Ischemic stroke: From pathological mechanisms to neuroprotective strategies

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Ischemic stroke (IS) has complex pathological mechanisms, and is extremely difficult to treat. At present, the treatment of IS is mainly based on intravenous thrombolysis and mechanical thrombectomy, but they are limited by a strict time window. In addition, after intravenous thrombolysis or mechanical thrombectomy, damaged neurons often fail to make ideal improvements due to microcirculation disorders. Therefore, finding suitable pathways and targets from the pathological mechanism is crucial for the development of neuroprotective agents against IS. With the hope of making contributions to the development of IS treatments, this review will introduce (1) how related targets are found in pathological mechanisms such as inflammation, excitotoxicity, oxidative stress, and complement system activation; and (2) the current status and challenges in drug development.

KEYWORDS
ischemic stroke, inflammation, excitatory toxicity, oxidative stress, complement system, thrombus

Introduction

Ischemic stroke is one of the most common cerebrovascular diseases. With the aging of society, personal underlying diseases (such as hypertension, diabetes, heart disease, and hyperhomocysteinemia), smoking, alcohol consumption, and other factors, the incidence of IS has been continuously rising. According to the World Health Organization (WHO), more than 1.1 million people die each year from IS (1), showing that IS is seriously endangering people’s health. The pathological mechanism of IS is very complex, including inflammation, excitotoxicity, oxidative stress, and the complement system, which eventually cause apoptosis and necrosis of neurons in the ischemic area. In the complex pathological mechanism of IS, inflammation was undervalued in the past because the brain was for a long time considered an immune-privileged organ, but its role is now more and more appreciated. Excitotoxicity is mainly caused by the increased glutamate and the subsequent calcium overload, which transformed the field of stroke research in the 1980s (2). Oxidative stress presents quite a challenge to ischemic tissue, particularly after reperfusion. Moreover, the activation of the complement system, thrombus formation, and pericyte death are important factors in triggering IS and subsequent neuronal death.
At present, intravenous thrombolysis and mechanical thrombectomy are the main treatment methods for IS, but they all have different restrictions. Intravenous thrombolytic therapy is usually represented by alteplase. Intravenous thrombolysis can be performed with alteplase within 4.5 h after an acute stroke, with the condition of excluding coagulation disorders and controlling blood pressure below 180/105 mmHg (3). The conventional regimen of clinical antplatelet therapy is a combination of clopidogrel and aspirin. This dual antplatelet treatment (a loading dose of 300 mg of clopidogrel plus 300 mg of aspirin, followed by a maintenance dose of 75 mg of clopidogrel and 75 mg of aspirin during the first 21 or 90 days), is effective in preventing IS after the onset of a transient ischemic attack (TIA) (4). Tenecteplase, a genetically modified variant of alteplase with increased fibrin specificity, shows similar safety and efficacy compared with alteplase in some clinical trials (5, 6).

Compared to alteplase, tenecteplase has more advantages, such as a longer half-life, greater ease of use administered as a bolus medication, lower cost in some settings, and a higher incidence of reperfusion when combined with thrombectomy (6–8). These strengths may make it more promising in the treatment of IS. Mechanical thrombectomy, performed within 6 h after the onset of stroke, is another first-line treatment strategy for patients with ischemic stroke (3). However, mechanical thrombectomy is limited to the treatment of basilar artery occlusion in hospitals with related equipment and conditions (9).

In general, there are certain deficiencies in conventional treatment regimens, because of the strict time window, difficulty in delivering drugs to the central nervous system (CNS), and the inability to reverse the neuronal death that has already occurred. Even after the thrombus is removed, there is still a blockage of capillaries attributed to the dead pericytes losing their ability to regulate blood vessels in the brain (10). Furthermore, researchers need to pay more attention to ischemia-reperfusion injury in the brain after revascularization.

Therefore, to overcome these difficulties and break these traditional constraints, researchers need to find potential targets and develop new neuroprotective agents for treatment strategies based on the pathological mechanism of IS. In this review, we mainly introduce the pathological mechanisms after IS such as inflammation, excitotoxicity, oxidative stress, complement system, and microcirculation.

Inflammation

The extremely complex inflammatory response in the CNS consists of immune cells derived from the lymphatic circulation, resident microglia, monocytes, neutrophils that originate from the peripheral circulation, and cytokines secreted by various inflammatory cells after stroke. Innate immunity is rapidly involved in post-IS inflammation. Damage-associated molecular patterns (DAMPs) such as heat shock protein (HSP), high mobility group protein B1 (HMGB1), and hepatoma-derived growth factor (HDGF) can be recognized by pattern recognition receptors (PRRs) on some effector cells, thereby activating associated transcription factors and stimulating effector cells to secrete inflammatory factors. As part of the innate immune system, antigen-presenting cells (APCs) play a key role in the initiation of adaptive immunity. Dendritic cells (DCs) are one type of APC. Langerhans cells (a type of immature DCs) mature in lymphoid tissue after ingesting and processing autoantigen released by damaged tissue and necrotic neurons. Then DCs express peptide–MHC complexes along with highly expressed B7 (CD80/CD86), which generates a double stimulus for T cells, thereby mediating adaptive immunity. Inflammatory factors and chemokines are mainly produced by activated immune cells, inducible peripheral monocytes and neutrophils to migrate and infiltrate into the ischemic penumbra. In humoral immunity, B lymphocytes can differentiate into memory B cells and plasma cells that can produce antibodies. Although B cells may be involved in post-stroke pathologies, such as their ability to mediate delayed cognitive impairment following stroke (11), their effect on neuroinflammation is significantly weaker than that of T cells after acute ischemic stroke (AIS) (12). Furthermore, some experiments have shown that in the middle cerebral artery occlusion (MCAO) model, mice deficient in B lymphocytes exhibit no significant changes in cerebral infarct size and neural function compared with the wild-type (12), suggesting that B lymphocytes may not be the key to affecting neuroinflammation after acute stroke. Here, we mainly describe several inflammatory cells (Figure 1) that play important roles in the acute inflammatory response after stroke.

Microglia

As a branch of the monocyte-phagocytic system, microglia are residents in the CNS, primarily responsible for immune surveillance and scavenging of pathogens and dying neurons (13). Microglia are the largest number of immune cells in the CNS, accounting for 5–10% of total brain cells (1–4). Microglia are involved in a variety of CNS diseases, including amyotrophic lateral sclerosis (ALS), Ischemic stroke (IS), Alzheimer’s disease (AD), meningitis inflammatory, and schizophrenia (15–18). Microglia show contradictory functions in post-stroke inflammation (19). This may be due to their different phenotypes. Usually, microglia can be divided into three types: M0 (surveillance), M1 (pro-inflammatory), and M2 (anti-inflammatory) (19). M0 is primarily responsible for surveillance, with characteristics of low phagocytosis and inactivity (20, 21). In acute inflammation after stroke, M1 is generally considered to be activated earlier than M2. In fact, in the early stages of IS, the M2 type is the first to be activated, and its main function is to remove necrotic debris and protect brain tissue. Then M1 type mainly involved in brain tissue damage is activated (22). M1 type plays
an important role in neuroinflammation after stroke, and the polarization of microglia to M1 has attracted a lot of attention. Many stimulatory factors cause the polarization of microglia toward M1, such as INF-γ secreted by Th1 cells activating the JAK/STAT pathway (22) or lipopolysaccharide stimulating Toll-Like receptor 4 (TLR4) on microglia (23). Activated M1 type can produce a variety of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, IL-23, IL-18, IL-12, CCL2, and CXCL10), reactive oxygen species (ROS), matrix metalloproteinase 9 (MMP9), and matrix metalloproteinase 3 (MMP3) leading to the apoptosis of neurons, the migrations of peripheral cells, the activation of immune cells, and the destruction of blood-brain barrier (BBB) (24–28). In contrast, M2 microglia mainly play an anti-inflammatory role and initiate neurogenesis, synaptogenesis, and neurovascular unit remodeling in the late stage of IS (29).

In addition, the different polarization patterns of microglia may be related to the microenvironment, age, gender, temperature, and diabetes (30–34).

Promoting microglia polarization toward M2 while inhibiting M1 has emerged as a therapeutic approach for AIS. Minocycline (Table 1), as a selective inhibitor of M1 microglia, can be capable of reducing inflammation and promoting neurogenesis (35). In an open-label and evaluator-blinded study, patients with acute stroke had a significantly better outcome with minocycline treatment compared with placebo. This finding suggested a potential benefit of minocycline in AIS (36). Exendin-4 (Table 1), as a glucagon-like peptide receptor 1 agonist, in addition to its usage in blood glucose control, can promote the polarization of M2 microglia thereby providing neuroprotection and improving the prognosis of MCAO mice (37). In addition, two clinical trials on Exendin-4 treating IS are under recruitment. Hyperglycemia has been shown to activate M1 microglia (38), and the neuroprotective effects of Exendin-4 may be based on an indirect inhibition. Furthermore, the microenvironment exerting its influence on microglia is important for post-IS inflammation. For example, lacking folic acid (Table 1) will activate microglia via Notch/NF-κB signaling in MCAO rats and BV-2 microglia after undergoing oxygen and glucose deprivation (OGD) (39). However, in two clinical trials of stroke, daily administration of folic acid, vitamin B6, and vitamin B12 did not seem to be more effective than a placebo in reducing the incidence of major vascular events, cognitive impairment, or cognitive decline (40, 41). In general, despite the difficulties in the transition to clinical, targeting microglia seems to be important in the treatment strategy of IS.

Macrophones

In rodents, according to the expression level of lymphocyte antigen 6 complex C1 (Ly6C) and chemokine receptors, monocytes are mainly divided into two subpopulations, namely pro-inflammatory subpopulations (Ly6C\text{high}CCR2\text{high}CX3CR1\text{low}) and anti-inflammatory subpopulations (Ly6C\text{low}CCR2\text{low}CX3CR1\text{high}) (42–44). The CCL2-CCR2 axis is key to driving peripheral monocytes to the infarct area (45). Targeting the CCL2-CCR2 axis appears to be an ideal anti-inflammatory regimen due to the high expression of CCR2 in pro-inflammatory subpopulations. However, anti-inflammatory subsets in CNS are mainly transformed by infiltrating pro-inflammatory subsets rather than derived from peripheral monocytes (42), which makes targeting CCL2-CCR2 unfavorable to the prognosis of patients with IS from this perspective. Microglia were previously thought to be originated from peripheral macrophages because they have the same surface markers: CD11b, F4/80, and Iba-1 (46). In addition, microglia and macrophages have similar phenotypes, resulting in difficulty to distinguish them for researchers (47).

Microglia are now thought to be derived from hematopoietic stem cells (HSCs), whereas microglia are the descendants of yolk sac erythromyeloid progenitors (EMP) (48). Researchers have found that, after IS, CXCR4 promotes monocyte infiltration and regional restriction of infarct tissue by macrophages derived from peripheral monocytes. Conversely, CXCR4 deficiency reduces the ability of monocytes to infiltrate the ischemic brain (49).

Macrophages are mainly derived from circulation, intestine, spleen, etc. In the early stage of IS, macrophages are induced to express triggering receptors expressed in myeloid cells 1 (TREM1), amplifying the inflammatory effects along with PRR (50). Interestingly, macrophages also contribute to neurogenesis. Mohle et al. believe that the reduction of neurons in the hippocampus is strongly correlated with the reduction of peripheral monocytes after oral antibiotics (51, 52). This suggests that macrophages may play a different role after a stroke. Macrophages derived from the spleen also get into CNS after IS. On the first day after cerebral infarction, the number of macrophages infiltrating CNS was significantly reduced in MCAO mice without spleen compared with the model group (53). Therefore, blocking the source of macrophages and preventing the differentiation of pro-inflammatory phenotype may be a strategy for the treatment of IS.

Neutrophils

Neutrophils are the first leukocytes to infiltrate the ischemic brain after stroke, peaking at 48–72 h in CNS (54). The role of neutrophils in IS mainly includes the following aspects. The first role is secretory effect. There are many active substances such as MMP9, ROS, RNS, chemokines, and pro-inflammatory factors secreted by neutrophils, which mediate inflammation and the disruption of the BBB (55). Second, promoting thrombosis and blocking cerebrovascular, then leading to a no-reflow phenomenon after stroke (56). The third aspect is the neutrophil extracellular traps (NETs). The primary role of NETs is to
TABLE 1 Clinical trials and pre-clinical studies of drugs.

| Generic name | Target or signaling pathways | Pre-clinical references | Applications | Clinical trial | Phase | Results/status of the clinical trial |
|--------------|------------------------------|-------------------------|--------------|----------------|-------|-------------------------------------|
| **Anti-inflammatory** |
| Natalizumab | Integrin α4β1 | (158) | Mice | NCT02730455 | 2 | Completed |
| Minocycline | Microglia | (159) | Mice | NCT00380020 | 4 | Terminated |
| Exendin-4 | Microglia | (37) | Mice | NCT03827076 | 2 | Active not recruiting |
| Folic acid/B12/B6 | Microglia | (39) | Rat | NCT08354081 | 3 | Completed |
| t6-FP | MAF | (89) | Mice | — | — | — |
| Tak242 | TLR4 | (160) | Rat/in vitro | — | — | — |
| Isoquercetin | TLR4 | (161) | Animals/in vitro | — | — | — |
| Dexametomidine | HMGB1/TLR4/NF-κB | (162) | Rat | NCT04916197 | 4 | Recruiting |
| **Anti-excitotoxicity** |
| NA-1 | GluN2B-PSD95-NOS | (163) | Animals/neuronal Cultures | — | — | — |
| ZL006 | GluN2B-PSD95-NOS | (164) | Mice/rat | — | — | — |
| ICS7201 | GluN2B-PSD95-NOS | (165) | In vitro | — | — | — |
| β-lactam antibiotics | GLT-1 | (103) | Mice/glial cell (gestation day 14–16 CD1 mice) | NCT05375240 | 2 | Not yet recruiting |
| Memantine | Extrasynaptic NMDARs | (125, 126) | Rat/mice | NCT02535611/ NCT02144584 | 3/0 | Completed/active not recruiting |
| Ketamine | NMDARs | (166) | Mice | NCT03223220 | 2, 3 | Unknown |
| Geniposide | GluN2A/AKT/ERK | (167) | Rat | — | — | — |
| Pseudoginsenoside-F11 | Akt-Creb | (168) | Rat | — | — | — |
| **Anti-oxidative stress** |
| Acetylcysteine | TNF-α, iNOS, GSH, System x(c)- | (112, 113) | Rat | NCT04918719/ NCT04920448 | 2/2 | Not yet recruiting/ |
| Edaravone | ROS, RNS | (169) | Mice | NCT02430350 | 3 | Completed |
| Uric acid | ROS | (170) | Mice | NCT08860366 | 2, 3 | Completed |
| Melatonin | ROS, MDA, TNF-α | (171) | OGD/R-induced neuron | NCT05247125 | 4 | Recruiting |
| tBHQ | Nrf2/ARE | (172) | Rat | — | — | — |
| Trans sodium crocetinate (TSC) | SIRT3/FOXO3α/SOD2 | (173) | Rat | NCT03763929 | 2 | Terminated |
| Genipin | UCP2-SIRT3 | (174) | Mice | — | — | — |
| **Complement system** |
| Human C1-esterase inhibitor | C1s, MASP-2 | (157) | Mice | NCT01694381 | 0 | Terminated |
| B4cry | IgM | (149, 150) | Mice | — | — | — |
| polyman2 | MBL, C1s | (156) | Rats | — | — | — |

Capture and neutralize invading microorganisms (57), yet it is involved in the formation and stabilization of thrombus after stroke, which can lead to persistent ischemia in the brain (58). The fourth aspect is the phagocytosis of neutrophils. Neutrophils contribute to the clearance of necrotic tissue (59). The fifth aspect is the neurorestorative function. There is growing evidence that reparative neutrophil subsets and their products can be deployed to improve neurological outcomes (60, 61).

Similar to microglia and macrophages, conflicting phenotypes are also present in neutrophils. Neutrophils affected by tumor microenvironments can differentiate into two
subtypes: N1 (anti-tumor) and N2 (pro-tumor) (62, 63). This contradictory phenotype also occurs in patients with stroke (55, 64). Targeting the neutrophil phenotype may also serve as an alternative to anti-neuroinflammation, and the ability of neutrophils to infiltrate the CNS also makes it a potential target for assisting drugs in getting into brain tissue (65).

**T cells**

T cells develop, differentiate, and mature in the thymus, undergoing processes such as TCR development, positive selection, and negative selection. Then the vast majority of T cells transform into single-positive T cells (TCRαβ+CD4+CD8− or TCRαβ+CD4−CD8+) mainly involved in adaptive immune, others mainly differentiate into TCRγδ+CD4−CD8− T cells involved in innate immune. T cells in the spleen are involved in the pathological process of IS, resulting in reduced spleen volume and histomorphological changes (66). The involvement of T cells is often thought to aggravate brain damage but in some experiments the performance of T cells is contradictory. In mice with splenectomy, the neurological function is improved at the early stages of IS, but long-term neurological recovery is detrimental (67). In addition, studies have found that neurogenesis in the hippocampus is significantly reduced in mice lacking a complete immune system, especially those lacking CD4+ T cells (68).

According to the leukocyte differentiation antigens, T cells are roughly divided into CD4+ T cells and CD8+ T cells, as well as mostly double-negative γδT (CD4−CD8−) cells.

**CD4+ T cells**

CD4+ T cells mainly include Th1, Th2, Th17, Th9, Th22, TFH, Treg, and other subpopulations. Th1 cells release the pro-inflammatory cytokines IFN-γ, IL-2, and TNF-α/β, and induce microglia and macrophage polarization toward M1 (69), which aggravates neuroinflammation after stroke. Contrary to Th1, Th2 promotes the polarization of microglia and macrophages toward the M2 type (70). Th1 and Th2 differ significantly in downstream cytokine lineages, and the Th1/Th2 mold can affect the outcomes of stroke (71). IL-33, as a member of the IL-1 family, improves MCAO mice’s neurological deficit scores and reduces infarction volume by reducing IFN-γ+ T cells and increasing Foxp3+ T cells in the spleen, thereby shifting Th1/Th2 mode to Th2 immune deviation and exerting a neuroprotective effect (70, 72). Similar to Th1, Th17 aggravates brain injury after a stroke. Intestinal Th17 are activated and then migrate into the meninges attributing to the CCL20–CCR6 axis after stroke (73). Furthermore, the CCR6–CCL20 axis can inhibit Treg differentiation and direct Tregs toward the pathogenic Th17-lineage (74). Targeting CCL20–CCR6 may be an ideal strategy for treating IS. Pioglitazone as a drug for the treatment of diabetes can reduce peripheral CCL20. Some animal experiments have shown that PG can reduce the inflammatory response after traumatic brain injury (TBI) (75), but whether PG can reduce neuroinflammation after stroke remains unclear. Treg is a special subset of Th2 that has been shown to negatively regulate neuroinflammation after stroke (76, 77). CD4+CD25+Foxp3+ Treg can inhibit neuroinflammation by producing the inhibitory cytokine TGF-β, IL-10, and IL-35 (78, 79). Treg cells have been a key topic in dealing with neuroinflammation after acute ischemic stroke in recent years.

**CD8+ T cells**

CD8+ T cells are mainly cytotoxic T lymphocytes (CTL), which are the key to the occurrence of neuroinflammation after stroke. CTL mainly mediates cellular immunity and exerts cytotoxic effects on target cells. The function process of CTL is as follows: first, CTL cells bind target cells. CTL cells are activated by identifying the peptide-MHC-I complexes on target cells. Then the active substances in CTL are transferred to the immune synapse which is structured with a ternary structure (TCR–MHC–peptide) and surrounding adhesion molecules. Finally, CTL launches a lethal attack and mediates apoptosis through the perforin-granzyme pathway, Fas/Fas pathway, and TNF-α pathway. Some studies have shown that consuming CD8+ T cells show a better neuroprotective effect than consuming CD4+ T cells, indicating that CD8+ T cells are more active than CD4+ T cells in post-IS neuroinflammation (70, 80, 81). In addition, after peripheral CD8+ T cells were depleted, the infiltration of macrophages, neutrophils, and CD4+ T cells into the infarcted brain tissue in transient middle cerebral artery occlusion (TMCAO) mice was correspondingly reduced (82). The toxic effects of CD8+ T cells can be achieved through the FASL-PDPK1 pathway and inhibition of PDPK1 can effectively improve neural function after stroke (83). Compared to CD4+ T cells, targeted therapy for CD8+ T cells may achieve a better outcome in acute IS.

**Other T cells**

After IS, non-specific immune T cells are also involved in the inflammatory response in the ischemic brain. γδT cells are distributed to the skin, intestines, airways, and other tissues after maturity in the thymus (84, 85), exerting an innate immune effect. Nasal-associated lymphoid tissue (NALT) may be one of the sources of γδT cells in the ischemic brain due to distance, but NALT ablation does not improve infarct size in stroke animals (84). After the proposal of the microbiome-gut-brain axis, γδT cells in the intestine are considered to be capable of migrating to the meninges after stroke, and the state of the microbiome in the gut can affect Treg/γδT cells ratio which is highly correlated with stroke outcomes (86). γδT cells predominantly secrete IL-17, mediating chronic inflammation after stroke, and promote
the migration of neutrophils and monocytes to the ischemic brain, exacerbating stroke outcomes (86–88). Compared to other T cells, mucosal-associated invariant T (MAIT) cells were involved earlier in neuroinflammation after stroke. TMCAO mice with a MAIT deficiency or MAIT inhibitory ligand drugs (isobutyryl 6-formylpterin, i6-FP, in Table 1) showed a smaller infarct size compared to the model group (89). NKT cells are also part of non-specific immune T cells. Although NKT cells in cancer, hepatitis, pneumonia, and sepsis are increasingly valued (90–92), their involvement in neuroinflammation after IS requires further investigation.

Inflammation has been increasingly studied in IS since researchers moved away from the dogma that the brain is an immune-privileged organ. Additionally, this theory of immune privilege may rest on the low permeability of the BBB. However, the integrity of the BBB is disrupted after stroke, which facilitates the migration of peripheral inflammatory cells to the CNS (93, 94). In fact, a damaged BBB is not the only way inflammatory cells enter the ischemic brain. Currently, more investigators tend to support the theory that the choroid plexus is the main route of peripheral lymphocytes getting into the ischemic brain (95). Chemokines and chemokine receptors play a key role in the migration of inflammatory cells to the ischemic brain, such as CCR2-CCL2 related to most T cells (95), CCR6-CCL20 related to 17+ T cells (74), and CXCL1/CXCR2 related to neutrophils. But not all chemokines and chemokine receptors exacerbate neuroinflammation. For example, the CXCR3/CXCL10 axis, which serves as the brain-homing mechanism for CD8+CD122+CD49d+ regulatory-like cells, can provide neuroprotection in MCAO mice (96). Therefore, regulating peripheral inflammatory cells or regulatory-like cell migration could serve as a strategy for treating IS. In addition, it is important to regulate the activation of inflammatory cells in the CNS. Although the emergence of the brain-gut axis theory may make the gut microbiota and pathogen-associated molecular patterns (PAMPs), the initiators of neuroinflammation, sterile inflammation triggered by DAMPs remain the main type after the onset of IS. DAMPs internalization was largely mediated by the class A scavenger receptors MSR1 that was regulated by the transcription factor Mafb (97). MSR1 and Mafb may be promising targets for treating IS. Finally, targeting inflammatory cells themselves or the inflammatory factors they produce can provide enlightenment for future drug development for IS.

## Production and metabolism of glutamate

Glutamate in the brain originates from multiple pathways (Figure 2). Glutamate in the periphery does not enter the brain under physiological conditions due to BBB. However, studies have shown that glutamate levels in the brain can be reduced by peritoneal dialysis after stroke (98). The main reasons for this phenomenon may be as follows. First, disrupted BBB. After the stroke, a high concentration of glutamate in brain tissue and a low concentration of glutamate in blood will form a gradient (99, 100). Disrupted BBB may facilitate glutamate to enter the periphery. Second, glutamate in the brain generates glutamine, which can directly cross BBB into the periphery. Then peripheral glutamate regenerated by glutamine is cleared by peritoneal dialysis. There is a glutamate-glutamine cycle in neurons and their neighboring astrocytes (Figure 2) (101, 102) and many targets in this cycle are important for extracellular glutamate production. Glutamate transporter GLT-1 is a member of excitatory amino acid transporters (EAATs), and it is capable of removing glutamate from the synaptic cleft. β-lactam antibiotics (Table 1) can stimulate the activity of GLT-1 in mice glial cells after OGD (103), Glutamine synthase (GS) is the speed limit of the glutamate-glutamine cycle (104) and can be degraded by reactive oxygen species (ROS) after stroke, resulting in the accumulation of glutamate (105). Targeting GS may be an ideal strategy for treating IS.

The glutamate/cystine antiporter system x(c)- transports cysteine into cells in exchange for neurotransmitter glutamate at a ratio of 1:1 (106, 107). Cystine ingested into the cell produces cysteine, which is used as a raw material for the synthesis of glutathione (GSH) and participates in the scavenging of intracellular free radicals (106, 107). System x(c)- relies on a gradient of glutamate, and when...
Inflammation in the ischemic brain. (1) Antigen (Ag) released by damaged neurons is ingested and processed by dendritic cells (DCs), then stimulates T cell activation (take CD4+ T cells as an example, CD4 T cells are activated by MHC II–peptide–TCR and CD28-B7). Activated T cells secrete pro-inflammatory factors and chemokines to participate in the inflammatory response. (2) Neurons are exposed to new epitopes after necrosis, which can be recognized by natural immunoglobulin IgM in brain tissue, thereby activating the classical pathway of the complement system and forming a membrane attack complex (MAC) eventually. Then cellular contents are released extracellularly by MAC, which exacerbates the inflammatory response. (3) Injured neurons release DAMPs to activate microglia and participate in the inflammatory response. (4) Chemokines secreted by microglia and other inflammatory cells promote peripheral phagocytes, neutrophils, CD4+ T cells, CD8+ T cells, and γδ T cells to migrate toward CNS.

extracellular glutamate concentration increases, the transfer of cystine into cells decreases, resulting in oxidative free radical damage \((107-109)\). Acetylcysteine (Table 1) contributes to the scavenging of this oxidative free radical by stimulating glutamate/cystine antiporter system x(c) and promoting the generation of GSH \((110, 111)\). Acetylcysteine protects against injury in a rat model of focal cerebral ischemia and ischemia/reperfusion, respectively \((112, 113)\). In addition, two clinical trials registered with acetylcysteine treating IS, are still ongoing \((114)\).

**Glutamate and glutamate receptors**

Glutamate exerts excitotoxic effects through glutamate receptors (NMDARs). NMDARs have a dual role in neurons, which may depend on the subtype of NMDARs \((115)\). NMDARs consist of two NR1 subunits and one other subunit (NR2A/B/C/D or NR3A/B). NMDARs containing NR2A subunits mainly promote neuronal survival, while NMDARs containing NR2B subunits mainly mediate neuronal excitotoxicity and promote apoptosis \((116)\). Another argument
FIGURE 2
Metabolism of glutamate in the brain. (1) Glutamate (Glu)–glutamine (Gln) cycle: when nerve impulses are transmitted to the postsynaptic membrane, the neurotransmitter glutamate (Glu) stored in synaptic vesicles is released into the synaptic cleft and binds with glutamate receptors (NMDARs). Glutamate transporters (GLT-1 shown in the figure) on astrocyte transport Glu together with Na$^+$ into astrocytes. Na$^+$ in astrocytes is transferred to extracellular by an ATP-dependent Na$^+$ pump. Glu in astrocytes is converted to Gln by GS, then Gln is taken up by synapses and converted to Glu in synapses. Glu is stored in synaptic vesicles again. (2) $\gamma$-aminobutyric acid (GABA) is taken up by GABA transporters (GATs) in astrocytes. Then GABA can be converted to Glu through the tricarboxylic acid cycle (TAC). (3) System x(c)- transports cystine into cells in exchange for the Glu. Cystine synthesizes glutathione (GSH) in astrocytes.

for the dual role of NMDARs has to do with their location. Studies have shown that there are two distinct subtypes of NMDARs: extrasynaptic NMDARs and intrasynaptic NMDARs (117). Stimulation of intrasynaptic NMDARs can activate CREBs in a variety of ways (115, 118). Activated CREB can enhance mitochondrial tolerance to cellular stress (119) and inhibit the pro-death transcription factor by promoting the expression of brain-derived neurotrophic factor (BDNF) (115), exerting an anti-apoptotic effect. Conversely, stimulating extrasynaptic NMDARs can dephosphorylate CREBs by inhibiting Ros/ERK1/2 pathway, which activates pro-apoptotic genes in the bcl-2 family, and thus induces apoptosis (120, 121).

In short, extrasynaptic NMDARs and intrasynaptic NMDARs play opposite roles. Various subtypes have different affinities with glutamate, and their positional relationships can both serve as explanations for glutamate excitotoxicity. In other words, physiological glutamate levels fail to activate extrasynaptic NMDARs (low affinity or low level of glutamate), but they can activate intrasynaptic NMDARs (high affinity or high level of glutamate) exerting a pro-survival signal. Extrasynaptic NMDARs can only be activated when the glutamate concentration rises above a certain threshold to exert a pro-death signal. Blocking NMDARs has been a potential target for inhibiting glutamate excitatory toxicity, particularly blocking extrasynaptic NMDARs and NMDARs containing NR2B subunit. Compared with intrasynaptic NMDARs, memantine (Table 1) can inhibit extrasynaptic receptors more effectively (122), and it has been clinically used in the treatment of AD in the US. However, memantine only shows efficacy in patients with severe and moderate AD (123). For mild AD, a meta-analysis found no difference between memantine and placebo in cognition, activities of daily living, or behavior (124). In a preclinical study of IS, memantine blunted the noxious effects of delayed thrombolysis on lesion volumes and neurological deficits in MCAO mice (125) and exerted synergistic neuroprotective effects with clenbuterol in MCAO rats (126). However, in vivo experiment of Trotman et al., higher doses of memantine (20 mg/kg/day) significantly increased injury. Similar results were also found in their in vitro experiments. Therefore, a proper dosage of memantine is significant in future clinical trials (127). Ifenprodil is capable of binding to NMDARs containing NR2B subunit with a high affinity (128, 129). Although clinical trials of ifenprodil are currently only conducted in idiopathic pulmonary fibrosis...
Glutamate toxicity

When glutamate binds with the receptor, an action potential is formed. After excitotoxicity occurs, glutamate continues to excite receptors and keeps Na\(^+\) channels open. Then intracellular osmotic pressure increases due to this persistently opened Na\(^+\) channel, resulting in acute neuronal death. When neurons are in a resting state, Ca\(^{2+}\) channels are blocked by Mg\(^{2+}\). When glutamate binds to NMDARs, Mg\(^{2+}\) is removed and Ca\(^{2+}\) channels are opened by the depolarized postsynaptic membrane. Intracellular Ca\(^{2+}\) is elevated by these opened Ca\(^{2+}\) channels, which contributes to the activation of a ternary structure (PSD95-NR2B-NOS) (130). The activated ternary structure then releases nitric oxide synthase (NOS) leading to the production of reactive nitrogen species (RNS). Cell in vitro experiments and animal experiments have demonstrated that disrupting this ternary structure can reduce glutamate-mediated excitotoxicity and improve neuronal tolerance to glutamate (131, 132). Furthermore, mitochondria are disturbed by calcium overload to release a large number of oxidative free radicals, which can promote neuronal apoptosis and aggravate calcium overload again through activated transient receptor potential melastatin-subfamily member 7 (TRPM7) and transient receptor potential melastatin-subfamily member 2 (TRPM2) (115). Recently, Zong et al. discovered that TRPM2 directly interacts with GluN2a/b of extrasynaptic NMDARs through the unique EE3 motif in its N-tail and the KKR motifs in the C-tail of GluN2a/b. This coupling mechanism plays an important role in the excitotoxicity of ischemic brain injury in mice (133). Therefore, in downstream of glutamate-mediated excitotoxicity, some ion channels that mediate calcium overload, some complexes that mediate oxidative stress, and the coupling of TRPM2 and extrasynaptic NMDARs are expected to be targets for the treatment of IS.

NMDAR-mediated excitotoxicity has been extensively studied in stroke; however, NMDAR antagonists face challenges now in the treatment of ischemic stroke in human patients. This may be attributed to some key targets and structures that have not been fully studied. Additionally, we hope to provide some inspiration for future drug development through the above summary of excitotoxicity.

Oxidative stress

Generation of oxidative free radicals

Physiologically, there is stable oxidation and antioxidant system in the body. In the process of reperfusion of the ischemic brain, glucose, and oxygen enter the brain again. Oxidative glycolysis of glucose produces a large amount of reduced NADH-H+ and FADH2 along with superoxide anion generated in the process of electron transfer, resulting in excessive ROS and RNS, as well as damage to the ischemic brain (134). It is currently believed that there are two oxidative respiratory chains (Figure 3 in mitochondria, one is NADH oxidation respiratory chain (NADH-complex I-CoQ-complex III-Cytc-complex IV-O2) and the other is the FADH2 oxidation respiratory chain, also called succinate oxidation respiratory chain (succinate -complex II-CoQ-complex III-Cytc-complex IV-O2). Studies have shown that FADH2 accumulation can be attributed to the reversal of succinate dehydrogenase (SDH) after stroke. In the early stage of perfusion, the accumulated FADH2 activates SDH and drives mitochondria to produce a large amount of ROS via reverse electron transfer (RET) (105). Excessive ROS and RNS promote lipid peroxidation, mitochondrial and DNA damage, protein nitrification and oxidation, and depletion of antioxidants (135). In addition to this, ROS and RNS lead to the overexpression of inflammatory genes, inflammatory and chemokine production, BBB disruption, leukocyte recruitment, and cerebral edema (136).

Removing ROS and RNS is a major strategy to treat IS and protect neurons. Edaravone (Table 1) is clinically used for the treatment of IS, which can scavenge oxidative free radicals and achieve the purpose of protecting ischemic neurons. Uric acid (Table 1) contributes to the scavenging of ROS, including nitrite, which can reduce cerebral infarct volume after stroke and improve neurological outcomes after transient or permanent cerebral ischemia in rodents (137–139).

Antioxidant enzyme system

There are various antioxidant enzymes and small molecule antioxidants (such as vitamin C/E, ubiquinone, β-carotene) in our body, which together constitute the antioxidant system. Superoxide dismutase (SOD) is widely distributed in the human body, responsible for catalyzing O2- and converting it to oxygen and hydrogen peroxide (H2O2), and it is an important part of the cellular antioxidant system (140). Catalase (CAT) has a strong catalytic ability to H2O2. However, CAT will inevitably generate OH- in the process of scavenging H2O2. Tri-manganese (III) salen-based cryptands, an analog of CAT, can minimize the production of OH- while removing H2O2 (141). Glutathione peroxidase (GPx) is the main enzyme in the body to scavenge ROS, removing H2O2 and other peroxides. Furthermore, GPx is the key to inhibiting neuronal ferroptosis (142). A selenocysteine-containing peptide, Tat SelPep, can increase GPx expression by binding to nuclear DNA and effectively improve stroke outcomes (143). Thioredoxin (Trx), also one of the body’s antioxidant enzymes, has been found to improve outcomes after stroke. Melatonin (MT), a neurohormone in the human body, can achieve anti-oxidation.
FIGURE 3
Generation of ROS and RNS. (1) NADH oxidation respiratory chain. NADH-H⁺ transfers electrons to complex I and is oxidized to NAD⁺. (2) Succinate oxidation respiratory chain. Succinate transfers electrons to complex II and is oxidized to fumarate. (3) The electrons in complex I/II are captured by ubiquinone (Q) to form QH₂. The electrons in QH₂ are transferred to complex III, then QH₂ that loses electrons becomes Q⁻ to participate in the electron transfer again. Electrons in complex III are transferred to complex IV via cytochrome c (Cytc). (4) O₂ obtains electrons from complex IV to generate ROS. (5) After ischemic/reperfusion injury, O₂⁻ accepts a single electron to generate superoxide anion (O₂⁻), O₂⁻ accepts a single electron to generate hydrogen peroxide (H₂O₂), and H₂O₂ accepts a single electron to be reduced to hydroxyl radical (•OH⁻). O₂⁻ can rapidly oxidize NO to produce nitrite (ONOO⁻).

by regulating Trx (144). However, the performance of most antioxidant drugs in animal experiments is not satisfactory, which may be related to the inability of antioxidants to target mitochondria, the birthplace of ROS and RNS (145). Therefore, this kind of antioxidant drug targeting mitochondria needs to be further considered in the future.

Scavenging oxidative free radicals and increasing the reserves of antioxidant enzymes have been one of the strategies for the treatment of IS. However, only edaravone is currently approved for the clinical treatment of IS. Additional mechanisms related to oxidative stress help resolve this dilemma. For example, ferroptosis is a new form of cell death caused by an increase in iron ion-dependent lipid peroxides (146). Although the specific mechanism of ferroptosis in IS has not been elucidated, inhibition of lipid peroxidation and regulation of iron metabolism is promising for treating IS.

Complement system

The complement system is composed of complement intrinsic components, complement regulatory proteins, and complement receptors, and its activation pathways are mainly three-fold: classical pathway (CP), bypass pathway (AP), and lectin pathway (LP). The complement system is part of innate immunity and is involved in the subsequent phases of humoral immunity. Since B lymphocytes are barely detectable in brain tissue within a week after stroke (11, 12), CP that rely on immune complexes (ICs) for activation may be limited in the acute phase of IS. However, there is a natural immunoglobulin M (IgM) in the brain, which can recognize a new epitope on damaged neurons and still activate CP to exert pathological damage after stroke (147, 148). The endpoint of the complement cascade is the formation of a membrane attack complex (MAC), resulting in cell rupture and death. In recent years, neuroprotective agents targeting C3 and C5 or complement fragments have entered our field of vision. As a fusion construct, B4cry inhibits IgM binding to the new epitope, C3 cleavage, and the activation of microglia, which can reduce complement deposition in ischemic lesions and improve neurological function after stroke (149, 150). C5a-C5AaR axis capable of promoting neutrophil migration (151) is widely involved in COVID-19-related coagulopathy, viral hepatitis, cancer, and myocarditis (152–155), and this axis may serve as a target for the treatment of IS. Mannan-binding lectin
(MBL) plays an important role in the activation of LP. Using polymeran2 (the synthesized mannosylated molecule selected for its binding to MBL) or anti-MBL antibody, can inhibit the activation of mannan-binding lectin-associated serine protease-2 (MASP-2) to block the subsequent complement cascade, and exert a neuroprotective effect on TMCAO or permanent middle cerebral artery occlusion (PMCAO) rats (156). Complement regulatory proteins are one of the long-term targets that complement drug developers are focusing on. C1 inhibitor (C1-INH) capable of inhibiting C1s or MASP-2 can inhibit complement cascade from the upstream of C3. Animal experiments have shown that compared with TMCAO mice, C1-INH-deficient TMCAO mice showed larger ischemic foci and worse neurobehavorial performance (157). There are many components in the complement system, but there is little research on the complement system in IS. Since the development of neuroprotective agents against IS has mostly failed, the complement system seems to be a good path.

**Thrombus and pericytes**

Thrombus is the fundamental factor causing cerebral vascular obstruction and cerebral ischemia and hypoxia. For thrombus formation, the importance of the involvement of ultra-large (UL) von Willebrand factor (ULVWF) and collagen–von Willebrand factor–glycoprotein Ib axis has been highlighted in many studies (175, 176). ULVWF multimers are regulated by a metalloproteinase Adamts13. Some studies have shown that low activity of Adamts13 is associated with an increased risk of IS and TIA (177). The interaction of platelets and neutrophils is critical in thrombosis. Platelets promote the formation of neutrophil extracellular traps (NETs) (178), which can be cleared by patients’ deoxyribonuclease-1 (DNase-1) in stroke treatment (179). Antithrombotic therapy has always been the main method for IS treatment. However, some limitations of thrombolysis, such as the therapeutic window and low efficacy of perfusion, are difficult to solve. In recent years, the combination of thrombolysis and neuroprotection seems to be an excellent strategy for treating IS. For example, uric acid helps to scavenge ROS. In a pre-clinical study by Romanos et al. (138), uric acid and recombinant tissue plasminogen activator (rtPA) showed a synergistic effect in the model of thromboembolic cerebral ischemia in rats. A clinical trial (NCT00860366) on the combination of rtPA and uric acid in the treatment of IS has been completed. Unfortunately, we have not found relevant research results. In addition, in a double-blind, placebo-controlled, phase 2b trial, the combination of rtPA and uric acid may prevent early ischemic deterioration after acute stroke in patients with thrombolysis (180). This strategy of thrombolysis combined with neuroprotection can be a good point in the treatment of IS in the future.

In addition to being interrupted by a thrombus, cerebral blood flow is also regulated by capillary pericytes. Ischemic pericytes constrict and compress capillaries after stroke, reducing cerebral blood flow. More importantly, pericyte death attributed to sustained ischemia and hypoxia can lead to irreversible constriction of capillaries and permanent interruption of blood flow (10). The time of pericyte resistance to ischemia and hypoxia may affect the time window of intravenous thrombolysis or mechanical thrombectomy. Preventing the shrinkage and death of pericytes and improving the tolerance of pericytes to ischemia and hypoxia may be of great significance to prolong this time window.

**How to deliver drugs to the CNS?**

We have described many promising drugs earlier, but all of these drugs have to face a common problem: It is difficult to deliver them to the CNS. The BBB is the main barrier preventing drugs from entering the CNS. It has been reported that <2% of small molecule drugs with CNS affects approved by the Food and Drug Administration (FDA) can pass through the intact BBB (181, 182). The existence of the BBB is necessary for maintaining the homeostasis of the cerebral microenvironment and ensuring the normal functions of the CNS. Nevertheless, the BBB also impedes the intracerebral delivery of therapeutic agents (183). Although studies have shown that the occurrence of IS can destroy and increase the permeability of the BBB, the BBB remains the main obstacle for drugs to overcome (184).

At present, scientists and industry have developed a variety of technologies to deliver drugs to the CNS. For instance, the emergence of nanoparticles provides a new strategy for drugs to enter the CNS. Nanoparticles have been proven to deliver a great variety of drugs across the BBB, and this mechanism of crossing BBB now appears to be receptor-mediated endocytosis of the brain capillary endothelial cells, followed by transcytosis (185, 186). By combining with different drugs, nanoparticles perform three major approaches for ischemic stroke therapy: recanalization, neuroprotection, and combination therapy (184). More importantly, nanoparticles are capable of increasing drug bioavailability, enhancing therapeutic efficacy, and reducing unwanted toxicity (184). In recent years, exosomes have been mentioned as a strategy for the treatment of IS. Exosomes are endosome-derived membrane-bound vesicles with diameters of 30–150 nm, and they are released by most cell types (187–189). Among the cargoes carried by exosomes, miRNAs are valued by researchers because they may be the core of the therapeutic effects of exosomes (189). Therefore, how to select miRNA contained in exosomes may become a strategy for treating IS. Additionally, in the treatment of IS, engineered exosomes that contain selected miRNA have been proven to be more effective compared with naïve exosomes (189). Although the ability of exosomes to directly cross the BBB is uncertain,
several studies have shown that some exosomes could cross the BBB in healthy and inflamed brains (189–191).

In addition to the aforementioned nanoparticles and exosomes, neurotropic virus mediation emerged as a strategy for the treatment of IS. Neurotropic viruses, with an affinity for nerve, can cross the BBB through multiple pathways, such as direct transcytosis, virus-infected immune cells, and retrograde transport from peripheral nerves to the CNS (192). Additionally, this property makes neurotropic viruses a CNS-targeting strategy. Carrier-mediated transcytosis (CMT) and receptor-mediated transcytosis (RMT) are long-term concerns of researchers. However, when delivering drugs to the CNS through RMT or CMT, the target receptor or carrier protein should be highly expressed in the endothelial cells of the cerebral vasculature, especially those in the microvascular (192). Therefore, this carrier protein that can match the receptors abundantly expressed in cerebral microvascular needs to be elaborately designed.

Crossing the BBB seems to be an inescapable obstacle for delivering drugs to the CNS, even though there are now many ways to bypass the BBB, such as highly invasive intracerebral injection, intranasal, retro-orbital, or intrathecal administration. However, these methods of bypassing the BBB are difficult to achieve clinically, which may be attributed to their operational complexity, high invasiveness, and low bioavailability. With the development of technology, RMT-based strategies, neurotropic virus-based approaches, nanoparticles, exosomes, etc. have shown great potential to deliver drugs to the CNS.

**Difficulties in clinical transformation**

At present, most neuroprotective drugs are facing difficulties in clinical transformation. These difficulties are caused by many aspects. Animal models of IS, where middle cerebral arteries are often blocked by nylon fibers, have been questioned because they do not reflect the occurrence of vascular embolism under natural conditions (193). Moreover, there are many differences between animal models and humans, such as age, species, and underlying diseases. The side effects of drugs are also important factors. For example, in the development of complement drugs, complement inhibitors inevitably inhibit the activity of serum complement while inhibiting complement activation, which increases the risk of infection (194). Therefore, it is necessary to develop more targeted drugs that have higher precision. More importantly, incomplete mechanism research will also lead to failure in drug development. For example, although the use of anti-IL-17A drugs has seen efficacy in the treatment of psoriasis, it is clinically invalid in the treatment of amyotrophic lateral sclerosis (ALS), rheumatoid disease, and experimental autoimmune encephalomyelitis (EAE) (195). This contradictory role in different diseases may be attributed to the incompleteness of the mechanism such as the possible duality of IL-17, the different pathogenicities of Th17, and the negative feedback of IL-17 (195, 196). In addition, the ability of the drug to penetrate the BBB, oral bioavailability, half-life, and statistical bias are also factors that determine whether the drug can be successfully translated into the clinic.

**Conclusion**

Since most clinical patients with ischemic stroke fail in conventional treatments such as intravenous thrombolysis and mechanical thrombectomy due to missed time windows, it is of great significance to find other strategies to protect CNS. In the complex pathological mechanisms of IS, we can obtain many methods and targets for the treatment of IS. It is very promising to develop new drugs for IS from these mechanisms and targets. Additionally, combining these new drugs with brain delivery technologies and more precise targeted therapy may go further in the clinical treatment of IS.

**Author contributions**

YJ and YT conceived the topic and determined the outline of this review. YJ, YT, and ZL contributed to the manuscript writing. YD and SS collected the literature and finished the figures and tables. YT, ZL, and YL critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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