Cerebral microvascular endothelial glycocalyx damage, its implications on the blood-brain barrier and a possible contributor to cognitive impairment

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Abstract

The socio-economic impact of diseases associated with cognitive impairment is increasing. According to the Alzheimer's Society there are over 850,000 people with dementia in the UK, costing the UK £26 billion in 2013. Therefore, research into treatment of those conditions is vital. Research into the cerebral endothelial glycocalyx (CeGC) could offer effective treatments.

The CeGC, consisting of proteoglycans, glycoproteins and glycolipids, is a dynamic structure covering the luminal side of the endothelial cells of capillaries throughout the body. The CeGC is thicker in cerebral micro vessels, suggesting specialisation for its function as part of the blood-brain barrier (BBB). Recent research evidences that the CeGC is vital in protecting fragile parenchymal tissue and effective functioning of the BBB, as one particularly important CeGC function is to act as a protective barrier and permeability regulator.

CeGC degradation is one of the factors which can lead to an increase in BBB permeability. It occurs naturally in aging, nevertheless, premature degradation has been evidenced in multiple conditions linked to cognitive impairment, such as inflammation, brain edema, cerebral malaria, Alzheimer's and recently Covid-19. Increasing knowledge of the mechanisms of CeGC damage has led to research into preventative techniques showing that CeGC is a possible diagnostic marker and a therapeutic target. However, the evidence is relatively new, inconsistent and demonstrated mainly in experimental models.

This review evaluates the current knowledge of the CeGC, its structure, functions, damage and repair mechanisms and the impact of its degeneration on cognitive impairment in multiple conditions, highlighting the CeGC as a possible diagnostic marker and a potential target for therapeutic treatment.

Key words

Cerebral endothelial glycocalyx; cognitive impairment; diagnostic marker

Contents

1. Introduction

The capillaries' role is not just to exchange the metabolites, proteins and other molecules from our bloodstream to their targets and vice versa, but to do so in a highly efficient and specialised manner to prevent damage and dysregulation of our biological systems by having additional components and functions. In unspecialised capillaries, such as in heart or lungs, this is achieved through paracellular transport between the intercellular spaces of the endothelial cells (ECs) that make up the capillary wall or by transcellular transport through the ECs (1, 2). However, for organs like the brain, the parenchymal tissue is fragile and requires a more robust cerebral microvascular structure to protect it from pathogens and other harmful agents – the blood-brain barrier (BBB). Consisting of proteoglycans, glycoproteins and glycolipids, the endothelial glycocalyx (eGC) covers the luminal side of the ECs of our capillaries within the BBB (3). This dynamic structure plays a key role in the regulation of endothelial permeability, microvascular and endothelial physiology, leukocyte adhesion and nitric oxide (NO) production (3-5).

The eGC has been a recent target of research due to its degradation and shedding (causality is still disputed) in inflammation, ischaemia and clinical conditions like sepsis and cerebral malaria (6), all of which are associated with cognitive impairment. And with the current COVID-19 pandemic, cases of prolonged cognitive dysfunction are being highly reported – 31% in a UK study (7) and 25% in Wuhan, China (8) – meaning a greater understanding of its pathogenic link to neuroinflammation and endothelial dysfunction could help to develop effective therapies that would ameliorate the symptoms.

However, due to technological limitations, the study of the cerebral eGC (CeGC) has only recently gained traction (9). As well as being an integral segment deep in our cerebral structure, it collapses and becomes thinner when studied *in vitro* (10). This has made it problematic to study, meaning attention has turned to other similarly functioning systems in the body to assess its possible structure and function, such as in the glomerulus in the kidney (11). To date, much research has focused on the glomerular eGC (11), though modern innovative techniques, such as two-photon microscopy, atomic force microscope or sidestream dark force imaging, have enabled research to start specifically focusing on CeGC. So far it has shown a high level of specialisation within the BBB and provided a prospective diagnostic marker and possible therapeutic intervention target (2, 12, 13).

This review will assess the current knowledge and techniques used to establish the structure and function of the CeGC. We will also try to establish its connection to a

multitude of clinical conditions through its degradation and shedding and discuss its therapeutic potential.

2. The capillaries, endothelium and the BBB

2.1 Capillaries and the cerebral endothelium

The capillary structure is highly conserved throughout the body with three distinct subdivisions – continuous, fenestrated and sinusoidal (13) – all appearing in various parts of the body, indicating a level of specialisation in function. However, further specialisation has emerged in select areas throughout the vasculature, creating a structure tailored to the demands of the individual tissue or organ (11). The normal capillary cell wall is built up of ECs and pericytes, with intercellular spaces for unrestricted passage to and from the bloodstream. However, in more complex organs like the brain, homeostasis regulation is essential for function. This is achieved with the addition of pericytes and astrocytic endfeet that form a complex neighbouring the ECs, which support the endothelial barrier and its properties (14). This assembly allows multifaceted reactions to a diverse range of stimuli, allowing them to participate as a biological interface (15).

Investigation into the cerebral capillary morphology of the CeGC (2, 16) indicates the ECs are interconnected by tight junctions to prevent the unsystematic nature of paracellular diffusion (17), resulting in cerebral capillaries forming a continuous EC membrane, with solutes transported by ECs expressed transport proteins (17). This causes a substantial restriction on passive transport, allowing active control over the passage of substances between the blood and the brain, creating the regulation of cerebral homeostasis. This structure is called the BBB and is essential for protection and regulation within the parenchymal tissue.

2.2 BBB role, structure and cellular properties

The healthy physiological functioning of the brain is highly dependent on the cerebral microvasculature's structure, due to its lack of energy reserves and rapidly changing metabolic rate. The BBB needs to be able to effectively regulate its permeability for its varying demands and need for a regulated homeostasis (18). The BBB has also a protective function, as the parenchymal tissue is susceptible to damage, by preventing neurotoxic plasma components or pathogens from entering the brain (19). Within the microcirculation, regulation of the blood flow through feedback mechanisms for the metabolic demands of the brain is a process called neurovascular recoupling (20), which coordinates the rate of exchange and delivery of the energy substrates and other substances across the BBB through various transport systems (21). These

plasma components then, either by crossing back across the BBB or via the perivascular spaces, re-enter the bloodstream (22).

Fundamentally, the BBB is characterised by its receptors, transporters, junctional proteins and basement membrane mechanisms, expressing low paracellular and transcellular permeability (23). However, research has shown that the BBB is more complex than first thought, and points to larger multifaceted incorporation of components. This has led to the introduction of a neurovascular unit (NVU) which is defined as the unit of the BBB (the central element) along with astrocytes, neurons, pericytes and microglia (24, 25).

Part of the BBB is the eGC, lining the luminal side of the capillaries, whose significance for the BBB function has been increasingly recognised.

3. Endothelial glycocalyx

The eGC can be defined from the viewpoint of its composition, structure and functions.

3.1 eGC composition

There is a consensus about the eGC composition (3, 24, 26-28). The eGC is a two-layer fibre matrix. The *dynamic luminal layer* – 460nm-1µm – is a porous gel-like outer luminal layer in contact with the blood, consisting mainly (90%) of glycosaminoglycans (GAGs), such as heparan sulphate (HS), hyaluronic acid (HA), chondroitin sulphate (CS), dermatan sulphate and keratin sulphate. The HA, though not bound to a core protein, is hydrophilic, forming the viscous solution on the eGC (29). GAGs carry a substantial number of negatively charged binding sites that, depending on sulphation, affect protein binding and thereby vascular permeability (30). They are covalently bound to proteoglycans such as syndecans (SDCs) which form the denser mesh-like *stable endothelial layer* – 200-300nm.This core protein is attached to the EC via transmembrane domain for SDCs or by a glycosylphosphatidylinositol anchor for glypicans and to the adherent HS and HA (31, 32) (see figure 1).

3.2 eGC structure

The dynamic interaction between the two layers – a stable endothelial layer, and a dynamic luminal layer (14) – determines the eGC function and mechanical properties (15). The differences in molecular structure allow the endothelial-derived molecules and plasma components to be incorporated and exchanged (14).

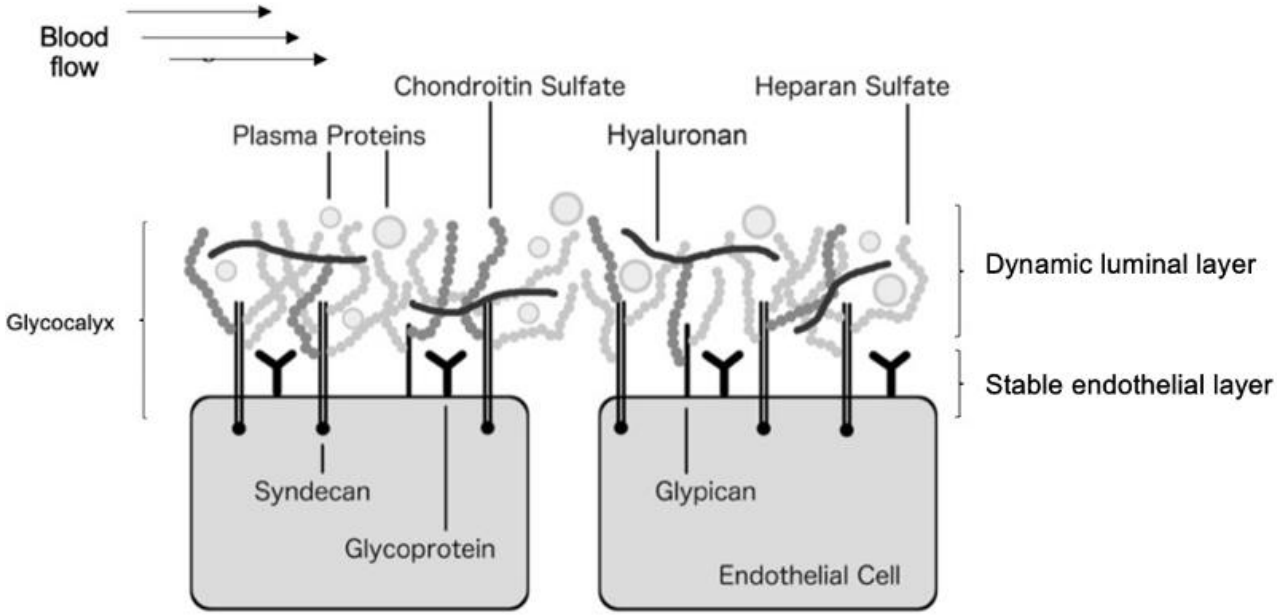

Figure 1 The structure and molecular components of the glycocalyx. The dynamic luminal layer consisting of Heperan Sulphate, Hyaluronan, Chondroitin Sulphate and Plasm proteins are covalently bonded to the more stable endothelial layer which consists of proteoglycans anchored to the endothelial cell (33) - adapted with author's permission

As a result of these structural differences, the eGC can exist in three distinct forms based on its rigidity and thickness – intact (soft and upright), collapsed (stiff and flat) and shed (softer and flat) – depending on the extracellular environment, namely the concentration of electrolyte sodium (Na^+) which regulates the body's fluid balance (34). High plasma Na^+ concentrations stiffen the endothelial cortex, decrease NO release and collapse eGC, which is a hallmark of endothelial dysfunction (34, 35).

An intact eGC (soft and upright) indicates healthy functioning eGC (36, 37). In a physiologically healthy extracellular environment, characterised by low Na^+, the eGC structure is relatively stable, however, it has a constant need to balance the biosynthesis of GAGs and the shear-dependent removal of its existing components (36, 37).

A collapsed eGC (stiff and flat) and shed eGC (softer and flat) signify a damaged eGC (36) and are likely to adversely affect the vascular system (37). Collapsed eGC is caused by the presence of high extracellular Na^+. Shed eGC is a result of heparanase or tumour necrosis factor α (TNFα) (37) where the pro-inflammatory cytokines interleukin-1β (IL-1β) and TNFα are released by high Na^+ (35).

As these eGC structural differences are linked to the ECs function, they are used as markers for assessing the healthy functioning of eGC (34).

3.3 eGC functions

Research has shown that eGC performs multiple roles (3) with further roles being investigated (11).

The eGC as a *vascular permeability regulator* prevents large molecule interaction with ECs thus protecting the BBB permeability (1, 3). The eGC structure has been identified as an element in maintaining the oncotic gradient (38), with cooperative working with junctional proteins and adhesion molecules facilitating this regulation (39). The eGC damage is marked by capillary permeability increase, showing barrier properties against not only water but also colloids (26). However, the mechanisms behind eGC damage impact on vascular permeability need establishing as there are two transport pathways: transendothlial and paracellular pathways (26, 40).

Another role is linked to *mechanotransduction and related sensoring shear stress*, as eGC acts as a mechanoreceptor that responds to the shear stress induced by the cerebral blood flow (CBF) (41). The higher shear stress increases albumin uptake, altering the eGC properties and increasing its thickness (26, 42) – seen in arteries with a much higher pressured CBF compared to capillaries – and NO production which dilates vessels leading to the reduction of the adhesion of leukocytes and platelets (26, 43).

An intact eGC has also *anticoagulant/anticlotting properties*, producing and releasing NO (44), and acting as a mechanotransductor affecting flow regulation (3). The ECs secrete HS, a large component of the eGC, which enhances the anticoagulant properties of the plasma circulating antithrombin, which binds to the HS in the eGC (26, 45), allowing a constant CBF.

An undamaged eGC also *regulates cell adhesion* by reducing the interaction of plasma cells with the ECs surface adhesion molecules – such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) – by limiting ligand-receptor interactions that promote the adhesion of leukocytes (46). During an infection, this leukocyte recruitment is a vital multi-step process in a host's immunological response (26). The eGC is targeted and shed by inflammatory mediators such as histones and proteases, allowing leukocyte adhesion. A healthy eGC would provide vascular protection (30) as the constant adhesion of these leukocytes would result in a systematic inflammatory state.

Lastly, the eGC is also an *immune cell regulator.* The eGC component implicated in this role is sialic acid, a monosaccharide present in eGC (47). The sialylated glycolipids, glycoproteins and the plasma proteins target immune cells (5, 48), thus

contributing to immune system downregulation (47). The desialylation of cancer cells was identified as a promising therapeutic target (47).

These findings show the eGC acts as a receptor for both chemical and physical stimuli, reacting to and inducing physiological responses in the vascular endothelium. These functions are likely due to its highly dynamic nature and nanomechanical property adaptability to minute changes in forces exerted by the bordering bloodstream – the shear stress – and any aggravations by vasoactive elements within the bloodstream (15).

The eGC has a vital role in our physiological functioning, therefore the mechanisms behind its degradation need to be understood. This could lead to potential therapeutic targets.

4. CeGC damage and amelioration - implications for BBB

CeGC damage is difficult to assess as it has been predominantly evaluated preclinically (49) and, though the CeGC implications in the BBB are proving increasingly essential, its study is still fairly limited (50). There is, however, a general understanding that the eGC is a dynamic and delicate structure, easily susceptible to damage (24, 51, 52).

4.1 Key CeGC components and mechanisms of their damage

Studies seem to indicate that the mechanisms of damage are linked to the HA, HS and syndecans, predominantly syndecan-1, which are the main shed components found circulating in plasma (53). The shed eGC components thus may serve as a potential marker for eGC damage and therefore EC injury, and as potential diagnostic and prognostic applications in disease states (34, 37). The eGC protection and its restoration, if already damaged, is a promising therapeutic target. As the dysfunction of the eGC is caused by the shedding, it is highly desirable to develop drugs which could increase the synthesis of eGC components, restore them or prevent their degradation by enzymes (30).

HA plays a vital role in eGC permeability, contributing to cell migration and proliferation (52); its abundance in the eGC highlights its impact on eGC health. HS stimulates cell adhesion and is essential for the regulation of cell interactions, with a shedding of HS increasing ECs activation by cytokines, leading to a switch to an inflammatory phenotype (3). Then lastly, the heparan sulphate proteoglycan (HSPG) syndecan-1 has mechanotranduction properties through shear stress mediation. As well as phenotypic regulation, its loss induces a pro-inflammatory phenotype in ECs (54).

The literature has so far demonstrated several mechanisms of eGC damage, mediated by enzymes or proteases. Heparinase is a group of enzymes that cleaves the heparin and HS chains through an elimination mechanism, i.e. the degradation of Heparin/HS polysaccharides into oligosaccharides, resulting in the HS loss from the eGC (15). The HS chains can also be degraded and shortened to an oligosaccharide with heparanase (55). The degradation of HA is initiated by a family of enzymes, hyaluronidase (HAse) (52). Lastly, metalloproteinases (MMPs) are known to not only cleave proteoglycans (such as syndecans-1) directly from the EC membrane, but also affect the cleavage of cell surface receptors (11).

Though these are so far the most researched components of the eGC, further research is still required, but also into the other eGC components. These mechanisms also heavily impact the results of studies which needs to be taken into consideration.

In addition, the eGC degradation also occurs as a result of the body's natural immunological inflammatory response, causing the release of inflammatory cytokines such as TNF-α and IL-1β (56). The physiological shear stress of the CBF is a vital component of the eGC structure, whilst also, over time, the cause of damage to its own mechanosensitive properties (57). The eGC appears to have both pro- and antiadhesive functions, making its nanochemical properties essential in leukocyte adhesion and inflammatory processes (15). The eGC damage is caused by an influx of Na^+ via the endothelial sodium channels resulting in a conformational change in the cortical actin, hardening the cortex and leading to a reduced NO release and leukocyte adhesion facilitation, indicating a development of vascular inflammation (35).

4.2 CeGC damage and implications for the BBB

The CeGC damage contributes to BBB breakdown which is considered to be an early biomarker of human cognitive impairment (58). Understanding of the factors and mechanisms behind how and why the eGC becomes damaged has led to the research into preventative therapies (52).

4.3 Amelioration of CeGC damage

The mechanisms of prevention are still not clear, however, promising results have been found.

The function of HA in eGC stability leads to the rationale of restoring it to protect a damaged eGC (59). A combination of exogenous HA and CS showed improved eGC thickness in an enzyme-mediated depleted hamster skeletal muscle model when applied together (60). In a rodent model of sepsis, HA administered intravenously reduced cytokine levels and sepsis-related injury (61).

Heparin exhibits neuroprotective, anticoagulant, anti-inflammatory and immunomodulatory properties that preserve and protect the CeGC (62) by being a heparinase inhibitor (51). Including unfractionated heparin, crystalloids and antibiotics reversed eGC damage in a septic shock model (63), suggesting heparin has a neuroprotective effect on the eGC. However, the evidence is still relatively new and inconsistent as to heparin's properties (51).

Antithrombin lll is a widely prescribed medication for the treatment of sepsis-induced disseminated intravascular coagulation (DIC). Though inconclusive, a trend in the reduction of mortality with patients receiving antithrombin was seen (64). However, in some cases it was administered alongside heparin, meaning there is a need for a large randomised controlled trial to decipher antithrombin lll's role, particularly in sepsis models and without heparin treatment (65).

Metabolic glycoengineering is a developing technique allowing the integration of 'non-natural' sugars into the eGC, providing a platform for the discovery of new 'sugar-based' drugs (66). Regulation of euvolemia and normoglycemia could help minimise eGC shedding preoperatively and has protected the eGC in experimental models. However, they have not been experimented on and used clinically.

The importance of studying how to maintain a healthy eGC is emphasised by the wide range of clinical conditions that accompany its degradation.

5. CeGC damage as a possible contributor to cognitive impairment

Under physiologically healthy conditions, the eGC is a stabilising barrier, preventing the leakage of plasma components and inhibiting platelet activation and leukocyte adhesion (67). The eGC deterioration is noted in a wide range of clinical conditions. Inflammatory conditions appear to be the initial cause of the eGC damage, resulting in vasodilation dysregulation, tissue edema and a harmful increase in vascular permeability (68). Alterations to the cerebral microvascular structure, BBB permeability and neurovascular coupling have also been associated with numerous neurological disorders such as AD (69).

5.1 Aging

BBB permeability increases as a normal part of the aging process. Though aging is linked to cognitive decline, it is not necessarily linked to cognitive impairment. It is, however, one of the factors that can contribute to it. Multiple eGC studies show a reduction in thickness with age (70), and deterioration in advanced age in cardiovascular disease (70). The main theories of the BBB permeability increase lie in hypertension and endothelial inflammation (71). With BBB damage accelerated in

individuals with mild cognitive impairment (72), aging negatively impacts ECs function in cerebral arteries and parenchymal arterioles (73). Within the cerebral microvasculature, it is particularly the BBB of the hippocampus (HP) that appears to be much more susceptible to damage and predisposed to age-related vascular dysfunction (72). Interestingly, another study, on the contrary, found the CeGC of the HP was actually thicker than other areas of cortical CeGC. This suggests not only a structural difference between CeGCs but also between them and the other eGCs throughout the body (74). There seem to be limited studies researching the direct role of the CeGC in aging.

5.2 Inflammation

Inflammation is a complex process in response to the body's immunological response to infection and damage. It occurs in many conditions linked to cognitive impairment, as the ECs are a central modulator to the inflammatory response and processes (75). CeGC research supports the hypothesis that inflammation is mediated by leukocyte adhesion to the ECs when the eGC has become degraded or shed (52). Studies on CeGC of rats showcased glial activation, adhesion molecules and proinflammatory cytokines in the HP and significantly higher levels of ICAM-1, VCAM-1 and cyclooxygenase-2 (COX-2) within the cerebral cortex after cardiac arrest (52). These findings support earlier work which evidenced that syndecan-1 plays a role in the endothelial phenotype regulation, as its decrease or loss leads to a switch to pro-inflammatory phenotypes (54), meaning that inflammation both contributes and is related to multiple other conditions.

5.3 Cerebrovascular/neurological conditions

5.3.1 Ischemia reperfusion injury and brain edema

Characterised as tissue damage due to a lack of oxygenation, ischemia reperfusion (I/R) injury is a reversible condition where the resumed oxygen supply increases reactive oxygen species (ROS) production. A combination of both oxygen deprivation and ROS increase results in membrane damage, altered membrane permeability, metabolic dysregulation, organ dysfunction and finally cell death (76). With eGC degradation being the earliest form of structural damage in I/R (77), a limited study into delayed cerebral ischemia (DCI) found increased levels of syndecan-1 and an increased adhesion of ICAM-1 and VCAM-1 to the ECs, facilitating the neuroinflammatory processes (78). I/R injury can lead to the development of a brain edema, categorised as a fluid build-up leading to swelling, further compounding the brain injury. Emerging evidence indicates that, as well as being linked to inflammation, the increased BBB permeability – potentially caused by CeGC damage - negatively affects the neurological outcome and exacerbates the brain edema symptomology

(52). Preservation of the CeGC seems to improve the neurological outcome of patients (52), and heparin ameliorates cerebral edema (51) and I/R injury (79, 80).

5.4 Responses to infection and infectious diseases

5.4.1 Cerebral malaria

Cerebral malaria (CM) is a parasitic infection with a multi-faceted pathogenesis. The parasite *Plasmodium.falciparum* appropriates erythrocytes resulting in endothelial activation and systemic inflammation leading to CeGC dysregulation (81, 82). CM arises when untreated malaria develops into severe malaria, with a comorbidity with multiorgan failure, metabolic deregulation and anaemia (83).The infections disrupt the BBB, a prerequisite for the development of CM (84). The eGC reduces binding between CD36-transfected cells with the CD36-binding *P.falciparum*-infected erythrocytes, with the eGC degradation leading to their adhesion to the receptors (85) (see figure 2). Image analysis of transmission electron microscopy (TEM) has indicated coagulation and inflammation dysregulation was due to specific endothelial receptor binding of endothelial protein C receptor (EPCR) and ICAM-1 (86). Plasma levels of HA and sulphated GAGs could be used as a proxy marker for eGC shedding (49). However, other microcirculations within the body also contain an eGC that can be shed in a stressful physiological state, meaning that the eGC shedding cannot be directly linked to CeGC shedding (87).

Three treatments have been tested to alleviate CM and its symptoms. Corticosteroids such as dexamethasone and adjunctive antithrombin-3 (AT3) are shown to prevent CM progression, reduce inflammation as well as inhibit eGC shedding. The third treatment of Batimastat (a BB94 treatment), though positive, is limited (88).

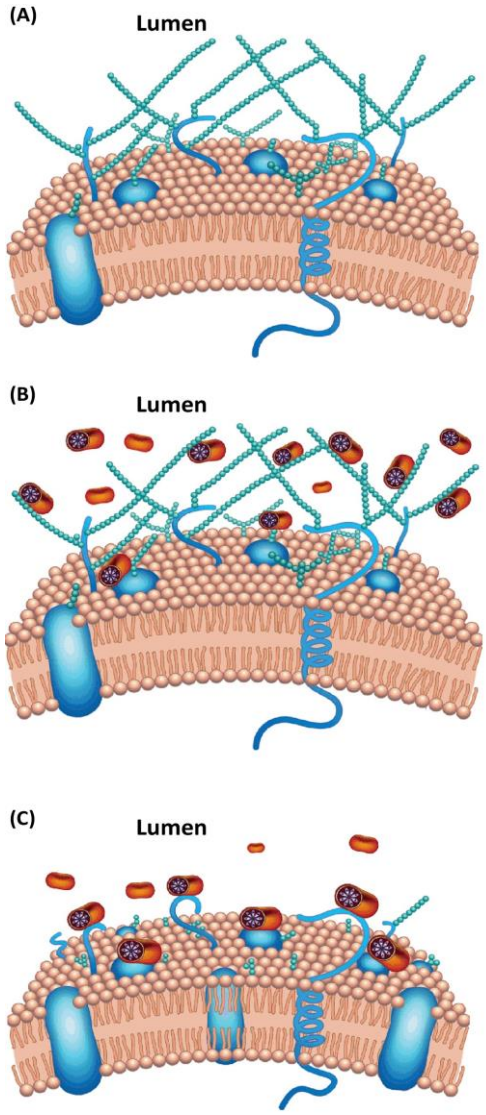

Figure 2 Breakdown of the eGC during malaria infection. The erythrocytes are in orange, with the purple nuclei representing the intracellular parasites. (A) Shows a healthy and intact eGC. (B) Shows the adhesion of the *Plasmadium*-infected erythrocytes, initially binding to the outer border of the eGC, onto the proteoglycans. (C) Shows a shedding and loss of the eGC, allowing direct adhesion to the glycoproteins and receptors on the endothelial surface. (89) – reproduced with author's permission

5.4.2 Sepsis

With three progressive stages – sepsis, severe sepsis and septic shock – this condition has a mortality rate above 50% for the most severe cases (90). This clinical syndrome results from a dysregulated and exponentially harmful immunological response to an infection, and is associated with acute organ dysfunction (91). Acute delirium, a cognitive impairment activated by BBB-penetrating cytokines (92) persists even after hospital discharge (93). Brain damage as a result of IL-1β-dependent neuroinflammation in early sepsis (94), showed decreased eGC thickness is a positive predictor for mortality in patients admitted with sepsis to ICU (6). The same study

revealed the eGC shedding as a principal pathophysiological mechanism, resulting in microvascular dysfunction and multiple organ failure (see figure 3). ROS such as NO and other proteases cause further eGC disruption, providing a significant site of sepsis-induced injury (26). After shedding, uncovered adhesion molecules on the endothelial surface induce leukocyte adhesion resulting in circulatory dysfunction, platelet aggregation and thrombus formation, (95). The eGC degradation also accelerates inflammation, hypercoagulation, capillary leakage and decreases vascular responsiveness. This negatively impacts blood flow, reducing oxygen delivery leading to organ failure (30).

Further evidence shows eGC fragments, such as HS hexa- and octasaccharides, lead to sepsis's cognitive dysfunction (96, 97). These fragments interact with growth factors and soluble proteins, such as brain-derived neurotropic factors (BDNF), and are heavily linked to memory, cognition and the HP, which suffers volume loss and BBB dysfunction in sepsis (96). Apart from HS as a superficial marker of eGC damage (98), there is a focus on syndecan-1 because it is a core transmembrane protein anchored and bound to HS (98), positively correlated with sepsis severity (99). There are conflicting results, with studies reporting HS levels rising proportionately quickly compared to syndecan-1 (100). whereas others found high syndecan-1 levels upon admission (101). These inconsistent results indicate different mechanisms to eGC damage and pathologies affecting the eGC and its components in diverse ways.

Alongside infectious diseases, there are neurological conditions that appear to be caused or exacerbated by these mechanisms.

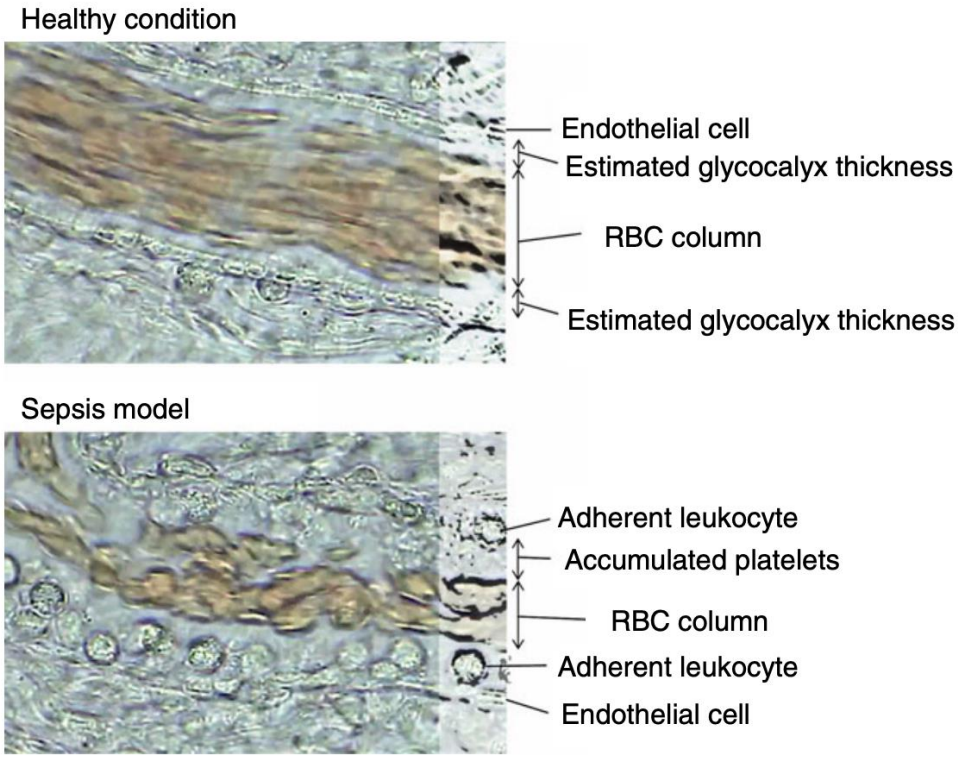

Figure 3 Comparison of the microcirculation between a healthy control vs. an endotoxin administration sepsis model. Using *in vivo* intravital microscopy, the healthy control shows clear boundaries for circulation under normal conditions. In the sepsis model, damage to the ECs can be seen with an adhesion of leukocytes and platelet accumulation showing a decrease in blood flow and suggests a degraded glycocalyx. (26) – reproduced with author's permission

5.4.3 COVID-19

The current coronavirus disease (COVID-19) pandemic, caused by the virus Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), is primarily a respiratory disease but has shown to have a wide-ranging spectrum of clinical presentations: from asymptomatic patients to critically ill cases with a high fatality rate (102). Alongside the lungs, it is able to inflict severe damage on multiple other organs in the human body. Most notably, the CNS and brain are appearing to seem to be secondary targets (103) with studies indicating a neurological disturbance in COVID-19 patients (103). Though knowledge of the pathogenesis of COVID-19 is still limited, studies are showing endothelial dysfunction being a central element in the more severe COVID-19 cases (104, 105).

COVID-19 primarily targets and binds to the angiotensin-converted enzyme-2 (ACE-2) receptors to attach and gain cell entry, therefore it is used as a significant determinant of viral entry and pathogenesis of COVID-19. ACE-2 has been found to be highly expressed in both endothelial cells (106, 107) and pericytes (108). Though, expressed in varying levels throughout the body, there is evidence indicating its

expression in various regions of the brain (109) such as stem-cell derived neurons (110), neuronal and glial cells (109) and the temporal lobe and HP (111). The latter two regions being highly involved in the pathology of AD in terms of affecting memory and cognition. It is worth nothing that two other extra entry receptor routes have been identified: Basigin (CD147) (112) and Neuropilin (NRP1) (113). Both of which coincidentally are also highly expressed in endothelial cells and pericytes (114).

Alongside inflammation from our body's immune response, there is evidence of neuroinflammation in particular, that is of interest due to the endothelial dysfunction found in severe COVID-19 cases (115). Elevated levels of cytokines have been located in the cerebrospinal fluid of patients reporting symptoms of neurological dysfunction (116). As well as post-mortem brain endothelial cells being detected close by to reactive gliosis markers could suggest this injury resulting from neuroinflammation (117). CeGC in relation to COVID-19 has been mentioned in some reviews (118). One of the closest measures to the CeGC was performed by Rovas (119) who has shown COVID-19 patients suffered both capillary and GC damage. With the use of SDF imaging, he found there was a marked density decrease of the sublingual micro-vessels.

With these results it is hard however to establish the initial cause of these neurological disturbances. The endothelial dysfunction could arise as a direct consequence of interaction with the virus. Or it could be achieved post-infection due the rise in cytokines and inflammatory response of the body against the virus (120).

5.5 Neurodegenerative disorders

There are more than 850,000 people with dementia in the UK. The cost of dementia in the UK in 2013 was £26 billion and is expected to grow as it is forecasted that the number of people with dementia in the UK could exceed two million by 2051 (121). Therefore, any research into possible treatment of those conditions is important.

Neurodegenerative disorders are mentioned together with inflammation and aging, but they have been rarely studied in terms of the CeGC, probably due to current techniques not directly measuring the CeGC (122) and the difficulty of studying it in isolation.

5.5.1 Subcortical vascular dementia

Subcortical vascular dementia (SVD) is the most common vascular dementia subtype. CeGC research indicates capillary stalling is increased and associated with CeGC loss in SVD (123), showing CeGC as a possible mediator for capillary stalling and possible target for SVD treatment (123).

5.5.2 Alzheimer's

Identification of the HP's BBB susceptibility to damage is of particular interest in research into AD, particularly the more frequent sporadic AD (SAD), with the condition's pathology tightly linked to the HP and the ever-increasing diagnosis of AD and other dementia subtypes (124). Observations suggest the initial problem is microvascular endothelial damage, causing increased BBB permeability allowing substrates to enter through the capillary wall, and inducing perivascular brain damage (125) which precedes and exacerbates the development of neurological conditions. Research indicates a positive correlation between HA accumulation and AD neuropathology (122). However, studies into BBB function in older individuals and patients with diagnosed cerebral microvascular disease produced varying results (126), with only one dementia study investigating the results by disease severity in the 1980's. However, with only 22 AD and 29 multi-infarct dementia participants, the study is limited, making its results inconclusive (127). Research into dementia is not straightforward with dementia subtypes overlapping (128). The role of HSPGs and HS in AD has been attracting attention (129), with HSPGs promoting Aβ and tau fibrilization and protecting against proteolytic breakdown (130), which suggests a relationship between AD features and heparinase expression (131).

To enable a more systematic study of the role of cerebral microvasculature in AD, a physiologically relevant three-dimensional *in vitro* human neural cell culture microfluidic AD model, which mimics BBB dysfunction, has been developed (132). However, it is not a model specifically for the CeGC.

5.5.3 Familial Alzheimer's disease

Familial Alzheimer's disease (FAD), with no known treatment (133), is a sub-category of AD with a prevalence of only 5% of AD cases (134) and a genetic predisposition to familial mutations (135) in at least three generations (134). FAD is caused by the mutation in three genes – amyloid precursor protein (APP), presenilin 1 (PSEN1) and 2 (PSEN2) – but the molecular reasons for this mutation are not clear (136). Research indicates that PSEN1 is the catalyst of the γ-secretase protease that produces Aβ from APP and the mutations are shown to increase $A\beta_{42}/A\beta_{40}$ ratio which leads to FAD (136-139).

BBB damage in FAD has been evidenced in several studies. Two *in vitro* studies using human cells showed that PSEN1 mutation alters the level of tight junctions' proteins and found Aβ deposits in the brain (140) identifying that PSEN1 mutation is more detrimental to BBB than PSEN2 mutation (141). Another *in vitro* study, using 5xFAD mice, showed the BBB damage at four months of age and that exosome derived from human neural stem cells reversed the BBB damage (142). However, to our knowledge

there are no studies of how CeGC dysfunction contributes to the pathogenesis of FAD, which is most likely due to the current challenges of techniques and limitations in the tissues available in research.

6. Challenges of researching CeGC and trends

6.1 Methods, techniques and subjects/tissues used to study CeGC

The main challenge of studying the CeGC is that it is highly fragile and very vulnerable to damage (15) which has complicated any examination to date.

Research has so far used several *methods* (*ex vivo, in vivo, in vitro*), *visualisation techniques* (e.g. staining, two-photon microscopy) and *subjects/tissues* (cells, animal models, humans) to assess the CeGC structure, composition and thickness. Only a brief review will be provided as more detailed overviews are provided elsewhere (3, 143, 144).

6.1.1 Methods and visualisation techniques

Current *ex vivo* techniques using post-mortem tissues to evaluate the CeGC and cerebral microcirculation mainly involve *microscopic evaluation of staining* to analyse structural changes (145-148). The most commonly used conventional stains are lanthanum nitrate (2), fluorescein isothiocyanate-linked wheat germ agglutinin (FITC-WGA) (146), Nissl (149) or a mixture of lanthanum and dysprosium (LaDy) (150). However, some are not CeGC specific (2) or they alter the CeGC (2, 148, 150). The staining is then analysed by, for instance, TEM (150) or light and electron microscopy (151). Recently there have been attempts to use frozen post-mortem samples without staining analysed by cryogenic transmission electron microscopy (cryo-TEM) (152-155) which could address the above issues. There are also efforts to develop models to reflect the *in vivo* actuality, such as *in situ* cells derived from the preparations of *ex vivo* tissue (156) or a development of three-dimensional cell culture models (157). *Ex vivo* techniques, though useful, are unable to provide functional analysis of both the eGC and microcirculation.

In vivo studies have seen the most recent advances. The spatial resolution of *confocal microscopy* enables detailed direct visualisation of the structure and composition of eGC (158) but the major challenge is the sample preservation by, e.g., formaldehyde which distorts the eGC (144). *Two-photon laser scanning microscopy (TPLSM)* has enabled direct visualisation of eGC both *ex vivo* and *in vivo* (1, 3, 143, 159). It records real-time images of single cortical capillaries, has good resolution, enhanced penetration depth (due to using long wavelength red photons which reduce scattering), low phototoxicity and an ability to section optical images (3, 143, 144, 160).

Sidestream dark force (SDF) imaging (161) enables visual mapping of microcirculations with a relatively safe, quick and straightforward non-invasive imaging technique, with the addition of Glycocheck software enabling analysis of the CeGC (14). Its limitation is that it measures the eGC indirectly by visualising the erythrocytes within the blood flow at the perfused boundary region (PBR) (1, 162). This allows unpredictable variables to distort the results, such as the effects of anaesthetics, any haemodynamic variations or intravascular volume variations (14). This is, however, an improvement, as previously the CeGC thickness was only sparsely evaluated due its collapse in *ex vivo* conditions. With most of the techniques mentioned focusing on the structure of the eGC, the molecular components still need to be studied. A versatile technique, *atomic force microscope (AFM),* is able to differentiate between the cell layers and, applied to eGC, it characterised the nanochemical properties on the eGC surface on a nanometre scale (163).

6.1.2 Tissues / subjects of eGC study

Further challenge is presented by comparing the CeGC thickness and structure using endothelial cells cultured *in vitro* with studying humans and animal, both *in vivo* and *ex vivo*.

In vitro studies of cells are valuable, however, they have shown the eGC composition and structure differ due to a lack of long-term shear stress in cells cultured *in vitro* (5, 164), with the eGC thickness substantially reduced *in vitro* (42, 165) or the slower recovery of eGC thickness *in vitro* suggesting that standard cell culture conditions do not provide the cellular conditions required for maintenance of the eGC *in vitro* (166). As a result, the validity of cultured cells has been recently questioned (144).

Post-mortem tissues used in *ex vivo* studies (see also above) have proved beneficial, but they cannot provide functional analysis (74). In addition, the eGC thickness is reduced due to damage caused by sample handling, and control of external variables is challenging. This was demonstrated by the comparison in mice using the two methods that showed the layer in the *in vivo* study was thicker, suggesting the eGC layer was damaged by the *ex vivo* method (159).

In vivo study of human subjects would be ideal but due to ethical and other considerations *the animal models* are often used instead. Animal tissues/subjects allow a higher degree of observation, manipulation and control, but results may not be fully applicable to humans (50). For instance, *AD mice models* (167), such as the commonly used 5xFAD mouse model, cannot encompass all the aspects of the AD pathology (168-171), even with an aggressive Aβ pathology and cognitive impairment. In addition, there are no standard operating procedures for identification of appropriate

AD models for different experiments, meaning results then depend on the used methods which can make the experiments difficult to repeat (168).

6.1.3 Assessment of cognitive function

Various tests can be used to determine the impact of the CeGC degradation on cognition. The *Mini-mental state examination (MMSE*) (135) is frequently used with human participants, though *Montreal cognitive assessment (MoCA)* is considered by some more sensitive (172). Regarding mice studies, *Morris water maze (MWM)* is a well-established test for measuring deficits in spatial memory and learning and has been routinely used with AD mice models such as 5xFAD (173-175).

6.1.4 Summary

With most research conducted on animal tissues *ex vivo*, it is becoming clear that to obtain precise and uninfluenced quantification of the CeGC we need to analyse living cells *in situ* and mainly humans *in vivo* (15), and techniques need to become more specific and sensitive (11).

Research currently focuses more on either the sublingual vasculature (SV) or the glomerulus (11). However, to progress our understanding of the cerebral microvasculature and CeGC, brain tissues should be used.

Though all the techniques have advantages and disadvantages, progress is being made in our understanding of the CeGC structure, mechanics and role.

6.2 Future research requirements

Future research should start determining variations in the CeGC components, what determines its permeability and dynamic nature, and what the mechanisms of dysregulation and repair are (74). There is also an issue of causality (51, 52) as we see a pattern of CeGC damage closely correlated with inflammation and other clinical conditions with cognitive impairment (6), yet it is still unclear whether eGC damage is the cause or a reaction. And though it is believed that the eGC thickness is proportional to its selectivity, this remains to be fully investigated. Ideally, BBB permeability changes before and during disease progression would be assessed in long-term human studies of patients predisposed to or at a higher risk of developing cerebral microvascular disease (126).

Overall, in the last half a decade, there has appeared to be a shift in focus towards our understanding of the BBB and its component, CeGC (9), with research shifting from more traditional neuronal factors towards a more integrative paradigm with an increasing emphasis on cell-cell signalling (176).

7. Conclusion

Interest in the BBB and its component, the CeGC, has been increasing in the last half a decade, shedding light on their functions and revealing their diverse and dynamic nature. The eGC structure and molecular properties enable its wide range of functions, all vital to the proper functioning of the vascular system and organs that depend on it. The eGC ability to adapt through the changes in its morphology between the different capillaries and locations show a specialisation to the varying demands of the body. Its natural degradation occurs as part of aging, alongside degradation related to infections, neurological disorders and cerebrovascular conditions, the symptomology of which has a recurring theme of cognitive impairment. This interest has most likely been sparked by the improvement in visualisation techniques, which have become clearer, more precise and less invasive, permitting the movement towards *in vivo* studies into the cerebral microcirculations so that the CeGC structure can be studied instead of inferred. From its understanding, a diagnostic biomarker as well as a potential therapeutic intervention for patients may be identified.

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