Review

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.

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 (Kassebaum et al., 2016; Lozano et al., 2012). In 2010, the global prevalence of stroke was estimated to be approximately 33 million, with 16.9 million individuals experiencing their first stroke (Mozaffarian et al., 2015). A significant proportion of stroke survivors are left with cognitive and physical disabilities (Douiri et al., 2013; Sun et al., 2014), or impaired language and verbal communication (Tatemichi et al., 1994). 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 (Beckett et al., 2008), diabetes (Khoury et al., 2013), hyperlipidaemia (Zavoreo et al., 2012), heart disease (Vasan et al., 2002), obesity (Suk et al., 2003) and reduced physical activity (Diep et al., 2010; Kelly-Hayes, 2010; McGinn et al., 2008). As an increasing risk factor, the aging world population is a major concern (Khare, 2016), with approximately 900 million people

aged over 60, rising to an expected 1.5 billion by the year 2050 (World Health Organization et al., 2011). 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. 4.5 h 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 DE-FUSE 3 clinical trials demonstrated that thrombectomy, when performed up to 16 to 24 h post-stroke symptoms, could improve outcomes in a subgroup of patients selected on the basis of the presence of potentially salvageable penumbral tissue (Albers et al., 2018; Nogueira et al., 2017). 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

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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 (Benjamin et al., 2017), 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 (Fig. 1) (Lee et al., 2000; Xing et al., 2012). The ischaemic cascade is influenced by the duration and severity of ischaemia (Woodruff et al., 2011), 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 (Ludtmann & Abramov, 2017), Alzheimer's disease (Magi et al., 2016), and Huntington's disease (Anandatheerthavarada et al., 2003; Bubber et al., 2005; Caspersen, 2005; Feigin et al., 2001; Gash et al., 2008; Huang et al., 2003; Sherer et al., 2007; Tabrizi et al., 1999; Zündorf & Reiser, 2011). Another consideration is that mitochondrial bioenergetics naturally decline with age, largely as a result of accumulated oxidative damage to mitochondrial DNA, proteins, and lipids (Gómez & Hagen, 2012; Grimm & Eckert, 2017; Payne & Chinnery, 2015; Yonutas et al., 2014). As a consequence, aged mitochondria are more likely to be vulnerable to the effects of cellular energy disturbances, as occurs in ischaemic stroke (Li et al., 2011). This has additional implications in stroke, as most strokes occur in individuals over the age of 65 (Thrift et al., 2017), 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

2.2.1. 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 (Attwell & Laughlin, 2001; Magistretti & Allaman, 2015). The resulting disruption to the mitochondrial electron transport chain (ETC) halts oxidative phosphorylation, forcing cells to rely on anaerobic glycolysis for ATP production (Kiewert et al., 2010). 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 (Erecinska & Silver, 1989), the resulting energy failure triggers the depolarisation of neurons and uncontrolled release of excitatory neurotransmitters, in particular glutamate, from pre-synaptic neurons (Kiewert et al., 2010). High extracellular levels of glutamate cause excitotoxicity due to the overstimulation of post-synaptic glutamate receptors (Loftis & Janowsky, 2003; Sattler & Tymianski, 2001), 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 (Bano & Nicotera, 2007; Gorter et al., 1997; Xiong et al., 2004) (Fig. 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 (Aarts et al., 2003; Bano & Nicotera, 2007; Hossmann, 2006; Malinski et al., 1993; Oliver et al., 1990). It is therefore not surprising that the inhibition of excitotoxic intracellular calcium influx is invariably neuroprotective (Duncan et al., 2010; Gouriou et al., 2013; Gulati et al., 2013; Nikonenko et al., 2005).

2.2.2. 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 (Cross et al., 2010; Friel, 2000; Hossmann et al., 1985; Sims & Muyderman, 2010). Mitochondria sequester calcium through several mechanisms including the mitochondrial calcium uniporter (MCU) (Cross et al., 2010; Hossmann et al., 1985; Liao et al., 2017), mitochondrial sodium-calcium exchanger (mNCX) (Williams et al., 2013), voltage-dependent anion channel (VDAC) (Görlach et al., 2015), "rapid-mode" of uptake (RaM) (Sparagna et al., 1995), and the mitochondrial-located ryanodine 1 receptor (mRyR) (Beutner et al., 2001; Jakob et al., 2014) (Fig. 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 (Nicholls, 2009; Starkov et al., 2004). Although mitochondria are capable of extruding excess calcium via the mNCX, this exacerbates the toxic cytosolic calcium loading (White & Reynolds, 1995; White & Reynolds, 1997). 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 (Pizzo & Pozzan, 2007). 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 (IP₃R) receptors (Jacobson & Duchen, 2002). 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 IP₃Rs (Fig. 1), causing prolonged release of calcium from the organelle, and thereby sustaining a toxic feedback loop know as calcium-induced calcium release (CICR) (Boehning et al., 2003). Ultimately, the excessive and sustained intra-mitochondrial accumulation
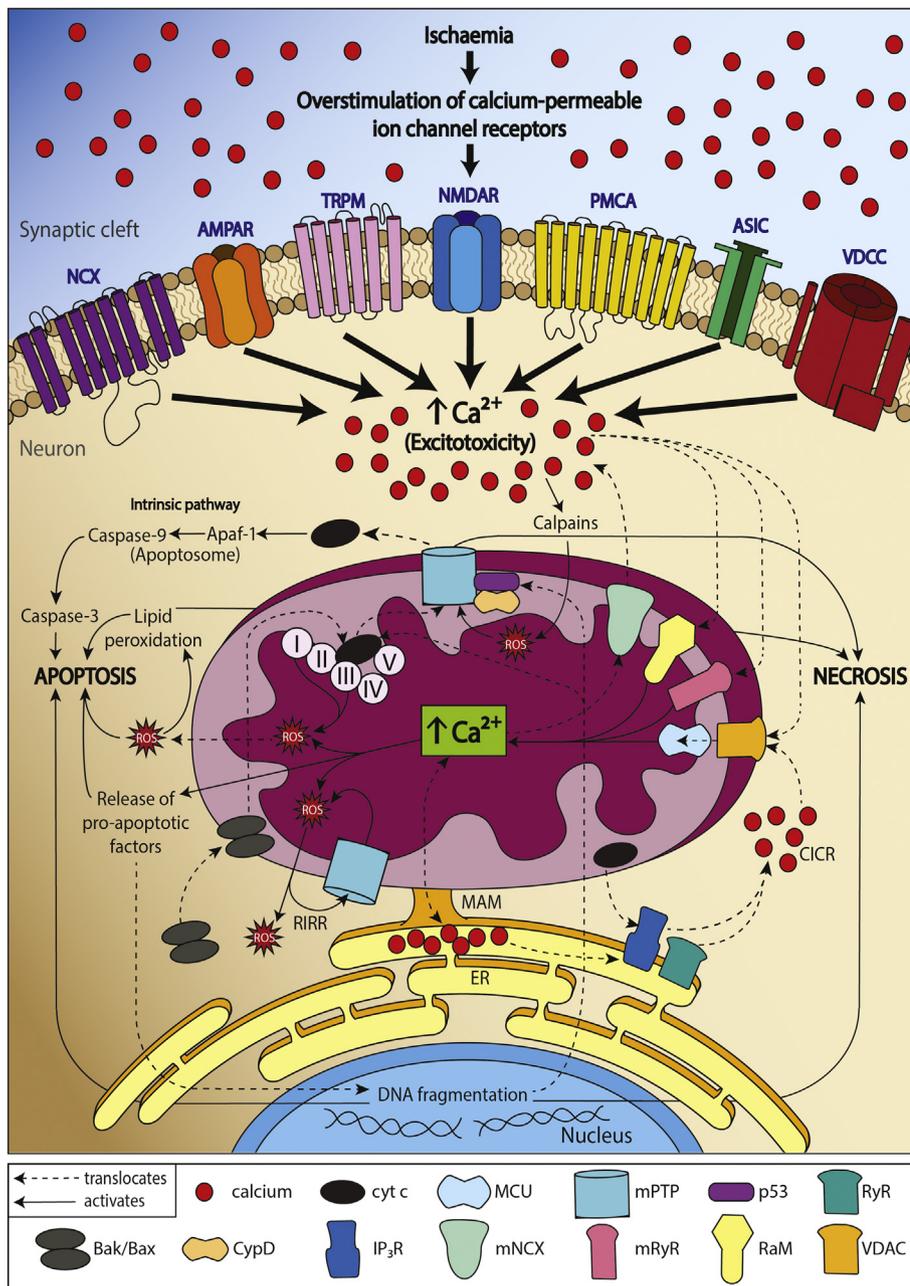
Fig. 1. Major biochemical pathways of the ischaemic cascade, which inevitably lead to irreversible cell death via apoptosis and necrosis. Insufficient oxygen for aerobic energy production results in diminished ATP levels, necessary for preservation of critical homeostatic functions, particularly maintenance of cellular ionic gradients. This disruption triggers mass release of excitatory neurotransmitters, which over-stimulates 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; IV, 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 Ca2 + -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.

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 (Fig. 2).

2.2.3. Oxidative and nitrosative stress and mitochondria

Stroke is associated with oxidative stress resulting from the over-production of reactive oxidative species (ROS) and reactive nitrosative species (RNS), which overwhelms endogenous neuronal antioxidant defence mechanisms, leading to damage to cellular components (Sies, 1997). During stroke, mitochondria generate an initial burst of ROS, including superoxide anion ($O_2^{·-}$), hydroxyl radical ($·OH$), and hydrogen peroxide (H_2O_2) (Cardoso et al., 2014; Kalogeris et al., 2014; Sanderson et al., 2013; Zorov et al., 2014). Such oxidants cause considerable damage to brain tissue due to the brain's large polyunsaturated fatty acid composition and limited antioxidant defensive capabilities (Friedman, 2011). Mitochondrial ROS generation is primarily derived from the ETC, with complexes I and III generating the bulk of ROS through 'electron leak' (Turrens, 1997). Auto-oxidation of

'leaked' electrons to oxygen forms the free oxygen radical $O_2^{·-}$ (Nohl et al., 2005; Sanderson et al., 2013), which is converted into H_2O_2 by endogenous manganese superoxide dismutase (MnSOD) (Holley et al., 2011). Mitochondrial catalase and peroxiredoxin can convert H_2O_2 into H_2O (Leak et al., 2013). 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 (Stehling et al., 2009), provide a bountiful source of iron for generation of $·OH$ from H_2O_2 via the Fenton reaction (Carroll et al., 2006) (Fig. 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 re-oxygenation 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 (Sanderson et al., 2013), which places additional stress on mitochondria already affected by ischaemia.
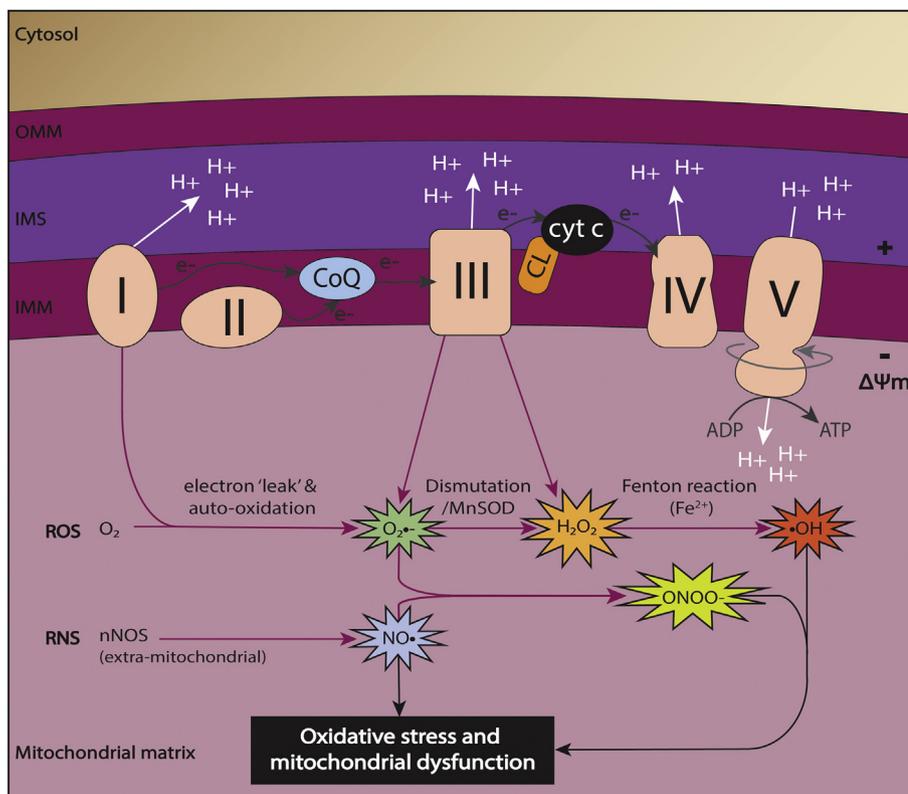
Fig. 2. Mitochondrial ROS generation contributes to oxidative stress during ischaemic stroke. The IMM contains mitochondrial complexes I to V, which comprise the ETC. Electrons are transferred along the ETC in a stepwise fashion via electron carriers, including CoQ and cyt *c*, thereby releasing protons (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$_2$·$^-$) formation, which further reacts with endogenous manganese superoxide dismutase (MnSOD), or undergoes spontaneous dismutation to form toxic levels of hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ 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$_2$·$^-$ 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$_2$O$_2$, hydrogen peroxide; IMM, inner mitochondrial membrane; I-V, mitochondrial ETC complexes; IMS, intermembrane space; NO, nitric oxide; O$_2$−, superoxide; OH, hydroxyl radical; OMM, outer mitochondrial membrane; ONOO−, peroxynitrite; $\Delta\psi$m, mitochondrial membrane potential.

Mitochondria are also the target of RNS due to the excessive production of nitric oxide (NO·) during stroke (Castillo et al., 2000; Di Meo et al., 2016). NO· plays an important role in excitotoxicity-mediated injury and is generated through neuronal nitric oxide synthase (nNOS) linked to neuronal NMDA receptor signalling (Brenman et al., 1996). During overactivation of NMDA receptors, calcium influx activates nNOS, producing NO·, which then interacts with mitochondrial-generated O$_2$·$^-$ to produce the highly volatile peroxynitrite species (ONOO$^-$) (Chen et al., 2011; Eliasson et al., 1999; Moskowitz et al., 2010). 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 (Eliasson et al., 1999; Gu et al., 2002; Nakamura et al., 2013) (Fig. 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 (Liu et al., 2009; Paradies et al., 2014).

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 (Gadicherla et al., 2012; Zhang et al., 2002). 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 (Paradies et al., 2014). 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 (Kagan et al., 2004). Furthermore, the interaction of oxidised cardiolipin with cyt *c* bestows peroxidase activity to the protein capable of catalyzing H$_2$O$_2$-dependent peroxidation, causing further oxidation of cardiolipin (Kagan et al., 2005).

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 (Adibhatla & Hatcher, 2010; Jena, 2012; Lee et al., 2005a).

2.2.4. 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 (Gupta & Ghosh, 2017). 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 (Endo & Kohda, 2002). 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 (Kulawiak et al., 2013; Qiu et al., 2013). 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 (Kühlbrandt, 2015) (Fig. 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 (Nakamoto et al., 2008; Qu et al., 2016). 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 (Nicholls & Budd, 2000).

During stroke, the excessive accumulation of calcium in the

mitochondrial matrix can trigger hyperpolarization, and subsequently depolarization of the IMM and dissipation of the $\Delta\psi m$ (Zorov et al., 2014), leading to impaired ATP production (Abramov & Duchen, 2008; Keelan et al., 1999). 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 (Martínez-Reyes et al., 2016; Walker, 2013). 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 (Fabbri et al., 2006). 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 (Kwok et al., 2010; Mehta et al., 2012; Wu et al., 2018).

2.2.5. 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 (Kinnally et al., 2011; Peixoto et al., 2010)). 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 (Hu et al., 2004). Oligomerization of Bad and Bax in the OMM triggers mitochondrial outer membrane permeabilization (MOMP), which releases pro-apoptotic factors such as cyt c (Yang & Cortopassi, 1998), apoptosis inducing factor protein (AIF) (Plesnila et al., 2004; Zhang et al., 2003), second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein with low pI (Smac/DIABLO), and endonuclease G (Endo G) (Lee et al., 2005b) from the intermembrane space into the cytosol (Tait & Green, 2010). 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 (Bolisetty & Jaimes, 2013) (Fig. 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 (Haworth & Hunter, 1979; Schild & Reiser, 2005). Sustained mPTP conduction also contributes to mitochondrial ROS release (Batandier et al., 2004), which in turn maintains mPTP opening in a self-propagating cycle known as ROS-induced ROS release (RIRR) (see review (Zorov et al., 2014)). 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 (Bernardi et al., 1992; Szabó et al., 1992). 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 (Bernardi et al., 2006; Izzo et al., 2016).

2.2.6. 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 (Yang et al., 2015), and thereby helps to preserve overall mitochondrial integrity (Chan, 2006; Tondera et al., 2009). 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 (Amchenkova et al., 1988; Rintoul & Reynolds, 2010; Skulachev, 2001). 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 (Grimm & Eckert, 2017). 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 (Otera & Mihara, 2011).

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 (Sesaki & Jensen, 1999), as well as Fis1 (Loson et al., 2013), 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 (Cho et al., 2009; Manczak & Reddy, 2012; Song et al., 2011).

With respect to stroke-mediated injury, NMDA receptor over-activation of cultured neurons is associated with excessive mitochondrial fragmentation, and promotion of mitochondrial fusion reduces excitotoxic cell death (Jahani-Asl et al., 2011). Similarly, Drp1 inhibition, which blocks mitochondrial fragmentation, reduces cell death in cortical neuronal cultures subjected to glutamate excitotoxicity (Young et al., 2010). 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 (Arnoult et al., 2005), 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 (Barsoum et al., 2006; Kumari et al., 2012; Niizuma et al., 2010; Sanderson et al., 2015). Taken together, it appears that strategies that promote mitochondrial fusion or inhibit excessive fission represent potential neuroprotective interventions to minimise 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 (Ginsberg, 2008). To date, while over 1000 novel potential neuroprotective therapeutics have been identified, many of which demonstrated efficacy in preclinical animal studies (O'Collins et al., 2006), 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

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

Peptide	Sequence[a]	Net charge (at pH7)	Arginine residues	Percentage Arginine
SS-peptides				
SS-01, SS-02, SS-20, SS-31, mtCPP-1	YrFK-NH$_2$, DmtrFK-NH$_2$, FrFK-NH$_2$, rDmtrKF-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$_2$	+7–+8	6–7	100%
C-s-s-C-R7	C-s-s-CRRRRRRR-NH$_2$	+8	7	77.80%
R9D	rrrrrrrrr-NH$_2$	+10	9	100%
R12	RRRRRRRRRRRR	+12	12	100%
R15	RRRRRRRRRRRRRRR	+15	15	100%
R18 and R18D	RRRRRRRRRRRRRRRRRR, rrrrrrrrrrrrrrrrrr	+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$_2$	+2–+3	2–3	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[b]	MSSDLRLTLLELVRRLNGNATIESGRLPGGRRRSPDTTTGTTGVTKTTEGPKECIDPTS**RPAPEGPGPQEEPLHDLRPRPANRKGAAVE**	+2	11	12.60%
PX3[c]	**SRPAPEGPQEEPLHDLRPRPANRKGAAVE-FRchaKFRChaK**	+4.2	6	16.20%

[a] Dmt = 2,6-dimethyltyrosine; O = ornithine; r = D-arginine; Ac and NH$_2$ = acetyl and amine side groups; cha = cyclohexyl-alanine.
[b] BVD-X protein region used for PX3 peptide (bold).
[c] BVD-X-derived peptide fused to cell and mitochondrial penetrating peptide (bold).
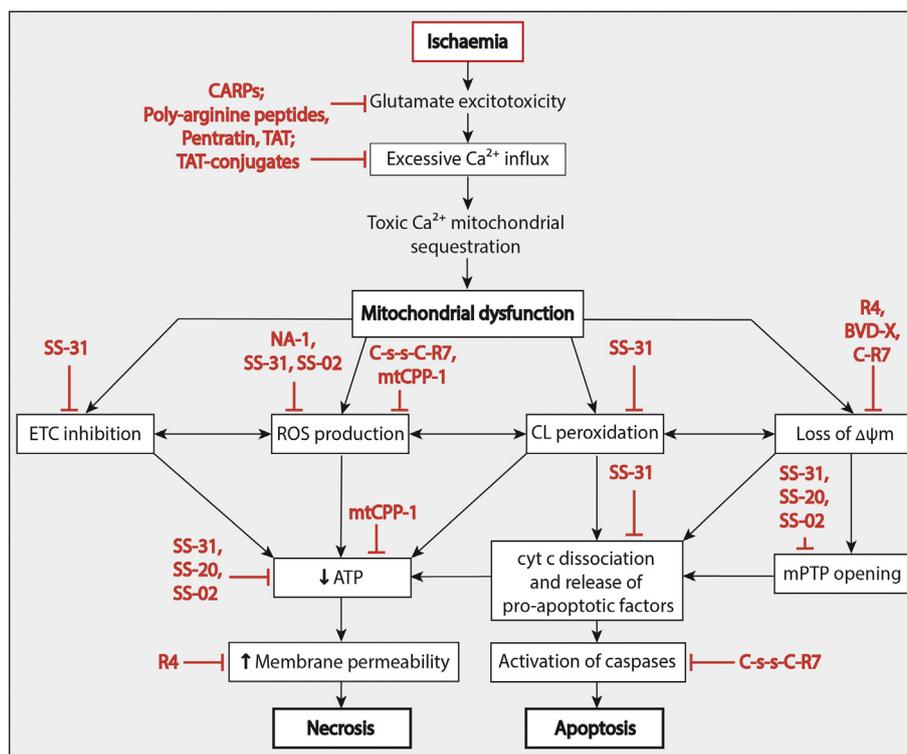
Fig. 3. Neuroprotective targets of CARPs during ischaemic stroke. A summation of documented and potential neuroprotective targets of CARPs that result from either direct and/or indirect mitochondrial protection, thereby preventing cell death.

disorders, as well as ischaemic injury in other tissues, including myocardial and renal tissue (Chiu et al., 2017; Edwards et al., 2017a; Ikeda et al., 2001; Meloni, 2017; Meloni et al., 2017; Milani et al., 2017a; Szeto et al., 2011).

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 (Herce et al., 2009; Herce et al., 2014) (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) (Munyendo et al., 2012). 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 (Chu et al., 1987; Horton et al., 2008; Nakase et al., 2012a; Takechi et al., 2012). 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 (Bogacheva et al., 2017; Fischer et al., 2004; Pantos et al., 2004; Rothbard et al., 2005; Vivès et al., 1997). 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 (Davoli et al., 2014; Herce et al., 2009; Herce et al., 2014; Tang et al., 2007), via both endocytic (e.g. macropinocytosis, caveolin/lipid-raft-mediated and clathrin-mediated endocytosis) (Duchardt et al., 2007; Nakase et al., 2004) and non-endocytic (e.g. passive diffusion, inverted micelle) mechanisms (Derossi et al., 1994; Futaki et al., 2001; Guterstam et al., 2009; Kawamoto et al., 2011; Vivès et al., 1997).

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 (Mitchell et al., 2000). Furthermore, peptide cellular uptake was enhanced when the arginine content was increased (Åmand et al., 2012). 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 (Mitchell et al., 2000). In addition, CARP membrane-traversing capabilities can be enhanced by presence of other residues, particularly the hydrophobic amino acid, tryptophan (Maiolo et al., 2005), and a high transmembrane potential (Rothbard et al., 2004). 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 (Rothbard et al., 2004). Regarding mitochondria, the $\Delta\psi m$ (Herce et al., 2009; Horton et al., 2008; Magzoub et al., 2005) and the presence of the negatively charged mitochondrial membrane phospholipid, cardiolipin (Marbella et al., 2013), 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 (Courderot-Masuyer et al., 1999; Giardino et al., 1998; Lass et al., 2002; Yildiz et al., 1998), making them perfectly suited to combat mitochondrial-generated ROS and/or cytosolic ROS originating from membrane-bound NADPH oxidase during ischaemia (Chen et al., 2009), which is encouraged by the deleterious crosstalk between the two sources (Rathore et al., 2008; Seung et al., 2006).

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: 24 h.	4 h pre-treatment.	SS-31: 10 μM.	Reduced cell death, cytochrome c release, oxidative stress, and caspase-3 activation; prevented p66Shc activation.	(Suk et al., 2003)
	H_2O_2 oxidative stress. G93A familial ALS model.	1 h post H_2O_2. IP: daily.	SS-31: 1, 10, 100 μM. SS-31: 5 mg/kg/day.	Reduced cell death. Improved survival and motor performance; reduced cell loss in spinal cord, and levels of lipid peroxidation and protein nitration.	(Sun et al., 2014) (Sun et al., 2014)
	Stroke (Rat): 30 min tMCAO.	IP: multiple injections at immediately post (0 h), 6, 24, & 48 h post-MCAO	SS-31 and SS-20: 2 mg/kg. SS-31: 2 or 5 mg/kg.	SS-31 only prevented ischaemia-induced cortical GSH depletion; reduced infarct volume	(Szelechowski et al., 2014)
Poly-arginine peptides	Glutamate excitotoxicity.	10-15 min 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.	(Li et al., 2010; Liu et al., 2009; Loftis & Janowsky, 2003; Szeto et al., 2011)
		10 min peptide pre-treatment at 1-6 h prior to 0-45 min post-glutamate exposure.		Protected against excitotoxicity up to 6 h prior to and 45 min post-glutamate.	(Plesnila et al., 2004)
	Oxygen-glucose deprivation.	Concurrent peptide incubation with OGD. 24 h exposure post-OGD. 15 min exposure post-OGD. 10 min exposure pre-OGD 1–3 h prior.	R6 to R15, R18 and tryptophan-containing poly-arginine peptides 0.5–15 μM.	Protected against OGD. Protected against OGD. Protected against OGD up to 2 h prior.	(Szeto et al., 2011) (Plesnila et al., 2004) (Plesnila et al., 2004)
	Stroke (Rat): 30 min pMCAO.	IV: 30 min post-MCAO onset.	R18 or R12W8: 1000 nmol/kg; R9D; 30 nmol/kg. R12, R15, or R18: 1000 nmol/kg; R18 and R18D: 1000 nmol/kg.	Reduced infarct volume and cerebral oedema; reduced hemisphere swelling. R12 and R18, and to a greater extent, R18D, reduced total infarct volume.	(Loftis & Janowsky, 2003; Plesnila et al., 2004) (Turrens, 1997; Walker, 2013)
	90 min tMCAO.	IV: 60 min post-MCAO onset.	R18: 100, 300, 1000 nmol/kg.	Reduced infarct volume and improved functional outcomes.	(Vasan et al., 2002)
	120 min tMCAO.	IV: 120 min post-MCAO onset.	R18: 1000 nmol/kg.	Reduced cerebral oedema and improved neurological score.	(Vaslin et al., 2009)
	180 min tMCAO.	IV: 150 min post-MCAO onset. ICA: 120 min post-MCAO onset. IV: 300 min post-MCAO onset.	R18: 30, 100, 300, 1000 nmol/kg. R18: 100 nmol/kg R18. R18: 1000 nmol/kg R18.	Reduced infarct volume and hemisphere swelling. Mild reduction in infarct volume. Mild reduction in cerebral oedema and improvement in neurological score with R18.	(Loson et al., 2013) (Vaslin et al., 2009) (Vaslin et al., 2009)
	NMDA excitotoxicity.	5 min prior and during NMDA exposure. Intravitreal injections (3 μL) of C-s-s-C-R7 peptide 2 h prior to successive injection with NMDA (20 nM).	R12: 1 or 2 μM R12. Cyclic poly-arginine peptides: 1.5, 3, 6 nM.	Protected against excitotoxicity. Inhibited NMDA-induced PARP-1 activation; reduced mitochondrial oxidative stress and attenuated mitochondrial membrane hyperpolarization.	(Plesnila et al., 2004) (Tu et al., 2010)
Penetratin, Protamine and Hexapeptides	Kainic acid excitotoxicity. Glutamate excitotoxicity.	15 min pre-treatment. 15 min prior to glutamic acid exposure. 10 min peptide incubation 0, 1, and 2 h prior to glutamic acid exposure.	R9: 1, 5, 10, 15 μM. Penetratin: 1, 5, 10, 15 μM. Protamine and Pmt1–4: 1, 2, 5 μM.	Protected against excitotoxicity. Protected against excitotoxicity. Protected against excitotoxicity up to 2 h prior to glutamate.	(Szeto et al., 2011) (Szeto et al., 2011) (Loftis & Janowsky, 2003)
	Kainic acid excitotoxicity. Oxygen-glucose deprivation.	15 min prior to kainic acid exposure. Concurrent peptide incubation with OGD.	Penetratin: 1, 5, 10, 15 μM. Penetratin: 1, 2, 5, 10 μM.	Protected against excitotoxicity. Protected against OGD.	(Szeto et al., 2011) (Szeto et al., 2011)
	Stroke (Rat): 30 min pMCAO.	IV: 30 min post-MCAO onset.	Protamine 1000 nmol/kg.	Reduced infarct volume and cerebral oedema; improved neurological score.	(Loftis & Janowsky, 2003)
	NMDA excitotoxicity.	Concurrent treatment with NMDA exposure for 20 min.	Hexapeptides: 10 μM.	Protected against excitotoxicity.	(Thrift et al., 2017)

Table 2 (*continued*)

	Injury model	Route and schedule of administration	Dose	Neuroprotection	Refs
TAT and TAT-fused peptides	Glutamate excitotoxicity.	10–15 min prior to or at intervals (0, 15, 30, 45, or 60 min) post glutamic acid exposure.	TAT or TAT-conjugated peptides, both L- and D-isoforms: 0.1–15 μM; TAT-NR2B9c: 1, 2, 5, 10 μM.	Protected against excitotoxicity.	(Plesnila et al., 2004; Szeto et al., 2011; Tait & Green, 2010; Takechi et al., 2012)
	Kainic acid excitotoxicity. Oxygen-glucose deprivation.	15 min prior to kainic acid exposure. Concurrent peptide incubation.	D-TAT and TAT-conjugates: 1–15 μM. D-TAT: 1, 2, 5, 10 μM.	Reduced calcium influx. Protected against OGD.	(Szeto et al., 2011) (Plesnila et al., 2004; Szeto et al., 2011)
	NMDA excitotoxicity.	Simultaneous TAT treatment or 15 min prior to and during NMDA exposure.	TAT and D-TAT pre-treatment: 5, 10 μM; TAT-NR2B9c: 2, 5, 10 μM.	Protected against excitotoxicity.	(Plesnila et al., 2004)
	Stroke (Rat): 30 min pMCAO.	IV: 60 min post-MCAO onset.	TAT-NR2B9c: 100, 300, 1000 nmol/kg.	Reduced infarct volume and improved functional outcomes.	(Vasan et al., 2002)
	90 min tMCAO.	IV: 150 min post-MCAO onset.	TAT-NR2B9c: 30, 100, 300, 1000 nmol/kg.	Mild reduction in infarct volume and hemisphere swelling.	(Loson et al., 2013)
	120 min tMCAO. 180 min tMCAO.	ICA: 120 min post-MCAO onset. IV: 300 min post-MCAO onset.	TAT-NR2B9c: 100 nmol/kg. TAT-NR2B9c: 1000 nmol/kg.	No neuroprotection. No neuroprotection; therapeutic window < 2 h.	(Vaslin et al., 2009) (Vaslin et al., 2009)
	Fas-mediated apoptosis.	10 μg/mL α-Fas antibody to stimulate Fas-induced apoptosis.	Transfection.	Reduced DX2-Fas-induced apoptosis.	(Zhao et al., 2004)
BVD-X protein and PX3 peptide	Glucose oxidase (GOX)-induced death-receptor-independent apoptosis.	1.5 or 2 h incubation with 2 mU/mL GOX.	Transfection.	Reduced receptor-independent, mitochondrial-dependent apoptosis.	(Zhao et al., 2004)
	MPTP PD neurodegeneration model (Mouse)	Stereotaxic injection with WT (LV-X$_{WT}$) or mutant X protein (X$_{A6A7}$) 2wk prior to MPTP intoxication model.	1.10⁶TUs of vectors in 1 μL at flow rate of 0.1 μL/min.	Reduced lesions and protection against MPTP-induced neurodegeneration.	(Zhao et al., 2013)
		ICV: PX3 daily, 1 day before IP MPTP intoxication and during first 4 days post-MPTP.	Transfection or PX3 peptide treatment (2 nM in 2 μL/injection at flow rate of 0.5 μL/min.	Reduced lesions and protection against MPTP-induced neurodegeneration.	(Zhao et al., 2013)
		Intranasal administration: PX3 daily, 1 day before IP MPTP intoxication and during first 4 days post-MPTP.	Transfection or PX3 peptide treatment.	Reduced lesions and protection against MPTP-induced neurodegeneration.	(Zhao et al., 2013)
	MPP+ toxicity.	WT (BVD$_{WT}$) or mutant (BDV-X$_{A6A7}$) X protein.	Transfection.	Reduced axonal fragmentation.	(Zhao et al., 2013)
	Rotenone-induced oxidative stress.	WT or mutant X protein (X$_{A6A7}$), or un-transfected neurons receiving PX3 peptide.	Transfection or PX3 peptide treatment	Increased mitochondrial filamentation and elongation, and reduced rotenone-induced fission, and reduced axonal fragmentation.	(Zhao et al., 2013)
		WT (BVD$_{WT}$) or mutant (BDV-X$_{A6A7}$) X protein.	Transfection.	Neuroprotection against axonal fragmentation and reduced rotenone-induced ROS and oxidative stress with WT only.	(Zhao et al., 2013)
		Neurons expressing non-mitochondrial X$_{A6A7}$ (control) or mutant X proteins (X$_{A2–4}$ and X$_{A4}$).	Transfection.	Near-complete prevention of rotenone-induced axonal fragmentation.	(Zorov et al., 2014)

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

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 (Apostolova & Victor, 2015). To date, a number of CARPs have been shown to target mitochondria, and have been examined in stroke and other brain injury models (Fig. 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 (Andrzejewski et al., 2014; Gilad & Gilad, 2000; Lee et al., 2002; Li et al., 2010; Pryor & Cabreiro, 2015).

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 (Butterworth et al., 2002; Jaques, 1949; Lee & Pardridge, 2001; Sarko et al., 2010). 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 (Nguyen et al., 2010; Sarko et al., 2010; Schartmann et al., 2018a). 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 (Leithold et al., 2016; Nguyen et al., 2010; Schartmann et al., 2018b). With regards to tissue targeting, CARPs and CARP-conjugates generally exhibit preferential distribution in kidney, liver, spleen, lung and to a lesser extent brain (Bullok et al., 2002; Delucia et al., 1993; Fawell et al., 1994; Kameyama et al., 2006; Lee & Pardridge, 2001; Nakase et al., 2012b; Sarko et al., 2010; Schwarze et al., 1999; Wunderbaldinger et al., 2002). 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 (Sarko et al., 2010). 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 (Gotanda et al., 2014).

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 (Edwards et al., 2017a; Fan et al., 2009; Meloni et al., 2015a; Meloni et al., 2017; O'Donnell et al., 2007), but also reduce neuronal surface expression of the NMDA receptor subunit protein NR2B (MacDougall et al., 2017). 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 (Borsello et al., 2003; Brittain et al., 2011; Brustovetsky et al., 2014; Cook et al., 2011; Fotin-Mleczek, 2005; François-Moutal et al., 2015; García-Caballero et al., 2014; Hirose et al., 2008; MacDougall et al., 2017; Planells-Cases et al., 2000; Sinai et al., 2010), 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 (Fotin-Mleczek, 2005), scavenge free radicals and reduce lipid peroxidation (Courderot-Masuyer et al., 1999; Giardino et al., 1998; Lass et al., 2002; Yildiz et al., 1998), inhibit the activity of the proteasome (Anbanandam et al., 2008; Gaczynska et al., 2003; Kloß et al., 2009), reduce inflammatory

responses (Aluganti Narasimhulu et al., 2014; Bolouri et al., 2014; Bosshart & Heinzelmann, 2002; Cheng et al., 2018; Hilchie et al., 2013; Kellett, 1965; Li et al., 2017), activate pro-cell survival signalling (Cook et al., 2011; O'Donnell et al., 2007; Yang et al., 2014), and inhibit pro-protein convertases that activate matrix metalloproteinases (Cameron et al., 2000; Fugere et al., 2006; Kacprzak et al., 2004).

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

3.5.1. 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 (Szeto, 2006). The SS peptides are synthesised with alternating cationic and aromatic residues, with five peptides being best characterised (SS-01: YrFK-NH2; SS-02: DmtrFK-NH2, SS-20: FrFK-NH2; SS-31: rDmtKF-NH2; mtCPP-1: rDmtOF-NH2; 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 (Szeto, 2008; Zhao et al., 2004). 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 (Birk et al., 2013). In addition, the ability of SS peptides to target and stabilize IMM cardiolipin is considered a mechanism that inhibits disengagement of cyt *c* from cardiolipin and its release from mitochondria into the cytosol (Zhao et al., 2004; Zhao et al., 2013). 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, ·OH and $ONOO^-$, and SS-02 quenches H_2O_2 (Petri et al., 2006; Zhao et al., 2004). The ROS-quenching effects of SS-31 have been demonstrated in a mouse model of hypertensive cardiomyopathy (Dai et al., 2011), and its ability to restore mitochondrial ATP production has also been shown in a murine model of ischaemic kidney injury (Szeto et al., 2011). Furthermore, positive results with SS-31 have been obtained in a mouse stroke model, as demonstrated by Cho et al. (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 h after a 30 min duration of MCAO significantly reduced infarct volume (Cho et al., 2007).

The most recently developed SS peptide, mtCPP-1, has superior mitochondrial uptake and superoxide scavenging abilities than SS-31 (Cerrato et al., 2015). 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 (Rigobello et al., 1995), which further highlights the critical

importance of arginine content for the mito-protective benefits of CARPs.

3.5.2. 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 (Meloni et al., 2014; Meloni et al., 2015a; Meloni et al., 2015b). 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 (Meade et al., 2010a; Meade et al., 2010b; Meloni et al., 2014; Vaslin et al., 2009; Xu et al., 2008). 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 (Ferrer-Montiel et al., 1998). 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 (Meloni et al., 2014). 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 (Meloni et al., 2015a). 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 excitotoxicity (Meloni et al., 2015b), and that CARPs can reduce excitotoxic neuronal calcium influx and cell surface expression of the NR2B NMDA receptor subunit protein (MacDougall et al., 2017). Interestingly, it appears that the ability of CARPs to traverse cellular membranes is correlated to peptide neuroprotective efficacy (Meloni et al., 2015a; Meloni et al., 2015b; Meloni et al., 2017). 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 (Delucia et al., 1993; Meade et al., 2010a; O'Donnell et al., 2007), 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-CRRRR-RRR-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, Δψm, 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) (Marshall et al., 2015).

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 (Edwards et al., 2017b; Milani et al., 2016a; Milani et al., 2016b; Milani et al., 2017a; Milani et al., 2017b; Milani et al., 2018), further confirming the efficacy of CARPs in a pre-clinical stroke setting.

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

The TAT peptide was first characterised for its cell penetrating properties by Frankel & Pabo, (1988). 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 (Cao et al., 2002; Doeppner et al., 2009; Kilic et al., 2003; Lai et al., 2005; Meade et al., 2010a; Nagel et al., 2008; Vaslin et al., 2009; Xu et al., 2008; Yin et al., 2006). 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 (Meloni et al., 2015a; Meloni et al., 2015b). 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 (MacDougall et al., 2017; Meloni et al., 2015a; Meloni et al., 2015b) 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) (Marshall et al., 2015; McQueen et al., 2017) (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 (Aarts et al., 2002; Asoh et al., 2002; Borsello et al., 2003; Choi et al., 2005; Fan et al., 2006; Fan et al., 2007; Ghosal et al., 2013; Guan et al., 2006; Guégan et al., 2006; Hirt et al., 2004; Kilic et al., 2003; Kim et al., 2005; Meloni et al., 2014; Pei et al., 2006; Ploia et al., 2011; Sik Eum et al., 2004; Wang et al., 2008). 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 (Marshall et al., 2015; Meloni et al., 2015b; Milani et al., 2016a; Milani et al., 2017a; Milani et al., 2017b).

3.5.4. 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 (Poenisch et al., 2009).

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 Δψm 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 (Szelechowski et al., 2014). A peptide derived from BVD-X (SRPAPEGPQEEPLHDLR-PRPANRKGAAVE) 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 (Szelechowski et al., 2014). 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 (Ferré et al., 2016).

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

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