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Pathogens Penetrating the Central Nervous System: Infection Pathways and the Cellular and Molecular Mechanisms of Invasion

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SUMMARY

The brain is well protected against microbial invasion by cellular barriers, such as the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). In addition, cells within the central nervous system (CNS) are capable of producing an immune response against invading pathogens. Nonetheless, a range of pathogenic microbes make their way to the CNS, and the resulting infections can cause significant morbidity and mortality. Bacteria, amoebae, fungi, and viruses are capable of CNS invasion, with the latter using axonal transport as a common route of infection. In this review, we compare the mechanisms by which bacterial pathogens reach the CNS and infect the brain. In particular, we focus on recent data regarding mechanisms of bacterial translocation from the nasal mucosa to the brain, which represents a little explored pathway of bacterial invasion but has been proposed as being particularly important in explaining how infection with *Burkholderia pseudomallei* can result in melioidosis encephalomyelitis.

INTRODUCTION

Microbial infection involving the central nervous system (CNS) is an important and relatively common presentation. CNS infections are frequently caused by viruses, such as the enteroviruses, which cause the majority of cases of aseptic meningitis and meningoencephalitis (1–3). Other neurotropic viruses, such as human cytomegalovirus, herpes simplex viruses, varicella-zoster virus, and the emerging viruses West Nile virus, henipaviruses, Japanese encephalitis virus, chikungunya virus, Ebola virus, and rabies virus, may also cause CNS infections (4–7). There are many bacterial pathogens that are associated with CNS invasion. Rapid detection of a bacterial agent in such presentations and the initiation of appropriate antibiotic therapy influence morbidity and mortality (8–10). The clinical presentations of bacterial CNS infection range from meningitis and meningoencephalitis to focal CNS syndromes.

Meningitis

Meningitis, or inflammation of the meninges, is usually acute but can also be subacute and most frequently presents with headache, fever, and neck stiffness (11). Meningitis can be pyogenic (pus forming), which is associated with common bacterial etiologies (described below), or aseptic, in which pyogenic bacteria are not isolated from the cerebrospinal fluid (CSF) and the causative agent may be viral or mycobacterial or there is a noninfective etiology. Features of CSF which would generally exclude a bacterial etiology for meningitis include a protein concentration of $<$600 mg/liter and a white cell count of $<90 \times 10^6$/liter (2). The classic triad of fever, neck stiffness, and a change in mental state is observed in 44% of patients with bacterial meningitis (11).

A prospective, laboratory-based surveillance study in 1986 defined the epidemiology of bacterial meningitis in a population of 34 million in the United States (12). This study reported an average of 6.7 cases of bacterial meningitis per 100,000 population across six states and demonstrated that *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* caused 77% of cases, which was consistent with a previous report by the National Surveillance of Bacterial Meningitis (13). More recently, it was reported that the incidence of bacterial meningitis in the United States declined by 31% during the surveillance period of 1998 to 2007, most likely due to the success of vaccine programs (14). *S. pneumoniae* was the most common cause of bacterial meningitis (58.0%), followed by *Streptococcus agalactiae* (group B streptococcus) (18.1%), *N. meningitidis* (13.9%), *H. influenzae* (6.7%), and *Listeria monocytogenes* (3.4%). *Staphylococcus aureus* and *Escherichia coli* are also important causes of bacterial meningitis (15), and *Streptococcus suis* is emerging as a zoonotic etiology of meningitis (16); however, it must be noted that meningitis can be caused by a plethora of different bacteria (17). The most common alternative bacterial cause of meningitis is *Mycobacterium tuberculosis*, which is recognized particularly in pediatric populations of the developing world (18, 19). These infections involve a clinical onset that is usually insidious, with hydrocephalus being a prominent feature.

Bacterial meningitis is usually preceded by nasopharyngeal or middle ear colonization, followed by (i) microbial invasion of the tissue and intravascular space and (ii) bacteremia. Meningeal invasion occurs following penetration of the cellular barriers of the CNS. High-level bacteremia was shown to be necessary for the development of meningitis in experimental animal models (20, 21), which is consistent with clinical observations in humans (22–24). Bacteria that commonly cause meningitis express antiphagocytic capsular polysaccharide, which enables survival/multiplication within the blood. Several species-specific factors that promote bacterial survival in blood, resistance to complement, and survival within phagocytes have been identified (25–30). Bacterial meningitis resulting from nonhematogenous spread is less common and is a consequence of infection preexisting in an adjacent intracranial site. Such infections include mastoiditis, frontal sinusitis, and rupture of an intracerebral abscess into the ventricles of the brain and may occur following head trauma or neurosurgical procedures.

Pneumococcal meningitis. There are no specific distinguishing clinical features of pneumococcal meningitis. While a rash is uncommon, it is occasionally seen in splenectomized patients with overwhelming sepsis. There is a close correlation between bacteremic and meningitic serotypes of *S. pneumoniae*, implicating hematogenous spread as the commonest mode of acquisition (31). *S. pneumoniae* is the most frequent cause of bacterial meningitis in the United States and has a case fatality rate of 14.7% (14). After the introduction of the 7-valent pneumococcal conjugate vaccine (which contains serotype antigens 4, 6B, 9V, 14, 18C, 19F, and 23F) in the United States, the incidence of pneumococcal meningitis declined by 30.1%, from 1.13 cases per 100,000 population in 1998 to 1999 to 0.79 cases per 100,000 population in 2004 to 2005 (32). In patients of $<$2 years and $>$65 years of age, the incidence of pneumococcal meningitis throughout the surveillance period declined by 64.0% and 54.0%, respectively. Across all age groups, the number of meningitis cases caused by *S. pneumoniae* serotypes

covered by the 7-valent vaccine dropped by 73.3% (32). Similar reductions in invasive pneumococcal disease caused by 7-valent serotypes have also been reported in Australia, England, and Wales (33–35); however, this has led to a replacement phenomenon, in which the rates of disease caused by non-7-valent *S. pneumoniae* serotypes have significantly increased (32, 34, 35).

Meningococcal meningitis. *N. meningitidis* is most likely to cause meningitis in children and adolescents and has a mortality rate of 10.1% in the United States (14). The serotypes most commonly implicated are A, B, C, W135, and Y (14, 36–38), and meningitis without shock is the most common presentation (38). The predominant clinical feature, which can distinguish *N. meningitidis* from other causes of bacterial meningitis, is the presence of a petechial rash. It can rapidly become purpuric, which in the presence of meningitis or sepsis strongly implicates *N. meningitidis* as the etiologic agent. These lesions are a consequence of meningococci adhering to the endothelial cells of the capillaries and small veins in the skin, thereby altering the antithrombotic surface of the endothelium. This results in the formation of clots and the extravasion of erythrocytes, which appear as skin hemorrhages. Lesions of >1.0 cm usually occur in patients developing shock, with high levels of circulating endotoxin (38).

Immunization against *N. meningitidis* serotypes A and C has resulted in a significant decrease in meningococcal disease. The United Kingdom was the first country to introduce routine immunization with a monovalent serotype C conjugate vaccine, in 1999. During the first follow-up surveillance period (2000 to 2001), the incidence of serotype C meningococcal disease fell by 80% in target age groups (39). Meningococcal serotype C vaccination programs in Australia have also led to a marked decrease in disease due to *N. meningitidis* serotype C. Of the 90% of meningococcal cases in Australia in 2010 that had serotype data available, *N. meningitidis* serotype B caused 84.0% of cases (40). In 2013, a recombinant meningococcus serotype B vaccine (Bexsero) was added to the Australian Register of Therapeutic Goods for use in individuals of >2 months of age and was also approved for use in Europe. In New Zealand, the introduction of an outer membrane vesicle vaccine against an epidemic *N. meningitidis* serotype B clone in New Zealand was reported to be 80.0% effective in fully immunized children aged 6 months to <5 years (41).

***H. influenzae*, *L. monocytogenes*, and other bacterial causes of meningitis.** *H. influenzae* as a cause of meningitis is now uncommon in most Western countries due to widespread immunization against *H. influenzae* capsular type B, which was previously responsible for the majority of cases of *H. influenzae* meningitis. As a cause of meningitis, *H. influenzae* has fallen from 45.0% in 1986 (12) to 6.7% in 2003 to 2007 (14). *L. monocytogenes* is also an infrequent cause of meningitis; however, it is associated with high mortality rates (17.0% to 28.0%) (14, 42, 43). *L. monocytogenes* meningitis occurs primarily in children of <2 months of age and in immunocompromised and elderly individuals (14, 42, 43). The signs and symptoms of *L. monocytogenes* meningitis are not different from those associated with other causes of community-acquired meningitis, but CSF findings may be atypical (43, 44). Listeriosis is associated with the consumption of contaminated food, implicating the gastrointestinal tract as the portal of entry to the bloodstream.

Group B streptococcus and *E. coli* are common causes of meningitis in neonates (45, 46), with group B streptococcus accounting for 86.1% of bacterial meningitis cases in patients of <2 months of age (14). Gram-negative aerobic bacteria, such as *Klebsiella pneumoniae*, *Serratia marcescens*, *Salmonella* spp., and *Pseudomonas aeruginosa*, can occasionally be responsible for meningitis in certain patient groups, including infants and patients with head injuries or those undergoing postneurosurgical procedures or immunosuppression (31, 47, 48). *M. tuberculosis* is a common cause of chronic or subacute meningitis (49) and is associated with nonspecific symptoms and an absence of neck stiffness during early disease (50). Tuberculosis meningitis occurs following hematogenous spread of bacilli from the lungs and the establishment of a caseating Rich focus (tuberculous granuloma) in the brain cortex, meninges, or choroid plexus. Bacteria are subsequently released from the foci and enter the subarachnoid space (51).

Encephalitis

In certain circumstances, acute meningitis can be clinically indistinguishable from acute encephalitis, which refers to inflammation of the brain parenchyma in association with neurologic dysfunction (52). Encephalitis is typically characterized by headache with an acute confusional state, with or without seizures. The California Encephalitis Program identified the clinical profiles and etiologies of encephalitis in a large cohort of immunocompetent patients from 1998 to 2005 (7). A confirmed or probable etiology was identified in only 15.8% of cases; among these, viruses were the causative agent in 68.5% of cases. Herpes simplex virus is the most common cause of encephalitis in Western countries (6, 53, 54). Enteroviruses, varicella-zoster virus, Epstein-Barr virus, measles virus, and arboviruses, such as Japanese encephalitis virus, West Nile virus, and Murray Valley encephalitis virus, have also been implicated as causes of encephalitis (6, 7, 54, 55). Beckham and Tyler provided a comprehensive list of other important and emerging viral causes of encephalitis (52). *L. monocytogenes* may cause meningoencephalitis, a syndrome where features of both meningitis and encephalitis are present.

Burkholderia pseudomallei, the causal agent of melioidosis, has a relatively uncommon association with CNS involvement, accounting for around 4% of cases in northern Australia (56, 57), with distinctive clinical features being brain stem encephalitis, often with cranial nerve palsies, together with peripheral motor weakness, or occasionally just flaccid paraparesis alone (myelitis), and presenting with urinary retention (56–58). Fevers are usually a prominent feature, and headaches are common, but neck stiffness is usually mild or absent. The computed tomography (CT) scan is often normal, but dramatic changes are seen on magnetic resonance imaging, most notably a diffusely increased T_2-weighted signal in the midbrain, brain stem, and spinal cord, with variable symmetry and enhancement with gadolinium. CSF shows high protein, normal or slightly decreased glucose, and, most notably, a pleocytosis with lymphocytes usually predominant. Bulbar palsy and decreased respiratory drive often necessitate ventilation, which may be required for many weeks (56, 59–62). In northern Australia, patients infected with the flavivirus Murray Valley encephalitis virus can present with clinical features indistinguishable from those seen with melioidosis encephalomyelitis.

Neurologic melioidosis is occasionally seen outside Australia, although it appears to be less common in melioidosis patients in Southeast Asia than in Australia. Rather than the distinctive brain stem encephalitis and/or myelitis seen in Australia, patients with neurological melioidosis in Southeast Asia usually present with

macroscopic cortical brain abscesses (63), suggesting that in these cases there has been bacteremic spread to the brain. Frank meningitis is rarely seen in melioidosis. A recent report described the first case of neurological melioidosis in the United States, in a 58-year-old male who presented with low-grade fever, nausea, vomiting, headaches, diplopia, left-sided ptosis, and transient episodes of marked somnolence, after recent travel to Cambodia (64). CSF demonstrated increased levels of glucose, protein, and lymphocytes; however, microbiological investigation consistently showed no organisms by Gram stain and failed to detect any organisms on culture medium. *B. pseudomallei* was diagnosed only following the culture of biopsy material collected during a suboccipital craniectomy. The mortality of neurological melioidosis is up to 30%, with ongoing residual neurological deficits in up to 50% of patients overall (56–58).

Focal Infections

The range of focal CNS infections includes brain abscesses, subdural empyema, and epidural abscesses. Brain abscess refers to an intracerebral infection that usually begins as localized cerebritis, which develops into a collection of pus that is surrounded by a well-vascularized capsule (65). The clinical presentations of brain abscesses are variable and depend on the anatomical area of the brain that is compressed or affected. Brain abscesses commonly originate from a contiguous focus of infection, such as the middle ear, mastoid cells, or paranasal sinuses. Brain abscesses may also occur following (i) hematogenous spread of bacteria from a distant focus of infection or (ii) brain trauma or neurosurgery (66). A recent systematic review and meta-analysis of 123 brain abscess studies reported between 1935 and 2012 demonstrated that *Streptococcus* and *Staphylococcus* spp. were the most frequently isolated microorganisms, causing 34.9% and 18.9% of infections, respectively (67). Gram-negative enteric bacteria were responsible for 15.0% of cases of brain abscess and were frequently associated with polymicrobial infections. Other, less common causative agents include anaerobic bacteria (*Bacteroides* spp., *Peptostreptococcus* spp., and *Fusobacterium* spp.), *Actinomycetales*, *Haemophilus* spp., and *Pseudomonas* spp. (66–68).

Subdural empyema refers to a collection of pus in the subdural space, which is located between the dura mater and arachnoid mater layers of the meninges (see Barriers of the CNS, below). In contrast, epidural abscesses are defined as a collection of pus between the dura mater and the skull or vertebral column (69). The etiologies of subdural empyema and epidural abscesses are similar to those of brain abscesses; however, due to the anatomical location of these infections, bacteria do not need to penetrate the blood-brain barrier (BBB) to establish disease.

BARRIERS OF THE CNS

The CNS is physically protected from injury by bony structures, such as the vertebrae and the cranium, and is also enveloped by the meninges and surrounded by CSF. The meninges comprise three layers: the dura mater, arachnoid mater, and pia mater. The arachnoid mater and pia mater are separated by the subarachnoid space, which is filled with CSF that is produced by the choroid plexus in the ventricles of the brain (Fig. 1) (70). The CSF provides mechanical support, regulates ion composition, and maintains chemical stability. Once it has circulated through the ventricles of the brain, the CSF enters the subarachnoid space and drains into the blood through arachnoid villi and granulations. CSF also drains through the cribriform plate into the lymphatics within the nasal mucosa (71–73).

The complex functions of the brain are dependent on homeostatic mechanisms that regulate the ionic composition of the interstitial fluid that surrounds neurons in the parenchyma (74). This homeostasis is maintained by two cellular barriers which separate the CNS from the systemic circulation: the BBB and the brain-CSF barrier (BCSFB) (Fig. 1). These barriers protect the brain from abrupt changes in blood biochemistry while at the same time allowing delivery of nutrients and removal of metabolites. Importantly, these barriers also protect the CNS from invasion by microbes that may be present within the blood.

The Blood-Brain Barrier

The BBB (Fig. 1) exists at the level of cerebral microvessels and represents the largest interface for blood-brain exchange (75). The BBB is composed of endothelial cells that line cerebral microvessels in association with pericytes, astrocytes, and the basement membrane (collectively referred to as a neurovascular unit, in association with neurons, microglia, and peripheral immune cells). Pericytes are attached to the abluminal surface of the endothelium and, together with the astrocytic end feet, form a contiguous membrane barrier. A physical barrier is formed at the interendothelial cleft by complex junctions that regulate the flux of ions, polar molecules, and macromolecules from the systemic circulation (74, 75).

At the interendothelial cleft, two types of junctions comprise the BBB: adherens junctions and tight junctions. These junctions block the transport of a wide range of molecules, restricting paracellular permeability across the BBB (75). In addition, endothelial cells forming the BBB lack fenestrations in their plasma membranes and have a reduced number of pinocytotic vesicles compared to endothelial cells in other tissues, which restricts transcellular flux (76). Despite this, certain solutes and macromolecules may be transported across the BBB via specific and nonspecific mechanisms, such as passive diffusion and receptor and/or adsorption-mediated transcytosis, or via ATP-binding cassette transporters and solute carrier transporters, which are expressed on cerebral microvessels (75, 77–79). The transcellular and paracellular routes of entry to the CNS are highly relevant for microbial pathogens.

The Blood-Cerebrospinal Fluid Barrier

The BCSFB (Fig. 1) is formed by tight junctions between (i) epithelial cells located at the choroid plexus, in the lateral, third, and fourth ventricles of the brain; and (ii) endothelial cells of the veins and venules within the subarachnoid space. Tight junctions between the epithelial cells of the arachnoid mater also contribute to the BCSFB by forming a barrier between the subarachnoid space and the leaky fenestrated blood vessels of the dura mater. Of these BCSFB locations, the choroid plexus has a large vascular surface area and represents the major interface between fenestrated blood vessels and CSF. Similar to the BBB, the BCSFB functions to protect the CNS and helps to maintain homeostasis; however, the choroidal epithelial cells of the BCSFB also secrete CSF from the choroid plexus into the brain ventricular system, and the tight junctions between the epithelial cells have a lower electrical resistance than that of BBB endothelial cells (80). Thus, the choroid plexus BCSFB may be more vulnerable to microbial penetration via paracellular mechanisms. The BCSFB at the arachnoid mater

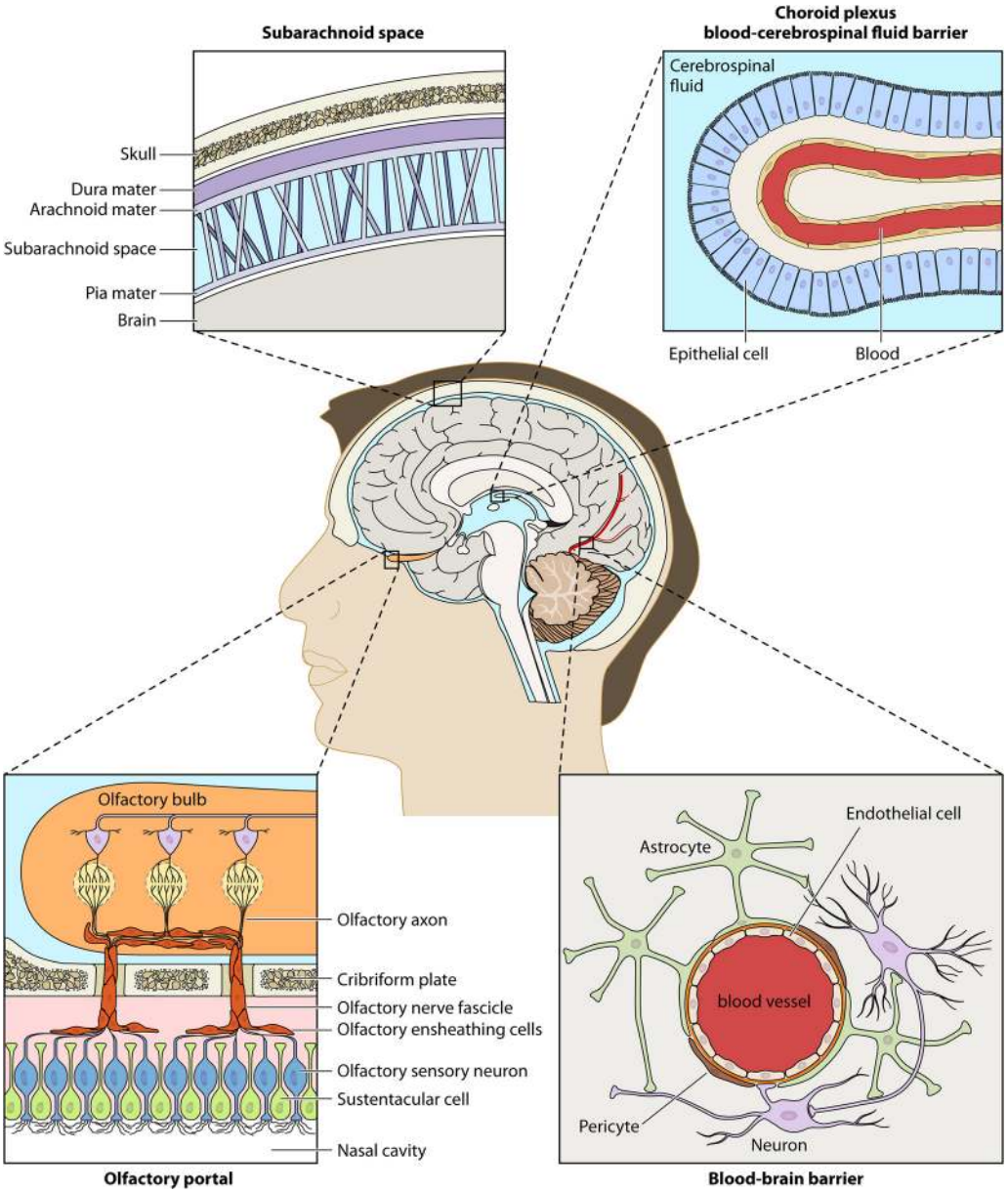

FIG 1 Anatomical locations and organizations of the blood-brain and blood-cerebrospinal fluid barriers and the olfactory portal. The skull and the meninges, which consist of the dura mater, arachnoid mater, and pia mater, protect the brain. The arachnoid mater and pia mater are connected by strands of connective tissue called arachnoid trabeculae, which course through the CSF-containing subarachnoid space. At the cellular level, the BBB and BCSFB maintain homeostasis of the brain. The BBB is formed by tight junctions between endothelial cells lining cerebral microvessels, in addition to pericytes and astrocytes. The BCSFB is formed by tight junctions between (i) epithelial cells at the choroid plexus, (ii) endothelial cells of the veins and venules within the subarachnoid space, and (iii) the epithelial cells of the arachnoid mater. The olfactory system bypasses the cellular barriers of the CNS and provides a direct portal from the nasal cavity to the olfactory bulb within the brain.

has a smaller total surface area than those of other barriers of the CNS and is not thought to be an important route for the entry of solutes into the brain (74). However, the arachnoid BCSFB may represent an important site of microbial entry.

Immunosurveillance of the CNS

The brain parenchyma and spinal cord are populated throughout by resident immune cells, the microglia, which are highly specialized tissue macrophages that are maintained through *in situ* self-renewal without reconstitution from the bone marrow (81–83). Microglia continuously survey the brain parenchyma by sampling their microenvironment with highly motile processes and protrusions (84). Recent data have also revealed that cells expressing CD11c are localized within the juxtavascular parenchyma of the healthy mouse brain and extend processes to the glia limitans (astrocytic foot processes at the parenchymal basement membrane, surrounding the brain under the pia mater) (85). In agreement with a previous study (86), this raises the possibility that dendritic cells, which are characterized by high-level CD11c expression, may reside within the brain parenchyma. However, a

range of cells of the myeloid lineage (including activated microglia) also express CD11c, albeit at lower levels. Thus, the CD11c-expressing cells described within the brain parenchyma may also represent a subpopulation of microglia (85).

Other resident immune cells of the CNS include perivascular macrophages in addition to small populations of blood-derived dendritic cells and macrophages within the choroid plexus and meninges. Dendritic cells constitute approximately 1% of immune cells within the choroid plexus and meninges (87, 88), but unlike the putative dendritic cells of the brain parenchyma, choroid plexus and meningeal dendritic cells are distinct from microglia and closely resemble classical dendritic cells that require FMS-like receptor tyrosine kinase 3 ligand for development (88). The CSF also contains a trafficking population of mononuclear cells, comprising T cells (90%), B cells (5%), monocytes (5%), and dendritic cells (<1%) (89).

Normal CNS immune surveillance occurs when central memory T cells migrate from the blood across the choroid plexus epithelium, enter the CSF, and scan the subarachnoid space. In the absence of recognizing ligands presented by local antigen-presenting cells, T cells exit the subarachnoid space and drain to the secondary lymphoid organs via the nasal mucosa (90, 91). In contrast, recognition of presented antigens results in the formation of immunological synapses and is accompanied by cellular signaling that results in the recruitment of peripheral inflammatory cells to the CNS via paracellular or transcellular diapedesis (92).

The production of cytokines, free radicals (such as reactive oxygen species), and matrix metalloproteinases by inflammatory cells within the CSF spaces can subsequently result in degradation of the glia limitans, BBB/BCSFB dysfunction, and cellular death (93–95). In bacterial meningitis, neuronal injury also occurs due to the direct effects of bacterial toxins and virulence factors (96–98). A recent study of human bacterial meningitis cases demonstrated that the profile of pro- and anti-inflammatory cytokines and matrix metalloproteinases within the CSF is dependent upon the causative pathogen (99). The sequelae of bacterial meningitis and the associated neuroinflammation include vasculitis and cerebral venous thrombosis, which can lead to cerebral ischemia. An increase in CSF outflow resistance in combination with interstitial edema results in raised intracranial pressure, which causes hypoxic brain damage and may lead to death (100).

MICROBIAL ENTRY TO THE CNS

Microbial pathogens may access the CNS (i) by penetrating the BBB or the BCSFB or (ii) via the olfactory and/or trigeminal nerves (101). Peripheral nerves other than the olfactory and trigeminal cranial nerves have also been implicated as routes of entry for some viruses, but these are not reviewed here. Penetrations of the BBB and BCSFB represent common routes of CNS invasion, resulting in encephalitis or brain abscesses and in meningitis, respectively. In contrast to a diverse group of neurotropic viruses for which mechanisms are described for invasion of the brain via the olfactory sensory nerves, bacterial infection via the olfactory epithelium and the olfactory nerve bundles has been described infrequently. Even less well understood is the fact that, in addition to the olfactory nerve, the trigeminal nerve may also present a potential pathway to the brain.

In Vivo and *In Vitro* Models of CNS Invasion

Much of our understanding of the mechanisms of microbial CNS invasion has been obtained from *in vivo* and *in vitro* studies. Mice and rats are the most common animals used to model CNS infections. These animal models have been used to investigate the sites of microbial entry (20, 102–106) and the roles of virulence determinants in CNS invasion and pathogenesis (107–111). Despite clear differences in the size and complexity of mouse and human brains, comparative transcriptomic studies between mice and humans have demonstrated that gene expression profiles of the healthy brain and choroid epithelium are highly conserved between the species (112–114).

There are, however, several important differences between the mouse and human CNS that are relevant to microbial invasion studies. In mice, the leptomeninges (comprising the arachnoid mater and pia mater) are significantly thinner and less vascular than the case in humans (115). Additionally, the human choroid plexus epithelium has more abundant fibrovascular stroma along the vascular channels and a relatively larger surface area than those of the mouse choroid epithelium. Mice also possess very large olfactory bulbs compared to humans (115), which may result in a higher incidence of CNS invasion via the olfactory route than the case in humans. Furthermore, mice lack several anatomically defined subclasses of astrocytes that are present in humans (116). These combined anatomical differences may influence the initial site of microbial attachment and subsequent CNS invasion.

In vitro cell culture systems have been used to model the BBB and to elucidate the cellular and molecular mechanisms of microbial CNS invasion. Primary human, rodent, and bovine brain microvascular endothelial cells have been used in some studies (97, 117–119); however, the isolation of these cells is technically demanding, and *in vitro* culture dramatically alters the transcriptome, leading to downregulation of genes involved in BBB function (120, 121). Human umbilical vein endothelial cells (HUVECs) (122–124), Caco-2 cells, and Madin-Darby canine kidney cells (125) have been used as surrogate models of the BBB, although the noncerebral origin of these cells may limit their relevance. The availability of immortalized brain endothelial cell lines (for example, human brain microvascular endothelial cells [HBMECs], hCMEC/D3, TY10, and BB19) has enabled the human BBB to be studied. When grown on Transwell membranes, HBMECs (i) form tight junctions, as measured by transendothelial electrical resistance; (ii) express the adherens junction and tight junction proteins zonula occludens-1, junctional adhesion molecule A, claudin-5, occludin, and vascular endothelial cadherin; and (iii) demonstrate negligible paracellular permeability (126–129).

It must be noted that brain microvascular endothelial cells grown in static monocultures represent a surrogate model for the human BBB, as they lack other components of the neurovascular unit that are required for the development of true BBB properties. Studies using pericyte-deficient mice have shown that pericytes (Fig. 1) are required for BBB integrity. Compared to wild-type mice, pericyte-deficient mice demonstrated an accumulation of water and tracer dyes within the brain, structural abnormalities in cerebral endothelial tight junctions, and increased expression of genes known to increase vascular permeability, endothelial vesicle trafficking, and immune cell recruitment (130–132). Astrocytes also play a key role in regulating the BBB (133–136), and as such, a range of coculture systems have been developed to more closely model the BBB properties of the neurovascular unit (137–139).

Choroid plexus epithelial cell lines that display the characteristic properties of the BCSFB have been described (140, 141); however, the majority of *in vitro* microbial invasion studies have been performed using monocultures of brain microvascular endothelial cells.

BACTERIAL INVASION THROUGH THE BLOOD-BRAIN AND BLOOD-CEREBROSPINAL FLUID BARRIERS

Sites of Entry

Classical bacterial meningeal pathogens may potentially enter the CSF by penetrating the BBB of cerebral microvessels and entering the extracellular fluid of the brain, which is continuous with the CSF. However, a more likely route involves direct entry to the CSF via penetration of the BCSFB at either the arachnoid or the choroid plexus (142). The highly vascularized choroid plexus has been favored as a major site of bacterial entry to the CSF due to lower electrical resistances between choroid epithelial cells. Autopsies of neonates who died from bacterial meningitis demonstrated that meningitis was frequently (84.0%) associated with an accumulation of a purulent exudate within the ventricles (ventriculitis) and the choroid plexus (plexitis) (143). Ventriculitis preceding meningitis would support the role of the choroid plexus as the initial site of bacterial invasion; however, ventriculitis frequently occurs as a complication of meningitis, and the site of bacterial CNS invasion remains incompletely understood.

In experimentally infected rhesus macaques, the choroid plexus was the site where the earliest histopathologic lesions were observed in *H. influenzae* meningitis (144). Following intranasal inoculation, *H. influenzae* was isolated from the CSF within the lateral cerebral ventricle, and this was associated with intravascular inflammatory infiltration and mild choroid plexitis (145). In 5- to 7-week-old pigs, *S. suis* was localized within the choroid plexus and caused disruption of the brush border of the choroid epithelium and loss of the apical cytoplasm (146). Interestingly, autopsy of a 2-month-old infant who died of fulminant meningococcemia prior to the onset of meningitis revealed adherent bacteria within the capillaries of the choroid plexus and, to a much lesser extent, the capillaries within the meninges (147). This would appear to support the choroid plexus as the preferential site of *N. meningitidis* entry to the CSF; however, no bacteria were found within or between choroid epithelial cells. In contrast, a study of autopsy material from symptomatic patients with meningococcal disease revealed the presence of *N. meningitidis* within the choroid plexus epithelium, interstitium, and blood vessels (148).

Neonatal rat models of *E. coli* meningitis have been used to identify the potential site of entry to the CSF. Parkkinen et al. demonstrated that *E. coli* binds to the luminal surfaces of choroid epithelial cells, vascular endothelial cells, and ependymal cells lining the brain ventricles (102). Similar findings were reported by Zelmer et al., who suggested that *E. coli* K1 enters the CSF via the choroid plexus, circulates to the subarachnoid space, and then adheres to the meninges (103). In contrast, Kim et al. reported that in the brains of 5-day-old rats, *E. coli* was localized within the perivascular areas of the subarachnoid space, not within the choroid plexus (20). Recently, the spatiotemporal events of CNS invasion were investigated in a BALB/c mouse model of pneumococcal meningitis (104). *S. pneumoniae* preferentially adhered to vessels within the subarachnoid space at 1 h postinfection and formed clusters around the endothelium at this location at later time points. Remarkably, pneumococci could be detected in the choroid plexus only at 8 h postinfection, suggesting that this is not the site of initial entry to the CSF.

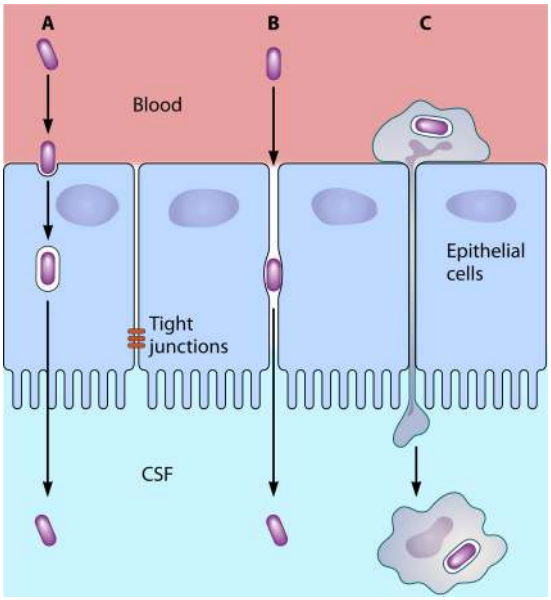

FIG 2 Mechanisms of blood-cerebrospinal fluid penetration by bacterial pathogens. Bacteria (purple) may invade the CNS via the BCSFB by transcellular penetration involving either pinocytosis or receptor-mediated mechanisms (A); by paracellular entry following the disruption of junctions (comprising tight junctions and adherens junctions) between choroidal epithelial cells, endothelial cells of veins/venules within the subarachnoid space, or the cells of the arachnoid membrane (B); and by the "Trojan horse" mechanism, where microbes may transmigrate with infected leukocytes (such as macrophages, as shown) (C).

Mechanisms of Entry

Bacteria may cross the BBB and BCSFB to access the CNS by transcellular penetration or paracellular entry or via infected leukocytes from the peripheral circulation (the "Trojan horse" mechanism) (Fig. 2). Transcellular penetration occurs following bacterial adhesion to endothelial or epithelial cells. Bacteria are subsequently translocated across these barriers by pinocytosis or receptor-mediated mechanisms. Alternatively, bacteria may invade the CNS paracellularly following disruption of the tight junctions between cells forming the BBB and/or BCSFB, resulting in increased permeability. Microorganisms may exploit more than one of these mechanisms to access the CNS.

Bacterial attachment to HBMECs is a prerequisite for transcellular and paracellular penetration. There has been significant research into the host-pathogen interactions that occur prior to bacterial invasion of brain microvascular endothelial cells (Table 1), including the identification of host receptors that mediate these interactions. *E. coli*, *H. influenzae*, *N. meningitidis*, and *S. pneumoniae* bind to HBMECs via the 37/67-kDa laminin receptor (149–152). The 37-kDa laminin receptor is a precursor for the mature, 67-kDa laminin receptor, which binds laminin-1 within the basement membrane on the abluminal surface of eukaryotic cells (153). Kim et al. (150) demonstrated that the 67-kDa laminin receptor is expressed on the basolateral side of HBMECs (i.e., the abluminal surface); however, cytoxic necrotizing factor 1 (CNF-1) of *E. coli* triggered clustering of the 67-kDa laminin receptor on

TABLE 1 Known bacterial ligands and their host receptors for adhesion to and invasion of the blood-brain barrier[a]

Bacterial pathogen	Ligand	HBMEC receptor	Function	Reference(s)
Escherichia coli (and other Gram-negative enterics)	FimH	CD48	Adhesion to HBMECs; FimH also promotes invasion by activation of RhoA, which induces host cytoskeletal rearrangements	154, 410
	Nlp1	Unknown	Adhesion to and invasion of HBMECs; Nlp1 activates host cytosolic phospholipase A_2	174
	OmpA	gp96 homologue	Loops 1, 2, and 3 of OmpA function in adhesion to and invasion of HBMECs; OmpA-mediated invasion is due to Rac1 activation and activation of host cytosolic phospholipase A_2	161, 162, 173, 178
	CNF-1	37/67-kDa laminin receptor	Invasion of HBMECs; CNF-1 activates RhoA and recruits focal adhesion kinase to the site of bacterial entry	149, 150, 171, 172
	IbeA	Vimentin, PSF	Invasion of HBMECs; activates Rac1	178, 411–413
	Ibe10	45-kDa Ibe10 receptor	Invasion of HBMECs	414, 415
	AslA	Unknown	Invasion of HBMECs	416
	TraJ	Unknown	Invasion of HBMECs	417
	YijP	Unknown	Invasion of HBMECs	418
	Flagella	Unknown	Adhesion to and invasion of HBMECs	419
Haemophilus influenzae	Phosphorylcholine	PAF receptor	Potentially involved in invasion of HBMECs	420
	OmpP2	37/67-kDa laminin receptor	Adhesion to HBMECs	151, 152
Listeria monocytogenes	InlA	E-cadherin	Invasion of polarized human choroid plexus epithelial cells	232
	InlB	gC1q-R, met receptor tyrosine kinase	Invasion of HBMECs and polarized human choroid plexus epithelial cells	213, 232, 421, 422
	Vip	gp96	Invasion of the brain *in vivo*	160
	IspC	Unknown	Adhesion to and invasion of sheep choroid plexus epithelial cells but not HBMECs	233
Mycobacterium tuberculosis	Rv0980c, Rv0987, Rv0989c, Rv1801	Unknown	Invasion of HBMECs	118
	HBHA	Heparan sulfate glycosaminoglycans	Adhesion to and invasion of BBMECs	234
	Rv0311, Rv0805, Rv0931c, Rv0986, MT3280	Unknown	Invasion of HBMECs	235
	PknD	Laminin	Attachment to, invasion of, and survival within HBMECs	158
Neisseria meningitidis	Type IV pili	Unknown	Adhesion to HBMECs; type IV pilus-mediated adhesion leads to the formation of cortical plaques and host membrane protrusions	122, 124
	PilQ and PorA	37/67-kDa laminin receptor	Adhesion to HBMECs	151, 152
	Opc	$\alpha_5\beta_1$ integrin (mediated by binding to the bridging molecules vitronectin and fibronectin)	Adhesion to and invasion of HBMECs; activates JNK1, JNK2, p38, and protein tyrosine kinase signaling	155, 191, 192
Staphylococcus aureus	YpfP and LTA	Unknown	Invasion of HBMECs	423
Streptococcus agalactiae	Lmb	Laminin	Invasion of HBMECs	159
	IagA and LTA	Unknown	Invasion of HBMECs; IagA anchors LTA to the streptococcal cell wall and is required for invasion	424
	FbsA	Fibrinogen	Adhesion to HBMECs	156
	SfbA	Integrins (via *S. agalactiae* binding to immobilized fibronectin)	Invasion of HBMECs	199

	PilA	$\alpha_2\beta_1$ integrin (mediated by *S. agalactiae* binding to immobilized collagen)	Adhesion to and invasion of HBMECs; PilA binding stimulates focal adhesion kinase phosphorylation and downstream phosphatidylinositol 3-kinase activation	109, 425
	PilB	Unknown	Invasion of HMBECs	425
	Srr-1	Fibrinogen (Srr-1 binds amino acids 283 to 410 of the fibrinogen α chain via a dock, lock, and latch mechanism)	Adhesion to and invasion of HBMECs	200, 201, 426
	HvgA	Unknown	Adhesion to HBMECs	198
	ACP	Glycosaminoglycans	Adhesion to and invasion of HBMECs; invasion potentially occurs following Rho GTPase-dependent actin rearrangements	157, 427
Streptococcus pneumoniae	CbpA	37/67-kDa laminin receptor	Adhesion to HBMECs	151
	Phosphorylcholine	PAF receptor	Invasion of HBMECs; binding to platelet activating factor receptor induces colocalization of β-arrestin	193, 197
	NanA	Unknown	Adhesion to and invasion of HBMECs	194, 195

[a] ACP, alpha C protein; AslA, aryl-sulfatesulfohydrolase; BBMEC, bovine brain microvascular endothelial cells; CbpA, choline binding protein A; CNF-1, cytotoxic necrotizing factor 1; FbsA, fibrinogen-binding protein; FimH, type 1 fimbrial adhesin; HBHA, heparin-binding hemagglutinin adhesin; HBMECs, human brain microvascular endothelial cells; HvgA, hypervirulent group B streptococcus adhesion; IbeA, invasion of brain endothelial cell protein A; IagA, invasion-associated gene A; InlA, internalin A; InlB, internalin B; LTA, lipoteichoic acid; Lmb, laminin-binding protein; NanA, neuraminidase A; Nlp1, new lipoprotein 1; OmpP2, outer membrane protein P2; Opc, outer membrane protein C; PAF, platelet activating factor; PilQ, pilus secretin protein; PorA, major outer membrane protein; PSF, polypyrimidine-tract-binding protein (PTB)-associated splicing factor; SfbA, streptococcal fibrinogen binding protein A; Srr-1, serine-rich repeat 1; Vip, virulence protein.

the apical side (i.e., the luminal surface), where it could potentially interact with bacteria within the cerebral microvasculature. In an *in vivo* model, Orihuela et al. reported that fluorescent microspheres coated with laminin receptor-binding adhesins (such as choline-binding protein A [CbpA] of *S. pneumoniae*) adhered to the cerebral endothelium of mice via the 37/67-kDa laminin receptor following intravascular injection, as demonstrated by cranial window imaging (151). Several bacterial meningeal pathogens have been shown to bind host glycoproteins, such as fibrinogen, vitronectin, fibronectin, laminin, and collagen, which may act as bridging molecules between the bacterium and HBMECs (109, 154–159). In addition, the heat shock protein gp96 and a gp96 homologue act as receptors for the surface-expressed virulence protein (Vip) of *L. monocytogenes* (160) and outer membrane protein A (OmpA) of *E. coli* (161, 162), respectively.

Transcellular Penetration of Brain Microvascular Endothelial Cells

Transcellular penetration of brain microvascular endothelial cells, mainly via receptor-mediated mechanisms (Fig. 2), has been demonstrated for several bacteria, including *E. coli*, *N. meningitidis*, *S. pneumoniae*, group B streptococcus, *S. suis*, *L. monocytogenes*, and *M. tuberculosis*.

E. coli. *E. coli* translocates from the upper compartment to the lower compartment in an HBMEC Transwell model without increasing cellular permeability, thus supporting a transcellular mechanism of penetration (163). Electron microscopy studies have demonstrated that *E. coli* invades HBMECs through a zipper-like, receptor-mediated endocytosis mechanism (164). Intracellular *E. coli* E44 bacteria were observed in membrane-bound vesicles within HBMECs at 30 min postinfection; by 45 min, the bacteria had translocated to the basolateral side (164). Both early (early endosome antigen 1 and transferrin receptor) and late (Ras-related protein 7 and lysosome-associated membrane protein 1) endosomal markers were recruited to vacuoles containing *E. coli* K1. However, the lysosomal hydrolytic enzyme cathespin D did not accumulate within the vacuoles, demonstrating that lysosomal fusion was prevented. This was found to occur in a capsule-dependent manner (165).

Several *E. coli* proteins contribute to the adhesion to and invasion of HBMECs (Table 1). Attachment of *E. coli* to HBMECs induces host cytoskeletal rearrangements and actin condensation below adherent bacteria (164). This is associated with a CNF-1-mediated recruitment of focal adhesion kinase and the cytoskeletal protein paxillin to the 67-kDa laminin receptor, which forms clusters that colocalize with adherent *E. coli* (150). Interactions between *E. coli* and HBMECs result in tyrosine phosphorylation of focal adhesion kinase and paxillin. The activity of focal adhesion kinase and its autophosphorylation site, tyrosine 397, were shown to be essential for *E. coli* invasion of HBMECs (166). Furthermore, the Src kinase-dependent activation of phosphatidylinositol 3-kinase and its interaction with focal adhesion kinase were required for *E. coli* K1 invasion and host cytoskeletal rearrangement (167, 168). Sukumaran and Prasadarao demonstrated that the association of phosphatidylinositol 3-kinase with focal adhesion kinase resulted in the downstream activation of protein kinase Cα (PKCα) in an Omp-A-dependent manner (169). Activated protein kinase Cα was recruited to the *E. coli* entry site, where it interacted with its substrate (myristoylated alanine-rich C kinase

substrate), leading to an accumulation of actin (169). The activated protein kinase Cα also interacted with caveolin-1 colocalized with condensed actin underneath the bacterial entry site to form caveolae, which are plasma membrane invaginations that are involved in endocytosis and signal transduction (170). CNF-1 (via the 37/67-kDa laminin receptor) and type 1 fimbrial adhesin (FimH; via CD48) have also been shown to induce cytoskeletal rearrangements through activation of the GTPase RhoA (149, 171, 172).

In an independent mechanism, *E. coli* OmpA and new lipoprotein 1 (Nlp1) promote the activation of host cytosolic phospholipase A_2 (173, 174). The activation of cytosolic phospholipase A_2 generated arachidonic acid metabolites, induced host actin cytoskeletal rearrangements, and was essential for *E. coli* K1 invasion of HBMECs (173, 175–177). OmpA and invasion of brain endothelial cell protein A (IbeA) also trigger the phosphorylation of STAT3, which results in the activation of Rac1, a Rho family GTPase that regulates host cytoskeletal rearrangements (178). Notably, OmpA is highly conserved between Gram-negative bacteria and may play a role in CNS invasion by those whose mechanisms of entry are unknown. Similarly, a *B. pseudomallei* toxin, *Burkholderia* lethal factor 1 (BLF-1), is structurally similar to *E. coli* CNF-1 and possesses a number of conserved residues that, in CNF-1, promote deamidation of glutamine and result in the activation of RhoA and subsequent host cytoskeletal rearrangements (179). Thus, OmpA and BLF-1 may be attractive targets for future studies of the mechanisms of *B. pseudomallei* CNS invasion.

N. meningitidis. The attachment of *N. meningitidis* to HBMECs is mediated by type IV pili. This initial attachment is inhibited by high cerebral microcirculation shear stress levels; however, once attached, *N. meningitidis* proliferates and is able to resist high blood velocities by forming microcolonies with strong bacterium-bacterium interactions (124) and bacterium-host interactions (122). CD46 was previously identified as the receptor for type IV pili (180); however, this was not corroborated by additional studies, and it is likely that the type IV pilus attaches to host cells independently of CD46 (181). The type IV pilus protein PilQ, in addition to the outer membrane porin PorA, was demonstrated more recently to bind to the 37/67-kDa laminin receptor on HBMECs (151). Furthermore, studies with human bronchial epithelial cells show that the meningococcal type IV pilus binds to the platelet activating factor (PAF) receptor and that synergy between a pilin-linked glycan and phosphorylcholine decorating moieties is required for pili to efficiently engage the receptor (182).

N. meningitidis is thought to invade HBMECs primarily by paracellular mechanisms (see below); however, *in vitro* studies have demonstrated that small numbers of both encapsulated and nonencapsulated meningococci are internalized by HUVECs and HBMECs (183–185). Following initial bacterial attachment, the meningococcal minor pilin protein, PilV, triggers a cellular response in which endothelial cells form protrusions around *N. meningitidis* (122). These cellular projections further protect the bacterial microcolony from the mechanical stresses associated with high-velocity blood flow (122) and also lead to the engulfment and internalization of *N. meningitidis* (186). The host signaling events that contribute to these interactions have been elucidated. Following type IV pilus-mediated attachment, *N. meningitidis* activates the host cell β2-adrenoceptor, leading to the translocation of β-arrestin to the site of bacterial attachment at the plasma membrane (187). Several host proteins, including ezrin and moesin, which regulate the cortical cytoskeleton through F-actin-binding sites, are then recruited to the site of bacterial attachment and accumulate in honeycombed molecular complexes, referred to as cortical plaques, underneath the meningococcal microcolonies (186, 188, 189). The translocation of β-arrestin also results in the docking and activation of the protein tyrosine kinase Src, which phosphorylates cortactin (187, 188). Cortical actin is then polymerized in a Rho GTPase- and Cdc42-dependent manner, which leads to the formation of cell membrane protrusions (186). In human bone marrow endothelial cells, the recruitment of cortactin and formation of membrane protrusions were also shown to be dependent on the activation of a phosphoinositide-3-kinase/Rac1 signaling pathway by lipooligosaccharide (190).

Once internalized, the membrane-bound vesicles containing meningococci associate with transferrin receptor and lysosome-associated membrane protein 1 endosomal markers (185). It is likely that lysosomal fusion is subverted, as live meningococci have been shown to translocate from the basolateral side to the apical side in a Transwell BCSFB invasion model (140). *In vitro* studies have demonstrated that capsular polysaccharide inhibits the invasion of *N. meningitidis* into HBMECs (191) and human choroid plexus epithelial cells (140) but is essential for survival and replication within the intracellular niche (185).

For nonencapsulated meningococcal strains, the invasion of HBMECs is dependent upon outer membrane protein C (Opc). Opc binds to the human serum factor fibronectin, which acts as a bridging molecule and anchors the bacterium to the $\alpha_5\beta_1$ integrin receptor of HBMECs (191). This interaction results in the activation of mitogen-activated protein kinase (c-Jun N-terminal kinase 1 [JNK1], JNK2, and p38) and protein tyrosine kinase signal transduction pathways (192). Pretreatment of cells with specific JNK1, JNK2, and protein tyrosine kinase inhibitors significantly reduced the internalization of *N. meningitidis* by HBMECs, without affecting bacterial adherence. Blocking of the $\alpha_5\beta_1$ integrin receptor of HBMECs also decreased JNK activation in the presence of *N. meningitidis*. Furthermore, the use of Opc-deficient mutant strains demonstrated that JNK signaling, but not p38 signaling, was mediated by Opc expression (192). A more recent study identified that Opc preferentially binds to activated vitronectin within human serum and that this interaction promotes *N. meningitidis* invasion of HBMECs (155). *In vitro*, the enhanced invasion of nonencapsulated *N. meningitidis* strains is thought to be due to the unmasking of Opc; however, the role of Opc *in vivo* has yet to be determined.

S. pneumoniae. *S. pneumoniae* is internalized by human and rat brain microvascular endothelial cells via receptor-mediated endocytosis. This uptake does not involve the formation of cellular membrane protrusions as discussed for *N. meningitidis* (193). The adhesion of pneumococci to HBMECs is mediated by interactions between CbpA and the 37/67-kDa laminin receptor (151) and between neuraminidase A (NanA) and an unknown cellular receptor(s) (194). Uchiyama et al. demonstrated that NanA, a surface-anchored sialidase, was necessary and sufficient to promote pneumococcal adhesion to and invasion of HBMECs (195). Following initial attachment, the laminin G-like domain of NanA initiates chemokine signaling and inflammatory activation of endothelial cells (194). The activation of endothelial cells results in the increased expression of PAF receptor—the receptor for pneumococcal phosphorylcholine, which is a component of the cell wall and acts as a molecular mimic of the chemokine PAF (196).

In HBMECs, *S. pneumoniae* within vacuoles may (i) transit

through the cell to the basolateral surface, (ii) recycle back to the apical surface, or (iii) be killed within the vacuole (193). Opaque *S. pneumoniae* variants, which express more capsular polysaccharide and less phosphorylcholine than transparent variants, were efficiently killed, suggesting that vacuoles containing these strains were targeted for lysosomal fusion. In contrast, transparent pneumococci may undergo some recycling back to the apical surface; however, by 5 h postinfection in a Transwell system, the majority of bacteria had translocated to the basolateral chamber (193). The invasion of HBMECs by transparent *S. pneumoniae* depends on phosphorylcholine, the PAF receptor, and β-arrestin, which targets G-protein-coupled receptors (such as PAF receptor) to clathrin-coated vesicles (193, 197). *S. pneumoniae* induces the translocation of β-arrestin from the cytosol to the plasma membrane, where it colocalizes with the PAF receptor. This interaction also stimulates mitogen-activated protein kinase signaling, which is required for pneumococcal uptake (197). Vacuoles containing *S. pneumoniae* colocalized with early and late endosomal markers; however, increased expression of β-arrestin subverted these vacuoles from lysosomal trafficking and promoted the transcytosis of viable bacteria. The small number of pneumococcus-containing vacuoles that underwent recycling to the apical surface demonstrated colocalization with Ras-related protein 11, which regulates endosome recycling. These findings suggest that the pneumococcus-induced interactions between PAF receptor and β-arrestin contribute to the transcellular penetration of HBMECs by *S. pneumoniae* (197).

Group B streptococcus. Several group B streptococcal proteins promote adhesion to and invasion of HBMECs (Table 1). The hypervirulent group B streptococcus adhesin (HvgA) was identified as a sequence type 17 (ST-17)-specific virulence factor which is anchored to the group B streptococcal cell wall by sortase A (198). ST-17 is a clonal complex belonging to the group B streptococcus capsular serotype III and is responsible for >80% of neonatal meningitis cases. Therefore, it was proposed that HvgA expression may contribute to the hypervirulence of ST-17. *In vitro* studies using an isogenic *hvgA* mutant demonstrated that HvgA was required for efficient group B streptococcal adhesion to HUVECs, primary rodent choroid plexus epithelial and brain microvascular endothelial cells, and immortalized HBMECs. Furthermore, in a murine model of hematogenous meningitis, HvgA was required for CNS invasion, although it had no effect on the levels of bacteremia (198).

A common characteristic of several other group B streptococcal adhesins/invasins is the ability to bind components of the HBMEC extracellular matrix, such as laminin, collagen, and fibronectin (109, 157, 159, 199). The group B streptococcal pilus tip adhesin (PilA) and the recently identified streptococcal fibronectin-binding protein A (SfbA) bind immobilized collagen and fibronectin, respectively. The collagen and fibronectin then associate with HBMECs via integrins, and these interactions facilitate the entry of group B streptococcus (109, 199). Similarly, the group B streptococcal serine-rich repeat 1 (Srr-1) glycoprotein binds the fibrinogen Aα chain within human blood through a dock, lock, and latch mechanism (200, 201). During this interaction, fibronectin docks between two IgG-like folds (N2 and N3 domains) of the binding region of the adhesin, which initiates a conformational change whereby a flexible region of the N3 domain latches to form a β-strand and completes a β-sheet within the N2 domain, effectively locking the ligand in place (200). Seo et al. demonstrated that deletion of the latch-like domain of the C-terminal end of the Srr-1 fibrinogen-binding region significantly reduced group B streptococcal adhesion to HBMECs (201). The fibrinogen-binding protein (FbsA) also mediates group B streptococcal adherence to HBMECs, by binding immobilized fibrinogen (156), although it is unknown if this interaction also occurs via a dock, latch, and lock mechanism.

Electron microscopy studies have demonstrated that *in vitro*, group B streptococcus associates closely with the cell membrane of HBMECs and is enveloped by microvillous structures (202). Group B streptococci are internalized within membrane-bound vesicles and translocate from the apical side to the basolateral side of HBMECs without a marked change in transendothelial electrical resistance. Serotype III group B streptococci were shown to invade HBMECs more efficiently than representative strains of serotypes Ia, Ib, II, and V; however, the serotype III capsule itself did not facilitate group B streptococcal invasion (202). Internalized group B streptococci did not undergo significant replication and survived within HBMECs for up to 20 h (202). Intracellular survival of group B streptococcus may be promoted by the CiaR-CiaH two-component regulatory system, which regulates several genes associated with stress tolerance and the subversion of host defenses (203). A serotype III strain that was deficient in the CiaR response regulator demonstrated adhesion and invasion levels similar to those of the wild-type group B streptococcus in HBMECs but was associated with a significant decrease in intracellular survival. Broth inhibition assays demonstrated that CiaR conferred resistance to antimicrobial peptides, lysozyme, and reactive oxygen species (203).

The host signal transduction pathways that are involved in the uptake of group B streptococcus by HBMECs are similar to those discussed for *E. coli*. *In vitro*, the trimolecular interactions between PilA, collagen, and $\alpha_2\beta_1$ integrins on HBMECs stimulate the phosphorylation of focal adhesion kinase at tyrosine 397, which then induces host cytoskeletal rearrangements and the uptake of group B streptococci via the recruitment and activation of phosphatidylinositol 3-kinase and paxillin (109, 204). It has also been demonstrated that group B streptococcus invasion occurs via cytoskeletal rearrangements that are induced following the activation of RhoA and Rac1 (205). Furthermore, group B streptococcus induces serine 505 phosphorylation of host cytosolic phospholipase A_2, which leads to the release of arachidonic acid metabolites, including cysteinyl leukotrienes (206). *In vitro* pharmacological inhibition studies demonstrated that the activation of cytosolic phospholipase A_2 was required for efficient group B streptococcal invasion of HBMECs. In addition, brain colonization by group B streptococcus was significantly reduced in mice deficient in cytosolic phospholipase A_2 compared to wild-type animals in a model of hematogenous meningitis. Following cytosolic phospholipase A_2 activation and the release of cysteinyl leukotrienes, downstream signaling activates protein kinase Cα, which is involved in the regulation of actin cytoskeletal rearrangements (206).

S. suis. The zoonotic pathogen *S. suis* adheres to but does not invade HBMECs *in vitro* (207). In contrast, invasion of porcine brain microvascular endothelial cells has been observed for this swine pathogen. Inhibition studies demonstrated that *S. suis* invasion of porcine brain microvascular endothelial cells required actin filaments but not microtubular cytoskeletal elements or active bacterial RNA or protein synthesis (208). Electron microscopy studies showed that *S. suis* adhered to porcine brain microvascular endothelial cells at 5 min postinfection, and at 2 h postinfection, the streptococci were in close contact with the cells, within invagi-

nated cell membrane structures and underneath the cell surface, behind the cell membrane. Unlike the case of group B streptococci, following internalization within membrane-bound vacuoles, the number of viable intracellular *S. suis* organisms steadily decreased over time, and viable cocci were not detected after 7 h (208). However, similar to the case with *S. pneumoniae* and group B streptococcus, the capsular polysaccharide of *S. suis* partially inhibited interactions with brain microvascular endothelial cells (208).

The *S. suis* proteins and virulence factors that mediate invasion of porcine brain microvascular endothelial cells remain largely unknown. Pretreatment of porcine brain microvascular endothelial cells with *S. suis* lipoteichoic acid reduced but did not abolish the adhesion and invasion of *S. suis*, in a dose-dependent manner, suggesting that lipoteichoic acid may play a role in *S. suis* interactions with porcine brain microvascular endothelial cells (209). Vanier et al. screened a transposon mutagenesis library of *S. suis* and identified several genes that may contribute to the invasion of porcine brain microvascular endothelial cells (210). These genes were characterized as encoding proteins belonging to the following groups: surface proteins, transport/binding proteins, regulatory functions, metabolism, amino acid synthesis, protein synthesis, and hypothetical proteins. However, the roles of these genes in *S. suis* invasion of porcine brain microvascular endothelial cells have not been confirmed using isogenic mutants.

As discussed above, the choroid plexus may be the site of entry to the CNS for *S. suis*. In order to study the interactions between *S. suis* and choroid plexus epithelial cells, Tenenbaum et al. developed an inverted Transwell model in which the translocation of *S. suis* from the basolateral, "blood" side to the apical, "CSF" side could be investigated (211). Following *S. suis* infection of the basolateral side, the transepithelial electrical resistance and paracellular permeability remained constant for up to 4 h. *S. suis* invaded porcine choroid plexus epithelial cells from the basolateral side in the inverted Transwell system; however, invasion was rare when *S. suis* was inoculated into the apical chamber in the standard Transwell model. These findings were also replicated in a human choroid plexus epithelial cell inverted Transwell model (140). This suggested that *S. suis* may interact with components of the extracellular matrix that are accessible on the basolateral side. The translocation of *S. suis* through porcine brain microvascular endothelial cells was inhibited when cells were treated with a phosphatidylinositol 3-kinase inhibitor, demonstrating that phosphatidylinositol 3-kinase may be involved in the uptake of *S. suis* (211).

***L. monocytogenes* and *B. pseudomallei*.** *L. monocytogenes* can enter the CNS by the Trojan horse mechanism (see below) or by transcellular penetration of HBMECs. Using HUVECs and HBMECs, it was demonstrated that *L. monocytogenes* can invade endothelial cells directly or via cell-to-cell spread from adherent, infected mononuclear phagocytes (212, 213). In HUVECs, *L. monocytogenes* attaches to the cellular surface and induces membrane ruffling, which leads to internalization (214). In HBMECs, *L. monocytogenes* invasion is preceded by intimate interactions between the bacterium and microvilli on the cell surface (215). The invasion of *L. monocytogenes* into HBMECs requires actin microfilaments (213), but unlike the case for other meningeal pathogens, it does not involve the activation of phosphatidylinositol 3-kinase (213) or cytosolic phospholipase A_2 (175).

Once internalized, *L. monocytogenes* degrades the phagosome via the activity of a pore-forming toxin (listeriolysin) and two phospholipases (PlcA and PlcB), and it escapes into the cytoplasm by using actin-based motility, which is driven by the actin assembly-inducing protein, ActA. Following escape into the cytoplasm, *L. monocytogenes* can replicate within HUVECs and HBMECs and use actin-based motility to spread to adjacent cells (212, 213), thus avoiding an extracellular lifestyle. However, *L. monocytogenes* can be isolated from the CSF (216), suggesting that translocation through the cellular barriers of the CNS also occurs.

B. pseudomallei has an intracellular lifestyle similar to that of *L. monocytogenes*: the type III secretion system of *B. pseudomallei* (particularly the BopE and BopA effector proteins and the Bsa translocation apparatus) is required for escape from the vacuole and the subversion of autophagy (217–222). In an epithelial cell line or a murine macrophage-like cell line, cell-to-cell spread then occurs by cell fusion, with the formation of multinucleated giant cells, in a type VI secretion-dependent process. BimA, which is necessary for actin-mediated motility, and Fla2, which is thought to mediate intracellular motility, are also required and are considered to mediate cell-to-cell contact prior to cell fusion (223–227). To our knowledge, the ability of *B. pseudomallei* to directly invade HBMECs has not been investigated, but it would be reasonable to hypothesize that the intracellular lifestyle of *B. pseudomallei* may contribute to the pathogenesis of CNS invasion. Interestingly, a recent study investigated the variable virulence factors of *B. pseudomallei* associated with melioidosis in Australia and reported that *B. pseudomallei* strains harboring a *bimA* allele that shares 95% homology with *B. mallei bimA* ($bimA_{Bm}$) (228) were significantly associated with neurological melioidosis (229). Patients that were infected with *B. pseudomallei* harboring the $bimA_{Bm}$ allele were found to be 14 times more likely to present with neurological involvement than patients infected with strains harboring the *B. pseudomallei bimA* variant ($bimA_{Bp}$) (229), indicating a role for actin-mediated motility in either transgression of the BBB/BCSFB or transit to the brain via the olfactory or trigeminal nerve pathways (see below).

Several studies have shown that internalin B (InlB) is required for *L. monocytogenes* invasion of HUVECs (212–214) and HBMECs (213, 215). Greiffenberg et al. demonstrated that although *L. monocytogenes* deficient in InlB could adhere to HBMECs at levels comparable to those of the wild-type strain, the invasive capability of the InlB mutant was reduced >100-fold (215). In contrast, one study reported that neither InlA nor InlB was required for *L. monocytogenes* invasion into HUVECs (230). It was suggested that these inconsistent findings may be attributable to differences in experimental conditions, especially involving the addition of normal human serum, which markedly affects InlB-mediated invasion due to the presence of anti-*Listeria* antibodies (231). In an inverted Transwell model of human choroid plexus epithelial cells, the cellular receptors for InlA and InlB (E-cadherin and met receptor tyrosine kinase, respectively) were expressed on the basolateral, "blood" side but not the apical, "CSF" side (232). In this model, *L. monocytogenes* invaded human choroid plexus epithelial cells exclusively from the basolateral side, and both InlA and InlB were required for efficient invasion.

Other *L. monocytogenes* virulence factors that may play a role in the attachment to and invasion of HBMECs include Vip and the autolysin IspC. Vip is a surface-expressed protein that is absent from nonpathogenic *Listeria* and binds to gp96 on host cells (160). A *vip* allelic replacement mutant was shown to be significantly less invasive than wild-type *L. monocytogenes* in Caco-2 cells and

L2071 mouse fibroblasts but was not investigated in HBMECs. However, in intravenously inoculated mice, the Δ*vip* strain was attenuated for virulence and associated with a significant decrease in bacterial loads within the brain (160). Similarly, IspC was also shown to contribute to *L. monocytogenes* invasion of the brain in a mouse model of hematogenous meningitis (233). Interestingly, the deletion of IspC did not affect the ability of *L. monocytogenes* to attach to and invade HBMECs *in vitro*. In contrast, the Δ*ispC* strain demonstrated a significant reduction in attachment and invasion in sheep choroid plexus epithelial cells (compared to wild-type *L. monocytogenes*), and purified IspC was capable of binding to these cells. Proteomic analyses revealed that IspC regulates the expression of other virulence factors, including ActA, InlC2, and a flagellin homologue (FlaA); therefore, the phenotype observed for the Δ*ispC* strain may be a result of the deletion of IspC in combination with a reduction in the expression of these factors (233).

M. tuberculosis. *M. tuberculosis* translocates from the apical chamber to the basolateral chamber in a Transwell model of HBMECs (118). *In vitro*, *M. tuberculosis* interacts with microvillus-like protrusions on HBMECs and is observed intracellularly but not paracellularly. Antibiotic protection assays demonstrated that *M. tuberculosis* invaded HBMECs. Furthermore, *M. tuberculosis* colocalized with actin on the cell surface. Treatment of HBMECs with cytochalasin D significantly decreased *M. tuberculosis* invasion, suggesting that actin polymerization is required for internalization (118).

The mechanisms and host-pathogen interactions involved in CNS invasion by *M. tuberculosis* remain incompletely understood. In an *in vitro* BBB model consisting of bovine brain microvascular endothelial cells (BBMECs) cocultured with rat astrocytes, the addition of purified recombinant heparin-binding hemagglutinin adhesin (rHBHA) induced actin filament rearrangement (234). This was shown to be dependent on the ability of rHBHA to bind to heparan sulfate glycosaminoglycans on the surfaces of BBMECs. The role of HBHA in brain microvascular endothelial cell invasion by *M. tuberculosis* has not been investigated in the context of live bacteria. Furthermore, gene expression profiling of *M. tuberculosis* associated with HBMECs *in vitro* (versus nonadherent bacteria) did not identify HBHA as a gene that was significantly upregulated during infection (118). Jain et al. demonstrated that 33 genes were upregulated >8-fold in *M. tuberculosis* associated with HBMECs. Transposon mutants were created to determine if these upregulated genes were associated with HBMEC invasion; of the 33 tested genes, 4 (*Rv0980c*, *Rv0987*, *Rv0989c*, and *Rv1801*) were required for efficient invasion and/or intracellular survival (118).

In vivo screening of transposon mutant libraries has also been employed to identify potential *M. tuberculosis* virulence factors that are associated with CNS invasion. Be et al. inoculated pools of *M. tuberculosis* mutants intravenously into BALB/c mice and determined that mutants deficient in the expression of 5 genes (*Rv0311*, *Rv0805*, *Rv0931c*, *Rv0986*, and *MT3280*) were significantly attenuated in the brain but not the lungs. *In vitro* studies confirmed that these mutants invaded HBMECs at significantly reduced levels compared to that of a negative-control transposon mutant (235). *Rv0931c* (also known as *pknD*) encodes a serine-threonine protein kinase and was also identified as a key microbial factor required for brain infection in a guinea pig model of hematogenous meningitis, from a library of 398 mutants (158). When studied independently in a mouse model of infection, the *pknD* mutant was associated with significant reductions in bacterial loads within the brain but not the lungs. *In vitro*, *pknD* was required for efficient invasion and intracellular survival within HBMECs; this phenotype was not observed in HUVECs, A549 lung epithelial cells, or J447 macrophages. Microspheres coated with *pknD* adhered to HBMECs at significantly higher levels than those of microspheres coated with bovine serum albumin, and it was proposed that *pknD* binds to HBMECs via interactions with laminin within the extracellular matrix (158).

Paracellular Penetration of Brain Microvascular Endothelial Cells

The breakdown of tight junctions between cells of the BBB and/or BCSFB may occur due to the activity of microbial virulence factors/toxins and/or inflammation-mediated processes. Early studies in intracisternally infected rats demonstrated that *E. coli* K1, *S. pneumoniae*, and *H. influenzae* type B caused an increase in pinocytotic vesicle formation in the cerebral capillary endothelium, in addition to the complete separation of intercellular junctions (236). Further experiments with *H. influenzae* showed that these cerebral capillary morphological changes were associated with a significant increase in BBB permeability to circulating ^{125}I-albumin (236). This suggests that bacterial meningeal pathogens may open a paracellular route of CNS entry, although these data should be interpreted cautiously, as intracisternal inoculation cannot be used to model the physiological events that occur during hematogenous meningitis because the inoculum is injected directly into the cisternal spaces between the arachnoid mater and pia mater. The development of *in vitro* models of the human BBB/BCSFB and *in vivo* models of hematogenous meningitis has enabled microbial invasion via the paracellular route to be studied in some detail. From these studies (reviewed below for specific pathogens), it appears that microbes may initially attach to and transcellularly invade brain microvascular endothelial cells and that these host-pathogen interactions may lead to a subsequent increase in barrier permeability and a loss of tight junctions. Thus, it is likely that both transcellular and paracellular mechanisms of CNS entry are exploited by bacterial pathogens. It is of considerable clinical importance that the breakdown of the BBB/BCSFB in bacterial meningitis also results in generally higher levels of antibiotics in the CSF.

E. coli. Several *E. coli* virulence factors promote the destruction of tight junctions between brain microvascular endothelial cells. In addition to triggering the internalization of *E. coli* K1 into HBMECs, the binding of OmpA to its gp96 homologue receptor stimulates inducible nitric oxide synthase and nitric oxide production, which enhances the generation of cyclic GMP (237). This increase in cyclic GMP activates protein kinase Cα (237), which leads to a Ras GTPase-activating-like protein (IQGAP1)-mediated dissociation of β-catenin from vascular endothelial cadherin at adherens junctions (238). Immunofluorescence experiments demonstrated that vascular endothelial cadherin was redistributed from the intercellular junctions to the sites of bacterial attachment (239). This resulted in a significant increase in HBMEC permeability to horseradish peroxidase and a parallel decrease in transendothelial electrical resistance (239). Furthermore, the activation of host cytosolic phospholipase A_2 by *E. coli* OmpA and Nlp1 may play a role in reducing the integrity of the BBB. Using cocultures of primary BBMECs and primary bovine retinal pericytes seeded onto Transwell membranes, Salmeri et al. demonstrated that the arachidonic acid released following cytosolic

phospholipase A_2 activation acts as a substrate for cyclooxygenase to produce prostaglandins, which trigger the synthesis of vascular endothelial growth factor by brain endothelial cells (119). This was associated with a significant decrease in transendothelial electrical resistance and a dramatic detachment of pericytes from the Transwell membrane. These effects were not observed when the pericyte vascular endothelial growth factor receptor 1 was blocked using a polyclonal antibody. These findings suggest that the vascular endothelial growth factor released by brain endothelial cells may bind to the vascular endothelial growth factor receptor 1 on adjacent pericytes and trigger ablation of the pericytes from the basal membrane, potentially opening a paracellular route (119).

In vitro studies using the mouse brain microvascular endothelial cell line Bend.3 have demonstrated a role for lipopolysaccharide (LPS; from *E. coli* O55:B5) in inducing BBB permeability. Similar to OmpA, LPS was shown to activate protein kinase C isoforms, which led to the activation of RhoA and the stimulation of downstream NF-κB signaling and myosin light chain phosphorylation (240, 241). These signaling events were associated with a decrease in the expression of the tight junction proteins claudin-5 and zonula occludens-1 and a reduction in transendothelial electrical resistance. LPS also induced changes in F-actin organization, leading to paracellular gaps and stress fiber formation (240). In Shiga toxin-producing *E. coli* associated with hemolytic-uremic syndrome and CNS involvement, both Shiga toxin 1 and Shiga toxin 2 induce cytotoxic effects that lead to a decrease in BBB integrity and to cellular injury *in vivo* and *in vitro* (242–245). LPS may mediate these effects (242, 245).

H. influenzae. Quagliarello et al. first demonstrated that *H. influenzae* causes an increase in BBB permeability and tight junction disruption in adult Wistar rats following intracisternal inoculation of approximately 10^6 CFU (236). Using this intracisternal inoculation model, the role of *H. influenzae* type B lipooligosaccharide (LOS) was investigated. Purified LOS and outer membrane vesicles containing non-cell-associated LOS induced a dose-dependent increase in BBB permeability to ^{125}I-bovine serum albumin, as well as CSF pleocytosis (246, 247). In leukopenic rats, no increases in BBB permeability and CSF leukocyte counts were observed following LOS or outer membrane vesicle challenge, suggesting that the decrease in BBB integrity is mediated by the intact host leukocyte response (246, 247). In contrast, Tunkel et al. demonstrated that *H. influenzae* type B LOS significantly increased the permeability of rat brain microvascular endothelial cells to ^{125}I-albumin in the absence of host inflammatory cells *in vitro* (248). Furthermore, in the absence of monocytic cells, purified LOS was cytotoxic to BBMECs. However, *in vivo* studies have shown that nitric oxide production within the CSF is required for LOS-mediated pleocytosis and increased BBB permeability (249) and that host PAF synergizes with *H. influenzae* LOS to augment these events (250).

Peptidoglycan within the *H. influenzae* cell wall may also contribute to BBB/BCSFB dysfunction *in vivo*. Intracisternally inoculated peptidoglycan induced an increase in BBB permeability to ^{125}I-albumin (251), as well as CSF leukocytosis (252), although the CSF leukocytosis was attenuated compared to that in animals challenged with LOS (252). It was subsequently hypothesized that *H. influenzae* cell wall components, such as peptidoglycan, activate brain microvascular endothelial cells and induce the separation of intercellular junctions. LOS may then augment this response by activating the recruited leukocytes to produce proinflammatory cytokines that further contribute to BBB breakdown (252); however, the mechanisms by which this may occur have not been elucidated. The *H. influenzae* type B porin may also contribute to these events. The inoculation of purified porin into the fourth cerebral ventricle of rats induced the expression of interleukin-1α (IL-1α), tumor necrosis factor alpha (TNF-α), and macrophage inflammatory protein 2, which preceded an increase in BBB permeability to serum proteins and leukocytes and an increased brain water content (108).

N. meningitidis. As discussed above, *N. meningitidis*-induced β2-adrenoceptor/β-arrestin signaling in HBMECs results in the formation of cortical plaques and membrane protrusions. In parallel, the type IV pilus-mediated translocation of β-arrestin to the cortical plaque also acts as a molecular scaffold for additional *N. meningitidis*-induced signaling events that open the paracellular route (187). These events include the recruitment of the Par3-Par6-PKCζ polarity complex to the cortical plaque, in a Cdc42-dependent manner, and the accumulation of adherens junction (vascular endothelial cadherin, p120-catenin, and β-catenin) and tight junction (zonula occludens-1, zonula occludens-2, and claudin-5) proteins underneath the site of bacterial adhesion (253). Labeling of vascular endothelial cadherin demonstated that *N. meningitidis* delocalized this protein from the adherens junctions between HBMECs and caused it to be redistributed to the cortical plaque. This depletion of junctional proteins was associated with an increase in barrier permeability to Lucifer yellow and the formation of gaps between cells, thus opening a paracellular route of entry (253). Interestingly, the recruitment of junctional components by *N. meningitidis* occurs only in endothelial cells, as it was demonstrated that meningococci do not activate the β2-adrenoceptor/β-arrestin signaling pathway in epithelial cells (254). Therefore, different host-pathogen interactions may occur at the BBB and BCSFB.

In an inflammation-mediated process, *N. meningitidis* stimulates HBMECs to produce matrix metalloproteinase-8, which was shown to cleave occludin and cause it to dissociate from tight junctions *in vitro* (255). The cleavage of occludin was prevented in the presence of matrix metalloproteinase-8 inhibitors and in cells transfected with matrix metalloproteinase-8-specific small interfering RNA (siRNA). Inhibition of matrix metalloproteinase-8 activity also significantly decreased the *N. meningitidis*-induced permeability of HBMECs to 40-kDa fluorescein isothiocyanate (FITC)-dextran at 24 h. Furthermore, the inhibition of matrix metalloproteinase-8 prevented the caspase-independent detachment of HBMECs from a Matrigel matrix on a solid support following *N. meningitidis* infection (255).

***Streptococcus* spp.** Group B streptococcus, *S. pneumoniae*, and *S. suis* invade brain microvascular endothelial cells via transcellular mechanisms; however, the associated production of proinflammatory cytokines, the activity of pore-forming toxins, and the induction of plasmin activity may also lead to a concurrent weakening of the BBB, potentially opening a paracellular route of entry. *In vivo* and *in vitro* studies have shown that group B streptococci, pneumococci, and *S. suis* induce the production of a range of cytokines and chemokines, including IL-1β, IL-6, IL-8, IL-10, TNF-α, macrophage chemoattractant protein 1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), Gro-α, and Gro-β, in brain microvascular endothelial cells (109, 256–258) and brain tissue (94, 259, 260). The stimulation of HBMECs with cytokines (including TNF-α, IL-1β, IL-6, and IL-17F) leads

to cytoskeletal rearrangements and a redistribution of tight junction and adherens junction proteins, resulting in a decrease in barrier integrity (261–267). Therefore, the host inflammatory response to group B streptococcus, *S. pneumoniae*, and *S. suis* meningitis may contribute to the disruption of tight junctions. In HBMECs, the group B streptococcal PilA-mediated activation of focal adhesion kinase induces bacterial uptake but also triggers a parallel mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, which leads to the secretion of IL-8 (109). Furthermore, the production of the IL-8 homologue (KC) in mice intravenously inoculated with group B streptococci resulted in the recruitment of neutrophils and an increase in BBB permeability to Evans blue and FITC-albumin (109). It is likely that disruption of the BBB/BCSFB by host inflammatory factors represents a common physiological event that occurs during bacterial CNS invasion.

Using a separate mechanism, group B streptococcus, *S. pneumoniae*, and *S. suis* may hijack the host plasminogen system to cause cellular injury and disrupt tight junctions between HBMECs. Plasminogen is found in plasma and extracellular fluids, and upon activation is converted to plasmin, which degrades the extracellular matrix and may upregulate matrix metalloproteinases that degrade tight junctions (268). *In vitro*, plasminogen binds to streptococcal glyceraldehyde-3-phosphate dehydrogenase (269–271). Enolase and choline-binding protein E have also been identified as additional pneumococcal plasminogen-binding proteins (272, 273). Once plasminogen is bound to its binding protein, it becomes activated and is subsequently converted to plasmin by exogenous tissue plasminogen activator and urokinase (269, 270, 274). Plasmin bound to the surfaces of group B streptococci enhanced the ability of bacteria to adhere to and invade HBMECs and induced a significant decrease in transendothelial electrical resistance (275). Furthermore, streptococcus-bound plasmin contributed to cellular injury, characterized by human brain microvascular cell detachment and lactate dehydrogenase release (275). In the human vascular endothelial cell line EaHy, plasmin bound to the pneumococcal cell surface cleaved vascular endothelial cadherin from adherens junctions and increased bacterial translocation across endothelial barriers (276). Taken together, group B streptococcus, *S. pneumoniae*, and *S. suis* may bind host plasminogen and hijack the proteolytic activity of plasmin to potentially open a paracellular route between cells. Plasminogen has also been shown to bind to *N. meningitidis* (277, 278), suggesting that this mechanism may be exploited by several common meningeal pathogens.

Group B streptococcus, *S. pneumoniae*, and *S. suis* produce pore-forming hemolysins, named β-hemolysin/cytolysin, pneumolysin, and suilysin, respectively. In HBMECs, these hemolysins are cytotoxic and promote lactate dehydrogenase release, the loss of cytoplasmic density, discontinuity of cytoplasmic membranes, clumping of nuclear chromatin, dilation of the endoplasmic reticulum, cell rounding, and detachment (97, 202, 207). These morphological changes most likely result in the formation of paracellular gaps. The effects of pneumolysin have also been investigated in rat astrocytes; the addition of pneumolysin to monolayers of primary astrocytes resulted in cell shrinkage and the subsequent separation of cells from each other (107). In rat brain slices, exposure to pneumolysin led to astrocytic process retraction and reorganization of astrocytes within the glia limitans. These pneumolysin-induced changes were associated with increased interstitial fluid retention and BBB permeability, which facilitated the penetration of macromolecules and bacteria into brain slices (107). These data suggest that in addition to mediating cytotoxic effects in brain microvascular endothelial cells, bacterial hemolysins may also induce morphological changes in other components of the neurovascular unit.

Trojan Horse Penetration of Brain Microvascular Endothelial Cells

Bacteria that are capable of surviving within host peripheral immune cells have the ability to invade the CNS via the Trojan horse route. This route of entry has been suggested for *L. monocytogenes* (279), *B. pseudomallei* (280), *S. suis* (146), and *M. tuberculosis* (281). In C57BL/6 × DBA/2 mice, *L. monocytogenes*-infected peripheral blood leukocytes disseminated to the CNS and were shown to induce brain colonization more efficiently than extracellular *L. monocytogenes* (279). Furthermore, CNS infection was not reduced following the elimination of extracellular *L. monocytogenes* by gentamicin delivered by surgically implanted osmotic pumps (282), suggesting that intracellular bacteria were responsible for neuroinvasion. Drevets et al. demonstrated that in C57BL/6 mice, *L. monocytogenes* associated with $CD11b^+$ monocytes, and further analysis revealed that most of these infected monocytes belonged to the $Ly\text{-}6C^{high}$ subset (283). Following systemic *L. monocytogenes* infection, 90% of $CD11b^+$ leukocytes isolated from the brain were $Ly\text{-}6C^{high}$, and the influx of these cells correlated with an increase in brain colonization, suggesting that $Ly\text{-}6C^{high}$ monocytes may act as Trojan horses *in vivo* (283). In BALB/c mice, bone marrow myelomonocytic cells ($CD31^+$ $Ly\text{-}6C^+$ $CD11b^+$ $Ly\text{-}6G^{low}$) may also transport *L. monocytogenes* to the CNS (284).

In a hematogenous model of melioidosis meningitis, *B. pseudomallei* established a primary focus of infection in the spleen, followed by migration to the bone marrow and subsequent spread to the brain (280). *B. pseudomallei* associated with $CD11b^+$ cells within the bone marrow and spleen, and it was hypothesized that these cells may serve as Trojan horses that facilitate bacterial spread to the brain (280). In adoptive transfer experiments, splenic $CD11b^+$ cells and bone marrow $CD11b^+$ cells harboring *B. pseudomallei* induced brain colonization. In contrast, bacterial burdens within the brain were attenuated following the adoptive transfer of *B. pseudomallei*-infected $CD11b^-$ cells, and equivalent numbers of extracellular *B. pseudomallei* were unable to colonize the brain. These data suggest that *B. pseudomallei*-infected $CD11b^+$ cells within the spleen and bone marrow may disseminate to the CNS and contribute to the pathogenesis of neurological melioidosis with meningitis. Further experiments demonstrated that the expression of selectin on *B. pseudomallei*-infected $CD11b^+$ cells was required for the development of meningitis in recipient mice (280).

THE OLFACTORY NERVE AS A PORTAL TO THE BRAIN

The olfactory system (Fig. 3A) comprises the odor-detecting sensory system that exists in the peripheral nervous system within the nasal cavity and, within the CNS, the olfactory bulb and higher processing centers of the brain. Within the nasal cavity, the olfactory epithelium lines the more dorsal and caudal regions of the cavity. Olfactory sensory neurons reside within the olfactory epithelium and project a dendritic knob-like swelling with 20 to 30 cilia into the mucous layer lining the nasal cavity. The olfactory

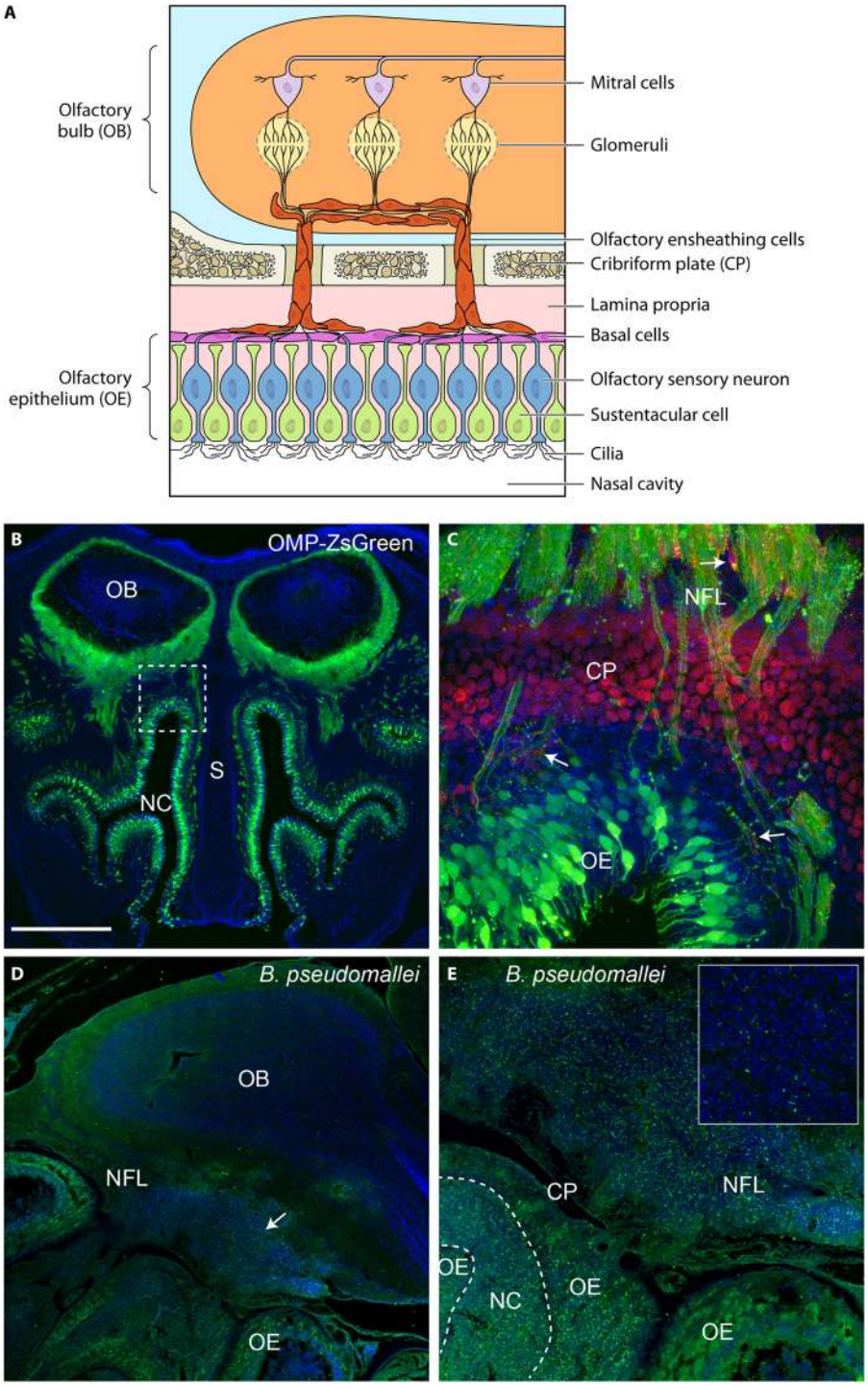

FIG 3 The olfactory system is a direct portal for bacterial pathogens to the brain. (A) The cilia of olfactory sensory neurons penetrate the nasal mucosa and provide a direct pathway from the external environment to the CNS. Olfactory sensory neurons in the olfactory epithelium are supported by sustentacular cells and replaced by proliferation and differentiation of basal stem cells, and their axons pass through the lamina propria and cribriform plate of the skull to synapse with mitral cells in the glomeruli of the olfactory bulb. Microbial pathogens can potentially access the brain through the olfactory epithelium via axonal transport, by travel within olfactory ensheathing cells that surround the axons, or external to these cells, within the perineural space and by passage through holes in the cribriform plate to access the subarachnoid space. (Also see references 101 and 428.) (B and C) Coronal section through the olfactory system of an OMP-ZsGreen × S100β-DsRed transgenic reporter mouse (429, 430). (B) Primary olfactory neurons (green) reside in the olfactory epithelium, which lines the nasal cavity (NC). S, septum. The neurons project axons to the olfactory bulb (OB) within the CNS. The boxed region is shown in panel C. (C) Bundles of olfactory axons project from the olfactory epithelium (OE) through the cribriform plate (CP; chondrocytes are bright red) and enter the nerve fiber layer (NFL), which forms the outer layer of the olfactory bulb. Olfactory ensheathing cells (dull red, arrows) surround the axon bundles. (D) Sagittal section through the olfactory bulb and nasal cavity of a mouse that was intranasally inoculated with *B. pseudomallei* (green), with invasion of the nerve fiber layer of the olfactory bulb in the region indicated by the arrow. (E) A higher-power view of the ventral nerve fiber layer shows bacterial infestation within the nasal cavity (NC) and the olfactory epithelium (OE) and invasion of the NFL by bacteria. (Inset) Higher-power view of *B. pseudomallei* (green) within the nerve fiber layer. The scale bar found in panel B is equivalent to 500 μm (B), 65 μm (C), 750 μm (D), 300 μm (E), and 70 μm (inset).

sensory neuron cilia possess odorant receptors (285), which bind a large spectrum of ligands (286). Binding of an inhaled odorant to its receptor results in signal amplification and the activation of a signal transduction pathway, leading to the generation of an action potential (287). The olfactory epithelium also comprises sustentacular cells, which are glia-like cells whose apical surfaces form the epithelial surface lining the nasal cavity. These supporting cells form tight junctions with each other and with the dendrites of the olfactory sensory neurons, forming the primary barrier from the environment (288). The olfactory epithelium also contains basal cells, which are stem and multipotent progenitors from which new olfactory sensory neurons are generated, and developing neurons, which are newly generated from the basal cells (289, 290). The axons of olfactory sensory neurons penetrate the basement membrane beneath the epithelium entering the lamina propria, where they are met by specialized glial cells, the olfactory ensheathing cells. Olfactory ensheathing cells surround multiple axons and bundle them together in larger fascicles that comprise the olfactory nerve (291, 292). Within the lamina propria are characteristic Bowman's glands of the olfactory mucosa, whose ducts penetrate the epithelium above to supply specialized mucus to the epithelium.

The axons of the olfactory nerve course through the lamina propria toward the brain, penetrating the skull through the cribriform plate and entering the brain at the olfactory bulb (Fig. 3A to C). Within the olfactory bulb, the sensory axons form specialized structures, the glomeruli, where they synapse with mitral cells, which carry the sensory signal to higher brain structures (293, 294). Olfactory sensory neurons are directly exposed to the external environment via the nasal cavity; therefore, microbes within the nasal cavity may potentially exploit the olfactory pathway and access the subarachnoid space and the olfactory bulb. From the olfactory bulb, viruses have been shown to migrate to higher brain regions, including the basal nuclei, thalamus, hypothalamus, cerebrum, and cerebellum, in animal models of infection (295–297).

THE TRIGEMINAL NERVE AS A PORTAL TO THE BRAIN

The trigeminal nerve is the largest cranial nerve, whose afferent branches carry touch, pain, and noxious stimuli from the face, the corneas of the eyes, and the oral and nasal cavities. The trigeminal nerve innervates the olfactory and respiratory epithelia of the nasal cavity via branches of the ophthalmic (V1) and maxillary (V2) nerves (Fig. 4A) (298). The trigeminal nerve endings are separated from the lumen of the nasal cavity via an apical tight junction complex at the epithelial surface (299, 300). The same trigeminal nerves that innervate the olfactory epithelium also branch to innervate the olfactory bulb, providing an alternative route for entry of pathogens (301). Lipid-soluble trigeminal irritants within the nasal cavity may reach their receptors on trigeminal nerve endings by diffusing across tight junctions, potentially by using paracellular mechanisms (302), or by interacting with specialized solitary chemosensory cells within the nasal and respiratory epithelia which form synaptic contacts with trigeminal nerve fibers (303). Solitary chemosensory cells isolated from mice were shown to detect bacterial signals, such as acyl-homoserine lactones, and to trigger downstream signaling pathways associated with bitter irritant transduction (304).

The trigeminal nerve fibers course to the brain, forming the trigeminal ganglion outside the brain stem but beneath the dura mater (298). From the trigeminal ganglion, the sensory axons innervate the trigeminal nucleus in the brain stem. Therefore, the trigeminal nerve, like the olfactory nerve, may provide a direct pathway by which bacteria within the nasal mucosa may access the brain independently of the blood. It should be noted that bacterial entry to the brain via the trigeminal nerve might not be limited to intranasal infection. For example, viruses such as herpes simplex virus type 1 access the trigeminal nerve through infection of the cornea (305), and potentially any epithelial infection of the facial skin or oral cavity could allow access to the trigeminal nerve.

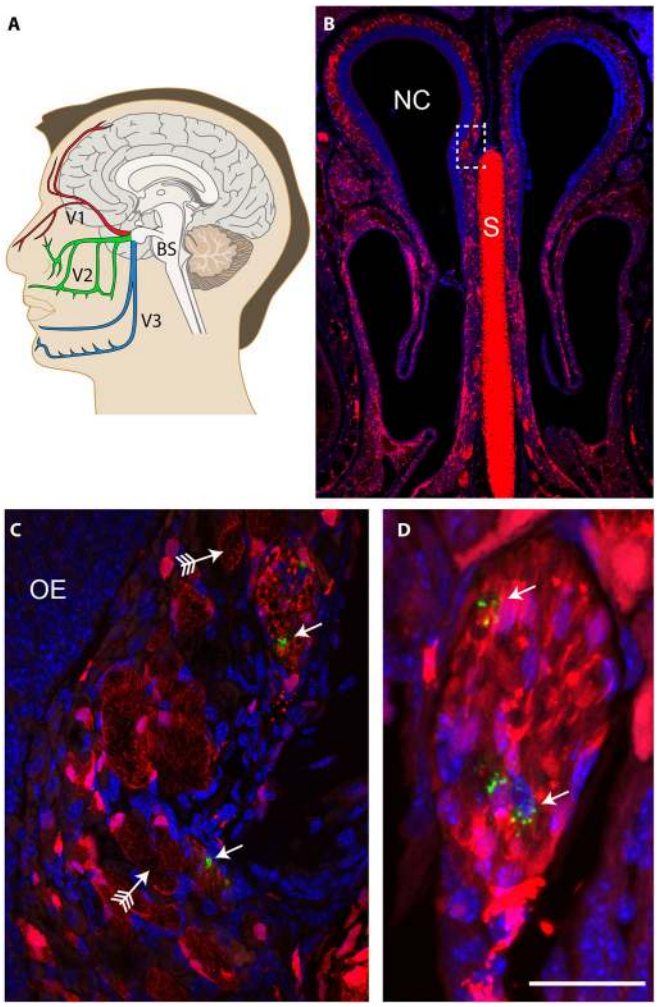

FIG 4 Trigeminal nerve route of entry. (A) Schematic showing the three branches of the trigeminal nerve: V1, V2, and V3. Branches V1 and V2 innervate the nasal cavity and project to the brain stem (BS). (B to D) In coronal sections of the mouse nasal cavity, *B. pseudomallei* (green) is localized within branches of the trigeminal nerve that innervate the septum (S) within the nasal cavity (NC). The boxed area in panel B is shown in panel C. Bacteria (arrows) are contained within the trigeminal nerve, while adjacent olfactory nerve bundles (arrows with tails) do not contain bacteria. (D) Higher-magnification view of the trigeminal nerve in a nearby section. The scale bar found in panel D is equivalent to 650 μm (B), 40 μm (C), and 15 μm (D). OE, olfactory epithelium.

PROTECTING THE CNS FROM MICROBIAL INVASION VIA THE NASAL CAVITY

The nasal cavity is constantly exposed to inhaled microbes, allergens, and particulate material. The epithelium lining the nasal

nares (306, 307) and the nasal cavity (308) harbor normal bacterial flora, and CNS pathogens, such as *S. aureus*, *S. pneumoniae*, *N. meningitidis*, and *H. influenzae*, may asymptomatically colonize the nasopharynx in some individuals (306, 309, 310). Therefore, the nasal cavity features innate defense mechanisms to filter inhaled air and prevent microbes from invading deeper tissues.

Goblet cells and submucosal glands secrete mucins (primarily MUC5AC and MUC5B) into the lumina of the airway and nasal cavity. MUC5AC and MUC5B cross-link to form the structural framework of a mucous barrier which floats on top of a periciliary layer of membrane-bound mucins (including MUC1 and MUC4) and glycoproteins (311). Through a mucociliary clearance process, inhaled bacteria and particulate matter become trapped in the mucous layer and are swept toward the pharynx by the coordinated beating of cilia located on the surfaces of epithelial cells. A recent study demonstrated that MUC5B, but not MUC5AC, is essential for mucociliary clearance activity and the prevention of bacterial spread from the nasal cavity to the lower respiratory tract (312). Mucus secretion and mucociliary clearance are enhanced in the presence of inflammatory mediators (313) and microbial pathogens (314–316) and following exposure to cigarette smoke (317). Antimicrobial substances, including enzymes (lysozyme), protease inhibitors (secretory leukoprotease inhibitor, elastase inhibitor, α1-antiprotease, and antichymotrypsin), antimicrobial peptides (β-defensins and L37), and oxidants (nitric oxide and hydrogen peroxide), are secreted by epithelial cells into the airway surface liquid and provide a further line of defense against microbes (318). Finally, tight junctions and adherens junctions between the epithelial cells lining the nasal cavity and airway create a cellular barrier that prevents microbial spread. Dendritic cells localized beyond the epithelium extend processes through these tight junctions to interact with ligands and collaborate with resident macrophages to remove foreign antigens (319).

Despite the host defenses that exist within the nasal cavity and upper airways, bacteria colonizing the epithelium may invade deeper tissues and cause disease. In the case of asymptomatic colonizers, the transition from a commensal to a pathogenic phenotype may be due to within-host evolution that results in genetic changes to the regulation of virulence factors (320, 321). Alterations in susceptibility to disease in immunocompromised individuals may also promote the spread of bacteria from the nasal cavity (318). Respiratory pathogens, such as *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, have been demonstrated to trigger the disassembly of tight junctions between epithelial cells to migrate to the lower respiratory tract (322, 323), whereas *N. meningitidis* redistributes junctional proteins at the olfactory epithelium (324). Harris et al. (325) demonstrated that the intact olfactory epithelium usually represents an effective barrier against infiltration of *S. aureus* into the nasal lamina propria. However, following mild detergent damage to the nasal epithelium of C57BL/6 mice, intranasally delivered *S. aureus* was detected in the olfactory epithelium, olfactory nerve, and olfactory bulb, as early as 6 h postinfection (325). Indeed, damage to the olfactory epithelium within the nasal cavity appears to be an important and common event in bacterial spread to the CNS via the olfactory nerve (324–327).

PATHOGENS THAT ENTER THE BRAIN THROUGH THE NOSE

Bacteria

Bacteria within the nasal cavity may potentially penetrate the olfactory epithelium by directly infecting olfactory sensory neurons; may enter via the ducts of Bowman's glands; or may enter after reactive inflammatory processes compromise the epithelial tissue, leading to extravasion and subsequent tissue damage after exposure to bacterial virulence factors. Bacterial signaling molecules, such as acyl-homoserine lactones, are detected by solitary chemosensory cells within the nasal mucosa that synapse with the trigeminal nerve, triggering trigeminally mediated reflex reactions (304). Mouse solitary chemosensory cells exposed to *P. aeruginosa* acyl-homoserine lactones responded with an increase in intracellular Ca^{2+}. Furthermore, *in vivo* studies demonstrated that mice exposed to acyl-homoserine lactones via the retronasal stream experienced significant reductions in respiratory rate, suggesting that interactions between bacterial acyl-homoserine lactones and chemosensory cells are capable of inducing responses indicative of nasal trigeminal irritants (304). The activation of nasal solitary chemosensory cells by acyl-homoserine lactones triggers a proinflammatory response (328); this response may damage the integrity of the epithelium and enable bacteria to access the trigeminal nerve endings. Once bacteria have penetrated the epithelium, they may potentially travel along the olfactory and/or trigeminal nerves to the brain, within axons, between the axons, within the surrounding glia (olfactory ensheathing cells or Schwann cells), or between the glia and the connective tissue sheath (perineurium) that contains the nerve bundle.

Meningeal pathogens, such as *S. pneumoniae* and *N. meningitidis*, unequivocally enter the CSF via the hematogenous route. Animal models of infection have also highlighted an alternative route of CNS entry, by which these bacteria may directly invade the olfactory bulb within the brain from the nasal mucosa. *B. pseudomallei* has also been shown to invade the brain via the olfactory and trigeminal nerves, whereas *L. monocytogenes* demonstrates a predilection for the trigeminal nerve and brain stem. The following section reviews the evidence for CNS invasion from the nasal cavity for these pathogens.

S. pneumoniae. Early studies by Rake demonstrated that *S. pneumoniae* rapidly enters the olfactory bulb in Swiss mice following intranasal inoculation (329). Remarkably, pneumococci were isolated from the olfactory bulbs of the brain at 1 min postinfection. At this time point, bacteria were not isolated from the blood, effectively excluding a hematogenous route of CNS invasion. At 2 min postinfection, pneumococci were observed between the sustentacular cells of the olfactory epithelium, within the perineural space of the olfactory nerve, and within the subarachnoid space (329). Similar findings were reported by van Ginkel et al., who demonstrated that intranasally delivered *S. pneumoniae* (strain EF3030) could be recovered from nasal washes, the olfactory nerve and epithelium, and the olfactory bulb at 24 h postinfection in CBA/CAHN/*xid* mice (330). These sites remained colonized throughout the duration of the experiment, until day 39 postinfection; however, this did not lead to extensive brain infection. In contrast, nonencapsulated pneumococci were unable to persist within the nasal cavity and olfactory epithelium and did not invade the olfactory bulb within the brain. Briles et al. confirmed that the pneumococcal capsular polysaccharide is an important mediator of olfactory bulb infection in CBA/CAHN/*xid* mice, as only opaque variants were isolated from the olfactory bulb following intranasal inoculation (331). In these studies, intranasally delivered *S. pneumoniae* did not lead to bacteremia, suggesting a role for direct brain invasion from the nasal cavity. *S. pneumoniae* was also recovered from the trigeminal ganglia, further suggesting that

the trigeminal nerve bundles may also be a potential route to the brain for this bacterial pathogen (330). However, it must be noted that these studies utilized CBA/CAHN/*xid* mice, which possess a mutation in Bruton's tyrosine kinase gene and thus do not respond to thymus-independent type II antigens (332). These mice also fail to respond to capsular polysaccharide; therefore, pneumococcal infection in this mouse strain is unlikely to accurately model human disease.

Incubation of *S. pneumoniae* with gangliosides prior to intranasal infection resulted in reduced colonization of the nasal mucosa, olfactory system, and brain (330). Phosphorylcholine residues on the pneumococcal cell wall have been shown to bind to gangliosides (333), suggesting that gangliosides may be an important target for *S. pneumoniae* attachment in the neuroepithelium. *In vitro* studies have also demonstrated that *S. pneumoniae* can invade olfactory ensheathing cells via mannose receptor-mediated endocytosis (334); however, the role of olfactory ensheathing cells in pneumococcal infection and the exact mode of transport of *S. pneumoniae* along the olfactory and/or trigeminal nerves have not yet been studied.

N. meningitidis. In cases of meningococcal meningitis, >60% of patients develop meningitis without septic shock (335), suggesting the possibility that *N. meningitidis* may also penetrate the CNS without prior bacteremia. In a mouse model of intranasal *N. meningitidis* infection, 20% of mice developed lethal meningitis; however, no bacteria were isolated from the blood (324). At 3 days postinfection, lesions and polymorphonuclear cells were observed within the olfactory epithelium. Furthermore, *N. meningitidis* infection was associated with significant damage to the olfactory epithelium and reduced the thickness of discrete, noncontinuous sections of epithelium by more than 50% compared to the case in controls. Immunofluorescence studies demonstrated *N. meningitidis* within the olfactory epithelium, basement membrane, and lamina propria, along the olfactory nerves in the cribriform plate, and within the nerve fiber layer of the olfactory bulb. Meningococci were also isolated from the CSF. Due to the absence of bacteremia, it was suggested that *N. meningitidis* may travel from the nasal cavity to the meninges and subarachnoid space via the olfactory nerves (324). In rhesus macaques, commensal neisseria bacteria (RM *Neisseria*) were shown to be transmitted between animals and naturally colonized the epithelium covering the cribriform plate (336), suggesting that migration of neisseria bacteria along the olfactory pathway is not a phenomenon that is observed only in animals experimentally inoculated with pathogenic *N. meningitidis*. In the African "meningitis belt," the onset of meningococcal meningitis epidemics coincides with harsh, dry harmattan winds. It has been hypothesized that these harsh environmental conditions may irritate the mucus membranes (such as the olfactory mucosa) and enable *N. meningitidis* to penetrate the epithelium and invade the CNS (337), although this has not yet been investigated.

Sjölinder and Jonsson demonstrated that the expression of the junctional protein N-cadherin in the olfactory epithelium and lamina propria was significantly reduced in mice intranasally infected with *N. meningitidis* (324). These data suggest that *N. meningitidis* may invade the olfactory epithelium via the destruction or rearrangement of cellular junctions between sustentacular cells and olfactory sensory neurons, although additional studies are required to confirm these findings. The mechanisms by which *N. meningitidis* may travel along the olfactory nerve pathway to invade the nerve fiber layer of the olfactory bulb, the meninges, and CSF are also unknown. The meninges and the subarachnoid space extend over the cribriform plate and further into the olfactory foramen, where the olfactory nerves pass through the cribriform plate (338, 339). Bacteria traveling along the olfactory pathway may potentially invade the meninges after traversing the cribriform plate, and subsequently enter the CSF. However, there is a positive pressure from the brain to the nasal lymphatics due to the drainage of CSF through the cribriform plate, and it is unknown how *N. meningitidis* could travel against this pressure gradient.

B. pseudomallei. The olfactory pathway has been identified as a route of CNS invasion by *B. pseudomallei*. Owen et al. used a capsule-deficient strain, which fails to survive within the blood, to demonstrate that bacteria were present in the olfactory epithelium and olfactory bulb of BALB/c mice at 24 h postinfection, in the absence of bacteremia. It was also shown that when colonization occurred in only one side of the nasal cavity, wild-type *B. pseudomallei* was detected in the olfactory epithelium and olfactory bulb of the infected side only. Combined, these data suggest that *B. pseudomallei* is able to directly invade the brain via the olfactory pathway, without blood-borne infection (105). These findings also demonstrate that the presence of the polysaccharide capsule is not a prerequisite for CNS invasion via the olfactory pathway, presumably in contrast to systemic invasion via the BBB/BCSFB. However, it might be the case that an absence of capsule does diminish translocation via the olfactory or trigeminal nerves, since it is not possible to definitively compare with the presence of capsule, due to hematogenous spread in the latter case. Figure 3D and E illustrate *B. pseudomallei* invasion of the nasal cavity, olfactory epithelium, and ventral nerve fiber layer of the olfactory bulb in a mouse model of acute melioidosis. It may be noted that mice, like most mammals, are naturally susceptible to melioidosis.

The mechanisms by which *B. pseudomallei* migrates along the olfactory nerves were recently investigated (326). In BALB/c mice, we demonstrated that intranasally delivered *B. pseudomallei* was associated with widespread crenellation of the olfactory epithelium and loss of the neuron cell bodies. This neuronal loss led to the degeneration of olfactory axons within 24 to 48 h, and the axon-devoid, hollow olfactory nerve fascicles, surrounded by olfactory ensheathing cells, provided an open conduit for bacterial passage from the nasal cavity through the cribriform plate and into the nerve fiber layer of the olfactory bulb. No bacteria were observed within the supporting sustentacular cells of the olfactory epithelium, nor within the Bowman's glands (326). By migrating within the nerve sheath, *B. pseudomallei* would be shielded from the flow of CSF out of the brain. This may also explain why *B. pseudomallei* is often not isolated from the CSF in cases of neurological melioidosis (56). During our initial studies in mice, we also demonstrated that *B. pseudomallei* was present in the brain stem, increasing in number for up to 48 h postinfection, indicating, though not definitively demonstrating, that bacteria may enter via the trigeminal nerve after intranasal inoculation (105). We have now confirmed that *B. pseudomallei* penetrates the intact respiratory epithelium in mice and migrates along Schwann cell-encased trigeminal nerve bundles to the cranial cavity (326). Figure 4B to D demonstrate *B. pseudomallei* localized within the branches of the trigeminal nerve. As noted above, clinical evidence points to a role for BimA, and hence actin-mediated motility, in neurological melioidosis (229); assuming inhalational acquisition to be associ-

ated with such cases, it remains to be determined where in the olfactory/trigeminal pathways such motility is utilized.

Although there is currently no direct evidence of *B. pseudomallei* penetration of the brain via the olfactory and/or trigeminal pathway in humans, two important considerations provide support for CNS invasion from the nasal mucosa. First, *B. pseudomallei* can colonize the nasal cavity in humans (340), and this may represent a potential reservoir of infection. Our unpublished data also demonstrate that following the inhalation of aerosolized *B. pseudomallei*, considered a major route of infection in humans, bacteria can be recovered in large numbers from the mouse olfactory epithelium. Second, for human cases of primary neurological melioidosis, Currie et al. reported that bacteremia was observed in only 3 of 14 patients (21%) (57). In addition to our animal studies, these data provide evidence that *B. pseudomallei* can invade the CNS by a nonhematogenous route. Furthermore, the trigeminal route of CNS invasion may explain the brain stem-related neurological presentations of melioidosis patients, without requiring frank encephalitis.

L. monocytogenes. In ruminants naturally infected with *L. monocytogenes*, bacteria migrate along several cranial nerves (including the trigeminal nerve) to the brain stem and cause encephalitis (341). Similar findings are observed in human cases of *L. monocytogenes* brain stem encephalitis, where inflammatory lesions may be observed in the nuclei and tracts of cranial nerves V, VII, IX, X, and XII, innervating the oropharynx (342). Of the cranial nerves, the trigeminal nerve pathway is thought to represent a primary route by which *L. monocytogenes* may invade the brain stem from the oropharynx (343, 344). It has been proposed that *L. monocytogenes* may bind to E-cadherin on Schwann cells surrounding trigeminal nerves and subsequently invade the trigeminal axons by receptor-independent cell-to-cell spread (345). However, binding of *L. monocytogenes* to E-cadherin-expressing Schwann cells has not yet been demonstrated.

In animals spontaneously infected and experimentally infected with *L. monocytogenes*, bacteria were observed within the axons of the trigeminal nerve, suggesting that these bacteria may use intraaxonal spread to migrate to the CNS (343, 344, 346). In a mouse model of brain stem encephalitis, *L. monocytogenes* was inoculated into the snouts of immunodeficient mice and was observed within the cytoplasm of trigeminal nerve cell bodies at 7 days postinfection (344). Following this, bacteria were then observed within the brain stem but not in other regions of the brain. In this model, gamma interferon (IFN-γ) release by natural killer cells was required for efficient control of *L. monocytogenes* neuroinvasion via the trigeminal route (344). *In vivo* and *in vitro* studies have demonstrated that colony-stimulating factor 1-dependent cells (including macrophages and dendritic cells) facilitate the neuronal spread of *L. monocytogenes* to the CNS (347, 348). Following replication and escape from the phagosome in colony-stimulating factor 1-dependent cells, *L. monocytogenes* may use actin-based motility to propel itself into adjacent nonphagocytic cells, such as axon terminals. Indeed, the presence of *L. monocytogenes* with actin tails has been demonstrated *in vivo* in the axons of sheep trigeminal nerves (346) and *in vitro* in mouse hippocampal neurons and hypothalamic neurons (349). Dramsi et al. showed that *L. monocytogenes* spreads from J774 macrophages to neurons *in vitro*, in a process that is dependent on actin polymerization by the bacterium (348), suggesting that the formation of actin tails is critical for neuronal spread in listeriosis.

Viruses

Data from animal studies and human cases have demonstrated that the olfactory and/or trigeminal nerve pathway represents a major route of CNS entry for several groups of viruses. *In vivo*, herpesviruses demonstrate a tropism for the olfactory epithelium but not the respiratory epithelium (350–353). Expression of the herpesvirus receptors heparan sulfate and nectin-1 on the apical side of the olfactory epithelium may facilitate binding to the neuroepithelium (350, 351). In the respiratory epithelium, these receptors are either expressed on the basal side of the epithelium (and are thus inaccessible) or not highly expressed (350, 351). Herpes simplex virus type 1 (354), bovine herpesvirus 5 (355), and equine herpesvirus 9 (352, 356) spread from the nasal mucosa to the CNS via the olfactory nerves in animal models of infection. In bovine herpesvirus 5 CNS invasion, the viral protein Us9 and the glycine-rich epitope region of glycoprotein E are required for transport from the olfactory sensory neurons to the olfactory bulb (353, 357). In suckling hamsters, equine herpesvirus 9 antigen was detected within olfactory sensory neurons 12 h after intranasal infection (352). At 48 h postinfection, viral antigen was detected within the olfactory nerve and olfactory bulb, and at 60 h postinfection, virus was observed within the frontal and temporal lobes of the cerebral cortex. Some positive staining occurred within the trigeminal nerve, the trigeminal ganglia, and the region where the trigeminal sensory nerve root connects to the brain stem, although this was observed at the later time points, suggesting that the olfactory nerve is likely to be the primary route of infection (352). Interestingly, Shivkumar et al. demonstrated that intranasally delivered herpes simplex virus type 1 targeted the olfactory epithelium of mice and subsequently traveled along the trigeminal nerve branches and reemerged peripherally within the facial skin after 5 days (350). In this model, the virus rarely reached the olfactory bulbs within the brain. However, in another study, herpes simplex virus type 1 was isolated from the olfactory bulbs and higher brain regions of mice 3 days after intranasal inoculation (354).

In a study of human autopsy material, Harberts et al. reported that herpesvirus 6 was detected at high frequencies within the olfactory bulb and tract (358). Herpesvirus 6B was the variant present in most positive samples, and quantitative real-time TaqMan PCR analysis of selected samples detected viral loads of 1×10^3 and 1×10^4 copies per million cells. In the same study, the prevalence of herpesvirus 6 within the nasal mucosa was determined in 3 cohorts of patients: (i) healthy controls, (ii) multiple sclerosis patients, and (iii) patients with a loss of smell. Overall, herpesvirus 6 DNA was detected in 41.3% of nasal mucus samples, and the prevalence did not differ between the patient cohorts. These findings suggest that the nasal cavity may be a reservoir for herpesvirus 6 and that virus within the nasal cavity may travel to the olfactory bulbs and tract via the olfactory pathway (358). Immunohistological evidence from fatal cases of herpes simplex encephalitis demonstrated that herpes simplex virus type 1 antigen was detected within the olfactory tract, the olfactory cortex, and regions of the limbic system that are connected by the olfactory pathway. In contrast, viral antigen was not detected within the trigeminal pathway (359). Combined, these studies suggest that the olfactory route of CNS entry is highly relevant in human cases of symptomatic and asymptomatic herpesvirus infections. The role of the trigeminal nerve as a portal of entry for herpesviruses in humans is less clear, although the sensory neurons of the trigem-

inal ganglia are the principal site of herpes simplex virus type 1 latent infection in humans (360, 361).

Several subtypes of influenza A virus, including H5N1 (362), WSN/33 (363), and H1N1 (364), invade the CNS via the olfactory nerve pathway in animal models of infection. In intranasally infected C57BL/6 mice, H1N1 (strain PR8) mRNA was detected within the olfactory bulb as early as 4 h postinfection, and viral antigen was visualized within the olfactory nerves and the glomerular layer of the olfactory bulb at 15 h (364). Iwasaki et al. demonstrated that hematogenous dissemination of H5N1 from the lungs to the CNS may not be an important entry mechanism, as viral antigen was detected within the brain prior to the lungs in a murine model (362). In ferrets intranasally infected with different H5N1 strains, three-dimensional (3D) imaging demonstrated that brain lesions were distributed (i) along the olfactory pathway, (ii) along the olfactory pathway and within the brain stem, or (iii) surrounding the brain vasculature (365). These data suggest that there may be different routes of entry used by H5N1 strains; however, the olfactory pathway was identified as the most common route used by the small number of strains that were investigated (365). Further studies in ferrets demonstrated that the Vietnam/1203/2004 H5N1 strain spread along the olfactory nerve filaments, passing through the cribriform plate and into the olfactory bulb (366). From the olfactory bulb, the virus migrated through the olfactory tract and into the anterior olfactory nucleus and anterior commissure, and subsequently to the pyriform lobe, cerebral cortex, and Ammon's horn (366). Schrauwen et al. demonstrated that the multibasic cleavage site in the hemagglutinin of H5N1 is required for virus spread from the nasal cavity to the olfactory bulb and the rest of the CNS (296).

The neurovirulence of influenza A virus subtypes may be influenced by the ability of the virus to disseminate from the olfactory bulb into other regions of the brain (367), which in turn may be controlled by the host immune response. *In vivo*, mouse olfactory sensory neurons infected with influenza A R404BP virus displayed apoptotic neurodegradation and were subsequently phagocytosed by Iba1-expressing microglia/macrophages (368). The infection was therefore restricted to the neuroepithelium and did not spread to the olfactory bulb. This suggested that apoptosis of olfactory sensory neurons might be a mechanism by which the host is protected from microbial invasion from the nasal cavity (368). Influenza A virus also stimulates a host proinflammatory cytokine response within the olfactory bulb (369), which may also act to protect the host from further CNS invasion (370). Autopsy of a severely immunocompromised 11-month-old infant revealed influenza A virus antigen within the olfactory bulb, olfactory tract, and gyrus rectus, which is located inferolaterally to the olfactory bulb (371). Viral antigen was not detected within any other regions of the CNS, the respiratory tract, or any other organs. Viral RNA was also not detected within plasma, suggesting that viremia was not present. These findings provide evidence for influenza A virus entry into the CNS via the olfactory route in a severely immunocompromised infant (371).

Paramyxoviruses, including Nipah virus, Hendra virus, and parainfluenza virus, may enter the CNS directly from the nasal mucosa. *In vivo*, the Sendai strain of parainfluenza virus infected mouse olfactory sensory neurons, but not sustentacular cells, and traveled to the glomeruli of the olfactory bulb (372, 373). Infection of second-order neurons and virus spread to the rest of the brain did not occur (372–374). The Sendai virus nucleoprotein gene was consistently detected within the olfactory bulb for up to 168 days postinfection, indicating that persistence may occur within the olfactory bulb (373). In hamsters, Nipah virus was detected in olfactory sensory neurons as they passed through the cribriform plate into the olfactory bulb, providing evidence of direct brain infection following intranasal infection (297). Similar results were reported for a porcine model of Nipah virus infection, in which Nipah virus antigen was detected within a cross section of the olfactory nerve (375). Temporal analysis demonstrated that Nipah virus entered the olfactory bulb within 4 days in mice (297), whereas the virus spread from the olfactory nerve to the granular cells of the olfactory bulb within 7 days in pigs (375). The related Hendra virus was also shown to target the olfactory pathway and to invade the brain directly from the nasal cavity (in the absence of viremia) in a mouse model of encephalitis (376). Weingartl et al. demonstrated that Nipah virus antigen was present within the endothelial cells and smooth muscle cells of the meningeal veins at 5 to 7 days postinfection (375), which is consistent with findings from autopsies of fatal human Nipah virus infection cases (377). Thus, it is likely that Nipah virus exploits both the hematogenous and olfactory routes of invasion.

Eastern, western, and Venezuelan equine encephalitis viruses can cause encephalitis in horses and humans and are transmitted by mosquitoes or following aerosol exposure. Using a bioluminescent western equine encephalitis virus, Phillips et al. demonstrated the progression of CNS invasion in intranasally infected mice (295). The bioluminescent signal was initially detected in the nasal turbinates and olfactory bulb and was amplified in the basal nuclei, thalamus, and hypothalamus. The distribution of lesions within the brain and the detection of viral antigen by immunohistochemistry supported the olfactory pathway as the route of infection and suggested that the trigeminal nerve may provide a secondary conduit to the brain (295). Venezuelan equine encephalitis virus also targeted both the olfactory (primary route) and trigeminal (secondary route) nerve pathways for CNS entry (378), whereas eastern equine encephalitis virus appeared to infect only the olfactory nerve (379). In CD-1 mice, ablation of the olfactory epithelium and the main olfactory bulb prevented invasion of Venezuelan equine encephalitis virus into the brain via the olfactory nerve; however, the virus was still able to spread to the CNS along the trigeminal nerve (378). Interestingly, replication of Venezuelan equine encephalitis virus within the nasal mucosa induced the expression of proinflammatory cytokines, matrix metalloproteinase-9, and intracellular adhesion molecule 1 within the olfactory bulb, which led to subsequent breakdown of the BBB (380). These events enabled circulating virus to penetrate the brain, suggesting that in addition to the olfactory and trigeminal routes of entry, Venezuelan equine encephalitis virus may also enter the CNS by a hematogenous route.

Among the members of the *Rhabdoviridae* family, rabies virus and vesicular stomatitis virus within the nasal cavity directly invade the olfactory bulbs within the brain. In a fatal human case of airborne rabies encephalitis, rabies virions were observed only within the nerve fibers of the olfactory bulb, not in any other regions of the brain (381). Data from animal studies have also demonstrated that intranasally delivered rabies virus selectively targets the olfactory epithelium and migrates to the olfactory bulb, including the glomeruli, mitral cells, and tufted cells (382). Rabies virus antigen was also detected in the mouse trigeminal nerve. The tropism of rabies virus to the olfactory epithelium may be due to the expression by olfactory sensory neurons of neural cell adhe-

sion molecule (383), which was identified as a receptor for rabies virus *in vitro* (384). Similar to rabies virus, intranasal vesicular stomatitis virus infected the olfactory epithelium, but not the respiratory epithelium, in a mouse model (385). By 6 h postinfection, viral antigen was observed within the olfactory sensory neurons. At 12 h, sustentacular cells and basal cells within the neuroepithelium were also infected, as were olfactory nerve bundles within the lamina propria, and at 24 h, the Bowman's glands and olfactory bulb were infected. In contrast to the case with rabies virus, the trigeminal nerve was not implicated as a portal of CNS entry for vesicular stomatitis virus (385).

Protozoa

Naegleria fowleri is a free-living amoeba that causes primary amoebic meningoencephalitis, a rare but almost always fatal disease in humans. *N. fowleri* is found in warm freshwater, and human infection occurs following the inspiration of contaminated water and is usually associated with swimming. Contaminated tap water used to reconstitute saline for nasal irrigation or for ablution of the nasal cavity has also been implicated as a source of infection (386, 387). Pathological investigations of fatal human cases revealed hemolytic, necrotic encephalitis of the olfactory area, the contiguous forebrain, and the cerebellum (388, 389). The suspected route of CNS entry was the olfactory route, due to the presence of amoebae and acute inflammation within the nasal mucosa and the olfactory nerve bundles (388, 389).

In murine models of the early stages of primary amoebic meningoencephalitis, intranasal *N. fowleri* invaded the cribriform plate and olfactory bulb and was observed within the olfactory nerves (390, 391). *In vivo* studies have shown that *N. fowleri* induces mucus production within the nasal cavity as early as 1 h postinfection and that, by 6 h, amoebae are covered by mucus and surrounded by neutrophils (392). In the mucin-producing cell line NCI-H292, *N. fowleri* induced the release of reactive oxygen species, which led to the activation of epidermal growth factor receptor, which in turn stimulated production of the mucin MUC5A and IL-8 secretion (314). Despite this host response, by 12 h, *N. fowleri* was observed adhering to and invading the olfactory epithelium in mice, suggesting that the amoeba may have pathogenic mechanisms to efficiently penetrate the mucous layer (392). *In vitro*, it was shown that both live trophozoites and crude total *N. fowleri* extracts demonstrated mucinolytic activity. A 37-kDa cysteine protease of *N. fowleri* was subsequently identified as being responsible for this mucinolytic activity (392); this most likely enabled the amoeba to penetrate the nasal mucosa and to invade the neuroepithelium. Penetration of the olfactory epithelium in mice occurred without cellular disruption or damage (390), and microscopy studies have shown that *N. fowleri* may be ingested by sustentacular cells within the epithelium or may migrate between the sustentacular cells (393). A recent study demonstrated that *N. fowleri* degraded the epithelial tight junction proteins claudin-1 and zonula occludens-1 *in vitro*, highlighting a potential paracellular mechanism of penetration (394).

Balamuthia mandrillaris, an opportunistic free-living amoeba that can cause granulomatous amoebic encephalitis, was also shown to enter the CNS directly from the nasal cavity after penetrating the olfactory epithelium and cribriform plate in immunodeficient mice following intranasal infection (395). However, the implications of these findings in humans are unclear, as *B. mandrillaris* is thought to cause CNS infections primarily due to hematogenous spread from the lungs or through cuts or skin abrasions (396).

Yeasts and Fungi

The encapsulated yeast *Cryptococcus neoformans* is an important cause of fungal meningoencephalitis worldwide and can enter the CNS by penetrating the BBB by use of transcellular, paracellular, and Trojan horse mechanisms following blood-borne dissemination from the lungs (as reviewed elsewhere [397]). Although the hematogenous route of CNS entry is well accepted for cryptococcal meningoencephalitis, some strains of *C. neoformans* isolated from the CSF of human patients were shown to be rhinotropic in mice (398, 399), and persistent *C. neoformans* colonization of the nasal cavity (90 days) was reported in one study (400). These findings prompted investigations into the possibility of an alternative direct route of CNS entry from the nose. Gomes et al. followed the progression of intranasal *C. neoformans* infection in immunodeficient mice and demonstrated colonization of the olfactory mucosa, invasion along the olfactory nerve, and meningeal involvement (401). In a study of the pathological features of three patients with AIDS and cryptococcal meningitis, *C. neoformans* was observed in the subarachnoid space around the olfactory tracts and bulbs and in the olfactory nerve fascicles (402). No cryptococci were located within the lamina propria or the olfactory epithelium, and thus the authors suggested that *C. neoformans* within the CSF may have been transported to the olfactory regions due to the drainage of CSF from the subarachnoid space through the cribriform plate (402). Using a guinea pig model, Lima and Vital provided further evidence that the olfactory route is unlikely to represent a portal of *C. neoformans* CNS entry, as intranasally instilled cryptococci were unable to penetrate the olfactory epithelium and were cleared from the olfactory mucosa (316).

In contrast, the fungal infection rhinocerebral mucormycosis has been demonstrated to spread to the brain via the trigeminal nerve. Rhinocerebral mucormycosis refers to infections caused by fungi within the order Mucorales and usually affects individuals with poorly controlled diabetes mellitus or the immunocompromised. These organisms display a predilection for the nasal cavity and paranasal sinuses; from these sites, the organisms typically invade blood vessel walls and then spread to the cavernous sinus, internal carotid artery, and brain (403–406). However, fungal hyphae and lesions have been demonstrated within the trigeminal nerve and the pons within the brain stem in the absence of leptomeningitis, suggesting that direct invasion occurred from the sinuses to the brain along the trigeminal nerve (407, 408). The perineural route of CNS entry was thought to be atypical; however, a study of the histologic features of patients with mucormycosis demonstrated that perineural invasion, characterized by fungal hyphae within the perineurium that surrounds the nerves, was a common feature that occurred concurrently with angioinvasion (409). The mechanisms of rhinocerebral mucormycosis CNS infection have not been investigated.

CONCLUDING REMARKS

A wide range of microbes can invade the CNS, and any organisms that can enter the CSF have the potential to cause meningitis. Many bacteria and other pathogens have developed sophisticated mechanisms to penetrate the BBB and/or BCSFB by transcellular and paracellular transport or via the Trojan horse route. The olfactory and trigeminal nerves represent pathways by which mi-

crobes enter the brain without encountering the BBB or the BCSFB. These nerves are well recognized as portals of entry for many viruses, as well as protozoa and fungi, and there is now evidence from animal models that some bacteria can infect the brain via the olfactory and trigeminal nerves. The purpose of this review is to highlight these alternative routes of entry that have thus far received little attention and may explain some of the pathological features observed in human disease. These routes of bacterial invasion require investigation in humans, especially in cases where the etiological agent is known to colonize the nasal mucosa. In such cases, clinical assessment of olfactory function and the nasal mucosa should be considered. Furthermore, several questions remain unanswered. First, what is the route for bacteria to travel along olfactory nerves—within the axons/nerve bundles, within the glia, or within the perineural space? We have demonstrated that *B. pseudomallei* causes destruction of the olfactory epithelium and axonal death, which provides an open conduit for migration to the olfactory bulb within the nerve fascicles (326). It is unknown if similar mechanisms of transport are used by other bacterial pathogens, such as *S. pneumoniae* or *N. meningitidis*. Remarkably, *L. monocytogenes* appears to travel intra-axonally. Second, which bacterial virulence factors are required for penetration of the olfactory epithelium and invasion of the brain via the olfactory and trigeminal nerves? This review has highlighted the requirement for additional research to characterize the roles of the olfactory and trigeminal nerves in bacterial penetration of the brain and to determine the molecular and cellular mechanisms by which bacterial pathogens may exploit these pathways.

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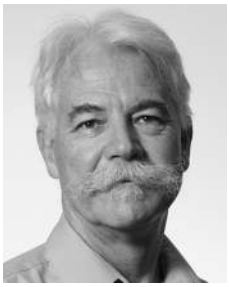

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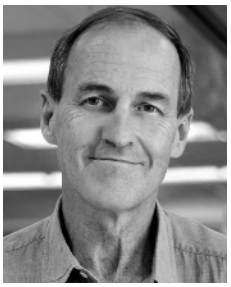

Bart J. Currie is Head of Infectious Diseases at Royal Darwin Hospital and Professor in Medicine at the Northern Territory Medical Program, Flinders and Charles Darwin Universities. He is also Program Leader for Tropical and Emerging Infectious Diseases in the Global and Tropical Health Division of the Menzies School of Health Research and Director of RHD Australia. His areas of interest include clinical and epidemiological aspects of tropical and emerging infections, development of treatment guidelines, and clinical toxinology. He initiated the Darwin Prospective Melioidosis Study in 1989, and this remains the basis for ongoing multidisciplinary collaborations on melioidosis.

James A. St. John obtained his Ph.D. in agricultural science at the University of Melbourne, Australia, in 1996. He then held full-time research fellowships at the University of Melbourne and The University of Queensland. He is currently a Senior Research Fellow at the Eskitis Institute for Drug Discovery, Griffith University. Since obtaining his Ph.D., he has worked in the field of mammalian olfactory nervous system development and regeneration and has published over 40 papers on olfactory axon guidance and the role of olfactory glia. He performs detailed microscopic anatomical studies of the olfactory system and has identified subpopulations of olfactory glia by using live-cell imaging of *in vitro* cultures. He is particularly interested in the role of olfactory glia in protecting the brain from bacterial infections. Together with Jenny Ekberg, he recently performed the majority of the work which identified the intranasal route of infection via the olfactory nerve by *Burkholderia pseudomallei*.

Jenny A. K. Ekberg obtained her Ph.D. in neuroscience/biomedical science from The University of Queensland in 2005. During her Ph.D. work and first postdoctoral appointment, also at The University of Queensland, she studied novel modulatory mechanisms of voltage-gated ion channels in neurons by using electrophysiological techniques. In 2008, she moved to Griffith University as a Research Fellow and is now focusing on neuron-glia interactions and neural regeneration in the olfactory nervous system. In 2012, she took a position as Senior Lecturer at Queensland University of Technology. She continues her work on neural repair and has expanded into the field of bacterial infections of the central nervous system. Together with James St. John and Ifor Beacham, she recently investigated how *B. pseudomallei* can invade the brain via the olfactory nerve. The focus of Dr. Ekberg's research is to investigate how cells in the olfactory nerve prevent microorganisms from entering the brain while simultaneously promoting nerve regeneration.

Michael Batzloff obtained his Ph.D. from Griffith University in 2001, after which he accepted a postdoctoral position at the Queensland Institute of Medical Research, Australia. He has been the recipient of two fellowships from the National Heart Foundation of Australia for his research into vaccine development for *Streptococcus pyogenes*. He was subsequently appointed the inaugural Head of the Bacterial Vaccines Laboratory at the Queensland Institute of Medical Research and recently accepted a position at the Institute for Glycomics at Griffith University. His research interests include neglected tropical diseases, focusing on pathogenesis and vaccine development for the bacterial pathogens *Streptococcus pyogenes* and *Burkholderia pseudomallei*.

Glen C. Ulett is an Associate Professor who received his Ph.D. from James Cook University (Australia) in 2001 and completed postdoctoral research at St. Jude Children's Research Hospital, the University of Queensland, and the University of Alabama at Birmingham. He is currently an Australian Research Council (ARC) Future Fellow in Microbiology at Griffith University, where he leads a research team studying bacterial pathogenesis and the mechanisms of host defense against infection. His laboratory focuses on infections related to the urogenital tract, programmed cell death, and mechanisms of virulence and disease associated with *Escherichia coli*, *Streptococcus agalactiae*, and *Burkholderia pseudomallei*. He has been a microbiology researcher in the field of bacterial pathogenesis for 17 years and is a two-time recipient of the George McCracken Infectious Disease Fellow Award from the American Society for Microbiology. His research program in microbiology and infectious diseases is supported by funding from the National Health and Medical Research Council of Australia.

Ifor R. Beacham undertook undergraduate studies in biochemistry at the University of Otago, New Zealand, and obtained his Ph.D. in microbial genetics from the University of Leicester, United Kingdom. He performed postdoctoral research at the University of Leicester, Washington University of St. Louis, and the University of California, Santa Barbara, and held teaching positions at the University of Wales, Aberystwyth, United Kingdom, and Griffith University, Australia. He is currently an Emeritus Professor and a Research Leader at the Institute for Glycomics, Griffith University. He worked in the general area of molecular microbiology on a variety of bacteria before undertaking work with *Burkholderia pseudomallei* 16 years ago. The latter studies were motivated by the endemicity of melioidosis in northern Australia, difficulties with genetic manipulation, and the enigmatic status of *B. pseudomallei* as a pathogen. He hopes his continuing work will contribute to a greater understanding of the molecular nature of the virulence of *B. pseudomallei*.