Mechanisms of Neuronal Cell Death in Ischemic Stroke and Their Therapeutic Implications

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ABSTRACT

Ischemic stroke caused by arterial occlusion is the most common type of stroke, which is among the most frequent causes of disability and death worldwide. Current treatment approaches involve achieving rapid reperfusion either pharmacologically or surgically, both of which are time-sensitive; moreover, blood flow recanalization often causes ischemia/reperfusion injury. However, even though neuroprotective intervention is urgently needed in the event of stroke, the exact mechanisms of neuronal death during ischemic stroke are still unclear, and consequently, the capacity for drug development has remained limited. Multiple cell death pathways are implicated in the pathogenesis of ischemic stroke. Here, we have reviewed these potential neuronal death pathways, including intrinsic and extrinsic apoptosis, necroptosis, autophagy.

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ferroptosis, parthanatos, phagoptosis, and pyroptosis. We have also reviewed the latest results of pharmacological studies on ischemic stroke and summarized emerging drug targets with a focus on clinical trials. These observations may help to further understand the pathological events in ischemic stroke and bridge the gap between basic and translational research to reveal novel neuroprotective interventions.

**KEYWORDS**

Ischemic stroke; Neuronal Death; Ferroptosis; Neuroprotection; Apoptosis

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1. INTRODUCTION

Stroke represents a significant threat to human life and is among the most frequent causes of disability and death worldwide\(^1,2\). When the brain experiences obstructed blood flow due to blockage or rupture of an artery, a stroke occurs. The cerebral energy supply is disrupted, causing damage to tissues and resulting in extensive neuronal death that can occur from 20 minutes to 10 days post-ischemic stroke\(^3\). The World Health Organization has suggested that an incidence of stroke occurs once in every 5 seconds worldwide\(^4\). Around 15 million cases of stroke occur each year, and with one-third of the affected individuals losing their life and another one-third becoming permanently disabled, it places a significant burden on families and communities.

Stroke can be broadly divided into ischemic and hemorrhagic stroke. Ischemic stroke occurs when a blood clot or other particles blocks the blood vessels that supply oxygen-rich blood to the brain; it accounts for about 71% of all strokes globally\(^5\). This type of stroke can be further divided into thrombosis (due to a locally formed blood clot), embolism (due to a mobilized clot in the blood), and global ischemia (systemic hypotension in the absence of a blood clot). Interestingly, due to differences in the vasculature, different brain regions have different susceptibilities for stroke-related events and different sensitivities.
to ischemic injury. Therefore, several experimental models, varying in terms of the ischemic region (local vs. global), the duration of the ischemic event (transient vs. permanent), and the relative levels of hypoxia and hypoglycemia, have been established to investigate ischemic neuronal damage. For example, the pathophysiology of the brain damage incurred due to global ischemia usually differs from that due to focal ischemia. Global cerebral ischemia usually lasts for a few minutes, and provided that reperfusion is adequate, neuronal damage is significantly delayed and mainly affects only vulnerable neurons. In contrast, focal ischemia is either long-lasting or permanent, affects the regions supplied by the occluded artery at an early stage, and invades the penumbra zone later. The cell death pathways involved can also differ in different animal models; however, this is usually overlooked during the drug development process. In fact, this could be one of the main reasons for the poor clinical translation of therapeutic drugs developed for ischemic stroke.

Ischemic tissues can be functionally grouped into those in the irreversibly damaged infarct core and those in the surrounding ischemic penumbra (Figure 1). The infarct core, consisting of dead or dying tissue, is in the central zone of the infarction area. Around the core of the infarct is tissue with less blood flow reduction that can be salvaged with early reperfusion, termed the ischemic penumbra or the peri-infarct zone. The penumbra is a dynamic and metastable zone that can account for up to half of the total lesion volume during ischemic stroke. Neurons in the penumbra activate survival signaling pathways shortly after the injury, and these pathways can remain activated from a few hours to several days. If the ischemic brain tissue remains without reperfusion for a prolonged period of time, the infarct core may extend to involve the penumbra. Subsequently, neurons in other areas of the brain may also die due to loss of contact with ischemic neurons (so-called secondary neuronal loss). Thus, salvaging of penumbral neurons is essential for neuroprotective therapy. It is
Currently, it is believed that multiple cell death pathways are involved in neuronal death in ischemic stroke; however, it remains unclear whether one or more pathways are of particular importance in this context.

Clinical treatment of ischemic stroke is limited to interventions that restore blood flow through either pharmacological thrombolysis or mechanical thrombectomy. However, due to the moderate recanalization rate, limited time window, and a number of thrombolytic contraindications, only 11% of ischemic stroke patients can receive intervention in the form of recombinant tissue plasminogen activator (r-tPA), and almost half of those patients fail to demonstrate any improvement\textsuperscript{14}. Therefore, we hope to illustrate the potential tractable target by reviewing our current understanding of the mechanisms of neuronal death in ischemic stroke, as well as the recent progress in drug development.

2. POTENTIAL NEURONAL CELL DEATH PATHWAYS IMPLICATED IN ISCHEMIC STROKE

2.1 Apoptosis

Apoptosis is the most common form of programmed cell death in multicellular organisms, and can be triggered through either the intrinsic or the extrinsic pathway (Figure 2). The initial morphological changes in apoptosis involve cell shrinkage and cytoplasmatic condensation, followed by nuclear membrane breakdown and formation of apoptotic bodies, all of which take place without any inflammatory response\textsuperscript{15}. Importantly, these features of apoptosis have also been observed in post-ischemic stroke neurons\textsuperscript{16,17}. Furthermore, it seems that apoptosis in neurons of the ischemic penumbra may be recoverable\textsuperscript{18}. Detailed evidence supporting the involvement of apoptosis in ischemic stroke and its therapeutic potential is reviewed below.
2.1.1 Apoptosis by the intrinsic/mitochondrial pathway

From the maintenance of ion homeostasis to proliferation, various cellular activities require adenosine triphosphate (ATP)\textsuperscript{19}, and cells become inactive and subsequently degrade in its absence. With glucose as the primary metabolic substrate, ATP in mammalian cells is mainly produced in two ways—oxidative phosphorylation, which occurs in mitochondria, is the main pathway for ATP production\textsuperscript{20}, whereas under anaerobic conditions, ATP is mainly produced by glycolysis\textsuperscript{21}.

When a stroke occurs, the transport of oxygen, glucose, and other substrates is severely limited. Consequently, neurons alter their glucose supply routes as well as the glucose metabolism pathway, which transitions from aerobic oxidation to anaerobic oxidation—available cytosolic glucose is metabolized by anaerobic oxidation and becomes the primary source of ATP\textsuperscript{22}. However, compared with glycolysis coupled to oxidative phosphorylation, this process is considerably less efficient: for thirty-eight ATP molecules produced under aerobic conditions, the anaerobic degradation of one molecule of glucose produces only two ATP molecules\textsuperscript{23}. Therefore, consumption quickly exceeds production, and the intracellular concentration of ATP drops. Such a drop reduces ionic gradients while also impairing Na\textsuperscript{+}/Ca\textsuperscript{2+} influx and K\textsuperscript{+} efflux\textsuperscript{24}. These cellular events further activate voltage-dependent Ca\textsuperscript{2+} channels, causing excitatory amino acids to be released into the extracellular space. Subsequently, intracellular Ca\textsuperscript{2+} appears to accumulate cytotoxically and trigger intrinsic apoptosis as well as several other cytoplasmic and nuclear events\textsuperscript{25} (Figure 3).

Calpain-mediated apoptosis

During cerebral ischemia, disrupted ionic gradients depolarize neurons and lead to the release of massive amounts of excitatory neurotransmitters, particularly
glutamate\textsuperscript{26}. Neuronal cells are also unable to remove excess glutamate due to energy depletion and the resulting functional disruption of reuptake transporters, which in turn leads to the accumulation of large amounts of glutamate in the extracellular space\textsuperscript{27}. Glutamate further activates ionotropic glutamate receptors, which act as excitotoxic channels and enable the rapid influx of ions\textsuperscript{28}.

The two major subtypes of ionotropic glutamate receptors are the N-methyl-D-aspartate receptors (NMDARs) and the \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs)\textsuperscript{29}. Pre-synaptic release of glutamate activates AMPARs, resulting in partial depolarization of the postsynaptic membrane while eliminating the \(\text{Mg}^{2+}\) blockade of the NMDAR channels\textsuperscript{30,31}. Once glutamate binds to NMDARs, \(\text{Ca}^{2+}\) channels are opened\textsuperscript{32}. Extracellular accumulation of excitatory glutamate can cause inappropriate activation of NMDARs and lead to an overload of \(\text{Ca}^{2+}\) in neurons\textsuperscript{29}. However, Johnson \textit{et al.} have shown that acid-sensing ion channels (ASICs) regulate the intracellular \(\text{Ca}^{2+}\) homeostasis imbalance after the reduction of extracellular pH\textsuperscript{33}. During cerebral ischemia, the extracellular pH in ischemic brain tissue is significantly reduced, which activates ASICs and further promotes ischemia-induced \(\text{Ca}^{2+}\) influx\textsuperscript{34,35}.

\(\text{Ca}^{2+}\) overload triggers calpain activation\textsuperscript{36}, and one of the essential substrates of calpain is the anti-apoptotic protein B-cell leukemia/lymphoma 2 (Bcl-2). Calpain cleaves Bcl-2 interacting domain (BID) to its truncated active form (tBID), which interacts with Bax on the mitochondrial membrane. Subsequently, Bax and tBID form a dimer and promote the formation of outer mitochondrial membrane pores called mitochondrial permeability transition pores (mPTPs), which are non-selectively permeable to solutes smaller than 1.5 kDa\textsuperscript{37}. This causes the release of various pro-apoptotic factors, including cytochrome C (Cytc) and apoptosis-inducing factor (AIF)\textsuperscript{38}. After entering the cytosol, Cytc
complexes form an apoptosome with apoptotic protein-activating factor-1 and procaspase-9, thereby activating effector caspases such as caspase-3\textsuperscript{39,40}. mPTP-induced neuronal injury has been implicated in ischemic stroke, and its specific inhibitor cyclosporine A has been reported to reduce neuronal death and infarct volume in animal models of focal cerebral ischemia\textsuperscript{41-43}.

Unlike Cytc, AIF can quickly translocate to the nucleus and mediate significant DNA fragmentation, which ultimately leads to caspase-independent cell death\textsuperscript{44}. Culmsee \textit{et al.} have demonstrated that knockdown of AIF by small inhibitory RNA reduces glutamate- and oxygen/glucose deprivation (OGD)-induced neuronal apoptosis\textsuperscript{45}; moreover, compared with wild-type littermates, mice that express lower AIF levels have smaller infarct volumes after middle cerebral artery occlusion (MCAO). The same study also found that inhibition of poly (ADP-ribose) polymerase (PARP) or BID can reduce nuclear AIF translocation, further supporting AIF as a target for preventing ischemic cell death. Studies have shown that small-molecule BID inhibitors can prevent glutamate- or OGD-induced mitochondrial release of AIF \textit{in vitro} and consequently ameliorate neuronal death, revealing the potential of these compounds as neuroprotective therapeutics for ischemic stroke\textsuperscript{46}.

\textit{Reactive oxygen species-mediated apoptosis}

When blood flow is restored after cerebral ischemia, a large amount of reactive oxygen species (ROS) is produced, which may be one of the causes of neuronal ischemia/reperfusion (I/R) injury\textsuperscript{47}. Neuronal cell damage by ROS may occur in association with apoptosis. For example, ROS can damage the plasma membrane, resulting in loss of membrane integrity\textsuperscript{48}. ROS also damage DNA, which can lead to strand breaks\textsuperscript{49}. Besides, the release of Cytc from the mitochondria into the neuronal cytosol is also affected by mitochondrial ROS\textsuperscript{50}.\hfill

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DNA damage-mediated apoptosis

During cerebral ischemia, DNA damage activates the nuclear pathway of cell death through the phosphorylation of p53 or translocation of nucleophosmin\(^{51}\). In neurons, nucleophosmin can activate Bax and then induce apoptosis through the mitochondrial pathway. Kerr et al. found that nucleophosmin that translocates to mitochondria can interact with Bax during apoptosis, and that the silencing of nucleophosmin can prevent neuronal cell death\(^{52}\). On the other hand, p53 is rapidly upregulated in ischemic brain tissue, and in turn regulates the transcription of pro-apoptotic genes such as Bax, p53-upregulated modulator of apoptosis (PUMA), and phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), thereby mediating neuronal cell death\(^{53-55}\).

However, research in the last decade has shown that p53 can also induce apoptosis by acting on mitochondria directly\(^{56-58}\). Cytoplasmic p53 is rapidly transferred to the surface of the outer mitochondrial membrane under stress, and is then combined with Bcl-2 family proteins to ultimately promote Bax/Bak-mediated permeabilization of the mitochondrial outer membrane. This triggers the release of Cytc as well as the activation of the caspase cascade\(^{56-58}\). Endo et al. have shown that pifithrin-alpha, a p53-specific inhibitor, can block the mitochondrial translocation of p53 and prevent its binding to Bcl-xL, thereby preventing delayed neuronal death in the cornu ammonis 1 after transient global cerebral ischemia\(^{59}\).

2.1.2 Apoptosis by the extrinsic/death receptor pathway

During cerebral ischemia, blood-brain barrier leakage and the signaling molecules (e.g., cytokines) released by astrocytes, microglia, and oligodendrocytes can cause inflammation\(^{60}\). Although many aspects of inflammation manifest themselves days or weeks after ischemia, the
inflammation cascade is activated immediately after a blood vessel is blocked. In rodent models of ischemic stroke, early activation of resident microglia activates the inflammatory response, causing a massive influx of macrophages, neutrophil polymorphs, and lymphocytes into the brain parenchyma. In the early stages of ischemic stroke, neutrophil accumulation occurs in the ischemic core, while microglial activation and proliferation occurs in the penumbra. The activation of immune cells during inflammation initiated by cerebral ischemia may release several factors that trigger neuronal cell death through the extrinsic apoptotic pathway, including pro-inflammatory cytokines such as interleukin 1β (IL-1β), chemokines, tumor necrosis factors (TNFs) such as TNF-α/β, Fas ligand (FasL), and TNF-related apoptosis-inducing ligand receptor (TRAIL-R) (Figure 4).

The extrinsic apoptosis pathway is triggered by the so-called plasma membrane ‘death receptors.’ Known receptors include TNF-receptor 1, apoptosis antigen-1 (APO1/FAS/CD95), and TRAIL-R. Extrinsic apoptosis can occur either independently or synergistically with the mitochondrial pathway. During an ischemic stroke event, one of the receptors mentioned above binds to a Fas-associated death domain (FADD) to create a death-inducing signaling complex with procaspase-8, which enables the activation of caspase-8. Once caspase-8 is activated, it activates downstream effector caspases (such as caspase-3) by direct proteolytic cleavage or indirect cleavage of BID to tBID, which mediates apoptosis through the mitochondrial-dependent pathway.

Plesniša et al. have shown that BID knockout neurons are resistant to death after OGD. In vivo, the infarct volumes decreased, and Cytc release was also less in BID knockout mice after MCAO. These findings indicate that Cytc release and caspase activation might be important for the contribution of BID in neuronal death after cerebral ischemia. Yin et al. also found that while the activation of BID as well as mitochondrial release of Cytc can be easily detected...
in wild-type brains after 3 hours of focal cerebral ischemia, until 24 hours later, no apparent Cytc release was detected in BID-deficient brains, which highlighted the importance of BID. This finding is further supported by the discovery that caspase-3, but not caspase-8, is deactivated in BID-deficient brains. In summary, these data indicate that BID is activated by caspase-8 in the early stage of neuronal ischemia; thus, it may be one of the earliest and most effective activators in the mitochondrial apoptosis pathway.

2.2 Necroptosis

For a long time, necrosis was considered to be a passive process caused by overwhelming stress\(^\text{37}\). In the 1990s, the concept of the purely unregulated nature of necrosis was challenged, and necrotic cell death has since then been at least partially proven to be a well-regulated and orchestrated event like apoptosis\(^\text{80,81}\). In contrast to apoptosis, necroptosis was a caspase-independent process that can be initiated by TNF-\(\alpha\), TRAIL, or FasL along with pan-caspase inhibitors such as Z-VAD-FMK\(^\text{82-85}\); moreover, the dying cells undergoing necroptosis do not exhibit the morphological characteristics observed in apoptosis. The TNF family of cytokines can recruit activate caspase-8 through the adaptor protein FADD, further triggering apoptosis; however, under certain conditions, suppression of caspase-8 or FADD increases the sensitivity to these cytokines, causing necroptosis\(^\text{86,87}\).

Necroptosis is regulated necrotic cell death that depends on the kinase activity of receptor-interacting protein kinase (RIPK) 1, RIPK3, and mixed lineage kinase domain-like pseudokinase (MLKL)\(^\text{88}\). Under necroptosis-inducing stimulus, activated RIPK1, RIPK3, and MLKL can form a complex termed the necrosome\(^\text{89}\). The cylindromatosis (CYLD) protein can deubiquitinate RIPK1, which is essential for necrosome formation in TNF-\(\alpha\)-induced necroptosis\(^\text{90}\).
Besides, CYLD is also a substrate for proteolysis by caspase-8, and caspase-8 inhibits necroptosis by processing CYLD\textsuperscript{91}. Necrostatin-1 (Nec-1) can specifically inhibit necroptosis\textsuperscript{92,93}.

Cerebral ischemia causes apoptosis of neuronal cells in the penumbra; however, necroptosis may occur even if the apoptotic signal is suppressed\textsuperscript{94}. Degterev \textit{et al.} showed that Nec-1 or its analogs could improve the neurological score and reduce the infarct volume in MCAO model mice\textsuperscript{93}. As cerebral ischemia occurs, microglial cells are activated and migrate to the ischemic regions before there is a response by any other cells in the brain. Activation of microglial cells releases cytokines such as TNF-\textalpha, TRAIL, and FasL, and binding of these death signals to their cell-surface membrane receptors triggers the recruitment of RIPK1 and other proteins to form complex I\textsuperscript{95} (Figure 5); subsequently, cellular inhibitor of apoptosis proteins promote the ubiquitination of complex I\textsuperscript{96-98}. At this time, the activity of caspase-8 determines the fate of neurons. However, caspase-8 activity is partially dependent on the level of ATP\textsuperscript{99}, which is insufficient for the maintenance of caspase-8 activity in the acute stage of cerebral ischemia due to the shortage of ATP production\textsuperscript{100}.

Thus, on one hand, caspase-8 inactivation prevents CYLD degradation, resulting in the deubiquitination of RIPK1\textsuperscript{91}, which is released from the deubiquitinated complex I to form complex II with FADD, TNF receptor 1-associated death domain protein, RIPK3, and caspase-8\textsuperscript{101}. On the other hand, activated caspase-8 cleaves and deactivates RIPK1 and RIPK3\textsuperscript{102}, which then bind to each other by the shared RIP homotypic interaction motif to cause oligomerization and autophosphorylation of RIPK3\textsuperscript{103}. Phosphorylated RIPK3 recruits and then phosphorylates MLKL\textsuperscript{104,105}, forming a complex termed the necrosome, which triggers the downstream signal cascade that eventually leads to necroptosis\textsuperscript{106}. Genetic and pharmacologic inhibition of RIPK1 can reduce...
ischemic brain injury\textsuperscript{107}; Nec-1 can only partially reduce infarct volume in MCAO model mice, but it has a synergistic effect with the anti-apoptotic peptide humanin, indicating that ischemic stroke injury is mediated by various forms of cell death\textsuperscript{108}.

2.3 Autophagy

Autophagy is the process by which organelles and cytosolic macromolecules are engulfed by membranes to form autophagosomes in which the engulfed materials are transported to the lysosome for digestion and recycling\textsuperscript{109}. All eukaryotic cells use autophagy to provide substrates for anabolism to prevent starvation and for use in various specialized purposes\textsuperscript{110,111}. It usually plays a role in preventing cell death, but if the level exceeds a certain threshold, it can cause cell death.

Autophagy can be induced by certain metabolic inhibitors as well as pathological conditions such as hypoxia or ischemia\textsuperscript{112}. It was found that autophagosomes accumulate in brain tissue shortly after ischemia-anoxia\textsuperscript{113,114}; moreover, in a rat model of severe neonatal cerebral hypoxia-ischemia, autophagy is significantly increased\textsuperscript{115}. It has been reported that autophagy occurs before apoptosis in PC12 neuronal cells following serum deprivation, a cell culture model of I/R\textsuperscript{116}. These observations suggest that autophagic processes occur after ischemic stroke.

During cerebral ischemia, insulin and amino acids are limited and cannot activate the primary inhibitor of autophagy, the mammalian target of rapamycin (mTOR) complex 1 (mTORC1)\textsuperscript{117-119}. The ratio between adenosine monophosphate (AMP) and ATP increases due to the same insufficient energy supply, activating AMP-activated protein kinase (AMPK) and thereby enhancing autophagy\textsuperscript{120}. On the other hand, mitochondrial dysfunction, accumulated ROS,
and subsequent DNA damage can occur during cerebral ischemia, which induces autophagy\textsuperscript{121}. Cerebral ischemia and hypoxia can also directly induce endoplasmic reticulum (ER) stress to increase the levels of unfolded proteins that enhance autophagy\textsuperscript{122,123}. Excessive intracellular Ca\textsuperscript{2+} in ischemia conditions also participates in autophagy by acting on the calmodulin-dependent protein kinase kinase/mTORC1 pathway\textsuperscript{124-126}. These regulatory pathways eventually induce neuronal autophagy in ischemic stroke (Figure 6).

2.3.1 mTOR signaling pathway-mediated autophagy

As the primary regulator of cellular metabolism, mTOR can promote cell growth following various environmental cues\textsuperscript{127}. mTOR forms two multiprotein complexes by binding with different companion proteins, namely mTORC1 and mTORC2\textsuperscript{127}. The phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling pathway plays a vital role in the acute neuronal injury observed in cerebral ischemia\textsuperscript{128-131}.

One of the recognized functions of mTORC1 is to promote cellular anabolism to provide the necessary foundation for cell growth and proliferation\textsuperscript{127}. mTORC1 can promote the synthesis of proteins, lipids, and nucleotides by integrating various stimuli and signaling networks and can block catabolic processes such as autophagy\textsuperscript{132,133}. Studies showed that oxygen, glucose, and growth factor levels are reduced after brain ischemia, inhibiting the activity of the PI3K/Akt pathway and leading to a decrease in mTORC1 activity\textsuperscript{134,135}. During cerebral ischemia, PI3K protein expression and the phosphorylation level of Akt were found to be significantly reduced\textsuperscript{136}.

In addition, inhibition of ATP production can also activate autophagy. Decreased ATP production stimulates AMPK, which is an early indicator of energy deprivation\textsuperscript{137}. A high AMP/ATP ratio can promote AMPK activation by
liver kinase B1\textsuperscript{138,139}. AMPK-mediated autophagy participates in the neuroprotection of ischemic preconditioning\textsuperscript{140}, indicating that the activation of AMPK plays a protective role in the ischemic brain\textsuperscript{141}, and that AMPK can be a target for preventing and treating ischemic stroke.

### 2.3.2 HIF-1α signaling pathway-mediated autophagy

Hypoxia-inducible factor 1 (HIF-1), which consists of an oxygen-regulated subunit HIF-1α and a constitutively expressed subunit HIF-1β\textsuperscript{142}, is essential for maintaining oxygen levels in mammalian cells\textsuperscript{143,144}. It is reported to be activated during ischemia in response to hypoxia\textsuperscript{145} and can induce the transcription of more than sixty genes. Many of these genes, such as those for vascular endothelial growth factor and erythropoietin (EPO), function to promote and increase oxygen delivery to hypoxic regions\textsuperscript{146,147}. HIF-1 responds to systemic oxygen levels by undergoing conformational changes and induces transcription by associating with hypoxia response elements in the promoters of hypoxia response genes\textsuperscript{148-152}. Moreover, HIF-1α can activate autophagy after ischemia by upregulating p53\textsuperscript{153}, possibly via the damage-regulated autophagy modulator pathway\textsuperscript{154}.

In the event of ischemic stroke, one crucial HIF-1α target is the gene for Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3), a member of a subfamily of Bcl-2 related proteins\textsuperscript{155}. BNIP3 can bind to and inhibit Rheb and activate autophagy by inhibiting the activation of mTORC1\textsuperscript{156}. BNIP3 is also a competitive substrate of Bcl-2 and Beclin-1 and therefore leads to the release of Beclin-1 and the activation of autophagy\textsuperscript{157,158}. It was observed that the Beclin-1 protein level increased as early as 4 hours and peaked at 24–72 hours after a rat model of severe neonatal cerebral hypoxia-ischemia\textsuperscript{159} and in rat ischemic brain tissue after MCAO\textsuperscript{160}. Moreover, Beclin-1 knockdown can
prevent neuronal injury after focal cerebral ischemia by inhibiting the activation of autophagy\textsuperscript{161}.

### 2.3.3 ER stress-mediated autophagy

Cell homeostasis can be disrupted due to various stimulating factors, due to which a series of cellular self-protecting events, including ER stress, are initiated for cell survival\textsuperscript{162}. As a consequence of cerebral ischemia\textsuperscript{163,164}, nutrient deficiencies and disruption of Ca\textsuperscript{2+} homeostasis may trigger ER stress, which can not only activate autophagy through the unfolded protein response, but also promote the release of Ca\textsuperscript{2+} from the ER into the cytoplasm\textsuperscript{165}, and is considered to be an important factor for neuronal ischemic injury\textsuperscript{123,166-168}.

### 2.4 Ferroptosis

Ferroptosis is defined as an iron-dependent form of regulated cell death\textsuperscript{169}. Morphologically, ferroptosis causes mitochondrial shrinkage, which is a unique feature that is distinguishable from other forms of cell death\textsuperscript{169}; moreover, it requires lipid peroxidation of polyunsaturated fatty acids (PUFAs)\textsuperscript{170,171}. In neurons, $\gamma$-L-glutamyl-L-cysteinylglycine (glutathione or GSH) peroxidase 4 (GPx4) can inhibit excessive lipid peroxidation, and inhibition of GPx4 activity triggers ferroptosis\textsuperscript{172-175}. Further study has shown that neuron-specific inducible knockout of GPx4 can trigger ferroptosis in motor neurons, which can be partially delayed by adding the lipid-based antioxidant vitamin E to diet\textsuperscript{176}.

During cerebral ischemia, there is rapid ATP loss and uncontrolled ion leakage across the cell membrane due to energy loss, resulting in membrane depolarization and release of neurotransmitters such as glutamate\textsuperscript{3,177,178}. Excessive release of glutamate and stimulation of its receptors leads to the activation of phospholipases\textsuperscript{3,179,180}, phospholipid hydrolysis, arachidonic acid
(AA) release\textsuperscript{181}, and the loss of GPx4 lipid peroxide repair capacity\textsuperscript{182,183}. The process also causes intracellular accumulation of redox-active iron\textsuperscript{184}. Ultimately, all of these may collectively lead to ferroptotic neuronal death after ischemic stroke (Figure 7).

Indeed, Tuo et al. have found that the level of soluble tau protein, which is implicated in Alzheimer’s disease and mediates iron transport\textsuperscript{184,185}, decreases in the ischemic region after transient focal ischemia in mice and rats, leading to iron accumulation and neuronal death\textsuperscript{184}. Ferroptosis inhibition (via ferrostatin-1 or liproxstatin-1) can prevent neuronal damage in MCAO model mice\textsuperscript{184}. Tat SelPep (a selenium-containing peptide) can inhibit ferroptosis by driving GPx4 expression and consequently reduce cerebral infarct volume significantly\textsuperscript{183}. Carvacrol, a natural monoterpene, can also protect hippocampal neurons against I/R injury in gerbils by increasing the expression of GPx4\textsuperscript{186}. Besides, one study has found that extract of Naotaifang, a compound traditional Chinese herbal medicine, can inhibit neuronal ferroptosis induced by acute cerebral ischemia in rats by increasing the levels of SLC7A11, GPx4, and GSH\textsuperscript{187}. These findings further suggest that ferroptosis may be essential in ischemic stroke.

### 2.4.1 Redox-active iron

Free iron and iron-containing lipoygenase (LOX) are crucial regulators of the oxidation of PUFAs-containing phospholipids (PL-PUFAs) that can form lipid ROS. The accumulation of iron has been implicated in several neurological disorders, including Alzheimer’s disease, Parkinson’s disease, and stroke\textsuperscript{188-192}. Excessive free intracellular iron and PL-PUFAs are the initiating factors of ferroptosis, while iron chelators, including deferoxamine (DFO), ciclopirox (CPX), and 2,2-bipyridyl (2,2-BP), have been shown to inhibit ferroptosis\textsuperscript{169,193,194}. Therefore, the regulation of iron is known to impact ferroptosis sensitivity.
The acidic environment in brain tissue after cerebral ischemia can inhibit the pH-dependent affinity of transferrin for iron, resulting in iron release from transferrin\(^\text{195}\). Unbound iron can then easily be taken up into neurons, causing intracellular iron accumulation\(^\text{196}\). Furthermore, decreased iron efflux following ischemic stroke may also be responsible for iron accumulation\(^\text{184,197-203}\). Clinical studies have shown that children with severe ischemia-hypoxia injury have significantly increased iron levels in multiple areas of the brain\(^\text{204}\).

Additional iron deposition may also occur more rapidly due to the direct damage of iron-catalyzed lipid peroxidation degradation products\(^\text{204}\). Studies have shown that iron deposition, lipid peroxidation, and neuronal death in the brain were significantly increased in an adult rat model of ischemic stroke\(^\text{205,206}\). The levels of ferritin and iron have also been found to increase significantly after focal cerebral I/R, and iron levels correlate with those of ferritin, especially in the macrophage- and microglia-positive regions where cell death or tissue necrosis was noted\(^\text{207}\). Different types of iron-chelating compounds or iron-transferring compounds have been tested in models of global or focal cerebral ischemia and have shown neuroprotective effects\(^\text{184,199,206,208-217}\), strongly supporting iron as a therapeutic target for ischemic stroke.

2.4.2 Loss of lipid peroxide repair

Iron-catalyzed PL-PUFA oxidation has a cytotoxic effect, which is why this process is strictly controlled. GPx4 is a selenoenzyme specifically utilized to reduce lipid hydroperoxides to lipid alcohols in membranes\(^\text{218}\). As it is an essential selenoprotein for normal mammalian development\(^\text{175,219}\), inactivation of this enzyme induces ferroptosis, and the deletion of GPx4 is lethal to mice\(^\text{173,220,221}\). Selenium can prevent the ferroptosis caused by increased GPx4 activity\(^\text{175}\); it can also upregulate GPx4 expression through driving the transcriptional activators TFAP2c and Sp1, resulted in ferroptosis inhibition\(^\text{175}\).
On the other hand, GPx4 activity also can be regulated by GSH. As an essential intracellular antioxidant itself, GSH is synthesized from glutamate, cysteine, and glycine in two steps through the ATP-dependent cytosolic enzymes glutamate-cysteine ligase and GSH synthetase\textsuperscript{190,192,193}. The availability of cysteine can limit the rate of GSH synthesis\textsuperscript{222}, and therefore cysteine starvation can result in a reduction of GPx4 activity\textsuperscript{223,224}. GPx4 converts the reduced form of GSH to its oxidized form while reducing lipid hydroperoxides to their corresponding alcohols\textsuperscript{225}. The same reaction can also reduce free hydrogen peroxide to water. The intracellular cysteine level mainly depends on extracellular cystine uptake by system X\textsubscript{c}\textsuperscript{−}\textsuperscript{226}, which consists of the light chain subunit SLC7A11 and the heavy chain subunit SLC3A2. The level of GSH is significantly reduced in mouse ischemic brain tissue after MCAO, and L-2-oxothiazolidine-4-carboxylic acid (OTC), a precursor of cysteine, can reduce neuronal injury after MCAO through restoring depleted GSH levels\textsuperscript{227}. The levels of SLC7A11 and GPx4 were also found to be significantly decreased in MCAO rats compared with those with sham operation\textsuperscript{187}.

Exogenous lipophilic antioxidants (e.g., ferrostatin-1, liproxstatin-1) can prevent the spread of oxidative damage in membranes, thereby effectively inhibiting ferroptosis\textsuperscript{169,220,228}. In short, a key trigger of ferroptosis is the loss of the repair system that eliminates lipid hydroperoxides from PUFA-PLs\textsuperscript{170}. Studies show that the levels of GSH and GPx4 are markedly reduced, while those of lipid peroxide are significantly increased, in rodent models of ischemic stroke\textsuperscript{186,187}. Small-molecule lipophilic antioxidants (e.g., vitamin E, CoQ10) also help detoxify membrane lipid ROS\textsuperscript{172,229,230}. In vivo studies have demonstrated that oral supplementation of \textalpha{-}tocotrienol, a natural form of vitamin E, protected against ischemic stroke\textsuperscript{231,232}. CoQ10 supplementation can also efficiently improve functional deficits and reduce cerebral infarct volumes after MCAO in rats\textsuperscript{233,234}.
2.4.3 Oxidation of PUFA-containing phospholipids

AA is a PUFA that can be obtained directly from dietary sources or indirectly from linoleic acid. AA is the precursor of eicosanoids, which are produced by oxidation through the cyclooxygenase (COX) and LOX pathways. Due to the pro-inflammatory and pro-ROS activities of eicosanoids, cells promote their esterification into cellular lipids in order to have minimal cytoplasmic fatty acids levels. Under physiological conditions, AA is usually esterified into the sn-2 position of glycerophospholipids, especially phosphatidylethanolamine (PE), phosphatidylcholine, and phosphatidylinositol. AA is an intermediate product of the membrane PL deacylation/reacylation cycle, in which it is released by cytosolic phospholipase A2α (cPLA2α). cPLA2α, a Ca²⁺-dependent cytosolic enzyme, can cleave AA to release free AA and lysophospholipid. Free AA is converted to arachidonoyl-CoA by acyl-CoA synthetase long-chain family member 4 and immediately incorporated into PLs by lysophosphatidylcholine acyltransferase.

Unlike saturated fatty acids and monounsaturated fatty acids, PUFAs have bis-allylic hydrogen atoms that are prone to free radical- as well as enzyme-mediated oxidation. PUFA oxidation is a critical step in ferroptosis during cellular response to diverse stimuli. The oxidation of arachidonoyl (C20:4) fatty acyl chains in the context of PE PLs (i.e., PUFA-PE) is linked explicitly with ferroptosis. PE PLs can assemble in a non-bilayer arrangement that facilitates pro-ferroptotic oxidation of PUFA-PE by 12/15-LOX. This non-heme iron-containing dioxygenase catalyzes the stereo-specific peroxidation of esterified PUFAs, resulting in the generation of bioactive lipid peroxides. It has been demonstrated that cells are resistant to ferroptosis when 12/15-LOX is knocked down using siRNAs. The expression and activity of 12/15-LOX increases significantly in the ischemic mouse brain.
moreover, 12/15-LOX colocalizes with MDA2 (a marker for oxidized lipids)\textsuperscript{246,247}, and neurons in 12/15-LOX knockout mice are protected against cerebral ischemic injury\textsuperscript{247,248}. These results indicate that 12/15-LOX is a critical regulator of ferroptosis in neuronal damage due to ischemic stroke, and can be considered as a therapeutic target for ischemic stroke.

cPLA2α phospholipid hydrolysis controls the availability of free PUFA to initiate the conventional LOX-based biosynthesis of lipid mediators\textsuperscript{249}. Clinical studies have shown that higher serum lipoprotein-associated phospholipase A2 (Lp-PLA2) activity in the acute period of ischemic stroke is a risk factor for recurrent vascular events\textsuperscript{250-253}, and that patients in the elevated Lp-PLA2 group had a significantly higher risk of all-cause mortality within one year of acute ischemic stroke\textsuperscript{254}. In mice, neuronal cPLA2α level was found to have doubled immediately after reperfusion and normalized 2 hours post-reperfusion, and the increase in ROS was significantly higher in the ischemic core of cPLA2α\textsuperscript{+/+} mice than that in cPLA2α\textsuperscript{-/-} mice\textsuperscript{255}. Furthermore, following cerebral ischemia and reperfusion, increased glutamate release activates NMDARs to increase neuronal Ca\textsuperscript{2+} flux, which may also contribute to the activation of cPLA2α\textsuperscript{256,257}. Consequently, cPLA2α knockout or inhibitor administration significantly limited infarction volume following cerebral ischemia\textsuperscript{180,258-262}. These results indicate that cPLA2α can modulate the early injury-related molecular responses after cerebral ischemia, suggesting that cPLA2α inhibitors have potential clinical value in the treatment of ischemic stroke.

In response to cerebral ischemia, AA released from phospholipids by cPLA2α is also immediately converted to prostaglandin H2 (PGH\textsubscript{2}) by COX (including COX-1 and COX-2), and short-lived PGH\textsubscript{2} is further rapidly converted into prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) that signals through four G protein-coupled receptors (GPCRs), EP1–EP4\textsuperscript{263}. Among the GPCRs, EP2 receptor level was found to be
highly elevated in neurons in the ischemic hemisphere, and postnatal deletion of the neuronal EP2 receptor in mice reduced cerebral ischemic injury\textsuperscript{264}. TG6-10-1, an antagonist of the PGE\textsubscript{2} receptor EP2, can reduce neurological deficits and infarct volumes after transient ischemia\textsuperscript{265}. Therefore, small molecules targeting the COX/prostanoid signaling cascade may guide current reperfusion therapy towards treating acute cerebral ischemia with higher specificity and a longer therapeutic window\textsuperscript{266}.

2.4.4 Oxytosis

In 2001, oxytosis was proposed as a new form of non-apoptotic regulated cell death, with a focus on highlighting the involvement of ROS accumulation\textsuperscript{267}. Further studies have shown that ferroptosis and oxytosis share several molecular features and pathways\textsuperscript{190,268}. For example, oxidative stress and GSH depletion are typical features of both oxytosis and ferroptosis\textsuperscript{269}. As with ferroptosis, oxytosis in HT-22 cells can be inhibited by iron chelators and exacerbated by different sources of iron\textsuperscript{270,271}. Thus, both oxytosis and ferroptosis are dependent on iron, indicating that they may be similar or even the same at the molecular level. Many of the results discussed previously can also be interpreted as evidence supporting the role of oxytosis in ischemic stroke.

2.5 Parthanatos

Parthanatos is a form of regulated cell death that depends on PARP1 and can be activated by oxidative stress-induced DNA damage and chromatinolysis\textsuperscript{272}. Unlike apoptosis, parthanatos does not result in the formation of apoptotic bodies and small DNA fragments\textsuperscript{273}. Parthanatos can also occur without cell swelling, but is accompanied plasma membrane rupture\textsuperscript{274}. PARP1, the critical mediator of parthanatos, is a nuclear, chromatin-associated protein that functions to recognize and then repair single- or double-strand DNA breaks\textsuperscript{275,276} by using
nicotinamide adenine dinucleotide (NAD\(^+\)) and ATP to trigger poly (ADP-ribose)-sylation\(^{277}\).

The translocation of AIF from mitochondria to the nucleus is necessary for parthanatos and chromatin degradation\(^{278}\). Poly (ADP-ribose), an enzymatic product of PARP1, can induce nuclear translocation of AIF, and causes the collapse of bioenergy homeostasis by inhibiting the glycolytic enzyme hexokinase, leading to necrosis\(^{279,280}\). NAD\(^+\) depletion caused by excessive PARP1 activity further impairs cellular metabolic processes, thereby promoting cell death\(^{279}\). During cerebral ischemia, excitotoxicity may generate peroxynitrite and other oxidants, which is followed by DNA damage and PARP1 activation. Studies in various animal models of ischemic stroke have shown that PARP1 inhibitors or PARP1 deletion can protect against neuronal death caused by cerebral ischemia\(^{281-287}\), suggesting that parthanatos may be a possible neuronal death mechanism in ischemic stroke (Figure 8).

During cerebral ischemia, the over-stimulation of NMDARs by glutamate causes a massive influx of Ca\(^{2+}\), which activates neuronal nitric oxide (NO) synthase (nNOS) with calmodulin\(^{288}\). nNOS is tethered to NMDARs by postsynaptic density protein-95 (PSD-95)\(^{289,290}\), and focal cerebral ischemia can induce the interaction of nNOS with PSD-95\(^{291}\). nNOS activation can also result in excessive NO generation, which in turn can promote reactive nitrogen species generation to cause oxidative or nitrosative injury to DNA\(^{292-294}\). Disruption of the nNOS-PSD-95 interaction can prevent glutamate-induced excitotoxicity and ischemic brain injury\(^{291,295,296}\).

NAD\(^+\) is a substrate for PARP-1, and when DNA damage occurs, PARP1 is promoted to bind acceptor proteins (hetero-modification) or PARP1 itself (auto-modification)\(^{297,298}\). AIF then binds to the PARP1 polymer, inducing AIF release from mitochondria. A PARP-1-dependent, AIF-associated nuclease,
migration inhibitory factor (MIF), is then recruited by AIF, and the complex translocates to the nucleus, where MIF cleaves the DNA, causing chromatin condensation and eventually leading to neuronal cell death. Targeting MIF (by depletion), its nuclease activity, or the AIF-MIF interaction have all been shown to reduce chromatinolysis and neuronal death induced by cerebral ischemia in mice.

2.6 Phagoptosis

Microglia are the resident mononuclear phagocytes of the central nervous system and can engulf whole neurons within hours. They continuously survey the brain parenchyma to detect any alterations in their microenvironment. Since it can reduce inflammation, it is beneficial for dead and dying neurons to undergo phagocytosis. Live neurons can also undergo the same process, to result in the death of the engulfed neurons, termed phagoptosis. The essential characteristic differentiating phagoptosis from other cell death pathways is that cell death can be prevented by the inhibition of phagocytosis.

Recognition, engulfment, and digestion are the three main steps in phagoptosis. Microglia constantly monitor the surface of neurons through direct somatic contacts and can rapidly recognize and engulf neurons that expose ‘eat-me’ signals. One of the most typical “eat me” signals is the presence of phosphatidylserine (PS) on the cell surface. PS is usually restricted to the inner leaflet of the plasma membrane, and its exposure may occur due to the inhibition of PS translocases due to oxidative stress, increased Ca$^{2+}$ levels, or ATP depletion. Neurons with exposed PS are recognized either by the opsonin milk fat globule EGF-like factor 8 (MFG-E8) and vitronectin receptors or the opsonin growth arrest-specific factor 6 and Mer...
receptor tyrosine kinase (MERTK) receptors\textsuperscript{308}. Alternatively, the presence of calreticulin on the cell surface, as a result of ER stress, can also serve as an ‘eat me’ signal\textsuperscript{320} by evoking phagocytosis via the microglial low-density lipoprotein-receptor-related protein\textsuperscript{321}. Moreover, microglia and astrocytes can produce C1q and C3, both of which can induce phagocytosis\textsuperscript{322,323} (\textit{Figure 9}).

Cerebral ischemia causes neuronal death directly in regions with deficient oxygen levels. However, in case of a mild ischemic event, neurons may survive under stress. Studies have shown that PS is exposed on the surface of neurons in the peri-infarct areas in a reversible manner\textsuperscript{324}. Neher \textit{et al.}\textsuperscript{308} found that inhibiting phagocytosis can prevent the death of functional neurons after transient cerebral ischemia. After focal cerebral ischemia \textit{in vivo}, microglia were found to transiently upregulate two phagocytic proteins, MERTK and MFG-E8, both of which mediate PS recognition. Surprisingly, due to the inhibition of neuronal phagocytosis, deficiency in either protein can completely prevent long-term functional motor deficits after cerebral ischemia and significantly reduce brain atrophy. \textit{In vitro} experiments have shown that non-toxic levels of glutamate lead to the transient exposure of neurons to PS, thereby promoting MERTK and MFG-E8-dependent phagocytosis. Alawieh \textit{et al.} have shown that B4Crry, a complement inhibitor, can prevent the phagocytosis of penumbral neurons after ischemic stroke\textsuperscript{325}. Therefore, blocking phagocytosis after mild ischemia appears to be beneficial.

2.7 Pyroptosis

In addition to direct neuronal death pathways, indirect pathways can also cause neuronal death during cerebral ischemia. Caspase-1 mediated microglial pyroptosis is accompanied by the release of a large number of proinflammatory factors that induce neuronal death\textsuperscript{326-328} (\textit{Figure 10}). IL-1β is one of the main proinflammatory cytokines mediating neuroinflammation in the brain, and can
directly induce neuronal cell death\textsuperscript{329}. Cerebral ischemia can initiate microglial inflammatory responses and result in the formation of multi-protein complexes known as inflammasomes, typically consisting of one of several sensor proteins (absent in melanoma 2 and the NLR family, pyrin domain containing [NLRP]1, NLRP3, and NLRP4 proteins), which then process these inflammatory signals to trigger an effector response\textsuperscript{330}. Inflammasomes recruit apoptosis-associated speck-like protein, an adaptor protein containing a caspase recruitment domain, which then recruits and activates pro-caspase-1 such that it autocleaves to give rise to active caspase-1\textsuperscript{330}. Once active, caspase-1 can cleave both pro-IL-1β and pro-IL-18 into biologically active, mature, pro-inflammatory cytokines that are released into the extracellular environment, thereby causing toxicity to neuronal cells\textsuperscript{331}. Inhibition or knockout of caspase-1 is neuroprotective in focal stroke models\textsuperscript{332-335}. In a mouse MCAO model, mice lacking IL-1β exhibited dramatically reduced ischemic infarct volumes compared with wild-type in MCAO model. Administration of a recombinant IL-1 receptor antagonist significantly reduced infarct volume in WT mice after MCAO\textsuperscript{336}.

3. POTENTIAL NEUROPROTECTIVE TARGETS FOR ISCHEMIC STROKE THERAPY

The primary goal of ischemic stroke therapy is to quickly restore cerebral blood flow, which is also a prerequisite for subsequent neuroprotective therapies. To date, r-tPA is the only FDA-approved therapeutic for ischemic stroke\textsuperscript{337}. Recent research has shown that r-tPA can improve long-term functional outcomes by promoting brain plasticity in a clinically relevant ischemic stroke model\textsuperscript{50}. However, the proteolytic activities of r-tPA can induce side effects such as blood-brain barrier breakdown and intracranial hemorrhage\textsuperscript{338}, and reduce the therapeutic time window\textsuperscript{339}. I/R injury is also a common occurrence after blood
flow is restored\textsuperscript{340-342}, which is why the identification of novel neuroprotectants remains imperative.

Currently, the major challenge in this context is that most neuroprotective agents that are efficacious in animal models have failed in clinical trials\textsuperscript{343}. As summarized above, several mechanisms that can potentially account for neuronal death may be appropriate therapeutic targets in this regard. However, many inhibitors of cell death have failed in trials where neuroprotection was the primary end-point, suggesting that the understanding of how neurons die due to ischemic stroke remains incomplete. It is possible that multiple pathways of cell death occur synergistically or sequentially, and that targeting these pathways individually may not be sufficient. There is, therefore, an urgent need to review the recent and current attempts at drug discovery, and to bridge the gap with clinical findings, to guide future stroke therapy.

3.1 Strategies targeting excitotoxicity

Excessive activation of NMDARs under cerebral ischemia leads to excitotoxicity and triggers neuronal death events\textsuperscript{344-346}. As a specific type of glutamate-mediated neurotoxicity, excitotoxicity may be a link between ischemia and neuronal death. Excitotoxicity causes free radical generation, mitochondrial dysfunction, and changes related to the functions of various transcription factors as activators of gene expression\textsuperscript{27}, which in turn activates cell death pathways. The synergistic effect of all these factors can cause extensive damage to cellular proteins, lipids, and DNA, leading to the deterioration of cellular architecture and signaling and resulting in various cell death forms depending on the severity of the insult and the relative speed of each process\textsuperscript{287,347-352}. However, proper NMDAR activation also is essential for the development and survival of neurons\textsuperscript{353-355}. Therefore, the overall inhibition
of NMDAR cannot effectively reduce the excitotoxicity caused by cerebral ischemia.\textsuperscript{356}

Recent therapeutic development has focused on blocking subtypes\textsuperscript{357,358} or subpopulations\textsuperscript{359} of NMDARs for neuroprotection. The tetrameric NMDAR consists of two essential GluN1 subunits and either two GluN2 or, in relatively rare cases, GluN3 subunits.\textsuperscript{360} Tu et al. have shown that death-associated protein kinase 1 (DAPK1) is recruited by the GluN2B subunit during cerebral ischemia.\textsuperscript{361} DAPK1 phosphorylates the GluN2B subunit at Ser-1303 and in turn enhances GluN1/GluN2B receptor channel conductance. Genetic ablation of DAPK1 or GluN2B(CT) (GluN2B C-terminal tail consisting of amino acids 1292–1304) administration can block harmful Ca\textsuperscript{2+} influx through NMDA channels at extrasynaptic sites and subsequently protect neurons from cerebral ischemic damage. Tang et al. have further shown that GluN2B mutation, which effectively disrupts the DAPK1-GluN2B interaction and inhibits extra-synaptic NMDAR currents, can prevent stroke-related damage \textit{in vitro} as well as \textit{in vivo}, thereby improving behavioral performance.\textsuperscript{362} It was also found that overexpression of miR-223 reduced the levels of GluA2 and GluN2B, inhibited NMDA-induced Ca\textsuperscript{2+} uptake in hippocampal neurons, and ameliorated ischemic injury.\textsuperscript{363} Therefore, selective inhibition of GluN2B or its downstream signaling pathways can be valid targets for ischemic stroke.

A few novel NMDAR antagonists, including ifenprodil, its derivative eliprodil, and traxoprodil, have been shown to protect against ischemic damage \textit{in vitro} and \textit{vivo}.\textsuperscript{357,358,364-368} These compounds were designed to effectively avoid the potential neurological side effects observed with traditional NMDAR antagonists.\textsuperscript{27} The injection of capsaicin, which causes the pungent taste of chili peppers and is known to reduce pain,\textsuperscript{369} directly into the peri-infarct area improved motor coordination function, recovered the neurological score, and

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reduced infarct volume in a MCAO/reperfusion rat model through reducing the expression of GluN2B\textsuperscript{370}.

ASIC1a gene knockout also protects the brain from ischemic injury\textsuperscript{371}. Specific ASIC1a blockade by the tarantula toxin psalmotoxin (PcTX) reduced infarct volume following ischemic stroke in rodents\textsuperscript{371,372}. Hi1a, a disulfide-rich spider venom peptide and an ASIC1a inhibitor, has also shown similar neuroprotection in MCAO rats\textsuperscript{373}. Similarly, β-estradiol has been shown to protect neurons against acidosis-mediated neurotoxicity and ischemic brain injury via suppressing ASIC1a protein expression and channel function\textsuperscript{374}.

Fluoxetine is a widely used antidepressant drug that can selectively block GluNR2B-containing NMDARs\textsuperscript{375} and has a protective effect in ischemic stroke\textsuperscript{376-378}. A double-blinded placebo-controlled trial found that early prescription of fluoxetine in combination with physiotherapy improved motor recovery three months after the onset of the disease in stroke patients with moderate to severe motor dysfunction\textsuperscript{379}. Moreover, a randomized controlled clinical study revealed that fluoxetine could significantly reduce the three-year recurrence rate when administered for 90 days\textsuperscript{380}. Collectively, this evidence suggests that NMDAR inhibition may be worth further clinical investigation.

The ENACT trial\textsuperscript{381} evaluated intravenous NA-1 (Tat-NR2B9c) treatment after endovascular surgery. NA-1 is a PSD-95 inhibitor that can link NMDAR to neurotoxic signaling in neurons\textsuperscript{382}. Supported by experimental evidence showing that NA-1 is neuroprotective for transient or permanent cerebral ischemia in non-human primates\textsuperscript{383}, the aim of the trial was to prevent glutamate-related toxicity in patients with intracranial aneurysms. Results from the ESCAPE-NA1 phase III clinical trial\textsuperscript{384} showed that NA-1 had a neuroprotective effect in the acute stroke group in which subjects were treated up to 12 hours after stroke symptom emergence; however, patients who had
been administered alteplase did not appear to benefit, likely due to a reduction of NA-1 plasma levels when alteplase was administered first. In the stratum that did not receive alteplase, 130 of 219 patients (59.3%) who received NA-1 achieved modified Rankin Scale (mRS) scores of 0–2, compared with 113 of 227 (49.8%) who received the placebo. The trial also showed that NA-1 reduced the mortality rate of stroke from 19% to 11%, highlighting its potential in stroke treatment.

3.2 Strategies targeting Ca\(^{2+}\) overload

Neuronal Ca\(^{2+}\) overload causes extensive cellular damage in ischemic stroke. Various Ca\(^{2+}\) channel blockers (e.g., lercanidipine, nicardipine, cilnidipine) have protective effects on ischemic brains in animal models of ischemic stroke\(^{385-388}\). Pertussis toxin, a G-protein blocker, was also shown to protect against ischemic stroke by blocking Ca\(^{2+}\) influx\(^{389}\).

Verapamil is a Ca\(^{2+}\) channel blocker that is used to treat cerebral vasospasm\(^{390,391}\). Intra-arterial administration of verapamil provided significant protection against neuronal damage in a mouse model of stroke when compared with intra-arterial vehicle injection immediately after middle cerebral artery recanalization\(^{392}\). The drug is well-tolerated and causes no hemorrhage or systemic side effects in mice\(^{392}\). A phase I clinical trial recently concluded that intra-arterial verapamil administration immediately after thrombectomy is safe and feasible and has direct dose-dependent benefits in cerebral ischemia\(^{393}\).

The expression and activity of Rho-associated protein kinase (ROCK), which is known to regulate Ca\(^{2+}\) intake\(^{394}\), are increased in axons at the early stage of cerebral ischemia\(^{395}\). Fasudil, a ROCK inhibitor, has been shown to prevent neuronal damage in vivo by restoring cerebral blood flow\(^{396}\). Hydroxyfasudil (the primary metabolite of fasudil) can also inhibit OGD-induced neuronal cell
death, and both compounds can independently reduce glutamate-induced neurotoxicity in vitro. However, fasudil treatment may cause certain adverse effects, as it can induce anxiety-like behaviors due to ROCK1 and/or ROCK2 inhibition.

3.3 Strategies targeting neuronal cell death pathways

3.3.1 Targeting apoptosis

In ischemic stroke, apoptosis can persist from a few days to several weeks after the onset of the ischemic event. Caspases are critical mediators of apoptosis and neurodegeneration in ischemic stroke. For example, estradiol, a known neuroprotective factor, can attenuate injury-mediated DNA fragmentation after MCAO by reducing caspase activity. Therefore, caspases are logical therapeutic targets for ischemic stroke, but they have never been evaluated clinically due to the lack of ideal drug candidates. The calpain inhibitor SNJ-1945 has good membrane permeability and water solubility, and can decrease infarct volume and improve neurological deficits when injected intraperitoneally 1 hour after MCAO. Moreover, its neuroprotective effects can be detected even up to 6 hours after MCAO.

The classic NMDAR antagonist memantine, an Alzheimer's disease drug, can also significantly reduce neurological deficits and cerebral infarction caused by ischemic stroke as well as neuronal death induced by ATP depletion. The neuroprotective effect of memantine is related to its ability to significantly inhibit the activation of the calpain/caspase-3 pathway and apoptosis such that it can attenuate brain damage and neuronal loss in MCAO rats. These results highlight the possibility of utilizing memantine for treating ischemic stroke.

Tongxinluo, a traditional Chinese medicine, is used for the treatment of vascular diseases in East Asia, and can effectively protect against I/R injury via
the calpain/caspase-3 pathway. Salvianolic acid A, a compound obtained from the Chinese herb *Salvia miltiorrhiza* Bunge (Labiatae), has also been reported to ameliorate neuronal damage after cerebral ischemia in parallel with decreased infarct volume and neurological deficits through inhibiting calpain proteolytic activity.

EPO has been shown to protect primary cultured neurons against NMDAR-mediated glutamate toxicity. Recombinant human EPO can markedly reduce cortical infarct volume as well as neurologic impairment after MCAO in rats, which may be due to a reduction in microglial activation and the number of apoptotic cells. Intraventricular EPO infusion reduced synaptic degeneration and neuronal loss in a gerbil model of forebrain ischemia. Adding soluble EPO receptor can rapidly reverse the neuroprotective effects of EPO infusion and result in exacerbation of the neuronal damage. Elevated Bcl-x and reduced caspase-3 and caspase-9 levels in neurons after EPO treatment are suggested to be involved in the protective effect. A randomized clinical trial has shown that the administration of high-dose EPO in the first 24 hours after stroke onset is efficient in reducing ischemic stroke complications. However, some results have been contradictory—a large-scale phase II/III clinical trial (the German Multicenter EPO Stroke Trial) has concluded that EPO treatment provides limited benefits in terms of the Barthel Index and other outcome parameters, while in an exploratory subgroup analysis, investigators found that stroke patients who do not undergo thrombolysis may benefit from EPO treatment during the recovery stage.

### 3.3.2 Targeting necroptosis

Necroptosis inhibitors mainly include the RIPK1 inhibitors Nec-1, 3, 4, 5; RIP3K inhibitors GSK’843 and GSK’872; and MLKL inhibitor necrosulfonamide. Intracerebroventricular administration of Nec-1 markedly
ameliorates neuronal damage after MCAO\textsuperscript{93}; Nec-1 can also significantly reduce the expression of phosphorylated RIPK1 in ischemic brains\textsuperscript{411}. DTIO, a novel analog of Nec-1, can both reduce infarct volume and improve neurological deficits during the acute phase after cerebral I/R, and attenuate brain atrophy and facilitate the functional recovery of neurons in the chronic phase\textsuperscript{412}. Nevertheless, clinical evidence regarding the efficacy of necroptosis inhibitors for ischemic stroke is lacking.

### 3.3.3 Targeting autophagy

As discussed previously, autophagy is known to be involved in regulating brain homeostasis in ischemic stroke. However, there is no consensus on the exact functions and influence of autophagy in cerebral ischemia. The autophagy inhibitor 3-methyladenine can significantly lower the expression of autophagy-related genes. In contrast, the autophagy inducer rapamycin (an mTOR inhibitor and autophagy activator) can reverse these effects during hypoxia/reoxygenation or I/R-induced brain injuries\textsuperscript{413}.

Melatonin acts as a neuroprotective agent in the central nervous system. Feng \textit{et al.} revealed significantly elevated ER stress and autophagy activation in the MCAO mouse model\textsuperscript{123}, and that pre-ischemic melatonin treatment can reduce the I/R-induced ER stress and autophagy. Melatonin significantly alleviated cerebral infarction, brain edema, and neurological deficits, whereas tunicamycin (an ER stress activator) or rapamycin reversed the neuroprotective effects. Therefore, pre-ischemic melatonin administration can significantly prevent cerebral injury after ischemic stroke through ER stress-dependent autophagy inhibition.
3.3.4 Targeting ferroptosis

Ferroptosis is also a form of neuronal death occurring in ischemic stroke, first reported in 2017\textsuperscript{184}. Interestingly, three hallmarks of ferroptosis—redox-active iron, loss of lipid peroxide repair, and oxidation of PUFA-containing phospholipids—were already being targeted for treatment of ischemic stroke without the knowledge of ferroptosis.

12/15-LOX catalyzes the stereo-specific peroxidation of esterified PUFAs to generate bioactive lipid peroxides. Many 12/15-LOX inhibitors (e.g., ML351, LOXBlock-1, zileuton, BW-B 70C, baicalein) have been shown to significantly reduce infarct volume and alleviate brain injury after ischemic stroke\textsuperscript{248,414-418}. Liu \textit{et al.} demonstrated that OTC (a precursor of cysteine) could protect neuronal cells from OGD-induced death\textsuperscript{227}. Furthermore, the administration of OTC can significantly reduce cerebral infarct injury through increasing GSH levels in the penumbral cortex in mice after I/R.

Because of the pathological and pathophysiological links between iron and ischemic stroke, iron chelators, including DFO, CPX, and 2,2-BP, have been extensively investigated for treating the disease\textsuperscript{209,211,212,214,215,419-422}. One of the most commonly studied chelators, DFO, has been shown to reduce brain injury and promote functional recovery in the MCAO model in rats and mice\textsuperscript{209,211}. In other studies, iron regulatory proteins such as transferrin and ceruloplasmin have also been shown to attenuate neuronal damage in models of ischemic stroke\textsuperscript{184,202,217}. However, in contrast to animal model studies, very few clinical studies have examined the use of iron chelators in stroke. One preliminary study on three ischemic stroke cases and four hemorrhagic stroke cases has shown that DFO treatment (500 mg per day for three days) is associated with lower serum levels of hydroperoxides and lipoperoxides\textsuperscript{423}. Unfortunately, a phase II clinical trial entitled “Thrombolysis and Deferoxamine in Middle Cerebral Artery
Occlusion” was completed in 2012 without the results being released (ClinicalTrials.gov identifier: NCT00777140). Thus, carefully designed large-scale studies are warranted to assess the safety and efficacy of iron chelation therapy in stroke patients.

After the discovery of ferroptosis, inhibition of the process was tested in animal models of stroke. Both ferrostatin-1 and liproxstatin-1 can significantly reduce infarct volume and improve neurological deficits in the MCAO mouse model\textsuperscript{184}. Selenium-containing compounds have also been shown to reduce infarct volume significantly in the MCAO mouse model\textsuperscript{183,424}. Guan et al. showed that carvacrol protected against cerebral I/R injury in gerbils by increasing the expression of GPx4\textsuperscript{186}. These findings further support the hypothesis that ferroptosis may be one of the main signal pathways of neuronal death in ischemic stroke, although this has not yet been clinically confirmed.

3.3.5 Targeting parthanatos

Propofol is a widely used anesthetic agent that can protect against cerebral I/R injury via suppression of parthanatos through amelioration of mitochondrial and ER swelling\textsuperscript{425}. Iduna, a PARP-binding protein, can provide \textit{in vivo} protection against MCAO in mice by interfering with poly (ADP-ribose)-dependent signaling events\textsuperscript{287}. The PARP inhibitor HYDAMTIQ can reduce infarct volumes and attenuate neurological impairment after MCAO; it can also decrease the post-ischemic accumulation of poly (ADP-ribose) in the ischemic cortex\textsuperscript{286}. These findings provide evidence to suggest that parthanatos could be an effective therapeutic target for ischemic stroke.
3.3.6 Targeting phagoptosis

The complement system plays a significant role in microglial phagocytosis\textsuperscript{426}. C1-INH was the first complement inhibitor to be approved for clinical use; it inhibits both classical and lectin-based pathways of the complement cascade\textsuperscript{427,428}. Studies have shown that C1-INH can reduce brain infarct volume and attenuate neurological deficits during the acute as well as later stages of ischemic stroke\textsuperscript{429-431}. Human recombinant C1-INH can significantly reduce neuronal damage up to 18 hours post transient ischemia and up to 6 hours post permanent ischemia, indicating a broad therapeutic window\textsuperscript{432}.

Alawieh \textit{et al.} have shown that the complement inhibitor B4Crry improves long-term motor and cognitive recovery\textsuperscript{325}, which is mediated by microglial phagocytosis of neurons that are stressed but salvageable. B4Crry can inhibit complement deposition both locally and transiently, which ultimately inhibits microglial activation. It was determined that the therapeutic window for B4Crry treatment could be up to 24 hours post-stroke in mice, which is promising for future clinical investigations.

3.3.7 Targeting pyroptosis

Activated caspase-1 in inflammasomes can trigger pyroptosis during cerebral ischemia\textsuperscript{330,433,434}. VX-765, a caspase-1 inhibitor, can ameliorate infarct volumes, neurological deficits, and neuronal injury after MCAO\textsuperscript{435}. The specific NLRP3 inflammasome inhibitor MCC950 significantly improved neurologic outcomes and reduced infarct volume when administered before or after MCAO\textsuperscript{436}. Studies have also shown that meisoindigo, a generally water soluble and well-tolerated second-generation indirubin derivative, reduces inflammation by inhibiting leukocyte chemotaxis and migration\textsuperscript{437,438}. Meisoindigo post-treatment alleviates brain damage induced by ischemic stroke \textit{in vivo} and in
\textit{in vitro} model experiments through blocking the activation of the NLRP3 inflammasome\textsuperscript{439}. These findings suggest, to some extent, that pyroptosis may be a therapeutic target for ischemic stroke.

4. CURRENT CLINICAL TRIALS AND FUTURE PERSPECTIVES

As summarized above, while a significant number of drug candidates have been preclinically tested for the treatment of ischemic stroke, only a small proportion have proceeded to clinical testing for neuroprotective efficacy. Of all the trials that have concluded trials in the last decade (Table 1, sourced from \textit{ClinicalTrials.gov}), only those on a few antioxidants and statins met their clinical endpoints, in addition to those on thrombolytic agents and anticoagulants in phase III or IV clinical trials.

Edaravone, a free radical scavenging antioxidant, was approved for treating acute ischemic stroke by the Japanese authorities in 2001 and was subsequently recommended in the Japanese Guidelines for Stroke in 2004. Edaravone can inhibit neuronal death, counteract microglia-induced neurotoxicity, and reduce long-term inflammation\textsuperscript{440}. It may also inhibit LOX, an essential player in ferroptosis, and therefore prevent the oxidation of low-density lipoprotein\textsuperscript{441,442}. Shinohara \textit{et al.} conducted a post-marketing clinical trial on edaravone and recruited a total of 401 patients to study the mRS score at three months after treatment initiation\textsuperscript{443}. The incidence of “grade 0–1” on the mRS was 57.1\% and 50.3\% in the edaravone and ozagrel (antiplatelet agent) groups, respectively, at the conclusion of the study. A recent large-scale retrospective cohort study of Japan's national administrative database has shown that early use of edaravone is significantly associated with better functional outcomes at hospital discharge. Edaravone also lowered in-hospital mortality and reduced intracranial hemorrhage after admission. Combination therapy with edaravone and emergent
endovascular reperfusion therapy, therefore, could be a promising therapeutic strategy for the treatment of acute ischemic stroke.\textsuperscript{444}

Statins, 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are a class of lipid-regulating medications that reduce cholesterol levels by inhibiting the HMG-CoA reductase, which is required for cholesterol synthesis.\textsuperscript{445} (Figure 11). It has been known that statins exert lipid-dependent effects on atherosclerosis by lowering the generation of serum LDL, oxidized LDL, and cholesterol.\textsuperscript{446} The Multiple Risk Factor Intervention Trial has found that cholesterol levels at baseline and the risk of ischemic stroke are positively correlated, and statins can reduce 25 to 30 percent in the rate of stroke.\textsuperscript{447} Hosomi et al. have performed a posthoc analysis in the J-STARS study (Japan Statin Treatment Against Recurrent Stroke) to define the appropriate LDL cholesterol levels for preventing stroke recurrence.\textsuperscript{448} Results indicated that 80 to 100 mg/dL LDL cholesterol after adjusting for pravastatin usage might reduce the risk of stroke events and transient ischemic attack. In another study, Tuttolomondo et al. have enrolled 42 patients with large arteries atherosclerosis stroke in a randomized parallel trial and found that atorvastatin (80 mg per day) treatment until discharge can lower the mean National Institutes of Health Stroke Scale (NIHSS) and mRS comparing to the control group.\textsuperscript{449} These findings provide a strong argument that atorvastatin is associated with a better functional and prognostic profile in stroke patients. In addition, reactive metals like Fe\textsuperscript{2+} can catalyze the lipid peroxidation of cholesterol,\textsuperscript{450} which may be related to ferroptosis. Therefore, whether statins have other molecular mechanisms conducive to the recovery of ischemic stroke needs further investigation.

The cell death pathways involved in ischemic stroke share several similarities and are known to cross-talk with each other (Figure 12). They all originate from

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the excitotoxicity caused by excessive NMDAR activation or the inflammation caused by excessive microglial activation. Since proper NMDAR and microglial activation is necessary for neuronal survival, the overall inhibition of NMDARs or inflammation is detrimental to neurons. Lately, an interaction between apoptosis and necroptosis in ischemic stroke was discovered, and the activation of RIPK1 is known to be critical for mediating both apoptosis and necroptosis after ischemic injury. RIPK1 activation has been shown to mediate two alternative cell death mechanisms depending on caspase-8 activity. TNFα stimulation can promote RIPK1 activation to mediate the execution of necroptosis under caspase-8 inhibition; however, under caspase-8 activation, TNFα stimulation can lead to the activation of RIPK1 kinase activity to promote RIPK1-dependent apoptosis by mediating the formation of complex IIa with FADD and caspase-8. Thus, administration of a RIPK1 inhibitor after stroke may protect neurologic function by inhibiting both necroptosis and apoptosis.

In fact, apoptosis, autophagy, and ferroptosis are also linked by the tumor suppressor p53. As a transcription factor, p53 can regulate several different cellular responses, including cell cycle arrest, senescence, DNA repair, and metabolism, by selective transcriptional regulation of target genes or by directly interacting with other proteins, thereby exerting its tumor-suppressive function. Once DNA is damaged during cerebral ischemia, p53 activates many pro-apoptotic genes, including PUMA, NOXA, Bax, and other pro-apoptotic members of the Bcl-2 family, and thus promotes neuronal apoptosis. Moreover, recent studies have shown that p53 can regulate autophagy through the AMPK/TSC2-mTOR signaling pathway. Importantly, damage-regulated autophagy modulator and ARF are two p53-inducing signaling molecules that participate in regulating the autophagy pathway and can also activate apoptosis. Therefore, p53 is believed to indirectly promote further development of apoptosis due to autophagy activation, especially in...
cancer cells\footnote{461}. Jiang \textit{et al.} first reported p53 induction in response to ferroptosis in 2015\textsuperscript{462}. Further studies have found that p53 can promote ferroptosis by inhibiting SLC7A11 expression or promoting spermidine/spermine N1-acetyltransferase 1 and glutaminase 2 expression\textsuperscript{463}. These results further indicate that p53 might be the central link of multiple cell death pathways. Besides, ROS-mediated autophagy can also increase intracellular iron levels through ferritin degradation and induction of transferrin receptor 1 expression, therefore leading to ferroptosis\textsuperscript{464}. Knockout or knockdown of autophagy-related 5 (Atg5) and Atg7 limit erastin-induced ferroptosis by decreasing intracellular ferrous iron levels and lipid peroxidation\textsuperscript{465}. Therefore, ferroptosis may be a type of autophagy-dependent cell death\textsuperscript{466}.

Whether the interaction of these cancer-related cell death pathways is also applicable to ischemic stroke requires further research; nevertheless, it does indicate that blocking a given cell death pathway may not be sufficient to prevent neuronal death in a stroke event. Therefore, to prevent neuronal damage caused by cerebral ischemia effectively, it is necessary to find a suitable target in a pathway that is further upstream. In addition to traditional anticoagulant drugs, drugs for treating ischemic stroke currently undergoing phase III/IV clinical trials are mainly antioxidant and anti-inflammatory drugs (Table 2). Although the effects of these drugs need to be further supported by appropriate clinical data, it has become clear that the candidates may not be specific, indicating the synergistic effect of cell death pathways.

In summary, we have reviewed here the evidence from preclinical and clinical studies supporting the involvement of intrinsic and extrinsic apoptosis, necroptosis, autophagy, ferroptosis, parthanatos, phagoptosis, and pyroptosis in the pathogenesis of ischemic stroke, while also highlighting that the importance of each cell death form and the sequence of the events involved have not yet
been completely clarified. Understanding these may facilitate the pursuit of a trackable neuroprotective target for ischemic stroke. It is also noted that, despite an overabundance of successful therapies in animal models of stroke, the benefits observed at the preclinical stage have largely failed to translate to the clinic; thus, new animal models may be needed to better characterize the complicated events in human ischemic stroke.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

PL conceived the review. QT prepared the first draft and figures. QT, SZ, and PL edited the manuscript.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Figure legends

**Figure 1** The schematic illustration of brain ischemia. Brain ischemia induces an infarction core that represents an irreversibly injured brain and an ischemic penumbra that may be clinically symptomatic but can be rescued if blood flow is restored.

**Figure 2** Apoptotic signaling cascades after cerebral ischemia. Apoptosis can be initiated by internal events involving the disruption of mitochondria and the release of the cytochrome C (CycC) or apoptosis-inducing factor (AIF), which execute caspase-dependent
or independent cell death, respectively. Alternatively, cell surface receptors can be activated by specific ligands that bind to “death receptors”.

**Figure 3 Intrinsic signaling cascade of apoptosis after cerebral ischemia.** Post-ischemic cytotoxic Ca\(^{2+}\) influx is mediated through the stimulation of N-methyl-D-aspartate (NMDA), D, L-α-amino-3-hydroxy-5-methyl-isoxazole propionic acid (AMPA) glutamate receptors, or acid-sensing ion channels (ASICs). Increased intracellular Ca\(^{2+}\) and ROS accumulation result in activation of calpains and mediate cleavage of Bid to truncated Bid (tBid), which integrates the different death pathways at the mitochondrial checkpoint of apoptosis. At the mitochondrial membrane, tBid interacts with Bax, which is usually neutralized by anti-apoptotic B-cell leukemia/lymphoma 2 (Bcl-2) family proteins Bcl-2 or Bcl-xL. Dimers of tBid and Bax form pores in the outer mitochondrial membrane that is mitochondrial permeability transition pore (mPTP), thereby releasing cytochrome C (Cytc) or apoptosis-inducing factor (AIF), which execute caspase-dependent or independent cell death, respectively. After released into the cytosol, cytochrome C complexes with apoptotic protein-activating factor-1 (Apaf-1) and pro-caspase-9 form the apopotosome, which further activates executor caspases, such as caspase-3. In contrast, AIF translocates rapidly to the nucleus, where it mediates large-scale DNA fragmentation and cell death in a
caspase-independent manner. Also, nuclear pathways of neuronal cell death are activated in response to DNA damage, for example, through phosphorylation (P) and activation of p53 or mitochondrial translocation of nucleophosmin.

**Figure 4** Extrinsic signaling cascade of apoptosis after cerebral ischemia. The extracellular death ligands (e.g., TRAIL, FasL, TNF-α) binds to death receptors (e.g., TRAILR, Fas, TNFR1), which triggers the recruitment of the Fas-associated death domain protein (FADD). FADD binds to procaspase-8 to create a death-inducing signaling complex (DISC), which activates caspase-8. Activated caspase-8 either mediates cleavage of Bid to truncated Bid (tBid) or directly activates caspase-3. At the mitochondrial membrane, tBid interacts with Bax, which is usually neutralized by anti-apoptotic B-cell leukemia/lymphoma 2 (Bcl-2) family proteins Bcl-2 or Bcl-xL. Dimers of tBid and Bax form mPTP in the outer mitochondrial membrane, thereby releasing cytochrome C (Cytc) or apoptosis-inducing factor (AIF), which execute caspase-dependent or independent cell death, respectively.
Figure 5 Necroptosis signaling pathway after cerebral ischemia. Binding of TNF-α to its receptor results in the formation of Complex I. RIPK1 and probably other components of Complex I were ubiquitinated by the cIAPs. Subsequently, RIPK1 and other components were deubiquitinated by deubiquitinating enzyme CYLD. Deubiquitinated RIPK1 is released from Complex I and combines with FADD, TRADD, RIPK3, and caspase-8 to form Complex II. Under conditions of ATP depletion during cerebral ischemia, inhibition of caspase-8 leads to the formation of a necrosome. RIPK1 phosphorylates and activates RIPK3, which in turn phosphorylates and activates MLKL, forming a complex termed the necrosome, which initiates a downstream signal cascade resulting in necroptosis.
Figure 6 Possible autophagy signaling pathway after cerebral ischemia. Growth factors can activate PI3K by binding to two types of receptors, RTKs or GPCRs. Then PI3K activated Akt through phosphorylation, subsequently activated Akt directly phosphorylates and thereby inhibits TSC1/2. The Akt-dependent phosphorylation results in dissociation of TSC1/2 from the lysosome, where Rheb is localized, promoting Rheb activation. Since GTP-bound Rheb is a potent mTORC1 activator, inhibition of TSC1/2 by AKT-dependent phosphorylation results in mTORC1 activation. Autophagy is inhibited by activated mTORC1. In addition, amino acids can activate Rag GTPases and recruit mTORC1 to the surface of the lysosome, where the kinase complex encounters its upstream effector Rheb, which activates the kinase complex. However, under conditions of growth factors or amino acid insufficiency during cerebral ischemia, mTORC1 activity is reduced and induces autophagy. Hypoxia caused by cerebral ischemia activates HIF-1α and induces autophagy through BNIP3 and p53. During cerebral ischemia, increased AMP/ATP ratio activates LKB1 kinase, what in turn, phosphorylates and activates AMPK kinase. AMPK mediates...
the induction of autophagy through inhibition of mTORC1. ER stress is triggered in cerebral ischemia, and can also induce autophagy. It is not only regulated by the accumulation and release of ER Ca\(^{2+}\), but also by the UPR signaling pathway, including PERK, IRE1α, and ATF6. Increased Ca\(^{2+}\) activates CaMKK, what in turn, phosphorylates and activates AMPK kinase. AMPK mediates the induction of autophagy through inhibition of mTORC1.

**Figure 7** Possible ferroptosis signaling pathway after cerebral ischemia. Post-ischemic Ca\(^{2+}\) influx is mediated through the stimulation of the NMDA receptor. Increased intracellular Ca\(^{2+}\) results in the activation of cPLA2α. cPLA2α can cleave arachidonic acid at the sn-2 position of glycerophospholipids to give free arachidonic acid and lysophospholipids. Subsequently, AA is converted to AA-CoA by ACSL4 and immediately incorporated into PLs by LPCAT3. PE PLs can form a non-bilayer arrangement that may facilitate pro-ferroptotic oxidation of PE-AA by 12/15-LOX, which is non-heme iron-containing dioxygenase that catalyzes the stereo-specific peroxidation of esterified AA generating bioactive PE-AA-O-OH. GPx4 is specialized for the reduction of lipid
hydroperoxides to lipid alcohols in membrane environments. High extracellular concentrations of glutamate inhibit cystine uptake and limit the biosynthesis of GSH. GPx4 is inactivated due to the lack of necessary substrates, which will cause the accumulation of toxic lipid ROS. In addition, reduction of soluble tau after cerebral ischemia prevents the dissociation of immature APP from the ER, abolishing the trafficking of APP to the neuronal surface, where APP interacts with FPN, allowing iron export from neurons. The absence of this interaction prevents iron from exiting neurons, leading to a toxic intracellular accumulation of iron and, ultimately, neuronal ferroptotic damage after ischemic stroke.

Figure 8 Parthanatos signaling pathway after cerebral ischemia. Over-stimulation of NMDA receptors by glutamate results in the influx of Ca$^{2+}$, which binds calmodulin and activates neuronal nNOS, to convert L-arginine to NO and L-citrulline. nNOS is tethered to the NMDA receptor via PSD-95. Excess NO can be neurotoxic, which is mediated by...
peroxynitrite (ONOO⁻) ions, a reaction product from NO and superoxide anion (O₂⁻). Peroxynitrite (a representative RNS) can damage DNA, which results in over-activation of PARP1. PAR generated by activation of PARP-1 translocates from the nucleus to the mitochondria, where it binds AIF, inducing AIF release from the mitochondria. AIF then binds MIF, which is a PARP-1-dependent AIF associated nuclease. This complex translocates to the nucleus to bind DNA and causes chromatin condensation and eventually leads to neuronal cell death. PAR polymer also binds HK and impairs its glycolytic activity, which accounts for the energy depletion due to activation of PARP-1.

**Figure 9** Mechanisms mediating microglial phagocytosis of stressed-but-viable neurons during cerebral ischemia. Cerebral ischemia leads to increased Ca²⁺, insufficient ATP content, and increased ROS production in neurons. Cerebral ischemia may cause PS to be reversibly exposed on the surface of stressed-but-viable neurons via Ca²⁺- or ROS-mediated activation of a PS scramblase (probably a TMEM16 protein) and/or inhibition of a PS translocase (probably ATP8A1 or ATP8A2), via ATP depletion-induced inhibition of translocase or via caspase-mediated activation of a distinct scramblase (probably XKR8). Exposed PS is bound by MFG-E8 or GAS6 that engage phagocytic...
receptor VNR and MERTK, respectively. In addition, neuron-exposed calreticulin (CRT) or neuron-bound C1q can induce phagocytosis by activating the microglial low-density lipoprotein receptor-related protein (LRP). C1q can also bind to glycoproteins from which sialic acid residues have been removed by the enzyme neuraminidase. C1q deposition on de-sialylated glycoproteins, in turn, leads to the conversion of C3 to the opsonin C3b, which activates neuronal phagocytosis via the microglial complement receptor 3 (CR3).

Figure 10 Caspase-1-mediated microglial pyroptosis is involved in neuronal death after ischemic stroke. Cerebral ischemia causes expression and activation of the inflammasome, causing activation of caspase-1, which cleaves pro-IL-1β to IL-1β, a key inflammatory cytokine, resulting in neuronal death.
**Figure 11 Cholesterol-biosynthesis pathway.** Starting from acetyl-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) is generated and further synthesizes mevalonate. This process is mediated by HMG-CoA reductase (HMGCR), the first rate-limiting enzyme in cholesterol biosynthesis. Mevalonate is then converted to farnesyl pyrophosphate (FPP), then squalene synthase (SQS) couples two FPP molecules to form squalene. Squalene is then converted to 2,3-epoxysqualene by squalene epoxidase (SQLE) and further to cholesterol. Reactive species can modify cholesterol at the 5,6 carbon-carbon double bond. In the presence of oxygen, cholesterol can be made into a peroxide at the 4, 5, 6, or 7 positions. This cholesterol peroxide can be further oxidized by ferrous iron to generate a reactive species that can propagate a free radical chain reaction.
Figure 12. Multiple neuronal cell death pathways are involved in ischemic stroke.

Cerebral ischemia causes excitotoxicity and inflammation, causing DNA damage and RIPK1 activation. The activation of RIPK1 can promote neuronal cell necroptosis under caspase-8 inhibition and RIPK1-dependent apoptosis under caspase-8 activation. Once DNA is damaged, p53 activates many pro-apoptotic genes, and thus promotes neuronal cell apoptosis; p53 can also regulate autophagy through AMPK/TSC2-mTOR signaling pathway, and promote ferroptosis by inhibiting SLC7A11 expression or promotion of spermidine/spermine N1-acetyltransferase 1 (SAT1) and glutaminase 2 (GLS2) expression.
Table 1. Completed Phase III/IV clinical trials for ischemic stroke reported between 01/01/2010 and 12/01/2020.

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TABLE 2. Ongoing Phase III/IV clinical trials for ischemic stroke.

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mRS: modified Rankin scale; NIHSS: National Institutes of Health Stroke Scale; MI: myocardial infarction; TIA: transient ischemic attack. Data sources are ClinicalTrials.gov updated on December 1, 2020.
NIHSS scores from baseline to 15 days in the two groups.

<table>
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<td>IV</td>
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<td>NCT03749</td>
<td>IV</td>
<td>Anticoagulant Rivaroxaban</td>
<td>Platelet activation incidence</td>
<td>1000</td>
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<td>NCT03639</td>
<td>III</td>
<td>Tyrosine kinase inhibitor Imatinib</td>
<td>Functional independence at 3 months as measured by mRS Score 0-2</td>
<td>1260</td>
<td>June 2022</td>
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<tr>
<td>NCT03844</td>
<td>III</td>
<td>Anticoagulant Eptifibatide</td>
<td>Platelet activation incidence</td>
<td>220</td>
<td>December 2020</td>
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Intracranial Hemorrhage within 48 hours after treatment

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<tr>
<th>NCT03181</th>
<th>Thrombolytic agent</th>
<th>Tenecteplase</th>
<th>Actoveg</th>
<th>Function</th>
<th>500</th>
<th>December 2022</th>
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<tr>
<td>360</td>
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<tr>
<th>NCT03545</th>
<th>Cell-based therapy</th>
<th>MultiStem</th>
<th>NA</th>
<th>Assessment</th>
<th>300</th>
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<td>607</td>
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<td>disability</td>
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Assessment of disability by examining the distribution of mRS scores evaluated by shift analysis at 90 days.
<table>
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<tr>
<th>NCT04047</th>
<th>III</th>
<th>Endothelin-B receptor agonist</th>
<th>PMZ-1620</th>
<th>Prevent mitochondrial dysfunction</th>
<th>Change in NIHSS at 90 days</th>
<th>August 2020</th>
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<td>NCT03192</td>
<td>III</td>
<td>Anticoagulant</td>
<td>Apixaban</td>
<td>Inhibit platelet activation</td>
<td>Incidence of recurrent stroke during 4 years</td>
<td>April 2022</td>
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<tr>
<td>NCT04041</td>
<td>III</td>
<td>Antioxidant</td>
<td>Alpha-lipoic acid (aLA)</td>
<td>Scavenge ROS</td>
<td>Number of participants with functional independence at 3 months</td>
<td>December 2021</td>
</tr>
<tr>
<td>NCT02898</td>
<td>III</td>
<td>Anti-inflammatory agent</td>
<td>Colchicine</td>
<td>Inhibit NLRP3 activity</td>
<td>Recurrence of non-fatal ischemic stroke at any time</td>
<td>October 2021</td>
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</tbody>
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within 60 months

mRS: modified Rankin scale; NIHSS: National Institutes of Health Stroke Scale. Data sources are ClinicalTrials.gov updated on December 1, 2020.
Author/s:
Tuo, Q-Z; Zhang, S-T; Lei, P

Title:
Mechanisms of neuronal cell death in ischemic stroke and their therapeutic implications

Date:
2022-01

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