

**PARIS-XI UNIVERSITY
FACULTY OF PHARMACY, CHÂTENAY-MALABRY**

**Graduate school ED 425 «Therapeutic innovation: *from fundamental to applied*»
Pharmacology - Toxicology**

Year 2012

Serial N° 1214

DISSERTATION

Presented at the
Pharmacology and Immunoanalysis service
CEA of Saclay

For the degree of
DOCTOR IN EXPERIMENTAL AND CLINICAL PHARMACOLOGY

by
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**Blood-Brain Barrier during Cerebral Maturation:
Impact of Neuro-Inflammation on the Regulation of Drug-Efflux/Influx Transporters**

Oral examination: December 5th, 2012

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Dedications

I thank god for granting me faith and determination to accomplish my doctoral thesis successfully.

I dedicate this work to my parents, **Awada Harati** and **Hala El Maiss Harati**. Your tremendous encouragement and precious prayers provided me with force and perseverance that kept me going forward.

I also dedicate this work to my brothers, **Mohamed** and **Khalil**, to my sister **Attorney Zeina Harati**, and to my grand-parents, **Widad** and **Khalil El Maiss**. This achievement would not have been possible without your prayers and support.

Acknowledgments

The works presented in this thesis were performed at the French Atomic and Alternative Energies Commission of Saclay (CEA de Saclay), in the Pharmacology and Immuno-analysis Service (SPI) within the Neurovascular Pharmacology group. The project was granted by the French National Agency for AIDS Research (ANRS).

I cordially thank **Dr. Christophe Créminon** and **Dr. Christophe Junot** for the opportunity they gave me to work in the Pharmacology and Immune-analysis Service at CEA.

I also thank my thesis supervisor, **Dr. Aloise Mabondzo**. Thank you for the interesting research project and for giving me the opportunity to conduct my thesis work in one of the leading research institutes in Europe; it is a once in a lifetime opportunity!

I extend my deep gratitude to my committee member, **Dr. Anne-Sophie Villégier** for sharing her knowledge and expertise throughout our collaboration, and for providing a cordial atmosphere in her laboratory at INERIS. I greatly enjoyed working with you!

My gratitude and thanks also go to the members of my defense committee **Pr. Saadia Kerdine-Römer, Pr. Reina Bendayan, Pr. Xavier Declèves and Dr. Jean-François Ghersi-Igea**.

Last but not least, my profound gratitude goes to the SPI members, specifically to **Mme Sophie Cholet, Mme Sandrine Blondel, Mme Armelle Martelet, Mme Florence Vizet, Dr. Ying Xu, Dr. Dhouha Darghouth, Dr. François Fenaille, Dr. François Becher, Dr. Emilie Brun, Dr. Henri Bénech**, and to my friends **Rana El Maiss** and **Attorney Maya Dghaidi**. Thank you all so much for believing in me and for all your encouragement, generosity and support.

Table of contents

| | |
|--|-----------|
| INTRODUCTION..... | 15 |
| 2.1 PHYSIOLOGICAL FUNCTION OF THE BLOOD-BRAIN BARRIER | 19 |
| 2.2 THE MATURE BLOOD-BRAIN BARRIER: GENERAL CONSIDERATIONS | 19 |
| 2.2.1 BASEMENT MEMBRANE..... | 22 |
| 2.2.2 PERICYTES | 22 |
| 2.2.3 ASTROCYTES..... | 23 |
| 2.2.4 <i>Neurons</i> | 23 |
| 2.2.5 BRAIN MICROVASCULAR ENDOTHELIAL CELLS..... | 23 |
| 2.2.5.1 INTER-ENDOTHELIAL JUNCTIONS..... | 24 |
| 2.2.5.1.1 TIGHT JUNCTIONS | 25 |
| 2.2.5.1.2 ADHERENCE JUNCTIONS | 29 |
| 2.2.5.2 DRUG TRANSPORTERS | 30 |
| 2.2.5.2.1 ABC TRANSPORTERS | 30 |
| 2.2.5.2.1.1 ABC B FAMILY | 31 |
| 2.2.5.2.1.2 ABC C FAMILY | 34 |
| 2.2.5.2.1.3 ABCG FAMILY | 35 |
| 2.2.5.2.2 SLC TRANSPORTERS | 37 |
| 2.2.5.2.2.1 SLC22A8/OAT3 | 39 |
| 2.2.5.2.2.2 SLCO1A4 /OATP1A4..... | 40 |
| 3.1 DEVELOPMENT OF THE BLOOD-BRAIN BARRIER..... | 42 |
| 3.1.1 VASCULOGENESIS PHASE | 42 |
| 3.1.2 ANGIOGENESIS PHASE: | 43 |
| 3.1.3 DIFFERENTIATION PHASE | 44 |
| 3.1.4 MATURATION PHASE | 45 |
| 3.1.5 THE ROLE OF WNT SIGNALING IN BBB DEVELOPMENT | 46 |
| 3.1.5.1 GENERAL ASPECT OF THE WNT CANONICAL SIGNALING | 46 |
| 3.1.5.2 WNT SIGNALING DURING ANGIOGENESIS | 47 |
| 3.1.5.3 WNT SIGNALING DURING DIFFERENTIATION..... | 48 |
| 3.1.5.4 WNT SIGNALING DURING MATURATION AND MAINTENANCE..... | 49 |
| 3.2 COMPONENTS OF THE DEVELOPMENTAL BLOOD-BRAIN BARRIER | 49 |
| 3.1.6 STRUCTURAL COMPONENTS | 50 |
| 3.1.7 DRUG TRANSPORTERS..... | 50 |
| 4.1 BBB AND PATHOLOGICAL DISEASES..... | 52 |
| 4.2 CNS INFLAMMATION | 55 |
| 4.2.1 MECHANISM OF INFLAMMATION | 56 |
| 4.2.2 MECHANISM OF INFLAMMATION IN IMMATURE BRAIN | 57 |
| 4.3 THE IMPACT OF CYTOKINES ON BBB COMPONENTS..... | 59 |
| 4.3.1 IL-1A..... | 60 |
| 4.3.2 IL-1B..... | 60 |
| 4.3.3 IL-6..... | 61 |
| 4.3.4 ENDOTHELIN-1..... | 62 |
| 4.4 THE IMPACT OF CHEMOKINES ON BBB COMPONENTS | 62 |
| 4.4.1 MCP-1 | 63 |

| | |
|---|------------|
| OBJECTIVES | 66 |
| RESULTS AND DISCUSSION..... | 70 |
| CHAPTER IA: DESCRIPTIVE BLOOD-BRAIN BARRIER ONTOGENESIS STUDY | 72 |
| CHAPTER IB: UNDERSTANDING THE MOLECULAR MECHANISMS OF BLOOD-BRAIN BARRIER ONTOGENESIS | 81 |
| CHAPTER II: REGULATION OF P-GLYCOPROTEIN AND BCRP EXPRESSION AND TRANSPORT ACTIVITY AT THE BLOOD-BRAIN BARRIER BY CEREBRAL INFLAMMATION TRIGGERED BY ENDOTHELIN-1..... | 88 |
| CHAPTER III: RESEARCH TOOLS..... | 98 |
| CONCLUSIONS & PERSPECTIVES..... | 122 |
| 1 DEVELOPMENT OF RESEARCH TOOLS: CONCLUSIONS AND PERSPECTIVES | 123 |
| 2 BLOOD-BRAIN BARRIER DESCRIPTIVE STUDY: CONCLUSIONS AND PERSPECTIVES...125 | |
| 3 UNDERSTANDING THE MOLECULAR MECHANISMS OF BBB ONTOGENESIS: CONCLUSIONS AND PERSPECTIVES..... | 127 |
| 4 COMPARATIVE STUDY OF THE IMPACT OF INFLAMMATION ON BBB DRUG EFFLUX TRANSPORTERS: CONCLUSIONS AND PERSPECTIVES..... | 128 |
| REFERENCES | 131 |

Abbreviations

- ABC:** Adenosine triphosphate-Binding Cassette
- AJ:** Adherent Junctions
- Ang:** Angiopoietin
- AP-1:** Activator Protein-1
- APC:** Adenomatosis Polyposis Coli
- AQP:** AQuaPorin
- ASD:** Autism Spectrum Diseases
- ATF-1:** Cyclic AMP-dependent Transcription Factor-1
- A β :** amyloid- β
- BBB:** Blood-Brain Barrier
- BCRP:** Breast Cancer Resistance Protein
- BIO:** 6-Bromo-Indirubin-3'Oxime
- BM:** Basement or Basal Membrane
- BMVECs:** Brain Microvascular Endothelial Cells
- cAMP:** Cyclic Adenosine MonoPhosphate
- CAR:** Constitutive Androstane Receptor
- CCL:** CC Chemokine Ligand
- CD31:** Cluster of Differentiation 31
- CNS:** Central Nervous System
- CRE:** cAMP Response Element.
- CREB-1:** cAMP Responsive Element Binding protein-1
- CSF:** CerebroSpinal Fluid
- DEP:** Diesel Exhaust Particles

ECM: Extracellular matrix

EGF: Epidermal Growth Factor

EM: Electron Microscopy

ER: Estrogen Receptors

ERE: Estrogen Response Element

ERK: Extracellular Regulated Kinase

ET-1: Endothelin-1

FcRn: neonatal Fc Receptor

Fzd: Frizzled homolog

GFAP: Glial Fibrillary Acid Protein

Glut-1: Glucose Transporter 1

GPR: orphan G Protein-coupled Receptor

GR: Glucocorticoid Receptor

GSK-3 β : Glycogen Synthase Kinase-3 β

hEGF: human recombinant Epidermal Growth Factor

HIV-1: Human Immunodeficiency Virus-1

HNF: Hepatocyte Nuclear Factor

Id: Inhibitor of Differentiation proteins

IFN: Interferon

IL: InterLeukin

INSR: INSulin Receptor

JAM: Junction-Associated Molecules

JNK /SAPK: c -Jun N-terminal Kinase / Stress-Activated Protein Kinase

LEF-1: Lymphoid Enhancer Factor-1

LIF: Leukemia Inhibitory Factor

LPS: LipoPolySaccharide

LRP: low-density Lipoprotein Receptor-related Protein

MAGI: Membrane-Associated Guanylate kinase Inverted

MAGUK: Membrane-Associated Guanylate Kinase

MAPK kinase: Mitogene-Activated PhosphoKinase

MCAF: Macrophage Chemoattractant and Activating Factor

MCP-1: Monocyte Chemotactic Protein-1

MDR: MultiDrug Resistance

MHCII: low Major Histocompatibility Complex class II

MIP-1 α : Macrophage Inflammatory Protein-1 α

MRP: Multidrug Resistance-associated Proteins

MW: Molecular Weight

NADPH: oxidase Nicotinamide Adenine Dinucleotide Phosphate-oxidase

NF- κ B: Nuclear factor-*kappa B*

NK: Natural killer

NO: Nitric Oxide

NOS: Nitric Oxide Synthase

NPCs: Neural Precursor Cells

NVU: NeuroVascular Unit

OAT: Organic Anion Transporters

OATP: Organic Anion Transporting Polypeptides

OSM: cytokine OncoStatin M

PAF: Platelet Activating Factor

PDGF-BB: Platelet Derived Growth Factor B

PECAM-1: Platelet Endothelial Cell Adhesion Molecule

P-gp: Permeability-glycoprotein

PKC: Protein Kinase C

PNVP: PeriNeural Vascular Plexus

PXR: Pregnane X Receptor

ROS: Reactive Oxygen Species

RT-PCR: Real-Time Polymerase Chain Reaction

SLC: SoLute carrier

Src: Sarcoma

SSeCKS: Src-Suppressed C-Kinase Substrate

TCF: T-Cell Factor

TEER: TransEndothelial Electrical Resistance

TFR: Transferrin

TGF β : Transforming Growth Factor beta

TJ: Tight Junctions

TLR-4: Toll-like receptor-4

TNF- α : Tumor Necrosis Factor- α

VE-cadherin: Vascular Endothelial-cadherin

VEGF: Vascular Endothelial Growth Factor

WHO: World Health Organization

ZO: Zonula Occludens

List of figures

Introduction

- Figure 1.** Timeline of the Blood-Brain Barrier research in adult and immature brain
- Figure 2.** The blood-brain barrier – A microvascular network
- Figure 3.** Morphology of brain microvessels
- Figure 4.** Structure of BBB tight junctions
- Figure 5.** Schematic diagram of the efflux transport mechanisms at the blood-brain barrier
- Figure 6.** BBB angiogenesis
- Figure 7.** BBB differentiation
- Figure 8.** The mature BBB
- Figure 9.** The Wnt/ β -catenin signaling pathway
- Figure 10.** Wnt signaling and TJ
- Figure 11.** Phases of inflammation in the immature brain after an acute hypoxic–ischemic or excitotoxic insult

Results and discussion

- Figure 12.** Relative mRNA expression of TJ components in isolated BMVs during brain maturation
- Figure 13.** Relative mRNA expression of ABCC transporters in isolated BMVs during brain maturation
- Figure 14.** Relative mRNA expression of SLC transporters in isolated BMVs during brain maturation
- Figure 15.** Regulation of P-gp and bcrp activity at the BBB level through activation of β -catenin *in vivo*.
- Figure 16.** Illustration of the experimental design

Figure 17. Relative mRNA expression of ETA in isolated BMVs during brain maturation

Figure 18. Relative mRNA expression of BBB TJ components in isolated BMVs

Figure 19. Relative mRNA expression of ABC transporters in isolated BMVs

Figure 20. Relative mRNA expression of SLC transporters in isolated BMVs

Figure 21. Relative mRNA expression of BBB receptors in isolated BMVs

Figure 22. Schematic illustration of the *in vitro* BBB model

Figure 23. Juvenile rat (P21) BMVECs two days after isolation (passage 0) observed in phase contrast microscope

Figure 24. Adult rat (P84) BMVECs two days after isolation (passage 0) observed in phase contrast microscope

Figure 25. Juvenile rat (P21) BMVECs twelve days after isolation (passage 0) observed in phase contrast microscope.

Figure 26. Adult rat (P84) BMVECs twelve days after isolation (passage 0) observed in phase contrast microscope

Figure 27. Fetal rat (P2) astrocytes, one day after seeding (passage 1) observed in phase contrast microscope

Figure 28. Fetal rat (P2) astrocytes, three (A) or twelve (B) days after seeding (passage 1) observed in phase contrast microscope

Figure 29. Illustration of experimental design

Figure 30. ET1 structure (Masaki, 2004)

Figure 31. Passage of baclofen across the BBB after ET-1 treatment in adult rats

Figure 32. Effect of ET-1 on glial activation in adult and juvenile brains

Figure 33. IL-1 β levels in adult brain after a single intracerebral injection of endothelin-1 for 24 h.

List of tables

Table 1. Abcb1 substrates

Table 2. Abcc transporters

Table 3. Alterations of BBB components in adult CNS diseases

Table 4. Alterations of BBB components in children CNS diseases

Table 5. BBB transporters

Foreword

Central Nervous System (CNS) disorders are disabling diseases affecting up to one billion people worldwide regardless of age. According to the World Health Organization (WHO), these disorders are the major medical challenge of the 21st century, because effective treatments for most of them aren't yet available, neither for children nor for adults (Neurological disorders: public health challenge, 2006).

One major reason of CNS pharmacotherapy's impediment is the existence of "barriers" between blood and CNS, especially the Blood-Brain Barrier (BBB), a neurovascular structure localized at the level of brain microvasculature. The BBB's major functions are protection of the CNS and maintenance of its homeostasis. But as the BBB ensures CNS tight protection, it strictly limits the entry of therapeutic drugs from the blood into the brain, which leads to treatment failure.

This barrier function is mainly due to: 1) the presence of Tight Junctions (TJ) between endothelial cells of the Brain MicroVessels (BMVs) which seal the spaces between neighboring cells and limit paracellular diffusion of substances, and 2) the numerous influx/efflux transport and carrier systems, expressed at the luminal and abluminal surface of the NeuroVascular Unit (NVU) that extrude neurotoxic substances, but also therapeutic drugs and prevent them from entering the brain and reaching their site of action.

To overcome drug efflux transporters, recent research in the field aims at identifying the intracellular signaling mechanisms that regulate these transporters in order to modulate their activity and improve pharmacotherapy of brain diseases.

For years, these transporters have been studied in adult organism. But, there is a wide spread belief among pediatricians, neurologists and neuroscientists that the BBB in the embryo, fetus, new born and infant is "immature", implying caution in giving drugs to infants (Saunders et al., 2012). However, current knowledge on the functional status of the BBB in immature organism remains very limited. Thus, from the clinical perspective of developing new drugs with enhanced efficacy in children's CNS, it is important to understand 1) the role of the BBB drug efflux transporters in children's brain and 2) the mechanisms that regulate their functional expression under normal and pathological conditions, mostly under inflammatory conditions,

because alterations in structural and functional components of the NeuroVascular Unit (NVU) have been reported in a long list of CNS pathologies, both in children and adults (see reviews: Stolp and Dziegielewska, 2009; Zlokovic, 2008).

The following introduction is divided into five chapters:

- The first chapter gives a brief historical background of BBB researches and localizes our project on these researches' timeline.
- The second chapter summarizes current knowledge on the mature BBB, its physiological function and components with an emphasis on their importance for the barrier properties, as well as mechanisms regulating their expression and function.
- The third chapter summarizes current knowledge on the developmental BBB, its ontogenesis and the suggested mechanisms.
- In the fourth part, I give a comprehensive review of the literature concerning BBB alterations and cerebral inflammation during CNS diseases. Indeed, BBB components are altered during almost all brain disorders. In all these pathologies, the CNS exhibit inflammatory features. There are growing evidences suggesting that the immune system, either through systemic or local inflammation, disturbs the brain barrier systems. This part summarizes current knowledge on cerebral inflammatory mechanisms during CNS diseases, with a focus on BBB alterations and more particularly those of drug efflux transporters during cerebral inflammation at different stages of brain development.
- The fifth chapter highlights the importance of our study.

INTRODUCTION

Chapter I

State of the art:

The situation of our project on the BBB researches' timeline

Our current understanding of the BBB is the result of 130 years of researches (Figure 1). In the 1880s, Paul Ehrlich, a German physician and scientist, discovered that the injection of certain dyes into the vascular system colors all organs except the brain (Ehrlich, 1885). Ehrlich interpreted these findings as a lack of dye affinity to the nervous system (Ehrlich, 1904). In 1900, M. Lewandowsky, a Berlin physician, discovered that the injection of Potassium Ferrocyanate, directly into the CNS was lethal at low doses, but when the product is injected systematically, 100-fold higher doses were required to obtain the same effect (Lewandowsky, 1990). This study led to the concept of a barrier's existence between the blood and the CNS, and the term *bluthirnschranke* (blood-brain barrier) was coined. Shortly after that, experiments by Ehrlich's student, Edwin E. Goldman, demonstrated that the injection of the same dyes used by Ehrlich directly into the cerebrospinal fluid stained the brain but not any other tissues (Goldmann, 1909; Goldmann, 1913). This suggests in turn, the existence of a physical barrier between the brain and the blood. These experiments performed in adults were soon followed by similar ones using embryos or newborns from various species. In 1917, Weed showed that the sodium ferrocyanide when injected into the neural tube of pig embryos was confined to the neural tube and did not penetrate out into rest of the embryo (Weed, 1917). Then several experiments using trypan blue were performed on the BBB in the developing brain from several species (rats, rabbits, pigs, human) and gave the same result as in adults (Gröntoft, 1954; Stern et al., 1929; Stern and Rapoport, 1928; Stern and Peyrot, 1927; Wislocki, 1920). In spite of the above studies clearly showing lack of penetration of small molecular compounds even into an embryonic brain, numerous reports claiming an absence or immaturity of the BBB in the fetus and neonate continue to be published (see Ek et al., 2012 for review; Sanders et al., 2012).

In conjunction to works on BBB in the developing brain, works on the adult barrier have succeeded, but the nature of this barrier, and even its existence, continued to be debated until the 1960s, with the advancement of Electron Microscopy (EM). In the late 1960s, studies done by

Reese, Karnovsky, and Brightman using EM, revealed that the structural base of the BBB is localized at the level of Brain MicroVessels (BMVs) and is formed because of the existence of TJ between adjacent Endothelial Cells (EC) of the BMVs that seal the space between neighboring cells (Reese and Karnovsky, 1967; Brightman and Reese, 1969). In the following years, *in vivo*, *ex vivo* and *in vitro* BBB models have been developed and have allowed a better understanding of the barrier physiology. Saikotos et al. were the first to report the isolation of BMVs in 1969 (Saikotos, 1969). In 1980, Betz et al. established an *in vitro* BBB model system (Betz et al., 1980). Since then, various *in vivo*, *ex vivo* and *in vitro* BBB models have been used in basic research (Lacombe et al., 2011; Hembury and Mabondzo, 2008; Miller et al., 2000; Abbott et al., 1992; Audus et al., 1990; Pardridge et al., 1990b; Pardridge et al., 1985; Goldstein et al., 1984).

The expanded studies on the BBB lead to a change in the concept of the BBB as an impermeable barrier. Now the BBB is described as a Neuro-Vascular Unit (NVU) filtering the passage of molecules at the Blood-brain interface because of a combination of “physical” (tight junctions), “transport” (transporters grouped into families based on molecular, genomic and protein sequence) and “metabolic” (enzymes metabolizing molecules in transit) processes. Moreover, the concept of the BBB as a static element has also changed, and now it has become very clear that the NVU components are regulated in both physiological and pathological conditions. Indeed, virtually all CNS degenerative disorders document BBB alterations in the disease process (see reviews: Zlokovic, 2008; Miller, 2010). Recent researches are focused on the role of these alterations in the CNS diseases’ process, and the determination of whether these alterations are a cause or a consequence of the disease. Nowadays, the BBB is considered to be the major obstacle to CNS entry of therapeutic drugs and is thus seen as the molecular basis for preclinical and clinical drug failure. However, even today, well with the expansion of genomics and proteomics innovations, overcoming the blood-brain barrier to treat disorders of the CNS system is still a challenge.

Recent researches in the field aim at a better understanding of the mechanisms that regulate structural and functional components of the BBB, under physiological and pathological conditions, in order to improve drug therapy of CNS disorders. And very recently, an increasing number of researches are focused on understanding the molecular mechanisms underlying development and maintenance of BBB as well as its function and regulation in immature brain

(Saunders et al., 2012; Grant et al., 1998; Staddon et al., 1995; Risau and Wolburg, 1990), in the perspective of improving drug therapy of CNS disorders in children. Indeed, now, it is believed that there are several fetal-specific mechanisms that have only recently begun to be described and are different from those in adults (e.g., fetal hemoglobin, Palis et al., 2010). These differences reflect adaptation to a fetal-specific environment rather than deficiencies compared to adult brain. However, at the BBB level, scientists emphasize the importance to consider evidence that BBB mechanisms, even if functionally effective in the developing brain, may be more susceptible to adverse circumstances than in adults and that damage to brain barrier mechanisms' during development may lead to neurological and neuropsychological dysfunction in later life (Saunders et al., 2012). Our work fits in this approach.

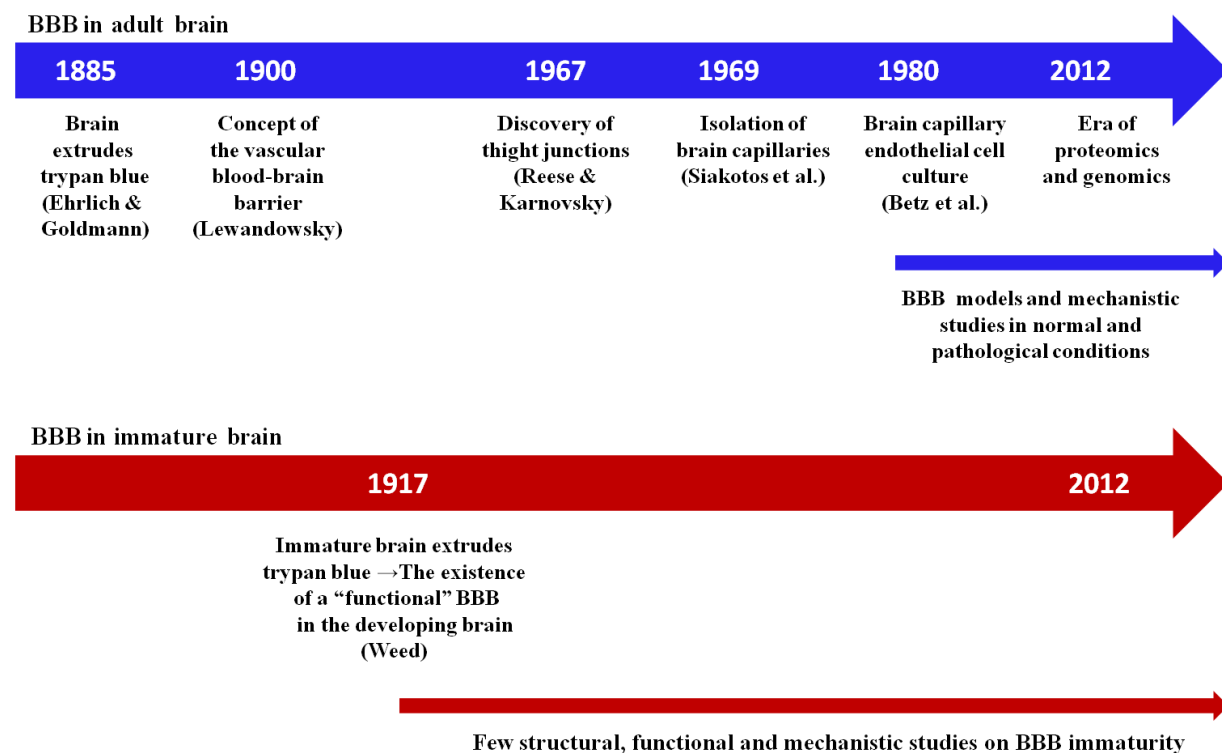


Figure 1. Timeline of the Blood-Brain Barrier research in adult and immature brain
(Timeline for adult BBB was adapted from Hartz, 2005)

Chapter II

The mature BBB

2.1 Physiological Function of the Blood-Brain Barrier

The BBB is by far the largest surface area of exchange between blood and brain (Pardridge, 2003). Its major functions are protection of CNS and maintenance of its homeostasis. The BBB protects the brain from many endo- or exogenous compounds circulating in the blood and potentially neurotoxic (like endogenous metabolites or proteins, xenobiotics ingested in the diet or acquired from the environment, etc.). It also isolates the brain from fluctuations in nutrients, hormones, metabolites, and other blood constituents (Bernacki et al., 2008). In addition of being a barrier to potential harmful molecules, the BBB maintains cerebral homeostasis required for proper CNS functions (Hawkins and Davis, 2005). In other terms, the BBB functions as a dynamic regulator of the high nutrients and energy demand of the brain by regulating ion balance and facilitating nutrient transport, and at the same time by eliminating metabolic wastes from the CNS, as well as protecting from potentially harmful molecules. Thus, the BBB is a “complex and fine-tuned transport machine that balances influx of nutrients and efflux of wastes, toxins, and drugs to maintain CNS homeostasis” (Hartz and Bauer, 2011).

2.2 The mature blood-brain barrier: General considerations

Brain MicroVessels (BMVs) are the structural basis of the BBB (Figure 2).

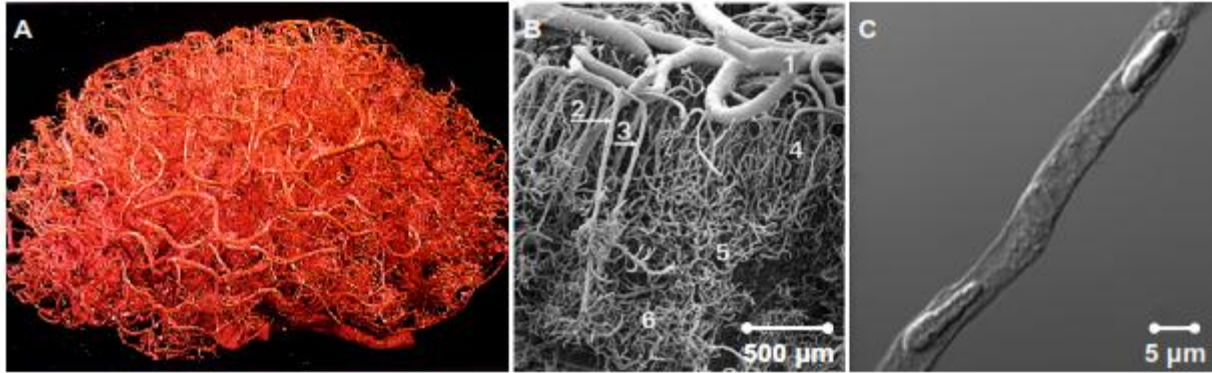


Figure 2. The blood-brain barrier – A microvascular network

A: Latex mold of the microvascular network of an adult human brain, from (Zlokovic and Apuzzo, 1998)

B: Scanning electron micrograph showing human cerebral cortex: (1) pial vessels, (2) long and (3) middle cortical arteries, (4) superficial, (5) middle and (6) deep capillary zone, from (Rodriguez-Baeza et al., 2003)

C: Differential interference contrast image of an isolated rat brain capillary (taken from the dissertation of Anika Hartz, 2005)

At the cellular level, the basic structural and functional unit of the mature and fully differentiated BBB is a NVU (Figure 3) composed of 4 cell types: endothelial cells of the brain microvessels (Brain Microvascular Endothelial Cells (BMVEC)), strictly associated to each other by TJ; pericytes, sharing with BMVEC a basal membrane; astrocytes, surrounding with their endfeet the brain capillaries; and neurons, innervating the microvasculature (Hartz and Bauer, 2011).

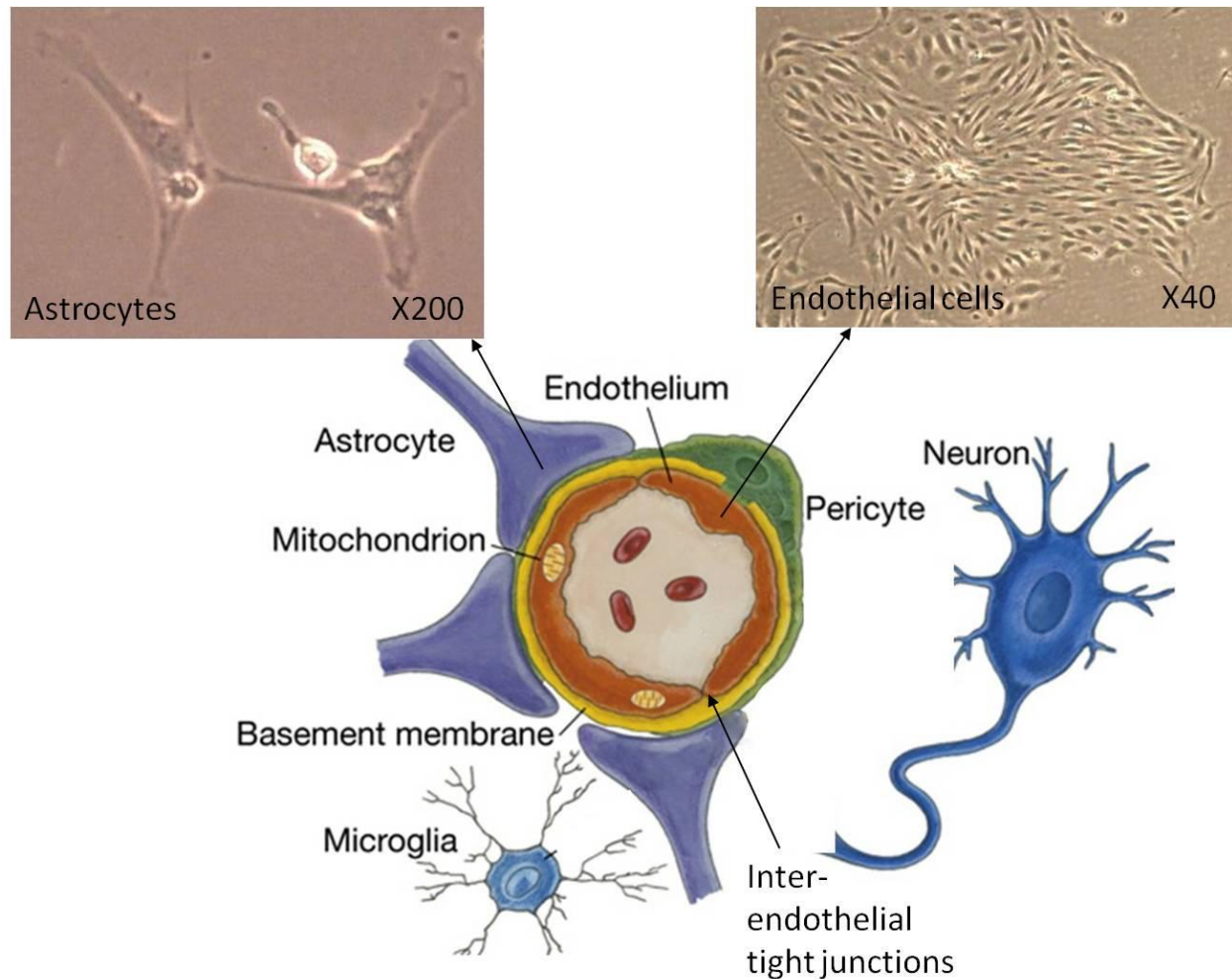


Figure 3. Morphology of the neuro-vascular unit

The neuro-vascular unit of the BBB is formed by endothelial cells of BMV, pericytes, a basement membrane, astrocytes and neurons. The inter-endothelial space is sealed by TJ. Phase contrast microscopic images of primary astrocytes (Magnification X200) and endothelial cells (Magnification X40) forming the blood-brain barrier (Adapted from Zlokovic, 2008).

The key element controlling the BBB permeability are the BMVECs (Pardridge, 1999), but interactions with other constituents of the neurovascular unit are essential for barrier function (Cardoso et al., 2010; Choi and Kim, 2008; Kaur and Ling, 2008; Zlokovic, 2008; Zozulya et al., 2008; Persidsky et al., 2006; Hawkins and Davis, 2005; Wolburg and Lippoldt, 2002). At the molecular level, several factors contribute to the unique functional role of the NVU: 1) The presence of TJ associating neighboring endothelial cells of BMV. These TJ seal the spaces between endothelial cells and limit paracellular diffusion into the brain of potentially neurotoxic substances carried in the blood. 2) The expression and polarization of a number of influx/efflux transport and carrier systems on the luminal and abluminal surface of the NVU to overcome TJ

and to meet the high nutrient and energy demand of the brain. Among these systems, ATP-binding cassette (ABC) and SoLute carrier (SLC) transporters facilitate brain uptake of nutrients (e.g. glucose, amino acids and ions) from the blood, extrude metabolic wastes and keep neurotoxic substances from entering the brain. But these efflux transporters also keep therapeutic drugs from entering the brain and reaching their site of action, which leads to treatment failure of several brain diseases (Hartz and Bauer, 2011). A chapter below is devoted to ABC and SLC transporters.

2.2.1 Basement membrane

The Basement or Basal Membrane (BM) is the acellular component of the NVU. In scanning electron microscopy, this basement membrane appears as thin, tightly interwoven protein sheets of 20–200 nm thickness (Engelhardt B and Sorokin, 2009; Timpl, 1989). It is constituted of three laminas containing different classes of extracellular matrix molecules (Weiss et al., 2009; Persidsky et al., 2006) including collagen type IV, elastin, fibronectin, laminin, heparan sulfate proteoglycans, nidogens cell adhesion molecules, signaling molecules, etc. (Carvey et al., 2009; Wolburg et al., 2009; Adibhatla and Hatcher, 2008). It also contains minor components such as BM40 (osteonectin); fibulin 1 and 2; collagen types VIII, XV, and XVIII; and thrombospondin 1 and 2 (Engelhardt B and Sorokin, 2009; hallman et al., 2009; Timpl, 1989). The BM, although understudied, is an important element of the NVU; it ensures the anchorage of the NVU in place and established the connection with the surrounding resident cells (Carvey et al., 2009). Its disruption has been reported in several pathological conditions and was associated with an increased BBB permeability (Carvey et al., 2009; Zlokovic, 2008; Hawkins and Davis, 2005). In effect, the BM disruption can alter the cytoskeleton of the BMVEC which causes modifications in TJ and thus alters the barrier integrity (Cardoso et al., 2010; Carvey et al., 2009).

2.2.2 Pericytes

Pericytes (also known as vascular smooth muscle cells, mural cells, or myofibroblasts) are important cellular constituents of the NVU (Cardoso et al., 2010; Dore-Duffy, 2008). They are small vessel wall-associated cells (Katychev et al., 2003) that sit on the top of BMVEC and are completely surrounded by the BM (Liebner et al., 2011). In the NVU, pericytes synthesizes

growth factor and most BM components (Dore-Duffy et al., 2006) and their association with the BMVEC essential for BBB structural maintenance, junctional integrity (Cardoso et al., 2010; Braun et al., 2007; Lai and Kuo, 2005). It has also been suggested that pericytes participate to the regulation of EC proliferation, migration and differentiation (Cardoso et al., 2010; Persidsky et al., 2006a) and play an important role in the mediation of inflammation.

2.2.3 Astrocytes

Glial cells are star-like cells and constitute the most numerous cell classes in the CNS (Liebner et al., 2011). They have numerous physiological functions. For example, they are important for the proper neural function (Persidsky et al., 2006a) and they are involved in maintenance of ionic homeostasis, mediation of signals from neurons to the vasculature and uptake of neurotransmitter, providing metabolic support to neurons (Liebner et al., 2011; Sofroniew and Vinters, 2010). At the BBB level, astrocytes end-feet are tightly attach to neurons from on one side and to blood vessels on the other, connecting thereby neurons and blood (Bernacki et la., 2008) and playing decisive role in the maintenance of the barrier properties, especially those of the BMVEC (Liebner et al., 2011; Abbott et al., 2010). It is believed that they also control the cerebral blood flow (Abbott et al., 2010), and participate in the induction of the differentiation of the specialized BBB phenotype of the brain endothelium (Bauer HC and Bauer H, 2000; Janzer and Raff, 1987; Davson and Oldendorf, 1967).

2.2.4 Neurons

Some evidence show that neurons can regulate the function of blood vessels in response to metabolic requirements, and this by inducing expression of enzymes unique for BMVEC (Persidsky et al., 2006a). Reciprocally, mature endothelium induces a stable brain microenvironment that enables proper neuronal activity (Cardoso et al., 2010; Choi and Kim, 2008).

2.2.5 Brain microvascular endothelial cells

BMVEC are the key cellular component of the NVU creating the barrier (Ueno, 2007). They have specific phenotipocally features differing them from their homologue in the periphery; their

most important features are 1) the presence of tight junctions between neighboring cells that restrict the paracellular diffusion of hydrophilic solutes (due to these TJ, BMVEC are 50–100 times tighter than peripheral microvessels), 2) They lack pinocytic vacuoles, 3) They have high number of cytosolic mitochondria suggesting their high energy metabolism and 4) They lack fenestrations in their plasma membranes (Abbott, 2005; Ballabh et al., 2004; De Vries et al., 1997; Chaudhuri, 2000; and Bernacki et al., 2008). Due to these features, brain endothelium is the most critical element preventing the entry of toxic substance from blood to brain.

2.2.5.1 Inter-endothelial junctions

The BMVEC lining at the BBB are stabilized by specialized cell junctions between adjacent cells (Cardoso et al., 2010). At the BBB level, there are two types of inter-endothelial junctions: Tight Junctions (TJ) and Adherent Junctions (AJ) (Figure 4).

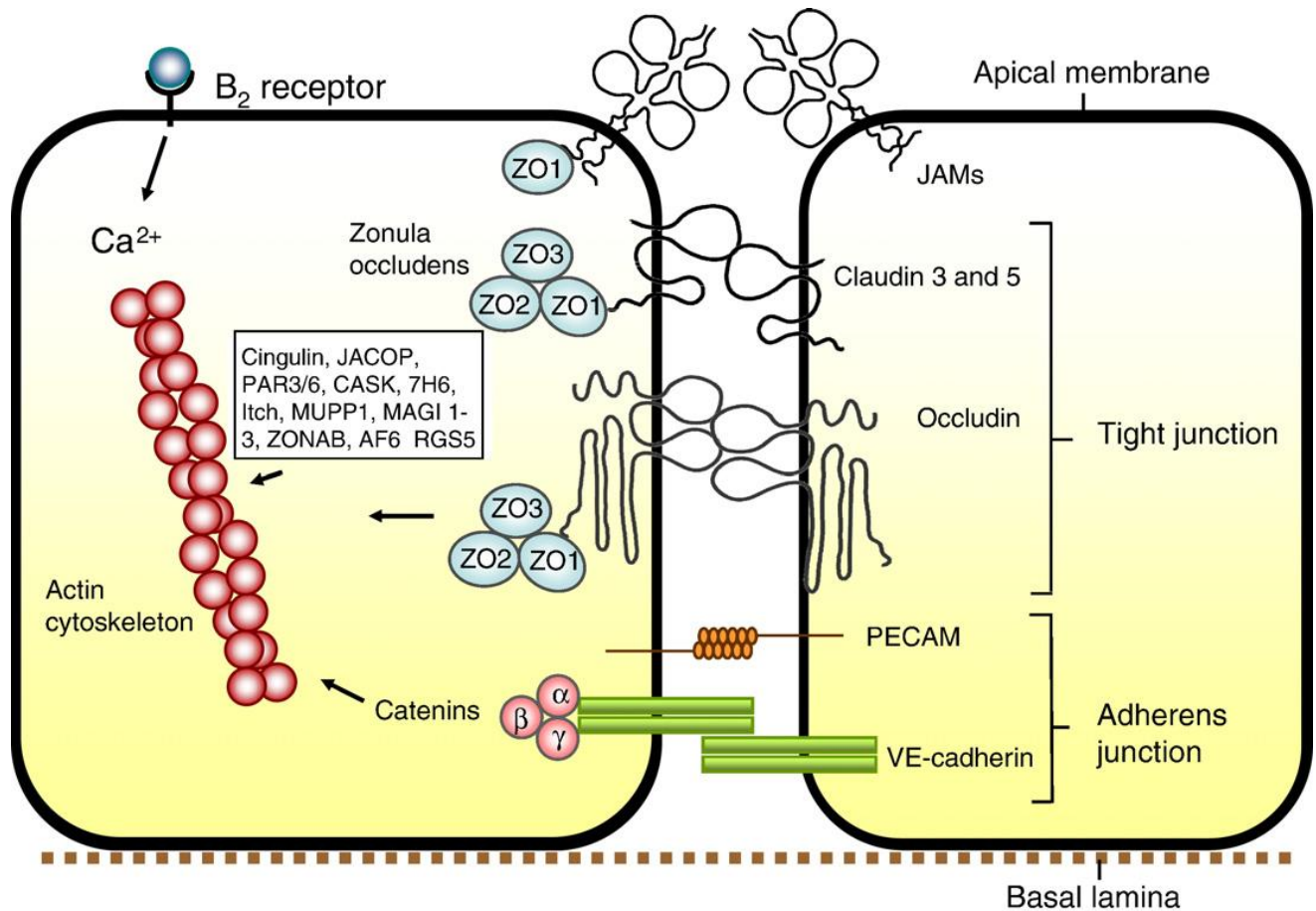


Figure 4. Structure of BBB inter-endothelial junctions

At the BBB level, there are two main types of inter-endothelial junctional complexes: tight junctions and adherens junctions. Tight junction complex comprises transmembrane proteins (occludin, claudins 3 and 5, possibly other claudins, and Junction-Associated Molecules (JAMs)) connected to the actin cytoskeleton via scaffolding proteins (Zonula-Occludens ZO1, ZO2, ZO3) linked in turn via cingulin dimers to the actin/myosin cytoskeletal system within the cell. Adherens junctions are located below the tight junctions and are composed of transmembrane proteins (cadherins) linked to the cytoskeleton by cytoplasmic proteins and giving place an adhesion belt (figure from Abbott et al., 2010 and adapted from Begley, 2007).

2.2.5.1.1 Tight junctions

In 1967 and 1969, the framework of Reese and Karnovsky (Reese and Karnovsky, 1967) and Brightman and Reese (Brightman and Reese, 1969) identified inter-endothelial tight junctions as the main structures responsible for the barrier properties. TJ are contacts between neighboring cerebral endothelial cells, located on the apical side of BMVEC. They seal the space between adjacent cells allowing only limited passage of water, ions and small molecules but keeping other substance from taking the paracellular pathway to enter the brain. Structurally, TJ are composed of transmembrane proteins (like occludin, claudins and junctional adhesion molecules

(JAMs)) and a cytoplasmic “plaque” of accessory and regulatory proteins (Vorbrodts and Dobrogowska, 2004; Vorbrodts and Dobrogowska, 2003;) connected to the actin cytoskeleton. Transmembrane proteins are integral membrane proteins that interact with those of neighboring plasmamembrane and form the TJ barrier. They include proteins like occludin, claudins and Junctional Adhesion Molecules (JAMs). The accessory proteins include proteins like Zonula Occludens (ZO), cingulin, 7H6 and AF-6, Membrane-Associated Guanylate kinase Inverted (MAGI) proteins, etc. These accessory or scaffolding proteins connect the junctional transmembrane proteins to actin filaments of the cytoskeleton (Weiss et al., 2009; Persidsky et al., 2006a; Lee et al., 2004; Matter and Balda, 2003b; Petty and Lo, 2002). They also recruit to the TJ several signaling proteins and transcription factors (Terry et al., 2010). The regulatory proteins include G proteins, atypical isoforms of Protein Kinase C (aPKC), symplektin, etc. Indeed, more than 40 proteins, including transmembrane, scaffolding and signaling proteins, have been found to be associated with TJ (González-Mariscal et al., 2003), forming multimeric protein complexes that receive and send regulatory signals (Cardoso et al., 2010; Terry et al., 2010) and involved in signaling to and from TJ (Matter and Balda, 2003b; Wolburg and Lippoldt, 2002).

Claudin

Claudins are 22 kDa integral membrane phosphoproteins family consisting of four transmembrane domains. It is believed that claudins are the primary constructive element of TJs and responsible for permeability restriction (Tsukita et al., 2001). Indeed, claudins overexpression can induce cell aggregation and formation of TJ-like structures (Yamamoto et al., 2008). Recent studies point a role of claudin family proteins in response to cellular stress (Romanitan et al., 2009), and in embryonic morphogenesis regulation (Gupta and Ryan, 2010). 24 members of this TJ protein family have been identified to date. In mammalian BMVEC, it has been reported the presence of claudin-1, -2,-3, -5, -11, -12 and -18 (Romanitan et al., 2009; Krause et al., 2008; Mahajan et al., 2008; Neuhaus et al., 2008; Bélanger et al., 2007; Petty and Lo, 2002). However, the presence of claudin-1 is still controversial (Lee et al., 2003). Claudin-5 is one of the most studied member of TJ at the BBB level; it has been shown to be expressed particularly in BMVECs (Sirotkin et al., 1997) and several important role have been assigned to this member in the NVU: 1) maintenance of normal BBB function (Vorbrodts and Dobrogowska,

2003) supported by the fact that down-regulation of claudin-5 expression correlated with breakdown of the BBB (in an experimental mouse model of autoimmune encephalomyelitis (Argaw et al., 2009), 2) an important role during angiogenesis and in disease processes (in conjunction with increased vessel permeability) (Wolburg and Lippoldt, 2002). Besides the endothelial cell-specific *cldn5*, *cldn3* has been shown to localize to endothelial TJ in primary human, rat and murine brain endothelial TJs generated (Mahajni et al., 2008; Coisne et al., 2005; Perriere et al., 2005). The loss of claudin-3 from the TJs in experimental allergic encephalomyelitis demonstrates that it may be important for determining permeability in pathological conditions (Wolburg et al., 2003). However, *cldn3* is specifically upregulated during BBB establishment and maintenance although the specific function of *cld3* for BBB TJs remains elusive (Liebner and Plate, 2010).

Occludin

Occludin is a 60 kDa phosphoprotein consisting as claudins of four transmembrane domains (Balda et al., 2000). Although overexpression of claudins is sufficient to induce TJ barrier properties, occludin is not essential for the formation of TJ (Hawkins and Davis, 2005). Indeed, results of several knockout and knockdown experiments indicate that loss of occludin can be compensated by normal expression and localization of other junctional proteins (Zlokovic, 2008). However, it has been suggested that occludins act as an additional support structure with regulatory functions (Hirase et al., 1997) that maintain electrical resistance and aqueous pore formation (Tsukita et al., 2001). In effect, high levels of occludin ensure decreased paracellular permeability (Huber et al., 2001) and high electrical resistance of the BMVEC monolayers (Persidsky et al., 2006a), which underlines an active role of occludin in BBB function (Yamamoto et al., 2008).

Junctional adhesion molecules (JAMs)

JAMs are 40 kDa immunoglobulins. In rodents' brain, three members of this family have been identified: JAM-1, JAM-2, and JAM-3. And it was demonstrated that JAM-1 and JAM-3 are localized at brain vessels (Vorbordt and Dobrogowska, 2004). Although the functions of JAM are still largely unknown in mature BBB, it has been suggested these proteins may play a role in

cell adhesion and monocyte migration through the BBB (Palmeri et al., 2000). It has also been showed that altered expression of JAM-1 affect the junctional tightness (Zlokovic, 2008).

Cytoplasmic proteins

Claudin and occludin transmembrane proteins of the TJ are linked to the cytoskeleton via cytoplasmic scaffolding proteins including zonula occludens proteins (ZO-1, ZO-2, and ZO-3), cingulin, 7H6, AF6, etc.

ZO proteins belong to the family of Membrane-Associated GUanylate Kinase (MAGUK) proteins (Persidsky et al., 2006a).

ZO-1

ZO-1, the first TJ-associated protein identified and characterized, is a 220-kDa phosphoprotein (Persidsky et al., 2006a) located in the cytoplasmic side of the BMVEC plasma membranes (Vorbrot and Dobrogowska, 2003). They connect transmembranous TJ proteins with the actin cytoskeleton and thus act as a central organizer of the TJ complex (Erickson et al., 2007). Though it is also expressed in other cell types that do not form TJ, there is no TJ without ZO-1. Loss or dissociation of ZO-1 from the junctional complexes is associated with increased barrier permeability (Cardoso et al., 2010; Choi and Kim, 2008).

ZO-2

ZO-2 is a 160-kDa phosphoprotein. Very much like ZO-1, ZO-2 binds to transmembranous proteins of the TJ (Persidsky et al., 2006a).

ZO3

ZO-3 has also homology to ZO-1 and ZO-2. The complex formed by ZO-1, ZO-2 and ZO-3 is considered to be involved in cadherin-based cell adhesion through their binding to α -catenin and to actin filaments (Cardoso et al., 2010).

Cingulin, 7H6 and AF6

Other cytoplasmic proteins are cingulin, AF-6 and 7H6. Cingulin is a 140–160 kDa phosphoprotein localized at the cytoplasmic side of TJs. 7H6 is a 155 kDa phosphoprotein correlated with TJ impermeability to ions and large molecules (Satoh et al., 1996). It has been shown that cingulin binds to ZO proteins, myosin, and AF6 (180 kDa), and this binding play a

role as a scaffold between transmembrane proteins and the cytoskeleton (Bernacki et al., 2008; Cordenonsi et al., 1999).

2.2.5.1.2 Adherence junctions

Adherens Junctions (AJ) are components of inter-endothelial junctions located below TJs in the basal region of lateral plasma membrane. AJ are formed mainly by a transmembrane protein, the Vascular Endothelial (VE)-cadherin linked to β -catenin and plakoglobin, which in turn bind via β -catenin, α -actinin, and vinculin to the actin cytoskeleton (Dejana et al., 2009; Engelhardt and Sorokin, 2009). AJ form a continuous belt so-called “the adhesion belt” (Petty and Lo, 2002). AJ play an important role in the adhesion of BMVEC to each other and in the regulation of paracellular permeability (Hawkins and Davis, 2005; Carvey et al., 2009).

VE-cadherin

VE-Cadherin is a Ca^{2+} -regulated protein considered as the major transmembrane components of AJ and an important determinant of microvascular integrity. It contains a plasma membrane domain and a cytoplasmic domain associated with catenins (Vorbrot and Dobrogowska, 2003).

Catenin

Catenins, and more particularly α - and β -catenin are proteins located in interendothelial junctions of BMVECs and required for cadherins to work as adhesion molecules. Their major role is to connect the cadherin complex to the actin cytoskeleton, but they also participate in cell and developmental signaling pathways. β -catenin can also serve as a transcription factor downstream of Wnt/ β -catenin signaling beside its function as a junctional component. Indeed, it has been shown that specific stabilization of β -catenin in endothelial cells *in vivo* during embryonic and postnatal development enhances BBB maturation by inducing the expression of claudin-3; whereas inactivation of β -catenin caused downregulation of claudin-3 (Liebner et al., 2008).

2.2.5.2 Drug transporters

Thanks to TJs, only lipid-soluble substances and substances possessing a transportation system can cross the BBB (Bernacki et al., 2008; O’Kane et al., 1999; Brightman and Reese, 1969). To meet the high nutrient and energy demand of the brain, influx transporters facilitate brain uptake of glucose, amino acids, ions, and other nutrients from the blood. On the other hand, efflux transporters extrude metabolic wastes into the blood, and also limit the entry of toxins and pathogens into the brain. To make these functions possible, a number of transport and carrier systems are expressed and polarized on the luminal or abluminal surface of the BBB. Among these systems, Adenosine triphosphate-Binding Cassette (ABC) transporters play a critical role in preventing neurotoxic substances from entering the brain, and in transporting toxic metabolites out of the brain (Loscher and Potschka, 2005). The SoLute Carrier family (SLC), another major group of membrane transporter proteins (Hagenbuch and Meier, 2004), controls the cellular uptake and efflux of nutrients, neurotransmitters, metabolites, and toxins (Engelhardt, 2003). These transporters are over-studied because of their contribution to MultiDrug Resistance (MDR) phenomenon, which plays a crucial role in the failure of treatments for several brain diseases such as sclerosis pathogenesis (Kooij et al., 2011) or Human Immunodeficiency Virus-1 (HIV-1) infection diseases (Jorajuria et al., 2004; Bousquet et al., 2009). Indeed, ABC and SLC transporters prevent from entering the brain, in addition to toxic xenobiotics, a large number of drugs which leads to CNS therapy failure.

2.2.5.2.1 ABC transporters

Adenosine triphosphate-Binding Cassette or ABC transporters are transmembrane proteins that import nutrients and other molecules into cells, and exports toxins and drugs. Their transport processes are ATP-driven (active transport); they use the binding and hydrolysis of ATP to translocate lipid-soluble compounds across membranes (Rees et al., 2009). ABC transporters are expressed in different compartments of the healthy organisms, e.g. liver, kidney, gastrointestinal tract, brain and others. In the brain, they are localized on the luminal and abluminal plasma membrane of the BMVECs and prevent the entry of neurotoxicants and therapeutic compounds in the CNS. Together with the endothelial tight junctions, these transporters considerably contribute to the barrier function of the NVU.

ABC transporters belong to one of the largest protein superfamilies (Holland et al., 2003; Ames et al., 1992; Higgins et al., 1992) and are highly conserved between species (Dean et al., 1995). In humans, 50 ABC transporters are known. According to their structural similarities, they are classified in seven families, among them: ABCB (or MDR (Multidrug Resistance Protein)), ABCC (or MRP (Multidrug Resistance-associated Proteins)), ABCCA, ABCD and ABCG2 (Leslie et al., 2005; de Lange, 2004).

At the BBB level, ABC transporters of greatest significance for efflux transport are mainly: ABCB1 (or P-glycoprotein (Pgp)), ABCC1, 2, 4, 5 and 6, and ABCG2 (or Breast Cancer Resistance Protein (BRCP)) (Abbott et al., 2010; Dauchy et al., 2008; Kamiie et al., 2008; Begley, 2004b).

2.2.5.2.1.1 ABC B Family

ABCB1 (also known as P-gp or MDR1) is the most studied transporter in the CNS of the ABC B family. Only few reports exist on other members in the brain (such as ABCB4, 6, 7, 8, 10) despite their expression in BMVECs (showed in human, cow, pig, rat and mouse brain) (Hartz and Bauer, 2011).

Abcb1/mdr1/P-glycoprotein

Historical Background

In 1976, Juliano and Ling identified a glycoprotein efflux pump unique to mutant cells that displayed altered drug permeability. They named it Permeability-glycoprotein (P-glycoprotein or P-gp) (Hartz, 2005). This discovery had a great importance because it explained the phenomenon of multidrug resistance in tumors (Juliano and Ling, 1976). In the mid to late 1980s, ABCB1 was found to be physiologically expressed in normal excretory and barrier tissues such as liver, kidney and intestine (Thiebaut et al., 1987). In 1989, P-gp was the first ABC transporter to be detected in BMVECs of human BBB (Cordon-Cardo et al., 1989; Thiebaut et al., 1987). Subsequently, it was localized in BMVECs of several species, including cow, pig, rat, hamster, mouse, dogfish, killifish, monkey, dog, and cat (Pekcec et al., 2009; Schlachetzki et al., 2003; Miller et al., 2002; Tsai et al., 2002; Nobmann et al., 2001; Samoto et al., 1994; Jette et al., 1993; Cordon-Cardo et al., 1989). Over the last decade, P-gp has been a main focus of BBB research.

Now it is believed that this transporter is the most important element in the BBB for preventing entry of xenobiotics and drugs. This role is mainly due to four factors: its expression, localization, potency, and multispecificity (Begley, 2004).

Abcb1 Expression

In the brain, mRNA and protein expression as well as functional P-gp has been detected in numerous cell types: BMVECs, perivascular astrocytes, parenchymal astrocytes, and neurons. It has been detected in human (Daood et al., 2008; Gazzin et al., 2008; Bendayan et al., 2006; Islam et al., 2005), monkey (Schlachetzki and Pardridge, 2003), sheep (Bougoin et al., 2008), rat (Niehof and Borlak, 2009; Bendayan et al., 2006; Ballerini et al., 2005; Ballerini et al., 2002; Decleves et al., 2000), and mouse (Wu et al., 2009; Rao et al., 1999). The transporter was also observed by electron microscopy in vesicles and the plasma membrane of pericytes from human brain sections (Bendayan et al., 2006) but functional studies in pericytes have not been reported (Hartz and Bauer, 2011). However, under physiological conditions, P-gp is predominantly expressed by BMVECs (Beaulieu et al., 1997; Biegel et al., 1995; Shirai et al., 1994; Tanaka et al., 1994; Tsuji et al., 1992; Sugawara, 1990).

Localization at the BBB

ABCB1 is localized at the luminal side of BMVECs (Hartz et al., 2010; Roberts et al., 2008; Bauer et al., 2006; Miller et al., 2000; Tatsuta et al., 1992; Tsuji et al., 1992; Sugawara et al., 1990).

Substrates

Abcb1 substrates range from small molecules such as morphine, verapamil, and loperamide (Molecular Weight (MW) 285-477 Da) to peptides such as amyloid-beta (MW 4200 Da) (Hartz et al., 2010; Kuhnke et al., 2007; Beaulieu et al., 1997; Biegel et al., 1995; Shirai et al., 1994; Tanaka et al., 1994; Tsujie t al., 1992; Sugawara et al., 1990). In general, these substrates are mainly organic cations and uncharged compounds with a molecular weight between 300-2000 Da, amphiphilic or lipophilic. Among P-gp substrates, there are many classes of drugs: opioids, steroids, antibiotics, calcium-channel blockers, chemotherapeutics, immunosuppressants, anti-

HIV drugs, antibiotics, and many more (Hartz and Bauer, 2011; Bauer et al., 2005; (Chen and Simon, 2000) (see table 1).

| Therapeutical use | Drugs |
|---------------------------|---|
| Analgesics | Asimadoline Morphine |
| Anti-cancer drugs | Vinca alkaloides (vinblastine, vincristine) Taxanes (paclitaxel, docetaxel) Anthracyclines (doxorubicine, daunorubicine, epirubicine) Anthracenes (bisantrone, mitoxantrone) Epipodophyllotoxines (etoposide, eniposide) Actinomycine D Methotrexate Topotecane |
| Inhibitor of HIV protease | Saquinavir Ritonavir Nelfinavir Indinavir Lopinavir Amprenavir |
| anti-diarrheal drug | Loperamide |
| Antiemetics | Domperidone Ondansetrone |
| calcium channel blocker | Verapamil |
| cardiac glycosides | Digoxin |
| immunosuppressive drugs | Cyclosporin A |
| corticoids | Dexamethasone Hydrocortisone Corticosterone Triamcinolone |
| Amebicides | Emetine |
| antibiotics | Erythromycin Gramicidin D Valinomycin |

Table 1. Abcb1 substrates
(Laurence Bousquet, 2008b)

Mechanisms of regulation

In the context of oxidative stress, signaling initiated by DEP (Diesel Exhaust Particles) for example, activates NADPH oxidase (Nicotinamide Adenine Dinucleotide Phosphate-oxidase) which produces ROS (Reactive Oxygen Species), stimulates TNF- α (Tumor Necrosis Factor release- α), and activate TNF-R1. In turn, TNF-R1 activates the transcription factor AP-1 (Activator Protein 1) leading to an increase in P-gp expression. In an inflammatory context, signaling initiated by TNF- α (Tumor Necrosis Factor) in the absence of ROS leads to ET-1 (Endothelin-1) release which binds to its receptors (ETA and ETB receptors), leading to an activation of NOS (Nitric Oxide Synthase), PKC (Protein Kinase C) and NF- κ B (nuclear factor-kappa B) which result in an increase in P-gp expression (Bauer et al., 2007). Abcb1 expression can also be increased by xenobiotics that bind directly to ligand-activated transcription factors, CAR (Constitutive Androstane Receptor) (Wang et al., 2010), PXR (Pregnane X Receptor) (Bauer et al., 2004) and GR (Glucocorticoid Receptor) (Narang et al., 2008)), which then translocate to the nucleus to increase gene expression.

In contrast, abcb1 activity is reduced in response to signaling occurring through Src kinase, PKC, and Estrogen Receptors (ER). For example, Exposing capillaries to low levels of LPS (LipoPolySaccharide), TNF- α or ET-1 causes a rapid and fully reversible loss of P-glycoprotein transport function with no change in protein expression; signaling involves ligand binding to TLR-4 (Toll-like receptor-4), TNFR1 or ETB receptor, followed by activation of NOS and PKC (Hartz et al., 2006; Hartz et al., 2004) (Miller et al., 2010).

2.2.5.2.1.2 ABC C family

The ABC C (or MRP) family consists of 13 members, 9 of which are transporters (ABCC1-6 and ABCC10-12) (Hartz and Bauer, 2011). At the BBB level, ABCC transporters of greatest significance for efflux transport are: ABCC 1, 2, 4, 5 and possibly 3 and 6 (this latest have only been detected at the mRNA level in the CNS) (Dauchy et al., 2008; Kamiie et al., 2008; Begley, 2004b). These transporters ensure the transport of organic anions and neutral organic drugs (Bortz et al., 2000). Although they confer protection against toxic compounds, several studies are devoted to study their expression, localization, function and regulation at the BBB level because of their important contribution to the MDR phenomenon. For example, ABCC2 (MRP2) confers

protection against toxic compounds but when overexpressed, it mediates resistance to numerous antitumor agents (e.g., vincristine and daunorubicin) (Rappa et al., 1999). MRP2 expression also restricts the transport of the anti-epileptic drug phenytoin through the BBB. The role of MRP4 in anti-epileptic drugs efflux has also been investigated. Furthermore, it has been shown that MRP4 limits the blood-to-brain and blood-to-CSF transport of substrate drugs (e.g., topotecan). MRP4 may also act to inhibit the brain penetration of toxic anionic compounds as well as therapeutic organic anions and transports toxic metabolites (e.g., 1-naphthol glucuronide) from the brain (Leggas et al., 2004). The table below summarizes the pharmacological substrates of ABCC transporters.

| Transporter | Human gene | Rodent gene | Subcellular Distribution | Physiological substrates | Function |
|----------------------------------|------------|-------------|--------------------------|---|----------------------|
| MRP-1 (ABCC1) | Abcc1 | Mrp-1 | Basolateral | Leukotriene C4; bilirubin | Drug resistance |
| MRP-2 (ABCC2) | Abcc2 | Mrp-2 | Apical | Bilirubin-glucuronides, acidic bile salts | Organic anion efflux |
| MRP-4 (ABCC4) | Abcc4 | Mrp-4 | Apical? | Cyclic nucleotides | Nucleoside transport |
| MRP-5 (ABCC5) | Abcc5 | Mrp-5 | basolateral | Cyclic nucleotides | Nucleoside transport |

Table 2. Abcc transporters

with their names, gene codes in humans and rodents, subcellular distribution, physiological substrates and function in drug resistance (adapted and modified from Lange 2004; Borst et al., 2002; Gottesmann et al., 2002)

2.2.5.2.1.3 ABCG family

Abcg2/bcrp

Historical Background

Breast Cancer Resistance Protein (BCRP) was discovered in 1997 in a human chemotherapy-resistant breast cancer cell line having cross-resistance to various anticancer agents with no overexpression of Pgp or MRPs (Doyle et al., 1998).

Abcg2 expression

In the CNS, ABCG2 mRNA and/or protein expression has been detected in a pericyte cell line (Shimizu et al., 2008), in primary cultured astrocytes, in the microglia MLS-9 cell line (Lee et al., 2007) and in cultured human neural progenitor and stem cells (Mouthon et al., 2006; Islam et al., 2005). At the BBB level, ABCG2 mRNA and/or protein has been detected in brain slices, BMVs, or in BMVECs isolated from human, pig, cow, rat, and mouse (Hartz et al., 2010; Warren et al., 2009; Daood et al., 2008; Lee et al., 2007; Aronika et al., 2005; Hori et al., 2004; Zhang et al., 2003; Eisenblatter et al., 2003; Cooray et al., 2002). Dauchy et al. found that, ABCG2 and ABCB1 were the two main ABC transporters expressed in BMVs from human cortex biopsies. These data were based on mRNA and protein levels (Dauchy et al., 2008).

Localization at the BBB

ABCG2 is localized in the luminal membrane of human and rat BMVs or BMVECs.

Substrates

The similarities with P-gp's localization in tissues suggest a possible role for BCRP in the protection from xenobiotics (Schinkel and Jonker, 2003). BCRP seems to have an important role in the pharmacokinetics and pharmacodynamics of certain xenobiotics and endogenous substrates (Cascorbi, 2006). It contributes to the resistance to many anticancer drugs such as doxorubicin, irinotecan, methotrexate, mitoxantrone, topotecan, many antihistamines, drugs such as cimetidine, as well as other substrates such as prazosin (Ito et al., 2005; Jonker et al., 2002; Borst et al., 1999). The lipophilic character of the majority of the BCRP substrates suggests that their sites of binding are in the intramembranous level.

Mechanisms of regulation

An example of signaling regulating Bcrp expression is the EGF (Epidermal Growth Factor), through activation of the MAPK kinases pathway (Mitogene-Activated PhosphoKinase) via phosphorylation of ERK 1 and 2 (Extracellular Regulated Kinase) and JNK / SAPK (c-Jun N-terminal Kinase / Stress-Activated Protein Kinase) (Meyer zu Schwabedissen et al., 2006).

An ERE (Estrogen Response Element) was discovered at the promoter sequence of BCRP (Ee et al., 2004). Estrogens such as estradiol-17 β regulate the expression of BCRP via the estrogen receptors (Imai et al. 2005; Ee et al., 2004).

2.2.5.2.2 SLC transporters

General considerations

The presence of TJ isolates the brain from many nutrients such as glucose and amino acids essential for proper neural function. To supply the CNS with these substances, BMVECs express transporters belonging to the Solute Carrier (SLC) family that transport a wide variety of solutes and nutrients into and out of the brain (Abbott et al., 2010).

SLCs are a superfamily of membrane transport proteins including over 300 members classified into 43 families (Kusuhara and Sugiyama, 2005). Among these numerous members, there are the Organic Anion Transporting Polypeptides (OATP/SLCO) and Organic Anion Transporters (OAT/SLC2A) families that have been identified at the BBB level where they play important roles in the transport of organic compounds across the BBB (Bernacki et al., 2008).

Function

Apart from lipophilic compounds, transporters are involved both in uptake and efflux in the transcellular transport from the brain to the blood-side.

Members of the solute carrier family (SLC) play an important role in the efflux transport, especially for organic anions include (OATP/SLCO) and (OAT/SLC2A) transporters. They cooperate with MDRs and MRPs in the removal of xenobiotics from the brain. It is believed that coordination of the members of SLC family with ABC transporters (such as P-gp, MRPs, and BCRP) allows an efficient transport across the endothelial cells to remove xenobiotics from the brain (Kusuhara and Sugiyama, 2005).

Localization at the BBB

Some of the SLC transporters are localized into either the luminal or abluminal membrane, others are localized into both membranes of the endothelial cells (Abbott et al., 2010; Bernacki et

al., 2008; Roberts et al., 2008; Ohtsuki and Terasaki, 2007; Nag and Begley, 2005; Begley and Brightman, 2003; Abbott, 2002; Mertsch and Maas, 2002; Begley, 1996; Betz et al., 1980).

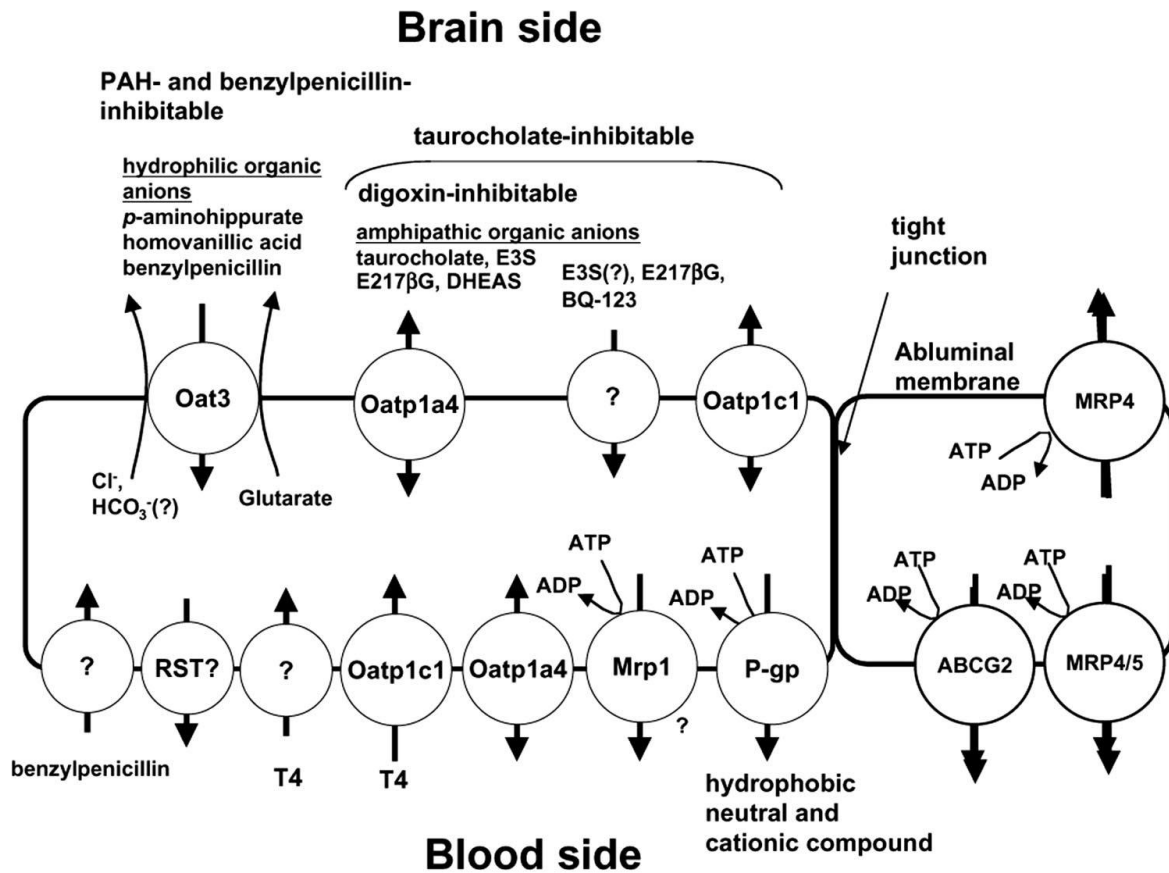


Figure 5. Schematic diagram of some SLC transporters at the BBB
 (Kusuhara and Sugiyama, 2005).

Members

The present manuscript focuses on the role of two SLC members at the BBB level: 1) *slc22a8* (synonym: *oat3*), member the *slc22* family, and 2) *slc1a4* (synonym: *oatp2* or *oatp1a4*), member of the *slc1/slc21* family.

2.2.5.2.2.1 Slc22a8/oat3

Slc22a8 (or oat3) was cloned from rat brain cDNA library as homolog of organic anion transporter 1. The cDNA consists of 536 amino acids with an apparent molecular mass of 92 kDa.

Expression

Oat3 is expressed in the liver, kidney, brain, and eye (Kusuhara et al., 1999). Moreover, mRNA for Oat3 was also detected in immortalized rat BMVECs (Ohashi et al., 2001).

Localization at the BBB

Immunochemical analysis of rat BMVs revealed abluminal localization of the Oat3; but weak signals were also visible at the luminal side (Kikuchi et al., 2003).

Substrates

Oat3 substrates include amphipathic organic anions (such as estrone sulfate and Dehydroepiandrosterone (DHEAS)), hydrophilic organic anions (such as Polycyclic Aromatic Hydrocarbons (PAH) and benzylpenicillin), and organic cations (such as cimetidine, ranitidine) (Nagata et al. 2004; Nagata et al. 2002; Kusuhara et al., 1999). In addition, it has been suggested that Oat3 is an exchanger driven by an outward concentration gradient of dicarboxylates (Bernacki et al., 2008; Sweet et al., 2003; Kusuhara et al., 1999).

Mechanisms of regulation

There is little information available on the molecular mechanisms regulating the gene expression OAT3. At the renal level, it has been shown that human OAT3 gene is regulated constitutively and inducibly via a cAMP (Cyclic Adenosine MonoPhosphate)-response element; CREB-1 (cAMP Responsive Element Binding protein-1) and ATF-1 (Cyclic AMP-dependent Transcription Factor-1) function as constitutive and inducible transcriptional regulators of the human OAT3 gene via CRE (cAMP Response Element). At the hepatic level, it has been

suggested that HNF-1 α/β and HNF-4 (Hepatocyte nuclear factor) play key roles in the transcriptional regulation of OAT3 (Saito, 2010).

2.2.5.2.2.2 Slco1a4 /Oatp1a4

Expression

Oatps are expressed in numerous tissues like the liver, choroid plexus, lung, heart, intestine, kidney, placenta, testis, and BBB (reviews: Hagenbuch and Meier, 2004; Hagenbuch and Meier, 2003; Tamai et al., 2000).

Localization at the BBB

Regarding the Oatp1a4 transporter, studies revealed abundant Oatp1a4 expression in the brain (Abe et al., 1998; Noe et al., 1997), where it is localized at the luminal and abluminal membranes of the BMVs (Gao et al., 1999).

Substrates

Numerous amphipathic organic solutes (e.g., bile salts, organic dyes, steroid conjugates, thyroid hormones, neuroactive peptides, and various drugs) are substrates for Oatps (Meier et al., 1997). Currently, the SLCO/SLC21 family includes fourteen members in rodents and human. Of the fourteen members of this family, expression of Oatp1a4 (Oatp2), Oatp1a5 (Oatp3), and Oatp1c1 (Oatp14) has been shown in BMVs. Its substrates include amphipathic organic anions (bile acids, steroid conjugates), cardiac glycosides (digoxin, ouabain), bulky organic cations (N-(4,4-azo-n-pentyl)-21-deoxyajmalinium, N-methylquinidine, N-methyl-quinine and rocuronium), and anionic peptides (BQ-123, [D-Pen²,D-Pen⁵]-enkephalin, deltorphin II) (Gao et al., 2000; Reichel et al., 1999; van Montfoort et al., 1999; Abe et al., 1998; Noe et al., 1997), DeHydroEpiAndroSterone (DHEAS) (elimination through the BBB) (Asaba et al., 2000; Gao et al., 1999), [D-penicillamine 2,5]-enkephalin and taurocholate (uptake) (Dagenais et al., 2001), steroids (such as estrone-3-sulfate, estradiol-17 β -glucuronide), thyroid hormones (T3 and T4), drugs (fexofenadine), cationic compounds (rocuronium, ADP-ajmalinium), and peptides exerting neuroactivity ([D-penicillamine 2,5]-enkephalin) (Hagenbuch et al., 2002; Dagenais et al., 2001; Sugiyama et al., 2001). Thus, at the BBB level, Oatp1a4 work as either 1) efflux pumps for both

substrates synthesized in the brain and waste products designated for excretion or 2) influx pumps for neuroactive compounds like opioid (Hagenbuch et al., 2002).

Mechanism of regulation

Little is known about mechanisms regulating *oatp1a4* at the BBB level. At the liver level, rat *oatp1a4* has been shown to be regulated by CAR and PXR signaling (Satudinger et al., 2003; Guo et al., 2002).

Chapter III

The developmental blood-brain barrier

3.1 Development of the blood-brain barrier

BBB development is a complex process comprising different alternatives phases: vasculogenesis, angiogenesis and barrierogenesis. This process, under the regulation of several cell signaling pathways, soluble factors and local environment, starts in early phases of pre-natal brain development and continues during postnatal development until full maturation of BBB is reached. Here I give a brief summary of current knowledge on the barrierogenesis during embryonic life. These development aspects of the BBB, although little studied, remain more studied than the maturational aspects occurring between birth and adulthood. These remain understudied.

3.1.1 Vasculogenesis phase

The development of the BBB starts with the vasculogenesis phase or the de novo formation of blood vessels. Migration of mesodermal hemangioblasts (bipotential stem cells that give rise to angioblasts (endothelial cells progenitors)) initiates the development of head vasculature. First, a so-called “blood-islands” are formed. Then they start their differentiation by fusion into a primary PeriNeural Vascular Plexus (PNVP) which is an external vascularization of the brain covering the entire surface of neural tube of the developing embryo (even before the onset of circulation), and which later will differentiate into veins and leptomeningeal arteries (review Liebner et al., 2011; Risau, 1986). This process is still poorly understood but it has been suggested that the differentiation of angioblast in endothelial cells, may occur through the release of soluble cytokines, cell-to-cell adhesion, and the synthesis of matrix proteins on which the endothelium adheres and grows (Ruhrberg et al., 2002; Luca Crecullo, 2009).

3.1.2 Angiogenesis phase:

After the de novo formation of blood vessels, BBB development continues with an angiogenesis phase or a neo-vascularization phase (Figure 6). It is a process by which new blood vessels are generated from existing ones, specifically from the external PNVP. In rodents' CNS, angiogenesis starts at embryonic day 9. A primitive capillary network expands from the PNVP, by growing along a preformed lattice of glial and Neural Precursor Cells (NPCs) (these latest give rise to differentiated neurons and astrocytes, which in turn will communicate with brain endothelium to form a functional NVU (Review: Liebner et al., 2011; Anthony et al., 2004)). The CNS angiogenesis phase forms an undifferentiated network of capillaries (Virgintino et al., 1998). In other terms, it generates leaky vessels, which then will interact with neural cells to form a functional BBB.

Several factors have been shown to be involved during this phase: VEGF-A (vascular endothelial growth factor) (where the vascular sprouts expand towards a concentration gradient of VEGF-A, produced by neuroectodermal cells located in the ventricular layer (Crameliet, 2005)), the Frizzled homolog-4 (Fzd-4), Angiopoietin-1 (Ang-1), the angiopoietin receptor Tie-2, the inhibitor of differentiation proteins Id-1 and Id-2 and the orphan G protein-coupled receptor GPR124 (Kuhnert et al., 2010) and Wnt proteins (3a, 5a, 7a/b) (review: Paolinelli et al., 2011). Regarding the implication of Wnt signaling, it has been shown that vessels in mice PNVP receive signals of the Wnt pathway at embryonic day 9.5, and this has a role in BBB induction (Reviews: Liebner et al., 2011; Liebner and Plate, 2010).

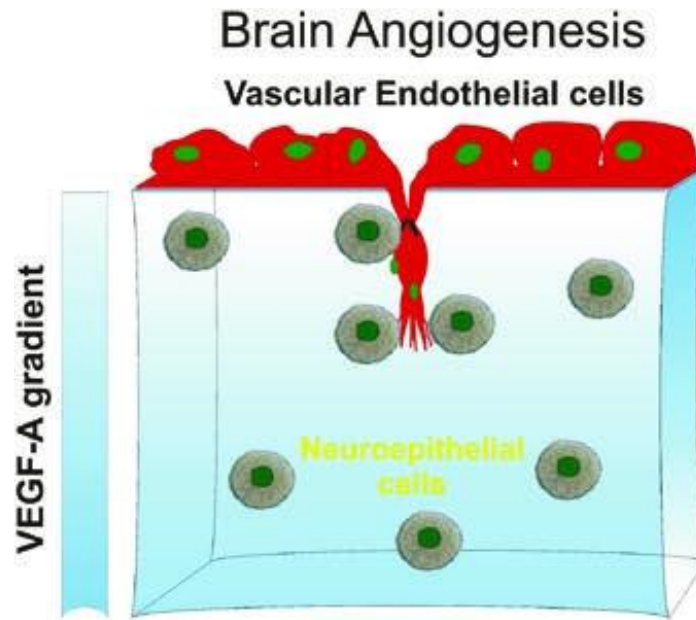


Figure 6. BBB angiogenesis

(From Cucullo, 2009)

3.1.3 Differentiation phase

After angiogenesis, the phenotype of BMVs evolves during a differentiation phase (Figure 7). In mice, the BBB begins to form between E11 and E17 (review: Abbott et al., 2009). In this phase, BMVECs switch from leaky vessels to endothelial cells having barrier characteristics. For example, they lose vesicles and fenestrations. In conjunction with their phenotypical changes, BMVECs establish during this phase close contact with pericytes and astroglial cells and thus form the NVU. BMVECs also present an upregulation of specific transporter systems such as Glucose Transporter-1 (Glut-1) (Dobrogowska and Vorbrodt, 1999), transferrin transporter as well as various amino acid transporters. In plus, de novo expression of ABC transporters can be observed. For example, ABC transporter *mdr2* can be detected in the brain by real time-PCR at E13. At E18, *mdr* isoforms like *mdr1a*, *mdr1b* and *mdr2* show strong signals (Scheingold et al., 2001). Regarding the evolution of the BBB structural components in this phase, TJs become more complex restricting paracellular movement of polar solutes and macromolecules and tight for small polar molecules. In effect, TJ are formed as blood vessels invade the brain, specifically at E10 in the mouse and E11 in the rat. But the high electrical resistance which is characteristic of an effective BBB, is not exhibited until E21 (review: Abbott et al., 2010; Butt et al., 1990;

Butt, 1995). However, these data indicate that functional tight junctions are formed prenatally. In conjunction with their phenotypical changes, BMVECs establish during this phase close contact with pericytes and astroglial cells and thus form the NVU.

Several factors are involved during this phase such as: Platelet Derived Growth Factor B (PDGF-B), N-cadherin, Angiopoietins-1 (Ang-1), the endothelial receptor Tyrosine kinase (Tie-2), Leukemia Inhibitory Factor (LIF) and Src-SupprESsed C-Kinase Substrate (SSECKS). Wnt proteins are also factors involved in BBB differentiation. Indeed, the Wnt/ β -catenin pathway is necessary and sufficient to induce BBB-type endothelial TJs *in vitro* and *in vivo* on a molecular and structural level (Liebner et al., 2011; Plate and liebner 2010).

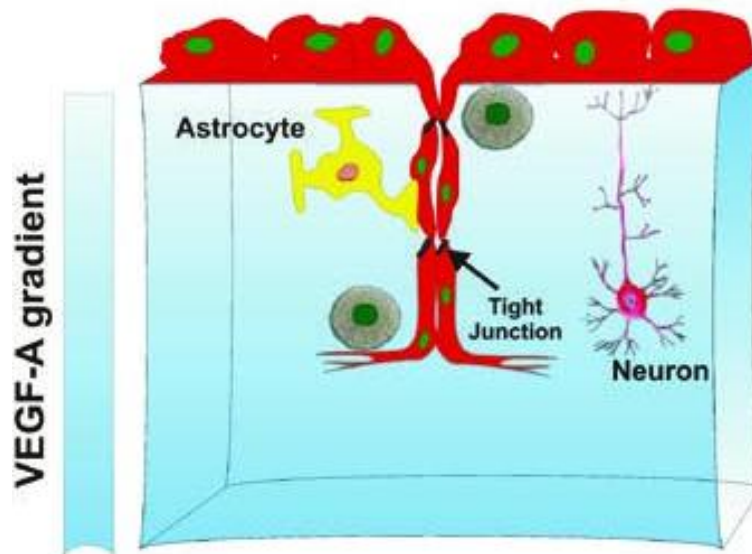


Figure 7. BBB Differentiation

(From Cucullo, 2009)

3.1.4 Maturation phase

Although the BMVECs form the barrier proper, close contact with pericytes, astrocytes and possibly neuronal cells is required for the maintenance of the BBB (Figure 8). The molecular mechanisms maintaining the BBB in the mature CNS remain unknown.

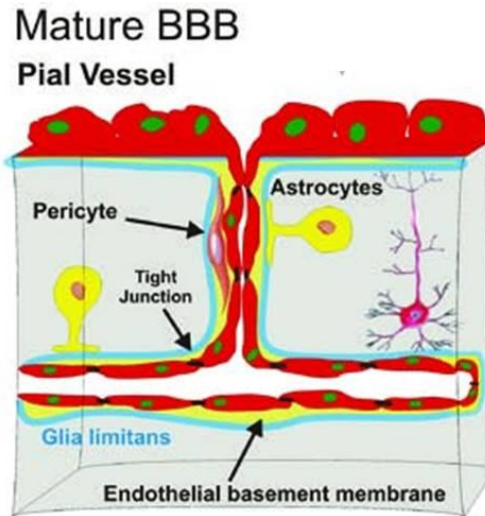


Figure 8. The mature BBB

(From Cucullo, 2009)

3.1.5 The Role of Wnt signaling in BBB development

Cellular and molecular pathways involved in the BBB development are still only superficially understood. Emerging evidences are pointing an important role of the Wnt signaling pathway in controlling BBB angiogenesis, differentiation, induction, and maintenance.

3.1.5.1 General aspect of the Wnt canonical signaling

The Wnt canonical or Wnt/ β -catenin signaling is a pathway regulating numerous physiological processes including fundamental aspects of development such as cell fate specification, proliferation, survival, and overall organogenesis (for review: Dejana, 2010; Angers and Moon, 2009; Franco et al., 2009; Klaus and Birchmeier, 2008; Clevers, 2006). The Wnt/ β -catenin pathway also mediates the regulation of cellular responses such as cell cycle, apoptosis, cell differentiation and communication (for review see Liebner and Plate, 2010).

Wnts are a family of 19 secreted ExtraCellular Matrix (ECM) glycoproteins. β -catenin (β -cat), another main component of the canonical Wnt signaling pathway (Wnt/ β -cat pathway), is a protein acting as a transcription factor, although it can be a part of the structure proteins constituting adherens junctions.

During canonical Wnt signaling, the ligands Wnt bind to their Frizzled/LRP (Low-density Lipoprotein Receptor-related Protein) cell surface receptor complexes. This binding causes a

stabilization of β -cat in the cytoplasm. Normally β -cat is phosphorylated by a destruction complex including Glycogen Syntase Kinase-3 β (GSK-3 β), the scaffolding protein Axin and Adenomatosis Polyposis Coli (APC) which targets the protein to be degraded. When β -cat is stabilized, it translocates into the nucleus, interacts with the Lymphoid Enhancer Factor-1 and T-Cell Factor (LEF-1/TCF) complexes, leading thus to the regulation of specific genes expressions (Gordon and Nusse, 2006; Logan and Nusse, 2004) involved in cell proliferation, differentiation, adhesion, morphogenesis and other processes involved in development, homeostasis and disease of many tissues and organs (Figure 9).

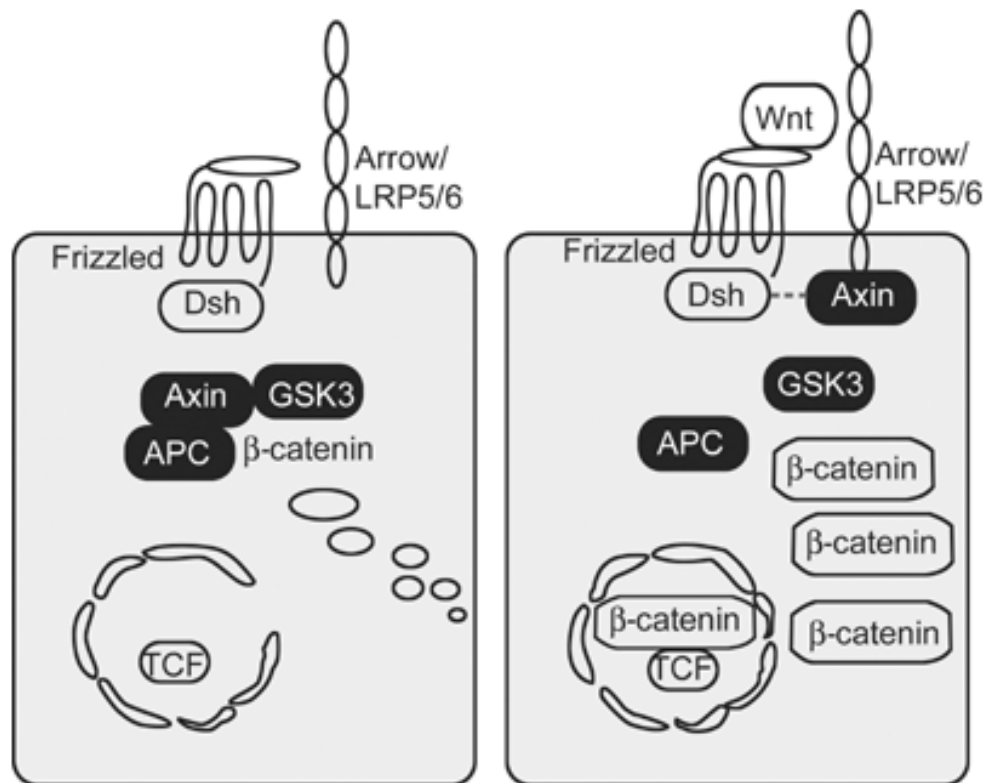


Figure 9. The Wnt/ β -catenin signaling pathway

(From Nusse, 2005)

3.1.5.2 Wnt signaling during angiogenesis

It is suggested that Wnt signaling regulate general endothelial cell function. Indeed, *in vitro* studies using endothelial cells immortalized from several different tissues showed that endothelial cells express Wnt ligands, receptors and secreted modulators. A member of Wnt

family, Wnt5a, regulates endothelial cell survival, proliferation and gene expression (Zerlin et al., 2008; Goodwin et al., 2006; Masckauchan, 2006).

In the CNS, BMVECs present high expression of several effector of Wnt signaling and this by comparison with their homologue in the periphery. Basing on these findings, an important role of Wnt signaling during angiogenesis has been suggested. Using reporter mice, Wnt signaling has been found particularly strong in vessels penetrating into the embryonic brain. This expression was still detectable during early post-natal period but declined in adult (Liebner and Plate, 2010; Liebner et al., 2008). Interestingly, this expression follows the same kinetics of that of brain angiogenesis and BBB development supporting the idea that Wnts are involved in brain vascularization as well as BBB properties induction (Paolinelli et al., 2011). Daneman et al. have demonstrated that canonical Wnt signaling is activated in CNS endothelial cells, but not in non neural endothelial cells, during development. Indeed, blocking Wnt/ β -cat signaling *in vivo* specifically disrupts CNS, but not non-CNS angiogenesis. These defects include reduction in vessel number, loss of capillary beds, and the formation of hemorrhagic vascular malformations that remain adherent to the meninges (Daneman et al., 2008). Liebner et al. demonstrated that the simultaneous ablation of the growth factors Wnt7a/7b leads to embryonic lethal neurovascular phenotypes, as it causes CNS-specific hemorrhages and disorganization of the neural tissue (reviews: Liebner et al., 2011; Liebner and Plate, 2010). Now, it has become clear that canonical Wnt/ β -cat signaling specifically acts on CNS vessels and is crucial for proper murine neurovascular development (Liebner et al., 2011).

3.1.5.3 Wnt signaling during differentiation

In addition to their function in brain angiogenesis, Wnts also participate in the differentiation of the endothelial cells to BBB phenotype. Indeed, it has been demonstrated that canonic Wnt signaling in endothelial cells is necessary and sufficient to induce BBB-type endothelial TJs *in vitro* and *in vivo* (Liebner et al., 2011; Liebner and Plate, 2010; Liebner et al., 2008): activation of β -cat transcriptional activity by Wnt3a-conditioned medium or infection of the cells with dominant active β -cat increase BBB characteristics such as TJs organization. In addition, it has been shown that Wnt7 α and β induce expression of the glucose transport, an early marker of BBB specialization (Daneman et al., 2009). Taken together, these data support the concept that Wnt signaling is involved in the regulation of BMVECs' differentiation (Paolinelli et al., 2011).

3.1.5.4 Wnt signaling during maturation and maintenance

Whether Wnt signaling is involved in barrier inducing/maintaining properties remain unclear.

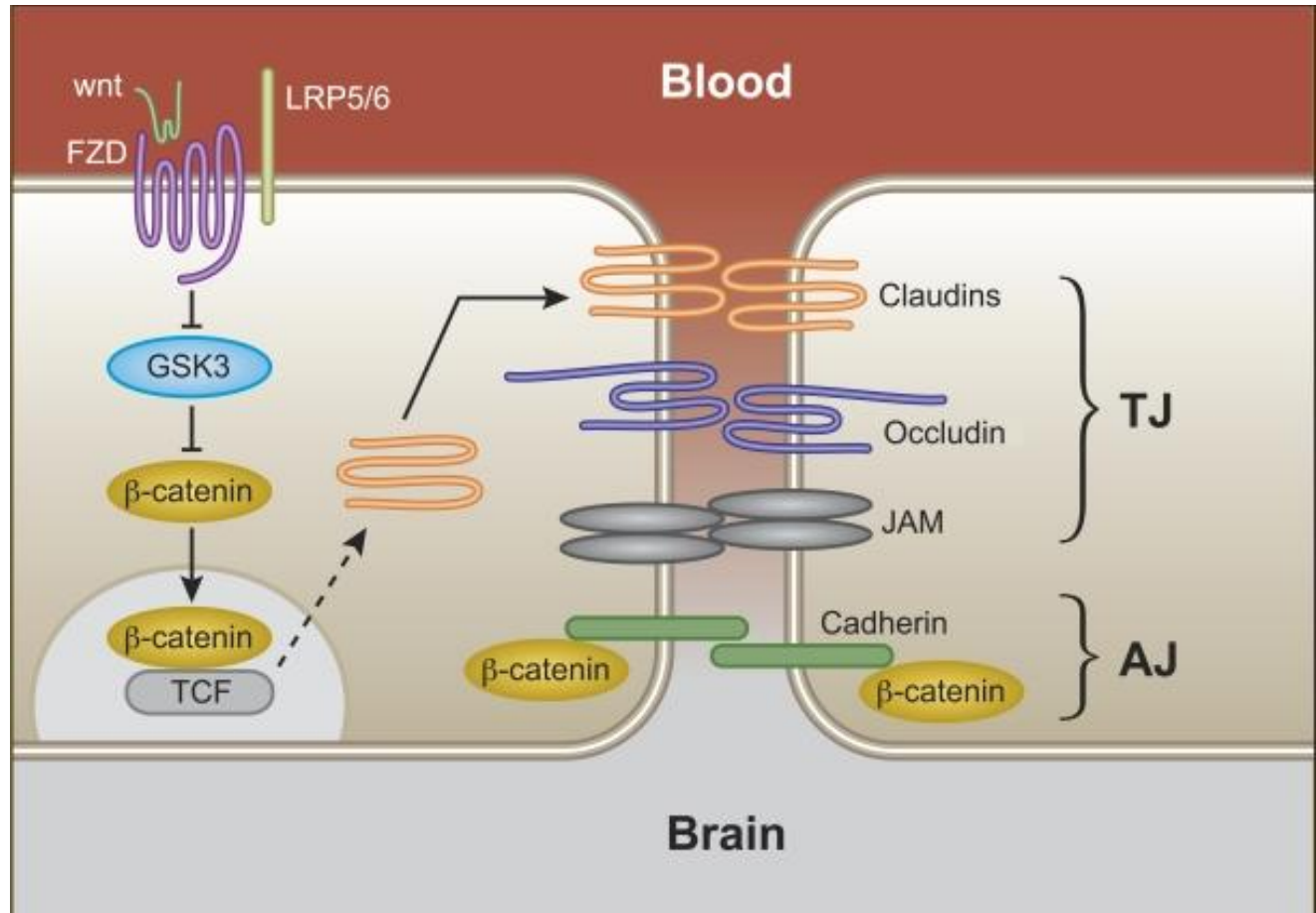


Figure 10. Wnt signaling and TJ

Activation of Wnt receptors FZD and LRP5/6 inhibits GSK3 to stabilize β -catenin that in turn enters the nucleus to activate T cell factor (TCF)-dependent transcription. This drives *Cldn3* gene activation either directly or indirectly (dashed line arrow), and the resulting *Cldn* protein reinforces the tight junction. JAM, junctional adhesion molecule (Polakis, 2008).

3.2 Components of the developmental blood-brain barrier

The BBB phenotype in the adult brain has been largely studied. BMVECs in embryonic brain have been little studied but aspects of the BBB during brain maturation between birth and

adulthood remains understudied. Here I give a summary on the brief existing literature about BBB aspects in the developing brain.

3.1.6 Structural components

There are several pieces of evidence suggesting that BBB develops during fetal life and that at birth it is well formed especially to proteins and macromolecules (Ballabh et al., 2004; Saunders et al., 2000; Preston et al., 1995; Keep et al., 1995; Moos and Møllgård, 1993; Saunders, 1992; Tauc et al., 1984; Olsson et al., 1968):

1- It has been demonstrated that TJ are well-formed and functionally effective in the early stages of brain vascularization; indeed, the BBB in embryos and neonates does protect the brain from low molecular weight molecules (Saunders et al., 2012; Daneman et al., 2010a; Ek et al., 2006; Johansson et al., 2006; Nitta et al., 2003).

2- In mammals, soon after blood vessels invade the brain, tight junctional proteins, such as claudin 5 and occludin are expressed at brain barrier interfaces (Daneman et al., 2010a; Ek et al., 2006; Johansson et al. 2006).

3- In the brain of rats having one week of age, BBB permeability to the tracer mannitol (a nonmetabolisable, but slowly BBB penetrant with a MW=182 Da) is identical to that of adult rats Preston et al. (1995)..

4- In human, there is nothing to suggest that the BBB is not at least as well formed at birth as it is in the rat . Indeed, occludin and claudin-5 expression are detected in BMVs of the 14 week human fetus and shows the same distribution at cell margins as seen in the adult (Virgintino et al., 2004). In stillborn human fetuses, there is a post-mortem BBB to trypan blue from at least the start of the second trimester, which is comparable to that of the adult human. Thus, in mammals, barrier is significantly tightened on the day of birth (Abbott et al., 2010; Butt et al., 1990; Grøntoft, 1954).

3.1.7 Drug transporters

In human fetuses, P-gp is expressed in BMVECs at 8 weeks gestation (Schumacher and Møllgård, 1997). Daood et al. reported that, in post-mortem CNS tissue from infants born 22^{0/7}-42^{0/7} week gestation and adults, P-gp/ABCB1 immunostaining was observed in BMVECs as

early as 22^{0/7} weeks and prevalence and intensity increase with maturation. However, they also reported that the intensity of BCRP/ABCG2 immunostaining did not vary notably with maturation (Daood et al., 2008). Ek et al., also demonstrated that practically no change in expression level for BCRP between the embryo and adult was found (Ek et al., 2010).

In mammals born in a relatively immature state, such as the rat and mouse, many of the characteristic BBB transport mechanisms may continue to mature and become fully expressed and functional only in the peri- or post-natal period (Jones et al., 1992). Gene expressions of P-gp and bcrp has been also established in the forebrain and brainstem at different developmental stages in rats: E13, E15, E18, P1, P14, P21 and adulthood. Results showed that both P-gp and bcrp were detected at E13. P-gp expression progressively increased during development (about 10–12-fold) in both the forebrain and brainstem, while Bcrp showed similar gene expression at all ages and in both brain regions (EK et al., 2010). Postnatally, expression of P-gp in the luminal rat BMVECs can be detected by immunoblotting at P7 and reaches a plateau of expression by P28 (Abbott et al., 2010; Matsuoka et al., 1999).

Little is known about the ontogeny of SLC transporters at the BBB level. Some studies on the ontogeny of oat3 have been performed in renal tissue. Fetal sheep were sacrificed at 80, 100, 120, 130, and 145 days of gestation, as well as P1 and P7. Renal tissue was extracted and the expression levels of oat3 were measured using RT-PCR. Compared with prenatal ages, oat3 mRNA was increased postnatally. The oat3 mRNA in renal cortex was increased in the more mature animals. Authors suggested that there is a developmental pattern of expression of oat3 in ovine renal cortex, and that the pattern of expression suggests that the function of this transporter is likely to be greater starting in late gestation (Wood et al., 2005). However, oat3 ontogenesis at the BBB level remains to be elucidated.

However, how the BBB mature post-natally, and what are the molecular mechanisms involved in BBB maturation and maintenance in the mature CNS remains unknown to date. Moreover, gene and functional protein expression of other ABC and SLC transporters such as p-gp, bcrp, oat3 and oatp1a4 in BMVECs, site of the BBB, as well as the molecular mechanisms underlying their functional expression at the BBB remain to be elucidated.

Chapter IV

BBB under inflammatory conditions

4.1 BBB and pathological diseases

Alterations in structural and functional components of the NVU have been reported in a long list of CNS pathologies, both in children and adults. This growing list include frequent pathologies affecting the ageing brain such as multiple sclerosis, Alzheimer's disease; HIV-associated dementia, and other diseases affecting also the immature brain such as schizophrenia, autism and epilepsy. Tables 3 and 4 below give some examples on BBB dysfunction in CNS diseases.

| CNS pathology | BBB dysfunction |
|----------------------------|--|
| Multiple sclerosis | <p>Breakdown of the BBB (Minagar and Alexander, 2003)</p> <p>Tight junction abnormalities (McQuaid et al., 2009)</p> <p>Downregulation of laminin in the basement membrane (Oki et al., 2004)</p> <p>Selective loss of claudin3 (shown with antibody vs Cl 1/3) in experimental autoimmune encephalomyelitis (Wolburg et al., 2003)</p> |
| HIV | <p>BBB tight junction disruption (Buckner et al., 2006; Berger and Avison, 2004; Dallasta et al., 1999)</p> |
| Alzheimer's disease | <p>Decreased glucose transport, downregulation of GLUT-1, upregulation of aquaporin-4 (AQP-4) expression (Kalaria, 1999)</p> <p>Loss of ABCA1 in brain endothelial cells associated with Aβ brain deposition and increased ABCA1 function in astrocytes reduces Aβ brain deposition (Dergunov et al., 2009; Fan et al., 2009; Minagawa, et al., 2009; Akanuma et al., 2008; ; Cao et al., 2007; Repa et al., 2007; Koldamova et al., 2005; Hirsch-Reinshagen et al., 2004; Panzenboeck, et al., 2002)</p> <p>Down-regulation of abcb1 in brain endothelial cells in Alzheimer's disease (Hartz et al., 2010; Bartels et al., 2009; Bell and Zlokovic, 2009; Pekcec, 2009; Tai et al., 2009; Kuhnke et al., 2007; Cirrito et al., 2005; Vogelgesang et al., 2005; Siegmund et al., 2002; lam et al., 2001)</p> <p>(<u>Note</u>: Accumulation of amyloid-β (Aβ), a key neuropathological feature of Alzheimer's disease, by decreased levels of P-gp transporter expression (Tai et al., 2009; Cirrito et al., 2005; Zlokovic, 2005)</p> |
| Brain tumours | <p>Breakdown of the BBB and downregulation of TJ protein claudin-3 (Wolburg, 2006; Papadopoulos et al., 2004; Warth et al., 2004; Davies, 2002; Liebner et al., 2000)</p> |
| Brain Cancer | <p>Abcb1 is up-regulated in BMVECs especially in glioblastoma (Breedveld et al., 2006; Calatuzzolo et al., 2005; Kemper et al., 2004; Kemper et al., 2003; Fellner et al., 2002; Spiegl-Kreinecker et al., 2002; Henson et al., 1993)</p> <p>Abcg2 is up-regulated in brain endothelial cells in glioblastoma</p> <p>(<u>Note</u>: Associated with tumor resistance for paclitaxel, topotecan, docetaxel, imatinib (Chen et al., 2009; Dean, 2009; Lagas et al., 2009; Polli et al., 2009; Zhou et al., 2008; de Vries et al., 2007; Breedveld et al., 2005)</p> |

Table 3. Alterations of BBB components in adult CNS diseases

(Adapted from Hartz and Bauer, 2011; Abbott et al., 2010)

| CNS pathology | BBB dysfunction |
|----------------------|---|
| Epilepsy | <p>Transient BBB opening in epileptogenic foci, and upregulated expression of P-gp and other drug efflux transporters in astrocytes and endothelium (Lazarowski et al., 2007; Marroni et al., 2003; Abbott et al., 2002)</p> <p>Abcb1 is up-regulated in brain endothelial cells and astrocytes in pharmacoresistant epilepsy (Note: abcb1 limits brain uptake of certain antiepileptic drugs) (Lazarowski et al., 2007; van Vliet et al., 2007; Kubota, 2006; Volk et al., 2005; Lazarowski, et al., 2004; Marchi et al., 2004; Ramos, 2004; Volk et al., 2004; Marroni et al., 2003; Sisodiya et al., 2002; Potschka and Loscher, 2001; Potschka, et al., 2001; Tishler et al., 1995)</p> <p>abcc1 is up-regulated in endothelial cells, astrocytes and neurons from epileptic patients (Ak et al., 2007; Kubota, et al., 2006 ; van Vliet et al., 2005 ; Aronica, et al., 2004; Marchi et al., 2004; Sisodiya, et al., 2002)</p> <p>abcc5 is up-regulated in human epileptic tissue (Ak et al., 2007; Kubota, et al., 2006; Marroni et al., 2003)</p> <p>abcg2 is up-regulated in astrocytes after seizures and in astrocytic tumors (Kubota et al., 2006; Hoffmann, et al., 2006; van Vliet, et al., 2005; Marroni et al., 2003; Potschka, H et al., 2003; Dombrowski et al., 2001)</p> |
| autism | <p>Epileptic symptoms in children with Autism Spectrum Diseases (ASD) were recently considered to be related to immune-mediated pathogenesis (Specchio et al., 2010). In fact, ASD are associated with some immune dysfunction, such as elevated antibody levels directed against the fetal brain (Goines et al., 2010; Enstrom et al. 2009; Theoharides et al., 2009; Wills et al., 2009) suggesting BBB disruption.</p> |
| Schizophrenia | <p>Increased plasma/CSF albumin ratio in 29% of schizophrenic patients tested. Increased albumin in CSF indicates increased BBB permeability in a subgroup of patients (Kirch et al., 1992)</p> |

Table 4. Alteration of BBB components in children CNS diseases

(Adapted from Hartz and Bauer, 2011; Abbott et al., 2010; Stolp and Dziegielewska, 2009)

Alterations in BBB do occur during adulthood and childhood CNS diseases, nevertheless, several questions are still unanswered: when do these alterations begin, what are the mechanisms implicated, are they a cause or a consequence in the disease process and what is their exact role in the contribution and the exacerbation of the pathogenesis? (Abbott et al., 2010; Persidsky et al., 2006)

One factor pointed to be involved in BBB alterations during CNS diseases is inflammation. Indeed, in all these neurological disorders, the CNS exhibit inflammatory features. There are growing evidences suggesting that the immune system, through systemic or cerebral inflammation, can disturb the brain barrier systems, and that this disturbance could be implicated as one of the leading causes in the pathogenesis of neurological diseases both in the young and ageing brain (Stolp and Dziegielewska, 2009; Hemmer et al., 2007; Stolp et al., 2005; Rite et al., 2007).

This second part of the introduction is devoted to give a comprehensive review of the literature concerning cerebral inflammation and its impact on BBB components and more particularly in TJ and drug efflux transporter expression both in children and adults.

4.2 CNS inflammation

Inflammation is a response of the organism to injury, ischaemia, autoimmune responses or infectious agents (Lucas et al., 2006). Normally, inflammation has benefit outcomes as it limits pathogens proliferation and promotes repair and recovery of affected tissues. However, when inflammation is extended it becomes highly detrimental (Allan et al., 2003). Indeed, in normal conditions, pro-inflammatory processes are highly regulated by anti-inflammatory processes. A perturbation in this anti/pro-inflammatory balance causes prolonged inflammatory response and this unregulated response becomes pathological (Szelenyi, 2001).

For a long time, the CNS has been considered as an ‘immune privileged’ organ that does not exhibit inflammatory features, and is largely unaffected by systemic inflammatory. This old view has been revised significantly and it is now appreciated that the CNS does exhibit local inflammatory responses, and it can be also affected by peripheral inflammation. But there is no doubt that brain inflammation differs significantly from that in other tissues, especially in its responses to pathogenic challenges, and even in its outcomes although under tight regulatory processes (Lucas et al., 2006). In other terms, the nature and number of cellular mediators of inflammation are quite distinct in brain tissue compared to the periphery, and it has been hypothesized that inflammation either in its acute or chronic aspect, alters structural and functional components of the BBB leading to modifications in the barrier permeability.

With the increasing evidences on the implication of neuro-inflammation in several important neurodegenerative diseases, the interest in CNS inflammation has grown rapidly.

Several studies have revealed that some inflammatory mechanisms are common to seemingly diverse CNS disorders. These studies have most importantly highlighted an impact of inflammation on structural and functional components of the NVU in adult organisms. The CNS inflammatory mechanism and its impact on adult brain are summarized below. In younger organisms, only few studies have been conducted; the interaction of inflammatory mediators with the developmental and maturational BBB has been little studied, despite the fact that inflammation as well as alterations in the BBB have been emphasized in several developmental and childhood CNS diseases (such as epilepsy and autism). A review of the brief existing literature on BBB alterations and cerebral inflammation during CNS diseases in the immature brain is summarized below following the overview from adult organisms.

4.2.1 Mechanism of inflammation

In the brain, the inflammatory response evolves two parts: the first is responsible for immediate action against pathogen agents called the “innate immune system, and the other, is a system that responds specifically to an external threat and requires plasticity and memory, called the “adaptive or acquired immune system.” Thus, rapidly, in response to infection or injury, microglia, major immune cells of the monocyte/macrophage lineage resident in the brain, are activated (Streit et al., 2002). Microglia are important phagocytic cells, and once activated they release numerous inflammatory molecules, such as eicosanoids, Platelet Activating Factor (PAF), Reactive Oxygen Species (ROS), Nitric Oxide (NO), and particularly pro and anti-inflammatory cytokines and chemokines (Hanisch et al., 2002) (Among pro-inflammatory molecules: Tumor Necrosis Factor- α (TNF- α), interleukin-1 β (IL-1 β), endothelin-1 (ET-1), interleukin-6 (IL-6) (Aloisi, 2001; EL Khoury et al., 1998; Kreutzberg et al., 1996), chemokines (IL-8, Macrophage Inflammatory Protein-1 α (MIP-1 α), MIP-1 β , Monocyte Chemotactic Protein-1 (MCP-1) (Ehrlich et al., 1998; Peterson et al., 1997), proteases (Nakanishi et al., 2003), and anti-inflammatory molecules such as transforming growth factor-beta (TGF- β) and IL-10 (Benveniste et al., 2001). Lately, astrocytes are activated (Ivey et al., 2009). Apart from being involved in a variety of physiologic processes, astrocytes rapidly react to different neurological insults. Upon activation, a series of changes occur in astrocytes, leading to the acquisition of macrophage differentiation markers and effector properties. One main feature of these changes is the increase in the number and size of Glial Fibrillary Acidic Protein (GFAP) expressing cells. GFAP is an intermediate

filament cytoskeletal protein expressed primarily by astrocytes and it is considered as the marker of astrocytes (Raine, 1999). Parallel to GFAP overexpression, astrocytes released many pro-inflammatory mediators and up-regulate the expression of several inflammatory molecules which contribute to the amplification of inflammation (Fraina et al., 2007). The GFAP overexpression, the release of many pro-inflammatory mediators and up-regulation of the expression of several adhesion molecules (such as selectins, integrins, adherins), all these factors contribute to the amplification of inflammation via leukocyte recruitment (Farina et al., 2007). Leucocytes, derived from blood, migrate into the CNS (Dietrich, 2002; Prat et al. 2001). The most studied route of entry of leukocytes into the CNS is the passage through the BBB (Man et al., 2007).

4.2.2 Mechanism of inflammation in immature brain

CNS inflammation during fetal life and neonatal life is the subject of some new studies. The figure below describes phases of inflammation in the developmental brain after an acute hypoxic–ischemic or excitotoxic insult (Hagberg et al., 2012). The innate and adaptive immune response inflammatory mechanisms implicated seems to be similar to that in adult regarding the sequence of inflammatory events:

- a pro-inflammatory phase characterized by microglial activation, cytokines and chemokines release in a time dependant-manner and astrocytes activation.
- An anti-inflammatory phase resulting in the resolution of inflammation or in a state of chronic inflammation.

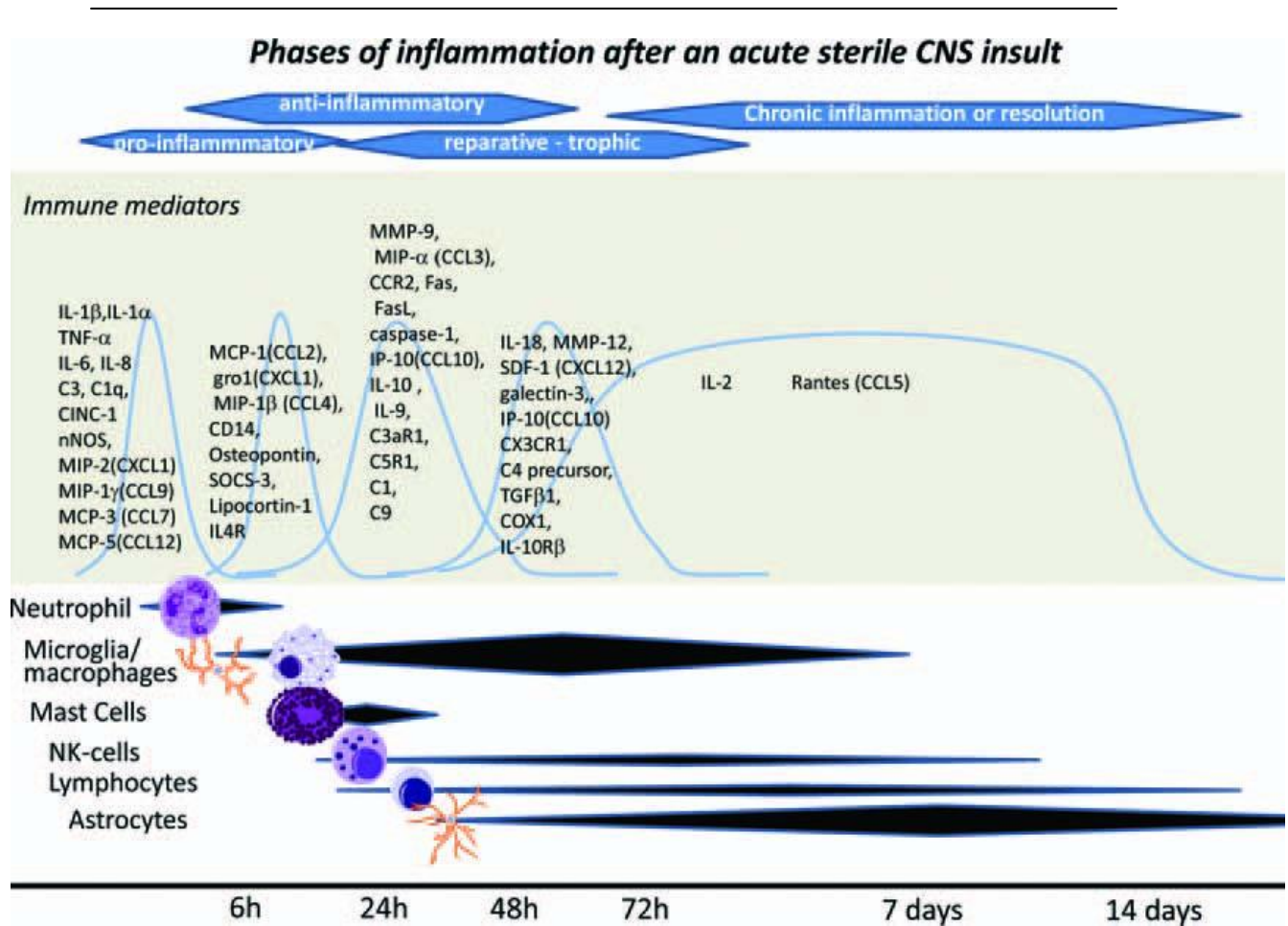


Figure 11. Phases of inflammation in the immature brain after an acute hypoxic–ischemic or excitotoxic insult

The figure is a summary based on various studies.^{2,54,56,59–63,68–70,73,171–180}. C = Complement; CD = Cluster of Differentiation; CINC = Cytokine-Induced Neutrophil Chemoattractant; COX = CycloOxygenase; IL = InterLeukin; MMP = Matrix Metalloproteinase; NK = Natural Killer; NOS = Nitrous Oxide Synthase; SOCS = Suppressor Of Cytokine Signaling; TGF = Transforming Growth Factor; TNF = Tumor Necrosis Factor (Taken from Hagberg et al., 2012).

Although the sequence of inflammatory mechanisms is similar, the microglial and astroglial activation as well as the panel of cytokines and chemokines released could be slightly different between adult and fetal brains. This point of view is supported by studies showing that neonatal microglia are different from adult microglia, as they have a slightly more activated phenotype under healthy conditions (higher expression of low Major Histocompatibility Complex class II (MHCII), Cluster of Differentiation 86 (CD86) and CD40, slightly different proliferative response to macrophage colony-stimulating factor and granulocyte-macrophage

colony-stimulating factor, and less dependence on astroglia for self-renewal (Hagberg et al., 2012; Santambrogio et al., 2001).

However, unlike brain inflammation in adults, the impact of inflammation in immature brain, and more specifically this impact on the “immature” BBB remain very little studied. Our study is devoted to understand the impact of pro- and anti-inflammatory cytokines and chemokines on the structural and functional components of the NVU. Thus, the part below is a brief review of some studies showing the impact of some cytokines and chemokines released during cerebral inflammation and found in high levels in the brain of patients with neurological disorders, on BBB components components and more particularly on tight junction and drug efflux transporter expression both in children and adults.

4.3 The impact of cytokines on BBB components

Cytokines are small protein molecules playing a role in signal transmission from the extracellular environment to the nucleus, and this through specific receptors or second messenger molecular pathways. In normal conditions, cytokines are present at very low levels in the brain and exercise normal CNS physiological functions (Pollmacher et al., 2002). After injury, cerebral ischemia, infectious or during autoimmune diseases, a number of cytokines are rapidly upregulated in the course of an inflammatory immune response. Cytokines are classified into pro-inflammatory cytokines generally viewed as neurotoxic, and anti-inflammatory cytokines viewed as neuroprotective. However, the effects of cytokines can depend on which cell type they act upon and whether it is a direct or indirect effect (Allan and Rothwell, 2001). Sites of action are determined by the presence of the appropriate receptor on the membrane of a particular cell. Most cytokine receptors have been described in the CNS (Allan and Rothwell, 2001; Szelenyi, 2001), including Proinflammatory cytokines: IL-1 α , IL-1 β , IL-2, IL-6, IL-8 and TNF- α , and anti-inflammatory cytokines: IL-4, IL-10, IL-13, TGF- β , InterFeroN (IFN- α) (Aloisi 2001). The main sources of cytokines in the CNS are microglia and astrocytes, but also infiltrating immune cells as well as CNS-associated macrophages (Raivich and Banati 2004). The mechanisms that control expression of different cytokines are often related; for example: TNF α stimulates expression of IL-1 and IL-6, and IL-1 can induce both IL-6 and TNF α . Thus, following an insult to the brain, initial upregulation of cytokines leads to infiltration of other. Previous work in our laboratory as well as studies from other laboratories have also shown that proinflammatory cytokines such as

TNF α and IL-1 β caused a released of endothelin-1 (ET- 1) at the BBB level (Bauer et al., 2007; Didier et al., 2003; Marsden et al., 1992). In turn, ET-1 causes the release of other cytokines and chemokines such as MCP-1 (Chen et al., 2001).

4.3.1 IL-1 α

In adults, high levels of IL-1 were found in brain of patients with Alzheimer's disease (Shaftel et al., 2008), HIV-associated dementia (Zhao et al., 2001) and ischemic brain injury (Luheshi et al., 2011). It has been demonstrated that Circulating IL-1 α cross the BBB via saturable transport (Banks et la., 1995). However, there is very little information describing the impact interleukin-1 α on BBB components.

IL-1 α expression has been found to be closely associated with areas of focal BBB breakdown and neuronal death, and this 24 h after ischemia induced by occlusion of the middle cerebral artery in mice (Luheshi et al., 2011). Curiously, Tohmi et al. showed that during early development, circulating IL-1 efficiently crosses the neonatal rat BBB and induces brain inflammatory reactions with influences on behavioral traits in later stages such those observed in schizophrenia, a developmental psychiatric disease. However, this effect of circulating IL-1 α was not observed when the cytokine was administrated in adult rats. The increases in brain IL-1 α immunoreactivity was less pronounced following IL-1 α administration and behavioral abnormalities were not manifested at the adult stage. When IL-1 α was administered to juveniles, BBB permeability was limited (Tohmi et al., 2007). This age-related difference in the impact of IL-1 α in the brain engenders unanswered questions: At the origin of this difference, age-related BBB differences or immune-response differences? If the IL-1 α was administrated directly in the brain, would the outcome of the triggered cerebral inflammation be the same irrespectively of age?

4.3.2 IL-1 β

In the human BMVECs cell line hCMEC/D3, IL-1 β significantly reduces BCRP mRNA and protein expression as well as its activity (Poller et al., 2010). In rat astrocytes culture, exposure to IL-1 β increased abcb1 protein expression (Ronaldson and Bendayan, 2006). The bacterial protein LipoPolySaccharide (LPS) affects the permeability of BBB TJs. This is mediated by the

production of free radicals, IL-6 and IL-1 β (Roberts and Goralski, 2008; Gaillard et al., 2003). Expression of IL-1 β in the brain induced BBB permeability (Argaw et al, 2008).

The impact of IL-1 β on SLC transporters at the BBB level remain poorly characterized. Some studies have been performed at the hepatic level. In human hepatocytes treated 24h with 1 ng/ml of IL-1 β , the mRNA levels of the following transporters were downregulated: OATP-B, OATP-C, and OATP8, MRP3, MRP4, and BCRP (LeVee et al., 2008).

4.3.3 IL-6

IL-6 has been found in high amounts in the CSF of adult patients with Alzheimer's and Parkinson's disease (Blum-Degen et al., 1995), stroke and brain trauma (Schwaninger et al., 1999). In CNS diseases affecting immature brain or having developmental etiology, IL6 expression is increased after seizures in epilepsy, and in serum during schizophrenia. Animals treated with cytokines including IL-6 as embryos or neonates exhibit schizophrenia-like behavioral abnormalities after puberty (Watanabe et al, 2010). Although it is suggested that IL-6 overexpression in CNS diseases may have protective effect, some studies performed in experimental murine model of cerebral palsy, showed that newborn treatment with IL-6 enhanced ibotenate excitotoxicity and exacerbate brain lesion (Dommergues et al, 2000).

At the BBB level, in pathological states, during stroke and trauma for example, experiments showed a production of a mediator of inflammation, the Bradykinin, which stimulates the release of IL-6 from murine astrocytes, and this lead to opening of the BBB (Shwaninger et al 1999). Another study showed that LPS infection affect the permeability of BBB and this is in conjunction with the production of several inflammatory mediators including IL-6 (Roberts and Goralski, 2008; Gaillard et al., 2003; Veldhuis et al., 2003). *In vitro*, LPS potentiated the release of IL-6 by mouse BMVECs and this IL-6 released from BMVECs acted at the luminal but not the abluminal surface to enhance HIV-1 transcellular transport across the BBB (Dohgu et al., 2011). IIL-6 has also an impact on ABC transporters: For example, IL-6 reduced ABCB1 mRNA, protein and activity in human hCMEC/D3 cell line (Poller et al., 2010) or porcine BMVECs (Von Wedel-Parlow et al., 2009). In human astrocytes cultures, IL-6 decreased abcb1 protein expression and function (Ronaldson and Bendayan, 2006).

The impact of IL-6 on BBB SLC transporters is little studied. Some data exist on the impact of IL-6 on SLC transporters in hepatocytes. Exposure of primary human hepatocytes to cytokine

Oncostatin M (OSM) (a member of the interleukin (IL-6 family) down-regulates mRNA levels of OATP1B1, OATP1B3, OATP2B1, OAT1 and OAT2, ABCC2 and ABCG2, without however repressing those of ABCB1, ABCC3 and ABCC4. The cytokine also reduced OATP1B1, OATP2B1 and ABCG2 protein expression (Le Vee et al., 2011).

4.3.4 Endothelin-1

Endothelin-1 (ET-1) is a 21 amino acid peptide hormone with diverse physiological functions (Rubanyi and Polokoff, 1994). In the vascular system, endothelins, have a vasoconstricting role and contributes to the development of vascular disease in hypertension and atherosclerosis (Barton, 2000; Schiffrin, 1999). They also affect physiology and pathophysiology of the immune system (Nett et al., 2006), liver (Berthiaume et al., 2005), glucose homeostasis (Lteif et al. 2007), and many other physiological and pathological functions (Barton and Yanagisawa, 2008).

The diverse biological actions of ET-1 are mediated by two different ET-1 receptor subtypes, ETA and ETB (Davenport, 2002) different in their tissue distribution, ligand binding and signal transduction (Offermanns, 2004). The ETA receptor is present in many tissues but it is highly expressed in aorta, heart and kidney (Webb, 1997). The ETB receptor is predominantly expressed in brain, lung, kidney and aorta (Webb, 1997; Ogawa et al., 1991). It has been described that in the vascular system, ETA and ETB exert opposite effects: ETA causes long-lasting vasoconstriction, whereas ETB transient vasodilation (Kedzierski and Yanagisawa, 2001; Levin, 1995).

At the BBB level, short term exposure of rat BMVs to ET-1 induces rapid and reversible reduction of ABCB1 transport activity, while no change in protein expression was observed (Hartz et al., 2006; Hartz et al., 2004). In the same context, long term exposure to ET-1 increases ABCB1 protein expression and transport activity (Bauer et al., 2007). ET-1 also induced ABCG2 protein expression in primary porcine BMVECs *in vitro* (von Wedel-Parlow et al., 2009).

4.4 The impact of chemokines on BBB components

Chemokines or 'chemotactic cytokines' are cytokines playing a major role in the recruitment of immune cells from the blood into the CNS and in the migration of some cells, including microglia. Structurally, they are a superfamily of small peptides (8–14 kDa) divided into four

subfamilies (CC, CXC, CX3C and C subfamilies) depending on their structural similarities. CNS chemokines are produced by adult microglia and astrocytes. Their biological effects are mediated via specific G-protein-coupled receptors (Vuillat-Savarin and Ransohoff, 2007; Ubogo et al., 2006).

4.4.1 MCP-1

MCP-1 (or Monocyte Chemoattractant Protein-1) also called MCAF (Macrophage Chemoattractant and Activating Factor) or CCL2 (CC Chemokine Ligand 2) (Matsushima et al., 1989, Yoshimura et al., 1989b) is a chemokine belonging to the CC subfamily and considered to be the most potent chemoattractant chemokine (Sozzani et al, 1994). It is able to attract to the site of inflammation cells expressing the receptor CCR2 (Siebert et al., 2000; Wang et al., 2002; Kuziel et al., 1997,), which includes, among others, monocytes, T cells, myeloid dendritic cells and Natural Killer (NK) cells (Nakamura et al., 1995; Taub et al., 1995a; Taub et al., 1995b; Allavena et al., 1994, Carr et al., 1994). During HIV infection, MCP-1 significantly enhances the transmigration of HIV-infected leukocyte across BMVECs of the BBB. This transmigration causes BBB disruption (Buckner et al., 2006).

Chapter V

Facts and Hypothesis

Several reasons emphasize the importance of comparing the functional status of juvenile BBB to the adult one, as well as revisiting the impact of inflammation on the BBB, either in the “immature” and in adults and this using both *in vivo* and *in vitro* approaches.

1- There are few pieces of evidence that BBB components under inflammatory impacts are regulated differently in juvenile organisms when compared to adults:

A study done by Stolp and al. reports that new born rats who received repeated intraperitoneal injections of LPS to produce prolonged period of inflammation, showed alterations in cellular localization of claudin-5 with no increase in BBB permeability. However, barrier permeability to sucrose was significantly increased in LPS treated animals at the adult stage but not at P20 or earlier. Behavioural tests showed that LPS treated animals at P20 exhibited alterations in behaviour different from those observed in adults. Stolp et al. suggested that an inflammatory insult during brain development can change BBB permeability and behaviour in later life. They have also suggested that the impact of inflammation can occur in several phases (short- and long-term) and that each phase might lead to different behavioural modifications (Stolp et al., 2012). These facts suggest a differential impact of inflammation on the juvenile BBB when compared to the adult one. This differential regulation may be due to different inflammatory mechanisms and/or cell signaling triggered in an age-dependent manner.

2- Another reason to study the maturational BBB is the evidence given by other studies on developmental BBB showing that pre- or neonatal inflammation sensitizes the brain to later insults. For example, a study done by Aden et al. who showed that systemic inflammation sensitizes the perinatal brain to an ischemic/excitotoxic insult and that the mechanisms involve is partly mediated by an imbalance between pro- and anti-inflammatory cytokines (Aden et al., 2010).

All these studies suggest that developmental inflammation may play an important role in the initiation of a number of neurological disorders, either by immediate death of cells during

susceptible periods of brain development, or by long-term alterations in the vasculature and function of the BBB, which may increase the susceptibility of the brain to further exposure to drugs and toxins. The outcome of developmental inflammation is likely to reflect the timing, magnitude and origin of the inflammatory response (Stolp and Dziegielewska , 2009; Saunders et al., 2008).

3- Although there is experimental evidence that the BBB is functionally tight during the earliest stages of brain development (Saunders et al., 2008; Ek et al., 2006), there are evidences that there are structural changes in TJ occurring as the brain matures (Anstrom et al., 2007; Virgintino et al., 2004). This may make BMVs vulnerable during development (Virgintino et al., 2004). However, the brain barriers are not affected by inflammation occurring during very early stages of development in the same manner as at later stages (Stolp et al., 2005; Anthony et al., 1997), thus this the vulneranility of BMVs in immature brain does not tell the whole story and further investigations on developmental differences in the inflammatory response need to be studies (Stolp and Dziegielewska , 2009).

OBJECTIVES

The BBB is a highly specialized neurovascular structure localized at the level of BMVs. Its major functions are protection of CNS and maintenance of its homeostasis. Several factors contribute to this unique functional role: 1) Neighboring endothelial cells of BMVs are associated by TJ that seal the spaces between endothelial cells and limit paracellular diffusion into the brain of potentially neurotoxic substances carried in the blood. 2) A number of influx/efflux transport and carrier systems are expressed and polarized on the luminal and abluminal surface of the NVU to overcome TJ and to meet the high nutrient and energy demand of the brain. Among these systems, ABC and SLC transporters facilitate brain uptake of nutrients (e.g. glucose, amino acids and ions) from the blood, extrude metabolic wastes and keep neurotoxic substances from entering the brain. But these efflux transporters also keep therapeutic drugs from entering the brain and reaching their site of action, which leads to treatment failure of several brain diseases. To overcome ABC and SLC transporters, recent research in the field aims at identifying the intracellular signaling mechanisms that regulate these transporters in the NVU in order to modulate their activity and improve pharmacotherapy of brain diseases.

For years, ABC and SLC transporters have been studied in adult organism. But, there is a wide spread belief among pediatricians, neurologists and neuroscientists that the BBB in embryo, fetus, new born and infant is “immature”, implying caution in giving drugs to infants. However, current knowledge on the functional status of the BBB in immature organism remains very limited (Saunders et al., 2012). Thus, from the clinical perspective of developing new drugs with enhanced efficacy in children’s CNS, it is important to understand 1) the role of the BBB drug efflux transporters in children’s brain and 2) the mechanisms that regulate their functional expression under normal and pathological conditions.

This study has several main objectives:

- 1- To study the ontogenesis of some structural and transporters components of the BBB in rats from birth to adulthood.
- 2- To investigate the role of the canonical Wnt signaling in the regulation of four drug efflux transporters: P-gp, bcrp, oat3 and oatp1a4.
- 3- To compare, between juvenile and adult rats, the impact of acute cerebral inflammation on the regulation of two of the most clinically relevant ABC transporters of the BBB: P-gp and bcrp.

Our working methodology was articulated as follows:

1- Development of research tools (see chapter III from the “results and discussion” section):

- *in vivo* models of acute cerebral inflammation in juvenile and adult rats: these models consist of an intra-cerebroventricular (icv) injection of endothelin-1, a neuropeptide known to trigger inflammatory reactions.
- An *in vitro* BBB model from juvenile rat cells: This model consists of a co-culture of primary astrocytes and BMVEC expressing endothelial junctional proteins and efflux transporters (e.g. P-gp and bcrp).

2- BBB ontogenesis study by profiling a number of its structural (inter-endothelial TJ) and functional (SLC and ABC transporters) components from birth to adulthood.

This study consists on the examination, using real time PCR and western-Blot techniques, of gene and protein expressions of a series of ABC and SLC efflux transporters, *ex vivo*, in isolated BMVs from new born, juvenile and adult rats. We also measured, *in vivo*, the activity of four BBB drug efflux transporters (P-gp, bcrp, oat3 and oatp1a4) by measuring the unbound partitioning coefficient $K_{p,uu}$ which determined the net influx and efflux across the BBB of known substrates which were administrated intravenously in catheterized rats. Furthermore, we investigated the role of an interaction between Wnt/ β -cat and ET-1 signaling in the regulation of these transporters at the BBB level in adult rats.

3- Impact of acute cerebral inflammation study by comparing, between juvenile and adult rats, the regulation of ABC transporters during acute cerebral inflammation mediated by ET-1.

This study consists at evaluating, *ex vivo* and *in vivo*, the impact of icv injection of ET-1, on the expression and activity of P-gp and bcrp. Under inflammatory conditions, BBB drug efflux transporters might be regulated differently in juvenile rats when compared to adult rats, due to age-related differences in the BBB components between juveniles and adults, or to a maturational difference in neuro-inflammatory response.

Our study will help to shed light on BBB changing properties during ontogenesis and to probably provide insights on the role of Wnt/ β -cat pathway in regulating ABC and SLC efflux transporters in adults. The determination of pathways modulating the activity of drug efflux transporters may open new therapeutic avenues to improve drug delivery into the brain, and this by targeting these pathways. It is also important to compare regulation of ABC efflux

transporters between juvenile and adult rats under inflammatory conditions. Indeed, giving the evidence of age-related differences in BBB components might emphasize the importance of taking into account the specific properties of the juvenile BBB and distinguishing it from the adult one in the aim of developing new drugs with enhanced efficacy in children's CNS.

RESULTS & DISCUSSION

This “Results and discussion” section is divided into three chapters:

Chapter IA: Descriptive Blood-Brain Barrier Ontogenesis Study

In this part, I summarize and discuss results of the descriptive study of BBB ontogenesis from birth to adulthood performed in rats (Harati et al., 2012a).

Chapter IB: Understanding the Molecular Mechanisms of Blood-Brain Barrier Ontogenesis

In this part, I summarize and discuss results of the mechanistic study of BBB ontogenesis from birth to adulthood performed in rats (Harati et al., 2012a).

Chapter II: Regulation of P-glycoprotein and Bcrp Expression and Transport Activity at the Blood-Brain Barrier by Cerebral Inflammation Triggered by Endothelin-1

This part summarizes and discusses the comparison, between juvenile and adult rats, of the impact of acute cerebral inflammation triggered by endothelin-1, on the expression and transport activity of P-gp and bcrp (*in vivo*) (Harati et al., 2012b).

Chapter III: Research Tools

This part describes and discusses research tools used to study BBB ontogenesis as well as the impact of inflammation on the regulation of structural and functional components of the BBB.

3.1. The *Ex Vivo* Blood-Brain Barrier Model

This part describes isolated rat BMVs used as an *ex vivo* model of the BBB.

3.2. The *In Vitro* Blood-Brain Barrier Model

This part describes the *in vitro* BBB models generated from adult and juvenile rat cells expressing endothelial junctional proteins and efflux transporters (e.g. P-gp and bcrp).

3.3. The *In Vivo* Model of cerebral inflammation

This part describes the *in vivo* models of acute brain inflammation in juvenile and adult rats.

Chapter IA

The Blood-Brain Barrier Ontogenesis: The Descriptive Study

This BBB ontogenesis study has been reported in an article published in the Journal of Molecular Pharmaceutics (September 2012) and untitled:

“P-glycoprotein, Breast cancer resistance protein, Organic anion transporter 3 and transporting peptide 1a4 during Blood-Brain Barrier Maturation: Involvement of Wnt/ β -Catenin and Endothelin-1 Signaling” (Harati et al., 2012a).

The descriptive BBB ontogenesis study has been presented as a poster in the 14th Symposium on Signal Transduction in the Blood-Brain Barriers, Istanbul, Turkey (September 2011):

“Functional Expression of ABC and SLC Transporters during Blood-Brain Barrier Maturation”.

1.1 Summary

Our current knowledge on the functional status of the BBB in maturational brain is very limited. However, from the clinical perspective of developing new drugs with enhanced efficacy in children’s CNS, it is important 1) to understand the role of the BBB drug efflux transporters in children’s brain and 2) to elucidate the mechanisms that regulate their functional expression under normal and pathological conditions. In this study, we established the gene expression profile of a panel of BBB structural and functional components during brain maturation.

Male Wistar rats at four stages of brain maturation were used in our experiments: Post-natal (P) P2 corresponding to preterm stage in human, P14 corresponding to human neonates, P21 corresponding to pediatric and P84 corresponding to adults in human.

Gene expression of BBB components were assessed *ex vivo* in BMVs, site of the BBB. This expression was measured by quantitative real-time PCR analysis. The experimental method is described in the article Harati et al., 2012a. Results on *abcb1*, *abcg2*, *sl22a8* and *slco1a4* are presented in figures 2a, 2c, 3a and 3c from our article Harati et al., 2012a. Supplemental findings on other BBB components are presented in figures 12, 13 and 14 below.

Gene expression of structural components during BBB maturation

Among the numerous BBB structural components, we assessed gene expression of three essential TJ components: Claudin-3, Claudin-5 and Zo1 (Figure 12).

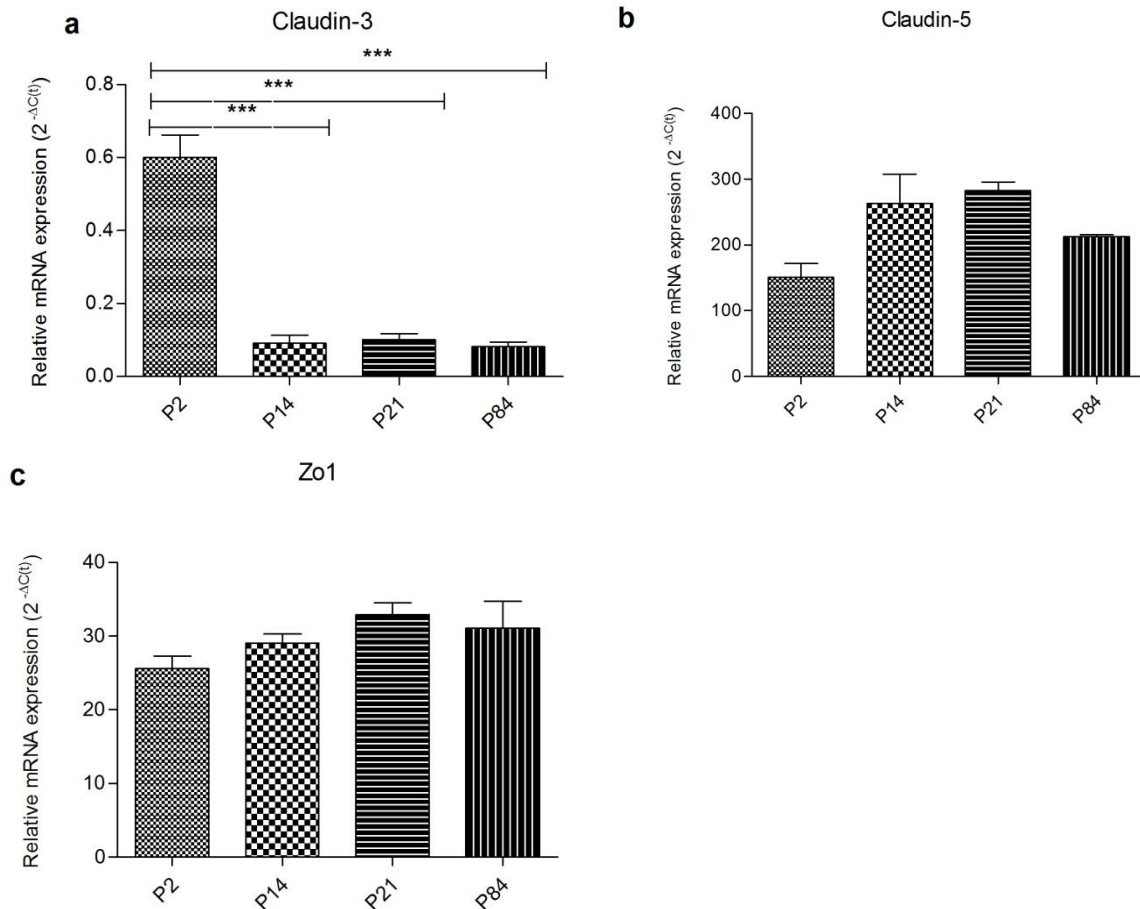


Figure 12. Relative mRNA expression of TJ components in isolated BMVs during brain maturation

Rats at different stages of brain development equivalent to the preterm (P2), neonate (P14), pediatric (P21), and adult (P84) human brain were used. mRNA transcription profiles for Claudin-3 (a), Claudin-5 (b) and Zo1 (c) were assessed by semi-quantitative real-time PCR performed in freshly isolated rat BMVs. Results are means \pm SEM for 3 batches of BMVs (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of BMVs. Variance analysis (one way ANOVA) was performed to assess the significant differences between different groups. Statistical significance was set at $p < 0.05$. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

Among the analyzed structural components, claudin-5 was more expressed than Zo1 and claudin-3 genes. Moreover, our results showed no tremendous significant variation of claudin-5 and Zo1 genes expressions during brain maturation; these genes are well expressed from birth

and keep almost the same level of expression as the brain matures. In contrast, claudin-3 had its higher expression at birth; at P14, P21 and P84, this gene was detected at very low levels.

Gene expression of ABCC family members during BBB maturation

We assessed gene expression of six ABCC transporters expressed at the BBB level: abcb1, abcg2 (respectively, figures 2a and 2c from our article Harati et al., 2012a), abcc1, abcc4, abcc5, and abcc6 (Figure 13 below).

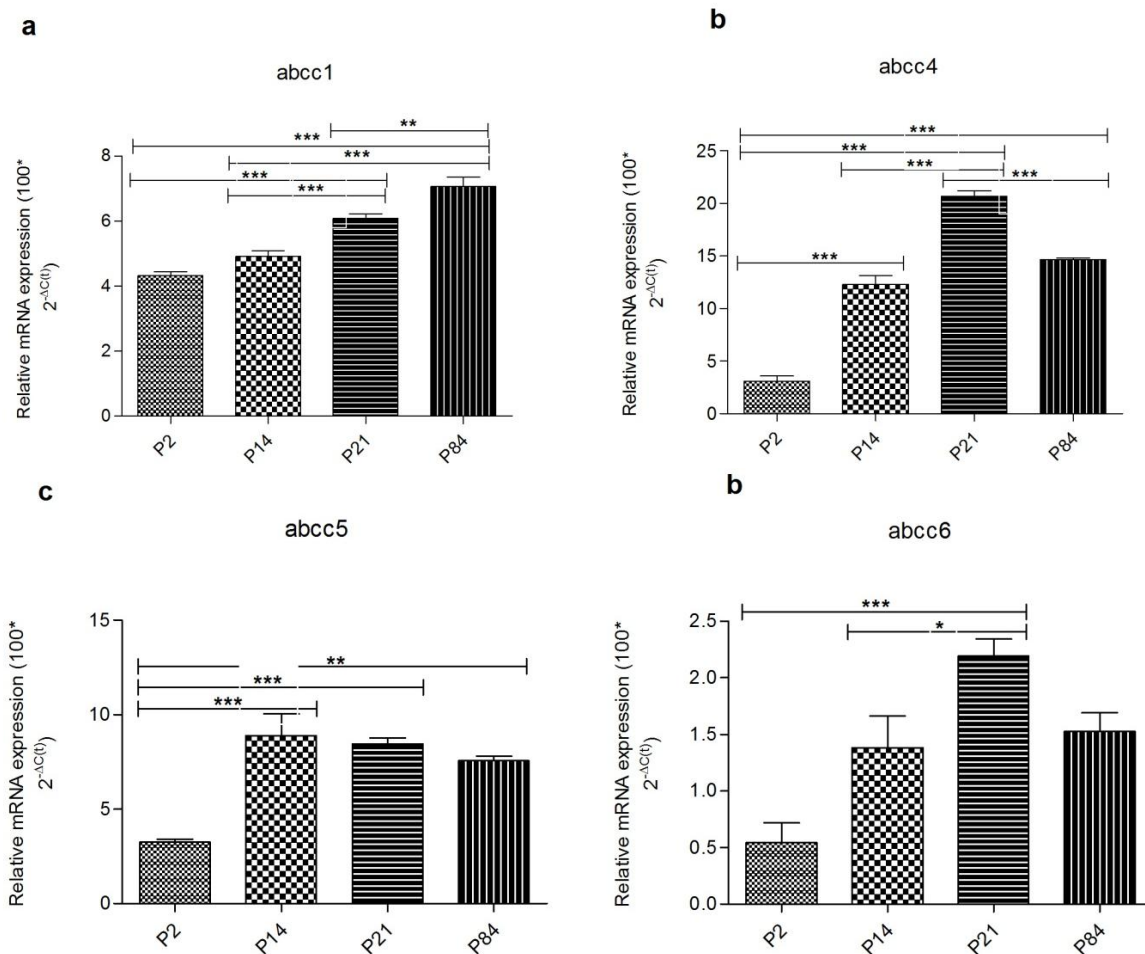


Figure 13. Relative mRNA expression of ABCC transporters in isolated BMVs during brain maturation

Rats at different stages of brain development equivalent to the preterm (P2), neonate (P14), pediatric (P21), and adult (P84) human brain were used. mRNA transcription profiles for abcc1 (a), abcc4 (b), abcc5 (c) and abcc6 (d) were assessed by semiquantitative real-time PCR performed in freshly isolated rat BMVs. Results are means \pm SEM for 3 batches of BMVs (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of BMVs. Variance analysis (one way ANOVA) was performed to assess the significant differences between different groups. Statistical comparisons: *P < 0.05, **P < 0.01, and ***P < 0.0001.

Among the analyzed ABC transporters, *abcb1* was the most expressed gene, more than *abcg2* and *abcc4* which have nearly same levels of expression; *abcc1* and *abcc5* were less expressed than *abcb1*, *abcg2* and *abcc1*. The lesser expressed gene was *abcc6*. During maturation, gene expression of the analyzed ABC transporters varied in an age-depend manner: most of the analyzed gene increase as the brain mature. In addition, our results showed that the analyzed genes can be grouped following the shape of their profile expression: *abcb1*, *abcc1* and *abcc5* can be grouped together for having the same expression profile (an increasing in expression as the brain mature), and *abcg2*, *abcc4* and *abcc6* can be grouped in another group for having the same expression profile (an increasing in expression from birth till P21, then we noted a slight decrease at P84); one exception: the *abcc4*, where we noted a significant decrease in its expression at P84.

Gene expression of SLC family members during BBB maturation

We assessed the gene expression of six SLC transporters expressed at the BBB level: *slc22a8*, *slc1a4* (respectively, figures 3a and 3c from our article Harati et al., 2012a), *slc2a1*, *slc15a2*, *slc7a7*, and *slc7a8* (Figure 14 below). Informations about *slc22a8* and *slc1a4* are available in the introduction part. *Slc2a1* is well studied at BBB level; it facilitates the transport of glucose from blood to brain. Little information exists about *Slc7a8* and *slc7a7* transporters at BBB level. *Slc7a7* is known to be cationic amino acid transporter, and *slc7a8* a neutral amino acid transporter. Regarding the *slc15a2* transporter, also known as Peptide Transporter-2 (PEPT2): It is a transporter involved in peptide transport and is expressed in brain by astrocytes, subependymal cells, ependymal cells and epithelial cells of choroid plexus (Berger and Hediger, 1999).

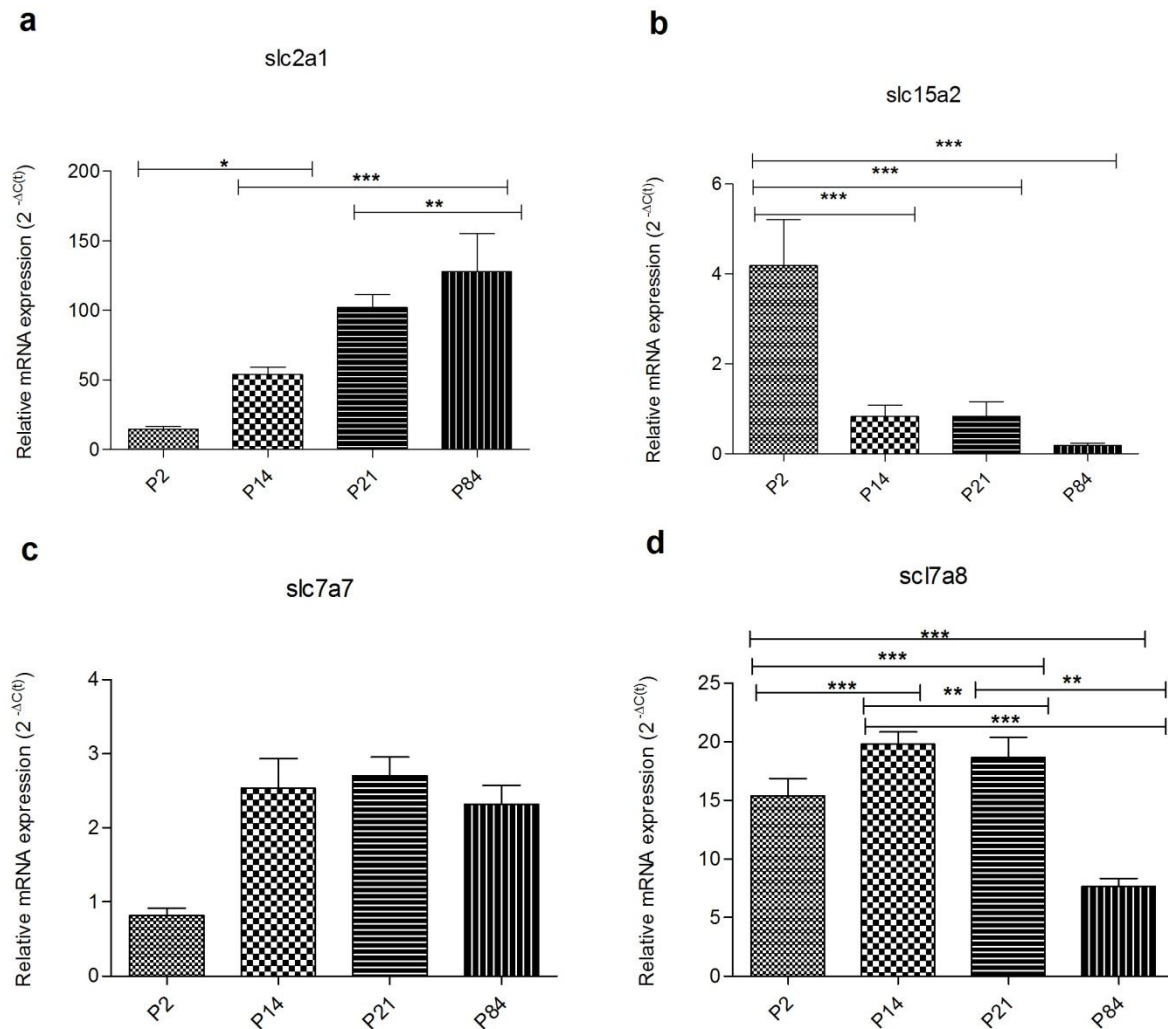


Figure 14. Relative mRNA expression of SLC transporters in isolated BMVs during brain maturation

Rats at different stages of brain development equivalent to the preterm (P2), neonate (P14), pediatric (P21), and adult (P84) human brain were used. mRNA transcription profiles for *slc2a1* (a), *slc15a2* (b), *slc7a7* (c) and *slc7a8* (d) were assessed by semiquantitative real-time PCR performed in freshly isolated rat BMVs. Results are means \pm SEM for 3 batches of BMVs (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of BMVs. Variance analysis (one way ANOVA) was performed to assess the significant differences between different groups. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

Among the analyzed SLC transporters, *slc1a4* was the most expressed gene, followed by *slc22a8* then *slc2a1* then *slc7a8*. The lesser expressed genes were *slc7a7*. *Slc15a2* was mainly detected at P2; at P14 and P21, it was barely detectable, and in adults it was not detected. During maturation, gene expression of the analyzed SLC transporters varied in an age-depend manner:

slc2a1, slco1a4 and scl22a8 increase as the brain matures. In contrast, slc7a8 decreased with aging, with no variation of slc7a7 gene expression.

Functional expression of the main drug efflux transporters during BBB maturation

To identify the impact of these variations on drugs' transport across the BBB, we focused our study on four drug efflux transporters: P-gp, bcrp, oat3 and oatp1a4; we established their protein as well as their activity profile during BBB maturation. Protein expression of the four drug efflux transporters were assessed *ex vivo* in BMVs, site of the BBB, and evaluated by Western-Blot technique. Efflux activity of the transporters was measured *in vivo* by calculating the unbound brain/plasma concentration ratio ($K_{p,uu}$) of known substrates for these transporters when administrated intravenously in catheterized rats. Experimental method is described in the article Harati et al., 2012a. Our results showed that gene and protein expressions of the analyzed transporters increase as the brain matures (Figures 2 and 3 from Harati et al., 2012a), with no variation in P-gp and bcrp activity (Figures 4a and 4b respectively from Harati et al., 2012 a), while transport activities of oat3 and oatp1a4 increase during brain maturation from preterm up to adulthood (See figures 4c and 4d respectively from Harati et al., 2012a).

1.2 Discussion

This BBB descriptive ontogenesis study had several major findings:

- (i) Claudin-5 and Zo1 genes are well expressed at birth and keep almost the same level of expression as the brain matures. No significant differences were detected between new-born and adult rats (figure 12).

Claudin-5 and Zo1 are capital elements of TJ where they play a key role in establishing the barrier for passive diffusion between blood and brain: Claudin-5 maintains normal BBB function (Vorbrodt and Dobrogowska, 2003) and Zo1 acts as a central organizer of the TJ complex where loss or dissociation of these components from the junctional complexes is associated with increased barrier permeability Cardoso et al., 2010; Choi and Kim, 2008). Our results showed that these two essential TJ components are highly expressed at birth, especially Claudin-5, with no significant differences in their gene expressions between new-born and adult rats. These findings are in agreement with the literature. Indeed, it has been demonstrated that the BBB

develops during fetal life and is well formed by birth, especially to proteins and macromolecules (Ballabh et al., 2004; Saunders et al., 2000; Keep et al., 1995; Preston et al., 1995; Moos and Møllgård, 1993; Saunders, 1992; Tauc et al., 1984; Olsson et al., 1968). Preston et al. (1995) also showed that BBB permeability to the mannitol (182 Da) in the brain of rats of 1 week of age is identical to that of adult rats. In human, Claudin-5 expression was detected in the brain capillary endothelium of 14 week human fetus (Virgintino et al., 2004). Pioneering studies by Grontoft (1954) in stillborn human fetuses from approximately 12 weeks gestation and from perinatal deaths have demonstrated that a post-mortem BBB to trypan blue is present from at least the start of the second trimester, which is comparable to that of adult human. It has also been demonstrated that BBB in embryos and neonates does protect the brain from low molecular weight molecules, and this is because TJ are well-formed and functionally effective in early stages of brain vascularization (Daneman et al., 2010a; Ek et al., 2006; Johansson et al., 2006; Nitta et al., 2003). Taken together, these studies suggest that, in mammals, barrier is significantly tightened on the day of birth (Butt et al., 1990), and our findings are in agreement with these data. Regarding the maturational profile of Claudin-5 and Zo1, our results are in agreement with previous works showing no significant differences in the expression of these two TJ components as the brain matures; for example, a study done by Kratzer et al. shows no significant differentiation in Claudin-5 and Zo1 expression between P9 and adult rats (Kratzer et al., 2012).

- (i) In contrast to Claudin-5 and Zo1, Claudin-3 had its higher expression at birth; at P14, P21 and P84, this gene was detected at very low levels (figure 12).

Claudin-3 is a TJ component, suggested to have a role in determining permeability in pathological conditions. However, Claudin-3 is known to be specifically upregulated during BBB establishment and maintenance although its specific function for BBB TJs remains elusive (Liebner and Plate, 2010). Its high expression at birth is in agreement with this role.

- (i) Gene expression of most of the analyzed ABC and SLC transporters varied in an age-depend manner (figures 13, 14 respectively).

For the first time, we elucidated gene and protein expression of these drug efflux transporters in BMVs, site of BBB during brain maturation. We showed variation of expression as the brain matures. To conclude on this variation, this gene study deserves to be completed by a functional one. Indeed, as these analyzed components play important role at the BBB level, especially in

multidrug resistance phenomenon, the variation observed in their expression during brain maturation emphasize the importance of conducting functional studies to establish their functional status in the maturational brain.

Regarding the gene *slc15a2*: It is a transporter involved in peptide transport and known to be expressed in brain by astrocytes, subependymal cells, ependymal cells and epithelial cells of choroid plexus (Berger and Hediger, 1999), but not at the BBB level. Studies on *Slc15a2* at the BBB level are rare. One study performed *in vitro* on human BMVECs cell line hCMEC/D3, showed that this cell line does not express *Slc15a2* which is consistent with previous studies obtained *in vivo* in the human BBB (Smith et al., 2004; Berger and Hediger, 1999). However, the profile of *slc15a2* during ontogenesis has never been studied, and for the first time, we showed that this transporter is expressed in immature brain especially at birth, which suggest that *slc15a2* might have a role in the developmental BBB during embryonic life. Further studies need to be performed on *slc15a2* expression in the developmental BBB, in order to clarify its eventual role at the BBB level.

- (ii) During rat brain maturation, protein expression of P-gp, *bcrp*, *oat3* and *oatp1a4*, like their gene products, increased with aging from preterm up to adulthood (Figure 2 and 3, Harati et al., 2012a).

For the first time, we elucidated gene and protein expression of these four drug efflux transporters in BMVs, site of the BBB during brain maturation. Previous research has established gene expression of P-gp and *bcrp* in the forebrain and brainstem at different developmental stages: E13, E15, E18, P1, P14, P21 and adulthood. These had shown that P-gp expression progressively increased during development (about 10–12-fold) in both the forebrain and brainstem, while *Bcrp* showed similar gene expression at all ages and in both brain regions in human (Ek et al., 2010; Daood et al., 2008). Daood et al. reported that, in post-mortem CNS tissue from infants born 22^{0/7} - 42^{0/7} week gestation and adults, P-gp/ABCB1 immunostaining was observed in BMVECs as early as 22^{0/7} weeks and prevalence and intensity increase with maturation. However, they also reported that the intensity of BCRP/ABCG2 immunostaining did not vary notably with maturation (Daood et al., 2008).

Our study was performed on BMVs, site of the BBB and our results suggest a potential age-related response to CNS drugs. Given the clinical importance and significance of these data,

we continued our study by evaluating functional status of these transporters during brain maturation.

- (iii) There was no variation in activity of BBB-P-gp and bcrp transporters between juvenile and adult rat while that of oat3 and oatp1a4 increase when passing from juvenile to adult stage (Figure 4, Harati et al., 2102a).

These results show that:

- 1) P-gp and bcrp transporters are functionally mature in childhood; however oat3 and oatp1a4 become fully mature only in adulthood. This means that: a) Children brain may be more vulnerable to neurotoxics than adult brain b) the response of CNS to drugs is age-related, and this require caution in giving drugs to infants who can not be assumed to “small adults”.

Regarding the increased in BBB transport activity mediated by oatp1a4, it is worth mentioning that this transporter is located on the luminal and abluminal side of the BMVECs suggesting the possibility that oatp1a4 mediates uptake and subsequent efflux process in the transcellular transport across blood-brain barrier. This is to say that the lower $K_{p,uu}$ for rosuvastatin to the higher expression of oatp1a4 could be expected to efflux substrate from brain to blood.

- 2) Transporters activity does not always correlate with their expression.

Indeed, there was no correlation between P-gp, bcrp and scl22a8 proteins expression and their transport activity at the BBB level from P21 and P84 rats (Figures 2, 3 and 4 from Harati et al., 2012a). This suggests that other factors may cause functional but not transcriptional regulation of BBB transporters in juvenile and adult BBB. This highlights the importance of evaluating drug transport activity during studies on drug efflux transporters development for better assessment of drug pharmacokinetic profiles.

Chapter I B

Understanding the Molecular Mechanisms of Blood-Brain Barrier Ontogenesis

1.1 Summary

The descriptive study sheds light on BBB changing properties during ontogenesis. However, the ultimate question that arises from this study relates to the mechanism underlying regulation of functional expression of P-gp, bcrp, oat3 and oatp1a4 during brain development as well as in adults 1) because the role of molecular mechanisms regulating the functional expression of these BBB drug efflux transporters are not fully understood, and 2) in the perspective of finding molecular targets that can be used to modulate BBB drug efflux transporters and thus enhancing drugs bioavailability in either adult and pediatric brain. A mechanism implicated in the regulation of BBB structural components during embryonic development is Wnt/ β -cat signaling (Liebner et al., 2008). In this pathway, interaction of Wnt proteins with cell surface receptors leads to inactivation of Glycogen Synthase Kinase-3 β (GSK-3 β) resulting in stabilization of β -cat and its translocation into the nucleus where it acts as component of a transcriptional signaling complex with members of the T Cell Factor (Tcf) (family of Transcription Factors) to activate target genes (Logan et al., 2004) (See figure 9, 10 and 11 from the introduction part). Interestingly, when we established the β -cat profil across the different maturational stages, we illustrated for the first time a downregulation of nuclear β -cat expression in BMVs, and this when P-gp, Bcrp, oat3 and oatp1a4 transporters are at their highest expression levels (See figure 5 from Harati et al., 2012) which suggest a link between protein transport expression and β -cat signaling. However, the role Wnt/ β -cat signaling in regulating the maturation of the BBB remains to be clarified. This was the other main target of the present study.

For that, we used an *in vivo* approach based on the activation of β -cat in rat brain, by intracerebroventricular injection (icv) of a GSK-3 β inhibitor, the BIO (6-Bromo-Indirubin-3'Oxime). Then we followed gene and protein expression of efflux transporters and cytoplasmic/nuclear β -cat in freshly isolated BMVs from rats of different ages, in the presence or absence of this β -cat activator. Our results showed that *in vivo* activation of nuclear β -cat in brain

enhances mRNA expression of P-gp, bcrp, oat3, and oatp1a4 (See figures 6 from Harati et al., 2012a). The potential modulation of activity of P-gp, bcrp, oat3 and oatp1a4 by β -cat/Tcf-4 signaling was also determined by the *in vivo* assessment of brain/plasma unbound partitioning coefficient ($K_{p,uu}$) of known influx and efflux transporter substrates (Hammarlund-Udenaes et al., 2008; Liu et al., 2005). Our results showed that *in vivo* activation of nuclear β -cat in brain enhances the activity of P-gp, bcrp, oat3, and oatp1a4 in adults (See figures 7 and 8 from Harati et al., 2012a). We also provided evidence on the role of a differential interaction between Wnt/ β -cat and ET-1 signaling in the regulation of these transporters at the BBB level in adult rats. Indeed, our laboratory has previously reported, *in vitro*, the role of ET-1, a vasodilator peptide (Wright et al., 1998; Yanagisawa et al., 1988) in the regulation of ABC transporters and more particularly of P-gp activity at the BBB level (Hawkins et al., 2010; Hembury et al., 2008; Bauer et al., 2007). For that, we investigated the hypothesis that regulation of efflux transporter expression during BBB maturation by β -cat signaling involves modulation of ET-1 signaling at the BBB level. In adults, inhibition of GSK3 β *in vitro* and *in vivo* results in increased ET-1-mRNA expression (See figure 9 from Harati et al., 2012). Interestingly, when we blocked the ETA receptor for ET-1, enhanced transporters' activity mediated by the activation of β -cat decreased (See figures 10 and 11 from Harati et al., 2012). We also showed that β -cat/Tcf-4 and ET-1 signalings could form a feedback loop to reduce/enhance both signaling pathways and regulate functional efflux transporter activities during BBB maturation in adult brain.

In juvenile rats, contrary to adults, *in vivo* inhibition of GSK-3 β decreased the functional expression of bcrp, with a tendency of decrease in P-gp activity (Figure 15 below).

Regulation of p-gp and bcrp activity at the juvenile BBB level through activation of β -catenin in vivo

We studied the regulation of P-gp and bcrp activity in juvenile rat brains after icv injection of GSK3 β by *in vivo* assessment of the brain/plasma unbound partitioning coefficient ($K_{p,uu}$) of known transporter substrates (Figure 15). The experimental method is described in our article Harati et al., 2012a.

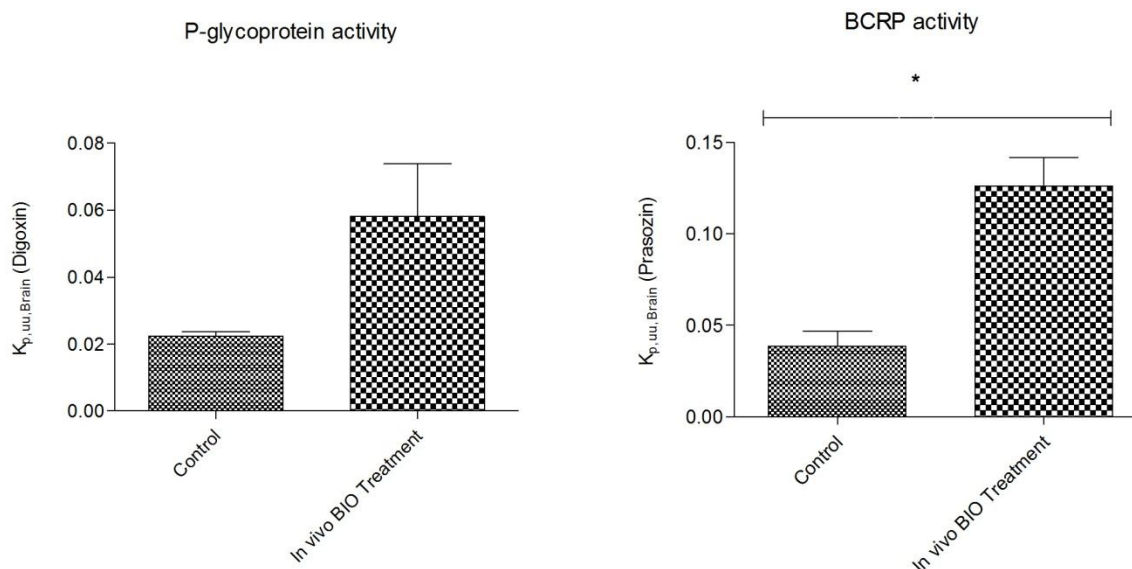


Figure 15. Regulation of P-gp and bcrp activity at the juvenile BBB level through activation of β -catenin *in vivo*.

Efflux transporter activity was assessed by determination of $K_{p,uu,brain}$ for each drug-substrate after BIO administration. Data represent the mean concentration for 3 rat brains and 3 rat plasmas; variability is given by bars \pm SEM. Data were analyzed using Student's t test. Statistical comparisons: $P < 0.05$, $**P < 0.01$.

1.3 Discussion

Our BBB mechanistic ontogenesis study had five major findings:

- (i) The expression of nuclear β -catenin varies in a counter manner to transporters' expression. Indeed, nuclear β -cat expression decreased significantly at postnatal day P21 and P84 compared with day P2 (Figure 5, Harati et al., 2012a).

Several signaling pathways have been demonstrated to be implicated in the regulation of the different BBB development stages from angiogenesis phase to differentiation phase. However, the mechanisms regulating BBB maturation and maintenance phases remain poorly understood. Among the numerous signaling pathways regulating BBB development, we were focused on one pathway: Wnt/ β -cat.

Several points triggered our study towards this pathway:

- 1) Wnt/ β -cat controls BBB development (Liebner et al., 2008). Indeed, endothelial specific stabilization of β -cat *in vivo* enhances barrier maturation, whereas inactivation of β -cat causes significant down-regulation of Claudin-3.
- 2) Recent evidence has demonstrated that β -cat is an efflux transporter's modulator, as shown by the correlation between activation of β -cat and expression of P-gp, MRP-4 and BCRP in an immortalized human brain cell line and primary monoculture of rat BMVECs (Lim et al., 2008).
- 3) However, the regulation of P-gp in mouse BMVECs cultured in Wnt3a conditioned medium were not confirmed by Liebner and colleagues. But they indicate that *abcg2* and the glucose transporter *Slc2a1* were notably up-regulated in mouse BMVECs by Wnt3a conditioned medium ((Liebner et al., 2008).

Based on these findings, we questioned about the role of Wnt/ β -cat in BBB maturation and maintenance. We started our investigation by establishing the maturational profil of nuclear β -cat. For the first time, we illustrated a downregulation of nuclear β -cat expression at both mRNA and protein levels, in BMVs with aging (Figure 5, Harati et al., 2012a).

This dowregulation occurs when P-gp, *bcrp*, *oat3* and *oatp1a4* transporters are at their highest expression levels. These counter correlation suggested a potential role of β -cat in regulation of BBB transporters expression. To formally demonstrate the role of β -cat in regulation of P-gp, *bcrp*, *oat3* and *oatp1a4* transporters at the BBB, rats were injected with GSK-3 β inhibitor (BIO), and expression of P-gp, *bcrp*, *oatp1a4* and *oat3* was measured in freshly isolated BMVs using immunoblotting, as well as transport activity of these efflux/influx transporters at the BBB, using the assessment of the $K_{p,uu,brain}$ which determined the net influx and efflux of drugs across BBB.

- (ii) In adult rats, increasing nuclear β -cat by *in vivo* administration of GSK-3 β inhibitor, enhanced the functional expression of *bcrp*, P-gp, *oat3* and *oatp1a4* in isolated BMVs (Figures 6, 7 and 8; Harati et al., 2012a).

These data suggest that treatment of animals by BIO which is associated with the overexpression of nuclear β -cat triggered an intracellular signal transduction pathways involved in the regulation of transporters activities.

These findings are in agreement with a previous report on the striking link between the increase of nuclear β -cat and the increase of P-gp function *in vitro* using an immortalized human endothelial cells line (Logan et al., 2004).

- (iii) In adult rats, modulation of bcrp, oat3, oatp1a4 and to a lesser extent of P-gp activity by β -cat at the BBB was strikingly linked to the modulation of ET-1 production (Figure 10 and Figure 11, Harati et al., 2012 a).

.We further demonstrated that *in vivo* inhibition of GSK-3 β enhanced nuclear β -cat (Figure 6, Harati et al., 2012 a) which strikingly coincided with ET-1 production in BMVs (Figure 9, Harati et al., 2012 a). This tended to show that the effect of β -cat on the regulation of Bcrp, oat3, oatp1a4 and to a lesser extent of P-gp involved at least the ET-1 regulator (Figure 10 and Figure 11, Harati et al., 2012a). Here, we also provided evidence of the role of an interaction between Wnt/ β -catenin and ET-1 signaling in the regulation of these transporters at the BBB level in adult rats. We have chosen the ET-1, a vasodilator peptide (Wright et al., 1998; Yanagisawa et al., 1988), because its role has been previously reported in the regulation of ABC transporters and more particularly of P-gp activity at the level of the BBB *in vitro* (Hawkins et al., 2010; Hembury et al., 2008; Bauer et al., 2007). For that, we investigated the hypothesis that regulation of efflux transporter expression during BBB maturation by β -cat signaling involves modulation of ET-1 signaling at the BBB level. Inhibition of GK-3 β *in vitro* and *in vivo* results in increased ET-1-mRNA expression (See figure 9 from Harati et al., 2012). Interestingly, when we blocked the ETA receptor for endothelin-1, transporters' activity mediated by the activation of β -cat decreased (See figures 10 and 11 from Harati et al., 2012). We also showed that β -cat/Tcf-4 signaling and ET-1 signaling could form a feedback loop to reduce/enhance both signaling pathways and regulate functional efflux transporter activities during BBB maturation in adult brain.

- (iv) In juvenile rats, increasing the nuclear β -cat by *in vivo* administration of GSK-3 β inhibitor decreased the functional expression of bcrp in isolated BMVs, with a tendency to a decrease of P-gp activity (Figure 15).

These data are different from with those observed in adults. This result, showing that BBB drug efflux transporters in juvenile brain are regulated differently than those in adults after β -cat

activation, needs to be complemented, as done in adults, by measuring transporters activity after ETA receptor blockade and β -cat activation. This information will help 1) to clarify the role of the interaction between β -cat signaling and ET-1 in regulating juvenile BBB transporters, and 2) to determine if ET-1 is the factor at the origine of differences observed between adult and juvenile rats. In addition, it is important to explore modulation of β -cat and ET-1 after GSK-3 β inhibition in juvenile brain, and to compare these aspects with those observed in adults. Corresponding experiments are in progress. The obtained data will help to further determine the factor at the origin of the differences observed between ages.

To further verify if ET-1 is the factor at the origin of the observed differences, it seemed important to compare the impact of ET-1 on BBB transporters between juvenile and adult rats. And this was the subject of the third objective of this thesis.

ARTICLE 1

**P-glycoprotein, Breast cancer resistance protein, Organic anion transporter 3
and transporting peptide 1a4 during Blood-Brain Barrier Maturation:
Involvement of Wnt/ β -Catenin and Endothelin-1 Signaling**

Molecular Pharmaceutics

P-Glycoprotein, Breast Cancer Resistance Protein, Organic Anion Transporter 3, and Transporting Peptide 1a4 during Blood–Brain Barrier Maturation: Involvement of Wnt/ β -Catenin and Endothelin-1 Signaling

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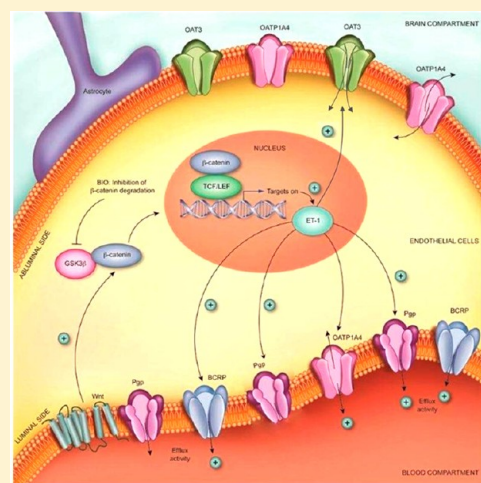
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Supporting Information

ABSTRACT: Our current knowledge about drug transporters in the maturational brain is very limited. In this study, we provide a comprehensive overview of the expression and activity profile of P-glycoprotein (P-gp), Breast Cancer Resistance Protein (bcrp), Organic Anion Transporter 3 (oat3), and Transporting Peptide 1a4 (oatp1a4) transporters during blood–brain barrier (BBB) maturation. Gene and protein expressions of the analyzed transporters increase as the brain matures, with no variation in their activity for P-gp and bcrp, while the transport activity of oat3 and oatp1a4 increases during brain maturation from preterm up to adulthood. For the first time, we illustrate a downregulation of nuclear β -catenin expression in brain capillaries when bcrp, P-gp, oat3, and oatp1a4 transporters are at their highest expression levels. *In vivo* activation of β -catenin in rat brains, by intracerebroventricular (ICV) injection of a GSK-3 inhibitor, enhances the activity of P-gp, bcrp, oat3, and oatp1a4. Interestingly, in an *in vitro* BBB model consisting of a coculture of primary endothelial brain cells with astrocytes or *in vivo*, activation of β -catenin enhances the mRNA expression of ET-1. Interestingly, blocking the ETA receptor for endothelin-1 *in vivo* by ICV injection of a ETA antagonist decreases transporter activity mediated by the activation of β -catenin. These findings shed light on the role of an interaction between β -catenin and endothelin-1 signaling in the regulation of these transporters at the BBB.

KEYWORDS: ontogenesis of blood–brain barrier, preterm and adulthood blood–brain barrier, β -catenin and endothelin-1 signaling, P-gp, bcrp, oat3 and oatp1a4 transporters



INTRODUCTION

The blood–brain barrier (BBB) maintains brain homeostasis and limits the entry of toxins and pathogens into the brain. A number of transport and carrier systems are expressed and polarized on the luminal or abluminal surface of the BBB to make these functions possible. Among these systems, Adenosine triphosphate-Binding Cassette (ABC) transporters play a critical role in preventing neurotoxic substances from entering the brain and in transporting toxic metabolites out of the brain.¹ The solute carrier family (SLC), another major group of membrane transporter proteins, controls the cellular uptake and efflux of nutrients, neurotransmitters, metabolites, and toxins (Engelhardt, 2003).^{2,3} These cited transporters have an affinity for a large number of therapeutic drugs and are involved in the emergence of multidrug resistance, which plays a crucial role in the failure of treatments for several brain diseases such as sclerosis pathogenesis and human immunodeficiency (HIV-1) infection.^{4–6}

Several pieces of evidence show that the specific mechanism of BBB transport such as ion regulation and probably efflux/influx transporter regulation may continue to mature and become fully expressed and functional in the peri- or postnatal period.⁷ Because intricate developmental processes take place during the prenatal and postnatal periods, when many organs including the brain are incompletely developed, we have hypothesized that BBB transporters undergo rapid and substantial changes in expression and activity during brain maturation. Modulation in the expression of P-glycoprotein (P-gp) with age has been reported. Daoood et al. reported that, in post-mortem CNS tissue from infants born 22^{0/7} to 42^{0/7} week gestation and adults, P-gp/ABCB1 immunostaining was observed

Special Issue: Drug Delivery across the Blood-Brain Barrier

Received: June 18, 2012

Revised: September 12, 2012

Accepted: September 18, 2012

in microvessel endothelial cells as early as 22^{0/7} weeks and prevalence and intensity increase with maturation. However, they also reported that the intensity of Breast Cancer Resistance Protein (bcrp)/ABCG2 immunostaining did not vary notably with maturation.⁸ In rats, gene expressions of P-gp and bcrp were established in the forebrain and brainstem at different developmental stages: E13, E15, E18, P1, P14, P21, and adulthood. Results showed that both P-gp and bcrp were detected at E1. P-gp expression progressively increased during development (about 10–12-fold) in both the forebrain and the brainstem, while bcrp showed similar gene expression at all ages and in both brain regions.⁹ Concomitant gene and protein expression studies associated with the assessment of transport activity for bcrp and SLC transporters such as Organic Anion Transporter 3 (oat3) and Organic Anion Transporting Peptide 1a4 (oatp1a4) in brain microvessels, site of the BBB, as well as the molecular mechanisms underlying their functional expression at the BBB remain to be elucidated. At the molecular level the role of Wnt/ β -catenin (β -cat) signaling in the development of the BBB has been suggested.¹⁰ In this pathway, interaction of Wnt proteins with cell surface receptors leads to inactivation of Glycogen Synthase Kinase-3 (GSK3) resulting in stabilization of β -cat and its translocation into the nucleus where it acts as component of a transcriptional signaling complex with members of the T Cell Factor (Tcf) (family of Transcription Factors) to activate target genes.¹¹ Recent evidence has demonstrated the efflux transporter modulator significance of β -cat, as shown by the correlation between the activation of β -cat and the expression of P-gp, multidrug resistance-associated protein 4 (MRP4) and bcrp in an immortalized human brain cell line and primary monoculture of rat brain endothelial cells.¹² However, the regulation of P-gp in mouse brain microvascular endothelial cells (MBE) cultured in Wnt3a conditioned medium was not confirmed by Liebner and colleagues. Abcg2 and the glucose transporter SLC2a1 were notably up-regulated in MBEs by Wnt3a conditioned medium.¹⁰ Thus, the primary role of β -cat in this process and the underlying mechanisms remain to be clarified. That was the main aim of the present study, in which we used an *in vivo* approach by following efflux transporters and cytoplasmic/nuclear β -cat gene and protein expression of freshly isolated brain vessels from rats of different ages in the presence or absence of GSK-3 inhibitor. Furthermore, the potential modulation of the functionality of P-gp, bcrp, oat3, and oatp1a4 by β -cat/Tcf-4 signaling was determined by the *in vivo* assessment of the unbound drug brain/plasma concentration ratio ($K_{p,uu}$) of known influx and efflux transporter substrates.^{13,14}

In addition, we and others have reported *in vitro* the role of endothelin-1 (ET-1), a vasodilator peptide,^{15,16} in the regulation of ABC transporters and more particularly of P-gp activity at the level of the BBB.^{17–19} Recent advances suggest that β -cat/Tcf-related transcription is correlated with expression of ET-1 in prostate cancer cells.^{20,21} We investigated the hypothesis that regulation of efflux transporter expression during BBB maturation by β -cat signaling involves modulation of ET-1 signaling at the BBB level. β -cat/Tcf-4 signaling and ET-1 signaling could form a feedback loop to reduce/enhance both signaling pathways and regulate functional efflux transporter activities during BBB maturation.

EXPERIMENTAL SECTION

Reagents. Bovine serum albumin (BSA), *N*- α -tosyl-L-lysine-chloromethylketone (TLCK), and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from Sigma (Saint Louis, MO, USA). Hank's buffered salt solution (HBSS), phosphate-buffered saline (PBS), and penicillin–streptomycin–neomycin (PSN) were from Invitrogen (Carlsbad, CA). Collagenase/Dispase and DNase I were from Roche (Indianapolis, IN, USA). Primary antibodies from Santa Cruz Biotechnologies were: anti-MDR1, anti-bcrp1, anti- β catenin. Primary antibody anti-oatp1a4 and anti-oat3 were from Alpha Diagnostic International (TX, USA). The secondary antibodies, goat antirabbit goat antimouse and antigoat IgGs, conjugated to horseradish peroxidase (HRP) were from Santa Cruz Biotechnologies.

Bumetanide (CAS 28395-03-1), digoxin (CAS 20830-75-5), and prazosin (CAS 19237-84-4) were from Sigma-Aldrich (St-Quentin-Fallavier, France). Rosuvastatin calcium (CAS 287714-41-4) was from Toronto Research Chemicals (North York, Canada). Internal standards vinblastine (CAS 143-67-9) and chlorpropamide (CAS 94-20-2) were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). 6-Bromoindirubin-3'-oxime (BIO) was from Calbiochem (Merck Chemicals, Nottingham; UK), the ETA-receptor antagonist JKC-301 from Alexis Biochemicals (San Diego, CA), and endothelin-1 (ET-1) from Bachem (Heidelberg, Germany).

Animals. Rats were from Janvier Laboratories (France). Four groups of male Wistar rats [postnatal day (P) 2, P14, P21, and P84 corresponding to the stages of BBB maturation were used in these experiments.²² Rats were maintained in a temperature- and humidity-controlled (19–23 °C) room under a 12:12 h light-dark cycle and were fed a standard diet (rodent rat chow) ad libitum with free access to tap water. Experiments were performed in accordance with the Declaration of Helsinki, the European community's council directives (86/609/EEC, November 24, 1986), and the French directives concerning the use of laboratory animals.

Stereotaxic Injections. Rat of different ages were weighed using a digital scale. All animals were then anesthetized with ketamine (87 mg/kg)/xylazine (13 mg/kg) and placed in a rodent stereotaxic apparatus. Skin and cranial fascia were resected and the skull exposed. A 33-gauge beveled injection needle (Coveto) was inserted into the right lateral cerebral ventricle (4 mm ventral to the dura) via a hole that was drilled in the cranium at 1.08 mm posterior and 1.5 mm lateral to the bregma. The tip of a Hamilton syringe needle was briefly lowered to a depth of 3.4 mm and immediately raised by 0.2 mm to create a trough. Each rat received a bilateral injection of BIO or the ETA-receptor antagonist JKC-301 at a dose of 3.5 μ g/kg and/or 25 pmol/kg, respectively. A total volume of 2.5 μ L of BIO or 2.6 μ L of ETA-receptor antagonist JKC-301 was injected in each ventricle at a rate of 1 μ L/min. Control rats received saline solution (0.9% NaCl) by icv. The needle was left in for 5 min following the end of the injection, after which it was withdrawn slowly, craniotomies were filled with gel-foam, and the scalp was sutured with wound clips. The wound was closed with self-dissolving sutures and swabbed with iodine. For pain control, lidocaine solution was applied. Animals were placed in a recovery cage with a heater, where they remained until the end of the study. Twenty-four hours after intracerebroventricular injection of adequate pharmacological agents, animals ($n = 3–5$) were euthanized by anesthetic

injection and decapitation. Brains were removed and brain capillaries isolated as described below.

Assessment of Brain/Plasma Concentration Ratio ($K_{p,uu,brain}$) of Known Influx and Efflux Transporter Substrates. *Drug Infusion.* The time-course distribution study of drug in plasma and brain of rats treated or not by pharmacological agents was conducted as previously described.²³ The *in vivo* plasma and brain exposure to tested efflux transporter substrates [digoxin (P-gp substrate), rosuvastatin (oatp1a4 substrate), bumetanide (oat3 substrate), and prazosin (bcrp substrate)] was assessed using unanesthetized rats catheterized in the femoral vein before a 4-h infusion (steady state) of the test solution at 1 mg/kg·h. Three or five rats per treatment were used. Blood was sampled from the abdominal aorta in Eppendorf tubes containing 20 μ L of a 1000 U/mL sodium heparinate solution and then centrifuged for 5 min at 3000 g at 4 °C to collect plasma. After collection, brains were weighed. Plasma samples and brains were stored at -20 °C for subsequent bioanalysis by mass spectrometry.

Transporter Substrate Quantification in Biological Samples. Brains were mixed with ultrapure water (2 mL/g of brain) using an Ultraturrax T65 system (IKA, Werke). Extract suspension (200 μ L) was submitted to protein precipitation with 1 mL of methanol previously spiked with internal standard (1 μ g/mL). After evaporation of the methanolic extracts to dryness, they were resuspended in 500 μ L of 1% NH_4OH . Plasma (200 μ L) was diluted with 200 μ L of 1% NH_4OH . Both brain extracts and diluted plasmas were submitted to solid-liquid extraction (SLE) on an Oasis cartridge (reference 186000366, Waters) as previously described (Videau et al., 2010). The eluates (2 \times 250 μ L) of 2% formic acid in methanol and 2 \times 250 μ L of acetonitrile/methanol (1:1 v/v) were evaporated to dryness and reconstituted in 100 μ L of 10 mM ammonium acetate/acetonitrile/formic acid (95:5:0.1 v/v), and 20 μ L was injected into the chromatographic system. Chromatography was performed using a Waters Acquity UPLC system on a BEH Shield RP18 column (2.1 mm \times 100 mm, 1.7 μ m) coupled with a BEH Shield RP18 1.7 μ m Van Guard Pre-Column (Waters, Saint-Quentin-en-Yvelines, France). Mobile phase solvent A comprised 0.1% formic acid in 10 mM ammonium acetate and mobile phase solvent B 0.1% formic acid in acetonitrile. The run time was 5 min, and analytes were eluted with the following gradient: from 0 to 1 min 5% solvent B, from 1 to 2.5 min the proportion of solvent B increased linearly from 5 to 80%, from 2.5 to 3 min a steady state of 80% solvent B was maintained, and from 3.1 to 5.0 min there was re-equilibration of initial conditions. The flow rate was set at 0.6 mL/min, the column temperature was maintained at 60 °C, and the autosampler was maintained at 4 °C.

Detection was performed with a triple quadrupole mass spectrometer Quattro Premier XE monitored by MassLynx software, version 4.1 (Waters, Saint-Quentin-en-Yvelines, France) equipped with an electrospray ionization source operating in positive or negative mode. Tuning parameters were: capillary voltage 3 kV, source temperature 120 °C, desolvation temperature 350 °C. The multiple reaction monitoring transitions for analytes were as follows: *m/z* bumetanide 364.9 > 240.2; *m/z* digoxin 325.2 > 779.4; *m/z* prazosine 384.1 > 247.2; and *m/z* rosuvastatin 482.0 > 258.1.

Analytes were quantified by means of calibration curves using vinblastine or chlorpropamide as internal standard. For plasma assay, calibration ranges were from 1.0 to 200 ng/mL for bumetanide, 1.0 to 200 ng/mL for digoxin, 0.4 to 170 ng/mL

for prazosine, and 0.5 to 200 ng/mL for rosuvastatin. For brain extract assay, calibration ranges were from 1.0 to 100 ng/mL for bumetanide, 1.0 to 100 ng/mL for digoxin, 0.5 to 100 ng/mL for prazosine, and 0.5 to 100 ng/mL for rosuvastatin.

The ratio between brain concentrations and plasma concentrations were used to estimate the delivery of drug to the brain.^{14,23} This ratio can be described by the partition coefficient, K_p :

$$K_{p,brain} = C_{SStot,brain} / C_{SStot,plasma}$$

where C_{SStot} is the steady-state concentration for total (bound and unbound) drug in brain and plasma.

Taking into account protein binding in plasma and brain tissue components of efflux/influx transporter substrates (digoxin prazosin, bumetanide, and rosuvastatin) measured using equilibrium dialysis in triplicate for each compound as described elsewhere,¹⁴ the unbound partitioning coefficient $K_{p,uu}$ which determined the net influx and efflux of drug across the BBB was calculated as follows:

$$K_{p,uu,brain} = C_{SStot,brain} \times f_{u,brain} / C_{SStot,plasma} \times f_{u,plasma}$$

where $f_{u,plasma}$ and $f_{u,brain}$ are the fractions of unbound drug (Table 1; see supplemental file) in plasma and brain tissue, respectively.

Brain Capillary Isolation. Rat brain capillaries were isolated as described previously.^{19,23} Five male rats from each group of age were used. Brains were extracted and stored in Hanks balanced salt solution (HBSS) supplemented with 1% (v/v) PSN on ice. The remainder of the isolation took place under aseptic conditions. The brains were cut sagittally into two halves and the cerebral cortices emptied of white matter. The meninges and the associated vessels were cleaned off by rolling on Whatman 3 mm chromatography paper. The homogenized tissue was pelleted by centrifugation at 1500 rpm for 5 min. The pellet containing the microvessels was digested in HBSS-1% PSN solution, 1 mg/mL collagenase Dispase, 10 U/ μ L DNase-I, and 1 μ g/mL TLCK for 1 h at 37 °C. Digested tissue was then pelleted by centrifugation at 1500 rpm for 5 min at 4 °C. To remove myelin, the pellet was resuspended in 20% (w/v) BSA in HBSS-1% PSN solution and centrifuged at 2800 rpm for 30 min. The resulting floating white matter and centrifugation medium were removed carefully, and the microvessel pellets were resuspended and further digested with the enzyme solution for 1 h at 37 °C, then pelleted by centrifugation at 1500 rpm for 5 min, and frozen at -80 °C.

Purity Control of Collected Brain Capillary. The purity of collected brain capillary was checked after RNA isolation and real-time PCR experiments by measuring the expression of cell-specific marker genes using specific primer (from Sabiosciences) for brain endothelial cells (CD31 or PECAM), for glial cells (glial fibrillary acid protein or GFAP), and for pericytes (α -actin). In addition, brain capillaries were immunostained with ZO-1 mAb as described in the immunostaining section.

Transcription Profiles of ABC Efflux and SLC Transporters. For a thorough evaluation, the mRNA expression profiles of *abcb1*, *abcg2*, *slc22a8*, and *slco1a4* were assessed on isolated brain microvessels as previously described.^{6,23}

RNA Extraction. RNA was isolated from rat brain microvessels using the RNeasy mini kit (Qiagen, Hilden, Germany) and Qiashredder homogenizer columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Samples were treated with DNaseI (Rnase-Free Dnase set; Qiagen SA)

to remove genomic DNA. The concentration and purity of the RNA samples were assessed spectrophotometrically at 260 and 280 nm using the Nanodrop ND-1000 instrument (NanoDrop Technologies, Wilmington, DE, USA). The A260/280 ratio ranged between 1.8 and 2.

Reverse Transcription. A sample of 0.5 μg of total RNA was converted to cDNA with random primers in a total volume of 10 μL using an RT² first strand kit (Superarray Bioscience Corporation, USA) according to the manufacturer's instructions. The cDNA was diluted with distilled water to a volume of 100 μL .

Quantitative Real-Time PCR. The quantitative expression of various tight junction components and ABC and SLC transporters was determined using 0.4 μM cDNA for each primer set in the RT² Pathway-Focused Profiler Array from SA Biosciences according to the manufacturer's recommended protocol. The RT² Profiler array consists of prevalidated qRT-PCR primer sets (1 μL) for relevant tight junctions, ABC and SLC transporters, and suitable housekeeping genes. Thermocycling was carried out in a CFX96 real-time PCR detection system (Bio-Rad) using SYBR green fluorescence detection. The final reaction mixtures contained 2 μL of diluted cDNA, 12.5 μL of Milli-Q water, and 9.5 μL of SYBR Green master mix (SA Biosciences, cat. no. PA-112). The specific amplification conditions were 2 min at 50 °C, 10 min at 95 °C followed by 40 amplification cycles at 95 °C for 0.5 min, and 60 °C for 1 min to reinitialize the cycle again. The specificity of each reaction was also assessed by melting curve analysis to ensure the presence of only one product. Relative gene expression values were calculated as $2^{-\Delta\text{CT}}$, where ΔCT is the difference between the amplification curve (CT) values for genes of interest and the housekeeping genes (hypoxanthine-guanine phosphoribosyltransferase or HPRT; glyceraldehyde phosphodehydrogenase or GAPDH). If the CT was higher than 35, we considered the expression level too low to be applicable.^{6,23,24}

Western Blot Analysis. To analyze protein expression, Western blot analysis was performed on rat cerebral microvessels or brain endothelial cells from the cell-based rat BBB model. Microvessels and cell lysates were prepared by homogenization on ice, in 300 μL of membrane protein lysis buffer (20 mM Tris-HCl pH = 8, 0.15 M NaCl, Igepal) and complete protease inhibitor cocktail (Roche diagnosis, Mannheim, Germany) in a 1.5 mL centrifuge tube. Samples were kept at 4 °C for 1 h and homogenized every 15 min. Homogenates were then centrifuged at 4 °C for 20 min at 12 000 rpm, and the resulting supernatants were stored at -80 °C. The nuclear and cytoplasmic fractions were separated using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Protein concentrations in each fraction were assayed by Bradford's method, using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein samples (15 μg) were electrophoresed in NuPage Novex 7% Tris-acetate with the aid of an XCell SureLock Mini-Cell gel running apparatus (Invitrogen, Carlsbad, CA). Proteins were transferred from the gel onto nitrocellulose membrane (0.45 μm pore size). The membranes were washed in Tris-buffered saline [10 mM Tris-HCl (TBS-T), 150 mM NaCl, pH 8.0], supplemented with 0.05% Tween 20, and blocked with 5% nonfat dry milk dissolved in TBS-T. To probe the membrane with primary antibodies, 5% of the milk solution was removed, and the membrane was incubated with the antibody, diluted in TBS-T,

5% milk overnight at 4 °C. The membranes were washed with TBS-T, and the secondary monoclonal antibody, diluted in TBS-T, was applied for 1 h at room temperature. The membranes were washed with TBS-T and protein bands were detected by enhanced chemiluminescence with the Amersham Biosciences Innovative ECL Kit and the following exposure to Hyperfilms (both GE Healthcare BioSciences). Optical density was quantified using VersaDoc analysis software.

In Vitro Cell-Based Rat BBB Model. Brain Endothelial Cell Isolation. For brain endothelial isolation, isolated brain capillaries were cultured in a 25 cm² flask coated with collagen type IV and fibronectin (0.1 mg/mL) in the presence of puromycin (2 $\mu\text{g}/\text{mL}$) in the endothelial basal medium containing 0.1% human recombinant epidermal growth factor (hEGF), 0.04% hydrocortisone, 0.1% human recombinant insulin-like growth factor, 0.1% ascorbic acid, 0.1% gentamicin, 0.1% amphotericin-BN, and 5% fetal bovine serum. On the third day, the cells received a new endothelial-specific medium without puromycin. When cells reached confluence (70%) at day 7, the purified endothelial cells were frozen and ready to be used for *in vitro* cell-based BBB genesis.

In Vitro Cell-Based Rat BBB Model. Glial cells (2×10^4 cells) were plated on Transwell plates (Costar, pore size 0.4 μm ; diameter 12 mm; insert growth area 1.12 cm², Dutscher SA, Brumath, France) in the glial-specific medium (α -MEM/F12) supplemented with 10 $\mu\text{g}/\text{mL}$ hEGF, 10 mg/mL insulin, 25 $\mu\text{g}/\text{mL}$ progesterone, 50 mg/mL transferrin, 50 mg/mL gentamicin, 50 $\mu\text{g}/\text{mL}$, 0.1% amphotericin-B, 1% human serum, and 5% fetal bovine serum. After 24–72 h, BECs (5×10^4 cells) were plated on the upper side of a collagen-coated polyester Transwell membrane (Costar, pore size 0.4 μm ; diameter 12 mm; insert growth area 1 cm²) in 0.5 mL of the endothelial basal medium-2 (EBM-2, LONZA, Walkersville, MD, USA) containing 0.1% human recombinant epidermal growth factor (hEGF), 0.04% hydrocortisone, 0.1% human recombinant insulin-like growth factor, 0.1% ascorbic acid, 0.1% gentamicin, 0.1% amphotericin-BN, and 5% fetal bovine serum (0.5 mL). The chambers containing glial cells and BECs were considered as the basolateral and apical compartment, respectively. The microplates were then incubated at 37 °C in a 5% CO₂ atmosphere. Under these experimental conditions, BECs formed a confluent monolayer within 15 days.

Checking the Integrity of the Primary Cell-Based Rat BBB Model. The integrity of the cell-based BBB models was demonstrated (i) by assessing the presence of tight junctions between the endothelial cells under a confocal microscope and (ii) by measuring the flux of the paracellular reference marker, [¹⁴C]-sucrose, through the monolayer.

For immunostaining, BEC monolayers were fixed with cytofix-cytoperm buffer (Pharmigen, France) and permeabilized with Perm/Wash buffer (Pharmigen) according to the manufacturer's procedure. The samples were washed with PBS and soaked in the blocking solution containing 4% BSA. They were then incubated with rabbit anti-Zonula occludens (ZO-1), anti- β -catenin mAb. After washing, the cells were incubated with the secondary FITC antibody. The preparations were examined with a fluorescence microscope.

BBB integrity was checked by assessment of sucrose permeability as a marker to validate the experiments before and after test compound incubations. Before use of the monolayers, BBB integrity was checked on 12% of monolayers. Transwells with HBMEC monolayers were transferred to new plates. T buffer (150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl₂,

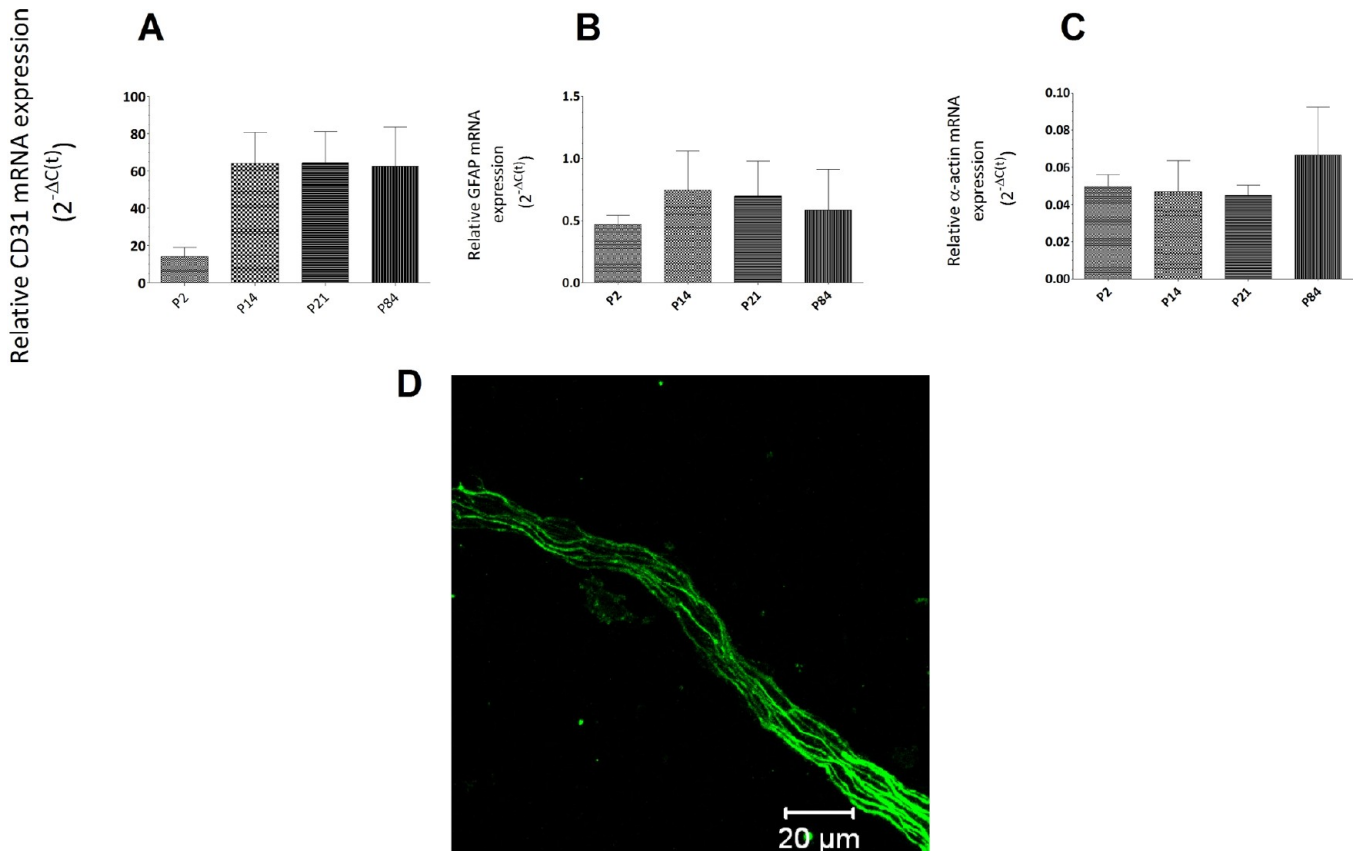


Figure 1. Checking the purity of freshly isolated rat brain endothelial microvessels. mRNA transcription profiles for CD31 (A), Glial fibrillary acid (B), and α -actin (C) by semiquantitative real-time PCR performed in freshly isolated rat brain endothelial microvessels. Results are means \pm SEM for 3 batches of brain endothelial microvessels (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of brain endothelial microvessels. (D) Fluorescence photomicrographs: rat brain endothelial microvessel cells stained for ZO-1. Scale bar: 20 μ m.

0.2 mM $MgCl_2$, 6 mM $NaHCO_3$, 2.8 mM glucose, and 5 mM HEPES) was added: 1.5 mL to the basolateral compartment (B) and 0.5 mL to the apical compartment (A), which also contained 0.37×10^{10} Bq/mL of [^{14}C]-labeled sucrose. After 60 min of incubation at 37 $^{\circ}C$, supernatants from both A and B compartments were collected, and the amount of tracer that passed through the endothelial monolayer was determined by scintillation counting. Monolayers were validated for sucrose permeability from A to B and B to A below 8×10^{-6} cm/s.

Pharmacological Interventions. Brain endothelial cells cultured as described above were grown to confluence in 12-well plates and then exposed, for 24 h, to various ET-1 or agents that activate β -cat signaling. The agent designed to activate β -cat signaling was 6-bromoindirubin-3'-oxime (BIO). Cells were subsequently harvested for real-time PCR analysis as outlined above.

Statistical Analysis. Statistical analysis was performed using the Prism 3.0 program (GraphPad Software, Inc., San Diego, CA) and the R statistical software (R Development Core Team, 2009). Comparisons between groups were performed using one-way analysis of variance (ANOVA) with the Student–Bonferroni post-test and the unpaired Student's *t* test. Data are presented as mean \pm SEM; statistical significance was set at $P < 0.05$.

RESULTS

ABC Transporter Expression during BBB Maturation.

First of all, we evaluated the purity of fresh brain endothelial capillary isolated from a pool of 10 rat brain samples at postnatal (P) days P2, P14, P21, and P84 corresponding to the stages of BBB maturation.²⁴ The purity of the microvessel preparation was evaluated by measuring the expression of cell-specific marker genes, like PECAM-1 (endothelial cells), α -actin (pericytes), and GFAP (astrocytes), and the relative abundance of the mRNAs in each sample was compared (Figure 1). PECAM-1 mRNA was more abundant in brain microvessels than GFAP and α -actin in the brain microvessel preparations, showing the acceptable purity of the rat brain microvessels. In addition, ZO-1 protein was expressed as showing an example of P84 rat brain microvessel immunostaining with anti-ZO1 monoclonal antibody (Figure 1b). These findings confirmed the purity of freshly isolated rat brain microvessels. Since *abcb1* and *abcg2* represent the major transporters of clinical relevance, we focused our study on the ontogenesis of both ABC transporters. Quantitative real-time PCR analysis of freshly isolated rat brain microvessel mRNA showed the presence of *abcb1* and *abcg2* mRNAs at all stages of BBB maturation (Figure 2a,c). In the freshly isolated rat brain microvessels, *abcb1* progressively increased during BBB maturation. Immunoblotting for P-glycoprotein demonstrated differences in protein expression between brain microvessels at P2, P14, P21, and P84 (Figure 2b, $P < 0.05$).

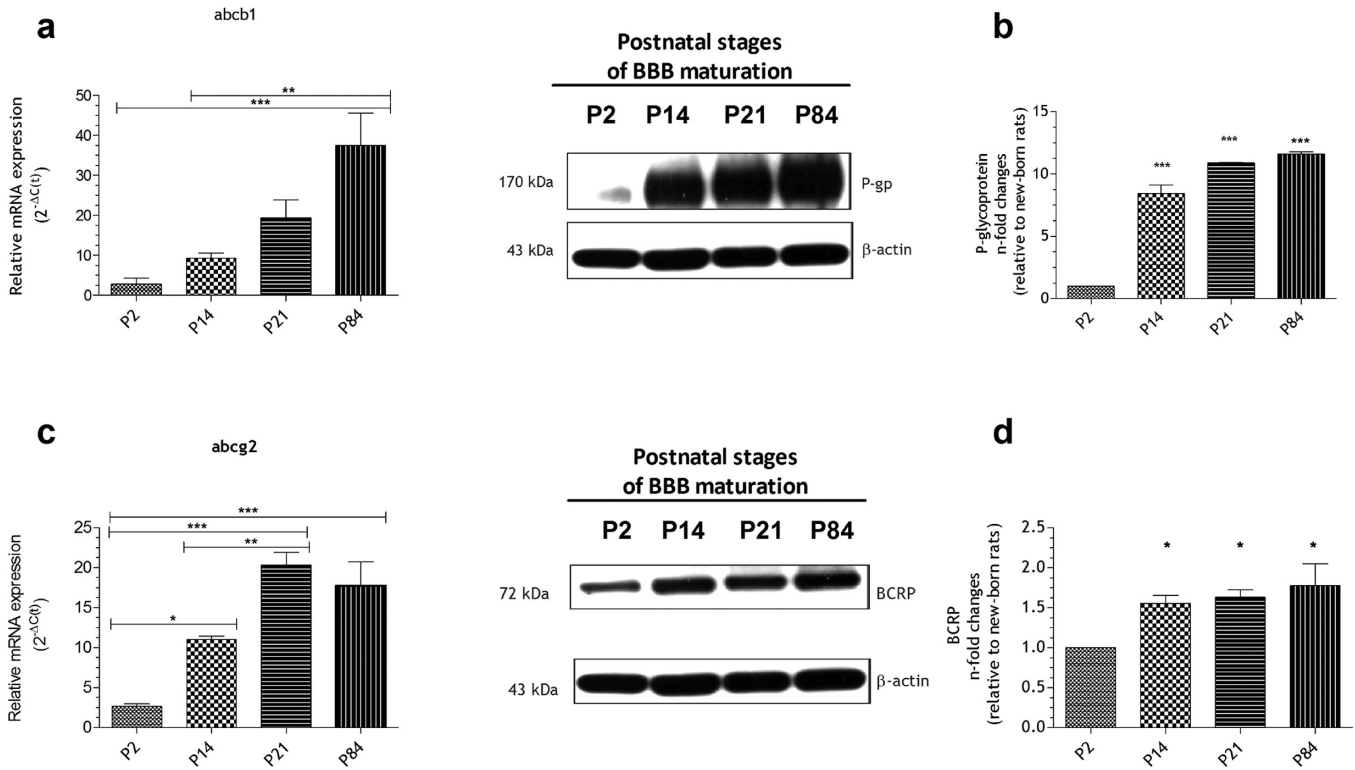


Figure 2. Developmental changes of abcb1 and abcg2, in freshly isolated rat brain microvessels. Rats at different stages of brain development equivalent to the preterm (P2), neonate (P14), pediatric (P21), and adult (P84) human brain were used. mRNA transcription profiles for abcb1 (a) and abcg2 (c) were assessed by semi-quantitative real-time PCR performed in freshly isolated rat brain endothelial microvessels. Results are means \pm SEM for 3 batches of brain endothelial microvessels (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of brain endothelial microvessels. P-glycoprotein (b) and bcrp (d) protein expression were assessed by Western blot. Immunoblot represents the mean \pm SEM of 3 batches of brain endothelial microvessels. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

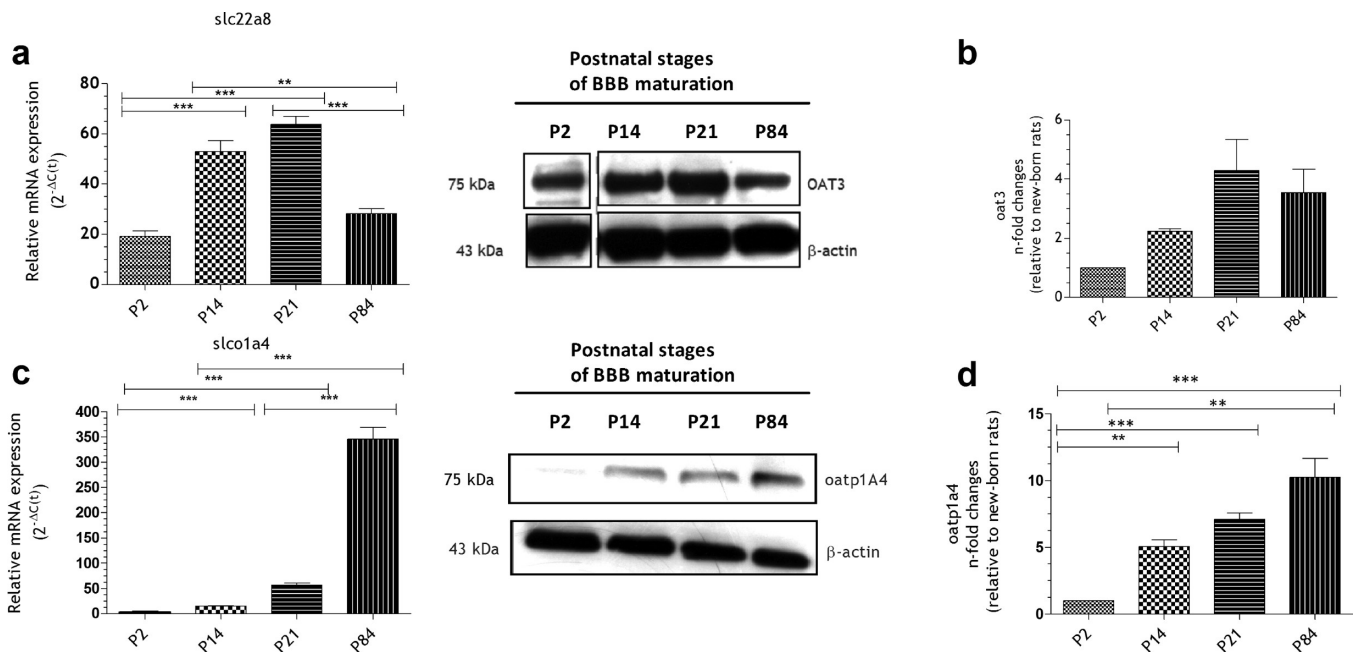


Figure 3. Developmental changes of slc22a8 and slco1a4, in freshly isolated rat brain microvessels. Rats at different stages of brain development equivalent to the preterm (P2), neonate (P14), pediatric (P21), and adult (P84) human brain were used. mRNA transcription profiles for slc22a8 (a) and slco1a4 (c) were assessed by semi-quantitative real-time PCR performed in isolated freshly rat brain endothelial microvessels. Results are means \pm SEM for 3 batches of brain endothelial microvessels (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of brain endothelial microvessels. Oat3 (b) and oatp1A4 (d) protein expression was assessed by Western blot analysis. Immunoblot represents the mean \pm SEM of 3 batches of brain endothelial microvessels. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

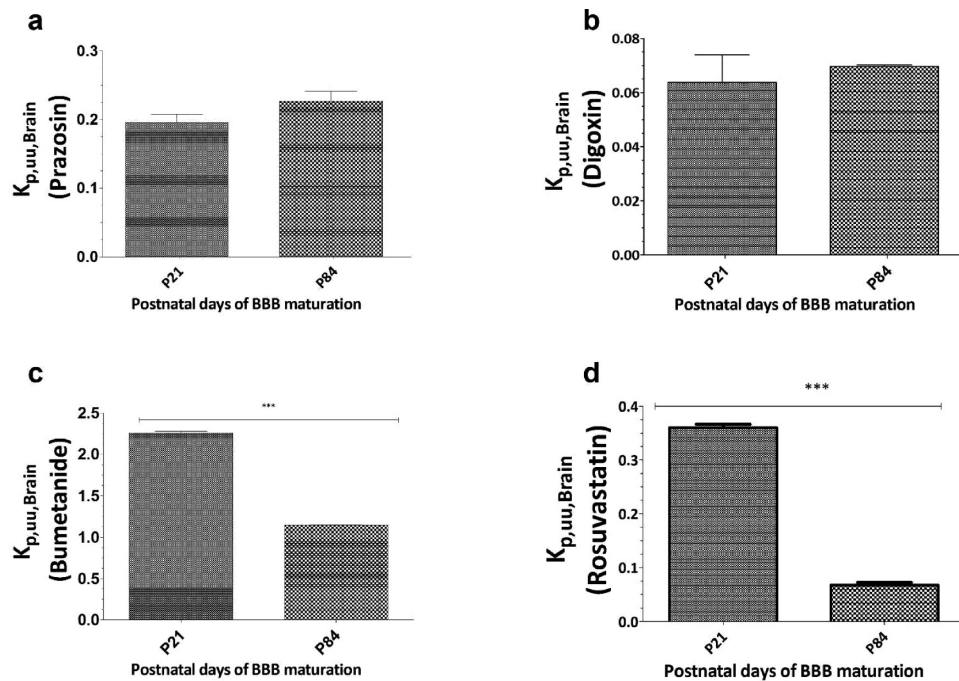


Figure 4. Age-related changes in P-gp, bcrp, oat3, and oatp1a4 function at the BBB. Rats at pediatric (P21) and adult (P84) stages of brain development were used. The *in vivo* plasma and brain exposure of tested efflux transporter substrates [a] prazosin (BCRP substrate): 0.25 mg/kg/h, b) digoxin (P-gp substrate): 0.5 mg/kg/h, c) bumetanide (oat3 substrate): 2.5 mg/kg/h, and d) rosuvastatin (oatp1a4 substrate): 1.75 mg/kg/h] was assessed using unanesthetized rats catheterized in the femoral vein. Drug infusion was performed for a period of 4 h (steady state) and then the unbound partitioning coefficient brain/plasma concentration ratio ($K_{p,uu,Brain}$) for each tested efflux transporter substrate was determined as described in the Experimental Section. Data represent the mean concentration for 3 or 5 rat brains and 3 or 5 plasmas; variability is given by bars \pm SEM. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

While mRNA transcription for bcrp showed a progressive increase during BBB maturation, with about a 6.73-fold increase between P2 and P84 ($P < 0.05$) (Figure 2c), immunoblotting for bcrp showed a lower level in brain microvessels at P2 than at P14, P21, and P84 (Figure 2d). No significant variation of bcrp expression was observed in brain microvessels between P14 and P84 (Figure 2d).

Oat3 and Oatp1a4 Transporter Expression during BBB Maturation. The expression profiles of the genes encoding two members of the SLC family, obtained by RT-qPCR, are represented in Figure 3. SLC14A4-mRNA encoding for oatp1a4 was expressed much more than SLC22A8-mRNA encoding for oat3, from P14 until P84.

The mRNA expression levels of slc22a8 in freshly isolated brain microvessels were not significantly different at P2 and P84. However, there was an increase from birth until P14 and P21, with a peak of 63.91-fold higher expression compared with P2 and P84 (Figure 3a). Immunoblotting for oat3 protein revealed its upregulation in the brain capillaries from P2 to P21 and a slight decrease at P84 (Figure 3b).

The mRNA for slc14a4 (Figure 3c) as well as of the corresponding oatp1a4 protein (Figure 3d) increased progressively from the postnatal stage (P2) to the adult stage (P84), with the most pronounced expression in the brain microvessels (Figure 3c,d).

Developmental Changes in BBB Transport Activity of bcrp, P-gp, Oat3, and Oatp1a4 in the Juvenile and Adult Brain. To demonstrate the transporter activity at the BBB, the unbound drug partitioning coefficient, which describes the unbound brain to plasma drug concentration ratio ($K_{p,uu}$) of known substrates prazosin (bcrp substrate²⁵), digoxin (P-gp substrate²⁶), bumetanide (oat3 substrate²⁷), and rosuvastatin

(oatp1a4 substrate²⁸), was determined in unanesthetized catheterized rats after 4 h. At steady state prazosin $K_{p,uu,Brain}$ did not differ between pediatric and adult BBB. The same conclusion could be drawn for digoxin (Figure 4a, b). However, the tremendous increase in the oat3 ($P < 0.0001$) and oatp1a4 ($P = 0.0008$) activity was noticed at the adult BBB compared with the juvenile BBB (Figure 4c,d). Indeed, the decrease in bumetanide (41%) and rosuvastatin (13%) brain concentrations, as the brain matures, suggests a maturational increase in oat3 and oatp1a4 efflux activities.

Downregulation of β -Catenin Expression during BBB Maturation. To test the hypothesis that endothelial β -cat signaling correlates with bcrp, P-gp, oat3, and oatp1a4 transporters during BBB maturation, RT-PCR of total mRNA and immunoblotting of nuclear β -cat in freshly isolated rat brain microvessels were performed. Throughout BBB maturation, β -cat expression decreased (Figure 5a,b). We found a significant difference in nuclear β -cat expression in freshly isolated brain microvessels between the P2, P14 and P21, P84 ($P < 0.05$) stages of BBB maturation. Our findings suggest that the level of expression of total and nuclear β -cat in freshly isolated brain microvessels is inversely correlated with the high expression of bcrp, P-gp, oat3, and oatp1a4 transporters, particularly at P21 and P84. The ultimate question that arises from our studies relates to the role of β -cat in the regulation of P-gp, bcrp, oat3, and oatp1a4 at the BBB at P21 and P84.

Bcrp, P-gp, Oat3, and Oatp1a4 *In Vivo* Expression through Activation of BBB β -Catenin. GSK-3 β normally phosphorylates β -cat on serine and threonine residues, causing β -cat to be targeted for degradation.¹¹ In the absence of GSK-3 β , β -cat escapes degradation and is translocated into the nucleus. Despite the striking difference in the level of

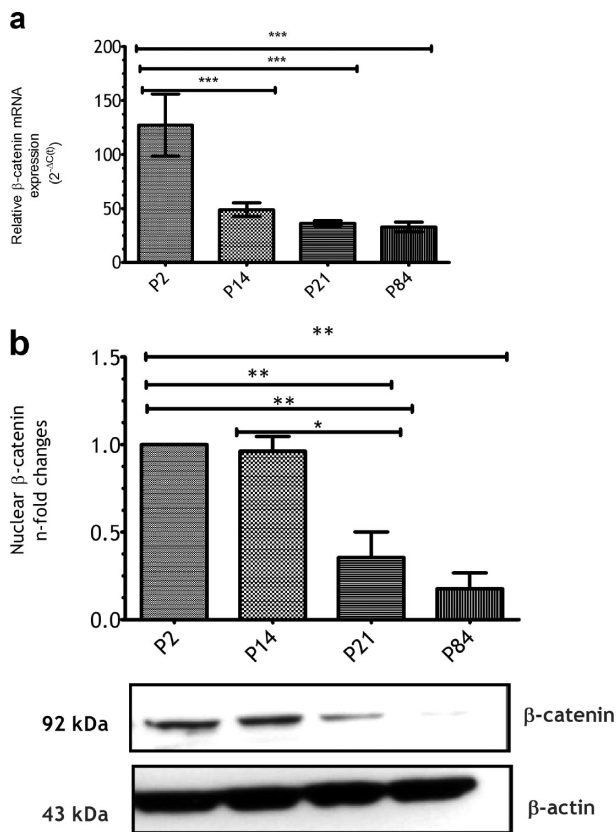


Figure 5. Age-related changes in β -catenin expression in freshly isolated rat brain microvessels. Rats at postnatal day 2 (P2), P14, pediatric (P21), and adult (P84) stages of brain development were used. Brain endothelial microvessels were isolated as described in the Experimental Section, and β -catenin expression was assessed by either RT-PCR (a) or Western blot (b). Data represent the mean for 3 batches of brain capillaries (each single preparation represents pooled tissue from 5 rats); variability is given by SEM bars. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

expression between β -cat and bcrp, P-gp, oat3, and oatp1a4 transporters in pediatric and adult BBB, we investigated one more time the direct impact of β -cat regulation on the expression of P-gp, bcrp, oat3, and oatp1a4 at the BBB. We studied whether the decreases in GSK-3 β induced by intracerebroventricular injection of [(2'-Z,3'-E)-6-bromindirubin-3'-oxime] or BIO, a cell-permeable bis-indolo (indirubin) compound that acts as a highly potent, selective, reversible, and ATP-competitive inhibitor of GSK-3 α/β , in rats could modulate the expression of β -cat and efflux transporters in freshly isolated brain microvessels. The results showed that the inhibition of GSK-3 β in rats by BIO resulted in enhanced mRNA (1.5-fold, $P < 0.001$) and protein (2-fold, $P < 0.05$) expression of β -cat (Figure 6a and b, respectively) as well as of abcg2 mRNA ($P < 0.01$, Figure 6c), slc22a8-mRNA ($P < 0.001$, Figure 6e), and slco1a4-mRNA ($P < 0.01$, Figure 6f) in isolated brain microvessels, whereas a slight increase was observed in the expression of abcb1-mRNA (Figure 6d).

In Vivo Relationship between β -Catenin Signaling and Bcrp, P-gp, Oat3, and Oatp1a4 BBB Transporter Activity. To demonstrate the role of β -cat in the regulation of bcrp, P-gp, oat3, and oatp1a4 transporter activity at the BBB, the brain/plasma concentration ratio $K_{p,brain}$ of known substrates (prazosin, digoxin, bumetanide, and rosuvastatin) was determined after intracerebroventricular injection of BIO in

rats. We monitored drug concentration in brain and plasma in unanesthetized catheterized P84 rats at steady state (4 h). LC/MS/MS analysis of brain tissues from rat pretreated with BIO for 24 h revealed a striking difference in transporter activity between bcrp, P-gp, and oat3 (Figure 7a,b,c). Notably, blocking GSK-3 β decreased unbound brain prazosin uptake [0.68 ± 0.10 ng/g of brain tissue (treated animals) vs 1.71 ± 0.78 ng/g of brain tissue (control animals)]. Consequently the decreased of the unbound partitioning prazosin coefficient [$K_{p,uu,brain} = 0.039 \pm 0.028$ (treated animals) vs $K_{p,uu,brain} = 0.14 \pm 0.07$ (control animals), $P = 0.01$, Figure 7A]. The change in prazosin uptake could suggest an increase in bcrp activity as for prazosin; a decreased in unbound brain uptake [2.07 ± 0.16 ng/g of brain tissue (treated animals) vs 4.00 ± 0.63 ng/g tissue (control animals)] of digoxin was observed after BIO treatment and consequently a decrease in the unbound digoxin partitioning coefficient [$K_{p,uu,brain} = 0.02 \pm 0.009$ (treated animals) vs $K_{p,uu,brain} = 0.08 \pm 0.023$ (control animals), $P = 0.004$, Figure 7B]. A similar conclusion could be drawn for the unbound bumetanide partitioning coefficient [$K_{p,uu,brain} = 0.74 \pm 0.00$ (treated animals) vs $K_{p,uu,brain} = 0.155 \pm 0.17$ (control animals), $P = 0.0012$, Figure 7c]. $K_{uu,brain}$ for rosuvastatin was not plotted since the brain concentrations measured in the presence of BIO were under the limit of quantification in LC/MS/MS.

LC/MS/MS analysis of plasma revealed a striking difference between rat pretreated with BIO and control animals. Figure 8A–D shows prazosin [18.13 ± 4.60 ng/mL (treated animals) vs 11.99 ± 1.22 ng/mL (animal controls, $P = 0.022$)] digoxin [92.79 ± 7.86 ng/mL (treated animals) vs 43.96 ± 13.19 ng/mL (animal controls, $P = 0.0012$)], bumetanide [1.58 ± 0.00 ng/mL (treated animals) vs 0.31 ± 0.008 ng/mL (animal controls, $P < 0.0001$)], and rosuvastatin [1.31 ± 0.5 ng/mL (treated animals) vs 7.72 ± 2.19 ng/mL (animal controls, $P = 0.0079$)] unbound concentrations in rat plasma. As drug concentrations are always significantly higher in the BIO-treated animals than in the control animals, we suggest that the enhancement of bcrp, P-gp, oat3, and oatp1a4 transporter expressions and activities coincided with the *in vivo* increase of nuclear β -cat under BIO conditions (Figure 6a,b). Taken together, these findings provide novel insight into the expression and function of ABC and SLC transporters under β -cat regulation.

In Vivo Inhibition of Gsk-3 β Results in Increased Endothelin-1 Expression in Rat Brain Microvessels. We investigated whether inhibition of GSK-3 β had an effect on the expression of ET-1 in freshly isolated brain microvessels from BIO-treated rats. Figure 9 shows that enhancement of β -cat mRNA (Figure 6a) or nuclear protein expression (Figure 6b) coincided with the increase of ET-1 mRNA synthesis in rat brain microvessels (Figure 9a, $P < 0.05$). Furthermore, using the *in vitro* cell-based rat BBB model, exhibiting *in vivo* BBB characteristics such as tight junctions sealing the endothelial cells, as highlighted by the immunofluorescence staining of ZO-1, and β -cat proteins, respective components of tight and adherent junctions (Figure 9b), we demonstrated that treatment of cells with BIO (5 μ M) increased ET-1 synthesis (Figure 9C), and in turn treatment of cells with ET-1 (1 nM) enhanced β -cat synthesis (data not shown). These results indicate that the inhibition of GSK-3 β results in increased ET-1 production via elevated β -cat and that β -cat/Tcf-4 and ET-1 signaling could form feedback loop regulation which influences bcrp, P-gp, oat3, and oatp1a4 transporter activity at the BBB.

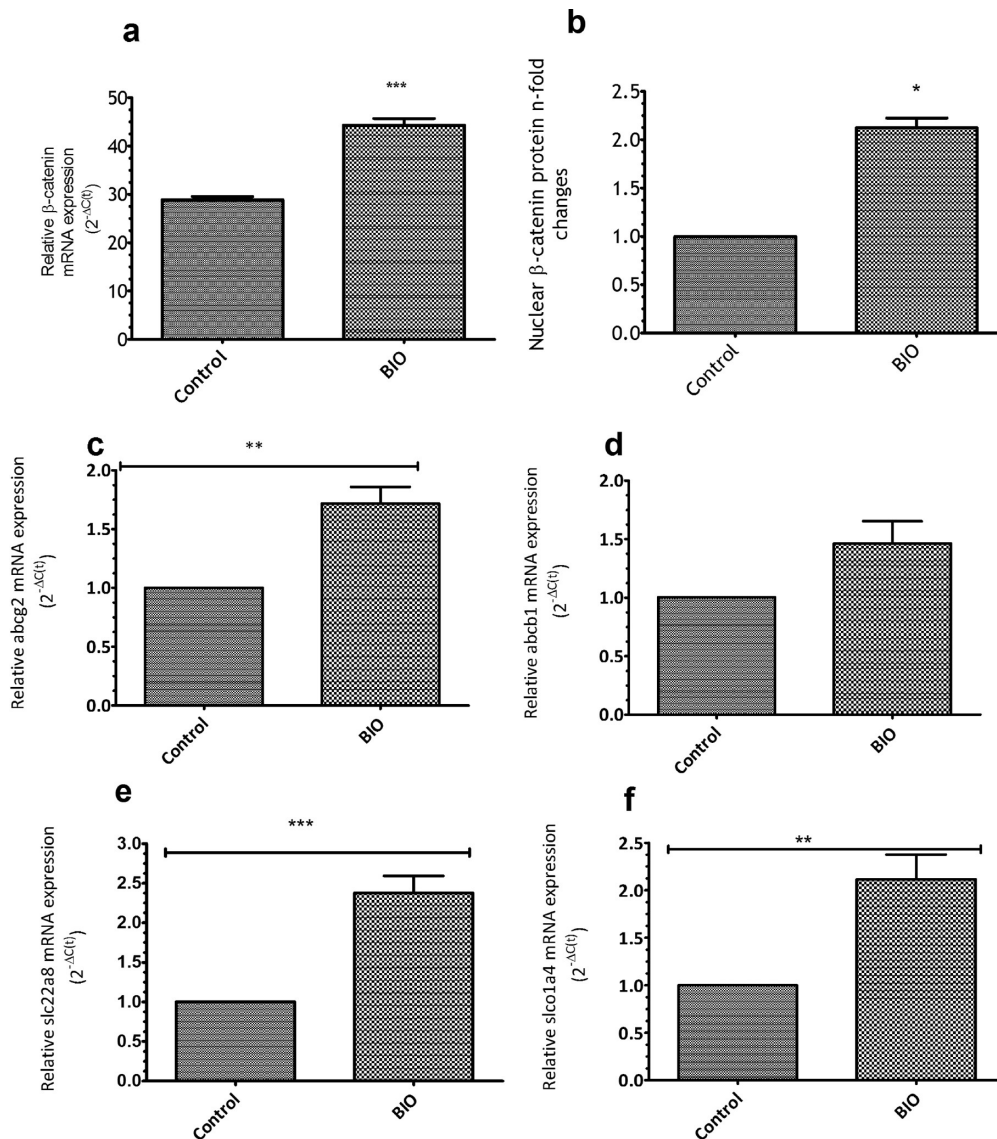


Figure 6. Inhibition of GSK-3 β in rat brain results in increased nuclear β -catenin expression in freshly isolated brain microvessels and regulation of efflux transporters. (a) mRNA transcription profiles for β -catenin, assessed by semiquantitative real-time PCR performed in freshly isolated rat brain endothelial microvessels. (b) β -catenin expression in isolated brain microvessels by Western blotting. Rats treated with BIO showed significantly higher ($P < 0.05$) expression of β -catenin mRNA and nuclear β -catenin protein than animals receiving control vehicle. Representative data from 3 batches of brain capillaries (each single preparation represents pooled tissue from 3 rats); variability is given by SEM bars. Assessment by RT-PCR of abcg2-mRNA(c), abcb1-mRNA (d), slc22a8-mRNA (e), and slco1a4-mRNA (f) synthesis in isolated brain microvessels after inhibition of GSK-3 β by BIO. Representative data from 3 batches of brain capillaries (each single preparation represents pooled tissue from 3 rat brains); variability is given by bars \pm SEM. * $P < 0.05$ and *** $P < 0.0001$.

Relationship between β -Catenin and ET-1 Signaling in the Modulation of Bcrp, P-gp, Oat3, and Oatp1a4 BBB Transporter Activity. Based on the data on the relationship between β -cat and ET-1 regulation, we hypothesized that ET-1 may be directly responsible for the modulation of bcrp, oat3, and oatp1a4 transporter activities at the BBB. To demonstrate the relationship between β -cat and ET-1 signaling in the modulation of BBB efflux transporter activity, *in vivo* experiments were performed. Rats were treated with either BIO or BIO in the presence of JKC-301 (antagonist of ETA receptor), and then the $K_{p,u,u,brain}$ was monitored by determination of brain and plasma drug concentration by mass spectrometry as described in the Experimental Section.

While inhibition of GSK-3 β increased nuclear β -cat expression, enhanced plasma concentration and decreased

$K_{p,u,u,brain}$ for prazosin, digoxin, bumetanide, and rosuvastatin (Figures 10 and 11), suggesting an enhancement of bcrp, P-gp, oat3, and oatp1a4 activity at the BBB, respectively, the coadministration of BIO with an antagonist of ET-1 receptor (ETA), the JKC-301, decreased plasma concentration of prazosin, bumetanide, and rosuvastatin but not of digoxin, by comparison with BIO-treated animals. A significant increase of the $K_{p,u,u,brain}$ for prazosin and a tendency to an increase for digoxin (Figure 11) were observed. These findings suggested a link between β cat and ET-1 signaling in the modulation of bcrp, P-gp, and oatp1a4 activity and to a lesser extent Pgp activity at the BBB, as summarized in Figure 12.

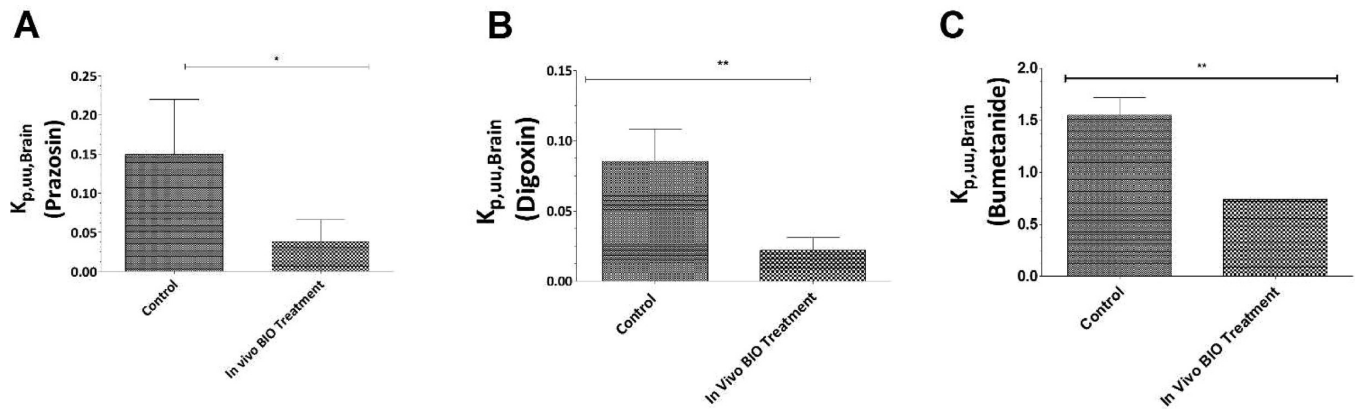


Figure 7. Functional expression regulation activity of bcrp (A), P-gp (B) and oat3 (C) at the BBB through activation of β -catenin. Efflux transporter activity was assessed by determination of $K_{p,uu,brain}$ for each drug-substrate after BIO administration. Data represent the mean concentration for 3 or 5 rat brains and 3 or 5 rat plasmas; variability is given by bars \pm SEM. Data were analyzed using Student's *t* test. Statistical comparisons: $P < 0.05$, $**P < 0.01$.

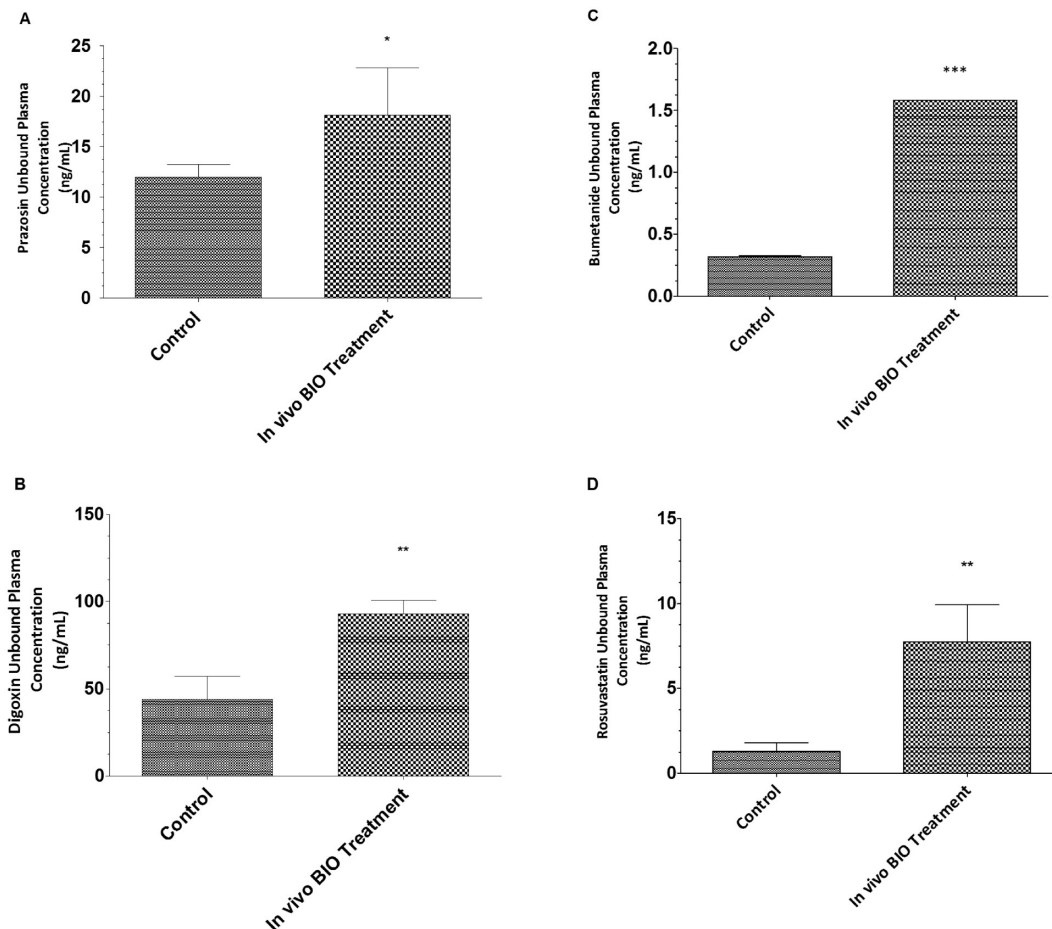


Figure 8. Downregulation of β -catenin mediated by BIO is associated with the increase of Prazosin (A), Digoxin (B), Bumetanide (C), Rosuvastatin (D) concentration in plasma rats. Rats were treated with BIO, and then the plasma drug concentration was determined by mass spectrometry as described in the Experimental Section. Data represent the mean concentration for 3 or 5 rat plasmas; variability is given by bars \pm SEM. Data were analyzed using Student's *t* test. Statistical comparisons: $P < 0.05$, $**P < 0.01$, $***P < 0.001$.

DISCUSSION

The BBB is a lipophilic membrane represented by cerebral capillary endothelial cells and characterized by the presence of tight junctions, low enzymatic metabolism, lack of intracellular vesicles and of endothelial fenestrae, and the presence of efflux/influx transporters. These properties, specific to cerebral

endothelial cells and not found in other endothelial cells, protect the brain from the free entry of toxins and pathogens coming from the blood circulation. This intrinsic system of protection may itself prove deleterious as regards brain bioavailability as it may significantly reduce brain uptake of drugs. The elucidation of the regulatory pathways of efflux/

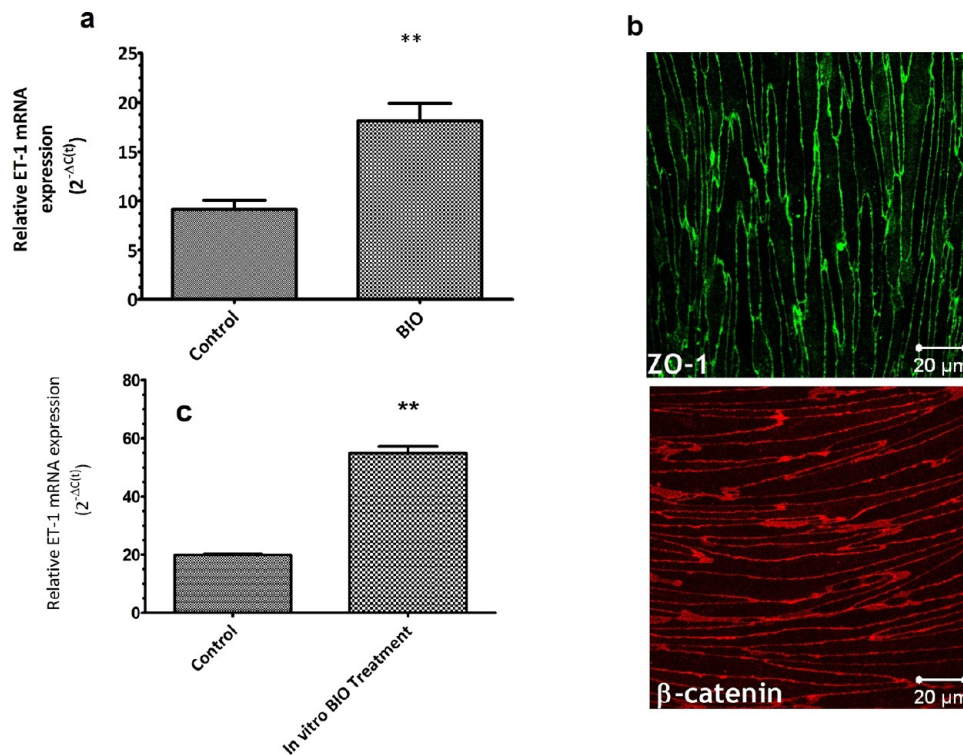


Figure 9. Inhibition of $GK3\beta$ *in vitro* and *in vivo* results in increased ET-1-mRNA expression. (a) Real-time PCR showed a significant ($P < 0.05$) increase in mRNA-ET-1 in freshly isolated rat brain microvessels 24 h after treatment of rat with BIO. Representative data from 4 batches of brain microvessels (each single preparation represents pooled tissue from 5 rat brains). (b) Fluorescence photomicrographs: Primary rat brain endothelial cells stained for ZO-1 and β -catenin. (c) Exposure of the *in vitro* cell-based rat BBB model to BIO increased ET-1-mRNA synthesis. Data are the average of three *in vitro* cell monolayer cultures in duplicate. Statistical comparison vs control: Student's *t* test, $**P < 0.01$.

influx transporters at the BBB may help enhance brain delivery and efficacy of central nervous system (CNS) therapeutics. Recent evidence has pointed out that the specific mechanism of BBB transport such as ion regulation and probably efflux/influx transporter regulation may continue to mature and become fully expressed and functional in the peri- or postnatal period and could modulate CNS drug disposition.^{7,29} A few studies have been reported on the ontogenesis of both ABC and SLC transporters during BBB maturation as well as the molecular mechanisms underlying functional BBB-efflux/influx transporter regulation during brain maturation. In this study, we provide for the first time a comprehensive overview of the expression profile of ABC and SLC transporters at the gene and protein levels as well as their activity during BBB maturation. We illustrate for the first time that the sustainable changes in efflux BBB transporter expression which occur during BBB maturation strikingly coincide with the regulation of β -cat expression. We tested the effect of loss of $GSK-3\beta$ in rat brain and subsequently the link between high expression of β -cat and efflux transporter expression and activity *in vivo* in rats. The involvement of ET-1 signaling in conjunction with β -cat/Tcf-4 signaling in the regulation of BBB functional expression of ABC and SLC transporters was also investigated.^{20,21}

Major findings for this study are: (i) The expression of ABC and SLC transporter genes and their gene products, bcrp, P-gp, oat3, and oatp1a4 increased in rats during brain maturation from preterm up to adulthood, suggesting a potential age-related response to CNS drugs; (ii) Unlike oat3 and oatp1a4, there was no variation in BBB-bcrp or P-gp transporter activity between pediatric and adult rats; (iii) Unlike the expression of ABC and SLC efflux/influx transporters, nuclear β -cat

expression decreased significantly at postnatal day P21 (pediatric human brain) and P84 (corresponding to adult human brain) compared with day P2 (corresponding to the preterm human brain); (iv) *In vivo* administration of $GSK-3\beta$ inhibitor increased the expression of nuclear β -cat expression; (v) Interestingly, the increase of β -cat strikingly coincided with the functional expression of bcrp, P-gp, oat3, and oatp1a4 in freshly isolated brain microvessels; (vi) Modulation of bcrp, P-gp, oat3, oatp1a4, and to a lesser extent of P-gp activity by β -cat at the BBB was strikingly linked to the modulation of ET-1 production. Taken together, our findings provide novel insight into the functional expression of ABC and SLC transporters under β -cat regulation and illustrate a major role of the inflammatory component ET-1 in the functional regulation of both ABC and SLC transporters at the BBB.

These findings are discussed below.

Using a rat model at different stages of brain development equivalent to the preterm (P1), neonate (P14), pediatric (P21), and adult (P84) human brain and the use of brain capillary isolation and immunoblotting techniques, we showed that abcb1 mRNA levels increased from P2 to P84. These findings are in agreement with previous observations using total brain but not freshly isolated brain microvessels.⁹ Immunoblotting shows the same pattern with a tremendous fold increase from P2 to P14 (8.41-fold increase P14 compared with P2) and no significant difference in the expression from P14 through P84. These findings are in agreement with a previous report on the maturation of P-gp in human brain.⁸ Abcg2 mRNAs and abcb1 mRNA are far more expressed in brain capillaries than abcc1, abcc4, abcc5, and abcc6 (see Supporting Information). A significant difference in bcrp protein expression as depicted in

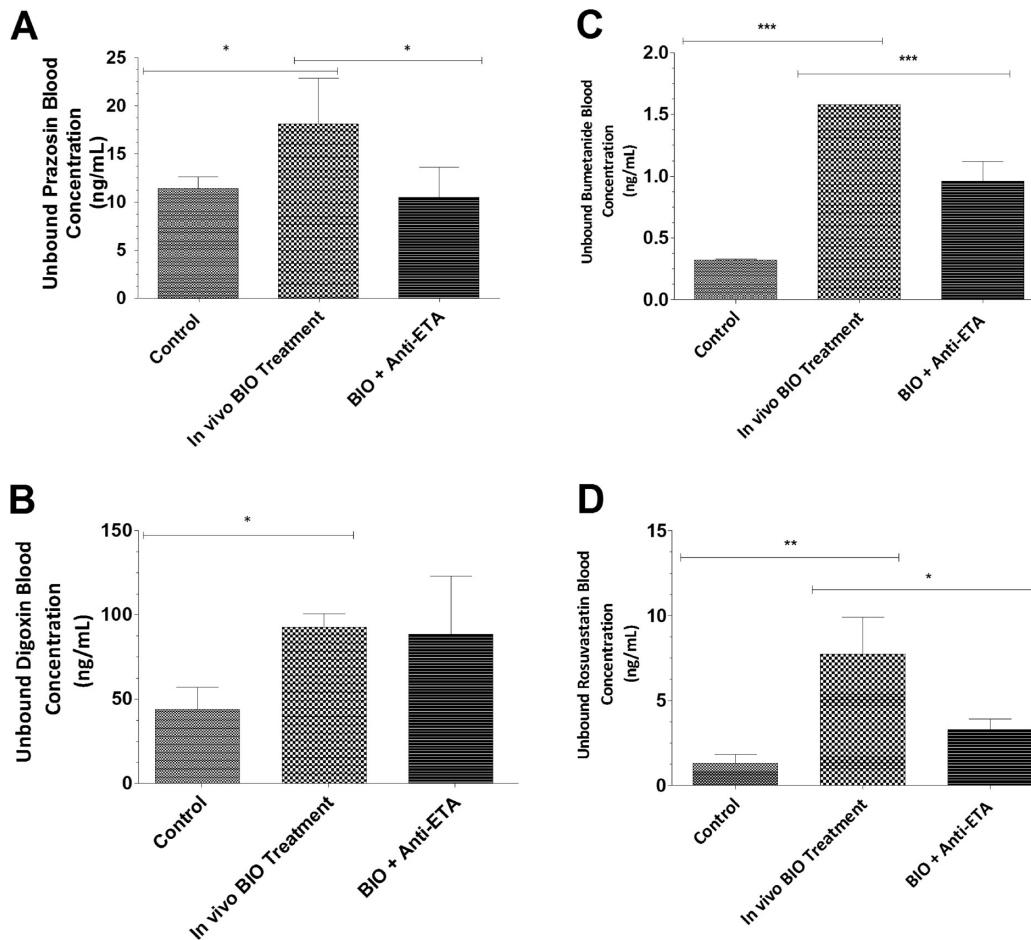


Figure 10. Downregulation of β -catenin mediated by BIO is associated with an increase of Prazosin (A), Digoxin (B), Bumetanide (C), and Rosuvastatin (D) concentration in plasma. Rats were treated with BIO in the presence or absence of JKC-301 (antagonist of ET-A receptor), and then the plasma drug concentration was determined by mass spectrometry as described in the Experimental Section. Data represent the mean concentration for 3 or 5 rat plasmas; variability is given by bars \pm SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. Statistical comparisons: $P < 0.05$, $**P < 0.01$, $***P < 0.001$.

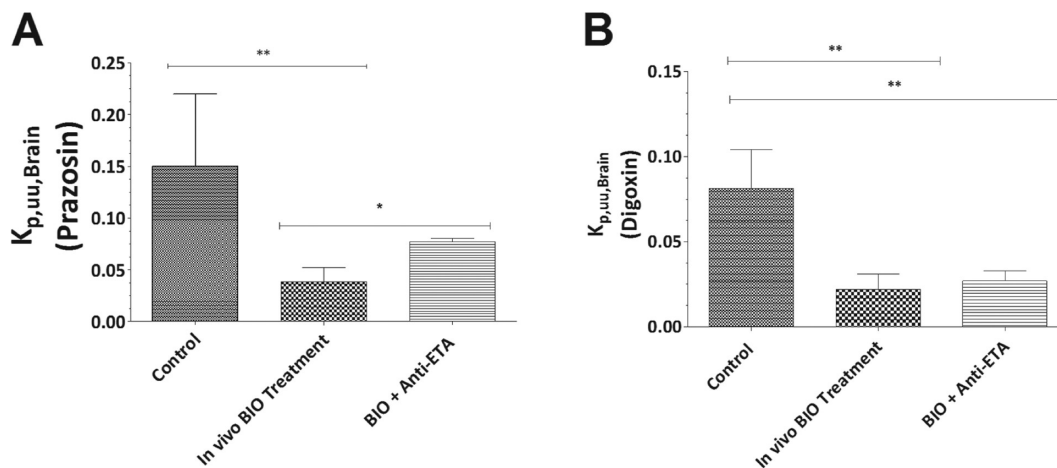


Figure 11. Functional expression regulation activity of bcpr (A) and P-gp (B) at the BBB through activation of β -catenin and endothelin-1 signaling. Efflux transporter activity was assessed by determination of $K_{p,uu,Brain}$ for each drug-substrate in rats treated with either BIO or BIO associated with an antagonist of ET-A receptor (JKC-301, antagonist of ETA). Data represent the mean concentration for 3 or 5 rat brains and 3 or 5 rat plasmas; variability is given by bars \pm SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test or Student's t test. Statistical comparisons: $P < 0.05$, $**P < 0.01$.

Figure 2d was observed between P2 and the other stages of brain development. These findings are in agreement with previous reports using human brain microvessels,³⁰ but not

with findings reported by Daood and colleagues.⁸ The discrepancies between results could be due by the fact that Daood and colleagues used the post-mortem tissue samples and

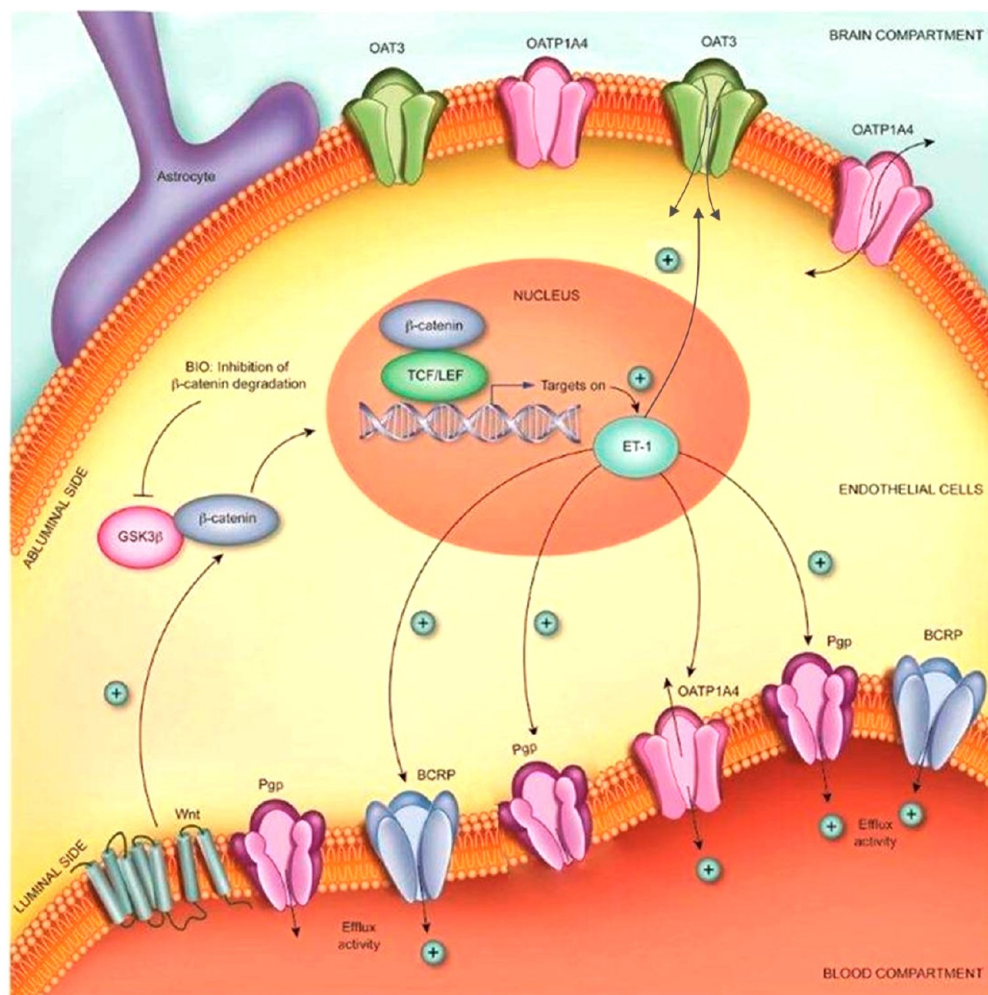


Figure 12. Model showing the relationship between Wnt/ β -catenin signaling, endothelin-1 signaling, and functional expression of P-gp, bcrp, oat3, and oatp1a4 at the BBB.

the pattern and level of transporter immunostaining could be modulated by conditions that precede patient's demise between death and post-mortem.

Slco1a4 mRNA expression is more reliable in brain capillary than expression of slc22a8, abcg2, and abcb1 mRNA. The developmental switch in efflux transporter oat3 expression in brain capillaries was most closely reflected between postnatal day P2 and P21, whereas oatp1a4 protein expression increased during brain maturation from preterm up to adulthood.

The distinct pattern of expression between both SLC family transporters may be related to their different mechanistic regulations.

In addition to the level of bcrp, P-gp, oat3, and oatp1a4 protein expression in brain capillaries, their functional activities were analyzed. Functional comparison of BBB-efflux transporter activities was undertaken only between P21 and P84 stages of BBB maturation since femoral vein catheterization for 4 h infusion of the tested compounds could not be performed on the younger rats.

Our findings demonstrate a lack of widespread related difference in CNS drug responses between pediatric and adult animals. Indeed, we observed no difference in protein expression at the BBB for P-gp, bcrp, and oat3 between pediatric and adult animals, while a tendency of increase in oatp1a4 expression was observed between the two ages.

In addition, our findings point out the lack of age-related developmental changes in bcrp and P-gp function in the rat BBB. Thus, there is a correspondence between P-gp and bcrp protein expression and transport activity at the BBB level from P21 and P84. In contrast to the oatp1a4, the increase in the BBB transport activity of oat3 between pediatric and adult rats (Figure 4c) does not correlate with its protein expression (Figure 3b), suggesting that other factors may cause functional but not transcriptional regulation of oat3 at the pediatric and adult BBB. Our findings emphasize the importance of considering activity profiles of transporter proteins during development for better assessment of drug pharmacokinetic profiles.

Regarding the increase in the BBB transport activity mediated by oatp1a4, it is worth mentioning that this transporter is located on the luminal and abluminal side of the brain endothelial cells, suggesting the possibility that oatp1a4 mediates the uptake and subsequent efflux process in the transcellular transport across the BBB.^{29–31} This is to say that the lower $K_{p,uu,brain}$ for rosuvastatin to the higher expression of oatp1a4 could be expected to efflux substrate from the brain to blood. The ultimate question that arises from our studies relates to the mechanism underlying the regulation of bcrp, P-gp, oat3, and oatp1a4 functional expression during brain development. The role of Wnt/ β -cat signaling in the develop-

ment of the BBB has been suggested.¹⁰ The regulation of some BBB properties by Wnt/ β -cat signaling was also suggested. This is the case for the expression of SLC2a1.³⁴ The endothelial specific β -cat mutants exhibited upregulation of glut1 in CNS parenchyma. It is likely that wnt/ β cat regulates endothelial cell expression of the BBB-specific transporter glut1.³⁴ Taking into account these findings, we endeavored to determine the link between β -cat expression and efflux/influx transporter expression during BBB maturation. The findings depicted in Figure 5b demonstrate that nuclear β -cat expression in freshly isolated brain capillaries was tremendous bcrp, P-gp, oat3, and oatp1a4 transporters were overexpressed, suggesting the potential role of β -cat in the expression of BBB transporters. To formally demonstrate the role of β -cat in the regulation of bcrp, P-gp, oat3, and oatp1a4 transporters at the BBB, rats were injected with GSK-3 β inhibitor (BIO), and expression of P-gp, bcrp, oatp1a4, and oat3 was measured in freshly isolated brain microvessels using immunoblotting, as was the transport activity of these efflux/influx transporters at the BBB, using the assessment of the $K_{p,u,u,brain}$ which determined the net influx and efflux of drug across the BBB. The findings depicted in Figures 6, 7, and 8 demonstrate that inhibition of GSK-3 β in rat by BIO resulted in enhanced protein expression of nuclear β -cat associated with the increase of abcg2, abcb1, slc22a8, and slco1a4 mRNA expression and function at the BBB. These findings suggest that treatment of animals by BIO which is associated with the overexpression of nuclear β -cat triggered an intracellular signal transduction pathways involved in the regulation of transporter activities.

Our findings are in agreement with a previous report on the striking link between the increase of nuclear β -cat and the increase of P-gp function *in vitro* using an immortalized human endothelial cell line.¹² With respect to the probe used to investigate the transport activity at the BBB level, it is worth mentioning that digoxin is indeed a substrate for transport by P-gp, and it is also a substrate for at least oatp1a4, two of which are believed to be expressed at the BBB. Digoxine is taken in charge by P-gp; however, it is likely to be taken in charge by oatp1a4. A cooperation between both transporter is to be taken into account on the efflux of digoxin compound at the level of the BBB.

We further demonstrated that the effect of β -cat on the regulation of bcrp, oat3, oatp1a4, and to a lesser extent of P-gp involved at least the ET-1 regulator (Figures 10 and 11). *In vivo* inhibition of GSK-3 enhanced nuclear β -cat (Figure 6) which strikingly coincided with ET-1 production in brain microvessels (Figure 9), suggesting cooperation between both partners in the potential regulation of BBB efflux/influx transporters. Data depicted in Figures 6, 9, 10, and 11 support this assertion.

In conclusion, while developmental changes of functional P-gp have been reported, this study examined in addition age-related functional expression of bcrp, P-gp, oat3, and oatp1a4 at the BBB.²⁹ We provide further evidence that, in contrast to oat3 and oatp1a4, the expression but not the function of P-gp and bcrp transporters increased particularly between pediatric and adult brains. In addition, we have evidence that the high expression of these transporters, particularly in adulthood, is associated with the down regulation of β -cat. Our findings provide further novel insights into the expression and activity of bcrp, oat3, oatp1a4, and to a lesser extent P-gp transporters in β -cat/ET-1 signaling (Figure 12).

■ ASSOCIATED CONTENT

● Supporting Information

Table of protein expression of prazosin, digoxin, bumetanide, and rosuvastatin. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank the Agence Nationale de Recherche sur le SIDA for financial support. The authors thank Richard Daneman for scientific advice and Aurélie Ghetas (CEA, iBiTec-S, Service de Pharmacologie et d'Immunoanalyse, Gif-sur-Yvette, France) for her technical skills in mass spectrometry.

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Chapter II

Regulation of P-glycoprotein, Bcrp, Oat3 and Oatp1a4 Expression and Transport Activity at the Blood-Brain Barrier by Cerebral Inflammation Triggered by Endothelin-1

This work has been reported in an article accepted for publication the Journal of Neuroinflammation and untitled:

“Susceptibility of Juvenile and Adult Blood-Brain Barrier to Endothelin-1: Regulation of P-glycoprotein and Bcrp Expression and Transport Activity” (Harati et al., 2012b).

2.1 Summary

In the first part of this thesis, we highlighted changing properties of the BBB during ontogenesis, as well as a differential regulation of BBB drug efflux transporters after β -cat activation. We hypothesized that regulatory mechanisms activated in adult brain may be different from those in juvenile brain, and that the factor at the origin of differences may be the ET-1, a cytokine peptide. On another hand, there is now a wealth of evidence that age could have a significant effect on response to cytokines, which in turn could modulate BBB efflux transporters expression and activity (Brochu et al., 2011; Miller, 2010; Salkeni et al., 2009, Stolp and Dzgieliewska, 2009; Hembury and Mabondzo, 2008; Ronaldson and Bendayan, 2006; Theron et al., 2003). Basing on these several findings, it seemed important to evaluate potential age-related differences in functional expression of BBB efflux transporters during brain maturation in the context of brain neuroinflammation triggered by ET-1, especially, that in response to nearly all CNS disorders, CNS exhibits inflammatory features (Miller, 2010).

That was the main aim of the second part of the thesis. In this study, we evaluated the impact of acute cerebral inflammation mediated by ET-1, on BBB efflux transporters with a comparison between juvenile and adult rats. Specifically, we evaluated, *ex vivo* and *in vivo*, the impact of intra-cerebroventricular injection of ET-1, on the expression and activity of two of the most clinically relevant BBB drug efflux transporters, the P-gp and the Bcrp transporters. We also investigated whether ET-1 triggers differential inflammatory reactions in juvenile organism when compared to adult, and evaluate whether this potential difference may be responsible of an

eventual differential regulation of BBB drug efflux transporters in the context of brain neuroinflammation. The scheme below presents an illustration of our experimental design:

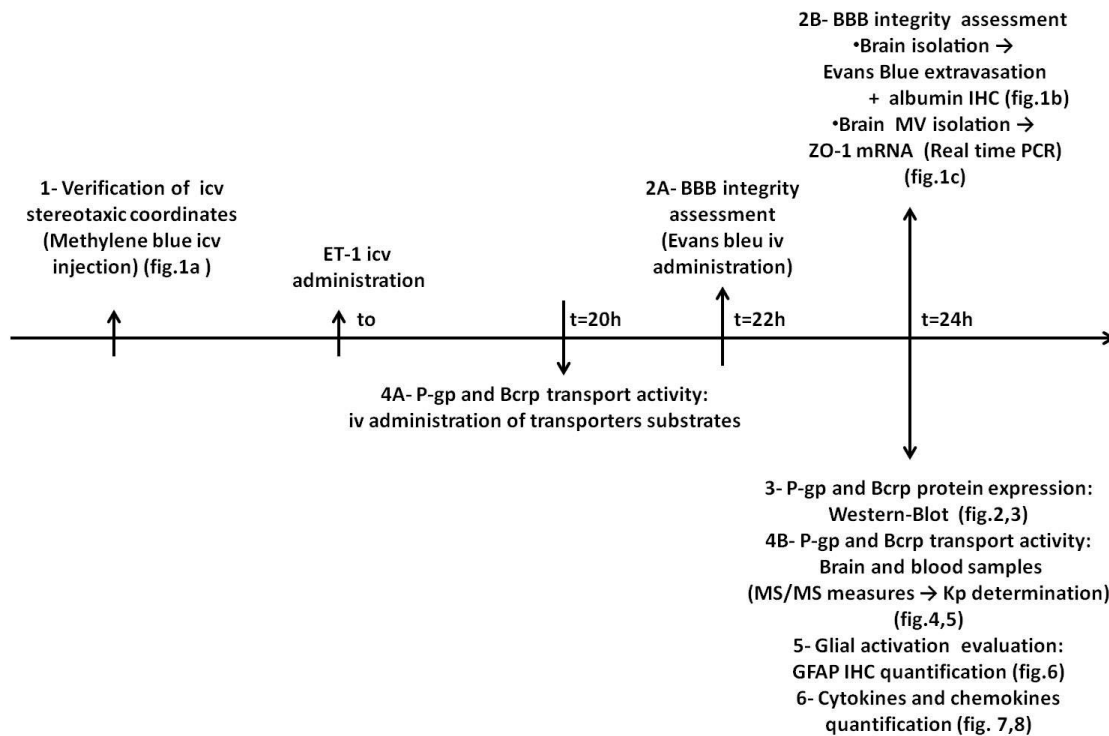


Figure 16. Illustration of the experimental design

Male Wistar rats at two stages of brain maturation were used in our experiments: Post-natal (P) P21 corresponding to the pediatric stage in human and P84 corresponding to the adult stage in human. The experimental design was articulated in several steps: 1. Verification of ventricular stereotaxic coordinates in both juvenile and adult rats: Methylene blue was injected intracerebroventricularly in order to visualize the stereotaxic implantation of the injection cannulae and the injection site into the brain lateral ventricles. 2. BBB integrity assessment by the Bleu Evan test, Baclofen test and the measure of mRNA relative expression of a tight-junction component, the Zo-1: ET-1 was administrated intracereboventricularly. Twenty-two hours later, a solution of 3% Evans Blue dissolved in 0.9% saline was administrated intravenously (4ml/kg). Two hours later, brains were isolated and the BBB integrity was assessed by verifying the absence of blue trace and by albumin immunohistochemical (IHC) detection in the cortex and the hippocampus. BBB integrity was further assessed by measuring using real-time PCR, mRNA relative expression of the ZO-1 in brain microvessels isolated 24h after ET-1 treatment. 3. Measure of P-gp and Bcrp protein expression in brain microvessels isolated 24h after ET-1 injection. 4. Measure of P-gp and Bcrp protein activity in vivo 24h after ET-1 injection: Twenty hours after ET-1 treatment, P-gp and Bcrp substrates were administrated intravenously during four hours (steady state (ss) achievement) at a concentration of 1 mg/kg/h. At the end of the infusion period, blood was sampled, brain was isolated and the administrated substrates were quantified in the two compartments using mass spectrometry. P-gp and Bcrp transport activity was estimated by the ratio between substrates brain concentrations (C) and plasma concentrations. This ratio is described by the partition coefficient, Kp,uu : $Kp,uu_{brain} = [C_{ss\ total, brain} / C_{ss\ total, plasma}] \times f_{ub}/f_p$. 5. Immunohistochemistry of Glial Fibrillary Acidic Protein (GFAP), a glial activation marker (Raine, 1999) in cortex and hippocampus of rat brains.

To evaluate whether potential developmental differences in neuroinflammatory responses could contribute to the age-specific patterns of BBB efflux transporter expression, rats at P21, and P84, corresponding to the juvenile and adult stages of human brain maturation, respectively,

were treated with ET-1 by intracerebroventricular route. Twenty-four hours after ET-1 icv administration, we measured P-gp (figure 2; Harati et al., 2012b) and bcrp (figure 3; Harati et al., 2012b) protein expression in isolated BMVs by immunoblotting, and we assessed their transport activity in vivo by measuring the partitioning coefficient ($K_{p,u\text{brain}}$) of known efflux transporter substrates (Hammarlund-Udenaes et al., 2008; Liu et al., 2005) administered intravenously. We found first of all that juvenile and adult BBBs exhibited similar P-gp and bcrp transport activities in the normal physiological conditions (Additional file 1; Harati et al., 2012b). However, long-term exposure of the juvenile brain to low-dose of ET-1 did not change BBB P-gp transport activity (figure 4; Harati et al., 2012b) but tended to decrease bcrp transport activity (figure 5; Harati et al., 2012b) in the juvenile brain, while a significant increase of the activity of both transporters was evidenced at the BBB in the adult brain.

After assessing BBB transport activity in adult and juvenile brains in the context of ET-1 treatment, we aimed at understanding the underlying mechanisms behind this differential regulation. We suspected a role of neuro-inflammatory response triggered by ET-1 because it is likely that cytokines secretion in adult and juvenile brains controls the regulation of BBB transporters (Miller et al., 2010; Hartz et al., 2004). To test whether the inflammatory response triggered by ET-1 can be responsible for the differential regulation of P-gp and bcrp transporters between juveniles and adults, we quantified two processes occurring during inflammation: 1) the glial activation marker, the GFAP in hippocampus and cortex of rat brains treated with ET-1, and 2) a panel of cytokines and chemokines that could be induced by ET-1 in both juvenile and adult rat brains. We found a difference in the GFAP basal levels between the two populations, but our results showed a similar pronounced glial activation in adult and juvenile cortex and hippocampus (Figure 6; Harati et al., 2012b) which coincided with cytokine/chemokine levels (Figures 7; Harati et al., 2012b). Our findings were that the following cytokines: IL6, IL-1 β , CCL2/MCP-1, TIMP-1 and IL-10 increased tremendously in adult brain compared with juvenile brain. To formally demonstrate that cytokine synthesis is a cause of the differential regulation of transporter activity between juvenile and adult BBB, we increased the amount of ET-1 administered after determining the lack of BBB breakdown. At the dose of 125 pmol/Kg of ET-1, the juvenile brain exhibited increases in IL-1 β , MCP-1, and IL6 (Additional file 2, Harati et al., 2012b). Despite the level of those cytokines in the brain, the juvenile BBB showed a decrease

in BCRP activity which strikingly coincided with the decrease of unbound plasma prazosin concentration (3.77 ± 1.58 ng/ml versus 8.56 ± 3.77 ng/ml, $P < 0.05$). In addition, the increase in the administered dose of ET-1 did not change the activity of P-gp in the juvenile BBB. We observed no significant decrease of digoxin concentration in unbound plasma of juvenile animals (23.50 ± 7.58 treated animals versus 47.88 ± 6.80 for control animals, $P < 0.05$). In adult brain, BBB at the dose of 125 pmol/kg of ET-1 (data not shown) showed the same profile regarding the increase of P-gp and BCRP activity compared with the dose of 25 pmol/kg. These findings suggest the involvement of other parameters in the differential regulation of P-gp and bcrp at the BBB, particularly in juvenile brain.

Thus, we hypothesized that, under inflammatory conditions, BBB drug efflux transporters are regulated differently in juvenile rats when compared to adults, due to:

- 1) Age-related differences in the BBB components between juveniles and adults, as shown in the descriptive BBB ontogenesis study.
- 2) Differences in neuro-inflammatory response related to GFAP basal levels. Since basal GFAP levels were different between juveniles and adults (Figure 6; Harati et al., 2012b), suggesting that differential regulation of drug efflux transporters observed between ages may be due in part to differences in GFAP activation.
- 3) Maturation differences in ET-1 receptors. For that, we established the profile of ETA, one of the two ET1 receptors, during brain maturation and found significant differences between juveniles and adults (Figure 17).

Our results showed significant difference of ETA expression between juvenile and adult brain.

- 4) Differences in signaling pathway activated differently in children compared to adults, and this depending on the ET-1 receptor implicated.

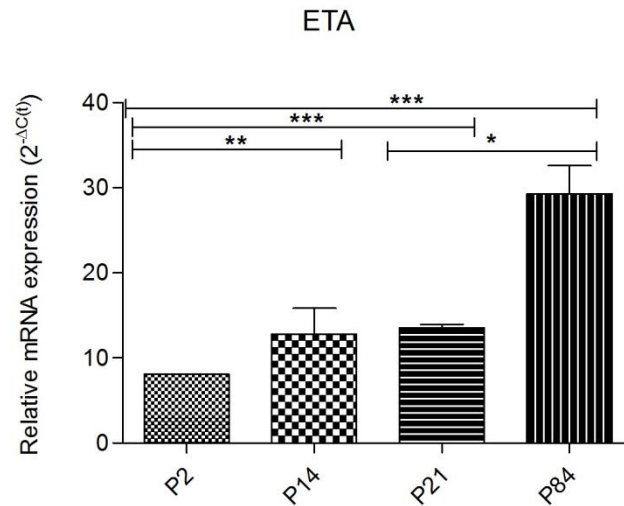


Figure 17. Relative mRNA expression of ETA in isolated BMVs during brain maturation

Rats at different stages of brain development equivalent to the preterm (P2), neonate (P14), pediatric (P21), and adult (P84) human brain were used. mRNA transcription profiles for ETA was assessed by semiquantitative real-time PCR performed in freshly isolated rat BMVs. Results are means \pm SEM for 3 batches of BMVs (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of BMVs. Variance analysis (one way ANOVA) was performed to assess the significant differences between different groups. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

2.2 Discussion

To resume, this study had four major findings:

- i. Transport activities of P-gp and bcrp are enhanced by ET-1 at the adult BBB, whereas no modification in P-gp transport activity and a tendency to decrease in bcrp transport activity were seen at the juvenile BBB (Figures 4 and 5, Harati et al., 2012b).

Under normal conditions, we found that juvenile and adult BBB have the same P-gp and bcrp transport activity unlike in the inflammatory context, where the response of drug transporters is age-dependent. In adults, ET-1 increases P-gp and bcrp transport activity. These findings are in accordance with previous studies; Indeed, Baur and colleagues (2007) have showed in rat brain capillaries that long-term exposure to ET-1 leads to an increase of P-gp activity (Bauer et al, 2007) which is in agreement with our results regarding the adult brain but not the juvenile brain. Unlike in adults, no variation of P-gp transport activity and a tendency to decrease in bcrp transport activity were seen at the juvenile BBB. Thus, these results gave evidence that P-gp and

bcrp transporters at the BBB level are regulated differently under pathological conditions in juvenile brain when compared to adult. These findings emphasize the importance of considering differential P-gp and bcrp transporter regulation mechanisms between juvenile and adult BBB in the context of pathological conditions.

ii. In conjunction with this differential regulation, juvenile and adult brain showed differences in their expression profiles of cytokines and chemokines (Figures 7, Harati et al., 2012b) despite similar glial activation mediated by ET-1 although basal levels of GFAP, a glial activation marker, were different in juvenile brain compared to adult (Figure 6, Harati et al., 2012b).

Several factors conduct our research to look for cytokines and chemokines profil, as well as glial activation in both ages:

- 1) During inflammation, a series of changes occur in astrocytes, leading to the acquisition of macrophage differentiation markers and effector properties. One main feature of these changes is the increase in the number and size of GFAP expressing cells.
- 2) ET-1 is known to be implicated in an inflammatory cascade; its released by several cytokines such as TNF- α , and in turns it triggers the release of a panel of inflammatory cytokines and chemokines. Previous work in our laboratory as well as studies from other laboratories have shown that proinflammatory cytokines such as TNF α and IL-1 β caused a released of ET-1 at the BBB level (Bauer et al., 2007; Didier et al., 2003; Marsden and Brenner; 1992). In turn, ET-1 causes the release of other cytokines and chemokines such as MCP-1 (Chen et al., 2001).
- 3) It is likely that cytokines secretion in adult and juvenile brains controls the regulation of BBB transporters (Slakeni et al., 2009). This point was discussed in the introduction part.

For this, to test whether the inflammatory response triggered by ET-1 can be responsible for the differential regulation of P-gp and bcrp transporters between juveniles and adults, we quantified the GFAP in hippocampus and cortex of rat brains treated with ET-1, and we quantified a panel of cytokines and chemokines that could be induced by ET-1 in both juvenile and adult rat brains. Our results showed a difference in the GFAP basal levels between the two populations, but our results showed a pronounced glial activation in adult and juvenile cortex and hippocampus coincided with cytokine/chemokine levels (Figures 6 and 7; Harati et al., 2012b).Our findings

were that IL6, IL-1 β , CCL2/MCP-1, TIMP-1 and IL-10 increased tremendously in adult brain compared with juvenile brain. These results emphasize the fact that brain development such as glial maturation is likely of paramount importance in the synthesis of specific cytokines such as IL6 for example. Indeed, IL6 is a well-known marker of glial activation (Ridet et al., 1997) and the autocrine action of this interleukin on glial cells might account for an increase of ABC transporters at the cell surface. This increase is important in the secretion of CCL2/MCP-1 by astrocytes upon Toll-like receptor 3 activation as reported recently (Kooji et al., 2011). Moreover, taking into account the differential effect of ET-1 on cytokine levels in juvenile and adult brains, we suggest that the increased levels of cytokines in the adult brain and more particularly IL-6 and IL-1 β may cause functional but not transcriptional regulation of P-gp and bcrp in adult BBB. These findings suggest that the differential regulation of BBB transporters under inflammatory impact in an age-dependant manner may be due in part to different inflammatory mechanisms triggered in response to ET-1 in juvenile when compared to adults. Indeed, our results showed no modulation of protein synthesis in bcrp and P-gp in adult rat brain microvessels. Modulation of transport activity for P-gp in response to peripheral pain inflammation with no increase in its protein expression has been previously reported (Seelbach et al., 2007)

iii. In juvenile brain, increased amount of ET-1 administered after determining the lack of BBB breakdown exhibited increases in IL-1 β , MCP-1, and IL6 (Additional file 2, Harati et al., 2012b), but despite the level of those cytokines in the brain, juvenile BBB showed a decrease in bcrp activity which strikingly coincided with the decrease of plasma prazosin. In addition, the increase in the administered dose of ET-1 did not change the activity of P-gp in the juvenile BBB. We observed a tremendous decrease of digoxin concentration in plasma of juvenile animals. In adult brain, BBB at the dose of 125 pmol/kg of ET-1 showed the same profile regarding the increase of P-gp and bcrp activity compared with the dose of 25 pmol/Kg. These findings formally demonstrate that cytokine synthesis is a cause of the differential regulation of transporter activity between juvenile and adult BBB and that the involvement of other parameters in the differential regulation of P-gp and bcrp at the BBB, particularly in juvenile brain.

iv. In adult brain, BBB at the dose of 125 pmol/kg of ET-1 showed the same profile regarding the increase of P-gp and bcrp activity compared with the dose of 25 pmol/Kg).

These findings suggest the involvement of other parameters in the differential regulation of P-gp and bcrp at the BBB, particularly in juvenile brain:

1) Age-related differences in the BBB components between juveniles and adults, as shown in the descriptive BBB ontogenesis study.

2) Differences in neuro-inflammatory response related to GFAP basal levels. Since basal GFAP levels were different between juveniles and adults (Figure 6; Harati et al., 2012b), suggesting that differential regulation of drug efflux transporters observed between ages may be due in part to differences in GFAP activation.

3) Maturation differences in ET-1 receptors. Indeed, we established the profil of ETA, one of the two ET1 receptors, during brain maturation and found significant differences between juveniles and adults (Figure 17). This difference may cause differential regulation of BBB transporters, especially that previous work have shown that P-gp regulation may be different depending on the ET-1 receptor activated (Bauer et al., 2007; Hartz et al., 2004).

4) Differences in signaling pathway activated. Indeed, it is also important to investigate the role of signaling pathways known to modulate expression and activity of ABC transporters at the BBB level. These pathways, such as signaling cascade via , PXR, CAR, NF- κ B and AP-1 (Miller, 2010) or NOS and PKC (Bauer et al., 2007), may be activated differently in children compared to adults, and this depending on the ET-1 receptor implicated.

ARTICLE 2

**Susceptibility of Juvenile and Adult Blood-Brain Barrier to Endothelin-1:
Regulation of P-glycoprotein and Bcrp Expression and Transport Activity**

Journal of Neuroinflammation

RESEARCH

Open Access

Susceptibility of juvenile and adult blood–brain barrier to endothelin-1: regulation of P-glycoprotein and breast cancer resistance protein expression and transport activity

Rania Harati¹, Anne-Sophie Villégier², William A Banks³ and Aloise Mabondzo^{1*}

Abstract

Background: P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) play a critical role in keeping neurotoxic substances from entering the brain. We and others have previously reported an impact of inflammation on the regulation of adult blood–brain barrier (BBB) efflux transporters. However, studies in children have not been done. From the pediatric clinical perspective, it is important to understand how the central nervous system (CNS) and BBB drug efflux transporters differ in childhood from those of adults under normal and inflammatory conditions. Therefore, we examined and compared the regulation of P-gp and BCRP expression and transport activity in young and adult BBB and investigated the molecular mechanisms underlying inflammatory responses.

Methods: Rats at postnatal day (P) P21 and P84, corresponding to the juvenile and adult stages of human brain maturation, respectively, were treated with endothelin-1 (ET-1) given by the intracerebroventricular (icv) route. Twenty-four hours later, we measured P-gp and BCRP protein expression in isolated brain capillary by immunoblotting as well as by transport activity *in vivo* by measuring the unbound drug partitioning coefficient of the brain ($K_{p,uu,brain}$) of known efflux transporter substrates administered intravenously. Glial activation was measured by immunohistochemistry. The release of cytokines/chemokines (interleukins-1 α , 1- β (IL-1 β), -6 (IL-6), -10 (IL-10), monocyte chemoattractant protein (MCP-1/CCL2), fractalkine and tissue inhibitor of metalloproteinases-1 (TIMP-1)) were simultaneously measured in brain and serum samples using the Agilent Technology cytokine microarray.

Results: We found that juvenile and adult BBBs exhibited similar P-gp and BCRP transport activities in the normal physiological conditions. However, long-term exposure of the juvenile brain to low-dose of ET-1 did not change BBB P-gp transport activity but tended to decrease BCRP transport activity in the juvenile brain, while a significant increase of the activity of both transporters was evidenced at the BBB in the adult brain. Moreover, juvenile and adult brain showed differences in their expression profiles of cytokines and chemokines mediated by ET-1.

Conclusions: BBB transporter activity during neuroinflammation differs between the juvenile and adult brains. These findings emphasize the importance of considering differential P-gp and BCRP transport regulation mechanisms between adult and juvenile BBB in the context of neuroinflammation.

Keywords: Juvenile and adult blood–brain barrier, Neuroinflammation, Endothelin-1, Brain-to-blood transport, Drug transport, P-glycoprotein, Breast cancer resistance protein

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Background

The blood–brain barrier (BBB) maintains brain homeostasis and limits the entry of toxins and pathogens into the brain. Adenosine triphosphate-binding cassette (ABC) transporters play a critical role in keeping neurotoxic substances from entering the brain and in transporting toxic metabolites out of the brain [1,2]. These transporters are largely responsible for the multidrug resistance (MDR) phenomenon, which plays a crucial role in treatment failure for several brain diseases such as seizure [3] and human immunodeficiency (HIV-1) infection disease [4,5]. In response to injury or brain diseases [6], the central nervous system (CNS) exhibits inflammatory features, which have effects on the expression and function of BBB efflux transporters in adults [7-9]. Because intricate developmental processes are taking place during the prenatal and postnatal periods, we hypothesized that BBB efflux transporters might also undergo important changes during brain maturation, and might possibly have age-related differences in the inflammatory response. There is now a wealth of evidence that age could have a significant effect on response to cytokines, which, in turn, could modulate BBB efflux transporters expression and activity [10-13] in an age-dependent manner.

Endothelin-1 (ET-1), an arterial vasoconstrictive and vasodilator peptide cytokine [14], is released in several CNS disorders [15-25]. Members of the endothelin family are released by various cell types in brain, including endothelial cells and some glial cells, especially during inflammation [26-29]. In effect, ET-1 is known to be a component of the brain's innate immune response. It is released during the activation of the brain's innate immune response [19-31] triggered by a variety of stimuli, including infection, trauma, disease, and cell stress and characterized mainly by glial activation and the release of proinflammatory cytokines and chemokines [32-36]. Previous works in our laboratory as well as studies from other laboratories have shown that proinflammatory cytokines such as TNF α and IL1 β cause a release of ET-1 at the BBB level [28-35]. In turn, ET-1 causes the release of other cytokines and chemokines such as MCP-1 [36] thus amplifying the inflammatory signals. ET-1 released in this inflammatory context is known to regulate the P-gp activity. Indeed, long-term exposure of isolated rat brain capillaries to the pro-inflammatory cytokine TNF α caused release of ET-1 and then an increase of P-gp transport activity [31,37]. As inflammation occurs in nearly all CNS disorders [8], it is important to understand how it alters the function of drug efflux transporters since these alterations will affect the efficacy of CNS drugs [37]. However, *in vivo* studies of the impact of ET-1 on the regulation of P-gp and other brain-to-blood ABC transporters such as breast cancer resistance protein (BCRP) in adults and more

particularly in children have not been done. From the clinical perspective of developing new drugs with enhanced efficacy in the CNS of children, it is important to understand how the BBB drug efflux transporters are regulated under inflammatory conditions in children's brains specifically. That is the main target of the present study. We examined and compared regulation of P-gp and BCRP expression and transport activity in young and adult BBB and investigated the molecular mechanisms underlying these processes. To evaluate whether potential developmental differences in neuroinflammatory responses could contribute to the age-specific patterns of BBB efflux transporter expression, rats at postnatal day (P) P21 and P84, corresponding to the juvenile and adult stages of human brain maturation, respectively, were treated with ET-1 by intracerebroventricular (icv) route. Twenty-four hours after ET-1 icv administration, we measured P-gp and BCRP protein expression in isolated brain capillary by immunoblotting, and we assessed their transport activity *in vivo* by measuring the unbound brain/plasma concentration ratios ($K_{p,uu, \text{brain}}$) of known efflux transporter substrates [38,39] administered intravenously. The findings are discussed in the context of glial activation leading to differential release of cytokines/chemokines, in particular interleukins-1 α , 1- β (IL-1 β), -6 (IL-6), -10 (IL-10), monocyte chemoattractant protein (MCP-1/CCL2), fractalkine, and tissue inhibitor of metalloproteinases-1 (TIMP-1). These inflammatory mediators were measured in the brain and serum of rats treated with ET-1 at two stages of brain maturation: P21 (pediatric stage in humans) and P84 (adult stage in humans).

Methods

Experimental design

Male Wistar rats at two stages of brain maturation were used in our experiments: Postnatal (P) P21, corresponding to the pediatric stage in human; and P84, corresponding to the adult stage in human. The experimental design was articulated in the following steps: 1) Verification of ventricular stereotaxic coordinates in both juvenile and adult rats; methylene blue was injected intracerebroventricularly in order to visualize the stereotaxic implantation of the injection cannulae and the injection site into the brain lateral ventricles. 2) BBB integrity assessment by the Evan's Blue test, the baclofen test, and the measure of mRNA relative expression of a tight-junction component, zonula occludens-1 (*ZO-1*); ET-1 was administered intracerebroventricularly. Twenty-two hours later, a solution of 3% Evan's Blue dissolved in 0.9% saline was administered intravenously (4 ml/kg). Two hours later, brains were isolated and the BBB integrity was assessed by verifying the absence of blue trace and by albumin immunohistochemical (IHC) detection in the cortex and the hippocampus. BBB integrity was further assessed by measuring (using

real-time PCR) mRNA relative expression of the *ZO-1* in brain microvessels isolated 24 h after ET-1 treatment. 3) Measure of P-gp and BCRP protein expression in brain microvessels isolated 24 h after ET-1 icv injection. 4) Measure of P-gp and BCRP protein activity *in vivo* 24 h after ET-1 icv injection; Twenty hours after ET-1 icv treatment, P-gp and BCRP substrates were administered intravenously during four hours (steady state (ss) achievement) at a concentration of 1 mg/kg/h. At the end of the infusion period, blood was sampled, brain was isolated and the administered substrates were quantified in the two compartments using mass spectrometry. P-gp and BCRP transport activity was estimated by the ratio between substrates unbound brain concentrations (C) and unbound plasma concentrations. This ratio is described by the unbound partitioning coefficient,

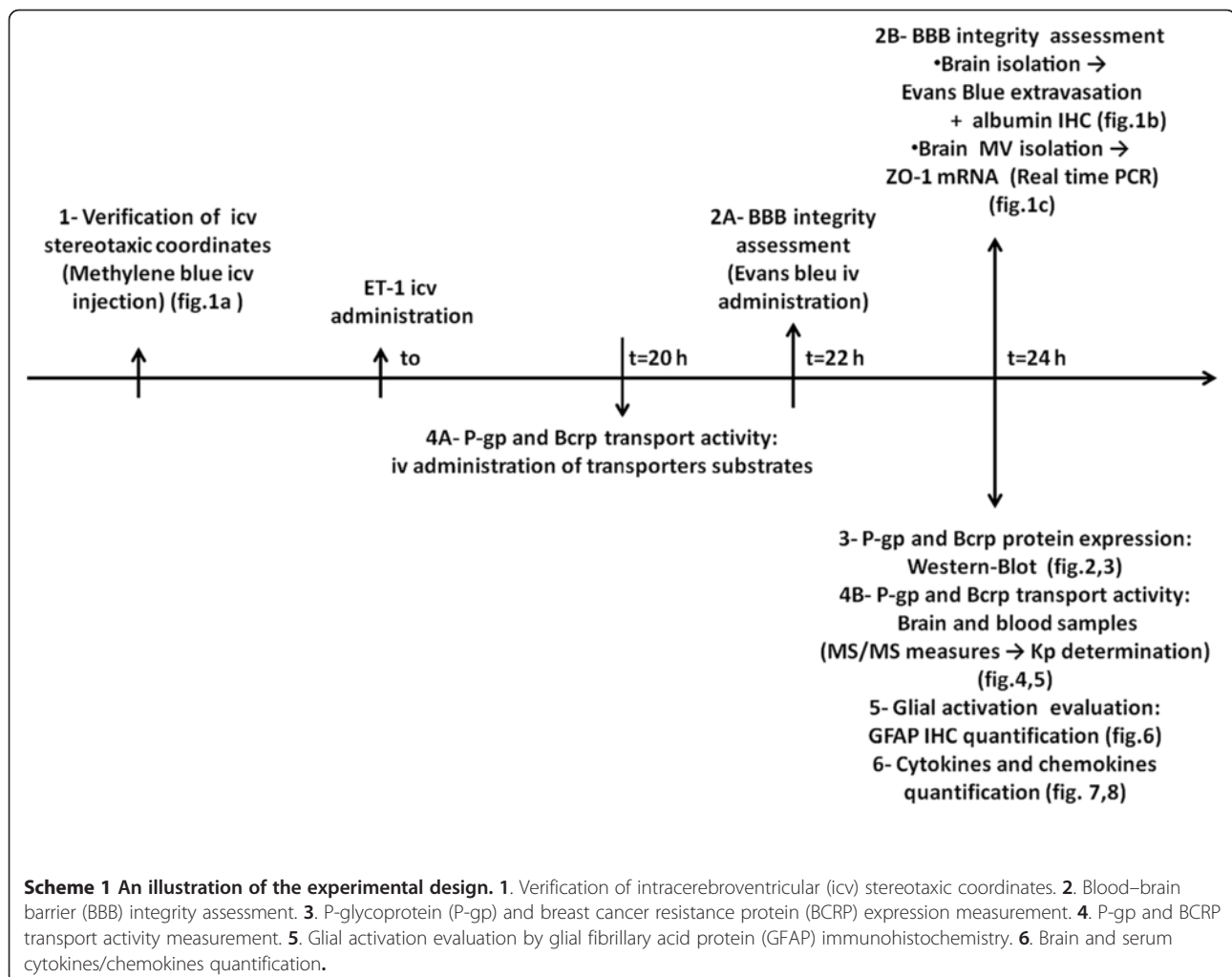
$$K_{p,uu,brain} = C_{ss,tot,brain} \times f_{u,brain} / C_{ss,tot,plasma} \times f_{u,plasma}$$

5) Immunohistochemistry of glial fibrillary acidic protein (GFAP), a glial activation marker [40], in cortex and

hippocampus of rat brains isolated 24 h after ET-1 treatment. 6) Brain and serum cytokines/chemokines quantification 24 h after ET-1 icv injection. The design of our research methodology is illustrated in Scheme 1.

Reagents

Bovine serum albumin (BSA), N-alpha-tosyl-L-lysiny-chloromethylketone (TLCK) and (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from Sigma (Saint Louis, MO, USA). Hank's buffered salt solution (HBSS), phosphate buffered saline (PBS) and penicillin-streptomycin-neomycin (PSN) were from Invitrogen (Carlsbad, CA, USA). Collagenase/dispase and DNase I was from Roche (Indianapolis, IN, USA). The NP-40 reagent (IGEPAL; CA-630) was from Sigma-Aldrich (St-Quentin-Fallavier, France). Primary antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) were: anti-MDR1 (sc-55510), anti-BCRP (sc-130933). Anti-actin (Mab 1501) was from Millipore (Molsheim, France). The secondary antibodies: goat anti-rabbit, goat anti-mouse and mouse anti-goat IgGs conjugated to



horseradish peroxidase (HRP) were from Santa Cruz Biotechnologies.

Digoxin (CAS 20830-75-5) and prazosin (CAS 19237-84-4) were from Sigma-Aldrich (St-Quentin-Fallavier, France). Internal standards vinblastine (CAS 143-67-9) and chlorpropamide (CAS 94-20-2) were from Sigma-Aldrich (Saint-Quentin-Fallavier, France). ET-1 was from Bachem (Heidelberg, Germany).

Animals

Rats were from Janvier Laboratories (Le Genest St Isle, France). Two groups of male Wistar rats [postnatal days 21 and 84] corresponding to the juvenile and adult stages of BBB maturation, respectively were used. Rats were maintained in a temperature and humidity controlled (19 C-23°C) room under a 12:12 h light–dark cycle, and were fed a standard diet (rodent rat chow) *ad libitum* with free access to tap water. Animal use in this research was approved in accordance with the Declaration of Helsinki, the European community's council directives (86/609/EEC, November 24, 1986) and the French directives concerning the use of laboratory animals.

Stereotaxic injections

Rat of different ages were weighed using a digital scale. All animals were then anesthetized with ketamine (87 mg/kg)/xylazine (13 mg/kg) and placed in a rodent stereotaxic apparatus. Skin and cranial fascia were resected and the skull exposed. A injection cannulae (from Cortat SA, Courrendlin, Switzerland) (length 25 mm, outer diameter 0.28 mm, internal diameter 0.18 mm) was inserted into the right lateral cerebral ventricle (4 mm ventral to the dura) via a hole that was drilled in the cranium at 1.08 mm posterior and 2 or 1.5 mm lateral to the bregma for P21 or P84 rats, respectively. The tip of a Hamilton syringe needle was briefly lowered to a depth of 3.8 or 4.2 mm for P21 or P84 rats, respectively, and immediately raised by 0.2 mm to create a trough. Each rat, received a double injection of ET-1 at a dose of 25 pmol/kg, or a vehicle control ((saline solution (0.9% NaCl)). The needle was left in for 5 minutes following the end of the injection, after which it was raised slowly, craniotomies were filled with gel-foam, and the scalp was sutured with wound clips. The wound was closed with self-dissolving sutures and swabbed with iodine. For pain control, lidocaine solution was applied. Animals were placed in a heated recovery cage, where they remained until the end of the study. Twenty-four hours after intracerebroventricular (icv) injection, animals were used for the assessment of unbound brain/plasma concentration of efflux drug substrates (see below) and/or euthanized by anesthetic injection and decapitated. Serum and brains were collected and frozen for further investigations.

Assessment of blood–brain barrier integrity

The integrity of the BBB was assessed by assessing the brain uptake of Evans blue dye and by following the expression of tight junctions on the brain microvessels. For that, ET-1 was administered icv. Twenty-two hours later, a solution of 3% Evans Blue dissolved in 0.9% saline was administered intravenously (iv, 4 ml/kg). Evans blue binds to serum albumin, giving rise to a high molecular complex which remains in intravascular spaces and diffuses to the extravascular space with BBB disruption [41]. Two hours after Evans blue iv administration, brains were isolated and the BBB integrity was assessed by verifying the absence of blue trace and by albumin IHC detection in the cortex and the hippocampus. BBB integrity was further assessed by measuring (using real-time PCR) mRNA relative expression of a tight-junction component, the *ZO-1*, in brain microvessels isolated 24 h after ET-1 treatment.

Isolation of brain microvessels

Rat brain microvessels were isolated as described previously [42]. The purity of collected brain microvessels was checked after RNA isolation and RT-PCR experiments by measuring the expression of cell-specific marker genes using specific primer for brain endothelial cells (CD31 or PECAM), for astrocytes (glial fibrillary acid protein or GFAP) and for pericytes (α -actin) as previously described [42].

To further evaluate changes in BBB integrity, the mRNA expression profile of the tight junction zonula occludens-1 (*ZO-1*) was assessed as described previously [5,42]. Quantitative expression of tight junction components was determined using 0.4 μ M of cDNA for each primer set in the RT² Pathway-Focused ProfilerTM Array from SABiosciences (T): catalog CAPR09279) (Frederick, Maryland, USA) according to the manufacturer's recommended protocol. The specific amplification conditions were 2 minutes at 50°C, 10 minutes at 95°C followed by 40 amplification cycles at 95°C for 0.5 minute and 60°C for 1 minute to reinitialize the cycle again. The specificity of each reaction was also assessed by melting curve analysis to ensure the presence of only one product. Relative gene expression values were calculated as $2^{-\Delta CT}$, where ΔCT is the difference between the amplification curve (CT) values for genes of interest and the housekeeping gene (hypoxanthine-guaninephosphoribosyltransferase, *HPRT*; glyceraldehyde phosphate dehydrogenase, *GAPDH*). If the CT was higher than 35, we considered the expression level too low to be applicable.

Western blot analysis

To analyze expression at the protein level, western blot analysis was performed on rat cerebral microvessels as reported elsewhere [31,37]. Optical density was quantified

using VersaDoc analysis software (BioRad Laboratories, Hercules, CA).

GFAP immunohistochemistry

Rats were anesthetized with isoflurane and subjected to intra-cardiac perfusion with 0.1% phosphate buffered saline (PBS). The brains were quickly removed and fixed in 4% paraformaldehyde (PFA) for 4 days, incubated in 30% sucrose solution, frozen in isopentane at -50°C , and stored at -80°C . Using a cryostat microtome, 40 μm sagittal brain slices were obtained and stored at -20°C in cryoprotectant solution until processing as free-floating sections. Brain slices were incubated in 30% H_2O_2 for 20 minutes before addition of normal goat serum blocking solution. Sections were incubated overnight (12 h) at 4°C with the primary antibody anti-GFAP (rabbit antibody, Abcam ab7260, 1/5000) (Paris, France) then for 30 minutes with the secondary biotinylated antibody (anti-rabbit IgG, ABC kit Abcam ab8627, 1/200). Sections were incubated with an avidine biotinyne solution (ABC kit Abcam ab 8627) for 30 minutes, and staining was revealed after 8-minute incubation with 3,3'-diaminobenzidine tetrahydrochloride (Sigma), 30% H_2O_2 . Sections were washed in PBS to stop the reaction.

GFAP image analysis was performed using Visilog 6.8 imaging software (Noesis, France), by measuring the percentage of stained surface over a manually defined area (optical density), excluding the interface of adjacent tissues (10 \times and 40 \times objectives). GFAP was assessed in the cortex and hippocampus.

Brain and serum cytokines/chemokines quantification

Protein extraction from brain tissue

Brain samples were weighed and homogenized with a Precellys 24 tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France) in 2 ml tubes containing 1.4 mm of ceramic beads (Cat 03961-1-003, Bertin Technologies). Then 500 μL of lysis buffer (20 mM TrisHCl pH 7.4, 0.15 M NaCl, 2 mM EDTA, 1 mM EGTA with protease inhibitor cocktail (Santa Cruz Biotechnologies, sc-29131)) was added to each tube. Samples were centrifuged (100 g) for 10 minutes at 4°C , and then the supernatant was removed and centrifuged a second time (20,000 g for 40 minutes at 4°C) to remove any remaining debris. Protein levels for all samples were quantified by the Bradford method and stored at -80°C .

Measurement of cytokines and chemokines in serum and brain supernatants All cytokines, IL-1 α , IL-1 β , IL-6, IL-10, MCP-1/ccl2, fractalkine, and TIMP-1 were simultaneously measured in brain and serum samples using the Agilent technology cytokine microarray (Tebu-bio, Le Perray-en-Yvelines, France). Data were calculated by generating a calibration curve obtained using reconstituted

cytokine standard in matrix of serum and buffer for brain samples. After incubation of samples with antibody cocktail and specific wash steps (as per manufacturer), 80 μL of Cy3 equivalent dye-conjugated streptavidin was added to each sample before incubation overnight at 4°C . The signals were visualized with a high-resolution microarray scanner (Agilent Technologies, Cat G2505B). Data extraction was done with the microarray analysis software GenePix 6.0. Quantitative data analysis was done with Quantibody[®] Q-Analyzer software (RayBiotech, Norcross, GA). All brain values were corrected for individual serum measurements as follows and described previously [6].

$$\begin{aligned} \text{Brain cytokine}(\text{pg}/\text{mg}) &= \text{Brain cytokine}(\text{pg}/\text{mg}) \\ &- [\text{Serum cytokine}(\text{pg}/\text{mg}) \\ & \times 0.02\%(\text{correction factor})] \end{aligned}$$

Assessment of unbound brain/plasma concentration ratio ($K_{p,uu,brain}$) of known efflux transporter substrates

A time-course distribution study of drug in plasma and brain of rats treated or not by pharmacological agents was conducted as previously described [42]. Substrates of specific efflux transporters (digoxin (P-gp substrate) and prazosin (BCRP substrate)) were infused via the femoral vein for 4 h, a time sufficient to achieve steady state, at a concentration of 1 mg/kg/h. Six rats per treatment were used. At the end of the infusion period, blood was sampled from the abdominal aorta in Eppendorf tubes (Eppendorf, Le Pecq, France) containing a sodium heparinate evaporated solution at 1000 U/ml, and then centrifuged for 5 minutes at 3000 g at 4°C to collect plasma. After blood collection, brains were collected and weighed. Plasma samples and brains were stored at -20°C , for subsequent bioanalysis by mass spectrometry.

Transporter substrate quantification in biological samples

Brains were mixed with ultrapure water (2 ml/g of brain) using an Ultraturax T65 system (IKA-Werke, Staufen, Germany) Extract suspensions (200 μL) were submitted to protein precipitation with 1 ml of methanol previously spiked with internal standard (1 $\mu\text{g}/\text{ml}$). After evaporation of the methanolic extracts to dryness, the dried extracts were resuspended in 500 μL of 1% NH_4OH . Plasma (200 μL) was diluted with 200 μL of 1% NH_4OH . Both brain extracts and diluted plasmas were submitted to solid-liquid extraction (SPE) on Oasis cartridges (reference 186000366 (Waters, Saint Quentin, France) as previously described [36]. The eluates (2 \times 250 μL) of 2% formic acid in methanol and 2 \times 250 μL of acetonitrile/methanol (1/1, v/v) were evaporated to dryness, reconstituted in 100 μL of 10 mM ammonium acetate/acetonitrile/formic acid, 95/5/0.1 v/v and 20 μL was injected into the chromatographic system.

Chromatography was performed using a Waters Acquity UPLC system on a BEH Shield RP18 column (2.1 mm × 100 mm, 1.7 μm) coupled with a BEH Shield RP18 1.7 μm Van Guard™ Pre-Column (Waters, Saint-Quentin-en-Yvelines, France). Mobile phase solvent A comprised 0.1% formic acid in 10 mM ammonium acetate, and mobile phase solvent B comprised 0.1% formic acid in acetonitrile. The run time was 5 minutes, and analytes were eluted with the following gradient: from 0 to 1 minute 5% solvent B, from 1 to 2.5 minutes the proportion of solvent B increased linearly from 5 to 80%, from 2.5 to 3 minutes a steady state of 80% solvent B was maintained, and from 3.1 to 5.0 minutes there was re-equilibration of initial conditions. The flow rate was set at 0.6 ml/min, the column temperature was maintained at 60°C and the autosampler at 4°C. The detection was performed with triple quadrupole mass spectrometer Quattro Premier XE monitored by MassLynx software, version 4.1 (Waters, Saint-Quentin-en-Yvelines, France) equipped with an electrospray ionization source operating alternatively in positive and negative mode. Tuning parameters were: capillary voltage 3 kV, source temperature 120°C, and desolvation temperature 350°C. The multiple reaction monitoring transitions for analytes were as follows: m/z digoxin 325.2 >779.4 and m/z prazosine 384.1 >247.2.

Analytes were quantified by means of calibration curves using vinblastine or chlorpropamide as the internal standard.

For plasma assay, calibration ranges were from 1.0 to 200 ng/ml for digoxin and from 0.4 to 170 ng/ml for prazosine. For brain extract assay, calibration ranges were from 1.0 to 100 ng/ml for digoxin and from 0.5 to 100 ng/ml for prazosine.

Measurement of the unbound partitioning coefficient of the drugs

The ratio between brain concentrations and plasma concentrations to estimate the delivery of drug to the brain was calculated [39,43]. This ratio can be described by the partition coefficient, K_p :

$$K_{p,brain} = C_{SS_{tot,brain}} / C_{SS_{tot,plasma}}$$

where $C_{SS_{tot}}$ is the steady-state drug concentration for total (bound and unbound) in brain and plasma.

Taking into account protein binding in plasma and brain tissue components of efflux/influx transporter substrates (digoxin and prazosin) measured using equilibrium dialysis in triplicate for each compound as described elsewhere (Table 1) [39], the unbound partitioning coefficient $K_{p,uu}$ which determined the net influx and efflux of drug across the BBB, was calculated as follows:

Table 1 Digoxin and prazosin unbound protein fraction in plasma ($f_{u,plasma}$) and brain tissue ($f_{u,brain}$)

| Compounds | $f_{u,plasma}$ | $f_{u,brain}$ | $f_{u,brain}/f_{u,plasma}$ ratio |
|-----------|----------------|---------------|----------------------------------|
| Prazosin | 0.204 ± 0.001 | 0.071 ± 0.004 | 0.349 |
| Digoxin | 0.379 ± 0.001 | 0.218 ± 0.027 | 0.576 |

$$K_{p,uu,brain} = C_{SS_{tot,brain}} \times f_{u,brain} / C_{SS_{tot,plasma}} \times f_{u,plasma}$$

where $f_{u,plasma}$ and $f_{u,brain}$ is the fraction of unbound drug (Table 1) in plasma and brain tissue, respectively.

Statistical analysis

Statistical analysis was performed using the Prism 3.0 program (GraphPad Software, Inc, San Diego, CA) and the R statistical software (R Development Core Team, 2009). Comparisons between groups were performed using one-way analysis of variance (ANOVA) with the Student-Bonferroni-post test and the unpaired Student's *t* test. Data are presented as mean ± S.E.M. Statistical significance was set at $P < 0.05$.

Results

Blood-brain barrier integrity assessment after intracerebroventricular injection of endothelin-1 in rats

An intact BBB simplifies the assessment of BBB-efflux transporter activity. We used Evans blue to determine whether ET-1 in our experimental conditions altered BBB integrity in juvenile or adult rats. Results depicted in Figure 1 demonstrate that in control animals as well as in ET-1-treated animals no albumin labeling or blue trace was detected in the cortex or in the hippocampus. These results showed no change in BBB permeability. Moreover, there was also no change in the quantitative real-time PCR analysis of *ZO-1* mRNA from freshly isolated rat brain microvessels after ET-1 treatment. Together, these results suggest that ET-1 under the conditions of these experiments does not change BBB permeability.

Comparison of P-glycoprotein and breast cancer resistance protein expression in juvenile and adult brain microvessels during brain inflammation mediated by endothelin-1

Previous *in vitro* results suggest that short-term exposure of adult rat brain endothelial cells to ET-1 decreases P-glycoprotein [31], whereas longer-term exposure (24 h) of adult human brain endothelial cells causes no changes in P-glycoprotein expression [7]. We found here that ET-1 administration into brain did not alter P-gp (Figure 2) or BCRP (Figure 3) expression in adult or in juvenile brain microvessels isolated twenty-four hours after ET-1 administration, as revealed by western blot.

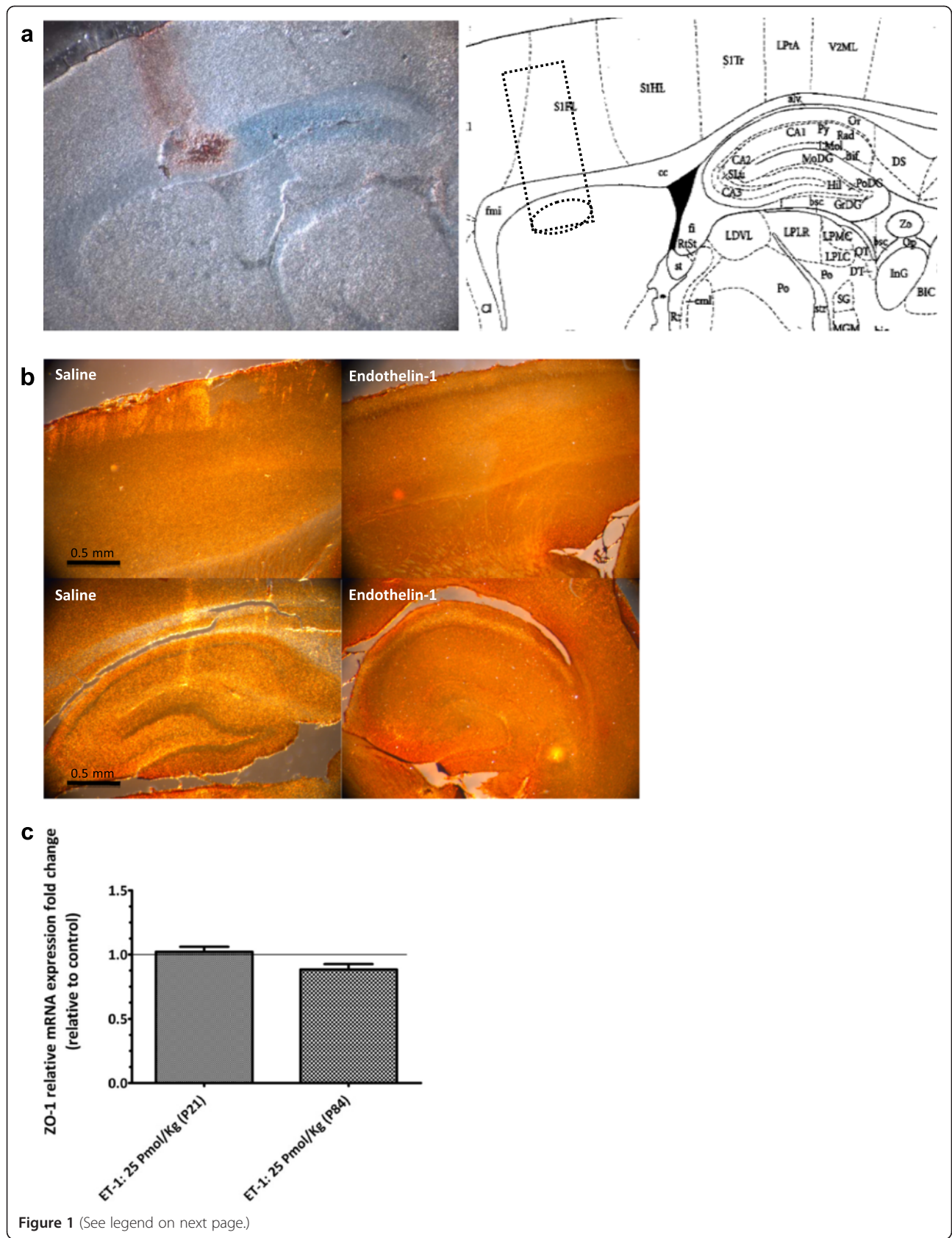


Figure 1 (See legend on next page.)

(See figure on previous page.)

Figure 1 Representative immunohistochemical staining of albumin in the adult and juvenile brains (scale bars, 5 μ m). **A**) Stereotaxic implantation of the cannulae was checked using intracerebroventricular (ICV) methylene blue-injected rats which permitted visualization of the stereotaxic implantation of the injection cannulae and the injection site into the brain lateral ventricles. Cannula implantation reached the lateral ventricle as shown by the blue color and cannula score (blood trace) (as referred to in Paxinos and Watson's stereotaxic atlas). Moreover, methylene blue diffused to the hippocampus. **B**) Blood-brain barrier (BBB) permeability after endothelin-1 (ET-1) injection was assessed using albumin immunohistochemical (IHC) in IV Evans blue injected rats. ET-1 was administered intracerebroventricularly. Twenty-two hours later a solution of 3% Evans Blue dissolved in 0.9% saline was administered intravenously (4 ml/kg). No albumin labeling or blue trace was detected in the cortex or in the hippocampus of adult or juvenile rat brains, suggesting a lack of BBB breakdown after icv injection of ET-1. **C**) BBB permeability after ET-1 injection was also assessed by measuring (using real-time PCR) mRNA relative expression of a tight-junction component, the *ZO-1* gene, in brain microvessels isolated 24 h after ET-1 treatment. Results represent mean \pm S.E.M from three batches of brain endothelial microvessels. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$.

Comparison of transporter activity in juvenile and adult blood-brain barrier during brain inflammation mediated by endothelin-1

To determine whether there were differential responses to ET-1 in transporter activity of P-gp and BCRP in the juvenile and adult BBBs, the unbound brain and unbound plasma concentration ratios ($K_{p,uu,brain}$) for known substrates of P-gp (digoxin) and BCRP (prazosin) were determined 24 h after ET-1 icv injection. In the untreated state, there were no differences between juveniles and adults for P-gp (Figure 4) or BCRP (Figure 5) activity. However, ET-1 used to trigger neuroinflammatory reactions characterized by cytokines/chemokines secretion, caused different regulation of P-gp and BCRP transport activity (Figures 4 and 5) between juvenile and adult rats. Notably, ET-1 induced an enhanced transporter activity of P-gp as assessed by digoxin efflux from brain [$K_{p,uu,brain} = 0.04 \pm 0.006$, for treated adult rat *versus* $K_{p,uu,brain} 0.07 \pm 0.01$, $P < 0.05$], whereas no significant change occurred in P-gp activity at the juvenile BBB (Figure 4). The decrease of brain uptake in the ET-1-treated adult animals strikingly coincided with the increases in the unbound plasma concentration of digoxin compared with the control (67.84 ± 22.74 ng/ml *versus* 47.75 ± 10.30 ng/ml), whereas no significant change in the plasma concentration of digoxin compared with the control was observed in juvenile rats (36.76 ± 6.50 ng/ml *versus* 47.75 ± 6.79 ng/ml). ET-1 also enhanced efflux of prazosin, the substrate for BCRP, at the adult BBB [$K_{p,uu,brain} = 0.09 \pm 0.01$, for treated adult rat *versus* $K_{p,uu,brain} 0.23 \pm 0.02$, $P < 0.0001$], with a corresponding arithmetic increase in unbound plasma concentration (10.06 ± 2.03 *versus* 7.40 ± 0.90 ng/ml) (Figure 5). Conversely, at the juvenile BBB, 24 h after ET-1 treatment, efflux of prazosin tend to decrease [$K_{p,uu,brain} = 0.30 \pm 0.07$, for treated adult rat *versus* $K_{p,uu,brain} 0.19 \pm 0.05$, for animal controls ($P = 0.11$)]. This tendency to decrease in BCRP activity at the juvenile BBB was strikingly associated with decreased unbound plasma prazosin concentration (4.60 ± 1.52 ng/ml *versus* 8.56 ± 1.75 ng/ml, $P < 0.05$) (Figure 5).

Comparative glial activation between juvenile and adult rat brain cortex and hippocampus under inflammation mediated by endothelin-1

We determined whether the activation of glial cells by ET-1 in juvenile and adult rat brains could be responsible for the differential effects of ET-1 on efflux transporter activity. Immunohistochemistry depicted in

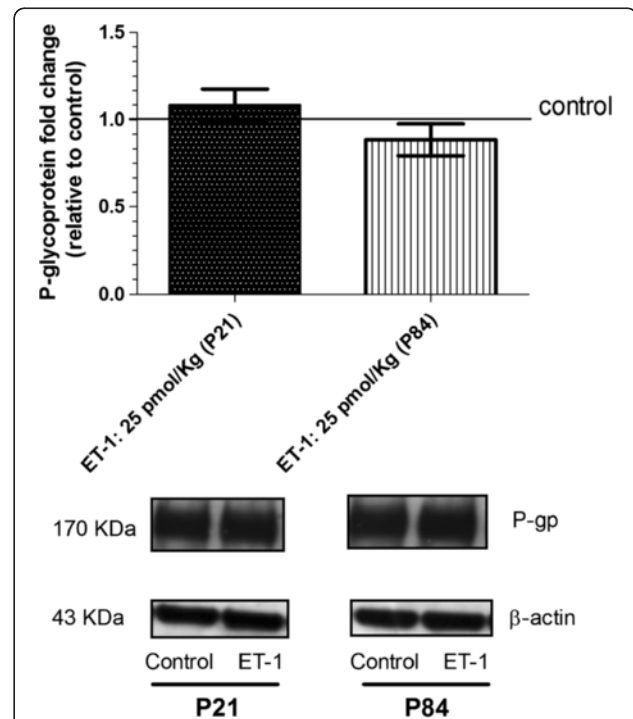


Figure 2 Expression of P-glycoprotein in freshly isolated rat brain microvessels after endothelin-1 treatment of juvenile and adult rats. Rats equivalent to pediatric (P21) and adult (P84) human brain were used. Twenty-four hours after intracerebroventricular (icv) injection of endothelin-1 (ET-1) was used to trigger inflammatory reactions, brain microvessels were isolated, and P-glycoprotein expression was assessed by western blot as reported in Methods. Results show no difference in P-glycoprotein expression after ET-1 treatment. Immunoblot represents mean \pm S.E.M from three batches of brain endothelial microvessels. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.0001$.

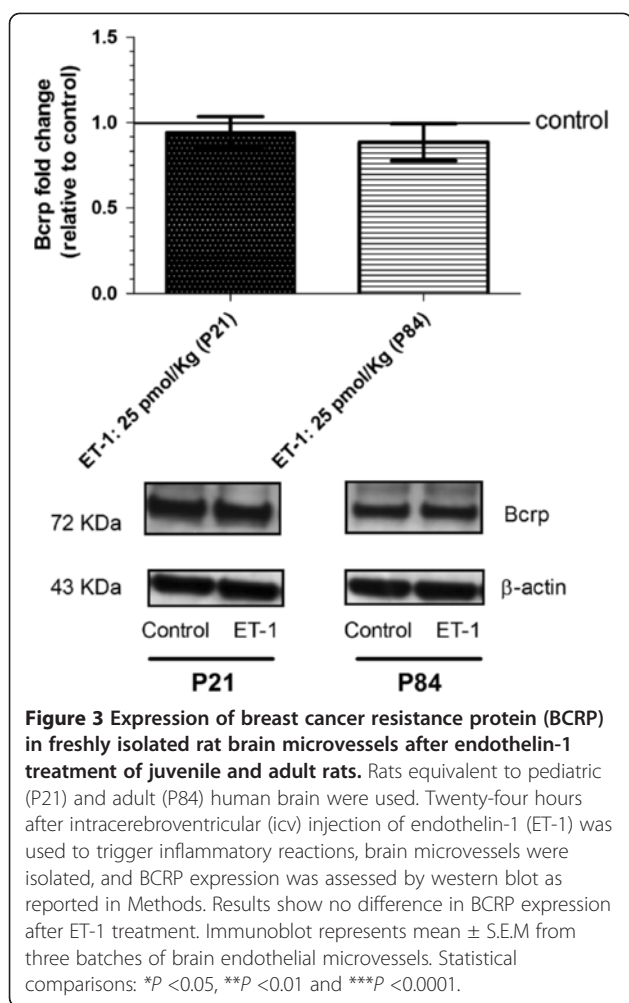


Figure 6 shows that ET-1 induced activation of glial cells as measured by GFAP. In cortex, ET-1 increased GFAP in glial cells in adults (1.94-fold), as well as in juvenile brain (1.78-fold, $P < 0.0001$). However, variance analysis (one-way ANOVA) with Bonferroni's multiple comparison post test showed difference in the basal glial activation between juvenile and adult brain cortex ($P = 0.0467$). In the hippocampus, the basal glial activation is statistically different between juvenile and adult ($P = 0.0013$), but ET-1 enhanced GFAP expression in glial cells of adult brain 2-fold and in juvenile brain 1.62-fold (Figure 6).

Cytokine and chemokine responses mediated by endothelin-1 in juvenile and adult brains

Cytokines and chemokines were measured in brain and serum 24 h after ET-1 injection (25 pmol/kg). Protein expression of cytokines and chemokines in the brain was calculated taking into account that 0.02% of the analyte serum value for serum is predicted to contribute to the value for the brain [6]. Expression patterns of all cytokines and chemokines are depicted in Figure 7 for

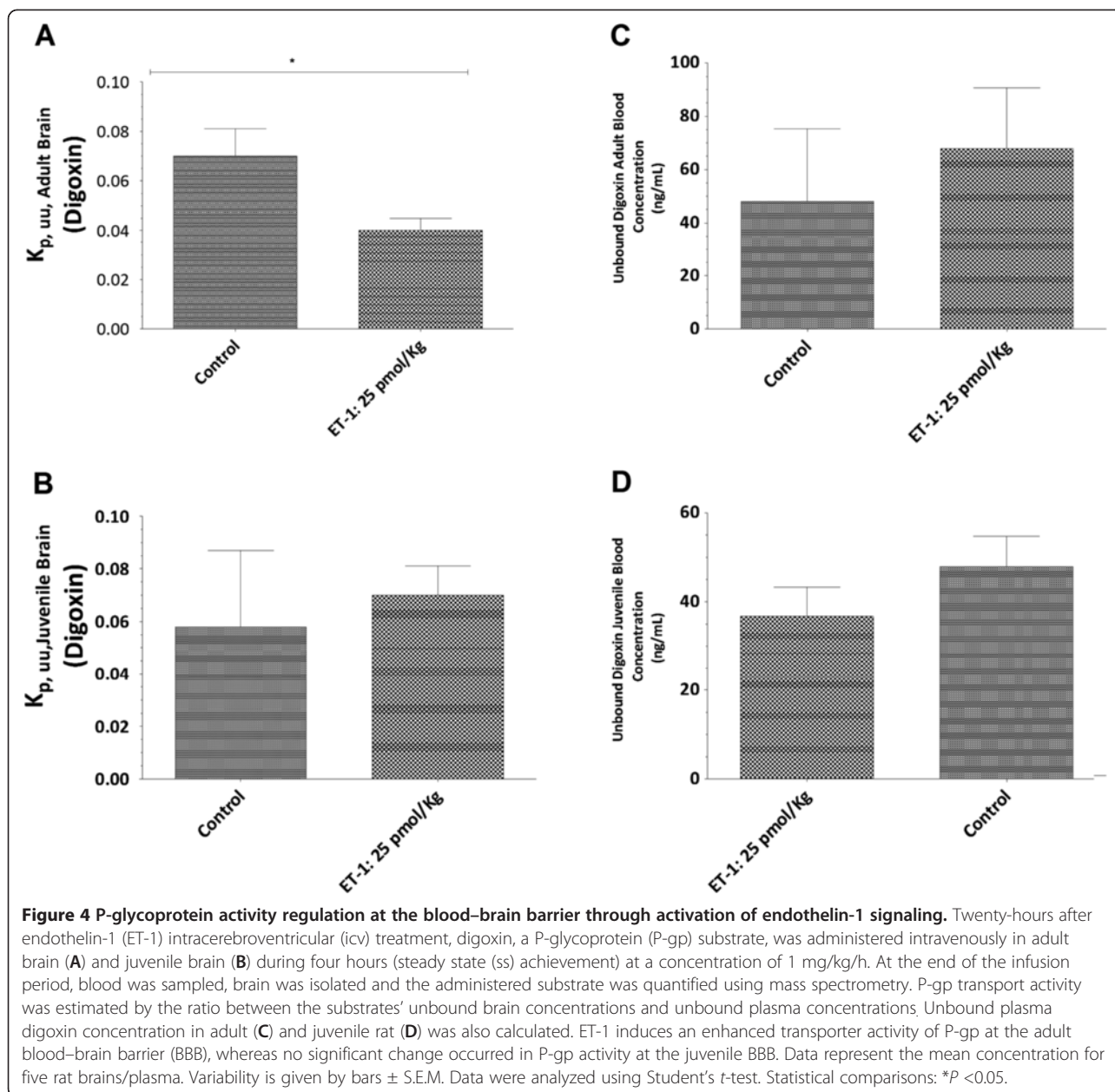
juvenile and adult rat brain, respectively. Among the panel of cytokines and chemokines tested, only IL-10, IL-6, CCL2/MCP-1, IL-1 α , IL-1 β , fractalkine, and TIMP-1 were detected. Cytokines that remained significantly elevated in adult brain were IL-10, IL-1 β , IL6, Ccl2/MCP1 and TIMP-1. Adult and juvenile rat brains showed different patterns for fractalkine, TIMP-1, IL-10, IL-1 β , and Ccl2/MCP-1. The expression of fractalkine, IL-10 and TIMP-1 was significantly decreased in juvenile brain, but increased in adult brain. In addition, we noted a striking difference in the magnitude of IL-1 β , at basal level and after ET-1 administration [3.23-fold increase ($P < 0.001$) versus 1.22 ($P < 0.01$) fold increase], and of IL-6 [3.46-fold increase ($P < 0.05$) versus 1.34-fold increase] secretion between adult and juvenile brain. IL-1 α showed the same pattern in juvenile and adult brain.

Discussion

CNS pharmacotherapy is impeded by the existence 'CNS barriers' at the interface between blood and neural tissue. As a result, many drugs fail as therapeutic agents for the CNS because they are pumped out of the brain. To overcome these drug efflux transporters, recent research in the field aims at identifying the factors and the intracellular signaling mechanisms implicated in their regulation in order to modulate their activity and improve pharmacotherapy of brain diseases. For years, BBB efflux transporters have been studied in the adult organism. But, there is a wide-spread belief among pediatricians, neurologists, and neuroscientists that the BBB in the embryo, fetus, newborn, and infant is 'immature', implying caution in giving drugs to infants [44]. Moreover, intricate developmental processes are taking place during the prenatal and postnatal periods, which might mean that BBB efflux transporters could also undergo important changes during brain maturation, and might possibly have age-related differences in the inflammatory response. There is growing evidence suggesting that the immune system, through systemic or cerebral inflammation, disturbs the BBB efflux transporters [45], and these alterations can affect the efficacy of CNS-acting drugs.

However, current knowledge on the functional status of the BBB in immature organism remains very limited. Thus, from the clinical perspective of developing new drugs with enhanced efficacy in both the adult and children CNS, it is important to understand 1) the role of the BBB drug efflux transporters in the CNS at the different stages of brain maturation and 2) the mechanisms that regulate their functional activity, under both normal and inflammatory conditions.

In this study, we evaluated the impact of acute cerebral inflammation mediated by ET-1 on BBB efflux transporters with a comparison between juvenile and adult rats. Specifically, we evaluated, *ex vivo* and *in vivo*,



the impact of intracerebroventricular (icv) injection of ET-1, on the expression and activity of two of the most clinically relevant BBB drug efflux transporters, the P-gp and the BCRP transporters.

Our results showed that, under inflammatory conditions, BBB drug efflux transporters are regulated differently in juvenile rats when compared to adult rats, and that this differential regulation may be due, in part, to a maturational difference in glial basal levels, and neuro-inflammatory response triggered by ET-1.

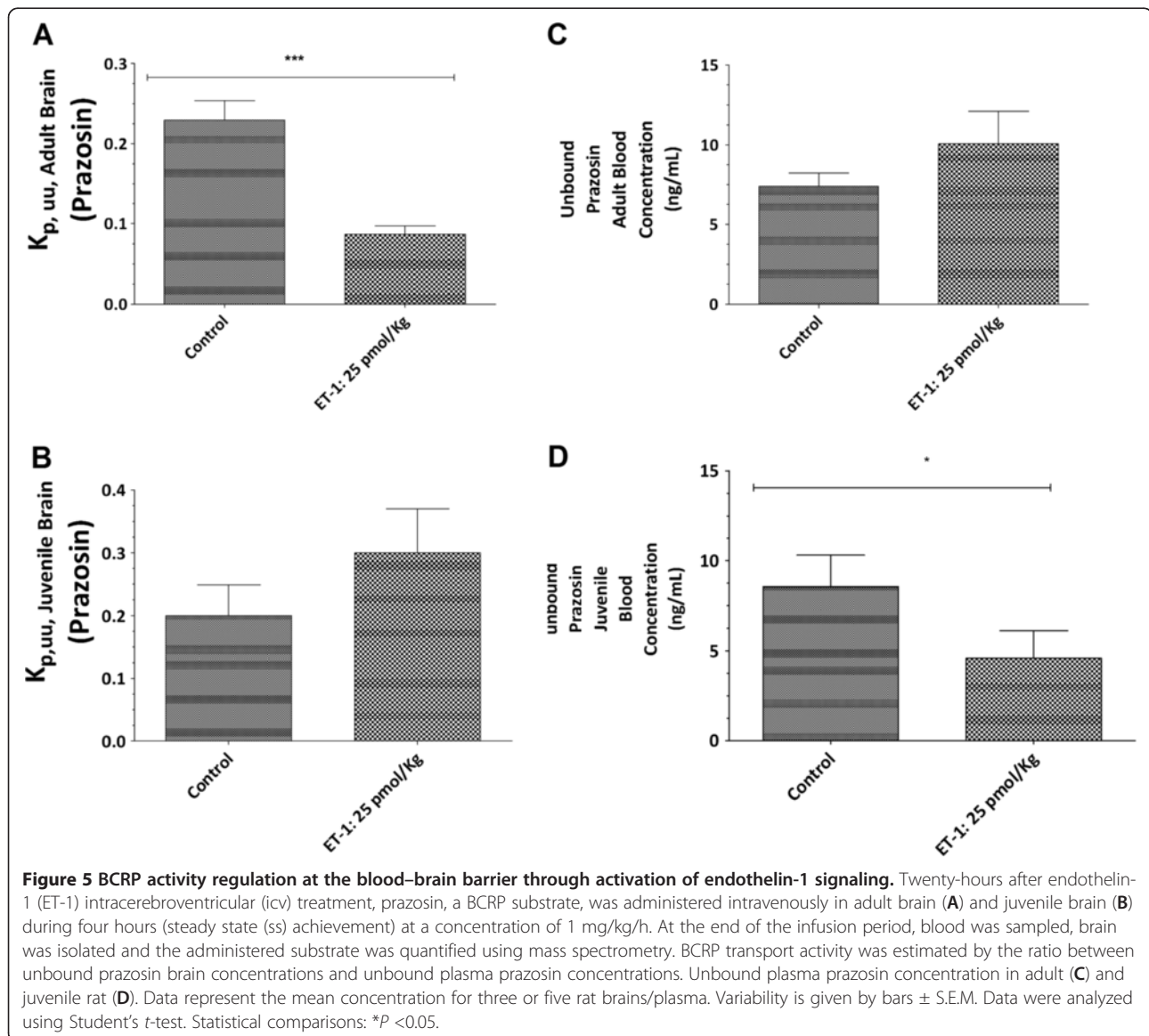
Since inflammation has been reported to influence BBB integrity [27,28], we first investigated in our experimental conditions whether icv injection of ET-1 changed

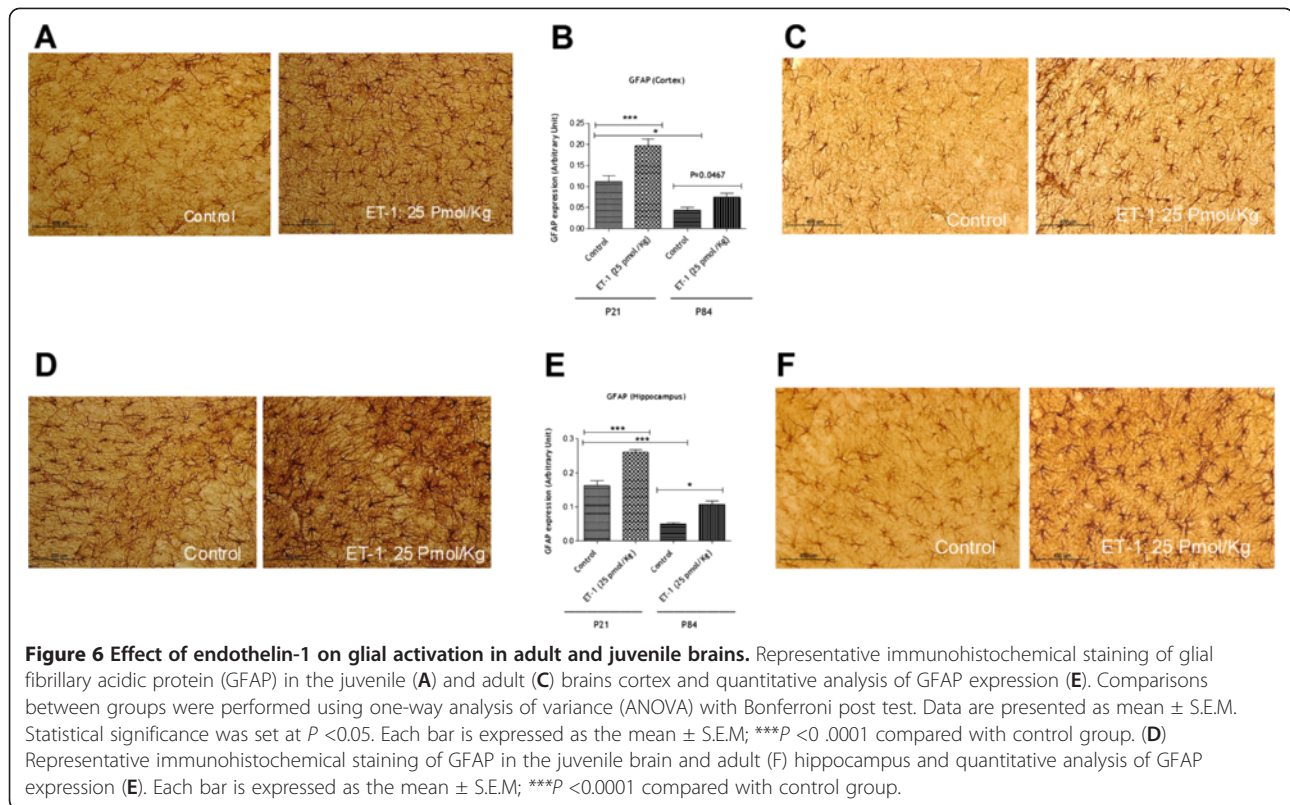
BBB permeability. BBB permeability was evaluated by assessing the brain uptake of Evans blue dye to ensure the functionality of the BBB to macromolecule entry after ET-1 treatment, and by following the expression of tight junctions on the brain microvessels to evaluate the integrity of tight junctions which restrict paracellular movement to small molecules across the BBB. Results depicted in Figure 1 demonstrate a lack of BBB permeability changes with the use of Evan blue as well as the use of baclofen, as small molecule which does not cross the BBB (data not shown). In addition, we found no modification in the *ZO-1* gene expression during the cerebral inflammation triggered by ET-1 treatment, and

this in both juvenile and adult brains. These findings allowed us to determine the impact of neuroinflammation on the expression and transport activity of BCRP and P-gp in adult and juvenile brains at the BBB. We first compared P-gp and BCRP transport activity between juvenile and adult BBB, and found that juvenile and adult BBB have the same P-gp and BCRP transport activity (Additional file 1). We also found evidence that the transport activities of P-gp and BCRP are enhanced by icv ET-1 at the adult BBB, whereas no significant modulation of P-gp transport activity and a tendency to decrease in BCRP transport activity were seen at the juvenile BBB (Figures 4 and 5). Thus, these results gave evidence that P-gp and BCRP transporters at the BBB level are regulated differently under pathological conditions in juvenile brain when compared to adult. These

findings emphasize the importance of considering differential P-gp and BCRP transporter regulation mechanisms between juvenile and adult BBB in the context of pathological conditions.

Second, after assessing BBB transport activity in adult and juvenile brains in the context of ET-1 treatment, we aimed at understanding the underlying mechanisms behind this differential regulation. We suspected a role of neuroinflammatory response triggered by ET-1 because it is likely that cytokine secretion in adult and juvenile brains controls the regulation of BBB transporters [9]. In the brain, the inflammatory response begins with recruitment of the innate immune system. Rapidly, in response to infection or injury, microglia, major inflammatory cells of the monocyte/microphage lineage that reside in the brain, are activated [46]. Microglia are important phagocytic cells,





and once activated they release numerous inflammatory molecules, particularly pro and anti-inflammatory cytokines and chemokines [47] (Proinflammatory molecules such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) [48-50], chemokines (IL-8, MIP-1 α , MIP-1 β , MCP-1) [51,52], proteases [53], and anti-inflammatory molecules such as TGF- β and IL-10 [54]). Later, astrocytes are activated [55]. Apart from being involved in a variety of physiologic processes, astrocytes rapidly react to different neurological insults. Upon activation, a series of changes occur in astrocytes, leading to the acquisition of macrophage differentiation markers and effector properties. One main feature of these changes is the increase in the number and size of glial fibrillary acidic protein (GFAP) expressing cells. GFAP is an intermediate filament cytoskeletal protein expressed primarily by astrocytes and it is considered as the marker of astrocytes [40]. Concomitant with GFAP overexpression, astrocytes release many proinflammatory mediators and upregulate the expression of several inflammatory molecules, contributing to the amplification of inflammation [34]. These facts allow us in this study, to test whether the inflammatory response triggered by ET-1 can be responsible for the differential regulation of P-gp and BCRP transporters between juveniles and adults. For that, we quantified 1) the glial activation marker, the GFAP in hippocampus and cortex of rat brains treated with ET-1, and 2) a panel of cytokines and

chemokines that could be induced by ET-1 in both juvenile and adult rat brains. We found a difference in the GFAP basal levels between the two populations either in hippocampus or in the brain cortex, but our results showed a pronounced glial activation in adult and juvenile cortex and hippocampus coincided with cytokine/chemokine levels (Figures 6 and 7). Our findings were that IL6, IL-1 β , CCL2/MCP-1, TIMP-1 and IL-10 increased significantly in adult brain compared with juvenile brain. These results emphasize the fact that brain development such as glial maturation is likely of paramount importance in the synthesis of specific cytokines such as IL6 for example. Indeed, IL6 is a well-known marker of glial activation [56] and the autocrine action of this interleukin on glial cells might account for an increase of ABC transporters at the cell surface. This increase is important in the secretion of ccl2/MCP-1 by astrocytes upon toll-like receptor 3 activation as reported recently [57]. Thus, a differential GFAP basal level and a differential secretion of cytokines might lead to a differential regulation of ABC transporters at the BBB level. Moreover, taking into account the differential effect of ET-1 on cytokine levels in juvenile and adult brains, we suggest that the increased levels of cytokines in the adult brain and more particularly IL-6 and IL-1 β may cause functional but not transcriptional regulation of P-gp and BCRP in adult BBB. Indeed, there was no modulation of protein synthesis in BCRP and P-gp in adult rat brain microvessels (Figure 5).

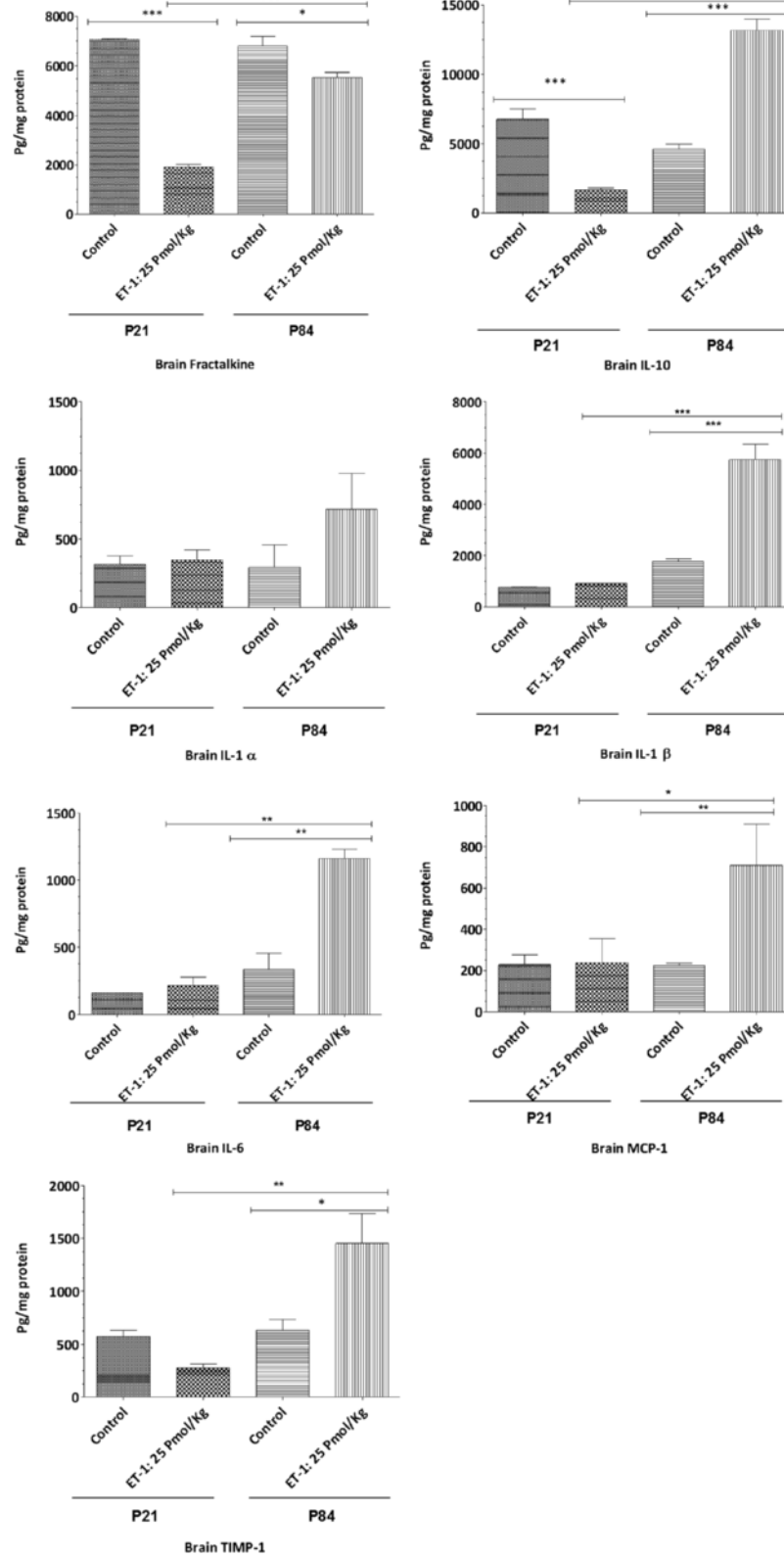


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Figure 7 Cytokines and chemokines levels for 24 hours in juveniles (P21) and adult (P84) rat brains after a single intracerebral injection of endothelin-1.

All cytokines, IL-1 α , IL-1 β , IL-6, IL-10, MCP-1/ccl2, fractalkine and TIMP-1 were simultaneously measured in brain and serum samples using the Agilent technology cytokine microarray (Tebu-bio). All brain values were corrected for individual serum measurements as described previously [6]. Comparisons between groups were performed using one-way analysis of variance (ANOVA) with the Student-Bonferroni post test. Data are presented as mean \pm S.E.M. Statistical significance was set at $P < 0.05$. Graph plotted as mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$ compared with animal controls. The number of animal tested: $n = 3$ or 5 rats for each group.

Modulation of transport activity for P-gp in response to peripheral pain inflammation with no increase in its protein expression has been previously reported [58]. We found no published results addressing whether cerebral inflammation as mediated by ET-1 regulates either P-gp or BCRP activity at the adult or juvenile BBB. Based on our results and the literature, we suggest that cytokine synthesis in the adult brain modulates BCRP and P-gp activity at the BBB by post-translational mechanisms such as phosphorylation and cellular localization of transporters. Inflammation regulates a number of intracellular signal transduction pathways [59-61] involved in the regulation of transporter activity. At the BBB, P-gp has been localized to plasma membrane surfaces as well as several subcellular sites, and there is overwhelming evidence suggesting that the localization of P-gp and its trafficking within brain endothelial cells contributes to its function [62,63]. To formally demonstrate that cytokine synthesis is a cause of the differential regulation of transporter activity between juvenile and adult BBB, we increased the amount of ET-1 administered after determining the lack of BBB breakdown. At the dose of 125 pmol/kg of ET-1, the juvenile brain exhibited increases in IL1 β , MCP-1, and IL6 (Additional file 2). Despite the level of those cytokines in the brain, the juvenile BBB showed a decrease in BCRP activity which strikingly coincided with the decrease of unbound plasma prazosin concentration (3.77 ± 1.58 ng/ml *versus* 8.56 ± 3.77 ng/ml, $P < 0.05$). In addition, the increase in the administered dose of ET-1 did not change the activity of P-gp in the juvenile BBB. We observed no significant decrease of digoxin concentration in unbound plasma of juvenile animals (23.50 ± 7.58 treated animals *versus* 47.88 ± 6.80 for control animals, $P < 0.05$). In adult brain, BBB at the dose of 125 pmol/kg of ET-1 (data not shown) showed the same profile regarding the increase of P-gp and BCRP activity compared with the dose of 25 pmol/kg. These findings suggest the involvement of other parameters in the differential regulation of P-gp and BCRP at the BBB, particularly in juvenile brain. Thus, further investigation is warranted to define more precisely the underlying mechanisms. It has been reported that the transcriptional activity of ABC transporters is under the control of orphan nuclear receptors such as steroid and xenobiotic receptors, and that their expression and function are regulated by environmental stimuli that induce stress. Recent studies show that increased transporter expression occurs in response to signals that activate specific transcription

factors including, PXR, CAR, NF- κ B and AP-1, and reduced transporter activity occurs rapidly and reversibly in response to signaling through Src kinase, protein kinase C and estrogen receptors [8]. Moreover, Bauer and colleagues have shown in rat brain capillaries that tumor necrosis factor alpha (TNF- α) binding to its receptor TNFR1 leads to the release of ET-1 which in turn acts through its receptor ETB to continue the signaling cascade via nitric oxide synthase (NOS) and protein kinase C (PKC) [31]. This long-term exposure leads to an increase of P-gp activity [31] which is in agreement with our results regarding the adult brain but not the juvenile brain even at low dose of ET-1 (25 pmol/kg) or at the dose of 125 pmol/kg. Thus, to determine the underlying mechanisms behind the differential regulation of the BBB P-gp and BCRP transporter further studies are required and these must be primarily focused on the role of these multiple signaling pathways modulating the expression and activity of ABC transporters at the BBB level in children compared to adults. In addition, difference in the ontogenesis of ET-1 receptor (ETA) evidenced (data not shown) in our laboratory at the level of BBB in juvenile and adult brain might be also taken into account to explain this differential functional regulation of P-gp and BCRP transporter.

Conclusions

In the present study, we found that BBB transporter activity during neuroinflammation is not affected in the same manner in juvenile brain as it is in the adult brain. This is the first report that illustrates the differential regulation of BBB transporter activity in pediatric and adult brains. In the pediatric brain long-term exposure to ET-1 leads to no modulation of BBB Pgp transport activity and BCRP transport activity tends to decrease while a significant increase of both transporters activity was evidenced in the adult brain. Further studies such as microglial activation, ontogenesis of endothelin receptors (ETB, ETA), NF κ B, IL, PKC isoforms, NOS, sphingolipid signaling pathways in the context of brain inflammation are warranted to understand accurately and precisely the differential regulation of BBB-ABC transporters in pediatric and adult brains. In this context, we suggest that potential selective reduction of transcriptional factors such as PKC and NOS isoform in the brain cortex could decrease BBB BCRP transport activity and lead to enhancement of CNS drug

pharmacotherapy in pediatric brain. Our study gives the evidence of age-related differences in the regulation of drug efflux transporters under inflammatory conditions, and emphasizes the importance of taking into account the specific properties of the juvenile BBB and distinguishing it from the adult one in the clinical perspective of developing new drugs with enhanced efficacy in children's CNS. Subsequently, it is highly important to determine the pathways modulating the activity of drug efflux transporters under pathological conditions, both in children and adult brains, because targeting these pathways may open new therapeutic avenues to improve drug delivery into the brain.

Additional files

Additional file 1: Age-related changes in P-gp, and bcrp function at the BBB.

Rats at pediatric (P21) and adult (P84) stages of brain development were used. The in vivo plasma and brain exposure of tested efflux transporter substrates [digoxin (P-gp substrate): 0.5 mg/kg h, and prasozin (bcrp substrate): 0.25 mg/kg h] was assessed using unanesthetized rats catheterized in the femoral vein. Drug infusion was performed for a period of 4 h (steady state), and then the unbound partitioning coefficient brain/plasma concentration ratio (K_{pu} , brain) for each tested efflux transporter substrate was determined as described in the Experimental Section. Data represent the mean concentration for 3 or 5 rat brains and 3 or 5 plasmas; variability is given by bars \pm SEM. Statistical comparisons: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$.

Additional file 2: Cytokines and chemokines levels in juvenile brain after a single intracerebral injection of endothelin-1 for 24 h at the dose of 125 pmol/kg.

All cytokines, IL-1 β , IL-6, IL-10, MCP-1/ccl2, fractalkine, TIMP-1, IL-13, conculin (CINC-1) were simultaneously measured in brain and serum samples using the Agilent technology cytokine microarray (Tebu bio). All brain values were corrected for individual serum measurements as follows and described previously [6]: Graph plotted as mean \pm S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with animal controls with $n = 3$ or 5 rats for each group.

Abbreviations

ABC: Adenosine triphosphate-binding cassette; BBB: Blood-brain barrier; BCRP: Breast cancer resistance protein; BSA: Bovine serum albumin; CNS: Central nervous system; CT: Amplification curve; ET-1: Endothelin-1; GAPDH: Glyceraldehyde phosphate dehydrogenase; GFAP: Glial fibrillary acidic protein; HBSS: Hank's buffered salt solution; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPRT: Hypoxanthine-guaninephosphoribosyltransferase; HRP: Horseradish peroxidase; IHC: Immunohistochemical; MCP: Monocyte chemoattractant protein; icv: Intracerebroventricular; iv: Intravenously; MDR: Multidrug resistance; P: Postnatal day; PBS: Phosphate buffered saline; PFA: Paraformaldehyde; P-gp: P-glycoprotein; PSN: Penicillin-streptomycin-neomycin; SS: Steady state; TIMP-1: Tissue inhibitor of metalloproteinases-1; TLCK: N-alpha-tosyl-L-lysinylnor-chloromethylketone; ZO-1: Zonula occludens-1.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RH carried out all experiments, stereotaxic injections, data acquisition, analysis, interpretation and participated in the manuscript's drafting. ASV was involved in analysis and data interpretation. WB and AM have been involved in the conception, rational, drafting manuscript critically and have given final approval of the version published. All authors have read and approved the final manuscript.

Acknowledgements

We would like to thank the Agence National de Recherche sur le SIDA for financial support. The authors thank Aurélie Ghetas (CEA, iBiTec-S, Service de Pharmacologie et d'Immunoanalyse, Gif-sur-Yvette, France) and Kelly Blazy (INERIS, 60550 Verneuil-en-Halatte, France) for their technical skills in mass spectrometry and immunohistochemical techniques, respectively.

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Received: 26 September 2012 Accepted: 7 November 2012

Published: 19 December 2012

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doi:10.1186/1742-2094-9-273

Cite this article as: Harati et al.: Susceptibility of juvenile and adult blood–brain barrier to endothelin-1: regulation of P-glycoprotein and breast cancer resistance protein expression and transport activity. *Journal of Neuroinflammation* 2012 **9**:273.

Chapter III

Research tools

Our study was performed in Wistar rats. Four groups of male Wistar rats [postnatal day (P) 2, P14, P21, and P84] corresponding to the stages of BBB maturation were used. P2 corresponds to embryonic life in humans, P14 to new born, P21 to childhood and P84 to adulthood in humans.

4.1 The *Ex Vivo* Blood-Brain Barrier Model

For the work presented in this thesis, isolated rat BMVs were used as an *ex vivo* BBB model to study BBB ontogenesis as well as transcriptional regulation of P-gp, Bcrp, oat3 and Oatp1a4 in the context of an acute cerebral inflammation triggered by ET-1 in juvenile rats compared to adult rats.

4.1.1 Isolation of Intact Rat Brain Microvessels

BMVs were isolated based on a method previously describe by Goldstein et al., Dallaire et al. and Miller et al. with modifications according to Lacombe et al., 2011 (Lacombe et al., 2011; Miller et al., 2000; Dallaire et al., 1991 and Goldstein et al., 1975). Brains were removed from euthanized rats and brain tissue was homogenized. Microvessels were then isolated from brain samples after enzymatic digestion with collagenase/ Dispase containing DNase1. Then BMVs were separated from myelin by density centrifugation (The experimental method of isolation is detailed in the article Harati et al., 2012a).

4.1.2 Purity of Isolated Rat Brain Microvessels

After isolation, the purity of collected BMVs was checked by measuring, using real-time PCR, the mRNA expression of cell-specific marker genes for BMVECs (CD31 (cluster of differentiation 31), also known as Pecam-1 (Platelet Endothelial Cell Adhesion Molecule)), for glial cells (Glial Fibrillary Acid Protein (GFAP) and for pericytes (α -actin) (The experimental methods of mRNA extraction, reverse transcription and real time PCR are detailed in the article

Harati et al., 2012a). Pecam-1 mRNA was more abundant than GFAP and α -actin in BMVs preparations, showing the acceptable purity of the freshly isolated rat BMVs (Figure 1, Harati et al., 2012a).

4.1.3 Detection of BBB components expressed in isolated Rat Brain Microvessels

Using real-time PCR, we studied the expression of a panel of structural and functional components of the BBB in the isolated BMVs.

Tight junctions mRNA

Among the structural TJ components, we studied the expression of two important element of TJ: claudin-5 and Zo1 (figure 18). Informations about these TJ components at the BBB level were provided in the introduction section.

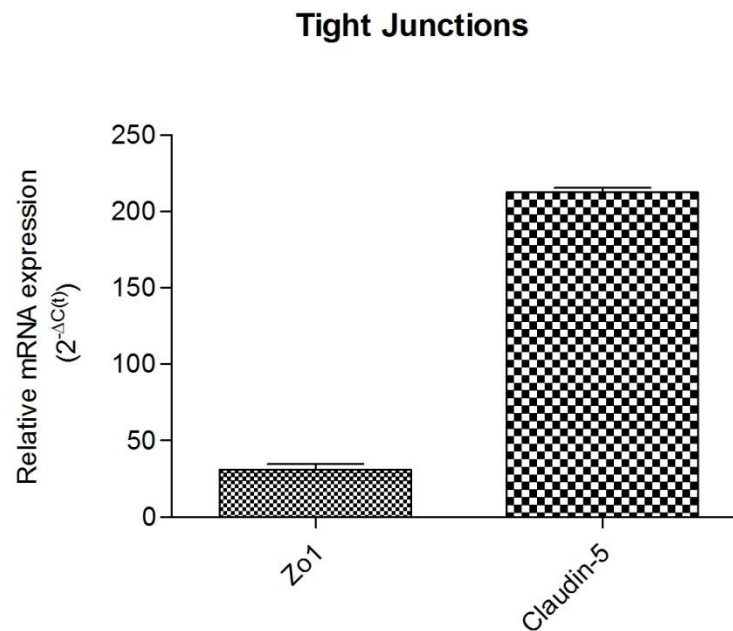


Figure 18. mRNA relative expression of BBB TJ components in isolated BMVs

Adult rats P84 were used. mRNA transcription profiles for claudin-5 and zo1 were assessed by semiquantitative real-time PCR performed in freshly isolated rat BMVs. Results are means \pm SEM for 3 batches of BMVs (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of BMVs.

Our results showed that these two essential TJ components are well expressed in our isolated BMVs which are highly enriched by claudin-5. Moreover, using immunostaining, we localized Zo1 in isolated BMVs along cell-cell contacts (Figure 1, Harati et al., 2012a).

ABC transporters mRNA

We studied the expression of six important ABC transporters at the BBB level: abcb1, abcg2, abcc1, abcc2, abcc4, abcc5 and abcc6 (figure 19). Informations about these ABC transporters at the BBB level were provided in the introduction section.

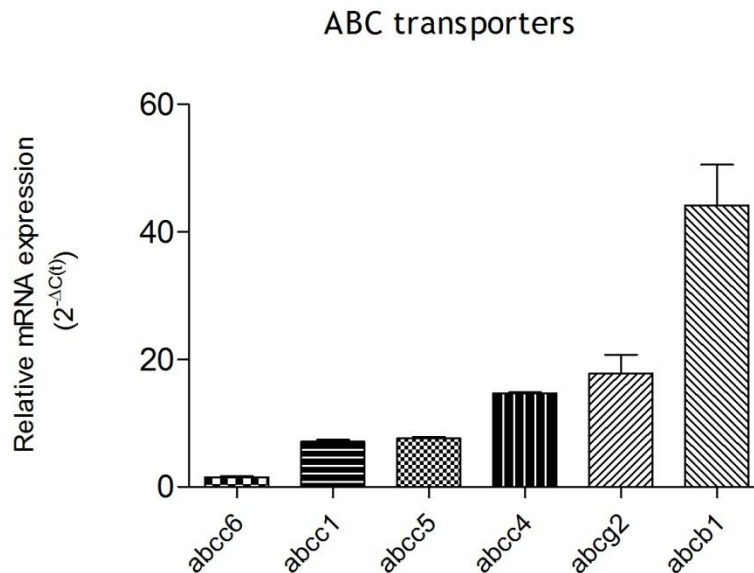


Figure 19. mRNA relative expression of ABC transporters in isolated BMVs

Adult rats P84 were used. mRNA transcription profiles for abcb1, abcg2, abcc1, abcc4, abcc5, abcc5 and abcc6 were assessed by semiquantitative real-time PCR performed in freshly isolated rat BMVs. Results are means ± SEM for 3 batches of BMVs (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of BMVs.

Our results showed that isolated BMVs express ABC transporters. Abcb1 has the higher expression, followed by abcg2 and abcc4, then by abcc5 and abcc1. The lesser expressed gene was abcc6. Abcc2 was not expressed in our BMVs.

SLC transporters mRNA

We studied the expression of six SLC transporters at the BBB level: slc2a1, slc15a2, slc7a7, slc7a8 and slc22a8 (figure 20). Informations about these SLC transporters at the BBB level are provided in the table 5.

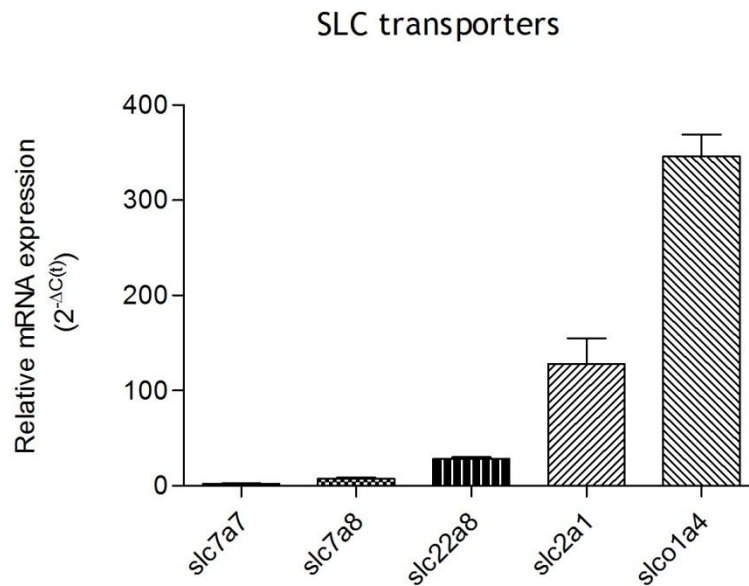


Figure 20. mRNA relative expression of SLC transporters in isolated BMVs

Adult rats P84 were used. mRNA transcription profiles for slc2a1, sl15a2, slc7a7, slc7a8, slc1a4 and slc22a8 were assessed by semiquantitative real-time PCR performed in freshly isolated rat BMVs. Results are means \pm SEM for 3 batches of BMVs (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of BMVs.

Our results showed that these SLC transporters are expressed in our isolated BMVs. Slc1a4 had the higher expression, followed by slc2a1 then slc22a8. Slc7a8 and slc7a7 had low expression levels.

Receptors mRNA

We studied the expression of seven receptors at the BBB level: lrp1 (Low density lipoprotein Receptor-related Protein-1), insr (Insulin Receptor), tfrc (TransFerine ReCeptor), Fc α n (the neonatal Fc receptor for IgG), cav1 (caveoline 1) and cav2 (caveoline 2) (figure 21). Caveolins are integral membrane proteins. They are the principal components of caveolae and are involved

in receptor-independent endocytosis. P-gp has been reported to be located in the caveolae of multidrug-resistant cells. It has been shown that caveolar microdomains isolated from bovine BMVECs are enriched in P-gp, cholesterol, caveolin-1, and caveolin-2, and that P-gp interacts with caveolin-1 and caveolin-2; it has been suggested that P-gp expressed at the BBB is mainly localized in caveolae and its activity may be modulated by interaction with caveolin-1 (Jodoin et al., 2003). This last is involved in the regulation of tight junction proteins and BBB permeability. Informations about lrp1, insr, tfr and FcRn receptors at the BBB level are provided in the table 5.

| Gene name | Abbreviation and/or transporter subtype | Orientation | Example of ligands | Reference |
|-----------|---|---|---|---|
| LRP1 | LDL-receptor related protein-1 | Bi-directional | Lipoproteins, Amyloid- β , lactoferrin, a | Herz and Marschang, 2003; Gaillard et al., 2005 |
| INSR | Insulin receptor | Blood to brain | insulin | Banks, 2004 |
| tfr | transferrin | Blood to brain | Fe-transferrin | Visser et al., 2004 |
| FcRn | rat neonatal Fc receptor (FcRn) | 'reverse transcytosis' of IgG in the brain to blood direction | IgG | Schlachetzki et al., 2002 |

Table 5. BBB receptors

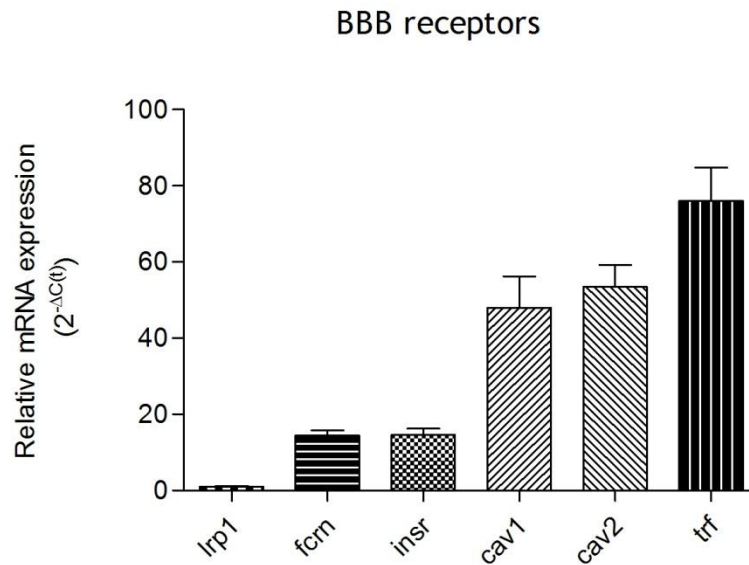


Figure 21. mRNA relative expression of BBB receptors in isolated BMVs

Adult rats P84 were used. mRNA transcription profiles for LRP1, AGER, INSR, TFRC, Fcrrn, CAV1 and CAV2 were assessed by semiquantitative real-time PCR performed in freshly isolated rat brain endothelial microvessels. Results are means \pm SEM for 3 batches of brain endothelial microvessels (each batch represents pooled tissue from 10 rats) and PCR performed in duplicate for each single preparation of brain endothelial microvessels.

Our results showed that these receptors are well expressed in our isolated BMV. Trf receptor, together with cav1 and cav2 are highly expressed, followed by insr and fcrrn. Lrp1 is expressed but has low expression level.

4.1.4 Discussion:

For the work presented in this thesis, we used isolated rat BMVs, rather than total brain tissue, as an *ex vivo* BBB model. In other terms, isolated BMVs were the source of the mRNA or protein used to study the BBB ontogenesis and the impact of inflammation on the regulation of the structural and functional components of BBB.

BMVs have now been isolated for more than 35 years. This isolation was performed from a variety of species such as killifish, shark, mouse, rat, pig, cow and human (Lee et al., 2005; Miller et al., 2002a; Miller et al., 2000; Nobmann et al., 2001; Dallaire et al., 1991; Choi and Pardridge, 1986; Pardridge et al., 1985; Goldstein et al., 1975). Nowadays, this *ex vivo* BBB model is considered to be a powerful tool to study transcriptional regulation of structural and functional components of the BBB as well as functional transport study. Indeed, BMV have been

used for the identification of mechanisms and biochemical signals implicated in the regulation of BBB functions (Collado et al., 2003; Catalan et al., 1996a; Catalan et al., 1996b; Catalan et al., 1993) in health and diseases (Naik and Cuculo, 2012; Guerin et al., 1990). Moreover, freshly isolated BMVs from pig, rat and fish have also been used to perform functional transport studies of efflux transporters (Bauer et al., 2004; Hartz et al., 2004; Miller, 2003; Miller et al., 2002a; Nobmann et al., 2001; Miller et al., 2000).

There are several major advantages of using isolated BMVs as an *ex vivo* BBB model:

- 1- Maintenance of structural and cellular characteristics and properties (Naik and Cuculo, 2012):

The expression of TJ proteins such as Zo1, occludin and claudin-5 were previously detected by western-blot technique (Nitta et al., 2003; Wolburg and Lippoldt, 2002; Kniesel and Wolburg, 2000). That means that isolated BMVs maintain their structural properties after isolation (Shivers et al., 1984; Goldstein et al., 1975). Moreover, several studies localized the Zo-1 along cell-cell contacts (Gao and Shivers, 2004; Virgintino et al., 2004; Lippoldt et al., 2000). In accordance with these data, we detected, in our isolated BMV, gene expression of Zo1 and claudin-5 by real-time PCR (Figure 18) and we showed Zo1 localization along cell cell contacts by immunofluorescence (figure 1, Harati et al., 2012a).

In addition to structural components, expression of functional BBB components (e.g. transporters and receptors) has also been detected in isolated BMVs. For example, ABC transporters such as bcrp were detected in BMVs from humans, pigs, rats and mice (Cisternino et al., 2004; Hori et al., 2004; Zhang et al., 2003a; Cooray et al., 2002; Eisenblatter and Galla, 2002), Mrp1 from rat (Kusuhara et al., 1998; Regina et al., 1998), Mrp4 from human, bovine, and mouse (Leggas et al., 2004; Nies et al., 2004; Zhang et al., 2000). In accordance with these data, we detected in our isolated BMVs, the gene expression of a panel of ABC (figure 19) and SLC (figure 20) transporters as well as many receptors (figure 21) known to be expressed at the BBB level.

- 2- The availability of well documented methods to obtain purified BMVs (Naik and Cuculo, 2012).
- 3- It is advisable to enrich for BMVs to avoid missing BBB derived transcripts and proteins (Calabria and Shusta, 2006).

For these reasons, we used isolated rat BMVs in our study as an *ex vivo* BBB model.

Our protocol was based on mechanical homogenization, enzymatic digestion followed by a density gradient centrifugation (Bauer et al., 2004; Hartz et al., 2004). Using this method, a fraction highly enriched in BMVs was obtained. We combined mechanical and enzymatic isolation because mechanical isolation yields BMVECs containing pericyte contamination as they share the same basement membrane. The enzymatic digestion has the potential to yield purer BMVs because the basement membrane is separated from the vessels during digestion. However, the enzymatic digestion process requires a significant amount of incubation time *ex vivo* at physiological temperature, and during this time the mRNA profile can change (Calabria and Shusta, 2006). Thus, we extended our study to *in vitro* and *in vivo* models. However, as our isolated BMVs express different BBB components: TJ (cldn5 and zo1), ABC (abcb1, abcg2, abcc1, abcc4, abcc5 and abcc6) and SLC (slco1a4, slc2a1, slc7a7 and slc7a8) transporters as well as BBB receptors (lrp1, tfr, fern, insr, cav1 and cav2), this reflect the validity of using this tool to study transcriptional regulation of BBB components.

However, informations from isolated BMVs reflect results obtained on BBB components expressed only in BMVECs as we showed that these isolated BMVs are composed mainly of BMVECs (Figure 1, Harati et al., 2012a). So, regulations of BBB components, during BBB ontogenesis and under the impact of inflammation, in the other cellular constituents of the NVU were missed. For that, in order to obtain a global overview of BBB components regulation in the NVU, we extended our study by developing *in vitro* and *in vivo* BBB models.

4.2 The *in Vitro* Blood-Brain Barrier Model

Our adult and juvenile *in vitro* BBB models consist of a co-culture system of primary rat BMVECs and astrocytes. The chambers containing astrocytes and BMVECs were considered as the basolateral and apical compartment respectively as shown by the figure 22 below. Adults *in vitro* BBB were generated from rats at P84, and juveniles *in vitro* BBB from rats at P21.

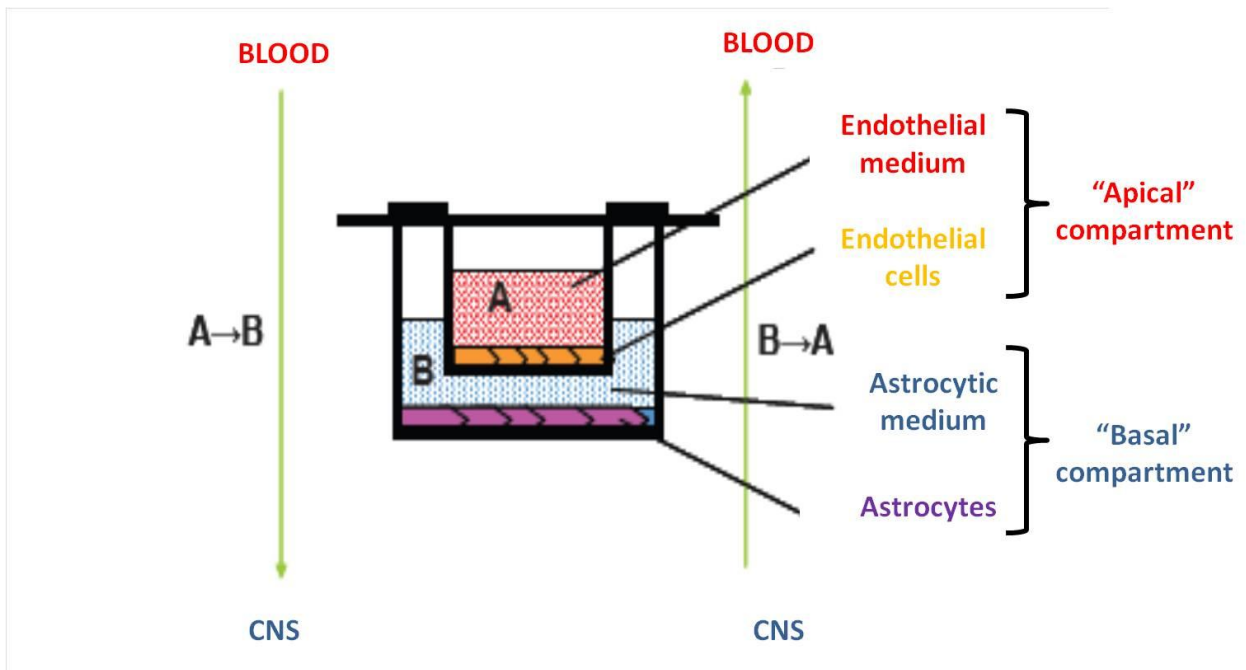


Figure 22. Schematic illustration of the *in vitro* BBB model

4.2.1 Brain microvascular endothelial cells isolation

For BMVECs isolation, isolated BMVs were cultured in a 25 cm² flask coated with collagen type IV and fibronectin (0.1 mg/mL) in the presence of puromycin (2 µg/mL) in the endothelial basal medium containing 0.1% human recombinant Epidermal Growth Factor (hEGF), 0.04% hydrocortisone, 0.1% human recombinant insulin-like growth factor, 0.1% ascorbic acid, 0.1% gentamicin, 0.1% amphotericin-BN and 5% fetal bovine serum. On the third day, the cells received a new endothelial-specific medium without puromycin. When cells reached confluence (70%) at day 7, the purified endothelial cells were frozen and ready to be used for *in vitro* cell-based BBB genesis. Photos of juvenile P21 and adult P84 rat BMVECs observed in phase contrast microscope two days after isolation (passage 0) are presented in figures 23 and 24 respectively. Photos of juvenile P21 and adult P84 rat BMVECs observed in phase contrast microscope six days after isolation (passage 0) are presented in figures 25 and 26 respectively. Endothelial cells appear like cells having Comma-like shape, and slightly oblong.

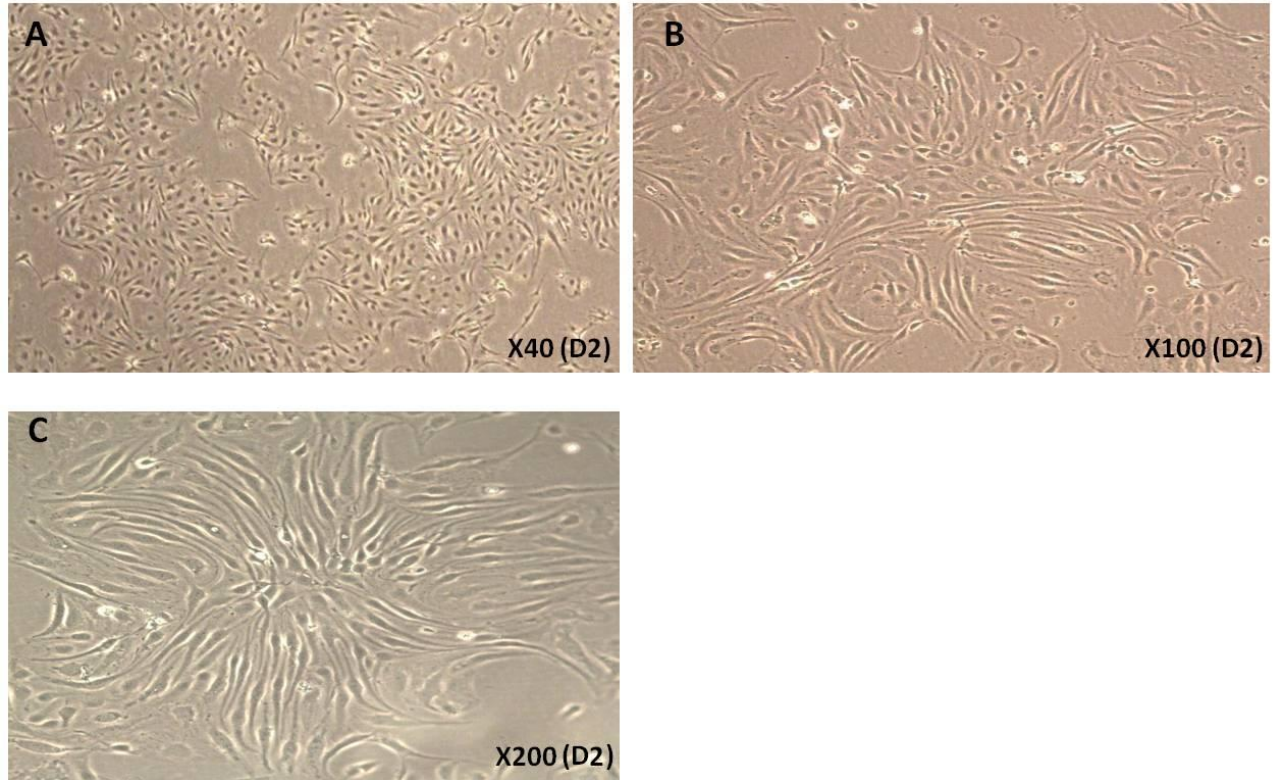


Figure 23. Juvenile rat (P21) BMVECs two days after isolation (passage 0) observed in phase contrast microscope.

A-cells observed with a magnification of 40 times, B-cells observed with a magnification of 100 times, C-cells observed with a magnification of 200 times.

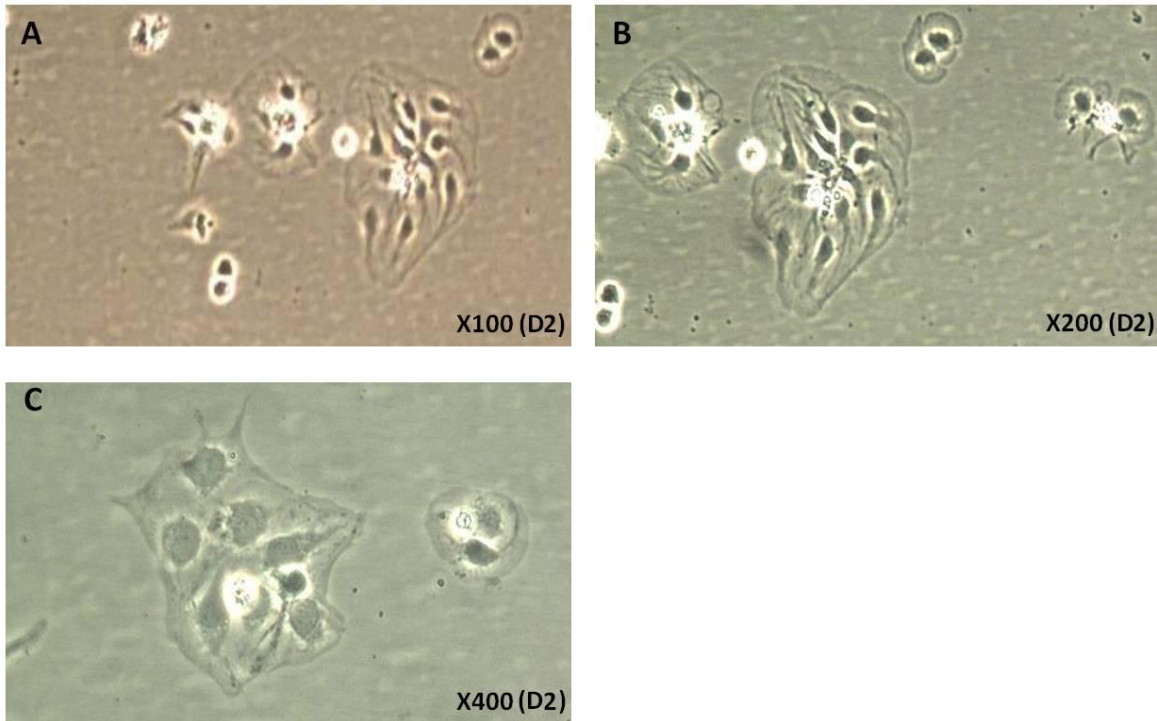


Figure 24. Adult rat (P84) BMVECs two days after isolation (passage 0) observed in phase contrast microscope A-cells observed with a magnification of 100 times, B-cells observed with a magnification of 200 times, C-cells observed with a magnification of 400 times.

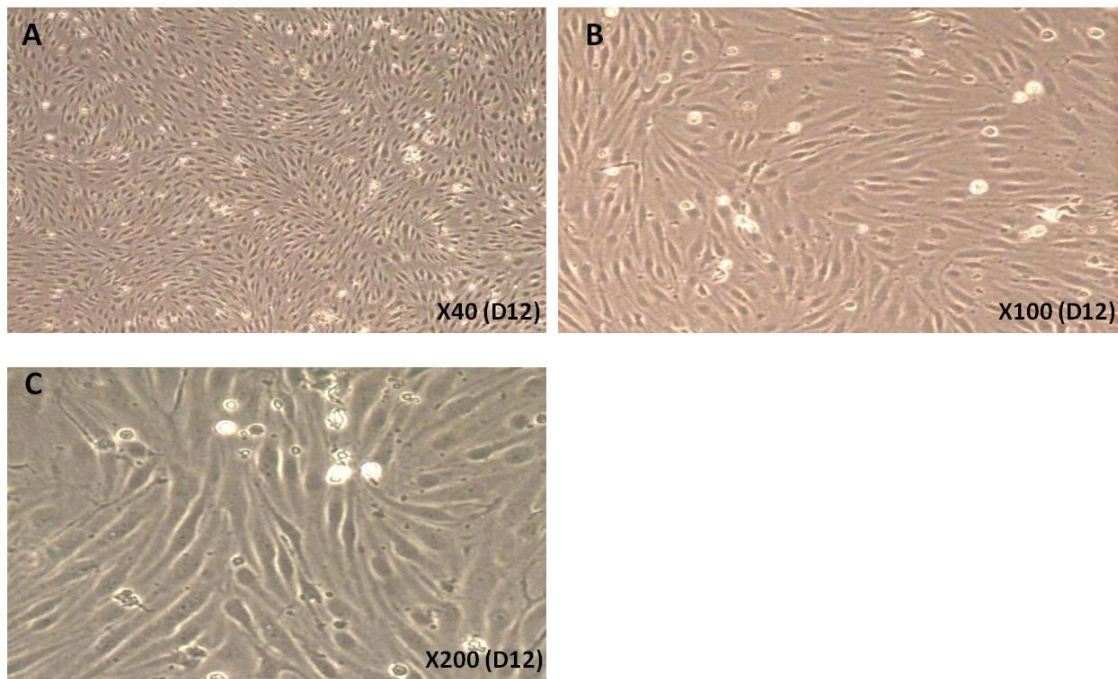


Figure 25. Juvenile rat (P21) BMVECs twelve days after isolation (passage 0) observed in phase contrast microscope. A-cells observed with a magnification of 40 times, B-cells observed with a magnification of 100 times, C-cells observed with a magnification of 200 times.

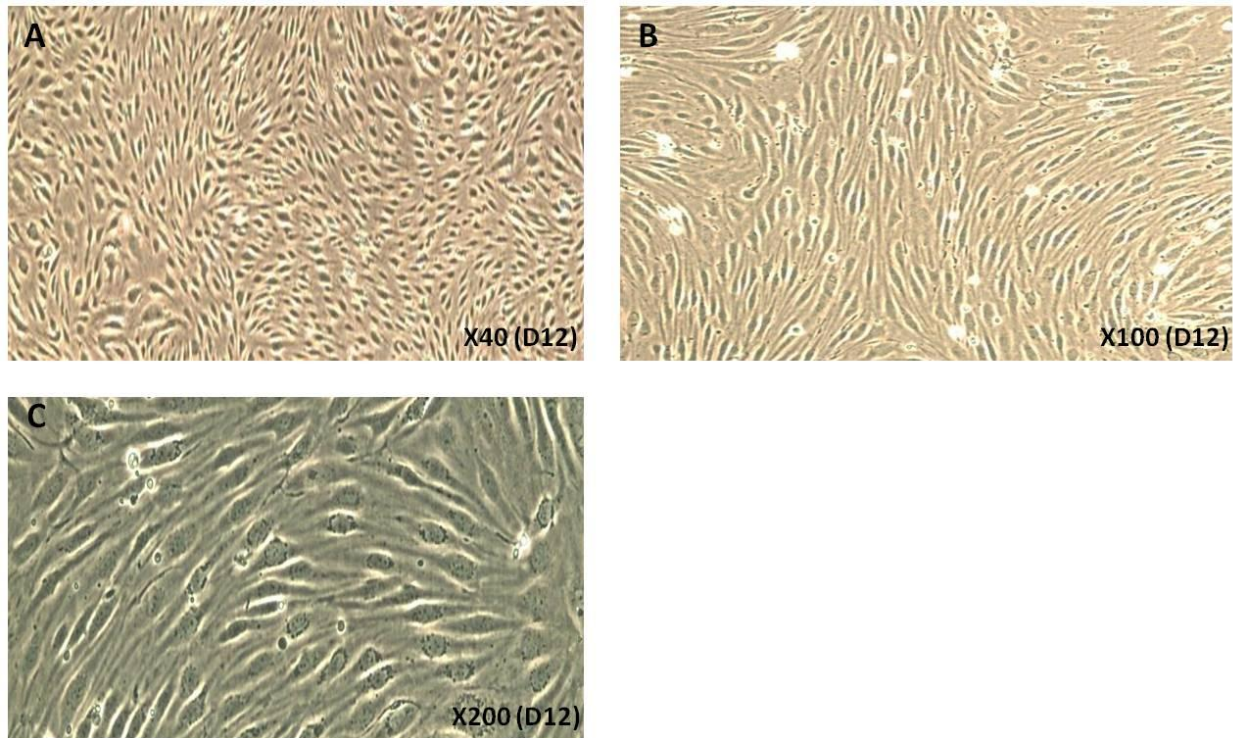


Figure 26. Adult rat (P84) BMVECs twelve days after isolation (passage 0) observed in phase contrast microscope

A-cells observed with a magnification of 40 times, B-cells observed with a magnification of 100 times, C-cells observed with a magnification of 200 times.

4.2.2 Astrocytes isolation

Glial cultures, consisting mainly of astrocytes are prepared from newborn rats and cultured on poly-L-lysine coated surfaces.

4.2.3 *In vitro* cell-based rat BBB model.

Glial cells (2×10^4 cells) were plated on Transwell^R plates (Costar, pore size $0.4 \mu\text{m}$; diameter 12 mm; insert growth area 1.12 cm^2 , Dutscher SA, Brumath, France) in the glial-specific medium α -MEM/ F12) supplemented with $10 \mu\text{g mL}^{-1}$, hEGF, 10 mg mL^{-1} insulin, $25 \mu\text{g mL}^{-1}$ progesterone, 50 mg mL^{-1} transferrin, 50 mg mL^{-1} gentamicin, $50 \mu\text{g mL}^{-1}$, 0.1% amphotericin-B, 1% human serum and 5% fetal bovine serum. Photos of fetal rat (P2) astrocytes observed in phase contrast

microscope at different days after isolation (passage 0) are presented in figure 26 and 27. Astrocytes are star-like, and when cultured, they develop a ramified morphology.

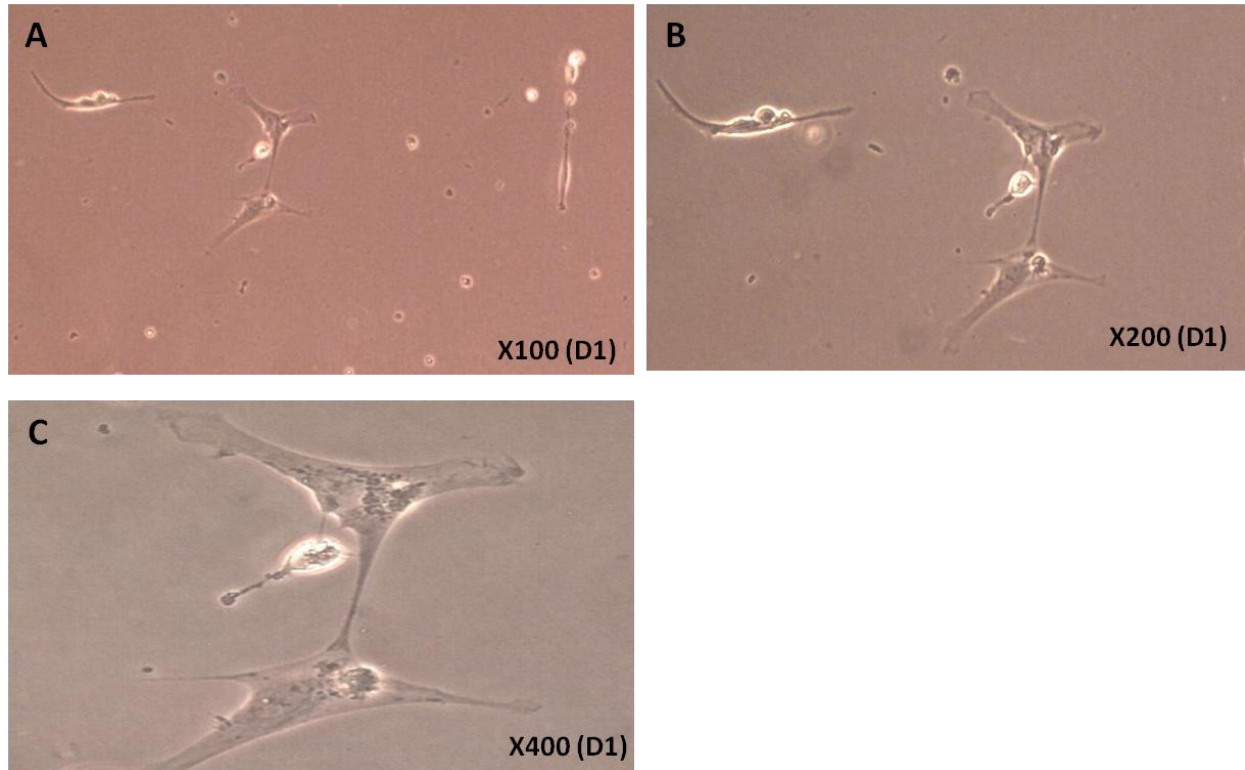


Figure 27. Fetal rat (P2) astrocytes, one day after seeking (passage 1) observed in phase contrast microscope.

A-cells observed with a magnification of 100 times, B-cells observed with a magnification of 200 times, C-cells observed with a magnification of 400 times.

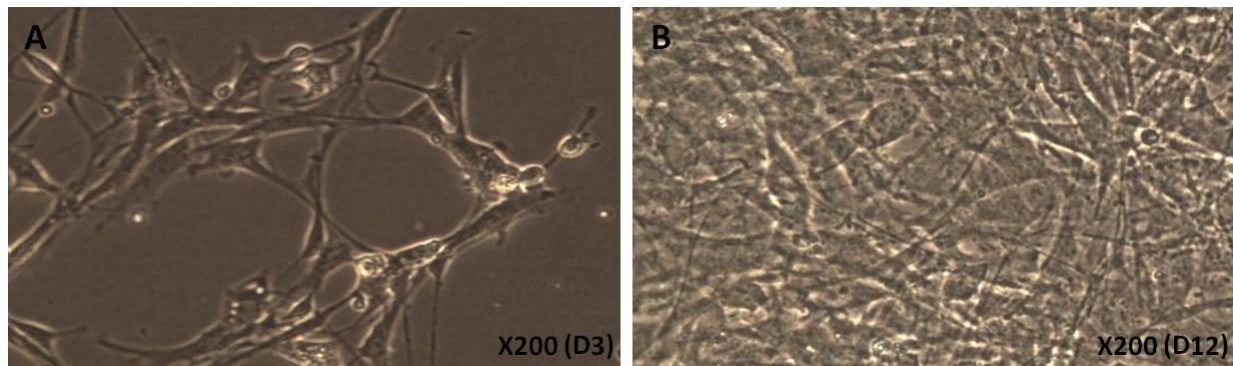


Figure 28. Fetal rat (P2) astrocytes, three (A) or twelve (B) days after seeking (passage 1) observed in phase contrast microscope. A and B-cells observed with a magnification of 200 times.

After 24-72 h, BECs (5×10^4 cells) were plated on the upper side of a collagen-coated polyester Transwell^R membrane (Costar, pore size 0.4 μm ; diameter 12 mm; insert growth area 1 cm^2) in 0.5 mL of the endothelial basal medium-2 (EBM-2, LONZA, Walkersville, MD, USA) containing 0.1% human recombinant epidermal growth factor (hEGF), 0.04% hydrocortisone, 0.1% human recombinant insulin-like growth factor, 0.1% ascorbic acid, 0.1% gentamicin, 0.1% amphotericin-BN and 5% fetal bovine serum (0.5 mL). The chambers containing glial cells and BECs were considered as the basolateral and apical compartment, respectively. The microplates were then incubated at 37°C in a 5% CO₂ atmosphere. Under these experimental conditions,

4.2.4 Checking the integrity of the primary cell-based rat BBB model.

The integrity of the cell-based BBB models was demonstrated by measuring, through the monolayer, the flux of three reference markers: [¹⁴C]-sucrose, [³H]-Propranolol and [³H]-Vinblastin.

BBB integrity was checked on 12% of monolayers before and after the use of the model. Transwells^R with BMEC monolayers were transferred to new plates. Transfer buffer (150 mM NaCl, 5.2 mM KCl, 2.2 mM CaCl₂, 0.2 mM MgCl₂, 6 mM NaHCO₃, 2.8 mM glucose and 5 mM HEPES) was added: 1.5 mL to the basolateral compartment (B) and 0.5 mL to the apical compartment (A). Radiolabelled products are added to the concentration of (0.1 uCi / ml) in the donor chamber (apical or basal compartment). After 60-minute incubation at 37°C, supernatants from both A and B compartments were collected from the acceptor chamber (basal or apical compartment, respectively) and the amount of tracer that passed through the endothelial monolayer was determined by scintillation counting. The permeability was calculated as follows:

From apical to basal:

$$\text{Recovery (\%)} = ((([\text{B}] \times 1500) + ([\text{A}] \times 1500)) / ([\text{initial}] \times 500)) \times 100$$

$$\text{Clearance (\mu L/min)} = ([\text{B}] \times 1500 / [\text{initial}])$$

$$\text{Passage speed (\mu L/min)} = ([\text{B}] \times 1500 / [\text{initial}]) / \text{time}$$

From basal to apical:

$$\text{Recovery (\%)} = ((([\text{B}] \times 1500) + ([\text{A}] \times 1500)) / ([\text{initial}] \times 500)) \times 100$$

$$\text{Clearance } (\mu\text{L}/\text{min}) = ([A] \times 1500 / [\text{initial}])$$

$$\text{Passage speed } (\mu\text{L}/\text{min}) = ([A] \times 1500 / [\text{initial}]) / \text{time}$$

Adult and juvenile BBB monolayers are validated and can be used if they meet the following criteria:

1 - The sucrose molecule cannot cross the TJ and it is not transported across the BBB. Its permeability coefficient must be less than $8 \cdot 10^{-6} \text{ cm} \cdot \text{s}^{-1}$. Our results obtained from three different experiments showed that sucrose permeability coefficient average = $(3.77 \pm 0.37) \cdot 10^{-6} \text{ cm} \cdot \text{s}^{-1}$.

2 - Propranolol is a molecule which undergoes a passive passage. The permeability coefficient must be greater than $20 \cdot 10^{-6} \text{ cm} \cdot \text{s}^{-1}$. Our results obtained from three different experiments showed that propranolol permeability coefficient average = $(35.55 \pm 6.86) \cdot 10^{-6} \text{ cm} \cdot \text{s}^{-1}$.

3 - Vinblastine is a substrat of efflux proteins which make it pass the barrier from A to B and B to A direction. The ratio of the permeability coefficients of direction B to A versus A to B must be greater than 2. Our results obtained from three different experiments showed that ratio of the vinblastine permeability coefficients average = $2.95 \pm 1.15 \text{ cm} \cdot \text{s}^{-1}$.

"Slope Pst (microL / min)" is the speed of passage of the compound of the donor compartment to the receiver compartment. "Slope Pst ($10^{-6} \text{ cm} / \text{sec}$)" takes into account the filter area (1.12 cm^2), it is the apparent permeability coefficient of the membrane (endothelial cells) + filter.

Our generated juvenile BBB model met all these criteria and thus showed that it expresses low paracellular permeation, well-developed tight junctions and functional efflux transporters, which proves that this model has *in vivo* BBB properties and is suitable for signaling studies, especially those involving astrocytes in addition to BMVECs.

4.1.1 Discussion

The important scientific and industrial interest in the physiology and pathology of the BBB led to the development of several *in vitro* models of the BBB engineered from primary cells or cell lines. The best *in vitro* models which mimic the best *in vivo* conditions are the co-culture models in which primary BMVECs are co-cultured with astrocytes. *In vitro* BBB models from several species have been engineered. The most widely used are BMVECs of rat, mouse, pig and bovine origin (Wilhelm et al., 2011; for review see: Deli et al. 2005). Our *in vitro* BBB models consist

of a co-culture system of primary rat BMVECs and astrocytes. There are two advantages for the use of rodent models: 1) Experimental animals are available and 2) these animals are the best characterized ones. However, due to their small size, relatively low amounts of endothelial cells can be obtained which makes the use of cells from other species such as pig or bovine endothelial cells more advantageous because of the large quantity of cells obtainable and good permeability properties. However, the access to these animals is restricted and they are not so well characterized from biochemical or molecular point of view. Regarding the use of human primary cells, the unavailability of experimental material restricts its use. Moreover, brain tissue used for BMVs isolation is usually obtained from surgical material (Persidsky et al. 1997, Bernas et al. 2010) which often cannot be considered as a “healthy” tissue (Wilhelm et al., 2011). For all these reasons, we choosed to performed our study on rat primary cells.

To ensure that the generated BBB models do exhibit *in vivo* BBB properties, several methods permit to determine the barrier properties of these *in vitro* models. Among these methods, there are electrical methods consisting on the measurement of TransEndothelial Electrical Resistance (TEER) and methods based ont the use of tracer substances with known molecular weight. ¹⁴C-Sucrose is a widely used tracer. To determine the barrier properties of our model, this last method was used. Our results showed that our generated BBB models, from juvenile as well as adult cells are characterized by low paracellular permeation, well-developed tight junctions and functional efflux transporters. These experiments have proven that our models mimic *in vivo* properties of the BBB and thus are suitable for our research, and can be an alternative tool for studying signals at the BBB level, especially those involving interaction between astrocytes and BMVECs.

4.2 The *In Vivo* Model of acute cerebral inflammation

Our *in vivo* model of acute cerebral inflammation consist of juvenile P21 and adults P84 rats having an intra-cerebroventricular (icv) injection of ET-1, a neuropeptide known to trigger inflammatory reactions. An illustration of our experimental protocol to generate these *in vivo* models is present in the figure below:

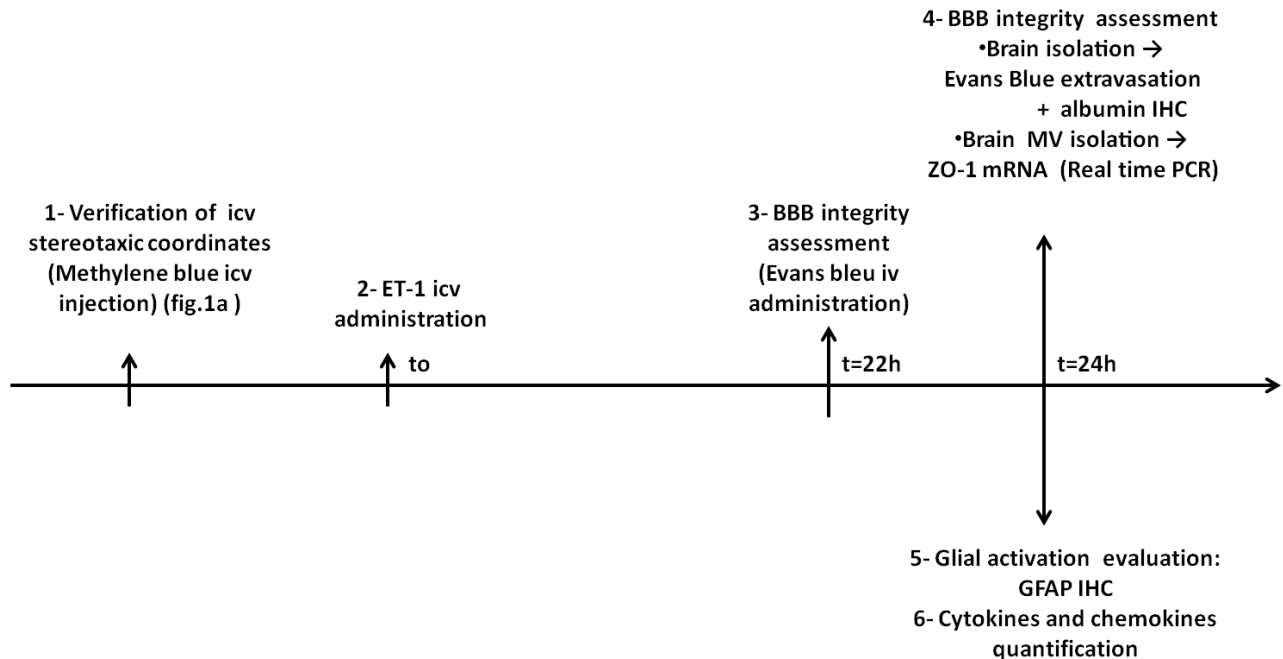


Figure 29. Illustration of experimental design.

Male Wistar rats at two stages of brain maturation were used in our experiments: Post-natal (P) P21 corresponding to the pediatric stage in human and P84 corresponding to the adult stage in human. The experimental design was articulated in several steps: 1. Verification of ventricular stereotaxic coordinates in both juvenile and adult rats: Methylene blue was injected intracerebroventricularly in order to visualize the stereotaxic implantation of the injection cannulae and the injection site into the brain lateral ventricles. 2. ET1 injection (25 pmol/kg) 3. BBB integrity assessment by the Bleu Evan test and 4. the measure of mRNA relative expression of a tight-junction component, the Zonula Occludens-1 (ZO-1): ET-1 was administrated intracerebroventricularly. Twenty-two hours later, a solution of 3% Evans Blue dissolved in 0.9% saline was administrated intravenously (4ml/kg). Two hours later, brains were isolated and the BBB integrity was assessed by verifying the absence of blue trace and by albumin immunohistochemical (IHC) detection in the cortex and the hippocampus. BBB integrity was further assessed by measuring using real-time PCR, mRNA relative expression of the ZO-1 in brain microvessels isolated 24h after ET-1 treatment. 5. Immunohistochemistry of Glial Fibrillary Acidic Protein (GFAP), a glial activation marker [40], in cortex and hippocampus of rat brains isolated 24h after ET-1 treatment. 6. Brain and serum cytokines/chemokines quantification 24h after ET-1 icv injection.

In the following section, I give general informations about ET1, its physiological and pathological levels, the choice of the ET1 dose to inject, and the validation of the model. This later consists of: 1. verification of cerebroventricular stereotaxic coordinates, 2. BBB integrity assessment, 3. Quantification of inflammatory markers: GFAP overexpression and the expression of cytokines.

4.2.1 ET-1: an inflammatory neuropeptide

General aspect

ET-1 is a 21 amino acid peptide hormone with diverse physiological functions (Rubanyi and Polokoff, 1994). In the vascular system, endothelins, have a vasoconstricting role and contributes to the development of vascular disease in hypertension and atherosclerosis (Barton, 2000; Schiffrin, 1999). They also affect physiology and pathophysiology of the immune system (Nett et al., 2006), liver (Berthiaume et al., 2005), glucose homeostasis (Lteif et al. 2007), and many other physiological and pathological functions (Barton and Yanagisawa, 2008). Three isoforms of the endothelin family have been identified: ET-1, ET-2 and ET-3. Endothelin-1 is the most abundantly and widely expressed, and the biologically most relevant member of the endothelins family (Luscher and Barton 2000; Kedzierski and Yanagisawa 2001).

Structure

Structure of ET-1 contains 2 disulfide bridges and has the appearance of a shepherd's crook (Figure 30) (Highsmith, 1998; Webb, 1997).

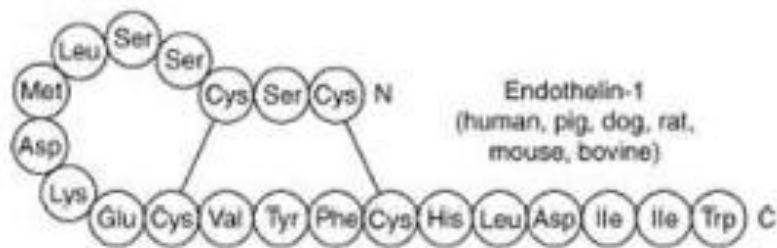


Figure 30. ET1 structure (Masaki, 2004)

Physiological function

Endothelins, of which ET-1 represents the predominant isoform and the biologically most relevant (Luscher and Barton 2000; Kedzierski and Yanagisawa 2001), can be considered ubiquitously expressed stress-responsive regulators working in a paracrine and autocrine fashion and having both beneficial and detrimental effects (Kedzierski and Yanagisawa 2001). Endothelins exert a number of physiological functions (Rubanyi and Polokoff 1994). In the vascular system, endothelin, has a vasoconstricting role and contributes to the development of

vascular disease in hypertension and atherosclerosis (Schiffrin 1999; Barton 2000). They also affect physiology and pathophysiology of the immune system (Lteif et al. 2007; Nett et al. 2006; Berthiaume et al. 2005). Endothelins are also involved in neural crest cell development and neurotransmission (Kedzierski and Yanagisawa 2001).

Signaling

The diverse biological actions of ET-1 are mediated by two different ET-1 receptor subtypes, ETA and ETB (Davenport, 2002) different in their tissue distribution, ligand binding and signal transduction (Offermanns, 2004). The ETA receptor is present in many tissues but it is highly expressed in aorta, heart and kidney (Webb, 1997). The ETB receptor is predominantly expressed in brain, lung, kidney and aorta (Webb, 1997; Ogawa et al., 1991). It has been described that in the vascular system, ETA and ETB exert opposite effects: ETA causes long-lasting vasoconstriction, whereas ETB transient vasodilation (Kedzierski and Yanagisawa, 2001; Levin, 1995).

4.2.2 ET-1: physiological and pathological doses

Previous work have been done by our team using ET-1 concentrations ranging from 40 to 400 nM, and for exposures of 24 hours on an *in vitro* BBB model from human cells (Didier et al., 2003). Hartz et al. (2004) studied the effect of ET-1 on an *in vitro* rat BBB model using doses of ET-1 from 1 to 100 nM. Studies on human monoculture BMVECs showed an optimal dose of ET-1 10 nM to see the maximum effect (Chen et al., 2001), while Zidovetzki et al. (1999), using the same type of cells, studied the action of ET-1 using a dose of 100 nM. Low doses of ET-1 (0.1 nM) induce the secretion of CXCL1 and IL-8 in human cells of melanoma. This phenomenon is concentration-dependent and the results were further studied for 10 nM ET-1 (Mangahas et al., 2005). In addition, our team has also found that, in an inflammatory context, when BMVECs are co-cultured with atherosocytes and exposed to TNF α , it follows secretion of ET-1 in the apical compartment (Didier et al., 2003). Thus, we chose concentrations of ET-1 to 1 nM, 10 nM and 100 nM. In the cerebrospinal fluid (CSF) of patients infected with HIV-1 encephalopathy, the concentration of ET-1 was 1.97 ± 2.33 pmol / L while it was 0.57 ± 0.67 pmol / L in patients showing no neurological dysfunction (Rolinski et al., 1999). Narushima et

al. consider that a moderate dose of ET-1 injected *in vivo* is about 10 pmol/kg for rats (Narushima et al., 2003). Thus, we chose the dose of 25 pmol/kg of ET-1.

4.2.3 Intracerebroventricular stereotaxic injection

Rats of different ages were weighed using a digital scale. All animals were then anesthetized with ketamine (87 mg/kg)/xylazine (13 mg/kg) and placed in a rodent stereotaxic apparatus. A 33-gauge beveled injection needle (Coveto) was inserted into the right lateral cerebral ventricle (4 mm ventral to the dura) via a hole that was drilled in the cranium at 1.08 mm posterior and 2 or 1.5 mm lateral to the bregma for P21 or P84 rats, respectively. The tip of a Hamilton syringe needle was briefly lowered to a depth of 3.8 or 4.2 mm for P21 or P84 rats respectively, and immediately raised by 0.2 mm to create a trough. To verify these coordinates and to ensure that the site of our injection is in the cerebroventricles, stereotaxic implantation of the cannulae was checked using icv methylene blue injected rats which permit to visualize the stereotaxic implantation of the injection cannulae and the injection site into the brain lateral ventricles. Cannula implantation reached the lateral ventricle as shown by the blue color and cannula scar (blood trace) (as referred to in Paxinos and Watson's stereotaxic atlas) (see figure 1a, Harati et al., 2102b).

4.2.4 Checking BBB integrity

- a) BBB permeability after ET-1 injection was assessed using albumin IHC in IV Evans blue injected rats. ET-1 was administered intracerebroventricularly. Twenty-two hours later a solution of 3% Evans Blue dissolved in 0.9% saline was administered intravenously (4ml/kg). Two hours later, brains were isolated and the BBB integrity was assessed by verifying the absence of blue trace and by albumin immunohistochemistry detection in the cortex and the hippocampus. No albumin labeling or blue trace was detected in the cortex or in the hippocampus of adult or juvenile rat brains, suggesting lack of BBB breakdown after icv injection of ET-1 (see figure 1B, Harati et al., 2102b).
- b) BBB permeability was also assessed using a molecule, baclofen.

The baclofen molecule does not cross the TJ very effectively and it is not transported across the BBB. Thus, we injected by icv route the ET-1 (25 pmol/kg). 20 hours later, we perfuse in catheterized rats the baclofen by icv route and this during 54 hours. At steady state, we extracted brain and plasma and measured baclofen in the two compartments in animals who received ET-1 compared to animal who received a saline solution. To estimate BBB permeability, we assessed the $K_{p,brain}$. Results are presented in the figure 31.

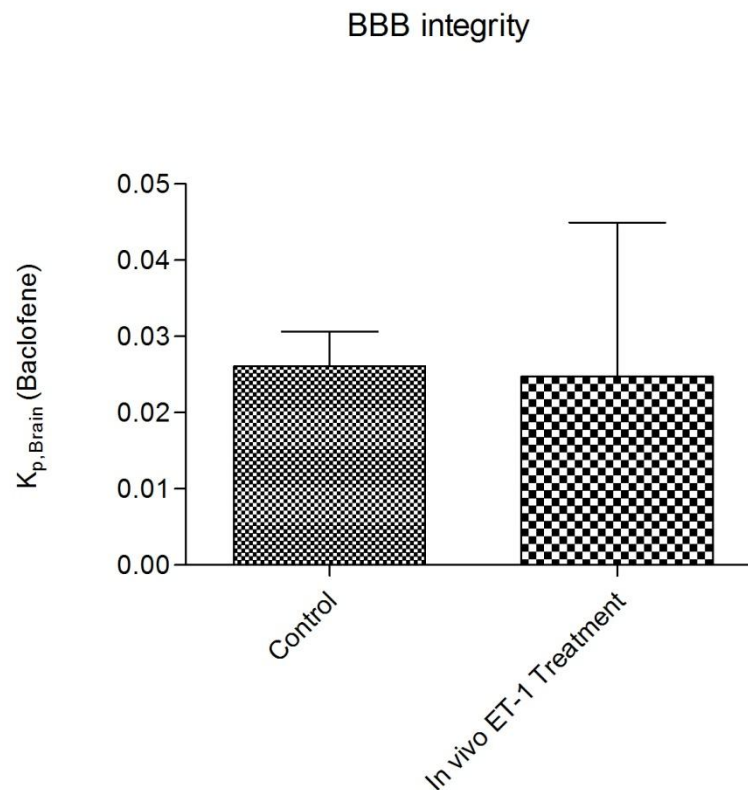


Figure 31. Passage of baclofen across the BBB after ET-1 treatment in adult rats.

Permeability was assessed by determination of $K_{p,brain}$ for baclofen after ET-1 administration. Data represent the mean concentration for 3 rat brains and 3 rat plasmas; variability is given by bars \pm SEM.

Our results showed that BBB permeability for baclofen was not affected by ET-1 treatment.

Thus, BBB integrity in our *in vivo* models is not affected suggesting that this model is suitable for the study.

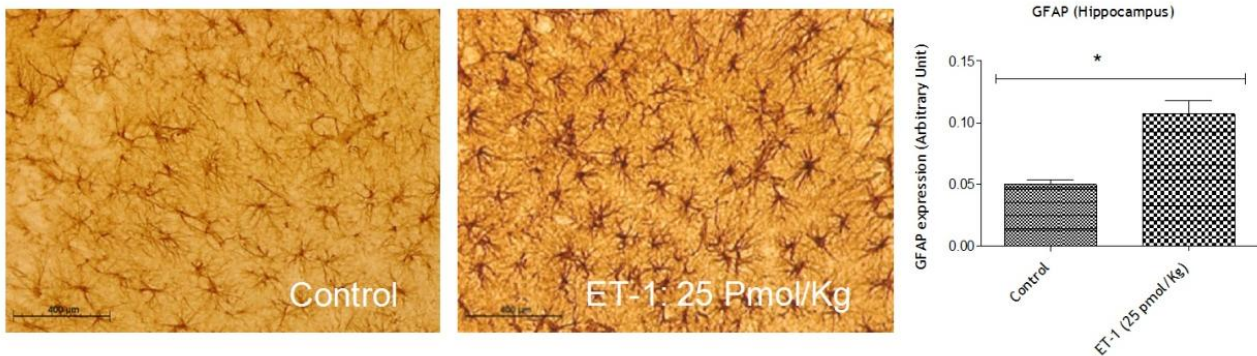
c) BBB permeability after ET-1 injection was also assessed by measuring, using real-time PCR, mRNA relative expression of a tight-junction component, the Zo-1 gene, in BMVs isolated

24h after ET-1 treatment. Results represent mean \pm S.E.M from 3 batches of brain endothelial microvessels (see figure 1C, Harati et al., 2102b).

4.2.5 GFAP quantification

We determined whether glial activation occurs after treatment of animals with ET-1. Immunohistochemistry shows that ET-1-induced activation of glial cells as measured by GFAP. In cortex, ET-1 increased GFAP in glial cells in adults as well as in juvenile brain (see figure 32 below and figure 6, Harati et al., 2102b).

a



b

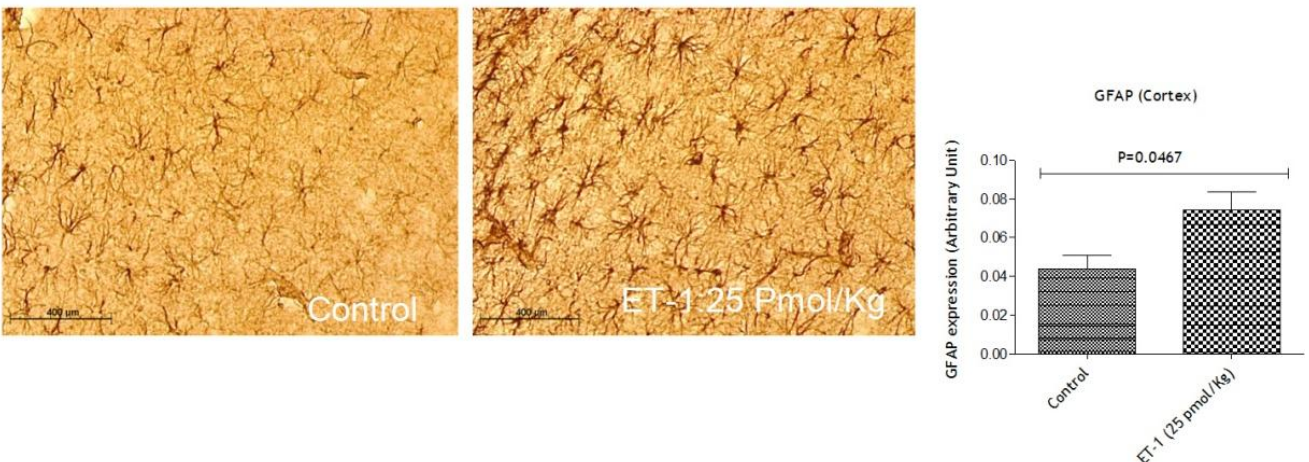


Figure 32. Effect of ET-1 on glial activation in adult and juvenile brains.

Representative immunohistochemical staining of GFAP in the adult (A) brains cortex and quantitative analysis of GFAP expression (B). Each bar is expressed as the mean \pm S.E.M; *** $P < .0001$ compared with control group. Representative immunohistochemical staining of GFAP in the adult (C) hippocampus and quantitative analysis of GFAP expression (D). In the cortex, ET-1 increased GFAP in glial cells in adults (1.94-fold). In the hippocampus, ET-1 enhanced GFAP expression in glial cells of adult brain 2-fold ($P < .0001$). Each bar is expressed as the mean \pm S.E.M; *** $P < .0001$ compared with control group.

4.2.6 Cytokines quantification

We have quantified a cytokine, the IL-1 β known to be an inflammation marker. Our results (Figure 33) show an increase in IL-1 β levels in adult brain 24h after ET-1 icv administration. The quantification was performed using specific ELISA assay IL-1 β Rat ELISA Kit (Source: Raybiotech; catalogue #: ELR-IL1beta-001; Purchased from Tebu-bio).

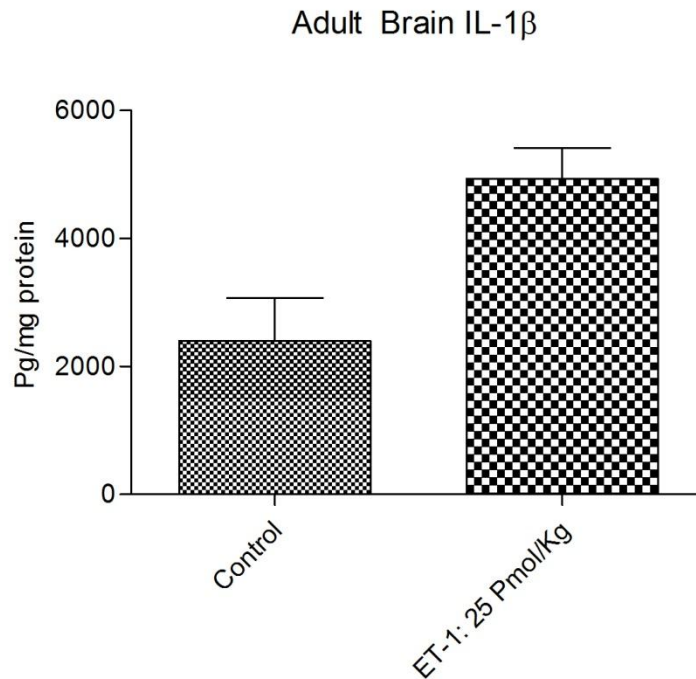


Figure 33. IL-1 β levels in adult brain after a single intracerebral injection of endothelin-1 for 24 h.

IL-1 β was measured in brain of adult rat using an ELISA kit. Results show an increase in IL-1 β levels in adult brain 24h after ET-1 icv administration.

4.2.5 Discussion

ET-1, an arterial vasoconstrictive and vasodilator peptide cytokine (Wright et al., 1988) is released in several CNS disorders (Jiang et al., 2006; Sasaki et al., 2006; Schinelli, 2006; Speciale et al., 2006; Hebert et al., 2004; Rolinskie t al., 1999; Nie and Olsson, 1996; Baron et al., 1995; Nakajima et al., 1994; Suzuki et al., 1992; Ziv et al., 1992), and known to be a component of the brain's innate immune response (Bauer et al., 2007; Chauchan et al., 2007;

Didier et al., 2003; Dider et al., 2002; Scinelli , 2002; Yoshimoto et al., 1999; Nie and Olsson 1996) triggered by a variety of stimuli, including infection, trauma, disease, and cell stress and characterized mainly by glial activation and the release of proinflammatory cytokines and chemokines (Farina et al., 2007; Allan, 2003; Rivest and Rothwell, 2003). Previous works in our laboratory as well as studies from other laboratories have shown that proinflammatory cytokines such as TNF α and IL-1 β caused a released of ET- 1 at the BBB level (Bauer et al., 2007; Didier et al., 2003; Marsden and Brenner; 1992). In turn, ET-1 causes the release of other cytokines and chemokines such as MCP-1 (Chen et al., 2001) amplifying thus the inflammatory signals.

For these reasons we have chosen the ET-1 as the inflammatory stimulus that causes acute cerebral inflammation. To validate our model we quantified in the brain cytokines such as IL-1 β and GFAP expression. We have choosen these three parameters for the following reasons:

In the brain, inflammatory response begins with recruitment of innate immune system. Rapidly, in response to infection or injury, microglia, major inflammatory cells of the monocyte/macrophage lineage resident in the brain, are activated (Streit, 2002). Microglia are important phagocytic cells, and once activated they release numerous inflammatory molecules, particularly pro and anti- inflammatory cytokines and chemokines (Hanisch, 2002) (Pro-inflammatory molecules such as TNF- α , IL-1 β , IL-6 (Aloisi, 2001; Elkhoury et al., 1998; Kreutzberg, 1996), chemokines (IL-8, MIP-1 α , MIP-1 β , MCP-1) (Peterson et al., 1997; Erlich et al. 1998), proteases (Nakanishi, 2003), and anti-inflammatory molecules such as TGF- β and IL-10 (Benveniste et al., 2001). Lately, astrocytes are activated (Ivey et al., 2009). Apart from being involved in a variety of physiologic processes, astrocytes rapidly react to different neurological insults. Upon activation, a series of changes occur in astrocytes leading to the acquisition of macrophage differentiation markers and effector properties. One main feature of these changes is the increase in the number and size of GFAP expressing cells. GFAP is an intermediate filament cytoskeletal protein expressed primarily by astrocytes and it is considered as the marker of astrocytes (Raine, 1999). Parallel to GFAP overexpression, astrocytes released many pro-inflammatory mediators and up-regulate the expression of several inflammatory molecules which contribute to the amplification of inflammation (Farina et al., 2007). Concerning the cascade of released cytokines/chemokines during brain inflammation, previous work in our laboratory as well as studies from other laboratories have shown that proinflammatory cytokines such as TNF α

and IL-1 β caused a released of ET-1 at the BBB level (Bauer et al., 2007; Didier et al., 2003; Marsden and Brenner; 1992). In turn, ET-1 causes the release of other cytokines and chemokines such as MCP-1 (Chen et al., 2001) amplifying thus the inflammatory signals.

In this study, to test whether ET1 triggered inflammatory reactions in the brain, we quantified the GFAP in hippocampus and cortex of rat brains treated with ET-1, and we quantified a cytokine, the IL-1 β that could be induced by ET-1 in adult rat brains. To validate our model, we have first ensured that icv ET-1 injection does not alter BBB integrity, and then we quantified an inflammatory marker the IL-1 β cytokine in cortex as well as GFAP expression, a glial activation marker, in hippocampus and cortex of rat brains treated with ET-1. Our results showed a pronounced glial activation in adult cortex and hippocampus that coincided with elevated IL1- β levels after ET-1 treatment. These data reflect a cerebral inflammation triggered by ET-1. Thus, these *in vivo* models are suitable to study the regulation of BBB components under acute brain inflammation.

CONCLUSIONS & PERSPECTIVES

Our study had three main objectives:

- 1- Studying the ontogenesis of a number of BBB structural and functional components in rats from birth to adulthood.
- 2- Investigating the role of the canonical Wnt signaling in the regulation of four drug efflux transporters: P-gp, bcrp, oat3 and oatp1a4.
- 3- Comparing, between juvenile and adult rats, the impact of acute cerebral inflammation on the regulation of two of the most clinically relevant ABC transporters of the BBB: P-gp and bcrp.

We articulated our working methodology as follows:

- 1- Development of research tools
- 2- Descriptive and mechanistic study of BBB ontogenesis
- 3- Impact fo acute cerebral inflammation study

1 Development of research tools: Conclusions and perspectives

Ex vivo, *in vitro* and *in vivo* research tools were used:

- *ex vivo* BBB model: cortical BMVs isolated from adult rats at P84 of age (and corresponding to adults in human).

BMVs are known to be a powerful tool to study BBB properties and to identify mechanisms and signals implicated in the regulation of BBB functions (Collado et al., 2003; Catalan et al., 1996a; Catalan et al., 1996b; Catalan et al., 1993). Moreover, it is advisable to enrich for BMVs to avoid missing BBB derived transcripts and proteins and this by comparison to the whole brain (Calabria and Shusta, 2006). In addition, there are many well documented methods to obtain purified BMVs (Naik and Cuculo, 2012).

For all these reasons, we started our study by isolating BMVs from adult rats and then we ensured that this model is a powerful tool for transcriptional studies on the regulation of BBB structural and functional components. For that, we first assessed the purity of isolated BMVs, and we established gene expression of a panel of essential BBB components. Our results showed that our isolated BMVs are enriched in TJ components such as claudin-5 and Zo1, and express as

well a panel of ABC drug efflux transporters such as *abcb1*, *abcg2*, *abcc1*, *abcc4*, *abcc5* and *abcc6*, in addition to numerous SLC transporters such as *slc22a8*, *slco1a4*, *slc2a1*, *slc7a7* and *slc7a8*, and BBB receptors such as *lrp1*, *insr*, *tfr*, *fcrr*, *cav1* and *cav2*. Some of these components known to be enriched at the BBB level were highly expressed in our BMVs after isolation such as claudin-5, *abcb1* and *slc2a1* for example. Moreover, several studies localized the *Zo1* along cell-cell contacts (Gao and Shivers, 2004; Virgintino et al., 2004; Lippoldt et al., 2000). In accordance with these data, we showed, in our isolated BMVs, *Zo1* along cell-cell contacts by immunofluorescence. Taken together, all these data validated that the isolated BMVs maintained their structural and cellular characteristics and properties, and thus can be used as an *ex vivo* BBB model to detect regulations of BBB components during ontogenesis and under the impact of brain acute inflammation.

However, informations from BMVs reflect results obtained on BBB components that are only expressed in BMVECs because we showed that these isolated BMVs are mainly composed of BMVECs (Figure 1, Harati et al., 2012a). So, regulations of BBB components, during BBB ontogenesis and under the impact of inflammation, in other cellular constituents of the NVU were missed. For that, in order to obtain a global overview of BBB components regulation in the NVU, we extended our study by developing *in vitro* and *in vivo* BBB models.

- *in vitro* BBB model from juvenile rat cells: This model consists of a co-culture of primary astrocytes and BMVECs expressing endothelial junctional proteins and efflux transporters (e.g. P-gp and bcrp).

To mimic in a best way *in vivo* conditions, we developed an *in vitro* BBB model from primary BMVECs cells isolated from juvenile rats at P21 of age (and corresponding to the pediatric stage in humans) and co-cultured with primary astrocytes isolated from new-born rats. This model was complementary to an *in vitro* BBB model previously established in our laboratory using adult rat cells (Lacombe et al., 2011). This latest model was validated by *in vitro/in vivo* correlation. Although not yet validated by such correlation, our generated juvenile BBB model expresses low paracellular permeation, well-developed tight junctions and functional efflux transporters, which proves that it has *in vivo* BBB properties and is suitable for signaling studies, especially those involving interactions between astrocytes and BMVECs. A better characterization of permeability properties of the generated juvenile *in vitro* BBB model will be performed by *in*

vitro/in vivo correlations study using drugs from many therapeutically classes; this objective is the subject of an actual PhD dissertation in our laboratory.

- *in vivo* models of acute cerebral inflammation in juvenile and adult rats: these models consist of an intra-cerebroventricular injection of ET-1, a neuropeptide known to trigger inflammatory reactions.

ET-1 is a peptide cytokine released in several CNS disorders (Jiang et al., 2006; Sasaki et al., 2006; Schinelli, 2006; Speciale et al., 2006; Hebert et al., 2004; Rolinskie t al., 1999; Nie and Olsson, 1996; Baron et al., 1995; Nakajima et al., 1994; Suzuki et al., 1992; Ziv et al., 1992) and known to be a component of the brain's innate immune response (Bauer et al., 2007; Chauchan et al., 2007; Didier et al., 2003; Didier et al., 2002; Scinelli , 2002; Yoshimoto et al., 1999; Nie and Olsson 1996). Previous works in our laboratory as well as studies from other laboratories have shown that proinflammatory cytokines such as TNF α and IL-1 β caused ET-1 release at the BBB level (Bauer et al., 2007; Didier et al., 2003; Marsden and Brenner; 1992). In turn, ET-1 causes the release of other cytokines and chemokines such as MCP-1 (Chen et al., 2001), amplifying thus the inflammatory signals. For these reasons we have chosen ET-1 as the inflammatory stimulus that causes acute cerebral inflammation. To validate our model, we ensured that icv ET-1 injection does not alter BBB integrity, and we quantified an inflammatory marker, the IL-1 β cytokine, in cortex as well as GFAP expression, a glial activation marker, in hippocampus and cortex of rats treated with ET-1. Our results showed a pronounced glial activation in cortex and hippocampus that coincided with elevated IL-1 β levels after ET-1 treatment. These data reflect a cerebral inflammation triggered by ET-1 proving that this *in vivo* model is suitable to study regulation of BBB components under acute brain inflammation.

2 Blood-brain barrier descriptive study: Conclusions and perspectives

The first aim for our study was to study BBB ontogenesis. For this, male Wistar rats at four stages of brain maturation were used: Post-natal (P) P2 corresponding to preterm stage in human, P14 corresponding to human neonates, P21 corresponding to pediatric and P84 corresponding to adults in human. First, we established *ex vivo*, in isolated BMVs, the maturational profile of a number of BBB structural (inter-endothelial TJ) and functional (SLC and ABC transporters)

components from birth to adulthood. Our first results showed no significant changes in BBB TJ components with age, which confirms previous works showing that TJ develop during fetal life and that BBB is functionally tight at birth (Olsson et al., 1968; Tauc et al., 1984; Saunders, 1992; Moos and Møllgård, 1993; Keep et al., 1995; Preston et al., 1995; Saunders et al., 2000; Ballabh et al., 2004). Concerning other BBB functional components, we showed age-dependant transcriptional variation for most of the studied transporters. Then, to evaluate the functional status of these transporters in “immature” brain when compared to adult, we measured, *in vivo*, the activity of four BBB drug efflux transporters (P-gp, bcrp, oat3 and oatp1a4). We showed that expression and function of oat3 and oatp1a4 increase as the brain matures. In contrast, just expression but not function of P-gp and bcrp transporters increased with aging and particularly between juvenile and adult brains; their function did not varied between ages.

These data suggest a potential age-related response to CNS drugs. They also suggest that other factors may cause functional but not transcriptional regulation of oat3 at the juvenile and adult BBB and this highlights the importance of evaluating the activity during studies on drug efflux transporters for a better assessment of drug pharmacokinetic profiles. Variations observed in the expression of these components during brain maturation emphasize the importance of conducting functional studies in order to establish their functional status in the maturational brain, especially because of their important roles at the BBB, in particular their contribution in the multidrug resistance phenomenon. As perspective for this descriptive study, it is important to evaluate functional status of the other studied transporters during brain maturation, in order to have a comprehensive overview on the BBB role in immature brain. These studies, in addition to our findings, will shed light on BBB changing properties during ontogenesis and will provide a clear answer 1) whether children brain is more vulnerable to neurotoxics than adult brain and 2) whether the response of CNS to drugs is age-related, which in turn will require caution in giving drugs to infants, and even more taking into account the specific properties of the juvenile BBB, especially during the definition of therapeutic strategies designed to treat childhood brain diseases.

3 Understanding the molecular mechanisms of BBB ontogenesis: Conclusions and perspectives

The descriptive study sheds light on BBB changing properties during ontogenesis. However, the ultimate question that arises from this study relates to the mechanism underlying regulation of functional expression of P-gp, bcrp, oat3 and oatp1a4 during brain development as well as in adults 1) because the role of molecular mechanisms regulating the functional expression of these BBB drug efflux transporters are not fully understood, and 2) in the perspective of finding molecular targets that can be used to modulate BBB drug efflux transporters and thus enhancing drugs bioavailability in either adult and pediatric brain.

A mechanism implicated in the regulation of BBB structural components during embryonic development is Wnt/ β -cat signaling (Liebner et al., 2008). Another factor known also to regulate BBB components, in particular P-gp, is the ET-1 (Hembury et al., 2008; Bauer et al. 2007). Thus, we investigated the role of Wnt/ β -cat, as well as its interaction with ET-1 signaling in the regulation of P-gp, bcrp, oat3 and oatp1a4 at BBB level in juvenile and adult rats.

In adults, we showed that *in vivo* inhibition of a kinase, the GSK-3 β , enhanced expression of nuclear β -cat, as well as ET-1 gene expression in BMVs, and increase the functional expression of P-gp, bcrp, oat3 and oatp1a4 at the BBB level. Interestingly, blocking the ETA receptor for ET-1 decreases the activation of transporters' activity observed following β -cat activation. This suggests that inhibition of GSK-3 β , activates β -cat and increase the expression of ET-1 which in turn upregulates drug efflux transporters (this mechanism is illustrated in the figure 12 of our article Harati et al., 2012a). In juvenile rats, contrary to adults, *in vivo* inhibition of GSK-3 β decreased the functional expression of bcrp, with a tendency of decrease in P-gp activity. This result, showing that BBB drug efflux transporters in juvenile brain are regulated differently than those in adults after β -cat activation, needs to be complemented, as done in adults, by measuring transporters activity after ETA receptor blockade and β -cat activation. This information will help 1) to clarify the role of the interaction between β -cat signaling and ET-1 in regulating juvenile BBB transporters, and 2) to determine if ET-1 is the factor at the origine of differences observed between adult and juvenile rats. In addition, it is important to explore modulation of β -cat and ET-1 after GSK-3 β inhibition in juvenile brain, and to compare these aspects with those

observed in adults. Corresponding experiments are in progress. The obtained data will help to further determine the factor at the origin of the differences observed between ages.

To further verify if ET-1 is the factor at the origin of the observed differences, we compared the impact of ET-1 on BBB transporters between juvenile and adult rats. And this was the subject of the third objective of this thesis.

In conclusion, while developmental changes of functional P-gp have been reported previously, this study examined, in addition to P-gp, functional expression of Bcrp, oat3 and oatp1a4 at the BBB during brain maturation. Moreover, our findings provided novel insights on a differential regulation, after β -cat activation, of ABC and SLC efflux transporters at BBB in adults when compared to juveniles. These data are very important because 1) they point molecular targets, particularly GSK-3 β and ET-1 that can be used to enhance drugs bioavailability in the adult brain, and 2) they tend to show that not only BBB properties may be different in juveniles when compared to adults, but also the involved mechanisms are different, and this fact, if confirmed, has to be well considered in the aim of developing new drugs with enhanced efficacy in children's brain.

4 Comparative study of the impact of inflammation on BBB drug efflux transporters: Conclusions and perspectives

In the first part of this thesis, we highlighted changing properties of the BBB during ontogenesis, as well as a differential regulation of BBB drug efflux transporters after β -cat activation. We hypothesized that regulatory mechanisms activated in adult brain may be different from those in juvenile brain, and that the factor at the origin of differences may be the ET-1, a cytokine peptide. On another hand, there is now a wealth of evidence that age could have a significant effect on response to cytokines, which in turn could modulate BBB efflux transporters expression and activity. Basing on these several findings, it seemed important to evaluate potential age-related differences in functional expression of BBB efflux transporters during brain maturation in the context of brain neuroinflammation, especially under impact of ET-1. For this, using the generated *in vivo* models of acute cerebral inflammation mediated by ET-1, we evaluated the impact of inflammation on P-gp and bcrp activity with a comparison between juvenile and adult rats. Our results showed that transport activities of P-gp and bcrp are enhanced by ET-1 at the adult BBB, whereas no variation of P-gp transport activity and a tendency to

decrease in bcrp transport activity were seen at the juvenile BBB (Figures 4 and 5, Harati et al., 2012b). Thus, we conclude that, under inflammatory conditions, BBB drug efflux transporters are regulated differently in juvenile rats when compared to adults, and this may be due to:

- 1) Age-related differences in the BBB components between juveniles and adults, as shown in the descriptive BBB ontogenesis study.
- 2) Differences in neuro-inflammatory response related to GFAP basal levels. Indeed, there are increasing evidences showing that inflammation modulate drug efflux transporters expression and activity. For that, we hypothesis that the observed differences may be due to a differential inflammatory response triggered by ET-1. Thus, we quantified a panel of inflammatory molecules in rats brain treated with ET-1, and found that the panel of released cytokines and chemokines were different. However, when we increased ET-1 doses in juvenile in a way to obtain a similar inflammatory profil in both ages, regulation of BBB drug efflux transporters remains different. To further test this hypothesis, we quantified GFAP levels, a marker of glial activation observed during inflammation, in rats' brains treated with ET-1. We found that GFAP levels increase in both ages. However, basal GFAP levels were different, suggesting that differential regulation of drug efflux transporters observed between ages may be due in part to differences in GFAP activation.
- 3) Maturation differences in ET-1 receptors. Indeed, we established the profil of ETA, one of the two ET1 receptors, during brain maturation and found significant differences between juveniles and adults. This difference may cause differential regulation of BBB transporters, especially that previous work have shown that P-gp regulation may be different depending on the ET-1 receptor activated (Bauer et al., 2007; Hartz et al., 2004).
- 4) Differences in signaling pathway activated. Indeed, it is also important to investigate the role of signaling pathways known to modulate expression and activity of ABC transporters at the BBB level. These pathways, such as signaling cascade via , PXR, CAR, NF- κ B and AP-1 (Miller, 2010) or NOS and PKC (Bauer et al., 2007), may be activated differently in children compared to adults, and this depending on the ET-1 receptor implicated.

To summarize, our study gave evidence that P-gp and bcrp transporters at the BBB level are regulated differently under pathological conditions in juvenile brain when compared to adult. These findings emphasize one more time the importance of considering differential P-gp and bcrp transporter regulation mechanisms between juvenile and adult BBB.

As perspective to this work, further investigations are warranted to explore the several pists that may be at the origine of the observed differences. This will permit to understand how drug efflux transporters are regulated at the juvenile BBB. These findings will also help to further emphasize the importance of considering differential P-gp and Bcrp transport regulation mechanisms between adult and juvenile BBB in the context of neuroinflammation.

In conclusion, our study with its three main objectives was performed in the clinical perspective of developing new drugs with enhanced efficacy in children's CNS. We highlighted the importance of considering an age-related response of CNS to drugs, which needs caution in giving drugs to infants, and even more taking into account the specific properties of juvenile BBB during definition of therapeutic strategies designed to treat childhood brain diseases. These findings may open new therapeutic avenues to improve drug delivery into children's brain and this by targeting the children's brain-specific pathways modulating the activity of juveniles' drug efflux transporters.

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