Impact of methamphetamine on the hippocampal neuropeptide Y system: Endothelial protective role

ARTIGO CIENTÍFICO

ÁREA CIENTÍFICA DE FARMACOLOGIA

Trabalho realizado sob a orientação de:
ANA PAULA SILVA MARTINS

MARÇO/2017
Impact of methamphetamine on the hippocampal neuropeptide Y system:  

Endothelial protective role

Fabiana Patrícia Nunes Ventura¹, Ricardo Alexandre Gomes Leitão¹,²,³, Vanessa Filipa Coelho-Santos¹,²,³, Ana Paula Pereira da Silva¹,²,³

1) Institute of Pharmacology and Experimental Therapeutics, Faculty of Medicine, University of Coimbra, Portugal; 2) Institute for Biomedical Imaging and Life Sciences (IBILI); 3) CNC.IBILI, University of Coimbra, Portugal.

fabi.ventura4@gmail.com
Impact of methamphetamine on the hippocampal neuropeptide Y system: Endothelial protective role

Abstract
Methamphetamine (METH) consumption is a worldwide health problem that can lead to ischemic stroke and psychiatric disturbances. Among several negative effects triggered by METH at the Central Nervous System, it is now well-established that METH also causes blood-brain barrier (BBB) dysfunction. Moreover, there are several studies showing that neuropeptide Y (NPY) has an important protective role by acting as dentate gyrus neurogenesis promoter, as an antiepileptic molecule, by preventing memory deficits, and by protecting against METH-induced neuronal and microglial cell death. Despite these beneficial effects of the NPY system, nothing is known about its role on endothelial cells properties and function. Therefore, the aim of our work was to clarify how METH interferes with the hippocampal NPY system with particular attention to the neurovasculature, and then to unravel its role as a target to counteract METH-induced endothelial cell death.

Thus, in order to study the NPY system expression in the hippocampus we performed immunohistochemistry in brain slices obtained from C57BL/6J mice treated with a METH binge protocol (4× 10mg/kg, 2h apart). Moreover, we used human brain microvascular endothelial cell lines (hCMEC/D3), an *in vitro* model of human BBB, to clarify if these cells express NPY and its receptors, and further understand their role under METH injury. TUNEL assay was performed to measure cell death induced by METH alone or in combination with NPY system modulators.

Our results show an upregulation of NPY immunoreactivity in the mice hippocampus and Y1R in both hippocampus and endothelial cells culture. Further, we demonstrate that human endothelial cells express Y2R that are able to protect against METH-induced endothelial cell
death. In sum, we unveiled the NPY system as a possible therapeutic target under conditions of neurovascular dysfunction induced by METH.

**Keywords:** Methamphetamine; Neuropeptide Y; Y1R; Y2R; Hippocampus; Endothelial cells; Blood-brain barrier; Cell death.
Abbreviations

**BBB**  Blood-brain barrier

**BSA**  Bovine serum albumin

**FBS**  Fetal bovine serum

**hCMEC/D3**  Human brain microvascular endothelial cell line

**i.p.**  Intraperitoneal

**METH**  Methamphetamine

**NPY**  Neuropeptide Y

**PBS**  Phosphate-buffered saline solution

**PFA**  Paraformaldehyde

**RT**  Room temperature

**S.E.M.**  Standard error of the mean

**TUNEL**  TdT-mediated dUTP-X nick end labelling

**Y1R**  NPY receptor 1

**Y2R**  NPY receptor 2
**Introduction**

Methamphetamine (METH) is a highly addictive psychostimulant largely consumed worldwide. During the past few years METH use has increased all over Europe (1), including in Portugal, mainly due to the easy access and low cost production. In turn, METH abuse leads to serious health problems including neurological and psychiatric disturbances, such as anxiety, psychosis and antisocial personality disorder. Moreover, its neurotoxicity has been related with oxidative stress, and both glutamatergic and dopaminergic systems dysfunction (2, 3). Recently, the impact of METH on blood-brain barrier (BBB) integrity was pointed as a crucial event on brain alterations triggered by this drug of abuse (4, 5). The BBB is a highly selective barrier responsible for brain homeostasis and protection against toxic molecules or organisms. Moreover, our group has already shown that a single high dose of METH increases BBB permeability by decreasing the levels of tight junction proteins (ZO-1, occludin and claudin-5) that control the paracellular transport across brain endothelium (6).

The neuropeptide Y (NPY) is a 36-amino acid peptide that belongs to the neuropeptide tyrosine family along with peptide YY, pancreatic polypeptide, and non-mammalian pancreatic peptide Y (7). NPY is widely expressed in the central and peripheral nervous systems, and its pleiotropic functions comprises the regulation of brain activity, mood, stress, metabolism, and both vascular and immune functions (8). Importantly, NPY effects can be exerted by one of the four G-protein couple receptors, termed Y1R, Y2R, Y4R and Y5R. In fact, Y1R and Y2R are the most expressed within brain parenchyma and their activities have been widely associated with psychiatric disorders such as anxiety, depression and post-traumatic stress disorder (9). Regarding animal studies, it was found that NPY through Y2R is involved in memory deficits induced by METH, which was prevented by Y2R blockade (3). Additionally, Sparrow and colleagues (10) demonstrated that binge-like ethanol drinking in C57BL/6J mice was attenuated by NPY, Y1R agonist, and Y2R antagonist. Still, NPY and Y1R immunoreactivity were
reduced in the central nucleus of amygdala in binge-like ethanol drinking group, which was reverted by drug abstinence. Moreover, NPY has antiepileptic effects (11, 12) and prevents neuronal apoptosis (13). \textit{In vitro} studies also demonstrated that NPY is a protective agent against METH-induced neuronal and microglial cell death (14), as well as a promoter of dentate gyrus neurogenesis (15).

Several observations highlight the importance of NPY as a potential target in psychiatric disorders treatment. Noteworthy, clinical trials are ongoing to test the effect of intranasal NPY administration on mood/anxiety in health male volunteers (NCT 00748956) and in posttraumatic stress disorder (PTSD) (NCT 01533519) (9). Despite the promising protective effects of this neuropeptide, nothing is known about the role of NPY system on BBB function. Thus, the aim of this work was to uncover the role of NPY and its receptors on METH-induced endothelial dysfunction.

\textbf{Materials and methods}

\textit{Animal treatments}

Male wild-type C57BL/6J mice (3 month old, Charles River Laboratories, Barcelona, Spain) were housed under controlled environmental conditions (12h light: 12h dark cycle, 24 ± 1°C) with food and water ad libium. Mice were divided in two different groups as follows: control group that received four injections of 0.9% NaCl intraperitoneal (i.p.), 2h apart; and METH binge group [(+)-methamphetamine hydrochloride, Sigma-Aldrich, St. Louis, MO, USA; 4×10 mg/kg, 2h apart, i.p.]. Animal were sacrificed 2h after the last administration. The present study was approved by the Institutional Animal Care and Use Committee (FMUC/CNC, University of Coimbra, Portugal). Experiments were performed by certified researchers in accordance with European Community Council Directives (2010/63/EU) and Portuguese law for care and use
of experimental animals (DL nº 113/2013). All efforts were made to minimize animal suffering and to reduce the number of animals used.

**Immunohistochemistry**

Mice were anesthetized with sodium pentobarbital (Sigma Aldrich) and intracardially perfused with 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline solution (PBS). Then, brains were removed, post fixed in 4% PFA and transferred to 30% sucrose in PBS at 4°C. Coronal sections (12 μm) were cut and mounted directly onto superfrost microscope slides (Thermo Scientific, Braunschweig, Germany). Slices were rinsed in 0.01 M PBS, blocked with 5% fetal bovine serum (FBS)/0.5% Triton X-100 in 0.01 M PBS for 1h at room temperature (RT), and incubated overnight at 4°C with rabbit anti-NPY (1:150, Sigma-Aldrich), sheep anti-Y1R (1:200, Serotec, Oxfordshire, UK), rabbit anti-Y2R (1:100, Abcam, Cambridge, UK) or goat anti-CD31 (1:150, R&D Systems, Abingdon, UK) antibodies. Afterwards, slices were incubated for 1h with anti-rabbit Alexa Fluor 594, anti-sheep Alexa Fluor 488, anti-rabbit Alexa Fluor 488 and anti-goat Alexa Fluor 594 secondary antibodies (all 1:200; Invitrogen, Inchinnan Business Park, UK), followed by staining with 5 μg/mL Hoechst 33342 (Sigma-Aldrich) for 5 min at RT in the dark. Finally, slices were mounted with Dako fluorescence medium (Dako North America, Carpinteria, USA), and images recorded using a LSM 710 Meta Confocal microscope (Carl Zeiss, Oberkochen, Germany).

**Cell cultures**

The human brain microvascular endothelial cell line (hCMEC/D3) is an in vitro model of BBB that mimics important characteristics of in vivo human brain endothelium (16, 17). This cell line was cultured in EBM-2 medium (Lonza, Walkersville, MD, USA), supplemented with 1 ng/mL basic fibroblast growth factor (bFGF, Sigma-Aldrich), chemically defined lipid
concentrate (1:100; Invitrogen), 1.4 µM hydrocortisone (Sigma-Aldrich), 5 µg/mL acid ascorbic (Sigma-Aldrich), 1% Penicillin–Streptomycin (Gibco, Paisley, UK), 5% FBS (Invitrogen), and 10 mM HEPES (Lonza). Medium was changed every 2 days until the cells reached confluence. hCMEC/D3 cells were seeded on culture plates coated with collagen type I (R&D Systems, Inc., Minneapolis, USA) and maintained at 37°C with 5% CO₂.

**Immunocytochemistry**

hCMEC/D3 cells were left untreated (control) or incubated with METH (100 µM) for 24h and then rinsed with 0.01 M PBS, fixed with 4% PFA during 30 min at RT, permeabilized with 0.25% Triton x-100 for 10 min and blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich) for 1h at RT. Afterwards, cells were incubated with sheep anti-Y1R (1:200, Serotec, Oxford, UK), rabbit anti-Y2R (1:100, Abcam), rabbit anti-NPY (1:150 Sigma-Aldrich) and goat anti-CD31 (1:150, R&D Systems), overnight at 4°C. After rinsed in PBS, cells were incubated with anti-sheep Alexa Fluor 488, anti-rabbit Alexa Fluor 488, anti-rabbit Alexa Fluor 594 and anti-goat Alexa Fluor 594 (all 1:200, Invitrogen), respectively, for 1h at RT. Finally, cultures were mounted with Dako fluorescent medium (Dako North America Inc.) and images were captured using a LSM 710 Meta confocal microscope (Carl Zeiss).

**Quantification of NPY receptors immunoreactivity in hCMEC/D3 cells**

Immunoreactivity quantification was performed in FIJI software 2.0. In sum, all photograph was considered as well as three different areas without staining (black) to be used for background subtraction. To determine the corrected total Y1R or Y2R fluorescence, we used the following formula: correct total fluorescence = (integrated intensity) − (area of picture × mean background). The results are expressed as mean of fluorescence intensity (arbitrary units) of 2-6 photos from three different cell cultures for each experimental group.
**TUNEL Assay**

In TUNEL assay (TdT-mediated dUTP-X nick end labelling; Roche Diagnostics GmbH, Mannheim, Germany), hCMEC/D3 cells were incubated with 3 mM METH (Sigma-Aldrich) for 24h. Pre-treatment with NPY (1 µM) or/and different NPY system modulators were performed as follows (1 µM for all): Y1R agonist ([Leu^{31},Pro^{34}]NPY, Tocris Cookson, UK) and antagonist (BIBP3226, Tocris), Y2R agonist [NPY(13-36), Tocris) and antagonist (BIIE0246, Tocris), as shown in figure legends. After the respective treatments, we collected the whole cell population, including in suspension and adherent cells. Then, hCMEC/D3 cells were fixed with 4% PFA and adhered to superfrost microscope slides (Thermo Scientific) by centrifugation (113×g, 5 min; Cellspin, Thermac GmbH, Waldsolms, Germany). Afterwards, cells were rinsed with 0.01 M PBS, permeabilized with 0.2% Triton X-100 for 30 min at RT, and incubated with terminal deoxynucleotidyl transferase for 1h at 37°C in a humidified chamber. Then, hCMEC/D3 cells were washed in terminal buffer (300 mM sodium chloride and 30 mM sodium citrate) for 15 min followed by 0.01 M PBS for 5 min. Incubation with fluorescein Avidin D (1:100; Vector Laboratories, Burlingame, CA, USA) was performed for 1h, and nuclei counterstain with 4 µg/mL Hoechst 33342 (Sigma-Aldrich) for 5 min. Slides were mounted in Dako fluorescent medium (Dako North America Inc.) and fluorescent images for cell counts were recorded using an Axiovert 200 M fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

![Figure 1](image.png)

**Figure 1.** Schematic representation of the experimental protocol to investigate the role of NPY system under conditions of METH-induced endothelial cells death (hCMEC/D3).
Data analysis

Statistical analysis of TUNEL assay was performed using an analysis of variance (one-Way ANOVA) followed by Bonferroni’s post-test, as indicated in the figure legends. Data are expressed as percentage of total cells. Statistical analysis of NPY receptors immunoreactivity was measured by using Mann-Whitney test, as indicated in the figure legends. Data are expressed as mean + S.E.M.

Results

METH increases NPY and Y1R immunoreactivity in the mice hippocampus

The expression of NPY, and both Y1R and Y2R in the mice hippocampus has already been demonstrated to be altered by a single high dose of METH (14). Also, it is known that NPY system has a protective role against METH-induced neuronal and microglial cell death (3, 14). Therefore, in order to test the acute effect of METH binge paradigm in the hippocampus, we used a well-established protocol, consisting of 4× 10mg/kg, 2h apart, known to cause BBB disruption and neuroinflammation (5).

Our data confirm the presence of NPY1R (Figure 2 and 3) and Y2R (Figure 4) in the mice hippocampus. Moreover, an upregulation of both NPY and Y1R immunoreactivity was observed after METH exposure (Figure 2), but with a more significant effect on Y1R. In fact, the basal protein levels of NPY are usually low and a significant increase is typically observed after an injury. Although Y2R are detected in control animals, no significant alterations were observed in METH exposed group (Figure 3).
Figure 2. METH increases NPY and Y1R immunolabeling in the mice hippocampus. Control group received four injections of 0.9% NaCl (i.p.) 2h apart, and METH binge group was exposed to 4× 10 mg/kg (i.p.) 2h apart. Mice were sacrificed 2h after the last injection. Immunohistochemistry studies were performed to identify NPY (red) and Y1R (green) in the hippocampus. METH triggered an increase in both NPY and Y1R immunoreactivity. Total brain sections were also stained with Hoechst 33342 (blue). Scale bar = 20 μm.

In order to clarify the expression of NPY receptors on brain microvasculature, we also double stained hippocampal slices with CD31 [also known as PECAM1 (platelet and endothelial cell adhesion molecule 1)], a marker of endothelial cells, with both NPY receptors. Thus, we showed the presence of Y1R (Figure 3) and Y2R (Figure 4) on mice cerebral vessels.
Figure 3. Y1R co-localized with hippocampal endothelial cells. Mice were administered with four injections of 0.9% NaCl i.p. 2h apart and sacrificed 2h after the last injection. To clarify if Y1R has a vascular location we performed double immunohistochemistry in mice hippocampus. In fact, the Y1R (green) co-localized with endothelial cells (CD31, red). Total brain sections were also stained with Hoechst 33342 (blue). Scale bar = 20 μm.

Figure 4. Y2R immunoreactivity is not affected by METH exposure. After METH administration (4× 10 mg/kg, i.p., 2h apart) immunohistochemistry studies were performed to evaluate the presence of
Y2R (green) in the hippocampal microvasculature. Despite the fact that Y2R immunoreactivity was not altered under METH conditions, we concluded that Y2R co-localized with endothelial cells (CD31, red). Total brain sections were also stained with Hoechst 33342 (blue). Scale bar = 20 μm.

**NPY, Y1R and Y2R are present in human cerebral microvascular endothelial cells**

It is well known that endothelial cells are the structural basis of the BBB, which is a dynamic and complex structure responsible for brain protection and homeostasis (18). To explore the role of NPY system in human brain endothelial cells, we used a well characterized endothelial cell line (hCMEC/D3) (16, 17). Herein, we show that endothelial cells express NPY (Figure 5), Y1R (Figure 6) and Y2R (Figure 7) corroborating our animal studies abovementioned. Moreover, no alterations in the NPY immunoreactivity (Figure 5) were detected in the presence of METH (100 μM for 24h).

**Figure 5. NPY immunoreactivity in human brain endothelial cells.** To study the effects of METH on endothelial NPY immunoreactivity, we used hCMEC/D3 cells as an *in vitro* model of BBB. Immunocytochemistry results revealed that NPY (red) is present in brain endothelial cells, nevertheless, it was unaffected by METH treatment (100 μM for 24h). Total cell nuclei were also stained with Hoechst 33342 (blue). Scale bar = 20 μm.
Interestingly, when we looked to NPY receptors, we identified a significant increase in Y1R immunoreactivity (Figure 6A), which was quantified (Figure 6B; **$P<0.01$ vs CTR). On the contrary, the levels of Y2R were not altered by METH exposure (Figures 6C and D).

**Figure 6. Y1R and Y2R are present in human brain endothelial cells.** hCMEC/D3 cells were exposed to 100 μM METH for 24h. Representative immunocytochemistry images for (A) Y1R (green), (C) Y2R (green), and (A, C) Hoechst (blue, nuclei). Scale bar = 20 μm. A significant increase in (B) Y1R immunoreactivity levels was observed in endothelial cells exposed to METH. On the contrary, no alterations in (D) Y2R immunoreactivity was observed after 24h of METH exposure. Data are expressed as arbitrary units mean ± S.E.M., **$P<0.01$**, significantly different from control using Mann-Whitney test.
The activation of NPY2R prevents endothelial cell death induced by METH

In order to evaluate whether NPY system could have a protective role against cell death induced by METH, we exposed hCMEC/D3 cells to a toxic concentration of METH (3 mM; Figure 7). In fact, we observed a significant increase in cell death after METH exposure (Figure 7A and B; ***P<0.001 vs CTR).

It is known that NPY has a neuroprotective role under several conditions (11, 12, 14, 15). Nevertheless, its effects on endothelial cells remain poorly understood. By itself, NPY (1 µM) exposure had no effect on cell viability. Notwithstanding, it exerted a significant protection against cell death induced by METH exposure (Figure 7A-B; ###P<0.001 vs METH). To identify which NPY receptor mediates this protective effect, selective Y1R and Y2R agonists [1 µM [Leu31,Pro34]NPY and 1 µM NPY(13-36), respectively] were used alone or in the presence of Y1R and Y2R antagonists (1 µM BIBP3226 and 1 µM BIIE0246, respectively). Interestingly, selective activation of Y2R protected hCMEC/D3 cells from the toxic effect induced by METH (Figure 7A and B; ###P<0.001 vs METH). To confirm this observation, we further showed that Y2R blockade abolished the protective effect of its agonist, presenting levels of cell death similar to METH alone (Figure 7B; ***P<0.001 vs CTR; $$$P<0.001 vs METH+Y2R agonist). On the contrary, Y1R agonist did not have any protective effect on endothelial cells (Figure 7B).
Figure 7. Y2R activation prevents METH-induced endothelial cell death. hCMEC/D3 cells were exposed to METH (3 mM for 24h) alone or with NPY (1µM), Y1R agonist (1µM [Leu31,Pro34]NPY) or Y2R agonist [1µM NPY(13-36)]. Additionally, Y1R antagonist (1µM BIBP3226) or Y2R agonist Y2R antagonist (1µM BIIE0246) were also used to block the effect of the respective agonists. (A) Representative fluorescence images of TUNEL-positive cells (green; pointed by the white arrows) and nuclei (Hoechst 33342; blue). Scale bar = 40 µm. (B) NPY protected against METH-induced endothelial cell death (hCMEC/D3 cell line). Only the activation of Y2R was able to prevent cell death induced by METH, and its selective effect was further proved by using the Y2R antagonist that completely blocked the protective effect of Y2R agonist. The results are expressed as percentage of total cells. ***P<0.001, significantly different from control; ###P<0.001, statistical significant when compared to 3 mM METH;
\*\*\*P<0.001, significantly different from METH+Y2R agonist, using one-Way ANOVA followed by Bonferroni’s post-test.

**Discussion**

It is well-established that METH causes neuronal death in several mice brain regions, such as striatum, hippocampus and cortex (4, 19). Moreover, the available literature shows that the hippocampus is one of the most vulnerable brain region to BBB disruption triggered by METH (6), and hippocampus is known to be related with depressive-like behaviours (20) and cognitive functions (3). Also, chronic human METH consumption leads to hippocampal atrophy, with consequent memory performance impairment (21). To counteract central negative effects including those associated with psychiatric disorders, NPY system, specifically Y1R activation or Y2R blockade were shown to prevent anxiety and depression-like behaviors, including in PTSD models (9).

The impact of drugs of abuse on the NPY system has been studied (2). In fact, our group has previously shown that a single high dose of METH (30 mg/kg) upregulated NPY, Y1R and Y2R protein levels in the mice hippocampus(3). Accordingly, we also demonstrated an upregulation of Y1R in the mice hippocampus. Additionally, the presence of NPY, as well as Y1R and Y2R, have been reported in peripheral vasculature endothelium in both animal and human samples (22-26). The importance of NPY on peripheral endothelium functions is related with angiogenesis promotion (23, 27, 28), vascular permeability regulation (29, 30), and secretion of endothelium-derived constricting factors (31-33). However, the expression and function of NPY system on cerebrovascular system is still poorly understood. Nevertheless, studies have shown the presence of NPY system in human cerebrovascular system (34, 35). Abounader and colleagues (41) detected Y1R mRNA in at least 67% of human pial vessels analysed, although no alterations were observed in endothelial cells (34). Also, Y2R receptor
mRNA was detected in approximately 20% of vascular cells, as well as in cultures of microvascular endothelial cells (34). The same authors suggested that Y1R is the most important NPY receptor subtype associated with human cerebral arteries, and it is mainly localized in smooth muscle cells. Others suggested the involvement of NPY on vascular permeability regulation through neurons and perivascular nerve fiber (36-39), and on vasodilatation mediated by nitric oxide (40, 41). Herein, we showed that human brain endothelial cells express NPY, Y1R and Y2R, and that METH upregulated Y1R immunoreactivity in endothelial cells.

Regarding the protective role of NPY, Gonçalves and colleagues have previously shown that Y1R activation partially prevented METH-induced microglia cell death (14). Here, although Y1R had no role on METH-induced endothelial cell death, we indeed observed an upregulation of its immunoreactivity, suggesting other role(s) at the vascular level. In fact, previous studies demonstrated anti-hyperalgesic effects of NPY in rat spinal dorsal horn via activation of Y1R under an inflammatory state (42). Importantly, Y1R knockout mice showed an increase in inflammatory processes and injury-related hyperreflexia (43).

Despite the few evidence showing the expression of Y2R on brain microvasculature, in this work we clearly demonstrated its presence in hippocampal vessels as well as on human brain endothelial cells. Thus, we hypothesized whether this NPY receptor could have a crucial role under conditions of METH-induced toxicity. First, we concluded that METH caused a statistically significant increase in endothelial cell death, but this only correspond to 5% of total cell population. On the contrary, the same METH concentration caused 40% of cell death on primary cultures of human endothelial cells (5). Such difference can be justified by the fact that cell lines usually show more resistance to injuries than primary cultures. Despite no alterations on Y2R immunoreactivity in the presence of METH, its activation prevented endothelial cells triggered by this psychostimulant. Accordingly, previous data showed that Y2R activation
protected neurons and microglia cells against METH-induced cell death (14), and that Y2R blockade inhibited the neuroprotective effect induced by NPY further proving that NPY-mediated effect was due to the selective activation of Y2R (14).

**Conclusion**

The present work shows an upregulation of NPY and Y1R in the mice hippocampus after METH binge administration. Moreover, we also demonstrated that endothelial cells express both Y1R and Y2R. Further, we concluded that NPY system, specifically via Y2R activation, has a protective role against METH-induced endothelial cell death. Nevertheless, we cannot exclude that Y1R can be involved in other cellular effects, but not in the prevention of METH-induced cell death.

**Acknowledgements**

This work was supported by Project PTDC/SAU-FCF/098685/2008 from Foundation for Science and Technology (FCT Portugal) co-financed by COMPETE and FEDER funds, Pest-C/SAU/UI3282/2013-2014 and Pest CNC.IBILI UID/NEU/04539/2013. I am truly grateful to Ana Paula Silva Martins and Ricardo Leitão for sharing their knowledge, time and guidance. I also would like to thank to Vanessa Santos for the technical support in hCMEC/D3 cell line management.

**References**


