low permeability and high electrical resistance of the BBB. Continuous strands of TJ are responsible for the almost complete sealing of the paracellular cleft between adjacent lateral endothelial membranes (Gloor *et al.*, 2001). Astrocytes are the largest subgroup of glial cells in the CNS and are involved in nourishing neurons, extracellular ion regulation, transmitter homeostasis and maintenance of the BBB. The astrocytic endfeet, part of the neurovascular unit, are close to the endothelial cells and help to stabilize and maintain BBB function (Abbott and Friedman, 2012). Additionally, adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), play an important role in the migration of activated peripheral blood T cells into the CNS, as adhesion molecules are released under inflammatory conditions.

The impact of METH on BBB has only recently started to be investigated. In fact, it is now known that METH can have a role by promoting BBB dysfunction through alterations in tight junction protein expression and conformation, and enzyme activation related to BBB cytoskeleton remodeling. Neuroinflammatory responses, such as gliosis and increase of inflammatory mediators, have also an important role on

BBB function (Martins *et al.*, 2013; Coelho-Santos *et al.*, 2015). These detrimental changes lead to increased permeability of the BBB and subsequent vulnerability of the brain to peripheral toxins (Kousik *et al.*, 2012) (Fig.1).

Physical exercise has recently started to receive attention of the scientific community with major interest in positive effects on cognitive functions, spatial learning and memory, and as a non-pharmacological method of maintaining brain health under conditions of neurodegenerative and/or psychiatric conditions (Cassilhas et al., 2016). Its positive correlation with aging has been the most explored topic since exercise plays a crucial beneficial role among senior citizens (Kirk-Sanchez and Mcgough, 2014). The neuroprotective effects of physical exercise on other conditions, such as stroke, cancer, CNS infection and ischemic brain injury, is also known (Guo et al., 2008; Zhang et al., 2011, 2013, 2015; Mota et al., 2012; Wang et al., 2014; De Senna et al., 2015; Wolff et al., 2015). Interestingly, it has been suggested a protective effect of exercise due to the modulation of inflammatory response and oxidative stress, with a possible consequence on BBB function (Li et al., 2005; Aksu et al., 2009; Wu et al., 2011; Mota et al., 2012). Noteworthy, Fontes-Ribeiro and collaborators (2011), suggested also that moderate physical exercise prior to METH administration may help to prevent its addiction (Fontes-Ribeiro et al., 2011). However, the beneficial/detrimental effects of exercise remain controversial.

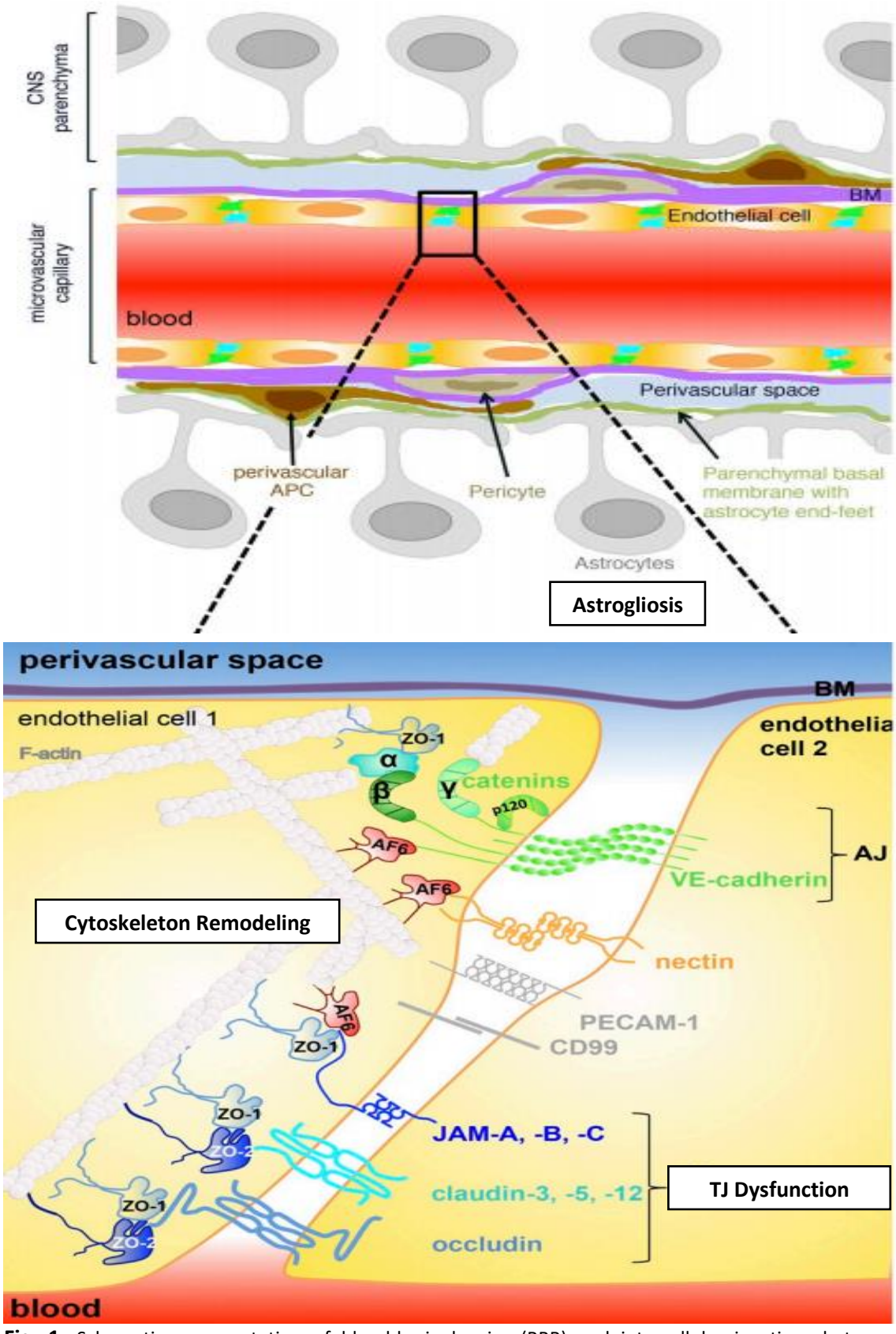

Fig. 1. Schematic representation of blood-brain barrier (BBB) and intercellular junctions between adjacent endothelial cells. Some targets of methamphetamine at the BBB level are highlighted in white boxes (TJ dysfunction, astrogliosis and cytoskeleton remodeling). Adapted from (Tietz and Engelhardt, 2015).

There is a lack of knowledge regarding the role of moderate exercise on BBB function under conditions of METH use. Also, not much is known about the underlying mechanisms involved in exercise-induced BBB alterations. One single study (Toborek *et al.*, 2013), using a voluntary wheel running protocol, suggested that endurance exercise training can protect against METH-induced BBB disruption by attenuating its effects on tight junctions. To date there is no effective therapy available to protect against METH neurotoxicity.

Therefore, the present work aims to evaluate the neurobiological effects of exercise on cerebrovascular toxicity of METH and, more specifically, to clarify some of the underlying cellular mechanisms responsible for METH-induced disruption of the BBB.

MATERIAL AND METHODS:

ANIMAL TREATMENT

Male C57BL/6J wild-type mice, aged three months (Charles River Laboratories, Inc, Barcelona, Spain) where housed under standard 12h light/dark cycle at room temperature (RT) of 21±1ºC, with *ad libitum* access to food and water. We used a binge protocol, with four doses of METH (4x 10 mg/kg; Sigma-Aldrich, St Louis, MO, USA) administered two hours apart, dissolved in a maximum volume of 100 µl of sterile 0.9% NaCl (intra-peritoneal injection). A total of 16 animals were used and sacrificed at different time points according to the experimental group. All procedures involving animals were performed by certified researchers (Portuguese National Authority for Animal Health "DGAV" and Federation for Laboratory Animal Science Associations, FELASA), and in accordance with both the European Community Council Directives (2010/63/EU) and the Portuguese law for the care and use of experimental animals (DL nº 113/2013). The experiments were approved by the institutional animal care and use committee in accordance with the ARRIVE guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used. The present study was supported by Pest-C/SAU/UI3282/2013-2014 and FCT-UID/NEU/04539/2013 (COMPETE-FEDER funds).

EXERCISE PROTOCOL

Mice were submitted to an exercise protocol with or without concomitant administration of METH (binge protocol, 4x 10 mg/kg i.p., 2h apart). Exercise training was performed on a motor treadmill (Treadmill Control, PanLab and LSI Letica) at a moderate intensity (16 cm/s) for 1 h/day, 5 days/week for 4 weeks, with a gradual increase in time of running (from 10 min to 1 h), corresponding to a 60-80% of VO_2max (Schefer and Talan, 1996; Perrino *et al.*, 2011). All animals were adapted to the procedure for one week (15 cm/s for 10 min) before beginning the exercise training

protocol. The sedentary animals were placed on the stationary treadmill during the period as for the other groups to provide the same stressful environment. The RT was controlled (21±2 ºC) to avoid effects caused by hyperthermia. Mice were divided in 5 different groups: group 1 – exercise training; group 2 – sedentary; group 3 – sedentary with administration of METH; group 4 – administration of METH 24h prior to the exercise training; group 5 – METH administration 24h after the exercise training. Mice were sacrificed 2h after METH administration and/or exercise training.

WESTERN BLOT ANALYSIS

The animals were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg, i.p.; Sigma-Aldrich) and transcardially perfused with 10 ml of 0.01 M phosphate buffered saline (PBS), pH 7.4, and the brain was immediately removed from the skull, placed on ice-cold dissection disc and the bilateral hippocampi and frontal cortex were dissected. The isolated tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 5 mM EGTA, 50 mM Tris, 1% (v/v) Triton, 0.1% sodium dodecyl sulfate (SDS) and 0.5% sodium deoxycholate), supplemented with protease inhibitor cocktail tablets (Roche Applied Sciences, Germany) in the ratio of 1 tablet/10 ml RIPA buffer. The homogenates were centrifuged at 14,000×g for 10 min, the supernatants were collected and protein concentration was determined by the Pierce bicinchoninic acid assay (BCA) Protein Assay Kit (Thermo Fisher Scientific, Northumberland, UK). Protein samples (50 µg or 100 µg) were separated by electrophoresis on 6–12% SDS-polyacrylamide gel electrophoresis (PAGE), and then transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Madrid, Spain). Membranes were then blocked for 1h at RT in blocking

solution, PBS containing 0.1% (v/v) Tween-20 (PBST) and 5% (w/v) non-fat dried milk, and incubated with primary antibodies overnight at 4°C as follows: albumin (1:2000, Bethyl Laboratories, Inc, Montgomery, TX, USA), claudin-5 (1:200, Invitrogen Lab), VCAM-1 (1:100; Zymed Lab) or GFAP (1:250; Zymed Lab). After washing 3×10 min with PBST, the membranes were incubated with alkaline phosphatase-conjugated secondary antibodies (1:20000; Amersham, GE Healthcare Life Science, USA) for 1 h at RT. The assessed proteins were detected using the Enhanced Chemifluorescence (ECF) reagent (Amersham, GE Healthcare Life Science, Buckinghamshire, UK) on the Typhoon FLA 9000 (GE Healthcare Bioscience AB, Uppsala, Sweden). The blots were stripped and reprobed with an antibody against GAPDH, which was used as loading control, (1:1000; Invitrogen). Band intensities were quantified using the ImageJ 1.44o software.

STATISTICAL ANALYSIS

Statistics were performed using a one-way ANOVA, followed by Dunnett's Multiple Comparison Test to detect differences between control and experimental groups, and Bonferroni's Multiple Comparison Test for comparisons among experimental groups. Results are expressed as mean + SEM, and the level of $p<0.05$ was accepted as statistically significant. “n” represents the number of experiments obtained from different animals.

RESULTS

Endothelial cells at BBB are linked by a junctional complex formed by the TJ and AJ, though it is primarily the TJ that are responsible for the low paracellular permeability and high electrical resistance of the BBB (Silva et al., 2010). In fact, alterations in the content and/or organization of TJ proteins, like ZO-1, claudin-5 and occludin, are usually associated with an impairment of the BBB and with an increase in permeability (Martins et al., 2011). However, the BBB is not formed only by endothelial cells but also by pericytes, extracellular matrix, astrocytes, microglia and neurons, which altogether form the neurovascular unit (Hawkins and Davis, 2005; Muoio *et al.*, 2014).

The impact of physical exercise on METH-induced BBB disruption is yet unknown. Moreover, different brain regions can have distinct responses to the same injury. Thus, in order to assess the BBB region-specific status, we analyzed the protein levels of Albumin, Claudin-5, Glial Fibrillary Acidic Protein (GFAP) and Vascular Adhesion Molecule – 1 (VCAM-1) in the hippocampus and pre-frontal cortex (PFC).

Hippocampus

In hippocampal region, we found an increase of albumin protein levels in Ex CTR, Sed METH and METH Ex groups, as follows: 181.20±24.91%, 186.50±25.83% and 214.70±37.88%, respectively (Fig. 2A). In the Ex METH group the result was not statistically significant (154.80±27.88%).

Regarding claudin-5 protein levels, we observed a significant decrease in Ex CTR and Sed METH groups (58.90±3.25% and 66.61± 5.72%, respectively; Fig. 2B), whereas there were no alterations in Ex METH and METH Ex groups (123.50±15.52%

and 107.7±9.670%, respectively). Thus, the exercise and METH by themselves seem to increase BBB permeability which can be explained, at least in part, by a downregulation of claudin-5. Interestingly, when exercise was present before METH, there was an upregulation of claudin-5 leading to a reestablishment of barrier properties. On the other hand, despite no alterations on claudin-5 in the group where METH was administered before exercise, there was an increase in albumin suggesting barrier alterations. In fact, several alterations at the BBB level can explain this observation, and the study of other intercellular proteins would be important.

Also, we observed an increase in GFAP protein levels in Ex CTR, Sed METH and Ex METH groups: 172.60±23.24%; 186.70±17.00%; 211.6±26.03%; respectively (Fig. 2C). Interestingly, METH Ex group (77.00±7.03%) when compared with Ex CTR or with Sed METH groups showed a significant decrease of GFAP protein levels. There was a clear astrogliosis promoted by exercise and METH by themselves that recovered to basal levels only when exercise was applied after METH administration. Here, the exercise did not have a preventive effect.

Concerning VCAM-1 protein levels, we observed a significant increase in Sed METH, Ex METH and METH Ex groups (167.50±13.09%, 161.40±16.54% and 200.00±27.51%, respectively; Fig. 2D). However, no differences were observed in Ex CTR group (72.76±8.23%). Noteworthy, exercise was not able to prevent or recovery the effect of METH.

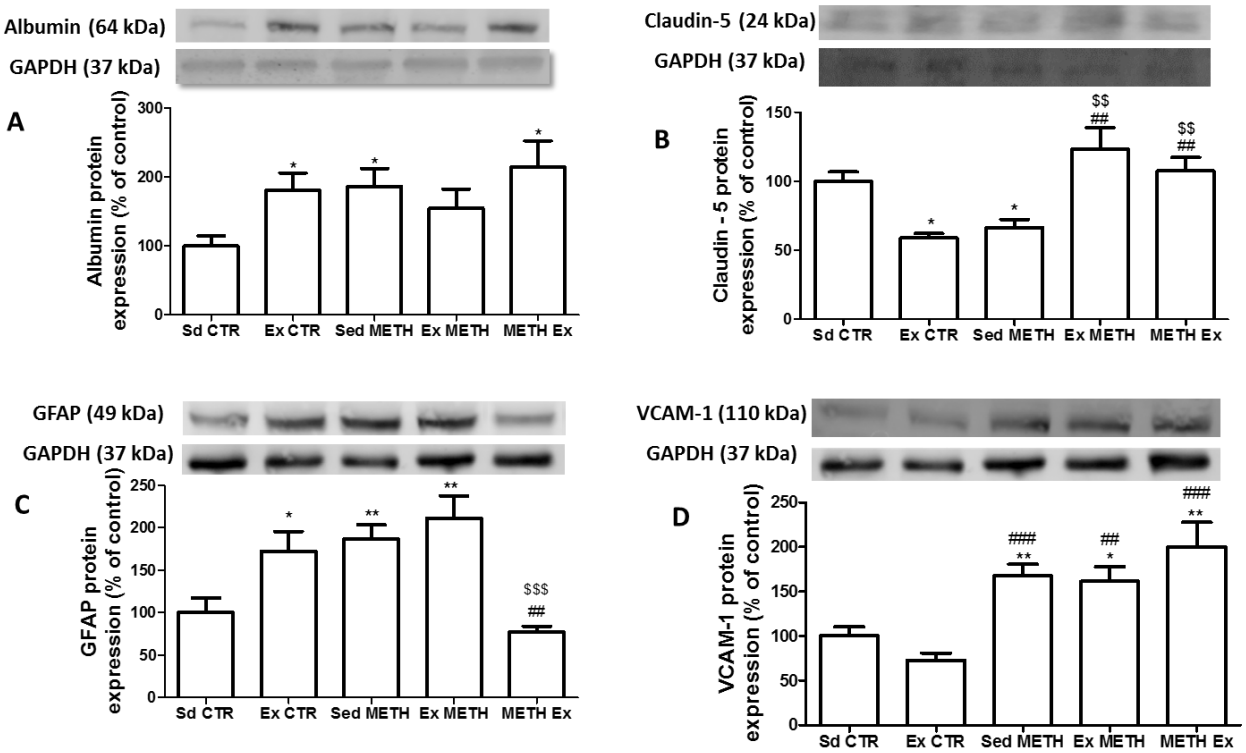

Fig. 2 – Hippocampal expression of albumin, claudin-5, GFAP and VCAM-1. (A) We observed a significant increase of albumin protein levels in Ex CTR, Sed METH and METH Ex groups, when compared with Sd control (n=7-14). **(B)** Regarding claudin-5, we found a decrease in Ex CTR and Sed METH groups when compared with control. (n=8-20). **(C)** Concerning GFAP expression, there was an increase in Ex CTR, Sed METH and Ex METH groups, when compared with control (n=9-18). **(D)** Finally, in VCAM-1 we observed an increase in Sed METH, Ex METH and METH Ex protein levels (n=7-12). Representative western blots for the tested protein and GAPDH (37 kDa) are shown above the bars. All results are expressed as mean ± S.E.M., *P<0.05, **P<0.01 when compared to Sd CTR; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 when compared to Ex CTR; $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001 when compared to Sed METH.

Pre-Frontal Cortex

In cortical brain region, we observed no differences in albumin protein expression as follows: Ex CTR (107.60±18.46%), Sed METH (118.40±16.88%), Ex METH (119.30±17.47%) and METH Ex (164.00±28.01%) (Fig. 3A). Nevertheless, there was a tendency to increase in the condition METH Ex.

When we looked to claudin-5 levels, we observed a significant increase in Ex CTR group (179.10±28.02%, Fig. 3B). However, there was no alterations in Sed METH, Ex METH and METH Ex groups (95.88±10.32%, 83.64±15.08% and 65.26±7.78%, respectively) when compared to control group. Regarding exercise impact, we observed a decreased claudin-5 expression when compared Ex METH and METH Ex groups to Ex CTR group showing that exercise by itself promoted the upregulation of claudin-5, and pre- and post-METH administration blocked exercise effect. Moreover, METH Ex group also showed a decreased expression when compared to Sed METH group. Thus, the tendency to increase albumin expression in the condition of METH followed exercise, can be explained by the tendency to decrease claudin-5 expression. Concerning GFAP protein levels, we did not observe differences as follows: Ex CTR, 96.28±8.33%; Sed METH, 100.50±8.41%; Ex METH, 96.09± 11.38%; and METH Ex, 90.06± 8.79% (Fig. 3C). Here, neither METH nor exercise caused astrocytic activation.

Finally, VCAM-1 expression showed a significant increase in Ex METH and METH Ex groups (141.20±14.75% and 211.50±29.66%, respectively; Fig. 3D), whereas in the Ex CTR and Sed METH groups did not show alterations (116.70±9.95%; 95.53±5.61%; respectively). Protein levels in Ex METH and METH Ex were also significantly increased when compared to Ex CTR and Sed METH. Therefore, our results suggests that exercise does not have preventive or harmful effect under METH conditions.

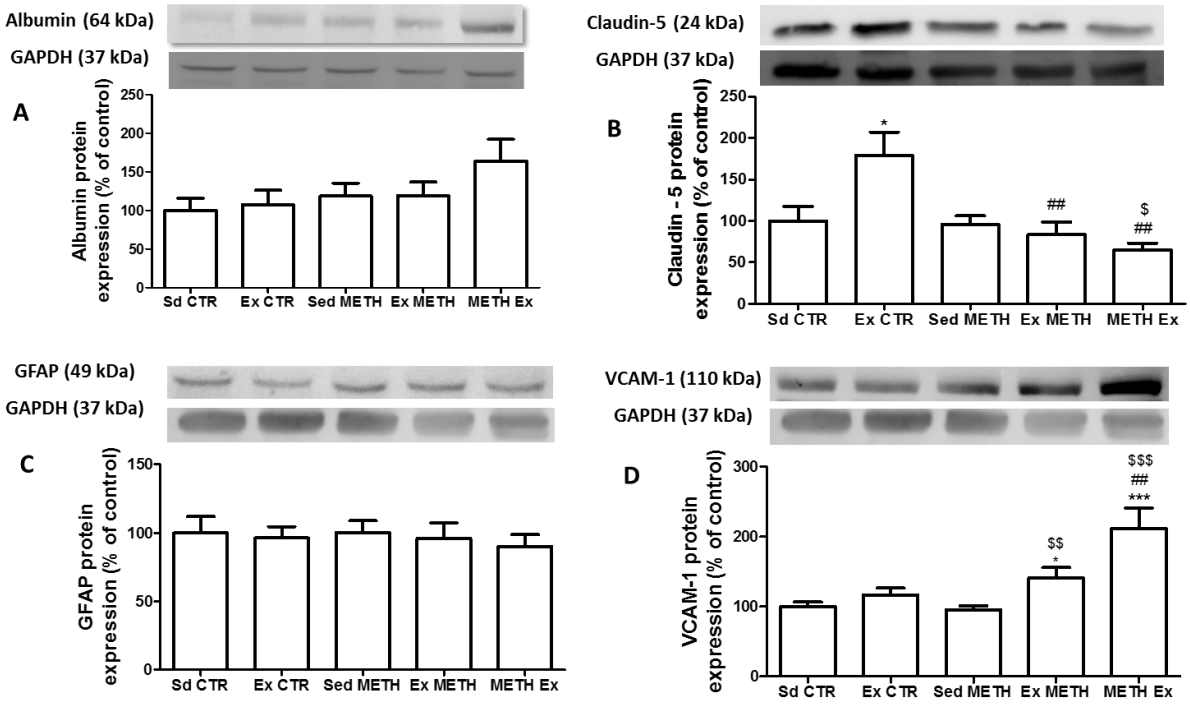

Fig. 4 – Pre-Frontal Cortex expression of albumin, claudin-5, GFAP and VCAM-1. (A) We did not observe significant differences of albumin protein level when compared with the control (n=7-14). **(B)** Regarding claudin-5, we found an increase in Ex CTR group when compared with control. This can also be observed when compared with Ex METH and METH Ex groups (n=8-20). **(C)** Concerning GFAP expression, it did not show significant differences when compared with the control (n=9-18). **(D)** Finally, in VCAM-1 we observed an increase in Ex METH and METH Ex protein levels (n=7-12). Representative western blots for the tested protein and GAPDH (37 kDa) are shown above the bars. All results are expressed as mean ± S.E.M., *P<0.05, ***P<0.001 when compared to Sd CTR, $^{\#\#}$P<0.01 when compared to Ex CTR, $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001 when compared to Sed METH.

DISCUSSION

Recently, METH has been suggested to lead to barrier impairment and different mechanisms were shown to be involved in this effect (Sharma and Kiyatkin, 2009; Gonçalves et al., 2014; Northrop and Yamamoto, 2015), including alterations on both transcellular and junctional/paracellular pathways across EC, involving oxidative stress and activation of matrix metalloproteinases (Martins et al., 2011; Coelho-Santos et al., 2015; Turowski and Kenny, 2015). Hyperthermia has also been suggested to promote BBB permeability (Watson et al., 2005; Maughan et al., 2007), astrogliosis and brain edema (Kiyatkin et al., 2007; Kiyatkin and Sharma, 2009; Kiyatkin, 2013). However, there are some contradictory results in the literature. Although hyperthermia seems to be an important factor in METH neurotoxicity, it was also shown that its consequences can be independent of body temperature alterations (Thomas et al., 2008; Herring et al., 2010).

Exercise has been identified as a non-pharmacological preventive and/or interventional treatment particularly due to its positive role on cognitive functions, including spatial learning and memory. A single bout of exercise can lead to improvements in CNS functions (Weinberg et al., 2014). However, increase in the BBB permeability has been observed during exercise (Watson et al, 2006; Nierwinska et al, 2008; Netto et al, 2006). Specifically, the authors used S100β protein (once used to assess BBB leakage) that was found to be also released by extra cerebral sources as activated adipocytes (Netto et al., 2006), by damaged muscle cells (Stocchero et al., 2010) and other tissues (Koh and Lee, 2014), contributing to the increased levels of the protein during physical activity. Other study using an alternative technique, such as

Evans blue extravasation into the brain parenchyma, showed increased BBB permeability in several brain regions after forced swimming (Sharma et al., 1996). A recent study suggested that exercise has the potential to increase BBB permeability without causing structural brain damage subsequent to a free radical-mediated impairment in the dynamic cerebral autoregulation (Bailey et al., 2011). Recently, Koh and Lee (2014) concluded that BBB can be compromised following exercise, with severity depending on the exercise intensity, wherein higher intensities lead to superior BBB impairment condition (Koh and Lee, 2014). In fact, under extreme conditions, such as high-intensity physical exercise, the antioxidant response may not be sufficient to keep the brain homeostasis (Aguiar et al., 2008). Despite these data, there are contradictory observations. Some authors showed that exercise performed at different strengths did not cause oxidative stress in the PFC, striatum and hippocampus (Acikgoz et al., 2006; Aksu et al., 2009). Others demonstrated that exercise has deleterious effects (Aguiar et al., 2010; Teixeira et al., 2009; Watson et al., 2006; Nierwinska et al., 2008). Increased vulnerability to oxidative damage of the striatum induced by high-intensity exercise is associated with the disruption of implicit memory in mice and accompanied by an alteration of signaling proteins involved in the plasticity of this brain structure (Aguiar et al., 2010). Moreover, using chronic intense exercise, it was verified deleterious effects on the striatal dopaminergic system that can be related to a higher consumption of oxygen during chronic intense exercise (Teixeira et al., 2009). It was also shown a disruption of the BBB after prolonged exercise in warm environments (Watson et al., 2006) and after forced swimming exercise (Nierwinska et al., 2008).

Stress induced by forced exercise should also be investigated in detail. One report proved that various stress modalities (exercise or restraint) have no repercussions on the BBB permeability (Ovadia et al., 2001), except in diencephalon (especially in the hypothalamus and thalamus) and cerebellum, where stress caused by restraint increases BBB permeability through activation of brain mast cells (Esposito et al., 2001). Morgan and collaborators (2015), showed that exercise induces numerous molecular and neuronal adaptations in the brain stem, hypothalamus and basal ganglia, mainly by using forced methods that may induce psychological stress. In contrast, studies using voluntary wheel running methods have identified a range of regional exercise-induced molecular neurophysiological mechanisms that may contribute to desirable changes in brain region specific functions, suggesting oxidative stress involvement (Morgan et al., 2015). Curiously, some authors demonstrated that by preventing oxidative stress, it was possible to avoid BBB METH-induced damage (Sharma et al., 1995, 2007; Ramirez et al., 2009; Gonçalves et al., 2010; Toborek et al., 2013; Zehendner et al., 2013). Studies involving both stress and METH variables, showed that stress enhances METH-induced early BBB disruption and prolongs the opening of the BBB, as compared to METH alone in a cyclooxygenase-dependent manner (Northrop and Yamamoto, 2012; 2015).

On the other hand, exercise by itself can have beneficial effects by its ability to avoid oxidative stress. One study, suggested that physical exercise training improves some cognitive functions in rats, with attenuation of oxidative stress damage in the brain (Radak et al., 2001). Others showed that long term exercise protects dopaminergic neurons (as in striatum and PFC) against inflammatory insult (Wu et al., 2011). A recent review also suggests a positive role of exercise by attenuating

neuroinflammation in neurological diseases (Spielman et al., 2016). In our study, we observed that in the hippocampus, both METH and exercise led to an upregulation of albumin expression that suggests an impairment of the BBB with repercussion on its permeability, since albumin (65 kDa molecular weight protein), a blood serum protein that does not cross the BBB under normal conditions, was identified in the brain. Regarding PFC results, we only observed a tendency to increase BBB permeability when METH preceded exercise. In a previous study, Martins and collaborators (2011) also showed that hippocampus was more susceptible to acute METH administration, since only this brain region showed BBB disruption.

METH has the ability to alter the BBB function through direct effects on endothelial cells by modulating the tight junction (TJ) proteins (Mahajan et al., 2008). Thereby, and for a better understanding of the mechanisms underlying the increased BBB permeability, we investigated possible alterations of TJ protein levels. As aforementioned, alterations of claudin-5, responsible for the primary seal of the BBB, could be related with BBB impairment (Haseloff et al., 2015). In hippocampus, we observed a decrease of hippocampal claudin-5 protein expression that could explain BBB leakage, except in METH Ex group where we did not have significant differences. Curiously, we noticed that exercise by itself seems to increase BBB leakage that can be explained by alterations in claudin-5 levels. Accordingly, Martins and collaborators (2011) showed that METH transiently increases the BBB permeability in the hippocampus due to downregulation of TJ proteins (namely claudin-5, occludin and zonula occludens-1) (Martins *et al.*, 2011). Furthermore, in the hippocampus we observed a possible protective role of exercise pre-METH administration by its ability to avoid the decrease of claudin-5 levels and consequently no significant upregulation

of albumin was observed. Accordingly, Coelho-Santos and collaborators (2015) proved that inhibition of inflammatory response prevented permeability induced by METH by restoring the levels of claudin-5 (Coelho-Santos et al., 2015). One report has shown beneficial effects of exercise after administration of METH by its attenuating effects in TJ (Toborek et al., 2013). Moreover, Siddharthan and collaborators (2007) stated that shear stress due to the effects in TJ proteins led to the maintenance of barrier properties of endothelial cells (Siddharthan et al., 2007). It was also demonstrated that METH can promote vesicular transport in brain ECs (Martins et al., 2013), suggesting that METH is able to increase BBB permeability by different mechanisms.

Concerning PFC, both exercise and METH had no significant effects on BBB leakage, corroborated with no alterations in claudin-5 protein levels. Interestingly, exercise group was the only one that showed claudin-5 level increase, which could be explained by exercise reinforcement/reorganization and/or by shear stress on this particular brain region. In fact, as abovementioned, Martins and collaborators (2011) showed no alterations on cortical BBB permeability (Martins et al., 2011). Here we only looked to claudin-5, but BBB is a very complex structure where several compensatory phenomena can occur. Thus, more studies are necessary to better clarify these alterations in each brain region and between them.

METH-induced neuroinflammatory response with increased release of pro-inflammatory cytokines is another proposed mechanism of BBB impairment (Gonçalves et al., 2010; Silva et al., 2010; Coelho-Santos et al., 2012; Erickson et al., 2012). Gonçalves and collaborators (2008) demonstrated METH early effects on hippocampus and PFC, with upregulation of pro-inflammatory cytokines (Gonçalves et al., 2008). In fact, METH triggered the release of TNF-α by astrocytes that will

negatively interfere with barrier function (Gonçalves et al., 2010; Coelho-Santos et al., 2015). GFAP and vascular adhesion molecule (VCAM-1) proteins alterations can be associated with neuroinflammatory conditions (Gonçalves et al., 2010; Santha et al., 2015). Regarding GFAP protein, it is expressed by astrocytes, which are the major glial cells in the CNS and play an important role in nourishing neurons, regulation of extracellular ion, neurotransmitters homeostasis and maintenance of the BBB (Muoio et al., 2014). Previous studies demonstrated that under normal conditions, astrocytosis have a beneficial role in BBB properties (Kuo and Lu, 2011). Astrocytes has been shown to be capable of maintaining the BBB characteristics since they produce several factors responsible for the endothelial cells to develop a BBB property (Kuo and Lu, 2011). In the present study, we observed an increase of GFAP expression in the hippocampus with both exercise and METH, except when exercise occurred post-METH administration. This is consistent with recent findings showing that exercise increases GFAP expression in the hippocampus (Saur et al., 2014). In METH Ex group there was no alteration of GFAP levels, which could suggest a possible protective role of exercise post-METH administration. In fact, Gonçalves and collaborators (2010) showed that by using an anti-inflammatory strategy it was possible to prevent METH-induced astrogliosis (Gonçalves et al., 2010). Moreover, no changes on total GFAP levels do not necessary mean that no astrocytic alterations are observed. In fact, Coelho-Santos and collaborators (2015) showed that METH promoted perivascular astrogliosis without alterations of GFAP protein levels. Moreover, exercise after METH administration could increase vascular circulation leading to an increased drug toxic effects and eventually causing astrocytic death. In fact, METH treatment causes neuronal cell death in several brain regions, including frontal cortex, striatum and hippocampus (Deng et al., 2001;

Bowyer and Ali, 2006; Sharma and Ali, 2006; Dietrich, 2009). Accordingly, Packer and Hoffman-Goetz (2015) found that acute exercise (treadmill running) in mice resulted in increased hippocampal levels of TNF-α, and increased apoptosis, as measured by elevated levels of caspase-3 and caspase-7 (Packer and Hoffman-Goetz, 2015). In PFC, we did not see alterations in GFAP expression. However, other technical approaches would be important to analyze other possible alterations in astrocytes, such as perivascular astrogliosis (Coelho-Santos et al., 2015).

Concerning VCAM-1 protein, it plays a role in leucocyte CNS infiltration through BBB (Steiner et al., 2010; Greenwood et al., 2011; Haarmann et al., 2015). Therefore our increased levels on hippocampus in groups with METH administration suggests vascular inflammatory status that can lead to infiltration of immune peripheral cells and BBB impairment. Here, we did not observe exercise protective/harmful effects. Curiously, in the PFC there was only a significant increase of VCAM-1 levels when METH was combined with exercise, which could be explained by increased cerebral blood circulation leading to an exercise drug-effects reinforcement.

CONCLUDING REMARKS

Distinct responses to METH were observed in different cell types, such as ECs and astrocytes (Coelho-Santos et al., 2015). Here we also found brain region-specific responses to the same aggressor (METH). Hippocampus seems to have higher permeability alterations justified by neuroinflammatory mechanisms including astrocytic activation, with exercise having a role in METH-induced BBB effects. In PFC we found only drug-mediated effects in adhesion molecules that play a crucial role on vascular infiltration, with no BBB leakage neither exercise influence.

Exercise itself seems to affect BBB integrity in the hippocampus due to its effects in ECs, namely in TJ claudin-5 levels and increased astrocytic activity, whereas in PFC there was no influence. METH itself triggered a neuroinflammatory response in the hippocampus and again with no effect in the PFC.

Exercise protective effect regarding claudin-5 levels was observed pre- and post-METH administration in the hippocampus. Interestingly, in the PFC we noticed a harmful exercise effect on neuroinflammatory response triggered by METH.

Taken together, these data suggest that hippocampus shows a higher susceptibility to METH-induced neuroinflammation, and also that there was a preventive role of exercise, mostly before METH administration. The highest susceptibility of the hippocampus to METH and/or exercise remains to be further investigated, and also remains unclear the reason why exercise has more beneficial effects in this cerebral region, whereas in PCF has the opposite effect. Therefore, more studies are needed to investigate the brain region-specific responses and its relation with exercise.

Since our results suggest an increase of the BBB permeability in the hippocampus, it may present an opportunity to enhance delivery of chemotherapeutic agents to the underlying neural tissue. As we know, BBB is at the same time the protector of the brain from harmful substances and a huge hurdle to drug pharmacotherapy development (Cecchelli et al., 2007). On the other hand, in neuroinflammatory diseases exercise may have a protective role.

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