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SECTION 1

Title: Molecular Physiology of the Brain and Brain Barriers Working Group Report

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Introduction

While the well-established term “blood-brain barrier” has served the purpose of emphasizing the unique characteristics of brain microvessels setting them apart from microvessels in other vascular beds, it all too often fails to inform that these cells provide a living dynamic interface between brain and blood. In fact, because brain microvascular endothelial cells provide a functional interface between blood and the CNS it would perhaps be more accurate to refer to the blood-brain barrier as the blood-CNS interface. As an integral part of the neurovascular unit, brain microvascular endothelial cells engage in intimate communications with neurons, astrocytes and pericytes. Studies within the last five years have also revealed that the myriad cellular and molecular components known to be vital to neuronal function are also present in various forms in blood-brain barrier endothelial cells. Blood-brain barrier research would vastly benefit from taking a broader neurovascular unit view and in particular, consider the processes occurring in neurons and astrocytes that are likely shared with the BBB endothelial cells. While much has been revealed by studies focusing on BBB endothelial cells over the years, there are a few issues that need more attention if we are to gain a more sophisticated understanding of this important blood-CNS interface. Perhaps the single most important question to address is “what are the molecular elements that determine, enable and/or support BBB function in any given physiological or pathophysiological state?” It is not sufficient to characterize tight junction structure, BBB receptors for chemical and cellular ligands and transendothelial transport of solutes. Rather, we must begin to clarify how these parts are integrated to provide the dynamic, functional blood-CNS interface that we know to be so critical in health and disease. One example of how our thinking must change is the common view that the BBB is either open or closed and that the tight junction proteins identified to date somehow create a very restrictive barrier. However, we know that the barrier is not simply a passive open or closed barrier but it is, in fact, quite dynamic with a range of permeabilities mediated by transcellular events as well as paracellular changes. We know that occludins and claudins play a major role in formation of tight junctions but we don’t really know how these and other proteins interact to create the effective tight junction. We also know very little about how all the molecular components that make the barrier are altered at different developmental stages, from fetal to old age, and in different disease states. Finally, more attention needs to be given to the fact that the blood-brain barrier is not the only important blood-CNS interface. Indeed, these interfaces also include the blood-CSF barrier, blood-retinal barrier, blood-nerve barrier and the blood-labyrinth barrier. The following sections touch upon some of the recent findings in the neuroscience field that should be considered in blood-CNS interface research, as well as some of the recent strides in our understanding of this important interface and suggestions for areas needing more aggressive investigation. It is certainly not possible to be inclusive in this discussion. Thus, the following are intended to serve only as highlights of questions at hand.

1. What are the scientific opportunities in the neuroscience field that may be applied to the brain barriers field and vice versa?

The neuroscience field has grown considerably in the past five years with respect to our understanding of the molecular components essential to neuronal and astrocyte function. Examples include the many discoveries about solute transporters, ion transporters and channels, water channels, receptors and signaling pathways. In the course of these studies, a
number of methodological advances have been made that have greatly facilitated investigations of neuronal and astrocyte molecular processes. An important advance in neuroscience research has been the use of genetic models, including knockout mice, conditional knockout mice, RNA interference and a variety of transgenic models. These have been used to analyze gene function and generate specific disease models. Methods have also been developed that allow cell-specific whole genome expression profiling. In addition, new methods for in vivo imaging of neural networks in awake behaving animals has allowed for a greater understanding of neural development and function. These and other technological advances arising from neuroscience research can and should be applied to brain barriers research in order to better understand function of the barriers during health and disease. For instance, very little is known about the molecules in CNS endothelial cells that form the physical blood-brain barrier to hydrophilic molecules. While genetic ablation of claudin 5 has shown that this molecule is necessary to limit movement of small molecules into the brain (Nitta et al., 2003), other studies have shown that claudin 5 is expressed in all endothelial cells, not just the blood-brain barrier. Studies have also shown that occludin is BBB-specific but is not required for the barrier (Saitou et al., 2000). Thus, it is still unknown why the barrier forms in CNS endothelial cells and not other tissues. Cell-specific gene profiling combined with genetic manipulation would be very helpful in identifying which genes are necessary for formation of the highly restrictive blood-brain barrier. Furthermore, in vivo live imaging of brain microvascular endothelial cells in disease models should be valuable in elucidating the cellular mechanisms that are responsible for BBB permeability changes in these diseases. In addition the use of model organisms including non-mammalian models such as drosophila and C. elegans, has enabled rapid genetic manipulation of the nervous system, greatly increasing the understanding of neural function. Understanding the evolutionary similarities of brain barriers in different organisms may provide similar advances in brain-CNS interface research (Stork et al., 2008).

2. What is the status of the science in the topic area, including key scientific advances made in the past 5 years? Are these advances relevant to the other field?

While much attention has focused on structure of the physical barrier in brain-CNS interfaces, research in recent years has begun to provide a better understanding of the molecular components underlying the function of the blood-CNS interface. A limited number of examples are provided in the following paragraphs. These include tight junctions, plasma membrane proteins functioning in ion and other solute transport, vesicular transport mechanisms, and also cell receptors and signaling pathways involved in regulating these and other brain microvascular endothelial cell functions.

Tight junctions
Several key advances have been made in the understanding of blood-brain barrier tight junctions, including evidence of the proteins involved in the makeup of the junctions both occludin and claudin as well as cytosolic proteins associated with tight junctions and other paracellular junctions (Hawkins and Davis, 2005; Nitta et al., 2003; Vorbrodt and Dobrogowska, 2003). A number of attempts have been made to identify CNS endothelial cell gene expression and protein composition related to barrier function. These studies have provided a template not only for understanding the components of the blood-brain barrier but also how the barrier is formed (Agarwal and Shusta, 2009; Calabria and Shusta, 2006; Enerson and Drewes, 2006).
However, further work is needed to elucidate the difference between blood-brain barrier endothelial cells and peripheral endothelial cells with respect to molecular components that form the paracellular junctions and provide the unique functional aspects of the BBB. This includes a consideration of how these junctional molecular elements, both their presence and the manner in which they interact to form the barrier, change in various physiological and pathophysiological states.

Several key studies have identified important CNS parenchymal cell-derived molecular signals, including AGT and Wnt, that appear to regulate blood-brain barrier formation and function (Daneman et al., 2009; Liebner et al., 2008; Stenman et al., 2008; Wosik et al., 2007). These studies have provided new insight into the mechanisms by which structure and function of the BBB are regulated. For instance, the finding that Wnt regulates both CNS-specific angiogenesis and induces specific BBB properties suggests that CNS angiogenesis and BBB formation are linked. Such findings are of great importance to gaining a better understanding of the phenotype of new vessels formed during diseases such as stroke, brain tumors and diabetic retinopathy.

Other key experiments have demonstrated that interactions between CNS endothelial cells and neural cells are important for the development of the CNS. For instance, endothelial cells provide a niche for neural stem cells and secrete molecular cues that regulate neural stem cell self-renewal as well as neuronal survival (Dugas et al., 2008; Shen et al., 2004; Shen et al., 2008). These studies demonstrate the importance of endothelial-neural cell interactions in the developing nervous system. Further work needs to identify how endothelial cells participate in the metabolism and function of neural circuits.

**Plasma membrane transport proteins**

Studies in recent years have also provided much insight into cellular aspects of the blood-brain barriers, in particular mechanisms that allow for regulated transcellular flux of solutes and ions. A few examples will be considered in the following paragraphs.

**Ion Transporters and channels**

Recent strides have been made in better understanding ion transport mechanisms of brain cells. However, the focus has been largely on whole brain and/or neurons and astrocytes, giving little or no attention to blood-brain barrier endothelial cells. These studies have included the discovery that a family of HCO₃⁻ transporters, the electrogenic and electroneutral Na/HCO₃ transporters (NBCe and NBCn, respectively) and Na-driven Cl/HCO₃ exchangers (NDCBE) are present in rat brain, with NBCs present in both neurons and astrocytes and NDCBEs predominantly in neurons (Bevensee and Boron, 2008; Chen et al., 2008a; Chen et al., 2008b; Majumdar et al., 2008). Much, too, has been learned about the roles of aquaporins (AQP) in water distribution within the brain both in health and disease (Amiry-Moghadam et al., 2004; Badaut and Regli, 2004; Brown et al., 2004; Gunnarson et al., 2004; Manley et al., 2004; Nagelhus et al., 2004; Nicchia et al., 2004; Papadopoulos et al., 2002). Both AQP1 and AQP4 play important roles in water distribution in the brain, with AQP4 phenomenally concentrated at the perivascular astrocytic endfeet (Amiry-Maghadam et al., 2003). AQP4 expression appears to be altered in disease, e.g., AQP4 abundance is increased in edematous meningiomas while it appears to be reduced, along with the Kir4.1 K channel, by vascular accumulation of amyloid in Alzheimer's disease. Evidence has also emerged that AQP1 and AQP4 both conduct CO₂ and NH₃
in addition to serving as water channels and that these and other AQP channels exhibit characteristic selectivities for CO₂ and NH₃ (Musa-Aziz et al., 2009).

Despite this, we know little about water and ion movement across blood-brain barrier endothelial cells. A number of studies have described the distribution of AQP1 as well as ion transporters at the blood-CSF barrier. Thus, we know that in choroid plexus, both Na-K-Cl cotransporter (NKCC1) and Na/K pump are found on the apical (CSF facing) membrane while the Cl/HCO₃ exchanger (AE2), the electroneutral Na/HCO₃ cotransporter (NBCn1) and the Na-dependent Cl/HCO₃ exchanger (NCBE) are found on the basolateral surface while AQP1 resides at both membranes (Praetorius and Nielsen, 2006). With respect to ion transporters of the blood-brain barrier, we know that BBB endothelial cells play an essential role in regulating the volume and composition of brain interstitial fluid, yet we know surprisingly little about the ion transporters and channels of these cells. BBB ion transporters and channels function not only in moving ions between blood and brain but also in many other processes, including e.g., regulation of intracellular pH and volume of the cells (Pedersen et al., 2006). Early studies demonstrated that BBB endothelial cells possess an abluminaly located Na/K pump, a luminal Na- and K-selective channel and less well-defined luminal Na transporters (Betz, 1986; Betz et al., 1980; Betz et al., 1994; Vigne et al., 1989). These studies laid the early groundwork for understanding how the BBB performs secretion of Na and Cl into the brain and absorption of K into the blood. By coupling activities of the abluminal Na/K pump with luminal cation channel the cells can absorb K from brain into blood as needed to maintain low extracellular brain [K] (Keep, 1993; Keep et al., 1995a; Keep et al., 1999; Keep et al., 1995b). Also, by coupling abluminal pump and luminal Na transporters, the BBB can secrete Na, Cl and water from blood into brain, accounting for the up to 30% of brain interstitial fluid generated by the BBB (Cserr et al., 1989; Keep, 1993).

Studies in recent years have begun to expand our understanding of BBB ion transporters. We now know that the cells possess a predominantly luminal membrane residing Na-K-Cl cotransporter (Foroutan et al., 2005; O'Donnell et al., 2005; O'Donnell et al., 2006; O'Donnell et al., 1995; O'Donnell et al., 2004b; Pedersen et al., 2006). Studies have also demonstrated that the cells possess a Na/H exchanger (Hsu et al., 1996; Kalaria et al., 1998; Kawai et al., 1997; Lam and O'Donnell, 2008; Nicola et al., 2008; Sipos et al., 2005; Spatz et al., 1997; Taylor et al., 2006; Vigne et al., 1991) and recent studies have shown that both NHE1 and NHE2 isoforms are present and reside primarily in the luminal membrane (Lam and O'Donnell, 2008). While the Na/H exchanger is known to function in pH regulation and both the cotransporter and exchanger can function in cell volume regulation, these BBB transporters are also known for their role in vectorial transport of ions (Pedersen et al., 2006). Thus, it has been hypothesized that these luminal Na transporters couple with the abluminal pump to transport Na across the barrier from blood into brain (Figure 1). Other ion transporters that have been reported to reside in BBB endothelial cells include Cl/HCO₃ exchange, Na/HCO₃ cotransport and also Na-dependent Cl/HCO₃ exchange (Nicola et al., 2008; Taylor et al., 2006). In addition, the cells possess volume-sensitive (swelling-activated) Cl channels, ATP-sensitive K channels, inward rectifying K channels (von Weikersthal et al., 1999) and non-selective cation channels (Vigne et al., 1989; Simard et al., 2007). (Hoyer et al., 1991; Janigro et al., 1993). The cellular location of these transporters and channels has yet to be determined.
**Other solute transporters**

Much attention has been given to nutrient transporters, e.g., GLUT 1 and MCT1 which transport glucose and lactate, respectively, and amino acid transporters (Hawkins et al., 2006; Simpson et al., 2007a). In general, advancing our understanding of plasma membrane proteins that function in solute transport across the blood-brain barrier is essential for a full understanding of how these cells function as a blood-CNS interface at different developmental stages as well as in health and disease. Here again, new methods developed in the neuroscience field for investigation of these and other solute transporters, including new imaging methods and development of genetically manipulated in vivo models, will be valuable when applied to brain barriers research. Drug transporters are, of course, a vital functional component of the blood-CNS interface. These will not be considered here, as they are the subjects of another report.

**Vesicular transport mechanisms: receptor-mediated transcytosis**

Transport of solutes across the blood-CNS interface includes vesicle-mediated pathways. A number of new findings have provided insight regarding components that make up these transcellular pathways and mechanisms by which they are regulated. Perhaps the majority of vesicular transport studies have been conducted from the perspective of identifying new strategies for delivering drugs to the brain. Many proteins that are targeted to the CNS are transported across the BBB by receptor-mediated transcytosis (Figure 2). The presence of these specific receptors on the surface of the BBB endothelial cells has allowed us to target and transport some therapeutic proteins to the CNS for the treatment of neurodegenerative diseases (Rubin and Staddon, 1999; Spencer and Verma, 2007). Well-characterized BBB receptors include the low-density lipoprotein receptor, transferrin receptor, and insulin growth factor receptor (Pardridge, 2005). The low-density lipoprotein receptor family is a group of cell surface receptors that bind lipoprotein complexes for internalization to the lysosomes. The family is comprised of approximately ten different receptors that are expressed in a tissue specific manner and primarily bind apolipoprotein complexes (Borén et al., 1998; Brown and Goldstein, 1986; Stefansson et al., 1995). The apolipoproteins, of which the two most prominent members are apolipoprotein B (ApoB) and apolipoprotein E (ApoE), function to bind lipids in the blood stream and target them for lysosomal degradation. Apolipoproteins bind to the low-density lipoprotein (LDL) receptor on the cell surface of the targeted cell, and then the complex is endocytosed. Conversion to an early endosome and subsequent lowering of the compartmental pH, results in release of the apolipoprotein and recycling of the receptor to the cell surface. In contrast, at the BBB, LDLR binds apolipoproteins, resulting in transcytosis to the abluminal side of the BBB where presumably, the apolipoprotein is released to be taken up by neurons and/or astrocytes (reviewed in Bickel et al., 2001; Hussain et al., 1999). This receptor target has been used successfully to deliver therapeutic proteins to the CNS via the use of the ApoB LDLR binding domain (Spencer and Verma, 2007).

**Receptors and signaling pathways**

A very large and growing body of evidence concerning plasma membrane and cytosolic/nuclear receptors and also signaling pathways of brain microvascular endothelial cells is now available. While these findings are too exhaustive to consider in this brief report, it should be noted that a broad range of receptors has been demonstrated in these cells, reflecting the fact that cells serve as an important interface between blood and the CNS, constantly sampling the vascular and neural environment and responding appropriately to the prevailing conditions. Examples of receptors present are those for steroid and peptide hormones, growth factors, neuropeptides
and cytokines (Abbott, 2000; Edvinsson, 2008; Ermisch, 1992; Langer and Natale, 2005; McCandless and Klein, 2007) and also receptors for elements present in the extracellular matrix (DelZoppo et al., 2006) as well as those for circulating cells, such as T-lymphocytes (Engelhardt, 2006). Many other signaling pathways have also been described in brain microvascular endothelial cells. These are beyond the scope of this report. However, it should be noted that studies in the neuroscience field, as well as the cardiovascular field, have largely guided investigations of brain-CNS interface receptors and signaling pathways. Future studies will benefit by taking advantage of new genetic models and imaging techniques developed by neuroscientists.

**BBB secretions**

Blood-brain barrier endothelial cells also actively secrete substances that regulate the function of other cells in the neurovascular unit and/or vascular space. These include, e.g., cytokines, nitric oxide, prostaglandins, endothelin (Dorheim et al., 1994; Faraci and Heistad, 1998; Ghersa et al., 2002; Mandi et al., 1998; McGuire et al., 2003; Shafer and Murphy, 1997; Spranger et al., 2006; Verma et al., 2006). The endothelial cells themselves have receptors for some of these secreted substances, suggesting the presence of autocrine signaling in some instances. As with blood-brain barrier receptors, the knowledge base of substances secreted by these cells is large and cannot be addressed adequately here. Suffice it to say that, as with receptors and signaling pathways, understanding the types of secretions produced by these cells is critical for understanding the function of the brain-CNS interface in health and disease.

**The BBB in disease**

For basic scientists and many interested in treating neurological diseases, the BBB is usually approached in terms of an impediment for drugs and proteins that could be used to treat neurological diseases. However, a large number of conditions including brain abscess, trauma, multiple sclerosis, diabetic ketoacidosis and stroke alter BBB permeability, with subsequent edema that may be life threatening in and of itself. Though some of the mechanisms of barrier dysregulation are becoming clearer, much more work and better treatments are needed. In addition, the mechanisms of BBB repair are not known and need study. For example, while the BBB dysregulation observed in stroke and traumatic CNS injury is followed by some degree of repair that may include angiogenesis, much is still unknown about these processes. Moreover, it is becoming very clear that abnormalities of the BBB either produce distinct disease entities or contribute to the pathogenesis of a disease. Discussing all of the diseases related to the BBB is outside the scope of this review. However, in this report we highlight recent findings related to this new field and tabulate some of these for easier reference (see Table 1). Virtually every aspect of the BBB may influence a disease or be a target for a treatment. For example, one of the newest treatments for multiple sclerosis is a monoclonal antibody that binds the alpha 4 integrin receptor found on leukocytes and prevents adhesion of the leukocytes to brain endothelial cells, thereby reducing the cytotoxic effects of these cells that lead to the demyelination and brain injury occurring in acute MS plaques (Goodin et al., 2008; Lutterotti and Martin, 2008). Molecules found on the luminal side of the BBB are targets for proteins associated with various infections that have a predisposition to infect the brain (de Lagerie et al., 2008; Unkmeir et al., 2002; Wang and Lin, 2008). Many transporters have also now been described on the luminal and abluminal membranes of BBB endothelial cells and astrocyte end feet. It seems likely that a large number of diseases will be associated with these genes or polymorphisms in these genes, some of which have been described (Blanz et al., 2007; Dean,
2005; Drozdzik et al., 2003; Kim et al., 2008; Klepper, 2008; Newmeyer et al., 2005; Suls et al., 2008; Weber et al., 2008; Westerlund et al., 2009) and many probably that are yet to be described. One of the most interesting and unexpected roles of transporters in a common disease is RAGE- and LRP-1-mediated influx and efflux of A beta, respectively, in Alzheimer’s disease. Though it seems these transporters are not a cause of AD, they might be targets for modulating bulk movement of A beta out the AD brain and could even be involved in antibody-mediated flux of amyloid out of AD brain. A variety of molecules, including IL17, IL22, perforin, tPA, VEGF and others, have been found to down regulate tight junction proteins and make the BBB leaky (Argaw et al., 2009; Kebir et al., 2007; Morgan et al., 2007; Reijerkerk et al., 2008; Suidan et al., 2008). Though corticosteroids have been used to decrease edema and BBB breakdown, only recently have potential mechanisms of action been suggested, including actions of the drugs on GRE promoters on tight junction proteins. It seems likely that other drugs targeted at other promoter elements on the TJ proteins might also be useful in inducing TJ proteins and decreasing leakiness at the BBB (Blecharz et al., 2008; Felinski et al., 2008; Harke et al., 2008).

**Stroke**

The mechanisms responsible for breakdown, or dysregulation, of the BBB following stroke have been the subject of intense study. Of the molecules involved, the metalloproteinases (MMPs), particularly MM9, have been studied the most. However, the roles of MMP9, MMP2 and other proteases have recently been questioned since they appear to open the BBB early and damage the BBB later after stroke (Lo, 2008). The BBB breakdown that occurs at 12-48 hours after a stroke can be prevented or at least ameliorated by MMP inhibitors (Candelario-Jalil et al., 2009; Lo, 2008; Lu et al., 2008; Rosenberg, 2009). The precise cell types that release MMP9 and other MMPs to open the barrier are still uncertain. However, a recent study from Gidday et al showed that BBB breakdown resulting from focal cerebral ischemia was blocked to a similar extent in MMP9 knockout mice (MMP-9/-) and in chimeras lacking leukocytic MMP-9 but not in chimeras with MMP-9-containing leukocytes (Gidday et al., 2005). The MMP-9 expression in the brain at 24 h of reperfusion was derived mainly from (Gidday et al., 2005). MMP-9/- mice had reduced leukocyte-endothelial adherence and reduced neutrophils plugging capillaries and infiltrating the ischemic brain during reperfusion. Collagen IV immunostaining, a marker for BBB basal lamina, was preserved in the MMP-9 knockout and chimeras lacking leukocytic MMP-9 (Gidday et al., 2005). Since MMP9 is expressed almost exclusively in neutrophils in peripheral blood (Du et al., 2006; Tang et al., 2006) these studies suggest that neutrophils are the major source of MMP9 that produce barrier breakdown following stroke. Moreover, since MMP9 and non-specific MMP inhibitors decrease BBB breakdown when given just prior to or just after stroke, and since these inhibitors probably do not cross the BBB, then these MMP inhibitors likely act on leukocytes (Lu et al., 2008). These findings suggest that endogenous brain derived MMP9 is not involved in barrier breakdown at early times following stroke. Given this, it seems surprising that almost every cell type in brain can be activated to induce and release MMP9.

It is possible that the sequential breakdown and subsequent repair of the BBB following stroke may involve release of MMPs by multiple cell types (Figure 3). In this regard, at early times after stroke (12-48h) neutrophils adhere to the capillary endothelium. The neutrophils release MMP9, which degrades tight junction proteins (claudin, occluding, JAM-A) and also the basement membrane (basal lamina) of the endothelial cells and astrocytes. This allows for migration of the neutrophils into the ischemic brain, and opening of the BBB resulting in vasogenic edema formation. In the core infarction areas it is possible that MMP9 is released by both neutrophils
and brain microglia. In this case, while the neutrophil MMP9 would break down the BBB, it is predicted that microglial MMPs would act, along with other molecules, to induce death of neurons and glial cells. Finally, once blood flow is restored, repair of the BBB would begin at the margins of the infarction. All cells including endothelial cells, glia and neurons would likely release MMPs and other proteases at lower concentrations to remodel the basement membrane and cell-cell contacts to allow for angiogenesis and gliogenesis that may occur to re-establish the BBB. Future studies in stroke and other diseases are needed to confirm or deny this synthesis of the current literature.

**White matter disease: cerebral amyloid angiopathy**

Two additional new important areas for investigation from a BBB perspective are cerebral white matter disease and cerebral amyloid angiopathy. Cerebral white matter disease is a poorly-understood entity first identified with the advent of brain MRI imaging, and is sometimes simply referred to by its MRI features: white matter hyperintensities. The prevalence of cerebral white matter disease dramatically increases with age. After the middle of the seventh decade, prevalence of white matter hyperintensities has been reported to be as high as approximately 95% (Fernando et al., 2004; Longstreth et al., 1996). Demyelination is a commonly observed pathologic characteristic (Simpson et al., 2007b), and the association of white matter hyperintensities with vascular risk factors and presence of hypoxia-regulated proteins have been interpreted as evidence of a unique cerebral ischemia syndrome (Fernando et al., 2006). A recent study demonstrated that brain regions with most extensive white matter hyperintensities also had reduced endothelial expression of both adhesion molecule CD-31 (platelet endothelial cell adhesion molecule-1) and blood-brain barrier constituent (and membrane transporter) P-glycoprotein, suggesting a substantial element of blood-brain barrier dysfunction in cerebral white matter disease (Young et al., 2008). Regarding cerebral amyloid angiopathy (one of the two most common causes of intracerebral hemorrhage of the elderly), recent MRI findings have demonstrated cerebral microbleeds in 18-38% of the population age 60 and older (Vernooij et al., 2008). These microbleeds usually represent remote areas of minimal bleeding and, when present in a cortical distribution, are thought to reflect presymptomatic cerebral amyloid angiopathy (Greenberg, 1998). The etiology of microbleeds is typically attributed to vascular injury at the arteriolar level (Fazekas et al., 1999). However, this prevailing view is based on rather limited pathologic studies, and presence of brain capillary amyloid β-protein in cerebral amyloid angiopathy (Thal et al., 2008) suggests that possible microvascular origin of cerebral microbleeds deserves attention. Thus, the scope of blood-brain barrier research may be significantly expanded by investigation into these common cerebrovascular disorders.

3. **What are the barriers to progress in the topic area? What are your recommendations for key steps to develop and advance knowledge in the topic area?**

A key barrier to progress in understanding BBB function during health and disease is lack of basic knowledge of the molecular components that make the endothelial barrier. Much remains unknown about the basic components of the barrier. If we are to truly understand this dynamic blood/CNS interface in both health and disease, we need to know more about the molecular elements that underlie the structure and function of the barrier. With respect to tight junctions, we do not yet understand why high resistance tight junctions form in CNS endothelial cells and not in endothelial cells in non-neural tissues. Nor do we understand how proteins that appear to
be involved in tight junction formation interact with each other to create a high resistance junction characteristic of the BBB. Further identification of the molecular components of the barrier and their interactions will lead to a greater understanding of why the BBB forms, and how it is disrupted during disease. We also need to consider other molecular aspects of the physical barrier such as the extracellular components important to structure/function of the barrier and of the neurovascular unit in general, including basal lamina components and glycocalyx of the endothelial cells. Further, we need to know more about all classes of proteins involved in transport across the barrier and among cells of the neurovascular unit. This includes ion transporters and channels, metabolite transporters, drug transporters and also proteins involved in vesicular transport mechanisms including receptor-mediated endocytosis, as well receptors and signaling pathways.

As another important step toward advancing brain barriers research, we need to recognize that this blood-CNS interface is not static nor is it simply “open or closed” and that changes in the properties of the barrier are likely to affect more than the BBB endothelial cells but also the architecture of the neurovascular unit, in particular the astrocytic endfeet and, e.g., signaling propagated along the endfeet. Further, it should be clarified that changes in permeability of the barrier are not the same in all cases, e.g., increased permeability induced by 1 M mannitol versus inflammation. Also, the frequently used term BBB breakdown would more aptly be identified as BBB dysregulation.

There is also a great need to better understand all aspects of BBB changes in traumatic injury to the brain and spinal cord. This is because it is likely that the window of therapeutic intervention includes the wound healing environment, which adds the complication of delivering therapeutics in regions that consist primarily of angiogenic vessels. To provide effective therapies, we need to know more about the transporter properties of angiogenic vessels and whether these properties are dependent on proximity to astrocytes and or the local proinflammatory microenvironment. We also need to know whether the glial scar influences vascular architecture and barrier phenotype. In this regard, questions that should be addressed include the following. First, what happens to the BBB in injury and when is the barrier open? Second, given that there appears to be an early BBB disruption followed by appearance of angiogenic vessels, what governs the behavior of the leaky angiogenic blood vessels that are formed after trauma-induced BBB disruption and appear to grow into a macrophage-rich environment? Further, what is the molecular basis of the leaky angiogenic phenotype? Finally, what is the molecular basis of phenotypic changes in brain microvessels that form in the local environment presented by other disease states?

With respect to traumatic injury as well as other diseases effort should be made to better understand the key molecules that from the barriers. Genetic models and in vivo imaging have been useful tools to better understand the barrier, especially tight junctions as the key paracellular barrier and some elements such as claudin 5 have been much studied. However, we must ask, are there other molecules that are important and what are the key molecular interactions that allow tight junctions to serve as the barrier? Thus, future studies of the BBB in other disease states, e.g., stroke, are needed to investigate the mechanisms of BBB breakdown and repair. This will be challenging because of intimate nature of all of the cells involved. Such studies would likely require cell specific conditional knockouts to answer each of the proposed steps/pathways.
For the purposes of better delivering therapeutic agents, we need to work toward a deeper understanding of all BBB transport systems, both plasma membrane transport proteins and vesicle-mediated transport. This includes targeting transport systems to increase delivery to the brain and/or reduce efflux from the brain, as well as to increase efflux of harmful agents from the brain, e.g., amyloid. With respect to BBB transcytosis mechanisms we do not yet understand how binding and transport of the targeted protein with its respective receptor results in transcytosis of the bound protein to the abluminal side rather than the more conventional targeting to the lysosome.

Another great need is to develop a better understanding of the functional interactions among cells within the neurovascular unit, how these may change in disease and how we might manipulate these interactions for therapeutic purposes (e.g. Interactions of cells within the neurovascular unit that determine structure and function of the barrier). This includes the functional and structural association of BBB with astrocytes, pericytes, neurons and circulating cells. In this regard we need to determine the evolving changes in molecular components of the BBB and neurovascular unit that occur with different disease states including for example, stroke, tumors, trauma, and epilepsy, as well as different stages of life, i.e., the developing versus aging brain.

One critical issue that needs to be addressed more rigorously, e.g., is that of metabolite flux among cells in the neurovascular unit. While much is known about glucose transport across the BBB, we know surprisingly little about the movement of glucose through the neurovascular unit. Studies have shown that abundant GLUT 1 is present on both luminal and abluminal BBB membranes and also in the dense encirclement of astrocytic endfeet surrounding the BBB yet we don’t really understand whether glucose gets to the brain parenchyma by going between endfeet or by getting taken up by astrocytes and distributed (Simpson et al., 2007a). Similarly, our knowledge of ion transporters and channels, as well as water channels, in brain cells is steadily increasing through ongoing studies. These transporters and channels have been studied in the context of their roles in, e.g., pH regulation, cell volume regulation and, for the BBB, also influx and efflux of ions and water across the barrier (Amiry-Maghaddam et al., 2003; Badaut et al., 2002; Brillault et al., 2007; Chang et al., 2007; Foroutan et al., 2005; Kintner et al., 2005; Kintner et al., 2004; Lam et al., 2005; O'Donnell et al., 2005; O'Donnell et al., 2004a; O'Donnell et al., 2006; O'Donnell et al., 1995; O'Donnell et al., 2004b; Papadopoulos et al., 2002; Pedersen et al., 2006; Su et al., 2002; Sun and Murali, 1998; Venero et al., 2001; Yuen et al., 2008). However, the focus of most studies has been limited to one cell type rather than the neurovascular unit as an integral whole. Future studies are needed to more fully understand the integrated function of the neurovascular unit in health and disease with respect to movement of metabolites, water and electrolytes. Developing appropriate therapies for CNS diseases requires an understanding of which pathways play a predominant role in neurovascular unit function under different physiological and pathophysiological conditions. As part of these studies, we also need to determine how the human neurovascular unit compares to those in various animal models currently in use.

Approaches used in neuroscience research should be applied to barrier research. This includes consideration of appropriate model systems employed by molecular physiologists and the importance of using multiple approaches to validate findings; including cell cultures, isolated
microvessels, brain slices and in vivo model. The insight and methods used by neuroscientists would be particularly helpful with respect to identifying, e.g., functional reporters of BBB function and dysfunction, assays that demonstrate the status of the BBB, and appropriate conditional knockout animals.

4. What are your recommendations for resources that are needed to advance the field?

Blood-brain barrier research is at the crossroads between the fields of vascular biology and neuroscience, and while recent data demonstrate that the development and function of the nervous system is intimately intertwined with the development of vascular system, leaders in these two fields rarely interact. In addition studies of the BBB are critical for understanding and developing treatments for many neurological diseases, including stroke, multiple sclerosis, Alzheimer’s disease, edema and brain traumas. Therefore, the issue of greatest importance for advancing brain barriers research is identifying and developing mechanisms to facilitate collaborative endeavors that will bring together researchers studying vascular biology, neural development, nervous system function and neurological diseases, as well as researchers skilled in different experimental approaches including cell biology, molecular biology, model system genetics, human population genetics, and clinical studies. This would greatly improve our ability to share knowledge and further the understanding of the role of brain barriers in the development, function and disease of the nervous system. Specific questions that need to be addressed include: 1) What are the molecular mechanisms that regulate CNS endothelial barrier properties, i.e., the BBB phenotype?; 2) How are these properties regulated by interactions with the CNS?; 3) Are barrier properties induced during development or is constant signaling required for maintenance?; 4) How does the BBB interact to regulate the development of the nervous system?; 5) What are the molecular mechanisms that regulate BBB breakdown during disease and repair following injury?; and 6) Does BBB dysfunction cause neurological disease? There is also great need for a transporter discovery program, not only a search for new transporters but also identification of what the protein/genes are and a study of their cellular and molecular biology.

Understanding what constitutes the functional blood-CNS interface under various physiology and pathophysiological conditions is paramount for developing appropriate therapies to address different disease states.
References


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Neuron. 57, 178-201.
Figure 1. Ion transport proteins of brain microvascular endothelial cells. Evidence has been provided for luminal Na-K-Cl cotransporter and Na/H exchanger and abluminal Na/K pump. K transport from brain to blood is thought to occur via functional coupling of Na/K pump and K channel. Na transport from blood into brain has been hypothesized to occur via functional coupling of the pump with luminal Na-K-Cl cotransporter and/or Na/H exchanger. Evidence has been provided for the presence of transporters shown in gray although their cellular locations have yet to be determined.
Figure 2. Receptor mediated transcytosis of proteins at the BBB
Figure 3. Hypothesized role of matrix metalloproteinases in BBB breakdown and repair following ischemic stroke. The BBB before (A) and 24 hours after ischemia at the margin (B) and core (C) of the infarct, with subsequent repair of the barrier at the margin of the infarct (C).
### Table 1. Diseases that affect the BBB or diseases impacted by the BBB.*

<table>
<thead>
<tr>
<th>Disease or Process</th>
<th>BBB Protein Affected – or mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neurodegenerative diseases</strong></td>
<td></td>
</tr>
<tr>
<td>Alzheimer disease</td>
<td>RAGE - receptor for advanced glycation end products – influx of Abeta (^1)</td>
</tr>
<tr>
<td></td>
<td>LRP-1: multi-ligand lipoprotein receptor – efflux of Abeta (^1)</td>
</tr>
<tr>
<td></td>
<td>P-glycoprotein (ABCB1) is reduced at blood-brain barrier and seems to play a critical role in clearing Abeta from brain (^2, 3, 4)</td>
</tr>
<tr>
<td></td>
<td>Changes in ABCG2 is related to cerebral amyloid angiopathy and controls blood-brain barrier transfer of Abeta (^5)</td>
</tr>
<tr>
<td>Parkinson disease</td>
<td>Polymorphism in P-glycoprotein drug transporter MDR1 gene association; ATP-binding cassette, sub-family B, member 1 (ABCB1) gene encoding the P-glycoprotein (P-gp) (^6, 7)</td>
</tr>
<tr>
<td><strong>Cerebrovascular diseases</strong></td>
<td></td>
</tr>
<tr>
<td>tPA and reperfusion induced hemorrhage</td>
<td>MMPs released by neutrophils and possibly endothelial cells degrade tight junction (TJ) proteins and BM – increase risk of hemorrhage (^8-11)</td>
</tr>
<tr>
<td>VEGF – blood brain barrier breakdown</td>
<td>Occludin and claudin-5: down regulation of mRNA and protein (^12)</td>
</tr>
<tr>
<td>Familial Cerebral Cavernous Malformations</td>
<td>CCM1/KRIT1, CCM2 or CCM3/PDCD10 localized in endothelial cells and perhaps astrocyte end feet- venous malformations with bleeding (^13, 14)</td>
</tr>
<tr>
<td>Ischemic brain edema</td>
<td>BBB breakdown due to MMP9 release by neutrophils which degrades occludin, claudins, JAM, basement membrane (BM) (^15-17)</td>
</tr>
<tr>
<td></td>
<td>SUR1-regulated NC(Ca-ATP) channel mediates ischemic cerebral edema (^18)</td>
</tr>
<tr>
<td>Acute mountain sickness and high altitude cerebral edema</td>
<td>Vasogenic edema (^19)</td>
</tr>
<tr>
<td><strong>Epilepsy and seizures</strong></td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>Mechanism</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Epilepsy and Exercise Induced Dystonia</td>
<td>GLUT1 mutations in brain endothelial cells (BEC) 20,22</td>
</tr>
<tr>
<td>Resistance to pharmacotherapy in some patients with epilepsy</td>
<td>Multidrug efflux pumps from the ATP Binding Cassette (ABC) superfamily (P-glycoprotein) at the BBB 23, 24</td>
</tr>
<tr>
<td>Alexander Disease – large brain, seizures, retardation</td>
<td>GFAP mutations – blood brain barrier abnormalities 25</td>
</tr>
<tr>
<td>Leukoencephalopathy with epilepsy</td>
<td>CIC-2 is a broadly expressed plasma membrane chloride channel – epilepsy, white matter degeneration, retinal degeneration in mice 26</td>
</tr>
</tbody>
</table>

**Infections**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV induction of STAT and/or rho HIV entry into brain endothelial cells</td>
<td>Down regulation of claudin-5, HIV increase in entry kinase in endothelial cells decreases ZO-1, and ZO-2 in endothelial cells - ? increase HIV entry 27,29</td>
</tr>
<tr>
<td>Susceptibility to certain types of brain infections – e.g., malaria and CNS listeria moncytogenes</td>
<td>MDR1A (ABCB1) deficiency at BBB-susceptibility to cerebral malaria; opc gene in Meningococcus produces protein that binds HBMECs via α 5 β 1 integrin receptors via fibronectin 30-32</td>
</tr>
<tr>
<td>CNS infections in general</td>
<td>Pathogens co-op BBB cellular machinery to enter the brain 33</td>
</tr>
<tr>
<td>NeuroAIDS in HIV</td>
<td>BBB efflux systems keep out antivirals from brain, fostering neuroAIDS 33</td>
</tr>
<tr>
<td>Malaria</td>
<td>Effect protein expression and permeability of human endothelial cells selectively from brain 34</td>
</tr>
</tbody>
</table>

**Neuroinflammation and brain tumors**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell oxidative stress causes adhesion to endothelium and transmigration across BBB</td>
<td>White blood cell (selectins, VLA-4, CD44,a4b7 integrin) and brain endothelial (selectin ligands, ICAM-1, VCAM-1, CD44) proteins mediate migration across BBB. 35-37</td>
</tr>
<tr>
<td>CD8+ cytotoxic T cell mediated BBB breakdown and edema</td>
<td>Perforin release degrades tight junction proteins 38</td>
</tr>
<tr>
<td>Brain Edema – tumor, inflammation, others</td>
<td>Aquaporins (astrocyte end feet) 39-40</td>
</tr>
<tr>
<td>Brain Edema – role of steroids; prednisone and dexamethasone decrease BBB leakage in acute MS plaques, tumors, and other pathologies</td>
<td>Steroids act on Glucocorticoid response elements (GRE), on promoter of tight junction genes (occludin, claudins, cadherin) to increase TJ proteins and increase BBB tightness 41-43</td>
</tr>
<tr>
<td>BBB break down in Multiple Sclerosis:</td>
<td>tPA induction of ERK1/2 in endothelial cells mediates</td>
</tr>
</tbody>
</table>
### Monocyte-endothelial interactions induce tPA in endothelial cells
- Monocyte transmigration across BBB and control breakdown of occludin

### BBB break down in MS: role of IL17 and IL22
- T(H)17 lymphocytes release IL17 and IL22 that act on receptors on BEC that results in degradation of TJ proteins and opening of the BBB

### Prevent leukocyte trafficking across the BBB – decreased Multiple Sclerosis relapses
- Monoclonal antibody to alpha4 integrin (Natalizumab) – adhesion molecule on leukocytes necessary to attach to and cross the BBB in EAE

### Inflammatory Pain – Cytokines mediate BBB breakdown
- Down regulation of Occludin and claudin-5

### Metabolic and psychiatric diseases

<table>
<thead>
<tr>
<th>Condition</th>
<th>Pathophysiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain Edema – associated with diabetic ketoacidosis and cerebral ischemia</td>
<td>Na-K-Cl cotransporter and Na/H exchanger at BBB</td>
</tr>
<tr>
<td>Adrenoleukodystrophy – abnormal white matter in brain with a wide range of neurological findings; retinal degeneration</td>
<td>ABCD1 gene mutation – ATP binding cassette disorder; ATP-binding cassette (ABC) superfamily</td>
</tr>
<tr>
<td>Obesity - leptin released from adipose tissue and binds to leptin receptor to modulate food intake</td>
<td>Deficient BBB transporter protein function - reduced leptin transport across the BBB</td>
</tr>
<tr>
<td>Imerslund-Gräsbeck syndrome- familial B12 malabsorption – dementia and white matter abnormalities</td>
<td>Amnionless (AMN) mutations- possible B12 transport into brain</td>
</tr>
<tr>
<td>Canavans Disease – large brain, seizures, retardation, white matter degeneration, other signs</td>
<td>Mutations in aspartoacylase lead to accumulation of N-acetylaspartate (NAA)</td>
</tr>
<tr>
<td>Mucopolysaccharidosis</td>
<td>Loss of the GUSB transporter with maturation underlies difficulty in treatment</td>
</tr>
<tr>
<td>Depression</td>
<td>Polymorphisms in the drug transporter gene ABCB1 predict antidepressant treatment response in depression</td>
</tr>
<tr>
<td>Hepatic encephalopathy</td>
<td>Affects potassium homeostasis in astrocytes, produces swelling and disrupts control of extracellular potassium</td>
</tr>
</tbody>
</table>
References for Table 1

5. Xiong, H. et al. ABCG2 is upregulated in Alzheimer’s brain with cerebral amyloid angiopathy and may act as a gatekeeper at the blood-brain barrier for Abeta(1-40) peptides. J Neurosci 29, 5463-75 (2009).
SECTION 2

Title: Intercellular Communication with the Neurovascular Unit Working Group Report

Co-Chairs: Luc Leybaert and Damir Janigro

Working group members: Giorgio Carmignoto, Greg del Zoppo, Christian Giaume, Aaron Johnson, Edith Hamel, Kyu-Won Kim, Eng Lo, Karoly Nikolich, MingMing Ning, Michael Pagel, Berislav Zlokovic
Introduction

A major notion that emerged from the neuroscience field over the past few years is the concept of ‘tripartite synapses’, denoting the fact that the two party structure of pre- and postsynaptic endings is adjoined by a third partner consisting of astrocyte extensions enwrapping these highly specialized structures. Tripartite synapses link the heart of the neurotransmitter signaling machinery to the network of surrounding cells that are of glial as well as vascular nature, together forming the neurovascular unit (NVU). Depending on the point of view, this entity has also been called the glio-vascular unit but the term neuro-glio-vascular unit is perhaps better suited to fully cover the different partners involved. The importance of the 3 party configuration of this unit is exemplified by the fact that it provides a linkage between neuronal communicative activity and the regulation of local blood flow and energy metabolism, two essential players in the integrated function -and dysfunction- of neural tissues. An additional player, historically recognized before the concept of tripartite synapse was born, is the capillary vessel with its ablumenal and luminal components, the latter including circulating white blood cells, erythrocytes and platelets. It is now clear that neurovascular signaling is of utmost importance in better understanding the data obtained by functional imaging approaches (BOLD-fMRI, MR spectroscopy and PET/SPECT).

The complexity of the BBB interface is enormous compared to tripartite synapses. First of all, the cells contributing include the endothelium (forming the barrier proper at capillary level), astrocyte endfeet, pericytes and blood cells as its closest neighbors, adjoined at some distance by nerve cells and vascular smooth muscle cells. All these cells engage in the functioning of the NVU, which would more appropriately be called the ‘extended NVU’ to stress the importance of the blood cells in its functioning (Fig. 1). The ‘sextapartite’ cell assembly, together with the basal lamina and extracellular matrix components, engage in a complex network of intercellular signals (fast and slow; active and trophic), membrane transport, cellular permeability (specific, selective or via leakage pathways) and intracellular metabolic cascades. The vascular niche is furthermore an important player in neurogenesis opening up interesting avenues to recruit its potential for neuroprotective actions. Above all, coupling-schemes between the diverse cell types orchestrate their collective functioning, with common examples being neurovascular and neurometabolic coupling or the recently proposed matrix-trophic coupling and neurobarrier coupling. Microglial cells are pathological sensors of brain function and recent insights into NVU functioning indicate that astrocytes have an even wider response scope, sensing both normal and abnormal function. If the consequent responses and signals make their way over the astrocyte-vascular bridge, then NVU-BBB signaling may prove to be of utmost importance in physiological BBB regulation, as an essential step in the development, maintenance or exacerbation of several brain diseases. We shall explore the current state and importance of integrated NVU-BBB signaling over the next few sections.
Figure 1. The extended NVU. A classical view of the NVU incorporates neurons, glial cells like astrocytes and microglial cells, and vascular cells like endothelial cells, pericytes and smooth muscle cells. In order to look at the full picture, it needs to be stressed that the blood cells, particularly white blood cells such as lymphocytes, monocytes and polymorphonuclear cells that interact with the BBB endothelium, are an integral part of this unit.

1) What are the scientific opportunities in the neuroscience field that may be applied to the brain barriers field and vice versa?

Signaling between neurons and glial cells, especially astrocytes, in response to electrical and synaptic activity is one of the key topics that emerged in the neuroscience field over the past decades. Astrocytes respond to neurotransmitters released from synaptic terminals with transient increases in cytoplasmic calcium concentration (calcium signals) that represent a calcium-based form of excitability in these cells. Astrocyte calcium responses to strong pathological stimuli and to lower physiological levels of neuronal activity have been well documented in vitro as well as in vivo. Astrocyte activation results in the release of extracellular messengers that act on presynaptic terminals and postsynaptic membranes to influence neuronal transmission. By propagating to endfoot extensions in contact with blood vessels and further extending over neighboring endfeet, astrocyte calcium signals contribute to controlling the local cerebral blood flow by releasing vasoactive messengers. In addition to neuron-triggered signals, astrocytes may also generate spontaneous calcium activity as recently reported in an in vivo study. Next to their role as key players in neurovascular communication, astrocytes play a pivotal role in controlling the composition of the extracellular milieu, and every action potential produced by neurons is translated into a significant raise in extracellular potassium followed by astrocyte depolarization and potassium distribution over the astrocyte network. Significantly, blockade or pathological impairment of this mechanism leads to seizures and loss of synaptic plasticity.
2) What is the status of the science in the topic area, including key scientific advances made in the past 4 years? Are these advances relevant to the other field?

Astrocytic calcium signals are involved in vascular responses and a key finding is that vasodilatory as well as vasoconstrictive vessel responses may result as a consequence, depending on the metabolic context: if oxygen availability is low, vasodilators prevail while if oxygen availability is high vasoconstrictors predominate. Several vasoactive messengers released by astrocytes have been proposed to mediate these actions, such as arachidonic acid (AA), PGE2, members of the epoxy-icosatrienoic (EET) acid family & nitric oxide (NO) as vasodilators and PG12 & OH-icosanoic-tetraenoic (HETE) acid products as vasoconstrictors. Astrocyte calcium signals propagate to endfeet that are in close juxtaposition to endothelial cells of the blood-brain barrier (BBB) but also make extensive contacts with smooth muscle cells at arteriolar level. The vasotropic actions of astrocyte calcium signals may therefore signal directly to smooth muscle cells or take an indirect pathway via BBB endothelial cells that secrete on their turn vasoactive substances like NO. The observation of neuronal activity-related calcium signals in endfeet located at the BBB interface strongly suggest that these signals may further extend to the BBB endothelial cells and pericytes that are within a reach of less than 0.1 μm.

Endfeet-endothelial cell communication is likely to influence BBB function, with possible consequences at the level of expression or function of transporters that shuttle for example glucose, transferrin or neurotransmitters over the BBB, thereby adapting BBB function to the neuronal state of activity. The mode and messengers of the astrocyte-endothelial communication link are poorly defined, especially when considering fast signaling events at a second/minute time scale. Astrocytes strongly express channel proteins like Kir4.1, aquaporin 4 and connexin 43 at their endfeet that are involved in the transport of ions, second messengers and water. Candidate messengers released by astrocytes include glutamate, ATP, D-serine, AA derived prostaglandins & EETs. For ATP and glutamate, the release mechanism has been thoroughly examined and both vesicular as well as non-vesicular pathways have been reported – the calcium dependency is currently best documented for vesicular release. Possible candidate messengers that may influence BBB function include PGE2, EETs and ATP. EP3-type PGE2 receptors have been reported on brain endothelium these receptors inhibit cAMP formation, which normally acts to increase barrier tightness. EETs trigger endothelial changes in cytoplasmic calcium that closely resemble those provoked by bradykinin, a well characterized mediator of BBB opening. Furthermore, 5,6-EET triggers calcium entry in vascular endothelial cells by activating TRPV4 channels – TRP family channels in BBB endothelia have recently been characterized and discussed. As to ATP, various receptors (P2Y1, P2Y2) have been reported in cultured brain endothelial cells but work in brain slices by the Nedergaard group did not find evidence for P2Y2 or P2Y4 receptors on endothelial cells. Work in other vascular beds (pulmonary artery) indicates that ATP may act to enhance endothelial barrier function. Obviously, messengers like nitric oxide (NO), cytokines (TNF-α, IL-1β & others), VEGF and matrix metalloproteinases (MMPs) may join the picture when considering pathological conditions. Also, changes in the activity of some neurotransmitter systems that interact closely with astrocytes and their endfeet, such as the noradrenergic, VIP-ergic and glutamatergic
systems\textsuperscript{32-34} may be particularly important regulators of neural activity-driven BBB adaptations, under both physiological and pathological states.

The list of brain disease states associated with altered BBB function is extensive and for the remainder of this chapter, we will focus on epilepsy, which is of particular interest because it generates strong astrocyte responses\textsuperscript{6} offering an interesting opportunity to investigate the communication toward the BBB and its consequences on BBB function. In addition, seizures change BBB function at multiple levels, for example permeability\textsuperscript{35} and GLUT-1 transporter activity\textsuperscript{36}, and conversely, BBB alterations may lead to seizures\textsuperscript{37,39}. Finally, and most importantly, BBB alterations may be induced by signals from the parenchymal (neuro-glial) side but surprisingly also from the blood side as recently evidenced in a rather provocative study\textsuperscript{37,39,40}.

Traditional experimental models of epilepsy (kainate, electrical stimulation/kindling, GABA-A antagonists) focus on neurons and, in general, address the issue of acute seizures. Perhaps not surprisingly, testing of new pharmacological treatments has been influenced, and perhaps limited, by the models used. Recent data suggest that BBB failure can lead to enhanced neuronal firing and seizures. The converging evidence from several labs has convincingly proven that even brief failure of the selective permeability of the BBB can profoundly alter neuronal behavior, even to the extent of promoting seizures\textsuperscript{38}. At a mechanistic level, the consequences of BBB disruption encompass increased paracellular leakage of large (albumin) and small (potassium) agents that due to a favorable osmotic and electrochemical gradient accumulate in the brain. Both albumin and potassium promote synchronous and excessive neuronal firing\textsuperscript{16,41}. Interestingly, the effects of albumin seem to be related to potassium buffering\textsuperscript{42}; thus, while leakage of potassium directly depolarizes neurons, the albumin-related loss of potassium buffering will indirectly increase the extracellular potassium concentration further enhancing excitability and synchronization.

More recent findings have shown that specific molecular mechanisms are activated by seizure-promoting agents. In particular, the cholinergic agonist pilocarpine was shown to cause seizures by activating adhesion molecules expressed in leukocytes and cerebrovascular endothelial cells\textsuperscript{39}. A fundamental issue remains to be resolved, i.e., what are the pathological triggers for these events in non-experimental seizure models? In other words, we have limited understanding of the events that trigger BBB dysfunction, leukocyte adhesion and extravasation of seizure-promoting molecules or ions in human pathology. Several possible scenarios exist: First, inflammation may take a center stage and lead to activation of white blood cells, hyperadhesion to the endothelium and subsequent BBB dysfunction. Second, hemodynamic changes such as hypoperfusion and venous stasis caused by the epileptic process\textsuperscript{13} may increase the likelihood of leukocyte-endothelial interactions, leading to BBB dysfunction without a frank inflammatory response. Third, the epileptic brain may be prone to inflammatory mediator overexpression (reviewed in ref. \textsuperscript{43}, which include IL-1β and TNF-α.

The clinical and therapeutic implications of these findings are potentially significant. Over the past three decades, dexamethasone has been widely used in the clinical management of patients with brain tumors because of its rapid (ranging from minutes to a few hours), often dramatic clinical effects on symptoms associated with intracranial neoplasms\textsuperscript{44}. If BBB dysfunction has a major etiological significance in epilepsy, then treatment with steroids should
be pursued also in patients where steroids are not the first line of treatment (e.g. West and Rasmussen’s syndrome\(^5\)). Rodent data have shown an unprecedented efficacy of immunosuppression or immunomodulation in the pilocarpine model of epilepsy\(^39,\ 40\). A preliminary clinical study has shown that dexamethasone is a powerful suppressant of drug resistant status epilepticus in patients who do not belong to the traditional category for steroidal treatment (inflammation, fever, etc.)\(^40,\ 46\).

3) What are the barriers to progress in the topic area? What are your recommendations for key steps to develop and advance knowledge in the topic area?

The BBB is a highly interactive structure, engaging with multiple neighbor cell partners including astrocytes, pericytes, neurons and blood cells like leucocytes. At the same time, BBB endothelial cells are extremely flat with a thickness of less than 0.5 \(\mu\)m outside the nuclear region\(^37\), comparing in size to dendritic spines - the core synaptic structures that use local restricted calcium signaling to control long-term potentiation\(^48\). In fact, the endfeet-endothelial interface strongly resembles the synaptic structure - except for the basal lamina not present in central synapses but present at the neuro-muscular junction. Cytoplasmic calcium is an important determinant of BBB function, adjoined by a large network of various other signals and kinases acting downstream\(^49\). Investigating calcium as well as non-calcium related signaling in BBB endothelial cells is therefore important but also puts up a major challenge because of the restricted size, the many cellular partners present and above all, the necessity to investigate the BBB \textit{in vivo}. In degree of complexity this compares to investigations on tripartite synapses but work at the BBB interface necessitates in addition an intact circulation, so as to preserve interactions with blood cell partners and maintain flow-related shear stress\(^50\). Only recently it has become possible to investigate calcium signals in single dendrite spines using \textit{in vivo} two-photon imaging of the exposed cortex of rodents\(^48,\ 51\). It is anticipated that a similar approach to the field of BBB signaling may bring up excitingly new information on the functioning of this unexploited area. In this respect, the development of GFP mice/rat with endothelial and specific markers of BBB functioning is a major challenge to allow further progress.

Next to the molecular and mechanistic aspects of NVU signaling, additional barriers related to brain disease, its diagnosis and treatment need special consideration. At first, a clear relationship between the effects of BBB disruption -more appropriately called BBB dysfunction- and acute or chronic brain disease is lacking, in spite of sporadic reports supporting this hypothesis (e.g. ref. \(^52\)). First, the assessment of BBB function is still a reductionist matter with a binary outcome, the barrier being intact/closed or permeable/open. Second, assessing the \textit{in vivo} BBB integrity is a complex, multistep procedure that requires radiologic (contrast CT or MRI), serologic (e.g. S100-\(\beta\)\(^53\)), or histologic (Evans Blue, FITC-albumin\(^38\)) assays. This is a significant obstacle to broader clinical and also experimental studies. Third, in stroke, difficulties in clinically establishing BBB function are the basis of our limited knowledge on the temporal, spatial and cellular profile of BBB responses during and after such insult. Improved insights into this matter may open up new avenues to define opportunities and time windows for anti-oxidant, MMP antagonistic and neuroprotective interventions. Fourth, the role, if any, that white blood cell extravasation plays in post-BBB disruption pathology is currently unclear\(^54,\ 55\).

4) What are your recommendations for resources that are needed to advance the field?

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Today, a large part of our knowledge on the BBB endothelium is based on observations obtained in *in vitro* systems. Methodological approaches like two-photon imaging are now available to focus on small structures such as synapses or endfeet-endothelial interfaces in the exposed brain of living animals. There is however a strong need for the development of new probes that report endothelial cell function and signaling, mainly because the classical ester-loaded reporters (mostly calcium reporter dyes) are rapidly cleared out of the cells by exchange or active transporter systems. Major efforts should be invested in developing new markers of cell function specifically designed to be taken up selectively by (lumenal) transporters or receptors on BBB endothelial cells and designing transgenic animals expressing fluorescent reporters under control of promoters specific to BBB endothelial cells. Development of new markers is equally important for astrocytes, for example to monitor potassium activity so as to better understand the dynamics of spatial buffering and clearance of potassium at the BBB. While specific targeting of probes is important, the dynamic range of the new probes, especially protein-based (FRET) probes (like cameleon calcium dyes) and GCaM-based indicators will be equally important in order to allow detection of spatially localized low amplitude signals.

Equally important as the probes are the animal models used. There is a strong need to establish consortia in order to develop cell-specific mouse/rat knockouts and to come up with a list of ‘representative’ disease models for major CNS disorders such as Alzheimer, Parkinson & Huntington disease, amyotrophic lateral sclerosis, stroke and vascular dementia. The use of zebrafish as an accessible model for mechanistic *in vivo* studies should also be further explored. These models can then be approached with optical technologies to systematically assess the role of BBB/NVU function. In the discovery process directed at unraveling cellular and molecular signaling it is important to direct the focus on events that can be either measured or approximated in a clinically meaningful setting. For example, a side-by-side comparison between laboratory data and MRI findings may be necessary. This latter technique has been promptly assimilated to the scientists’ armamentarium also because of the development of new generation of contrast and cell tracking agents (e.g. ref. ). Availability and concentration of dedicated imaging facilities is an important issue here because this will allow to use, compare and interprete data from various imaging approaches including multi-photon, nanospect and microPET imaging in laboratory animals, ideally combined with electrophysiological measurements as well.

The issue of interaction of circulating white blood cells with the BBB and its consequent translation to altered function of the NVU and BBB alike, urgently needs further elucidation by a concerted action of basic, preclinical and clinical research. Combining the cellular and molecular aspects of NVU/BBB signaling described above with the important insight that signaling can also originate from the lumenal side brings us to propose the following event sequence (Fig. 2): activated white blood cells interact with endothelial adhesion molecules (ICAM-1, VCAM-1, E-selectin), initiating intra-endothelial signaling that alters BBB function. This results in the entry of potassium and albumin the brain interstitium, turning the tissue into a seizure-prone medium. Activated astrocytes pick-up the increased neuronal activity and transmit signals toward the BBB that promote the expression of adhesion molecules, thereby facilitating its interactions with leucocytes and turning the sequence into a vicious circle that maintains and exacerbates the pathological state.
Figure 2. Proposed signaling cascade in the extended NVU. The sequence order is based on data available from the epilepsy field but may be further explored in the context of other brain diseases as well, including stroke and Alzheimer disease. Astrocyte-endothelial signaling may be bi-directional, with signals from the BBB contributing to glial/neuronal dysfunction and cell death.

The continuation of such an aberrant loop, by astrocyte-to-endothelial cell signaling, may contribute to the active role that has been proposed for astrocytes in epileptogenesis\textsuperscript{62-64}. Many additional links may join the picture, for example, intra-endothelial signaling may directly stimulate adhesion molecule expression, endothelial cell to astrocyte signaling may add its part, and enhanced astrocytic calcium signals may lead to neuronal excitotoxic cell death\textsuperscript{6} further worsening the situation. The proposed cascade involves issues thoroughly studied in the context of Multiple Sclerosis – in the context of seizures, the entry point is pilocarpine (based on the recent data presented in ref. 39) that triggers inflammation and leucocyte activation, initiating the cascade leading to seizures. Interestingly, stroke is also associated with an inflammatory component and with BBB dysfunction, making it worth to consider the proposed event sequence in this particular condition as well. Finally, microglial cells form an integral part of this scheme, contributing to dysfunction of the NVU/BBB but also delivering neuroprotective influence as well, as recently demonstrated in axonal injury models, Parkinson’s disease and amyotrophic lateral sclerosis\textsuperscript{65}. Collaborative efforts of neurocentric and gliocentric scientist alike, together with immunologists and cellular/molecular biologist will be indispensable in better understanding the concerted signaling and interaction of all cellular players involved in this complex picture.
References


SECTION 3

Title: Transport Biology in the Brain and Brain Barriers Working Group Report

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Introduction

The blood-brain barrier is a highly dynamic capillary endothelial interface between blood and brain that controls what goes in and comes out of the CNS. The blood-cerebrospinal fluid barrier resides within the choroid plexus epithelium. For both, influx transporters facilitate uptake of ions, amino acids, glucose, and other nutrients from the blood to meet the high nutrient and energy demand of the brain. Efflux transporters on the other hand extrude metabolic wastes into the blood, and form a selective, active barrier, protecting the CNS by limiting xenobiotics, including toxins and a large number of drugs, from entering the brain. In the brain capillary endothelium, tight junctions that seal the spaces between neighboring endothelial cells represent a passive barrier that restricts paracellular diffusion from blood to brain. Thus, brain barriers are complex and fine-tuned transport machines that balance influx of nutrients and efflux of wastes, toxins, and drugs to maintain CNS homeostasis. The transporter proteins of this transport machinery are the subject of the present report. An appendix on basic transport processes is provided for context and background.

Status of the science and key scientific advances made in the past 5 years

Major scientific advances have been made in the brain barriers transport biology field in the past 5 years. Key advances include the discovery and localization of new blood-brain barrier transporters, the identification of several signaling pathways that regulate transporters, the finding that several brain barrier transporters play an important role in disease, and the development of new models and technologies.

Discovery and localization of new transporters

Recent studies show that 10-15% of all blood-brain barrier proteins are transporters, and in 2003 it was estimated that only about 50% of blood-brain barrier transporters had been identified (Enerson and Drewes, 2006; Pardridge, 2003). Since then, several new transporters have been detected and localized in both blood-brain barrier and choroid plexus (blood-CSF barrier, Table 1). These include active (ATP-driven) transporters of the ABC (ATP-binding cassette) transporter families A, B, C, and E, and facilitated transporters of the SLC (solute carrier) family such as organic anion transporters (Oats), organic anion transporting polypeptides (Oatps), organic cation transporters (Octs), and monocarboxylic acid transporters (Mct). In addition, RLIP76, an ATP hydrolyzing non-ABC transporter, has been found at the blood-brain barrier and two receptors, RAGE and LRP, that are critical in Aβ transport across the brain capillary endothelium were detected. For details on new transporter proteins and their localization in the brain barriers see Table 1. In the neuroscience field, new discoveries include structural insights into coupled transporters, e.g. GAT/NET, EAAT, and ABC transporters. These findings improve understanding of the physiological and biophysical mechanisms underlying transport function and are largely discussed in the context of single neuron function (Bridges and Esslinger, 2005; Bunch et al., 2009; Gonzalez and Robinson, 2004; Torres-Salazar and Fahlke, 2007). Here it should also be noted that the recently launched website of the Allen Brain Atlas (www.brain-map.org) provides a vast amount of in situ hybridization data at the tissue (not cellular!) level on mRNA expression of transporters in the CNS of the developing and adult mouse brain and the human cerebral cortex. Figure 1 shows the current picture of transporters in the neurovascular unit.
Identification of signaling pathways that regulate transporters

Research aimed at identifying signaling pathways that regulate brain barrier transporters is relatively new. This area of research holds the promise of finding the molecular switches that turn on or off, regulating up- or down, transporters to improve brain protection and drug delivery. For example, turning off blood-brain barrier P-glycoprotein for a short period would provide a window-in-time during which normally non-penetrating drugs could be delivered into the brain with minimal disturbance of protective barrier function. Targeted up-regulation of LAT1 could even further increase L-DOPA brain uptake for the treatment of Parkinson’s disease. Down-regulation of P-glycoprotein and/or BCRP at the blood-brain and brain-tumor barriers could increase uptake of chemotherapeutics in tumor tissue and improve therapeutic efficacy in brain cancer. Up-regulation of efflux transporters could increase brain protection and minimize central side effects during pharmacotherapy of peripheral diseases. Finally, targeted modulation of the blood-brain barrier transporters and receptors that have been identified to play a key role in a CNS disorder could underlie a new therapeutic strategy. Consider Alzheimer’s disease where LRP and RAGE are critical in Aβ transport across the brain capillary endothelium and other transporters, potentially P-glycoprotein and BCRP, may also play a significant role. In this case, down-regulation of RAGE, up-regulation of LRP, and modulation of P-glycoprotein (and possibly BCRP and other not yet identified transporters and receptors that may be involved) could reduce Aβ brain burden.

Over the past 5 years, multiple signaling pathways that regulate brain barrier transporters have been mapped. These pathways are triggered by inflammatory mediators (Bauer et al., 2007; Hartz et al., 2004; Hartz et al., 2006, Rigor et al., 2010), oxidative stress (Hartz et al., 2008; Nwoazuzu et al., 2003), neurotransmitters (Bankstahl et al., 2008a; Bauer et al., 2008; Liu et al., 2008; Pekcec et al., 2009; Zibell et al., 2009), xenobiotic-nuclear receptor activation (AhR, ER, GR, PXR, CAR; (Bauer et al., 2004; Bauer et al., 2008; Bauer et al., 2006; Dauchy et al., 2008; Hartz et al, 2010a; Hartz et al., 2010b; Narang et al., 2008; Ott et al., 2009; Wang et al., 2010), insulin (Liu et al., 2008), HIV-1 Tat and gp120 protein (Hayashi et al., 2006; Ronaldson and Bendayan, 2006; Ronaldson and Bendayan, 2008), astrocyte-derived soluble factor(s) (Hori et al., 2004), or by yet unknown stimuli (Andrews et al., 2009). Several of the pathways share common signaling elements (TNF-α, TNF-R, NOS, PKC, NF-κB, oxidative stress), some of which are potential therapeutic targets (TNF-α, NF-κB, COX-2, GR). Figure 2 shows a summary of pathways signaling to brain barrier transporters that have been identified in the past 5 years.

The pathways outlined in Figure 2 are likely to also prove relevant for the neuroscience field because most CNS disorders are accompanied by inflammation, oxidative stress, and neurotransmitter release; these are all signals that trigger changes in brain barrier transporters. HIV-1 Tat and gp120 protein effects on transporters could impact brain HIV therapy, and HIV protease inhibitor treatment could activate nuclear receptors (PXR) that regulate transporters in the brain barriers and possibly impair treatment over the longer term (Perloff et al., 2007; Zastre et al., 2009). The recent discoveries about ABC transporter regulation in the blood-brain barrier will also be beneficial for studies in other cell types of the neurovascular unit since a number of ABC and SLC transporters have been detected in astrocytes, microglia, and neurons (Figure 1; (Dahlin et al., 2009; Dallas et al., 2006).
On the other hand, advances in the neuroscience field on astrocyte and neuron signaling and especially intercellular communication will certainly benefit brain barriers transport biology. These advances will help us to understand the interplay between cells of the neurovascular unit during development, in health and disease, and may both raise and answer questions concerning the role of intercellular communication in transporter regulation. In addition, the discovery of new transporters in cells of the neurovascular unit and the identification of signaling pathways that regulate these transporters may answer the question about a secondary barrier, or possibly even a tertiary barrier, behind the primary endothelial barrier (Dallas et al., 2004; Dallas et al., 2003; Ronaldson et al., 2004).

**Role of brain barrier transporters in disease**

Over the past 5 years, brain barrier transporters have been found to play a role in major CNS diseases. In addition to LRP and RAGE, several studies report involvement of blood-brain barrier P-glycoprotein and possibly BCRP in Alzheimer’s disease and cerebral amyloid angiopathy. Some of the findings suggest that in addition to LRP, P-glycoprotein could also play an important role in clearing Aβ from the brain, which provides new areas for transporter research (Cirrito et al., 2005; Hartz et al., 2010c; Kuhnke et al., 2007; Tai et al., 2009; Vogelgesang et al., 2004; Xiong et al., 2009). AD research could significantly benefit from these findings given that they provide new insights into disease etiology and potentially new therapeutic strategies.

In brain cancer, ABC drug efflux transporters have been known for some time to underlie multi-drug resistance at the levels of the blood-brain barrier and brain tumor cells themselves. An emerging concept suggests that one of the principal protection mechanisms of cancer stem cells is drug efflux mediated by ABC transporters. For example, P-glycoprotein, Mrp1 and BCRP are overexpressed in cancer stem cells (Dean, 2009). This is particularly important given that, after surgical removal of the primary tumor, chemotherapy is the treatment of choice to remove cancer cell and stem cell remnants. This suggests that brain cancer stem cells that overexpress ABC transporters will not respond to chemotherapy but will be able to regenerate into larger and more aggressive tumors, which poses a tremendous challenge. Moreover, new evidence shows that BCRP and P-glycoprotein work in concert at the blood-brain barrier and possibly also in brain tumors (Breedveld et al., 2006; Chen et al., 2009; de Vries et al., 2007; Polli et al., 2009). This novel concept initiated the design of new transporter inhibitors that block two or more efflux transporters. These dual inhibitors block P-gp and Mrp1 (CBT-1; (Robey et al., 2008) or P-gp and BCRP (nilotinib and sunitinib; (Shukla et al., 2009; Tiwari et al., 2009). One such inhibitor, JAI-51, that blocks blood-brain barrier P-gp and BCRP has been shown to delay glioblastoma growth in mice (Boumendjel et al., 2009). Another new type of transporter inhibitors is NSC73306 that inhibits BCRP and kills P-gp-overexpressing cancer cells (Wu et al., 2007). It remains to be seen whether any of these novel drugs will be effective in brain cancer chemotherapy. In addition to this strategy, the targeting of signaling pathways that regulate efflux transporters at the blood-brain and blood-tumor barriers and in brain cancer stem cells could provide an interesting approach.

Several blood-brain barrier drug efflux transporters including P-glycoprotein, MRPs, BCRP, MVP, and RLIP76 are also up-regulated in epilepsy and appear to work in concert to
contribute to antiepileptic drug resistance (Awasthi et al., 2005; Dombrowski et al., 2001; Lazarowski et al., 2007; Sisodiya and Bates, 2006; van Vliet et al., 2005). Although the complete role of most of these transporters in epilepsy is still not fully understood, studies indicate that P-glycoprotein could be involved in seizure generation (Marchi et al., 2004) and could at least in part be responsible for antiepileptic drug resistance (Loscher and Potschka, 2005). Indeed, recent studies show that seizures induce up-regulation of blood-brain barrier P-gp through a glutamate signaling pathway involving NMDAR, COX-2 and EP1; all of these provide potential therapeutic targets that could reduce seizure-induced antiepileptic drug resistance (Bankstahl et al., 2008a; Bauer et al., 2008; Pekcec et al., 2009; Zibell et al., 2009).

Finally, studies suggest that the blood-brain barrier leptin transporter plays an important role in obesity (Banks, 2008; Banks et al., 2004). Leptin is part of a negative feedback loop between adipose tissue and the brain, and the leptin transporter in the brain capillary endothelium is part of this feedback loop. However, in obesity leptin movement across the blood-brain barrier is impaired due to a failure of the leptin transporter, causing leptin resistance. This suggests communication between the CNS and the periphery, and this concept should be considered in future research on brain barrier transporters.

Development of new models and technologies

Currently used models in the brain barriers transport biology field include in vitro brain capillary endothelial cell cultures, ex vivo isolated capillaries and brain slices, several in vivo models (e.g. brain perfusion), as well as novel models (e.g. Drosophila melanogaster; Culot et al., 2008; Hartz et al., 2010c; Mayer et al., 2009; Parepally et al., 2006). Currently used methods mostly include standard biochemistry techniques (e.g. Western blotting, immunohistochemistry, immunoprecipitation, ELISA), physiology methods to measure transport and determine transport activity (e.g. uptake and transwell assays, membrane vesicles, isolated brain capillary assays, brain perfusion, brain uptake index), various imaging techniques (e.g. confocal microscopy, PET), and more specialized techniques such as brain microdialysis.

A major strength of the neuroscience field is the development of new and innovative molecular techniques (e.g. RNA interference) and animal models (e.g. animal disease models for brain tumors, AD, PD, epilepsy, etc) including constitutive and conditional (tissue- and cell-specific) knockout models, constitutive knockin models, transgenic (humanized) models, and comparative models that facilitate genetic and genomic studies (e.g. Drosophila melanogaster). The brain barriers transporter field will potentially greatly benefit from these advances.

Among the technologies that have been developed in the last 5 years are molecular imaging approaches such as the eXplore Optix system used to measure BCRP-mediated transport of fluorescence-labeled Aβ (Xiong et al., 2009). Several groups have used [11C]verapamil for PET imaging of P-glycoprotein function at the blood-brain barrier of rodents, non-human primates, and humans to study drug-resistant epilepsy and to predict drug-drug interactions (Hsiao et al., 2008; Langer et al., 2007; Lee et al., 2006; Luurtsema et al., 2005). In the neuroscience field, two-photon in vivo imaging is used to monitor events in intact animals (Tian et al., 2006) and represents a unique approach to studying blood-brain barrier transporters in live animals (Takano et al., 2007). More technologies for imaging the blood-brain barrier are subject of the report “Imaging the Brain and Brain Barriers: Structure, Function, and Dynamics”.

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Other new technologies that will potentially be important for brain barriers transport biology include approaches to construct the membrane proteome. In this regard, Agarwal and Shusta (2009) recently used multiplex expression cloning, a novel and innovative technology for conducting moderate throughput membrane protein analyses of the blood-brain barrier. An approach to determine absolute expression of blood-brain barrier membrane transporter proteins was developed by (Kamiie et al., 2008) using highly sensitive simultaneous LC/MS/MS tandem analysis. These technologies for analyzing the protein and transporter composition of the blood-brain barrier will help identify new transport proteins and may potentially be useful in mapping the “brain barriers and CNS transportomes”.

New drug delivery technologies have also been developed including several genetically engineered fusion proteins consisting of a peptide drug that is fused with a barrier-permeable receptor ligand or peptidomimetic monoclonal antibody, which enters the brain via receptor-mediated transport (Pardridge, 2008). Angiochem/Regina et al. (2008) developed ANG1005 to shuttle paclitaxel into the brain. ANG1005 is a conjugate of 3 paclitaxel molecules and Angiopep-2, a rationally designed 19 amino acid peptide that targets LRP in the brain capillary endothelium. Thus far, both approaches have shown potential but it remains to be seen how far they will go. Drugs conjugated to cell-penetrating peptides represent another new technology for passive brain drug delivery (Foerg and Merkle, 2008; Herve et al., 2008). However, cell-penetrating peptides lack specificity for brain-targeted transport, which may limit their application. Advances have also been made with various other delivery systems (e.g. liposomes, microspheres, nanoparticles, nanogels, and bionanocapsules) that have been used to enhance drug delivery to the brain (Koziara et al., 2004; Patel et al., 2009). Whether these approaches will actually be able to deliver sufficient drug for a physiological or therapeutic response in the clinic remains to be seen.

Scientific opportunities in the neuroscience field that may be applied to the brain barriers field and vice versa

Scientific opportunities in the neuroscience field that have the potential to benefit research in the brain barriers transporter field include new molecular techniques and methods, new animal disease and genetic models, and novel findings on cells of the neurovascular unit.

One new resource is the Allen Brain Atlas (www.brain-map.org) that provides a large amount of information on mRNA expression and localization of transporters (and other genes) at the tissue level in the entire mouse brain and the human cerebral cortex. Using in situ hybridization data from the Allen Brain Atlas, (Dahlin et al., 2009) recently constructed a brain expression map for mouse Slc (solute carrier) genes that includes the blood-brain and blood-CSF barriers (choroid plexus). Thus, the brain barriers transporter field and the whole brain barriers field could benefit from this resource.

One of the new molecular techniques that has been used in the neuroscience field is RNA interference (Hommel et al., 2003). Although this technique has been applied in the brain barriers transporter field (Campbell et al., 2008; Zhang et al., 2003), there is potential for use in several areas of transporter research. For example, RNA interference could help unravel
signaling pathways and the role transporters play in CNS disease. This technique could also potentially be used as a therapeutic strategy. New imaging methods including in vivo two-photon imaging and PET, that have been developed and used in the neuroscience field could help in advancing brain barriers transporter research (Newberg and Alavi, 2005; Tian et al., 2006). Combining in vivo two-photon imaging with an animal disease or genetic model could prove to be a very unique and powerful approach to answer important questions in the transporter field. Given that blood-brain barrier transporters and receptors may be involved in AD and depression, behavioral testing should also be combined with approaches to modulate transporters in animal models of these diseases. This has the potential to further provide physiological and behavioral evidence for transporter involvement in diseases such as AD and depression.

Recent research findings made in the neuroscience field should also be applied in the brain barriers transporter field. For example, findings on the role astrocytes play in glutamate release and its effect on hypersynchronous firing during epileptic seizures (Tian et al., 2005). This could help explain transporter up-regulation at the blood-brain barrier and how cells of the neurovascular unit communicate. A second example from the neuroscience field is the discovery of brain cancer stem cells (Singh et al., 2003) that overexpress drug efflux transporters. These transporters render multi-drug resistance to stem cells and prevent effective and successful chemotherapy after surgical removal of a primary tumor.

On the other hand, research on blood-brain barrier transporters provides opportunities for the neuroscience field. Experience gained with modulators of efflux transporters may be useful for delivering not only chemotherapeutics into tumors and brain cancer stem cells, but also for shuttling drugs into the brain for other CNS disorders (Bankstahl et al., 2008b; Lagas et al., 2009). Similarly, current knowledge on brain barriers transporter regulation through inflammatory mediators, oxidative stress, and neurotransmitters may prove to be useful in multiple CNS diseases (Miller et al., 2008). Such knowledge may also help to answer the question of whether transporters in astrocytes and other cells of the neurovascular unit present a secondary barrier behind the primary endothelial barrier. In addition to research in the brain barriers transporter field, understanding the molecular regulation of tight junctions may provide therapeutic opportunities in diseases where barrier integrity is disrupted (Hawkins and Davis, 2005). Such knowledge could also be used to specifically modulate tight junctions for barrier opening over a short period of time to increase passive paracellular transport of therapeutic drugs into the brain, a procedure that is now used experimentally in the clinic (Angelov et al., 2009).

**Barriers to progress in the topic area**

Barriers to progress in the CNS transport field are divided into scientific barriers and infrastructural/logistical/systemic barriers. *Scientific barriers to progress* include that the “brain barriers and CNS transportomes” have not yet been fully described. In addition, the exact subcellular location of many of the transporters that are already known to be expressed in the brain and brain barriers is still unclear. For example, while it is accepted that P-glycoprotein is largely expressed in the luminal membrane of the capillary endothelium, the extent and function of intracellular and abluminal transporter is still unclear (Bendayan et al., 2006). At the
blood-CSF barrier there is even more uncertainty with regard to P-glycoprotein localization and function (Rao et al., 1999). Transporters of the MRP-family provide other examples where their exact localization at the blood-brain barrier is still not clear (Nies et al., 2004; Roberts et al., 2008; Zhang et al., 2004). The discussion of whether a transporter is expressed in a specific cell and where it is localized also should be expanded to include astrocytes, other cells of the neurovascular unit, and choroid plexus. Remembering that localization implies function will be most critical when transporters will be considered in the context of CNS therapy. Indeed, it is important for us to recognize that these answers may also be species-, model-, age-, and potentially method/technique-dependent. Two additional barriers to progress that also impact these particular issues are a lack of specific antibodies for most of the brain barrier transporters and a lack of affordable high resolution in vivo imaging technologies needed to pinpoint transporter localization. Although high resolution structured illumination microscopes (HR-SIM) and photoactivated localization microscopes (PALM) have recently become commercially available, such microscopes are expensive and it will take a long time before these technologies will be readily available in research laboratories. Finally, the brain barriers transporter field needs to move towards more mechanistic, disease-related, and translational research using more relevant in vivo and animal disease models in conjunction with appropriate and optimized in vitro cell culture models.

Infrastructural/logistical/systemic barriers to progress include the low visibility of the blood-brain barrier field in the larger neuroscience area. This may be related to the fact that the cerebral vasculature is often not considered to be “neuro”. Low visibility limits opportunities for interaction between neuroscience researchers and brain barrier researchers. As a consequence, it is often difficult to publish reports from the brain barriers transporter field in higher impact neuroscience journals. One has the sense that brain barriers researchers cannot call any journal “home” to their research areas.

Another barrier to progress is the lack of true brain barriers research centers. This may be a consequence of the small size of the field, but it clearly affects the development and exchange of new ideas as well as the supply of young scientists (pre/post-doctoral level) who have basic understanding of transporter physiology and are interested in brain barriers transporter research. We need to establish a pipe-line through which innovative and enthusiastic young scientists can be trained and encouraged to stay in the field.

Recommendations

Historically, the goal of the brain barriers transport biology field has been to increase brain uptake of CNS drugs by facilitating drug transport across the blood-brain barrier through passive diffusion, by targeting influx transporters, or by blocking efflux transporters. Key findings in recent years suggest new roles for transporters. These are reflected in the overall recommendations for the brain barriers transport biology field:

Advance knowledge of transporter expression, localization and regulation to target transporters in the neurovascular unit for CNS drug delivery, brain protection, and prevention of CNS disease.
Key steps to develop and advance knowledge in the brain barriers transport field

- Identify new transporters using recently available technologies
- Localize transporters with high resolution imaging techniques
- Unravel transporter regulation within the NVU in development, health, and disease
- Study molecular mechanism to identify therapeutic targets
- Combine molecular approaches with whole animal approaches
- Use knockout animal models to study mechanism and disease models to understand pathophysiological changes
- Find answers to the following research questions:
  - Do transporters work in concert?
  - Do transporters regulate other transporters/other proteins?
  - Does metabolism drive transport in the brain barriers?
  - What is the role of transporters during blood-brain barrier opening in disease?
  - Does communication between CNS and periphery affect brain barrier transporters or are brain barrier transporters involved in this communication?

Recommendations to advance the brain barriers transport field

- Propose generation of transporter-specific antibodies to neuromab.org (NIH-subsidized)
- Support advances in high resolution imaging as well as acquisition of such equipment
- Establish a negative results and a brain barriers methods database
- Generate a database for brain barriers-specific genes with matching KO and transgenic mice
- Generate a database of patients with altered brain barrier function
- Agree on standard methodological procedures (“Good BBB Research Practice”)
- Bring together researchers from different disciplines to increase interactions
  - Invite researchers from other fields to present at brain barrier meetings
  - Invite clinicians to create contacts for translational brain barriers research
- Establish 2-3 brain barrier research centers in the U.S.
  - Enhance collaborative research
  - Foster communication and the exchange of ideas
  - Connect with other disciplines and clinics
- Promote brain barriers transporter field to increase visibility
  - Organize symposia at major meetings of other research fields
  - Hold joint meetings with researchers of other fields on topics of common interest
- Enhance recruitment, training, mentoring, and involvement of young researchers

Perspectives
Based on what brain barriers transporter researchers have found in the past years and based on the field’s status quo, the future (5-year perspective) of the brain barriers transporter biology field revolves around the following 5 points:

1) Understanding transporter regulation under normal, physiological conditions.

2) Understanding transporter changes and regulation in disease.

3) Understanding the role of the NVU in brain barriers transporter regulation.

4) Developing methods, techniques, and protocols for human application.

5) Translation into the clinic.
References


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**Table 1: Recently detected and localized transporters in the brain barriers.**

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Gene</th>
<th>Type</th>
<th>Localization</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mrp1</td>
<td>ABCC1</td>
<td>active</td>
<td>BBB: luminal, abluminal CP: basolateral Neurons</td>
<td>human, rat, mouse</td>
<td>Nies et al., 2004; van Vliet et al., 2005; Sugiyama et al., 2003</td>
</tr>
<tr>
<td>Mrp2</td>
<td>ABCC2</td>
<td>active</td>
<td>BBB: luminal</td>
<td>human, rat, mouse</td>
<td>Kubota et al., 2006; Bauer et al., 2008</td>
</tr>
<tr>
<td>Mrp4</td>
<td>ABCC4</td>
<td>active</td>
<td>BBB: luminal, abluminal CP: basolateral</td>
<td>human, bovine, rat, mouse</td>
<td>Nies et al., 2004; Leggas et al., 2004; Zhang et al., 2004; Roberts et al., 2008</td>
</tr>
<tr>
<td>Mrp5</td>
<td>ABCC5</td>
<td>active</td>
<td>BBB: luminal</td>
<td>human, bovine, rat</td>
<td>Nies et al., 2004; Zhang et al., 2004; Dazert et al., 2006</td>
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<tr>
<td>RLIP76</td>
<td>RALBP1</td>
<td>active</td>
<td>BBB: luminal</td>
<td>human, mouse</td>
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</tr>
<tr>
<td>LRP</td>
<td>LRP1</td>
<td>endocytosis</td>
<td>BBB: abluminal Neurons</td>
<td>mouse</td>
<td>Deane et al., 2004</td>
</tr>
<tr>
<td>RAGE</td>
<td>AGER</td>
<td>endocytosis</td>
<td>BBB: luminal Neurons</td>
<td>mouse</td>
<td>Deane et al., 2003; Deane et al., 2004</td>
</tr>
<tr>
<td>MCT8</td>
<td>SLC16A2</td>
<td>facilitated</td>
<td>BBB: luminal, abluminal</td>
<td>human, rat, mouse</td>
<td>Roberts et al., 2008</td>
</tr>
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<td>SLC22A8</td>
<td>facilitated</td>
<td>BBB: abluminal Neurons</td>
<td>rat, mouse</td>
<td>Mori et al., 2004; Ohtsuki et al., 2004; Roberts et al., 2008</td>
</tr>
<tr>
<td>Oatp1a4</td>
<td>SLCO1A4</td>
<td>facilitated</td>
<td>BBB: abluminal</td>
<td>Rat</td>
<td>Roberts et al., 2008</td>
</tr>
<tr>
<td>Oatp1c1</td>
<td>SLCO1C1</td>
<td>facilitated</td>
<td>BBB: luminal, abluminal</td>
<td>rat, mouse</td>
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<td>Taut</td>
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<td>BBB</td>
<td>mouse</td>
<td>Kamiie et al., 2008</td>
</tr>
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Figure 1: Current picture of the primary transporters in the neurovascular unit (only transporters that have been detected at the protein level are included).

(Modified with permission from Hartz and Bauer, 2010, and Hartz and Bauer, 2011)
**Figure 2:** Signaling pathways that regulate transporters in the neurovascular unit.
APPENDIX

This section provides a brief overview of the basic principles and language of biological transport with special reference to brain barrier tissues.

Every process involving movement of a solute across a cell membrane or a cell layer, either via a transcellular or a paracellular route, is considered transport. Such processes can be passive (simple diffusion), facilitated (carrier-mediated), or active (directly ATP-driven). The driving force behind simple diffusion is a concentration gradient along which a solute moves through the cell membrane of the paracellular space; solute movement can go in either direction depending on the direction of the gradient. With a few exceptions, essentially all currently used CNS drugs cross the blood-brain barrier into the brain via passive diffusion. This leaves little room for therapeutic manipulation other than changing dose level and dosing frequency.

Facilitated diffusion of a solute requires transport proteins (facilitated or carrier-mediated transport) which accelerate movement through membranes. True facilitated diffusion is still passive, with solute moving down its concentration gradient. All mediated transport processes exhibit both saturation at high substrate concentrations and competition by structurally similar chemicals. At the blood-brain barrier, many facilitative transport proteins are nutrient transporters that mediate influx of their endogenous substrate down its concentration gradient (e.g., GLUT-1 and glucose). A special class of facilitative transporters utilizes gradients established by ATPases to indirectly drive solute uphill transport (against a concentration gradient). For example, nutrient uptake transporters can achieve high levels of cellular substrate accumulation through coupled uptake with Na⁺. The energy needed for this process is supplied by the Na-gradient that is generated by the Na⁺/K⁺-ATPase (secondary active transport). Facilitative transporters can be uniporters (transport of one solute in one direction), symporters (co-transport of two solutes in the same direction), or antiporters (counter-transport in opposite directions). Prominent examples of facilitative transporters at the blood-brain and blood-CSF barriers are members of the solute carrier (SLC) family including glucose transporters (GLUT), amino acid transporters, monocarboxylic acid transporters (MCT), organic anion transporters (OAT), organic anion transporting polypeptides (OATP), and organic cation transporters (OCT).

Active transport of a solute is directly ATP-driven (primary active transport) and can, therefore, be directed against a solute’s concentration gradient. Since ATP hydrolysis takes place inside the cell, primary active transport is always outwards-directed efflux transport. Examples for primary active transporters at the blood-brain barrier include the ATP-binding cassette (ABC) transporters P-glycoprotein (P-gp, ABCB1), multidrug resistance proteins 1, 2, 4, and 5 (MRP, ABCC-family), and breast cancer resistance protein (BCRP, ABCG2). These transporters limit xenobiotics including toxins and a large number of drugs from entering the brain, and thus, can cause drug resistance.
Section 4

**Title:** Neurodevelopment and the brain barriers

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Introduction

The barriers in the developing brain establish a specialized environment for growth and maturation, as well as acting as an important interface with the rest of the body. This report will be based on three hypotheses that seek to integrate knowledge about brain barrier mechanisms into more general concepts of how the brain develops and may be affected by adverse intrauterine and postnatal circumstances. These hypotheses are:

I. That the presence of the blood-brain barrier from the earliest stages is an essential component of normal brain development.

II. That perturbation of the fetal blood-brain barrier during pregnancy or in the postnatal period may contribute to neonatal and adult disease by altering fundamental steps of brain development.

III. Developmental principles aid understanding the mature CNS, particularly in the context of repair following insult.

The fundamental hypotheses outlined above can be broken down into specific predictions that form the basis of our understanding of brain development and the role of the cerebral vasculature in developmental processes in health and disease.

I. The presence of barrier mechanisms provides the brain with a specialised internal environment. Specific features of this internal environment, not yet fully understood, are likely to be essential for normal brain development.
   a. The morphological basis of the barrier mechanisms are tight junctions present in the brain barrier interfaces from the earliest stages of development.
   b. The surrounding neural environment influences the characteristics of the endothelial cells that establish the brain barrier mechanisms.
   c. The molecular structure and specific function of the brain barriers vary relative to their roles in brain development.
   d. The vasculature has a complex interaction with the neural environment, including shared molecular mechanisms, which influence the growth and maturation of the developing brain.
   e. The behaviour of the neurovascular unit and neurovascular coupling will be dependent on the stage of brain development.

II. Although effective brain barrier mechanisms are established from the earliest stages of brain development, some of these mechanisms may render the developing brain more vulnerable to pathological (including toxicological) events during pregnancy or in the postnatal period. This is in addition to an intrinsic vulnerability of the brain due to sensitivity of complex developmental processes such as neurogenesis, migration and cell differentiation.

III. Developmental principles may aid understanding of the mature CNS, particularly in the context of repair following insult. However, it is not yet clear to what extent repair in the adult does recapitulate developmental programs. One example of where this appears to be the case is given below. It is not clear how general this
recapitulation is; it may be that the local brain environment is so different in the adult that the processes involved are not always similar.

1) **What are the scientific opportunities in the neuroscience field that may be applied to the brain barriers field and vice versa?**

Substantial studies have been carried out on angiogenesis in the central nervous system in recent years, including the human fetal brain. In addition, research on vascular, immune and neuronal development has been converging over the last few years. There are three major reasons for this: (i) evidence for shared molecules and mechanisms during the development of these systems. (ii) interactions between blood vessels and neurogenetic compartments in embryonic and adult brains and (iii) embryonic and perinatal brain injuries require understanding of both fields. Moreover, understanding of these interactions might hold the key to establishing the causal links between environmental interactions and genetic susceptibility in the aetiology of complex cognitive disorders (autism, schizophrenia, ADHD). An additional consideration lies in the similarity of some immune and neural molecular mechanisms during development.

The importance of interactions between the nervous, immune and vascular systems is being increasingly recognised, therefore this report includes a brief outline of the present status of angiogenesis in the developing brain, *However, the remit of this report principally covers properties of barrier interfaces in the developing brain and their relation to brain development. Although this requires in vivo studies of these mechanisms in relation to specific features of brain development, this has rarely been undertaken.* It may be that the concept of “the blood-brain barrier”, which currently describes exchange mechanisms across the blood-brain interfaces (Figure 1) should be extended to include reciprocal interactions between blood vessels and nervous tissue. In any event, information from studies of angiogenesis and the molecular mechanisms of interactions between the nervous, immune and vascular systems provides important background for the design of studies of the interaction between brain barrier mechanisms and nervous system development.

**Angiogenesis:** During normal development, the growth of cerebral vessels takes place by angiogenesis (Conway *et al* 2001; Gerhardt and Betsholtz 2003). In the classical model of angiogenesis, a temporally regulated sequence of events includes: detachment of vascular pericytes from the vessel wall, vascular basement membrane degradation, endothelial cell migration (sprouting) on a transitory extracellular matrix, endothelial cell proliferation, interendothelial junction re-assembling, vascular lumen formation and finally, recruitment of pericytes (Folkman, 1971; Bergers and Benjamin, 2003; Rundhaug, 2005; Tam and Watts, 2010). It has been recently suggested that alternative modes of cerebral angiogenesis exist during normal brain vascularization, thus angiogenesis in pathological conditions may also be diversely executed.

Detailed analysis of the angiogenic process during normal human brain vascularization reveals that an initial sprouting of pericytes, rather than of endothelial cells, provides a set of pioneering cells forming vascular cords (Virgintino *et al* 2007a; Virgintino *et al* 2008). These results, primarily based on the analysis ‘in situ’ by high resolution confocal microscopy of distribution and cell-cell/cell-matrix relationships of microvessel pericytes expressing the transmembrane
proteoglycan NG2, led to the hypothesis that at least a subset of newly formed vascular sprouts may arise from NG2+ pericytic seamless cords that become unsheathed by a collagen-IV-enriched basal lamina. These then guide migrating endothelial cells from the
parental vessel, where they proliferate, inwards to target sites of vascular anastomosis and develop lumina (Virgintino et al 2007a; Virgintino et al 2008). The angiogenically activated endothelial cells of the forming microvessels are guided by an intimate interplay with immature NG2+ pericytes. They exhibit an heterogeneous phenotype characterized by the expression of growth factor receptors (e.g., PDGFR-β) and basement-membrane degrading enzymes (e.g., metalloproteinase-2) together with features of the blood-brain barrier, such as glucose transporter protein type-1 (GLUT-1) and claudin-5 (Virgintino et al 2007a, 2008). NG2 null mice showed aberrant vascularisation of syngeneic tumours, thus reinforcing the importance of NG2+ pericytes in normal vascularisation of the brain (Huang et al 2010). The role of pericytes in normal vasculogenesis and in pathological conditions in the adult has been recently reviewed by Bronkowski et al (2011).

During human corticogenesis, immunolocalization and detailed confocal microscopic analysis of blood-brain barrier and angiogenesis-specific markers reveal that a constant, exuberant ‘phenotype remodelling’ takes place in the cell components of the neurovascular unit (NVU). Tight junction-associated transmembrane proteins, such as occludin and claudin-5, are already expressed by endothelial cells at 12 weeks of gestation in the human and at mid gestation a typical junctional. A linear pattern is observed together with the presence of blood-brain barrier-specific transporters, such as GLUT-1 and P-glycoprotein (Virgintino et al 2004, 2007b, 2008). In rodents, claudin-5 is present in cerebral endothelial cells at least as early as E18.5 (Nitta et al., 2003) and GLUT-1 is expressed and present even earlier at E11 (Bauer et al 1995) as is the efflux protein MDR1α (P-glycoprotein) at E10.5 (Qin and Sato, 1995). At this time, radial vessel branching and intense vascular sprouting characterize the process of cortex vascularisation.

**Shared molecules in vascular and neuronal development:** The global anatomical similarities between the vascular and nervous systems have long been recognized but are just recently leading to research on the interface between the two systems. Recently, it has been discovered that developing blood vessels and neurons share similar cellular and molecular mechanisms. This is highlighted by the discovery of numerous molecules previously associated with neuronal specification and axon guidance as being involved in the guidance and patterning of blood vessels (Carmeliet and Tessier-Lavigne, 2005; Vasudevan et al 2008; Vates et al 2005). Thus, shared processes and signalling mechanisms for maturation are leading to further examination on the common mechanisms and processes shared during development (Tam and Watts, 2010).

**Interactions between blood vessels and neurogenic compartments:** Recent studies suggest not only that the vasculature can provide guidance cues but also exert effects on, for example, mitosis in surrounding cells at the vascular branch points. Co-culture experiments by Shen and colleagues suggest that this mechanism may be mediated via soluble growth factors released from endothelial cells maintaining neural stem cells in a proliferative state (Shen et al 2004). A
supportive neurovascular niche has been described in the subventricular zone during adult neurogenesis (Riquelme et al 2008). Recent work from the Kriegstein and Molnár laboratories showed that a similar “vascular niche” for neural intermediate progenitor cells exists during development (Javaherian and Kriegstein, 2009; Stubbs et al 2009; Nie et al 2010). In addition, endothelial cells also provide neuronal survival signals (Dugas et al 2008).

**Shared molecular mechanisms in developing neural and immune systems:** Recent evidence suggests that neural development and immune development share similar molecular mechanisms (Filipovic and Zecvic, 2008). Several molecules have been shown to regulate both immunological and neural development. The presence of the blood-brain barrier during development may provide the crucial separation of immune and neural compartments to allow for differential regulation of these molecules in each compartment. For instance, endogenous thrombospondins regulate synapse formation and MHC molecules and complement proteins regulate synapse elimination (Christopherson et al 2005, Huh et al 2000, Stevens et al 2007, Boulanger et al 2001). However, the inappropriate presence of these factors from the periphery could result in altered synapse formation and pruning during development. Thus, the consequences of a defective blood-brain barrier during brain development may be far reaching with regards to causing dis-equilibrium of key regulators in neural network maturation. As one example, the chemokine CXCL1 influences the development of oligodendrocyte progenitors (Filipovic and Zecvic, 2008), however, when inappropriately expressed can lead to damage of CNS components including the blood-brain barrier (Anthony et al 1998). It is now accepted that T-cells constantly monitor the CNS and it is speculated that dendritic cells along the vascular wall may serve as the antigen-presenting cell. Thus the ability of the brain barrier systems to segregate and regulate the movement of these and other molecules between the blood and the brain is a critical component of brain development that requires further investigation. Moreover, there is ample evidence that neural activity plays a role in the development of the brain (Katz and Shatz, 1996), and thus the role of the blood-brain barrier in clamping CNS ion concentrations to allow for proper neuronal function to orchestrate this development appears likely. Although the experimental evidence available is limited, the question can be raised whether a developmental brain barrier dysfunction causes improper circuit formation, which in turn may lead to altered neurobehavioral functioning?

**Embryonic and perinatal brain injuries:** Both epidemiological and experimental studies have identified maternal infection (Leviton et al 2005; Meyer and Feldon 2009; Boksa, 2010), fetal brain ischemia-hypoxia (Hagberg et al 2002), or hypoxia (Gerstner et al 2008) as potential causes of neonatal brain injury resulting in white matter tract damage. Only recently has the possibility of involvement of blood-brain barrier dysfunction in systemic inflammatory effects on white matter been studied (Stolp et al 2005a, b, 2007). The list of genetic susceptibility genes for complex cognitive disorders (autism, schizophrenia, ADHD) is rapidly increasing, but it is also becoming evident that environmental interactions and genetic susceptibility are BOTH required and are the driving force behind the pathology (Harrison, 2007). The link between the cerebral vasculature and neurogenesis, together with the vulnerability of the blood-brain barrier during early stages of brain development, might hold the key to these interactions. Insults such as inflammation may cause major alterations in neural and vascular development leading to a number of neurodevelopmental disorders, such as autism and cerebral palsy, or an increased susceptibility to adult neurological disorders such as schizophrenia, Alzheimer’s disease, Parkinson’s disease and multiple sclerosis (Stolp & Dziegielewska, 2009; Stolp et al 2011).
2) What is the status of the science in the topic area, including key scientific advances made in the past 5 years? Are these advances relevant to the other field?

_Hypothesis I_. The presence of the blood-brain barrier is an essential component of normal brain development from the earliest stages.

**Blood-brain interface.** The evidence for this hypothesis comes from a strong body of work conducted over the last 40 years using traditional physiological measures. As a result we know that ion gradients between the cerebrospinal fluid (CSF) and the blood are established early in development and may change during development (Bito and Myers, 1970; Bradbury et al 1972; Amtorp and Sorensen, 1974). We also know that many of the influx transport mechanisms across the barriers are present and functional early in brain development (e.g. glucose, amino acids), and may even, in some cases, be more active than in the adult (Partridge and Meitus, 1982; Vannucci et al 1994; Bauer et al 1995). The establishment of ion gradients and the presence of influx transporters suggest that a functional barrier (tight junctions) is present in the developing brain.

This is confirmed by the observations that as early as E12 in the mouse, cerebral endothelial cells express tight junction related characteristics such as claudin 5, occludin and zonula occludens proteins (Daneman _et al_ 2010a). Before the neural tissue is vascularized in the early embryo, neuroepithelial cells fulfil the task of creating a homeostatic environment in neural tissues. Membrane specialisations are also seen between the cells at the ventricular borders of the intraneural domains. (Bauer _et al_ in prep). These intercellular junctions are sometimes described as tight junctions (e.g. Götz & Huttner, 2005); however, the literature cited does not appear to support this. Thus Manabe _et al_ (2002) specifically state that the ventricular zone does not contain tight junctions. It seems likely that these junctions are in fact “strap” junctions (Møllgård _et al_ 1987). A combined ultrastructural (especially freeze fracture) and molecular characterisation of these junctions is long overdue.

In the last 5 years, unequivocal evidence of both structural and functional barriers in the developing brain has been put forward. Use of new, small molecular weight markers has shown that functionally effective tight junctions are present as soon as blood vessels begin to

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**Figure 2.** Cartoon depicting the relationship between the cellular and local environment of the brain and the vascular environment separated by the blood-brain barrier (BBB).
penetrate the early CNS parenchyma and as soon as epithelial cells begin to differentiate (Ek et al 2006; Johansson et al 2008) providing the basis for selective barrier interfaces. Efflux transporters (e.g. P-glycoprotein, MRPs, BCRP) that can reduce the accumulation of drugs and toxins in the brain environment are expressed in cerebral endothelial and choroid plexus epithelial cells early in development (Kalabis et al 2007; Gazzin et al 2008; Ek et al 2010). This has shown that the efflux transporters are different at these two interfaces indicating that their individual roles to protect the brain are not the same (Gazzin et al 2008; Ek et al 2010). Further work is required to show whether these transporters are also functional at these early stages of brain development. An aspect of cerebral endothelial cell function in the developing brain that has been neglected is the possible role of transendothelial transport mechanisms such as receptor-mediated transcytosis. A key distinguishing feature of adult cerebral endothelial cells has for long been held to be a lack of pinocytotic vesicles; however, this was based on studies using horseradish peroxidase, which is taken up by fluid phase endocytosis which may be stimulated by horseradish peroxidase; ultrastructural studies of cerebral endothelial cells in young and adult brains not exposed to horseradish peroxidase have revealed numerous vesicular profiles (Møllgård et al 1988). Further studies will be required to determine whether there are developmental differences in these subcellular transport-related features of cerebral endothelial cells.

Recent key advances have also come from the application of molecular techniques. Three groups (Liebner et al 2008; Stenman et al 2008; Daneman et al 2009) have recently shown that Wnt/β-catenin signaling drives cerebral (but not non cerebral) angiogenesis and that cerebral angiogenesis and blood-brain barrier formation are inextricably linked (e.g. expression of the blood-brain barrier-specific glucose transporter GLUT-1, Daneman et al 2009). The Wnt ligands are expressed by cells in the ventricular zone of the developing CNS, identifying neural stem cells and neural progenitor cells as inducers of blood-brain barrier properties (Daneman et al 2009).

A combination of experiments using microarray data and pericyte deficient knockout mice has established the role of pericytes in influencing blood-brain barrier properties, in the developing brain (Daneman et al 2010a). It has been known for sometime that pericytes can induce barrier properties, but, until recently, technical limitations have hampered the study of pericyte function. The newly identified haemostatic regulatory role of human brain pericytes in the embryonic period raises a number of important questions relating to the development of the brain microvasculature and the neurovascular unit. For example, embryonic pericyte dysfunction, rather than astrocyte dysfunction, alters the integrity of the blood-brain barrier in adulthood (Armulik et al 2010; Daneman et al 2010a) but effects on neural development have yet to be determined. Lack of pericytes leads to

Figure from Stubbs et al. (2009). E14.5 sections show Tbr2 reactive presumed intermediate progenitors (red). Tbr2 immunoreactivity has been the most intensive along a band through cortical SVZ. Blood vessels are stained with IB4 (green) and mitotic cells (phospho-Histone H3 immunoreactive, appear yellow). Quantitative analysis demonstrated that blood vessels are closer to Tbr2 reactive mitotic profiles than chance would suggest.
endothelial hyperplasia and abnormal vascular morphogenesis in the brain (Hellstrom et al 2001; Armulik et al 2010; Daneman et al 2010a). The dominance of pericytes in development might be expected to profoundly affect the neurovascular unit and neurovascular coupling.

We now have the opportunity to study the neurovascular coupling by fMRI in utero, providing insights into critical age-related changes. Regional variations in the control of cerebral blood flow occur during neuronal activity, however, to date, no one has systemically investigated age-related changes in the fMRI signal. We hypothesize that pericytes may assume the role of astrocytes in the neurovascular coupling during development, but it is entirely unclear whether the mechanisms for controlling flow (mGluR5- and PG-dependent) are present during development.

To understand how the CNS regulates blood-brain barrier development and function, it is crucial to understand which aspects of the blood-brain barrier are regulated chronically by maintenance signals, which aspects are induced at a single time point during development, or which aspects show a developmental progression. Endothelial cells removed from the CNS still express a variety of blood-brain barrier specific genes including occludin, Pgp and GLUT-1; however, these isolated cells fail to form high electrical resistance barriers. Thus it appears that some signals are required chronically for maintenance of the high resistance barriers, while other signals are only required once during development to induce blood-brain barrier specific gene expression. A model consistent with the data is that neural stem/progenitor cells induce blood-brain barrier specific genes during angiogenesis, and astrocyte/pericyte signals influence the function of the barrier after vessels are formed. The identification of neural stem cell derived Wnt and other factors that regulate blood-brain barrier formation and function will be very important for the development of methods to repair the blood-brain barrier during neurological insults.

Wnt/β-catenin experiments (Liebner et al 2008; Stenman et al 2008; Daneman et al 2009) suggest that neural stem cells may play an important role in inducing barrier properties as the cerebral vasculature forms. The marked failure of brain growth following blocking of Wnt/β-catenin signaling has been suggested to indicate a dependence of brain growth on signals from endothelial cells that is complementary to the earlier established dependence of cerebral endothelial characteristics on interaction with neural stem cells (see Daneman et al 2009). The neural stem/progenitor cell-expressed Wnt acts as a migration factor, calling endothelial cells into the CNS (Daneman et al 2009). Thus the same signals that bring vascular cells into the CNS also induce barrier properties and situate the vessels in progenitor rich areas of the developing nervous system.

Further support for the importance of these interactions has come from recent work by the groups of Molnar and of Kriegstein, who have independently shown a novel relationship between neural progenitor cells and developing endothelial cells in the embryonic cortex (Javaherian and Kriegstein, 2009; Stubbs et al 2009; Nie et al 2010). The studies go further and investigate the relationship of the Tbr2 positive intermediate cortical progenitors and the developing blood vessels that defines a vascular neurogenic niche in the embryonic subventricular zone. Additional studies are included on early interactions between vasculature and newly born neurons. The Tbr2 positive intermediate progenitor neurons have a key role in
the generation and differentiation of cortical neurons (in particular for the differentiation of the upper layers, Arnold et al 2008).

The structural and functional modulation of the brain barrier mechanisms during development, and the role of these in brain function have been under increasing focus in the past 5 years. Developmental and region specific timetables of tight junction protein expression have been identified in the human brain (Virgintino et al 2004; Anstrom et al 2007). The choroid plexus specifically transfers proteins into the CSF in an age specific manner (Liddelow et al 2009), which is likely to play a role in ventricular expansion (Johansson et al 2008; Liddelow et al 2010) and may also be a source of neurotrophic factors for ventricular zone neurogenesis. Fgf signalling is known to be of crucial importance for neural development, and Fgf2 has been shown to be one of the important players in modulating cortical development. Expression of Fgf2 is detected early in choroid plexus development both in rodents and humans, and FGFR expression in the choroid plexus is developmentally regulated (Reid and Ferretti, 2003, Greenwood et al 2008). The possible role of Fgf-mediated cross talk between the developing neuroepithelium and the choroid plexus begs investigation. Furthermore, a role for Fgf 2 and 5 in regulating the blood-brain barrier in vivo has also been suggested (Reuss et al 2003).

The very recent paper of Daneman et al (2010b) provides comprehensive data on gene expression in isolated cerebral endothelial cells and pericytes from adult and postnatal (P2-8) mice, with comparisons with endothelial cells from adult lung and liver. This provides essential background for better understanding of brain barrier properties and function both in the adult and developing brain. However, these studies need to be extended to the embryonic period both for the blood-brain barrier and blood-CSF barrier (choroid plexus).

More research is required to determine the consequence of stage-specific changes in barrier structure and function for regulation of brain development. New MRI technology, recently explored (Preece et al 2009) will allow functional magnetic resonance imaging of small animals, with which the relationship between neurovascular coupling and barrier function can be assessed in the developing brain.

**Blood-CSF interface.** The choroid plexuses are sites of the other major brain-barrier interface, the blood-cerebrospinal fluid (CSF) barrier that contributes to regulation of the internal environment of the brain. The blood-CSF barrier appears to be especially important during development as the choroid plexuses are functional and restrict paracellular passage at a time in development when the brain parenchyma has low levels of vascularity (Johansson et al 2008). Despite the potentially great importance of the choroid plexuses in normal brain development, relatively little is known about their specification and regulation of functional aspects such as barrier formation and fluid secretion. It has been suggested that the choroid plexuses play a role in both the nutrition of the brain as well as cortical development as a consequence of the expansive pressure generated by the secreted fluid and trophic factors present in CSF that can regulate cortical progenitor proliferation and differentiation (Mashayekhi et al 2002; Martin et al 2006; Johansson et al 2008). Recent studies have shown the importance of transeellular transport of macromolecules across the choroid plexus, which in the developing brain appears to be specific for some proteins in plasma that are transported into CSF; they or their ligands may then be taken up into the developing brain (Liddelow et al 2009, 2010).
In regards to the specification and differentiation of the choroid plexus from the roof plate, recent work has shown the importance of the bone morphogenetic proteins (Furuta et al 1997; Hebert et al 2002) and the potential involvement of the transcription factor E2F5 in the regulation of epithelial cell maturation (Swetloff and Ferretti, 2005). In mutant mice, in which the dorsal midline invaginates, but the choroid plexus does not form (Hebert et al 2002; Yoshida et al 2006; Imayoshi et al 2008) the impact on cortical development has not been investigated. However, in a hem deficient mouse in which the choroid plexus is virtually absent, the cerebral ventricles appear smaller compared to wild-type yet no difference is seen in cortical layering at embryonic day 12.5 (Yoshida et al 2006). In the queue courte rat, which has a mutation in the Lmx1 locus, the choroid plexus is hypoplastic, the ventricles are almost absent, and the brain is disorganised, although this mutation is not specifically localised to the telencephalic dorsal midline (Kuwamura et al 2005).

Defective cortical development and a reduction in the size of the choroid plexus has been recently reported in mouse mutants such as the tumor suppressor gene Pml−/− mouse (Regad et al 2009) and the Zic1/Zic3 compound mutant mouse (Inoue et al 2007) where cortical defects seem largely due to abnormal growth of neural progenitors. It has been suggested that in the Zic1/3 mouse reduced secretory activity might contribute to the cortical phenotype. In these models, reduction in choroid plexus size is likely to be due to defective neuroepithelial cell growth/differentiation, though reduced secretion by the hypoplastic choroid plexus could, in turn, affect cortical development. Given our limited understanding of choroid plexus development, it cannot be ruled out that, in some cases, reduced choroid plexus size may be the primary defect leading to cortical abnormalities. The tumour suppressor ASPP2 co-localizes with some components of the apical junctional complex in choroid plexus epithelium. In mutants, the junctional complexes are disrupted. In addition, in this mutant there are direct effects on neurogenesis, which appear to be mediated by disruption of the intercellular junctions in the proliferative ventricular zone, resulting in extensive morphological abnormalities and hydrocephalus (Sottocornola et al 2010).

Functionally effective CSF secretion could not occur without a blood-CSF barrier as the gradients of proteins and ions, between plasma and CSF that drive fluid secretion could not occur in the absence of a tight epithelium. Further studies, focused on the impact of the choroid plexuses on brain development are needed.

Hypothesis II. That perturbation of the fetal blood-brain barrier during pregnancy or in the postnatal period contributes to adult disease by altering fundamental steps of brain development.

Antenatal environmental stressors can alter the activity of the adult immune system and decrease adult neurogenesis (Lemaire et al 2000; Koo et al 2003; Kippin et al 2004; Mirescu et al 2004; Karten et al 2005). Importantly, these antenatal effects on the activity of the adult immune system can be elicited by the administration of LPS or IL-1 antenatally or neonatally, which mimicks infection or injury during pregnancy (Reul et al 1994; Shanks et al 1995; del Rey et al 1996; Shanks, 2000; Hodgson et al 2001; Ellis et al 2005); autonomic nervous system aspects of these immune responses are reviewed in Karrow (2006). These early periods of life are regarded as vulnerable periods where elements of the immune system, the HPA axis for example, can be fine-tuned (Reul et al 1994; Hagberg and Mallard, 2005; Karrow, 2006).
Inflammatory insults in the developing brain result in specific changes in the permeability of the blood-brain barrier (Stolp et al 2005a). The increased permeability occurs primarily in the white matter and results in delayed myelination of these tracts (Stolp et al 2005a, 2005b, 2009). Long-term consequences of the early inflammatory response include an increased permeability to small molecules (Stolp et al 2005b) and a number of behavioural changes (Shi et al 2003; Meyer et al 2006; Pang et al 2006; Meyer and Feldon, 2009; Boksa, 2010; Stolp et al 2011). Additional studies have suggested that injury-induced neurogenesis in the immature and adolescent brain is dependent upon an inflammatory response. Whether this involves changes in the blood-brain barrier for infiltration of systemic factors or is dependent upon resident immune cells is not known; however, it does demonstrate a critical regulatory component during different stages of development. The sensitivity of the developing brain to inflammatory insults reiterates the critical importance of an intact blood-brain barrier in preventing blood-borne inflammatory factors from penetrating the brain parenchyma. Long-term or latent changes following a maternal inflammatory response have been identified by the finding of a diminished level of neurogenesis in adult offspring following antenatal administration of LPS to dams (Pitossi and Anthony personal communication). Similarly, antenatal LPS administered to dams resulted in reduced and abnormal neurogenesis in their embryos (Stolp et al submitted). No data are available on the mechanisms underlying this effect, but the administration of LPS and IL-1 in the antenatal period is known to affect the integrity of the fetal blood-brain barrier. It is now important to discover the relationship between antenatal barrier dysfunction, fetal neurogenesis, and long-term changes in the neurogenic capability in adult offspring. There is no doubt that early structural changes during development (for example neurogenesis or vascularisation) or long-lived epigenetic changes that are induced by barrier dysfunction in utero may have profound implications for the development of neurological disorders in adulthood. At present, there is very little information available on this important issue, but recent technological advances have provided the ability to investigate the functional consequences of perinatal barrier dysfunction on the development of adult disease or in senescence of barrier function that will enable Hypothesis II to be tackled systematically. As described above, interactions between the vasculature and neural components of the brain have important consequences for embryonic and perinatal brain injuries and may contribute to long term or latent changes in brain function. Considering the interrelated development of the vasculature and blood-brain barrier mechanisms with the neural component of the brain it is likely that effects of damage to these barrier mechanisms on changes in brain development are more widespread than is currently understood.

Understanding the dynamics and importance of brain barrier mechanisms for brain development and long-term adverse events is critical for assessing the contribution of barrier disruption by early exposure. The general belief in the “immaturity” of the blood-brain barrier has led to an assumption that the developing brain is differentially sensitive to chemical or drug exposure due to a greater permeability of the immature blood-brain barrier. Alternatively, if a chemical or drug causes a disruption to the blood-brain barrier, it is assumed that greater exposure to the brain would occur. These assumptions have been established prior to data available to test such assumptions. With regards to therapeutic interventions, the lack of available data leads to the recommendation to minimize drug exposure during pregnancy. However, this itself can be harmful depending on the nature of the illness and the impact of loss of therapeutic intervention as would occur with serious medical conditions such as epilepsy, diabetes or hypertension. Testing for adverse effects during pregnancy is not a regulatory
requirement for drugs. It has been estimated that data on adverse effects for around 90% of drugs administered to pregnant women are not available (Lo and Friedman, 2002). Virgintino et al (2007b) suggest that it is time to re-evaluate the prevailing dogma (e.g. Ginsberg et al 2004; Pollard, 2007) that fetal brains are more vulnerable to neurotoxic effects because their barrier mechanisms are absent or not fully functional during development. There is currently a body of data that can be used to more accurately reflect the human health risk involved thus allowing for greater guidance to physicians and patients, although more systematic research is required.

Environmental toxicants may show a different pattern; however, it is still critical that the most accurate and up to date data be employed for any risk assessment. With regards to heavy metals that continue to be a problem with developmental exposure and may expand given the use of metals as plastic stabilizers and the release of such compounds in recycling efforts, understanding the impact on barrier mechanisms in the developing brain is likely to be of critical importance for formulating appropriate risk assessments. There is evidence that some normal inward transfer mechanisms are more active in the fetus than in the adult and since some (amino acid carriers) are known to be carriers for toxic metals such as mercury (particularly in the form of methyl mercury) and lead, this may mean that an exposed fetus is more vulnerable because of a normally functioning mechanism (Saunders et al 2010). Several metals bind to plasma proteins that are transported across the choroid plexus epithelial cells by what appears to be a protein-specific mechanism in the fetus (Liddelow et al 2009). The slow turnover of CSF results in a high concentration of proteins in CSF in the developing brain (Johansson et al 2008), which would be expected to increase the exposure of the fetal brain to metals or other toxins bound to these proteins. Some heavy metals (e.g. mercury, Chang and Hartmann, 1972; lead, Moorhouse et al 1988) may directly damage the blood-brain barrier in the developing brain. Some of the potentially protective efflux mechanisms appear to be present in the immature brain, but the extent to which they are functionally active seems not to have been much studied (see Saunders et al 2010 for review). This is a research field that is much in need of detailed studies using modern methods, as much of the literature is from an earlier era.

**Hypothesis III. Developmental principles may aid understanding of the mature CNS, particularly in the context of repair following insult.**

Barrier dysfunction in neurological disease is well documented (see review by Neuwelt et al 2007). As more emphasis is placed on discovering molecules that regulate barrier development, understanding the role of these newly discovered molecules in disease becomes paramount. For example, it would be worth investigating how Wnt/β-catenin signalling is regulated in circumstances of barrier dysfunction, such as in stroke or neurodegenerative disease. Furthermore, it has been reported that capillary density is reduced in the aged brain (Abernethy et al 1993; Uspenskaia et al 2004). An accompanying decrease in cell density might be expected with increasing age given the reduction in the number of vessels, but this is not the case. The previous studies suggesting that there is neuronal loss with aging have now been shown to be incorrect; there is a difference in tissue volume but not in actual neuronal number. However, the relative proportions of individual cell populations within any specific brain region may be altered, which may result in a reduction in the energy demands of the aged brain. Fluorodeoxyglucose uptake, a marker of the level of brain energy metabolism, is reduced in aged mice compared with the mature animals (Uecker et al., 2000). It remains unclear whether the loss of vessels precedes these functional deficits or is a consequence of them, but this is an issue that requires investigation. Cerebrovascular small vessel disease (SVD), which is the first
step in the development of vascular cognitive impairment and cerebrovascular occlusions, is increasing in incidence in our aging population. SVD results in low blood flow and metabolic insufficiency in the brain.

As has been considered for many components of the nervous system, regeneration after injury has been thought to recapitulate development. While this may not be as direct an association as previously thought, understanding the dynamics that permit the development of a system may provide a greater understanding of the mechanisms that could be employed to promote successful regeneration of vascular network recovery. This could represent the induction of a specific cell-signaling event, involvement of other cells within the brain, reversal of senescence of the system, or a silencing of an inhibitory signal. It is an imperative that we understand the processes that lead to the loss of vessel density in the ageing brain and how revascularization of the brain might be encouraged. To fit with this assumption, revascularization after ischemia seems to recapitulate developmental processes. Bone marrow-derived cells (BMDC) are known to contribute to revascularization after experimental brain ischemia, but, until recently, the fate of these cells was unknown. It has now become clear that the majority of the BMDC are pericytes, which seem to stabilize vessels in a manner that is very reminiscent of angiogenesis during development (Kokovay et al 2006).

3) What are the barriers to progress in the topic area? What are your recommendations for key steps to develop and advance knowledge in the topic area?

There still remains a perception in the field of blood-brain barrier physiology, and therefore in the wider area of neurobiology, that the barrier systems in the developing brain are immature in structure and function. The result of this is that many developmentally variant observations are ascribed uncritically and without evidence to "incomplete" formation of the blood-brain barrier, thereby failing to fully understand the biological process under investigation (for a recent example see Foust et al 2009). This leads to confusion in the field that distracts from progress. A different obstacle to progress in this area is a combination of the very small number of laboratories worldwide studying the development of barrier mechanisms, the lack of knowledge of current data on the developing barrier systems, the lack of understanding of the complex interaction between vasculature and neurogenesis in the developing brain as well as the neurological basis of behaviour during development. Neuropathology resulting from combinations of genetics and perinatal insult is likely to be subtle, with small changes in cell signalling and neurotransmitter release, superimposed on subtle malformations of structure and connectivity in a number of brain regions. In depth, collaborative research by experts in a number of neuroscience fields will be required to identify the pathological changes responsible for behavioural abnormalities frequently identified in animal models of human disease. The recent evidence in support of barrier function in the developing brain, along with the introduction of some exciting new scientists in the field, will help to overcome both of these particular barriers to progress. In addition, systematic studies of the functional effectiveness of brain barrier efflux mechanisms are required to understand the extent to which the brain is vulnerable to exposure to drugs and toxins. This should lead to a regulatory framework to provide evidence for the rational use of drugs in pregnant women and their offspring.

4) What are your recommendations for resources that are needed to advance the field?

- Specific calls for funding this particular area (EU, Wellcome Trust, NIH).
• Improved animal models appropriate for studies of relation between barrier mechanisms and brain development with application of modern techniques (e.g. imaging, targeted gene deletion).
• Development of clinically relevant animal models.
• Develop screening methods for BBB function on mutants derived from mutagenesis programs.
• Support for clinical-preclinical collaborative studies.
• Training opportunities for young researchers.
• Practical courses for new researchers in the field to learn correctly how to use currently available techniques.
• Joint sessions with developmental neurobiologists at international meetings (FENS, SFN).
• Encouragement of workshops with regulatory agencies (FDA, EPA, National Toxicology Program) regarding the actual data on the developing brain barrier systems and how the database needs to be expanded.
References


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SECTION 5

Title: Imaging the Brain and Brain Barriers: Structure, Function, and Dynamics
Working Group Report

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Introduction

A generally accepted definition of imaging is the visual representation of an object for the purpose of medical diagnosis or data collection, using any of a variety of imaging techniques. For the purpose of this report, imaging has been considered a spectrum of enabling technologies essential to answering pivotal scientific questions related to the brain and blood-brain barrier (BBB) function in space and time. Therefore, the topic area covers a wide spectrum of technologies and approaches that span from a nanoscale molecular imaging of cells to functional MRI imaging in patients. The brain is a highly complex organ, both anatomically and physiologically, requiring an impressive arsenal of technological tools to study it. From landmark studies by Ehrlisch and Goldman, the use of dyes, a precursor to cellular imaging, has played a critical role in the understanding of how the BBB interacts with cells of the central nervous system (CNS) and how it adapts or is broken down in the presence of pathology. Modern advances in brain imaging – ex vivo and in vivo – offer the opportunity to elucidate the integrated molecular and functional complexity of cells, tissues, organs, and even whole bodies both in health and in disease. Imaging techniques are now capable not only of visualizing chemically diverse biologically active species, dispersed and compartmentalized in discrete regions of tissues, but also of measuring the dynamics of their appearance and activity. In recent years there have been unparalleled methodological advances in imaging of brain activity from measuring neuronal activity (e.g., membrane potential, ion and neurotransmitter fluxes), energy metabolism (glucose and oxygen consumption) and functional hyperemia (blood flow, volume, oxygenation) to detecting changes in specific molecules within diseased brain. Imaging technologies are important in aiding the fundamental understanding of the brain and neurovascular function and are among key enablers of discovery translation into clinical applications.

Status of the science and key scientific advances made in the past 5 years

The last several years have seen a rapid progress in developing imaging techniques for pre-clinical and clinical applications. These advances have provided an unprecedented insight into the cellular and molecular interactions at the BBB and are expected to facilitate biomarker validation in animal models of disease. Some of these techniques have already been used for examining BBB disruption, therapeutic brain delivery and molecular imaging of brain vessels.

Imaging of genes, molecules and signaling networks

Molecular imaging of brain cells in culture and brain tissues using optical techniques has advanced immensely with developments of confocal- and time-lapse microscopies and other techniques that can simultaneously tag/visualize multiple molecular targets (Raj et al., 2008). Imaging techniques at the atomic level and “label-less” techniques such as Raman spectroscopy have also been developed (Freudiger et al., 2008). Imaging mass spectroscopy (IMS) permits the direct analysis and determination of the distribution of multiple molecules in tissue sections. With this technique, tissues are analyzed intact and thus spatial localization of molecules within a tissue is preserved (Seeley and Caprioli, 2008; Figure 1). IMS is becoming a powerful method for imaging tissue (bio)markers that define particular regions and for following ‘biomarker’ responses to disease, pharmacological treatment, electrical stimulation, etc. (Seeley and
Caprioli, 2008). IMS does not require a target-specific reagent. A significant advantage of IMS is the capability to distinguish molecular species not easily achievable by other means, for example, truncated forms of beta-amyloid peptides, or drug metabolites in brain tissue. The mass spectral images obtained with IMS can be correlated with conventional magnetic resonance imaging (MRI) techniques (Sinha et al., 2008). Fluorescent murine transgenic reporter systems have gained in popularity, mostly utilizing green fluorescent protein (GFP) as a reporter. GFP has been utilized to track exogenously-added cells (i.e. tumor cells, immune cells, and progenitor cells), as well as proxy reporters for endogenous genes (i.e. transgenic mice) (Zhuo et al., 1997; Todman et al., 2005; Turney and Lichtman, 2008). Development of an array of fluorescent protein-reporter systems has enabled a multi-color recombination (‘brainbow’ strategies) in various neuronal cell types in vitro and in vivo aiding in visualization of the brain connectome (Lichtman JW et al., 2008). These approaches have enhanced greatly our understanding of the trafficking of inflammatory cells across the BBB in models of ischemic brain injury and autoimmune demyelination, among others (Priller et al., 2001). In addition, the recent introduction of optogenetic approaches (Miesenbock, 2009) will allow investigators to examine discrete neuronal signaling events in the context of the NVU, providing a degree of in vivo analytical power previously achievable only using in vitro systems.

More recently, firefly luciferase has been identified as a practical and efficient reporter in transgenic mice that eliminates the need for fluorescence excitation, penetrates deeper into tissues (2-3 cm), possesses low intrinsic backgrounds of bioluminescence, and is readily detectable with deep cooled CCD cameras, provided available ATP and administration of appropriate substrate (Massoud et al., 2007). Transgenic mice expressing luciferase under the control of the vascular endothelial growth factor receptor 2 promoter (Ryan et al., 2005) and glial fibrillar acidic protein (Luo et al., 2008) have been used to monitor vascular remodeling and astrogliosis non-invasively. The application of such strategies is in the early stages of what promises to be a powerful gene activity imaging modality, compatible with non-invasive imaging.

**Molecular imaging in vivo**

The Society for Nuclear Medicine broadly defines molecular imaging as the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living systems. Molecular imaging agents are probes used to visualize, characterize, and measure biological processes in living systems. Both endogenous molecules and exogenous probes can be molecular imaging agents.

Most molecular imaging applications for CNS diseases are developed for radioactivity-dependent PET and SPECT modalities, although the last several years have seen a rapid progress in the development of non-invasive optical molecular imaging techniques for pre-clinical applications (Abulrob et al., 2008; Figure 2). The molecular imaging agents used are typically small molecules (receptor ligands) with short circulation half-lives that readily penetrate across the BBB. PET has many clinical imaging applications; although microPET has some limitations regarding spatial resolution in experimental animal models, recently developed nanoSPECT/CT imaging systems provide a better spatial resolution to combine precise structural anatomy with molecular data. High resolution SPECT is economical, requires less radiation burden than PET and is foreseen as a potentially important technology in advancing preclinical translational research of CNS disorders and their treatments (Sharma and Ebadi, 2008).

Some of the notable advances in the molecular imaging domain in the last five years have been development and clinical testing of the Pittsburgh compound B (PiB) for PET imaging.
of Alzheimer’s disease (Villemagne et al., 2008), advances in receptor distribution/activation studies using PET ligands in various neurodegenerative and neuropsychiatric conditions (Herholtz et al., 2007; Elsinga et al., 2006), as well as application of Pgp-modifying agents to enable delivery of PET tracers into the brain (Elsinga et al., 2005). Some aspects of BBB functions have been recently estimated using 11C-Loperamide - an avid substrate for P-glycoprotein (Liow et al. 2009). Initial applications in MR-molecular imaging have been driven by the development of superparamagnetic nanoparticles, such as USPIO, that have been applied for imaging neuroinflammation (Engberink et al., 2008), satellite metastatic brain tumors (Enonch et al., 1999) and for cell tracking (Budde and Frank 2009) Various other novel nanoparticles are also being designed for example using DNA (Chisholm et al.,2009; Modi et al., 2009) or recombinant adenovirus (Singh et al., 2009)) for imaging gene expression ( Waerzeggers et al., 2009) or cell physiology. An inherent problem with non-invasive in vivo imaging techniques using targeted nano-particles (e.g., SPIO, USPIO) is the difficulty in their gaining access to target sites across cell membranes and the blood-brain barrier and their potential non-specific retention (bound, internalized or incorporated into basement membrane) within the cerebrovascular compartment. The validation and translation to the clinic of imaging such nanoparticles will require a thorough characterization of their properties including pharmacokinetics and cellular distribution in animal models.

**Dynamic and functional imaging in vivo**

Modern imaging tools allow microscopic scale visualization of changes in brain structure as well as assessment of the brain function at multiple length and timescales. Two-photon laser scanning microscopy is an ideal tool for high-resolution fluorescence imaging in intact organs of living animals (Kerr and Denk, 2008). Two- and multi-photon imaging has the advantage that time-lapse images of the same subject can be taken over even weeks apart. By re-imaging the same animal it will be possible to perform statistically powerful comparisons of paradigms designed to affect cerebrovascular and BBB function in conjunction with astrocytic and neuronal responses (Iadecola and Nedergaard, 2007; Takano et al., 2007; Brown et al., 2008; Li and Murphy, 2008). Related tools that allow targeted disruption of the BBB have been developed that may provide a unique animal model for localized BBB failure (Nishimura et al., 2006).

In the last decade, functional MRI has had a tremendous impact on enhancing our understanding of CNS processing of human cognitive and motor functions under both normal and pathophysiological conditions (Jezzard and Buxton 2006; Mathews et al., 2006). The human applications of functional MR imaging range from classification and localization of disease such as Alzheimer’s, headache or epilepsy to understanding the mechanisms of brain reorganization following brain injury or stroke (Ward 2006). Functional MRI signal intensity changes are detected as a result of alterations in neuronal activity being closely coupled to changes in local blood flow and oxygenation within the activated part of the brain (Logothetis, 2008). The opportunity exists to apply this technology as well as other cerebral perfusion based brain mapping methods (Bandettini 2009; Dolan 2008, Shibasaki 2008) to advance our understanding of neuro-glio-vascular coupling and BBB pathophysiology.

Although there are a number of methods for assessing BBB permeability in vivo, spatial resolution and sensitivity are currently lacking. *Qualitative* evaluation of BBB permeability in humans is available using non-invasive imaging modalities such as MRI and computerized tomography (CT), following the peripheral administration of non-permeable contrast agents. *Quantitative* measures of BBB permeability are also possible using blood pool contrast agents and theoretical modeling of contrast accumulation in the brain (Tofts et al. 1999; Knight et al.,
2005; Zaharchuk 2007; Tomkins et al. 2008). With the advent of high performance gradients and parallel imaging, it is now possible to use dynamic contrast-enhanced MRI techniques to quantify permeability measurements across the brain endothelium in 3D at relatively high speed and good coverage. Although quantitative detection of contrast enhancement using MRI can generally provide good spatial resolution, MRI is relatively insensitive for detecting small changes in permeability and the temporal resolution of quantitative measures is also suboptimal (e.g. seconds to minutes).

**Scientific opportunities in the neuroscience field that may be applied to the brain barriers field and vice versa**

*Advance the understanding of the biology and pathobiology of the BBB*

The advances in various microscopy techniques and the explosion of available fluorescent dyes offer the opportunity to develop more sensitive tissue-based methods for optical evaluation of BBB permeability and for quantifying blood-to-brain diffusion. This methodology may have a significant number of advantages in that there is a multitude of dyes all having different functions and or affinity for tissue and organelles that can be used to determine BBB function and BBB interactions with CNS cells. This methodology has the potential to resolve images of permeability changes to less than a micron in both tumor and normal brain (Stemmer et al., 2008). Gene reporter models utilizing GFP or luciferase that have the capacity to follow the temporal component of gene signaling networks could yield a wide range of research tools with relevance to studies of the BBB. For example, vascular and BBB remodeling can be followed in mice expressing GFP under the regulation of promoter of genes related to vascular or BBB development. A challenge for the future is to develop systems that can analyze the rapid kinetics of signaling networks (i.e. turnover). To this end, studies of circadian rhythms and deliberately de-stabilized reporters have led to important insights into the kinetics of complex signaling networks (Robertson et al., 2008), which will ultimately guide research into novel areas of BBB signaling. Dynamic analyses of neuronal-glial-vascular interactions enabled by multi-photon microscopy (Hirase, 2005) can be used to extend previous intravital microscopy studies to resolve individual capillaries and fine subcellular structures such as dendrites and their spine synapses to understand how barrier integrity affects dynamic remodeling of these structures (Zhang and Murphy, 2007; Figure 3). We anticipate that the technology will initiate advancements in understanding and assessing barrier function in areas such animal models of blood brain barrier mutations or polymorphisms, combinations of BBB integrity assays with single cell measures of function such as calcium imaging of somatosensory evoked responses (Stosiek et al., 2003) and neurovascular coupling in models of disease, particularly stroke.

Lacking in BBB research is an understanding of regional changes in permeability and their relation to altered CNS function. Thus, fMRI (a measure of neuronal activity), in addition to MR perfusion (a measure of vascular hemodynamic status) imaging, could be used to provide a combined measure of the neurovascular unit when studying BBB pathophysiology and its relationship to neuronal activation in health and disease. Recent studies in experimental animals further suggested a role for altered transport across the endothelium in direct control on astrocytic properties, neuronal excitability and plasticity (Ivens et al. 2007; Maggio et al. 2008). Diffusion tensor imaging (DTI) provides a measure of both the magnitude and direction of water diffusibility that is represented in tensor form for each voxel (Bammer et al. 2005; Mori and
Zhang 2006; Talos et al. 2006; Nucifora et al. 2007). Such information is important in that water diffusion within tissues is generally anisotropic, particularly within axons. Whether BBB permeability differs in white matter tracts or is differentially affected in white matter by disease or other conditions is not known. It is possible that DTI, again in combination with BBB permeability imaging, can be used to identify selective white matter changes. It is also possible that alterations in diffusivity or fractional anisotropy, with and without contrast, could provide more sensitive or other measures of altered permeability.

**Multimodal Imaging of the BBB**

Since no single method can cover the several orders of magnitude in temporal and spatial resolutions and at the same time capture cellular and vascular events, one of the key opportunities to be harnessed in the future is a combination and integration of data and knowledge obtained through multi-modal imaging. There are many different imaging technologies that have been or can be combined to provide novel information regarding brain function (Hyder, 2009) and BBB permeability (Tomkins et al., 2001; Figure 4). Technologies available include optical imaging (e.g. near infrared imaging), CT, MRI, PET, MEG, TMS, EEG and MEG (Otte and Halsband 2006; Shibasaki 2008; Eliassen et al. 2008; Kerr and Denk 2008; Pichler et al. 2008a). Several of these imaging systems have been engineered for use in combination and several different types of multimodal systems are available commercially. Table 1 summarizes various biological properties that can be analyzed by a combination of MRI and PET (modified from Heiss, 2008) to achieve a more complete and integrative understanding of brain/neurovascular unit function.

**BBB biomarkers for molecular imaging applications**

Brain vasculature is affected by and functionally implicated in a majority of brain diseases. Examples include cerebrovascular diseases (stroke), AD, neuroinflammatory diseases (MS), brain tumors (angiogenesis) etc. In many cases, changes in molecular ‘make-up’ (or functional properties) of brain vasculature are an early ‘sign’ of disease and could potentially be exploited as image-able biomarker for early diagnosis or monitoring disease progression. The added advantage of such biomarkers is their accessibility for targeted molecular imaging agents that do not cross the BBB. Yet, with the exception of brain tumors where angiogenic biomarkers have been targeted for molecular imaging (Iqbal et al., 2010; Hsu et al., 2007; Cai et al., 2006), cerebrovascular biomarkers have not yet been fully exploited for molecular imaging of brain diseases. Endothelial progenitor cells and cells relevant to inflammatory response and BBB function are being imaged in animals (e.g. control of monocyte diapedesis across the endothelium) (Suidan et al. 2008; Reijerkerk et al. 2008). Thus labeling cells with optical or MR contrasts to allow their imaging in humans is a research area for development to provide new insights clinically.

**Drug delivery to the CNS and approaches for molecular imaging agents**

Developments in label-free mass spectroscopy imaging techniques could be applied to analyze drug and biologics delivery into the brain without the need to modify drug or the target. These techniques could be used to simultaneously answer questions such as: Does the intended therapeutic cross the BBB? Is the therapeutic differentially distributed in different brain regions? Does the therapeutic bind to or reach intended target and what are the consequences to the target upon interacting with the therapeutic? Which metabolites/degradation products of the therapeutic are present in different brain regions? Which proteins/peptide/metabolites are

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changed in the presence of the therapeutic? etc. IMS technologies could be applied to address the above questions in a more ‘integrated’ manner, creating an opportunity for advancements in both BBB research and in the CNS drug development in general. Even though lipophilicity is often thought to enable drug entry into the brain, many lipophilic drugs show negligible brain uptake because they are substrates for transporters such as P-glycoprotein (P-gp), multidrug–resistance associated protein (MRP) and organic anion transporting polypeptides (OATPs). The action of these carrier systems results in rapid efflux of xenobiotics from the central nervous system (CNS) (Hermann and Bassetti, 2007). Classification of candidate drugs as substrates or inhibitors of such carrier proteins is of crucial importance in drug development. Positron emission tomography (PET) can play an important role in the screening process by providing in vivo information, after the putative drug has passed in vitro tests. Studies in experimental animals have indicated that it is possible to assess P-glycoprotein function in the BBB and its effect on the uptake and binding of drugs within the intact CNS, using suitable P-gp modulators labeled with positron emitters (Elsinga et al., 2005; Figure 5). Provided that radiopharmaceuticals (and P-gp modulators) can be developed for human use, several exiting fields of study may be explored, viz. (i) direct evaluation of the effect of modulators on the cerebral uptake of therapeutic drugs; (ii) assessment of mechanisms underlying drug resistance in epilepsy; (iii) examination of the role of the BBB in the pathophysiology of neurodegenerative and affective disorders; and (iv) exploration of the relationship between polymorphisms of transporter genes and the pharmacokinetics of test compounds within the CNS. In addition to Pgp modulators, some emerging receptor- and adsorptive- mediated trancytosis carriers for BBB delivery (Partridge, 2006), most of which are biologics, could be exploited to deliver imaging agents targeted to specific molecular recognition sites in the brain or for developing biologics-based imaging agents.

**Barriers to progress in the topic area**

**Technological/scientific**

- Pervasive ‘dogma’ in the neuroscience field that the BBB exists in two states – open and closed – in contrast to actual evidence that the BBB is a dynamic, controlled in time and space, and tightly coupled to and influenced by the mural cells (smooth muscle cells, pericytes) and brain parenchymal cells (neuro-glio-vascular unit).
- Lack of incentive for imaging technology to be optimized for BBB measures, as currently there is greater clinical and scientific interest in assessing neuronal function.
- Lack of knowledge of dynamic changes in BBB permeability in concert with altered astrocyte/neural function.
- Difficulty in quantifying very low and spatially constrained permeability of the BBB accurately to detect small yet potentially physiologically significant changes. Further improvement in sensitivity and resolution of imaging techniques for these measures is a never-ending challenge.
- Lack of clinically relevant and valid models for barrier disruption. A lack of understanding of how the BBB fails in humans (so that it could be well modeled). Lack of standardization of methods and models.
- Lack of knowledge of detailed (and specific) changes in BBB permeability under disease states.
- Lack of early and predictive image-able biomarkers for CNS diseases, particularly neurodegenerative diseases.
Lack of validated and clinically translatable BBB delivery technologies/approaches. *Delivery across the BBB hinders the development of both novel molecular imaging approaches and therapeutics.* The majority of emerging targeting molecules and treatments for neurodegenerative diseases are biopharmaceuticals - growth factors, genes and vaccines. The most important impediment for clinical translation of such treatments is their delivery to the brain or neurons affected by disease.

Lack of targeted contrast agents with sufficient sensitivity and specificity. Although a significant progress has been made in developing molecular imaging agents for PET/SPECT, similar agents are lacking for more accessible MRI modalities, as well as for rapidly developing, cheaper optical imaging modality.

Difficulties in integration of data and knowledge gained across imaging disciplines and modalities (micro to macro scale; fast dynamics to long prospective studies; anatomical molecular and functional; *ex vivo* with *in vivo* imaging modalities).

Advanced imaging techniques often are very costly and require very specialized training

Lack of large multicenter clinical trials to validate the power (i.e. sensitivity and specificity) of the newly developed methods and translating those into meaningful clinical tools

**Systemic:**

- Fragmentation/compartmentalization of the efforts in the CNS imaging and BBB imaging fields.
- Lack of appropriate (interdisciplinary) education and training at the interface of vascular biology, neuroscience, and multimodal imaging.
- Integration of processes and projects across diverse groups (academic, industrial, etc.) – need for multidisciplinary, large-scale science.

**Recommendations:**
The principal identified need and the recommendation of the Working Group in this field is to:

*Develop and apply integrated, quantitative imaging approaches with high spatial and dynamic resolution to understand physiological regulation of the neurovascular unit and its changes in disease states.*

**Key steps to develop and advance knowledge in the topic area**

- Enhance the understanding of the cellular and molecular biology of the BBB in health and disease through the development of novel investigative tools and through the application of existing technology to the study of the BBB
  - Pose clear question(s) that can be answered by a combination of fundamental neuroscience and imaging
  - Advance methods – such as fMRI – to build base knowledge of how normal neurovascular unit functions
  - Develop imaging methods to understand transporter function in normal BBB (cotransport involving multiple transporters, substrate specificity, polymorphism, drug influx/eflux, etc)
o Identify hits from genetic screens that can be imagable. Define (minimal) read-outs for BBB function ‘phenotype’ that can be identified using genetic or phenotypic screens (e.g., mutagenesis studies).

• Develop more standardized and clinically relevant animal models of disease and the imaging tools to appropriately study them. Animal models that mimic the various failure pathways of the BBB in humans (e.g. global, chronic vs. local, acute failure) are needed.
• Use imaging as a validation tool for diseases biomarkers/targets; couple peripheral biomarkers with imaging biomarkers
• Identify approaches that are potentially translatable
• In parallel with studies in animal models, develop translational approaches to study the human BBB in health and disease.
• Couple imaging with effective drugs
• Focus on quantitation and reproducibility
• Create training opportunities to attract young researchers to the field.

*Resources that are needed to advance the field*

• Support for the formation of multidisciplinary teams focused on developing new multimodal imaging technologies optimized for BBB research (Centers for BBB discovery).
• Support the exchange of ideas and approaches among relevant disciplines by facilitating the communication between centers studying the BBB.
• Support for funding for ‘key’ recommendations identified by the Working Group(s).
References:


Modi S, M.G. Swetha, D Goswami, GD. Gupta, S Mayor & Y Krishnan (2009) A DNA nanomachine that maps spatial and temporal pH changes inside living cells. Nature Nanotechnology Published online: 6 April 2009


Pichler BJ, Judenhofer MS, Pfannenberg C (2008a) Multimodal imaging approaches: PET/CT and PET/MRI. Handb Exp Pharmacol 109-132


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Table 1. Complementary assessment of various biological properties using MRI and PET (modified from Table 1, Heiss W-D, Eur J Nucl Med Mol Imaging, 2008).

<table>
<thead>
<tr>
<th>MRI</th>
<th>PET</th>
</tr>
</thead>
<tbody>
<tr>
<td>•Morphology</td>
<td>•Flow ($H_2\textsuperscript{15}$O)</td>
</tr>
<tr>
<td>•Water motion in time (DWI)</td>
<td>•Metabolism (FDG)</td>
</tr>
<tr>
<td>•Vascular anatomy (MRA)</td>
<td>•Blood volume (C$\textsuperscript{15}$O)</td>
</tr>
<tr>
<td>•Perfusion (PWI, DCT-MRI)</td>
<td>•Oxygen consumption ($\textsuperscript{15}$O)</td>
</tr>
<tr>
<td>•Tissue metabolites (MRS)</td>
<td>•Vascular permeability</td>
</tr>
<tr>
<td>•Functional activation (fMRI)</td>
<td>•Nucleic acid synthesis (FLT)</td>
</tr>
<tr>
<td>•Cerebral fibre tracts (DTI)</td>
<td>•Transmitters</td>
</tr>
<tr>
<td>•Oxygen consumption</td>
<td>•Receptors</td>
</tr>
<tr>
<td>•Migration of cells</td>
<td>•Enzymatic activity</td>
</tr>
<tr>
<td></td>
<td>•Angiogenesis (e.g., $\textsuperscript{18}$F-RGB)</td>
</tr>
<tr>
<td></td>
<td>•Distribution and kinetic of tracers and drugs</td>
</tr>
<tr>
<td></td>
<td>•BBB transporter activity</td>
</tr>
<tr>
<td></td>
<td>•Beta-amyloid (PIB)</td>
</tr>
<tr>
<td></td>
<td>•Cell tracking</td>
</tr>
</tbody>
</table>

DWI – Diffusion-weighted imaging
MRA – MR angiography
DCT-MRI - Dynamic contrast-enhanced MRI
PWI – Perfusion- weighted imaging
MRS – Magnetic resonance spectroscopy
DTI – Diffusion tensor imaging
Figures

Figure 1. A-D. Principles and workflows applied for Imaging Mass Spectroscopy (IMS) of serial brain sections. Left: an example of a tissue distribution of 4 different peptides in the brain sections determined by IMS. (The figure is a combination of Figure 1 and Figure 2 from Seeley and Caprioli, PNAS, 2008)
Figure 2. *In vivo* non-invasive imaging of the BBB disruption in left middle cerebral artery occlusion (MCAO, 2 h) followed by a 24 h reperfusion using time-domain near-infrared in vivo imaging and optical tomography (from Abulrob et al., Molecular Imaging, 2008).
**Figure 3.**

**In vivo imaging of plasma extravasation and loss of dendritic spines after rose bengal (RB) photoinjurious ischemia** (from Zhang and Murphy, PLOS Biol, 2007).

(a) Low-magnification two-photon image of vasculature (texas red-dextran) before and after photoactivation of RB. 24% percent of vessels were clotted over an ischemic area of ~0.05 mm².

(b) Images from the boxed region in (a) showing the vasculature (red) and YFP-labeled dendrites (green) before and 1, 4 and 5 h after photoactivation of RB. The red fluorescence in the tissue indicates extravasation (leakage of plasma) containing tennessee red-dextran.

(c) A higher-magnification view of a dendritic segment (box in b) showing no significant damage or loss of spines despite extravasation.

(d) Quantification of extravasation and spine number for this animal. Extravasation was quantified by determining the percentage of vessel texas red-dextran fluorescence intensity present in the tissue.

(e) Group data from 9 animals showing relative changes in extravasation and spine number (data aligned by the time point when the maximal rate of extravasaton was observed; set as the 0 time point) after RB stroke. We did not observe a significant change in spine number when the rate of extravasation was maximal.

(f) There was no significant correlation between the rate of spine loss, change in spine number between consecutive 1 h time points and extravasation at different times (r²=0.02, p=0.75; nonparametric Spearman correlation coefficient; same data as (e) analyzed differently).
Figure 4. Multi-modal imaging in patients. fMRI-EEG imaging of evoked visual stimulation activity responses. A) The pattern-reversal checkerboard visual stimulation, B) fMRI activation map with a corrected threshold p<0.01, and C) the global field power of VEP and the dynamic cortical source distribution at three VEP latencies (76, 112, 212 ms after the visual onset) imaged from EEG alone (1st row), or fMRI-EEG integration using our proposed adaptive wiener filter (2nd row) and the conventional 90% fMRI weighted algorithm (3rd row). Both the source images and the fMRI activation map are visualized on an inflated representation of cortical surface (from Liu Z and He B, Neurimage, 2008)
Figure 5. Micro PET images of the head biodistribution of calcium channel blocker

Micro PET images of the head of a Wistar rat, showing the biodistribution of the calcium channel blocker, \(^{11}\text{C}\) verapamil injected systemically, either alone (Control) or after pre-treatment of the animal with the Pgp inhibitor Cyclosporin A. \(^{11}\text{C}\) verapamil, a substrate for the blood-brain barrier efflux transporter P-glycoprotein, gains access to the brain only after Pgp inhibition by Cyclosporin A. Images are courtesy of Dr. P. Elsinga, University Medical Center Groningen, The Netherlands.