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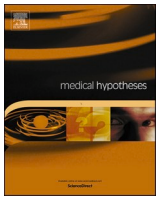

Long-term neurological implications of severe acute respiratory syndrome coronavirus 2 infections in neonates: Innate immune memory and chronic neuroinflammation

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection can cause long-term neurological complications in adults. However, the mechanisms by which early-life SARS-CoV-2 infection increases the risk of abnormal neurodevelopment remain unknown.

Recent studies have shown an association with chronic proinflammatory cytokine/chemokine production in the central nervous system (CNS). Therefore, it was hypothesised that innate immune activation and induction of innate immune memory may play a potential role in the neonatal brain. Haematopoietic stem cells in the bone marrow are exposed to SARS-CoV-2, SARS-CoV-2 envelope protein (E protein), lipopolysaccharide (LPS)-bound spike proteins (S1 and S2 proteins), and damage-associated molecular patterns (DAMPs). Myeloid progenitors enter the stroma of the choroid plexus and are further directed to incessantly supply the brain parenchyma with resident innate immune cells. The S proteins-LPS complex can cross the blood–brain barrier and plays an important role in microglial and astrocytic inflammatory responses and innate immune memory. Persistently activated microglia with memory release pro-inflammatory cytokines/chemokines which contribute to abnormal synaptic development in the frontal lobe and cerebellum, potentially leading to long-term neurological complications, similar to those observed in autism spectrum disorder (ASD). In addition, this hypothesis suggests that bacterial and fungal products may act as adjuvants to S proteins and may also explain why S proteins alone are insufficient to induce neuroinflammation in neonates.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can cause neurological diseases [1]. Recent findings have demonstrated that even mild respiratory SARS-CoV-2 infection in mice can trigger reactive microglia and sustained chemokine production within the central nervous system (CNS). This pathological response subsequently results in oligodendrocyte loss and impaired neurogenesis [2], and its potential impact on neonates cannot be understated.

Although no studies have reported a high incidence of severe neurological complications in neonates with SARS-CoV-2 infection, an increasing number of infants and children with neurological manifestations have been reported [2]. Moreover, it is unclear whether SARS-CoV-2 infection causes the same subtle brain dysfunction in neonates as in adults, as infection-related neurological complications are sometimes undetected until the affected neonates reach a certain age. A significant concern is the potential impact of future mutant SARS-CoV-2 variants on the developing brain. As new variants continue to emerge,

Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; CNS, central nervous system; E protein, envelope protein; LPS, lipopolysaccharide; S1 protein, S1 subunit of spike protein; S2 protein, S2 subunit of spike protein; S proteins, S1 protein and S2 protein; DAMPs, damage-associated molecular patterns; ASD, autism spectrum disorder; BECs, brain endothelial cells; TLRs, Toll-like receptors; hBMVECs, human brain microvascular endothelial cells; NF, nuclear factor; NOD, nucleotide-binding oligomerisation domain-containing protein; TNF, tumour necrosis factor; IL, interleukin; CCL2, CC chemokine ligand 2; CXCL, CXC motif chemokine ligand; CSF, cerebrospinal fluid; ACE, angiotensin-converting enzyme; BCSFB, blood-cerebrospinal fluid barrier; NLRP3, NLR pyrin containing 3; ER, endoplasmic reticulum; DCs, dendritic cells; mRNA, messenger RNA; HMGB1, high mobility group box1; MMPs, matrix metalloproteinases; C/EBP, CCAAT/enhancer-binding protein; CaMKII, calcium/calmodulin-dependent protein kinase II; MeCP2, methyl-CpG binding protein 2; COVID-19, coronavirus disease; ACC, anterior cingulate cortex; PCC, posterior cingulate cortex.

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the recurrence of pandemics and the occurrence of neonatal neurological complications may intensify.

The choroid plexus is vital for relaying peripheral inflammation to the CNS upon SARS-CoV-2 infection [3], and 18 % of infected neonates show neurological manifestations [4]. Neonates from mothers without antibodies against SARS-CoV-2 potentially have an infection risk; however, those from infected mothers may experience neurodevelopmental problems [5] and cytokine storms during pregnancy. Early-life SARS-CoV-2 infection may increase the risk of autism spectrum disorder (ASD) [6]. However, the possible mechanisms remain unknown.

The hypothesis

This study hypothesises that in neonatal SARS-CoV-2 infection, SARS-CoV-2 envelope protein (E protein) and lipopolysaccharide (LPS)-bound S proteins (S1 and S2 proteins) activate brain endothelial cells (BECs) and stromal macrophages in the choroid plexus through Toll-like receptor 2 (TLR2)-and TLR4-mediated inflammatory signalling and upregulate chemokine expression, thereby increasing blood–brain barrier and blood-cerebrospinal fluid barrier (BCSFB) permeability and directing viral invasion into the CNS. Moreover, innate immune training of myeloid progenitors in the bone marrow and the choroid plexus plays an important role in the continuous supply of activated myeloid cells to the CNS. Thus, innate immune memory is induced, resulting in chronic neuroinflammation and abnormal synaptic development that causes ASD-like neurological sequelae.

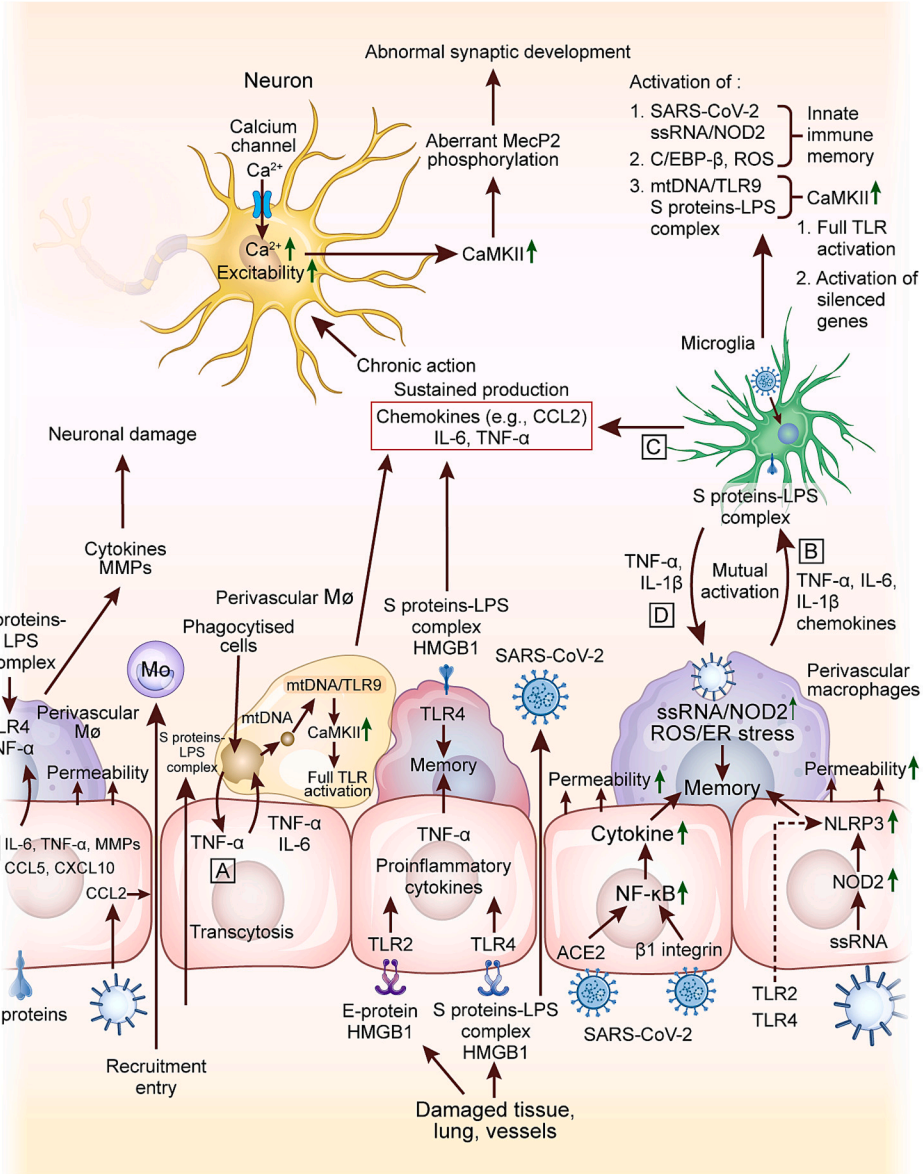

Fig 1. Activation of brain endothelial cells and induction of innate immune memory in the central nervous system. (SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TLRs, Toll-like receptors; NOD, nucleotide-binding oligomerisation domain-containing protein; ssRNA, single-stranded RNA; CaMKII, calcium/calmodulin-dependent protein kinase II; CCL2, CC chemokine ligand 2; E protein, envelope protein; S proteins, spike protein S1 and S2; S1 protein, S1 subunit of S protein; S2 protein, S2 subunit of S protein; LPS, lipopolysaccharide; MeCP2, methyl-CpG binding protein 2; TNF, tumour necrosis factor; IL, interleukin; CXCL, CXC motif chemokine ligand; NF, nuclear factor; NLRP3, NLR pyrin containing 3; ER, endoplasmic reticulum; HMGB1, high mobility group box1; MMPs, matrix metalloproteinases; C/EBP, CCAAT/enhancer-binding protein; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; Mo, monocytes; MΦ, macrophages).

Evaluation of the hypothesis

SARS-CoV-2 infection in neonates activates BECs via TLR2 through E protein and TLR4 through the formation of the S proteins-LPS complex

SARS-CoV-2 infects the human brain's microvascular endothelial cells (hBMVECs) and disrupts tight junction integrity [7]. Human cerebral endothelial cells express TLR2, 3, 4, and 6 [8], and microglia express TLR1–9 [9], with E protein activating TLR2 [10].

TLR2 expression is highest in the striatum on postnatal day (P) 1 [11]; however, its activation in the CNS is crucial for neuroinflammation and neuronal injury [12]. Early postnatal exposure to peripheral TLR2 activation can lead to learning and memory impairments later in life [13]. Therefore, irreversible brain damage may occur if neonates are infected with SARS-CoV-2 variants that overactivate TLR2 in the CNS. Moreover, while S proteins could not be clearly shown to induce inflammatory responses via TLR4 because of contamination issues with reagents [14], the S proteins-LPS complex induces inflammatory reactions through activation of cluster of differentiation 14/TLR4-myeloid differentiation factor 2 receptor complex/nuclear factor (NF)-κB signalling with its variable activity between SARS-CoV-2 variants [15]. This complex formation and inflammation of mucosal tissue may indeed occur in SARS-CoV-2 infection [14]. In a SARS-CoV-2 infected hBMVECs, signalling pathways involving nucleotide-binding oligomerization domain (NOD)-like receptors, TLR, cytokines such as tumour necrosis factor (TNF) and interleukin (IL)-6, and chemokines are significantly upregulated [7]. Through these innate immune signalling pathways, SARS-CoV-2 first activates BEC through the E protein and S proteins-LPS complex (Fig. 1).

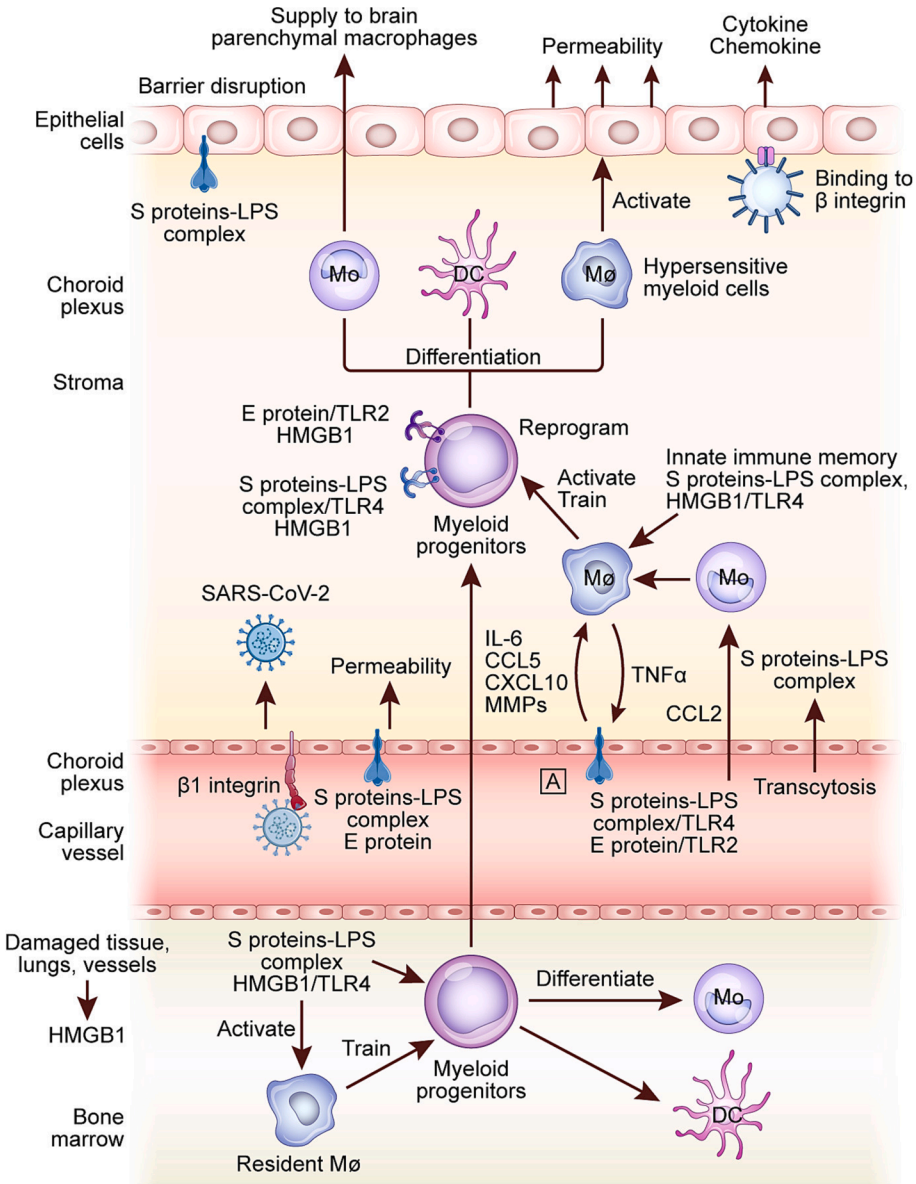

Fig 2. Choroid plexus as a reservoir of hypersensitive innate immune cells arising from reprogrammed myeloid progenitors induced by neonatal SARS-CoV-2 infection. (SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TLRs, Toll-like receptors; CCL, CC chemokine ligand; E protein, envelope protein; S proteins, spike protein S1 and S2; S1 protein, S1 subunit of S protein; S2 protein, S2 subunit of S protein; LPS, lipopolysaccharide; TNF, tumour necrosis factor; IL, interleukin; CXCL, CXC motif chemokine ligand; DC, dendritic cell; HMGB1, high mobility group box1; Mo, monocytes; MΦ, macrophages; MMPs, matrix metalloproteinases).

SARS-CoV-2 infection causes neuroinflammation through developmentally enhanced TLR2, CC chemokine ligand 2 (CCL2), and NOD2 signalling in the neonatal brain

TLR2 [11] and CCL2 [16] in the CNS are developmentally upregulated in the CNS of neonates. Peripheral TLR2 activation via Pam3CSK4 in neonatal mice upregulates chemokine genes, including *CCL2* and *CXC motif chemokine ligand 2* (*CXCL2*), in the choroid plexus [17]. Both chemokines increase in the cerebrospinal fluid (CSF) seven weeks post-infection in mild respiratory SARS-CoV-2 infection model using 6–12-week-old mice [18]. Furthermore, SARS-CoV-2 infects choroid plexus epithelial cells via the angiotensin-converting enzyme 2 (ACE2) receptor and enters the CNS [19]. Additionally, endothelial cells in capillary vessels are potentially susceptible to ACE2/NF-κB-mediating inflammatory reactions in SARS-CoV-2 infection, leading to capillary permeability [20].

Therefore, it is hypothesised that E protein may activate endothelial cells and stromal macrophages in the choroid plexus in neonates through TLR2-mediated inflammatory signalling and upregulate chemokine expression, thereby increasing blood–brain barrier and BCSFB permeability and directing viral invasion into the CNS (Figs. 1 and 2). CCL2 production can also be further activated, thus contributing to the entry of monocytes into the CNS. Therefore, when SARS-CoV-2 infects neonates, TLR2-activating E protein may contribute to the increased penetration of pro-inflammatory cytokines, proteins, and monocytes into the CNS.

NOD1 recognises meso-diaminopimelic acid-containing peptidoglycan found predominantly in gram-negative bacteria and contributes to rapid innate immune responses [21]; NOD2 plays an important role in anti-viral immunity in single-stranded RNA viral infection [22] and NLR pyrin-containing 3 (NLRP3) inflammasome activation in BECs [23]; and NOD-1, -2 signalling mediates endoplasmic reticulum (ER) stress-induced inflammation [24]. *Nod1* expression increases at P7 and peaks at P21 in the mouse striatum [21]. In contrast, *Nod2* expression increases from P1 to P7 [11]. Thus, the increase in the rate of Nod2 expression in the early postnatal days may play an important pathogenic role in SARS-CoV-2 infection.

Moreover, N protein activation promotes NLRP3 inflammasome activation in SARS-CoV-2-infected macrophages and dendritic cells (DCs) and the production of IL-1β and IL-6 [25]. Furthermore, TLR signalling is essential for inflammasome activation in the CNS [26]. Therefore, TLR- and NOD2-mediated signalling in BECs may lead to the further activation of NLRP3. Furthermore, *Nod2* expression was relatively higher in males than in females of all ages, and *Nod1* and *Nod2* expression were significantly higher in males than in females at P3 and P1, respectively [11]. Therefore, male neonates may have an increased risk of neurological sequelae compared to female neonates after neonatal SARS-CoV-2 infection.

SARS-CoV-2 infection in neonates induces innate immune responses and neuroinflammation

Neonatal SARS-CoV-2 infection may induce enhanced inflammatory reactions in the CNS through the following mechanisms:

Direct activation of perivascular macrophages by the S proteins-LPS complex

S1 protein crosses the blood–brain barrier via adsorptive transcytosis after binding to the surface receptor and is distributed to the brain parenchyma [27]. The same transport may also occur in the capillary vessels in the choroid plexus. S proteins have been immunohistochemically detected in capillary vessels in brain pathology [28]. S proteins can bind to LPS, boosting inflammatory reactions [15]. Furthermore, peripheral administration of LPS to eight-week-old mice increases the levels of IL-1β messenger RNA (mRNA) expression with microglial activation in the frontal and parietal cortex, hippocampus, and striatum [29]. Therefore, LPS-bound S proteins can hypothetically activate BECs and cross the blood–brain barrier and might directly activate perivascular macrophages, stromal macrophages of the choroid plexus, and microglia, thereby inducing an innate immune response (Figs. 1 and 2).

High mobility group box1 (HMGB1) and E protein/TLR2-mediated blood–brain barrier disruption

HMGB1 is an endogenous alarm signal released from damaged tissues that induces inflammatory reactions through TLR-2, -4, and -9 and receptors for advanced glycation end-product-mediated signalling [30]. Peripheral TLR2 activation via Pam3CSK4 in neonatal mice increases the influx of neutrophils and monocytes into the brain, along with increased blood–brain permeability in the cortex and cerebellum, followed by the midbrain and brainstem [31]. Therefore, enhanced TLR2 expression in neonates suggests that, along with E proteins, HMGB1 released from damaged peripheral tissues (e.g. vessels and lungs) contributes to increased blood–brain barrier permeability in the cerebellum and frontal cortex and cytokine/chemokine production, leading to neuroinflammation (Fig. 1). This may lead to disruption of the blood–-brain barrier and neuroinflammation in neonatal SARS-CoV-2 infection.

β1-integrin-mediating inflammatory signalling in BECs and choroid plexus

S protein has an integrin-binding arginyl-glycyl-aspartic acid motif [32] and induces inflammation in endothelial cells through integrin α5β1/NF-κB signalling [33]. Integrin β1 is highly expressed in the CNS, including the choroid plexus, and plays an essential role in congenital cytomegalovirus infection [34]. Therefore, SARS-CoV-2 can bind to cell surface β1 integrins in BECs, capillary endothelial cells, and choroidal epithelial cells in the choroid plexus, activating and disrupting the integrity of both barriers (Figs. 1 and 2), as in congenital cytomegalovirus infection. The choroid plexus is a possible entry route for SARS-CoV-2 into the CNS [19], and peripheral TLR2 activation in neonates preferentially increases permeability in the BCSFB over the blood–brain barrier [31] because capillary endothelial cells are more susceptible to peripheral inflammatory stimulation [20]. Therefore, SARS-CoV-2 variants with a high binding affinity to β1 integrins could threaten foetuses and neonates.

Increasing blood–brain barrier and BCSFB permeability by mutual activation between endothelial cells and macrophages initiated by SARS-CoV-2 proteins

S1 and S2 proteins, led to upregulation of gene expression for the pro-inflammatory cytokines IL-6, CCL5, CXCL10, and matrix metalloproteinases (MMPs) in hBMVECs, inducing a pro-inflammatory response [35]. Once the barrier integrity of BECs and capillary endothelial cells is impaired, endothelial cells and perivascular macrophages around BECs, as well as capillary endothelial cells and stromal macrophages of the choroid plexus, may be activated by pro-inflammatory cytokines such as TNF-α (Figs. 1A and 2A). Thus, monocytes are persistently recruited along with blood–brain barrier and BCSFB activation; resident macrophages are constantly replaced by activated macrophages, and pro-inflammatory chemokines/cytokines are produced in the brain parenchyma.

Amplifying inflammatory reactions between perivascular macrophages and microglia

Stimulation of BECs by TNF-α and IL-1β leads to their exaggerated release by perivascular macrophages, activating microglia [36]. Similarly, BECs activated by SARS-CoV-2 and the S proteins-LPS complex can hypothetically lead to increased production of TNF-α and IL-1β as well as IL-6, CCL5, and CXCL10 by perivascular macrophages, activating microglia (Fig. 1B). Microglia release pro-inflammatory cytokines/chemokines that damage neurones (Fig. 1C) and further activate perivascular macrophages (Fig. 1D). Particularly, microglia and perivascular macrophages could hypothetically be mutually activated through TNF-α (Fig. 1D and 1B). Acute encephalopathy can occur if

mutually activating inflammatory responses in the blood–brain barrier rapidly and intensively exceed or overshoot thresholds.

SARS-CoV-2 can induce innate immune memory in the CNS

Four possible mechanisms by which neonatal SARS-CoV-2 infection induces innate immune memory and persistent inflammatory reactions in perivascular macrophages and microglia are discussed below:

S proteins-LPS complex and HMGB1

Peripheral stimulation caused by LPS leads to innate immune memory in microglia, mediated by brain-resident macrophages alone, without the penetration of monocytes or LPS [37]. Moreover, a single peripheral administration of LPS epigenetically primed and trained inflammation-associated genes in microglia and re-stimulation leads to the upregulation of gene clusters in innate immune reactions and the regulation of cytokine production [38]. The S proteins-LPS complex can induce innate immune memory and immune priming in microglia by a similar mechanism. In the process of direct invasion and endothelial transcytosis, the S proteins-LPS complex might directly interact with perivascular macrophages and myeloid progenitors in the choroidal stroma, hypothetically undergoing training and priming and producing pro-inflammatory cytokines/chemokines in these cells (Figs. 1 and 2). As HMGB1 can induce innate immune memory [30], HMGB1 released from damaged tissue and the S proteins-LPS complex not only leads to a vicious cycle of HMGB1 production and inflammatory reactions but also enhances the memory effect of the S proteins-LPS complex.

Transcription factor CCAAT/enhancer-binding protein (C/EBP)-β-mediating mechanism

A single peripheral administration of LPS induces trained immunity via persistent epigenetic modifications in haematopoietic stem cells through C/EBP-β [39]. C/EBP homologous proteins are upregulated during ER stress [40] and induced in SARS-CoV-2-infected cells [41]. Thus, SARS-CoV-2-infected BECs, perivascular macrophages, and microglia are trained during cell stress responses, and C/EBP-β activation can be hypothesised (Fig. 1). Moreover, SARS-CoV-2 infection causes mitochondrial dysfunction [42], and mitochondrial reactive oxygen species chronically stress endothelial cells [43]. Therefore, reactive oxygen species production and chronic oxidative stress caused by SARS-CoV-2 infection may contribute to the induction of innate immune memory in BECs and perivascular macrophages.

Single-stranded RNA/NOD2-mediating innate immune memory in the CNS

As discussed above, NOD2 mediates the ER stress response [24]. Therefore, activating NOD2 signalling as an anti-viral immune reaction in SARS-CoV-2 infection can lead to innate immune memory through ER stress. Moreover, cytosolic bacterial muramyl dipeptide and Bacillus Calmette-Guérin [44] lead to trained immunity in monocytes through NOD2-mediating signalling, increased cytokine production, and protection against reinfection [30]. The Bacillus Calmette-Guérin/NOD2/NF-κB pathway contributes to increased histone 3 lysine trimethylation in IL-6 and TNF-α promoters in macrophages and antigen non-specific, cross-protective, and long-lasting innate immune reactions [45]. Similarly, the NOD2/NF-κB pathway-mediated immune memory may be induced in perivascular macrophages and microglia in preparation for SARS-CoV-2 invasion (Fig. 1). Therefore, upon increased expression of NOD2, TLR2, and CCL2 in the neonatal brain, naïve innate immune cells of the CNS have innate immune memory in microglia, perivascular macrophages, or DCs through epigenetic mechanisms.

TLR-mediating calcium/calmodulin-dependent protein kinaseII (CaMKII) activation

SARS-CoV-2 damages mitochondria, releases mitochondrial DNA into the cytoplasm, and activates the TLR9/NF-κB pathway, leading to endothelial dysfunction [46]. TLR3, 4, and 9 activations in macrophages cause an increase in intracellular calcium, and CaMKIIα activation contributes to the full activation of TLR signalling to promote pro-inflammatory cytokine production [47]. Therefore, extracellular release of mitochondrial DNA from damaged or apoptotic BECs and the S-proteins-LPS complex can activate perivascular macrophages via the TLR9- and TLR4-CaMKII pathways, respectively, leading to enhanced inflammatory cytokine production (Fig. 1).

Furthermore, activation of CaMKII induces methyl-CpG-binding protein 2 (MeCP2) release from repressed promoters of tumour suppressor genes into the cytoplasm without promoter DNA methylation or histone modifications, probably by changing MeCP2 phosphorylation sites, which leads to the epigenetic reactivation of silenced tumour suppressor genes in cancer cells [48]. Neonatal SARS-CoV-2 infection can hypothetically reactivate downregulated pro-inflammatory cytokine/chemokine genes in innate immune cells of the CNS without promotor DNA methylation/histone modifications.

Thus, perivascular macrophages, choroid plexus stromal macrophages, and microglia may hypothetically be repeatedly trained or reactivated with epigenetic modifications in inflammatory signalling and durably produce pro-inflammatory cytokines and chemokines, even without further stimulation from peripheral inflammation.

Role of innate immune training in the bone marrow and choroid plexus stroma in the persistence of neuroinflammation

The choroid plexus contains immature myeloid progenitors in neonatal rats and may serve as a reservoir for macrophages in the brain [49]. Therefore, myeloid progenitor cells can be constantly generated from the bone marrow to the choroid plexus stroma to maintain the homeostasis of innate immunity in the CNS.

The choroid plexus plays a crucial role in transmitting peripheral inflammation to the CNS after SARS-CoV-2 infection [3]. Haematopoietic stem cells/progenitor cells are activated and differentiate into myeloid cells via epigenetic mechanisms mediated by molecular cascades including LPS/TLR4/myeloid differentiation factor 88 and Toll/IL-1 receptor domain-containing adaptor inducing interferon β signalling pathway, as well as β-glucan/dectin-1/IL-1β signalling pathway [50]. Therefore, training of myeloid progenitors in the choroid plexus may be essential in neonatal SARS-CoV-2 infection because when they are trained by the S proteins-LPS complex and HMGB1/TLR4, they differentiate into activated DCs, macrophages, and monocytes. Moreover, the activation of peripheral TLR2 in neonates has been shown to enhance the permeability of the BCSFB compared with the blood–brain barrier [27]. This activation is facilitated by the E protein, in conjunction with the S proteins-LPS complex, which activates choroid plexus capillary endothelial cells and promotes the infiltration of myeloid progenitor cells into the choroid plexus stroma. This process results in their subsequent myeloid differentiation, contributing to a continuous supply to the brain parenchyma (Fig. 2). Additionally, the epigenetic mechanisms involved in the training of endothelial cells through activation of NLRP3 and NF-κB also contribute to these processes [51]. Therefore, SARS-CoV-2 and SARS-CoV-2 proteins may train choroid plexus capillary endothelial cells and BECs. Resident macrophages and epithelial cells chronically produce pro-inflammatory cytokines, enhancing myeloid progenitor memory and increasing BCSFB permeability.

As discussed, a single peripheral TLR4 stimulation leads to permanently trained immunity in stem cells via stress response and C/EBP-β activation. Moreover, bone marrow haematopoietic stem/progenitor cells are epigenetically trained by peripheral inflammation through IL-1β, such as periodontitis; myelopoiesis is sustained; and hypersensitive myeloid cells are constantly released [52]. Similarly, myeloid progenitor cells in the bone marrow could be trained by the S-protein-LPS complex, hypersensitive myeloid progenitor cells and myeloid cells may be persistently released into the systemic circulation. In addition, HMGB1 released from damaged tissues, such as the lungs and blood vessels, may

hypothetically augment the innate immune memory of bone marrow cells, including myeloid progenitors and surrounding macrophages through TLR4. Thus, trained myeloid progenitor cells, DCs, and monocytes released from the bone marrow upon SARS-CoV-2 infection may enter the choroid plexus stroma and contribute to the induction of innate immune training in resident myeloid precursor cells and macrophages (Fig. 1). Abnormalities in circulating myeloid progenitors and myeloid cells can be speculatively observed during long-term coronavirus disease (COVID-19) after neonatal SARS-CoV-2 infection. Chronic activation of the bone marrow and choroid plexus capillary vessels, peripheral monocytes, DCs, and myeloid progenitors released from the bone marrow constantly replaces and activates the resident cells in the choroidal plexus stroma. Trained myeloid progenitor cells penetrating the choroidal stroma may be activated by neighbouring resident macrophages by pro-inflammatory cytokines such as IL-6 and TNF-α and then differentiate into DCs and macrophages with hyper-responsiveness to inflammatory stimuli. In addition to the direct interaction between SARS-CoV-2 and the S proteins-LPS complex, chronic activation of choroid plexus epithelial cells by pro-inflammatory cytokines produced by stromal macrophages compromises the integrity of epithelial tight junctions. Thus, monocytes with enhanced pro-inflammatory cytokine/chemokine production are released from the choroid plexus stroma into the brain parenchyma, microglia are chronically activated, and neuroinflammation persists (Fig. 2).

Neonatal SARS-CoV-2 infection leads to the continuous production of pro-inflammatory cytokines/chemokines in the CNS. Thus, persistent inflammatory reactions, as observed in mild respiratory COVID [18], may have been caused by this hypothetical model.

Mechanisms causing an abnormal synaptic development after neonatal SARS-CoV-2 infection

CaMKII

CCL2 enhances neural hyperexcitability and neural transmission [53]. Calcium influx via neural activity triggers autophosphorylation of CaMKII by binding to calcium/calmodulin [54]. Moreover, CaMKII selectively binds to the beta subunits of L-type calcium channels and phosphorylates them, thereby facilitating their functions [55]. Thus, chronic CCL2 production induced by neonatal SARS-CoV-2 infection can impair the synaptic development and function modulated by CaMKII-mediated MeCP2 phosphorylation (Fig. 1).

Thus, microglial training following neonatal SARS-CoV-2 infection, which causes CCL2 production and excessive neuronal excitability, might lead to persistently increased intracellular calcium levels in neurones, non-physiological long-term CaMKII activation, and aberrant MeCP2 phosphorylation, leading to synaptic maldevelopment (Fig. 1).

Chemokines

In a mild respiratory COVID mice model, microglia/macrophages remained reactive, and sustainably increased CCL2, CCL5, CCL11, CXCL2, and CXCL10 levels in the CSF were observed at seven weeks post-infection [18], which is critical in neonatal brain development. First, CCL2 is expressed in Purkinje cells from week 27 of gestation to two years of age, promoting the growth of dendrites and synapses [56]. Second, the S1 and S2 proteins increase *CCL5* gene expression in hBMVECs [35]. Third, CCL11 promotes the migration and proliferation of neural progenitor cells in neonates [57]. Sustained elevated levels of these chemokines may be caused by choroid plexus stromal macrophages, perivascular macrophages, and microglia, which have memory functions (Figs. 1 and 2). Thus, chronic exposure to these chemokines from neonates to infancy, beyond developmentally regulated levels, may be the pathological basis for neurodevelopmental disorders.

Neonatal SARS-CoV-2 infection leads to abnormal synaptic development, followed by ASD-like neurological sequelae

Cytokine/chemokine production and sustained neuroinflammation in each brain region contribute to abnormal synaptic development.

Anterior cingulate cortex (ACC)

Mice stressed by the peripheral administration of LPS at P14 undergo depression by inducing microglial priming and excessive microglial engulfment of dendritic spines around the ACC [58]. TLR4 activation by the S proteins-LPS complex may lead to a similar pathology in neonates with ACC.

ACC dysfunction also causes social impairments in ASD [59]. Neonatal SARS-CoV-2 infection can contribute to chronic monocyte recruitment from the bone marrow into the ACC, potentially leading to the same brain pathology in ASD because tissue protein levels of CCL2 and CCL7 in the ACC and CCL2 in the cerebellum are increased in the brains of patients with ASD [60].

Posterior cingulate cortex (PCC)

The PCC has a relatively higher expression of the SARS-CoV-2 receptor ACE2 than other brain regions [61]. The strength of connectivity between the PCC and the frontal and limbic systems is associated with social functioning and restricted/repetitive behaviours [62]. Neonates exposed to postnatal stress have fewer connections between the PCC and other limbic systems [63]. Thus, innate immune activation may damage the default mode network and limbic system functions connecting the PCC during SARS-CoV-2 infection in neonates.

Cerebellum

E proteins [10] can activate TLR2; however, blood–brain barrier permeability preferentially increases in the frontal cortex and cerebellum via peripheral TLR2 activation in neonates, as discussed above. SARS-CoV-2 infection may lead to neurological complications similar to those of ASD by damaging Purkinje cells, which are vulnerable and important in ASD pathology [64].

Importance of "adjuvant" proteins from co-bacterial and fungal infection for neuroinflammation in neonatal SARS-CoV-2 infection

Given the rare occurrence of acute encephalopathy due to SARS-CoV-2 infection, viral proteins alone would not be sufficient to hyperactivate innate immunity and disrupt the integrity of the blood–brain barrier and BCSFB. Moreover, the synergy of TLRs and NODs in SARS-CoV-2 infection by co-infection of bacterial/fungal infection plays an important role in the cytokine storm [65]. Thus, only in the presence of co-infection with bacterial infection or DAMPs, including HMGB and mtDAMPs, do they synergistically cause excessive innate immune activation, inducing neuroinflammation and innate immune memory in neonates. Therefore, although most SARS-CoV-2-infected neonates appear to make a full recovery without neurological complications, activation of multiple innate immune signalling and "adjuvant" or "bystander" activation of innate immunity by bacterial co- or super-infection with SARS-CoV-2 would result in over-activation of innate immunity, such that co-infected mothers/neonates would be at the highest risk of neurological complications. Activation of TLR2 and TLR4, independent of SARS-CoV-2 proteins, may also be involved.

Furthermore, extending beyond SARS-CoV-2 infections, any viral infection rarely causes acute encephalopathy and neurological complications at any age. This may be explained by similar mechanisms, including the viral protein-adjuvant bacterial/fungal products complex, activation of multiple innate immune systems such as TLRs and inflammasomes, and direct entry of the complex into the CNS via transcytosis.

Another mechanism is that acute encephalopathy may occur, particularly in those who have been previously primed and have innate

immune memory from previous viral, bacterial and fungal infections.

Use of acetaminophen in neonates during SARS-CoV-2 infection may potentially augment neurological damage

A recent study suggested that postnatal use of acetaminophen in males may increase the risk of ASD [66]. Acetaminophen inhibits transcription factor NF-erythroid 2-related factor-2, which plays an essential role in innate defence and antioxidant pathways and contributes to hepatotoxicity via the TLR4/NF-κB/Mitogen-activated protein kinase pathway and inflammasome activation [67]. NF-erythroid 2-related factor-2 blocks pro-inflammatory cytokine transcription in macrophages [68] and protects against hypoxic-ischaemic brain injury in neonates [69]. In the neonatal brain, acetaminophen may enhance NOD2 activation by SARS-CoV-2 infection. Therefore, it is necessary to investigate whether using acetaminophen to treat SARS-CoV-2 infection augments the severity of neurological sequelae, including ASD, in neonatal mouse models.

Moreover, acetaminophen use in pregnancy decreased oestradiol levels in the placenta [70]. This might affect the neuroendocrine system and foetal brain development because placental oestrogen synthesis plays an important role in the oestrogen/hypothalamus–pituitary–adrenal axis in the foetal brain [71]. Acetaminophen might activate TLR4-mediating inflammatory reactions in neonatal SARS-CoV-2 infection. It will be necessary to make a strict distinction between neurological complications caused by a neonatal SARS-CoV-2 infection and those caused by acetaminophen taken during pregnancy.

S proteins alone in mRNA vaccines are unlikely to induce neuroinflammation

The formation of S protein-adjuvant complexes during vaccination under pathological conditions, such as co-infection with bacteria, is an important factor in neurological complications. Therefore, SARS-CoV-2 mRNA vaccines themselves are safe for adults. However, since neonatal brains can potentially be susceptible to even relatively minor TLR2 activation, SARS-CoV-2 vaccination against spike proteins seems risky from a neonatal neurological perspective.

Maternal immunisation is considered safe, as both, spike mRNA and protein are not detectable in cord blood and do not induce inflammation in the placenta [72]. These results suggest that maternal immunisation is unlikely to induce neuroinflammation in the foetal brain.

Discussion

Depending on the future variants, the SARS-CoV-2 infection may present as more than simply a common cold for neonates and infants. It may be dangerous for brain development if viral infection results in persistent innate immune activation and calcium signalling in the CNS. Thus, whether novel SARS-CoV-2 protein mutants strongly activate TLRs and NODs, either alone or through increased binding activity to co-infected pathogen-derived products and DAMPs, induce innate immune-mediated inflammation and memory, or lead to epigenetic up- or downregulation of synaptic proteins and neurotransmitter molecules.

Therefore, based on developmental mechanisms, we should be cautious and aim to conduct predictive rather than retrospective research on the effects of any mutant strain on the neonatal brain. Thus, developing methods using *in silico* analysis and artificial intelligence may help assess whether emerging variants are dangerous for foetal and early postnatal brain development.

Therefore, experiments using a mild respiratory SARS-CoV-2 infection model [18] using neonatal mice are necessary, and neurodevelopment can also be tested.

Conclusion

SARS-CoV-2 infection in neonates can cause ASD and neurodevelopmental problems by inducing long-lasting subclinical neuroinflammation and innate immune memory in the CNS. Therefore, emerging variants that influence postnatal brain development should be carefully monitored.

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Consent statement/ethical approval

Not required.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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