Regulation of blood-brain barrier tightness and polarity following ischemic stroke

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LIST OF ABREVIATIONS

Aβ	amyloid-beta
ABC transporter	ATP binding cassette transporter
ACA	anterior cerebral artery
ACAT	acyltransferase enzyme
ACE	angiotensin converting enzyme
ACcomA	anterior communicating artery
AJ	adherens junction
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANG1	angiopoetin 1
ANOVA	analysis of variances
AP-1	activator protein-1
Apaf-1	apoptosis-activating factor-1
ApoE	apolipoprotein E
ApoER2	apolipoprotein E receptor 2
ASA	American Stroke Association
ASA	acetylsalicylic acid
AT1R	Angiotensin II type 1 receptor
AT2	angiotensin 2
ATP	adenosine triphosphate
BA	basal artery
BBB	blood-brain barrier
BCRP	breast cancer resistance protein (ABCG2; MXR)
BDNF	brain-derived neurotrophic factor
BCSFB	blood cerebrospinal fluid barrier
bFGF	basic fibroblast growth factor
BH1 – BH4	Bcl-2 homology domains 1 – 4
BHT	2,6-di-tert-butyl-4-methylphenol
BMI	body mass index
BSA	bovine serum albumin
CASK	dependent serine protein kinase
CCA	common carotid artery
CD31	see also PECAM-1
CDB	Cdc42 binding domain of WASP

CINC	cytokine-induced neutrophil chemo-attractant protein
CNTF	ciliary neurotrophic factor
CNS	central nervous system
Cox-2	cyclooxygenase-2
CSF	cerebrospinal fluid
CSLM	confocal scanning laser microscopy
DAPI	4,6-diaminido-2-phenylidole
DNA	deoxyribonucleic acid
EAAT1–3	excitatory amino acid transporters 1–3
ECA	external carotid artery
ECL	enhanced chemoluminescence
EDTA	ethylenediaminetetraacetic acid
ELAM-1	endothelial leukocyte adhesion molecule-1
ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric-oxide synthase
ER	endoplasmic reticulum
ERK-1/2	extracellular-signal-regulated kinases-1/2
ESAM	endothelial selective adhesion molecule
ET1	endothelin 1
17ßE	17ß-estradiol
17ßE-d3	^{3}D-labeled 17ß-estradiol
17ßEG	17ß-estradiol-17ß-D-glucuronide
FK506	synonym for tacrolimus (immunosuppressant)
FKBP	FK506 binding proteins
FMOCCl	9-fluorenylmethyl chloroformate
GABA	gamma aminobutyric acid
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDNF	glial-derived neurotrophic factor
GDP	guanine diphosphate
GEF	guanine exchange factor
GFAP	glial fibrillary acidic protein
GLUT1	glucose transporter 1
GSH	reduced glutathione
GS	glycosylation sites

GSNO	S-nitrosoglutathione
[^{15}N]-GSNO	^{15}N-labeled S-nitrosoglutathione
GSSG	glutathione disulfide
GST	glutathione S-transferase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HIF-1α	hypoxia-inducible factor-1α
HIV-TAT PTD	human immunodeficiency virus-RAR protein transductions domain
HMG-CoA	hydroxymethylglutaryl-coenzyme A (HMG-CoA inhibitors = statins)
H_2O_2	hydrogenperoxide
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
5-HT, serotonin	5-hydroxytryptamine
ICA	internal carotid artery
ICAM-1	intracellular adhesion molecule-1
IFN-γ	interferon
IgE	immunoglobuline E
IGF-1	insuline-like growth factor-1
IL-1ß	interleukin-1ß
IL-6	interleukin-6
i.a.	intra-arterial
i.p.	intra-peritoneal
IRF1	interferon regulatory factor 1
i.v.	intra-venous
JACOP	junction-associated coiled-coil protein
JAM	junctional adhesion molecules
JIP1/2	Jun N-terminal kinase-1/2 -interacting protein-1/2
JNK	c-Jun NH_2-terminal kinases
JNK-1/2	c-Jun N-terminal kinase-1/2
LAT1	L-system for large neutral amino acids
LC-MS	liquid chromatography-mass spectrometry
LDF	Laser Doppler flow

LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein receptor
LIF	leukemia inhibitory factor
LSD	least significant differences
LTC_4	leukotriene C_4
LTCC	L-type calcium channel
LXR	Liver X receptors
MAPK	mitogen-activated protein kinase
MAGI-1 / -2 / -3	membrane-associated guanylate kinase with inverted orientation of protein–protein interaction domains
MCA	middle cerebral artery
MCP-1	monocyte chemotactic protein-1
Mdr-1	multidrug resistance protein-1 (p-glycoprotein; ABCB1)
mdr-1a	*abcb1a* gene
mdr-1b	*abcb1b* gene
MKK-7	mitogen-activated protein kinase (MAPK) kinase-7
MKP-7	phosphatase MAPK phosphatase-7
MIB	microvessel isolation buffer
MLC	light-chain of myosin
MLCP	myosin light-chain phosphatase
MMP	matrix metalloproteinases
MPP+	1-methyl-4-phenylpyridinium
Mrp-1	multidrug resistance-associated protein 1 (ABCC1)
mrp-1	*abcc1* gene
MS	multiple sclerosis
MSD	membrane-spanning domains
MUPP1	multi-PDZ-protein 1
MX	Mitoxantrone
NAD^+	nicotinamide adenine dinucleotide
$[^{15}N]NaNO\underline{2}$	^{15}N-labeled sodium nitrite
NBD	nucleotide-binding domains
NDS	normal donkey serum
NeuN	neuron-specific nuclear protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

NGF	nerve growth factor
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS I, nNOS	neuronal NO synthase
NOS II, iNOS	inducible NO synthase
NOS III, eNOS	endothelial NO synthase
NT-4/5	neurotrophins 4/5
OATP	organic anion-transporting polypeptide
ONR	orphan nuclear receptors
OxLDL	oxidized LDL
PAR3/6	partitioning defective proteins 3 and 6
PARP	poly-(ADP-ribose) polymerase
PBS	phosphate-buffered saline (0.1 *M*; pH 7.4)
PBS-T	phosphate-buffered saline containing 0.3% Triton-X-100
PCA	posterior cerebral artery
PComA	posterior communicating artery
PD	Parkinson's disease
PECAM-1	platelet and endothelial cell adhesion molecule-1 (marker for endothelial cells; CD31)
PFA	paraformaldehyde
P-gp	P-glycoprotein
PKB/Akt	protein kinase B
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PPA	pterygopalatin artery
PXR	pregnane X receptor
P2Y2	purinergic receptor
RBD	Rho binding domain
RGS5	regulator of G-protein signalling 5
Rif	rifampicin (antibiotic agent)
ROCK	Rho-associated coiled-coil containing protein kinase
ROS	reactive oxygen species
RNS	reactive nitrogen species

RT	room temperature
RTK	Rhotekin
rt-PA	recombinant tissue-plasminogen activator
RT-PCR	real-time polymerase chain reaction
SAPK	stress-activated protein kinase
SDHA	succinate dehydrogenase complex subunit A
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
SLC	solute carriers
SNPs	single nucleotide polymorphisms
STAT3	signal transducer and activator of transcription 3
SXR	steroid and xenobiotic receptor
TBS	tris buffered saline (0.1 M, pH 7.6)
TBS-T	tris buffered saline containing 0.1% Tween 20
TCA	trichloroacetic acid
TGF-ß	transforming growth factor-ß
TIE2	endothelium-specific receptor tyrosine kinase 2
TJ	tight junctions
TMD	transmembrane domain
TNF- α	tumor necrosis factor-α
TQD	tariquidar (XR-9756)
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling
UGT	uridine diphosphate
VA	vertebral artery
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
WASP	Wiskott-Aldrich syndrome protein
WHO	World Health Organization
ZO-1 / -2 / -3	zonula occludens protein-1 / -2
ZONAB	ZO-1-associated nucleic acid-binding protein

FIGURE TITELS

ABSTRACT

Neuroprotective therapies have made little progress in the recent past. Vascular components of injury contributing to the development and progression of neuronal injury, the inability of drugs to pass the blood-brain barrier (BBB) and unsuitable animal models used for mimicking stroke pathophysiology have been recognized as major factors responsible of treatment failure. The molecular mechanisms underlying disturbances of BBB function after stroke are poorly understood. Microvascular cells are polarized cells forming a tight barrier, which is complemented by membrane-bound ATP-binding cassette (ABC) transporters both on the luminal and abluminal endothelial membrane, giving rise to two key features, BBB tightness and polarity. Both BBB tightness and polarity are dysregulated upon stroke. Our study was carried out to study the underlying molecular mechanisms regulating BBB integrity and polarity. As such, the relevance of liver X receptor (LXR), the role of the apolipoprotein ApoE and effects of hypercholesterolemia, a highly prevalent risk factor for vascular diseases were investigated.

By means of pharmacological studies using a LXR agonist, T0901317, using Western blots analysis, protein interaction studies and pull-down assays, we showed that LXR activation preserved BBB integrity and decreased BBB leakage after focal cerebral ischemia induced by middle cerebral artery (MCA) occlusion, representing a promising strategy to prevent post-ischemic brain swelling, a frequent complication of ischemic stroke. On the molecular level, the anti-edematous effect was caused by deactivation of microvascular calpain-1/2 and MMP-2/9 protease activity, resulting in the stabilization of p120 catenin, thereby decreasing RhoA and increasing Cdc42 activity, thus modulating the expression, phosphorylation and assembly of tight junction (TJ) proteins. BBB enhanced integrity did not influence BBB polarity, as upon LXR activation the expression of the ABC transporters ABCB1, ABCC1, ABCA1 and ABCG1 expression was similarly increased.

By means of genetic knockout, protein delivery and pharmacological signal transduction inhibition experiments, we demonstrated that ApoE controls the polarized expression changes of ABC transporters following stroke, presumably via its receptor ApoER2, which is mainly expressed at the abluminal side of brain

capillaries and acts as sensor of BBB leakage. As such, ApoE binding to ApoER2 deactivates Jun-Kinase-1/2 (JNK-1/2) and c-Jun, thus transcriptionally increasing luminal endothelial ABCB1 and decreasing abluminal ABCC1 expression, thereby reducing the accumulation of the pharmacological model compound FK506 in the ischemic brain. Interestingly, BBB tightness, as evaluated by measuring by serum IgG extravasation, was not influenced by ApoE deficiency. Based on our data, ApoE regulates an endogenous cellular response, which prevents the accumulation of potentially toxic blood-derived molecules in the ischemic brain, but also impedes brain pharmacotherapies. Deactivation of ApoER2, based on our data may represent a promising strategy to ameliorate drug delivery to the stroke brain.

Hypercholesterolemia is a major risk factor of ischemic stroke. By means of Western blots, protein-protein interaction and pull-down assays, we showed that hypercholesterolemia increases BBB leakage upon stroke without influencing ABC drug transporters. This increased permeability was accompanied by and exacerbated activation of calpain-1/2, MMP-2/9 and downregulation of TJ proteins under hypercholesterolemic conditions. The effect of hypercholesterolemia on BBB permeability was associated with the overexpression of the angiotensin II type 1 receptor (AT1R), with overactivation of its downstream target RhoA and RhoA's activator, the leukemia associated Rho GEF (LARG). Our results show that BBB tightness and polarity are regulated by separate mechanisms. Our results offer new perspectives for the treatment of cerebrovascular diseases. As such, LXR agonists might be used for the treatment of brain edema, ApoE regulation or ApoER2 blockade might be a novel strategy to enhance neuroprotective drug entry. The effects of hypercholesterolemia on BBB integrity should be taken more thoroughly in account in translational stroke studies.

1 INTRODUCTION

1.1 Blood-brain barrier (BBB) in central nervous system (CNS) diseases

The central nervous system (CNS) is a particularly vulnerable system. In contrast to other peripheral systems in the human body, the CNS is very sensitive to a wide range of chemicals, xenobiotics, plasma composition fluctuations and metabolites in the blood circulation, which can be very harmful and toxic for the CNS (Hawkins and Davis, 2005). The regulation of many important ions such as Na^+, K^+, and Ca^{2+}, and other molecules like amino acids, oxygen and glucose, is crucial for neuronal survival and function (Hawkins and Davis, 2005). Therefore, tightly controlled brain homeostasis and narrowly regulated brain microenvironment are essential for a proper function of the brain.

This drastic control and regulation of the CNS homoeostasis and microenvironment unraveled the existence of a complex and dynamic interface between the CNS and the blood circulation. This interface that acts as an ion balance regulator, a nutriments transport facilitator and a physical barrier that protects the CNS from harmful and toxic molecules (Hawkins and Davis, 2005), is the blood-brain barrier (BBB).

The presence of the BBB was first noticed by Ehlrich (Ehlrich, 1885), who observed that water soluble dyes almost stained all organs with two exceptions, the brain and the spinal cord. Ehlrich's explanation was that the CNS has not, or has very low, affinity for this water soluble dye (Ehlrich, 1904). Later, experiments conducted by his student Goldmann showed that when a blue dye was administered directly in the cerebrospinal fluid (CSF), this dye stained all CNS and did not stain any peripheral system or organ (Goldmann, 1913). These experiments confirmed the presence of a specialized physical barrier separating the CNS from the blood circulation, limiting and restricting the dye exchange between the CNS and the blood circulation. This propriety of the BBB in strongly and narrowly controlling brain homeostasis, regulating brain microenvironment, and protecting the brain from exogenous molecules under physiological conditions is now considered to be the main challenge in CNS diseases.

As mentioned above, in the CNS the BBB primarly plays a role in controlling brain homeostasis and regulating brain microenvironment. It constitutes a special tight physical barrier separating the blood circulation and CSF, contributes in proper function of the brain by allowing the entry of oxygen and nutriments, and protects the brain from harmful molecules by limiting their entry and accumulation into the brain (Hawkins and Brown, 2005). Due to the crucial role of the BBB in CNS function, the dysfunction of the BBB represents the centre of development and progression of many CNS diseases. As such, increased permeability is observed in ischemic stroke and brain trauma (IIzecka, 1996; Morganti-Kossmann et al., 2002) leading to brain edema and exacerbating neuronal injury. In multiple sclerosis (MS) the BBB dysfunction participates directly in the pathophysiology of MS (Minagar and Alexander, 2003), and contributes in the development of the disease. In brain cancers like glioblastomas, the BBB becomes very leaky with exaggerated angiogenesis leading to brain edema and enhancing tumor survival (Anderson et al., 2008). Moreover, many other risk factors and peripheral diseases contribute in BBB loss of function, like obesity, hypercholesterolemia, diabetes and atherosclerosis (Iso et al., 2002; O'Regan et al., 2008; Hebert et al., 1995; Hebert et al., 1997; Dormandy et al., 2005; Methia et al., 2001; Hafezi-Moghadam et al., 2007). In addition to these pathophysiological events that compromise the function and the integrity of the BBB upon many CNS diseases, the BBB itself represents a major challenge in the context of brain pharmacotherapies.

In CNS diseases, the BBB prevents drugs from brain entry and accumulation (Pardridge, 2007a). The BBB does not act only as passive diffusion barrier, but rather it expresses different active and dynamic transporters that prevent and eliminate drugs from brain entry, and thereby profoundly influence brain drug levels (Dallas et al., 2006; Löscher and Potschka, 2005). The great advances achieved in medicine increased the human longevity, but in counterpart CNS diseases treatment did not follow this tendency due to multiple factors like neuronal regeneration limitations and challenges, and especially the lack of efficient drug delivery strategies to the diseased brain. Therefore, the need now for efficient clinical therapeutics that can offer a long-term treatment for CNS diseases is crucial. The role of the BBB in drug transport and elimination represents the major challenge in the development and the elaboration of efficient brain targeting drugs.

This fact contributed in the decline observed in CNS drug market because most drugs do not reach the diseased brain in sufficient quantities and are eliminated by the BBB (Pardridge, 2002). Only few lipophilic molecules that have very low molecular weight between 400-500 Da are able to cross the BBB (Pardridge, 2001), and only a small number of CNS diseases respond to such molecules under low doses like depression and pain. On the other hand other serious CNS diseases do not respond at all to these small molecules like stroke, Alzheimer and brain cancers (Ajay, Bemis and Murcko, 1999; Ghose et al., 1999). Therefore, understanding the pathophysiology of the BBB and the governing molecular events that compromise the function of the BBB, as well as the regulation of the drug transport systems existing on the BBB, constitute major axis that will allow in the future the development of efficacious strategies for successful CNS diseases treatment.

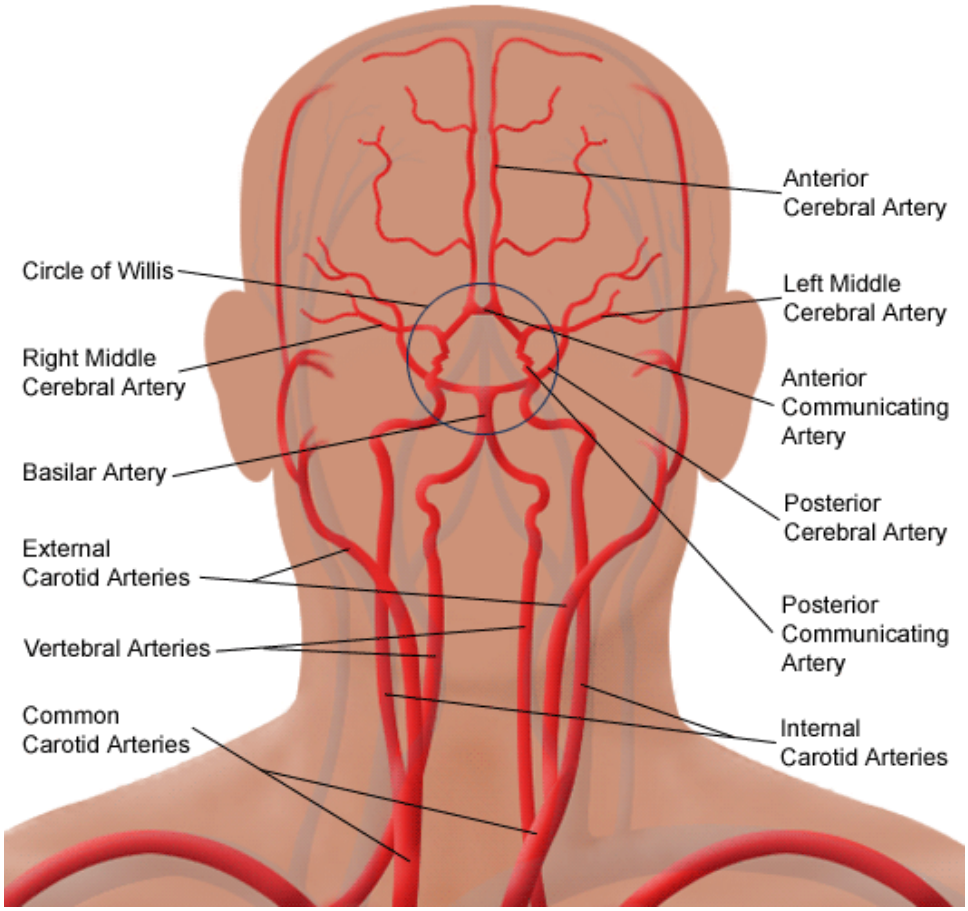

Figure 1. Blood supply to the brain: Scheme illustrating brain blood supply by main brain arteries. (Source: University of Rochester, Medical Center).

1.2 Biology of the BBB

The BBB is a selective barrier constituted by specialized endothelial cells forming the endothelial cell layers that dynamically interact with other cells in the brain especially pericytes, astrocytes and neurons. Compared to other peripheral vascular systems, the BBB adopts a special phenotype characterized by tight endothelial cell-cell contacts, providing high transendothelial electrical resistance (Zlokovic, 2008; Bazzoni and Dejana, 2004), which limit almost all large exogenous and endogenous molecules in the blood circulation from brain entry and accumulation, except some small and lipophilic molecules (Pardridge, 2001).

The endothelial cell – cell contacts are constituted by specialized junctional complexes, the tight junctions (TJ) and adherens junctions (AJ) (Kniesel and Wolburg, 2000). TJ are formed by transmembrane proteins, such as occludin, claudins and junctional adhesion molecules (JAM), that to establish endothelial cell-cell contacts tightly interact with accessory cytoplasmic proteins such as membrane associated guanylate kinase like proteins (MAGUK), e.g. zonula occludens proteins (ZO-1, ZO-2, ZO-3) (Del Maschio et al., 1999; González-Mariscal et al., 2000; Hawkins and Davis, 2005), and other proteins such as cingulin, afadin (AF6) and 7H6 (Citi et al., 1988; Zhong et al., 1993). AJ are constituted by the membrane proteins cadherins. The main cadherins are cadherin-E, -P, and -N, which are single pass transmembrane glycoproteins that, for establishing cell-cell contacts, interact with the cytoplasmic proteins catenins, constituted by catenin-α, -β, -γ and p120 catenin (Takeichi, 1995; Schulze and Firth, 1993).

In contrast to other catenins, p120 catenin function remains unclear. Unlike catenin-β, p120 catenin does not interact with catenin-α, implying more complex role in of p120 catenin in cell-cell contacts (Daniel and Reynolds, 1995). It has been suggested a role of p120 catenin in signal transduction (Daniel and Reynolds, 1999). It has been shown that p120 catenin expression is deregulated in many tumors, and linked to the degree of tumor aggressivity (Shimazui et al., 1996; Dillon et al., 1998; Jewhari et al., 1999). Moreover, a novel role of p120 catenin has been suggested in controlling cell membrane cytoskeleton and cellular motility (Daniel and Reynolds, 1999).

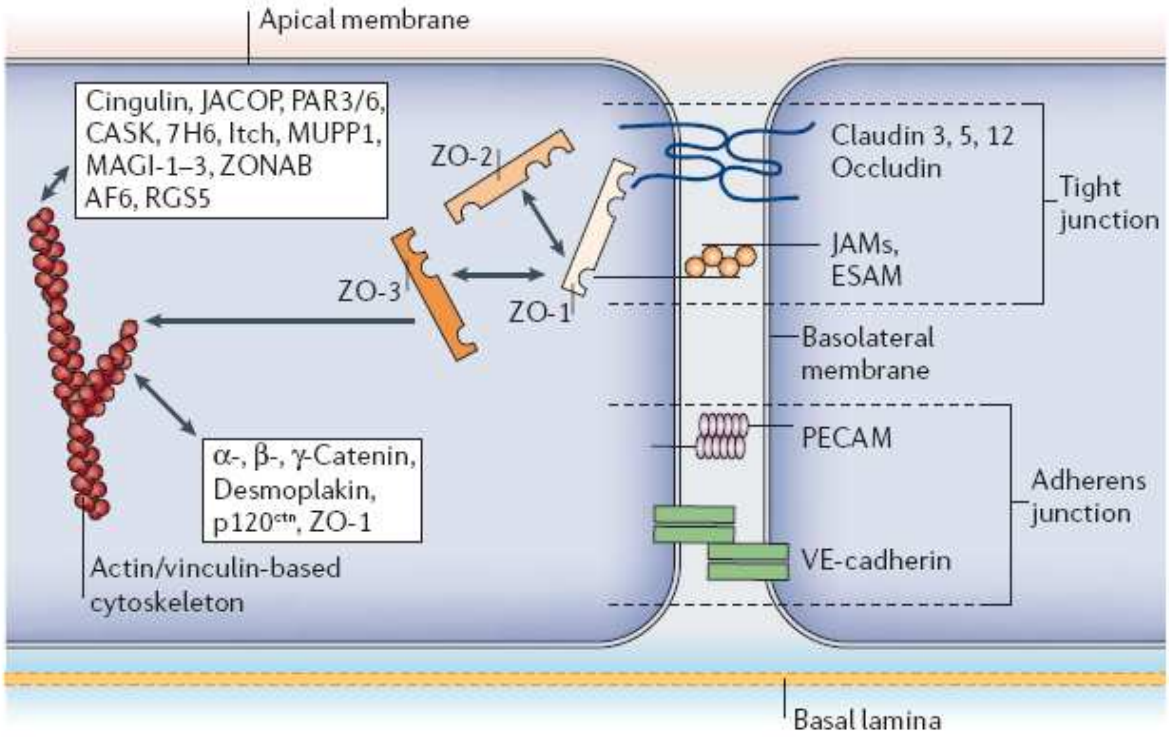

Figure 2. Molecular composition of endothelial cell-cell contacts: Scheme illustrating brain endothelial cell contacts. In addition to JAM and endothelial selective adhesion molecule (ESAM), which are members of the immunoglobulin superfamily, the two most important proteins of TJ are occludin and claudins, which have four transmembrane domains and two extracellular loops. In the cytoplasm there are many important adaptor proteins such as zonula occludens (ZO-1, -2, -3) and Ca^{2+} dependent serine protein kinase (CASK), which bind to the intramembrane of transmembrane proteins. Other adaptor molecules are expressed such as cingulin and junction-associated coiled-coil protein (JACOP). Other signaling and regulatory proteins are expressed such as multi-PDZ-protein 1 (MUPP1), the partitioning defective proteins 3 and 6 (PAR3/6), membrane-associated guanylate kinase with inverted orientation of protein–protein interaction domains (MAGI-1, -2, -3), ZO-1-associated nucleic acid-binding protein (ZONAB), AF6, and regulator of G-protein signaling 5 (RGS5), which control the interaction of the membranous components with endothelial cell cytoskeleton. AJ are very important in establishing endothelial cell contacts constituted mainly by cadherins such as vascular endothelial cadherin (VE-cadherin) and the platelet–endothelial cell adhesion molecule (PECAM) that mediates homophilic adhesion. The catenins, including p120 catenin, are very important for linking AJ to the cytoskeleton. (Source: Abbott et al. 2006).

In addition to the physical proprieties of the BBB, endothelial cells that form this barrier express variety of transmenbrane proteins that play an important role in BBB transport (Schinkel, 1999), such as ATP binding cassette (ABC) transporters; solute carriers (SLC) (Dahlin et al., 2009), e.g. SLCO, SLC22A; lipid related receptors, e.g. LDLR and ApoER2; and endocytosis related receptors. All these transmembrane proteins regulate the dynamic exchange between the brain and the blood circulation, which assure a proper function of the brain by controlling many aspects of brain homeostasis (de Boer et al., 2003; Pardrige et al., 1985; Pardrige and Fierer, 1985; Zhang and Pardridge, 2001).

1.2.1 Neurovascular unit

At the anatomical level the BBB is constituted by cerebral endothelial cells, astrocytes, pericytes, neurons, and the extracellular matrix, forming all together the neurovascular unit. Recently, the concept of neurovascular unit has emerged and has been adapted in investigating CNS physiology and pathophysiology, as it constitutes the functional unit of the BBB. Therefore, cell-cell interaction, crosstalk and signaling at the neurovascular unit have been shown to be crucial and essential for the function of the BBB. At the cellular level, brain endothelial cells forming the BBB differ from other endothelial cells by the high quantity of mitochondrial content (Oldendorf et al., 1977), the very low rate of endocytosis (Ballabh et al., 2004; Pardrige, 2002), the lack of membrane fenestrations (Fenstermacher et al., 1988), and the basal pinocytotic activity (Sedlakova et al., 1999).

Structurally, the neurovascular unit is formed by endothelial cells that interact with pericytes attached at the basal side of the endothelium. In addition to pericytes, endothelial cells are ensheathed by the basal lamina composed mainly of collagen type IV, heparin sulfate proteoglycans, laminin, fibronectin (Farkas and Luiten, 2001), constituting the extracellular matrix that provides a physical support for endothelial cells and pericytes. Brain percicytes are different from other pericytes in peripheral systems (Nehls and Drenckhahn, 1991) and cover up to 30% of brain microvessel circumference, and play an important role in regulating endothelial cell proliferation, survival and migration (Hellström et al., 2001). Moreover, percicytes have been shown to modulate the branching of brain microvessels (Hellström et al., 2001).

In addition to pericytes, the basal lamina is directly interacting with astrocyte end-feet that ensheathe more than 90% of brain microvessels, and this interaction plays a crucial and essential role in the function of the BBB (Davson and Oldendorf, 1967). Astrocytes act also as scaffold cell by guiding neurons during development (Jacobs and Doering, 2010) and by directing newly formed brain microvessels (Zhang et al., 1999)

Cell-cell interaction at the neurovascular unit plays a crucial role in regulating the function of the BBB. Therefore, the special phenotype of brain endothelial cells alone is not sufficient to explain the specificity of the BBB. It is well established that when isolated brain microvessel endothelial cells are cultured alone in normal culture medium, they form a leaky monolayer that does not reflect BBB phenotype *in vivo*. In contrast, when astrocytes and/or pericytes are added to the culture medium, the monolayer shows higher electric resistance, increased integrity and decreased permeability, thus reflecting to some level BBB phenotype *in vivo* (Neuhaus et al., 1991). In parallel, polarized astrocytes induce endothelial cell polarization, and endothelial cell polarity is required to maintain astrocyte polarity, which translate a reciprocal role in maintaining the function of the neurovascular unit (Abbott et al., 1992). Based on these observations, it is now well established that endothelial cell interaction sites with the endfeet of polarized astrocytes represent the major signaling sites at the neurovascular system. Interestingly, some studies have shown that even pericytes are able to induce BBB phenotype like astrocytes (Minakawa et al., 1991).

1.2.2 Signal transduction at the neurovascular unit

As described above, the long term effect and crosstalk between astrocytic endfeet and endothelial cells are crucial for the proper function of the neurovascular unit and for inducing BBB phenotype. Beside the long term effect of astrocytes on endothelial cell, evidence pointed at similar important short term effect of astrocytes on endothelial cell mediated by different signaling molecules and ligands produced and secreted by astrocytes. As such, astrocytes secrete different signaling molecules like growth factors, cytokines, and neurotransmitters that act directly not only on neurons but also on endothelial cells and other components of the neurovascular unit.

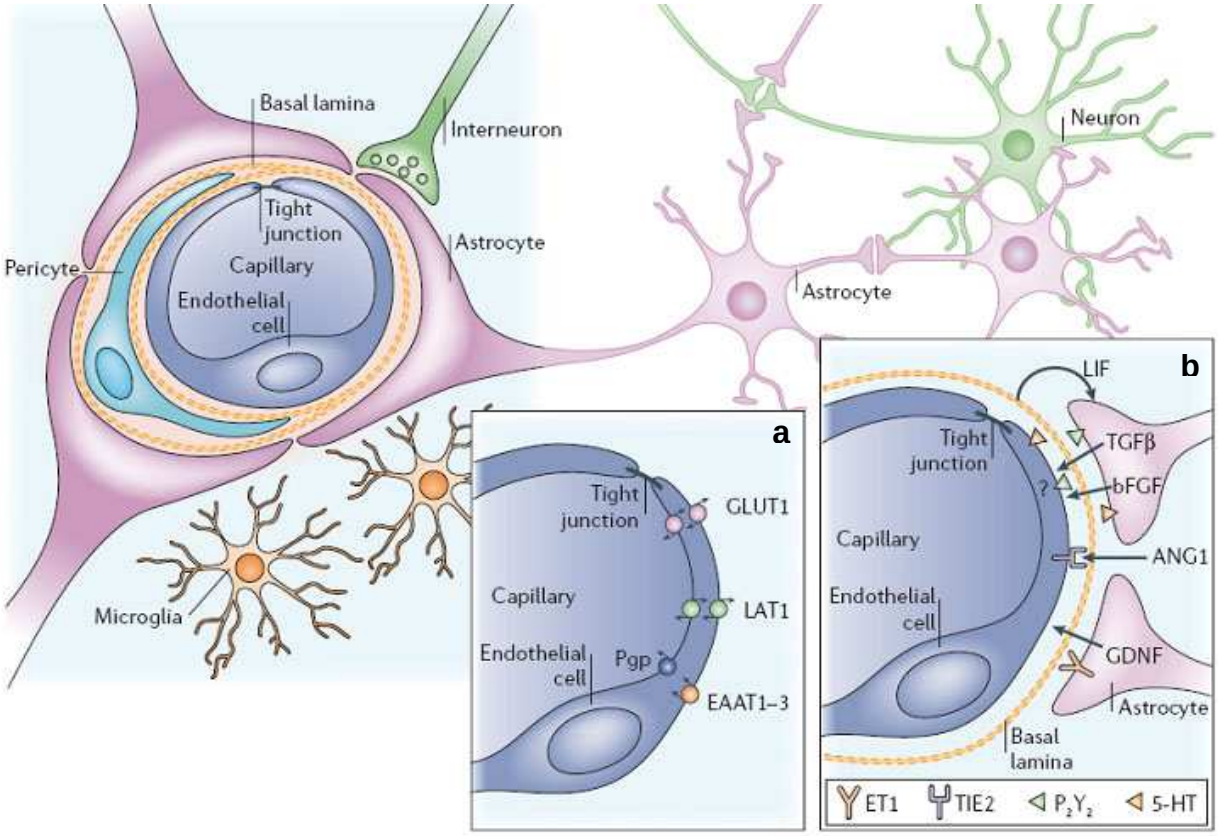

Figure 3. Crosstalk and signaling at the neurovascular unit: Scheme illustrating signaling pathways at the neurovascular unit. The neurovascular unit is formed by capillary endothelial cells and pericytes that are surrounded by the extracellular matrix that forms the basal lamina, and astrocytic endfeet. Astrocytic endfeet link the neurovascular unit to neurons. It represents an important site of signaling of the BBB. **a.** In cell culture the capillary endothelial cells express different transporters and receptors, such as excitatory amino acid transporters 1–3 (EAAT1–3), glucose transporter 1 (GLUT1), L-system for large neutral amino acids (LAT1) and P-glycoprotein (P-gp). **b.** Crosstalk between astrocytic endfeet and endothelial cells is necessary to induce and maintain the BBB. Different molecules secreted by astrocytes and endothelial cells play role in this signaling process through different receptors expressed at astrocytic endfeet and endothelial cells such as 5-hydroxytryptamine (5-HT, serotonin), angiopoetin 1 (ANG1), basic fibroblast growth factor (bFGF), endothelin 1 (ET1), glial cell line-derived neurotrophic factor (GDNF), leukemia inhibitory factor (LIF), purinergic receptor (P2Y2), transforming growth factor-β (TGFβ), endothelium-specific receptor tyrosine kinase 2 (TIE2) (Begley and Brightman, 2003; Schinkel, 1999; Mi et al., 2001). (Source: Abbott et al., 2006).

The importance of the crosstalk between astrocytes and endothelial cells at the BBB has been demonstrated by many *in vitro* experiments. It has been shown that astrocyte-derived signaling molecules such as transforming growth factor-β (TGF- β), glial cell line-derived neurotrophic factor (GDNF), interleukin-6 (IL-6) and basic fibroblast growth factor (bFGF) modulate the morphology and the integrity of the BBB (Tran et al., 1999; Utsumi et al., 2000)

As such, endothelial cells express many receptors (Mi et al., 2001), which upon activation by astrocyte-derived signaling molecules, regulate endothelial cell-cell contacts by modulating TJ and AJ formation and assembly, affect the transport systems existing at the BBB, and regulate the activities of many enzymes at the BBB (Abbott and Romero, 1996). Interestingly, this mechanism is not unidirectional, as in parallel endothelium-derived leukemia inhibitory factor (LIF) that are secreted by endothelial cells has been shown to play an important role in inducing astrocytic differentiation (Mi et al., 2001). Besides astrocytes, recent evidence showed that pericytes also play a role in BBB integrity and genesis by mainly regulating matrix metalloproteinase-9 (MMP-9) (Elfont et al., 1989; Healy et al., 1993; Dehouck et al., 1997).

1.2.2.1 Regulation of BBB tightness

Endothelial paracellular permeability at the BBB is mainly maintained by TJ and AJ, which are regulated by different complex mechanisms, among these mechanisms phopshorylation of TJ and AJ proteins (McCarthy et al., 1996; Hirase et al., 2001). TJ and AJ are phosphoproteins that can be phosphorylated on amino acid serine, threonine, and tyrosine residues. Although the exact phosphorylation sites of TJ and AJ proteins remain unknown, their phosphorylation state directly influences their assembly, dissociation and subcellular distribution, therefore controlling the integrity of the BBB (Sakakibara et al., 1997; Andreeva et al., 2001; Rao et al., 2002).

It has been shown that the phosphorylation of TJ and AJ depends on the type of stimulus as well as the neurovascular microenvironment. A proper interaction between TJ components such as the interaction between occludin and ZO-1 is essential for maintaining the tightness of TJ complex, thus the tightness of the BBB. Occluding and ZO-1 interaction is dependent on the phosphorylation state of these

two proteins. For example, vascular endothelial growth factor (VEGF) induces serine / threonine phosphorylation and redistribution of occludin and ZO-1 in mouse brain endothelial cells, and oxygen mediators induce primarily tyrosine phosphorylation and reorganization of the TJ complex (Harhaj et al., 2006; Kago et al., 2006; Haorah et al., 2007). Moreover, AJ proteins such as VE-cadherin and β-catenin tend to be serine / threonine and tyrosine phosphorylated during brain endothelial barrier opening (Kirk et al., 2003; Potter et al., 2007).

VE-cadherins represent the major protein of AJ, and play an important role in vascular integrity. VE-cadherins interaction with catenin-α, -β, -γ and p120 catenin is essential for AJ complex stability. The exact mode of interaction between AJ components remains not fully understood, but it seems that catenin-β and catenin-γ forms a complex with cadherins and catenin-α, whereas the latter forms a bridge coupling the complex to actin and therefore endothelial cells cytoskeleton (Bazzoni et al., 2000). The role of the last member of catenin family, p120 catenin, remains unclear but many reports have shown that this protein plays an important role in modulating signal transduction (Nagafuchi et al., 2001).

As shown above, many molecular mechanisms control and modulate TJ and AJ expression, distribution and assembly. Recently, Rho GTPases have caught attention and have been shown to be major regulators of TJ formation, maintenance and disruption. Rho GTPases are members of the Ras superfamily small proteins, which act as molecular switches, regulating many essential molecular mechanisms, including actin dynamics, gene transcription, cell cycle progression and cell adhesion. The most studied Rho GTPases are RhoA, Rac1 and Cdc42; they exist in two conformational states: active guanosine triphosphate (GTP)-bound state and inactive guanosine diphosphate (GDP)-bound state. In the active GTP-bound state, Rho GTPases recognize, bind and activate specific target proteins called effectors, inducing specific signaling pathway and generating specific cell responses (Etienne-Manneville and Hall, 2002).

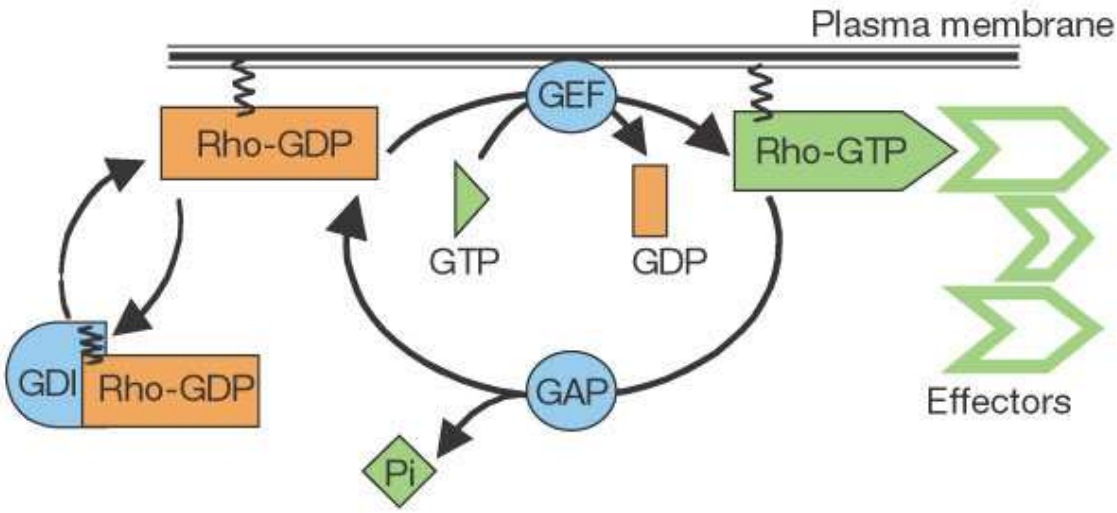

Figure 4. Rho GTPase activation and deactivation cycle: Rho GTPases switch between an inactive (GDP)-bound and active (GTP)-bound forms. The activation of Rho GTPases is initiated by guanine exchange factor (GEF) that stimulates GDP exchange by GTP. The active GTP-bound form binds and activates specific effector proteins initiating a signal transduction response. Translational modification with lipid moieties, e.g. farnesyl, geranyl-geranyl, is also an important factor in Rho GTPases activity, as it allows the attachment of the active GTP-bound form to the plasma membrane. Afterwards, GTPase activating protein (GAP) stimulates the hydrolysis of GTP into GDP and P_i, turning back Rho GTPases to the inactive GDP-bound form, terminating the signal transduction response. The GDP dissociation inhibitor (GDI) maintains the inactive GDP-bound form of Rho GTPases in the cytosol, therefore making them inaccessible for further activation by GEF. (Source: Etienne-Manneville and Hall, 2002).

Upon activation by the active GTP-bound form of RhoA, RhoA effector the Rho-associated coiled-coil containing protein kinase (ROCK) promotes phosphorylation of the regulatory light-chain of myosin (MLC) on serine-19 and threonine-18 through phosphorylation of the myosin light-chain phosphatase (MLCP), therefore blocking MLC dephosphorylation. This site-specific phosphorylation of MLC in turn elevates myosin ATPase activity, leading to acto-myosin contraction (Nelson et al., 2004; Noma et al., 2006), causing long-lasting alterations in endothelial permeability.

On the other hand, Cdc42 activation controls the polarity of actin and microtubule cytoskeleton and promotes BBB integrity by enabling the assembly of TJ proteins, such as occludin and ZO-1 (Ramchandran et al., 2008; Cau and Hall, 2005; Fukuhara et al., 2003), and by the restoration AJ of endothelial barrier, and it was suggested that activating Cdc42 therapeutically may represent a possible strategy for endothelial barriers restoration (Broman et al., 2007; Kouklis et al., 2004). Besides these molecular mechanisms and signaling pathways, calcium Ca^{2+} homeostasis and signaling are crucial in endothelial cells function and BBB integrity. For example, endothelial cells express different receptors such as chemokine receptors (CXCR4), monocyte chemotactic protein-1 (MCP-1) receptor (CCR2) that controls calcium homeostasis via the L-type calcium channel (LTCC), leading to endothelial cell function disruption (Foresti et al., 2009). Recently, MCP-1 has been shown to induce endothelial permeability by inducing proteases and enzyme activation and directly downregulating TJ proteins expression, localization and distribution (Stamatovic et al., 2003).

1.2.2.2 Regulation of BBB polarity

Structural, biochemical and functional studies have shown that brain endothelial cells have two functionally distinct compartments; luminal compartment (i.e. apical membrane) that faces and interacts with the blood circulation and abluminal compartment (i.e. basal membrane) that faces and interacts with brain parenchyma (Betz et al., 1980). The polarity of cerebral endothelial cells is crucial for BBB function in controlling brain homeostatic and microenvironment. Due to this polarity, some channels, receptors and transporters expressed at the BBB are exclusively localized at the luminal membrane of endothelial cells and others exclusively at the abluminal membrane of endothelial cells (Betz et al., 1980; Roberts et al., 1993; Roberts et al., 2008). As such, the polarized distribution of the apical Na^+ channels and the basolateral Na^+-K^+-ATPase is crucial for endothelial cells isosmotic fluids transport (Betz et al., 2008). This sub-cellular distribution and polarity of these transporters and receptors are deeply influenced by BBB pathophysiological conditions.

Although many studies have shown that TJ proteins contribute in the establishment and maintenance of this luminal - abluminal membrane polarity

(Förster, 2008), it is still controversial whether TJ are the main responsible of BBB polarity or just a consequence of an already established polarity (Förster, 2008). TJ alone cannot explain totally the polarity of the BBB, because some studies showed that TJ play an important role in abluminal protein polarity but does not play any role in luminal protein polarity (Roberts and Sandra, 1993). In parallel, astrocytes interacting with endothelial cells have been shown to play an important role in mediating BBB tightness and polarity (Janzer and Raff, 1987; El Hafny et al., 1996; El Hafny et al., 1997; Bauer and Bauer, 2000).

1.3 Transport across the BBB

As described above, the BBB constitutes a physical and selective barrier between the CNS and blood circulation. The presence of the special TJ between endothelial cells forming the BBB is responsible for the high electrical resistance that has been estimated to be in the range of 8000 Ω× cm^2 *in vivo* (Smith and Rapoport, 1986), which is far more higher than the electrical resistance of any other peripheral vascular system, where the average electrical resistance is in the range of 3-33 Ω× cm^2 (Crone and Christensen 1981; Butt et al., 1990).

This high electrical resistance restricts and limits the passage of a big number of molecules and compounds across the BBB. In parallel, the brain is characterized by its high metabolic demands, consuming up to 20% of total oxygen and almost 25% of glucose (Rolfe and Brown, 1997). As such, a proper exchange between the brain and the blood circulation is crucial for the function of the brain. Although the BBB constitutes a tight physical barrier separating the brain and the blood circulation, the exchange is assured by special transport systems that are specific for the BBB. Therefore, to enter the brain, endogenous and/or exogenous compounds have to cross the BBB, either by passive diffusion for compounds and molecules that have low molecular weight (< 500 Da) and small lipophilic compounds, in addition to oxygen and CO_2, or by receptor mediated endocytosis for larger compounds and molecules, e.g. insulin and leptin that are very important for a proper brain function (Pardridge, 2001; Pardridge, 2002).

In addition to these conventional ways of molecule passage, the BBB expresses specialized transporters and carriers like ABC transporters and solute carriers (SLC) that contribute in molecule transport and exchange across the BBB. On one hand ABC transporters act mainly as efflux transporters using energy to pump out of cells harmful molecules and limiting their brain entry. On the other hand, SLC carriers play an important role in the selective uptake of vital molecules that are necessary for the proper functioning of the brain. Importantly, ABC transporters represent a big challenge in CNS diseases pharmacotherapies by restricting protective drugs from brain entry and accumulation (Hermann and Bassetti, 2007).

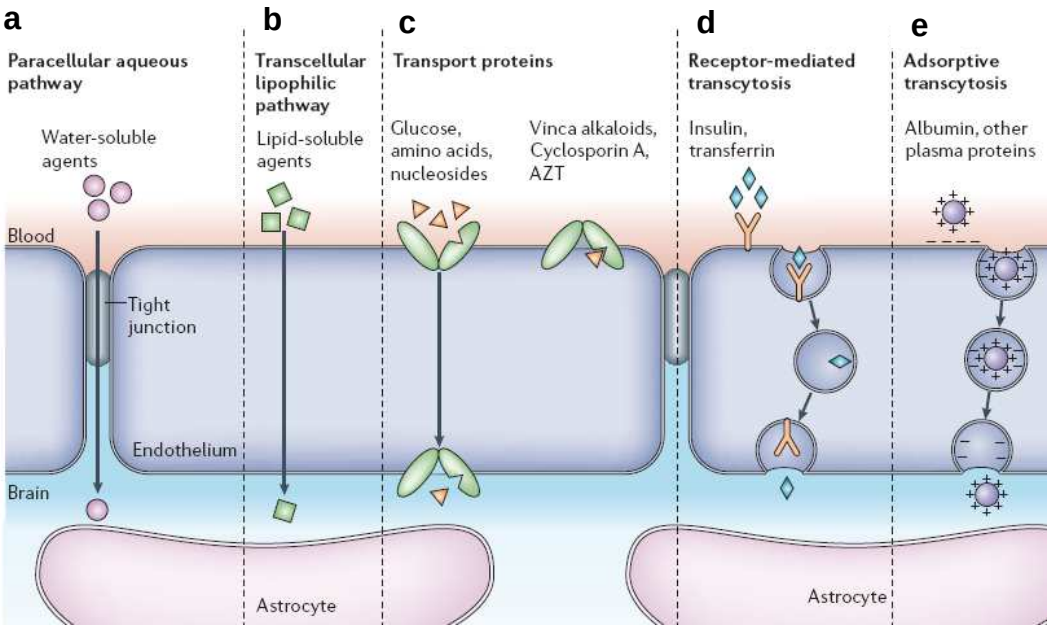

Figure 5. Different ways of transport across the BBB: The BBB restricts and controls the transport of molecules into the brain. The main routes of molecular transport across the BBB are **a.** the paracellular route, which is restricted by TJ for most hydrophilic molecules, **b.** the lipid membranes of the endothelium as a passive diffusion route for lipophilic molecules, **c.** endothelial cells express different transporters and carriers for glucose, amino acids, purine bases, nucleosides, choline and other substances, some of these transporters are energy-dependent, e.g. P-glycoprotein and act as efflux transporters, **d.** some proteins, like insulin and transferrin, are transported by specific receptor-mediated endocytosis and transcytosis, **e.** native plasma membrane proteins like albumin are poorly transported, but cationization can increase their uptake by adsorptive-mediated endocytosis and transcytosis. Drug delivery across the BBB is dependent on routes **b**-**e.** (Source: Abbott et al., 2006).

1.3.1 ATP binding cassette (ABC) transporters

ABC transporters have been discovered initially by oncologists, after observing a cross-resistance of cancers treated with different anti-cancer drugs to different cytotoxic agents to which they had never been exposed, resulting in chemotherapy resistance. Moreover, adding cells from these multi-drug resistant tumors into normal tissue culture resulted in chemotherapy resistance (Biedler and Riehm, 1970). For example, cancer cells obtained from Chinese hamsters have been shown to develop resistance to many drugs *in vitro*, like mithramycin, puromycin, vincristin, vinblastin, daunorubicin, mitomycine C and demecolcine after they had been pretreated with actinomycin D (Biedler and Riehm, 1970). Based on that, this selection of tumour cells by only one cytotoxic agent resulted in a cross resistance to various anti cancer drugs, which had been named multidrug resistance (MDR).

Afterwards, many transmembrane protein transporters have been identified to play a role in multidrug resistance. Interestingly, they are expressed in cancer cells, and in different healthy tissues in multiple organs, like liver, kidney, gastrointestinal tract, brain. ABC transporters represent a big part of these transmembrane protein transporters.

1.3.1.1 Structure of ABC transporters

In humans, so far 48 ABC transporters have been identified and described, and based on domains organization and gene homology, they have been classified into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White) (de Lange,2004; Leslie et al., 2005). ABC transporters seem to play an important role in the function of many organs and systems, as such it has been shown that mutations in many of these transporters are the main cause of many genetic disorders such as bleeding disorders (Albrecht et al., 2005), eye and liver diseases (Martinez-Mir et al., 1998; Jacquemin, 2000), where ABC transporters fail to extrude specific ligands across cell membranes.

In order to be functional, ABC transporters require a minimum of four domains. Two transmembrane domains (TMD) form the ligand binding sites that provide specificity, and two nucleotide binding domains (NBD) that bind and hydrolyze ATP to execute the translocation of the bound ligand. Importantly, the NBD, and not the TMD, are homologous throughout the family and have many characteristic motifs including the Walker A and B motifs that are common to many NBD, and others like the ABC signature, stacking aromatic D, H, and Q loops, which are unique to the family, and they have different glycosylation sites (GS) (Borst and Elferink, 2002; Gottesmann et al., 2002; Löscher and Potschka, 2005).

In general, ABC transporters are structurally closely related and they differ mainly by the number of TMD and NBD and the GS. Therefore, ABC transporters can be classified into three major groups based on their structure models; similar to multi-drug resistance transporter (Mdr-1; i.e. ABCB1), the multi-drug resistance-associated protein (Mrp-4, -5; i.e. ABCC4, 5) constitute the first group, which spins the plasma membrane with two membrane-spanning domains (MSD), and each is composed of six TMD, one NBD located within the intracellular segment and a second NBD at the MSD COOH-terminal end (Borst and Elferink, 2002; Gottesmann et al. 2002; Löscher et al.,2005). The second group, including Mrp-1, -2, -3 and -6 (i.e. ABCC1, 2, 3, 6), is similar to the first structure model except they have a third MSD composed of five TMD at their NH_2-terminal end that does not have a NBD. The third group is similar to the one of the half transporter breast cancer resistance protein (BCRP; termed sometimes MXR for mitoxantrone-resistance gene, officially termed ABCG2), consisting of only one MSD and one NBD located at the NH_2-terminal end. It has been shown that ABCG2 play a role in drug efflux when it forms homodimers and/or heterodimers with another half transporters.

In general, all ABC transporters have a biochemical common feature as they are all glycosylated on their extracellular segments (Borst and Elferink, 2002; Gottesmann et al. 2002; Löscher and Potschka, 2005).

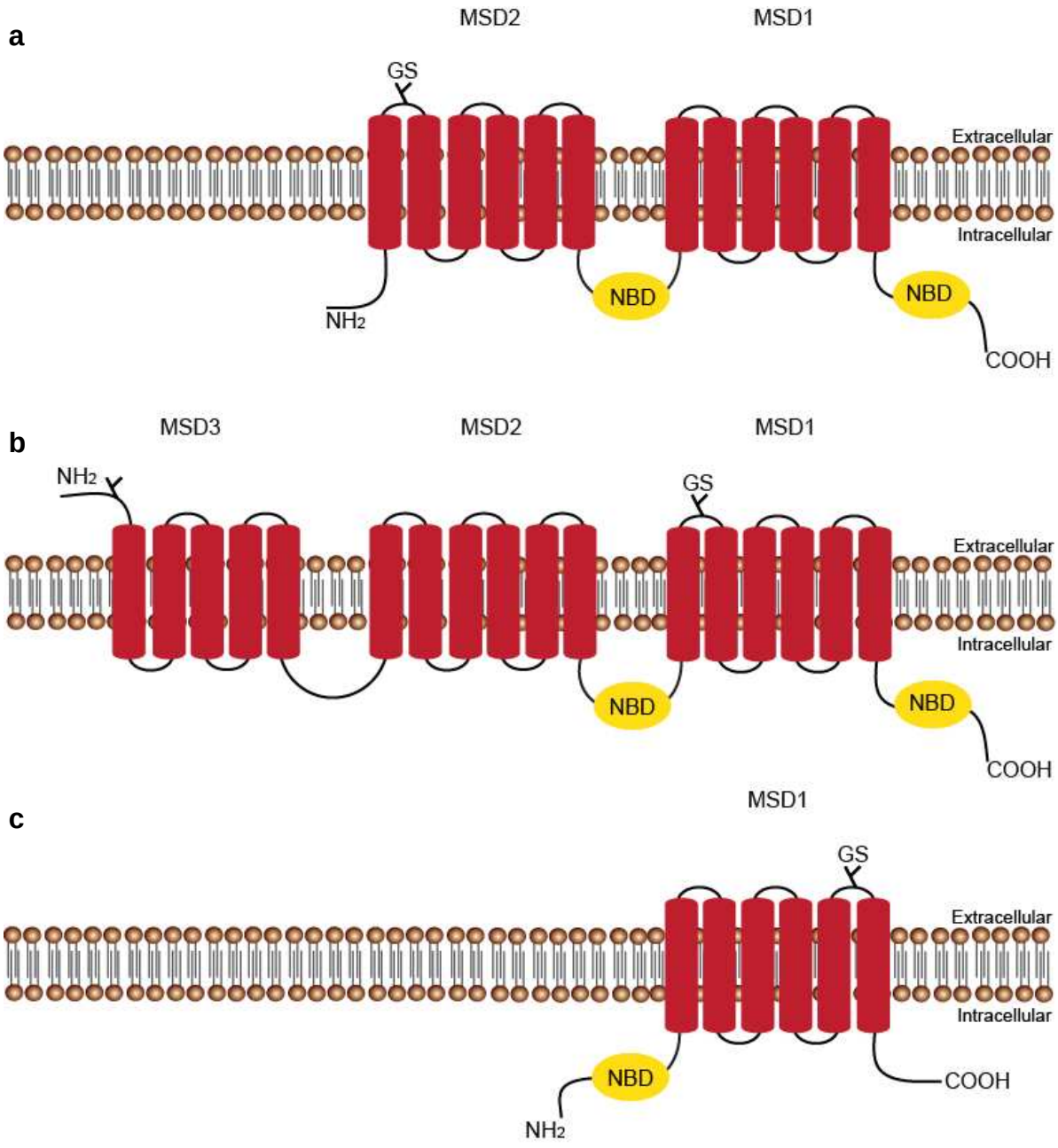

Figure 6. Structure models of ABC transporters: ABC transporters, generally, can be divided into three groups depending on their structure **a.** with two MSD and two NBD, **b.** with three MSD and two NBD, **c.** with one MSD and one NBD.

1.3.1.2 Function of ABC transporters

The substrate binding and transport by ABC transporters are still not totally understood, but it is accepted now that the ATP switch model is the mode of action that ABC transporters adopt (Higgins and Linton, 2004). In this model, binding of a substrate to its high affinity site on the TMD, induces a decrease in the activation of energy for the ATP dependent NBD dimerization. Moreover, binding of two ATP molecules at the dimer interface induces a conformational change in the TMD, resulting in the formation of a closed, sandwich like configuration, of NBD dimers that directly affect the substrate binding properties. These changes in the physical conformation of TMD and NBD decrease the affinity of the substrate to its binding site, inducing its release into the extracellular compartment. Afterwards, the bound ATP is hydrolyzed to ADP and P_i destabilizing and opening the closed NBD dimers, and the basal configuration of NBD and TMD is restored and ready for a new substrate binding and transport. Based on this ATP switch model, ABC transporters are active and energy dependent transporters where ATP binding and hydrolysis is crucial for proper transport mechanism, as ATP binding is necessary for drug transport and ATP hydrolysis is necessary for transporter restoration.

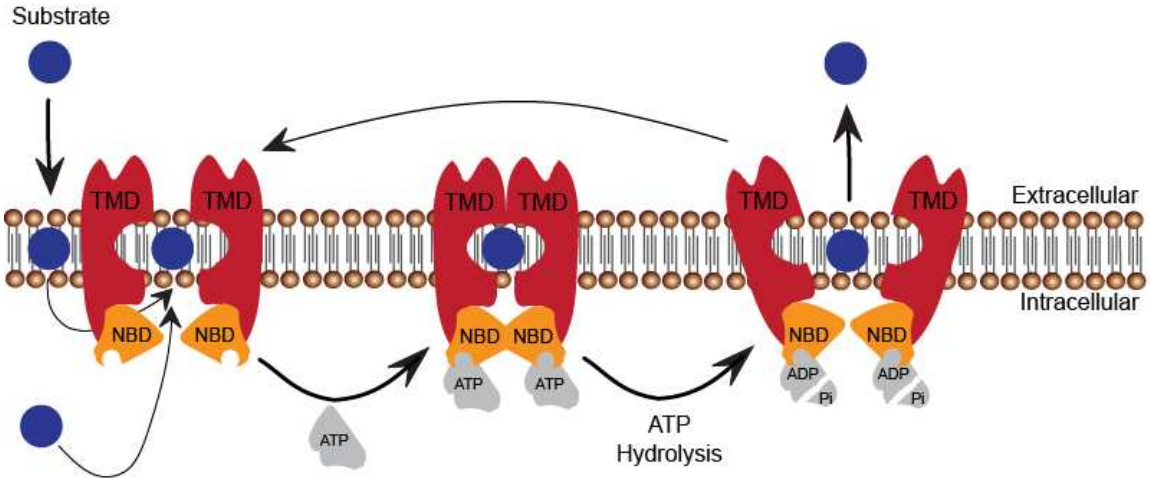

Figure 7. ABC transporter function: Representative scheme of ABC transporter function. ABC transporters are energy-dependent. They carry molecules (drugs) using energy generated by ATP hydrolysis.

1.3.2 Physiological role of ABC transporters

All known ABC transporters have overlapping substrate affinity for many lipophilic and amphipathic natural products, such as bacterial, herbal and fungal toxins, as well as carcinogens, therefore considered cell detoxification systems (Leslie et al., 2005). In some organs, like brain, testis and placenta, ABC transporters protect these organs actively from toxic compounds, whereas in other organs, like intestine and kidney, they transport biologically active compounds produced in these organs into the circulation.

The exact physiological role of Mdr-1 remains unclear, but it is known that it protects organs against toxic xenobiotics. Some studies, in parallel, found out that Mdr-1 functions also as flippase and is able to transport lipids, like phospholipids and simple glycoshingolipids from plasma membrane into the extracellular space (Orlowski et al., 2006; Sharom et al., 2005). Interestingly, other studies showed that Mdr-1 may contribute in the membrane associated hydrophobic amyloid-beta (Aβ) peptide clearance (Vogelgesang et al., 2004; Burk et al., 2005). Mdr-1 absence in mice did not reduce the viability of *mdr-1a*$^{-/-}$ mice, suggesting no essential role of Mdr-1 in cell survival.

None the less, *mdr-1a*$^{-/-}$ mice brain showed increased sensitivity to the neurotoxic compounds ivermectin, and to the cytostatic drug vinblastinme compared to wildtype mice (Schinkel et al., 1994), and elevated levels of many drugs in different organs (Schinkel et al., 1995; Schinkel et al., 1996; Smit et al., 1999). Like Mdr-1, Mrp-1's exact physiological role remains unclear, but based on observations that Mrp-1 has high affinity and transports leukotriene C_4 (LTC_4), it has been suggested that it could function as physiological transporter for LTC_4, because in *mrp-1*$^{-/-}$ mice LTC_4 release from eosinophils and mast cells in response to IgE mediated inflammation is reduced (Wijnholds et al., 1997). Mrp-1 has also been suggested to have a role in cell detoxification, as it has been shown that in *mrp-1*$^{-/-}$ mice the passage of Mrp-1 substrates from the blood to the cerebrospinal fluid increased (Wijnholds et al., 2000).

1.3.3 Brain distribution of ABC transporters

ABC transporters are expressed in many different cell types where they contribute in the transport of various substances (Glavinas et al., 2002), and control many aspects of cell microenvironment (Ye and al., 2008). In CNS, many ABC transporters are expressed, mainly drug transporters, e.g. Mdr-1, Mrp-1, BCRP (Borst and Elferink, 2002; Löscher et al., 2005), preventing toxic compounds uptake, and facilitating toxic compounds elimination. In the brain, ABC transporters are mostly expressed on endothelial cells (Demeule et al., 2001; Soontornmalai et al., 2006). The subcellular localization of ABC transporters is very important for their activity and function, for example, ABC transporters involved in multidrug resistance are predominately localized to the luminal membrane of cells (van Helvoort et al., 1996; Abele and Tampé, 1999), but the accurate subcellular localization of different ABC transporters expressed on endothelial cells of the BBB remains unclear clear. Moreover, CNS pathophysiological conditions play an important role in ABC transporters expression, function and subcellular localization. As such in some CNS diseases, the expression of ABC transporters was found in addition to endothelial cells, in astrocytes, microglia and neurons (Pradridge et al., 1997; Zhang et al., 1999; Lee at al., 2001; Seegers et al., 2002).

The existing data on ABC transporters expression and subcellular localization at the BBB showed that Mdr-1, Mrp-2, Mrp-4 and MXR (BCRP; ABCG2) are predominantly expressed and located at the luminal (i.e. apical) plasma membrane of endothelial cells forming the blood brain barrier, whereas Mrp-1, Mrp-3 and Mrp-5 are expressed and located at the abluminal (i.e. basolateral) plasma membrane of endothelial cells (Borst and Elferink, 2002). Other studies have shown luminal expression and localization of Mrp-1 and Mrp-5, and abluminal expression for Mrp-4 (Löscher et al., 2005). Moreover, detailed studies using high resolution immunofluorescence staining (Soontornmalai et al., 2006) showed that Mdr-1 and Mrp-5 are both expressed and localized at the luminal plasma membrane of endothelial cells, and Mrp-1 at the abluminal plasma, whereas Mrp-2 and Mdr-3 were not detectable.

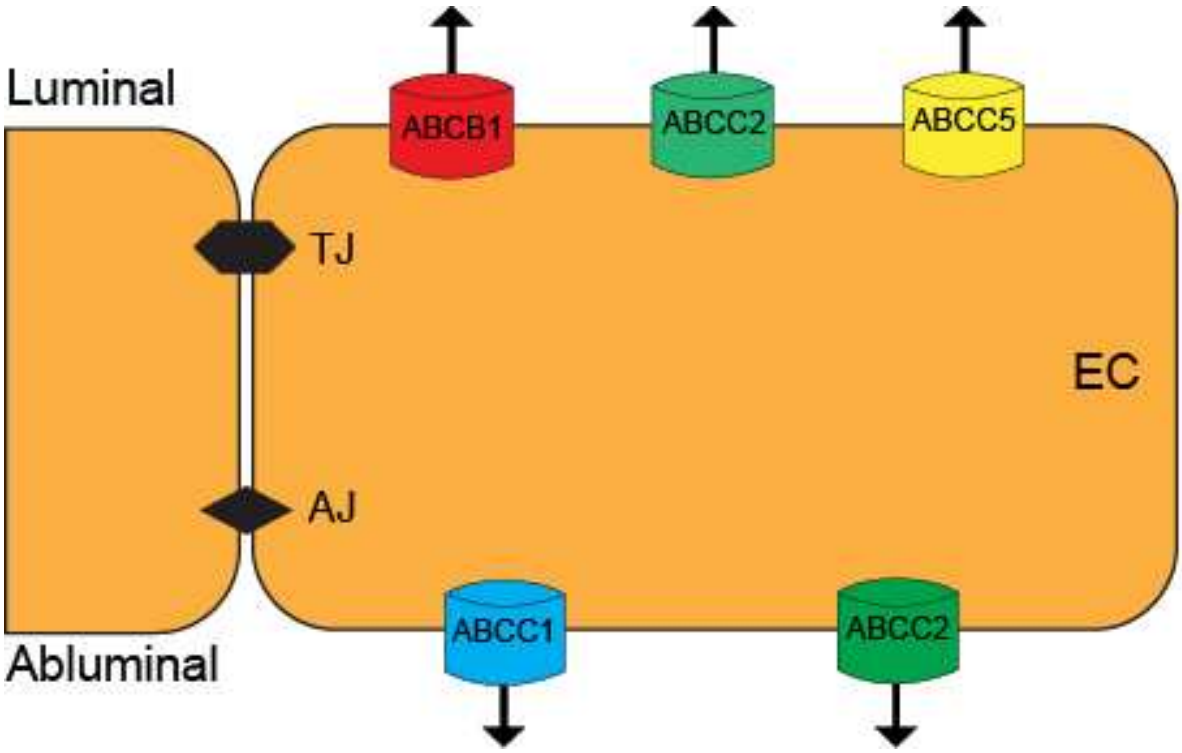

Figure 8. ABC transporter at the BBB: Many ABC transporters have been identified and shown to be expressed at the BBB. Their function is tightly dependent on the polarity of endothelial and their sub-cellular localization, **a.** some of them are expressed exclusively at the luminal side such as ABCB1, ABCC5 **b.** others exclusively at the abluminal side such as ABCC1 **c.** and some at both sides such as ABCC2.

1.3.4 ABC transporter regulation at the BBB

The regulation of ABC transporters at the BBB is very complex. The molecular mechanisms that control the physiological expression of ABC transporters are largely unknown. Under pathophysiological conditions, many unspecific signaling pathways have been shown to contribute to some level in ABC transporters regulation, such as the extracellular-signal-regulated kinases-1/2 (ERK-1/2), protein kinase C (PKC), c-Jun N-terminal kinase-1/2 (JNK-1/2), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), protein kinase B (PKB/Akt) and cyclooxygenase-2 (Cox-2) that upregulate ABCB1, and the tumor suppressor p53 that downregulates ABCC1 expression (Wang and Beck, 1998).

Interestingly, some studies showed that ABCB1 is specifically regulated by a member of the orphan nuclear receptors (ONR) superfamily, such as steroid and xenobiotic receptor (SXR) in humans and the pregnane X receptor (PXR) in rodents (Bauer et al., 2004; Löscher et al., 2005; Bauer et al., 2006). Moreover, it has been shown that PXR activation induces ABCB1 expression at the BBB, transporting amyloid-beta (Aβ) peptide and reducing the number of Aβ plaques in Alzheimer's disease (Vogelgesang et al., 2004; Burk et al., 2005).

Recently, another member of the ONR superfamily, the liver X receptor (LXR) has caught attention. LXRs act as cholesterol modulators that upregulate the expression of ABC transporters involved in lipid transport such as ABCA1, ABCG1 and ABCG8 (Morales et al., 2008). LXRs are members of the ONR family acting as transcription factors. They exist in two isoforms NR1H3 (LXRα) and NR1H2 (LXRβ), which are activated by their endogenous ligands, oxysterols (Schroepfer, 2000). Initially, LXR have been described as sterol sensors protecting cells from cholesterol overload by regulating genes involved in reverse cholesterol transport (Naik et al., 2006).

The activation of LXR by synthetic agonists has been shown to provide protection against glucose intolerance, diabetes and atherosclerosis (Efanov et al., 2004; Bradley et al., 2007), to provide anti-inflammatory effects under conditions of atherosclerosis and ischemic stroke (Bradley et al., 2007; Morales et al., 2008), and to regulate the expression of VEGF in macrophages (Walczak et al., 2004)

All these studies suggested a potent and profound effect of LXR activation on the vascular system by controlling molecular mechanisms in endothelial cells and regulating endothelial cell gene expression (Joseph et al., 2002). This observation was supported by recent results showing that LXRs are also involved in regulating the expression and activity of some drug metabolizing enzymes, therefore suggesting a broader function of LXR signaling beyond being sterol sensors (Wada et al., 2008).

1.4 BBB in ischemic stroke

Ischemic stroke is caused mainly by the occlusion of a blood vessel, due to arterial embolism or thrombosis, in a specific region of the brain disturbing blood supply, initiating the ischemic cascade which leads to neuronal death and rapid loss of brain functions (Dirnagl et al. 1999). Ischemic stroke represents approximately 85% of strokes. Stroke is the main cause of adult disability in the United States and Europe (Feigin, 2005), and It is now the second leading cause of death in the world after heart disease (Donnan et al., 2008).

Recently, new report released by the American Stroke Association (ASA) indicated that stroke would become the first leading cause of death in the world by 2030 (http://www.theheart.org/article/749039.do). In this report, statistics performed by World Health Organization (WHO) showed that out of 58 million worldwide deaths in 2005, 7.6 million were caused by heart diseases; 5.7 million were caused by cerebrovascular disease and 7.6 million caused by all cancers. Moreover, this report highlighted striking statistics that the number of deaths caused by all cancers will fall in the West, due to the progress in cancers treatments; on the other hand, deaths caused by stroke will continue to rise worldwide, mainly due to the lack of efficient treatments.

As mentioned above, the occlusion of the brain blood vessel initiates the ischemic cascade, leading to the activation of many signaling pathways that compromise cell survival and function, such as glutamate mediated excitotoxicity, Ca^{2+} overload, oxidative stress and BBB dysfunction, inflammation and cell death (Mehta et al., 2007).

The dysfunction of the BBB represents, by itself, an important factor in worsening ischemic stroke insult. As such, at the site of the infraction, ischemic stroke challenges BBB function mainly by the creation of an oxidative stress environment that generates free radicals and reactive oxygen species (ROS) production, which lead to endothelial cells apoptosis and necrosis (Toda et al., 2009), activation of set of molecular events that compromise endothelial cell function (Cosentino et al., 2001).

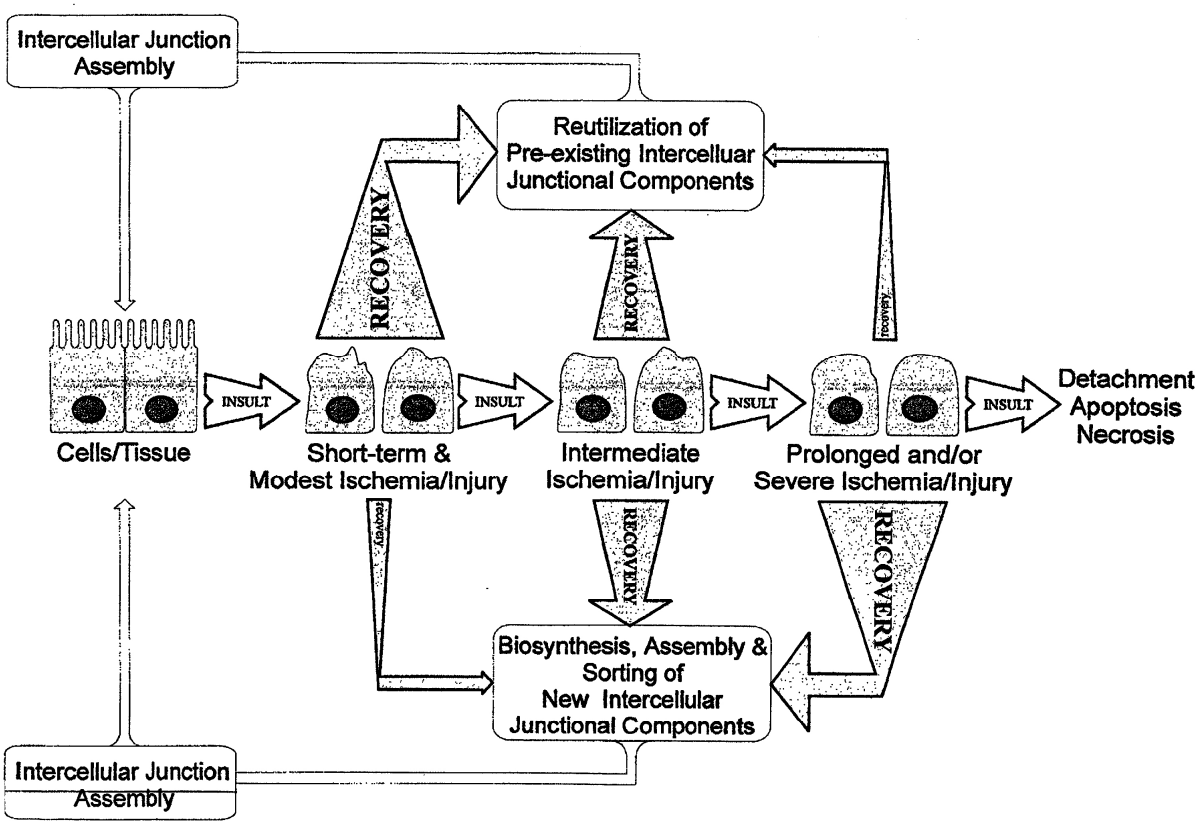

Figure 9. BBB pathophysiology in ischemic stroke: The response of endothelial cells forming the blood-brain barrier to ischemic stroke is dependent on injury severity.

The stress signaling pathways initiated by ischemic stroke challenges the BBB integrity by disturbing the expression of TJ proteins such as occludin and ZO-1 (Huber et al., 2001), in altering the phosphorylation of TJ proteins and negatively modulating TJ proteins assembly and interaction (Takenaga et al., 2009), thus decreasing endothelial cell-cell contacts, increasing endothelial cell detachment at the BBB, therefore increasing endothelial wall permeability. At the acute phase of ischemic stroke, the high influx of Ca^{2+} into endothelial cells activates many signaling pathways and molecular events (Mehta et al., 2007) especially calpain proteases (Cao et al., 2007) and matrix metalloproteinases (MMP) (Zhao et al., 2004; Zhao et al., 2006; Gu et al., 2002), which are widely regarded as hallmarks of the breakdown of the BBB.

Calpains are calcium-dependent non-lysosomal cysteine proteases that are ubiquitously expressed. Calpains consist of two isoforms, mu-calpain and m-calpain (calpain-1 and -2, respectively), both representing heterodimers of a common 28-kDa subunit with two different 80-kDa subunits (Goll et al., 2003). Calpain-1/2 activity is tightly controlled by calpastatin, its endogenous specific inhibitor. Calpastatin expression has been shown to decrease after stroke, increasing calpain-1/2 activity (Sedarous et al., 2003).

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are zinc-dependent endopeptidases, promoting extracellular matrix degradation (Park et al., 2009). MMP-2/9 are activated upon cerebral hypoxia and ischemia, increasing brain edema and exacerbating neuronal injury (Park et al., 2009; Zhao et al., 2004). MMP-9 activity is negatively regulated by calpain inhibitors (Tsubokawa et al., 2006), suggesting that calpain mediates matrix protease activation. While the molecular actions of calpains and MMPs are comparably well understood, not much is known about whether and how these proteases mediate BBB permeability.

a **b**

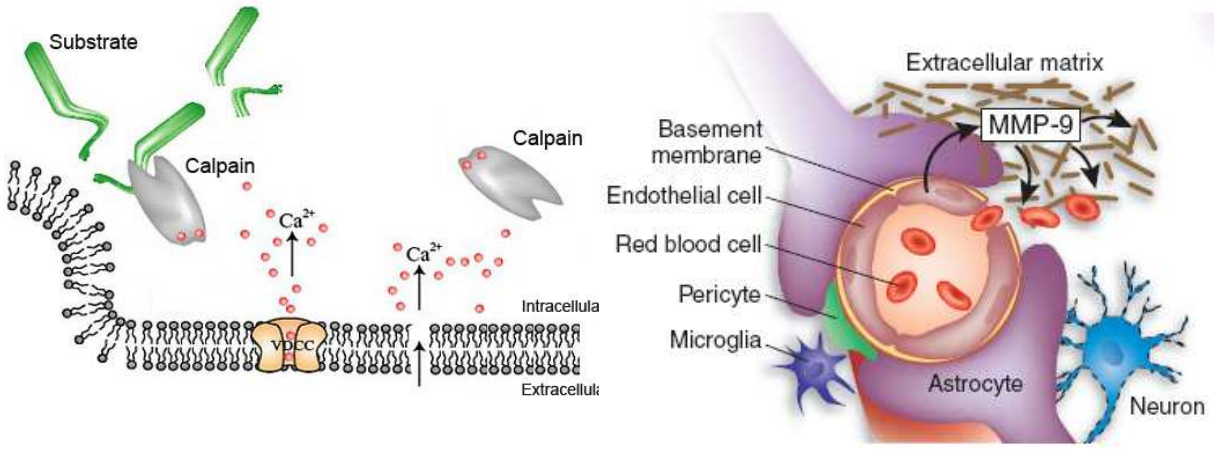

Figure 10. Calpain and MMP-9 contribution in the pathophysiology of the BBB: Calpains are cysteine protease activity that requires Ca^{2+} **a.** ischemic stroke elevates Ca^{2+} activating calpians **b.** at the acute phase of ischemic injury, MMP-9 activation contributes in destabilizing the extracellular matrix contributing in the breakdown of the BBB. (Source: Okayama University, Department of Physiology; Zolkovic, 2006).

At the molecular level, many signaling pathways are activated at the acute phase of stroke contributing to BBB damage. As such, it has been suggested that Rho GTPases induce at least part of BBB permeability changes upon stroke (Schreibelt et al., 2007; Chrissobolis and Sobey, 2006).

Rho GTPases are small G-proteins transducing signals by binding and activating downstream effectors (Etienne-Manneville and Hall, 2002). The most studied are RhoA, Rac1 and Cdc42. In endothelial cells, RhoA activation induces stress fiber formation, thus destabilizing TJ and increasing BBB leakage (Schreibelt et al., 2007; Hirase et al., 2001). Cdc42 activation controls cell polarity and promotes BBB integrity by enhancing cell-cell contact (Ramchandran et al., 2008; Cau and Hall, 2005; Fukuhara et al., 2003).

All these factors contribute directly and indirectly in BBB damage, compromising BBB function, thus abolishing the protective role of the BBB in controlling the microenvironment at the site of infraction. This increased permeability and loss of function of the BBB causes the secondary progression of brain injury by increasing cerebral edema, promoting the recruitment and migration of macrophages and neutrophils into the infracted region, and increasing the inflammatory responses (Charo and Ransohoff, 2006).

Besides these molecular events that affect BBB tightness, ischemic stroke induces the regulation of the ABC transporters expression at the BBB. As such, the ischemic cascade induces the activation of many signaling pathways that have been shown to contribute in the regulation of ABCB1 and ABCC1 expressions. We have previously shown that ABC transporters exhibit profound expression changes on the luminal and abluminal endothelial membrane of ischemic cerebral microvessels, which impede drug accumulation in the stroke brain. As such, the luminal transporter ABCB1 (previously: multidrug resistance transporter-1), which extrudes pharmacological compounds from the brain into blood, is upregulated (Spudich et al., 2006), whereas the abluminal transporter ABCC1 (previously multidrug resistance-associated protein-1), which carries drugs in the opposite direction from the blood to brain, is downregulated in response to stroke (Kilic et al., 2008).

Deactivation of ABCB1 by pharmacological inhibition or genetic deletion increased concentrations of neuroprotective compounds more strongly in ischemic than non-ischemic brain tissue by up to an order of magnitude (Spudich et al., 2006), whereas ABCC1 blockade decreased tissue levels by as much as hundred-fold (Kilic et al., 2008). The alterations in pharmacodistribution were associated with the gain or loss of survival-promoting drug activities (Spudich et al., 2006; Kilic et al., 2008), demonstrating that changes in ABC transporter expression influence drug efficacy in a clinically relevant way. The mechanisms responsible in the observed upregulation of ABCB1 (Spudich et al., 2006), and the downregulation of ABCC1 (Kilic et al., 2008) at the blood-brain barrier of ischemic brains and their respective implication in neuroprotective drug entry and accumulation in the brain, are still not well understood.

Figure 11. Brain drug accumulation upon ischemic stroke: Upon stroke, ABCB1 (i.e. Mdr-1) and ABCC1 (i.e. Mrp-1) balance seems to be highly important in controlling drug entry and accumulation into ischemic brain. Post-ischemic manipulation of this balance would contribute in enhancing blood-to-brain drug transport.

1.5 BBB in hypercholesterolemia

It is well established that high cholesterol level in blood is a major risk factor for stroke, as the vessels show exacerbated atherosclerosis (Suk et al., 2003). Recently, some studies have shown also a direct correlation between increased risk of stroke and obesity revealed by body mass index (BMI). Stroke therapies have made little progress in recent years. Several pharmacological compounds stabilizing BBB integrity or promoting neuronal survival in animal models of ischemic stroke were tested in stroke patients (O'Collins et al., 2006; Stroke Therapy Academic Industry Roundtable, 1999). Apart from thrombolytics, none of these compounds turned out to be effective in humans (O'Collins et al., 2006; Stroke Therapy Academic Industry Roundtable, 1999).

A major limitation of animal stroke studies is that mostly young animals without risk factors are evaluated (O'Collins et al., 2006, Hossmann, 2009). Such studies hardly mimic the pathophysiology of ischemic stroke in humans, which often affects elderly subjects with unfavourable life habits or cerebrovascular risk factors (O'Collins et al., 2006, Hossmann, 2009). Beside being risk factors for stroke, high levels of blood lipids contribute directly to ischemic injury, therefore worsening ischemic stroke outcome, by increasing lipids peroxidation catalyzed by reactive oxygen species (ROS) production, enhancing the oxidative stress and endothelial cells apoptosis (Takabe et al., 2010). Moreover, it has been shown that high cholesterol levels in blood promote cerebral microvessel extravasation of apo B lipoprotein-Aβ (Takechi et al., 2008), and that some lipid lowering agents like atorvastatin and pravastatin ameliorate the exaggerated BBB dysfunction caused by saturated fats and cholesterol (Cheng et al., 2009; Kalayci et al., 2005).

The exposure of endothelial cells to low-density lipoprotein (LDL) and oxidized LDL (OxLDL) causes endothelial dysfunction by activating the lectin-like oxidized LDL receptor-1 (LOX-1), increasing endothelin and angiotensin II production and secretion (Chen et al., 2002; Galle et al., 2000), increasing the activation of MMPs leading to occludin degradation (Casas et al., 2010), enhancing endothelial nitric-oxide synthase (eNOS) removal and preventing the production of nitric oxide (NO) (Kincer et al., 2002). Moreover, hypercholesterolemia increases the level of oxLDL in serum, therefore worsening the ischemic injury (Uno et al., 2005).

Although many ABC transporters are lipid transporters; the regulation of ABC transporters at the blood brain barrier by high cholesterol levels remains unclear. For example, some studies showed that when cholesterol levels became high, Mdr-1 expression increases and plays a role in transporting the excess of cholesterol that can be toxic for endothelial cells from the phospholipid bilayer into the endoplasmic reticulum (ER) to be esterified by the acyltransferase enzyme (ACAT) to cholesteryl esters, that are less toxic, and therefore enhancing endothelial cells survival (Luker et al., 1999).

1.6 BBB in brain tumors

Brain tumors are characterized by exagerated vascular leakage (Ewing et al., 2006), causing edema mainly due to TJ disruption and to the excessive angiogenesis (Davies, 2002). Moreover, brain tumors are very resistant to chemotherapies (Szakacs et al., 2006). Interestingly, both the tumor cells and the vasculature express ABC transporters at high level (Kemper et al., 2004; Regina et al., 2001).

The chemoresistance in cancers is complex due to the fact that this drug resistance can be intrinsic or acquired depending on the causes of ABC transporters expression, as cancer cells could constitutively express ABC transporters, and pharmacotherapies can induce ABC transporters expression. As mentioned above, most chemotherapeutics are lipophilic molecules that show a very high affinity for ABCB1 (Gottesmann et al., 2002). It has been shown using specific inhibitors for ABCB1 or ABCB1 ablation in *mdr-1a/b*$^{-/-}$ mice increase brain drug entry and accumulation increased in both healthy and tumors tissues (Kemper et al., 2003).

These strategies were efficacious in increasing the anti cancer drug paclitaxel entry and accumulation, which led to a significant decrease in tumor volumes (Fellner et al., 2002). Therefore, ABCB1 expression and activity regulation strategy is regarded as promising in enhancing brain tumor chemotherapies.

1.7 BBB in epilepsy

It has been shown that ~ 30% of epilepsy patients continue to get seizures and that despite optimized drug treatment (Löscher and Potschka, 2005). Interestingly, in epileptic patients exhibiting pharmacoresistance, elevated levels of Mdr-1 and Mrp-1 expression have been observed in brain capillaries as well as astroglial endfeet around epileptogenic focuses in medically intractable epilepsy (Tishler et al., 1995; Sisodiya et al., 2001; Sisodiya et al., 2002). These observations support the hypothesis of a possible role of ABC transporters in drug resistance. This hypothesis is reinforced by the fact that many widely used anti-epilptics, which are in majority lipophilic to enbale brain entry, are substrates of Mdr-1 (Löscher and Potschka, 2005). Unfortunately, brain levels of these anti-epileptics were not much increased after Mdr-1 inhibition in experimental studies and only less than twofold concentration increases are observed (Potschka et al., 2001; Rizzi et al., 2002).

The major problem with respect to drug resistance experiments in epilepsy is the lack of appropriate control conditions from areas located outside the epileptogenic focus and from epileptic patients that are responsive to anti-epileptics. Controlled experimental studies in kainic acid-induced limbic seizures have revealed a transient increase in mdr-1 mRNA expression levels in the epileptic mouse hippocampus (Rizzi et al., 2002), indicating that epileptic seizures themselves may induce Mdr-1 expression. The upregulation of Mdr-1 in mice exhibiting limbic seizures has previously been shown to have decreased levels of anti-epileptic drugs, such as phenytoin (Rizzi et al., 2002). In contrast the inhibition of Mdr-1 with verapamil has not only elevated anti-convulsant levels in rat brains with limbic seizures, but also enhanced the response of the seizures to oxcarbacepine (Clinckers et al., 2005). All these data indicate that Mdr-1 may be causatively involved in drug resistance. In view of the moderate effects of Mdr-1 inhibitors of brain drug biodistribution, some skepticism remains whether Mdr-1 is indeed the major and predominant factor responsible of drug resistance in epilepsy. Understanding the molecular mechanisms involved in Mdr-1 and Mrp-1 expression in brain capillaries could be very valuable in this context.

1.8 BBB in multiple sclerosis

Multiple sclerosis (MS) is a complex inflammatory disease associated with chronic inflammation of the CNS due to the autoimmune attacks directed against components of the myelin sheath, which leads to demyelination, axonal loss, and brain atrophy. MS lesions are characterized by the presence of mononuclear inflammatory infiltrates and reactive gliosis (Brosnan and Raine, 1996). It is now widely accepted that the BBB breakdown is the main key and the initial step in the development of inflammatory lesions (Minagar and Alexander, 2003). Although the mechanisms involved in BBB breakdown and dysfunction remain unknown, it is supposed that this loss of function is associated with T cells and macrophages activation that secrete pro-inflammatory cytokines interleukin 1, 12 (IL-1, -12), tumor necrosis factor-α (TNF- α), and interferon- γ (IFN-γ), reactive oxygen and reactive nitrogen species (RNS) (Brosnan and Raine, 1996). These molecular events are involved in MS pathogenesis and contribute in the development of the disease. Moreover, the T cells activation alters TJ at the BBB increasing its permeability and allowing the infiltration of these cells into brain (Suidan et al., 2008).

Mitoxantrone (MX) is a type II topoisomeras inhibitor that acts as an antineoplastic agent, and it used in many cancers treatment. MX is also used in MS treatment, mainly by decreasing the secondary progressive MS lesion and it contributes in extending the time between relapses in relapsing-remitting MS and progressive relapsing MS (Fox, 2006). The therapy of active MS with MX is limited because of its dose-dependent side effects.

Moreover, MX is a substrate for the efflux carriers ABCB1 and ABCG2 that are expressed at the BBB (Spudich et al., 2006; Zhang et al., 2003), and it has been shown that there is a correlation between the single nucleotide polymorphisms (SNPs) in these two transporters and MX dose response, and therefore it was proposed that MX dosing could be assessed individually by using the (SNPs) of ABC transporters as a pharmacogenetic markers associated with clinical response to MX therapy in MS (Cotte et al., 2009).

1.9 BBB in Parkinson’s disease

Parkinson's disease (PD) is a degenerative disorder of the CNS that impairs motor skills, behavior and speech (Rodriguez-Oroz et al., 2009). The BBB dysfunction in PD remains unclear. A study, using PD rat model, found out that BBB was disrupted in the basal ganglia of these animals (Westin et al., 2006), whereas in other study using primates no disruption of the BBB was observed (Astradsson et al., 2009). Although BBB dysfunction is not causative in PD progression, a interesting study showed that at an advanced stage of PD, ABCB1 expression was decreased and was associated with severe BBB disruption (Bartels, et al 2008a), this study suggested a possible role of PD in reducing ABCB1 expression (Bartels, et al. 2008b), increasing toxins accumulation in PD brain and contributing in PD progression.

2 AIM OF THE STUDY

2.1 Part I: Effect of LXR activation on BBB tightness

Experimental stroke research has focused mainly on basic cellular mechanisms that cause irreversible neuron damage and on strategies that make neurons less vulnerable and more resistant to stroke. Such strategies were not successful. Recently, many reports have shown that cerebrovascular system represents an important target in stroke research and treatment. Therefore, understanding the molecular mechanisms involved in BBB dysfunction and preserving BBB function upon stroke represent new promising strategy for stroke treatment.

The molecular mechanisms involved in BBB dysfunction upon ischemic stroke are diverse. Ischemic stroke downregulates TJ proteins (Hubert et al., 2001), decreases BBB tightness, increases the activity of proteases (Cao et al., 2007; Zhao et al., 2004; Gu et al., 2002), increases endothelial membrane and basal lamina degradation (Lo et al., 2003), increases endothelial apoptosis (Lo et al., 2003) and deregulates endothelial active transports (Spudich et al., 2006; Kilic et al., 2008), leading to endothelial cell wall permeability and edema formation (Charo and Ransohoff, 2006). Although these molecular pathophysiological mechanisms are known to be activated upon stroke contributing in BBB damage, the upstream signaling pathways that control and regulate these pathophysiological mechanisms are not well characterized.

In this study we used C57Bl6/j submitted to 30 min focal cerebral ischemia to dissect the key molecular mechanisms involved in BBB dysfunction. In view of the profound effect of nuclear receptors on ABC transporter expression (see chapter 1.3.4), we were interested in the effect of LXR activation on BBB tightness and polarity. As such, we examined how the LXR agonist T0901317 influences brain edema, BBB permeability and ABC transporter expression in ischemic brain microvessels. In these studies we wanted to understand whether BBB tightness and polarity share common mechanisms of regulation or whether both features of the BBB are differentially regulated.

2.2 Part II: Effect of apolipoprotein-E (ApoE) on BBB polarity

A large number of neuronal survival promoting molecules known from different *in vitro* and *in vivo* studies were not successful in clinical trials (Danton and Dietrich, 2004; Hoyte et al., 2004). Drug access to the diseased brain tissue is a major hampering factor for CNS – targeting therapies. The upregulation of ABC transporters at the luminal endothelial membrane is thought to contribute to neuroprotection failure by preventing drugs from reaching therapeutic tissue level. Indeed, we have previously shown that ABCB1 inhibition strategies (Spudich et al., 2006) improve neuroprotective drug accumulation in the ischemic brain upon stroke enhancing stroke outcome. In addition, we have previously shown that ABCC1 acts as gateway for drug accumulation in the brain (Kilic et al., 2008) is downregulated at the abluminal endothelial membrane upon stroke. These results showed the importance of BBB polarity in drug transport, as ischemic stroke specifically upregulates ABCB1 at the luminal side and downregulates ABCC1 at the abluminal side impeding drug accumulation in the brain.

The molecular mechanisms underlying the polarity changes of ABC transporters at the BBB upon ischemic stroke are unknown. Understanding these mechanisms might offer insights into the functioning of the BBB, and it may allow identifying targets via which drug access to the ischemic brain may be improved. In the preparation studies for this thesis using apolipoprotein-E knockout $(ApoE)^{-/-}$ mice, which originally were studied in order to characterize expression changes of ABC transporters in the atherosclerotic brain, we observed that adolescent $ApoE^{-/-}$ mice that were held on regular diet and therefore did not exhibit atherosclerosis, revealed a fundamentally altered expression pattern of ABCB1 and ABCC1 upon stroke, ABCB1 being downregulated whereas ABCC1 being upregulated in response to stroke.

We therefore characterized in a more systematic way ApoE's role in the regulation of ABC transporters, demonstrating that ApoE itself controls the polarity of ABC transporter expression.

2.3 Part III: Effect of hypercholesterolemia on the ischemic BBB

Hypercholesterolemia induced by excess or high-calory food is a highly prevalent condition in ischemic stroke patients. In large clinical trials or patient registries, ~45-60% of patients exhibit elevated serum cholesterol levels (Sacco et al., 2008; Röther et al., 2008). Cholesterol levels above 7,0 mmol/l are associated with an elevated stroke risk (Leppälä et al., 1999), which in clinics may successfully be reduced, by cholesterol-lowering drugs (Amarenco et al., 2006). In view of the huge importance of hypercholesterolemia for stroke pathology; it is surprising that not much is known from experimental studies about how dietary influences affect acute stroke development. Moreover, in caucasians, prior use of statins has recently been shown to be associated with enhanced stroke outcome (Leppälä et al., 1999). In that study, the beneficial effects were explained by pleiotropic effects of statins, as such leaving the question open as whether elevated cholesterol levels influence cerebrovascular injury.

Moreover, in mice exhibiting enhanced atherosclerosis induced by apolipoprotein-E (ApoE)$^{-/-}$, enhanced BBB permeability has previously been reported upon cortex trauma and ageing when animals were placed on a cholesterol-rich diet (Methia et al., 2001; Hafezi-Moghadam et al., 2007). In these studies the authors did not observe BBB breakdown in wildtype animals undergoing cortex trauma (Methia et al., 2001), suggesting that the ApoE$^{-/-}$ was responsible for the dietary effects. The question whether and how hypercholesterolemia influences BBB integrity after stroke was so far unknown.

In view of the lack of evidence, we examined the effect of a cholesterol-rich (so-called Western) diet on brain edema, BBB tightness and polarity and investigated the molecular mechanisms involved, using C57Bl6/j and ApoE$^{-/-}$ mice submitted to 30 min MCA occlusion. With these experiments, we wanted to understand, whether unfavorable dietary habits, which are highly prevalent in stroke patients, affect BBB functioning after stroke.

3 MATERIALS AND METHODS

3.1 Animal groups and pharmacological experiments

Animal experiments were done according to the NIH guidelines for the care and use of laboratory animals.

For part I: Six weeks old C57BL6/j mice (20-25 g) were divided into two groups that were intraperitoneally treated one week prior to stroke with (a) 5% ethanol in 0.1 M phosphate-buffered saline (PBS; vehicle) and (b) the LXR agonist T0901317 (1.25 mg/ animal; 270-309-M050; Alexis Biochemicals, San Diego, CA, USA) dissolved in 5% ethanol in 0.1 M PBS (n = 8 animals each).

For part II: In a first set of studies, Six weeks old C57BL6/j wildtype and ApoE$^{-/-}$ mice (20-25 g) were subjected to 30 min MCA occlusion followed by 24 hours reperfusion. These animals were used for a detailed analysis of cell signaling changes of ischemic cerebral microvessels by Western blotting, immunoprecipitation and immunohistochemical analysis (n = 8 animals each) and qRT-PCR (n = 4 animals each). To evaluate the relevance of the signal changes for ABC transporter expression, a second set of ApoE$^{-/-}$ mice were submitted to 30 min MCA occlusion. These animals were treated with (a) 500 µl of normal saline containing 20% dimethylsulfoxide (DMSO) (vehicle), (b) recombinant human ApoEε3 (50 µg; Invitrogen, Karlsruhe, Germany) diluted in 500 µl normal saline/ 20% DMSO, or (c) the JNK-1/2 pathway inhibitor SP600125 (1.25 mg) (Calbiochem, Bad Soden, Germany) diluted in 500 µl normal saline/ 20% DMSO (n = 4 animals each), which were intraperitoneally administered twenty-two hours after reperfusion, i.e., two hours before animal sacrifice. And to study the functionality of the ABCB1 transporter, a third set of C57Bl6/j and ApoE$^{-/-}$ mice underwent 30 min of MCA occlusion. Nineteen hours after reperfusion, animals were treated intraperitoneally with the ABCB1 substrate FK506 (3 mg kg^{-1}, diluted in 150 µl 0.9% NaCl / 10% ethanol / 1.6% Tween 80; obtained from Fujisawa, Munich, Germany). Three hours later, one set of ApoE$^{-/-}$ animals (n = 5 animals) received intraperitoneal injections of recombinant human ApoEε3 (50 µg, diluted in 150 µl 0.9% NaCl; Invitrogen, Karlsruhe, Germany), all

other animals being treated with vehicle (150 µl 0.9% NaCl) (n = 5 animals / group). Animals were sacrificed three hours after this injection.

For part III: Six weeks old C57BL6/j (20-25 g) were divided into two groups: a) mice fed with normal diet chow (Normal Diet), b) mice fed western diet (TD.88137) chow (Western Diet) for 6 weeks as hypercholesterolemia model. Mice were subjected to 30 min MCA occlusion followed by 24 hours reperfusion (n = 8 animals each). These animals were used for a detailed analysis of cell signaling changes of ischemic cerebral microvessels.

3.2 Induction of focal cerebral ischemia

For the induction of focal cerebral ischemia, animals were anesthetized with 1% isoflurane (30% O_2, remainder N_2O). Rectal temperature was maintained between 36.5 and 37.0℃ using a feedback-controlled heating system. Laser Doppler flow (LDF) was monitored using a flexible 0.5 mm fiberoptic probe (Perimed, Stockholm, Sweden) attached to the intact skull overlying the MCA territory (2 mm posterior / 6 mm lateral from bregma). LDF changes were measured during 30 min of MCA occlusion and up to 15 min after reperfusion onset. Focal cerebral ischemia was induced using an intraluminal filament technique (Figure 12).

Briefly, a midline neck incision was made, and the left common and external carotid arteries were isolated and ligated. A microvascular clip (FE691, Aesculap, Tuttlingen, Germany) was temporarily placed on the internal carotid artery. A 8-0 nylon monofilament (Ethilon; Ethicon, Norderstedt, Germany) coated with silicon resin (Xantopren, Bayer Dental, Osaka, Japan; diameter of the coated suture: 180-200 µm) was introduced through a small incision into the common carotid artery and advanced 9 mm distal to the carotid bifurcation for MCA occlusion. Thirty minutes after MCA occlusion, reperfusion was initiated by withdrawal of the monofilament. After the surgery, wounds were carefully sutured, anesthesia discontinued and animals allowed to recover.

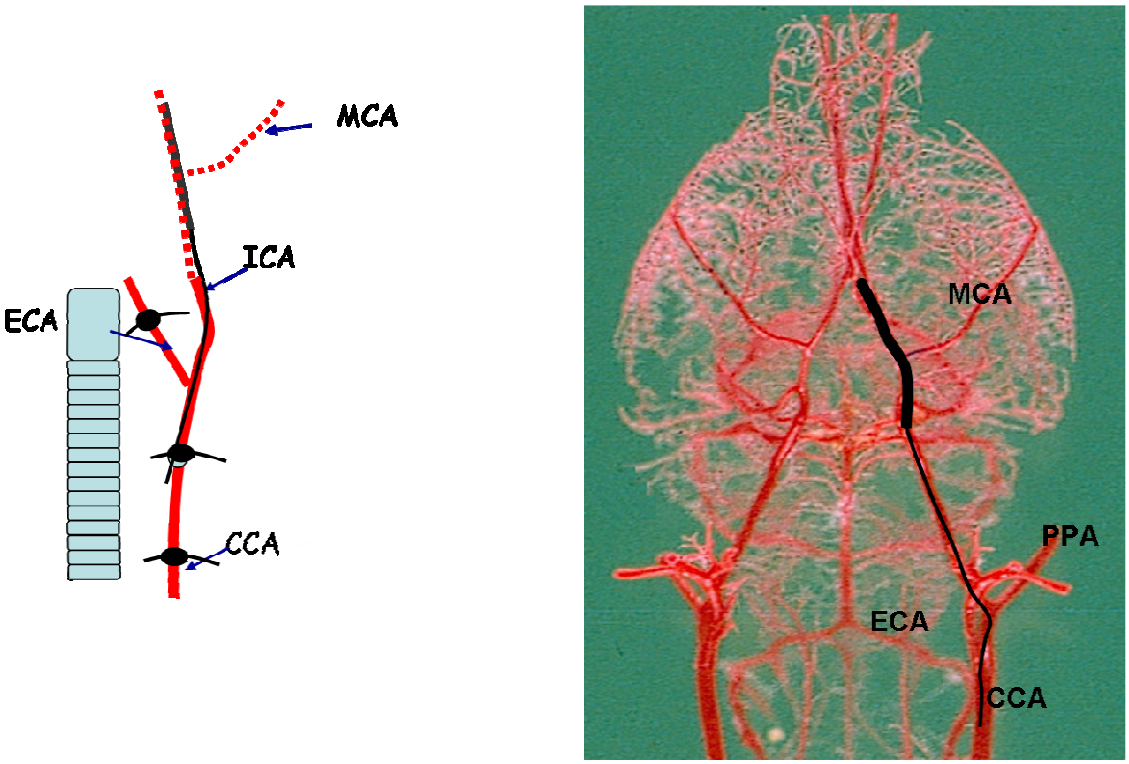

Figure 12. Middle cerebral artery (MCA) occlusion model: Representative scheme where the nylon microfilament is introduced via the common carotid artery (CCA) up to the middle cerebral artery (MCA), obstructing blood circulation.

Twenty-four hours after reperfusion, animals were reanaesthetized, and sacrified by transcardiac perfusion with 20 ml heparinised normal saline solution followed by 20 ml of normal saline. For drug biodistribution studies animals were first anaesthetized and blood was collected from heart in EDTA-coated tube and kept in -20°C. Animal were then sacrified by transcardiac perfusion with 10 ml heparinised normal saline solution followed by 10 ml of normal salinemice.

Brains were removed and cut on a cryostat into 20 µm thick coronal sections that were retrieved at the rostrocaudal level of the bregma, which represents the level of the midstriatum, at which MCA infarcts are most reproducible. In addition, tissue samples were collected from the ischemic and contralateral non-ischemic MCA territory (striatum and overlying parietal cortex) for Western blotting, immunoprecipitation, pull down, co-precipitation experiments and proteases activity assays.

3.3 Infarct and edema size evaluation studies

To evaluate infarct and edema size, representative brain sections were stained with 0.5% cresyl violet. On these sections, the border between injured and non-injured, healthy tissue was outlined with image analysis software (Image j; NIH, Bethesda, MD) and the lesion area was calculated by subtracting the area of non-lesioned tissue on both sides of the brain, and brain swelling area by subtracting the area of ipsilateral hemisphere from the contralateral hemisphere.

3.4 IgG extravasation studies

Upon ischemic stroke, BBB damage and opening lead to the extravasation of serum constituents as serum proteins and serum IgG into brain. IgG entry and extravasation is correlated with the severity of BBB damage. Serum IgG immunohistochemisrty and quantification assess the degree of blood-brain barrier damage.

For this purpose, the 20 µm thick cryostat brain sections obtained from the midstriatum level were used. Sections were rinsed slightly and gently for 5 min at room temperature in 0.1 M PBS to not remove endogenous intravascular IgG, and fixed for 15 min at 4 ℃ with 4% paraformaldehyde / 0.1 M PBS (Kilic et al., 2006). Due to the presence of endogenous peroxidase, brain sections were first treated with methanol/ 0.3% H_2O_2 to block the endogenous peroxidase. Afterwards, treated brain sections were immersed in 0.1 M PBS containing 5% bovine serum albumin and normal goat serum (1:1000), sections were incubated for 1 h in biotinylated goat anti-mouse IgG (sc-2039; Santa Cruz Biotechnology, Nunningen, Switzerland) and stained with an avidin peroxidase kit (Vectastain Elite; Vector Labs., Burlingname, CA) and diaminobenzidine (DAB; Sigma, Deisenhofen, Germany).

Finally brain sections were immersed in alcohol bath and finally in xylen, and after coverslipping, sections were scanned and evaluated by analyzing the IgG extravasation in brain.

3.5 Microvessel isolation and protein extraction

Brain homogenates are not appropriate for studying protein expression and interaction at the BBB, because many proteins are exclusively expressed in brain microvessels. In total brain homogenates these proteins will be present in very low concentration. Moreover, some proteins are expressed in the whole brain, in the BBB and other cells, thus making it difficult to interpret any changes in protein expression. Therefore we firstly isolated brain microvessels using dextran separation technique, which is the most robust technique in microvessels isolation allowing the isolation of 99% pure microvessels.

Tissue samples from animals belonging to the same group were pooled and mildly homogenized in a glass Teflon® homogenizer in ice-cold microvessel isolation buffer (MIB; 15 mM HEPES, 147 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$ and 12 mM $MgCl_2$), supplemented with 5% protease inhibitor cocktail (P8340; Sigma, Deisenhofen, Germany) and 1% phosphatase inhibitor cocktail 2 (P5726; Sigma). Homogenates were centrifuged at 3,200 rpm for 10 min at 4°C. The resulting pellets were resuspended in 20% dextran (MW 64,000-76,000; D4751, Sigma) in MIB. Suspensions were centrifuged at 6,500 rpm for 20 min at 4°C. The resulting crude microvessel rich pellets were resuspended in MIB and filtered through two nylon filters of 100 µm and 30 µm mesh size (Millipore, Schwalbach, Germany).

The quality of trapped microvessels in 30 µm filters was checked. Microvessels were stored at -80°C until further use. Isolated microvessels were homogenized and lysated in NP-40 lysis buffer supplemented with 5% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail 2. Lysate samples were sonificated over 2 cycles lasting 20 seconds each at 4℃ at 40% power. Protein concentrations were measured using Bradford assay kit with an iMark Microplate Reader (Bio-Rad, Hercules, Ca, USA).

3.6 Western blot analysis

To evaluate total and phosphorylated protein expression levels, the semi-quantitative Western blot was used. For all experiments, 5X SDS loading buffer was added to lysate samples or blood serum samples containing equal protein quantities (20 µg). These samples were heated for all protein analysis studies except ABCB1 and ABCC1, for which samples were loaded without heating to avoid aggregation of the highly glyosylated trans-membrane proteins. The loaded samples were subjected to sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by Western blot analysis, using primary antibodies diluted 1:100 for ABCC1 and 1:1,000 for all other proteins in 5% skim milk (Sigma) and 0.1 M tris buffered saline-Triton-X100 (TBS-T).

For total and phosphorylated protein analysis by Western blot analysis the following antibody were used: Anti-ApoE (sc-6385, detecting murine ApoE), anti-human ApoE (sc-6383, detecting human ApoE), anti-ABCB1 (sc-8313), anti-LXRαβ (sc-1000), anti-calpain-1/2 (sc-58326), anti-calpastatin (sc-20779), anti-ABCG1 (sc-11130), anti-occludin (sc-27151), anti-RhoA monoclonal (sc-418), anti-RhoA polyclonal (sc-179), anti-Cdc42 (sc-6083) and anti-CD31 (sc-8306), anti-LARG (sc-25638) anti-goat-anti-rabbit-HRP (sc-2004), anti-goat-anti-mouse-HRP (sc-2005) and anti-donkey-anti-goat (sc-2020) were purchased from Santa Cruz Biotechnology (Nunningen, Switzerland). Anti-p120 catenin (4989), anti-cleaved caspase-3 (Asp175) (9661), anti-total-JNK1/2 (9252), anti-phosphorylated JNK1/2$^{Thr183/Tyr185}$ (9255), anti-total MMP-2 (4022), anti-total MMP-9 (3852) and anti-β-actin (4967) were purchased from Cell Signaling (Allschwil, Switzerland). Anti-ApoER2 (ab52905) was obtained from Abcam (Cambridge, U.K.), anti-JIP1/2 (34-5300) and anti-GFAP (18-0063) from Invitrogen anti-ABCA1 (NB400-105) from Novus Biologicals (Cambridge, UK), anti-ZO-1 (40-2300) from Invitrogen (Karlsruhe, Germany), and anti-ABCC1 (ALX-801-007-c250) from Alexis Biochemicals (San Diego, CA, USA). Primary antibodies were detected with horseradish peroxidase (HRP)-conjugated secondary IgG that were diluted 1:5000 in 5% skim milk and TBS-T and revealed by enhanced chemiluminescence plus (ECL) solution (Amersham International, Buckinghamshire, England). Films were digitized and quantified using ImageJ (NIH, U.S.A.) image processing software.

3.7 Rho GTPase affinity binding (=pull down) assays

Rho GTPases pull down assay is an *in vitro* technique used to study Rho GTPases activation (i.e. GTP-bound) in cell lysate samples. The active GTP-bound form of Rho GTPases binds specific effectors that are tagged by glutathione S-transferase tail (GST-Effectors) and immobilized on glutathione agarose or sepharose beads (GST-Effectors). The Bead-GST-Effectors bind only the active GTP-bound form of Rho GTPases, which can be quantified afterwards.

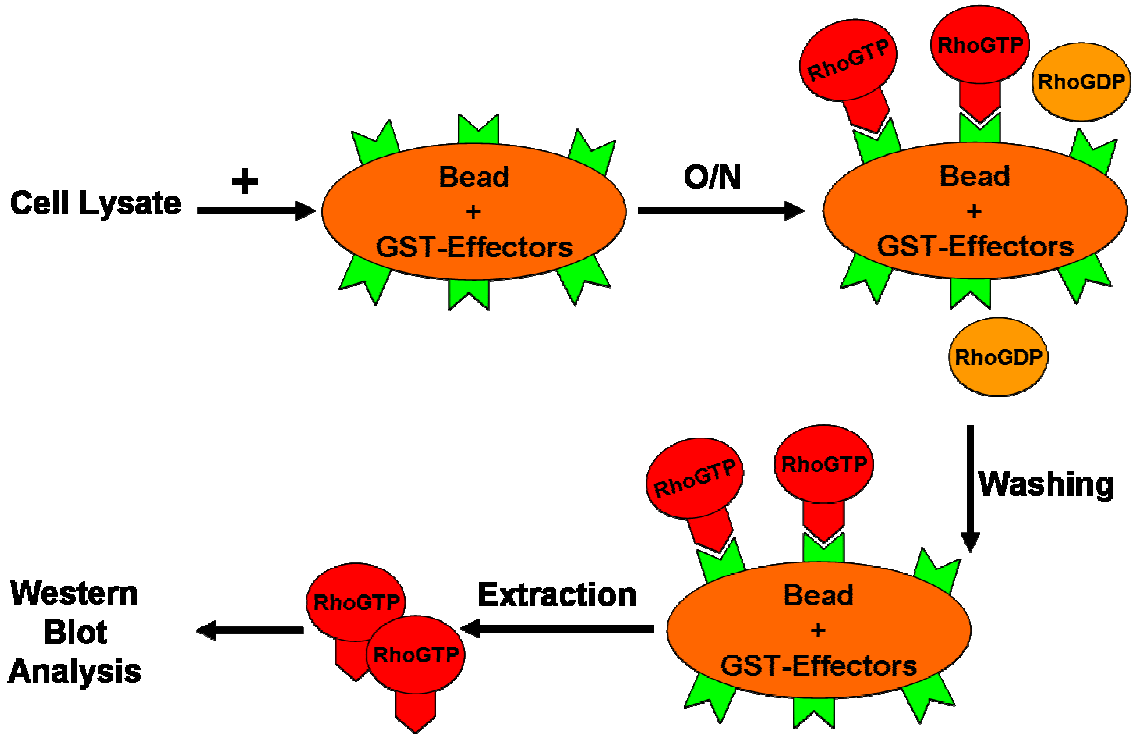

Figure 13. Affinity binding assay: GST-Effectors immobilized on beads are loaded in cell lysate samples. GST-Effectors effectors bind only the active GTP-bound form of Rho GTPase, which can be quantified and analyzed by Western blot.

To make quantitative analysis, equal protein quantities (800 µg) of lysates obtained using a $MgCl_2$ lysis buffer containing 50 mM Tris base, 100 mM NaCl, 2 mM $MgCl_2$ and 1% NP-40 (pH 7.4) were added in Eppendorf tubes and complemented to a total volume of 1 ml by adding $MgCl_2$ lysis buffer. To study and quantify Cdc42 activation, Cdc42 affinity binding assay was applied. A volume of 20 µl (20 µg) of recombinant Wiskott-Aldrich syndrome protein (WASP) - Cdc42 binding domain of WASP (CDB) - glutathione S-transferase (GST), GST-WASP-CDB, (WS03;

Cytoskeleton Inc, Frankfurt, Germany) prefixed on agarose beads were loaded into prepared lysates and incubated under slight rotation overnight at 4℃. The next day, samples were centrifuged for 30 seconds at 15,000 rpm, supernatants removed and pellets washed three times in ice cold $MgCl_2$ lysis buffer. A volume of 20 µl of 2X SDS plus 5% 2-mercaptoethanol loading buffer was added to each sample and boiled for 5 minutes (Heater Plate, Eppendorf, Germany), followed by a short centrifugation at 4,000 rpm to precipitate beads. Supernatants from each sample were subjected to SDS-PAGE using 12.5% acrylamide-bis gel. To study and quantify RhoA activation, RhoA affinity binding assay was applied, where a volume of 50 µl (20 µg) of recombinant Rhotekin (RTK) - Rho binding domain (RBD) - glutathione S-transferase (GST), GST-RTK-RBD, (RT02; Cytoskeleton Inc) prefixed on beads were loaded according to the same protocol of Cdc42 binding assay. For total Cdc42 and RhoA detection, lysate samples were loaded in 12.5% SDS-PAGE, followed by Western blot analysis using anti-Cdc42 (sc-6083; Santa Cruz Biotechnology) or anti-RhoA (sc-418; Santa Cruz Biotechnology). Films were digitized and densitometrically assessed.

3.8 Co-precipitation assays with GST tagged recombinant proteins

Upon activation and / or under specific conditions, some proteins interact specifically with other proteins to initiate signaling pathway or to modulate molecular mechanisms. In order to investigate this protein-protein interaction and signal transduction at the BBB, co-precipitation assay was applied. Co-precipitation assay permits a quantitative analysis of protein-protein interactions, and offers a tool to study the interaction between two or more proteins especially when at least one of these proteins is present in very low quantity, which is compensated by adding exogenously this protein, by using recombinant proteins that are tagged glutathione S-transferase. For these experiments brain microvessel samples, equal protein quantities (600 µg) of lysates obtained using a $MgCl_2$ lysis buffer containing 50 mM Tris base, 100 mM NaCl, 2 mM $MgCl_2$ and 1% NP-40 (pH 7.4) were added in Eppendorf tubes and complemented to a total volume of 1 ml by adding $MgCl_2$ lysis buffer.

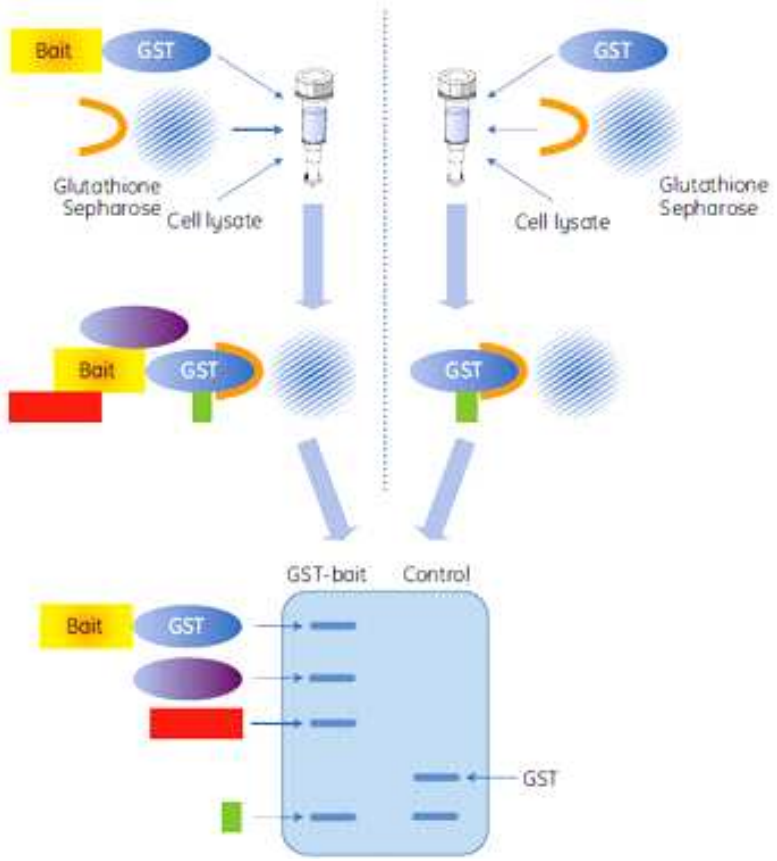

Figure 14. Co-precipitation assay: GST-Bait is loaded into cell lysate samples, the proteins of interest bind the GST-Bait, which can be quantified and analyzed by Western blot. (Source: GE Healthcare, life science).

Co-precipitation with GST-Cdc42: To study the interaction between inactive Cdc42 with p120 catenin, 50 µl (50 µg) of recombinant GST-Cdc42-(T17N) (C17G01; Cytoskeleton Inc) prefixed on agarose beads were added to $MgCl_2$ lysate samples, as described in the affinity binding assay protocol. Samples were subjected to SDS-PAGE using 10% acrylamide-bis gel, followed by Western blot analysis, using anti-p120 catenin (4989; Cell Signaling, Allschwil, Switzerland).

Co-precipitation with GST-RhoA: To investigate the upstream signalling pathway that contributed in RhoA activation, 50 µl (50 µg) of recombinant GST-RhoA-(wildtype) (RHG01; Cytoskeleton Inc) prefixed on agarose beads were processed with $MgCl_2$ lysate samples, as described in the affinity binding assay protocol. Samples were subjected to SDS-PAGE using 7% acrylamide-bis gel, followed by Western blot analysis for LARG (sc-25638; Santa Cruz Biotechnology). Films were digitized and densitometrically assessed.

3.9 Immunoprecipitation and co-immunoprecipitation assays

To investigate intracellular signaling pathway, endogenous protein-protein interaction were studied. For this purpose immunoprecipitation and co-immunoprecipitation assays were applied. As such, equal protein quantities (800 µg) of lysates obtained using a NP-40 lysis buffer containing 150 mM NaCl, 1% NP-40, 50 mM Tris base (pH 8.0) were supplemented with sodium orthovanadate (final concentration: 1 mM) and complemented with three equal volumes of NET buffer (100 mM Tris, 200 mM NaCl, 5 mM EDTA, 5% NP-40, pH 7.4). Six µg of anti-RhoA antibody (sc-179, Santa Cruz Biotechnology), 2 µg of ApoER2 (sc-20746; Santa Cruz Biotechnology), 10 µg of occluding (sc-27151; Santa Cruz Biotechnology) or 6 µg of p-120 catenin (4989; Cell Signaling) were added to each sample, and incubated overnight at 4℃ under slight rotation.

The next day 20 µl of protein A/G plus-agarose (sc-2003; Santa Cruz Biotechnology) were added to the samples and incubated over one hour at 4℃. Finally samples were centrifuged for 30 seconds at 15,000 rpm at 4℃. Supernatants were dispersed and pellets washed three times in ice cold NET buffer. 20 µl of 2X SDS loading buffer was added to each pellet and boiled for 5 minutes (Heater Plate), followed by a short centrifugation at 4,000 rpm to precipitate beads.

Supernatants were subjected to SDS-PAGE using 10% acrylamide-bis gel followed by Western blot analysis using anti-p120 catenin (4989; Cell Signaling), anti-ZO-1 (40-2300; Invitrogen), anti-phospho-tyrosine (sc-508; Santa Cruz Biotechnology), phospho-threonine (sc-81527; Santa Cruz Biotechnology), anti-phosphorylated JNK1/2$^{Thr183/Tyr185}$ (9255; Cell Signaling), anti-JIP1/2 (34-5300; Invitrogen), anti-MKP-7 (sc-98872; Santa Cruz Biotechnology), anti-MKK-7 (4172; Cell Signaling) or anti-Vav2 (sc-20803). Films were digitized and densitometrically assessed.

3.10 Immunofluorescence staining

In order to investigate the sub-cellular localization and co-localization of proteins expressed at the BBB, 20 µm thick cryostat brain sections obtained from were fixed in ice-cold acetone for 10 min, washed three times in 0.1M ice-cold phosphate-buffered saline (PBS), and blocked for 1 h in 0.1M PBS containing 0.25% Triton X-100 (PBS-T), 10% normal donkey serum (NDS) and 1% bovine serum albumin (BSA). After three more washes in 0.1M ice-cold PBS, sections were incubated overnight at 4℃ with polyclonal rabbit a nti-ApoER2 (sc-20746; Santa Cruz Biotechnology) diluted to 1:50 in 0.1 M PBS-T and 2% Normal Donkey Serum (NDS), rat anti-CD31 (RM5200; Invitrogen, Karlsruhe, Germany) diluted to 1:200 in 0.1 M PBS-T and 2% NDS and mouse anti-GFAP-Alexa Fluor® 647 conjugated (3657, Cell Signaling). The next day sections were washed three times in ice-cold 0.1M PBS and incubated for 1 h with secondary donkey anti-rat-FITC (712-095-150; Jackson Immunoresearch, Suffolk, U.K.) and donkey anti-rabbit-TRITC (711-025-152; Jackson Immunoresearch), diluted 1:200 in 0.1M PBS-T and 2% NDS. Finally sections were mounted and coverslipped. Sections were microscopically evaluated and microphotographs taken from the ischemic striatum using confocal microscopy (Leica microsystems, Wetzlar, Germany).

3.11 Caseinase microplate assay for calpain-1/2

Calpain-1/2 enzyme are caseinase, therefore to investigate calpain-1/2's role in LXR's effects on the BBB, we developed a caseinase microplate assay, which evaluates caseinase activity in the presence of calpain-1/2's endogenous inhibitors. Twenty µg of protein extracts from each sample, obtained using NP-40 lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl and 1% NP-40 (pH 7.4), were added into 96 well microplate and complemented with calibrating buffer containing 20 mM Tris-HCl, 1 mM EDTA, 100 mM KCl and 0.1% 2-mercaptoethanol (pH 7.4), resulting in volumes of 60 µl in each well.

Two solutions were prepared containing casein at 20 µg / ml: (i) Solution A (= activation) consisted of 20 mM Tris-HCl, 1 mM EDTA, 10 mM Ca^{2+}, 100 mM KCl and 0.1% 2-mercaptoethanol (pH 7.4), allowing to study the enzymatic activity of caplain-

1/2 owing to the fact that samples contained Ca^{2+} that is required for calpain-1/2 to exert its protease activity. (ii) Solution I (= inhibition) contained 20 mM Tris-HCl, 50 mM EDTA, 100 mM KCl and 0.1% mercaptoethanol (pH 7.4). In this solution calpain-1/2 was inactive as the sample lacked Ca^{2+} ions. Experiments were conducted in triplicate for each sample, 100 µl of solution A being added to the first set of wells containing protein samples, and 100 µl of solution I being added to the next set, increasing the total volume in each well to 160 µl (60 µl + 100 µl). As such, the assay was run four times for statistical comparisons.

Each microplate was incubated for 2 hours at room temperature. Then, 120 µl of 1X G-250 dye (Quick Start Bradford Protein Assay; Biorad) were added simultaneously to each well and incubated for 5 min at room temperature, the G-250 dye forming a stable complex upon binding proteins shifting light absorbance from 470 nm to 595 nm. The dye does not bind proteolytic fragments of digested proteins like small peptides or amino acids, assessing the enzymatic activity of calpain-1/2, the higher the activity the less absorbance values are.

The absorbance was read at 595nm using the iMark microplate reader and calpain-1/2 caseinase activity was calculated for each sample by subtracting absorbances between solution I and solution A. Calpain-1/2 protease activity was expressed as absorbance difference at 595 nm units (AD_{595nm}).

3.12 Gelatin polyacrylamide gel zymography for MMP-2/9

MMP-2/9 enzyme are gelatinase, therefore to examine the role of MMP-2/9 in LXR's actions on brain microvessels, we used both gelatin polyacrylamide gel zymography (Zechariah et al., 2010) and a gelatinase microplate assay, which we established in analogy to the caseinase microplate assay presented above. For polyacrylamide gel zymography, 25 µg proteins were mixed with 5X non reducing loading buffer for 15 min at room temperature and subjected to sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 9% acrylamide-bis gel containing 0.1% gelatin (Sigma). Gels were removed and washed, incubated for 1 hr at room temperature with slight shaking in modified enzymatic activation buffer (50 mM Tris-HCl, 6 mM CaCl2, 1.5 µM ZnCl2, pH 7.4) containing

2.5% Triton X-100 to remove SDS and restore gelatinase activity. Gels were then incubated for 24 hr at 37°C in modified enzymatic activation buffer. Gels were stained in Coomassie brilliant blue R-250 (Bio-Rad), followed by immersion in destaining solution (40% methanol, 10% acetic acid, 50% water) and 2% acetic acid. Gels were dried and digitized. A total of four experiments were run, which were densitometrically analyzed.

The limitation of gelatin zymography in our study is that the corresponding band of MMP-2 in stained gel is very fad and it is very hard to assess and quantify such band, therefore the gelatin zymography was adopted to study MMP-9 activity, and gelatinase microplate assay was adopted to study MMP-2 and MMP-9 activities.

3.13 Gelatinase microplate assay for MMP-2/9

For studying MMP-2/9 gelatinase activity in presence of their endogenous inhibitor, the gelatinase microplate assay was used. For this purpose the above caseinase microplate assay was adopted with the following changes: 5 µg of proteins obtained by using the same lysis buffer as above were added to a 96 well microplate, complemented with a calibrating buffer containing (50 mM Tris-HCl, 6 mM $CaCl_2$, pH 7.4) to have volumes of 60 µl in each well. Two solutions were prepared containing gelatin at a concentration of 20 µg/ml: (i) Solution A (= activation) (50 mM Tris-HCl, 6 mM $CaCl_2$, 1.5 µM $ZnCl_2$, pH 7.4) and (ii) solution I (= inhibition) (50 mM Tris-HCl, 6 mM $CaCl_2$, 50 mM EDTA, pH 7.4), the two samples differing in terms of the presence and absence of $ZnCl_2$, which is a co-factor needed for gelatinase to exert its enzymatic activity.

Experiments were again conducted in triplicate for each sample. The assay was run four times. Each microplate was incubated for 2 hours at 37℃. Then, G-250 dye was added, the absorbance red, and MMP-2/9 gelatinase activity calculated by subtracting absorbance between solution I and solution A. MMP-2/9 protease activity was again expressed as absorbance difference at 595 nm units (AD_{595nm}).

3.14 Reverse transcriptase (RT) - polymerase chain reaction (PCR) analysis

To investigate the molecular mechanisms involved in regulating ABCB1 and ABCC1 at the transcriptional level at mice brain microvessels, we applied RT-PCR techniques to quantify mRNA of ABCB1 and ABCC1. For this purpose, isolated brain microvessel samples were harvested in RNA stabilization reagent RNAlaterTM (Qiagen GmbH, Hilden, Germany). Total RNA was extracted using SV RNA-Isolation Kit (Promega GmbH, Mannheim, Germany) according to the manufacturer's protocol. Total RNA purity and concentration were assessed using the NanoPhotometerTM system (Implen GmbH, Munich, Germany). Simultaneously, the expression of *abcb1* ,*abcc1* and the housekeeping gene succinate dehydrogenase complex subunit A (SDHA) were studied in individual animals using the one-step TaqMan® RT-PCR system with FAM-BHQ probe and ROX (passive reference) in 48 microplates (Applied Biosystems, Foster City, USA).

For each sample, duplicates of 10 ng total RNA were processed in 15 µl volume reactions of the QTaq™ one-step qRT-PCR master mix kit (Clontech-Takara, Saint-Germain-en-Laye, France), which contains Hotstart Taq DNA polymerase and optimised reaction buffer with 5 mM MgCl2, 2.5-3.5 mM nucleotides (including dUTP), reverse transcriptase and RNAse inhibitor. NCBI GenBank Accession Number and primers used are shown in Supplementary Table 1. The PCR regime consists of a reverse transcriptase reaction (20 min at 48℃), followed by hotstart Taq DNA polymerase activation (10 min at 95℃), and fol lowed by 45 cycles (15 sec at 95℃ – 1 min at 60℃). The following primers were u sed: ***abcb1*** (Genbank accession number: NM_011075.2) fwd: CACAGAAAGCAA GACCAAGAGA, rev: TTTAGATTTAGGATCCGCCAAA, TM: 5'FAM GCACATCTT CATCCACAGCCTCTT-3'BHQ, ***abcc1*** (Genbank accession number: NM_008576.2) fwd: GAACTTGGACCCTTTCAGTCAG, rev: GACACAAAGCCCTTTAGGTGAG TM: 5'FAM-TCTGATGAAGAAGTCTGGAT GGCC-3'BHQ.For each gene in different samples, the delta threshold cycle (ΔCт) and delta-delta threshold cycle (ΔΔCт) values were calculated (ΔCт = Cт of the gene of interest - the Cт of *SDHA*; ΔΔCт = ΔCт control – ΔCт ischemic), followed by Fold Change calculation (Fold Change = $2^{(-1)*\Delta\Delta C_T \text{ gene of interest}}$).

3.15 Enzyme-linked immunosorbent assay (ELISA) for FK506

To translate the role of ApoE in controlling ABCB1 expression on brain drug accumulation, we performed pharmacological studies using FK506 (tacrolimus) which is a lipophilic compound and a well-known substrate of ABCB1. To investigate FK506 accumulation and biodistribution in mice brain, we used PRO-Trac^{TM} II tacrolimus ELISA kit (Diasorin Deutschland GmbH, Dietzenbach, Germany) as described (Spudich et al., 2006). Briefly, whole blood samples were collected from mice heart in ethylene diamine tetraacetic acid (EDTA)-coated tubes. Weighted brain samples taken from the MCA territory both ipsilateral and contralateral to the stroke were harvested in ice cold normal saline solution (100 mg brain tissues in 1.5 ml normal saline solution). The PRO-Trac^{TM} II tacrolimus ELISA kit was only recommended for the analysis of FK506 in human blood, and the direct analysis of FK506 in mice brain homogenate failed probably due to the interfering mouse proteins, e.g. IgG.

Therefore FK506 has first to be extracted from mice brain homogenate (Yokogawa et al., 1999). For this purpose, brain homogenates and blood samples were pretreated with PRO-Trac^{TM} tacrolimus digestion reagent, and FK506 was extracted with n-hexane containing 2.5% isoamyl alcohol (1 volume homogenate: 9 volumes n-hexane / 2.5% isoamyl alcohol). The extracted FK506 was diluted in PRO-Trac^{TM} conjugate diluent and after competitive binding with FK506-horseradish peroxidase conjugate, substrate reaction was measured using iMark Microplate Reader (Bio-Rad) at 450/630 nm wavelength. Positive controls were provided in the ELISA kit, negative samples obtained from the brain and blood of untreated mice.

Data were computed and concentrations calculated using 4PL curve fitting program (MPM6 software, Biorad). From the concentrations determined, brain-to-blood concentration ratios were calculated and were expressed as ng.mg^{-1} (brain tissue) per µg.ml^{-1} (blood).

3.16 Statistics

Results are presented as means ± S.D. Data were analyzed by unpaired t-tests (comparisons between two groups) or one-way ANOVA followed by least significant differences (LSD) tests (comparisons between ≥4 groups) using SPSS for Windows. For the latter data, two-way ANOVA were also computed, of which condition interaction effects (mouse line x hemisphere or treatment x hemisphere) were reported in the graphs. In cases multiple comparisons were made (i.e., for ABCB1 and ABCC1), significance levels were adapted using Bonferroni corrections. *P* values less than 0.05 were considered significant.

4 RESULTS

4.1 Part I: Effect of LXR activation on BBB tightness

Liver X receptors (LXR) are sensors of cell metabolism. Their role in the maintenance of BBB tightness was unknown. In this study, mice receiving the LXR agonist T0901317 (1.25 mg, i.p.) or vehicle were submitted to transient focal cerebral ischemia. Twenty-four hours after reperfusion, ischemic injury, brain edema and serum IgG extravasation were assessed. Signaling pathways controlling BBB tightness were evaluated by Western blots, activity assays and protein interaction experiments.

4.1.1 Effect of LXR activation on ischemic injury and vascular permeability

To evaluate ischemia reproducibility, Laser Doppler flow (LDF) during and after MCA occlusion did not differ between vehicle treated and T0901317 treated animals. As such, LDF values decreased to 20.32±9% and 22.93±12% of baseline within 5 min after onset of ischemia in untreated and treated mice respectively, remaining stable throughout the MCA occlusion period. After reperfusion, LDF values rapidly recuperated to 82.5±17% and 86.4±18.8% of baseline.

To evaluate effects of LXR activation on brain edema, vascular tightness and ischemic injury, we analyzed cresyl violet stainings and histochemistries against extravasated serum IgG. Reduced brain swelling (Figure 15A) and decreased serum IgG extravasation (Figure 15B) were noticed in ischemic animals receiving T0901317 treatment, indicating preservation of the BBB by the LXR agonist. Infarct area did not show any difference between groups (Figure 15C).

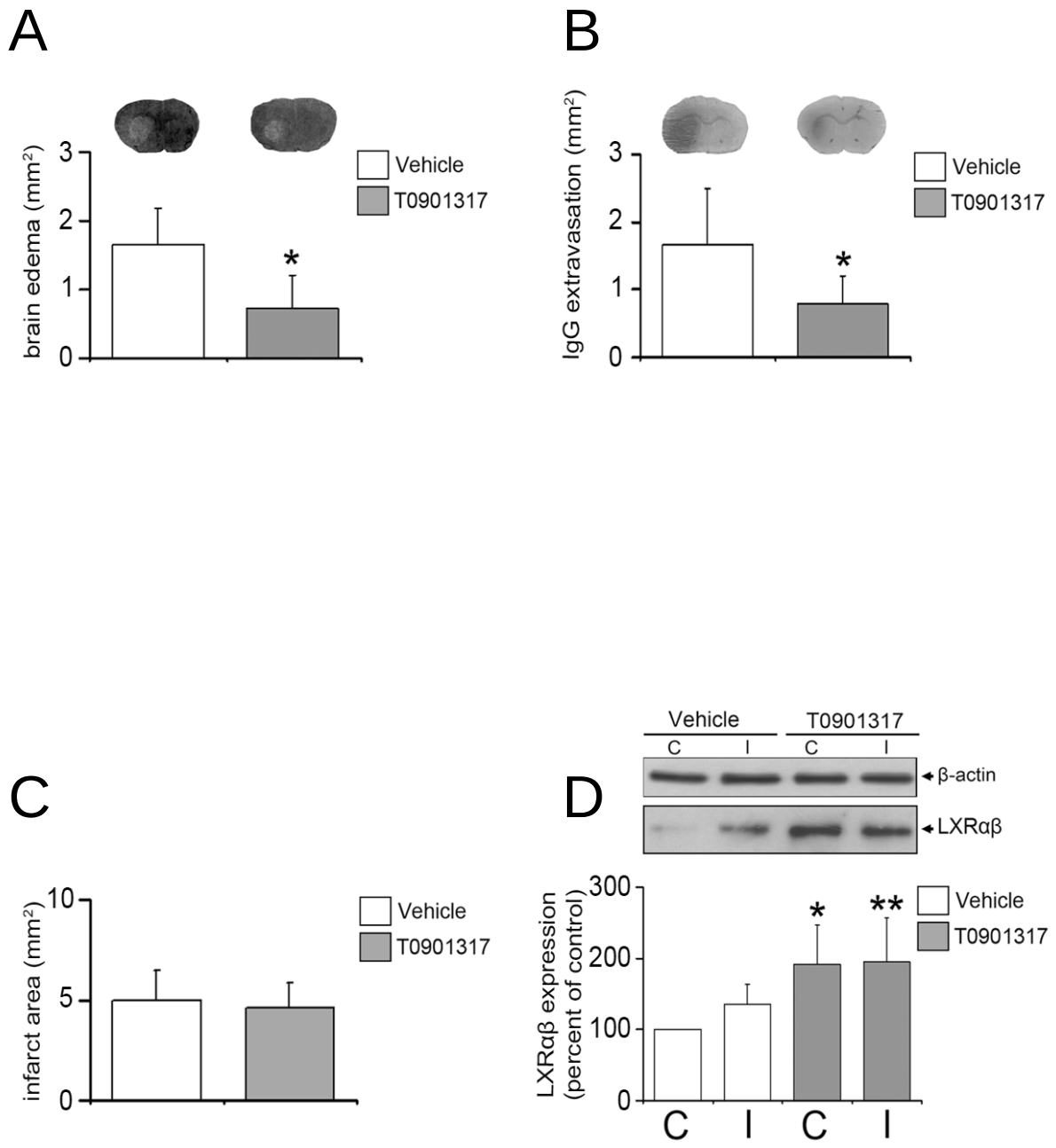

Figure 15. LXR agonist decreases brain edema and leakage: Histochemical studies analyzing brain swelling (A) and serum IgG extravasation (B), which are both reduced in animals receiving the LXR agonist. Note that infarct area (C) is not influenced by LXR activation. Western blots using brain capillary extracts show that T0901317 robustly increases LXRαβ expression (D). Data are means ± SD (*n*=4 Western blots). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p<0.05/**p<0.01 compared with non-ischemic vehicle (A, B, C: unpaired two-tailed t-tests, D: one-way analysis of variance (ANOVA) followed by LSD tests)

4.1.2 Effect of T0901317 on LXRαβ expression

LXR activation by T0901317 potently increases LXRαβ expression (Whitney et al., 2001). To verify T0901317 administration efficacy, we checked LXRαβ levels in brain microvessels using Western blot analysis. We showed that LXRαβ expression is indeed increased by T0901317 in both the ischemic and non-ischemic brain tissue (Figure 15D).

4.1.3 Calpain-1/2 and MMP-2/9 activities regulation after LXR activation

To elucidate the role of calpain-1/2 and MMP-2/9 in BBB preservation induced by LXR activation, we evaluated their expression and activity in cerebral microvessels by Western blots, calpain-1/2 caseinase microplate assay, gelatine zymography and MMP-2/9 gelatinase microplate assay. Western blots revealed that the overall expression of calpain-1/2 and MMP-2/9 is neither influenced by ischemia nor by T0901317 (Figure 16A).

The activities of calpain-1/2 and MMP-2/9, on the other hand, strongly increased after stroke (Figure 16B-D), which is in line with earlier studies from other groups (Cao et al., 2007; Zhao et al., 2006; Tsubokawa et al., 2006). Importantly, calpain-1/2 and MMP-2/9 activities were attenuated by LXR agonist (Figure 16B-D). In case of MMP-2 and -9, inhibition of protease activity was detected by using gelatine zymography and MMP-2/9 gelatinase microplate assay (Figure 16C,D) that evaluate MMP-2/9 activity in the absence and presence of endogenous inhibitors, which, based on our studies, are not essential for LXR's inhibitory influence on MMP-2/9.

4.1.4 T0901317 increases the expression of LXR's target gene calpastatin

To identify factors responsible of calpain-1/2 deactivation, we studied the expression of its specific inhibitor calpastatin, which is transcriptionally regulated by LXR (Hummasti et al., 2004), by means of Western blots. Reduced expression levels of calpastatin were observed in ischemic microvessels of vehicle-treated mice (Figure 17A). T0901317 increased calpastatin expression above levels in non-ischemic vessels (Figure 17A). Our data suggest that calpastatin is responsible for the inhibition of calpain-1/2 by the LXR agonist.

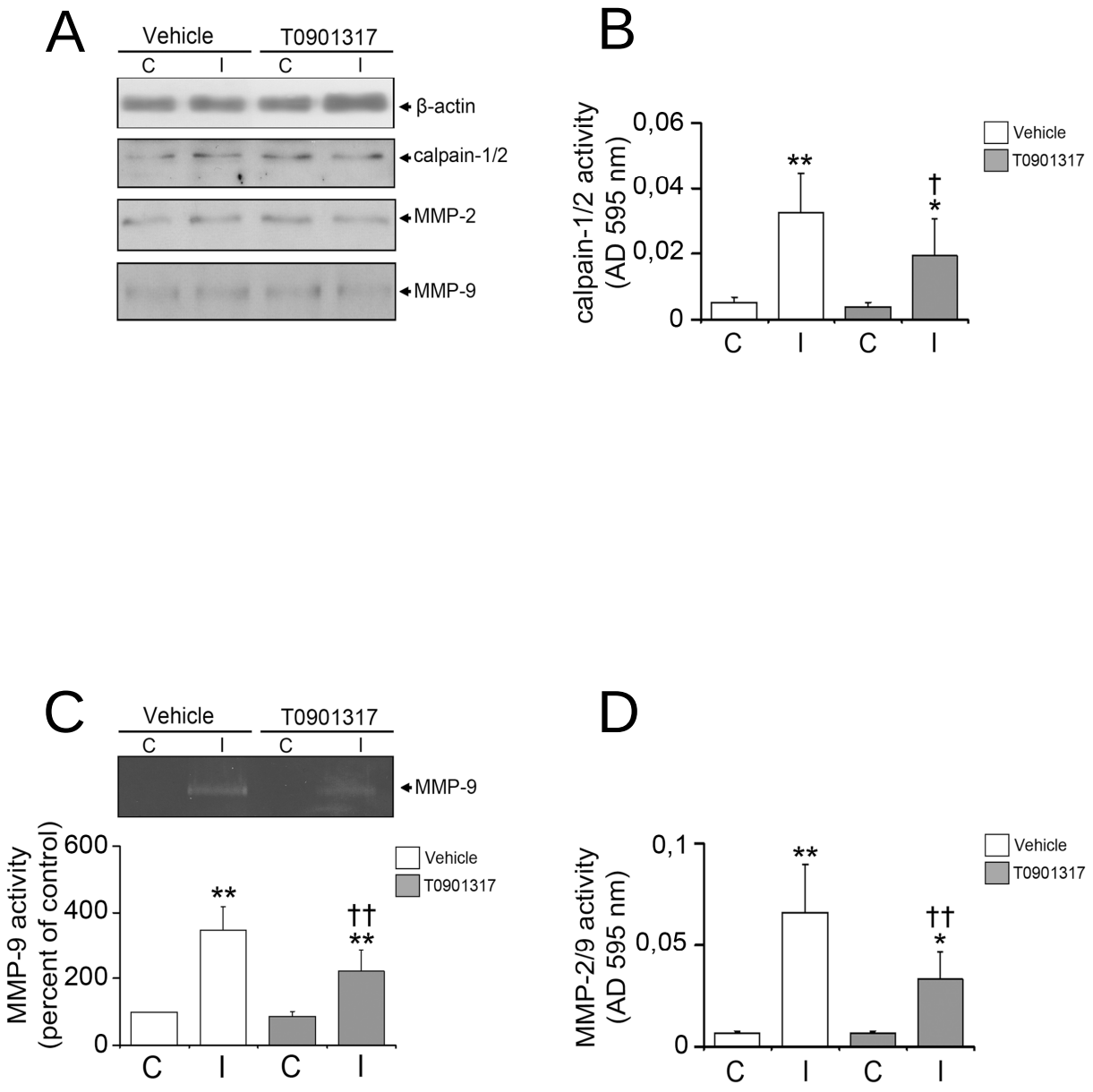

Figure 16. LXR agonist decreases calpain-1/2 and MMP-2/9 activities: Western blots (A,), calpain-1/2 caseinase assay (B), MMP-9 gelatine zymography (C), and MMP-2/9 gelatinase assay (D) showing that calpain-1/2 and MMP-2/9 activity (B-D) but not expression (A) are reduced in ischemic microvessels of T0901317 treated mice. Data are means ± SD (*n*=4 Western blots or assays). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p<0.05/**p<0.01 compared with non-ischemic vehicle/ †p<0.05/††p<0.01 compared with ischemic vehicle (one-way analysis of variance (ANOVA) followed by LSD tests).

4.1.5 LXR activation stabilizes p120 catenin

To define downstream targets of calpain-1/2 that might mediate BBB preservation induced by LXR activation, we assessed the expression of p120 catenin, a target protein degraded by calpain-1/2 (Ohno et al., 2007), using Western blot analysis. As a consequence of calpain-1/2 activation, p120 catenin expression was reduced in ischemic microvessels of vehicle-treated mice (Figure 17B). Conversely, treatment with T0901317 that inhibited calpain-1/2 elevated p120 catenin expression in ischemic brain capillaries even above levels in non-ischemic vessels (Figure 17B).

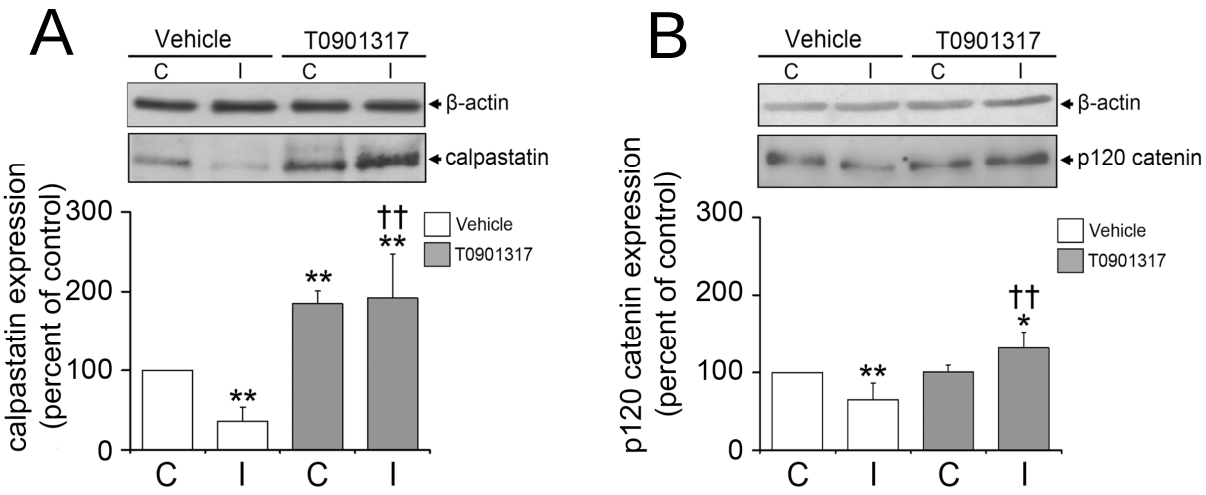

Figure 17. LXR agonist induces calpastatin expression, stabilizing p120 catenin: Western blots (A, B) show calpain-1/2 endogenous inhibitor calpastatin is overexpressed in the presence of the LXR agonist (A). In response to the reduced activity of calpain-1/2, p120 catenin expression is preserved (B). Data are means ± SD (*n*=4 Western blots or assays). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p<0.05/**p<0.01 compared with non-ischemic vehicle/ †p<0.05/††p<0.01 compared with ischemic vehicle (one-way analysis of variance (ANOVA) followed by LSD tests).

4.1.6 LXR activation differentially regulates RhoA and Cdc42 activation

To understand whether and how LXR activation influences the activity of Rho GTPases involved in the regulation of BBB tightness, we next performed pulldown assays for RhoA and Cdc42. These studies revealed that both RhoA and Cdc42 are activated upon ischemia in cerebral microvessels (Figure 18A, B). Interestingly, T0901317 treatment inhibited RhoA (Figure 18A), at the same time further elevating Cdc42 activity in ischemic, but not in non-ischemic vessels (Figure 18B).

4.1.7 Differential regulation of RhoA and Cdc42 activity in ischemic microvessels

Since p120 catenin acts as GDI for RhoA (Wildenberg et al., 2006; Anastasiadis et al., 2000) and also binds GEFs that specifically activate Cdc42 (Noren et al., 2000), we investigated the role of p120 catenin in the differential regulation of RhoA and Cdc42 by T0901317.

In immunoprecipitation studies we showed that RhoA binds physically to p120 catenin in vehicle treated, non-ischemic cerebral microvessels, whereas this RhoA interaction with p120 catenin is absent in ischemic brain capillaries (Figure 18C). T0901317 restored the RhoA binding to p120 catenin in ischemic microvessels (Figure 18C), indicating that RhoA interaction with this GDI is responsible for the RhoA inhibition by the LXR agonist.

On the other hand, we found that inactive GST-Cdc42 binds p120 catenin in T0901317 treated, but not in vehicle treated non-ischemic and ischemic microvessels (Figure 18D), most likely as a result of the p120 catenin expression loss. Active GST-Cdc42 did not reveal interaction with p120 catenin (not shown). Moreover, we observed that Vav2 interacts with p120 catenin (Figure 18D), suggesting that p120 catenin promotes Cdc42 activation by enhancing the access of Cdc42 to its GEF.

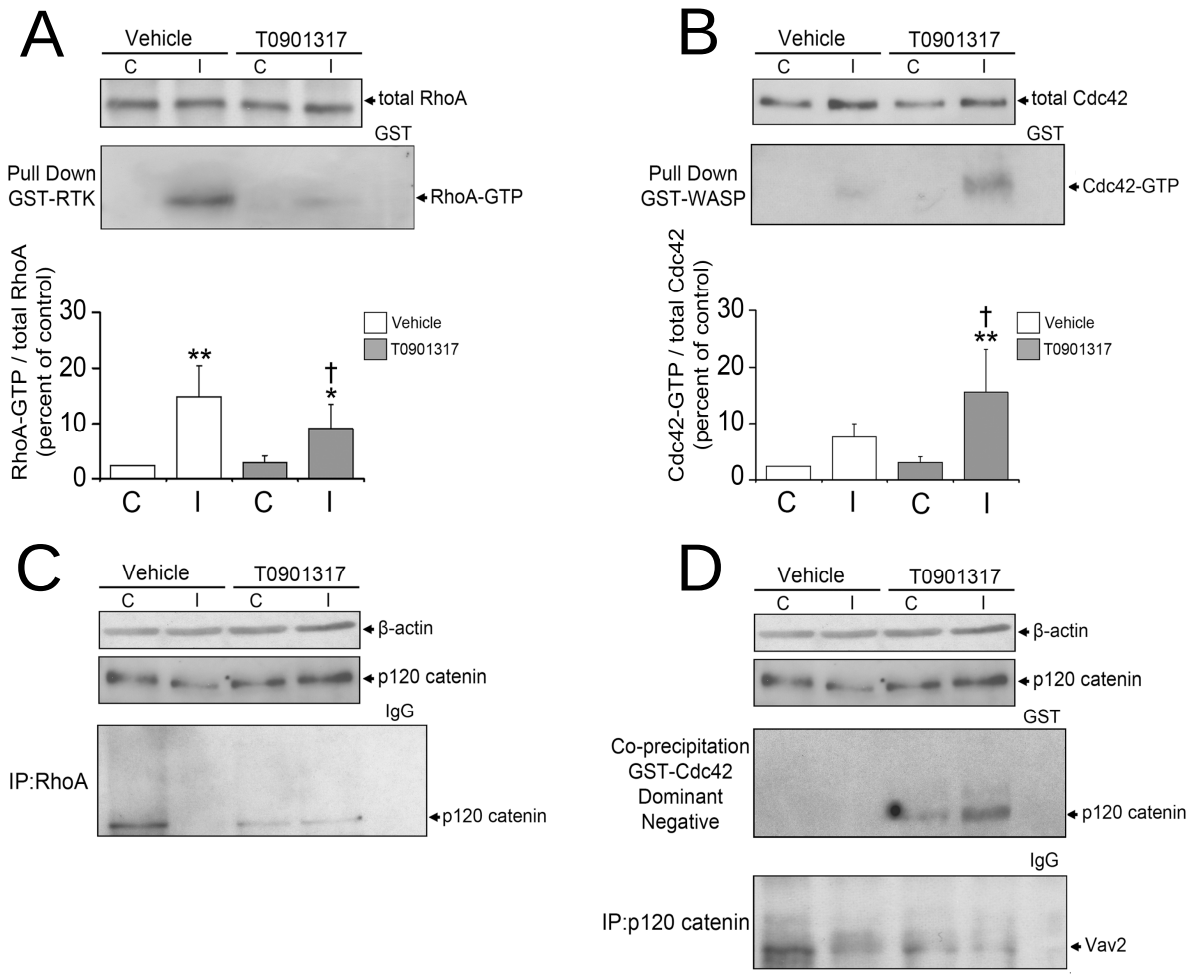

Figure 18. p120 catenin controls RhoA and Cdc42 activation: Pull-Down assays detecting GTP-bound (i.e., activated) RhoA and Cdc42 reveal that LXR induction reduces RhoA activation (A) and increases Cdc42 activation (B) in ischemic microvessels. Western blots of total RhoA and Cdc42 expression reveal no differences among groups (A, B). Immunoprecipitation experiments demonstrate that RhoA binding to p120 catenin is abolished upon ischemia, but restored upon T0901317 treatment (C). Co-precipitation studies show that inactive Cdc42 does not bind p120 catenin neither under normal, nor ischemic microvessels of vehicle-treated mice (D). Interestingly, LXR activation induces binding of inactive Cdc42 to p120 catenin, which also binds Vav2, a Cdc42 GEF (D). Data are means ± SD (*n*=4 pulldown assays). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p<0.05/**p<0.01 compared with non-ischemic vehicle/ †p<0.05 compared with ischemic vehicle (one-way analysis of variance (ANOVA) followed by LSD tests).

4.1.8 Regulation of occludin and ZO-1 after LXR activation

To further elucidate how LXR activation influences BBB integrity, we studied the expression, phosphorylation and interaction of the tight junction proteins occludin and ZO-1 by Western blots and immunoprecipitation studies. In accordance to previous studies (Takenaga et al., 2009; Chen et al., 2000), the overall expression and threonine phosphorylation of occludin decreased, the tyrosine phosphorylation of occludin increased and the association of occludin with ZO-1 decreased upon ischemia in vehicle treated microvessels (Figure 19A, B; Figure 20A, B), reflecting the breakdown of BBB integrity following stroke. Interestingly, T0901317 selectively increased occludin and ZO-1 expression on ischemic microvessels (Figure 19A, B), at the same time reversing occludin threonine phosphorylation and abolishing occludin tyrosine phosphorylation, thus enhancing the assembly of occludin with ZO-1 (Figure 20A, B).

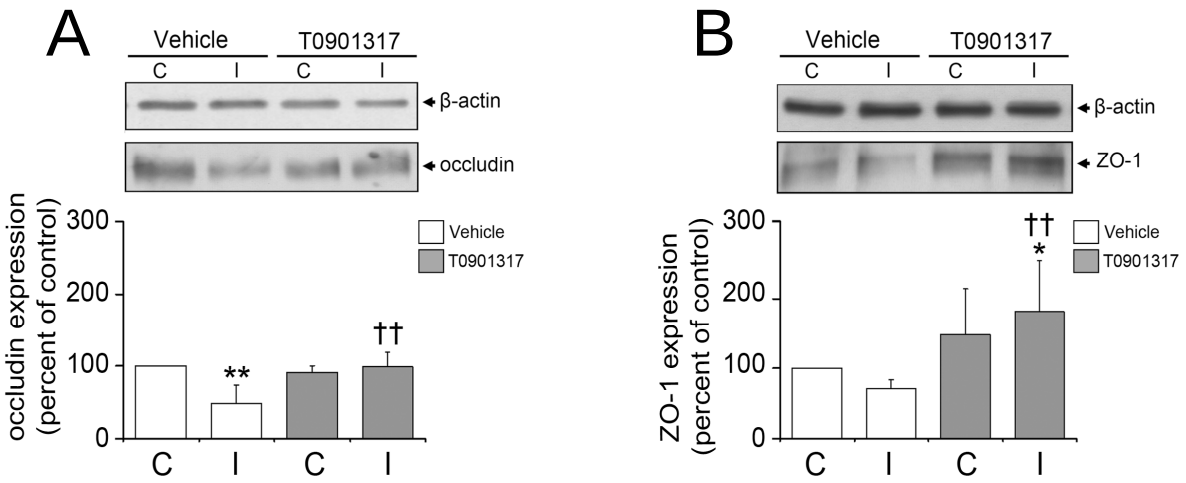

Figure 19. LXR activation modulates the expression of occludin and ZO-1: Western blot analysis (A, B) demonstrating that T0901317 restores occludin and ZO-1 expression in ischemic microvessels that are otherwise reduced in vehicle treated mice (A, B). Data are means ± SD (*n*=4 Western blots). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p<0.05/**p<0.01 compared with non-ischemic vehicle/ ††p<0.01 compared with ischemic vehicle (one-way analysis of variance (ANOVA) followed by LSD tests).

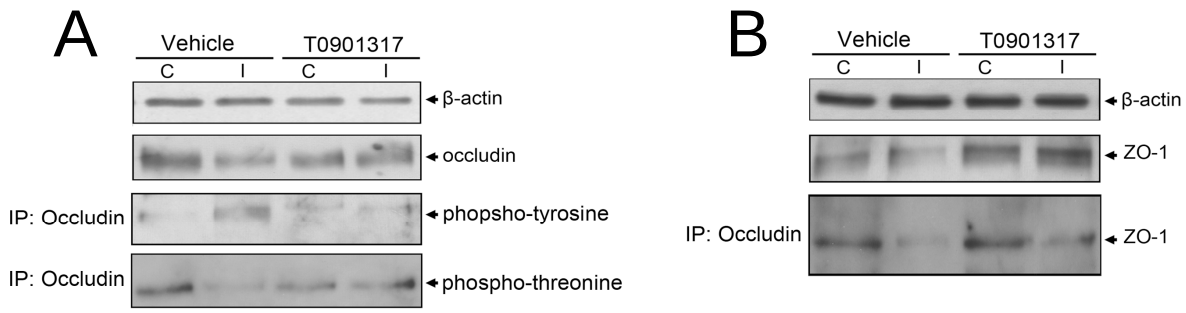

Figure 20. LXR activation modulates the phosphorylation of occludin, and its assembly with ZO-1: Immunoprecipitation experiments (C, D) demonstrating that T0901317 decreases occludin tyrosine phosphorylation (C), increasing occludin threonine phosphorylation (C) and enhancing occludin interaction with ZO-1 (D). Data are means ± SD (*n*=4 Western blots). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p<0.05/**p<0.01 compared with non-ischemic vehicle/ ††p<0.01 compared with ischemic vehicle (one-way analysis of variance (ANOVA) followed by LSD tests).

4.1.9 LXR activation deactivates JNK1/2 pathways and caspase-3

The activation of Jun N-terminal kinase-1/2 (JNK-1/2) via phosphorylation represents a cellular stress signal that promotes endothelial apoptosis (Schulz et al., 2008). To analyze how LXR activation influences JNK1/2 phosphorylation state, we performed Western blots using antibodies detecting either phosphorylated or total (i.e., phosphorylated and unphosphorylated) JNK1/2. In vehicle treated mice, JNK phosphorylation was decreased in ischemic microvessels (Figure 21A). T0901317 further reduced JNK1/2 phosphorylation levels (Figure 21A). It has been shown that calpain-1/2 facilitates the activation of caspase-3, thus promoting neuronal apoptotic injury (Blomgren et al., 2001). To investigate how T0901317, which deactivates calpain-1/2, influences caspase-3 activity, we examined the expression of cleaved, i.e., activated caspase-3 by Western blots. Our results showed that caspase-3, which is strongly activated upon ischemia, is deactivated by LXR agonist (Figure 21B). Our data point towards a survival-promoting effect of T0901317 on cerebral microvessels.

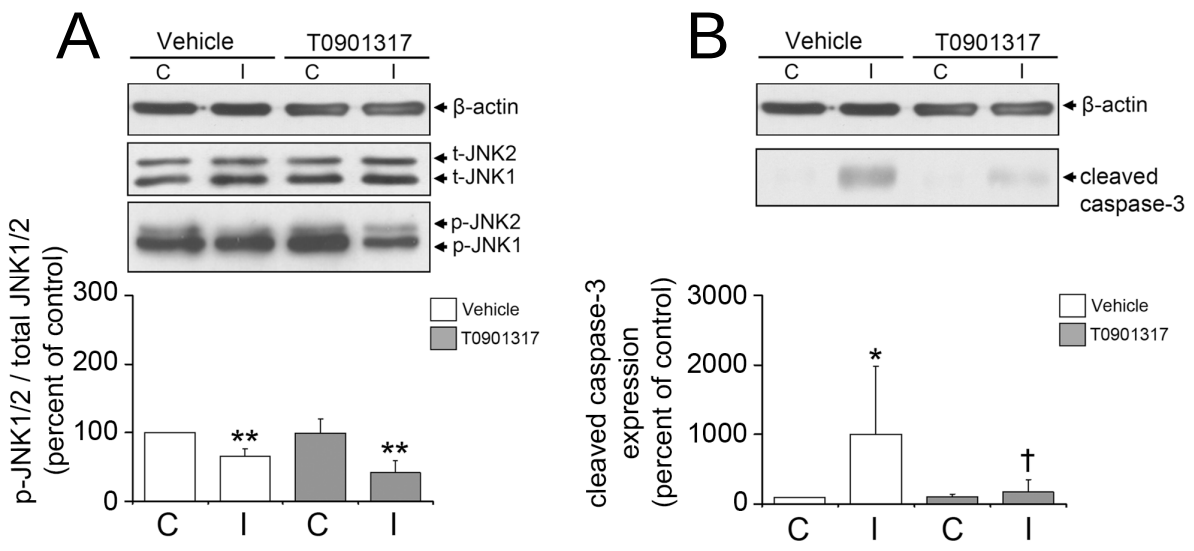

Figure 21. LXR agonist deactivates JNK1/2 and caspase-3: Western blot analysis shows that LXR activation decreases JNK phosphorylation (A) and caspase-3 cleavage (B) in ischemic microvessels. Data are means ± SD (*n*=4 Western blots). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p<0.05/**p<0.01 compared with non-ischemic vehicle/ †p<0.05 compared with ischemic vehicle (one-way analysis of variance (ANOVA) followed by LSD tests).

4.1.10 T0901317 regulates ABC transporters at the BBB, without influencing their polarity

We have previously shown that the ABC transporter ABCB1 is upregulated on the luminal surface (Spudich et al., 2006), whereas ABCC1 is downregulated on the abluminal surface of ischemic endothelial cells (Kilic et al., 2008), presumably as part of endogenous attempt protecting the brain against blood-derived toxins. By means of Western blots we now observed that the lipid transporters ABCA1 and ABCG1, known LXR targets (Naik et al., 2006), are upregulated on cerebral microvessels upon T0901317 treatment (Figure 22A, B). Strikingly, LXR activation also elevated ABCB1 expression (Figure 22C) and restored the reduced ABCC1 expression that was observed in ischemic vehicle treated mice (Figure 22D). As such, there was an overall upregulation pattern of ABC transporters – both of luminally located and abluminal ones – by LXR activation, which did not affect the polarity of the transporters. We interpret the elevated ABC transporters levels as a part of mechanism strengthening neurovascular integrity.

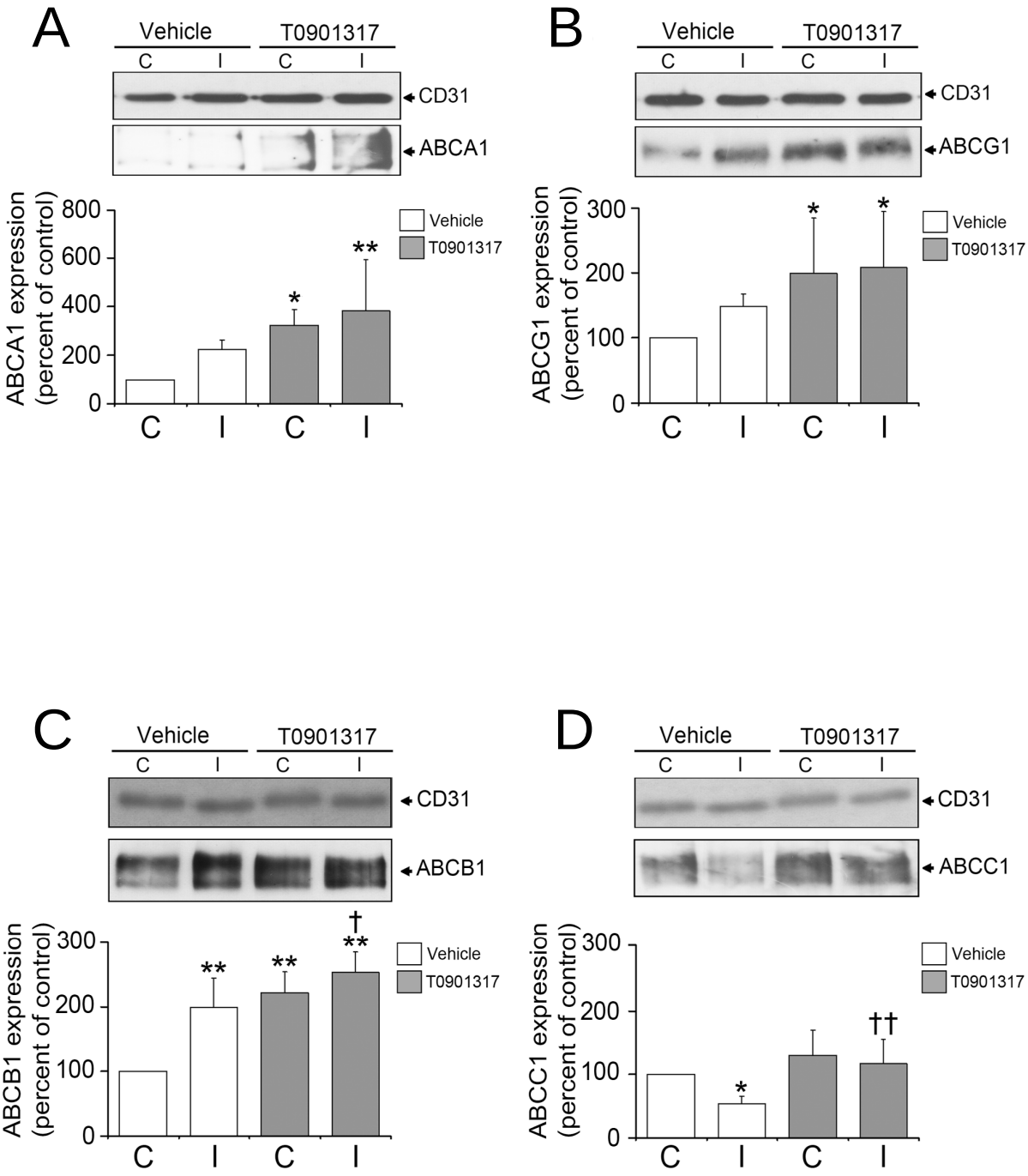

Figure 22. LXR activation upregulates ABC transporters: Western blot analysis revealing that the LXR agonist increases expression levels of the lipid transporters ABCA1 (A) and ABCG1 (B) on brain microvessels, at the same time elevating expression levels of the drug transporters ABCB1 (C) and ABCC1 (D) on ischemic capillaries. Data are means ± SD (*n*=4 Western blots). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p<0.05/**p<0.01 compared with non-ischemic vehicle/ †p<0.05/††p<0.01 compared with ischemic vehicle (one-way analysis of variance (ANOVA) followed by LSD tests).

4.2 Part II: Effect of apolipoprotein-E (ApoE) on BBB polarity

By means of protein and gene expression studies that we combined with genetic knockout, pharmacological and protein delivery experiments, we investigated the role of ApoE in the regulation of BBB polarity, studying the post-ischemic expression of ABCB1 and ABCC1 and examining drug accumulation in the ischemic brain.

4.2.1 Severity of injury does not differ between wildtype and ApoE$^{-/-}$ mice

Laser Doppler flow (LDF) during and after MCA occlusion did not differ between wildtype and ApoE$^{-/-}$ animals that were held on normal diet. As such, LDF values decreased to 20.2±5.2% and 21.4±4.1% of baseline within 5 min after onset of ischemia in C57BL6/j and ApoE$^{-/-}$ mice, remaining stable throughout the MCA occlusion period. After reperfusion, LDF values rapidly recuperated to 87.2±15.5% and 89.0±12.1% of baseline. Twenty-four hours after the stroke, localized brain lesions were observed in the mouse striatum. Infarct areas at the level of the mid-striatum were similar in both mouse lines (4.30±0.51 vs. 4.21±0.47 mm2).

4.2.2 ABCB1 and ABCC1 are inversely regulated upon ischemia in wildtype and ApoE$^{-/-}$ mice

To study ABC transporters expression, we used brain microvascular extracts, which did not reveal any GFAP protein expression (Figure 23A), indicating absence of astrocytic endfeet in these samples. Importantly, ABCB1, which is upregulated on ischemic cerebral microvessels of wildtype mice (Spudich et al., 2006), was downregulated (Figure 23B), whereas ABCC1, which is expressed at reduced level on ischemic brain capillaries of wildtype mice (Kilic et al., 2008), was overexpressed upon ischemia in ApoE$^{-/-}$ animals (Figure 23C). Real time-polymerase chain reaction analysis (RT-PCR) demonstrated that *abcb1* mRNA was upregulated upon stroke in wildtype, but not ApoE$^{-/-}$ mice, whereas *abcc1* mRNA was elevated after ischemia in ApoE$^{-/-}$ but not wildtype mice (Figure 23D), thus confirming differential expression of both ABC transporters on the transcriptional level.

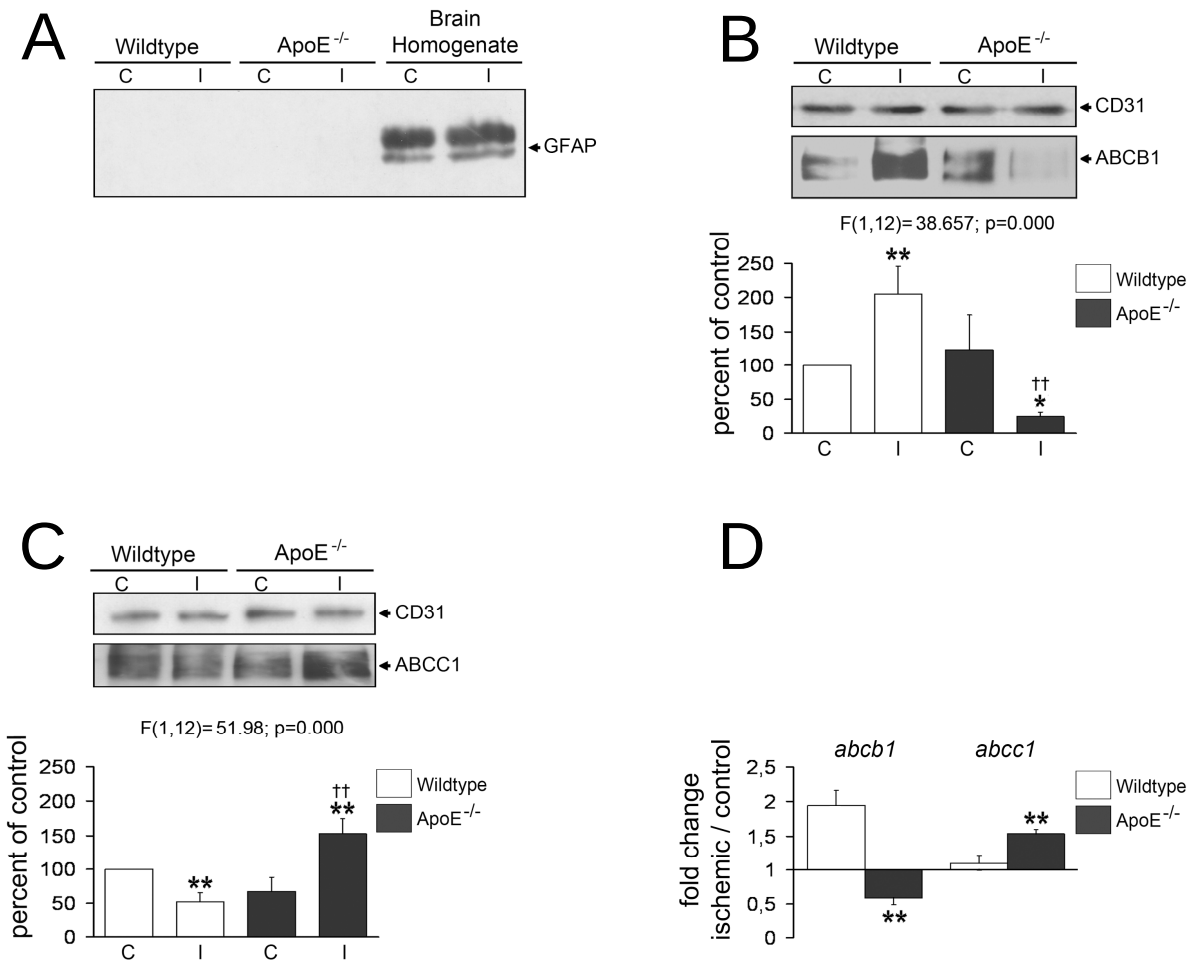

Figure 23. ABC transporters are inversely regulated upon ischemia in the presence and absence of ApoE: Western blot analysis using brain capillary extracts showing that (A) GFAP is expressed neither in non-ischemic nor in ischemic microvessels of wildtype mice and ApoE$^{-/-}$, and (B) ABCB1, which is upregulated in wildtype animals, is downregulated, whereas (C) ABCC1, which is expressed at reduced level in wildtype mice, is overexpressed following stroke in ApoE$^{-/-}$ mice. RT-PCR studies reveal that (D) *abcb1* mRNA expression is induced in the ischemic cerebral microvessels of wildtype but not ApoE$^{-/-}$ mice, whereas *abcc1* mRNA is reduced in ApoE$^{-/-}$ but not wildtype mice. Data are means ± SD (n = 4 separately processed Western blots/ RT-PCR). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p< 0.05 / **p< 0.01 compared with non-ischemic wildtype // ††p< 0.01 compared with non-ischemic ApoE$^{-/-}$ (B, C: one-way analysis of variance (ANOVA) followed by LSD tests; two-way ANOVA were also computed, from which mouse line x hemisphere interaction effects are shown; D: unpaired two-tailed t-tests).

4.2.3 ApoE and its receptor, ApoE receptor-2 (ApoER2), are present on ischemic brain capillaries

To evaluate whether ApoE is present in cerebral microvessels, Western blots and immunohistochemistry were used. These studies revealed that ApoE is *de novo* present on ischemic brain capillaries (Figure 24A) together with its receptor ApoER2, which is constitutively expressed on microvascular cells of wildtype and ApoE$^{-/-}$ mice, namely on the abluminal endothelial surface, in between the CD31+ luminal endothelial surface and GFAP+ astrocytic endfeet (Figure 24B).

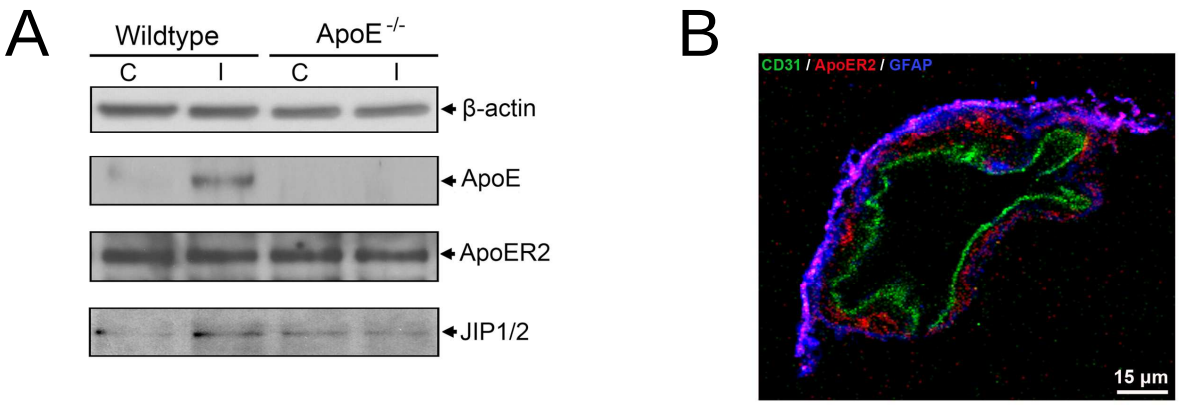

Figure 24. ApoE and its receptor ApoER2 are expressed on cerebral microvessels: Western blotting (A) and immunohistochemical (B) analysis examining the presence and absence of ApoE and ApoER2 in mouse cerebral capillary extracts. In wildtype mice, ApoE appears on cerebral microvessels after MCA occlusion together with its receptor ApoER2 (A), which is constitutively expressed on endothelial cells, particularly on their abluminal surface, i.e. in between the luminal CD31+ endothelial membrane and GFAP+ astrocytic endfeet (B). C, contralateral non-ischemic microvessels; I, ischemic microvessels. Bar, 15 µm.

4.2.4 ApoER2 binds Jun kinase-1/2 (JNK1/2), its kinase and phosphatase via JNK1/2-interacting protein-1/2 (JIP1/2)

ApoER2 has previously been demonstrated to form complexes with Jun N-terminal kinase-1/2 (JNK1/2)-interacting protein-1/2 (JIP1/2) in rat total brain extracts (Verhey et al., 2001; Stockinger et al., 2001). To assess whether ApoER2 also binds JIP1/2 in brain capillaries, co-immunoprecipitation studies were performed. These experiments revealed that ApoER2 indeed forms complexes with JIP1/2, furthermore binding JNK1/2 (Figure 25A), JNK1/2's kinase mitogen-activated protein kinase (MAPK) kinase-7 (MKK7) and JNK1/2's phosphatase MAPK phosphatase-7 (MKP7) (Figure 25B).

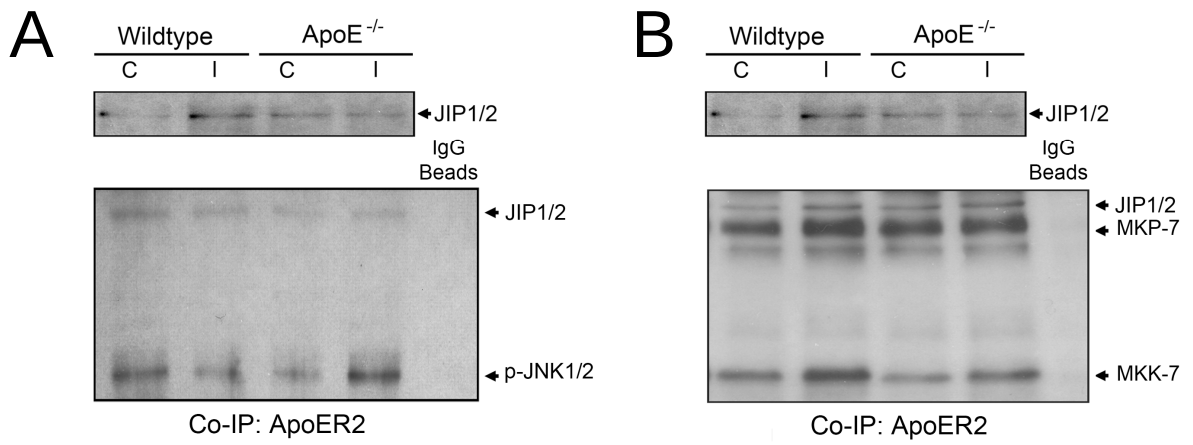

Figure 25. ApoER2 signaling complex at the cerebral microvessels: Western blotting (C, D), and co-immunoprecipitation (C, D) analysis examining the expression of JIP-1/2 (C,D) and the interaction of ApoER2 with JIP1/2, phosphorylated JNK1/2, MKP-7 and MKK-7 in mouse cerebral capillary extracts. ApoER2 constitutively forms a complex with JIP1/2 (C, D), furthermore binding JNK1/2 (C), its kinase MKK7 and phosphatase MKP7 (D) in ApoE$^{-/-}$ and wildtype cerebral capillary extracts. C, contralateral non-ischemic microvessels; I, ischemic microvessels.

4.2.5 ApoE$^{-/-}$ abolishes post-ischemic deactivation of JNK1/2, thereby overactivating c-Jun

To examine whether the presence or absence of ApoE affects the phosphorylation (i.e activation) of JNK1/2 and its downstream signal, the transcription factor c-Jun using Western blots were used. These studies revealed that in the presence of ApoE, levels of phosphorylated JNK1/2 were reduced in ischemic microvessels (Figure 26A). As a consequence, levels of phosphorylated (i.e. activation) c-Jun were low, indicating that the transcription factor was inactive (Figure 26B). In the absence of ApoE, levels of phosphorylated JNK1/2 increased (Figure 26A), which went along with increased phosphorylation, i.e., overactivation of c-Jun (Figure 26B).

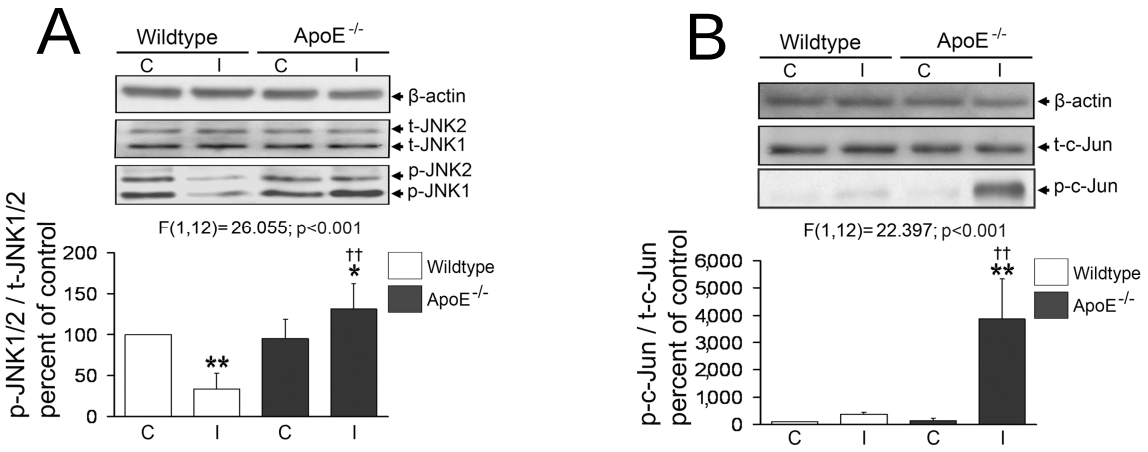

Figure 26. ApoE depletion abolishes post-ischemic deactivation of JNK1/2, thereby overactivating the transcription factor c-Jun: Western blot analysis shows that in the presence of ApoE, phosphorylated JNK1/2 is reduced (A) and phosphorylated c-Jun is low (B). In the absence of ApoE, levels of phosphorylated JNK1/2 increase (A), which goes along with elevated phosphorylation of c-Jun (B). Data are means ± SD (n = 4 separately processed Western blots). C, contralateral non-ischemic microvessels; I, ischemic microvessels. *p< 0.05 / **p< 0.01 compared with non-ischemic wildtype // ††p< 0.01 compared with non-ischemic ApoE$^{-/-}$ (one-way analysis of variance (ANOVA) followed by LSD tests; two-way ANOVA were also computed, from which mouse line x hemisphere interaction effects are shown).

4.2.6 Delivery of recombinant human ApoEε3 or of JNK1/2 inhibitor restores ABC transporter levels

To test whether ApoE is indeed responsible for the coordinated expression changes of ABCB1 and ABCC1 after stroke, mice submitted to 30 min MCA occlusion were intraperitoneally treated either with recombinant human ApoEε3 (50 µg) or with the pharmacological JNK1/2 pathway inhibitor SP600125 (1.25 mg). Indeed, both recombinant human ApoEε3 and SP600125 attenuated the phosphorylation of JNK1/2 (Figure 27A) and c-Jun (Figure 27B) induced by ApoE$^{-/-}$ and restored ABCB1 (Figure 28A) and ABCC1 (Figure 287B) levels. These studies confirmed that ApoE regulates both ABC transporters in the ischemic brain.

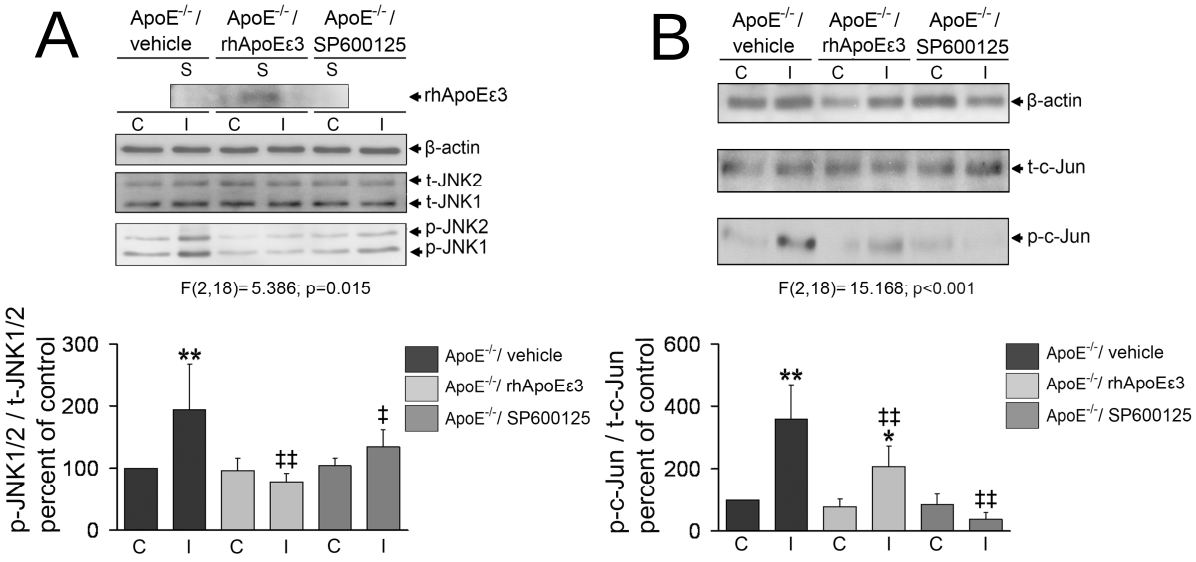

Figure 27. Recombinant human ApoEε3 and pharmacological JNK1/2 blockade restore JNK1/2 activation: Intraperitoneal delivery of recombinant human ApoEε3 (50 µg) or of the JNK1/2 pathway inhibitor SP600125 (1.25 mg) to ApoE$^{-/-}$ mice attenuates the post-ischemic phosphorylation (i.e., activity) of JNK1/2 (A) and c-Jun (B), C, contralateral non-ischemic microvessels; I, ischemic microvessels; S, serum. *p< 0.05 / **p< 0.01 compared with non-ischemic ApoE$^{-/-}$ // ‡p< 0.05 / ‡‡p< 0.01 compared with ischemic ApoE$^{-/-}$ (one-way ANOVA followed by LSD tests; treatment x hemisphere interaction effects from two-way ANOVA are also shown).

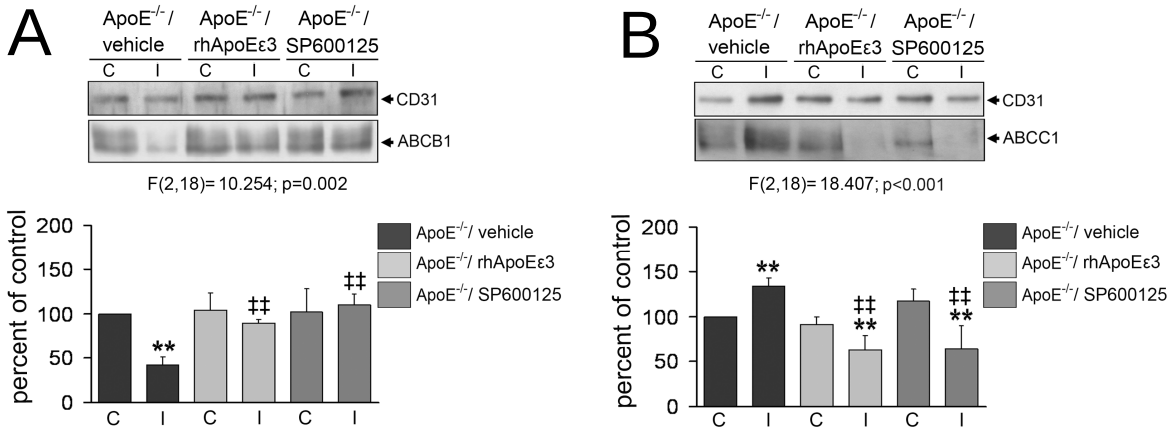

Figure 28. Recombinant human ApoEε3 and pharmacological JNK1/2 blockade restore ABC transporters expression: Intraperitoneal delivery of recombinant human ApoEε3 (50 µg) or of the JNK1/2 pathway inhibitor SP600125 (1.25 mg) to ApoE$^{-/-}$ mice replenishes ABCB1 (C) and mitigating ABCC1 (D) levels. These data show that ApoE controls ABC transporter expression in a JNK1/2-dependent way. Data are means ± SD (n = 4 Western blots). C, contralateral non-ischemic microvessels; I, ischemic microvessels; S, serum. *p< 0.05 / **p< 0.01 compared with non-ischemic ApoE$^{-/-}$ // ‡p< 0.05 / ‡‡p< 0.01 compared with ischemic ApoE$^{-/-}$ (one-way ANOVA followed by LSD tests; treatment x hemisphere interaction effects from two-way ANOVA are also shown).

4.2.7 ApoE deficiency enhances FK506 accumulation in ischemic brain, whereas ApoE delivery partly restores drug elimination

To evaluate whether the downregulation of ABCB1 in ApoE$^{-/-}$ mice increases the brain passage of FK506 (tacrolimus), a well-known ABCB1 substrate, enzyme-linked immunosorbent assays (ELISA) (Spudich et al., 2006) were used. Whole blood FK506 concentrations were very similar in wildtype and ApoE$^{-/-}$ mice, both when vehicle and recombinant human ApoEε3 were delivered (Figure 29A). Importantly, ApoE$^{-/-}$ increased the brain-to-blood concentration ratio of FK506 in the ischemic tissue by more than five times (Figure 29B). Conversely, recombinant human ApoEε3 delivery reduced the brain uptake (Figure 29B). Our data demonstrate that by regulating ABC transporter levels, ApoE controls drug accumulation in the ischemic brain.

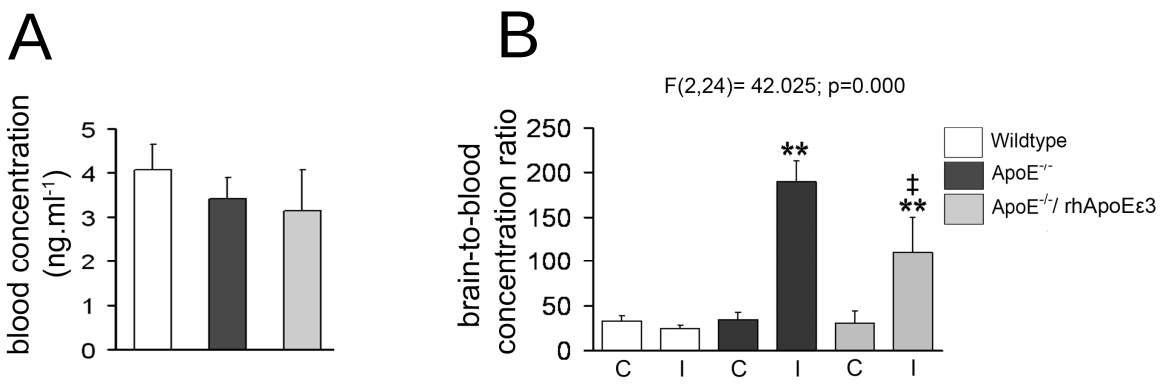

Figure 29. ApoE depletion enhances ABCB1 substrate (FK506) accumulation in the ischemic brain that is partly reversed by human ApoEε3: ELISA for the ABCB1 substrate FK506 showing no difference of FK506 levels in whole blood samples of vehicle treated wildtype mice, vehicle treated ApoE$^{-/-}$ mice and recombinant human ApoEε3 treated ApoE$^{-/-}$ mice (**A**). Interestingly, ApoE$^{-/-}$ increases brain FK506 levels in the ischemic, but not non-ischemic MCA territory by more than five times (**B**). This elevation is attenuated by recombinant human ApoEε3 (**B**). Data are means ± SD (*n* = 5 animals per group). C, contralateral hemisphere; I, ischemic hemisphere. **p< 0.01 compared with ischemic wildtype // ‡p< 0.05 compared with ischemic ApoE$^{-/-}$ (one-way ANOVA followed by LSD tests; treatment x hemisphere interaction effects from two-way ANOVA are also shown).

4.3 Part III: Effect of hypercholesterolemia on the ischemic BBB

To elucidate the effect of hypercholesterolemia on BBB tightness and polarity after stroke, we subjected C57Bl6/j mice fed with regular (i.e., normal) diet or cholesterol-rich (so-called Western) diet to 30 min MCA occlusion. Using protein expression, interaction studies and enzymatic activity assays, we investigated the effect of hypercholesterolemia on ischemic injury, brain edema and BBB function.

4.3.1 Increased brain swelling and IgG extravasation in animals receiving cholesterol-rich (Western) diet

To evaluate the reproducibility of ischemia, LDF measurements were analyzed. LDF values decreased to 20.32±9% and 18.1±10% of baseline within 5 min after onset of ischemia in animals receiving normal and Western diet, remaining stable throughout the MCA occlusion period. After reperfusion, LDF values rapidly recuperated to 82.5±17% and 91±21 of baseline.

Twenty four hours after reperfusion, focal ischemic injury was observed after animal sacrifice mainly in the striatum. As such, mild brain edema (Figure 30A) associated with modest IgG extravasation (Figure 30B) was noticed in animals on normal diet. Western diet markedly increased brain swelling and IgG extravasation (Figure 30A, B). Interestingly Infarct areas did not differ between groups (Figure 30C).

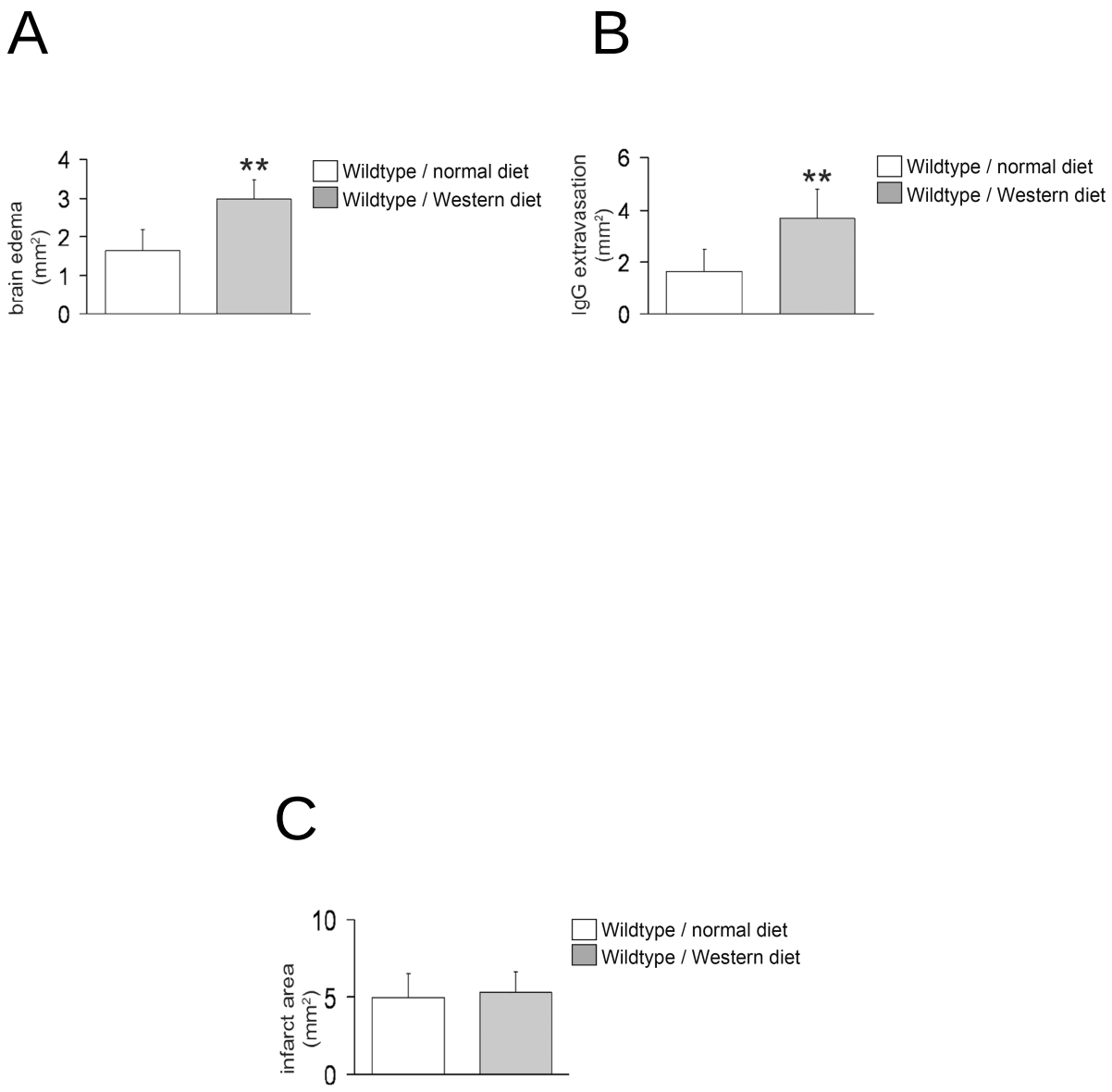

Figure 30. BBB permeability is increased in hypercholesterolemic mice submitted to MCA occlusion: Brain edema (A), serum IgG extravasation (B), and infarct area (C) measured at the level of the midstriatum 24 hours after reperfusion in wildtype mice receiving regular (i.e., normal) or cholesterol-rich (i.e., Western) diet. Note the absence of infarct area (C) changes in Western diet mice. Data are means ± SD (*n* = 6 animals per group). **p< 0.01 compared with normal diet (unpaired two-tailed t-tests).

4.3.2 Overactivation of calpain-1/2 and MMP-2/9 in ischemic microvessels of hypercholesterolemic mice

To elucidate the effects of hypercholesterolemia on calpain-1/2 and MMP-2/9 activity, which contribute to BBB breakdown (Chaitanya and Babu, 2008; Kamada et al., 2007), microplate assays and gelatine zymography were used. Calpain-1/2 and MMP-2/9 activities did not differ between groups in non-ischemic tissues (Figure 31A). Upon ischemia, calpain-1/2 protease activity increased in cerebral microvessels of normal diet mice (Figure 31A). This activation was augmented by the Western diet (Figure 31A).

Since the microplate assay evaluates protease activity in the presence of endogenous inhibitors, we checked the expression of calpain-1/2's endogenous inhibitor calpastatin by Western blots. Thus, calpastatin was downregulated by ischemia, more strongly in animals receiving Western than normal diet (Figure 31B), thus providing an explanation for the overactivation of calpain-1/2.

It has been shown that MMP-9 activity is negatively regulated by calpain-1/2 inhibitors (Tsubokawa et al., 2006), suggesting that calpains mediate, at least partly, MMPs activation. Therefore in addition to calpian-1/2, the activation of MMP-2/9 was investigated using gel zymography and gelatinas microplate assays. Similarly to calpain-1/2, MMP-2/9 activity was increased in ischemic microvessels, as shown in gel zymography (Figure 31C) and microplate assay (Figure 31D), more strongly in animals receiving Western than normal diet. Our data suggest that both MMP-2/9 and calpain-1/2 may contribute to the elevated BBB permeability induced by hypercholesterolemia.

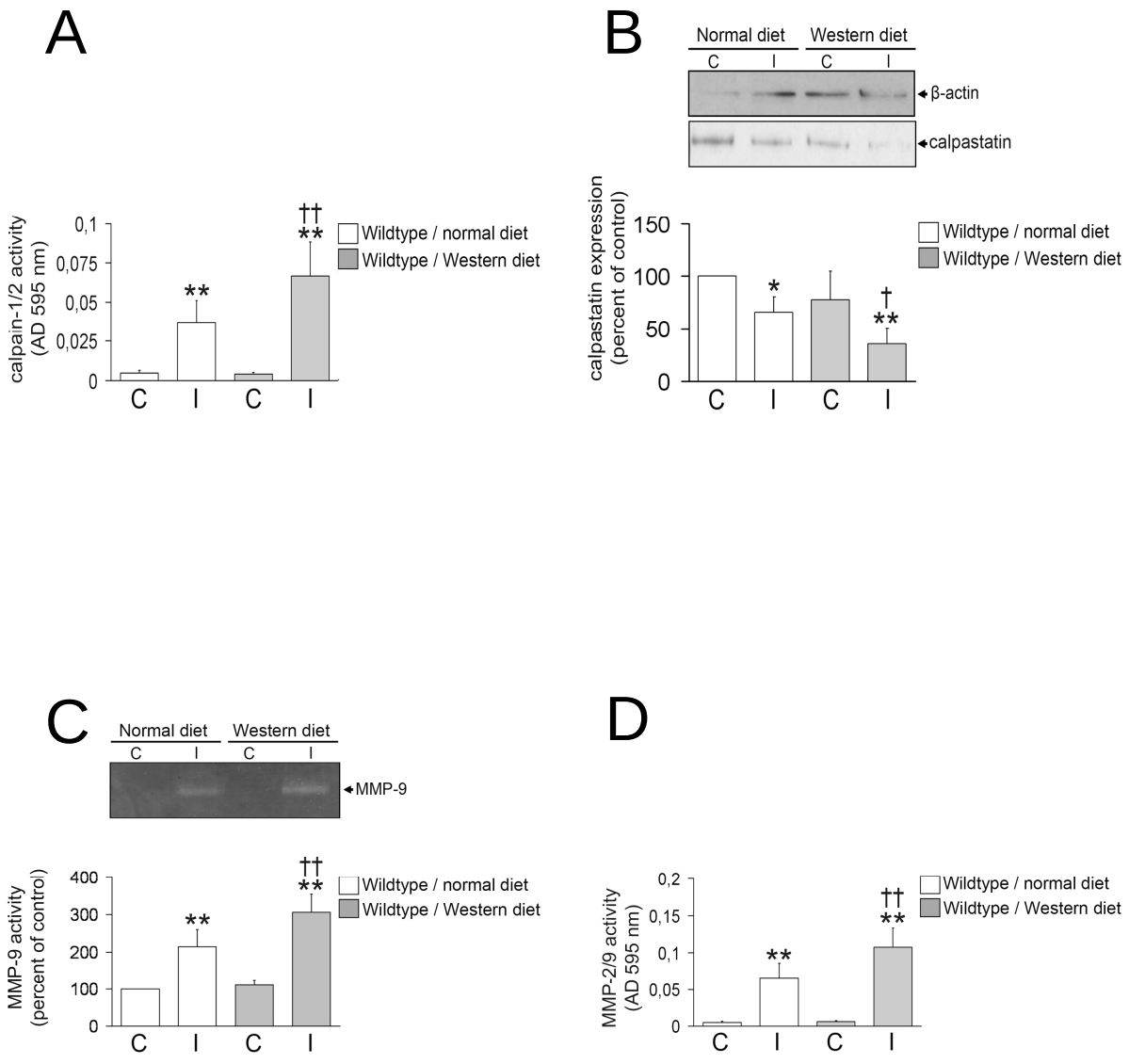

Figure 31. Calpain-1/2 and MMP-2/9 activities are elevated in cerebral microvessels of ischemic mice receiving cholesterol-rich diet: Caseinase microplate assay for calpain-1/2 (A), Western blot for calpain-1/2's endogenous inhibitor calpastatin (B), gelatine gel zymography for MMP-9 (C) and gelatinase microplate assay for MMP-2/9 (D) using brain capillary extracts from mice submitted to MCA occlusion. Note that in parallel with the activation of calpain-1/2 (A) and MMP-2/9 (C, D) calpain-1/2's endogenous inhibitor calpastatin, which is downregulated by Western diet. Data are means ± SD (*n* = 4 assays, zymographies or blots per group). C, contralateral non-ischemic; I, ischemic. *p< 0.05 / **p< 0.01 compared with corresponding non-ischemic/ †p< 0.05 / ††p< 0.01 compared with ischemic normal diet (one-way analysis of variance (ANOVA) followed by LSD tests).

4.3.3 Cholesterol-rich diet enhances the activation of RhoA and downregulates tight junction protein occludin

RhoA is a member of the Rho GTPases proteins family that has previously been shown to destabilize tight junctions, thus promoting BBB breakdown (van Nieuw Amerongen et al., 2000; Schreibelt et al., 2007). To elucidate whether RhoA is responsible for the induction of BBB permeability by hypercholesterolemia, we used Western blots, pull-down assay experiments. Western blots analysis showed that ZO-1 expression was not altered in response to stroke, neither under conditions of normal nor Western diet (Figure 32A). On the other hand, the expression of occludin decreased upon ischemia in cerebral microvessels of normocholesterolemic mice (Figure 32A). Hypercholesterolemia further reduced occludin expression (Figure 32A). Using pull-down assays, we showed that RhoA activity increased upon ischemia in brain microvessels of normocholesterolemic mice (Figure 32B). Western diet further increased RhoA activation (Figure 32B).

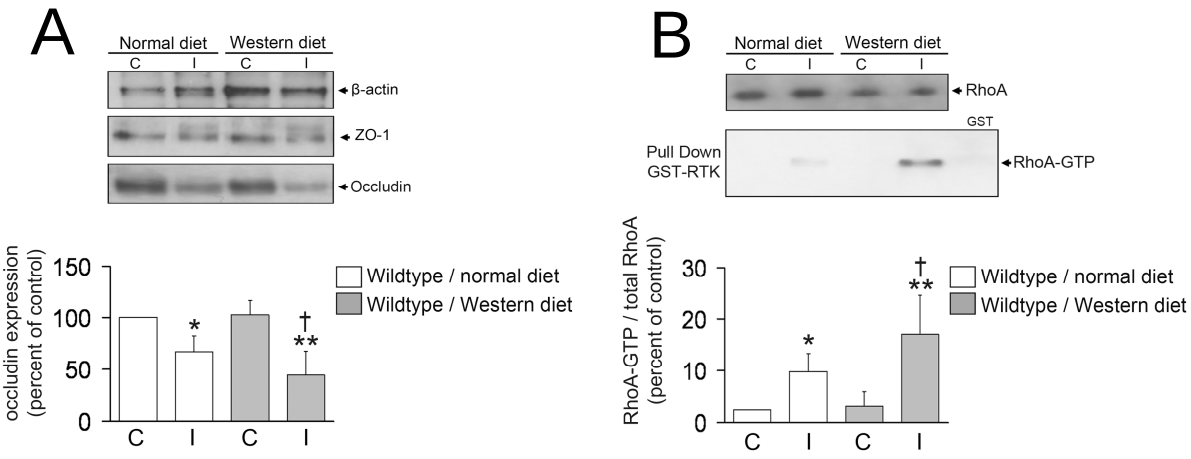

Figure 32. Western diet activates RhoA, resulting in tight junction protein occludin downregulation: Western blots for the tight junction proteins occludin and zona occludens (ZO)-1 (A). Western blots and pull-down assay for RhoA (B). Data are means ± SD (*n* = 4 Western blots, pull-down assays). C, contralateral non-ischemic; I, ischemic. *p< 0.05 / **p< 0.01 compared with corresponding non-ischemic/ †p< 0.05 compared with ischemic normal diet (one-way analysis of variance (ANOVA) followed by LSD tests).

4.3.4 Angiotensin II type 1 receptor (AT1R) upregulation in cholesterol-rich diet enhances the activation of RhoA in ischemic brain microvessels

To investigate part of the upstream signaling pathways that contributes in RhoA activation, Western blots and co-precipitation experiments were used. Angiotensin II type 1 receptor (AT1R) activation by angiotensin II induces vasoconstriction in many vascular systems by at leats partly induicing RhoA activation (Ito et al., 1991). It has been shown that hypercholesterolemia and hypertension increase the expression of AT1R, leading to endothelial cell wall dysfunction (Watanabe et al., 2005). Using Western blot analysis, we show here that ischemic stroke did not influence AT1R expression, whereas the expression of AT1R has increased in ischemic cerebral microvessels of hypercholesterolemic mice (Figure 33A).

Moreover, AT1R has been shown to induce RhoA activation by directly enhancing RhoA access and activation by its GEF, leukemia associated Rho-GEF LARG (Ying et al., 2006). Western blots analysis show an increased expression of LARG in ischemic cerebral microvessels of hypercholesterolemic mice (Figure 33B). Using co-precipitation experiments with glutathione-S-transferase tagged wildtype RhoA (GST-RhoA), showed an enhanced interaction between RhoA and LARG (Figure 33C) in ischemic cerebral microvessels of hypercholesterolemic mice, providing a link between AT1R upregulation and RhoA overactivation upon stroke under hypercholesterolemia conditions.

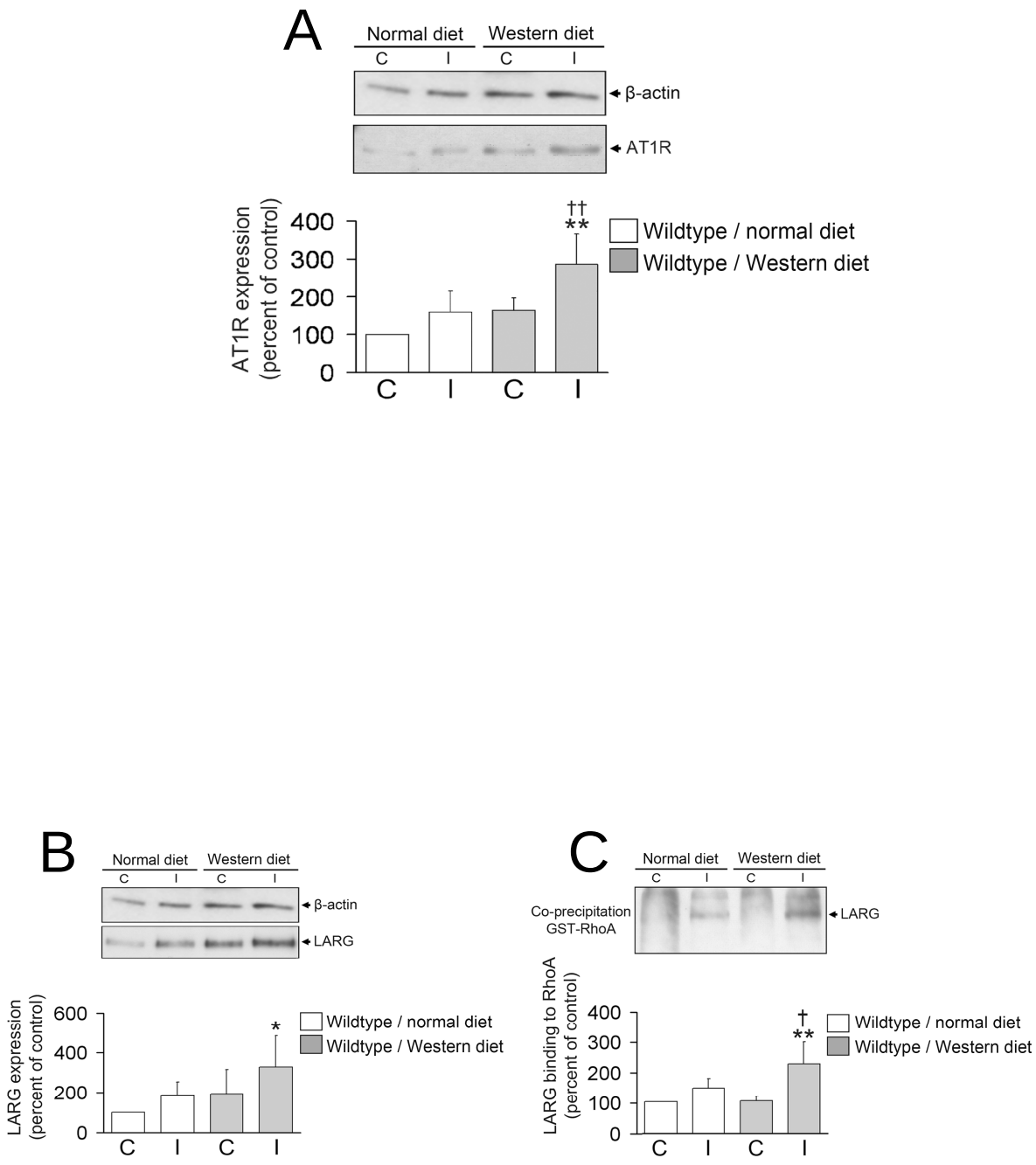

Figure 33. AT1R is upregulated in Western diet, overactivating RhoA through its GEF, LARG: Western blot for AT1R (A), Western blot for LARG (B), co-precipitation study exhibiting interaction of RhoA and LARG following Western diet (C). Data are means ± SD (*n* = 4 Western blots and co-precipitation studies per group). C, contralateral non-ischemic; I, ischemic. *p< 0.05 / **p< 0.01 compared with corresponding non-ischemic/ †p< 0.05 compared with ischemic normal diet (one-way analysis of variance (ANOVA) followed by LSD tests).

4.3.5 Hypercholesteroleia does not influence ABC transporter expression

Many ABC transporters play an important role in the transport of lipids. ABC transporters regulation at the BBB by lipid serum levels remains unclear. Besides the drug transporters ABCB1 and ABCC1, we investigated the expression of two important lipid transporters ABCA1 and ABCG1 at the BBB. By using Western blot analysis, we showed that the expression of ABCA1, ABCG1, ABCB1 and ABCC1 is similar in the ischemic microvessels of normal and hypercholesterolemic mice (not shown). This result shows that BBB polarity upon stroke is not influenced by hypercholesterolemia.

5 DISCUSSION

5.1 General aspects

BBB tightness and polarity are crucial for a proper function of the CNS. We show in this study that BBB tightness and polarity both respond to ischemic stroke, and that both features are differentially regulated on the molecular level. As such, the molecular mechanisms involved in controlling BBB tightness do not regulate BBB polarity and vice versa. As such, protecting the tightness of the BBB offers new approaches in limiting incidence and severity of many cerebrovascular diseases, such as stroke, and controlling BBB polarity may allow to develop new approaches in delivering drugs to the ischemic brain.

By using LXR agonist we showed a novel mode of action of LXR at the BBB, i.e. preservation of BBB tightness. As such, we showed that LXR activation reduces brain swelling and promotes BBB tightness, without influencing the polarity of ABC transporter expression after stroke. Our results reveal a hitherto unknown vascular protective mechanism, which potentially offers itself for clinical use under conditions of stroke-related brain edema in patients with malignant stroke. In these patients, brain edema leads to a rise in intracranial pressure that critically affects the patients' prognosis. Until now there are still no therapies available allowing to antagonize BBB breakdown.

By using knockout mice not expressing the lipoprotein ApoE, we furthermore demonstrated that ApoE controls BBB polarity presumably through ApoER2 and regulates the post-ischemic expression of ABCB1 and ABCC1, thereby controlling drug accumulation in the ischemic brain. As such, our study unraveled a novel target via which the access of systemically delivered drugs into the brain may be improved. The blood-brain access of drugs is impeded by ABC transporters, which, as we previously showed are regulated upon stroke in a coordinated way that makes drug access difficult (Spudich et al., 2006; Kilic et al., 2008). In our studies we furthermore showed that the regulation of BBB polarity by ApoE does not go along with changes in BBB tightness. Hence, different mechanisms control both features of brain endothelial cells.

Stroke occurs mainly in persons with vascular risk factors, such as hypercholesterolemia. For this purpose we examined the effect of a cholesterol-rich diet on the BBB after stroke, showing an increased permeability but unchanged polarity of the ischemic BBB in hypercholesterolemic animals. Our observations are highly relevant with respect to translational studies in ischemic stroke, which so far are mainly performed in animals on a regular, cholesterol-restricted diet. Such animals do not adequately reflect the pathophysiology of BBB changes under conditions of hypercholesterolemia, as we now demonstrate.

5.2 Part I: LXR activation enhances BBB tightness but not polarity in the ischemic brain

There is now a large body of evidence indicating the importance of endothelial integrity in BBB. At the acute phase of ischemic stroke, the excessive Ca2+ influx into endothelial cell activates many molecular mechanisms that compromise BBB tightness, exacerbating neuronal injury. Therefore, there is now a growing interest in BBB as a therapeutic target. It is an intriguing idea that reversion of BBB function might be used for therapeutic purposes in CNS diseases, where neuronal injury is consequence of vascular disturbances, or where neuronal injury is exacerbated by BBB damage.

For this purpose, we investigated the effect of LXR activation, which has been previously shown to upregulate ABC transporters on BBB tightness, by using mice submitted to focal cerebral ischemia. Using protein expression and interaction studies, we demonstrate, for the first time, that LXR activation promotes post-ischemic BBB tightness by mechanisms involving upregulation of LXR's target gene calpastatin, deactivating calpain-1/2 and stabilizing p120 catenin. p120 catenin inhibits RhoA and activates Cdc42, thus restoring the post-ischemic expression and phosphorylation of TJ proteins and promoting their assembly. Moreover, LXR activation induces ABC transporters expression at the BBB without affecting BBB polarity. Our data provide a detailed analysis of signaling pathways involved in BBB integrity regulation after stroke.

It has previously been shown that the cysteine protease calpain-1/2 promotes brain injury and exacerbates edema, thus presumably increasing BBB permeability (Cao et al., 2007; Sedarous et al., 2003). The signal pathways via which this protease leads to BBB breakdown remained unknown. By showing that calpain-1/2, which is activated upon ischemia as a consequence of the downregulation of its endogenous inhibitor calpastatin (Goll et al., 2003; Sedarous et al., 2003), reduces p120 catenin levels in ischemic microvessels, resulting in the loss of RhoA binding to p120 catenin, a known GDI for RhoA (Anastasiadis et al., 2000; Noren et al., 2000), thus inducing RhoA overactivation, we now provide such mechanism. Indeed, the delivery of LXR agonist T0901317 restored calpastastin, a known LXR target based on transcriptional profiling studies (Hummasti *et al.*, 2004), deactivated calpain-1/2 and stabilized p120 catenin, reconstituting p120 catenin interaction with RhoA and Cdc42, which resulted in RhoA deactivation and, as p120 catenin binds GEFs specifically activating Cdc42, such as Vav2, in Cdc42 overactivation. That LXR activation deactivates calpain-1/2 is new, and modulation of Rho GTPases by LXR agonists to the best of our knowledge has not yet been shown.

In migrating cells RhoA activation is followed by Cdc42 activation (Machacek et al., 2009). That both Rho GTPases respond conversely to LXR activation is noteworthy and may offer a clue about why the LXR agonist treatment promotes BBB tighntess. RhoA and Cdc42 have opposite effects on BBB tightness. RhoA activation destabilizes tight junction complexes via stress fibre formation, thereby inducing BBB leakage (Schreibelt et al., 2007; Chrissobolis and Sobey, 2006; Hirase et al, 2001), whereas Cdc42 activation promotes the assembly of tight junction proteins, thus enabling BBB tightness (Ramchandran et al., 2008; Cau et al., 2005; Fukuhara et al., 2003). In our studies, the shift between RhoA and Cdc42 balance was accompanied by profound changes in the expression, phosphorylation and interaction of tight junction proteins occludin and ZO-1. We presume that the differential response of RhoA and Cdc42 to the LXR agonist is a consequence of specific interactions with p120 catenin, which has differential effects on activation of both small GTPases (Anastasiadis et al., 2000; Noren et al., 2000). Besides modulating the activation of Rho GTPases, T0901317 also deactivated MMP-2/9, JNK1/2 and caspase-3 in cerebral microvessels, which may at least partly represent downstream signals of the reduced calpain-1/2 activity. Previous studies already suggested a role of calpains in MMP-9 activation in the stroke brain (Tsubokawa et al., 2006). In microvascular

endothelial cells, calpain-1 was shown to translocate into mitochondria upon activation, inducing oxidative bursts activating MMP-2/9 (Moshal et al., 2006). Indeed, calpain-1 inhibitors blocked the formation of reactive oxygen species and prevented the MMP-9 activation (Tsubokawa et al., 2006). Regarding caspase-3, the proteolytic capacity of calpain-2 to facilitate the activation this executioner caspase is well-known. As such, calpain-2 degrades the caspase-3 full length proform into a 29kDa fragment that may subsequently be cleaved more easily to active forms (Blomgren et al., 2001). This degradation has been shown to contribute to neonatal hypoxic-ischemic neuronal injury (Blomgren et al., 2001).

More likely as a direct consequence of the activation of LXR, T0901317 treatment increased expression levels of ABC transporters after stroke. As such, not only the lipid transporters ABCA1 and ABCG1, which are known targets of LXR based on transcriptional profiling studies (Naik et al., 2006; Hummasti et al., 2004) and which have been shown to be induced by T0901317 in non-cerebral vessels (Efanov et al., 2004; Bradley et al., 2007), but also the multidrug resistance transporters ABCB1 and ABCC1, which control the distribution of pharmacological compounds at the blood-brain interface and for which regulation by LXR has so far not been reported, were elevated in cerebral microvessels upon LXR activation. We interpret the elevation of ABC transporters as part of a coordinated response aiming at the maintenance of cerebral homeostasis. Interestingly, both ABC transporters, ABCB1 which is exclusively expressed at the luminal endothelial membrane and ABCC1 which is exclusively expressed at the abluminal endothelial membrane, were upregulated at the BBB. This observation shows that LXR activation does not affect BBB polarity upon stroke, therefore suggesting other mechanisms had to be involved in regulating BBB polarity, which still needed to be assessed.

The preservation of BBB tightness by T0901317 offers new perspectives for the treatment of cerebrovascular diseases, where LXR agonists might be used for the treatment of vasogenic edema. Since LXR activation promotes BBB tightness, at the same time providing vascular protection, LXR agonists possess a particularly promising profile that might render them suitable for therapeutic purposes.

5.3 Part II: Apolipoprotein-E controls BBB polarity without influencing BBB tightness

It has previously been shown that ABC transporters are regulated in a coordinated way on ischemic brain microvessels. As such, the luminal transporter ABCB1 was upregulated (Spudich et al., 2006) while the abluminal transporter ABCC1 was downregulated in response to stroke (Kilic et al., 2008). BBB polarity is an importing aspect in ABC transporters expression and function. As such, the luminal upregulation of ABCB1 allows drug extrusion from the ischemic brain, whereas the abluminal downregulation of ABCC1 decreases drug uptake by the ischemic brain. The mechanisms involved in BBB polarity and in the differential expression of ABC transporters upon stroke are largely unknown.

By using protein and gene expression studies we demonstrated that ABCB1 and ABCC1 are regulated on the transcription level after focal cerebral ischemia and that ApoE regulates the coordinated expression changes of both ABC transporters on the luminal and abluminal endothelial membrane, thereby controlling the accumulation of pharmacological compounds in the brain tissue.

That the lipoprotein ApoE, which is involved in reverse cholesterol transport in the blood, regulates BBB polarity is new. ApoER2 is strongly expressed on brain capillaries, besides neurons (Korschineck et al., 2001). In neurons, ApoER2 has been shown to control synaptic plasticity, acting as receptor for the extracellular matrix molecule reelin (Beffert et al., 2005). ApoER2's role on vascular cells remained elusive until now.

Interestingly, ApoER2 was particularly strongly expressed on the abluminal surface of brain capillary cells in our study. This might explain why ApoE controls ABC transporter expression in ischemic, but not in non-ischemic brain tissue. As such, ApoER2 might act as a sensor for post-ischemic BBB breakdown, regulating ABC transporters when blood-derived proteins, namely ApoE, enter the brain parenchyma, thus contributing to the maintenance of tissue homeostasis.

That ApoER2 forms complexes with JNK1/2 via the scaffold protein JIP1/2 in mouse cerebral microvessels, has already been demonstrated by bidirectional

immunoprecipitation studies in rat total brain extracts (Verhey et al., 2001; Stockinger et al., 2001). JIP1/2 in turn targets MKK-7 and MKP-7 to either phosphorylate or dephosphorylate JNK1/2, as previously shown in vitro (Willoughby et al., 2003). JNK1/2 negatively regulates ABCB1 (Liu et al., 2007) and positively regulates ABCC1 (Shinoda et al., 2005) transcription, as demonstrated by others in non-small and small cell lung adenocarcinoma cells. Transcription regulation by JNK1/2 is mediated by c-Jun binding to the activator protein-1 binding site of the promoter of both genes (Shinoda et al., 2005; Liu et al., 2007). We herein confirmed that c-Jun is indeed phosphorylated, i.e., overactivated upon ischemia in ApoE$^{-/-}$ cells, thus downregulating ABCB1 and upregulating ABCC1.

In biodistribution studies we show that the expression changes of ABCB1 and ABCC1 have a profound impact on drug biodistribution, as revealed after systemic delivery of the neuroprotectant FK506, an ABCB1 substrate (Spudich et al., 2006). As such, the reduced expression of ABCB1 in ApoE$^{-/-}$ mice was associated with a five times increased access of FK506 into the ischemic brain tissue, an observation that was partly reversed after delivery of human ApoEε3.

That ApoE controls drug biodistribution offers fascinating perspectives for translation studies to human stroke patients (Figure 34). Future studies will have to identify, if the regulation of human ABC transporters resembles that of mice. In the case, the receptor ApoER2 might be targeted to increase the accumulation of pharmacological compounds in the brain tissue.

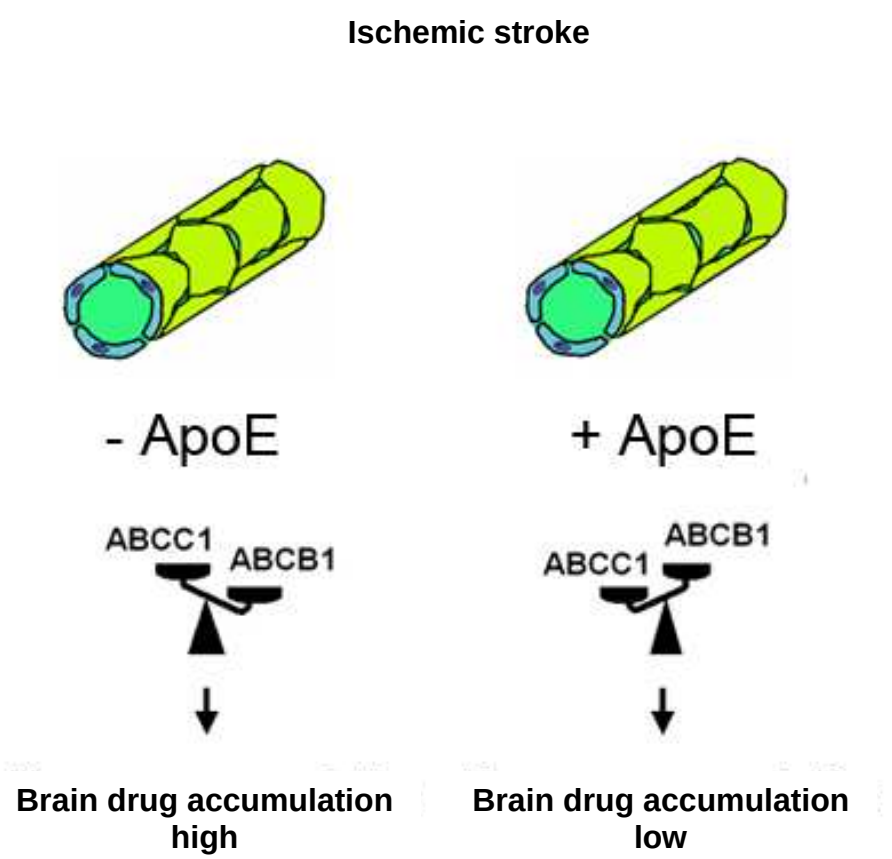

Figure 34. ApoE regulates the expression of ABC transporters at the BBB: Upon ischemic stroke ABCB1 is upregulated at the luminal side of BBB and ABCC1 is downregulated at the abluminal side of BBB, therefore decreasing drug uptake by the brain. Our findings show that upon ischemic stroke ApoE is expressed on brain microvessels, and controls ABCB1 and ABCC1 via its receptor ApoER2 that is constitutively expressed at the BBB.

5.4 Part III: Hypercholesterolemia impairs post-ischemic BBB tightness but not polarity

The accumulation of lipids into endothelial walls forming vessels is one of the most important factors contributing to vascular dysfunction (Adams et al., 2000). The effect of hypercholesterolemia on the cardiovascular system is well studied (Paterick and Fletcher, 2001), where lipid accumulation into artery walls causes artery thickening, therefore hardening vessels wall, affecting directly blood flow and inducing multiple inflammatory responses, which compromise endothelial function (Baldassarre et al., 2002).

Until now there are no reports that explicitly investigated the direct effect of hypercholesterolemia on BBB integrity after ischemic stroke. In our present studies we showed that hypercholesterolemia enhances vascular permeability without altering the expression of ABC transporters along the BBB, presumably via mechanisms involving the overactivation of calpain-1/2 and MMP-2/9.

In the acute phase of stroke, calpain-1/2 are activated mainly by the intracellular accumulation of Ca^{2+} and the degradation of its endogenous inhibitor calpastatin (Cao et al., 2007), therefore leading to cell cytoskeleton degradation (Zaidi and Narahara, 1989; Stalker et al., 2003). Similarly, MMP-2/9 are activated, contributing in BBB disruption and leakage (Gu et al., 2002; Zhao et al., 2006). Calpain-1/2 and MMP-2/9 are involved in TJ degradation, compromising BBB integrity (Gu et al., 2002; Zhao et al., 2006; Cao et al., 2007). In agreement with these earlier studies, we observed in our here presented experiments that upon ischemia, calpain-1/2 and MMP-2/9 are activated in brain microvessels. Interestingly, hypercholesterolemia exacerbated the activation of both proteases, thus inducing occludin degradation, and contributing to BBB leakage. The increased activation of calpain-1/2 and MMP-2/9 upon ischemic stroke in hypercholesterolemia might be explained by the increased Ca^{2+} intracellular entry, accumulation and endothelial dysfunction that were observed in peripheral vascular system (Bialecki et al., 1991; Perrault et al., 2000).

Interestingly, we found an increased expression of the G protein coupled receptor AT1R in the ischemic brain under hypercholesterolemic conditions. Upon activation, AT1R has a powerful vasoconstriction function (Van Linthout et al., 2009). Vasoconstriction is mediated by the downstream activation of RhoA signalling (Bregeon et al., 2009). Indeed, we observed in our here presented experiments that focal ischemia induces RhoA activation. Since hypercholesterolemia similarly activated a RhoA specific GEF, LARG (Ying et al., 2006; Ying et al., 2009), which is known to be a target of AT1R (Ying et al., 2006), we hypothesize that AT1R might have played a role in mediating BBB permeability induced by hypercholesterolemia.

The induction of BBB permeability by hypercholesterolemia is an important observation with respect to translational studies, as the vast majority of animal experiments is presently conducted with animals receiving a cholesterol-restricted regular diet. Our data suggest that such animals poorly reflect the pathophysiology of a stroke in hypercholesterolemia. Animal models carrying vascular risk factors should more stringently be assessed in the stroke field in the future.

6 CONCLUSION AND OUTLOOKS

In the recent past, the reasons for the failure of clinical trials in ischemic stroke have intensively been discussed. As such, it has been suggested that the poor translation of experimental stroke research and the failure of clinical trials may be attributed to clinically irrelevant animal models and to the focus on clinically irrelevant pathways. It was suggested that more emphasis should be placed on the cerebrovascular system, especially on the key molecular mechanisms involved in the evolution of injury and the mechanisms involved in the secondary injury, such as brain edema (Hossmann, 2009).

As a consequence of such ideas, we now deciphered molecular mechanisms regulating two key features of the cerebrovascular system, i.e., the paracellular tightness of the BBB and the polarity of ABC transporters expressed on the luminal and abluminal membranes of the brain endothelium, showing that both features are independently regulated from each other, the nuclear receptor LXR regulating BBB tightness and the apolipoprotein ApoE changes in the luminal versus abluminal expression of ABC transporters upon stroke.

Originally, LXR have been described as oxysterol sensors playing an important role in cholesterol homeostasis. LXR activation has broader effects on endothelial cell. Our results showed that LXR activation strategy, using synthetic agonists, is an interesting tool that provides endothelial protection and BBB restoration, thereby decreasing brain edema. This novel role of LXR activation might be useful in other CNS diseases, where edema is observed.

ABC transporters might play a predominant role in maintaining brain homeostasis and protecting brain from harmful metabolites generated after cerebral ischemia. As such, it appears reasonable that a luminal transporter, ABCB1, which eliminates toxic molecules from the brain, is upregulated, whereas an abluminal transporter, ABCC1, which carries molecules from the blood to brain, is downregulated after stroke. Our results showed that ApoE controls this elegant defence system, thereby contributing to brain protection against toxins. Notably, ABC transporter expression was not influenced by hypercholesterolemia.

In view of the relevance of this system in preserving BBB from toxins, long term inhibition using molecules that bind ABCB1 and alter its function as carrier efflux would increase the concentration of toxins, which could be problematic for cell survival. From this point of view, ischemic stroke, which is an acute disease, may represent a particularly suitable condition for ABC transporter modulation strategies. ApoER2 in this context represents a promising target, which may synergistically alter the expression and functionality of sets of ABC transporters. We predict that by modulating the activity of ApoER2, the drug accumulation and efficacy may be improved in the ischemic brain in humans.

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8 PERMISSIONS AND IMAGE SOURCES

Part II of this thesis is the accepted version of the manuscript, published in Science Signaling, before Science's copy editing and production. Citation: A. ElAli, D.M. Hermann, Apolipoprotein E Controls ATP-Binding Cassette Transporters in the Ischemic Brain. *Sci. Signal.* **3**, ra72 (2010). Link: http://stke.sciencemag.org/cgi/content/abstract/sigtrans;3/142/ra72

Figure 1 : University of Rochester Medical Center. Link: http://www.urmc.rochester.edu/encyclopedia/images/ei_2397.gif

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Figure 4 : Etienne-Manneville S, Hall A (2002) Rho GTPases in cell biology. *Nature*. 420:629-35. License Number: 2550681309299

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Figure 10 b : Zlokovic BV (2006) Remodeling after stroke *Nat Med.* 12:390-1. License Number: 2550671215356

Figure 14 : GE Healthcare, Life Science. Link: http://www.gelifesciences.co.jp/newsletter/protein_sciences/tag_interaction.asp

9 CURRICULUM VITAE

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Education and qualifications

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10 PUBLICATIONS

1. Daubon T, Chasseriau J, **ElAli A**, Rivet J, Kitzis A, Constantin B, Bourmeyster N (2008) Differential motility of p190bcr-abl- and p210bcr-abl-expressing cells: respective roles of Vav and Bcr-Abl GEFs. ***Oncogene***.

2. Kilic E*, **ElAli A***, Kilic U, Guo Z, Ugur M, Uslu U, Bassetti CL, Schwab ME, Hermann DM (2010) Role of Nogo-A in neuronal survival in the reperfused ischemic brain. ***Journal of Cerebral Blood Flow and Metabolism***. * *equal contribution*

3. Zechariah A, **ElAli A**, Hermann DM (2010) Combination of tissue-plasminogen activator with erythropoietin induces blood-brain barrier permeability, extracellular matrix disaggregation, and DNA fragmentation after focal cerebral ischemia in mice. ***Stroke***.

4. **ElAli A**, Hermann DM (2010) Apolipoprotein E controls ATP-binding cassette transporters in the ischemic brain. ***Science Signaling***.

5. **ElAli A**, Hermann DM (2010) ATP-binding cassette transporters and their roles in protecting the brain. ***The Neuroscientist***. Review. *in press*

6. Reitmeir R, Kilic E, Kilic U, Bacigaluppi M, **ElAli A**, Salani G, Pluchino S, Gassmann M, Hermann DM (2010) Post-acute delivery of erythropoietin induces stroke recovery by promoting peri-lesional tissue remodeling and contralesional pyramidal tract plasticity. ***Brain***. *in press*

7. Doeppner TR, Kaltwasser B, **ElAli A**, Zechariah A, Hermann DM, Bähr M (2010) Acute hepatocyte growth factor treatment induces long-term neuroprotection and stroke recovery via mechanisms involving neural precursor cell proliferation and differentiation. ***Journal of Cerebral Blood Flow and Metabolism***. *in press*

11 ERKLÄRUNG

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema *„Regulation of blood-brain barrier tightness and polarity following ischemic stroke“* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von *(Ayman EL ALI)* befürworte.

Essen, den 23.06.2010

Unterschrift d. wissenschaftl. Betreuers/Mitglieds der Universität Duisburg-Essen

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema *„Regulation of blood-brain barrier tightness and polarity following ischemic stroke“* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von *(Ayman EL ALI)* befürworte.

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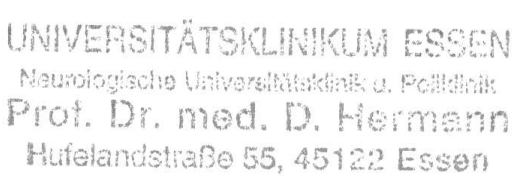

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat. Fakultäten zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 23.06.2010 Ayman EL ALI

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Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat. Fakultätenzur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, den 23.06.2010 Ayman EL ALI

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