Cortical Organotypic Slice Cultures as a Tool to Analyze the Neurovascular Unit in Hypoxia/Ischemia and Hypothermia-Induced Neuroprotection

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SUMMARY

Neurons and glial cells of the central nervous system (CNS) communicate and work together to function and execute an array of complex tasks. In addition to them a third cell type which also works to keep the brain alive are the cerebral endothelial cells that create the vascular system which supply and deliver oxygen and nutrients. The cerebral endothelium is also specialized with a blood-brain barrier (BBB) that is important for protecting the CNS from harmful substances and for regulating access only to certain ions and nutrients for optimal maintenance and support of CNS activities. A complex of tight junction proteins which include occludin, Claudin-1/3, Claudin-5, and ZO1-3 are thought to keep the endothelium impermeable, while a system of transporters, such as GLUT1 and P-gp are involved in regulating the molecular trafficking across the BBB. The endothelium is also characterized by a low pinocytotic activity compared to vessels in peripheral organs. A lot is known on the formation and composition of the BBB, but less is understood on the maintenance. This is probably due to the heterogeneity of the cerebral endothelium, which makes it difficult to study. However, the cerebrovascular function is supported by a combination of interactions with glia, pericytes, and nerve cells, known together as the neurovascular unit (NVU). A number of *in vitro* studies show that co-culturing endothelial cells with astrocytes and pericytes, as well as nerve tissue is capable of expressing tight junction proteins and a tight endothelial barrier, suggesting that cell-cell interactions and production of essential factors such as FGF and TGF-β are involved in BBB maintenance.

Disruption of the BBB is often associated with brain injury and considered to be detrimental to the recovery process. Although the mechanisms are poorly understood, breakdown of the BBB has been suggested to be due to production of matrix-metalloproteinases, growth factors and cytokines. Inflammatory mediators have been well known to modulate BBB permeability. Since inflammation typically follows excitotoxicity in the ischemic cascade to cell death, BBB disruption may be secondary in the injury process. Whether the vascular damage is precipitated by neurodegeneration is unclear. Understanding how the neurovasculature might be affected is important for prevention and treatment of neurovascular diseases, such as stroke or neonatal asphyxia since neuroprotectants, except for therapeutic mild-hypothermia, have failed in the clinics.

Cooling of the head or body down to about 33°C for up to 3 days has so far been the most effective neuroprotective strategy for treating cerebral ischemia observed in stroke or perinatal asphyxia. Along with reducing energy demand and energy consumption, mild hypothermia seems to affect many aspects of cellular injury but overall is very efficient at stopping cell death.

A small group of proteins are expressed during mild-hypothermia. One of them is the RNA binding motif protein 3 (RBM3) and based on *in vitro* studies it is involved in cell proliferation and survival. Whether RBM3 is involved in hypothermia-induced neuroprotection deserves investigation as it may provide some hints into the mechanisms of how hypothermia prevents cell death.

A useful *in vitro* system to study aspects of neurodegeneration that is close to the *in vivo* situation is the organotypic slice culture system, which has been very well established for cortical, hippocampal, and cerebellar tissue. Previously our lab has established an *in vitro* BBB model utilizing the cortical organotypic slice culture system, which preserves tight junction proteins for more than a week under the presence of FGF2. The main objective of my PhD thesis was to analyze the expression of the neuronal and vascular elements of the blood-brain barrier (BBB) under normal and stressed conditions and to study the cell-cell interaction at the biochemical and cellular level utilizing the organotypic slice culture system established in our laboratory. In the first project we have studied the expression of transporter proteins in cortical organotypic slice cultures (COSCs). We could show that transporters such as glucose transporter 1 (GLUT-1) and the ATP-binding cassette (ABC) transporter, P-glycoprotein (P-gp) were present and functional in the blood vessels of COSCs. In the second project we used entorhino-hippocampal organotypic slice cultures (EHOSCs) derived from newborn mice to study the effect of oxygen-glucose deprivation (OGD) as well as excitotoxicity on neurodegeneration and accompanying neurovascular changes. We could show that changes in BBB integrity and vascular remodeling were linked to neurodegeneration. Selective loss of the neurovasculature in CA1 of the hippocampus was preceded by neuronal death indicating that not OGD by itself, but the OGD-induced neurodegeneration was responsible for the loss of local blood vessels. Finally, in the third project we have explored a potential function of the cold-inducible RNA binding motif protein 3 as a neuroprotectant in response to mild hypothermia that is employed after neonatal asphyxia. We could show that RBM3 expression was strongly induced in COSCs upon hypothermia and was required and sufficient for neuroprotection of dissociated PC12 cells from induced apoptosis.

ABBREVIATIONS

ABC ATP-binding cassette

AD Alzheimer's disease

AED Anti-epileptic drug

AF-6 All-1 fusion partner from chromosome-6

AJ adherens junction

ALS Amyotrophic Lateral Sclerosis

AMPA (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMT adsorptive-mediated transport

ANG angiopoietin

AQP4 aquaporin-4

ATP adenosine-5'-triphosphate

BBB blood-brain barrier

BCRP breast cancer resistance protein

BCSFB blood-cerebrospinal fluid barrier

BDNF brain derived neurotrophic factor

CA cornu Ammonis

CIRP cold-inducible RNA-binding protein

Cld5 claudin-5

CNQX 6-cyano-7-nitroquinoxaline-2, 3-dione

CNS central nervous system

COSC cortical organotypic slice culture

CSF cerebrospinal fluid

CSP cold shock protein

CS-RBD consensus sequence RNA-binding domain

DCC Delected in Colorectal Cancer

DCX doublecortin

DG dentate gyrus

DIV days *in vitro*

DNA deoxyribonucleic acid

DND delayed neuronal death

E embryonic day

EAAT excitatory amino acid transporter

EC entorhinal cortex

ECM extracellular matrix

EGL external granular layer

EHOSC entorhino-hippocampal organotypic slice cultures

Eph ephrin receptor

FDA Food and Drug Adminstration

FFA free fatty acid

FGF fibroblast growth factor

FVIII Factor-8

GABA γ-aminobutyric acid

GDNF glial derived neurotrophic factor

GFAP glial fibrillary acidic protein

GluR glutamate receptor

GLUT1 glucose transporter-1

GRP glycine-rich RNA-binding protein

GUK guanyl-kinase-like domain

HIF hypoxia-inducible factor

HIE hypoxia-ischemia encephalopathy

hnRNP heterogeneous ribonucleoprotein

IgG immunoglobulin G

IGL internal granular layer

IL interleukin

JAM junctional adhesion molecule

KA kainic acid

KA1 kainate receptor-1

LAM laminin

MAGUK membrane-associated guanylate kinase-like-protein

MCAO middle cerebral artery occlusion

MDR multidrug resistance

mGluR metabotropic glutamate receptor

miRNA micro-RNA

ML molecular layer

mRNA messenger RNA

MRP multidrug resistance-associated protein

MS Multiple Sclerosis

NeuN neuronal nuclear

NG2 chondroitin sulfate proteoglycan

NGF nerve growth factor

NMDA N-methyl-D-aspartate

NO nitric oxide

NR NMDA receptor

NT-3 neurotrophin-3

NVU neurovascular unit

OGD oxygen-glucose deprivation

OHC organotypic hippocampal slice culture

P postnatal age

$p75^{NGFR}$ p75 nerve growth factor receptor

PC12 pheochromocytoma-12 cell line of rat adrenal medulla

PD Parkinsin's Disease

PDGF platelet derived growth factor

P-gp P-glycoprotein

PI propidium iodide

PI3K phosphoinositide 3-OH-kinase

RBD RNA binding domain

RBM3 RNA binding protein motif 3

RG arginine-glycine

RMT receptor-mediated transport

RNA ribonucleic acid

RNP ribonucleoprotein

Robo round about

RRM RNA recognition motif

SE status epilepticus

SGZ subgranular zone

siRNA small interfering RNA

SLC solute carrier

STS staurosporine

SVZ subventricular zone

TBI traumatic brain injury

TEER transendothelial electrical resistance

TGF transforming growth factor

TIA transient ischemic attack

TJ tight junction

TLE temporal lobe epilepsy

TNF tumor necrosis factor

t-PA tissue-type plasminogen activator

TTX tetrodotoxin

Unc5 Uncoordinated-5

VE vascular endothelial

VEGF vascular endothelial growth factor

vWF von Willebrand Factor

WHO World Health Organization

Wnt Wingless-type

ZO zonula occluden

CHAPTER I: NERVOUS AND VASCULAR NETWORKS AND PATHOLOGY

1.1 SIMILARITIES BETWEEN NERVES AND VESSELS

The anatomical patterning of nerves and vessels and their intimate contacts with one another in the human body have been recognized since the late 1800's, as illustrated by the Belgian anatomist, Andreas Vesalius (Larrivée et al., 2009). From veins to arteries to capillaries the vascular network is strikingly complex with numerous interconnected branches extending in various directions to deliver and exchange blood borne substances throughout the body. Similarly, neuronal networks are arranged in a complex fashion in order to connect information from various parts of the brain to the periphery. These commonalities at the anatomical level imply that nerves and vessels may require similar instructions for patterning of branches. Indeed, it has been known for a while that nerves and vessels utilize the same routes and modes of migration (Speidel, 1933; Martin and Lewis, 1989). More recent and elegant genetic studies in mice and zebrafish have only deepened this relationship at the cellular and molecular level revealing similarities in chemical cues and in sensing mechanisms (Carmeliet, 2003; Tam and Watts, 2010). The frequent alignment of blood vessels and nerve fibers of peripheral tissue suggest that cell-cell interactions may also be necessary in determining their patterning (Larrivée et al., 2009). In the skin, growth factor released by Schwann cells may determine vessel branching along nerves (Mukouyama et al., 2002). The patterning of sympathetic neurons is dependent on artemin released by blood vessels (Honma et al., 2002). In development, the co-occurrence of vascular and neuronal formation in branching, coupling, and guidance within target tissues have also suggested that neurons and endothelial cells build their systems in coordination to achieve a working neurovascular system (Park et al., 2003). In the adult brain angiogenesis and neurogenesis continue to take place in neurogenic zones further supporting this cellular coordination (Palmer et al., 2000). Indeed, a number of growth factors and guidance molecules are shared by both the nervous and vascular systems (Table 1; Ward and LaManna, 2004: Park et al., 2003). For example vascular endothelial growth factor (VEGF), a well known angiogenic factor that induces angiogenesis and vasculogenesis, is also important for neuronal development, patterning, and growth (Ferrara, 2000; Carmeliet and Storkebaum, 2002; Jin et al., 2002). In addition to these structural parallels, both networks communicate bidirectionally, where information via electrical impulses in nerves is sent back and forth along axons and dendrites, while oxygen and nutrients carried by blood flow are exchanged between veins and arteries. Furthermore, analogous to the glia of the nervous system, which aid in neural function, the mural cells of the vascular system aid in vessel stability (Shima and Mailhos, 2000; Ward and LaManna, 2004). Despite these commonalities existing between

nerves and vessels little is known about the coordination and interaction between the two systems, especially at the interface between the vascular and nervous systems of the neurovascular unit (NVU). The physiologic interactions between nerves and vessels may offer insights into endogenous protective responses that the brain activates once an injury occurs.

Table 1.1: Effects of growth factors and guidance molecules on nervous and vascular systems (adapted and modified from Ward and LaManna, 2004; Park et al., 2003)

	Nervous System	Vascular System	Refs
Growth factor:			
VEGF	neurogenic neuroprotective neuron/ axon growth	vasculogenesis angiogenesis EC cell survival/ proliferation/ migration	Ward and LaManna, 2004
bFGF	neurogenesis	angiogenesis	Marchal et al., 2009; Murakami and Simons, 2008
Angiopoetins	neurite outgrowth neurite patterning neuroprotection	EC cell survival/ migration pericyte recruitment anti-permeability	Ward and LaManna, 2004 Park et al., 2003
PDGF	oligodendroglial/ neuronal development neuroprotection	angiogenesis vessel stability pericyte recruitment/ migration/ proliferation	Raff et al., 1988; Williams et al., 1997; Winkler et al., 2010
TGF-β	neurogenesis	vasculogenesis angiogenesis	Choi and Bellermann, 1995; Dünker and Kriegelstein, 2000;
Erythropoietin	neurogenesis neuroprotection	endothelial growth factor	Park et al., 2003
GDNF	neuroprotection	BBB formation	Park et al., 2003
Artermin	GDNF family member	vascular guidance of sympathetic nerves	Park et al., 2003
BDNF	neuronal survival	EC cell survival vessel stabilization secreted by EC	Ward and LaManna, 2004
NGF	neuronal survival/ differentiation neurite outgrowth	angiogenic	Ward and LaManna, 2004
NT-3	neuronal survival/ differentiation	angiogenic	Ward and LaManna, 2004
$p75^{NGFR}$	co-receptor for high affinity Neurotrophin binding to Trk	vascular tone/ smooth muscle cell apoptosis	Ward and LaManna, 2004
Nogo	inhibitor of axon regeneration	EC migration inhibition of SMCs	Ward and LaManna, 2004
Guidance molecule:			
Semaphorin/Plexin/ Neuropilin	axon guidance attraction/ repulsion repulsive growth cone guidance	coronary vessel EC marker VEGF164/5 receptor	Ward and LaManna, 2004
Ephrin/Eph	guidance cues for growing axons migration of neural crest cells	angiogenesis arterial versus venous identity	Ward and LaManna, 2004
Robo/Slits	axon guidance	vessel stability	Wang et al., 1999; Jones et al., 2009
Netrin/Unc5/DCC	axon guidance	vessel tip cell repulsion	Dickson et al., 2002; Larrivée et al., 2007

1.2 HIPPOCAMPUS

The hippocampus is located in the temporal lobe and is one of the most extensively studied regions of the brain. Mainly, due to its single layer of principle cells (pyramidal neurons) and the laminated organization of synaptic inputs to well defined dendrites, the hippocampus has been an attractive model to study general neuronal and systems properties of the brain. The simplicity in architecture has made identifying neuronal cell bodies and dendrites of mammalian origin possible and their synaptic inputs and population discharges recordable. Methods for growing hippocampal neurons in culture (Banker and Cowan, 1977) and organotypic slices (Li and McIlwain, 1957; Gähwiler, 1981), in particular transverse hippocampal slices (Zimmer and Gähwiler, 1984), have helped to advance progress in neurobiology. Although hippocampal cells in culture can be individually identified, easily manipulated, and recorded, hippocampal slices provide the cytoarchitecture of the characteristic laminated synaptic inputs and their dendrites. Field potential recordings are easily performed and assessed in the slices, just as well as in freely moving animals (Bliss and Richards, 1971; Anderson et al., 1972). In addition to the special hippocampal anatomy, the hippocampus is involved in learning and memory and selectively vulnerable to certain types of neurological disorders (e.g Alzheimer's disease, epilepsy and stroke).

1.2.1 STRUCTURE AND FUNCTION

The hippocampal formation is composed of the entorhinal cortex (EC), hippocampus proper, and subiculum. C*ornu Ammonis*1 (CA1), CA2, and CA3 pyramidal neurons, and the dentate gyrus (DG) granule cells, that are organized respectively into a laminated structure of stratum pyramidale and stratum granulosum, make up the subfields of the hippocampus proper (Fig. 1A; Cajal, 1901). In the mammalian brain glutamate is the major excitatory neurotransmitter, while γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter. Pyramidal and granule cells make up 90% of glutamatergic hippocampal neurons and GABAergic interneurons make up the remaining 10% in the hippocampus (Fritschy et al., 1998). Two types of glutamate receptors exist, ionotropic, which are ligand-gated ion channels, and metabotropic, which are G-protein coupled receptors that modulate glutamate release as well as GABA release (de Blas et al., 1988). The most prominent ionotropic receptors in the adult hippocampus are NMDA receptors: NR1 and NR2A/B subunits, AMPA receptors: GluR1 and GluR2 subunits, and kainate receptors (GluR5,-6,-7, KA1 and KA2). There are 8 metabotropic receptor subtypes, mGluR1-8, and all are expressed in the hippocampus except for mGluR6 (Shigemoto et al., 1997). Other receptors

such as acetylcholine nicotinic receptors, noradrenaline receptors, and serotonin receptors are also present in CA1; therefore other neurotransmitters are present as well (Vizi and Kiss, 1998).

The hippocampus is part of the limbic system and has many important functions including memory formation, navigation, emotional response, and spatial orientation. Injury to the hippocampus proper and EC seems to be associated with anterograde and retrograde amnesia, while only loss of CA1 pyramidal neurons occurs in anterograde amnesia (Alvarez and Squire, 1994). Unlike most of the neuronal circuitry within the neocortex, which is bidirectional, the circuitry of the hippocampal formation is unidirectional. The pathway of input to the hippocampus comes from the major glutamatergic afferents of neuronal projections from the EC. The axons of EC project to CA1 or along towards the so-called perforant path, which project to dendrites of DG granule cells. The mossy fibers, which are the axonal projections of DG granule cells, interact with dendrites of pyramidal cells of the CA3 region. From there, the Schaffer collateral axons of CA3 pyramidal cells project to the dendrites of CA1 subfield. The final and major excitatory output is then made by the efferent projections of CA1 to the subiculum or to the EC, where the hippocampal processing loop ends and the information is sent to other cortical regions (Amaral and Witter, 1989).

The transverse hippocampal arteries, of the longitudinal hippocampal artery, which branches from the posterior cerebral artery, supply the blood to the hippocampus in rat (Fig.1B; Coyle, 1976). Arteries in CA1 have been reported to be smaller in diameter, shorter, and fewer in major branches than the internal transverse arteries of CA3 (Coyle, 1976). Of the transverse hippocampal veins, the internal transversal hippocampal veins are the longest and most abundant (Fig.1C; Coyle, 1976). Based on the observation of segmentally arranged and an alternating pattern of transverse arteries and veins within CA3, the transversely directed hippocampal vessels have been postulated to be involved in hippocampal lamellar neuronal organization (Coyle, 1976). Unfortunately, no follow-up studies investigating this theory have been reported, to date. Nevertheless, differences in hippocampal vascularity have been observed (Imdahl and Hossmann, 1986; Gerhart et al., 1991). Capillary density within stratum pyramidale of CA1 has been reported to be reduced and shown to be selectively decreased following cerebral ischemia (Imdahl and Hossmann, 1986; Jingtao et al., 1999). This uniquely low density of the vascular network and possibly low blood supply to CA1 suggests for regional differences in requirement of blood supply (Lokkegaard et al., 2001). Additionally, differences in density of perfused capillaries along the septotemporal axis of rat hippocampus have been observed (Fig.1D-E; Grivas et al., 2003).

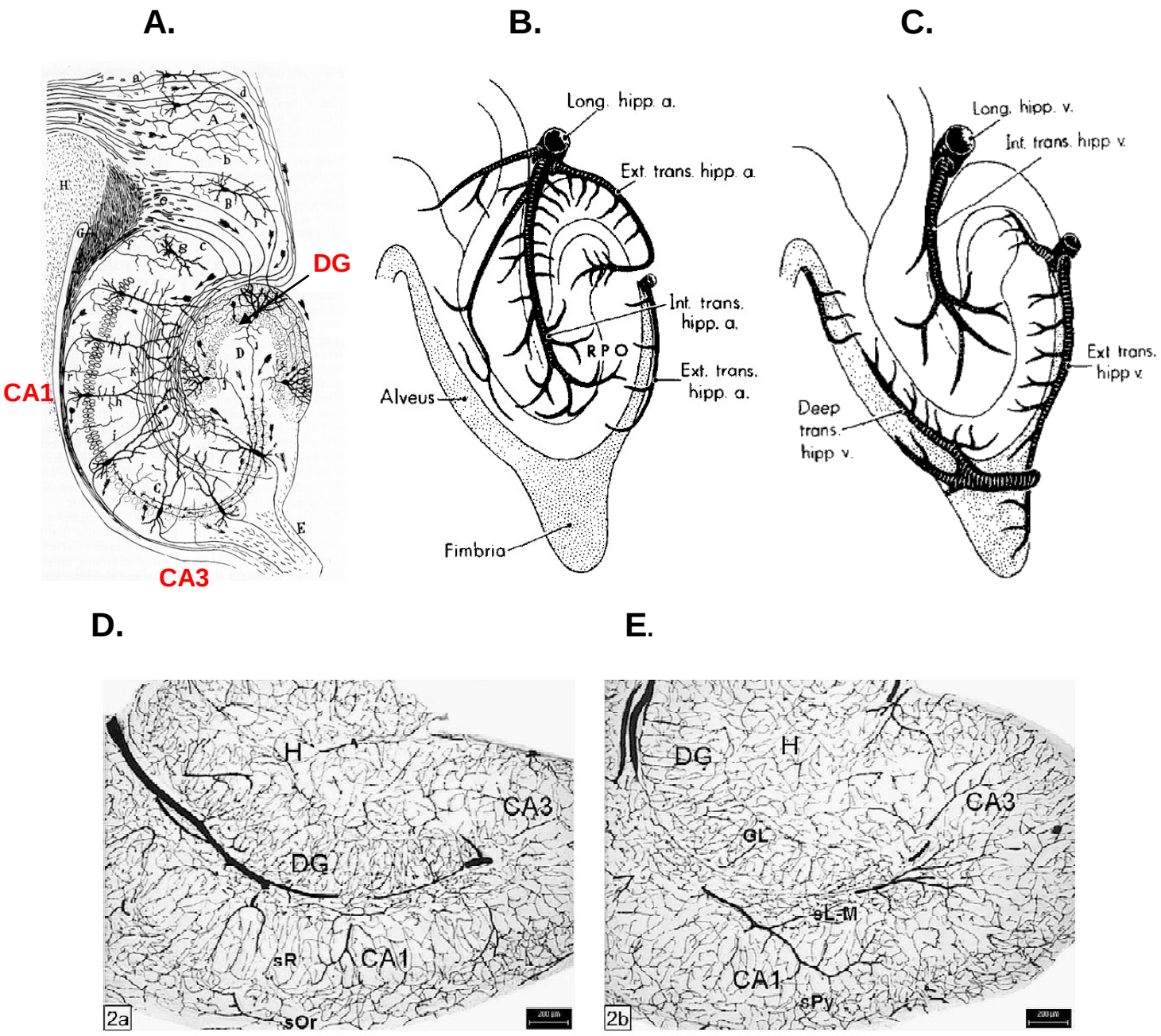

Figure 1. Structure of nerves and vessels of rodent hippocampus. The hippocampal formation and neuronal network (A), major hippocampal arteries (B), major hippocampal veins (C), dorsal hippocampal vasculature (D) and ventral hippocampal vasculature (E). (Cajal, 1901; Coyle, 1976; Grivas et al., 2003)

1.2.2 NEUROVASCULAR NICHE

The hippocampus also contains a neurogenic region in the dentate gyrus. There are two neurogenic zones in the adult brain, the subventricular zone which produces new interneurons in the olfactory bulb and the subgranular zone (SGZ), which generates new granule cells within dentate gyrus of the hippocampus (Palmer et al., 2000). It is not completely understood why adult neurogenesis is restricted to these two regions. Interestingly, in the SGZ, 37% of the cells proliferating are endothelial precursor cells. Because angiogenesis is also present, these zones have been termed as 'neurovascular niches' (Palmer et al., 2000; Louissaint et al., 2002). The blood vessels and neuroprogenitor cells appear in close proximity to each other (Palmer et al., 2002), which is reminiscent of neuronal and endothelial precursors that are found together during embryogenesis and postnatal neurogenesis. Since the two events are temporally associated, the coordinated interactions between neurogenesis and angiogenesis have been suggested to influence development and responses to the environment and following injury (Ward and LaManna, 2003; Park et al., 2003). Interestingly, angiogenic factors have been reported to induce and sustain neural stem cells, suggesting the importance of angiogenesis in maintaining a neurogenic environment (Shen et al., 2004). As previously mentioned, during development neural cells and endothelial cells respond by production of growth factors and their receptors. Regional production of VEGF in the neurovascular niche attract growth and migration of vessels as well as stimulate neurogenesis in part by activating endothelial cells to express brain derived neurotrophic factor (BDNF; Louissant et al., 2002; Barami, 2008; Shen et al., 2004). Thus, VEGF has been considered as a potent inducer of both neuronal and endothelial proliferation and differentiation (Jin et al., 2002; Schänzer et al., 2004; Sun et al., 2006; Segi-Nishida et al., 2008). This functional overlap of growth factors and receptors suggest that neurogenesis and angiogenesis are coordinated in a co-dependent manner sharing common molecular triggers and signaling pathways (Park et al., 2003). Whether the production of growth factors are shared between nerves and vessels (paracrine signaling) or utilized separately within their respective compartments (autocrine signaling) remains to be determined.

1.3 NEUROVASCULAR DISEASES

Neurovascular diseases involve damage to blood vessels that supply blood to the brain or spinal cord, such as stroke. Abnormalities to the blood vessels include narrowing of arteries, which reduces blood flow, or weakening of arteries, which leads to brain aneurysms and hemorrhaging, but ironically, most of the research on neurovascular diseases has been focused on understanding molecular processes of neuronal excitotoxicity, oxidative stress, and cell

death. Yet neuron-targeted therapeutic approaches have been mostly unhelpful in clinical trials. Due to this neurocentric view, less has been reported on the impact of non-neuronal cells, in particular endothelial cells. Previously endothelial cells were viewed as quiescent cells which simply make up blood vessels mainly to supply the brain with oxygen and nutrients. However, with advancements in vascular biology, endothelial cells have shown to be metabolically active, involved in wound healing, vascular tone, blood coagulation, inflammatory responses, immune surveillance, and endocrine processes, all of which are necessary in maintaining a balanced homeostatic microenvironment (Nachman and Jaffe, 2004). Today, neurological disorders that were previously viewed as soley due to neuronal dysfunction have been included as neurovascular diseases caused by or associated with cerebral endothelial dysfunction. For example, observations of reduced endothelial glucose transport capacity (Kalaria and Harik, 1989) and regulation of Aβ trafficking, by influx and efflux transporters at the blood-brain barrier (Deane et al., 2004) suggests a pathological status of the endothelium to contribute to initiation or progression of AD. Indeed, the number of neurodegenerative diseases which are accompanied by vascular abnormalities have grown, some of which include Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), traumatic brain injury (TBI), neuro-AIDS and different types of brain tumors (Abbott et al., 2006). As either a precipitating or secondary event of injury, it may well be that cerebral endothelial dysfunction is associated with most, if not all diseases (Aird, 2005).

1.3.1 STROKE AND CEREBRAL ISCHEMIA

The brain utilizes about 20% of oxygen and receives about 15% of blood pumped by the heart (Rolfe and Brown, 1997). The blood supply to the brain is physiologically demanding. Since neurons have such a high rate of metabolism a reduction in oxygen and glucose, from a disruption in blood supplied by arteries and capillaries would cause neuronal dysfunction, as in stroke. Stroke or transient ischemic attack (TIA) involves impairment of blood vessels either by a vessel occlusion (85% of stroke cases are cerebral ischemia) or cerebral bleeding. Embolisms account for 75% of vessel occlusions and are mainly caused by focally-obstructed blood flow within the brain (Mergenthaler et al., 2004). Stroke is the second leading cause of mortality with about 6 million deaths worldwide each year and some of the risk factors for stroke include smoking, diabetes, hypertension, high cholesterol, and a sedentary lifestyle (WHO Global Infobase; Moskowitz et al., 2010; Woodruff et al., 2011).

Stroke is a vascular disorder with neurological consequences. Brain lesions followed by cerebral ischemia is variable depending on the brain region that is affected. White matter appears to be

more resistant than gray matter to ischemic cell damage (Mattson et al., 2001). Also, in certain neuronal populations some are selectively vulnerable to ischemic cell death while others are not, such as CA1 pyramidal neurons and dentate granule neurons of the hippocampus, respectively (Mattson et al., 2001). Additionally, following cerebral ischemia, alterations in the cerebral vasculature occur, which include blood-brain barrier breakdown, upregulation of endothelial adhesion molecules, apoptosis and angiogenesis (Moskowitz et al., 2010). Although stroke causes ischemic cell death it also triggers a regenerative response in the penumbra, which is the area adjacent to the injured cells that belong to the ischemic core. Cell death ensues in the ischemic core because of severe loss of blood flow, reduction in ATP levels and energy stores, ionic imbalance and metabolic failure. The ischemic penumbra zone is defined by an area of reduced blood flow from collateral blood vessels but with brain tissue surviving for a few hours as a consequence of milder insults (Astrup et al., 1981). If the blood flow is not restored within this short time frame, the penumbra becomes a part of the ischemic core (Green et al., 2003; Durukan and Tatlisumak 2007). In response to low oxygen levels, endothelial cells proliferate and angiogenesis occurs primarily in the penumbra. The growth of new blood vessels is thought to act as a natural defense mechanism in restoring oxygen and nutrient supply to the damaged site (Beck and Plate, 2009). A greater density of microvessels in the penumbra has shown to be correlated with longer survival in stroke patients (Krupinski et al., 1994). The simultaneous neuronal and vascular responses in the ischemic region has been suggested to occur as coordinated events as was proposed during embryogenesis and postnatal development (del Zoppo, 2009; Park et al., 2003).

1.3.1.1 Developing brain

Neonatal asphyxia affects about 1 to 3 out of 1000 live births (Ferriero, 2004). Asphyxia is lack of oxygen (hypoxia) or perfusion (ischemia), which predominantly affects newborn infants however it can arise from anytime before, during, or after birth. It is due to a disruption in maternal oxygenation, placental blood flow or impairment in pulmonary gas exchange leading to poor oxygenation and eventually perinatal brain injury (Badawi et al., 1998; Ferriero, 2004). Infants born prematurely with very low birth weight usually exhibit abnormal or underdeveloped lungs and heart; therefore suffer from chronic hypoxia. As a consequence of the lack of oxygen or perfusion, various organs are damaged and most vulnerable to injury is the brain which can lead to hypoxia-ischemia encephalopathy (HIE). Lesions in the basal ganglia and thalamus are frequently observed (Ferriero, 2004). Neonatal encephalopathy is a very devastating condition of which 20% result in fatality before age 1 and 25% that survive are neurologically impaired

including mental disabilities, cerebral palsy, motor and vision problems, and epilepsy (Finer et al.1981; Vannucci and Perlman, 1997; Ferriero, 2004).

1.3.1.2 Animal models

Knowledge about stroke pathophysiology has been gained through studying various experimental animal models, mostly in rodents. *In vivo* models of stroke are of three types: focal cerebral ischemia, global cerebral ischemia, and hypoxia/ischemia as a model used in young animals combining vessel occlusion and hypoxic breathing (Cimino et al., 2005). Within these stroke models there is also a permanent or transient form of ischemia, where reperfusion can be studied in the latter. Focal cerebral ischemia is usually induced by middle cerebral artery occlusion (MCAO). MCAO has been developed because it closely mimics ischemic stroke patients which exhibit a thrombotic or embolic occlusion in a large cerebral artery such as the middle cerebral artery (del Zoppo et al., 1992; Durukan and Tatlisumak, 2007). Lesions by focal cerebral ischemia are complex characterized by an ischemic core of necrotic cells, surrounded by an ischemic penumbra of cells that are metabolically compromised, which will eventually die by apoptosis and increase the size of the infarct (Durukan and Tatlisumak, 2007). The cells of the penumbra are of interest since they would be targeted for therapy. On the other hand, global cerebral ischemia is clinically relevant to cardiac arrest and asphyxia in humans. Its duration of insult to cause neuronal damage is shorter (5-10 minutes) than in focal cerebral ischemia (90-120 minutes). Global cerebral ischemia is induced commonly by four-vessel occlusion, bilateral arterial occlusion or less commonly, due to high mortality, by cardiac arrest (Woodruff et al., 2011). Lesions by global cerebral ischemia are associated with delayed neuronal death in the hippocampus, in particular CA1 region (Kirino, 1982). The neuronal death has been accepted as a consequence of excitotoxicity since inhibitors of glutamate receptors and GABA agonists reduce neuronal injury following ischemia (Choi, 1992). Although, experiments conducted to prevent and relieve brain injury have successfully been performed on animal models unfortunately neuroprotective strategies, except for therapeutic hypothermia, have not been successfully translated in the clinics,

1.3.1.3 In-vitro models

Most *in vitro* models employ primary cultures of neurons or glia from the cortex, hippocampus or cerebellum of newborn rats or mice. Usually, O_2 is replaced by N_2 gas in a hypoxia chamber, and cultures incubated in medium with or without glucose are transferred there. However, the

deprivation of oxygen (hypoxia) or oxygen and glucose (ischemia) seems to require a longer time to induce neuronal death *in vitro* than *in vivo* (Woodruff et al., 2011). Nevertheless, as a very simplified system, it can still offer some information on how a particular cell reacts which certainly contributes towards identifying possible mechanisms involved in ischemia,

Another *in vitro* model for stroke which has been comprehensively studied is the organotypic hippocampal slice culture (OHC; Laake et al., 1999; Frantseva et al., 1999; Rytter et al., 2003). In addition to the advantages of a preserved neuronal network and contacts to native *in vivo* partners, the duration of OGD-induced neuronal death in slice cultures is shorter (15-30 minutes) than in dissociated cell cultures. Moreover, neuronal death is localized to CA1 of hippocampus which closely resembles the *in vivo* model of global cerebral ischemia displaying NMDA receptor mediated cell death (Rytter et al., 2003). Whether non-NMDA ionic glutamate receptors are involved in OGD induced neuronal death is not as clear but 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA antagonist seems to suggest that they contribute to neuronal death induced by mild OGD (Noraberg et al., 2005). A notable difference that is observed in OHCs is that the kinetics of delayed neuronal death seems more accelerated in slice cultures (24-48 hours) than *in vivo* (48-96 hours; Noraberg et al., 2005). Cellular uptake of the fluorescent dye propidium iodide (PI) is often used to measure cell death in the slice cultures. Although a number of mechanisms are elicited by OGD, excitotoxicity has been the most popular to study using OHCs.

1.3.2 EPILEPSY

Another sequela of brain injury by excitotoxicity is epilepsy. It is a very serious common neurological disorder that affects approximately 1% of the population (Pitkänen and Sutula, 2002). Epilepsy is characterized by the generation of seizures due to a long-term predisposition and co-morbidity of neurobiological, psychological, cognitive, and social deficits (Fisher et al., 2005). Epileptic seizures are abnormal patterns of neuronal activity, altering neuronal excitability which can induce plastic changes in the cortical circuitry (Pitkänen and Lukasiuk, 2011). Status epilepticus (SE) is characterized by a recurrence of seizures in the brain. The etiology of epilepsy is unknown, but it is frequently acquired after a primary injury to the brain (i.e. trauma, infection, tumor, stroke, etc.). Temporal lobe epilepsy (TLE) is the most common form of focal epilepsy and is characterized by hippocampal sclerosis and vascular malformation (Weissberg et al., 2011). Stroke appears to be a major cause of TLE suggesting a neurovascular role in the etiology of epilepsy (Hauser et al., 1996).

Unfortunately, there is no cure for epilepsy. Although, there are anti-epileptic drugs (AEDs) designed to inhibit seizures by enhancing the inhibitory neurotransmitter GABA or by inhibiting activation of Na^+ channels to lower firing of action potentials. Most AEDs, however, are not very effective because they are prevented from gaining access to or remaining in the CNS by blood-brain barrier transporters, mainly P-glycoprotein, a multi-drug resistant transporter which is specialized in drug efflux (Löscher and Potschka, 2005a, b; Bauer et al., 2008). In rare cases is resection of the epileptic region performed and even then patients still depend on AEDs.

1.3.2.1 Developing brain

The incidence of seizures is higher in the developing brain than the adult brain (Holmes and Ben-Ari, 2001). CNS disorders such as HIE, trauma, infection, congenital defects in cortical growth, neurovascular malformation, or a previous episode of febrile seizures are some causes of epilepsy in the infant and it seems that any disturbances in the development of the immature brain, such as abnormal neuronal migration or synaptic maturation may give rise to epilepsy (Ben-Ari and Holmes, 2006). Interestingly, the immature brain appears to be more resistant than the mature brain to seizure-induced neuronal injury (Ben-Ari and Holmes, 2006), which has been also observed in SE models of younger animals less than 2 weeks of age (Holopainen, 2008).

1.3.2.2 Animal models

Rodent models have contributed a great deal to understanding the mechanisms underlying the formation of seizure-generating neuronal circuits (Pitkänen and Lukasiuk, 2011). The two most common models are the poststatus epilepticus model and kindling model. The poststatus epilepticus model induces seizures by exposing systemically or intracerebrally to excitotoxic agents, such as kainic acid or pilocarpine (Walker et al., 2002). The kindling model uses repeated weak electrical stimulation to excite neurons by implanting electrodes to certain brain regions such as the amygdala or hippocampus (Sayin et al., 2003). Eventually, the administration of kainic acid or pilocarpine or the weak electrical stimulations alter the neuronal activity and are able to produce spontaneous seizures that later result in neuronal loss in the hippocampus. The neuronal loss in CA1, CA3, and hilus of hippocampus of the poststatus epilepticus model in adult animals is similar to the human form of disease (Gorter et al., 2003) and therefore is a common animal model of TLE. Recently, angiogenesis and BBB disruption have been observed in a pilocarpine-induced SE rodent model (Rigau et al., 2007; van Vliet et al., 2007).

1.3.2.3 In-vitro models

Organotypic slice cultures have been also used as *in vitro* models of epilepsy. Since the hippocampal lesions are often associated with epilepsy, such as TLE, OHCs have been preferred. Glutamate and its analogues such as NMDA, KA, and AMPA have been used to induce neural excitotoxicity and neuronal death in OHCs derived from 6-7 day old postnatal animals (Noraberg et al., 2005). Frequently, KA is used to induce neuronal damage in OHCs since the regional specific neuronal death (CA1 and CA3) and reorganization of the hippocampal circuitry are reported to be similar to epileptic models of SE (Scharfman, 1994; Okazaki et al., 1999; Holopainen et al., 2004). More recently, angiogenesis and absence of blood-brain barrier tight junction protein, ZO-1 have been reported in OHCs exposed to KA (Morin-Brureau et al., 2011).

1.4 NEURONAL DEATH

The evolution of injury and cell death in part depends on the brain region, the cellular constituents, and the extent of insult. In hypoxia-ischemia as well as in status epilepticus, neuronal death occurs. In ischemia, the physiological disruption by oxygen and glucose deprivation does contribute to cell death but the cascade of neuronal damage begins at the onset of excitotoxicity which is mediated by activation of glutamate receptors (Fig.2; Dirnagl et al., 1999). Cell dysfunction and a majority of neuronal damage is due to accumulation of glutamate in the extracellular matrix that activates glutamate receptors (NMDA, AMPA, and KA) allowing toxic increases in intracellular calcium in the CNS (Choi, 1992). The ionic imbalance in calcium and activation of AMPA receptors result in high intracellular levels of sodium which is detrimental as it can impair cell function and induce cell death at earlier time points after ischemia (Won et al., 2002). Multiple signaling pathways however can be activated by calcium or by activation of calcium dependent enzymes which may signal release of free radicals leading up to cell death (Wang et al., 2005). This variation in type of cell death suggests that the consequences of cerebral ischemia are complex. In fact, the events leading up to cell death trigger a fairly well-defined spatio-temporal pattern of pathophysiologic events, known as the ischemic cascade, which begins with and not strictly in order but overlapping, excitotoxicity, spreading depression, formation of oxygen free radicals, tissue acidosis, followed by inflammation and cell death by apoptosis (Dirnagl et al., 1999; Durukan and Tatlisumak, 2007).

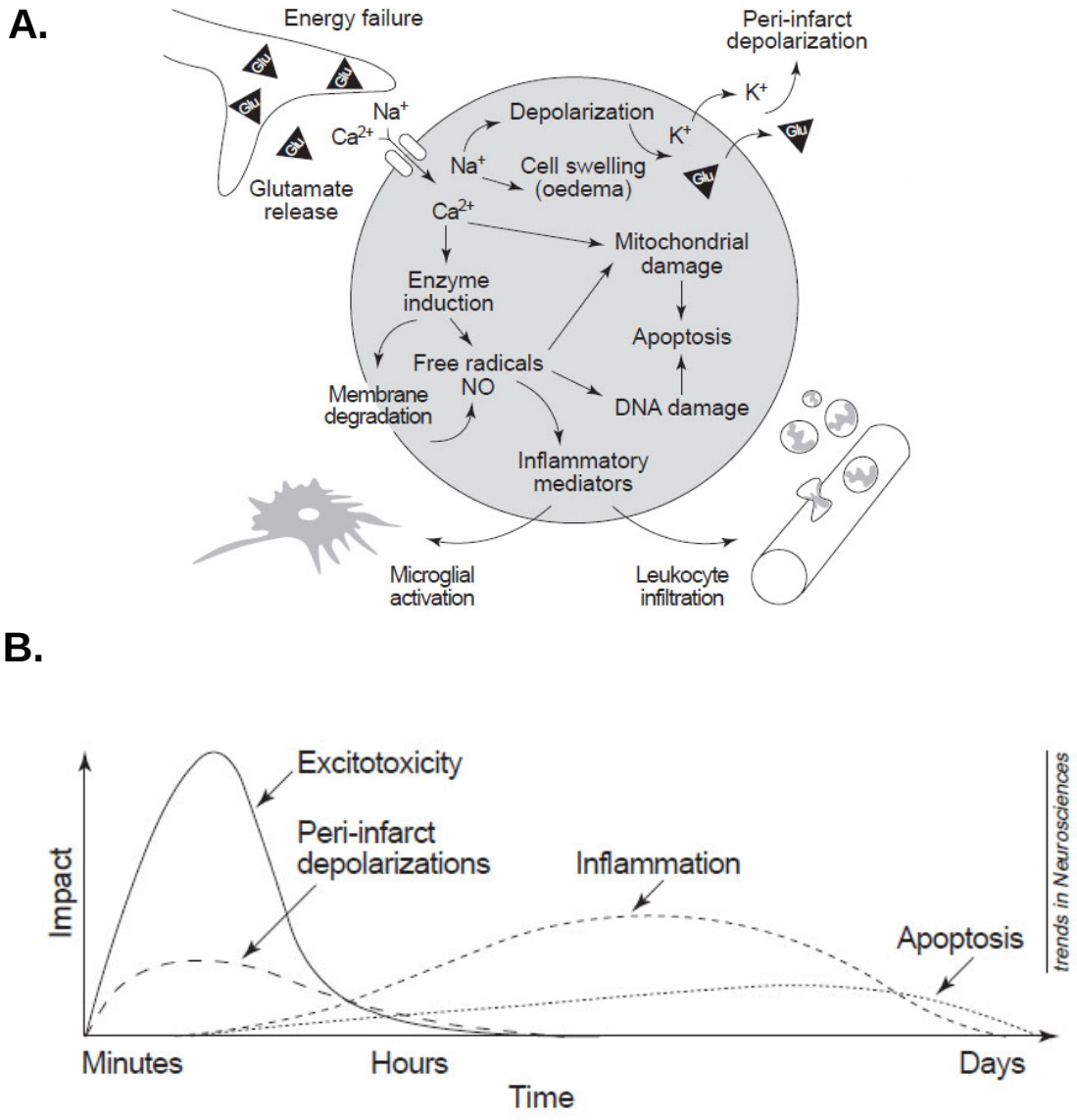

Figure 2. Ischemic Cascade. A number of mechanisms occur in ischemia leading up to cell death including excitotoxicity, depolarization, calcium overload, production of free radicals, tissue acidosis, mitochondrial dysfunction, microglial activation, and inflammation (A). The ischemic cascade is spatio-temporal occurring from hours-to-days (B) (Dirnagl et al., 1999)

1.4.1 NECROSIS AND APOPTOSIS

Ischemic cell death may arise in two forms: necrosis and apoptosis. Depending on the extent and duration of injury the cells will not only suffer a functional but also a structural breakdown. Disturbances in ion homeostasis can be accompanied by water influx which creates an imbalance in the cell volume and forces the cell to swell and lyse, undergoing necrosis. Apoptosis is a physiological mechanism of which cell death is programmed to respond to a

particular insult and can be triggered by a number of processes, including excitotoxicity, free-radical formation, inflammation, mitochondrial and DNA damage, and cytochrome c release from mitochondria (Fisher and Schaebitz, 2000; Mergenthaler et al., 2004; Sugawara et al., 2004). In apoptosis, cell membranes and organelles remain intact, while the cell shrinks along with chromatin condensation, DNA fragmentation, and cleavage into small membrane bound bodies (Love, 2003; Mergenthaler et al., 2004). Ischemia-induced neuronal death has been characterized by a mixture of necrosis and apoptosis, however, in focal cerebral ischemia necrosis is predominant in the ischemic core while apoptosis is found in the penumbra (Smith, 2004). Increasing morphological and biochemical data have indicated that in the ischemic brain both apoptosis and necrosis may occur simultaneously and since both processes may not always be mutually exclusive a hybrid term for the continuum of the two forms of cell death 'aponecrosis' has been proposed (MacManus and Buchan, 2000; Unal-Cevik et al., 2004).

1.4.2 DELAYED NEURONAL DEATH

Depending on the type of insult certain subfields of the hippocampus is particularly prone to damage. Pyramidal neurons in the hippocampal CA1 subfield have been extensively studied and found to be selectively vulnerable to injury by ischemia in experimental animals and in humans after ischemic injury (Ito et al., 1975; Kirino, 1982; Petito et al., 1987). Although, the selective CA1 vulnerability to ischemia has not been completely understood, intrinsic differences between the hippocampal subfields have been proposed to be involved (Kirino et al., 1984). However, CA1 cell death does not immediately appear following brief ischemia but develops slowly, taking at least 2 days to occur (Kirino, 1982; Kirino and Sano, 1984). Furthermore, energy metabolism (Arai et al., 1986) and electrical activities (Suzuki et al., 1983) in neurons appear normal and unaffected up to 24 hours. However, between 2 and 4 days after ischemia the cell undergoes an irreversible change which does not allow it to be rescued by reperfusion and therefore dies. As a result, this slow development in CA1 cell death has been labeled as delayed neuronal death or DND (Kirino and Sano, 1984). This type of cell death has been characterized as apoptotic (Nitatori et al., 1995).

1.5 BLOOD-BRAIN BARRIER

The blood-brain barrier (BBB) is vitally important to the CNS. Analogous to the function of a cell membrane which maintains and balances the composition of the intracellular fluid, the BBB maintains and balances the extracellular milieu of the nervous system. The BBB includes the blood-cerebrospinal fluid barrier (BCSFB), blood-retinal barrier, and blood-spinal cord barrier.

Other barriers interfaces that separate the blood from the CNS are located in the arachnoid epithelium and the choroid plexus epithelium. There is no BBB in the circumventricular organs (neurosecretory or chemosecretory sites) but the ependyma and the surrounding glia contain tight junctions that prevent leakage into the brain or CSF (Abbott, 2005).

Experiments performed by Paul Ehrlich in 1885 were the first to show that a barrier existed between the blood and the brain in the CNS. Evans blue dye was intravenously injected in rats which demonstrated all regions except the brain to be infused with the dye (Ehrlich, 1904). However, not until 1913, when his graduate student Edwin E. Goldmann injected the dye in the cerebrospinal fluid (CSF) and showed the opposite effect where only the CNS was infused with the dye and not the blood vessels that his hypothesis for a BBB existing was truly corroborated. But it was in 1898 that Lewandowsky introduced the term 'blood-brain barrier' after he and colleagues observed neurotoxic compounds to selectively affect the CNS by intracerebral injection and not by intravenous administration. Remarkably, it was not until over half a century later in 1967 that Reese and Karnovsky identified by electron microscopy that the endothelium was the main component making up the BBB. The endothelium of the CNS is unlike the one of the periphery because it lacks fenestrations and contains tight junction proteins (Bauer et al., 1993; Stewart and Hayakawa, 1994).

Importantly, the BBB functions to protect the CNS from toxins and blood-borne pathogens that may travel by way of the vascular system. It contains both transcellular and paracellular pathways for non-permeable substances to pass through the brain but allows only essential molecules, such as oxygen and glucose to enter the brain, as well as maintaining ion homeostasis in the CNS (Fig. 3). In addition the BBB prevents neurotransmitters from escaping the brain (synaptic regions are highly vascularized) and regulates immune surveillance with reduced inflammation and cell damage (Abbott, 2005).

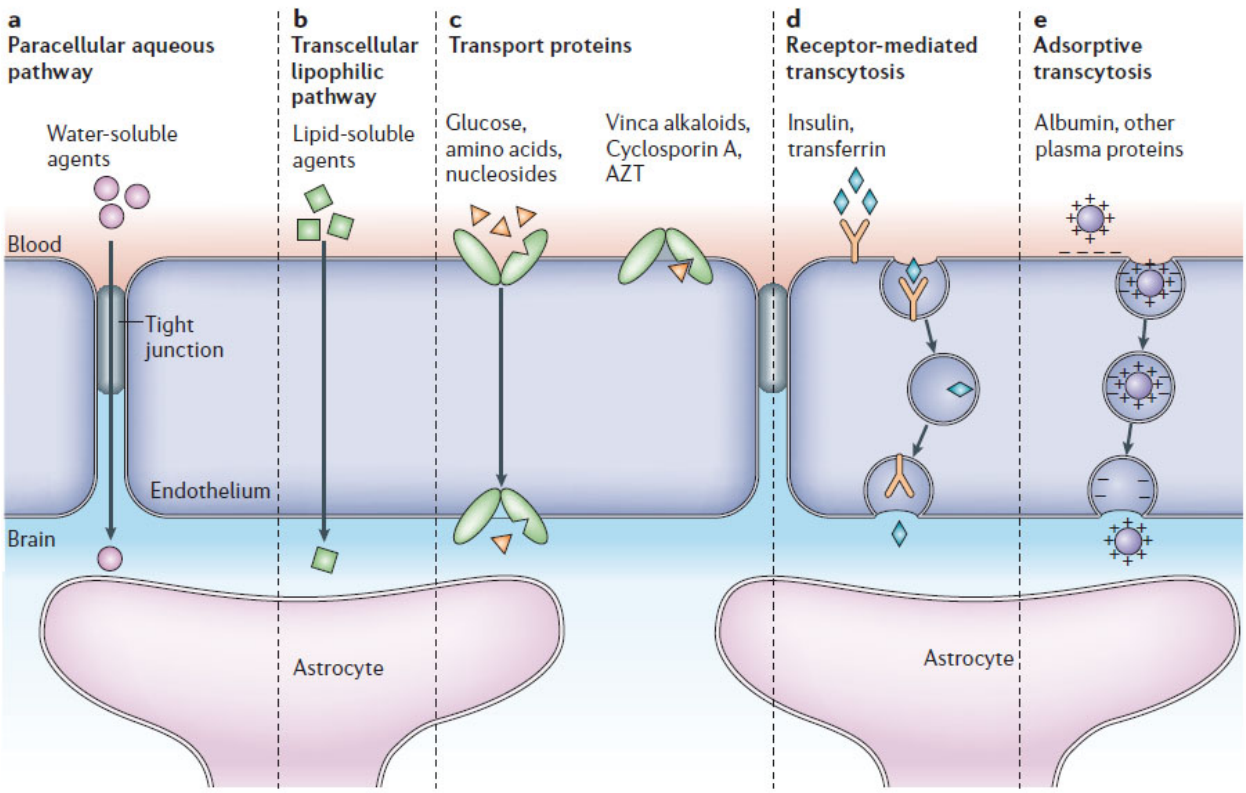

Figure 3. Paracellular and Transcellular pathways of the Blood-brain barrier. The BBB protects the brain by regulating the accessibility of molecules to the CNS. The paracellular pathway is regulated by tight junction proteins that allow passage of certain water soluble molecules (a). The transcellular pathway is regulated by various transporters and receptor-mediated and adsorptive-mediated systems, which control the passage of other essential molecules that support to maintain an optimal environment for cerebral functions (b, c, d, and e) (Abbott et al., 2006)

1.5.1 BBB INDUCTION

As mentioned previously, the endothelial cells of BBB lack fenestrations and additionally they have low pinocytotic vesicles, and contain more mitochondria than endothelial cells of the periphery (Fenstermacher et al., 1988; Sedlakova et al., 1999; Oldendorf et al., 1977). Grafting experiments of embryonic quail tissue revealed that induction of capillaries in non-vascularized brain tissue transplanted to the coelomic cavity possessed lower pinocytotic vesicles and higher mitochondrial content compared to somite grafts of dorsal mesoderm transplanted to the cerebral ventricles, suggesting that the origin of the BBB is neuronal (Stewart and Wiley, 1981). Recently, the induction and specialization of the CNS vascular system and BBB has been shown to be controlled by the signaling between the neuroepithelium and the vascular endothelium via the canonical wingless-type protein (Wnt) and β-catenin pathway (Stenman et

al., 2008; Liebner et al., 2008; Daneman et al., 2009; Liebner and Plate, 2010). Mice generated to develop without *wnt7a/wnt7b* expressed in the neuroepithelium or beta-cateninin in endothelial cells displayed CNS specific abnormal angiogenesis. The mice died around E12.5 with severe CNS hemorraging. The endothelial and pericytes were strongly decreased in number and the vascular morphology was malformed. BBB formation with respect to the expression of GLUT1 and tight junction proteins were also reduced. The fact that Wnt signaling is critical for the proper development and function of the CNS; that it also modulates the formation of blood vessels and the specialization of BBB further underlines the importance of the relationship between then nervous and vascular systems (Stewart and Wiley, 1981).

1.5.2 BBB COMPOSITION

The endothelial cells that make up the inner lining of the vasculature express tight junction proteins (e.g. occludin, claudin 1/3 and 5, and zonula occluden-1 to 3) and transporter proteins such as glucose transporter 1 and P-glycoprotein allow close inter-endothelial cell connections and regulate transport across the endothelium, respectively (Gonzalez-Mariscal et al., 2003; Guo et al., 2005: Schinkel et al., 1999). The molecular composition of the BBB, in particular the tight junctions (TJs) and adherens junctions (AJs) have been sought after for over a decade (Furuse et al., 1993, 1998a, b; Ando-Akatsuka et al., 1996; Morita et al., 1999; Balda and Matter, 2000; Tsukita and Furuse, 1999, 2000; Heiskala et al., 2001; Fig. 4). Most of the findings have been based on epithelial and endothelial cells of non-neuronal origin. In freeze-fracture replicas, the TJ particles appear similar to connexons of gap junctions (Wolburg and Lippoldt, 2002). The assembly of TJs occurs along the outer leaflet between adjacent endothelial membranes, where the extracellular domains of TJs form homo- and heterodimers interconnecting the plasma membranes merging into “kiss” points (Kniesel et al., 1996). In freeze fracture replicas, the outer leaflet is referred to as the protoplasmic fracture face (P-face) and the inner leaflet is the exo-cytoplasmic fracture face (E-Face) of endothelial or epithelial cells. The P-face has been associated with a more mature endothelial barrier with a high transendothelial electrical resistance (TEER) than the E-face (Butt et al., 1990). Therefore, the complexity of strands and their association with the P-face or E-face have been considered as parameters in determining the functional quality of tight junction proteins (Wolburg and Lippoldt, 2002). Depending on the vascular bed, peripheral endothelial cells possess a lower level of P-face strands than epithelial cells, whereas brain endothelial cells have the highest P-face association (Wolburg and Lippoldt, 2002). Despite the large amount of research that has been conducted on elucidating the structural components involved in formation and maintenance of

the BBB, the cellular and molecular mechanisms controlling its maintenance are still poorly understood.

1.5.2.1 Tight junctions

With the exception of small (< 400 Da) and lipophilic molecules, endothelial cells of brain capillaries are able to restrict movement of blood borne solutes to the brain due to TJs. Indeed TJs represent one of the main constituents that distinguish the endothelium in the CNS from the periphery as it is 50-100 times tighter (Rubin and Staddon, 1999; Abbott et al., 2006). TJ proteins are considered to regulate the permeability of the BBB and maintain endothelial cell polarity, known as the “barrier” and “fence” functions, respectively (Bazzoni and Dejana, 2004). The cell-cell juncture is complexed with other transmembrane proteins, such as adherens junctions (AJ), gap junctions, and desmosomes, but TJs are the most apical. The carboxy termini of the junctional proteins are linked to cytoplasmic accessory proteins which recruit the actin filaments of the endothelial cells forming a continuous capillary structure (Fig. 4).

Occludin is a 65 kDa phosphoprotein with 4 transmembrane spanning domains, 2 extracellular and 1 intracellular loops and a cytoplasmic amino and carboxyl terminus. It was the first tight junction protein to be identified (Furuse et al., 1993). The function of occludin in the paracellular seal has not been determined but appears to have a minor role as barrier formation was normal in occludin-deficient mice (Saitou et al., 2000), although, in some diseases, BBB dysfunction has been associated with reduced occludin expression (Bolton et al., 1998; Huber et al., 2002; Brown and Davis, 2005). Claudins are 20-27 kDa proteins with two extracellular and intracellular loops (Mitic et al., 2000). Claudin-1/3, 5, and 12 have been reported to be associated with the BBB (Morita et al., 1999; Liebner et al., 2000; Lippoldt et al., 2000). Claudin-5 deficient mice die within 10 hours after birth from fatal hemorrhage, suggesting that it necessary for BBB formation (Nitta et al., 2003). Zonula occludens (ZO-1, -2, and -3) are membrane-associated guanylate kinase-like-protein (MAGUKs) with 3 PDZ domains, 1 SH3 domain, 1 guanyl kinase-like domain (GUK), which are all important for protein binding. They are the third major type of BBB TJs and are the largest in size between130-220 kDa (Haskins et al., 1998).They are phosphoproteins expressed in endothelial, epithelial and astroglial cells (Howarth et al., 1992). The TJs are linked to the actin cytoskeleton by ZO-1 (Fanning et al., 1998). ZO-1 is 220 kDa and functions also as a signaling molecule conveying information about the TJs to the interior of the cell (Hawkins and Davis, 2005). ZO-2 is 160 kDa, similar in sequence to ZO-1 and can functionally substitute ZO-1, *in vitro* (Gumbiner et al., 1991; Umeda et al., 2004). Under stress conditions and cell proliferation both ZO-1 and -2 translocate into the nucleus, *in vitro* (Gottardi et al., 1996; Islas et al., 2002). ZO-3 is 130 kDa and binds to claudins, but less is known of its expression at the BBB

(Itoh et al., 1999). Additional accessory proteins include cingulin, which is a myosin-like protein and binds to ZO-1, junctional adhesion molecule 1 (JAM-1), and myosin at the globular domain and to other cingulin proteins and actin at the tail (Citi et al., 1989). Less well known accessory proteins but are part of the TJ complex are AF-6 and 7H6 (Yamamoto et al., 1999; Zhong et al., 1994)

1.5.2.2 Adherens junctions

Along the blood vessels at the junctional zone are also present, the adherens junctions. The adherens junctions assist in stabilizing the cell-cell contact. They are formed by cadherins, which are transmembrane adhesion proteins. In general the cadherins bind to catenins with their carboxy-terminal domain to link on with anchoring proteins that bind to actin. It was shown that in order for tight junction proteins to develop and be organized at the cell-cell juncture, the formation of adherens junctions was a prerequisite (Dejana, 2004). Vascular endothelial (VE) cadherin and cadherin-10 are Ca^{2+} regulated proteins that mediate cell-cell adhesion and have been shown to be involved in development and maintenance of BBB and BCSFB (Williams et al., 2005; Taddei et al. 2008). The cadherins maintain vessel integrity through activation of phosphoinositide 3-kinase (PI3K) which signals actin cytoskeletal rearrangement or by complexing with VEGFR2 to signal VEGF expression (Lampugnani et al., 2002; Carmeliet and Collen, 1999). Disruption of AJs can lead to BBB permeability (Abbruscato and Davis, 1999), but TJs are more involved in restricting the paracellular route (Romero et al., 2003).

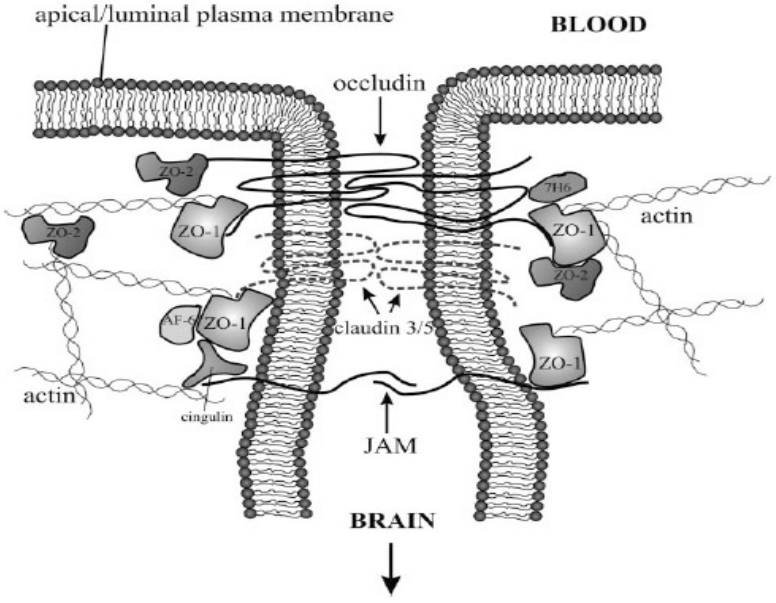

Figure 4. Tight junction proteins of the Blood-Brain Barrier. A tight endothelial barrier is due to the homo- and heterodimerization of TJs (occludin, claudin 3/5, JAM) forming a junctional complex linked to the actin cytoskeletal by scaffolding proteins (ZO1-3 and cingulin) (Hawkins and Davis, 2005).

1.5.2.3 Transporters

Since tight junctions and adherens junctions keep molecules from passing through the BBB paracellularly, possible routes for compounds to cross the BBB are through the transcellular pathways. As briefly mentioned the BBB consists of a system of transporters that are associated with the transcellular pathway (Fig. 5). The transporter proteins are present on the luminal and abluminal endothelial membranes and are responsible for regulating the traffic of molecules entering and exiting the CNS. A substantial portion of metabolic substrates crossing the BBB are hydrophilic (e.g. glucose and amino acids) and are able to pass the BBB by specialized Solute Carrier (SLC) transporters, but xenobiotics such as drugs and toxins are pumped out by the ATP-binding cassette (ABC) transporter systems. However, lipophilic and hydrophobic compounds of less than 400 Da (e.g. ethanol, caffeine, and nicotine) are able to efficiently cross the BBB by lipid-mediated transport which is passive diffusion (Pardridge, 1999). Some macromolecules (peptides and proteins) cross the BBB by receptor or adsorptive-mediated transcytosis (Abbott et al., 2006).

There are a number of BBB SLC transporters since many solutes and nutrients are polar molecules (e.g. glucose, amino acids, nucleosides, and monocarboxylic acids) that cannot cross cell membranes by passive diffusion (Abbott et al., 2010). For example, the transport of glucose across the BBB is mediated by the endothelial transporter GLUT-1 through facilitated diffusion (Fig. 5). Dick et al. (1984) were the first to identify GLUT1 in the BBB of rats. GLUT1 is a member of the SLC2 gene family which consists of 13 members and is a uni-porter which only carries glucose. The 55 kDa isoform of GLUT1 is found in the BBB and is more glycosylated than the 45 kDa GLUT1 isoform expressed in astrocytes (Simpson et al., 2007). The expression of GLUT1 is vital for brain and vascular development (Stenman et al., 2008; Liebner et al., 2008). It has been demonstrated that changes in GLUT1 activity in endothelial cells can be activated by signals released from neuronal cells in rats that have been seizure-induced (Cornford et al., 2000). The increased transporter expression in the endothelial plasma membrane is a result of increased stability of the GLUT-1 mRNA or by translocation of transporters from the intracellular pool (Farrell and Pardridge, 1991; Simpson et al., 2001). The transport of glucose across the BBB is passive and not rate limiting under physiologic conditions. However, under pathological conditions such as after hypoxia or ischemia, transport capacity of GLUT1 can be modulated (Simpson et al., 2007; Boado and Pardridge, 2002). Also, AD neurodegeneration has been proposed to be associated with GLUT1 downregulation (Kalaria and Harik, 1989). Examples of other solute carriers are provided by Abbott et al. (2010).

Some transporters may not always be symmetrically distributed on the plasma membranes of endothelial cells. Efflux transporters are generally located on the luminal (apical or blood) side of cerebral endothelial cells. Due to this polarization of transporters along the endothelium, the uptake of xenobiotics in the brain is thought to be hindered. The major efflux transporters of the BBB are P-glycoprotein (P-gp), members of multidrug resistance-associated protein (MRP1-6) family, and breast cancer resistance protein (BCRP) (Begley, 2004; Löscher and Potschka, 2005b, Dauchy et al., 2008). They belong to the ABC family because they contain an ATP binding domain allowing them to bind and hydrolyze ATP for energy to pump out compounds against a concentration gradient. They are endogenously expressed in normal cells and overexpressed in tumor cells. Therefore anti-cancer or tumor agents have not been able to work effectively, hence the names of the efflux transporters, “multidrug” and “breast cancer” resistance proteins. Constitutive expression of P-gp seems necessary to protect the brain from potential toxic compounds since P-gp deficient mice show an increase in neurotoxicity of various drugs (Schinkel et al., 1994). Furthermore, P-gp inhibitors appear to improve drug penetration and seem promising to overcome drug resistance however more studies are necessary before being clinically relevant (Löscher and Potschka, 2005b). Other BBB transporters include the excitatory amino acid transporters (EAAT1-3), which remove glutamate out of the brain, but for more information on other BBB transporters refer to the review by Abbott et al., (2010).

1.5.2.4 Enzymes

The BBB also contains a variety of intracellular and extracellular enzymes that neutralize drugs and nutrients, supporting a ‘metabolic barrier’. For example, peptidases and nucleotidases, which are ecto-enzymes metabolize peptides and ATP, respectively and monoamine oxidase and cytochrome P450 which are intracellular enzymes inhibit neuroactive and toxic compounds (Abbott et al., 2006). Also, some enzymes are located on the luminal surface (e.g. alkaline phosphatase and γ-glutamyl transpeptidase) and some on the abluminal surface (e.g. Na^+-K^+ ATPase and sodium-dependent neutral amino acid transporter) thus are also responsible for maintaining the polarity of the endothelial membrane. Overall, this enzymatic barrier functions to prevent harmful substances from entering the CNS (Abbott et al., 2006).

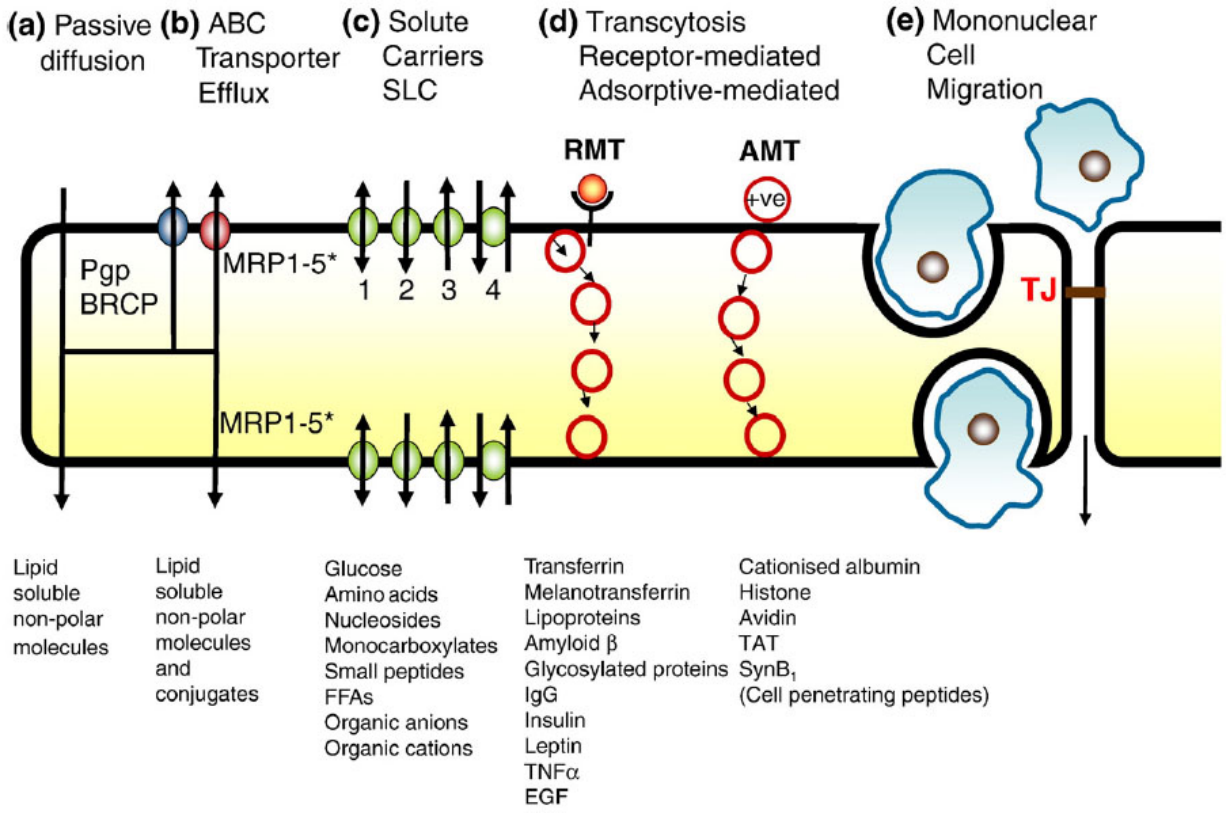

Figure 5. Transporters at the Blood-Brain Barrier. The transcellular pathways of the BBB include (a) passive diffusion (b) ABC transport efflux (c) solute carrier mediated transport (d) receptor mediated (RMT) and adsorptive mediated transport (AMT) and (e) monocyte transmigration. A list of molecules is given below each transport system. Arrows indicate the direction of transport. (Abbott et al., 2010)

1.6 NEUROVASCULAR UNIT

The term neurovascular unit (NVU) was introduced in 2001 as a physiological unit made up of neurons, non-neuronal cells (e.g. astrocytes and microglia) and the endothelium (Lo et al., 2003; Iadecola, 2004; Hawkins and Davis, 2005). It is a conceptual framework that connects neuronal function with the vasculature as a structural unit linked by astrocytes (Fig. 6; del Zoppo, 2012). The NVU includes the endothelium attached by pericytes to the abluminal membrane ensheathed by the basal lamina that is surrounded by astrocytic end-feets which wrap around the cerebral capillaries (Hawkins and Davis, 2005). It has been known for a while that soluble factors released by astrocytes and by contact-mediated mechanisms support formation and maintenance of the BBB (Rubin et al., 1991; Tao-Cheng et al., 1987). Less is known regarding pericytes, neurons, and perivascular microglial cells but they have been

suggested to be involved in changing vascular properties and function (Hawkins and Davis, 2005).

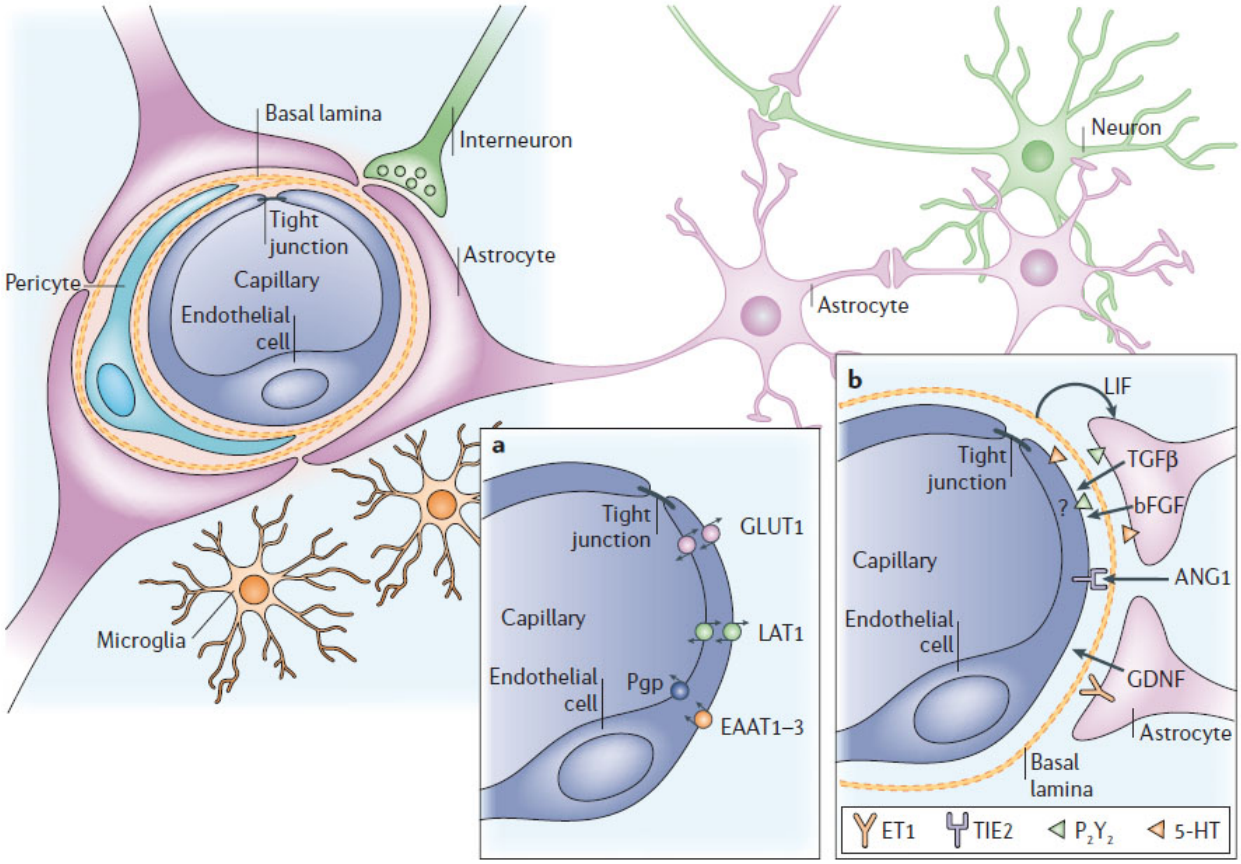

Figure 6. The Neurovascular Unit at the Blood-Brain Barrier. The cerebral capillary endothelium of the BBB is supported by a basal lamina of extracellular matrix proteins and is in constant interaction with astrocytes, pericytes, neurons and perivascular microglia that release factors involved in maintaining a tight endothelial barrier (Abbott et al., 2006).

1.6.1 STRUCTURE: CELL-CELL INTERACTION

Camillo Golgi was the first to observe that glial cells were in contact with blood vessels. The close distance between astrocytic endfeets and cerebral endothelial cells strongly suggest astrocytes to have a major influence in BBB function. Many *in vitro* studies have indicated the importance of astrocytes in formation and maintenance of tight junction proteins (Bauer and Bauer, 2000). They have been shown to release many kinds of growth factors such as basic fibroblast growth factor (bFGF/FGF2), transforming growth factor-beta (TGF-β), glial derived neurotrophic factor (GDNF), and angiopoietin 1 (ANG-1) that are effective in modulating the endothelium as well as regulating the permeability of the BBB (Abbott et al., 2006). Since

astrocytes arise after birth when angiogenesis is finished, they are considered to be not essential for angiogenesis but rather important for BBB formation and maintenance (Bauer and Bauer, 2000). The fact that astrocytes can communicate with nerves and vessels also indicates that they function in mediating signals between the nervous and vascular systems. Furthermore, astrocytes have water channels known as aquaporin4 (AQP4), which are involved in regulating the water content in the brain. They are found along specialized membrane domains, such as the astrocytic endfeets that are in contact with blood vessels or astrocytic membranes that ensheathe glutamatergic synapses (Nielsen et al., 1997; Nagelhus et al., 2004). More recently, expression of AQP4 by immunohistochemistry was found along blood vessels and in astrocytes. Furthermore, it was expressed in a laminar-specific pattern within the mouse hippocampus (Hsu et al., 2011). Interestingly, AQP4 was highly expressed in CA1 and DG subfields suggesting the importance of water regulation in these regions (Hsu et al., 2011). Additionally, astrocytes have glutamate transporters for neurotransmitter signaling and processing (Rothstein et al., 1996). Under pathological conditions they can become reactive known as gliosis and activate an inflammatory response by releasing cytokines (Pekny and Nilsson, 2005).

However, in much closer contact to the cerebral endothelial cells are pericytes. Like astrocytes, these cells which were once viewed as bystanders have recently been considered quite essential for vessel development and BBB formation. They are referred also to as mural cells and are related to vascular smooth muscle cells which function to stabilize the vasculature. The precursor cells of pericytes express platelet derived growth factor receptor-beta (PDGFR-β) and are recruited to the endothelium by expression of PDGF-β in endothelial cells (Leveen et al., 1994; Betsholtz et al., 2001). Expression of TGF-β is upregulated once the two cells are in contact and the pericyte precursors differentiate into the mature form aiding in the proper formation and stabilization of the vasculature (Bergers and Song, 2005). Interestingly, the CNS contains the most abundant number of pericytes and this is thought to be important for maintaining the BBB. Recently, pericytes were shown involved in the polarization of astrocytic endfeets during BBB development (Armulik et al., 2010). Furthermore, PDGF-β knockout mice revealed their involvement in regulating blood flow, which was reminiscent of hypoperfusion that occurs in AD brains (Bell and Winkler et al., 2010).

In order to meet the metabolic needs of the nervous system, nerves and vessels must communicate in a coordinated fashion to direct blood flow accordingly, although the exact mechanisms remain elusive. VEGF released by neurons can act in an autocrine and paracrine manner affecting both vascular and neuronal cells (Ogunshola et al., 2002). As mentioned previously neuronal signaling can also be transferred via astrocytic endfeets to the endothelial

cells. Whether neurons are involved in BBB induction is not known. However, a higher γ-glutamyl transpeptidase activity which correlates with BBB maturation has been reported in co-culturing experiments of cerebral endothelial cells with either neurons or neuronal plasma membranes than with glial cells (Tontsch and Bauer, 1991). As mentioned neurons and vessels share similar growth factors and guidance molecules in neurogenesis and angiogenesis. In neurovascular niches, neuroblasts have been reported to migrate along vessel walls (Palmer et al., 2000). Neural stem cells proliferate and self-renew when co-cultured with endothelial cells implying that the vessel environment supports neural stem cells (Shen et al., 2004). Indeed, the expression of neuroprogenitor cells and endothelial precursor cells together during neurogenesis and angiogenesis suggest that they may have an important interaction and this will be critical to understand in repair after brain injury. Direct signaling between blood vessels and nerves is found in the vaso vasorum and the perivascular autonomic nerve fibers (Kummer and Haberberger, 1999; Schratzberger et al., 2000).

Brain macrophages consist of microglia and perivascular macrophages. They are important in CNS defense, in particular with immune surveillance. They can release cytokines which signal an immune response to upregulate adhesion molecules in endothelial cells for recruitment of leukocytes from the blood into the CNS (Zlokovic, 2008).

Cell-cell contacts of the NVU are maintained by the extracellular matrix (ECM) of the basement membrane which is also known as the basal lamina that is composed of mostly collagen type IV, laminin, and fibronectin (Wolburg and Lippoldt, 2002; Chen and Shuaib, 2007). Perlecan, nidogen, and other proteoglycans are present to a lesser extent. Integrin receptors of cerebral endothelial cells link onto the ECM proteins which anchors the endothelial cells to the basement membrane. Thus cell-cell signaling within the NVU is maintained and TJ proteins are preserved by the basal lamina (Hawkins and Davis, 2005). Heparin sulfate proteoglycan agrin has been shown to be important in maintenance of BBB (Barber and Lieth, 1997). Absence of collagen type IV, laminin, and fibronectin as observed in focal cerebral ischemia/reperfusion results in BBB permeability leading to cerebral hemorrhage (Hamann et al., 1995). Also, present in the ECM are noncellular proteolytic systems including plasmin, cathepsins, and matrix metalloproteinases (MMPs) secreted by cells of the NVU which appear involved in degrading the basal lamina and remodeling of the vasculature during ischemia (Chen and Shuaib, 2007; Zlokovic, 2006). Impairment of the NVU and BBB breakdown is thought to be associated with these proteases (Fig. 7; Lo et al., 2003).

1.6.2 FUNCTION

The NVU is involved in regulating many processes including hemodynamic neurovascular coupling, microvascular permeability, matrix interactions, neurotransmitter inactivation, neurotrophic coupling, and angiogenic / neurogenic coupling. The execution of these activities seems dependent on the paracrine signaling between the different cell types of the NVU (Zlokovic, 2008). The signaling mechanisms between the various cells will be important to elucidate as well as the roles that each play during development, maturation, maintenance, and disease. As cell-cell signaling is important for the preservation of the NVU any disruption of the cellular interaction leads to vascular damage and BBB breakdown.

1.7 BLOOD-BRAIN BARRIER DISRUPTION

Under physiological conditions the BBB is rather impermeable to many physical and chemical insults. In contrast, BBB dysfunction is characterized by increased permeability of cerebral vessels, disassembly of junctional protein complexes, and alterations in matrix adhesion receptors (Hawkins and Davis, 2005; Sandoval and Witt, 2008; Milner et al., 2008). Depending on the type of brain injury, BBB permeability has been conjectured to occur as a primary event such as with multiple sclerosis (Floris et al., 2004) or as a consequence of the pathology as in ischemic stroke (Ilzecka, 1996; Albayrak et al., 1997) and traumatic brain injury (Morganti-Kossmann et al., 2002) or even independent of excitotoxic nerve damage (Chen et al., 1999). Although, the mechanisms of BBB dysfunction are not completely understood, the accompaniment of BBB breakdown with CNS injuries suggests that the endothelial barrier plays an important role that exacerbates disease or may contribute to development of other disorders (Oby and Janigro, 2006). BBB impairment results in decrease of nutrients, increase of toxins, ionic imbalance and inflammation in the CNS. In an *in vitro* model of ischemia, an upregulation of matrix proteases have been reported to alter the integrity of BBB by breaking down matrix adhesion receptors and inducing loss of cell-cell interactions between endothelial cells and astrocytes (Milner et al., 2008). Interestingly, endothelial cells can release permeabilizing factors such as nitric oxide (NO) and ATP, which can act on their associated endothelial receptors in an autocrine manner (Abbott et al., 2006; 2005). Disturbances in BBB TJs (Abbruscato and Davis, 1999) and increased permeability through the transcellular route (Plateel et al., 1997; Cipolla et al., 2004) have also been reported following hypoxia and hypoxia/reperfusion. Activation of transcription factors, nuclear factor-kappaB and hypoxia-inducible factor-1 (Witt et al., 2005) and upregulation of growth factors such as VEGF induce BBB permeability, *in vivo* (van Bruggen et al., 1999). Inflammatory mediators have been well known for modulating BBB permeability

(Abbott, 2005). However, the CNS is an immune privileged site and cytokines produced in response to peripheral damage are not able to enter the CNS unless it has been compromised by a primary injury or infection. As part of BBB immune surveillance, astrocytes and microglia may respond by releasing inflammatory cytokines, such as IL-1β, TNF-α, IL-6 and cycloxygenase-2 that affect TJ stability (Abbott, 2005). The BBB disruption may allow immune cells to enter as part of the inflammatory response through monocyte transmigration (Persidsky et al., 2006) as well as making the CNS vulnerable to toxins and pathogens that do further harm. As cells of the NVU are exposed to activated macrophages, the inflammatory response persists and BBB disruption continues (Persidsky et al., 1999). In experimental models of MS, T cells and monocytes appear to cause BBB disruption (Seeldrayer et al., 1993). Thus depending on the disease as in MS, the impairment of BBB may occur as an early event (Floris et al., 2004). Nonetheless, the preservation of junctional complexes and the cells of the NVU are integral to BBB function (Fig. 7) and suggest that promising therapies should be targeted to protecting all these components for treatment and prevention of neurological diseases.

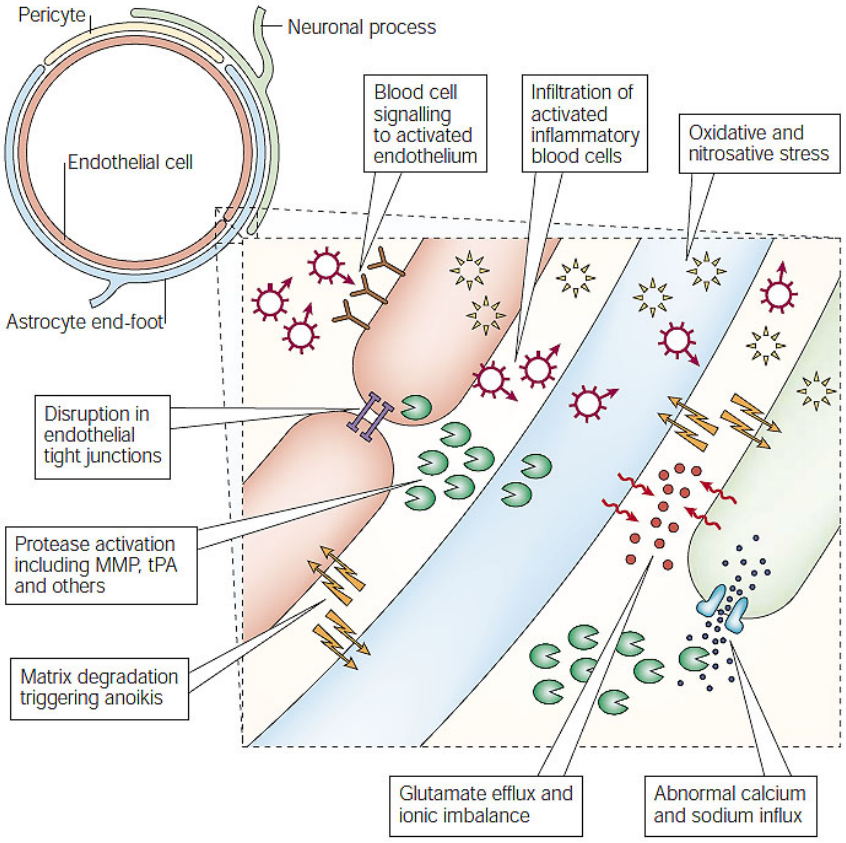

Figure 7. Contributing factors to loss of NVU and BBB dysfunction (Lo, 2003)

1.8 INTERVENTIONS FOR CEREBRAL ISCHEMIA

The aim of most interventions for cerebral ischemia is mainly neuroprotection. There are a number of ways to promote neuronal survival which is to reduce the infarct volume and death of vulnerable cells. Following cerebral ischemia, the cascade of pathophysiological events leading to cell death is complex. As discussed depolarization and activation of voltage-gated calcium channels and release of excitatory amino acids, such as glutamate occur, followed by activation of glutamate receptors that continue increasing intracellular calcium leading to release of enzymes producing free radicals and eventually neuronal death (Dirnagl et al., 1999). Therefore, inhibiting intracellular calcium, activation of enzymes and reducing glutamate excitotoxicity have all been a predominant focus in designing therapies for cerebral ischemia. However, successful experiments carried out in animal models have not been able to yield promising outcomes in the clinics.

1.8.1 ANTI-THROMBOTICS

In acute ischemic stroke, early restoration of blood flow accompanied by anti-thrombotics is a common treatment. During the 1960's, complications with brain hemorrhaging occurred with thrombolytic drugs, such as streptokinase, which was discouraging (Wahlgren and Ahmed, 2004). However, tissue-type plasminogen activator (t-PA), a protein that is naturally produced and dissolves blood clots is currently the only approved anti-thrombotic therapy by the Food and Drug Administration (FDA) for acute ischemic stroke (NINDS, 1995). Restoring blood flow by tPA is critical for survival when given within 3 hours after stroke onset but is associated with a high risk of hemorrhage into the ischemic tissue if the treatment is provided after this time window, suggesting the primary importance of rescuing the cerebral vasculature by re-establishing blood flow in limiting ischemia. Currently about 2-5 % of stroke patients are eligible for this thrombolytic therapy (Donnan et al., 2011). In experimental models tPA may induce excitotoxicity (Nicole et al., 2001) or blood-brain barrier disruption by activating matrix metalloproteinases (Wang et al., 2003). Combination therapies have been considered to lengthen the tPA therapeutic window while reducing adverse effects of reperfusion and plasminogen activation (Liu et al., 2004; Cheng et al., 2006).

1.8.2 NEUROPROTECTANTS

Since anti-thrombotics are mainly effective during acute ischemic injury other interventions are necessary for those stroke patients which are uneligible for tPA therapy. Furthermore, the evolution of brain injury in a majority of stroke patients is rather slow occurring over a period of

hours-to-days following an attack. As previously eluded, neurons of the ischemic core are necrotic, and neurons of the penumbral region can be protected if blood flow and oxygenation are resupplied in time (Green et al., 2003; Durukan and Tatlisumak 2007). Therefore, these neurons of the penumbral region are of interest for designing neuroprotective agents (Ginsberg et al., 1999). Many experimental studies have been conducted in various animal models of stroke showing incontrovertible positive effects in reduced infarct size and mortality rate with different types of pharmacological agents yet disappointingly have been ineffective in clinical trials (Ginsberg, 2008). If *in vivo* models are suppose to provide the proof-of-principle then why should the treatments not work in humans? There are a number of reasons for this discrepancy. Mainly, the treatments given in clinical trials are not comparable to controlled animal studies. Timing of administration is a critical issue and for some neuroprotective agents a delay in the treatment decreases a drug's efficacy. Incorrect dosage and slow bioavailability of the drug could also account for a poor outcome. Moreover, inadequacies of attaining prognostically important baseline variables because of unrandomized techniques and using small sample sizes in trials are other possible explanations (Wahlgren and Ahmed, 2004). On the other hand, in the laboratory setting the animal models used for studying may not be suitable and representative of stroke in humans. Young and healthy rather than old animals are preferred. Also, stroke is not only a disorder due to ischemic damage but involves other co-morbities such as atherosclerosis, which is a very important risk factor and animal models typically show one type of disorder (Wahlgren and Ahmed, 2004). Unfortunately, in many instances some agents have been subjected to clinical trials without even enough solid data from pre-clinical ischemia studies (Ginsberg, 2008). Despite these discrepancies, clinical trials are currently still on-going for some potential neuroprotective candidates (Table 2. Minnerup et al., 2012).

Neuroprotective treatments currently investigated in phase II and phase III clinical trials.

Treatment	Mode of action
Magnesium Sulfate	Anti-excitotoxic, NMDA ion channel blocker
Albumin	Antioxidant, Hemodiluiting agent
Cyclosporin A	Anti-inflammatory, anti-excitotoxic
Dapsone (diamino-diphenyl sulfone, DDS)	Anti-inflammatory, antioxidant
Deferoxamine mesylate	Iron chelator, antioxidant
Ebselen	Antioxidant, free radical scavenger
GM602	Anti-apoptotic and anti-inflammatory
Hypothermia	Reduce cerebral oxygen metabolism, synaptic inhibitor
Lovastatin	Antioxidant, HMGCoA inhibitor
Minocycline	Anti-inflammatory, antioxidant
PG2 (Polysaccharides of Astragalus membranaceus)	Chinese Herb, assumed antioxidative and anti-inflammatory
Simvastatin	Antioxidant, HMGCoA inhibitor
Spheno-Palatine Ganglion (SPG) stimulation	Induction of cerebral vasodilatation
THR-18	Synthetic plasminogen activator inhibitor
Transcranial laser therapy	Mitochondrial stimulation

Information on ongoing clinical studies gathered from the databases Clinicaltrials.gov (August 2012) and Strokecenter.org (August 2012)

Table 2. Neuroprotective compounds, currently in clinical trials (Minnerup et al., 2012).

1.8.3 THERAPEUTIC HYPOTHERMIA

Other than by pharmacological treatment, a very promising neuroprotective intervention is mild therapeutic hypothermia. It has been around since the late 1940's as a neuroprotective strategy for various clinical problems (Hoesch and Geocadin, 2007). Still, it is the most effective treatment at preventing brain damage and secondary injury by reperfusion in patients suffering from transient global cerebral ischemia (Hypothermia after Cardiac Arrest Study Group; 2002; Bernard et al., 2002). Although, the molecular mechanisms underlying the protective effect have not been established, mild hypothermia seems to inhibit many aspects of cellular injury along with reducing energy demand and energy consumption. Cooling of the head or the body after injury lessens cell death by slowing down cellular metabolism, inhibiting glutamate release, by disrupting the apoptotic pathway, and even preventing cellular ionic imbalance (Drury et al., 2010). Furthermore, it has been shown effective at ameliorating reperfusion injury due to oxidative stress (Katz et al., 2004) and preventing BBB breakdown and cerebral edema (Baumann et al., 2009). The pleiotropic actions of hypothermia suggest that targeting multiple rather than individual death pathways provides better protection.

For hypothermia to be positively effective, however, the timing and duration of administration after injury appears critical. The therapeutic time window for hypothermia seems optimally

effective when administered early and no later than 6 hours after injury. Moreover, experimental evidence show partial or temporary neuroprotection when the duration of hypothermia is less than 6 hours, while a longer duration of up to 72 hours offers a better improvement in recovery and neurological performance (Drury et al., 2010). The severity of the insult is also another important factor that may influence the efficacy of hypothermia in delaying further damage (Iwata et al., 2007). Adverse effects are few but include shivering, hypotension, slowing of heart rate, pneumonia, and infection (Minnerup, et al., 2012).

1.9 SCOPE OF THESIS

Brain homeostasis is severely impaired by a decrease in oxygen and/ or glucose resulting in disturbances in neuronal activity and function and leading to cell death. Excitotoxicity by overactivation of GluRs is a major contributing factor of neurodegeneration induced by hypoxia or ischemia (Choi, 1992). Unfortunately, there are no neuroprotectants, except for therapeutic hypothermia, that have been clinically effective in treating brain injury after stroke (Gladstone et al., 2002). The function of the BBB is to protect the CNS from toxins and pathogens and only allow important nutrients to pass. The BBB is made of tight junction and transporter proteins that form the paracellular and transcellular pathways, respectively. NVU is a conceptual framework that has been proposed to maintain neurovascular properties and BBB function (Lo, 2003; Iadecola, 2004). The NVU is composed of neuronal, glial, and vascular elements, which together provide the optimal matrix for sustaining BBB function. A number of studies have reported BBB disruption followed by brain injury. *In vitro* data suggest that tight junction proteins may be compromised (Hawkins and Davis, 2005). However, few *in vitro* studies have brought together more than two cellular partners (Bendfeldt et al., 2007; Nakagawa et al., 2009; Al Ahmad et al., 2011). Interestingly, in animal models of global cerebral ischemia, BBB leakage and capillary damage were found in the hippocampus, suggesting the vascular bed to be associated with regional vulnerability to ischemic damage (Suzuki et al., 1983; Imdahl and Hossmann, 1986, Jingtao et al., 1999, Won et al., 2011).

The hippocampal organotypic slice culture system is an excellent tool for studying cellular and molecular mechanisms of neuronal death and for testing pharmacological compounds that may be related to neuronal survival. Age dependent differences can be analyzed by changing the culturing time and preparing slices of different age groups. Furthermore, both physiological and pathophysiological factors can be studied. Under such a well controlled system, many aspects from development, plasticity, injury, recovery, and to neurogenesis can be studied (Noraberg et al., 2005). Functional, morphological, and pharmacological properties can be better understood.

In the following studies we have taken advantage of the organotypic slice culture system in several ways: first we continued the characterization of COSCs as an *in vitro* BBB model with the objective of determining the presence and function of BBB transporters as they also make up the cerebrovascular system and control the influx and efflux of molecules within the brain. Second, we studied BBB disruption and neurovascular remodeling after oxygen and glucose-deprivation seeking evidence for a crosstalk between neurons and endothelial cells. Third, we studied potential mechanisms of therapeutic hypothermia induced neuroprotection that has been effective in treating neurological injury caused by neonatal asphyxia with a focus on the role of the RNA binding protein, RBM3.

CHAPTER II: PRESERVATION OF GLUT1 AND P-GP TRANSPORTERS USING IN-VITRO BBB MODEL

2.1 EXPRESSION AND FUNCTION OF GLUT1 AND P-GP

Two of the best known transporters of the BBB are GLUT1 and P-gp. Since the brain is an extremely metabolic organ, a constant glucose supply is essential. GLUT1 is expressed in endothelial cells and astrocytes. It is the main glucose transporter of the BBB (Simpson et al., 2007). The distribution of GLUT1 may determine the status of BBB function (Dermietzel et al., 1992; Garbuzova-Davis et al., 2007). It is an integral membrane protein which is expressed asymmetrically along luminal and abluminal membranes of the endothelium as evidenced by immunogold electron microscopy in rat and bovine brains (Farrell and Pardridge, 1991; Simpson et al. 2001). In brain capillaries, P-gp carries substrates that have entered the endothelial membrane back into the blood. As mentioned previously, hydrophobic molecules < 400 Da easily cross the BBB and apparently P-gp accounts for this size restriction. The multidrug resistance P-gp is involved in effluxing a variety of drugs including chemotherapeutics, opioids, steroids, antibiotics, calcium-channel blockers, immunosuppressants, and etc. (Bauer et al., 2005). The ABC transporter typically develops later after the BBB has been established (Qin and Sato, 1995; Matsuoka et al., 1999; Schumacher and Mollgard, 1997). Lipophilic substances such as narcotics, alcohol, and anticonvulsants are able to pass the BBB, however, most pharmacological drugs that are not lipophilic fail because they are not able to cross the BBB due to tight junction proteins and/or cannot remain in the CNS due to efflux by P-gp. Generally, efflux transporters are located on the luminal membrane which is thought to effectively prevent xenobiotics from entering the CNS. Whether the localization of P-gp on the endothelial membrane is preferably luminal or abluminal is not clear from immunolocalization studies, although functional analyses of P-gp suggest that it is located on the luminal membrane (Bauer et al., 2005). Since the capillary structure is complex, both GLUT1 and P-gp have been studied in endothelial cell culture models (El Hafny et al., 1997; Janigro et al., 1999; Bauer and Bauer, 2000) however have not been previously assessed in organotypic slice cultures.

2.2 BBB MODELS

As mentioned earlier, BBB is maintained by the NVU, which is composed of several cell types: astrocytes, pericytes, nerve cells, and perivascular microglia surrounding the endothelium with their processes along with the basal lamina (Abbott et al., 2010; Armulik et al., 2010). *In vivo* models have the main advantage of preserving the anatomical structure of the neurovascular

unit and BBB interface. Furthermore, we can study the regional variation that evidently exists within the brain. The study of the BBB has been focused by many on the permeability. Brain perfusion techniques and intravenous administration of compounds of different sizes have been utilized as tracers for measuring transport and permeability across the BBB (Grant et al., 1998). This method is only sufficient to observe BBB disruption. Since the BBB is a dynamic structure assessing the kinetics of BBB opening is rather important. Therefore, *in vitro* models were established by isolating and culturing brain endothelial cells with astrocyte conditioned medium or by co-culturing methods with astrocytes and more recently with pericytes to establish BBB function with a high TEER, to express tight junction proteins, and to display transport properties (Grant et al., 1998; Nakagawa et al., 2007; Abbott and Romero, 1996). The molecular and cellular mechanisms responsible for function and maintenance of the BBB under physiological or pathophysiological conditions could be studied. Therefore, a lot of BBB research has been based on developing in-vitro systems.

Many utilize transwell tissue culture plates designed for co-culturing. These systems allow the endothelial cells to be cultured on a porous membrane with a plastic support that is able to fit within a well containing a separate culture of either astrocytes or pericytes. This interaction, between endothelial cells and astrocytes in this co-culturing system should mimic the organization of BBB at the level of the astrocytic end-feet and the endothelial cell interface. Furthermore, the endothelial monolayer facing the cells is considered as the abluminal side and the one facing away is the luminal side. Indeed, this model has helped the BBB field tremendously. The permeability of many compounds can be tested by using this model however the specificity of the BBB in transporters and the TEER are often not physiologically relevant (Grant et al., 1998). Therefore, translating in-vitro to in-vivo studies has been unsatisfactory and the data of many in-vitro BBB studies has been questioned.

Another important discrepancy between the in-vivo situation and many co-culture systems is that dissociated cells do not provide the 3-dimensional aspect of the BBB, composed of a vascular lumen with the abluminal side surrounded by pericytes and enclosed by a polarized network of astrocytic endfeets linking the communication between the nerves cells and the blood vessels. To resolve this disparity between in-vitro and in-vivo BBB systems, our lab previously established an alternative BBB model utilizing cortical organotypic slice cultures (COSCs) of newborn mouse brains and in the presence of FGF2 BBB tight junction proteins are well preserved (Bendfeldt et al., 2007).

2.3 BBB PRESERVATION IN COSCs

This slice culture system allows the study of the different cell types making up the NVU taking in to account their 3-dimentional structural connectivity. In the presence of low amounts of FGF2, vascular structures can be preserved at 37°C up to 10 days in the absence of blood flow. With this tool it is possible to study many components of the BBB in a more realistic *in vitro* environment, with cell-cell interactions between natural *in vivo* partners. COSCs are derived from neonatal mice of postnatal age 3 (P3). In the presence of 5 ng/mL FGF2 the density of vessels is higher than in controls without growth factor or in cultures with a higher FGF2 concentration. Moreover, the expression of BBB markers: occludin, claudin-5, claudin-3, and ZO-1 are all retained (Bendfeldt et al., 2007).

2.4 ARE BBB TRANSPORTERS EXPRESSED IN COSCs?

Tight junction proteins are not the only ones involved in controlling the permeability of the BBB. Other important determinants for BBB function are transporter proteins expressed by endothelial cells which modulate and regulate transport across the endothelium. GLUT1 allows for transport of glucose and the ABC transporter, P-gp, effluxes drugs across the BBB. Although, we showed that in the presence of FGF2 tight junction proteins such as claudin-3,-5, ZO-1, and occludin in blood vessels were well preserved (Bendfeldt et al., 2007) we did not examine at that time how transporters are expressed in COSCs. Therefore, in a follow up study, we analyzed the expression of GLUT-1 and P-gp in slice cultures derived from young and old mice. Furthermore, we performed transport studies in order to test the functionality of P-gp. Rhodamine 123, a substrate of P-gp, can be seen collected into the lumen of blood vessels in live slices within 2 hours. The specificity of the transporter was examined by applying an inhibitor of P-gp, verapamil, which resulted in the absence of Rhodamine 123 in the lumen of the blood vessels.

2.5 PRESERVATION OF TRANSENDOTHELIAL GLUCOSE TRANSPORTER 1 AND P-GLYCOPROTEIN TRANSPORTERS IN A CORTICAL SLICE CULTURE MODEL OF THE BLOOD-BRAIN BARRIER

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PRESERVATION OF TRANSENDOTHELIAL GLUCOSE TRANSPORTER 1 AND P-GLYCOPROTEIN TRANSPORTERS IN A CORTICAL SLICE CULTURE MODEL OF THE BLOOD–BRAIN BARRIER

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Abstract—A variety of neurological diseases are characterized by disturbances of the blood–brain barrier (BBB) and its transporters. We recently introduced fibroblast growth factor treated cortical organotypic slice cultures of mice as a model for *in vitro* studies of the blood–brain barrier and have now further characterized the maintenance and function of transport-proteins typically expressed in the endothelium of cerebral blood vessels. The glucose transporter GLUT-1 is present in blood vessels of slice cultures derived from postnatal day 4 to 21 mice after 3 days *in vitro*. The endothelial multidrug resistance P-glycoprotein (P-gp) which is involved in the control of pharmacological substance transport across the blood–brain barrier is also maintained in blood vessels, most prominently in slice cultures derived from postnatal day 14 and 21 mice. To assess P-gp function, we tested rhodamine 123 transport in presence or absence of the P-gp inhibitor verapamil. Rhodamine 123-fluorescence accumulated rapidly in the vascular lumen both in acute slices and in slices cultured for 3 days *in vitro*. Our results provide evidence that endothelial transporters and their functional properties can be maintained in organotypic cortical slices cultures, thus making it an attractive model system for the study of the blood–brain barrier.

Key words: brain vasculature, endothelial cells, P-glycoprotein, glucose transporter 1, cortical organotypic slice cultures, mouse.

Disturbances of the integrity and transport function of the blood–brain barrier (BBB) are typical for neuropathologies such as stroke, Alzheimer's disease and multiple sclerosis. The BBB separates the nervous tissue from the general circulation, thereby ensuring an optimal environment for cerebral functions. The unique BBB phenotype characterized by the tightest endothelium is the result of the continuous influence from the surrounding nervous tissue, including pericytes embedded in the basement membrane, perivascular microglia, astrocytes that surround the basement membrane with their processes, and the basal lamina consisting of type IV collagen; fibronectin and laminin (Abbott, 2002).

To date, useful *in vitro* BBB models which take the structural connectivity of the different BBB elements into account are rare. We recently introduced fibroblast growth factor (FGF-2) treated cortical organotypic slice cultures (COSCs) of newborn mice as a new model for *in vitro* studies of the BBB (Bendfeldt et al., 2007). In the proposed model both the viability and the structural connectivity of the different brain tissue elements are maintained for several days *in vitro*. In addition, persistence of the tight junction proteins occludin, zonula occludens protein-1, claudin-3 and claudin-5 suggests preservation of BBB integrity at the level of the tight junction.

BBB function, however, is not only based on physical restrictions secured by interendothelial tight junctions, but also on the function of highly efficient transport proteins (Abbott and Romero, 1996; Begley and Brightman, 2003). Different selective transporters provide for blood to brain permeability of certain highly hydrophilic solutes (e.g., glucose and amino acids), or hydrophobic foreign chemicals (Miller et al., 2008). The glucose transporter 1 (GLUT-1) is expressed selectively in the capillary endothelium of the brain (Guo et al., 2005) and the distribution of GLUT-1 is considered a sensitive indicator of normal or abnormal BBB function (Dermietzel et al., 1992; Garbuzova-Davis et al., 2007).

P-glycoprotein (P-gp), a 170–180 kDa plasma membrane-associated protein, is a product of the multidrug resistance gene (mdr) (Ling, 1997) and belongs to the ATP-binding cassette (ABC) superfamily (Cordon-Cardo et al., 1989). Brain capillaries express particularly high levels of P-gp. Because of its location in the luminal membrane, its high expression level, as well as its ability to transport drugs and several endogenous compounds, P-gp is considered as a primary control for penetration

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Abbreviations: ABC, ATP-binding cassette; BBB, blood–brain barrier; BS, blocking solution; COSCs, cortical organotypic slice cultures; DIV, day *in vitro*; FGF-2, fibroblast growth factor -2; GFAP, Glial Fibrillary Acidic Protein; GLUT-1, glucose transporter 1; mdr, multidrug resistance gene; NeuN, anti-neuronal nuclei; P, postnatal day; PBS, phosphate buffered saline; P-gp, P-glycoprotein; R123, rhodamine 123; TBST, Tris–Buffered Saline containing 0.5% Triton X-100.

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of chemical substances across the BBB (Schinkel et al., 1995; Lin and Yamazaki, 2003). Furthermore recent data indicate that P-gp is involved in the excretion of amyloid beta, a peptide that is associated with Alzheimer's disease (Cirrito et al., 2005).

The aim of the present study was twofold: to determine the preservation of the vasculature in COSCs derived from more mature mice (postnatal day 8–21) and to search for the presence of the BBB transporters GLUT-1 and P-gp. In addition, here we investigated transport function of P-gp in blood vessels of acute cortical slices and COSC's of the mouse brain.

EXPERIMENTAL PROCEDURES

Culture protocol

COSCs were prepared and cultivated with the classical technique (Stoppini et al., 1991) with slight modifications (Radojevic and Kapfhammer, 2004; Bendfeldt et al., 2007). Postnatal day (P) 4, P8, P14 and P21 C57Bl/6 mice were decapitated. After rapidly removing the brain, the cortex was dissected together with parts of the underlying striatum or diencephalon, and 250-μm-thick frontal slices were cut on a tissue chopper. Slices were separated, placed on Millicell-CM 0.4 μm culture plates (six slices per membrane; Millipore, Zug, Switzerland) in six-well dishes and were maintained in culture medium at 37 °C in a humidified CO_2-enriched atmosphere for a minimum of one day *in vitro* (DIV). The medium consisted of HEPES-buffered minimal essential medium (50%), Hank's Balanced Salt Solution (HBSS) (25%), and heat-inactivated horse serum (25%) complemented with glutamax (2 mM; Invitrogen, Eugene, OR, USA), Glucose (1 g/l) and adjusted to pH 7.3. FGF-2 (Peprotech, London, UK) was added to the medium (5 ng/mL) and medium was changed every other day. No antibiotics or antimitotics were used. All animal experiments were performed with permission of the local animal care committee and in accordance with international guidelines on handling laboratory animals and the present Swiss law.

Preparation for immunohistochemistry

The cellular integrity of the slices, the formation of blood vessels and the presence of transporters were analyzed by immunofluorescence of relevant marker proteins. Cultures were fixed for 3 h at room temperature (RT) with 4% paraformaldehyde in phosphate buffered saline (PBS), rinsed three times with 0.1 M PBS, and incubated in blocking solution (BS) containing Tris–Buffered Saline containing 0.5% Triton X-100 (TBST) and 3% normal goat serum for 90 min at RT. Primary antibodies were diluted in BS as follows: the neuronal marker anti-Neuronal Nuclei (mouse anti-NeuN 1:250; Chemicon, Temecula, CA, USA), the astrocyte marker Glial Fibrillary Acidic Protein (rabbit anti-GFAP 1:250; Dako, High Wycombe, UK), the glucose transporter 1 (rabbit anti-GLUT-1 1:1000; Thermo, Fremont, CA, USA) and P-glycoprotein (mouse anti-P-gp 1:1000; Calbiochem, Darmstadt, Germany). Architecture of blood vessels was visualized by labeling for the basal membrane constituent laminin (rabbit anti-Lam 1:200 or mouse anti-Lam 1:2000; Sigma, St. Louis, MO, USA), and the tight junction was visualized by labeling for the integral membrane protein claudin-5 (rabbit anti-Cl-5 1:200 or mouse anti-Cl-5 1:50; Zymed, San Francisco, CA, USA). Cultures were incubated with primary antibodies overnight at 4 °C, rinsed with TBST at RT, and incubated with Alexa Fluorochromes (Invitrogen, Eugene, OR, USA) linked goat-anti-rabbit (1:1000) or goat-anti-mouse (1:1000) secondary antibodies in BS for 2 h at RT. Unspecific staining was controlled by omitting the primary antibody. The slices were mounted on glass slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and viewed on a Zeiss Axio Imager.Z1 (Zeiss, Oberkochen, Germany). High magnification imaging was performed using a laser-scanning confocal microscope, Zeiss LSM 510 Meta, (Carl Zeiss AG, Oberkochen, Germany) and then processed by Bitplane Imaris (Bitplane AG, Zurich, Switzerland). Recorded images were adjusted for brightness and contrast with Photoshop (Adobe, San Jose, CA, USA) and ImageJ (Rasband W.S., U. S. National Institutes of Health, Bethesda, MD, USA).

Transport activity of P-glycoprotein in acute and cultured slices

Tissue dissection was the same as described above. Slices were separated, placed in 6-well dishes and were maintained in culture medium, at 37 °C in a humidified CO_2-enriched atmosphere for either 30 min for the experiments with the acute slices or 3 days for the experiments with cultured slices. The medium consisted of PBS and heat-inactivated horse serum (2.5%) complemented with glutamax (2 mM; Invitrogen, Eugene, OR, USA) and glucose (1 g/l) and adjusted to pH 7.3. Slices were incubated with 0.5 μM of rhodamine 123 (R123; Sigma), a P-gp substrate, up to 120 min and to control for specificity of rhodamine uptake by P-gp, slices were treated with a P-gp inhibitor, verapamil (100 μM; Sigma). Images were taken with a Zeiss Axio Imager.Z1. For confocal imaging studies organotypic slices of 3 days *in vitro* were cultured on porous membranes (Millipore), incubated for 120 min with either R123 or R123 and verapamil and then transferred onto glass bottom dishes (MatTek Corporation, Ashland, MA, USA) before imaging was performed on a laser-scanning confocal microscope (Zeiss, LSM 510 Meta).

Confocal microscopy

The sections were imaged with a Zeiss LSM 510 Meta scanning confocal microscope system equipped with an argon ion laser for the green channel and a helium-laser for the red channel. A series of optical sections (63× or 100× magnification) was acquired at 0.5 μm intervals through the entire thickness of the specimen (approximately 10–25 μm) and then projected into a single-focus image with the Bitplane Imaris (Bitplane AG).

RESULTS

Maintenance of blood–brain barrier characteristics in slice cultures derived from mouse pups up to postnatal day 21

In a prior study we concentrated on cultures obtained from P3 and P4 mice (Bendfeldt et al., 2007). Here, we have studied cultures derived from more mature mice (P8, P14 and P21). Cultures from cortical slices of P4, P8, P14 and P21 mice remained well preserved in the presence of FGF-2 (Figs. 1 and 2). The neuronal DNA-binding protein NeuN revealed survival and differentiation of nerve cells in the cultures prepared from brains of P4, P8, P14 and P21 mice (Fig. 1B, D, F, H). GFAP-staining showed characteristically shaped astrocytes. A dense network of astrocytic endfeet engulfed the cerebral microvasculature (Fig. 2A, B1–4).

Immunolabeling of the extracellular matrix protein laminin showed a dense network of blood vessels in cultures derived from P4, P8, P14 and P21 mice in the presence of FGF-2 (Figs. 1 and 2A). Labeling for the tight junctional

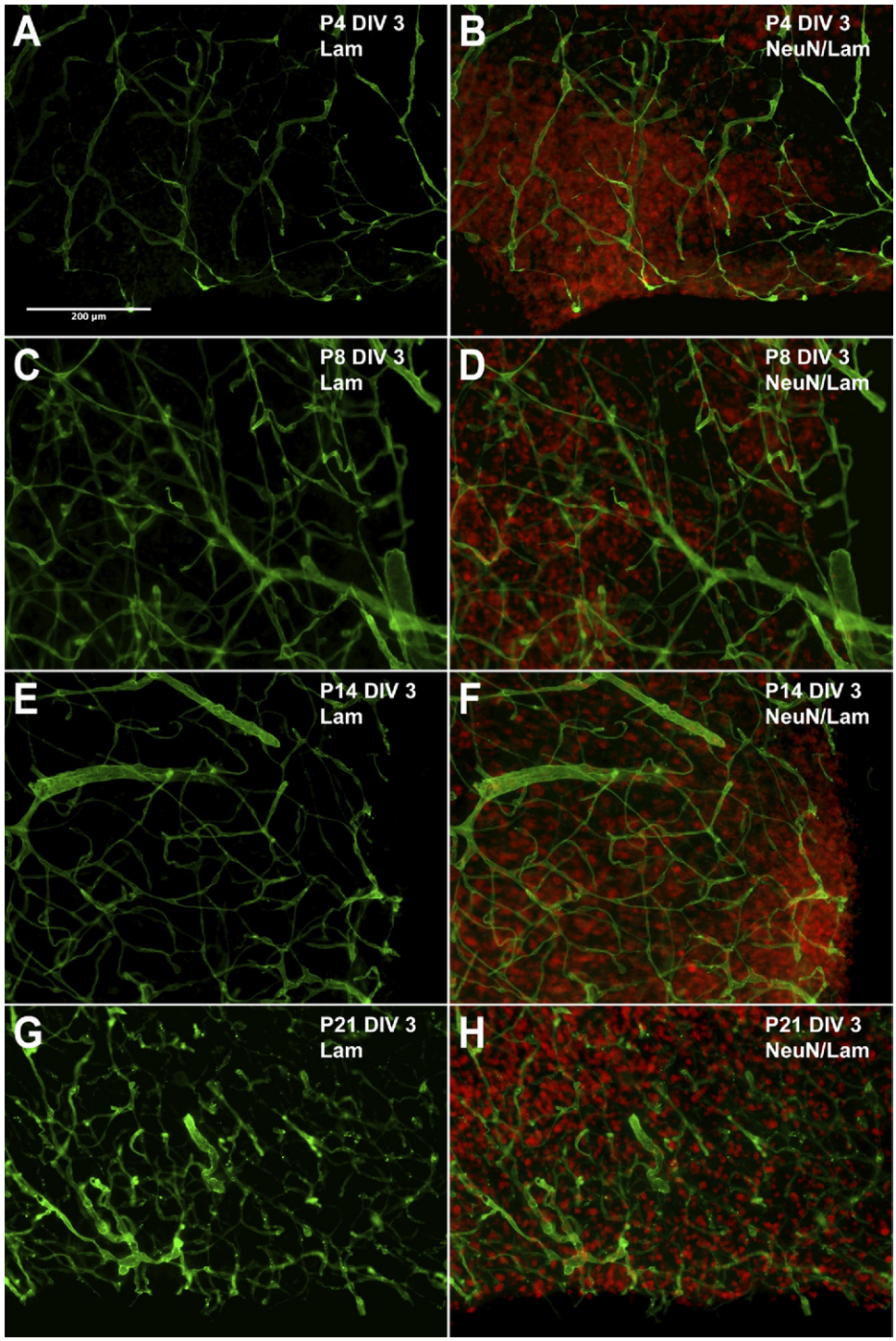

Fig. 1. Demonstration of cerebral microvessels and neurons in cortical organotypic slice cultures at DIV 3 from postnatal day 4–21 mice under FGF-2 treatment. Immunofluorescence images labeled for laminin (green) revealed the presence of microvessels in COSCs after incubation with 5 ng/mL FGF-2 and show survival and complexity of the vascular network in P4 (A, B), P8 (C, D), P14 (E, F) and P21 (G, H) mice. Double-immunolabeling for laminin and the neuronal DNA-binding protein NeuN (red) revealed well-differentiated nerve cells in cultures derived from P4 (B), P8 (D), P14 (F) and P21 (H) mice.

integral membrane protein claudin-5 indicated the morphological integrity of the BBB at the level of the tight junction after DIV 3 in cultures derived from P4, P8, P14 and P21 mice (Fig. 2C–F).

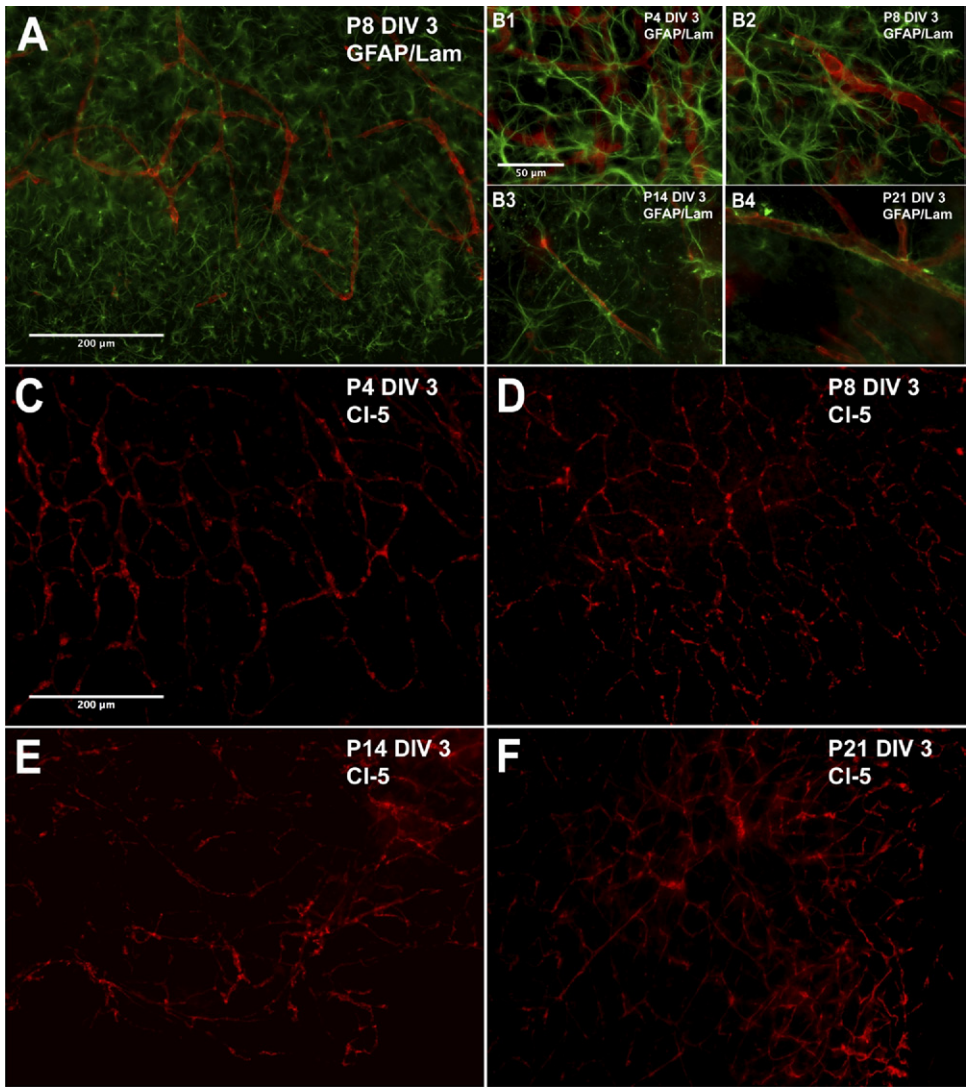

Fig. 2. Astrocytes and tight junctions in cortical organotypic slice cultures at DIV 3 from postnatal day 4–21 mice under FGF-2 treatment. Immunofluorescence images double labeled for laminin (red) and for GFAP (green) show normally shaped astrocytes as well as a dense network of astrocytic endfeet in close apposition to the cerebral microvasculature (A, B1–4). Immunolabeling for claudin-5 indicated the morphological integrity of the BBB at the level of the tight junction in cultures derived from P4 (C), P8 (D), P14 (E) and P21 (F) mice.

The GLUT-1 transporter is specifically expressed in blood vessels in cortical organotypic slice cultures derived from various postnatal ages

We have studied the presence of GLUT-1 protein in COSCs derived from P4, P8, P14 and P21 mice after DIV 3. This allowed us to evaluate whether the maturity of the cortical tissue would affect GLUT-1 expression. A high density of GLUT-1 immunoreactivity was found in slices from all age groups (Fig. 3). Cultures from younger mice (P4, P8) showed a more discontinuous pattern of GLUT-1 staining as compared to the older mice (P14, P21). When GLUT-1 was co-stained with laminin, a majority of blood vessels showed colocalization. By confocal microscopy the GLUT-1 expression in blood vessels of slices from P14 animals was not as consistent as from P21 animals. GLUT-1 expression could be found along the inner and outer walls of small vessels (Fig. 3E–G) in P14 cultures. GLUT-1 expression was more continuous in slices derived from P21 mice, where it was present in the wall but not the lumen of the blood vessels consistent with its presence in the plasma membrane of the laminin labeled vessels (Fig. 3H–J). Localization to the vessel wall is confirmed in the orthogonal view, tracing the lumen of a blood vessel (Fig. 3H, right part).

Specific expression of P-glycoprotein in blood vessels of cortical organotypic slice cultures

Another important endothelial transport protein typically present in cerebral blood vessels is P-gp, a product of the mdr gene, which is critically involved in the control of drug penetration into the CNS tissue. Immunostainings were performed on cultures derived from P4, P8, P14 and P21 mice (DIV 3).

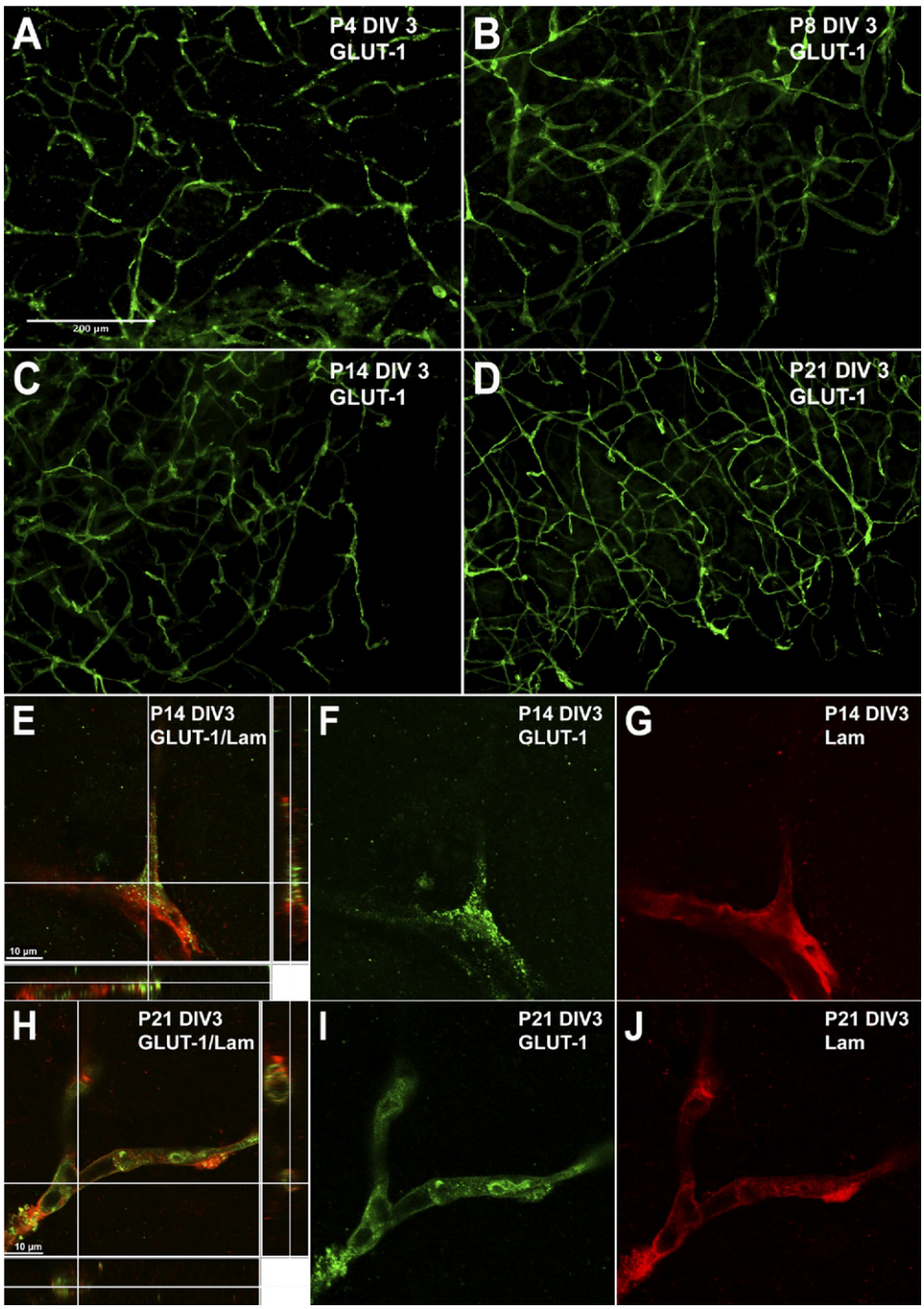

Fig. 3. Demonstration of the glucose transporter GLUT-1 in brain endothelial cells of cortical organotypic slice cultures in the presence of FGF-2. GLUT-1 staining was present in the majority of blood vessels and preferentially in smaller vessels in a rather homogenous fashion. In cultures from younger mice (P4, P8) (A, B), GLUT-1 showed a slightly discontinuous staining pattern as compared to the older mice (P14, P21) (C, D). Doublestaining of GLUT-1 (green) and laminin (red) was performed with confocal microscopy and showed that GLUT-1 was exclusively located in blood vessels (E–J). Orthogonal reconstructions of double-labeled microvessels as viewed in the *x*–*z* (bottom) and *y*–*z* (right) planes show that GLUT-1 expression is maintained along the inner and outer borders of the vessels (E, H).

P-gp immunofluorescencewas confined to endothelial cells as verified in colocalization studies with laminin (Fig. 4) or claudin 5 (not shown) and was much more pronounced in vessels from older mice than in those from younger mice (Fig. 4). It was preferentially detected in capillaries where it appeared rather in patches than continuously along the vessel walls. In P21 cultures, P-gp staining appeared most intense in capillaries and in the endothelium of larger vessels (Fig. 4E, F). In general, the expression pattern of P-gp was found to be more variable compared to GLUT-1. By confocal microscopy, both cultures showed P-gp expression either along the inner walls

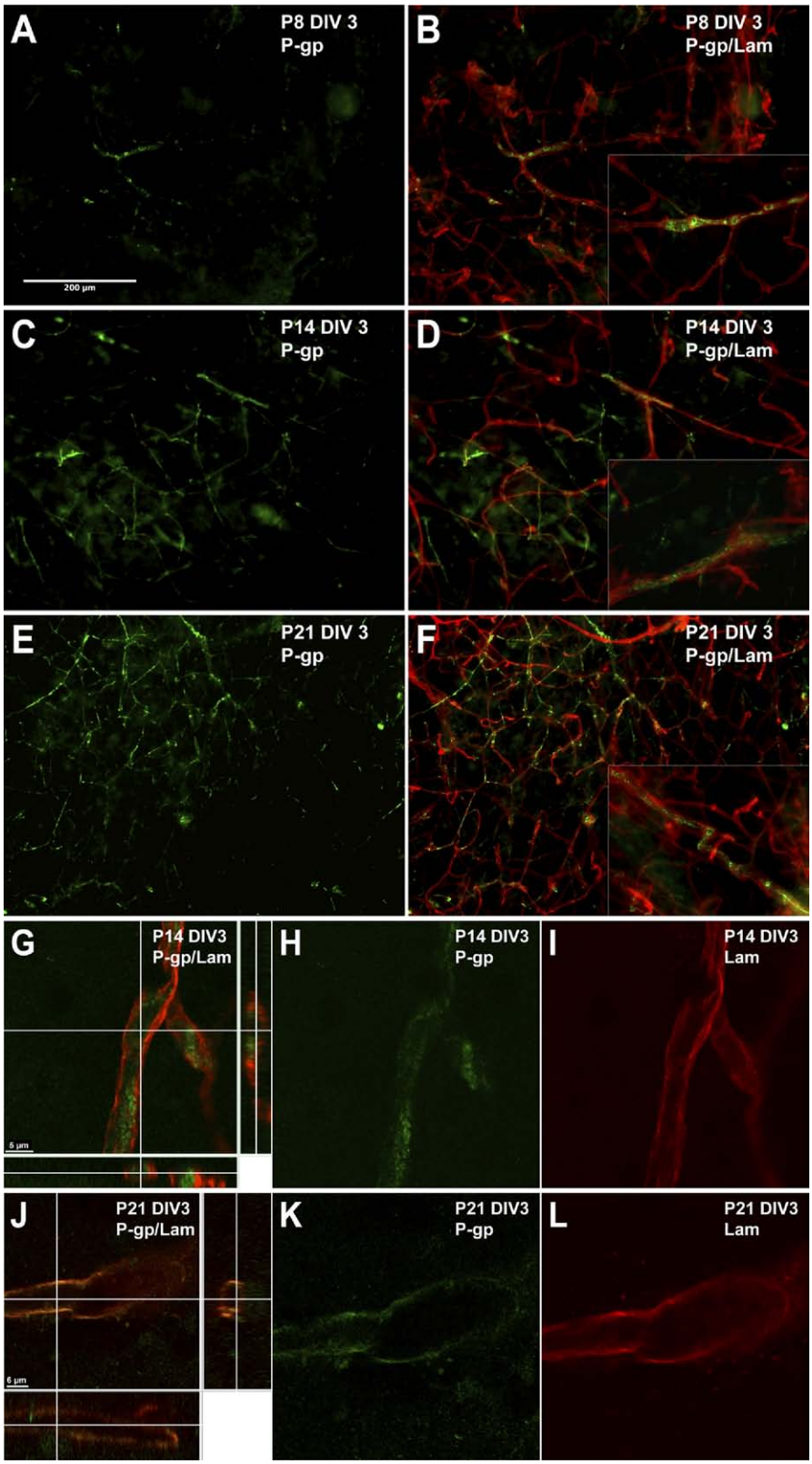

Fig. 4. Immunolocalization of the efflux transporter P-gp (green) in vascular endothelial cells of cortical organotypic slice cultures in the presence of FGF-2. In general, P-gp staining was barely detectable in cultures of the younger mice (P8) (A, B) where it appeared rather in patches than continuously along the vessel walls. In older mice P14 (C, D, G–I) and at P21 (E, F, J–L), however, P-gp staining was stronger and rather homogenous along the vessel wall. A close colocalization of P-gp and laminin (red) staining was preferentially detectable in capillaries. P-gp staining was most intense in P21 cultures (E, F, J–L). Orthogonal reconstructions of double-labeled microvessels confirmed that P-gp is localized along the wall (G) and within the borders of the endothelium as stained by laminin (J).

of laminin labeled vessels (Fig. 4G–I) or colocalized with the plasma membrane (Fig. 4J–L), similar to GLUT-1 expression. However, unlike GLUT-1, P-gp expression was never found along the outer borders of large vessels, but was located along the inner borders (Fig. 4G).

P-glycoprotein mediated transport in acute and cultured cortical slices of newborn mice

Accumulation of the fluorescent dye R123 on the luminal side of brain microvessels is frequently used to determine the transport capacity of P-gp in cultured endothelial cells and blood vessels. This is the first study that has investigated R123 transport across blood vessels of acute and cultured cortical slices either in the absence or presence of the P-gp inhibiting agent verapamil (Bogman et al., 2001).

Uptake of R123 occurred in capillaries and small blood vessels of acute (Fig. 5A) and cultured slices (Fig. 5B–F) consistent with the distribution pattern of P-gp in the cultures (Fig. 4). In capillaries, R123 accumulated in the vascular lumen (Fig. 5A–D). In small vessels and in larger vessels R123 accumulation appeared in patches in the endothelial cells (Fig. 5E, F). In acute slices R123 uptake occurred in a time dependent manner with more luminal R123 accumulation as the incubation time period increased with a maximum at 120 min (Fig. 5A). With confocal microscopy, a higher magnification than 63× was not possible without bleaching out the fluorescence. In DIV 3 cultures of P14 and P21 mice R123 seemed to be present in the luminal space of small vessels as shown in the orthogonal views of the confocal images (Fig. 5B–F). Although background staining was more prominent in cultures as compared to the acute slices they clearly demonstrate that the transport capacity of P-gp is maintained in slice cultures for several days. In larger vessels, the accumulation of R123 was found in the luminal space (Fig. 5F) as well, but also accumulated in the cytoplasm of endothelial and smooth muscle cells that encompass the vessels (Fig. 5E). The presence of the P-gp inhibitor verapamil completely prevented R123 transport into large and small networks of vessels for both acute (Fig. 5G) and cultured slices (Fig. 5H), indicating the specificity of the transport function.

DISCUSSION

Here we studied, in COSCs of different aged mice (P4–P21), in the presence of 5 ng/mL FGF-2 persistence and function of vascular networks. Structural integrity of the BBB is shown, in particular the presence of the endothelial transport proteins GLUT-1 and P-gp, thereby extending previous own work focusing on tight junctions (Bendfeldt et al., 2007). As shown before in 3–4 days old mice, in cultures prepared from adolescent animals (P21), cerebral blood vessels maintain BBB associated tight junctions and transporters.

Relevance of the model as compared to other *in-vitro* systems

The BBB is challenged in a variety of infectious and neurodegenerative diseases of the CNS. Drug access to the brain parenchyma, however, is limited due to the presence of drug-efflux-proteins, for example, P-gp, in the BBB impeding pharmacotherapy of those diseases. To date most "*in vitro*" models of the BBB rely on monolayers of isolated endothelial cells (Huwyler et al., 1996; Gutmann et al., 1999; Török et al., 2003), or use isolated rat brain capillaries to study regulation and modulation of BBB transport function (Cecchelli et al., 1999; Poller et al., 2010) in order to gain insight into barrier properties to be able to devise strategies for improved CNS pharmacotherapy. However, isolated cells are prone to lose some of their *in vivo* properties (Colgan et al., 2008) due to loss of regulating factors from surrounding astrocytes and glia cells. For *in vitro* models consisting of isolated cells important features of the intact BBB are lacking which might impair functional mechanistic studies. Isolated capillaries, on the other hand, can be maintained in a physiological saline for up to 8 h without loss of metabolism-driven, concentrative transport (Miller et al., 2000; Bauer et al., 2004, 2008), but are removed from their natural environment to artificial culture conditions as well. Thus, to date, "*in vivo*" studies in animal models remain the gold standard for investigations on BBB disturbances.

Recently, we suggested FGF-2 treated COSCs as a new model for reliable *in vitro* studies of the BBB (Bendfeldt et al., 2007). Those cultures take the complex environment of the brain into account, meanwhile maintaining tight junction proteins critical for BBB structure and function. Compared with other models, COSCs have the advantage that blood vessels and their membrane components remain *in situ*, thus allowing cell–cell interactions between natural *in vivo* partners.

Although a number of vessels might perish in those cultures with time because of the absence of intraluminal blood flow, two early ultrastructural studies in human and newborn rat brain tissue (Wolff et al., 1974; Hauw et al., 1975) indicated survival of vessels in explanted brain tissue. Furthermore, more recent immunohistochemical approaches demonstrated the viability of vessels, for example, in the presence of vascular endothelial growth factor in rat cortical cultures (Moser et al., 2003).

Localization of transport proteins

The present study shows for the first time in cultured cortical brain slices of mice, maintenance of BBB transport proteins in cerebral blood vessels for several days *in vitro*.

Firstly, we assessed the presence of GLUT-1 protein in COSCs. Density of GLUT-1 staining was high in endothelial of COSCs for up to 3 days *in vitro* in all examined age groups (P4, P8, P14 and P21). The central dogma of cerebral energy metabolism is that glucose is the obligate energetic fuel of the mammalian brain and the only substrate able to completely sustain neural activity (Siesjo, 1978). The potential relationship between rates of cerebral

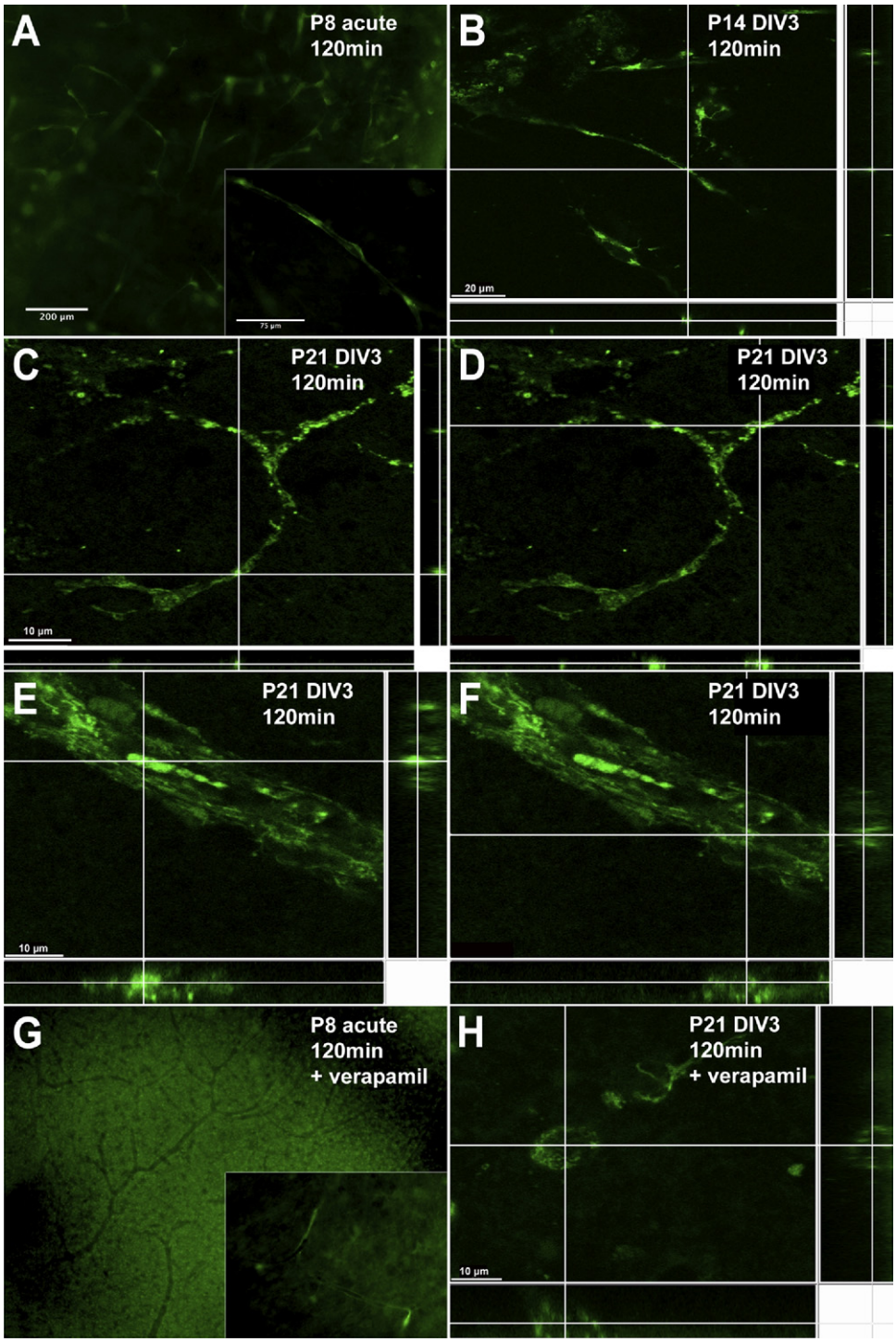

Fig. 5. Accumulation of rhodamine 123 from the abluminal to the luminal side of brain vessels in cortical organotypic slice cultures at DIV 3 from P8, P14 and P21 mice after 120 min. Slices were incubated with rhodamine 123 in the absence (A–F) or presence of verapamil (G, H) for 120 min. Orthogonal reconstructions of the microvessels as viewed in the x–z and y–z planes showed accumulation of rhodamine 123 in the microvasculature of capillaries (B–D). Rhodamine 123 accumulation was more pronounced in small vessels (A–D) than in larger vessels (E–F). In the latter, uptake was also present in endothelial and smooth muscle cells (E–F). Accumulation appeared in patches both in the endothelial cells and in the vascular lumen. Presence of the P-gp inhibitor verapamil completely prevented rhodamine 123 uptake in the vascular lumen (G) but in few areas of the slices accumulation of rhodamine was not completely abolished as seen by its occasional presence in the soma of neurons under high magnification (H).

glucose utilization and the expression of GLUT-1 transporters has been demonstrated in normal adult rat brain (Vannucci et al., 1998). GLUT-1 is expressed selectively at a high level in the capillary endothelium of the BBB, and is responsible for glucose transfer across the BBB (Guo et al., 2005). The 55 kDa form of GLUT-1 is found exclusively in the microvascular endothelial cells that make up the BBB, where it is present in both the luminal and abluminal membranes (Dick et al., 1984; Simpson et al., 2007).

In addition to GLUT-1, the current study shows the presence of the multidrug resistance transporter P-gp in COSCs. P-gp immunoreactivity was more pronounced in slices obtained from older mice (P21, P14) than in those from younger mice (P4, P8). This is consistent with previous data, where a developmental pattern of enhanced P-gp expression during maturation has been shown in the mouse (Ying and Thomas, 1995), rat (Yasuji et al., 1999) and human BBB (Schumacher and Mollgård, 1997). P-gp expression in newborn mouse brain is low, but increases during postnatal maturation and by P21 the adult expression level is reached (Tsai et al., 2002).

There has been some controversy regarding the immunolocalization of P-gp in human brain capillaries. While in some studies P-gp was localized to pericytes (Golden et al., 1997), other studies have suggested, that it might be restricted to endothelial cells (Jette et al., 1993; Seeta et al., 1998). Results for rat and pig are consistent with the latter, which also suggest that P-gp is expressed on the basal abluminal surface of the capillary (Miller et al., 2000). In human and mouse brain (Sugawara et al., 1990; Tatsuta et al., 1992; Beaulieu et al., 1997; Bendayan et al., 2002), P-gp was shown to be localized on the luminal membrane of endothelial cells, where an ATP-driven xenobiotic transport from cell to blood prevents xenobiotic entry into the CNS, and P-gp acts both as an excretory pump and a barrier to drug entry (Bauer et al., 2005). Double-staining of P-gp and laminin in our own study also suggest a luminal localization of P-gp.

P-glycoprotein-mediated transport function

In the present study we assessed P-gp mediated transport across the BBB by examining the accumulation of the fluorescent dye R123 (Fontaine et al., 1996) into the lumen of cerebral blood vessels. R123 was administered to the culture medium either alone or in the presence of the P-gp mediated transport inhibiting agent, verapamil (Bogman et al., 2001).

Although rhodamine is a vital dye for staining mitochondria, the uptake of R123 was mostly specific to cerebral endothelial cells. R123 appeared in patches both in endothelial cells and in the vascular lumen, thereby supporting the hypothesis of P-gp expression on both the luminal surface and in astrocytic endfeet on the abluminal surface of mouse brain vessels. R123 appeared both in endothelial cells and in the vascular lumen of brain capillaries, indicating that P-gp is mainly located on the capillary endothelium. In line with our findings are Western blot studies that showed strong enrichment of P-gp in the brain capillary luminal membranes compared to brain capillaries (17-fold) and whole brain membranes (400–500-fold) (Beaulieu et al., 1997).

This is in agreement with results from transport experiments in isolated microvessels of rat brain (Miller et al., 2000). R123 accumulation was reduced after incubation with verapamil, consistent with findings from other studies where transcellular levels of R123 dramatically decreased upon addition of verapamil in cultured cerebral capillary endothelial cells of mice (Hegmann et al., 1992). Our results indicate functional activity and specific inhibition of P-gp both in acute brain slices and in slice cultures incubated for several days. They show that the slice cultures are an appropriate model system not only for studying morphological, but also functional aspects of the blood brain barrier *in vitro*.

The BBB contributes to brain homeostasis by protecting the brain from potentially harmful endogenous and exogenous substances. Both inflammation and altered barrier function are known to be associated with a number of neurological diseases as in epilepsy, for example (Tomkins et al., 2007). There, increases in endothelial P-gp expression occur in regions of the brain with seizure activity (Bauer et al., 2008).

P-gp can limit the penetration of drugs into the brain and thus modulate effectiveness and CNS toxicity of numerous drugs (Deeley et al., 2006; Girardin, 2006). Consequently, CNS disorders are difficult to treat since the majority of drugs do not cross the BBB. In effect, BBB blocks delivery of more than 98% of CNS-acting drugs (Taylor, 2002). Adequate BBB penetration is a prerequisite for the design of both CNS-acting drugs and non-CNS-acting drugs in advance of clinical usage.

Studies with MDR1 knockout mice have revealed the importance of P-gp in preventing the entry of many drugs into the brain, indicating that complete knockout of P-gp leads to an increase in the accumulation of P-gp substrate in the body (Schinkel et al., 1995; Mealey, 2004). The functional similarity in the gene members of human and mice P-gps makes COSCs or acute slices from mice a useful model to assess the importance of P-gp in drug absorption, distribution and clearance.

CONCLUSION

Our data indicate that the COSC-model is a valuable tool for BBB studies, in particular for studies of tight junction regulation or studies on GLUT-1- or P-gp-mediated transport function. Because of the relative simplicity of the system, COSCs can be readily manipulated and subjected to different insults such as hypoxia, glucose deprivation, and pharmacological treatments to assist in understanding BBB disturbances. The present study demonstrates an intact cytoarchitecture of the BBB in COSCs derived from more mature mice (P21). GLUT-1 and P-gp transport proteins were localized in the cortical vascular system. Furthermore, transport activity of P-gp in slices suggests BBB function *in vitro*. Future studies will show, whether this transport activity persists for longer *in vitro* incubation intervals.

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CHAPTER III: SELECTIVE NEURONAL DEATH AND NEUROVASCULAR REMODELING FOLLOWED BY OGD AND EXCITOTOXICITY USING IN-VITRO BBB MODEL

3.1 VULNERABILITY OF THE HIPPOCAMPUS TO HYPOXIA AND ISCHEMIA

In cerebral ischemia, a decrease in the supply of oxygen and glucose to the brain induces neuronal impairment and even cell death. The hippocampus is very well known for its structure, function, neuronal circuitry, and vulnerability to cell death. A majority of the neuronal connections in this brain region are excitatory, responding to glutamate. Pyramidal neurons in CA1 of the hippocampus are especially vulnerable. Humans and animal models of transient global brain ischemia all display selective and delayed neuronal death in the hippocampus (Petito et al., 1987; Suzuki et al., 1983; Kirino et al., 1984; Nitsch et al., 1989). This selective neurodegeneration has been also observed in hippocampal organotypic slice cultures (Laake et al., 1999; Rytter et al., 2003) suggesting the *in vitro* system comparable to *in vivo* models.

3.2 NEUROVASCULAR REMODELING AND NEURODEGENERATION

Neurodegeneration and BBB damage are often associated together after brain injury, but it is not clear whether the events are separate or linked. Since endothelial cells make up the vasculature of the BBB and maintain the neuronal 'milieu', it is not surprising that BBB dysfunction and neurodegeneration are found associated with one another. In fact, mounting evidence has projected a crucial role of the neurovascular unit in BBB dysfunction in neurodegenerative diseases (Zlokovic, 2010; del Zoppo, 2012). Disrupted endothelial tight junctions, glia reactivity and neuron death have been associated with excessive glutamate levels that arise in CNS disorders (Massieu et al., 2000). Moreover, following hypoxia or ischemia, endothelial cells release trophic factors such as VEGF which is involved in processes of angiogenesis and inflammation, BBB permeability but also in neuronal survival as it is also a neurotrophin (Zhang et al., 2000; Storkebaum et al., 2004; Prat et al., 2002). Interestingly, in temporal lobe epilepsy an increase in vessel density has been reported (Rigau et al., 2007) and the overexpression of VEGF by neurons and astrocytes has been suggested as a mediator for BBB damage and vascular remodeling after excitotoxicity (Morin-Brureau et al., 2011). Whether the vasculature is remodeled as a direct effect of neurodegeneration is debatable. Therefore, we adapted our COSC model to entorhino-hippocampal organotypic slice cultures to study vascular remodeling in the presence of neurodegeneration.

3.3 NEUROVASCULATURE OF ENTORHINO-HIPPOCAMPAL ORGANOTYPIC SLICE CULTURES

Since CA1 of hippocampus is most vulnerable to neuronal death in ischemic animal models as well as in humans (Kirino, 1982; Petito et al., 1987), we were interested in exploring BBB disruption and neurovascular remodeling in entorhino-hippocampal organotypic slice cultures (EHOSCs). Therefore, we initially had to establish the expression profile of vessels in EHOSCs before performing neurodegenerative studies. Both GLUT-1 and Cld-5 along with laminin and von Willebrand factor (or FVIII) were ubiquitously expressed, suggesting the presence of an intact BBB. Regional vessel densities were quantified and the dentate gyrus contained a higher density of vessels than other regions (CA1, CA3, and EC), which was consistent to *in vivo* data of vessels in the rat hippocampus (Ndode-Ekane et al., 2010). Fewer vessels in CA1 than in CA3 have been reported in rodents (Imdahl and Hossmann, 1986; Gerhart et al., 1991; Shimada et al., 1992) and the nature of this difference in vessel density is unknown but has been postulated to be due to differences in glucose utilization as well as cerebral blood flow (Gerhart et al., 1991). Although, blood flow was absent in organotypic cultures and oxygen and glucose were equally distributed to all areas, the regional differences in vessel density were still apparent, suggesting that other factors may be involved. It is plausible that the cellular differences between CA1 and CA3 such as Ca^{2+} load, mitochondrial function, glutamate receptor expression, and proteosomal degradation contribute to the differences in vessel density.

3.4 IS THE VASCULATURE SELECTIVELY VULNERABLE?

Here, we used EHOSCs derived from newborn mice to induce selective neuronal death by OGD or excitotoxicity and examined how it may affect the local vasculature. Induced asphyxia by OGD was applied on to the slice cultures for 15 minutes. Along with neuronal death, components of the BBB and vascular damage were analyzed at 48 hours after injury. In line with *in vivo* studies, OGD induction caused selective cell death in the hippocampal CA1 field only. There was a reduction in the density of blood vessels, which was also regionally restricted, a finding not previously reported in slice cultures. Exposure to tetrodotoxin (TTX) or to an AMPA antagonist (CNQX) to protect the neuronal cells from neurodegeneration prevented reduction of blood vessels in the CA1 region. We hypothesized that the vessel reduction was not simply due to the OGD insult but dependent on neuronal death. To corroborate our hypothesis we induced neuronal death by over activation of AMPA receptors in the slice cultures that is to force neuronal excitotoxicity to occur by stimulating the opening of AMPA specific calcium ion

channels to allow for accumulation of calcium and release of glutamate. Neuronal death was more pronounced in the CA1 region as compared with OGD and had even spread to CA3. Vessel loss was induced in the CA1 region but surprisingly, although many neurons also died in CA3, the vessels there were not reduced. On the other hand in DG region, neurons were resistant to excitotoxicity and the vessel density was increased. This suggested that a more complex interaction between neurons and vessels was taking place such that the fate of the vessels was dependent on which region of the hippocampus the vessels were residing in.

3.5 SUBFIELD SPECIFIC NEUROVASCULAR REMODELING IN THE ENTORHINO-HIPPOCAMPAL ORGANOTYPIC SLICE CULTURE AS A RESPONSE TO OXYGEN-GLUCOSE DEPRIVATION AND EXCITOTOXIC CELL DEATH

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ORIGINAL ARTICLE

Subfield-specific neurovascular remodeling in the entorhino-hippocampal-organotypic slice culture as a response to oxygen–glucose deprivation and excitotoxic cell death

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Transient ischemia causes delayed neurodegeneration in selective brain areas, particularly in the CA1 field of the hippocampus. This is accompanied by neurovascular impairment. It is unknown whether neurodegeneration is the cause or consequence of vascular changes. In an entorhino-hippocampal-organotypic slice culture system with well-preserved blood vessels, we studied the interplay between neurodegeneration and neurovasculature. Short-term oxygen and glucose deprivation (OGD) resulted in upregulation of hypoxic markers and with a delay of 24 to 48 hours in selective nerve cell death in CA1. In parallel, local vessel density decreased as detected by markers of endothelial cells and of the extracellular matrix. Claudin-5, a tight junction protein and marker of the blood–brain barrier was reduced. Preventing neuronal death with tetrodotoxin or 6-cyano-7-nitroquinoxaline-2,3-dione rescued blood vessels, suggesting that vessel loss is not due to OGD per se but a consequence of neuronal death. Induction of excitotoxic neuronal death with AMPA caused widespread neurodegeneration, but vessel reduction was confined to CA1. In dentate gyrus without neuronal loss, vessel density increased. We propose that neuronal stress and death influence maintenance, loss and remodeling of the neurovasculature and that the type of vascular response is in addition determined by local factors within the hippocampus.

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Keywords: angiogenesis; blood–brain barrier; epilepsy; hippocampus; selective neuronal death; transient ischemic attack

INTRODUCTION

The cerebral vasculature supplies oxygen and nutrients to the brain as well as eliminates and protects the nervous system from toxins and pathogens. Endothelial cells assembling the inner lining of the vasculature release trophic factors, support the maintenance of the blood–brain barrier (BBB), and are involved in processes of angiogenesis and inflammation.[1,2] A decrease in oxygen and glucose by a dysfunction in the cerebral vasculature can lead to neuronal impairment and cell death. Unfortunately, introducing pharmacological neuroprotectants before or after injury to prevent cell death or secondary damage to the central nervous system has so far not met clinical expectations. A better understanding of the proper functioning of the vasculature and its endothelium might provide new ways on how to preserve or restore the vasculature as one of the prerequisites for maintenance of brain activities.

It is a well-known phenomenon that different areas of the brain react differently to ubiquitously present noxious events as hypoxia or exposure to toxins. Reasons for selective vulnerability have been considered to be either due to specific properties of nerve cells (the *special pathoclysis* concept of Cecile and Oskar Vogt[3]) or to regional differences in vascularization.[4] This controversial issue has not yet been resolved conclusively. The hippocampus with its different subfields is especially interesting in this context: while the CA1 area exhibits a high susceptibility to hypoxia/ischemia in humans[5] and in animal models,[6–8] the CA3 area together with the hilus of the dentate gyrus is much more involved in epilepsy-associated hippocampal sclerosis[9] and in animal models of temporal lobe epilepsy.[10]

In case of ischemia, the display of transmitter receptors and ion channels, but also control of oxidative and proteasomal stress[11,12] have been proposed as the basis for the differential vulnerability of regions and/or subpopulations of neurons. In addition, local characteristics of glial cells[13] are considered to play a role in the regional selectivity of neurodegeneration. Interestingly, capillary damage and higher susceptibility of BBB leakage in the CA1 region but not the CA3 region in the gerbil and the rat model of cerebral ischemia–reperfusion have been reported.[6,14,15] In the case of temporal lobe epilepsy, not vascular loss, but angiogenesis has been described[10,16] and the overexpression of vascular endothelial growth factor (VEGF) by neurons and astrocytes has been suggested to be the mediator for BBB damage and vascular remodeling after excitotoxicity.[17] Whether the vasculature is remodeled as a direct effect of neurodegeneration is debatable.

Hippocampal-organotypic slice cultures challenged with oxygen and glucose deprivation (OGD) are *in vitro* models affording reproducibility and representing closely to *in vivo* models by mimicking cerebral ischemia that occurs after stroke or cardiac arrest.[18,19] Hypoxic conditions in this slice culture system with or without additional glucose deprivation induce a comparable

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selective neurodegeneration in the CA1 region.[19] We have previously demonstrated the feasibility of the cortical organotypic slice culture as a BBB model system.[20,21] In the present studies we have adapted this model to entorhino-hippocampal-organotypic slice cultures (EHOSCs), enabling us to study vascular effects in the CA1 region elicited by OGD and neuronal death.

Up to now, little data are available linking neurodegeneration and neurovascular remodeling. We investigated whether in the hippocampus, remodeling of the cerebral vasculature after ischemia is dependent on the selective neurodegeneration in the CA1 region and whether excitotoxic nerve cell death in other fields of the hippocampal formation has similar effects. A better understanding of not only the mechanisms of neuronal damage but also the diversity in brain regions in their reaction to cerebral injury as well as of the accompanying vascular damage is necessary for proper development of therapeutics.

MATERIALS AND METHODS

Preparation of Entorhino-Hippocampal-Organotypic Slice Cultures

All animal experiments were carried out according to the international guidelines on handling laboratory animals as well as the present Swiss law for animal experiment and were approved by the animal care committee of the Canton of Basel. Cultures were prepared as described previously.[20,21] Briefly, C57Bl/6CRL mice were decapitated at postnatal day 4 (P4) and the brains were extracted and placed in ice-cold preparation medium containing minimal essential medium, 1% glutamax (Life Technologies, Zug, CH, Switzerland), pH 7.3. Meninges around the cortical hemispheres were gently removed without disturbing the hippocampus. The hippocampus together with the entorhinal cortex (EC) was dissected and 400 μm transversal hippocampal slices were sectioned using a McIllwain tissue chopper under aseptic conditions. Slices were carefully separated and laid over 0.4 μm Millicell-CM, 30 mm diameter culture inserts (Millipore, Zug, CH, Switzerland) in six-well plates with 1 mL/per well of incubation medium composed of: HEPES-buffered minimal essential medium (minimal essential medium, 50%), Hank's-buffered salt solution (25%), and heat-inactivated horse serum (25%) supplemented with glutamax (Life Technologies), glucose (1 g/L), pH 7.3. Plates were stored in a humidified incubator with 5% CO_2 at 37°C. The medium was changed the next day and then every other day up to 1 week.

In Vitro Hypoxia and Oxygen–Glucose Deprivation with Reperfusion

Entorhino-hippocampal-organotypic slice cultures were cultured for 5 days (DIV5) before exposure to hypoxia or OGD. Oxygen and glucose deprivation medium was prepared as detailed by Rytter *et al.*[19] using Neurobasal medium containing: 2% B27 and 1% glutamax (Life Technologies). Two six-well tissue culture plates were filled with 1 mL/well of OGD medium, perfused with N_2 for 1 hour in a hypoxic chamber (Billups-Rothenberg, Del Mar, CA, USA) and then sealed overnight to equilibrate the medium. Anaerobic strips (Sigma-Aldrich, Buchs, CH, Switzerland) were used as hypoxic indicators when the color of the strips changed from pink to white. Before the slices were exposed to OGD, the OGD medium was perfused again with N_2 for 30 minutes. Propidium iodide (PI; Sigma-Aldrich, 2 μg/mL) was added to the slices for 30 minutes and only healthy slices were selected for experimentation as indicated by low numbers of PI-positive cells. The schedule of OGD exposure and reperfusion is indicated in Figure 2A. Briefly, slices were either deprived of oxygen only (hypoxia) or oxygen and glucose for 15 minutes, then replaced with oxygenated Neurobasal medium and allowed to recover for 3, 24, or 48 hours. Propidium iodide was added before the insult to establish the baseline of cell death and then added again during the recovery periods.

Pharmacological Interventions

At DIV5, EHOSCs with low number of PI-positive cells were selected for exposure with several compounds prepared in Neurobasal medium: 100 μmol/L (RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; R&D Systems, Oxford, UK), 100 μmol/L 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; R&D Systems), 1 μmol/L tetrodotoxin (TTX; R&D

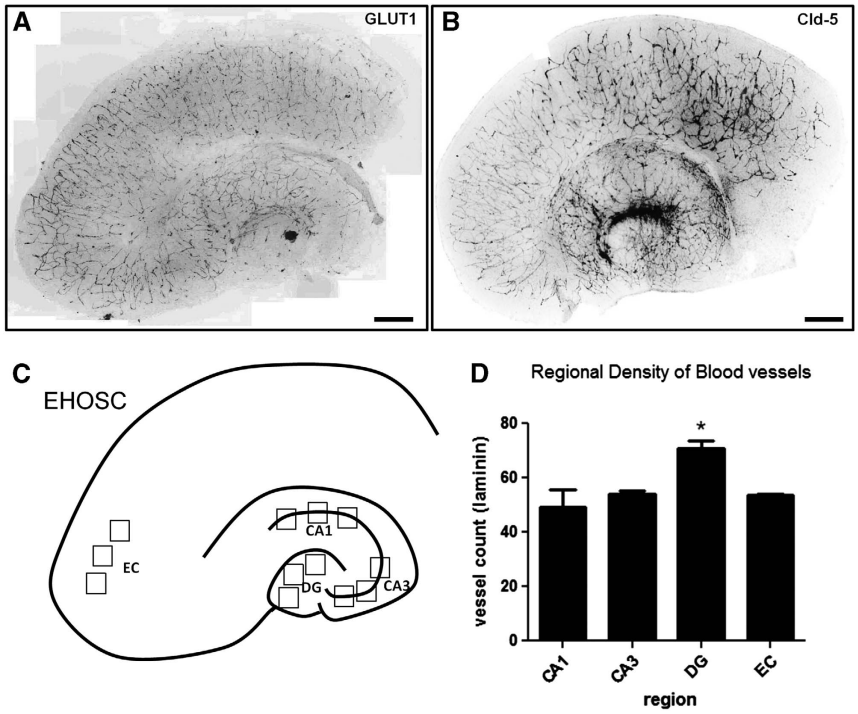

Figure 1. Preservation of blood vessels in entorhino-hippocampal-organotypic slice cultures (EHOSCs). (**A**, **B**) Immunohistochemistry of a transversal slice of the hippocampus along with the entorhinal cortex (EC) shows the expression of glucose transporter 1 (GLUT1) and claudin-5 (Cld-5), at DIV7. Scale bar = 200 μm. (**C**) Schematic diagram of an EHOSC, indicating location of the vessel counting grids shown as squares in the single regions (see Materials and methods). (**D**) The average vessel density in subfields of the hippocampus (CA1, CA3, DG) and the EC. Results are mean vessel counts from three independent experiments ($n = 9$) and significance was determined by analysis of variance (ANOVA) for each group, bars ± s.e.m., *$P < 0.05$ as compared with the other three regions.

Systems) for 30 minutes in normoxic slices and then kept for 48 hours until fixation and immunohistochemistry. Treatment experiments were carried out with CNQX and TTX during or directly after the insult.

Immunohistochemistry

We used only hippocampal slices of the septal half because they displayed consistent cell death. Slices were fixed in freshly made 4% paraformaldehyde overnight at 4°C. Paraformaldehyde was removed the following day and the slices were washed three to four times with phosphate-buffered saline. Slices were carefully removed from the culture inserts for immunohistochemistry of free-floating sections. Each slice was placed in a well of a 96-well plate containing 100 μL of blocking buffer: 3% normal goat serum in phosphate-buffered saline for blocking and 2% Triton-X100 for permeabilization, respectively. The plates were placed on an orbital shaker and the slices were incubated in the blocking buffer for 2 hours at room temperature. Primary antibodies were prepared in phosphate-buffered saline containing 1% normal goat serum and 0.5% Triton-X100. The primary antibodies used for detecting blood vessels were: polyclonal laminin (Sigma-Aldrich) 1:200, polyclonal rabbit anti-human Von Willebrand Factor (Dako, Glostrup, Denmark) 1:250, polyclonal glucose transporter 1 (Thermo Scientific, Fremont, CA, USA) 1:1,000, and monoclonal Claudin-5 (Life Technologies) 1:50; and for detecting neurons: microtubule-associated protein 2 (MAP2; Abcam, Cambridge, UK) 1:500. DAPI (1:500) was occasionally used to verify the total cell population. Incubation with primary antibodies was for 48 hours at 4°C and then washed with 0.5% Triton-X100 for 3 to 4 times. Then, slices were incubated for 3 hours at room temperature with secondary antibody prepared in 1% normal goat serum and 0.5% Triton-X100 of either goat anti-rabbit or goat anti-mouse conjugated to Alexa fluorochromes 350 or 488 (Life Technologies). Afterwards, slices were washed with tris-buffered saline (TBS) three times; mounted on glass slides and cover slipped with Mowiol. The stained slices were viewed by an Olympus AX-70 microscope (Olympus, Hamburg, Germany) installed with a Spot digital camera. The images were recorded and adjusted for brightness by Adobe Photoshop (Adobe Systems, Zurich, Switzerland) or ImageJ (NIH, Bethesda, MD, USA).

Western Blot

Four to five slices of the septal half of the hippocampal region were sonicated and lysed in 1 × RIPA buffer: 50 mmol/L Tris–HCl (pH 7.4), 1% NP-40, 0.25% deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA. In total, 150 μg of protein was boiled for 5 minutes in Laemmli buffer and then loaded in an sodium dodecyl sulfate polyacrylamide gel electrophoresis. The gel was transferred onto a nitrocellulose membrane. The membrane was incubated at room

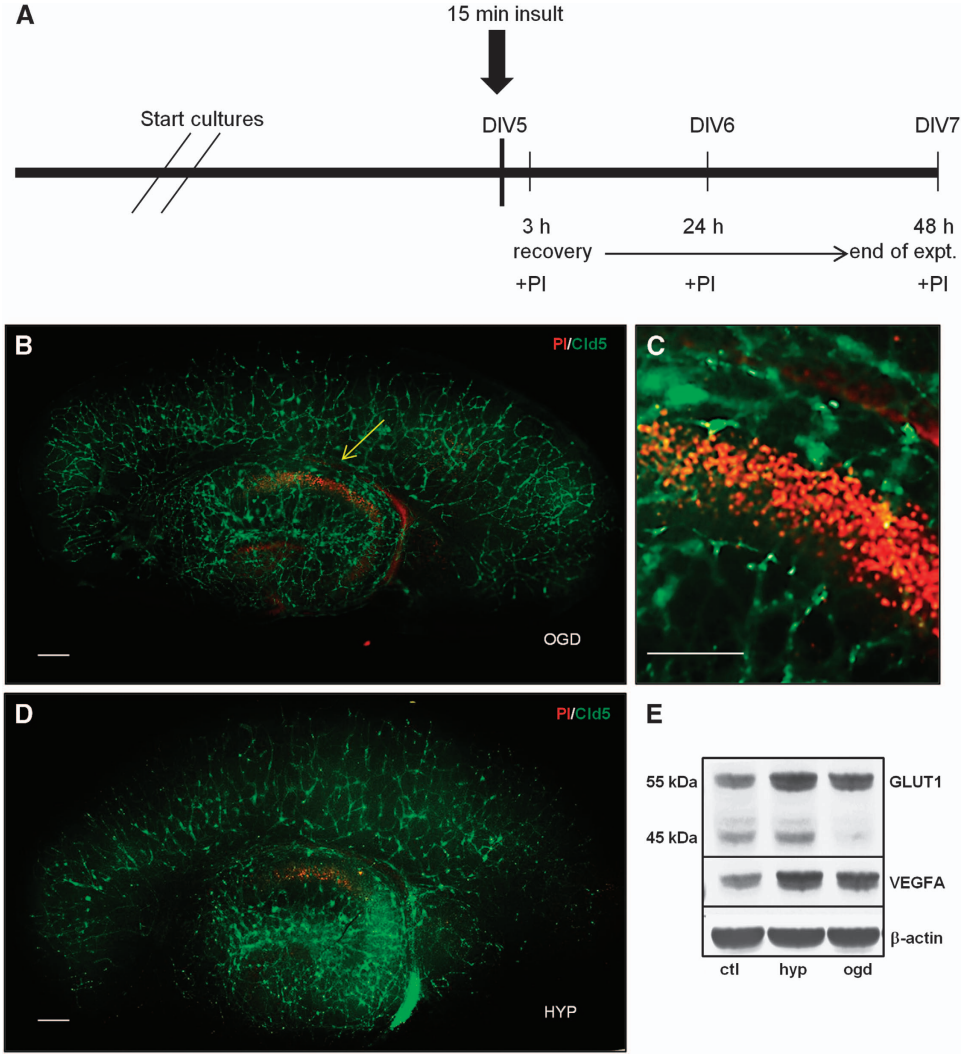

Figure 2. Entorhino-hippocampal-organotypic slice cultures (EHOSCs) submitted to either oxygen and glucose deprivation (OGD) or hypoxia. (**A**) Treatment schedule for hypoxia or OGD. EHOSCs were cultured for 5 days (DIV5) before being submitted to hypoxia or OGD of 15 minutes duration followed by recovery periods (see Materials and methods). (**B**) Propidium iodide (PI) (red) and Cld-5 (green) staining after 15 minutes of OGD and 48 h of recovery. The arrow points to the degenerating neurons in CA1. Scale bar = 200 μm. (**C**) Higher magnification of the CA1 region in (**B**). Scale bar = 100 μm. (**D**) PI and Cld-5 staining after hypoxia and 48 hours of recovery. Scale bar = 200 μm. (**E**) A representative immunoblot of protein expression of the hypoxic markers vascular endothelial growth factor (VEGF) and GLUT1.ctl, control; hyp, hypoxia.

temperature on an orbital shaker for 1 hour in blocking solution containing TBST (TBS and 0.1% Tween20), and 5% bovine serum (Sigma-Aldrich). After blocking, the membrane was probed with either polyclonal GLUT1 (Thermo Scientific) 1:5,000, polyclonal VEGF 164 (VEGFA; Abcam) 1:1,000, or monoclonal β-actin (Millipore) 1:8,000 overnight at 4°C and washed the next day for 5 to 10 minutes with TBST before incubating in secondary goat anti-rabbit or goat anti-mouse conjugated to AP (alkaline phosphatase; Jackson ImmunoResearch Europe, Suffolk, UK) for 1 hour. Then, the secondary antibody was washed several times with TBST for 30 minutes and the signal was detected by AP Conjugate Substrate Kit system (Bio-Rad, Cressier, FR, Switzerland).

Vessel Density and Statistics

The vessel density was quantified based on vessel crossings as described by Bendfeldt *et al.*[20] Recorded images of $\times 10$ magnification were superimposed with three 6×6 grids, where each grid is equal to $100 \times 100\,\mu$m per square and a total field of $0.25\,mm^2$ over the CA1, CA3, DG, and EC region (Figure 1C). The average of the crossings from the three grids for each region was calculated. Countings were based on a minimum of nine animals (three independent experiments, which each included data from three animals, each providing three slices). Statistical analysis was performed by analysis of variance with Bonferroni correction as *post hoc* test ($P<0.05$ was defined as significant).

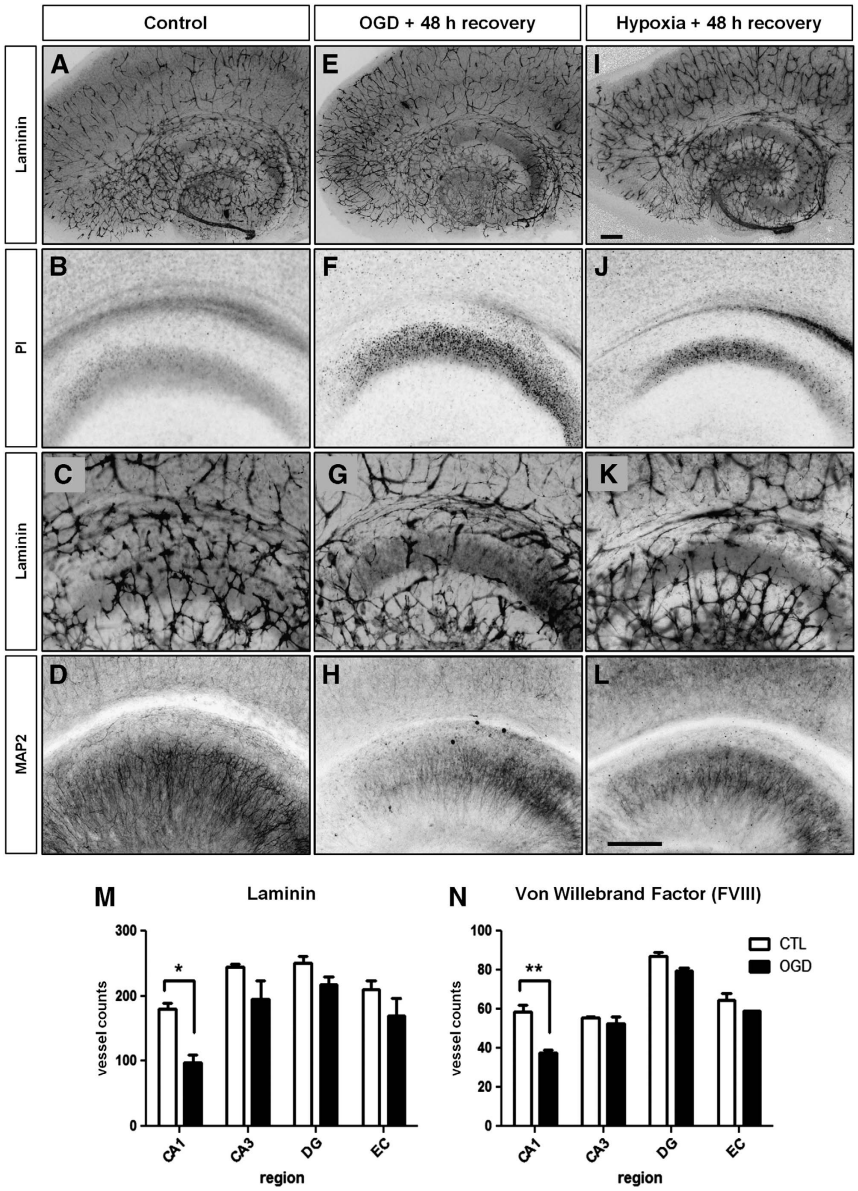

Figure 3. Oxygen and glucose deprivation (OGD) induces cell death, along with neuronal loss and vessel reduction in the CA1 region of the hippocampus. (**A**) A control slice labeled with laminin. (**B**–**D**) Higher magnification of the CA1 field labeled with propidium iodide (PI), laminin, and microtubule-associated protein 2 (MAP2). (**E**) An OGD-treated slice, after 48 hours of recovery showing reduced laminin-positive vessels, specific to CA1. (**F**–**H**) Higher magnification of CA1 shows increased cell death, reduced vessel density and neuronal disintegration. (**I**) A hypoxia-treated slice, after 48 hours of recovery shows less reduction in laminin-positive vessel in CA1 as compared with OGD. (**J**–**L**) Higher magnification of CA1 region shows a mild increase in cell death, reduced vessels, and neuronal loss. (**M**) Vessel density was determined by counting vessels immunolabeled with either laminin or (**N**) von Willebrand factor (FVIII) in the CA1, CA3, DG, and entorhinal cortex (EC) region of entorhino-hippocampal-organotypic slice cultures (EHOSCs) 48 hours after OGD along with their respective controls (CTL). Results are mean vessel counts from three independent experiments ($n=9$) and significance was determined by analysis of variance (ANOVA) for each group, bars ± s.e.m., $^*P<0.05$, $^{**}P<0.01$. Scale bars = 200 μm.

RESULTS

Blood Vessels and Tight Junction Markers Are Preserved Under Normal Culture Conditions in Entorhino-Hippocampal-Organotypic Slice Cultures

Entorhino-hippocampal-organotypic slice cultures prepared of postnatal day 4 (P4) C57Bl/6CRL mice showed preservation of local blood vessels, which exhibited a stable expression of glucose transporter 1 (GLUT1) and claudin-5 (Cld-5) for 1 week (DIV7) in culture (Figures 1A and 1B). Vessel counts were performed in the single subfields of the EHOSCs (Figure 1C). Vessel densities as examined by laminin staining were similar for the CA1, CA3, and EC region but significantly higher in DG (Figure 1D).

Entorhino-Hippocampal-Organotypic Slice Culture as a Model for Studying the Blood–Brain Barrier in Short-Term Oxygen and Glucose Deprivation with 48 hours of Recovery

Since the presence of tight junction proteins and blood vessel densities remained stable for >1 week in culture, we conducted all experiments within this time period (Figure 2A). Organotypic slices were cultured for 4 to 5 days and cell death was assayed in living slices by PI signal. Only slices with low constitutive PI staining were selected for experimentation. Oxygen and glucose deprivation caused cell death along the pyramidal layer of the CA1 sector, where strong PI staining was observed at 48 hours of recovery (Figures 2B and 2C). In parallel, staining for markers of the cerebral vasculature as Cld-5 (Figures 2B and 2C), von Willebrand factor (not shown) and laminin (see Figure 3) was massively reduced in the CA1 sector only. For all other subfields of the EHOSCs, the integrity of blood vessels appeared intact and resistant to short-term OGD. Hypoxia had the same effect on EHOSC but to a lesser extent: nerve cell death in CA1 was less pronounced and vessel reduction less severe (Figure 2D).

The protein expression in the EHOSCs of vascular endothelial growth factor-A (VEGFA) and the 55-kDa GLUT1 isoform were upregulated after short-term hypoxia or OGD followed by 48 hours recovery (Figure 2E). Both proteins are well known to be hypoxia regulated. Interestingly, the 45-kDa GLUT1 isoform that is expressed in glial cells was found to be downregulated specifically after OGD but not after hypoxia (Figure 2E).

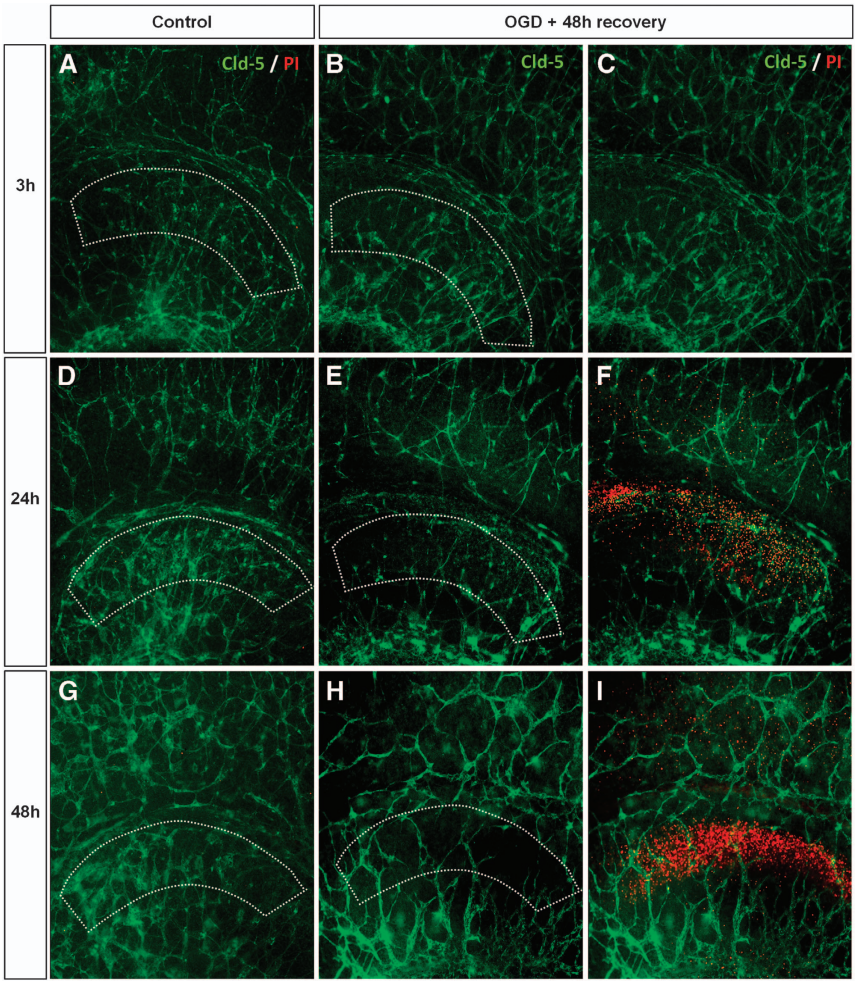

Figure 4. Time course of cell death and change in the vessel architecture at 3, 24, and 48 hours after oxygen and glucose deprivation (OGD). (**A**) A control slice colabeled with Cld-5 (green) and propidium iodide (PI) (red) for the 3-hour recovery time point. (**B**) OGD-treated slice showing vessels labeled with Cld-5. (**C**) OGD-treated slice with vessels colabeled with Cld-5 and PI. Vessels are highly branched and continuous with no cell death occurring. (**D**) A control slice colabeled with Cld-5 (green) and PI (red) for the 24-hour recovery time point exhibits highly branched vessels with no PI-positive cells. (**E**, **F**) In all, 24 hours after OGD, vessels appear disintegrated along with the presence of PI-positive cells becoming apparent. (**G**) A control slice colabeled with Cld-5 (green) and PI (red) for 48 hours recovery time point. (**H**, **I**) After 48 hours of recovery, Cld-5-stained vessels have greatly diminished and cell death has increased in OGD-treated slices in the CA1 region. White dotted lines represent the stratum pyramidale of the CA1 area.

Selective Vessel Loss in the CA1 Region Upon Short-Term Oxygen and Glucose Deprivation Followed by 48 hours Recovery

Despite the induction of cell death in the CA1 region by OGD or hypoxia in our model, overall the integrity of blood vessels in EHOSCs was preserved: the vasculature appeared well branched as observed by the abundance of Cld-5 or laminin immunolabeling (Figures 2B, 2D, 3A, 3E, and 3I). Solely in the CA1 region, blood vessels were reduced compared with the control (Figures 3C, 3G, and 3K). Cell death seen with PI staining was pronounced in CA1 of the OGD or hypoxia-treated samples compared with the control (Figures 3B, 3F, and 3J) and in parallel pyramidal neurons exhibited a disintegration of cytoskeleton as visualized with less MAP2 immunostaining (Figures 3D, 3H, and 3L). These effects in the CA1 field were milder after hypoxia than OGD (Figures 3J, 3K, and 3L). Other regions of the EHOSC (CA3, DG, EC) were not affected, neither PI increase nor MAP2 decrease was found there after short-term OGD or hypoxia followed by 48 hours recovery. The quantitation of vessel density for CA1, CA3, DG, and EC indicated a general decrease in the vessel number for all hippocampal regions, but the reduction of vessels became statistically significant only in the CA1 region where $\sim$50% of the vessels were lost 48 hours after short-term OGD (Figures 3M and 3N). This holds true for the endothelial marker, von Willebrand factor (FVIII), as well as the extracellular matrix marker of blood vessels, laminin.

Since short-term OGD did affect vessels specifically in the CA1 region, we performed a time course analysis to see whether the change in vessel architecture began before, in parallel, or was preceded by neuronal death. Immunohistochemistry of Cld-5 revealed that at 3 hours of recovery, vascular architecture in CA1

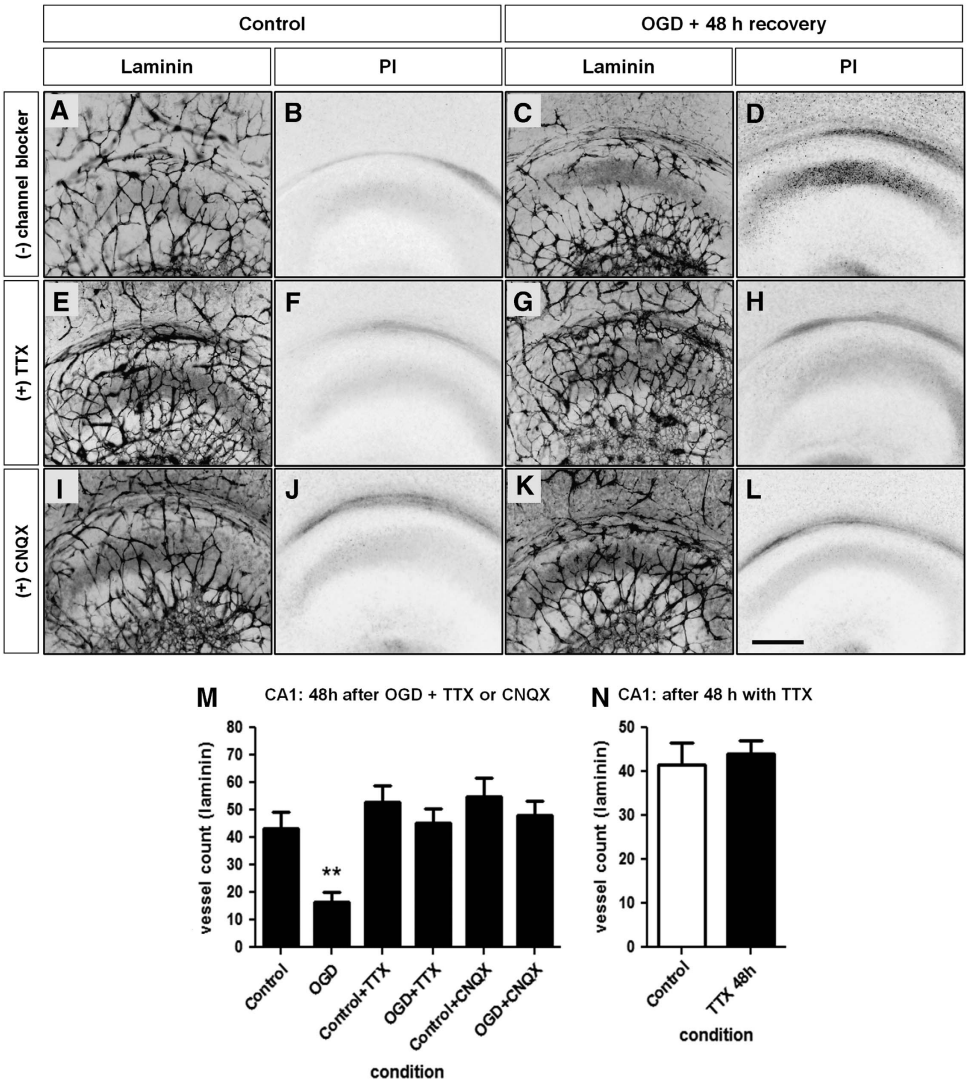

Figure 5. Neuronal cell death and vessel loss are rescued by applying either 1 μmol/L tetrodotoxin (TTX) or 100 μmol/L 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) during oxygen and glucose deprivation (OGD). (**A**, **B**) Laminin and propidium iodide (PI) staining of CA1 region of a control slice culture. (**C**, **D**) Laminin was reduced and PI staining was increased after OGD exposure. (**E**, **F**) Blockage of neuronal activity by TTX did not change laminin staining as compared with control. (**G**, **H**) Vessel loss and neuronal death induced by OGD was blocked by TTX. (**I**, **J**) CNQX exposure of a control slice had no effect on laminin or PI staining. (**K**, **L**) Vessel loss and neuronal death induced by OGD was blocked by CNQX. (**M**) Vessel density in CA1 of entorhino-hippocampal-organotypic slice cultures (EHOSCs) treated with or without 1 μmol/L TTX or 100 μmol/L CNQX during OGD and after 48 hours of recovery. Results are from three independent experiments ($n = 9$). (**N**) Vessel density in CA1 region of EHOSCs treated with only 1 μmol/L TTX for 48 hours to silence neuronal activity without inducing neuronal death. Results are from two independent experiments ($n = 6$) and significance was determined by analysis of variance (ANOVA) for each group, bars $\pm$ s.e.m., $^{**}P < 0.01$. Scale bars = 200 μm.

did not differ from controls showing the presence of highly branched vessels and no PI-stained cells (Figures 4A–4C). As the recovery period lengthened to 24 hours, cell death became evident in the OGD-treated slices as PI-positive cells were appearing and, in parallel, the network of vessels started to become disintegrated (Figures 4E and 4F). At 48 hours of recovery, the CA1 region was virtually cleared of vessel coverage (Figures 4H and 4I). The temporal increase of PI signal from 24 to 48 hours suggests an inverse relationship occurring between the development of cell death and the decrease of vessels. No changes were evident in the control slices (Figures 4A, 4D, and 4G). These findings indicate that the vessel changes neither occur before or after, but rather developed in parallel with a close temporal correlation to the development of neuronal death.

Protection of Neurons and Vasculature by Tetrodotoxin and CNQX After Short-Term Oxygen and Glucose Deprivation with 48 hours of Recovery

Next, we questioned whether the loss of vasculature after OGD is due to a direct effect of OGD on the vessels or due to the OGD-induced neurodegeneration. Furthermore, is blockade of neuronal

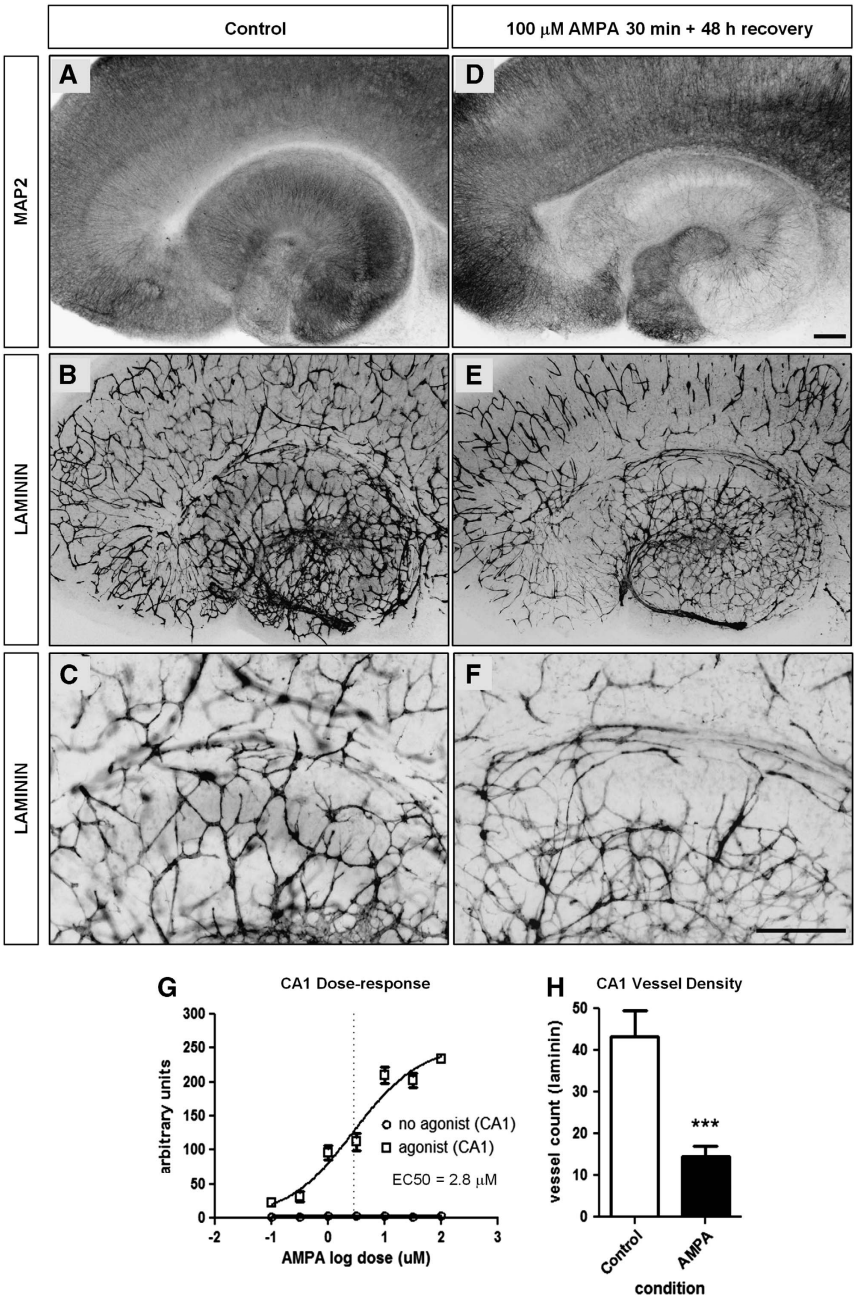

Figure 6. Entorhino-hippocampal-organotypic slice cultures (EHOSCs) treated with amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) for 30 minutes and allowed to recover for 48 hours. (**A**, **B**) Microtubule-associated protein 2 (MAP2) and laminin expression of a control slice. (**C**) High magnification of blood vessels in CA1 region in (**B**). (**D**, **E**) MAP2 and laminin expression of a slice treated with 100 μmol/L AMPA for 30 minutes with 48 hours of recovery. (**F**) High magnification of blood vessels in CA1 region in (**E**). (**G**) A dose response for the AMPA agonist. Boxes and circles show mean propidium iodide (PI) intensity in CA1 area with and without the agonist, respectively, bars ± s.e.m. (**H**) The density of vessels in the CA1 region was significantly reduced in samples of three to six animals, bars ± s.e.m. (*$P < 0.05$). Scale bars = 200 μm.

activity sufficient to induce vessel loss, or must neurodegeneration take place to cause a disintegration of the vasculature? We tested this by using the blocker of fast sodium spikes TTX or the AMPA receptor antagonist CNQX. In control slices with TTX only, with neuronal activity silenced, vessel architecture was similar to controls (Figures 5A, 5B, 5E, and 5F). With 1 μmol/L TTX, CA1 neurons were strongly protected from cell death induced by OGD and the vessel architecture did not change compared with the OGD only (Figures 5C, 5D, 5G, and 5H). In control slices with CNQX only, the vessel architecture was also not affected (Figures 5I and 5J). Very similarly, 100 μmol/L CNQX also protected neurons from OGD (Figures 5C, 5D, and 5K, and 5L). Quantification of vessel density clearly established that both neuroprotecting compounds, TTX and CNQX preserved the blood vessel morphology and density (Figure 5M). Furthermore, vessel density in CA1 did not change between controls and slices treated with TTX, where neuronal activity was blocked for 48 hours (Figure 5N). These data clearly show that vascular loss after OGD is due to neurodegeneration.

CA1 Vessel Loss by Short-Term Exposure to AMPA

Since TTX and CNQX could rescue neurons and blood vessels from OGD-induced cell death and disintegration, we wondered whether AMPA receptor-triggered excitotoxicity in the absence of OGD might also induce blood vessel disintegration. Microtubule-associated protein 2 and laminin staining show a well-preserved appearance of neuronal cytoskeletal and vascular architecture, respectively, in a control slice culture (Figures 6A and 6B). A dose response for AMPA indicated strong cell death in the CA1 region to occur after a 30-minute exposure to 100 μmol/L (Figure 6G). In contrast to hypoxia or OGD, there was no delay in nerve cell death; it was already present after 3 hours (data not shown) to a massive degree. A 30-minute exposure to AMPA followed by a 48-hour recovery period had eliminated the majority of MAP2-positive neurons in the cornu ammonis (Figures 6A, 6D, 7A, and 7E). At the same time, there was also a clear reduction of vessel density in CA1, indicating that the AMPA-treatment induced neurodegeneration was sufficient to provoke vessel loss (Figures 6B, 6C, 6E, 6F, 7B, and 7F). Vessel counts confirmed that excitotoxic nerve cell death reduced the vessel density in the CA1 region (Figure 6H). Blocking the AMPA-induced excitotoxicity by 100 μmol/L of CNQX partially rescued neurons and preserved the vessels almost to control levels, even when applied as posttreatment (Figures 7C, 7D, 7G, and 7H, quantification in Figure 7I). Taken together, these experiments prove that vessel loss in the hippocampal CA1 field is due to excitotoxic neurodegeneration.

Regional Variations in Blood Vessel Plasticity After AMPA-Induced Excitotoxicity

Neurodegeneration induced by AMPA is not confined to the CA1 region. Microtubule-associated protein 2 staining revealed loss of neurons in CA3 and survival of neurons in the dentate gyrus (Figures 7A and 7E). In contrast to CA1, blood vessels were not lost or were even enriched with more numerous branches in CA3 (Figures 7B and 7F, quantification in Figure 7I). In the DG, there was a consistent increase in blood vessel density, which could be blocked by posttreatment with CNQX (Figures 7F and 7H, quantification in Figure 7I). Thus, nerve cell death is not always accompanied by vessel loss but rather can induce a variety of changes in neurovasculature. The actual type of modification is probably determined by regional features together with the mode of induction of neurodegeneration.

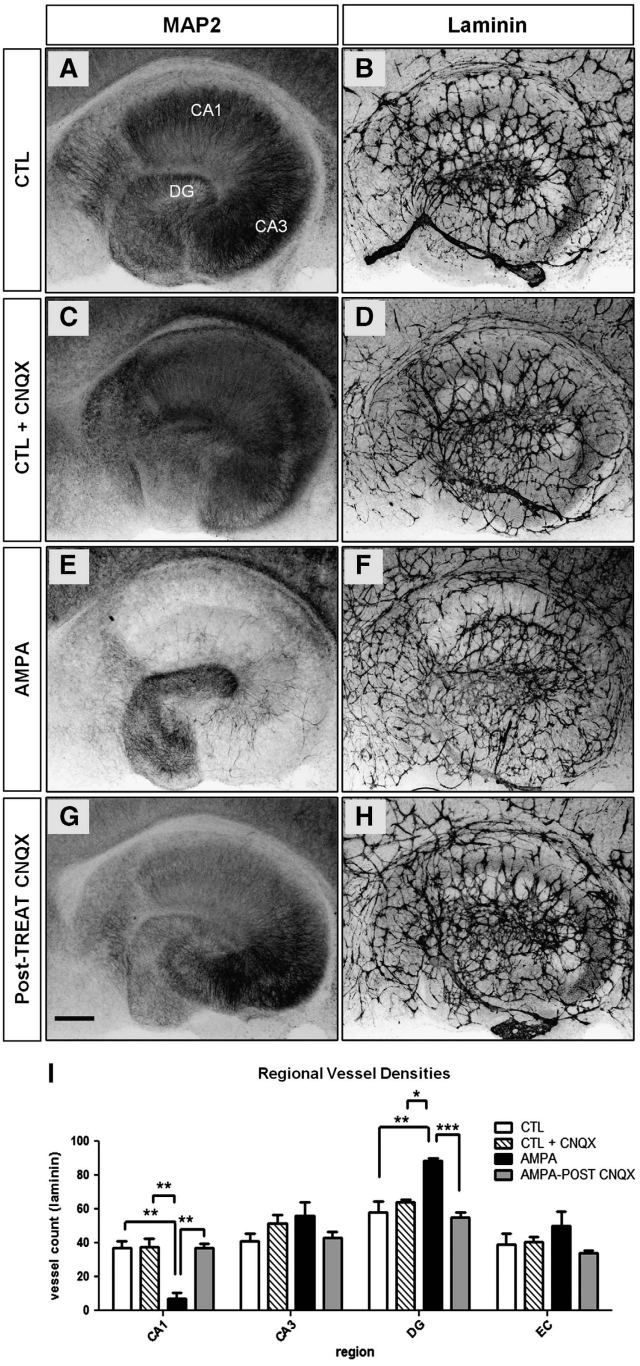

Figure 7. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) applied after exposure to amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) maintains the vascular architecture and blocks neuronal death in the hippocampus proper. (**A**–**D**) Control slices in the absence or presence of 100 μmol/L CNQX show similar microtubule-associated protein 2 (MAP2) and laminin staining. (**E**, **F**) In all, 48 hours after recovery from 30 minutes of exposure to 100 μmol/L (RS)-AMPA reduced MAP2 labeling in CA1 and CA3, but the vessel marker laminin was only reduced in CA1. (**G**, **H**) Vessel reduction and neuronal death are blocked by 100 μmol/L CNQX applied subsequently to the 30-minute AMPA insult. (**I**) The vessel densities were compared regionally for the respective treatments. Results are from three independent experiments ($n = 9$) and significance was determined by analysis of variance (ANOVA), bars $^{*}P < 0.05$, bars ± s.e.m., $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bar = 200 μm.

DISCUSSION

We have adapted an *in vitro* neocortical BBB model[20,21] to the hippocampal formation to investigate the effects of ischemia and recovery on neurovascular integrity in the most vulnerable brain region, the hippocampal CA1. Entorhino-hippocampal-organotypic slice cultures behave similar to cortical organotypic slice cultures with respect to the preservation of blood vessels in the control situation. Short-term hypoxia or OGD induced a selective cell death in the CA1 region, delayed by 24 to 48 hours. This is accompanied by a significant reduction in vessel density. The blocker of fast sodium spikes TTX or the AMPA receptor antagonist, CNQX, prevented both, neuronal death and vascular loss in the CA1 region. Induction of excitotoxicity by AMPA caused neuronal death in CA1 and CA3 regions. In contrast to a diminished vasculature in neuron-depleted CA1, in CA3 nerve cell loss did not reduce vessel density. Nerve cells in the EC and DG were resistant to excitotoxic cell death and in DG the vessel density even increased. These contrasting effects could be prevented by CNQX and TTX. We conclude that neuronal stress and neuronal death control maintenance, loss and remodeling of the neurovasculature and that the vascular response is in addition determined by region-specific factors.

Studying Hypoxia/Ischemia Using Entorhino-Hippocampal-Organotypic Slice Cultures

Hippocampal-organotypic slice cultures have been used to study neural development, neural function, neurodegeneration, as well as neuroprotection.[18,19,22] Ischemia induced in *in vitro* models produce consistent cell death in specific regions within the hippocampus.[18,19] In our study, we used the OGD medium proposed by Rytter *et al.*,[19] which caused a delayed and selective cell death to CA1 without damaging CA3 after 15 minutes of OGD exposure. Septal and temporal regions have been known to function distinctively from each other.[23] Notably, the vulnerability to cell death appears to be more associated with the septal half, a phenomenon that we also observed (preliminary data). To avoid a possible interference from the position of the slice along the septotemporal axis, we used only hippocampal slices of the septal half because they displayed consistent cell death in addition to the reduced vessel density.

In models of hypoxia or ischemia, an increase in induction of VEGF and GLUT1 has been reported.[24] In fact, we were able to observe VEGFA and GLUT1 upregulation after hypoxia or OGD. Intriguingly, we found that unlike the increase in expression of the 55-kDa GLUT1 isoform that resides in endothelial cells, the expression of the 45-kDa GLUT1 isoform that resides in glial cells[25] was decreased after OGD. The functional relevance of the GLUT1 isoforms is largely unknown, but the glycosylated form facilitates glucose transport.[26] Nevertheless, the observed decrease in expression of the 45-kDa GLUT1 isoform suggests an impairment of glucose transport capacity in glial cells, adding to the potency of the OGD medium proposed by Rytter *et al.*:[19] it was reported to induce stronger cell death after glucose was reintroduced in the recovery period. Furthermore, the increase of the 55-kDa GLUT1 isoform indicates that despite the absence of blood flow, endothelial cells respond to OGD, rendering their functionality in EHOSCs. All in all, the results of this study suggest that our *in vitro* model of short-term OGD using EHOSCs is valid and representative of hypoxia or ischemia occurring *in vivo*.

Selective Cell Death and Vessel Loss in CA1

Neurodegeneration and BBB damage often cooccur after cerebral ischemia, but it is not clear whether the events are interconnected or separate entities. Neuronal death has been shown to be associated with BBB leakage in the context of transient ischemia,[6,14,15] suggesting neuronal damage as the primary event of injury. However, this idea was challenged by the finding that intracerebral injection of kainic acid could induce BBB damage without neuronal death in knockout mice resistant to excitotoxic injury, supporting that BBB damage and neuronal death are separate events.[27] Thus, depending on the context of CNS injury, the question of whether neuronal death and BBB leakage are separate or linked events is debatable, which encouraged us to perform the present study.

In brain slices, techniques for measuring the permeability of the BBB do not exist. Indirect measures are the detection of tight junction proteins. In addition, investigating the expression of components of the cellular matrix of the neurovascular unit is reasonable, since functioning of the BBB also depends on the survival of the endothelium, basal lamina, astrocytes, and pericytes.[2,28,29] In the present study, we have provided evidence of a link between neuronal death and impairment of BBB: along with the glial impairment in glucose transport and the upregulation of GLUT1 in the endothelium, the main tight junction protein of the BBB, Cld-5, is markedly reduced in CA1 24 hours after OGD, that is, at the onset of neurodegeneration, with a further reduction at 48 hours. Yet, not only tight junction proteins are reduced with neurodegeneration, the whole neurovascular unit is impaired as evidenced by the loss of the extracellular matrix protein laminin. Previous studies on rodents submitted to cerebrovascular ischemia have also seen capillary damage in the CA1 region.[14] Earlier studies in vessel density in control animals have indicated fewer vessels in CA1 than CA3[14,30,31] and more recently a higher vascular density in the molecular layer of the dentate gyrus than in the rest of the hippocampus was demonstrated.[32] Differences in vessel density have also been observed across the septotemporal axis,[33] suggesting that the organization of the vasculature in the hippocampus is dependent on regional factors. The regional variation in vessel density has been considered to be caused by differences in glucose utilization.[30] Also in so-called neurovascular niches, such as in the DG, angiogenesis is more frequent as it relies on the same trophic factors that are involved in neurogenesis.[32]

In organotypic cultures, without a need for blood flow as oxygen and glucose are equally distributed to all areas, regional differences in vessel density were nevertheless still apparent. The cellular organization in the single brain region might determine vessel density. Since pyramidal cells in CA1 and CA3 are different in terms of calcium load, mitochondrial function, glutamate receptor expression, and proteosomal degradation, it might be possible that factors produced by or associated within CA1 influence vessel density. Because in our *in vitro* model of global ischemia, vessel loss was specifically observed in CA1, we propose that the loss of vessels arises as a consequence of CA1 pyramidal cell death.

Blocking Neuronal Death and Vascular Loss in CA1

Neuronal death after hypoxia or ischemia is mainly contributed to glutamate excitotoxicity.[34] The glutamate release has been linked to sodium and calcium influx as a consequence of ionic imbalance and neuronal depolarization. Blocking the activation of either sodium channels by TTX or ionic glutamate receptors with NMDA or AMPA antagonists before OGD improves neuronal resistance.[35] In our OGD model, TTX as well as the AMPA receptor antagonist CNQX prevented neuronal death proving that it is due to glutamate excitotoxicity. This neuroprotective effect of TTX or CNQX was associated with a preservation of blood vessels. Moreover, in control experiments with TTX only, the vessel density was similar to controls. Since TTX can inhibit neuronal activity without affecting cell viability, the stability in vessel expression in the presence of TTX implied that it is not the absence of neuronal activity but that indeed neuronal death is responsible for vessel loss.

We used an AMPA antagonist, CNQX, rather than a NMDA antagonist in our study, as it has been shown to be more effective in preventing delayed neuronal death.[36] Moreover, we wanted to use an antagonist that was specifically affecting neurons. Evidence for the presence and function of glutamate receptors in cerebral endothelial cells is controversial. Cerebral endothelial cells derived from rat or human brain have been contended as having no expression or function of glutamate receptors,[37] but subsequent studies showed glutamate receptors in cerebral blood vessels and even suggested NMDA receptors to be involved in BBB regulation.[38] On the other hand, AMPA receptors are only expressed at very low levels in cerebral endothelial cells and the application of DNQX, an AMPA receptor antagonist, but not MK-801, an NMDA receptor antagonist, failed to counteract BBB permeability induced by glutamate excitotoxicity, suggesting that AMPA receptors may have little importance in BBB regulation.[38] In light of these published findings, we presumed that CNQX had little direct effect on vessels, if at all.

Glutamate Excitotoxicity Leads to a Selective Modulation of Vessel Architecture in the Hippocampal Formation

Since AMPA receptors seemed to be involved in OGD-induced neurodegeneration, we challenged this finding by directly exposing EHOSCs to a high dose of (RS)-AMPA agonist for a short duration followed by 48 hours of recovery to mimic the glutamate excitotoxicity induced by OGD. A widespread neuronal death together with vessel loss in the CA1 region was observed. Both neurodegeneration and vessel loss could be reversed when the agonist was blocked by CNQX providing additional evidence that excitotoxic neuronal death in the CA1 region is accompanied by a concomitant loss of blood vessels. However, the other subfields of the hippocampus did not react in the same way. In the CA3 region, excitotoxic neuronal death did not induce major changes of blood vessel density or architecture. In the DG with neuronal survival, there was an increase of blood vessel density and in the EC neurons survived and there were no evident changes in the blood vessels. Interestingly, in four different areas we thus have observed four different types of neuronal and vascular responses to excitotoxic challenge highlighting the importance of local factors for determining the final cellular responses.

In *in vivo* and *in vitro* models of epilepsy, an increase of vessel density has been demonstrated.[10,16,17,32] It has been linked to seizure-induced mossy fiber sprouting and neurogenesis, which is prominent in the neurovascular niche of the DG.[39] An upregulation of VEGF expression by neurons and astrocytes as well as its receptor, VEGFR2, in endothelial cells was present in *in vitro* kainate seizure-induced vessel remodeling.[17] In our model, we also did observe VEGF expression to be upregulated. Further work is necessary to determine whether the selective AMPA-induced excitation mimics on the one hand in DG seizures resulting in angiogenesis, and on the other hand in CA1 ischemia-induced glutamate toxicity resulting in massive neurodegeneration and accompanying vessel loss.

Possible Mechanisms of Vascular Damage Induced by Neuronal Death

A primary mechanism underlying ischemia-induced neuronal death of pyramidal cells is excitotoxicity, which we observed 48 hours after injury by OGD and by excessive stimulation of AMPA-type glutamate receptors. However, the neuronal death was accompanied by microvessel loss confined to the CA1 region. Ischemia-induced neuronal death has been characterized by a mixture of apoptosis and necrosis. Neuronal death includes a large number of changes such as the loss of ATP, production of free radicals, loss of mitochondrial membrane potential, release of cytochrome c, and production of reactive oxygen species.[40] The mechanisms by which death of pyramidal neurons mediate vessel loss, are not known. The present results lay the groundwork for further study of the biological crosstalk between pyramidal neurons and the vasculature, in particular, the role for AMPARs in ischemia-induced neuronal death. Understanding the regional differences of the hippocampus and especially the interaction between pyramidal cells and their surrounding vasculature might be an important aspect for developing better treatments for neurovascular disorders in particular cerebral ischemia after stroke or short-term cardiac arrest.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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CHAPTER IV: RBM3 IS INVOLVED IN HYPOTHERMIA-INDUCED NEUROPROTECTION

4.1 NEONATAL ASPHYXIA AND CELL DEATH

The process of brain injury following neonatal asphyxia is complex lasting from the beginning of insult and until the period of recovery. Lack of oxygen and glucose trigger cells to switch from oxidative phosphorylation to glycolysis or anaerobic metabolism reducing high-energy phosphate levels (including ATP) and increasing lactate production. The energy loss and metabolic acidosis unleash a biochemical cascade of deleterious events that lead to cell dysfunction and finally cell death. Furthermore, damage of brain metabolism by increases in oxidative stress lead to reperfusion injury (see review by Lai and Yang, 2011).

It has been proposed that neurodegeneration in response to excitotoxicity or hypoxia-ischemia in the neonatal brain of rodents display an apoptosis-necrosis "continuum" (Portera-Calliau et al., 1997; Northington et al., 2007), which both forms of cell death are activated. Cell death after hypoxia-ischemia has been also described to occur in two phases. During the initial injury which is the primary phase that takes place in the first few hours, cell death is predominantly necrotic, while 12-48 hours later after energy supply and cellular pH have recuperated to baseline levels from reperfusion and reoxygenation, a second energy loss and a delayed phase of injury occurs, which apoptosis appears to be more apparent (Towfighi et al., 1995; Northington et al., 2001). However, it is also possible that mitochondrial dysfunction and cytochrome *c* release during the second energy failure is caused by increases in calcium influx, glutamate excitotoxicity, or oxygen free radicals. Nevertheless, it is this delayed phase of injury that determines the outcome of neurodevelopment. Whether necrosis and apoptosis occur simultaneously or at distinct time points following neonatal asphyxia is a matter of debate. They both contribute to brain injury and preventing both types of cell death before the second injury event would be a good therapeutic strategy.

4.2 THERAPEUTIC HYPOTHERMIA AS A TREATMENT FOR NEONATAL BRAIN INJURY

There is no cure for encephalopathy followed by neonatal asphyxia; therefore effective therapies of neuroprotection have been to reduce or prevent further cerebral injury. Therapeutic hypothermia has been the most effective treatment at reducing brain damage and in preventing neurodevelopmental disabilities (Azzopardi et al., 2009). As mentioned above, brain injury after asphyxia is biphasic. The optimal time window to commence therapeutic hypothermia

comprises a few hours after the initial insult. This window is during the recovery period and precedes the second phase of injury which ultimately results in delayed neuronal death. Cooling of the head or the whole body of the newborn to 33-34°C within 6 hours after injury for 72 hours has shown to improve recovery; reducing the outcome of death and neurological complications (Drury et al., 2010).

How does hypothermia reduce brain injury? As mentioned earlier, hypothermia has a pleiotropic effect. It can affect many aspects of cellular function from inhibiting metabolism, delaying protein synthesis, to disrupting the cascade of events leading to cell death. However, since apoptosis is a major contributor to cell death in neonatal asphyxia, hypothermia could potentially disrupt the apoptotic pathway. It was first reported in newborn pigs with transient hypoxia-ischemia that mild hypothermia (34.9°C) decreased the number of apoptotic cingulated neurons rather than necrotic neurons during the second phase of injury (Edwards et al., 1995). The specific inhibition of apoptotic and not necrotic cells by hypothermia was not determined in this study, however, it was postulated that the biochemical cascade leading to apoptosis could be disrupted by hypothermia while not in necrosis as the cell death was too great to be rescued. The activation of caspase-3 following hypoxia-ischemic brain injury was observed to be prominent in immature neurons and declining in mature neurons (Hu et al., 2000). Caspase-3 is a cysteine protease that cleaves cytoskeletal proteins, kinases, and DNA repair enzymes. It is also an effector caspase downstream of the initiator caspases (caspase-2, -8, -9, and -10) in the apoptotic pathway and when caspase-3 is activated cells undergo apoptosis. Later studies conducted in neonatal rats exposed to hypothermia after injury by hypoxia-ischemia found caspase-3 was inhibited (Fukuda et al., 2001). This suggested that blocking the apoptotic pathway by hypothermia could reduce secondary brain injury. Indeed, when caspase-3 was specifically inhibited exogenously by intracerebroventricular injection into hypoxic-ischemic neonatal brain injured rats, only apoptotic neurons were rescued and brain injury was reduced (Han et al., 2002). A subsequent study by Zhu et al. (2004) carried out further biochemical analyses in the apoptotic pathway, revealed that inhibition of cytochrome *c*, which is upstream of caspase-3 was also involved in hypothermia induced neuroprotection. Although, the previous studies indicate that therapeutic hypothermia offers neuroprotection by disrupting the apoptotic pathway, the exact molecular mechanisms underlying this protective effect are still not fully understood. The complexity in cell death and the pleiotropic effects of hypothermia on neuroprotection suggest a more complicated interplay of molecular mechanisms.

4.3 COLD INDUCIBLE PROTEINS

As mild hypothermia can downregulate the expression of certain proteins (e.g. cytochrome *c* and caspase-3), it can upregulate a select group of proteins (e.g. cold-shock proteins or CSPs) as well, which are important determinants in the adaptation to growth and survival during low temperatures. CSPs, unlike heat-shock proteins (HSPs) have been less extensively studied even though the cold shock response seems to be important for the skin, testis, organ storage, hibernation, and treatment of brain damage.

The RNA-binding motif protein 3 (Rbm3) and cold-inducible RNA-binding protein (Cirp, also referred as A18 hnRNP) are the only two mammalian CSPs that have been well characterized (Danno et al., 1997; Nishiyama et al., 1997). Both are highly conserved and induced by mild stress including hypothermia. They belong to the glycine-rich RNA-binding protein (GRP) family. Their amino termini are defined by a consensus sequence RNA-binding domain (CS-RBD); also known as RNA recognition motif (RRM), ribonucleoprotein motif (RNP), or RNP consensus sequence, which is important for RNA binding. Their carboxy termini contain a glycine-rich domain important for strengthening protein-protein and or protein-RNA interactions. Mouse Cirp, which is 99% identical in CS-RBD to human CIRP, is also 77% structurally related to human RBM3 and with an overall 64% similarity in amino acid sequence (Nishiyama et al., 1997; Danno et al., 1997). The function of these two CSPs are still currently being elucidated, however they have been postulated to be involved in posttranscriptional regulation of gene expression indicated by studies on proteins with CS-RBD (Burd and Dreyfuss, 1994; Danno et al., 1997; Fujita, 1999). Both have been found to be expressed in the testis, pancreas, placenta, adrenal gland, and brain. However, only Cirp was expressed in the heart (Derry et al., 1995; Danno et al., 1997)

In mammalian cells, proliferation and growth are generally reduced during mild hypothermia or at 27-32°C but are not arrested unless below 27°C (Fujita, 1999; Roobol et al., 2008). Interestingly, in terms of recombinant protein production in cultured mammalian cells, mild hypothermia seems to prolong cell viability (Al-Fageeh and Smales, 2006). The inhibition of metabolism by reducing cell proliferation during hypothermia is thought to be a protective mechanism, lowering protein synthesis (Lleonart, 2010).

In-vitro experiments assessing cell proliferation and protein synthesis, independent of mild hypothermia, have revealed some aspects into the function of Cirp and Rbm3. Over-expression of Cirp in mouse fibroblasts (BALB/3T3) reduced growth rate and prolonged the G1 phase of the cell cycle (Nishiyama et al., 1997), which suggested that it could be responsible for slowing

down metabolic events seen at low temperatures. On the other hand, Rbm3 does not reduce cell growth (Fujita, 1999).

Since both CSPs are upregulated in response to protein synthesis inhibitors and protein synthesis is reduced at low temperatures the cold shock response in mammalian cells is thought to be dependent on a temperature sensory mechanism in ribosomes, moreover, that expression of Rbm3 and Cirp may facilitate ribosomal functions by serving as RNA chaperones, increasing mRNA stability and preventing formation of stable secondary structures, which has been previously posed for non-homologous bacterial CSPs (Jones et al., 1996; Danno et al., 1997; Fujita, 1999).

Hypothermia not only exposes cells to cold stress due to changes in temperature but also exposes them to hypoxic stress due to changes in dissolved oxygen levels at low temperatures. Both types of cell stress have been reported to affect cell metabolism and interestingly the transduction pathway of cold stress has been suggested to share similar components specific to the hypoxic transduction pathways and not the hyperoxic pathways (Gon et al., 1998). In fact, Cirp and Rbm3 have been reported to be expressed in-vitro under hypoxic conditions in a HIF (hypoxia-inducible factor) independent manner (Wellmann et a., 2004).

4.4 CAN RBM3 BE NEUROPROTECTIVE?

Although, their functions have not been well-characterized, CSPs have been examined in a variety of mammalian cells and suggested to participate in coordinating many cellular events involved in transcription, translation, the cell cycle, metabolism, and cytoskeleton organization (Al-Fageeh and Smales, 2006). Resolving the function of CSPs will be important in understanding the cold shock response especially in hypothermia-induced neuropotection.

In the brain the physiological function of Cirp and Rbm3 in neuronal cells is currently unknown. Cirp was reported to be constitutively expressed in the hippocampus (Xue et al., 1999). By Northern blot analysis, Cirp mRNA expression was reduced in the hippocampus followed by transient forebrain ischemia at 3 and 6 hours (Xue et al., 1999). Elevation in body temperature and generation of reactive oxygen species were speculated by the authors to inhibit Cirp expression. A similar study performed a decade later showed an increase of Cirp expression at 24 hours but not at 6 hours followed by transient ischemia in the cortex only. Cirp expression was significantly increased at 6 hours followed by ischemia only when hypothermia was introduced (Liu et al., 2010). The results of this study suggested that ischemia alone was not able to induce Cirp. Additionally, Cirp did not directly affect metabolic processes as lactate production could not be influenced by Cirp suggesting that the neuroprotective effects of

hypothermia-induced Cirp were rather on gene regulation. Whether Cirp expression influenced neuronal death followed by ischemia was not concluded in these studies.

Unlike Cirp, Rbm3 has been proposed to promote protein synthesis under mild hypothermia (Danno et al., 1997; Fujita, 1999). Expression of Rbm3 has been well documented in cancer cell lines and its induction seems to be necessary for cell survival and proliferation (Sureban et al., 2008; Wellmann et al., 2010). Rbm3 transcripts have also been reported to be expressed in the cerebrum, cerebellum, and olfactory bulb of adult mice (Danno et al., 2000; Smart et al., 2007). Two isoforms of Rbm3 that differ by a single arginine in the RG region have been identified in rat and mouse brain. Both seem to influence protein synthesis strongly however may still function differently since they were localized to different neuronal compartments. Depending on the splice variant they could be either expressed in the nuclei or dendrites (Smart et al., 2007). The existence of the two isoforms needs to be clarified, but the difference in localization suggests that they might be important for specifying local translation. Interestingly, in glia cells, Rbm3 Arg+ and not Arg-, was expressed (Smart et al., 2007).

Little is known on Rbm3 in neuroprotection but so far the literature suggests that it seems beneficial, preventing cell death and increasing protein synthesis. Overexpression of Rbm3 rescued PC12 cells from polyglutamine-induced apoptosis of mutated Huntington's disease (Kita et al., 2002). In neuroblastoma N2a cells, overexpression of Rbm3 increased protein synthesis by lowering microRNA (miRNA) levels (Dresios et al., 2005). Furthermore recent studies in mammalian cells show Rbm3 to be critical for cell survival (Sureban et al., 2008; Wellmann et al., 2010). Therefore we hypothesized that Rbm3 might be able to mediate hypothermia induced neuroprotection in neonatal asphyxia. In COSCs, cultured from postnatal age 4 (P4) animals, Rbm3 was highly concentrated in nuclei after hypothermia (32°C) in contrast to slices at normothermia (37°C). P4 and P21 slices showed upregulation of Rbm3 at the mRNA and protein level after mild hypothermia, but this increase was 2-3 fold higher in cultures from the younger animals. Upregulation of Rbm3 was also observed in primary neurons under hypothermia. Here, we tested whether mild hypothermia was neuroprotective after induced apoptosis by staurosporine (STS) and indeed at 32°C the cells were protected from degeneration. Knockdown of the expression of Rbm3 by RNA interference through small-interfering RNA (siRNA) did not protect the cells by mild hypothermia from induced apoptosis. Furthermore, in the absence of mild hypothermia, vector-driven upregulation of Rbm3 rescued PC12 cells from degeneration suggesting that upregulation of this protein is required for neuroprotection.

4.5 THE RNA-BINDING PROTEIN RBM3 IS INVOLVED IN HYPOTHERMIA-INDUCED NEUROPROTECTION

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The RNA-binding protein RBM3 is involved in hypothermia induced neuroprotection

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ABSTRACT

Induced hypothermia is the only therapy with proven efficacy to reduce brain damage after perinatal asphyxia. While hypothermia down-regulates global protein synthesis and cell metabolism, low temperature induces a small subset of proteins that includes the RNA-binding protein RBM3 (RNA-binding motif protein 3), which has recently been implicated in cell survival. Here, immunohistochemistry of the developing postnatal murine brain revealed a spatio-temporal neuronal RBM3 expression pattern very similar to that of doublecortin, a marker of neuronal precursor cells. Mild hypothermia (32 °C) profoundly promoted RBM3 expression and rescued neuronal cells from forced apoptosis as studied in primary neurons, PC12 cells, and cortical organotypic slice cultures. Blocking RBM3 expression in neuronal cells by specific siRNAs significantly diminished the neuroprotective effect of hypothermia while vector-driven RBM3 over-expression reduced cleavage of PARP, prevented internucleosomal DNA fragmentation, and LDH release also in the absence of hypothermia. Together, neuronal RBM3 up-regulation in response to hypothermia apparently accounts for a substantial proportion of hypothermia-induced neuroprotection.

Introduction

The only available therapy for neonatal asphyxia is cooling the infant's body to about 33 °C (mild hypothermia) for 48–72 h. A series of randomized multicenter trials have established a protective effect of therapeutic hypothermia in reducing long-term sequelae (Edwards et al., 2010; Jacobs et al., 2007; Schulzke et al., 2007; Shah et al., 2007).

Hypoxic-ischemic brain damage following asphyxia occurs in a biphasic manner, where the early phase (first hours) is classified as necrosis and the later phase (by 48 h) display characteristics of neuronal apoptosis (Northington et al., 2001a, 2001b). Thus, the primary target of hypothermia is the mitigation of early cell death and the prevention of delayed neuronal apoptosis (Robertson et al., 2009).

In neurons, hypoxia-ischemia triggers an intrinsic apoptotic pathway (Ferrand-Drake and Wieloch, 1999; Perez-Pinzon et al., 1999; Zhu et al., 2003), characterized by a biochemical cascade leading to activation of caspases, cleavage of poly ADP-ribose polymerase (PARP), internucleosomal DNA fragmentation, and eventually cell death with release of intracellular enzymes, such LDH (Liu et al., 1996; Susin et al., 1999a, 1999b). Hypothermia ultimately appears to stop cell death (Edwards et al., 1995; Fukuda et al., 2001; Han et al., 2002; Zhu et al., 2004) but the precise molecular events of how and where hypothermia retards this biochemical cascade are poorly understood.

The RNA-binding protein RBM3 belongs to a very small group of cold inducible proteins being synthesized in response to either hypothermia or other conditions of mild stress (Lleonart, 2010). RBM3 is evolutionary highly conserved (Derry et al., 1995) and has been suggested to play a role in hibernation (Fedorov et al., 2009; Williams et al., 2005). While a temperature drop from 37 to 32–34 °C is sufficient to induce the expression of RBM3 (Danno et al., 1997), elevated temperature decreases the expression of RBM3 in the setting of therapeutic hyperthermia (Zeng et al., 2009) and in cryptorchid

Abbreviations: RBM3, RNA-binding motif protein 3; CIRP, cold inducible protein; RRM, RNA-recognition motif; COSCs, Cortical organotypic slice cultures; PC12, Rat pheochromocytoma cells; DCX, Doublecortin; NGF, Nerve growth factor; PARP, poly ADP-ribose polymerase; STS, Staurosporine; EGL, External granular cell layer; IGL, Internal granular cells layer; ML, Molecular layer; PCL, Purkinje cell layer; SGL, Subgranular layer; NeuN, Neuron-specific nuclear protein; SGD, Serum-glucose deprivation.

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testis, a condition in which the testes do not descend from the 37 °C abdominal compartment to the 33–34 °C scrotal environment (Danno et al., 2000).

Recent studies have attributed RBM3 a role in cell survival. Blocking RBM3 signaling by siRNAs triggers cells mitotic catastrophe in colon carcinoma cells (Sureban et al., 2008) and causes loss of proliferation and ultimately cell death in human embryonic kidney cells (HEK-293) (Wellmann et al., 2010). In contrast, exogenous over-expression of RBM3 inhibits apoptosis in neurons transfected with mutated huntingtin (Kita et al., 2002) and increases protein synthesis in mouse neuroblastoma N2a cells (Dresios et al., 2005; Smart et al., 2007).

These findings on RBM3 prompted us to hypothesize that the cold-inducible RBM3 might serve as a candidate to mediate hypothermia-induced neuroprotection after neonatal asphyxia, via its up-regulation in response to hypothermia. Therefore, we first determined RBM3 expression in the developing brain of mice. Second, we studied RBM3 induction in response to hypothermia in cortical organotypic slice cultures (COSCs), primary neurons and rat pheochromocytoma cells (PC12). Third, we investigated whether in PC12 cells neuroprotection by hypothermia might involve RBM3, employing siRNA knockdown and vector-driven RBM3 over-expression experiments.

Material and methods

Animals, slice preparations, cortical organotypic slice cultures, and primary neurons

All animal experiments were performed with permission of the local animal care committee and in accordance with international guidelines on handling laboratory animals and current Swiss law.

C57BL/6J mice from postnatal days 1, 10, 19, and adult (4 months old) were used. Mice were anesthetized with pentobarbital (Vetanarcol®, 0.04 g/kg ip) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were dissected and immersed in 4% paraformaldehyde overnight. Serial sagittal 40 μm sections were cut with a vibratome and collected in cold Tris-phosphate buffer (TBS, 0.05 M).

COSCs were prepared and cultivated with the classical technique (Stoppini et al., 1991) with modifications as published recently by our group (Camenzind et al., 2010). Briefly, brains of C57Bl/6J postnatal day 4 (P4) or P21 mice were removed and coronal slices of the cortex with a thickness of 400 μm were prepared on a tissue chopper. The slices were placed on 0.4 μm Millicell-CM, 12 mm diameter culture inserts (Millipore, Zug, Switzerland) in six-well dishes and cultured in medium containing HEPES-buffered minimal essential medium (50%), Hank's buffered salt solution (HBSS, 25%), and heat-inactivated horse serum (25%) complemented with glutamax (2 mM; Invitrogen, Basel, Switzerland), glucose (1 g/l) in a humidified incubator with 5% CO_2 at either 32 °C or 37 °C for 72 h. Medium was changed 24 h after the COSCs were prepared.

Primary neuronal cultures were obtained from the cerebral cortex of C57Bl/6 J mice gestational stage E14 as previously described (Ogunshola et al., 2002). Briefly, dissected cortices were dissociated in HBSS containing trypsin and DNase I for 5 min at 37 °C. Neurons were seeded on poly-L-lysine coated petri dishes (1.2×10^6 cells per 60 mm dish) in Dulbecco's modified Eagle's medium (DMEM)/Glutamax medium containing: B27 supplement (1X), albumax (0.25 g/ml), sodium pyruvate (1%) and 100 U/ml penicillin streptomycin, (Invitrogen, Basel, Switzerland). Neurons were maintained in culture for 6 days in a humidified incubator at 37 °C with 5% CO_2.

Immunohistochemistry

Immunohistochemistry of free-floating (adult and P19) and mounted sections (younger ages) was carried out as described previously (Maetzler et al., 2007). Sections were washed thoroughly and solubilized with 0.4% Triton X-100 in 0.1 M phosphate-buffered saline (PBS) for 90 min. Non-specific binding was blocked for 2 h in normal goat serum (5% in PBS containing 0.1 M lysine). Then sections were incubated with different preparation of the RBM3 antibody (rabbit, 1:500 up to 1:5000, (Wellmann et al., 2010) overnight at room temperature. Biotinylated goat-anti rabbit IgG (1:100; Antibodies, Davis, CA, USA), and the avidin-biotin complex (Vector, REACTOLAB, Servion, Switzerland) were applied for 90 min each. Immunoreaction was visualized with 0.05% diaminobenzidine (DAB) and 0.1% H_2O_2 with nickel enhancement (1% $NiSO_4$ in the DAB solution). Serial sections were immunoreacted for doublecortin (DCX, rabbit polyclonal anti-DCX diluted 1:500 to 1:1000; #4604 Cell Signaling) and developed as indicated above. All sections were mounted on coated slides, dehydrated in graded ethanol, cleared with xylol and coverslipped in Eukitt (Kindler, Freiburg, Germany).

For co-labeling in COSCs (Camenzind et al., 2010), monoclonal anti-NeuN (diluted 1:250, Chemicon, Hofheim, Germany) was incubated together with rabbit polyclonal anti-RBM3 (diluted 1:100, ProteinTech Group, Chicago, IL, USA) and DAPI (Invitrogen) overnight at 4 °C. Secondary antibodies were anti-rabbit Alexa-488 and anti-mouse Alexa-568 (both diluted 1:500, Invitrogen). Slides were incubated for 2 h at room temperature, mounted with Vectamount (Vector) and photographed.

Cell culture

PC12 were purchased from the German Collection of Microorganisms and Cell cultures (DSMZ, Braunschweig, Germany, http://www.dsmz.de/) and were maintained in DMEM supplemented with 5% heat-inactivated fetal calf serum (FCS), 10% heat-inactivated horse serum and 100 units penicillin/streptomycin per mL (herein after referred to as complete medium, all from Oxoid AG, Pratteln, Switzerland), at 37 °C and 5% CO_2 in a fully humidified incubator.

Induction of apoptosis and hypothermia

PC12 cells, primary neurons and COSCs were treated with various concentrations of bacterial alkaloid staurosporine (STS; Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) in fresh medium supplemented with 1% horse serum and 0.5% FCS, as indicated. In some PC12 experiments, as indicated, apoptosis was induced by serum and glucose deprivation (SGD) for 6 h. Forty-eight hours after transfection and seeding of PC12 cells in complete media, cells were washed twice with PBS, then incubated with PBS for 6 h (SGD), followed by rescue with complete medium. For immunoblotting of cleaved PARP, cells were harvested 4 h after addition of STS 0.8 μM; for assessment of LDH release, cell viability and internucleosomal DNA fragmentation cells were harvested 24 to 48 h after addition of 0.2 or 0.4 μM STS, as indicated. Hypothermia of 32 °C was achieved in a Mini Galaxy A incubator (Sysmex Digitana, Horgen, Switzerland) and applied for varying times starting either prior (24 h) or synchronous with induction of apoptosis as indicated.

RNA-extraction and real-time RT-PCR analyses

RNA preparation and Reverse Transcription (RT) were performed as described (Wellmann et al., 2004). For quantitation, cDNA was amplified by use of SYBR GreenER qPCR SuperMix Universal (Invitrogen) and real-time PCR was carried out on a Rotor-Gene 6000 (Corbett Life Science, Sydney, Australia) as previously described (Wellmann et al., 2008). Primers for detection of the following mouse mRNA molecules have been published previously, *RBM3* and reference gene *RPL13A* (Wellmann et al., 2004), *DCX* and *p35* (Ge et al., 2006). For detection of rat *RBM3* mRNA the mouse RBM3 primers could be used due to 100% homology and the primers for the

rat reference gene *RPL13A* have been published previously (Wellmann et al., 2008). All primers were purchased from Microsynth (Microsynth, Balgach, Switzerland).

Protein extraction and immunoblot analyses

Total cell protein extraction, quantification and immunoblotting were performed as described previously by our group (Shalapour et al., 2006; Wellmann et al., 2004). The following antibodies were used: goat polyclonal anti-RBM3 (#EB09201, Everestbiotech, Oxfordshire, UK), rabbit polyclonal anti-cleaved PARP (Asp214, Cell Signaling, Danvers, MA, USA), and rabbit polyclonal anti-DCX (#4604 Cell Signaling). As an internal control, blots were stripped and reprobed with rabbit polyclonal anti-ß-actin (#4967, Cell Signaling). Bound antibodies were detected using secondary antibodies conjugated with horseradish peroxidase rabbit anti-goat (Invitrogen) and goat anti-rabbit (Cayman, Ann Arbor, MI, USA). Finally the enhanced chemiluminescence system (ECL, Amersham, GE Healthcare, Glattbrugg, Switzerland) was applied.

RBM3 over-expression and short interfering RNA

The sequence of the rat RBM3 gene GenBank NM_053696 was optimized for increased mammalian expression, re-synthesized chemically by GENEART (GENEART company, Regensburg, Germany) and cloned into the expression vector pCEP4 (Invitrogen). 2×10^6 PC12 cells were transfected either with the RBM3 containing vector or the empty vector, serving as control. For transient down-regulation of rat RBM3 mRNA 2×10^6 PC12 cells were transfected either with RBM3 specific siRNA sequences (Rn_Rbm3_1 and Rn_Rbm3_4; Qiagen, Hombrechtikon, Switzerland) or control siRNA molecules (AllStars Negative Control siRNA; Qiagen). All transfections were performed by use of Nucleofector technology according to the manufacturer's instructions for PC12 cells (Cell Line Kit V; Lonza, Basel, Switzerland). After transfection PC12 cells were plated in four to six collagen-coated 20 mm dishes (BD-Biosciences) with complete medium. Then, 24 h after transfection, plates were subjected to the various experiments as indicated. Previously, two RBM3 isoforms have been described differing in one arginine at position 137, encoded by the first three bases of exon 6, isoform Arg+ and Arg- (Smart et al., 2007). Our RBM3-overexpression vector codes for the complete RBM3 protein, including arginine at position 137. The siRNA-sequences used here target specific sequences downstream of exon 6, within the untranslated region of exon 7 and therefore silence RBM3 signaling of both isoforms, Arg+ and Arg-.

Cell viability, LDH release, and internucleosomal DNA fragmentation

Viability of cells was examined by use of the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] tetrazolium assay according to the manufacturers recommendations (Cell Titer96 Aqueous, Promega, Dubendorf, Switzerland). LDH release was examined by Cytotoxicity Detection Kit^{PLUS} LDH and internucleosomal DNA fragmentation by Cell Death Detection ELISA^{PLUS} (Roche, Rotkreuz, Switzerland). In experiments with primary neurons, LDH release is given as absorbance (490–620 nm), in some experiments with PC12 cells LDH release is given as fold change relative to control and in some as percentage of total cell lysis, as indicated. Total cell lysis was achieved by adding supplied lysis reagent to control cells cultivated in parallel according to the manufacturers recommendations.

Statistical analysis

Statistical significance was determined using the matched pairs test. Student's *t* test was performed for each pair of data separately. Significance with a $p<0.05$ is reported.

Results

RBM3 is expressed in the developing mouse brain in a temporally and spatially restricted pattern

During postnatal murine brain development, RBM3 levels were found to be high immediately after birth (P1) and low in adult brain, paralleled by a very similar DCX and p35 mRNA and protein expression dynamic (Figs. 1A and B). DCX is a microtubule associated protein and is expressed in newborn and migrating neurons as well as in neuronal stem cells of the adult brain (for review of DCX and p35 see Feng and Walsh, 2001). The decrease in mRNA expression alongside maturation was stronger for *DCX* than for *RBM3*, while p35 exhibited only a moderate decrease (Fig. 1A).

At the immunohistochemical level, RBM3 was expressed in most neurons of the brain during the first postnatal days (not shown). In the course of development, RBM3 expression decreased in parallel with the maturity of the neurons. Thus, in the somatosensory cortex of P10 mice early generated neurons of the deep layers expressed RBM3

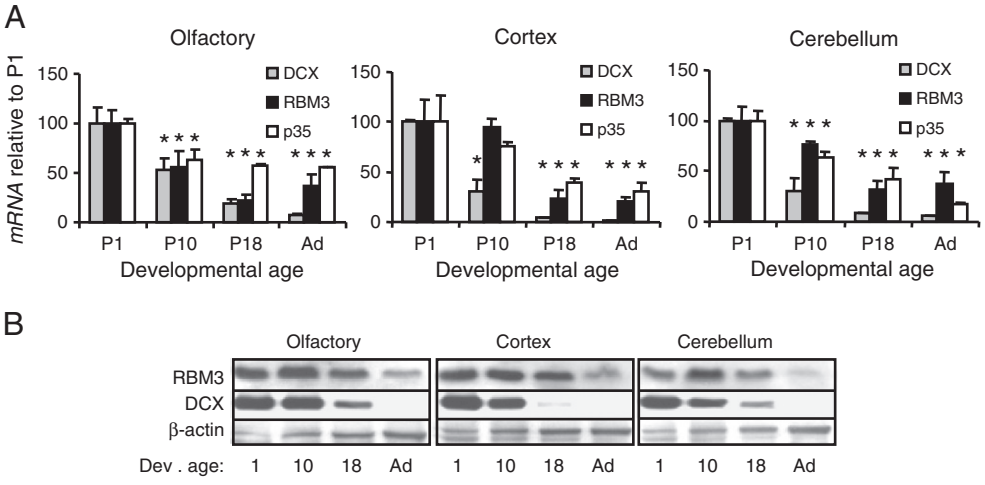

Fig. 1. RBM3 mRNA and protein expressions during postnatal brain development. Panel A: mRNA expression of *DCX* (gray columns), *RBM3* (black), and *p35* (white) is given relative to P1 and was determined in three brain regions as indicated. In each postnatal day of assessment (developmental age), indicated with P1, P10, P18, and Ad for adult, 3–5 animals were investigated. Columns, mean mRNA expression in samples of 3–5 animals performed in triplicates, bars, ± s.d. * $p<0.05$ relative to P1. Panel B: Representative immunoblot for the indicated antigens of the various brain regions and postnatal days of assessment (Dev. age, developmental age) as given.

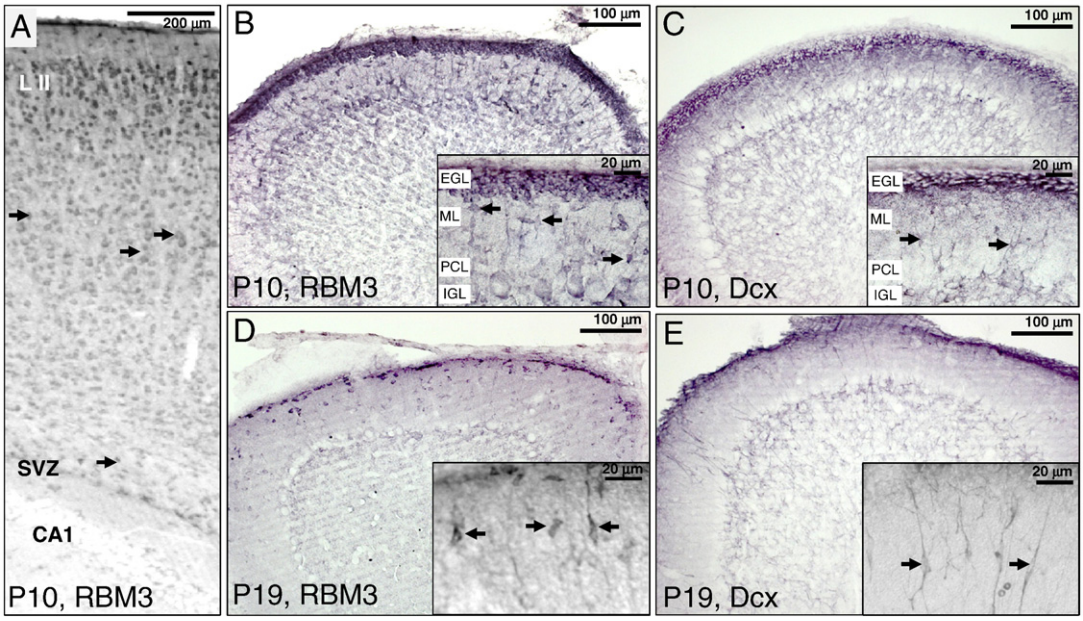

Fig. 2. Presence of RBM3 in the postnatal mouse brain. A: In the developing (P10) somatosensory cortex overlying the hippocampus (CA1), increased expression is found in cells of the subventricular zone (SVZ), in recently migrated neurons (arrows) and in numerous cells of the second cortical layer (L II). B and C: In the early developing cerebellar cortex (P10), RBM3 and DCX are expressed in the external granular cell layer (EGL) as shown in neighboring sections. Similar to DCX, RBM3 is present in single cells (arrows in the high magnification insets) crossing the molecular layer (ML) and the Purkinje cell layer (PCL) on the way to the internal granular cell layer (IGL). D and E: At P19, few neurons (arrows) still migrate to their final destination in the IGL. These migrating neurons express RBM3 and DCX (arrows). In contrast to DCX, RBM3 is present in Purkinje cells in early development (see B) but not at later stages (see D).

at a low level, while later generated neurons in the upper layers exhibited stronger RBM3 immunoreactivity (Fig. 2A). In the early developing cerebellar cortex, RBM3 was present in the external granule layer (EGL) where the granule cells are generated. The centripetal migrating neurons (Figs. 2B and D) which cross through the molecular layer (ML) and the Purkinje cell layer (PCL) were RBM3 positive. At the place where they reach their final destination, the internal granule cell layer (IGL), RBM3 expression was absent (Figs. 2B and D). DCX was found to stain the same type of cells in the same time pattern as RBM3 (Figs. 2C and E).

In contrast, in adult brain, RBM3 expression was restricted to very few neurons in the hippocampus and the forebrain. The localisation of these cells in the subgranular layer of the adult hippocampus together with a very similar staining pattern found for DCX in parallel sections, suggests that neuronal stem cells of the dentate gyrus express RBM3 (Figs. 3A and B). The same is true for the rostral stream, where the

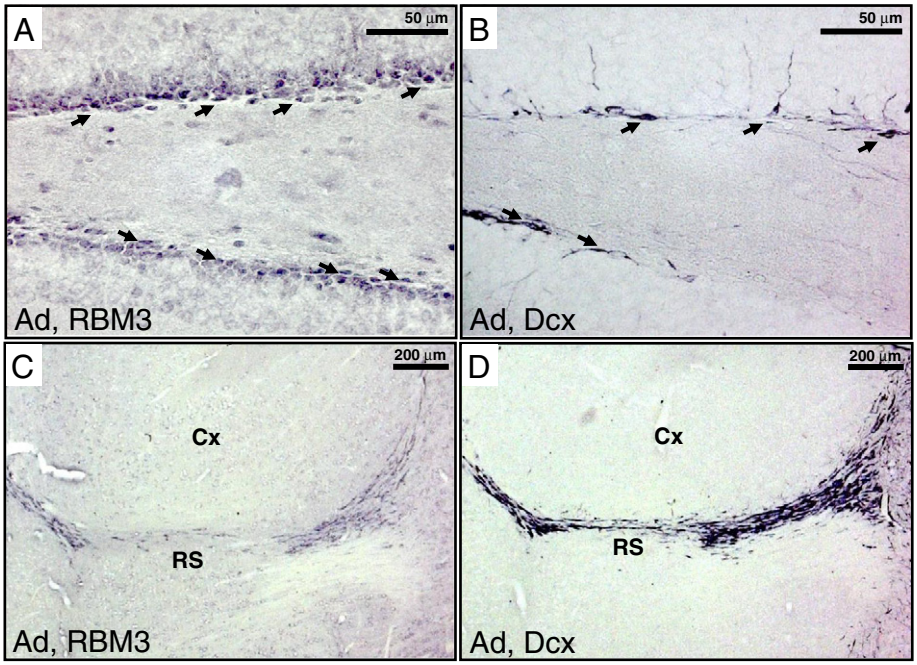

Fig. 3. Presence of RBM3 in the adult mouse brain. A and B: In adult hippocampus, neuronal stem cells, within the subgranular layer (SGL), express RBM3 and DCX. C and D: Progenitor cells for the olfactory bulb are present in the rostral migratory stream (RS), which separates the prefrontal cortex (Cx) from the underlying basal forebrain, express RBM3 and DCX.

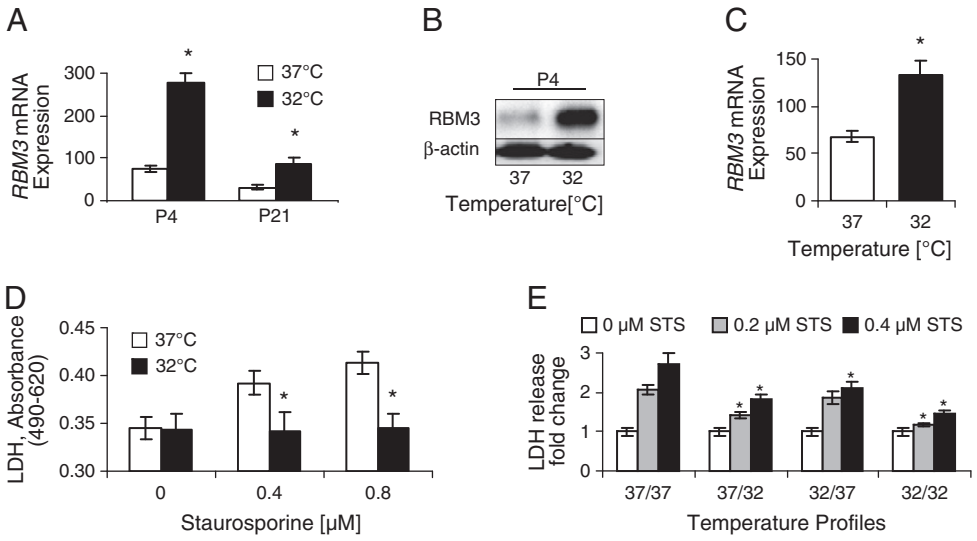

Fig. 4. Effects of hypothermia on RBM3 expression and apoptosis in murine COSCs, primary neurons, and PC12 cells. Panel A: *RBM3* mRNA expression normalized to *RPL13* mRNA in cortical slices when cultured 72 h at 37 or 32 °C, postnatal day P4 and P21, as indicated. Columns, mean mRNA expression in samples of 6 animals performed in triplicates, bars, ± s.d. * $p<0.05$ relative to COSCs maintained at 37 °C of each group. Panel B: Representative immunoblot for RBM3 and β-actin of COSCs derived from postnatal day P4 and cultured as indicated. Panel C: *RBM3* mRNA expression normalized to *RPL13* mRNA in primary neurons, exposed to 37 °C or 32 °C for 24 h. Columns, mean of 3 experiments performed in triplicates, bars, ± s.d. * $p<0.05$ relative to neurons maintained at 37 °C. Panel D: LDH release of primary neurons treated with various concentrations of STS either at 37 °C or 32 °C for 48 h. Columns, mean of 3 experiments performed in triplicates, bars, ± s.d. * $p<0.05$ relative to neurons maintained at 37 °C of each group. Panel E: PC12 cells were exposed to four different temperature (°C) profiles prior (24 h) and subsequent (48 h) STS treatment as indicated. LDH release is given as fold change relative to STS-untreated PC12 cells. Data are representative of three independent experiments; columns, mean of quintuplicates, bars, ± s.d. Significance was calculated between corresponding STS-treated PC12 cells of the 37/37 group and the three other groups, * $p<0.05$.

neuronal progenitor cells for the olfactory bulb are residing (Figs. 3C and D). Cells of the subventricular zone were also found to express RBM3 (see Fig. 2).

Mild hypothermia (32 °C) induces RBM3 expression in cortical organotypic slice cultures and primary neurons

Next, we investigated RBM3 in murine COSCs drawn from temporal cortex of newborn (P4) and adult littermates (P21). Basal *RBM3* mRNA expression (at 37 °C) was found to be about 3-fold higher in newborn, as compared to young adult mice (Fig. 4A). In response to 72 h of mild hypothermia (32 °C), *RBM3* mRNA was up-regulated in the P21 mice (2.8-fold) and in the P4 mice (5-fold, Fig. 4A). Analyses of RBM3 protein expression confirmed this finding (Fig. 4B). Primary murine neurons exhibited a robust increase in *RBM3* mRNA expression even after 24 h of mild hypothermia (Fig. 4C).

Immunohistochemistry of COSCs showed upregulation of RBM3 in response to hypothermia for 72 h as compared to normothermia (Fig. 5). RBM3 was particularly expressed in neurons as shown by double-labeling with NeuN. In addition, RBM3 upregulation was present in immature neurons as exemplified by the staining of the subventricular zone (SVZ), which is not labeled by the marker for mature neurons NeuN (Fig. 5). A few unidentified cells were also positive for RBM3 which may represent immature neurons or glial cells.

Induced apoptosis in primary neurons and PC12 cells is diminished by mild hypothermia (32 °C)

STS is an established agent for inducing classical (caspase-dependent) apoptosis in neurons. Primary neurons as well as PC12 cells when cultured under mild hypothermic (32 °C) conditions were significantly less vulnerable to STS as shown by determination of LDH release (Figs. 4D and E, respectively).

The effect of hypothermia on alleviating STS induced apoptosis was most intense when hypothermia commenced 24 h prior to induction of apoptosis and continued throughout the experiment, irrespective of the STS concentrations employed (Fig. 4E, temperature profile 32/32). When comparing either exclusive pre- (32/37) or post-cooling (37/32), the anti-apoptotic effect of hypothermia was more effective in the experiments with post-cooling, irrespective of the used STS concentration (Fig. 4E).

siRNA-mediated RBM3 down-regulation impedes the protective effect of mild hypothermia (32 °C)

Down-regulation of *RBM3* mRNA expression to less than 90% of control levels was achieved in PC12 cells by combining two siRNA sequences directed against rat *RBM3*. After transfection (up to 72 h) of PC12 cells with either siRNA directed against *RBM3* or nonsense mRNA, there was no significant difference with respect to cell viability (MTS assay) and apoptosis (DNA fragmentation and LDH release, Fig. 6A). However, when PC12 cells were cultured after siRNA transfection in mild hypothermic conditions (32 °C) and were exposed to STS, the protective effect of hypothermia was almost completely abolished in the RBM3-silenced PC12 cells. This was shown by cleavage of PARP, internucleosomal DNA fragmentation, and determination of LDH release (Figs. 6B, C, and D, respectively).

Vector-based RBM3 over-expression mimics the protective effect of mild hypothermia (32 °C)

Two days after transfection of PC12 cells with either the RBM3-pCEP4 expression-vector or the empty control vector, cells were treated either with STS (Figs. 7A–B) or with SGD for 6 h following complete medium for 42 h (Fig. 7C). RBM3 over-expressing cells had less cleavage of PARP, internucleosomal DNA fragmentation, and LDH release, as compared to controls.

Discussion

Here, we present several lines of evidence for an involvement of the cold-inducible RNA binding protein RBM3 in mediating hypothermia-induced neuroprotection. First, RBM3 is widely expressed in

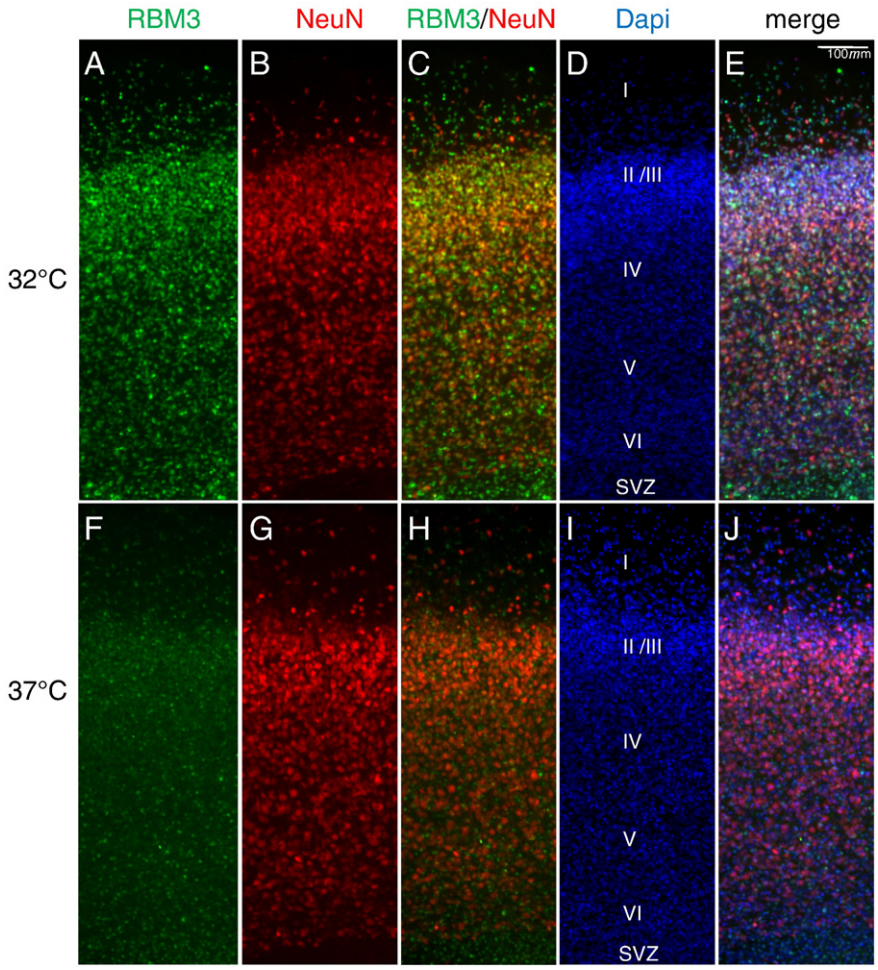

Fig. 5. In COSC obtained from postnatal day 4 mice, RBM3 is present after 3 days in culture. In slices exposed to hypothermia (32 °C) RBM3 is highly concentrated in nuclei (A), as compared to slices cultured at normothermia (37 °C; F). NeuN, a marker of differentiated neurons, exhibits a similar expression at 32 °C (B) and 37 °C (G) and is strongly colocalized with RBM3 (C and H). The nuclear stain DAPI allows the identification of the cortical layers (D and I) and shows that under hypothermia, RBM3 is not only present in differentiated neurons but also in the subventricular zone (SVZ) and in unidentified cells of the developing cortex (E and J).

juvenile neurons of murine brain. Second, mild hypothermia of 32 °C causes significant RBM3 mRNA and protein up-regulation in juvenile as well as in adult neurons. Third, the degree of neuronal apoptosis is inversely related to the level of RBM3 expression. Fourth, blocking RBM3 over-expression that normally follows mild hypothermia (32 °C) in cultured neuronal cells diminishes the protective effect of hypothermia. Fifth, inducing RBM3 expression even under euthermic conditions (37 °C) endows neuronal cells with more resistance against induction of apoptosis.

Our observations on the spatiotemporal RBM3 protein expression during murine postnatal brain development are in line with the recent findings in rats (Pilotte et al., 2009). An overall high neuronal RBM3 expression in the first postnatal week of mice (Figs. 1 and 2) corresponds to a climax in RBM3 protein expression in the second postnatal week of rats (Pilotte et al., 2009). In both species, RBM3 expression becomes restricted to the remaining neuronal proliferative zones of the olfactory bulb and the hippocampus when animals grow up (Fig. 3). Thus, RBM3 appears to be among several other RNA-binding proteins exhibiting a common dynamic expression pattern, peaking early after birth and then declining (Hambardzumyan et al., 2009; McKee et al., 2005). This coordinated temporal expression wave parallels neuronal requirements of translational rate (Pilotte et al., 2009). Within the group of RNA-binding proteins, RBM3 and the highly related cold inducible RNA-binding protein (CIRP) stand out due to their cold-inducibility (Lleonart, 2010).

On the one hand we performed a developmental study in mice from P1 up to adulthood. P10 and 19 are just representative examples (Fig. 2). On the other hand, to demonstrate RBM3 expression in response to hypothermia, we used COSCs from early postnatal animals (P4) to mimic the situation in the newborn infant (Figs. 4A, B, and 5). P21 animals are the oldest animals from which COSCs can be generated. Therefore, we selected this age to show the reaction in a brain as mature as possible (Fig. 4A).

STS, a broad spectrum protein kinase inhibitor, induces cell death by triggering the intrinsic apoptotic pathway involving cytochrome c release from mitochondria (Johansson et al., 2003). Exposure of PC12 cells to STS has been studied extensively to investigate either neuronal differentiation by application of low concentrations (range of 10 nM) (Demeneix et al., 1990; Rasouly et al., 1996) or induced apoptosis when applied at high concentrations (up to 1 µM) (Xia et al., 1995). In response to nerve growth factor (NGF), PC12 cells differentiate into neuronal cells with neurite extensions (Greene, 1978). In our experiments presented here, naïve PC12 cells were used due to the following considerations: First, for transfection, cells had to be collected according to the nucleofector protocol and then replated in new dishes, thus in case of prior differentiation cells may lose their differentiated phenotype including neurites. Second, the effect of transient siRNA transfection is too short to render the possibility of a subsequent differentiation by NGF over a couple of days. Third, it is unknown how treatment of NGF affects RBM3 expression. However,

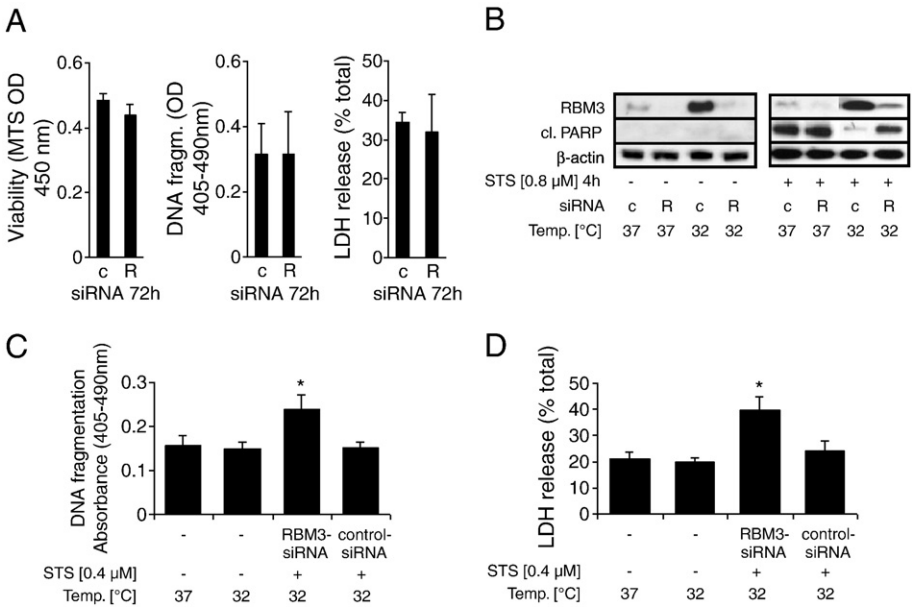

Fig. 6. Influence of RBM3 silencing on apoptosis and neuroprotection by hypothermia in PC12 cells. PC12 cells were transfected either with siRNA targeting *RBM3* mRNA (R) or control mRNA (c) as indicated. Panel A shows the effect of RBM3 down-regulation without further treatment. Exposure of PC12 cells to either hypothermia (32 °C) or normal temperature (37 °C) started 24 h prior to STS treatment as indicated (panel B, C, and D). For immunoblotting of the indicated antigens (RBM3, cleaved PARP (cl. PARP), and β-actin) cells were harvested already 4 h after STS treatment, panel B. For assessment of viability (MTS assay, panel A), DNA fragmentation (panel A and C) and LDH release (panel A and D) cells were harvested 48 h after STS treatment. All data shown are representative of at least three independent experiments and columns, average of quintuplicates, bars, ± s.d. Student's t-tests were conducted in A, C and D using a criteria of $p<0.05$ for significance, marked by asterisks.

to fully understand the role of RBM3 in cold-induced neuroprotection appropriate *in vivo* experiments with transgenic animals are required.

The use of LDH release assay to monitor apoptosis in neurons is still on debate. However, while LDH release was originally used to measure neuronal cell death occurring from necrosis (Koh and Choi, 1987), in more recent publications it was shown, that by means of LDH release neuronal apoptosis in cortical cultures is accurately detected (Gwag et al., 1995; Koh and Cotman, 1992). In a comparative study of LDH and MTT assays in cortical cell cultures with induced neuronal apoptosis by exposure to various pro-apoptotic stimuli some benefits were noted when using LDH release assays (Lobner, 2000). We decided not to use MTT assay as this assay quantifies mitochondrial activity which might be reduced by hypothermia, thus negatively affecting comparability of cooled and uncooled samples.

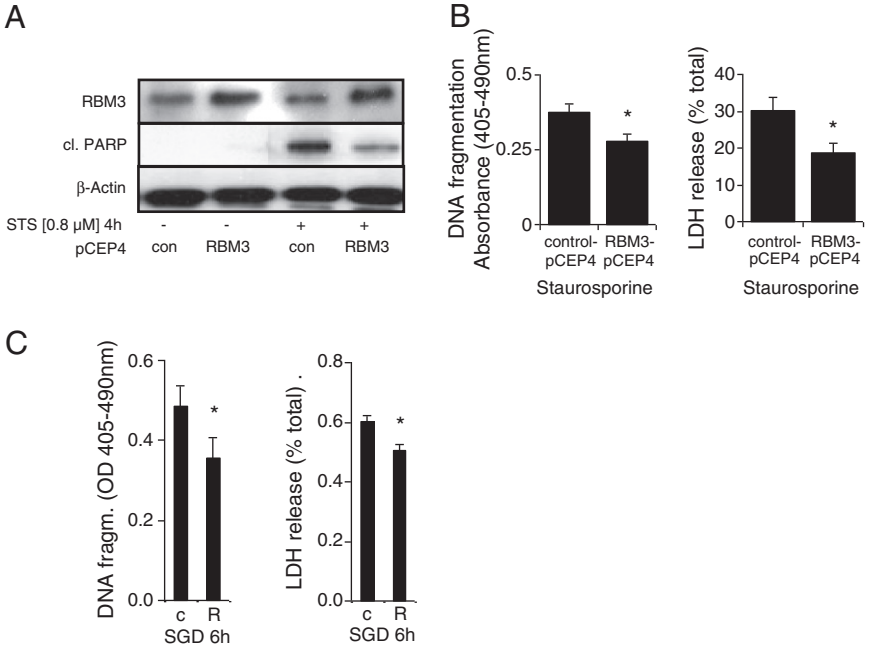

Fig. 7. Influence of RBM3 over-expression on apoptosis and neuroprotection in PC12 cells. PC12 cells were transfected with empty (con) or RBM3 pCEP4 expression vector. For immunoblotting of the indicated antigens (RBM3, cleaved PARP (cl. PARP), and β-actin) cells were harvested already 4 h after STS treatment, panel A. For assessment of DNA fragmentation or LDH release cells were harvested 48 h after STS treatment (panel B) or after 6 h serum-glucose deprivation (SGD) following 42 h complete medium (panel D). All data shown are representative of at least three independent experiments and columns, average of quintuplicates, bars, ± s.d.; Student's t-tests were conducted in B, C and D using a criteria of $p<0.05$ for significance, marked by asterisks.

The boundaries between apoptosis, necrosis and other forms of cell death are less distinct than previously thought (Vandenabeele et al., 2010), particularly so in neurons (Yuan et al., 2003).

Further investigations will have to elucidate the mechanisms of RBM3 action, including the physical RBM3 interaction partners and the target molecules of RBM3 signaling in neuroprotection. RBM3 belongs to a small group of RNA-binding proteins that contains only one single RNA-recognition motif (RRM) (Lleonart, 2010). RRMs are extremely diverse in terms of both structure and function and bind RNA species as well as proteins (Maris et al., 2005). Evidence suggests that two or more RRMs located within the same protein are required for full action (Clery et al., 2008). Moreover, it is now known that regulated gene expression at the post-transcriptional level is based on a network of interactions among RNA-binding proteins operating within multifactorial ribonucleoprotein complexes (Glisovic et al., 2008). Therefore, it is not surprising that several RNA-binding proteins exhibit a common dynamic expression pattern during postnatal brain development (Hambardzumyan et al., 2009; McKee et al., 2005). Previous reports found RBM3 to be associated with ribosomal subunits (Dresios et al., 2005; Smart et al., 2007) and at least one heterogenous ribonucleoprotein (Sureban et al., 2008).

Exposure of mammalian cells to mild hypothermia causes a general inhibition of protein synthesis (Burdon, 1987). The observation that RBM3 has a positive effect on protein synthesis suggests that RBM3 may prevent a more dramatic reduction of protein synthesis under conditions of hypothermia (Dresios et al., 2005; Smart et al., 2007). However, it is unlikely that RBM3 rescues neuronal cells from apoptosis by merely stabilizing the translational machinery. Thus, we propose that in a fashion similar to the specific stabilization of *cyclooxygenase-2* (*COX-2*) mRNA in colon carcinoma cells (Cok et al., 2004; Sureban et al., 2008), a number of specific target mRNAs are stabilized by RBM3 in neuronal cells. The cold inducible protein CIRP, also known as heterogenous ribonucleoprotein A18, shares high homology with RBM3, especially in the RRM (Lleonart, 2010). It has recently been found to induce the expression of thioredoxin and thereby protecting cells from oxidative stress and apoptosis through quenching reactive oxygen species (Yang et al., 2006). Furthermore, a role for CIRP has recently been established in preserving the stemness of neural stem cells and suppressing apoptosis of the cells in conditions of moderate low temperature (32 °C) (Saito et al., 2010).

Our experiments performed on the COSCs are the first to show the effect of hypothermia on RBM3 expression in intact brain tissue. Ultimate proof of the neuroprotective action of RBM3 will require *in vivo* experiments employing RBM3 transgenic animals which are not available at present. The signaling cascades upstream and downstream of RBM3 also remain to be elucidated.

While RBM3 is expressed in brain neurons in a spatiotemporally restricted fashion, its hypothermia-mediated upregulation may rescue neuronal cells from apoptosis and thus contribute to the clinical efficacy of cooling asphyxiated newborn infants.

Acknowledgments

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5.1 FINAL DISCUSSION

The hippocampus is remarkably unique in structure, important for learning and memory as well as vulnerable to various neurological disorders, in particular cerebral ischemia by stroke or neonatal asphyxia. The mechanisms underlying CA1 neuronal death following cerebral ischemia are not well understood. Nevertheless, a large proportion of research on cerebral ischemia has been centered on neuroprotection. *In vivo* and *in vitro* models have provided a wealth of data, which strongly suggest that ischemic-induced neuronal death is due to glutamate excitotoxicity (Choi et al., 1992; Rytter et al., 2003). It is believed that upon release of glutamate, activation of glutamate receptors that are ionotropic and metabotropic such as NMDA, AMPA, KA, and mGluRs, allows Ca^{2+} entry leading to a cascade of signaling pathways activated by Ca^{2+} that induce processes of either neuronal survival or death (Dirnagl et al., 1999).

Therefore, efforts have been made towards neuroprotection inhibiting activation of glutamate receptors with antagonists to certain receptor types in order to prevent glutamate-induced neuronal death. The glutamate receptor antagonists have shown to be effective in both *in vivo* and *in vitro* models, but unfortunately have not been proven to be effective drugs in clinical practice (Wahlgren and Ahmed, 2004), implicating the need for re-examination of the physiological relevance of experimental models used, both *in vivo* and *in vitro* and further highlighting the complexity of neuronal death. Neuronal death by glutamate excitotoxicity may contribute to a substantial portion of the initial phase of injury, but may not be the only active event. The neurovasculature is also affected when blood flow is blocked or reduced by an occlusion. BBB damage ensues during the secondary phase of injury by reperfusion, when oxygen and glucose are being resupplied to the brain (Kuroiwa et al., 1988, Belayev et al., 1996; del Zoppo et al., 2003). Dysfunction of BBB contributes to CNS injury by further decreasing nutrient supply and allowing toxins to invade the brain, interfering with the CNS microenvironment (Murakami et al., 1997). Moreover, increased cerebrovascular permeability can lead to the development of cerebral edema and disturb the CNS ion gradient (Betz et al., 1994).

The BBB is found only in cerebral endothelial cells that make up the brain microvasculature, composed of TJs, AJs, and transporters that maintain the paracellular and transcellular pathways in BBB (Abbott et al., 2010). As the BBB is important for cerebral homeostasis and brain function a lot of research has been conducted in understanding its development and function. Of particular interest is the paracellular pathway not only for understanding BBB mechanics but for designing pharmaceutical drugs to be delivered into the CNS as it is also a rate limiting step.

Genetically modified animal models, such as Cld-5 knockout mice (Nitta et al., 2003), have revealed a certain amount of detail on the relevance of tight junction proteins as key players in maintaining an impermeable barrier. Still these *in vivo* models limit BBB research to investigating mostly developmental mechanisms. Less is known about how tight junction proteins are regulated after disease. A major obstacle in BBB research is the difficulty of gaining direct access to these anatomical features of BBB *in vivo*. Even with the advent of *in vitro* models, many studies have utilized epithelial cells, endothelial cells of non-neuronal origin, or co-cultures of cerebral endothelia and glia of different species, such that translating the data from in-vitro to in-vivo has been very difficult. Furthermore, BBB function is maintained by cell-cell signaling between neuronal, glial, and vascular elements that make up the neurovascular unit (Abbott et al., 2006). The endothelial and glial co-culture systems that allow the fine details of TJs and transporters to be examined lack the structural connectivity and influences by the pericytes, neurons, and basement membrane encompassing this neurovascular unit which may also be required for a more accurate analysis of the cellular and molecular mechanisms of the BBB.

In our lab an alternative *in vitro* BBB model was developed which incorporates culturing cortical organotypic slices (COSCs) of newborn mice in the presence of FGF2 (Bendfeldt et al., 2007) with the aim of narrowing the gap between *in vitro* and *in vivo* BBB data. Since tight junction proteins occludin, ZO-1, Cld-5, and -3 were well preserved in COSCs derived from newborn mice (P3-4), the aims of the current study were to examine the preservation of transporters GLUT-1 and P-gp that are also a part of the BBB and whether they were present in COSCs derived from older mice (P21). We postulated that in the presence of FGF-2 these transporter proteins would also be preserved. Furthermore, we investigated the function of P-gp transport to further characterize the *in vitro* system.

Unlike tight junction proteins which maintain the paracellular pathway sealed, transporters are responsible for regulating the exchange of molecules along the transcellular pathway of BBB. GLUT-1 is a very essential transporter, since glucose is a major nutrient providing energy to the brain (Maher et al., 1993). It has been considered as a sensitive indicator of vascular integrity and BBB function (Dermietzel et al., 1992; Garbuzova-Davis et al., 2007). Furthermore, it is an important cerebral endothelial protein as it has been recently shown to be necessary in normal development of the cerebrovasculature and the BBB (Stenman et al., 2008; Liebner et al., 2008). In our study, GLUT-1 expression was observed in both the luminal and abluminal membranes of vessels, which is comparable to observations found *in vivo* (Dick et al., 1984; Simpson et al., 2007). The expression of GLUT1 persisted for at least 3 days in cultures derived from mice of P4 to P21, thus strengthening the COSC model as a suitable *in vitro*

system to study BBB at the level of glucose transport and at different developmental time points. Additionally, we examined the expression of multidrug resistance P-gp, an ATP-dependent efflux pump; another important BBB transporter in preventing the entry of xenobiotics or toxic metabolites and facilitating them out of the brain parenchyma (Löscher and Potschka, 2005; Hermann and Bassetti, 2007). At DIV3, P-gp transporters were expressed along the luminal membrane of vessels and became more strongly expressed as the slices were derived from older mice, which is consistent with the developmental pattern of P-gp expression *in vivo* (Tsai et al., 2002). Also, P-gp was demonstrated to be functional in slices, derived from P8 to P21 mice, as rhodamine 123, a P-gp substrate, was able to specifically accumulate into the lumen of large and small blood vessels of both acute and cultured slices. P-gp transport capacity was previously shown in cerebral endothelial cell cultures (Hegmann et a., 1992), however our study is the first to demonstrate P-gp transport function in organotypic slice cultures which could also be blocked by verapamil, a P-gp inhibitor, underlining the potential value of this simple model system in studying mechanisms of BBB transport and furthermore as a potentially useful tool in the pharmaceutical industry for exploring drug penetration into the brain.

In an effort to continue and exploit our in-vitro BBB model we employed oxygen-glucose deprivation to examine the components of the BBB under a pathological condition, with the development of neurodegeneration. In terms of the BBB, cell-to-cell interactions between the endothelial and perivascular cells are necessary to maintain its barrier properties (Abbott et al., 2006). As mentioned before the integrity of BBB is often compromised in brain injuries. Dysfunction of non-neuronal cells along with loss of the neurovascular unit that maintains the BBB may be involved in the development of neurodegeneration (Zlokovic, 2008). Up to now the evidence linking neurodegeneration and neurovascular alterations have been circumstantial (Zlokovic, 2010). *In vivo* data have been controversial in showing whether alterations in the BBB and neurodegeneration are linked or separate events (Albayarak et al., 1999; Chen et al., 1999). Furthermore, evidence available on the vascular component during neuronal injury is rather scarce (Zlokovic, 2010). With this in mind, the aim of the second study was to use our in-vitro BBB model to follow the development of neurodegeneration induced by hypoxia, oxygen-glucose deprivation or excitotoxicity; in parallel, to determine whether components of the neurovascular unit were also disrupted by analyzing the expression of both BBB and neuronal markers.

When EHOSCs were submitted to 15 minutes of hypoxia or oxygen-glucose deprivation cell death was specific to CA1 which developed in a delayed manner, 24 and 48 hours later, consistent with other studies done in hippocampal organotypic slice cultures (Rytter et al., 2003) although *in vivo* transient global ischemia models usually exhibit a longer delay at 48-96 hours

(Kirino, 1982; Chen et al., 1998). The mechanism for this delayed neuronal death is currently not understood.

Astrocytes have been known to be reactive and become immunopositive for glial fibrillary acidic protein (GFAP) following transient ischemia (Soltys et al., 2003). In terms of the BBB, their endfeet envelop the endothelium, which is suggested to maintain the unique endothelial phenotype of the BBB (Hawkins and Davis, 2005). Initially, we examined the morphology of astrocytes and observed noticeable features of astrogliosis by GFAP immunohistochemistry as well as loss of astrocytic contacts to blood vessels (preliminary data). Since the differentiation between reactive and non-reactive astrocytes is not easily assessed in organotypic slices because there is a high background expression of GFAP due to the primary lesion made from the preparation of brain slices, we did not continue any further analyses on this cell type. However, we did perform some biochemical analyses on GLUT1 and VEGF, two well known markers, which are known to be upregulated upon hypoxia (Ran et al., 2005) and indeed both were upregulated in EHOSCs by hypoxia or OGD. Because two isofoms of GLUT1 exist: 55 kDa isoform that is endothelial cell specific and the 45 kDa isoform that is glial cell specific (Leino et al., 1997), we could observe that the glial cell specific GLUT1 isoform was downregulated only by OGD and not by hypoxia, which confirmed the presence of glial cell impairment. All in all, our *in vitro* model of short-term OGD in EHOSCs shows neuronal and glial impairments as well as selective vulnerability of CA1 making it a valid and comparable *in vitro* model to transient global ischemia that occurs *in vivo*.

The selective cell death in CA1 was accompanied by a significant reduction in vessel density that was not observed in other subfields of the hippocampus raising the question whether the reduction of vessels in CA1 was a direct effect of OGD or whether it might be due to the CA1-specific neurodegeneration. In rodent models of transient global ischemia, neuronal death, BBB leakage, and capillary damage in CA1 have been reported (Suzuki et al., 1983; Imdahl et al.,1986; Jingtao et al., 1999; Won et al., 2011). The density of vessels in the hippocampus has been quantified by perfusion staining techniques and has indicated less vascularity in the normal CA1 (Imdahl et al., 1986; Jingtao et al., 1999). Also, the cell density in CA1 was observed to be inversely related to vessel density and vessel loss in CA1 was more pronounced after ischemia, suggesting that the microcirculation in CA1 was especially vulnerable to pathological conditions (Imdahl et al., 1986; Jingtao et al., 1999). Vascular reduction in CA1 appeared to correlate with neurodegeneration in our *in vitro* model, but it was not clear whether the vessel changes preceded, paralleled, or followed neuronal death. Since CA1 pyramidal cell death in the hippocampus does not occur immediately after OGD a time course was performed following the development of neurodegeneration and revealed that reduction of claudin-5

expression neither occurred before or after the development of neuronal death, but in parallel with the onset of neurodegeneration, at 24 hours, suggesting that the two events (neurodegeneration and neurovascular loss) were linked.

Blocking Na^+-channels by TTX or ionic glutamate receptors by NMDA or AMPA antagonists has been reported to effectively prevent OGD-induced neuronal death, indicating excitotoxicity as the culprit of neurodegeneration (LoPachin et al., 2001; Rytter et al., 2003). Indeed, with the application of glutamate blockers during ischemia, neuronal death was reduced but also vessel reduction was prevented. Whether glutamate receptors are present in cerebral endothelial cells is controversial (Morley et al., 1998; Andras et al., 2007). Our results suggest that vessel preservation was a consequence of neuronal protection. We utilized CNQX, an AMPA antagonist since it has been shown to be more effective in ameliorating delayed neuronal death (Xue et al., 1994). The evidence regarding the importance of AMPA receptors in cerebral endothelial cells have not been convincing since AMPA antagonists have not been effective in maintaining BBB integrity after glutamate exposure or OGD, *in vitro* and *in vivo* (Andras et al., 2007; Liu et al., 2010). Therefore, the preservation of the neurovasculature in CA1 seems to be secondary to neuronal protection. Furthermore, vessel density was unaffected by silencing of neuronal activity rather than inducing neuronal death by TTX. All in all, these findings suggest that OGD-induced neurodegeneration is required for obtaining vessel loss and therefore is primary to neurovascular remodeling.

Alternatively, to delineate whether neuronal death might be responsible for vessel reduction, neurodegeneration was provoked by applying a high dose of an AMPA agonist to over stimulate AMPA-type glutamate receptors in slice cultures. It has been observed in hippocampal organotypic slice cultures that the severity and location of neuronal damage induced by glutamate and its analogues (NMDA, KA, and AMPA) are dependent on concentration and duration (Kristensen et al. 2001). In our experiments, exposure to 100 μM of AMPA for 30 minutes caused widespread neurodegeneration in CA1 and CA3 but reduced vessels selectively in CA1. Unexpectedly, the vessel density in CA3 was not substantially altered. Furthermore, in other subfields the neuronal and vascular responses were dissimilar. Neurons were present and viable in dentate gyrus but vessel density was significantly increased, while in the entorhinal cortex AMPA excitotoxicity was not effective at either inducing neuronal death or vessel changes. Interestingly, increases in vessel density have been observed in models of epilepsy both *in vitro* and *in vivo* (Rigau et al., 2007; Hellsten et al., 2005; Morin-Brureau et al., 2011; Ndode-Ekane et al., 2010). Upregulation of VEGF by neurons and astrocytes has been suggested to increase vessel density (Morin-Brureau et al., 2011). In DG of seizure-induced animal models, mossy fiber sprouting and neurogenesis have been observed (Parent and

Murphy, 2008), which may release neurotrophic factors, promoting angiogenesis that might have also occurred in our model since VEGF was also upregulated. All these responses were specific to AMPA as they could be prevented by CNQX. These findings under AMPA excitotoxic challenge suggest that the final vascular response is rather complex and not simply dependent on a single phenomenon, such as neuronal death, but due to additional local factors associated to the particular subfield. Further studies are necessary to disentangle the single factors influencing vessel loss and angiogenesis after an excitotoxic insult.

Indeed, a primary mechanism underlying ischemia-induced neuronal death of pyramidal cells is excitotoxicity. However, stroke is not simply a neuronal disorder but a neurovascular disorder as it involves dysfunction of blood vessels. The role of the neurovasculature in neurodegeneration remains elusive and the present results suggest further study of the biological crosstalk between the vasculature and pyramidal neurons and in particular, of the role of AMPARs in ischemia-induced neuronal death. Moreover, research on vascular components might provide a better understanding of how certain injuries in the CNS are brought about and could help to improve therapeutic strategies that have been mainly viewed as neuroprotective.

In this regard, therapeutic hypothermia has shown the most promising results in neuroprotection in neonates, reducing cerebral injury and also preventing the possibility for neurodevelopmental disabilities that may arise later in the life of a child who has suffered from neonatal asphyxia (Edwards et al., 2010; Jacobs et al., 2007; Schulzke et al., 2007; Shah et al., 2007). Mild-hypothermia is a broad-spectrum approach to neuroprotection influencing multiple pathways (Corbett and Thornhill, 2000; Lo et al., 2003; Lo, 2009). However, the cellular mechanisms underlying this protective effect by mild-hypothermia have not been established. Upon mild-hypothermia, a small group of proteins is upregulated and one of them is Rbm3 (Fujita et al., 1999; Danno et al., 1997). Emerging evidence suggest Rbm3 to be involved in cell survival (Kita et al., 2000; Sureban et al., 2008; Wellmann et al., 2010). Therefore, the aim of the final part of my studies was to examine potential mechanisms of mild-hypothermia that is used to treat brain injury after neonatal asphyxia with a focus on the role of Rbm3.

Previously, Rbm3 protein has been shown to be expressed highly in the developing rat brain and to a lesser extent in the adult (Pilotte et al., 2009). We showed here that a similar spatio-temporal expression pattern of Rbm3 protein was observed in mouse from P1 to adult in the olfactory bulb, cortex, and cerebellum. Interestingly, in P10, Rbm3 protein was localized to cells of the subventricular zone and in migrating neurons of the 2nd cortical layer and of the external granule cell layer of the cerebellum. Furthermore, Rbm3 colocalized with doublecortin (DCX), a marker for newborn and migrating neurons. In the adult brain, Rbm3 protein was scarcely expressed and mainly restricted to the subgranular layer in the dentate gyrus of the

hippocampus, where neural stem cells reside, and in the rostral migratory stream, which contains progenitor cells of the olfactory bulb. Our findings in mouse confirm the results obtained in rat by Pilotte et al. (2009) and provide a foundation for further studies on Rbm3 in mouse brain as it is an attractive animal model for genetic manipulations.

Currently, the function of Rbm3 is unknown, but it is a cold-inducible protein, which is upregulated in mammalian cells upon mild-hypothermia (Danno et al., 1997). Recently, it has been further suggested to regulate micro-RNA biogenesis (Pilotte et al. 2011). We found that in response to mild-hypothermia, Rbm3 mRNA and protein levels were upregulated in primary neurons, PC12 cells, and in COSCs. Again, we took advantage of the COSC system to study mild hypothermia, since maintaining precise brain temperature is rather difficult *in vivo.* Rbm3 protein was strongly expressed in nuclei at 32°C compared to slices cultured at 37°C and was 2-3 times higher in slice cultures derived from newborn mice than older mice. Furthermore, mild-hypothermia was indeed neuroprotective as primary neurons and PC12 cells were rescued from cell death induced by staurosporine, a general protein kinase inhibitor which activates apoptosis by the intrinsic pathway (Johannsson et al., 2003). These findings suggest Rbm3 is functional in neuronal cells and is neuroprotective.

In non-neuronal cells, Rbm3 has been shown to be involved in proliferation and cell survival, triggering mitotic catastrophe in colon carcinoma cells (Sureban et al., 2008) or loss of proliferation leading to cell death in human embryonic kidney cells (Wellmann et al., 2010) if it is knocked down by siRNAs. In line with these observations, when Rbm3 expression was knocked down by siRNA in PC12 cells the protective effect of mild-hypothermia followed by induced apoptosis was diminished. Intriguingly, over-expression of Rbm3 in neuroblastoma cells expressing a long polyglutamine form of huntingtin has been reported to prevent cell death (Kita et al., 2000). We also found that exogenous expression of Rbm3 in PC12 cells rescued them from forced apoptosis in the absence of mild-hypothermia, additionally supporting the role of Rbm3 in cell survival.

Our findings provide evidence for a positive role of Rbm3 in cell survival, particularly in neuroprotection induced by mild-hypothermia. Further studies are necessary, as the mechanism of the function of Rbm3 in mild-hypothermia still remains to be elucidated. Particular studies directed towards understanding the signalling upstream and downstream of Rbm3 will be able to determine its mechanism of action and should provide better insight into the signalling pathways of Rbm3. *In vivo* models suitable for examining the function of Rbm3 should be investigated. However, until i*n vivo* models are available, the COSC system has shown to be a useful tool and should be used to analyze the role of Rbm3 in cell survival especially in the setting of hypothermia-induced neuroprotection. The protective effect of therapeutic

hypothermia should be explored as it is the best currently available treatment for ameliorating brain injury after neonatal asphyxia. The finding that Rbm3 is important for hypothermia-induced neuroprotection is a first step towards elucidating the protective action of hypothermia.

5.2 OUTLOOK AND CLOSING REMARKS

In the presence of FGF2 blood vessels including tight junctions and transporters were preserved and functional in slice cultures without blood flow suggesting that endothelial cells along with neurons and glia are reactive and just as viable *in vitro*. Initially, immunostaining of pericytes was performed but unfortunately so far the commercially available antibodies for NG2 and α-smooth muscle actin have not been yielding satisfactory staining results in COSCs. Although pericytes were not examined in these studies, their contribution in maintaining vessels in COSCs should be explored since they have been reported to contribute to BBB maintenance (Abbott et al., 2010). Recently, pericytes have been reported to be involved in controlling cerebral blood flow and their absence in the adult brain of pericyte deficient mice induces vascular damage, reducing blood flow leading to neurodegeneration and inflammation (Bell et al., 2010). It will be interesting to observe whether pericytes survive in the absence of blood flow in COSCs, but since endothelial cells do they most likely will also. Moreover, cultured pericytes seem to perform better at maintaining barrier properties of cerebral endothelial cells *in vitro* than astrocytes (Al-Ahmad et al., 2009).

Inducing selective neuronal death in CA1 of EHOSCs by OGD suggested that our *in vitro* model was comparable to *in vivo* models of transient global ischemia. This was certainly not a novel finding as it has been reported before in many studies but the selective vascular loss accompanying neurodegeneration was previously not observed in organotypic hippocampal cultures. Vessel loss was reversible by neuroprotection through glutamate receptor blockers suggesting a remodeling of the neurovasculature, *in vitro*. The vessel loss appeared to be preceded by neuronal death, but the type of death mechanism (necrosis or apoptosis) was not determined. Short-term OGD induced a delayed form of cell death rather than immediate, which suggest that apoptosis was predominant. In AMPA-induced excitotoxic neurodegeneration, the timing of cell death was more rapid occurring within 3 hours after a brief exposure to a high dose of agonist, which suggested that necrosis was predominant. However, the timing of cell death alone does not exclude the possibility that both types of death mechanisms occurred. Further studies on the death mechanisms should be undertaken because the factors involved in necrosis or apoptosis or both processes may contribute to remodeling of the neurovasculature.

Differences in the vulnerability to ischemia in different hippocampal subfields have been suggested to be due to intrinsic differences between these regions of the hippocampal formation (Kirino et al, 1984). Responses in neurons and more recently glial cells of CA1 have been investigated (Stanika et al., 2010; Tsuchiya et al., 2011; Xu et al., 2010). We observed that neurodegeneration and the response of the local blood vessels after AMPA-induced excitotoxicity was different in each of the hipppocampal subfields. These findings suggest that regional differences were involved in the final vascular response. Therefore, investigations directed towards examining local factors related to endothelial signaling as observed for neuronal and glial cells may offer more hints on the nature of neurovascular remodeling in the hippocampus and in particular why CA1 is selectively vulnerable to the loss of vessels.

Our results on Rbm3 were promising as we could provide evidence in various *in vitro* models that this cold-inducible protein may be involved in hypothermia-induced neuroprotection. Its ability to prevent apoptotic cell death induced by staurosporine or serum-glucose deprivation suggests a role in cell survival and makes it an attractive candidate in reducing long-term sequelae after neonatal asphyxia. Studies directed towards elucidating its role in neuronal survival should determine which part of the survival pathway Rbm3 is involved in. Therefore, further examining Rbm3 under various forms of stress induced by neonatal asphyxia, such as excitotoxic neuronal death or inflammation may provide more clues on the mechanisms of Rbm3 neuroprotection. At present, there are only few *in vivo* studies on Rbm3 expression in brain (Pilotte et al., 2009; Chip et al., 2011). We have only characterized the expression of Rbm3 in developing and adult brain of mouse. Unlike the *in vitro* models presented here, where Rbm3 was knocked down by siRNA to show its neuroprotective effect, the absence of Rbm3 *in vivo* may offer a better understanding of its role in the complex brain environment. Both constitutive and conditional Rbm3 knockout mice should be investigated. If Rbm3 is important in neuronal survival, then we would expect to see a slower recovery rate from neuronal injury in Rbm3 knockout mice. Slice cultures from Rbm3 deficient mice should be considered as a complement to *in vivo* data. Until Rbm3 knockout mice are available, the expression of Rbm3 in brain exposed to hypoxia-ischemia should be examined since Rbm3 has been reported to be upregulated in hypoxia, *in vitro* (Wellman et al., 2004). Neuroprotection by ischemic preconditioning has been observed in animal models (Narayanan et al., 2012) and perhaps Rbm3 is also involved here.

In conclusion, the brain is an organ composed of many cell types and to understand its function under physiological or pathological conditions requires studying more than one cell-type. Research which integrates cell-cell interactions in the specialization of the BBB has not been well-established yet. The main goal of my studies was to utilize organotypic slice cultures of

mouse brain as a model system to study cell-cell interactions that are present within the nervous system. The focus was on the neurovascular unit that encompasses the interaction of vascular elements (endothelial and pericytes) with the nervous tissue (neurons and astrocytes), which maintain the BBB. Our data show that the neurovasculature of organotypic brain slices is preserved with a variety of components of the BBB, including transporter proteins which remain functional. Moreover, under pathological conditions of oxygen-glucose deprivation, blood vessel remodeling in CA1 was observed without blood flow and this was dependent on death or survival of the neurons. Lastly, we demonstrated that Rbm3 is involved in neuronal survival and postulate that it may be involved in therapeutic hypothermia-induced neuroprotection.

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