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# Methamphetamine promotes α-tubulin deacetylation in endothelial cells: The protective role of acetyl-L-carnitine

S. Fernandes [a,b,c,d], S. Salta [a,b,c], T. Summavielle [*,a,b,c]

[a] Rua Alfredo Allen, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, 4200-135 Porto, Portugal
[b] Rua do Campo Alegre, 823, Addiction Biology Group, Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, 4150-180 Porto, Portugal
[c] Rua Valente Perfeito, 322, School of Allied Health Sciences – Polytechnic Institute of Porto (ESTSP-IPP), 4400-330 Vila Nova de Gaia, Portugal
[d] Alameda Prof. Hernâni Monteiro, Faculdade de Medicina da Universidade do Porto (FMUP), 4200-319 Porto, Portugal

## HIGHLIGHTS

- Methamphetamine induces α-tubulin deacetylation in endothelial cells.
- Acetyl-L-carnitine prevents methamphetamine-induced deacetylation of microtubules.
- Acetyl-L-carnitine modulates the activity of HDACs at the post translational level.

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## ABSTRACT

Methamphetamine (METH) is a powerful psychostimulant drug used worldwide for its reinforcing properties. In addition to the classic long-lasting monoaminergic-disrupting effects extensively described in the literature, METH has been consistently reported to increase blood brain barrier (BBB) permeability, both *in vivo* and *in vitro*, as a result of tight junction and cytoskeleton disarrangement. Microtubules play a critical role in cell stability, which relies on post-translational modifications such as α-tubulin acetylation. As there is evidence that psychostimulants drugs modulate the expression of histone deacetylases (HDACs), we hypothesized that in endothelial cells METH-mediation of cytoplasmatic HDAC6 activity could affect tubulin acetylation and further contribute to BBB dysfunction. To validate our hypothesis, we exposed the bEnd.3 endothelial cells to increasing doses of METH and verified that it leads to an extensive α-tubulin deacetylation mediated by HDACs activation. Furthermore, since we recently reported that acetyl-L-carnitine (ALC), a natural occurring compound, prevents BBB structural loss in a context of METH exposure, we reasoned that ALC could also preserve the acetylation of microtubules under METH action. The present results confirm that ALC is able to prevent METH-induced deacetylation providing effective protection on microtubule acetylation. Although further investigation is still needed, HDACs regulation may become a new therapeutic target for ALC.

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## 1. Introduction

Methamphetamine (METH) is a powerful psychostimulant used worldwide for its reinforcing properties that leads to long-lasting deleterious effects (Gold et al., 2009; Krasnova and Cadet, 2009). METH toxicity is characterized by the disruption of the dopaminergic system, concomitant with terminal degeneration and eventual neuronal death (Conant et al., 2011; Krasnova and Cadet, 2009). However, METH has been increasingly recognized to impact also the blood-brain-barrier (BBB), causing the release of inflammatory mediators and astrogliosis (Gold et al., 2009; Goncalves et al., 2010; Northrop and Yamamoto, 2012; Ramirez et al., 2009). METH-induced permeability at the BBB level has been consistently reported both *in vivo* and *in vitro* (Conant et al., 2011; Martins et al., 2011; Urrutia et al., 2013), as a result of tight junction and cytoskeleton disarrangement

* Corresponding author at: Addiction Biology Group, Instituto de Biologia Molecular e Celular (IBMC), Rua do Campo Alegre 823, 4150-180 Porto, Portugal. Tel.: +351 226 074 900.
*E-mail addresses:* silvia.fernandes@ibmc.up.pt (S. Fernandes), sofia.salta@ibmc.up.pt (S. Salta), tsummavi@ibmc.up.pt (T. Summavielle).

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(Dietrich, 2009; Kousik et al., 2012; Park et al., 2013). In endothelial cells, METH was also shown to trigger nitric oxide (NO)-mediated transcytosis (Martins et al., 2013). Recently, we showed, also in endothelial cells, that exposure to METH leads to disruption of actin filaments concomitant with claudin-5 translocation to the cytoplasm, promoted by MMP-9 activation in association with ILK overexpression (Fernandes et al., 2014).

Similarly to the actin filaments, microtubules play a critical role in cell stability and dynamics. Proper regulation of microtubule components relies on post translational modifications such as α-tubulin acetylation (Hammond et al., 2008). Microtubule deacetylation is carried out by histone deacetylase (HDAC) 6, a class II HDAC and the class III HDAC sirtuin 2 (SIRT2), which form a complex that allows them to bind to tubulin (Hubbert et al., 2002; Nahhas et al., 2007; Sadoul et al., 2011; Yang and Seto, 2008). Of note, it was recently shown that interfering with HDAC6 is sufficient to prevent microtubule deacetylation (Gold et al., 2015). There is growing evidence that HDACs inhibition is strongly associated with decreased cell mobility, which is of particular interest in the oncology field (Hrabeta et al., 2014; Ocker and Schneider-Stock, 2007). Although there are several studies showing that METH and other psychostimulants affect the expression of HDACs (Cassel et al., 2006; Host et al., 2011; Martin et al., 2012; Omonijo et al., 2014), and in particular the expression of HDAC6 (Omonijo et al., 2014), the effect of METH in microtubules acetylation was not yet explored. Therefore, we hypothesized that METH-induced regulation of HDACs activity, and in particular of HDAC6, may also mediate the structural loss observed in METH-exposed endothelial cells. Moreover, since we recently showed that a pretreatment with acetyl-L-carnitine (ALC) was able to prevent METH-induced activation of MMP-9, preserving the actin structural arrangement in the endothelial cells (Fernandes et al., 2014), and ALC was shown to have the potential to interact with HDAC activity (Huang et al., 2012), we reasoned that ALC could also preserve the acetylation of microtubules under METH action. ALC is a natural occurring compound that was seen to be protective by different mechanisms in several neurological conditions, including BBB dysfunction (Alves et al., 2009; Haorah et al., 2011; Muneer et al., 2011; Pettegrew et al., 2000).

To verify our hypothesis, we exposed the endothelial cell line bEnd.3 to increasing doses of METH and evaluated the individual and combined action of METH and ALC on α-tubulin acetylation. Trichostatin A (TSA), a natural product isolated from *Streptomyces hygroscopicus* commonly used as an inhibitor of class I/II HDACs known to promote microtubule acetylation, was also assayed (Dompierre et al., 2007; Harrison and Dexter, 2013).

## 2. Material and methods

### 2.1. In vitro model and cell culture

The immortalized bEnd.3 cells are derived from mouse brains and known to mimic some of the BBB characteristics. The cell line bEnd.3 was obtained from ATCC (American Type Cell Culture-CRL-2299, Manassas, VA) and cultures were maintained in DMEM (1×)/Glutamax (GIBCO®, Life Technologies, Paisley, UK), containing 1% penicillin and streptomycin (GIBCO®, Life Technologies) and 10% fetal bovine serum (GIBCO®, Life Technologies). Purity of the cell line was checked using an anti-CD31 antibody (Abcam 7388, rat monoclonal, 1:1000), which showed 100% enrichment of cells on the adhesion marker. For immunocytochemistry, cells were plated on 24-well plates (80,000 cells/well) containing glass cover slips. To obtain protein extracts or mRNA, bEnd.3 cells were cultured in petri dishes (1 million of cells). Cell culture media was changed every 3 days until cells were confluent.

### 2.2. Drug regimen

ALC-hydrochloride was kindly provided by Sigma-Tau S.p.A (Pomezia, Italy). TSA was purchased from Promega (#G6560) and METH hydrochloride from Sigma–Aldrich (St. Louis, MO, Cat. M-8750).

Immortalized bEnd.3 cells at confluence were treated with 0.5 mM and 1 mM of METH. METH doses were predetermined in our laboratory in agreement with previous studies using METH in similar cell models (Fernandes et al., 2014; Jin et al., 2002). ALC 1 mM was added 30 min before METH. TSA was used in a 100 nM concentration. The selected TSA and ALC doses were previously shown to be safe for the cells and are below the range of doses previously used in similar works (Huang et al., 2012; Hubbert et al., 2002; Tu et al., 2014).

### 2.3. Immunostaining procedure

For immunocytochemistry, bEnd.3 cells were cultured on glass cover slips in 24 well plates until 90–100% confluence. Cells were then treated with 0.5 and 1 mM of METH in the presence or absence of 100 nM TSA or 1 mM ALC, for 24 h. To evaluate α-tubulin, cells were washed with PBS, fixed during 10 min in methanol and permeabilized in 0.1% Triton X-100 during 10 min. After a blocking of 45 min in 10% NGS (normal goat serum), cells were incubated overnight at 4 °C with respective primary antibodies – Mouse anti-α-tubulin (Sigma, clone AA13 IgG1, #T8203, 1:500) and Mouse anti-acetyl-α-tubulin (Sigma, clone 6-11B-1 IgG2b, #T7451, 1:500). For secondary antibody incubation we used Anti-Mouse IgG Alexa-Fluor 488® Conjugate (Life Technologies, #A11001, 1:1000) for acetylated form and Anti-Mouse IgG Alexa-Fluor 568® Conjugate (Life Technologies, #A11004, 1:1000) for total α-tubulin, for 1 h at room temperature in the dark. Coverslips were then mounted onto glass slides with immunomount (Fluorescent Mounting Medium, CA, USA) containing 4′,6-diamidino-2-phenylindole (DAPI), and then fluorescence microphotographs were captured using Axio Imager Z1 fluorescence microscope (Carl Zeiss, Germany).

### 2.4. Morphometric analysis

Changes observed in α-tubulin acetylation by immunofluorescence assays were evaluated using the Fiji Software version 2.0. A total of four independent experiments were performed. From each coverslip, ten images were blindly captured and analyzed through measurement of fluorescence intensity.

### 2.5. Protein expression analysis by western blot

Confluent bEnd.3 cells cultured in petri dishes were scrapped and lysed with TEN buffer (50 mM Tris–HCl, 2 mM EDTA, 150 mM NaCl, 1% NP-40, supplemented with phosphatases and proteases inhibitors) and then centrifuged at 14,000 × *g* for 15 min at 4 °C. Protein concentration of the cell lysate was estimated in the supernatant by the Bradford method (Bio-Rad Protein Assay, Munich, Germany). Proteins were loaded at 25 μg per lane and resolved by SDS-PAGE on 12% Bis/acrilamide gels and then transferred onto PVDF membranes. After blocking with dry milk 5%, membranes were incubated with mouse anti-acetyl-α-tubulin antibody (Sigma, clone 6-11B-1 IgG2b, #T7451,1:1600). Membranes were washed in Stripping Buffer (Restore™ Western Blot, Thermo Scientific) and then incubated with mouse anti-α-tubulin (Sigma, clone AA13 IgG1, #T8203, 1:1000). Secondary antibody HRP (horseradish peroxidase)-conjugated were used for 1 h of incubation. Chemiluminescent signal detection was achieved using the Immun-Star HRP kit (Bio-Rad Laboratories, USA). A

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