Mechanisms of regulation of P-glycoprotein and breast cancer resistance protein at the blood-brain barrier : focus on the role of morphine, and P-glycoprotein activation

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Mechanisms of regulation of P-glycoprotein and Breast Cancer Resistance Protein at the Blood-Brain Barrier: Focus on the role of morphine, and P-glycoprotein Activation.

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Dr. Renata SILVA	Universidade do Porto (Portugal)

If you can keep your head when all about you
Are losing theirs and blaming it on you,
If you can trust yourself when all men doubt you,
But make allowance for their doubting too;
If you can wait and not be tired by waiting,
Or being lied about, don't deal in lies,
Or being hated, don't give way to hating,
And yet don't look too good, nor talk too wise:

If you can dream—and not make dreams your master;
If you can think—and not make thoughts your aim;
If you can meet with Triumph and Disaster
And treat those two impostors just the same;
If you can bear to hear the truth you've spoken
Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to, broken,
And stoop and build 'em up with worn-out tools:

If you can make one heap of all your winnings
And risk it on one turn of pitch-and-toss,
And lose, and start again at your beginnings
And never breathe a word about your loss;
If you can force your heart and nerve and sinew
To serve your turn long after they are gone,
And so hold on when there is nothing in you
Except the Will which says to them: 'Hold on!'

If you can talk with crowds and keep your virtue,
Or walk with Kings—nor lose the common touch,
If neither foes nor loving friends can hurt you,
If all men count with you, but none too much;
If you can fill the unforgiving minute
With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And—which is more—you'll be a Man, my son!

Rudyard Kipling, from "*Rewards and Fairies*"

To my Parents, Family, Friends,
and all those who contributed to
the construction of the person I am today

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ABSTRACT

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The blood-brain barrier (BBB) is the main interface of molecular exchange between the bloodstream and the central nervous system (CNS), where it plays an essential role on the control over the bi-directional passage of endogenous and exogenous compounds. At the BBB, P-glycoprotein (P-gp) and Breast Cancer Resistance Protein (BCRP) are the most important ABC drug efflux transporters preventing the entry into the brain of toxic compounds, drugs and xenobiotics circulating in the blood. There is increasing interest in understanding the molecular mechanisms underlying the modulation of P-gp and BCRP expression and function in order to control CNS accumulation of neurotoxicants and to overcome pharmacoresistance phenomena.

Recent studies showed that morphine, itself a substrate of P-gp, is implicated in the up-regulation of P-gp expression, which may contribute to its poor brain penetration and tolerance. However, it was unknown the mechanism underlying P-gp induction by morphine and its role on BCRP expression. Rats were used as an animal model for the study of the amplitude and the kinetics of the modulation of P-gp and Bcrp expressions at the BBB following a subchronic morphine treatment, in an escalating morphine dose regimen. Freshly isolated rat brain microvessels were used as BBB model to study P-gp and Bcrp contents following the *in vivo* treatment, while the hCMEC/D3 cell line was occasionally used for complementary studies. Our results demonstrated that a 5-day subchronic morphine regimen up-regulated both P-gp and Bcrp 12 to 24h after the last dose of morphine, which was not registered at earlier time-points of animal sacrifice, nor with a single dose of morphine. The animal treatment with a glutamatergic NMDA receptor antagonist, or a COX-2 inhibitor abolished the subchronic morphine-induced P-gp and Bcrp protein up-regulation, 24h after the last dose of morphine, suggesting that both are implicated in the morphine-dependent P-gp and Bcrp up-regulation.

Since the registered up-regulation only occurred from 12h after the last dose of morphine-onwards, we investigated whether it was a direct effect of continued exposure to morphine, or rather a consequence of the morphine withdrawal developed after discontinuation of treatment. Rats were treated either with a constant morphine infusion (5 days), or two chronic morphine regimens where withdrawal was precipitated by naloxone administration: an escalating dose (5 days) or a constant dose morphine regimen followed by a withdrawal period (2 days) and resume of the treatment for 3 additional days. Continuous i.v. morphine did not change P-gp and Bcrp levels in rat brain microvessels, it does not have a direct consequence on the cascade of regulation of these transporters at the BBB. Naloxone-precipitated withdrawal after escalating or chronic morphine dose regimen increased *Mdr1a* and *Bcrp* mRNA levels, but protein expression and activity

remained unchanged after naloxone administration. This latter result discrepancy may be due to posttranslational regulation or naloxone action at non-opioid receptors hampering P-gp and Bcrp up-regulation.

Subsequently, we did a large screening of the expression of several neurotransmitter receptors at the rat BBB, many of them implicated in the inflammatory cell-cell signaling, and which may have a role in the modulation of these ABC transporters. Also, we compared two different approaches of isolation of rat brain microvessels, mechanical dissection and enzymatic digestion, to assess which yield the purest microvessel fraction for the BBB study. The enzymatic digestion provided the highest enrichment of endothelial cells and pericytes, and the least contamination with astrocyte and neuron markers. Among the neurotransmitter receptors, rat brain microvessels have low expression of the adenosine receptor A_1, the adrenoceptors α_{2A}, β_1, and cannabinoid receptors, while the ATP receptors $P2Y_1$ and $P2X_7$, and the adenosine receptor A_{2A} assume a very significant expression, and so may play an important role in the cell signaling at the BBB.

Additionally, newly synthetized thioxanthonic derivatives, which previously demonstrated to directly increase P-gp activity without necessarily increase its expression in Caco-2 cells, were tested for P-gp activation in an *in vitro* BBB model, RBE4 cells, and it was evaluated whether they would afford protection against mitoxantrone-induced toxicity. Most of these compounds showed a prompt increase of Rho 123 efflux out of RBE4 cells, suggesting to be efficient P-gp activators in such model. However, when co-incubated with mitoxantrone, these compounds did not confer an increased protection against the mitoxantrone-induced cytotoxicity in RBE4 cells in culture for 24h. Still, thioxanthones remain as interesting drug candidates for an antidote strategy against the toxicity induced by harmful P-gp substrates, even though P-gp activation phenomenon should be interpreted carefully.

In summary, the present work developed under this PhD dissertation explored important pathways of regulation of both the expression and activity of the two major ABC transporters present at the BBB, and thus can be valuable tools to either overcome pharmacoresistance in the treatment of neurological diseases or to revert the neurotoxicity of substrates.

Keywords: Blood-brain barrier, P-glycoprotein, Breast Cancer Resistance Protein, Morphine, Regulation, Activation.

RESUMO

RESUMO

A barreira hemato-encefálica (BHE) representa a principal interface entre a corrente sanguínea e o sistema nervoso central (SNC), desempenhando um papel essencial no controlo da passagem sangue-cérebro de diversos compostos endógenos e exógenos. A glicoproteina P (P-gp) e a proteína de resistência ao cancro da mama (BCRP) são os principais transportadores de efluxo da família ABC presentes ao nível da BHE, limitando a passagem cerebral de compostos tóxicos, fármacos e xenobióticos circulantes na corrente sanguínea. Actualmente, regista-se um crescente interesse na comunidade científica para a melhor compreensão dos mecanismos moleculares subjacentes à modulação quer da expressão quer da função da P-gp e BCRP, no sentido de desenvolver medidas mais eficazes quer para prevenção da acumulação de compostos neurotóxicos no SNC, quer para superar fenómenos de farmacorresistência associados à terapêutica.

Estudos recentes evidenciam que a morfina, por si só um substrato da P-gp, está envolvida na indução da expressão da P-gp, o que poderá contribuir para a sua menor penetração cerebral, bem como para o desenvolvimento de tolerância. No entanto, não se conhece o mecanismo subjacente a tal indução da P-gp pela morfina, nem o seu eventual papel na expressão da BCRP. Com efeito, na condução da presente dissertação, realizamos um estudo da amplitude e a cinética da regulação da expressão da P-gp e BCRP ao nível da BHE na sequência de um tratamento subcrónico com morfina, em regime de doses crescentes, usando o rato como modelo animal. Para o efeito, foram isolados os capilares cerebrais dos animais sujeitos a tratamento, *in vivo*, enquanto que a linha celular hCMEC/D3 foi ocasionalmente utilizada para estudos complementares. Os nossos resultados demonstraram que um tratamento subcrónico com morfina (5 dias) foi capaz de induzir tanto a P-gp como a Bcrp 12 a 24 horas após a última dose de morfina administrada, mas não para tempos de sacrifício anteriores, bem como tal indução não foi registada quando a morfina foi administrada de forma aguda. O tratamento animal com um antagonista do receptor glutamatérgico NMDA, ou com um inibidor da COX-2 anulou este efeito de indução da P-gp e Bcrp pela administraçãosubcrónica de morfina, o que sugere o envolvimento destes dois componentes na indução da P-gp e Bcrp dependente da morfina.

Uma vez que este aumento da expressão só surgiu a partir de 12h após a última dose de morfina, decidimos investigar se tal seria um efeito direto da exposição continuada à morfina, ou por outro lado, uma consequência do síndrome de abstinência à morfina, desenvolvido após a descontinuação do tratamento. Desta forma, os animais foram tratados por um lado com uma infusão contínua de morfina (5 dias), ou sujeitos a dois

diferentes regimes de exposição crónica à morfina, após os quais o síndrome de abstinência foi provocado pela administração de naloxona. A administração de morfina em contínuo, via i.v., não alterou os níveis de P-gp e BCRP nos capilares cerebrais de rato, o que indica a ausência de uma consequência directa da morfina na cascata de regulação destes transportadores ao nível da BHE. O síndrome de abstinência opióide provocado pela naloxona aumentou os níveis de mRNA *Mdr1a* e *Bcrp*, mas tanto a expressão e atividade proteicas mantiveram-se inalteradas após a administração de naloxona. Esta discrepância de resultados pode-se dever ou a um regulamento pós-translacional, ou a uma acção inespecífica da naloxona em receptores não opiáceos, impedindo a indução da P-gp e Bcrp.

Num outro estudo, foi feito um *screening* da expressão de vários receptores de neurotransmissores na BHE de rato, muitos deles envolvidos na sinalização célula-célula em processos inflamatórios, e que podem ter um papel na modulação destes transportadores ABC. Além disso, foram ainda comparadas duas técnicas de isolamento de capilares cerebrais de rato, o método de dissecção mecânica e o método de digestão enzimática, de modo de apurar qual a metodologia que permite a obtenção da fracção capilar mais enriquecida para o estudo da BHE. A digestão enzimática gerou um maior enriquecimento em células endoteliais e pericitos, com mínima contaminação em astrócitos e neurónios. Quanto aos receptores de neurotransmissores, os capilares cerebrais de rato exibiram uma baixa expressão de receptores de adenosina A_1, adrenérgicos α_{2A}, β_1, e canabinóides, enquanto que os receptores de ATP $P2Y_1$ e $P2X_7$, bem como o receptor A_{2A} de adenosina assumem uma expressão muito significativa, pelo que poderão desempenhar um papel importante na sinalização celular ao nível da BHE.

Por fim, um conjunto de compostos tioxantónicos, que anteriormente haviam demonstrado aumentar directamente a actividade da P-gp sem um aumento da sua expressão na linha celular Caco-2, foram testados com vista a activação da P-gp num modelo de BHE *in vitro*, células RBE4, e protecção celular contra a toxicidade induzida pela mitoxantrona. A maioria destes compostos mostrou aumentar rapidamente o efluxo da Rho 123 das células RBE4, sugerindo que tais compostos são activadores de P-gp igualmente eficientes neste modelo. No entanto, quando co-incubados com mitoxantrona, estes compostos não conferiram um aumento da protecção contra a citotoxicidade induzida pela mitoxantrona nas RBE4 em cultura durante 24h desde o início da exposição. Ainda assim, as tioxantonas permanecem como interessantes candidatos no desenvolvimento de estratégias antidotais contra a toxicidade de substratos da P-gp, embora este fenómeno de ativação da P-gp deva ser interpretado com cuidado.

Em resumo, o presente trabalho desenvolvido no âmbito desta tese de doutoramento explorou importantes vias de regulação, quer ao nível da expressão como da actividade

dos dois principais transportadores ABC presentes na BHE, e que, portanto, poderão ser ferramentas úteis quer na tentativa de contornar fenómemos de farmacorresistência no tratamento de doenças neurológicas, como na reversão da neurotoxicidade de substratos.

Palavras-chave: Barreira-hematoencefálica, Glicoproteína P, Proteína de Resistência ao Cancro da Mama, Morfina, Regulação, Activação.

RÉSUMÉ

RÉSUMÉ

La barrière hémato-encéphalique (BHE) représente la principale interface d'échange moléculaire entre la circulation sanguine et le système nerveux central (SNC), où elle joue un rôle essentiel sur le contrôle du passage bidirectionnel de composés endogènes et exogènes. À la BHE, la P-glycoprotéine (P-gp) et Breast Cancer Resistance Protein (BCRP) sont les transporteurs d'efflux ABC les plus importants, empêchant l'entrée de composés toxiques, des médicaments et des xénobiotiques circulant dans le sang dans le cerveau. Il y a un intérêt croissant pour la compréhension des mécanismes moléculaires sous-jacents à la modulation de l'expression et de la fonction de la P-gp et BCRP, afin de pouvoir contrôler l'accumulation de substances neurotoxiques dans le SNC et de surmonter les phénomènes de pharmaco-résistance.

Des études récentes ont montré que la morphine, elle-même un substrat de la P-gp, est impliquée dans l'augmentation de l'expression de la P-gp, qui peuvent contribuer à sa faible pénétration dans le cerveau et pour le développement de la tolérance. Cependant, le mécanisme sous-jacent à l'induction de la P-gp par la morphine, bien comme son rôle sur l'expression de BCRP était inconnu. Des rats ont été utilisés comme modèle animal pour l'étude de l'amplitude et la cinétique de la modulation de la P-gp et Bcrp à la BHE, après un traitement morphinique subchronique, en utilisant un protocole d'escalade de doses. Des microvaisseaux cérébraux isolés ont été utilisés comme modèle pour étudier la BHE, et les contenus en P-gp et Bcrp après le traitement *in vivo*, tandis que la lignée cellulaire hCMEC/D3 a parfois été utilisé pour des études complémentaires. Nos résultats ont montré qu'un régime subchronique de traitement à la morphine pendant 5 jours a induit la P-gp et Bcrp 12 à 24 heures après la dernière dose de morphine, un effet qui n'a pas été enregistrée lors des précédentes temps de sacrifices des animaux, ni avec une traitement aigue à la morphine. Le traitement des animaux avec un antagoniste de du récepteur glutamatergique NMDA, ou avec un inhibiteur de la COX-2 a aboli l'induction protéique de la P-gp et Bcrp par la morphine-subchronique, ce qui suggère que les deux facteurs sont impliqués dans l'up-régulation morphine-dépendante de la P-gp et BCRP.

Sachant que l'induction a été enregistrée seulement à partir de 12h après la dernière dose de morphine, nous avons examiné si elle était un effet direct de l'exposition continue à la morphine, ou plutôt une conséquence du sevrage à la morphine développé après l'arrêt du traitement. Les rats ont été traités soit avec une perfusion constante de morphine (5 jours), soit avec deux schémas chroniques de morphine lorsque le sevrage a été précipité par l'administration de naloxone: un régime de doses croissantes (5 jours) ou un régime de doses constantes de morphine. La perfusion en continue de morphine n'a pas changé les niveaux de P-gp et Bcrp dans les microvaisseaux cérébraux de rat, et du

coup n'a pas une conséquence directe sur la cascade de régulation de ces transporteurs à la BHE. Le sevrage provoqué par la naloxone a augmenté les niveaux d'ARNm pour le *Mdr1a* et *Bcrp*, mais l'expression et de l'activité protéiques sont restées inchangées après l'administration de naloxone. Cette disparité peut être dû soit à un effet de la régulation post-traductionnelle, soit à l'action de la naloxone dans des récepteurs non-opioïdes, qui peut entraver l'induction de la P-gp et Bcrp.

Par la suite, on a fait un large *screening* de l'expression de plusieurs récepteurs de neurotransmetteurs chez la BHE de rat, beaucoup d'entre eux impliqués dans la signalisation inflammatoire, et qui peut jouer un rôle dans la modulation de ces transporteurs ABC. Aussi, nous avons comparé deux approches différentes de l'isolement des microvaisseaux cérébraux de rat, la dissection mécanique et la digestion enzymatique, pour évaluer ce qui donne la fraction la plus pure des microvaisseaux pour l'étude de la BHE. La digestion enzymatique a donné le plus haut enrichissement en cellules endothéliales et pericytes, et la moindre contamination avec des marqueurs d'astrocytes et des neurones. Parmi les récepteurs de neurotransmetteurs, les microvaisseaux rat ont montré une faible expression du récepteur d'adénosine A_1, adrénergiques α_{2a}, β_1 et cannabinoides, tandis que les récepteurs d'ATP $P2Y_1$ et P2X7 et les récepteurs d'adénosine A_{2A} assument une expression très significative, et peut donc jouer un rôle important dans la signalisation cellulaire au niveau de la BHE.

Finalement, des nouvelles composés dérivés des thioxanthones, qui ont précédemment montré d'être capables d'augmenter directement l'activité P-gp, sans augmentation de l'expression dans les cellules Caco-2, ont été testés pour l'activation P-gp dans un modèle de BHE *in vitro*, les cellules RBE4, et ont été évalués dans le but de la protection cellulaire contre la toxicité induite par la mitoxantrone. La plupart de ces composés ont augmenté rapidement l'efflux de la Rho 123 des cellules RBE4, suggérant d'être activateurs de la P-gp efficaces dans ce modèle. Cependant, quand co-incubées avec la mitoxantrone, ces composés ne confèrent pas une protection contre la cytotoxicité induite par la mitoxantrone dans les cellules RBE4 en culture pendant 24h. Pourtant, les thioxanthones restent comme candidats intéressants dans une stratégie antidotal contre la toxicité induite par des substrats nocives de la P-gp, même si le phénomène d'activation de la P-gp doit être interprétée avec prudence.

En résumé, le présent travail au sein de cette thèse de doctorat a exploré des voies importantes de régulation de l'expression et de l'activité des deux principaux transporteurs ABC présents à la BHE, et peuvent donc être des outils précieux soit dans la tentative de contourner des phenomènes de résistance aux médicaments dans le traitement des maladies neurologiques, soit dans le renversement de la neurotoxicité liée à des substrats.

Mots-clés: Barrière hémato-encéphalique, P-glycoprotéine, Breast Cancer Resistance Protein, Morphine, Regulation, Activation.

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ABBREVIATIONS LIST

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ABC (transporters) – ATP-binding cassette (transporters)
Aβ – β amyloid peptide
AhR – Aryl hydrocarbon receptor
Akt – Protein kinase B
ALS – Amyotrophic lateral sclerosis
AJ – Adherens junction
AMT – Adsorptive-mediated transcytosis
AP – Alkaline phosphatase
AP-1 – Activator protein 1
AQP4 – Aquaporin-4
ATP – Adenosine triphosphate
AUC – Area under the curve
BBB – Blood-brain barrier
BCRP – Breast cancer resistance protein
BCSFB – Brain-cerebrospinal fluid
BEC – Brain endothelial cell
bFGF – Basic fibroblast growth factor
BM – Basement membrane
cAMP – Cyclic AMP
CAM – Cell adhesion molecule
CAR – Constitutive androstane receptor
CNS – Central nervous system
COX – Cyclooxygenase
CPA – Conditioned place aversion
cPLA2 – Cytosolic phospholipase A2
CSF – Cerebrospinal fluid
CYP – Cytochrome P450
DHEA – Dehydroepiandrosterone
ECE – Endothelin converting enzyme
ECM – Extracellular matrix
EP1R – Prostaglandin E receptor 1
ER – Estrogen receptor
ERK – Extracellular signal-regulated kinase
ET – Endothelin
ETR – Endothelin receptor
FTC – Fumitremorgin C
GDNF – Glial-derived neurotrophic factor
GFAP – Glial fibrillary acidic protein
GLUT-1 – Glucose transporter-1

GPCR – G-protein coupled receptor
GR – Glucocorticoid receptor
GSK – Glycogen synthase kinase
HBEC – Human brain endothelial cells
hCMEC/D3 – Human cerebral microvessel endothelial cell line D3 clone
HIV – Human immunodeficiency virus
iNOS – Inducible nitric oxide synthase
I.C.V. – Intracerebroventricular
I.V. – Intravenous
I.M. – Intramuscular
I.P. – Intraperitoneal
IL - Interleukin
ISF – Interstitial fluid
JAM – Junctional adhesion molecule
JNK – C-Jun N-terminal kinase
LDL – Low-density lipoprotein
LPS – Lipopolysaccharide
LTD – Long-term depression
LTP – Long-term potentiation
M3G – Morphine-3-glucoronide
M6G – Morphine-6-glucoronide
MAPK – Mitogen-activated protein kinase
MDR – Multidrug resistance
miRNA – MicroRNA
NBD – Nucleotide-binding domain
Nfr2 – Nuclear factor (erythroid-derived 2)-like 2
NMDA – N-methyl-D-aspartate
NOP – Nociceptin or orphanin
NR1 – NMDA-NR1 subunit
NR2A – NMDA-NR2 subunit
NVU – Neurovascular unit
ORL – Receptor-like orphan receptor
OST (transporters) – Organic solute carrier (transporters)
PAG – Periaqueductal gray
PCN – 16α-carbonitrile
PECAM-1 – Platelet endothelial cell adhesion molecule-1
PET – Positron emission tomography
PGE2 – Prostaglandin-E2
P-gp – P-glycoprotein
PhIP – 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

PI3-K – Phosphatidylinositide-3-kinase
PKA – Protein kinase A
PKC – Protein kinase C
PPAR – Peroxisome proliferator-activated receptor
PQ – Paraquat
PTEN – Phosphatase and tensin homolog
PXR – Pregnane X receptor
qRT-PCR – Quantitative real time-polymerase chain reaction
RBE4 – Rat brain endothelial cell line clone 4
ROS – Reactive oxygen species
Rho 123 – Rhodamine 123
RMT – Receptor-mediated transcytosis
RVM – Rostral ventromedial medulla
S1P – Sphingosine-1-phospate
SAPK – Stress-activated protein kinase
S.C. – Subcutaneous
SLC (transporters) – Solute carrier (transporters)
SNP – Single nucleotide polymorphism
TACE – TNF-α converting enzyme
TCDD – 2,3,7,8-tetrachlorodibenzo-p-dioxin
TEER – Transendothelial electrical resistance
TGF-β1 – Transforming growth factor-β1
TJ – Tight junction
TKI – Tyrosine kinase inhibitor
TLR4 - Toll-like receptor 4
TM – Transmembrane
TMD – Transmembrane domain
TMH – Transmembrane helices
TNF-α – Tumor necrosis factor α
TX – Thioxanthone
UGT – Uridine diphospho-glucuronosyltransferase
UNODC – United Nations Office against Drugs and Crime
VDR – Vitamin D receptor
VEGF – Vascular endothelial growth factor
WB – Western blot

OUTLINE OF THE DISSERTATION

OUTLINE OF THE DISSERTATION

- **Part I – General Introduction on the blood-brain barrier, ABC Transporters and Regulation**

In this section, a review on the existing literature on the blood-brain barrier, the main ABC transporters, and the known mechanisms of regulation of their expression at this barrier is presented, in order to provide a good basis for understanding the objectives and the obtained results of the experimental studies.

- **Part II – Objectives**

The general objectives set for the preparation of the present PhD dissertation are presented in this dedicated section.

- **Part III – Experimental section**

In part III, the manuscripts published or submitted for publication in the scope of this dissertation are presented.

- **Part IV – Discussion and Conclusions**

In this section, an integrated discussion of the results obtained in the scope of this dissertation is presented. The discussion of their potential relevance and their connection with existing scientific reports is also addressed here. Moreover, part III includes the main conclusions taken from the work of the present dissertation and the future perspectives of research.

- **Part V – References**

In this final part, all the literature references that were used in the introduction and discussion sections are listed.

PART I

I. GENERAL INTRODUCTION

1. The Blood-Brain Barrier

1.1. The Central Nervous System: a homeostatic need

The central nervous system (CNS), which comprises the brain and the spinal cord, is generally considered as one of the most important and complex systems in the human body. Through its coordination with the peripheral nervous system, the CNS is vital to the control and function of all the other systems present in the organism. The CNS contains two distinctive liquid compartments, the interstitial fluid (ISF) and the cerebrospinal fluid (CSF). The brain ISF bathes the neurons and the neuroglia (*i.e* microglia, astrocytes, oligodendrocytes, ependymocytes), whereas the CSF fills the ventricles and surrounds the meninges that protect the external surface of the brain. In order to ensure the proper functioning of the CNS and its neuronal signaling and cell:cell communication, it is essential to closely regulate the extracellular microenvironment and the composition of these extravascular fluids, and thus maintain local homeostasis. Simultaneously, the body needs to guarantee a proficient supply of oxygen and nutrients to the brain, and on the other hand prevent the accumulation of metabolic waste products and toxins through an effective clearance system.

In the human brain, the so-called CNS barriers represent barrier layers between the blood and the CNS and play an essential role in ensuring this constant supply of important nutritive elements, ions, amino acids and energy, and the removal of metabolic products, while at the same time, protect the brain against noxious substances (Daneman and Prat 2015; Zlokovic 2008).

1.2. CNS barrier layers

The first time there was evidence of the existence of a physical barrier between the CNS and the peripheral circulation was described by Paul Ehrlich in 1885, who noted that a dye injection into the blood circulation stained peripheral organs but not the brain and the spinal cord (Ehrlich 1885). Later in 1913, Ehrlich's student Edwin Goldmann showed that an injection of trypan blue directly into the CSF stained cells within the CNS and not in the periphery (Goldmann 1913). Further support was later found by electron microscopy by Reese and Karnovsky, who demonstrated that when horseradish peroxidase was intravenously administrated it did not pass the capillary lumen, and thus evidencing the

existence of a solute exchange barrier between the blood and the brain (Reese and Karnovsky 1967).

Three CNS barrier layers are described to be present in the human brain and spinal cord. A first interface is formed by the highly specialized endothelium of parenchymal microvessels comprising the blood-brain barrier (BBB), partitioning the blood and brain ISF. The combined surface area of these capillaries (between 150 and 200 $cm^2.g^{-1}$ tissue) represents the largest CNS interface for blood-brain exchange, resulting in a total area for exchange in the average human adult brain of between 12 and 18 m^2 (Abbott et al. 2010; Nag et al. 2005).

The second interface is provided by the epithelium of the choroid plexus (modified ependymal lining of the brain ventricles), which secretes the CSF into the brain ventricular system and forms the blood-CSF barrier (BCSFB) (Abbott et al. 2010; Brown et al. 2004).

As a third interface present in the brain we have the arachnoid epithelium, which constitutes the middle layer of the meninges forming the outer covering of the CNS and separates the blood from the subarachnoid CSF. Due to its avascular nature and relatively small surface area, it does not represent a significant surface for exchange between the blood and the CNS (Abbott 2013; Abbott et al. 2010).

In this dissertation, we will give special focus to the BBB and its ability to restrict the brain entry of xenobiotics and other blood-circulating harmful compounds into the CNS.

1.3. Overview of the BBB and its function

It is well established that the human brain consumes over 20% of total body oxygen and energy, while it only corresponds to approximately 2% of total body mass (Shulman et al. 2003). Of the three mentioned CNS barriers, the BBB is distinguished as the most important interface for molecular exchanges between blood and the brain parenchyma, due to its dense network of microvessels throughout the brain and the proximity of their finest branches to individual neurons (Abbott et al. 2010). This network is composed of approximately 100 billion capillaries with diameters as small as 3 to 7 μm (Zlokovic and Apuzzo 1998), and the distance between capillaries is as short as 40 μm (Rodriguez-Baeza et al. 2003). These characteristics ensure that almost every neuron is perfused by its own blood capillary and therefore guarantee an efficient oxygen and nutrient supply to such high brain demand. No brain cell is over 25 μm from a brain capillary, meaning that once the solute diffuses through the BBB, it is considerably close to the neurons or glial cells. Despite the huge number of capillaries, they occupy only 0.1% of the brain volume

(Pardridge 1991; Pardridge 2003). The BBB is present in all brain regions, except in specialized and very size-limited sites of physiological cross-talks between the brain and the periphery, i.e. the circumventricular organs, where blood vessels allow diffusion of blood-borne molecules across the vessel wall (Ballabh et al. 2004; Cardoso et al. 2010; McKinley et al. 2003; Weiss et al. 2009).

BBB structure is formed by brain endothelial cells (BECs) composing the capillaries, which differ from endothelial cells present in the rest of the body due to the lack of fenestrations, and tight regulation of the movement of molecules, ions and cells across this barrier (Ballabh et al. 2004; Daneman 2012). The BBB exerts its barrier function at three different levels: (1) physical, through the extensive expression of tight junctions (TJs) along adjacent endothelial cells, reducing the paracellular pathway, (2) transporter-dependent passage, due to the existence of specific transport mechanisms mediating solute flux, and (3) metabolic, given the enzymes present in the BECs are capable of processing molecules in transit (Abbott et al. 2010). The resulting neurovascular coupling allows the maintenance of brain homeostasis by providing for the energy demands of neuronal activity, as well as it protects the CNS from toxins, pathogens, inflammation, injury, and disease, in association with various perivascular cells such as pericytes, microglia, astrocytes, and specialized cellular compartments such as the endothelial glycocalyx (Stanimirovic and Friedman 2012). In this way, the BBB plays a crucial role by exerting a bi-directional control over the passage of a large diversity of regulatory proteins, nutrients and electrolytes, as well as potential neurotoxins, and maintain a strict extracellular environment around synapses and axons. The BBB functions can be described as follows:

1. *Brain nutrition*: the BBB expresses specific transport systems in the luminal and abluminal membranes of BECs in order to ensure the appropriate supply of essential water-soluble nutrients (Abbott et al. 2010);
2. *Regulation of ion homeostasis and preservation of neural signaling*: the BBB provides a combination of specific ion channels and transporters to keep the optimal ionic composition for synaptic signaling function, as well as maintain the central and peripheral neurotransmitter pools separate, minimizing “cross-talk” (Abbott et al. 2010; Bernacki et al. 2008);
3. *Control molecular trafficking*: the BBB functions as a shield preventing neurotoxins (endogenous metabolites or ingested/environmental xenobiotics) and many macromolecules in the blood circulation from entering in the brain. The protein content of the CSF is much lower and markedly different to that of plasma, and the

leakage of several serum macromolecules into the brain can result in serious pathological consequences (Abbott et al. 2010). In this way, the BBB minimizes neuronal cell death, and preserves neural connectivity and immune quiescence.

Thus, the BBB guarantees that the CNS environment is not easily affected by peripheral changes under physiological conditions. However, the BBB remains a dynamic interface that acknowledges the necessity to meet the demands of the whole organism, which are not static.

1.4. The Neurovascular Unit: the architecture of the BBB

The properties of the BBB are mostly defined by the BECs, but these are regulated and maintained by crucial interactions with the basement membrane and neighboring cells, such as microglia and astrocytes, as well as neurons and perivascular pericytes (Cardoso et al. 2010; Zlokovic 2008). The close proximity of these cells results in an effective unit of paracrine regulation critical for normal CNS functioning, often called the neurovascular unit (NVU) (see *Figure 1*).

1.4.1. Brain Endothelial Cells

The brain microvascular endothelium constitutes the most critical element of the BBB. BECs have been identified as morphologically and metabolically distinct of those present in the peripheral circulation. In comparison to those cells, BECs are characterized by (1) the presence of a narrow junctional complex at their adjacent margins, eliminating gaps or spaces between cells and preventing any free paracellular diffusion of blood-borne substances into the brain parenchymal space, (2) lack of fenestrations typically present in peripheral capillaries, (3) fewer pinocytic vesicles, and (4) higher number of cytosolic mitochondria, suggesting important metabolic activity (Abbott 2005; Bearer and Orci 1985; Correale and Villa 2009; Dorovini-Zis et al. 1991; Lane et al. 1992; Villegas and Broadwell 1993). In fact, BECs are said to be 50 to 100-fold tighter than those in peripheral microvessels (Abbott 2002) due to the expression of this elaborated junctional complex that includes mainly TJ and adherens junction (AJ) proteins (Hawkins and Davis 2005) [*presented in more detail in section 1.4.1.1.*]. Gap junctions have also been identified at the BBB, but their role in the barrier function is not clear (Zlokovic 2008). Additionally, the luminal surface of the BECs is covered by the so-called glycocalyx, which corresponds to a negatively charged mesh of proteoglycans, glycosaminoglycans, glycoproteins and glycolipids, making anionicity another determinant factor for BBB permeation (de Boer and

Gaillard 2006). Ultrastructural cytochemistry and immunocytochemistry revealed a functional polarity of the brain microvascular endothelium, characterized by an asymmetric distribution of the majority of the receptors and transporters present in the luminal and abluminal BECs plasma membranes, indicating that luminal and abluminal membranes of endothelial cells are functionally distinct (Correale and Villa 2009; Farrell and Pardridge 1991).

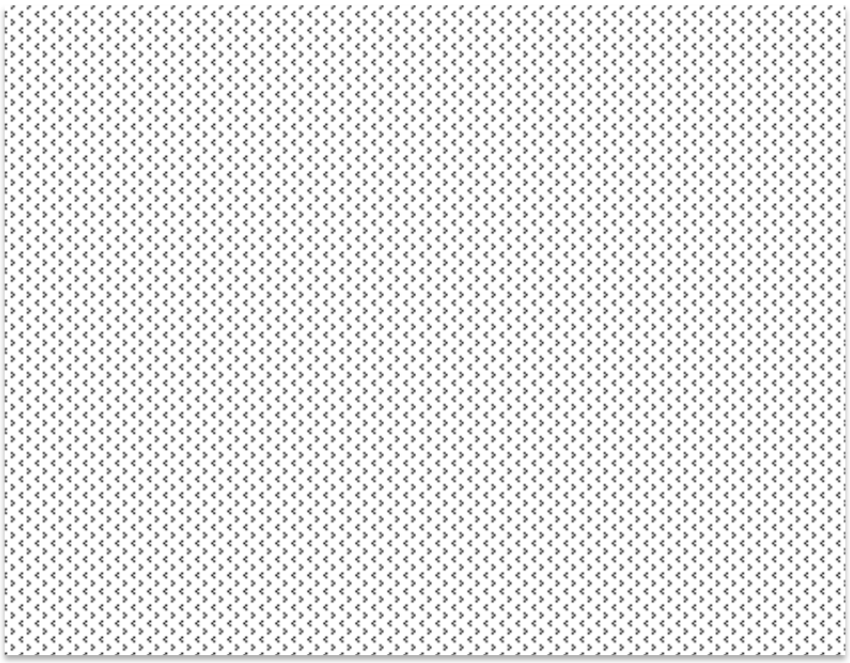

Figure 1. Representation of the BBB and the cells belonging to the NVU, an elaborate interplay of central and peripheral cells.

The endothelium's abluminal surface is covered by a basement membrane in which pericytes are embedded. Astrocyte-foot processes encircle the abluminal side of the vessel at great extent. Neurons and microglia are also members of the NVU as they interact with core elements of the BBB and influence barrier functions. Image taken from (Obermeier et al. 2013)

More than an impermeable wall, the BECs express a particular pattern of receptors and specific transport systems [to be further characterized in *section 1.5.*], which on one hand facilitate the uptake of essential nutrients and hormones, and on the other hand help to regulate the concentrations of ions, metabolites and xenobiotics in the brain (Weksler et al. 2005; Zlokovic 2008).

Lastly, the brain endothelium also forms a metabolic barrier once it expresses a specialized set of enzymes, such as alkaline phosphatase (AP), c-glutamyl transpeptidase, aromatic acid decarboxylase and several cytochrome P450 (CYP) isoenzymes and phase 2 conjugation enzymes that metabolize numerous xenobiotics, and whose expression is much higher than in non-neuronal capillaries (Decleves et al. 2011; Persidsky et al. 2006; Shawahna et al. 2011).

BECs can be identified by several specific cell markers, including the von Willebrand factor VIII related antigen, which mediates platelet adhesion to injured vessel walls and serves as a carrier and stabilizer for coagulation factor VIII, and the glucose transporter-1 (GLUT-1), which facilitates the transport of glucose across the brain endothelium into the CNS (Ballabh et al. 2004; Wilhelm et al. 2011).

1.4.1.1. Intercellular Junctions

The presence of intercellular junctions along the brain endothelium results in a physical barrier component to macromolecules and most polar solutes, significantly reducing the permeation through paracellular diffusion between endothelial cells from the blood plasma to the brain ECF. A junctional complex consists of all the transmembrane proteins and accessory cytoplasmic proteins located on the apical side of the endothelium (Stamatovic et al. 2008). The existent junctional complexes at the BBB include AJs and TJs. In peripheral endothelial cells, TJs are placed apically and separated from AJs (Staddon and Rubin 1996), but in the brain endothelium they are close to each other, forming the junction complex between adjacent endothelial cells (Stamatovic et al. 2008). TJs seal the BECs forming a continuous tubular structure, while AJs play a major role in the initiation and maintenance of BEC contact. TJ integrity depends highly on intact AJ, where cell-cell adhesion molecules are found (see *Figure 2*).

- *Tight Junctions*

TJs are responsible for the severe restriction of the paracellular diffusional pathway between the luminal and abluminal domains of the BECs, and effectively block penetration of macromolecules and polar solutes and ions by this route. Also, this enables the asymmetric distribution of membrane constituents, conferring cell polarity to the BECs (Cardoso et al. 2010; Hawkins and Davis 2005). This junctional system consists in a transmembrane protein complex spanning the intercellular cleft composed by occludin and claudins acting together with junctional adhesion molecules (JAMs). Occludin and claudins are linked to a number of cytoplasmic scaffolding and regulatory proteins zonula occludens protein 1 (ZO-1), ZO-2, ZO-3 and cingulin (Wolburg and Lippoldt 2002; Wolburg et al. 2009). The intracellular ZO-1, ZO-2 and ZO-3 regulate the effectiveness of TJs, which link claudin and occludin via cingulin to intracellular actin and the cytoskeleton (Wolburg and Lippoldt 2002; Wolburg et al. 2009). Selective loss of claudin-3 from the TJs has been shown to be associated with a loss of BBB integrity together with some functional barrier loss (Wolburg et al. 2003). Also, lack of claudin-5 severely compromises

the BBB in genetically modified mice (Nitta et al. 2003). The signaling at the TJs is bi-directional, meaning that signals are transmitted from the cell interior to existing TJs to regulate its assembly and function, while TJs also receive and transfer information back to the cellular environment to regulate gene expression and subsequent cellular responses (Terry et al. 2010).

The proper function of these TJs in not only related to the expression of these proteins spanning the intercellular cleft, but it is also influenced by how these proteins are organized and interact (Hamm et al. 2004), and by CNS-produced and circulating factors (Wolburg et al. 2003). Many of the cell types associated with brain microvessels, including pericytes, astrocytes, and nerve terminals adjacent to the endothelial ECM release several active factors that can modify tight junction assembly and barrier permeability (Abbott et al. 2010; Abbott et al. 2006).

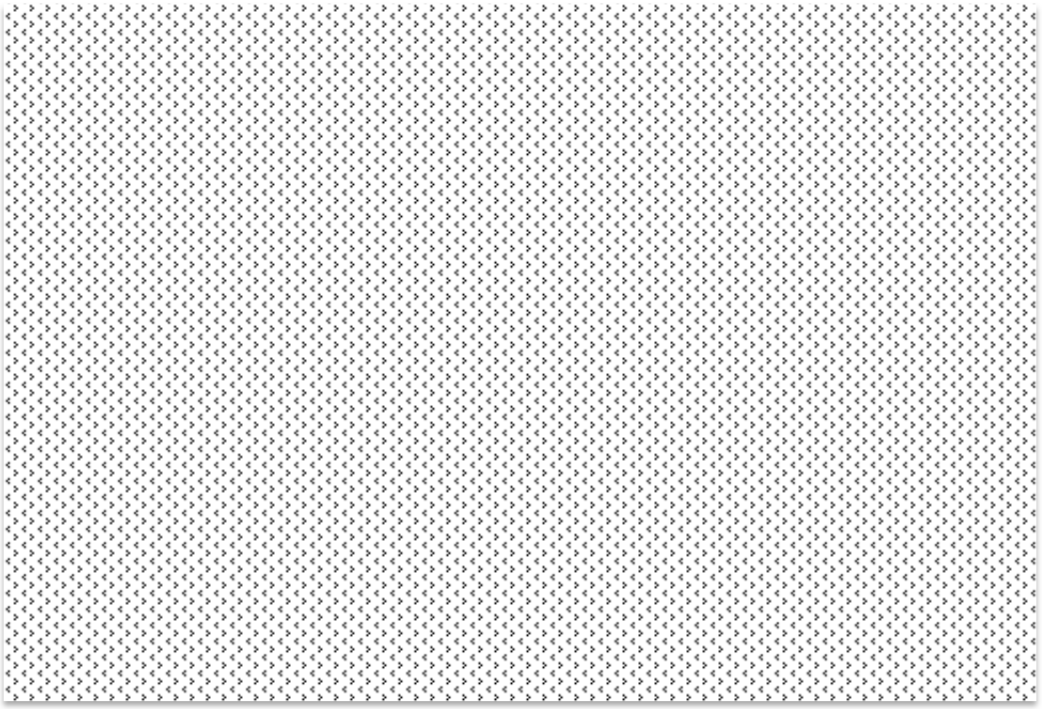

Figure 2. Schematic representation of the intercellular junctions present at the endothelial cells of the BBB.

TJ proteins (occludin, claudins, JAMs) are anchored to the cell cytoplasm by ZO-1, ZO-2, ZO-3 and actin cytoskeleton. Cadherins of the AJs work to form adhesive contacts between cells, providing structural integrity, and are anchored to actin cytoskeleton via catenins. Actin cytoskeleton may be activated via an increase of intracellular calcium, which may change the claudins and occludin configuration, and thus modify the tight junctional properties. Image taken from (Abbott et al. 2010)

- *Adherens Junctions*

The AJs hold the cells together giving the tissue structural support, and are essential for contact inhibition during vascular growth and remodeling, initiation of cell polarity, and

formation of TJs (Hawkins and Davis 2005). Its disruption leads to barrier disruption itself (Wolburg and Lippoldt 2002). In these junctions, transmembrane cadherin proteins (in particular, VE-cadherin) span the intercellular cleft to form adhesive contacts between cells, and join the actin cytoskeleton via intermediary proteins, namely alpha-, beta- and gamma-catenin. In addition, studies have shown that human AJs express platelet endothelial cell adhesion molecule-1 (PECAM-1), which takes part in shaping AJs via interactions with beta-catenin (Matsumura et al. 1997). Although its function is still unclear, a new catenin termed p120ctn was recently discovered, presenting a high affinity binding to VE-cadherin, which might suggest it has relevance in BBB permeability and function (Bazzoni 2006). TJ and AJ components are known to interact and cooperate, particularly ZO-1 and catenins, once more suggesting the two intercellular junctions work in concert (Matter and Balda 2003).

1.4.2. Basement membrane

The basement membrane (BM) surrounds the brain endothelium and separates it from the neighboring cells, namely pericytes and astrocytes, and all these cells cooperate for its own production and maintenance (Persidsky et al. 2006; Weiss et al. 2009). It is composed of different extracellular matrix (ECM) structural and specialized proteins, such as type IV collagen, elastin, fibronectin and laminin, and proteoglycans (Erickson and Couchman 2000). The BM also includes matrix adhesion receptors, known as cell adhesion molecules (CAM), which are expressed on vascular cells, neurons and supporting glia (Del Zoppo et al. 2006). This BM provides an anchor for many signaling processes at the brain vasculature, and its disruption by matrix metalloproteinases can lead to BBB dysfunction and leukocyte infiltration, often observed in many different neurological disorders (Daneman and Prat 2015).

1.4.3. Astrocytes

Astrocytes are a major glial cell type, that closely interact with BECs, and whose end-feet processes cover almost completely the basolateral surface of the vascular tube, playing an essential role in the regulation and maintenance of specific BBB characteristics. Astrocytes release factors that modulate and maintain the BBB phenotype and a proper differentiation of BECs, such as the glial-derived neurotrophic factor (GDNF), transforming growth factor-β1 (TGF-β1), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6) and angiopoietin 1 (Abbott et al. 2006; Bernacki et al. 2008; Bicker et al. 2014; Correale and Villa 2009). In fact, the close interaction between astrocytes and the brain endothelium up-

regulates many BBB features, namely the expression of TJs, specialized enzyme systems and polarized transporter location (Abbott et al. 1992; Bicker et al. 2014; Hayashi et al. 1997; Persidsky et al. 2006; Sobue et al. 1999) and helps control the cerebral blood flow (Koehler et al. 2009). This includes regulating the contraction/dilation of vascular smooth muscle cells surrounding arterioles as well as pericytes surrounding capillaries.

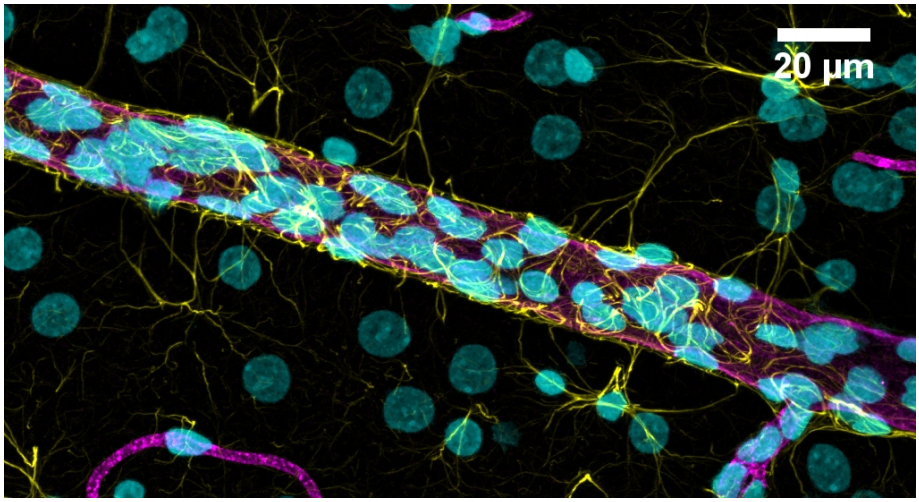

Figure 3. Confocal microscopy image of a rat brain microvessel surrounded by astrocytic end-feet processes.

Cell nuclei are stained in blue (TOPRO3), glial fibrillary acidic protein (GFAP), an astrocyte end-feet process marker, is stained in yellow (anti-Gfap), and P-glycoprotein, used as a marker of the luminal membrane of the brain microvascular endothelial cells, is stained in purple (anti-P-gp). Image provided by Dr. Bruno Saubaméa (*Plateforme d'imagerie cellulaire, Faculté de Pharmacie, Université Paris Descartes*).

The astrocytical end-foot processes show high density of purinergic P2Y receptors, potassium channel Kir4.1, and water-channel protein aquaporin-4 (AQP4), indicating key roles in the gliovascular signaling and in the regulation of brain water and electrolyte metabolism under normal and pathological conditions (Abbott et al. 2006). In addition, the high density of AQP4 is also related to the expression of the dystroglycan-dystrophin complex in the end-feet processes, which helps link the cellular cytoskeleton to the BM by binding to agrin (Daneman and Prat 2015; Wolburg et al. 2011). Agrin, a heparin sulphate proteoglycan of the ECM, accumulates whenever there is BBB tightening (Abbott et al. 2006; Bernacki et al. 2008), suggesting a vital role for the ECM in the cross-influence of astrocytes and endothelium.

Astrocytes are in close contact with both neuronal cell bodies and brain capillaries, providing a cellular link between these elements, and underlining their importance for functional neuronal activity and NVU (Attwell et al. 2010; Daneman and Prat 2015; Persidsky et al. 2006).

1.4.4. Pericytes

Pericytes are small vessel-associated cells sharing the BM with BECs, whose numerous cytoplasmic processes encircle capillaries, and existent gap junctions provide contact spots between the two cell types (Cuevas et al. 1984). Given such close relationship, pericytes are ideally suited to have many influences on brain microcirculation. Pericyte-to-endothelia ratio in the brain is 30-fold higher compared to that of striated cells, and the endothelium coverage by pericytes, oscillating between 22 and 32% (Kim et al. 2006), varies considerably between different microvessel types (Allt and Lawrenson 2001). Pericytes show rich contents of α-smooth muscle actin, which is typical of a vascular smooth muscle cell, suggesting a contractile ability of pericytes (Cardoso et al. 2010; Peppiatt et al. 2006). The physical contact of pericytes' cellular processes over inter-endothelial junctions, together with the α-smooth muscle actin contents, point to a functional role of pericytes in controlling cerebral blood flow (Peppiatt et al. 2006), but also in regulating junctional permeability (Edelman et al. 2006).

In fact, it is believed that pericytes are linked to the regulation of BEC proliferation, migration and differentiation (Persidsky et al. 2006), and to the stabilization of small vessel architecture, neovascularization and angiogenesis (Bicker et al. 2014). The loss of such cell type leads to an abnormal vascular morphogenesis, endothelial hyperplasia and increased BBB permeability (Bicker et al. 2014; Hellstrom et al. 2001). Pericytes and BECs communicate through several apparatuses such as gap junctions, TJs, adhesion plaques (Allt and Lawrenson 2001) and soluble factors. Pericytes influence vessel stability as they synthesize most elements of the BM and activate signals that promote BECs differentiation and quiescence (Armulik et al. 2005; Dore-Duffy et al. 2006). In addition, pericytes release a great number of growth factors and angiogenic molecules which regulate microvascular permeability and angiogenesis (Dore-Duffy and LaManna 2007) and, therefore, play an important role in the maintenance of the BBB.

1.4.5. Microglia

The role of microglia in endowing BBB phenotype is still controversial. Microglia, which are CNS-resident parenchymal immune cells, occupy a strategic position in the BBB allowing control of innate and adaptive immune responses in the brain (Zenker et al. 2003), surveying local microenvironment and changing the phenotype in response to homeostatic disturbance of the CNS. In the absence of pathology, the resting microglia have small bodies and long, thin processes; when activated, they assume a phagocytic morphology with short processes, accompanied by changes in surface antigen expression

and cytokine release (Zlokovic 2008). This way, these cells are fully competent in antigen recognition, antigen presentation and co-stimulation, supporting a possible role for these cells in perivascular inflammation regulation in the human CNS (Fabriek et al. 2005). As microglia are found in the perivascular space, it is thought that their interactions with BECs may contribute to the BBB properties (Choi and Kim 2008; Zenker et al. 2003).

1.4.6. Neurons

Although little is known on the effect of neurons on BBB properties, there is evidence that they can induce the expression of BECs regulatory enzymes (Persidsky et al. 2006), and also induce the stabilization of the endothelium TJs (Schiera et al. 2003). On the other hand, mature endothelium induces a stable brain microenvironment that enables proper neuronal activity (Cardoso et al. 2010; Choi and Kim 2008).

1.5. Transport across the BBB

In addition to structural elements assuring BBB tightness, metabolizing enzymes and transport systems constitute additional barriers. However, nutrients and some water-soluble compounds like ions, amino acids, vitamins and proteins vital for proper brain function need to cross such barrier. Therefore, the BBB keeps various specific transport processes that allow entry into the brain to supply its needs. The different transport mechanisms existing in microvessel endothelial cells of the BBB are schematically presented in *Figure 4*. Depending on its intrinsic physicochemical properties (degree of ionization, water solubility, size, spatial conformation), the concentration gradient, its plasma protein binding and its affinity for a particular transporter, a particular molecule will be transported by one of these transport pathways to cross the BBB.

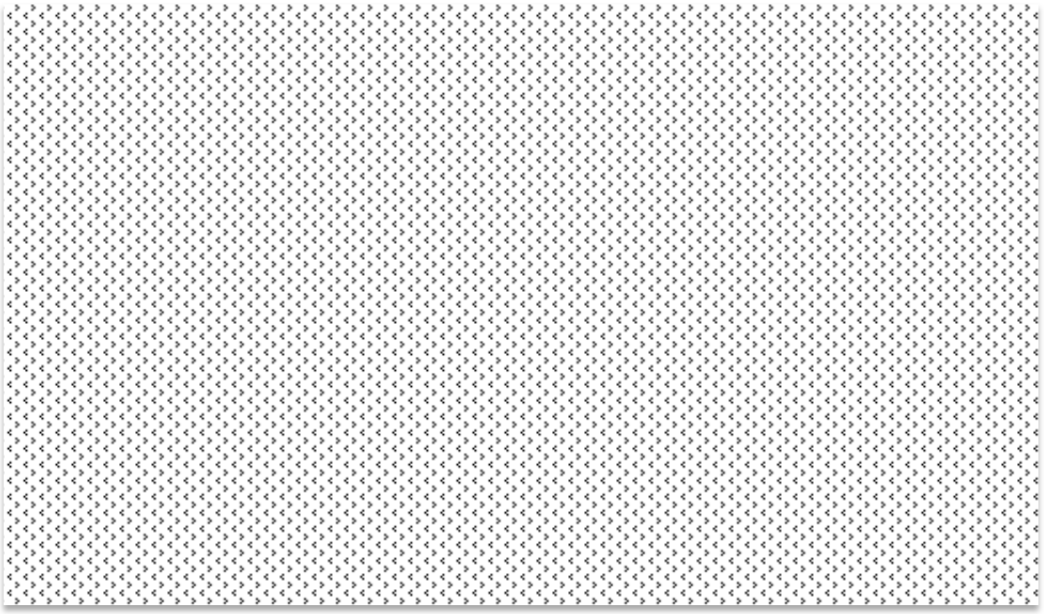

Figure 4. Major routes of molecular and cell trafficking across the endothelium of the BBB.
Small lipophilic molecules and solutes may cross the BBB by passive diffusion. Various essential nutrients and ions are transported into the CNS through a carrier-mediated influx system, whereas several noxious substances, metabolites and xenobiotics are brain-to-blood pumped through a carrier-mediated efflux system. Macromolecules, such as peptides, can cross the endothelium through receptor-mediated transcytosis; in particular, positively charged macromolecules can non-specifically induce adsorptive-mediated transcytosis. Image taken from (Serlin et al. 2015).

1.5.1. Paracellular pathway

This transport mechanism relies on passive diffusion of molecules, and so it is limited to lipophilic or low molecular mass solutes, which are dependent on electrochemical, hydrostatic and osmotic gradients. This pathway is essentially determined due to the presence of TJs at the brain microvasculature, and is solute concentration-dependent hence not involving energy consumption. The transport of O_2 and CO_2 across the BBB is diffusive and blood-flow dependent, and move according to their concentration gradients. Also, a wide range of lipid-soluble molecules can diffuse through the BBB and enter the brain passively (Liu et al. 2004). However, these molecular and physicochemical properties are not always an absolute indication for CNS penetration, as well as there are a few effective CNS active molecules that do not comply with the general rules for BBB penetration.

1.5.2. Carrier-mediated transport

The carrier-mediated pathway is a saturable transport mechanism that can be energy-dependent or independent, mediating the exchange of substrates between the systemic

circulation and the brain parenchyma, and it relies on the polarized expression of molecular carriers at both luminal and/or abluminal membranes of the BBB. Carrier-mediated transport tend to be highly stereospecific in order to selectively vehicle nutrients and some small molecules, like amino acids, glucose, nucleoside amines and vitamins (Pardridge 2005). These transporters carry the influx (blood-to-brain) or efflux (brain-to-blood) of molecules across the membrane of BECs by either facilitated diffusion (i.e. according to the concentration gradient) or by active transport (i.e. against the concentration gradient). For the latter, the energy required can be provided either by the hydrolysis of ATP (primary active transport) or by the co-transport of an ion or another molecule (secondary active transport). Membrane transport proteins can be classified into: 1) *water channels*, 2) *ion channels*, 3) *ATP pumps*, 4) *ATP-binding cassette transporters* and 5) *solute carrier transporters* (Hediger et al. 2004).

Ion transporters like the sodium-potassium-two chloride co-transporter and the sodium-proton exchanger, predominantly expressed in the luminal side of the BBB (O'Donnell et al. 2006), and the chloride-bicarbonate exchanger, expressed at both sides, play critical roles in regulating the intracellular pH of the endothelium (Taylor et al. 2006). Influx transporters are grouped under two main families known as solute carrier (SLC), also known as solute linked carrier, and organic solute carrier (OST) transporters, whereas, the majority of the efflux transporters are grouped under a family known as ATP-binding cassette (ABC) transporters.

1.5.2.1. Solute Carrier Transporters in the BBB

The expression of SLCs in the cell membrane of BECs allows the transport of essential polar nutrients necessary for cell metabolism (Zhang et al. 2002). Some SLC transporters allow the entry of their substrates by facilitated diffusion, such as glucose and amino acids. Other SLCs operate as secondary active transporters: carrying their substrate against the concentration gradient, the energy required for transport is provided through co-transport of an ion or another molecule in the same direction (symport) or in the opposite direction (antiport). The main SLC transporters expressed at the BBB are GLUT-1/SLC2A1 (Glucose Transporter), LAT-1/SLC7A5 (L-aminoacid transporter), OAT3/SLC22A8 (organic anion/cation transporter) and OATP1A4/SLC21A4 (organic anion transporting polypeptides) (Abbott et al. 2010; Kusuhara and Sugiyama 2005; Shawahna et al. 2011). Some significant SLCs expressed in the BBB are listed in *Table 1* (adapted from (Abbott et al. 2010).

Once the expression of some of these SLC transporters is polarized, and thus present in the luminal or abluminal membranes only, or in both, it results in preferential transport of substrates into or across the endothelial cell, and either blood-to-brain or brain-to-blood (Nag et al. 2005; Ohtsuki and Terasaki 2007).

1.5.2.2. ATP-Binding Cassette Transporters in the BBB

The ABC transporters present at the BBB function as active efflux pumps of a wide range of lipid-soluble compounds, and so are responsible for the removal of potentially neurotoxic endogenous or xenobiotic molecules from the brain (Dallas et al. 2006). The ABC transporters family will be described with further detail in a dedicated section [*see section 2*].

1.5.3. Transcytosis

The majority of membrane transporters only carry small molecules (< 1000 Da). Therefore, the influx of large proteins into the brain, such as transferrin, low-density lipoprotein (LDL) and insulin occurs through a transport mechanism known as transcytosis (Pardridge 2005). Transcytosis can be a receptor-mediated (RMT) or an adsorptive-mediated (AMT) phenomenon.

In receptor-mediated process, a circulating ligand interacts with a specific receptor at the luminal side of the BECs, and once bound it promotes the formation of coated pits that eventually engulf to form an endocytic vesicle (Brown and Greene 1991; Scherrmann 2002). These vesicles then travel to the abluminal side of the polarized endothelial cell, where they are released. Caveolae are these vesicular invaginations of the plasma membrane involved in molecular transport, cell adhesion and signal transduction, being caveolin the principal structural component (Frank et al. 2003; Parton and Richards 2003). BECs express several transport systems for neuroactive peptides such as arginine-vasopressin, enkephalins, hormones, and some cytokines and chemokines.

Table 1. Non-exhaustive list of some SLC transporters expressed in the human BBB.

Adapted from (Abbott et al. 2010)

SLC Transporter (Gene Name/Abbreviation)	**BBB Location**	**Orientation**	**Endogenous Substrates/mechanism of transport**
SLC2A1/GLUT1	Luminal Abluminal	Blood to brain	Glucose (facilitative, bi-directional)
SLC5A1/SGLT1	Abluminal	Brain to endothelium	Glucose (sodium-dependent)
SLC5A3/SMIT *SLC2A13/GLUT13*	Luminal	Blood to endothelium	Myoinositol (sodium-dependent)
SLC7A1/CAT1 *SLC7A3/CAT3*	Luminal	Blood to endothelium	Basic L-amino acids Lysine, arginine (sodium-dependent)
SLC7A5/LAT1	Luminal Abluminal	Blood to brain	Asparagine, glutamate, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine (facilitative, bi-directional
SLC7A6/LAT2	Luminal Abluminal	Blood to brain	Arginine, lysine, ornithine (facilitative, bi-directional)
SLC38A2/SNAT2	Abluminal	Brain to endothelium	Small neutral amino acids: alanine, asparagine, proline, serine, glycine (sodium-dependent)
SLC38A3/SNAT3 *SLC38A5/SNAT5*	Abluminal	Brain to endothelium	Asparagine, glutamine, histidine, serine (sodium-dependent)
SLC1A4/ASCT1 *SLC1A5/ASCT2*	Abluminal	Brain to endothelium	Alanine, cysteine, glycine, isoleucine, leucine, methionine, serine, threonine, valine (sodium-dependent)
SLC1A3/EAAT1 *SLC1A2/EAAT2*	Abluminal	Brain to endothelium	Anionic amino acids: glutamate, aspartate (sodium-dependent)
SLC29A1/ENT1 *SLC29A2/ENT2*	Luminal	Blood to endothelium	Nucleosides, nucleotides, nucleobases (facilitative, equilibrative)
SLC28A1/CNT1 *SLC28A2/CNT2*	Abluminal	Endothelium to brain	Nucleosides, nucleotides, nucleobases (sodium-dependent)
SLC16A1/MCT1 *SLC16A2/MCT8*	Luminal Abluminal	Blood to brain	Ketone bodies T3 thyroid hormone (facilitative)
SLC22A7/OAT2 *SLC22A8/OAT3*	Luminal	Blood to brain	Dicarboxylate exchange with α-cetoglutarate, bicarbonate, Cl^-
SLC22A2/OCT2 *SLC22A3/OCT3*	Luminal	Blood to endothelium	Organic cation/proton exchange
SLC22A5/OCTN2	Luminal Abluminal	Blood to endothelium	Organic cation/proton exchange
SLC29A44/PMAT	Abluminal	Brain to endothelium	Organic cation/proton exchange, MPP^+, serotonin, dopamine

While in RMT there is binding of a ligand to a specific receptor, in AMT excessive positive charged molecules bind to negatively charged sites on the plasma membrane. This process lacks specific targeting and may lead to widespread absorption (Bickel et al. 2001; Correale and Villa 2009; Sauer et al. 2005).

In both cases, to successfully achieve transcytosis of a molecule, the lysosomal compartment within the cell must be avoided. Routing away the formed endosome from the lysosome may be a specialized feature of the BBB endothelium, where the intact transcytosis of a significant number of macromolecules is needed, as it does not occur in many peripheral endothelia (Abbott et al. 2010; Nag et al. 2005). Once transcytosis is neither size-limited nor lipophilicity-dependent, and also safe and effective due to its high specificity, it has been proposed as a reference target pathway for CNS drug delivery.

1.5.4. Cell passage across the BBB

During the embryonic development, cells from the bone-marrow derived monocyte lineage enter into the brain, and after differentiation become resident immunologically-competent microglia (Glezer et al. 2007). However, in the adult brain and as a result of BBB maturation, leukocyte infiltration into the CNS is low compared to other tissues. Under normal healthy conditions, leukocyte traffic across the BBB is strictly regulated, with low expression of luminal adhesion molecules minimizing the first stages of leukocyte adhesion, in cooperation with other cells of the NVU (Engelhardt and Ransohoff 2012). Mononuclear cells are able to be recruited to the CNS and play roles complementary to those of microglia (Davoust et al. 2008), entering by diapedesis directly through the cytoplasm of BECs, and thus without TJ disruption (Wolburg et al. 2005). In pathological events such as inflammation, trauma or ischemia, TJs may be opened as a result of cytokines and other agents released by mononuclear cells, facilitating the entry by both transcellular and paracellular routes (Konsman et al. 2007; Scholz et al. 2007). In addition, during CNS inflammatory states, like multiple sclerosis, the brain endothelium exhibits an enhanced expression of adhesion molecules, such as ICAM-1 and VCAM-1, which are essential for T cell crawling on the BBB prior to diapedesis (Abadier et al. 2015; Engelhardt 2008). This leads to an increase in the leukocyte trafficking into the CNS, and consequently to enhanced central inflammation.

1.6. BBB study approaches

It is undeniable that the BBB represents a unique interface in the human organism, resultant of a perfect and intimate connection of the brain endothelium with the neighboring brain parenchymal cells, playing a critical role in the regulation of brain microenvironment and functioning as a shield against neurotoxic agents. Considering its vital function, there is a considerable interest in the scientific and health community to study the BBB, which can be enumerated: 1) study of the BBB physiology (development, maintenance, regulation), 2) how does the BBB behave and its characteristics in pathological conditions, and 3) BBB permeability to compounds and CNS drug delivery.

It is increasingly recognized that the BBB is not a static but a dynamic system, capable of responding to local changes and requirements, and able to be regulated and modified by different mechanisms and cell types, in both physiology and pathological conditions. This is crucial so the BBB can matches its activities to the requirements of the brain, whether for protection from circulating agents, adjustment of nutrient supply, or modification to facilitate local repair (Abbott et al. 2010). On the other hand, the BBB might be vulnerable to modification and see its role affected when a CNS disorder takes place. BBB dysfunction has been described in a variety of neurological diseases, not only as a late event but also as putatively involved in the early steps of disease progression (Weiss et al. 2009). It is estimated that approximately 1.5 billion people suffer from CNS disorders (Tosi et al. 2008), which corresponds to 6.3% of the global burden of disease, and this number is projected to rise in the coming years due to an aging population (Patel et al. 2012). In addition, it is believed that 50% of the total worldwide population will show Alzheimer's symptoms by the end of the 21st century (Tosi et al. 2008), leading to increasing concern by health organizations worldwide. In fact, recent studies have shown that BBB impairment is present in neurological diseases such as Alzheimer and Parkinson's diseases, epilepsy, AIDS-provoked dementia or traumatic brain injury (van Vliet et al. 2007a; Zlokovic 2008), which can in turn contribute to progressive nerve cell dysfunction in such disorders. This is a matter that dramatically draws the attention of the pharmaceutical industry in search for novel therapeutic approaches to abrogate such disease processes. However, due to the referred outstanding properties of the BBB, it is estimated that only 2% of drug candidates are able to cross such barrier and reach the CNS to exert a therapeutic effect (Pardridge 2003), making this field of research one of the most stimulating challenges for the scientific world.

Altogether, these facts have attracted great interest over the study of the BBB. In order to better understand the physiology and functioning of the BBB, different study approaches

were developed. It is generally accepted that the similarity of an experimental system to the *in vivo* situation is directly related to its degree of complexity (Cardoso et al. 2010). There are currently different animal or human models and experimental approaches available, and depending on the intended purpose, with their own advantages and disadvantages. These will be briefly described below.

1.6.1. In vivo

Considering *in vivo* studies are conducted in biological systems that preserve the whole brain microenvironment and perfectly functional biological processes, these provide the most reliable reference information for testing and validating other models. Due to ethical considerations and sensitivity of the CNS, all human molecular data concerning the BBB either came from patients or was generated from postmortem donors. To date, the effect of disease states and postmortem changes are still not fully understood, hence questioning the validity of the data. Taking this into account, the selection of animal experimental models that mimic as closely as possible the human BBB is important for studying BBB physiology and permeability to compounds and new agents of interest. Several animal models have been used to generate *in vivo* data including mice, rats, pigs, and upper primates. Such studies showed variable results and suggested essential interspecies differences (Alanne et al. 2009; Cutler et al. 2006; Syvanen et al. 2009).

In vivo studies have been used to assess BBB-associated feature, such as TJ functioning (Yamamoto et al. 2008), activity of ABC transporters (Cisternino et al. 2004; Seleman et al. 2014; van Vliet et al. 2007b), permeability properties, as well as behavior patterns, in knockout animals for specific BBB proteins (Cardoso et al. 2010). Also, use of imaging techniques has been applied to this study, where large sample sizes and appropriate controls are needed to detect modest but clinically relevant BBB changes in early stages of cerebral microvascular disease and any subsequent progression (Farrall and Wardlaw 2009). Positron emission tomography (PET) is an non-invasive imaging technique used to study the drug transport across the BBB. It requires the administration of a short half-life labeled compound containing a positron-emitting isotope, and it provides real time monitoring of the body distribution of the radiolabeled compound, including its brain distribution. In this way, it allows the study of the cerebral passage of radiolabeled products, and their possible brain efflux, and thus the activity of ABC transporters, by using known substrates and inhibitors.

The *in situ* brain perfusion is an invasive technique suitable in rodents for measuring the BBB permeability of a compound. The perfusion of artificial saline via the heart or the

internal carotid, in a flow rate- and time-controlled manner, allows the measurement of the uptake into different brain regions and the accurate pharmacokinetic parameters of a compound that penetrates relatively fast.

However since these studies can be highly demanding in technical and economic terms, other alternatives have been also used.

1.6.2. Ex vivo

Since *ex vivo* studies are performed on living tissue outside the organism with minimum alteration of natural conditions, experiments can be conducted under highly controlled conditions impossible in the intact organism. *Ex vivo* studies may also be performed on post-mortem collected tissues, which can be used to circumvent the limited availability of human brain tissue. This has been valuable for the study of BBB structure and function, namely for study of TJ (Ballabh et al. 2005), basement membrane assembly (Buttner et al. 2005), and the expression of brain microvessel efflux transporters (Boer et al. 2008). Nevertheless, proteins may suffer rapid degradation if the collection of autopsy specimens doesn't follow the right time and storage protocol, and such changes may render difficult the establishment of conclusive results.

Isolated brain slices are one of the most common *ex vivo* methods for studying the structure of the BBB, where the interactions and the cell architecture of all components of the NVU are highly preserved. Its use allows the study of regional-dependent patterns associated with some brain diseases, as well as the exploration of molecular pathways, the study of their modulation with pharmacological agents, the screening of therapeutic molecules (Cardoso et al. 2010; Cho et al. 2007; Lossi et al. 2009), and specially the immunolocalization of many enzymes and transporters within the different cells composing the NVU by fluorescence immunohistochemistry.

1.6.2.1. Isolation of brain capillaries

A widely used technique consists in the isolation of brain microvessels from the cortical gray matter of the brain tissue. Different studies were carried out on isolated microvessels from different species like mouse, rat, monkey and human (Dauchy et al. 2008; Ito et al. 2011; Shawahna et al. 2011; Uchida et al. 2011; Yousif et al. 2008). The characterization of gene and protein expression in isolated microvessels has gained considerable attention in molecular biology research. In order to isolate brain microvessels from fresh biopsies and/or post-mortem tissues, different techniques were developed, as here summarized.

- *Mechanical dissection*

Mechanical dissection allows the isolation of brain microvessels through a mechanical disruption of the cortical gray matter followed by a gradient separation (dextran or albumin, typically), and filtration of the separated portion of the solution that contains the microvessels using a series of successive filters (Goldstein and Betz 1986; Yousif et al. 2007). This protocol permits an important elimination of cell debris and contamination from other associated cell types in the NVU; however, pericytes, embedded in the basement membrane, and some astrocytic end-feet processes remain attached to the endothelium and cannot be eliminated. Inexpensive and quick to implement, this technique enables the study of gene and protein expressions of BBB microvessels. This method was broadly used in the development of the present thesis work to assess the effects of different exposure protocols conducted *in vivo* in rats, on the expression of BBB markers.

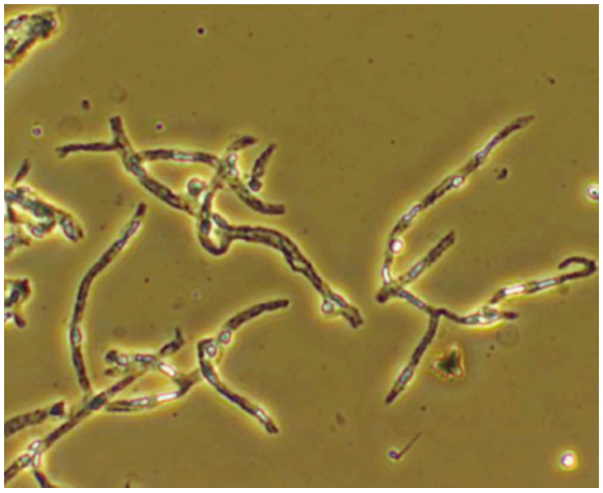

Figure 5. Contrast-phase microscopic visualization of brain microvessels isolated by mechanical dissection of rat brain cortical grey matter.
Image taken from (Yousif et al. 2007).

- *Enzymatic dissociation*

With enzymatic dissociation, the isolation of brain microvessels differs from the first as enzymatic digestion, usually with a cocktail of enzymes such as collagenase, dispase and DNase, replaces the mechanical disruption of the isolated brain gray cortical matter (Bowman et al. 1981; Ishikawa et al. 1999).

- *Laser microdissection*

Unlike the first two, this method involves a delicate cutting and isolation of brain microvessels under optical control with a laser, allowing a more precise isolation of

microvessels from a specific cerebral region (Emmert-Buck et al. 1996). Therefore, isolated microvessels are closer to those in *in vivo* condition and less variant to protein and mRNA transcript quantities.

1.6.3. In silico

The development of technology and of advanced algorithms gave rise to the creation of mathematical models using computer simulators to predict the BBB permeability of a particular molecule. Accurate *in vivo* measurements of BBB permeability have permitted testing of *in silico* predictive models based on understanding the role of physico-chemical properties of the compounds (solubility, lipophilicity, molecular weight, hydrogen bonding and electric charge) (Abbott 2013). This approach has drawn much attention in the development of new pharmaceutical compounds, since innovative molecules can be quickly and easily evaluated if they are likely good drug candidates for crossing the BBB in an early phase of development, even before synthesis (Naik and Cucullo 2012). It is also very useful in prediction of transporter properties (Demel et al. 2008), channels (Huber et al. 2009), protein activity (Maruszak et al. 2009) and toxicity evaluation (Piotrowski et al. 2007), among others. Still, *in silico* is a good prediction tool but currently insufficiently accurate to determine whether and how a compound crosses the BBB, as well as not providing quantitation of permeation. In addition, the complex nature of BBB is neglected, which leads to uncertain results and impairs the establishment of solid conclusions (Cardoso et al. 2010; Mehdipour and Hamidi 2009).

1.6.4. In vitro

In vitro modeling of the BBB results in a simplification of the *in vivo* situation, allowing the conduction of more controlled experimental system, often impossible to be carried out *in vivo* (Cecchelli et al. 2007), while also raising less ethical concerns and producing significant economical savings (Tajes et al. 2014).

An ideal *in vitro* BBB model should be simple, reproducible, and mimic as closely as possible the *in vivo* human barrier either functionally or anatomically, in order to allow the drawing of proper conclusions concerning BBB-related issues in normal and pathological conditions. However, this is not fully achievable in practice, since cells are taken out from their natural brain microenvironment and grown in a simplified system, lacking many of the influences they would normally experience from both the CNS and the periphery. In particular, the *in vitro* cell model must reproduce functional and structural BBB properties, and therefore 1) preserve endothelial cell morphology with high expression of junctional

proteins, 2) present restrictive paracellular passage and selective permeability, 3) possess a realistic cell architecture, 4) display functional expression of transporter mechanisms, and 5) maintain a high transendothelial electrical resistance (TEER) as a marker of BBB maturity and integrity (Booth and Kim 2012; Naik and Cucullo 2012). Yet, *in vitro* approaches are somehow limited by the absence of *in vivo* signaling and intercellular contacts, and subjected to the *in vitro* differentiation and phenotypic modification that occurs when cells are isolated and kept in culture (Cardoso et al. 2010). Nevertheless, recent improvements in cell culture made available several models, which can provide very useful information concerning BBB basic physiology, BBB permeability to compounds and new therapeutics and permeation mechanisms, as well as for mimicking some aspects of the barrier in pathology (Abbott 2013; Ogunshola 2011; Shah et al. 2012). Furthermore, they may constitute the first approach in routine toxicity and pharmacological testing, thus reducing the number of animals used (Cardoso et al. 2010). Most *in vitro* BBB models derived from isolated brain capillary endothelial cells, considered to be the anatomic basis of the BBB, are used as primary cultures or as immortalized brain endothelial cell lines. Most models rely on brain tissue from rat, mouse, porcine, bovine and human. However, the obtained results must take into account interspecies differences, which should be considered when correlations between *in vitro* and *in vivo* approaches are done (Alanne et al. 2009; Cecchelli et al. 2007).

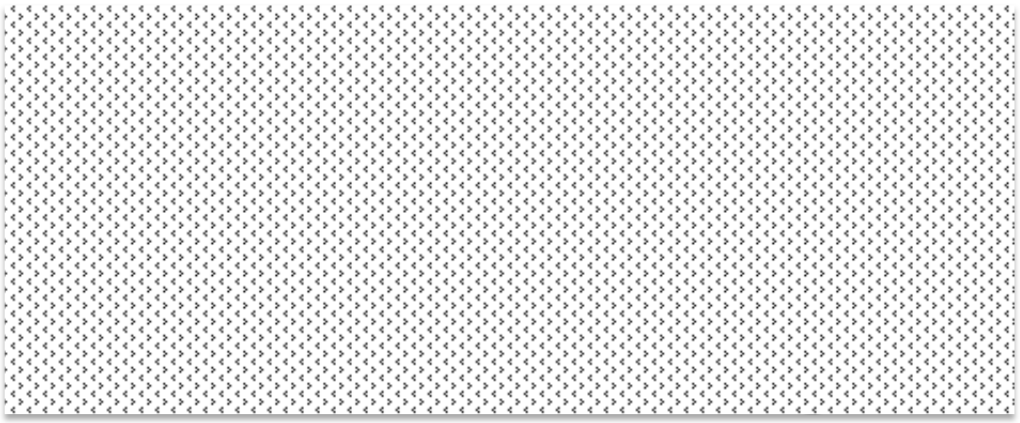

Figure 6. Static VS Dynamic Models for the study of the BBB.

In both static (A) and dynamic (B) BBB models, BECs (1), from either primary or immortalized cell lines, grow in the upper or inner compartment, respectively, of these culture systems. Other cell types composing the NVU, such as astrocytes, pericytes and microglia (2), can be co-cultured in the lower compartment of the static model, or in the surrounding compartment of the dynamic model. Images adapted from (Weiss et al. 2009).

Up to date, rat BEC monolayer culture is the simplest *in vitro* cell screening experiment and has proved to be useful in early stages of drug development (Garberg et al. 2005; Tajes et al. 2014). Different categories of BEC cultures can be used, comprising primary

cell cultures and immortalized cell lines: primary cultures more likely represent a simplified model of the *in vivo* condition but require considerable technical resources and are more time consuming; cell lines are less complex, but less resemble the *in vivo* condition (Cardoso et al. 2010). Also, both static and dynamic models are available, the latter consisting on subjecting endothelial luminal surface to a flowing fluid (Naik and Cucullo 2012), and thus more closely mimicking the blood flow to which the cells are subjected in *in vivo* conditions (see *Figure 6*). They can range from simple monolayer of cells to co-cultures of endothelial cells with other cell types belonging to the NVU. All together, these *in vitro* BBB models are a valuable tool for the screening of the permeability of new drugs, as well as to unravel the molecular mechanisms of BBB control in physiological and dysfunctional BBB in various CNS pathological states.

- *Primary culture of brain endothelial cells*

Even though technically challenging to isolate and maintain, primary cell cultures of BECs are the closest possible phenotype to the *in vivo* BBB, providing a convenient model and the optimal choice for BBB research (Smith et al. 2007). Although some cultures are conducted using human brain tissue (Bernas et al. 2010; Siddharthan et al. 2007), it is not a feasible option due to its limited availability, quality and uniformity. On the other hand, the yield of microvessels from small rodents commonly used for *in vivo* studies (mouse, rat) is low (Avdeef 2011). Subsequently, porcine and bovine tissues as a source of BECs are seen as a good alternative (Cecchelli et al. 2007).

Primary cultures of BECs usually preserve well TJ proteins and transporter mechanisms, but it remains difficult to obtain a pure endothelial culture, as the basement membrane surrounding the microvascular endothelium also encloses pericytes (Calabria et al. 2006). They also quickly lose their specific characteristics in culture, undergoing dedifferentiation and senescence even upon limited passaging (Nakagawa et al. 2009), thus hampering usefulness as *in vitro* model of the BBB.

- *Immortalized cell lines*

Immortalization of brain microvessel endothelial cells are of great help for BBB study, as they achieve confluence in a very short time, allowing cell amplification and providing a large amount of cells per experience. Such cell lines are also available for many species, but are usually less tight than primary cultured cells, and may be deficient in some tight junctional proteins, enzymes and transport systems (Wilhelm et al. 2011). Cell lines have been generated mostly by SV40 T antigen (Stins et al. 1997), but also by human

papilloma E6E7 gene (Prudhomme et al. 1996), Rous sarcoma virus (Mooradian and Diglio 1991), as well as by incorporating human telomerase (Weksler et al. 2005). BBB studies profit from the existence of human cell lines, such as the hCMEC/D3 (human cerebral microvascular endothelial cell/D3 clone) cell line, which retains most of the morphological and functional features of BECs (Schreibelt et al. 2007; Weksler et al. 2005). Despite their obvious advantages, immortalized cell lines have usually a poorly differentiated phenotype compared to the *in vivo* situation, as well as commonly present leaky intercellular junctions and lack paracellular barrier properties (Cardoso et al. 2010). Two of these immortalized cell lines, of human (hCMEC/D3 cells) and rat (RBE4 cells) origins, have been used in the development of the present thesis work.

- *Co-culture systems*

The interplay between different cellular components of the NVU on BBB structure and function has led to the establishment of more complex *in vitro* models, such as co-culture systems (de Boer and Gaillard 2006). Co-culture models have demonstrated to be more suitable than monocultures since their permeability is more selective (Tajes et al. 2014). Most co-culture systems are based on a BEC-astrocyte/pericytes/neuron/microglia two-chamber cell culture system (Nakagawa et al. 2007), allowing a better understanding of the cross talk between the brain endothelium and the neighboring elements of the NVU and a closer reproduction of the *in vivo* condition (see *Figure 7*). Recently, there have also been studies with triple cultures of BEC, astrocytes and pericytes/neurons (Nakagawa et al. 2009; Nakagawa et al. 2007; Schiera et al. 2003), but its complexity is limiting the wide use of such *in vitro* models.

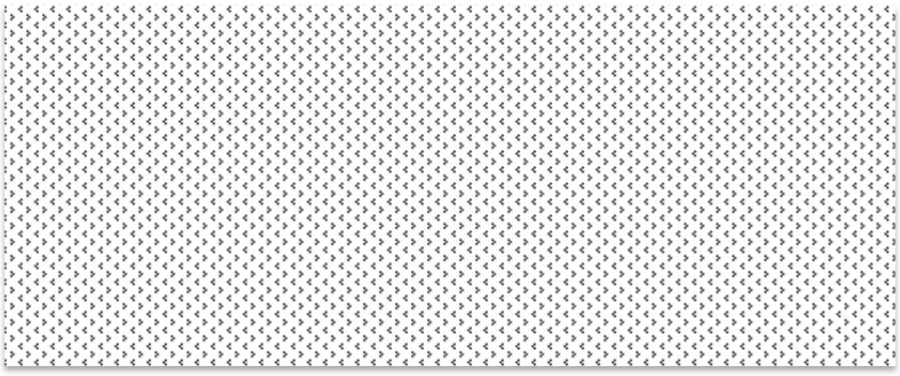

Figure 7. Schematic representation of a static co-culture model for the BBB study.
In A) it is exemplified a co-culture system with cultured endothelial cells on a plate insert and astrocytes at the bottom of the plate well; in B), a triple co-culture system is exemplified, where endothelial cells and pericytes are cultured on the top and bottom of the insert, respectively, and astrocytes grow on the bottom of the plate well. Image taken from (Cardoso et al. 2010).

- *Dynamic models*

Given the shear stress provoked by the capillary blood flow influences the BECs and the tightness of the BBB, dynamic *in vitro* models more closely mimic the *in vivo* situation, than the static ones. It has been shown that the flow-induced forces as well as the presence of glial cells, or of factors released by them, improve the barrier properties and tightness of endothelial cells, thus rendering this approach a better model to study the BBB (Siddharthan et al. 2007). However, most screening tests are still done on static systems, considering that dynamic systems, even though reliable and promising, are technically demanding.

1.6.4.1. hCMEC/D3 cell line

The model was developed from cerebral microvessels obtained from a cortectomy performed on a patient with epilepsy. The immortalization was performed by double transfection with SV40 T antigen and human telomerase (hTERT) (Poller et al. 2008).

The human immortalized hCMEC/D3 cell line retains most of the morphological and functional characteristics of BECs, namely the expression of TJs and the polarized expression of multiple active transporters, receptors and adhesion molecules, even if not co-cultured with glia or astrocytes (Schreibelt et al. 2007; Weksler et al. 2005). This cell line has shown consistent expression of a considerable number of BBB endothelial cell markers genes such as CD31, VE-cadherin, and von Willebrand factor (Poller et al. 2008; Weksler et al. 2005), as well as expresses fully functional drug efflux transporters (Dauchy et al. 2009; Weksler et al. 2005). Despite being an immortalized cell line, the paracellular pathway is considerably more restricted than in other cell lines, making it a promising candidate not only for microscopy, transcriptomic and proteomic studies, but also for measuring the passage of molecules from one compartment to another and, thus, drug transport. The model also permits the study of substrate affinity to a certain transporter with or without the presence of a specific inhibitor (Poller et al. 2008).

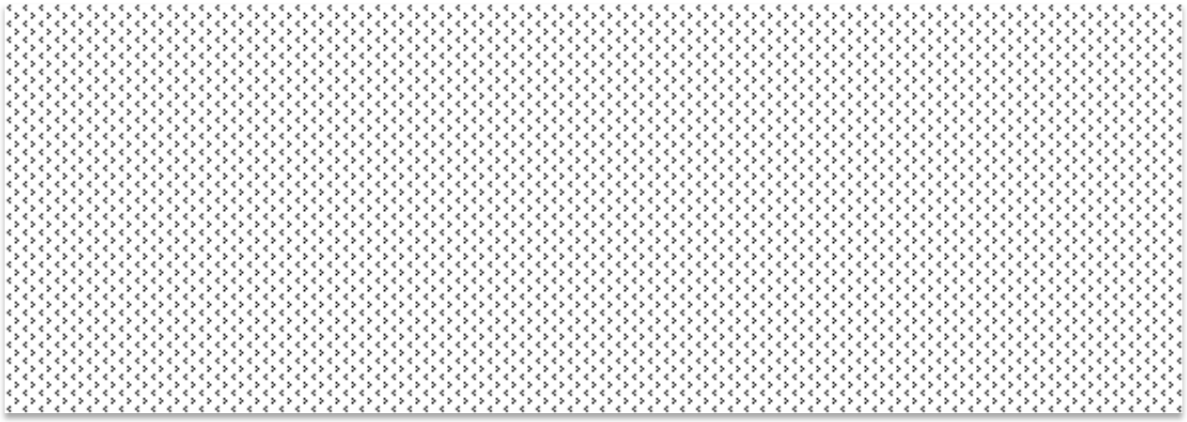

Figure 8. Phase-contrast microscopic view of the immortalized hCMEC/D3 and RBE4 cells.
A) human hCMEC/D3 cell line (taken from (Weksler et al. 2005)) and B) rat RBE4 cell line (taken from (Wilhelm et al. 2011)). The highly similar morphology of these two brain microvascular endothelial cells is noteworthy.

1.6.4.2. RBE4 cells

The rat RBE4 cells are a rat immortalized cell line isolated and cultured for the first time using the Hughes and Lantos (Hughes and Lantos 1986) method with some modifications (Roux et al. 1989). The immortalization was obtained by transfection of the plasmid pE1A/neo carrying the E1A region of Adenovirus 2 (Roux et al. 1994). The rat RBE4 cell line has been extensively used in different types of studies, namely of transport (Faria et al. 2010; Vilas-Boas et al. 2013b) and subcellular localization of transporters (Babakhanian et al. 2007), signaling pathways (Zhang et al. 2009a) and inflammatory cascades (Pan et al. 2007b), toxicity (dos Santos et al. 2010) and toxicity modulation (Marreilha dos Santos et al. 2008), among others. According to the morphological and biochemical studies performed, the RBE4 cell line exhibit features that are representative of an *in vivo* BBB situation, namely the ability to form a confluent monolayer, polarized expression of BBB specific transporters, as well as the presence of BBB cell surface markers, such as TJ and AJ proteins (Bendayan et al. 2002; Friedrich et al. 2001; Huwyler et al. 1999; Regina et al. 1998; Regina et al. 1997; Roux et al. 1994). The major limitation of this model appears to be its relatively high paracellular permeability to small molecules, thus limiting their use for permeability studies (Bendayan et al. 2002; Roux and Couraud 2005).

2. The ATP-binding cassette transporters in the BBB – Focus on P-glycoprotein and Breast Cancer Resistance Protein

P-glycoprotein (P-gp, ABCB1, MDR1) and Breast Cancer Resistance Protein (ABCG2, BCRP) are the most critical efflux transporters, among the ABC transporters expressed in the BBB. These two transporters are present in the luminal, blood-facing side of the endothelial cells of brain capillaries, and thereby critical in the prevention of the entry of toxic molecules, drugs and other xenobiotics circulating in the blood into the brain (Cooray et al. 2002; Graff and Pollack 2004). On the other hand, P-gp and BCRP can also prevent the entry of CNS-acting drugs, and this barrier role may be even exacerbated in pathological conditions. In this section, the ABC transporter family will be explored in detail, and special focus will be given to the role of the expression and activity of P-gp and BCRP at the BBB.

2.1. General Considerations on the ABC Transporter Family

The cellular transport mechanisms, and particularly the membrane transporters, importantly contribute to cell homeostasis, and to the inter-individual variation in the bioavailability and vulnerability of a broad range of drugs, toxins, metabolic products and xenobiotics, playing a prominent role in their pharmacokinetics (i.e. absorption, distribution, metabolism and excretion) (Sai 2005). Within the human body, this is greatly determined by the balance of the uptake and efflux phenomena, along with metabolic activity. Among the efflux transporters, the ABC transporters are the most extensively studied.

Multidrug resistance (MDR) was first described in the early seventies during the observation of treatment cross-resistance with different anticancer drugs and cytotoxic agents. Cells from these drug-resistant tumors were resistant to drugs with different structures and with different mechanisms of action (Biedler and Riehm 1970; Dano 1973). This phenotype of MDR was later associated with the overexpression of membrane proteins belonging to the family of ABC transporters. The term “ABC” standing for “ATP-Binding Cassette” was introduced for the first time in 1992 following the discovery that these transporters used the energy of ATP hydrolysis to translocate solutes across cellular membranes, through the highly conserved ATP-binding cassette, the most characteristic feature of this superfamily of proteins (Higgins 1992). In the meantime, several proteins have been recognized to play a role in MDR, and of those, many

represent transporters belonging to this transporter superfamily. Thus, ABC transporters form one of the largest of all protein families, and are central, not only in the aforecited cancer resistance, but also in drug disposition and response (Gottesman and Ambudkar 2001). In contrast to the above-mentioned SLC transporters, which essentially allow the entry of many endogenous compounds and xenobiotics within the cell, ABC transporters are able to efflux xenobiotics and, thus, act as key elements in the absorption, distribution and elimination of several molecules in the human organism. ABC transporters are selectively expressed in strategic tissues, such as the brain, intestine, kidney, liver, placenta and testis to determine drug absorption and disposition (DeGorter et al. 2012; Hagenbuch and Meier 2003; Sai 2005).

At the absorption level, ABC transporters extrude drugs, xenobiotics and metabolites into the lumen of the intestine, thereby preventing their absorption into the blood or lymph circulation and protecting the body against acute and chronic toxicity (Ambudkar et al. 1999; Schinkel and Jonker 2003). These transporters are also responsible for excretion of xenobiotics and metabolites *via* bile and urine, contributing to renal and biliary clearance (Hesselson et al. 2009; Schinkel et al. 1996). Yet, and not least important is their function as key determinants in drug and xenobiotic distribution, particularly in tissues protected by blood-tissue barriers, such as the placental barrier, the blood-testis barrier and, mostly important for the present subject, the BBB (DeGorter et al. 2012). The ABC transporter superfamily is responsible for the efflux of xenobiotics from the brain into the bloodstream, making some ABC transporters key elements in controlling the brain penetration of many drugs and, thus, their CNS effects (Abbott et al. 2010; Begley 2004; Scherrmann 2005).

The great importance of the main ABC transporters expressed in the BBB relies on three critical characteristics that they all share: 1) localization in the luminal plasma membrane of brain capillary endothelial cells, at the blood-CNS interface, 2) highly effective and potent ATP-driven efflux transport against the concentration gradient of their substrates, and 3) a remarkably low substrate specificity that covers a wide range of structurally unrelated therapeutic drugs (Hartz and Bauer 2010).

Therefore, the BBB not only acts as a physical barrier, but also actively eliminates a large number of molecules from the brain, protecting the brain from intoxicants but simultaneously restricting the entrance therapeutic drugs, and thus in some cases impairing effective CNS pharmacotherapy.

2.1.1. Nomenclature and Structure

In the human genome, 49 genes encoding ABC transporters have been identified and divided into seven different subfamilies, A-G, based on their amino acid sequence similarities and evolutionary divergence (Miller 2015; Moitra and Dean 2011): ABCA (12 members; previously ABC1), ABCB (11 members; previously MDR/TAP), ABCC (13 members; previously MRP/CFTR), ABCD (4 members; previously ALD); ABCE (1 member; previously OABP); ABCF (3 members; previously GCN20) and ABCG (6 members; previously White) (see *Table 2*). The three ABCB, ABCC, ABCG subfamilies comprise the transporters involved in the efflux of xenobiotics.

Investigation using topological models predicted the structure for most of the ABC family members. In general, ABC transporters display two hydrophobic transmembrane domains (TMDs), usually each containing multiple (typically six) membrane-spanning α-helices – which are involved in the recognition of substrates – and two hydrophilic cytoplasmic nucleotide-binding domains (NBDs) located in the cytoplasm – which in turn couple conformation changes induced by ATP binding and hydrolysis, and ADP release to the transport process (Higgins and Linton 2004; Jones and George 2004) (see *Figure 9*). Some members do not follow this typical structure as they display either fewer or additional TMDs and/or NBDs. In fact, ABC transporter proteins can be classified into either full transporters (when containing at least two TMDs and two NBDs) or half transporters (containing one of each domain), the latter being assembled into homodimers or heterodimers to form functional units (Hyde et al. 1990). Such is the case of ABCG2, which is a half transporter consisting in a singular TMD and NBD (Haimeur et al. 2004), while ABCC1, ABCC2, ABCC3, ABCC6 and ABCC10 have an extra amino terminal TMD (Couture et al. 2006). The NBD share three conserved domains: the Walker A and B domains, which are not ABC transporter-specific as they can be found in other nucleotide-binding proteins, and the LSGGQ signature C motif, located upstream of the Walker B site, which, by being ABC transporter-specific, determines the affiliation of a protein to the ABC transporter family (Hyde et al. 1990).

Table 2. Classification of the ABC transporter family transporters according to the Human Genome Organization nomenclature.

Adapted from (Chaves et al. 2014)

Subfamilies	**ABCA**	**ABCB**	**ABCC**	**ABCD**	**ABCE**	**ABCF**	**ABCG**
Alternative names	ABC1	MDR/TAP	MRP/CFTR	ALD	OABP	GCN20	White
Number of members	12	11	13	4	1	3	6

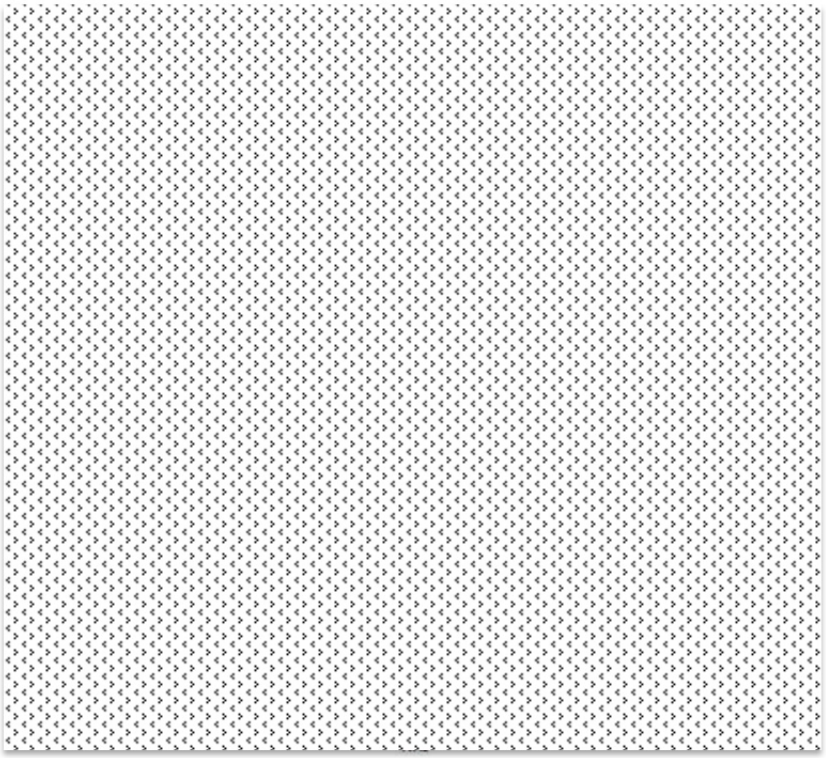

Figure 9. Topological models for the structure of most ABC transporters.
A) Generally, ABC transporters display 2 TMDs and 2 NBDs located in the cytoplasm (e.g. P-gp, ABCC4, ABCC5). B) Some members display 3 TMDs and 2 NBDs (e.g. ABCC1, ABCC2, ABCC3, ABCC6), or alternatively, C) ABC transporters can constitute half transporters, with 1 TMD and 1 NBS (e.g. BCRP, ABCG1). Image taken from (ElAli and Hermann 2011)

2.1.2. Mechanism of Action

Every ABC transporter uses the energy released by ATP hydrolysis for the efflux of various endogenous compounds and xenobiotics against the concentration gradient. The transport involving these efflux carriers is considered a multistep process involving communication via conformational changes, in both directions, between the NBDs and TMDs. Within the membrane, the TMDs are organized to form an inverted “V” pocket, where substrates either in the cytoplasm or in the inner leaflet of the cytoplasmic membrane can bind and establish multiple interactions. The substrate binding to this pocket increases the affinity of NBDs for ATP (Ramachandra et al. 1998; Wang et al. 1998). ATP binding to the NBDs and its hydrolysis induces a conformational change in the TMDs, leading to the opening of the inverted “V” to the opposite side, allowing substrate release into the extracellular space. Upon ATP hydrolysis, the transporter is then restored to its original inward facing orientation, able to begin a new cycle of transport (see *Figure 10*) (Fukuda and Schuetz 2012; Martin et al. 2001). However, the exact mechanism

regarding the coupling of ATP hydrolysis to unidirectional substrate translocation is not yet completely clear.

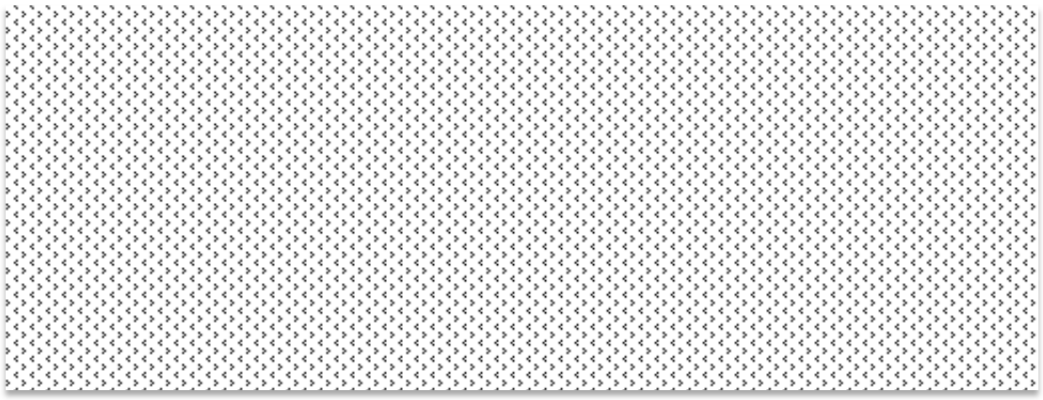

Figure 10. Schematic representation of the mechanism of ABC transporter function.
Members of the ABC superfamily exert their transport function upon substrate binding, and subsequent ATP binding to the NBD. ATP hydrolysis induces a conformational change in the TMDs, which allows the transport of substrates to the extracellular space. Image taken from (ElAli and Hermann 2011)

2.1.3. Physiological and pharmacological implications of the ABC Transporters

ABC transporters are key elements in the maintenance of essential physiological functions in the human organism, including the extrusion of harmful compounds, transport of ions and peptides, and cell signalling (DeGorter et al. 2012; Gottesman and Ambudkar 2001; Gottesman et al. 2002). In fact, the improper functioning of these transporters may have severe clinical repercussions. As such, it has been shown that loss-of-function mutations in many genes coding for ABC transporters are linked to several human genetic diseases, including adrenoleukodystrophy (ABCD1) (Mosser et al. 1993), juvenile macular degeneration (ABCA4) (Allikmets et al. 1997), cystic fibrosis (ABCC7) (Teichgraber et al. 2008), Tangier disease (ABCA1) (Bodzioch et al. 1999), among others (Gottesman and Ambudkar 2001).

ABC transporters belonging to the subfamilies ABCB, ABCC, ABCG play a major role in protecting the body from xenobiotics – by opposing their intracellular accumulation, ABC transporters help reduce their potential harmful effects within the body. For example, the ABCB1 and ABCG2 provide the efflux of toxic compounds such as benzo[a]pyrene, a pro-carcinogenic compound, and some of its metabolites and conjugates (Ebert et al. 2005; Yeh et al. 1992).

Since a considerable number of therapeutic drugs fall as substrates of some ABC efflux transporters, the distribution and bioavailability of drugs can be compromised, and thus, the drug concentrations at the pharmacological target may be under the therapeutic level. Therefore, ABC transporters are often related to the development of a MDR phenotype, particularly evident in cancer or epilepsy treatment (Potschka 2010b; Qosa et al. 2015).

2.1.4. Role of ABC Transporters in the brain microvessels

The localization and the oriented transport of ABC transporters present at the BBB provides support to its barrier function considering the CNS vulnerability to many stressors, such as ischemia, trauma or toxins, and the lack of self-renewal capacity and, therefore, cell replacement. Yet, experimental studies give no complete consensus regarding the distribution of the ABC transporters at the BBB. Whereas the use of isolated brain capillaries cannot ensure that the detected genes or proteins belong to the BECs, due to incomplete removal of pericytes and glial end-feet, BEC in culture do not suffer this contamination but the expression and activity of some genes are frequently influenced by culture conditions (Scherrmann 2005). In addition, the polarity exhibited by the brain microvessel endothelial cells is also critical in the role of ABC transporters at the BBB level, since these are mostly expressed in the luminal side of the BECs. Some of the ABC transporters functions in the brain can be briefly described as 1) protection of cerebral microvessels through elimination of glutathionized organic molecules from endothelial cells, 2) brain protection through elimination of organic toxins and elimination of the β-amyloid (Aβ) peptide from the brain, 3) regulate cell membrane composition by translocating lipids from the cytosolic to exoplasmic leaflet, and 4) control of neural precursor cell proliferation and differentiation into neurons and astrocytes (ElAli and Hermann 2011).

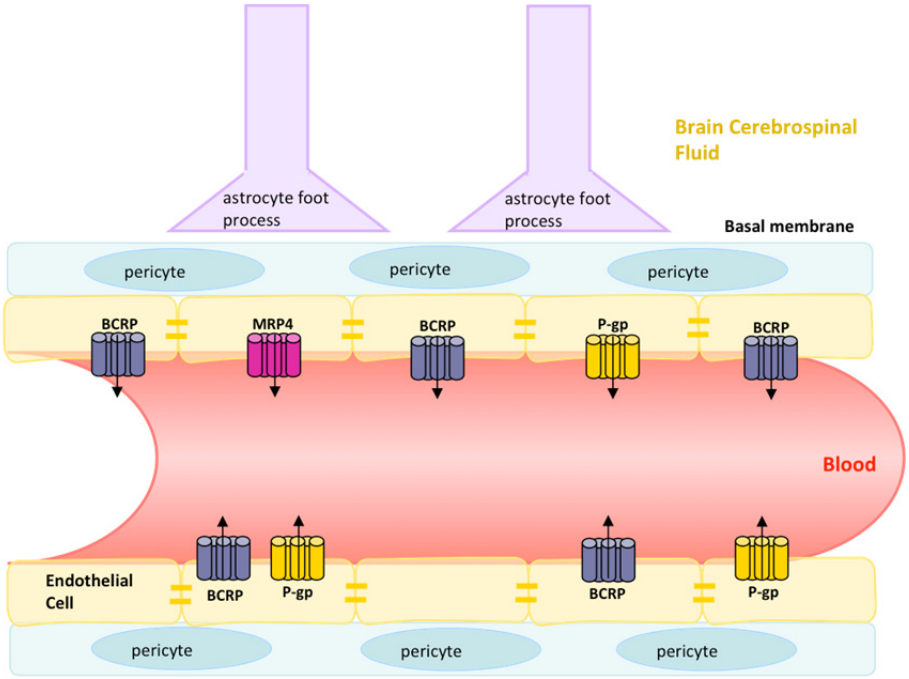

Figure 11. Representation of the major ABC transporters expressed at the human BBB.
ABC transporters are mainly present at the luminal side of the BECs, where P-gp and BCRP play the major role in the brain-to-blood efflux of neurotoxicants and xenobiotics. Image taken from (Chaves et al. 2014)

2.2. P-glycoprotein

2.2.1. P-glycoprotein tissue distribution and main physiological roles

P-glycoprotein was originally identified in 1976 as a membrane glycoprotein capable of altering membrane permeability of colchicine in drug-resistant ovary cells from Chinese hamsters (Juliano and Ling 1976). The presence of this efflux transporter was later identified in human (Thiebaut et al. 1987) and rodent (Croop et al. 1989) normal tissues, as well as in many cultured cells of mammalian and human origins, including 39 of 60 tumor cell lines used by the United States National Cancer Institute in the discovery of new anti-cancer drugs (Alvarez et al. 1995). By the end of the 80's, it was established that P-gp was expressed in several human tissues emphasizing its strong involvement in the pharmacokinetics and toxicology of xenobiotics. In fact, P-gp was found to be expressed at considerable high levels at the apical surface of epithelial cells of the intestine, liver bile and pancreatic ducts and kidney proximal tubules (Chin et al. 1989; Fojo et al. 1987; Thiebaut et al. 1987), suggesting its physiological role in the elimination of xenobiotics and endogenous metabolites. It is also found in blood-tissue interfaces, such as the placenta, blood testis barrier and the BBB (Cordon-Cardo et al. 1990; Cordon-Cardo et al. 1989; Thiebaut et al. 1987), in turn suggesting its organ-protective role against toxic compounds circulating in the blood. The discovery of P-gp constitutes a major event since it brought, for the first time, a biological explanation for the frequently observed anticancer drug resistance phenomenon, in addition to clarifying its functions in the protection of susceptible organs like the brain, testis as well as the fetus from potential harmful compounds, and in the secretion of metabolic byproducts and xenobiotics to bile, urine and into the lumen of the gastrointestinal tract. To date, P-gp remains the most studied transporter of the ABC family.

P-gp exists in different isoforms, which have more than 70% sequence homology and are encoded by a small number of closely related genes. In humans, P-gp is encoded by two *ABCB* genes, *ABCB1/MDR1* and *ABCB4/MDR2*, which arose from a duplication event, and are located in the chromosome 7 (7q21) (Callen et al. 1987; Chin et al. 1989). The MDR phenotype is associated with the *ABCB1* isoform, while *ABCB4* encodes for a protein that carries out phosphatidylcholine transport into the bile (Hennessy and Spiers 2007; Sharom 2006). In rodents, P-gp is encoded by three different genes: *Abcb1a/Mdr1a* and *Abcb1b/Mdr1b*, which both encode drug transporters and correspond to the *ABCB1* human analog, and *Abcb4/Mdr2* which correspond to the *ABCB4* human analog (Sharom 2006). In the following sections of this dissertation, the term P-gp will be used to indicate the *ABCB1* gene product (or *Abcb1a/Abcb1*b gene products in studies performed in rodents).

2.2.2. P-glycoprotein expression and function in the BBB

P-gp was the first ABC transporter to be detected in the endothelial cells of the human BBB by immunochemistry (Cordon-Cardo et al. 1989). Over the course of time, genes encoding P-gp have been also identified in the cerebral endothelial cells of most living species, including rodents, rabbits, pigs, dogfish, cattle and monkeys, by several experimental approaches (Cisternino et al. 2001; Fricker et al. 2002; Jette et al. 1993; Miller et al. 2002; Thiebaut et al. 1987), suggesting that P-gp may serve as a general defense mechanism in the mammalian BBB, protecting the brain form potentially harmful lipophilic compounds from natural sources and other lipophilic xenobiotics that otherwise would penetrate the BBB by simple diffusion without any limitation (Schinkel 1999). The immunolocalization of P-gp in the human endothelial cells of the BBB has been extensively reported using brain sections, isolated brain microvessels and primary cultures of human brain endothelial cells.

The localization of P-gp in capillary blood vessels of human brain tissue was evidenced by immunoelectron microscopy studies, being specifically expressed on the luminal surface of the endothelium whereas no staining was observed in neurons and glial cells (Tanaka et al. 1994). In the human cerebral microvascular endothelial cell line hCMEC/D3 the expression and functional activity of P-gp was clearly demonstrated (Dauchy et al. 2009; Poller et al. 2008; Weksler et al. 2005), as well as in primary cultures of human brain microvessel endothelial cells (HBEC), co-cultured or not with astrocytes (Lee et al. 2007; Megard et al. 2002), while P-gp expression was not found in primary human astrocytes cultured alone (Megard et al. 2002). Supporting these data, studies performed using human isolated microvessels and human brain sections evidenced strong immunocytochemical staining for P-gp, but no detection of this transporter was seen in neurons and glial cells (Seetharaman et al. 1998; Virgintino et al. 2002). In line with these results, two studies using isolated human brain microvessels showed high P-gp expression (Shawahna et al. 2011; Uchida et al. 2011), but given that during the isolation procedure there are still astrocyte foot-processes that remain attached to the microvessels, which cannot be removed, it is difficult to conclude that the expression of P-gp is only due to brain endothelial cells and exclude its expression in astrocytes.

However, P-gp does not seem to be exclusively expressed by the endothelial cells of the NVU. The research group of R. Bendayan has also identified P-gp at luminal and abluminal membranes of capillary endothelial cells, and likewise in adjacent pericytes and astrocytes of human and rat BBB, using immunogold cytochemistry at the electron microscope level (Bendayan et al. 2006). In agreement with this astrocytical presence, there is additional data suggesting that astrocyte foot processes are important sites of

microvascular localization for P-gp, and that it is present on the abluminal side of the human brain microvasculature (Golden and Pardridge 1999; Pardridge et al. 1997). Using human brain sections, immunodetection of P-gp was also possible in the microvascular pericytes that closely envelop the brain capillary endothelial cells (Virgintino et al. 2002). In another study using healthy human brains, P-gp was not detectable in neurons (Aronica et al. 2003; Tishler et al. 1995).

In the rodent brain, both the *Abcb1a* and *Abcb1b* are present, but only *Abcb1a* is localized in brain capillaries of mice, while *Abcb1b* is only present in the brain parenchyma (Demeule et al. 2002). In the normal rat brain, a differential expression of the two codifying P-gp genes has been reported, with *Abcb1a* being expressed in all brain regions, whereas Abcb1b was predominantly expressed in the hippocampus (Kwan et al. 2003).

These data are in agreement that P-gp is highly expressed on the luminal membrane of the endothelial cells of the BBB, and to a much lesser extent in the brain parenchyma, neuronal, and glial cells. Therefore, when a P-gp substrate enters in the BECs it is immediately pumped back into the bloodstream. The lack of concordant results evidencing the presence of P-gp in other brain cells than the endothelium suggests that P-gp expression in the brain parenchymal cells is nominal, although it may be significantly enhanced in pathologic conditions, such as amyotrophic lateral sclerosis (ALS) (Jablonski et al. 2012), epilepsia (Bauer et al. 2008), focal cortical dysplasia (Ak et al. 2007), and brain tumours (Haar et al. 2012). In fact, a cause-relationship may exist between brain pathologies as epilepsy, glioma and cortical dysplasia and the particular up-regulation of the expression of P-gp in astrocytes (Calatozzolo et al. 2005; Chengyun et al. 2006; Sisodiya et al. 2001; Tishler et al. 1995).

The important role of P-gp in barrier function and brain protection is best highlighted when comparing brain drug distributions of wild-type and P-gp genetic knockout mice, as well as in studies in which transporter function is abolished by using P-gp selective inhibitors. *In vivo* dosing studies using P-gp knockout mice show 5 to 50-fold increased brain-to-plasma ratios of a large number of therapeutic drugs that are P-gp substrates and normally cannot cross the BBB and exert their pharmacologic effect (Decleves et al. 2011). For example, the brain penetration of ivermectin is increased up to 100-fold, and that of vinblastine up to 3-fold in *Abcb1a* deficient mice when compared to wild-type mice (Schinkel et al. 1994). In line with this, the blockade of BBB P-gp through local application of P-gp inhibitors significantly increased the brain concentration of several drugs (Loscher and Potschka 2005a). The selective inhibition of P-gp increased paclitaxel accumulation in the brain, substantially reducing tumour volume by 90% in nude mice with cerebrally implanted glioblastoma, while paclitaxel showed to be ineffective without the chemical knockout

(Fellner et al. 2002). Furthermore, the implementation of studies by positron emission tomography using P-gp substrates or inhibitors are powerful tools for studying the *in vivo* functionality of this transporter in the human BBB. Using ^{11}C-verapamil and ^{11}C-desmethyl loperamide, recent studies have shown that P-gp is functional to the BBB in humans (Kreisl et al. 2010; Muzi et al. 2009; Seneca et al. 2009; Wanek et al. 2013).
All accounted, P-gp is nowadays considered the most prominent element of selective, active barrier function that limits xenobiotics from entering the brain.

2.2.3. P-glycoprotein structure and mechanisms of drug efflux

The human P-gp is a single polypeptide of 1280 amino acids weighing approximately 170 kDa (Schinkel 1999). It contains two homologous but not identical TMDs each one containing six α-helices, linked by a highly charged extracellular portion, and two intracellular ATP-binding sites, the NBDs (Aller et al. 2009; Zhou 2008). The amino and carboxyl terminals of this polypeptide are also located intracellularly, while the first N-glycosylated loop is extracellular (Zhou 2008). The 12 transmembrane (TM) segments fold together to form a barrel-like structure that traverses the plasma membrane, and the hydrolysis of ATP that follows the ATP binding to the NBDs provides the necessary energy for drug transport, as for all the ABC transporters (Schinkel 1999).

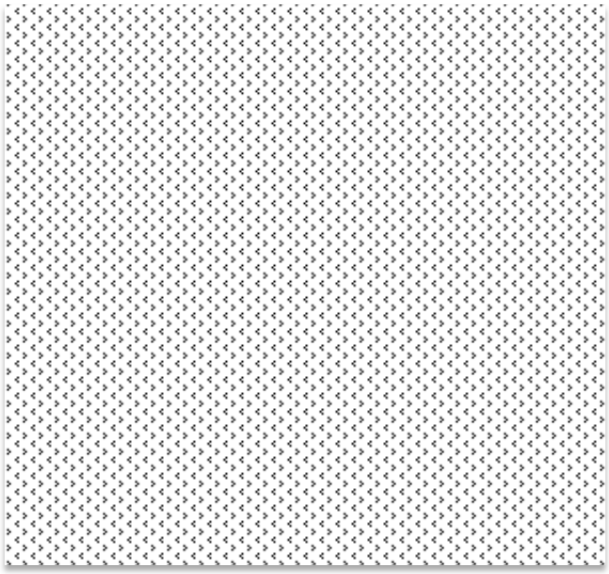

Figure 12. Crystal structure of mouse P-gp. The N- and C-terminal halves of the molecule are colored yellow and blue, respectively. The TMDs and NBDs are also identified within the structure. The shaded area corresponds the approximate positioning of the lipid bilayer. Image taken from (Aller et al. 2009)

The exact mechanism of drug transport has not been elucidated yet, but its structure rendering suggests that P-gp recognizes its substrates in the plasma membrane (Aller et al. 2009; Loo and Clarke 2005b). Considering the clinical relevance of this ABC transporter, the full structure characterization of mouse P-gp, which was reported to share 87% sequence identity with human P-gp in a drug-binding competent state, brought important insights in this field of research (Aller et al. 2009). The characterized P-gp

structure presents a nucleotide-free inward-facing conformation arranged as two “halves” with pseudo two-fold molecular symmetry spanning ~136 Å perpendicular to and ~70 Å in the plane of the bilayer. The NBDs are separated by ~30 Å. The inward facing conformation, formed from two bundles of six helices (TMs 1-3,6,10,11 and TMs 4,5,7-9,12), results in a large internal cavity open to both the cytoplasm and the inner leaflet. Also, two portals - formed by transmembrane helices (TMHs) 4/6 and 10/12 – seem to allow the access for the entry of hydrophobic molecules directly from the membrane (Aller et al. 2009). The volume of the internal cavity within the lipid bilayer is substantial (~ 6,000 $Å^3$) and can accommodate at least two compounds simultaneously (Loo et al. 2003b). The inward-facing structure does not allow substrate access from the outer membrane leaflet nor the extracellular space. This conformation, in a pre-transport state, likely represents an active state where the binding of ATP, stimulated by a substrate, likely causes a dimerization in the NBDs and produces large structural changes, resulting in an outward facing conformation. Depending on the specific compound, substrates could either be released as a consequence of decreased binding affinity caused by changes in specific residue contacts between the protein and drug going from the inward to outward facing conformation or, alternatively, facilitated by ATP hydrolysis. In either case, ATP hydrolysis likely disrupts NBD dimerization and resets the system back to inward facing reinitiating the transport cycle (Aller et al. 2009; Tombline et al. 2005).

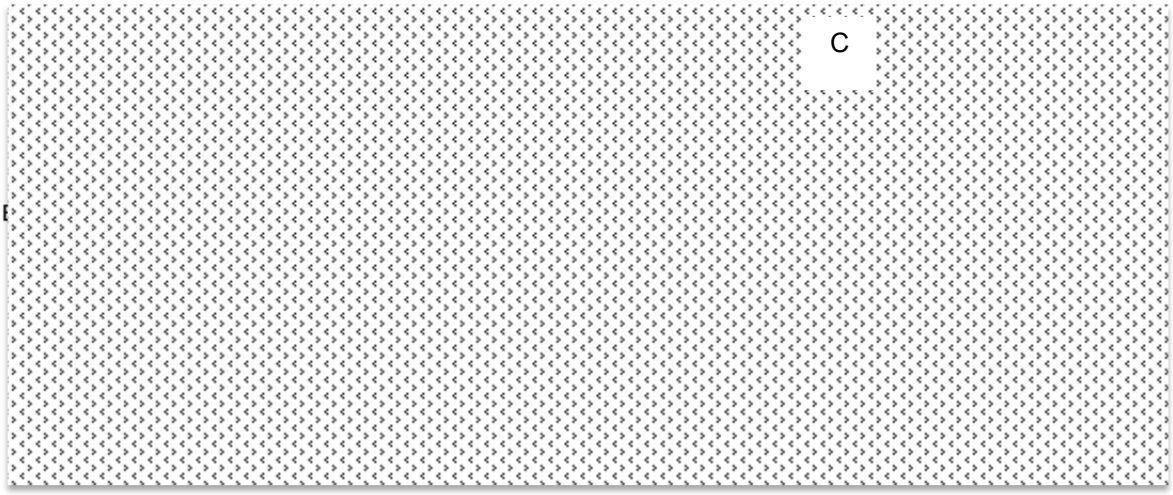

Figure 13. Representation of a consensual model of P-gp substrate transport.

A) P-gp is represented in its substrate-binding conformation, of two inward facing bundles of six TMHs, and its large internal cavity is open to both the cytoplasm and the inner leaflet of the lipid bilayer. Substrate-P-gp interactions facilitate ATP binding to the NBDs. B) ATP binding to the NBDs leads to a conformational change, resulting in an outward-facing conformation. This arrangement leads to the release of substrates into the extracellular space or to the outer leaflet of the lipid bilayer. Image taken from (O'Brien et al. 2012). C) General view of the substrate-binding cavity in the inward-facing conformation of a homology model of human P-gp. Image taken from (Pajeva et al. 2009).

This reported 3D structure for mouse P-gp was used to describe a model of human P-gp for the inward-facing conformation of the protein, which confirmed the role of TMDs 4, 6, 10 and 12 as entrance gates to the internal cavity and describes differences in their functions (Pajeva et al. 2009). Furthermore, the analysis of human P-gp interactions with the QZ59 stereoisomers confirms the multi-specific binding of P-gp with its ligands (Pajeva et al. 2009). In fact, P-gp can distinguish between the stereoisomers of cyclic peptides, resulting in different binding locations, orientation and stoichiometry (Aller et al. 2009; Pajeva et al. 2009).

2.2.3.1. Mechanisms of drug efflux by P-glycoprotein

The exact mechanism by which P-gp couples the ATP hydrolysis to allow the movement of drugs across the plasma membrane, as well as the exact site of substrate interaction with the transporter, are not clarified. However, several models have been proposed on the mechanism by which P-gp is able to efflux its substrates, namely the “hydrophobic vacuum cleaner”, “flippase” and “pore” models (Hennessy and Spiers 2007; Varma et al. 2003). The first two models are the most widely accepted (Hennessy and Spiers 2007; Sharom 2011; Varma et al. 2003) and both models are consistent with the tertiary structural data for P-gp, indicating that substrates gain access to the pore from the lipid phase of the membrane (Rosenberg et al. 1997; Rosenberg et al. 2001).

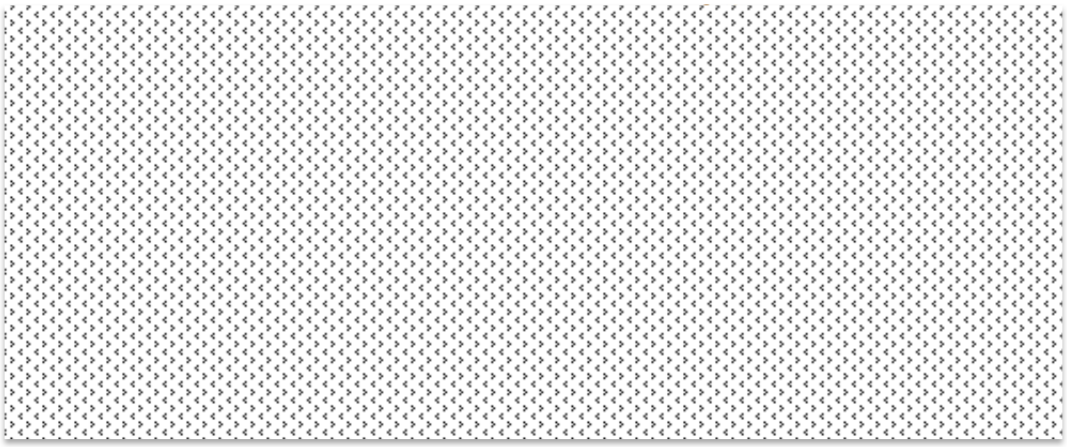

Figure 14. Proposed models of P-gp substrate efflux mechanisms.
According to the “vacuum cleaner” model, P-gp recognizes hydrophobic substrates that previously partitioned into the lipid bilayer, and drives them out of the membrane directly to the external aqueous medium. In the “flippase” model, P-gp substrates are transported from the inner leaflet of the lipid bilayer, to either the outer leaflet of the plasma membrane or to the extracellular space. Image taken from (Sharom 2011).

- *“Hydrophobic vacuum cleaner”*

The compounds that interact with P-gp are relatively hydrophobic and readily soluble in lipid bilayers, and it is now widely accepted that they partition into the membrane before

interacting with the protein (Seelig and Landwojtowicz 2000). Moreover, P-gp substrate binding sites appear to be contained within its TMDs, and drugs gain access to these sites after partitioning into the lipid bilayer (Raviv et al. 1990). Thus, the idea that P-gp acts a "hydrophobic vacuum cleaner", that expels lipophilic molecules from the membrane into the extracellular medium, was firstly suggested by Higgins and Gottesman to account for the lipophilic nature of P-gp substrates (Higgins and Gottesman 1992) and has found widespread acceptance. The general consensus of the “hydrophobic vacuum cleaner” model relies on the principle that P-gp recognizes hydrophobic compounds embedded in the inner leaf of the plasma membrane (after they have partitioned into the bilayer), and pumps them out of the membrane directly to the external aqueous medium (Higgins and Gottesman 1992). This pumping action gives rise to a concentration gradient across the plasma membrane, with a higher drug concentration in the external aqueous phase. This way, the transporter is able to intercept substrates before they have an opportunity to enter the cytosol, and therefore protecting the cell from exposure to potentially toxic molecules (Sharom 2011). Additionally, P-gp 3D conformation changes upon nucleotide binding to the intracellular NBD have been reported (Rosenberg et al. 2003). In the absence of nucleotide, the two transmembrane domains form a single barrel with a central pore that is open to the extracellular surface and spans much of the membrane depth, while upon nucleotide binding the TMDs reorganize into three compact domains that open the central pore along its length in a manner that could allow access of hydrophobic drugs directly from the lipid bilayer to the central pore of the transporter (Rosenberg et al. 2003). The recent P-gp X-ray crystal structures also support this model, which demonstrated that two peptide stereoisomers are bound deep within the TMDs, suggesting they may gain access to P-gp from within the lipid bilayer (Aller et al. 2009).

- *The “flippase” model*

This model assumes that P-gp substrates are flipped from the inner leaflet of the lipid bilayer, to either the outer leaflet of the plasma membrane or directly to the extracellular space (Higgins and Gottesman 1992). This means that drug substrates should be located at the leaflet of the bilayer, rather than at the hydrophobic core of the membrane. According to this model, a low rate of spontaneous movement of substrates between the two bilayer leaflets is also necessary, to allow P-gp to generate a drug concentration gradient. Actually, P-gp has the ability to bind lipid-like drugs and platelet-activating factors (Eckford and Sharom 2006), apart from also translocating fluorescently-labeled phospholipids across the membrane in an ATP-dependent way (Romsicki and Sharom 2001). Therefore, this transporter may function as a translocase or ‘flippase’ for lipophilic

molecules (Higgins and Gottesman 1992), moving them from the cytoplasm to the extracellular membrane leaflet. The rate of spontaneous transbilayer movement of many P-gp substrates is relatively low and, as they appear to be discretely localized in one membrane leaflet, the transporter would be able to maintain a higher substrate concentration in the outer leaflet (Sharom 2011).

It should be noted that both these models described above assume that P-gp substrates partition into the lipid phase prior to interacting with the transporter. In fact, this may help to explain the P-gp unusually broad substrate specificity, since the primary determinant of specificity would be the ability of a substrate to appropriately intercalate into the lipid bilayer, with the subsequent substrate-binding site interaction being a secondary matter (Hennessy and Spiers 2007). Also, the P-gp substrate binding pocket is located within the TM regions that contact with the cytoplasmic membrane leaflet (Aller et al. 2009; Lugo and Sharom 2005b; Qu and Sharom 2002; Shapiro and Ling 1997a), which is consistent with both models. However, experimentally, it is difficult to distinguish between the “hydrophobic vacuum cleaner” and “flippase” models (Hennessy and Spiers 2007).

2.2.4. P-glycoprotein substrates, substrate-binding sites and pharmacoresistance: clinical implication

2.2.4.1. P-glycoprotein substrates

As P-gp was the first ABC transporter to be described, it is also by far the best characterized, where a broad range of chemically diverse drugs have been identified as substrates. They are significantly different in size, structure, and function and include chemotherapeutic agents such as anthracyclines, vinca alkaloids, and tyrosine kinase inhibitors (TKIs), human immunodeficiency virus (HIV) protease inhibitors and HMG-CoA reductase inhibitors. Given the heterogeneity of P-gp substrates, the structural requirements for a substance to fall in a P-gp substrate category are difficult to define. However, a typical P-gp substrate is hydrophobic or amphipathic, presents a planar mainly aromatic ring system, and is usually positively charged at physiological pH (Sharom 1997). Still, P-gp is able to transport neutral compounds (digoxin, cyclosporine A), negatively charged molecules (fexofenadine, atorvastatin) and hydrophilic drugs (methotrexate, peptides) (Choong et al. 2010). Other xenobiotics recognized and transported by P-gp include opioid analgesics (Dagenais et al. 2004; King et al. 2001; Thompson et al. 2000), anti-psychotics and antidepressants (Uhr et al. 2003; Uhr et al. 2000), anti-emetics (Schinkel et al. 1996), and an important number of chemotherapeutic, anti-retroviral and anti-epileptic drugs. A summary of these and other substrates of P-gp is

displayed in *Table 3*, and is evidence of the remarkable number of CNS and other drugs that can be transported by P-gp, and of its contribution to pharmacoresistance.

2.2.4.2. P-glycoprotein role in pharmacoresistance

While on one hand the presence of P-gp at the BBB plays a role in the protection of the brain parenchyma from the potentially toxic effects of blood-circulating xenobiotics, on the other hand, it may represent a real obstacle to drug brain penetration. This is of particular concern for therapeutic drugs targeting CNS diseases as it can lead to the emergence of pharmacoresistance. Here we focus on the implication of P-gp in the MDR phenotype in pathophysiological states where the brain is involved.

- *Brain tumors*

In general, brain tumours respond poorly to chemotherapy, and the development of a MDR phenotype is frequently observed. A great number of anti-cancer drugs have shown to be P-gp substrates: anthracyclines such as doxorubicine and daunorubicine (Kubota et al. 2001), etoposide (Lagas et al. 2010), tyrosine kinase inhibitors imatinib and gefitinib (Breedveld et al. 2005; Kawamura et al. 2009), taxanes (paclitaxel) (Fellner et al. 2002), and vinca alkaloids (vinblastine, vincristine) (Cisternino et al. 2003; Ushigome et al. 2000). This makes P-gp a serious cause in the resistance to anti-cancer treatment, particularly in brain tumours. Furthermore, P-gp is highly expressed in brain tumour cells, and especially in brain tumour microvessels (Calatozzolo et al. 2005; Demeule et al. 2001).

- *HIV-associated neurocognitive disorders*

P-gp at the BBB is also involved in the resistance of brain HIV to pharmacotherapy. The brain is known to be a site of viral replication for HIV and thus is an important target tissue for antiretroviral agents. HIV enters the brain early on in the course of infection, and infects brain parenchymal cells, causing the release of toxins and excitatory neurotransmitters, which, in turn, cause inflammation and cell death. The majority of HIV protease inhibitors (indinavir, saquinavir, nelfinavir), which brought considerable progress in the treatment of HIV infection, are also important P-gp substrates, as studies show that these drugs reach significantly higher brain levels in *Abcb1a* knockout mice when compared to wild-type mice (Kim et al. 1998; Washington et al. 2000). The remarkable increase in abacavir brain distribution showed in *Abcb1a*-deficient mutant mice over wild-type mice suggests that P-gp may also play a significant role in restricting the reverse transcriptase inhibitor abacavir distribution to the CNS (Shaik et al. 2007). P-gp was also

demonstrated to be involved in the brain efflux of amprenavir and ritonavir (Edwards et al. 2002; van der Sandt et al. 2001). Thus, P-gp-mediated efflux of several HIV protease inhibitors limits virus eradication from the brain, and is involved in the brain resistance to HIV pharmacotherapy.

- *Epilepsy*

It is believed that pharmacologic treatment fails to effectively control and prevent seizures in about a third of patients with epilepsy. This is a most likely multifactorial phenomenon, where different explanations are pointed to justify therapeutic failure (Potschka 2010b). In fact, there is considerable evidence that a large number of antiepileptic drugs are transported by P-gp: cell lines transfected with the human P-gp gene confirmed that P-gp transported the active metabolite of carbamazepine (carbamazepine-10,11-epoxide) as well as eslicarbazepine acetate and oxcarbazepine, transports that were blocked by P-gp inhibitors tariquidar and verapamil (Zhang et al. 2011a); a similar study was conducted to show that phenytoin and phenobarbital are as well P-gp substrates (Zhang et al. 2010). Another hypothesis is linked to the overexpression of ABC transporters at the BBB, namely P-gp. Tishler and colleagues were the first to report that the expression of the gene encoding for P-gp in humans was markedly increased in the majority of patients with medically intractable temporal lobe epilepsy (Tishler et al. 1995). Moreover, the expression of P-gp seems to be increased in brain capillary endothelial cells and in glial end-feet covering the blood vessels of the epileptic foci (Dombrowski et al. 2001; Marchi et al. 2004; Sisodiya et al. 2002). Neuronal P-gp expression in epileptic patients has also been reported (Lazarowski et al. 2004a; Lazarowski et al. 2004b; Volk et al. 2004). Once more, these data point out the role that P-gp may play in drug resistance in refractory epilepsy, especially if epilepsy patients exhibit over-expression of P-gp at the BBB.

- *Psychiatric disorders*

A high percentage of patients with depression, psychosis and other psychiatric disorders respond inadequately to drug therapy, despite adequate choice of therapeutic drugs at maximum tolerated doses. The mechanisms underlying this resistance are still poorly understood, and as in epilepsy are most likely multifactorial. Still, some antidepressant and anti-psychotic drugs are known to be P-gp substrates, such as amitriptyline and paroxetine (Uhr et al. 2003; Uhr et al. 2000), and their penetration is enhanced in mice lacking P-gp. These data showing that brain penetration of anti-depressant drugs is dependent on the presence of P-gp have been proposed as an explanation for the observed resistance to treatment. Furthermore, several anti-epileptic drugs that are P-gp

substrates, such as carbamazepine or lamotrigine, are also used to treat psychiatric diseases like bipolar disorder (Rogawski and Loscher 2004).

Given the implication of P-gp in the development of drug resistance, different strategies are being considered in order to circumvent this problem (Agarwal et al. 2011a). One strategy considers the inhibition of functional and active P-gp through the co-administration of a P-gp inhibitor. A second strategy, of more recent debate, proposes the direct targeting of intracellular signalling pathways known to be involved in the regulation of P-gp expression. By targeting these pathways, the aim is to prevent a pathology-associated overexpression of P-gp. Although P-gp targeting in order to decrease drug resistance seems to be a very promising approach, it must be taken into account that P-gp is not the only ABC transporter expressed in the brain involved in MDR phenomena. Thus, especial attention should be also given to other major ABC transporters expressed at the BBB, in particular to BCRP, which is also known to be involved in drug resistance phenomenon.

2.2.4.3. P-glycoprotein substrate-binding sites

The presumptive drug-binding pocket comprises mostly hydrophobic and aromatic residues (Aller et al. 2009). Still, P-gp is able to recognize and transport an impressive broad range of substrates, ranging in size from approximately 250 Da (cimetidine) to more than 1850 Da (gramicidin D) (Schinkel 1999). While drug binding is known to occur within the TMD of P-gp, where and how substrates bind to this protein is not clear. The presence of multiple drug binding sites on P-gp could provide an explanation for the wide range of compounds known to interact with this protein. In fact, the existence of multiple drug binding sites that interact allosterically has been suggested, based on measurements of radiolabeled drug binding to P-gp (Martin et al. 2000). It was also proposed that both the N- and C-terminal halves of P-gp contain binding sites, and these two sites may generate a single region in the overall protein structure (Loo et al. 2003b; Morris et al. 1994), being this drug-binding pocket large enough to accommodate more than one substrate (Loo and Clarke 2001). Indeed, several research studies have demonstrated that two different substrates can bind to P-gp at the same time (Lugo and Sharom 2005a) (Loo et al. 2003a).

On the other hand, there is evidence for P-gp allosteric sites distinct from transport sites, as some compounds showed to confer allosteric control of the P-gp binding site for substrate transport (Martin et al. 1997). Therefore, binding sites can be classified as both transport and modulating sites (Martin et al. 2000; Shilling et al. 2006), and have the

ability to switch between high and low affinity states to accommodate substrates/inhibitors (Wang et al. 2003a).

Table 3. List of some endogenous compounds and xenobiotics that are substrates of P-gp.
Adapted from (Chaves et al. 2014; Loscher and Potschka 2005a; Nies 2007; Qosa et al. 2015; Robey et al. 2011; Urquhart and Kim 2009). TKIs, Tyrosine kinase inhibitors

P-glycoprotein substrates	
Therapeutic Class	**Compounds**
Anti-cancer drugs	doxorubicine, daunorubicine, vinblastine, vincristine, etoposide, teniposide, paclitaxel, methotrexate, irinotecan, imatinib, gefitinib and other TKIs
Anti-emetics	domperidone, ondansetron
Corticoids	dexamethasone, hydrocortisone, corticosterone
Anti-retroviral drugs	amprenavir, indinavir, saquinavir, abacavir, nelfinavir, ritonavir, lopinavir
Opioids and analgesics	morphine, methadone, oxycodone, fentanyl, loperamide, norbuprenorphine
Antidepressants and antipsychotics	amitriptyline, nortryptiline, venlafaxine, fluoxetine, paroxetine, sertraline, amisulpride, risperidone
Anti-epileptic drugs	phenytoin, carbamazepine, lamotrigine, phenobarbital, felbamate, gabapentin, topiramate
Antibiotics	erythromycin, rifampicin, gramicidin A, valinomycin, tetracyclines, fluoroquinolines
Immunosuppressants:	Cyclosporine A, Tacrolimus, Sirolimus
Anti-helminthic drugs	ivermectin, abamectin
Others:	verapamil, colchicine, cimetidine, digoxin, fexofenadine, amiodarone, lovastatin, simvastatin
Endogenous compounds:	β-Amyloid peptide, bilirubin, cortisol, aldosterone

Thus, P-gp appears to bind multiple drugs by having a highly flexible binding cavity, which can accommodate several compounds in different locations by an induced fit mechanism. Biochemical cross-linking and fluorescence studies had already pointed to a substrate-binding region with these properties (Loo and Clarke 2005a). The polyspecific nature of

the P-gp-binding pocket and its ability to bind more than one drug molecule simultaneously makes the rational design of specific high-affinity inhibitors a challenging problem.

2.2.5. Modulation of P-glycoprotein at the BBB: inhibition, induction, and activation

2.2.5.1. P-glycoprotein inhibition

As previously mentioned, there is currently important evidence that ABC transporter-mediated drug efflux, namely by P-gp at the BBB, is responsible for limiting brain drug delivery of several CNS drugs, thereby leading to treatment failure in brain disorders, including brain cancer, epilepsy and depression.

Through the use of P-gp transporter inhibitors, one might overcome the function of the efflux transporter-mediated barrier and improve drug delivery to the brain. The importance of BBB P-gp was established when P-gp knockout mice exhibited up to 100-fold greater CNS exposure to ivermectin, resulting in serious CNS toxicity at dose levels safely used in wild-type animals (Schinkel et al. 1994). Since then, P-gp-mediated drug resistance has been considered a major clinical issue leading to a joint research effort, and as a result a large number of compounds have been screened for their ability to inhibit the major BBB ABC drug efflux transporters, particularly P-gp, and thus reverse MDR. Many of these P-gp inhibitors are currently used in therapy for other clinical indications (see *Table 4*). Here we will present an overview of the different P-gp inhibitors and their corresponding mechanisms of action.

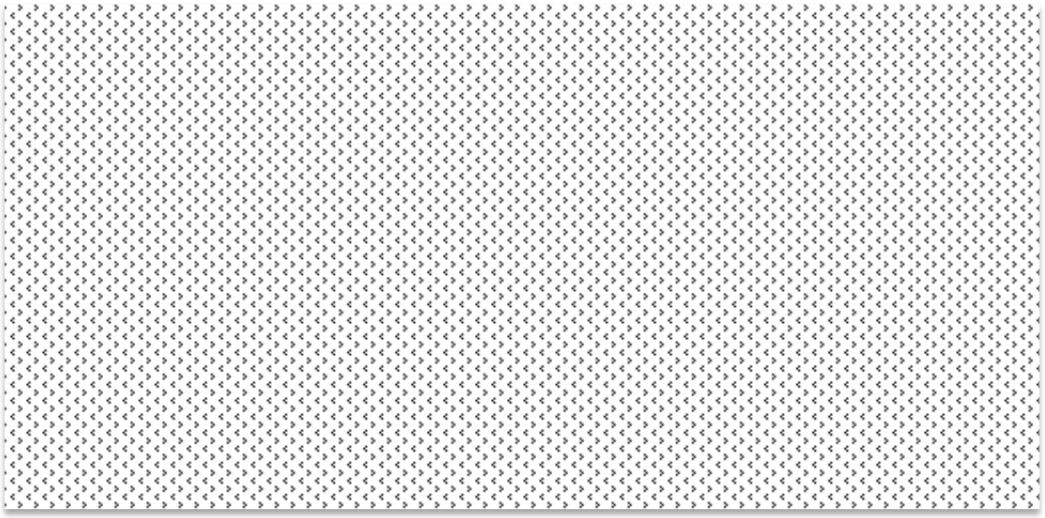

Figure 15. Schematic representation of mechanisms of P-gp inhibition.

P-gp may be inhibited through a) interference with the substrate-binding sites, b) alteration of the cell membrane integrity, c) interaction with the ATP-binding sites. Other mechanisms of limiting P-gp efflux activity may comprise ATP depletion and decrease P-gp expression. Image taken from (Akhtar et al. 2011)

Different mechanisms are said to be involved in P-gp inhibition: 1) competitive, non-competitive or allosteric drug binding site blockage, 2) interference with ATP hydrolysis and 3) change in cell membrane lipids integrity (Akhtar et al. 2011). Verapamil and cyclosporine A are P-gp substrates that competitively inhibit the efflux pump by blocking the drug binding sites (Miller et al. 1991; Slater et al. 1986). Still, the presence of multiple binding sites hampers the development of conclusive structure-activity relation studies for substrates or inhibitors. On the other hand, compounds that inhibit ATP hydrolysis, such as quercetin, could perform better as P-gp inhibitors, since they are unlikely to be transported by P-gp, and will require a lower dose (Varma et al. 2003). Finally, commonly used pharmaceutical surfactants, such as sodium dodecyl sulphate and Tween-20, are emerging as a different class of P-gp inhibitors, which act by altering integrity of membrane lipids may contribute to modifications in P-gp secondary and tertiary structure and ultimately to its loss of function (Varma et al. 2003).

2.2.5.2. P-glycoprotein inhibitors

P-gp inhibitors can be classified into four generations according to their potency, selectivity and drug-drug interaction potential (see *Table 4*) (Palmeira et al. 2012a).

- *First generation P-gp inhibitors*

First-generation inhibitors include the classic P-gp inhibitors (verapamil or cyclosporine A) but also all pharmacological active compounds, already in clinical use or under investigation for other therapeutic indications, other than P-gp inhibition, irrespective of the date of discovery (Palmeira et al. 2012a; Varma et al. 2003). These include anti-hypertensives, like reserpine, anti-arrhythmics, such as quinidine; and antidepressants, such as paroxetine. Many of these compounds are themselves P-gp substrates and, therefore, act through competition with another P-gp substrate for efflux (Varma et al. 2003). The clinical use of these compounds is however limited since high serum concentrations, and thus toxic, are needed to achieve P-gp inhibition, given the low binding affinity for the transporter (Varma et al. 2003). Many of them are also substrates and inhibitors of other transporters and of drug-metabolizing CYP enzymes, which produce unpredictable pharmacokinetic interactions in the presence of other substrates (Breedveld et al. 2006). To overcome these limitations, several novel analogs were developed.

Table 4. List of some known P-gp inhibitors.

Adapted from (Chaves et al. 2014; Loscher and Potschka 2005a; Nies 2007; Palmeira et al. 2012a; Qosa et al. 2015; Robey et al. 2011; Sharom 2011; Urquhart and Kim 2009; Zhou 2008)

P-gp inhibitors	Class	Compounds
1st Generation	*Anticancer drugs*	Tamoxifen, bicalutamide, fefitibin, lapatinib, erlotinib, tipifamib, vinblastine
	Anti-retroviral drugs	Ritonavir, nelfinavir, saquinavir
	Antidepressant / antipsychotic drugs	Amoxapine, loxapine, sertraline, paroxetine, fluoxetine, perphenazine, chlorpromazine, haloperidol
	Cardiovascular drugs	Amiodarone, quinidine, verapamil, nifedipine, diltiazem, dipyridamole, reserpine, prazosin
	Immunosuppressant drugs	Cyclosporin A, tacrolimus, sirolimus
	Others	Methadone, disulfiram, pentoxifyline, midazolam
2nd Generation		Dexverapamil, 1,8-dibenzoyl-rifampicin (DiBenzRif) dexniguildipine, valspodar (PSC-833), biricodar (VX-710), timcodar (VX-853), toremifene, dofequidar (MS-209)
3rd Generation		Zosuquidar (LY335979), tariquidar (XR9576), elacridar (GF120918), laniquidar (R101933), ontogen (OC144-093)
4th Generation	*Natural products*	Flavonoids (Quercetin, nobiletin), coumarins (conferone, cnidiadin, rivulobirin A), cannabinoids (cannabidiol), terpenes (portlanquinol, jolkinol B, uvaol, oleanolic acid, sipholenol A)
	Surfactants and lipids	Pluronic P85, tween-20, triton X-100, crmophor EL, PEG-300, nonidet P40
	Dual ligands	Dual inhibitors of P-gp and tumor cell growth [aminated thioxanthones such as 1-[2-(1H-benzimidazol-2yl)ethanamine]-4-propoxy-9H-thioxanthen-9-one]

- *Second generation P-gp inhibitors*

Second generation inhibitors are analogues of the initial agents to which structural modifications were applied in order to decrease their main therapeutic activity and increase P-gp inhibitory activity, resulting in less toxicity and greater potency, in comparison to the corresponding first-generation inhibitors (Palmeira et al. 2012a). Consequently, these compounds lack the pharmacological activity of the first-generation compounds, and usually, possess a higher P-gp affinity (Varma et al. 2003). Non-immunosuppressive analogues of cyclosporin A, such as valspodar (PSC-833), the D-isomer of verapamil, dexverapamil, and other compounds such as biricodar (VX-710),

timcodar (VX-853) and dofequidar (MS-209) are some of the second generation inhibitors. The synthetic rifampicin derivative, 1,8-dibenzoyl-rifampicin (DiBenzRif), has also recently showed to be a P-gp inhibitor, by depleting the ATP intracellular levels and increasing membrane fluidity (Vilas-Boas et al. 2013a). Nevertheless, they retain some first generation features that limit their clinical usefulness, since they lack P-gp selectivity and may, thus inhibit other ABC transporters, as well as may significantly inhibit the metabolism and excretion of cytotoxic agents, leading to unacceptable toxicity, complex and unpredictable drug-drug interactions (Fischer et al. 1998; Varma et al. 2003; Wandel et al. 1999).

- *Third generation P-gp inhibitors*

Several third-generation P-gp inhibitors were developed through quantitative structure-activity relationship (QSAR) and combinatorial chemistry studies (Palmeira et al. 2012a), to overcome the limitations of previous P-gp inhibitor generations, and obtain the most selective and potent P-gp inhibitors known to date. In fact, the inhibitory effect of these compounds largely exceeds those of first and second generation in what concerns to potency and duration of action (Varma et al. 2003), and were primarily developed for MDR tumors treatment improvement. Another important feature of this third generation is that these compounds do not affect cytochrome P450 3A4 at relevant concentrations (Coley 2010). Zosuquidar (LY335979), elacridar (GF120918), laniquidar (R101933) and tariquidar (XR9576) are among the most studied (Palmeira et al. 2012a; Varma et al. 2003).

Tariquidar inhibits P-gp by a non-competitive mechanism, particularly through ATPase activity inhibition, and binds to P-gp with a greater affinity than that of the transported substrates (Fox and Bates 2007), although it is not clear whether it binds directly to the ATP binding site or to an allosteric location (Martin et al. 1999).

Elacridar acts by binding to the allosteric site of P-gp and reverses the drug resistance at the nanomolar range (Akhtar et al. 2011). However, tariquidar and elacridar were reported to also bind and inhibit the BCRP transporter (Kelly et al. 2011; Kruijtzer et al. 2002; Robey et al. 2004), thus increasing the potential for pharmacokinetic interactions.

On the other hand, other third-generation agents, such as zosuquidar, demonstrated to be more specific for P-gp, rather than for other ABC transporters (Palmeira et al. 2012a). Zosuquidar, which is among the most potent P-gp modulators known to date, inhibits P-gp at nanomolar concentrations, both *in vitro* and *in vivo* (Dantzig et al. 2001; Green et al. 2001), and there is no evidence that it may interact with MRPs or BCRP (Palmeira et al. 2012a). Although a non-competitive inhibitory mechanism has already been suggested, as

this transporter cannot transport it (Dantzig et al. 1996), its mechanism of P-gp inhibition is still unclear. Despite the strong potency of third-generation P-gp inhibitors, there is still no commercial drug for use in MDR therapy, and this is due to disappointing results obtained in either preclinical or clinical trials. In what concerns targeting P-gp activity in the BBB, the existing differences in the sensitivity of P-gp located in different cells and blood–tissue barriers need to be considered. In fact, P-gp in the BBB proved to be more resistant to inhibition than P-gp in other tissues (Choo et al. 2006). This resistance can be overcome by a sufficiently high dose of an inhibitor, but this raises further concerns regarding tolerability of the approach.

- *Fourth generation P-gp inhibitors*

Given the several side effects and the pharmacokinetic interactions observed with previous generations, new strategies were employed to find new P-gp inhibitors, such as compounds extracted from natural origins and their derivatives, surfactants and lipids, peptidomimetics and agents with dual activity (Palmeira et al. 2012a), to give rise to the fourth generation of inhibitors.

The knowledge that some food components interfere with the oral bioavailability of many drugs, which may involve P-gp, lead to the search of molecules obtained for the first time from natural sources, to which several chemical modifications have been performed to generate novel, selective, and high affinity P-gp inhibitors. Among these new natural products, several flavonoids, alkaloids, coumarins, cannabinoids, ginsenosides, terpenes, among others, have been identified and tested for P-gp inhibition, with very promising results (Coley 2010; Palmeira et al. 2012a).

Many commonly used pharmaceutical surfactants are also emerging as a different class of P-gp inhibitors, which by altering the integrity of membrane lipids, seem to cause modifications in P-gp secondary and tertiary structure, resulting in the loss of P-gp functionality function due to interruption in hydrophobic environment by the surfactant molecule (Hugger et al. 2002). Furthermore, surfactants such as Pluronic P85, Tween-20, Triton X-100 and Cremophor EL can inhibit P-gp-mediated efflux, with no appreciable effect on the transbilayer movement of drugs (Palmeira et al. 2012a), and thus capable of a transporter-specific interaction rather than unspecific membrane permeabilization (Regev et al. 2007).

Growing attention has also arisen in the development of multifunctional drugs, which have the ability to interact with multiple targets related to a specific pathological condition (Morphy and Rankovic 2009). For example, several aminated thioxanthones were reported as dual inhibitors of cell growth and P-gp (Palmeira et al. 2012b), setting a new

opportunity for MDR reversal. Additionally, other strategies have been adopted, such as the design of inhibitors of more than one transporter from the ABC superfamily (Palmeira et al. 2012a), as in some situations alternatively targeting multiple efflux transporters may possess a higher therapeutic efficacy than a specific drug (Roth et al. 2004).

2.2.5.3. P-glycoprotein induction

Since P-gp is one of the major drug efflux transporters expressed at the BBB, and with a wide range of substrates, a tight regulation of the expression of this transporter is crucial. The identification of the regulatory mechanisms of P-gp, how its expression can adapt to the changing environmental conditions, the elements that can trigger such adaptation, and the time and extent of such response are valuable, in order to be able to choose the best P-gp-modulating approach, as a new mechanism of enhancing the efficacy of drug therapy or to improve the clinical outcome of a disease progression state.

- *P-gp regulation in the periphery*

Disease processes, such as inflammation, can influence the expression of multiple hepatic ABC transporters, including P-gp (Ho and Piquette-Miller 2006; McRae et al. 2003). Due to its role in multidrug resistance in cancer, the signals implicated on P-gp regulation in tumor cells have been widely explored. For the human *ABCB1* gene, several promoter elements have been identified, such as GC-box, Y-box, p53, pregnane X receptor (PXR), inverse MED1, activator protein 1 (AP-1), NF-kB, and heat shock protein elements (Labialle et al. 2002; Scotto 2003). These promoter elements are binding sites for transcription factors that respond to environmental stimuli, such as oxidative stress, inflammation, hypoxia, xenobiotics and others. Furthermore, recent findings also suggest that P-gp is subject to microRNA (miRNA)-mediated gene regulation (Haenisch et al. 2014). MiRNAs, small noncoding RNAs, which regulate the expression of their target genes post-transcriptionally by RNA interference, can directly or indirectly interfere with P-gp expression, and are associated to both down-regulation (Feng et al. 2011; Zhu et al. 2013) and up-regulation (Zhao et al. 2010; Zhu et al. 2008) phenomena.

- *P-gp regulation in the brain*

Since the BBB represents an interface between the periphery and the CNS, it is exposed to normal and pathological signals from both the blood and the CNS, which may likely influence transporter expression and overall BBB function. In fact, currently, there is convincing evidence that P-gp expression at the BBB can be altered during disease, such

as in epilepsy and stroke, and susceptible to a number of physiological and pathological signals (Miller et al. 2008).

The expression of P-gp at the BBB has shown to be target of induction by many well-known drugs, including dexamethasone, rifampicin and the herbal antidepressant St John's wort (Bachmeier et al. 2011; Bauer et al. 2006; Narang et al. 2008; Zastre et al. 2009). Also, some studies have indicated that anti-epileptic drugs might also induce P-gp (Potschka 2010a). Treatment with carbamazepine or valproic acid was associated with higher BBB P-gp expression rates when compared to treatment with lamotrigine or topiramate (Wang-Tilz et al. 2006), although these differences might be related to varying effects on seizure control. Also, carbamazepine, phenobarbital and phenytoin were reported to induce P-gp and other transporters in rat BEC lines via an interaction with the PXR and the constitutive androstane receptor (CAR) (Lombardo et al. 2008).
Moreover, P-gp may not only be induced by several compounds, but also by other environmental factors, such as X-irradiation, UV-irradiation, cytokines, oxygen free radicals, tumor suppressor genes and heat shock (Chin et al. 1990b; Hu et al. 2000; Kioka et al. 1992b; Miyazaki et al. 1992; Ohga et al. 1998; Uchiumi et al. 1993; Wartenberg et al. 2005; Zastawny et al. 1993; Zhou 2008). Some of these inducers are summarized in *Table 5*.

Table 5. List of some known P-gp inducers at the BBB.
Adapted from (Chaves et al. 2014; Silva et al. 2014f)

P-glycoprotein Inducers	
Class	**Compounds**
Antibiotics and derivatives	Reduced rifampicin derivative (RedRif), rifampicin
Anti-retroviral drugs	Abacavir, amprenavir, atazanavir, daurunavir, efavirenz, lopinavir, nevirapine, ritonavir
Opioids	Morphine, oxycodone
Antiepileptic drugs	Carbamazepine, phenytoin, phenobarbital
Steroids	1α,25-dihydroxyvitamin D4, pregnenolone-16α-carbonitrile, retinoic acid
Others	Benzo[a]pyrene, venlafaxine, dexamethasone, hyperforin, insulin

2.2.5.4. P-glycoprotein activation: an emerging P-gp-modulator class

Very recently, a new class of compounds that interact with P-gp to increase its activity without increasing its protein expression has been denominated as P-gp activators (Silva

et al. 2014f; Sterz et al. 2009; Vilas-Boas et al. 2013b). A P-gp activator is a compound that binds to P-gp and induces a conformational change that stimulates the transport of a substrate bound on another binding site (Silva et al. 2014f), while a P-gp inducer acts by increasing the protein expression from which an associated increase in its activity is expected. This activation mechanism promotes P-gp transport function without interfering with protein expression levels, making it a more rapid process than P-gp induction (Silva et al. 2014f; Vilas-Boas et al. 2013b). Moreover, this is in line with the previous described functional P-gp model where, at least, two distinct sites for drug binding and transport are identified, which interact in a positively cooperative manner (Shapiro and Ling 1997b).

In fact, it has long been known that there are compounds that bind to P-gp and stimulate the transport of a substrate on another binding site. Hoechst-33342 and Rhodamine (Rho) 123 were shown to act by this cooperative mode of action (Shapiro and Ling 1998), and such was also suggested for prazosin and progesterone (Shapiro et al. 1999). Furthermore, a four-P-gp-binding site model was proposed, with three transport sites and a regulatory one, where the latter allows an allosteric change of conformation of the substrate-transport binding sites, altering its affinity, and thus increasing the rate of translocation (Martin et al. 2000). In line with this, it has long been suggested that the adaptation and survival mechanisms of living beings have allowed the binding of several xenobiotics at the same time to P-gp (Safa 1993; Safa 1998), increasing the transport of each other, not competing, but instead activating the transport cycle (Safa 2004).

The most recently proposed models of P-gp drug binding suggest a large, flexible drug-binding region, confirmed by the high-resolution crystal structure (Aller et al. 2009), containing a number of sub-sites where drugs can bind. The flexibility of the binding pocket would allow induced fit of multiple drugs via hydrophobic interactions, hydrogen bonding, and electrostatic interactions with residues lining the binding pocket. The number and strength of these interactions would dictate the affinity of drug binding to the protein.
In another study, several small molecules, first designed as inhibitors of the p53 protein, were able to stimulate both doxorubicin and Rho 123 efflux in a P-gp-dependent manner (Kondratov et al. 2001). However, this dramatic stimulation of P-gp-mediated efflux of doxorubicin and Rho 123 by the identified compounds was accompanied by suppression of P-gp-mediated efflux of other substrates, such as paclitaxel or Hoechst 33342, indicating that they act as modulators of P-gp substrate specificity. Consistently, these P-gp modulators altered the pattern of cross-resistance of P-gp-expressing cells to different P-gp substrates: an increase in resistance to doxorubicin, daunorubicin, and etoposide was accompanied by cell sensitization to vinca alkaloids, gramicidin D, and paclitaxel (Kondratov et al. 2001). The effect of these modulators seemed to depend at least

partially on the substrate-binding site postulated by Shapiro and Ling (Shapiro and Ling 1997b).

The P-gp modulating properties of 27 different imidazobenzothiazoles and imidazobenzimidazoles were also investigated (Sterz et al. 2009). Most of the compounds were able to stimulate P-gp-mediated efflux of daunorubicin and Rho 123 in a concentration-dependent manner, although some of the compounds also displayed weak inhibitory effects. These novel compounds seem to bind to the P-gp H site and activate the efflux of specific substrates of the R site in a positive cooperative manner, whereas binding of H-type substrates is competitively inhibited. This hypothesis was further confirmed by the observation that these modulators do not influence hydrolysis of ATP or its affinity towards P-gp (Sterz et al. 2009).

Additionally, a synthetic derivative of rifampicin (a reduced derivative, RedRif), which showed to modulate P-gp expression and activity in a rat BBB model (RBE4 cells), also increased P-gp activity at time points when no increase in protein content had occurred yet, thus acting as a P-gp activator (Vilas-Boas et al. 2013b). *In silico* studies with this molecule consistently confirmed the previously described activation mechanism (Vilas-Boas et al. 2013b). Furthermore, it was demonstrated that several thioxanthonic compounds were capable of rapidly and significantly increase P-gp activity, as assessed by the Rho 123 efflux assay performed in the presence of these thioxanthones (TXs) during a short 45 min efflux phase (Silva et al. 2014c). These results during such short cell exposure to TXs during the Rho 123 efflux phase cannot reflect a possible contribution of an increased P-gp protein expression, indicating the existence of a mechanism of P-gp activation. The registered increase in P-gp activity in the presence of TXs resulted in a significant protection against paraquat (PQ) cytotoxicity, an effect that was reversed upon incubation with a specific P-gp inhibitor (Silva et al. 2014c). Consequently, these thioxanthonic compounds may have protective effects against PQ intoxications, by favoring its P-gp-induced efflux. Similar results in terms of P-gp activation and protection against PQ cytotoxicity were obtained with dihydrolylated xanthones (Silva et al. 2014d).

Therefore, P-gp activators that exert various effects on the intracellular accumulation of distinct P-gp substrates are useful tools for investigating the interactions between multiple drug binding sites of this transport protein (Sterz et al. 2009).

2.2.6. P-glycoprotein polymorphisms: implications in drug therapy and disease

The human *ABCB1* gene, located on the long arm of chromosome 7, is particularly characterized to be polymorphic, as well being a target of several mutations (Zhou 2008). To present date, more than 50 single nucleotide polymorphisms (SNPs) are known to occur in the *ABCB1* gene (Sharom 2008; Sharom 2011). Some of these polymorphisms are known to change mRNA and protein expression, and P-gp function (Sharom 2008; Sharom 2011). Considering the important role of P-gp in the pharmacokinetics and bioavailability of many drugs, metabolites and xenobiotics, it is then predictable that P-gp polymorphisms can seriously contribute to the observed inter-individual variability to drug responses and vulnerability, and, consequently influence the outcome of drug treatment. This has raised a considerable interest in the study of P-gp polymorphisms in recent years.

SNPs can result in a change in the amino acid coding sequence (non-synonymous) or not (synonymous; silent) (Sharom 2008). The first polymorphism to be reported in the human *ABCB1* gene was the G2677T variant, a non-synonymous SNP that results in a change in the amino acid sequence, Ala893Ser (Ser893 polymorphism) (Marzolini et al. 2004).

Among the most common and frequently described variants identified to date, the functional C3435T (exon 26, synonymous), G2677T (exon 21, non-synonymous) and C1236T (exon 12, synonymous) SNPs have been associated with an altered expression and function of P-gp in human tissues (Hitzl et al. 2001; Hoffmeyer et al. 2000). Unfortunately the results remain controversial: some studies show a relationship between a drug pharmacokinetics and an *ABCB1* polymorphism, while others do not (Lee et al. 2010). Most of the work was conducted at the intestinal barrier, and thus there are few data on the possible correlation between *ABCB1* polymorphisms and activity of the transporter at the BBB. The synonymous C3435T polymorphism was reported to be linked with reduced P-gp mRNA expression in the duodenum and associated to increased oral absorption and higher plasmatic levels of digoxin (Hoffmeyer et al. 2000). This was later contradicted with subsequent studies with other P-gp substrates, such as tacrolimus, fexofenadine and cyclosporine A, which failed to confirm this association (Leschziner et al. 2007). Another study demonstrated that C3435T polymorphism resulted in a slightly different P-gp tertiary structure, which altered interaction with drugs and modulators, despite of having the same amino acid sequence (Kimchi-Sarfaty et al. 2007). Furthermore, it was demonstrated that reduced fexofenadine uptake could be associated with the G2677T SNP, suggesting that this variant has increased activity *in vivo* (Kim et al. 2001). Also, the non-synonymous mutations of G2677T/A/C, which result in the amino acid changes Ala893Ser, Ala893Thr, and Ala893Pro, resulted in changes in both

substrate specificity and ATPase kinetic properties, as evaluated with 41 different compounds (Sakurai et al. 2007). This suggests that the polymorphism at the amino acid 893, located within the second intra-cellular loop in the C-terminal half of P-gp, is potentially implicated in the alteration of drug disposition and therapeutic efficacy.

Recent research has focused on the association of protein expression level with haplotypes (a set of SNPs). The haplotype combination 3435C–2677G–1236T within the *ABCB1* gene was found to be associated with a more severe depressive symptomatology, related to a poor response to antidepressants, and this may be due a higher expression of P-gp or to more active protein forms (Kato et al. 2008). Similarly, it was shown that the attention-deficit/hyperactivity disorder improvement caused by treatment with guanfacine was less evident in children with the T/T *ABCB1* C3435T genotype, compared to either C/T or C/C genotypes (McCracken et al. 2010). Thus, a higher expression of P-gp would result in lower brain levels of CNS drugs that are P-gp substrates, and consequently in resistance to pharmacotherapy. In a recent study, the C3435T SNP, and the TT genotype (TT3435) linked to this SNP, were shown to be significantly more prevalent in Japanese patients with major depressive disorder than in the controls, suggesting that polymorphisms of *ABCB1* may result in susceptibility to this disorder (Fujii et al. 2012). In addition, it was reported a higher frequency of the 1236C–2677G haplotype among individuals with Parkinson's disease, where an altered P-gp expression might contribute to an accumulation of drugs and neurotoxins in harmful amounts (Westerlund et al. 2009). Therefore, this P-gp variant seems to be a susceptibility factor in Parkinson's disease, particularly in such cases where there is an exposure to known risk factors for the development of this disease, such as exposure to pesticides. Three studies showed an increased risk of developing Parkinson's disease after exposure to pesticides in individuals carrying certain *ABCB1* alleles (Drozdzik et al. 2003; Dutheil et al. 2010; Zschiedrich et al. 2009). However, the association between haplotype and P-gp expression need to be further explored, in line with other factors, such as medication, diet, inter-individual differences in drug metabolism and disease, which may render results interpretation more difficult.

Nevertheless, polymorphisms occurring in the *ABCB1* gene are associated with increased susceptibility to several human diseases, and such knowledge, allied with substantial further investigation in this field, may help in the adoption of the appropriate interventions to reduce the likelihood of a particular disease.

2.3. Breast Cancer Resistance Protein

2.3.1. BCRP tissue distribution and main physiological roles

Three independent research teams conducted the discovery of this ABC transporter, almost simultaneously. BCRP was cloned for the first time in the breast carcinoma human cell line MCF-7 (Doyle et al. 1998). Shortly after, a new ABC transporter was identified in the placenta, which was then called ABCP for “placenta-specific ABC transporter” (Allikmets et al. 1998). Finally, a mitoxantrone resistance gene (MXR, mitoxantrone resistance protein) was also described in a colon carcinoma cell line (Miyake et al. 1999). Once BCRP/ABCP/MXR identified genes were sequenced, they have been found to be identical and to correspond to the exact same gene. This gene was then classified in the ABC transporter G subfamily, and the ABCG2 name was assigned.

Such discovery arose the need to determine the location, expression and possible physiologic role of BCRP. Meanwhile, BCRP was reported to be expressed in many tissues at both gene and protein level. In humans, BCRP is expressed in the CNS, placenta, liver, adrenal gland, prostate, testis, endometrium, as well as in the small and large intestine, stomach, kidney, pancreas and lung (Fetsch et al. 2006; Maliepaard et al. 2001a). In these tissues, BCRP was noted to be present at the venous and capillary endothelium (Maliepaard et al. 2001a).

The tissue distribution of BCRP shows extensive overlap with that of P-gp, suggesting that both transporters should similarly confer protection from potentially harmful xenobiotics in various tissues (Schinkel and Jonker 2003). Indeed, similarly to P-gp, BCRP is also highly expressed in organs that determine absorption (small intestine), elimination (liver and kidney), and distribution (blood-brain and placental barriers), which increasingly recognizes its important role in drug disposition and tissue protection (Ni et al. 2010; Polgar et al. 2008; Robey et al. 2009). BCRP was identified to be present in hematopoietic stem cells of the human bone marrow (Scharenberg et al. 2002), where it is believed to exert a protective role against xenobiotics (Zhou et al. 2002). Importantly, BCRP expression was found to be strongly induced during lactation in the mammary gland of mice, cows and humans, and responsible for the secretion and concentration of clinically and toxicologically important substances such as the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and the chemotherapeutic agent topotecan into milk (Jonker et al. 2005). Also, BCRP is involved in the secretion of the B vitamin riboflavin, required for the metabolism of fats, into the milk, emphasizing its likely physiological role in lactation (van Herwaarden et al. 2007). In the placenta, BCRP expression was shown to play an important role in the protection of the developing fetus from possible toxins in the maternal space, as supported by studies carried out with

topotecan, nitrofurantoin and PhIP (Jonker et al. 2000; Myllynen et al. 2008; Zhang et al. 2007). Thus, the physiological roles of BCRP are likely to provide tissue protection against endogenous toxins or xenobiotics, and to regulate cellular homeostasis of physiologically important endogenous compounds (Ni et al. 2010).

The *ABCG2* gene is highly conserved and has been found in all sequenced vertebrates to date (Robey et al. 2009). In most species there is only a single gene present, with the exception of rodents, which contain one or more copies of a closely related gene, *Abcg3*, and fish, which have 3 or more *ABCG2* genes (Annilo et al. 2006; Robey et al. 2009).

2.3.2. BCRP expression and function in the BBB

P-gp and BCRP are the most extensively reported ABC transporters at the BBB. Shortly after BCRP discovery in human and rat brain capillaries, it became clear that this transporter also plays a crucial role in brain-to-blood efflux of xenobiotics, metabolites and endogenous compounds (Cooray et al. 2002; Hori et al. 2004). Several studies have used specific antibodies for BCRP to show that this transporter is abundant in vein and capillary endothelium cells of many normal tissues and organs, particularly those that express P-gp (Fetsch et al. 2006; Maliepaard et al. 2001a). BCRP was first identified at the BBB in 2003 (Eisenblatter et al. 2003), and is now a well-recognized BBB component. Bcrp was shown to be much more abundant in the luminal membrane of mice cerebral endothelial cells than in the total cortex (Cisternino et al. 2004), and the same holds for rats (Lee et al. 2007), pigs (Eisenblatter et al. 2003) and humans (Cooray et al. 2002).

The presence of BCRP was showed in human brain tissue, which is consistently expressed in lysates of human temporal cortex and hippocampus (Aronica et al. 2005). *ABCG2* gene transcripts were detected in several regions of the human brain (Dutheil et al. 2009). Expression and immunolocalization of BCRP was observed in human frozen brain sections, which strongly suggested that the fluorescent signals were limited to the microvessel endothelium (Cooray et al. 2002). Supporting evidence was obtained from human brain isolated microvessels, suggesting that BCRP is the major ABC transporter expressed at the BBB (Dauchy et al. 2008; Shawahna et al. 2011). The expression at mRNA and protein level and functional activity of BCRP have been confirmed in primary cultures of human brain endothelial cells (Lee et al. 2007), in the immortalized human brain endothelial cell line BB19 (Kusch-Poddar et al. 2005), and in hCMEC/D3 cells (Dauchy et al. 2009). Regarding the membrane localization within the endothelial cells, there is considerable proof that BCRP displays localization in the luminal side of the endothelium of the normal human brain (Aronica et al. 2005; Cooray et al. 2002; Maliepaard et al. 2001a). BCRP expression in the brain seems to be limited to the blood-

brain vessels, as no neuronal or glial BCRP expression was detected (Aronica et al. 2005). However, in a different study, the expression of BCRP was detected in both human brain microvessel endothelial cells and fetal human astrocytes, although in the latter in low levels (Zhang et al. 2003). A very limited expression of BCRP was demonstrated in the choroid plexus epithelial cells of the porcine and rat blood-cerebrospinal fluid, which may mean a similar but less significant function of this transporter in this brain barrier (Eisenblatter et al. 2003; Reichel et al. 2011). However, BCRP expression in the human BCSFB is currently unknown.

For a long time, it was believed that P-gp was the major ABC transporter at the human BBB. This belief was substantiated by the findings that the P-gp was 3-fold more expressed than Bcrp in isolated mouse brain capillaries (Kamiie et al. 2008). However, porcine and human brain tissues evidenced that the expression of BCRP seemed higher compared to other ABC transporters, such as P-gp and ABCCs (Aronica et al. 2005; Cooray et al. 2002; Eisenblatter et al. 2003; Zhang et al. 2003). In contrast to previous studies in rodents, the expression profiles of ABC transporter genes on human cerebral microvessels showed that *P-gp* was about 7-fold less expressed than *BCRP* (Dauchy et al. 2008). These data were recently confirmed by quantitative proteomic analysis of isolated human cerebral capillaries, where BCRP expression was shown to be 1.6 times superior than that of P-gp in human brain microvessels (Shawahna et al. 2011; Uchida et al. 2011). In fact, the proteomics of ABC transporters present in microvessels isolated from frozen human brain samples were recently compared to the protein amounts of transporter homologues in mice (Uchida et al. 2011). Interestingly, their results showed that the human BCRP was 2-fold more than that of the mouse Bcrp, whereas the human P-gp were almost 2-fold less than the mouse Abcb1a (Uchida et al. 2011). This is indicative of the substantial interspecies differences, underlying the need to keep in mind that the expression of different ABC transporters at the BBB is variable among different species. In fact, P-gp seems to be the more abundant of the two transporters at the BBB of smaller species, like rat and mouse, whereas BCRP is more abundant than P-gp at the BBB of larger species, such as monkey and human (Ball et al. 2013).

Mounting evidence suggests that BCRP helps in the restriction of brain penetration of substrate compounds. This was demonstrated by using P-gp knockout mice, in which Bcrp was inhibited by elacridar, resulting in enhanced brain uptake of prazosin and mitoxantrone (Cisternino et al. 2004). In addition, Bcrp (-/-) mice showed a 9.2-fold increase in brain penetration of genistein, a phytoestrogen, compared to wild-type mice (Enokizono et al. 2007). Another study using BCRP knockout mice showed an

augmentation of brain accumulation of the tyrosine kinase inhibitor imatinib up to 2.5-fold as compared to wild-type mice (Breedveld et al. 2005).

Interestingly, P-gp knockout mice had approximately 3 times more BCRP in the brain microvessels than wild-type mice, suggesting an up-regulation of this transporter to compensate the lack of P-gp in the BBB (Cisternino et al. 2004). It also suggests that BCRP cooperates with P-gp at the BBB to restrict the passage of their common substrates into the brain. Another study using *Mdr1a/b* and *Bcrp* knockout mice clearly shows how the two drug transporters act in concert to limit the brain penetration of topotecan (de Vries et al. 2007). Indeed, the deficiency or inhibition of one of the two transporters is not always accompanied by an increase of the brain penetration of a common substrate: in Bcrp-deficient mice (but in which P-gp is present), P-gp alone is sufficient to prevent the brain penetration of the substrate; Similarly, mice deficient in P-gp (but in which the Bcrp is present), Bcrp alone is sufficient to limit the brain entry of the substrate (Kawamura et al. 2010). These data clearly show that these two ABC transporters compensate each other and act together to reduce the *in vivo* brain penetration of their common substrates.

As with P-gp, studies have focused on the involvement of BCRP in the pathophysiology of neurodegenerative diseases. In what concerns Alzheimer's disease, studies show that BCRP is implicated in the transport of the Aβ peptide, in human brain endothelial cells used as *in vitro* model (Tai et al. 2009). This is supported by an *in vivo* study, where the intracerebral accumulation of the Aβ peptide was lower in wild-type mice compared to the *Abcg2* knockout mice (Xiong et al. 2009). These data suggest that the expression of BCRP is also an important factor contributing to the protection against the development of Alzheimer's disease.

2.3.3. BCRP structure and mechanisms of mechanisms of drug efflux

BCRP is an approximately 72 kDa protein composed of 665 amino acids. BCRP structure differs from the common ABC transporters, like P-gp, as it is a half-transporter composed of a single N-terminal NBD, followed by a linker region and a single C-terminal TMD containing 6 TM α-helices (see *Figure 16*) (Ni et al. 2010). Its second unique feature is that BCRP, being composed by one NBD and TMD, requires dimerization to express activity. It may work as homo-oligomer or hetero-oligomer held together by disulfide bonds (Cucullo et al. 2007; Ni et al. 2010), and even as a homodimer, since the amplification of *Abcg2* gene alone is sufficient to circumvent drug toxicity in drug-selected cell lines (Knutsen et al. 2000; Miyake et al. 1999).

The latest membrane topology of BCRP evidences that: Asp^{590}, which is the only N-linked glycosylation site in BCRP (Diop and Hrycyna 2005; Mohrmann et al. 2005) is located in the extracellular loop connecting TM5 and TM6; Cys^{603}, responsible for intermolecular disulfide bond formation is also in the extracellular loop; and Arg^{482}, which is crucial for substrate specificity and overall transport activity of BCRP, is located in TM3 near the cytosolic membrane interface. Cys^{592} and Cys^{608}, which are also in the extracellular loop, reportedly form intramolecular bonds and appear to have impact in the ability of the transporter to traffic and transport properly if mutated (Henriksen et al. 2005). Also, a GxxxG motif found in the BCRP protein structure, and shown to be involved in the dimerization of other membrane proteins (Russ and Engelman 2000), seems to be necessary for proper transport function, as the subsequent mutation of the glycines to leucines results in impaired function but not expression of BCRP (Polgar et al. 2004).

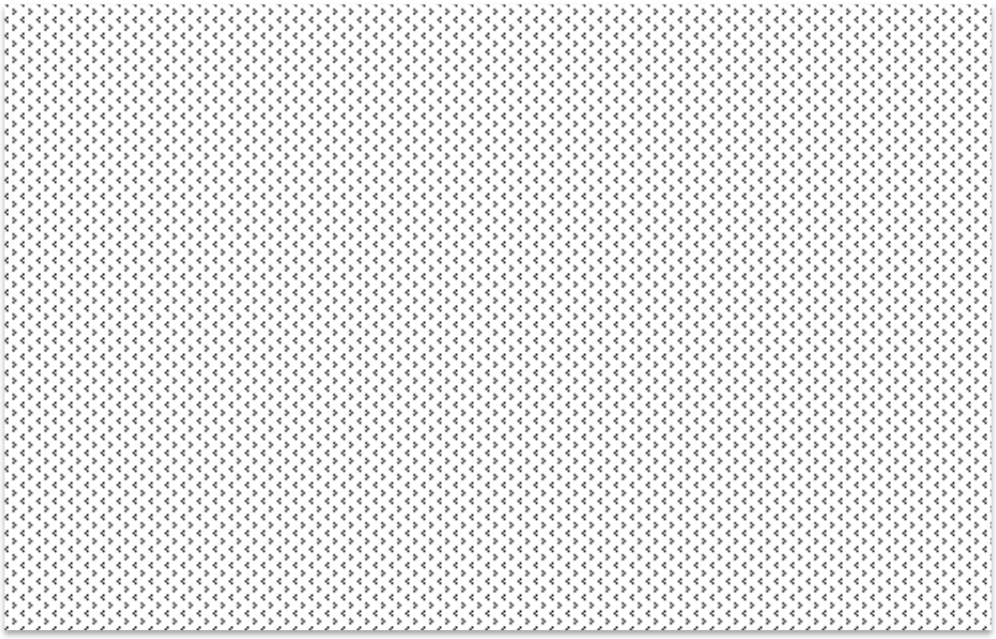

Figure 16. Proposed membrane topology of P-gp and BCRP (ABCG2) transporters.
It is noteworthy that BCRP is a half-transporter, composed by a single N-terminal NBD and a single C-terminal TMD containing 6 TM α-helices, while P-gp is composed by two NBDs (positioned on the C-terminal), and two TMDs. Image taken from (Sarkadi et al. 2004) (e.c. = extracellular, i.c. = intracellular)

Despite the fact that BCRP shows low protein sequence identity in NBDs (~20%) and essentially no protein sequence identity in TMDs with P-gp or even MRP1, transfection of cells with BCRP cDNA confirmed its ability to confer resistance to a variety of chemotherapeutic agents such as mitoxantrone and topotecan (Doyle et al. 1998; Miyake et al. 1999). Like P-gp, BCRP performs ATP hydrolysis-dependent efflux transport of a large number of structurally and chemically unrelated compounds that also include non-chemotherapy drugs and xenobiotics (Ni et al. 2010; Robey et al. 2009).

Three homology models of BCRP representing different conformational states have been generated. The first model, which used the MsbA structure as template (Ward et al. 2007), represents the substrate-unbound nucleotide-free inwardfacing open apo conformation. The second model, using the mouse P-gp structure as template (Aller et al. 2009), represents the substrate-bound nucleotide-free inward-facing closed apo conformation. The third model, which used the Sav1866 structure as template (Dawson and Locher 2006), represents the nucleotide-bound outward-facing conformation. These authors proposed that substrate binding to the open apo form promotes the closure of TMs, which in turn, sends a signal to the NBDs, allowing the formation of the ATP sandwich in the outward-facing conformation upon nucleotide binding (Ni et al. 2010) (see *Figure 17*).

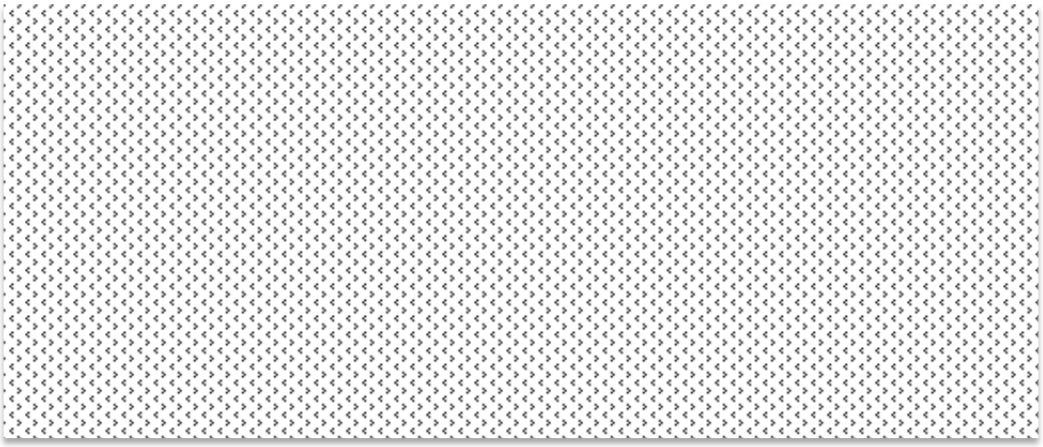

Figure 17. Representation of the homology models of BCRP.

A) Substrate-unbound nucleotide-free inward-facing open apo conformation, based on the MsbA structure; B) Substrate-bound nucleotide-free inward-facing closed apo conformation, based on the mouse P-gp structure; C) Nucleotide-bound outward-facing conformation, based on the Sav1866 structure. The two monomers of the BCRP dimer are represented in different colors. Image taken from (Ni et al. 2010)

The development of these BCRP homology models lead to the identification of several unique features (Ni et al. 2010):

I. The intracellular entry of the two inward-facing apo forms is large enough to allow access of a bulk of BCRP substrates from either the inner lipid leaflet of the plasma membrane or cytosol.

II. The extracellular loop 1 connecting TM1 and TM2 forms intermolecular contacts with the extracellular loop 3 between TM5 and TM6, thus possibly stabilizing the dimeric structure.

III. Comparing the open and closed inward-facing apo forms, there is a rotation of ~30° for TM4 and TM5 of BCRP, which forces TMs 1-3 and 6 to similarly change their positions, resulting in a more closed intracellular entry in the closed apo structure as compared with the open apo structure. Such a domain rearrangement would alter

the shape of the substrate-binding cavity which is formed primarily by residues in the TMs by making it more compact in the presence of substrate binding.

IV. In the outward-facing form of BCRP, there are even more drastic conformational changes, with the intracellular entry completely closed and the wide open V-shaped gap may allow the release of substrates from BCRP after ATP binding and hydrolysis. Once the intracellular entry in the outward-facing form is completely closed, the two NBDs of the BCRP dimer form the so-called ATP sandwich.

V. When comparing the membrane topology of P-gp and BCRP, the extracellular loop between TM5 and TM6 in BCRP is much larger than the corresponding region in P-gp (Rosenberg et al. 2010). Recent studies suggest that this extracellular loop may be critical in modulating substrate binding (Ozvegy-Laczka et al. 2008) as well as stability and ubiquitin-mediated degradation of BCRP (Wakabayashi et al. 2007).

Still, further insights into the structure of BCRP will be of help to better understand the function of this transporter, providing tools towards the design of more potent and specific inhibitors.

2.3.4. BCRP substrates and substrate-binding sites

2.3.4.1. BCRP substrates

A large number of chemotherapeutic and non-chemotherapeutic molecules have been shown to be substrates of BCRP, since its discovery as a drug efflux transporter contributing to the multidrug resistance phenotype of cancer cell lines. The BCRP substrate spectrum includes physiological compounds, common dietary xenobiotics, and several cancer and non-cancer drugs, a list of substrates that is progressively expanding. Particularly in the case of cancer medication, BCRP covers a spectrum of several structurally unrelated classes of anti-cancer agents, highlighting the importance of BCRP as multidrug resistance protein. Despite the broad range of BCRP substrates, several common characteristics can be found: 1) hydrophobic, lipophilic and hydrophilic compounds can be transported, but all of them contain hydrophobic regions, 2) the substrates usually range from 7-20 carbon atoms, 3) all substrates contain aromatic groups, 4) not all substrates have a planar structure but, all of them can assume a flat conformation, 5) most substrates contain charged groups, and both positively and negatively charged molecules are transported, 6) substrates have oxygen-containing, and often nitrogen-containing functional groups, and 7) most side functional groups can be donors and/or acceptors of hydrogen bonds (Szafraniec et al. 2014).

Table 6. List of some endogenous compounds and xenobiotics that are substrates of BCRP.
Adapted from (Chaves et al. 2014; Loscher and Potschka 2005a; Nies 2007; Qosa et al. 2015; Robey et al. 2011; Urquhart and Kim 2009) DHEA, dehydroepiandrosterone

ABC Transporter	Therapeutic Class	Compounds
BCRP	*Anti-cancer drugs*	Considerable overlap with P-gp; anthracylines, camptothecins, indolocarbazoles, mitoxantrone, methotrexate, imatinib, gefitinib, erlotinib
	HMG-CoA reductase inhibitors	Rosuvastatin, pitavastatin, cerivastatin
	Anti-retroviral drugs	Zidovudine, lamivudine, abacavir
	Antibiotics	Ciprofloxacin, ofloxacin, norfloxacin, erythromycin, rifampicin, nitrofurantoin
	Immunosuppressants:	Cyclosporine A, Tacrolimus, Sirolimus
	Others:	Sulfasalazine, cimetidine, riboflavin, dipyridamole, prazosin
	Endogenous compounds:	Estrone-3-sulfate, 17β-oestradiol, DHEA sulfate Glucoronide, glutathione and sulphate conjugates

Resistance to mitoxantrone is the hallmark of cells expressing BCRP, as well as resistance to camptothecin derivatives (9-aminocamptothecin, topotecan, irinotecan and SN-38, its active metabolite) (Doyle and Ross 2003; Maliepaard et al. 2001b; Yang et al. 1995). Other chemotherapeutic substrates have been shown to be transported by BCRP, like the TKIs imatinib, gefitinib and nilotinib (Brendel et al. 2007; Burger et al. 2004; Elkind et al. 2005), and indolocarbazoles and antifolates such as methotrexate (Chen et al. 2003). Several other substrate classes have been described, including antivirals (Pan et al. 2007a; Wang et al. 2003b; Wang et al. 2004), HMG-CoA reductase inhibitors (Hirano et al. 2005; Huang et al. 2006; Matsushima et al. 2005), carcinogens (Ebert et al. 2005; van Herwaarden et al. 2003; van Herwaarden et al. 2006) and antibiotics (Janvilisri et al. 2005; Merino et al. 2006; Merino et al. 2005). A summary of some of the cancer and non-cancer drugs transported by BCRP is given in *Table 6*. The expanding list of BCRP substrates highlights the fact that this transporter may play a significant role in brain pharmacology and protection.

Furthermore, BCRP shows considerable overlapping substrate and inhibitor specificity with P-gp and ABCCs (Szakacs et al. 2006). For example, BCRP substrates such as glyburide, imatinib, methotrexate, mitoxantrone, and prazosin are also P-gp substrates,

although some of them such as methotrexate are poor substrates of P-gp (Dohse et al. 2010; Ni et al. 2010; Schinkel and Jonker 2003). Furthermore, in addition to hydrophobic substrates such as mitoxantrone, BCRP can also transport hydrophilic conjugated organic anions, particularly the sulfated conjugates with high affinity, whereas P-gp generally transports hydrophobic compounds (Ni et al. 2010).

Thus, this cross substrate specificity between BCRP and P-gp results in a synergistic effect of the transporters in limiting drug penetration across the BBB, but also renders difficult the estimation of the clinical implications of BCRP.

2.3.4.2. BCRP substrate-binding sites

Docking studies on the previously discussed BCRP homology models for various BCRP substrates suggest the existence of multiple substrate binding sites in the central cavity primarily formed by TM α-helices (Cai et al. 2010). In fact, it was demonstrated that different substrates, such as topotecan and daurorubicin did not reciprocally inhibit the efflux of each other (Nakanishi et al. 2003), suggesting that there are multiple drug binding sites in BCRP that are not, or are only partially, overlapping. To evidence drug binding in BCRP, direct drug binding interactions with BCRP has been investigated using photo-affinity labeling (Alqawi et al. 2004; McDevitt et al. 2006; Shukla et al. 2006). Photo-affinity labeling of purified BCRP combined with trypsin digestion and mass spectrometry analysis of peptide fragments may provide valuable information for the drug-binding sites in BCRP (Ni et al. 2010). At present, the residues potentially involved in interactions of the photoactive substrates with BCRP have not been identified (Ni et al. 2010). Some other studies concordantly suggest that there are multiple drug binding sites in BCRP with doxorubicin, prazosin and daunomycin binding clustered in one region and Rho 123 and methotrexate binding in different regions (Clark et al. 2006; McDevitt et al. 2008; Pozza et al. 2006).

In the BCRP protein structure, Arg^{482} has been extensively analyzed by site-directed mutagenesis and was found to be crucial for substrate specificity and transport activity (Miwa et al. 2003; Ozvegy-Laczka et al. 2005; Robey et al. 2003). In the homology models, Arg^{482} in TM3 is located in the central cavity close to the cytosolic membrane interface with the side chain pointing towards the drug translocation pathway (Ni et al. 2010). Cells with a glycine or threonine instead of Arg^{482} were shown to readily transport doxorubicin and Rho 123 (Honjo et al. 2001; Lee et al. 1997; Rabindran et al. 1998). Also, docking studies indicated that mitoxantrone and Hoechst 33342, but not prazosin and SN-38, may directly interact with Arg^{482}, which is consistent with previous studies that show that a muation in this amino acid increases resistance to mitoxantrone but not to SN-38 or

prazosin (Miwa et al. 2003; Robey et al. 2003). Still, to date, it what really determines the substrate specificity of BCRP remains elusive.

2.3.5. Modulation of BCRP transport: BCRP inhibitors and inducers

2.3.5.1. BCRP inhibition

Since BCRP was discovered to be present in brain endothelial cells, the long-standing opinion that P-gp is the only important transporter responsible for drug efflux at the BBB changed considerably.

As with P-gp, BCRP has a considerable broad list of substrates, particularly a great number of chemotherapeutic drugs. Thus, it is expected that BCRP should also have a great impact on limiting brain drug delivery of such drugs, and consequently on treatment of brain disorders, namely brain tumors. However, BCRP expression at the BBB has not been unequivocally correlated to low brain penetration of all BCRP substrates. In fact, the brain penetration of several BCRP substrates, such as dehydroepiandrosterone (DHEA) sulfate, mitoxantrone, abacavir and zidovudine did not increase when using *Bcrp* knockout mice (Giri et al. 2008; Lee et al. 2005; Pan et al. 2007a). In contrast, and as mentioned before, it was promptly demonstrated that BCRP limits prazosin and mitoxantrone entry into the brain (Cisternino et al. 2004), as well as that the brain distribution of several drugs, such as sorafenib or imatinib, increased significantly in *Bcrp1* knockout mice (Agarwal et al. 2011b; Breedveld et al. 2005; Enokizono et al. 2008). The controversy regarding the role of BCRP at the BBB was somewhat clarified with the use of P-gp/Bcrp knockout mice. This provided the opportunity to study the combined impact of these two drug efflux transporters on the CNS drug delivery. Indeed, the brain uptake of topotecan, a substrate for both P-gp and BCRP, was not increased in mice lacking BCRP, was slightly increased by 1.5-fold in P-gp knockout mice, but in mice lacking both transporters was increased by more than 12-fold (de Vries et al. 2007). Similar findings were obtained with lapatinib, dasatinib, gefitinib and sorafenib (Agarwal et al. 2010; Agarwal et al. 2011b; Chen et al. 2009; Polli et al. 2009). These data show that, even though these drugs are substrates of both transporters, the absence of only one of the transporters did not significantly increase brain drug delivery, unless both transporters were absent or inhibited at the BBB. This suggests that inhibition of either P-gp or BCRP can be compensated by the other respective transporter, and that both transporters cooperate in the prevention of drug entry into the brain (Agarwal et al. 2011a).

Therefore, P-gp but also BCRP can significantly affect drug delivery to the brain, and thereby influence drug efficacy, as also their combined inhibition should be an attractive therapeutic strategy to improve delivery and efficacy of dual substrate drugs in the CNS.

Consequently, the use of combined inhibition for both P-gp and BCRP should have its greatest impact in the treatment of brain cancers, even though the overlap of P-gp and BCRP substrates is not limited to chemotherapeutic drugs.

2.3.5.2. BCRP inhibitors

In general, BCRP inhibitors can be classified according to their capacity to act specifically against BCRP, or with an overlap of activity against two transporters, usually P-gp and BCRP. When comparing specific P-gp and BCRP inhibitors, the former usually contains more nitrogen atoms and aromatic moieties (Szafraniec et al. 2014).

There are several natural and synthetic compounds that have shown the ability to inhibit BCRP activity. Fumitremorgin C (FTC), a tremorgenic mycotoxin, was one of the first BCRP inhibitors to be discovered (Rabindran et al. 2000). FTC probably acts as competitive inhibitor, due to its structure resemblance to mitoxantrone and doxorubicin. However, its clinical application *in vivo* is limited due to neurotoxic side effects. This lead to the development of its tetracyclic analogs, Ko132, Ko134 and, the most potent, Ko143, which also show high, and even more potent inhibitory activity against both human and mouse BCRP/Bcrp1 (Allen et al. 2002), while being less toxic and suitable for use *in vivo*. Several steroids, such as 17β-estradiol, vitamin K3 and plumbagin showed to specifically block the BCRP-mediated efflux of mitoxantrone (Imai et al. 2002b; Shukla et al. 2007). Additional classes of BCRP inhibitors include pyridines and dihydropyridines, such as nimodipene and nicardipene; flavonoids such as quercetin, gensitein, chrysin and tectochrysin; taxane derivatives, bisindolylmaleimides and indolcarbazole kinase inhibitors (Polgar et al. 2008; Robey et al. 2009).

Recent efforts have focused on TKIs such as imatinib, nilotinib, gefitinib, and erlotinib, which directly interact with BCRP at the substrate binding site and that block ATPase activity of the transporter, being both effective chemotherapeutics and potent BCRP inhibitors/substrates (Shukla et al. 2008). Even though some of these TKIs may show much higher influence on BCRP transport than on other ABC transporters, several studies showed that most of these TKIs, like erlotinib, gefitinib, lapatinib and sunitinib, are dual P-gp/BCRP substrates that competitively inhibit both transporters (Agarwal et al. 2011a). On the other hand, and given the cooperation of P-gp and BCRP at the BBB, this suggests their potential use in combination therapy to improve drug pharmacokinetics and increase drug brain delivery for the treatment of CNS diseases.

Elacridar has shown to be a dual P-gp/BCRP inhibitor, and it has been used in several pre-clinical studies to inhibit both transporters at the BBB with the purpose of enhancing brain distribution of simultaneously administered compounds (Cutler et al. 2006; Jin et al.

2011; Oostendorp et al. 2009). Indeed, similarly to what was demonstrated with double P-gp/Bcrp1 knockout mice, elacridar showed to considerably increase dasatinib, gefitinb, sorafenib, and topotecan brain uptake (Agarwal et al. 2010; Agarwal et al. 2011b; Chen et al. 2009; de Vries et al. 2007). Thus, elacridar, which displays low toxicity *in vivo*, seems to be a promising candidate to increase brain delivery of drugs that are dual P-gp/BCRP substrates.

Table 7. List of some inhibitors of BCRP.

Adapted from (Agarwal et al. 2011a; Chaves et al. 2014; Cutler et al. 2006; Jin et al. 2011; Ni et al. 2010; Oostendorp et al. 2009; Szafraniec et al. 2014). TKIs, Tyrosine Kinase Inhibitors.

	Specificity	Compounds
BCRP inhibitors	*BCRP-specific*	Fumitremorgin C and analogs (Ko132, Ko134), 17β-estradiol, vitamin K3, plumbagin
	Dual P-gp/BCRP inhibitor	Elacridar (GF12918), cyclosporine A, TKIs

2.3.5.3. BCRP induction

As with P-gp and other ABC transporters, the study of the regulatory mechanisms of BCRP is important to understand how the BBB responds to the changes in its environment, to identify the factors that urge a specific response, and the time and extent of such response. The identification of the regulatory mechanisms of this and other ABC transporters in health and pathological conditions can be of value in order to change disease progression or to enhance the efficacy of drug therapy.

Most studies focusing on the regulation of BCRP are interested in the transcriptional mechanisms that regulate the expression of this transporter.

- *BCRP regulation in the periphery*

In the promoter region of the *ABCG2* gene, response elements to hypoxia, estrogen, progesterone, xenobiotics, and to the transcription factor peroxisome proliferator-activated receptor (PPAR) γ have been identified (Robey et al. 2009; Tompkins et al. 2010). It was also proposed that Aryl hydrocarbon Receptor (AhR) up-regulates the expression of BCRP, and thus its regulation is associated with cellular defense, but the response element has not yet been identified in the promoter region (Robey et al. 2009). Treatment of Caco-2 cells with AhR agonists such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), benzo[a]pyrene, indolo[3,2-b]carbazole and benzo[k]fluoranthene resulted in increased BCRP mRNA and protein levels and a 180% increase of benzopyrene-3-sulfate efflux, a BCRP substrate (Ebert et al. 2005). Bcrp was reported to be up-regulated under hypoxic

conditions to reduce haem or porphyrin accumulation, thereby facilitating cell survival (Krishnamurthy et al. 2004). Likewise, Caco-2 cells continuously exposed to imatinib showed an up-regulation of BCRP expression, resulting in a decreased of imatinib accumulation into the cells (Burger et al. 2005).

Table 8. List of some BCRP inducers.

Adapted from (Robey et al. 2009; Xia et al. 2005) TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin

	Class	Compounds
BCRP inducers	*AhR agonists*	TCDD, benzo[a]pyrene, indolo[3,2-b]carbazole, benzo[k]fluoranthene
	Steroids	Progesterone, testosterone, 17β-estradiol
	Others	Dexamethasone, imatinib

- *BCRP regulation in the brain*

Given its more recent discovery, the control of BCRP expression in the brain or at the BBB has been less studied than for P-gp. Nevertheless, there are a few studies that demonstrated induction or repression of expression of BCRP at the BBB.

In rodents, exposure to dexamethasone, which acts at both PXR and GR nuclear receptors, has shown to increase *Bcrp* gene expression, protein expressions, as well as its functional activity in primary cultures of cerebral endothelial cells (Narang et al. 2008). On human cerebral endothelial cells, exposure to agonists of the nuclear receptor PPAR γ induced both genomic and protein expression of BCRP (Hoque et al. 2012). Also using human cerebral endothelial cells, exposure to pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) decreased the expression of BCRP and its transport activity (Poller et al. 2010). The role of sex hormones, such as testosterone, which showed to induce Bcrp1 expression in the male mouse liver, and oestradiol, which showed to suppress Bcrp expression in rat kidneys, in the regulation of BCRP expression at the BBB still remains controversial, as it seems to be cell-dependent (Imai et al. 2005; Wang et al. 2006; Yasuda et al. 2006). Still, the *ex vivo* incubation of mice brain microvessels with 17β-estradiol for 6 hours decreased BCRP protein expression and transport activity (Mahringer and Fricker 2010).

Besides transcriptional regulation, BCRP is also susceptible to epigenetic regulation: depending on the cell lines, the BCRP promoter may be methylated and/or acetylated. Methylation of the BCRP promoter region has shown to repress BCRP expression, while its acetylation may result in an increase of BCRP expression (Robey et al. 2009; To et al. 2006; Turner et al. 2006). In addition, as for P-gp, BCRP is also a target of miRNA-

mediated gene regulation (Haenisch et al. 2014). Several miRNAs, such as miR-200c, miR-328, miR-519c, and miR-520h are linked to a decrease in BCRP expression, in human melanoma cell lines and in the human breast cancer cell line MCF-7 (Li et al. 2011; Liu et al. 2012).

2.3.6. BCRP polymorphisms: implications in drug therapy and disease

The human *ABCG2* gene is located on chromosome 4, and extends over 66 kb containing 16 exons and 15 introns (Robey et al. 2009). It is highly polymorphic, given over 80 naturally occurring SNPs have been reported in the *ABCG*2 gene (Tamura et al. 2007), which may have significant physiological and pharmacological relevance. Some of these genes polymorphisms can results in a change of the protein expression and/or function. Of these, the non-synonymous Q141K SNP, where a lysine replaces a glycine, and which affects the codon 421 (421C>A), is the most extensively studied, and has been found to be associated with inter-individual variations in the pharmacokinetics, response or toxicity of drugs (Keskitalo et al. 2009a; Keskitalo et al. 2009b; Sissung et al. 2010). This SNP has also been associated to genetic diseases, such as gout (Woodward et al. 2009). In particular, the Q141K SNP has been linked to decreased plasma membrane expression of BCRP, decreased drug transport or reduced ATPase activity (Imai et al. 2002a; Mizuarai et al. 2004; Morisaki et al. 2005; Tamura et al. 2006). Indeed, human subjects carrying the Q141 variant often had higher plasma levels of BCRP substrate drugs than the subjects carrying wild-type BCRP (Sparreboom et al. 2004; Sparreboom et al. 2005).

Recently, research has developed interest in evaluating the clinical relevance of the Q141K SNP in patients undergoing chemotherapy. In these studies, elevated plasma concentration of gefitinib (Li et al. 2007), diflomotecan (Sparreboom et al. 2004), and increased bioavailability of oral topotecan (Sparreboom et al. 2005) were found. The Q141 mutation has also been associated with increased adverse effects in response to gefitinib treatment (Cusatis et al. 2006). A recent study has revealed that Q141K undergoes increased lysosomal and proteasomal degradations compared to wild-type BCRP, which probably explains the lower level of protein expression of the variant (Furukawa et al. 2009). This variant occurs in Japanese and Chinese populations at high allele frequencies (30-60%) and in Caucasians and African-American populations at relatively low allele frequencies (5-10%) (Lepper et al. 2005), which consequently leads to higher drug toxicity in some patient populations than in others.

As previously mentioned, BCRP is involved in Aβ transport at the BBB, as well as it was found to be significantly up-regulated in patients with Alzheimer's Disease (Xiong et al. 2009). In fact, the C421A polymorphism has shown to be associated with an increased

susceptibility to the disease, since the wild type ABCG2 C/C genotype has shown to have higher transcription activity, and thus increased expression of BCRP, in Alzheimer's Disease patients in comparison with the variant allele containing genotypes (CA and AA) (Feher et al. 2013). However, these results are contradictory, given the suggested protective role of BCRP in this neurodegenerative disorder. Considering the importance that BCRP at the BBB for the protection against unwanted substances including the Aβ peptides, it was expected that the variant A allele, with reduced transcription activity, should have shown better correlation with the development of this disorder.
Furthermore, it was recently reported that diplotypes of two linked polymorphisms in the *ABCG2* gene were associated with higher area under the curve (AUC) and C_{max} of erlotinib (Rudin et al. 2008), but this remains to be confirmed in a larger cohort of patients. Nevertheless, these data underline the importance of the analysis of haplotypes as well as of individual polymorphisms in the prediction of drug pharmacokinetics, efficacy and toxicity.

In conclusion, there is no doubt that P-gp and BCRP are the two main ABC transporters expressed at the human BBB, and the two main gatekeepers involved in the efflux transport of xenobiotics, endogenous molecules and metabolites. The high expression levels of these two drug efflux transporters has encouraged a deep exploration of their involvement at this brain-blood interface, and in particular, the signaling pathways involved in the regulation of their expression.

3. Transcriptional Regulation of ABC transporters: Focus on P-glycoprotein and BCRP induction mechanisms

Given the drawbacks of direct transport inhibition, research has also focused on elucidating the intracellular signaling pathways that control ABC transporters, namely P-gp and BCRP, at the BBB. While most currently used inhibition mechanisms occur mainly at posttranslational level, resulting in a decrease of the functional activity of the transporter, the induction phenomena occur mainly at the transcriptional level. In what concerns specifically to the P-gp gene expression, it may be up-regulated either by an increase in the amount of *ABCB1* mRNA through transcriptional regulation, or stabilization of the mRNA (Yague et al. 2003). Many studies have sought to identify the signaling pathways and regulatory networks that control the expression of ABC transporters, and several of them have been identified at the BBB, particularly for P-gp. ABC transporter expression and transport activity at the BBB can be altered by multiple factors, including disease, stress, diet, therapy and toxicant exposure (Miller 2010; Miller 2014). The up-regulation of ABC transporters at the BBB through the action of a number of ligand-activated receptors can lead to selective tightening of the barrier to both harmful neurotoxicants and therapeutic drugs, providing on one hand increased neuroprotection, but reduced drug delivery on the other hand (Miller 2010; Miller 2015). The full understanding of the molecular mechanisms underlying P-gp and BCRP regulation would allow the manipulation of their transcriptional activation to either increase or prevent it, and consequently reduce the intracellular accumulation of toxic xenobiotics and other compounds, or overcome the MDR phenomenon, respectively. Such an approach may have different advantages: 1) targeting transporter regulation allows subtler changes in transporter activity than direct transport inhibition, so transporters can be turned off for brief, controlled periods of time and, thus, reducing the risk of harmful toxicants entering the brain; 2) unlike direct inhibition, it can be used specifically to increase transporter expression and/or activity, and therefore increase barrier function for therapeutic purposes, and 3) knowing that BBB ABC efflux transporters are implicated in CNS disorders, targeting the signaling pathways that control these transporters could be a useful therapeutic strategy (Hartz and Bauer 2010).

Here we will focus on the main P-gp and BCRP regulatory pathways that may occur in the brain, and/or in the BBB.

According to recent research we can define two signaling patterns that alter ABC transporter expression and/or activity at the BBB: 1) brain capillary endothelial cells express multiple nuclear receptors that can be activated by endogenous metabolites, nutrients, and xenobiotics, driving to increases in expression of multiple ABC transporters; 2) upon ligand binding, certain receptors indirectly alter transporter expression through other downstream transcription factors (Miller 2015).

3.1. Ligand-activated Receptors

3.1.1. Direct action of ligand-activated nuclear receptors

At the BBB, multiple ligand-activated receptors function as sensors for metabolites, drugs and toxicants. These receptors contain multiple functional domains, including ligand and DNA binding domains, and often activation function domains. They classically affect gene expression through translocation of the receptor-ligand complex to the nucleus, where it binds to the promoter regions of target genes. The conjugation of the receptor with its partner protein within the nucleus leads to binding of the heterodimer to the DNA, facilitating the assembly of the transcription complex (Novac and Heinzel 2004; Olefsky 2001). Current data shows that several ligand-activated receptors are expressed in brain capillary endothelial cells, namely PXR, CAR, AhR, PPARα, vitamin D receptor (VDR), and glucocorticoid receptor (GR) (Miller 2014). Ligands for these receptors include a number of highly prescribed drugs, dietary constituents, and nutraceuticals (PXR, CAR, PPARα, VDR and GR) and persistent environmental contaminants (AhR) (Qosa et al. 2015). This broad substrate specificity is in accordance with the function of these nuclear receptors in the activation of proteins that are involved in the detoxification of xenobiotics from the body, such as metabolizing enzymes and efflux transporters. There is currently evidence that the *in vivo* exposure to receptor ligands for PXR (Bauer et al. 2006; Chan et al. 2013), CAR (Wang et al. 2010), AhR (Wang et al. 2011), VDR (Durk et al. 2012) and GR (Narang et al. 2008) is able to increase P-gp expression at the BBB, and reduce drug delivery to the brain.

Although the DNA-binding domain of PXR is highly conserved across species, the ligand-binding domain is not, and consequently substantial species differences in ligand affinities for rodent versus human PXR can be found. In isolated rat brain capillaries, 16α-carbonitrile (PCN), a specific rodent PXR ligand, was able to increase the P-gp protein expression and increase P-gp-mediated transport of a fluorescent cyclosporine A derivative (Bauer et al. 2004). Consistent with this finding, transgenic mice expressing human PXR, and treated with the human PXR activator rifampicin, showed an increase in

P-gp expression and transport activity in brain capillaries and a reduced central antinociception to the P-gp substrate methadone (Bauer et al. 2006). Isolated rat brain capillaries exposed to the CAR activators phenobarbital and [1,4-bis[2-(3,5-dichloro-pyridyloxy)] benzene] also demonstrated an increased transport activity and protein expression of both P-gp and BCRP, which was corroborated by *in vivo* studies (Wang et al. 2010). Concerning the AhR receptor, isolated brain capillaries from rats exposed to TCDD showed increased P-gp and BCRP transport activity and protein expression (Wang et al. 2011). In accordance with increased P-gp expression in capillaries from TCDD-dosed rats, *in situ* brain perfusion indicated significantly reduced brain accumulation of verapamil, a P-gp substrate (Wang et al. 2011). In opposition, a recent study conducted in in the hCMEC/D3 cell line demonstrated that exposure to TCDD had no effect on the protein expression or functional activity of P-gp nor BCRP (Jacob et al. 2015), which raises some controversy regarding the role of the AhR receptor in the regulation of P-gp and BCRP at the BBB. Treatment of primary rat brain microvascular endothelial cells with dexamethasone induced both P-gp and Bcrp expression and function, in a time- and dose-dependent manner, which was partially abolished by GR antagonist RU486 (Narang et al. 2008). One problem in assessing the mechanism of action of receptor ligands and receptors arises from the possibility that certain ligands could interact with more than one receptor (Miller 2015).

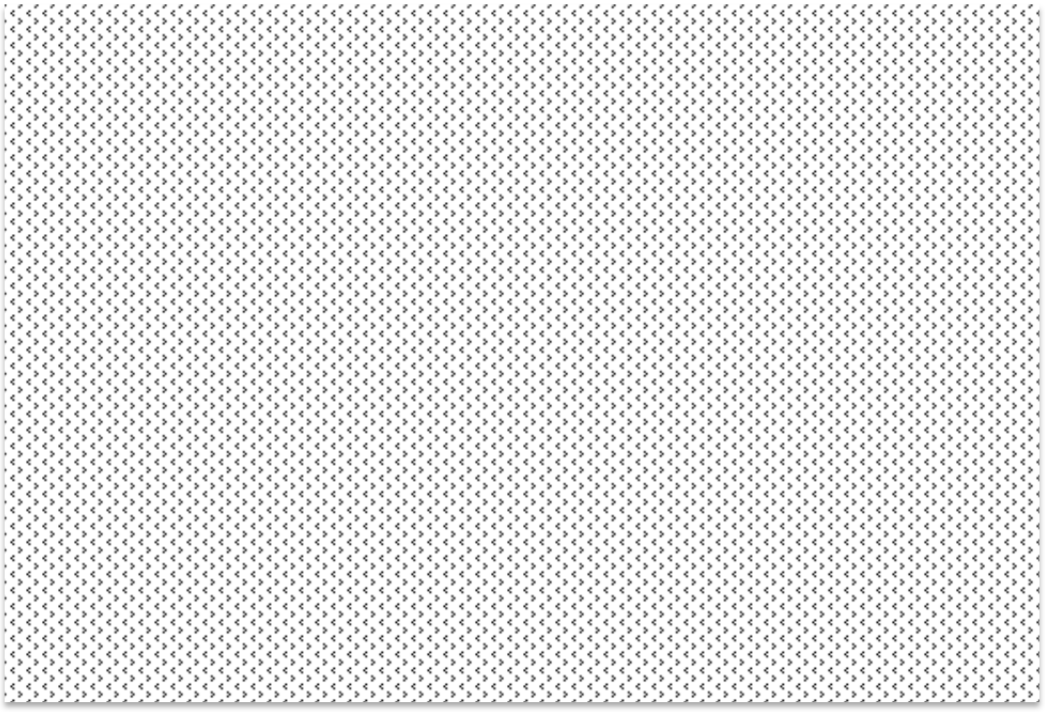

Figure 18. Regulation of ABC transporters present at the BBB by direct action of ligand-activated nuclear receptors.

Nuclear receptor activation by drugs, xenobiotics and endogenous compounds enhances the release of nuclear receptor from co-suppressors and translocation into the nucleus to stimulate ABC efflux transporter

expression. Short-term exposure to 17β-estradiol (E2 in the figure) reduces BCRP activity through activation of ERs, whereas long-term exposure to this molecule leads to BCRP degradation via ERβ and a PTEN/PI3-K/Akt/GSK3 pathway. Image taken from (Qosa et al. 2015).

3.1.1.1. Regulation of BCRP activity through estrogen signaling

Although most nuclear receptors activate the expression of P-gp and BCRP at the BBB, short-term exposure of male or female rat and mouse brain capillaries to estradiol rapidly and reversibly reduces BCRP transport activity without altering protein expression, in a mechanism involving both estrogen receptor (ER) ERα and ERβ (Hartz et al. 2010b). Several studies show that BCRP transporter protein can undergo protein trafficking away from the plasma membrane, with subsequent degradation, and even reduced transporter transcription in both mouse and rat brain capillaries when exposed to 17β-estradiol (Hartz et al. 2010a; Hartz et al. 2010b; Mahringer and Fricker 2010). In fact, rodent brain capillaries have shown to express both ERα and ERβ, but the ERβ expression dominates at both the mRNA and protein levels (Hartz et al. 2010a; Hartz et al. 2010b; Miller 2015). When rat and mouse brain capillaries are exposed short-term to subnanomolar concentrations of 17β-estradiol, BCRP-mediated transport is reduced, with no alteration on its protein expression (Hartz et al. 2010a; Hartz et al. 2010b), occurring through intracellular vesicle trafficking. When either ERα-null mice or ERβ-null mice are exposed to this compound, none shows reduced BCRP transport, suggesting that both ER are involved.

Extended exposure to 17β-estradiol for more than 6 hours decreases BCRP expression, which is mediated by ERβ but not ERα. In order to decrease BCRP expression, ERβ signals through phosphatase and tensin homolog (PTEN), phosphatidylinositide-3-kinase (PI3-K), protein kinase B (Akt), and glycogen synthase kinase-3β (GSK-3β) (Hartz et al. 2010a; Mahringer and Fricker 2010). Such signaling increases ubiquitination of BCRP protein, which leads to transporter protein degradation at the proteasome (Mahringer and Fricker 2010). These studies suggest two estrogen-based strategies for reducing basal BCRP activity at the BBB, with ERα-specific agonists rapidly and reversibly reducing transport activity, while ERβ-specific agonists initially reducing transport activity and later sending BCRP protein to the proteasome for degradation (Miller 2015).

Overall, these studies show that activation of most nuclear receptor at the BBB increases brain capillary localized ABC transporter and activity, resulting in barrier tightening. It is currently unknown whether changes in nuclear receptor–mediated transporter expression play a role at the BBB in humans, whether such changes could cause drug-drug side

effects in the brain capillaries in humans, or whether this approach could be used therapeutically (Hartz and Bauer 2010). Accumulating clinical data indicates, however, that nuclear receptors participate in the regulation of ABC transporters in the intestine, liver, and kidney. It is possible that this will also be the case at the human BBB. For example, transplant patients taking St. John's wort, whose main constituent is hyperforin – a potent human PXR activator, in combination with the immunosuppressant cyclosporine A see an increase of the expression of metabolizing enzymes and drug efflux transporters, leading to altered pharmacokinetics of co-administered drugs (Karliova et al. 2000; Moore et al. 2000).

3.1.2. Increased expression by receptor-driven signaling

Whereas direct ligand-activated nuclear receptor signaling is a relatively simple mechanism, the increase in ABC transporter expression can involve more complicated mechanisms through extended signaling from the plasma membrane or cytoplasm to transcription factors not directly activated by ligands. These transcription factors are often maintained in a cytoplasmic complex that facilitates poly-ubiquitination and guides the transcription factor to the proteasome for degradation. Upon activation, the transcription factor translocates to the nucleus, where it binds to specific sequences in target gene promoters. In fact, the transcription factor NF-κB appears to be such a factor, and a downstream point of convergence of stressed-induced signals (inflammation, epileptic seizures, oxidative stress) at the BBB, implicated in the regulation of ABC transporters (Miller 2015).

3.1.2.1. Wnt/β-catenin signaling

β-catenin is another modulator that has been noted to play a role in drug-induced alterations of the expression of ABC transporters at the BBB (Potschka 2010b). This protein acts as an adhesion molecule but also as a signaling factor in the Wnt-β-catenin pathway (McCarty 2009). The interaction of Wnt proteins with cell surface receptors leads to inactivation of GSK-3, and consequent cytosolic stabilization of β-catenin (Lim et al. 2008). Consequently, β-catenin accumulates and translocates into the nucleus of the cell, where it will be able to modulate the effect of transcription factors. There is evidence that this signaling pathway can enhance P-gp and BCRP expressions (Lim et al. 2008). In fact, lithium chloride is a CNS active drug that is known to inhibit GSK-3, and proven to up-regulate P-gp (Lim et al. 2008).

3.1.2.2. Inflammation

Brain inflammation is involved in several CNS disorders, such as stroke, multiple sclerosis, Alzheimer's and Parkinson's diseases (Hartz and Bauer 2010). The resulting release of pro-inflammatory cytokines is known to influence gene expression in the BBB, including changes in the expression of ABC transporters. In fact, the administration of lipopolysaccharide (LPS) has shown to trigger cytokine-induced inflammation and reduce P-gp function in the brain, in both rats and mice (Goralski et al. 2003; Salkeni et al. 2009). However, the signaling can be complex, depending on the nature, the dose, and the time of exposure to the stimulus (Roberts and Goralski 2008). One of the most important inflammatory mediators that participate in the regulation of ABC transporters is the pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α). In rat brain capillaries, a long-term (> 6h) TNF-α exposure activates TNF-R1 and induces release of big-endothelin-1 (ET-1), which is in turn converted to ET-1 by extracellular ET converting enzyme (ECE). ET-1 signals through the endothelin receptor B (ET_BR), which leads to the downstream activation of the inducible form of nitric oxide synthase (iNOS), protein kinase C isoform β2 (PKCβ2) and NF-κB, sequentially (see *Figure 19, B*) (Bauer et al. 2007; Rigor et al. 2010). On the other hand, short-term exposure of isolated rat brain capillaries (1h) to TNF-α decreased P-gp activity without affecting its expression, while expression and activity of BCRP was unchanged (see *Figure 19, A*) (Hartz et al. 2004; Hartz et al. 2006). Actually, TNF-α regulates both the induction and reduction of P-gp activity at the BBB, since signaling branches at the PKC isoforms: activating PKCβ1 initiates rapid loss of activity, with no change in expression, and activating PKCβ2 independently initiates a delayed increase in transporter expression (Hartz et al. 2006; Miller 2015). Thus, P-gp transport activity is reduced after short exposure to TNF-α but increased with longer exposure times, indicating a complex, time-dependent regulatory mechanism.

Thus far, little is known about the regulation of other ABC drug efflux transporters at the BBB during inflammation. However, a recent study demonstrates that TNF-α and IL-1β both decrease BCRP protein expression and function in brain capillary endothelial cells (von Wedel-Parlow et al. 2009), which was previously demonstrated with ET-1 treatment (Bauer et al. 2007). The suppression of BCRP by IL-1β, IL-6, and TNF-α was also demonstrated in the human hCMEC/D3 BBB cell model (Poller et al. 2010).

These studies are evidence that brain inflammation plays a role in the modulation of BBB ABC transporters in a complex, context-dependent way that will determine either the increase or decrease of the expression of these transporters.

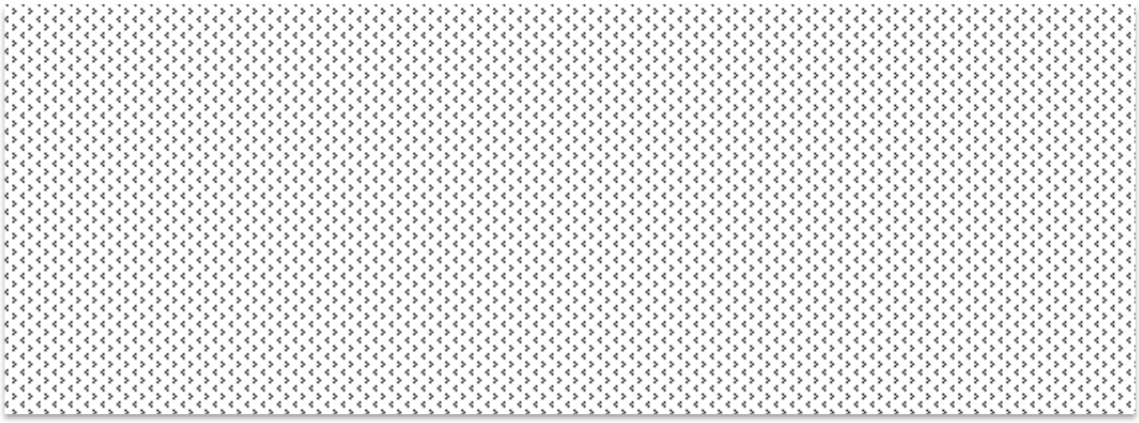

Figure 19. Regulation of ABC transporters present at the BBB by inflammation and oxidative stress.
A) Effect of a short-term and B) long-term exposure to TNF-α on the regulation of P-gp and the molecular mediators of each signaling pathway. C) Regulation of P-gp during oxidative stress through a signaling pathway that is triggered by ROS, such as H_2O_2. D) Regulation of P-gp through a signaling pathway that is triggered by diesel exhaust particles (DEP). Image taken from (Hartz and Bauer 2010)

3.1.2.3. Oxidative Stress/Ischemia

Brain inflammation is often accompanied by oxidative stress. Recent studies show that both microglia and brain capillary endothelial cells are able to produce reactive oxygen species (ROS) in response to cellular stress, and ultimately affect the expression of ABC transporters at the BBB (Hartz et al. 2008; Miller et al. 2008). Exposure of primary endothelial cells to H_2O_2 has shown to increase P-gp expression and function through extracellular signal-regulated kinase 1/2 (ERK 1/2), stress-activated protein kinase (SAPK), Akt and PKC (see *Figure 19, C*) (Felix and Barrand 2002; Nwaozuzu et al. 2003). Oxidative stress likely mediates P-gp induction through a receptor signaling mechanism, where the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) seems to be implicated (Copple 2012). It was recently found that rats dosed with sulforaphane, a compound that was known to increase the expression of multiple ABC transporters in the live, also increased P-gp and BCRP expression in brain capillaries, and reduced the brain delivery of a P-gp substrate (Wang et al. 2014). Experiments with rat and mouse brain capillaries showed that Nrf2 acts indirectly, and thus requires p53, p38 and NF-κB to increase P-gp and BCRP expressions (Wang et al. 2014). Although these factors work in concert to increase transporter expression, to date it is unknown how this happens.

In addition, oxidative stress induced by exposure to environmental toxins, such as diesel exhaust particles, seems to activate a signaling pathway involving NADPH oxidase and ROS production, which initiates TNF-α converting enzyme (TACE)-mediated TNF-α release. Here, TNF-α signals through TNF-R1, c-Jun N-terminal kinase (JNK), and nuclear translocation of another transcription factor, AP-1, resulting in increased P-gp

expression in isolated rat and mouse brain capillaries, *in vitro* (see *Figure 19, D*) (Hartz et al. 2008). This pathway and the pro-inflammatory pathway both involve signaling through TNF-R1, diverging at the level of the transcription factor, where in a pro-inflammatory context TNF-R1 signals through NF-κB and in an oxidative context it signals through AP-1 (Miller 2014). Diesel exhaust particles exposure also increases BCRP expression, but it is not clear whether the identified signaling pathway is responsible for this increase or if other pathways may be involved (Hartz and Bauer 2010). Altogether, these findings point to the important role that oxidative stress have in the regulation of the expression of ABC transporters, resulting in BBB tightening and reduction of drug penetration into the brain.

3.1.2.4. Signaling mechanisms for decreased P-gp activity

Two signaling pathways that rapidly and reversibly reduce P-gp transport activity, without change of protein expression, have been described, in both rat and mouse brain microvessels.

In isolated rat brain capillaries, vascular endothelial growth factor (VEGF) demonstrated to acutely and reversibly reduce P-gp transport activity, with no alteration of its protein expression or opening of TJs (Hawkins et al. 2010). VEGF seems to have such effect via activation of the VEGF receptor Flk-1 and Src kinase, considering the effect on P-gp activity was blocked when inhibitors of both the receptor and the kinase were used. Additionally, the Src-mediated phosphorylation of caveolin-1 may play a role in the down-regulation of P-gp activity (Hawkins et al. 2010). These findings were confirmed *in vivo* by *in situ* brain perfusion, since an intracerebroventricular (i.c.v.) injection of low doses of VEGF resulted in an increased brain distribution of P-gp substrates, such as morphine and verapamil, without any effect on the tight junction marker, sucrose, and blocked by the systemic administration of a Src kinase inhibitor, PP2 (Hawkins et al. 2010). However, VEGF has multiple effects on the BBB at higher doses, including TJ disruption, and thus rendering its clinical use in P-gp activity modulation unlikely (Miller 2015). Still, the identification of the downstream elements of VEGF signaling is important for the development of possible targeting strategies for acute modulation of P-gp activity and brain drug delivery improvement.

A second signaling pathway that has been described to decrease P-gp activity is a rather complex mechanism, and involves three signaling modules: pro-inflammatory, sphingolipid, and protein kinase (Miller 2015). Firstly, brain capillaries exposed to TNF-α release big-ET-1, which is later cleaved into active ET-1 by an extracellular ET converting enzyme and binds to the ET_BR, as it is described in inflammatory conditions (Hartz et al. 2006). The subsequent signaling leads to the activation of iNOS and PKCβ1. Secondly,

sphingosine is converted to sphingosine-1-phospate (S1P), which is transported out of the endothelial cells by MRP1, activating S1P receptor 1 (Cartwright et al. 2013), which then signals via PI3-K and Akt to activate mTOR, resulting in reduced P-gp activation (Miller 2015). The activation of this pathway can transiently reduce P-gp activity at the rodent BBB, and thus is a promising approach to improve drug delivery to the brain. Still, it remains to be demonstrated whether this same signaling pathway occurs in the human BBB, and consequently if it would be practical in a clinical setting (Miller 2015).

3.2. Epilepsy

Epilepsy is one of the most common neurological disorders and affects more than sixty million people worldwide (Kotsopoulos et al. 2005). It is characterized by the recurrent manifestation of seizures, which can cause brain damage, and despite the advances in drug development and the introduction of novel antiepileptic drugs in the past years, about 30-40% of the patients fail to respond to the treatment due to the development of pharmacoresistance (Loscher and Potschka 2005b), putting these patients in a life-threatening situation. Patients with drug-resistant epilepsy have a seven-fold higher mortality compared to the general population or epileptic patients who respond to pharmacotherapy (Sperling et al. 1999). In general, nonresponsive patients experience a low quality of life; despite advances in pharmacotherapy and neurosurgery, drug-resistant epilepsy remains a major clinical problem (Piazzini et al. 2007). In fact, the cause for anti-epileptic drug resistance in epilepsy is not yet fully understood, but several hypotheses for the underlying resistance are proposed. As already mentioned in a previous section, the expression of ABC transporters, such as P-gp and BCRP, seems to be increased in the brain of epileptic patients, and might contribute to anti-epileptic pharmacoresistance. The first time ABC transporters were suggested to contribute to anti-epileptic drug pharmacoresistance was in 1995, when a high expression of P-gp was detected in the capillary endothelial cells isolated from the brain tissue of refractory epileptic patients (Tishler et al. 1995). Since then, overexpression of P-gp, and other ABC efflux transporters such as BCRP have been confirmed either through *in vitro*, *in vivo* or clinical studies (Aronica et al. 2012; Potschka et al. 2004; Sisodiya et al. 2002). Furthermore, *in vivo* and *in vitro* studies showed that many anti-epileptic drugs are substrates of ABC transporters, namely of P-gp (Luna-Tortos et al. 2008). Consistent with these findings, the inhibition of these transporters increases the brain uptake and efficacy of several anti-epileptic drugs in animal seizure models (van Vliet et al. 2006). Prevention of seizure-induced transporter expression has also demonstrated to improve anti-epileptic drug

efficacy in drug-resistant epilepsy animal models (Schlichtiger et al. 2010). This body of evidence led to the conduction of numerous studies, over the past 10 years, to understand the regulatory mechanisms that stand behind the ABC transporter up-regulation at the BBB in epilepsy, and thus the development of pharmacoresistance to antiepileptic drugs. These studies were mainly focused on the mechanism driving the increases in P-gp expression. Recent *in vitro* and *in vivo* studies have suggested that overexpression of ABC transporters is complex and involves several elements of pathological alterations observed in epilepsy, such as inflammatory responses, oxidative stress, ligand-activated nuclear receptors and glutamate (Potschka 2010b).

Glutamate is an excitatory neurotransmitter that contributes to excitotoxicity in several neurological diseases, like epilepsy, where its excessive release from neurons and glial cells occurs (Bankstahl et al. 2008). Glutamate signaling via ionotropic, N-Methyl-D-aspartate (NMDA) receptors is major to the pathophysiology of such disorders (Barnes and Slevin 2003). In accordance with high glutamate levels in the epileptic brain, it has been shown that seizures induce overexpression of P-gp at the BBB in endothelial cells through glutamate by an NMDA receptor and cyclooxygenase-2 (COX-2)-dependent mechanism (Bauer et al. 2008). In fact, exposing isolated rodent brain capillaries to glutamate increased P-gp expression and transport activity, which were both abolished when blocking transcription or translation, substantiating that these effects depend on *de-novo* protein synthesis (Bauer et al. 2008). As with inflammation, NF-κB appears to drive the increase in P-gp expression, but it is the excess of glutamate release that starts the signaling, through its binding to NMDA receptor, in a seizure-provoked situation. This presumably increases Ca^{2+} flow into the cell and activates phospholipase A2 (cPLA2), which produces arachidonic acid. This metabolite is then converted to prostaglandin-E2 (PGE2) by COX-2, which is released to the extracellular space by efflux mediated by an MRP, likely MRP4 (Bauer et al. 2008). Extracellular PGE2 binds to a prostaglandin E receptor 1 (EP1R), which then signals NF-κB activation and increases P-gp expression (see *Figure 20*) (Bankstahl et al. 2008; Bauer et al. 2008; Pekcec et al. 2009; van Vliet et al. 2010; Zhu and Liu 2004). Consistent with this signaling pathway, seizure-induced P-gp up-regulation was blocked when rats were treated with celecoxib, a specific COX-2 inhibitor (van Vliet et al. 2010; Zibell et al. 2009), and with SC-51089 (Fischborn et al. 2010), a specific EP1R inhibitor. In line with this, COX-2 inhibition resulted in prevention of P-gp up-regulation and enhanced brain uptake of phenytoin in rats with recurrent seizures (van Vliet et al. 2010). Furthermore, seizure-induced P-gp expression in rats also showed to be NMDA receptor and COX-2 dependent, and the inhibition of these elements of the signaling pathway improved the efficacy of certain anti-epileptic drugs (Potschka and Luna-Munguia 2014). A recent publication shows that a similar signaling pathway (NMDA

receptor, COX-2) up-regulates P-gp activity in capillaries from the human brain (Avemary et al. 2013).

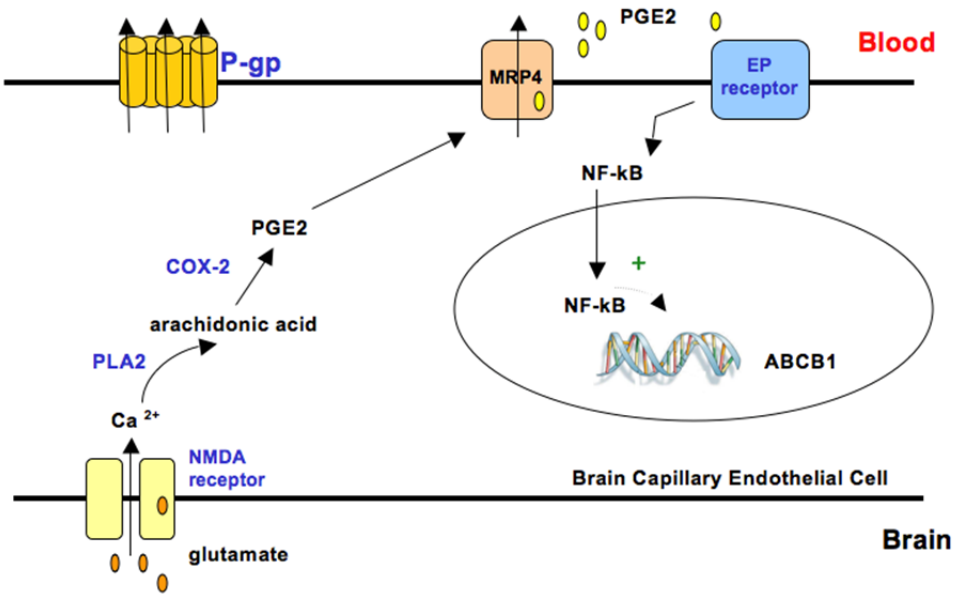

Figure 20. Glutamatergic transmission as a trigger of transcriptional regulation of P-glycoprotein. Excessive extracellular glutamate signals Ca^{2+} intake, via endothelial NMDA receptors with consequent release of arachidonic acid from the plasmatic membrane, which is converted to PGE2 by COX-2. PGE2 acts through the EP1 receptor to have an effect on P-gp expression via NF-κB activation. Adapted from (Potschka 2010b)

In contrast to the amount of data concerning the effect of glutamate on P-gp expression, its role on BCRP expression at the BBB is not clear. A recent report showed that glutamate exposure decreased BCRP expression and activity in isolated porcine capillaries, and provided evidence that NMDA receptor/COX-2 contribute to the down-regulation this transporter (Salvamoser et al. 2015). Similar results concerning BCRP down-regulation were obtained when using human capillaries isolated from surgical specimens of epileptic patients (Salvamoser et al. 2015).

4. Morphine and the Blood-Brain Barrier: Interplay with ABC transporters

"*…here was the secret of happiness, about which philosophers had disputed for so many ages, at once discovered; happiness might now be bought for a penny, and carried in the waistcoat-pocket; portable ecstasies might be had corked up in a pint-bottle; and peace of mind could be sent down by the mail*."

— Thomas de Quincey, *Confessions of an English Opium Eater*

4.1. Historical and general overview of Morphine

Opiates, and morphine in particular, have been used by humans for millennia, either for their euphoric and recreational properties, or for their analgesic effect, which has made them an important part of current medical care (Brownstein 1993).

Morphine is the principal active alkaloid, and the most abundant opiate found in opium, which is obtained from the seedpods of the opium poppy plant, *Papaver Somniferum* (see *Figure 22, A*). A first widespread use of opium was seen in the Ancient Era, since it was the most potent form of pain relief then available. Its widespread and worldwide recreational use began in the 15th century, as it became a powerful and valuable tool of international trade between major world empires, namely the Ottoman, Chinese and British Empires (Brownstein 1993). Morphine was extracted and isolated from the opium poppy plant for the first time in 1805, by Friedrich Sertürner, who named morphine after the god of sleep and dreams in Greek mythology, Morpheus (Brownstein 1993). Its commercial production began shortly after, in 1827 by the pharmaceutical company Merck.

Morphine is considered a leader molecule among opioids and endowed with powerful analgesic properties. Despite the development of several powerful synthetic opioids, morphine occupies a prominent place in both severe acute and chronic pain management, particularly cancer-related pain. Unfortunately, morphine and its opioid derivatives, associated with a long-term use, have the potential to cause substance dependence. Due to its severe and harmful consequences, opiates are on the top of the list of problem drugs that cause the most burdens of disease and drug-related deaths worldwide. According to the latest World Drug Report (2014) by the United Nations Office against Drugs and Crime (UNODC), it is estimated that between 28.6 and 38 million people

worldwide are opioid users, including heroin and prescription painkillers. It is also estimated that approximately 69 thousand people die from opioid overdose each year.

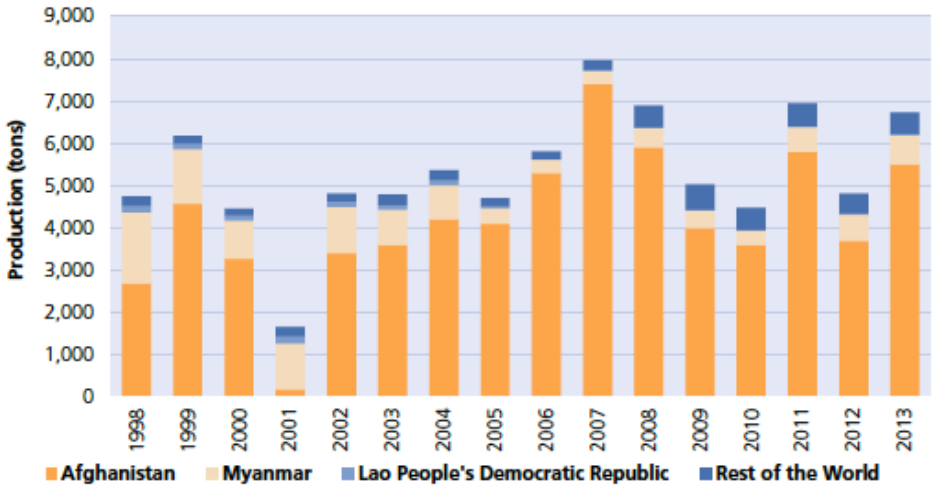

Figure 21. Global potential opium production since 1998 until 2013.

Data obtained from the World Drug Report (2014) produced by the United Nations Office on Drugs and Crime. The present graph shows that, since 2001, Afghanistan is the world leader of opium production and supply. The potential production of opium in 2013 is estimated at 6.883 tons, which is a return to the levels observed in 2011 and 2008. The opium production in Afghanistan accounts for 80 per cent of the global opium production (5.500 tons).

Morphine and codeine are the best-known opiate alkaloids, naturally occurring in opium, and their semi-synthetic derivatives, oxycodone and heroin, and full synthetic opioids, such as methadone, are also of valuable therapeutic use. The term *opiate* is refers to the natural opium alkaloids and their semi-synthetic derivatives, while the term *opioid* refers to the fully synthetic molecules. The endogenous opioid peptides, such as endorphins and enkephalins, have an opioid structural-related tyrosine terminal, as well as related morphine moieties, that account for their resembling biological effects of opioids.

Morphine is a phenanthrene pentacyclic alkaloid with a piperidine ring (see *Figure 22, B*). Its molecular formula is $C_{17}H_{19}NO_3$ and has a molecular weight of 285.35 g/mol. Since morphine is sparingly soluble in water, pharmaceutical companies produce mainly hydrochloride and sulfate salts for use in human medicine, both of which are about 300 times more water-soluble than the parent molecule (Mazak et al. 2009). Therefore, morphine sulphate, in particular, is presented as a white crystalline powder or, in the case of morphine hydrochloride, it is presented as colorless silky needles of bitter taste. It presents an amphoteric character, due to its basic tertiary amino and phenol functional groups. In the blood plasma, at pH 7.4, a substantial portion (7.9%) of morphine is in the noncharged form, and thus can most likely penetrate biological membranes, including the BBB (Mazak et al. 2009).

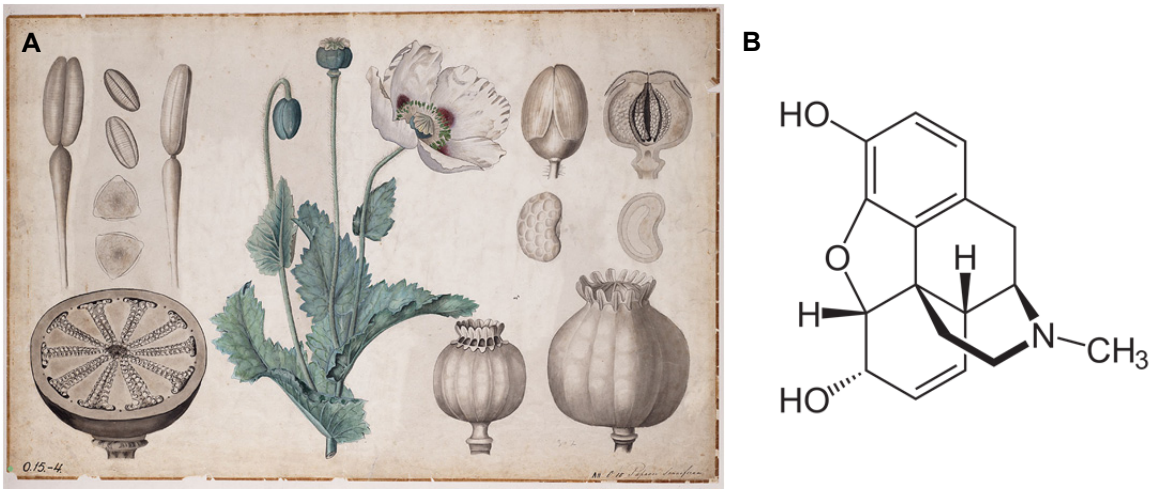

Figure 22. Opium poppy plant and its principal active alkaloid, morphine.
A) Opium poppy (*Papaver somniferum*) botanical drawing (University of Amsterdam, 20th century). B) Molecular structure of morphine, a phenanthrene pentacyclic molecule with a piperidine ring.

It is generally accepted that oxygen, sunlight, UV radiation and metal ions can catalyze the degradation of morphine in aqueous solutions (Mazak et al. 2009), and once it contains a phenolic hydroxyl group, its stability in aqueous solution is pH-dependent.

4.2. Pharmacokinetics and Pharmacological Considerations

4.2.1. Morphine Pharmacokinetics in the human body

Absorption. Morphine can be administered via several routes, including oral, intravenous (i.v.), subcutaneous (s.c.), intramuscular (i.m.), intrathecal and epidural. IV and subcutaneous continuous-infusion morphine are among the most common routes of administration, and are equally effective in producing analgesia. Its oral administration is not an especially effective pain-relieving drug when administered in a single dose, owing to its limited bioavailability (Brunk and Delle 1974). Conversely, the effectiveness of repeated doses seems to result from the presence of the enteropathic circulation, which allows a recirculation of morphine and its metabolites (Hanks et al. 1987).

Peak plasma levels are achieved within 15-20 min of intramuscular and subcutaneous administration, and within 30-90 min after oral. Peak levels after oral administration are much lower than after parenteral routes, since oral morphine undergoes extensive first-pass metabolism in the liver (Glare and Walsh 1991).

Distribution. After absorption, morphine is rapidly and widely distributed and crosses barrier interfaces, such as the placenta and the BBB. At therapeutic doses, plasma protein binding is only 20-35%, predominantly to albumin and to a lesser extent to α_1-acid

glycoprotein, and the volume of distribution is 1-6 L/kg (Glare and Walsh 1991). P-gp, which is expressed in the intestinal epithelial and at the BBB, as well as ABCCs are involved in brain efflux clearance of morphine (Tunblad et al. 2003; Xie et al. 1999).

Metabolism. As previously stated, in humans, the mean bioavailability after oral administration of morphine is low (20-30%) due to extensive hepatic first-pass metabolism (Hanks et al. 2001). Morphine administered via the oral route results in higher metabolite production compared with other routes of administration that avoid first-pass metabolism (Faura et al. 1998). Experimental studies carried out in animal show that morphine is glucuronidated in the liver and intestinal mucosa (Dahlstrom and Paalzow 1975). Morphine has three major metabolites: morphine-3-glucoronide (M3G) morphine-6-glucoronide (M6G), and normorphine (Glare and Walsh 1991; Labella et al. 1979; Osborne et al. 1986; Zaw-Tun and Bruera 1992). M3G is the main metabolite constituting 45-55% of morphine's metabolites, while 10-15% of morphine is converted into M6G (Christrup 1997). Uridine diphospho-glucuronosyltransferases (UGT) 2B7, involved in the formation of both metabolites but at different amounts (5 times more M3G than M6G), and UGT1A3, for the formation of M3G, are the major enzymes involved in the glucuronidation of morphine (Coffman et al. 1997). In addition to the liver, human brain homogenates have been shown to metabolize morphine at nanomolar concentrations to M3G and M6G (Yamada et al. 2003). Consequently, M6G can be formed directly in the CNS, and so it does not need to cross the BBB to exert a greater central effect than the M6G produced in the liver. Although analgesically inactive, M3G has been reported to mildly antagonize morphine and produce stimulatory effect, such as myoclonus, seizure and allodynia (Smith 2000). On the other hand, M6G has analgesic activity (Christrup 1997), and it greatly contributes to morphine analgesic effect. In fact, its higher potency and less marked respiratory depressive effects makes of M6G an attractive analgesic even when compared to the parent drug. However, its analgesic power following systemic administration is markedly lower than morphine, due to poor BBB permeability (De Gregori et al. 2012). Approximately 5% of morphine is N-demethylated into normorphine via CYP3A4 and CYP2C8 (Lalovic et al. 2006).

Excretion. Morphine half-life is relatively short, which on a repeated dosing reaches a steady state level in 10-12 hours, while the duration of its action stands for approximately 4 to 6 hours. Approximately 10% of a morphine dose is really excreted as unchanged morphine, while 45-55% is excreted as M3G, 10-15% as M6G, and 5% as minor metabolites (Christrup 1997). The elimination of morphine metabolites is mainly performed via urine (around 90% in 24 hours) in a combination of glomerular filtration and tubular secretion. Fecal elimination of these conjugates is relatively low (<10%) (Inturrisi 2002). Morphine clearance decreases in patients over 50 years old, which explains elderly

patients' higher sensitivity to the drug (Kaiko 1980). This clinical observation implies that younger patients may need larger doses to achieve the same analgesic effect (Ventafridda et al. 1989).

4.2.2. Therapeutic applications and Pharmacological Effects

Morphine and other opioid derivatives are generally indicated to treat moderate to severe pain, either acute or chronic, and are among the most effective drugs available for pain management (Kalso et al. 2004). Neuropathic pain is particularly challenging to treat, and manifests as spontaneous pain (continuous or intermittent) and abnormally evoked pain (usually by touch or movement), typically characterized by numbness, tingling, burning and other unpleasant sensations (Dworkin et al. 2003; Ossipov and Porreca 2005). The abnormal pain syndromes that are most commonly associated with neuropathic pain are tactile or mechanical allodynia, meaning that touch is abnormally perceived as painful, and hyperalgesia, where perception of noxious stimuli is exaggerated. Neuropathic pain can arise from direct nerve damage due to trauma or surgery, or can be associated with viral infection, cancer, diabetes or chemotherapy (Dworkin et al. 2003; Ossipov and Porreca 2005). In such cases, sensory neurons within the pain pathway (e.g., in the spinal cord dorsal horn) become hyperexcitable, signaling pain despite the absence of painful stimuli (Millan 1999; Woolf and Salter 2000). The effective analgesic dose of morphine and other opioids varies considerably among patients, which is dependent on pain severity, pain perception by the patient, among other factors.

However, the use of morphine is associated with a considerable number of side effects, which may be considered as desirable or undesirable. The development of some of these adverse effects markedly limits the use of opioids in pain management. The most common adverse effects are sedation, nausea and vomiting, constipation, and respiratory depression. Other adverse effects include confusion, hallucinations, nightmares, urinary retention, multifocal myoclonus, dizziness, and dysphoria (Bruera and Kim 2003). Here is a brief description of some of the most common adverse effects provoked by morphine:

- *Respiratory depression*: morphine acts on brainstem respiratory centers to produce a dose-dependent respiratory depression. At the same time, as CO_2 accumulates, it stimulates central chemoreceptors, resulting in a compensatory increase in respiratory rate that masks the degree of respiratory depression. Tolerance develops rapidly to this effect with repeated drug administration.
- *Euphoria*: morphine and other opioids can trigger feelings of euphoria and well-being, which contributes to its addictive potential.

- *Sedation*: morphine produces sedation and drowsiness, which may be useful in certain clinical situations, like preanesthesia, but undesirable in ambulatory patients. Like with respiratory depression, this is particularly characteristic in an early phase of the treatment with morphine, and it tends to disappear with the development of tolerance.
- *Nausea and vomiting*: such effects occur due to morphine's action on the medullary chemoreceptor trigger zone.
- *Constipation*: the most common adverse effect of morphine is constipation. It acts in multiple sites in the gastrointestinal tract and spinal cord to decrease intestinal secretions and peristalsis. Tolerance develops slowly to the smooth muscle effects of opioids, so constipation tends to persist when morphine is used for chronic pain.
- *Urinary retention*: As opioids increase smooth muscle tone, they can cause bladder spasm and an increase in sphincter tone, leading to urinary retention. This is most common in elderly patients.
- *Inhibition of immune responses*: morphine has shown to suppress a variety of immune responses that involve major cell types including natural killer cells, T cells, macrophages and polymorphonuclear leukocytes. This action is mediated by pharmacological activation of δ-receptors, which are present in immune cells (Lockwood et al. 1994), but possibly also through an *in vivo* neural-immune circuit through which morphine acts to depress the function of all cells in the immune system (Roy et al. 2011).
- *Tolerance and opioid-induced hyperalgesia*: tolerance develops in most patients receiving opioid analgesics chronically, and it is characterized by a reduced effect of a given dose of morphine, and so a larger dose is required to produce the original effect (McQuay 1999). Preclinical studies suggest that apparent opioid tolerance may result from excitatory CNS changes that facilitate transmission of pain and increase sensitivity to pain (Angst and Clark 2006). This appears to result from the up-regulation of pro-nociceptive systems and this condition is termed opioid-induced hyperalgesia.

In summary, opioid analgesics like morphine are widely used clinically for the management of acute to chronic pain, but their long-term use is compromised by the prevalence of undesirable side effects involving the CNS and gastrointestinal system. Furthermore, the prolonged use of morphine and related opioid drugs is linked to the development of analgesic tolerance, as well as with the development of physical dependence, which leads to severe withdrawal symptoms when opioid administration is suspended (Ballantyne and Mao 2003).

4.3. Mechanism of action

The pain-relieving properties of morphine have been extensively studied. The pharmacological properties of morphine are a result of its interaction with endogenous opioid receptors, which were reported to be existent in the human body in 1973 (Pert and Snyder 1973; Simon 1973; Terenius 1973). The possibility of the existence of multiple subtypes of the opioid receptor through purely pharmacological studies was later proposed (Martin et al. 1976). Because the opioid receptors are widely distributed throughout the entire nervous system, opioids may exert their antinociceptive activity through different mechanisms.

Opioid receptors are G-protein coupled receptors (GPCR), with seven TMDs, and are located in high levels in the brain and spinal cord (Ossipov et al. 2004). These receptors play a central role in basic physiological functions of the majority of known neurotransmitters and hormones, and can be activated by either endogenous or exogenous opioids, such as morphine. To date, four major opioid receptors families have been identified and cloned, and each has unique central pharmacological actions. The existence of genetic and physiological variations that influence the sensitivity of opioid receptors towards different opioid ligands, and the difference in their potency across various species and animal strains (Pasternak 2004), led to the proposal that receptors could be classified into subtypes:

- μ (mu, MOR), which has three subtypes, μ1, μ2 and μ3 (Wang et al. 1994). Agonists of μ-opioid receptor generally produce analgesia, euphoria, respiratory depression, and are largely responsible for the physical dependence associated with opioids (Kieffer 1999). Activation of the μ receptors also leads to bradycardia, hypothermia and locomotor hyperactivity (Gourlay 2005).
- κ (kappa, KOR), which has four subtypes, κ1a, κ1b, κ2 and κ3 (Mansson et al. 1994). κ-opioid agonists provide analgesia and show little dependence liability; still, they are poor therapeutic targets, as they produce intense dysphoric reactions (Hasebe et al. 2004). Other resultant effects of the activation of these receptors are miosis, sedation, and dysphoria (Gourlay 2005).
- δ (delta, DOR), with two subtypes, δ1 and δ2 (Kieffer et al. 1992). Agonists of the δ-opioid receptor also seem to be poor therapeutic targets, since their activation in rodents has shown to produce convulsions (Comer et al. 1993).
- Nociceptin or orphanin (NOP) receptor, or the receptor-like orphan receptor (ORL), which was later added to the list of opioid receptors (Mollereau et al. 1994) (Feng et al. 2012).

Central μ-opioid receptors are those that are most responsible for the analgesic effects of morphine and with less severe side effects, and thus remain the primary choice in the clinical setting. In fact, morphine has an excellent affinity for the μ-opioid receptors, which is much superior compared to the affinities to the other opioid receptors. The pharmacological activity of morphine is mostly correlated with the anatomical distribution of these receptors, and indeed, it was showed that morphine had no effects on mice whose gene encoding for the μ-opioid receptor was abolished (Kieffer 1999). Yet, in another study, morphine was able to induce analgesic activity via stimulation of the κ-opioid receptors, in *MOR* knockout mice (Yamada et al. 2006).

The distribution and anatomical localization of the opioid receptors have been extensively explored throughout the preceding decades. Morphine sites of action can be basically classified in spinal and supraspinal sites of action.

Opioid receptors, namely μ-opioid receptors, are concentrated in the outer laminae of the dorsal horns of the spinal cord, whereas δ-opioid receptors are diffusely distributed throughout the dorsal horn (Ossipov et al. 2004). The interaction of morphine with the opioids receptors at the spinal levels results in 1) closure of the calcium channels on the neuronal presynaptic terminal, resulting in a reduced release of neurotransmitters such as glutamate, acetylcholine, norepinephrine and serotonin, and 2) decreased activity of postsynaptic neurons due to an induced hyperpolarization by the opening of the potassium channels, which leads to a reduction in the nociceptive transmission (Goodsell 2005).

Descending inhibitory projections from supraspinal sites that are activated by opioid receptors also modulate nociceptive signals entering at the spinal cord level. Regions that were shown to express the opioid receptors include the frontal cortex, nucleus accumbens, hippocampus, thalamus, and hypothalamus (Ossipov et al. 2004). Two prominent regions in opioid-mediated antinociception, the periaqueductal gray (PAG) and the rostral ventromedial medulla (RVM) were identified as expressing opioid receptors. The PAG was found to be rich in μ-opioid receptors, whereas levels of δ- or κ-opioid receptors were low or undetectable (Mansour et al. 1995; Mansour et al. 1987). The RVM, which is defined as the region of the medulla including the nucleus raphe magnus, the nucleus gigantocellularis pars α, and surrounding reticular neurons, was found to express all three subtypes of the opioid receptors (Mansour et al. 1995; Mansour et al. 1987). Additionally, opioid receptors have also been identified in the locus coeruleus, which also plays a role in modulation of nociceptive inputs (Van Bockstaele et al. 1996). The action of morphine on the opioid receptors at the supraspinal level results in the activation of inhibitory bulbo-spinal pathways that project to the dorsal horn of the spinal cord, and are likely to reduce the level of transmission of the nociceptive message.

The efficacy of morphine is believed to be a result of a synergistic interaction between its activity at both spinal and supraspinal sites (Ossipov et al. 2004).

4.4. Morphine Tolerance, Addiction and Withdrawal Syndrome

4.4.1. Development of Tolerance to Morphine

Frequent or chronic opioid intake leads to the development of the tolerance phenomenon in the CNS. Opioid tolerance is characterized by a reduced responsiveness to an opioid agonist such as morphine, and it usually reflects the need to use increasing doses of the drug to achieve the desired effect. Clinically, more than 10-fold dose escalations of opioid dose in chronic pain management are common (Buntin-Mushock et al. 2005), and illicit opioid addicts can consume daily doses tens to hundreds of fold higher than acutely effective doses (Christie 2008). Tolerance is a result of adaptive mechanisms not only at the level of the drug target (in this case the opioid receptors and the μ-opioid receptor, in particular), but also at the cellular, synaptic and network levels, where adaptations due to homeostatic mechanisms tend to restore normal function despite the continued perturbations produced by opioid agonists (Christie 2008). In general, opioid intake induces adaptive changes in opioid receptors that may differ if following acute (desensitization and/or internalization phenomena) or chronic (adaptive tolerance and receptor down-regulation) opioid administration (Harrison et al. 1998; Mayer and Hollt 2006). Several intracellular molecules of signal transmission have been identified to be involved in opioid tolerance and dependence, including G proteins, cyclic AMP (cAMP), Mitogen-activated protein kinases (MAPK), and transcription factors, the latter responsible for the modification of gene expression, which may be responsible for the long-lasting neural plasticity induced by opioids (Christie 2008; Przewlocki 2004).

4.4.2. Opioid Addiction

Over the past 30 years, the clinical use of opioid has become widespread, with the development of different routes of administration to improve its safety, as well as through the development of extended-release, and immediate-release opioid formulations, which proved to be effective and with a leading role for the treatment of moderate to severe acute and chronic pain conditions. However, and despite its benefits and the widespread rational guidelines for their appropriate use, these opioid formulations are often inappropriately marketed and prescribed, which partially precipitates the current epidemic of recreational opioid use and addiction (Shurman et al. 2010).

Drug addiction, as in the case of morphine addiction, is a chronic relapsing disorder characterized by: 1) compulsive behavior to seek and take the drug, 2) loss of control in limiting intake, and 3) the emergence of a negative emotional state when the access to the drug is prevented (dependence) (Koob and Le Moal 2008). Drug addiction can be conceptualized as a disorder that progresses from impulsive drug use to uncontrollable, compulsive drug use in a cycle comprising three stages: 1) preoccupation/anticipation, 2) binge/intoxication, and 3) withdrawal/negative affect (Koob and Le Moal 1997). With the development of opioid addiction, a compulsive pattern of drug taking evolves, which is accompanied by the development of tolerance and its resulting escalation in drug intake to accomplish the original effect. The escalated drug use enhances the negative emotional symptoms of abstinence, which in turn results in profound dysphoria, irritability, sleep disturbances, anxiety, emotional pain, and craving. Craving often precedes somatic signs of withdrawal, and it relates with obtaining the drug and anticipation of its rewarding effects, as well as with anticipation of the aversive effects of withdrawal. Consequently, it is experienced as a need to obtain and take the drug to avoid the severe dysphoria, discomfort, and psychic stress experienced during withdrawal and abstinence (Shurman et al. 2010).

The mechanisms underlying neurological disorder caused by opioid addiction are not currently clear. The neuronal basis of positive reinforcement relies on activation of dopaminergic neurons, resulting in an increased dopamine release in the mesolimbic brain structures. Certain aspects of opioid dependence and withdrawal syndrome are also related to noradrenergic and serotonergic systems, as well as to both excitatory and inhibitory amino acid systems (Feng et al. 2012).

4.4.3. Opioid Withdrawal

As so far discussed, opioid addiction is a complex disorder that progresses with the setup of tolerance, drug-seeking, physical dependence and development of withdrawal syndrome. The severity of opioid dependence and the somatic symptoms triggered by withdrawal are major contributors to the compulsive drug-taking behavior, short-term relapse, and thus the addictive potential of opioids (Ouyang et al. 2012).

In humans, the typical opioid withdrawal syndrome is characterized by the manifestation of signs and symptoms like stomach cramps, diarrhea, rhinorrhea, sweating, elevated heart rate and increased blood pressure, irritability, dysphoria, hyperalgesia, and insomnia (Morgan and Christie 2011). After abrupt cessation of heroin or morphine use, the withdrawal syndrome develops within 24 hours and generally persists with declining severity for 1 week to 10 days (Morgan and Christie 2011). The withdrawal syndrome

contributes to opioid addiction presumably because repeated dosing is maintained or escalated to avoid the withdrawal symptoms, and thus leading to the development of more profound tolerance and dependence (Koob and Le Moal 1997; Morgan and Christie 2011). The described signs and symptoms found in human opioid withdrawal can be promptly replicated in animal models of compulsive drug use (Morgan and Christie 2011; Ouyang et al. 2012), and therefore are valid instruments for prediction of opioid dependence in humans. Opioid withdrawal signs are readily observed and quantified following the administration of opioid antagonists, such as naloxone (termed “naloxone-precipitated withdrawal”) or after abrupt cessation of treatment with relatively short-acting opioids (termed “spontaneous withdrawal”) (Morgan and Christie 2011). Opioid antagonist-precipitated withdrawal, through its peripheral administration, is the most common means of studying this phenomenon in animals. Upon opioid withdrawal in rats and mice, the manifested signs include “wet-dog” shakes, jumps, burrowing, hyperactivity, abnormal posture, teeth chatter, piloerection, ptosis, lacrimation, rhinorrhea, diarrhea, abrupt weight loss, and sweating (Laschka et al. 1976; Morgan and Christie 2011). As would be expected from the highly unpleasant nature of these symptoms, avoiding or relieving withdrawal is an important factor in continued drug use. In addition to its acute effect, learned associations between the withdrawal and environmental cues may be acquired upon opioid withdrawal (O'Brien 2008). In a phenomenon termed conditioned place aversion (CPA), animals will learn to spend less time in a previously neutral environment, which was later paired with aversive periods of an unconditional negative stimulus, such as naloxone-opioid withdrawal (Stinus et al. 2000). CPA can be produced with such low doses of naloxone that even physical withdrawal signs are not elicited (Gracy et al. 2001), which indicates that a negative affective state, rather than physical symptoms per se is an enough stimulus for learning (Schulteis et al. 1998). There is also evidence that withdrawal-associated stimuli can prime opioid self-administration in dependent animals, meaning that learned aversive cues may also impact drug taking behavior (Hellemans et al. 2006; Kenny et al. 2006).

Opioid withdrawal is believed to result from multiple adaptations within the CNS, whose cellular and molecular mechanisms are still not fully understood. The PAG, in particular, seems to be implicated in the expression of many signs and symptoms of opioid withdrawal (Ouyang et al. 2012). Opioid receptor-effector uncoupling has been implicated in physical dependence, but it does not fully account for withdrawal signs and symptoms or rebound responses in neurons after the administration of opioid receptor antagonists (Ingram et al. 1998; Ouyang et al. 2012). Currently, there is evidence that glial activation occurs over repeated morphine administration in several brain regions and spinal cord,

and that it should importantly account for the development of tolerance, dependence and precipitation of opioid withdrawal (DeLeo et al. 2004; Watkins et al. 2005; Watkins et al. 2007). In summary, chronic treatment with morphine has shown to activate glia in rodent models, evidenced by the up-regulation of glia-specific activation markers and release of pro-inflammatory cytokines, which may oppose the analgesic actions of opioids and contribute to opioid abuse liability and withdrawal symptoms upon cessation of opioid treatment (Song and Zhao 2001; Tawfik et al. 2005). Immunosuppressive treatments have shown to attenuate glial activation, and to reduce the precipitation of morphine dependence (Dougherty et al. 1986). Hence, treatments that reduce glial activation may be therapeutically useful to improve the efficacy of morphine, by reducing the development of tolerance, the abuse liability, and the potential withdrawal symptoms (Ledeboer et al. 2007).

4.4.3.1. Cellular, molecular and neurotransmission adaptations during opioid addiction and withdrawal. The case of the glutamatergic transmission.

Although the neurophysiology underlying these opioid-related phenomena is still not fully understood, recent progress has been made in the identification of molecular and cellular adaptations in opioid-sensitive neurons which can potentially lead to opioid tolerance, dependence and opioid-withdrawal behavior. It requires identification of adaptations that can drive cellular excitation or synaptic plasticity that are related to identifiable behavioral outputs including those involved in compulsive use of opioids.

During chronic opioid exposure, excessive stimulation or inhibition of a signal downstream the μ-opioid receptor can result in a homeostatic adjustment of the signaling system. Tolerance, in particular, arises when the downstream signal of the μ-opioid receptor activation directly controls neural excitability, as it is the case of cAMP in some nerve terminals (Ingram et al. 1998). Both the cAMP/PKA and MAPK signaling cascades seem to be of interest, as they are directly downstream the μ-opioid receptor activation. Removal of the inhibitory stimulus on the hypertrophied cAMP signaling during withdrawal can lead to an overshoot in downstream signaling mechanisms and neural excitation, and thus lead to the opioid withdrawal manifestations (Christie 2008). Other kinase cascades may be implicated in tolerance and withdrawal at cellular level, including PKC (Bailey et al. 2006) and other signaling systems (Bailey and Connor 2005; Johnson et al. 2005). Furthermore, mechanisms of cellular tolerance can also develop as a general cellular homeostatic response to depression of neural excitation by opioid agonists, such as altered TM chloride gradients, calcium homeostasis and synaptic structure and strength (Christie 2008; Rich and Wenner 2007).

A great number of studies has also focused on the early and transient transcriptional activation and altered gene expression during morphine withdrawal, such as of members of the Fos, Jun and Krox families (Couceyro and Douglass 1995; Erdtmann-Vourliotis et al. 2000; Georges et al. 2000), components involved in receptor-coupled signaling, neuropeptides, and other genes associated with metabolic function, cell protection and synaptic plasticity (Ammon-Treiber and Hollt 2005).

Several neurotransmitter systems, including the dopaminergic (Harris and Aston-Jones 1994), cholinergic (Bristow et al. 1997), noradrenergic (Maldonado 1997; Van Bockstaele et al. 2001), and glutamatergic (Siggins et al. 2003; Zhu and Barr 2001) have been also shown to play an important role in opioid withdrawal. In fact, there is evidence that glutamate and the NMDA receptor in particular play a well-established role in opioid tolerance, dependence and withdrawal (Mao 1999; Mayer et al. 1999; Trujillo 2000; Zhu and Barr 2001). Functional NMDA receptors are required for the full development and expression of dependence. The NMDA receptor is a tetrameric heteromer composed of the essential NMDA-NR1 subunit (NR1) in combination with NMDA-NR2 subunits (NR2), of which there are four subtypes (NR2A–D), to form a glutamate-responsive NMDA receptor (Glass 2010).
It has been shown that administration of NMDA receptor antagonists attenuates physical withdrawal symptoms in opioid-dependent mice (McLemore et al. 1997), guinea pigs (Tanganelli et al. 1991), rats (Trujillo and Akil 1991), and humans (Bisaga et al. 2001). Moreover, in rodent models, NMDA antagonists also suppress the above-mentioned CPA of naloxone-precipitated withdrawal (Glass 2010; Higgins et al. 1992).

On the other hand, chronic treatment with opioids and its subsequent withdrawal also influences the glutamatergic transmission. An elevation of the extracellular glutamate levels has been demonstrated in the locus coeruleus after a naloxone-precipitated withdrawal from morphine (Zhang et al. 1994b). Studies have demonstrated that either the naloxone-precipitated withdrawal or the natural morphine withdrawal resulted in a significant increase in the concentrations of glutamate and aspartate (Sepúlveda et al. 2004; Sepulveda et al. 1998).

NMDA receptor activation has shown to modulate numerous intracellular signaling processes including protein kinase activity (Haddad 2005), protein transport (Shi et al. 1999), transcription factor activity, and gene expression (Yoneda et al. 2001), as well as epigenetic events (Lubin et al. 2008). These properties of NMDA receptor activation may have profound effects on cellular and behavioral plasticity (Tsien 2000), such as long-term potentiation (LTP) and long-term depression (LTD) (Kullmann et al. 2000), in addition to whole-animal learning and memory (Shapiro and Eichenbaum 1999; Walker and Davis

2002), processes that play a role in opioid dependence. Furthermore, NMDA receptors and μ-opioid receptors are strategically co-positioned for postsynaptic modulation of glutamate signaling in several brain regions, such as the central nucleus amygdala (Glass 2010). In addition, there is evidence of an elevation in extracellular fluid levels of glutamate and, to a lesser extent, aspartate within the locus coeruleus during naltrexone- or naloxone-precipitated withdrawal in morphine-dependent rats (Aghajanian et al. 1994; Zhang et al. 1994a). Glutamate i.c.v. injection was also able to dose-dependently precipitate withdrawal signs in morphine-dependent rats, but not in non-dependent animals (Tokuyama et al. 1996), suggesting that a rapid release of glutamate may be a trigger or key factor for the expression of withdrawal signs from opioids.

4.5. Morphine and the BBB

4.5.1. Transport of morphine across the BBB – role of P-glycoprotein

In order to exert its pharmacological properties, morphine must cross the BBB to reach the CNS and activate the central opioid receptors. Although morphine is a lipophilic molecule, the BBB does not seem to be as permissive to its passage as expected.

Pharmacokinetic studies indicate that morphine reaches the brain through an active process (Bourasset and Scherrmann 2006). In addition, the co-administration of probenecid with morphine resulted in a 1.4-fold reduction in the brain-to-blood clearance of morphine in comparison to control animals, as well as it was seen an increase in the half-life of morphine in brain (Tunblad et al. 2003). Systemic clearance of morphine also decreased upon probenecid co-administration, suggesting morphine is a substrate for the probenecid-sensitive transporters at the BBB.

Present evidence suggests that the ABC transporters expressed at the BBB, and specifically P-gp, may contribute to the active efflux of morphine from the brain, and thus, to the development of central tolerance to opioids. The interaction of P-gp with natural and synthetic opioids was first reported in 1993 (Callaghan and Riordan 1993). Since then, P-gp mediated efflux of morphine at the BBB has been demonstrated using *in vitro* and *in vivo* approaches (Letrent et al. 1998; Letrent et al. 1999a; Letrent et al. 1999b; Mercer and Coop 2011; Schinkel et al. 1995; Xie et al. 1999; Zong and Pollack 2000). The first time morphine was identified to be a P-gp substrate was *in vitro* through the use of radiolabeled morphine across MDR cells (Callaghan and Riordan 1993; Huwyler et al. 1998). It was later confirmed to be a P-gp substrate *in vivo*, using P-gp knockout mice (Schinkel et al. 1995). Subsequent *in vivo* studies in rats showed that the inhibition of P-gp with elacridar resulted in significantly elevated antinociceptive effects following morphine administration (Letrent et al. 1998). Similar results concerning the increase of antinociceptive effects of morphine were also later confirmed in mice pre-treated with the P-gp inhibitor verapamil (Shimizu et al. 2004). A microdialysis study performed using knockout and wild-type mice in order to assess the role of P-gp in the transport of morphine across the BBB also corroborated these data, showing that morphine is transported across the BBB by P-gp (Xie et al. 1999). In fact, they showed that morphine brain/plasma concentration ratio was significantly higher in P-gp knockout mice in comparison to wild-type mice. In line with these findings, using the tail flick and hot plate analyses, P-gp knockout mice showed a greater morphine antinociception effect than the wild-type mice, with an ED_{50} more than 2-fold lower in knockout mice in comparison to wild-type mice (Thompson et al. 2000; Zong and Pollack 2000).

Further studies were later conducted to determine the extent to which morphine is transported out of the brain by P-gp. Morphine was found to be a weak P-gp substrate in Caco-2 (Crowe 2002), HEK293 (Tournier et al. 2010) and L-MDR1 (Wandel et al. 2002) cells, which showed a efflux:influx ratio of 1.5, which was later confirmed in *in situ* animal studies (Dagenais et al. 2004).

The assessment of morphine efflux transport at the BBB and its CNS effects has also been conducted in humans. A study conducted with healthy human volunteer subjects, pretreated with quinidine, a P-gp inhibitor, showed that when morphine was given intravenously they did not experience enhanced CNS opioid effects (Skarke et al. 2003), whereas when morphine was given orally, human volunteers showed sustained increased plasma concentrations (Kharasch et al. 2003). These results suggest that P-gp plays a role in orally administered morphine, probably by interfering with its intestinal disposition. However, a clinical study also conducted in humans demonstrated that P-gp inhibition by PSC-833 did not significantly alter the pharmacokinetic and pharmacodynamics profile of morphine (Drewe et al. 2000).

More recently, it was demonstrated that the variability of P-gp expression in the brain cortex of mice and the analgesic effect of morphine were correlated (Hamabe et al. 2007). Furthermore, this study demonstrated a negative correlation between the expression of P-gp or its ATPase activity and the antinociceptive effect of morphine, suggesting that the inter-individual variability of the morphine effect could be due to a difference in the expression and/or function of P-gp in the brain (Hamabe et al. 2007).

The development of morphine pharmacokinetic modeling also helped to study the influence of P-gp on morphine brain distribution. One of these studies showed that morphine brain distribution is essentially determined by three factors: 1) limited passive diffusion across the BBB, 2) active efflux at the BBB, which is reduced by 42% when P-gp is inhibited, and 3) low active uptake capacity (Groenendaal et al. 2007). Such study clearly demonstrated that P-gp modulates the distribution of morphine within the biophase and so helps delay its CNS effect (Groenendaal et al. 2007).

Moreover, inter-individual variability in the pain relief of patients with cancer has been associated with variants of the *ABCB1* gene that alter the expression and/or activity of P-gp (Campa et al. 2008; Lotsch et al. 2010). Not surprisingly, different studies suggest that the overexpression of P-gp at the BBB may contribute to the development of tolerance to morphine, by decreasing brain concentrations of morphine (Ambudkar et al. 2003; Mercer et al. 2007).

4.5.2. Morphine influence on the BBB

The fact that morphine may exert an influence on the expression of protein specifically expressed at the BBB is of great interest, given there is data that suggest a possible correlation between the BBB modulation and the development of tolerance to morphine.

Data concerning the influence of morphine on the BBB is still limited. However, some studies show that this compound is able to modify certain features of the BBB. Morphine has shown to affect BBB permeability.

A significant increase of the brain concentration of sodium fluorescein, used as a permeability marker, was shown following an acute s.c. or i.c.v. morphine administration in mice (Baba et al. 1988). This increased permeability was inhibited by the administration of naloxone, suggesting that the μ-opioid receptors are involved in the regulation of the permeability of the BBB. More recently, the effect of a morphine chronic treatment, daily administered at a dose of 10mg/kg administered via intraperitoneal (i.p.), for 12 days, on the BBB permeability to large molecule tracers, such as Evans blue albumin and radioiodine, in several rat brain regions was investigated (Sharma and Ali 2006). They showed that morphine caused no significant BBB opening on the 12th day of morphine dependence. On the other hand, spontaneous withdrawal of morphine on day 1 resulted in profound stress symptoms, which were much more intense on the second day of morphine withdrawal. During morphine withdrawal, a marked increase in the BBB permeability to these protein tracers was seen in several regions of the brain, and this was most pronounced on the second day of morphine withdrawal (Sharma and Ali 2006). These findings suggested that morphine, its associated withdrawal syndrome, and subsequently provoked stress are capable to modify BBB function.

Another study demonstrated that an acute morphine treatment (12 mg/kg i.p.) did not alter blood-to-brain transport of IL-1α, IL-2 or TNF-α, whereas both spontaneous and precipitated (injection of 1mg/kg of naltrexone) morphine withdrawal following a chronic morphine treatment had increased blood-to-brain transport of IL-2, with no change in blood-to-brain transport of IL-1α or TNF-α (Lynch and Banks 2008). The permeability of the BBB to IL-2 is usually dominated by brain-to-blood efflux, with only limited blood-to-brain transport. With this study it was found that chronic morphine and withdrawal from morphine did not alter brain-to-blood efflux, but induced a novel saturable blood-to-brain transport system (Lynch and Banks 2008).

Treatment of a primary culture of human brain microvessel endothelial cells with both morphine and the HIV-1 viral protein tat showed to induce the release of pro-inflammatory cytokines, which could trigger common intracellular pathways to regulate TJ protein expression, and, consequently, the structural and the functional integrity of the BBB

(Mahajan et al. 2008). Treatment of these cells with a combination of morphine and tat resulted in a significant decrease in ZO-1 and occludin gene expression in comparison to untreated control cells. Morphine alone and in combination with tat also increased JAM-2 gene expression.

The research group supporting the research of the present dissertation has also studied the effect of a chronic, 5-day protocol treatment with morphine in rats, a protocol that has previously demonstrated to induce opioid dependence (Noble et al. 2000), on the expression of several genes encoding for proteins critically involved in the maintenance of BBB properties, such as junction proteins, influx and efflux transporters (Yousif et al. 2008). The expression of occludin registered a slight increase (1.23-fold induction) in isolated brain microvessels of morphine-treated rats in comparison to control rats (Yousif et al. 2008). However, the induction of this gene did not seem to have an effect on BBB tightness, as morphine-treated and saline-treated animals did not register any difference in terms of the brain accumulation of the radiolabeled sucrose, which was *in situ* brain perfused and used as a marker of BBB integrity (Yousif et al. 2008). These findings seem to be in agreement with the previous conducted study by Sharma and Ali (Sharma and Ali 2006).

4.5.2.1. Focus on the role of Morphine on the expression of ABC transporters

Morphine is used in the control of pain in patients with cancer. Therefore, it is often co-administered with several anticancer agents, and the majority of these agents are substrates for P-gp. The modulation of this ABC transporter at the BBB by the administration of morphine can modify the efflux, the distribution and efficacy of these chemotherapeutic agents and, thus, cancer therapy.

There are a few studies that evidenced that morphine is also able to influence the expression and activity of the transport systems at the BBB, namely that of the ABC transporters. In 2000, a chronic administration of morphine in rats resulted in decreased antinociceptive response, which was accompanied by a 2-fold increase in P-gp protein expression in whole brain homogenates, determined by Western Blot (WB) (Aquilante et al. 2000). Rats were subjected to a 5-day treatment with morphine, where they received morphine at 10mg/kg in the first 2 days, and then 20mg/kg in the last 3 days of treatment, which resulted in a decrease of the antinociceptive effect of morphine. It was hypothesized that the registered P-gp up-regulation was an important cause for the enhanced morphine efflux from the brain, and, thus, reducing its pharmacological activity and contributing to the development of morphine tolerance (Aquilante et al. 2000). A similar study was later conducted in 2003 by Zong and Pollack, who demonstrated that

mice treated with morphine for 3 days showed an increase in the P-gp expression of about 1.4-fold in comparison to control mice (Zong and Pollack 2003).

In 2004, a study explored the effect of short-term (72 h) morphine exposure on the P-gp expression of several human cancer cell lines and of the mouse fibroblast NIH-3T3 cell line, as well as its effects on cellular resistance to the known P-gp substrates, vinblastine and colchicine (Pajic et al. 2004). This study has shown that the P-gp regulation may be cell-type dependent, as the morphine treatment induced P-gp expression in the mouse fibroblast cell line, but not in any of the human cancer cell lines.

ABCB1 gene expression was also reported to increase in human brain microvessel endothelial cells when exposed to either morphine (100 nM) or the HIV-1 protein tat, alone and in combination (Mahajan et al. 2008).

The previously mentioned study, conducted by the research group supporting the research of the present dissertation, demonstrated that a chronic treatment with morphine for 5 days was able to induce the gene expression of several ABC efflux transporters, such as *Abcb1a*, *Abcg2*, and *Abcc1*, in rat brain microvessels (Yousif et al. 2008). However, no change was registered following this protocol on the P-gp protein expression or functional activity, assessed by WB and *in situ* brain perfusion, respectively. Still, chronic treatment with morphine showed to induce the expression of P-gp in two brain regions, the brain cortex and hippocampus, as evidenced at both mRNA level by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), and protein level by *in situ* hybridization (Yousif et al. 2008). These findings are in agreement with the earlier results presented by Aquilante and her group (Aquilante et al. 2000). One should note, these results were obtained from morphine-treated rats that were sacrificed 12h after the last dose of morphine. Different studies have demonstrated that morphine withdrawal symptoms start to appear as early as 12h, and evolve for the following 24-72h after the last dose of morphine (Desjardins et al. 2008; Sharma et al. 2006). Therefore, such results on the modulation of P-gp and other BBB markers may be not only linked to the chronic morphine treatment applied, but can also be a result of the developing morphine withdrawal syndrome.

PART II

II. OBJECTIVES

P-gp and BCRP are the main drug efflux transporters present at the rat and human BBB, having the most significant expression levels among all ABC transporters at the BBB, and also exhibiting the broadest list of substrates known at present time. Secondly, P-gp and BCRP share a considerable number of substrates, and so work in concert in the efflux of various drugs, neurotoxicants and xenobiotics out of the brain, cooperating in the task of brain protection and maintenance of CNS homeostasis. Therefore, a change on the expression of these two ABC transporters can have a dramatic impact on the brain levels of several compounds, and alter CNS vulnerability. The discovery of the exact mechanisms by which P-gp and BCRP are regulated enables the development of the best strategies 1) to either prevent disease-associated up-regulation and/or to down-regulate these efflux transporters, and thus improve the clinical prognosis and brain drug delivery and treatment efficacy of CNS disorders, or 2) to increase P-gp and BCRP activities as a mechanism of enhanced brain protection and antidote against harmful P-gp/BCRP substrates.

Since morphine, as previously demonstrated, is be capable of inducing P-gp expression at the rat BBB, the first main objective of this dissertation is to evaluate if **morphine exposure in the rat is also able to influence Bcrp expression at the BBB**, as well as to **study the mechanism behind morphine-induced P-gp and Bcrp up-regulation**. For this purpose, the following specific objectives were set in order to accomplish the present aim:

- To evaluate at what **time and extent the expression of P-gp and Bcrp in the rat BBB is altered following a subchronic morphine treatment**.
- To explore the **regulatory signaling mechanism underlying morphine treatment effects on the levels of P-gp and Bcrp at the rat BBB**. For the matter, we focused on one of the mechanisms of P-gp transcriptional up-regulation described in the literature, namely the glutamatergic pathway, which is thought to be involved in the overexpression of P-gp in epilepsy.
- To evaluate the **role of the opioid withdrawal syndrome developed following a subchronic morphine treatment on the modulation of P-gp and Bcrp levels at the rat BBB**. For this purpose, the animal study model was subjected to different morphine protocol treatments, following which the development of a spontaneous or naloxone-precipitated withdrawal syndrome was conditioned.

One of the most widely used model and valuable tool for the study of BBB structure and function is the isolation of brain microvessels from the brain cortical gray matter. Another

main objective of the present dissertation consists in a methodological study, to **compare two techniques of brain microvessel isolation, mechanical dissection and enzymatic dissociation, and evaluate which technique gives the purest and the most enriched brain capillary endothelial cell fraction**. This methodological study is crucial regarding the choice of the best approach to isolate brain microvessels for subsequent molecular biology and functional studies on the BBB. The further **characterization of this BBB model in terms of the expression of several neurotransmitter receptors** is also attained, in order to better understand how the BBB is able to answer to the external stimuli.

Considering the important physiological role of P-gp in the efflux and elimination of harmful compounds out of the brain, therefore providing significant protection to such a vital organ, and the already evidenced capacity of quickly increasing its transport activity in other study models, the second main objective of the present dissertation is to **test promising drug candidates as P-gp activators in an *in vitro* rat BBB model, and thus as potential antidotes against neurotoxic P-gp substrates**. For that purpose, the following specific objectives were set:

- To evaluate the **P-gp activation capacity of thioxanthonic derivatives in an *in vitro* rat BBB model (RBE4 cells)**. These compounds have shown in a previous study to be suitable P-gp activators in the human intestinal epithelial Caco-2 cells (colorectal adenocarcinoma). Therefore, our aim is to evaluate if these data can be extrapolated to other tissue barriers, such as the BBB.
- To assess whether the **tested compounds identified as potential P-gp activators are able to confer an effective protection against neurotoxic P-gp substrates in RBE4 cells**. For this matter, mitoxantrone was selected as the neurotoxic P-gp substrate, and the protection effectiveness of potential P-gp activators against its cytotoxicity was evaluated by simultaneous cell exposure to the potential antidote and the toxic P-gp substrate.

PART III

III. Manuscript 1

Induction of P-glycoprotein and Bcrp at the rat blood-brain barrier following a subchronic morphine treatment is mediated through NMDA/COX-2 activation.

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Rationale and Objectives:

Our group had previously revealed that a subchronic morphine treatment was responsible for an increase of P-gp expression in rat brain cortex vessels, 12h after the last dose of morphine was administered (Yousif et al. 2008). Moreover, since several studies indicate that morphine is a substrate of P-gp in both rodents and humans (Hamabe et al. 2007; Letrent et al. 1999a; Zong and Pollack 2000), and thus play a role in its brain-to-blood active efflux at the BBB, P-gp induction following a chronic exposure to morphine may potentiate its poor brain penetration and the fast tolerance development. Yet, the mechanism by which chronic morphine treatment lead to the P-gp induction was not explored and remained unknown, as well as little was known about its effect on the expression of BCRP, another major ABC transporter present at the BBB.

Therefore, in this study we proposed to explore the amplitude and the kinetics of the modulation of P-gp and Bcrp expressions following a subchronic morphine treatment, using a escalating morphine dose regimen (10-40 mg/kg, i.p.) in rats. For that purpose, animals were sacrificed at different times after the treatment suspension, and freshly isolated rat brain microvessels and large vessels were used to analyze P-gp and Bcrp contents, at both mRNA and protein level. We also proposed to analyze the role of glutamate, the glutamatergic NMDA receptor and COX-2 in the morphine-induced regulation of such ABC transporters as a hypothetical mechanism of regulation. Indeed, brain extracellular levels of glutamate have shown to be increased during morphine withdrawal (Sepulveda et al. 2004). Also, different studies showed that glutamate increased P-gp expression in rat brain microvessels through action on the NMDA receptor, and subsequent COX-2 activity (Bauer et al. 2008; Zhu and Liu 2004; Zibell et al. 2009). For this second purpose of this study, animals were also exposed to an NMDA receptor antagonist, MK-801, or to a COX-2 inhibitor, meloxicam, at the end of the subchronic morphine treatment. Subsequently, P-gp and Bcrp protein contents were assessed in rat brain microvessels and large vessels, once animals were sacrificed 24h after the last dose of morphine.

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ORIGINAL ARTICLE

Induction of P-glycoprotein and Bcrp at the rat blood–brain barrier following a subchronic morphine treatment is mediated through NMDA/COX-2 activation

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Abstract

Subchronic morphine treatment induces P-glycoprotein (P-gp) up-regulation at the blood–brain barrier. This study investigates the rate and extent to which P-gp and breast cancer-resistance protein (Bcrp) increase at the rat blood–brain barrier following subchronic morphine treatment. Rats were given increasing doses of morphine (10–40 mg/kg) or saline i.p. twice daily for 5 days. The brain cortex large vessels and microvessels were then mechanical isolated 6, 9, 12, 24, and 36 h after the last injection. The gene and protein expression of P-gp and Bcrp in morphine-treated and control rats were compared by qRT-PCR and western blotting. The levels of *Mdr1a* and *Bcrp* mRNAs were not significantly modified 6 h post morphine, but the *Mdr1a* mRNA increased 1.4-fold and Bcrp mRNA 2.4-fold at 24 h. P-gp and Bcrp protein expression in brain microvessels was unchanged 6 h post morphine and increased 1.5-fold at 24 h. This effect was more pronounced in large vessels than in microvessels. However, extracellular morphine concentrations of 0.01–10 μM did not modify the expressions of the *MDR1* and *BCRP* genes in hCMEC/D3 human endothelial brain cells *in vitro*. MK-801 (NMDA antagonist) and meloxicam (cyclo-oxygenase-2 inhibitor) given after morphine treatment completely blocked P-gp and Bcrp up-regulation. Interestingly, misoprostol and iloprost, two well-known agonists of prostaglandin E2 receptors induced both *MDR1* and *BCRP* mRNA levels in hCMEC/D3. Thus, morphine does not directly stimulate P-gp and Bcrp expression by the brain endothelium, but glutamate released during morphine withdrawal may do so by activating the NMDA/cyclo-oxygenase-2 cascade.

Keywords: Bcrp, blood–brain barrier, glutamate, morphine, P-glycoprotein.

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Morphine, especially its subchronic and chronic use, is associated with the development of phenomena such as addiction, tolerance, and the appearance of withdrawal symptoms when it is stopped. The mechanisms underlying these events involve complex molecular pathways, most of which have been attributed to regulation of the main morphine target, the opioid receptors. However, there is increasing evidence that the blood–brain barrier (BBB) is also involved (Tournier *et al.* 2011). Morphine crosses the BBB to reach the brain parenchyma, but it does so less well than other opioid drugs like fentanyl and methadone

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Abbreviations used: ABC, ATP-binding cassette; BBB, blood–brain barrier; BCRP, breast cancer-resistance protein; BSA, bovine serum albumin; CNS, central nervous system; COX-2, cyclooxygenase-2; HBSS, hank's buffered salt solution; Mdr, multidrug resistance; P-gp, P-glycoprotein; qRT-PCR, quantitative RT-PCR; TBP, TATA box-binding protein; TKI, tyrosine kinase inhibitors.

(Dagenais *et al.* 2004). This relatively poor brain penetration of morphine has been linked, in part, to its active efflux from the brain to the blood by the P-glycoprotein (P-gp, MDR1, ABCB1) at the BBB (Zong and Pollack 2000; Hamabe *et al.* 2006). P-gp is the best known of the many drug transporters at the BBB (Scherrmann 2005). The breast cancer-resistance protein [breast cancer-resistance protein (BCRP), ABCG2], another drug efflux transporter, is also abundant at the BBBs of monkeys and humans (Dauchy *et al.* 2008; Ito *et al.* 2011; Shawahna *et al.* 2011) and has been shown to efflux some substrates that it has in common with P-gp (Decleves *et al.* 2011). P-gp and BCRP belong to the ATP-binding cassette (ABC) efflux transporter superfamily and they limit the access of many drugs to the CNS (Urquhart and Kim 2009). The effects of opioids on BCRP activity have been poorly evaluated. Recently, we identified buprenorphine and its metabolite norbuprenorphine as inhibitors of BCRP (Tournier *et al.* 2010). However, the effect of exposure to subchronic opioid treatment on the synthesis of BCRP at the BBB has never been studied. In contrast, there have been several studies on the P-gp-mediated transport of morphine in rodents and humans (Callaghan and Riordan 1993; Letrent *et al.* 1999b; King *et al.* 2001). Inhibiting P-gp increased morphine-induced analgesia in rats (Letrent *et al.* 1999a), and morphine has an increased anti-nociceptive effect in P-gp-deficient knockout mice (Thompson *et al.* 2000; Zong and Pollack 2000). Moreover, variation in the pain relief in patients with cancer has been associated with variants of the *ABCB1* gene which alter the expression and/or activity of P-gp (Campa *et al.* 2008; Lotsch *et al.* 2009). The pharmacokinetic–pharmacodynamic relationships between the plasma concentration of morphine and its CNS effect EEG were clearly demonstrated by Groenendaal *et al*. 2007 who showed that P-gp modulates the distribution of morphine within the biophase and so helps delay its CNS effect by (Groenendaal *et al.* 2007). Therefore, P-gp is a key element in the regulation of the effect of morphine on the CNS. An increase in P-gp expression during morphine treatment may thus lead to a timely decrease in the brain uptake of morphine, which may explain the tolerance frequently observed in the CNS effects of morphine. Two studies have shown that P-gp is induced in the brains of mice (Zong and Pollack 2003) and rats (Aquilante *et al.* 2000) by chronic exposure to morphine. Recently, we showed that subchronic morphine treatment induced P-gp expression in rat brain cortex vessels 12 h after the last morphine dose (Yousif *et al.* 2008), but rate and amplitude of this stimulation and the mechanism underlying it remain unknown. It may be because of at least two mechanisms: (i) the direct induction of P-gp by morphine *via* increased transcription of the gene encoding P-gp, increased translation, and/or post-translational processing, (ii) an indirect effect of morphine during the withdrawal syndrome occurring after subchronic treatment. In this study, we have firstly investigated the kinetics of P-gp and Bcrp mRNA and protein levels in rat brain vessels at different times after the last dose of subchronic morphine treatment and then evaluated the roles of glutamate and cyclooxygenase-2 (COX-2) in this induction. During morphine withdrawal, there is an increase in the excitatory amino acid neurotransmission in the CNS area, particularly the glutamate and aspartate concentrations (Sepúlveda *et al.* 2004). Glutamate acts at four receptor subtypes: NMDA, AMPA, kainate, and metabotropic glutamate receptors, and this neurotransmitter increases P-gp expression in rat brain endothelial cells (Zhu and Liu 2004). P-gp was found to be regulated by a pathway that involves glutamate signaling through NMDA receptors and COX-2 activity, which have both been found in the brain capillary endothelial cells (Bauer *et al.* 2008; Zibell *et al.* 2009). In this way, a glutamate–NMDA receptor–COX-2 pathway could be involved in this morphine-mediated induction.

Materials and methods

Animals

The adult male 8-week-old Sprague–Dawley rats weighing 200–240 g used in this study were purchased from Charles River laboratory (L'arbresle, France). They were housed in groups of four animals per cage in a temperature- and humidity-controlled room with 12 : 12 h light/dark conditions (light from 8:00 AM to 8 PM) and access to food and water *ad libitum*. They were acclimated for at least 3 days prior to experimentation. The care and treatment of animals conformed to the standards and guidelines promulgated by the European Union Council Directive (2010/63/EU).

Drug treatment

Acute and subchronic morphine dosing

We used two treatment protocols. For acute treatment, rats were given a single dose (10 mg/kg or 40 mg/kg, ip) of morphine (or saline) and killed 3, 6, and 24 h later to study the levels of *Mdr1a* and *Bcrp* mRNAs in cerebral vessels. For subchronic treatment, animals were given several doses of morphine (or saline) over 5 days. The test rats were given morphine i.p. twice daily: 10 mg/kg on day 1, 20 mg/kg on day 2, 30 mg/kg on day 3, and 40 mg/kg on days 4 and 5. The increasing doses of morphine were used to overcome any tolerance developed and to better simulate addiction to morphine. Control rats were given physiological saline (1 mL/kg i.p.) twice daily. Rats in both groups were killed at 6, 9, 12, 24, and 36 h post-treatment (day 5).

Treatment with MK-801

We measured the levels of P-gp and Bcrp by western blotting in rats conditioned by subchronic morphine treatment and then given dizocilpine maleate (MK-801, Sigma-Aldrich, Lyon, France), an NMDA antagonist, to determine the influence of glutamate on the

morphine-induced increases in P-gp and Bcrp. The rats were given two injections of 1 mg/kg MK-801 i.p. one 1 h and the other 4 h post morphine or saline treatment, because its half-life of elimination is only 2 h. They were killed 24 h after the last morphine or saline injection.

Treatment with Meloxicam

We studied the influence of COX-2 on the increases in P-gp and Bcrp induced by morphine treatment by giving them each a single i.p. injection of meloxicam (10 mg/kg), a COX-2 inhibitor, immediately after the last morphine or physiological saline injection. They were killed 24 h after the last morphine or saline injection.

Isolation of large vessels and microvessels from rat brain cortex

The brain cortex vessels were isolated from treated rats according to Yousif *et al.* 2007. Briefly, rats were anesthetized with isoflurane and decapitated. Their brains were immediately removed and placed in ice-cold Hank's buffered salt solution (HBSS). The cerebella, meninges, brainstems, and large superficial blood vessels were removed from the brains and the remaining cortices were minced in ice-cold HBSS (4 mL per gram of tissue). The minced samples were then homogenized in a Potter–Thomas homogenizer (Kontes Glass, Vineland, NJ, USA) (0.25 mm clearance), using 15–20 up-and-down strokes at 400 rpm. The resulting homogenates were centrifuged at 1000 *g* for 10 min. Each pellet was suspended in 17.5% dextran (64–76 kDa, Sigma-Aldrich, Lyon, France) and centrifuged for 15 min at 4400 *g* at 4°C in a swinging-bucket rotor. The resulting pellets were suspended in Hank's Buffered Salt Solution containing 1% bovine serum albumin (BSA), while the supernatants containing a layer of myelin were centrifuged once more for 15 min at 4400 *g*. The two pellets from each sample were pooled, suspended in HBSS with 1% BSA, and this suspension passed through a 100 μm nylon mesh. Large vessels (mainly 20–30 μm) were collected from the fraction retained on the 100 μm nylon mesh. The filtrate was passed through a 20 μm nylon mesh which retained the microvessels (mainly 4–6 μm).

Measurement of transcript levels

Extraction of RNA from brain tissues

Total RNA was obtained from the isolated large vessels and microvessels by lysing the surrounding basal lamina with proteinase K and then extracting the RNA using the RNeasy Micro-Fibrous Tissue micro-kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The isolated RNA samples were purified from contaminating genomic DNA by treatment with DNase (RNase-Free DNase Set, Qiagen, SA). The concentration and purity of the RNA samples obtained were assessed spectrophotometrically using a Nanodrop spectrophotometer (Nanodrop ND-1000; Nano-Drop Technologies, Wilmington, DE, USA), and the integrity of the RNA was checked by electrophoresis through 0.8% agarose gels.

Reverse transcription

Total RNA samples (1 μg) from brain vessels or cultured hCMEC/D3 cells were reverse transcribed into cDNA in a final volume of 20 μL. The mixture consisted of 1 μg total RNA, 500 μM of each deoxyribonucleotide triphosphate, 10 mM dithiothreitol, 1.5 μM random hexa-nucleotide primers, 20 U RNAsin ribonuclease inhibitor, and 100 U Superscript II Rnase reverse transcriptase. Reagents were purchased from Invitrogen (Cergy-Pontoise, France). Reverse transcription was performed on a programmable thermal cycler (PTC-100 programmable thermal controller; MJ Research Inc., Waltham, MA, USA). Hexamers were annealed at 25°C for 10 min, the products were extended at 42°C for 30 min, the reaction terminated by heating at 99°C for 5 min, and samples were quick-chilled to 4°C.

Real-time quantitative PCR

The effect of morphine on the transcripts levels was investigated by qRT-PCR as previously described (Yousif *et al.* 2007). Specific primer sequences were designed using OLIGO 6.42 software (Medprobe, Oslo, Norway) (Table 1) and these primers were synthesized by Invitrogen Life Technologies (Invitrogen). Fluorescent PCR reactions were performed on a Light Cycler thermal cycler (Light-Cycler® instrument; Roche Diagnostics) using the LC-FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Meylan, France). The PCR reaction mixture contained 1 μL LC-FastStart DNA Master SYBR Green 1 mix, 1.2 μL of 10 mM $MgCl_2$, 0.5 μL of each upper and lower primer (final concentration 0.5 μM), and 1.8 μL water. The cDNAs were diluted 50-fold and 5 μL aliquots were mixed with an equal volume of PCR mixture to give a final volume of 10 μL. The thermal cycling conditions were 8 min at 95°C, followed by 40 amplification cycles at 95°C for 5 s, 64°C for 5 s, and 72°C for 5 s. Gene expression in each sample was normalized on the basis of its *β-actin* gene expression (rat samples) or TATA box-binding protein (TBP) gene expression (human samples). The change was calculated from the ratio of the expression of the gene of interest to that of the housekeeping gene (*β-actin* or *TBP*) in morphine-treated and control samples.

Western blotting

Immunoblots were performed on protein extracted from both large vessels and microvessels isolated at different times after the

Table 1 Sequences of primers used for qRT-PCR

	Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Length (bp)
Rat	*β-actin*	CTGGCCCGGACCTGACAGA	GCGGCAGTGGCCATCTCTC	132
	Mdr1a	CAACCAGCATTCTCCATAATA	CCCAAGGATCAGGAACAATA	97
	Bcrp	CAGCAGGTTACCACTGTGAG	TTCCCCTCTGTTTAACATTACA	75
Human	*TBP*	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA	132
	MDR1	CACCCGACTTACAGATGATG	GTTGCCATTGACTGAAAGAA	81
	NR1	TCGGACAAGAGCATCCA	GACACGCATCATCTCAAACC	87
	BCRP	TGACGGTGAGAGAAAACTTAC	TGCCACTTTATCCAGACCT	122

last dose of morphine or saline to measure, semi-quantitatively, the amounts of P-gp and Bcrp. β-actin was the reference protein. After its extraction, the protein content from samples of large or microvessels was determined with the Bradford reagent (Sigma-Aldrich, Lyon, France) and a BSA calibration curve. Equal amounts of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8% acrylamide/bisacrylamide gels and the separated proteins were transferred electrophoretically to a nitrocellulose membrane (2 h at 80 V). Free sites on the membrane were blocked by incubation overnight at 4°C with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20. The membranes were immunoblotted by incubation overnight at 4°C with mouse monoclonal antibody C219 against rat P-gp (diluted 1 : 100) (Abcam, Cambridge, UK), or rat monoclonal anti-rat Bcrp antibody BXP-53 (diluted 1 : 150) (Abcam) The membranes were then washed and incubated for 1 h at 25°C with horseradish peroxidase-conjugated anti-mouse IgG (diluted 1 : 20 000) or anti-rat IgG (1 : 10 000) (GE healthcare, Buckinghamshire, UK). The membranes were washed again and exposed to Amersham ECL blotting detection reagent (ECL, Amersham Biosciences Europe GmbH, Orsay, France). Signals were revealed using the Bio-Rad ChemiDoc® XRS imaging device (Marnes La Coquette, France). The blots were then stripped and reprobed with monoclonal mouse anti-β-actin antibody AC-74 (Sigma-Aldrich, Lyon, France) (diluted 1 : 10 000). Proteins were quantified using the Quantity One data analyser software v. 4. 6. 1 (Bio-Rad) and normalized to β-actin.

Effect of morphine, glutamate, iloprost, and misoprostol on the expression of P-gp and BCRP in the hCMEC/D3 cells

The hCMEC/D3 cells were kindly donated by Dr. P.O. Couraud of the Institut Cochin, University Paris Descartes, Paris, France. The immortalized hCMEC/D3 cells were cultured as previously described (Dauchy *et al.* 2009). Cells were grown on plates coated with rat-tail collagen type I in EBM-2 medium supplemented with 1 ng/mL bFGF, 2.5% FCS, ascorbate acid, 10 mM HEPES, penicillin–streptomycin at 37°C in a saturated atmosphere of 5% CO_2, 95% air. The culture medium was changed every 3–4 days.

Cells were cultured for 24 h in medium alone (controls) or in medium containing morphine (0.01, 0.1, 1, or 10 μM), glutamate (10 or 100 μM), iloprost (1 or 10 μM), or misoprostol (1 or 10 μM). Total RNA was extracted from the hCMEC/D3 cells using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, including DNAse I treatment to remove genomic DNA. The concentrations and purity of the RNA samples were assessed. The expressions of the *MDR1* and *BCRP* genes were evaluated by qRT-PCR using the procedure outlined above. The reference genes were *TBP* and β-actin.

Statistical analysis

Data were analyzed using GraphPad Prism® 4.0 software (San Diego, CA, USA). The results are expressed as means ± SD. Student's unpaired *t*-test was used to identify significant differences between *in vivo* morphine and saline groups. The effect of morphine on hCMEC/D3 cells was analyzed by one-way ANOVA and a *post hoc* Tukey's test. All the tests were two-tailed and statistical significance was set at $p < 0.05$.

Results

Expression of *Mdr1a* and *Bcrp* gene in the cortex vessels of morphine-treated rats

The levels of *Mdr1a* and *Bcrp* mRNAs were determined in cortex large vessels and microvessels of rats 3, 6, and 24 h after a single dose of saline or morphine ($n = 4$ per group). The levels of neither the *Mdr1a* nor the *Bcrp* gene were significantly altered by morphine at any of the times tested (data not shown).

We also determined the effect of a 5-day morphine treatment on the levels and kinetics of *Mdr1a* and *Bcrp* mRNAs in cortex microvessels (Fig. 1a and 2a) and large vessels (Fig. 1b and 2b). The amounts of *Mdr1a* and *Bcrp* transcripts in both microvessels and large vessels were unchanged 6 h after the last dose of morphine. But, the levels of both *Mdr1a* and *Bcrp* genes increased starting at 9 h after the last morphine dose. The amounts of *Mdr1a* transcripts in microvessels were significantly increased by 22% at 12 h and by 36% at 24 h after the last dose of morphine (Fig. 1a). *Mdr1a* mRNA levels increased more in large vessels than in microvessels. The levels of *Mdr1a* transcripts 12 h after the last morphine dose were increased by 60% in large vessels and by 22% in microvessels (Fig. 1a and b). However, levels of *Mdr1a* transcripts in both types of vessels returned to baseline 36 h after the last dose of morphine (Fig. 1a and b).

The levels of *Bcrp* transcripts in both the microvessels and large vessels of morphine-treated rats were roughly double those of controls, with the increase occurring earlier in large vessels (Fig. 2a and b). Thus, the amount of *Bcrp* transcript was 120% greater than that of controls 12 h after the last morphine injection in large vessels, whereas it was only 40% greater in microvessels. *Bcrp* gene expression was still significantly greater than in controls 36 h after the last dose of morphine, in both the large vessels and microvessels, unlike *Mdr1a* expression (Fig. 2b). These data indicate that the levels of both *Mdr1a* and *Bcrp* transcripts were increased in rat brain vessels from 12 h after the last dose of a subchronic morphine treatment.

P-gp and Bcrp in the cortex vessels of subchronic morphine-treated rats

Western blot experiments with the anti-P-gp C-219 antibody revealed an immunoreactive protein of about 170 kDa, corresponding to P-gp, in isolated large vessels and microvessels, as previously reported (Yousif *et al.* 2007). The levels of P-gp were no higher than those of controls 6 h after the last dose of morphine. But, its level in both microvessels and large vessels was 1.5-times that of controls 24 h after the last dose of morphine. Like mRNA levels, morphine increased the expression of P-gp more in large vessels than in microvessels. The P-gp expression in microvessels decreased back to baseline at 36 h (Fig. 3).

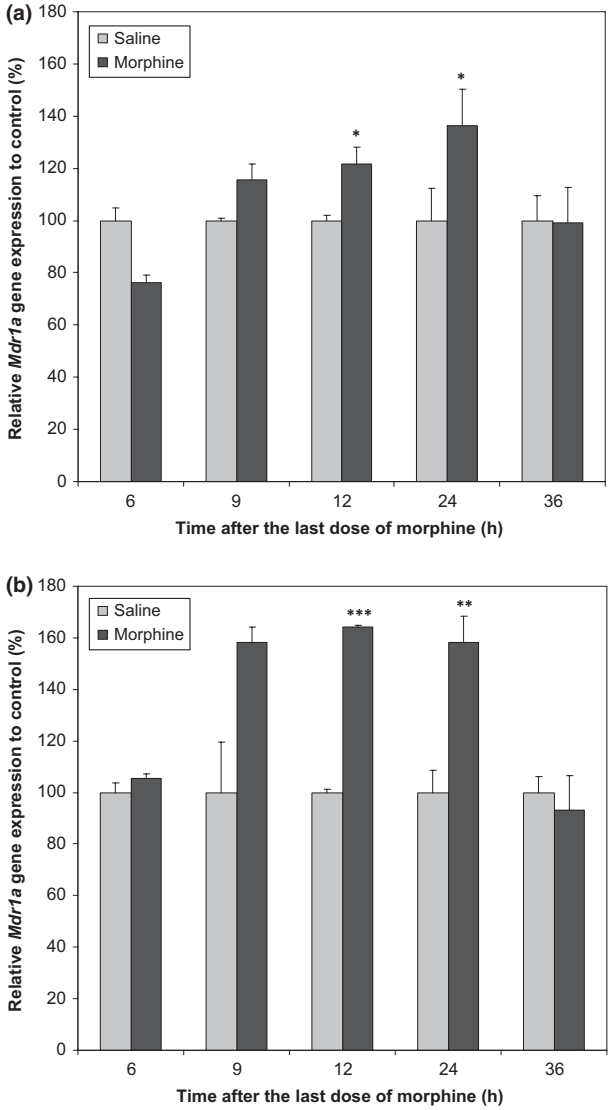

Fig. 1 Changes in *Mdr1a* gene expression over time in morphine-treated rats after the last dose of morphine compared to control rats (baseline fixed at 100%) in small cerebral vessels (a) and large cerebral vessels (b). All data have been normalized to β-actin mRNA in the same sample and are expressed as means ± SD ($n = 4$ rats per time point). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's *t*-test).

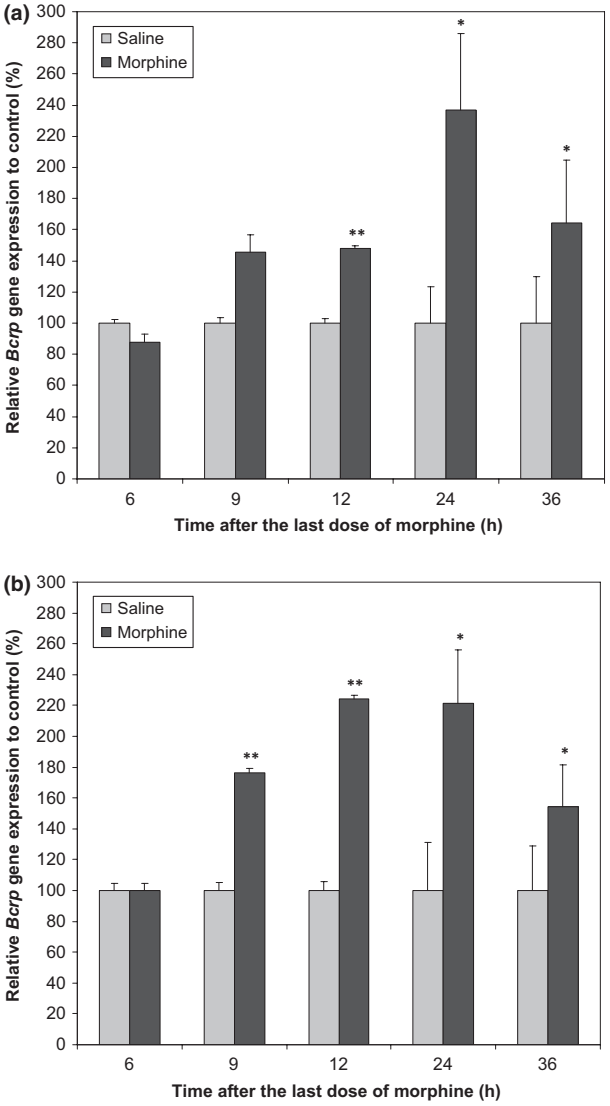

Fig. 2 Changes in breast cancer-resistance protein (*Bcrp*) gene expression over time in morphine-treated rats after the last dose of morphine compared to control rats (baseline fixed at 100%) in small cerebral vessels (a) and large cerebral vessels (b). All data have been normalized to β-actin mRNA in the same sample and are expressed as means ± SD ($n = 4$ rats per time point). *$p < 0.05$, **$p < 0.01$ (Student's *t*-test).

We also determined the kinetics of Bcrp expression in the samples that were used for P-gp analysis. Just like its mRNA, the amount of Bcrp protein was unaltered 6 h after the last dose of morphine, but had increased 1.45-fold in large vessels and 1.6-fold in microvessels by 24 h post treatment (Fig. 4) and the amount of Bcrp in morphine-treated rats was still higher than in controls 36 h post treatment.

Thus, the levels of P-gp and Bcrp proteins were increased in brain vessels of morphine-treated rats from 24 h post treatment, as were the levels of *Mdr1a* and *Bcrp* transcripts from 12 h after the last morphine dose.

Effect of morphine on the levels of *MDR1* and *BCRP* transcripts in human hCMEC/D3 cells

We tested the effect of morphine on immortalized human brain capillary endothelial (hCMEC/D3) cells to determine whether the up-regulation of P-gp and Bcrp in brain vessels by morphine involved only the vascular endothelial cells or the complete neurovascular unit (Weksler *et al.* 2005). Cells were exposed to concentrations of morphine from 0.01 to 10 μM for 24 h and the amounts of *MDR1* and *BCRP* gene transcripts were determined by qRT-PCR. Morphine did not

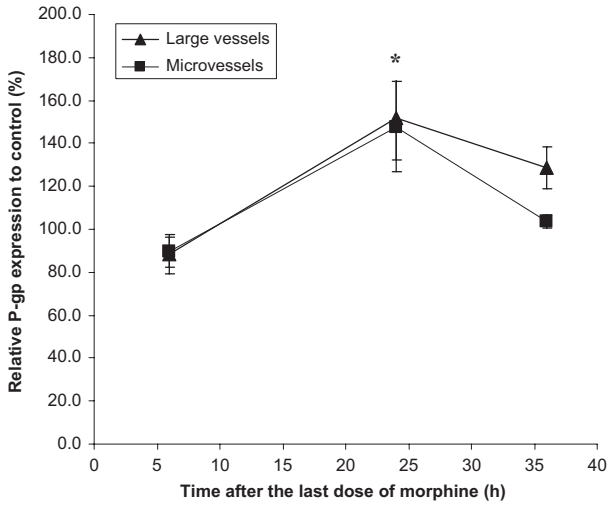

Fig. 3 Kinetics of P-gp expression in morphine-treated rats after the last dose of morphine compared to control rats (baseline fixed at 100%) in cortex microvessels and large vessels. All data have been normalized to β-actin protein in the same sample and are expressed as means ± SD (n = 4 rats). *p < 0.05 (Student's t-test).

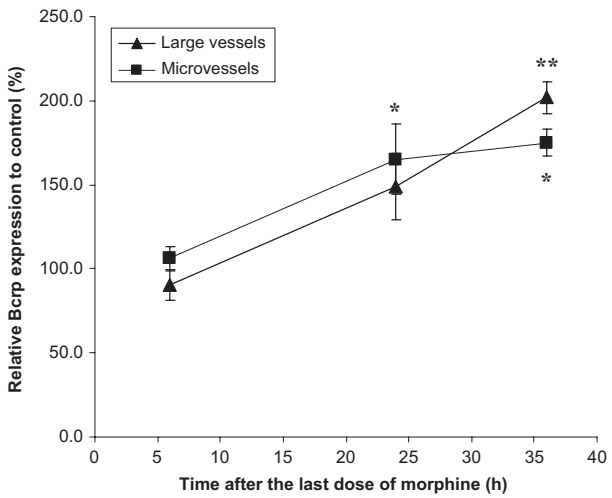

Fig. 4 Kinetics of Bcrp expression in morphine-treated rats after the last dose of morphine compared to control rats (baseline fixed at 100%) in cortex microvessels and large vessels. All data have been normalized to β-actin protein in the same sample and are expressed as means ± SD (n = 4 rats in each group) *p < 0.05, **p < 0.01 (Student's t-test).

alter the levels of either *MDR1* or *BCRP* gene transcripts in hCMEC/D3 cells at any of the concentrations used (Fig. 5).

Effect of MK-801 and meloxicam on the expression of P-gp and Bcrp in cortex vessels

As endothelial cells may not be the direct target of morphine to increase P-gp and BCRP expression, we looked for the pathway by which it might act in rat cortex vessels. It was recently shown that exposure of isolated rat brain microvessels

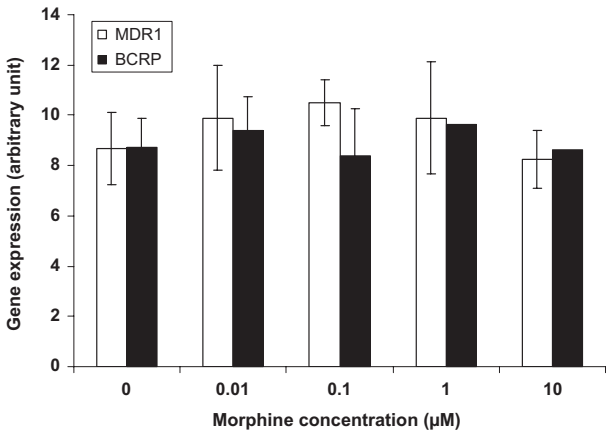

Fig. 5 Expression of *MDR1* and *BCRP* genes in morphine-treated hCMEC/D3 cells. Cells were cultured without (0) or with medium containing 0.01, 0.1, 1, or 10 μM morphine for 24 h. Data represent relative changes in the expression of the *MDR1* and *BCRP* genes in morphine-treated cells and control cells. All data have been normalized to TATA box-binding protein mRNA in the same sample and are expressed as means ± SD (n = 3 independent cell culture experiments in triplicate).

to glutamate *ex vivo* significantly increased P-gp expression by activation of the NMDA/COX-2 pathway (Bauer *et al.* 2008). We therefore treated rats with morphine with or without the NMDA antagonist MK-801 to determine whether activation of NMDA receptors by glutamate was involved in the up-regulation of P-gp and Bcrp. The amounts of P-gp and Bcrp measured by western blotting indicated that MK-801 totally blocked the morphine-dependent up-regulation of P-gp and Bcrp in large vessels and microvessels 24 h after the last morphine dose; their expressions in rats given morphine plus MK-801 were comparable to those in control rats (Fig. 6 and 7). MK-801 had a less pronounced effect on Bcrp expression in large vessels than in microvessels. Similarly, meloxicam, a COX-2 inhibitor, totally blocked the morphine-dependent up-regulation of P-gp and Bcrp in large vessels as well as in microvessels 24 h after the last morphine dose (Fig. 6 and 7). Neither MK-801 nor meloxicam given alone changed the expression of *Mdr1a* and *Bcrp;* their activities were the same as in saline-treated rats (data not shown). Taken together, these results suggest that NMDA/COX-2 pathway is implicated in morphine-dependent Bcrp and P-gp induction.

Effect of glutamate, iloprost, and misoprostol on the levels of *MDR1* and *BCRP* transcripts in human hCMEC/D3 cells

We tested the effect of glutamate on *MDR1* and *BCRP* transcripts in hCMEC/D3 cells to determine whether glutamate was directly involved in the regulation of P-gp and BCRP. Cells were exposed to 10 and 100 μM of glutamate for 40 min, washed, and then incubated in glutamate-free medium for 5.3 h before the amounts of *MDR1* and *BCRP* gene transcripts were analyzed by qRT-PCR. The relative

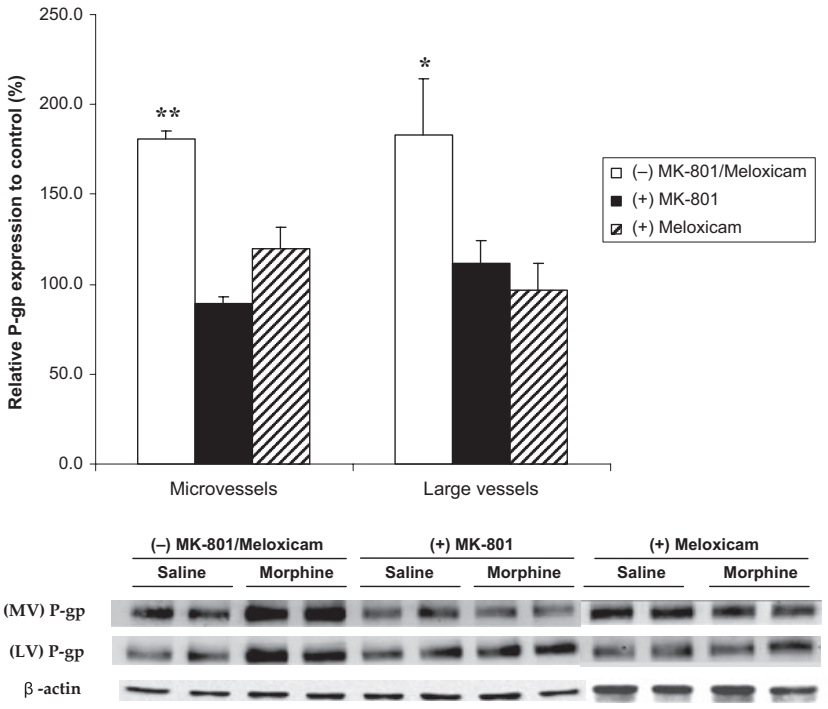

Fig. 6 Effect of MK-801 and Meloxicam on morphine-dependent P-gp induction. Rats were dosed with subchronic morphine treatment for 5 days (see Materials and Methods). Rats were then dosed with 1 mg/kg MK-801 or 10 mg/kg meloxicam after the last morphine dose and cortex microvessels (MV) and large vessels (LV) were isolated 24 h after the morphine dose. P-gp expression was determined by western blotting and compared with control group (baseline fixed at 100%) in cortex microvessels and large vessels. All data have been normalized to β-actin protein in the same sample and are expressed as mean ± SD ($n = 4$ rats in each group). $*p < 0.05$, $**p < 0.01$ (one-way ANOVA with Tukey's Test).

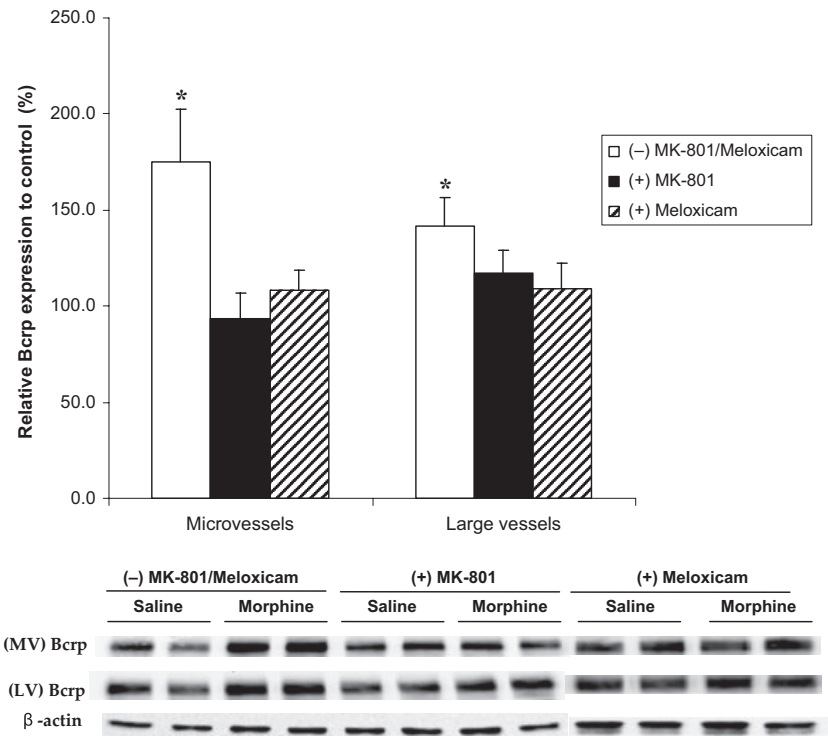

Fig. 7 Effect of MK-801 and meloxicam on morphine-dependent Bcrp induction. Rats were subjected to a subchronic morphine treatment for 5 days and then given 1 mg/kg MK-801 or 10 mg/kg meloxicam after the last morphine dose. Their cortex microvessels and large vessels were isolated 24 h after the last morphine dose. The amount of P-gp in cortex microvessels and large vessels were determined by western blotting and compared with those in control rats (baseline fixed at 100%). All data have been normalized to β-actin protein in the same sample and are expressed as means ± SD ($n = 4$ rats in each group). $*p < 0.05$ (one-way ANOVA with Tukey's Test).

expressions of *MDR1* and *BCRP* were not significantly different from those in non-treated cells (Fig. 8a and b). We also measured the transcript levels of the NR1 gene of the NMDA receptor in hCMEC/D3 cells. NR1 transcript levels were very low in hCMEC/D3 cells (cycle threshold > 32 for a dilution 1 : 20 of the cDNA reversed transcribed from 1 μg of total RNA), that may explain the absence of effect of glutamate on hCMEC/D3 cells.

As prostaglandin E2 (PGE2) is the major product of COX-2, we also tested the effect of two PGE2 analogs, iloprost and misoprostol, on hCMEC/D3 cells to determine whether COX-2 activity is involved in the regulation of P-gp and

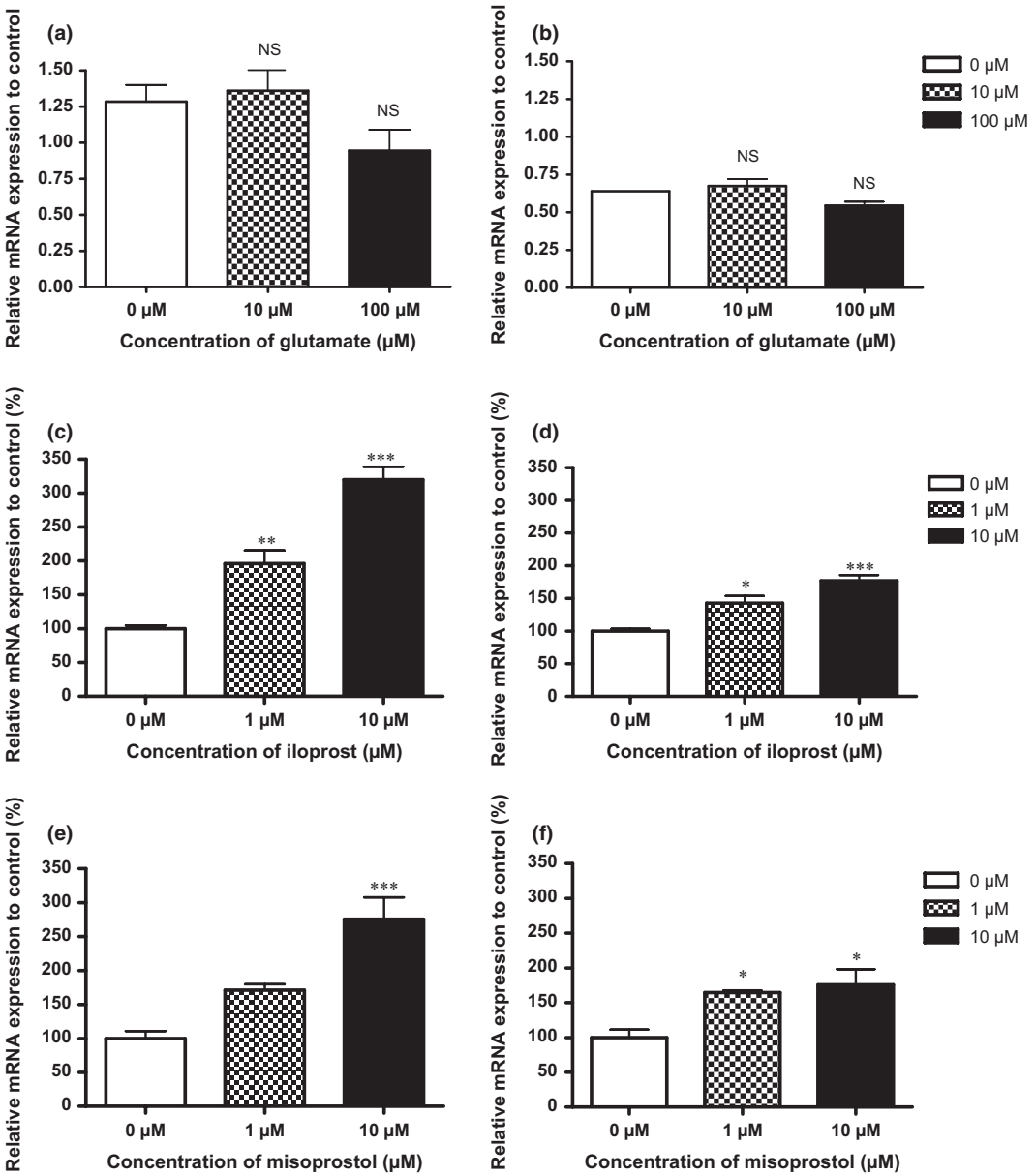

Fig. 8 Effects of glutamate, iloprost, and misoprostol on *MDR1* and *BCRP* gene expression. hCMEC/D3 cells were incubated with medium containing 0, 10 μM, or 100 μM of glutamate at 37°C for 40 min, or with medium containing 0, 1 μM, or 10 μM of iloprost or misoprostol for 6 h. Data represent relative changes in the expression of the *MDR1* (a, c, e) and *BCRP* (b, d, f) genes in treated and control cells. All data have been normalized to β-actin mRNA in the same sample and are expressed as means ± SD ($n = 2$ independent cell culture experiments in duplicate). NS: not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA with Tukey's Test).

BCRP expression. Cells were exposed to 1 μM or 10 μM of iloprost or misoprostol for 6 h and the amounts of *MDR1* and *BCRP* gene transcripts were also determined by qRT-PCR. Both concentrations of iloprost significantly increased the concentrations of *MDR1* and *BCRP* gene transcripts. Thus, 10 μM iloprost increased *MDR1* transcripts three-fold over controls and *BCRP* transcripts two-fold (Fig. 8c and d). Misoprostol had weaker effects on the levels of *MDR1* and *BCRP* transcripts; 10 μM misoprostol produced just a significant increase in *MDR1* expression over the control and a weaker increase in *BCRP* expression.

Discussion

Opioids, particularly morphine, are the standard analgesics used in the clinical management of chronic and severe pain. However, patients require greater and greater doses to maintain the same level of analgesia as they become tolerant

to their central effects, a phenomenon known as central tolerance. The animal treatment protocol, with frequent increasing doses reflects the typical pattern of opioid abuse. The subchronic morphine treatment, twice daily for 5 days, allows the serum concentration of morphine to fluctuate, with peaks and troughs of morphine. These fluctuations generate withdrawal after the last dose of morphine as already reported in our laboratory (Desjardins *et al.* 2008). This profile of drug administration is also responsible for the development of tolerance to morphine (Way *et al.* 1969; Kalivas and Duffy 1987). P-gp, a key-element in the control of the brain distribution of several opioids at the BBB, including morphine, may be responsible for this tolerance, as its expression is also increased by morphine. There have been two reports of P-gp induction in the whole brains of mice (Zong and Pollack 2003) and rats (Aquilante *et al.* 2000) after subchronic exposure to morphine. Aquilante *et al*. 2000 measured the amount of P-gp the day after the last dose of morphine, while Zong and Pollack 2003 did so 24 h after the last morphine dose. But, neither of these studies distinguished the effect of morphine during treatment from that occurring during withdrawal. We have previously used isolated rat brain microvessels to show that the amount of P-gp at the BBB increased as early as 12 h after a 5-day morphine treatment; this treatment was similar to that used in the studies cited above (Yousif *et al.* 2008). The subchronic morphine treatment used in this study is widely used for behavioral and neurobiological studies in our laboratory. We showed, recently, that it triggers withdrawal that started as early as 9 h after the last dose of morphine (Desjardins *et al.* 2008). The working hypothesis for this study was that the induction of P-gp and Bcrp in the rat BBB was because of the withdrawal syndrome following subchronic morphine treatment rather than from the direct effect of morphine at the BBB during the morphine treatment. A single dose of morphine (10 or 40 mg/kg, i.p.) did not increase the levels of P-gp mRNA in either type of rat brain vessels at 3, 6, and 24 h. Similarly, the levels of P-gp transcripts and protein were not modified 6 h after a 5-day morphine treatment, suggesting that morphine itself does not stimulate P-gp synthesis during this treatment. Conversely, the levels of *Mdr1a* transcripts and P-gp protein increased between 9 h and 24 h after this morphine treatment. Therefore, these *in vivo* experiments suggest that morphine does not directly induce P-gp up-regulation at the brain endothelial, but triggers other mechanisms during withdrawal that follows its chronic administration which then induce P-gp synthesis. This is supported by our *in vitro* experiments showing that morphine did not stimulate the synthesis of *MDR1* transcripts in hCMEC/D3 human endothelial cells, although P-gp synthesis by these cells is increased by rifampin (Zastre *et al.* 2009). Our data are also supported by other *in vitro* experiments demonstrating the absence of P-gp induction in several cancer cell lines exposed to morphine (Pajic *et al.* 2004). In contrast, Pal *et al*. 2011 have shown that prolonged exposure (15 days) of caco-2 cells to 3 μM morphine increased their *MDR1* mRNA contents, suggesting that effect of morphine on P-gp expression may depend on tissue of origin and length of exposure (Pal *et al.* 2011).

We therefore postulate that morphine-mediated stimulation of P-gp synthesis occurs during the spontaneous withdrawal syndrome that follows the end of a 5-day morphine treatment rather than to a direct effect of morphine on P-gp expression. *Guo et al.* (2005) found that the extracellular concentration of glutamate in the hippocampus of mice determined by microdialysis was decreased after acute or subchronic morphine treatment. But, they also showed that the extracellular glutamate concentration was significantly increased in an artificial morphine withdrawal situation induced by the opioid antagonist naloxone (Guo *et al.* 2005). There is also good evidence from studies on rodents with experimental epilepsy that the massive release of glutamate or intracerebroventricular injection of kainate results in the activation of glutamate receptors in the hippocampus and the increase in P-gp expression (Zhu and Liu 2004; Potschka 2010). Glutamate also increases P-gp expression in primary cultures of brain endothelial cells (Zhu and Liu 2004). It has also been shown recently that exposure of isolated rat brain microvessels to glutamate *ex vivo* significantly increases P-gp synthesis through the activation of NMDA receptors, which increases the activity of COX-2, which, in turn produces prostaglandin E2. It is believed that the effect of COX-2 on P-gp expression is mediated by PGE2 acting on E-type prostanoid receptor (EP) 1 receptors. PGE2 is a major product of COX-2 signaling in the brain. MRP4, a member of the multidrug-resistance proteins (MRPs) belonging to the ABC protein superfamily, takes part in the brain-to-blood efflux transport of PGE2 at the BBB. It is thus likely to be involved in the release of PGE2 from the endothelial cells and so to act as a signaling molecule (Reid *et al.* 2003; Akanuma *et al.* 2010). It acts on four different G protein-coupled receptors (EP1, EP2, EP3, and EP4), which mediate diverse effects (Hata and Breyer 2004). EP1 receptor expression has already been found in isolated rat brain capillaries by (Pekcec *et al.* 2009), and its blockade by SC-51089, a specific antagonist of EP1 receptor, abolished the glutamate-induced increase of P-gp expression in brain capillaries, suggesting that the EP1 receptor plays a key role in the signaling events that regulate P-gp expression via glutamate.

The regulation of P-gp gene transcription at the BBB is rather complex and is far from being completely understood (Miller 2010). Several factors that are known to be involved in the regulation of the *MDR1* promoter. NF-kB is a ubiquitous transcription factor that has been shown to up-regulate P-gp expression and function in several cancer cells (Bentires-Alj *et al.* 2003). Upon activation of NF-kB, PI3K/Akt are upstream signals which lead to the phophorylation of IkB

proteins by IkB kinase. The phosphorylated IkB allows the release of NF-kB, which is translocated to the nucleus, where it activates its target genes (Karin 1999). EP1 receptor signaling is coupled to the phospholipase C/inositol triphosphate pathway, leading to activation of PI3K/Akt and mobilization of intracellular calcium (Asbóth *et al.* 1996). PGE2 also activates NF-kB through EP1 receptors in colonocytes and strongly induces IkB phophorylation, (Kim *et al.* 2007). Hence, we postulate that these mechanisms might be present at the BBB and the downstream effect of COX-2 on the regulation of P-gp and BCRP expression may be through a PGE2-EP1-PI3K/Akt-NF-kB signaling pathway. The β-catenin signaling pathway might also be behind the effect of NMDAr/COX-2 on the regulation of P-gp and BCRP expression. Lim *et al*. 2008. showed that stabilizing and activating β-catenin signaling increases P-gp expression and that this was functionally significant (Lim *et al.* 2008). Moreover, manipulating β-catenin signaling also affects the expression of other ABC transporters, such as BCRP (Scotto 2003). The EP1 receptor contributes significantly to the PGE2-mediated stabilization of β-catenin by activating phospholipase C and PI3K, and thus strongly increases the amount of β-catenin in the nucleus (Lee *et al.* 2004). The stabilization and subsequent translocation of β-catenin leads to the activation of the Tcf-/Lef-1 transcription factor, which alters the expression of several genes, including the *MDR1* gene (Fujino *et al.* 2002).

Thus, these data on the release of glutamate and its effect on P-gp support our hypothesis that the induction of P-gp synthesis in brain large vessels and microvessels of rats treated with morphine for 5 days involves the glutamatergic system and is because of the glutamate released during spontaneous morphine withdrawal. This induction driven by morphine withdrawal may occur through activation of the NMDA receptor by glutamate and COX-2 activity. We tested the direct effect of glutamate on *MDR1* and *BCRP* transcript production by incubating hCMEC/D3 cells with two concentrations of glutamate (10 and 100 μM). Glutamate had no influence on *MDR1* and *BCRP* expression, the transcript levels were the same as in the control (Fig. 8). We also checked the mRNA levels encoding for the NR1 subunit of the NMDA receptor by qRT-PCR on cDNA from hCMEC/D3 cells. There was very low expression of the NR1 subunit of the NMDA receptor in these cells, which agrees well with the finding that glutamate had no effect on *MDR1* and *BCRP* mRNA levels in hCMEC/D3.

As we observed the greatest expression of P-gp in brain vessels 24 h after the last morphine dose, we dosed morphine-treated rats with two injections of 1 mg/kg MK-801 or a single injection of meloxicam after the last dose of morphine to see whether the NMDA/COX-2 pathway could be involved. Both MK-801 and meloxicam completely blocked the over-expression of P-gp in both microvessels and large vessels. Although meloxicam is not a very specific inhibitor of COX-2, it consistently proved to be a preferential inhibitor of COX-2 rather than of COX-1, in contrast to a group of standard NSAIDs (Engelhardt 1996; Degner *et al.* 1998). COX-1 has not been shown to be constitutively expressed in the endothelial cells of the human BBB (Tomimoto *et al.* 2002), whereas there is evidence that COX-2 is (Zibell *et al.* 2009). This indicates that the observed effect of meloxicam is more likely to be because of inhibition of COX-2 than of COX-1. As hCMEC/D3 cells express no NMDA receptor, exposing this human cell line to MK-801 or meloxicam should have no effect on the expression of *MDR1* and *BCRP* genes. We incubated hCMEC/D3 cells with PGE2 analogs, iloprost and misoprostol, to determine the involvement of COX-2 in the regulation of P-gp and BCRP in these cells and to study the downstream cascade. Iloprost is a structural analog of prostacyclin whose affinity for EP1 receptors is similar to that of PGE2, whereas misoprostol is a PGE1 analog whose agonist activity is mediated by EP2, EP3, and EP4 receptors (Abramovitz *et al.* 2000). Iloprost increased the amounts of both *MDR1* and *BCRP* gene transcripts, whereas the highest concentration tested of misoprostol increased *MDR1* transcription but had little effect on *BCRP* expression (Fig. 8c to 8f). These findings also point to COX-2 activity mediated by PGE2 should act via EP1 receptor rather than via EP2–EP4 receptors. We believe this is clear evidence that the glutamate release and COX-2 activity after the last morphine dose are involved in the induction of P-gp up-regulation. We therefore suggest that repeated doses of morphine without the withdrawal syndrome may not affect P-gp synthesis during exposure to morphine. However, the glutamate released during the spontaneous morphine withdrawal syndrome does affect P-gp expression and may be part of the mechanism underlying morphine tolerance, even though the small increase in Mdr1a mRNA expression found here cannot be the major cause of tolerance as morphine is a poor substrate of P-gp. Schinkel *et al.* 1995 showed that the concentration of morphine in the brains of Mdr1a 'knockout' mice was only 1.7-fold greater than that in the brains of normal mice (Schinkel *et al.* 1995). These pre-clinical data may have clinical relevance as patients treated with opiates for pain relief (oxycodone, morphine, hydromorphone) or heroin maintenance (methadone) suffer from withdrawal syndrome. This could increase P-gp expression and thus decrease the pharmacological effect of opiates that are P-gp substrates, but most importantly decrease the efficacy of anti-cancer drugs that are frequently given with morphine, and of methadone for heroin addiction, as these drugs are better substrates of P-gp than morphine.

We have also measured the kinetics of P-gp induction in large vessels and microvessels. P-gp synthesis is not uniform throughout the rat brain vasculature, as there is less P-gp in small arteries than in microvessels and small veins of the brain cortex (Saubamea *et al.* 2012). Our results show that the increase in P-gp expression was more pronounced in large vessels than in microvessels, suggest-

ing that small arteries and veins are more sensitive to the released glutamate. We also find that the expression of Bcrp, like that of P-gp, is increased following subchronic morphine treatment. There is a lack of information on the effects of opioids on BCRP activity despite increasing evidence that BCRP at the BBB modulates the brain distribution of drug substrates. We showed recently that buprenorphine and its main metabolite in human, norbuprenorphine, are inhibitors of BCRP, whereas opioids appeared to be poor substrates of BCRP (Tournier *et al.* 2010). However, we clearly showed that the concentration of Bcrp in rat brain vessels was doubled 36 h after the last dose of a 5-day morphine treatment. The increase in Bcrp synthesis was higher than that of P-gp and more prolonged. The concentration of Bcrp did not return to baseline 36 h after the last dose of morphine, unlike that of P-gp. This difference in Bcrp and P-gp induction might mean that there is a distinct transcriptional mechanism or post-transcriptional regulation during the activation of the *Mdr1a* and *Bcrp* genes, although they both involve NMDA receptor activation and COX-2 activity. These higher concentrations of Bcrp protein are positively correlated with greater *Bcrp* gene expression 36 h after the last dose of morphine, in contrast to the findings for *Mdr1a* and P-gp. These distinct results could also be related to the difference in the half-lives of these two transporters: P-gp has a half-life of 14–17 h (Muller *et al.* 1995), whereas the estimated half-life of BCRP is longer around 60 h (Peng *et al.* 2010). Thus, *de novo* synthesized P-gp might be eliminated faster than BCRP. This is, to our knowledge, the first time that Bcrp expression has shown to be regulated *via* glutamate-NMDA-COX-2 activation. The increase in BCRP expression that follows morphine treatment may thus decrease the penetration of drugs that are BCRP substrates into the brain. The substrates of BCRP that are used to treat humans are mainly anti-cancer drugs like cytotoxic drugs and tyrosine kinase inhibitors. Erlotinib and gefitinib, two tyrosine kinase inhibitors used to treat several solid cancers, are well-known substrates of BCRP and their brain distributions are restricted because of the high concentration of BCRP at the BBB (Chen *et al.* 2011; de Vries *et al.* 2012). This low brain penetration is a major issue in the treatment of brain metastasis that is often observed in patients suffering lung cancer and metastatic renal cancer. There is also evidence that the expression of BCRP in isolated human brain microvessels is higher than that of P-gp (Shawahna *et al.* 2011), making BCRP a key element in controlling the access of its substrates to the brain. As morphine is widely prescribed to manage severe pain in patients with cancer, the up-regulation of BCRP following intermittent morphine treatment and withdrawal syndromes may increase the expression of BCRP in the human BBB, which could restrict the distribution within the CNS of any anti-cancer drugs that are substrates of BCRP.

In conclusion, we have clearly shown that the expression of P-gp and Bcrp at the rat BBB is not increased at the end of a subchronic morphine treatment that triggers tolerance to the analgesic effects of morphine. Thus, our data do not support the concept that P-gp auto-induction is involved in the mechanism of tolerance often observed with morphine. Conversely, the expression of P-gp and Bcrp begins to increase 9 h after the last dose of a subchronic morphine treatment, which coincides with the appearance of the morphine withdrawal syndrome. We suggest that morphine withdrawal syndrome occurring after a subchronic morphine treatment leads to the activation of the NMDA/COX-2 pathway whose consequences are increases in P-gp and Bcrp expression at the rat BBB.

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The authors have no conflict of interest to declare.

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General outcome:

The results obtained in the present publication demonstrated, for the first time, that subchronic morphine exposure was able to up-regulate P-gp but also Bcrp expression at the rat BBB. Furthermore, it was also the first time that a glutamate-NMDA receptor-COX-2-PGE_2 signaling pathway was pointed and demonstrated as being implicated in the morphine-induced up-regulation of both ABC transporters.

In fact, a 5-day subchronic morphine regimen was able to up-regulate both P-gp and Bcrp 12-24h after the last dose of morphine, an effect that was not registered at earlier time-points of animal sacrifice (6h, 9h). This was confirmed at both mRNA and protein level. The peak of *Abcb1a* and *Abcg2* mRNA induction was registered at 24h, with a 1.4-fold and a 2.4-fold increase, respectively. Similarly, P-gp and Bcrp protein levels were 1.5-fold and 1.6-fold increased 24h after the last dose of morphine. Such effects were more pronounced in rat brain large vessels than in microvessels.

On the other hand, neither a single dose administration of morphine in rats, nor the direct exposure of the hCMEC/D3 endothelial cell line to morphine were able to modify the mRNA levels of *Abcb1a/ABCB1a* and *Abcg2/ABCG2*. This suggests that changes in the expression of these ABC transporters are more likely linked to a prolonged exposure to morphine, and that it must involve other cells of the NVU other than only endothelial cells, in a cell-cell interaction complex mechanism.

The NMDA receptor antagonism, as well as COX-2 inhibition resulted in the abolition of the subchronic morphine-induced P-gp and Bcrp protein up-regulation, 24h after the last dose of morphine. These results suggest that the NMDA receptor, and thus its ligand, glutamate, and COX-2 are implicated in the morphine-dependent P-gp and Bcrp up-regulation.

Since the induction of P-gp and Bcrp was only registered from 12h after the last dose of morphine-onwards, which is timely-coincident with the development of opioid withdrawal following a similar subchronic morphine regimen (Desjardins et al. 2008), together with the fact that some studies showed that brain extracellular glutamate levels are considerably increased during morphine withdrawal (Guo et al. 2005; Sepulveda et al. 2004), lead us to consider that the development of the morphine withdrawal could drive the modulation of the expression of P-gp and Bcrp at the rat BBB, rather than the direct effect of morphine exposure. The following presented study has focused on the research of this particular subject.

III. Manuscript 2

Effect of subchronic intravenous morphine infusion and naloxone-precipitated morphine withdrawal on P-gp and Bcrp at the rat blood-brain barrier.

Journal of Pharmaceutical Sciences, Accepted for publication

Rationale and Objectives:

The results obtained *in vivo* evidenced that P-gp and Bcrp expressions in rat brain microvessels were increased following a 5-day, twice daily, subchronic morphine administration. However, this up-regulation was not registered during the treatment or once it was interrupted, but only several hours after the last administration of morphine, at a timely coincident point with the development of a spontaneous morphine withdrawal syndrome. These results strongly encouraged us in the pursuit of a better understanding of the mechanism involved in the modulation of P-gp and Bcrp by morphine, namely by trying to understand whether it is a direct consequence of continued exposure to morphine, or rather a result of the morphine withdrawal which is further developed after discontinuation of treatment, such as the previous results seem to point to.

Thus, the aim of the present study was to evaluate the effects, on one hand, of a subchronic continuous morphine infusion, and on the other hand, of naloxone-precipitated morphine withdrawal on the expression and activity of P-gp and Bcrp at the rat BBB. For this purpose, rats were subjected to three different protocols:

i. a continuous i.v. infusion of morphine for 120h (5 days), to attain a theoretical plasmatic concentration of morphine of 100 ng/mL (study 1). Animals were sacrificed immediately, once the morphine infusion was interrupted;
ii. Triggering of a precipitated morphine withdrawal with an injection of a single dose of naloxone (1 mg/kg, s.c.), a strong μ-opioid receptor antagonist, 1h after an escalating morphine dosing regimen (10-40 mg/kg, i.p., twice daily for 5 days) (study 2). Rats were sacrificed 12h after naloxone administration;
iii. a chronic morphine regimen (10 mg/kg s.c., twice daily for 5 days), followed by a withdrawal period (2 days), and treatment was resumed for 3 additional days. Similarly, a single administration of naloxone (1 mg/kg, s.c.) was given in order to trigger an intense precipitated opioid withdrawal (study 3). Rats were sacrificed at two different time points: 4h and 24h after naloxone administration.

The administration of naloxone provokes an intense precipitated withdrawal in morphine-dependent rats, which was measured through the observation of a set of behavioral signs. Control rats were given physiological saline in an equal protocol of administration in the 3 studies. Once more, animals were sacrificed to obtain freshly isolated brain microvessels, which were subsequently used to analyze P-gp and Bcrp expressions, at both mRNA and protein level, following each of the described treatment protocols.

Effect of subchronic intravenous morphine infusion and naloxone-precipitated morphine withdrawal on P-gp and Bcrp at the rat blood-brain barrier

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Abbreviations: ABC, ATP-binding cassette; BBB, blood-brain barrier; BCRP, breast cancer resistance-protein; BSA, bovine serum albumin; EC, endothelial cell; HBSS, hank's buffered salt solution; LC/MS-MS, liquid chromatography tandem mass spectrometry; MRM, multiplexed reaction monitoring; P-gp, P-glycoprotein; qRT-PCR, quantitative real time-polymerase chain reaction; WB, Western Blot.

Abstract:

Rationale: Chronic morphine regimen increases P-glycoprotein (P-gp) and breast cancer resistance protein (Bcrp) expressions at the rat blood-brain barrier (BBB) but what drives this effect is poorly understood.

Objectives: Assess subchronic continuous morphine infusion and naloxone-precipitated morphine withdrawal effects on P-gp/Bcrp contents and activities at the rat BBB.

Methods: Rats were treated either with (i) a continuous i.v. morphine for 120h, (ii) escalating morphine dosing (10-40 mg/kg, i.p., 5 days), (iii) a chronic morphine regimen (10 mg/kg s.c., 5 days) followed by a withdrawal period (2 days) and treatment for 3 additional days. Animal behavior was assessed after naloxone-precipitated withdrawal (1mg/kg, s.c.). P-gp/Bcrp expressions and activities were determined in brain microvessels by qRT-PCR, WB, UHPLC-MS/MS, and *in situ* brain perfusion of P-gp or Bcrp substrates.

Results: Continuous i.v. morphine did not change P-gp/Bcrp protein levels in rat brain microvessels, whereas naloxone-precipitated withdrawal after escalating or chronic morphine dose regimen increased *Mdr1a* and *Bcrp* mRNA levels by 1.4-fold and 2.4-fold, respectively. Conversely, P-gp/Bcrp protein expressions remained unchanged after naloxone administration, and brain uptake of [^{3}H]-verapamil (P-gp) and [^{3}H]-mitoxantrone (Bcrp) was not altered.

Conclusions: Subchronic morphine infusion and naloxone-precipitated morphine withdrawal have poor effect on P-gp/Bcrp levels at the rat BBB.

Keywords: Blood-brain barrier, Morphine, Opioid withdrawal, P-glycoprotein, Breast Cancer Resistance Protein

1. INTRODUCTION

Opioids represent one of the most powerful analgesics used for the treatment of moderate to severe chronic pain.[1,2] On the other hand, opioids such as morphine and heroin, due to their euphoria and pleasure inducing properties, are also well-known substances of abuse. Their misuse is a major public health concern as it leads to the development of phenomena like physical dependence, opioid tolerance, and ultimately to severe withdrawal syndrome when the consumption is abruptly suspended.[3] Central nervous system (CNS) effects of morphine depend on both pharmacokinetic and pharmacodynamic parameters. From a pharmacokinetic point of view, it is well demonstrated that morphine CNS effects are linked to its ability to cross the blood-brain barrier (BBB) that is partially embedded by its brain-to-blood efflux mediated by the ATP-binding cassette (ABC) transporter P-glycoprotein (P-gp, Abcb1a, Mdr1a).[4,5] Thus, modulation of P-gp at the BBB may enhance or decrease CNS distribution of P-gp substrates such as morphine, respectively. There is considerably increasing evidence that chronic exposure to morphine induces adaptive modifications including those of gene regulation and brain plasticity.[6-8] Interestingly, twice daily subchronic morphine exposure has been shown to increase P-gp content in rat,[9-11] and mouse whole brain.[5] Actually, we previously showed that P-gp expression in rat brain microvessels was only up-regulated several hours after, and not immediately after, treatment suspension, following a twice daily subchronic morphine administration, which timely coincided with the beginning of a spontaneous morphine withdrawal.[11] Breast cancer-resistance protein (BCRP, ABCG2), another drug efflux transporter, has been shown to share a significant number of substrates with P-gp, and it is more expressed than P-gp at the human BBB, unlike in rodents.[12] Like P-gp, Bcrp was induced in rat brain microvessels following a subchronic morphine treatment.[11] However, it remains unknown if P-gp and Bcrp induction at the rat BBB is directly triggered by morphine, or indirectly through its subsequent morphine withdrawal. To answer this question, we either intravenously and continuously infused rats with morphine for 5 days, or provoked a strong morphine withdrawal with naloxone, an opioid receptor antagonist, after two different subchronic morphine regimens. P-gp and Bcrp expression in rat brain microvessels and functional activities at the BBB were evaluated in the different animal treatment protocols.

2. EXPERIMENTAL PROCEDURES

2.1. Animals

Adult male Sprague–Dawley rats weighing 200 to 250g (5 to 7 weeks old) were purchased from Janvier laboratory (Le Genest-Saint-Isle, France). They were housed in groups of three animals per cage under standard 12h light/dark conditions (light from 8:00 a.m. to 8 p.m.) in a temperature- and humidity-controlled room. The animals had access to food and water *ad libitum* and were acclimated for a minimum of 7 days prior to conducting experiments. All animal experiments complied with the standards and guidelines promulgated by the latest European Union Council Directive (2010/63/EU) and were approved by the local ethics review committee (Paris Descartes University n°12-186).

2.2. Animal treatments:

(±)-Morphine hydrochloride was obtained from Francopia (Paris, France). (±)-Naloxone hydrochloride was purchased from Sigma (St Quentin-Fallavier, France). All drugs were diluted in isotonic and sterile sodium chloride. Animals were treated using a volume of 0.1 mL/100 g of body weight (b.w.) when injected either subcutaneously (s.c.) or intraperitoneally (i.p.). We used three treatment protocols of chronic exposure to morphine (Fig. 1).

***Study 1*:** Rats were infused with either morphine (15.6 mg/kg/day) or saline for 120h (5 days), at a constant flow rate of 1 mL/kg/h, via the jugular vein cannula (i.v.). Considering a total plasma clearance of morphine in the rat as 108 ± 32 mL/min/kg,[13] the concentration of the infused morphine solution – 0.65 mg/mL – and the rate of infusion, it is expected to attain a theoretical morphine concentration in the plasma of 100 ng/mL, at steady-state,

***Study 2*:** Rats were treated with either saline (control group) or escalating doses of morphine (test group) over a period of 5 days. The test group was given morphine i.p. twice daily: 10 mg/kg on day 1, 20 mg/kg on day 2, 30 mg/kg on day 3 and 40 mg/kg on day 4 and 5, as previously described.[11] Rodents chronically exposed to morphine have shown to develop a conditioned-place preference.[14] To provoke an intense morphine withdrawal, a single dose of naloxone (1mg/kg, s.c.) was given 1h after the administration of the last dose of morphine (day 5), and animals were sacrificed 12h after naloxone injection. Opioid withdrawal signs were visible as soon as naloxone was injected.

***Study 3*:** Animals were given s.c. morphine 10 mg/kg twice daily, over the course of 5 plus 3 additional days, with a two-day spontaneous withdrawal of morphine between the two periods of administration. To cause an acute and strong morphine withdrawal, rats received a single injection of naloxone 1 mg/kg (s.c.) 1h after the last dose of morphine (day 10). Rats were sacrificed 4h or 24h after naloxone injection.

Control rats were given physiological saline in an equal protocol of administration in the 3 studies.

2.3. Behavioral Studies

Spontaneous (18h and 36h after the last dose of morphine of the first period of administration – study 3) and naloxone-precipitated (30s after the administration of s.c. naloxone 1 mg/kg – study 2 and study 3) withdrawal syndromes were observed for 25 min in circular Plexiglas observation boxes (30 cm wide; 40 cm high). Two classes of withdrawal signs were measured: counted signs and observed signs. The number of teeth chattering, jumps and wet dog shakes were counted and the sum is a score for each counted signs. Ptosis, defecation, mastication, salivation and abnormal posture were observed over periods of 5 min, with one point being given for the presence of each sign during each period. The number of periods showing the sign was then counted (maximum score 5). Global withdrawal score was calculated as the sum of scores for all signs. B.w. was measured during the treatment and before observation period.

2.4. Quantification of morphine concentrations in rat plasma

Plasma samples collected during the 5-day morphine infusion (study 1) were used to determine the concentrations of morphine after solid-phase extraction procedure by high performance liquid chromatography tandem mass spectrometry (LC/MS-MS) (TSQ Quantum Ultra®, Fisher). Separation was carried out on a Hypersil Gold aQ® column (100 mm x 2.1 mm, 3 µm) (Fisher Scientific, Illkirch, France) maintained at 40°C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with gradient program. Briefly, morphine was extracted using Oasis HLB® solid-phase extraction cartridges (1 cc, 30 mg, Millipore-Waters, Guyancourt, France). Aliquots of rat plasma (100 µL) were added to 800 µL of human plasma and 100 µL of internal standard (morphine-d3, 200 ng/mL, Cerilliant, LGC, Molsheim, France) and 100 µL ammonium sulfate (0.5 M, pH 9.3), and loaded onto extraction columns that had been preconditioned with methanol LC-MS grade, followed by H_2O LC-MS grade, under gentle vacuum. The extraction columns were then washed with H_2O LC-MS grade and dried under vacuum for 10 min. Morphine and the internal standard were then eluted in an acetonitrile/H_2O (1:4) mix with 0.1% formic acid. After evaporation under nitrogen flow at 30°C, the residue was dissolved in 70 µL of H_2O LC-MS grade with 0.1% formic acid, centrifuged at 13 000 rpm for 10 min and 10 µL were used for injection. Detection in triple quadrupole mass spectrometer used an electrospray ionization (ESI) probe and operated in the positive ion mode. The multiple reaction monitoring transitions used for quantification were 286.08/201.04 for morphine and 289.10/164.97 for morphine-d3. The

retention times of morphine and internal standard were approximately 2.4 and 2.5 min, respectively. The linear calibration ranges were 0.15 ng/mL-200 ng/mL in plasma (r^2 >0.999).

2.5. Isolation of microvessels from rat brain cortex

All steps were carried out at 4°C. The brain cortex vessels were isolated from rat brain cortices as previously described.[15] Rats were euthanized by CO_2 inhalation and decapitated. Brain microvessels were obtained after mechanical dissection, centrifugation in a dextran (64–76 kDa, Sigma) gradient buffer and filtered through two successive 100-µm and a 20-µm nylon meshes.

2.6. RNA extraction and reverse transcription (RT)

Total RNA was isolated through homogenization and cell lysis in a TissueLyser system (Qiagen, Courtaboeuf, France), extraction using the RNeasyQiagen Micro kit according to the manufacturer's instructions (Qiagen), and treatment with DNase (RNase-Free DNase Set, Qiagen). The concentration and purity of the RNA samples obtained were assessed spectrophotometrically using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Total RNA samples were reverse transcribed into cDNA in a final volume of 20 µL. RT reagents were purchased from Invitrogen (Cergy-Pontoise, France). RT was performed on a programmable thermal cycler (PTC-100 programmable thermal controller; MJ Research, Waltham, MA, USA).

2.7. Quantitative Real-Time PCR

Specific nucleotide primer sequences (Table 1) were designed using OLIGO 7 software (Medprobe, Oslo, Norway) and synthesized by Eurogentec (Angers, France). Amplification was detected by SYBR® Green fluorescence on an ABI PRISM® 7900HT sequence detection system (Applied Biosystems, Courtaboeuf, France). The thermal cycling conditions were 2 min at 50°C then 10 min at 95°C followed by 40 amplification cycles at 95°C for 15s, at 60 or 65°C for 45s and at 95°C for 15s. Results were expressed as the ratio between the expression of the gene of interest to that of the housekeeping gene (*β-actin*) in treated and saline groups, as previously described.[11]

2.8. Western blot (WB)

Immunoblots were performed on protein extracted from isolated microvessels to measure, semi-quantitatively, the amounts of P-gp and Bcrp as previously described.[11] P-gp, Bcrp and β-actin expressions were revealed using the following primary antibodies: mouse

monoclonal antibody C219 against rat P-gp (Abcam, Cambridge, UK), rat monoclonal anti-rat Bcrp antibody BXP-53 and mouse monoclonal anti-β-actin (AC-15) antibody (HRP) (Abcam).

2.9. Protein Quantification by UHPLC MS/MS

The protein quantification of P-gp and Bcrp was performed using a QTAP approach, as previously described[16] and is detailed in supplementary methods. The denatured and alkylated proteins were precipitated with a methanol-chloroform-water mixture before digestion). The standard isotope-labeled (SIL) peptide mixture was added and samples were analyzed with an ACQUITY UPLC H-Class® System coupled to a Waters Xevo® TQ-S mass spectrometer (Waters, Manchester, UK) operated in MRM mode. Data was analyzed using Skyline software and the QuaSAR plugin[17] was used to evaluate performance and obtain linear regression equations from calibration points. Finally, a *home-developed* R script allowed the calculation of the peptide amounts from the light to heavy ratios from these equations.

We take into account the microvascular endothelial cell (EC) enrichment of each sample by analyzing the relative amount of the EC marker Pecam-1. A list of specific peptides for Pecam-1 were selected, according to the previously defined *in silico* criteria.[18] A preliminary UPLC-MS/MS sample analysis allowed us to select the three peptides with the highest signal from this list, and include them in the MRM analysis method. P-gp and Bcrp levels in each sample were normalized by dividing the calculated expression values by the sum of the areas of the three Pecam-1 peptides. Indeed, previous studies have shown that the concentration of a protein is directly proportional to the sum of the peak-areas of the three most intense peptides. [19,20]

2.10. *In situ* Rat Brain Perfusion

2.10.1. Surgery and Perfusion

The brain transport of [^{3}H]-verapamil (Perkin Elmer, Courtaboeuf, France) and [^{3}H]-mitoxantrone (Moravek, Brea, CA, USA), P-gp and Bcrp substrates respectively, was measured by *in situ* brain perfusion as previously described.[23] Rats were anaesthetized 18h after naloxone administration in rats receiving saline or morphine according to study 3. Animals were initially perfused with tracer-free Krebs-carbonate buffer for 30s and then with [^{3}H]-verapamil or [^{3}H]-mitoxantrone (0.5 µCi/mL) and [^{14}C]-sucrose (0.1 µCi/mL) for 90s at 10 mL/min. Perfusion was finished by decapitating the rat. Samples were digested in 2 mL of Solvable (Perkin Elmer) and mixed with 9 mL of Ultima gold XR (Perkin Elmer). Dual label counting was performed in a Tri-Carb counter (Perkin Elmer).

2.10.2. Calculation of the luminal BBB transport parameters

All calculations have been previously described.[23] The brain vascular volume (V_v; µL/g) was estimated from the tissue distribution of [^{14}C]-sucrose using the following equation:

$$V_v = \frac{X^*}{C^*_{perf}}$$

where X* (dpm/g) is the amount of [^{14}C]-sucrose in the right brain hemisphere and C^*_{perf} (dpm/µL) is the concentration of [^{14}C]-sucrose in the perfusion fluid.

The apparent brain volume of distribution (V_{brain}; µL/g) was calculated from:

$V_{brain} = \frac{X_{brain}}{C_{perf}}$ where X_{brain} (dpm/g) is the calculated amount of the [^{3}H]-compound in the right cerebral hemisphere and C_{perf} (dpm/µL) is the concentration of [^{3}H]-compound in the perfusion fluid.

Brain tissue radioactivity of the [^{3}H]-compound was corrected for vascular contamination with the following equation:

$X_{brain} = X_{tot} - V_v C_{perf}$ where X_{tot} (dpm/g) is the total quantity of [^{3}H]-compound measured in the brain tissue (vascular + extravascular).

Initial brain transport rate is expressed as a K_{in} (µL/g/s) and was calculated from:

$$K_{in} = \frac{V_{brain}}{T}$$

where T is the perfusion time (s).

2.11. Pharmacokinetic calculations and statistical analysis

Morphine plasma total clearance (mL/min/kg) was calculated for each rat as the rate of morphine infusion (mg/min/kg) divided by the mean of morphine plasma concentrations (ng/mL) determined at each time point, assuming that steady-state plasma concentrations of morphine were achieved from 24h after the start of morphine infusion. Mean ± SD of morphine plasma clearance was thus calculated for all rats of the study 1. Data were analyzed with GraphPad Prism® 4.0 software (San Diego, CA, USA). The results are expressed as means ± SEM. Student's unpaired t-test was used to identify significant differences between *in vivo* morphine and saline groups. All the tests were two-tailed and statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. STUDY 1 – Effect of constant morphine i.v. infusion on brain microvessel P-gp and Bcrp expressions

In the study 1, rats were infused *via* i.v. for 5 days at a constant infusion rate (1 mL/kg/h) with a morphine solution of a known concentration (0.65 mg/mL). Steady-state morphine plasma levels were achieved from 24h after the beginning of the infusion and remain reasonably stable until the 5th day of infusion (Fig. 2). The mean steady-state of morphine plasma levels was 182 ± 27 ng/mL, given a mean systemic plasma morphine clearance of 61 ± 6 mL/min/kg. UHPLC-MS/MS and WB results showed no significant modification of either P-gp or Bcrp contents in brain microvessels from rats infused by morphine, when compared to the saline-infused group (Fig. 3, Study 1, A and B) (Table 3). This clearly showed that morphine at such a constant plasma concentration for 5 days does not modulate the expression of P-gp and Bcrp in the rat BBB. This raises the question of whether or not a strong naloxone-precipitated morphine withdrawal could be responsible of P-gp and Bcrp modulation in the rat BBB.

3.2. STUDY 2 – 5-day protocol treatment with naloxone-precipitated opioid withdrawal

3.2.1. Validation of withdrawal syndrome development – behavioral studies

The development of a spontaneous withdrawal syndrome in study 2 was previously described by our research team.[24] Here, we described behavioral signs using the same morphine protocol after the precipitation of opioid withdrawal with the injection of the μ-receptor antagonist naloxone. Animals showed typical opioid withdrawal behavioral signs, including abnormal posture, diarrhea, mastication, wet-dog shakes, ptosis, salivation and jumping. The global withdrawal score (Fig. 4) was highly significant in morphine-pretreated rats (34.4 ± 2.2) as compared to saline-pretreated rats (1.6 ± 0.4) ($p < 0.001$).

3.2.2. Effect of naloxone-precipitated opioid withdrawal on *Mdr1a*/P-gp and *Bcrp*/Bcrp contents in rat cortical microvessels

Rats were sacrificed 12h after the administration of a single injection of naloxone (1 mg/kg) following the last morphine dose (day 5). In the morphine-treated rats *Mdr1a* and *Bcrp* transcript levels increased by 1.4-fold and 2.5-fold, respectively (Table 2). However, in opposition to the qPCR data, UHPLC-MS/MS and WB analyses did not evidence the expected up-regulation of P-gp and Bcrp proteins in brain microvessels of morphine-

pretreated animals as compared to saline-pretreated animals (Fig. 3, Study 2, A and B) (Table 3).

3.3. STUDY 3 – 10-day protocol treatment with both spontaneous and precipitated opioid withdrawal

3.3.1. Validation of withdrawal syndrome development – behavioral studies

Since study 3 has never been validated before as being capable of implementing an opioid dependent-state and the subsequent withdrawal – neither a spontaneous nor a precipitated one –, we conducted a set of behavioral studies similar to the ones conducted previously for study 2. Physical dependence to opioids was induced in rats by administration of morphine 10 mg/kg s.c. twice daily. The b.w. gain was measured every 12h during the course of the treatment (Fig. 5a). By the end of the first 5 days of treatment, control rats receiving physiological saline s.c. showed a 12% gain in b.w. while rats receiving morphine gained as little as 6% in b.w. ($p < 0.001$). After the first period of spontaneous withdrawal and just before starting re-dosing rats with morphine (day 8), it was clear that the weight gain during this period in the morphine-treated group was considerably weaker to that of the saline-treated group (14% in the morphine group *vs* 27% in the control group, $p < 0.001$); b.w. gain remained lower in the rats treated with morphine until the end of the treatment protocol.

Withdrawal signs were monitored at the two time-points when morphine was withdrawn. The calculated scores for the spontaneous and precipitated withdrawals are presented in Fig. 5b and Fig. 5c, respectively. Animals in spontaneous morphine withdrawal manifested typical behavioral signs, including diarrhea, teeth chattering, mastication, wet-dog shakes and ptosis. Withdrawal signs were observed 18h and 36h from the last injection of morphine after the first 5 days of treatment, and global withdrawal scores of morphine-treated rats were significantly higher than those of saline-treated rats at both 18h (9.4 ± 2.0 *vs* 2.5 ± 0.4) and 36h (5.9 ± 0.8 *vs* 1.8 ± 0.3) ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 5b). However, these manifestations became fewer and less intense at the 36h-time point of assessment of spontaneous morphine withdrawal. Animals in a naloxone-precipitated withdrawal at the end of the treatment protocol (day 10) showed a very similar behavior to that observed in study 2 once the withdrawal was precipitated: they exhibited the previously mentioned spontaneous signs of opioid withdrawal plus abnormal posture, salivation and jumping. It is clear that the naloxone-precipitated withdrawal induced a much stronger abnormal behavior (Fig. 5c) than the opioid spontaneous one (Fig. 5b). As shown in Fig. 5c, the global withdrawal score is 14-times higher in morphine-treated rats (27.0 ± 2.9) than in saline-treated rats (1.9 ± 0.2) ($p < 0.001$).

3.3.2. Effect of naloxone-precipitated opioid withdrawal on *Mdr1a*/P-gp and *Bcrp*/Bcrp contents in rat cortical microvessels

In order to evaluate the impact of the precipitated opioid withdrawal on the expression of P-gp and Bcrp in brain microvessels in study 3, qRT-PCR were performed on brain microvessels from treated rats sacrificed 4h (t=4h) and 24h (t=24h) after the administration of naloxone (1 mg/kg) (day 10). Morphine-treated group registered a significant 1.4- and 2.3-fold increase in the levels of *Mdr1a* and *Bcrp* transcript levels, respectively (Table 2). 24h after the naloxone-precipitated opioid withdrawal, *Mdr1a* mRNA levels in brain microvessels were no longer significantly different between saline- and morphine-treated rats in contrast to those of *Bcrp* which remained significantly higher in the morphine-treated group (Table 2). However, in contrast to the results obtained by qPCR at t=4h (for P-gp and Bcrp) and at t=24h (for Bcrp), UHPLC-MS/MS and WB experiments did not evidence any significant change in P-gp and Bcrp protein contents in the morphine plus naloxone-treated group compared to the saline plus naloxone-treated group (Fig. 3, Study 3, A and B) (Table 3).

3.3.3. Effect of naloxone-precipitated opioid withdrawal on the activity of P-gp and Bcrp at the rat BBB

The brain transport rate (K_{in}) of [^{3}H]-verapamil, a P-gp substrate, did not show any statistical difference 18h after naloxone dosing between saline- (1.61 ± 0.15 µL/g/s; n=5) and morphine-treated (1.81 ± 0.23 µL/g/s; n=4) animals. Any significant modification between the two groups was neither observed for the brain transport of [^{3}H]-mitoxantrone, a Bcrp substrate, (0.56 ± 0.02 µL/g/s; n=5, for saline plus naloxone-treated rats *vs* 0.56 ± 0.02 µL/g/s; n=4, for morphine plus naloxone-treated rats). These results showed that P-gp and Bcrp transport activities in brain microvessels were not modified by the implemented treatment.

Low molecular weight sucrose is commonly used as a marker of vascular space and BBB integrity once it does not significantly cross plasma membranes in a short time and its paracellular diffusion is hampered by existing tight junctions between ECs in the BBB. The sucrose whole brain volumes (V_v) in the saline plus naloxone- (16.1 ± 1.2 µL/g; n=10) and morphine plus naloxone-treated (16.9 ± 1.2 µL/g; n=9) rats were not significantly different. These sucrose spaces also agreed with the physiological vascular space,[23] showing that the physical integrity of the BBB in both groups of rats was maintained.

4. DISCUSSION

Among the described effects of morphine at the BBB, it has been shown that P-gp and Bcrp were transiently up-regulated following an exposure to morphine during 5 days, with an escalating dose regimen (10-40 mg/kg, i.p).[11,25] The aim of this new work was to know whether morphine itself or its subsequent precipitated withdrawal syndrome was responsible for P-gp and Bcrp up-regulation in the rat BBB.

First, we maintained morphine plasma levels stable at 182 ± 27 ng/mL for 4 days in rats. These plasma concentrations are close to those reached in patients continuously exposed to morphine (112 ± 50 ng/mL) for pain control.[26] The calculated plasma clearance for morphine in this study (61 ± 6 mL/min.kg) also approaches the ones found in the literature.[27] Despite a continuous morphine exposure, P-gp and Bcrp expressions were not modified in brain microvessels, which clearly demonstrates that morphine exposure by itself has no effect on these transporters in the BBB. Therefore, the increase in P-gp and Bcrp brain contents after subchronic morphine exposure reported in the literature[5,9-11] was more likely due to morphine withdrawal than to morphine itself. Since the elimination half-life of morphine is roughly 1h in both plasma and brain in the rat,[28,29] it is completely eliminated from the body in the rat 6h after morphine dosing. Therefore, induction of P-gp and Bcrp that starts 24h after the last morphine dose of a subchronic treatment[5,9] could be due to the morphine withdrawal.

In opposition to the morphine continuous infusion protocol, studies 2 and 3 consisted on a subchronic exposure to morphine that triggers a series of plasma morphine peaks and troughs of undetectable plasma concentrations of morphine. This peak and trough plasma morphine alternation is favourable to the installation of physical dependence and withdrawal once morphine treatment is suspended.[24] To test the effect of a strong precipitated morphine withdrawal on P-gp and Bcrp expressions, we applied study 2 and dosed rats with naloxone, a strong inducer of morphine withdrawal, after the last morphine injection. The protocol adopted for study 3 contrasts with study 2 by having an additional spontaneous withdrawal over a 2-day period, which increased the naloxone-precipitated withdrawal syndrome. In both studies 2 and 3, *Mdr1a* and *Bcrp* mRNA levels in brain microvessels were increased with similar folds to the ones found in the previous work during a spontaneous morphine withdrawal.[11] Protein expression of P-gp and Bcrp were studied by WB and further validated by UHPLC-MS/MS using a QTAP approach.[16] The expression levels in all samples (Supplementary Table 2) are within the same magnitude, in line with previously reported values for rat brain microvessels,[16,22] hence validating our analysis. Three specific peptides for Pecam-1 were included in the MRM method for the MS analysis in order to evaluate the EC enrichment of each sample. We observed a

heterogeneous level of the EC marker Pecam-1 across the samples (data not shown), which indicate differences on EC enrichment. The expression levels of P-gp and Bcrp were, thus, normalized by dividing the expression values by the sum of the areas of the three Pecam-1 peptides in each sample (Fig. 3 A1 and B1),[19,20] The analysis of the resulting data showed that the induction of P-gp and Bcrp was not confirmed at protein level. This difference could be explained by posttranslational regulation, as has been previously suggested.[30] The absence of enhanced P-gp or Bcrp activities in brain microvessels was yet confirmed by *in situ* brain perfusion. We previously showed that P-gp and Bcrp were up-regulated in brain microvessels not before 12h after the suspension of the morphine treatment (day 5).[11] Interestingly, the onset of the registered induction coincided with the initial development of an opioid withdrawal syndrome in the rat,[24] suggesting that these two phenomena could be mechanistically related. Furthermore, we showed that morphine-mediated P-gp and Bcrp up-regulation depended on the activation of the NMDA glutamatergic receptor and COX-2 activity.[11] In fact, naloxone-precipitated morphine withdrawal showed to provoke a 2-fold increase (from 10 μM to 21 μM, approximately) on the extracellular glutamate levels in the locus coeruleus in rats.[31] Similar extracellular glutamate levels and fold increase were observed in the hippocampus of morphine-dependent mice treated with naloxone, while no significant change was observed in saline-treated mice.[32] Corroborating these results, Sepulveda et al have demonstrated that both naloxone-precipitated withdrawal and spontaneous morphine withdrawal in the rat resulted in a significant increase in the concentrations of glutamate and aspartate in the nucleus accumbens.[33] Glutamate has been shown to increase both P-gp expression and activity in rodent brain microvessels, when exposed to relatively high concentrations (50-100 μM).[34-36] Conversely, when glutamate was used at concentrations higher than 150 μM it resulted in a reduction on P-gp expression and activity, likely due to cell toxicity.[34] Even though naloxone-precipitated withdrawal did not seem to drive such an augmentation on brain extracellular levels of glutamate, these studies evidence a potential contribute of morphine withdrawal and the subsequent glutamate release on the up-regulation of P-gp at the BBB. Still, we did not observe such P-gp and Bcrp induction in brain cortex microvessels once a strong precipitated morphine withdrawal has been triggered. It is known that there are several signaling pathways implicated in the cellular regulation of P-gp and BCRP at the BBB.[37] Thus, even though naloxone is a selective antagonist of the μ-opioid receptors, the absence of P-gp and Bcrp up-regulation in brain microvessels following the naloxone-precipitated opioid withdrawal may be explained by its effect on other non-opioid receptors,[38,39] which may also be involved in the regulation of these ABC transporters following a morphine chronic treatment. For example, naloxone showed to have a low-affinity for the NMDA receptor in a *Xenopus oocytes* model.[40]

However, to efficiently block NMDA receptors, naloxone had to be used in high (300 μM) concentrations,[40] while plasma naloxone concentrations only reached low micromolar values in *in vivo* studies similar to our experimental settings.[41] Moreover, it is unlikely that naloxone may have a direct effect on the NMDA receptor at the currently administered dose, as NMDA receptor antagonism is related to anti-nociception and prevention of withdrawal,[42,43] which are opposite to the naloxone-precipitated effects. Naloxone also proved to reverse neuropathic pain in rats via inhibition of toll-like receptor 4 (TLR4) signaling.[44,45] Activation of TLR4 leads to downstream production and release of pro-inflammatory cytokines like TNF-α and Il-1β, which are signaling factors known to play a role in the regulation of P-gp and Bcrp at the BBB.[46,47] However, naloxone only showed to inhibit TLR4 at doses 100-fold superior (100 mg/kg) to the ones used in the present study (1 mg/kg), while 1 mg/kg and 10 mg/kg doses failed to produce such effect.[45] Thus, P-gp and Bcrp up-regulation following a subchronic morphine treatment[11] may result from a sum of multiple mechanisms and signaling pathways implicated in the modulation of both transporters at the BBB, and naloxone may act at non-opioid receptors, linked for instance to an inflammatory component of morphine withdrawal, to partially hamper P-gp and Bcrp up-regulation.

Conclusion: In summary, we showed that P-gp and Bcrp protein contents and activity were not up-regulated in the rat when morphine is intravenously and continuously infused for several days nor when a strong morphine withdrawal is pharmacologically precipitated by naloxone. Thus, although we have registered an induction which temporally coincided with the development of the withdrawal syndrome following a subchronic morphine treatment in the rat,[11] our present results show that a strong precipitated morphine withdrawal has poor effects on P-gp and Bcrp at the rat BBB, suggesting that brain distribution of P-gp and Bcrp substrates may not be altered following this treatment. Still, further studies are needed in order to understand the signalling mechanisms differentially activated upon triggering spontaneous or precipitated opioid withdrawal that may regulate P-gp and BCRP at the BBB.

5. AUTHOR DISCLOSURE

Role of funding source: This study was granted by Catarina Chaves' PhD grant provided by Fundação para a Ciência e Tecnologia (FCT), reference number SFRH/BD/79196/2011. FCT had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Conflict of interest: All authors declare that they have no conflicts of interest.

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6. FIGURES

Figure 1

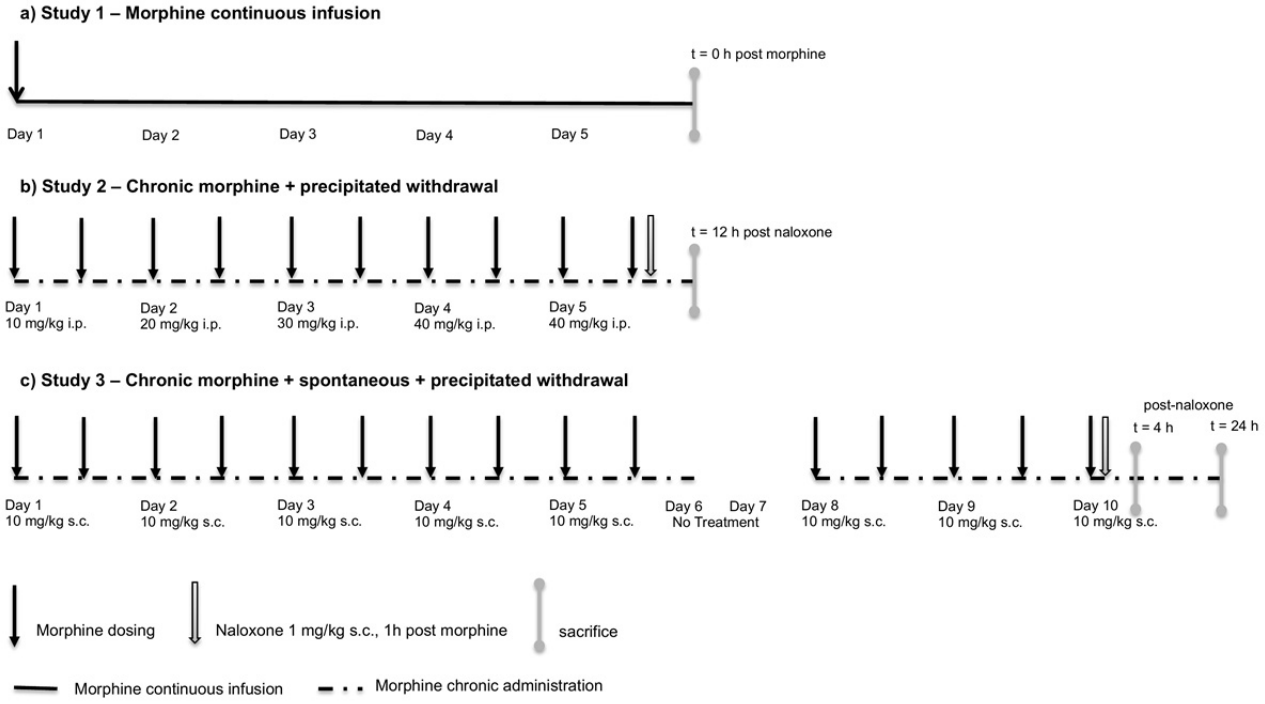


Fig. 1. Schematic representation of morphine treatment protocols**.** a) Study 1: animals received a constant i.v. morphine infusion of 0.65 mg/kg/h for 120h, and were immediately sacrificed. b) Study 2: animals were treated with morphine on a regimen of twice daily injections with escalating doses over a period of 5 days, and morphine withdrawal was precipitated by naloxone (1mg/kg, s.c.) 1h following the last morphine injection. Rats were sacrificed 12h after the injection of naloxone. c) Study 3: animals were treated with 10 mg/kg of morphine twice daily over a period of 5 plus 3 days, with a two-day interval between the two periods of administration. Morphine withdrawal was precipitated by naloxone (1mg/kg, s.c.) 1h following the last morphine injection, and rats were sacrificed 4h and 24h after.

Figure 2

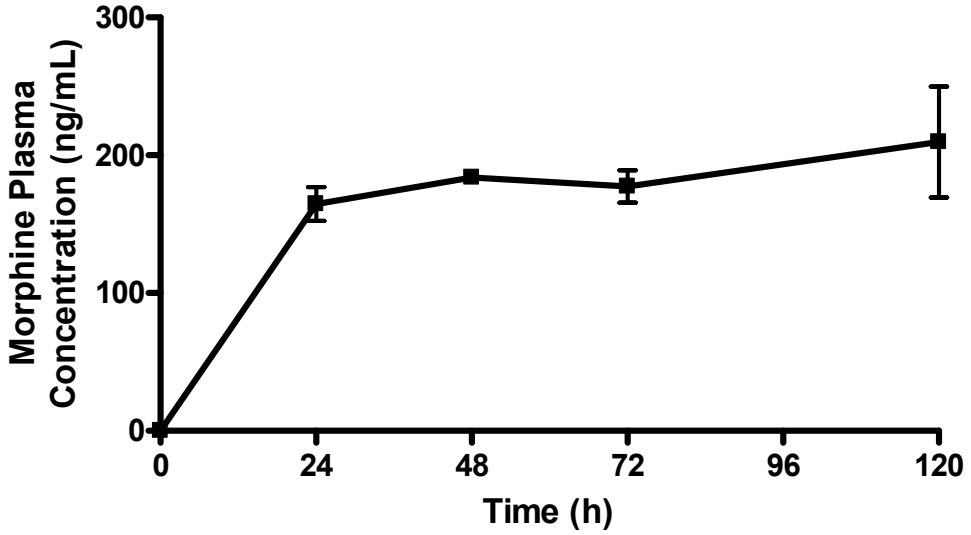
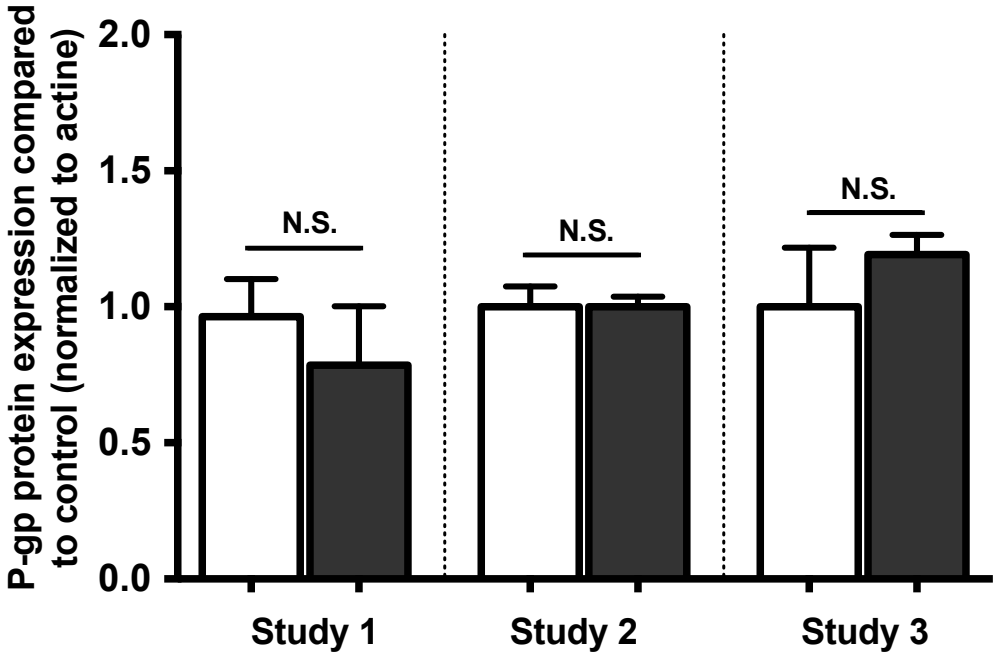


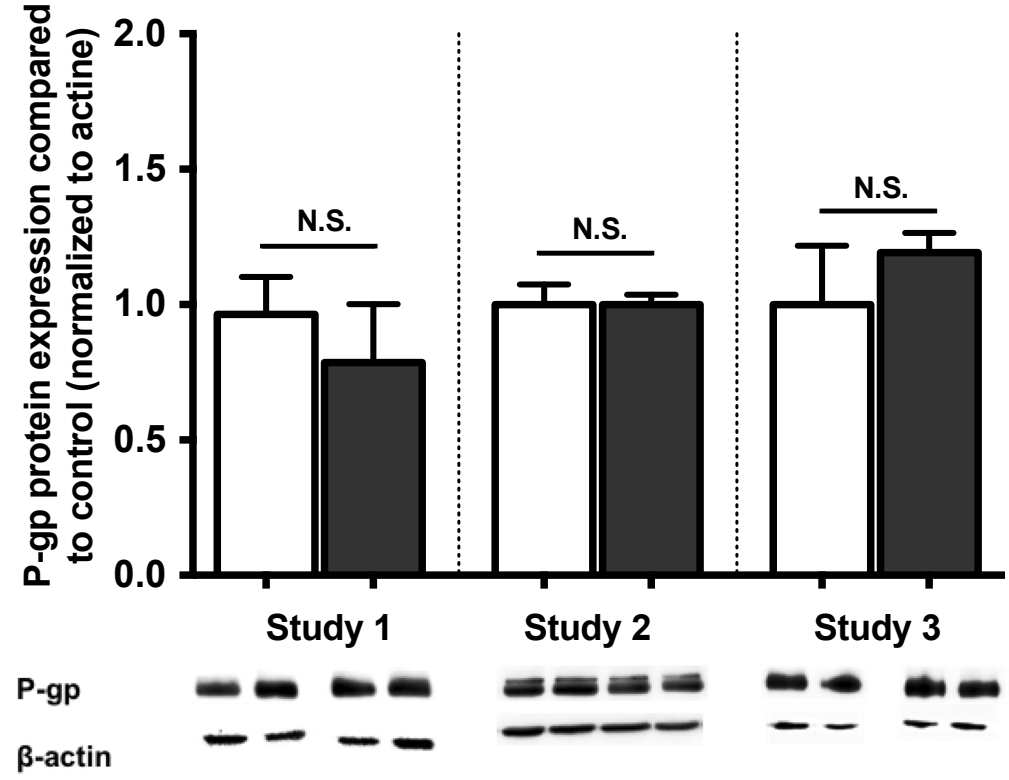
Fig. 2. Plasma concentrations of morphine versus time (mean ± SEM) during constant rate infusion of a morphine solution at 0.65 mg/kg/hr (n=4). The total infusion time was 120h.

Figure 3

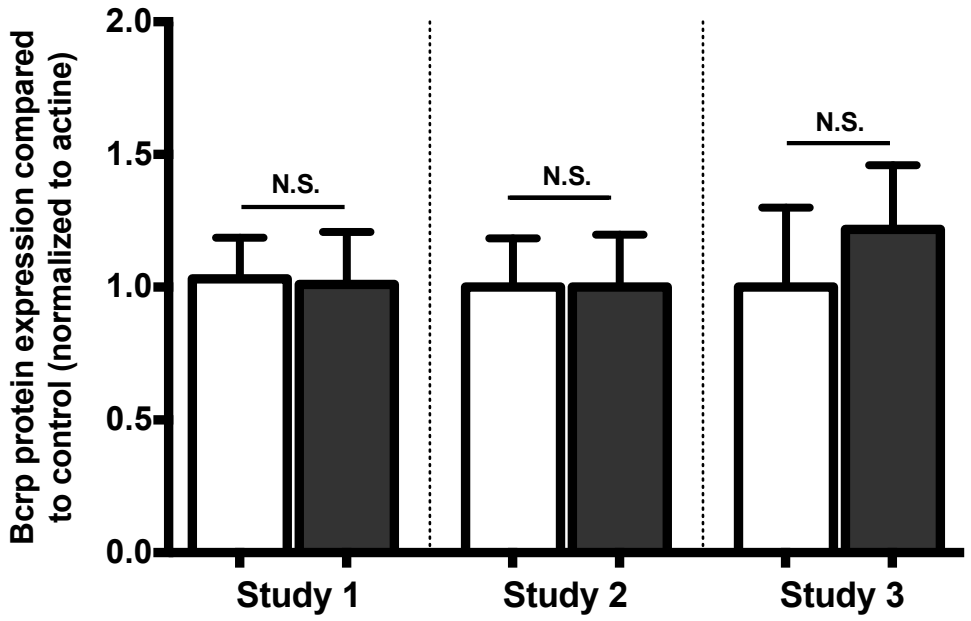
A1) UHPLC-MS/MS



A2) Western Blot



B1) UHPLC-MS/MS



B2) Western Blot

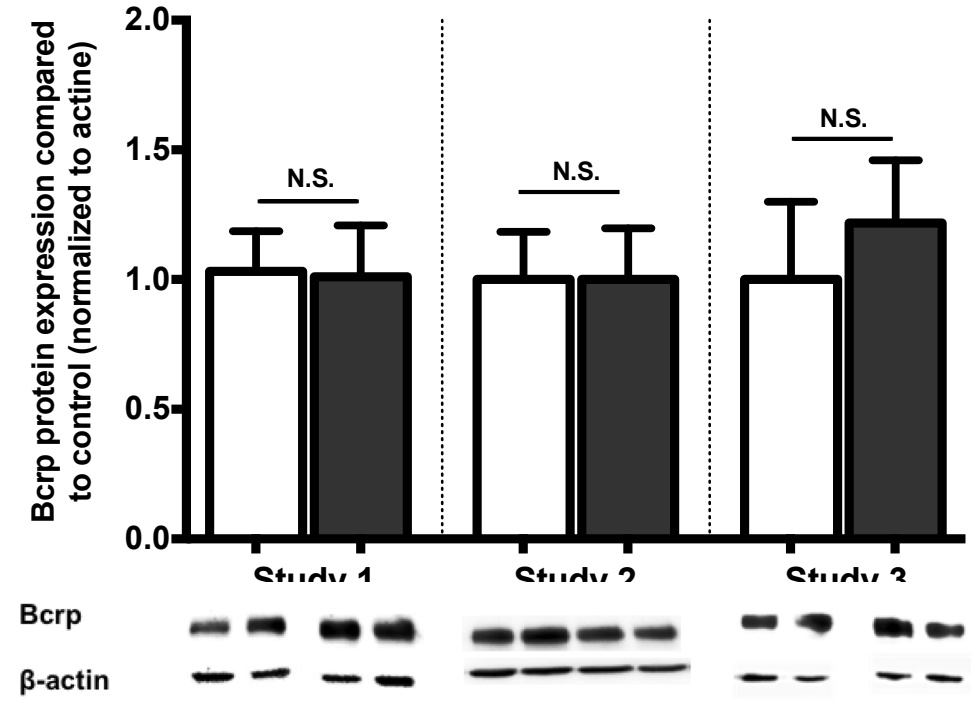


Fig. 3. Effect of continuous i.v. infusion of morphine (study 1), naloxone-precipitated opioid withdrawal following a 5 day escalating-dose morphine regimen (study 2), or naloxone-precipitated opioid withdrawal following a 5 plus 2 day fixed-dose morphine regimen (study 3) on the protein expression of P-gp (A1, A2) and Bcrp (B1, B2) in cortical

microvessels. On study 1, rats were treated over a period of 5 days and immediately sacrificed upon cessation of treatment, whereas on study 2 and study 3, rats were sacrificed 12h and 4h, respectively, after the administration of naloxone. A1 and B1 show quantification of P-gp and Bcrp specific tryptic peptides analyzed by UHPLC-MS/MS (MRM mode); protein expression values (fmol/µg) have been normalized to Pecam-1. A2 and B2 show P-gp and Bcrp expression levels analyzed by WB, where protein expression data have been normalized to β-actin protein. All generated data are compared to control group (baseline fixed at 1) and are expressed as mean ± SEM (*n* = 4-6 rats per group). Unpaired t-student test with Welch's correction. NS: non-significant.

Figure 4

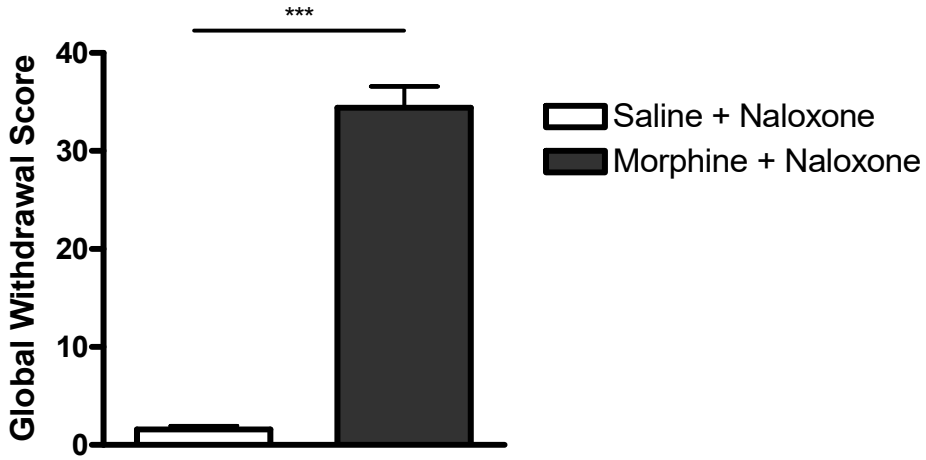


Fig. 4. Global withdrawal score in naloxone-precipitated opioid withdrawal in saline- or morphine-treated rats (study 2). Rats were subjected to observation immediately after the administration of naloxone. Two independent experiments were achieved. Results are expressed as mean ± SEM (n=6 rats per treatment). Unpaired t-student test with Welch's correction (***$p < 0.001$).

Figure 5

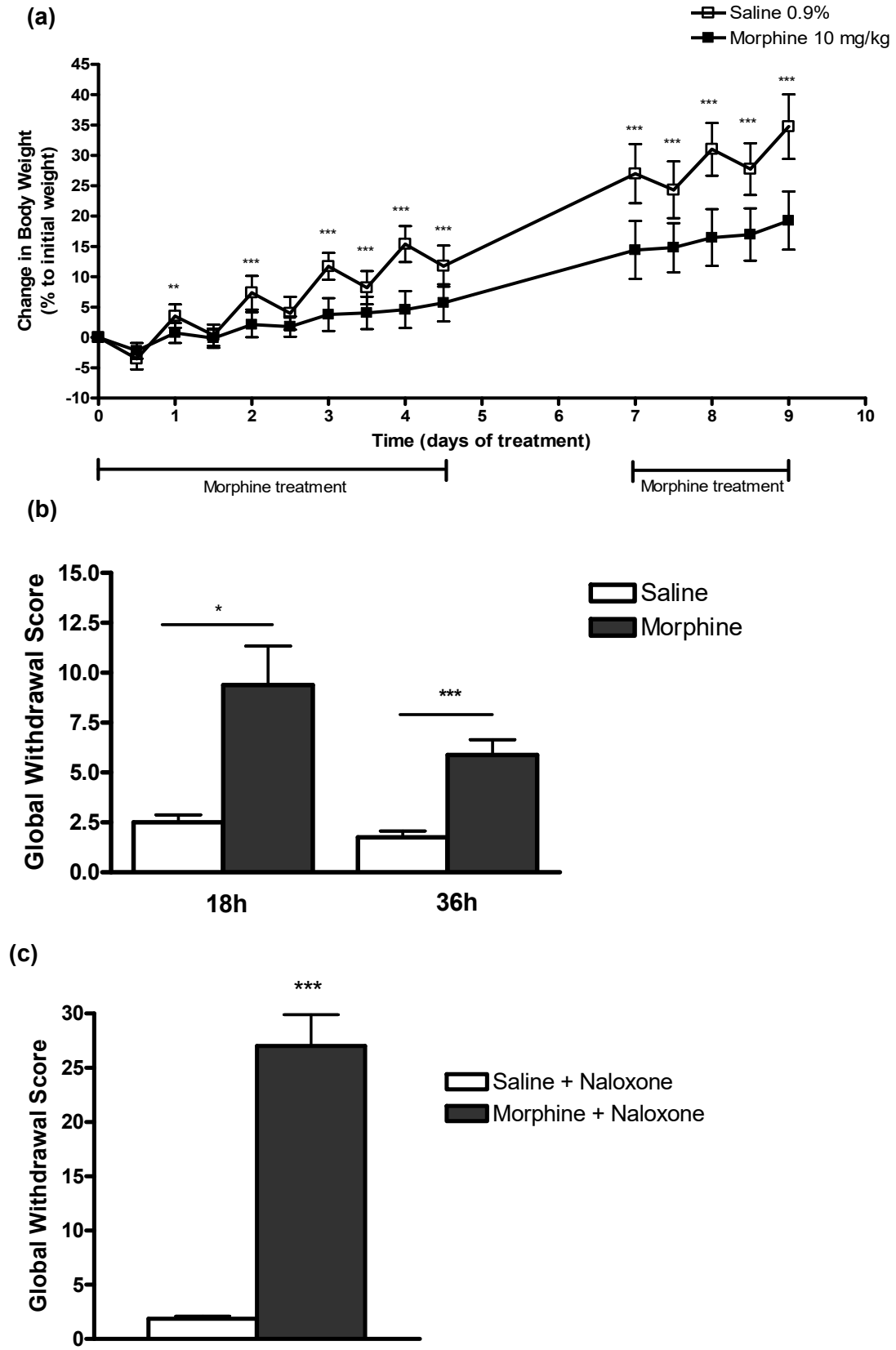


Fig. 5. (a) Evolution of weight variation as compared to weight before the first injection of morphine (study 3). Body weight of each rat was measured before the injection of

morphine in a constant dose-regimen, twice daily for 5 plus 3 days. Three independent experiments were achieved. Results are expressed as mean ± SEM. (n=12 rats per treatment) two-way repeated measure ANOVA **(**$p < 0.01$, ***$p < 0.001$); (b) Global opioid withdrawal score in the developed spontaneous opioid withdrawal (study 3). Rats were subjected to observation at 18h and 36h after the last injection of morphine over 5 days of treatment. Results are expressed as mean ± SEM (n=8 rats per treatment). Unpaired t-student test (*$p < 0.05$; ***$p < 0.001$); (c) Evolution of global withdrawal score in naloxone-precipitated opioid withdrawal. Rats were subjected to observation during 5 periods of 5 min, immediately after the administration of naloxone. Results are expressed as mean ± SEM (n=8 rats per treatment). Unpaired t-student test with Welch's correction (***$p < 0.001$).

7. TABLES

Table 1. Primer sequences used for SYBR Green-based qRT-PCR

Gene	Forward Primer (sense) (5'-3')	Reverse Primer (antisense) (5'-3')	Length (bp)
β-actin	CTGGCCCGGACCTGACAGA	GCGGCAGTGGCCATCTCTC	132
Abcb1a (Mdr1a)	CAACCAGCATTCTCCATAATA	CCCAAGGATCAGGAACAATA	97
Abcg2 (Bcrp)	CAGCAGGTTACCACTGTGAG	TTCCCCTCTGTTTAACATTACA	75

Table 2. Effect of naloxone-precipitated opioid withdrawal after subchronic treatment with morphine on the mRNA levels of the selected genes in cortical microvessels (studies 2 and 3). Rats were treated over the course of 5 days and sacrificed 12h after the administration of naloxone (study 2), or over a period of 5 plus 3 days and sacrificed 4h or 24h after the administration of naloxone (study 3). P-gp and Bcrp mRNA levels were analyzed by qRT-PCR and presented as fold expression towards saline group. All data have been normalized to β-actin mRNA and are expressed as mean ± SEM. Unpaired t test with Welch's correction (n=6 rats per group) (*p < 0.05, **p < 0.01, ***p < 0.001 vs saline-treated group).

	Gene	Saline + Naloxone (Mean ± SEM)	Morphine + Naloxone (Mean ± SEM)
Study 2		***Time point: 12h***	
	Abcb1a (Mdr1a)	1.00 ± 0.10	1.40 ± 0.08*
	Abcg2 (Bcrp)	1.00 ± 0.19	2.51 ± 0.30**
Study 3		***Time point: 4h***	
	Abcb1a (Mdr1a)	1.00 ± 0.05	1.35 ± 0.06**
	Abcg2 (Bcrp)	1.00 ± 0.11	2.26 ± 0.14***
		Time point: 24h	
	Abcb1a (Mdr1a)	1.00 ± 0.07	1.18 ± 0.07
	Abcg2 (Bcrp)	1.00 ± 0.11	1.87 ± 0.33*

Table 3. Protein Expression in cortical microvessels of rats treated with morphine or saline solution according to the studies 1, 2 and 3 (see Experimental Section). Protein levels were obtained by QTAP quantification by UHPLC-MS/MS.

	Proteins	Expression (fmol / µg of total protein)	
		Saline (Mean ± sd)	**Morphine (Mean ± sd)**
Study 1	P-gp (Abcb1a)	16.3 ± 1.64	22.4 ± 4.40
	Bcrp (Abcg2)	0.99 ± 0.17	1.64 ± 0.06
Study 2 (+ Naloxone)	P-gp (Abcb1a)	13.2 ± 3.15	15.8 ± 1.38
	Bcrp (Abcg2)	0.98 ±0.59	1.2 ± 0.66
Study 3 (+ Naloxone)	P-gp (Abcb1a)	26.3 ± 3.00	28.2 ± 1.93
	Bcrp (Abcg2)	3.6 ± 0.52	4.24 ± 2.29

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SUPLEMMENTARY DATA

EXPERIMENTAL PROCEDURES

1. Study 1: Animal surgery, treatment and blood sampling

This study was conducted in the laboratory of Bertin Pharma Company (Orléans, France). Rats underwent jugular and femoral vein cannulation while under general anesthesia with 4% isoflurane: 96% oxygen. Following surgery, the animals were allowed to recover overnight before starting the experiments. Groups of rats received an infusion for 120h (5 days), at a constant flow rate of 1 mL/kg/h, via the jugular vein cannula (i.v.) of either morphine (15.6 mg/kg/day) or saline. If we consider a total plasma clearance of morphine in the rat as 108 ± 32 mL/min/kg,[1] the concentration of the infused morphine solution – 0.65 mg/mL – and the rate of infusion were chosen to obtain, at steady-state, a theoretical morphine concentration in the plasma of 100 ng/mL.

Blood samples (200 µL) were collected twice daily during 5 days of morphine infusion, via the femoral vein cannula, in order to prevent possible contamination from the morphine infusion. Blood samples were subsequently centrifuged, and plasma was collected and stored at -20°C, before they were used for quantification of morphine concentration by LC/MS-MS.

2. Protein Quantification by UHPLC MS/MS

2.1. Quantitative targeted proteomics (QTAP) method

P-gp and Bcrp were quantified using a QTAP approach, as previously described [2]. The protein expression is calculated according to the measured concentration of surrogate tryptic peptides determined by using UHPLC MS/MS in multiplexed reaction monitoring (MRM) mode. These target peptides were previously reported [3] (for further information, consult Supplementary Table 1). The standard isotope-labeled (SIL) peptides were added to samples prior to analysis. The UHPLC-MS/MS parameters optimization was performed with the assistance of Skyline® software [4]. The standard solutions of peptides were either provided by Pepscan (Lelystad, The Netherlands), or synthetized by Pr. M. Vidal and Dr. W. Q. Liu (UMR 8638, Paris, France). The former standard solution concentrations were determined after acid hydrolysis and amino-acid analysis (Dr. E. Thioulouse, Hôpital Trousseau, Paris, France).

Enzymes and ProteaseMAX detergent were purchased from Promega (Charbonnières-les-Bains, France), solvents were obtained from VWR (Strasbourg, France) and all other reagents were from Sigma Aldrich (Saint Quentin Fallavier, France).

2.2. Protein digestion

Protein samples were digested as previously described [2,5]. Briefly, protein extracts were diluted in denaturing buffer (7 mol.L^{-1} guanidine hydrochloride, 10 mmol.L^{-1} EDTA, 500 mmol.L^{-1} Tris pH 8.5), reduced by DTT and alkylated by iodoacetamide. The resulting denatured and alkylated proteins were precipitated with a methanol-chloroform-water mixture. The protein pellets were then ressuspended in an urea solution at 8 mol.L^{-1} and further diluted to 1.2 mol.L^{-1} by using 0.1 mol.L^{-1} Tris pH 8.5. Samples were first pre-digested for 3h at RT with rLysC endoprotease (enzyme:protein ratio = 1:50). Proteins were further digested overnight at 37°C using trypsin (enzyme:protein ratio = 1:100) with 0.05% (W/W) ProteaseMAX detergent. The SIL peptide mixture was added and digestion was stopped by adding formic acid to a final concentration of 2.5% (V/V).

2.3. Protein quantification

Samples were analyzed with an ACQUITY UPLC H-Class® System (Waters, Manchester, UK) coupled to a Waters Xevo® TQ-S mass spectrometer (Waters, Manchester, UK). The column was an Acquity Peptide BEH® C18 Column (300Å, 1.7 µm, 2.1 mm X 100 mm) supplied by Waters (Guyancourt, France). Peptides were separated in a 24 min gradient going from 100 % aqueous mobile phase (water and 0.1% formic acid (V/V)) to 35% of apolar mobile phase (ACN with 0.1% formic acid (V/V)), at a flow-rate of 0.5 mL/min, at 30°C. The Xevo TQ-S was operated in positive electrospray ionization (ESI+) mode using a capillary voltage of 2.80 kV, desolvation gas at a flow-rate of 800 L/h, at 650°C. Peptides were detected by a multiplexed MRM-mode method using three to four transitions per native- or heavy-labeled peptide (Supplementary table 1). The peak areas were integrated using the Skyline software. The resulting calibration curves were analyzed with the Skyline's QuaSAR plugin [6] to evaluate performance and obtain the linear regression equations. Finally, a *home-developed* R script allowed the calculation of the peptide amounts from the light to heavy ratios from these equations.

Supplementary table 1. Target peptides and selected ions used in the UHPLC-MS/MS multiplexed SRM method analysis.

Protein	Peptide Sequence	Isotope Type	Precursor M/z	Precursor Charge	Product M/z	Product Charge	Fragment Ion Type
Bcrp	SSLLDVLAAR	light	522.8	2	757.5	1	y7
					644.4	1	y6
					529.3	1	y5
					430.3	1	y4
	SSLLDVLA[+4]AR	heavy	524.8	2	761.5	1	y7
					648.4	1	y6
					533.4	1	y5
					434.3	1	y4
P-gp	NTTGALTTR	light	467.8	2	719.4	1	y7
					618.4	1	y6
					561.3	1	y5
					490.3	1	y4
	NTTGALTTR[+10]	heavy	472.8	2	729.4	1	y7
					628.4	1	y6
					571.3	1	y5
					500.3	1	y4

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General outcome:

Study 1 was conducted with the purpose of maintaining a continuous, uninterrupted exposure to morphine, and thus evaluate if morphine itself has a direct effect on the expression of P-gp and Bcrp at the rat BBB. The results obtained with such study demonstrated that, even though animals were continuously exposed to morphine and had attained significant plasmatic concentrations of morphine throughout the treatment, P-gp and Bcrp protein expressions did not change in rat brain microvessels. This suggests that the observed up-regulation of both ABC transporters in the previous study (Yousif et al. 2012) was not a direct consequence of morphine on the cascade of regulation of P-gp and Bcrp at the BBB.

Since the induction of P-gp and Bcrp has been reported to appear 12h to 24h after the last morphine dose of a subchronic dosing regimen (Aquilante et al. 2000; Yousif et al. 2012; Zong and Pollack 2003), it seems likely that morphine withdrawal can be responsible for this modulation. Therefore, studies 2 and 3 are two different protocols that favor the installation of morphine physical dependence, and were developed in order to induce an intense opioid withdrawal, precipitated by naloxone. The considerable high global withdrawal scores observed confirm the success of the implemented protocols to attain the development of morphine dependence and the subsequent precipitated withdrawal. Naloxone-precipitated withdrawal after escalating or chronic morphine dose regimens increased *Mdr1a* and *Bcrp* mRNA levels by 1.4-fold and 2.4-fold, respectively. Such results are of similar magnitude to the previously obtained during a spontaneous morphine withdrawal (Yousif et al. 2012). However, induction of P-gp and Bcrp was not confirmed at protein level, neither by WB nor by UHPLC-MS/MS. P-gp or Bcrp transport activities at the rat BBB, analyzed by *in situ* brain perfusion, also remained unchanged, which corroborated such findings.

The P-gp and Bcrp modulation discrepancy found at mRNA and protein levels following naloxone-precipitated withdrawal protocols could be due to posttranslational regulation, like it has been previously suggested (Vogel and Marcotte 2012), as well as it could mean that naloxone may act at non-opioid receptors and hamper P-gp and Bcrp up-regulation, such as receptors linked to an inflammatory component of morphine withdrawal. The following presented study does a large screening of several neurotransmitter receptors, many of them implicated in inflammatory cell-cell signaling, as potential receptors expressed at the rat BBB, and which may have a role in the modulation of these ABC transporters.

III. Manuscript 3

Comparative analysis of the expression of several neurotransmitter receptors in the rat brain cortex, and in rat isolated brain microvessels

Unsubmitted manuscript

Rationale and Objectives:

The regulation and maintenance of the BBB structure and properties is a product of the close interaction between cells constituting the NVU, particularly the BECs, astrocytes, pericytes, and microglia, and the presence of multiple modulating factors in both the brain ISF and the bloodstream, such as neurotransmitters, hormones, ions and cytokines. Currently, there is little data on the expression of major neurotransmitter receptors in the different cell types composing the BBB. The exploration of the expression of neurotransmitter receptors present at the BBB would allow a better understanding of some of the active cell:cell communication mechanisms at the BBB, which are most likely implicated in neurovascular signaling and in the modulation of BBB structure and function. Furthermore, this can bring further insight into some of the already partially known mechanisms of regulation of ABC transporters, such as the one that morphine is implicated in. For such study, it is crucial to select the most appropriate BBB model that more closely preserves the original BBB structure and features.

Therefore, the subsequently presented study had two main aims:

- To compare two different approaches of isolation of rat brain microvessels, mechanical dissection and enzymatic digestion, for the yield of the purest microvessel fraction for the study of the BBB. For this purpose, the expression of several specific cell markers for the different cell constituents of the NVU was compared between samples obtained by either mechanical dissection or enzymatic digestion. The expression of such markers in isolated microvessels was also compared against the expression in rat brain cortex as a measure of enrichment on the BBB cell markers.
- To assess the expression of 11 neurotransmitter receptors in both rat brain cortex samples and in isolated rat brain microvessels: adrenergic receptors α_{2A}, β_1 and β_2 (*Adra2a*, *Adrb1*, *Adrb2*), adenosine receptors A_1, A_{2A} and A_3 (*Adora1*, *Adora2a*, *Adora3*), ATP receptors $P2X_7$ and $P2Y_1$ (*P2rx7*, *P2ry1*), cannabinoid receptors CB_1 and CB_2 (*Cnr1*, *Cnr2*), and the glutamate receptor subunit *NMDAr1*.

The expression of different genes of interest was evaluated using the qRT-PCR technique.

Comparative analysis of the expression of neurotransmitter receptors in the rat brain cortex, and in rat isolated brain microvessels

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Abbreviations: ABC, ATP-binding cassette; BBB, Blood-brain barrier; BCRP, Breast Cancer Resistance Protein; BSA, Bovine Serum Albumin; CB, cannabinoid receptors; CNS, Central Nervous System; CT, Cycle Threshold; ECs, Endothelial Cells; HBSS, Hank's buffered salt solution; NVU, Neurovascular Unit; qRT-PCR, quantitative real time-Polymerase Chain Reaction; RT, Reverse Transcription; TBP, TATA box-binding protein; WB, Western Blot

Abstract:

The blood-brain barrier (BBB) is crucial to the maintenance of the correct neural homeostasis and microenvironment. The dynamic interplay between the cells composing the neurovascular unit (NVU), as well as the input of neurotransmitters, hormones, ions and cytokines, coming from both the nervous tissue and the bloodstream, allows the regulation of the proper BBB structure and function. In this study, we performed the isolation of rat brain microvessels, a powerful tool for the BBB study, through two different approaches, mechanic and enzymatic, and compared the resulting mRNA levels of each specific brain cell markers in microvessels obtained by each technique and to rat brain cortex, by qRT-PCR. In addition, also by qRT-PCR, the expression of 11 neurotransmitter receptors (adrenergic receptors α_{2A}, β_1 and β_2, adenosine receptors A_1, A_{2A} and A_3, ATP receptors $P2X_7$ and $P2Y_1$, cannabinoid receptors CB_1 and CB_2, and glutamate receptor subunit NMDAr1) was analyzed in both rat brain cortex samples and in isolated rat brain microvessels obtained by these two techniques.

An enrichment of EC (2-fold for *glut-1*) and pericyte (4.8-fold and 1.2-fold for *ng-2* and *pdgfrb*, respectively) markers was observed in microvessels isolated enzymatically in comparison to the mechanic isolation. Also, a general reduction of the expression of the selective markers for the other brain cell types (*gfap*, *s100b* 5-fold, *mbp* 1.6-fold, *syp* 10-fold reduction) was registered from microvessels isolated mechanically to enzymatically, suggesting that the enzymatic approach yield a more pure fraction of brain capillaries than the mechanic approach. According to the obtained mRNA levels, the NMDA receptor subunit NR1, and the ATP receptors $P2Y_1$ and $P2X_7$ are the most expressed genes in cortex samples, being 257-, 156- and 85-fold more expressed than the least expressed gene tested in cortex, which is the adrenoceptor β_2, followed by the CB_2 and the adrenoceptor α_{2A} as the least expressed receptors in the rat cortex. Among the analyzed neurotransmitter receptors, rat brain microvessels demonstrate to poorly express the adenosine receptor A_1, the adrenoceptors α_{2A} and β_1, as well as both CB receptors, while the ATP receptors $P2Y_1$ and $P2X_7$, and the adenosine receptor A_{2A} assume a very important expression among the tested genes. These results suggest that purinergic receptors $P2Y_1$ and $P2X_7$ and adenosine A_{2A} are highly express at the rat BBB, and so may play an important role in the cell signaling between the bloodstream and the CNS, and in the regulation of BBB function.

Keywords: Blood-brain barrier, isolated microvessels, neurotransmitter, receptors, RT-PCR

1. INTRODUCTION AND OBJECTIVES

The blood-brain barrier (BBB) is an essential element required for the maintenance of the adult central nervous system (CNS) proper homeostasis and function. Communication between the CNS and the vasculature is mediated through the constitutive components of the BBB, microvessel endothelial cells (ECs) closely connected by tight junctions, surrounded by pericytes that share the same basal membrane with ECs, and astrocyte end-feet processes. The dynamic interplay between these cells within the neurovascular unit (NVU) allows the regulation of the BBB homeostasis, CNS blood flow, entry and clearance of compounds into/out of the CNS. The brain access of many compounds, including many drugs used in therapy, is amply limited by ATP-binding cassette (ABC) efflux transporters present at the luminal membrane of the brain microvessel ECs (Chaves et al. 2014; Coureuil et al. 2014).

The neural microenvironment is crucial for the neurovascular development and maintenance of BBB function and properties. The overall exact mechanisms underlying the preservation of normal function of the NVU, and how cells of the NVU are involved in these mechanisms are still unknown, but are without doubt a result of the neurotransmitter, hormone, ionic and cytokine input coming from both the nervous tissue and the bloodstream (del Zoppo 2008; Iadecola and Nedergaard 2007; Paemeleire 2002). Recent studies have uncovered critical insights about neural and vascular communication within the NVU, particularly related to extracellular matrix-mediated signaling, as well as through calcium-mediated signaling (McCarty 2009). At present time, little is known about the role of major neurotransmitter receptors in the neurovascular signaling, and particularly on the expression of these receptors in the different cell-types composing the BBB, namely in brain ECs, astrocytes, pericytes, and microglia. While the presence of some neurotransmitter receptors in cerebral vessels, such as the adrenergic receptors, which serve to regulate vasoconstriction and vasodilation as in the systemic vasculature, is consensual (Durieu-Trautmann et al. 1991), the expression and role of neurotransmitter receptors such as the purine, cannabinoid and glutamate receptors remain controversial. Furthermore, cerebral vessels of different size and nature (arteries, veins, and capillaries) may considerably differ regarding the presence/absence and level of expression of such receptors.

In the present study, we used isolated grey cortical matter of rat brain, and isolated rat brain cortical microvessels in order to check and compare the expression of specific cell markers for each constituent of the NVU in these samples, and therefore evaluate the purity of the obtained isolated microvessels by quantitative real time-polymerase chain reaction (RT-PCR). Furthermore, the expression of 11 neurotransmitter receptors in either rat brain cortex samples and in isolated rat brain microvessels was assessed: adrenergic

receptors α_{2A}, β_1 and β_2 (*Adra2a*, *Adrb1*, *Adrb2*), adenosine receptors A_1, A_{2A} and A_3 (*Adora1*, *Adora2a*, *Adora3*), ATP receptors $P2X_7$ and $P2Y_1$ (*P2rx7*, *P2ry1*), cannabinoid receptors CB_1 and CB_2 (*Cnr1*, *Cnr2*), and the glutamate receptor subunit *NMDAr1*.

2. MATERIALS AND METHODS

2.1. Animals

Adult male Sprague–Dawley rats weighing 150 to 200g (4-5 weeks old) were purchased from Janvier laboratory (Le Genest-Saint-Isle, France). They were housed under standard 12h light/dark conditions (light from 8:00 a.m. to 8 p.m.) in a temperature- and humidity-controlled room, and had access to food and water *ad libitum*. All animal experiments complied with the standards and guidelines promulgated by the latest European Union Council Directive (2010/63/EU) and were approved by the local ethics review committee (Paris Descartes University n°12-186).

2.2. Isolation of rat brain cortex samples and of microvessels from rat brain cortex

All steps were carried out at 4°C. Rat brain cortex vessels were isolated according to (Yousif et al. 2008), which minimizes contamination from astrocyte and neuron mRNA. Rats were euthanized by CO_2 inhalation and decapitated. Brains were immediately removed and placed in ice-cold Hank's buffered salt solution (HBSS) supplemented with 10 mM HEPES. Brain cortices were isolated and either collected for homogenization and RNA extraction (< 30 mg), or used to isolate brain microvessels. To obtain brain microvessels, two different approaches, an enzymatic and a mechanic method, were adopted.

Mechanic method: Total isolated cortices were minced in HBSS, and homogenized in a Potter-Thomas homogenizer (0.25 mm clearance). The resulting homogenates were centrifuged at 2000g for 10 min at 4°C, and each pellet was suspended in 17.5% dextran (64–76 kDa, Sigma) and centrifuged at 4400g for 15 min at 4°C. The resulting pellets were suspended in HBSS containing 1% bovine serum albumin (BSA), and passed through a 100-µm nylon mesh. The filtrate was then passed through a 20 µm nylon mesh where microvessels (mainly 4–6 µm) were retained and immediately collected.

Enzymatic method: Brain cortices were isolated and minced as described in the mechanic method, and centrifuged at 600g for 5 min at 4°C. The remaining pellets were enzymatically digested and homogenized in a buffer solution containing Liberase DL (0.195 Wunsch U/mL) and Dnase I (20 Kunitz U/mL) for 45 min at 37°C. The resulting homogenates were re-centrifuged and each pellet was then suspended in HBSS containing 18% BSA and centrifuged at 1500g for 15 min at 4°C. The resulting pellets were suspended in HBSS containing 1% BSA and passed through a 10 µm nylon mesh.

The fraction retained in the mesh was subsequently collected and used for further analysis.

2.3. Extraction and dosage of total RNA from rat brain cortex and rat brain microvessels

Total RNA was isolated through tissue homogenization and lysis in a TissueLyser system (Qiagen, Courtaboeuf, France) and extraction using the RNeasyQiagen Micro kit (for rat brain microvessel samples) and MiniPlus kit (for rat brain cortex samples) according to the manufacturer's instructions (Qiagen). The concentration and purity of the RNA samples obtained were assessed spectrophotometrically using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.4. Reverse Transcription and Quantitative Real-Time PCR

cDNA synthesis: All RNA samples were reverse transcribed in the same Reverse Transcription (RT) from 1 µg of total RNA into cDNA in a final volume of 20 µL. Reverse transcription reagents were purchased from Invitrogen (Cergy-Pontoise, France). RT was performed on a programmable thermal cycler (PTC-100 programmable thermal controller; MJ Research, Waltham, MA, USA).

RT-PCR theoretical principles: The transcript levels for each target gene were investigated by qRT-PCR as previously described (Coureuil et al. 2010; Di Tullio et al. 2004). Relative expressions of target genes were determined for samples with a gene Cycle Threshold (CT) lower than 33. Target genes were considered to be unquantifiable when CT values were above 33. To ensure the validity of the comparison between the 11 neurotransmitter mRNA expressions, efficiency of PCR (E) was checked and it was greater than 95% for all the tested genes.

The ΔΔCT method was used to compare the expression of a gene of interest in rat brain microvessels and cortex, which were normalized to that of the housekeeping gene TATA box-binding protein (TBP). This comparison was possible because the expression of the TBP gene in cortex and microvessels was not statistically different in any of the tested samples.

PCR amplification: Specific nucleotide primer sequences (see Table 1) were designed using OLIGO 7 software (Medprobe, Oslo, Norway) and synthesized by Eurogentec (Angers, France). Amplification was detected by SYBR® Green fluorescence on an ABI PRISM® 7900HT sequence detection system (Applied Biosystems, Courtaboeuf, France). Thermocycling was carried out in a final volume of 20 µL. The thermal cycling conditions

were 2 min at 50°C then 10 min at 95°C followed by 40 amplification cycles at 95°C for 15s, at 60 or 65°C for 45s and at 95°C for 15s.

2.5. Statistical Analysis

Data were analyzed with GraphPad Prism® 6.0 software (San Diego, CA, USA). The results are expressed as means ± SEM. One-way and two-way ANOVA comparisons with the Bonferroni's post-test adjustment were used to compare the expression of genes in different analyzed tissues. Statistical significance was set at $p < 0.05$ for all the tests.

3. RESULTS

3.1. Expression profiles of cell markers for endothelial cells (*glut1*), pericytes (*ng2*, *pdgfrb*), astrocytes (*gfap*, *s100b*), microglia (*cd11b*), oligodendrocytes (*mbp*) and neurons (*syp*)

The mRNA expression of several genes considered as CNS cell markers for ECs (*glut1*), pericytes (*ng2, pdgfrb*), astrocytes (*gfap, s100b*), microglia (*cd11b*), oligodendrocytes (*mbp*) and neurons (*syp*) was evaluated by qRT-PCR, and their relative abundances between samples – brain cortex homogenates, rat brain microvessels isolated by an enzymatic method and by a mechanic method – were compared (Table 2). The marker used for ECs, *glut1*, was significantly enriched in isolated microvessels when compared to brain cortex, registering 8.5-fold and 15.6-fold enrichment in microvessels isolated mechanically and enzymatically, respectively ($p < 0.05$). Similar results were found for *ng-2* and *pdgfrb*, both selective markers used for pericytes, which are in strict contact with the BBB endothelium: *ng-2* was 15.2-fold ($p < 0.01$) and *pdgfrb* 12-fold ($p < 0.001$) more expressed in brain microvessels isolated enzymatically than in the cortex. In contrast, the relative amounts of *gfap* and *s100b* (representing contamination by astrocytes) were significantly reduced in isolated microvessels when compared to their presence in brain homogenates: when we compare microvessels isolated enzymatically to brain homogenates, *gfap* expression was reduced 25.6-fold while *s100b* was reduced 67.7-fold ($p < 0.001$). As expected, *syp* mRNAs, tracing contamination by neurons, were massively lowered in microvessels isolated enzymatically. In brain microvessels obtained enzymatically, a reduction of 4.3-fold ($p < 0.05$) in the expression of *mbp*, a selective marker used to detect oligodendrocytes, was also registered, when compared to the brain cortex.

Comparing the two methods of isolation of rat brain microvessels, there is an enrichment of EC and pericyte cell markers when the enzymatic method is used – *glut1* is approximately 2-fold, *ng-2* 4.8-fold ($p < 0.01$) and *pdgfrb* 1.2-fold higher in microvessels isolated enzymatically than in the ones isolated mechanically. This suggests that microvessels isolated with an enzymatic approach yield a more pure fraction of brain capillaries than microvessels isolated with a mechanic approach. In agreement with these data, a general reduction of the expression of the selective markers for the other cell types (*gfap*, *s100b* 5-fold, *mbp* 1.6-fold, *syp* 10-fold reduction) was registered from microvessels isolated mechanically to enzymatically, evidencing a clear reduction on the contamination coming from other cell elements surrounding the brain capillaries in these microvessel isolates.

3.2. Expression profiles of genes encoding neurotransmitter receptors: *Adra2a*, *Adrb1*, *Adrb2*, *Adora1*, *Adora2a*, *Adora3*, *P2rx7*, *P2ry1*, *Cnr1*, *Cnr2*, *NMDAr1*

The expression of 11 neurotransmitter receptor genes – adrenergic (*Adra2a*, *Adrb1*, *Adrb2*), adenosine (*Adora1*, *Adora2a*, *Adora3*), ATP (*P2rx7*, *P2ry1*), cannabinoid (*Cnr1*, *Cnr2*), and glutamate receptor *NMDAr1* – was investigated by qRT-PCR in samples from rat brain cortex homogenates and rat brain microvessels isolated mechanically and enzymatically. The expression of all the tested genes was detectable and quantifiable in all samples. The amount of each transcript in the three types of samples was normalized to the smallest amount of mRNA of one target gene detectable and quantifiable in the same kind of sample (to the target gene the least expressed is given the arbitrary expression of 1).

Among the tested, *NMDAr1*, *P2ry1* and *P2rx7* are the most expressed genes in cortex samples, being 257-, 156- and 85-fold more expressed than the least expressed gene tested in cortex, which is *Adrb2* (Fig. 1, a). *Adrb2* together with *Adrb1*, *Cnr2* and *Adra2a* are the least expressed receptors in the rat cortex.

The least expressed gene in rat brain microvessels isolated enzymatically was found to be *Adora1*, followed by *Cnr1* (2.1-fold), *Adrb1* (6.4-fold), *Cnr2* (6.8-fold) and *Adra2a* (7.2-fold). In microvessels isolated enzymatically, it was also found that *P2ry1* (788-fold of the least expressed gene, *Adora1*), *Adora2a* (454-fold), *P2rx7* (264-fold) and *Adrb2* (21-fold) assume a very important expression among the tested genes (Fig. 1, b).

In rat brain microvessels isolated mechanically, while *Adora1* and *Adra2a* are the least expressed genes, followed by *Adora3* (1.1-fold), *Cnr2* (1.6-fold), *Adrb1* (3.2-fold) and *Cnr1* (5-fold), and similarly to what was found in microvessels isolated enzymatically, *P2rx7* (150-fold), *Adora2a* (126-fold) and *P2ry1* (80-fold) are also among the most expressed genes (Fig. 1 c).

3.3. Comparison of expressions of studied neurotransmitter receptors genes between rat brain isolated microvessels and rat brain cortex

Once the expression of the selected neurotransmitter receptors was determined in rat brain cortex homogenates and in rat brain microvessels, their expression among tissues was compared (Table 3). Comparisons subsequently described will mainly focus on the expression levels found in rat brain cortex and in rat brain microvessels isolated using the enzymatic approach, since this technique yielded the purest capillary fraction, and thus should best reflect the expression of neurotransmitter receptors at the BBB. The transcript levels found for *Adora1*, *Cnr1* and *NMDAr1* in rat brain microvessels are approximately

50-fold lower ($p < 0.001$) to those found in brain cortex samples (Table 3, Fig. 2), suggesting that these genes are barely expressed in rat brain capillaries, or even that they might come from neuronal contamination. In rat brain microvessels isolated mechanically the lower expression for these genes is less substantial, being *Adora1* 25-fold, *Cnr1* 8.5-fold and *NMDAr1* 4.2-fold less expressed than in brain cortex ($p < 0.001$). The higher expression of *Adora1*, *Cnr1* and *NMDAr1* in rat brain microvessels isolated mechanically than in the capillaries isolated enzymatically may suggest that cells which closely surround the capillaries, such as astrocytes and pericytes, may express these receptors. The expressions of *Adrb1* and *Adora3* in rat brain microvessels isolated enzymatically were found to be approximately 3.5-fold ($p < 0.01$) and 8.5-fold ($p < 0.001$) inferior to ones found in brain cortex samples, respectively. The adrenergic receptor *Adrb2* and the adenosine receptor *Adora2a* are the only neurotransmitter receptors studied seeing their expression to be increased from rat brain cortex samples to rat brain microvessels, being 7-fold and 5-fold higher, respectively (Table 3, Fig. 2). In turn, the adrenergic receptors *Adra2a*, ATP receptors *P2rx7*, *P2ry1* and the cannabinoid receptor *Cnr2* do not see their expressions considerably altered from brain cortex to brain microvessel samples. However, we should note that *Adra2a* and *Cnr2* are among the least expressed genes in the brain cortex, whereas *P2rx7* and *P2ry1* are among the most expressed, meaning that the expression of these latter genes in the microvessels is very significant, as shown in Fig. 1 b, c, and in Fig. 2. This suggests that these receptors are present in the NVU, and so may play a role in the cell signaling between the bloodstream and the CNS.

4. DISCUSSION

Purine receptors are membrane-bound receptors that have extracellular nucleosides (P1 receptors) or nucleotides, such as ATP (P2 receptors) as receptor ligands, and are thought to be important signaling intervenients for the normal function of the NVU (Iadecola and Nedergaard 2007). Such receptors are involved in regulation of vasodilatation and of BBB permeability (Peterson et al. 2010; Sipos et al. 2000). Adenosine (P1) receptors consist of four known subtypes (A_1, A_{2A}, A_{2B}, A_3), and their activation leads to modification of adenylyl cyclase signaling, by regulating the intracellular levels of cAMP (Fredholm et al. 1994) (Fredholm et al. 2001). Even though the expression of A_1 and A_3 receptors has been detected in astrocyte preparations (Alloisio et al. 2004; Verkhratsky et al. 2009), their expression in the cerebrovasculature remains controversial (Di Tullio et al. 2004; Ngai et al. 2001). On the other hand, A_{2A} receptors seem to be reasonably well expressed in the blood-brain vessels (Koehler et al. 2006; Ngai et al. 2001). In the present study, we demonstrated that among the three adenosine receptors tested, A_1, A_{2A} and A_3, A_3 has the lowest expression in the rat brain cortex. This is in agreement with a previous study, which showed that adenosine receptors A_3 have low expression in the brain (Rebola et al. 2005). Focusing on their expression in the brain microvasculature, whereas the expression of the adenosine receptors A_1 and A_3 assume a less important relative expression, the A_{2A} receptors see their relative expression greatly increased in comparison to that of brain cortex (Table 3, Fig. 1 and 2). This suggests that the A_{2A} adenosine receptor in particular may play a role in the BBB signaling system, which can be stimulated by either the adenosine signaling coming from astrocytes and other neighboring cells of the CNS, or alternatively by agonists circulating in the blood. In a study using bovine brain capillary ECs, the presence of A_{2A} and A_3 adenosine receptors was demonstrated by RT-PCR and radioligand binding assays, while A_1 was absent and the presence of the A_{2B} receptor remained uncertain (Schaddelee et al. 2003). The protein expression of both A_1 and A_{2A} adenosine receptors was evidenced by Western Blot (WB) on primary ECs isolated from mice brains and on the human brain endothelial cell line hCMEC/D3 (Carman et al. 2011; Mills et al. 2011), supporting that the BBB is able to directly respond to extracellular adenosine. In fact, it was also demonstrated that the activation of the adenosine receptors A_1 and A_{2A} at the brain ECs facilitated the entry of intravenously administered macromolecules, such as dextrans and anti-β-amyloid antibody, into the murine brain (Carman et al. 2011).

The P2 receptors are subdivided into ligand-gated ion channels P2X receptors, and G-protein-coupled P2Y receptors (von Kugelgen 2006), and so far little is known about their

expression at the BBB and their hypothetical role for the maintenance of the BBB properties. Two different RT-PCR experiments evidenced the expression of mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ in primary rat BBB ECs (Anwar et al. 1999), and the expression of the G protein-coupled receptors $P2Y_2$, $P2Y_6$, $P2Y_{11}$ as well as the ionotropic $P2X_4$, $P2X_5$ and $P2X_7$ receptors in the hCMEC/D3 cells (Bintig et al. 2012). Our present study demonstrates that $P2X_7$ and $P2Y_1$ receptors are among the most expressed neurotransmitter receptors of all tested genes, either in the rat brain cortex or in isolated microvessels. Interestingly, both purinergic receptors do not see their expression considerably altered from samples of brain cortex to isolated brain microvessels (Table 3), indicating that these receptors may play an important signaling role at the BBB.

In the brain, $P2X_7$ receptor is predominantly expressed in the microglia (Harry 2013), and it is known that its activation promotes the release of IL-1α and IL-1β (Bernardino et al. 2008; Ferrari et al. 1997; Mingam et al. 2008), TNF-α (Hide et al. 2000), superoxide (Parvathenani et al. 2003), and plasminogen (Inoue et al. 1998). Microglial $P2X_7$ receptor activation also seems to be involved in the protection of neurons against glutamate-induced neurotoxicity (Suzuki et al. 2004). $P2X_7$ receptor is also expressed in other brain cell types, such as astrocytes, and thus it may play a role in the modulation of glutamate release from astrocytes and in neuroexcitability, since astrocytes react with neurotransmitters released from the synapse (Duan et al. 2003). However, to date, little is known about the role of ATP and of the $P2X_7$ receptors expressed in the ECs of the BBB. Nevertheless, microglial $P2X_7$ receptor has shown to regulate BBB properties. It was recently demonstrated that the selective blockade of the activation of microglial $P2X_7$ receptors was able to prevent basal lamina degradation and BBB leakage provoked by 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) via $P2X_7$ receptor-microglial activation (Rubio-Araiz et al. 2014).

$P2Y_1$ receptor is widely expressed in the different regions of the mammalian brain, and in different brain cell types, including Purkinje cells, oligodendrocytes, astrocytes, microglia and neurons (Weisman et al. 2012). This purinergic receptor has shown to be implicated in the regulation of glial cell functions (Moran-Jimenez and Matute 2000), providing neuroprotection against oxidative stress, and contributing to brain development and repair (Fujita et al. 2009; Mishra et al. 2006). $P2Y_1$ receptor activation in microglia has shown to promote the down-regulation of pro-inflammatory response, as its activation increases the release of the anti-inflammatory cytokine, IL-10 (Seo et al. 2008). Similarly to $P2X_7$ receptor, little data is available concerning the role of $P2Y_1$ receptor at the brain microvasculature. Still, recent studies indicate that activation of $P2Y_2$ receptors in the brain vascular endothelium can increase the transendothelial permeability of leukocytes

(Kukulski et al. 2010; Seye et al. 2002; Seye et al. 2003), suggesting that the activation of $P2Y_2$ receptors, and possibly of $P2Y_1$, in brain microvessels may function as a novel target for increasing drug delivery into the brain.

The adrenoceptors are a class of G protein-coupled receptors, targets of catecholamines, such as norepinephrine and epinephrine, comprising α (α_{1A}, α_{1B}, α_{1D}, and α_{2A}, α_{2B}, α_{2C}) and β (β_1, β_2, and β_3) receptor subtypes. Our present results evidence that the adrenoceptors α_{2A}, β_1 and β_2 are poorly expressed in either the rat brain cortex or in isolated rat brain microvessels (Fig.1 and 2). Nevertheless, we demonstrate that even though the expression of the β_2 adrenoceptor is low in brain microvessels, it sees its expression being enriched up to 7-fold when microvessels are purified from total brain cortex (Table 3, Fig. 2), whereas the expression of the other two adrenoceptor sub-types tested remain unchanged or are even lower than in brain cortex. The β adrenoceptors in the ECs are responsible for controlling vascular homeostasis and regulation of receptor desensitization, internalization and signaling via β-arrestins (DeWire et al. 2007). The expression of β_1 and β_2 adrenergic receptors was firstly demonstrated in a bovine primary culture of brain ECs (Durieu-Trautmann et al. 1991). The expression of the β_2 adrenoceptor in the BBB was also evidenced to mediate the interaction of the *Neisseria meningitidis* pathogen with the brain endothelium and to facilitate its crossing through the BBB (Coureuil et al. 2010). These data are in line with the present results obtained in this study.

Cannabinoid receptors essentially mediate distinct effects: type 1 cannabinoid receptors (CB_1) mediate the psychoactive effects of cannabinoids and are mainly expressed in the CNS, whereas type 2 cannabinoid receptors (CB_2) mostly mediate anti-inflammatory effects and are predominantly expressed in cells of the immune system and hematopoietic cells (Cabral et al. 2008; Miller and Stella 2008). In the brain, the expression of CB_2 receptors was found in brain microvascular ECs. The endocannabinoid system is implicated in neuroprotection, modulation of the inflammatory response, reduction of brain edema and induction of neuroprotective hypothermia (Cabral and Griffin-Thomas 2009). Activation of CB_1 receptors can protect against excitotoxicity, by control of the release of excitatory neurotransmitters from neurons (Marsicano et al. 2003), and promote neurogenesis (Aguado et al. 2007). On the other hand, activation of CB_2 receptors reduces oxidative stress and neuroinflammation by attenuating microglial activation and production of proinflammatory mediators (Campbell and Gowran 2007; Horvath et al. 2012). It is generally accepted that CB_2 receptors are predominantly expressed by immune cells and resident microglia, whereas the presence of CB_2 receptors in neurons

remains controversial (Atwood and Mackie 2010; Van Sickle et al. 2005; Walter et al. 2003). Our study shows that CB receptors are expressed at very low levels in either rat brain cortex or rat brain microvasculature. In a study conducted using human cerebromicrovascular ECs, the mRNA and protein expression of both CB_1, CB_2 receptors was demonstrated (Golech et al. 2004). Little CB_2 receptor immunoreactivity in healthy brain endothelium or in human brain microvascular ECs has also been evidenced (Ramirez et al. 2012), and seem to be up-regulated in pathologies and in response to inflammation and stress (Schley et al. 2009; Zhang et al. 2011b). A recent study showed that CB_1 receptor mRNA levels are found in both human brain microvessel ECs and human astrocytes, but CB_2 receptor are only found in astrocytes (Hind et al. 2015). Furthermore, this study demonstrates that several endocannabinoids are able to regulate the BBB permeability *in vitro* via CB_2 receptors. Considering the low expression of these receptors at the mammalian BBB, and their overall role in the CNS, it is expected that CB receptors present in the ECs have a small role in the regulation of BBB permeability and control of neuroinflammation, by working in concert with the other cell types and modulators that constitute the NVU.

Concerning the presence of the NMDA receptor in brain ECs, its expression has been a matter of study over the past decade, but the resulting data has generated controversy regarding its actual expression at the BBB and its role. The gene expression of several subunits of the NMDA receptor was first demonstrated in cultured rat brain ECs in 1998 (Krizbai et al. 1998). Since then, several studies using molecular-biology techniques for direct localization of this glutamate receptor have brought little consensual results. Gene expression and functional activity of main NMDA receptor subunits failed to be demonstrated in rat and human cortical microvessels and in cultured cerebrovascular ECs (Morley et al. 1998). Furthermore, NMDAr1 subunit expression was not detected in a rat brain microvascular EC culture by WB (Domoki et al. 2008). However, in 2003, a research group was able to demonstrate by RT-PCR, WB and immunoprecipitation that immortalized human ECs expressed the NMDAr1 subunit (Sharp et al. 2003). The expression of NMDAr1 and NMDAr2 subunits were also evidenced by WB, in primary cultures of rat brain ECs (Andras et al. 2007); similarly, in mouse brain microvessel ECs, the expression of NMDAr1 was evidenced by RT-PCR, immunostaining, and WB (Legros et al. 2009). The presence of the NMDAr1 subunit was also reported by immunofluorescence, WB and qPCR, in the hCMEC/D3 cell line (Reijerkerk et al. 2010). In our current study, the *NMDAr1* expression in the enzymatic isolation of microvessels, which is the technique that yields a purest fraction of brain ECs, is relatively poor in

comparison to the other tested genes. However, its mRNA levels are still fairly identified, and thus supporting that this receptor is expressed at the rat brain BBB.

The presence of the NMDA receptor in the brain microvasculature has been recently indicated as an element for the regulation of the expression of the ABC drug efflux transporters (Bauer et al. 2008; Salvamoser et al. 2015; Zibell et al. 2009), namely P-glycoprotein and Breast Cancer Resistance Protein (BCRP). Additionally, it was also evidenced that in a murine BBB model, the NMDA receptor mediates the hyperhomocysteinemia effect on the increase of the BBB permeability, by regulating the expression of adherens and tight junctions present at the brain ECs (Beard et al. 2011). Using the same murine BBB model, this receptor also showed to mediate the up-regulation of the glucose uptake transporter (glut-1) during oxygen/glucose deprivation and subsequent reoxygenation in brain ECs (Neuhaus et al. 2012).

Conclusion: the analysis of the mRNA levels of markers for the different cell types of the CNS present in brain microvessels isolated using a mechanic and an enzymatic approach allow us to conclude that the enzymatic approach is the method which yields the purest fraction of brain capillary ECs. This method renders a capillary cellular fraction more highly enriched in pericyte and endothelial cell markers, and with less other cell contaminants than what is obtained with the mechanic isolation from the rat brain cortex, and thus should be preferentially used for the study of the BBB physiopathology and properties. In addition, the current study gives an original look over the expression of several neurotransmitter receptors in both rat brain cortex and in rat brain isolated microvessels, as well as providing a comparative analysis of their relative amounts among these tissues. Therefore, the present study allows us to understand which neurotransmitter receptors are more specifically expressed along the BBB, such as the ATP receptors $P2X_7$ and $P2Y_1$, as well as the adenosine receptor A_{2A}, suggesting their important role in the signalling system for the normal functioning of the NVU.

5. AUTHOR DISCLOSURE

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Conflict of interest: All authors declare that they have no conflicts of interest.

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6. TABLES

Table 1. Primer sequences used for SYBR Green-based real-time quantitative polymerase chain reaction.

Gene (name)	Forward Primer (5'-3')	Reverse Primer (5'-3')	Length (bp)
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA	132
Adra2A **(ADr α_{2A})**	CGGCAATAGCAGCTGGAACGG	GCCAAACACGGTGAACAGCAT	127
Adrb1 **(ADr β_1)**	CTGCTACAACGACCCCAAGTG	AACACCCGGAGGTACACGAA	120
Adrb2 **(ADr β_2)**	CCTTGGCGTGTGCTGATCTA	GTCCAGAACTCGCACCAGAA	109
Adora1 **(Ar A_1)**	AAGATCCCTCTCCGGTACAAG	ACTCAGGTTGTTCCAGCCAA	123
Adora2a **(Ar A_{2A})**	GCCTGTTTTGTCCTGGTCCT	ACCAAGCCATTGTACCGGAG	107
Adora3 **(AR A_3)**	TGTCCTGTGTGCTTCTGGTC	AGTGGTAACCGTTCTATATCTGACT	113
Grin1 **(NMDAr1)**	GCAGGTGGAGTTGAGTACCATGTAC	CAGCTTGTTGTCCCGCACA	94
Cnr1 **(CB_1)**	CGCACAGCCTCTAGACAACAG	TGCTCTTGATGCAGCTCTCC	104
Cnr2 **(CB_2)**	GCCACCCAGCAAACATCTATGC	CATGGACAGACAGGCTTTGGCT	89
P2rx7 **(ATPr $P2X_7$)**	ATCGGTGTGCTTTCTTCGG	CAGGCTCACTCTGTTTCGGC	96
P2ry1 **(ATPr $P2Y_1$)**	CAAGCAGAACGGAGACACAAG	GTGCATCAGGTCATCTCAGGG	96
gfap	AGGCAGAAGCTCCAAGATGAAAC	TCTCCAGATCCACACGAGCCAA	109
s100b	CACCGAAGCCAGAGAGGACT	TGGAAGACATCAATGAGGGCAAC	99
glut-1	CCCAGGTGTTCGGCTTAGAC	CTCTCAGGGCAGAAGGGCAAC	118
ng-2	ACCAGCTAGAGGTAGTCCAGG	GACACCATCACCAAGTAGCCAG	94
pdgfrb	ACCCAGCCACTTGAACCTGA	ATCTACAGATTCATCCTTGCTCA	73
cd11b	AGGCTCAGACAGAGACCAAAG	TGATGAGAGCCAAGAGCACCAG	102
mbp	CTCCCTGCCCCAGAAGTCG	GGGGTGTACGAGGTGTCACAAT	86
syp	GCCACGGACCCAGAGAACATTA	TTCAGGAAGCCAAACACCACT	125

Table 2. Relative expression (mRNA) of endothelial (*glut1*), astrocyte (*gfap, S100b*), pericyte (*ng-2, pdgfrb*), microglia (*cd11b*), oligodendrocytes (*mbp*) and neuron (*syn*) markers in samples from rat microvessels isolated enzymatically or mechanically compared to rat brain cortex. The tissue sample with the lowest expression of a certain gene was given an arbitrary expression value of 1. All data have been normalized to TBP mRNA and are expressed as means ± SEM of 3 independent experiments. Unpaired two-tailed t-student test *p< 0.05, **p< 0.01, ***p<0.001 compared to rat brain cortex samples.

	Endothelial cell	Astrocyte		Pericyte		Microglia	Oligo dendrocyte	Neuron
Cell marker	*glut-1*	*gfap*	*s100b*	*ng-2*	*pdgfrb*	*cd11b*	*mbp*	*synaptophysin*
Cortex	1.00 ± 0.05	25.55 ± 2.45	67.72 ± 5.09	1.00 ± 0.07	1.00 ± 0.08	2.61 ± 0.33	43.00 ± 11.85	533.45 ± 45.13
Microvessels enzymatic	15.61 ± 3.61*	1.00 ± 0.38***	1.00 ± 0.14***	15.20 ± 2.51**	12.02 ± 1.01***	1.80 ± 0.70	1.00 ± 0.38*	1.00 ± 0.23***
Microvessels mechanic	8.38 ± 1.72*	5.73 ± 0.72**	5.50 ± 0.47***	3.22 ± 0.30**	9.81 ± 0.54***	1.00 ± 0.10**	15.81 ± 1.20	10.28 ± 4.33***

Table 3. Relative expression (mRNA) of several neurotransmitter receptors in samples from rat microvessels isolated enzymatically or mechanically in comparison expression levels in the rat brain cortex (receptor expression in brain cortex was given an arbitrary expression value of 1). All data have been normalized to TBP mRNA and are expressed as means ± SEM of 3 independent experiments. One-way ANOVA with Bonferroni's Multiple Comparison post-test adjustment *p< 0.05, **p< 0.01, ***p<0.001.

	Cortex	Microvessels enzymatic	Microvessels mechanic
Adrenergic receptors			
Adra2A	1.00 ± 0.07	1.08 ± 0.10	0.40 ± 0.17*
Adrb1	1.00 ± 0.02	0.29 ± 0.04**	0.40 ± 0.13**
Adrb2	1.00 ± 0.10	6.99 ± 1.34**	7.03 ± 0.52**
Adenosine receptors			
Adora1	1.00 ± 0.09	0.02 ± 0.01***	0.04 ± 0.01***
Adora2A	1.00 ± 0.05	5.32 ± 0.79**	4.02 ± 0.67*
Adora3	1.00 ± 0.05	0.12 ± 0.04***	0.13 ± 0.04***
ATP receptors			
P2rx7	1.00 ± 0.09	1.48 ± 0.20	2.24 ± 0.77
P2ry1	1.00 ± 0.07	1.68 ± 0.31	0.47 ± 0.13
Cannabinoid receptors			
Cnr1	1.00 ± 0.08	0.02 ± 0.01***	0.12 ± 0.05***
Cnr2	1.00 ± 0.12	1.34 ± 0.45	0.87 ± 0.09
Others			
NMDAr1	1.00 ± 0.01	0.02 ± 0.01***	0.24 ± 0.13***

7. FIGURES

Fig. 1. **Relative abundance of the studied neurotransmitter receptors (mRNA) compared to the least expressed receptor in each analyzed tissue, in (a) rat brain cortex samples, (b) rat brain isolated microvessels isolated enzymatically, and (c) rat brain isolated microvessels isolated mechanically.** All data have been normalized to TBP mRNA and are expressed as mRNA-fold expression to the least expressed gene in the analyzed tissue (expression level set as 1). Results are means ± SEM of 3 independent samples.

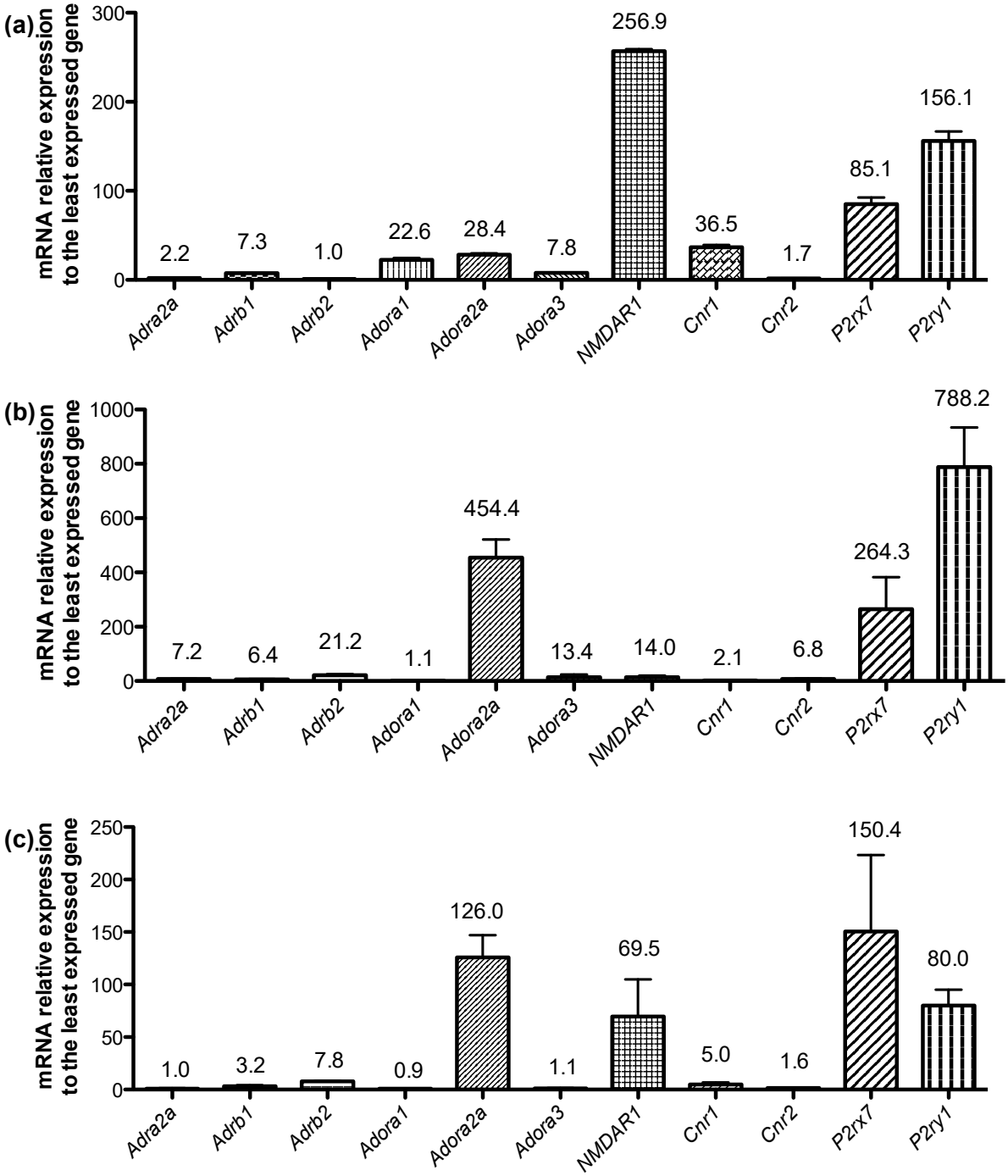
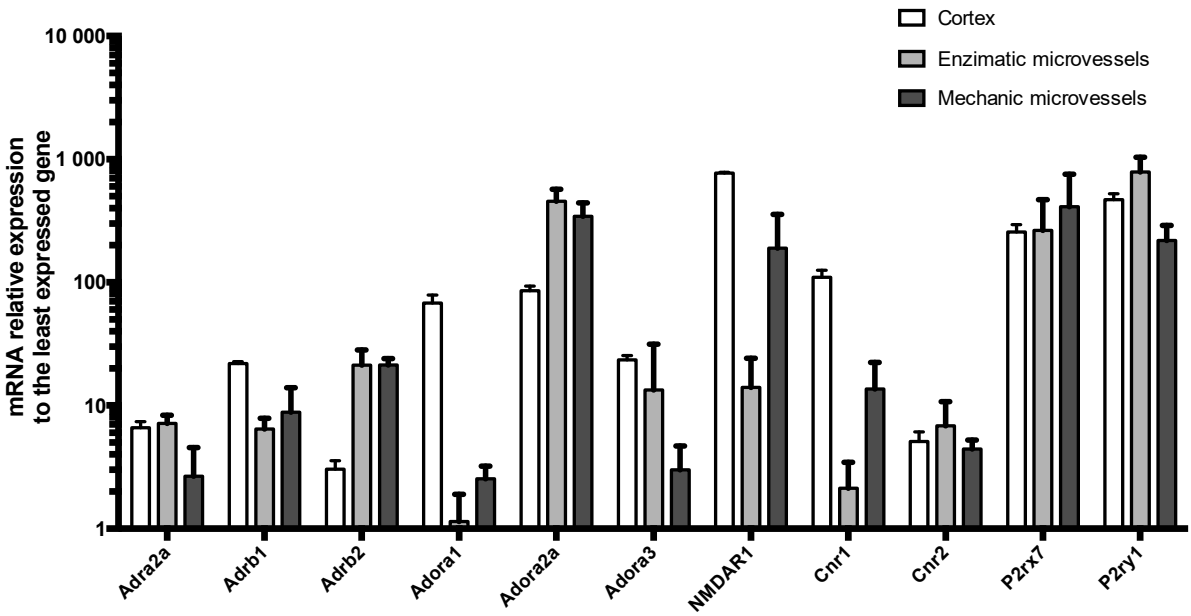


Fig. 2. Overview of the mRNA relative expression of the studied neurotransmitter receptors between samples from rat brain cortex and rat brain isolated microvessels isolated enzymatically and mechanically. Transcript amounts for each target gene were normalized to TBP mRNA and are expressed as n-fold expression towards the smallest amount of one target gene mRNA detectable and quantifiable (*Adora1* in rat brain microvessels isolated enzymatically, expression level set as 1). Results are means ± SD of 3 independent experiments.



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General outcome:

In this present work, we performed an original study concerning the development of BBB models, bringing new insights for the choice of a best approach to isolate brain capillaries for the study of the BBB. When the expression and relative abundance of several CNS cell markers, such as *glut1* (BECs) *ng2, pdgfrb* (pericytes), *gfap, s100b* (astrocytes), *cd11b* (microglia), *mbp* (oligodendrocytes) and *syp* (neurons) was compared between samples, we conclude that there is an higher enrichment of BEC and pericyte cell markers with the enzymatic digestion approach, in comparison to the mechanical dissection method. In fact, *glut1* is approximately 2-fold, *ng-2* 4.8-fold, and *pdgfrb* 1.2-fold higher in microvessels isolated enzymatically than in microvessels isolated mechanically. Furthermore, the expression of specific markers for astrocytes, oligodendrocytes and neurons was considerably reduced when microvessels were isolated with the enzymatic approach compared to the brain microvessels isolated mechanically. The resulting data point to a clear reduction on the contamination coming from other cell elements surrounding the brain capillaries in the microvessel isolates coming from the enzymatic digestion technique. Thus, microvessels isolated with an enzymatic approach yield a more pure fraction of brain capillaries than microvessels isolated with a mechanic approach, indicating that this technique should be preferentially adopted for studying the BBB properties in the rat.

Among the 11 neurotransmitter receptor genes – adrenergic (*Adra2a*, *Adrb1*, *Adrb2*), adenosine (*Adora1*, *Adora2a*, *Adora3*), ATP (*P2rx7*, *P2ry1*), cannabinoid (*Cnr1*, *Cnr2*), and glutamate receptor *NMDAr1*, *P2ry1* (788-fold of the least expressed gene, *Adora1*), *Adora2a*, *P2rx7* and *Adrb2* showed to assume an important expression level in rat brain microvessels. In addition, transcript levels found for *Adora1*, *Cnr1* and *NMDAr1* in rat brain microvessels were approximately 50-fold lower to those found in brain cortex samples, suggesting that such receptors are poorly expressed in rat brain capillaries, and likely come from neuronal contamination. On the other hand, *Adrb2* and *Adora2a* are the only neurotransmitter receptors with an increased expression in rat brain microvessels in comparison to brain cortex, while *Adra2a*, *Cnr2* and the ATP receptors *P2rx7*, *P2ry1* do not register any considerable change of expression from brain cortex to brain microvessel samples. Furthermore, *P2rx7* and *P2ry1* showed to be among the most expressed genes in the rat brain cortex, and thus the microvessel expression of such genes is particularly important. These findings suggest that ATP receptors $P2X_7$ and $P2Y_1$, as well as the adenosine receptor A_{2A} are present at the BBB, and so may play a central role in cell signaling at the NVU and in the normal function of the BBB.

III. Manuscript 4

Effect of newly synthetized thioxanthones on the activity of P-glycoprotein in RBE4 cells: a new approach to minimize the cytotoxicity of xenobiotics?

Unsubmitted Manuscript

Rationale and Objectives:

In the objectives set for the present thesis dissertation, we primarily focused on a mechanism of modulation of P-gp and BCRP gene and protein expressions at the BBB. Still, it is difficult to estimate the impact of such P-gp and BCRP up-regulation on the activity of these drug efflux transporters, and thus, to predict the extent of this change on the pharmacokinetics and pharmacodynamics of drugs or other xenobiotics. On the other hand, the direct activation of ABC transporters, namely of P-gp, where an increase in its activity can be seen without any change in the gene or protein expression, is gaining increasing interest as a mechanism of modulation of transport across physiological barriers, since it directly affects the transport of its substrates and its impact can be easily estimated. Very recently, a set of thioxanthonic compounds has demonstrated to rapidly increase the transport activity of P-gp in Caco-2 cells (Silva et al. 2014c). Furthermore, the cytotoxicity induced by the toxic herbicide PQ was significantly reduced in the presence of most of these thioxanthonic derivatives, a protective effect that was reversed upon incubation with a specific P-gp inhibitor. These results encouraged us to investigate if such modulation could be attained in a BBB model, in order to use such compounds, named as P-gp activators, as antidotes to revert neuronal damage and its consequences in potential situations of acute intoxication with harmful P-gp substrates, in a fast and efficient manner.

Therefore, the main objective of the following study is to uncover if six different thioxanthonic compounds can be used as P-gp activators at the BBB, and revert the mitoxantrone-induced cytotoxicity. For this purpose, RBE4 cells were used as *in vitro* BBB model, which were incubated for a short time with each thioxanthonic derivative and the efflux transport activity was evaluated, by measuring the intracellular accumulation of Rho 123, a fluorescent P-gp substrate. Additionally, RBE4 cells were incubated with a broad range of concentrations of mitoxantrone, a toxic P-gp substrate, in the presence or absence of each tested thioxanthone. Cell death was then evaluated after 4h, 8h and 16h of incubation in order to estimate the protection conferred by each thioxanthonic compound against the mitoxantrone-induced toxicity.

Effect of newly synthetized thioxanthones on the activity of P-glycoprotein in RBE4 cells: a new approach to minimize the cytotoxicity of xenobiotics?

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Abbreviations:
ABC, ATP-binding cassette; BBB, Blood-brain barrier; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; NR, Neutral Red; P-gp, P-glycoprotein; Rho 123, Rhodamine 123; TMD, Transmembrane domain

Abstract:

The activation of P-gp is a fast approach to increase the efflux of substrates, which could confer protection in an intoxication scenario. Some thioxanthonic derivatives were recently described to activate P-gp, protecting Caco-2 cells against paraquat toxicity. The aim of this study is to screen six thioxanthonic derivatives as potential P-gp activators and to evaluate whether they would afford protection against the cytotoxicity induced by mitoxantrone, a P-gp substrate,, in a blood-brain barrier (BBB) model, RBE4 cells.

Thioxanthonic derivatives were synthesized and their cytotoxicity on RBE4 cells was assessed by the NR uptake and MTT reduction assays 24h after exposure. P-gp ATPase activity was determined in the presence of these derivatives to evaluate their potential as P-gp substrates. The effect of thioxanthones on the efflux of Rhodamine123 in RBE4 cells was evaluated by flow cytometry. Mitoxantrone-induced cytotoxicity was evaluated in the absence/presence of the thioxanthones.

All thioxanthones showed no cytotoxicity at 10 µM, except TX129. None showed to be a clear P-gp substrate. TXA4, TXA3, TX105 and TX129 (10 µM) increased the efflux of Rhodamine123 up to 168%, reflecting an activation of P-gp transport in RBE4 cells. However, thioxanthones did not significantly reduce the cytotoxicity of mitoxantrone.

In conclusion, this study demonstrates the potential of thioxanthonic compounds in the activation of P-gp at the BBB. However, the observed increase in P-gp activity did not protect cells against mitoxantrone cytotoxicity. Thus, the results highlight the absence of a mandatory correlation between higher P-gp activity and lower toxic effects of its substrates.

Keywords: Blood-brain barrier, RBE4 cells, P-glycoprotein, Thioxanthones, P-glycoprotein activation, Mitoxantrone

1. INTRODUCTION

The blood-brain barrier (BBB) plays an important role in brain homeostasis and protection from potentially toxic substances, since it determines the bioavailability of a wide variety of compounds, including endogenous substances, drugs and xenobiotics in the brain (Chaves et al. 2014; DeGorter et al. 2012). ATP-binding cassette (ABC) transporters, in particular, are key elements present at the BBB implicated in the efflux of xenobiotics, which enable the extrusion of a large number of structurally unrelated substrates, and therefore regulate CNS drug efficacy and toxicity (Chaves et al. 2014; DeGorter et al. 2012; Gottesman et al. 2002). A differential expression of ABC transporters contributes to the inter-individual vulnerability to xenobiotics and pharmacoresistance (Gottesman et al. 2002). The induction of these drug efflux transporters can be understood as an antidote approach for limiting the toxicity of their substrates, through their augmented efflux and limited drug intracellular accumulation (Silva et al. 2014f).

Our research group recently showed that newly synthesized (thio)xanthonic derivatives protected Caco-2 cells against the cytotoxicity of paraquat (Silva et al. 2014b; Silva et al. 2014e). Protective effects of P-gp inducers are dependent on their ability to induce the synthesis of a functional protein, which may not be observed whenever an increased protein expression is registered. Interestingly, these newly synthesized compounds seem to be capable of rapidly increase P-gp activity with no need to increase its expression, representing a new class of drugs, known as P-gp activators. *In silico* studies showed interactions between (thio)xanthones and P-gp in the presence of paraquat, which suggested that a co-transport mechanism might be operating (Silva et al. 2014b; Silva et al. 2014e).

In this way, we decided to test the thioxanthonic derivatives (Fig. 1) that showed to be promising P-gp activators in Caco-2 cells (TX87, TXA4, TXA3, TXOH), as well as new ones synthetized (TX105, TX129) and characterized *in-house*, on an *in vitro* BBB model – RBE4 cells – in order to understand if these compounds can also function as P-gp activators in such model, by conferring cytoprotection against a P-gp toxic substrate, mitoxantrone, and thus be used as a potential antidotal mechanism.

2. EXPERIMENTAL PROCEDURES

2.1. Material

Minimum essential medium, nutrient mixture F-10 Ham, sodium bicarbonate, 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES), neomycin sulfate, neutral red (NR) solution, methylthiazolyldiphenyl-tetrazolium bromide (MTT), rhodamine 123 (Rho 123), cyclosporine A and zosuquidar were obtained from Sigma (St. Louis, MO, USA). Reagents used in cell culture, including fetal bovine serum (FBS), antibiotic solution (10 000 U/mL penicillin G, 10 000 µg/mL streptomycin sulphate), rat tail collagen type I, Hanks' balanced salt solution (HBSS) (either with or without Ca^{2+} and Mg^{2+} salts), 0.25% trypsin-EDTA solution, were obtained from Gibco Laboratories (Lenexa, KS). Basic fibroblast growth factor (bFGF) was from Invitrogen.

Flow cytometry reagents and propidium iodide were obtained from Becton, Dickinson and Company (BD Biosciences, San Jose, California, USA). All other reagents used were of analytical grade or of the highest available grade.

2.2. Synthesis of thioxanthones

The synthesis of the thioxanthonic derivatives TX87, TXA4, TXA3 and TXOH was performed according to the described procedure (Palmeira et al. 2012) by Ullman cross-coupling between 1-chloro-4-propoxy-9*H*-thioxanthen-9-one and an amine in alkaline medium using methanol as solvent and conventional heating (TX129) or *N*-methylpirrrolidone (NMP) and microwave heating (TX87, TXA3, TX105 and TXA4), or by dealkylation of 1-chloro-4-propoxy-9*H*-thioxanthen-9-one with boron bromide, BBr_3 (TXOH). Thioxanthones were characterized by spectroscopic methods according to described procedures (Palmeira et al. 2012). Figure 1 shows the chemical structures of the six thioxanthones used in this study. The purity of each compound was determined by HPLC–DAD analysis using an isocratic elution of MeOH:H2O basified with TEA (1 %) (TX129, TX87, TXA3, TX105 and TXA4) or acidified with CH3COOH (1 %) (TXOH) at a constant flow rate of 1.0 mL/min (Palmeira et al. 2012). All tested compounds had a purity of at least 95 %.

2.3. Cell culture

Immortalized rat brain microvessel endothelial cells, RBE4 (Roux et al. 1994) were grown in minimum essential medium/Ham's F10 (1:1) supplemented with 300 µg/mL neomycin, 10% FBS, 1 ng/mL bFGF, 100 U/mL penicillin G, 100 µg/mL streptomycin, 25 mM sodium bicarbonate and 25 mM HEPES. This mixture will herein be referred to as cell culture medium. RBE4 cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. The

cell culture medium was changed every 48 to 72 h. For sub-culturing, cells were dissociated with 0.25% trypsin-EDTA, which was neutralized with culture medium, and sub-cultured in 75 cm^2 flasks. In all experiments, cells were seeded at a density of 25.000 cells/cm^2 and used 3 days after seeding, once confluence was reached.

2.4. Cytotoxicity assays

Cells were seeded in 96-well plates at a density of 10.000 cells per well, and the tested compounds cytotoxicity was evaluated three days after seeding. Thioxanthones (0–50 μM) cytotoxicity was evaluated 24h after exposure by the MTT reduction and by the NR uptake assays.

2.4.1. *MTT reduction assay*

At the selected time point (24h), cell culture medium was removed, followed by the addition of fresh cell culture medium containing 0.5 mg/mL MTT and incubation at 37 °C in a humidified, 5% CO_2 atmosphere for 1h. After this incubation period, the cell culture medium was removed and the formed formazan crystals dissolved in 100% DMSO. The absorbance was measured at 550 nm in a multi-well plate reader (PowerWaveX, BioTek Instruments, VT, USA). The percentage of MTT reduction relative to that of control cells was used as the cytotoxicity measure.

2.4.2. *NR uptake assay*

At the end of the incubation period, cells were incubated with NR (50 μg/mL in cell culture medium) at 37 °C, in a humidified, 5 % CO_2 atmosphere, for 90 min. After this incubation period, cell culture medium was removed, and the dye absorbed by viable cells extracted with ethyl alcohol absolute/distilled water (1:1) with 5% acetic acid. The absorbance was measured at 540 nm in a multi-well plate reader (PowerWaveX, BioTek Instruments). The percentage of NR uptake relative to that of control cells was used as the cytotoxicity measure.

2.5. Mitoxantrone cytotoxicity assays

Mitoxantrone cytotoxicity was evaluated in RBE4 cells by using the NR uptake assay, simultaneously incubated with the tested thioxanthones (10 μM) or with a well-known and potent P-gp inhibitor, zosuquidar (Shepard et al. 2003; Starling et al. 1997), at a non-cytotoxic concentration (5 μM). Briefly, cells were seeded onto 96-well plates to obtain confluent monolayers at the day of the experiment. After reaching confluence, cells were

exposed to mitoxantrone (0.1-50 μM) in fresh cell culture medium in the presence or absence of the studied thioxanthones or with zosuquidar for 4h, 8h and 16h, and once the incubation period was finished it was replaced with fresh cell culture medium. Cytotoxicity was evaluated 24 h after the beginning of exposure by the NR uptake assay as described in section 2.4.2. Each experiment was performed in triplicate and repeated with at least three independent experiments.

2.6. Evaluation of P-glycoprotein transport activity

The P-gp transport activity was evaluated by flow cytometry using 1 μM Rho 123 as a P-gp fluorescent substrate, as previously described (Silva et al. 2014c). For that purpose, Rho 123 efflux was assessed in the presence of thioxanthones. RBE4 cells were seeded onto 75 cm^2 flasks and, after reaching confluence, washed twice with HBSS (-/-) and harvested by trypsinization (0.25 % trypsin/1 mM EDTA) to obtain a cellular suspension. This cell suspension was then divided into aliquots of 500.000 cells/mL. The cells were centrifuged (300*g*, for 10 min, at 4°C), suspended in PBS buffer (pH 7.4) containing 10 % heat-inactivated FBS, 1 μM Rho 123 and the P-gp inhibitor cyclosporine A (10 μM), and incubated at 37°C for 30 min to allow maximum Rho 123 accumulation. After this inhibited accumulation (IA) phase, cells were submitted to an efflux phase, where the energy-dependent P-gp function was re-established by removing the P-gp inhibitor (cyclosporine A) and by adding supplemented cell medium. For that purpose, cells were washed twice with ice-cold PBS buffer with 10% heat-inactivated FBS, centrifuged (300 *g* for 10 min, at 4°C) and resuspended in cell culture medium, with or without the tested thioxanthones (10 or 20 μM), and incubated for 45 min at 37 °C. Cells were then washed twice with ice-cold PBS buffer with 10 % heat-inactivated FBS and resuspended in ice-cold PBS buffer immediately before analysis. Fluorescence measurements of isolated cells were performed using a BD ACCURI™ C6 flow cytometer. Green fluorescence of FITC-Rho 123 was measured by a 530 ± 15 nm band-pass filter (FL1). Acquisition of data for 15.000 cells was gated to include viable cells on the basis of their forward and side light scatters and the propidium iodide (2.5 μg/mL) incorporation (propidium iodide interlaces with a nucleic acid helix with a resultant increase in fluorescence intensity emission at 615 nm). Logarithmic fluorescence was recorded and displayed as a single parameter histogram. The mean of fluorescence intensity for 15.000 cells was the parameter used for comparison (calculated as percentage of control). Non-labeled cells (with or without thioxanthones) were also analyzed in each experiment by a 530 ± 15 nm band-pass filter (FL1) in order to detect a possible contribution from cells auto-fluorescence to the analyzed fluorescence signals. P-gp activity was expressed as percentage of control of

Rho 123 efflux ratio, which was in turn obtained by calculating the difference between mean fluorescence intensity values of intracellular Rho 123 before (in the presence of Cyclosporine A) and after the efflux phase.

2.7. Evaluation of P-glycoprotein ATPase activity

P-gp ATPase activity was evaluated using the P-gp-Glo™ assay kit, according to the manufacturer's instructions (Promega, USA). Briefly, P-gp enriched membrane vesicles (25 µg/well) were incubated with 5 mM MgATP and with either sodium orthovanadate (Na_3VO_4, 100 µM), verapamil (200 µM) or each of the tested thioxanthonic compounds (10 µM) for 120 min at 37 °C. Verapamil was used as a positive control for P-gp ATPase activity stimulation, while sodium orthovanadate acts as a P-gp ATPase activity inhibitor. Pgp ATPase reaction was stopped by adding 50 µL of developer solution to each well, and samples were incubated for an additional 20 min at room temperature, to allow signal developing. The remaining unmetabolized ATP was detected as a luciferase-generated luminescent signal, read in a multi-well plate reader (PowerWaveX, BioTek Instruments). Basal P-gp ATPase activity is a result of the difference of luminescence of untreated (NT) samples and sodium orthovanadate-treated controls (no P-gp ATPase activity), and are expressed as change in luminescence (ΔRLU). P-gp activity in the presence of the tested compounds (TC) is a result of the difference of luminescence of samples treated with either verapamil or thioxanthones and sodium orthovanadate-treated controls. Change in luminescence was calculated using the formula ΔRLU (TC) = RLU (Na_3VO_4) – RLU (TC), where TC stands for the verapamil-treated or thioxanthone-treated membranes. Two independent experiments in duplicate were performed for this assay.

2.8. Docking of mitoxantrone and thioxanthones into a P-gp model

For the structure-based study, the 3D structures of six thioxanthones and mitoxantrone were drawn using HyperChem 7.5 (Froimowitz 1993), and minimized by the semi-empirical Polak-Ribiere conjugate gradient method (RMS<0.1 kcal.$Å^{-1}$. mol^{-1}) (Zhang et al. 2006). Docking simulations between mouse P-gp (pdb code 4Q9H) and thioxanthones, or mitoxantrone, or thioxanthones together with mitoxantrone were undertaken in AutoDock Vina (Scripps Research Institute, USA) (Seeliger and de Groot 2010; Trott and Olson 2009). Parallel docking simulations were performed using a previously built P-gp model based on sav1866 (Silva et al. 2014c; Silva et al. 2014d; Vilas-Boas et al. 2013b). AutoDock Vina considered the target conformation as a rigid unit, while the ligands were allowed to be flexible and adaptable to the target. Vina searched for the lowest binding affinity conformations and returned nine different conformations for each ligand. AutoDock

Vina was run using an exhaustiveness of eight and a grid box with the dimensions 37.0, 30.0, 40.0, engulfing the channel formed by the transmembrane domains (TMDs). Conformations and interactions were visualized using PyMOL version 1.3 (Lill and Danielson 2010).

2.9. Statistical analysis

Data were analyzed with GraphPad Prism® 6.0 software (San Diego, CA, USA). Normality of the data distribution was assessed by three different tests: KS normality test, D'Agostino & Pearson omnibus normality test and Shapiro–Wilk normality test. For data with parametric distribution, statistical comparisons were made using the parametric method of one-way ANOVA, followed by the Bonferroni's multiple comparisons post hoc test. For data with nonparametric distribution, statistical comparisons were estimated using the nonparametric method of Kruskal–Wallis (one-way ANOVA on ranks), followed by the Dunn's post hoc test. In experiments with two variables, statistical comparisons between groups were made using two-way ANOVA, followed by the Sidak's multiple comparisons post hoc test. In the mitoxantrone cytotoxicity assays, concentration–response curves were fitted using least squares as the fitting method and the comparisons between curves (LOG EC_{50}, TOP, BOTTOM, and Hill Slope) were made using the extra sum of squares *F* test. Details of the performed statistical analysis are described in each Figure legend. In all cases, *p* values lower than 0.05 were considered statistically significant.

3. RESULTS

3.1. Thioxanthones cytotoxicity assays

Thioxanthones cytotoxicity was evaluated by the MTT reduction and the NR uptake assays, in order to select a noncytotoxic working concentration. According to the NR uptake assay, after 24h of incubation, no cytotoxicity was observed for TXA3 at any of the tested concentrations, while TX87, TXOH and TX105 only showed to be cytotoxic at 50 μM, and TXA4 and TX129 for concentrations higher than 20 μM (Fig. 2, a). The MTT reduction assay demonstrates no cytotoxicity registered at any of the tested concentrations for the TX87, TXA3, TXOH and TX105 derivatives, and confirms TXA4 cytotoxicity at 20 and 50 μM, and TX129 cytotoxicity from 10 μM (Fig. 2, b).

For subsequent experiments, thioxanthones were tested at 10 μM, a noncytotoxic concentration for all the compounds, with the exception of TX129, which was the only compound that showed a slight cytotoxicity (89% cell viability) at this concentration on the MTT reduction assay.

3.2. P-glycoprotein transport activity

Rho 123 is a P-gp fluorescent substrate widely used for the evaluation of P-gp activity (Silva et al. 2014c; Vilas-Boas et al. 2013b). In the present study, Rho 123 efflux was evaluated in the presence of the tested thioxanthones, at 10 μM and 20 μM, during an efflux step of 45 min, in order to evaluate their immediate effects on P-gp activity as a result of a direct activation of the transporter. The thioxanthonic derivatives TXA4, TXA3, TX105 and TX129 were able to produce a significant increase in the Rho 123 efflux from RBE4 ells (Fig. 3, a). At 20 μM, all the compounds provoked a significant increase in Rho 123 efflux, with the exception of TXOH, when compared to control cells (Fig. 3, b). TXA4, TXA3, TX105 and TX129 revealed to be equally efficient as potential P-gp activators at 10 μM, since they increased Rho 123 efflux by 159, 167, 161 and 168%, respectively. Rho 123 efflux was only slightly increased to 170% when the compounds are used at 20 μM. The derivative TX87, although to a lower extent, also increased Rho 123 efflux to 126%, thus also indicating P-gp activation.

3.3. P-glycoprotein ATPase activity in the presence of thioxanthones

P-gp ATPase activity was assessed in order to determine if the tested thioxanthones in the present study are able to stimulate P-gp ATPase activity, and thus work as P-gp substrates. Basal P-gp ATPase activity was estimated based on the difference in luminescence of untreated samples towards sodium orthovanadate-treated samples, which inhibits P-gp ATPase activity. Verapamil was used as a positive control for

stimulation of P-gp ATPase activity, which rendered a change in luminescence of around 31.000 RLU, representing an increase in luminescence of 4-fold in comparison to basal ATPase activity ($p < 0.001$) (Fig. 4). None of the thioxanthonic derivatives, at 10 μM, provoked a statistical significant increase on the P-gp ATPase activity in comparison to the untreated samples ($p > 0.05$).

3.4. Docking Studies

Since thioxanthones need to interact with the P-gp protein structure in order to promote its activation, it was hypothesized that these compounds could bind to the drug-binding pocket formed by the TMD interface, activating the drug efflux mechanism. Also, as we aim to test the role of thioxanthones against the cytotoxicity provoked by mitoxantrone, we performed docking simulations for mitoxantrone and thioxanthones in this binding pocket of P-gp (Table 1). A visual inspection of thioxanthones alone or complexed with mitoxantrone in the TMD interface of P-gp was performed (see Fig. 5). Mitoxantrone binds to a cavity formed by TMDs 2, 3, 10, and 11 (Fig. 5B), described as *H*-site for being the preferential binding site for Hoechst 33342 (Ferreira et al. 2013). Mitoxantrone has shape, size, and stereoelectronic complementarity to P-gp binding pocket forming a stable complex with P-gp with a negative energy of -5.3 kJ/mol and was predicted to establish hydrogen interactions with Arg-144, and Asn-179.

When docked individually, all of the tested thioxanthones are predicted to have more affinity to P-gp than mitoxantrone (more negative docking scores, from -5.9 to -6.6 $kcal.mol^{-1}$) (Table 1). The TX129 is the top ranking thioxanthone, with a docking score of -6.6 $kcal.mol^{-1}$. Thioxanthones are predicted to dock to the same binding site as mitoxantrone (Fig 5C), but with higher affinity, suggesting a competitive mechanism of action. Thioxanthones and mitoxantrone, besides being able to bind individually to the same or to different binding pockets on P-gp, may bind simultaneously to the same cavity on the TMD by the establishment of stacking interactions between the two small molecules (Fig 5D). This non-covalent complex (Fig 4C) binds to P-gp with higher (TXA3, TXA4, TXOH, and TX129) or lower (TX105, TX87) affinity than the small molecules docked individually. The non-covalent complex occupies a larger volume in the substrate-binding cavity, interacting with residues from several TMDs. The two-ligand complex establish polar interactions with residues such as Thr-941, Gln-343, and Gly-868, described as being part of the translocation pathway and/or involved in drug binding (Ferreira et al. 2013).

3.5. Protective effects of thioxanthones against mitoxantrone-induced cytotoxicity

Firstly, with the purpose of verifying the impact of P-gp on limiting the mitoxantrone-induced toxicity in RBE4 cells, cells were exposed to a wide range of concentrations of mitoxantrone (0.1-50 μM) in the presence or absence of a P-gp specific inhibitor, zosuquidar (LY335979). Zosuquidar was used at a 5 μM concentration, since this concentration did not show to be cytotoxic in this cell line following 24h of incubation, according to the MTT and NR uptake assays (Sup. Fig. 2), and it was previously reported to be an efficient concentration to inhibit P-gp activity (Durmus et al. 2015; Liu et al. 2014). Mitoxantrone-induced cytotoxicity was evaluated after 4h, 8h and 16h of exposure in co-incubation or not with zosuquidar by the NR uptake assay. For every time-point tested, the concentration-response (death) curves obtained show that the co-incubation with the P-gp inhibitor (MTX + ZQ) resulted in a leftwards shift of the curve (Fig. 6), which reflects an increase in the cytotoxicity of mitoxantrone when P-gp is inhibited. For all the fitted curves, no significant differences in the maximal cell death (TOP) and in the baseline (BOTTOM) were observed (Table 2). For that reason, the EC_{50} values, which represent the half-maximum-effect concentrations from the fitted curves, were used for statistical comparison. As shown in Table 2, the EC_{50} values of the MTX + ZQ curves significantly decreased in comparison to the EC_{50} of the MTX curve after 4h, 8h and 16h incubation. After 4h of incubation the EC_{50} value in cells exposed to both mitoxantrone and zosuquidar decreased from 3.48 μM to 0.92 μM ($p < 0.0001$), and after 8h and 16h it decreased from 2.08 μM to 0.65 μM ($p < 0.0001$) and from 4.31 μM to 1.76 μM ($p < 0.01$), respectively.

Afterwards, and in order to check whether the observed increases in P-gp activity could result in an effective protection of RBE4 cells against mitoxantrone-induced toxicity, cells were exposed to a wide range of concentrations of mitoxantrone (0.1–50 μM), and cytotoxicity was evaluated with or without simultaneous exposure to the tested thioxanthonic derivatives (10 μM) for 4h, 8h, and 16h. Mitoxantrone-induced cytotoxicity was evaluated 24h after the beginning of exposure by the NR uptake assay. Figure 4 shows the concentration–response curves obtained with mitoxantrone alone and with simultaneous incubation with thioxanthones (MTX + TXs). For all the tested thioxanthones, any significant differences were observed in the mitoxantrone-induced cell death, and so a rightwards shift of the mitoxantrone concentration–response curves was not observed as it would if thioxanthone-protective effect was present (Fig. 7). For all the fitted curves, no significant differences in the maximal cell death (TOP) and in the baseline (BOTTOM) were observed (Table 3). EC_{50} values were then used for statistical

comparison. No significant differences were observed for the EC_{50} values of the fitted curves, when compared to the EC_{50} of the MTX curve, with the exception of TXA4 and TX105 for the 4h time point, where the EC_{50} value registered a decreased to 1.564 µM and 1.411 µM, respectively (Table 3B, 3C), in comparison to the EC_{50} of the MTX curve (2.455 µM) ($p < 0.05$).

4. DISCUSSION

The data here presented show that, even though four of the tested thioxanthones – TXA4, TX105, TXA3 and TX129 – successfully increased P-gp activity, they failed to confer protection to RBE4 cells against mitoxantrone-induced cytotoxicity.

The widely accepted *in vitro* BBB model used in the present study, the RBE4 cell line, is one of the best characterized brain endothelial cell lines, which retains many BBB characteristics, particularly the expression of P-gp (Regina et al. 1998; Roux et al. 1994). This cell line has been largely used for the study of the BBB physiology and modulation, namely in signaling studies (Fabian et al. 1998; Krizbai et al. 1995; Smith and Drewes 2006; Zhang et al. 2009b), cell migration (Barakat et al. 2008), permeability studies (Pan et al. 2005), and regulation of P-gp expression and activity (Pilorget et al. 2007; Yu et al. 2007).

Thioxanthones (dibenzo-γ-thiopyrones) are a group of compounds that have risen increasing interest in the field of medicinal chemistry. These compounds have shown to have remarkable biological properties, such as antitumor activity and P-gp modulation (Paiva et al. 2013; Palmeira et al. 2012b; Silva et al. 2014c). Very recently, it was demonstrated that thioxanthonic derivatives were able to significantly increase both P-gp expression and activity in Caco-2 cells (Silva et al. 2014c). In addition, an important decrease on the Rho 123 intracellular accumulation, reflecting an increase on P-gp activity, could be seen only after a 45 min incubation period of Caco-2 cells with these thioxanthonic compounds (Silva et al. 2014c). This increase of P-gp activity does not reflect an effect from increased P-gp expression, since such a short time of Caco-2 cells incubation with thioxanthones is most probably not enough time to allow an induction on the protein expression to take place.

In our present study, we tested four of the most promising thioxanthonic compounds according to the previous study performed in Caco-2 cells (Silva et al. 2014c), together with two newly synthetized thioxanthones, TX105 and TX129 in RBE4 cells. When the Rho 123 efflux was evaluated in the presence of the tested thioxanthones (10 μM) during an efflux step of 45 min, the thioxanthonic derivatives TXA4, TXA3, TX105 and TX129 were able to produce a significant increase in the Rho 123 efflux from RBE4 cells, and thus inducing an activation of P-gp efflux transport. At 20 μM, all the compounds, except TXOH, provoked a significant increase in Rho 123 efflux. These four thioxanthonic compounds increased Rho 123 efflux by 160-170%, which means that the intracellular levels of the P-gp substrate were significantly reduced in the presence of these molecules. We further analyzed the impact of such compounds on the ATPase activity of recombinant human P-gp in a cell membrane fraction. Compounds that interact with P-gp can be

identified as stimulators or inhibitors of its ATPase activity. Stimulators of the ATPase activity are likely substrates for P-gp-mediated efflux, which will also competitively inhibit transport of other P-gp substrates (Ambudkar et al. 1999). Our results show that, at the concentration tested (10 μM), all the thioxanthones poorly increased the ATPase activity of human P-gp. These findings suggest that these compounds may work as poor P-gp substrates, and thus unlikely participate in competition phenomena and inhibit the transport of P-gp substrates. Docking studies explored the binding configurations of the tested thioxanthonic derivatives, using an X-ray P-gp structure based on the mouse P-gp (Szewczyk et al. 2015). Recent data shows that, unlike the symmetric Sav1866 template (already described in previous studies (Silva et al. 2014a; Silva et al. 2014d; Vilas-Boas et al. 2013a), mouse P-gp has a high degree of asymmetry, with only 44% similarity between the two halves of TMDs (Pan and Aller 2015), which likely may play a major role in the overall function and mechanism of the transporter. In fact, it is thought that mouse P-gp should favor closed TMDs and open nucleotide-binding domains, whereas Sav1866 should favor the exact opposite configurations, as well as the hydrophobicity of mouse P-gp in the drug-binding pocket is substantially larger than that of Sav1866 (Pan and Aller 2015). Given that rat P-gp should have a closer homology to mouse P-gp than to the Sav1866 template, we decided to preferentially adopt the mouse P-gp model for docking studies. Indeed, when comparing the results retrieved with the Sav 1866 (see Sup. Data) and the mouse P-gp model, we can observe that all thioxanthones require a higher free energy to bind to P-gp in the mouse model than in the Sav1866, probably due to the higher hydrophobicity of its drug-binding pocket. Furthermore, thioxanthones are described to bind to different TMDs and with different affinities between models. Still, docking studies in both models allowed us to understand that thioxanthones bind to P-gp with a considerable high affinity, with a preferential binding pocket in P-gp, engulfed by TMD 2, 3, 10 and 11 according to the mouse model, further supporting that these compounds most probably interact with P-gp for its activation. According to the experimental results, thioxanthones activate P-gp efflux when simultaneously incubated with Rho 123, suggesting that, the former should bind at a different location, and increase the P-gp efflux of the later by allosteric modulation (Ghosh et al. 2006).

Considering the observed effects of the tested thioxanthones on the P-gp activity and the unlikely scenario that they might competitively inhibit the P-gp substrate efflux, we evaluated the impact of these compounds on the mitoxantrone-induced cytotoxicity in RBE4 cells. Mitoxantrone is an antineoplastic agent, used in the treatment of several types of cancer, mostly metastatic breast cancer, acute myeloid leukemia, and non-Hodgkin's lymphoma (Parker et al. 2010). P-gp and BCRP have shown to team up at the BBB for the efflux transport of mitoxantrone (Agarwal et al. 2011a; Kodaira et al. 2010;

Lee et al. 2005). When RBE4 cells were co-incubated with mitoxantrone and the third generation P-gp inhibitor, zosuquidar, we observed a leftwards shift of the concentration-response (cell death) curves, accompanied by a decrease in the EC_{50} values, in comparison to cells incubated with mitoxantrone alone. The results indicate that mitoxantrone successfully demonstrated to be a P-gp substrate in this cell line, since the P-gp inhibition resulted in a reduced efflux of mitoxantrone and thus an increase in the mitoxantrone-induced cytotoxicity. Therefore, RBE4 cells were further co-incubated with mitoxantrone and each test compound (10 μM) for 4h, 8h and 16h, and mitoxantrone-induced cytotoxicity was evaluated 24h after the beginning of the exposure. Surprisingly, it was not observed a rightwards shift of the mitoxantrone concentration–response curves in the cell samples co-incubated with the thioxanthonic compounds, in comparison to cells incubated with mitoxantrone alone, as well as it was not observed a significant difference on the EC_{50} values of the fitted curves. These findings suggest that none of these compounds was able to confer additional protection against mitoxantrone-induced cytotoxicity in RBE4 cells, unlike what was observed in a previous study for paraquat in Caco-2 cells (Silva et al. 2014c). Docking results using the mouse P-gp model show that all thioxanthones bind with higher affinity to P-gp (lower docking scores) than mitoxantrone. This suggests that thioxanthones may compete with mitoxantrone for one of P-gp drug binding sites, even though thioxanthonic compounds showed to have alternative binding sites to that of mitoxantrone, nor should inhibit substrate transport by competition, since no increase on the P-gp ATPase activity was registered. Besides, since mitoxantrone is a common substrate of P-gp and BCRP, P-gp is not the only transporter implicated in the efflux of mitoxantrone in this experimental setting, which can explain the absence of a P-gp activation-mediated protection against its cytotoxicity. Several studies show that a double knockout or inhibition of P-gp and BCRP frequently results in an increase in a greater brain accumulation of common substrates than the additive effects of single knockout or inhibition, which suggests that when the function of one of the transporters is compromised it tends to compensated by the other with an increase in its expression and/or activity (Cisternino et al. 2004; de Vries et al. 2007). Also, there is evidence that BCRP expression can be down-regulated in cell models overexpressing P-gp (Bark et al. 2008). Therefore, it cannot be put aside that an eventual change on Bcrp expression and/or activity may occur when P-gp is activated in the present experimental model, and thus that a P-gp/BCRP cooperation may imply that the activation of P-gp alone may not result in an appreciable decrease in cell accumulation of dual substrates.

On the other hand, it should also be considered that the likely reduction on the intracellular accumulation of mitoxantrone as a result of P-gp activation by thioxanthones may not be sufficient to see an actual reduction in its induced cytotoxicity. In fact, the P-gp

activation induced by thioxanthones might be a transient phenomenon, resulting in a reduction of mitoxantrone intracellular accumulation only for a short period of time, which may not be enough to confer cell protection over a period of exposure of 4h, 8h or 16h. Furthermore, in the previous study conducted by Silva et al (2014), thioxanthonic derivatives have shown to not only promote an immediate activation of P-gp but also to up-regulate the P-gp protein expression in Caco-2 cells (Silva et al. 2014c), which was not explored in the present study. An increase of the expression of P-gp at the cell membrane is more likely to produce long-lasting consequences in terms of the drug efflux of substrates over the course of the incubation period, which may explain the differences found between the present study and the previously conducted in Caco-2 cells. In fact, since the two models, Caco-2 and RBE4 cells, represent two different species, and considering the significant species differences of some of the P-gp regulatory pathways, particularly between ligand-binding and activation profiles for orphan nuclear receptors (Wang and LeCluyse 2003), we cannot guarantee that the mechanism by which thioxanthones up-regulate P-gp protein expression in the Caco-2 cell model is activated in the RBE4 cells. Besides, drug binding involves an induced fit (Loo et al. 2003c), and in order to produce a conformational change that induces ATP hydrolysis drug binding has to occur in certain sites, and in a certain orientation (Aller et al. 2009). As previously mentioned, the tested thioxanthonic compounds establish interactions with TMDs of the mouse P-gp model that are distinct from those established with the Sav1866 model. In addition, previous studies already evidenced different P-gp structure and activity depending on the animal model, indicating important interspecies differences (Pan and Aller 2015; Xia et al. 2006). In this way, thioxanthones may bind differently to the rat and to the human P-gp due to structural differences, which may explain the discrepant results regarding the protection against the cytotoxicity of P-gp substrates that these compounds produced in the present (rat) model and the previous one (Silva et al. 2014c) (Caco-2 cells, human cell line). Furthermore, since P-gp has multiple and overlapping binding sites (Safa 2004), the mechanism of action of these thioxanthones may be inhibitory or activator, depending on the compounds that are co-incubated and their binding mechanisms (Litman et al. 1997).

In conclusion, even though these compounds failed to demonstrate an increased protection against mitoxantrone-induced cytotoxicity in RBE4 cells, they remain as interesting drug candidates for an antidote strategy against the toxicity induced by P-gp substrates. Most of the thioxanthones showed to promptly activate P-gp efflux transport, achieved through a change in the protein conformation, which, in comparison with the induction phenomenon, results in a much faster manner to reduce the cytotoxicity of harmful P-gp substrates. However, as an increase in the protein expression of P-gp does

not always reflect an increase in its activity, an increase in the efflux of a P-gp substrate due to P-gp activation does not always translate into an overall increase in the efflux of all its substrates, and therefore it does not necessarily confer protection against the toxicity of any harmful P-gp substrate.

5. AUTHOR DISCLOSURE

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Contributors: Fernando Remião, Renata Silva and Catarina Chaves designed the study and wrote the protocol. Catarina Chaves performed the experiments. Andreia Palmeira, Emília Sousa and Madalena Pinto were responsible for the synthesis and docking studies. All authors contributed to and have approved the final manuscript.

Conflict of interest: All authors declare that they have no conflicts of interest.

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6. FIGURES

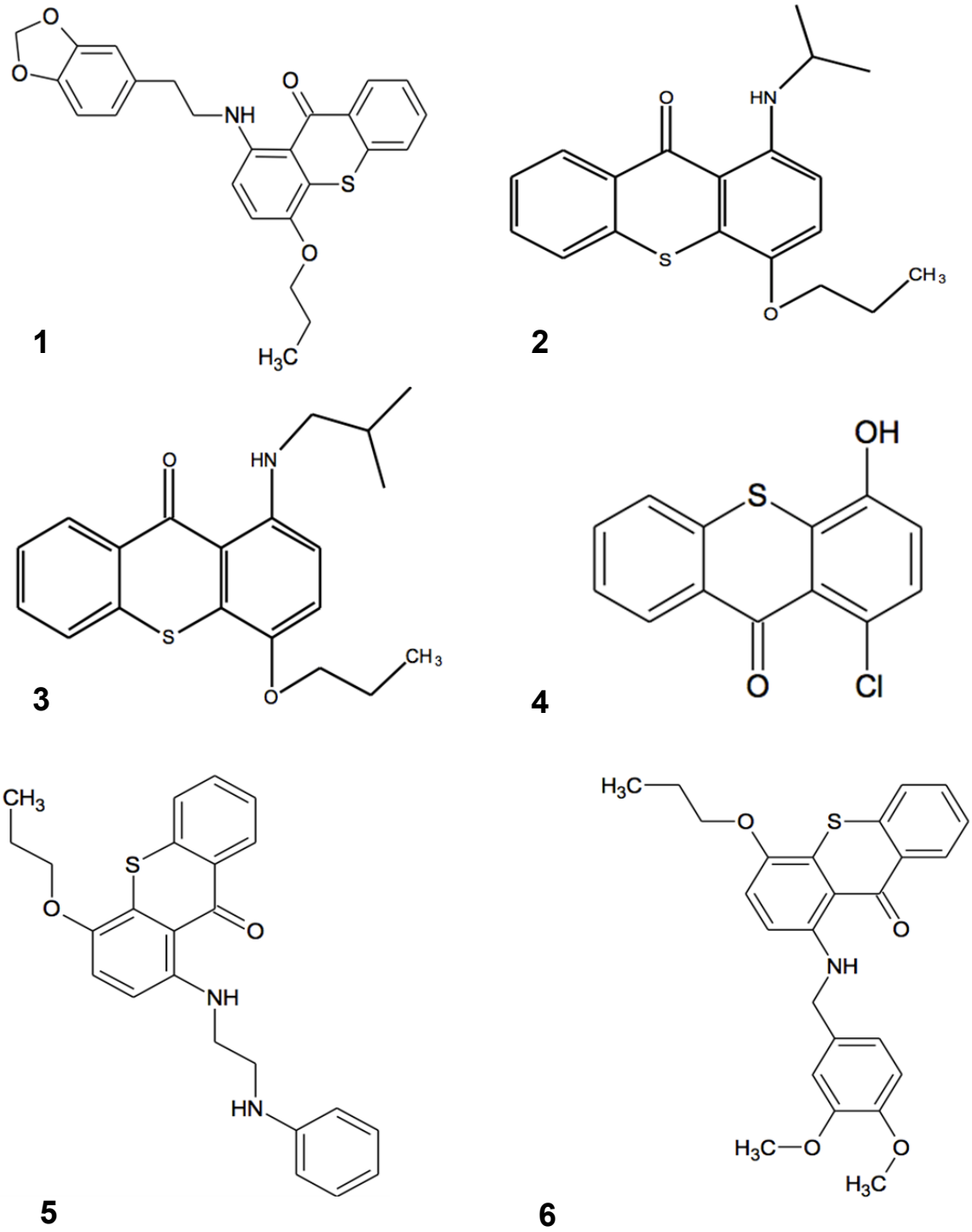


Fig. 1. Chemical structures of the six thioxanthones (TXs) investigated in this study.

(1) TX87 (1-{[2-(1,3-benzodioxol-5-yl)ethyl]amino}-4-propoxy-9*H*-thioxanthen-9-one),

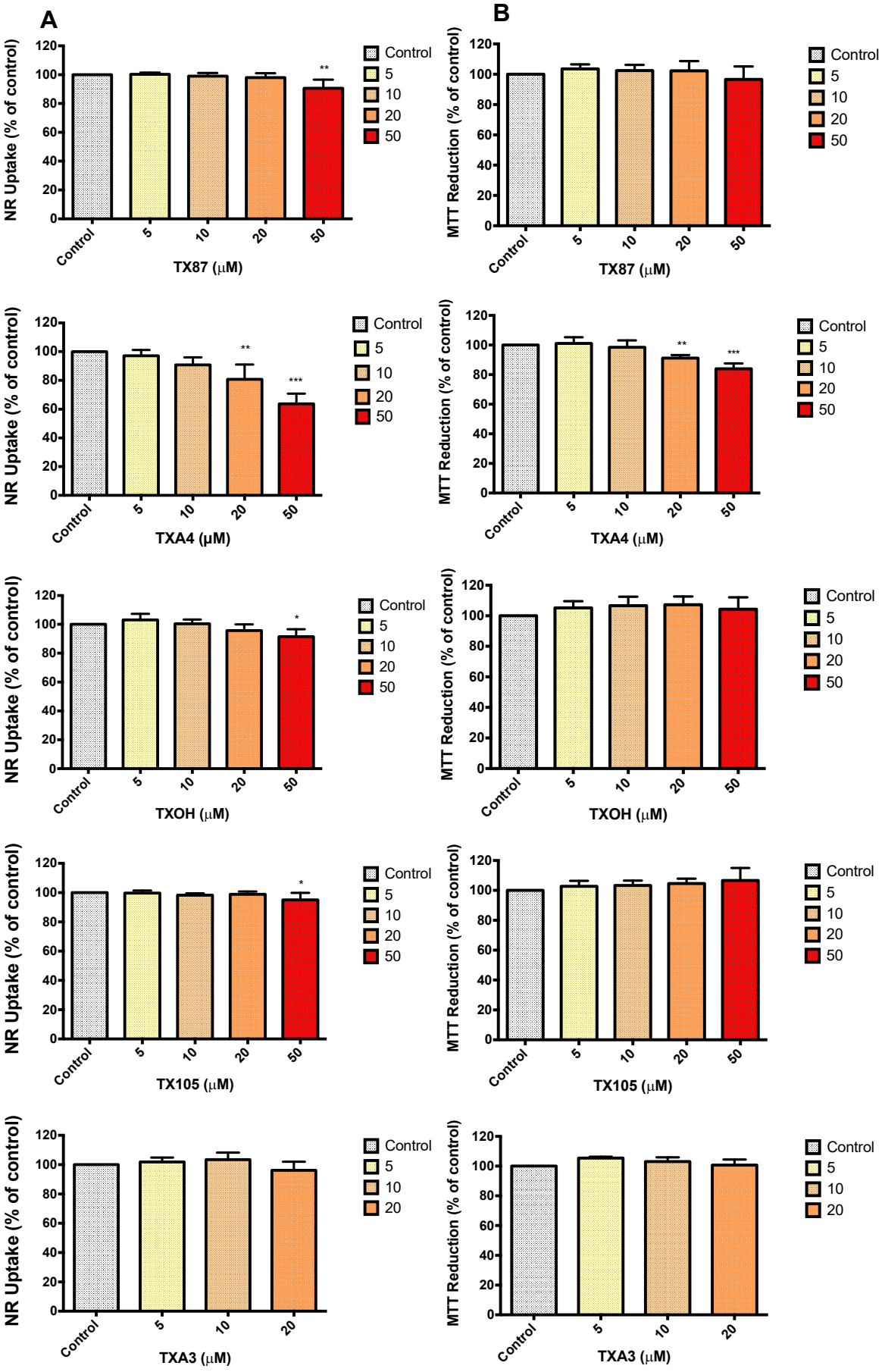
(2) TXA4 (1-(propan-2-ylamino)-4-propoxy-9*H*-thioxanthen-9-one),

(3) TXA3 (1-[(2-methylpropyl)amino]-4-propoxy-9*H*-thioxanthen-9-one),

(4) TXOH (1-chloro-4-hydroxy-9*H*-thioxanthen-9-one),

(5) TX105 (1-{[2-(phenylamino)ethyl]amino}-4-propoxy-9*H*-thioxanthen-9-one,

(6) TX129 (1-[(3,4-Dimethoxybenzyl)amino]- 4-propoxy-9*H*-thioxanthen-9-one.


A
B
NR Uptake (% of control)
MTT Reduction (% of control)
Control
5
10
20
50
TX87 (μM)
TXA4 (μM)
TXOH (μM)
TX105 (μM)
TXA3 (μM)
0
20
40
60
80
100
120
**
***
*

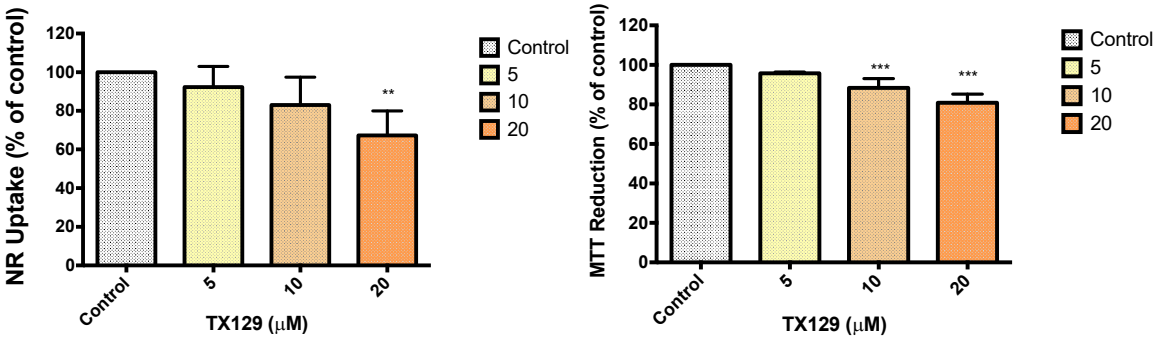


Fig. 2: Cytotoxicity profiles of all the tested thioxanthones, assessed by the NR uptake (A) and the MTT reduction (B) assays. Cytotoxic effect was evaluated 24h after exposure to the compound in concentrations ranging from 5 to 50 µM. Results are shown as mean ± SD of four independent experiments. Differences between control and treated cells were estimated using Kruskal-Wallis test (one-way ANOVA on ranks) followed by Dunn's multiple comparison post hoc test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *vs* control).

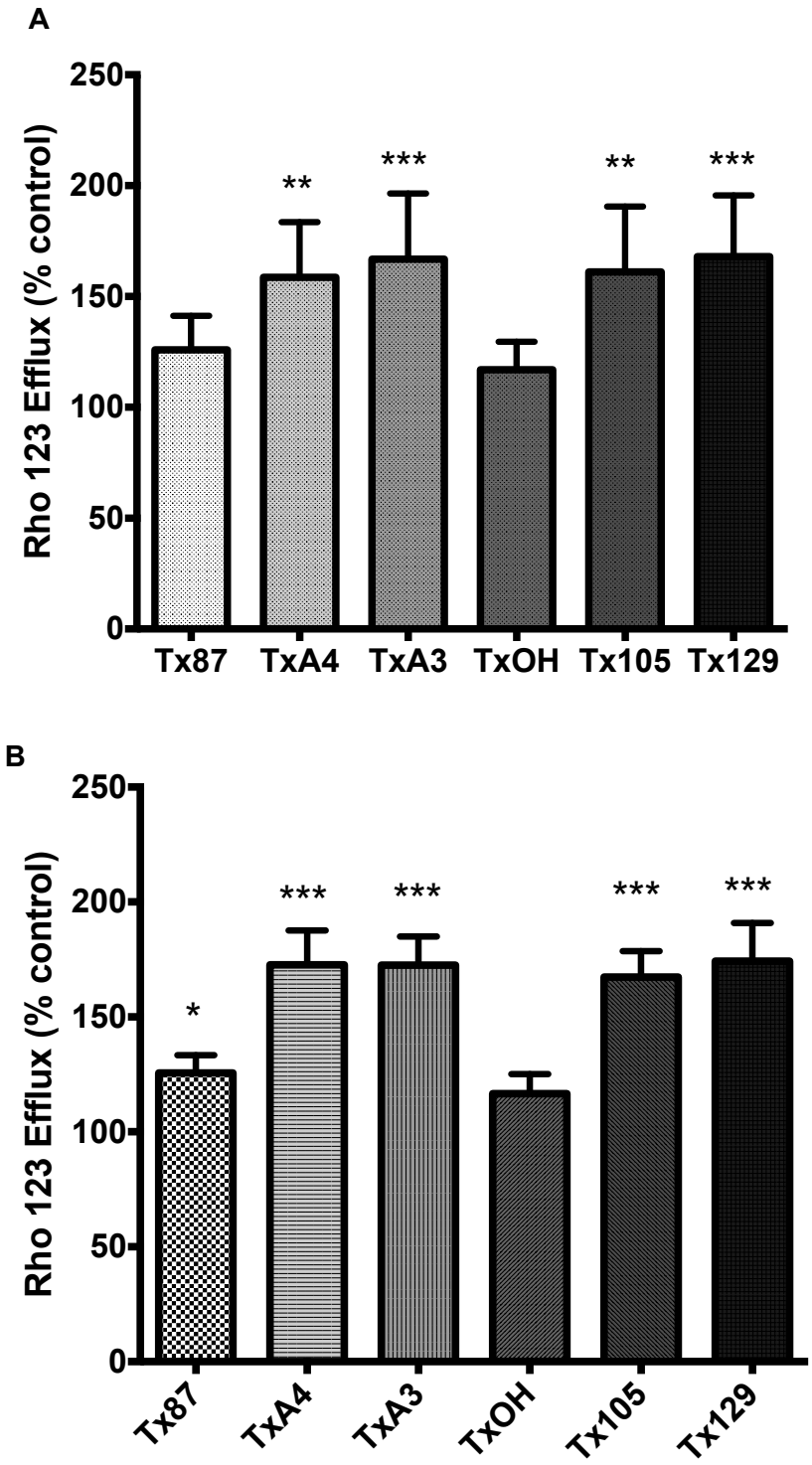


Fig. 3: P-glycoprotein activity evaluated through the Rho 123 efflux in the presence of thioxanthones, 10 µM (A) or 20 µM (B) during the Rho 123 efflux phase. Results are shown as mean ± SD from five independent experiments (performed in triplicate). Statistical comparisons were estimated using the nonparametric method of Kruskal-Wallis (one-way ANOVA), followed by Dunn's post hoc test (** $p < 0.01$; *** $p < 0.001$ *vs* control).

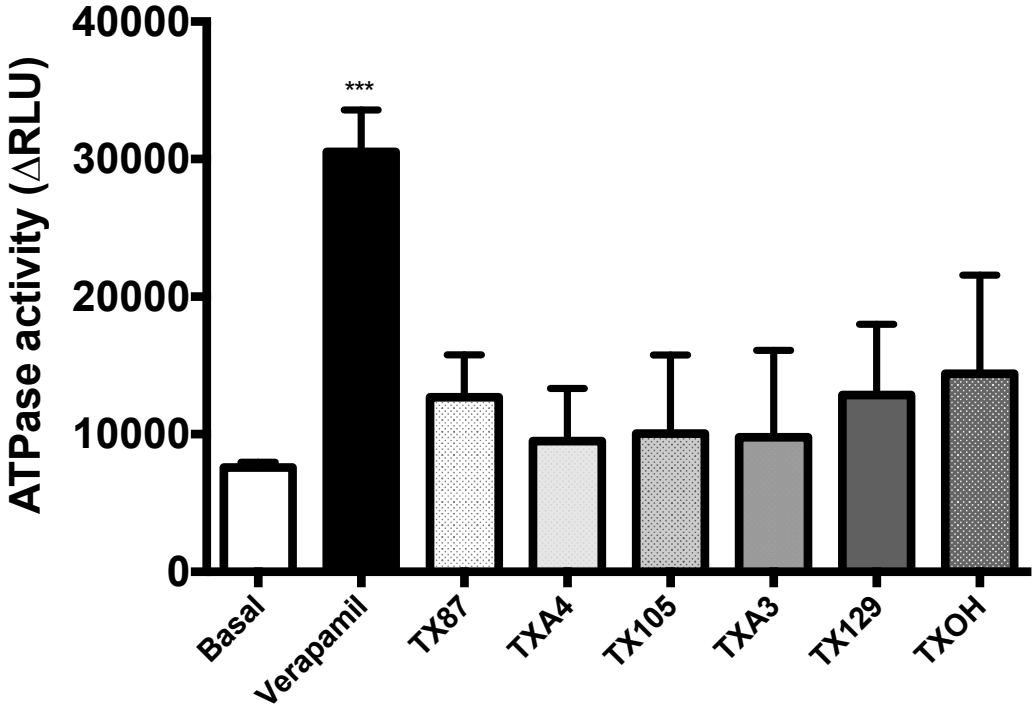


Fig. 4. Effect of several thioxanthones on the ATPase activity in membrane vesicles enriched with human P-gp. Verapamil was used as a positive control of the stimulation of P-gp ATPase activity and P-gp substrate. Results are presented as change in luminescence (ΔRLU) and are expressed as mean ± SD from two independent experiments, performed in duplicate. Differences between basal, verapamil and thioxanthone-treated membranes were estimated using one-way ANOVA, followed by the Bonferroni's multiple comparisons post hoc test (*** $p < 0.001$ *vs* basal P-gp ATPase activity).

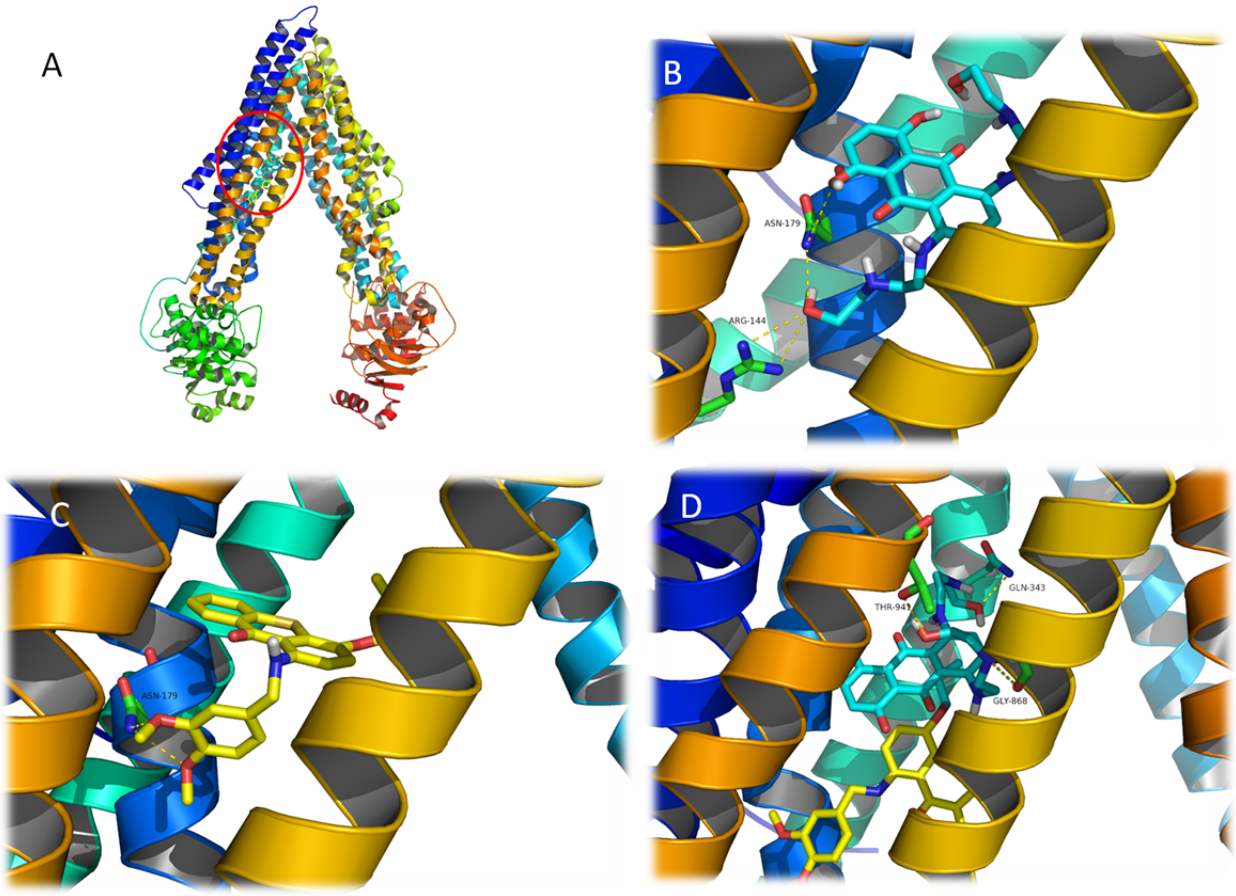


Fig. 5. P-gp 3D structure with thioxanthones/mitoxantrone binding site surrounded by a red circle (A); Mitoxantrone (B) and TX129 (C) docked individually into P-gp TMDs; mitoxantrone and TX129 (C) docked simultaneously into P-gp TMDs. Mitoxantrone and TX129 are represented as blue and yellow sticks, respectively. Polar interactions are represented with yellow dashes.

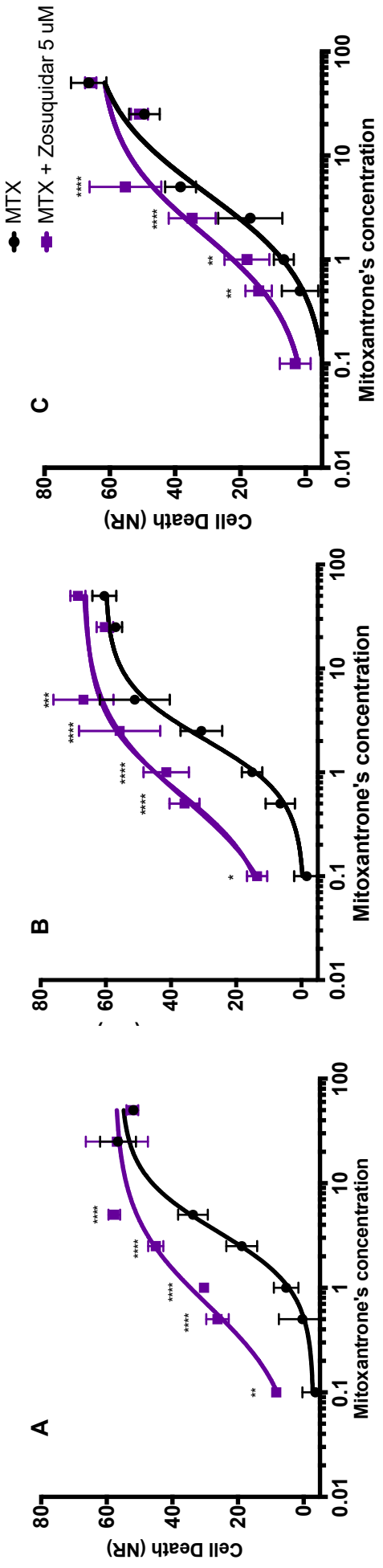
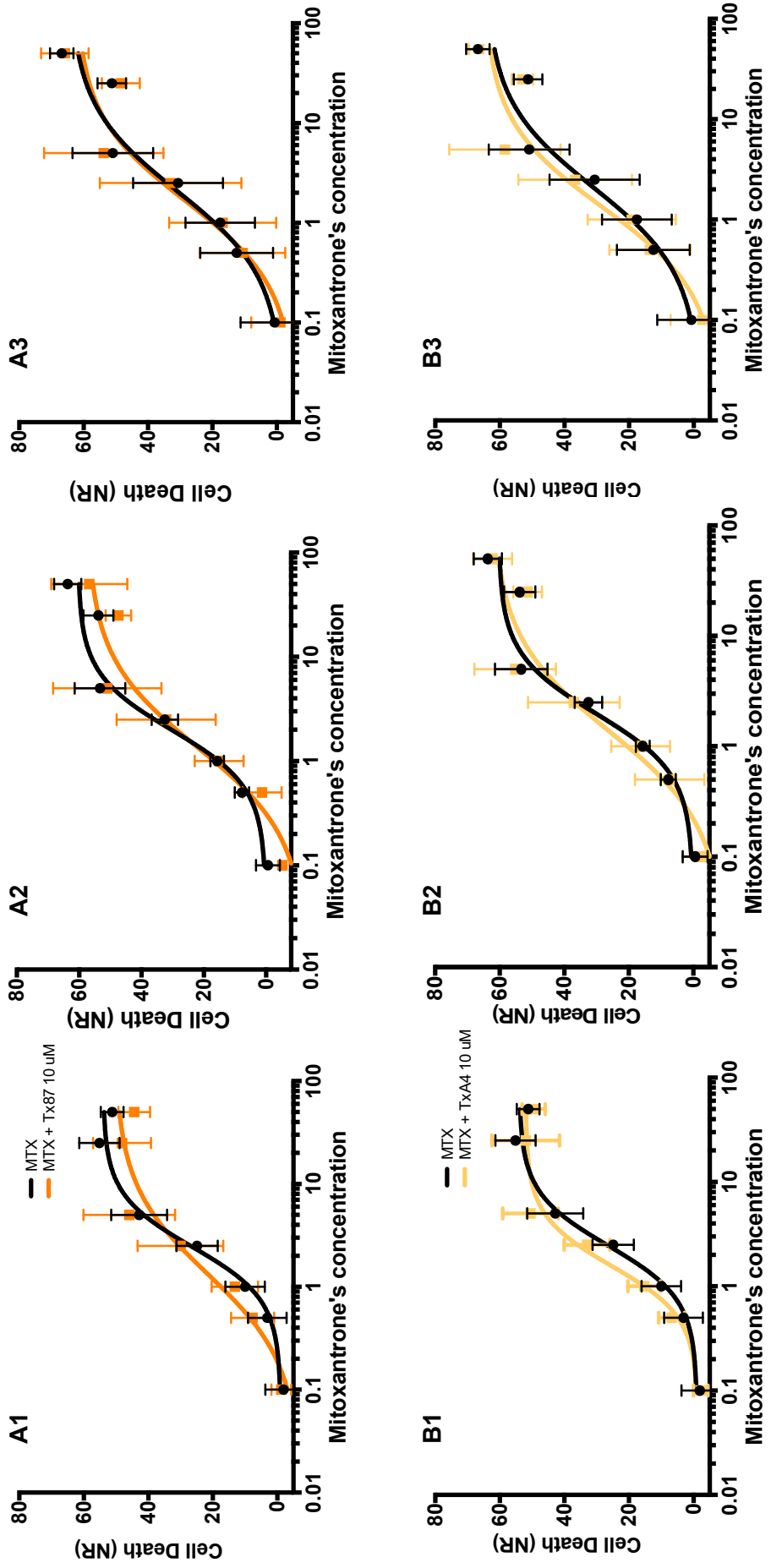
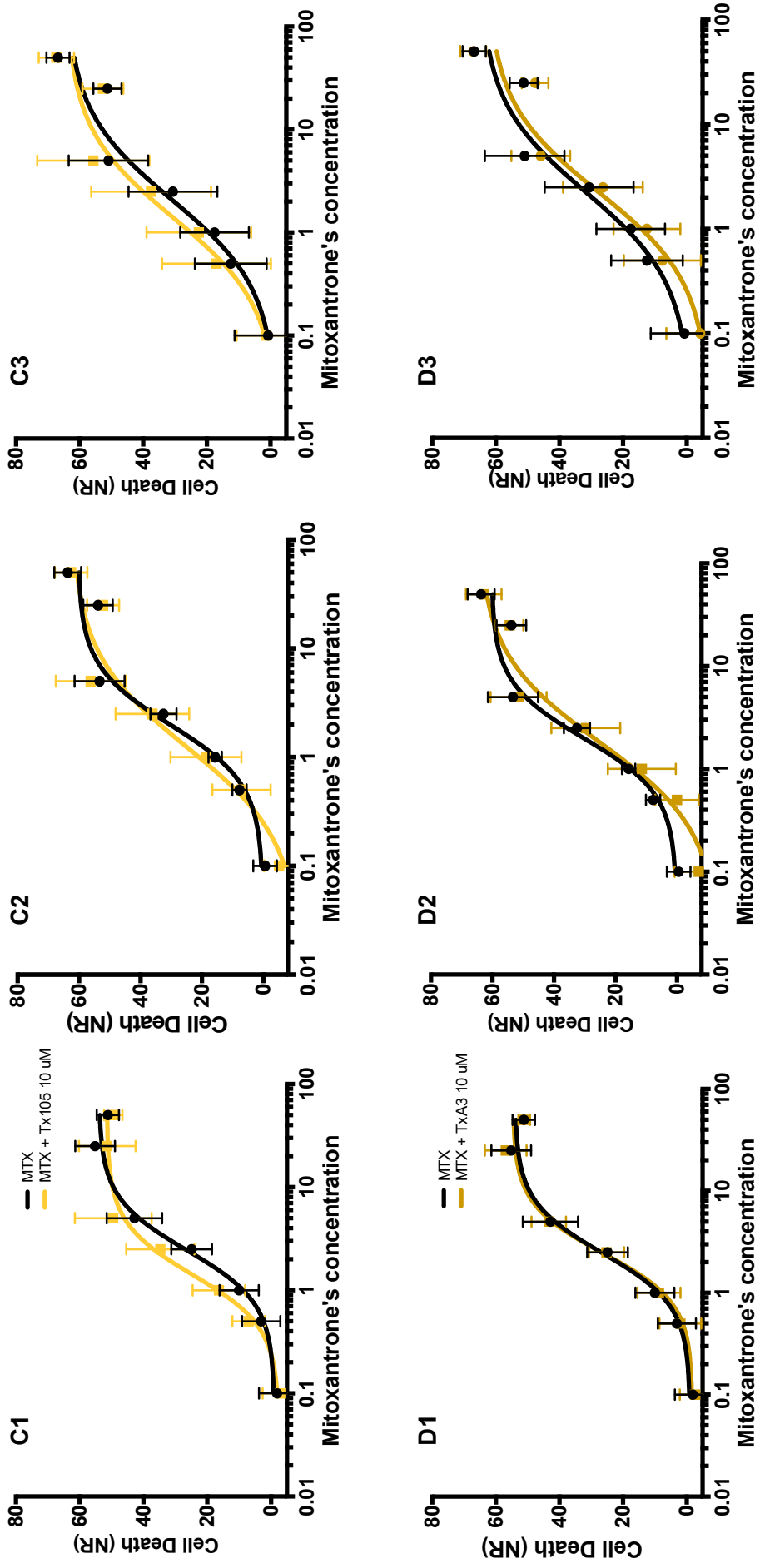


Fig. 6. Mitoxantrone concentration–response (cell death) curves in the absence (MTX) or presence of the specific P-gp inhibitor, zosuquidar (5 µM) (MTX + ZQ) (A-4h, B-8h, or C-16h of incubation in the presence of MTX +/- ZQ, in a total of 24 h of incubation). Results are shown as mean ± SD from two independent experiments (performed in triplicate). Concentration-response curves were fitted using least squares as the fitting method and the comparisons between MTX and MTX + TXs curves (LOG EC_{50}, TOP, BOTTOM, and Hill Slope) were made using the extra sum-of-squares *F* test. Statistical comparisons were made using two-way ANOVA, followed by Sidak's multiple comparisons post hoc test (**p < 0.01; ****p < 0.0001 vs. MTX).


A1
A2
A3
B1
B2
B3
Cell Death (NR)
Mitoxantrone's concentration
0.01
0.1
1
10
100
0
20
40
60
80
MTX
MTX + Tx87 10 uM
MTX + TxA4 10 uM


C1
C2
C3
D1
D2
D3
Cell Death (NR)
Mitoxantrone's concentration
0
20
40
60
80
0.01
0.1
1
10
100
MTX
MTX + Tx105 10 uM
MTX + TxA3 10 uM

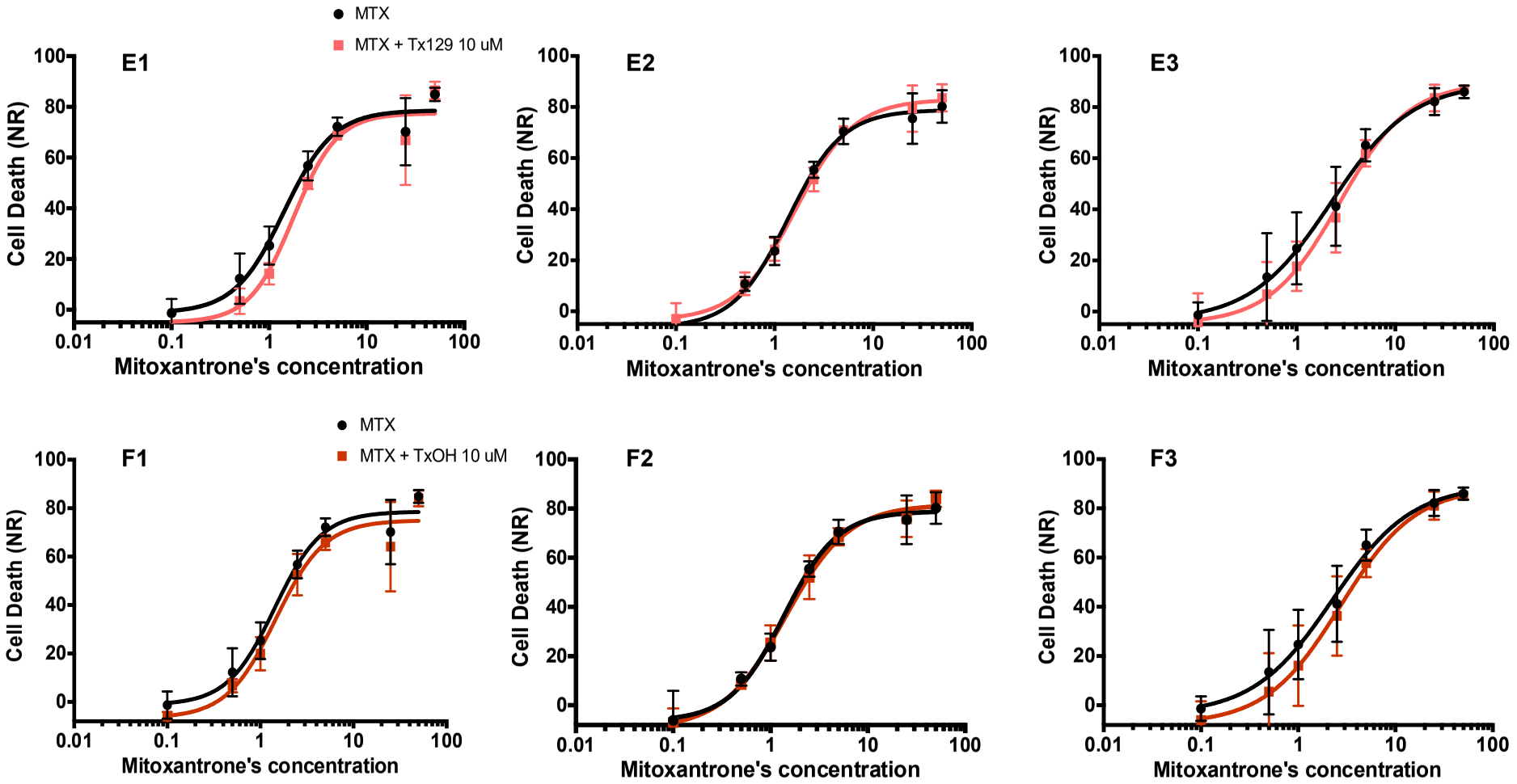


Fig. 7. Mitoxantrone concentration–response (cell death) curves in the absence (MTX) or presence of thioxanthones (10 μM) (MTX + TXs) (4h, 8h, or 16h of incubation in the presence of MTX +/- TXs, in a total of 24 h of incubation). A) TX87, B) TXA4, C) TX105, D) TXA3, E) TX129, F) TXOH, and 1) 4h, 2) 8h and 3) 16h. Results are shown as mean ± SD from at least three independent experiments (performed in triplicate). Concentration-response curves were fitted using least squares as the fitting method and the comparisons between MTX and MTX + TXs curves (LOG EC_{50}, TOP, BOTTOM, and Hill Slope) were made using the extra sum-of-squares *F* test. Statistical comparisons were made using two-way ANOVA, followed by Sidak's multiple comparisons post hoc test.

7. TABLES

Table 1. Docking scores (kcal.mol^{-1}) of thioxanthones and mitoxantrone.

	Docking score (kcal.mol^{-1})		Docking score (kcal.mol^{-1})
TX105	-6.1	**Mitoxantrone + TX105**	-5.9
TX129	-6.6	**Mitoxantrone + TX129**	-6.9
TX87	-6.3	**Mitoxantrone + TX87**	-5.8
TXA3	-5.8	**Mitoxantrone + TXA3**	-6.1
TXA4	-6.2	**Mitoxantrone + TXA4**	-6.3
TXOH	-5.9	**Mitoxantrone + TXOH**	-7.2
Mitoxantrone	-5.3		

Table 2. EC_{50} (half-maximum-effect concentrations), TOP (maximal effect), BOTTOM (baseline) and Hill Slope values of the MTX concentration-response curves, with (MTX + ZQ) or without (MTX) simultaneous exposure to a specific P-gp inhibitor, Zosuquidar (5 µM) for 4h, 8h and 16h. Concentration-response curves were fitted using the least squares as the fitting method and the comparisons between MTX and MTX + ZQ curves (EC_{50}, TOP, BOTTOM, Hill Slope for 4h and 8h time points, and EC_{50}, TOP, BOTTOM for 16h time point) were made using extra sum-of-squares *F* test. (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ *vs* the MTX curve).

Time of exposure	**4h**		**8h**		**16h**	
	MTX	**MTX + ZQ**	**MTX**	**MTX + ZQ**	**MTX**	**MTX + ZQ**
EC_{50} (µM)	3.48	0.92****	2.08	0.65***	4.31	1.76**
TOP	55.84	56.34	60.27	66.52	67.51	63.77
BOTTOM	-3.01	8.36	-0.83	8.82	-6.88	-1.15
Hill Slope	1.54	1.40	1.54	1.18	-	-
Curve *p* value (comparison between MTX and MTX + TXs curves)	< 0.0001		< 0.0001		< 0.0001	

Table 3. EC_{50} (half-maximum-effect concentrations), TOP (maximal effect), BOTTOM (baseline) and Hill Slope values of the mitoxantrone concentration-response curves, with (MTX + TXs) or without (MTX) simultaneous exposure to the tested thioxanthones (10 µM) for 4h, 8h and 16h. Concentration-response curves were fitted using the least squares as the fitting method and the comparisons between MTX and MTX + TXs curves (EC_{50}, TOP, BOTTOM, Hill Slope for 4h and 8h time points, and EC_{50}, TOP, BOTTOM for 16h time point) were made using extra sum-of-squares *F* test. (* $p < 0.05$ *vs* the MTX curve).

3A) TX87

Time of exposure	4h		8h		16h	
	MTX	**MTX + TX87**	**MTX**	**MTX + TX87**	**MTX**	**MTX + TX87**
EC_{50} (µM)	2.46	1.65	1.99	1.54	2.10	1.65
TOP	54.04	47.42	60.38	53.45	64.36	62.48
BOTTOM	-1.00	0.34	0.20	-5.59	-2.07	-5.82
Hill Slope	1.71	1.85	1.60	1.72	-	-
Curve *p* value (comparison between MTX and MTX + TXs curves)	0.24		0.42		0.97	

3B) TXA4

Time of exposure	4h		8h		16h	
	MTX	**MTX + TXA4**	**MTX**	**MTX + TXA4**	**MTX**	**MTX + TXA4**
EC_{50} (µM)	2.46	1.56*	1.99	1.58	2.10	1.34
TOP	54.04	52.05	60.38	58.29	64.36	64.79
BOTTOM	-1.00	-1.21	0.20	-2.00	-2.07	-8.02
Hill Slope	1.71	1.79	1.60	1.72	-	-
Curve *p* value (comparison between MTX and MTX + TXs curves)	0.06		0.90		0.65	

3C) TX105

Time of exposure	4h		8h		16h	
	MTX	**MTX + TX105**	**MTX**	**MTX + TX105**	**MTX**	**MTX + TX105**
EC_{50} (µM)	2.46	1.41*	1.99	1.42	2.10	1.42
TOP	54.04	51.54	60.38	59.28	64.36	64.24
BOTTOM	-1.00	-2.43	0.20	-5.54	-2.07	-2.61
Hill Slope	1.71	1.72	1.60	1.49	-	-
Curve *p* value (comparison between MTX and MTX + TXs curves)	0.06		0.76		0.61	

3D) TXA3

Time of exposure	4h		8h		16h	
	MTX	**MTX + TXA3**	**MTX**	**MTX + TXA3**	**MTX**	**MTX + TXA3**
EC_{50} (µM)	2.46	2.46	1.99	1.97	2.10	2.43
TOP	54.04	54.66	60.38	60.19	64.36	63.43
BOTTOM	-1.00	-1.72	0.20	-7.05	-2.07	-6.37
Hill Slope	1.71	1.80	1.60	1.59	-	-
Curve *p* value (comparison between MTX and MTX + TXs curves)	0.98		0.30		0.43	

3E) TX129

Time of exposure	4h		8h		16h	
	MTX	**MTX + TX129**	**MTX**	**MTX + TX129**	**MTX**	**MTX + TX129**
EC_{50} (µM)	1.42	1.77	1.37	1.63	2.24	2.74
TOP	78.61	77.46	78.93	83.06	90.59	93.61
BOTTOM	-1.27	-5.03	-6.44	-3.66	-5.06	-8.86
Hill Slope	1.72	1.93	1.58	1.46	-	-
Curve *p* value (comparison between MTX and MTX + TXs curves)	0.25		0.77		0.54	

3F) TXOH

Time of exposure	4h		8h		16h	
	MTX	**MTX + TXOH**	**MTX**	**MTX + TXOH**	**MTX**	**MTX + TXOH**
EC_{50} (µM)	1.42	1.46	1.37	1.43	2.24	2.76
TOP	78.61	75.02	78.93	81.60	90.59	91.21
BOTTOM	-1.27	-6.68	-6.44	-9.39	-5.06	-10.08
Hill Slope	1.72	1.65	1.58	1.35	-	-
Curve *p* value (comparison between MTX and MTX + TXs curves)	0.57		0.94		0.35	

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SUPPLEMENTARY DATA

I. Experimental Section

1.1. Docking of mitoxantrone and thioxanthones into a P-gp model built based on Sav1866

For the structure-based study, the 3D structures of six thioxanthones and mitoxantrone were drawn using HyperChem 7.5 (Froimowitz 1993), and minimized by the semi-empirical Polak-Ribiere conjugate gradient method (RMS<0.1 kcal.Å^{-1}. mol^{-1}) (Zhang et al. 2006). Docking simulations between a previously built P-gp model [successfully used in the discovery of P-gp modulators in (Palmeira et al. 2012b)] and thioxanthones, or mitoxantrone, or thioxanthones together with mitoxantrone were undertaken in AutoDock Vina (Scripps Research Institute, USA) (Seeliger and de Groot 2010; Trott and Olson 2009). AutoDock Vina considered the target conformation as a rigid unit while the ligands were allowed to be flexible and adaptable to the target. Vina searched for the lowest binding affinity conformations and returned nine different conformations for each ligand. AutoDock Vina was run using an exhaustiveness of 8 and a grid box with the dimensions 37.0, 30.0, 40.0, engulfing the channel formed by the TMDS. Conformations and interactions were visualized using PyMOL version 1.3 (Lill and Danielson 2010). Validation of the docking of P-gp activators using known P-gp activators (Palmeira et al. 2012b; Sousa et al. 2013; Sterz et al. 2009) has already been described (Silva et al. 2014a; Silva et al. 2014d; Vilas-Boas et al. 2013a).

II. Results

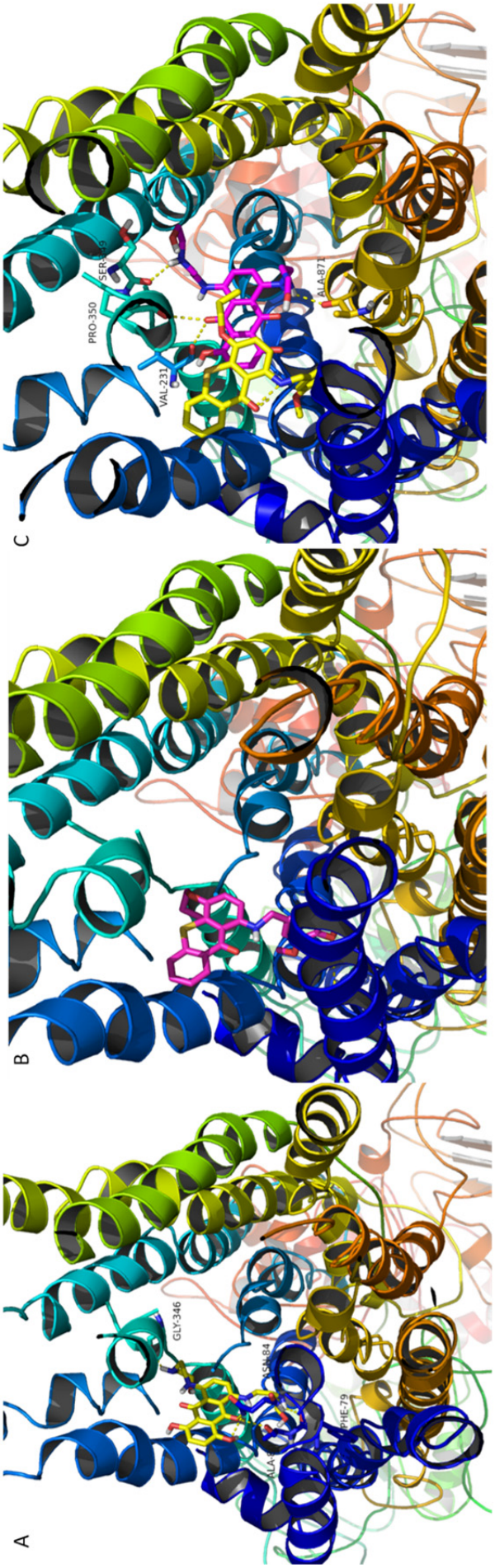
2.1. Docking studies: results with a Sav P-gp model

Mitoxantrone binds to a cavity formed by TMDs 4, 5, 8-10 and 12 (Sup. Fig 1A), and it has shape, size, and stereoelectronic complementarity to P-gp binding pocket forming a stable complex with P-gp with a negative energy of -7.5 kJ/mol and was predicted to establish hydrogen interactions with Gly-346, Asn-84, Ala-80, and Phe-79.

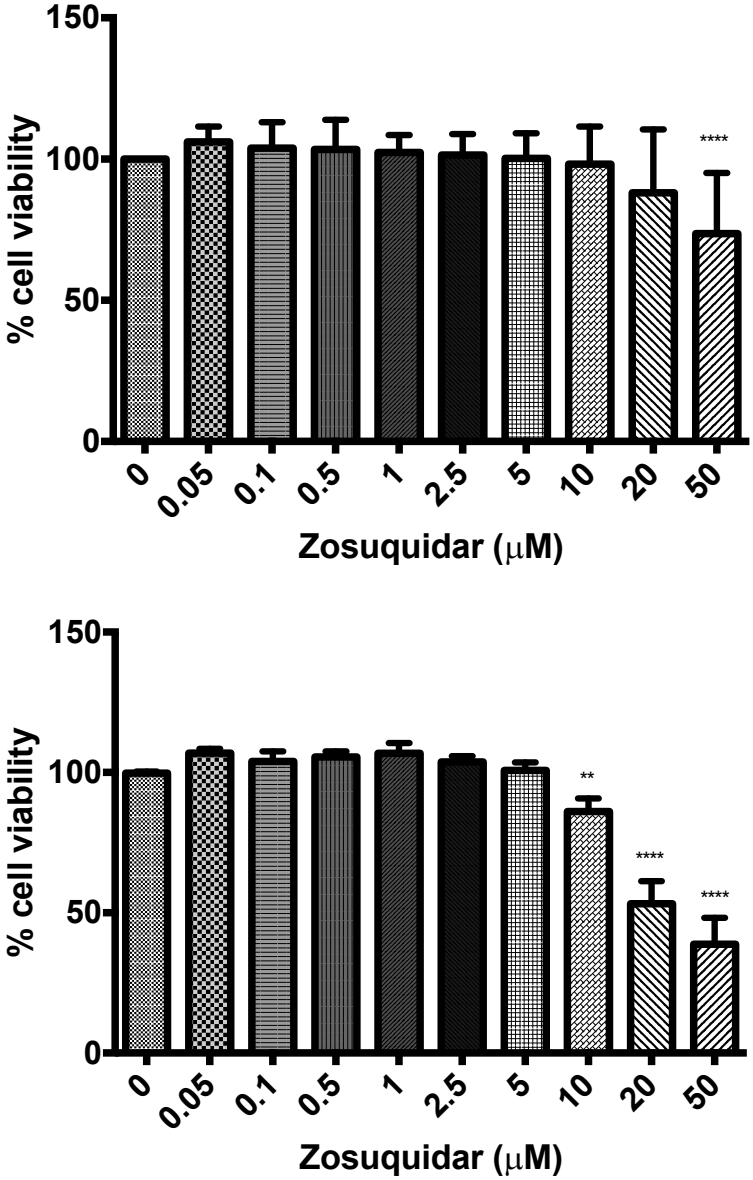
When docked individually, five of the six thioxanthones are predicted to have more affinity to P-gp than mitoxantrone (more negative docking scores) (Sup. Table 1). The TX87 is the top ranking thioxanthone, with a docking score of -9.3 kcal.mol^{-1}. Thioxanthones have two preferential binding pockets in P-gp, engulfed by TMDs 4, 5, 8-10 and 12, or by TM 1-3, 6, 7, and 11. Thioxanthones and mitoxantrone, besides being able to bind individually to the same or to different binding pockets on P-gp, may bind simultaneously to the same cavity on the TMD by the establishment of stacking interactions between the two small molecules (Sup. Fig. 1C). This non-covalent complex (Sup. Fig. 1C) binds to P-gp with

higher (TXOH, TXA3, TX105, and TX129) or lower (TXA4, TX87) affinity than the small molecules docked individually. The non-covalent complex occupies a larger volume in the substrate-binding cavity, interacting with residues from several TMDs. The two-ligand complex establish polar interactions with residues such as Val-231, Ser-349, Pro-350, and Ala-871 residues, described as being part of the translocation pathway and/or involved in drug binding (Loo et al. 2009; Loo and Clarke 1999; Loo and Clarke 2000).

Docking studies explored the binding configurations of the tested thioxanthonic derivatives using a model built based on Sav1866, an ABC transporter from *S. aureus* (Palmeira et al. 2012b). These docking studies allowed us to understand that thioxanthones bind to P-gp with a considerable high affinity, with two preferential binding pockets in P-gp, engulfed by TMD 4, 5, 8-10 and 12, or by TM 1-3, 6, 7, and 11, further supporting that these compounds most probably interact with P-gp for its activation.



Supplementary Fig. 1. Mitoxantrone (A) and TX129 (B) docked individually into P-gp TMDs; mitoxantrone and TX129 (C) docked simultaneously into P-gp TMDs. Mitoxantrone and TX129 are represented as yellow and pink sticks, respectively. Polar interactions are represented with yellow dashes



Supplementary Fig. 2. Cytotoxicity profiles of zosuquidar assessed by the MTT reduction (A) and NR uptake (B) assays. Cytotoxic effect was evaluated 24h after exposure to the compound in concentrations ranging from 0.05 to 50 μM. Results are shown as mean ± SD of 4 independent experiments. Differences between control and treated cells were estimated using Kruskal-Wallis test (one-way ANOVA on ranks) followed by Dunn's multiple comparison post hoc test. **p<0.01; ****p<0.0001

Supplementary Table 1. Docking scores (kcal.mol^{-1}) of thioxanthones and mitoxantrone.

	Docking Score		Docking Score
TX87	-9.3	**Mitoxantrone + TX87**	-8.7
TXA4	-7.7	**Mitoxantrone + TXA4**	-7.4
TX105	-8.6	**Mitoxantrone + TX105**	-8.9
TXA3	-7.5	**Mitoxantrone + TXA3**	-7.8
TX129	-8.8	**Mitoxantrone + TX129**	-9.2
TXOH	-7.4	**Mitoxantrone + TXOH**	-7.8
Mitoxantrone	-7.5	**-**	-

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General outcome:

The experimental work here presented explores, for the first time, the potential of six different thioxanthonic compounds as P-gp activators. The activation of P-gp drug efflux transport is of increasing interest as an approach to quickly increase the efflux of toxic compounds out of vital organs, such as the brain. Thus, these new class of compounds, which interact with the P-gp protein structure to induce a change in its conformation and promote the efflux of substrates, can confer an increased brain protection in acute intoxications or in potentially dangerous situations following an exposure to an harmful P-gp substrate.

In this study, we demonstrated that four of the six thioxanthones tested were able to increase the efflux of Rho 123 in the RBE4 cell line, following a very short period of incubation (45 min). Such increase could not be due to a protein up-regulation given the short time of exposure, thus evidencing an induction of P-gp activity. These compounds also show to have high affinities to the P-gp protein structure according to the findings obtained by docking studies based on a rat P-gp model. On the other hand, they did not show to stimulate ATPase activity of recombinant human P-gp, therefore suggesting that these thioxanthones are unlikely to be P-gp substrates and to inhibit the transport of P-gp substrates by competition. However, and contrary to what was expected, thioxanthones did not seem to confer additional protection against mitoxantrone-induced cytotoxicity in RBE4 cells, unlike what was observed in a previous study for paraquat in Caco-2 cells (Silva et al. 2014c). In fact, we did not observe a rightwards shift of the mitoxantrone concentration–cell death curves when cells were co-incubated with the thioxanthonic compounds, in comparison to cells incubated with mitoxantrone alone, nor a significant difference on the EC_{50} values of the fitted curves. Since mitoxantrone is a common substrate of P-gp and BCRP, and thus P-gp is not the only transporter implicated in the efflux of mitoxantrone in this experimental setting, mitoxantrone may not be the ideal drug candidate to perform this analysis. Therefore, we aim to reproduce this experiment with another drug that is preferentially, if not exclusively, transported by P-gp in order to exclude any potential interference by BCRP or another ABC transporter.

Still, our results show that thioxanthonic compounds may be promising candidates for an antidote strategy against the toxicity induced by P-gp substrates through P-gp activation, resulting in a much faster manner to reduce the cytotoxicity of harmful P-gp substrates.

III. Co-written review manuscript

Human ABC transporters at blood-CNS interfaces as determinants of CNS drug penetration

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Human ABC Transporters at blood-CNS Interfaces as Determinants of CNS Drug Penetration

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Abstract: Since the discovery of P-glycoprotein (P-gp) in brain microvessels composing the human blood-brain barrier (BBB), ATP-binding cassette (ABC) transporters have been recognized as bottlenecks in the development and delivery of neuropharmaceuticals. ABC transporters are expressed predominately at the plasma luminal membrane of brain capillary endothelial cells. These ABC transporters are responsible for the efflux of their substrates from the endothelial cells to the bloodstream against the concentration gradient and thus limit the entry of some drugs within the central nervous system (CNS). Advanced quantitative molecular biology tools allowed gene and protein quantification of the components of microvessels isolated from different species including human. Recently, positron emission tomography using radiolabelled probes that are substrates of ABC transporters allowed the determination of their functional activity at the human BBB. Here, we summarized new information regarding the relative expression, substrate recognition pattern for CNS drugs and functional activity of ABC transporters that are quantitatively expressed at the human BBB.

Keywords: ATP-binding cassette transporters, blood-brain barrier, blood-cerebrospinal fluid barrier, P-glycoprotein, breast cancer resistance protein, multidrug resistance protein.

1. INTRODUCTION

Differential expression of transporters and metabolizing enzymes is one of the main factors that significantly contribute to the inter-individual variation in the bioavailability and vulnerability to drugs and xenobiotics. ATP-binding cassette (ABC) transporters, solute carrier (SLC) transporters and phase I and phase II metabolizing enzymes play a prominent role in modulating the pharmacokinetics (i.e. absorption, distribution, metabolism and excretion) of a broad range of endogenous and exogenous compounds. ABC and SLC transporters are selectively expressed at interfaces in brain, intestine, kidney, liver, placenta and testis where they have an impact on drug absorption, disposition and elimination [1, 2].

SLC transporter superfamily comprises more than 370 *SLC* genes in the human genome. These transporters play a vital role in maintaining the homeostasis of the human body *via* selective and regulated transport of nutrients, hormones, electrolytes, metal ions, and metabolites [3, 4]. While SLC transporters essentially allow the entry of substrates into the cell down their gradient concentration, transporters of the ABC superfamily efflux substrates outside the cell and thus act as a key element in the pharmacokinetics of several molecules. In the intestine, ABC transporters extrude substrates back into the lumen and hamper their absorption into the blood or lymph circulation [5, 6]. These transporters are determinants of the renal and biliary clearance since they are also responsible for excretion of xenobiotics and metabolites *via* bile and urine [7, 8]. In protected tissues like brain, placenta and testis, ABC transporters are expressed at barriers where they limit substrate uptake and distribution by effluxing their substrates from the tissue into the bloodstream [9].

The primary interfaces between the peripheral circulation and the central nervous system are the blood-brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB). Since the early concept of the BBB proposed by Ehrlich and Goldmann at the beginning of the last century, the development of molecular biology and biochemistry tools including quantitative transcriptomic and proteomic approaches considerably brought new insights into the phenotype of the human BBB. The BBB is a dynamic neurovascular unit with delicate cellular configuration. The brain capillary endothelial cells (BMECs) sealed by tight and adherent junctions are in dynamic interaction with the pericytes that share the basal membrane with the BMECs and the astrocyte end-foot processes with the neuron axonal projections that almost entirely surround the brain microvessels. These specific features make the endothelial cells of the brain microvessels very different from those of peripheral tissues. The tight junctions restricts paracellular diffusion and the presence of specific transport proteins make the BBB a physical and a biochemical barrier that controls the entry of endogenous and exogenous compounds into the brain. The BCSFB is composed of a tight monolayer of cuboidal epithelial cells known as choroid plexus epithelial cells, which function as a barrier in the choroid plexus (CP). The CP is a highly vascularized structure located in the four brain ventricles and its blood capillaries are relatively permeable due to the non-tight junction architecture [10]. Also, the arachnoid barrier, a brain membrane envelope formed by a multilayered epithelium with tight junctions between the cells, is a barrier which only allows CSF movement out of the brain to blood; as it is non-vascularized, the arachnoid membrane is not an important route for the transport of molecules into the brain [11]. The ABC transporter superfamily is responsible for the efflux of xenobiotics from the brain into the bloodstream, making some ABC transporters key elements in controlling the brain penetration of many drugs and thus their central nervous system (CNS) effects [11-13]. Typically, ABC transporters display low substrate specificities which enable them to extrude a large number of structurally unrelated substrates. Therefore, the BBB not only acts as a passive barrier, but also actively efflux a large number of molecules from the brain. In this review we will focus on recent data concerning the expression, relative abundance and activity of ABC transporters in the human BBB.

2. HUMAN ABC TRANSPORTER GENES, PROTEINS AND ACTIVITIES IN THE BBB

2.1. Classification, Nomenclature, Structure and General Functions of Human ABC Transporters

ABC transporter superfamily members are grouped into subfamilies based on their amino acid sequence similarities and

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phylogeny. In human, there are 48 ABC members grouped into 7 subfamilies A-G. The subfamilies are: ABCA (12 members; previously ABC1), ABCB (11 members; previously MDR/TAP), ABCC (13 members; previously MRP/CFTR), ABCD (4 members; previously ALD), ABCE (1 member; previously OABP), ABCF (3 members; previously GCN20) and ABCG (5 members; previously White) [14, 15]. Investigations using topological models predicted the structure for most of the ABC family members. A common protein structure for the majority of ABC transporters can be described as two hydrophobic transmembrane domains (TMDs), each consisting of multiple membrane-spanning α-helices involved in the recognition of substrates, and two hydrophilic cytoplasmic nucleotide-binding domains (NBDs) which couple conformational changes induced by ATP binding, ATP hydrolysis and ADP release to the transport process [16, 17]. Some members deviate from this common structure by deficiency or additional TMDs and/or NBDs. For example ABCG2 is a half transporter consisting of 1 TMD and 1 NBD that dimerize or multimerize to form functional units. However, ABCC1-3, ABCC6, and ABCC10 have an additional amino terminal TMD, a total of three TMDs [18, 19].

ABC transporters are membrane proteins that use the energy release by hydrolysis of ATP to transport various substrates across the cell membrane. The TMDs form an internal cavity that binds substrates through multiple interactions. Within the membrane, the TMDs are organized to build an inverted "V" pocket capable of accepting substrates from either the cytoplasmic side or extracellular space. Substrate binding to this pocket increases the affinity of the NBDs to ATP [20, 21]. Binding and hydrolysis of ATP by the NBDs induces a conformational change in the TMDs, leading to the opening of the inverted "V" to the opposite side, allowing substrate release into the extracellular space. Upon ATP hydrolysis, the transporter is then restored to its original inward facing orientation, able to initiate a new cycle of transport ([22]; reviewed in [23]). However, the exact mechanism of unidirectional substrate translocation through ATP hydrolysis coupling is not completely clear so far.

This review is focused on ABC transporters expressed in normal tissues and that are implicated in the efflux transport of drug and toxic molecules either into the main bloodstream or in excretion organs like liver, kidney and gut, depending on the tissue-interface where these transporters are expressed [24, 25]. ABC transporters are major players in the multidrug resistance (MDR) phenotype that has been described in multidrug resistance cancer cells [26] and contribute actively to the pharmacoresistance observed in CNS diseases like epilepsy, depression and brain cancers [27-29]. Researchers in academia and industry have been putting effort to determine the role of ABC transporters in the transport of neuropharmaceuticals across the BBB. These efforts have been facilitated by the concomitant development of sophisticated molecular biology techniques like quantification of mRNA (qRT-PCR) and proteins (quantitative proteomics using mass spectrometry), confocal immunofluorescence, isolation of brain microvessels from human tissues and positron emission tomography (PET) using radiolabelled substrates. These studies enabled regional and cellular localization, relative abundance and activity of some ABC transporters in human, and provided new insight into the respective role of ABC transporters in controlling the CNS distribution of drugs.

2.2. ABC transporters in the human blood-brain interfaces

2.2.1. Expression and Localization of ABC Transporters at Human Blood-brain Interfaces

The functions of ABC transporters at the human BBB are of major concern for researchers in academia and industry. Nevertheless, ABC transporters are particularly implicated in physiopathology and pharmacoresistance. Many studies have examined the gene and protein expressions of ABC transporters at the BBB in many species. These studies brought new insights into the abundance of ABC transporters at the human BBB, which will be discussed with other studies in the following paragraphs.

a. P-glycoprotein (ABCB1/MDR1)

P-glycoprotein (ABCB1, MDR1, P-gp) was discovered in 1976 [30] and it was the first ABC transporter detected in the endothelial cells of the human BBB by immunochemistry [31]. The human ABCB1 involved in the efflux of xenobiotics at the BBB is encoded by the *ABCB1* (*MDR1*) gene. Using different techniques, the genes encoding P-glycoprotein have been identified in the cerebral endothelial cells of most living species, including rodents, rabbits, pigs, dogfish, cattle and monkeys [25, 32-35]. The immunolocalization of ABCB1 in the human endothelial cells of the BBB has been extensively reported using brain sections, isolated brain microvessels and primary cultures of human brain endothelial cells. Tanaka *et al.* showed by immunoelectron microscopy studies that the localization of ABCB1 in the walls of capillary blood vessels in human brain tissue was specifically on the luminal surface of the endothelium [36] (Fig. **1**). However, no staining for ABCB1 was observed in neurons and glial cells. In the human cerebral microvascular endothelial cell line hCMEC/D3 the expression and functional activity of ABCB1 was demonstrated [37-39]. Similarly, expression was shown in primary cultures of human brain microvessel endothelial cells (HBEC) co-cultured or not with astrocytes [40, 41]. No ABCB1 expression was found in primary human astrocytes cultured alone [41]. Supporting these data, studies performed using human isolated microvessels and human brain sections showed a strong immunocytochemical staining for ABCB1, but no staining was seen in neurons and glial cells [42, 43]. In line with these results, two studies using isolated human brain microvessels showed the high expression of ABCB1 [44, 45]. However, the isolation procedure did not ensure astrocyte foot-processes removal which remain attached to the microvessels. Therefore, it is difficult to conclude that the expression of ABCB1 occurs only in the brain endothelial cells and to exclude its expression in astrocytes. Using microscopic immunogold cytochemistry Bendayan *et al* identified ABCB1 at both luminal and abluminal membranes of capillary endothelial cells, but also in adjacent pericytes and astrocytes of human BBB [46]. In accordance with this study, there is additional data suggesting that ABCB1 is present on the abluminal side of the human brain microvasculature, more specifically on. astrocyte foot processes [47, 48]. Also, a cause-relationship may exist between brain pathologies as epilepsy, glioma and cortical dysplasia and the up-regulation of the expression of ABCB1 in astrocytes [49-52]. Using human brain sections, immunodetection of ABCB1 was also possible in the microvascular pericytes that closely envelop the brain capillary endothelial cells [43]. In other study using healthy human brains, ABCB1 and ABCC1 were not detectable in neurons [51, 53]. Rao *et al* found functionally active ABCB1 expression in the epithelia of choroid plexus in the human brain. It has been suggested that ABCB1 paradoxically prevents trafficking of substrates out of the cerebrospinal fluid (CSF) in choroid plexus epithelia [54]. Still, the presence and function of P-gp in the human CP remains questionable since Abcb1a/P-gp is much less expressed than Abcc1/Mrp1 in rat CP while Abcb1a/P-gp expression is higher than Abcc1/Mrp1 at the rat BBB [55] (Fig. **2**). Further studies are needed to clearly demonstrate the role of ABCB1 in the human CP.

b. Breast Cancer Resistance Protein (ABCG2/BCRP)

ABCG2 (BCRP) has been identified for the first time in a breast cancer cell line exhibiting pharmacoresistance to cytotoxic drugs [56]. ABCG2 differs from common ABC transporters as it is a half-transporter composed of a single nucleotide-binding domain with six transmembrane domains. ABCG2 may also function as homodimers, homo-oligomer or hetero-oligomer held together by disulfide bonds [57]. Several studies showed the abundance of ABCG2 in the endothelium cells of capillaries and veins of many normal tissues and organs, particularly those expressing ABCB1 [58, 59]. ABCG2 was first identified at the BBB by Hans-Joachim

Galla's group [60] and is now a well-recognized BBB component. Abcg2 is much more abundant in the luminal membrane of cerebral endothelial cells than in the total cortex in mouse [61], rats [40], pigs [60] and humans [62].

Aronica *et al* showed the presence of ABCG2 protein in human brain tissue, which is consistently expressed in lysates of human temporal cortex and hippocampus [63]. *ABCG2* gene transcripts were detected in several regions of the human brain [64]. Expression and immunolocalization of ABCG2 was observed in human frozen brain sections, which strongly suggested that the fluorescent signals were limited to the microvessel endothelium [62]. Supporting evidence was obtained from human brain isolated microvessels, suggesting that ABCG2 is the major ABC transporter expressed at the BBB [44, 65]. Interestingly, ABCG2 expression was about twice that of ABCB1 in isolated human brain microvessels, suggesting that it may be of interest to study its functional role at the human BBB in limiting CNS drug delivery. The expression at mRNA and protein level and functional activity of ABCG2 have been confirmed in primary cultures of human brain endothelial cells [40], in the immortalized human brain endothelial cell line BB19 [66], and in hCMEC/D3 cells [37]. Regarding the membrane localization within the endothelial cells, there is considerable proof that ABCG2 displays localization in the luminal side of the endothelium from normal human brain [59, 62, 63] (Fig. **1**). According to Aronica *et al*, ABCG2 expression in the brain is limited to the blood brain vessels, as no neuronal or glial ABCG2 expression was detected [63]. However, Zhang *et al* detected the expression of ABCG2 protein in both human brain microvessel endothelial cells and fetal human astrocytes, although in the latter at levels considered low [67]. A very limited expression of ABCG2 was demonstrated in the choroid plexus epithelial cells of the porcine and rat BCSFB, which may mean a similar but less significant function of this transporter in this brain barrier [60, 68]. However, ABCG2 expression in the human BCSFB is currently unknown.

c. Multidrug Resistance-associated Proteins (ABCCs/MRPs)

The multidrug resistance-associated proteins, MRP (ABCC) are a group of 13 efflux transporters belonging to the C subfamily. Some ABCCs are able to transport drugs [18]. The ABCCs are presently divided into two groups depending on their structure. The "long" ABCCs are ABCC1-3, ABCC6 and ABCC7. They contain three transmembrane domains, TMD0, TMD1 and TMD2. The "short" ABCCs include ABCC4, ABCC5, ABCC8, and possibly ABCC9, which do not contain TMD0. The ABCC isoforms differ in their tissue distribution, substrate specificity, and proposed physiological functions [69]. The ABCCs are involved in a wide range of specific and essential physiological activities, particularly in the transport of organic anions either unconjugated or conjugated with glutathione, glucuronate, sulphate, or phosphate [70, 71]. Moreover, it is now clear that these proteins are involved in the transport of drugs across lipid membranes from inside to outside the cell contributing to multidrug resistance. In contrast to the distribution profiles of ABCB1 and ABCG2 in the BBB, ABCCs distribution is still controversial because of the large interspecies differences. This may explain the conflicting recently published results regarding their abundance and/or function at the BBB.

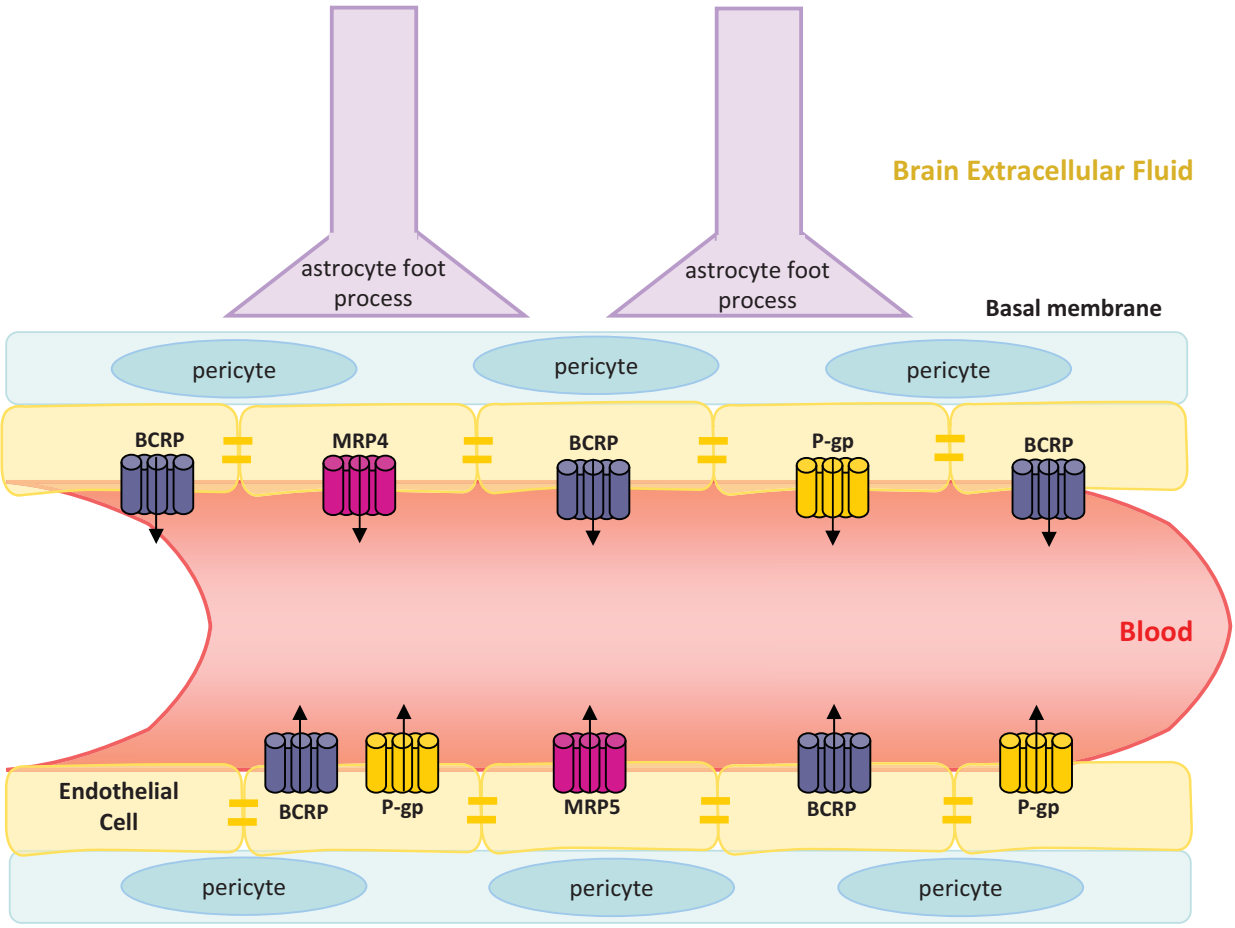

Fig. (1). Schematic representation of the blood-brain barrier and the ABC transporters expressed in the human brain capillary endothelial cells. The ABC transporters are expressed at the luminal side of the membrane of endothelial cells, and ABCG2/BCRP seems to be the highly expressed transporter, followed by ABCB1/P-glycoprotein and ABCC4/MRP4.

ABCC1 was the first identified member of the ABCC/MRP subfamily. Like other family members, it is involved in the efflux of many organic anionic substrates [71]. ABCC1 is usually present in cells of blood-tissue barriers protecting different body organs and limiting tissue distribution of certain substrates. In the brain, the localization of ABCC1 in the human BBB and thus its contribution in drug efflux are still controversial. *ABCC1* mRNA has been detected in human BMECs [65, 72, 73], but the expression of ABCC1 protein remains unclear. ABCC1 couldn't be detected in the human BBB in most studies [44, 53, 62, 72, 74]. However, Dauchy *et al.* found that *ABCC1* gene expression was well demonstrated in hCMEC/D3 cells as compared to the rest of *ABCCs* [37], suggesting culturing conditions of human brain endothelial cells may increase *in vitro* the expression of ABCC1 that is probably not expressed *in vivo* in human. Nies *et al* detected a weak immunostaining for ABCC1 in the luminal membrane of human brain endothelial cells [37, 75]. ABCC1 immunocytochemical expression and activity was found in human epithelial cells of the choroid plexus, and it is located in the basolateral membrane of choroid plexus epithelial cells [53, 54] (Fig. **2**). These findings indicate that ABCC1 contributes to the basolateral broad-specificity drug-permeation barrier in choroid plexus, preventing the accumulation of several organic anions in the CSF. Studies reported that normal human astrocytes cultured *in vitro* expressed high amounts of ABCC1. The expression was shown mainly in foot-processes as observed by immunofluorescence when using astrocytes grown in chamber slides. This expression might indicate a physiological transport function of ABCC1 in normal astrocytes [76]. In contrast, ABCC1 protein was hardly detected in normal human astrocytes *in situ* [75]. However, ABCC1 has not been detected in isolated human brain microvessels showing that its expression is weak in brain endothelial cells and astrocyte foot processes of human origin. Considering the inconsistent and low-level expression of ABCC1, the function of ABCC1 at the human BBB may be limited.

hCMEC/D3 cells expressed *ABCC2* and *ABCC3* [37], in accordance with those reported by Carl *et al* where they have shown that hCMEC/D3 cells expressed these *ABCCs* [77]. In contrast, qRT-PCR studies showed that *ABCC2* and *ABCC3* are not expressed at the human BBB [44]. These results are consistent with the immunolocalization studies using human brain and cultured human brain capillary endothelial cells, in which neither ABCC2, ABCC3 nor ABCC6 were detected. These observations substantiate the conclusion on the absence of these ABCCs at the human BBB [75, 78, 79]. However, rodent data showed Abcc2 expression in isolated brain microvessels, suggesting interspecies differences and questioning the reliability of using animal models in the study of brain uptake of ABCC2 substrates [80]. Nevertheless the most probable absence of these ABCCs at the human BBB, ABCC1 and ABCC2 may gain importance in restricting the entry of organic anions into the brain under certain pathophysiological conditions like in epilepsy.

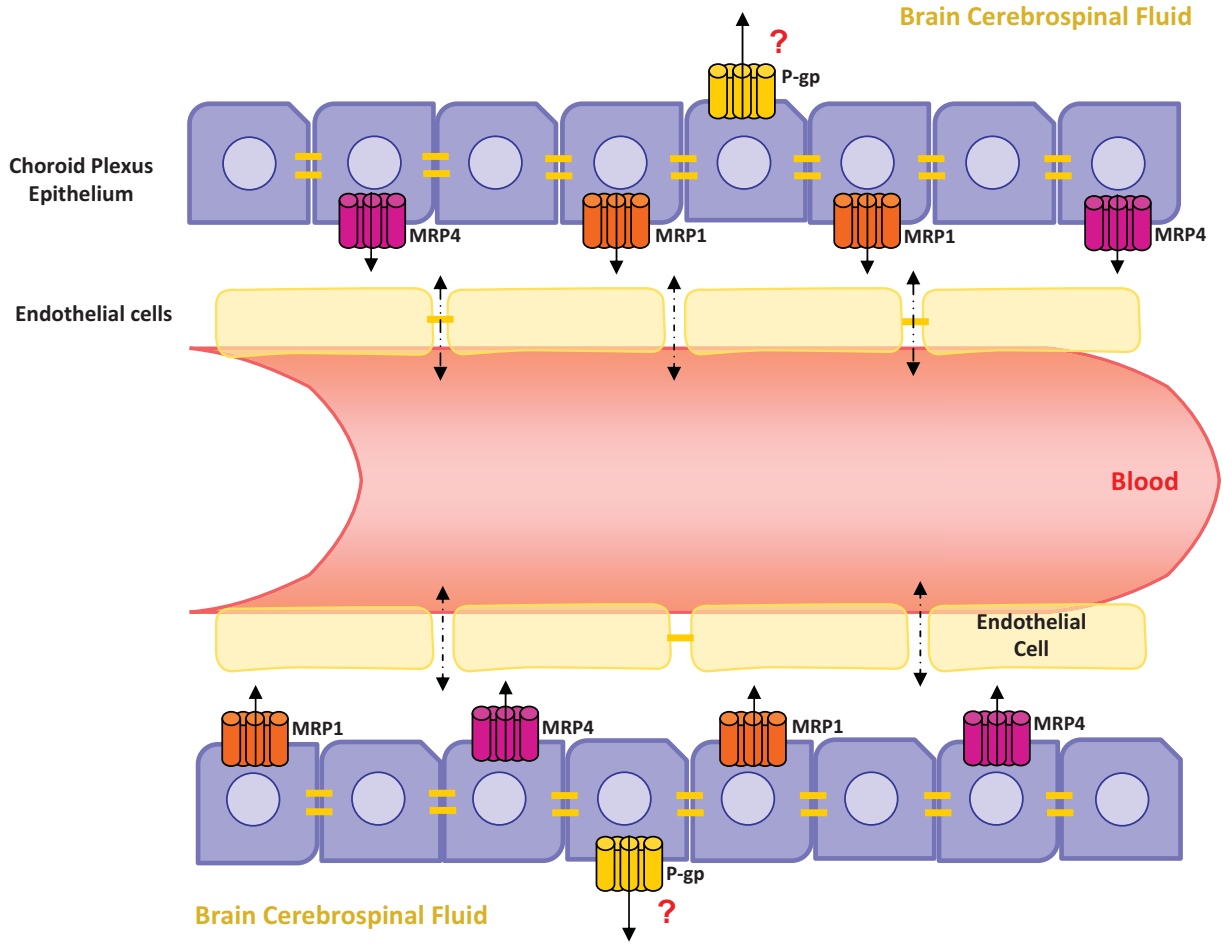

Fig. (2). Schematic representation of the blood-cerebrospinal fluid barrier and the ABC transporters present in the human choroid plexus epithelial cells. ABCC1/MRP1 and ABCC4/MRP4 are expressed in the basolateral membrane of the choroid epithelium, contributing to the prevention of the accumulation of several organic anions, drugs and metabolites in the CSF. Paradoxically, ABCB1/P-gp evidenced an apical localization, which prevents trafficking of substrates out of the cerebrospinal fluid.

Gene and protein studies using human brain freshly isolated microvessels showed the expression of *ABCC4*/ABCC4, and it was shown by absolute quantitative proteomics that only ABCC4 protein quantity was above the limit of quantification (LOQ), whereas the rest of ABCCs were below the LOQ [44, 65]. In accordance, hCMEC/D3 cells expressed significant amounts of *ABCC4* gene transcripts [37]. ABCC4 was demonstrated *in situ* at the human BBB and BCSFB, where it was localized in the luminal side of capillary endothelial cells [75, 81], and in the basolateral membrane of choroid plexus epithelial cells [75, 81]. These observations indicate an important function of ABCC4 in limiting drug brain penetration and in the efflux of organic anions from brain to blood. ABCC4 was also immunolocalized in astrocytes of human brain slices [75].

ABCC5 is involved in the efflux of cyclic nucleotides. *ABCC5* gene transcripts were detected, in human brain freshly isolated microvessels and in hCMEC/D3 cells [37, 65]. A recent study corroborated these data, and also established that *ABCC5* is the most abundant ABCC family member expressed in human BMECs [73]. Nevertheless in the quantitative proteomic study of Shawahna *et al.* the amount of ABCC5 was below the limit of quantification [44], human brain *in situ* studies reported its localization in the luminal membrane of capillary endothelial cells [75]. Human pyramidal neurons and human astrocytes showed detectable ABCC5 amounts [75].

Few studies investigated ABCC6, ABCC10-ABCC12 expression in human BBB. A major problem is the lack of selective antibodies against these ABCC. Two independent studies have reported mRNAs levels of *ABCC10* and *ABCC11* in homogenates of human brain [82, 83]. *ABCC6*, *ABCC11* and *ABCC12* mRNAs have been detected in the human brain cortex and in the isolated microvessels of the corresponding human brains [65]. However, any of the corresponding proteins have been detected by quantitative proteomics [44, 45] and no mRNAs coding for these transporters was detected by RT-PCR in hCMEC/D3 immortalized human cerebral microvascular endothelial cells [37]. Nevertheless, the high limit of quantification of quantitative proteomics did not allow excluding protein expression of ABCC6, ABCC10 and ABCC11 at the human BBB. ABCC11 was localized in axonal membrane of neurons in human cerebral cortex, but it was not detected in tumor cells and gliomas [84].

d. Other ABC Transporters Expressed at the Blood-CNS Interfaces

In addition to the expression of the above mentioned ABC transporters, it was also shown that microvessels also express some transporters belonging to the ABCA subfamily in the human BBB, namely ABCA2 and ABCA8 [44, 174]. It is known that ABCA transporters are responsible for the transport of steroids, such as cholesterol and derivatives, which are important components of the CNS. However, in addition to the role that ABCA transporters play in maintaining the steroid homeostasis in the brain, it is yet to be discovered if they may also be involved in the efflux of drugs and xenobiotics whose structure is essentially similar to steroid or lipid structure, and thus contribute to the transport from the brain to the bloodstream.

2.2.2. Relative Abundance of ABC Transporter Proteins and their Activities in the BBB

While the abundance of some ABC transporters in the BBB is still a matter of debate, ABCB1, ABCC4-5 and ABCG2 proteins are believed to be the main ABC transporters in human and mouse BMECs so far [65, 85] (Fig. **1**). ABCB1 is also functional in the human BBB, as indicated by PET-imaging studies using ^{11}C-verapamil [86, 87], and ^{11}C-desmethyl-loperamide [88]. ABCB1 activity is quite uniform in all brain regions protected by the BBB and, consequently, ABCB1 inhibition results in strong increase in substrate accumulation throughout the healthy human brain [87]. The ABCB1 also plays in concert with the ABCG2 at the BBB to restrict the passage of their common substrates into the brain. Thus the deficiency or inhibition of one of the two transporters is not accompanied by an increase of the brain penetration of a common substrate: in Abcg2 deficient mice (but in which Abcb1 is present), the Abcb1 alone is sufficient to prevent the brain penetration of the substrate. Similarly, mice deficient in Abcb1 (but in which the Abcg2 is present), the Abcg2 alone is sufficient to limit the brain entry of the substrate [89]. These data clearly show that these two ABC transporters compensate for each other and act together to reduce the *in vivo* brain penetration of their common substrates. This synergistic effect of Abcb1 and Abcg2 at the BBB has been clearly established in mouse for anticancer drugs that are substrates of both ABC transporters [90] but remains to be demonstrated in human.

Unfortunately, no similar PET studies are presently available on the functions of ABCG2 and ABCC4-5 at the human BBB. So we still do not know whether ABCG2 and ABCC4 are functional in the human BBB. Since ABCG2 is believed to be more abundant than ABCB1 at the human BBB, PET studies should focus on the function of ABCG2 at the human BBB. However, no PET radiolabelled probes that are selective for ABCG2 function evaluation have been described, partly because of its recent discovery and due to the high overlapping substrate specificities of ABCG2 and ABCB1 [91]. ^{11}C-desmethyl-loperamide is a case of a radiolabelled PET probe that is highly selective for ABCB1, but not for ABCG2 or ABCC1 [92]. Recently, ^{11}C-gefitinib and ^{11}C-tariquidar showed to be promising PET radiotracers suitable to evaluate the function of ABCG2 by either genetic or chemical knockout of ABCB1 in the study model [93, 94]. Also, Tournier *et al.* validated an *in vitro* model using MDCKII cells transfected with human *ABCB1* or *ABCG2* genes to assess the transport of selected PET ligands by the concentration equilibrium technique [95]. The model showed that the monoamine oxidase A antagonist befloxatone was exclusively transported by ABCG2, and not by ABCB1 [95], suggesting that it may be useful to study the activity of ABCG2 at the human BBB.

The identification of gene polymorphisms involving ABC transporters is gaining considerable importance regarding their relative expression and functional activity at the BBB. To the present date, more than 50 single nucleotide polymorphisms (SNPs) have been identified for the ABCB1 gene, and particularly the functional C3435T, G2677T and C1236T SNPs have been associated to an altered expression and function of ABCB1 in human tissues [96, 97]. Kato *et al* found that the haplotype combination 3435C–2677G–1236T within the *ABCB1* gene was associated with a more severe depressive symptomatology, related to a poor response to antidepressants, and this may be due to a higher expression of ABCB1 or to more active protein forms [98]. Similarly, it was shown that the attention-deficit/hyperactivity disorder (ADHD) improvement caused by treatment with guanfacine was less evident in children with the T/T *ABCB1* C3435T genotype, compared to either C/T or C/C genotypes [99]. Thus, a higher activation of ABCB1 would result in lower brain levels of CNS drugs that are ABCB1 substrates, and consequently in resistance to pharmacotherapy. In a study conducted in Japan by Fujii *et al* the C3435T SNP, and the TT genotype (TT3435) linked to this SNP, were shown to be significantly more common in patients with major depressive disorder than in the controls, suggesting that polymorphisms of ABCB1 may give susceptibility to this disorder [100]. In addition, it was reported that a higher frequency of the 1236C–2677G is evident among individuals with Parkinson's disease. Here, altered ABCB1 expression might result in accumulation of drugs and/or toxins in harmful amounts, and therefore contributing to the development of disease [101].

Nevertheless, we still need to know the relative amounts of each ABC transporter at the BBB in order to better understand how they influence brain drug distribution. ABCB1 and ABCG2 are the

most extensively reported ABC transporters at the BBB. For a long time, it was believed that ABCB1 was the major ABC transporter at the BBB. This belief was substantiated by the findings that Abcb1 was 3-fold more expressed than Abcg2 in isolated mouse brain capillaries [85]. However, human brain tissues showed that the expression of ABCG2 seemed higher compared to other ABC transporters, such as ABCB1 and ABCCs [62, 63]. The expression profiles of the genes encoding the main ABC transporters at the human BBB were recently established by studies on human cerebral microvessels isolated from the brains of patients undergoing neurosurgery (glioma or epileptic patients). It has to be noted that biopsies were as far as possible from the brain lesions suggesting that this part of the brain could be considered as healthy. In contrast to previous studies in rodents, Dauchy *et al* showed that *ABCB1* was about 7-fold less expressed than *ABCG2* in human brain microvessels, whereas *ABCC4* and *ABCC5* were much less expressed [65]. Also, ABCG2 is higher expressed than ABCB1 in total human brain homogenates [67]. At the human BBB, these data were recently confirmed by quantitative proteomic analyses of isolated human cerebral capillaries, in collaboration with the group led by Drs. S. Ohtsuki and T. Terasaki (Tohoku University, Sendai, Japan), where the ABCG2 expression was also higher than that of ABCB1 in human brain microvessels [44, 45]. The ABCG2 expression was shown to be 1.6 times superior than ABCB1 expression and to be 12.8 times inferior than GLUT-1 expression, where ABCG2 absolute protein amount was found to be about 6.15 ± 1.41 fmol/µg of protein while ABCB1 protein was about 3.98 ± 0.88 fmol/µg of protein [44]. This is indicative of the substantial interspecies differences and questions the use of rodents as representative models in studying drug efflux at the BBB. In the Uchida *et al* study, they investigated the proteomics of ABC transporters in microvessels isolated from frozen human brain samples [45], where it was compared to the protein amounts of transporter homologues in mouse. Interestingly, their results showed that the human ABCG2 was 2-fold of the mouse Abcg2, whereas the human ABCB1 and ABCC4 were almost 2 and 8 times less than the mouse Abcb1a and Abcc4, respectively [45]. Thus, it is essential to keep in mind that the expression of different ABC transporters at the BBB is highly variable among different species.

3. CNS DRUG SUBSTRATES OF ABC TRANSPORTERS EXPRESSED AT THE HUMAN BBB

The BBB, as a protecting mechanism built to hamper endogenous and exogenous threatening molecules from entering and damaging the brain, also prevents therapeutic drugs to reach the CNS, thus potentially reducing their efficacy. Of the 48 human ABC transporters that have been described, there are three particularly predominant at the human BBB, and which thus have been linked to a potential role in narrowing CNS drug penetration, these are ABCB1, ABCG2 and ABCC4. In this review we simply analyze the impact of these three ABC transporters, as they are believed to be the major ABC transporters involved in the regulation of brain uptake and extrusion of drugs at the human BBB. A list of some substrates of ABCB1, ABCG2 and ABCC4 are represented in Table **1**, together with some of their inducers, inhibitors and physiological substrates, which is a representative list of the chemical diversity of molecules recognized and transported by these ABC transporters.

a. P-glycoprotein

As ABCB1 was the first ABC transporter to be described, it is also by far the best characterized. ABCB1 limits the brain penetration of a broad range of chemically diverse substrate drugs. Its substrates vary greatly in size, structure, and function. Substrates include chemotherapeutic agents as anthracyclines, vinca alkaloids, tyrosine kinase inhibitors, HIV protease inhibitors and HMG-CoA reductase inhibitors. Several chemotherapeutic agents have been shown to be substrates of ABCB1, namely anthracyclines such as doxorubicine and daunorubicine [102], etoposide [103], the tyrosine kinase inhibitors imatinib and gefitinib [93, 104] and vinca alkaloids (vinblastine, vincristine) [105, 106]. ABCB1 over-expression is now considered a major player in the development of resistance to anticancer treatment, particularly in brain tumors. The majority of human immunodeficiency virus (HIV) protease inhibitors (indinavir, saquinavir, nelfinavir), which brought considerable progress in the treatment of HIV infection, are also important ABCB1 substrates. Studies show that these drugs reach significantly higher brain levels in *Abcb1a* knockout mice when compared to wild-type mice [107, 108]. The remarkable increase in abacavir brain distribution showed in Abcb1-deficient mutant mice over wild-type mice by Shaik *et al* suggests that ABCB1 may also play a significant role in restricting the nucleoside analog reverse transcriptase inhibitor abacavir distribution to the CNS [109]. Thus, ABCB1 is also involved in the brain resistance to HIV pharmacotherapy. There is also considerable evidence that a large number of antiepileptic drugs are transported by this ABC transporter. Cell lines transfected with the human *ABCB1* gene confirmed that ABCB1 transported the active metabolite of carbamazepine (carbamazepine-10,11-epoxide) as well as eslicarbazepine acetate and oxcarbazepine. Their transport was blocked by the ABCB1 inhibitors tariquidar and verapamil [110]. A similar study was conducted to show that phenytoin and phenobarbital are as well ABCB1 substrates [111]. Results indicated the possible role of ABCB1 in drug resistance in refractory epilepsy, especially if epilepsy patients exhibit over-expression of ABCB1 at the BBB. Other xenobiotics recognized and transported by ABCB1 include opioid analgesics [112-114], antidepressants [115, 116] and anti-emetics [8]. Interestingly, a significant clinical drug-drug interaction exists between loperamide, a well-known substrate of ABCB1, and quinidine, a well-known inhibitor of ABCB1. The co-ingestion of these two drugs has produced significant CNS adverse effect due to the increase in brain uptake of loperamide [117]. Recently, we evidenced that norbuprenorphine, the main metabolite of buprenorphine (an opioid used in heroin addiction and pain), was a strong substrate of human ABCB1 [118] and that brain uptake of norbuprenorphine was significantly decreased by Abcb1a at the mouse BBB, thus controlling its respiratory adverse effect [119]. A summary of ABCB1 substrates is displayed in Table **1**.

b. ABCG2/BCRP

Since ABCG2 was first identified as a drug efflux protein contributing to the multidrug resistance phenotype of cancer cell lines, a large number of chemotherapeutic and non-chemotherapeutic molecules have been shown to be substrates of this transporter. The ABCG2 substrate spectrum includes physiological compounds, nutrients, and several cancer and non-cancer drugs. Particularly in the case of non-cancer drugs, ABCG2 covers a spectrum of several structurally unrelated classes of anti-cancer agents, increasing the importance of ABCG2 as multidrug resistance protein.

Resistance to mitoxantrone is the hallmark of cells expressing ABCG2, as well as resistance to camptothecin derivatives (9-aminocamptothecin, topotecan, irinotecan and SN-38, its active metabolite) [120-122]. Other chemotherapeutic substrates have been shown to be transported by ABCG2 as the tyrosine kinase inhibitors imatinib, gefitinib and nilotinib [123-125], indolocarbazoles and antifolates such as methotrexate [126]. Cells with a glycine or threonine instead of an arginine at the amino acid 482 of the ABCG2, considered to be a "hotspot" for mutation, readily transport doxorubicin and rhodamine 123 in addition to mitoxantrone [127-129]. Several other substrate classes have been described including antivirals [130-132], HMG-CoA reductase inhibitors [133-135], carcinogens [136-138] and antibiotics [139-141]. A summary of some of the cancer and non-cancer drugs transported by ABCG2 is given in Table **1**. The expanding list of ABCG2 substrates highlights the fact that this transporter may play a significant role in brain pharmacology and protection.

Table 1. List of some endogenous compounds and xenobiotics that are substrates, inducers and inhibitors of ABC transporters expressed at the human BBB.

ABC Transporter	Xenobiotic substrates	Endogenous compounds	Inducers	Inhibitors
ABCB1	*Anticancer drugs*: doxorubicine, daunorubicine, vinblastine, vincristine, etoposide, teniposide, paclitaxel, methotrexate, irinotecan, imatinib, gefitinib *Anti-emetics*: domperidone, ondansetron *Corticoids*: dexamethasone, hydrocortisone, corticosterone Anti-retroviral drugs: amprenavir, indinavir, saquinavir, abacavir, nelfinavir *Opioids*: morphine, methadone, oxycodone, fentanyl, loperamide *Antidepressants*: amitriptyline, nortryptiline, venlafaxine, fluoxetine, paroxetine, sertraline *Antipsychotics*: amisulpride, risperidone *Antiepileptic drugs*: phenytoin, carbamazepine, lamotrigine, phenobarbital, felbamate, gabapentin, topiramate *Antibiotics*: erythromycin, valinomycin, tetracyclines, fluoroquinolines *Anthelminthic agents*: ivermectin, abamectin *Others*: verapamil, colchicines, cimetidine, digoxine, fexofenadine	β-Amyloid peptide, bilirubin, cytokines, cortisol, aldosterone	Rifampin, dexamethasone, Phenobarbital, TCDD	Verapamil, cyclosporine A, amlodipine, quinidine
ABCG2	*Anticancer drugs*: considerable overlap with P-gp, anthracylines; camptothecins, indolocarbazoles, mitoxantrone, methotrexate, imatinib, gefitinib, erlotinib *Anti-retroviral drugs*: zidovudine, lamivudine, abacavir *HMG-CoA reductase inhibitors*: rosuvastatin, pitavastatin, cerivastatin *Antibiotics*: ciprofloxacin, ofloxacin, norfloxacin, erythromycin, rifampicin, nitrofurantoin *Glucoronide, glutathione and sulphate conjugates* *Others*: sulfasalazine, cimetidine, riboflavin, dipyridamole	Estrone-3-sulfate, 17β-oestradiol, DHEA sulfate	TCDD	Fumitremorgin C, TKIs, flavonoids
ABCC4	Anti-retroviral drugs: Adefovir, tenofovir Anticancer drugs: methotrexate, topotecan, 6-mercaptopurine, thioguanine Others: dehydroepiandrosterone sulphate, furosemide	DHEA sulfate, cyclic nucleotides, bile acids + GSH, Prostaglandin E2, Thromboxane B2	Rifampin, clofibrate, cyclophosphamide	Celecoxib, diclofenac

For reference see [19, 28, 175, 176]; DHEA, dehydroepiandrosterone; GSH, reduced glutathione; TCDD, tetrachlorodibenzodioxin; TKI, tyrosine; kinase inhibtor

c. ABCC4/MRP4

ABCC4/MRP4 is known to participate in the extrusion of organic anions, the transport of diverse antiviral and has also been implicated in multidrug resistance during chemotherapy. Among the anticancer drugs known to be substrates of this ABC transporter are 6-mercaptopurine, methotrexate, 6-thioguanine [142-144] and topotecan [81], emphasizing the importance that this transporter may have in determining the CNS distribution of these drugs and contributing to cancer pharmacoresistance. ABCC4 confers resistance to purine-based antiretroviral agents as ganciclovir [145], adefovir and tenofovir [146], and thus may influence the efficacy of antiviral therapy. Other drugs that are apparently transported by ABCC4 are the pharmacologically active form of oseltamivir [147], furosemide and hydrochlorothiazide [148].

4. ABC TRANSPORTERS AS DETERMINANTS OF CNS DRUG PENETRATION

Considering the previously discussed and largely documented expression of ABC transporters at the blood-CNS interfaces, namely the BBB and the BCSFB, allied to the wide list of drugs which correspond to their substrates, it is clear the significant impact of ABC transporters on the CNS penetration of drugs used for the treatment of several diseases such as epilepsy, HIV, humour disorders, and brain tumors. Moreover, the occurrence of changes in the expression of ABC transporters at the human brain is an evident event in several pathologies, including ischemia, glioma and epilepsy. These changes may result in an increased or decreased expression of the usually present ABC transporters and may even induce the expression of an efflux transporter that is not normally expressed in the healthy brain. Tishler *et al* were the first to describe an up-regulation of *ABCB1*/ABCB1 gene and protein expres-

sions in epileptogenic brain regions, either in the capillary endothelium or in astrocytes [51]. Following reports demonstrated that not only ABCB1 but also several ABCCs, such as ABCC1, ABCC2 and ABCC5 are over-expressed in brain capillary endothelial cells and/or astrocytes of CNS-pharmacoresistant patients [50, 53, 74, 149, 150]. Additionally, Lazarowski *et al* observed in the epileptogenic regions increased levels of ABCG2, in refractory epilepsy patients [151]. According to some studies, the expression of ABC transporters can also be up-regulated upon ischemia [152, 153], despite the fact that Patak *et al* didn't find any changes of ABCB1 and ABCC1 expressions immediately after hypoxia, in hCMEC/D3 cells [154]. The efficacy of chemotherapy for malignant brain tumors is very limited, since many anticancer drugs are substrates for multidrug transporters (see Table **1**), and due to the presence of ABC transporters at the BBB. The expression of drug efflux transporters as *Abcb1b*, *Abcc1*, *Abcc4* and *Abcc5* in glioma cells are a true evidence of the limitations of the anticancer drug therapy nowadays [155]. Therefore, the over-expression of ABC transporters has logical consequences on the permeability of drugs across the BBB, leading to pharmacoresistance phenomena to the CNS-acting drugs.

Considering the anatomical and molecular characteristics of the BBB and BCSFB, there is a pressing need to develop strategies to circumvent the efflux capacity of ABC transporters, and thus enhance the permeability of CNS-acting drugs into the brain and improve their brain distribution and bioavailability. Thus, designing of effective drugs that are not substrates, ABC transporter inhibitors, increase of BBB permeability through pharmacological or physical disruption of the BBB are imperative strategies to develop by the pharmaceutical industry to increase brain distribution of drugs [156, 157]. Some studies showed the potential of the use of nanocarriers such as nanoparticles, liposomes and solid lipid nanoparticles (SLN) as promising vehicles facilitating the transport and delivery of CNS-acting drugs into the brain through endocytic pathways or inhibition of ABC transporters expressed at the BBB and BCSFB [74, 158-162]. The use of different types of ABC transporter inhibitors in several studies also revealed a good correlation in terms of improving the brain delivery and bioavailability of CNS-acting agents that are substrates of ABC transporters [163-167]. Nevertheless, we have to consider the ubiquitous presence of ABC transporters in the human body and once these inhibitors are not CNS-specific, their use may lead to undesirable alteration of pharmacokinetics, and consequently to a high risk of drug toxicity and unpredictable drug-drug interactions. Likewise, the development of prodrugs formed by molecular conjugates of suitable functional groups with more favourable lipophilicity has arisen as a strategy to improve BBB passage by passive diffusion [163, 168, 169]. Once these prodrugs cross the endothelial cells of the BBB, they would subsequently be hydrolyzed to the active drug form within the brain.

On the contrary, an inverse strategy may also be to increase the function of ABC transporters to limit CNS drug distribution and/or to efflux toxic compounds, thus limiting CNS drug adverse effects. Interestingly, ABC transporters are also known to be transcriptionally regulated by several transcription factors like pregnane-X-receptor, constitutive androstane receptor and aryl hydrocarbon receptor that are expressed at the BBB [37, 170-172]. Therefore, up-regulating ABC transporters may be a strategy to limit CNS distribution of drugs with CNS adverse effects or to efflux toxic endogenous compounds as it has been postulated for β-amyloid peptide by ABCB1 [173]. Regulation of ABC transporters at the blood-brain interfaces may also occur under pathophysiological conditions that were described elsewhere in this special issue.

5. CONCLUSION

ABC transporters are key elements at the BBB, where they help to maintain the homeostasis and physiological functions of the CNS. The presence of these ABC transporters at the BBB has implications not only for protection from toxin-induced damage but also for hampering brain penetration of pharmacological agents used for the treatment of several brain diseases. Due to the differences between animal and human transporter profiles, and even due to recurrent disparities in transporter expression between different human study models, there is an increasing need of a specific human model to clarify the ABC transporters' substrate status of the drugs, as well as to predict the variability in the brain distribution.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

ABC	=	ATP-binding cassette
ADHD	=	Attention-deficit/hyperactivity disorder
BBB	=	Blood-brain barrier
BCSFB	=	Blood-cerebrospinal fluid barrier
BMEC	=	Brain microvessel endothelial cell
CNS	=	Central nervous system
CP	=	Choroid plexus
CSF	=	Cerebrospinal fluid
HBEC	=	Human brain endothelial cell
HIV	=	Human immunodeficiency virus
LOQ	=	Limit of quantification
MDR	=	Multidrug resistance
MDD	=	Major depressive disorder
NBD	=	Nucleotide-binding domain
P-gp	=	P-glycoprotein
PET	=	Positron emission tomography
SLC	=	Solute carrier
SLN	=	Solid lipid nanoparticles
SNP	=	Single nucleotide polymorphisms
TMD	=	Transmembrane domain

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PART IV

IV. GENERAL DISCUSSION

The experimental work conducted under the present dissertation has as a common thread the study of the mechanisms of regulation of ABC transporters present at the BBB, in particular P-gp and BCRP. It includes methodological approaches as well as expression and modulatory studies, which enroll for a better understanding of the BBB. We adopted several BBB study models, being the isolation of brain microvessels the predominantly used model, but some studies were also complemented with *in vitro* models, such as the human hCMEC/D3 cell line and the rat RBE4 cell line.

As previously presented in the literature review in a dedicated section, there are currently several animal and human models available to characterize and study BBB maintenance and regulation. Since the BBB is a dynamic system, which is regulated and modified by different signaling factors released by different surrounding cell types, it is ideal to choose an experimental model that closely preserves the brain microenvironment and its biological processes.

The use of freshly isolated rat brain microvessels, a technique that was largely resorted to in the course of the experiments conducted for the present dissertation, represents a valuable tool for studying the BBB physiology, and particularly the characterization of gene and protein expression at the BBB. *In vivo* treatments can be carried out in a rat animal model, and their consequences on the BBB physiology and features can be studied while preserving brain biological processes and signaling pathways fully functional. Thus, given that this model preserves the high degree of complexity of the CNS and NVU cellular interaction, it closely mimics the human BBB, providing reliable data for the BBB study. However, this model has some limitations, since several studies already demonstrated some important interspecies differences between rodent and human BBB models (Cutler et al. 2006; Syvanen et al. 2009; Uchida et al. 2011), which render difficult the extrapolation of the obtained results to the human reality. Still, due to ethical considerations and the difficulty to have access to samples from human origin, the nature of the work conducted for the present dissertation would be impracticable in humans, and so was conducted in an accessible rat model.

For this purpose, we used a mechanical isolation technique, which involves the homogenization of samples from rat brain cortical gray matter, and subsequent successive centrifugations and filtrations. This technique was previously validated in our laboratory (Yousif et al. 2007). The isolation of microvessels from the cortical gray matter of the brain tissue allows a considerable elimination of cell debris and contamination from other associated cell types in the NVU, in an expensive and fast manner. However, it does

not render a pure fraction of brain capillary endothelial cells since pericytes, which are embedded in the basement membrane and closely in contact with endothelial cells, and also some astrocytic end-feet processes, remain attached to the endothelium. The immediate use of the rat brain for microvessel isolation ensured limited mRNA and protein degradation.

1. Consequences of a subchronic morphine treatment in the expression of P-gp and Bcrp at the rat BBB: a kinetic study

The regulation of ABC transporters present at the BBB by drugs and xenobiotics is currently a subject of study of growing importance. This regulation is often associated with a change in the expression or functional activity of these transporters, which may therefore change the BBB features. In the experimental works performed for the present dissertation, we particularly focused on the effects of morphine exposure on the expression of P-gp and BCRP at the BBB. This subject is of particular interest since some data suggest that there is a possible correlation between the induction of P-gp by morphine and the development of tolerance to this substance. Furthermore, morphine is often co-administered with anticancer drugs, which are, in a great number, substrates for P-gp, and thus P-gp up-regulation by morphine may alter the distribution and efficacy of these anticancer agents. Previous studies conducted in mice and rats registered an increase of P-gp expression following a subchronic administration of morphine, with reduced antinociceptive response (Aquilante et al. 2000; Zong and Pollack 2003). A previous study, conducted by our research group, evidenced an induction of the gene expression of several ABC efflux transporters, such as *Abcb1a*, *Abcg2*, and *Abcc1*, in rat brain microvessels, following a subchronic treatment with morphine for 5 days (Yousif et al. 2008). Additionally, it induced the expression of P-gp in the rat brain cortex and hippocampus at both mRNA and protein level (Yousif et al. 2008), in morphine-treated rats that were sacrificed 12h after the last dose of morphine. Furthermore, little was known about the effect of exposure to morphine on the expression levels of BCRP at the BBB.

In sequence with the previous studies conducted by our team, we had interest in analyzing the kinetics of the modulation of P-gp, but also, of Bcrp in the rat BBB following a subchronic morphine treatment, of 5 days, in an escalating dose regimen. For this purpose, rats were sacrificed at different times after the last dose of morphine: 6h, 9h, 12h, 24h and 36h, and both large and microvessels from rat brain cortex were isolated. β-actin was chosen as housekeeping gene or protein in the qRT-PCR and WB analyses, since it is greatly expressed in these samples, and does not suffer any modification in its expression with the applied protocols. At the 6h-time point, the *Mdr1a* and *Bcrp* mRNA

levels did not suffer any change with morphine treatment in comparison to control. An up-regulation of the *Mdr1a* and *Bcrp* mRNA levels was registered from the 9h-time point onwards. *Mdr1a* mRNA expression registered a significant increase 12h and 24h after the last dose of morphine, in both large and microvessels, and returned to baseline expression at the 36h-time point. Similarly, *Bcrp* transcript levels registered an up-regulation in rat brain microvessels as early as 9h after the last dose of morphine, and such up-regulation was verified until the last time-point tested (36h). The greatest increase observed in rat brain microvessels was at 24h after the last dose of morphine, where *Mdr1a* and *Bcrp* transcript levels increased 40% and 140%, respectively. These results were corroborated at protein level, where P-gp and Bcrp protein expression remained unchanged 6h after the last dose of morphine, and both registered an increase of approximately 1.5-fold, in both large and microvessels, at the 24h-time point. To date, this is the first time it is demonstrated that morphine exposure is capable of inducing the expression of Bcrp, in addition to P-gp, at the BBB. Also, these results suggest that tolerance to morphine may be increased following a subchronic exposure to morphine also due to the observed up-regulation of P-gp.

2. Regulatory signaling pathways behind P-gp and Bcrp up-regulation following a subchronic treatment: the involvement of a glutamate-NMDA receptor-COX-2 cascade

On the other hand, there are three major results obtained in our study that point to a non-direct involvement of morphine in the regulation of P-gp and Bcrp: 1) the expression of *Mdr1a* or *Bcrp* was not altered when rats were subjected to a single dose administration of morphine, 2) nor immediately after the treatment suspension in the subchronic morphine regimen, since P-gp and Bcrp expression were never seen to be altered at the 6h time-point of sacrifice; 3) in addition, when hCMEC/D3 were exposed to several concentrations of morphine (0.01, 0.1, 1 and 10 μM) for 24h, no change in the *MDR1* and *BCRP* transcript level was registered, indicating that morphine has no direct effect on the brain capillary endothelial cells.

Indeed, every previously conducted study that showed an up-regulation of P-gp by morphine followed a subchronic morphine exposure protocol. Since the subchronic or chronic use of morphine is associated with the development of phenomena such as dependence, craving, and withdrawal syndrome, it is likely that some of these phenomena, and the underlying brain molecular changes, might be implicated in the registered up-regulation. In fact, the exact same morphine protocol applied in this study showed to trigger withdrawal symptoms as early as 9h after the last dose of morphine

(Desjardins et al. 2008), meaning that such phenomenon is already present by the time an induction on P-gp and Bcrp expressions is registered. In addition, its development seems to coincide with the onset of the regulation of the mRNA levels of these ABC transporters. Therefore, cellular mechanisms triggered during the development of morphine withdrawal may subsequently induce both P-gp and Bcrp at the rat BBB.

Increasing evidence demonstrates that opioids significantly alter the function of the glutamatergic system. Morphine-dependent rats showed a 2-fold increase of the extracellular fluid levels of glutamate in the locus coeruleus when morphine withdrawal was precipitated by the administration of naloxone (Zhang et al. 1994a). In a second study, glutamate concentration in the extracellular fluid of the hippocampus was decreased following either an acute or chronic treatment of morphine in mice (Guo et al. 2005). In turn, when morphine withdrawal was precipitated by naloxone in dependent mice, extracellular glutamate concentrations increased significantly in the hippocampus while no significant change was observed in saline-treated mice (Guo et al. 2005). In line with these results, the administration of naloxone or the cessation of morphine administration resulted in increased concentrations of glutamate in the nucleus accumbens of rats (Sepulveda et al. 2004). Naloxone-precipitated morphine withdrawal yielded such increase in just a few minutes after naloxone administration. Spontaneous morphine withdrawal also increased concentrations of glutamate in this brain region. When single doses of morphine were administered to rats previously withdrawn from chronic morphine treatment, the glutamate concentrations in the nucleus accumbens were restored to normal levels (Sepulveda et al. 2004). In addition, the glutamatergic NMDA receptor seems to be necessary for the development of physical withdrawal symptoms in several opioid-dependent animal models (Glass 2010; Higgins et al. 1992; McLemore et al. 1997; Trujillo and Akil 1991), and humans (Bisaga et al. 2001).

In turn, glutamate, an excitatory neurotransmitter, has shown to be in high extracellular levels in the epileptic brain, since it is massively released from neurons and glial cells (Bankstahl et al. 2008). Likewise, it has been shown that epileptic seizures induce P-gp up-regulation at the brain capillary endothelial cells through glutamate by an NMDA receptor and COX-2-dependent mechanism (Bauer et al. 2008). In this study, isolated rat or mouse brain capillaries exposed to glutamate for 15 to 30 min showed an increased P-gp expression and transport activity hours later. These increases were blocked by dizocilpine maleate (MK-801), an NMDA receptor antagonist, and by celecoxib, a selective COX-2 inhibitor. Furthermore, the intracerebral microinjection of glutamate provoked a local increase on P-gp expression in rat brain capillaries (Bauer et al. 2008).

Considering that glutamate has been shown to increase P-gp expression in rodent brain microvessels (Bauer et al. 2008; Zhu and Liu 2004), together with the previous described

findings pointing to a considerable link between morphine withdrawal and increased extracellular glutamate levels in the brain, we built the hypothesis that the development of morphine withdrawal, and the consequent increase of glutamate levels, lead to the observed up-regulation of both P-gp and Bcrp at the rat brain microvessels. In order to test if a similar signalling pathway is involved in the morphine-dependent up-regulation of P-gp and Bcrp, we subjected the animals to a similar morphine treatment regimen as we used before (5 days, twice daily, in an escalating dose regimen), and at the end of this treatment protocol, rats were treated either with or without an NMDA receptor antagonist, MK-801, or a COX-2 inhibitor, meloxicam. Animals were sacrificed 24h after the last dose of morphine, since it was the time-point that gave the highest up-regulation levels for both ABC transporters among the previously tested. Morphine-dependent rats to which the NMDA receptor antagonist was given had P-gp and Bcrp protein levels comparable to those of saline-treated rats. Rats subchronically treated with morphine but not with MK-801 registered a P-gp and Bcrp overexpression in both large and microvessels 24h after the last dose of morphine, confirming the previously obtained results. These data show that MK-801 blocked the morphine-dependent up-regulation of P-gp and Bcrp in large vessels and microvessels, indicating that the NMDA receptor, and thus its ligand, glutamate, are implicated in the subchronic morphine up-regulation of P-gp and Bcrp at the rat BBB. Similarly, the COX-2 inhibitor, meloxicam, also blocked the observed morphine-dependent up-regulation of P-gp and Bcrp in large and microvessels, which together indicate that, like in epilepsy, a glutamate-NMDA receptor-COX-2 pathway must be implicated in the regulation of the expression of P-gp and Bcrp at the rat BBB following a subchronic morphine exposure.

As previously discussed in the introductory section, in this cascade of regulation, glutamate acts on the NMDA receptors to presumably increase the intracellular Ca^{2+}, activating cPLA2 with increased production of arachidonic acid, which is then converted to PGE2 by COX-2, which subsequently signals through four different G protein-coupled receptors, EP1, EP2, EP3, and EP4 (Hata and Breyer 2004). Therefore, we decided to test the effect of two PGE2 analogs, iloprost and misoprostol, on the expression P-gp and BCRP on the hCMEC/D3 cell line, and further support the involvement of COX-2 in this cascade of regulation. Iloprost is a structural analog of prostacyclin whose affinity for EP1 receptors is similar to that of PGE2, while misoprostol is a PGE1 analog whose activity is mainly mediated by EP2, EP3, and EP4 receptors (Abramovitz et al. 2000). The use of PGE2 analogs, which have different affinities for the several EP receptors, served the purpose of uncovering the EP receptor(s) that should be preferentially implicated in this signaling cascade. Iloprost increased both *MDR1* and *BCRP* transcript levels, whereas misoprostol increased *MDR1* mRNA levels but had little effect on BCRP expression.

These findings also point to COX-2 activity mediated by PGE2, which should preferentially act via EP1 receptor. EP1 receptor expression has already been evidenced in isolated rat brain capillaries (Pekcec et al. 2009). Further studies are in support of the involvement of such signaling pathway in the regulation of P-gp: in *status epilepticus* animal models, seizure-induced P-gp up-regulation in brain capillaries was blocked when rats were treated with the COX-2 inhibitor celecoxib (van Vliet et al. 2010; Zibell et al. 2009), and with SC-51089 (Fischborn et al. 2010; Pekcec et al. 2009), a specific EP1 receptor antagonist. In contrast to the amount of data concerning the effect of glutamate on P-gp expression, little data is available concerning its role on BCRP expression at the BBB. However, and contrary to the results hereby presented, a recent study reported a down-regulation on BCRP expression and activity in isolated porcine and human brain capillaries exposed to glutamate, the latter obtained from surgical specimens of epileptic patients (Salvamoser et al. 2015). The mechanism underlying this down-regulation also seems to involve the NMDA receptor and COX-2 (Salvamoser et al. 2015). The down-regulation of BCRP expression and activity observed in this study, and thus contrasting with our study, might be a result from the high glutamate concentrations (100 μM) to which brain capillaries were exposed (Salvamoser et al. 2015), which may be cytotoxic and accelerate the decline in the capillary cell viability in these animal models.

3. Opioid withdrawal syndrome on the regulation of P-gp and Bcrp expressions at the rat BBB: what role?

We therefore postulated that repeated exposure to morphine might not affect P-gp and BCRP expression at the BBB unless a subsequent withdrawal syndrome takes place. In the latter case, the glutamate released during spontaneous morphine withdrawal syndrome should in turn up-regulate P-gp and BCRP expressions, and may be part of the mechanism underlying morphine tolerance, even though it cannot be the major cause of tolerance since morphine is a poor substrate of P-gp (Schinkel et al. 1995).

The pursuit of a better understanding of what drives the modulation of P-gp and Bcrp at the BBB by morphine motivated the second publication of the present PhD dissertation. This publication particularly focused on uncovering whether such modulation is a direct consequence of continued exposure to morphine, or rather a result of the morphine withdrawal that is further developed after discontinuation of treatment. Sprague-Dawley rats were treated according to different protocols of exposure to morphine and the expression of P-gp and Bcrp at the rat BBB was evaluated once rat brain microvessels were freshly isolated:

- In study 1, animals were subjected to a continuous i.v. infusion of morphine for 120h, at a constant infusion rate. This protocol was implemented in order to evaluate if morphine itself, following a prolonged, uninterrupted exposure, could be responsible for an up-regulation of P-gp and Bcrp expression at the rat BBB. The infusion flow rate (1 mL/kg/h) and the daily dose of morphine (15.6 mg/kg/day) were chosen to attain a theoretical plasmatic concentration of morphine of 100 ng/mL, based on the plasma clearance of morphine in the rat found in the literature (Ekblom et al. 1993; South et al. 2001). The target morphine plasma concentration was chosen based on the correspondent plasma concentrations reached in patients who are continuously infused with morphine for pain control (Wolff et al. 1996). Animals were sacrificed immediately once morphine infusion was interrupted, to avoid the development of withdrawal symptoms. The results obtained with this study demonstrated that, even though animals were continuously exposed to morphine and had attained significant plasmatic concentrations of morphine throughout the treatment, P-gp and Bcrp protein expressions remain unaltered in rat brain microvessels. This suggests that the observed up-regulation of both ABC transporters in the previous study (Manuscript no. 1) was not a direct consequence of morphine on the cascade of regulation of P-gp and Bcrp at the BBB.
- In study 2, rats were subjected to an escalating morphine dosing regimen (10-40 mg/kg, i.p., twice daily for 5 days) followed by an injection of a single dose of naloxone (1 mg/kg, s.c.), a strong μ-opioid receptor antagonist, 1h after in order to precipitate withdrawal. The peak and trough of morphine plasmatic concentrations, which is produced with this type of intermittent protocol, favours the installation of physical dependence and withdrawal once morphine treatment is suspended (Desjardins et al. 2008). Opioid withdrawal signs were visible as soon as naloxone was injected, and animals showed typical opioid withdrawal behavioral signs, such as diarrhea, mastication, wet-dog shakes, ptosis, salivation and jumping. The global withdrawal score was significantly higher in morphine-pretreated rats than in saline-pretreated rats, confirming the success of the present protocol in the development of dependence to morphine and of the precipitated withdrawal. Rats were sacrificed 12h after naloxone administration. Since the onset of the opioid withdrawal immediately follows the injection of naloxone in this study, the time of sacrifice was chosen given the difference between the onset of a spontaneous morphine withdrawal syndrome (i.e. approximately 12h after the last dose of morphine) and the time-point at which it was registered the highest increase in P-gp and Bcrp expression in our previous study (24h after the last dose of morphine, and therefore 12h after the theoretical onset of the spontaneous morphine withdrawal) (Manuscript no.1). Morphine-treated rats

exhibited an increase in *Mdr1a* and *Bcrp* transcript levels of 1.4-fold and 2.5-fold, respectively, which are of similar magnitude to the previously obtained 24h after the cessation of a 5-day subchronic treatment with morphine (Manuscript no. 1). However, proteomic and WB analyses did not evidence the expected up-regulation of P-gp and Bcrp proteins in brain microvessels of morphine-pretreated animals.

- In study 3, animals were submitted to a chronic morphine regimen (10 mg/kg s.c., twice daily for 5 days), followed by a withdrawal period (2 days), and treatment was resumed for 3 additional days. Similarly to study 2, a single administration of naloxone (1 mg/kg, s.c.) was given in order to trigger an intense opioid withdrawal. This protocol contrasts with the previous one since it has a first spontaneous withdrawal over a 2-day period, which increases the naloxone-precipitated withdrawal syndrome. At the end of the treatment protocol (day 10), animals showed similar behavior and withdrawal signs to those observed in study 2 once the withdrawal was precipitated. Naloxone-precipitated withdrawal clearly induced a much stronger abnormal behavior than the spontaneous withdrawal. Rats were sacrificed at two different time points: 4h and 24h after naloxone administration. As in the study 2, *Mdr1a* and *Bcrp* transcript levels registered similar increasing rates 4h after the naloxone injection, but once more UHPLC-MS/MS and WB analyses evidenced that P-gp and Bcrp protein contents remained unchanged in the morphine-treated group compared to the control animals. Furthermore, when the brain transport of [^{3}H]-verapamil, a P-gp substrate, or of [^{3}H]-mitoxantrone, a preferential Bcrp substrate, were assessed by *in situ* brain perfusion, there were no significant differences in their transport across the BBB between saline- and morphine-treated rats, 18h after naloxone was administered. These results show that P-gp and Bcrp activities in brain microvessels did not suffer any change by the implemented treatment, corroborating the findings obtained at protein level.

Contrary to what was expected, the present set of studies (Manuscript no. 2) did not allow us to establish a direct relationship between the up-regulation of P-gp and Bcrp at the BBB and the development of an opioid withdrawal in the rat. On one hand, P-gp and Bcrp modulation discrepancy found at mRNA and protein levels following naloxone-precipitated withdrawal could be explained by the occurring posttranslational regulation, as it has been previously suggested (Vogel and Marcotte 2012). Also, and even though naloxone is a selective antagonist of the μ-opioid receptors, the absence of P-gp and Bcrp up-regulation in brain microvessels following the naloxone-precipitated opioid withdrawal may be due to its effect on other non-opioid receptors (Liu et al. 2000a; Liu et al. 2000b), which may also be involved in the regulation of these ABC transporters following a morphine chronic

treatment, and thereby hamper P-gp and Bcrp up-regulation. In fact, naloxone has proved to inhibit TLR4 signaling (Hutchinson et al. 2008), even though it was produced with doses 100-fold superior to the ones used in the present study (Manuscript no. 2). Activation of TLR4 leads to downstream production and release of pro-inflammatory cytokines like TNF-α and IL-1β. As reviewed in the introductory section, these pro-inflammatory cytokines are implicated in the regulation of P-gp and BCRP at the BBB in both inflammatory and oxidative stress cases (Hartz et al. 2008; Hartz et al. 2006; Miller 2015; Poller et al. 2010).

In addition, our premise is based on the fact that during morphine withdrawal there is a massive increase in extracellular levels of glutamate in the brain parenchyma, as suggested by previous studies (Guo et al. 2005; Sepulveda et al. 2004; Zhang et al. 1994a), and that this excitatory amino acid is able to up-regulate the expression of P-gp at the BBB (Bankstahl et al. 2008; Bauer et al. 2008; Zhu and Liu 2004), and presumably that of BCRP. However, even though such studies demonstrate that glutamate concentrations are excessively high in the hippocampus, locus coeruleus or nucleus accumbens during an opioid withdrawal, none comprises an analysis of the glutamate levels in the brain cortex, which is the brain region we used for the study of the modulation of the expression of these ABC transporters at the BBB. Therefore, it would be of interest to perform a kinetics study of the concentrations of glutamate in the parenchymal extracellular fluid in the rat brain cortex during the subchronic morphine protocols we applied and following the development of both spontaneous and precipitated morphine withdrawal. This study could be performed by brain microdialysis. The microdialysis technique is widely used in neuroscience, and enables sampling and quantification of small molecular weight substances, such as neurotransmitters, peptides, and hormones in the brain ISF (Chefer et al. 2009). However, this is an invasive technique, which may alter tissue morphology including the BBB integrity (Morgan et al. 1996), with some practical limitations, and requires a high expertise in the implementation of the method. Furthermore, brain extracellular levels of glutamate produced during opioid withdrawal (approximately 20 μM) (Guo et al. 2005; Sepulveda et al. 2004; Zhang et al. 1994a) are relatively inferior to those that have shown to increase P-gp expression in brain capillary isolates (50-100 μM) (Bauer et al. 2008; Zhu and Liu 2004; Zibell et al. 2009), suggesting that this mechanistic pathway should contribute, but in parallel with other activated signaling pathways, to the up-regulation of P-gp and Bcrp. Thus, P-gp and Bcrp up-regulation following a subchronic morphine treatment (Yousif et al. 2012) (Manuscript no. 1) might result of a sum of multiple mechanisms and signaling pathways implicated in the modulation of both transporters at the BBB, and naloxone may act at non-opioid receptors, such as TLR4 or even neurotransmitter receptors, linked for instance to an

inflammatory component of morphine withdrawal, to partially hamper P-gp and Bcrp up-regulation.

4. Characterization of isolated brain microvessels as a BBB study model: a comparison between techniques, and neurotransmitter receptor expression by qRT-PCR

In the work developed for the elaboration of the present dissertation, we had also exposed the hCMEC/D3 cell line to several glutamate concentrations, in order to test the effect of glutamate on the expression of P-gp and BCRP in this BBB *in vitro* model (Manuscript no. 1), and further support the involvement of this neurotransmitter in the regulatory mechanism of these transporters. Cells were exposed to 10 and 100 μM of glutamate for 40 min, and then incubated in glutamate-free medium for 5h30min before the amounts of *MDR1* and *BCRP* transcripts were analyzed by qRT-PCR. Yet, glutamate did not significantly alter the mRNA expression of any of these transporters in comparison to non-treated cells. Considering this result, we questioned the expression of the glutamate receptor NMDA in this cell line. To clarify this, we checked the mRNA expression of the gene encoding for the NR1 subunit of the NMDA receptor in hCMEC/D3 cells. The *NR1* transcript levels found were very low and unquantifiable, which might explain the absence of effect of glutamate on *MDR1* and *BCRP* mRNA levels in the hCMEC/D3 cell line. Still, the presence of the NMDA receptor in brain endothelium has been a matter of study over the past decade, with general controversy and little consensus regarding its actual expression and role at the BBB. The gene expression of several subunits of the NMDA receptor was demonstrated in cultured rat BECs for the first time in the late nineties (Krizbai et al. 1998). At the same time, in another study, gene expression and functional activity of the main NMDA receptor subunits was not demonstrated in either rat or human cortical microvessels and in cultured cerebrovascular endothelial cells (Morley et al. 1998). The NR1 subunit protein expression was also not detected in a rat brain microvascular endothelial cell primary culture (Domoki et al. 2008). In turn, in 2003, the expression of the NR1 subunit was found in an immortalized human endothelial cell line using RT-PCR, WB and immunoprecipitation approaches (Sharp et al. 2003). Later on, the NR1 and NR2 subunits of the NMDA receptor were also showed to be expressed in primary cultures of rat brain endothelial cells (Andras et al. 2007); similarly, the expression of NR1 subunit was evidenced by qRT-PCR, immunostaining, and WB in mouse brain microvessel endothelial cells and in the hCMEC/D3 cell line (Legros et al. 2009; Reijerkerk et al. 2010). This later study clearly contrasts with our results, since they report

the expression of the NMDA receptor subunit using a similar technical approach and the same human cell line we used.

The third study conducted under the preparation of this dissertation (Manuscript no. 3) comprehended a large screening of several receptors for their expression at the rat BBB, including the NMDA receptor subunit NR1 and many other neurotransmitter receptors implicated in the inflammatory cell-cell signaling, and which may have a role in the modulation of P-gp and BCRP efflux transporters. This study was performed in order to bring further insight into some of the already known mechanisms of regulation of ABC transporters, such as the one that morphine is implicated in. Many of these receptors may also have a general role in the regulation and maintenance of the BBB structure and properties, if present in the NVU cells, particularly in the BECs, astrocytes, pericytes, and microglia.

In this study we first compared two different techniques of isolation of rat brain microvessels – mechanical dissection and enzymatic digestion – to determine which yielded the purest microvessel fraction for the study of the BBB. Hence, the relative expression, evaluated by qRT-PCR, of several specific cell markers for the different cell types that are part of the NVU – *glut1* (BECs) *ng2, pdgfrb* (pericytes), *gfap, s100b* (astrocytes), *cd11b* (microglia), *mbp* (oligodendrocytes) and *syp* (neurons) – was compared between samples obtained by either mechanical dissection or enzymatic digestion. The expression of such markers in isolated microvessels was also compared against the expression in rat brain cortex as a measure of enrichment on the BBB cell markers. The results led us to conclude that there is a higher enrichment of BEC and pericyte cell markers with the enzymatic digestion approach, in comparison to the mechanical dissection method. In fact, *glut1*, *ng-2*, and *pdgfrb* registered higher expressions in microvessels isolated enzymatically than in microvessels isolated mechanically. Furthermore, the enzymatic technique results in a higher reduction on the contamination coming from other cell elements surrounding the brain capillaries as the expression of specific markers for astrocytes, oligodendrocytes and neurons was considerably reduced when microvessels were isolated with the enzymatic approach compared to the brain microvessels isolated mechanically. These results indicate that, since microvessels isolated with an enzymatic approach yield a more pure fraction of brain capillaries than microvessels isolated with a mechanical approach, this technique should be preferentially adopted for studying the BBB properties in the rat. Our previously presented studies used isolated microvessels obtained by mechanic disruption, which would not be the ideal approach, given these comparative results. However, we mainly focused on the study of the expression of ABC transporters in the brain microvessels, whose expression is essentially limited to the brain capillary endothelial cells. Data from

previous studies reported an induction of P-gp by other xenobiotics in parenchymal cells, such as astrocytes and neurons (van Vliet et al. 2004; Volk et al. 2005; Volk et al. 2004). However, previous observations obtained by our research group using immunohistochemistry on brain sections of animals following a subchronic morphine treatment, exclude this occurrence as no presence of P-gp was detected neither in astrocytes nor in neurons (Yousif et al. 2008). Hence, even though rat brain microvessels isolated by mechanic disruption might have some cell contamination from other elements surrounding the capillaries, it does not have an important impact on the yielded results concerning the expression of P-gp and Bcrp.

The expression of 11 neurotransmitter receptors in both rat brain cortex samples and in isolated rat brain microvessels was then assessed: adrenergic receptors α_{2A}, β_1 and β_2 (*Adra2a*, *Adrb1*, *Adrb2*), adenosine receptors A_1, A_{2A} and A_3 (*Adora1*, *Adora2a*, *Adora3*), ATP receptors $P2X_7$ and $P2Y_1$ (*P2rx7*, *P2ry1*), cannabinoid receptors CB_1 and CB_2 (*Cnr1*, *Cnr2*), and the glutamate receptor subunit *NMDAr1*.

Among these neurotransmitter receptors, *P2ry1* (788-fold of the least expressed gene, *Adora1*), *Adora2a*, *P2rx7* and *Adrb2* showed to assume an important expression level in rat brain microvessels, while *Adora1*, followed by *Cnr1*, *Adrb1*, *Cnr2* and *Adra2a* are among the least expressed receptors. The comparisons of expressions between samples from brain cortex and from brain microvessels took into account the expression found in rat brain microvessels isolated using the enzymatic approach, since this was the technique that yielded the purest capillary fraction, and thus should best reflect the expression of neurotransmitter receptors at the BBB. In rat brain microvessels, the *Adora1*, *Cnr1* and *NMDAr1* transcript levels showed to be approximately 50-fold lower than those found in brain cortex samples, suggesting that these receptors are poorly expressed in rat brain capillaries, and are likely derived from neuronal contamination. *Adrb2* and *Adora2a* are the only neurotransmitter receptors with an increased expression in rat brain microvessels in comparison to brain cortex, while *Adra2a*, *Cnr2* and the ATP receptors *P2rx7*, *P2ry1* do not register any considerable change of expression from brain cortex to brain microvessel samples. Yet, it is important to note that while *Adra2a* and *Cnr2* are among the least expressed genes in the brain cortex, *P2rx7* and *P2ry1* are among the most expressed, meaning that the expression of these latter genes in the microvessels is very significant. Thus, the ATP receptors $P2X_7$ and $P2Y_1$, as well as the adenosine receptor A_{2A} are highly expressed at the BBB, and so may play a central role in the cell signaling at the NVU and in the normal function of the BBB. Concordant with our findings, the expression of A_{2A} receptors in the brain vasculature was already demonstrated in a few studies (Koehler et al. 2006; Ngai et al. 2001). This is further supported by studies performed on a primary culture of mice brain endothelial cells and in

hCMEC/D3 cells, where the protein expression of A_{2A} adenosine receptors was evidenced (Carman et al. 2011; Mills et al. 2011). Our results, which are supported by the literature, indicate that the BBB is able to directly respond to extracellular adenosine, either coming from astrocytes and other neighboring cells, or from the bloodstream. However, to present, any connection between the activation of this adenosine receptor and the regulation of the expression of P-gp and BCRP is unknown.

Regarding the ATP receptors, it is known that $P2X_7$ receptors are predominantly expressed in the microglia (Harry 2013), and promote the release of pro-inflammatory cytokines, such as IL-1α, IL-1β and TNF-α (Bernardino et al. 2008; Ferrari et al. 1997; Hide et al. 2000; Mingam et al. 2008), and the ROS superoxide (Parvathenani et al. 2003). In fact, pro-inflammatory factors, such as TNF-α, and ROS have been suggested as central elements implicated in the up-regulation of P-gp at the BBB during inflammation/oxidative stress phenomena (Felix and Barrand 2002; Nwaozuzu et al. 2003) (Bauer et al. 2007; Rigor et al. 2010), as extensively reviewed in the introductory section. Hence, the activation of this receptor in the microglia or in the endothelium may favor the installation of inflammatory and/or oxidative stress cascades, which may in turn interfere with the regulation of P-gp and BCRP at the BBB. In opposition to $P2X_7$, the ATP receptor $P2Y_1$ activation in microglia has shown to promote the down-regulation of the pro-inflammatory response, since its activation increases the release of the anti-inflammatory cytokine, IL-10 (Seo et al. 2008). In this way, by limiting the inflammatory signaling propagation at the NVU, and by providing neuroprotection against oxidative stress, $P2Y_1$ may also play a role in the control of P-gp and BCRP levels at the BBB. Recent studies also indicate that activation of $P2Y_2$ receptors in the brain vascular endothelium can increase the transendothelial permeability of leukocytes (Kukulski et al. 2010; Seye et al. 2002; Seye et al. 2003). This suggests that the activation of $P2Y_2$ receptors in brain microvessels, and possibly of $P2Y_1$, may function as a novel target for increasing drug delivery into the brain.

Similarly to $P2Y_1$ receptors, CB_2 receptors, predominantly expressed in microglia, are implicated in the reduction of oxidative stress and neuroinflammation by attenuating microglial activation and production of proinflammatory mediators (Campbell and Gowran 2007; Horvath et al. 2012). In our study (Manuscript no. 3), we demonstrated very little expression of this receptor in rat brain microvessels, which likely comes from small contamination from microglia. This is in agreement with the literature, as little CB_2 receptor immunorreactivity has been detected in the healthy brain microvasculature (Ramirez et al. 2012). However, the expression of CB_2 receptors seems to be up-regulated in pathologies and in response to inflammation and stress (Schley et al. 2009; Zhang et al. 2011b).

According to our study, the *NR1* subunit expression is relatively poor in comparison to the other tested genes in rat brain microvessels isolated enzymatically. Considering that, among the tested receptors, the NMDA receptor was the receptor that registered, by far, the highest transcript levels in the rat cortex, it is likely that the expression of *NR1* found in microvessels might represent a small contamination from the brain parenchyma. This poor *NR1* expression in brain microvessels is in line with the absence of this NMDA receptor subunit found in the hCMEC/D3 cell line in one of our previous studies (Manuscript no. 1). Despite recent studies have indicated the presence of the NMDA receptor in the brain microvasculature as an element in the regulation of the expression of ABC transporters (Bauer et al. 2008; Salvamoser et al. 2015; Zibell et al. 2009), the expression of this receptor at the brain endothelium is controversial among the scientific community. Thus, it is more likely that the NMDA receptor takes part of a mechanism that involves the complete NVU, and not only the BECs, concerning the regulation of P-gp and BCRP at the BBB.

5. P-gp activation at the BBB: thioxanthonic derivatives as P-gp activators and evaluation of their antidote properties against the substrate-induced neurotoxicity

Several studies focusing on the regulation of ABC transporters at the BBB analyze their modulation in terms of gene and protein expression, but lack to confirm their correlation with an increased or decreased transport function, which is what ultimately may contribute to an altered brain distribution and therapeutic effect/toxicity of their substrates. However, it is generally accepted nowadays that a registered increase in protein expression may not necessarily result in a proportional increase in the protein function (Kosztyu et al. 2014). This is particularly true for P-gp, where several studies showed that an induction of the transporter did not always translate into an increase in its efflux activity (Silva et al. 2011; Silva et al. 2014a; Takara et al. 2009). On the other hand, there is rising evidence that several compounds are able to increase P-gp transport activity without increasing its protein expression (Silva et al. 2014c; Silva et al. 2014d; Sterz et al. 2009; Vilas-Boas et al. 2013b). These studies gave rise to a new emerging class of compounds, known as P-gp activators. In fact, the activation of ABC transporters, a phenomenon that reflects the increased efflux of their substrates, has been recently brought to discussion and is gathering attention, given the important impact it may have in the rapid modulation of the transport of substrates of P-gp and other ABC transporters, and their therapeutic effectiveness/organ toxicity. The study and development of novel P-gp activators is a complex approach involving the establishment of quantitative structure-activity

relationships, since these molecules will act through a direct interaction with the ABC transporter to induce a change in its conformational position, and favor the transport of substrates.

It was long known that several compounds could bind to P-gp and stimulate the transport of substrates on another binding site. As was reviewed in the introductory section, P-gp is suggested to have at least three drug binding sites, comprising an allosteric binding site which may exhibit a regulatory function (Shapiro et al. 1999). A fourth binding site was also proposed, with three transport sites and a regulatory one, which allows a change in the conformation of the transporter altering the affinity for substrates and increasing the rate of translocation (Martin et al. 2000). In addition, recent crystallography studies on the structure of the mouse P-gp evidenced a large drug-binding cavity, where at least two substrate molecules may bind simultaneously (Aller et al. 2009). The exposure of Caco-2 cells to several NSAIDs, such as mefenamic acid, sulindac, naproxen, and meloxicam, significantly reduced intracellular accumulation of Rho 123, with no change in the *MDR1* mRNA levels (Takara et al. 2009). Some catechins were also reported to increase the P-gp mediated transport of LDS, a fluorescent P-gp marker substrate, without affecting the daunorubicin or Rho 123 transport (Wang et al. 2002). In addition, several compounds primarily synthetized to act as p53 inhibitors and derivatives, generated different effects on the intracellular accumulation of P-gp substrates: these molecules stimulated the efflux transport of anthracyclines and Rho 123 by P-gp, whereas the P-gp mediated efflux of vinblastine and colchicine was inhibited (Kondratov et al. 2001; Sterz et al. 2009). It was therefore suggested that these modulating compounds should bind to the P-gp *H* site and stimulate the efflux of *R* site-binding substrates, while the transport of *H* site-binding substrates is competitively inhibited.

Recently, it was shown in the RBE4 cell line that a synthetic derivative of rifampicin, RedRif, was able to increase both P-gp expression and activity (Vilas-Boas et al. 2013b). Yet, the increase on P-gp transport activity was registered at early points where no up-regulation on P-gp expression had been observed: RedRif increased P-gp expression after 48h of exposure, while the efflux activity was increased after only 24h of exposure. The increased P-gp activity registered in RBE4 cells following a 24h pre-exposure or simultaneous exposure to RedRif resulted in higher cell protection against PQ-induced cytotoxicity (Vilas-Boas et al. 2013b). In fact, the simultaneous exposure to both the rifampicin derivative and PQ yielded the more efficient protection against PQ cytotoxicity.

Recent studies performed in Caco-2 cells demonstrated that five newly synthetized thioxanthonic derivatives (Silva et al. 2014c) and five dihydroxylated xanthones (Silva et al. 2014d) significantly increased both P-gp expression and activity. Indeed, the 24-hour simultaneous incubation of Caco-2 cells with the (thio)xanthonic derivatives and PQ

resulted in a significant reduction in the PQ-induced cytotoxicity (Silva et al. 2014c; Silva et al. 2014d). The protection that these compounds conferred through P-gp activation against PQ cytotoxicity was further confirmed since this increased protection was abolished in the presence of a P-gp inhibitor, elacridar (Silva et al. 2014c; Silva et al. 2014d). Importantly, it was also observed that these compounds rapidly increased the activity of this ABC transporter, assessed by the Rho 123 efflux assay, where Caco-2 cells are incubated with Rho 123 in the presence of (thio)xanthones during a short 45 min Rho 123 efflux phase. These findings indicate an increased P-gp activity that is not a result of an increased P-gp protein expression, given the short time of exposure of cells to such compounds. Thus, these (thio)xanthonic derivatives showed to increase P-gp expression in Caco-2 cells, but also to produce an expression-independent increase in the activity of P-gp.

Given their demonstrated ability to promptly activate P-gp efflux transport in Caco-2 cells, and considering their promising source of antidotes against the cytotoxicity of harmful P-gp substrates, we decided to further evaluate if this mechanism of rapid activation could be triggered in a BBB model, using the RBE4 cell line (Manuscript no. 4). For this purpose, we selected some of the previously tested TXs in Caco-2 cells, such as TX87, TXA4, TXA3 and TXOH, and some new thioxanthonic derivatives, TX105 and TX129. The assays performed in RBE4 cells to evaluate the cytotoxicity profile of each thioxanthonic compound lead us to conclude that any compound showed to be toxic in a 10 μM concentration, with the exception of TX129, which according to the MTT reduction assay lowered the cell viability to 88%. When the P-gp transport activity was assessed by the Rho 123 efflux assay, where RBE4 cells were exposed to each thioxanthonic compound (10 μM) for a short period of 45 min during the Rho 123 efflux phase, most of the compounds – TXA4, TXA3, TX105 and TX129 – produced a significant reduction on the Rho 123 intracellular accumulation. TXA4, TXA3, TX105 and TX129 showed to be equally efficient as potential P-gp activators at this concentration, since they increased Rho 123 efflux by 159, 167, 161 and 168%, respectively. When the concentration of such compounds was elevated to 20 μM, not only these compounds but also TX87 revealed to significantly activate P-gp. Once more, most of the thioxanthonic compounds showed to produce a significant increase in the P-gp-mediated efflux of a substrate, now in an *in vitro* BBB model upon a considerable short period of incubation (45 min), and where no increase on the P-gp protein expression is likely to occur.

When the impact of thioxanthonic compounds on the ATPase activity of recombinant human P-gp was analyzed at the 10 μM concentration, all thioxanthones showed to poorly increase ATPase activity, suggesting that they may only work as poor P-gp substrates. Stimulators of ATPase activity are likely substrates for P-gp-mediated efflux, and thus also

competitively inhibit transport of other P-gp substrates (Ambudkar et al. 1999). Therefore, thioxanthones unlikely participate in competition phenomena and inhibit the transport of P-gp substrates. Nevertheless, some of these thioxanthones have been previously tested for their capacity to stimulate or inhibit the P-gp ATPase activity, and at 20 μM concentration, TXA4, TXA3 and TXOH produced a significant increase (Silva et al. 2014c). This indicates that the concentration at which such molecules are used can be determinant in their role within the interaction with P-gp, namely in acting as potential substrates and in enrolling in competition phenomena.

P-gp activators typically bind in the drug-binding pocket formed by the TMD interface. Since our aim was to test the effect of these compounds against the mitoxantrone-induced cytotoxicity, binding modes of the tested thioxanthonic derivatives, alone or with mitoxantrone, as well as of mitoxantrone alone, were further explored by a docking study, using an X-ray P-gp structure based on the mouse (Szewczyk et al. 2015). Recent data shows that, unlike the symmetric Sav1866 template (described and used in previous studies (Silva et al. 2014a; Silva et al. 2014d; Vilas-Boas et al. 2013a), mouse P-gp has a high degree of asymmetry, with only 44% similarity between the two halves of TMDs (Pan and Aller 2015), which likely play a major role in the overall function and mechanism of the transporter. In fact, it is thought that mouse P-gp should favor closed TMDs and open nucleotide-binding domains, whereas Sav1866 should favor the exact opposite configurations, as well as the hydrophobicity of mouse P-gp in the drug-binding pocket is substantially larger than that of Sav1866 (Pan and Aller 2015). Given that rat P-gp should have a closer homology to mouse P-gp than to the Sav1866 template, we decided to preferentially adopt the mouse P-gp model for docking studies.(Palmeira et al. 2012b) Even though they showed to be poor P-gp substrates, docking studies allowed us to understand that thioxanthones bind to P-gp with a considerable high affinity, with two preferential binding pockets in P-gp, and a preferential binding pocket engulfed by TMD 2, 3, 10 and 11, further supporting that these compounds most probably interact with P-gp for its activation.

Considering the observed effects of the tested thioxanthones on P-gp activity in RBE4 cells, we further aimed to elucidate if they could confer additional protection to this cell line against the cytotoxicity of a P-gp substrate, mitoxantrone, as it was demonstrated in Caco-2 cells. In fact, in the previous study where these thioxanthonic compounds showed to efficiently activate P-gp in Caco-2 cells according to the Rho 123 efflux assay, cell simultaneous incubation with these thioxanthonic derivatives and PQ, a toxic P-gp substrate, resulted in a significant reduction in PQ cytotoxicity (Silva et al. 2014c). Mitoxantrone was chosen as the substrate of study, since it is transported by both P-gp and BCRP (Agarwal et al. 2011a; Kodaira et al. 2010; Lee et al. 2005). Given our interest

in studying the modulation of P-gp activity by these compounds, but it is also in perspective to study that of BCRP, mitoxantrone would thus allow us to understand, in a single assay, if Bcrp activity in RBE4 cell could be also modulated upon incubation with thioxanthones. Therefore, the cytotoxicity of mitoxantrone was assessed by the neutral red uptake assay, in the presence or absence of each test compound for 4h, 8h and 16h, and evaluated 24h after the beginning of the exposure. However, and surprisingly, no significant differences were observed in the overall comparison of the fitted curves (TOP, BOTTOM and EC_{50} value), meaning that none of these compounds was able to confer additional protection against mitoxantrone-induced cytotoxicity in RBE4 cells, unlike what was observed for PQ in Caco-2 cells. These results indicate that thioxanthones did not promote a biologically important increase in the activity of P-gp, and most likely neither in that of Bcrp, so that a protection against the cytotoxicity of mitoxantrone could be observed.

Different biological scenarios can be at the origin of the observed results. In fact, it was already observed that the PQ-induced cytotoxicity was not reduced with all thioxanthones that showed to produce an increase in the P-gp activity in the Rho 123 assay, in Caco-2 cells (Silva et al. 2014c). The different affinities between P-gp activators and P-gp substrates for the drug-binding pocket of P-gp may determine if P-gp activators can confer protection or not the cell models. Still, other scenarios can be pointed to justify the results obtained in this manuscript.

Firstly, mitoxantrone may not have been a good drug candidate in this study, since it is a common substrate of P-gp and BCRP, and thereby P-gp is not the only transporter implicated in the efflux of mitoxantrone in this experimental setting. Previous studies demonstrated the important contribute of P-gp in the efflux transport of this molecule. The C_{brain}/C_{plasma} mitoxantrone markedly increased in double knockout *Mdr1a*/*1b*(-/-)/*Bcrp*(-/-) mice compared with wild-type mice, or even to *Mdr1a*/*1b*(-/-), and/or *Bcrp*(-/-) mice, as well as in *Mdr1a*/*1b* (-/-) in comparison to wild-type mice (Kodaira et al. 2010). We also successfully demonstrated the impact of P-gp inhibition on the increase of mitoxantrone-induced cytotoxicity in the RBE4 cell line (Manuscript no. 4), further confirming that mitoxantrone is a substrate of P-gp, and that this transporter is implicated in its efflux in this BBB *in vitro* model. Still, it is suggested that when the activity of either P-gp or BCRP is primarily compromised, the other transporter can compensate through an increase in its expression and/or activity. For example, double knockout studies or inhibition of both transporters frequently results in a greater increase in the brain accumulation of common substrates than the additive effects of single knockout or inhibition (Cisternino et al. 2004; de Vries et al. 2007). In addition, there is evidence that BCRP expression can be down-regulated in cell models overexpressing P-gp (Bark et al. 2008). Thus, if P-gp activation

has been met in the performed study, we cannot put aside that an eventual change, and in the present case, down-regulation, in Bcrp expression and/or activity could have occurred. As a result, due to the P-gp/BCRP cooperation, an increase in P-gp activity alone may not translate into an appreciable decrease in cell accumulation of dual substrates, as it is the case of mitoxantrone.

Secondly, the P-gp activation induced by thioxanthones in our study might be a transient phenomenon, resulting in a reduction of mitoxantrone intracellular accumulation only for a short period of time, which may not be enough to confer cell protection against mitoxantrone-induced cytotoxicity over a period of exposure of 4h, 8h or 16h. Furthermore, our study differs from the previous one conducted by Silva et al (2014), as in the latter thioxanthonic derivatives have shown to promote an immediate activation of P-gp but also to induce an up-regulation on the P-gp protein expression in Caco-2 cells (Silva et al. 2014c), which was not explored in ours. In fact, an increase of the expression of P-gp at the cell membrane is more likely to produce long-lasting consequences on the drug efflux of substrates over the course of the incubation period, which may explain the different thioxanthones-mediated effects found between the study conducted on RBE4 cells and the previously conducted in Caco-2 cells.

Thirdly, important interspecies differences may contribute to these differential results among studies. Given that there are significant species differences of some of the pathways implicated in the regulation of P-gp expression, such as the ligand-binding and activation profiles for orphan nuclear receptors like PXR and CAR (Wang and LeCluyse 2003), we cannot guarantee that the mechanism by which thioxanthones up-regulate P-gp protein expression in the Caco-2 cell model is activated in the RBE4 cells. Also, previous studies show that the P-gp structure and activity is different depending on the animal model (Pan and Aller 2015; Xia et al. 2006). Since Caco-2 cells express human P-gp and RBE4 cells express rat P-gp, thioxanthones and P-gp substrates may bind to the rat and to the human P-gp in a different arrangement due to structural differences. Indeed, there are interspecies differences in the affinities of P-gp activators and P-gp substrates for the drug-binding pockets of P-gp. This may explain the distinct results given by thioxanthones regarding the protection against the cytotoxicity of P-gp substrates in the rat model used in our study and in the previous one (Silva et al. 2014c).

Still, even though thioxanthones failed to demonstrate an increased protection against the mitoxantrone-induced cytotoxicity in RBE4 cells, they remain as interesting drug candidates for an antidote strategy against the toxicity induced by P-gp substrates. Most of the thioxanthones showed to promptly activate P-gp efflux transport, achieved through a change in the protein conformation, which, in comparison with the induction phenomenon, results in a much faster manner to reduce the cytotoxicity of harmful P-gp

substrates. P-gp activators are able to immediately increase P-gp activity with no increase in its expression, since they act by binding to a specific ligand-binding site in the P-gp structure, which induces a conformational change in the P-gp structure. This stimulates the efflux of substrates bound onto another ligand-binding site (Vilas-Boas et al. 2013b). Indeed, it is believed that adaptation and survival mechanisms in living beings has permitted the establishment of multiple binding sites for xenobiotics in P-gp, thus favoring the co-transport of substrates by activating the transportation cycle (Safa 2004). Noteworthy, we can say that an increase in the protein expression of P-gp does not always reflect into an increase in its activity, and in the same way an increase in the efflux of a P-gp substrate due to P-gp activation does not always translate into an overall reduction of cytotoxicity of its substrates. Therefore, the P-gp activation phenomenon should be interpreted carefully, and studied case by case, since it does not necessarily mean that it may confer protection against the toxicity of any harmful P-gp substrate.

GENERAL CONCLUSIONS

The work conducted in the present thesis dissertation resulted in some useful further insight regarding the use of different approaches for the study of the BBB, and especially into the mechanisms of modulation of drug efflux transporters present in this barrier. The further understanding of the molecular mechanisms that regulate P-gp and BCRP provided by these studies represents an interesting tool for the development of strategies to manipulate their expression and activity, depending on the main therapeutic goal: transporter expression and/or activity can be up-regulated to achieve a selective tightening of the barrier and reduce the brain accumulation of harmful neurotoxicants, or, on the other hand, transcriptional activation of ABC transporter genes can be controlled in order to increase drug delivery into the brain, and prevent pharmacoresistance phenomena, and CNS disorder-induced P-gp and/or BCRP up-regulation. The major conclusions of these studies can be summarized as follows:

- A subchronic morphine treatment transiently up-regulates the expression of both P-gp and Bcrp at the rat BBB, observed at mRNA and protein level. However, the increased levels of both ABC transporters are not immediately observed once treatment is suspended, but only 9h-12h, and lasting for 24h-36h after the last dose of morphine was given, being timely-coincident with the development of a morphine withdrawal syndrome.
- P-gp and Bcrp up-regulation following a subchronic morphine administration is dependent on the NMDA receptor activation by glutamate and the production of prostaglandins like PGE2 by COX-2, since the antagonism of the NMDA receptor and inhibition of COX-2 counteracted the increase of P-gp and Bcrp expressions at the rat BBB. Interestingly, these elements are also linked to the P-gp overexpression in epileptic patients.
- A continuous infusion of morphine for 5 days, and thus morphine exposure by itself, does not modify the protein contents of P-gp and Bcrp in rat brain microvessels.
- Naloxone-precipitated opioid withdrawal following subchronic morphine exposure protocols increased the mRNA expression of P-gp and Bcrp, but failed to produce an increase on their protein expression and activity. The glutamatergic pathway is probably not the only signalling mechanism activated upon morphine withdrawal driving the up-regulation of ABC transporters at the BBB, and naloxone may hamper other important pathways implicated in such regulation.
- When the mRNA levels of specific cell markers for the different cells representing the NVU and the CNS were compared between brain microvessels obtained by two different techniques – a mechanic and an enzymatic approach – it lead us to conclude

that the enzymatic approach yielded the purest fraction of brain capillary endothelial cells. Indeed, brain microvessels isolated enzymatically are more highly enriched in pericyte and endothelial cell markers and with less other cell contaminants, and thus should be preferably used to the mechanic approach in the upcoming studies using brain microvessels for analysing BBB physiopathology and properties.

- ATP receptors $P2X_7$ and $P2Y_1$, as well as the adenosine receptor A_{2A} are highly specific receptors expressed at the rat BBB, suggesting they may have an important role in the signalling system for the normal function of the NVU. On the other hand, the adenosine receptor A_1, as well as the cannabinoid receptor 1 and the NMDA receptor subunit NR1 expressions in rat brain microvessels were approximately 50-fold lower to those found in brain cortex samples, indicating that these receptors likely come from neuronal contamination.
- Thioxanthonic compounds showed to promptly activate P-gp, and increase the efflux of P-gp substrates, like Rho 123, in the RBE4 brain endothelial cell line, even without any increase on the P-gp protein contents. However, when co-incubated with mitoxantrone, these compounds did not confer an increased protection against the mitoxantrone-induced cytotoxicity in RBE4 cells in culture for 24h. Still, thioxanthones remain as interesting drug candidates for an antidote strategy against the toxicity induced by harmful P-gp substrates, but P-gp activation phenomenon should be interpreted carefully, and studied case by case, since it does not necessarily mean that it may confer protection against the toxicity of every P-gp substrate.

FUTURE PERSPECTIVES

In summary, the present work developed under this PhD dissertation explored important pathways of regulation of both the expression and activity of the two major ABC transporters present at the BBB, and thus can be valuable tools to either overcome pharmacoresistance in the treatment of neurological diseases or to revert the neurotoxicity of substrates. Nevertheless, our research has some shortcomings that need to be addressed, and that we intend to further investigate to better clarify the outcome of the here presented results:

- Given that the isolation of brain microvessels using the enzymatic digestion has shown to yield a richer fraction of brain capillary endothelial cells than the mechanical dissection, and with less cell contaminants from other NVU and CNS cell-types, in the future, studies on the BBB properties and regulation of its transporters should be conducted with this technical approach;
- Since the glutamatergic pathway is probably only one among other signalling pathways contributing to the up-regulation of P-gp and BCRP at the BBB during morphine withdrawal, the role and activation of other well-known regulatory pathways, such as the oxidative stress and inflammation-linked pathways, should be a matter of study following a subchronic morphine treatment;
- Glutamate extracellular levels should be investigated in the rat brain cortex following a subchronic morphine treatment and during both spontaneous and precipitated-morphine withdrawal. In fact, we systematically analysed the effect of drug treatment on the expression of ABC transporters from rat brain cortical microvessels, and to our knowledge it remains to be demonstrated that there is a glutamate overshoot in this brain region, even though it has been demonstrated in other brain regions like the hippocampus, locus coeruleus and nucleus accumbens, during opioid withdrawal;
- To confirm that thioxanthones are able to successfully activate P-gp in RBE4 cells and increase the efflux of mitoxantrone, the intracellular accumulation of mitoxantrone should be analysed following its incubation in the presence and in the absence of the different thioxanthonic derivatives;
- In parallel to the study of the activation of P-gp by these thioxanthonic derivatives, we should investigate if these compounds are also able to increase the P-gp protein expression in the RBE4 cell line, like it was demonstrated in the human Caco-2 cell line, and therefore verify if similar mechanisms of action are present in both models.

PART V

V. REFERENCES

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