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REVIEW

Ischemic stroke and intervention strategies based on the timeline of stroke progression: Review and prospects

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Abstract Ischemic stroke (IS), a leading cause of morbidity and mortality worldwide, primarily results from blood clot formation in cerebral vessels, leading to vessel occlusion, reduced cerebral blood flow, and subsequent tissue ischemia. While thrombolytic therapies and mechanical thrombectomy remain cornerstone treatments for restoring blood flow, their clinical efficacy is significantly limited by the narrow therapeutic window, which underscores the critical need for novel, safe, and effective therapeutic strategies. In this review, we present an intensive analysis of four pathophysiological stages of IS progression and their intervention targets, and evaluate both established and emerging therapeutic strategies with the molecular mechanisms underpinning these methods, aiming to enhance the understanding of IS intervention. Additionally, we discuss current challenges in IS therapy, emphasizing the importance of timely, stage-specific approaches to optimize therapeutic outcomes. Finally, we highlight some promising research directions and innovations to advance IS field.

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1. Introduction

Ischemic stroke (IS), also called cerebral infarction, is a devastating cerebrovascular disease that is characterized by the formation of a thrombus in cerebral blood vessels. It can lead to blood vessel occlusion and reduced blood flow to cause ischemia and hypoxia of the brain tissue; this results in neurological deficits and, in severe cases, death[1,2]. Trial of ORG 10172 in acute stroke treatment (TOAST) classifies IS into five different subtypes, as follows: (1) large artery atherosclerosis (LAAS), (2) cardioembolic stroke (CEI), (3) lacunar infarction (LAC), (4) stroke of other determined etiology (ODE), and (5) stroke of undetermined etiology (UDE)[3]. Among these, IS is chiefly caused by atherosclerosis and cardiogenic embolism of large arteries, while lacunar stroke is another important subtype that accounts for 15%−25% of all ISs. As per statistics reports, more than 13.7 million people worldwide suffer from stroke every year, which is currently the second leading cause of death globally. The incidence of stroke is expected to increase further by 3.4 million by the year 2030[4]. Of note, IS accounts for more than 87% of the total incidence of stroke[5]. Given the high morbidity, mortality, the rate of disability, and recurrence of stroke and considering population aging, patients with stroke comorbidities such as hypertension and hyperlipidemia typically face worse clinical outcomes, resulting in a heavy socioeconomic burden[6-9].

As shown in Fig. 1, IS can be divided into four stages, termed the hyperacute, acute, recovery, and sequela stages[10]. The hyperacute stage typically occurs within 6 h of stroke onset is characterized by a sudden reduction or interruption of cerebral blood flow, leading to a deficiency of glucose and oxygen. This triggers mitochondrial dysfunction within minutes, impairing neuronal energy supply[11]. Specifically, mitochondrial dysfunction manifests as the collapse of mitochondrial membrane potential, impaired oxidative phosphorylation, and reduced ATP production. These changes disrupt cellular energy homeostasis, leading to increased glutamate release, intracellular calcium overload, and the generation of reactive oxygen species (ROS), which collectively exacerbate neuronal damage. Additionally, mitochondrial dysfunction contributes to the opening of the mitochondrial permeability transition pore (mPTP), further promoting cell death pathways such as apoptosis and necrosis[11]. The ischemic tissue consists of the infarct core and the surrounding ischemic penumbra[5]. Although the damage to the infarct core is irreversible, the area of reduced blood flow around it, called the penumbra, can be salvaged by reperfusion and is critical for treatment[12-14]. The acute stage occurs about a week after the incidence of stroke and is characterized by the disappearance of the penumbra, mainly due to inflammation triggered by the infiltration of immune cells into the brain tissue[15]. A clear demarcation does not exist between the recovery and sequelae stages of IS in terms of duration, albeit each has different characteristics. The recovery stage usually refers to the period from one week to six months after the onset of stroke. There are various treatment options during this period, which is important for the recovery of normal functioning of the patients. The sequela stage usually refers to the period after six months of stroke onset, during which patients may still face some complications; the use of certain drugs and physical exercise is beneficial to improve the patient's functioning during this time. Defining the concept of time window based on the abovementioned facts is very valuable and allows early intervention for the treatment of IS.

The primary treatment for the hyperacute stage of IS is vascular recanalization, including mechanical thrombectomy and intravenous thrombolysis[16-20]. Mechanical thrombectomy is limited to large artery occlusions[21,22], while intravenous recombinant tissue plasminogen activator (rt-PA) remains the only US Food and Drug Administration (FDA)-approved drug for acute ischemic stroke (AIS) that is capable of rapidly restoring cerebral blood flow[23]. However, the narrow therapeutic window for its application renders only 5% of the patients eligible for rt-PA treatment, and its use is associated with the risk of reperfusion injury, which can result in adverse effects[24,25]. The primary therapeutic goal in the acute stage is to reduce neuroinflammation to minimize further brain damage. During the recovery stage, emphasis is placed on promoting neuroregeneration and revascularization to improve neurological function. Stroke management in the sequelae stage focuses on alleviating functional impairments through nutritional support and exercise, as specific targeted medications are not available. This review provides a summary of the targeted interventions and therapeutic strategies for IS across different stages of disease progression and aims to propose a comprehensive, stage-specific therapeutic strategy for the treatment of IS.

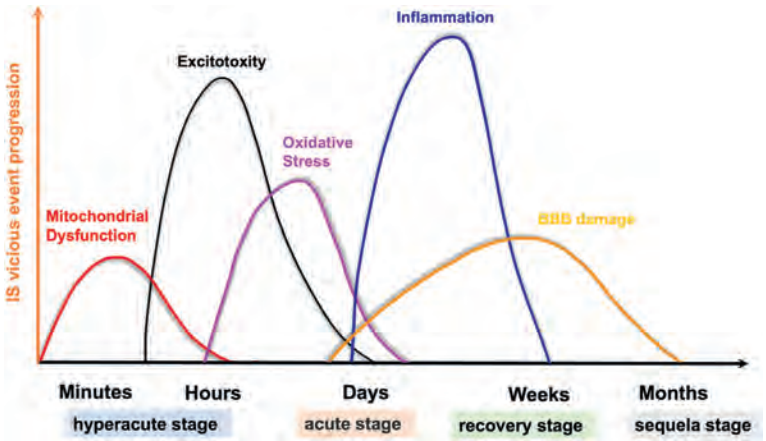

Figure 1 Summary of the pathological progression of IS across four stages.

2. Pathological characteristics and intervention targets in different stages of temporal progression

2.1. Hyperacute stage

As previously established, mitochondrial dysfunction manifests within minutes following IS onset, significantly exacerbating neuronal damage through impaired cellular energy metabolism. To address mitochondrial dysfunction, several therapeutic strategies have been explored. Mitochondrial protectants, such as coenzyme Q10 and idebenone, have shown promise in stabilizing mitochondrial membrane potential, enhancing ATP production, and reducing oxidative stress[26]. Additionally, inhibitors of the mPTP, such as cyclosporine A, have been investigated for their ability to prevent mitochondrial swelling and subsequent cell death[27]. Emerging therapies, including mitochondrial-targeted antioxidants (*e.g.*, MitoQ) and compounds that enhance mitochondrial biogenesis (*e.g.*, resveratrol), are also being actively studied for their neuroprotective potential in the hyperacute phase[28,29]. While vascular recanalization remains the cornerstone therapeutic intervention during the hyperacute phase of IS, while vascular recanalization remains the cornerstone therapeutic intervention during the hyperacute phase of IS, only a minority of patients benefit from it. This therapeutic limitation has driven substantial research investment in novel neuroprotective strategies, including remote ischemic conditioning (RIC), normobaric hyperoxia (NBO) therapy, and targeted temperature management through therapeutic hypothermia (TH). Here, we review the main therapies for hyperacute IS, including emerging therapies and vascular recanalization.

2.1.1. Remote ischemic conditioning (RIC)

RIC refers to "preconditioning" the ischemic area for IS by repeatedly applying intermittent ischemic stimuli to enhance the brain's tolerance for ischemia/reperfusion injury, protect ischemic tissue, reduce neural damage, promote vascular remodeling, and improve cerebral microcirculation; RIC includes remote ischemic preconditioning (RIPreC), remote ischemic preconditioning (RIPerC), and remote ischemic postconditioning (RIPostC); these correspond to the pre-, mid-, and post-ischemia phases, respectively (Fig. 2). Preclinical studies suggest that RIC may exert protective effects by mitigating ischemic reperfusion injury, promoting the opening of collateral circulation, and improving mitochondrial function[30]. These findings further support the potential application of RIC in the subacute and chronic phases, such as in reducing vasospasm (*e.g.*, following subarachnoid hemorrhage) and delaying cognitive decline in cerebral small vessel disease[30]. Multiple clinical trials have revealed that this approach results in effective protection against brain injury[31-34]. Currently, 14 clinical studies are ongoing in patients with AIS, 9 have been completed and 5 are ongoing. For example, a single-center, randomized, placebo-controlled trial (ISRCTN 86672015) demonstrated the good tolerance and safety of RIC following AIS[35,36]. A Phase IIb Randomized Controlled Trial in Hyperacute Stroke further confirms the feasibility of RIC in AIS (NCT02779712). We can find that RIC can play a protective role in AIS in different time windows. However, there were still two negative results, probably due to the short duration of RIC treatment (NCT02189928)[37,38]. Subsequent analysis of the RICAMIS trial reveals that RIC exhibits significant efficacy in AIS patients without a history of stroke or transient ischemic attack (TIA). Specifically, the proportion of patients achieving favorable functional outcomes at 90 days, defined as a modified Rankin Scale (mRS) score of 0−1, was significantly higher in the RIC group compared to the control group[39]. However, no significant differences were observed in patients with a prior history of stroke or TIA. Furthermore, long-term repeated applications of RIC in patients with intracranial atherosclerosis have been shown to reduce stroke recurrence and improve cerebral perfusion[40]. However, the standardization of RIC's timing, duration, and dosage remains elusive, hindered by the absence of validating biomarkers.

2.1.2. Normobaric hyperoxia (NBO) therapy

The non-invasive treatment method NBO is usually initiated in an ambulance or emergency room (and continued uninterrupted during endovascular treatment) at the onset of IS in combination with other vascular reperfusion therapies. In the setting of experimental stroke and brain trauma, NBO significantly reduces infarction size and improves neurological function by increasing cerebral oxygen content, restoring mitochondrial function, and alleviating blood-brain barrier damage. Compared to Hyperbaric Oxygen Therapy (HBO), NBO exhibits higher safety and greater potential for translation into clinical practice[41]. A clinical trial (NCT03620370) demonstrated the safety and efficacy of NBO in

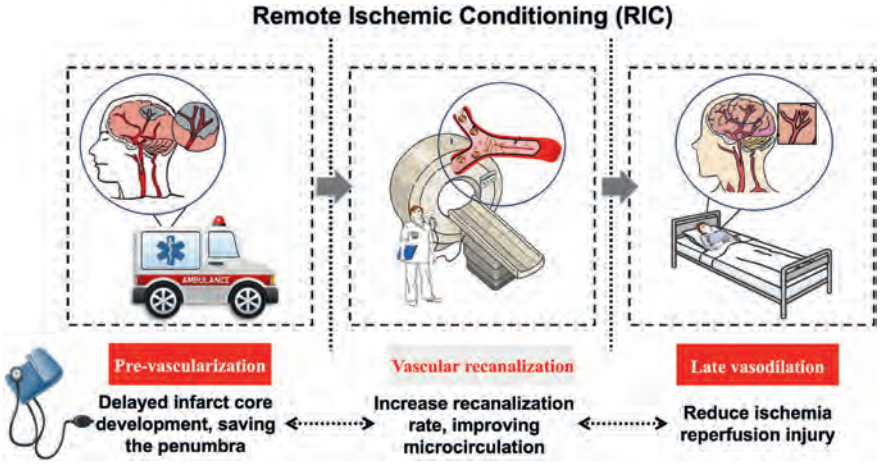

Figure 2 Beneficial effects of RIC before and after revascularization in patients with IS.

combination with endovascular therapy (EVT) in patients with IS, which effectively improved cerebral oxygenation, protected the penumbra, and widened the reperfusion therapeutic window for IS[42]. Further investigations on the optimal duration for NBO therapy in patients with IS revealed greater efficacy of NBO treatment given for 4 and 6 h during endovascular treatment (NCT05404373)[43].

2.1.3. Therapeutic hypothermia (TH)

A treatment with rapid, mild, and short-term hypothermia as an adjuvant therapy for moderate to severe stroke can affect multiple physiological and pathological processes of IS. Previous studies have found that intervention-targeted hypothermic brain protection can increase the local concentrations of neuroprotective drugs, thereby reducing reperfusion injury in patients with IS[44]. Cooling helmets are among the most frequently employed methods for inducing cooling but do not allow a rapid reduction in temperature within a short duration after the onset of IS[45]. Intra-arterial selective cooling infusion (IA-SCI) can result in rapid cooling of the brain within a few minutes, but the operation is very invasive[46]. Chen et al.[47] reported a selective intracranial brain cooling technology that can rapidly lower brain temperature while maintaining normal body temperature; this method circumvents the need for invasive surgery while maintaining cooling efficiency, avoids the systemic side effects of reduced therapeutic intervention, and may provide clinical benefits. This method of nasal cooling can be applied to multiple phases of IS development and can be employed in an ambulance during the hyperacute phase, potentially extending the window for recanalization treatment[47]. Therefore, nasal cooling may be used as a complementary therapy for vascular recanalization.

2.1.4. Vascular recanalization

Revascularization has proven to be beneficial for functional outcomes in clinical AIS patients[48]. However, issues such as re-occlusion, lack of reflux (microvascular obstruction), and hemorrhagic transformation (HT) inevitably occur after vascular recanalization, eventually leading to adverse outcomes[22]. HT refers to the coagulation dysfunction that occurs upon the restoration of blood flow by reperfusion and is a consequence of the destruction of blood−brain barrier (BBB) permeability. The risk factors for HT following rt-PA therapy in thromboembolic AIS have long been reported in the European Cooperative Acute Stroke Study. APLT-PA is a platelet biomimetic nanodrug that can improve the targeted delivery of tPA to the site of thrombosis and reduce the risk of HT[49]. However, whether HT increases the benefits of thrombolytic therapy for the treatment of AIS remains to be determined. The activation of the cyclic GMP−AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway has been demonstrated to impact the development of HT following venous thrombolysis[50]. Consequently, inhibiting the cGAS−STING pathway may hold promise in mitigating the side effects associated with thrombolysis.

Timely thrombolytic therapy is closely associated with improved long-term outcomes, yet overcoming the challenge of the limited therapeutic window for effective thrombolysis remains a significant issue[51]. The intravenous administration of recombinant tissue plasminogen activators within 3 h of the onset of stroke can result in excellent thrombolysis and a significant reduction in the incidence of disability in the patients over the succeeding 6−12 months; however, only 11% of the patients benefit from this treatment, chiefly those with small-vessel occlusion[52,53]. Clinical studies have shown that applying near-infrared spectroscopy (NIRS) -guided cerebral oximetry monitoring to measure cerebral oxygenation during acute reperfusion therapy may be beneficial for predicting the prognosis of AIS patients treated with rt-PA[54]. Recently, the application of computed tomography (CT) perfusion and perfusion-diffusion magnetic resonance imaging (MRI) has allowed the extension of the therapeutic window for intravenous thrombolysis to 9 h, offering more precise guidance for thrombolytic therapy[55]. Addressing the challenge of extending the therapeutic window for thrombolysis requires a multifaceted approach and innovative solutions. This includes deepening basic research to explore the mechanisms of thrombolytic drugs, collecting clinical data from patients undergoing thrombolytic therapy to establish comprehensive databases, optimizing the allocation and distribution of medical resources, and developing a robust stroke care system. Such integrated efforts hold the potential to provide greater hope and recovery opportunities for stroke patients.

Intravenous thrombolysis, thrombectomy, or stent placement are typically employed for the treatment of stroke patients with large vessel occlusion[56,57]. SYNTHESIS expansion experiments carried out in patients with AIS caused by an occlusion in the proximal anterior intracranial artery have revealed that intra-arterial therapy combined with rt-PA was safer and more effective than rt-PA therapy within 6 h after onset of AIS[22]. Thrombectomy after intravenous thrombolysis improves clinical outcomes in stroke patients, according to follow-up results at different stroke centers. However, in the Multicenter Randomized Clinical Trial of Endovascular Treatment for AIS in the Netherlands (MR CLEAN) with the expertise for carrying out mechanical thrombectomy found that recanalization rates reached 80% in patients treated with retrievable stents, compared with 32% in the standard treatment group[56,57]. These results suggest that a combination of intravascular therapy (including thrombectomy and stenting) with intravenous thrombolysis may prove beneficial for the treatment of AIS patients with large vessel occlusion and reduce the risk of reperfusion[58]. However, few stroke centers can boast of the expertise to carry out MT; patients with AIS are therefore unlikely to receive vascular recanalization in a timely manner[59].

In addition, the accurate identification of the infarct core and penumbra as well as the effective prevention of ischemia and hypoxia are important challenges for the treatment of IS. Therefore, there is an urgent need for developing novel methods for monitoring the penumbra to achieve visual treatment for clinical stroke and substantiate the diagnosis, treatment, and improvement in the prognosis of stroke patients. The expression of CircOGDH, a circular RNA derived from oxoglutarate dehydrogenase, correlated positively with the size of the penumbra *in vitro* experiments; in addition, CircOGDH inhibited the expression of *COL4A4* (Gallus collagen, type IV, alpha IV) and reduced neuronal damage, suggesting that CircOGDH could be exploited as a potential biomarker to monitor the size of the penumbra[60]. Moreover, COL4A4 is a promising target for developing molecular therapeutics for AIS and reducing the apoptosis of ischemic anoxic neurons. In addition, the occurrence of stroke is associated with an imbalance in cellular metabolism due to insufficient energy and supply of nutrients; ion balance is also affected due to the disruption of ion transport. X-ray fluorescence imaging was used to label the changes in the elemental levels (mainly of calcium, chlorine, and sodium[61]) of the penumbra, infarct core, and normal tissues during cell metabolism, which can also be exploited as a marker to track the size of the penumbra region and judge the degree of infarction[62].

Given that various complications can negatively impact the clinical outcome of stroke, an interdisciplinary approach has been established for the treatment of IS. The involvement of cardiologists is crucial for enhancing the prognosis of IS patients and preventing the incidence of stroke[59,63,64]. There is optimism regarding the feasibility of achieving comprehensive stroke care in the future.

2.2. *Acute stage*

Monitoring the penumbra region and achieving timely reperfusion of the ischemic tissue can be a potential strategy for the treatment of IS. However, the process of reperfusion is typically accompanied by the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which leads to oxidative stress[65]. This may be accompanied by lipid peroxidation, protein misfolding, mitochondrial dysfunction, DNA damage, and neuronal apoptosis, causing a series of injuries to neurovascular units (NVU)[66]; examples include the destruction of the BBB, injury to endothelial cells and the formation of thrombus, and activation of the inflammatory response, which further causes the death of ischemic neurons[67]. Oxygen and glucose transport are inhibited during the early stage of stroke and ischemia, resulting in depolarization of the cell membrane and increased inflow of calcium ions (Ca^{2+}) into the cells; this, in turn, leads to enhanced release of excitatory neurotransmitters such as glutamic acid and subsequently, the apoptosis of the neurons[68]. The continued accumulation of ROS leads to mitochondrial dysfunction, activation of exogenous apoptosis signaling cascades, the release of cytochrome C (Cytc) and apoptosis-inducing factor (AIF) resulting in DNA damage, and finally, apoptosis, necrosis, and autophagy *via* programmed cell death (PCD) signaling[69]. Thus, the activation of the innate and adaptive immune responses leads to inflammation, resulting in the destruction of the BBB, brain edema, and the death of ischemic neurons. The study of the pathophysiological mechanisms of the acute stage of IS has opened the door to several potential intervention strategies for stroke such as vascular recanalization, neuroprotection, and protection of the BBB.

Herein, we review several targets for neuroprotective intervention during the acute phase of IS; these include excitotoxicity, oxidative and nitrosative stress, modulation of autophagy, inhibition of ferroptosis, protection of the BBB, and immune intervention (Fig. 3).

2.2.1. *Neuroprotection*

At present, the main approach for the treatment of stroke focuses on the rapid restoration of blood flow to the brain, particularly to the ischemic penumbra region; however, the timing of recanalization is critical in cases of AIS. Research on neuroprotective interventions for stroke management is gaining increasing interest among the scientific community, especially given the need to address issues such as the limited therapeutic window. The beneficial effects of several neuroprotective agents in the treatment of IS has been previously demonstrated, and combining these with revascularization strategies could potentially widen the therapeutic window for thrombolytic therapy[70]. These observations underscore the necessity for developing novel neuroprotective drugs, preventing reperfusion injury, and enhancing the application of thrombolytic agents for treating window stenosis in IS. Neuroprotection involves strategies aimed at preserving the ischemic nerve cells by mitigating the ischemic cascade and the underlying molecular processes[71]. Herein, we summarize the existing neuroprotective strategies and highlight certain novel insights.

2.2.2. *Attenuation of excitotoxicity*

The deficiency of oxygen and glucose in the brain upon the onset of stroke results in insufficient energy supply to nuerons[72]. This leads to depolarization of the cell membrane, increased release and decreased uptake of glutamate, activation of *N*-methyl-D-aspartic acid (NMDA) receptors (NMDAR), enhanced influx of Ca^{2+} ions, and a series of downstream signaling cascades that can cause mitochondrial dysfunction and trigger neuronal death[73,74]. This process is termed excitotoxicity. Glutamate, an important excitatory neurotransmitter, regulates intercellular communication and synaptic plasticity. In general, excessive excitatory glutamate during IS activates postsynaptic glutamate receptors, triggering cascade of downstream cell death signals[75]. The chief glutamate receptors hitherto identified in the central nervous system (CNS) include NMDARs, kainate receptors (KARs), and α-amino-3-hydroxy-5-methyl-4-isooxazolepropionic acid (AMPA) receptors[76]. NMDAR is an important mediator of glutamate induced excitatory effects, which is divided into the subtypes GluN2A and GluN2B. The former is positively associated with neuronal survival and neural function and the latter, typically with the induction of neuronal apoptosis[77-79].

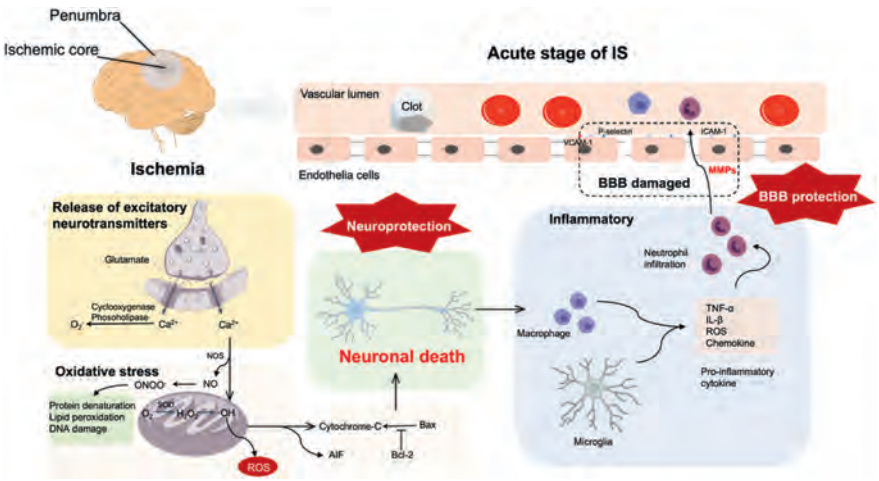

Figure 3 Overview of pathophysiological processes and effective treatment strategies for the acute stage of IS.

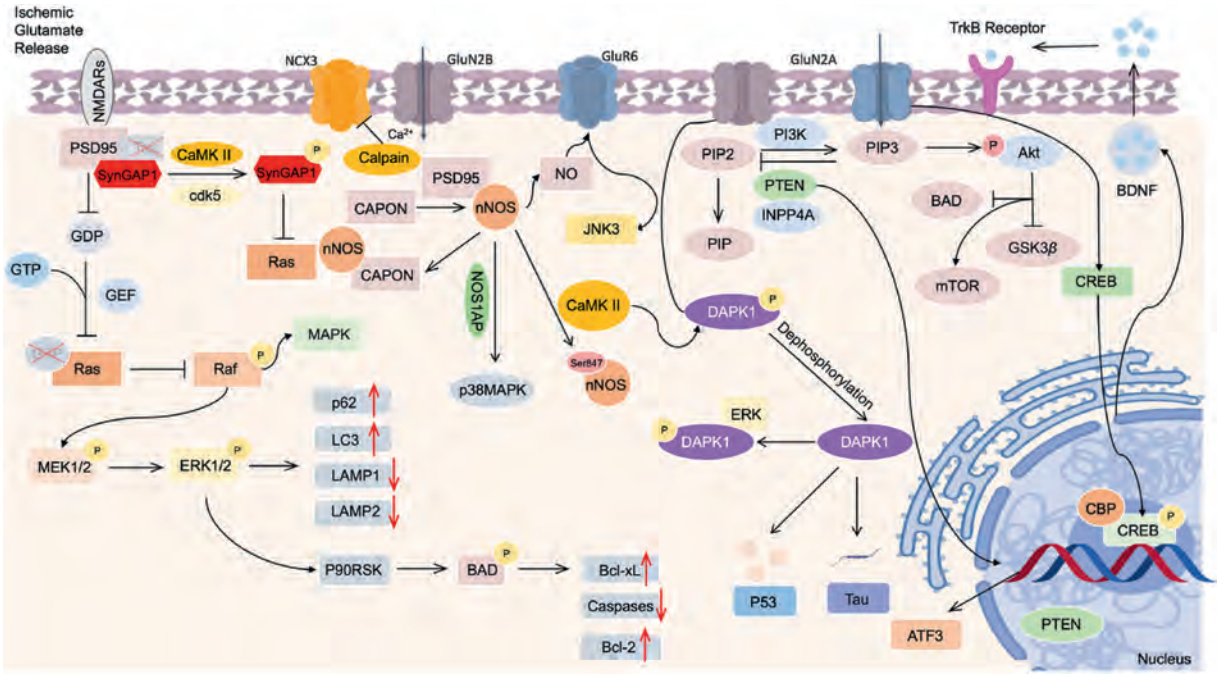

Figure 4 Excitatory synaptic transmission in IS and downstream signaling pathways associated with NMDAR.

To date, the treatment of excitotoxicity is mainly dependent on the following four strategies: (1) inhibition of glutamate release, (2) regulation of glutamate receptors, (3) promotion of glutamate uptake, and (4) regulation of downstream signaling pathways associated with the glutamate receptors. Therefore, an investigation of the mechanism underlying the neuronal death caused by excitotoxicity is of great significance for the clinical treatment of cerebral IS and the development of novel therapeutic drugs for the condition. Herein, we present a summary of the current interventions that target the signaling pathways downstream the activated glutamate receptor, which we hope provides valuable insights for devising therapeutic strategies for IS and developing novel drugs toward this end (Fig. 4).

2.2.2.1. Inhibition of NMDAR−PI3K/Akt−mTOR signaling. The neuroprotective activity of NMDAR is mediated *via* the activation of Akt, a serine/threonine kinase that is also known as PKB. The important role of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway in neuronal survival is widely documented (Fig. 5)[80]. On the one hand, the autophosphorylation of numerous neurotrophic factors and transmembrane receptors such as the insulin receptor substrate 1 (IRS-1) and tyrosine kinases triggers the activation of PI3K, which in turn facilitates the generation of phosphatidylinositol 3,4,5-triphosphate (PIP3) and the phosphorylation of Akt (to generate p-Akt)[81]. The activated Akt can be translocated to the nucleus, where it contributes to neuronal survival. Previous studies have demonstrated that the activation of NMDAR enhances the PI3K/Akt signaling pathway and confers protection against hypoxia and/or excitotoxicity-induced neuronal death under *in vitro* conditions[82]. On the other hand, the well-known tumor suppressor phosphatase and tensin homolog (PTEN) is a phosphatase that negatively regulates the PI3K/Akt signaling pathway by dephosphorylating PIP3 into phosphatidylinositol 4,5-bisphosphate (PIP2), thereby inhibiting the phosphorylation of Akt on the cell

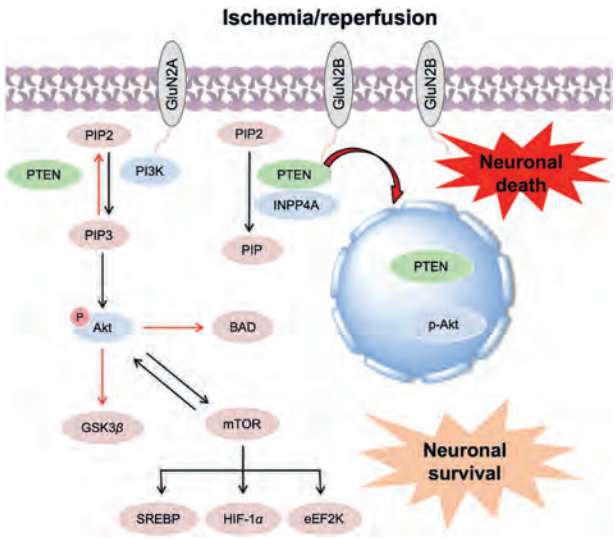

Figure 5 PI3K/Akt signaling in neuronal survival.

membrane. Additionally, inositol polyphosphate phosphatase 4A (INPP4A) facilitates the dephosphorylation of PIP2 into the neuroprotective phosphatidylinositol monophosphate (PIP)[83]. Concurrently, PTEN interacts with the C-terminal region of GluN2B to induce its nuclear translocation, leading to excitotoxicity and neuronal death[84].

Notably, there is extensive evidence for the role of mTOR as a prominent downstream effector of PI3K/Akt signaling and its potential to reciprocally activate Akt. Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) JAK2/STAT3−PI3K/AKT/mTOR axis emerges as a key neuroprotective cascade in cerebral ischemia, wherein STAT3 activation upregulates p-Akt to dually suppress: (1) apoptotic pathways *via* Bcl-2/Bax modulation, and (2) GSK-3β-mediated excitotoxicity

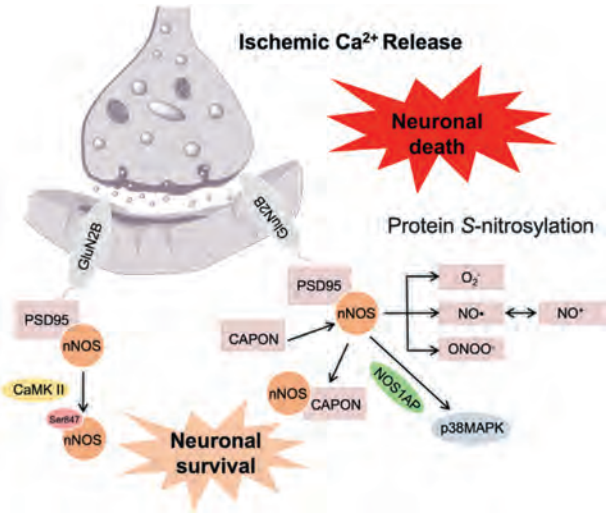

Figure 6 NMDAR/PSD95/nNOS signaling pathway.

through Tau hyperphosphorylation inhibition[85,86]. The downstream effectors of mTOR signaling include sterol regulatory element−binding protein (SREBP), hypoxia-inducible factor 1α (HIF-1α), forkhead box transcription factor (FoxO), nuclear elongation factor-2 kinase (eEF2K), and P70S6K1/2[87-92]. Consequently, the development of BBB-penetrant allosteric modulators to mitigate Tau hyperphosphorylation is of great significance for the protection of ischemic neurons.

2.2.2.2. Inhibition of NMDAR/PSD95/nNOS signaling. The primary role of nitric oxide synthase (NOS) is to catalyze the synthesis of nitric oxide (NO) from L-arginine. Mammals possess three distinct subtypes of NOS, namely, endothelial (eNOS, NOS3), neuronal (nNOS, NOS1), and inducible (iNOS, NOS2) NOS[93]. Among these subtypes, nNOS is predominantly found within the CNS and peripheral nervous system and exhibits a strong correlation with neurological function[94]. Postsynaptic density protein-95 (PSD95) belongs to the membrane-associated guanylate kinase family of proteins; it serves as the core scaffolding protein of the postsynaptic membrane and has three PDZ domains. Under physiological conditions, PSD95 binds NMDARs while its PDZ domain binds nNOS[95,96]. The incidence of IS leads to an increased influx of Ca^{2+} and the association of the GluN2B subunit of NMDAR with PSD95 and nNOS to form the complex GluN2B−PSD95−nNOS; this leads to the subsequent production of peroxyanions (O_2^-), nitric oxide radicals (NO·), and peroxynitrite ($ONOO^-$), ultimately leading to neuronal death. The three main types of NO include the nitrogen monoxide radical (NO·), nitrosonium cation (NO^+), and nitrosyl anion (NO^-)[97]. The NO^+ break the disulfide bonds in sulfhydryl-containing proteins and cause the *S*-nitrosylation (S-NO) of proteins, which activates cell death pathways[98]. Endogenous Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) can induce the phosphorylation of nNOS at Ser847, thereby decreasing nNOS activity in a rat model of ischemia/reperfusion. Previous studies have shown that the inhibition of nNOS expression can inhibit ischemia-induced excitotoxicity and ischemic brain injury[99]. The protein CAPON colocalizes with nNOS and competes with PSD95 for nNOS binding in neurons. The overexpression of CAPON leads to reduced formation of the complex GluN2B−PSD95−nNOS[100]. In addition, the deletion of exon 2 of the *NOS1* gene prevents the formation of the GluN2B−PSD95−nNOS complex, thereby providing resistance against the damage caused by cerebral ischemia. Li et al.[101] reported the activation of p38 mitogen-activated protein kinase (p38 MAPK) downstream of nNOS; NMDAR can induce the binding of nNOS to the MKK3-binding protein NOS1AP, which mediates excitotoxicity by activating the p38 MAPK signaling pathway. Zhang et al.[102] reported the formation of the nNOS−Sox2 complex following the nuclear translocation of nNOS, leading to the activation of Sonic hedgehog (*Shh),* a direct target of Sox2. The expression of nNOS, Sox2, and Shh was enhanced in the rat model of cerebral ischemia, suggesting that neurons may be protected from excitotoxicity by modulating the nNOS−Sox2−Shh pathway. We propose the following four targeted strategies to protect neurons from NO-mediated cell death (Fig. 6): (1) Developing small-molecule inhibitors that specifically target PSD95 or nNOS, (2) Identifying activators of the CAPON protein, (3) Selective denitrosation of target proteins, and (4) Developing inhibitors for NOS1AP.

2.2.2.3. Inhibition of NMDAR/Ras/Raf/MEK/ERK signaling. The Ras/Raf/MEK/ERK signaling pathway is a well-established MAPK pathway that transmits signals from cell surface receptors such as NMDAR to mediate the expression of cell-specific proteins, resulting in excitotoxic brain injury (Fig. 7)[103]. The GDP-

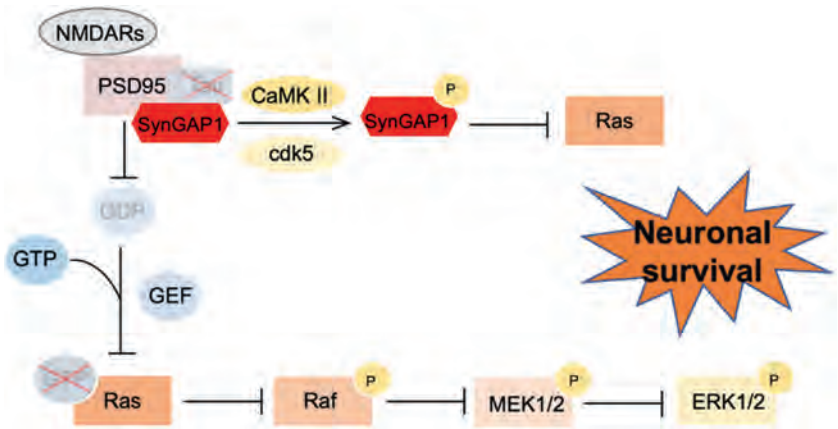

Figure 7 Ras/Raf/MEK/ERK signaling pathway.

bound form of Ras is typically inactive, and the Raf-mediated binding of GTP to Ras is brought about by guanine nucleotide exchange factor, resulting in Ras activation. The activated Ras further activates the MEK/ERK pathway *via* the phosphorylation of Raf to regulate cell proliferation, differentiation, apoptosis, autophagy, and other cellular processes. Further, the activated ERK1/2 activates P90RSK, phosphorylates the pro-apoptotic protein BAD, and increases the expression of the antiapoptotic proteins Bcl-2 or Bcl-xL, thus inhibiting neuronal apoptosis[104,105].

Reduced activation of the excitotoxic Ras/ERK signaling cascade downstream of NMDAR and increased activation of the postsynaptic Ras-inhibiting GTPase-activating protein SynGAP1 were observed in Tau$^{-/-}$ mice, suggesting the region-specific inhibition of Ras[106]. Tau, which is expressed in neuronal axons, is chiefly a microtubule-associated protein that maintains the stability of tubulin. However, targeting the Tau protein for the treatment of IS is challenging. Despite several studies on signaling pathways that are activated downstream of Tau, noteworthy progress in targeting Tau for the treatment of IS remains to be accomplished. Achieving Tau knockouts in the mouse model of middle cerebral artery occlusion (MCAO) has been well documented to reduce brain damage and neurological deficits[107], highlighting the potential benefits of developing small molecule inhibitors of Tau protein to regulate its expression and activation under *in vivo* conditions. SynGAP1 protects the ischemic neurons from damage in Tau$^{-/-}$ mice by binding PSD95 to form the PSD95/SynGAP1 complex that exhibits postsynaptic accumulation, thereby inhibiting Ras expression and preventing ERK activation[106]. Additionally, the phosphorylation of SynGAP1 by CaMKII and cyclin-dependent kinase 5 (Cdk5) results in the inactivation of Ras and protection against excitotoxicity. Inhibition of Tau protein expression reduces transient middle cerebral artery occlusion (tMCAO)-induced excitotoxic brain damage, which has been demonstrated in several animal models[108].

2.2.2.4. Inhibition of NMDAR/CREB/BDNF signaling. The increase in Ca^{2+} levels in the astrocytes upon the onset of IS facilitates the release of various glial transmitters (including glutamate and adenosine triphosphate[109,110]) that regulate the growth and differentiation of neurons and improve synaptic function. Glutamate uptake affords protection to the neurons against excitotoxicity, but it can also induce inflammation to aggravate brain damage. BDNF is a neurotrophic protein that is chiefly secreted in the form of its precursor. During IS, an increase in the influx of Ca^{2+} ions promotes CREB phosphorylation by activating downstream calcium/calmodulin-dependent kinase IV, upregulates BDNF expression and promotes its binding to tyrosine kinase B (TrkB) receptors, activates the Ras/MAPK/ERK1/2 signaling pathway, and upregulates the expression of CREB transcription factors[111]. Increased expression of the antiapoptotic protein Bcl-2 protects the nerve cells from damage and also promotes axon development by activating the PI3K/Akt signaling pathway[112]. Therefore, the activation of the CREB/BDNF/TrkB signaling pathway can protect neurons during IS, presenting another promising avenue of exploration for the treatment of stroke (Fig. 8). Moreover, the application of the TrkB antagonist ANA-12 has been demonstrated to reverse the protective effects of baicalin on neurons *via* the BDNF/TrkB pathway in an oxygen−glucose deprivation/reperfusion (OGD/R) coculture model of astroglia with neurons[113].

2.2.2.5. Inhibition of NMDAR−DAPK1−CaMKII signaling. Death-associated protein kinases are members of the Ca^{2+}/calmodulin-dependent serine/threonine protein kinase family[114]. Death-associated protein kinase 1 (DAPK1) is a pro-apoptotic kinase that is mainly involved in the regulation of neuronal activity, which forms the focus of the current review. During stroke, increased inflow of Ca^{2+} ions mediated by the GluN2B subunit of the NMDAR receptor leads to the dephosphorylation of DAPK1 and further accumulation of CaMKII at excitatory synapses[115,116]. An increased activity of DAPK1 was observed in the tMCAO mouse model for IS; however, the volume of intracranial infarction was significantly reduced following DAPK1 knockout. DANGER (a membrane protein) regulates the release of Ca^{2+} ions by binding IP3R; it also directly binds DAPK1 to inhibit DAPK1 activity[117]. Ginsenoside-Rd has also been reported to inhibit DAPK1 and protect ischemic neurons under *in vitro* conditions[118]. N-myc downstream-regulated gene 2 (NDRG2) is a DAPK1 substrate, and overexpression of DAPK1 phosphorylates NDRG2, increasing neuronal cell death. In the presence of DAPK1 inhibitors, it has been shown to significantly reduce neuronal cell death and eliminate NDRG2 phosphorylation[119]. The presynaptic protein Caytaxin was also found to prevent neuronal cells apoptosis by inhibiting DAPK1 under *in vivo* conditions in a mouse model of MCAO[120]. The activation of DAPK1 also leads to endoplasmic reticulum (ER) stress,

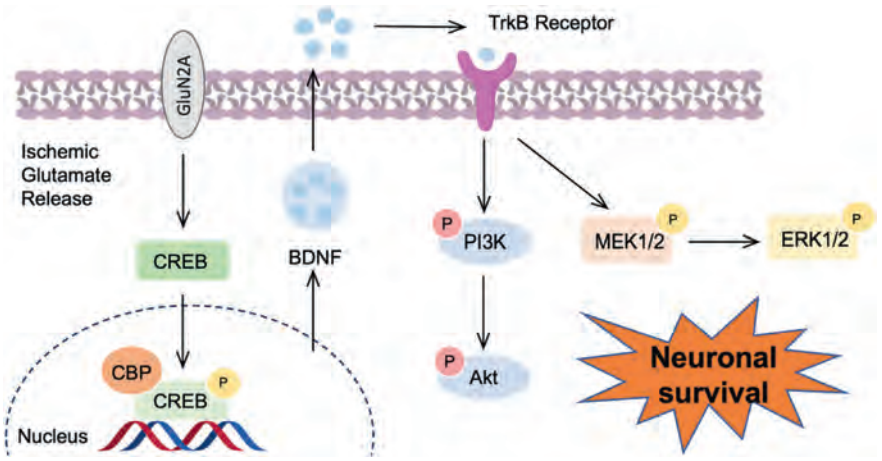

Figure 8 Specific mechanisms targeting NMDAR/CREB/BDNF.

phosphorylation of p53 at Ser23 and activation of related apoptotic factors, as well as the promotion of neuronal apoptosis. The peptide Tat-p53DM was found to effectively block the interaction of DAPK1 with p53. DAPK1 was found to activate the apoptosis regulators Bax and cyclophilin D (CypD) *via* the phosphorylation of p53 in the primary cortical neurons of mice[121,122]. DAPK1 was also found to mediate Tau phosphorylation at Ser262, exacerbating the brain damage caused by excitotoxicity[106]. The application of the peptide TAT-R1D inhibits DAPK1 activity and interferes with Tau phosphorylation to significantly reduce the neuronal damage caused by IS[123]. Thus, DAPK1 is a key modulator of cell death, and inhibition of DAPK1 activity is likely to protect the ischemic neurons. In addition, the signaling factor ERK that functions downstream NMDAR can directly interact with DAPK1 and mediates the phosphorylation of DAPK1 at Ser735, thereby activating DAPK1 and promoting neuronal death[124].

Targeting the NMDAR−DAPK1−CaMKII signaling pathway is expected to be a promising approach for stroke therapy (Fig. 9). There are four potential strategies toward this end: (1) Inhibition of DAPK1 dephosphorylation *via* the inhibition of CaMKII, (2) Inhibition of the assembly of the NMDAR−DAPK1 death-signaling complex, (3) Development of selective DAPK1 inhibitors, which has been the focus of multiple studies, and (4) Development of novel drugs targeting the DAPK1 mRNA. However, most of the currently-employed DAPK1 inhibitors interfere with multiple pathways that exert beneficial as well as harmful effects; the development of target-specific selective DAPK1 inhibitors is therefore an urgent necessity and appears promising for the treatment of IS.

2.2.2.6. Inhibition of NMDAR-calpain signaling. There are numerous proteases and proteolytic complexes that occur in biological systems; of these, calpains are Ca^{2+}-activated neutral proteases that are directly activated by Ca^{2+} ions, which act as an important second messenger in signal transduction pathways[125]. Calpain cleaves a variety of intracellular proteins and mediates excitotoxicity[126]. At the onset of stroke, the activation of NMDAR results in an increased influx of Ca^{2+} ions. In turn, this leads to the activation of calpain and subsequent excitotoxicity, which causes synaptic dysfunction. Activated calpain further inhibits sodium−calcium exchanger 3 (NCX3) on the plasma membrane to induce neuronal death. In fact, a significant reduction in the infarct core volume in the ischemic region of the brain tissue was reported in a rat model of MCAO following the intra-arterial injection of the calpain inhibitor AK295, proving that inhibition of calpain activity can protect the ischemic neurons from damage[127]. The microtubule-associated protein Tau is known to be hydrolyzed by calpain during stroke. The expression of the 17-kDa Tau was significantly increased in an OGD/R model under *in vitro* conditions, which can lead to synaptic damage. Therefore, the Tau protein shows promise as a potential marker for predicting the occurrence of IS[107].

Notably, promoting the hydrolysis of all proteins is hardly beneficial; for example, the neuroprotective proteins NCX and metabotropic glutamate receptor (mGluRα-1) promote neuronal survival, but their hydrolysis results in a loss of their neuroprotective activities[128,129]. The tyrosine kinase Src family plays an important role in the growth and development of neurons. Calpain1 induces excitotoxicity by cleaving Src and inhibiting Akt. Therefore, the development of selective small-molecule inhibitors of calpain1 that target-specific death-signaling proteins holds great promise for the treatment of stroke in the future.

2.2.2.7. Inhibition of nNOS/glutamate receptor 6 (GluR6)/c-Jun N-terminal kinase (JNK) signaling. During cerebral ischemia, the activation of nNOS followed by increased production of NO leads to *S*-nitrosylation of GluR6 and subsequent activation of JNK *via* the GluR6−PSD95−MLK3 signaling pathway[130]. JNKs, as members of the MAPK family, are involved in the regulation of cellular stress responses and a variety of cellular processes, including cell proliferation, differentiation, and apoptosis[131]. JNK3 activation was observed in the rat model of induced ischemia/reperfusion. The nuclear translocation of JNK results in the phosphorylation of c-Jun and enhances Fas L (Fas Ligand) expression, leading to apoptosis. Moreover, JNK induces the release of cytochrome *c* (cyt-c) *via* the regulation of mitochondrial Bcl-2, causing neuronal apoptosis[132]. Hence, the nNOS/GluR6/

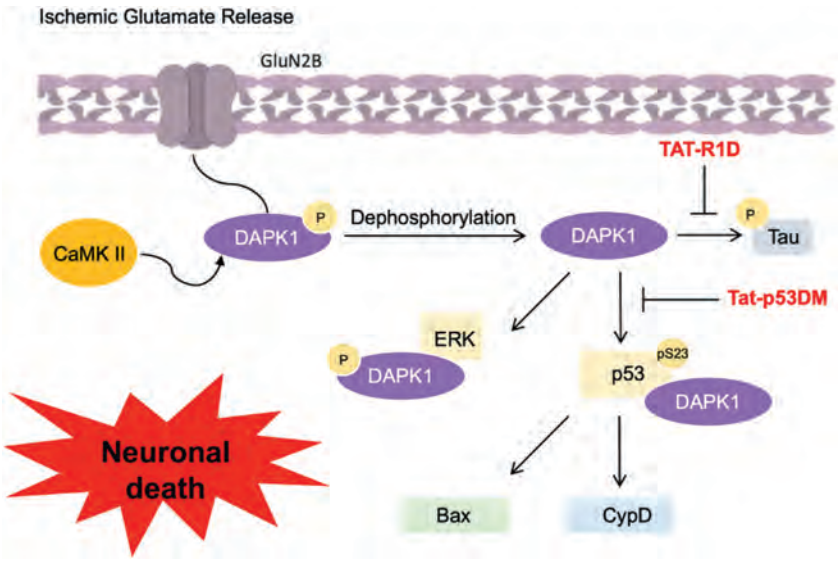

Figure 9 DAPK1 signaling pathway.

JNK signaling pathway can also be explored as a potential target for stroke treatment.

2.2.2.8. Other treatment strategies. Drugs that directly target NMDAR for the treatment of IS have not been developed to date, and research on the feasibility of exploiting the downstream signaling effectors of NMDAR as potential targets for the treatment of IS has gained increasing traction. Besides exploiting the above pathways, there are several potential strategies for the treatment of IS injury: (1) Application of calcium channel blockers (CCBs) to modulate the signaling pathways downstream of the glutamate receptor[133], (2) Stable ion pump function[134], and (3) Use of TREK-1 channel blockers. TREK-1 is a potassium channel that is highly expressed in astrocytes; moreover, the expression of TREK-1 is increased after cerebral ischemia[135], and the use of TREK-1 channel blockers can significantly inhibit glutamate release and protect neurons from injury. In addition, the anti-neuroinflammatory effects of certain natural drugs such as thujaorientalissemen (TOS) have also been documented[136].

2.2.3. Targeting oxidative stress

The rapid accumulation of large quantities of ROS and RNS after vascular reperfusion leads to oxidative/nitrosative stress, which results in intracellular Ca^{2+} overload, protein denaturation, lipid peroxidation, mitochondrial dysfunction, and DNA damage; these processes have been identified as the fundamental mechanisms responsible for brain injury after stroke[137]. Hydrogen peroxide (H_2O_2), O_2^-, NO, hypochlorous acid (HOCl), and $ONOO^-$ constitute the main types of ROS and RNS[65]. This article summarizes the main pathways associated with oxidative stress during AIS and discusses the potential exploitation of the concerned receptors and enzymes as therapeutic targets, which may aid the development of small-molecule drugs for IS and provide novel treatment options for stroke in the future (Fig. 10).

2.2.3.1. Inhibition of nicotinamide adenine dinucleotide phosphate oxidase (NOX) signaling. Under normal physiological conditions, ROS is generated *in vivo* by the activity of NOX and is required for the normal functioning of cells[138]. NOX is a membrane-bound enzyme complex with seven isoforms. Of these, NOX1 has been shown to be expressed in microglia, while NOX2 and NOX4 are expressed in the neurons, astrocytes, and microglia. The expression of NOX2 was found to be upregulated during AIS; it mediates the production of ROS and activates NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3), a cytosolic inflammasome complex that regulates innate immune and inflammatory signaling pathways, leading to the induction of inflammation[139]. The application of a NOX inhibitor can reverse the increase in ROS concentrations in the brain, suggesting that NOX inhibition can afford protection to the ischemic neurons[138]. Presently, isoform-selective NOX inhibitors have been successfully validated using cellular model systems, and certain microRNAs (miRNAs) such as miR-320 have been shown to modulate neuronal oxidative stress during IS by inhibiting the NOX2/ROS pathway[128,140]. However, whether NOX is the main ROS-generating enzyme during IS remains unclear. Ye et al.[141,142] investigated the impact of NOX2-mediated ROS production on cerebral vascular regeneration. The results revealed that during the recovery stage of stroke, the ROS generated by NOX2 can inhibit the formation of NLRP3 inflammasomes, promote vascular regeneration, affect vascular remodeling, and play a role in the activation of autophagy. Therefore, NOX2/ROS appears to be a double-edged sword, with a dual role in the different stages of stroke[143]. Taken together, the data suggests that appropriate isoform-selective small-molecule inhibitors of NOX can be exploited to inhibit ROS overproduction and prevent neuronal apoptosis after the onset of stroke. In addition, interventions that target the activation of the NLRP3 inflammasome can also provide novel opportunities for the treatment of IS.

2.2.3.2. Inhibition of Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2−related factor 2 (Nrf2)/ARE signaling. Organisms avoid damage to the brain by modulating a series of signals associated with oxidative stress. The transcription factor Nrf2 plays a key role in IS by regulating the antioxidant response. The activation of Nrf2 during the acute stage of stroke has a neuroprotective effect owing to the transcriptional activation of antioxidant genes such as heme

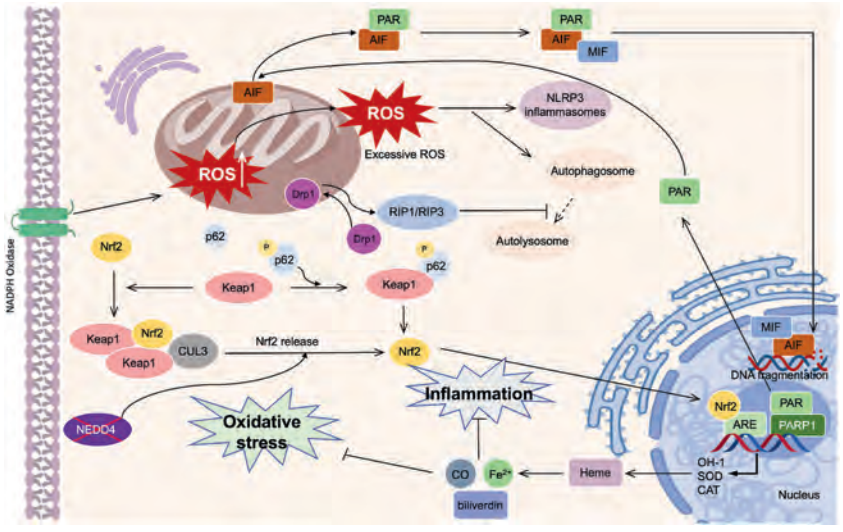

Figure 10 Major pathways associated with oxidative stress during AIS.

oxygenase-1 (HO-1)[144,145]. In addition, HO-1 plays a role in heme metabolism; it degrades heme to generate carbon monoxide (CO), biliverdin, and iron (Fe^{2+}) and has antioxidant, anti-inflammatory, and metabolic regulatory functions[31]. Keap1 is a substrate for E3 ubiquitin ligase. Under normal physiological conditions, Nrf2 binds Keap1 in the cytoplasm and is subsequently ubiquitylated by the E3 ubiquitin ligase to form the complex Keap1−CUL3−E3 ubiquitin ligase. Oxidative stress inhibits the activation of Nrf2, resulting in the translocation of cytosolic Nrf2 to the nucleus and the activation of the Nrf2−ARE pathway. This, in turn, promotes the expression of HO-1 and antioxidant enzymes such as superoxide dismutase and catalase (CAT). However, Nrf2 activators do not appear to affect the dissociation of Keap1 from Nrf2. Dynamin-related protein 1 (Drp1), which is essential for mitochondrial fission, and p62, an autophagic adaptor protein, are newly-discovered regulatory factors that play important roles in oxidative stress. Under normal conditions, Drp1 is localized in the cytoplasm. However, ischemia causes the translocation of Drp1 from the cytoplasm to mitochondria, which disrupts mitochondrial homeostasis and leads to the accumulation of ROS within the mitochondria; this inhibits autophagy *via* the receptor-interacting protein kinase 1/3 (RIP1/RIP3) pathway[146,147]. Nrf2 is chiefly expressed in astrocytes and directly binds the protein neuronal precursor cell−expressed developmentally downregulated 4 (NEDD4), which is a type of E3 ubiquitin ligase that mediates Nrf2 ubiquitination. A knockout of NEDD 4 can inhibit Nrf2 ubiquitination, reduce oxidative stress, and protect damaged neurons[148]. Therefore, the modulation of Nrf2 ubiquitination pathway is a promising approach for the clinical treatment of patients with cerebral ischemia.

2.2.3.3. Inhibition of NMDAR-nNOS-PARP1 signaling. Poly (ADP-ribose) polymerase 1 (PARP1) is a nuclear protease that plays a crucial role in various biological processes, including DNA repair, inflammatory response, and gene regulation[149,150]. The occurrence of stroke causes an increased glutamate release in the neurons of the ischemic brain, which leads to the production of NO, $ONOO^-$, and ROS *via* the NMDAR−nNOS pathway; this results in DNA damage accompanied by PARP1 hyperactivation. Additionally, the activation of PARP1 generates large quantities of poly (ADP-ribose) or PAR in the presence of nicotinamide adenine dinucleotide (NAD^+)[120]. Poly (ADP-ribose) (PAR) acts as a cell death signal capable of transmitting signals to the mitochondria and facilitates the release of AIF from the mitochondria[151]. Microphase migration inhibitory factor (MIF) as a PARP1-dependent AIF-associated nucleotide (PAAN), which can be recruited to the nucleus by AIF, inhibits The nucleotide activity of MIF results in DNA damage and ultimately, cell death[152,153]. Furthermore, the mitochondrial apoptotic pathway has been demonstrated to play a significant role in the cascade of events leading to IS.

While PARP1 targeting has been extensively explored in oncology, its therapeutic potential in IS remains underdeveloped[154]. Emerging evidence indicates that PARP1 inhibition (*e.g.*, PJ34[155]) attenuates neuroinflammation by restoring NAD^+ levels and blocking PAR-mediated mitochondrial AIF/MIF nuclear translocation, thereby reducing DNA fragmentation in preclinical models[156,157]. Notably, MIF knockout mice exhibit attenuated PARP1-induced DNA damage, suggesting that disrupting the AIF-MIF interaction may circumvent the risks associated with systemic PARP1 inhibition (*e.g.*, impaired DNA repair) while maintaining neuroprotection (Fig. 11)[152].

2.2.3.4. Inhibition of ER stress. The accumulation of ROS during IS activates protein kinase R-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme type 1 (IRE1) by enhancing ER stress signaling[158]. PERK induces the expression of C/EBP homologous protein (CHOP) *via* the phosphorylation of eukaryotic initiation factor 2α (eIF2α). CHOP induces apoptosis *via* the upregulation of Bax/Bcl-2 signaling[159]. The protein hairy and enhancer of split-1 (Hes1) was shown to inhibit the PERK−eIF2α−CHOP pathway, suggesting that Hes1 can be exploited as a potential target for the treatment of stroke[160]. The binding of IRE1α with tumor necrosis factor receptor-associated factor 2 (TRAF2) can activate the JNK pathway. Recent studies have shown that inhibitors of phosphodiesterase 4 (PDE4) inhibit apoptosis by hindering the interaction between IRE1α and TRAF2. The glutathione (GSH) pathway has an important antioxidant function and scavenges the accumulated ROS in cells to protect cells from oxidative stress−induced damage. Recent studies have shown that the oral administration of exogenous GSH can significantly reduce oxidative stress and the release of inflammatory factors in rat models of IS, thereby displaying neuroprotective effects[161]. Despite the good neuroprotective effects of GSH in animal models, the rapid metabolism of GSH before it can reach the site of action and the existence of the BBB pose challenges for the exploitation of these neuroprotective effects of GSH for the clinical treatment of IS. The precursor of GSH, γ-glutamylcysteine (γ-GC), is not limited by the BBB and can be converted to GSH by the enzyme GSH synthetase (GSS)[162]. The application of γ-GC significantly reduced the infarct core volume and protected neurons in the mouse model of MCAO[163]. Furthermore, γ-GC supplementation reduced neuronal apoptosis in the *in vitro* OGD/R model by increasing GSH and decreasing ROS concentrations. Supplementation with γ-GC also increased the activities of antioxidant enzymes such as SOD[164].

2.2.3.5. Inhibition of SIRT/FOXO3a/SOD2 signaling. SIRTs belong to a family of NAD^+-dependent histone deacetylases and include seven members (SIRT1−7) in mammals. They participate in a variety of physiological and pathological processes such as apoptosis and oxidative stress[165]. Silent information regulator 1 (SIRT1) is mainly localized in the nucleus. FOXO3a, a forkhead transcription factor, is one of the substrates of SIRT1. FOXO3a can bind SIRT1 and translocate to the nucleus, which promotes the posttranslational modification of FOXO3a. Activated FOXO3a regulates the expression of mitochondrial ROS scavengers such as SOD2 and protects nerve cells from oxidative stress−induced damage. However, the autophosphorylation of FOXO3a at Ser253 leads to its translocation to the cytoplasm and subsequent inactivation, thereby inducing cell death. Sirtuin-3 (SIRT3), a protein localized in the mitochondrial matrix, is typically modified by SUMOylation, resulting in suppression of its deacetylation activity[166]. Under pathological conditions such as oxidative stress, the mitochondrial translocation of sentrin-specific protease 1 (SENP1) induces the deSUMOylation of SIRT3; this aspect of SIRT3 deSUMOylation by SENP1 provides a novel perspective of apoptosis[167]. The deSUMOylated SIRT3 can bind SOD2 to protect neurons from ischemic injury and inhibit cell apoptosis[168].

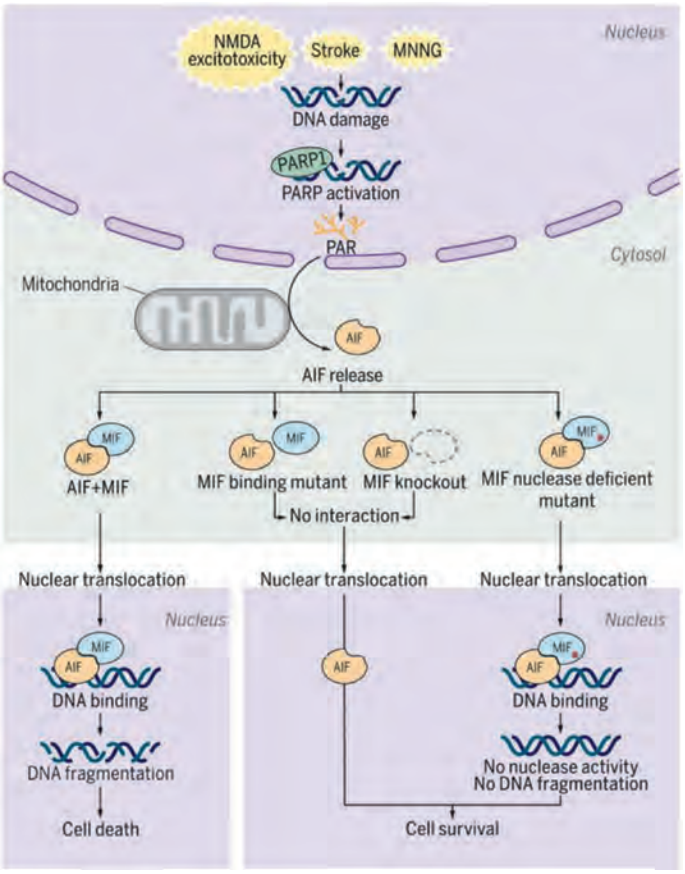

Figure 11 PARP1 signaling in AIS. PARP1 hyperactivation triggered by ischemic glutamate surge exacerbates neuronal DNA damage through NMDAR-nNOS-mediated ROS/$ONOO^-$ overproduction, while PAR polymer generated during PARP1 catalysis induces mitochondrial AIF release and MIF-dependent nuclear DNA degradation, collectively amplifying the apoptotic cascade in IS[152]. Reprinted with the permission from Ref. 152. Copyright © 2016, American Association for the Advancement of Science.

Trans sodium crocetinate (TSC) is a small-molecule drug that has been shown to exhibit neuroprotective effects in the rat model of induced cerebral ischemia[169]. A previous study revealed that TSC treatment significantly increased SIRT3 activity and SIRT3 and SOD2 protein levels. Therefore, the SIRT/FOXO3a/SOD2 pathway is expected to be a new target for IS therapy. Moreover, an increase in SIRT3 expression is closely associated with angiogenesis[170]. The development of SIRT3 activators is of great significance for modulating the antioxidant response and inflammation-induced damage in nerve cells.

2.2.3.6. Activating erythropoietin (EPO) receptor. EPO is an important hypoxia-related transcription factor that can bind the homodimeric erythropoietin receptor (EPO-R) to promote erythropoiesis and protect ischemic nerve cells from oxidative stress−induced damage[90]. In addition, it can also bind the heterodimeric tissue protective receptor (TPR) of EPO. TPR activates a series of signaling molecules such as STAT5, PI3K, and MAPK to inhibit apoptosis[171]. Previous studies have shown that the upregulation of EPO expression protects neurons against ischemia/reperfusion injury owing to the upregulation of HIF-1α[172]. Moreover, HIF-1α has been shown to inhibit apoptosis by stimulating the expression of EPO in hypoxic retinal cells[173]. The beneficial effects of EPO on IS continue to be investigated, supporting the premise that EPO may have an important regulatory role in stroke treatment by protecting damaged neurons and improving the prognosis of stroke patients.

2.2.4. Modulation of autophagy

Autophagy is believed to be a double-edged sword in ischemia and preconditioning. Autophagy can be considered a process of self-degradation. Physiological autophagy eliminates harmful protein aggregates and damaged organelles in cells and thus limits the transmission of harmful signaling, playing a neuroprotective effect and promoting cell death in patients with IS[174]. However, increased autophagy is not always beneficial to neuronal health[175]. An appropriate regulation of the extent of autophagy in ischemic cells may represent a potential strategy for the treatment of IS. The proteins p62 and LC3 have been employed as markers for monitoring and evaluating the extent of autophagy. The mitochondria play an important role in the regulation of oxidative stress, calcium homeostasis, and apoptosis. Mitophagy refers to the specific autophagic elimination of mitochondria and can regulate mitochondrial homeostasis by removing the dysfunctional mitochondria. Recent studies have shown that deoxyhypusine hydroxylase (DOHH), a basic enzyme, catalyzes the posttranslational hypusination modification of eukaryotic translation initiation factor 5A (eIF5A). Brazilin (BZ) is a naturally-occurring small molecule that enhances mitochondrial function *via* the allosteric activation of DOHH followed by the activation of

DOHH/eIF5A signaling pathway[176]; it can protect ischemic neurons by activating mitophagy in the *in vitro* and *in vivo* model systems OGD/R and MCAO mice, respectively. The modulation of the DOHH/eIF5A/mitophagy signaling pathway may present yet another novel approach for the treatment of neuronal injuries in IS. The design and employment of novel neuroprotective agents that mediate the allosteric activation of DOHH represents a potential therapeutic strategy for the treatment of IS.

A large number of noncoding RNAs, including miRNA, circular RNA (circRNA), and long noncoding RNA (lncRNA), have been shown to regulate autophagy and protect neurons from injury in animal models of IS[177]. The miRNAs have been reported to play an important role in the pathogenesis of stroke and the comorbidities in IS. miR-30d induces autophagy and promotes apoptosis in astrocytes *via* the inhibition of Beclin1[178]. Aberrantly-expressed circRNAs can manipulate intracellular processes, participate in immune responses, and improve synaptic dysfunction by targeting miRNAs[179]. The inhibition of circHECTD1 expression can inhibit astrocyte activation and autophagy, while improving neurological functions in the MCAO mouse model for stroke[180]. A knockout of circVRK1 reduced cell death, oxidative stress, and inflammation-induced injury in an OGDR model of human brain microvascular endothelial cells by regulating the miR-150-5p/MLLT1 axis[181]. circHECTD1 can be exploited as a novel biomarker and potential therapeutic agent for cerebral ischemia. lncRNAs are noncoding RNAs with a length of over 200 nucleotides and are mainly expressed in the CNS. The abnormal expression of lncRNAs results has very complex outcomes in terms of the impacted functions. The potential mechanism(s) underlying the role of lncRNAs in IS remains unclear despite considerable research. Recent studies have shown that several lncRNAs are highly expressed during IS and play an important role in the regulation of autophagy and apoptosis[182]; these include metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and lncRNA paternal expressed gene 11 antisense (PEG11as). Cao et al.[183] discovered that MALAT1 competitively binds miR-18c-5p, which in turn upregulates the expression of high-mobility group box1 (HMGB1) protein and exacerbates inflammation-induced damage during stroke. The downregulation of MALAT1 inhibits autophagy and reduces cell death[182]. The lncRNA PEG11as is chiefly localized in the cytoplasm, and the downregulation of PEG11as protects neurons from ischemia/reperfusion injury *via* the miR-342-5p/profilin 1 (PFN1) axis[184].

In addition, ROS and RNS also play important roles in autophagy, and targeting ROS/RNS to regulate autophagy may be a suitable approach for the treatment of IS. Puerarin activates the adenosine monophosphate−activated protein kinase/mammalian target of rapamycin/UNC-51-like kinase 1 (AMPK/mTOR/ULK1) signaling pathway and alleviates autophagy in the rat MCAO model of ischemia/reperfusion injury[185]. AMPK is a conserved serine/threonine (Ser/Thr) protein kinase and plays a role in autophagy, apoptosis, and oxidative stress. The depletion of ATP under conditions of ischemia/hypoxia leads to the activation of AMPK, which is an upstream regulator of mTOR; activated AMPK inhibits mTOR and phosphorylates ULK1 at Ser317 to induce autophagy[177].

2.2.5. *Inhibition of ferroptosis*

Ferroptosis is a recently-identified form of programmed cell death that is closely associated with the iron-dependent peroxidation of membrane lipids and cellular metabolism[186]. A disruption of iron homeostasis and lipid peroxidation are recognized as significant indicators of iron-dependent cell death and is associated with the excessive accumulation of ROS[187]. There is mounting evidence implicating iron-dependent cell death in conditions such as IS, cancer, doxorubicin-induced cardiomyopathy, and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease[188]. The inhibition of ferroptosis has emerged as a promising approach for the development of therapeutics for the abovementioned diseases; numerous studies have revealed positive outcomes from such an approach under *in vitro* as well as *in vivo* conditions.

The GSH−GPX4 (GSH peroxidase 4) axis plays a central role in the regulation of iron homeostasis and protection of cells from the damage caused by lipid peroxidation. The inhibition of GPX4 or depletion of GSH induces ferroptosis, as does the disruption of the cystine/glutamate antiporter system X_c^-[189]. Ferritin, a protein complex responsible for iron storage, is crucial for the maintenance of iron homeostasis and is an important antioxidant in cells. The autophagy-mediated degradation of ferritin, which is facilitated by nuclear receptor coactivator 4 (NCOA4), is essential for iron homeostasis. Inhibitors targeting NCOA4 or its interaction with FTH1 are being explored as a novel strategy for the treatment of neurological diseases such as IS[190].The cGAS−STING pathway has been reported to play a role in regulating cell death following IS. The inactivation of GPX4 leads to the obstruction of the cGAS−STING signaling pathway, thereby impeding the smooth transmission of downstream signals[191]. Recent research has uncovered that NCOA4-mediated autophagy-mediated degradation of ferritin enhances the production of reactive oxygen species (ROS) by inducing the Fenton reaction[192]. These ROS can further oxidize the C147 cysteine residue of STING, thereby inhibiting the cGAS−STING pathway and subsequent interferon production[193]. Further investigation into the interplay between the cGAS−STING pathway and oxidative stress is of paramount importance, as it may offer novel insights into the regulatory mechanisms underlying cell death, and potentially identify new targets for therapeutic strategies aimed at addressing IS in the future.

2.2.6. *BBB protection and immune intervention*

The BBB acts as a critical defensive barrier between the CNS and peripheral circulation, protecting the brain from damage and preventing the entry of harmful substances into the organ[194]. The neurovascular unit (NVU) plays a key role in maintaining the integrity of the BBB and is composed of neurons, endothelial cells, astrocytic endfeet, pericytes, blood vessels, and the basement membrane[195-199]. Given the complexity of stroke pathology, there is a pressing need for employing multitarget therapeutic approaches. Previous studies have revealed a dysfunction of the BBB to varying degrees in several neurodegenerative diseases, including stroke and Alzheimer's disease[195,200,201]. Such dysfunction is primarily driven by three factors: (1) Inflammation, a major contributor to leakage of the BBB, (2) Disruption of tight junction (TJ) structures, and (3) Imbalance in ion pump transport systems (Fig. 12)[202].

Immune cell trafficking is closely tied to the integrity of the BBB. Inflammatory responses triggered by IS lead to the activation of immune cells, including microglia and peripheral immune cells such as the T-cells and macrophages, which release cytokines and chemokines that exacerbate the breakdown of the BBB; this immune-mediated disruption further increases the permeability of the BBB, allowing the infiltration of potentially harmful

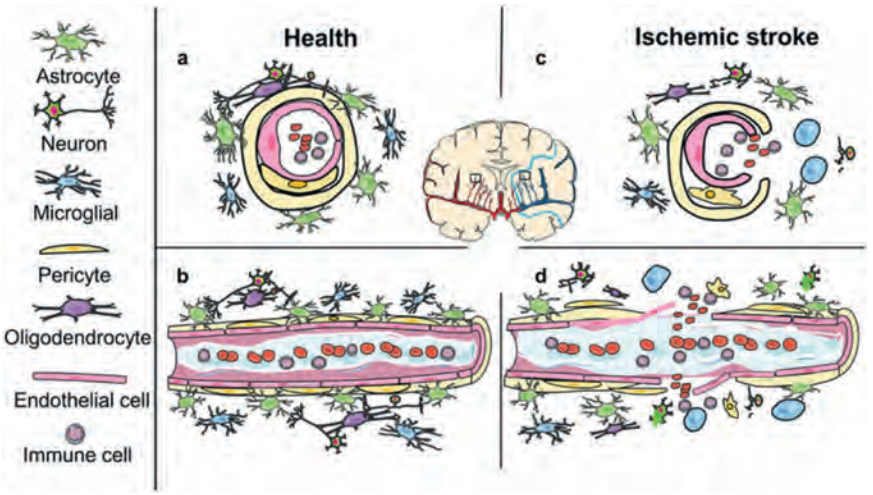

Figure 12 NVU in healthy brain and after IS[202]. Reprinted with the permission from Ref. 202. Copyright © 2023 Wiley-VCH GmbH.

substances into the CNS and worsening neuronal damage. Additionally, pro-inflammatory cytokines weaken the TJs of endothelial cells, further compromising the selective permeability of the BBB.

Herein, we summarize key therapeutic targets associated with the dysfunction of BBB, with particular emphasis on strategies aimed at mitigating immune-mediated damage. This is anticipated to provide useful insights into the repair of the BBB after stroke and delineate potential directions for the development of novel therapies for stroke in the future.

2.2.6.1. Inhibition of neuroinflammation. Inflammation is an important aspect of the physiology and pathology of stroke. The occurrence of AIS leads to the activation of microglia, the only immune cells in the brain, resulting in the release of ROS, pro-inflammatory cytokines (such as tumor necrosis factor-α[147], interleukin-6[202], and interleukin-17[203-206]), and matrix metalloproteinases (such as MMP-9). This results in the disruption of the BBB. The inhibition of TNF-α signaling has been demonstrated in preclinical studies to reduce infarct core volume in ischemic regions of the brain. However, certain studies have shown that activated microglia also play a beneficial role by removing cell debris, protecting ischemic neurons, and promoting nerve regeneration during ischemia; this opposing effect is attributable to the differential polarization of microglia[207].

Current research on the role of microglia in IS is mainly focused on two aspects; these include inhibiting the activation of microglia and regulating their polarization. AZD1390, an ataxia telangiectasia mutated (ATM) specific inhibitor, has been shown to suppressed NF-κB signaling pathway to alleviate ischemic brain injury in IS, and attenuated microglia activation and reduced the level of pro-inflammatory cytokines[208]. Toll-like receptor 4 (TLR4) is a type I transmembrane protein that is expressed on the surface of a variety of immune cells, including monocytes, macrophages, and regulatory T-cells. Previous studies have demonstrated that the activation of TLR4 can promote the polarization of microglia, increase the expression of pro-inflammatory factors, and aggravate neuronal damage. The activation of microglia leads to the production of IL-17 *via* the activation of the TLR2/IL-23/IL-17 signaling pathway. IL-17 is mainly produced by the $CD4^+$ T-cells and plays an important role in inducing neuroinflammation after IS[206,209]. TLR4 functions as a receptor for HMGB1 during IS and modulates the functions of various inflammatory mediators such as NF-κB *via* the HMGB1−TLR4 pathway to induce neuroinflammation[210]. Cerebrolysin (CBL) is a neuropeptide preparation with demonstrated neuroprotective effects in multiple clinical trials as well as in animal models. Guan et al.[211] found that the administration of CBL promotes CREB phosphorylation, increases the expression of peroxisome proliferator−activated receptor gamma coactivator-1α (PGC-1α), promotes the M2 polarization of microglia, and exerts anti-inflammatory effects. Moreover, the potential role of Polyphyllin I (PPI), a steroidal saponin, in cerebral ischemia has been elucidated. Research has demonstrated that PPI modulates microglial polarization towards an anti-inflammatory M2 phenotype in MCAO mice *in vivo*, as well as following OGD/R *in vitro*[212].

Similarly, the cGAS−STING pathway, as an integral component of innate immunity, plays a non-negligible role in neuroinflammation. Recently, numerous publications have outlined that excessive activation of the cGAS−STING pathway can exacerbate neuroinflammation in IS[50]. Kong et al.[213] employed a model of middle cerebral artery occlusion (MCAO) in C57BL/6 mice *in vivo* and the BV2 microglia OGD/R model *in vitro* to discover that STING is significantly upregulated in microglia and promotes their polarization towards the M1 phenotype. Administration of C-176, a small-molecule pharmacological inhibitor of STING, resulted in improvements in neurological function and cognitive impairments, as well as the alleviation of neuroinflammation. Therefore, targeting the cGAS−STING pathway emerges as a promising immunotherapeutic approach for the treatment of IS.

Sirtuin 5 (SIRT5) is highly expressed in microglia following an IS episode, further inducing neuroinflammation[214]. MC3482, an inhibitor of SIRT5, has demonstrated significant potential in the treatment of IS. Xia et al.[215] discovered that MC3482 can regulate microglial activity by enhancing the level of succinylation modification of Annexin-A1. Annexin-A1 is a protein with anti-inflammatory properties, and its succinylation may potentiate these anti-inflammatory functions, thereby reducing neural damage after an IS event.

As an important regulator of lymphocytes (such as adoptive transfer of regulatory T-cells (Tregs) and $\gamma\delta$ T cells). The Tregs continue to accumulate during IS and produce the anti-

inflammatory cytokine IL-10, promote neurogenesis, and reduce inflammation by activating the ERK/STAT3 pathway[216]. However, a few studies have shown that Tregs can aggravate ischemic brain damage[217]. The role of Tregs in stroke and the underlying mechanisms remain unclear and warrant further studies.

Ion transporters such as $Na^+/K^+-ATPase$ are chiefly responsible for ion transport across the BBB. The stimulation of $Na^+-K^+-Cl^-$ and Na^+/H^+ exchange during ischemia increases the influx of extracellular Na^+, leading to its accumulation in the cells, which induces brain edema. A correlation between brain edema and disruption of the BBB has been uncovered *via* MRI of stroke patients[218]. Brain edema is considered the main cause of pre-stroke. The destruction of the BBB after IS is closely associated with brain edema and hemorrhagic transformation. Aquaporin 4 (AQP4) is one of the major aquaporins in the endfeet of astrocytes and is closely associated with the occurrence of brain edema following stroke[219]. Recent studies have shown that AQP4 knockout can promote astrocyte autophagy by affecting Na^+/Ca^{2+} exchange, thereby reducing the release of inflammatory factors and protecting neurons[220]. A recent study by Wang et al.[221] revealed significant upregulation of MALAT1 (described previously in section 2.2.3.) in IS and its interaction with miR-145. The inhibition of MALAT1 downregulated AQP4 expression and protects ischemic neurons from injury.

In addition, activated astrocytes also mediate neuronal death *via* the production of free radicals such as NO, inactivation of NF-κB, and increased expression of iNOS. The activated astrocytes induce neutrophil infiltration into the brain parenchyma by releasing chemokines such as CCL2, CCL20, and CXCL10.

Neutrophils are the most potent source of MMP-9 that can induce neuroinflammatory responses, leading to the destruction of the BBB. Neutrophil infiltration into the ischemic region requires adhesion, which is orchestrated by adhesion molecules such as vascular cell adhesion protein 1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, and P-selectin. Concomitantly, the apoptotic cells in the ischemic region release damage-associated molecular patterns (DAMPs) that are recognized by pattern recognition receptors (PRRs)[222]. For instance, the TLRs and scavenger receptors (CD36) trigger inflammatory signal transduction and are involved in ischemic brain injury[223]. Previous studies in the rat model of permanent MCAO (pMCAO) have shown that the TLR2/4 signaling pathway may initiate the inflammatory cascade[224]. Therefore, the development of selective TLR2/4 antagonists and targeting the downstream signaling molecules represents a promising strategy for the treatment of IS and warrants further exploration.

In addition, the communication of the gut microbiota with the brain *via* the "gut−brain" axis during IS is associated with adverse outcomes[205]. The immune cells such as $CD4^+$ T-cells (that secrete IL-17A) and IL-17^+ $\gamma\delta$ T cells migrate into the brain, accelerate the infiltration of cytotoxic immune cells into the brain, promote the production of pro-inflammatory factors, and induce neuronal apoptosis[203]. The "gut−brain" and "oral−gut" axes have become the focus of increasing attention. Changes in intestinal flora may be closely associated with ischemic brain injury[203]. Sodium butyrate (NaB), a short-chain fatty acid produced by intestinal microorganisms, reduces infarct core volume and neuronal apoptosis by activating the GPR41−G$\beta\gamma$−P13K/Akt pathway[225]. The species *Lactobacillus helveticus* and *Lactobacillus brevis* of the microbiota are very closely associated with stroke treatment and can alleviate the accumulation of branched-chain amino acids, thereby affecting Akt/STAT3/NF-κB signaling and alleviating microglia-induced neuroinflammation[226]. Research on "gut−-brain" and "oral−gut" communication may provide important clues for the development of novel treatment strategies for IS in the future, including protection of the BBB, regulation of neurogenesis, and alleviation of adverse outcomes in stroke patients.

Adenosine and its receptors have long been recognized as potential therapeutic targets for IS. However, the complexity of their role is compounded by the fact that adenosine receptors are distributed variably throughout the brain, and their effects can be spatially or temporally opposed, leading to the possibility of off-target effects even when directly targeting specific adenosine receptors[227]. This "double-edged sword" makes adenosine receptors a potential and challenging target. While the administration of specific adenosine receptor agonists during early life may confer protective effects, the same drugs may exacerbate ischemic damage when administered later during a stroke[228]. Through strategies such as precision medicine, novel drug development, multi-target combination therapy, and in-depth investigation of mechanisms of action, we can harness this target more effectively to treat IS.

2.2.6.2. Inhibition of TJ proteins. TJ proteins are key junction proteins that are found in the tight junctions (TJs) between the microvascular endothelial cells of the brain; they include occludin, claudins, and junctional adhesion molecules. Of these, claudin-5 and occludin are the major transmembrane TJ proteins. The proteins zonula occludens-1/2/3 (ZO-1, ZO-2, and ZO-3) and cingulin function as scaffold proteins for TJs and regulate TJ function by binding cytoskeletal proteins such as actin[229]. During IS, TJ proteins undergo a series of modifications, translocations, and degradation that lead to increased permeability of the blood-brain barrier; this facilitates the entry of foreign materials across the BBB and results in damage to the brain. Therefore, TJ proteins play an important role in maintaining the integrity of the BBB[230]. Previous studies have shown that decreased modification of the TJ proteins as well as changes in the endothelial actin cytoskeleton can protect the BBB in patients with stroke[230]. Therefore, the modification of TJ proteins has also been proposed as a strategy for protecting the BBB. In addition, estrogen has been shown to improve ischemia-induced dysfunction of the BBB by reducing TJ disruption in the microvascular endothelial cells of the brain[231]. In addition, autophagy and ubiquitin−proteasome system (UPS) have also been implicated in the degradation of TJ proteins[231]. The expression of ubiquitin-specific protease 14 (USP14), a deubiquitinating enzyme (DUB) increases in the brain tissue during IS. *In vivo* studies carried out by Hou et al.[232] revealed that the administration of the USP14 inhibitor IU1 decreased the degradation of the TJ proteins and protected the integrity of the BBB (Fig. 13)[233].

2.2.6.3. Inhibition of c-Jun N-terminal kinase (JNK) signaling. There are three subtypes of JNKs. Of these, JNK1 and JNK2 are associated with apoptosis while JNK3 is mainly expressed in the nervous system. Previous studies have shown that mice with JNK3 knockout do not exhibit neuronal death arising from excitotoxicity[234]. The adipocyte fatty acid binding protein (A-FABP) is an adipokine that is mainly expressed in adipocytes, macrophages, and endothelial cells[235]. The concentration of A-FABP increases during IS, which protects the BBB by enhancing JNK/c-Jun signaling axis and inhibiting the expression

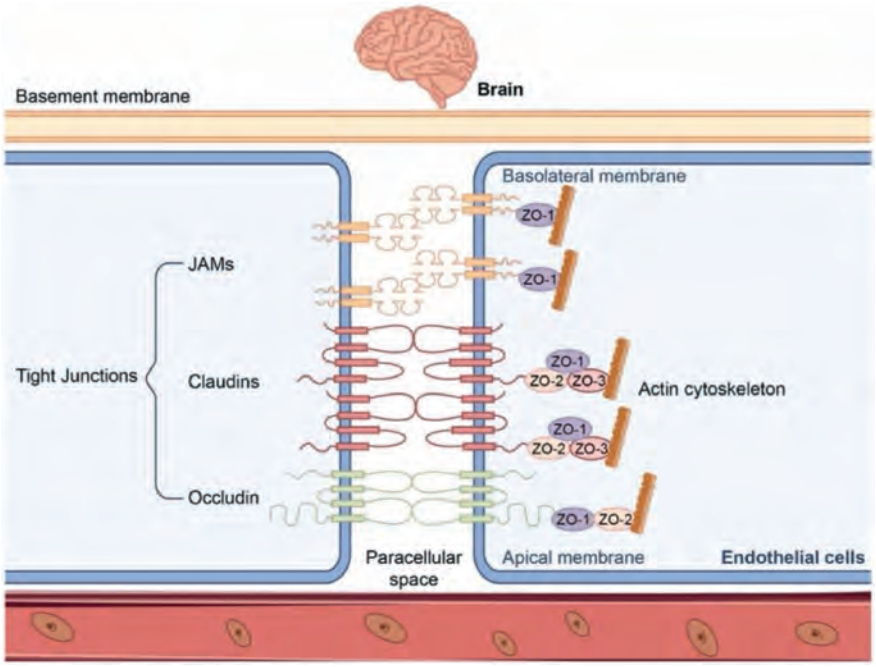

Figure 13 Structure and molecular composition of TJs[233]. With permission of the Creative Commons CC-BY-NC-ND.

of MMP-9, thereby mitigating adverse outcomes in stroke patients[236]. A-FABP−induced MMP-9 expression is dependent on the activity of the activator protein 1 (AP1). Mutations in two sites on AP1 (AP1a and 1b) can eliminate the A-FABP−mediated activation of the MMP-9 promoter, and targeted drugs can be developed for these two sites on AP-1. Liao et al.[237] recently reported that a monoclonal antibody 6H2, capable of blocking circulating A-FABP, significantly ameliorated IS-induced injury in mice with transient MCAO. The development of novel A-FABP inhibitors is another potential strategy for the treatment of cerebral ischemia.

2.2.6.4. Inhibition of protein kinase Cδ (PKCδ)/myristoylated alanine-rich C-kinase substrate (MARCKS)/myosin light chain (MLC) signaling. Like ubiquitination, neddylation is also an important posttranslational modification for proteins. The major ubiquitin-like protein neural precursor cell−expressed developmentally downregulated protein 8 (NEDD8) is expressed in neuronal precursor cells and plays a key role in the regulation of cerebral ischemia. Recent studies have shown that MLN4924, a small molecule inhibitor of NEDD8-activating enzyme E1 (NAE1), inhibits neddylation process and upregulates the PKCδ/MARCKS/MLC signaling pathway, which plays an important role in maintaining the integrity of the BBB, thereby mitigating adverse outcomes in stroke patients[238]. Thus, the inhibition of protein neddylation (involves covalent binding of NEDD8 protein to substrate protein) may be explored as a potential therapeutic strategy for IS. It is expected to uncover novel treatment avenues for Alzheimer's disease, atherosclerosis, cancer, and other diseases.

2.3. Recovery stage

The use of neuroprotective agents during the recovery stage of IS has been demonstrated to inhibit cell death in the ischemic penumbra and interrupt the cascade of neuronal death. This approach may reduce the limitations associated with the currently-employed vascular recanalization approach and improve patient outcomes. Consequently, scientific research has increasingly focused on neuroprotection and neurorepair as key strategies for intervention in IS. Neuronal damage represents the primary mechanism underlying the pathology of IS. Neurological recovery during the post-stroke period follows a nonlinear logarithmic pattern. The molecular signaling pathways involved in neuronal injury are the subject of intensive research. Processes such as synaptic remodeling, axonal regeneration, neuronal transformation, and nerve repair are critical for enhancing neuronal recovery in stroke patients[239]. Recent years have witnessed a growing interest in targeting non-neuronal cells such as astrocytes and microglia as potential therapeutic strategies for IS. Astrocytes, a major component of NVU and the most abundant cell type in the brain, are activated during IS and regulate neuronal activity by releasing various gliotransmitters[109]. Herein, we provide an overview of current neuroprotective targets and intervention strategies during the recovery phase of IS, highlighting potential areas for future research (Fig. 14)[240].

2.3.1. Nerve repair and regeneration

Previous studies have established that glial cells can improve synaptic plasticity and the release of gliotransmitters[241]. Newborn neurons exhibit a significant extent of plasticity. ATP, a crucial gliotransmitter, plays a highly dynamic role during the process of synaptic remodeling as well as synaptic transmission among neurons[242].

The current strategies for promoting the repair of nerves damaged due to IS focus on enhancing angiogenesis and vascular regeneration, axon regeneration, and cell therapy. Angiogenesis and vascular regeneration refer to the processes of generating new blood vessels to provide neurotrophic factors to the damaged neurons after stroke[243]. Several vascular growth factors such as vascular endothelial growth factor (VEGF), platelet-derived

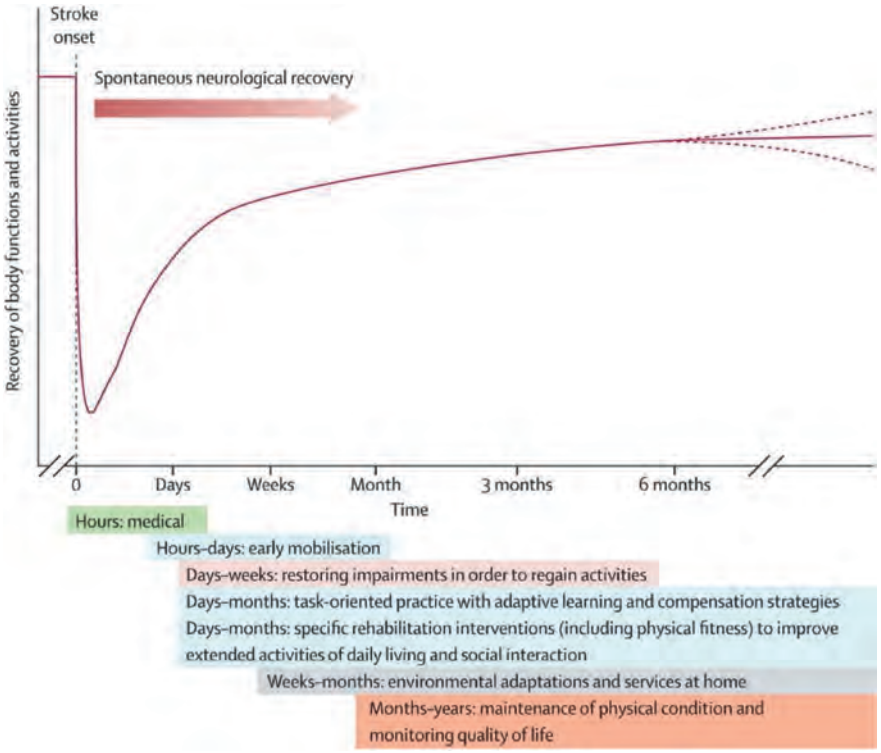

Figure 14 Hypothetical pattern of recovery after stroke, with the timing of intervention strategies[240]. Reprinted with the permission from Ref. 240. Copyright © 2011 Elsevier Ltd. All rights reserved.

growth factor, angiopoietin 1/2 (Ang1 and Ang2), transforming growth factor β (TGF-β), NOS, and EPO can stimulate the formation of blood vessels. Angiogenesis is a multistep process that occurs after cerebral ischemia and involves endothelial cell proliferation, migration, angiogenic sprouting and lumen formation, as well as endothelial network maturation (Fig. 15)[244]. A ginkgolide extract XQ-1H has exhibited significant benefits in promoting cell proliferation and angiogenesis under *in vitro* and *in vivo* conditions[245]. In addition, XQ-1H exerts a neuroprotective effect by regulating microglial polarization *via* the peroxisome proliferator−activated receptor γ (PPARγ) pathway[246]. Ginkgolide B (GB) promotes angiogenesis in IS and has been extensively studied. GB binds creatine kinase B (CKB) and inhibits its enzymatic activity, thereby restoring cerebral blood flow and reconstructing blood vessels after IS by modulating the CCT/TRIC-SK1 axis[247].

2.3.2. Inhibition of cannabinoid receptor (CB1R) signaling

CB1R is a G protein-coupled receptor that is highly prevalent in the CNS[248]. The increased influx of Ca^{2+} ions into the neurons in the early stages of stroke leads to an increased release of the postsynaptic endocannabinoids (eCBs). The eCBs then regulate the activity of presynaptic CB1R, ultimately inhibiting the release of neurotransmitters[249]. However, eCBs have the opposite effect in glial cells and enhance synaptic functions in health and disease[250]. The signaling pathways that are activated downstream of eCB−CB1R binding need further investigation. Promoting the release of astrocyte neurotransmitters by increasing the release of postsynaptic eCBs holds promise as a therapeutic strategy for IS.

2.3.3. Inhibition of Notch1 signaling

Mesenchymal stem cells (MSCs) are multipotent stem cells that occur throughout the body and can differentiate into multiple cell types. Among these, the bone marrow- and adipose tissue-derived MSCs (BM-MSC and AT-MSC, respectively) exhibit the highest potential for neural differentiation, making these cells the primary focus of research[251]. Previous studies have shown that fibroblasts can be converted into neurons through the activation of specific transcription factors, underscoring the potential for reprogramming non-neuronal cells into neurons. Recent studies have demonstrated that astrocytes can also be reprogrammed into neurons[252,253]. Achaete-scute homolog 1 (Ascl1) and neuropoietin-2 are crucial transcription factors that facilitate the transformation of astrocytes into neurons. This transformation has been found to rely on the Notch1 signaling pathway and can be accomplished by suppressing the activity of Notch1 or using small-molecule inhibitors of Notch1 such as DAPT[252].

The Notch pathway is a highly conserved signaling pathway that facilitates intercellular communication. Notch, a receptor that functions downstream of HIF in the signaling pathway, is activated upon the binding of specific ligands and relies on proteolytic cleavage by specific enzymes for signal transmission[254]. Delta-like 4 (Dll4) is expressed upon the activation of VEGF signaling[255,256]. Dll4 can activate Notch1, leading to the cleavage and release of its notch intracellular domain (NICD) *via* the activity of γ-secretase and subsequent translocation of the NICD into the nucleus[257]. In the nucleus, NICD binds to the transcription factor CBF1/Suppressor of Hairless/Lag-1 (CSL), promoting the expression of downstream target genes such as Hes-1, Hes-3, Hes-5, and Hey-1, thereby promoting angiogenesis and neuronal

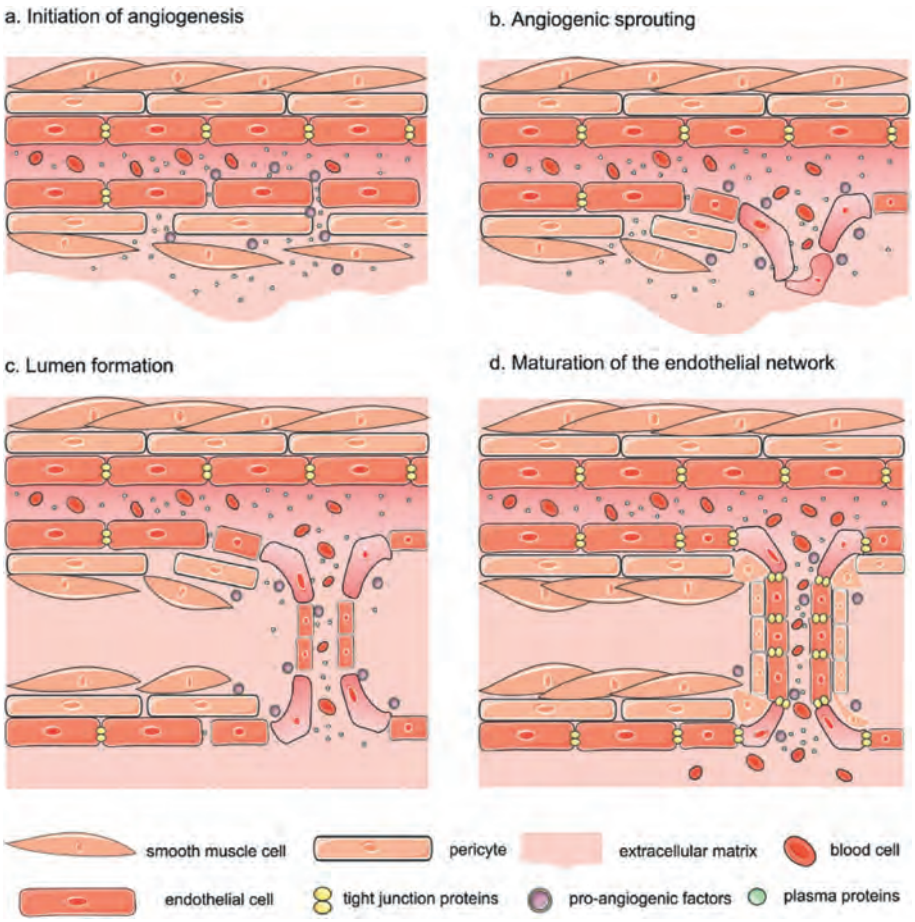

Figure 15 Schematics of key steps in angiogenesis after IS[244]. Reprinted with the permission from Ref. 244. Copyright © 2023. Under exclusive licence to Shanghai Institute of Materia Medica, Chinese Academy of Sciences and Chinese Pharmacological Society.

differentiation[258]. Recent studies have shown that the administration of *Danhong* injection (DHI) along with tPA can activate Notch-1 and its downstream target Hes-1; this reduces the expression of Dll-4, promotes the expression of VEGF, stimulates angiogenesis, and enhances vascular repair after stroke[259].

2.3.4. Inhibition of VEGF/VEGF receptor (VEGFR) signaling

The formation of new blood vessels after stroke can help compensate for occluded vessels, restore cerebral blood flow, and rescue neurons in the ischemic penumbra region[260]. These aspects are crucial for stroke patients. HIF-1α is a regulator of oxygen homeostasis and is activated under conditions of hypoxia/ischemia. Moreover, it translocates to the nucleus and promotes angiogenesis by increasing the expression of VEGF[261]. VEGF is an important pro-angiogenic factor. VEGF has contextual effects that can be both beneficial and deleterious according to dosage and timing[262]. In the acute phase (0−24 h after stroke), intrinsic upregulation of VEGF may exacerbate tissue damage by inducing BBB disruption, promoting vascular leakage of inflammatory factors, and contributing to post-stroke brain edema, as well as life-threatening intracranial hypertension[263]. In the late phase (48 h after stroke), low-dose VEGF is generally recognized for its neuroprotective effects, which include facilitating collateral vessel formation, promoting vasodilation, enhancing angiogenesis, and supporting neurogenesis[264]. A study using the MCAO mouse model revealed significant increase in the number of new blood vessels in the ischemic penumbra within 2−3 days of vascular occlusion[265]. VEGF promotes the proliferation of endothelial cells *via* its interaction with VEGFR-1/2[266]. In addition, VEGF can activate the Flk-1 receptor *via* the MAPK pathway, promoting axonal growth and enhancing neuronal plasticity[267,268]. Placental growth factor (PLGF) is a member of the VEGF family and is widely expressed in neurons; it can enhance the pro-angiogenic effects of VEGF by activating VEGFR-1[269]. *In vitro* studies have shown a significant increase in the expression of PLGF in mice following MCAO, reaching a peak after three days[270]. These studies suggest that an increased expression of VEGF may provide neuroprotective benefits in patients with stroke. Angiogenesis is commonly considered an important aspect of collateral circulation and plays an important role in IS caused by large vessel occlusion[271]. The expression of plasma factor VII activating protease (FSAP) significantly increases following stroke[272]. High-molecular weight hyaluronic acid (HMW-HA) functions as an inhibitor of FSAP and can activate the Wnt5a signaling pathway (Fig. 16)[272]. The activation of this pathway promotes the expression of VEGFR and VEGF, leading to effective angiogenesis and neuroprotection[273]. FSAP has been suggested as a potential biomarker for the clinical diagnosis and monitoring of stroke. HMW-HA, as a potential neuroprotective agent, plays a crucial role in improving outcomes in patients with IS caused by

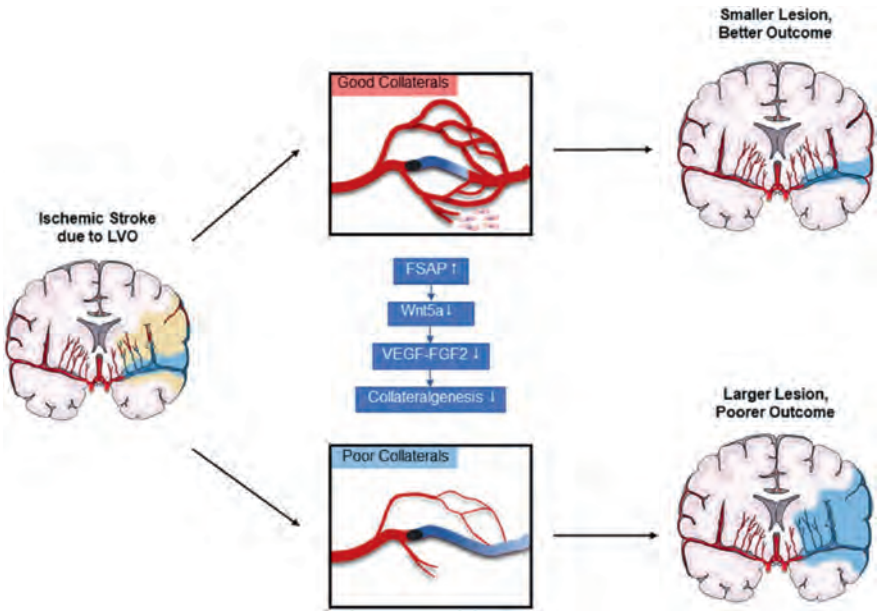

Figure 16 Role of FSAP in angiogenesis following IS[272]. With permission of the Creative Commons CC-BY.

large vessel occlusion. Moreover, it helps improve endothelial dysfunction, reduce infarct core volume, improve microcirculation, and protect the BBB.

2.3.5. Activation of eNOS/NO/cyclic guanosine monophosphate (cGMP)/cGMP-dependent protein kinase 1 (PKG1) signaling

As mentioned previously, the three major subtypes of NOS include nNOS, eNOS, and iNOS. Studies carried out in animal models have shown that supplementation with L-arginine enhance eNOS expression and promotes blood vessel formation at the ischemic site[274]. NO produced by the activity of eNOS increases the intracellular concentration of cGMP by activating soluble guanylyl cyclase (sGC) in smooth muscle cells, thereby activating PKG1 and relaxing the blood vessels[274]. NO is an important regulator of vascular angiogenesis and participates in various stages of the process[275]. NO donors such as sodium nitroprusside can promote endothelial cell proliferation and migration under *in vivo* conditions, but the effect was abolished upon pretreatment with the NO inhibitor L-NAME[275,276].

2.3.6. Inhibition of Rho/Rho-associated protein kinase (ROCK) signaling

Rho and ROCK are members of the Rho family of GTPases and a family of serine/threonine kinases, respectively. ROCK1 and ROCK2 are the two isoforms of ROCK. Of these, ROCK 2 is mainly expressed in the brain and skeletal muscle[277]. Numerous studies have found that the Rho/ROCK pathway is associated with the physiological and pathological process of IS. In the animal model of MCAO, ROCK is activated by Rho in the ischemic region, which then restrain the PI3K/Akt pathway and causes neuronal apoptosis. Sonic hedgehog (Shh) is secreted by astrocytes during IS[278]. Shh is a soluble protein that can upregulate vascular growth factors and mediate angiogenesis by inhibiting the RhoA/ROCK signaling pathway, suggesting that ROCK is a potential therapeutic target for stroke.

2.3.7. Others

Stem cell therapy is a novel therapeutic approach that has proven extremely beneficial for nerve repair after stroke. Bone marrow MSCs (BMSCs) are a type of stem cells that exhibit high differentiation potential; they can differentiate into neuronal cells and play an important role in promoting angiogenesis, neurogenesis, and vascular repair[279]. Synergistic effects are anticipated upon the administration of BMSCs along with drugs that promote angiogenesis and cell proliferation. Other studies have shown that the inhibition of Notch1 signaling plays a key role in the reprogramming of activated astrocytes into neurons; the pericytes can also differentiate into cells of neural lineage during ischemia[252].

Exosomes are currently emerging as a rising star in IS therapy and play an indispensable role in nerve repair by regulating a variety of intercellular signaling pathways after IS. Exosomes typically transport miRNAs to recipient cells. The mesenchymal stem cell−derived exosomes (MSCs-Exo) in particular contribute to improved prognosis of stroke patients by promoting angiogenesis, vascular regeneration, and neurogenesis by transferring miR-21-5p to target cells[280]. In addition, Ding et al.[281] found that the administration of berberine inhibited neuroinflammation *via* the miR-182-5p/Rac1 axis during cerebral ischemia.

2.4. Sequelae stage

As IS progresses to the recovery stage, some patients may experience the sequelae stage. Comprehensive nursing interventions are primarily adopted for this stage, and specific drugs for treatment are not available at present. Moreover, specific symptoms in different patients are treatable with antiplatelet drugs or lipid-lowering medications. In addition, studies have shown that exercise can improve the neuroprotective effects exerted by glial cells[282]. BDNF is a widely-distributed neurotrophin that acts in conjunction with insulin-like growth factor-I (IGF-I) and nerve growth factor to promote neurogenesis after physical activity/

exercise[283]. Therefore, appropriate physical activity is potentially an additional treatment for neuroprotection during the recovery and sequelae stages of stroke.

2.5. *Multiple temporal progression*

2.5.1. *Regulation of circadian rhythm*

The circadian rhythm is widely involved in various pathological process influenced by changes in the time dimension. The circadian rhythm is regulated by circadian clocks whose main components are primarily located in the suprachiasmatic nucleus (SCN) of the hypothalamus, also known as the "pacemaker"[284]. The SCN includes molecular clocks that operate within each neuron and other types of brain cells. The circadian rhythm is regulated by the transcription and translation of specific genes in parallel with the day−night cycle. These genes are present in various tissues and cells, including the neurons and astrocytes[285]. The changes in the pattern of time dimension and the expression of the "clock" genes ultimately affect the outcome of stroke. The molecular and cellular basis of the circadian rhythm "clock" involves a transcription−translation feedback loop mechanism activated by the Bmal1-Clock activator[286]. The activator can bind E-box sequences and regulate the transcription of various genes, including circadian rhythm inhibitors (Period [Per 1−3]), cryptochromes (Cry 1/2), and Rev−Erb, which is a transcriptional target of Bmal1 and can inhibit the transcription of "clock" genes (Bmal1-Clock)[287].

Normobaric hyperoxia, the free-radical scavenger α-phenyl-butyl-*tert*-nitrone (αPBN), and the NMDA antagonist MK801 were found to reduce the infarct core volume in rodent (mice and rat) models of focal cerebral ischemia during the day (inactive period) but failed to exert any such effect during the night (active period)[288]. Another study revealed that disrupting the circadian rhythm by phase advance significantly increased the infarct size, impaired vasculogenesis, and reduced the recovery of neurological functions in mice subjected to MCAO-induced IS compared to those in the control group[289,290]. Furthermore, the incidence of stroke typically occurs in the morning after the patients awaken, while stroke severity and adverse outcomes worsen at midnight[291]. A nationwide multicenter observational cohort study in Korea evaluated the influence of circadian rhythm on stroke severity; the results revealed that strokes that occurred during the night exhibited greater neurological severity and worse three-month functional outcomes compared to those that occurred during the day[292]. These findings confirm the correlation between disruptions in endogenous circadian rhythms and infarct core volume as well as prognosis in IS, underscoring the fact that circadian rhythms control multiple aspects of vasculogenesis; in fact, the "clock" genes (especially Cry1/2) regulate different stages of vasculogenesis[293]. The main focus of treatment in the hyperacute and acute stages of ischemia is the reopening the blocked blood vessels, while the focus in the chronic recovery stage of ischemia is on the formation of new blood vessels[294]. Neovascularization in the ischemic penumbra and surrounding vessels is an important mechanism for recovery after IS as it can improve local blood flow by rebuilding collateral circulation, increase cerebral blood flow, and reshape synapses to promote neural repair[295]. Several neuroprotective agents are yet to be successfully transitioned into clinical application. We are left to ponder whether this lack of success evidenced in *in vitro* animal models and mismatched clinical populations is influenced by the circadian rhythm in addition to other factors such as age and hypertension. Reassessing the underlying mechanisms of IS and the numerous therapeutic targets in rodent models with appropriate circadian backgrounds may enhance the successful transition of neuroprotective agents into clinical application (Fig. 17).

2.5.2. *Activation of NO/cGMP/PKG/G-substrate (GS)/protein phosphatase 2A (PP2A) signaling*

Previous studies have shown that late-night light stimulation can promote the secretion of glutamate. Glutamate is the main excitatory neurotransmitter secreted at the synaptic terminals of the retinohypothalamic tract (RHT) and is associated with light-dependent circadian rhythms[296]. Increased secretion of glutamate resulting from the activation of NMDAR in SCN neurons leads to an increase in the intracellular concentration of Ca^{2+} ions,

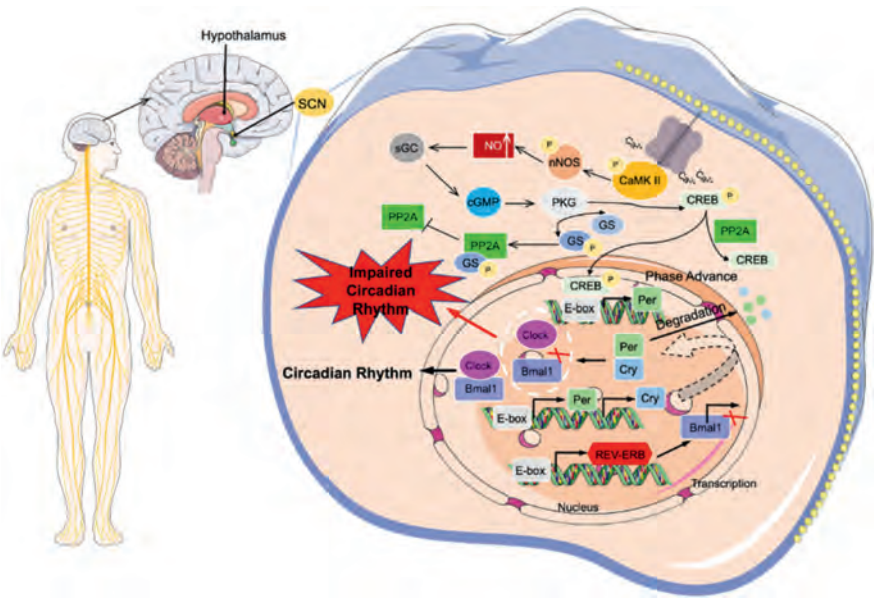

Figure 17 Molecular mechanisms of circadian clocks involved in IS.

resulting in the activation of various enzymatic cascade reactions mediated by CamKII, nNOS, and sGC to generate cGMP and NO[297]. Phosphorylated CaMKII can increase the activity and phosphorylation of nNOS, thereby increasing NO concentration. Endogenous NO is an important regulator of the biological clock and circadian rhythms; it increases the concentration of cGMP by activating sGC, subsequently enhancing the activity of PKGs and phosphorylation of CREB at Ser142[296,298]. A recent study reported that GS is a novel substrate for PKG and is phosphorylated by the enzyme[299]. Phosphorylated GS (p-GS) interacts with PP2A to inhibit its activity. Phosphorylated CREB (p-CREB) translocates to the nucleus and interacts with E-box sequences, leading to increased transcription of Per 1/2, ultimately resetting the biological clock[300]. Additionally, PP2A has been identified as the main phosphatase mediating the dephosphorylation of p-CREB.

2.5.3. Inhibition of VEGF/Notch signaling

VEGF/Notch−mediated formation of the vascular endothelium is closely associated with Bmal1 activity. According to the environmental-circadian disruption model, overexpression of the core "clock" gene Bmal1 has a positive impact on vascular formation and wound healing[290]. This suggests that Bmal1 is a potential intervention target for vascular regeneration following ischemia/reperfusion injury and functional recovery after stroke.

3. Summary of marketed drugs

The development of drugs for the treatment of IS has long been a major focus of medical research. However, very few drugs have been demonstrated to exert precise therapeutic effects in stroke patients. Here, we summarize the currently marketed drugs for IS, aiming to provide research ideas for the design and development of new anti-ischemic stroke drugs in the future.

Alteplase (rt-PA, **1**) is the only drug that is currently approved by the FDA for the treatment of AIS. It exhibits significant thrombolytic effects but its clinical application is limited due to the short treatment window and various adverse reactions. Tenecteplase (TNK, **2**) is a genetically-engineered modified rt-PA that demonstrates higher safety and thrombolytic efficacy; moreover, only a single dose administered *via* the intravenous route is sufficient for IS therapy. Currently approved by the FDA for thrombolytic therapy, TNK features amino acid substitutions at positions 103, 117, and 296−299 of rt-PA and is applied for the treatment of different types of thrombotic diseases, including IS (Fig. 18)[301]. Urokinase is a traditional thrombolytic drug marketed in China for several years. The drug is widely applied for acute myocardial infarction, cerebral thrombosis, cardiac embolism, and other cardiovascular diseases.

Edaravone (**3**), an antioxidant and free-radical scavenger, was first marketed in Japan by Mitsubishi Tanabe Pharmaceuticals in 2001 and has been widely utilized for the treatment of AIS. Studies have shown that **3** can alleviate the increase in hydroxyl radicals (·OH) in the brain and elevate the concentration of hyperoxide radicals ($O_2 \cdot^-$) in animal models of ischemia. The intravenous administration of **3** following cerebral ischemia or ischemia/reperfusion inhibits further expansion of the infarct core in the ischemic area, prevents oxidative damage to nerve and vascular endothelial cells, and protects ischemia-damaged neurons while improving neurological symptoms[302]. Additionally, **3** exhibits antiapoptotic, anti-necrotic, and anti-inflammatory effects by targeting various intracellular signaling pathways, inhibiting the release of pro-inflammatory cytokines, and reducing the infiltration of inflammatory cells into the brain. Edaravone dexborneol (**4**) is a novel neuroprotective agent consisting of edaravone and (+)-borneol; it was approved for the treatment of AIS in China in 2020 and offers superior efficacy while maintaining a safety profile comparable to that of edaravone (Supporting Information Fig. S1)[303].

3-*n*-Butylphthalide (NBP, **5**) was approved for use as an anti-IS drug by the China Food and Drug Administration in 2002 (Supporting Information Fig. S2). The potential mechanism underlying the action of **4** in the treatment of IS may involve the targeting of various pathophysiological processes. A few studies have shown that **5** can scavenge free radicals, exert antiplatelet aggregant, antithrombotic, and antiapoptotic effects, protect mitochondrial function, and improve cerebral microcirculation and neuroprotection[304]. **5** not only improves the symptoms of IS but also contributes to long-term neurological recovery. However, the administration of a single drug for IS therapy is not an ideal scenario. Currently, **5** is often used clinically in combination with other drugs or **5** derivatives (including donepezil, clopidogrel, edaravone and aspirin) to enhance its therapeutic effects.

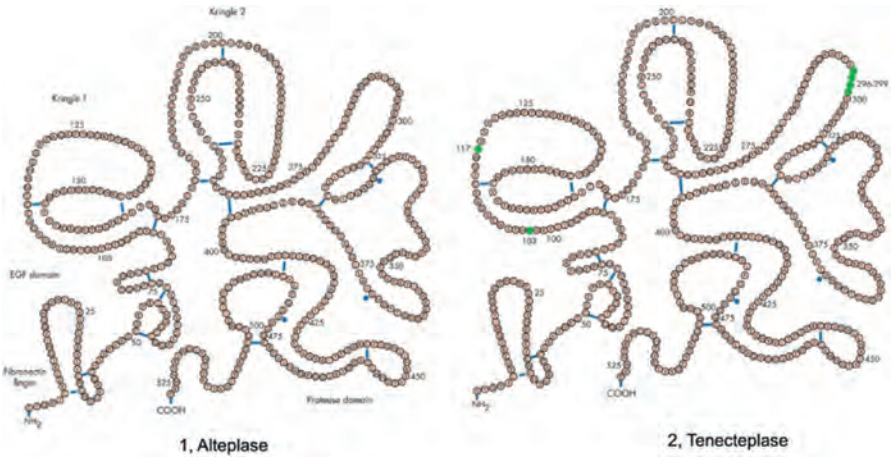

Figure 18 Structure of rt-PA and TNK, the currently-approved drugs for IS[301]. Reprinted with the permission from Ref. 301. Copyright © 2003. Published by BMJ Publishing Group Ltd.

Cinepazide maleate (**6**), developed and marketed by Sanofi-Aventis in France in 1974, was approved for the treatment of IS and marketed in China in 2002 (Supporting Information Fig. S3). Many patients with IS have benefited from the use of this drug. As a weak calcium channel blocker and vasodilator, **6** can prevent the entry of Ca^{2+} ions into vascular smooth muscle cells, thereby relaxing vascular smooth muscles and effectively improving blood supply in ischemic areas. In addition, **6** can also inhibit cAMP phosphodiesterase to improve microcirculation.

4. Summary of investigational drugs

Most of the drugs that are currently being investigated for IS therapy are interventions that can be applied for the acute and recovery stages. The pathological mechanisms underlying neuroinflammation in the acute stage are quite intricate, prompting in-depth investigations of this aspect. While the recovery stage occurs later during the temporal progression of stroke, it remains an essential aspect of treatment. Recent investigations have therefore been focused on interventions for this stage as well. A limited selection of thrombolytic medications for the acute stage is under investigation, underscoring the significant potential for further research in this field. In contrast, specific therapeutic drugs for the sequelae stage are not being developed at present.

A few drugs have exhibited strong therapeutic effects on cerebral ischemia under *in vivo* and *in vitro* conditions as well as in clinical trials. Certain drugs with anti-excitotoxic, antioxidant/nitrosative, and anti-inflammatory activities, hormones, monoclonal antibodies, stem cell−based therapies, and natural medicines have been extensively studied (Table 1). Of these, drugs such as verapamil, SY-007, NA-1, JPI-289, SP-8203, DDFPe, minocycline, and clopidogrel have shown efficacy in clinical trials, improving the prognosis of stroke patients and providing new impetus for the development of neuroprotective agents in the future.

Table 1 Representative clinical trials for AIS that have been completed as of 7/31/2024.

Trial number	Clinical phase	Stage of IS	Proposed molecular mechanism(s)	Intervention	Sample size
NCT01422616	III	Hyperacute	Thrombolytic	rt-PA	4700
NCT01387113	II	Hyperacute	Thrombolytic	Alteplase	48
NCT05752916	IV	Hyperacute	Thrombolytic	rhTNK-tPA	568
NCT02133521	II/III	Hyperacute	Thrombolytic	DLBS1033	160
NCT00630396	I/II	Hyperacute	Thrombolytic	Minocycline	60
NCT02869009	I	Hyperacute	Thrombolytic	Clopidogrel/aspirin	3000
NCT02776540	IV	Hyperacute	Thrombolytic	Clopidogrel	201
NCT02586233	I/II	Hyperacute	Thrombolytic	DS-1040b	96
NCT02478294	IV	Hyperacute	Thrombolytic	Anticoagulant/warfarin	474
NCT00859885	IV	Hyperacute	Thrombolytic	Aspirin/clopidogrel	427
NCT02912663	I	Acute	Anti-excitotoxic	Verapamil	10
NCT01220622	IV	Acute	Anti-excitotoxic	Nimodipine	656
NCT02535611	III	Acute	Anti-excitotoxic	Memantine	47
NCT02258204	I/II	Acute	Anti-excitotoxic	Ketamine	50
NCT02767999	IV	Acute	Anti-excitotoxic	Fluoxetine	60
NCT02683213	III	Acute	Anti-excitotoxic	Fluoxetine	1500
NCT02831088	II	Acute	Anti-excitotoxic	Neu2000	210
NCT05041010	III	Acute	Anti-excitotoxic	Salfaprodil	165
NCT04111523	I	Acute	Anti-excitotoxic	SY-007	78
CTR20231033	II	Acute	Anti-excitotoxic	ZL006	−
NCT02930018	III	Acute	Anti-excitotoxic	NA-1	1105
NCT02396069	I	Acute	Antioxidant	JPI-289	24
NCT03062397	II	Acute	Antioxidant	JPI-289	110
NCT00061022	II	Acute	Antioxidant	NXY-059	3306
NCT00860366	III	Acute	Anti-nitrosative	Uric acid	421
NCT00200356	IV	Acute	Antioxidant	Edaravone	401
NCT03320018	II/III	Acute	Antioxidant	Hydrogen/Minocycline	15
NCT02002390	II	Acute	Anti-neuroinflammatory	Fingolimod	87
NCT04479449	II	Acute	Anti-neuroinflammatory	SP-8203	178
NCT05200728	I	Acute	Anti-neuroinflammatory	AER271	68
NCT00639249	II	Acute	Anti-neuroinflammatory	SA4503	60
NCT05439356	III	Acute	Anti-neuroinflammatory	Colchicine	8343
NCT06388772	I	Acute	Anti-neuroinflammatory	QHRD106	74
NCT02963376	I	Acute	Oxygen transporter	DDFPe	24
NCT03090113	IV	Acute	Multiple mechanisms	Shuxuetong injection	2416
NCT02178657	II	Recovery	Cell-based therapy	Autologous bone marrow mononuclear cell	76
NCT01678534	II	Recovery	Cell-based therapy	Allogenic mesenchymal stem cells	20
NCT02176395	IV	Acute and recovery	Multiple mechanisms	DanHong injection	46

"−": Not reported.

As stated previously, given that the hyperacute and sequelae stages of IS do not form the priority for therapeutic targeting, our review focuses on the investigational anti-IS drugs that confer partial neuroprotection in the acute and recovery stages, aiming to present novel research ideas for the design and development of anti-IS drugs in the future.

4.1. Acute stage intervention

4.1.1. Inhibition of excitotoxicity

Glutamate-mediated excitotoxicity is one of the important mechanisms of IS and involves a variety of enzymes, receptors, and ion channels. Herein, we review *Calcium channel blockers* (CCBs), NMDAR antagonists, PTEN inhibitors, and drugs affecting nNOS−PSD95 interaction.

4.1.1.1. Calcium channel blockers. Verapamil (**7**) is a calcium channel blocker that reduces excitotoxicity by inhibiting the influx of Ca^{2+} ions (Supporting Information Fig. S4). It is an FDA-approved drug that dilates constricted cerebral vessels after thrombectomy, reduces the infarct volume, improves neurobehavioral outcomes, and exerts neuroprotective effects in mice models for MCAO[305]. A phase I clinical trial (NCT02235558) found that the administration of **7** after endovascular thrombectomy was a safe and feasible adjunct to intra-arterial thrombolysis. In addition, the intra-arterial administration of **7** in conjunction with magnesium sulfate treatment has also been validated in preclinical animal models of IS as an effective therapy following thrombectomy and is capable of significantly reducing the mean infarct volume. Currently, a phase I human clinical trial (NCT02912663) has shown that the combined administration of **7** and magnesium sulfate after endovascular thrombectomy is beneficial for improving intracranial hemorrhage.

Nimodipine (**8**), a fat-soluble dihydropyridine calcium channel blocker, was marketed by Bayer in 1985 (Fig. S4). It can effectively cross the BBB and selectively act on cerebral vessels and neurons, especially cerebral capillaries. Thus, **8** represents a novel approach to the treatment of ischemic cerebrovascular disease, and its potential clinical benefits can be easily envisaged[306]. Early-phase clinical trials have revealed that **8** reduces the infarct volume within 24 h of stroke onset and improves cerebral blood flow to the ischemic area. However, several recent large-scale clinical trials have shown that **8** does not exert beneficial effects in IS. For example, a double-blind, placebo-controlled, randomized trial (NCT01220622) revealed that the administration of **8** within seven days of AIS did not result in improved cognitive functions over those of the placebo-treated group, suggesting that **8** does not prevent functional decline in cases with mild cognitive impairment; nevertheless, it may exert effects on specific cognitive domains. The potential benefits associated with the application of **8** have been speculated to result from the lowering of blood pressure (BP), which is associated with poor prognosis for patients with stroke. The Intravenous Nimodipine West European Stroke Trial also confirmed the association between **8**-induced drop in BP and adverse outcomes in acute stroke[307]. Therefore, the administration of **8** in conjunction with vasodilators may prove to be far more beneficial for patients with stroke than treatment with just **8**.

4.1.1.2. NMDAR antagonists. Memantine (**9**), an NMDA receptor antagonist, selectively blocks the NMDA receptor *via* noncompetitive binding to prevent its overactivation (Supporting Information Fig. S5). Numerous *in vitro* and *in vivo* studies have shown that **9** exerts neuroprotective effects. In the rat model for MCAO and reperfusion, **9** was found to significantly reduce the infarct volume, reduce apoptosis, and improve neurological score[308]. In addition, **9** protected primary hippocampal neurons from death induced by ATP depletion[110]. A phase III clinical trial (NCT02535611) evaluating the impact of memantine treatment on the outcome of IS has been completed. Further clinical trials are warranted to evaluate the potential application of **9** for improving outcomes in stroke patients.

Ketamine (**10**), a lipid-soluble enantiomeric derivative of phencyclidine, is commonly used as an anesthetic and analgesic agent (Fig. S5). Numerous preclinical studies have found that **10** can rescue injured neurons, increase cerebral blood flow, upregulate dendritic spine density, and exert neuroprotective effects in various models of nerve injury[309]. Therefore, this old, cheap drug needs to be evaluated in a new light for the purpose of repurposing. An ongoing phase II/III clinical trial (NCT02636218) is focused on exploring the capacity of the anesthetic **10** to improve outcomes and reduce neuronal damage in patients with neurological deficit. Gavestinel (**11**) and dizocilpine (MK-801, **12**) have been shown to exert neuroprotective effects in different animal models of stroke[310]; however, they are yet to receive approval for clinical trials, which may be attributable to the narrow therapeutic window and the failure to render emergency medical care for stroke patients in time. Ifenprodil (**13**) and its derivative Eliprodil (**14**) are selective inhibitors of GluN2B and represent a class of drugs with great anti-ischemic potential[311]. In addition, fluoxetine (**15**), an antidepressant, can also selectively block GluN2B and has been recently found to exert potential neuroprotective effects after stroke[312].

Nelonemdaz (Neu2000, **16**) and salfaprodil (**17**) are multitarget drugs that can block NMDAR-mediated excitotoxicity and combat oxidative stress, thereby exhibiting dual neuroprotective effects (Fig. S5). In animal models of cerebral ischemia, **16** and **17** exhibited better efficacy and extended therapeutic window than those of NMDA antagonists or antioxidants[313]. A phase II, double-blind, randomized, placebo-controlled, multicenter study (SONIC trial, NCT02831088) has been completed and the endpoints were met satisfactorily. An overall reduction in mortality over a 12-week duration were observed in the groups treated with **16** compared to those treated with placebo, while adverse events were not observed[314].

4.1.1.3. PTEN inhibitors. **SY-007** (KEIVSRNKRRYQEDGRKKRRQRRR, **18**) is an interfering peptide that can interact with NMDARs, trigger the nuclear translocation of PTEN, and cause neuronal death. Preclinical studies have shown that **18** exerts significant neuroprotective effects on ischemia-injured neurons under both *in vitro* and *in vivo* conditions[315]. Given that **18** is a potential neuroprotective drug for the treatment of IS, a phase I clinical trial (NCT04111523) was first conducted in 2022 to evaluate its safety, tolerability, and pharmacokinetics in humans; the results show that the intravenous administration of **18** at a dosage of less than 60 mg/kg appears to be safe and well tolerated in patients with IS[315].

4.1.1.4. Small-molecule inhibitors targeting nNOS−PSD95 interaction. 4-([3,5-Dichloro-2-hydroxybenzyl) amino)-2-hydroxybenzoic acid (**ZL006**, **19**) is a selective small-molecule inhibitor that targets the interaction between nNOS and PSD95 but does not inhibit NMDAR function (Supporting Information

Fig. S6). **19** not only exerts neuroprotective activity under *in vitro* conditions but also significantly reduces the infarct size and improves neurological deficit in mice and rats models of MCAO and reperfusion in a dose-dependent manner[96]. The compound **19** is currently being evaluated in an ongoing phase II clinical trial (CTR20231033) and appears, so far, to be a potential candidate small-molecule drug for the treatment of cerebral ischemia. However, its rapid metabolism and low permeability across the BBB limit its application. Therefore, Chen et al.[316] designed and synthesized six analogs of **19** and investigated their metabolism. The results revealed that the permeability of ester derivatives of **19** (**19-1**, **19-2**, and **19-3**) across the BBB was better than that of **19**. Of these derivatives, **19-3** is a prodrug of **19** that exhibits great potential for the treatment of cerebral ischemia.

Nerinetide (NA-1, Tat-NR2B-9c, **20**) is a peptide consisting of 20 amino acids (YGRKKRRQRRR-KLSSIESDV) that exhibits the capacity to penetrate cell membranes and disrupt the interaction of NMDAR with PSD95; it inhibits the binding of NR2A and NR2B with PSD95 with IC_{50} concentrations of 0.5 and 8 μmol/L, respectively. Therefore, **20** can inhibit excitotoxic signals transmitted by neurons and alleviate brain damage. A phase III trial (NCT02930018) further evaluated the safety and efficacy of **20**; however, beneficial effects of the administration of **20** in combination with rt-PA were not evident compared to placebo treatment. In contrast, patients who received **20** alone and not rt-PA exhibited significantly improved functional outcomes.

4.1.2. Inhibition of oxidative and nitrosative stress

PARP1 inhibitors During cerebral ischemia, the oxidative stress−induced DNA damage activates repair enzymes such as PARP1. However, multiple studies over the past decade have found that PARP1 activation is likely to exert harmful effects during ischemia; moreover, several PARP inhibitors have been shown to exert neuroprotective effects (Supporting Information Fig. S7)[202]. **3-AB** (**21**) is a classic PARP inhibitor. Studies have shown that it can protect the brain from ischemic injury by reducing neutrophil infiltration and infarct core volume during cerebral ischemia; however, it exhibits weak PARP-inhibitory activity[317]. **PJ34** (**22**) is a pharmacological inhibitor of PARP1 that can reduce cerebral infarct volume and improve neurological deficit in the rat model of ischemia/reperfusion by inhibiting excessive activation of the inflammatory response. Although its PARP-inhibitory activity is somewhat better than that of **21**, further studies are needed to evaluate its safety profile and address its poor cellular permeability[202,318]. Fujio et al.[319] designed and synthesized a novel water-soluble PARP1 inhibitor **MP-124** (**23**) that can competitively inhibit PARP1 activity (K_i = 16.5 nmol/L) and exert strong neuroprotective effects in both *in vivo* and *in vitro* model systems. **JPI-289** (**24**) is one of the few PARP1 inhibitors that are currently being investigated for the treatment of AIS. In the monkey stroke model of transient middle cerebral artery occlusion (tMCAO), **24** reduced infarct volume by 32% compared to **23**. A first-in-human, phase I, randomized, double-blind, placebo-controlled clinical trial (NCT02396069) that was completed in 2015 revealed that **24** was safe and well tolerated, causing minimal adverse events[320].

4.1.2.1. Nrf2 agonists. Icariside II (ICS II, **25**) is a natural flavonoid found in herba Epimedii and is considered a promising activator of Nrf2 (Supporting Information Fig. S8). In the mouse model of MCAO, **25** was found to directly bind Nrf2, which promotes its nuclear translocation and transcriptional activity, thus exerting potent neuroprotective effects. Similarly, *Clausena lansium* (Lour.) Skeels, a plant of the Rutaceae family, is a source of various small-molecule compounds with anti-inflammatory, antioxidant, anticancer, hepatoprotective, and neuroprotective properties. Claulansine F (**26**), a carbazole alkaloid extracted from *C. lansium*, exhibits significant antioxidant and free radical−scavenging activities to exert strong neuroprotective effects (Fig. S8). However, its aldehyde group may pose cardiotoxic risks. To address this, Zang et al.[321] designed **CZ-7** (**27**), a derivative of **26** with enhanced free radical−scavenging capacity and the ability to penetrate the BBB, although its solubility is poor (Fig. S8). Additionally, **CZK** (**28**), a new derivative with significantly higher hydroxyl radical−scavenging capacity than edaravone and the potential to bind the Kelch domain of Keap1, leading to the release and nuclear translocation of Nrf2; this is responsible for its enhanced antioxidant activity and potential therapeutic benefits for IS (Fig. S8). Caffeic acid (**29**) is a phenolic acid widely found in plants. Li et al.[322] found that **29** can upregulate the expression of Nrf2, inhibit iron-mediated cell death and inflammation, and exert neuroprotective effects (Fig. S8).

4.1.3. Inhibition of ferroptosis

Fang et al.[323] reported the synthesis of a novel ferroptosis inhibitor **DPT** (**30**), which targets NCOA4. They also designed a series of **30** derivatives, with compound **3f** (**31**) demonstrating potent anti-ferroptotic activity. Unlike the classic inhibitors of the GSH−GPX4 axis, **31** inhibits ferroptosis by increasing the expression of FSP1, decreasing the concentration of coenzyme Q10 (CoQ10), and inhibiting ferritin. Notably, **31** can penetrate the BBB, exhibit significant anti-ferroptotic activity, and exert protective effects against brain injury in a rat model of IS (Supporting Information Fig. S9).

The amino acid segment 383−522 of the NCOA4 protein, also referred to as NCOA4383-522, has been reported to interact with FTH1[324]. Fang et al[325]. successfully developed and synthesized novel benzimidazole derivatives; of these, compound **9a** (**32**) is the first known ferritin inhibitor to directly interact with the NCOA4383-522 segment and disrupt NCOA4−FTH1 interaction, exhibiting an IC_{50} concentration of 3.26 μmol/L (Fig. S9).

4.1.4. Free radical scavengers

NXY-059 (**33**), a compound with demonstrated free radical−scavenging activity, has been shown to effect a reduction in the cerebral infarct volume and significant improvement in neurological function in various animal models of IS (Supporting Information Fig. S10). However, a phase II clinical trial failed to demonstrate significant effects of **33** in IS**.** This lack of efficacy may be attributed to the instability of **33**, which can be degraded into *N-t*-butyl hydroxylamine (NtBHA, **34**)[326]. **34** has been shown to inhibit the production of ROS, reduce mitochondrial damage, and exert neuroprotective effects in both *in vivo* and *in vitro* animal models. Additionally, **34** can be oxidized to 2-methyl-2-nitrosopropane (MNP, **35**), which generates NO and functions as a vasodilator[326].

Uric acid (UA, **36**), a byproduct of xanthine oxidase−catalyzed purine metabolism, serves as an endogenous antioxidant that is depleted during ischemia (Fig. S10)[327]. The antioxidant properties of **36** have been hypothesized to be beneficial for neuronal survival. Studies have indicated that elevating serum UA levels *via* exogenous administration positively impacts the prognosis of stroke patients. A meta-analysis involving 8131 patients with IS revealed that high concentrations of UA, which was employed as a biomarker, were associated with better prognosis in patients with AIS[328].

Furthermore, a multicenter observational study involving 4621 patients with AIS revealed that decreased serum concentrations of UA were associated with adverse outcomes in patients with AIS[329].

4.1.5. Inhibition of neuroinflammation

4.1.5.1. Sphingosine 1-phosphate receptor (S1PR) modulators. Fingolimod (FTY720, **37**) is a S1PR antagonist (IC_{50} = 0.033 nmol/L). **37** is a potent immunomodulator that has been approved by the FDA for the treatment of multiple sclerosis in 2010 (Supporting Information Fig. S11). Recently, **37** has been shown to improve neurological functions in animal models of stroke[330]. Its efficacy and safety were further evaluated in a phase II clinical trial (NCT02002390).

4.1.5.2. Inhibitors of MMPs. MMPs are members of a family of zinc-dependent endopeptidases. The expression of MMP-9 increases several hours following the onset of IS, leading to a reduction in endothelial cell TJ, an increase in the permeability of the BBB, and nerve damage[331,332]. The therapeutic potential of MMP-9 in cerebral ischemia has been confirmed. Several naturally-occurring small-molecule compounds have shown excellent therapeutic potential for stroke, including the natural flavonoid compounds baicalin (**38**) and oxymatrine (OMT, **39**) (Supporting Information Fig. S12). **38** participates in the $ONOO^-$/MMP-9 signaling pathway, scavenges $ONOO^-$, reduces the production of 3-nitrotyrosine (3-NT), inhibits the activity of MMP-9, and protects the BBB, demonstrating strong therapeutic efficacy for IS under both *in vitro* and *in vivo* conditions[333]. By contrast, **39** downregulates CAV1, inhibiting the expression of MMP-9 and exerting potent neuroprotective effects in a rat model of MCAO[334]. Unfortunately, MMP-9 inhibitors are currently not being investigated in any clinical trial. However, Ji et al.[332] identified L13, a specific MMP-9-neutralizing antibody that reduces the activity of MMP-9 in IS patients; this effect is abolished in *Mmp9*-knockout mice. Moreover, L13 protects the BBB and exerts significant neuroprotective effects, opening the avenue to its potential application for the treatment of IS. Otaplimastat (SP-8203, **40**) is an inhibitor of MMPs, which is employed in studies of cerebral ischemic injury (Fig. S12). A phase II clinical trial (NCT02787278) revealed that safety concerns and adverse events did not occur in subjects who received **40**[335]. In addition, a combination therapy of **40** and rt-PA resulted in significant neurological improvement.

4.1.5.3. Inhibitors of aquaporin 4 (AQP4). George et al.[336] employed cell-based high-throughput screening to discover **AER-270** (**41**), a novel class of AQP4 inhibitors with limited solubility. Subsequent synthesis of the prodrug **AER-271** (**42**) increased the aqueous solubility of **41** by 5000-fold (Supporting Information Fig. S13). The administration of **42** was found to reduce brain edema and improve prognosis in animal models of IS[336]. **42** is currently under evaluation in a phase I clinical trial (NCT05200728). Laquinimod (LAQ, **43**) is a novel oral immunomodulator that has been shown to exert potent neuroprotective effects in various autoimmune diseases, and its potential therapeutic application for IS has recently been uncovered (Fig. S13). Animal studies have shown that **43** can inhibit the expression of MMP-9, thereby reducing damage to the BBB[337].

4.1.5.4. Sigma-1 receptor (σ-1R) agonists. σ-1R is mainly present in the ER and plays a role in the recovery of stroke-related neuronal plasticity. The activation of *σ-1R* can affect several physiological pathways, including those involved in oxidative stress response. Cutamesine (SA4503, **44**) is a selective agonist of σ-1R and can improve functional recovery after stroke in both *in vivo* and *in vitro* animal models (Supporting Information Fig. S14)[338]. A phase II clinical trial (NCT00639249) revealed the safety and absence of serious adverse events associated with the oral administration of 1 and 3 mg/day of **44** compared to the placebo group.

4.1.5.5. Others. Colchicine (**45**) is gaining attention owing to its broad anti-inflammatory activity (Supporting Information Fig. S15). A phase I clinical trial (NCT02551094) revealed that it significantly reduces the risk of ischemic cardiovascular events, including stroke, compared with the placebo group. A subsequent phase III clinical trial (NCT05439356) revealed that the administration of low doses of **45** did not significantly alter stroke recurrence over a 90-day duration compared to placebo treatment[339]. In addition, QHRD106 injection represents a new class of drugs independently developed by Qianhong Pharmaceutical; it is a novel long-acting tissue-type kallikrein preparation that inhibits the release of HMGB1 at ischemic sites *via* bradykinin (BK) receptor B2 (B2R) and reduces neuroinflammation as well as neuronal apoptosis[340]. A phase I clinical trial (NCT06388772) has been completed; the results show that QHRD106 is safe, well tolerated, and expected to alleviate the adverse clinical outcomes of stroke.

4.1.6. Rev-Erbα agonist

Rev-Erbα, also known as NR1D1, is a nuclear hormone receptor that plays a dominant role in regulating circadian rhythms and is closely associated with conditions such as inflammation, Alzheimer's disease, cancer, and metabolic diseases. **SR9009** (**46**) is a highly lipid-soluble, dual Rev-Erb agonist that is capable of crossing the BBB (Supporting Information Fig. S16). In mice model of MCAO, the administration of **46** reversed the ischemia-induced decline in circadian gene expression while also inhibiting the excessive production of inflammatory cytokines and reducing the concentration of MDA in mouse serum. Additionally, **46** was found to attenuate oxidative stress *via* the upregulation of Nrf2 and HO-1[341].

In summary, excitotoxicity is a pivotal mechanism underlying neuronal damage following cerebral IS. Drug development targeting the GluN2B−PSD95−nNOS complex has garnered considerable attention, with Gavestinel, an NMDA receptor antagonist, aiming to mitigate excitotoxicity by blocking the GluN2B subunit. However, despite demonstrating neuroprotective effects in animal studies, Gavestinel failed to meet its primary efficacy endpoints in Phase III clinical trials. This failure is attributed to various factors, including potentially inadequate therapeutic time windows in clinical trials to fully manifest drug efficacy and limitations of animal models in mimicking human stroke conditions. Furthermore, preclinical studies have shown neuroprotective potential with DAPK1 inhibitors, such as (4*Z*)-4-(3-pyridylmethylene)-2-styryl-oxazol-5-one (IC_{50} = 69 nmol/L) *in vivo*[342]. Despite promising preclinical results, no DAPK1 inhibitors have advanced to clinical trials, possibly due to concerns regarding safety, efficacy, and off-target effects. These challenges represent bottlenecks in the translation of excitotoxicity-related drugs. The development of ultra-acute biomarkers coupled with prehospital drug delivery systems holds promise for future clinical

Table 2 Ongoing clinical trials of pharmacological interventions for patients with AIS.

Trial number	Clinical trial phase	Stage of stroke	Proposed molecular mechanism(s)	Intervention	Primary Outcome	Estimated completion
NCT06447415	I	Hyperacute	Thrombolytic	HRS-7450	Symptomatic intracranial hemorrhage (sICH) within 36 h of the first administration	2025
NCT06403267	II	Hyperacute	Thrombolytic	NoNO-42	Modified Rankin scale (mRS) scores on day 90	2026
NCT06226805	II	Hyperacute	Thrombolytic	BB-031	sICH	2027
CTR20233526	III	Hyperacute	Thrombolytic	Milvexian	Time to first IS onset within 41 months	2026
NCT06322394	II	Hyperacute	Anti-excitotoxic	BXOS110	mRS scores on day 90	2025
NCT02018406	I/II	Acute	Anti-neuroinflammation	Erythropoietin/ granulocyte colony stimulating factor (GCSF)	Days 5 and 30, 6 months post-pretest vital signs	2025
NCT06472921	II	Acute	Anti-neuroinflammation	Butylphthalide	Proportion of participants with an mRS score of 0−2	2025
NCT06299579	III	Acute	Anti-neuroinflammation	GD-11	mRS scores on day 90	2025
NCT06517173	III	Acute	Multiple mechanisms	Y-3	mRS scores on day 90	2025
NCT06371495	−	Acute and recovery	Nerve regeneration	Rosuvastatin	Carotid duplex performed at the IS onset and after 1 and 3 months of onset	2025

"−": Not reported.

translation in this field. Edaravone stands as the sole globally approved Nrf2 activator for the treatment of IS. Conversely, NXY-059, another agent acting as a radical scavenger, encountered clinical failure. This outcome is intimately tied to the timing of target activation and the intrinsic metabolic properties of the molecule within the body. Distinct from classic GSH−GPX4 axis inhibitors, ferroptosis inhibitor **31** suppresses ferroptosis by enhancing FSP1 expression. Meanwhile, ferroptosis inhibitor **32** represents the first agent to disrupt the NCOA4−FTH1 interaction. These molecules circumvent the gastrointestinal toxicity potentially associated with systemic GPX4 inhibition, hinting at promising clinical translation potential. Targets related to circadian rhythms may exhibit time-dependency, as studies indicate that PKG activation is only effective at night (correlated with the Bmal1 rhythm), suggesting the necessity of timed dosing in clinical practice[343].

In addition to the aforementioned points, this article also summarizes the biological activity data of the investigated drugs in clinical settings, including molecular weight, half-life, and blood-brain barrier permeability.

Notably, an increasing number of neuroprotective agents have been developed and evaluated *via* preclinical trials. Moreover, increasing numbers of pharmacological interventions targeting cerebral ischemia are being evaluated in clinical trials, many of which are currently ongoing (Table 2).

4.2. *Hyperacute stage*

4.2.1. *Thrombolytic agents*

Thrombolytic therapy is one of the effective treatments for AIS and has the potential to restore cerebral blood flow and reduce nerve cell damage. The clinical trial application for **NoNO-42** (YGRKKRRRQRRRKLSSIESDV, **47**) was submitted in April 2024; an ongoing phase II clinical trial aims to evaluate the efficacy and safety of intravenous thrombolytic therapy with **47** within 3 h of the onset of IS. HRS-7450, developed by Fujian Shengdi Pharmaceutical, has been approved for clinical use in September 2023; an ongoing phase I clinical trial (NCT06447415) aims to evaluate its safety, tolerability, and thrombolytic efficacy in patients with stroke, which is expected to extend the time window of thrombolytic therapy.

4.2.2. *Inhibitors of blood coagulation pathway*

Milvexian (**48**) is an orally-administered small-molecule antagonist of factor Xla and was independently developed by Bristol Myers Squibb; it exhibits K_i values of 0.11 and 0.38 nmol/L for humans and rabbits, respectively (Supporting Information Fig. S17). An ongoing phase III clinical trial (NCT05702034) aims to assess the therapeutic potential of **48** for improving outcomes in patients with IS.

4.2.3. *Inhibitors of platelet adhesion*

von Willebrand factor (vWF) is a key structural component of thrombus and plays an important role in thrombosis. Studies have revealed significantly higher levels of vWF in stroke patients compared to those who have never had stroke. BB-031 is an inhibitor of vWF and is being developed by Basking Biosciences; it binds vWF and inhibits its activity without affecting normal blood vessels. BB-031 was found to rapidly exert thrombolytic effects and restore cerebral blood flow in various animal models of IS. BB-031 is currently being evaluated in a phase II clinical trial (NCT06226805). In addition, BB-025, which neutralizes the pharmacological activity of BB-031 and stops thrombolysis in time to prevent bleeding or other complications in patients; this compound is currently under investigation.

4.3. *Acute stage intervention*

BXOS110 is a class 1 innovative polypeptide drug developed by Biocells for affording neuroprotection in the hyperacute phase of

AIS. A phase II clinical trial (NCT06322394) is currently underway to evaluate the efficacy and safety of different doses of BXOS110 when administered within 3 h of the onset of IS.

A phase II clinical trial (NCT06472921) is currently underway for exploring the optimal window (within 3 h *versus* 3−6 h of the onset of AIS) for the administration of NBP to elicit neuroprotection in patients with AIS; the trial is expected to be completed in 2025. However, existing neuroprotective agents do not appear to fundamentally resolve the problem of neurobiological reduction. Certain cytokines such as EPO and GCSF have potential antiapoptotic effects on neurons and facilitate neuroregeneration. A clinical trial (NCT02018406) is presently underway to investigate the safety and effectiveness of the combined administration of EPO and GCSF in patients with IS. GD-11 is an innovative drug that is under development by Jiangsu Wangao Pharmaceutical for the treatment of AIS. An ongoing phase III clinical trial (NCT06299579) focuses on the efficacy and safety of GD-11 administration within 48 h of stroke onset in patients with IS.

4.4. Recovery stage intervention

Statins have been shown to alleviate adverse outcomes in patients with IS, which may be associated with their ability to enhance endogenous angiogenesis and promote neurogenesis[344,345]. A clinical trial (NCT06371495) is presently underway to determine the role of statins in AIS by assessing carotid intima thickness in patients with AIS before and after the administration of rosuvastatin (**49**) (Supporting Information Fig. S18).

4.5. Interventions with multiple targets and mechanisms of action

Y-3 is an *N*-benzylaniline derivative developed by NeuroDawn as a small-molecule nNOS/PSD95 uncoupler; it inhibits the signaling pathways downstream NMDAR by interfering with the interaction of nNOS with PSD95. Y-3 concomitantly activates α2-GABAAR and reduces excitotoxic damage. An ongoing phase III clinical trial (NCT06517173) aims to assess the efficacy and safety of Y-3 administration within 48 h of the onset of stroke. In addition, owing to its good permeability across the BBB, a combination therapy of Y-3 with thrombolytic drugs may be more beneficial for improving the prognosis of patients with IS.

5. Perspectives

The past few decades have witnessed significant progress in the treatment of IS, particularly in acute-phase management and intervention strategies. The advent of proteomics, metabolomics, and RNA sequencing technologies has led to the identification of specific anti-stroke molecular mechanisms and novel pathophysiological targets, laying a robust foundation for the discovery of novel therapeutic agents.

5.1. Rational analysis of the clinical failure of existing neuroprotective drugs

Neuroprotective strategies for cerebral ischemia have demonstrated limited successes but lots of failures in clinical trials. The underlying mechanisms for such failures are often associated with factors such as circadian rhythm variations, limitations of therapeutic time windows, the singular mechanisms of action of neuroprotective agents, and discrepancies between animal models and clinical conditions. Future clinical trials should carefully consider the influence of circadian rhythms on therapeutic efficacy, such as grouping treatments based on different time periods, like daytime or nighttime[288]. Extending the therapeutic window may be achieved by improving the pharmacokinetic properties of drugs or developing novel formulations to prolong their effective duration *in vivo*[346]. Developing neuroprotective drugs or therapies targeting multiple steps of the ischemic cascade may enhance therapeutic outcomes. Additionally, constructing animal models of metabolic diseases that better mimic the physiological and pathological characteristics of clinical populations is essential. Moreover, individual differences and comorbidities among patients must be fully considered to improve trial design[72]. These strategies are expected to provide more accurate and reliable guidance for future clinical trials, thereby promoting the development of neuroprotective drugs and therapeutic approaches.

5.2. Pay attention to heterogeneity in stroke patients

The heterogeneity among stroke patients is an important factor, that cannot be overlooked as it is closely related to variables such as age, comorbidities, and lifestyle. These factors significantly influence treatment responses and recovery trajectories, underscoring the necessity of tailoring current and future therapeutic approaches to accommodate this diversity. To address this variability, personalized and precision-based treatment strategies are critical. Continuous exploration of novel therapies and technologies, such as the identification of reliable and broadly applicable biomarkers, is essential for accurately monitoring stroke recovery and predicting long-term outcomes[347]. Young IS patients often exhibit stronger recovery potential, whereas elderly patients are more likely to face additional challenges due to comorbidities. For young patients, treatment strategies may emphasize aggressive acute-phase interventions. In contrast, treatment plans for elderly patients must carefully account for the potential risks and complexities posed by comorbidities such as hypertension, diabetes, and cardiovascular diseases, ensuring both safety and efficacy[348]. Looking ahead, advances in medical technology and deeper research into stroke mechanisms may pave the way for innovative approaches such as gene therapy and immunotherapy, offering improved therapeutic prospects for stroke patients.

5.3. Timely intervention and tailored therapeutic approaches

There is still a lack of effective drugs in pre-hospital treatment, and enhancing prehospital stroke management and systems of care presents the most practical strategy for improving IS patients experiencing such as RIC, TH, and NBO, which holds significant promise for advancing stroke care by providing more timely and effective treatment.

An effective early intervention for IS hinges on a timely and accurate diagnosis. A variety of biomarkers have been identified for the prediction and diagnosis of stroke. Future research should therefore be focused on the identification and utilization of advanced biomarkers and imaging technologies that accurately predict the onset of stroke, monitor its progression, and assess treatment response. Such developments have the potential to significantly improve treatment efficacy and patient prognosis. Additionally, these biomarkers could provide critical insights into the underlying pathophysiological conditions and aid in tailoring personalized treatment strategies.

While imaging techniques remain the gold standard for stroke diagnosis, their limitations in time and space often hinder timely diagnosis and management. Biomarkers, particularly microRNAs (*e.g.*, miR-124, miR-9), inflammatory markers (*e.g.*, CRP, IL-6), and neurodegenerative proteins (*e.g.*, GFAP, S100B), have emerged as promising tools for early stroke detection due to their rapid response to ischemic injury and stability in blood[349-351]. Metabolomic and lipidomic changes, such as lysophosphatidylcholine, further enrich the biomarker landscape, offering a comprehensive approach to capturing stroke-related pathophysiological alterations[351].

However, the complexity of stroke pathophysiology poses a significant challenge, as single biomarkers often lack sufficient sensitivity and specificity for clinical use[352]. To address this, multi-marker panels combining miRNAs, inflammatory markers, and neurodegenerative proteins have been developed, demonstrating improved diagnostic accuracy and predictive power[353]. Advances in high-throughput technologies (*e.g.*, genomics, proteomics, metabolomics) and artificial intelligence (AI) are expected to enhance biomarker discovery and validation, paving the way for their integration into clinical practice[354]. The success of such AI-driven technologies is expected to be largely dependent on interdisciplinary collaboration and rapid technological progress. A key challenge involves the effective collection of long-term follow-up data using AI and big data analytics. This involves identifying the most efficient combinations of biomarkers, integrating data on underlying comorbidities, and iteratively refining diagnostic models to enhance stroke detection rates and optimize treatment outcomes. Additionally, the development of biocompatible and degradable contrast agents could provide critical insights into underlying pathological conditions and facilitate personalized treatment strategies[355]. These advancements, combined with the exploration of neuroimaging techniques, hold the potential to revolutionize stroke management by enabling rapid diagnosis, accurate prediction of stroke onset, and tailored therapeutic interventions.

An effective treatment strategy for IS should prioritize early intervention to prevent the progression of brain damage. This requires a dynamic, stage-specific therapeutic strategy that addresses the evolving pathophysiological mechanisms associated with the different phases of stroke, including excitotoxicity, oxidative stress, neuronal apoptosis, and inflammation, etc. Given the time-sensitive nature of stroke, the adoption of individualized treatment approaches based on the specific phase of stroke progression becomes crucial. For instance, the rapid pharmacological or non-pharmacological interventions immediately after the diagnosis of stroke in an ambulance setting could significantly reduce the time interval between symptom onset and treatment, thereby improving clinical outcomes.

Regenerative medicine, particularly stem cell therapy, holds significant promise for repairing damaged brain tissue, offering novel perspectives and possibilities for IS treatment[356]. Mesenchymal stem cell (MSC)-based therapies have emerged as a breakthrough strategy for IS treatment[356]. Umbilical cord-derived MSCs, already applied in treating ischemic conditions like diabetic foot ulcers and lower limb ischemia, are increasingly being explored for their potential in stroke therapy. Clinical evidence supports the efficacy of MSC therapy in promoting angiogenesis, enhancing blood perfusion, and restoring the function of damaged tissues[357]. Therefore, prioritizing adult stem cells, such as bone marrow-derived MSCs and umbilical cord blood stem cells—sources that are relatively abundant and less ethically contentious—offers great promise for addressing neurological deficits in IS patients and represents a key frontier in stroke treatment.

An integration of genetic data, biomarker profiles, and various clinical characteristics allows the formulation of more precise and individualized treatment strategies for patients with IS. Additionally, the application of machine learning and AI algorithms to large datasets enables the identification of variations in treatment responses across different patient groups, facilitating personalized medicine approaches. The utility of AI extends beyond the early detection and diagnosis of stroke; it encompasses stroke risk assessment, surgical planning, simulation of surgical procedures, and postoperative monitoring, thus providing comprehensive support through the complete stroke care continuum.

Ensuring the safety and efficacy of treatment in the hyperacute stage of IS involves a rapid assessment of the patients' condition, determining the eligibility for thrombolytic therapy, and tailoring treatment based on factors such as patient age, weight, and comorbidities. Given the narrow therapeutic window in this phase, multimodal imaging techniques such as CT and MRI or emerging methods of penumbra detection can facilitate real-time treatment decisions based on visual data. During the acute stage, the focus shifts to disease stabilization, continuous monitoring, and the administration of neuroprotective agents to limit further damage (Table 3).

5.4. Management of chronic stroke and rehabilitation strategies

While remarkable progress has been achieved in the interventions for acute cerebral ischemia, it is imperative to acknowledge the paramount importance of patients' long-term prognosis and rehabilitation strategies. The management of chronic stroke focuses on long-term recovery and prevention of recurrent events. While rehabilitation therapies like mirror therapy, physical, occupational, and speech therapy are essential for functional recovery, the real challenge lies in improving outcomes for patients with significant residual deficits[358]. To offer a more holistic view that incorporates the chronic phase of stroke management into patient care, future research endeavors and clinical practices must delve more profoundly into the following facets. Emerging pharmacological approaches targeting neuroinflammation, neuroplasticity, and neuronal survival show promise in modifying the chronic stroke process[359,360]. Moreover, secondary prevention remains a cornerstone of stroke management, emphasizing the control of vascular risk factors such as hypertension, diabetes, and dyslipidemia[361]. Future strategies should prioritize individualized care, integrating both rehabilitation and pharmacological interventions tailored to each patient's unique needs. As research continues to uncover new molecular targets and therapeutic agents, innovations like stem cell therapy and advanced neuroprotective drugs may further enhance recovery, offering hope for more effective treatments in chronic stroke management. The integration of continuity between acute and chronic management, with a specific emphasis on the synergistic effects between acute-phase interventions and chronic-phase rehabilitation, represents a transformative approach in stroke care. By comprehensively considering acute interventions, long-term prognosis, and rehabilitation strategies, we can provide patients with more comprehensive and personalized care plans, thereby enhancing their survival rates and quality of life. This not only complements and improves upon current research but also embodies a holistic approach to patient care.

Table 3 The bio-activity data of above-mentioned investigational drugs.

Compound ID	Research progress	Molecular weight	Activity data	Half-life	References
3	Phase III	179.3	IC_{50} of ~1 μmol/L at −60 mV	60−100 h	346
6	Preclinical	221.3	K_d = 37.2 nmol/L	52.31 min	347
7	Preclinical	325.44	K_i = 14.80 nmol/L	2.42 h	348
14	Phase III	347.85	IC_{50} = 0.5 μmol/L for NR2A IC_{50} = 8 μmol/L for NR2B	−	349
15	Preclinical	309.33	IC_{50} = 5.4 μmol/L	−	350
23	Preclinical	384.36	K_i = 16.5 nmol/L IC_{50} = 20.8 nmol/L cLog*P*: 1.50 pK_a: 8.0−8.4	−	321
24	Preclinical	379.88	IC_{50} = 18.5 nmol/L	1.88−3.05 h	322
31	Preclinical	442.52	EC_{50} = 1.7 μmol/L Pe = (15.3 ± 0.9) × 10^{-6} cm/s	−	325
37	Phase II	307.471	IC_{50} = 0.033 nmol/L	−	205
40	Phase II	534.61	High affinity K_d = 0.2 μmol/L Low affinity K_d = 28 μmol/L	−	351
44	Phase II	368.52	σ-1R (K_i = 17 nmol/L) σ-2R (K_i = 1800 nmol/L)	−	352
46	Preclinical	437.94	IC_{50} = 670 nmol/L for Rev-Erbα IC_{50} = 800 nmol/L for Rev-Erbβ K_d = 800 nmol/L for Rev-Erbα	−	353

"−": Not reported.

5.5. *Innovations and integration in thrombolytic therapy*

Innovations and integration in thrombolytic therapy are essential for the effective treatment of AIS. While thrombolytic therapy has proven successful in restoring blood flow to the ischemic regions by reopening occluded vessels, the limited therapeutic window for its application significantly constrains its neuroprotective potential. Research efforts should therefore be focused on identifying novel thrombolytic agents and evaluating the safety and efficacy of combining thrombolytic therapy with the administration of other anti-stroke agents. Early anticoagulant therapy may be considered in specific cases that present minimal risk of hemorrhage and high likelihood of severe cerebral embolism. This decision must be based on a thorough assessment of factors such as lesion size, BP, as well as liver and kidney function. The potential agents for such interventions include fibrinolysin, urokinase, lumbrokinase, and tenecteplase, etc.

5.6. *Drugs with multiple mechanisms of action*

The etiology of brain damage in IS involves a complex array of biological processes and molecular pathways. A thorough understanding of the pathogenic mechanisms underlying stroke is essential for improving the precision and efficacy of therapeutic interventions. Traditional strategies that are focused on targeting a single mechanism or pathway have proven insufficient for the comprehensive treatment of stroke. Consequently, there is an increasing emphasis on the development of drugs that target multiple mechanisms of the ischemic cascade. Significant efforts have been made toward devising therapeutic interventions of greater efficacy that can be applied at various stages of stroke pathology. Current research is primarily focused on exploring the potential of NMDAR antagonists, CCBs, inhibitors of iron-induced cell death, agents that preserve the functions of the BBB, modulators of circadian rhythms, and noncoding RNAs. This multitarget approach not only provides neuroprotection but also facilitates improved recovery after cerebral infarction, offering a more holistic strategy for stroke management.

5.7. *Interdisciplinary collaboration and integration of technology*

Effective research on the management of IS requires multidisciplinary collaboration, encompassing fields such as cardiology, integrative clinical medicine, biology, and computer science[362]. Partnerships with the engineering sector are particularly valuable as they can allow the development of cutting-edge devices and instruments. Innovations such as advanced brain-imaging technologies and minimally-invasive surgical tools have the potential to greatly improve the diagnosis, treatment, and overall care of stroke patients. These cross-disciplinary efforts are essential for driving progress in stroke management and achieving better therapeutic outcomes.

Additionally, nanotechnology-based drug delivery systems are emerging as promising tools to enhance the precision and efficacy of therapeutic interventions, particularly in crossing the blood−brain barrier to target ischemic brain regions. Furthermore, the application of wearable devices and telemedicine platforms is transforming post-stroke care by facilitating real-time monitoring of patients' recovery progress and enabling remote rehabilitation programs. These technologies not only improve the timeliness and accessibility of stroke care but also hold immense potential for personalized treatment strategies. As research progresses, the integration of these cutting-edge technologies into clinical practice is expected to significantly enhance patient outcomes and reshape the landscape of IS management.

6. Conclusions

In conclusion, ongoing scientific and technological advancements are steadily expanding the therapeutic options for IS patients,

albeit significant challenges remain. A key insight from recent research progress is the need for timely interventions that target the distinct pathophysiological characteristic of four stages of IS. From the vascular recanalization technology in the hyperacute stage to the neuroprotective strategies in the acute, recovery and sequelae stages, tailored approaches are essential to improve patients' therapeutic outcomes.

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Author contributions

Anning Xu and Honghua Zhang contributed equally. Anning Xu wrote and edited this paper. Honghua Zhang contributed to review and edit the paper. Yihua Zhang reviewed, edited the paper and provided perspective. Jianbing Wu and Zhangjian Huang contributed to the writing-review & editing of the manuscript. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2025.07.026.

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