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REVIEW ARTICLE

Cell Death Mechanisms in Stroke and Novel Molecular and Cellular Treatment Options

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Abstract: As a result of ischemia or hemorrhage, blood supply to neurons is disrupted which subsequently promotes a cascade of pathophysiological responses resulting in cell loss. Many mechanisms are involved solely or in combination in this disorder including excitotoxicity, mitochondrial death pathways, and the release of free radicals, protein misfolding, apoptosis, necrosis, autophagy and inflammation. Besides neuronal cell loss, damage to and loss of astrocytes as well as injury to white matter contributes also to cerebral injury. The core problem in stroke is the loss of neuronal cells which makes recovery difficult or even not possible in the late states. Acute treatment options that can be applied for stroke are mainly targeting re-establishment of blood flow and hence, their use is limited due to the effective time window of thrombolytic agents. However, if the acute time window is exceeded, neuronal loss starts due to the activation of cell death pathways. This review will explore the most updated cellular death mechanisms leading to neuronal loss in stroke. Ischemic and hemorrhagic stroke as well as subarachnoid hemorrhage will be debated in the light of cell death mechanisms and possible novel molecular and cellular treatment options will be discussed.

Keywords: Ischemic stroke, hemorrhagic stroke, apoptosis, necrosis, autophagy, pyroptosis, neuroprotective therapies.

1. INTRODUCTION

Stroke is one of the leading causes of death worldwide and affects nearly 17 million individuals per year [1]. It is divided into mainly two types, namely occlusive' with 'ischemia (80-85% of total cerebral strokes [2]) and hemorrhagic strokes. Brain physiology and blood flow are two processes that play an important role in stroke. Ischemic strokes result from obstructed cerebral arteries and are caused *via* thrombus formation or emboli or atherosclerosis. Hemorrhagic stroke can be divided as intracerebral hemorrhage hemorrhage (ICH) and subarachnoid hemorrhage (SAH). ICH is mostly due to long lasting increased blood pressure (hypertension). The current treatment for ischemic stroke in the acute time window is reperfusion with recombinant tissue plasminogen activator (rtPA) *via* i.v. administration within 4.5 hours of onset or intravascular cloth retrieval with devices [3]. However, only 5% of ischemic stroke patients are eligible for this treatment [4]. Altogether, stroke leads to brain damage which causes long-term/lifelong disabilities and or even death. Current research seek for long-term therapeutics primarily to restore post-ischemic neuronal damage. But in order to establish novel treatment options, it is crucial to understand involved cell death mechanisms. In this review we attempt to emphasize post-stroke inflammation and the most updated cell death mechanisms in stroke and discuss several molecular and cellular mechanisms that are potential candidates for novel treatment options.

2. POST-STROKE INJURY PROPAGATED BY INFLAMMATION

Ischemic tissue follows a series of secondary events including vascular, cellular and molecular alterations. The vascular response to ischemia activates endothelial cells and upregulates circulating leukocytes [5] and adhesion molecules including E- (endothelial surface) and P- (platelet surface) and L- (leukocyte surface) selectins, ICAM-1 and integrins. Leukocytes can travel across endothelial cells to the brain by interacting *via* these adhesion molecules and secrete pro-inflammatory cytokines into the brain. The acute inflammatory response after stroke therefore leads to the interactions between platelets, leukocytes, lymphocytes and endothelial cells that are thereupon responsible for blood-brain barrier (BBB) injury and infiltration of immune cells into the brain parenchyma [6]. The injured BBB can further exacerbate leakage into the brain causing edema and worsen tissue

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injury. In physiological conditions, injured regions attract inflammatory cascades with an attempt to recover the damaged site. In stroke injury this is also the case, although, with respect to the severity of the injury, the infarct size and area at stake, the harmful cascades may weight more than the recovery processes which disturb the balance of the cellular microenvironment leading to the activation of deleterious pathways including different cell death mechanisms. The inflammatory response to the injured site is therefore not always beneficial but on the contrary can have a catalytic effect on the ongoing post-ischemic injury. Most importantly, inflammation in the brain initiates the release of cytokines and free radicals which lead to cellular injury. Next to these processes, as a secondary event of inflammatory responses, the damaged tissue is removed by the defending immune system and synaptic remodeling is established.

3. POST-STROKE CELL DEATH EXACERBATED BY MANY OVERLAYING MECHANISMS

Next to the role of inflammation, also other cells and factors serve to cerebral injury after stroke. Glial cells play an important role in promoting the regulation of the BBB, angiogenesis and synaptogenesis in physiological conditions but during stroke they may cause a glial scar at the site of damage and thereby prevent further plasticity [7]. Furthermore, the role of calcium, mitochondrial integrity and its response, the release of free radicals and oxidative stress, the role of stressed endoplasmic reticulum (ER) on protein misfolding, white matter injury, glial and astrocytic response and disrupted BBB integrity during inflammation are of high importance in the progress of cell death during post-ischemic stroke [8]. Hence, many of these mechanisms overlap intrinsic pathways and may co-exist in post-stroke injury [9]. The dual role of inflammation as well as the fine crossroad of the activation of different cell death pathways is highly dependent on the individual's physiological condition and the extent of injury. In fact, this fine tuning of signal transduction both beneficial as deleterious, is complex and may need to be addressed on many levels simultaneously, hence that renders treatment therapies very difficult.

4. CELL DEATH MECHANISMS IN STROKE

Several pathways are involved in post-stroke injury which are dependent on the delicate balance between restoration and deleterious pathways. If more damage is accomplished than restored, cell death mechanism may be initiated, these include apoptosis, necrosis, autophagocytosis, necroptosis and pyroptosis. Many of these pathways are extensively discussed in literature, see Table **1**. Next, we will highlight the most crucial mechanisms involved in cellular death in stroke.

5. EXCITOTOXIC CELL DEATH

Cell death activated by excitotoxicity can be initiated at several moments of post-injury processes [10]. First of all, when neurons get less blood supply due to an ischemic event especially in the core region of ischemia, the significantly decreased oxygen supply leads to hypoxia hence decreases ATP production. A decreased ATP production leads to failure of the Na^+/K^+ pump and the plasma membrane Ca^{2+}/ATP-pump. Normally, Ca^{2+} is always leaking in a low amount into the cell, however if ATP production is decreased, calcium extruders (plasma-membrane associated Ca^{2+}/ATPase and Na^+/Ca^{2+} exchanger) stop working and the intracellular calcium concentration, $[Ca^{2+}]_i$, increases. Membrane transporters involved in calcium homeostasis at the presynaptic neuronal membrane are illustrated in Fig. (**1**). Secondly, increased and unstoppable intracellular calcium binds to synaptotagmin, located at the vesicular membranes floating in the axon terminal, which in turn causes the vesicles to fuse with the membrane and releases glutamate into the synaptic cleft. Excessive available glutamate leads to hyper excitation of the glutamate receptors namely NMDA, AMPAR and kainate receptors on the post-synaptic membrane. Their hyperexcitaton increases intracellular calcium load and hence, events leading to cell death start. These cascade of events lead to postsynaptic cell death mediated by mainly glutamatergic pathways. Thirdly, continuous glutamate presence leads to continuous depolarization and therefore continuous Ca^{2+} influx. In normal resting state, the NMDAR contains a Mg^{2+} in its core that pops out when glutamate binds to the non-GluN1 subunits. Excessive influx of Ca^{2+} *via* the NMDAR activates calpein [11, 12] in the post-synaptic neuron which in turn inhibits the Na^+/Ca^{2+}X3 receptor. To this end, calcium concentrations get even higher in the neuron. Moreover, massive calcium influx causes activation of calpein which causes proteolysis of cytosolic substrates and therefore leads to cell death. Calpastatin is a useful drug that can decrease neuronal death on this step [13]. Calpastatin is a naturally occurring protein which upon specific binding to activated calpein, can inhibit calpein activity. This calpastatin-calpain interaction is the most relevant mechanism responsible for the regulation of Ca^{2+}-induced proteolysis [14-16].

Besides the activation of glutamate vesicles, the excessive $[Ca^{2+}]_i$ causing mitochondrial membrane depolarization leads to disturbed mitochondrial functions which in turn activate the release of free radicals, enzymes and proteases that also lead to cellular death pathways activation in the neurons of ischemic tissue [10, 17]. Other less prominent routes include possible involvement of dysfunctional glutamate receptors on the pre-synaptic membrane or absent and/or dysfunction of glutamate degrading enzymes in the synaptic cleft which normally maintain physiological concentrations of free glutamate. Also, dysfunctional astrocytes and glial cell that normally mediate in restoring extracellular calcium levels play a role in neuronal cell loss after stroke injury.

One way or another, the most crucial molecular event that mediates the progressive activation of cell death pathways after stroke injury is the excessive intracellular calcium amount that in physiological conditions is constantly being maintained in low levels in the environment either by the neuron or by surrounding supportive astrocytes/glial cells. However, destroyed Ca^{2+} homeostasis activates either programmed cell death (apoptosis) or collapses due to lost control of cell mechanisms (necrosis), and in some areas of ischemia (especially in penumbral region) both mechanisms of cell death pathways are activated depending on the energy status of the cell. The downstream effects of increased Ca^{2+} influx is accompanied by the activation of Ca^{2+}-dependent

Table 1. Post-stroke events leading to inflammation, neurodegeneration stress and/or cell death.

Factors	Affected Cell/Organelle	Involved Pathways	Outcome
ATP depletion	Neurons and glial cells [20, 21]	Dysfunctional Ca^{2+} extruders	Apoptosis
		Excitotoxicity	Apoptosis, necrosis
		Cytoskeletal degradation	Necrosis
	ER [22-26]	Failure of SERCA	ER Ca^{2+} depletion, Stress
		IRE1 oligomerization/downstream kinases	Apoptosis
		Misfolded protein accumulation	Necrosis, apoptosis, autophagy
Misfolded proteins	ER [20, 22-24, 27-29]	PERK/eIF2α kinase phosphorylation	Autophagy
		Increased chop expression	Apoptosis
		Protein synthesis inhibition	Ribophagy
		GRP78 chaperone capacity exceeds	Apoptosis
		Excessive glutamate release	Apoptosis, autophagocytosis, necrosis
Calcium influx	Mitochondria [30-32]	mtPTP opening	Apoptosis
		ROS production	Oxidative stress, apoptosis, necrosis
		Free radical release	Oxidative stress
	Neurons [12, 33-35]	Phospholipases and esterases activation	Necrosis
		Excitotoxicity	Hyperexcitation, dysfunction, apoptosis, necrosis
		T253D-αCaMKII	Excitotoxic cell death
		O-GlcNAcylation of nNOS	Apoptosis
	Astrocytes [36]	Reactive astrocytes	Glial scar formation
		Pro-inflammatory cytokine release	Inflammation
	Microglia [37, 38]	Dysfunction	Demyelination and white matter loss, neuronal death
	Oligodendrocytes [39]	NOS/NO/$ONOO^-$/excessive PARP-1	Mitochondrial stress, apoptosis, inflammation
Free radicals	Neurons and glial cells [40]	NADPH oxidase	ROS formation
		PI3-kinase/Akt pathway activation	Necrosis
		NF-kB upregulation	Inflammation
		TRPM7 channel activation	Increased Ca^{2+} influx
		Parkin	Mitophagy
		ROS	Apoptosis
Inflammatory responses	Microglia [41]	TNF-α and IL-1β release	Increase inflammation and CAM
	Astrocytes [36, 42]	MMPs and MPO release	BBB breakdown
		IL-15	Lymphocyte toxicity
	Dendritic cells [43]	Cytokine release	Inflammation
	Endothelial cells [44]	ICAM-1, P-selectin, E-selectin	Attract leukocytes to ischemic area, inflammation

(Table 1) contd….

Factors	Affected Cell/Organelle	Involved Pathways	Outcome
Inflammatory responses	Lymphocytes [45, 46]	Pro-inflammatory cytokines	Inflammation
	Macrophages [47-49]	MMP-3 and MMP-9 release	BBB breakdown and degradation of extracellular matrix
		TLR 2 and TLR4/IL-23	Neural cell death
	Neutrophils [50, 51]	iNOS release	Toxic levels of NO
	Reactive astrocytes [52]	P2X7 receptor activation and MMP-9 release	Ca^{2+} overload and mitochondrial depolarization, BBB breakdown
	Oligodendrocytes [53]	IL-6	Inflammation
	Brain resident cells and astrocytes [54, 55]	MCP-1/CCL2	Migration of leukocytes, monocytes/macrophages/microglia to the ischemic core
	Neurons [56-58]	Bax/AIF	Pro-apoptotic
		Drp1/mitochondrial dysfunction	Apoptotic cell death
α-Syn aggregation	Neurons [59-62]	LRRK2	Apoptotic cell death
		GSK-3β/Tau hyperphosphorylation/downregulation of Nrf2	Apoptotic cell death
		Nrf2/NDP52	Autophagy

Abbreviations: ER, Endoplasmic reticulum; **SERCA**, Sarco/endoplasmic reticulum Ca2+-ATPase; **IRE1**, Inositol-requiring enzyme 1; **PERK**, PKR-like ER kinase; **eIF2α**, Eukaryotic translation initiation factor 2A; **mtPTP**, Mitochondrial permeability transition pore; **ROS**, Reactive oxygen species; **T253D-αCaMKII,** Phosphomimic form of calcium/calmodulin-dependent protein kinase type II subunit alpha; **O-GlcNAcylation of nNOS**, Ser(Thr)-O-linked β-N-acetylglucosamine glycosylation of neuronal nitric oxide synthase; **NOS**, Nitric oxide synthase; **NO**, Nitric oxide; **ONOO$^-$**, Peroxynitrate; **PARP-1**, Poly [ADP-ribose] polymerase 1; **NADPH**, Nicotinamide adenine dinucleotide phosphate-oxidase; **PI3-kinase**, Phosphoinositide-3 kinase; **Akt,** Protein kinase B; **NF-kB**, Nuclear factor-kB; **TRPM7**, Transient receptor potential cation channel, subfamily M, member 7; **TNF-α**, Tumor necrosis factor alpha; **IL**, Interleukin; **CAM**, Cell adhesion molecule; **MMP**, Matrix metalloproteinase; **MPO**, Myeloperoxidase; **ICAM-1**, Intercellular adhesion molecule 1; **TLR**, Toll-like receptor; **iNOS**, Inducible nitric oxide synthase; **P2X7**, P2X purinoceptor 7; **CCL2**, Chemokine (C-C motif) ligand 2; **Bax**, Bcl-2 associated X protein; **AIF**, Apoptosis-inducing factor; **Drp1**, Dynamin related protein 1; **LRRK2**, Leucine-rich repeat kinase 2; **GSK-3β**, Glycogen synthase kinase 3 beta; **Nrf2**, Nuclear factor erythroid 2-related factor 2; **NDP52**, Nuclear Domain 10 Protein 52.

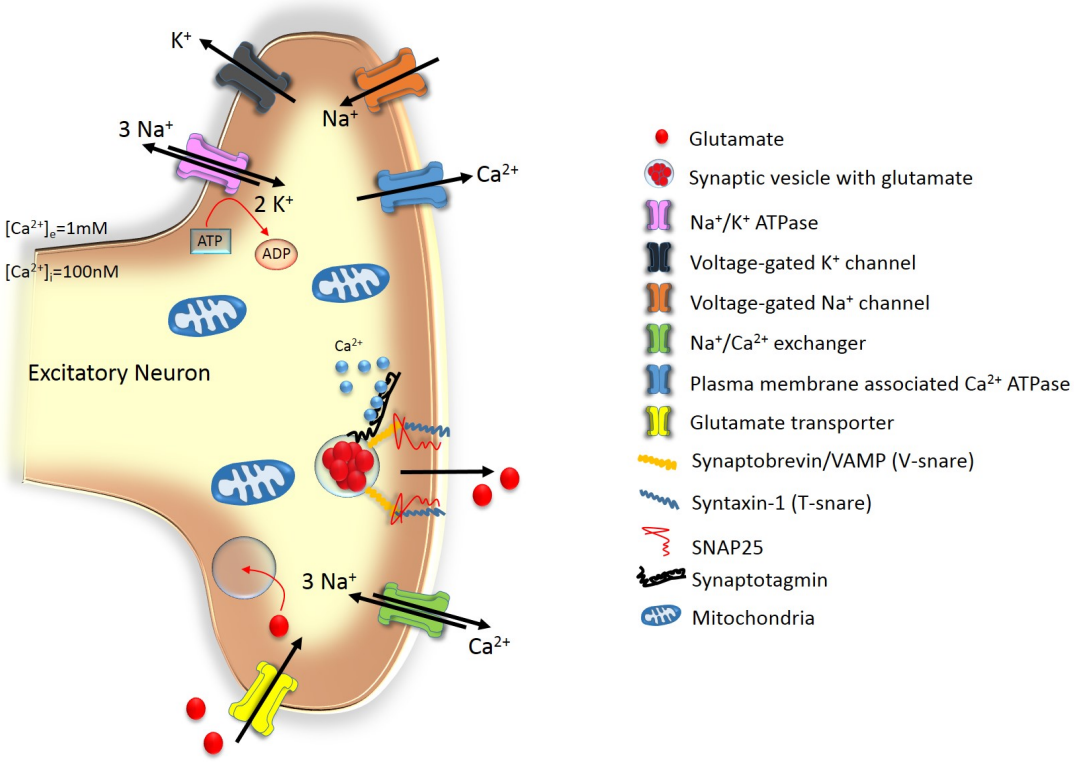

Fig. (1). The axon terminal of a neuron with membrane transporters and intracellular components which are prone to excitotoxicity.

catabolic processes such as proteases, phospholipases, Poly [ADP-ribose] polymerase 1 (PARP-1) and nucleases which lead to cellular toxicity lasting in neuronal death [18, 19].

6. APOPTOSIS

Excessive $[Ca^{2+}]_I$, is normally removed by mitochondria but in ischemic tissue excessive accumulation of calcium in these mitochondria leads to its membrane depolarization and dysfunction. Dysfunctional mitochondria releases cytochrome C *via* the mitochondrial permeability transition pore (mtPTP). Cytochrome C, then activates caspase that leads to DNA fragmentation and eventually cell death. Caspases are the main drivers of apoptosis. Moreover, caspases are also involved in the cross-talk between apoptosis and autophagy [63]. Apoptosis is a process that needs energy and hence is prominently present in neurons in the ischemic penumbral region where there is energy production although its amount is decreased significantly [64].

Mitochondrial apoptosis is mainly regulated by B-cell lymphoma-2 (Bcl-2) family proteins which are either pro-apoptotic (*e.g*. Bax, Bok) or anti-apoptotic (*e.g*. Bid, Bcl-2) [65]. As a response to danger signals or stress, the expression and activation levels of Bcl-2 family members determine whether programmed cell death will be initiated or restrained [66, 67]. This family of protein members control neuronal apoptosis by acting on the integrity of the mitochondrial outer membrane and energetics as well as regulate Ca^{2+} homeostasis in mitochondria and ER. *Via* use of gene targeting, several studies have demonstrated that a single deletion of the pro-apoptotic *Bax* gene is sufficient to be neuroprotective [68, 69]. After pro-apoptotic proteins are released into the cytoplasm, they initiate the activation of a cascade of caspase enzyme family. Caspase-3 is the last and executive enzyme in the cascade for apoptosis. This enzyme cuts proteins, enzymes and nucleotides leading to dysfunction in the cell homeostasis and hence cell death. In apoptosis, the cell membrane structure is preserved until the cell eventually dies. In contrast to apoptosis, necrosis starts with cell swelling and cell membrane disintegration. Next to mitochondria, also stressed ER induces activation of caspase proteins leading to neuronal apoptosis [27].

Interestingly, several chronic neurodegeneration-related proteins such as α-Synuclein, Parkin, PTEN-induced putative kinase 1 (PINK 1), Parkinson disease protein 7 (DJ-1) and leucine-rich repeat kinase 2 (LRRK2) are also involved in neuronal cell death after ischemic stroke [70]. The post-stroke condition with inflammation, oxidative stress and ER stress altogether provides an optimal environment for α-Synuclein aggregation that promotes inflammation. Especially reactive oxygen species (ROS) enhance α-Synuclein expression and aggregation [71]. Knockdown of α-Synuclein leads to attenuated ischemic markers such as mitochondrial dysfunction, oxidative stress, apoptosis and autophagy [72]. Several studies have shown that increased presence of DJ-1 expression after focal ischemia is neuroprotective by preventing ischemic neuronal death by suppressing ROS production [73-77]. Furthermore, LRRK2-mediated apoptosis in cerebral ischemia is propagated by the modulation of tau phosphorylation in the presence of α-Synuclein. Parkin and PINK 1 are known for their neuroprotective functions after stress-induced mitochondrial injury [78-80]. However, inactivation of these factors due to oxidative and nitrosative stress after stroke injury depletes this protective pathway signaling which leads to neurodegeneration and apoptotic cell death.

7. NECROSIS

In ischemic core area, blood flow decreases significantly (below 20% of baseline values). This decrease leads to significant reduction in the amount of ATP. ATP is essential in the maintenance of neuronal membrane potential *via* Na^+/K^+-ATPase pump. In ischemia, this pump fails and the regulation of the concentrations of Na^+ and K^+ inside and outside of the neuronal cell membrane is lost. To this end, Na^+ accumulates inside the cell leading to the formation of cellular edema. If this event continuous, the cell membrane will start to rupture and release its content to the extracellular environment and finally the nuclei will degrade. Degradation and release of cellular components to the extracellular space leads to inflammatory reaction around the dying cell. Ca^{2+} overload, excessive ROS and reactive nitrogen species (RNS) formation also lead to mitochondrial swelling and thereby contribute to neuronal death. Necrosis is recognized by morphological changes such as vacuolation of the cytoplasm, disrupted cell membrane integrity and loss of cell content, presence and release of pro-inflammatory molecules and inflammation in and around the dying cell [81]. The neurons in the ischemic core are prominently subjected to necrosis [64]. On the other hand, astrocytes die by delayed necrosis as demonstrated by Gürer *et al.* [82]. At penumbral area, astrocytes (although swelled at the beginning) resisted to ischemic conditions better than neurons and showed less apoptotic signaling than necrosis.

8. NECROPTOSIS

At early times of stroke studies, necrosis was thought to be distinct from apoptosis. In recent years, however, it was shown that necrotic cell death can also be driven by defined molecular pathways. Necroptosis, a type of necrotic cell death [83] dependent on receptor-interacting protein kinase-1 (RIPK1) has been recognized and demonstrated in stroke [84].

In apoptosis, especially caspase-3 and 8 are activated. On the other hand, necroptosis requires that the function of caspase-8 is inhibited or disrupted. The downstream mediators in the necroptosis pathway are currently incompletely understood, but the rapid swelling of necroptotic cells that results in plasma membrane rupture are resembling necrosis. In accordance with this knowledge, previously it has been shown by Kilinc *et al.* and Unal-Cevik *et al.* that ischemic neurons showed both necrotic and apoptotic features [85, 86]. The authors found lysosomal rupture, lysosomal enzymes spilling into the cytoplasm immediately after ischemia, suggesting that lysosomal membrane integrity was rapidly lost as occurs in necrosis. The same neurons also exhibited caspase-3 and Bid cleavage and cytochrome-C release as markers of apoptosis.

In general, death receptors mediate apoptosis, however these death receptors can also activate necrotic cell death [87]. Increased expression of ligands for death receptors in

the ischemic penumbra are Fas ligand (FasL) and tumor necrosis factor alpha (TNF-α) which in apoptosis-eligible cells activate downstream kinases. However, in apoptosis deficient cells, these ligands are not capable of activating caspases but instead activate RIP1 kinase and necroptosis [88]. While, RIP1 is a death-domain containing kinase, its kinase activity is not essential in the propagation of apoptosis. Studies have shown that necrostatins, inhibitors of necroptosis [89], have no effect on apoptosis. How the activation of RIP1 kinase leads to programmed necrosis is not yet revealed, however it seems that Bcl-2 modifying protein (Bmf), one of the Bcl-2 family protein members, plays a key role in mediating necroptosis [90] by inducing necrotic mitochondrial damage. Besides RIP1, also PARP1, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and calpains have been assigned a role in mitochondrial mediated necroptosis [91].

Recently, Zille *et al.* demonstrated that cell death mechanisms in cultured neurons exposed to hemoglobin or hemin showed simultaneous features of both necroptosis and ferroptosis (an iron- and ROS-dependent form of regulated cell death [92]) in their ICH model [93]. The authors also recommended future treatment approaches to decrease the signaling of both pathways to below their threshold in order to achieve cell survival.

9. AUTOPHAGY IN STROKE: GOOD AND BAD

Autophagy is another programmed cell death pathway in mammalian cells that regulates the bulk degradation of aggregated macromolecules and damaged organelles (associated with several stress environments) in the cytosol *via* the lysosomal system [94]. This pathway is interestingly essential for healthy cells on long term [95]. Autophagy is divided into three pathways including chaperone-mediated autophagy, microautophagy and macroautophagy [96] from which macroautophagy is the most demonstrated pathway in mammalian systems such as ischemic stroke [97], ICH [98] and SAH [99]. Several evidence has proven that autophagy is involved in ischemic brain tissue [100, 101] and hemorrhagic stroke [98, 102]. Some experiments have located autophagy in the ischemic penumbra [103]. Although some studies propose protective involvement of autophagy in cerebral ischemia [104], other increasing evidence shows increased neuronal cell death in models of ischemic brain injury [95]. Also, interactions between autophagy and apoptosis appear to decide the fate of the cells. The signaling pathway of autophagy is driven on mechanistic target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) pathways, which leads to autophagy inhibition [105] and initiation respectively [106]. Autophagy basically starts with the formation of autophagosomes (bilayer membrane vesicles) which engulf damaged organelles from the cytosol, then fuse with lysozomes and form autolysozomes which degrade the engulfed content. Furthermore, the selective removal of damaged mitochondria from the injured cell through autophagosome is called 'mitophagy' and this unique mechanism plays an important role in cerebral ischemia [107] as well as in several neurodegenerative diseases. An increased accumulation of autophagosomes in the cytosol is mainly observed together with upregulated LC3-II (autophagosomal membrane component). The extent to which autophagy is present in a system is prominently based on the LC3-II/I ratio. It is proposed that autophagy could only be beneficial if the autophagy stream is intact, and that detrimental effects could be caused if the autophagy flux was impaired mainly leading to cell death. Moreover, a moderate activation of autophagy seems beneficial in post-stroke condition, however, decreased or increased autophagy proteins suggest a pro-death role of autophagy. Also, ischemic excitotoxicity participates in the autophagy stream.

Following ischemic and hemorrhagic stroke, an overload of ferritin causes brain damage and cell death which are mediated by a process called 'ferritinophagy' [108]. This is also a form of autophagy, only the selective turnover of ferritin is accomplished by the autophagosomes. The nuclear receptor coactivator 4 (NCOA4) is the targeted receptor in this pathway [109].

Post-stroke brain damage and neuronal cell death is likely for a major part caused by loss of supporting astrocytes. Recently, it was revealed that autophagy also contributes to cell death of ischemic astrocytes [110]. Both in neurons and non-neural cells, lysosomal proteases are involved in mediating Cyt-c release and caspase activation leading to apoptosis. Although the exact mechanism of autophagy in stroke is unclear, yet it was found that inhibition of autophagy causes lysosomal membrane stabilization that retains the signaling of lysosomal proteases such as cathepsins which subsequently inhibit the truncated BH3 interacting domain death agonist (tBid)-mitochondrial apoptosis pathway in ischemic astrocytes [111].

Furthermore, autophagy is also involved in post-stroke inflammatory response where microglia regulate cytokine production [112]. Together with apoptosis, autophagy also effects p53-mediated cell death pathway after ischemic stroke/reperfusion insult [113].

Weis *et al*. showed lately that autophagy in the brain of neonates is dependent on gender and location [114]. In their study, autophagy and apoptosis were studied in the brain following hypoxia-ischemia induction in rats and it was found that the lysosome activity decreased whereas caspase-3 activity increased in the cortex of female rats compared to male rats [114]. Also, the researchers found that autophagy occurred at specific locations only, namely the cortex and hippocampus [114].

In general, it is believed that autophagy is time-dependent and has dual roles with turnovers from being beneficially anti-apoptotic enhanced at basal levels to deterioration into pro-apoptotic favor under ischemic or hypoxic conditions. However, discrepancies have arisen about whether autophagy should be enhanced or diminished for treatment approaches.

10. PYROPTOSIS: AN INFLAMMATORY CELL DEATH MECHANISM

Defined as host cell death and inflammation, pyroptosis is morphologically and mechanistically distinct from other types of cell death. One of the features of pyroptosis is the dependence on caspase 1 [115]. Cells that undergo pyropto-

sis show morphological caspase-1 dependent features such as rapid plasma-membrane rupture and release of pro-inflammatory intracellular contents, DNA damage and cell lysis, destruction of actin cytoskeleton and eventually cell death by an yet unknown mechanism [116, 117].

Damage associated molecular patterns (DAMPs) released by dying cells and some pro-inflammatory cytokines such as interleukin (IL)-1α released by necrotic cells [118], initiate the inflammatory response. As a response to tissue injury, toll-like receptors (TLRs) initiate a signaling cascade that leads to the cellular activation and production of inflammatory cytokines (TNF, IL-6, IL-8 and IFNs). Furthermore, the nucleotide-binding oligomerization domain-containing 1 (NOD1) and NOD2 of nod-like receptors (NLRs) also trigger signaling cascades leading to inflammatory cytokine production. Both TLRs and NOD1 and NOD2 mediate cells to undergo caspase 1 activation and produce cytokine IL-1β.

DAMPs activate intracellular pattern recognition receptors (PRRs) which lead to the formation of multiprotein complexes called inflammasomes such as NLR Pyrin domain containing 1 (NLRP1) and NLRP3 [119]. Moreover, it is suggested that plasma membrane PRRs on neurons and glial cells can play an important role in activating nuclear factor kappa B (NFκB) and mitogen activated protein kinase (MAPK) pathways [120].

Inflammasomes, expressed abundantly in the brain and immune cells, may play important roles in detecting cellular damage and mediating inflammatory responses to aseptic tissue injury during ischemic stroke [119]. Signaling through NLRP1 and NLRP3 inflammasomes produces cleaved caspase-1 [121, 122], which in turn both initiates the formation and regulates the release of the pro-inflammatory cytokines IL-1β and IL-18 that are released into the extracellular environment [123-125]. The evidence for the role of inflammasomes and pyroptosis in stroke pathology is well defined in several studies [126-128]. This pro-inflammatory programmed cell death pathway is facilitated by caspases (caspase 1 or caspase 4/5/11) [129-131]. Caspase 1 deficiency, or pharmacological inhibition, provides protection against inflammation, cell death and organ dysfunction that are associated with these diseases, making caspase 1 an attractive therapeutic target against pyroptosis in post-stroke injury [132-134].

11. MICROGLIA AN ACTIVE PLAYER IN STROKE

Brain resident cells, microglia, are the first active responders to ischemia. While these cells produce and release many pro-inflammatory factors by producing reactive species, attracting immune cells, phagocytosis, and producing inflammatory mediators such as IL-1β, TNF-α, IL-6 and matrix metalloproteinases (MMPs) *etc*., these microglia also generate anti-inflammatory mediators such as IL-4, IL-10, and transforming growth factor beta (TGF-β) during the restoration phase of the inflammation [135, 136]. The ischemic brain triggers microglia activation which has been linked to worsen stroke outcomes. Other brain cells and brain entered leukocytes are in close relationship with microglia involved in the infarct region. Hence, the influence of activated microglia on post-stroke inflammation and brain damage is not fully understood. Similar to activated microglia, activated astrocytes also produce pro- and anti-inflammatory cytokines. Therefore both, microglia ad astrocytes are prominent regulators of neuronal cell death after ischemic brain injury and are potential target candidates for stroke treatment [128, 137-141].

One of the mediators of acute brain injury is the pro-inflammatory cytokine IL-1. Together with activated microglia and other inflammatory cells (neutrophils, macrophages, lymphocytes) the post-stroke inflammatory response further propagates [142] and covers the area around the ischemic core, so called 'peri-infarct area' or 'inflammatory penumbra'. After initiating a cascade of immune cells, post-stroke inflammation is ultimately enhancing either recovery or cell death. Inflammatory cytokines, produced by microglia, such as IL-6, TNF-α and adhesion molecules are involved in the early stages of neurological impairment and ischemic infarct volume [143]. Moreover, it has been revealed that IL-6 predicts both the severity of the stroke lesions and functional outcome of patients [144, 145]. The plasma IL-6 concentrations associated with the severity of acute ischemic stroke in humans could therefore serve as a prognostic marker [53].

12. THE BIPHASIC RESPONSE OF NEUROVASCULAR UNIT INJURY AND PROTECTION

Whereas excitotoxicity and oxidative stress is present during the first hours till days after stroke injury, inflammation can last for months. The members of the neurovascular unit (endothelia, neurons, astrocytes, and pericytes) together with microglia, oligodendrocytes, neutrophils, monocytes and lymphocytes are involved in post-stroke injury [146]. The neurovascular unit cells are the first main responders (minutes to hours) to the vascular occlusion while immune cells initiate a robust inflammatory response that will be needed later in the delayed recovery phase (days to months). In the acute phase after stroke onset, lost cell-cell crosstalk between the cells of the neurovascular unit mediates both acute injury and delayed recovery. Moreover, the neurovascular unit mediators (NMDA, MMPs, HMGB1, VEGF, ROS, TNFα *etc*.) are deleterious in the acute phase after stroke onset causing immune cell recruitment, BBB breakdown and cell death mainly in the core area after stroke injury, however are on the contrary involved beneficially in the recovery phase by mediating angiogenesis, axonal sprouting and neurogenesis in the preserved peri-infarct area [146-148]. For example, the vascular endothelial growth factor (VEGF) increases BBB permeability in the acute phase, and facilitates angiogenesis and neurogenesis in the delayed stroke phase [149, 150]. Stroke pathophysiology therefore shows a biphasic phenomenon [146-148] which makes it rather difficult for stroke treatment.

13. EPIGENETICS ROLE IN CELL DEATH AND INFLAMMATION AFTER STROKE

Epigenetic processes such as DNA methylation, post-translational modifications of histone proteins and microRNAs (miRNAs) which regulate gene expression without directly changing the sequence of DNA have recently emerged as important regulators of increased plasticity during the recovery phase of stroke [151-153]. Global DNA methyla-

tion levels change dramatically after ischemic injury and contribute to cell death by silencing the neuroprotective genes [154-156]. On the other hand, it is also shown that DNA methylation is involved in restorative processes such as adult neurogenesis and synaptic plasticity [157]. Upon this, several DNA methylation and methyltransferase inhibitors such as Zebularine and Resveratrol have been suggested as therapeutic epigenetic targets against cerebral ischemia [156, 158].

Another epigenetic mechanism, histone deacetylase enzyme, deacetylates specific lysine residues in histone tails which leads to chromatin condensation and gene repression. The extent of histone deacetylase expression after stroke injury is dependent on cell type and location [159]. There are several types of histone deacetylases enzymes, from which stroke causes a global reduction in acetylation levels of histones H3 [160, 161] and H4 [162] in the ischemic brain. Histone deacetylase starts within hours of stroke onset and can last for two weeks [163]. Histone deacetylase inhibitors can specifically change gene expression and ameliorate ischemic damage by increasing the expression of neuroprotective proteins such as Bcl-2 and Hsp70 and thereby become potential therapeutic targets for ischemic stroke therapy [164].

Furthermore, ischemic stroke injury dramatically alters the expression of miRNAs that are involved in neurogenesis and brain repair [165]. One of the miRNAs that is downregulated after stroke is miR-124a, which normally acts to reduce the proliferation of neural progenitor cells in the subventricular zone of adult animals [166]. Downregulated miR-124a leads to increased cellular proliferation and neurogenesis [166] and is therefore a beneficial endogenous brain repair mechanism after stroke injury.

The ability for dynamic and reversible epigenetic modifications in neurons makes this approach very suitable for stroke treatment. While preliminary studies show modulation of neural cell regeneration and promoted brain repair and functional recovery after cerebral ischemia, yet these epigenetic strategies are far from clinical level and need more investigations.

14. NOVEL MOLECULAR AND CELLULAR TREATMENT STRATEGIES

Treatment in the acute phase of stroke, is prominently based on quick recovery of the blood flow or by neuroprotective agents aiming to disrupt the progression of the deleterious pathways [167]. On long-term, current approaches seek to achieve both to minimize the damage to the peri-infarct area and simultaneously maximize the restoration potential of lost neuronal cells. Post-stroke restoration occurs mainly by increased spine formation, axonal spouting and plasticity when the brain reorganizes after the injury [168, 169]. However, this is generally not enough to restore back to physiologically functional level. Also, neuronal circuitry can be imbalanced for a long period after the stroke accident. Cortical stimulation therapies can reorganize this inhibition-excitation balance and restore the neuronal circuitry [170, 171]. With regard to the post-stroke inflammation, in order to inhibit components of the inflammation cascade after stroke, some potential molecular and cellular treatment modalities could include ER stress inhibitors, microglia derived cytokines, free radical scavengers, cyclooxygenase-2 (COX-2) inhibitors and inhibitors of cell death pathways. Also, immunotherapy targeting potential receptors, cytokines, and adhesion molecules as well as epigenetic mechanisms have lately been gaining more scientific attention. A list of several potential targets for stroke recovery is provided in Table **2**.

Potential drugs that can be used along with thrombolytic treatments are agents that may provide inhibition on cell death pathways like caspase-3, calpein, and cathepsin inhibitors [172] and agents balancing profound autophagy. Although there are a number of potential drugs, unfortunately they can't penetrate to brain tissue due to the BBB. For example, caspase-3 inhibitors provide significant protection if given *via* intracerebral application. But using targeted drug delivery strategies can overcome the BBB issue. Karatas *et al.* clearly demonstrated that intravenously applied nanoparticles loaded with z-DEVD-FMK (a caspase inhibitor) provided significant neuroprotection after stroke and associated also with clinical improvement [173].

Both autophagy and apoptosis in neurons in the penumbral area after ischemic stroke can be evaluated for LC3-II and cleaved caspase-3 expression, respectively. While numerous studies have revealed that apoptosis and autophagy are co-occurring simultaneously in the penumbral area, Deng *et al.* showed recently that during the first 5 hours both autophagy and apoptosis are upregulated while after 72 hours of permanent ischemic stroke model, autophagy is still upregulated but apoptosis downregulated [174]. With regard to the latter, the study underlined that therapeutic strategies should rely on the dynamic activation status of both pathways.

Dhungana *et al.* showed that the lack of endothelial collagen XV is neuroprotective after ischemic stroke in collagen XV (member of the multiplexin family) knock-out mice and that this was correlative with rtPA treatment mechanism causing increased unbound collagen XV in plasma of wild-type mice [191]. Also, the study showed that the protective behavior of the absence of collagen XV may be mediated by the increased type A VEGF production after stroke onset [191]. VEGF-A is particularly of interest due to its abilities to mediate neuroprotection, angiogenesis, neurogenesis, the migration and survival of neurons and axon guidance [196].

In a recent study, Wei-Na *et al.* revealed that microglia depletion aggravates brain inflammation and brain injury after ischemia. In their middle cerebral artery occlusion (MCAO) model of mice, they used a colony-stimulating factor I receptor (CSF1R) inhibitor that inhibits microglia survival and proposed that microglia have neuroprotective effects after stroke by inhibiting the response from astrocytes in the post-ischemic inflammatory condition [227].

Interestingly, necroptosis inhibitors can reduce programmed necrotic cell death by selectively targeting the inactive form of the RIP1 kinase [89]. This necrostatin-1 was shown neuroprotective against ischemic brain damage. Moreover, recently researchers also demonstrated that intracerebroventricular injection of necrostatin-1 can improve neurologic outcomes in mice following ICH [228].

The cell death mechanism, pyroptosis, mainly mediated by NACHT, LRR and PYD domains-containing protein 3

Table 2. Potential neuroprotective targets for post-stroke brain damage and neuronal loss.

Therapeutic Candidate	Targeted Cell	Outcome	Study Model
Endogenous/Drug Therapies			
Uric acid	Neuronal mito-chondria	Reduces excessive intracellular calcium, reduces excito-toxicity	*In vitro* rat hippocampal neurons [175]
GRP78	Neuronal ER	Ca^{2+} homeostasis, protects against excitotoxicity and apoptosis	*In vitro* rat hippocampal neurons [176]
Arundic Acid (ONO-2506)	Astrocytes	Diminishes activation of astrocytes, reduces toxic levels of S-100β	*In vitro* astrocytes [177], permanent focal ischemia or transient focal ischemia models in rodents [178], patients with acute ischemic stroke [179]
Resveratrol	Neurons	Prevents oxidative stress, attenuates neuronal death	Rats subjected to global cerebral ischemia [180]
Coumestrol	Neurons	Neuroprotective, prevents long-term neuronal death	Rat model of global ischemia [181, 182]
TNFα and TNFβ	Neurons	Maintenance of calcium homeostasis	*In vitro* embryonic rat hippocampal, septal, and cortical neurons [183]
GDNF	Neurons	Protects against NMDA-induced cell death	*In vitro* mouse cortical neurons and astro-cytes and glial cells [184]
TAT-GDNF	Stroke volume	Transports GDNF across the BBB, reduces caspase 3 activity, increases viable neurons	Mouse model of focal cerebral ischemia [185]
IgG-GDNF and IgG-TNFR combination therapy	Stroke volume	Transports GDNF and TNFR across the BBB, reduces stroke volume in acute ischemic stroke	Mouse model of focal cerebral ischemia [186]
BDNF-MAb	Stroke volume	Transports BDNF across the BBB, reduction in stroke volume and an improvement in functional outcomes	Rat model of permanent MCAO [187]
IGF-1	Microglia and neural stem cells	Axonal growth and neurogenesis, tissue repair, cell pro-liferation, migration	*In vitro* neural progenitor cells, primary microglial culture [188]
Fumarate	Monocytes	Increases the level of neuroprotective IL-10, improves functional outcome, decreases edema volume after stroke	Mouse model of permanent MCAO [189]
TGF-β1	Microglia	Microglial phenotype changes from pro-inflammatory to anti-inflammatory phenotype, functional recovery	Murine model of ICH and plasma TGF-β1 levels of patients after ICH [190]
Absence of Collagen XV	Endothelial cells	Neuroprotective	Mouse model of ischemic stroke [191]
Humanized monoclonal anti-E/P-selectin	Endothelial cells	Neurovascular protective	Primate model of cerebral ischemia [192]
P-selectin	Endothelial cells	Reduces BBB breakdown	P-selectin deficient mice, transient ischemic stroke [193]
β2-integrin blocking	Neutrophils	Reduces neutrophil recruitment and decreases neutrophil mediated inflammation	Mouse model of renal ischemia-reperfusion [194]
GCSF	Monocytes	Reduces monocyte recruitment, reduces integrin expression on monocytes	Mouse model of focal brain ischemia [195]
VEGF-A	Neurons	Pro-angiogenesis, neurogenesis, neuroprotective	Rat model of MCAO [196]
HSPG and CSPG	Reactive astrocytes	Reduces glial scar formation	Rat model of chronic stroke [197, 198]
Perlecan domain V	Endothelial cells	Neuroprotective, improves stroke-affected motor function, and increases the post-stroke angiogenic response	Mouse and rat model of stroke [199]

Table 2. contd….

Therapeutic Candidate	Targeted Cell	Outcome	Study Model
Endogenous/Drug Therapies			
Inhibition of caspase-1 or caspase-11	Neurons, astrocytes and microglia	Decreases apoptosis and pro-inflammatory cytokines, promotes cell survival	Rat model of permanent MCAO [132], organotypic rat slices [133], mouse model of MCAO [134]
MFGE8	Neurons and glia	Inhibits inflammasome-induced IL-1β	Mouse model of permanent focal cerebral ischemia [200]
IL-1β antagonist	Neurons	Neuroprotective	Rat model of transient cerebral ischemia [123]
IVIg	Neurons	Suppresses NLRP1 and NLRP3 inflammasome mediated neuronal death	Rat model of ischemic stroke [125]
Beclin, Parkin	Neuronal ER and mitochondria	Mitophagy, cell survival	*In vitro* primary neuron culture and mouse model of transient MCAO [26], rat model of MCAO [201]
Necrostatin-1	Neurons	Inhibits necroptosis, suppresses apoptosis and autophagy, reduces brain edema and blood–brain barrier disruption, improves neurological outcome	Mouse model of MCAO, primary mouse cortical neuron culture [202], mouse ICH model [203], rat ICH model [204]
mtPGAM5	Neurons	Mitophagic protection against necroptosis	*In vitro* MEF cells and mouse model of focal middle carotid artery occlusion/reperfusion [205]
TRPM7 gene silencing	Neurons	Neuroprotective, neuronal survival	*In vitro* hippocampal and cortical neurons [206], rat model of ischemia [207], primary neuronal culture and neonatal mice model of hypoxia–ischemia [208]
Genetic inhibition of RIPK1	Neurons	Reduces acute neuronal death and improves functional outcome	Mice models of ICH [209]
PSD-95 inhibitor	Neurons	Inhibits PSD-95/nNOS/NMDA receptor-induced excitotoxicity [210], reduces infarct volumes, improves neurobehavioral outcome	Rat model of pial vessel occlusion or transient or permanent MCAO [211], neonatal mice pups with hypoxic-ischemia model [212]
miR-181a antagomiR	Astrocytes	Enhances estrogen receptor-α mediated stroke protection in females, reduces infarction size and improves behavioral outcome	*In vitro* cortical male and female astrocyte culture and female mice model of MCAO [213], mice model of MCAO [214]
miR-365 antagomiR	Reactive astrocytes	Modulation of PAX6-mediated astrocyte-to-neuron conversion, reduces neurological deficits and cerebral infarct volume	Rat model of MCAO [215]
miR-3473b antagomiR	Microglia	Reduces infarct damage, reduces microglia-mediated neuroinflammation	Mouse model of MCAO [216]
miR-15a/16-1 cluster antagomiR	Stroke volume	Reduces pro-inflammatory responses, upregulates anti-apoptotic proteins, improves neurobehavioral performance	Mouse model of MCAO [217]
miR-30d-5p antagomiR	Neurons	Increases autophagy and decreases apoptosis, decreases infarct volume, improves neurological performance	Rat model of cerebral hypoxic-ischemia [218]
Cell Therapies			
IPS cells	Neurons	Replace lost neurons	Mouse and rat models of stroke [219]
Exogenous MSCs	Glial cells	Reduce microglia/macrophages, enhance gliogenesis, reduce scar thickness	Retired breeder rats subjected to MCAO [220, 221]

Table 2. contd….

Therapeutic Candidate	Targeted Cell	Outcome	Study Model
Cell Therapies			
Autologous MSCs	Stroke volume	Less prominent atrophy, improved functional outcome	Patients with cerebral infarct at the middle cerebral arterial territory and with severe neurological deficits [222]
Exogenous MSCs	Proliferating cells and oligodendrocytes	Facilitate axonal sprouting and remyelination	Adult rat model of MCAO [223]
NT2N, ReNeurons	Exogenous neural cells	Neuroprotective, restoration of damaged areas	Rat model of stroke [224], rodent models of stroke model and human patients after stroke injury [225]
Autologous $CD34^+$ cells	Stroke volume	Improved clinical functional outcomes, reduced lesion volumes, promotion of angiogenesis and neurogenesis	Patients with acute ischemic stroke [226]

Abbreviations: GRP78, 78-kDa glucose-regulated protein; **S-100β**, Glial specific protein; **TNF**, Tumor necrosis factor; **MCAO**, Middle cerebral artery occlusion; **GDNF**, Glial cell line-derived neurotrophic factor; **TAT**, 86-amino acid protein; **IgG**, Immunoglobulin G; **NMDA**, N-methyl-D-aspartate receptor; **BDNF**, Brain-derived neurotrophic factor; **IGF-1**, Insulin-like Growth Factor 1; **IL**, Interleukin; **TGF-β**, Transforming growth factor beta; **GCSF**, Granulocyte colony stimulating factor; **VEGF-A**, Vascular endothelial growth factor A; **CSPG**, Chondroitin sulfate proteoglycan; **HSPG**, Heparan sulfate proteoglycans; **MFGE8**, milk fat globule-epidermal growth factor 8; **IVIg**, Intravenous immunoglobulin; **mtPGAM5**, Mitochondrial protein PGAM5; **MEF**, Mouse embryonic fibroblast; **TRPM7**, Transient receptor potential cation channel, subfamily M, member 7; **RIPK1**, Receptor-interacting protein kinase-1; **PSD-95**, Postsynaptic density protein 95; **IPS**, Induced pluripotent stem cell; **MSC**, Mesenchymal stem cell; **NT2N**, Teratocarcinoma-derived Ntera2/D1 neuron-like cells; **$CD34^+$**, Hematopoietic progenitor cell antigen.

(NLRP3) inflammasome contributes to ischemic brain injury. Regulation of the activation of this NLRP3 inflammasome at the molecular level may lead to potential novel therapeutic approaches [229]. Inhibitors of this inflammasome have revealed neuroprotection, however, many aspects of this approach need to be solved before it can be translated to the clinical level.

Next to targeting cellular factors, also focus can be led on the restoration of the neurovascular unit, especially the role of pericytes are crucial in ischemic injury. Namely, pericytes contribute to rapid and localized proteolytic degradation of the BBB during cerebral ischemia [230], and therefore can be suitable targets for the recovery of the capillary flow by antioxidative/antinitrative treatments.

Selectins can be a therapeutic target for neurovascular protection since they have important roles in cell death mechanisms of stroke [192, 231-233]. For example, P-selectin mediates the recruitment of leukocytes in the early phase after stroke and is a potential target for reducing BBB breakdown [193]. Furthermore, a humanized monoclonal anti-E/P-selectin antibody investigated in nonhuman primate stroke model was demonstrated to be safe and a potential effective clinical treatment for human stroke [192]. The study showed that, immediately after the onset of 1 hour of temporary ischemia, trends showed reduced polymorphonuclear leukocyte infiltration into ischemic cortex, reduced infarct volumes (by 41%), improved neurological score (by 35%), and improved ability to self-care (by 39%) [192].

Due to the infiltration of monocytes into the brain that correlates with brain edema and stroke outcome, recently, Weise *et al.* investigated the response of monocytes to high-dose of granulocyte colony stimulating factor (GCSF) and found out that this cytokine/hormone reduces monocyte infiltration into the ischemic brain [195]. The study further showed that GCSF decreases integrin expression on circulating inflammatory monocytes after stroke in an IL-10 dependent way. Thus, reduced presence of adhesion molecules leads to reduced infiltration of monocytes into the brain and can be neuroprotective after stroke injury.

Specific miRNAs are also emerging as new molecular targets in both prevention of stroke as well as in recovering post-stroke injury. miRNAs are endogenously expressed noncoding short single-stranded RNAs that play a role in the regulation of gene expression at the post-transcriptional level, *via* degradation or translational inhibition of their target mRNAs [234]. Since stroke treatment has not succeeded with targeting single genes due to complex overlapping pathways, miRNAs are especially useful because of their ability to simultaneously regulate many target genes. miRNAs which are silencing their target mRNA are called antagomiRs. During stroke prevention, the use of synthetic antagomiRs may reduce focal cerebral damage [235]. Additionally, even in post-stroke injury antagomiRs have shown promising results *in vivo* [214, 215]. One of these antagomiRs, miR-181, administered 2 hours after MCAO to mice reduced the infarct volume and improved long-term recovery. Besides, post-stroke inflammation was reduced and anti-apoptotic BCL2 and apoptosis inhibitor XIAP were increased, indicating a neuroprotective outcome. In another study, it was found that the overexpression of miR-365 in ischemic brain and hypoxic cultured astrocytes caused a decreased PAX6 expression leading to reduced astrocyte-to-neuron conversion. Following this result, the miR-365 antagomiR was investigated and the modulation of astrocyte-to-neuron conversion was targeted by knockdown of miR-365 in a rat model of ischemic injury. The inhibition of miR-365 caused an increase in PAX6 expression and enhanced the conversion of astrocytes into mature neurons in rat brain after MCAO. Overall, the use of antagomiRs may be quite promising in enhancing post-stroke neurogenesis and brain repair.

15. CELL THERAPIES

Stem cell therapy is a potential therapy for stroke, however, many issues are addressed within this area including the therapeutic dose, type of cell therapy, and thorough understanding of the molecular and cellular mechanism [236].

Cell therapies for post-stroke recovery have different mechanism as target. In fact, endogenous neural stem cells, located in the subventricular zone and the dentate gyrus, can be targeted for increased proliferation and/or migration to the damaged site to compensate for and/or replace the lost neurons (Table **2**). This approach can be established by using factors such as glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), GCSF, insulin-like Growth Factor 1 (IGF-1), drugs such as indomethacin, non-coding RNA and hormones such as erythropoietin for neural stem cell proliferation, and stromal derived factor 1, integrin β1, and extracellular matrix manipulations for neural stem cell migration (8). Exogenous cell replacement therapies aim to deliver, teratocarcinoma-derived Ntera2/D1 neuron-like cells (NT2N) [225], ReNeuron and neural progenitor cells to the damaged areas in the brain. Stem cells can act as immunoregulators, promote angiogenesis and help restore the BBB. They further achieve synapse formation, dendritic branching and axonal plasticity. Currently, induced pluripotent stem (IPS) cells are very promising in reprogramming cells to the desired neural cells which may be potential candidates in replacing the lost tissue in post-ischemic injury. But these cell therapies [237] are on their baby steps yet and much effort and enormous studies are necessary in order to provide translational approaches for stroke treatment.

CONCLUSION

Many novel cellular treatment strategies lead to failures in human strokes and this can result from several factors. One of these factors is related to the translation of bench-to-bed data. Novel treatment modalities are tested in very well structured animal models with controlled physiological parameters including temperature, O_2 and CO_2 levels. Additionally, all human strokes and time to hospital admissions are different from each another showing high inter-individual variability. Besides, in many preclinical stroke models treatments are given at a certain time point after injury onset, however patients admitted to hospital after stroke may not be suitable for application of novel drugs due to late admission and results are not as homogenous as pre-clinical studies. These abovementioned variations may alter the therapeutic response in human strokes. Also, preclinical studies usually utilize minimum numbers of animals and conditions that may vary between laboratories. To this end, the Stroke Treatment Academic Industry Roundtable (STAIR) [238] recommends consolidation of data from different laboratories into a mega merged databank to advance both preclinical as well as clinical experimental design and outcome. Finally, it is also recommended to establish targeting multiple mechanisms simultaneously instead of meeting a solo pathway after stroke injury. Namely, crosstalk between different cell death pathways determines the fate of an injured cell after ischemic or hemorrhagic stroke. There is

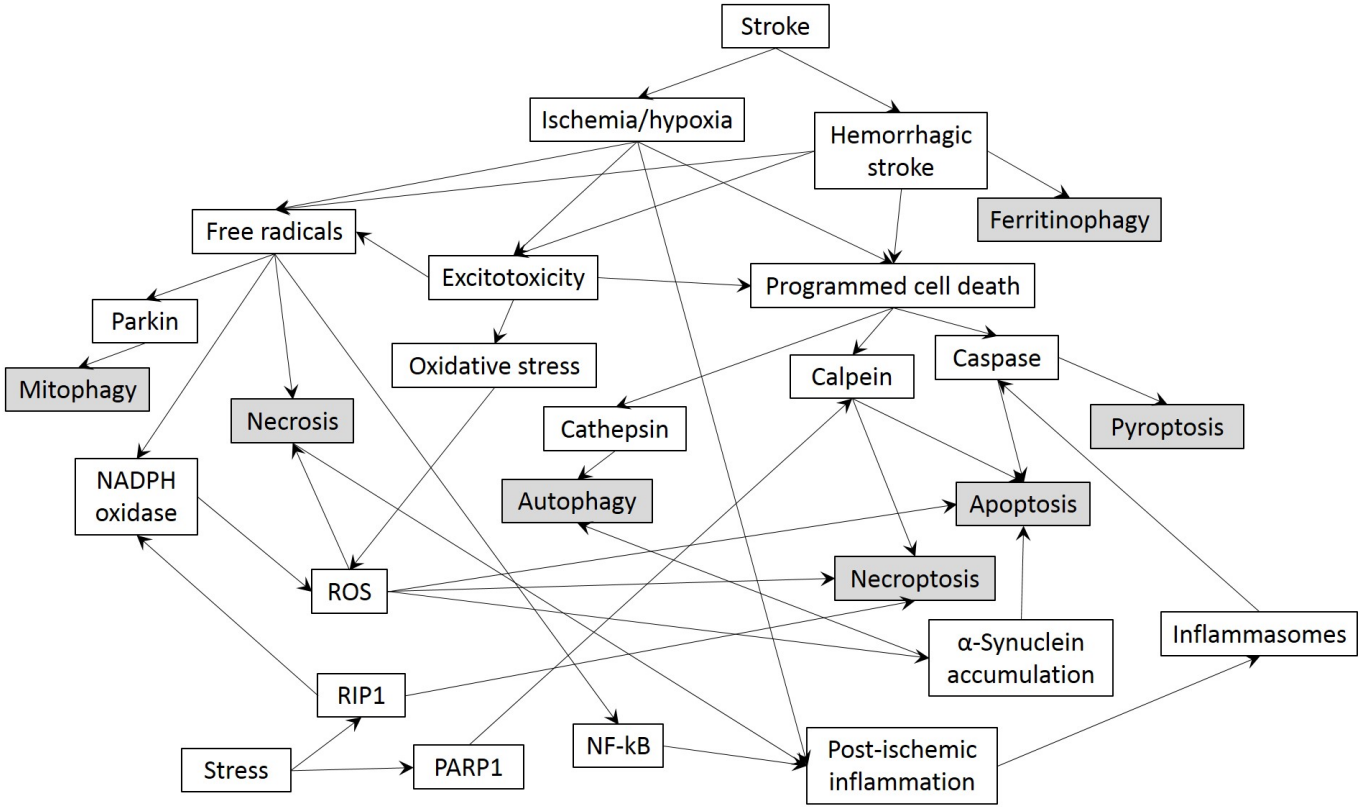

Fig. (2). Schematic overview of different and complex cell death pathways involved in stroke.

increasing evidence that multiple cell death pathways are simultaneously present in the ischemic core and penumbral area. Moreover, these pathways are fine-tuned and have either beneficial, deleterious or dual roles in the progression of post-stroke brain damage. The final death or survival state of a stroke-injured cell is likely dependent on the threshold of incoming and outgoing signaling cascades. Many treatment modalities incorporate the interruption of solo signaling cascades leading to suppression of the activation of cell death mechanisms. However, the cell death pathways are overlapping each other in several steps of the cascades and share common features which may end up in opposite cell fates when activated or inhibited, rendering a rather difficult system to regulate (Fig. **2**). If the signaling mechanism of these cell death pathways could be enlightened for the interfering parts, or possible combination therapies may be developed for targeting multiple cascades at the same time, it might actually bring us a step closer to a promising approach for stroke treatment.

LIST OF ABBREVIATIONS

AMPK	=	AMP-Activated Protein Kinase
BBB	=	Blood-Brain Barrier
Bcl-2	=	B-Cell Lymphoma-2
BDNF	=	Brain-Derived Neurotrophic Factor
Bmf	=	Bcl-2 Modifying Protein
COX-2	=	Cyclooxygenase-2
CSF1R	=	Colony-Stimulating Factor 1 Receptor
DAMP	=	Damage-Associated Molecular Patterns
DJ-1	=	Parkinson Disease Protein 7
ER	=	Endoplasmic Reticulum
FasL	=	Fas Ligand
GABA	=	Gamma-Aminobutyric Acid
GCSF	=	Granulocyte Colony-Stimulating Factor
GDNF	=	Glial Cell Line-Derived Neurotrophic Factor
ICH	=	Intracerebral Hemorrhage
IGF-1	=	Insulin-like Growth Factor 1
IL	=	Interleukin
IPS	=	Induced Pluripotent Stem Cells
LRRK2	=	Leucine-Rich Repeat Kinase 2
MAPK	=	Mitogen Activated Protein Kinase
MCAO	=	Middle Cerebral Artery Occlusion
miRNA	=	microRNA
MMP	=	Matrix Metalloproteinase
mTOR	=	Mechanistic Target of Rapamycin
mtPTP	=	Mitochondrial Permeability Transition Pore
NADPH	=	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NCOA4	=	Nuclear Receptor Coactivator 4
NFκB	=	Nuclear Factor Kappa B
NLR	=	Nod Like Receptors
NLRP1	=	NACHT, LRR and PYD Domains-Containing Protein 1
NLRP3	=	NACHT, LRR and PYD Domains-Containing Protein 3
NOD	=	Nucleotide-Binding Oligomerization Domain
NT2N	=	Teratocarcinoma-Derived Ntera2/D1 Neuron-Like Cells
PARP-1	=	Poly [ADP-Ribose] Polymerase 1
PINK 1	=	PTEN-Induced Putative Kinase 1
PRR	=	Pattern Recognition Receptors
RIPK1	=	Receptor-Interacting Protein Kinase-1
RNS	=	Reactive Nitrogen Species
ROS	=	Reactive Oxygen Species
rtPA	=	Recombinant Tissue Plasminogen Activator
SAH	=	Subarachnoid Hemorrhage
tBid	=	Truncated BH3 Interacting Domain Death Agonist
TGF-β	=	Transforming Growth Factor Beta
TLR	=	Toll Like Receptor
TNF-α	=	Tumor Necrosis Factor Alpha
VEGF	=	Vascular Endothelial Growth Factor A

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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