The Role of Astrocyte-Derived Factors in the Regulation of Multidrug Resistance at the Developing Blood-Brain Barrier

by

Stephanie Baello

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Department of Physiology
University of Toronto

The Role of Astrocyte-Derived Factors in the Regulation of Multidrug Resistance at the Developing Blood-Brain Barrier

Stephanie Baello

Doctor of Philosophy

Department of Physiology
University of Toronto

2016

Abstract

P-glycoprotein (P-gp; encoded by *Abcb1*) at the blood-brain barrier (BBB) plays an important role in regulating the movement of exogenous and endogenous substrates into the developing brain. P-gp levels in brain endothelial cells (BECs), which form the capillaries of the BBB, increase dramatically in late gestation and early post-natal life. During this period, glial precursors differentiate into astrocytes and begin to ensheathe brain microvessels. However, little is known regarding the effect of astrocytes on *Abcb1*/P-gp at the developing BBB. This thesis investigated the effects of astrocyte-derived factors on regulating *Abcb1*/P-gp at the developing BBB.

In particular, the studies in this thesis demonstrated the upregulatory effect of transforming growth factor-beta1 (TGF-β1), a growth factor secreted by astrocytes in late gestation, on P-gp at the BBB. This effect was attenuated as gestation progressed. During this time in pregnancy, it is common for women to receive synthetic glucocorticoids (sGC). This thesis has demonstrated that sGC treatment matures the BBB as it increases P-gp and tight junction function. These BECs also display attenuated responsiveness to TGF-β1 stimulation, an effect similar to that seen in post-natal guinea pig BECs obtained following normal pregnancies.

By utilizing a co-culture model using guinea pig BECs and astrocytes from two distinct time-points in gestation, the studies in this thesis have demonstrated that astrocytes enhance levels of *Abcb1* mRNA and P-gp function in BECs via astrocyte-derived factors. However, post-natal astrocytes induce a more prominent increase in P-gp at the developing BBB compared to fetal astrocytes. This effect was correlated with higher levels of secreted proteins by post-natal astrocytes compared to fetal astrocytes. Thus, compromised astrocyte maturation may dysregulate P-gp function and expression at the BBB, which may contribute to the pathogenesis of neurodevelopmental disorders. This new knowledge will be critical in the development of future therapies to counteract these effects.

Acknowledgments

First and foremost, I would like to express my gratitude to my supervisor Dr. Stephen G. Matthews. He has been a constant source of encouragement, critique, wisdom and insight through my PhD studies. Under his guidance, I have excelled both professionally and personally. I am also very grateful to Dr. William Gibb for his insight and invaluable suggestions during our weekly lab meetings. As painful as it was to sit through our 3-hour meetings (sometimes 4), I owe much of the success of my PhD project to the discussions and all the head-scratching that took place during these meetings.

I would also like to thank the members of my supervisory graduate committee, Dr. Lee Adamson, Dr. Steffen-Sebastian Bolz and Dr. Martin Wojitowicz, whose insightful criticism and hard questions have guided my PhD project and strengthened my critical thinking skills.

To the members of the Matthews' lab, past and present, including: Majid, Mel, Mohsen, Vasilis, Alice, Enrrico, Sophie, Tam, Elena, the new guys and the new kids, I am truly thankful for your support and friendship. Thank you to Alice and Mohsen for our "fresh air" breaks and keeping a constant grin on my face; to Vasilis and Enrrico for their feedback and help 24/7. I would like to especially thank my mentor, Majid Iqbal, for taking me under his wing as a new masters student and for his continued support and input through my studies.

Finally, I would like to thank my parents and my sister for their unconditional love and encouragement through my academic journey. They have instilled in me the importance of a strong work ethic, to be persistent, and to follow my passion. To Mateusz Ziobrowski, thank you for standing by my side and keeping me balanced during this journey. To Sarah Montanaro, thank you for being a true friend through the ups and downs.

Table of Contents

Other Publications

1. Iqbal M, **Baello S**, Audette MC, Javam M, Gibb W, and Matthews SG. Multidrug Resistance in the Developing Blood-Brain Barrier: Interactions between Glucocorticoids and Cytokines. *Journal of Neuroendocrinology*, Submitted 2015.

2. Lam J*, **Baello S***, Iqbal M, Kelly LE, Javam M, Shannon PT, Matthews SG, Chitayat D, Koren G. Ontogeny of P-glycoprotein in the developing human blood brain barrier- Implications for toxicity in neonates. *Pediatric Research*, April 2015. **indicates co-first authorship*

List of Tables

List of Figures

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Abbreviations

ABC; ATP-binding cassette

ACM; astrocyte-conditioned medium

ALK; activin like kinase

ANOVA; analysis of variance

AP-1; activator protein-1

AQP4; aquaporin-4

ASD; autism spectrum disorder

ATP; adenosine triphosphate

BBB; blood-brain barrier

BECs; brain endothelial cells

BCRP; breast cancer resistance protein

DMEM; dulbecco's modified eagle medium

BMP; bone morphogenic protein

BVM; brain arteriovenous malformations

C/EBPβ; CCAAT/enhancer-binding protein beta

CCL2; chemokine (C-C motif) ligand 2

CNS; central nervous system

COX; cyclooxygenase

CSF; cerebrospinal fluid

DEX; dexamethasone

DNA; deoxyribonucleic acid

E; embryonic day

EAAT; excitatory amino acid transporters

EC; endothelial cell

ECM; extracellular matrix

ERK; extracellular-signal-regulated kinases

FITC; fluorescein isothiocyanate

FMRP; fragile X mental retardation protein

GABA; gamma-aminobutyricacid

GD; gestational day

GFAP; glial fibrillary acidic protein

GLAST; glutamate aspartate transporter

GLUT-1; glucose transporter-1

GM-CSF; granulocyte-macrophage colony-stimulating factor

GR; glucocorticoid receptor

GSH; glutathione

GW; gestational week

H; hour

HBSS; hank's balanced salt solution

HSE; heat shock element

IL; interleukin

INF; interferon

invMED1; inverted mediator-1 element

LAMs; leukocyte-adhesion molecules

LC-MS/MS; Liquid chromatography-tandem mass spectrometry

LPS; lipopolysaccharide

MAPK; mitogen-activated protein kinases

MeCP2; methyl CpG binding protein 2

MiRs; microRNAs

MMP; matrix metalloproteinases

MR; mineralocorticoid receptor

MRP; multidrug resistance protein

NBD; nucleotide binding domain

NES; nestin

NF-Y; nuclear factor-Y

NGS; normal goat serum

OAPs; orthogonal arrays of particles

P-gp; p-glycoprotein

PAI-1; plasminogen activator inhibitor-1

PAPs; peripheral astrocytic processes

PI3K; phosphoinositide 3-kinase

PND; post-natal day

RA; retinoic acid

RNA; ribonucleic acid

RT-PCR; real-time polymerase chain reaction

sGC; synthetic glucocorticoids

Shh; sonic hedgehog

siRNA; small interfering ribonucleic acid

SIS3; specific inhibitor of SMAD3

SODs; superoxide dismutases

Sp; specificity protein

SXR; steroid xenobiotic receptor

TEER; transendothelial electrical resistance

TCF/LEF; t-cell factor/lymphoid enhancer-binding factor

TGF-1β; transforming growth factor-β1

TGFBR; TGF-β receptor

TMD; transmembrane domain

TNF; tumor necrosis factor

TSP; thrombospondins

TSS; transcription start site

UCM; unconditioned media

UTR; untranslated region

VEGF; vascular endothelial growth factor

VEH; vehicle

VPL; verapamil

VWF; von willebrand factor

YB-1; y-box binding protein

ZO-1; zonula occludens-1

CHAPTER 1: INTRODUCTION

1.1 P-glycoprotein (P-gp)

1.1.1 History

P-glycoprotein (P-gp) is a membrane-transporter belonging to the ATP-binding cassette (ABC) superfamily of transporters, which comprises a large number of functionally diverse transmembrane proteins. P-gp was the first "multidrug resistant" protein to be identified in cancer cells and so was deemed multidrug resistance protein 1 (MDR1). It was initially discovered in 1976, by Juliano and Ling, as a 170-kilodalton (kDa) glycoprotein that conferred colchicine-resistance in cultured Chinese hamster ovary cells[1]. They named this protein "permeability-glycoprotein", commonly referred to

as P-gp.

1.1.2 Physiological Role

Since its discovery in cancer cells, P-gp was identified in many normal tissues, such as the surface of epithelial cells of the intestine, liver bile ductules and kidney proximal tubules[2-4]. Organs with specialized barriers, such as the blood-brain, blood-testes and blood-placental barriers were also found to highly express P-gp[5-7]. The tissue localization of P-gp suggests that the protein plays a physiological role in the protection of vital organs, such as the brain and testis, and in the secretion of metabolites and xenobiotics into bile, urine and the lumen of the gastrointestinal tract. Evidence for the role of P-gp in normal physiology is highlighted by P-gp-deficient mice, as they display an increased susceptibility to ivermectin toxicity at doses that do not harm normal mice.[8,9]

1.1.3 Protein Structure

P-gp is synthesized in the endoplasmic reticulum as a 150-kDa intermediate[10]. It is subsequently glycosylated in the Golgi apparatus before it is shuttled to the cell membrane. Human P-gp consists of 1276-1280 amino acids with a molecular mass of approximately 170 kDa. In the human, P-gp is encoded by a single gene *ABCB1* while rats and mice have two genes, *Abcb1a/Abcb1b.* The Matthews' lab, in collaboration with Moshe Syfe's lab, has demonstrated that guinea pig P-gp is encoded by a single gene, *Abcb1*, and shares approximately 87% homology with human *ABCB1*[11]. P-gp protein has

a duplicated structure, each half consisting of six transmembrane domains (TMD) and a nucleotide-binding domain (NBD) (Fig. 1-1)[12]. The two transmembrane regions are joined by a highly charged linker region.

P-gp is a post-translationally processed protein, yet the effects of these modifications on P-gp function are unclear. N-glycosylation occurs on 3 sites on the first extracellular loop[13,14]. Inhibiting glycosylation of P-gp leads to improper localization, folding or stability of P-gp protein [10,15-19]. Yet some studies show that there is no effect on P-gp function. P-gp can also be phosphorylated by protein kinase A and C at 4 serine residues in the highly charged linker region[20,21]. However, the effect of phosphorylation on P-gp function is unclear as P-gp mutants lacking all phosphorylation sites exhibit normal P-gp transport function[22,23].

P-gp transports a wide variety of structurally unrelated substances, varying in size from small organic cations and carbohydrates to polysaccharides and proteins[24]. Most of these substrates are generally relatively hydrophobic, weakly amphipathic, and often contain aromatic rings and a positively charged nitrogen atom[25]. P-gp substrates include a wide variety of exogenous compounds, such as chemotherapeutics (e.g. etoposide, doxorubicin, and vinblastine), cardiac glycosides (e.g. digoxin) and antiretroviral drugs (e.g. ritonavir)[24,26,27]. P-gp is also responsible for the efflux of endogenous compounds such as steroids (e.g cortisol), chemokines (the chemokine [C-C motif] ligand 2 [CCL2]) and cytokines, interleukin (IL)-4, IL-1β, IL-2, IL-6, interferon (IFN)-γ, granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α (Table 1-1)[28-34].

The exact mechanism by which P-gp effluxes its substrates is not well-defined. These substrates are thought to pass though a funnel-shaped structure formed by the two transmembrane regions of P-gp from the inner leaflet of the lipid bilayer[35]. This funnel contains multiple substrate binding sites, some of which are involved in the direct transport of substrates, while others are thought to be binding sites for allosteric modulation of P-gp function[36]. The transport of these substrates is dependent on ATP binding to NBD regions, as its binding causes a conformational change in P-gp protein. This induces an "outward-facing" conformation in which there is low substrate-binding affinity and exposes the substrate-binding site to the extracellular environment[37]. Subsequent hydrolysis of ATP is assumed to "reset" the transporter into an "inward-facing" conformation, with the high-substrate binding sites exposed to the cytoplasmic leaflet. However, the exact mechanism of how this energy is used to drive transport is unclear. Unlike most ATP-driven transporters, P-gp displays constitutive ATPase activity, even in the absence of substrate[38]. Upon substrate-binding, studies indicate that ATPase activity is upregulated 3-4 fold. Additionally, it is uncertain whether hydrolysis of one or two ATP molecules is required for transport of one substrate.

1.1.4 Genomic and Epigenetic Regulation of Human *ABCB1*

The human *ABCB1* promoter lacks the classical TATA-box upstream of the transcription start site (TSS) that majority of genes transcribed by RNA polymerase II contain. Constitutive expression of *Abcb1* relies on the "GC" elements (GC-box) and the inverted CCAAT sequence (Y-box), which are found in the majority of TATA-less promoters[39,40]. Mutation or removal of these promoter elements leads to loss of

transcription, indicating that they are needed for constitutive expression of *ABCB1*[41]. Each of these elements can interact with different transcription factor families, as shown in studies with epithelial and cancer cell lines. It has been proposed that transcription is initiated by NF-Y (nuclear factor Y) binding to the Y-box[42] (Fig. 1-2). Assembly of RNA-polymerase II complex to the Y-box is facilitated by specificity-protein (Sp)-1 and -3 transcription factors, which bind to the GC-box in the *ABCB1* promoter[43]. Two GC-rich regions (GC-box) have been found in the human *ABCB1* promoter. Recent studies have shown that Sp3 binds preferentially to the GC-box on the *ABCB1* promoter in brain endothelium[44]. In contrast, Sp1 preferentially associates with the GC-box in the colon carcinoma cell line, Caco-2. Both NF-Y and Sp transcription factors are essential for constitutive expression of *ABCB1*.

Other promoter elements found in the *ABCB1* promoter include a p53 element, an inverted mediator-1 element (invMED1), an activator protein-1 (AP-1) element, a heat shock element (HSE), and a steroid xenobiotic receptor (SXR) element[45-48]. Consequently, several transcription factors are associated with increased expression levels of *ABCB1* (Fig. 1-2). These include heat-shock transcription factor 1 (HSF-1), AP-1, CCAAT/enhancer-binding protein beta (C/EBPβ or NF-IL-6, nuclear factor for IL-6 expression), early growth response protein 1 (EGR-1), and Y-box binding protein 1 (YB-1). Adding to this complexity, the regulation of *Abcb1* expression by these transcription factors is species specific. For example, the AP-1 site in the mouse has been shown to repress transcription of *Abcb1a/b*, while in the hamster and human the AP-1-binding elements were shown to activate transcription of *Abcb1*[49,50].

The level of *ABCB1* mRNA expression can also be controlled through epigenetic

mechanisms. In particular, methylation and histone acetylation have been shown to regulate *ABCB1* mRNA expression in various cancer cell lines and cancer tissues. Demethylation of the *ABCB1* promoter was shown to be associated with increased *ABCB1* mRNA expression in cancer cell lines, including promyelotic leukemia cell line HL-60 and breast cancer cell line MCF-7[51,52]. Similar evidence was also found in human T-cell leukemia cells, acute myelogenous leukemia clinical samples, and adult acute lymphocytic leukemias compared to non-malignant tissue[53,54]. Additionally, histone acetylation can also modulate *ABCB1* mRNA expression. The use of histone deactylase C inhibitor, trichostatin, was shown to regulate *ABCB1* mRNA levels in human colon carcinoma cells, SW620, indicating a relationship between increased histone acetylation and increased levels of *ABCB1* mRNA[48]. Further evidence for this stems from MES-SA cells, which were found to have a 20-fold increase in acetylated H3 in the nucleosomes within the 968-bp region of the *ABCB1* promoter[55]. However, very little is known about how epigenetic processes regulate *ABCB1* expression in normal tissues.

MicroRNAs (miRs) have also been shown to target *ABCB1*. MiRs are small non-coding RNA molecules of 20-24 nucleotides and work in concert with transcriptional and epigenetic gene expression. The mechanism by which miRs regulate translation of proteins is by binding to 3'-untranslated region (UTR) of target mRNA to either degrade or inhibit translational process of target mRNA. The majority of studies investigating the interaction of miRs and *ABCB1* have been conducted using cancer cell lines. For example, in doxorubicin-resistant human breast cancer cells (MCF-7/DOX), in which P-gp expression is upregulated, the level of miR-451 was found to be almost undetectable. When these cells were transfected with miR-451, doxorubicin sensitivity was re-

established in MCF-7/DOX cells[56]. However, more than one miR is able to regulate *ABCB1,* as similar results were associated with miR-27a, miR-145, miR-298 and miR-1253 in other cancer cell lines[57-59]. MiRs can also indirectly modulate *Abcb1* expression by binding to mRNA encoding for transcription factors that regulate *ABCB1*. For instance, miR-137 was shown to bind to transcription factor, YB-1 in MCF-7 cells. This was found to prevent YB-1 interactions with the promoter region of *ABCB1*, resulting in downregulation of *ABCB1* compared to untransfected MCF-7/DOX cells[60]. Adding to the complexity of miRs, some are capable of increasing P-gp expression, as is the case with miR-138 and miR-296[61,62]. However, the mechanism by which these miRs interact with *ABCB1* is currently unknown. It is also unclear if these miRs are expressed in normal tissues.

1.2 Blood-Brain Barrier

1.2.1 Overview

The blood-brain barrier (BBB), composed primarily of brain endothelial cells (BECs), is an interphase that regulates movement of endogenous and exogenous molecules between blood and brain extracellular fluid compartments. BECs are extremely thin cells, measuring approximately one quarter of a micron, which is 40% less thick than endothelial cells (ECs) found in muscle[63]. Moreover, unlike the peripheral vasculature, BECs express specialized tight junctions that strictly limit movement of small hydrophilic molecules and even small ions within the paracellular space. BECs also have low rates of transcellular transport, such as endocytosis, and lack fenestrations. Low expression of

leukocyte-adhesion molecules (LAMs) prevents transcytosis of immune cells into the brain parenchyma. BECs also possess increased number of mitochondria compared to peripheral ECs, in order to support the activity of metabolizing enzymes and transporters. ABC-transporters are located on the luminal surface of BECs. Of these transporters, P-glycoprotein (P-gp) is the highest expressed at the BBB[64-66]. BECs interact dynamically with surrounding glial cells (such as microglia and astrocytes), pericytes, and neurons[67] (Fig. 1-3). The basement membrane surrounding BECs is secreted by BECs themselves, pericytes and astrocytes. However, BECs in particular interact intimately with astrocytes as astrocytic-end feet ensheathe 99% of the abluminal surface of brain capillaries[68].

1.2.2 Development and Maturation of the BBB

The development of the BBB is categorized into three stages: angiogenesis, differentiation and maturation. The central nervous system (CNS) acquires its vasculature solely by the process of angiogenesis, as blood vessels arise from the primary vascular plexus, forming the perineural vascular plexus[69]. The perineural vascular plexus encases the surface of the developing brain[70]. At embryonic day (E)11 in the mouse[71,72] and around gestational week (GW)8 in humans[73-75], vascular sprouts of the perineural vascular plexus invade the CNS parenchyma. This process is initiated by neural precursor cells and radial glial cells, which produce a vascular endothelial growth factor (VEGF) gradient, guiding vascular sprouts from the pial surface of the brain to the ependymal cell layer[76,77].

As these nascent vessels invade the CNS, they differentiate and begin to exhibit

characteristics of a primitive BBB, expressing P-gp and tight junctions proteins, claudin-5 and claudin-3, and are capable of restricting the movement of blood-derived proteins in the developing CNS[78]. These BECs also lose vesicles and fenestrations. Using ultrastructural methods, it has been shown that tight junctions present very early in brain development resemble tight junctions in the adult. This was demonstrated as early as GW8 in human fetuses (term is 40 weeks), at 13 days gestation in fetal mice (term is 21 days) and post-natal day (PND) 5 in Monodelphis opossums, when blood vessels first appear in the developing neocortex[79-82]. During this stage, tight junctions prevent the movement of serum proteins into the developing brain. Previous studies that concluded leakiness of the developing BBB to serum proteins were shown to have used large injection volumes or concentrations of tracer molecules, such as Evan's Blue Dye[83,84]. Tight junction function has been shown to increase as gestation progresses and continues into post-natal life. In the developing rat brain, transendothelial electrical resistance (TEER), a measure of tight junction function, increased from 310 $\Omega \bullet cm^2$ at E17 to 1462 $\Omega \bullet cm^2$ at PND7[85]. Other aspects of the BBB, such as transporter and enzymatic activity, also display stage specific properties that vary with gestation. Generally, BBB specific properties are enhanced in late gestation and post-natal life, a stage termed BBB maturation. For example, the activity of gamma-glutamyl-transferase, a BBB-specific enzyme, continuously increases to birth and plateaus in post-natal life[86]. The level of *Abcb1*/P-gp at the BBB also displays a similar pattern through development, which is further discussed in the following section.

1.2.3 Expression and Function of P-gp at the BBB

In the mature BBB, P-gp is mainly localized to the luminal surface (blood-facing side) of BECs (Fig. 1-4). This allows P-gp to efflux blood-derived substances that move into BECs back into the blood circulation, as is the case for xenobiotics, drugs and toxins. Additionally, P-gp can also efflux brain-derived substances that are actively transported or diffuse into BECs. Recent studies indicate that this is the case for β-amyloid, a molecule associated with the cognitive decline and neuroinflammation in Alzheimer's disease[87]. P-gp has also been implicated in trafficking of immune cells into the brain parenchyma by regulating BECs secretion of chemokine (C-C motif) ligand 2 (CCL2)[88].

P-gp also plays a vital role in the protection of the developing brain. Specifically, P-gp at the BBB becomes essential in late gestation as levels of P-gp at the placenta dramatically decrease, which has been shown in mice, guinea pigs and humans (mouse placenta; Fig. 1-5) [89-91]. Our research group has shown, in mice and guinea pigs, that in early gestation brain microvessels express very low amounts of P-gp that dramatically increases with advancing gestation (guinea pig microvessels; Fig. 1-6) [92,93]. The presence of P-gp in the developing brain microvasculature is readily detectable early in gestation at the onset of brain angiogenesis[82]. However, immunohistochemical studies in brains of early human fetuses (approximately GW8) demonstrate that P-gp is diffusely expressed in the cytoplasm, indicating that early in gestation P-gp may not serve a role in brain protection[94]. As gestation progresses, levels of P-gp dramatically increase in late gestation and post-natal life, and its localization becomes predominantly on the luminal side of BECs. In collaboration with Dr. Koren, our lab has recently demonstrated that P-

gp protein levels in the brain reach adult levels by post-natal age of 3-6 months in humans[95].

A number of studies have depicted the functional importance of P-gp at the developing BBB. Following administration of P-gp substrate, digoxin, our laboratory observed a greater digoxin brain accumulation at E15.5 in mice compared to E18.5, reflecting an increase in P-gp activity as gestation progressed[93]. This is of clinical significance as drug use during pregnancy is a common occurrence in developed countries, with approximately 30-93% of pregnant women, depending on the country[96]. Prescribed drugs are used to treat chronic diseases, such as diabetes, systemic lupus erythematosus, depression and epilepsy. Drugs are also used to ease the symptoms associated with pregnancy, such as vomiting and nausea, and to aid in fighting infections[97]. However, many of these prescribed drugs are potentially teratogenic and can lead to fetal loss, congenital malformations or have effects on development later in childhood[98]. Moreover, numerous drugs are also substrates of P-gp and so risk of fetal exposure to these drugs are dependent on P-gp function at the placenta and BBB. A recent study among the mothers of children registered by EUROCAT Northern Netherlands (NNL), demonstrated that approximately 10% of drugs taken by these women during pregnancy were indeed P-gp substrates[99]. This study also showed a correlation between higher user rate of these drugs and increased rate of fetal anomalies. Thus, due to declining levels of P-gp at the placenta as gestation progresses, the importance of BBB P-gp becomes essential in protecting the developing brain.

1.3 Astrocytes

1.3.1 Overview

Astrocytes are the most abundant cell type in the mammalian brain. Among their many functions, they are essential for many metabolic processes in the brain such as potassium buffering, release of gliotransmitters, release of glutamate by calcium signaling, and control of brain pH[100,101]. Astrocytes are also responsible for the metabolization of dopamine and other substrates by monoamine oxidases, uptake of glutamate and gamma-aminobutyricacid (GABA) by specific transporters and production of antioxidant compounds like, glutathione (GSH) and enzymes such as superoxide dismutases (SODs)[102]. Recent evidence indicates that astrocytes also regulate vascular perfusion to match neuronal activity, a process termed neurovascular coupling[103,104]. Additionally, BBB characteristics of the adult brain are reliant on the influence of astrocytes through astrocyte-derived factors, such as retinoic acid, transforming growth factor (TGF)-β and sonic hedgehog (shh)[105,106]. Astrocyte polarity and proper ensheathment of the brain microvasculature by astrocytic endfeet also contribute to the maintenance of the BBB phenotype[107]. However, far less is known about the role of astrocytes in regulation of the BBB properties, such as P-gp function and expression in the developing brain.

1.3.2 Astrocyte Development

Astrocytes differentiate from neural stem cells originating from the neuroectoderm at E18 through to the first week of life in mice[108]. In contrast, astrocyte

differentiation is entirely an *in utero* process commencing at gestational day (GD) 45 and GW14 in the guinea pig and human, respectively[109,110]. Recent evidence suggests that astrocyte differentiation is regulated by early differentiated neurons or late-stage neural stem cells that have been shown to secrete signaling molecules such as, ciliary neurotrophic factor, cardiotrophin-1 and leukemia inhibitory factor[111,112]. These molecules activate important signaling pathways, such as the janus kinase (JAK)/STAT[113], bone morphogenic protein (BMP)-SMAD[114,115], notch[116] and nuclear factor IA (NFIA)[117], that initiate astrocyte differentiation in neural stem cells. These signals also induce epigenetic changes that open chromatin regions, allowing transcription factor binding to astroglial gene promoters glial fibrillary acidic protein (GFAP), S-100β and glutamate aspartate transporter (GLAST)[114,115,117,118].

Subsequent to astrocyte differentiation is astrocyte maturation, whereby differentiated astrocytes form endfeet that extensively ensheathe microvessels of the BBB. This process occurs entirely during post-natal development in mice and rats starting at PND14[119,120]. In humans and guinea pigs, astrocytes begin to mature at approximately GW23 and GD55, respectively[109,110]. One of the hallmarks of astrocyte maturation is the formation of peripheral astrocyte process (PAPs). PAPs are ultra fine processes that allow insertion of various membrane proteins such as ion channels, ligand receptors and transporters. These include astroglial genes, aquaporin-4 (AQP4), GLAST[121,122], connexin 30 and 43[123] and inwardly rectifying potassium channel, Kir 4.1[124,125], which were shown to be induced at PND21-28 in mice. However, the mechanisms underlying astrocyte maturation are unclear. Evidence suggests that this process is driven by surrounding neurons, as astrocytes express many neurotransmitter receptors, allowing

them to respond to neural signals[126,127]. Additionally, co-culture studies indicate that neurons induce the expression of many of these genes in astrocytes[128-131].

1.3.3 Astrocytes & BBB

In the mature BBB, 99% of the brain microvasculature is ensheathed by astrocytic-endfeet, which are in a poised position to modulate properties of the BBB[132]. As such, astrocyte-BEC interaction regulates vascular quiescence, angiogenesis, EC morphology and barrier conditions at the adult BBB. Conversely, less is known about how astrocytes regulate the developing BBB[68,105,133-135]. During fetal development, astrocyte maturation, a process by which astrocytic processes or endfeet ensheathe the brain endothelium, is temporally correlated with BBB maturation. This process is species specific. In rats and mice, astrocyte maturation occurs entirely post-natally, while it is mostly an *in utero* process in humans and guinea pigs. The upregulation in P-gp expression at the developing BBB follows this species-specific temporal pattern, providing strong evidence of an astrocyte-induced regulation of P-gp expression during development[92,93,95].

The first evidence demonstrating that BECs did not have intrinsic BBB properties was shown by an elegant study conducted by Stewart and Wiley[136]. In this study, avascular tissue from 3-day-old quail brain was transplanted it into the coelomic cavity of chick embryos. They found that chick endothelial cells that vascularized the quail brain grafts form a competent BBB. In contrast, when avascular embryonic quail coelomic grafts are transplanted into embryonic chick brain, the chick endothelial cells that invade

the mesenchymal tissue grafts form leaky capillaries and venules. Following this study, in 1987, the first clear demonstrations of the BBB-inducing properties of astrocytes were provided by two independent groups, Raff and Brightman. These two groups utilized *in vivo* grafting and *in vitro* cell culture models, derived from adult animal tissues, to demonstrate that morphological and functional BBB-specific characteristics are dependent on perivascular astrocytes[137,138]. Further corroborating these studies, Rubin et al. demonstrated that isolated BECs lose their barrier capacity as measured by transendothelial electrical resistant (TEER) and tracer molecule assays[139]. However, the presence of astrocytes or astrocyte-conditioned (ACM) was able to rescue these properties in BECs[106,107,140]. More recent studies have shown that rat astrocytes can induce BBB-specific properties, such as P-gp and tight junction expression, in human umbilical vein endothelial cells or non-neural endothelial cells in co-culture[141]. Similar results were found using an *in vitro* co-culture model utilizing immortalized human endothelial cells (ECV 304) and by rat astrocytes[142]. These results demonstrate that BBB-specific properties are not an innate property of BECs, but are induced by surrounding perivascular astrocytes.

1.3.4 Astrocyte-Derived Factors

Since co-culturing astrocytes and BECs in a non-contact manner induces BBB characteristics and ACM can mimic these effects, it is plausible that soluble astrocyte-derived factor(s) are implicated in this interaction. The multifaceted levels of communication and signaling events that occur between astrocytes and BECs makes it a difficult task to identify these factors. Many factors have been implicated in the

regulation P-gp function and expression in the adult BBB, epithelial tissues and cancer cell lines, some of which are secreted by astrocytes during development. These molecules have been shown to activate signaling pathways including the sonic hedgehog (Shh), Wnt/β-catenin canonical pathway, retinoic acid (RA) and TGF-β superfamily[106,143-149].

RA has been proposed as an important molecule in the early induction of BBB characteristics in the developing vasculature[149,150]. Studies using human fetal brains indicate that radial glial cells express the final enzyme involved in retinoic acid biosynthesis, while endothelial cells express RA-receptor β (RARβ)[149]. Moreover, the importance of RA signaling in BBB development was highlighted by treatment of mice with RAR antagonist from E10.5 to E16.5 of gestation. This treatment led to downregulation of several BBB characteristics[149]. These results were corroborated by *in vitro* studies which demonstrated that activation of RARβ in mouse BECs upregulated TEER, and increased expression levels of VE-cadherin, P-gp and ZO-1, while permeability factor, VEGF, was reduced[107].

The Shh signaling cascade is important in both the developing and adult CNS by regulating neuronal guidance and brain angiogenesis[151]. Astrocytes secrete Shh and BECs express the hedgehog (Hh) receptor, Patched-1, the signal transducer Smoothened (Smo), and transcription factors of the Gli family[107]. Activation of the Shh pathway upregulates TEER and expression of junctional proteins in human primary BECs. Moreover, blocking the Shh pathway *in vivo* disrupted the BBB, indicated by increased brain accumulation of serum proteins[106]. Mice lacking Smo also mimicked this phenotype[107]. In cancer cells, activation of the Shh pathway is correlated with increased function and expression of P-

gp and BCRP[148]. However, much less is known about how astrocyte-derived Shh affects BBB-properties in the developing brain.

The Wnt/β-catenin canonical pathway has also emerged as a prominent signaling pathway important in promoting angiogenesis and inducing hallmark characteristics of the BBB, including the expression and function of P-gp. The *ABCB1* promoter contains binding sites for T-cell factor/lymphoid enhancer-binding factor (TCF/LEF), the downstream transcription factor activated by Wnt/β-catenin pathway[152]. Intestinal expression of the human *ABCB1* and mouse *Abcb1a* genes have been associated with nuclear β-catenin accumulation. There is also evidence for the importance of Wnt/β-catenin pathway activation in regulating P-gp and other ABC transporter levels in the adult BBB. Activation of β-catenin signaling, through inhibition of glycogen synthase kinase-3 (GSK-3), was shown to upregulate levels of *ABCB1* mRNA and P-gp function in the human brain endothelial cell line hMEC/D3, as well as in adult rat BECs[153]. Expression of other ABC transporters at the BBB are also regulated by Wnt/β-catenin, as expression of *ABCC4* (encodes MRP4 protein) and *ABCG2* (encodes BCRP protein) were also upregulated in these cells. However, there is presently no evidence for the role of Wnt signaling in the development of the BBB in fetal and post-natal life.

Another major regulatory pathway that has been implicated in BBB development is TGF-β1. TGF-β1 is a multifunctional growth factor involved in many cellular processes, such as growth and differentiation, during embryogenesis[154]. In the CNS, TGF-β1 is secreted by astrocytes and has been shown to regulate BBB characteristics in BECs[146]. It was shown to attenuate leukocyte transmigration across adult mouse BECs by downregulating BECs expression of leukocyte adhesion molecules (LAMs)[155]. Moreover,

TGF-β1 was shown to enhance tight junction and P-gp function in mono-cultured adult rat BECs[146]. However, the role of astrocyte-derived TGF-β1 in protection and immunomodulation of the CNS remains controversial. Astrocytic expression of TGF-β1 is increased in neurodegenerative disorders such as, Multiple Sclerosis, Alzheimer's disease, stroke, tumors and neuro-AIDS[156,157]. Evidence also indicates that TGF-β1 has a role in the aberrant transformation of astrocytes in epilepsy[158]. Its highly pleiotropic nature may be due to the numerous signaling pathways activated, which depends upon which TGF-β receptors are expressed. For example, it is well established that TGF-β1 can have opposite effects on the angiogenic state of ECs[159]. ECs express two isoforms of the TGF-β1 type I receptor, ALK1 and ALK5. Activation of the ALK1 pathway stimulates proliferation and migration of ECs, while the ALK5 pathway facilitates the opposite. Thus, the effect of astrocyte-derived TGF-β1 on the adult BBB is unclear and nothing is known about how it modulates characteristics of the developing BBB.

1.3.5 Astrocyte Polarity and Extracellular Matrix

The extracellular space between BECs and astrocytes contains components of extracellular matrix (ECM) and matrix adhesion receptors required for appropriate interaction between cells[132]. Physical interaction between these cells is essential in regulation of the adult BBB. This is highlighted by pathological conditions such as, stroke and multiple sclerosis, in which BBB disruption is associated with reduction in astrocytic endfeet coverage of the brain microvasculature[160-163]. Additionally, tight junction and BBB-transporter expression is increased in BECs co-cultured with astrocytes in contact compared to those co-cultured in a non-contact manner[164-166].

Evidence suggests that the mechanism by which this occurs is through maintenance of astrocyte polarity and proper composition of ECM.

The development of astrocyte polarity and formation of astrocyte endfeet is essential in regulating tight junction function at the adult BBB. The astrocyte endfeet membrane contains numerous square arrays or intramembranous orthogonal arrays of particles (OAPs), containing membrane proteins such as AQP4, Kir4.1 and connexins 30 and 43[167]. The membrane structure of astroglial endfeet is maintained by the dystrophin–dystroglycan complex, which is a molecular array of proteins in muscle and brain cells (Fig. 1-7) [168]. Interestingly, the adaptor proteins of the dystrophin-dystroglycan complex, which are co-located with these membrane proteins, have also been implicated in BBB regulation. Mice lacking these scaffolding proteins demonstrate dysregulated astrocyte polarity, attachment of astrocyte endfeet to brain endothelium, decreased expression of AQP4 and leaky BBB[169,170]. Additionally, astrocyte endfeet contain connexin 30 and 43, which are gap junction proteins[123,171]. These proteins also appear to play a role in maintaining astrocyte polarity, as connexin30/43 knockout animals display loss of astroglial AQP4 and dystroglycan and consequently have permeable BBB[134].

The structure and composition of the ECM surrounding astrocytes and BECs during CNS development is critical for interaction between these cells. Two basement membranes separate the endothelium from astrocytes. The endothelial basement membrane is composed of fibronectin, collagen type IV, perlecan, and laminin α4 and α 5 chains, while the parenchymal or astroglial basement membrane contains fibronectin, agrin and laminin α1 and α2 chains[85,172,173]. The role of agrin in BBB maturation is of particular interest, as knockout animals display a compromised BBB[174]. However, the

mechanism by which agrin mediates this effect is unclear. Evidence suggests that agrin influences astrocyte polarity, as loss of agrin results in redistribution of AQP4 and disappearance of OAPs[175]. Alternatively, agrins may directly influence composition of tight junctions. A correlative study using microvessels extracted from human glioblastoma demonstrated that microvessels with the least agrin expression also had decreased expression of tight junction proteins[107].

Astrocytes also secrete proteins involved in ECM regulation, which are implicated in BBB induction. Astrocytes secrete thrombospondins (TSP), matrix proteins that mediate cell-cell and cell-matrix interactions by binding with other ECM proteins and with an array of membrane receptors and cytokines[176]. In particular, astrocyte-derived TSP-1 and 2, have well-established roles in supporting synapse formation during brain development[177,178]. With respect to the BBB, these astrocyte-secreted TSPs have been shown to inhibit EC proliferation and have been suggested to promote vascular maturation[179]. The mechanism by which TSPs facilitate the anti-angiogenic state of BECs may be through regulation of matrix metalloproteinases (MMPs), as TSP-2 deficient animals display increased levels of MMPs and dysregulated ECM remodeling[180].

In conclusion, it is clear that physical contact between astrocytes and BECs is essential in maintaining proper function of tight junctions at the adult BBB. Evidence suggests that astrocyte polarity and ECM components play significant roles in this regulation. However, very little is known about how these components regulate the developing BBB and how these factors may effect other BBB-specific characteristics, such as P-gp function and expression.

1.3.6 Astrocytes in Neurodevelopmental Disorders

Neurodevelopmental disorders encompass a wide range of pathologies associated with impaired growth and development of the CNS. Recent studies have implicated astrocytes in the pathogenesis of these disorders. In particular, disruption in astrocyte development may contribute to the pathogenesis of autism spectrum disorder (ASD), which represents a major category of neurodevelopmental disorders. Using post-mortem brain samples from autistic patients, immunohistochemical studies have shown dysregulation in expression of astrocyte markers. These findings include increased GFAP in cerebellar cortex, increased excitatory amino acid transporters (EAAT)1/2 in cerebellum, increased connexin 43 in superior frontal cortex and decreased AQP4 in cerebellum[181-184]. Moreover, mutations in astrocyte potassium channel, Kir4.1, have been found in a subset of autistic patients[184]. Recent studies have also highlighted the role of astrocytes in Rett Syndrome and Fragile X syndrome, both of which resemble typical autistic types. Due to monogenic nature of these neurodevelopmental disorders, mouse models have been generated and shown to display the cognitive and behavioral aspects of these disorders[185-187].

Rett Syndrome is caused by a loss-of-function mutation in the gene encoding methyl CpG binding protein 2 (MeCP2)[188]. Co-culture studies using astrocytes from $MeCP2^{-/-}$ mice show that these astrocytes do not support growth of normal neurons[189]. ACM from these astrocytes mimicked these effects, indicating that a soluble factor is responsible for mediating these effects. Moreover, loss of MeCP2 has been shown to change expression of several astroglial genes, including Kir4.1, AQP4 and GFAP[190]. Re-

expression of MeCP2 in astrocytes, via cre-loxP system, improves behavioral and cognitive function of MeCP2$^{-/-}$ mice[191]. Similar studies have been performed using Fragile X Mental Retardation Protein (FMRP)$^{-/-}$ mice, a model of Fragile X Syndrome, which is the most common inherited form of mental retardation. Astrocytes from FMRP$^{-/-}$ mice have been shown to induce developmental delays in normal hippocampal neurons, indicated by abnormal dendrite maturation and synaptic protein expression[192,193]. This was shown to be mediated by increased neuotrophin-3 secretion from FMRP$^{-/-}$ astrocytes. Additionally, dysregulated levels of genes strongly induced during maturational process of astrocytes, such as glutamate transporter GLAST, is reduced in brains of FMRP$^{-/-}$ mice[194,195].

Alterations in astrocyte phenotype have also been associated with adult-onset psychiatric disease, such as depression. Reduction in GFAP levels in the prefrontal cortex and cortiolimbic areas were found in a rat model of depression[196]. Post-mortem samples of human patients with depression showed decreases in glial density in the amygdala[197]. Moreover, coverage of blood vessels by astrocytic endfeet markers, AQP4 and GFAP, was reduced in post-mortem samples of patients diagnosed with Major Depressive Disorder[198]. Dysregulated astrocyte phenotype has also been demonstrated in sporadic Amyotrophic Lateral Sclerosis. Post-mortem brain samples showed loss of up to 95% of astroglial EAAT2 expression, depending on the brain region analyzed[199].

In summary, it is clear that the maturation of astrocytes, as indicated by alterations in astrocyte markers, is disrupted in neurodevelopmental disorders, such as Rett Syndrome and Fragile X Syndrome. Evidence from *in vitro* and *in vivo* studies demonstrate astrocytes may contribute to the pathogenesis of these disorders by stunting

the growth and development of surrounding neurons. However, currently nothing is known about the effect of disrupted astrocyte phenotype on P-gp function or expression at the developing BBB.

1.4 Transforming Growth Factor-β1

1.4.1 Overview

TGF-β1 is a member of the TGF-β superfamily, comprised of over 30 members including activins, nodals, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs). TGF-β1 was first discovered in 1981 in hepatic tissue and named due to its presence in transformed fibroblasts[200]. Generation of mice lacking TGF-β1 demonstrated that it is essential in vasculogenesis in embryogenesis. Recent evidence suggests its importance in the formation and maturation of the CNS. As such, altered TGF-β1 levels are implicated in various neurodevelopmental disorders.

1.4.2 TGF-β1 Synthesis and Bioavailability

TGF-β1 is synthesized as a large precursor molecule containing a propeptide on its N-terminal, called latency associated peptide (LAP) (Fig. 1-8). Intracellular furin cleaves the propeptide into C-terminal pro-region, LAP and N-terminal peptide, mature TGF-β1 (25 kDa)[201]. However, LAP remains non-covalently bonded to mature TGF-β1 to form a complex known as small latent complex (SLC). Still inside the cell, SLC will form a larger latent binding complex (LLC) with latent TGF-β binding protein (LTBP), which is then secreted from the cell[202]. This form of TGF-β1 cannot bind to its receptors,

so it is deemed latent. The majority of secreted LLCs bind to the extracellular matrix via LTBP[203]. The precise multistep activation of TGF-β1 has yet to be elucidated but requires the dissociation of TGF-β1 from its latent complex. A number of different processes have been proposed to activate latent TGF-β, including heat, acidic pH, reactive oxygen species, various proteases and thrombospondin-1[204-206].

1.4.3 TGF-β1 Signaling

Typically, TGF-β1 binds to TGF-β receptor type 2 (TGFBR2), which phosphorylates and recruits a TGF-β receptor type 1 (TGFBR1) that is responsible for eliciting signal transduction[207]. These receptors are glycoproteins and belong to a class of transmembrane serine/threonine kinase receptors. Phosphorylation of the type 1 receptor occurs at a highly conserved region known as the GS domain, where there are clusters of serine and threonine residues. Endothelial cells express two TGFBR1 isoforms: ALK5, which is expressed in a variety of cell types and ALK1, which is expressed exclusively in endothelial cells[159]. These type 1 receptors phosphorylate SMAD2/3 and SMAD1/5 respectively, which in turn form a complex with SMAD4 and translocate to the nucleus to effect gene transcription [208,209]. There is a co-dependency between these TGFBR1 isoforms as membrane-bound ALK5 is required for ALK1 signaling to occur[210].

In addition, there are transmembrane auxiliary receptors (type III receptors) of TGF-β signaling, betaglycan and endoglin, which are modulators of cellular responsiveness to TGF-β1. These receptors do not participate directly in signaling function but are involved in receptor complex formation and modulate ligand access to

receptors. Two isoforms exist, long (L) and short (S), which differ in the length of the intracellular domain, tissue distribution and degree of phosphorylation[211]. L-Endoglin is highly expressed by endothelial cells and has been shown to potentiate ALK1 signaling[212]. The ALK1/endoglin route has also been shown to inhibit TGF-β1/ALK5/Smad2,3 in endothelial cells[213-215]. Similarly, betaglycan regulates cellular responsiveness to TGF-β1. It has been shown to dampen cellular responsiveness to TGF-β1 by disrupting the complex formed by TGFBR2 and TGFBR1[216,217].

Canonical TGF-β signaling occurs via SMAD proteins, latent cytoplasmic transcription factors that become directly activated by serine phosphorylation, described above. There are three groups of SMAD proteins: receptor (R)-SMADs, common (Co)-SMAD, and inhibitory (I)-SMADs. R-SMADs (SMAD1-3, 5, 8) are those that are receptor regulated and become directly phosphorylated by type 1 receptors. I-SMADs (SMAD6, 7) are inhibitory SMADs that antagonize TGF-β/SMAD signaling via binding to TGFBR1 and preventing R-SMAD phosphorylation. Transcription of these I-SMADs is increased upon activation of TGF-β signaling and acts as a negative auto-regulatory feedback system. Lastly, co-SMAD or common SMAD is SMAD4, which forms a complex with SMAD 2/3 or SMAD 1/5. These complexes accumulate in the nucleus, where they regulate gene expression[218]. It is also well established that TGF-β1 can exert its biological effects through activation of SMAD-independent pathways, including the Notch, Wnt, Akt, mitogen-activated protein kinase (MAPK), and hippo signaling pathways[219].

1.4.4 Role of TGF-β1 in Vascular and CNS Development

The phenotypes of TGF-β1 knockout mice or mutations of the TGF-β1 pathway support the role of TGF-β1 in the development and maintenance of vasculature in fetal life. Transgenic mice lacking TGF-β1 die by midgestation due to deficient vasculogenesis[220]. Targeted deletion of *alk1, alk5, TGFβR2, endoglin* or *smad 5* are embryonic lethal, as a result of cardiovascular defects[221]. In humans, heterozygous mutations of either *endoglin* or *alk1* cause hereditary hemorrhagic telangiectasia (HHT)-1 or HHT-2[222]. Both are characterized by vascular anomalies such as enlarged vessels, edema, and arterio-venous malformations, which lead to pulmonary, liver and neurological problems.

Recent evidence suggests that glial-cell derived TGF-β1 has an essential role in the structure and function of developing synapses. In the periphery, Schwannn cells have been shown to secrete TGF-β1, which plays a critical role in the induction of synaptogenesis by regulating clusters of acetylcholine receptors at the neuromuscular junction[223]. The role of TGF-β1 as a mediator of the synaptogenic effects of glial cells in the CNS was confirmed by the generation of transgenic mice overexpressing TGF-β1 in astrocytes. These animals exhibited increased levels of AMPA and NMDA receptor subunits[224]. There is also compelling evidence for the role of TGF-β1 in the formation of excitatory synpases in the CNS. Rodent and human astrocytes were shown to increase the number of excitatory synpases in the cortex by increasing secretion of TGF-β1[225]. This effect was shown to be mediated by increased levels of d-serine amino acid, as pharmacological/genetic manipulation of the levels of d-serine completely inhibited the synaptogenic properties of the astrocyte-conditioned medium and TGF-β1[226].

1.4.5 Role of TGF-β1 in Neurodevelopmental Disorders

Studies utilizing human subjects have demonstrated that TGF-β1 levels are altered in various neurodevelopmental disorders, such as Down's syndrome and ASD. However, the exact nature of its participation in these diseases is unknown. ASD are complex neurodevelopmental disorders that are characterized by impairments in social interaction, deficits in verbal and non-verbal communication, and restricted repetitive and stereotyped patterns of behavior and interests. Recent studies have demonstrated that children with ASD have significantly lower plasma TGF-β1 levels compared with age-matched controls and compared with children with developmental disabilities other than ASD[227]. There were also significant correlations between psychological measures and TGF-β1 levels demonstrating that lower TGF-β1 levels were associated with decreased adaptive behaviors and worse behavioral symptoms[227]. Additionally, altered plasma levels of TGF-β1 may persist into adulthood as adult patients with autism were shown to have decreased levels compared to the control group[228]. Moreover, another study showed that TGF-β1 levels were also decreased in brain tissue of subjects with autism[229].

Table 1-1. List of exogenous and endogenous P-gp substrates.

Substrates of P-gp	
Chemotheraputics	**Antiretroviral Drugs**
Doxorubicin	Indinavir
Etoposide	Ritonavir
Mitomycin C	Saquinavir
Paclitaxel	
Tamoxifen	**Steroids**
Vinblastine	Cortisol
Vincristine	Dexamethasone
Antibiotics	**Cytokines/Chemokines**
Cefazolin	CCL2
Cefoperazon	GM-CSF
	IL-1beta
Immunosupressants	IL-2
Cyclosporin A	IL-4
Tacrolimus	IL-6
	INF-gamma
Calcium Blockers	TNF-alpha
Diltiazem	
Nicardipine	**Other Signaling Molecules**
Verapamil	Cholesterol
	Phosphatidylcholine
Cardiacs	Phosphatidylserine
Propafenone	Platelet Activating Factor
Amiodaron	Sphyngosine-1-phosphate
Quinidine	
Digoxin	

Figure 1-1. Topological model of P-gp. P-gp is comprised of two homologous halves, each with one transmembrane domain (TMD) and one nucleotide binding domain (NBD) located on the cytoplasmic side of the membrane. Modified with permission from Silva R et al., *Pharmacology and Therapeutics* (2015).

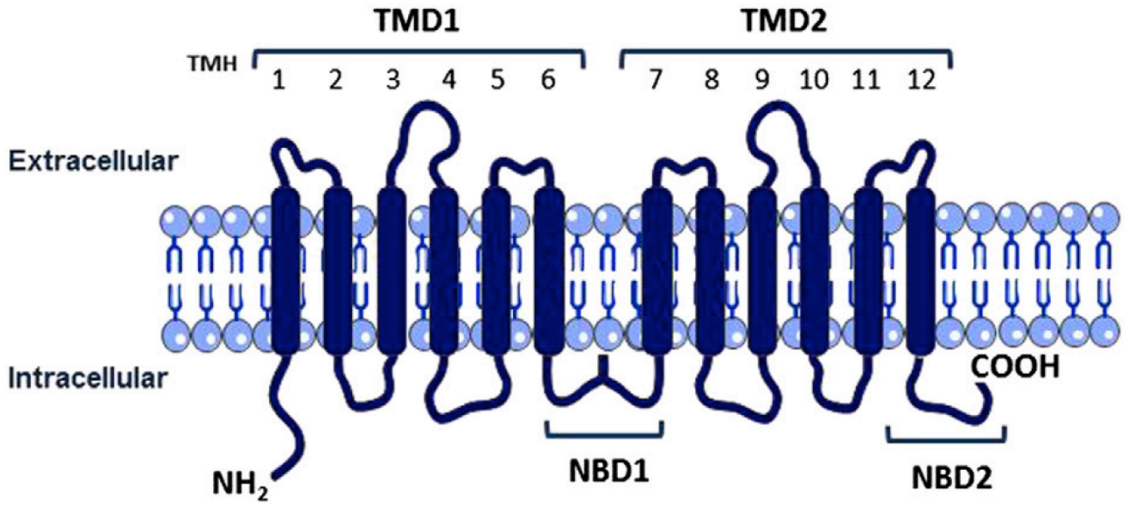


Figure 1-2. Diagram of the human *ABCB1* promoter with transcriptions factors known to regulate its expression. All factors displayed interact directly with DNA and some binding sites overlap. Adapted with permission from Scotto KD, *Oncogene* (2003).

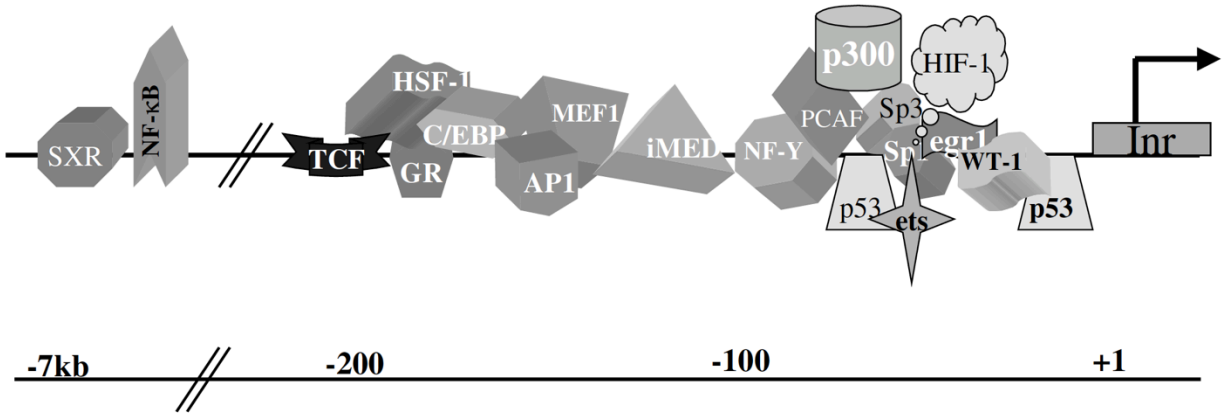


Figure 1-3. Schematic of the cellular interactions at the blood-brain barrier. Astrocyte endfeet extensively ensheath the abluminal side of the brain microvaculature, while pericytes cover ~30% of the abluminal surface. In contrast, there is very little innervation of the brain microvasculature by neurons and contact by microglia. Adapted with permission from Obermeier et al., *Nature Medicine* (2013).

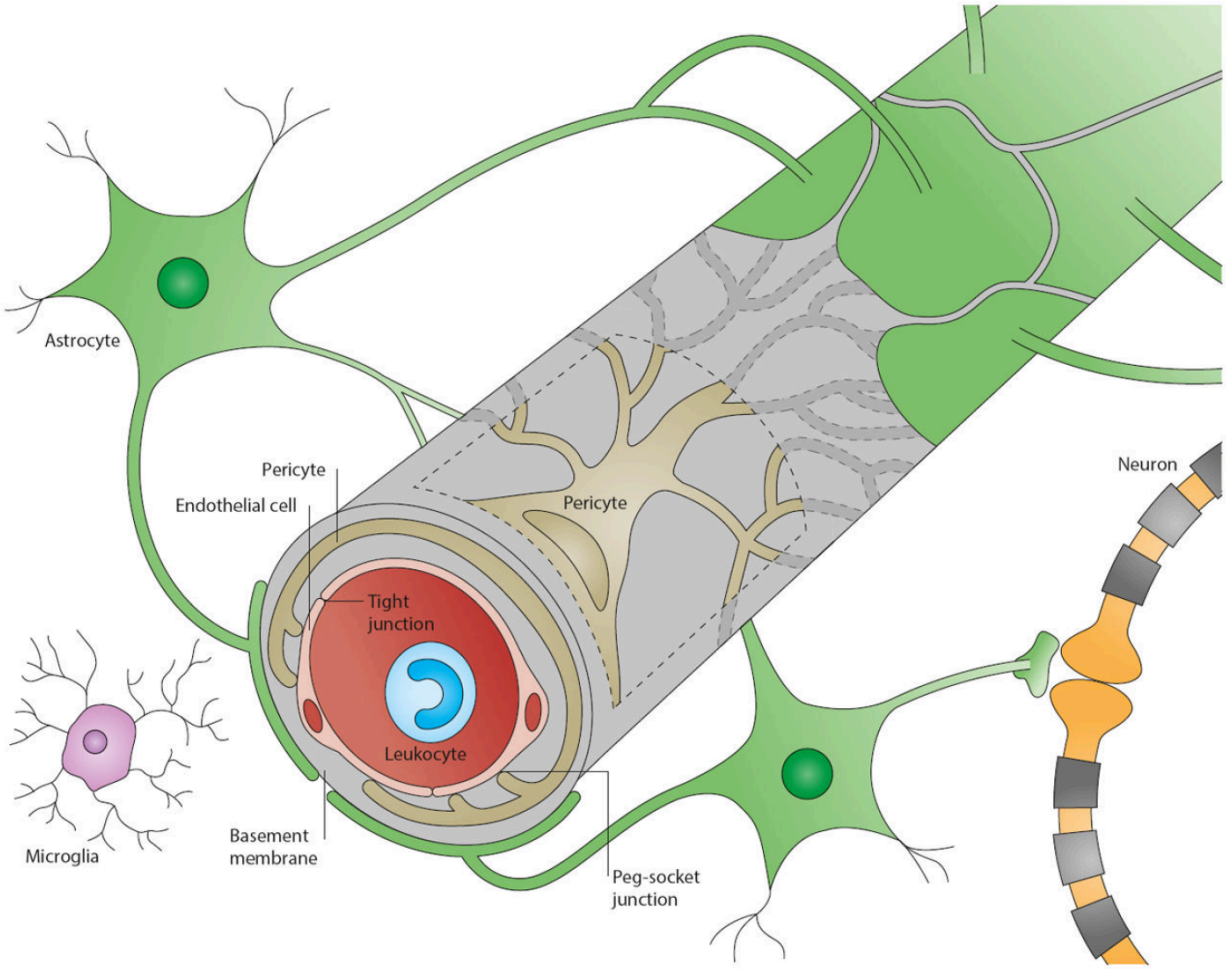


Figure 1-4. Schematic of P-gp localization at the blood-brain barrier. P-gp is located on the luminal, blood-facing, surface of brain endothelial cells and substrates are effluxed into blood cirulcation.

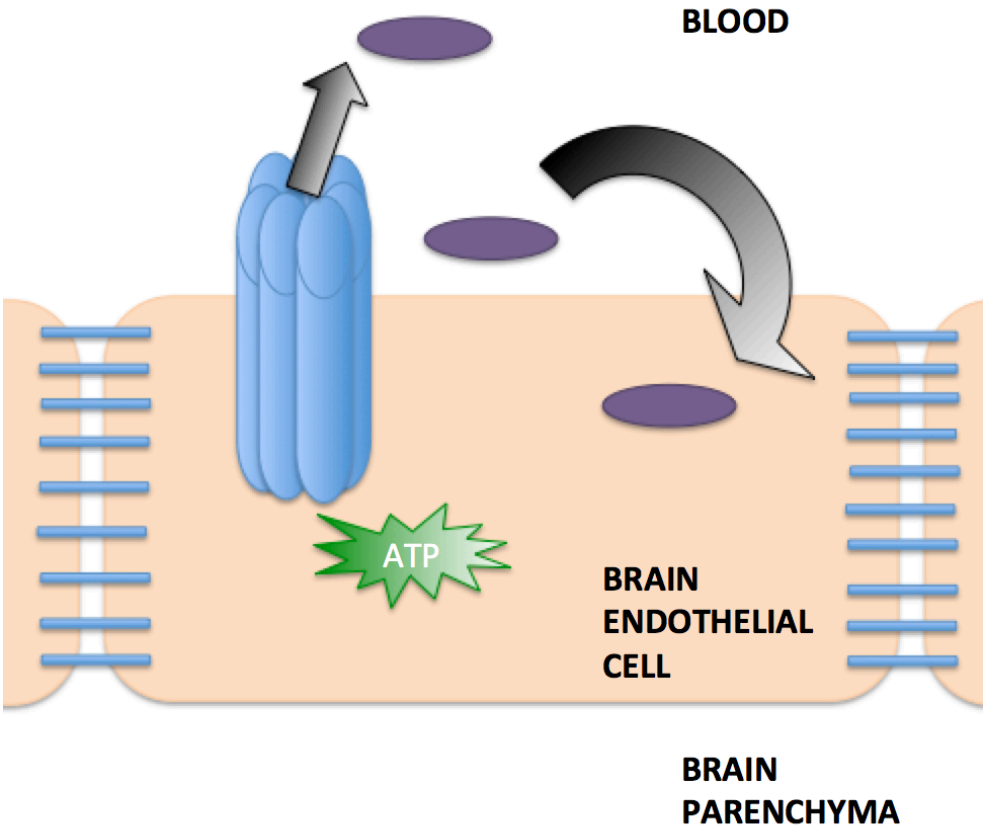


Figure 1-5. Ontogenic expression of *Abcb1* mRNA in mouse placenta. A dramatic decrease in *Abcb1* mRNA is demonstrated with advancing gestation. *Abcb1b* is the predominant isoform expressed in the placenta. (*) $P<0.05$, (**) $P<0.01$ compared to embryonic day 12.5. Modified with permission from Kalabis GM et al., *Biology of Reproduction* (2005).

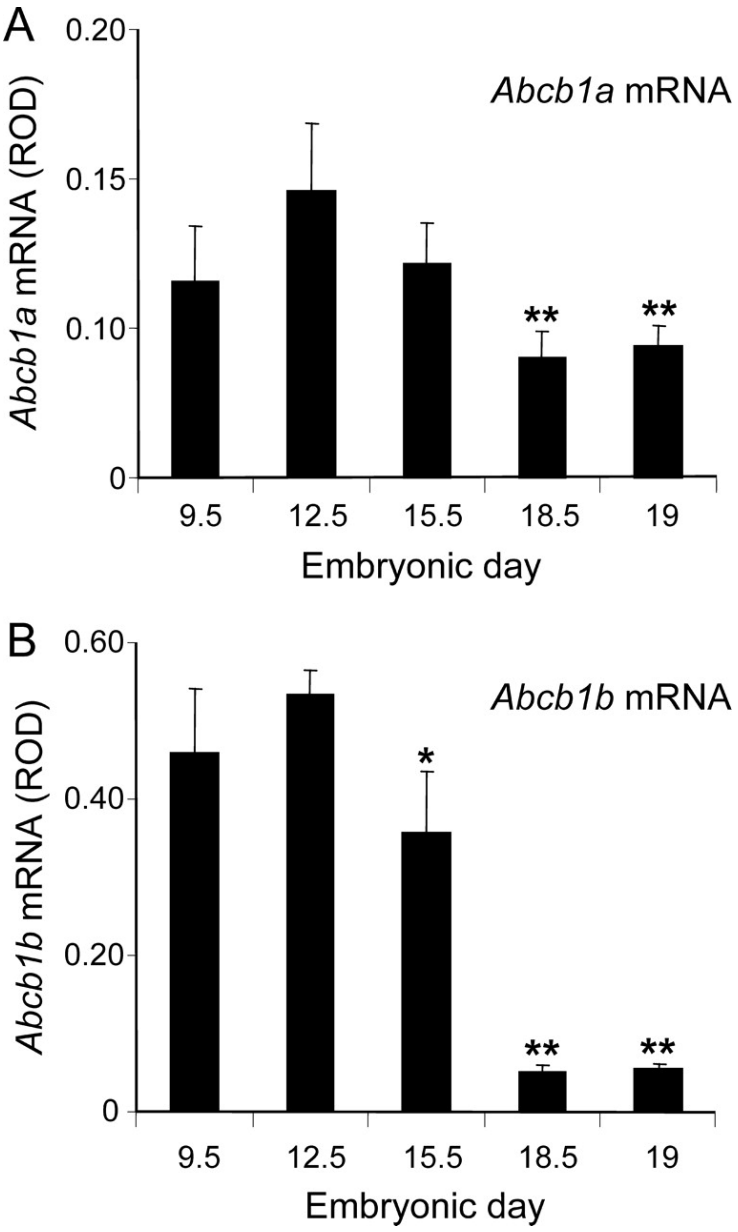


Figure 1-6. Developmental expression of P-gp protein in isolated guinea pig brain microvessels. Levels of P-gp protein dramatically increase in late gestation and post-natal life in both male and female guinea pigs. A significant difference from GD40 of the same sex indicated by (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$. Modified with permission from Iqbal et al., *Endocrinology* (2011).

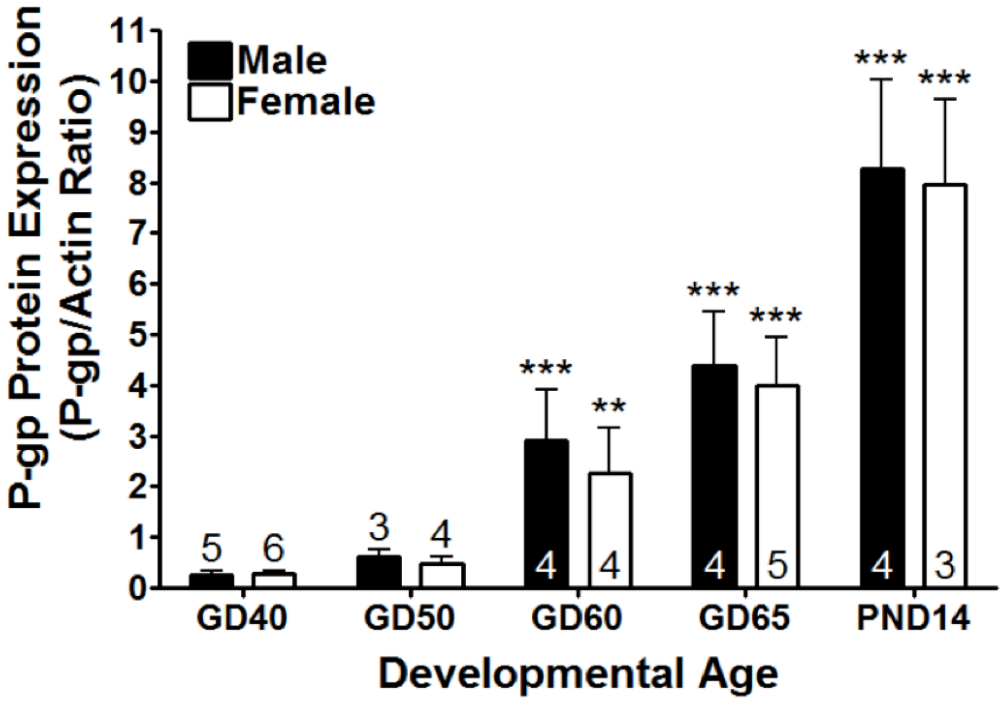


Figure 1-7. Schematic of the molecular organization of the dystrophin-dystroglycan complex at the astrocytic endfoot. Laminin and agrin of the basal lamina interact with the dystroglycan–dystrophin complex. The complex is also connected to AQPs and the inwardly rectifying potassium Kir4.1 channels via syntrophins and is located in lipid rafts. Dp71 is a glial form of dystrophin. These proteins are concentrated at astrocytic endfeet and contribute to astrocyte polarity. Adapted with permission from Dityatev et al., *Trends in Neuroscience* (2010).

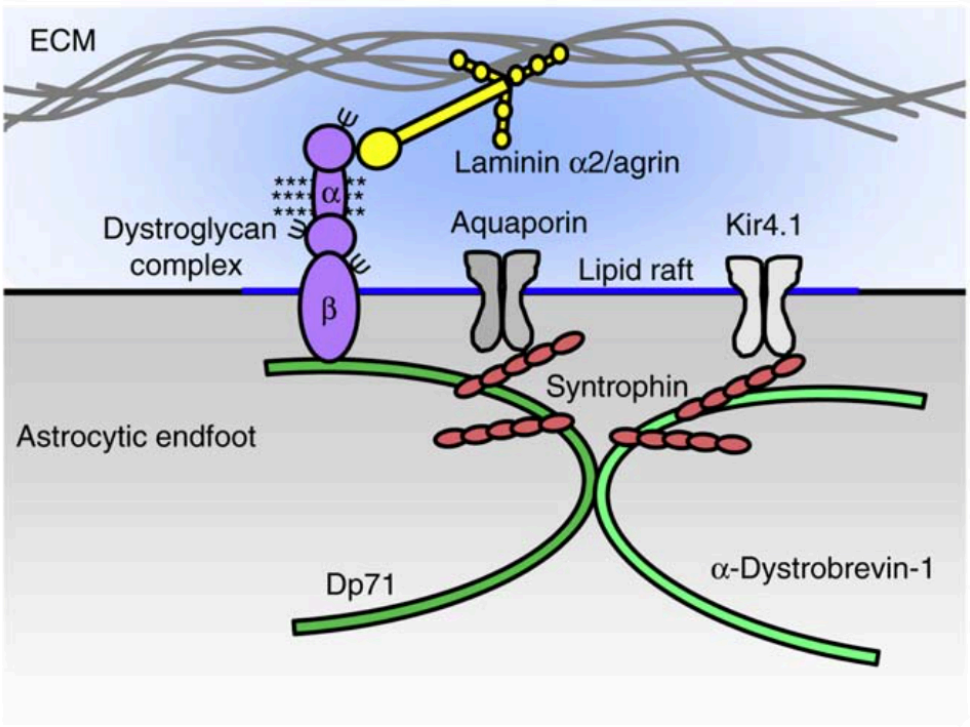


Figure 1-8. Synthesis of latent TGF-β1. Pro-TGF-β1 is cleaved intracellularly by a furin protease, producing a noncovalently-bound dimeric complex of latency-associated propeptide (LAP) and TGF-β1, referred to as the small latent complex (SLC). These complexes are bound to latent TGF-β binding protein (LTBPs) forming the large latent complex (LLC) for secretion and subsequent incorporation into the matrix. (RDG; Arg–Gly–Asp motif) Adapted with permission from Lafyatis, *Nature Reviews Rheumatology* (2014).

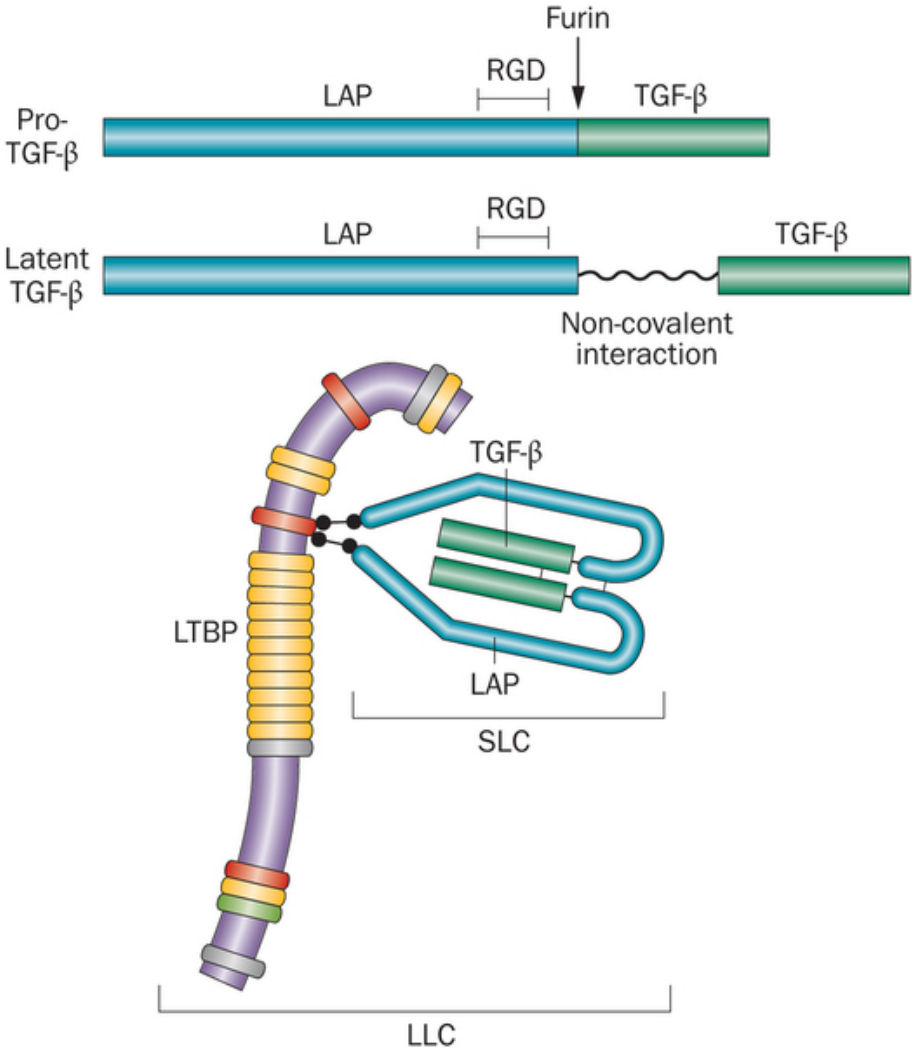
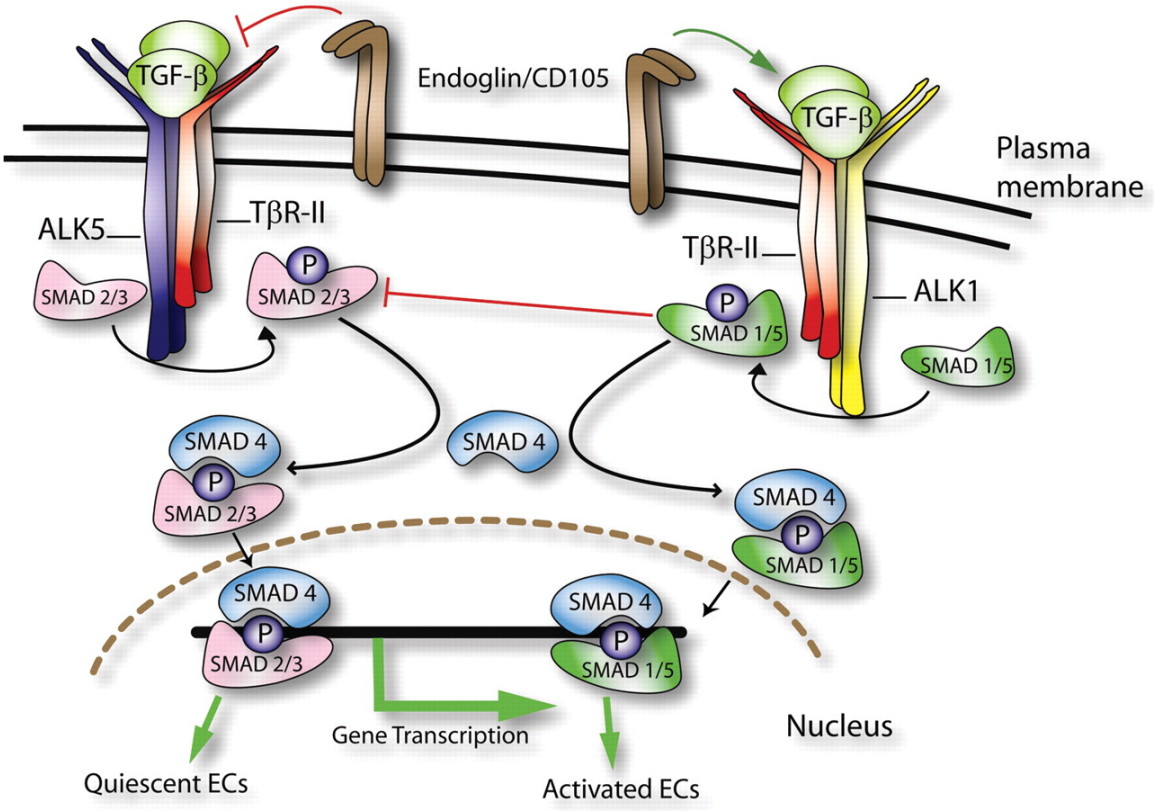


Figure 1-9. Schematic of TGF-β1/SMAD signaling. TGF-β1 binds to the TGFBR2 which recruits and phosphorylates a TGF-β type I receptor. Endothelial cells express two isoforms, ALK1 and ALK5. ALK5 phosphorylates SMAD2/3, while ALK1 phosphorylates SMAD1/5. These SMADs form a complex with SMAD4 and move into the nucleus. Modified with permission from Fonsatti et al., *Cardiovascular Research* (2009).



CHAPTER 2:
OBJECTIVES & HYPOTHESES

2.1 RATIONALE

Astrocyte differentiation during fetal development is temporally correlated with the surge in P-gp function and expression at the developing BBB, suggesting a functional role of astrocytes in the establishment and maintenance of the developing BBB. There is also extensive physical interaction between BECs and astrocytes in the mature brain microvasculature, as 99% of the abluminal surface is ensheathed by astrocyte-endfeet. Compelling evidence from co-culture studies, utilizing tissue from adult animals, have demonstrated that the presence of astrocytes enhances BBB characteristics in BECs.

Astrocytes have also been shown to induce BBB characteristics in non-neural endothelial cells. However, very little is known about how astrocytes modulate multidrug resistance via P-gp at the developing BBB.

This is a clinically relevant question since astrocyte dysfunction is evident in brains of individuals diagnosed with neurodevelopmental disorders, such as Rett syndrome and Fragile X mental retardation. During these disease-states, astrocytes may release different factors or levels of these factors compared to normal astrocytes. This could ultimately lead to perturbed P-gp regulation at a critical time in brain development and result in substantial changes in fetal brain exposure to xenobiotics, such as prescription drugs and pesticides, and other P-gp substrates. Thus, it is critical to investigate the role of astrocytes in regulating multidrug resistance via P-gp, as it will provide insight into the possible link between brain protection and neurodevelopmental diseases.

2.2 OBJECTIVES

The studies described in this thesis explore the effect of astrocyte-derived factors in the regulation of P-gp function and expression at the BBB during brain development. In Chapter 4, I investigated the effect of TGF-β1, an astrocyte-derived growth factor, on P-gp expression and function at the developing BBB. In Chapter 5, I examined how prenatal dexamethasone treatment altered TGF-β1-induced upregulation in P-gp activity and expression at the developing BBB. The second half of my thesis focused on establishing a co-culture model to investigate the role of astrocytes in modulating P-gp

function and expression at the developing BBB (Chapter 6). I also began to characterize the astrocyte-derived factors responsible for this effect.

2.3 OVERALL HYPOTHESIS

Factors released by astrocytes drive the developmental upregulation in P-gp function and expression at the developing BBB.

2.4 SPECIFIC HYPOTHESES

1. TGF-β1, a growth factor secreted by astrocytes, increases P-gp activity and levels of *Abcb1* mRNA in brain endothelial cells. The magnitude of this effect will vary with gestational age.

2. Prenatal dexamethasone treatment blunts responsiveness to TGF-β1-induced upregulation in P-gp activity and levels of *Abcb1* mRNA.

3. Astrocytes increase P-gp function and levels of *Abcb1* mRNA in brain endothelial cells. The magnitude of this effect will vary with developmental stage of the astrocyte.

CHAPTER 3:
GENERAL METHODS

3.1 RATIONALE

The guinea pig is an ideal animal model for investigating the interaction of astrocytes and BECs in regulating BBB characteristics. The use of this animal model allowed me to derive primary BECs and astrocytes for monocultures and co-cultures. Utilizing this i*n vitro* culture system, I determined the effect of astrocyte-derived factors in regulating P-gp function and levels of *Abcb1* mRNA in BECs derived from fetal and post-natal guinea pigs. Isolation of BECs and astrocytes from different stages of brain development allowed for the characterization of regulatory mechanisms involved in the modulation of P-gp expression and activity. The isolation and characterization procedures used to establish these *in vitro* models are described below.

3.2 ANIMAL MODEL

Our research group has utilized mouse and guinea pig models for previous studies, as human brain tissue is very limited. Using the mouse model allowed us to demonstrate the function of drug transporters through gestation at the placental and BBB interfaces[89,90,92,93,230]. Its relatively small body size permitted us to use radioactively labeled tracer molecules specific to these drug transporters, which demonstrated the importance of these transporters in fetal brain protection. However, there are challenges to using the mouse to develop an *in vitro* model. Fetal mouse brains are very small in size, making it challenging to derive both primary BECs and astrocyte cultures. Moreover, in the mouse, gliogenesis occurs mostly during the post-natal period, which makes it difficult to investigate the effect of *in utero* events on gliogenesis[231-234]. Also, unlike the human and the guinea pig, two genes encode for P-gp. Lastly, the gestation period of the mouse is relatively short in length (~19 days). This allows for limited windows for *in vivo* manipulation and time-points in gestation from which primary cells can be derived at.

In contrast to the mouse, the guinea pig has a relatively long gestation period (~68 days) and has similar placentation to humans. Our lab has shown that unlike other rodents, P-gp is encoded by a single gene, *Abcb1* in the guinea pig. Moreover, guinea pig brain development follows a similar pattern to human brain development, such that the fetal brain growth spurt, due to rapid gliogenesis, occurs *in utero*[110,235]. As such, the effect of *in vivo* manipulation on astrocyte development can be investigated using the guinea pig. There are also many advantages to using guinea pigs for establishing an *in*

vitro co-culture model of the BBB. Guinea pigs give birth to large fetuses (~100 g at term) that provide sufficient brain tissue for *in vitro* analysis. Fetal brain tissue derived at mid-gestation is sufficient to yield both primary BECs and astrocyte cultures. These characteristics have allowed us to generate a co-culture model using primary BECs and astrocytes derived from guinea pigs at various stages of fetal and neonatal development. All studies were carried out in accordance with protocols approved by the Animal Care Committee at the University of Toronto and in accordance with the Canadian Council on Animal Care.

3.3 BRAIN ENDOTHELIAL CELL CULTURE

3.3.1 Overview

BECs were isolated from guinea pigs at different time points in gestation as previously described[92]. These cells were characterized using the endothelial marker, Von Williebrand Factor, via immunocytochemistry. BECs also express BBB-specific markers such as, ZO-1, occludin, glucose transporter-1 (Glut-1), P-gp and BCRP. Moreover, BECs derived from different stages of gestation retain their developmental characteristics. Since there were no sex-differences in the developmental pattern of P-gp protein levels, only cells from male guinea pigs were utilized in this thesis[92].

3.3.2 Procedure

Isolation of BECs from GD50 and PND14 guinea pigs was carried out as previously described[92]. These time-points in development were chosen as our group has

found that P-gp expression is relatively low at GD50 and becomes upregulated at PND14 at the BBB[92]. Briefly, guinea pigs were anesthetized using isoflurane (Baxter Corp Mississauga, Ontario, Canada). Once anesthetized, guinea pigs were decapitated and brain tissue was excised, meninges removed, and brain tissue halved. One half was used for BEC isolation, while the other half was used to isolate astrocytes as outlined below. All subsequent steps were completed in a biological safety cabinet. Brain tissue was minced and homogenized (Potter-Elvehjem Tissue Grinder; Sigma, St. Louis, Missouri, USA). Homogenate was suspended in dextran solution (17.5%; Sigma) and centrifuged (4000 g for 15 min). The microvessel pellet was mixed with type I collagenase solution (1 mg/ml; Sigma) and digested for 30 min at 37°C. At the end of digestion, the solution was centrifuged at 1000 g for 10 minutes. The pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM; Wisent Inc., Saint-Jean-Baptiste, Quebec, Canada) supplemented with 20% fetal bovine serum (Wisent Inc.) and plated on 0.5% gelatin-coated 75 mm^2 tissue culture flasks (Becton-Dickinson (BD) Biosciences, Franklin Lakes, New Jersey, USA) and grown at 37°C in 5% CO_2/air. BECs were previously characterized[92]. BECs were frozen in liquid nitrogen.

3.4 ASTROCYTE CULTURE

3.4.1 Overview

Astrocytes were extracted from GD50 and PND14 guinea pigs using a modified protocol[236]. These cells were characterized by expression of GFAP via

immunocytochemistry. Like BECs, astrocytes derived from different stages of brain development retain developmental characteristics as described in section 3.4.3.

3.4.2 Procedure

Brain tissue was cut into small pieces using scissors and digested in papain (2.5 U/ml; Worthington Biochemical Corp., Lakewood, New Jersey, USA) at 37°C for 30 min. The mixture was agitated every 10 min by pipetting up and down with a Pasteur pipette. After incubation, papain was removed by centrifuging the solution at 1000 g for 10 minutes. The pellet was resuspended in Neurobasal A medium supplemented with B27 (Invitrogen, Carlsbad, California, USA) and loaded onto a column containing glass beads (5 mm; Thermo Fisher Scientific, San Jose, CA, USA). The filtrate was plated on rat tail collagen-coated 75 mm^2 tissue culture flasks (BD Biosciences) and grown at 37°C in 5% CO_2/air. Phase-contrast images of astrocytes from GD50 and PND14 guinea pigs are shown in figure 3-1. Astrocytes were frozen in liquid nitrogen until use.

3.4.3 Immunocytochemical characterization of astrocytes

To validate astrocyte isolation, cells were characterized by positive expression of astrocyte markers and lack of expression for markers of other CNS cells. Astrocytes from GD50 and PND14 guinea pigs were plated on 8-well tissue culture slides (BD Biosciences). At 80% confluence, media was removed and cells were washed with HBSS (Invitrogen). Cells were fixed by room temperature incubation with 4% paraformaldehyde (Sigma) for 30 min. Following fixation, cells were washed with in tris-

buffered saline (0.01M, pH7.5; Sigma) containing Triton X-100 (0.1% TBS-Triton; Sigma) and blocked in normal goat serum (NGS; 5% in TBS Triton; Invitrogen) for 2h. Primary antibody (diluted in NGS/TBS-Triton) was added after blocking. Primary antibodies: rabbit anti-von Willebrand Factor (vWF; 1:200; Sigma), mouse anti-glial fibrillary acidic protein (GFAP; 1:100; Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), rabbit anti-melanoma-associated chondroitin sulfate proteoglycan (Ng2; 1:100; Cell Signaling Technology, Inc.), mouse anti-nestin (1:200; abcam, Cambridge, United Kingdom), rabbit anti-aquaporin 4 (AQP4; 1:200; abcam), mouse anti-synaptophysin (1:100; Millipore, Billerica, Massachusetts, USA). After overnight incubation at 4°C, cells were washed and subsequently incubated with Alexa Fluor 488- or 555-conjugated goat secondary antibody (1:500 in NGS/TBS-Triton; Invitrogen) raised against respective primary antibody species. As a negative control, cells were incubated with mouse IgG or rabbit IgG followed by secondary antibody. After final washes, cells were mounted using mounting media with DAPI (Vector Laboratories, Burlingame, California, USA) and cover-slipped.

The majority of cells (>95%) derived from GD50 and PND14 guinea pigs stained positive for astrocyte-marker, GFAP, with PND14 cells expressing more GFAP compared to GD50 cells (Fig. 3-2). In contrast to PND14 cells, cells from GD50 guinea pigs (>95%) expressed nestin, which is an intermediate filament expressed by immature astrocytes. Similarly, aquaporin-4 (AQP4), a marker for mature astrocytes, was expressed by PND14 cells but not in GD50 cells. Both populations of cells stained negative for markers for other cells in the CNS: synaptophysin (neuronal), von-willie brand factor (BECs) and NG2 (pericytes).

3.5 CO-CULTURE

3.5.1 Overview

Co-cultures are commonly used as an *in vitro* BBB model. I established co-cultures to investigate the effect of astrocytes on P-gp function and expression in BECs by comparing to mono-cultured BECs. Our co-cultures are unique in that both primary astrocytes and BECs are derived from a single animal. This is in contrast to majority of studies that have utilized a mixture of cell lines, cells derived from different species or different aged animals to establish co-cultures. However, since the cells are not in contact in this co-culture model, only soluble factors secreted by astrocytes were investigated.

3.5.2 Procedure

BECs and astrocytes were plated on 75 mm^2 tissue culture flasks (BD Biosciences) and grown at 37°C in 5% CO_2/air. Once confluent, cells were used for co-cultures. To establish co-cultures using GD50 and PND14 BECs and astrocytes, astrocytes were plated in the basolateral compartment of Transwell plate (Corning, Corning, New York, USA) at a seeding density of 1×10^4 cells/cm^2 and BECs were plated on the Transwell PET insert containing 0.45 μm pores (Corning) at a seeding density of 1×10^4 cells/cm^2 (Fig. 3-3).

3.5.3 Characterization of co-culture

Tight junction and P-gp function and expression were analyzed in co-cultured BECs. These parameters were compared in mono-cultured BECs. The results of these experiments are found in Chapter 6.

3.6 Quantification of mRNA expression

Cells were washed twice with HBSS and total RNA was extracted using TRIzol reagent (Invitrogen) as per the manufacturer's protocol. Samples were digested with a ribonuclease-free deoxyribonuclease (Ambion-Austin, TX). RNA purity and concentration were assessed by the A260/A280 ratio via spectrophotometric analysis. RNA integrity was verified using gel electrophoresis. Total RNA was subjected to reverse-transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Hammonton, NJ) as per the manufacturer's protocol. Samples were incubated at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min using the C1000 Thermal Cycler (Bio-Rad, Hercules, CA).

Primer sequences were designed using Autoprime (Gunnar Wrobel & Felix Kokocinski; Cambridge, United Kingdom) based on transcript ID (Ensemble Genome Browser; Guinea Pig) and synthesized by Integrated DNA Technologies (Coralville, Iowa; Table 3-1). For each primer set, a standard curve was generated by serial dilution of a pooled reference sample with an efficiency of 100% ± 10. Levels of mRNA were quantified using Real-time PCR. cDNA (100 ng) was mixed with respective primer probes and SsoFast EvaGreen Supermix (Bio-rad Laboratories, Inc., Hercules, California,

USA) and loaded onto CFX96 Real-Time System (Bio-Rad). All samples were run in triplicate. No-template and no-RT negative controls were included in all runs. Relative mRNA expression was calculated as gene of interest expression normalized [ΔΔc(t)] to reference gene expression.

3.7 CONCLUSIONS

Using the guinea pig as our model enabled us to establish mono-cultured and co-cultured BECs from different stages of development. Using BECs mono-cultures, we were able to determine the effect of a specific astrocyte-derived factor, TGF-β1, on P-gp function and expression in BECs derived from different developmental stages (Chapters 4 & 5). Additionally, the utilization of co-cultures enabled us to investigate the differential effect of fetal and post-natal astrocyte-derived factors on P-gp function and expression (Chapter 6).

Figure 3-1. Phase-contrast images of brain endothelial cells (A, B) and astrocytes (C, D) derived from GD50 and PND14 male guinea pigs. Images were taken at 40X magnification.

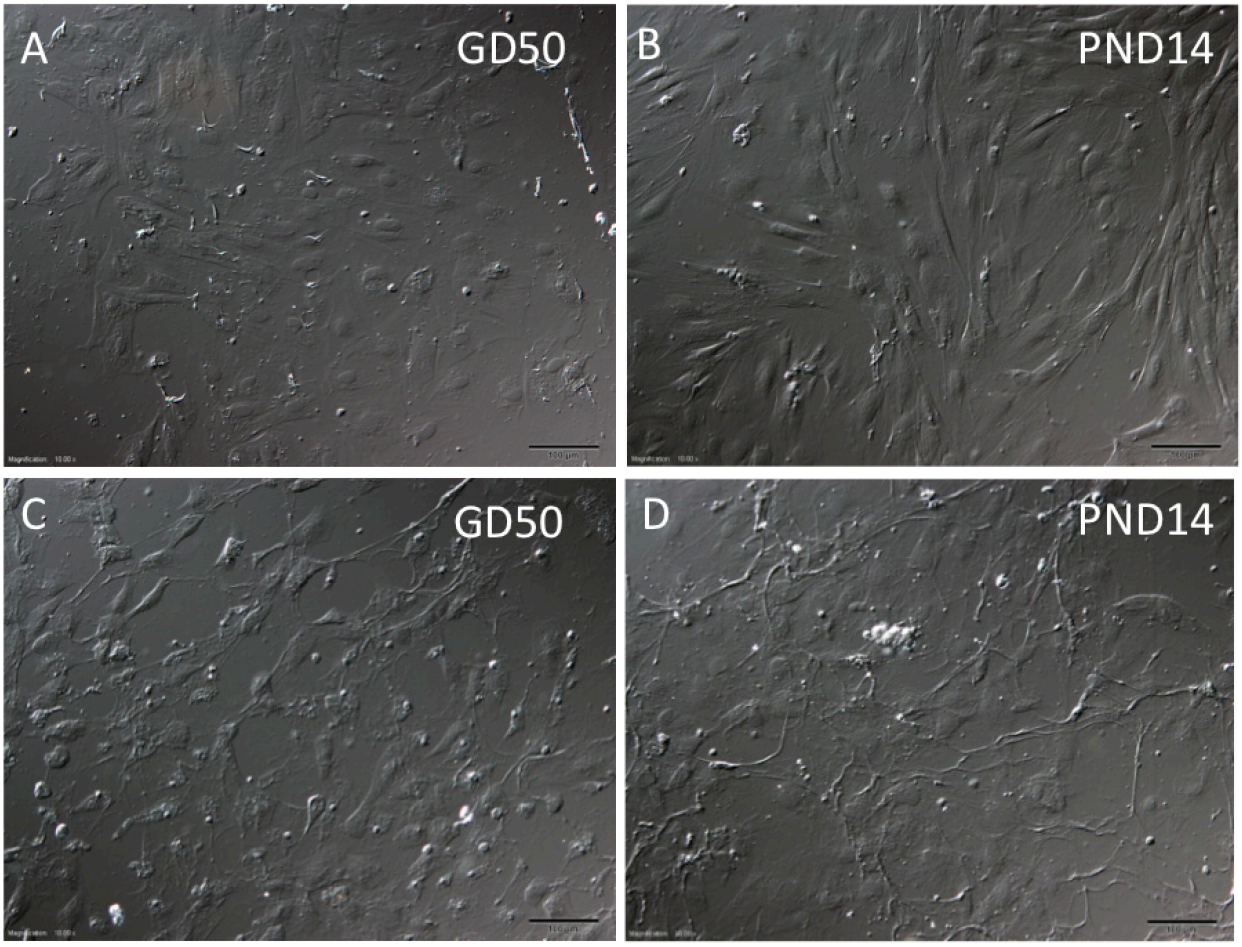


Figure 3-2. Fluorescent immunocytochemical images of astrocytes derived from GD50 (A-C) and PND14 (D-F) male guinea pigs. Cells were stained for nestin (nes), GFAP and AQP4. Cell nuclei were stained by 4′,6-diamidino-2-phenylindole (*blue*).

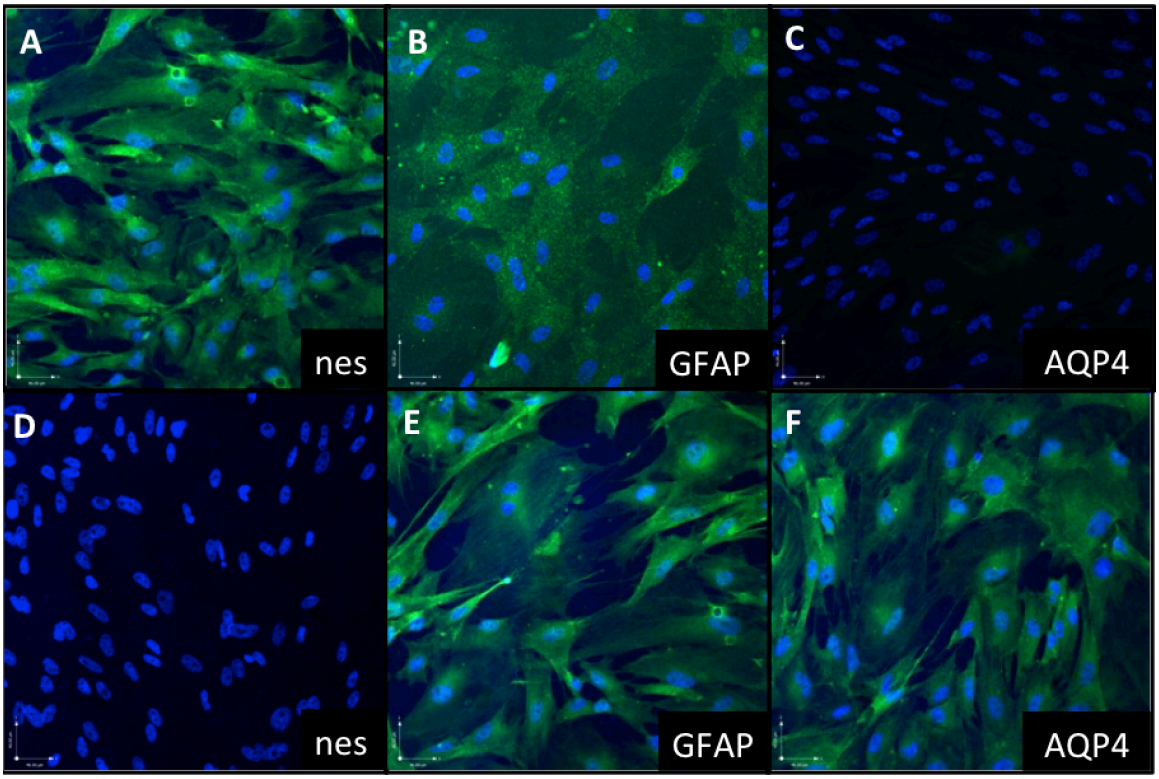


Figure 3-3. Schematic of co-culture model consisting of primary guinea pig BECs and astrocytes. On day 0, astrocytes are plated in the basolateral compartment. Two days later, BEC are plated on porous membrane.

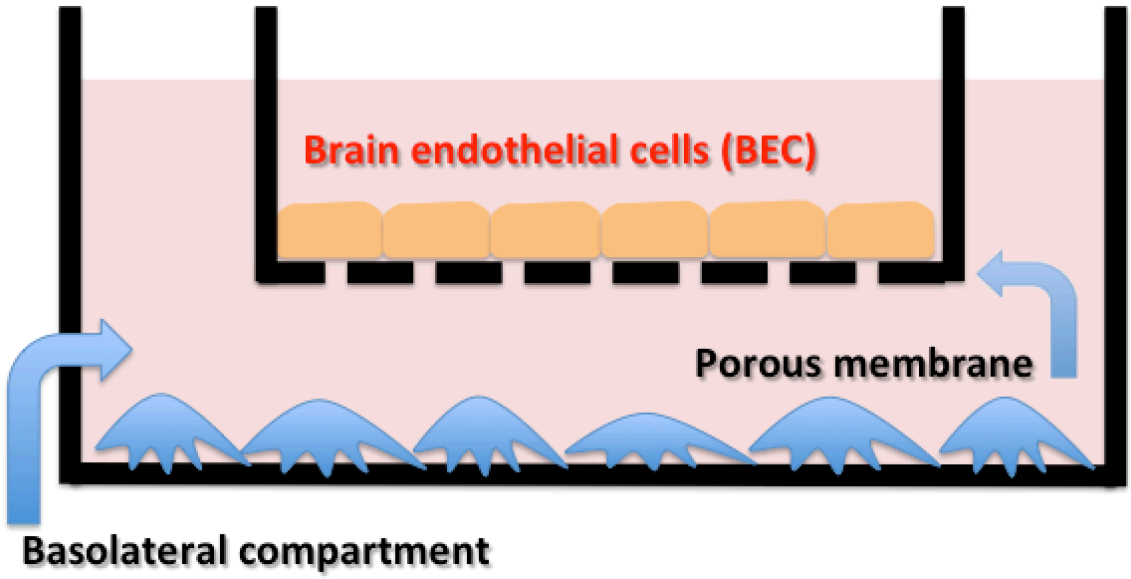


Table 3-1. Primer details. Forward and reverse primer sequence and annealing temperature listed for each target gene.

Target	Primer	Temperature (Celsius)	Ensembl Transcript ID
P-glycoprotein	F. 5'-CAA TCT GGG CAA AGA TAC TG-3' R. 5'-CAA GTT CTT TGC TTT GTC CTC-3'	51	ENSCPOT00000012540
Occludin	F. 5'-CCTGATGAATTCAAACCAAATC-3' R. 5'-AAGGAATATGCTGGCTGAGAC-3'	61	ENSCPOT00000005836
VE-cadherin	F. 5'-AGC ACT TTC TGG ATG TCT TTG-3' R. 5'-AAG TTC TGC ATG TTT GGT CTC-3'	60	ENSCPOT00000005821
ZO-1	F. 5'-CGA ATT AAG CTT TAT CAG AGC AC-3' R. 5'-GTT CCC ATA TAG CTG TTT CCT C-3'	57	ENSCPOT00000011029
Beta-actin	F. 5'-TTT ACA ATG AAT TGC GTG TG-3' R. 5'-ACA TGA TCT GGG TCA TCT TC-3'	58	ENSCPOT00000013600
TGFBR2	F. 5'-GTA TGG CGG AAG AAT GAT G-3' R. 5'-CAG GAG CAC ATA AAG AAG GTC-3'	52	ENSCPOG00000014487
ALK5	F. 5'-CGA AGG CAT TAC AGT GTT TC-3' R. 5'-TGT GAT AGA GAC AAA GCA GAG G-3'	52	ENSCPOG00000014890

ALK1	F. 5'-ACA ACA TCC TAG GCT TCA TC-3' R. 5'-CCT CTG CAG GAA GTC ATA GAG-3'	60	ENSCPOG00000001660
Endoglin	F. 5'-TCC ATC TGG CCT ATG ACG-3' R. 5'-GAA GGT AAA TGG TGG CAT CTC-3'	60	ENSCPOG00000000436
Betaglycan	F. 5'-GGG AAG ATC AAG TGT TTC CTC-3' R. 5'-TGG CTG GAC ATC ACA CAC-3'	52	ENSCPOG00000007217
Cadherin2	F. 5'-CCC TGC TCC AGG CAT CTG-3' R. 5'-CTG CCC TTC ATG CAC ATC C-3'	58	ENSCPOG00000012391
Nedd9	F. 5'-CTC CTA TGA AAG GGA TCT ATG-3' R. 5'-TGG AGG AGG GAA ATC ATAC-3'	55	ENSCPOT00000012524
SMAD7	F. 5'- AAC TGC AGA CTG TCC AGA TG-3' R. 5'- GTC TTC TCC TCC CAG TAT GC-3'	55	ENSCPOT00000021317

CHAPTER 4:

TGF-β1 Regulation of Multidrug Resistance P-glycoprotein in the Developing Male Blood-Brain Barrier

4.1 PUBLICATIONS (arising from studies presented in Chapter 4)

Stephanie Baello, Majid Iqbal, Mohsen Javam, Enrrico Bloise, William Gibb and Stephen Matthews (2014). *TGF-β1 Regulation of Multidrug Resistance P-glycoprotein in the Developing Male Blood-Brain Barrier.* Endocrinology **155**(2): 475-84.

4.2 INTRODUCTION

Previous studies have implicated TGF-β1 in the regulation of P-gp expression in endothelial cells derived from the adult brain. *In vitro*, TGF-β1 was found to increase P-gp activity in BECs derived from the adult mouse brain[146]. TGF-β1 is secreted by differentiating astrocytes[237,238] and plays an essential role in brain development[239]. Co-culture studies of astrocytes and BECs have shown that there is an increase in *Abcb1* mRNA expression in co-cultured BECs compared to monocultured BECs[164]. This increase is most likely due to factors secreted by astrocytes since the BECs do not require physical contact with astrocytes for this upregulation to occur[240-242].

To date, it is not known whether TGF-β1 affects P-gp function in the developing BBB and, if so, which downstream TGF-β1 signaling pathway(s) mediates the effect of TGF-β1 on P-gp function. Therefore, the objectives of this study were to determine the effect of TGF-β1 on P-gp function and *Abcb1* expression in BECs during fetal and neonatal development and to investigate the TGF-β1 signaling pathways involved. I hypothesized that TGF-β1 treatment would increase P-gp function and that the magnitude of this effect would vary with the developmental age at which the BECs were derived.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Twelve-week-old female Dunkin-Hartley-strain guinea pigs were purchased from Charles River Canada, Inc. (St. Constant, Quebec, Canada) and bred as described previously[243]. Pregnant females were untreated during pregnancy. Two-week-old male

guinea pigs were purchased from Charles River. All studies were carried out in accordance with protocols approved by the Animal Care Committee at the University of Toronto and in accordance with the Canadian Council on Animal Care.

4.3.2 Guinea Pig Primary Brain Endothelial Culture

BECs were isolated from GD40, GD50, GD65 male fetuses and PND14 male guinea pigs, as described in Chapter 3. Time-points were chosen as P-gp protein expression is detectable at GD40 in guinea pig brain microvasculature. As gestation progresses, P-gp protein expression dramatically increases, approximately 17- and 32-fold at GD65 and PND14 compared to levels at GD40[92]. Cell viability following isolation was 99% as assessed by using trypan blue staining (Sigma). Cells were then frozen in liquid nitrogen until use in the following experiments.

4.3.3 TGF-β1 Treatment and P-gp Functional Assay

BECs derived from GD40, GD50, GD65 and PND14 guinea pigs were plated on gelatin-coated 96-well culture plates (Becton Dickinson Biosciences) at a seeding density of $1x10^4$ cells/cm^2. Cells were grown 37°C in 5% CO_2/air for 5 days. At confluence, media was replaced with phenol-red free DMEM (Wisent Inc.) containing 20% charcoal stripped FBS (Wisent Inc.). Twenty-four hours after media change, cells were treated with TGF-β1 (0.001-10ng/ml; Invitrogen, Carlsbad, California, USA) for 2, 4, 8, and 24h. Cell viability following TGF-β1 treatment was 99% as assessed by using trypan blue staining (Sigma). These TGF-β1 doses were selected as maternal plasma levels of TGF-

β1 range from 1-30ng/ml[244]. After treatment, cells were washed twice with warm Tyrodes' salt solution (Sigma) and P-gp activity was measured using an established Calcein-AM Assay[92]. Briefly, cells were incubated with calcein-AM (10^{-6} M; 1h). After incubation, cells were washed twice with ice-cold Tyrodes' solution (Sigma), lysed and calcein was measured using a fluorescent plate reader (ex/em: 485/510 nm). Mean background fluorescence was subtracted from all control and treated readings.

4.3.4 TGF-β1 Treatment and P-gp Specificity

BECs derived from PND14 male guinea pigs were grown to confluence in 96-well plates, as described above. Cells were treated with phenol red-free medium containing stripped fetal bovine serum and 10 ng/ml TGF-β1 (8h). Cells were washed with Tyrodes' solution and then subsequently incubated for 1h with either 10^{-6} M calcein-AM or 10^{-6} m calcein-AM with 10^{-4} m verapamil (VPL) (Sigma). Verapamil is an L-type calcium channel blocker that has been shown to be a competitive inhibitor of P-gp[245]. Cells were then washed, lysed, and calcein was measured, as described above.

To further validate that the effects of TGF-β1 were specific to P-gp, an alternative P-gp substrate, rhodamine 123 (Sigma), was used. BECs were treated for 8h with either phenol red-free medium containing stripped fetal bovine serum and TGF-β1 (10ng/ml). Cells were washed before incubation with 10^{-5} M Rho123 for 30 min. After lysis, rhodamine123 accumulation was measured (Ex/Em: 485/528 nm).

4.3.5 Quantification of mRNA expression

To investigate whether functional changes in P-gp elicited by TGF-β1 corresponded to changes to *Abcb1* mRNA expression, PND14 BECs were cultured on 10 cm^2 gelatin-coated tissue dishes (Becton Dickinson Biosciences) at a seeding density of $1x10^4$ cells/cm^2. Cells were grown at 37°C in 5% CO_2/air for 5 days. At confluence, BECs were treated with TGF-β1 (10 ng/ml) for 2, 4, 8 and 24h. Total RNA was collected, reverse transcribed to cDNA and levels of specific transcripts were analyzed via real-time PCR as described in Chapter 3. In addition, RNA was isolated from cells at GD40, GD50, GD65 and PND14 to quantify TGF-β receptors (*tgfbr2, alk1, alk5, endoglin, betaglycan*). Relative mRNA expression was calculated as gene of interest expression normalized [ΔΔc(t)] to reference gene expression (β-*actin*). β-*actin* was not differentially regulated across gestation or altered by TGF-β1, SIS3, BMP-9, dorsomorphin or SB-431542 treatment (data not shown).

4.3.6 Signaling Pathways involved in TGF-β1 regulation of P-gp

To investigate signaling pathways mediating TGF-β1 effects on P-gp function, BECs derived at GD50 and PND14 were treated with various ALK1 and ALK5 inhibitors and agonists. We investigate signaling on GD50 and PND14 because levels of signal transducing receptor, *alk1*, varied with gestational age. To investigate the involvement of ALK5 signaling, BECs were pre-treated (1h) with SB-431541 (ALK5 antagonists; 10μM and 25μM; Sigma) or specific inhibitor of smad3 (SIS3; 30 μM; Sigma). Cells were then treated with TGF-β1 (10ng/ml) in the presence of the respective inhibitor for 8h. To investigate the role of ALK1 signaling, BECs were treated (2, 8 and 24h) with BMP-9

(ALK1 agonist; 0.001-10ng/ml; R&D Systems, Minneapolis, MN). In another experiment, BECs were also pre-treated (1h) with dorsomorphin (ALK1 inhibitor; 1, 8, and 40 μM; EMD Millipore, Billerica, MA) and subsequently treated with TGF-β1 (10 ng/ml) and inhibitor for 8h. P-gp activity was assessed after treatment, using Calcein-AM, as described above. The various treatments had no significant effect on cell viability, which was determined using trypan blue (Sigma; data not shown). Following treatment, *Abcb1* mRNA levels were analyzed via qRT-PCR. SMAD3 activation upregulates *cadherin2* mRNA and thus levels were quantified as a positive control.

4.3.7 Statistical analysis

All statistical analyses were performed using Prism (GraphPad Software, Inc., San Diego, CA). TGF-β1 associated receptors (*tgfbr2, alk1, alk5, endoglin, betaglycan*) and *Abcb1* mRNA data were analyzed using one-way ANOVA, followed by Newman-Keuls *post hoc* test. Samples were run in triplicates for RT-PCR experiments. Functional P-gp data were analyzed using one-way ANOVA, followed by Dunnett's (for comparisons against the control group) and Newman-Keuls (for comparisons against other treatment groups) *post hoc* analyses. Functional P-gp data are displayed as percent change in activity from controls. Significance was set at $P < 0.05$. Each treatment group consisted of 6-8 animals.

4.4 RESULTS

4.4.1 TGF-β1 regulation of P-gp Function During Development

TGF-β1 significantly increased P-gp function in BECs derived from fetal (GD40, GD50, GD65) and young (PND14) male guinea pigs (Fig. 4-1). This increase in function occurred within 2h of treatment. However, at 24h, no effect of TGF-β1 on P-gp activity was detected apart from at the highest concentration (10ng/ml) with BECs derived from GD40 fetuses. BECs derived from GD40 and GD50 male fetuses were more responsive to TGF-β1 treatment when compared with PND14 BECs (Fig. 4-1).

4.4.2 Effect of TGF-β1 is P-gp Specific

In order to demonstrate that this effect of TGF-β1 was indeed specific to P-gp, BECs derived from PND14 BECs were exposed TGF-β1 in the presence of P-gp inhibitor, verapamil (VPL). Treatment of BECs derived at PND14 with TGF-β1in the presence of VPL attenuated the effects of TGF-β1 on P-gp function (Fig. 4-2A). To further demonstrate the effect of TGF-β1 is P-gp specific, we replicated the TGF-β1-induced increase in P-gp function using an alternative substrate of P-gp, rhodamine 123 (Fig. 4-2B).

4.4.3 TGF-β1 regulates Abcb1 mRNA in BECs

TGF-β1 increased *Abcb1* mRNA levels in BECs derived from PND14 guinea pigs (Fig. 4-3). The effect of TGF-β1 (10ng/ml) on *Abcb1* mRNA was biphasic. Within 2h, *Abcb1* mRNA increased 3-fold compared to control ($P<0.001$) and returned to baseline

levels at 4h. However, *Abcb1* mRNA then increased by approximately 2-fold compared to control at 8h ($P<0.01$) returning to control levels by 24h. The changes in *Abcb1* mRNA at 8 and 24h mirrored the functional changes in P-gp (Fig. 4-1).

4.4.4 Developmental expression of TGF-β1 associated receptors

The relative expression of TGF-β type II receptor (*tgfbr2*) mRNA (GD40 1.2±0.33, GD50 1.1±0.49, GD65 1.0±0.19, PND14 1.0±0.27), *alk5* mRNA (GD40 1.1±0.25, GD50 0.7±0.29, GD65 1.1±0.20, PND14 0.7±0.2), and the TGF-β1 type III receptor (*endoglin*) mRNA (GD40 1.4±0.54, GD50 1.5±0.7, GD65 1.1±0.32, PND14 1.2± 0.28) did not change through development. In contrast, *alk1* and *betaglycan* mRNA levels were significantly higher in BECs derived at GD65 and PND14 compared to those derived at GD40 ($P<0.01$; Fig. 4-4A and $P< 0.001$; Fig. 4-4B). Since the response to TGF-β1 significantly decreased in PND14 compared to BECs derived at GD50 (Fig. 4-2), while *alk1* and *betaglycan* mRNA levels increased, we examined the signaling mechanisms involved in TGF-β1 regulation of P-gp function at GD50 and PND14.

4.4.5 Role of ALK5 in TGF-β1-induced increase in P-gp

BECs derived at GD50 and PND14 were treated with the ALK5 inhibitor SB-431542 in order to examine if activation of ALK5 is required for P-gp regulation. SB-431542 is a small molecule that inhibits the intracellular kinase domains of ALK5[246]. Both doses of SB-431542 prevented the TGF-β1-induced increase in P-gp function in

BECs derived at GD50 and PND14 indicating that ALK5 is required for TGF-β1 regulation of P-gp function (Fig. 4-5A, B).

To further define the role of ALK5, BECs derived from PND14 male guinea pigs were treated with specific inhibitor of SMAD3 (SIS3). SMAD3 is a signal transduction molecule that is phosphorylated as a result of ALK5 activation, and SIS3 blocks this action[247]. Treatment of BECs derived at GD50 and PND14 with TGF-β1 in the presence of SIS3 did not reduce P-gp activity or *Abcb1* mRNA compared to cells treated with TGF-β1 alone (Fig. 4-6A, B, D, E), indicating that SMAD3 was not involved in ALK5 mediated regulation of P-gp. As a positive control, the effect of SIS3 on TGF-β1-induction of *cadherin 2* mRNA was determined since TGF-β1 acting via SMAD3 increases *cadherin 2* expression[248]. As expected, TGF-β1-induction of *cadherin 2* mRNA was prevented by SIS3 treatment (Fig. 4-6C, F).

4.4.6 Role of ALK1 in the TGF-β1-induced increase in P-gp

The contribution of ALK1 activation to changes in P-gp function was investigated. ALK1 signals through SMAD1/5. BECs derived at GD50 and PND14 were treated with the ALK1 agonist BMP-9[249,250]. Treatment with BMP-9 caused an increase in P-gp activity in BECs derived at both GD50 and PND14 (Fig. 4-7A-D). However, there was a discrepancy between the effects of BMP-9 (Fig. 4-7) and TGF-β1 (Fig. 4-1). Treatment with BMP-9 (10ng/ml; 24h) stimulated an increase in P-gp function in cells derived at GD50 ($P<0.001$) and PND14 ($P<0.05$). In contrast, there was no effect of TGF-β1 on P-gp function at 24h in BECs derived from PND14 guinea pigs (Fig. 4-1).

One explanation is that TGF-β1 is no longer active after 24h, in contrast to the agonist BMP-9. To investigate this, BECs derived at PND14 were exposed to TGF-β1 for 24h. After 24 hours, this media was transferred to new BECs derived at PND14 and P-gp activity was accessed. After 2h treatment, p-gp activity remained unchanged.

To confirm that activation of ALK1 is required for TGF-β1 mediated regulation of P-gp, BECs derived at GD50 and PND14 were treated with dorsomorphin (ALK1 inhibitor)[251]. Dorsomorphin antagonized the TGF-β1-induced increase in P-gp function in BECs derived at PND14 indicating that ALK1 is required for TGF-β1 regulation of P-gp function (Fig. 4-8B). However, the same doses of dorsomorphin did not inhibit the TGF-β1-induced increase in P-gp function in BECs derived at GD50 (Fig. 4-8A).

4.5 DISCUSSION

This is the first study to show that TGF-β1 is a potent modulator of P-gp function in BECs derived in late gestation and the early post-natal period. Effects were greater in BECs derived at earlier stages of development. Moreover**,** we have shown that the effect of TGF-β1 on P-gp is dependent upon ALK5 activation. However, the regulatory effects of TGF-β1 on P-gp function and *Abcb1* mRNA do not appear to involve classical ALK5/SMAD3 signaling. In addition, activation of the ALK1 pathway mimicked the TGF-β1-induced regulation of P-gp function and we have shown that this pathway is dependent on the maturity of BEC.

There was generally good correlation between P-gp function and *Abcb1* mRNA following stimulation with TGF-β1. *Abcb1* mRNA levels increased at 2h and 8h time points and decreased at 24h, correlating with respective functional data at these time points. The biphasic effect of TGF-β1 on *Abcb1* mRNA (indicated by the lack of effect of TGF-β1 on *Abcb1* mRNA at 4h) may be due to both direct and indirect mechanisms. It is known that TGF-β1 modulates gene expression by affecting transcriptional activation and mRNA turnover rate[252]. TGF-β1 has been shown to enhance the stability of *COX-2* mRNA in intestinal epithelial cells and human lung fibroblasts[253,254], and products of this enzyme have potent regulatory effects on P-gp function[255]. Moreover, TGF-β1 may stimulate endothelial cells to secrete various factors, potentially affecting P-gp function[146]. Thus, the biphasic effect of TGF-β1 on *Abcb1* mRNA may also result from the production of TGF-β1-induced factors from the endothelium.

The present study has identified the downstream signaling pathways by which TGF-β1 regulates *Abcb1* mRNA levels and P-gp activity in the developing BBB. Through inhibition of the ALK5 intracellular kinase domain, we have shown that activation of ALK5 is required for TGF-β1 regulation of P-gp. Moreover, by inhibiting SMAD3, we demonstrated that ALK5-associated SMAD3 is not required for TGF-β1 regulation of *Abcb1* mRNA expression and P-gp function in BECs. However, it remains possible that P-gp is regulated through ALK5 non-SMAD signaling pathways such as, MAPK and PI3K [256,257]. BECs also express ALK1, a type I receptor activated by TGF-β1. To our knowledge, this is the first study to demonstrate that activation of ALK1, with BMP-9, induces similar effects on P-gp function to those of TGF-β1. Also similar to TGF-β1, BMP-9-induced effects on P-gp were reduced in BECs derived near term

compared with those derived earlier in gestation. This decreasing cellular responsiveness to BMP-9 may be attributed to an increase betaglycan mRNA, as betaglycan has been shown to be a negative regulator of BMP signaling [216,217]. Moreover, signaling through the ALK1 pathway is dependent on maturity of BEC since ALK1 inhibitor, dorsomorphin, markedly reduced the TGF-β1-induced increase in P-gp function in BECs derived at PND14, but not those derived at GD50. We speculate that this is may be due to low *alk1* mRNA expression in BECs derived at GD50 compared to those derived at PND14, and that TGF-β1 affects in early development are primarily mediated by ALK5. These findings are consistent with studies demonstrating low *alk1* mRNA expression in microvessels derived from mouse forebrain at embryonic day 9[258].

The balance between *alk1* and *alk5* mRNA expression is crucial for healthy brain development as aberrations in these receptor levels contribute to the pathogenesis of congenital conditions, such as brain arteriovenous malformations (BVM). The pathogenesis of BVM, the primary cause of intracranial hemorrhage, is poorly understood. Human studies have shown that there is a decrease in *ALK1* mRNA expression and an increase in *ALK5* mRNA expression in BVM[259]. This imbalance of receptor levels correlates with the lower expression of *ABCB1* mRNA in BVM when compared to normal human brain samples[260]. Therefore, it is possible that compromised brain protection through reduced levels of P-gp activity may contribute to the pathogenesis observed in BVM.

Expression of the TGF-β1 associated receptors, *tgfbr2*, *alk5*, and *endoglin* did not change in BECs derived from GD40 to PND14, suggesting that these receptors are not responsible for the decrease in BEC responsiveness to TGF-β1 with advancing gestation.

However, *betaglycan* mRNA, an accessory receptor to TGF-β1 signaling, dramatically increased in late gestation. Previous studies have demonstrated that betaglycan-mediated changes in TGF-β1 responsiveness vary with cell-type and state[261]. Mesenchyme-derived cells, including mesangial cells, are generally poorly responsive to TGF-β1 and express high levels of betaglycan [262]. Studies have shown that membrane-bound betaglycan decreases cellular responsiveness to TGF-β1 by preventing TGFBR2 from recruiting and activating TGFBR1[216,217]. Betaglycan may function through a similar mechanism in BECs, which may explain why increasing levels of betaglycan in late gestation and post-natal BECs, correlate with decreasing responsiveness to TGF-β1.

The present study also demonstrated that *alk1* mRNA levels were higher in BECs derived from GD65 and PND14 guinea pigs compared to those derived at GD40 and GD50. It has been shown that at earlier stages of development, TGF-β1 acting through the ALK5 receptor is provasculogenic. However, later in development when the endothelial cells have differentiated and both *alk1* and *alk5* are expressed, there is a shift towards an angiogenic state[263]. In terms of the brain vasculature of the developing guinea pig, the highest rate of brain growth occurs from GD40 to PND14[110], which is accompanied by increasing oxygen demand by this tissue. This demand is met by increasing blood flow to the brain via angiogenesis. Thus, the rise in *alk1* mRNA expression BECs derived at PND14 correlates with increasing rates of angiogensis in the brain vasculature of the neonatal guinea pig. Our studies have shown that TGF-β1, at least partially, mediates P-gp function through ALK1 and so it might be expected that TGF-β1 regulation of P-gp would be more potent in BECs derived from late gestation and neonatal guinea pigs. However, as discussed above, betaglycan expression increases

and has been shown to disrupt the interaction between TGFBR2 and TGFBR1.Therefore, an increase in betaglycan may counteract the expected increase in TGF-β1 responsiveness associated with an increase in ALK1 expression. However, the direct relationship between betaglycan and TGF-β1 signaling in BECs requires further investigation.

Based on our data, we can conclude that the timing of TGF-β1 activation at the developing blood-brain barrier is likely important for brain homeostasis. This activation can occur as a result of TGF-β1 derived from blood or brain extracellular fluid. Increased levels of TGF-β1 in maternal plasma levels have been described in gestational diabetes and preeclampsia [264,265]. Since TGF-β1 can cross the placenta[266], this will result in altered TGF-β1 levels in the fetal circulation. Additionally, perturbations in TGF-β1 levels caused by delayed or early gliogenesis, such as that observed in fetal alcohol syndrome and autism [267,268], may effect brain protection and consequently contribute to disease pathogenesis. This may, in turn, result in substantial changes in fetal brain exposure to xenobiotics and other P-gp substrates, many of which have teratogenic properties and thus may contribute to disease progression.

In vitro, we have shown that a single dose of TGF-β1 elicits rapid effects on *Abcb1* mRNA expression and P-gp function, which disappear after 24 hours. Our results infer that TGF-β1 contributes to dynamic regulation of P-gp and that short-term perturbations in TGF-β1 do not result in permanent changes in P-gp function. However, *in vivo*, the release of many astrocyte-derived factors occurs in a pulsatile fashion in response to neuronal activation[269]. The release of TGF-β1 from astrocytes may occur in this manner, which may result in a constant regulation of P-gp. BECs and astrocyte co-culture studies are required to further investigate this important relationship.

In conclusion, TGF-β1 potently regulates P-gp activity and *Abcb1* mRNA at the developing BBB, but the magnitude of these effects is age-dependent. We have shown, for the first time, that ALK5 signaling through SMAD3 is not essential for TGF-β1 regulated P-gp function in fetal BECs. Moreover, we have identified that TGF-β1 signaling through the ALK1 pathway represents an important route in the regulation of P-gp function in the developing BBB, particularly near term. P-gp in the fetal BBB protects the developing brain, preventing a wide spectrum of endogenous and exogenous factors from entering the fetal brain. Aberrations in TGF-β1 levels, either as a result of delayed or early glial cell differentiation, may lead to substantial changes in fetal brain exposure to P-gp substrates, triggering profound consequences with respect to brain development.

Figure 4-1. TGF-β1 increases P-gp activity in fetal and post-natal BECs. P-gp activity in brain endothelial cell (BEC) cultures derived at gestational day (GD)40 (A-D), GD50 (E-H), GD65 (I-L) and post-natal day (PND)14 (M-P) after treatment with TGF-β1 (0.001-10 ng/ml) for 2, 4, 8 or 24h. Activity is displayed as percent change from untreated control cells mean ± SEM (N=6-8/group). A significant difference from control indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

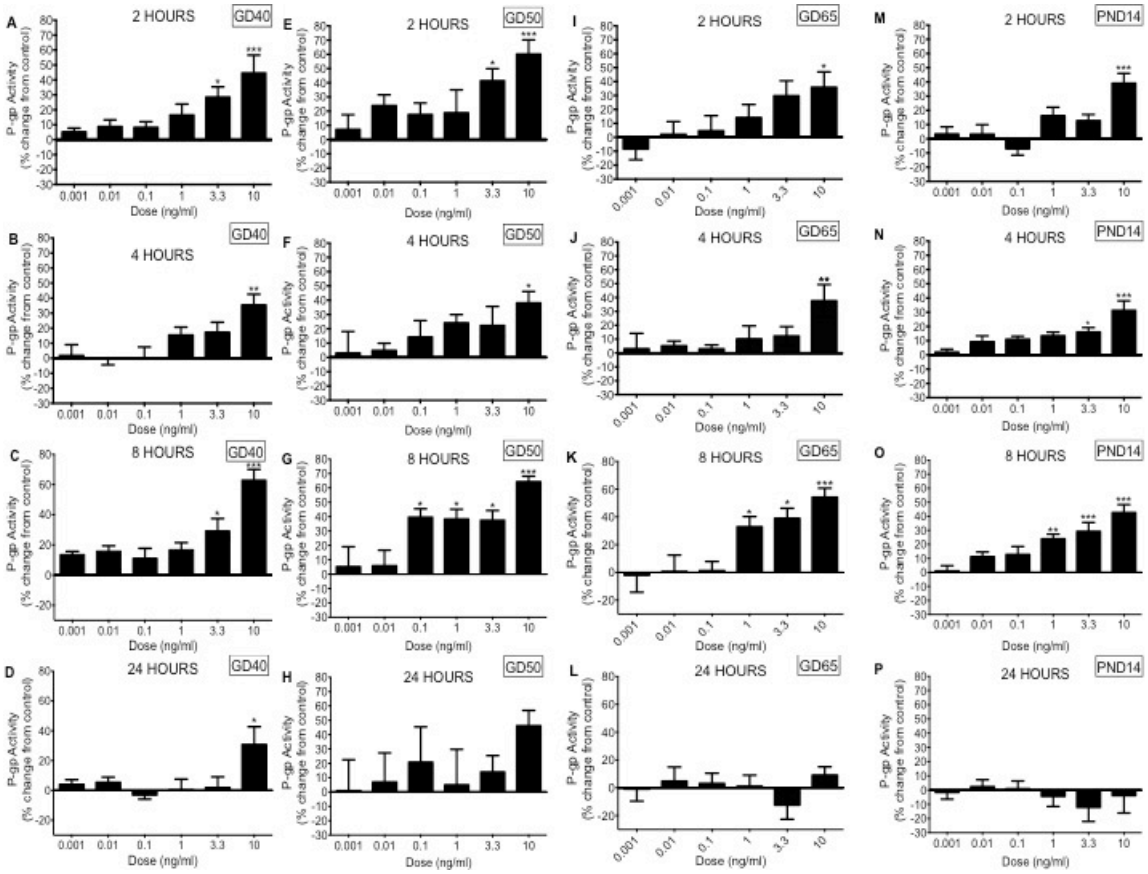


Figure 4-2. Effect of TGF-β1 is specific to P-gp. P-gp activity after treatment with (A) TGF-β1 (10ng/ml for 8h) with or without inhibition of P-gp by 10^{-4} m verapamil (VPL); and (B) P-gp activity (using rhodamine 123 as a P-gp substrate) after an 8h treatment with TGF-β1 (10 ng/ml). P-gp activity is displayed as percent change from untreated control cells (*zero line*). Values displayed as mean ± SEM (N =6-8/group). A significant difference from control indicated by **, $P < 0.01$; ***, $P < 0.001$.

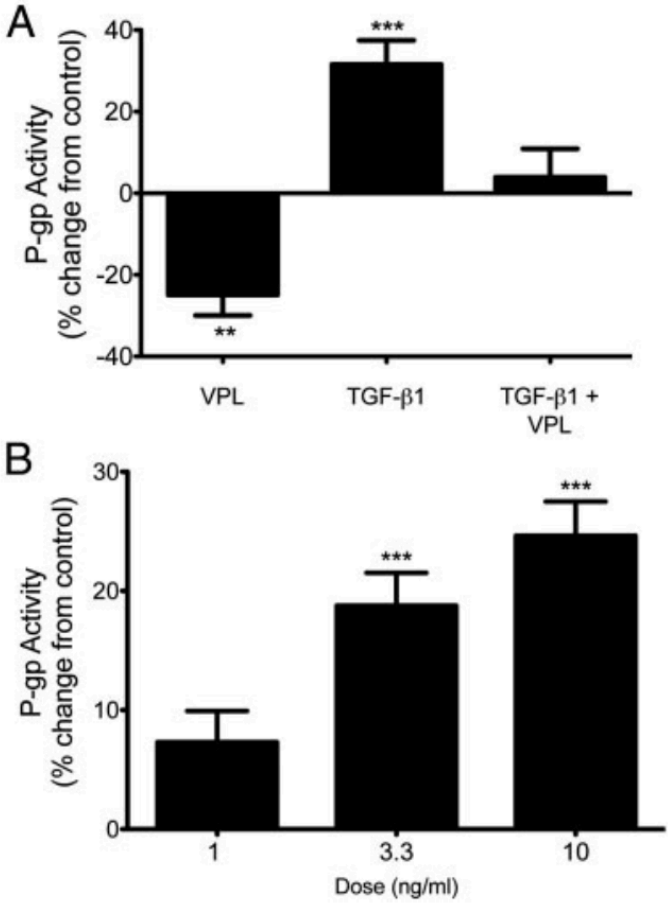


Figure 4-3. TGF-β1 increases *Abcb1* mRNA in BECs. The effect of TGF-β1 (10ng/ml for 8h) treatment on *Abcb1* expression in brain endothelial cell (BEC) cultures derived at post-natal (PND)14. *Abcb1* mRNA expression shown relative to *β-Actin*. Values displayed as mean ± SEM (N =6-8/group). A significant difference from control indicated by **, $P < 0.01$; ***, $P < 0.001$.

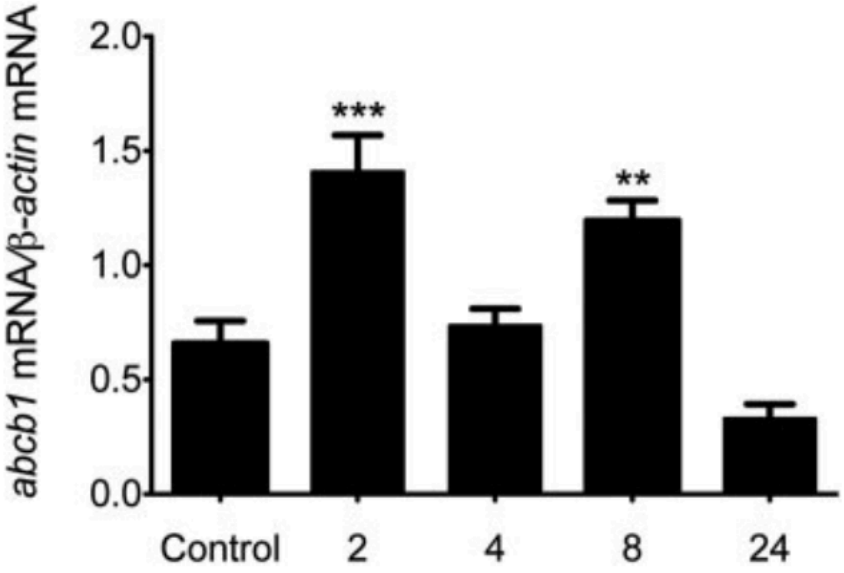


Figure 4-4. *Alk1* and *betaglycan* mRNA expression in BECs increases in late gestation. The developmental profile of (A) *alk1* mRNA (type I receptor) (B) *betaglycan* mRNA (type III accessory receptor) in brain endothelial cells (BECs) derived at gestational (GD)40, GD50, GD65 and post-natal day (PND)14. Expression was normalized to β-actin. Values displayed as mean ± SEM (*N* =6-8/group). A significant difference from GD40 indicated by **, $P < 0.01$; ***, $P < 0.001$.

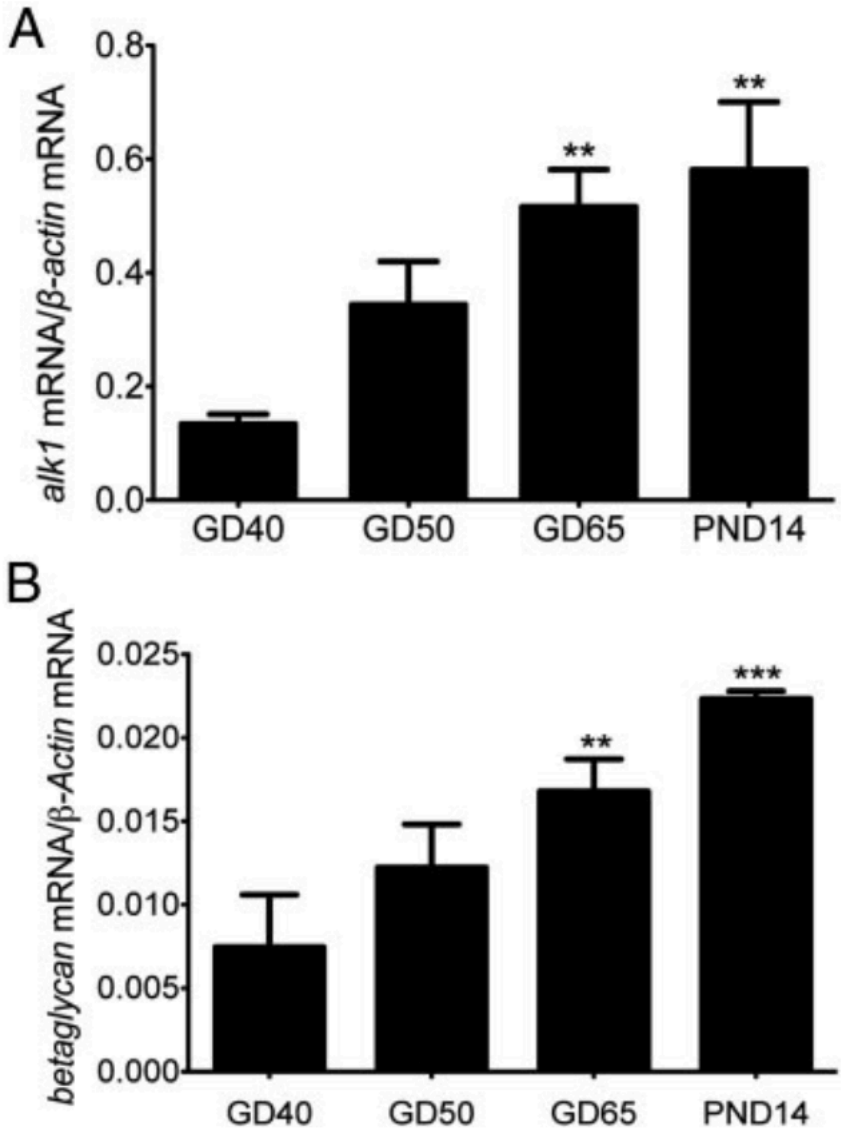


Figure 4-5. SB-431542 attenuates effect of TGF-β1 on P-gp function in BECs. P-gp activity in brain endothelial cell (BEC) cultures derived at (A) gestational (GD)50 and (B) post-natal day (PND)14 after treatment with TGF-β1 (10 ng/ml) and SB-431542 (SB) for 8h. P-gp activity is displayed as percent change from untreated control cells mean ± SEM (N=6-8/group). A significant difference from control indicated by ***, $P < 0.001$. Difference between treatments are indicated by †, P<0.001.

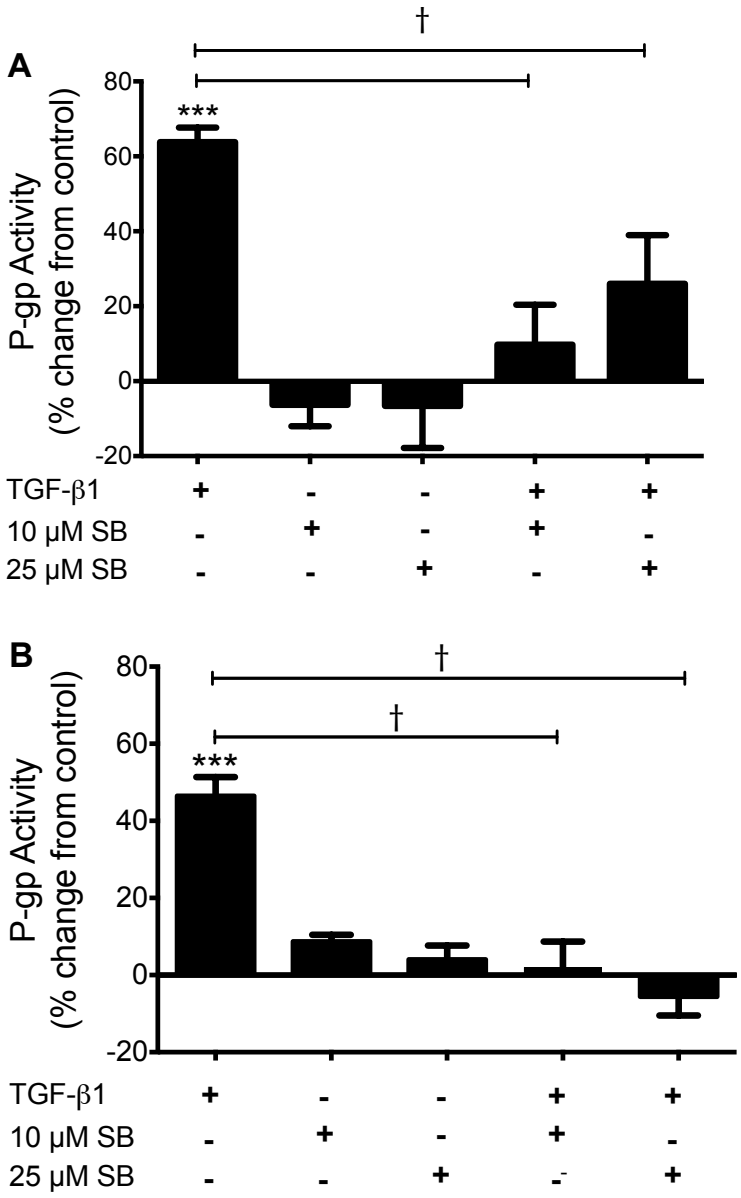


Figure 4-6. SIS3 has no effect on TGF-β1 mediated increase in P-gp function in BECs. P-gp activity in brain endothelial cell (BEC) cultures derived at gestational (GD)50 (A-C) and post-natal day (PND)14 (D-F) treated with TGF-β1 (10 ng/ml) and Specific Inhibitor of SMAD3 (SIS3) for 8 h. (A, D) P-gp activity is displayed as percent change from control cells. (B, E) *Abcb1* mRNA shown relative to β-Actin. (C, F) *Cadherin2* mRNA shown relative to β-Actin. Values displayed as mean ± SEM (N= 6-8/group). Significant difference from control indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Difference between treatments are indicated by †, P<0.001.

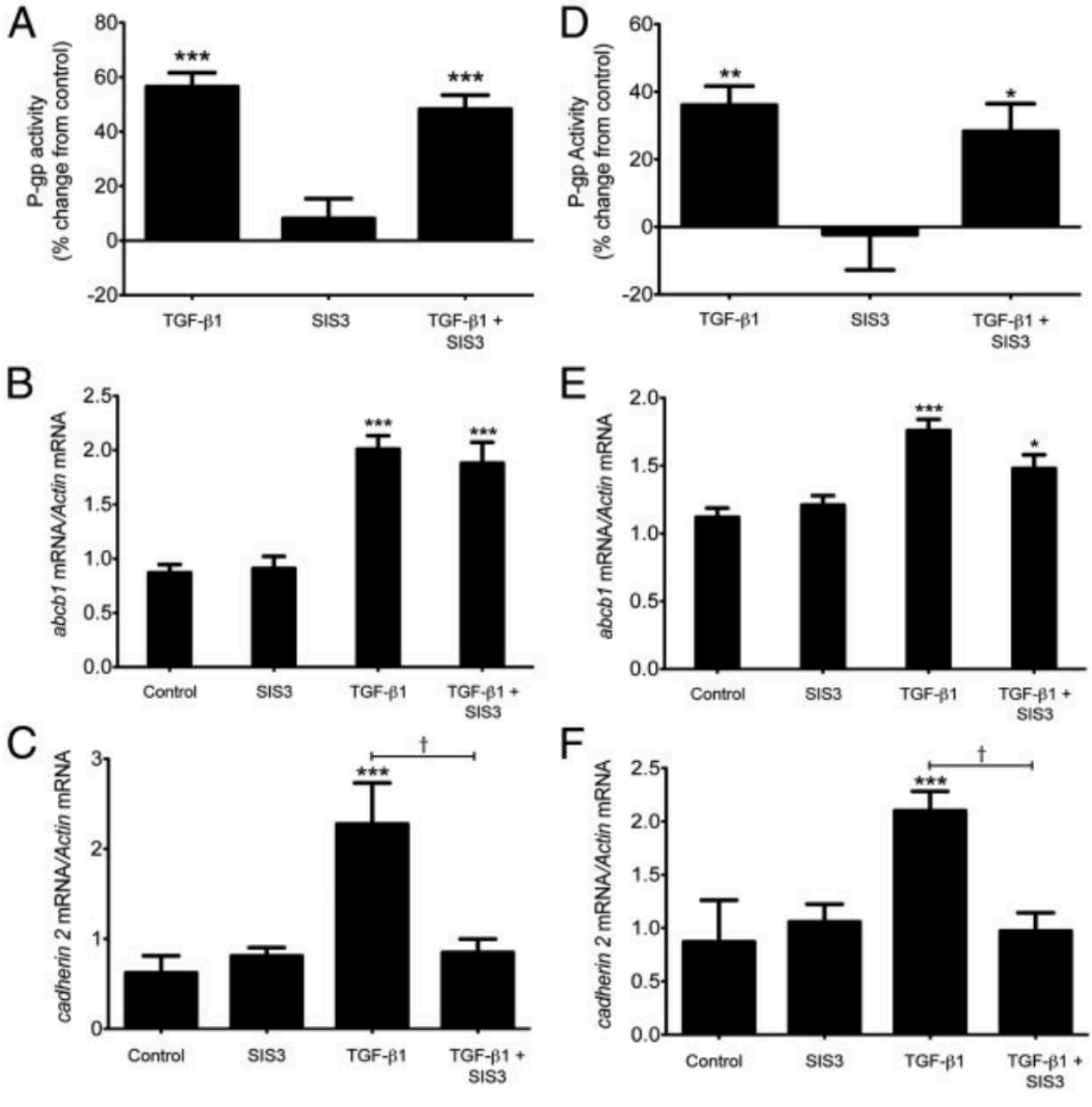


Figure 4-7. BMP-9 increases P-gp activity in BECs. P-gp activity in brain endothelial cell (BEC) cultures derived at (A,B) gestational day (GD)50 and (C, D) post-natal day (PND)14 after treatment BMP-9 (0.001-10 ng/ml) for 8 or 24 h. P-gp activity is displayed as percent change from untreated control cells mean ± SEM (N=6-8/group). A significant difference from control indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

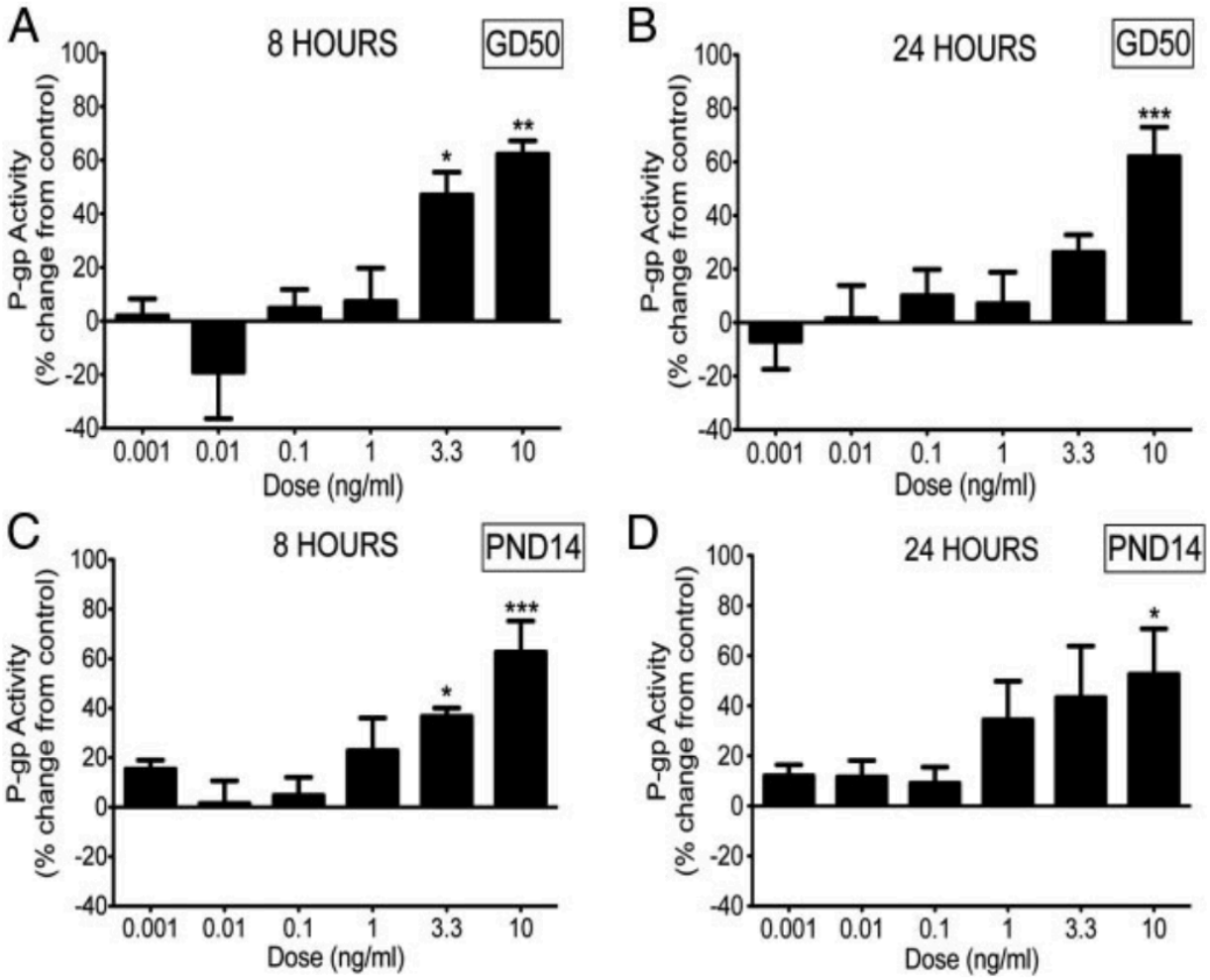
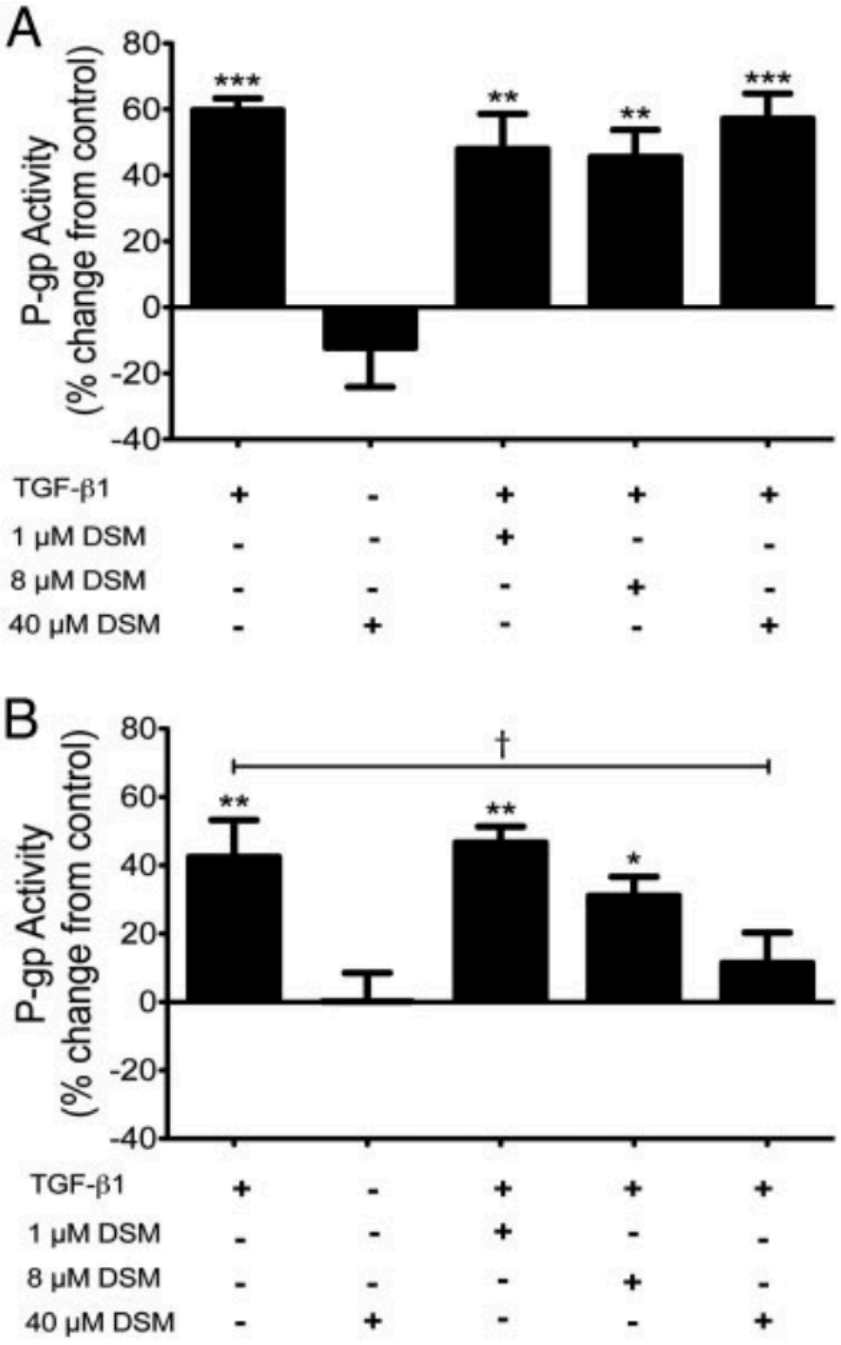


Figure 4-8. Age-specific effect of Dorsomorphin on TGF-β1 mediated increase in P-gp function in BECs. P-gp activity in brain endothelial cell (BEC) cultures derived at (A) gestational day (GD)50 and (B) post-natal day (PND)14 treated with TGF-β1 (10 ng/ml) and Dorsomorphin (DSM) for 8 h. P-gp activity is displayed as percent change from untreated control cells mean ± SEM (N=6-8/group). A significant difference from control indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Difference between treatments are indicated by †, P<0.05.



CHAPTER 5:

Glucocorticoids Modify Effects of TGFβ1 on Multidrug Resistance in the Fetal Blood-Brain-Barrier

5.1 PUBLICATIONS (arising from studies presented in Chapter 5)

Stephanie Baello, Majid Iqbal, Samantha Kearney, Shikha Kuthlia, Enrrico Bloise, William Gibb and Stephen Matthews (2015). Glucocorticoids Modify Effects of TGF-β1 on Multidrug Resistance in the Fetal Blood-Brain Barrier. *Growth Factors* (manuscript submitted).

5.2 INTRODUCTION

P-gp expression at the BBB is low during the first half of gestation but dramatically increases in late gestation and post-natal life in mice, guinea pig and humans[92,93,95]. This upregulation in P-gp at the developing BBB coincides with the rapid phase of astrocyte differentiation in the fetal brain[237,238,270]. TGF-β1 is a multifunctional growth factor released by astrocytes that modulates vascular and neural cells of the CNS. Recently, it was shown that astrocyte-derived TGF-β1 induces inhibitory synapse formation in mouse cerebral cortex neurons, a crucial event occurring late in brain development[226]. Moreover, we have also shown that TGF-β1 has a role in the developing BBB as it induces brain protection via P-gp in fetal and neonatal BECs - an effect that was attenuated with advancing age (Chapter 4).

Pregnant women who are at risk of preterm birth (approximately 10% of all pregnancies) are prescribed sGC, to mature fetal lungs and reduce neonatal risk of respiratory distress syndrome. How such a treatment might influence P-gp function in the developing BBB and therefore protection of the late gestation fetal brain is largely unexplored. In rabbits, maternal sGC treatment decreased vascular endothelial growth factor (VEGF) and increased TGF-β levels in the fetal brain[271]. Prenatal sGC exposure has also been shown to increase expression of TJ proteins in the fetal brain microvasculature[272,273]. We have recently shown that prenatal sGC exposure matures the BBB by increasing levels of *Abcb1* mRNA and P-gp function in BECs (*M Iqbal, S Baello, M Javam, M Audette, W Gibb & SG Matthews, manuscript submitted*). However, very little is known as to how prenatal exposure to sGCs might impact TJ function and modulate the responsiveness of P-gp to astrocyte-derived factors such as TGF-β1 in fetal

BECs. We hypothesized that prenatal exposure to sGC would mature (increase) TJ function in the developing BBB and decrease BECs responsiveness to TGF-β1.

5.3 MATERIALS AND METHODS

5.3.1 Animals, Breeding and Synthetic Glucocorticoid Treatment

Twelve-week-old female Dunkin-Hartley-strain guinea pigs were purchased from Charles River Canada, Inc. (St. Constant, Quebec, Canada). Female guinea pigs were bred as described previously.[92] Pregnant guinea pigs were treated with dexamethasone (DEX; 1mg/kg; Vetoquinol Lavaltrie, Quebec, Canada) or saline (vehicle; VEH) on GD48 and GD49 and euthanized on GD50 (term approximately 68-70 days) (N=5 per treatment group) as described in (M Iqbal, S Baello, M Javam, M Audette, W Gibb & SG Matthews, manuscript submitted). The dosing regimen was based on clinical situations wherein mothers presenting with threatened preterm labor receive two injections of synthetic glucocorticoids, 24 hours apart. Pregnant women usually receive a dose of synthetic glucocorticoids of approximately 0.25 mg/kg[274]. However, in guinea pigs, the glucocorticoid receptor has 4-fold lower affinity to DEX[275]. Thus, a dose of 1 mg/kg was used in the current study. All studies were carried out in accordance with protocols approved by the Animal Care Committee at the University of Toronto and in accordance with the Canadian Council on Animal Care.

5.3.2 Guinea Pig Primary Brain Endothelial Culture

Pregnant guinea pigs (N=6-8 per treatment group) were anaesthetized (isoflurane; Baxter Corp., Mississauga, Ontario, Canada) and euthanized on GD50 and BECs were isolated from male fetal guinea pigs, as described in Chapter 3[92,276]. Cell viability was assessed by trypan blue (Sigma) staining and was determined to be >95%. Cells were frozen in liquid nitrogen until use in the following experiments.

5.3.3 TGF-β1 Treatment and P-gp Functional Assay

BECs derived from DEX-exposed and control male GD50 guinea pigs were plated on gelatin-coated 96-well culture plates (Becton Dickinson Biosciences) at a seeding density of $1x10^4$ cells/cm^2. Cells were grown until confluent (37°C; 5% CO_2/air) and the media was changed to phenol-red free DMEM with charcoal stripped FBS (20%, Wisent Inc.). Cells were treated with TGF-β1 (1-10ng/ml for 8h; Invitrogen) and P-gp function was assessed using the calcein-AM assay, as we have previously described in Chapter 4[92]. TGF-β1 doses and treatment duration were selected based on previous studies that had demonstrated upregulation of P-gp activity and *Abcb1* mRNA levels in fetal- and post-natally-dervied BECs[276]. Cell viability was >95% as assessed by trypan blue (Sigma) staining following TGF-β1 treatment.

5.3.4 TGF-β1 Treatment and Tight Junction Function

BECs derived from DEX and VEH-exposed male GD50 guinea pigs were plated on gelatin-coated 24-well CoStar Transwell Membrane (0.4 μm pore; Fisher Scientific,

Hampton, New Hampshire, USA) at a seeding density of $1x10^4$ cells/cm^2. Once cells were confluent and treated with TGF-β1 (10 ng/ml, 8h; Invitrogen), tight junction function was measured using FITC-dextran permeability assay. BECs were washed with HBSS (Invitrogen) and incubated with HBSS (0.5ml) containing FITC-Dextran (70 kDa, 1.0 mg/mL, Sigma), which was placed into upper chamber and 1.5 mL of HBSS into the lower chamber. After incubation (37°C; 1h) the FITC-Dextran concentration in the lower chamber was assessed at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

5.3.5 Quantification of mRNA expression

Abcb1 mRNA expression (P-gp), expression of genes that encode tight junction proteins (*Ocln* and *Tjp1*) and TGF-β1-inducible genes (*Smad7*, *Cdh2* and *Nedd9)*[248,277] were assessed in BECs following TGF-β1 treatment. Expression of TGF-β associated receptors (*Tgfbr2, Alk1, Alk5, Endoglin, Betaglycan*) were also measured in BECs from VEH- and DEX-exposed fetuses. For mRNA analysis, BECs were cultured on 10 cm^2 gelatin-coated tissue dishes (Becton Dickinson Biosciences) at a seeding density of $1x10^4$ cells/cm^2. BECs at confluence were treated with TGF-β1 (10 ng/ml; 8h) and total RNA extracted, converted to cDNA and level of specific transcripts were quantified using real-time PCR as described in Chapter 3. Relative mRNA expression was calculated as gene of interest expression normalized [ΔΔc(t)] to reference gene expression (β-*actin*). β-*actin* was not altered in BECs following *in vivo* DEX or saline exposure or *in vitro* TGF-β1 treatment (data not shown).

5.3.6 Statistical analysis

All statistical analyses were performed using Prism (GraphPad Software, Inc., San Diego, CA). Two-way ANOVA followed by Bonferonni's test was utilized to compare the effect of TGF-β1 on P-gp and tight junction function, as well as A*bcb1*, *Tjp1*, *Ocln, Smad7, Nedd9 and Cdh2* mRNA levels in BECs derived from DEX-exposed and control fetuses. TGF-β associated receptors (*Tgfbr2, Alk1, Alk5, Endoglin, Betaglycan*) mRNA data were analyzed between BECs from DEX-exposed and control fetuses using an unpaired t-test. Significance was set at $P < 0.05$. Each treatment group consisted of 6-8 male fetuses from independent pregnancies.

5.4 RESULTS

5.4.1 TGF-β1 effects on Abcb1 mRNA and P-gp function in BECs

An 8h exposure to TGF-β1 (10 ng/ml) significantly increased P-gp function in BECs derived from control fetuses (Fig. 5-1A, $P<0.001$). However, there was no increase in P-gp activity after TGF-β1 treatment of BECs derived from DEX-exposed fetuses (Fig. 5-1B). *Abcb1* mRNA was significantly higher ($P<0.05$) in BECs derived from DEX-exposed fetuses compared to controls (Fig. 5-1C). Exposure to TGF-β1 (10ng/ml; 8h) resulted in a significant increase ($P<0.01$) in *Abcb1* mRNA in BECs derived from control fetuses but not those derived from DEX-exposed fetuses (Fig 5-1C).

5.4.2 TGFB1 effects on tight junction function and related mRNA expression in BECs

TGF-β1 significantly decreased FITC-dextran permeability in BECs derived from control fetuses (Fig. 5-2A; $P<0.001$), indicating an increase in TJ function. BECs derived from DEX-exposed fetuses displayed decreased baseline FITC-dextran permeability compared to controls indicating increased TJ function (Fig. 5-2A; $P<0.001$). TGF-β1 caused no further decrease in FITC-dextran permeability in this group. TGF-β1 had no effect on *Ocln* or *Tjp1* mRNA levels in BECs derived from control fetuses (Fig. 5-2B, C). Prenatal DEX-treatment resulted in significant increases in baseline *Ocln* and *Tjp1* mRNA levels ($P<0.001$) but TGF-β1 did not significantly modify the levels of these transcripts (Fig. 5-2B, C).

5.4.3 Effects of Prenatal Dexamethasone on TGFB-Associated Receptors in BECs

As BECs derived from DEX-exposed fetuses displayed decreased responsiveness to TGF-β1 compared to BECs from control fetuses (Fig. 5-1B), expression of TGF-β1-associated receptors was assessed. TGF-β receptor 2 (*tgfbr2*) and *Alk1* mRNA levels were significantly higher in BECs derived from the DEX-exposed fetuses compared to controls (Fig. 5-3A & B; $P<0.01$). In contrast, the expression of *Alk5*, *Betaglycan* and *Endoglin* mRNA was not different between BECs derived from the treatment groups (Fig. 5-3C-E).

5.4.4 Prenatal Dexamethasone Effects on TGF-β1-Associated Intracellular Signaling

SMAD7 transcription is induced by TGF-β family members and is an established marker of cellular responsiveness to TGF-β1[277]. TGF-β1 treatment (10ng/ml; 8h) resulted in an approximate 3-fold increase in *Smad7* mRNA levels in BECs derived from the control fetuses (Fig. 5-4A; $P<0.01$). However, there was no increase in *Smad7* mRNA levels in response to TGF-β1 treatment in BECs derived from the DEX-group.

Levels of *Cdh2* and *Nedd*9 mRNA, TGF-β1 responsive genes, were also assessed in BECs[248]. Treatment with TGF-β1 (10ng/ml; 8h) upregulated *Cdh2* ($P<0.05$) and *Nedd9* ($P<0.001$) mRNA levels in BECs derived from the control fetuses (Fig. 5-4B and 4C). *Nedd9* mRNA levels were higher in BECs derived from the DEX-exposed fetuses compared to the controls but was not altered by TGF-β1 treatment.

5.5 DISCUSSION

We have shown that a single course of maternal sGC treatment appears to decreases responsiveness of fetal BECs to TGF-β1. P-gp and TJ function are increased in BECs derived from control fetuses but not those from DEX-exposed fetuses. This insensitivity to TGF-β1 was associated with failure to upregulate the TGF-β1 responsive genes, *Smad7*, *Nedd9* and *Cdh2* mRNA levels in BECs derived from DEX-exposed fetuses. Together, these results suggest that prenatal exposure to sGC modifies the effect of TGF-β1 on multidrug resistance at the developing BBB. The implications of these

findings for the developing fetal brain are profound and yet complex as P-gp effluxes drugs and toxins but also endogenous compounds, such as hormones and cytokines.

A major finding in this study is the differential effect of TGF-β1 on P-gp function and *Abcb1* mRNA levels in BECs derived from DEX-exposed and control fetuses. TGF-β1 may not have had an effect on P-gp function and *Abcb1* mRNA levels due to the upregulatory effect that sGC has on *Abcb1*/P-gp, which we have recently demonstrated (*M Iqbal, S Baello, M Javam, M Audette, W Gibb & SG Matthews, manuscript submitted*). Thus, our findings demonstrate that there is no synergistic effect of *in vitro* treatment of TGF-β1 and *in vivo* prenatal DEX on *Abcb1*/P-gp. Additionally, this decrease in the responsiveness of BECs to TGF-β1 is similar to that which we have previously shown in BECs derived from juvenile guinea pigs compared to those derived from GD50 fetuses. The effect of TGF-β1 on P-gp activity was considerably greater in BECs derived from GD50 fetuses compared to those derived on PND14[276]. Thus, prenatal DEX treatment appears to mature the fetal BBB by increasing levels of *Abcb1* mRNA and P-gp activity and decreasing responsiveness to TGF-β1.

We also demonstrated that prenatal DEX treatment may mature the BBB by increasing the expression of genes that encode TJ proteins, occludin and ZO-1 in BECs, which was correlated with an increase in TJ function. Our results are consistent with previous studies which demonstrated that a single course of prenatal DEX increased occludin and ZO-1 protein levels in the cortex and cerebellum of fetal sheep [272,273]. These effects may vary within brain regions as prenatal betamethasone treatment has been shown to decrease TJ function in the paraventricular nucleus of post-natal mice [278]. However, it is difficult to compare these studies due to the utilization of different animal

models, prenatal sGC dosing regime and different sGCs. Future studies should investigate whether the effect of prenatal sGC treatment on P-gp in the brain is also region specific.

We found that TGF-β1 intracellular signaling may be aberrant in BECs derived from DEX-exposed fetuses. Baseline levels of *Smad7* mRNA in BECs were unchanged with prenatal DEX treatment. However, when BECs were exposed to TGF-β1 for 8h, those from DEX-exposed fetuses failed to upregulate *Smad7* mRNA levels. This is indicative of a blunted response to TGF-β1 as *Smad7* mRNA is expressed by most cell types and is upregulated in response to TGF-β1. This may be due to an interaction between glucocorticoid and TGF-β signaling pathways since recent investigations have shown that glucocorticoids regulate TGF-β-induced SMAD signaling pathways. High levels of cortisol inhibited the SMAD2/3 pathway in immortalized human fetal hippocampal progenitor cell line, which was mediated by glucocorticoid receptor (GR)[279]. DEX is a potent activator of GR and may be acting in a similar fashion in the present study, resulting in failure of BECs exposed to prenatal DEX to upregulate *Smad7* mRNA levels.

In the present study, the expression of TGF-β1-associated receptors, *Tgfbr2* and *Alk1* mRNA was increased in BECs derived from DEX-exposed fetuses. This is somewhat counterintuitive given that TGF-β1 failed to upregulate *Smad7* mRNA in BECs derived from DEX-exposed fetuses. However, it is tempting to speculate that these receptors may be upregulated in order to compensate for the blunted response to TGF-β1. It is well established that TGF-β1 is involved in the regulation of cell growth and proliferation. Further, there is evidence of blunted responsiveness to TGF-β in fibroids and cancer cells[280-282]. These cells also express increased levels of various TGF-β-

associated receptors[283-285]. Moreover, malignant cells generally express higher levels of *ABCB1* mRNA and P-gp protein compared to normal tissue. Further studies are required to elucidate the intracellular mechanisms that cause the decrease in responsiveness to TGF-β1 in BECs following prenatal DEX expsure.

Prenatal sGC exposure has been linked to peripheral microvascular dysfunction. Femoral microvessels from fetal sheep prenatally exposed to sGC showed increased sensitivity to vasoactive agents, such as nitric oxide, endothelin-1 and bradykin[286]. Another study in fetal sheep reported a single-course of DEX to decrease myosin light chain isoform expression and alter contractile dynamics in the carotid artery[287]. These studies hypothesize that prenatal exposure to sGC makes the fetus more susceptible to cardiovascular disease in adult life. Less is known about the impact of prenatal sGC exposure on cerebrovasculature and its long-term effect. Interestingly, TGF-β1 levels have been shown to be elevated in the cortex of rabbit pups that received a single course of betamethasone[271]. TGF-β1 is important in development of vasculature and surrounding extracellular matrix. Blunted responsiveness to TGF-β1 may explain some of the adverse effects of prenatal sGC on the developing vasculature. It was recently shown that rabbit pups exposed to a single-course of prenatal sGC exhibited decreased angiogenesis and enhanced pericyte coverage of the cerebrovasculature. An altered ratio of *Alk1* to *Alk5* expression in endothelial cells has been shown to affect the migration and proliferation ability in these cells[159]. In accordance with this, we found that *Alk1* mRNA expression was increased in BECs derived from DEX-exposed fetuses.

An impairment of the TGF-β1 signaling pathway has also been implicated in Alzheimer's Disease, particularly, to the early phase of the disease[288]. Several animal and

cellular investigations have indicated that β-amyloid elimination from the brain is partially mediated through active transport by P-gp at the level of the BBB[289]. Lowered P-gp function at the BBB is observed in patients with neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease[290-292]. Analysis of P-gp expression and β-amyloid deposition in the cerebral vessels of 243 non-demented, elderly adults found that vessels with low P-gp contained increased β-amyloid whereas there was no β-amyloid accumulation in vessels with high P-gp [293]. Since prenatal sGC alters responsiveness to TGF-β1 and levels of *Abcb1* mRNA and P-gp protein at the BBB, future studies should investigate whether sGC-exposed fetuses are more likely to suffer from neurodegenerative disease, such as Alzheimer's disease, later in life.

In conclusion, this study is the first to show that single-course of prenatal DEX treatment alters the effect of TGF-β1 on *Abcb1* mRNA and P-gp activity at the developing BBB. TGF-β1 increased *Abcb1* mRNA and P-gp function as well as TJ proteins in BECs derived from control fetuses. However, these effects did not occur in BECs derived from sGC-exposed fetuses. TGF-β1 associated receptors *Alk1* and *Tgfbr2* mRNA expression were upregulated in BECs following prenatal sGC exposure, but TGF-β1 treatment failed to upregulate *Smad7* mRNA in these BECs. Insensitivity to TGF-β1 may have implications on the developing cerebrovasculature as TGF-β1 is a prominent regulator of angiogenesis. This study has also identified novel mechanisms by which antenatal sGC exposure matures the fetal BBB. These alterations may lead to substantial changes in fetal brain exposure to P-gp substrates, triggering profound consequences with respect to brain development and subsequent life-long brain function.

Figure 5-1. TGF-β1 increases P-gp activity in BECs derived from VEH but not DEX fetuses. P-gp activity in BECs derived from fetuses prenatally exposed to either saline (VEH; A) or dexamethasone (DEX; B) following TGF-β1 treatment for 8 hours (N=6-8/gp). P-gp activity is displayed as percent change from untreated control cells (zero line). (C) Levels of *Abcb1* mRNA in BECs derived from VEH- and DEX-exposed fetuses after treatment with TGF-β1 (10ng/ml; 8h; N=6-8). Open bars represent non-TGF treated BECs and solid bars represent TGF-β1 treated BECs. *Abcb1* mRNA expression shown relative to *β-Actin*. Values displayed as mean ± S.E.M. A significant effect of TGF-β1 treatment is indicated by (**) P<0.01; (***) P<0.001. A significant difference between non-TGF treated VEH- and DEX BECs represented by (#) P<0.05.

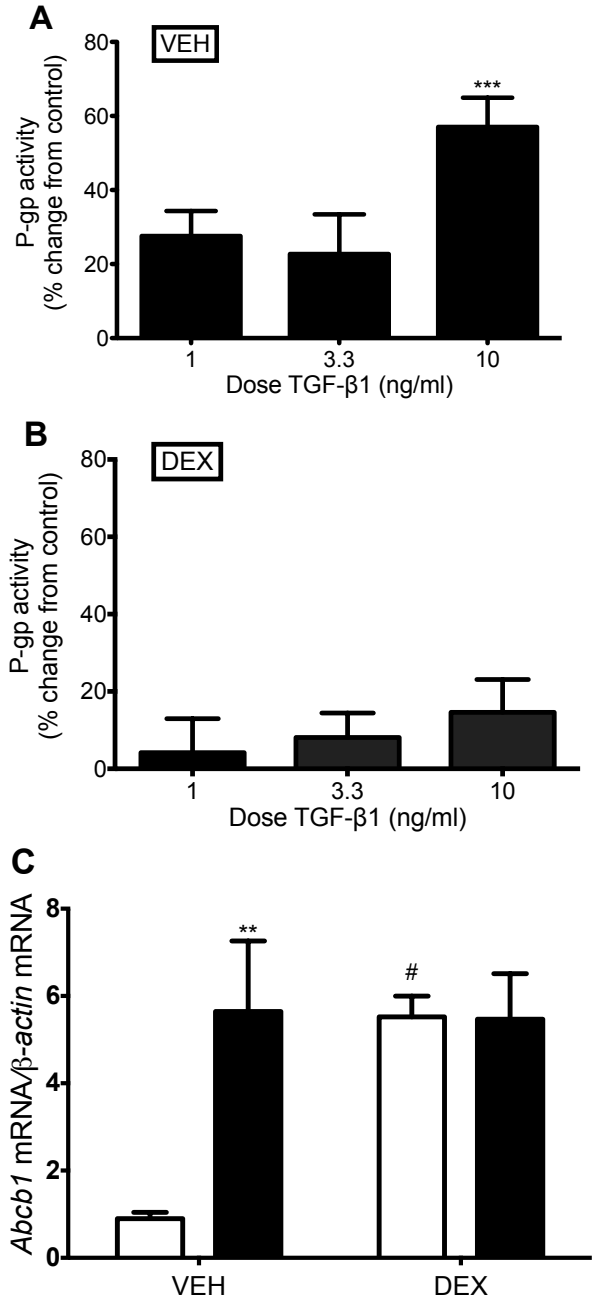


Figure 5-2. **TGF-β1 increases tight junction function and expression in BECs from VEH but not DEX exposed fetuses.** (A) Tight junction function in BECs derived from VEH and DEX-exposed fetuses with and without TGF-β1 stimulation (10 ng/ml; 8h; N=6-8). Expression of genes that encode tight junction proteins B) occludin (*Ocln*) and C) ZO-1 (*Tjp1*) in BECs derived from VEH and DEX-exposed fetuses in the presence or absence of TGF-β1 treatment (10 ng/ml; 8h; N=6-8). Values displayed as mean ± S.E.M. Open bars represent non-TGF treated BECs and solid bars represent TGF-β1 treated BECs. Expression was normalized to β-actin. A significant effect of TGF-β1 treatment is indicated by (***) P<0.001. A significant difference between non-TGF treated VEH- and DEX BECs represented by (#) P<0.001.

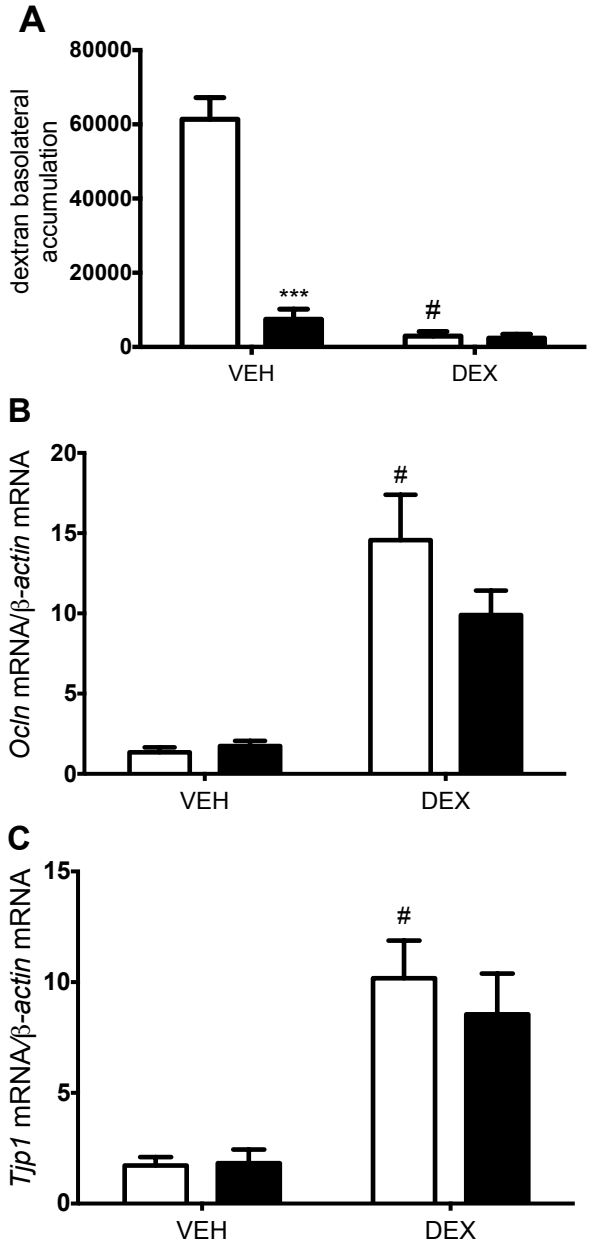


Figure 5-3. Prenatal sGC exposure increases Alk1 and Tgfbr2 mRNA in BECs. Effect of prenatal DEX exposure on mRNA levels for TGF-β1 associated receptors in BECs. Levels of (A) *TGFB receptor type 2* (*Tgfr2*), (B) *Alk1,* (C) *Endoglin,* (D) *Betaglycan,* and (E) *alk5* mRNA in BECs derived from VEH and DEX-exposed fetuses (N=6-8). Expression was normalized to β-actin (n=6-8 per treatment group). All values are displayed as mean ± S.E.M. A significant difference from VEH indicated by (**) $P<0.01$.

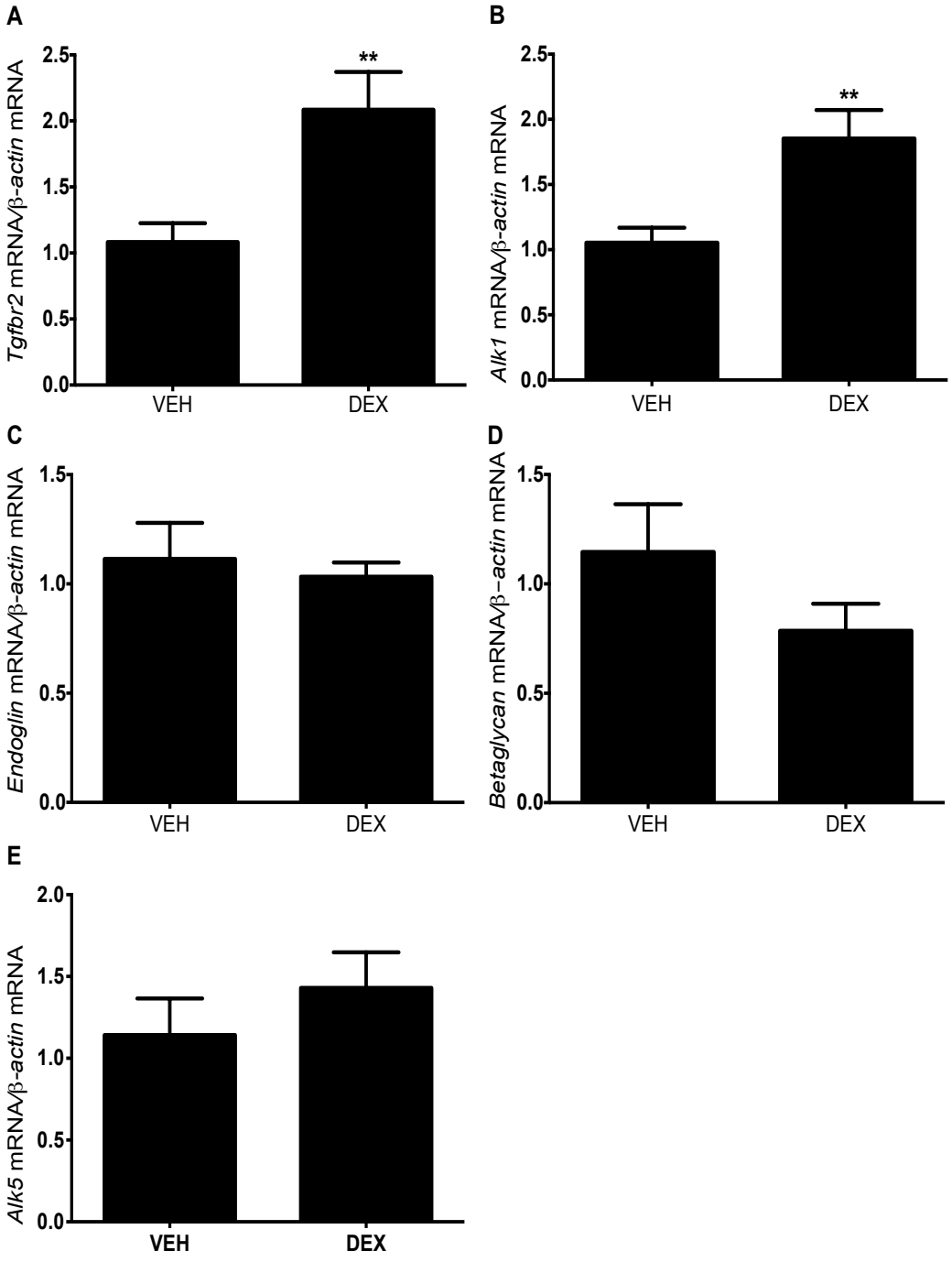
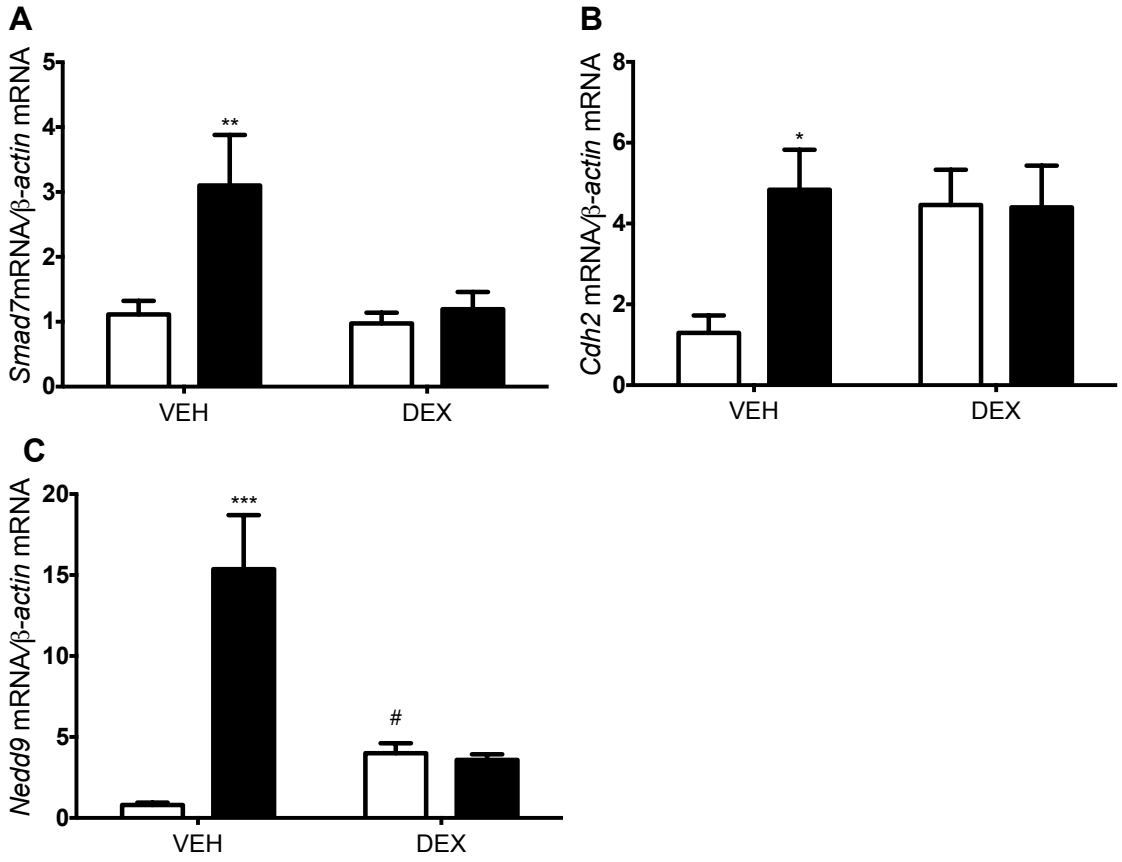


Figure 5-4. BECs from DEX-exposed fetuses display decreased cellular responsiveness to TGF-β1. Levels of (A) *Smad7,* (B) *Cdh2 and* (C) *Nedd9* mRNA in BECs derived from VEH and DEX-exposed fetuses in the presence or absence of TGF-β1 treatment (10 ng/ml) for 8h (N=6-8). Open bars represent non-TGF treated BECs and solid bars represent TGF-β1 treated BECs. Expression was normalized to β-actin. Values displayed as mean ± S.E.M. A effect of TGF-β1 treatment is indicated by (*) P<0.05; (**) P<0.01; (***) P<0.001. A significant difference between non-TGF treated VEH- and DEX BECs represented by (#) P<0.05.



CHAPTER 6:

Astrocyte-Derived Factors Differentially Regulate Multidrug Resistance at the Developing Blood-Brain Barrier: Age-Dependent Effects

6.1 PUBLICATIONS (arising from studies presented in Chapter 6)

Stephanie Baello, Majid Iqbal, William Gibb and Stephen Matthews (2015). Astrocyte-Derived Factors Differentially Regulate Multidrug Resistance at the Developing Blood-Brain Barrier: Age-Dependent Effects. *The FASEB* Journal (manuscript submitted).

6.2 INTRODUCTION

In this study, we investigated the effect of astrocytes on P-gp function and expression at the developing BBB. As described in Chapter 3, we have developed an *in vitro* BBB model comprised of BECs and astrocytes derived from fetal and post-natal guinea pigs. We utilized this model to investigate and characterize astrocyte-derived factors responsible for upregulating P-gp at the developing BBB. We hypothesized that astrocytes would enhance P-gp function and expression in BECs, but that the magnitude of this effect would vary with developmental age.

6.3 METHODS

6.3.1 Animals

Twelve week-old female Dunkin-Hartley-strain guinea pigs were purchased from Charles River Canada Inc. (St. Constant, Quebec, Canada) and were bred as described previously [243]. Two-week old male Dunkin-Hartley-strain guinea pigs were also purchased from Charles River Canada Inc. Animal protocols used in the following studies were approved by the Animal Care Committee at the University of Toronto and in accordance with the Canadian Council on Animal Care.

6.3.2 Isolation and culture of guinea pig primary brain endothelial cells

Isolation of BECs from GD50 and PND14 guinea pigs was carried out as described in Chapter 3[92]. These time-points in development were chosen as our group has found that P-gp expression is low at GD50 and high at PND14 at the BBB[92]. BECs were

frozen in liquid nitrogen. BECs isolated and cultured in this way have been previously characterized [92].

6.3.3 Isolation and culture of guinea pig primary astrocytes

Astrocytes were extracted from GD50 and PND14 guinea pigs using a protocol as described in Chapter 3 [236]. Astrocytes were frozen in liquid nitrogen until use.

6.3.4 Guinea pig co-culture and measurement of tight junction function

BECs and astrocytes were plated on 75 mm^2 tissue culture flasks (BD Biosciences) and grown at 37°C in 5% CO_2/air. To establish co-cultures using GD50 and PND14 BECs and astrocytes, astrocytes were plated in the basolateral compartment of Transwell plate (Corning, Corning, New York, USA) at a seeding density of 1×10^4 cells/cm^2 and BECs were plated on the Transwell insert containing 0.45 μm pores (Corning) at a seeding density of 1×10^4 cells/cm^2. TEER measurements were undertaken every 24h using Millipore Millicell ERS probe MERSSTX01 and ERS-2 Epithelial volt-ohm meter (Millipore, Billerica, Massachusetts, USA). Cells were washed twice with tyrode solution (Sigma). Chopstick electrodes were inserted in the donor and receiver chambers. Final resistance was calculated by subtracting the resistance of blank filters. Cell number between treatments was constant as measured by a hemocytometer.

TJ function was also assessed using a permeability assay with FITC-dextran (70 kDa; Sigma). Cells were washed twice with HBSS (Invitrogen). FITC-dextran was added

to the apical compartment (1 mg/ml) and after 1h at 37°C, FITC-dextran concentration was assessed in the basolateral compartment (Ex/Em: 485/530 nm).

6.3.5 P-gp functional assay

P-gp activity was measured using calcein-AM as described in Chapter 4[92,276]. BECs were washed twice with Tyrode solution (Sigma) incubated at 37°C. BECs plated on inserts were transferred to new 24-well tissue culture plates (BD Biosciences) and washed. Subsequently, BECs were incubated with calcein-AM (10^{-6} M calcein-AM; Sigma) for 1h at 37°C in 5% CO_2/air. After incubation, BECs were placed on ice, washed twice with tyrode solution (Sigma), and lysed with ice-cold 1% Triton X-100 (Sigma) lysis buffer. Intracellular calcein was measured using a spectrophotometer (Ex/Em: 485/510 nm).

6.3.6 Quantification of mRNA expression

BECs were mono-cultured or co-cultured on 24-well Transwell inserts as described above (Corning). On Day 3 of co-culture, BECs were trypsinized (0.05% trypsin-EDTA (Invitrogen)) and centrifuged (1000 g, 5 min). Total RNA was extracted, reverse transcribed to cDNA and levels of specified transcripts were measured via real-time PCR as described in Chapter 3. Relative mRNA expression was calculated as gene of interest expression normalized [$\Delta\Delta c(t)$] to reference gene expression, β-actin, and expressed as fold-change from control. Expression levels of β-actin did not change with age or co-culturing conditions.

6.3.7 Collection of astrocyte-conditioned medium

Astrocytes from GD50 and PND14 guinea pigs were plated on 150 mm^2 petri dishes at a seeding density of 1×10^4 cells/cm^2. Once confluent, astrocytes were washed twice with HBSS (Invitrogen) and media was changed to phenol-red free Neurobasal A medium (Invitrogen). After 24h, astrocyte conditioned medium (ACM) was collected, centrifuged at 1000 g for 10 minutes and passed through a 0.22 μM filter. ACM was frozen at -80°C until use.

6.3.8 Heat-inactivation, protease treatment and crude exosome-depletion of astrocyte-conditioned medium

ACM was subjected to heat-inactivation at 100°C for 4h. For protease treatment, ACM was incubated with trypsin (200 μg/ml; Sigma) at 37°C for 1h and subsequently incubated with trypsin inhibitor (400 μg/ml; Sigma) at 37°C for 30 minutes. As a control, trypsin and trypsin inhibitor were mixed and incubated at 37°C for 1h and 30 minutes and then added to ACM. Crude exosome-depletion was carried out by centrifuging ACM at 100000 g for 6h and the supernatant collected.

6.3.9 Fractionation of astrocyte-conditioned medium

To obtain fractions of ACM containing soluble factors of varying molecular weights, ACM was fractionated using Centrifugal Filter Devices with different MWCO (Molecular Weight Cut-Offs) Ultracel membranes (Millipore). First, 15 mL of ACM was

loaded onto Centrifugal Filter Device with Ultracel membranes with a 100 kDa cut-off and centrifuged at 2700 x g for 35 min. The retentate was collected and resuspended in in the same volume of Neurobasal A medium (Invitrogen) as the loaded sample. The filtrate was collected and resuspended in in the same volume of Neurobasal A medium as the loaded sample and subsequently loaded onto a column with a 30 kDa membrane cut-off and centrifuged at 2700 x g for 35 min. The retentate and filtrate were again collected and resuspended in the same volume of medium as loaded sample. The filtrate was loaded onto a column with a 3 kDa membrane cut-off and centrifuged at 2700 x g for 35 min. BECs were then treated with these fractions of ACM containing proteins of varying molecular weights and P-gp function was assessed.

6.3.10 In-solution digestion

ACM was sent to the SickKids Proteomics, Analytics, Robotics & Chemical Biology Centre (SPARC BioCentre) for in-solution digestion. The SPARC BioCentre protocol for in-solution digestion is described. Proteins in ACM were denatured, reduced and alkylated prior to digestion and LC-MS/MS. Briefly, protein (10 mg) was incubated with urea (8M, Sigma), 50mM Tris-HCl (Sigma; pH 8), and 4mM DTT (Sigma) at 60°C for 30 minutes and then incubated with iodoacetamide (100mM; Sigma, 22C, 15 mins). The samples were subsequently diluted five times with 0.1 M Tris–HCl pH 8.3 (Sigma) and digested using trypsin (1:50 w/w; Sigma; 37°C, 16 h). Samples were desalted using Pierce C18 Spin Tips (Thermo Fisher Scientific) as per manufacturer's protocol.

6.3.11 LC-MS/MS analysis

ACM was sent to the SickKids SPARC BioCentre for LC-MS/MS. Tryptic digests were analyzed on an Orbitrap analyzer (Q-Exactive, Thermo Fisher Scientific) outfitted with a nanospray source and EASY-nLC nano-LC system (Thermo Fisher Scientific). Lyophilized peptide mixtures were dissolved in 0.1% formic acid and loaded onto a 75µm x 50cm PepMax RSLC EASY-Spray column filled with 2µM C18 beads (Thermo Fisher Scientific) at a pressure of 800 Bar. Peptides were eluted over 60 min at a rate of 250nl/min using a 0 to 35% acetonitrile gradient in 0.1% formic acid. Peptides were introduced by nano-electrospray into the Q-Exactive mass spectrometer (Thermo Fisher Scientific). The instrument method consisted of one MS full scan (400–1500 m/z) in the Orbitrap mass analyzer with an automatic gain control (AGC) target of 1e6, maximum ion injection time of 120 ms and a resolution of 70,000 followed by 10 data-dependent MS/MS scans with a resolution of 17,500, an AGC target of 1e6, maximum ion time of 120 ms, and one microscan. The intensity threshold to trigger a MS/MS scan was set to 1.7e4. Fragmentation occurred in the HCD trap with normalized collision energy set to 27. The dynamic exclusion was applied using a setting of 10 seconds.

6.3.12 Protein Identification

Raw data generated by LC-MS/MS (peaklists generated by Xcalibur 2.2) were analyzed using Xcalibur (Thermo Fisher Scientific) for ion current analysis, and were searched against the guinea pig SwissProt UniProt protein-database (containing 20392 entries) at the SickKids SPARC BioCentre. Database searching was undertaken using

SEQUEST version 1.4.0.288 (through Proteome Discoverer, Thermo Fisher Scientific). The data was then imported into Scaffold 4.3.4 (Proteome Software, Portland, OR, USA) and X!Tandem CYCLONE (2010.12.01.1) database searching was performed. These databases were searched with a parent ion tolerance of 10.0 PPM, and a fragment mass tolerance of 0.6Da. Peptide identifications were accepted if they could be established at greater than 95% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95% probability and contained at least 3 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [294].

6.3.13 Protein Quantification

Proteins were quantified based on the average MS signal response for the three most intense tryptic peptides for each protein and expressed as percentage of total protein. Briefly, the MS files were searched as listed above and the SEQUEST result files were used to generate a spectral library in Skyline 2.1.0.4936 [295] using the guinea pig sequences from SwissProt as background proteome. Employing the same parameters as for the SEQUEST search, the three most abundant peptides for each protein were manually chosen from all peptides available in the spectral library. After data import, the chromatographic traces (extracted ion chromatograms) were manually inspected and adjusted where needed to correct wrongfully assigned peaks. Proteins identified with less than two peptides were not included in the quantification. The relative abundance of proteins quantified in three samples was calculated as the average MS intensity for the two peptides for each protein divided by the sum of the average signal for all quantified

proteins in the sample. Relative abundance of protein displayed as fold change from protein levels found in GD50 ACM.

6.3.14 Statistics

Statistical analyses were performed using Prism (GraphPad Software, Inc., San Diego, CA). Effect of co-culture or ACM on TJ function and *Abcb1*/P-gp was analyzed using Student's Unpaired T-test. Effect of heat-inactivated and exosome-depleted ACM was also analyzed via Student's Unpaired T-test. Effect of age-match and different-aged co-culture, protease-treated ACM and fractionation of ACM data were analyzed using one-way ANOVA, followed by Newman-Keuls *post hoc* analyses. Significance was set at $P<0.05$. Each treatment group consisted of 6-8 animals.

LC-MS/MS data were analyzed using MSstats (3.1.4; Vitek Lab, Purdue University). Three biological replicates of ACM were collected at each gestational age. Proteomic differences between GD50 and PND14 ACM were evaluated for statistical significance ($P<0.05$) by Student Unpaired T-Tests, and corrected for multiple testing using the Benjamini–Hochberg correction.

6.4 RESULTS

6.4.1 Effect of astrocytes on tight junction function in BECs

Co-culture of age-matched cells increased TEER in GD50 ($P<0.01$) and PND14 ($P<0.05$) BECs compared to mono-cultured cells after 48h and 72h (Fig. 6-1A, B). After

72h in culture, co-cultured PND14 BECs attained a TEER of approximately 452 Ω•cm^2 while co-cultured GD50 BECs displayed a TEER of 109 Ω•cm^2 (Fig. 6-1A, B). These results were corroborated with FITC-dextran permeability assay. Co-cultured GD50 and PND14 BECs also demonstrated decreased permeability to FITC-dextran after 72h in culture compared to mono-cultured BECs, approximately 60% and 30% respectively (*P<0.05*, *P<0.001*) (Fig. 6-1C, D).

6.4.2 Effect of astrocytes on P-gp activity and levels of *Abcb1* mRNA in BECs

Since TJ function was maximal after 72h in culture, P-gp activity and levels of *Abcb1* mRNA were measured in mono-cultured and co-cultured BECs at this time-point. P-gp activity in GD50 and PND14 BECs co-cultured with age-matched astrocytes increased by approximately 2-fold (*P<0.01*) and 7-fold (*P<0.001*) respectively, compared to mono-cultured BECs (Fig. 6-2A, B). This corresponded to a 3-fold increase in *Abcb1* mRNA (*P<0.05*) in co-cultured PND14 BECs compared to mono-cultured PND14 BECs (Fig. 6-2D). However, there was no statistically significant change in *Abcb1* mRNA levels in co-cultured GD50 BECs compared to mono-cultured GD50 BECs (Fig. 6-2C).

6.4.3 Effect of age of astrocytes on P-gp activity and levels of Abcb1 mRNA in BECs

Co-culture induced more dramatic changes in P-gp activity and levels of *Abcb1* mRNA in PND14 cultures compared to GD50 cultures. Therefore GD50 BECs were co-

cultured with PND14 astrocytes and vice versa. Co-culturing GD50 BECs with PND14 astrocytes induced a greater increase in P-gp function compared to co-culturing with GD50 astrocytes (*P<0.01*; Fig. 6-3A). However, despite the enhancement in P-gp activity in GD50 BECs after co-culturing with PND14 astrocytes, there was no statistically significant increase in levels of *Abcb1* mRNA compared to GD50 BECs that were mono-cultured or co-cultured with GD50 astrocytes (Fig. 6-3C). Moreover, co-culturing PND14 BECs with GD50 astrocytes did not increase in P-gp function or levels of *Abcb1* mRNA in PND14 BECs (Fig. 6-3B, D).

6.4.4 Effect of astrocyte-secreted factors on P-gp function in BECs

To determine if the effects of co-culture on P-gp function were due to soluble astrocyte-derived factors, we exposed PND14 BECs to ACM from PND14 astrocytes. P-gp activity was increased in PND14 BECs after 24h of exposure compared to control BECs exposed to unconditioned medium (UNM) (*P<0.05*; Fig 6-4A). Surprisingly, the effects of ACM on P-gp activity were not observed at the 48h or 72h time-points (Fig. 6-4B, C). However, when ACM was changed every 24h, the effects were rescued at the 48h and 72h time-points (*P<0.001, P<0.001*; Fig. 6-4D, E).

To investigate whether the astrocyte-secreted factors responsible for increasing P-gp activity were proteins, ACM was subjected to heat-inactivation and trypsin (protease) treatment. Heat-inactivated ACM did not increase P-gp activity in PND14 BECs after 24h of exposure compared to BECs exposed to UNM (Fig. 6-5A). Treatment of ACM with trypsin also prevented the increase in P-gp activity and the stimulatory-effect was

restored by inhibiting the protease (*P<0.001*; Fig. 6-5B). We also determined the potential role of exosome-mediated communication between astrocytes and BECs (Fig. 6-5C). Exosome-depletion of ACM did not affect its stimulatory affect on P-gp activity (*P<0.001*) indicating that exosome protein cargo is not responsible for the stimulatory effect.

6.4.5 Effect of ACM fractions on P-gp function in BECs

To determine the approximate size of the protein(s) responsible for upregulating P-gp function in BECs, ACM was fractionated using successive filtration (molecular weight ranges: < 3kDa, 3-30 kDa, 30-100 kDa, >100 kDa). P-gp activity was increased in PND14 BECs that were exposed to ACM containing 3-30 kDa and 30-100 kDa proteins, by approximately 4-fold (*P<0.001, P<0.001*; Fig. 6-6) but was not significantly altered by ACM containing <3kDa or >100 kDa protein fractions.

6.4.6 Differential expression of proteins in GD50 and PND14 ACM

Using LC-MS/MS, 467 proteins were identified in conditioned medium from GD50 and PND14 astrocytes. Of these proteins, 85 were found to be differentially secreted by PND14 and GD50 astrocytes, all of which were elevated in PND14 ACM compared to GD50 ACM (*P<0.05*; Table 6-1). The five principal proteins were Y-box binding protein-1 (YB-1), high-density lipoprotein binding protein, melanoma differentiation-associated gene 20, SERPINE1 MRNA Binding Protein 1 and Actin, Beta-Like 2.

We further analyzed the differentially secreted proteins using GO Term Finder based on cellular component (Fig. 6-7A). Of these 85 proteins, 59 were identified as being extracellularly located and 54 were associated with extracellular exosomes. GO analysis also associated 74 proteins with cytoplasmic localization, with some proteins related to cytoskeletal and cell-junction related proteins. Proteins were also analyzed based on GO function (Fig. 6-7B). Most proteins (~92%) were classified as ligand proteins, capable of binding to other molecules, such as small molecules (i.e. monosaccharaides), organic cyclic compounds, actin, nucleic acids, and macromolecules.

6.5 DISCUSSION

This is the first study to establish a co-culture model using BECs and astrocytes from two distinct developmental ages. Using this co-culture model, we demonstrated that astrocytes enhance TJ and P-gp function in BECs. However, post-natal astrocytes can upregulate P-gp function in fetal BECs, while fetal astrocytes had no significant effect on P-gp activity in post-natal BECs. Thus, the BBB-inducing properties of astrocytes are dependent on developmental stage at which astrocytes were derived. We also found that ACM could mimic these effects on P-gp function, suggesting a mechanism that involves soluble astrocyte-derived factors. Using heat-inactivation and protease treatment, we demonstrated that these astrocyte-derived factors are dependent upon proteins. Fractionation of ACM indicated that these factors are in the molecular weight range of 3-100 kDa. Moreover, LC/MS-MS identified 85 proteins that were significantly upregulated in PND14 ACM compared to GD50 ACM.

We have previously shown that isolated BECs retain their developmental characteristics. In the present study, we demonstrate that astrocytes also maintain properties appropriate to the age at which they were derived. Astrocytes derived at GD50 and PND14 express astrocyte-markers appropriate for their developmental age. Nestin is expressed by GD50 astrocytes but not PND14 astrocytes. This is consistent with astrocyte development *in vivo* as nestin is expressed by immature, developing CNS cells [296,297]. Nestin expression is downregulated and is transiently co-expressed with GFAP during astrocyte differentiation [298]. Astrocytes derived at PND14 but not GD50 express AQP4. AQP4 is the major water channel expressed in brain perivascular astrocyte processes and its expression is upregulated *in vivo* as differentiating astrocytic endfeet make contact with the brain microvasculature [299]. GFAP was expressed in both GD50 and PND14 astrocytes. GFAP may be important in astrocyte-mediated upregulation of P-gp expression and function at the BBB since GFAP KO mice exhibit a structurally and functionally impaired BBB [300].

The majority of published studies that have investigated the effects of astrocytes on the BBB have utilized co-culture models composed of BECs and astrocytes from different ages, usually from adult tissues, or cells from different animal species. These studies demonstrated that astrocytes enhanced many BBB characteristics in co-cultured BECs compared to mono-cultured BECs [141,143,164]. However, our study highlights the importance of the maturational status of astrocytes in the regulation of P-gp developing BBB. We utilized a novel co-culture model using BECs and astrocytes derived from the same animal at two precise time-points in development to show that post-natal and fetal astrocytes can enhance P-gp function in age-matched BECs. However, post-natal

astrocytes increased P-gp function in fetal BECs but fetal astrocytes had no effect on post-natal BECs. Other studies have shown that the maturational state of astrocytes also affects neuronal development. Astrocytes derived from newborn and adult rats were shown to differ in their capacity to sustain neurite outgrowth in retinal ganglion cells in co-culture [301,302]. Thus, the maturational status of astrocytes appears to influence both neuronal and BBB development.

A novel finding of our study was that PND14 astrocytes exhibit different effects on P-gp function and levels of *Abcb1* mRNA in GD50 and PND14 BECs. There was strong correlation between *Abcb1* mRNA levels and P-gp function in PND14 co-cultures. However, in GD50 BECs, enhancement in P-gp activity in GD50 BECs as a result of co-culturing with age-matched or different-aged astrocytes, did not correspond to statistically significant increases in *Abcb1* mRNA levels. This indicates that there may be different mechanisms involved in upregulation of P-gp function in GD50 and PND14 BECs. It is well-established that P-gp function can be enhanced without changes at the level of mRNA. This includes post-translational modifications to P-gp protein, such as glycosylation and phosphorylation, both of which lead to an increase in P-gp function [16,21,23]. P-gp function can also be increased by the recruitment of intracellular storage of P-gp in vesicles to the cell membrane [10]. Additionally, the epigenetic status of the *Abcb1* promoter may vary between GD50 and PND14 BECs. The density of methylation in the *Abcb1* promoter is negatively correlated with *Abcb1* expression in cancer cells [303,304]. Our lab has shown that global DNA methylation in brain microvessels significantly decreases from GD50 to PND14 (M. Iqbal, J. Pappas, S.G. Matthews; *unpublished observations*). However, it has yet to be determined if these global changes in methylation include

specific changes in promoter methylation of the *Abcb1* gene. Early in gestation, increased methylation of the *Abcb1* promoter may prevent binding of transcription factors, activated by astrocytes-derived molecules, from increasing *Abcb1* mRNA levels in GD50 BECs. Moreover, GD50 and PND14 BECs may respond differently to astrocyte-derived signals from PND14 astrocytes simply because they express different types or levels of receptors.

Utilizing ACM protease treatment and heat-inactivation, we demonstrated that the astrocyte-derived factor(s) is indeed a protein or dependent on a protein. We also showed that 3-30 kDa and 30-100 kDa PND14 ACM fractions can upregulate P-gp function in PND14 BECs. These data indicate that more than one factor is likely responsible for this effect. Moreover, of the 85 proteins that were found to be significantly upregulated in PND14 ACM compared to GD50 ACM, 63 proteins fall into these molecular weight ranges. This suggests that astrocytes may regulate P-gp function and *Abcb1* mRNA levels via multiple signaling pathways, which are activated by various astrocyte-secreted proteins. Consistent with this hypothesis, studies have implicated many astrocyte-derived factors in regulation of P-gp at the adult BBB. These pathways include sonic hedgehog (shh), Wnt/β-catenin canonical pathway, retinoic acid and transforming growth factor-β (TGF-β) superfamily [68,106,107,145,146,149,276]. However, our studies do not conclusively rule out the role of lipid or fatty acid derivatives in regulating P-gp. For example, the stability of retinol, from which retinoic acid is derived, is dependent on proteins [305,306] and retinol-binding protein was identified in conditioned media from GD50 and PND14 astrocytes. Moreover, GO analysis classified most of the significantly upregulated proteins in PND14 ACM (Table 6-1) as ligand proteins, capable of binding to small molecules,

nucleic acids, macromolecules and organic cyclic compounds. Further studies are needed to elucidate the identity of the factor(s) involved in astrocyte-mediated upregulation in P-gp function and expression at the fetal and post-natal BBB.

The most differentially expressed protein between GD50 and PND14 astrocytes was Y-box binding protein (YB-1), which was secreted approximately 18-fold more by PND14 astrocytes compared to GD50 astrocytes. YB-1 is a transcription factor that regulates genes such as *Abcb1* [307,308]. However, recent studies indicate that many cell types release YB-1 into the extracellular space [309,310]. Nanomolar concentrations were shown to stimulate proliferation and migration of rat mesangial cells and human kidney cells [309]. This effect was mediated by YB-1 interaction with EGF-repeats of the Notch3 receptor, consequently activating canonical Notch signaling [311,312]. Currently, very little is known about how Notch signaling regulates P-gp function and expression. The only evidence of such regulation stems from cancer cells, in which Notch signaling is generally over-activated and is associated with increased P-gp function [313,314]. For example, knockdown of Notch1 receptor leads to decreased *ABCB1* and *ABCC1* mRNA in cultured intrahepatic cholangiocarcinoma cells [313]. Moreover, overexpression of Notch1 resulted in an increase in *ABCB1* mRNA in gastric human cancer cell lines [315]. Future studies are needed to investigate the role of Notch signaling in regulating P-gp in normal tissues.

High-density lipoprotein binding protein (HDLBP) was also found to be increased by approximately 16-fold in PND14 ACM compared to GD50 ACM. HDLBP binds high-density lipoprotein (HDL), which transports cholesterol, phospholipids and triglycerides. A positive association between cellular cholesterol content and P-gp

function has been demonstrated in various cancer cell lines [316,317]. However, the mechanism by which this occurs is unclear. HDLBP may mediate this effect as it is a secreted protein that can become anchored to the cell membrane through glycosylphosphatidylinositol-enriched domains [318]. Binding of cholesterol to HDLBP via HDL may elicit various signaling pathways [319]. Consistent with this hypothesis, brain cholesterol content is maximal in late gestation and post-natal life, which is coincident with the surge in P-gp at the BBB [320]. Additionally, HDLBP can bind to RNA and prevent its degradation [319]. PND14 astrocytes may secrete more HDLBP, resulting in the stabilization of *Abcb1* mRNA in BECs. The link between cholesterol and P-gp warrants further investigation, especially in the context of the developing BBB.

` We demonstrated that exosome-mediated communication may not be responsible for the astrocyte-mediated effect on P-gp function in co-culture. We attempted to remove exosomes from ACM by centrifuging ACM for 100000 g for 6h. However, a limitation of this technique is that smaller exosomes may not have been pelleted as efficiently as larger exosomes. Thus, future studies should use optimized exosome capturing techniques, such as nanoparticle tracking analysis, to rule out the role of exosomes in the communication between astrocytes and BECs.

We also found that ACM has short-term effects on P-gp function in BECs, as these effects disappear at 48h and 72h. However, when ACM was replaced every 24h, the effect was rescued at 48h and 72h, indicating that BECs are responsive to these signals and that these soluble factors may be labile or metabolized. However, the *in vivo* induction of P-gp at the BBB by astrocytes is constant, as the release of many astrocyte-secreted factors are dependent on neuronal activity [103]. Recently, astrocytes have been

shown to be responsible for neurovascular coupling, a process by which blood flow is matched to neuronal activity [321]. This coupling may regulate BBB properties through development. However, very little is known about how this process occurs in the developing brain or how it may affect BBB development.

It is clear that disruptions in astrocyte-neuron interaction contribute to the pathogenesis of neurodevelopmental disorders [193,322]. However, this study highlights the importance of astrocyte-BBB interaction. Disruptions in astrocyte development may also dysregulate P-gp at the developing BBB, which may contribute to the CNS dysfunction in neurodevelopmental disorders. Moreover, many conditions have been shown to alter the timing of astrocyte maturation. Prenatal chronic hypoxia and inflammation can delay the maturation of astrocytes in the fetal brain – an effect that has been shown to persist later in life [323,324]. Further investigation is needed to determine the effect of compromised astrocyte maturation on P-gp function at the developing BBB.

In conclusion, astrocytes are key regulators of P-gp expression and function at the developing BBB, but the magnitude of this effect is dependent on maturational state of astrocytes. Aberrations in astrocyte maturation may alter levels of astrocyte-derived factors and dysregulate P-gp at the BBB. It is common for women to take prescribed drugs during pregnancy, many of which are P-gp substrates. Altered levels of P-gp at the developing BBB may result in increased fetal brain exposure to drugs and xenobiotics. Understanding how events during fetal life compromise astrocyte maturation and consequently P-gp at the BBB will be critical in the development of future therapies to counteract these effects.

Table 6-1. List of proteins found to be upregulated in PND14 ACM compared to GD50 ACM (n=3/group).

Accession	Protein	Molecular Weight (kDa)	Fold Change
B5ABI3	Y box binding protein 1	36	18.75
H0V7G7	High density lipoprotein binding protein	141	16.92
H0UW86	Melanoma Differentiation-Associated Gene 20	18	14.09
H0VZ48	SERPINE1 MRNA Binding Protein 1	45	8.51
H0VI92	Actin, Beta-Like 2	42	6.26
H0VDC5	Enolase 2 (Gamma, Neuronal)	47	6.17
H0WE19	KH Domain Containing, RNA Binding, Signal Transduction Associated	48	6.1
H0W4Y4	C-Myc-binding protein	40	5.79
H0UTU8	Tropomyosin 2	33	5.78
H0UZV6	Calsyntenin 2	107	5.5
H0VA39	Fructose-bisphosphate aldolase	39	5.14
H0V1C6	Reticulocalbin 3	37	5.04
H0VAU1	MARCKS-Like1	20	5.02
H0UU38	Profilin	15	4.75
H0W7U7	Heat Shock 27kDa Protein 1	23	4.56
H0V7E4	Galectin	15	4.49
H0VP81	Von Hippel-Lindau Binding Protein 1	23	4.45
H0V318	Neuropilin 2	105	4.18
H0V795	Microtubule-associated protein 1B	271	4.11
H0VP72	Tropomyosin alpha-4 chain	50	4
H0UWQ4	Nestin	177	3.99
H0V0S9	radixin	69	3.96
H0V880	GTPase Activating Protein (SH3 Domain) Binding Protein 1	52	3.89

H0UT42	Transketolase	68	3.86
H0W1R5	Small Nuclear Ribonucleoprotein 70kDa	52	3.86
H0VUB7	Calponin	34	3.85
H0VRJ3	ATPase Inhibitory Factor 1	12	3.72
H0VUC2	Heat Shock 10kDa Protein 1	11	3.7
H0WBK0	14-3-3 protein beta/alpha	28	3.65
H0W469	Myosin, Light Chain 4	22	3.46
H0VTP6	Lipoma-Preferred Partner	66	3.46
H0VGT2	Ependymin Related 1	25	3.46
H0VHL2	Phosphoribosylaminoimidazole Carboxylase	47	3.41
H0VBB2	Dihydropyrimidinase-Like 3	62	3.37
H0VVN0	Actin-Related Protein 2	45	3.31
H0VN17	Proteasome subunit alpha type	26	3.21
H0VR64	Eukaryotic Translation Elongation Factor 1 Beta 2	25	3.18
H0VKC2	Thioredoxin Reductase 1	71	3.16
H0VDQ3	Septin 11	49	3.12
H0VPA8	Septin 2	41	3.04
H0W7J9	Ribosomal Protein L30	13	3
H0V5Q1	A kinase (PRKA) anchor protein 12	191	2.98
H0VSD7	Splicing Factor Proline/Glutamine-Rich	76	2.91
H0VP94	Proteasome subunit alpha type	26	2.88
H0UYE7	Alanyl-TRNA Synthetase	107	2.84
H0V2K4	Peroxiredoxin 4	31	2.83
H0VBD5	Fascin actin-bundling protein 1	55	2.71
H0UYD2	PDZ And LIM Domain 7 (Enigma)	50	2.69
H0VG97	14-3-3 protein gamma	28	2.68
H0VL04	Protein disulfide-isomerase	57	2.67

H0VRR0	PDZ and LIM domain 5	64	2.61
H0UTV0	Talin-1	270	2.61
H0UW91	Proteasome subunit beta type	25	2.6
H0VH33	Endoplasmic Reticulum Protein 29	29	2.6
H0VL82	Triosephosphate isomerase	31	2.6
H0UUL6	Fibromodulin	43	2.59
H0VLN5	Dachsous Cadherin-Related 1	346	2.55
H0VIH6	Calcium Activated Nucleotidase 1	45	2.54
H0UZ34	Filamin A, alpha	281	2.53
H0VLM3	Binding Immunoglobulin Protein	72	2.52
H0UVL7	Serine/Threonine Kinase Receptor Associated Protein	38	2.5
H0VQH8	Phospholipase B domain containing 2	65	2.5
H0W6M2	Immunoglobulin Superfamily Containing Leucine-Rich Repeat	46	2.49
H0VLM1	Septin 7	51	2.45
H0VHD0	Collagen, Type I, Alpha 2	129	2.44
H0W8N8	Zyxin	61	2.43
H0VBS1	Protein disulfide-isomerase	57	2.4
H0UZG7	Tropomodulin-3	40	2.39
H0UTQ3	Myosin, Heavy Chain 9, Non-Muscle	227	2.37
H0VL30	Collagen Triple Helix Repeat Containing 1	26	2.36
Q8CHK7	NAD(P)H dehydrogenase [quinone] 1	31	2.34
H0V758	Adenosine Deaminase	41	2.25
H0VL18	SH3 domain-binding glutamic acid-rich-like protein	128	2.24
H0VSU2	Proteasome subunit beta type	29	2.22
H0V2J1	Lipopolysaccharide-Associated Protein 1	71	2.2
H0VBA9	Capping Protein (Actin Filament), Gelsolin-Like	38	2.18
H0VCZ6	Guanine Nucleotide Binding Protein (G Protein),	35	2.17

H0W2A1	Nucleoside Diphosphate Kinase	17	2.16
H0VW15	14-3-3 protein zeta/delta	28	2.15
H0VSV5	Proteasome subunit alpha type	30	2.08
H0W6T7	Microtubule-associated protein RP/EB family member 1	30	1.95
H0VAZ3	Cartilage Acidic Protein 1	71	1.9
H0VHL7	Malate dehydrogenase	36	1.87
H0VEJ5	Chaperonin Containing TCP1, Subunit 8 (Theta)	60	1.83
H0VCE7	UDP-glucose pyrophosphorylase 2	57	1.78

Figure 6-1. Astrocytes increase tight junction function in BECs. Tight junction function in monocultured (MONO) or co-cultured (CO) BECs derived from gestational day (GD) 50 and post-natal day (PND) 14 guinea pigs (n=6-8/group). Tight junction function was assessed using transendothelial electrical resistance (TEER) after 24h, 48h, and 72h in culture (A, B). Data from TEER experiments is displayed as mean ± SEM. After 72h in culture, FITC-dextran was also used to measure tight junction function (C, D). Data are expressed as % dextran accumulation from control (mono-cultured BECs). A significant difference from mono-cultured cells is indicated by (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

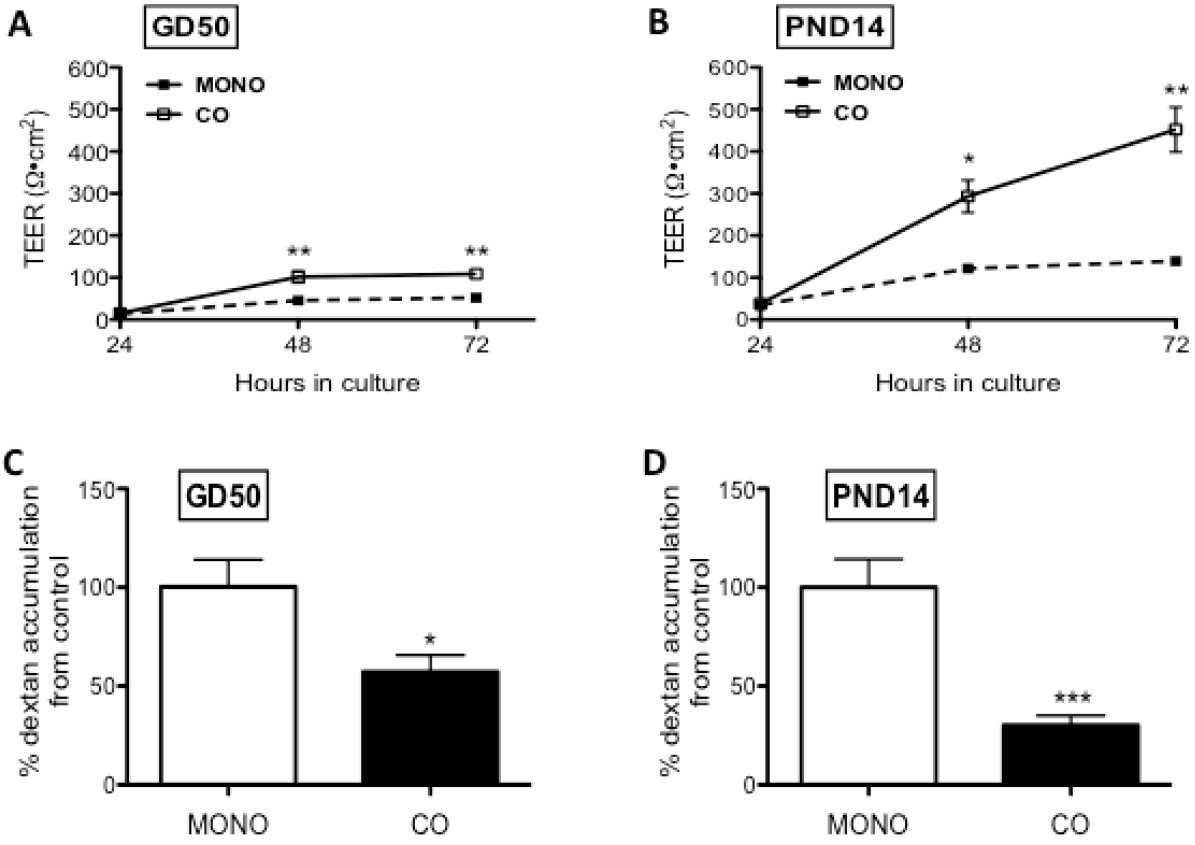


Figure 6-2. Astrocytes increase P-gp activity and *Abcb1* mRNA in BECs. P-gp activity (A, B) and levels of *Abcb1* mRNA (C, D) in mono-cultured or co-cultured BECs derived at gestational day (GD) 50 and post-natal day (PND) 14 (n=6-8/group). P-gp activity is displayed as fold change from control (mono-cultured BECs). *Abcb1* mRNA expression was normalized to *β-Actin* and shown as fold change from mono-cultured BECs. Data is displayed as mean ± SEM. A significant difference from mono-cultured cells is indicated by (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

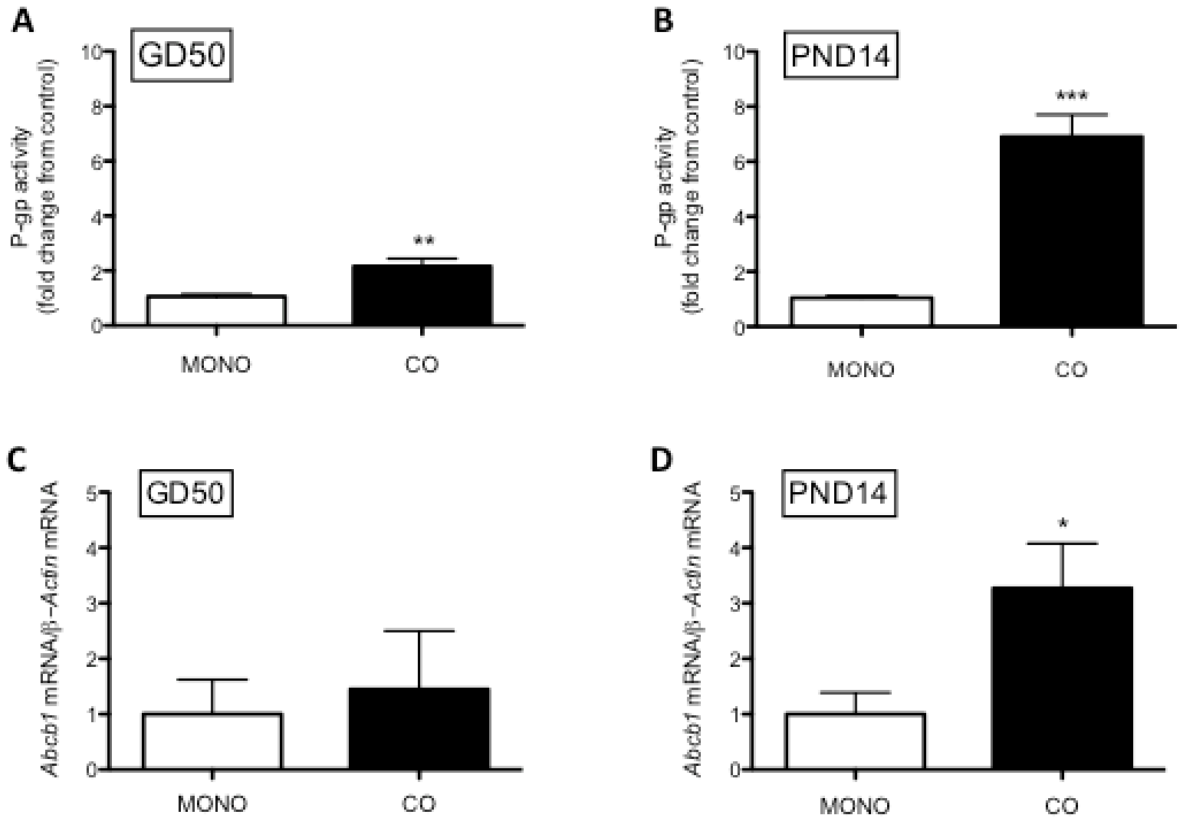


Figure 6-3. **Post-natal astrocytes increase P-gp function in fetal BECs while fetal astrocytes have no effect on post-natal BECs.** P-gp activity (A, B) and levels of *Abcb1* mRNA (C, D) measured in gestational day (GD) 50 and post-natal day (PND) 14 BECs after mono-culture, co-culture with age-matched astrocytes or co-culture with different-aged astrocytes (n=6-8/group). P-gp activity is displayed as fold change from control (mono-cultured BECs). *Abcb1* mRNA expression was normalized to *β-Actin* and shown as fold change from mono-cultured BECs. Data is displayed as mean ± SEM. A significant difference from mono-cultured BECs is indicated by (*) $P < 0.05$; (***) $P < 0.001$. A significant difference between two co-culture treatments is indicated by (#) $P < 0.01$.

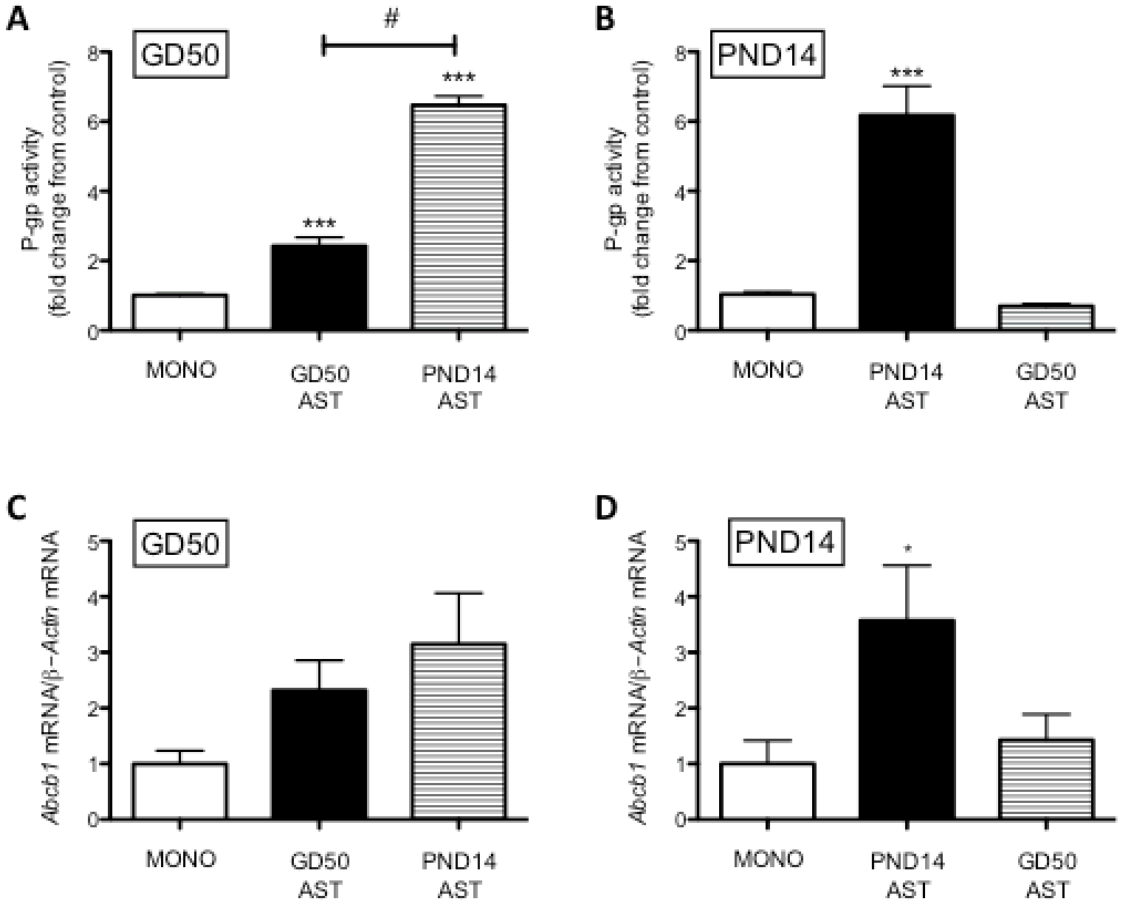


Figure 6-4. ACM increases P-gp activity in BECs. P-gp activity in post-natal day (PND) 14 BECs exposed to unconditioned (UCM) or astrocyte conditioned media (ACM) for 24h (A), 48h (B) and 72h (C). P-gp activity was also measured in PND14 BECs to UCM or ACM, with the media being changed every 24h (D, E). P-gp activity is displayed as fold change from control (mono-cultured BECs) (n=6-8/group). Data is displayed as mean ± SEM. A significant difference from control is indicated by (*) $P < 0.05$; (***) $P < 0.001$.

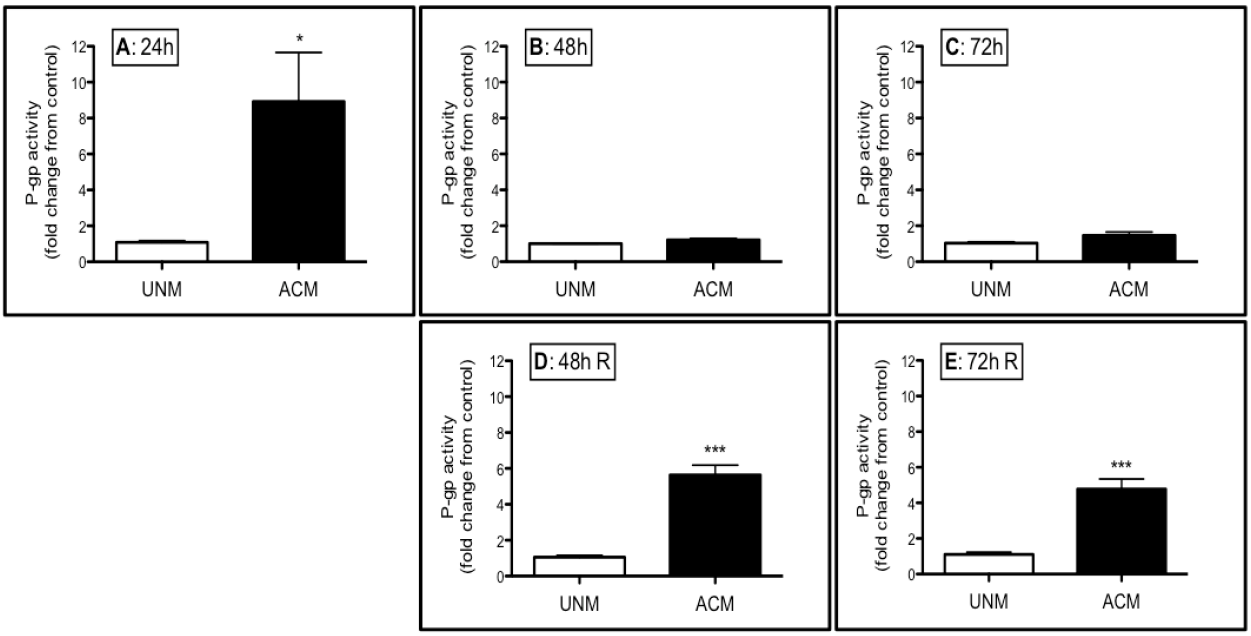


Figure 6-5. Astrocyte secreted factor is dependent on a protein(s) and is not mediated by exosomes. P-gp activity in post-natal day (PND) 14 brain endothelial cells (BECs) exposed to heat-inactivated (A), protease-treated (B), or exosome-depleted (C) astrocyte-conditioned medium (ACM). ACM was subjected to heat-inactivation at 100°C for 4h. For protease treatment, ACM was incubated with trypsin (200 μg/ml; Sigma) at 37°C for 1h and subsequently incubated with trypsin inhibitor (400 μg/ml; Sigma) at 37°C for 30 minutes. As a control, trypsin and trypsin inhibitor were mixed and incubated at 37°C for 1h and 30 minutes and then added to ACM. To eliminate exosomes, ACM was centrifuged at 100000 g for 6h and the supernatant collected. P-gp activity is displayed as fold change from control (BECs exposed to unconditioned medium [UCM]) (n=6-8/group). Data is displayed as mean ± SEM. A significant difference from control is indicated by (***) $P < 0.001$.

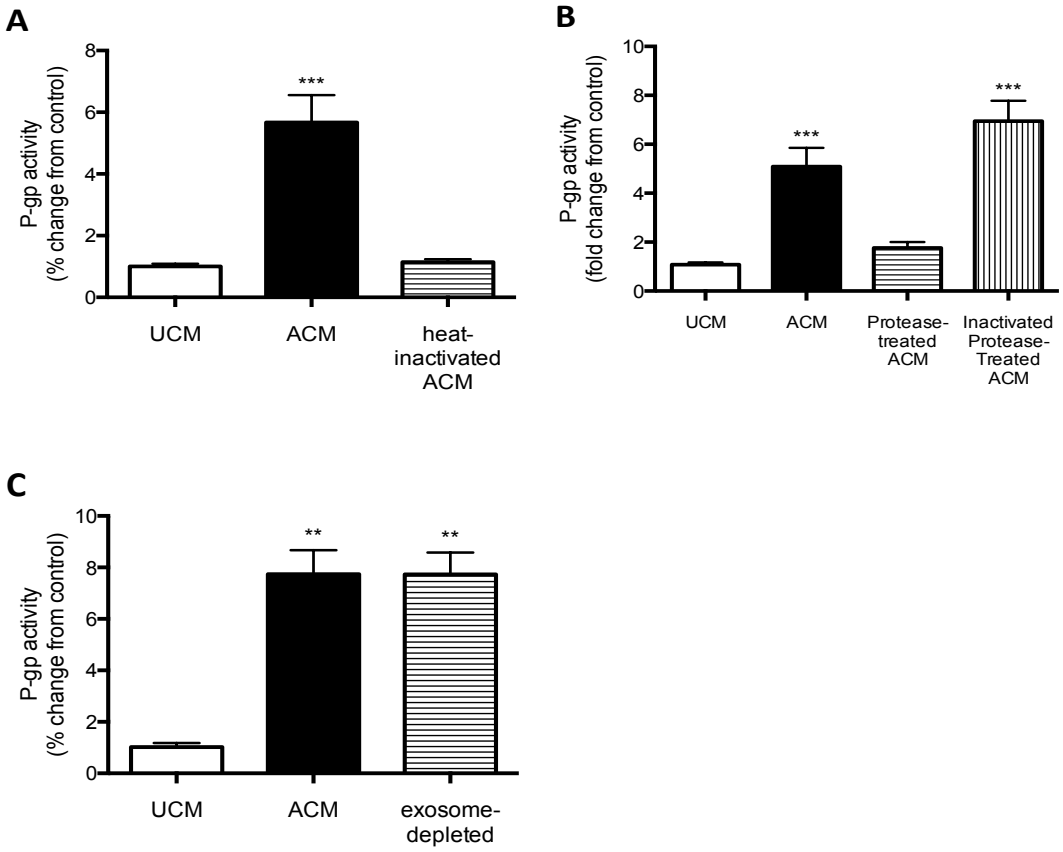


Figure 6-6. Molecular weight of astrocyte-secreted factor is between 30-100 kDa. P-gp activity in post-natal day (PND) 14 BECs exposed astrocyte-conditioned medium (ACM) containing soluble factors of varying molecular weights. ACM was fractionated using Centrifugal Filter Devices with different MWCO (Molecular Weight Cut-Offs) Ultracel membranes. P-gp activity is displayed as fold change from control (mono-cultured BECs) (n=6-8/group). Data is displayed as mean ± SEM. A significant difference from control is indicated by (***) $P < 0.001$.

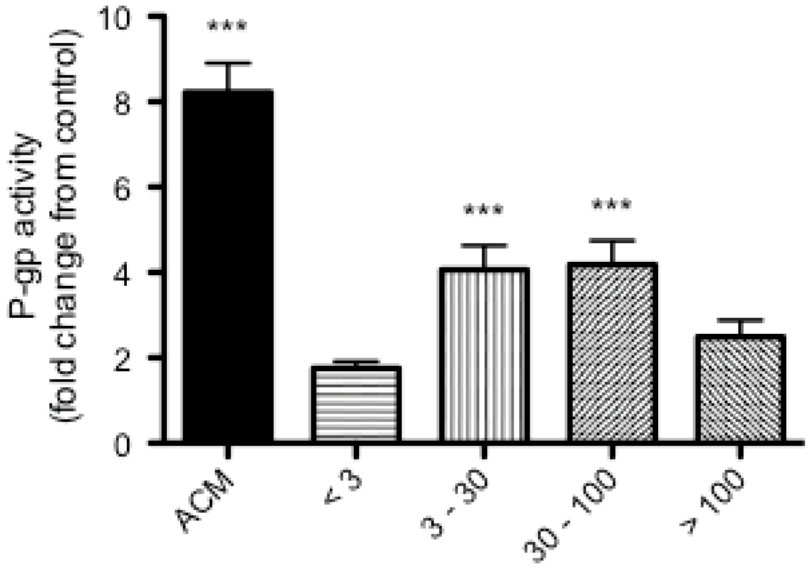
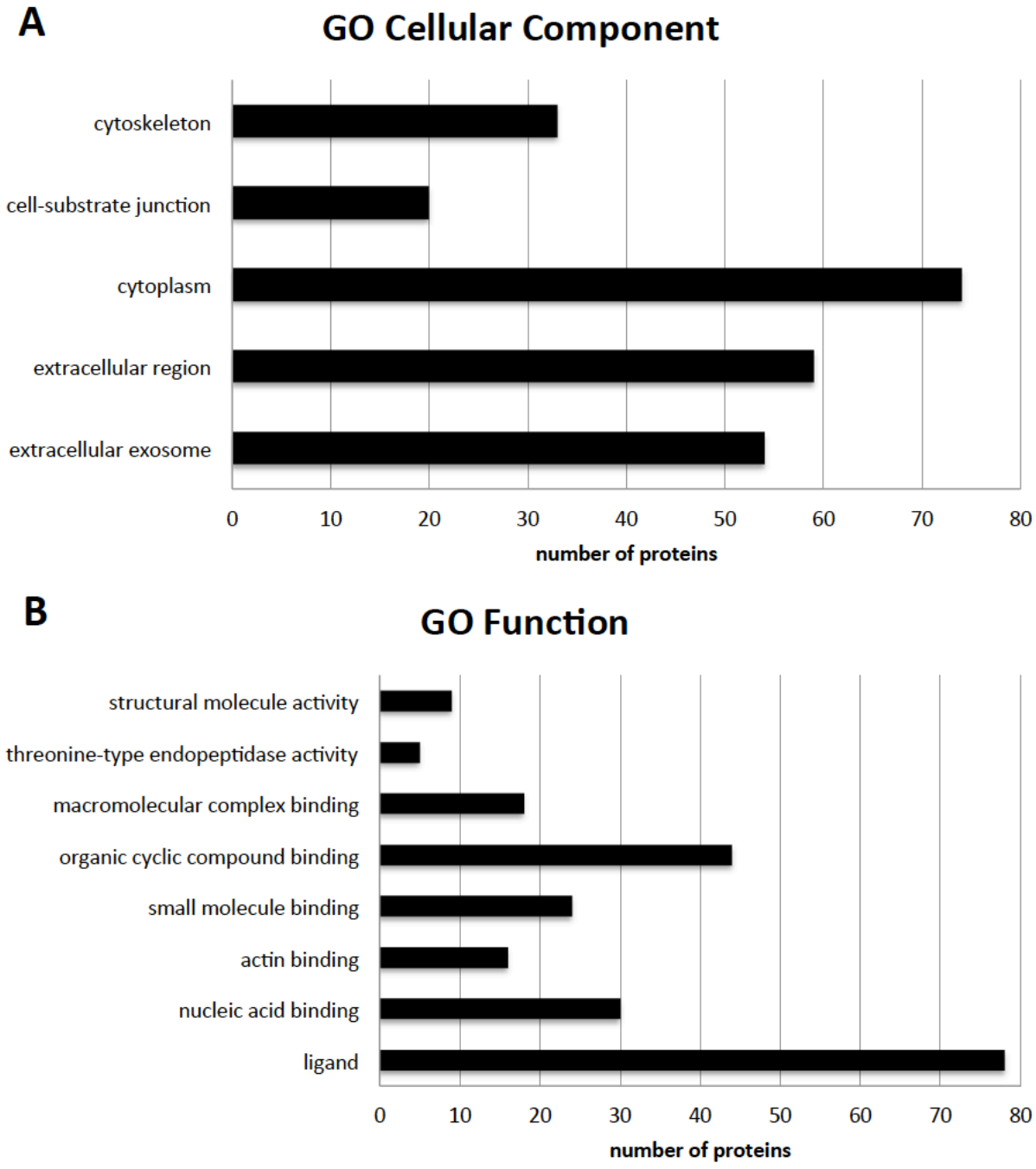


Figure 6-7. Bioinformatic analysis of differentially secreted proteins. Proteins were clustered based on gene ontology (GO) cellular component (A) and GO Function (B) using GO Term Finder. GO terms with $P < 0.01$ are listed in the figure.



CHAPTER 7:
GENERAL DISCUSSION

7.1 Overview

The studies outlined in this thesis contribute significantly to our understanding of the role of astrocytes in the regulation of P-gp at the fetal and post-natal BBB. The first studies of this thesis focused on investigating the effect of TGF-β1, an astrocyte-derived factor, on P-gp function in monocultured BECs and how prenatal synthetic glucocorticoid exposure may attenuate this effect. The studies in the second-part of this thesis explored the differences in fetal and post-natal astrocytes in regulating P-gp at the BBB. I also

began to elucidate the identity of the astrocyte-derived factor(s) that could be responsible for this regulation.

7.2 Astrocyte-Derived Factors and P-gp

Data from this thesis has demonstrated that astrocytes have a profound effect on P-gp activity and levels of *Abcb1* mRNA in BECs, and that this effect depends on maturational state of astrocytes. To our knowledge, this is the first report of the age-specific effect of astrocytes on *Abcb1*/P-gp at the BBB, using a co-culture system comprised of cells from the same species at two specific time-points in development. The mechanism by which this occurs is at least partially dependent on a protein(s) secreted by astrocytes, as these effects are mimicked by ACM and prevented by protease treatment or heat-inactivation. Previous studies have attempted to identify BBB-inducing molecules secreted by astrocytes[325-327]. The majority of these studies have used mouse or rat astrocytes isolated from one time-point in development, specifically during post-natal life as astrocyte differentiation begins just before birth (~E18) in these species. In contrast to these studies, this was the first study to identify differentially secreted proteins by guinea pig astrocytes derived at two distinct time-points in development. By comparing ACM at two developmental time-points that elicit different effects on *Abcb1*/P-gp in BECs, I was able to identify 85 proteins in ACM from PND14 astrocytes that were at a higher concentration compared to the ACM of GD50 astrocytes. However, identification of the specific factor(s) or pathways implicated in upregulating *Abcb1*/P-gp at the BBB by astrocytes warrants further investigation.

Studies in this thesis have also demonstrated that BECs responsiveness to astrocyte-derived factors changes with developmental age. Co-culturing GD50 BECs

with PND14 astrocytes led to a higher increase in P-gp activity compared to co-culturing these cells with GD50 astrocytes. In contrast, co-culturing PND14 BECs with GD50 astrocytes led to no significant increase in P-gp activity. Since astrocytes express increased levels of TGF-β1 as they mature, these data suggest that TGF-β1 could be one of the factors secreted by PND14 astrocytes. However, TGF-β1 was not identified in ACM from GD50 or PND14 guinea pigs via LC-MS/MS. This discrepancy may be due to the stability of TGF-β1 in ACM. In Chapter 4, I found that the effect of TGF-β1 on P-gp function in BECs was acute, maximal at 8h of exposure but disappeared by 24h. Since ACM was only collected at one time-point, it is possible that degradation of TGF-β1 had occurred before identification via LC-MS/MS. There is evidence suggesting the presence of TGF-β1 in ACM as TGF-β binding proteins were identified in ACM from PND14 astrocytes. To resolve this discrepancy, ACM could have been collected at multiple-time points and proteins at these time-points identified by LC-MS/MS. However, due to resource contraints, this was not possible.

Our findings regarding the effect of astrocyte-derived factors on P-gp at the developing BBB is clinically relevant as astrocytes are implicated in numerous neurodevelopmental and neurodegenerative disorders[108,171]. In particular, recent evidence suggests that astrocytes contribute to neuronal dysfunction observed in Rett's Syndrome and Fragile X Syndrome, which are monogenic neurodevelopmental disorders. Rett's Syndrome is caused by a mutation in the gene encoding the methyl binding CpG protein (MeCP2), while Fragile X Syndrome is caused by a mutation in the gene encoding the fragile X mental retardation protein (FMRP)[185,328]. Co-culturing mutant mouse astrocytes, derived from mice that lack either of these proteins, with wild-type mouse neurons stunts

axonal and dendritic growth in these neurons compared to co-culturing with wild-type mouse astrocytes[190,191,193,195]. However, nothing is known about the effect of astrocyte dysfunction observed in these disorders on *Abcb1*/P-gp levels at the BBB. Based on the studies in this thesis, it is plausible that astrocyte dysfunction seen in these disorders, could dysregulate *Abcb1*/P-gp at the developing BBB. P-gp effluxes a wide variety of exogenous molecules, such a xenobiotics, drugs and pesticides. However, it also regulates the entry of endogenous molecules, including cytokines, chemokines and hormones, into the brain. Thus, future studies should investigate the contribution of altered *Abcb1*/P-gp at the developing BBB to the pathogenesis seen in neurodevelopmental disorders.

7.3 Prenatal Synthetic Glucocorticoids

Prenatal synthetic glucocorticoids are commonly administered to pregnant women at risk of pre-term delivery (~10% of pregnancies). Previous studies in our lab have shown that prenatal sGC matures the BBB by increasing P-gp function and levels of *Abcb1* mRNA in BECs (Fig. 7-1). The studies in this thesis further support this hypothesis. In Chapter 5, it was demonstrated that tight junction function and expression of genes that encode for tight junction proteins, ZO-1 and occludin, were upregulated in BECs derived from dexamethasone-exposed fetuses compared to vehicle-exposed fetuses. Moreover, these BECs show decreased responsiveness to astrocyte-derived factor, TGF-β1, which is similar to the response seen in BECs derived from GD65 and PND14 guinea pigs. Thus, exposure to prenatal sGC appears to mature the fetal BBB.

Glucocorticoids are powerful transcriptional regulators that signal through two types of receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Astrocytes are highly responsive to glucocorticoids as they express high levels of MR and GR[329]. A number of glucocorticoid responsive genes, those that contain a glucocorticoid responsive element (GRE), have been identified in astrocytes, including glutamine synthetase and GFAP[330,331]. Since prenatal sGC seem to mature the BBB, it is temping to hypothesize that prenatal sGC can also speed up the maturation of astrocytes. Astrocytes are derived from neural precursor cells, which have the ability to differentiate into glial and neurons. Glucocorticoids have been shown to regulate the rate of proliferation and differentiation of these neural precursor cells, which is mediated by GR. Studies using human neural precursor cells demonstrated that dexamethasone inhibits neural differentiation in these cells and instead facilitates glial formation (GFAP positive cells)[332]. Thus, antenatal sGC may facilitate astrocyte maturation at an earlier time-point in gestation. This may contribute to the increase in *Abcb1*/P-gp in microvessels and in BECs derived from dexamethasone-exposed fetuses described in Chapter 5. The consequence of a premature upregulation in *Abcb1*/P-gp at the BBB on brain development is unclear. From a toxicology perspective, an increase in *Abcb1*/P-gp is beneficial to the developing brain as it would protect the brain from various drugs, pesticides and toxins present in the environment. P-gp also effluxes hormones, chemokines and cytokines, many of which have essential roles in normal brain development. Ultimately, our *in vitro* findings must be validated *in vivo* to determine the consequence of a premature enhancement in *Abcb1*/P-gp at BBB on brain development.

7.4 Limitations

7.4.1 Co-culture Model

The studies described in this thesis were conducted using a novel monoculture and co-culture system comprised of BECs and astrocytes to model the fetal and post-natal guinea pig BBB. The strengths of these systems were highlighted throughout this thesis. However, as with any *in vitro* model of the BBB, there are limitations to this model.

We have demonstrated that astrocytes retain characteristics appropriate for the developmental stage at which they were derived. Fetal astrocytes express high levels nestin and lower levels of GFAP compared to post-natal astrocytes (Fig. 3-2). In contrast, post-natal astrocytes are characterized by increased expression of GFAP and high expression of AQP4. However, our co-culture model assumes that astrocytes are a homogenous population of cells. Recent evidence suggests that astrocytes are a heterogeneous population of cells. There are at least 11 classes of astrocytes, 8 of which make contact with the BBB *in vivo*[105]. Astrocytes across brain regions as well as within the same brain region show major differences in gene expression[333,334]. This translates into functional differences in electrophysiological properties, calcium dynamics and gap junction coupling[335-337]. This heterogeneity has been shown to affect surrounding neurons *in vitro*. Cultured astrocytes from different brain regions varied in capacity to stimulate neurite growth and branching in co-cultured neurons[338,339]. However, very little known about the consequence of astrocyte heterogeneity on the BBB. There is some evidence for BBB heterogeneity across brain regions. Enzymatic assays in homogenates of different regions from adult rat brain showed that gamma-glutamyl transpeptidase (GGTP), a highly enriched BBB enzyme, activity varies considerably in different areas of rat

brain[340]. Future studies should investigate whether P-gp function and expression at the BBB demonstrates this heterogeneity and to what extent astrocytes influence this property.

This experimental model also lacks the influence of physical contact in regulating BBB characteristics. *In vivo*, astrocytes and BECs share a surrounding ECM, composed of fibronectin, agrin and various laminins[173,341]. Astrocytes establish close interaction with the brain microvasculature through their endfeet which express high levels of anchoring transmembrane proteins, AQP4 and KIR4.1 and adaptor molecules, syntrophin, dystrphin and dystrobrevin[167]. Astrocytes are *polarized cells* as these proteins are highly expressed by astrocyte endfeet and to a lesser extent in astrocyte body[342]. Studies have shown that deletion of any of these proteins results in disrupted astrocyte polarity, as evidenced by redistribution of many of these proteins and subsequent tight junction dysfunction[175,299,343]. Moreover, co-culture studies utilizing adult rat BECs and astrocytes demonstrate the importance of this physical interaction, as BBB characteristics are upregulated in contact co-culture models compared to non-contact co-culture models[165]. However, we have not been able to optimize a contact co-culture model due to limited adhesion of astrocytes to the basolateral side of a Transwell Insert.

Another limiting factor to this experimental model is the absence of other CNS cell types. Endothelial cells of the BBB are influenced by a number of cell types. The focus of this thesis was the interaction between BECs and astrocytes because astrocyte maturation is temporally correlated with the upregulation in *Abcb1*/P-gp at the BBB[92,270]. Moreover, 99% of the abluminal surface of brain microvasculature is ensheathed my astrocyte-endfeet in the adult BBB. However, studies have shown that neurons and

pericytes also influence characteristics of the BBB, especially tight junction function and expression. Subsequently, a limitation of our co-culture model is that BECs do not attain the same tight junction function *in vitro* compared to *in vivo* measurements. of As stated in Chapter 6, co-cultured PND14 BECs attained a TEER of approximately 452 $\Omega \bullet cm^2$ while co-cultured GD50 BECs displayed a TEER of 109 $\Omega \bullet cm^2$. In the developing rat brain, *in vivo* TEER measurements increase from E17 to PND7, from 310 $\Omega \bullet cm^2$ to 1462 $\Omega \bullet cm$[285].

7.4.2 Guinea Pig

The guinea pig is an excellent animal model to study CNS and BBB development as described in Chapter 3. However, genomic and proteomic databases for the guinea pig are not as well established compared to those available for mice and humans. Due to the lack of guinea pig protein and cDNA resources, the majority of the gene models are based on Genewise alignments of proteins from mammals. These gene models were assessed by generating sets of potential orthologs to genes from other mammalian species. As such, attaining antibodies specific to a protein of interest in the guinea pig is a challenge since most of the commercially available antibodies are designed and validated for human and mouse tissues. Currently, we have not been able to optimize these commercially available antibodies specific for P-gp in BECs derived from guinea pig.

7.4.3 Other Transporters at the BBB

P-gp is the one of the highest expressed efflux transporters at the BBB. However, P-gp has been shown to work in concert with other groups of ABC transporters expressed at the BBB. In particular, breast cancer resistance protein (BCRP) and the transporters belonging to the multidrug resistance protein (MRP) family have been shown to significantly contribute to brain protection.

BCRP is a "half-transporter" composed of 665 amino acids with a molecular weight of approximately 72-kDa. BCRP has one transmembrane region along with a single nucleotide binding-domain and there is evidence that homodimerization of BCRP is essential for function[344]. Like P-gp, BCRP is highly expressed by BECs on the luminal side and has a significant role in brain protection[27,345]. Studies indicate that BCRP and P-gp work synergistically, as demonstrated by brain accumulation of Lapatinib in knockout mice[346]. *Abcb1a/b* knockout mice display 4-fold higher brain accumulation of Lapatinib compared to wild-type controls, while *bcrp* knockout mice display 3-fold higher compared to wild-type controls. However, double knockout of transporters results in 40-fold increase in brain accumulation of Lapatinib compared to control mice. Later, it was found that this discrepancy was due to a compensatory mechanism, in which knockout of one transporter led to the upregulation of the other transporter[347]. Future studies should investigate whether changes in *Abcb1*/P-gp in co-cultured BECs also affects levels of BCRP in these cells.

BECs of the developing BBB also express MRPs, though the presence and distribution of these transporters seem to vary with species. MRPs represent a large subfamily of ABC-transporters, composed of 13 members encoded by *ABCC1-13*

respectively. MRPs transport small organic anions, some of which are also transported by P-gp and BCRP[348]. The expression and localization of MRPs at the BBB may be species specific. In capillary enriched fractions of bovine brain, it was found that mRNAs coding for MRP1, MRP4, MRP5, and MRP6 were highly expressed, as well as low levels of MRP3 mRNA, whereas MRP2 mRNA was absent[349]. However, studies using isolated capillaries from rat and pig brain, demonstrated immunohistochemical staining of P-gp and MRP2 on the luminal surface of the brain endothelium[350]. In human BECs, high expression levels of mRNAs coding for MRP1 and low expression levels of mRNAs coding for MRP2, MRP3, and MRP5 were found using microarray[351]. Thus, at least six MRPs are expressed at the BBB of different species. However, the exact subcellular localization and functional significance of most of these MRP transporters in BECs remains to be elucidated. Furthermore, there have been few studies undertaken to investigate the localization and expression of these transporters in the developing brain.

7.4.4 Other Barriers in the Developing Brain

Many of these ABC-transporters are also expressed at the blood-cerebrospinal fluid (CSF) barrier and meningeal barriers (Fig. 7-2). The blood-CSF barrier is found at the level of the epithelial cells of the choroid plexus, while the meningeal barrier is located at the epithelial cells of the arachnoid membrane. Compared to the BBB, there have been few studies on the contribution of the blood-CSF and meningeal barriers to brain protection.

In the rat choroid plexus tissue, mRNA analysis and western blotting revealed a strong expression for MRP1, which is encoded by *Abcc1*[352]. Using functional and immunohistochemical analysis, MRP1 was found to be located on the basolateral side (brain-facing side) of epithelial cells of the choroid plexus[353]. In contrast, immunohistochemical and western blot analysis have demonstrated relatively low expression of P-gp at the choroid plexus compared to the BBB[5]. Its basolateral localization on epithelial cells of the choroid plexus suggests that it does not act in a similar fashion to P-gp at the BBB[353]. However, very little is known about the contribution of either MRP1 or P-gp at the choroid plexus to brain protection.

Even less is known about ABC transporters at the meningeal barrier. To date, only one study has investigated the expression of ABC transporters at the arachnoid barrier. This recent study has identified expression of P-gp and BCRP in primary rat arachnoid barrier cells using immunohistochemical analysis[354]. Using fluorescent substrate assays, it was also demonstrated that these cells express functional levels of P-gp and BCRP. However, the functional significance of ABC transporters at the arachnoid barrier *in vivo* is unknown. The function and developmental expression of these ABC transporters at the choroid plexus and meningeal barriers should be investigated in future studies.

7.5 Potential Mechanisms

7.5.1 Astrocyte-Secreted Factors

Evidence from literature suggests that the astrocyte-mediated effect on *Abcb1*/P-gp is not caused by a single protein, but perhaps is dependent on a signaling pathway

activated by multiple proteins. Many signaling molecules are capable of enhancing *Abcb1*/P-gp, such as retinoic acid, TGF-β1, Wnts, and shh[144,148,276,355]. These molecules activate various signaling pathways such as mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinases (ERK), SMAD, Wnt/β-catenin, and Hedgehog/Patched-Gli pathways[356]. In Chapter 6, I found that proteins upregulated in PND14 ACM were potentially capable of activating multiple signaling pathways and most of them were able to elicit more than one pathway. Furthermore, P-gp function was enhanced by ACM fractions containing 3-30 kDa and 30-100 kDa proteins.

To add to this complexity, there is extensive crosstalk between these signaling pathways. During embryonic development, there is complex but delicate interactions between the TGF-β, Wnt, Hedgehog, Notch, MAPK, and other pathways crucial for stem cell maintenance, cell differentiation, body axis patterning, and organogenesis. In Chapter 4, I highlight the importance of the TGF-β1 signaling pathway in the regulation of *Abcb1*/P-gp at the developing BBB. Typically, TGF-β1 binds to the TGF-β type II receptor, the ligand binding receptor and it will subsequently recruit a TGF-β type I receptor. In endothelial cells, there are two isoforms of the type I receptor, ALK1 and ALK5, which are responsible for signal transduction[159]. These type I receptors activate the canonical TGF-β signaling pathway involving SMADs. In Chapter 4, we demonstrate that both ALK5 and ALK1 essential in TGF-β1 increase in *Abcb1*/P-gp. However, using an inhibitor for SMAD3, we found that signaling through the SMAD3 pathway is not essential for this effect. Thus, non-canonical TGF-β signaling may be involved in regulating *Abcb1*/P-gp at the developing BBB.

One of the most extensively studied signaling crosstalk is between TGF-β and Wnt pathways, which occurs at multiple levels. First, TGF-β and Wnt reciprocally regulate the production and secretion of their ligands[357]. Additionally, activation of TGF-β/Smad pathway leads to nuclear translocation of Wnt/β-catenin[358]. Perhaps these pathways all contribute to the astrocyte-mediated upregulation in *Abcb1*/P-gp in BECs. The redundancy in these pathways may be a defense mechanism, allowing one pathway to compensate for another in times of environmental stress.

7.5.2 Pericytes and the BBB

Other cells of the CNS heavily influence BBB-characteristics in BECs. Neurons may have an indirect effect on BECs, perhaps by influencing pericytes and astrocytes, as there is very little innervation of the BBB by neurons[359,360]. In contrast, pericytes make extensive contact with the brain microvasculature, covering up to 30% of the abluminal surface[132,361]. Pericytes are found adjacent to nascent blood vessels as they invade the brain parenchyma early in gestation[120]. Thus, pericytes are thought to be involved in the initial induction of BBB-characteristics in brain endothelium[362-364]. However, pericytes may indirectly influence the upregulation in *Abcb1*/P-gp at the developing BBB as studies demonstrate that pericytes can influence the function and morphology of astrocytes. Astrocytes that have been co-cultured with pericytes show enhanced polarity, as they display increased expression levels of ligand-receptors and ion channels localized to their endfeet[365]. This increase in polarity may affect astrocytes' capability to influence *Abcb1*/P-gp at the BBB, since alterations in astrocyte polarity affect tight junction

function in BECs[170,299,300,343]. Thus, future studies should investigate the role of pericytes in regulating *Abcb1*/P-gp at the developing BBB.

7.5.3 Epigenetics and miRNAs

Studies in cancer cell lines and malignant tissue have made it increasingly apparent that epigenetic mechanisms modulate transcriptional changes in *Abcb1* mRNA. These studies show that methylation status of the *Abcb1* gene inversely correlates with gene expression[51,55,303]. Moreover, histone acetylation has been correlated with increase *Abcb1* mRNA expression[48,55]. However, currently it is unknown whether these epigenetic processes affect *Abcb1* mRNA expression at the fetal or adult BBB.

The upregulation in P-gp function and expression by astrocytes may be attributed to epigenetic changes at the level of the *Abcb1* promoter. Preliminary evidence from our lab, in collaboration with Moshe Syzfe's lab at McGill University, has shown significant changes in global methylation status in brain microvessels derived from GD50 and PND14 guinea pigs (Fig. 7-3). We found that global methylation is decreased by 7% in brain microvessels from GD50 to PND14. However, it is unclear if methylation of the *Abcb1* promoter is specifically altered during these time-points. Decreased methylation in the promoter region of *Abcb1* would allow for increased transcription factor binding and subsequent increase in *Abcb1* mRNA expression. Moreover, signaling pathways implicated in BBB development such as TGF-β1 have been shown to elicit epigenetic modifications in various cell types. For example, TGF-β1 secreted by surrounding neurons, has been shown decrease methylation status of promoter regions of GFAP, S-

100β and other genes important for astrocyte function in neural precursor cells[366,367]. This allows for the transcription of these genes and facilitates the differentiation of astrocytes from neural precursor cells. A similar interaction may occur between astrocytes and the developing BBB, whereby signals from astrocytes elicit demethylation of the *Abcb1* promoter in BECs, allowing increased transcription of *Abcb1* and subsequently increased P-gp function.

The rise in *Abcb1* expression in BECs elicited by astrocytes may also be mediated by miRs. miRs can increase or decrease expression of target mRNAs. A number of miRs have been shown to regulate *Abcb1* expression. In Caco-2 cells, an intestinal epithelial cancer cell line, deletion of the miR-145 binding site within *ABCB1* 3'-UTR led to an increase in *ABCB1* mRNA expression and P-gp function[368]. Overexpression of either miR-200c or miR-451 attenuated levels *Abcb1* mRNA expression in colorectal cancer cells[369,370]. Preliminary studies in our lab indicate that these miRs may regulate developmental levels of *ABCB1* mRNA in human placenta (Fig. 7-4). In human placenta, levels of *ABCB1* mRNA decreases from first trimester to third trimester[91,371]. We found that this is correlated with increase in expression of miR-145, miR-451 and miR-200c in third trimester placenta tissue compared to first trimester. Astrocytes may also regulate *Abcb1*/P-gp via miRs at the BBB. A recent study has demonstrated that ACM can affect levels of numerous miRs in human BECs[372]. However, the mechanism of action and specific mRNA targets of these miRs in BECs warrants further investigation.

7.6 Significance to Current Knowledge

The studies presented in this thesis have greatly advanced our understanding of the regulation of fetal brain protection via *Abcb1*/P-gp at the developing BBB. Levels of *Abcb1*/P-gp surge at the BBB in late gestation and post-natal life of the guinea pig. This is temporally correlated with astrocyte maturation, a process in which astrocyte endfeet begin to ensheathe the abluminal surface of developing brain microvessels. Utilizing a co-culture model of guinea pig BECs and astrocytes from two distinct time-points in gestation, the studies in this thesis demonstrate how astrocyte maturation may regulate *Abcb1*/P-gp at the developing BBB. Prior to the studies presented in this thesis, little was known regarding the astrocyte-mediated effect on the developing BBB.

In particular, this thesis provides evidence that TGF-β1, an astrocyte-derived factor essential in neurogenesis and angiogenesis, is capable of enhancing *Abcb1*/P-gp at the fetal and post-natal BBB. Preceding this thesis, little was known regarding the effect of TGF-β1 on *Abcb1*/P-gp at the fetal or post-natal BBB. The first evidence indicating a possible link between multidrug resistance and TGF-β1 stemmed from studies with cancer cells, which generally display over activated TGF-β signaling and demonstrate high levels of P-gp activity[207]. Subsequently, Dohgu et al. (2004) demonstrated the stimulatory effect of TGF-β1 on P-gp and tight junction function at the adult rat BBB[146]. Data from this thesis also demonstrates that prenatal dexamethasone exposure may reduce BBB responsiveness to TGF-β1. This was the first study to show the effect of prenatal sGC exposure and altered cerebrovascular response to an essential factor involved in vascular development. Prior studies had shown a link between prenatal sGC and altered peripheral vasculature responsiveness to vasoactive agents, such as nitric

oxide, endothelin-1 and bradykin[286]. These factors are important in regulating blood pressure in peripheral vasculature and thus led to the hypothesis that prenatal sGC may lead to cardiovascular disease in adulthood. Much less is known about the effect of prenatal sGC on the cerebrovasculature. In sheep and mice, prenatal sGC exposure has been shown to decrease vascular density in various regions of the CNS. This may be attributed to decreased responsiveness to TGF-β1 as proper activation of the TGF-β1 pathway is essential in vascular development. Moreover, TGF-β1 has been shown to work in concert with other vascular factors such as, vascular endothelial growth factor (VEGF) and plasminogen activator inhibitor-1 (PAI-1), to regulate angiogenesis in the brain and peripheral organs[373,374]. Moreover, very little is known regarding the effects of prenatal sGC on astrocyte development. Since our studies indicate the crucial role of astrocytes in regulating the developing BBB, future studies should investigate how prenatal sGC may alter astrocyte maturation and how this may affect astrocyte secretome.

The majority of published studies investigating the effects of astrocytes on the BBB have utilized co-culture models composed of BECs and astrocytes from different ages, usually from adult, or cells from different animal species. Overall, these studies demonstrated that astrocytes enhanced many BBB characteristics in co-cultured BECs compared to moncultured BECs[141,143,164]. Our findings led to similar conclusions, but highlight the importance of the maturational state of astrocytes in the regulation of *Abcb1*/P-gp at the developing BBB, as astrocytes from fetal and post-natal age have different effects on *Abcb1*/P-gp in BECs. Moreover, this thesis is the first to demonstrate that the astrocyte secretome profile changes with maturational state. In accordance with the age-specific effects of astrocytes on P-gp function, we found that ACM from post-

natal astrocytes contained increased levels of astrocyte-derived proteins. However, more work is needed to elucidate which factor(s) or pathway(s) may be important in this astrocyte-mediated upregulation in *Abcb1*/P-gp.

7.7 Conclusions

We now know that astrocytes are key regulators of *Abcb1*/P-gp at the developing BBB and likely contribute to the surge in P-gp in late gestation and post-natal life. The mechanism by which this may occur is through astrocyte-derived factors, which are secreted at different levels depending on the maturational state of astrocytes. In late gestation, the fetal brain is more dependent on P-gp levels at the BBB, as placental expression and function of P-gp decrease. It is common for women take prescription drugs during pregnancy, many of which are P-gp substrates and have teratogenic potential. The studies in this thesis indicate that aberrations in astrocyte function, associated with neurodevelopmental disorders such as Rett's Syndrome and Fragile X Syndrome, may dysregulate P-gp at the BBB in late gestation, making the brain more susceptible to P-gp substrates. Thus, future studies should focus on understanding how compromised astrocyte maturation may dysregulate *Abcb1*/P-gp at the BBB, which may contribute to the pathogenesis of neurodevelopmental disorders. This new knowledge will be critical in the development of future therapies to counteract these effects.

7.8 Future Studies

7.8.1 Identifying astrocyte-secreted factor

Our studies indicate that astrocytes induce *Abcb1*/P-gp in BECs via a soluble factor(s). The studies in this thesis have begun to characterize this factor(s). We have demonstrated that it is a factor(s) secreted at higher levels by PND14 astrocytes compared to GD50 astrocytes. The factor(s) is a protein or its stability relies on a protein with an approximate molecular size of 3-100 kDa. Bioinformatics pathway analysis of these 85 significantly upregulated implicated signaling pathways such as Wnt and Notch. Pharmacological inhibition or small-interfering (si) RNA could be used to antagonize these pathways in BECs in co-culture. After treatment, P-gp function could be assessed using the calcein-AM assay. This study would determine which signaling pathways upregulate P-gp function in BECs that have been co-cultured with astrocytes.

Recent evidence suggests that astrocytes are a heterogenous population of cells, varying in morphology and function across brain regions. Since the studies in this thesis have highlighted in the importance of astrocytes on P-gp function and expression at the BBB, future studies should investigate whether levels *Abcb1*/P-gp in brain microvessels varies across brain regions and how this is regulated through development. To investigate this, brain microvessels could be collected from different regions of the brain, such as the cortex, cerebellum and brain stem, from GD40, GD50, GD65 and PND14 guinea pigs. Protein and RNA could be extracted from these microvessels in order to measure P-gp protein levels and *Abcb1* mRNA via qRT-PCR. Additionally, P-gp function could be measured *in vivo* using radio-labeled substrates. This would entail injecting pregnant or PND14 guinea pigs with 50 μg/kg [^{3}H]-digoxin (10 μCi/dam) 1h prior to euthanasia to

assess P-gp function. Digoxin is considered the 'gold standard' substrates to assess P-gp function. It is not readily metabolized and the recovery rate of intact [^{3}H]digoxin in the mouse is 95% after 4 hours[375]. Fetal plasma, amniotic fluid and fetal brains can be collected and homogenized. To assess regional brain levels of P-gp function, brains can be separated into cortex, cerebellum and brain stem prior to homogenization. Tissue homogenate can then be counted using a beta-radiation scintillation counter, and normalized to fetal plasma or amniotic fluid[93]. By comparing accumulation of [^{3}H]-digoxin in different regions of the brain, we could determine if P-gp function varies across brain regions.

7.8.2 Compromised astrocyte maturation

The data from this thesis indicates that the extent of astrocyte maturation, determined by markers nestin, AQP4 and GFAP, affects the astrocyte-mediated effect on *Abcb1*/P-gp at the developing BBB. Thus, aberrations in astrocyte maturation may dysregulate P-gp at the developing BBB. Prenatal chronic inflammation and hypoxia have been demonstrated to cause changes in astrocyte markers in the fetal brain. Previous studies demonstrate that chronic hypoxia delays astrocyte maturation, as demonstrated by an increase in nestin expression and decreases in GFAP and GLAST (glutamate transporter) expression - resembling a more immature phenotype[323]. Similarly, nestin expression was increased in astrocytes of rats exposed to prolonged systemic inflammation *in utero*. Also, hypoxia-ischemia was shown to downregulate AQP4 expression in cultured astrocytes from neonatal rats. However, very limited information currently exists about how prenatal chronic inflammation or hypoxia may regulate P-gp

at the developing BBB. This is clinically relevant as these conditions are common during pregnancy, such as obesity, smoking and diabetes.

To investigate this, co-cultures could be exposed to lipopolysaccharide (LPS), which elicits inflammatory signaling pathways, or hypoxia via an oxygen controlled incubator. Once BECs reach confluence, co-cultures could be exposed to control media or media containing LPS (1-1000 μg/ml) for 24 h, 48 h or 72 h. Previous studies have shown that these doses of LPS do not have effect on BBB properties in BEC but cause astrogliosis and release of pro-inflammatory cytokines in primary cultures of astrocytes[376,377]. To model chronic hypoxia, co-cultures could be exposed to 0.1%, 0.5%, 2% O_2 and normoxic conditions for 24, 48 and 72 hours. Normal oxygen concentrations in the fetal brain range from 2-5% oxygen[378]. These conditions were chosen as exposing astrocytes to 0.5% O_2 for 48 h was shown to decrease GFAP expression and increase nestin expression, which resembles an immature astrocyte phenotype[323]. Following each treatment, P-gp function in BECs can be assessed by measuring cellular accumulation of calcein-AM. RNA can also be collected to measure levels of *Abcb1*. Astrocyte phenotype can be analyzed before and after treatment using immunocytochemistry.

In vivo, chronic inflammation could be modeled by injecting pregnant guinea pigs with LPS (Fig. 7-5). Pregnant guinea pigs at GD50 or 65 would be euthanized 24 h following multiple injections (1 dose/day for 5 days; chronic infection) of 100 ug/kg LPS or saline (VEH). This dosing regime was shown to increase pro-inflammatory cytokine levels in fetal guinea pig brain and resulted in fetal brain injury without significant alterations to fetal blood gases or mean arterial pressure.[379-381]. Chronic hypoxia could be simulated by performing maternal uterine artery ligation (Fig. 7-6). Unilateral ligation of

the maternal uterine artery could be done at GD30 and GD45. Briefly, pregnant guinea pigs would be anesthetized. The mesometrium of the right uterine horn would be exposed via a midline incision and the uterine artery would be ligated with silk sutures near the cervical end of the arterial cascade. This model was shown to induce white matter damage in fetal guinea pigs, with near-term insults resulted in less brain damage[382,383]. Fetuses could be delivered twenty days after hypoxic insult, at GD50 and GD65 by cesearan section. Subsequently, brain microvessels could be collected to measure P-gp at the mRNA level. At time points that yield significant changes in *Abcb1* mRNA, P-gp function could be evaluated via fetal brain accumulation of P-gp substrate, [^{3}H]digoxin as described in the section above. These studies will determine the effect of disturbed astrocyte maturation, specifically by prenatal chronic inflammation and hypoxia, on regulation of P-gp at the fetal BBB.

7.8.3 Long-term effect

Emerging evidence suggests that early insults during fetal life, such as chronic inflammation or chronic hypoxia, can downregulate P-gp function and expression at the BBB[384,385]. These effects have been correlated with an increased risk of neurodevelopmental disorders and neuropathology in adult life, such as schizophrenia and Alzheimer's disease[386,387]. The mechanism by which this occurs may be through disturbed astrocyte maturation, by chronic inflammation or hypoxia, in the developing fetal brain, as these effects can persist to adult life. Studies have shown that adult mice treated with E.coli as neonates have decreased astrocytes compared to controls[388]. Fetal guinea pigs that were exposed to *in utero* hypoxia also had decreased astrocyte density

compared to controls, an effect that occurred acutely during fetal development and that persisted into adulthood[389]. However, no study has considered whether these early insults that cause astrocyte loss or dysfunction in adulthood also affect P-gp function and expression at the BBB.

To determine the long-term effect of prenatal chronic inflammation and hypoxia, levels of *Abcb1* could be measured in brain microvessels derived from offspring at PND14, 4 months and 12 months of age following fetal insults (chronic inflammation or hypoxia; Fig. 7-7). The timing in pregnancy in which fetal insults would be undertaken would be based on time-points that yield significant changes on P-gp expression and function in short-term studies as outlined above. We can also assess β-amyloid brain accumulation and astrocyte morphology using immunohistochemistry in brain slices. β-amyloid is the main component of extracellular deposits found in the brains of patients with Alzheimer's disease and there is growing evidence that P-gp mediates the efflux of β-amyloid from the brain. If P-gp function is decreased as a result of these prenatal insults, we would expect there to be more β-amyloid accumulation compared to control animals. To investigate how these effects are propagated later in life, we would investigate transcription factor binding (via chromatin-immunoprecipitation-sequencing) and methylation status of *Abcb1* promoter (via Na-bisulfite sequencing). These proposed studies would provide a mechanism by which fetal brain insults, by inflammation and hypoxia, may alter P-gp at the BBB and make the brain more susceptible to neurodegenerative disorders later in life. Understanding how events during fetal life compromise astrocyte maturation and consequently P-gp at the BBB will be critical in the development of future therapies to counteract these effects.

Figure 7-1. Effect of prenatal dexamethasone on *Abcb1*/P-gp levels at the developing guinea pig blood-brain barrier. Characterization of *Abcb1*/P-gp at the blood-brain barrier Levels of *Abcb1* mRNA expression in brain endothelial cells (A) and P-gp protein levels brain microvessels (B) in gestational day 50 guinea pigs prenatally exposed to either saline (VEH) or dexamethasone (DEX). Both *Abcb1* mRNA and P-gp protein levels are elevated in BECs derived from DEX-exposed fetuses. A significant difference from VEH indicated by (*) $P < 0.05$. (Iqbal et al., *manuscript in submission* 2015)

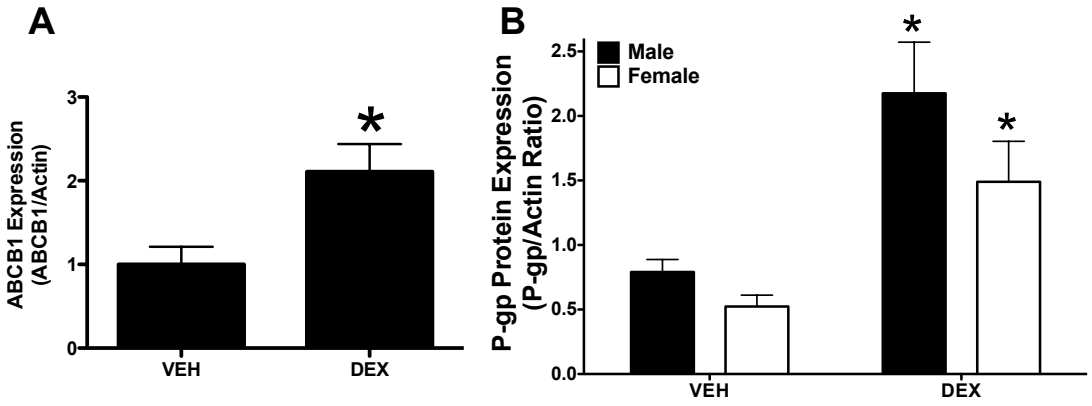


Figure 7-2. Schematic of barrier interfaces in the developing brain. Note the barrier forming cellular layers at each interface are coloured purple. Adapted with permission from Ek et al., *Neurotoxicology* (2012).

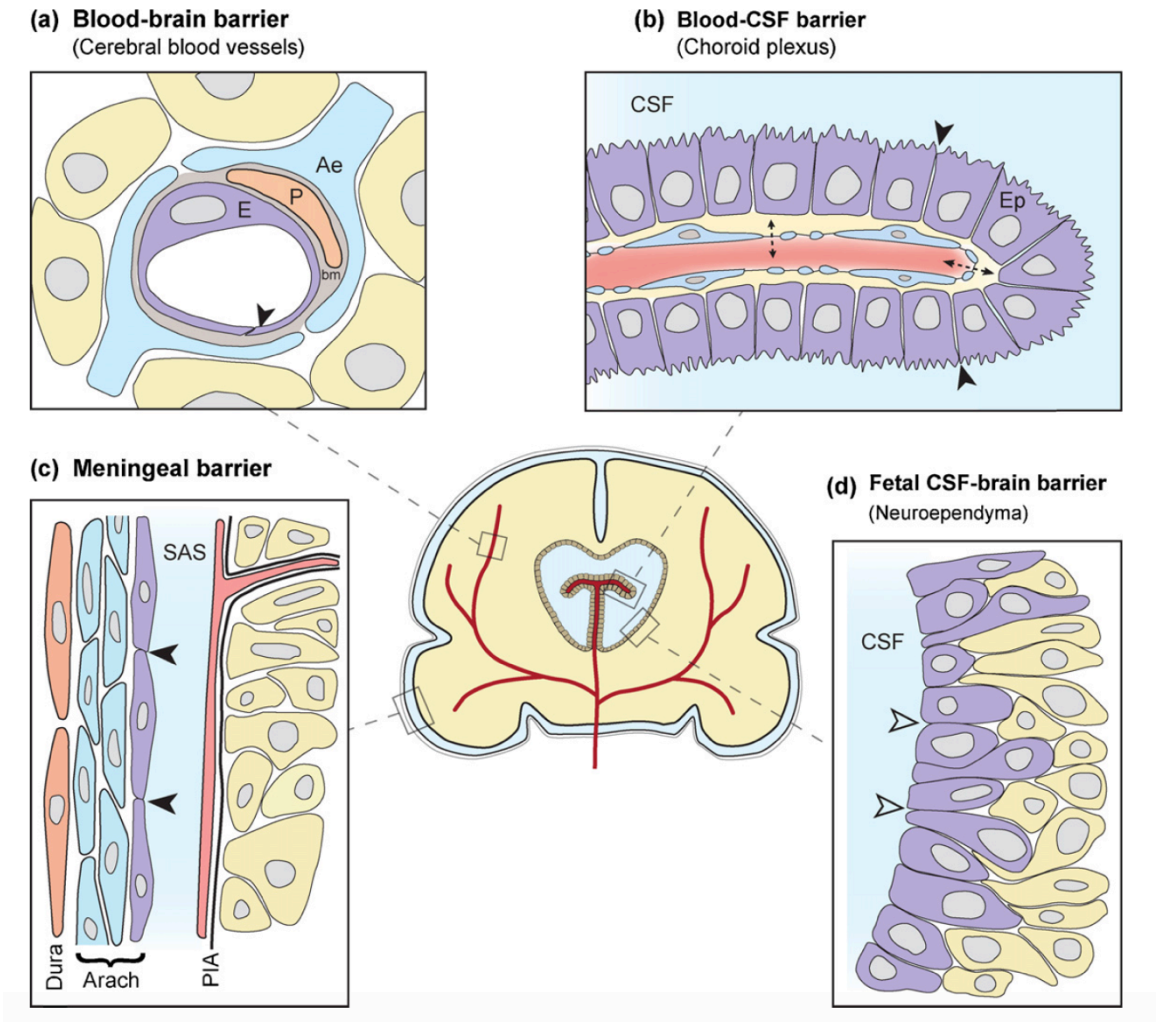


Figure 7-3. Developmental levels of global DNA methylation in guinea pig brain microvessels. Global DNA methylation decreases with development in isolated brain microvessels. Luminometric methylation assay (LUMA), a method to assess global DNA methylation, was performed on brain microvessels derived from GD50 and 65, and PND14 male and female guinea pigs. Data is expressed as mean percent global methylation ± S.E.M. Significant differences from GD50 of the same sex indicated by (**) P<0.01; (***) P<0.001. (Iqbal et al., *unpublished data*)

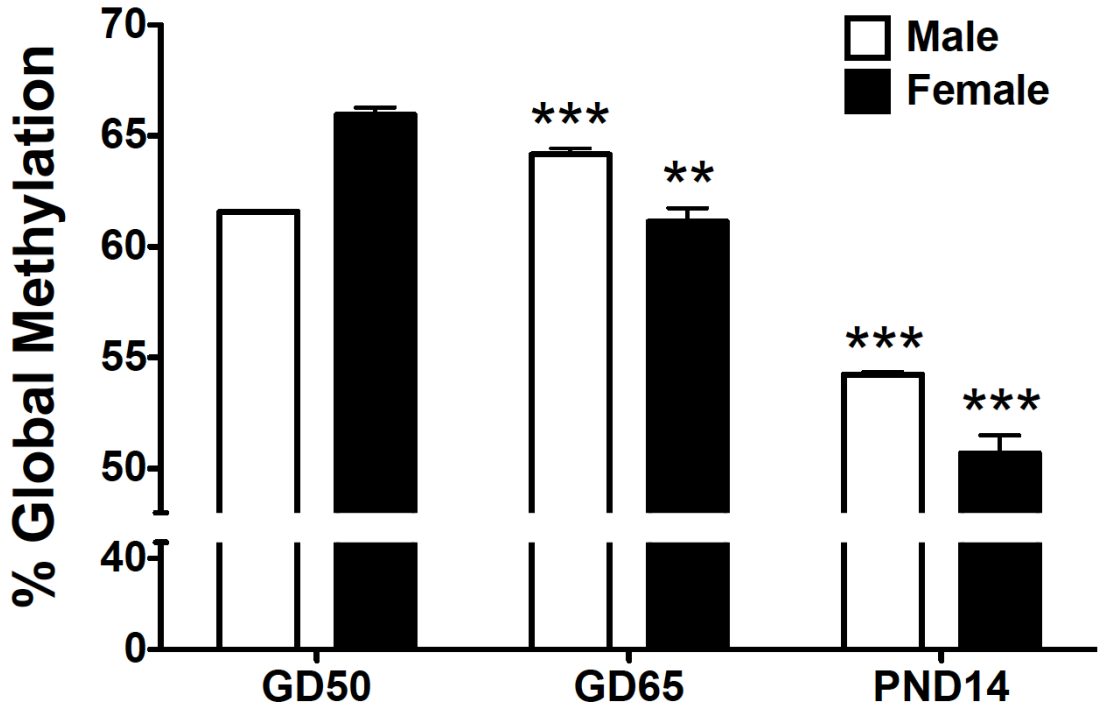


Figure 7-4. Placental levels of miRNAs known to regulate *Abcb1*. Levels of miR-451 (A), miR-145 (B) and miR-200c (C) are elevated in third trimester human placental explants compared to first trimester explants. (Imperio et al., *unpublished data*)

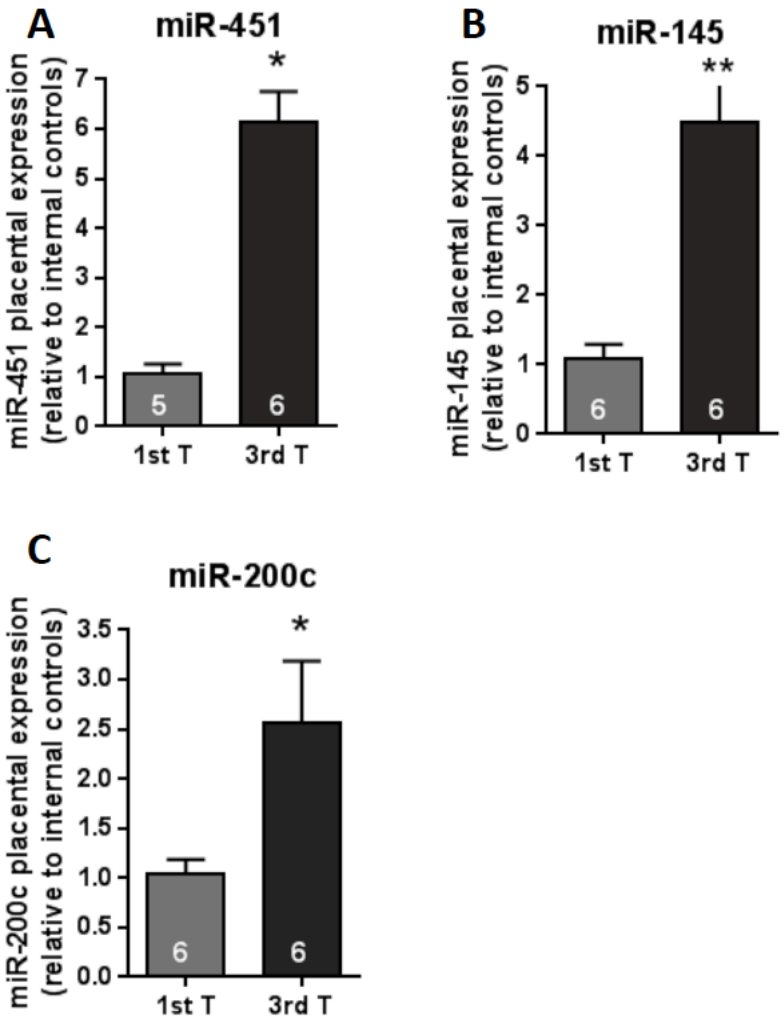


Figure 7-5. Paradigm for the experiments outlined to investigate effect of compromised astrocyte maturation, induced by inflammation, on *Abcb1*/P-gp at the BBB. Pregnant guinea pigs will either be treated with multiple doses of either VEH or LPS, prior to GD50 or 65. Pregnant guinea pigs will receive injections every day for 5 days, prior to euthanasia. Mothers will be injected with [3H]-digoxin (P-gp substrate) 1h prior to euthanasia. Fetal microvessels will be collected to measure mRNA expression of various genes of interest. Fetal brains will be collected to measure accumulation of [3H]-digoxin in order to assess changes in drug transporter function.

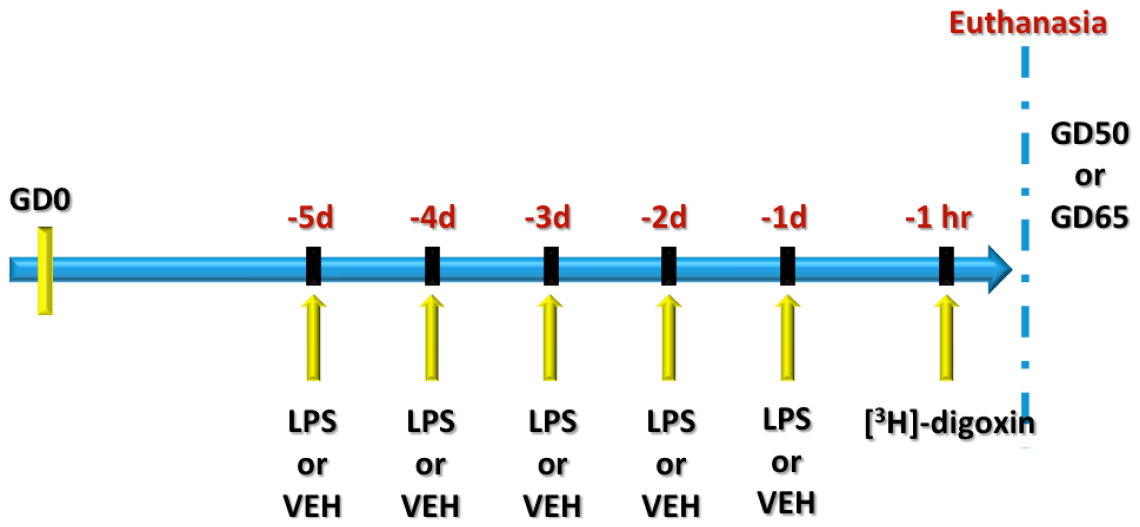


Figure 7-6. Paradigm for the experiments outlined to investigate the effect of compromised astrocyte maturation, induced by hypoxia, on *Abcb1*/P-gp at the BBB. Pregnant guinea pigs will undergo unilateral ligation of maternal uterine artery 20 days prior to GD50 or 65. Mothers will be injected with [3H]-digoxin (P-gp substrate) 1 h prior to euthanasia. Fetal microvessels will be collected to measure mRNA expression of various genes of interest. Fetal brains will be collected to measure accumulation of [3H]-digoxin in order to assess changes in drug transporter function.

Figure 7-7. Paradigm for the experiments outlined to investigate long-term effect of dysregulated astrocyte maturation on *Abcb1*/P-gp at the BBB. Following prenatal insult described in short-term studies, microvessels will be collected at PND14. RNA will be extracted to assess changes in *Abcb1* mRNA. If changes are seen, the latter will be repeated on offspring at 4 months and 12 months of age. β-amyloid will also be assessed as a measurement of P-gp activity and neurodegeneration.

References

1 Juliano, R. L. & Ling, V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochimica et biophysica acta* **455**, 152-162 (1976).

2 Koren, G., Woodland, C. & Ito, S. Toxic digoxin-drug interactions: the major role of renal P-glycoprotein. *Veterinary and human toxicology* **40**, 45-46 (1998).

3 Thiebaut, F. *et al.* Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 7735-7738 (1987).

4 Cordon-Cardo, C. *et al.* Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* **38**, 1277-1287 (1990).

5 Cordon-Cardo, C. *et al.* Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 695-698 (1989).

6 Holash, J. A., Harik, S. I., Perry, G. & Stewart, P. A. Barrier properties of testis microvessels. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 11069-11073 (1993).

7 Moscow, J. A. *et al.* Expression of anionic glutathione-S-transferase and P-glycoprotein genes in human tissues and tumors. *Cancer research* **49**, 1422-1428 (1989).

8 Lankas, G. R., Wise, L. D., Cartwright, M. E., Pippert, T. & Umbenhauer, D. R. Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. *Reproductive toxicology (Elmsford, N.Y.)* **12**, 457-463 (1998).

9 Schinkel, A. H. *et al.* Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. *Cell* **77**, 491-502 (1994).

10 Fu, D. & Arias, I. M. Intracellular trafficking of P-glycoprotein. *The international journal of biochemistry & cell biology* **44**, 461-464, doi:10.1016/j.biocel.2011.12.009 (2012).

11 Pappas, J. J. *et al.* The multidrug resistance 1 gene Abcb1 in brain and placenta: comparative analysis in human and guinea pig. *PLoS One* **9**, e111135, doi:10.1371/journal.pone.0111135 (2014).

12 Loo, T. W. & Clarke, D. M. Drug rescue distinguishes between different structural models of human P-glycoprotein. *Biochemistry* **52**, 7167-7169, doi:10.1021/bi401269m (2013).

13 Molinari, A. *et al.* Subcellular detection and localization of the drug transporter P-glycoprotein in cultured tumor cells. *Current protein & peptide science* **3**, 653-670 (2002).

14 Ambudkar, S. V., Kimchi-Sarfaty, C., Sauna, Z. E. & Gottesman, M. M. P-glycoprotein: from genomics to mechanism. *Oncogene* **22**, 7468-7485, doi:10.1038/sj.onc.1206948 (2003).

15 Loo, T. W. & Clarke, D. M. The human multidrug resistance P-glycoprotein is inactive when its maturation is inhibited: potential for a role in cancer chemotherapy. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **13**, 1724-1732 (1999).

16 Draheim, V., Reichel, A., Weitschies, W. & Moenning, U. N-glycosylation of ABC transporters is associated with functional activity in sandwich-cultured rat hepatocytes. *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences* **41**, 201-209, doi:10.1016/j.ejps.2010.06.005 (2010).

17 Schinkel, A. H., Kemp, S., Dolle, M., Rudenko, G. & Wagenaar, E. N-glycosylation and deletion mutants of the human MDR1 P-glycoprotein. *The Journal of biological chemistry* **268**, 7474-7481 (1993).

18 Kramer, R. *et al.* Constitutive expression of multidrug resistance in human colorectal tumours and cell lines. *British journal of cancer* **67**, 959-968 (1993).

19 Wojtowicz, K. *et al.* Inhibitors of N-glycosylation as a potential tool for analysis of the mechanism of action and cellular localisation of glycoprotein P. *Acta biochimica Polonica* **59**, 445-450 (2012).

20 Chambers, T. C., Pohl, J., Raynor, R. L. & Kuo, J. F. Identification of specific sites in human P-glycoprotein phosphorylated by protein kinase C. *The Journal of biological chemistry* **268**, 4592-4595 (1993).

21 Chambers, T. C., Pohl, J., Glass, D. B. & Kuo, J. F. Phosphorylation by protein kinase C and cyclic AMP-dependent protein kinase of synthetic peptides derived from the linker region of human P-glycoprotein. *The Biochemical journal* **299 (Pt 1)**, 309-315 (1994).

22 Goodfellow, H. R. *et al.* Protein kinase C-mediated phosphorylation does not regulate drug transport by the human multidrug resistance P-glycoprotein. *The Journal of biological chemistry* **271**, 13668-13674 (1996).

23 Germann, U. A. *et al.* Characterization of phosphorylation-defective mutants of human P-glycoprotein expressed in mammalian cells. *The Journal of biological chemistry* **271**, 1708-1716 (1996).

24 Zhou, S. F. Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. *Xenobiotica; the fate of foreign compounds in biological systems* **38**, 802-832, doi:10.1080/00498250701867889 (2008).

25 Bain, L. J., McLachlan, J. B. & LeBlanc, G. A. Structure-activity relationships for xenobiotic transport substrates and inhibitory ligands of P-glycoprotein. *Environmental health perspectives* **105**, 812-818 (1997).

26 Lagas, J. S., Vlaming, M. L. & Schinkel, A. H. Pharmacokinetic assessment of multiple ATP-binding cassette transporters: the power of combination knockout mice. *Molecular interventions* **9**, 136-145, doi:10.1124/mi.9.3.7 (2009).

27 Leslie, E. M., Deeley, R. G. & Cole, S. P. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicology*

and applied pharmacology **204**, 216-237, doi:10.1016/j.taap.2004.10.012 (2005).

28 Kim, W. Y. & Benet, L. Z. P-glycoprotein (P-gp/MDR1)-mediated efflux of sex-steroid hormones and modulation of P-gp expression in vitro. *Pharmaceutical research* **21**, 1284-1293 (2004).

29 Rao, U. S., Fine, R. L. & Scarborough, G. A. Antiestrogens and steroid hormones: substrates of the human P-glycoprotein. *Biochemical pharmacology* **48**, 287-292 (1994).

30 Pawlik, A., Baskiewicz-Masiuk, M., Machalinski, B., Safranow, K. & Gawronska-Szklarz, B. Involvement of P-glycoprotein in the release of cytokines from peripheral blood mononuclear cells treated with methotrexate and dexamethasone. *The Journal of pharmacy and pharmacology* **57**, 1421-1425, doi:10.1211/jpp.57.11.0007 (2005).

31 Bleier, B. S. *et al.* P-glycoprotein functions as an immunomodulator in healthy human primary nasal epithelial cells. *International forum of allergy & rhinology* **3**, 433-438, doi:10.1002/alr.21166 (2013).

32 Kooij, G. *et al.* Adenosine triphosphate-binding cassette transporters mediate chemokine (C-C motif) ligand 2 secretion from reactive astrocytes: relevance to multiple sclerosis pathogenesis. *Brain : a journal of neurology* **134**, 555-570, doi:10.1093/brain/awq330 (2011).

33 Yates, C. R. *et al.* Structural determinants of P-glycoprotein-mediated transport of glucocorticoids. *Pharmaceutical research* **20**, 1794-1803 (2003).

34 Moisiadis, V. G. & Matthews, S. G. Glucocorticoids and fetal programming part 2: Mechanisms. *Nature reviews. Endocrinology* **10**, 403-411, doi:10.1038/nrendo.2014.74 (2014).

35 Loo, T. W., Bartlett, M. C. & Clarke, D. M. The drug-binding pocket of the human multidrug resistance P-glycoprotein is accessible to the aqueous medium. *Biochemistry* **43**, 12081-12089, doi:10.1021/bi049045t (2004).

36 Globisch, C., Pajeva, I. K. & Wiese, M. Identification of putative binding sites of P-glycoprotein based on its homology model. *ChemMedChem* **3**, 280-295, doi:10.1002/cmdc.200700249 (2008).

37 Sharom, F. J. The P-glycoprotein efflux pump: how does it transport drugs? *The Journal of membrane biology* **160**, 161-175 (1997).

38 Sauna, Z. E. & Ambudkar, S. V. About a switch: how P-glycoprotein (ABCB1) harnesses the energy of ATP binding and hydrolysis to do mechanical work. *Molecular cancer therapeutics* **6**, 13-23, doi:10.1158/1535-7163.mct-06-0155 (2007).

39 Sundseth, R., MacDonald, G., Ting, J. & King, A. C. DNA elements recognizing NF-Y and Sp1 regulate the human multidrug-resistance gene promoter. *Molecular pharmacology* **51**, 963-971 (1997).

40 Goldsmith, M. E., Madden, M. J., Morrow, C. S. & Cowan, K. H. A Y-box consensus sequence is required for basal expression of the human multidrug resistance (mdr1) gene. *The Journal of biological chemistry* **268**, 5856-5860 (1993).

41 Cornwell, M. M. The human multidrug resistance gene: sequences upstream and downstream of the initiation site influence transcription. *Cell growth &*

differentiation : the molecular biology journal of the American Association for Cancer Research **1**, 607-615 (1990).

42 Hu, Z., Jin, S. & Scotto, K. W. Transcriptional activation of the MDR1 gene by UV irradiation. Role of NF-Y and Sp1. *The Journal of biological chemistry* **275**, 2979-2985 (2000).

43 Cornwell, M. M. & Smith, D. E. SP1 activates the MDR1 promoter through one of two distinct G-rich regions that modulate promoter activity. *The Journal of biological chemistry* **268**, 19505-19511 (1993).

44 Gromnicova, R., Romero, I. & Male, D. Transcriptional Control of the Multi-Drug Transporter ABCB1 by Transcription Factor Sp3 in Different Human Tissues. *PLoS ONE* **7**, e48189, doi:10.1371/journal.pone.0048189 (2012).

45 Johnson, R. A., Ince, T. A. & Scotto, K. W. Transcriptional repression by p53 through direct binding to a novel DNA element. *The Journal of biological chemistry* **276**, 27716-27720, doi:10.1074/jbc.C100121200 (2001).

46 Ince, T. A. & Scotto, K. W. A conserved downstream element defines a new class of RNA polymerase II promoters. *The Journal of biological chemistry* **270**, 30249-30252 (1995).

47 Miyazaki, M. *et al.* Activation of human multidrug resistance-1 gene promoter in response to heat shock stress. *Biochemical and biophysical research communications* **187**, 677-684 (1992).

48 Jin, S. & Scotto, K. W. Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y. *Molecular and cellular biology* **18**, 4377-4384 (1998).

49 Daschner, P. J., Ciolino, H. P., Plouzek, C. A. & Yeh, G. C. Increased AP-1 activity in drug resistant human breast cancer MCF-7 cells. *Breast cancer research and treatment* **53**, 229-240 (1999).

50 Ikeguchi, M., Teeter, L. D., Eckersberg, T., Ganapathi, R. & Kuo, M. T. Structural and functional analyses of the promoter of the murine multidrug resistance gene mdr3/mdr1a reveal a negative element containing the AP-1 binding site. *DNA and cell biology* **10**, 639-649 (1991).

51 Desiderato, L., Davey, M. W. & Piper, A. A. Demethylation of the human MDR1 5' region accompanies activation of P-glycoprotein expression in a HL60 multidrug resistant subline. *Somatic cell and molecular genetics* **23**, 391-400 (1997).

52 Tada, Y. *et al.* MDR1 gene overexpression and altered degree of methylation at the promoter region in bladder cancer during chemotherapeutic treatment. *Clinical cancer research : an official journal of the American Association for Cancer Research* **6**, 4618-4627 (2000).

53 Nakayama, M. *et al.* Hypomethylation status of CpG sites at the promoter region and overexpression of the human MDR1 gene in acute myeloid leukemias. *Blood* **92**, 4296-4307 (1998).

54 Garcia-Manero, G. *et al.* DNA methylation patterns at relapse in adult acute lymphocytic leukemia. *Clinical cancer research : an official journal of the American Association for Cancer Research* **8**, 1897-1903 (2002).

55 Chen, K. G. *et al.* Genetic and epigenetic modeling of the origins of multidrug-resistant cells in a human sarcoma cell line. *Cancer research* **65**, 9388-9397, doi:10.1158/0008-5472.can-04-4133 (2005).

56 Kovalchuk, O. *et al.* Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. *Molecular cancer therapeutics* **7**, 2152-2159, doi:10.1158/1535-7163.mct-08-0021 (2008).

57 Feng, D. D. *et al.* Down-regulated miR-331-5p and miR-27a are associated with chemotherapy resistance and relapse in leukaemia. *Journal of cellular and molecular medicine* **15**, 2164-2175, doi:10.1111/j.1582-4934.2010.01213.x (2011).

58 Uchino, K., Ochiya, T. & Takeshita, F. RNAi therapeutics and applications of microRNAs in cancer treatment. *Japanese journal of clinical oncology* **43**, 596-607, doi:10.1093/jjco/hyt052 (2013).

59 Bao, L. *et al.* Increased expression of P-glycoprotein and doxorubicin chemoresistance of metastatic breast cancer is regulated by miR-298. *Am J Pathol* **180**, 2490-2503, doi:10.1016/j.ajpath.2012.02.024 (2012).

60 Zhu, X. *et al.* miR-137 restoration sensitizes multidrug-resistant MCF-7/ADM cells to anticancer agents by targeting YB-1. *Acta biochimica et biophysica Sinica* **45**, 80-86, doi:10.1093/abbs/gms099 (2013).

61 Zhao, X., Yang, L., Hu, J. & Ruan, J. miR-138 might reverse multidrug resistance of leukemia cells. *Leukemia research* **34**, 1078-1082, doi:10.1016/j.leukres.2009.10.002 (2010).

62 Hong, L. *et al.* The prognostic and chemotherapeutic value of miR-296 in esophageal squamous cell carcinoma. *Annals of surgery* **251**, 1056-1063, doi:10.1097/SLA.0b013e3181dd4ea9 (2010).

63 Coomber, B. L. & Stewart, P. A. Morphometric analysis of CNS microvascular endothelium. *Microvascular research* **30**, 99-115 (1985).

64 von Moltke, L. L. *et al.* Interaction of triazolam and ketoconazole in P-glycoprotein-deficient mice. *Drug metabolism and disposition: the biological fate of chemicals* **32**, 800-804 (2004).

65 van Asperen, J. *et al.* Altered pharmacokinetics of vinblastine in Mdr1a P-glycoprotein-deficient Mice. *Journal of the National Cancer Institute* **88**, 994-999 (1996).

66 Kemper, E. M. *et al.* Increased penetration of paclitaxel into the brain by inhibition of P-Glycoprotein. *Clinical cancer research : an official journal of the American Association for Cancer Research* **9**, 2849-2855 (2003).

67 Abbott, N. J. & Friedman, A. Overview and introduction: the blood-brain barrier in health and disease. *Epilepsia* **53 Suppl 6**, 1-6, doi:10.1111/j.1528-1167.2012.03696.x (2012).

68 Abbott, N. J. Astrocyte-endothelial interactions and blood-brain barrier permeability. *Journal of anatomy* **200**, 629-638 (2002).

69 Risau, W., Hallmann, R., Albrecht, U. & Henke-Fahle, S. Brain induces the expression of an early cell surface marker for blood-brain barrier-specific endothelium. *The EMBO journal* **5**, 3179-3183 (1986).

70 Gerhardt, H. *et al.* Neuropilin-1 is required for endothelial tip cell guidance in the developing central nervous system. *Developmental dynamics : an official*

publication of the American Association of Anatomists **231**, 503-509, doi:10.1002/dvdy.20148 (2004).

71 Risau, W. & Wolburg, H. Development of the blood-brain barrier. *Trends in neurosciences* **13**, 174-178 (1990).

72 Risau, W. Mechanisms of angiogenesis. *Nature* **386**, 671-674, doi:10.1038/386671a0 (1997).

73 Bar, T. The vascular system of the cerebral cortex. *Advances in anatomy, embryology, and cell biology* **59**, I-vi,1-62 (1980).

74 Ballabh, P., Hu, F., Kumarasiri, M., Braun, A. & Nedergaard, M. Development of tight junction molecules in blood vessels of germinal matrix, cerebral cortex, and white matter. *Pediatric research* **58**, 791-798, doi:10.1203/01.pdr.0000180535.14093.fb (2005).

75 Norman, M. G. & O'Kusky, J. R. The growth and development of microvasculature in human cerebral cortex. *Journal of neuropathology and experimental neurology* **45**, 222-232 (1986).

76 Breier, G., Albrecht, U., Sterrer, S. & Risau, W. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development (Cambridge, England)* **114**, 521-532 (1992).

77 Hogan, K. A., Ambler, C. A., Chapman, D. L. & Bautch, V. L. The neural tube patterns vessels developmentally using the VEGF signaling pathway. *Development (Cambridge, England)* **131**, 1503-1513, doi:10.1242/dev.01039 (2004).

78 Wolburg, H. & Lippoldt, A. Tight junctions of the blood-brain barrier: development, composition and regulation. *Vascular pharmacology* **38**, 323-337 (2002).

79 Ek, C. J., Dziegielewska, K. M., Stolp, H. & Saunders, N. R. Functional effectiveness of the blood-brain barrier to small water-soluble molecules in developing and adult opossum (Monodelphis domestica). *The Journal of comparative neurology* **496**, 13-26, doi:10.1002/cne.20885 (2006).

80 Mollgard, K. & Saunders, N. R. The development of the human blood-brain and blood-CSF barriers. *Neuropathology and applied neurobiology* **12**, 337-358 (1986).

81 Mollgoard, K. & Saunders, N. R. Complex tight junctions of epithelial and of endothelial cells in early foetal brain. *Journal of neurocytology* **4**, 453-468 (1975).

82 Bauer, H. C. *et al.* Neovascularization and the appearance of morphological characteristics of the blood-brain barrier in the embryonic mouse central nervous system. *Brain research. Developmental brain research* **75**, 269-278 (1993).

83 Saunders, N. R., Habgood, M. D. & Dziegielewska, K. M. Barrier mechanisms in the brain, II. Immature brain. *Clinical and experimental pharmacology & physiology* **26**, 85-91 (1999).

84 Dziegielewska, K. M. *et al.* Studies of the development of brain barrier systems to lipid insoluble molecules in fetal sheep. *The Journal of physiology* **292**, 207-231 (1979).

85 Engelhardt, B. & Sorokin, L. The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. *Seminars in immunopathology* **31**, 497-511, doi:10.1007/s00281-009-0177-0 (2009).

86 Betz, A. L. & Goldstein, G. W. Developmental changes in metabolism and transport properties of capillaries isolated from rat brain. *The Journal of physiology* **312**, 365-376, doi:10.1113/jphysiol.1981.sp013633 (1981).

87 Kuhnke, D. *et al.* MDR1-P-Glycoprotein (ABCB1) Mediates Transport of Alzheimer's amyloid-beta peptides--implications for the mechanisms of Abeta clearance at the blood-brain barrier. *Brain pathology (Zurich, Switzerland)* **17**, 347-353, doi:10.1111/j.1750-3639.2007.00075.x (2007).

88 Kooij, G. *et al.* P-glycoprotein regulates trafficking of CD8(+) T cells to the brain parenchyma. *Acta Neuropathol* **127**, 699-711, doi:10.1007/s00401-014-1244-8 (2014).

89 Kalabis, G. M. *et al.* Multidrug resistance phosphoglycoprotein (ABCB1) in the mouse placenta: fetal protection. *Biology of reproduction* **73**, 591-597, doi:10.1095/biolreprod.105.042242 (2005).

90 Kalabis, G. M., Petropoulos, S., Gibb, W. & Matthews, S. G. Multidrug resistance phosphoglycoprotein (ABCB1) expression in the guinea pig placenta: developmental changes and regulation by betamethasone. *Canadian journal of physiology and pharmacology* **87**, 973-978, doi:10.1139/y09-087 (2009).

91 Lye, P. *et al.* Effect of oxygen on multidrug resistance in the first trimester human placenta. *Placenta* **34**, 817-823, doi:10.1016/j.placenta.2013.05.010 (2013).

92 Iqbal, M., Gibb, W. & Matthews, S. G. Corticosteroid regulation of P-glycoprotein in the developing blood-brain barrier. *Endocrinology* **152**, 1067-1079, doi:10.1210/en.2010-1227 (2011).

93 Petropoulos, S., Gibb, W. & Matthews, S. G. Developmental expression of multidrug resistance phosphoglycoprotein (P-gp) in the mouse fetal brain and glucocorticoid regulation. *Brain research* **1357**, 9-18, doi:10.1016/j.brainres.2010.08.016 (2010).

94 Virgintino, D. *et al.* Fetal blood-brain barrier P-glycoprotein contributes to brain protection during human development. *Journal of neuropathology and experimental neurology* **67**, 50-61, doi:10.1097/nen.0b013e31815f65d9 (2008).

95 Lam, J. *et al.* The ontogeny of P-glycoprotein in the developing human blood brain barrier: Implication for opioid toxicity in neonates. *Pediatric research*, doi:10.1038/pr.2015.119 (2015).

96 Daw, J. R., Hanley, G. E., Greyson, D. L. & Morgan, S. G. Prescription drug use during pregnancy in developed countries: a systematic review. *Pharmacoepidemiology and drug safety* **20**, 895-902, doi:10.1002/pds.2184 (2011).

97 Bakker, M. K. *et al.* Drug prescription patterns before, during and after pregnancy for chronic, occasional and pregnancy-related drugs in the Netherlands. *BJOG : an international journal of obstetrics and gynaecology* **113**, 559-568, doi:10.1111/j.1471-0528.2006.00927.x (2006).

98 Andrade, S. E. *et al.* Prescription drug use in pregnancy. *American journal of obstetrics and gynecology* **191**, 398-407, doi:10.1016/j.ajog.2004.04.025 (2004).
99 Daud, A. N. A. *et al.* P-Glycoprotein-Mediated Drug Interactions in Pregnancy and Changes in the Risk of Congenital Anomalies: A Case-Reference Study. *Drug Safety* **38**, 651-659, doi:10.1007/s40264-015-0299-3 (2015).
100 Sofroniew, M. V. & Vinters, H. V. Astrocytes: biology and pathology. *Acta Neuropathologica* **119**, 7-35, doi:10.1007/s00401-009-0619-8 (2010).
101 Parpura, V. & Verkhratsky, A. Astrocytes revisited: concise historic outlook on glutamate homeostasis and signaling. *Croatian medical journal* **53**, 518-528, doi:10.3325/cmj.2012.53.518 (2012).
102 Aschner, M., Sonnewald, U. & Tan, K. H. Astrocyte Modulation of Neurotoxic Injury. *Brain Pathology* **12**, 475-481, doi:10.1111/j.1750-3639.2002.tb00465.x (2002).
103 Otsu, Y. *et al.* Calcium dynamics in astrocyte processes during neurovascular coupling. *Nature neuroscience* **18**, 210-218, doi:10.1038/nn.3906
http://www.nature.com/neuro/journal/v18/n2/abs/nn.3906.html - supplementary-information (2015).
104 Petzold, Gabor C. & Murthy, Venkatesh N. Role of Astrocytes in Neurovascular Coupling. *Neuron* **71**, 782-797, doi:10.1016/j.neuron.2011.08.009.
105 Abbott, N. J., Ronnback, L. & Hansson, E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nature reviews. Neuroscience* **7**, 41-53, doi:10.1038/nrn1824 (2006).
106 Alvarez, J. I. *et al.* The Hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. *Science (New York, N.Y.)* **334**, 1727-1731, doi:10.1126/science.1206936 (2011).
107 Prat, A., Biernacki, K., Wosik, K. & Antel, J. P. Glial cell influence on the human blood-brain barrier. *Glia* **36**, 145-155 (2001).
108 Molofsky, A. V. *et al.* Astrocytes and disease: a neurodevelopmental perspective. *Genes & development* **26**, 891-907, doi:10.1101/gad.188326.112 (2012).
109 Wilkinson, M., Hume, R., Strange, R. & Bell, J. E. Glial and neuronal differentiation in the human fetal brain 9-23 weeks of gestation. *Neuropathology and applied neurobiology* **16**, 193-204 (1990).
110 Dobbing, J. & Sands, J. Comparative aspects of the brain growth spurt. *Early human development* **3**, 79-83 (1979).
111 Barnabe-Heider, F. *et al.* Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1. *Neuron* **48**, 253-265, doi:10.1016/j.neuron.2005.08.037 (2005).
112 Freeman, M. R. Specification and morphogenesis of astrocytes. *Science (New York, N.Y.)* **330**, 774-778, doi:10.1126/science.1190928 (2010).
113 Bonni, A. *et al.* Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science (New York, N.Y.)* **278**, 477-483 (1997).
114 Stipursky, J., Francis, D. & Gomes, F. C. Activation of MAPK/PI3K/SMAD pathways by TGF-beta(1) controls differentiation of radial glia into

astrocytes in vitro. *Developmental neuroscience* **34**, 68-81, doi:10.1159/000338108 (2012).

115 Nakashima, K. *et al.* BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 5868-5873, doi:10.1073/pnas.101109698 (2001).

116 Nagao, M., Sugimori, M. & Nakafuku, M. Cross talk between notch and growth factor/cytokine signaling pathways in neural stem cells. *Molecular and cellular biology* **27**, 3982-3994, doi:10.1128/mcb.00170-07 (2007).

117 Deneen, B. *et al.* The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron* **52**, 953-968, doi:10.1016/j.neuron.2006.11.019 (2006).

118 Stipursky, J. & Gomes, F. C. TGF-beta1/SMAD signaling induces astrocyte fate commitment in vitro: implications for radial glia development. *Glia* **55**, 1023-1033, doi:10.1002/glia.20522 (2007).

119 Cassella, J. P., Lawrenson, J. G., Allt, G. & Firth, J. A. Ontogeny of four blood-brain barrier markers: an immunocytochemical comparison of pial and cerebral cortical microvessels. *Journal of anatomy* **189**, 407-415 (1996).

120 Daneman, R., Zhou, L., Kebede, A. A. & Barres, B. A. Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* **468**, 562-566, doi:10.1038/nature09513 (2010).

121 Sutherland, M. L., Delaney, T. A. & Noebels, J. L. Glutamate transporter mRNA expression in proliferative zones of the developing and adult murine CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **16**, 2191-2207 (1996).

122 Schlag, B. D. *et al.* Regulation of the glial Na+-dependent glutamate transporters by cyclic AMP analogs and neurons. *Molecular pharmacology* **53**, 355-369 (1998).

123 Nagy, J. I., Patel, D., Ochalski, P. A. & Stelmack, G. L. Connexin30 in rodent, cat and human brain: selective expression in gray matter astrocytes, co-localization with connexin43 at gap junctions and late developmental appearance. *Neuroscience* **88**, 447-468 (1999).

124 Seifert, G. *et al.* Analysis of astroglial K+ channel expression in the developing hippocampus reveals a predominant role of the Kir4.1 subunit. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 7474-7488, doi:10.1523/jneurosci.3790-08.2009 (2009).

125 Higashimori, H. & Sontheimer, H. Role of Kir4.1 channels in growth control of glia. *Glia* **55**, 1668-1679, doi:10.1002/glia.20574 (2007).

126 Cahoy, J. D. *et al.* A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 264-278, doi:10.1523/jneurosci.4178-07.2008 (2008).

127 Lovatt, D. *et al.* The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**, 12255-12266, doi:10.1523/jneurosci.3404-07.2007 (2007).

128 Yang, Y. *et al.* Presynaptic regulation of astroglial excitatory neurotransmitter transporter GLT1. *Neuron* **61**, 880-894, doi:10.1016/j.neuron.2009.02.010 (2009).
129 Swanson, R. A. *et al.* Neuronal regulation of glutamate transporter subtype expression in astrocytes. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **17**, 932-940 (1997).
130 Koulakoff, A., Ezan, P. & Giaume, C. Neurons control the expression of connexin 30 and connexin 43 in mouse cortical astrocytes. *Glia* **56**, 1299-1311, doi:10.1002/glia.20698 (2008).
131 Rouach, N., Glowinski, J. & Giaume, C. Activity-dependent neuronal control of gap-junctional communication in astrocytes. *The Journal of cell biology* **149**, 1513-1526 (2000).
132 Mathiisen, T. M., Lehre, K. P., Danbolt, N. C. & Ottersen, O. P. The perivascular astroglial sheath provides a complete covering of the brain microvessels: an electron microscopic 3D reconstruction. *Glia* **58**, 1094-1103, doi:10.1002/glia.20990 (2010).
133 Al Ahmad, A., Taboada, C. B., Gassmann, M. & Ogunshola, O. O. Astrocytes and pericytes differentially modulate blood-brain barrier characteristics during development and hypoxic insult. *J Cereb Blood Flow Metab* **31**, 693-705, doi:10.1038/jcbfm.2010.148 (2011).
134 Ezan, P. *et al.* Deletion of astroglial connexins weakens the blood-brain barrier. *J Cereb Blood Flow Metab* **32**, 1457-1467, doi:10.1038/jcbfm.2012.45 (2012).
135 Pardridge, W. M. Blood-brain barrier biology and methodology. *Journal of neurovirology* **5**, 556-569, doi:10.3109/13550289909021285 (1999).
136 Stewart, P. A. & Wiley, M. J. Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: a study using quail--chick transplantation chimeras. *Developmental biology* **84**, 183-192 (1981).
137 Janzer, R. C. & Raff, M. C. Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* **325**, 253-257, doi:10.1038/325253a0 (1987).
138 Tao-Cheng, J. H., Nagy, Z. & Brightman, M. W. Tight junctions of brain endothelium in vitro are enhanced by astroglia. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **7**, 3293-3299 (1987).
139 Rubin, L. L. *et al.* A cell culture model of the blood-brain barrier. *The Journal of cell biology* **115**, 1725-1735 (1991).
140 Neuhaus, J., Risau, W. & Wolburg, H. Induction of blood-brain barrier characteristics in bovine brain endothelial cells by rat astroglial cells in transfilter coculture. *Annals of the New York Academy of Sciences* **633**, 578-580 (1991).
141 Hayashi, Y. *et al.* Induction of various blood-brain barrier properties in non-neural endothelial cells by close apposition to co-cultured astrocytes. *Glia* **19**, 13-26 (1997).
142 Kuchler-Bopp, S., Delaunoy, J. P., Artault, J. C., Zaepfel, M. & Dietrich, J. B. Astrocytes induce several blood-brain barrier properties in non-neural endothelial cells. *Neuroreport* **10**, 1347-1353 (1999).

143 Cecchelli, R. *et al.* A stable and reproducible human blood-brain barrier model derived from hematopoietic stem cells. *PLoS One* **9**, e99733, doi:10.1371/journal.pone.0099733 (2014).
144 Correa, S. *et al.* Wnt/beta-catenin pathway regulates ABCB1 transcription in chronic myeloid leukemia. *BMC cancer* **12**, 303, doi:10.1186/1471-2407-12-303 (2012).
145 Liebner, S. *et al.* Wnt/beta-catenin signaling controls development of the blood-brain barrier. *The Journal of cell biology* **183**, 409-417, doi:10.1083/jcb.200806024 (2008).
146 Dohgu, S. *et al.* Transforming growth factor-beta1 upregulates the tight junction and P-glycoprotein of brain microvascular endothelial cells. *Cellular and molecular neurobiology* **24**, 491-497 (2004).
147 Heinemann, U., Kaufer, D. & Friedman, A. Blood-brain barrier dysfunction, TGFbeta signaling, and astrocyte dysfunction in epilepsy. *Glia* **60**, 1251-1257, doi:10.1002/glia.22311 (2012).
148 Sims-Mourtada, J., Izzo, J. G., Ajani, J. & Chao, K. S. Sonic Hedgehog promotes multiple drug resistance by regulation of drug transport. *Oncogene* **26**, 5674-5679, doi:10.1038/sj.onc.1210356 (2007).
149 Mizee, M. R. *et al.* Retinoic acid induces blood-brain barrier development. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33**, 1660-1671, doi:10.1523/jneurosci.1338-12.2013 (2013).
150 Paschaki, M. *et al.* Retinoic acid-dependent signaling pathways and lineage events in the developing mouse spinal cord. *PLoS One* **7**, e32447, doi:10.1371/journal.pone.0032447 (2012).
151 Pierani, A., Brenner-Morton, S., Chiang, C. & Jessell, T. M. A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-915 (1999).
152 Yamada, T. *et al.* Transactivation of the multidrug resistance 1 gene by T-cell factor 4/beta-catenin complex in early colorectal carcinogenesis. *Cancer research* **60**, 4761-4766 (2000).
153 Lim, J. C. *et al.* Activation of beta-catenin signalling by GSK-3 inhibition increases p-glycoprotein expression in brain endothelial cells. *Journal of neurochemistry* **106**, 1855-1865, doi:10.1111/j.1471-4159.2008.05537.x (2008).
154 Roberts, A. B. & Sporn, M. B. Differential expression of the TGF-beta isoforms in embryogenesis suggests specific roles in developing and adult tissues. *Molecular reproduction and development* **32**, 91-98, doi:10.1002/mrd.1080320203 (1992).
155 Takeshita, Y. & Ransohoff, R. M. Inflammatory cell trafficking across the blood-brain barrier (BBB): Chemokine regulation and in vitro models. *Immunological Reviews* **248**, 228-239, doi:10.1111/j.1600-065X.2012.01127.x (2012).
156 Eddleston, M. & Mucke, L. Molecular profile of reactive astrocytes--implications for their role in neurologic disease. *Neuroscience* **54**, 15-36 (1993).

157 Wyss-Coray, T., Lin, C., Sanan, D. A., Mucke, L. & Masliah, E. Chronic Overproduction of Transforming Growth Factor-β1 by Astrocytes Promotes Alzheimer's Disease-Like Microvascular Degeneration in Transgenic Mice. *The American Journal of Pathology* **156**, 139-150 (2000).

158 Cacheaux, L. P. *et al.* Transcriptome profiling reveals TGF-beta signaling involvement in epileptogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 8927-8935, doi:10.1523/jneurosci.0430-09.2009 (2009).

159 Goumans, M. J. *et al.* Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *The EMBO journal* **21**, 1743-1753, doi:10.1093/emboj/21.7.1743 (2002).

160 Brosnan, C. F. & Raine, C. S. The astrocyte in multiple sclerosis revisited. *Glia* **61**, 453-465, doi:10.1002/glia.22443 (2013).

161 Alvarez, J. I., Cayrol, R. & Prat, A. Disruption of central nervous system barriers in multiple sclerosis. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* **1812**, 252-264, doi:http://dx.doi.org/10.1016/j.bbadis.2010.06.017 (2011).

162 Ito, U., Hakamata, Y., Kawakami, E. & Oyanagi, K. Temporary focal cerebral ischemia results in swollen astrocytic end-feet that compress microvessels and lead to focal cortical infarction. *Journal of Cerebral Blood Flow & Metabolism* **31**, 328-338, doi:10.1038/jcbfm.2010.97 (2011).

163 Wagner, S., Tagaya, M., Koziol, J. A., Quaranta, V. & del Zoppo, G. J. Rapid disruption of an astrocyte interaction with the extracellular matrix mediated by integrin alpha 6 beta 4 during focal cerebral ischemia/reperfusion. *Stroke; a journal of cerebral circulation* **28**, 858-865 (1997).

164 Berezowski, V., Landry, C., Dehouck, M. P., Cecchelli, R. & Fenart, L. Contribution of glial cells and pericytes to the mRNA profiles of P-glycoprotein and multidrug resistance-associated proteins in an in vitro model of the blood-brain barrier. *Brain research* **1018**, 1-9, doi:10.1016/j.brainres.2004.05.092 (2004).

165 Ma, S. H., Lepak, L. A., Hussain, R. J., Shain, W. & Shuler, M. L. An endothelial and astrocyte co-culture model of the blood-brain barrier utilizing an ultra-thin, nanofabricated silicon nitride membrane. *Lab on a chip* **5**, 74-85, doi:10.1039/b405713a (2005).

166 Jeliazkova-Mecheva, V. V. & Bobilya, D. J. A porcine astrocyte/endothelial cell co-culture model of the blood-brain barrier. *Brain research. Brain research protocols* **12**, 91-98 (2003).

167 Wolburg, H., Wolburg-Buchholz, K., Fallier-Becker, P., Noell, S. & Mack, A. F. Structure and functions of aquaporin-4-based orthogonal arrays of particles. *International review of cell and molecular biology* **287**, 1-41, doi:10.1016/b978-0-12-386043-9.00001-3 (2011).

168 Noell, S. *et al.* Evidence for a role of dystroglycan regulating the membrane architecture of astroglial endfeet. *The European journal of neuroscience* **33**, 2179-2186, doi:10.1111/j.1460-9568.2011.07688.x (2011).

169 Nico, B. *et al.* Severe alterations of endothelial and glial cells in the blood-brain barrier of dystrophic mdx mice. *Glia* **42**, 235-251, doi:10.1002/glia.10216 (2003).
170 Lien, C. F. *et al.* Absence of glial alpha-dystrobrevin causes abnormalities of the blood-brain barrier and progressive brain edema. *The Journal of biological chemistry* **287**, 41374-41385, doi:10.1074/jbc.M112.400044 (2012).
171 Yang, Y., Higashimori, H. & Morel, L. Developmental maturation of astrocytes and pathogenesis of neurodevelopmental disorders. *Journal of neurodevelopmental disorders* **5**, 22, doi:10.1186/1866-1955-5-22 (2013).
172 Hamann, G. F., Okada, Y., Fitridge, R. & del Zoppo, G. J. Microvascular basal lamina antigens disappear during cerebral ischemia and reperfusion. *Stroke; a journal of cerebral circulation* **26**, 2120-2126 (1995).
173 Sixt, M. *et al.* Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. *The Journal of cell biology* **153**, 933-946 (2001).
174 Berzin, T. M. *et al.* Agrin and microvascular damage in Alzheimer's disease. *Neurobiology of aging* **21**, 349-355 (2000).
175 Wolburg, H., Noell, S., Wolburg-Buchholz, K., Mack, A. & Fallier-Becker, P. Agrin, aquaporin-4, and astrocyte polarity as an important feature of the blood-brain barrier. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry* **15**, 180-193, doi:10.1177/1073858408329509 (2009).
176 Crawford, D. C., Jiang, X., Taylor, A. & Mennerick, S. Astrocyte-derived thrombospondins mediate the development of hippocampal presynaptic plasticity in vitro. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**, 13100-13110, doi:10.1523/jneurosci.2604-12.2012 (2012).
177 Christopherson, K. S. *et al.* Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* **120**, 421-433, doi:10.1016/j.cell.2004.12.020 (2005).
178 Lu, Z. & Kipnis, J. Thrombospondin 1—a key astrocyte-derived neurogenic factor. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **24**, 1925-1934, doi:10.1096/fj.09-150573 (2010).
179 Bornstein, P. Thrombospondins function as regulators of angiogenesis. *Journal of Cell Communication and Signaling* **3**, 189-200, doi:10.1007/s12079-009-0060-8 (2009).
180 Krady, M. M. *et al.* Thrombospondin-2 Modulates Extracellular Matrix Remodeling during Physiological Angiogenesis. *Am J Pathol* **173**, 879-891, doi:10.2353/ajpath.2008.080128 (2008).
181 Laurence, J. A. & Fatemi, S. H. Glial fibrillary acidic protein is elevated in superior frontal, parietal and cerebellar cortices of autistic subjects. *Cerebellum (London, England)* **4**, 206-210, doi:10.1080/14734220500208846 (2005).

182 Purcell, A. E., Jeon, O. H., Zimmerman, A. W., Blue, M. E. & Pevsner, J. Postmortem brain abnormalities of the glutamate neurotransmitter system in autism. *Neurology* **57**, 1618-1628 (2001).
183 Fatemi, S. H., Folsom, T. D., Reutiman, T. J. & Lee, S. Expression of astrocytic markers aquaporin 4 and connexin 43 is altered in brains of subjects with autism. *Synapse (New York, N.Y.)* **62**, 501-507, doi:10.1002/syn.20519 (2008).
184 Gadow, K. D., Roohi, J., DeVincent, C. J., Kirsch, S. & Hatchwell, E. Glutamate transporter gene (SLC1A1) single nucleotide polymorphism (rs301430) and repetitive behaviors and anxiety in children with autism spectrum disorder. *Journal of autism and developmental disorders* **40**, 1139-1145, doi:10.1007/s10803-010-0961-7 (2010).
185 Guy, J., Hendrich, B., Holmes, M., Martin, J. E. & Bird, A. A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nature genetics* **27**, 322-326, doi:10.1038/85899 (2001).
186 Chen, R. Z., Akbarian, S., Tudor, M. & Jaenisch, R. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nature genetics* **27**, 327-331, doi:10.1038/85906 (2001).
187 The Dutch-Belgian Fragile, X. C. *et al.* Fmr1 knockout mice: A model to study fragile X mental retardation. *Cell* **78**, 23-33, doi:http://dx.doi.org/10.1016/0092-8674(94)90569-X (1994).
188 Amir, R. E. *et al.* Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nature genetics* **23**, 185-188, doi:10.1038/13810 (1999).
189 Ballas, N., Lioy, D. T., Grunseich, C. & Mandel, G. Non-cell autonomous influence of MeCP2-deficient glia on neuronal dendritic morphology. *Nature neuroscience* **12**, 311-317, doi:10.1038/nn.2275 (2009).
190 Okabe, Y. *et al.* Alterations of gene expression and glutamate clearance in astrocytes derived from an MeCP2-null mouse model of Rett syndrome. *PLoS One* **7**, e35354, doi:10.1371/journal.pone.0035354 (2012).
191 Lioy, D. T. *et al.* A role for glia in the progression of Rett's syndrome. *Nature* **475**, 497-500, doi:10.1038/nature10214 (2011).
192 Jacobs, S. & Doering, L. C. Astrocytes prevent abnormal neuronal development in the fragile x mouse. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**, 4508-4514, doi:10.1523/jneurosci.5027-09.2010 (2010).
193 Jacobs, S., Nathwani, M. & Doering, L. C. Fragile X astrocytes induce developmental delays in dendrite maturation and synaptic protein expression. *BMC neuroscience* **11**, 132, doi:10.1186/1471-2202-11-132 (2010).
194 Matsugami, T. R. *et al.* From the Cover: Indispensability of the glutamate transporters GLAST and GLT1 to brain development. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 12161-12166, doi:10.1073/pnas.0509144103 (2006).
195 Higashimori, H. *et al.* Astroglial FMRP-dependent translational down-regulation of mGluR5 underlies glutamate transporter GLT1 dysregulation in

the fragile X mouse. *Human molecular genetics* **22**, 2041-2054, doi:10.1093/hmg/ddt055 (2013).

196 Gosselin, R. D., Gibney, S., O'Malley, D., Dinan, T. G. & Cryan, J. F. Region specific decrease in glial fibrillary acidic protein immunoreactivity in the brain of a rat model of depression. *Neuroscience* **159**, 915-925, doi:10.1016/j.neuroscience.2008.10.018 (2009).

197 Altshuler, L. L. *et al.* Amygdala astrocyte reduction in subjects with major depressive disorder but not bipolar disorder. *Bipolar disorders* **12**, 541-549, doi:10.1111/j.1399-5618.2010.00838.x (2010).

198 Rajkowska, G., Hughes, J., Stockmeier, C. A., Javier Miguel-Hidalgo, J. & Maciag, D. Coverage of blood vessels by astrocytic endfeet is reduced in major depressive disorder. *Biological psychiatry* **73**, 613-621, doi:10.1016/j.biopsych.2012.09.024 (2013).

199 Bristol, L. A. & Rothstein, J. D. Glutamate transporter gene expression in amyotrophic lateral sclerosis motor cortex. *Annals of neurology* **39**, 676-679, doi:10.1002/ana.410390519 (1996).

200 Roberts, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M. & Sporn, M. B. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 5339-5343 (1981).

201 Wakefield, L. M., Smith, D. M., Flanders, K. C. & Sporn, M. B. Latent transforming growth factor-beta from human platelets. A high molecular weight complex containing precursor sequences. *The Journal of biological chemistry* **263**, 7646-7654 (1988).

202 Gleizes, P. E., Beavis, R. C., Mazzieri, R., Shen, B. & Rifkin, D. B. Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-beta binding protein-1 that mediates bonding to the latent transforming growth factor-beta1. *The Journal of biological chemistry* **271**, 29891-29896 (1996).

203 Munger, J. S. *et al.* The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* **96**, 319-328 (1999).

204 Benzoubir, N. *et al.* HCV core-mediated activation of latent TGF-beta via thrombospondin drives the crosstalk between hepatocytes and stromal environment. *Journal of hepatology* **59**, 1160-1168, doi:10.1016/j.jhep.2013.07.036 (2013).

205 Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M. & Sporn, M. B. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. *The Journal of biological chemistry* **258**, 7155-7160 (1983).

206 Shi, M. *et al.* Latent TGF-beta structure and activation. *Nature* **474**, 343-349, doi:10.1038/nature10152 (2011).

207 Massague, J., Blain, S. W. & Lo, R. S. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* **103**, 295-309 (2000).

208 Massague, J. TGF-beta signal transduction. *Annual review of biochemistry* **67**, 753-791, doi:10.1146/annurev.biochem.67.1.753 (1998).

209 Shi, Y. & Massague, J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700 (2003).
210 Goumans, M. J. *et al.* Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. *Molecular cell* **12**, 817-828 (2003).
211 Nassiri, F. *et al.* Endoglin (CD105): a review of its role in angiogenesis and tumor diagnosis, progression and therapy. *Anticancer research* **31**, 2283-2290 (2011).
212 Velasco, S. *et al.* L- and S-endoglin differentially modulate TGFbeta1 signaling mediated by ALK1 and ALK5 in L6E9 myoblasts. *Journal of cell science* **121**, 913-919, doi:10.1242/jcs.023283 (2008).
213 ten Dijke, P., Goumans, M. J. & Pardali, E. Endoglin in angiogenesis and vascular diseases. *Angiogenesis* **11**, 79-89, doi:10.1007/s10456-008-9101-9 (2008).
214 Lebrin, F. & Mummery, C. L. Endoglin-mediated vascular remodeling: mechanisms underlying hereditary hemorrhagic telangiectasia. *Trends in cardiovascular medicine* **18**, 25-32, doi:10.1016/j.tcm.2007.11.003 (2008).
215 Lastres, P. *et al.* Endoglin modulates cellular responses to TGF-beta 1. *The Journal of cell biology* **133**, 1109-1121 (1996).
216 Kaname, S. & Ruoslahti, E. Betaglycan has multiple binding sites for transforming growth factor-beta 1. *The Biochemical journal* **315 (Pt 3)**, 815-820 (1996).
217 Lopez-Casillas, F., Payne, H. M., Andres, J. L. & Massague, J. Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. *The Journal of cell biology* **124**, 557-568 (1994).
218 Massague, J., Seoane, J. & Wotton, D. Smad transcription factors. *Genes & development* **19**, 2783-2810, doi:10.1101/gad.1350705 (2005).
219 Derynck, R. & Zhang, Y. E. Smad-dependent and Smad-independent pathways in TGF-[beta] family signalling. *Nature* **425**, 577-584 (2003).
220 Kulkarni, A. B. *et al.* Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 770-774 (1993).
221 Bottinger, E. P. & Kopp, J. B. Lessons from TGF-beta transgenic mice. *Mineral and electrolyte metabolism* **24**, 154-160 (1998).
222 McDonald, J., Bayrak-Toydemir, P. & Pyeritz, R. E. Hereditary hemorrhagic telangiectasia: An overview of diagnosis, management, and pathogenesis. *Genet Med* **13**, 607-616 (2011).
223 Feng, Z. & Ko, C. P. Schwann cells promote synaptogenesis at the neuromuscular junction via transforming growth factor-beta1. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 9599-9609, doi:10.1523/jneurosci.2589-08.2008 (2008).
224 Bae, J. J. *et al.* Increased transforming growth factor-β1 modulates glutamate receptor expression in the hippocampus. *International Journal of Physiology, Pathophysiology and Pharmacology* **3**, 9-20 (2011).

225 Diniz, L. P., Matias, I. C., Garcia, M. N. & Gomes, F. C. Astrocytic control of neural circuit formation: highlights on TGF-beta signaling. *Neurochemistry international* **78**, 18-27, doi:10.1016/j.neuint.2014.07.008 (2014).

226 Diniz, L. P. *et al.* Astrocyte transforming growth factor beta 1 promotes inhibitory synapse formation via CaM kinase II signaling. *Glia* **62**, 1917-1931, doi:10.1002/glia.22713 (2014).

227 Ashwood, P. *et al.* Decreased transforming growth factor beta1 in autism: a potential link between immune dysregulation and impairment in clinical behavioral outcomes. *Journal of neuroimmunology* **204**, 149-153, doi:10.1016/j.jneuroim.2008.07.006 (2008).

228 Okada, K. *et al.* Decreased serum levels of transforming growth factor-beta1 in patients with autism. *Progress in neuro-psychopharmacology & biological psychiatry* **31**, 187-190, doi:10.1016/j.pnpbp.2006.08.020 (2007).

229 Vargas, D. L., Nascimbene, C., Krishnan, C., Zimmerman, A. W. & Pardo, C. A. Neuroglial activation and neuroinflammation in the brain of patients with autism. *Annals of neurology* **57**, 67-81, doi:10.1002/ana.20315 (2005).

230 Sun, M. *et al.* Expression of the multidrug resistance P-glycoprotein, (ABCB1 glycoprotein) in the human placenta decreases with advancing gestation. *Placenta* **27**, 602-609, doi:10.1016/j.placenta.2005.05.007 (2006).

231 Burns, K. A., Murphy, B., Danzer, S. C. & Kuan, C. Y. Developmental and post-injury cortical gliogenesis: a genetic fate-mapping study with Nestin-CreER mice. *Glia* **57**, 1115-1129, doi:10.1002/glia.20835 (2009).

232 Jacobsen, C. T. & Miller, R. H. Control of astrocyte migration in the developing cerebral cortex. *Developmental neuroscience* **25**, 207-216, doi:72269 (2003).

233 Mission, J. P., Takahashi, T. & Caviness, V. S., Jr. Ontogeny of radial and other astroglial cells in murine cerebral cortex. *Glia* **4**, 138-148, doi:10.1002/glia.440040205 (1991).

234 Rowitch, D. H. & Kriegstein, A. R. Developmental genetics of vertebrate glial-cell specification. *Nature* **468**, 214-222, doi:10.1038/nature09611 (2010).

235 Byrnes, M. L., Reynolds, J. N. & Brien, J. F. Brain growth spurt-prenatal ethanol exposure and the guinea pig hippocampal glutamate signaling system. *Neurotoxicology and teratology* **25**, 303-310 (2003).

236 Babu, H. *et al.* A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. *Frontiers in neuroscience* **5**, 89, doi:10.3389/fnins.2011.00089 (2011).

237 Sousa Vde, O., Almeida, J. C., Eller, C. M. & Gomes, F. C. Characterization of TGF-beta1 type II receptor expression in cultured cortical astrocytes. *In vitro cellular & developmental biology. Animal* **42**, 171-175, doi:10.1290/0602013.1 (2006).

238 de Sampaio e Spohr, T. C., Martinez, R., da Silva, E. F., Neto, V. M. & Gomes, F. C. Neuro-glia interaction effects on GFAP gene: a novel role for transforming growth factor-beta1. *The European journal of neuroscience* **16**, 2059-2069 (2002).

239 Gomes, F. C., Sousa Vde, O. & Romao, L. Emerging roles for TGF-beta1 in nervous system development. *International journal of developmental neuroscience : the official journal of the International Society for*

Developmental Neuroscience **23**, 413-424, doi:10.1016/j.ijdevneu.2005.04.001 (2005).

240 Boveri, M. *et al.* Induction of blood-brain barrier properties in cultured brain capillary endothelial cells: comparison between primary glial cells and C6 cell line. *Glia* **51**, 187-198, doi:10.1002/glia.20189 (2005).

241 Sobue, K. *et al.* Induction of blood-brain barrier properties in immortalized bovine brain endothelial cells by astrocytic factors. *Neuroscience research* **35**, 155-164 (1999).

242 Tontsch, U. & Bauer, H. C. Glial cells and neurons induce blood-brain barrier related enzymes in cultured cerebral endothelial cells. *Brain research* **539**, 247-253 (1991).

243 Dean, F. & Matthews, S. G. Maternal dexamethasone treatment in late gestation alters glucocorticoid and mineralocorticoid receptor mRNA in the fetal guinea pig brain. *Brain research* **846**, 253-259 (1999).

244 Power, L. L. *et al.* Immunoregulatory molecules during pregnancy and at birth. *Journal of reproductive immunology* **56**, 19-28 (2002).

245 Weiss, J. *et al.* Inhibition of P-glycoprotein by newer antidepressants. *The Journal of pharmacology and experimental therapeutics* **305**, 197-204, doi:10.1124/jpet.102.046532 (2003).

246 Inman, G. J. *et al.* SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Molecular pharmacology* **62**, 65-74 (2002).

247 Jinnin, M., Ihn, H. & Tamaki, K. Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression. *Molecular pharmacology* **69**, 597-607, doi:10.1124/mol.105.017483 (2006).

248 Wu, X., Ma, J., Han, J. D., Wang, N. & Chen, Y. G. Distinct regulation of gene expression in human endothelial cells by TGF-beta and its receptors. *Microvascular research* **71**, 12-19, doi:10.1016/j.mvr.2005.11.004 (2006).

249 Larrivee, B. *et al.* ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. *Developmental cell* **22**, 489-500, doi:10.1016/j.devcel.2012.02.005 (2012).

250 David, L., Mallet, C., Mazerbourg, S., Feige, J. J. & Bailly, S. Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood* **109**, 1953-1961, doi:10.1182/blood-2006-07-034124 (2007).

251 Hao, J. *et al.* Dorsomorphin, a selective small molecule inhibitor of BMP signaling, promotes cardiomyogenesis in embryonic stem cells. *PLoS One* **3**, e2904, doi:10.1371/journal.pone.0002904 (2008).

252 Dibrov, A., Kashour, T. & Amara, F. M. The role of transforming growth factor beta signaling in messenger RNA stability. *Growth factors (Chur, Switzerland)* **24**, 1-11, doi:10.1080/08977190500365995 (2006).

253 Diaz, A., Chepenik, K. P., Korn, J. H., Reginato, A. M. & Jimenez, S. A. Differential regulation of cyclooxygenases 1 and 2 by interleukin-1 beta, tumor necrosis factor-alpha, and transforming growth factor-beta 1 in human lung

fibroblasts. *Experimental cell research* **241**, 222-229, doi:10.1006/excr.1998.4050 (1998).

254 Sheng, H. *et al.* Transforming growth factor-beta1 enhances Ha-ras-induced expression of cyclooxygenase-2 in intestinal epithelial cells via stabilization of mRNA. *The Journal of biological chemistry* **275**, 6628-6635 (2000).

255 Patel, V. A., Dunn, M. J. & Sorokin, A. Regulation of MDR-1 (P-glycoprotein) by cyclooxygenase-2. *The Journal of biological chemistry* **277**, 38915-38920, doi:10.1074/jbc.M206855200 (2002).

256 Cordenonsi, M. *et al.* Integration of TGF-beta and Ras/MAPK signaling through p53 phosphorylation. *Science (New York, N.Y.)* **315**, 840-843, doi:10.1126/science.1135961 (2007).

257 Olieslagers, S., Pardali, E., Tchaikovski, V., ten Dijke, P. & Waltenberger, J. TGF-beta1/ALK5-induced monocyte migration involves PI3K and p38 pathways and is not negatively affected by diabetes mellitus. *Cardiovascular research* **91**, 510-518, doi:10.1093/cvr/cvr100 (2011).

258 Lopez-Coviella, I. *et al.* Developmental pattern of expression of BMP receptors and Smads and activation of Smad1 and Smad5 by BMP9 in mouse basal forebrain. *Brain research* **1088**, 49-56, doi:10.1016/j.brainres.2006.02.073 (2006).

259 Chen, G. Z. *et al.* [Expression of TGFbeta1 and its type I receptors ALK1 and ALK5 mRNA in brain arteriovenous malformation]. *Nan fang yi ke da xue xue bao = Journal of Southern Medical University* **26**, 675-677 (2006).

260 Vogelgesang, S. *et al.* Expression of multidrug transporters in dysembryoplastic neuroepithelial tumors causing intractable epilepsy. *Clinical neuropathology* **23**, 223-231 (2004).

261 Eickelberg, O., Centrella, M., Reiss, M., Kashgarian, M. & Wells, R. G. Betaglycan inhibits TGF-beta signaling by preventing type I-type II receptor complex formation. Glycosaminoglycan modifications alter betaglycan function. *The Journal of biological chemistry* **277**, 823-829, doi:10.1074/jbc.M105110200 (2002).

262 Wiater, E. & Vale, W. Inhibin is an antagonist of bone morphogenetic protein signaling. *The Journal of biological chemistry* **278**, 7934-7941, doi:10.1074/jbc.M209710200 (2003).

263 Mallet, C., Vittet, D., Feige, J. J. & Bailly, S. TGFbeta1 induces vasculogenesis and inhibits angiogenic sprouting in an embryonic stem cell differentiation model: respective contribution of ALK1 and ALK5. *Stem cells (Dayton, Ohio)* **24**, 2420-2427, doi:10.1634/stemcells.2005-0494 (2006).

264 Madazli, R., Aydin, S., Uludag, S., Vildan, O. & Tolun, N. Maternal plasma levels of cytokines in normal and preeclamptic pregnancies and their relationship with diastolic blood pressure and fibronectin levels. *Acta obstetricia et gynecologica Scandinavica* **82**, 797-802 (2003).

265 Enquobahrie, D. A., Williams, M. A., Qiu, C., Woelk, G. B. & Mahomed, K. Maternal plasma transforming growth factor-beta1 concentrations in preeclamptic and normotensive pregnant Zimbabwean women. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal*

Societies, the International Society of Perinatal Obstet **17**, 343-348, doi:10.1080/14767050500132450 (2005).

266 Letterio, J. J. *et al.* Maternal rescue of transforming growth factor-beta 1 null mice. *Science (New York, N.Y.)* **264**, 1936-1938 (1994).

267 Gressens, P., Lammens, M., Picard, J. J. & Evrard, P. Ethanol-induced disturbances of gliogenesis and neuronogenesis in the developing murine brain: an in vitro and in vivo immunohistochemical and ultrastructural study. *Alcohol and alcoholism (Oxford, Oxfordshire)* **27**, 219-226 (1992).

268 McCaffery, P. & Deutsch, C. K. Macrocephaly and the control of brain growth in autistic disorders. *Progress in neurobiology* **77**, 38-56, doi:10.1016/j.pneurobio.2005.10.005 (2005).

269 Winship, I. R., Plaa, N. & Murphy, T. H. Rapid astrocyte calcium signals correlate with neuronal activity and onset of the hemodynamic response in vivo. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**, 6268-6272, doi:10.1523/jneurosci.4801-06.2007 (2007).

270 Dobbing, J. & Sands, J. Growth and development of the brain and spinal cord of the guinea pig. *Brain research* **17**, 115-123 (1970).

271 Vinukonda, G. *et al.* Effect of prenatal glucocorticoids on cerebral vasculature of the developing brain. *Stroke* **41**, 1766-1773, doi:10.1161/strokeaha.110.588400 (2010).

272 Sadowska, G. B., Malaeb, S. N. & Stonestreet, B. S. Maternal glucocorticoid exposure alters tight junction protein expression in the brain of fetal sheep. *American journal of physiology. Heart and circulatory physiology* **298**, H179-188, doi:10.1152/ajpheart.00828.2009 (2010).

273 Duncan, A. R., Sadowska, G. B. & Stonestreet, B. S. Ontogeny and the effects of exogenous and endogenous glucocorticoids on tight junction protein expression in ovine cerebral cortices. *Brain research* **1303**, 15-25, doi:10.1016/j.brainres.2009.09.086 (2009).

274 Ballard, P. L. & Ballard, R. A. Scientific basis and therapeutic regimens for use of antenatal glucocorticoids. *American journal of obstetrics and gynecology* **173**, 254-262 (1995).

275 Keightley, M. C., Curtis, A. J., Chu, S. & Fuller, P. J. Structural determinants of cortisol resistance in the guinea pig glucocorticoid receptor. *Endocrinology* **139**, 2479-2485, doi:10.1210/endo.139.5.5982 (1998).

276 Baello, S. *et al.* TGF-beta1 regulation of multidrug resistance P-glycoprotein in the developing male blood-brain barrier. *Endocrinology* **155**, 475-484, doi:10.1210/en.2013-1472 (2014).

277 Yan, X., Liu, Z. & Chen, Y. Regulation of TGF-beta signaling by Smad7. *Acta biochimica et biophysica Sinica* **41**, 263-272 (2009).

278 Frahm, K. A. & Tobet, S. A. Development of the blood-brain barrier within the paraventricular nucleus of the hypothalamus: influence of fetal glucocorticoid excess. *Brain structure & function*, doi:10.1007/s00429-014-0787-8 (2014).

279 Anacker, C. *et al.* Glucocorticoid-related molecular signaling pathways regulating hippocampal neurogenesis. *Neuropsychopharmacology : official*

publication of the American College of Neuropsychopharmacology **38**, 872-883, doi:10.1038/npp.2012.253 (2013).

280 Ciarmela, P. *et al.* Activin-A and myostatin response and steroid regulation in human myometrium: disruption of their signalling in uterine fibroid. *The Journal of clinical endocrinology and metabolism* **96**, 755-765, doi:10.1210/jc.2010-0501 (2011).

281 Nicolas, F. J. & Hill, C. S. Attenuation of the TGF-beta-Smad signaling pathway in pancreatic tumor cells confers resistance to TGF-beta-induced growth arrest. *Oncogene* **22**, 3698-3711, doi:10.1038/sj.onc.1206420 (2003).

282 Tobin, S. W. *et al.* Consequences of altered TGF-beta expression and responsiveness in breast cancer: evidence for autocrine and paracrine effects. *Oncogene* **21**, 108-118, doi:10.1038/sj.onc.1205026 (2002).

283 Kleeff, J. & Korc, M. Up-regulation of transforming growth factor (TGF)-beta receptors by TGF-beta1 in COLO-357 cells. *The Journal of biological chemistry* **273**, 7495-7500 (1998).

284 Kawakami, T. *et al.* Increased expression of TGF-beta receptors by scleroderma fibroblasts: evidence for contribution of autocrine TGF-beta signaling to scleroderma phenotype. *The Journal of investigative dermatology* **110**, 47-51, doi:10.1046/j.1523-1747.1998.00073.x (1998).

285 Leask, A. & Abraham, D. J. TGF-beta signaling and the fibrotic response. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **18**, 816-827, doi:10.1096/fj.03-1273rev (2004).

286 Molnar, J., Nijland, M. J., Howe, D. C. & Nathanielsz, P. W. Evidence for microvascular dysfunction after prenatal dexamethasone at 0.7, 0.75, and 0.8 gestation in sheep. *American journal of physiology. Regulatory, integrative and comparative physiology* **283**, R561-567, doi:10.1152/ajpregu.00031.2002 (2002).

287 Hai, C. M., Sadowska, G., Francois, L. & Stonestreet, B. S. Maternal dexamethasone treatment alters myosin isoform expression and contractile dynamics in fetal arteries. *American journal of physiology. Heart and circulatory physiology* **283**, H1743-1749, doi:10.1152/ajpheart.00281.2002 (2002).

288 Caraci, F. *et al.* Dysfunction of TGF-beta1 signaling in Alzheimer's disease: perspectives for neuroprotection. *Cell and tissue research* **347**, 291-301, doi:10.1007/s00441-011-1230-6 (2012).

289 Cirrito, J. R. *et al.* P-glycoprotein deficiency at the blood-brain barrier increases amyloid-beta deposition in an Alzheimer disease mouse model. *The Journal of clinical investigation* **115**, 3285-3290, doi:10.1172/jci25247 (2005).

290 van Assema, D. M. *et al.* Blood-brain barrier P-glycoprotein function in Alzheimer's disease. *Brain : a journal of neurology* **135**, 181-189, doi:10.1093/brain/awr298 (2012).

291 Kortekaas, R. *et al.* Blood-brain barrier dysfunction in parkinsonian midbrain in vivo. *Annals of neurology* **57**, 176-179, doi:10.1002/ana.20369 (2005).

292 Deo, A. K. *et al.* Activity of P-Glycoprotein, a beta-Amyloid Transporter at the Blood-Brain Barrier, Is Compromised in Patients with Mild Alzheimer

Disease. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* **55**, 1106-1111, doi:10.2967/jnumed.113.130161 (2014).

293 Vogelgesang, S. *et al.* Deposition of Alzheimer's beta-amyloid is inversely correlated with P-glycoprotein expression in the brains of elderly non-demented humans. *Pharmacogenetics* **12**, 535-541 (2002).

294 Nesvizhskii, A. I., Keller, A., Kolker, E. & Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Analytical chemistry* **75**, 4646-4658 (2003).

295 MacLean, B. *et al.* Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics (Oxford, England)* **26**, 966-968, doi:10.1093/bioinformatics/btq054 (2010).

296 Laywell, E. D., Rakic, P., Kukekov, V. G., Holland, E. C. & Steindler, D. A. Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13883-13888 (2000).

297 Lim, J. C., Wolpaw, A. J., Caldwell, M. A., Hladky, S. B. & Barrand, M. A. Neural precursor cell influences on blood-brain barrier characteristics in rat brain endothelial cells. *Brain research* **1159**, 67-76, doi:10.1016/j.brainres.2007.05.032 (2007).

298 Sergent-Tanguy, S., Michel, D. C., Neveu, I. & Naveilhan, P. Long-lasting coexpression of nestin and glial fibrillary acidic protein in primary cultures of astroglial cells with a major participation of nestin(+)/GFAP(-) cells in cell proliferation. *Journal of neuroscience research* **83**, 1515-1524, doi:10.1002/jnr.20846 (2006).

299 Nicchia, G. P. *et al.* The role of aquaporin-4 in the blood-brain barrier development and integrity: studies in animal and cell culture models. *Neuroscience* **129**, 935-945, doi:10.1016/j.neuroscience.2004.07.055 (2004).

300 Liedtke, W. *et al.* GFAP is necessary for the integrity of CNS white matter architecture and long-term maintenance of myelination. *Neuron* **17**, 607-615 (1996).

301 Smith, G. M., Rutishauser, U., Silver, J. & Miller, R. H. Maturation of astrocytes in vitro alters the extent and molecular basis of neurite outgrowth. *Developmental biology* **138**, 377-390 (1990).

302 Bahr, M., Przyrembel, C. & Bastmeyer, M. Astrocytes from adult rat optic nerves are nonpermissive for regenerating retinal ganglion cell axons. *Experimental neurology* **131**, 211-220 (1995).

303 Baker, E. K. & El-Osta, A. Epigenetic regulation of multidrug resistance 1 gene expression: profiling CpG methylation status using bisulphite sequencing. *Methods in molecular biology (Clifton, N.J.)* **596**, 183-198, doi:10.1007/978-1-60761-416-6_9 (2010).

304 Fryxell, K. B., McGee, S. B., Simoneaux, D. K., Willman, C. L. & Cornwell, M. M. Methylation analysis of the human multidrug resistance 1 gene in normal and leukemic hematopoietic cells. *Leukemia* **13**, 910-917 (1999).

305 Klaassen, I., Brakenhoff, R. H., Smeets, S. J., Snow, G. B. & Braakhuis, B. J. Considerations for in vitro retinoid experiments: importance of protein interaction. *Biochimica et biophysica acta* **1427**, 265-275 (1999).

306 N'Soukpoe-Kossi, C. N., Sedaghat-Herati, R., Ragi, C., Hotchandani, S. & Tajmir-Riahi, H. A. Retinol and retinoic acid bind human serum albumin: stability and structural features. *International journal of biological macromolecules* **40**, 484-490, doi:10.1016/j.ijbiomac.2006.11.005 (2007).

307 Shen, H. *et al.* Upregulation of mdr1 gene is related to activation of the MAPK/ERK signal transduction pathway and YB-1 nuclear translocation in B-cell lymphoma. *Experimental hematology* **39**, 558-569, doi:10.1016/j.exphem.2011.01.013 (2011).

308 Dolfini, D. & Mantovani, R. Targeting the Y/CCAAT box in cancer: YB-1 (YBX1) or NF-Y? *Cell death and differentiation* **20**, 676-685, doi:10.1038/cdd.2013.13 (2013).

309 Frye, B. C. *et al.* Y-box protein-1 is actively secreted through a non-classical pathway and acts as an extracellular mitogen. *EMBO reports* **10**, 783-789, doi:10.1038/embor.2009.81 (2009).

310 Raffetseder, U. *et al.* Extracellular YB-1 blockade in experimental nephritis upregulates Notch-3 receptor expression and signaling. *Nephron. Experimental nephrology* **118**, e100-108, doi:10.1159/000324209 (2011).

311 Rauen, T. *et al.* YB-1 acts as a ligand for Notch-3 receptors and modulates receptor activation. *The Journal of biological chemistry* **284**, 26928-26940, doi:10.1074/jbc.M109.046599 (2009).

312 D'Souza, B., Meloty-Kapella, L. & Weinmaster, G. Canonical and non-canonical Notch ligands. *Current topics in developmental biology* **92**, 73-129, doi:10.1016/S0070-2153(10)92003-6 (2010).

313 Wu, W. R. *et al.* Notch1 is overexpressed in human intrahepatic cholangiocarcinoma and is associated with its proliferation, invasiveness and sensitivity to 5-fluorouracil in vitro. *Oncology reports* **31**, 2515-2524, doi:10.3892/or.2014.3123 (2014).

314 Capaccione, K. M. & Pine, S. R. The Notch signaling pathway as a mediator of tumor survival. *Carcinogenesis* **34**, 1420-1430, doi:10.1093/carcin/bgt127 (2013).

315 Hang, Q., Sun, R., Jiang, C. & Li, Y. Notch 1 promotes cisplatin-resistant gastric cancer formation by upregulating lncRNA AK022798 expression. *Anti-cancer drugs* **26**, 632-640, doi:10.1097/cad.0000000000000227 (2015).

316 Troost, J., Lindenmaier, H., Haefeli, W. E. & Weiss, J. Modulation of cellular cholesterol alters P-glycoprotein activity in multidrug-resistant cells. *Molecular pharmacology* **66**, 1332-1339, doi:10.1124/mol.104.002329 (2004).

317 Belli, S., Elsener, P. M., Wunderli-Allenspach, H. & Kramer, S. D. Cholesterol-mediated activation of P-glycoprotein: distinct effects on basal and drug-induced ATPase activities. *Journal of pharmaceutical sciences* **98**, 1905-1918, doi:10.1002/jps.21558 (2009).

318 Beigneux, A. P. *et al.* Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 plays a critical role in the lipolytic processing of chylomicrons. *Cell metabolism* **5**, 279-291, doi:10.1016/j.cmet.2007.02.002 (2007).

319 Fidge, N. H. High density lipoprotein receptors, binding proteins, and ligands. *Journal of lipid research* **40**, 187-201 (1999).
320 Dietschy, J. M. & Turley, S. D. Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. *Journal of lipid research* **45**, 1375-1397, doi:10.1194/jlr.R400004-JLR200 (2004).
321 Devonshire, I. M. *et al.* Neurovascular coupling is brain region-dependent. *NeuroImage* **59**, 1997-2006, doi:10.1016/j.neuroimage.2011.09.050 (2012).
322 Williams, E. C. *et al.* Mutant astrocytes differentiated from Rett syndrome patients-specific iPSCs have adverse effects on wild-type neurons. *Human molecular genetics* **23**, 2968-2980, doi:10.1093/hmg/ddu008 (2014).
323 Raymond, M., Li, P., Mangin, J. M., Huntsman, M. & Gallo, V. Chronic perinatal hypoxia reduces glutamate-aspartate transporter function in astrocytes through the Janus kinase/signal transducer and activator of transcription pathway. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**, 17864-17871, doi:10.1523/jneurosci.3179-11.2011 (2011).
324 Tilleux, S. & Hermans, E. Down-regulation of astrocytic GLAST by microglia-related inflammation is abrogated in dibutyryl cAMP-differentiated cultures. *Journal of neurochemistry* **105**, 2224-2236, doi:10.1111/j.1471-4159.2008.05305.x (2008).
325 Greco, T. M., Seeholzer, S. H., Mak, A., Spruce, L. & Ischiropoulos, H. Quantitative mass spectrometry-based proteomics reveals the dynamic range of primary mouse astrocyte protein secretion. *Journal of proteome research* **9**, 2764-2774, doi:10.1021/pr100134n (2010).
326 Haseloff, R. F., Blasig, I. E., Bauer, H. C. & Bauer, H. In search of the astrocytic factor(s) modulating blood-brain barrier functions in brain capillary endothelial cells in vitro. *Cellular and molecular neurobiology* **25**, 25-39 (2005).
327 Lafon-Cazal, M. *et al.* Proteomic analysis of astrocytic secretion in the mouse. Comparison with the cerebrospinal fluid proteome. *The Journal of biological chemistry* **278**, 24438-24448, doi:10.1074/jbc.M211980200 (2003).
328 Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium. *Cell* **78**, 23-33 (1994).
329 Vielkind, U., Walencewicz, A., Levine, J. M. & Bohn, M. C. Type II glucocorticoid receptors are expressed in oligodendrocytes and astrocytes. *Journal of neuroscience research* **27**, 360-373, doi:10.1002/jnr.490270315 (1990).
330 Khelil, M., Rolland, B., Fages, C. & Tardy, M. Glutamine synthetase modulation in astrocyte cultures of different mouse brain areas. *Glia* **3**, 75-80, doi:10.1002/glia.440030110 (1990).
331 Rozovsky, I. *et al.* Transcriptional regulation of glial fibrillary acidic protein by corticosterone in rat astrocytes in vitro is influenced by the duration of time in culture and by astrocyte-neuron interactions. *Endocrinology* **136**, 2066-2073, doi:10.1210/endo.136.5.7720656 (1995).
332 Moors, M. *et al.* Dickkopf 1 mediates glucocorticoid-induced changes in human neural progenitor cell proliferation and differentiation. *Toxicological*

sciences : an official journal of the Society of Toxicology **125**, 488-495, doi:10.1093/toxsci/kfr304 (2012).

333 Doyle, J. P. *et al.* Application of a translational profiling approach for the comparative analysis of CNS cell types. *Cell* **135**, 749-762, doi:10.1016/j.cell.2008.10.029 (2008).

334 Yeh, T. H., Lee da, Y., Gianino, S. M. & Gutmann, D. H. Microarray analyses reveal regional astrocyte heterogeneity with implications for neurofibromatosis type 1 (NF1)-regulated glial proliferation. *Glia* **57**, 1239-1249, doi:10.1002/glia.20845 (2009).

335 McKhann, G. M., 2nd, D'Ambrosio, R. & Janigro, D. Heterogeneity of astrocyte resting membrane potentials and intercellular coupling revealed by whole-cell and gramicidin-perforated patch recordings from cultured neocortical and hippocampal slice astrocytes. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **17**, 6850-6863 (1997).

336 Matthias, K. *et al.* Segregated expression of AMPA-type glutamate receptors and glutamate transporters defines distinct astrocyte populations in the mouse hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**, 1750-1758 (2003).

337 Blomstrand, F., Aberg, N. D., Eriksson, P. S., Hansson, E. & Ronnback, L. Extent of intercellular calcium wave propagation is related to gap junction permeability and level of connexin-43 expression in astrocytes in primary cultures from four brain regions. *Neuroscience* **92**, 255-265 (1999).

338 Denis-Donini, S., Glowinski, J. & Prochiantz, A. Glial heterogeneity may define the three-dimensional shape of mouse mesencephalic dopaminergic neurones. *Nature* **307**, 641-643 (1984).

339 Garcia-Abreu, J., Moura Neto, V., Carvalho, S. L. & Cavalcante, L. A. Regionally specific properties of midbrain glia: I. Interactions with midbrain neurons. *Journal of neuroscience research* **40**, 471-477, doi:10.1002/jnr.490400406 (1995).

340 Wolff, J. E., Belloni-Olivi, L., Bressler, J. P. & Goldstein, G. W. Gamma-glutamyl transpeptidase activity in brain microvessels exhibits regional heterogeneity. *Journal of neurochemistry* **58**, 909-915 (1992).

341 Aranmolate, A., Shan, X. & Colognato, H. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, doi:10.1523/jneurosci.3678-13.2014.

342 Nielsen, S. *et al.* Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **17**, 171-180 (1997).

343 Lien, C. F. *et al.* Expression of alpha-dystrobrevin in blood-tissue barriers: sub-cellular localisation and molecular characterisation in normal and dystrophic mice. *Cell and tissue research* **327**, 67-82, doi:10.1007/s00441-006-0241-1 (2007).

344 Kage, K. *et al.* Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent

homodimerization. *International journal of cancer. Journal international du cancer* **97**, 626-630 (2002).

345 Ek, C. J. *et al.* Efflux mechanisms at the developing brain barriers: ABC-transporters in the fetal and postnatal rat. *Toxicology letters* **197**, 51-59, doi:10.1016/j.toxlet.2010.04.025 (2010).

346 Polli, J. W. *et al.* An unexpected synergist role of P-glycoprotein and breast cancer resistance protein on the central nervous system penetration of the tyrosine kinase inhibitor lapatinib (N-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-({[2-(methylsulfonyl)ethyl]amino }methyl)-2-furyl]-4-quinazolinamine; GW572016). *Drug metabolism and disposition: the biological fate of chemicals* **37**, 439-442, doi:10.1124/dmd.108.024646 (2009).

347 Agarwal, S., Hartz, A. M. S., Elmquist, W. F. & Bauer, B. Breast Cancer Resistance Protein and P-glycoprotein in Brain Cancer: Two Gatekeepers Team Up. *Current pharmaceutical design* **17**, 2793-2802 (2011).

348 Seetharaman, S., Barrand, M. A., Maskell, L. & Scheper, R. J. Multidrug resistance-related transport proteins in isolated human brain microvessels and in cells cultured from these isolates. *Journal of neurochemistry* **70**, 1151-1159 (1998).

349 Zhang, Y., Han, H., Elmquist, W. F. & Miller, D. W. Expression of various multidrug resistance-associated protein (MRP) homologues in brain microvessel endothelial cells. *Brain research* **876**, 148-153 (2000).

350 Miller, D. S. *et al.* Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. *Molecular pharmacology* **58**, 1357-1367 (2000).

351 Dombrowski, S. M. *et al.* Overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy. *Epilepsia* **42**, 1501-1506 (2001).

352 Wijnholds, J. *et al.* Multidrug resistance protein 1 protects the choroid plexus epithelium and contributes to the blood-cerebrospinal fluid barrier. *The Journal of clinical investigation* **105**, 279-285, doi:10.1172/jci8267 (2000).

353 Rao, V. V. *et al.* Choroid plexus epithelial expression of MDR1 P glycoprotein and multidrug resistance-associated protein contribute to the blood-cerebrospinal-fluid drug-permeability barrier. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3900-3905 (1999).

354 Yasuda, K. *et al.* Drug Transporters on Arachnoid Barrier Cells Contribute to the Blood–Cerebrospinal Fluid Barrier. *Drug Metabolism and Disposition* **41**, 923-931, doi:10.1124/dmd.112.050344 (2013).

355 Geick, A., Eichelbaum, M. & Burk, O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *The Journal of biological chemistry* **276**, 14581-14587, doi:10.1074/jbc.M010173200 (2001).

356 Aggarwal, S. *et al.* Nonclassical Action of Retinoic Acid on the Activation of the cAMP Response Element-binding Protein in Normal Human Bronchial Epithelial Cells. *Molecular Biology of the Cell* **17**, 566-575, doi:10.1091/mbc.E05-06-0519 (2006).

357 Guo, X. & Wang, X.-F. Signaling cross-talk between TGF-[beta]/BMP and other pathways. *Cell research* **19**, 71-88 (2009).
358 Jian, H. *et al.* Smad3-dependent nuclear translocation of beta-catenin is required for TGF-beta1-induced proliferation of bone marrow-derived adult human mesenchymal stem cells. *Genes & development* **20**, 666-674, doi:10.1101/gad.1388806 (2006).
359 Brightman, M. W. Morphology of blood-brain interfaces. *Experimental eye research* **25 Suppl**, 1-25 (1977).
360 van Deurs, B. Structural aspects of brain barriers, with special reference to the permeability of the cerebral endothelium and choroidal epithelium. *International review of cytology* **65**, 117-191 (1980).
361 Armulik, A. *et al.* Pericytes regulate the blood-brain barrier. *Nature* **468**, 557-561, doi:10.1038/nature09522 (2010).
362 Cuevas, P. *et al.* Pericyte endothelial gap junctions in human cerebral capillaries. *Anatomy and embryology* **170**, 155-159 (1984).
363 Dore-Duffy, P. Pericytes: pluripotent cells of the blood brain barrier. *Current pharmaceutical design* **14**, 1581-1593 (2008).
364 Nakagawa, S. *et al.* Pericytes from brain microvessels strengthen the barrier integrity in primary cultures of rat brain endothelial cells. *Cellular and molecular neurobiology* **27**, 687-694, doi:10.1007/s10571-007-9195-4 (2007).
365 Gundersen, G. A., Vindedal, G. F., Skare, O. & Nagelhus, E. A. Evidence that pericytes regulate aquaporin-4 polarization in mouse cortical astrocytes. *Brain structure & function* **219**, 2181-2186, doi:10.1007/s00429-013-0629-0 (2014).
366 Asano, H. *et al.* Astrocyte differentiation of neural precursor cells is enhanced by retinoic acid through a change in epigenetic modification. *Stem cells (Dayton, Ohio)* **27**, 2744-2752, doi:10.1002/stem.176 (2009).
367 Sanosaka, T. *et al.* Identification of genes that restrict astrocyte differentiation of midgestational neural precursor cells. *Neuroscience* **155**, 780-788, doi:10.1016/j.neuroscience.2008.06.039 (2008).
368 Ikemura, K. *et al.* MicroRNA-145 post-transcriptionally regulates the expression and function of P-glycoprotein in intestinal epithelial cells. *Molecular pharmacology* **83**, 399-405, doi:10.1124/mol.112.081844 (2013).
369 Sui, H. *et al.* miR200c attenuates P-gp-mediated MDR and metastasis by targeting JNK2/c-Jun signaling pathway in colorectal cancer. *Molecular cancer therapeutics* **13**, 3137-3151, doi:10.1158/1535-7163.mct-14-0167 (2014).
370 Bitarte, N. *et al.* MicroRNA-451 is involved in the self-renewal, tumorigenicity, and chemoresistance of colorectal cancer stem cells. *Stem cells (Dayton, Ohio)* **29**, 1661-1671, doi:10.1002/stem.741 (2011).
371 Lye, P. *et al.* Impact of bacterial and viral challenge on multidrug resistance in first- and third-trimester human placenta. *Am J Pathol* **185**, 1666-1675, doi:10.1016/j.ajpath.2015.02.013 (2015).
372 Reijerkerk, A. *et al.* MicroRNAs regulate human brain endothelial cell-barrier function in inflammation: implications for multiple sclerosis. *The Journal of*

neuroscience : the official journal of the Society for Neuroscience **33**, 6857-6863, doi:10.1523/jneurosci.3965-12.2013 (2013).

373 Ferrari, G., Cook, B. D., Terushkin, V., Pintucci, G. & Mignatti, P. TRANSFORMING GROWTH FACTOR-BETA 1 (TGF-β1) INDUCES ANGIOGENESIS THROUGH VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)-MEDIATED APOPTOSIS. *Journal of cellular physiology* **219**, 449-458, doi:10.1002/jcp.21706 (2009).

374 Balsara, R. D. & Ploplis, V. A. Plasminogen Activator Inhibitor-1: The Double Edged Sword in Apoptosis. *Thrombosis and haemostasis* **100**, 1029-1036 (2008).

375 Schinkel, A. H. *et al.* Normal viability and altered pharmacokinetics in mice lacking mdr1-type (drug-transporting) P-glycoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 4028-4033 (1997).

376 Lieberman, A. P., Pitha, P. M., Shin, H. S. & Shin, M. L. Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 6348-6352 (1989).

377 Xie, Z., Morgan, T. E., Rozovsky, I. & Finch, C. E. Aging and glial responses to lipopolysaccharide in vitro: greater induction of IL-1 and IL-6, but smaller induction of neurotoxicity. *Experimental neurology* **182**, 135-141 (2003).

378 Zhang, K., Zhu, L. & Fan, M. Oxygen, a Key Factor Regulating Cell Behavior during Neurogenesis and Cerebral Diseases. *Frontiers in molecular neuroscience* **4**, 5, doi:10.3389/fnmol.2011.00005 (2011).

379 Dickinson, M. A., Harnett, E. L., Venditti, C. C. & Smith, G. N. Transient lipopolysaccharide-induced cytokine responses in the maternal serum and amniotic fluid of the guinea pig. *American journal of obstetrics and gynecology* **200**, 534.e531-536, doi:10.1016/j.ajog.2008.12.034 (2009).

380 Harnett, E. L., Dickinson, M. A. & Smith, G. N. Dose-dependent lipopolysaccharide-induced fetal brain injury in the guinea pig. *American journal of obstetrics and gynecology* **197**, 179.e171-177, doi:10.1016/j.ajog.2007.03.047 (2007).

381 Duncan, J. R. *et al.* Chronic endotoxin exposure causes brain injury in the ovine fetus in the absence of hypoxemia. *Journal of the Society for Gynecologic Investigation* **13**, 87-96, doi:10.1016/j.jsgi.2005.12.003 (2006).

382 Mallard, C., Loeliger, M., Copolov, D. & Rees, S. Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth-restriction. *Neuroscience* **100**, 327-333 (2000).

383 Tolcos, M. *et al.* Intrauterine growth restriction affects the maturation of myelin. *Experimental neurology* **232**, 53-65, doi:10.1016/j.expneurol.2011.08.002 (2011).

384 Pop, V. *et al.* Early brain injury alters the blood-brain barrier phenotype in parallel with [beta]-amyloid and cognitive changes in adulthood. *J Cereb Blood Flow Metab* **33**, 205-214, doi:http://www.nature.com/jcbfm/journal/v33/n2/suppinfo/jcbfm2012154s1.html (2013).

385 Jullienne, A. *et al.* Juvenile traumatic brain injury induces long-term perivascular matrix changes alongside amyloid-beta accumulation. *J Cereb Blood Flow Metab*, doi:10.1038/jcbfm.2014.124 (2014).

386 Mallard, E. C., Rehn, A., Rees, S., Tolcos, M. & Copolov, D. Ventriculomegaly and reduced hippocampal volume following intrauterine growth-restriction: implications for the aetiology of schizophrenia. *Schizophrenia research* **40**, 11-21 (1999).

387 Zhang, X. *et al.* Prenatal hypoxia may aggravate the cognitive impairment and Alzheimer's disease neuropathology in APPSwe/PS1A246E transgenic mice. *Neurobiology of aging* **34**, 663-678, doi:10.1016/j.neurobiolaging.2012.06.012 (2013).

388 Lieblein-Boff, J. C. *et al.* Neonatal E. coli infection causes neuro-behavioral deficits associated with hypomyelination and neuronal sequestration of iron. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33**, 16334-16345, doi:10.1523/jneurosci.0708-13.2013 (2013).

389 Blutstein, T. *et al.* Differential responses of hippocampal neurons and astrocytes to nicotine and hypoxia in the fetal guinea pig. *Neurotoxicity research* **24**, 80-93, doi:10.1007/s12640-012-9363-2 (2013).