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From the blood to the brain: avenues of eukaryotic pathogen dissemination to the central nervous system

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Abstract

Infection of the central nervous system (CNS) is a significant cause of morbidity and mortality, and treatments available to combat the highly debilitating symptoms of CNS infection are limited. The mechanisms by which pathogens in the circulation overcome host immunity and breach the blood–brain barrier are active areas of investigation. In this review, we discuss recent work that has significantly advanced our understanding of the avenues of pathogen dissemination to the CNS for four eukaryotic pathogens of global health importance: *Toxoplasma gondii*, *Plasmodium falciparum*, *Trypanosoma brucei*, and *Cryptococcus neoformans*. These studies highlight the remarkable diversity of pathogen strategies for trafficking to the brain and will ultimately contribute to an improved ability to combat life-threatening CNS disease.

Introduction

The central nervous system (CNS) is protected by a formidable and unique barrier system. Remarkably, several pathogens have evolved mechanisms to breach this barrier and cause disease. Pathogens circulating in the bloodstream may access the brain parenchyma by crossing the blood–brain barrier (BBB) or through other portals of entry, such as the peripheral nerve root ganglia or the choroid plexus, which generates the cerebrospinal fluid (CSF). The blood vessels of the BBB are comprised of densely packed endothelial cells that are linked by tight junctions and surrounded by pericytes and astrocyte end-feet [1]. Collectively, these cells function to restrict the passage of molecules, pathogens, and leukocytes into the parenchyma. In contrast, endothelial cells of the choroid plexus are fenestrated, permitting molecules from the bloodstream into the choroid plexus stroma. Tight junctions interconnect plexus epithelial cells, which line the choroid plexus and form the blood–CSF barrier (BCB) [2]. These barriers physically protect the brain from microbial invasion and toxins and mediate the selective permeability of nutrients and ions. As discussed below, the pathogens that can overcome this unique barrier and enter the CNS often cause severe, life-threatening disease.

In this review, we will highlight recent findings on four eukaryotic pathogens of global health relevance. Although many researchers have made valuable contributions to the field

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of CNS infection, the focus for this review will be on research published in the past three years. Interestingly, the pathogens discussed here appear to use distinct routes for trafficking to the CNS (Figure 1) and cause a diverse range of disease symptoms. *Toxoplasma gondii* establishes a chronic infection in the parenchyma, and parasite reactivation can cause encephalitis in immune compromised individuals. *Trypanosoma brucei* invasion of the brain leads to the neurological disorders and sleep disturbances that characterize African sleeping sickness. In contrast, red blood cells infected with *Plasmodium falciparum* do not enter brain tissue, but instead become sequestered in the cerebral microvessels, resulting in vascular obstruction, a defining feature of cerebral malaria (CM). The fungal pathogen *Cryptococcus neoformans* causes a respiratory infection that can undergo hematogenous spread to the brain and is a major cause of CNS disease in HIV/AIDS patients. The outcomes of these CNS infections vary depending on the pathogen, the immune status of the host, and the stage of infection that occurs in the brain. Recent work in these fields and the use of intravital imaging technologies have significantly expanded our understanding of pathogen entry into the brain as well as revealed areas that require further investigation.

T. gondii entry into the brain: free parasites or ‘Trojan horse’?

Infection by *T. gondii* typically occurs via the oral route through the ingestion of parasite cysts. Evidence suggests that the circulation is a major avenue for *T. gondii* dissemination to distal organs, including the brain. The replicative tachyzoite form of the parasite multiplies in the small intestine [3] and encounters an influx of neutrophils and inflammatory monocytes [4]. Within days after oral infection of mice with tissue cysts, *T. gondii* can be detected both inside monocytes and as extracellular parasites in the blood [5]. Systemic inflammation is characteristic of *in vivo T. gondii* infection, and increased BBB permeability has been associated with the development of toxoplasmic encephalitis [6]. Ultimately, the parasites enter the brain parenchyma and establish a chronic infection as bradyzoite-containing tissue cysts.

The precise mechanism by which *T. gondii* breaches the BBB remains unknown; however, several possibilities have been investigated. Extracellular *T. gondii* tachyzoites can adhere to human vascular endothelium in conditions of shear stress by using the parasite adhesin MIC2 [7]. After adhesion, tachyzoites may either transmigrate across the endothelial barrier [8] or invade endothelial cells [9], and invasion appears to predominate [7]. Interestingly, human brain endothelial cells are more permissive to *T. gondii* replication than neurons or microglia [10]. The extracellular parasite can also migrate through multiple tissue layers of the human retina [11]. Infection enhances the expression of the host adhesion molecules ICAM-1, VCAM-1, and ALCAM in the CNS [6,12]. In particular, ICAM-1 interacts with MIC2 to facilitate the migration of extracellular tachyzoites across epithelium [13] and retinal endothelium [8] without disrupting monolayer integrity.

Recent studies have also expanded our understanding of the ‘Trojan horse’ mechanism of dissemination, by which the extravasation of parasitized leukocytes facilitates tachyzoite translocation across barriers. Following oral infection of mice, *T. gondii* are found in the brain in $CD11b^{+}/CD11c^{-}$ monocytes [5], and a direct comparison of GFP^{+} intracellular and $DsRed^{+}$ extracellular parasites injected into mice resulted in a significantly greater

number of GFP^+ tachyzoites in the brain [14]. More recently, infected human dendritic cells (DCs) and murine monocytes were shown to efficiently transmigrate across retinal and brain endothelium, respectively [12,15], supporting the idea that infected cells may facilitate *T. gondii* entry into the CNS.

T. gondii infection induces hypermotility in migratory cells, and this phenotype correlates with cytoskeletal rearrangement in infected DCs [16] and monocytes [17]. Interestingly, the DC hypermotility is linked to signaling by the neurotransmitter GABA [18••]. The degree to which hypermotility plays a role in crossing the BBB is still not well understood. Under fluidic shear stress, *T. gondii* delays the firm adhesion of infected human monocytes on vascular endothelium [17] and enhances their subsequent crawling [19]. This could be due in part to changes in adhesion molecules, since infection impairs integrin clustering [16,17]. *T. gondii*-infected monocytes appear to undergo TEM (transendothelial migration) via the paracellular route *in vitro* by a process involving the monocyte surface integrin Mac-1 (CD11b/CD18) and its binding partner ICAM-1 [19]. These findings are consistent with the up-regulation of ICAM-1 on brain endothelial cells and the accumulation of $CD11b^+$ monocytes in the brain during *T. gondii* infection *in vivo* [12]. Taken together, hypermotility may facilitate the mobilization of parasitized leukocytes to the CNS while altering the dynamics of their adhesion and motility in the cerebral vasculature. Additionally, since inflammation and vascular permeability change during the course of infection, *T. gondii* may access the CNS using distinct mechanisms at different stages of the infection. A major focus in the coming years will be to translate many of the *in vitro* findings described above into *in vivo* studies. Experiments employing intravital imaging of the brain during acute infection will significantly improve our understanding of the parasite's remarkable ability to colonize the CNS.

Sequestration of *Plasmodium*-infected red blood cells in cerebral vessels

In contrast to *T. gondii*, infection with *Plasmodium* spp. occurs through an insect vector, during a blood meal of the female *Anopheles* mosquito. Cerebral malaria (CM) is a severe and often fatal complication of *P. falciparum* infection, and most cases occur in children. Notably, CM is characterized by vascular dysregulation rather than entry into the brain parenchyma. The cytoadherence of infected red blood cells (iRBCs) in the brain microvasculature is a hallmark of this disease and is associated with vessel obstruction. Post-mortem brain tissue analysis of Malawian children with clinically and pathologically defined CM revealed that iRBC sequestration is associated with myelin and axonal damage, breakdown of the BBB, and glial cell responses [20]. iRBC sequestration is a defining feature of infection with *P. falciparum*. These parasites harbor the highly polymorphic *var* genes that encode the virulence factor erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is exported to the knob structures on the surface of the iRBC through a process that relies on various parasite proteins, including the *Plasmodium* translocon of exported proteins (PTEX) [21] and PfEMP1 trafficking protein 1 (PfPTP1) [22].

PfEMP1 mediates iRBC attachment to vascular endothelium, and both ICAM-1 [23] and CD36 [24,25] have been identified as receptors. *P. falciparum* isolates expressing the domain cassettes 8 and 13 of PfEMP1 are associated with severe childhood malaria, including

severe anemia and CM [26]. Recently, domain cassettes 8 and 13 were found to bind to the endothelial protein C receptor (EPCR) and interfere with the binding of its natural ligand, protein C [27••,28•], which functions in endothelial cytoprotection and the anticoagulant pathway. By examining children in Malawi with CM, Moxon *et al.* found that EPCR is lost from endothelial cells at sites of iRBC sequestration [29], and in a study of children in Benin, high levels of soluble EPCR in the blood positively correlated with pediatric CM and mortality [30]. These data suggest a link between EPCR dysregulation and coagulation during acute malaria in children.

Rosetting, the binding of multiple uninfected RBCs to sequestered iRBCs, contributes to the formation of aggregates and vascular obstruction. Based on studies using human brain microvascular endothelial cells, distinct domains of PfEMP1 were found to mediate rosetting and cytoadhesion [31]. STEVOR, which is encoded by a multi-gene family, is expressed on the iRBC surface and interacts with Glycophorin C to mediate rosette formation independently of PfEMP1 [32]. The binding of pentameric IgM to iRBCs is also associated with rosetting, and recent data suggest that IgM may function by binding to PfEMP1 and strengthening the interactions between RBCs and iRBCs [33].

There has been debate about the relevance of rodent models of experimental cerebral malaria (ECM) for studying the histopathology of human CM, since the predominant model of ECM, *P. berghei* ANKA (PbA) infection in susceptible mouse strains, is associated more with leukocyte accumulation in postcapillary venules than iRBC sequestration [34]. Indeed, recent work in ECM models has focused on the role of $CD8^+$ T cells in vessel obstruction. Intravital imaging of PbA-infected mice revealed extensive vascular leakage in postcapillary venules that is associated with platelet margination, leukocyte adhesion, the deposition of fibrin, and death from ECM [35]. This was prevented by treatment with anti-LFA-1 or with the sphingosine analog FTY720, which inhibits lymphocyte egress from lymph nodes [35]. The recruitment of $CD8^+$ T cells, macrophages, and neutrophils to postcapillary venules restricts blood flow, increasing intracranial pressure, and potentially contributing to ECM [36•]. In the PbA model, cross-presentation of an immunodominant $CD8^+$ T cell epitope from glideosome-associated protein 50 (GAP50) was found to contribute to BBB breakdown and ECM development [37]. Despite the notable differences in the pathogenesis of human malaria and rodent models of ECM, an improved understanding of the host response to PbA infection may provide avenues of inquiry for investigation in human cell systems or clinical studies to inform our understanding of human disease.

Two-step invasion by *T. brucei* spp.: the blood–brain barrier versus the blood–cerebrospinal fluid barrier

T.b. gambiense and *T.b. rhodesiense* are vector-borne diseases that are transmitted by the tsetse fly and cause African sleeping sickness in humans. Unlike *Toxoplasma* and *Plasmodium*, which are obligate intracellular parasites, the trypanosomes are extracellular, free-swimming parasites. The parasite surface is decorated with many copies of a variant surface glycoprotein (VSG) coat that under-goes antigenic variation during the blood stage to evade humoral immunity [38]. CNS infection induces human African trypanosomiasis,

and the mechanism by which trypanosomes enter the CNS is a topic of active investigation and debate [39]. The long-standing model suggests a 'two-step' mode of invasion, by which parasites in the blood initially cross the BCB in the choroid plexus but ultimately penetrate the BBB and enter the brain parenchyma at a late stage of infection. In infected mice, *T.b. brucei* migration across the BBB may follow the extravasation of lymphocytes. TNF-α and IFN-α/β release upon TLR-mediated recognition of the parasite up-regulates ICAM-1 and VCAM-1 expression in the CNS and leads to the accumulation of T cells and parasites in the parenchyma [40••]. This could be due in part to MyD88-induced production of IFN-γ and CXCL10, which are upregulated in blood vessel-associated cells during infection [41]. *In vitro* studies have suggested that *T.b. gambiense* potentially migrates across human brain endothelium via both paracellular and transcellular pathways [42]. Brucipain, a cysteine protease secreted by *T.b. gambiense*, enhances parasite TEM, possibly by targeting GPCR-mediated calcium flux in endothelium to dysregulate barrier permeability [43].

Emerging reports build upon the above model by further dissecting the avenues of *T. brucei* spp. migration into the CNS. Wolburg *et al.* used an electron microscopy approach to visualize the sequential stages of *T.b. brucei* brain infection in rats [44•]. Beginning around 20 days post-injection, trypanosomes were found inside the fenestrated vessels, the stroma, and the ventricle of the choroid plexus and within the meninges, near the pial cells. Parasites were found in the blood vessels but not in the surrounding parenchyma. The authors thus concluded that the direct access to the parenchyma by the bloodstream form is unlikely and that *T.b. brucei* likely migrates out of the choroid plexus and to the leptomeninges, either by crossing the BCB into the CSF current to the subarachnoid space or via the Virchow–Robin space, which extends from the pia mater alongside blood vessels into the brain [44•]. Frevert *et al.* utilized intravital microscopy in mice to characterize *T.b. brucei* interactions in the brain. They visualized parasite entry into the parenchyma as early as 5 hours post-intravenous (IV) injection and captured subsequent parasite replication in the parenchyma [45•]. Notably, parasite transmigration occurs at postcapillary venules even before the onset of host inflammation, leukocyte recruitment, and vascular leakage [45•]. These contrasting studies are just beginning to broaden our appreciation for the complexity of trypanosome entry into the CNS.

The observations by Wolberg *et al.* and Frevert *et al.* could be reconciled by differences between early and late stages of encephalitic trypanosomiasis as well as by recent findings demonstrating the dynamic and cyclical nature by which *T.b. brucei* appears in the CSF [46]. While the majority of trypanosomes likely invade the CNS via the choroid plexus, it is possible that a subset of parasites extravasates across the BBB, triggering the production of glial cell cytokines and chemokines that promote endothelial permeability [41]. Trafficking across the BCB may not be a requirement for reaching the meninges, and this is supported by the detection of *T.b. brucei* in the meningeal blood vessels and in the extravascular space by intravital microscopy within days post-IV injection in mice [47]. However, it is important to note that the stromal matrix, which is easily accessed via the fenestrated vessels, is also populated by resident macrophages and DCs [48], which could amplify host innate immune responses upon parasite recognition by TLRs. Therefore, *T. brucei* spp. may employ a variant of the two-step invasion model: in combination with direct migration across the BBB, entry via the choroid plexus may represent the path of least resistance into

the CNS. Further work will certainly lead to a better understanding of this important aspect of *T. brucei* spp. infection and of how CNS infection ultimately leads to chronic cerebral trypanosomiasis.

Transcellular migration: *C. neoformans* penetration of the brain vascular endothelium

C. neoformans is a prevalent yeast fungal pathogen and the leading cause of CNS infection in HIV/AIDS patients. The pathology of cryptococcal meningitis has been well reviewed elsewhere [49]. Unlike the pathogens discussed above, transmission of *Cryptococcus* occurs through the airways. Following the inhalation of fungal spores, *C. neoformans* establishes infection in the lungs. During reactivation due to immune compromise, *C. neoformans* can spread in the blood as extracellular yeast cells. The infection most frequently presents as meningitis, but parenchymal infection also occurs. Transcytosis of the yeast can occur across human brain microvascular endothelial cells, and in a mouse model of IV infection, *C. neoformans* associates with endothelial cells in the brain [50]. The molecular interactions that mediate CNS infection are of particular interest. *C. neoformans* utilizes inositol, an abundant metabolite in the CNS, to synthesize hyaluronic acid [51], which decorates the yeast capsule [52]. CD44, the hyaluronic acid receptor on brain endothelial cells, induces the lipid raft-mediated endocytosis of *C. neoformans* [53]. Consistent with these findings, mice deficient in CD44 have reduced *C. neoformans* brain invasion and fungal burden and enhanced survival [50,54•,55]. Similarly, the ablation of inositol metabolism by yeast cells decreases the formation of cystic lesions in the parenchyma and enhances survival in mice [51]. Additionally, transcytosis of *C. neoformans* may be accompanied by modification of the endothelium by yeast factors [56,57,58••,59].

Advances in intravital imaging have permitted visualization of yeast cells lodging in brain capillaries, frequently at branch points in the vessels, prior to crossing the BBB [56]. Although *C. neoforms* can undergo passive uptake by endothelial cells [55], studies indicate that entry into the CNS is actively driven by the pathogen: BBB penetration by *C. neoformans* is dependent on yeast urease, which can lead to breakdown of the endothelium via the production of highly toxic ammonia from urea [56]. Additionally, *C. neoformans*-derived serine proteases compromise barrier resistance of bovine [60] and human brain endothelium [59,61] *in vitro*. Mpr1, a newly identified secreted fungal metalloprotease, facilitates *C. neoformans* cytoadherence and transendothelial migration (TEM) across human cerebral microvascular endothelium [58••]. Yeast deficient in Mpr1 fail to induce brain pathology, which prolongs survival in mice. Strikingly, mutants lacking Mpr1 establish a fungal burden comparable to that of wild-type yeast, suggesting that Mpr1 is a virulence factor specific for CNS invasion [58••].

The host cell response to the uptake of *C. neoformans* is an area of active study. A proteomic survey of human brain endothelial cells following exposure to *C. neoformans* demonstrated marked changes in the expression of metabolic and cytoskeletal factors, and analysis by transmission electron microscopy revealed structural damage to the organelles as well as cell injury and death [62•]. Rearrangement of host actin may be a critical step in *C. neoformans*

transcellular passage across the BBB, and evidence supports the targeting of host Rho GTPases and downstream activation of focal adhesion kinase and ezrin by yeast, possibly due to binding to CD44 [63]. Furthermore, the *C. neoformans* phospholipase Plb1 associates with and activates the Rho GTPase Rac1 for TEM across human brain endothelium [57]. Collectively, these studies suggest that *C. neoformans* traffics *through* brain endothelial cells, disrupting endothelial barrier integrity, and that the pathogen possesses a diverse set of highly evolved virulence factors for facilitating this process.

Concluding remarks

There is a tremendous interest in defining the mechanisms of entry into the brain for pathogens that cause debilitating CNS disease. Recent studies have yielded valuable insight into the diverse routes of eukaryotic pathogen entry into the CNS, and as noted for *T. gondii* and *T. brucei*, some pathogens may utilize more than one mechanism to breach the CNS barrier. Despite the growing body of work in this area, more studies are needed to precisely define the molecular interactions mediating CNS infection. Future studies using transgenic or knock-out mice, genetic manipulation of the pathogen, and high resolution intravital imaging techniques will undoubtedly provide novel insight into this important area of pathogenesis.

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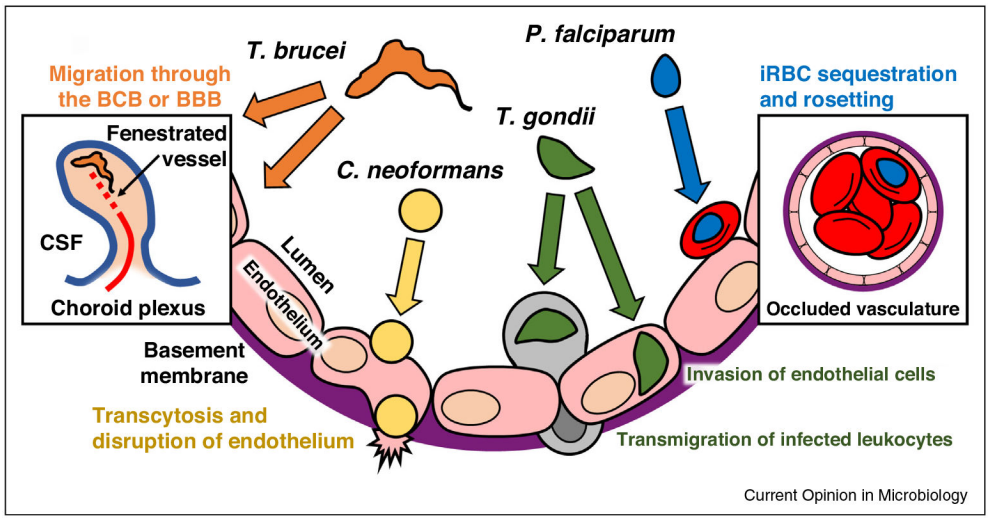

Figure 1.

Eukaryotic pathogens utilize a diverse range of strategies to migrate from the blood to the CNS. A schematic shows the cross section of a blood vessel of the BBB and potential pathways of CNS entry for four global pathogens. *T. gondii* can invade and replicate in brain endothelial cells and may undergo transendothelial migration either as a free tachyzoite or inside an infected leukocyte. Cerebral malaria is associated with sequestration of *P. falciparum*-infected red blood cells (iRBCs) in the brain microvasculature and binding of iRBCs to uninfected RBCs in a process known as rosetting. This leads to the obstruction of blood flow and may contribute to breakdown of the BBB and vascular leakage. *T. brucei* likely crosses the BCB via fenestrated vessels inside the choroid plexus, followed by trafficking to the meninges. Some evidence suggests that enhanced vascular permeability induced by host inflammatory responses may allow the parasite to directly cross the BBB. *C. neoformans* can cytoadhere to endothelium, often at narrow points in the blood vessels and undergo transcytosis. Endothelial cells that internalize yeast lose their structural integrity, resulting in cell stress and injury.