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# Blood–brain barrier genomics and proteomics: elucidating phenotype, identifying disease targets and enabling brain drug delivery

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**The blood–brain barrier (BBB) regulates the passage of material between the bloodstream and the brain. Recent genomic and proteomic studies of the BBB have identified some of the unique molecular characteristics of this vascular bed, and have reinforced the concept that the BBB is heavily involved in brain function. Genomic and proteomic techniques have also been used to analyze the molecular events underlying diseases that have BBB involvement, such as multiple sclerosis, Alzheimer's disease, stroke and HIV-1 infection. It is expected that a better understanding of the complex mechanisms that link the BBB to neurological disease will ultimately lead to the development of innovative treatments.**

Brain tissue is separated from the bloodstream by specialized endothelial cells (EC) that form an impermeable vasculature known as the blood–brain barrier (BBB). Because of its barrier properties, the BBB helps prevent the entry of harmful blood-borne materials into the brain, and actively removes lipophilic drugs or other substances that might have gained access to the brain. Although referred to as a barrier, the BBB is actually selectively permeable and plays important roles in regulating the uptake and efflux of ions, nutrients and metabolites. Paul Ehrlich first observed barrier properties in the early 1900s when, after intravenous injection, vital dyes stained nearly the entire body but were excluded from the brain [1,2]. Later studies ascribed this phenomenon to brain microvessel endothelial cells (BMEC) that lack fenestrae and are linked by complex intercellular tight junctions [3–5].

Many of the unique BMEC attributes result from cues provided by the local brain microenvironment (Fig. 1). Astrocytic endfeet are highly invested in the basement membrane that encloses the brain microvessels [6], and several *in vivo* studies have illustrated the importance of astrocytes in conferring barrier properties to BMECs [7,8]. Neurons and pericytes are also intimately associated with BMECs and are thought to be involved in the differentiation and regulation of the BBB [9–12]. The BMEC–perivascular-cell composite shown in Figure 1 has been coined the neurovascular unit to emphasize the tremendous amount of cellular interplay.

With its location at the interface between the brain interior and the bloodstream, the BBB plays a major role in disease pathology. Stroke, neurodegenerative diseases, microbial infections and head injuries can all lead to alterations in BBB permeability. Neurodegenerative diseases such as Alzheimer's disease (AD) [13] and multiple sclerosis (MS) [14,15] are often associated with BBB compromise, which further compounds symptoms. As another example, HIV-infected lymphocytes and/or monocytes are able to traverse the BBB and gain entry to the brain [16] causing disorders such as HIV-related encephalitis or HIV-associated dementia. Although treatments for some of these diseases exist, there is a great demand for improved drugs and novel therapeutic targets.

From the standpoint of drug delivery, the BBB constitutes a major hurdle in disease treatment because it is impermeable to many drug molecules. The BBB restricts brain access for a large fraction of small-molecule pharmaceuticals, and nearly all gene and protein biopharmaceuticals are unable to cross the BBB [17]. Therefore, innovative brain drug delivery systems are needed to complement drug discovery efforts.

Genomic and proteomic approaches have expanded our knowledge of BBB function, by allowing simultaneous analysis of thousands of genes and proteins. This review will focus specifically on the impacts that these two technologies have had on

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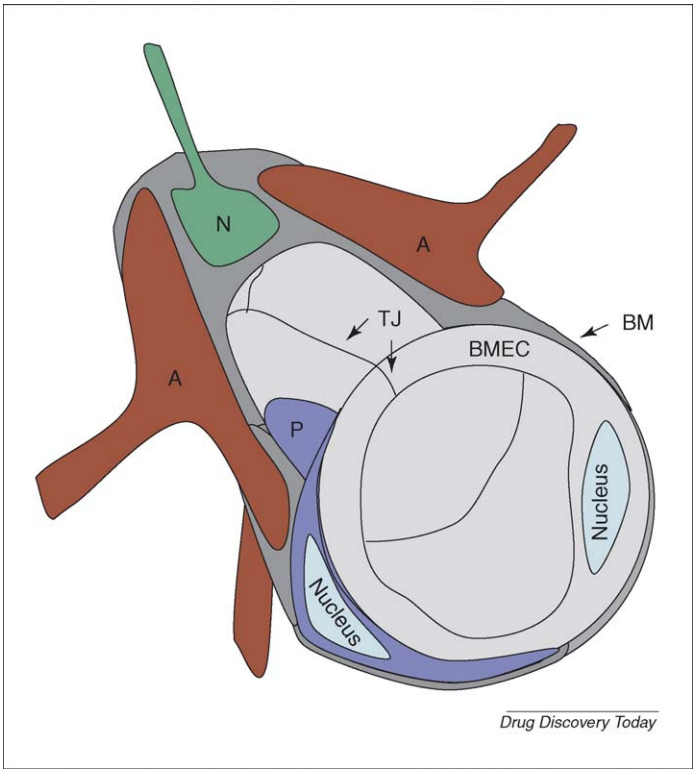


**FIGURE 1**

**Illustration of a brain capillary cross-section showing the major components of the neurovascular unit.** Brain microvessel endothelial cells (BMEC) form the vessel lumen, and BMEC are adjoined by complex tight junctions (TJ, solid lines). BMEC are ensheathed by a dense basement membrane (BM) that is shared with pericytes (P). Astrocytic endfeet (A) encircle the majority of the capillary surface and neuronal processes (N) also have direct contact with the vessel structure.

the aforementioned health, disease and drug delivery aspects of BBB research.

## Genomic and proteomic methods in blood–brain barrier research

A variety of genomic and proteomic techniques have been used in BBB research, and each technique has its inherent advantages and disadvantages (Table 1). Genomic methods used in BBB research include gene microarray technologies, serial analysis of gene expression (SAGE) and suppression subtractive hybridization (SSH). Gene microarray technologies are useful for generating semiquantitative data regarding gene expression across an entire genome. SAGE generates information about the entire gene expression profile by parsing mRNAs into short nucleotide fragments called tags, which allow for the quantitative cataloging of all expressed genes in cells or tissues [18]. Finally, SSH is a PCR-based method for identifying differentially regulated (up- or down-regulated) and tissue-specific (not detectable in compared samples) gene transcripts [19].

Although gene-expression profiles are often used to characterize cellular responses, the levels of mRNA and protein in cells do not always correlate [20]. Therefore, proteomic methods have been developed to examine proteins, the true actors in many cell functions. A widely used technique for creating differential proteomic profiles is 2D polyacrylamide gel electrophoresis (2D PAGE). This technique separates proteins according to charge and mass allowing for the resolution of up to 10,000 individual protein spots on a single gel [20]. Mass spectrometry is often used in conjunction with this method to identify the resolved proteins. Another proteomic technique employed in BBB research uses isotope coded affinity tags (ICAT) [21]. Labeling of protein samples with discrete isotope tags allows for a semiquantitative

**TABLE 1**

**A comparison of global profiling methods utilized in blood–brain barrier research**

| Techniques | Advantages | Disadvantages |
|---|---|---|
| **Genomic methods** | | |
| Gene microarray | Semiquantitative<br>Broad genome coverage<br>Highly optimized technique<br>Differential profiling | Limited sensitivity<br>Most arrays not comprehensive |
| Serial analysis of gene expression (SAGE) | Quantitative<br>Comprehensive genome coverage<br>Detection of rare and unknown sequences | Extensive DNA sequencing required<br>Differential profiling only when SAGE catalogs for compared tissues also available |
| Suppression subtractive hybridization (SSH) | Detection of rare and unknown sequences<br>Differential profiling | Not quantitative<br>Low sample throughput |
| **Proteomic methods** | | |
| 2 Dimensional polyacrylamide gel electrophoresis (2D PAGE) | Highly optimized technique<br>Reasonable proteome coverage | Poor compatibility with membrane proteins<br>Poor detection of low abundance proteins |
| Isotope-coded affinity tags (ICAT) | Compatibility with membrane proteins<br>Quantitative<br>Broad proteome coverage<br>Detection of low abundance proteins | Mass spectroscopy expertise required<br>Bioinformatics expertise required |
| Protein array | Moderate compatibility with membrane proteins<br>Detection of low abundance proteins<br>Uses traditional biochemical techniques | Minimal coverage of proteome<br>Limited selection of commercial arrays<br>Requires antibodies and/or proteins with high selectivity |

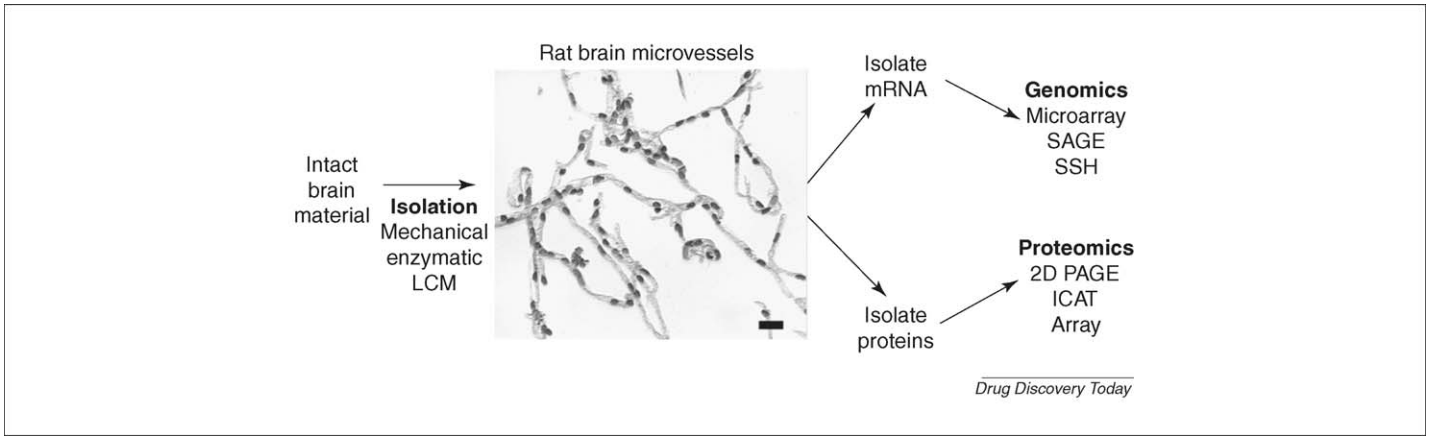


**FIGURE 2**

**Schematic of methods used for blood–brain barrier genomics and proteomics.** Brain microvessels are purified from total brain material and mRNA or proteins are isolated from the vessels. Subsequently, differential gene- and protein-profiling is performed. The image shows rat brain microvessels isolated by mechanical homogenization and stained with *o*-toluidine blue. Scale bar represents 20 μm. Abbreviations: LCM, laser-capture microdissection; SAGE, serial analysis of gene expression; SSH, suppression subtractive hybridization; 2D PAGE, 2 dimensional polyacrylamide gel electrophoresis; ICAT, isotope-coded affinity tagging.

comparison of protein expression using mass spectrometry [22,23]. Finally, similar to genomic arrays, proteomic arrays can be used to evaluate differential protein expression. One type of protein array used in BBB research is the antibody array. In this technology, antibodies are immobilized at high concentrations on a substrate to capture antigens from protein mixtures such as cell lysates [24].

Brain microvessels, rather than total brain tissue, are often the source of the mRNA or protein used in such studies. Because the microvasculature represents only 0.1% of the total brain volume, it is advisable to enrich for brain microvessels to avoid missing BBB-derived transcripts and proteins. The isolation of microvessels from total brain can be accomplished by various means including mechanical homogenization [25] or enzymatic digestion [26] followed by density centrifugation and filtration steps, or laser-capture microdissection [27] (Fig. 2). It should be noted that mechanically isolated microvessels invariably contain pericyte contamination because the BMEC and pericytes share the same robust basement membrane (Fig. 1), and laser-captured vessels will also have cellular contamination because of technical constraints. Thus, data resulting from global profiling techniques will actually contain information regarding multiple cell types, making for challenging interpretation. Microvessel isolation using enzymatic digestion has the potential to yield purer BMEC isolates because the basement membrane is separated from the vessels during digestion. However, the enzymatic digestion process requires a significant amount of incubation time *ex vivo* at physiological temperature, and during this time the mRNA profile can change. Thus, whereas mechanical or laser-capture isolation can yield *in-vivo*-like mRNA samples as a result of microvessel harvest at cold temperatures, the purer enzymatic isolates yield mRNA that is less like that found *in vivo*. Although brain microvessels are the optimal starting point for performing genomic and proteomic techniques aimed at analyzing the BBB, restricted sets of high-abundance vessel-related genes and proteins have sometimes been identified using total brain material. If BMEC cell lines or primary culture are used for genomic and proteomic study, the resultant data need to be carefully interpreted and conclusive findings would require a link to the actual *in vivo* situation. Taken together, the isolation technique and BMEC source can greatly influence the outcome of global profiling experiments, and the choice of approach needs to take these variables into account. After the appropriate BBB source has been chosen, mRNA or protein can be purified [28,29] and analyzed using genomic and proteomic methods (Fig. 2). In the following sections, BBB studies employing various combinations of genomics and proteomics techniques will be detailed.

## Unique attributes of the brain microvasculature

As described earlier, the brain microvascular endothelium substantially differs from the peripheral microvasculature, and the molecular basis for of these differences has been illuminated by several genomic studies. Gene expression of human brain microvessels was compared with that of liver and kidney tissues using the SSH methodology [30]. This study identified 38 brain microvascular genes that were upregulated at the BBB, 20 of which were known genes whose protein products are involved in cellular processes, such as angiogenesis, neurogenesis, molecular transport, immune response and tight junction formation (Table 2). Using the same method, Li and colleagues [31,32] analyzed rat brain microvessels and found an additional set of genes that were differentially upregulated at the brain microvasculature compared with the liver and kidney. In these two studies, 67 genes that had upregulated expression at the BBB were identified. These genes represented known genes, novel genes with unknown function and expressed sequence tags (ESTs). The proteins encoded by known genes from these two studies function in vascular remodeling, signal transduction, transport, cytoskeletal development and junctional assembly, among others. The genomic studies described above illustrate the unique attributes of the brain microvascular endothelium and its importance to the development and maintenance of the brain (Table 2). These studies were complemented by a proteomic analysis of the BBB, where a subtractive antibody-expression cloning method was used to detect brain microvessel-specific membrane proteins. To date, this method has led to the identification of

**TABLE 2**

**[a]Functional groupings of genes upregulated at the blood–brain barrier (BBB) compared with liver or kidney (studies 1–3[a]), and a listing of whether or not these genes were also found in the comprehensive BBB SAGE catalog (study 4[a])**

| Gene Functional Category | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| **GROWTH FACTORS** | | | | |
| Platelet-derived growth factor receptor β (PDGF-Rβ) | | | X | X |
| Insulin-like growth factor 2 (IGF-2) | | X | | X |
| Fibroblast growth factor 19 (FGF19) | X | | | |
| Heparin affinity regulatory peptide (HARP) | X | | | X |
| Insulin-like growth factor binding protein 3 (IGF-BP-3) | X | | | |
| Tomoregulin | X | | | |
| **SIGNAL TRANSDUCTION** | | | | |
| Guanine nucleotide exchange factor HERC2 | X | | | |
| Grb-2-associated binder-2 (Gab2) | X | | | |
| AU-rich RNA binding factor (LaAUF-1) | X | | | X |
| G-protein-signaling regulator-5 (Rgs5) | | X | X | X |
| Prostaglandin D synthase (Pgds) | | | X | |
| S100 calcium-binding protein | | X | | X |
| **TRANSPORT** | | | | |
| Caveolin-1α | | | X | X |
| Organic anion transporting peptide type 2 (oatp2) | | X | X | X |
| Na, K ATPase α2 | X | | X | X |
| Monocarboxylate transporter 1 (MCT1) | X | | | X |
| BBB-specific anion transporter type 1 (BSAT1) (oatp14) | | X | X | X |
| Cationic amino acid transporter 1 (CAT1) | | | X | X |
| FXYD domain-containing ion transport regulator 5 (FXYD5) | | | X | X |
| Transferrin receptor (TfR) | | X | | X |
| **IMMUNOLOGY** | | | | |
| Major histocompatibility complex I (MHCI) | | X | | X |
| Podocalyxin-like protein | X | | | X |
| [b]Membrane cofactor protein, CD46 (B) | | | | |
| [b]52 kDa ribonucleoprotein, Ro52 (B) | | | | |
| Platelet endothelial cell-adhesion protein (PECAM-1) | | | X | |
| **TIGHT JUNCTIONS, EXTRACELLULAR MATRIX** | | | | |
| Endothelial cell-selective adhesion molecule (ESAM) | | | X | X |
| Claudin5 | X | | | X |
| Prominin | | | X | X |
| Serglycin | X | | | |
| [b]Lutheran (B) | | | | |
| **TRANSCRIPTION FACTORS** | | | | |
| ElonginA | | | X | |
| Vascular endothelial cell-specific protein 14 | | | X | |
| Enhancer of zeste homolog 1 (EZH1) | | X | | |
| Inhibitor of nuclear factor-κB (IκB) | | X | | X |
| Human homolog of yeast SW12 transcription factor | | X | | |
| B-cell translocation gene-2 | X | X | | X |
| **SECRETION** | | | | |
| Carboxypeptidase E (Cpe) | | X | X | X |
| Secretory granule proteoglycan core protein precursor (Pgsg) | | | X | X |
| **AMYLOID** | | | | |
| Amyloid precursor-like protein 2 (APLP2) | | | X | X |
| Sperm membrane protein related to A4 amyloid protein, YWK-II | | | X | X |
| Integral membrane protein 2a (Itm2a) | X | | X | |
| **HEMOSTASIS** | | | | |
| Factor 8 | | | X | X |
| Serine protease inhibitor 4 (Spi4) | | | X | X |
| Tissue plasminogen activator (tPA) | | | X | X |
| **MYELIN** | | | | |
| Myelin basic protein (MBP) | | X | X | X |
| Protein zero-related protein 1, PZR related | | | X | X |
| Proteolipid protein 1 (PLP-1) | | | X | X |
| **LIPIDS** | | | | |
| Phospholipid transfer protein (PLTP) | | | X | X |
| Paraoxonase | X | | | |
| α2-macroglobulin | X | | | |
| Stearoyl-CoA desaturase 2 | | | X | X |
| **VASCULAR REMODELING** | | | | |
| Vascular endothelial growth factor receptor type 1 (Flt-1) | | X | X | X |
| Hypoxia-inducible factor 2α (HIF-2α) | | | X | X |
| Osteonectin | X | | | X |
| Vascular endothelial receptor-type protein tyrosine phosphatase (VE-PTP) | | | X | |
| **CYTOSKELETON** | | | | |
| Regulatory myosin light chain isoform C (MLC20) | | | X | |
| Connexin-45 | | X | | |
| Utrophin | | X | | X |
| β-actin | X | | | X |

[a] Study 1: human SSH [30]. Studies 2 and 3: rat SSH [31,32]. Study 4: rat SAGE [36].
[b] Also included are the three BBB proteins isolated in the bovine subtractive antibody-expression cloning studies (B, Refs [33–35]).

three BBB-specific membrane proteins: Lutheran membrane glycoprotein [33]; CD46 complement regulator [34]; and an autoantigen in Sjogren's syndrome (Ro52) [35].

A recent study by Enerson and Drewes [36] represents the first reported attempt at generating a comprehensive profile of gene expression at the BBB using SAGE. Unlike the SSH studies that assessed differential gene expression, the SAGE study yielded a complete listing of genes expressed at the BBB. The rat SAGE catalog contained >100,000 tags, and 33% of these matched known genes, 51% corresponded to ESTs and 16% represented

unknown genes. By comparing the rat BBB SAGE catalog with SAGE profiles derived from rat cortex and hippocampus, 864 genes were identified as being selectively expressed at the BBB. These genes encoded proteins belonging to a variety of functional groups, including signal transduction (17%), transport (11%), structural proteins (10%), receptors (5%) and proteins involved in vesicle trafficking (4%). Similar to the SSH results, the nature of these functional groups underlines the distinct and considerable role of the BBB as the transport and communication interface between the brain and the bloodstream (Table 2). As one would expect, most of the genes identified as being differentially upregulated at the BBB (compared with the liver and kidney) were also shown to be expressed at the BBB in the comprehensive SAGE profiling study, but several SSH-derived genes were not found in the SAGE study. This could be a result of several factors including species differences, because many of the genes in Table 2 that were not identified in the rat SAGE study originated from human BBB studies. Also, although the SAGE study involved sequencing 100,000 tags, the list of expressed genes was probably not comprehensive; an important factor for low expression level genes such as transcription factors isolated in SSH screens (Table 2).

Gene-expression comparisons have also been used to examine the differences between the closely related BBB and blood–retinal barrier (BRB) of the eye [37,38]. Although many attributes of these barriers are believed to be similar, each participates in unique functions suggesting differences in gene expression. These studies utilized differential display, a technique very similar to SSH, and found eight genes to be preferentially expressed at the BRB [37], whereas 12 genes were specifically expressed at the BBB [38]. The differentially expressed genes were related to cell–cell adhesion and to the regulation of molecular transport, confirming that these two structures possess distinct physical and functional characteristics.

Finally, the results obtained by genomic studies are being verified by proteomic studies that indicate significant differences between BMECs and those EC found in the periphery. In particular, 2D gel electrophoresis profiles of freshly isolated luminal EC membranes allowed analysis of EC surface proteins from the brain, heart, lung, liver and kidney [39]. Although the resultant BBB protein-expression profile was not examined in detail (protein spots not sequenced), the BBB profile was substantially different from those seen for the peripheral organs. These data indicate that the global differences between BMECs and other ECs, seen at the genomic level, correlate with differences in the resultant protein-expression profiles.

## The blood–brain barrier and disease

Given its location at the interface between the brain and the bloodstream, it is not surprising that the BBB is affected by neurological diseases and involved in their progression. Genomic and proteomic technologies provide a means whereby BBB-disease links can be studied in a comprehensive fashion. In these studies, the healthy BBB is usually compared with BBB from the diseased state to determine the molecular changes taking place, with the hope that this information will lead to the identification of therapeutic targets. To date, genomic methods have been utilized more extensively than proteomic methods to analyze BBB function in disease, but the number of reports using both sets of techniques is rapidly increasing. The survey below is not an exhaustive discussion of the BBB and disease, but focuses on those studies that have employed genomics or proteomics.

### Metabolic imbalance

The integrity of the BBB is crucial for proper brain function. Many neurodegenerative diseases can cause BBB dysfunction and often compromise the barrier, leading to additional symptoms and an accelerated disease progression. One disease that is thought to affect the BBB is glutaric aciduria type I, an inherited neurometabolic disorder caused by a deficiency in glutaryl-CoA dehydrogenase (GCDH). This deficiency results in the buildup of 3-hydroxyglutaric acid (3-OH-GA), which is thought to have neurotoxic effects. To better understand the pathologic effects of 3-OH-GA, a microarray study compared total brain material from GCDH-deficient and control mice [40]. This analysis uncovered genes related to vascular regulation [e.g. vascular endothelial growth factor (VEGF) and angiogenin], and further experiments suggested that 3-OH-GA can decrease endothelial integrity and increase vascular permeability. In addition, cholinergic imbalances are detected at the onset of neurodegenerative diseases and after brain trauma, and can cause impaired BBB functioning [41]. Microarray analysis of the prefrontal cortices of transgenic mice overexpressing synaptic acetylcholinesterase and strain-matched wild-type mice revealed a higher expression of genes relating to water (aquaporin 4) and ion transporters (potassium, chloride, calcium and sodium) in the transgenic mice. Although not all of the transporters identified are expressed at the BBB, it was suggested that their overexpression affected BBB maintenance and resulted in the impaired BBB functioning [41].

### Multiple sclerosis

BBB disruption is also a major pathological feature of MS [42], an autoimmune disease characterized by the demyelination and death of neurons in the central nervous system (CNS). The molecular mechanisms underlying BBB breakdown were examined by analyzing differential gene and protein expression in the brain microvascular endothelium of mice afflicted with experimental autoimmune encephalomyelitis (EAE), the animal model of MS [43]. Oligonucleotide microarray and 2D PAGE proteomic analyses identified 128 genes and six proteins with altered expression levels in the EAE-afflicted brains, as compared with healthy brains. The identified genes and proteins are involved in numerous cellular functions, including leukocyte recruitment and extracellular matrix regulation, and potentially provide novel targets on the brain microvasculature for the treatment of MS. Because tumor necrosis factor-α (TNF-α) has been suggested to play an important role in MS and can promote an inflammatory response at the BBB, the effects of TNF-α on human BMECs were studied *in vitro* [44]. Oligonucleotide microarray and 2D PAGE analyses revealed BMEC-specific responses of the urokinase plasminogen activator system and alterations of the BMEC cytoskeleton as a result of TNF-α stimulation. Another study investigated the administration of erythropoietin (EPO) as a method for controlling the inflammatory response of the BBB in MS [45]. A human cytokine gene array was used to examine the effects of recombinant EPO on TNF-α-activated human BMECs *in vitro*. Interleukin (IL)-6 and CXC receptor 4 genes were downregulated when cells were treated

for 24 h with TNF-α and recombinant EPO, as compared with TNF-α alone, and these results suggested that EPO could be useful in stabilizing the BBB.

## Amyloid diseases

BBB dysfunction is also observed in AD. Neurovascular unit abnormalities include thickening of the basement membrane and degenerating tight junctions, as well as a reduced capillary density that can contribute to the pathogenesis of AD. Using a cDNA microarray of human BMECs, Wu and colleagues [46] identified differential expression of 34 genes in AD microvessels. One downregulated gene encoded the homeodomain transcription factor mesenchyme homeobox 2 (MEOX2), and subsequent experiments revealed that reduced expression of MEOX2 could disrupt the cerebrovascular architecture leading to the lowered brain capillary density and cerebral blood flow observed in AD patients. The authors therefore suggested MEOX2 as a potential target for treating AD [46]. AD pathology is similar to that of a rare autosomal dominant disorder known as hereditary cerebral hemorrhage with amyloidosis-Dutch type, because both diseases induce cerebral amyloid angiopathy. To better understand the effects of amyloid-β (Aβ) peptides on BMECs, an oligonucleotide microarray analysis examined cultured human BMEC response to Dutch $A\beta_{1–40}$ peptide exposure [47]. This study identified 1270 genes whose expression levels were altered. These genes are involved in biological networks relating to angiogenesis, tumorigenesis, atherosclerosis, cellular migration and proliferation. Many of the genes altered in response to Dutch $A\beta_{1–40}$ encoded proteins that possess similar functions to those found in the study by Wu and colleagues [46], and offer clues regarding pathological activity of Aβ on the BBB.

## Stroke

Cerebral ischemia and hypoxia as a result of stroke can affect the integrity of the BBB. To better understand the underlying mechanisms associated with these processes, the cerebral capillaries of stroke-prone spontaneously hypertensive rats (SHRSP) were compared with stroke-resistant spontaneously hypertensive rats using SSH [48]. Among the genes differentially expressed in SHRSP rats were the upregulated sulfonylurea receptor 2B and the downregulated G-protein-signaling regulator-5. Effects of stroke were also examined using an SSH analysis of total brain tissue from the cold-induced hypertensive rat stroke model [49]. Seventy-six genes involved in energy metabolism, signal transduction, transcription and translation were overexpressed in stroke brain tissue. Another study of cerebral ischemia was recently performed using ICAT [50]. The protein-expression profiles of laser-microdissected brain microvessels from rats subjected to a transient global cerebral ischemia were analyzed after reperfusion for 1, 6 or 24 h. A collection of 57 genes was found to be differentially expressed with lowered expression of ion pumps, nutrient transporters, cell structure proteins and motility proteins, and upregulation of proteins functioning as transcription factors and signal transduction proteins.

## Aberrant angiogenesis

Hyperhomocysteinemia, a condition marked by increased homocysteine (Hcy) levels in the body, has been connected with cerebrovascular diseases [51,52], in part owing to the inhibition of angiogenesis by Hcy [53,54]. The molecular mechanisms triggering this response were examined using an angiogenesis antibody array to analyze protein expression in mouse BMECs cultured with and without Hcy [55]. Hcy treatment of BMECs reduced the expression of the following angiogenic proteins: leptin, IL-6, IL-8, placenta growth factor, fibroblast growth factor-α, and VEGF. Increased expression was observed for the antiangiogenic factors IL-12, interferon-inducible protein IP-10 and tissue inhibitor of metalloproteinase 1, and Hcy reduced vascular tube formation. These results suggested that the clinical effects of ischemic insult could be exacerbated by hyperhomocysteinemia because an angiogenic injury response would be disrupted. Angiogenesis-related problems are also associated with human brain arteriovenous malformations (BAVMs), a rare disorder causing stroke in young adults. mRNA samples isolated from total human brain were studied utilizing the Affymetrix Human Gene Set U95Av2 array, and 1781 genes were found to be differentially expressed, compared with control brain samples [56]. Increased gene expression was found for several angiogenesis-related genes including VEGF, VEGF receptors (Flt-1 and Flk-1), and angiopoietins 1 and 2.

## Microbial and viral infection

Cerebral insult can also occur as a result of microbial infections. To gain access to the brain, microorganisms must first traverse or disrupt the BBB and, thus, pathogens are likely to interact with BMECs. One type of brain infection is bacterial meningitis, and the majority of infections are caused by the meningeal pathogen group B *Streptococcus* (GBS). An oligonucleotide microarray study of the initial response of an *in vitro,* human BBB model to GBS indicated that a subset of genes involved in inflammatory response was upregulated (cytokines, chemokines and adhesion molecules) [57]. A more recent study of microbial–BBB interactions examined the cytotoxic effect of *Acanthamoeba* (causes fatal granulomatous encephalitis) on human BMECs *in vitro* [58]. A gene array designed for detailed analysis of the cell cycle revealed the reduced expression of genes encoding proteins involved in cell-cycle progression. The microarray also implicated increased expression of those genes important for cell-cycle arrest, providing a better understanding of the pathogenesis of this organism. Another recent study investigated the pathogenesis of HIV-1 infection. This study used gene microarray analyses to show that the HIV-accessory-protein Nef (negative factor) induced the expression of various proapoptotic genes in primary human BMECs [59]. The Nef protein was therefore identified as a potential target for reducing the toxicity and degree of brain entry of HIV-1.

The recent studies described in this review illustrate the utility of global proteomic and genomic analysis techniques in elucidating the numerous molecular events that are altered at the BBB by disease. A better understanding of the molecular mechanisms of various BBB pathologies will probably be valuable for identifying novel therapeutic targets.

## Functional proteomics and brain drug delivery

The selectively permeable BBB restricts brain uptake of circulating drugs to those that are small and lipophilic [17]. Therefore, a longstanding goal of brain drug delivery research has been to develop methods that allow noninvasive delivery of small molecules, proteins and DNA. Endogenous nutrient transport systems

present at the BBB can be used to shuttle drug cargo from the blood into the brain. Peptides or antibodies that specifically bind to these transport systems can often act as artificial transporter substrates that mediate blood-to-brain trafficking. Therefore, when conjugated to therapeutics, BBB-targeting peptides and antibodies represent new routes for noninvasive brain drug delivery via the bloodstream. To identify such drug delivery portals, a growing collection of protein-profiling techniques, classified here as functional proteomics, can be exploited. Unlike traditional proteomics, which simply measures the presence and/or abundance of proteins, the power of BBB functional proteomics lies in the coordinate identification of targeting reagents and BBB-specific molecular transporters and/or targets. As an example of functional proteomics, the subtractive antibody-expression cloning methodology mentioned earlier allows for the identification of membrane proteins that are selectively expressed at the BBB, as compared with peripheral tissues [33]. In this way, it might be possible to identify molecular transporters that are specific to the BBB and have potential use as delivery portals. Although this method is antibody-based, the antibodies are in the form of a polyclonal antiserum that recognizes diverse targets and, thus, cannot be used as a specific delivery reagent. By contrast, other functional proteomic methods allow for the simultaneous identification of the cognate targeting reagent. For example, a large peptide library was screened *in vivo* to identify peptides that specifically targeted bone marrow, skin, fat, skeletal muscle and prostate tissues. The results indicated that tissue vasculatures have unique 'molecular addresses' that could ultimately be used for drug targeting by employing the extracted peptides [60]. Of particular relevance to brain drug delivery, phage display combined with an *in vitro* subtractive panning protocol was used to identify llama single-domain antibodies (sdAb) that specifically bind to (and transmigrate across) the BBB [61]. The ability of phage-displayed sdAbs to mediate brain uptake via vesicular transport mechanisms was also demonstrated [61,62]. Yeast-surface display, like phage display, is another powerful tool whose utilities include cell-panning selections. Yeast displaying model single-chain antibodies have been demonstrated to interact with the surface of brain ECs [63], and these results indicate that BBB-binding antibodies could also be isolated from antibody libraries. Although the area of BBB functional proteomics research is in its nascent stages, the studies presented above hold promise for the development of new brain drug delivery systems.

## Conclusions and future directions

This review has focused on recent advances in the BBB field that have resulted from genomic or proteomic approaches. Genomics and proteomics have provided us with information about the molecular-level differences of the BBB as compared with peripheral vascular beds, and the SAGE study has provided a catalog of transcript abundance at the BBB. Global techniques also yield insights regarding the role of the BBB in neurodegenerative diseases, and the goal of many of these studies has been to identify novel therapeutic targets for the improved treatment of diseases such as MS, AD, stroke and HIV-1 infection. Because these studies have all been completed very recently, they have not yet provided a significant breakthrough in neurological disease treatment. However, it is probable that some of the early hits described above will offer promising therapeutic alternatives. As genomic and proteomic techniques continue to improve and evolve, their utility is expected to grow. In particular, functional proteomic techniques have proven valuable for the identification of new transport systems that might be useful for delivering drugs across the BBB. Although global profiling techniques result in a wealth of new data, traditional biochemical and cellular analyses are still required for detailed confirmation of these results. In addition, as our ability to manage, compare and interpret large datasets continues to improve, global profiling techniques could further accelerate the process of understanding the BBB from the standpoints of health, disease and treatment.

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## References

1 Ehrlich, P. (1885) *Das sauerstoff-bedürfnis des organismus: eine farbenanalytische studie*. Habilitation thesis, Hirschwald
2 Ehrlich, P. (1906) Ueber die beziehungen von chemischer constitution, vertheilung, und pharmakologischen wirkung. In *Collected Studies on Immunity*, pp. 404–442, John Wiley & Sons
3 Reese, T.S. *et al.* (1971) Electron microscopic study of the blood-brain and blood-cerebrospinal fluid barriers with microperoxidase. *J. Neuropathol. Exp. Neurol.* 30, 137–138
4 Reese, T.S. and Karnovsky, M.J. (1967) Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* 34, 207–217
5 Brightman, M.W. and Reese, T.S. (1969) Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* 40, 648–677
6 Kacem, K. *et al.* (1998) Structural organization of the perivascular astrocyte endfeet and their relationship with the endothelial glucose transporter: a confocal microscopy study. *Glia* 23, 1–10
7 Stewart, P.A. and Wiley, M.J. (1981) Developing nervous tissue induces formation of blood-brain barrier characteristics in invading endothelial cells: a study using quail–chick transplantation chimeras. *Dev. Biol.* 84, 183–192
8 Janzer, R.C. and Raff, M.C. (1987) Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* 325, 253–257
9 Lee, E.J. *et al.* (1999) Early alterations in cerebral hemodynamics, brain metabolism, and blood-brain barrier permeability in experimental intracerebral hemorrhage. *J. Neurosurg.* 91, 1013–1019
10 Petty, M.A. and Wettstein, J.G. (2001) Elements of cerebral microvascular ischaemia. *Brain Res. Brain Res. Rev.* 36, 23–34
11 Hatashita, S. and Hoff, J.T. (1990) Brain edema and cerebrovascular permeability during cerebral ischemia in rats. *Stroke* 21, 582–588
12 Hori, S. *et al.* (2004) A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation *in vitro*. *J. Neurochem.* 89, 503–513
13 Jellinger, K.A. (2002) Alzheimer disease and cerebrovascular pathology: an update. *J. Neural Transm.* 109, 813–836
14 Miller, D.H. *et al.* (1998) The role of magnetic resonance techniques in understanding and managing multiple sclerosis. *Brain* 121, 3–24
15 Davie, C.A. *et al.* (1994) Serial proton magnetic resonance spectroscopy in acute multiple sclerosis lesions. *Brain* 117, 49–58
16 Toborek, M. *et al.* (2005) Mechanisms of the blood-brain barrier disruption in HIV-1 infection. *Cell. Mol. Neurobiol.* 25, 181–199
17 Pardridge, W.M. (2005) The blood-brain barrier: bottleneck in brain drug development. *NeuroRx* 2, 3–14
18 Velculescu, V.E. *et al.* (1995) Serial analysis of gene expression. *Science* 270, 484–487
19 Diatchenko, L. *et al.* (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. U. S. A.* 93, 6025–6030
20 Anderson, L. and Seilhamer, J. (1997) A comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 18, 533–537

21 Gygi, S.P. *et al.* (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17, 994–999
22 Yu, L.R. *et al.* (2002) Proteome analysis of camptothecin-treated cortical neurons using isotope-coded affinity tags. *Electrophoresis* 23, 1591–1598
23 Moseley, M.A. (2001) Current trends in differential expression proteomics: isotopically coded tags. *Trends Biotechnol.* 19 (Suppl. 10), 10–16
24 Wilson, K.E. *et al.* (2004) Functional genomics and proteomics: application in neurosciences. *J. Neurol. Neurosurg. Psychiatry* 75, 529–538
25 Bowman, P.D. *et al.* (1981) Primary culture of capillary endothelium from rat brain. *In Vitro* 17, 353–362
26 Deli, M.A. *et al.* (2003) N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine increases the permeability of primary mouse cerebral endothelial cell monolayers. *Inflamm. Res.* 52 (Suppl. 1), 39–40
27 Mojsilovic-Petrovic, J. *et al.* (2004) Development of rapid staining protocols for laser-capture microdissection of brain vessels from human and rat coupled to gene expression analyses. *J. Neurosci. Methods* 133, 39–48
28 Weiler-Guttler, H. *et al.* (1989) cDNA cloning and sequence analysis of the glucose transporter from porcine blood-brain barrier. *Biol. Chem. Hoppe Seyler* 370, 467–473
29 Boado, R.J. and Pardridge, M.M. (1991) A one-step procedure for isolation of poly(A)+ mRNA from isolated brain capillaries and endothelial cells in culture. *J. Neurochem.* 57, 2136–2139
30 Shusta, E.V. *et al.* (2002) Vascular genomics of the human brain. *J. Cereb. Blood Flow Metab.* 22, 245–252
31 Li, J.Y. *et al.* (2001) Blood-brain barrier genomics. *J. Cereb. Blood Flow Metab.* 21, 61–68
32 Li, J.Y. *et al.* (2002) Rat blood-brain barrier genomics. II. *J. Cereb. Blood Flow Metab.* 22, 1319–1326
33 Shusta, E.V. *et al.* (2002) Vascular proteomics and subtractive antibody expression cloning. *Mol. Cell. Proteomics* 1, 75–82
34 Shusta, E.V. *et al.* (2002) Subtractive expression cloning reveals high expression of CD46 at the blood-brain barrier. *J. Neuropathol. Exp. Neurol.* 61, 597–604
35 Shusta, E.V. *et al.* (2003) The Ro52/SS-A autoantigen has elevated expression at the brain microvasculature. *Neuroreport* 14, 1861–1865
36 Enerson, B.E. and Drewes, L.R. (2005) The rat blood-brain barrier transcriptome. *J. Cereb. Blood Flow Metab.* doi:10.1038/sj.jcbfm.9600249
37 Tomi, M. *et al.* (2004) Retinal selectivity of gene expression in rat retinal versus brain capillary endothelial cell lines by differential display analysis. *Mol. Vis.* 10, 537–543
38 Ohtsuki, S. *et al.* (2005) Dominant expression of androgen receptors and their functional regulation of organic anion transporter 3 in rat brain capillary endothelial cells; comparison of gene expression between the blood-brain and -retinal barriers. *J. Cell. Physiol.* 204, 896–900
39 Oh, P. *et al.* (2004) Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature* 429, 629–635
40 Muhlhausen, C. *et al.* (2004) Vascular dysfunction as an additional pathomechanism in glutaric aciduria type I. *J. Inherit. Metab. Dis.* 27, 829–834
41 Meshorer, E. *et al.* (2005) Chronic cholinergic imbalances promote brain diffusion and transport abnormalities. *FASEB J.* 19, 910–922
42 Petty, M.A. and Lo, E.H. (2002) Junctional complexes of the blood-brain barrier: permeability changes in neuroinflammation. *Prog. Neurobiol.* 68, 311–323
43 Alt, C. *et al.* (2005) Gene and protein expression profiling of the microvascular compartment in experimental autoimmune encephalomyelitis in C57Bl/6 and SJL mice. *Brain Pathol.* 15, 1–16
44 Franzen, B. *et al.* (2003) Gene and protein expression profiling of human cerebral endothelial cells activated with tumor necrosis factor-alpha. *Brain Res. Mol. Brain Res.* 115, 130–146
45 Avasarala, J.R. and Konduru, S.S. (2005) Recombinant erythropoietin down-regulates IL-6 and CXCR4 genes in TNF-alpha-treated primary cultures of human microvascular endothelial cells: implications for multiple sclerosis. *J. Mol. Neurosci.* 25, 183–189
46 Wu, Z. *et al.* (2005) Role of the MEOX2 homeobox gene in neurovascular dysfunction in Alzheimer disease. *Nat. Med.* 11, 959–965
47 Paris, D. *et al.* (2005) Anti-angiogenic activity of the mutant Dutch A(beta) peptide on human brain microvascular endothelial cells. *Brain Res. Mol. Brain Res.* 136, 212–230
48 Kirsch, T. *et al.* (2001) Altered gene expression in cerebral capillaries of stroke-prone spontaneously hypertensive rats. *Brain Res.* 910, 106–115
49 Zhu, Z. *et al.* (2004) Differentially expressed genes in hypertensive rats developing cerebral ischemia. *Life Sci.* 74, 1899–1909
50 Haqqani, A.S. *et al.* (2005) Characterization of vascular protein expression patterns in cerebral ischemia/reperfusion using laser capture microdissection and ICAT-nanoLC-MS/MS. *FASEB J.* 19, 1809–1821
51 Loscalzo, J. (2002) Homocysteine and dementias. *N. Engl. J. Med.* 346, 466–468
52 Fridman, O. (1999) Hyperhomocysteinemia: atherothrombosis ad neurotoxicity. *Acta Physiol. Pharmacol. Ther. Latinoam.* 49, 21–30
53 Rodriguez-Nieto, S. *et al.* (2002) Anti-angiogenic effects of homocysteine on cultured endothelial cells. *Biochem. Biophys. Res. Commun.* 293, 497–500
54 Wang, H. *et al.* (1997) Inhibition of growth and p21ras methylation in vascular endothelial cells by homocysteine but not cysteine. *J. Biol. Chem.* 272, 25380–25385
55 Shastry, S. *et al.* (2004) Proteomic analysis of homocysteine inhibition of microvascular endothelial cell angiogenesis. *Cell Mol. Biol. (Noisy-le-grand)* 50, 931–937
56 Hashimoto, T. *et al.* (2004) Gene microarray analysis of human brain arteriovenous malformations. *Neurosurgery* 54, 410–423
57 Doran, K.S. *et al.* (2003) Group B streptococcal beta-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. *J. Clin. Invest.* 112, 736–744
58 Sissons, J. *et al.* (2004) Acanthamoeba induces cell-cycle arrest in host cells. *J. Med. Microbiol.* 53, 711–717
59 Acheampong, E.A. *et al.* (2005) Human Immunodeficiency virus type 1 Nef potently induces apoptosis in primary human brain microvascular endothelial cells via the activation of caspases. *J. Virol.* 79, 4257–4269
60 Arap, W. *et al.* (2002) Steps toward mapping the human vasculature by phage display. *Nat. Med.* 8, 121–127
61 Muruganandam, A. *et al.* (2002) Selection of phage-displayed llama single-domain antibodies that transmigrate across human blood-brain barrier endothelium. *FASEB J.* 16, 240–242
62 Abulrob, A. *et al.* (2005) The blood-brain barrier transmigrating single domain antibody: mechanisms of transport and antigenic epitopes in human brain endothelial cells. *J. Neurochem.* 95, 1201–1214
63 Wang, X.X. and Shusta, E.V. (2005) The use of scFv-displaying yeast in mammalian cell surface selections. *J. Immunol. Methods* 304, 30–42