2018

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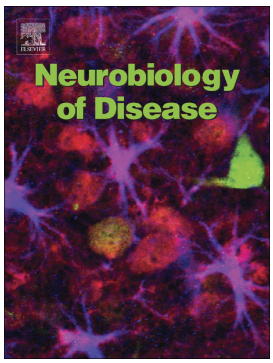
This article was originally published as:
MacDougall, G., Anderton, R. S., Mastaglia, F. L., Knuckey, N. W., & Meloni, B. P. (2018). Mitochondria and neuroprotection in stroke: Cationic arginine-rich peptides (CARPs) as a novel class of mitochondria-targeted neuroprotective therapeutics. *Neurobiology of Disease, 121*, 17-33.

Original article available here:
10.1016/j.nbd.2018.09.010

This is the accepted manuscript version of the article published as:

MacDougall, G., Anderton, R.S., Mastaglia, F.L., Knuckey, N.W., and Meloni, B.P. (2018) Mitochondria and neuroprotection in stroke: Cationic arginine-rich peptides (CARPs) as a novel class of mitochondria-targeted neuroprotective therapeutics. *Neurobiology of Disease, 121,* 17-33. doi: 10.1016/j.nbd.2018.09.010

This article has been published in final form at https://doi.org/10.1016/j.nbd.2018.09.010



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PII: S0969-9961(18)30575-8
DOI: doi:10.1016/j.nbd.2018.09.010
Reference: YNBDI 4277

To appear in: *Neurobiology of Disease*

Received date: 14 May 2018
Revised date: 26 August 2018
Accepted date: 11 September 2018

Please cite this article as: Gabriella MacDougall, Ryan S. Anderton, Frank L. Mastaglia, Neville W. Knuckey, Bruno P. Meloni , Mitochondria and neuroprotection in stroke: Cationic arginine-rich peptides (CARPs) as a novel class of mitochondria-targeted neuroprotective therapeutics. Ynbdi (2018), doi:10.1016/j.nbd.2018.09.010

Mitochondria and Neuroprotection in Stroke: Cationic Arginine-Rich Peptides (CARPs) as a Novel Class of Mitochondria-Targeted Neuroprotective Therapeutics

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Abstract

Stroke is the second leading cause of death globally and represents a major cause of devastating long-term disability. Despite sustained efforts to develop clinically effective neuroprotective therapies, presently there is no clinically available neuroprotective agent for stroke. As a central mediator of neurodamaging events in stroke, mitochondria are recognised as a critical neuroprotective target, and as such, provide a focus for developing mitochondrial-targeted therapeutics. In recent years, cationic arginine-rich peptides (CARPs) have been identified as a novel class of neuroprotective agent with several demonstrated mechanisms of action, including their ability to target mitochondria and exert positive effects on the organelle. This review provides an overview on neuronal mitochondrial dysfunction in ischaemic stroke pathophysiology and highlights the potential beneficial effects of CARPs on mitochondria in the ischaemic brain following stroke.

Keywords

Stroke · Ischaemia · Cationic arginine-rich peptides · Mitochondria · Mito-protection · Oxidative stress · Membrane potential · Permeability transition · Apoptosis

1. Stroke Background: Epidemiological 'Snapshot' and Need for Neuroprotection

Acute stroke is a global epidemic responsible for the second and third leading causes of death and disability respectively [1, 2]. In 2010, the global prevalence of stroke was estimated to be approximately 33 million, with 16.9 million individuals experiencing their first stroke [3]. A significant proportion of stroke survivors are left with cognitive and physical disabilities [4, 5], or impaired language and verbal communication [6]. The burden of stroke to the global community is continuing to increase due to the aging world population and the increasing prevalence of risk factors for stroke, such as hypertension [7], diabetes [8], hyperlipidaemia [9], heart disease [10], obesity [11] and reduced physical activity [12–14]. As an increasing risk factor, the aging world population is a major concern [15], with approximately 900 million people aged over 60, rising to an expected 1.5 billion by the year 2050 [16]. To make matters worse, there are currently no clinically available neuroprotective agents for the acute treatment of stroke to minimise brain injury, and current therapies are mainly restricted to endovascular recanalisation interventions for a selected subgroup of ischaemic stroke patients. However, most patients are ineligible for such interventions due to narrow therapeutic time windows (e.g. 4.5h for tPA thrombolysis) and/or the need for specialised tertiary hospital facilities to perform the procedure (e.g. mechanical thrombectomy). Notwithstanding, the recent DAWN and DEFUSE 3 clinical trials demonstrated that thrombectomy, when performed up to 16 to 24 hours post-stroke symptoms, could improve outcomes in a subgroup of patients selected on the basis of the presence of potentially salvageable penumbral tissue [17, 18]. For this reason it is now recommended that a “tissue window” rather than a “time window” be used when considering therapeutic interventions, such as thrombectomy and neuroprotection. Furthermore, it highlights the need for a safe neuroprotective agent that can be administered early after stroke onset to slow brain infarction and preserve penumbral tissue.

Consequently, there is an urgent requirement for the development of new and more widely applicable neuroprotective therapies that lessen the neurological impact of stroke and increase the number of patients eligible for endovascular treatments. To this end, due to the lack of success in the past, there is growing sentiment that new approaches in the discovery and development of acute neuroprotection therapies will be required.

The main purpose of this review is to examine the role of mitochondrial dysfunction in stroke pathophysiology, and in doing so highlight the beneficial effects that neuroprotective cationic

arginine rich peptides (CARPs) may exert on mitochondria in neurons in the ischaemic brain following stroke.

2. Mitochondrial Dysfunction in Stroke Pathophysiology

2.1 Underlying stroke injury mechanisms: The ischaemic cascade

Ischaemic stroke results from the acute occlusion of a cerebral artery by thrombosis or embolism, and constitutes the bulk of stroke events, accounting for approximately 80% of cases [19], and as such, will be the main focus of this review. Ischaemic stroke pathophysiology is initiated by a sudden reduction in blood flow to the affected brain tissue, causing a deficiency in oxygen and glucose supply, triggering what is known as the ischaemic cascade (Figure 1) [20, 21]. The ischaemic cascade is influenced by the duration and severity of ischaemia [22], which usually culminates in the time-dependent death of neuronal tissue. Acute injury occurs within minutes in severely affected brain tissue in the ischaemic core, which represents an area of infarcted or irreversibly damaged brain tissue, and cannot be salvaged. Surrounding the ischaemic core is the potentially salvageable tissue known as the penumbra, which is affected by ischaemia, although to a lesser extent than in the core, and consequently has not succumbed to cell death. Brain injury in the penumbra can proceed over many hours, and is the target of endovascular recanalisation interventions (tPA and thrombectomy) and neuroprotective treatments. However, in the absence of adequate reperfusion or a neuroprotective therapy, the on-going and cumulative cellular injury, including the demise of mitochondria, reaches a critical threshold, and neurons become irreversibly committed to dying and are incorporated into the ischaemic core.

Mitochondria are key regulators in determining the fate of ischaemic brain tissue, as they are both affected by and are propagators of ischaemic injury. Furthermore, neuronal mitochondrial disturbances have also been identified as key contributors to the pathophysiology of several chronic neurological disorders, including Parkinson's disease [23], Alzheimer's disease [24], and Huntington's disease [25–33]. Another consideration is that mitochondrial bioenergetics naturally decline with age, largely as a result of accumulated oxidative damage to mitochondrial DNA, proteins, and lipids [34–37]. As a consequence, aged mitochondria are more likely to be vulnerable to the effects of cellular energy disturbances, as occurs in ischaemic stroke [38]. This has additional implications in stroke, as

most strokes occur in individuals over the age of 65 [39], and age-related decline in mitochondrial function may result in an increased susceptibility to ischaemic brain injury. In this context, mitochondria represent critical targets for the development of neuroprotective strategies for stroke and other neurological disorders, and as such, their role in the ischaemic cascade is described in detail below.

2.2 Biomolecular mechanisms underlying mitochondrial dysfunction during stroke

Energy failure and excitotoxicity: Glutamate and calcium

During ischaemic stroke, neuronal mitochondrial function is severely compromised due to the reduced availability of glucose and oxygen necessary for ATP production, a situation which is exacerbated by the high energy demand of neuronal tissue [40, 41]. The resulting disruption to the mitochondrial electron transport chain (ETC) halts oxidative phosphorylation, forcing cells to rely on anaerobic glycolysis for ATP production [42]. However, due to the limited supply of glucose, glycolysis is also severely compromised. Since ATP is required for the maintenance of ion gradients across neuronal plasma membranes [43], the resulting energy failure triggers the depolarisation of neurons and uncontrolled release of excitatory neurotransmitters, in particular glutamate, from pre-synaptic neurons [42]. High extracellular levels of glutamate cause excitotoxicity due to the overstimulation of post-synaptic glutamate receptors [44, 45], including N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, thereby causing prolonged depolarisation and toxic accumulation of calcium and sodium within neurons [46–48] (Figure 1). High levels of intracellular calcium are particularly toxic to neurons due to its involvement in the activation of proteolytic enzymes and lipases, free radical formation and oxidative stress, mitochondrial dysfunction, cell death signalling, and cytotoxic oedema [47, 49–52]. It is therefore not surprising that the inhibition of excitotoxic intracellular calcium influx is invariably neuroprotective [53–56].

Mitochondria and intracellular calcium

As a consequence of excitotoxic intracellular calcium disturbances, mitochondria can act as a buffer to help regulate cytosolic calcium concentrations and minimise toxic effects [57–60]. Mitochondria sequester calcium through several mechanisms including the mitochondrial calcium uniporter (MCU) [59–61], mitochondrial sodium-calcium exchanger (mNCX) [62],

voltage-dependent anion channel (VDAC) [63], "rapid-mode" of uptake (RaM) [64], and the mitochondrial-located ryanodine 1 receptor (mRyR) [65, 66] (Figure 1). However, during severe excitotoxicity and ongoing ionic disturbances, the buffering capabilities of mitochondria can become overwhelmed, leading to excessive intra-mitochondrial calcium concentrations and disruption of mitochondrial function [67, 68]. Although mitochondria are capable of extruding excess calcium via the mNCX, this exacerbates the toxic cytosolic calcium loading [69, 70]. Mitochondria also co-operate with the endoplasmic reticulum (ER) to help maintain cellular calcium homeostasis via the mitochondria-associated ER membrane (MAM), allowing the organelles to collaborate in calcium ion exchange [71]. In spite of this, the ER can be an additional source of cytosolic calcium following release of excess calcium via the ryanodine (RyR) and inositol triphosphate (IP_3R) receptors [72]. Furthermore, cytochrome *c* (cyt *c*), a protein involved in the mitochondrial ETC and apoptosis, is released from dysfunctional mitochondria and can translocate to the ER to selectively bind IP_3Rs (Figure 1), causing prolonged release of calcium from the organelle, and thereby sustaining a toxic feedback loop know as calcium-induced calcium release (CICR) [73]. Ultimately, the excessive and sustained intra-mitochondrial accumulation of calcium significantly impacts mitochondrial function, and is a potent trigger for mitochondrial reactive free radical species generation, which further impacts the function of the organelle and cell viability (Figure 2).

Oxidative and nitrosative stress and mitochondria

Stroke is associated with oxidative stress resulting from the overproduction of reactive oxidative species (ROS) and reactive nitrosative species (RNS), which overwhelms endogenous neuronal antioxidant defence mechanisms, leading to damage to cellular components [74]. During stroke, mitochondria generate an initial burst of ROS, including superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) [75–78]. Such oxidants cause considerable damage to brain tissue due to the brain's large polyunsaturated fatty acid composition and limited antioxidant defensive capabilities [79]. Mitochondrial ROS generation is primarily derived from the ETC, with complexes I and III generating the bulk of ROS through 'electron leak' [80]. Auto-oxidation of 'leaked' electrons to oxygen forms the free oxygen radical $O_2^{\bullet-}$ [75, 81], which is converted into H_2O_2 by endogenous manganese superoxide dismutase (MnSOD) [82]. Mitochondrial catalase and peroxiredoxin can convert H_2O_2 into H_2O [83]. However, the abundance of iron sulfur clusters and iron containing heme groups associated with ETC proteins, such as those present in complexes I, II and III [84], provide a bountiful source of iron for generation of $^{\bullet}OH$ from

H_2O_2 via the Fenton reaction [85] (Figure 2). Mitochondria are also major contributors to the ROS generation that occurs during spontaneous reperfusion or following therapeutic endovascular recanalization after stroke. Although timely restoration of cerebral blood flow is of primary importance in preventing further injury during stroke, the sudden reoxygenation of brain tissue can trigger what is known as 'reperfusion injury'. Reperfusion reintroduces molecular oxygen to oxygen-starved tissue and provides a fresh source of substrates causing a surge in ROS formation [75], which places additional stress on mitochondria already affected by ischaemia.

Mitochondria are also the target of RNS due to the excessive production of nitric oxide (NO•) during stroke [86, 87]. NO• plays an important role in excitotoxicity-mediated injury and is generated through neuronal nitric oxide synthase (nNOS) linked to neuronal NMDA receptor signalling [88]. During overactivation of NMDA receptors, calcium influx activates nNOS, producing NO•, which then interacts with mitochondrial-generated $O_2^{\bullet-}$ to produce the highly volatile peroxynitrite species ($ONOO^-$) [89–91]. Both NO• and $ONOO^-$ react with mitochondrial membranes and other lipid membranes, as well as eliciting S-nitrosylation and activation of pro-death proteins, including caspases and matrix metalloproteinases [89, 92, 93] (Figure 2). Furthermore, ROS/RNS-induced damage of ETC components, particularly the ETC anchoring phospholipid, cardiolipin, exacerbates ROS generation arising from enhanced electron 'leak', resulting in mitochondria not only acting as propagators of ROS formation, but also targets of ROS-induced injury [94, 95].

Free radical oxidation of cardiolipin is particularly detrimental as the phospholipid plays an important role in maintaining inner mitochondrial membrane (IMM) structure and anchoring ETC complexes to the IMM to ensure optimal mitochondrial bioenergetics [96, 97]. Furthermore, due to cardiolipin's high unsaturated fatty acid content and its close proximity to the site of mitochondrial ROS production, it is highly susceptible to oxidative damage [95]. As a consequence, oxidised cardiolipin can lead to further destabilisation of the ETC, as well as promoting the detachment of cyt *c* from the IMM and its release from mitochondria [98]. Furthermore, the interaction of oxidised cardiolipin with cyt *c* bestows peroxidase activity to the protein capable of catalyzing H_2O_2-dependent peroxidation, causing further oxidation of cardiolipin [99].

Together, excessive mitochondrial ROS/RNS generation perpetuates cellular injury by attacking mitochondrial and cytoplasmic lipid membranes, proteins and DNA, causing cellular damage that contributes to neuronal cell death [100–102].

The mitochondrial membranes and mitochondrial membrane potential

The outer mitochondrial membrane (OMM) is a selectively permeable phospholipid bilayer that controls the transport of substances in and out of the organelle via membrane-bound transporter complexes. For example, the OMM voltage-dependent anion channel (VDAC) facilitates the exchange of various ions and small uncharged molecules [103]. Similarly, selective cytosolic protein and peptide import across the OMM is achieved via multi-subunit conductor complexes known as 'translocase of outer mitochondrial membrane' (TOM) channels [104]. The inner mitochondrial membrane (IMM) is also a phospholipid bilayer that is extensively folded and is embedded with proteins comprising the ETC, as well as other conductance protein channels known as 'translocase of the inner membrane' (TIMs), which form a supercomplex with TOMs that communicate across the intermembrane space, and are responsible for the exchange of pre-proteins from the intermembrane space into the mitochondrial matrix [105, 106]. Crucially, the ETC is responsible for generating the electrochemical gradient across the IMM, known as the mitochondrial membrane potential ($\Delta\psi m$), which is necessary for energy production via oxidative phosphorylation [107] (Figure 2). The $\Delta\psi m$ is an integral part of the proton motive force (Δp) generated by the ETC and used by ATP synthase to generate ATP [108, 109]. The $\Delta\psi m$ is also critical for mitochondrial function and cell viability, as it is a primary factor that determines mitochondrial respiration, calcium sequestration capacity, and ROS production, all of which play a critical role in determining the fate of neurons during ischaemia [110].

During stroke, the excessive accumulation of calcium in the mitochondrial matrix can trigger hyperpolarization, and subsequently depolarization of the IMM and dissipation of the $\Delta\psi m$ [78], leading to impaired ATP production [111, 112]. In an attempt to maintain the multitude of $\Delta\psi m$-dependent processes critical for mitochondrial function, mitochondrial ATP synthase acts in reverse (F1Fo-ATPase) and hydrolyzes ATP to conserve the $\Delta\psi m$. However, due to the ongoing effects of ischaemia, the depletion of mitochondrial and cytosolic ATP further exacerbates loss of mitochondrial integrity and activation of cell death cascades [113, 114]. It is considered that the complete dissipation of the $\Delta\psi m$ in a large proportion of mitochondria

within a cell is one of the earliest indicators of irreversible cell death [115]. It is for these reasons that interventions that restore and/or preserve the $\Delta\psi m$ can improve the likelihood of cell survival, and therefore represent potent therapeutic interventions for neuroprotection [116–118].

Mitochondrial outer membrane permeabilization and cell death signalling

Neuronal mitochondrial dysfunction culminates in mitochondrial membrane permeabilization, which is a critical event in promoting pro-death signalling pathways via the release of cell death-promoting molecules from the intermembrane space into the cytosol. Mitochondrial-mediated cell death can proceed by two different mechanisms depending on either apoptotic or necrotic stimuli (see comprehensive reviews by [119, 120]). Classic mitochondrial-mediated apoptotic cell death is instigated by the recruitment of pro-death Bcl-2 family member proteins, Bad and Bax, to the OMM [121]. Oligomerization of Bad and Bax in the OMM triggers mitochondrial outer membrane permeabilization (MOMP), which releases pro-apoptotic factors such as cyt *c* [122], apoptosis inducing factor protein (AIF) [123, 124], second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein with low pI (Smac/DIABLO), and endonuclease G (Endo G) [125] from the intermembrane space into the cytosol [126]. The release of the pro-cell death factors together orchestrates an interconnected process resulting in the dismantling of cell structures, contributing to the demise of the cell [127] (Figure 1).

Additionally, mitochondria play a role in necrotic cell death associated with elevated ROS generation and mitochondrial calcium loading. The toxic effects of ROS and calcium influx on mitochondria causes permeabilization of the IMM, known as permeability transition (PT), and results in the formation of the highly conductive mitochondrial permeability transition pore (mPTP) spanning the inner and outer mitochondrial membranes. The mPTP facilitates influx of calcium and other solutes into the mitochondrial matrix [128, 129]. Sustained mPTP conduction also contributes to mitochondrial ROS release [130], which in turn maintains mPTP opening in a self-propagating cycle known as ROS-induced ROS release (RIRR) (see review [78]). Influx of calcium into the matrix causes equilibration of the proton gradient and collapse of the $\Delta\psi m$, further enhancing ROS generation and metabolic inhibition [131, 132]. Furthermore, the increasing osmotic pressure from mitochondrial calcium accumulation causes the matrix to swell beyond the limits of the OMM, eventually rupturing the OMM and liberating calcium in addition to the aforementioned mitochondrial pro-death factors into the

cytosol. This stimulates proteases, nucleases, and lipases, causing subsequent degradation of cellular proteins, DNA, and membrane lipids, leading to necrotic cell death [133, 134].

Mitochondrial fusion and fission

As an adaptive response to metabolic and oxidative stress, mitochondria can rapidly alter their structural architecture through a process of fusion and fission. Mitochondrial fusion involves the joining of mitochondria in order to allow compensatory functions between healthy and metabolically challenged mitochondria [135], and thereby helps to preserve overall mitochondrial integrity [136, 137]. Fused mitochondria have a filamentous morphology comprising a large interconnected metabolic network that can span over a hundred microns across a single cell to enable the supply of ATP to energy-deficient cellular regions [138–140]. The ability of mitochondria to fuse is particularly important in the context of post-mitotic cells, such as neurons, as it provides a means of minimising the toxic effects of dysfunctional mitochondria without triggering cell death [35]. The process of mitochondrial fusion is controlled through the actions of the dynamin GTPase family members, mitofusins (Mfn1, Mfn2) and optic atrophy 1 (Opa1), which act on the OMM and IMM, respectively [141].

Similarly, mitochondrial fission represents a mechanism whereby the organelle can divide in an attempt to segregate mitochondrial damage, and eliminate dysfunctional mitochondria by mitophagy. Mitochondrial fission is mediated by the dynamin GTPase family member, Drp1 [142], as well as Fis1 [143], which act on the OMM and IMM respectively. However, mitochondrial fragmentation resulting from excessive mitochondrial fission, as well as the altered ability of cells to undergo mitochondrial fission, have been linked to neuronal injury and neurodegeneration [144–146].

With respect to stroke-mediated injury, NMDA receptor overactivation of cultured neurons is associated with excessive mitochondrial fragmentation, and promotion of mitochondrial fusion reduces excitotoxic cell death [147]. Similarly, Drp1 inhibition, which blocks mitochondrial fragmentation, reduces cell death in cortical neuronal cultures subjected to glutamate excitotoxicity [148]. In addition, it has been demonstrated in HeLa cells that the release of cyt *c* from mitochondria is accelerated as a consequence of down-regulation of OPA1, a dynamin-like GTPase required for mitochondrial fusion, and precedes mitochondrial fragmentation [149], indicating a temporal link between induction of mitochondrial pro-death

signalling and mitochondrial fragmentation. This is corroborated by several reports demonstrating mitochondrial fragmentation coinciding with ischaemic neuronal injury [150–153]. Taken together, it appears that strategies that promote mitochondrial fusion or inhibit excessive fission represent potential neuroprotective interventions to minimize neuronal death following stroke and possibly other acute and chronic neurodegenerative disorders.

3. CARPs as Mitochondria-Targeted Neuroprotective Therapeutics

3.1 Neuroprotection in stroke and CARPs: A brief history

In the context of stroke, a 'neuroprotective agent' refers to any compound that has the capacity to slow and/or halt the progression of infarct development by inhibiting neurodamaging processes and/or by stimulating cell survival pathways [154]. To date, while over 1000 novel potential neuroprotective therapeutics have been identified, many of which demonstrated efficacy in preclinical animal studies [155], none have returned positive outcomes in clinical trials. While there are many reasons for previous failures to translate into clinical efficacy (e.g. late administration of the agent after stroke, undesirable side-effects, ineffective drug targets, single pathway/mechanism of action, and study design), as mitochondria are a central component in many of the critical aspects of the ischaemic cascade, they represent a key target in the development of neuroprotective strategies. To this end, a class of inherently neuroprotective peptides, known as CARPs have emerged as candidates for mitochondria-targeted therapeutics in stroke and other acute and chronic CNS disorders, as well as ischaemic injury in other tissues, including myocardial and renal tissue [156–162].

3.2 Features and membrane-traversing properties of CARPs

CARPs typically range in size from 4 to 30 amino acids, and are positively charged due to the presence of cationic arginine residues, as well as cationic lysine and to a lesser extent histidine residues [163, 164] (Table 1). An important feature of CARPs is their ability to traverse cell membranes and enter cells and mitochondria, and for this reason they are also known as cell-penetrating peptides (CPPs) [165]. The ability of CARPs to traverse cell membranes is a direct result of peptide arginine content, namely the positive charge provided by the guanidinium head that is unique to the arginine amino acid [166–169]. To this end, the

guanidinium head group can form bidentate hydrogen electrostatic interactions with sulphate (e.g. in heparin sulphate proteoglycans), phosphate (e.g. in phospholipid head groups) and carboxylate (in phospholipid phosphatidylserine and protein receptors) moieties present on the plasma membrane or organelle membrane structures, such as those present in mitochondria, Golgi apparatus, endoplasmic reticulum, or the nucleus [170–174]. The electrostatic interactions between arginine residues and anionic structures present on membranes is a critical requirement for CARPs to cross typically impervious membranes, including the blood-brain barrier [163, 164, 175, 176], via both endocytic (e.g. macropinocytosis, caveolin/lipid-raft-mediated and clathrin-mediated endocytosis) [177, 178] and non-endocytic (e.g. passive diffusion, inverted micelle) mechanisms [173, 179–182].

CARP membrane-traversing efficacy is dependent on peptide arginine content, by increasing peptide positive charge and guanidinium head group electrostatic interactions with membrane anionic moieties. This was demonstrated by significantly reduced translocation capabilities of the TAT CPP when arginine residues were replaced with the alternative cationic amino acids, ornithine, histidine, and lysine, which are less basic compared to arginine and lack a guanidinium head group [183]. Furthermore, peptide cellular uptake was enhanced when the arginine content was increased [184]. With respect to arginine content and peptide length, CARP transduction efficiency across the plasma membrane, at least for poly-arginine peptides, is detectable with R6 (6 mer arginine) and peaks with R15 [183]. In addition, CARP membrane-traversing capabilities can be enhanced by presence of other residues, particularly the hydrophobic amino acid, tryptophan [185], and a high transmembrane potential [186]. The cell penetrating properties of CARPs are particularly useful in the context of neuroprotection, as one of the main obstacles in drug delivery to the CNS is transport across the blood-brain barrier and entry into the brain.

Interestingly, and of particular importance in the context of mitochondria-targeted peptides, is that the presence of a transmembrane potential is a crucial element for ability of guanidinium-rich CARPs to traverse membranes [186]. Regarding mitochondria, the $\Delta\psi m$ [163, 166, 187] and the presence of the negatively charged mitochondrial membrane phospholipid, cardiolipin [188], provides the ideal electrostatic attractive forces that enable CARPs to target this organelle. The guanidinium head groups of CARPs is also the likely source of their antioxidant capabilities, due to their innate free radical scavenging properties [189–192], making them perfectly suited to combat mitochondrial-generated ROS and/or cytosolic ROS

originating from membrane-bound NADPH oxidase during ischaemia [193], which is encouraged by the deleterious crosstalk between the two sources [194, 195].

By targeting mitochondria, CARPs can exert mito-protective effects on the organelle and by extension help maintain cell viability during times of stress, as occurs following ischaemia [196]. To date, a number of CARPs have been shown to target mitochondria, and have been examined in stroke and other brain injury models (Figure 3, Table 2), several of which are discussed in detail below. It should be noted that while they are outside the scope of this review, there are several non-peptide neuroprotective agents that potentially overlap mechanistically with CARPs by way of their guanidinium head groups, including metformin, phenformin and agmatine [197–201].

3.3 Pharmacokinetics of CARPs

While free CARPs are likely to have a serum half-life of several minutes, CARPs bound to serum proteins are likely to have a serum half-life of several hours [202–205]. For example, serum proteins such as albumin and α1-acid glycoprotein bind CARPs, providing a reservoir of the peptide that prolongs serum half-life and potentially extending peptide therapeutic duration [203, 206, 207]. Furthermore, various peptide structural modifications such as cyclization and use of D-enantiomer amino acids can enhance resistance to serum proteases, and thus improve serum stability [206, 208, 209]. With regards to tissue targeting, CARPs and CARP-conjugates generally exhibit preferential distribution in kidney, liver, spleen, lung and to a lesser extent brain [203, 204, 210–216]. While the ability of CARPs to target brain tissue is relatively modest, this may be advantageous in terms of reducing undesirable neurological side effects, while still providing the desired therapeutic effects [203]. Furthermore, the increased permeability of the blood-brain barrier during ischaemic stroke is likely to increase uptake into the brain and prolong the presence of CARPs within ischaemic tissue [217].

3.4 Non-mitochondrial neuroprotective mechanisms of CARPs

There is growing evidence that CARPs, in addition to possessing mito-protective properties are plurifunctional in terms of their neuroprotective mechanisms of action. For example, studies in our laboratory have demonstrated that CARPs, including the TAT-fused NA-1

peptide (TAT-NR2B9c), have the capacity to not only reduce glutamic acid induced excitotoxic neuronal death and intracellular calcium influx [160, 161, 218–220], but also reduce neuronal surface expression of the NMDA receptor subunit protein NR2B [221]. CARPs also have the capacity to reduce the activity and/or surface expression of other ion channels and receptors (e.g. AMPAR, NCX, TRPV1, CaV2.2, CaV3.3) that may exacerbate excitotoxic ionic disturbances in the brain following stroke [221–231], and is one likely mechanism whereby the peptides reduce the damaging effects of glutamate-induced excitotoxicity. In addition to the confirmed neuroprotective properties of CARPs, they possess other properties that may be beneficial following stroke; they induce the internalization of cell surface TNF receptors [231], scavenge free radicals and reduce lipid peroxidation [189–192], inhibit the activity of the proteasome [232–234], reduce inflammatory responses [235–241], activate pro-cell survival signalling [218, 229, 242], and inhibit pro-protein convertases that activate matrix metalloproteinases [243–245].

3.5 Preclinical and clinical efficacy of CARPs as mitochondria-targeted therapeutics for stroke

Szeto-Schiller (SS) peptides

Szeto-Schiller (SS) peptides possess cytoprotective and mitoprotective properties and consist of four amino acids in length, with a net positive charge of +3 due to the presence of one arginine and one lysine or ornithine residue, and C-terminal amidation [246]. The SS peptides are synthesised with alternating cationic and aromatic residues, with five peptides being best characterised (SS-01: YrFK-NH_2; SS-02: DmtrFK-NH_2, SS-20: FrFK-NH_2; SS-31: rDmtKF-NH_2; mtCPP-1: rDmtOF-NH_2; r = D-arginine, Dmt = 2,6-dimethyltyrosine, O = ornithine). It is likely that the combination of the arginine residue and cationic charge are the reason that SS peptides are able to traverse plasma and mitochondrial membranes.

In vitro studies examining the cytoprotective properties of SS-peptides have revealed several potential mechanisms of actions. With respect to mitochondrial targeting, it has been estimated that SS peptides can translocate within mitochondria at a 100-5000-fold higher concentrations compared to the extramitochondrial cytosolic compartment [247, 248]. Furthermore, SS peptides concentrate in the IMM, where they are believed to interact with and stabilize cardiolipin during oxidative stress, and thereby help preserve the IMM bioenergetics and reduce ROS production [249]. In addition, the ability of SS peptides to

target and stabilise IMM cardiolipin is considered a mechanism that inhibits disengagement of cyt *c* from cardiolipin and its release from mitochondria into the cytosol [248, 250]. While the exact mechanisms for the beneficial effects of SS peptides on IMM cardiolipin are not fully known, the ability of the cationic guanidinium group present on arginine residues to interact with anionic phosphate groups of cardiolipin (−2 net charge) may be involved.

There is also evidence that SS peptides have direct antioxidant properties, which would also reduce mitochondrial ROS levels. For example, *in vitro* studies have demonstrated that SS-31 quenches the ROS H_2O_2, $^{\bullet}OH$ and $ONOO^-$, and SS-02 quenches H_2O_2 [248, 251]. The ROS-quenching effects of SS-31 have been demonstrated in a mouse model of hypertensive cardiomyopathy [252], and its ability to restore mitochondrial ATP production has also been shown in a murine model of ischaemic kidney injury [157]. Furthermore, positive results with SS-31 have been obtained in a mouse stroke model, as demonstrated by Cho et al. (2007) whereby intraperitoneal administration of SS-31 (2 and 5 mg/kg; 3000 and 7150 nmol/kg) immediately, as well as 6, 24, and 48 hours after a 30 minute duration of MCAO significantly reduced infarct volume [253].

The most recently developed SS peptide, mtCPP-1, has superior mitochondrial uptake and superoxide scavenging abilities than SS-31 [254]. The mtCPP-1 peptide differs from SS-31 by the substitution of the lysine (K) residue with an ornithine (O) residue. While no explanation has been provided for the improved mito-protective effects of mtCPP-1 over SS-31, Rigobello et al. (1995) demonstrated in isolated rat liver mitochondria that the ornithine tetramer (OOOO; O4) was more effective than the lysine tetramer (KKKK) at maintaining mitochondrial permeability transition and membrane potential induced by calcium. Importantly, the arginine tetramer (RRRR; R4) was even more effective than O4 at inhibiting the toxic effects of calcium on mitochondrial permeability transition and membrane potential [255], which further highlights the critical importance of arginine content for the mito-protective benefits of CARPs.

Poly-arginine and related CARPs

In addition to SS peptides, longer chain CARPs, including poly-arginine peptides and cell penetrating peptides (CPPs), exhibit neuroprotective properties [219, 256, 257]. Initial reports from our laboratory and other laboratories demonstrated that the cationic arginine-rich CPP, TAT_{48-57} (TAT: GRKKRRQRRR; net charge +9; arginine residues 6), displayed modest

neuroprotective actions in both *in vitro* and *in vivo* stroke models [256, 258–261]. These findings were in line with an earlier study by Ferrer-Montiel et al (1998), demonstrating that cationic arginine-rich hexapeptides (net charge +3 - +7; Arginine residues 2 - 6) were neuroprotective in a hippocampal neuronal NMDA excitotoxicity model [262]. Subsequent studies in our laboratory showed that poly-arginine-9 (R9; 9 mer of arginine) and penetratin were 17-fold and 4.6-fold respectively, more neuroprotective than TAT in a cortical neuronal glutamate excitotoxicity model [256]. Further studies in our laboratory revealed that other poly-arginine peptides and CARPs as a group are highly neuroprotective, with efficacy increasing with increasing peptide arginine content and positive charge, peaking at R15 to R18 for poly-arginine peptides [219]. In addition, we have demonstrated that different amino acids can increase (e.g. tryptophan) or decrease (e.g. alanine) the neuroprotective efficacy of CARPs following glutamate excitoxicity [257], and that CARPs can reduce excitotoxic neuronal calcium influx and cell surface expression of the NR2B NMDA receptor subunit protein [221]. Interestingly, it appears that the ability of CARPs to traverse cellular membranes is correlated to peptide neuroprotective efficacy [161, 219, 257]. While the exact reason for this correlation is not fully known, it may be related to the ability of CARPs to modulate plasma receptor function or expression levels [216, 218, 258], and/or the ability of CARPs to reach therapeutic concentrations within the cell cytosol and organelles (e.g. mitochondria, nucleus, endoplasmic reticulum) by virtue of their cationic charge.

The neuroprotective properties of CARPs, including poly-arginine peptides, was further validated by Marshall et al (2015), who demonstrated the ability of CARPs to reduce NMDA-induced retinal ganglion cell death in the retina of rats. In addition, an R7 poly-arginine peptide with a cysteine di-sulphide bridge (C-s-s-C-R7: C-s-s-CRRRRRRR-NH2) reduced mitochondrial oxidative stress in retinal ganglion cells following NMDA exposure, and localised to mitochondria in HEK293 cells. Furthermore, in HEK cells R7 and C-s-s-C-R7 reduced mitochondrial respiration, $\Delta\psi m$, and ROS generation, and it was proposed that these effects on mitochondria would be neuroprotective for neurons during metabolic stress (e.g. excitotoxicity and ischaemia) [264].

With respect to stroke, we have demonstrated in experimental studies in the rat that the poly-arginine peptides R9D, R12, R18, R18D (D = D-isoform) and protamine all reduce infarct volume following permanent and/or transient middle cerebral artery occlusion (MCAO) and

perinatal hypoxia-ischaemia, and also reduce the severity of traumatic brain injury [162, 265–269], further confirming the efficacy of CARPs in a pre-clinical stroke setting.

TAT and other cationic arginine-rich CPP-fused neuroprotective peptides

The TAT peptide was first characterised for its cell penetrating properties by Frankel and Pabo (1988) [270]. Since then, many studies have used TAT and related cationic arginine-rich CPPs (CCPPs) (e.g. R9, penetratin) fused to "putative" neuroprotective peptides for application in stroke and other acute CNS injury pre-clinical neuroprotection studies, with most returning positive results [258, 260, 261, 271–276]. However, given the unequivocal neuroprotective properties of CARPs, several years ago we proposed that the neuroprotective effects of "putative" neuroprotective peptides fused to CCPPs, which are CARPs, was largely, if not exclusively mediated by the carrier peptide [219, 257]. In this context, it is also likely that the cargo peptide can further enhance the neuroprotective efficacy of CCPPs by providing additional arginine residues, other positively charged amino acids (e.g. lysine) or amino acids that increase CARP neuroprotective efficacy (e.g. tryptophan), as well as providing structural stability. Importantly, studies in our laboratory [219, 221, 257] and by Marshall et al. (2015), and more recently McQueen et al. (2017), support a CARP-mediated neuroprotective mechanism for "putative" neuroprotective peptides fused to CCPPs (e.g. TAT-NR2B9c and TAT-NR2Bct) [264, 277] (for more detail on this subject, see Meloni et al. 2015b). Therefore, based on the findings from our and other laboratories, we believe that past neuroprotective studies that have utilized CCPPs need to be critically re-evaluated.

Despite the confounding neuroprotective effects of the carrier peptide in studies using "putative" neuroprotective peptides fused to CCPPs, it is likely that due to their cationic and arginine-rich properties, these peptides have the capacity to target and exert beneficial effects on neuronal mitochondria following stroke. While it will be important for future studies to confirm the mito-protective properties of neuroprotective peptides fused to CCPPs, it is encouraging that so many studies have returned positive results in stroke and other acute and chronic neuronal injury models [226, 256, 274, 278–291]. To this end, the TAT-fused neuroprotective peptide TAT-NR2B9c, now known as NA-1, is currently being assessed in two Phase III clinical stroke trials (ESCAPE-NA-1 and FRONTIER). It is noteworthy in this regard that based on studies from our and other laboratories, other CARPs including poly-arginine peptides (e.g. R18) display even greater neuroprotective efficacy than NA-1 in stroke and related neuronal injury models [162, 257, 264, 265, 267].

Borna disease virus X protein

The X protein derived from the Borna virus (BVD-X) is a cationic protein containing 11 arginine residues that has mitochondriotropic and mito-protective capabilities. It was demonstrated to localise in mitochondria in rat C6 astroglioma cells, and was shown to prevent apoptosis in response to both extrinsic (10 μg/mL α-Fas) and intrinsic (50 μM peroxynitrite) pathway stimuli in HEK293T and mouse L929 cells, respectively [292].

In relation to neuroprotection, Szelechowski et al. (2014) demonstrated that in neurons over-expressing BVD-X, the protein localised to mitochondria and reduced rotenone–induced axonal fragmentation. Furthermore BVD-X preserved mitochondrial bioenergetics by preventing rotenone-induced loss of $\Delta\psi m$ and subsequent ROS generation. In addition, it was demonstrated that when administered stereotaxically into the substantia nigra pars compacta, BVD-X provided neuroprotection in a mouse MPTP Parkinson's disease model [293]. A peptide derived from BVD-X (SRPAPEGPQEEPLHDLRPRPANRKGAAVE) and fused to a CCPP and mitochondrial penetrating peptide (FRchaKFRchaK; cha = cyclohexyl-alanine), termed PX3, (net charge +4.1, arginine residues = 6), when administered intranasally also reduced MPTP-induced neurodegeneration in mice. PX3 also accumulated within mitochondria of cultured neurons and protected axons against rotenone-induced axonal fragmentation, which corresponded to reduced levels of mitochondrial fission and the retention of a greater proportion of filamentous over fragmented mitochondria within neurons. Interestingly, both BVD-X and PX3 were shown to reduce Drp1 phosphorylation in neurons exposed to rotenone, which is a signalling event known to stimulate mitochondrial fission [293]. In a subsequent study, the same group demonstrated that introduced mutations of the BVD-X protein designed to enhance mitochondrial localization resulted in greater efficacy against rotenone-induced mitochondrial stress in cortical neuronal cultures, an effect that correlated with enhanced mitochondrial filamentation and reduced fragmentation [294].

It is important to note that while the efficacy of the BVD-X derived peptide PX3 has yet to be explored in stroke injury models, its neuroprotective actions in terms of maintaining mitochondrial structural integrity provides further insight into the potential mechanisms whereby CARPs may be neuroprotective in stroke.

4. Conclusions and Future Perspectives

With ischaemic stroke constituting one of the leading causes of global morbidity and mortality, the development of effective neuroprotective therapeutics is of paramount importance and an urgent priority. Mitochondria are an exciting focus for developing novel neuroprotective agents, particularly in view of the critical role they play in homeostatic cellular processes, including the generation of cellular ATP and regulation of intracellular calcium homeostasis. Furthermore, as a consequence of cerebral ischaemia, damaged mitochondria play a key role in contributing to oxidative stress and the activation of pro-death signalling cascades in neurons post-ischaemia. At present, CARPs represent an exciting new class of pluripotent therapeutic for acute stroke due to their mitochondria-targeting and mitoprotective properties, as well as other neuroprotective actions. In particular, poly-arginine peptides (such as R18 and R18D), which have been shown to have potent neuroprotective properties in experimental stroke models, warrant further preclinical evaluation prior to progressing to clinical trials. In addition, due to their multiple neuroprotective mechanisms of action, CARPs may have broader potential clinical application for the treatment of other forms of acute brain injury, as well as chronic neurodegenerative disorders.

Declaration of conflicting interests

B.P. Meloni and N.W. Knuckey are named inventors of several patent applications regarding the use of arginine-rich peptides as neuroprotective agents. The other authors declare no conflict of interest.

Acknowledgments

This review in part was supported by the Department of Neurosurgery, Sir Charles Gairdner Hospital, and by a Neurotrauma Research Program of Western Australia research grant. The University of Notre Dame Australia also provided support through a University Postgraduate Award (UPA).

Figure Legends

Figure 1. Major biochemical pathways of the ischaemic cascade, which inevitably lead to irreversible cell death via apoptosis and necrosis. Insufficient oxygen for aerobic energy production results in diminished ATP levels, necessary for preservation of critical homeostatic functions, particularly maintenance of cellular ionic gradients. This disruption triggers mass release of excitatory neurotransmitters, which overstimulates calcium-permeable ion channel receptors on post-synaptic neurons, causing massive calcium influx. This in turn triggers a number of key destructive events, collectively known as the ischaemic cascade, and include activation of calcium-sensitive proteases, free radical formation, and mitochondrial dysfunction. Mitochondria are central modulators in this cascade, and are both targets and contributors of the resulting injurious effects.

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ASIC, acid-sensing ion channel; Apaf-1, apoptotic protease activating factor 1; CICR, calcium-induced calcium release; cyt c, cytochrome c; CypD, cyclophilin D; ER, endoplasmic reticulum; I-V, mitochondrial ETC complexes; IP_3 R, inositol triphosphate receptor; MCU, mitochondrial calcium uniporter; MAM, mitochondria associated membranes; mNCX, mitochondrial sodium-calcium exchanger; mPTP, mitochondrial permeability transition pore; mRyR, mitochondrial ryanodine receptor; NCX, sodium-calcium exchanger; NMDA, N-methyl-D-aspartate; PMCA, plasma membrane Ca^{2+} -ATPase; RaM, rapid mode calcium uptake; RyR, ryanodine receptor, TRPM, transient receptor potential ion channel; Smac, second mitochondria-derived activator of caspase; VDAC, voltage-dependent anion channel.

Figure 2. Mitochondrial ROS generation contributes to oxidative stress during ischaemic stroke. The IMM contains mitochondrial complexes I to V, which comprise the ETC. Electrons are transferred along the ETC in a stepwise fashion via electron carriers, including CoQ and cyt *c*, thereby releasing protons (H^+) into the IMS. This accumulation of cations comprises the mitochondrial membrane potential ($\Delta\psi m$), which is required for ATP production by complex V. This process naturally generates low levels of ROS as a by-product, which are metabolized by endogenous catabolic enzymes. However, during ischaemic stroke additional cellular stress turns mitochondria into the largest contributor of ROS in the brain. Consequently, electron 'leak' from complexes I and III are responsible for excess superoxide radical ($O_2 \bullet^-$) formation, which further reacts with endogenous manganese superoxide dismutase (MnSOD), or undergoes spontaneous dismutation to form toxic levels of hydrogen peroxide (H_2O_2). H_2O_2 subsequently reacts with transitional metals to further form hydroxyl radicals (•OH) via the Fenton reaction. Furthermore, NO• generated from nNOS as a result of calcium influx during ischaemic stroke are able to translocate to the mitochondria and react with $O_2 \bullet^-$ to form the highly toxic peroxynitrite ($ONOO^-$). Collectively, the overproduction of these ROS plays a key role in cell death during stroke.

CL, cardiolipin; CoQ, Coenzyme Q; e^-, electrons; H_2O_2, hydrogen peroxide; IMM, inner mitochondrial membrane; I-V, mitochondrial ETC complexes; IMS, intermembrane space; NO•, nitric oxide; $O_2 \bullet^-$, superoxide; •OH, hydroxyl radical; OMM, outer mitochondrial membrane; $ONOO^-$, peroxynitrite; $\Delta\psi m$, mitochondrial membrane potential.

Figure 3. Neuroprotective targets of CARPs during ischaemic stroke. A summation of documented and potential neuroprotective targets of CARPs that result from either direct and/or indirect mitochondrial protection, thereby preventing cell death.

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Table 1. CARP sequences, cationic charge and arginine content of peptides named in review.

Peptide	Sequence[1]	Net charge (at pH 7)	Arginine residues	Percentage Arginine
SS-peptides				
SS-01, SS-02, SS-20, SS-31, mtCPP-1	YrFK-NH_2, DmtrFK-NH_2, FrFK-NH_2, rDmtKF-NH_2, rDmtOF-NH_2	3	1	25%
Poly-arginine peptides				
R4	RRRR	4	4	100%
R6 - R7	RRRRRR-NH_2 - RRRRRRR-NH_2	-1	7-Jun	100%
C-s-s-C-R7	C-s-s-CRRRRRRR-NH_2	8	7	77.80 %
R9D	rrrrrrrrr-NH_2	10	9	100%
R12	RRRRRRRRRRR	12	12	100%
R15	RRRRRRRRRRRRRR	15	15	100%
R18 and R18D	RRRRRRRRRRRRRRRRR, rrrrrrrrrrrrrrrrr	18	18	100%

Other CARPs				
Penetratin	RQIKIWFQNRRMKWKK-NH_2	8	3	18.80 %
Protamine	PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR	21	21	65.60 %
Hexapeptides				
Hexapeptides	Ac-LCRRKF-NH_2, Ac-RRWWIR-NH_2, Ac-MCRRKR-NH_2	-1	3-Feb	33.3 - 50%
R4W2	RRRRWW-NH_2	5	4	66.70 %
r4W2	rrrrWW-NH_2	5	4	66.70 %
R5W	RRRRRW-NH_2	6	5	83.30 %
r5W	rrrrrW-NH_2	6	5	83.30 %
TAT and TAT-fused peptides				
TAT_{48-57} (TAT)	GRKKRRQRRR	8	6	60%
TAT-NR2B9c (NA-1)	YGRKKRRQRRR-KLSSIESDV	7	6	31.60 %
TAT-NR2Bct	GRKKRRQRRR-NRRRNSKLQHKKY	14.1	9	39.10 %

BVD-X protein and PX3 peptide				
BVD-X[2]	MSSDLRLTLLELVRRLNGNATIESGRLPGGRRRSPDTTTGTTGVTKTTEGPK ECIDPTSRPAPEGPQEEPLHDLRPRPANRKGAAVE	2	11	12.60 %
	SRPAPEGPQEEPLHDLRPRPANRKGAAVE-FRchaKFRChaK			
PX3[3]		4.2	6	16.20 %

[1]Dmt = 2,6-dimethyltyrosine; O = ornithine; r = D-arginine; Ac and NH_2 = acetyl and amine side groups; cha = cyclohexyl-alanine. [2] BVD-X protein region used for PX3 peptide (bold). [3]BVD-X-derived peptide fused to cell and mitochondrial penetrating peptide (underlined).

Table 2. Neuroprotective CARPs utilized in stroke and related CNS injury models.

	Injury model	Route and schedule of administration	Dose	Neuroprotection	Refs
SS-peptides	Hypoxia: 24h.	4h pre-treatment.	SS-31: 10 μM.	Reduced cell death, cytochrome c release, oxidative stress, and caspase-3 activation;	[250]
	H_2O_2 oxidative stress.	1h post H_2O_2.	SS-31: 1, 10, 100 μM.	prevented p66Shc activation.	[251]
			SS-31: 5 mg/kg/day.	Reduced cell death.	[251]
	G93A familial ALS model.	IP: daily.	SS-31 and SS-20: 2 mg/kg.	Improved survival and motor performance; reduced cell loss in spinal cord, and levels of lipid peroxidation and protein nitration.	[253]
	Stroke (*Rat*): 30min tMCAO.	IP: multiple injections at immediately post (0h), 6, 24, & 48h post-MCAO	SS-31: 2 or 5 mg/kg.	SS-31 only prevented ischaemia-induced cortical GSH depletion; reduced infarct volume	
Poly-arginine peptides	Glutamate excitotoxicity.	10-15min peptide pre-treatment and/or during glutamate exposure.	R6 to R15, R18 and tryptophan-containing poly-arginine peptides 0.5-15 μM.	Protected against excitotoxicity and reduced calcium influx.	[156, 160, 161, 256]
				Protected against excitotoxicity up to 6h prior to and 45min post-glutamate.	[219]
	Oxygen-glucose deprivation.	10min peptide pre-treatment at 1-6h prior or 0-45min post-glutamate exposure.	R6 to R15, R18 and tryptophan-containing poly-arginine peptides 0.5-15 μM.	Protected against OGD.	[219]
				Protected against OGD.	[256]
				Protected against OGD up to 2h prior.	[219]
	Stroke (*Rat*): 30min pMCAO.		R18 or R12W8: 1000 nmol/kg; R9D; 30 nmol/kg.	Reduced infarct volume and cerebral oedema; reduced hemisphere swelling.	[219] [161, 219]
				R12 and R18, and to a greater extent, R18D, reduced total infarct volume.	
		Concurrent peptide incubation with OGD.	R12, R15, or R18: 1000 nmol/kg; R18 and R18D: 1000 nmol/kg.	Reduced infarct volume and improved functional outcomes.	[265, 269]
		24h exposure post-OGD.	R18: 100, 300, 1000 nmol/kg.	Reduced cerebral oedema and improved neurological score.	[266]
	90min tMCAO.		R18: 1000 nmol/kg.	Reduced infarct volume and hemisphere swelling.	[267] [162]
	120min tMCAO.	15min exposure post-OGD.	R18: 30, 100, 300, 1000 nmol/kg.	Mild reduction in infarct volume.	[267]
		10min exposure pre-OGD 1-3h prior.	R18: 100 nmol/kg R18.	Mild reduction in cerebral oedema and improvement in neurological score with R18.	[267]
	180min tMCAO.		R18: 1000 nmol/kg R18.	Protected against excitotoxicity.	[219]
	NMDA excitotoxicity.	IV: 30min post-MCAO onset.	R12: 1 or 2 μM R12. Cyclic poly-arginine peptides: 1.5, 3, 6 nM.	Inhibited NMDA-induced PARP-1 activation; reduced mitochondrial oxidative stress and attenuated mitochondrial membrane hyperpolarization.	[264] [256]
				Protected against excitotoxicity.	
	Kainic acid excitotoxicity.	IV: 60min post-MCAO onset.	R9: 1, 5, 10, 15 μM.		
		IV: 120min post-MCAO onset.			
		IV: 150min post-MCAO onset.			
		ICA: 120min post-MCAO onset.			
		IV: 300min post-MCAO onset.			
		5min prior and during NMDA exposure.			
		Intravitreal injections (3 μL) of C-s-s-C-R7 peptide 2h prior to			

		successive injection with NMDA (20 nM). 15min pre-treatment.			
Penetratin, Protamine and Hexapeptides	Glutamate excitotoxicity. Kainic acid excitotoxicity. Oxygen-glucose deprivation. Stroke (*Rat*): 30min pMCAO. NMDA excitotoxicity.	15min prior to glutamic acid exposure. 10min peptide incubation 0, 1, and 2h prior to glutamic acid exposure. 15min prior to kainic acid exposure. Concurrent peptide incubation with OGD. IV: 30min post-MCAO onset. Concurrent treatment with NMDA exposure for 20min.	Penetratin: 1, 5, 10, 15 µM. Protamine and Pmt1-4: 1, 2, 5 µM Penetratin: 1, 5, 10, 15 µM. Penetratin: 1, 2, 5, 10 µM. Protamine 1000 nmol/kg. Hexapeptides: 10 µM.	Protected against excitotoxicity. Protected against excitotoxicity up to 2h prior to glutamate. Protected against excitotoxicity. Protected against OGD. Reduced infarct volume and cerebral oedema; improved neurological score. Protected against excitotoxicity.	[256] [161] [256] [256] [161] [262]
TAT and TAT-fused peptides	Glutamate excitotoxicity. Kainic acid excitotoxicity. Oxygen-glucose deprivation. NMDA excitotoxicity. Stroke (*Rat*): 30min pMCAO. 90min tMCAO. 120min tMCAO. 180min tMCAO.	10-15min prior to or at intervals (0, 15, 30, 45, or 60min) post glutamic acid exposure. 15min prior to kainic acid exposure. Concurrent peptide incubation. Simultaneous TAT treatment or 15min prior to and during NMDA exposure. IV: 60min post-MCAO onset. IV: 150min post-MCAO onset.	TAT or TAT-conjugated peptides, both L- and D-isoforms: 0.1-15 µM; TAT-NR2B9c: 1, 2, 5, 10 µM. D-TAT and TAT-conjugates: 1-15 µM. D-TAT: 1, 2, 5, 10 µM. TAT and D-TAT pre-treatment: 5, 10 µM; TAT-NR2B9c: 2, 5, 10 µM. TAT-NR2B9c: 100, 300, 1000 nmol/kg. TAT-NR2B9c: 30, 100, 300, 1000 nmol/kg. TAT-NR2B9c: 100 nmol/kg. TAT-NR2B9c: 1000 nmol/kg.	Protected against excitotoxicity. Reduced calcium influx. Protected against OGD. Protected against excitotoxicity. Reduced infarct volume and improved functional outcomes. Mild reduction in infarct volume and hemisphere swelling. No neuroprotection. No neuroprotection; therapeutic window <2h.	[219, 256, 258, 259] [256] [219, 256] [219] [266] [162] [267] [267]

		ICA: 120min post-MCAO onset. IV: 300min post-MCAO onset.			
BVD-X protein and PX3 peptide	Fas-mediated apoptosis.	10μg/mL α-Fas antibody to stimulate Fas-induced apoptosis.	Transfection.	Reduced DX2-Fas-induced apoptosis.	[291]
	Glucose oxidase (GOX)-induced death-receptor-independent apoptosis.	1.5 or 2h incubation with 2mU/mL GOX.	Transfection.	Reduced receptor-independent, mitochondrial-dependent apoptosis.	[291]
	MPTP PD neurodegeneration model (*Mouse*)	Stereotaxic injection with WT ($LV\text{-}X_{WT}$) or mutant X protein (X_{A6A7}) 2wk prior to MPTP intoxication model. ICV: PX3 daily, 1 day before IP MPTP intoxication and during first 4 days post-MPTP. Intranasal administration: PX3 daily, 1 day before IP MPTP intoxication and during first 4 days post-MPTP.	1.10^6TUs of vectors in 1 µl at flow rate of 0.1 µl/min. Transfection or PX3 peptide treatment (2 nM in 2 µL/injection at flow rate of 0.5 µl/min. Transfection or PX3 peptide treatment. Transfection. Transfection or PX3 peptide treatment Transfection. Transfection.	Reduced lesions and protection against MPTP-induced neurodegeneration. Reduced lesions and protection against MPTP-induced neurodegeneration. Reduced lesions and protection against MPTP-induced neurodegeneration. Reduced axonal fragmentation. Increased mitochondrial filamentation and elongation, and reduced rotenone-induced fission, and reduced axonal fragmentation. Neuroprotection against axonal fragmentation and reduced rotenone-induced ROS and oxidative stress with WT only. Near-complete prevention of rotenone-induced axonal fragmentation.	[292] [292] [292] [292] [292] [293]
	MPP+ toxicity.				
	Rotenone-induced oxidative stress.				
		WT (BVD_{WT}) or mutant ($BDV\text{-}X_{A6A7}$) X protein. WT or mutant X protein (X_{A6A7}), or un-transfected neurons receiving PX3 peptide. WT (BVD_{WT}) or mutant ($BDV\text{-}X_{A6A7}$) X protein. Neurons expressing non-mitochondrial X_{A6A7} (control) or mutant X			

proteins (X, $X_{\Delta 2\text{-}4}$ and X_{A4}).

ICA = intracerebral artery; ICV = intracerebral vessel; IP = intraperitoneal; IV = intravenous; MCAO = middle cerebral artery occlusion; OGD = oxygen-glucose deprivation; PD; Parkinson's disease.

Highlights

- There are currently no clinically effective neuroprotective therapeutics available for stroke patients.
- Mitochondria have been highlighted as a critical central mediator in determining the cellular fate of neuronal and glial cells during stroke.
- Cationic arginine-rich peptides (CARPs) are an emerging class of novel neuroprotective agents that have demonstrated potent neuroprotection by targeting mitochondria during stroke.
- Mito-protection may represent a new avenue of targeted drug development, and translate to better clinical outcomes in stroke trials.

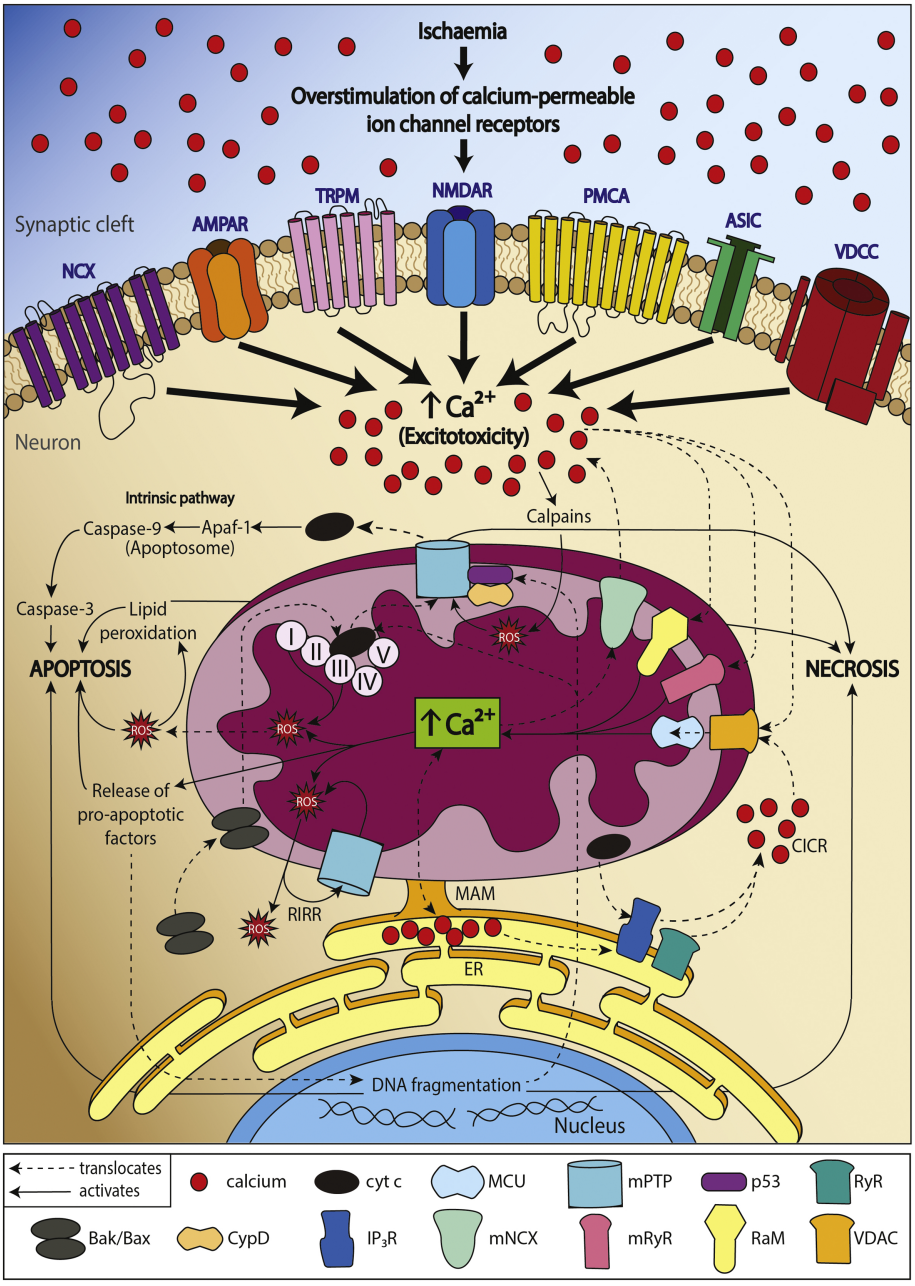
Ischaemia
Overstimulation of calcium-permeable ion channel receptors
Synaptic cleft
AMPAR
TRPM
NMDAR
PMCA
ASIC
VDCC
NCX
Neuron
↑ Ca2+ (Excitotoxicity)
Intrinsic pathway
Caspase-9 (Apoptosome)
Apaf-1
Calpains
Caspase-3
Lipid peroxidation
APOPTOSIS
NECROSIS
I
II
III
IV
V
ROS
↑ Ca2+
Release of pro-apoptotic factors
CICR
RIRR
MAM
ER
DNA fragmentation
Nucleus
translocates
activates
calcium
cyt c
MCU
mPTP
p53
RyR
Bak/Bax
CypD
IP3R
mNCX
mRyR
RaM
VDAC

Figure 1

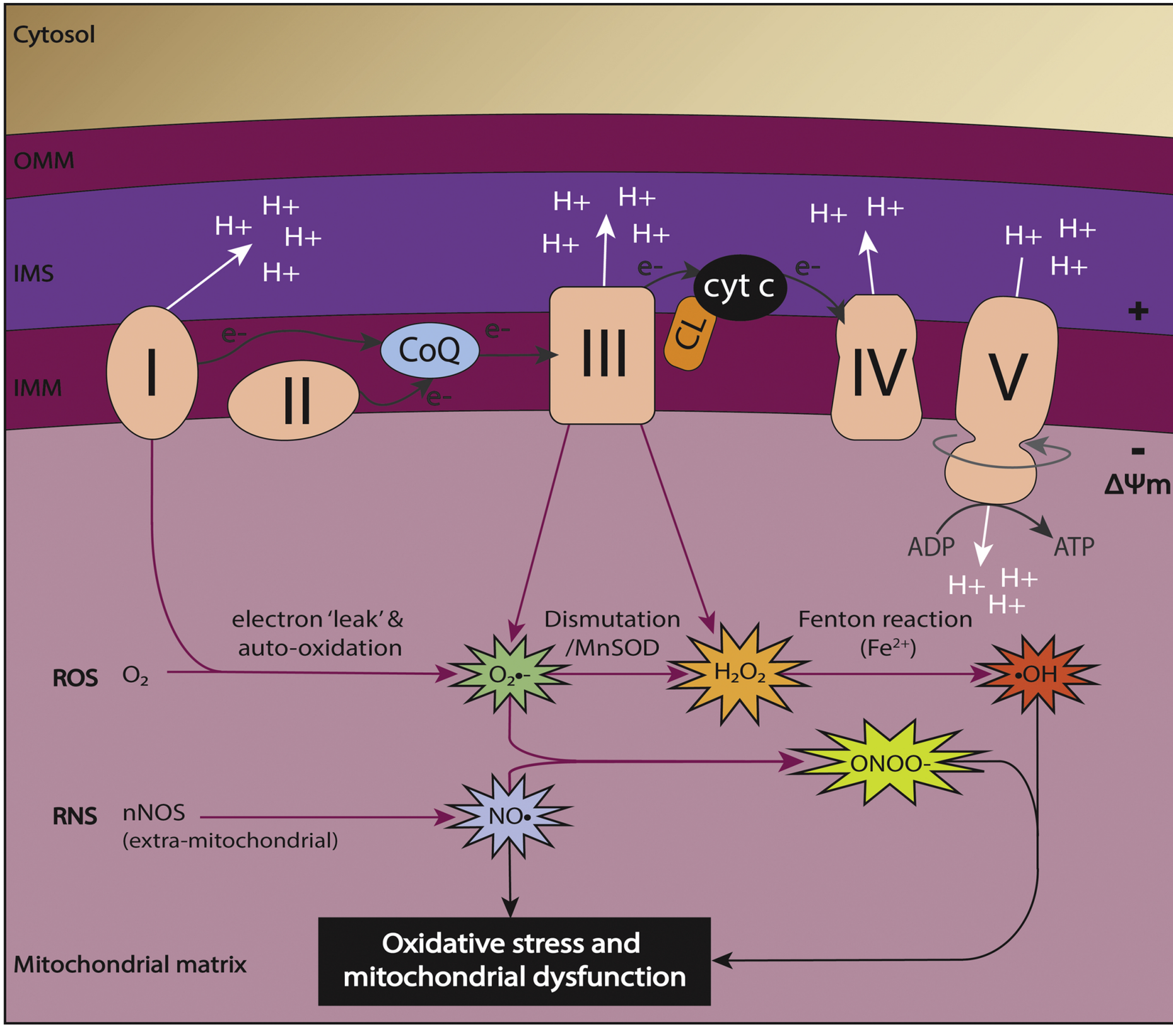
Cytosol
OMM
IMS
IMM
Mitochondrial matrix
H+
e-
I
II
CoQ
III
CL
cyt c
IV
V
+
-
ΔΨm
ADP
ATP
electron 'leak' &
auto-oxidation
Dismutation
/MnSOD
Fenton reaction
(Fe2+)
ROS
O2
O2•-
H2O2
•OH
ONOO-
RNS
nNOS
(extra-mitochondrial)
NO•
Oxidative stress and
mitochondrial dysfunction

Figure 2

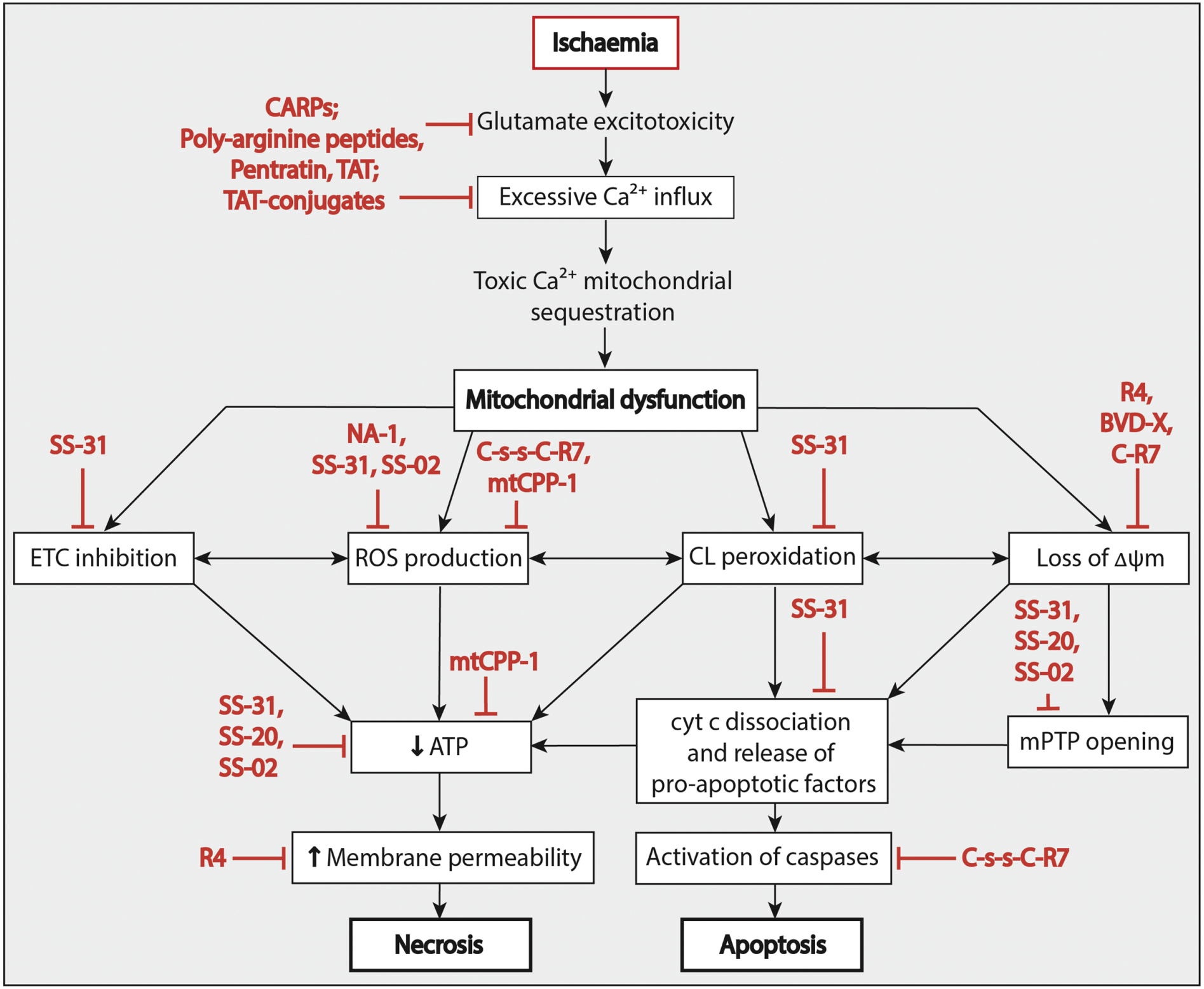
Ischaemia
CARPs;
Poly-arginine peptides,
Pentratin, TAT;
TAT-conjugates
Glutamate excitotoxicity
Excessive Ca^{2+} influx
Toxic Ca^{2+} mitochondrial sequestration
Mitochondrial dysfunction
SS-31
NA-1,
SS-31, SS-02
C-s-s-C-R7,
mtCPP-1
SS-31
R4,
BVD-X,
C-R7
ETC inhibition
ROS production
CL peroxidation
Loss of Δψm
SS-31
SS-31,
SS-20,
SS-02
mtCPP-1
SS-31,
SS-20,
SS-02
↓ ATP
cyt c dissociation
and release of
pro-apoptotic factors
mPTP opening
R4
↑ Membrane permeability
Activation of caspases
C-s-s-C-R7
Necrosis
Apoptosis

Figure 3