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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 antiplatelet therapy is a combination of clopidogrel and aspirin. This dual antiplatelet 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, inducing 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 (14). Microglia are involved in a variety of CNS diseases, including amyotrophic lateral sclerosis (ALS), IS, Alzheimer's disease (AD), meningeal inflammation, 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 $\text{INF-}\gamma$ 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-1 β , 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- κ B 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.

Macrophages

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^{high}CCR2⁺CX3CR1^{low}) and anti-inflammatory subpopulations (Ly6C^{low}CCR2⁻CX3CR1^{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). Monocytes are now thought to be derived from hematopoietic stem cells (HSCs), whereas microglia are the descendants of yolk sac erythromyeloid progenitors (EMPs) (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 $\alpha 4\beta 1$	(158)	Mice	NCT02730455	2	Completed
Minocycline	microglia	(159)	Mice	NCT00930020	4	Terminated
Exendin-4	Microglia	(37)	Mice	NCT03287076	2	Active not recruiting
Folic acid/B12/B6	Microglia	(39)	Rat	NCT00354081	3	Completed
i6-FP	MAIT	(89)	Mice	—	—	—
Tak242	TLR4	(160)	Rat/ <i>in vitro</i>	—	—	—
Isoquercetin	TLR4	(161)	Animals/ <i>in vitro</i>	—	—	—
Dexmedetomidine	HMGB1/TLR4/NF-kB	(162)	Rat	NCT04916197	4	Recruiting
Anti-excitotoxicity						
NA-1	GluN2B-PSD95-NOS	(163)	Animals/neuronal Cultures	—	—	—
ZL006	GluN2B-PSD95-NOS	(164)	Mice/rat	—	—	—
IC87201	GluN2B-PSD95-NOS	(165)	<i>In vitro</i>	—	—	—
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/ Not yet recruiting
Edaravone	ROS, RNS	(169)	Mice	NCT02430350	3	Completed
Uric acid	ROS	(170)	Mice	NCT00860366	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/FOXO3a/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 ($\text{TCR}\alpha\beta^+\text{CD4}^+\text{CD8}^-$ or $\text{TCR}\alpha\beta^+\text{CD4}^-\text{CD8}^+$) mainly involved in adaptive immune, others mainly differentiate into $\text{TCR}\gamma\delta^+\text{CD4}^-\text{CD8}^-$ 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 $\gamma\delta\text{T}$ ($\text{CD4}^-\text{CD8}^-$) 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). $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$ 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/FasL 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. $\gamma\delta\text{T}$ 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 $\gamma\delta\text{T}$ 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, $\gamma\delta\text{T}$ 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/ $\gamma\delta\text{T}$ cells ratio which is highly correlated with stroke outcomes (86). $\gamma\delta\text{T}$ 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 (isobutyril 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 served as the brain-homing mechanism for $CD8^+CD122^+CD49d^{lo}$ T 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 Mafk (97). MSR1 and Mafk 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.

Excitatory toxicity

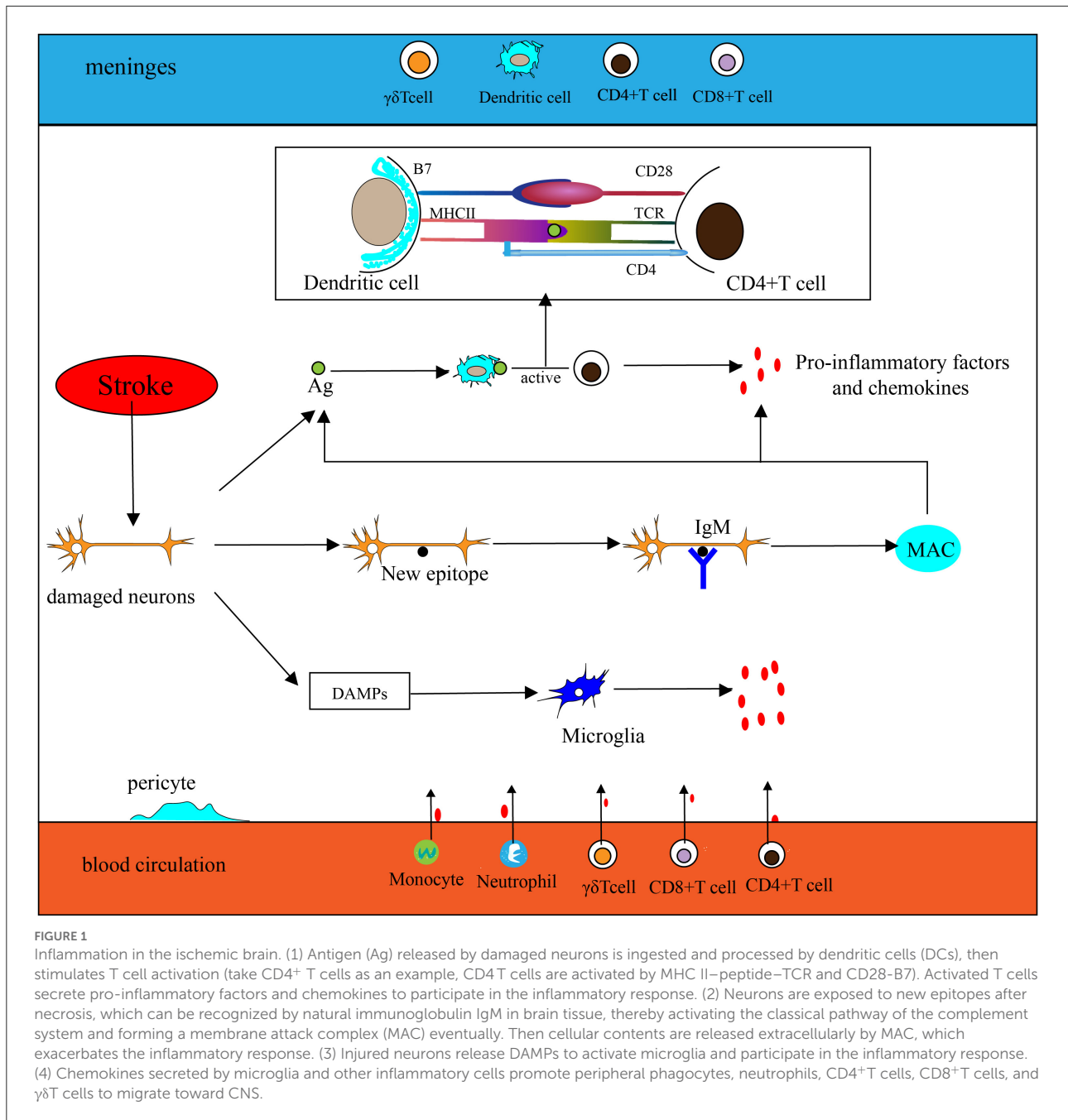
Excitatory toxicity is mainly caused by the increased glutamate (Glu) in the ischemic brain, which leads to neuronal necrosis and apoptosis by a series of biochemical cascades. After an acute stroke, mitochondrial ATP production decreases due to cerebral ischemia and hypoxia. Then intracellular

and extracellular ion disorders (intracellular: Na^+ , Ca^{2+} , Cl^- increase; extracellular: K^+ increase) caused by the dysfunctional ATP-dependent ion pump will eventually lead to an increase in glutamate in the extracellular or synaptic cleft. For example, intracellular transport of glutamate dependent on a normal Na^+ gradient (extracellular Na^+ are more than intracellular) is regulated by an ATP-dependent Na^+ pump. The normal Na^+ gradient is reversed (intracellular Na^+ are more than extracellular) when ATP synthesis is reduced and the ATP-dependent Na^+ pump is deactivated, which ultimately increases glutamate in the extracellular or synaptic cleft. As glutamate-mediated excitotoxicity severely affects prognosis in patients with stroke, studying the production and metabolism of glutamate and the downstream pathways mediated by glutamate receptors have great potential for the development of neuroprotective drugs against 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.

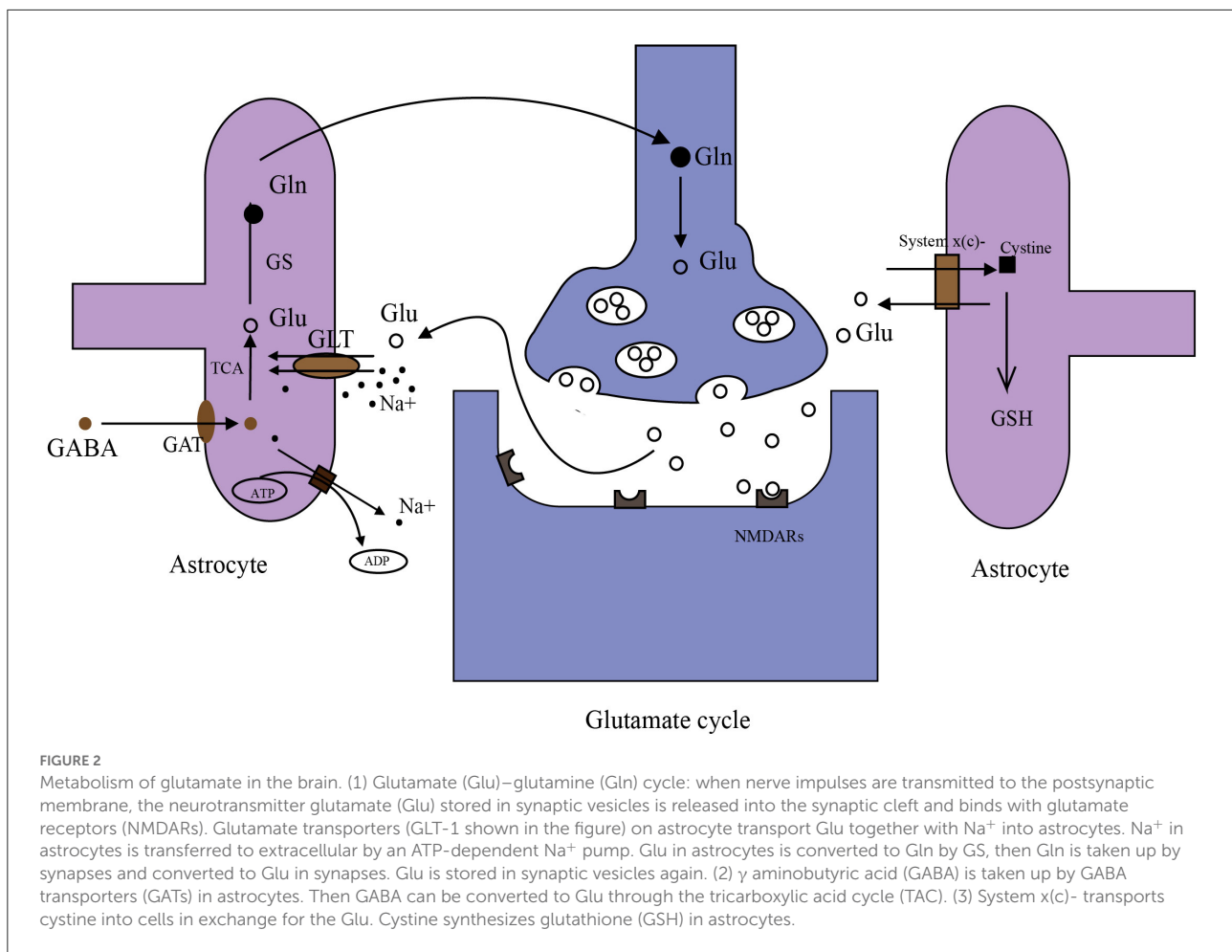
The glutamate/cystine antiporter system $x(c)^-$ transports cystine into cells in exchange for neurotransmitter glutamate at a ratio of 1:1 (106, 107). Cystine ingested into the cell produces cysteine, which is used as a raw material for the synthesis of glutathione (GSH) and participates in the scavenging of intracellular free radicals (106, 107). System $x(c)^-$ relies on a gradient of glutamate, and when



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



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

(IPF)/corona virus disease 2019 (COVID-19)/post-traumatic stress disorder (PTSD), using ifenprodil in treatment against IS remains promising.

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, Ca²⁺ channels are blocked by Mg²⁺. When glutamate binds to NMDARs, Mg²⁺ is removed and Ca²⁺ channels are opened by the depolarized postsynaptic membrane. Intracellular Ca²⁺ is elevated by these opened Ca²⁺ 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

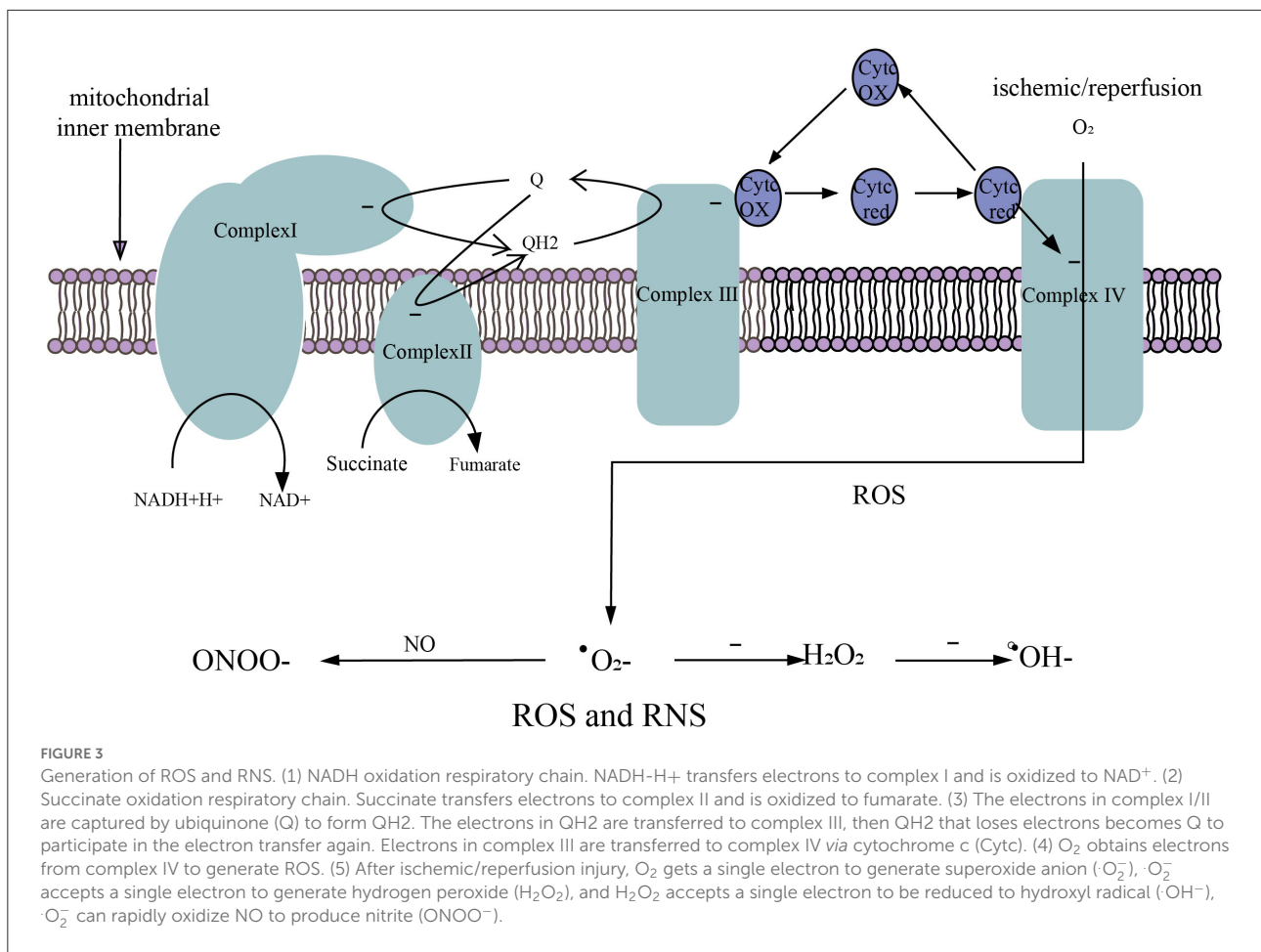
Physiologically, there is stable oxidation and antioxidant system in the body. In the process of reperfusion of the ischemic brain, glucose, and oxygen enter the brain again.

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 nitration 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



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 polyman2 (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 neurobehavioral 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 reperfusion, 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 effects 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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