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Review: Neuroprotective peptides fused to arginine-rich cell penetrating peptides: Neuroprotective mechanism likely mediated by peptide endocytic properties

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ABSTRACT

Several recent studies have demonstrated that TAT and other arginine-rich cell penetrating peptides (CPPs) have intrinsic neuroprotective properties in their own right. Examples, we have demonstrated that in addition to TAT, poly-arginine peptides (R8 to R18; containing 8-18 arginine residues) as well as some other arginine-rich peptides are neuroprotective *in vitro* (in neurons exposed to glutamic acid excitotoxicity and oxygen glucose deprivation) and in the case of R9 *in vivo* (after permanent middle cerebral artery occlusion in the rat). Based on several lines of evidence, we propose that this neuroprotection is related to the peptide's endocytosis-inducing properties, with peptide charge and arginine residues being critical factors. Specifically, we propose that during peptide endocytosis neuronal cell surface structures such as ion channels and transporters are internalised, thereby reducing calcium influx associated with excitotoxicity and other receptor-mediated neurodamaging signalling pathways. We also hypothesise that a peptide cargo can act synergistically with TAT and other arginine-rich CPPs due to potentiation of the CPPs endocytic traits rather than by the cargo-peptide acting directly on its supposedly intended intracellular target. In this review, we systematically consider a number of studies that have used CPPs to deliver neuroprotective peptides to the central nervous system (CNS) following stroke and other neurological disorders. Consequently, we critically review evidence that supports our hypothesis that neuroprotection is mediated by carrier peptide endocytosis. In conclusion, we believe that there are strong grounds to regard arginine-rich peptides as a new class of neuroprotective molecules for the treatment of a range neurological disorders.

Keywords:

Arginine-rich peptides

Cell penetrating peptides

Endocytosis

Excitotoxicity

Neuroprotection

Poly-arginine peptides

Stroke and cerebral ischaemia

TAT peptide

Contents

Abbreviations:

AIP, autocamtide-2-related inhibitory peptide; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APOE, Apolipoprotein E; APLP, amyloid precursor-like protein; APP, amyloid precursor protein; CaMKII, calcium/calmodulin-dependent protein kinase II; CaM-KIIN, calcium/calmodulin-dependent protein kinase II inhibitor; CaV2.2, voltage-gated, N-type calcium channel; Cav2.3; voltage-gated, R-type calcium channel; CaV3.3, voltage-gated, T-type calcium channel; CBD, calcium channel-binding domain; cGMP, cyclic guanosine monophosphate; CGRP, calcitonin gene related peptide; CNQX, central nervous system; 6-cyano-7-nitroquinoxaline-2,3-dione; CPP, cell penetrating peptide; CRMP, collapsing response mediator protein; DAPK1, death-associated protein kinase 1 protein; DM, DNA-binding motif; D1R-D2R, Dopamine D1-D2 receptor; DRG, dorsal root ganglion; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; FGFR, fibroblast growth factor receptor; GluR6, glutamate receptor 6; HSPG, heparan sulphate proteoglycan; HIV-TAT, human immunodeficiency virus-type 1 trans-activator of transcription; Insig-1, insulin-induced gene 1; JNK, c-Jun N-terminal kinase; JIP-1, c-Jun N-terminal kinase interacting protein-1; kFGF, Kaposi fibroblast growth factor; mGluR1, metabotropic glutamate receptor 1; MCAO, middle cerebral artery occlusion; ND2.1, NADH dehydrogenase subunit 2; NADPH, nicotinamide adenine dinucleotide phosphate; NCX, sodium calcium exchanger; NMDA, N-methyl-D-aspartate; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; NR2B, NMDA receptor subunit 2B; OGD, oxygen glucose deprivation; PDZ, PSD-95, and *Drosophila* disc large tumor suppressor, and zonula occludens-1 protein; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PKC protein kinase C; PNS, peripheral nervous system; PSD-95, protein postsynaptic density-95; PTPσ, Protein tyrosine phosphatase σ; PTD, protein transduction domain; SCAP, SERBP cleavage-activating protein, SCI, spinal cord injury; sEPSC, spontaneous excitatory post-synaptic currents; siRNA, small interfering RNA; SERBP-1, sterol regulatory element-binding protein-1; TAT, trans-activator of transcription; TNFR, tumor necrosis factor receptor; VGCC, voltage-gated calcium channel; VR1, vanilloid receptor 1.

1. Introduction

1.1 Neuroprotective peptides and cell penetrating peptides

In recent years there has been an increased interest in the use of specifically designed peptides targeting cyto-damaging or cyto-protective pathways as neuroprotective agents. There are several reasons why this interest arose, including: i) peptide sequences critical for neurodamaging or neuroprotective intracellular protein-protein interactions can be easily identified and used as competitive inhibitors of target proteins (e.g. JNKI-1 peptide); ii) small peptides (2 – 40 amino acids) can be synthesised relatively cheaply using commercial sources; and iii) the development of cell penetrating peptides (CPPs), also referred to as protein or peptide transduction domains (PTDs), has provided a way to deliver peptides and other cargos (incl. proteins, nucleic acids and drugs) into cells and across the blood brain barrier.

The discovery of CPPs has led to studies on the ability of a number of peptides and proteins to act as neuroprotection agents, as well as providing a means to explore the role of protein/protein interactions in brain function in health and disease (viz. neurological and non-neurological disorders). The main focus of this review is the use of arginine-rich CPPs (mainly TAT) for the delivery of neuroprotective peptides (<40 amino acids) particularly in cerebral ischaemia and stroke. The recent observation that CPPs have intrinsic neuroprotective properties in their own right has led us to question the conclusions of other studies. Here, we critically reappraise previous studies that have used putative neuroprotective peptides fused to CPPs as agents in cerebral ischaemia and other models of CNS injury, and examine the mechanism whereby arginine rich-peptides exert their neuroprotective effects. Importantly, we highlight that many past studies on neuroprotective peptides that have used cationic CPPs for CNS delivery may need to be reinterpreted in the light of the intrinsic neuroprotective effects of the carrier-peptide.

1.2 Cell penetrating peptides

Cell penetrating peptides (CPPs) are small peptides (typically 5 - 25 amino acids) that are commonly used to facilitate the delivery of normally non-permeable cargo molecules such as other peptides, proteins, nucleic acids or drugs into cells, and across the blood brain barrier. The development of CPPs as drug vehicles was sparked by the discovery of the PTD within the human immunodeficiency virus-type 1 trans-activator of transcription (HIV-TAT) protein (Frankel and Pabo, 1988; Green and Loewenstein, 1988). The active transporting peptide sequence within the HIV-TAT protein was isolated ($TAT_{48-57:}$ GRKKRRQRRR) and is now referred to as the TAT

peptide or TAT (Becker-Hapak et al., 2001). Subsequently, over 100 CPPs have been identified (Milletti, 2012).

By far the most commonly used CPP peptide is TAT, especially to deliver various cargo molecules to the brain, including neuroprotective peptides and proteins. Other CPPs include penetratin (also known as antennapedia), poly-arginine peptides (R8 to R12; where R refers to arginine residues), Pep-1 and transportan. The amino acid sequences for these peptides, as well as of some less commonly used CPPs, are shown in Table 1. TAT, poly-arginine and penetratin are cationic arginine-rich CPPs.

1.3 Arginine-rich cell penetrating peptides and intrinsic neuroprotection

Potential neuroprotective peptides fused to CPPs have been assessed in cultured neurons and animal models that mimic neural injury mechanisms seen in a variety of disorders, including cerebral ischemia, spinal cord injury, traumatic brain injury, epilepsy, Parkinson's disease and Alzheimer's disease (Lai et al., 2005; Liu et al., 2006; Arthur et al., 2007; Colombo et al., 2007; Nagel et al., 2008; Meade et al., 2009). However, several years ago, we and others demonstrated that TAT possesses intrinsic neuroprotective properties both *in vitro* in neurons exposed to excitotoxicity and oxygen-glucose deprivation (OGD) and *in vivo* following cerebral ischemia in P12 rats after intraventricular injection (Xu et al., 2008; Vaslin et al., 2009b; Meade et al., 2010ab; Craig et al., 2011). We subsequently showed that poly-arginine-9 (R9), penetratin and Pep-1 also display neuroprotective actions in *in vitro* excitotoxic and/or OGD models (Meloni et al., 2014). Furthermore, our data showed that R9 and penetratin were 17- and 4.6-fold respectively more neuroprotective than TAT (Meloni et al., 2014).

The higher potency of R9 relative to TAT and penetratin led us to explore the *in vitro* neuroprotective potency of other poly-arginine peptides (R1, R3, R6 - R15 and R18), as well as, other arginine-rich peptides (Meloni et al., 2015). These studies confirmed that poly-arginine and arginine-rich peptides as a group are highly neuroprotective, with efficacy increasing with increasing arginine content, peaking at R15 (Meloni et al., 2015). We also showed that arginine-rich peptides have the capacity to reduce glutamic acid-induced neuronal calcium influx and are neuroprotective with a single treatment several hours before glutamic acid or OGD exposure. Furthermore, neuroprotective efficacy was shown to be directly related to peptide positive net charge conferred by the positively charged arginine (R) and lysine (K) amino acids residues, which could be blocked by fusion with a negatively charged glutamic acid (E9) poly-peptide (e.g. R9/E9 peptide) or by incubation with the highly negatively charged molecule heparin. The latter finding strongly suggests that peptides bind to negatively charged cell surface molecules such as heparin

sulphate proteoglycans (HSPGs), chondroitin sulfate proteoglycans (CSPGs) or sialic acid residues present in glycosphingolipids to initiate and stimulate peptide endocytosis (Kim et al., 2012; Ravindran et al., 2013; Favretto et al., 2014; Wallbrecher et al., 2014) a process crucial for neuroprotection (Meloni et al., 2015). In this context, others have demonstrated that the nature of the peptide interaction with HSPGs determines a CPPs endocytic properties (Wallbrecher et al., 2014).

With respect to endocytosis, studies have demonstrated that peptide charge conferred by arginine and lysine residues (note: arginine and lysine are the only two strongly positively charged amino acids, with histidine being only weakly positively charged, whereas glutamic acid and aspartic acid are the only two negatively charged amino acids) facilitate HSPG binding, and that mainly arginine residues trigger the endocytic process (Amand et al., 2012; Wallbrecher et al., 2014; Yang et al., 2014). Consistent with our proposed endocytic neuroprotective mechanism, we have demonstrated that poly-lysine (K10) is only weakly neuroprotective in a cortical neuronal glutamic acid excitotoxicity model (Meloni et al., 2015). It is also likely that other amino acids can influence the endocytic properties of cationic peptides in both a positive and negative manner as has been demonstrated for tryptophan (W; Rydberg et al., 2012; Bechara et al., 2013) and alanine (A; Yang et al., 2014), respectively. Indeed, we have now confirmed that tryptophan and alanine amino acids within arginine-rich peptides respectively increase and decrease neuroprotective efficacy in a glutamic acid excitotoxicity model (Fig. 1).

1.4 Proposed neuroprotective mechanism of action used by arginine-rich peptides

Based on our recent findings we hypothesised that arginine-rich peptides exert their neuroprotection effects by inducing the endocytic internalisation of cell surface ion channels, thereby reducing the damaging effects of excitotoxicity (see Fig 2). This is a novel hypothesis that essentially identifies arginine-rich peptides as a new class of neuroprotective molecule. There are several lines of evidence based on our findings and those of others that support our endocytosis hypothesis. Arginine-rich peptides, including so called “neuroprotective peptides” fused to TAT have been shown to: i) reduce neuronal calcium influx (Meloni et al., 2015) and interfere with ion channel function (NMDA receptor: Ferrer-Montiel et al., 1998; Tu et al., 2010; Sinai et al., 2010; Brittain et al., 2011b; Brustovetsky et al., 2014, VR1: Planells-Cases et al., 2000, CaV2.2: Brittain et al., 2011ab; Feldman and Khanna, 2013; Brustovetsky et al., 2014; sodium calcium exchanger [NCX], CaV3.3: Garcia-Caballero et al., 2014); ii) cause internalisation of neuronal ion channels (Sinai et al., 2010; Brustovetsky et al., 2014); and iii) require endocytosis as a prerequisite for neuroprotection (Vaslin et al., 2011; Meloni et al., 2015). Interestingly, other TAT-fused peptides

have also been shown to interfere with the function of neuronal receptors (D1R-D2R; Pei et al., 2010; PTPσ: Lang et al., 2015). In this context, it is important to note that endocytosis is a known mechanism used by cells to internalise cell surface receptors (Höller and Dikic, 2004; Maxfield and McGraw, 2004; Marchese, 2014).

Neuroprotective efficacy, at least for poly-arginine peptides (Meloni et al., 2015), appears to correlate with peptide transduction efficacy (Mitchell et al., 2000), a process known to occur by endocytosis (El-Sayed et al., 2009; Appelbaum et al., 2012; Bechara et al., 2013). Furthermore, it is important to note that the rapid and transient (lasting up to 4 hours with peptide pre-treatment) nature of the neuroprotection induced by poly-arginine peptides (Meloni et al., 2015) corresponds closely to the timeframes of endocytosis and endosomal receptor re-cycling (Gundelfinger et al., 2003; Maxfield and McGraw, 2004; Yashunsky et al., 2009). Importantly, it is known that TAT, penetratin and R9 can induce the internalisation of EGFR and TNFR in HeLa cells (Fotin-Mleczek et al., 2005). Our hypothesis also links endocytosis as a common neuroprotective mechanism of action for a diverse range of arginine-rich peptides (including TAT-fused peptides), all of which are likely to have endocytic inducing properties.

This neuroprotective mechanism we propose is also consistent with the link between neuronal cell surface-HSPGs (Litwack et al., 1994) and endocytic activity (Vaslin et al., 2009a), which are known to promote endosomal uptake of cationic CPPs (Nakase et al., 2007; Vaslin et al., 2009a, 2011). It is also possible that other negatively charged cell surface receptors such as CSPGs and glycosphingolipids can promote cationic CPP endocytosis and neuroprotection. As mentioned above, positively charged poly-arginine and arginine-rich peptides are known to bind negatively charged HSPGs to initiate endocytosis. It is important to note that any neuroprotective peptide fused to a CPP and internalised by endocytosis must escape the endosome to interact with its intended cytoplasmic target. However, endosomal escape appears to be a highly inefficient process (Appelbaum et al., 2012; Qian, et al., 2014) (and rarely confirmed) and as a result, due to the cargo's inability to engage with its intracellular target it is unlikely to have a significant impact within the cytoplasm.

In light of our recent findings, the aim of this review is to critically re-examine studies in the literature that have used neuroprotective peptides fused to cationic CPPs (i.e. TAT, R9) and present evidence supporting our hypothesis that the neuroprotective actions of these peptides is primarily, if not exclusively, due to the endocytic properties of the peptide *per se*.

2. Examination of studies using CPP-fused to neuroprotective peptides in neuronal injury models

To date, over a dozen neuroprotective peptides fused to CPPs have been described (Tables 2 - 4). Three of the most intensely studied peptides developed as potential neuroprotective agents for stroke/cerebral ischaemia are NR2B9c, JNKI-1 and CBD3 (Tables 2 - 4). This review in particular critically examines the use of these three peptides in a neurological setting, and provides evidence suggesting that the critical neuroprotective and functional structural elements of these peptides are arginine and lysine residues in the carrier and cargo-peptides. The remainder of the review focuses on a range of other peptides to further highlight possible biological effects mediated by the TAT carrier peptide.

2.1 NR2B9c and Tat-NR2B9cpeptide (also known as NA-1)

NR2B9c is a 9 amino acid peptide (KLSSIESDV$_{1479\text{-}1484}$) derived from the intracellular terminal carboxyl region of the N-methyl-D-aspartate (NMDA) receptor NR2B subunit protein (Aarts et al., 2002). This region of the NR2B subunit was selected for peptide design because of its high binding affinity to the cytoplasmic signaling/adaptor protein postsynaptic density-95 (PSD-95) via one of its three PDZ domains (PDZ: PSD-95, and *Drosophila* disc large tumor suppressor, and zonula occludens-1 protein: three proteins that share this signaling domain). PSD-95 couples the NR2B subunit to intracellular signaling proteins and enzymes, subsequent to NMDA receptor activation. For example, following receptor activation by the neurotransmitter glutamate, PSD-95 binds to the NR2B subunit and the enzyme neuronal nitric oxide synthase (nNOS), resulting in the production of nitric oxide (NO), which under normal conditions serves as a signaling molecule in neuronal, glia and vascular cells. As a consequence, the NR2B9c peptide has the potential to act as a competitive inhibitor of PSD-95 binding to the NR2B subunit, and in doing so blocks down-stream signaling associated with the interaction of these two proteins.

In cerebral ischaemia, one neuro-damaing event linked to NMDA receptor over-activation and PSD-95 signalling is nNOS over-stimulation and the excessive production of NO. In addition to the direct intracellular toxic effects of NO, a major target for this free radical is stress-activated protein kinase p38, which is known to be involved in ischaemic brain injury (Barone et al., 2001). The inhibition of NO over-production is thought to be the basis of the neuroprotective action of the TAT fused NR2B9c peptide (TAT-NR2B9c).

In vitro studies: In initial *in vitro* neurophysiological (using hippocampal slices or cortical neuronal cultures for measuring synaptic activity, calcium influx and cGMP production) and NMDA induced injury (using cortical neuronal cultures) studies, TAT-NR2B9c was used at a concentration of 0.05µM (Aarts et al., 2002). A concentration of 0.05µM is considerably lower than the concentration required to achieve neuroprotection for other TAT-fused peptides and

arginine-rich peptides (Meloni et al., 2014, 2015). It is therefore hardly surprising that when tested at 0.05µM, TAT-NR2B9c did not affect synaptic responses in brain slices or NMDA induced calcium influx in cortical neurons. By contrast, it is surprising that at 0.05µMTAT-NR2B9c was effective in reducing cortical neuronal death and cGMP levels (increases after nNOS activation) following NMDA exposure (20 - 100µM) after either a 1-hour pre-exposure or continuous post-insult exposure. One possible explanation to account for the neuroprotective efficacy of TAT-NR2B9c at 0.05µM following NMDA exposure may relate to the lower level of excitotoxicity induced by the model. The model incorporates 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and nimodipine in the culture medium during NMDA exposure to prevent secondary activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors and voltage-gated calcium channels, respectively. In a subsequent study using a NMDA model in the presence of the voltage-gated calcium channel blocker nifedipine, TAT-NR2B9c at 2µM was shown to be neuroprotective following exposure to 20 - 60µM NMDA, but not 100µM (Soriano et al., 2008). Interestingly, although individually the TAT-NR2B9c (2µM) nor JNKI-1-TATD (2µM) peptides were effective following 100µM NMDA exposure, together they had a synergistic positive effect (Soriano et al., 2008).

In their initial study of TAT-NR2B9c, Aarts et al. (2002) used a variety of controls including: i) a mutated TAT-NR2B9c peptide (KLSSIEADA; TAT-NR2B9c-AA) incapable of binding to PSD-95 (negative control); ii) a TAT-fused truncated PSD-95 protein (pTAT-PDZ1-2) containing PDZ domains 1 and 2 that is known to competitively block PSD-95 binding to the NR2B subunit and: iii) a pTAT-GK protein containing the PSD-95 guanylate kinase-like domain that does not interfere with PSD-95 binding to the NR2B subunit. At 0.05µM, none of the controls blocked neuronal calcium influx following NMDA exposure (in the presence of CNQX and nimodipine), while only the pTAT-PDZ1-2 protein (like the TAT-NR2B9c peptide) was neuroprotective following NMDA exposure. Unlike the TAT-NR2B9c peptide, the TAT-NR2B9c-AA peptide did not inhibit cGMP formation.

In a recent study (Chen et al., 2015), TAT-NR2B9c was shown to inhibit neuronal NMDA induced superoxide production by blocking the activation of the membrane-bound NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) complex. It was proposed that the TAT-NR2B9c peptide by inhibiting the PSD-95/NR2B9c interaction blocked PSD-95 adaptor protein APPL1 (adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1) coupling with PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase), thereby inhibiting PKCζ (protein kinase C) to phosphorylate the NADPH oxidase subunit protein $p46^{phox}$, which is required for activation of the complex. Interestingly, the TAT-fused arginine-rich NADPH oxidase complex inhibitor peptide gp91ds-TAT (CSTRIRRQL-TAT-NH2; net charge with NH2 group +12) inhibited

neuronal NMDA induced superoxide production to a greater extent than TAT-NR2B9c, while the TAT-fused non arginine-rich scrambled peptide (CSFNSYELGSLCY-TAT) did not. The Chen et al. (2015) study also showed that TAT-NR2B9c did not inhibit NMDA induced neuronal calcium influx.

In our laboratory, we have shown that TAT-NR2B9c is not neuroprotective in cultured neurons exposed to glutamic acid induced excitotoxicity, even at high concentrations (20μM), while modest neuroprotection is evident in a milder NMDA excitotoxicity model (Meloni et al., 2015). In addition, we have also shown that at 5μM, TAT-NR2B9c can reduce neuronal calcium influx following glutamic acid exposure, but not to the same extent as a poly-arginine peptide (Fig. 3 and Meloni et al., 2015).

In vivo studies: Studies utilising the TAT-NR2B9c peptide in different *in vivo* injury models are presented in Table 2. Animal studies have generally yielded positive outcomes with TAT-NR2B9c being shown to be neuroprotective at doses ranging from 30 to 3000nmol/kg. Neuroprotection was seen in the macaque when TAT-NR2B9c treatment was commenced 3 hours after stroke onset, in a 3.5 hour transient middle cerebral artery occlusion (MCAO) model. While some positive results have been obtained in permanent MCAO models, several studies have reported a lack of neuroprotection even at a dose of 3000nmol/kg. In the few studies that have investigated the TAT-NR2B9c-AA negative control, no neuroprotection was evident (See Table 2).

Of particular interest is a study in humans using TAT-NR2B9c (renamed NA-1 in the study) following endovascular treatment to repair ruptured or unruptured intracranial aneurysms (ENACT: Evaluating Neuroprotection in Aneurysm Coiling Therapy). The ENACT trial confirmed that TAT-NR2B9c is safe, and showed that while two-thirds of the trial participants (peptide; n = 92/saline; n = 93) had small ischaemic stroke lesions detectable by MRI, patients treated with peptide had significantly fewer brain infarcts.

Is the neuroprotective action of TAT-NR2B9c mediated by TAT or the NR2B9c peptide? Based on recent observations on the neuroprotective properties of cationic CPPs, we view it as likely that TAT itself is the active neuroprotective component in TAT-NR2B9c. Like TAT alone, TAT-NR2B9c has only modest neuroprotective efficacy *in vitro*, which can easily be overcome by increasing the severity of excitotoxicity (Martel et al., 2009). The fact that the level of neuroprotection is only modest for TAT-NR2B9c is hardly surprising as the NR2B9c peptide contains one positively charged (lysine; K) and two negatively charged amino acids (glutamic acid; E and aspartic acid; D), resulting in a peptide net charge (+7; hereafter net charge is at pH 7) lower than TAT (+8). Given the importance of peptide charge in peptide neuroprotection (Meloni et al., 2015), the NR2B9c peptide is unlikely to potentiate the neuroprotective properties of TAT by way of cationic charge. However, the NR2B9c peptide is larger in size, which may contribute to peptide

stability and thereby improve peptide efficacy to a small degree. We believe that TAT-NR2B9c has modest neuronal cell endocytic penetrating properties compared to poly-arginine peptides, (e.g. R12 and R15) and therefore is likely to have a modest capacity to reduce the level of surface receptors (e.g. NMDA and VGCC). This would explain why TAT-NR2B9c has a lower capacity to inhibit glutamate induced calcium influx compared to poly-arginine peptides (Fig. 3 and Meloni et al., 2015) and why neuroprotection provided by the peptide is easily overcome by increasing the severity of excitotoxicity (Martel et al., 2009; Soriano et al., 2008; Meloni et al., 2015).

Given the above, it appears likely that the modest neuroprotective actions of TAT-NR2B9c simply reflect the modest level of neuroprotection achievable with TAT. This may also explain why other NDMA receptor activated pathways not associated with NR2B/PSD-95 signaling, such as, JNK and CREB are not inhibited by TAT-NR2B9c (Soriano et al., 2008). Furthermore, the TAT-fused mutant peptide TAT-NR2B9c-AA is not neuroprotective both *in vitro* and *in vivo*. The lack of neuroprotection of TAT-NR2B9c-AA is consistent with a study demonstrating that alanine can significantly impede CPP-HSPG binding, a critical step in the process of endocytosis (Yang et al., 2014).

While it cannot be precluded that one element in the neuroprotective actions of TAT-NR2B9c is mediated by its inhibition of the PSD-95/NR29Bc interaction within the cytoplasm, we view this as highly unlikely as this would require the intact release of the peptide from endosomes following cellular uptake. As discussed previously, CPP endosomal escape is known to be a highly inefficient process, and endosomal fusion to lysosomes leads to the degradation of peptides and proteins (Maxfield and McGraw, 2004). To this end, no evidence for the endosomal release of TAT-NR2B9c, or other CPP-fused neuroprotective peptides (TAT-JNKI-1, TAT-CBD3), has ever been directly demonstrated as is the case for all of the cargo peptides fused to CPPs discussed in this review.

Another issue relevant to this discussion is that TAT-NR2B9c mainly targets synaptically located NMDA receptors where there is an abundant pool of PSD-95 protein. It is generally accepted that extra-synaptic NMDA receptor activation is cell death promoting, whereas synaptic NMDA receptor activation is pro-survival (Hardingham and Bading, 2010). There is the possibility that TAT-NR2B9c may act to some degree by blocking synaptically induced neuronal NO production, which could in theory have a negative impact on neuronal survival, as it could lead to the inhibition of NO-induced blood vessel vasodilation (Garthwaite, 2008; Lourenço et al., 2014), thereby potentially exacerbating the reduction in blood flow seen in stroke.

It has been demonstrated that a TAT-NR2B9c peptide with a serine (S) to theronine (T) substitution (KLSSIE<u>T</u>DV; TAT-NR2B9c$_{TDV}$) is as effective as TAT-NR2B9c as a neuroprotective agent following permanent MCAO (Sun et al., 2008). The proposed mechanism of action of TAT-

NR2B9c involves its specific binding to a peptide sequence within the PSD-95 protein, thereby blocking NR2B/PSD-95 engagement (Aartes et al., 2002). Given the high specificity of protein-protein interactions, it is difficult to reconcile this putative mechanism with the fact that the TAT-NR2B9c$_{TDV}$ mutated peptide is capable of inhibiting the NR2B/PSD-95 interaction, at least to the same level as the TAT-NR2B9c peptide.

Recently, it was reported that TAT-NR2B9c reduces synaptically induced neuronal superoxide production (Chen et al., 2015) by disrupting a signaling cascade initiated by NR2B/PSD-95 coupling and activation of the NADPH oxidase membrane complex. However, it is more likely that the TAT-NR2B9c peptide through its endocytosis-inducing properties acts by reducing the levels of neuronal NMDA receptors and/or the NADPH oxidase membrane complex, thereby also reducing superoxide production.

2.2 JNKI-1 peptide and JNKI-TAT (also known as XG-102)

JNKI-1 is a 20 amino acid peptide (RPKRPTTLNLFPQVPRSQDT$_{157-175}$) derived from the signaling adaptor protein c-Jun N-terminal kinase interacting protein-1 (JIP-1) (Borsello et al., 2003). The region of JIP-1 that provides the basis for JNKI-1 peptide design is the JNK binding domain. Consequently, the JNKI-1 peptide has the ability to competitively inhibit JNK interaction with JIP-1, thereby block JNK activation (phosphorylation) and JNK downstream signaling (Borsello et al., 2003).

JNK is a protein kinase involved in the final steps of a stress-activated signaling pathway that leads to cell death. JNK is highly expressed in neurons and is activated in the brain in pathological states associated with excitotoxicity, including trauma, epilepsy and stroke (Borsello and Bonny, 2004). Activated JNK can affect cell death pathways by altering protein post-translational structure or by stimulating the expression of pro-death proteins. The inhibition of JNK activation is thought to be the mechanism by which the TAT-JNKI-1 peptide exerts its neuroprotective effects.

In vitro studies: The first *in vitro* study (Borsello et al., 2003) explored the neuroprotective effects of TAT-JNKI-1 in both the L- and D-isoforms (i.e. peptide synthesized with protease resistant D-amino acids, retro-inversely). In cortical neuronal cultures exposed to NMDA excitotoxicity, peptides at a concentration of 2µM were highly neuroprotective after 12 hours, but only the D-isoform protected neurons for an extended period (24 - 48h). TAT-JNKI-1 treatment in the NMDA model also inhibited phosphorylation of the JNK target protein c-Jun. TAT and JNKI-1 as well as a TAT fused mutant JNKI-1 peptide with three alanine substitutions (RPKRPTAANAFPQVPRSQD-TAT) were not neuroprotective in cells exposed to NMDA excitotoxicity (Borsello et al., 2003).

A smaller version of JNKI-1 ($RPKRPTTLNLF_{157-167}$) fused to TAT (TAT-TIJIP) has also been assessed for neuroprotection in cultured neurons exposed to glutamic acid excitotoxicity (Arthur et al., 2007). At 2μM TAT-TIJIP was highly neuroprotective at 24 hours post-insult, even in the presence of the mRNA transcription inhibitor actinomycin D. This latter observation suggests that TAT-TIJIP neuroprotection does not involve the inhibition of expression of pro-death proteins. In addition, TAT-TIJIP reduced neuronal cytosolic calcium levels following glutamic acid exposure, suggesting that the mechanism of neuroprotection may involve improved mitochondrial function and calcium storage capacity, rather than the direct blocking of intracellular influx.

The *in vitro* neuroprotective findings reported for JNKI-1-TATD are consistent with findings from our laboratory using this peptide in neuronal excitotoxicity models (Meade et al., 2010ab; Craig et al., 2011).

In vivo studies: Studies using the JNKI-1-TATD peptide in different *in vivo* animal ischaemic injury models are presented in Table 3. As with the NR2B9c peptide, most animal studies using JNKI-1-TATD have yielded positive results. Studies in younger rats (P7 - P12) have produced the best results in terms of neuroprotective efficacy and therapeutic time windows. The effective dose ranges appears to be between 0.076 to 760nmol/kg via the IV route and 7.6 to 2800nmol/kg when administered via the IP route. Studies yielding negative results have usually been ones that have used more severe models of ischaemic injury (permanent MCAO) or pre- or late-treatment peptide administration time-points.

Is the neuroprotective action of TAT-JNKI-1 mediated by TAT or the JNKI-1/TIJIP peptides? We view it as most likely that the arginine and lysine residues in the JNKI-1 and TIJIP peptides potentiate the neuroprotective actions of TAT. The TAT-JNKI-1 and TAT-TIJIP peptides are highly cationic with a net charge of +11. In addition, while it has been shown that the TAT-JNKI-1 peptide does not block NMDA induced calcium influx (Centeno et al., 2007), our laboratory has shown that this peptide is capable of reducing neuronal calcium influx following glutamic acid exposure (Meloni et al., 2015). Other studies have shown that the TAT-TIJIP peptide reduces neuronal intracellular calcium influx following glutamic acid exposure via a mechanism that is proposed to be associated with an increased capacity of mitochondria to store incoming calcium (Arthur et al., 2007). Given that the TAT fused JNKI-1/TIJIP peptides have the ability to reduce neuronal excitotoxic intracellular calcium levels, it is hardly surprising that associated down-stream pathological processes, such as activation of calpain, JNK and c-Jun have consistently been shown to be blocked by this peptide (Borsello et al., 2003; Bessero et al., 2010; Meade et al., 2010a). It has never been explained, however, how the TAT-JNKI-1 peptide has the capacity to block the activation of the calcium-sensitive protease, calpain following an excitotoxic insult (Meade et al., 2010a) if the peptide has no effect on neuronal calcium influx (Centeno et al., 2007).

As discussed above, the presence alanine residues is known to impede CPP endocytosis. Consequently, the lack of neuroprotection seen with a TAT fused mutant JNKI-1 peptide containing three alanine substitutions (RPKRPTAANAFPQVPRSQD-TAT) in cultured neurons exposed to NMDA excitotoxicity may well be due to the reduced endocytic traits of this peptide. In this context, the studies by Vaslin et al. (2009a, 2011) suggest that neuronal endocytic uptake of the TAT-JNKI-1 peptide is an essential prerequisite for neuroprotection, a view reinforced by studies in our laboratory (Meloni et al., 2015). However, as discussed elsewhere, our view is that the endocytic process is the key to the neuroprotective mechanism and that the interaction of the TAT-JNKI-1 peptide with its cytoplasmic target (JIP scaffold protein) is unlikely to play a major role due to poor endosomal peptide escape and/or endosomal/lysosomal peptide degradation.

2.3 CBD3 and TAT-CBD3 peptides

CBD3 is a 15 amino acid peptide (ARSRLAELRGVPRGL$_{484-498}$) derived from the calcium channel-binding domain (CBD) of collapsing response mediator protein 2 (CRMP2). The direct binding interaction of CRMP2's CBD with the first intracellular loop (L1) and the distal carboxyl terminus (Ct-dis) of the alpha-1 subunit protein CaV2.2, of the N-type voltage-gated calcium channel was the factor that led to the identification and isolation of the CBD3 peptide (Brittain et al., 2011b). The peptide was originally developed to suppress inflammatory and behavioral hypersensitivity associated with CaV2.2 activity.

Along with CRMP2's other four family members (viz. CRMP1 and CRMP3-5), CRMP2 is predominantly expressed in the nervous system during development where its microtubule interacting functions play important roles in axonal formation and nerve terminal growth cone collapse (Charrier et al., 2003). CRMPs are also involved in regulating the function of ion channels and following brain trauma their degradation contributes to the neurodegenerative process (Zhang et al., 2007). There is also evidence that CRMP2 is involved in endocytosis, as siRNA mediated knockdown in neurons has been shown to inhibit endocytosis of the trans-membrane cell adhesion protein L1 (Nishimura et al., 2003).

It is thought that the CBD3 peptide competitively inhibits the interaction of CRMP2 with the intracellular domains of CaV2.2 and other ion channels (e.g. CaV2.3), as well as glutamate receptors (e.g. NMDA receptor), and calcium transporters (e.g. NCX3), in doing so altering their plasma membrane location and/or function and thereby their calcium influx properties. Consequently, the TAT-CBD3 peptide's neuroprotective mechanism of action is commonly thought to be related to its ability to suppress the excitotoxic influx of calcium.

In vitro studies: TAT-CBD3 was first shown to reduce calcium currents by around 60% in cultured dorsal root ganglion (DRG) neurons via a process that was not blocked further by a CaV2.2 inhibitor (Brittain et al., 2011b). Other *in vitro* studies using DRG and cortical neurons, spinal cord slices and cortical slices showed that TAT-CBD3 also reduced several electrophysiological processes (e.g. sEPSC; spontaneous excitatory post-synaptic currents and CGRP release; calcitonin gene-related peptide) associated with CaV2.2 activation (Brittain et al., 2011ab). Interestingly, in rat spinal cord slices following stimulation of the vanilloid receptor subtype 1 (VR1; also known as TRPV1) with capsaicin, TAT-CBD3 reduced CGRP release. This was not considered to be the result of direct inhibition of VR1 because of evidence that TAT-CBD3 has no effect on VR1 recordings in DRG neurons.

In a subsequent study, a modified TAT-CBD3 peptide with an alanine to lysine amino acid substitution (TAT-CBD3A6K) reduced T- and R-type voltage-dependent calcium currents in DRG neurons (Piekarax et al., 2013). Replacing the TAT peptide with poly-arginine-9 (R9) produced an even greater effect than for TAT-CBD and TAT-CBD3A6K in terms of inhibition of neuronal calcium influx and neuropathic pain (Feldman and Khana, 2013; Ju et al., 2013).

In terms of neuroprotection, TAT-CBD3 has been demonstrated to reduce glutamate- and NMDA-induced calcium influx in cortical neurons as well as reducing cortical neuronal death following glutamate exposure (Brittain et al., 2011a). The peptide also induced the internalisation of the NMDA subunit protein NR2B in the dendritic spines (but not cell somas) of cortical neurons, and inhibited NMDA receptor currents in hippocampal neurons (Brittain et al., 2011a). siRNA down-regulation of CRMP2 in cortical neurons reduced the ability of TAT-CBD3 to inhibit NMDA induced calcium influx in cortical neurons, which the authors concluded supported the idea that TAT-CBD3 antagonises a function of CRMP2 (Brittain et al., 2011a). It is of interest therefore that in a latter study by this group (Brittain et al., 2012), it was shown that when the TAT-CBD3 peptide was applied intracellularly, it failed to block hippocampal neuronal NMDA receptor calcium currents.

A further study has also shown that TAT-CBD3 inhibits glutamate- and NMDA-induced calcium influx in cultured hippocampal neurons (Brustovetsky et al., 2014), as well as disrupting a CRMP2-NMDA receptor complex interaction, however it did not appear to induce internalisation of the NR2B protein. The same study demonstrated that TAT-CBD3 also inhibits NCX-mediated calcium influx, and that CRMP2 can interact with NCX3 but not with NCX1. Surprisingly, TAT-CBD3 strengthened the CRMP2-NCX3 interaction rather than inhibiting it and also induced NCX3 internalisation in hippocampal neurons. siRNA down-regulation of CRMP2 in hippocampal neurons blocked the ability of TAT-CBD3 to cause the NCX protein to be internalised.

In many experiments a TAT fused non-arginine containing scrambled peptide (TAT-scramble; TAT-WEAKEMLYFEALVIE; net charge +5) with no amino acid content relationship to the CBD3 peptide was used as a negative control (Brittain et al., 2011ab; Brustovetsky et al., 2014). In experiments using the TAT-scrambled peptide, negative results similar to the vehicle control were obtained.

*In vivo studies: S*tudies utilising the TAT-CBD3 peptide in different animal models of injury/disease are presented in Table 4. The CBD3 peptide was identified as a potential therapeutic for suppressing inflammatory and neuropathic hypersensitivity associated with activation of the calcium channel CaV2.2 (Brittain et al., 2011b). Initial *in vivo* studies established that TAT-CBD3 reduces: i) capsaicin induced CGRP vasodilation in rat dura; ii) pain responses in rat hind-paw and eye following formalin and capsaicin exposure respectively; and iii) neuropathic pain associated with HIV reverse transcriptase therapy. As mentioned previously, the two variants (TAT-CDB3:A6K and R9-CDB3) of TAT-CBD3 displayed an increased ability to reduce neuropathic pain (Ju et al., 2013).

Following the initial *in vivo* studies, the neuroprotective properties of TAT TAT-CBD3 peptide were assessed in traumatic brain injury and animal models of stroke. In a cortical impact mouse injury model, IP administration of TAT-CBD3 5-minutes after injury was shown to reduce hippocampal granular neuronal death (Brittain et al., 2011a). Similarly, in transient MCAO in the P10 rat, IP administration of TAT-CBD3 1-hour prior to occlusion significantly reduced infarct volume (Brittain et al., 2013). In a similar fashion, a 14 amino acid peptide derived from CRMP-2 (GVPRGLYDGPVCEV$_{493\text{-}506}$; underlined sequence overlaps with CDB3 peptide) and fused to TAT (TAT-CRMP-2) when administered intra-cerebroventricularly 6-hours prior to MCAO also reduced infarct volume (Bu et al., 2011).

Is the neuroprotective action of TAT-CBD3 mediated by TAT or the CBD3? It is highly likely that the CBD3 peptide acts to potentiate the neuroprotective action of TAT. The CBD3 contains four arginine residues, resulting in a net charge for the TAT-CBD3 peptide of +11, as compared to +8 for TAT. In our opinion, difference in net charge should have the effect of increasing neuroprotective potency (Meloni et al., 2015). The importance of cationic charge and the presence of arginine residues in TAT-CDB3 peptide is further highlighted by evidence of increased efficacy with respect to inhibition of evoked calcium influx and pain suppression following substitution of an alanine by a lysine residue (TAT-CBD3-A6K: +12) and replacement of TAT with R9 (R9-CDB3: +12) (Felfmam and Khana, 2013). The A6K modification results in increased binding affinity of CDB3:A6K Cav2.2, while replacement of TAT with R9 improves peptide cell transduction. Based on our findings for poly-arginine and arginine-rich peptide, both these modifications, especially the R9 substitution, would be expected to increase neuroprotective

potency, most likely by enhancing the peptides' endocytic properties. In this context, R9-CDB3 has been shown to be more potent than TAT-CBD3-A6K.

The control peptide used in most CBD3 studies has been a TAT-scrambled peptide (TAT-WEAKEMLYFEALVIE) that possesses no arginine residues and has a peptide charge of +5 due to the presence of several negatively charged glutamic acid residues. Due to its lower cationic charge and fewer number of arginine residues compared to TAT-CBD3, it is hardly surprising that this peptide was shown not to be neuroprotective or inhibitory to neuronal calcium influx. A more appropriate control would have been a scrambled CBD3 peptide fused to TAT, which we predict would possess neuroprotective properties due to its higher arginine content. However, in one study, a CDB3 peptide in reverse amino acid sequence fused to TAT peptide (TAT-LGRPVGRLEALRSRA) (which would alter CRMP2 peptide binding affinity) was used as a control in a DRG neuron depolarization-evoked calcium influx studies. While the more potent R9-CDB3 peptide at 10µM reduced peak calcium influx by 50%, the reverse control peptide at the same concentration had no inhibitory effect. Based on the chemistry of the CBD3 reverse peptide, it is possible that higher concentrations may have had an inhibitory effect.

More remarkably, and in line with a non-specific endocytic-mediated down-regulation of cell surface structures, was the demonstration that TAT-CBD3 and TAT-CBD3:A6K peptides interfere with the function of N- (Cav2.2), T- (Cav3.1-3.3) and R-type (Cav2.3) calcium ion channels, the NMDA receptor and the NCX transporter. Moreover, there is evidence of TAT-CBD3 induced internalisation of the NMDA receptor subunit NR2B and NCX proteins (Brittain et al., 2011a; Brustovetsky et al., 2014). The finding of internalisation of NR2B by TAT-CBD3 was not confirmed in another study (Brustovetsky et al., 2014), possibly due to the fixation of cells (the initial study used live cells), a process which is likely to have altered the distribution of endosomal contents (Lundberg et al., 2003), including NR2B protein within the cell.

It should also be noted that siRNA down-regulation of CRMP2, which was used to confirm the specific action of the TAT-CBD3 peptide would be expected to suppress endocytosis (Nishimura et al., 2003). Consequently, in studies on CRMP2 down-regulation, the inability of TAT-CBD3 to induce NCX neuronal internalisation and attenuate NCX activity may well have been due to the suppression of CRMP2-associated endocytosis of the peptide rather than TAT-CBD3 not being able to interact with its intracellular target, CRMP2. Another anomalous finding was that TAT-CBD3 appeared to strengthen the CRMP2-NCX interaction rather than inhibit it (Brustovetsky et al., 2014), which is the opposite to what would be expected if the peptide was interfering with this protein-protein interaction.

2.4 mGluR1 and TAT-mGluR1 peptides

The 14 amino acid mGluR1 peptide (VIKPLTKSYQGSGK$_{928-942}$) is derived from the metabotropic glutamate receptor mGluR1. The peptide sequence is located at the intracellular carboxyl region of the mGluR1 protein and was initially selected based on its calpain cleavage site (Xu et al., 2007). Following glutamate-induced excitotoxicity, calpain cleavage of mGluR1 results in truncation of the protein at serine residue 936 (Xu et al., 2007). While truncation of mGluR1 does not inhibit the receptor's ability to increase cytosolic calcium, it does inhibit receptor-mediated PI3K-Akt signaling, a process known to be associated with neuroprotection. In theory, blockage of calpain-mediated mGluR1 cleavage following excitotoxicity should enable neurons to better withstand excitotoxic insults due to neuroprotective signalling. Consequently, the TAT fused peptide TAT-mGluR1 was developed as a cell penetrable peptide to competitively inhibit calpain cleavage of the mGluR1 receptor.

In vitro and in vivo studies: In cultured cortical neurons exposed to NMDA excitotoxicity the TAT-mGluR1 peptide reduced cell death in a dose-dependent manner (Xu et al., 2007). The peptide was also neuroprotective in a hippocampal slice exposed to OGD. In other models (mouse kainic acid excitotoxicity and rat perinatal hypoxia-ischaemia; Table 5) pre-treatment with TAT-mGluR1 reduced mGluR1 truncation and neuronal degeneration.

Interesting, the developers of TAT-mGluR1 were one of the first groups to recognise the intrinsic properties of the TAT peptide itself and concluded that TAT and mGluR1 are likely to act synergistically via independent mechanisms (Xu et al., 2008). These authors postulated that the TAT peptide exerted its neuroprotective effects by altering membrane proteins such as the NMDA receptor and affecting their function and/or structure.

Is the neuroprotective action of TAT-mGluR1 mediated by TAT or the mGluR1? Xu et al. (2008), who first developed the TAT-mGluR1 peptide have suggested that the TAT and mGluR1 peptides are likely to act synergistically to produce a neuroprotective effect. We believe this view is partly correct in that the mGluR1 peptide acts to potentiate the neuroprotective action of TAT, and that it is less likely the mGluR1 peptide is interacting with its cytoplasmic target. The two lysine residues in the mGluR1 peptide increase TAT/TAT-mGluR1 peptide net charge from +8 to +11, thereby increasing the peptide's endocytic properties. The increase in peptide charge and the potentially increased stability due to increased length would more than likely result in greater neuroprotective potency, independently of any intracellular action of the mGluR1 peptide.

2.5 NR2Bct and TAT-NR2Bct peptides

NR2Bct is a 14 amino acid peptide (KKNRNKLRRQHSY$_{1292-1304}$) derived from the NMDA receptor NR2B subunit. The peptide sequence is located at the intracellular carboxyl region of NR2B protein and was initially selected based on its binding affinity for the death-associated protein kinase 1 protein (DAPK1) (Tu et al., 2010). DAPK1 is a calcium-calmodulin regulated protein activated in neurons following NMDA receptor over-stimulation as occurs in ischaemia mediated excitotoxicity. The DAPK1 protein has been shown to interact with extra-synaptic NMDA receptors containing the NR2B subunit, a process that is thought to induce and/or exacerbate injurious calcium influx (Tu et al., 2010). In addition, activated DAPK1 is associated with other signaling pathways linked to ischaemic cell death (Pei et al., 2014; Wang et al., 2014). TAT-NR2Bct competitively inhibits activated DAPK1 binding to the NR2B subunit protein and thereby blocks subsequent downstream damaging cellular events caused by NMDA receptor over-activation.

In vitro and in vivo studies: In vitro TAT-NR2Bct is reported to block cortical neuron calcium influx following NMDA stimulation and following OGD exposure, whereas *in vivo* it reduces infarct volume following transient MCAO in the mouse (Tu et al., 2010; Table 5). By contrast, a TAT scrambled control peptide (TAT-NR2Bcts: TAT-NRRRNSLKLQHKKY) does block NMDA or OGD induced calcium influx and is equally ineffective when administered before MCAO.

The TAT-NR2Bct peptide as well as a modified version containing an additional peptide motif (CTK: KFERQKILDQRFFE; NR2Bct-CTM) that directs the peptide for lysosomal degradation were also shown to reduce neuronal injury following transient MCAO in the rat with TAT-NR2Bct-CTM being more effective than TAT-NR2Bct (Fan et al., 2014; Table 5). The rationale for incorporating the CTK motif was to promote degradation of NR2Bct-CTM:DAPK1 complexes and thereby improve the peptide's ability to block the action of DAPK1.

Is the neuroprotective action of TAT-NR2Bct and TAT-NR2Bct-CTM mediated by TAT or NR2Bct and NR2Bct-CTM? TAT-NR2Bct has been shown to reduce neuronal calcium influx following excitotoxic insults (NMDA and OGD) suggesting that it is via this mechanism that it exerts its neuroprotective action. This raises the question of whether the reduction of calcium influx is due to the interaction of TAT-NR2Bct with the intracellular domain of the NMDA receptor subunit NR2B or is mediated via internalisation of calcium channels. In this context, OGD is known to activate neuronal calcium influx pathways other than via NMDA receptors (e.g. VGCC and reverse NCX activity) and it is difficult to reconcile this with the fact that TAT-NR2Bct is so effective at blocking net calcium influx in neurons exposed to OGD. Alternatively, if TAT-NR2Bct only acts by blocking the secondary injurious calcium influx following the interaction of activated DAPK1with the NMDA receptor, the kinase should in theory still be available to stimulate other non-NMDA receptor associated cell death pathways resulting in neuronal degeneration.

Based on our hypothesis, the high arginine content and positive charge (+14.1) of the TAT-NR2Bct would predict that the peptide is neuroprotective and has neuronal calcium influx inhibiting properties. Consequently, it is surprising that it is reported (Tu et al., 2010) that the TAT fused scrambled NR2Bct peptide control (TAT-NR2Bcts) does not display any neuroprotective or calcium influx inhibitory properties despite having a similar arginine content and charge to the TAT-NR2Bct peptide. This anomaly led us to re-assess the effectiveness of the scrambled TAT-NR2Bcts peptide in cultured cortical neurons exposed to glutamate excitotoxicity, and as predicted our data showed that in our hands the peptide is neuroprotective (Fig. 4).

2.6 GluR6 and TAT-GluR6 peptides

The 12 amino acid GluR6 peptide (RLPGKETMA$_{900\text{-}908}$) is derived from the carboxyl terminal of kainic acid receptor GluR6 subunit, which binds to the PDZ1 domain of the PSD-95 protein (Pei et al., 2006; Table 5). As a result, the peptide has the capacity to inhibit GluR6 receptor-PSD-95 signaling, which following over-receptor stimulation can result in activation of JNK and other cell death associated pathways.

In vitro studies: Electrophysiological studies have shown that TAT-GluR6 (0.05µM) has no inhibitory effects on kainic acid receptor function and is neuroprotective *in vitro*, significantly reducing hippocampal neuronal death following kainic acid exposure (Pei et al., 2006). By contrast, a control peptide in which the carboxyl ETMA peptide motif is replaced by the amino acids AADD (RLPGKAADD; TAT-GluR6AA) is not neuroprotective.

In vivo studies: Treatment with TAT-GluR6 but not the control TAT-GluR6AA peptide reduced MLK3/PSD-95 assembly, signaling events associated with JNK activation (JNK and c-jun phosphorylation, Fas ligand expression) and CA1 hippocampal injury in rats in rats following intracerebroventricular administration of the peptides 40 minutes before the onset of global cerebral ischaemia (Pei et al., 2006; Table 5).

Is the neuroprotective action of TAT-GluR6 mediated by TAT or GluR6? The TAT-GluR6 peptide has a charge of +9, while the TAT-GluR6AA peptides has a charge of +8, which is the same as the TAT peptide. Consequently, it is predicted that an additional charge should increase the neuroprotective potency of TAT-GluR6 peptide. However, the fact that the TAT-GluR6AA control peptide is not neuroprotective despite having the same charge as TAT is problematic, but may be explained by the presence of two introduced alanine residues, which may well have a negative impact on the peptide's neuroprotective properties by impeding peptide endocytosis (Yang et al., 2014).

2.7 p53DM and TAT-p53DM peptides

p53DM is a 12 amino acid peptide (RVCACPGRDRRT$_{271-282}$) derived from the p53 tumour suppressor protein (Wang et al., 2014). The peptide sequence is located within a p53 DNA-binding motif (DM) that also binds activated DAPK1, which can subsequently phosphorylate p53 at serine-23. Thus, the p53DM peptide acts to inhibit the DAPK1-p53 binding interaction and p53 phosphorylation, with phosphorylated p53 being known to induce the expression of pro-apoptotic proteins (e.g. Bax) and cell death.

In vitro and in vivo studies: Treatment of cortical neurons with a TAT-fused p53DM peptide (TAT-p53DM) has been shown to reduce the number of terminal deoxynucleotidyl transferase dUTP nick end labelled and propidium iodide positive neurons following OGD compared to a scrambled control peptide (TAT-s-p53DM; TAT-CCPGECVRTRRR) (Pei et al., 2014). However, in experiments involving OGD exposure, a vehicle control was not used. Treatment of mice with TAT-p53DM (but not the TAT-fused scrambled control peptide, TAT-s-p53DM) 3 or 6 hours after the commencement of transient MCAO (60min) similarly reduces brain injury and improves functional outcomes (Wang et al., 2014; Table 5).

Is the neuroprotective action of TAT-p53DM mediated by TAT or p53DM? Both TAT-p53DM and TAT-s-p53DM are arginine-rich and have a net charge of +10.9 and therefore would be predicted to both have neuroprotective properties. Studies in our laboratory have confirmed that TAT-s-p53DM is neuroprotective *in vitro* in cortical neurons exposed to glutamic acid excitotoxicity (Fig. 4). TAT-s-p53DM peptide was however, slightly less effective than the TAT-p53DM peptide. One possible reason for the differential neuroprotective effects of the two peptides is that in s-p53DM, all arginine residues are located at the C-terminus of the peptide away from the TAT arginine (and lysine) residues. In contrast, p53DM has one of its arginine residues at the N-terminus adjacent to the TAT peptide and therefore may have a greater capacity to enhance the neuroprotective efficacy of TAT.

2.8 CN21, AIP, TAT-CN21 and TAT-AIP peptides

CN21 is a 21 amino acid peptide (KRPPKLGQIGRSKRVVIEDDR$_{43-63}$) derived from the calcium/calmodulin-dependent protein kinase II inhibitor (CaM-KIIN) protein. As its name suggests, CaM-KIIN is an inhibitor of kinase calcium/calmodulin-dependent protein kinase II (CaMKII). The CN21 is also a specific inhibitor of CaMKII (Vest et al., 2007). AIP (autocamtide-2-related inhibitory peptide) is a 13 amino acid peptide (RKKLRRQEADAL) derived from but not

homologous to the auto-regulatory domain of CaMKII, that can inhibit multiple members of the CaMKII-kinase family (Smith et al., 1990; Ishida et al., 1995).

The CaMKII protein is a mediator of many calcium associated signaling pathways. In the brain, CaMKII is involved in physiological glutamate receptor signaling but following receptor over-stimulation, the kinase is activated and autophosphylated, processes believed to be involved in cell death pathways (Liu et al., 2012).

In vitro and in vivo studies: The TAT fused CN21 peptide (TAT-CN21) was shown to be neuroprotective in *in vitro* following both glutamic acid and NMDA excitotoxicity in hippocampal and cortical neuronal cultures (Vest et al., 2010). By contrast, a TAT-fused CN21 reverse sequence (RDDEIVVRKSRGIQGLKPPRK) and a scrambled CN21 peptide (VKEPRIDGKPVRLRGQKSDRI) at 5µM were shown to be equally ineffective as neuroprotective agents following glutamate excitotoxicity. In another study, both TAT-CN21 and TAT-AIP were established to be neuroprotective in cultured cortical neurons following glutamic acid excitotoxicity (Ashpole and Hudmon, 2011), with the TAT-AIP peptide having slightly greater potency than TATCN21. In the same study, TAT peptide (10µM) was shown to be neuroprotective, while a control mutated TAT-CN21 peptide (TAT-CN21Ala; with seven alanine amino acid and one tryptophan substitutions: KAPAKAAWAAASKRVVIEDDR) had significantly lower neuroprotective properties. TAT-CN21 has also been shown to reduce infarct volume in a transient MCAO (60min) mouse stroke model when administered after reperfusion (Vest et al., 2010; Table 5).

Is the neuroprotective action of TAT-CN21 and TAT-AIP mediated by TAT or CN21/AIP? TAT-CN21 and TAT-AIP have net peptide charges of +12 and +11, respectively compared to +8 for the TAT peptide. Consequently, based on our hypothesis, it would be predicted that when fused to TAT, the additional cationic charge provided by CN21 and AIP should increase the neuroprotective potency of TAT. As discussed, it appears that TAT-AIP is slightly more neuroprotective than TAT-CN21. This may be due to the fact that all the positively charged arginine and lysine residues in AIP are located at its N-terminal region adjacent to the TAT peptide, whereas CN21 has a more even distribution of these residues across its structure. It is also hardly surprising that the TAT-CN21Ala control peptide displayed significantly lower neuroprotective properties compared to TAT-CN21 given its high alanine content and reduced charge (+9), factors of which we would be expected to lead to reduce peptided endocytic efficiency (Yang et al., 2014).

2.9 Indip and TAT-Indip peptides

Indip is a 10 amino acid peptide ($GEPHKFKREW_{152-161}$) derived from insulin-induced gene 1 (Insig-1) protein (Taghibiglou et al., 2009). One of the roles of Insig-1 is to regulate the intracellular trafficking of sterol regulatory element binding protein-1 (SERBP-1). In non-stimulated neurons, SERBP-1 forms a complex with SERBP cleavage-activating protein (SCAP), which is retained in the endoplasmic reticulum (ER) due to the interaction of SCAP with the ER membrane protein Insig-1. Following NMDA receptor activation, Insig-1 lysine residues 156 and 158 are ubiquitinated and the protein degraded by the proteosome, thereby releasing SCAP from the ER membrane and allowing the protein to chaperone SERBP-1 to the Golgi apparatus. Within the Golgi apparatus, SERBP-1 is proteolytically cleaved to its transcriptional active N-terminal form (nt-SERBP-1), after which it translocates to the nucleus to stimulate the expression of genes containing sterol regulatory elements. The exact mechanisms associated with activated SERBP-1 and its role in neuronal injury are not known, however blocking Insig-1 degradation with the TAT-Indip peptide has been shown to be neuroprotective in NMDA induced excitotoxicity and OGD in cortical cultures (Taghibiglou et al., 2009). The Indip peptide was initially designed to competitively block ubiquitination of Insig-1 lysines residues 156 and 158, and thereby inhibit its degradation allowing maintenance of SERBP-1's anchorage to the ER membrane.

In vitro studies: The TAT fused Indip peptide (TAT-Indip) is reported to be neuroprotective (2μM) *in vitro* in cultured neurons in both NMDA excitotoxicity and OGD (Taghibiglou et al., 2009). By contrast, a TAT fused mutated Indip peptide (2μM: TAT-GEPHRFRREW; $TAT\text{-}Indip_{K\text{-}R}$), in which lysine residues 156 and 158 are replaced with arginine residues, was shown to be ineffective.

In vivo studies: Treatment of rats with the TAT-Indip peptide 45 minutes before MCAO (90min) or 30 minutes after reperfusion significantly reduces infarct volume (Taghibiglou et al., 2009; Table 5). The control peptide $TAT\text{-}Indip_{K\text{-}R}$ administered 30 minutes after reperfusion was shown to be ineffective resulting in infarct volumes equivalent to those in vehicle treated rats.

Is the neuroprotective action of TAT-Indip mediated by TAT or Indip? Both the TAT-Indip and $TAT\text{-}Indip_{K\text{-}R}$ peptides have a net peptide charge +9.1 compared to +8 for the TAT peptide, which based on our hypothesis, leads us to predict that the $TAT\text{-}Indip_{K\text{-}R}$ due to its higher arginine content should be more neuroprotective than TAT-Indip. The fact that the opposite was observed is surprising. This led us to re-examine the potency of the two peptides in cortical neurons exposed to glutamate excitotoxicity. In our hands, both peptides are neuroprotective with the $TAT\text{-}Indip_{K\text{-}R}$ peptide appearing to be more potent than the TAT-Indip peptide as we predicted (Fig. 4).

2.10 Src, Src40-49-TAT and TAT-Src40-58 peptides

Src40-49 is a 10 amino acid peptide (KPASADGHRG$_{39-48}$ equivalent to the mouse sequence peptide region 39-48) and Scr40-58, a 19 amino acid peptide (PASADGHRGPSAAFVPPAA$_{40-58}$), derived from the protein kinase Src protein. It has been reported that the Src peptides can bind to the NADH dehydrogenase subunit 2 (ND2.1) protein (Liu et al., 2008). The Src protein can use ND2.1 as well as PSD-95 as adaptor proteins to indirectly bind to the NR2B NMDA receptor subunit. Intracellular signaling events following tissue injury and inflammation can trigger the Src protein to bind to and phosphorylate the NR2B subunit (at tyrosine residue 1472). This process can inhibit NMDA receptor endocytosis, and thus promote increased surface expression of the NMDA receptor (Zhang et al., 2008). Increased NMDA receptor expression/activity can be associated with chronic pain, pain hypersensitivity and emotional memory. The rationale for designing the Src40-49 peptide was to block the Src-ND2 protein interaction and thereby inhibit NMDA receptor phosphorylation and hyperactivity without disrupting normal receptor function (Liu et al., 2008). The TAT-Src40-58TAT peptide was designed to block the interaction of Src with the PSD-95/NR2B protein complex and thereby reduce NMDA receptor surface expression.

In vitro studies: Studies using the longer TAT-Src40-58 peptide (TAT-PASADGHRGPSAAFVPPAA) were performed in mouse amygdala neuronal cultures and brain slices (Sinai et al., 2010). Since it had been previously demonstrated that inhibiting phosphorylation of tyrosine (Y1472) on the NMDA receptor subunit NR2B causes receptor endocytosis (Zhang et al., 2008), it was predicted that adding TAT-Src40-58TAT to the neuronal cultures would decrease NR2B surface expression. As predicted, treatment of the neuronal cultures with peptide (20µM) resulted in NR2B internalisation, resulting in a >50% reduction in receptor protein surface expression in dendritic processes compared to controls (Sinai et al., 2010). Similarly, treating brain slices with the peptide reduced amygdalar long-term potentiation in the lateral nuclei of the basolateral complex. Controls consisting of TAT only or scrambled TAT-Srr40-58 peptides were not used in the experiments.

In vivo studies: IV administration of Src40-49-TAT (KPASADGHRG-TAT) 45 minutes before hind paw formalin injection, or intrathecal administration at the lumbar level 30 minutes before hindpaw injection reduced phase 2 NMDA receptor-dependent flinching (Liu et al., 2008; Table 5). By contrast, a scrambled sSrc40-49 peptide (GAAKPRSDGH-TAT; sSrc40-49-TAT) and the TAT peptide alone did not have any effect. Moreover, IV and/or intrathecally administered Scr40-49-TAT, but not vehicle or sScr40-49-TAT, also reduced inflammatory pain and pain behaviour in a model of peripherial nerve injury (Liu et al., 2008). Coincidentally, Src40-49-TAT has also been shown to reduce Src binding to ND2.1 and NR2B phosphorylation in rat brain and spinal cord

lysates, following IV or intrathecal peptide treatment in normal rats or rats subjected to inflammatory and neuropathic pain (Liu et al., 2008).

In another study, intravenous injection of the longer TAT-Scr40-58 peptide (TAT-PASADGHRGPSAAFVPPAA) (but not vehicle control or scrambled TAT-Scr40-58) reduced NR2B tyrosine phosphorylation in the mouse amygdala compared to vehicle control and scrambled TAT-Scr40-58 treated rats (Sinai et al., 2010; Table 5). IV administered TAT-Scr40-58 also impaired amygdala-dependent cued fear conditioning and non-associative social recognition tasks in mice. It was concluded that both responses were linked to TAT-Scr40-58 mediated reduced NMDA receptor surface expression.

Are the neurological effects induced by Src40-49TAT and TAT-Src40-58 mediated by TAT? The Src40-49TAT and TAT-Src40-58 peptides are intriguing as their active components (Src40-49 and Src40-59 peptides) are predicted to cause NR2B/NMDA receptor endocytosis and reduced surface expression. Similarly, we have proposed that TAT-mediated peptide cellular uptake may result in NMDA receptor internalisation and reduced surface expression. There are several pieces of evidence suggesting that the latter mechanism is occurring. In both studies described above, the authors were unable to adequately reconcile why their peptides were so effective at reducing Src kinase NMDA receptor mediated responses when the closely related Src family member Fyn is another kinase capable of phosphorylating the NR2B subunit at tyrosine 1472, including when associated with neuropathic pain (Abe et al., 2005; Nakazawa et al., 2001). That is, Fyn kinase activity, which is not blocked by the Src40-49TAT and TAT-Src40-58 peptides, would be capable of phosphorylating NR2B and inhibit endocytosis of the NMDA receptor, and allow pain to occur. However, this was not the case and thus it is more likely that a TAT-mediated peptide mechanism was responsible for NMDA receptor endocytosis and reduced pain.

Moreover, it has been shown that Src, in addition to binding the ND2 protein can bind the PSD-95 protein to facilitate phosphorylation of tyrosine NR2B, a process that would not be blocked by the Src40-49TAT and TAT-Src40-58 peptides. Given this, we argue that the reduced NMDA receptor activated processes reported above are the result of the TAT peptide's endocytic properties. These endocytic effects may have been enhanced by the actions of the Src peptide to increase TAT's stability or endocytic uptake independent of the cargo-peptide's potential indirect cytoplasmic inducing endocytic effects.

3. Examination neuroprotective arginine- (and lysine-) rich peptides used in neuronal injury models

It is beyond the scope of this review to include every study that has used a neuroprotective TAT-fused peptide. For completeness, other *in vivo* studies using TAT-fused peptides are listed in Table 5. Evidently, several studies have identified arginine- and lysine-rich peptides not fused to TAT or a CPP as being neuroprotective. We argue that it is possible that these peptides are intrinsically neuroprotective via mechanisms unrelated to their proposed action on a specific cell surface receptor or intracellular target. Like TAT, the neuroprotective effects of poly-arginine and arginine-rich peptides are likely to be related to their endocytosis-inducing properties. To pursue this idea further, here we review in detail the properties of two arginine- and lysine-rich peptides that meet these criteria, apolipoprotein E (APOE) derived peptides and an amyloid precursor protein (APP) derived peptide. Animal studies that have used these peptides in CNS injury models are presented in Table 6.

3.1 APOE peptides - APOE141-149, COG133 and COG1410

The APOE141-1491 and COG133 peptides are derived form the APOE protein ($LRKLRKRLL_{141-149}$ and $LRVRLASHLRKLRKRLL_{133-149}$) and represent a region within the protein's receptor binding domain. Interestingly, an early study using a dimer of the APOE141-1491 peptide showed that the peptide was capable of inducing significant increases in intracellular calcium influx in cultured neurons, especially at concentrations of ≥5μM (Wang and Gruenstein, 1997). By contrast, the APOE141-1491 monomer peptide was less effective at inducing intracellular calcium influx even at high concentrations (100μM). The mechanism associated with the COG1410 peptide induced calcium influx was proposed to be linked to peptide binding to the low-density lipoprotein receptor (Wang and Gruenstein, 1997).

A subsequent study showed that the COG133 peptide reduced cell death and calcium influx in mixed cortical-glial cultures following NMDA excitotoxicity (Aono et al., 2003). Neuroprotection was seen when the COG133 peptide was added to cortical cultures prior to or concurrently with NMDA but not a scrambled peptide of the identical size and amino acid composition. It is of interest that poly-arginine and arginine-rich peptides behave in a similar fashion to COG133 with respect to calcium influx and neuroprotection following glutamic acid excitotoxicity (Meloni et al., 2015).

In other studies it has been shown that intravascular administration of the COG133 peptide reduced the systemic and brain inflammatory responses induced by cytokines (e.g. TNF-α) and led to improved outcomes after closed head injury in the mouse (Lynch et al., 2003, 2005). Similarly, intraperitoneal administration of the COG133 peptide reduced disability and inflammatory infiltrates in to the spinal cord of a mouse model of multiple sclerosis (Li et al., 2006). A slightly

shorter and modified COG133 peptide (COG1410: acetyl-AS-Aib-LRKL-Aib-KRLL-amide; Aib = 2-Aminoisobutyric acid) with superior anti-inflammatory properties has also been developed, and has shown to reduce vasospasm and/or lead to improved outcome in subarachnoid and intracerebral haemorrhage in the mouse (Gao et al., 2006; Laskowitz et al., 2012). In addition, COG1410 peptide improves functional and/or histological outcomes in different traumatic brain injury models (Hoane et al., 2007; Laskowitz et al., 2007; Hoane et al., 2009; Kaufman et al., 2010; Jiang and Brody, 2012), in transient MCAO (Tukhovskaya et al., 2009) and perinatal hypoxia-ischaemia models (McAdoo et al., 2000). Finally in a transgenic *Drosophila* model of Alzheimer's disease, COG133 alone or when fused to the CPP penetratin (COG12) has been shown to reduce neurodegeneration and restore cognitive functions.

The mechanism whereby COG133 and its variant peptides exert their neuroprotective, calcium influx inhibitory and anti-inflammatory effects is not fully understood, but is assumed to be associated with the peptide's binding to APOE receptors (e.g. low-density lipoprotein; LDL, lipoprotein receptor-related protein; LRP, apolipoprotein E receptor 2).

What is the neuroprotective mechanism of APOE141-149, COG133 and COG1410 peptides? Due mainly to their arginine and lysine residues the COG133 and COG1410 peptides have a net charge of +7.1 and +6.1 respectively, and therefore we would predict that these peptides should possess mild endocytic properties and as has been reported to be neuroprotective and reduce calcium influx following excitotoxicity (Aono et al., 2003). Indeed our own assessment of the COG133 peptide in cultured cortical neurons exposed to glutamate excitotoxicity showed that the peptide has mild neuroprotective effects (Fig. 4).

3.2 APP peptide - APP96-110

The APP96-110 is a 15 amino acid peptide (NWCKRGRKQCKTHPH) derived from the heparin-binding site within the D1 domain of the APP (Corrigan et al., 2014). The APP96-110 peptide is thought to represent one of the neuroprotective regions (the other being the D6a domain) within the secreted amyloid-β precursor protein-α. The D1 domain is considered a growth factor-like domain and interestingly it has been hypothesised that its neuroprotective properties may be related to its ability to bind HSPGs (Corrigan et al., 2011).

The APP96-110 peptide has been assessed for neuroprotective effects in traumatic brain injury models in APP-/- knock-out mice and normal rats (Corrigan et al., 2014). Intra-cerebroventricular peptide administration was shown to improve both histological and functional outcomes, and was effective whether administered pre- or post-insult. By contrast, a mutated form of the peptide (mAPP96-110: NWCNQGGKQCKTHPH) specifically designed to reduce the ability of the peptide

to bind heparin, by replacing positively charged arginine and lysine residues, was ineffective in traumatic brain injury in the rat.

What is the neuroprotective mechanism of APP96-100? The APP96-110 peptide was synthesised with an acetylated N-terminal and an amidated C-terminal to minimise peptide proteolytic degradation and has a net charge of +5.1. The mutated form of the peptide has a net charge of +2.1. As mentioned above, it was hypothesised by the developers of APP96-110 that the peptide's neuroprotective mechanism is related to its HSPG binding properties. The developers of APP96-110, have confirmed that this peptide had a strong binding affinity for the highly sulphated glycosaminoglycan molecule, heparin (Corrigan et al., 2014). By contrast, the mutated peptide mAPP96-110 has significantly reduced binding affinity for heparin. Despite the heparin binding properties of the APP96-110 peptide, its mechanism of action is not exactly known, although some form of interaction with cell surface receptors (e.g. FGFRs, APP, APLP1, APLP2) and the activation of signaling pathways has been proposed (Corrigan et al., 2014). However, it appears likely that APP96-100 behaves in a similar fashion to neuroprotective poly-arginine and arginine-rich peptides by binding cell surface HSPGs, and in doing so triggering peptide endosomal uptake. On this basis, we conclude that the endocytic properties of APP96-110 are integral to the peptide's neuroprotective mechanism of action.

4. Discussion and concluding remarks

The main purpose of this review is to describe the neuroprotective properties of peptides fused to the arginine-rich CPP, TAT and in doing so provide evidence supportive of our hypothesis that neuroprotection is mediated not by the cargo molecule but largely by TAT itself. This hypothesis crystallised for us following the analysis of the neuroprotective and calcium influx inhibiting properties of a diverse set of peptides including: 1) arginine-rich CPPs (TAT, penetratin); 2) poly-arginine peptides (R3, R6, R7-15, R18); 3) several CPP-fused peptides (e.g. TAT-JNKI-1, TAT-PYC36, kFGF-JNKI-1); 4) several arginine-rich peptides (e.g. BEN0254, BEN1079, NCXBP3, XIP); 5) poly-lysine-10 (K10) and a poly-arginine-9 fused poly-glutamic acid-9 peptide (E9/R9); and 6) a non-arginine, non-endocytic CPP (kFGF) (Meloni et al., 2015). What became increasingly clear from this analysis was that peptide neuroprotection is associated with peptide cationic charge. In particular, arginine and to a lesser extent lysine and tryphophan amino acid residues appear to be important determinants of neuroprotective potency.

Other studies have added weight to our hypothesis. For example, evidence that endocytosis is essential for TAT-JNKI-1 neuroprotection (Vaslin et al., 2011) and the observation that CPP-fused CDB3 peptides can induce internalisation of NR2B, NCX and CaV2.2 membrane proteins.

Another interesting and largely unexplained finding is that most TAT-fused and arginine-rich peptides are effective as neuroprotectants in neuronal excitotoxicity models in the µM range (e.g. 1-10µM) rather than the nM range (e.g. 10-100nM or 0.01-0.1µM). Our interpretation of this is that in order to be neuroprotective, a peptide must reach a critical concentration at the plasma membrane in order to induce HSPG mediated endocytic internalisation of cell surface structures.

Recent findings suggests that cationic peptide charge conferred by arginine and lysine residues facilitates electrostatic interactions with cell surface HSPGs followed by arginine induced heparin sulphate clustering and endocytosis (Amand et al., 2012; Wallbrecher et al., 2014; Yang et al., 2014). Additionally, there is evidence that tryptophan residues within basic peptides can also promote proteoglycan binding and endocytosis (Rydberg et al., 2012; Bechara et al., 2013), while alanine resides have been shown to impede peptide-proteoglycan binding (Yang et al., 2014). Interestingly, the replacement of 1 to 3 arginine residues in poly-arginine-6 with the equivalent number of tryptophan residues increases the ability of the peptide to block NMDA receptor activity in amphibian oocytes (Ferrer-Montiel, et al., 1998). Consistent with these observations, we have demonstrated that a 15mer consisting of 10 arginine and 5 tryptophan resides (WRRRWRRRRWWRRRW) is more neuroprotective than R15 in a cultured cortical neurons exposed to glutamic acid excitotoxicity, with an arginine /alanine peptide (ARRRARRRRAARRRA) being slightly less effective (Fig. 1).

There is emerging evidence that the presence of other amino acids or the amino acids sequence itself may decrease or increase peptide endocytosis and neuroprotective efficacy. In an earlier study, we reported that only 5 out of 19 TAT-fused peptide displayed significant neuroprotection in cortical neurons exposed to glutamic acid excitotoxicity (Meade et al., 2010b). Similarly, the addition of three amino acids to TAT (PKIGRKKRRQRRRG; AM8D-TAT) significantly increases peptide *in vitro* efficacy (Meade et al., 2010a). Therefore, it appears likely that in addition to arginine content and peptide charge, other amino acids most likely by influencing peptide secondary and tertiary structure can influence the endocytic and neuroprotective properties of arginine-rich and TAT-fused peptides. Ultimately, however, we recognise that additional *in vitro* and *in vivo* studies are required to unequivocally prove our hypothesis. Moreover, we can not rule out the possibility that other peptide induced neuroprotective mechanisms are also operating, since it has been demonstrated that CPPs can exert intracellular biological effects by altering gene expression and the activity of kinases and proteolytic enzymes (Brugnano et al., 2010).

Finally, we speculate that by altering amino acid residue content and sequence within arginine-rich peptides it may be possible to develop peptides with a greater capacity to target specific cell surface structures within the CNS and PNS, thereby providing a way to improve drug specificity for a range of neurological disorders such as epilepsy, pain, depression, Alzheimer's disease and

Parkinson's disease. It may equally be possible to develop arginine-rich peptides that can target cell surface receptors associated with the pathogenesis of non-neurological disorders.

Conflict of Interest

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.

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Figure Legends

Fig. 1. Glutamic acid excitotoxicity model; peptide dose response experiments. Peptides present in neuronal cultures for 10 min before and during (half concentration) 5-min glutamic acid exposure. Peptides: R15, R10/A5: ARRRARRRRAARRRA and R10/W5: WRRRWRRRRWWRRRW. Neuronal viability measured 24 h following glutamic acid exposure. Concentration of peptide in μM. MTS data were expressed as percentage neuronal viability with no insult control taken as 100% viability and glutamic acid control taken as 5% (mean ± SE; n = 4; $*P < 0.05$). For additional methodological details see Meloni et al. (2015).

Fig. 2. Diagrammatic representation of proposed model of arginine-rich CPPs inducing internalisation of neuronal cell surface structures. Note: model applies to neuronal synaptic and extra-synaptic plasma membranes and potentially the plasma membrane of astrocytes, pericytes, brain endothelial cells, oligodentrocytes and microglia. NMDAR: N-methyl-D-aspartate receptors; AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; NCX: sodium calcium exchanger; VGCC: voltage-gated calcium channels (e.g. CaV2.2, CaV3.3); ASIC: acid-sensing ion channels; TRPM2/7: Transient receptor potential cation channels 2 and 7: mGluR: metabotropic glutamate receptors; VR1: vanilloid receptor 1 or transient receptor potential cation channel subfamily V member 1; TNFR: tumour necrosis factor receptors; FASR: FAS receptor; EAAT: excitatory amino-acid transporters; AQP4: Aquaporin 4; Trk: tropomyosin-receptor-kinase receptors. Adapted from Maxfield and McGraw (2004).

Fig. 3. Inhibition of intracellular calcium influx by R15 and TAT-NR2B9c peptides assessed using Fura-2 AM kinetics (5μM; 20min pre-loading) following glutamic acid exposure in neuronal cultures; Fluorescent Fura-2 AM tracers showing change in fluorescence ratio (Δ F-ratio; F340/F380nm) in neuronal cells 2 min before and for 5 min following addition of glutamic acid (100μM final concentration). Peptides (5μM) were added to neuronal cultures for 10 min before cover slips were removed from culture wells and washed in a balanced salt solution before glutamic acid addition (arrow; time 0). Normal control received no peptide or glutamic acid, while the glutamate control only received glutamic acid. Values are mean ± SE; n = 8-21 cells for each treatment group. Note: fluctuations in Δ F-ratio for TAT-NR2B9c peptide, while for the R15 peptide Δ F-ratio remained more stable over the examination time period.

Fig. 4. Glutamic acid excitotoxicity model; peptide dose response experiments. Peptides present in neuronal cultures for 10 min before and during (half concentration) 5-min glutamic acid exposure. Neuronal viability measured 24 h following glutamic acid exposure. Concentration of peptide in μM. MTS data were expressed as percentage neuronal viability with no insult control taken as 100% viability and glutamic acid control taken as 5% (mean ± SE; n = 4; $*P < 0.05$). For additional methodological details see Meloni et al. (2015).

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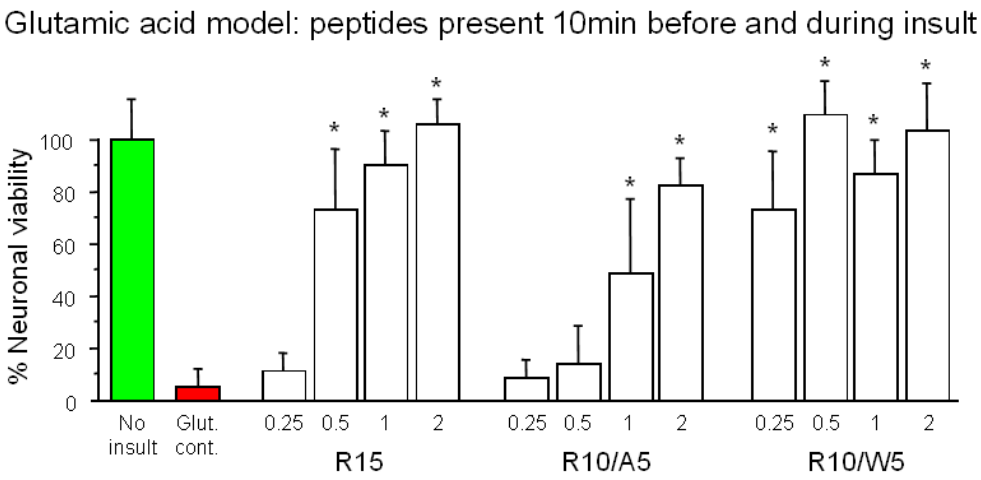

Figure 1

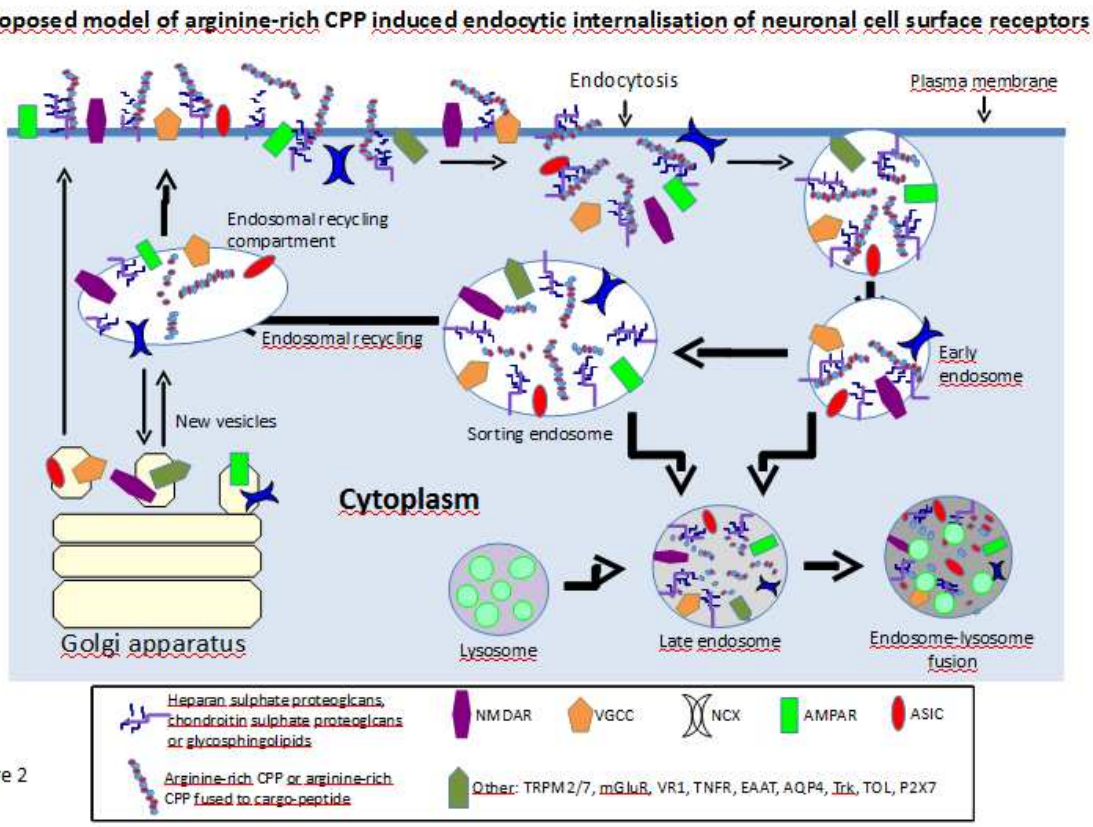

Figure 2

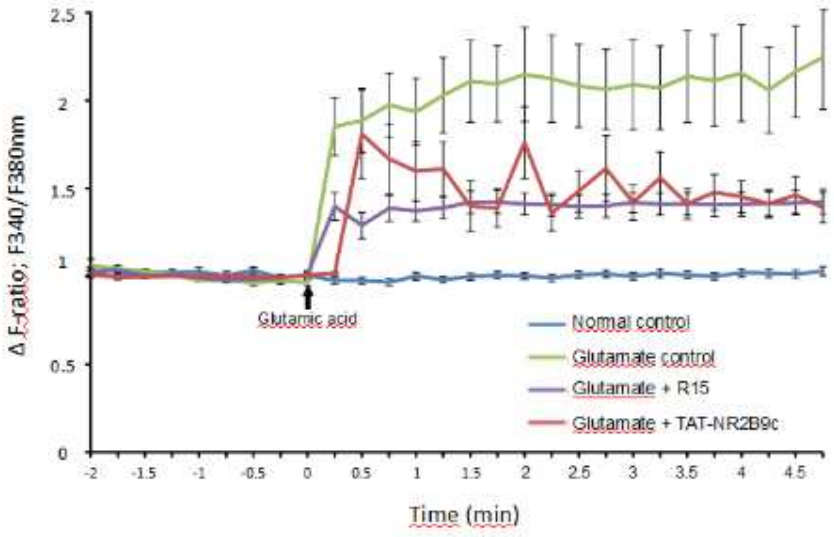

Figure 3

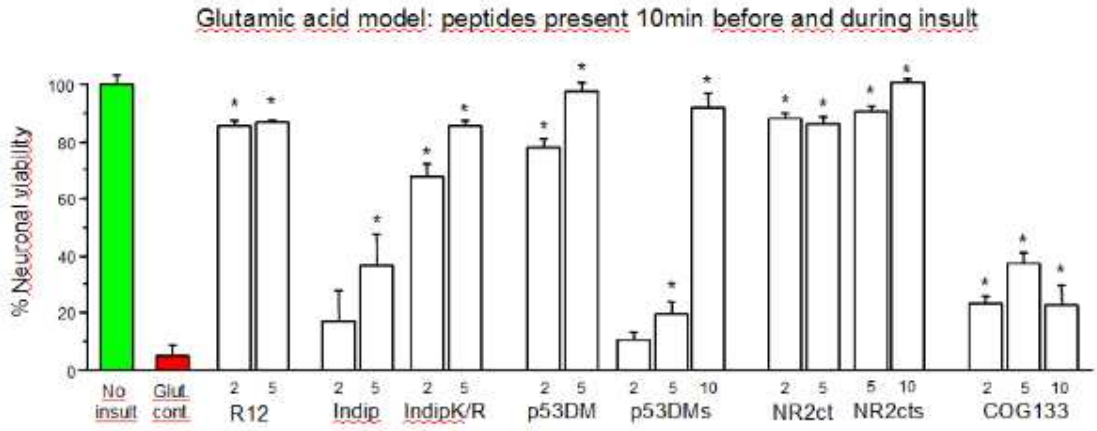

Figure 4

Table 1
Examples of commonly used cell penetrating peptides.

Peptide	Sequence[1]	Amino acids: MW (Da)	Net charge at pH 7
TAT	GRKKRRQRRR	10: 1,397	8
TAT-D	rrrqrrkkrG	10: 1,397	8
R9 – R15	RRRRRRRRR - RRRRRRRRRRRRRRR	9: 1,423 – 15: 2,360	9 - 15
Penetratin*	RQIKIWFQNRRMKWKK	16: 2,245	7
Pep-1	KETWWETWWTEWSQPKKKRKV	21: 2,848	3
HSV-1 VP22	DAATATRGRSAASRPTERPRAPARSASRPRRVD	33: 3,548	6
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	27: 2,841	4
kFGF*	AAVALLPAVLLALLAP	16: 1,516	0
MAP	KLALKLALKALKAALKLA	18: 1,877	5
MPG	GALFLGWLGAAGSTMGAPKKKRKV	24: 2,445	5

[1] Sequences are in standard single letter code with L-isoform amino acid residues represented in uppercase and D-isoform amino acid residues (sequences in retro-inversed form) represented in lowercase. *Penetratin is also known as antennapedia peptide and kFGF (Kaposi fibroblast growth factor) is also known as MTS (membrane translocating sequence).

Table 2
Studies using NR2B9c peptide fused to TAT in cerebral ischaemia/stroke and other CNS injury models.

Peptide name & sequence[1]	Injury model[2]	Route & treatment schedule	Dose	Neuroprotection: reduced infarct volume or brain injury[3]	Study
TAT-NR2B9c: KLSSIESDV					
Control: TAT-NR2B-ADA; KLSSIEADA	Rat (SD): tMCAO; 90min	IV: 45min before or 1h after MCAO	3000nmol/kg	Yes. No, ADA peptide	Aarts et al., 2002
Control: TAT-NR2B9c-ADA	Rat (SD): 3 pPVO	IV: 1h after PVO	300 or 3000nmol/kg	Yes, 3000nmol/kg. No, ADA peptide	Sun et al., 2008
Control: TAT-NR2B9c-TDA; KLSSIETDV	Rat (SD): pMCAO	IV: 1h after MCAO	300 or 3000nmol/kg	Yes. Yes TDA peptide	
	Rat (SD): tMCAO; 90min	IV: 1.5h after reperfusion	30, 300, 1000 or 3000nmol/kg	Yes	
Control: TAT-NR2B9c-ADA		IV: 1.5h after reperfusion	300nmol/Kg	Yes, at 62 days. No ADA peptide	
	Rat (SD): 3 PVO	IP: 15min before PVO	3000nmol/kg	Yes	Soriano et al., 2008
	Rat 12 day (SD): pMCAO + 90min CCAO	IV: 30min before MCAO	3000nmol/kg	No	
Control: TAT-NR2B-ADA	Rat (SD): Status epilepsy (SE): IP pilocarpine.	IV: 10min after SE onset IV: 3h after termination of SE	3000 or 9000nmol/kg 3000nmol/kg	No Yes. No ADA peptide	Dykstra et al., 2009
	Macaque: tMCAO; 90 min	IV: 1h after MCAO	1083nmol/kg	Yes	Cook et al., 2012
	Macaque: tMCAO; 4.5h	IV: 1h after MCAO		Yes	
	Macaque: tMCAO; 3.5h	IV: 3h after MCAO		Yes	
	Rat (W): pMCAO	IV: 1h after MCAO	3000nmol/kg	Yes	Bråtane et al., 2011
	Mouse: pMCAO	IV: 30min after MCAO	3000nmol/kg	No	Bach et al., 2012
	Human: intracranial aneurysm surgery	IV: After endovascular treatment	1083nmol/kg	N/S reduction in ischaemic lesions	Hill et al., 2012
	Rat (SD): pMCAO/pACA (anterior branches)	IV: 5min before MCAO	3000nmol/kg	Yes (Evoked Field Potential, EEG Power)	Srejic et al., 2013
	Rat (SD): 3 PVO	IV: 15min prior to PVO	3000nmol/kg	Yes	Bell et al., 2013
TAT-NPEG4-(IETDV)2					

	Mouse: pMCAO	IV: 30min after MCAO	3000nmol/kg	Yes	Bach et al., 2012

[1] TAT = GRKKRRQRRR, ADA = TAT-NR2B9c-ADA, TDA = TAT-NR2B9cTDA. [2] SD = Spargue Dawley, W = Wistar. tMCAO = transient middle cerebral artery occlusion, PVO = pial vessel occlusion (permanent), pMCAO = permanent middle cerebral artery occlusion, CCAO = common carotid artery occlusion. [3] Reduced Infarct volume or brain injury.

Table 3
Studies using JNK peptides fused to TAT in cerebral ischaemia/stroke and other CNS injury models.

Peptide & sequence[1]	Injury Model[2]	Route & treatment schedule[3]	Dose	Neuroprotection[4]	Study
JNKI-1-TATD: tdqsrpvqpflnlttprkpr-pp-tat, **TAT-JNKI-1L**: TAT-PP-RPKRPTTLNLFPQVPRSQDT or **TAT-JIP-1:** RPKRPTTLNLF					
	Mouse: tMCAO; 30min Rat (W) 14d: pMCAO	ICV: 1h before or 3, 6 or 12h after MCAO IP: 30min before or 6 or 12h after MCAO	15.7ng 2800nmol/kg	Yes, except 12h after Yes	Borsello et al., 2003
	Mouse: pMCAO	ICV: 3 or 6h after MCAO	150ng	Yes, 3h. No 6h	Hirt et al.2004
TAT-JIP-1	Mouse: tMCAO; 60min	ICV: 15min before or 30min after MCAO	3000ng	Yes	Gao et al., 2005
TAT-JIP-1	Rat (SD): tGCI; 15min/4VO	ICV: 40min before or 60min after 4VO	100,000ng	Yes	Guan et al., 2006
	Rat (W) 14d: pMCAO + 90min CCAO	IP: 30min before MCAO	2800nmol/kg	Yes	Repici et al., 2007
	Mouse: tMCAO; 30min	IV: 6h after MCAO	0.0076 to 760nmol/kg	Yes, all doses	Wiegler et al., 2008
	Rat (SD): tMCAO; 90min	IV: 3h after MCAO	7.6, 25 or 76nmol/kg 25nmol/kg	Yes, 25nmol/kg (at 3d) No at 6d (N/S) or 10d	Esneault et al., 2008
	Rat (SD) 12d: pMCAO pMCAO + 90min/CCAO	IP: 6h after MCAO IV: 30min before or 6h after MCAO	76nmol/kg 76nmol/kg	Yes No	Soriano et al., 2008
	Mouse: TBI	IP: 10min after TBI	2800nmol/kg	Yes	Ortolano et al., 2009
	Rat (SD) 7d: 2h hypoxia + CCAO	IP: 30min before & 3, 5, 8 12 & 20h after HI	76nmol/kg	No	Ginet et al., 2009
TAT-JNKI-1L	Rat (W) 7d: 2h hypoxia + CCAO	IP: 0 & 3h, 3 or 6h after HI	2550nmol/kg	Yes. No after 6h	Nijboer et al., 2010
	Mouse: tMCAO; 45min	IV: 3h after MCAO	25nmol/kg	Yes	Benakis et al., 2010
	Rat (SD): Epilepsy; IP KA	IP: 2h after KA	76nmol/kg	Yes	Spigolon et al., 2010
	Rat (SD): tMCAO; 30min tMCAO; 30min tMCAO; 90min	IP: 30min before MCAO IP: 3h after MCAO IP: 3h after MCAO	5100nmol/kg 125 or 510nmol/kg 510nmol/kg	No Yes, 510nmol/kg Yes	Liu et al., 2010
	Mouse: ICH	IV: 3h after ICH	25nmol/kg	Yes (day 2 post ICH)	Michel-Monigadon et al., 2010
	Rat (SD): NMDA retinal injections	Intravitreal: 40min before NMDA	19600ng	Yes	Bessero et al., 2010
	Rat (SH): tMCAO; 90 min	IV: 10min, 1 or 2h after reperfusion	255nmol/kg	No	Gow et al., 2011
Controls: D-JBD19; dqsrpvqpflnlttprkpr TATD	Rat (W) 12d: pMCAO	IP: 6h after MCAO IP: 4h before MCAO ICV: after MCAO	0.076 to 2800nmol/kg 25 or 2800nmol/kg 15.7ng 15.7 or 1570ng: D-JBD19 290ng: TATD	Yes, 7.6, 76, 2800nmol/kg No Yes Yes, 1570ng No	Vaslin et al., 2011

Control: scrambled; lpsvfgdvgapsrlpevsls-pp-tat	Rat (SD): pMCAO	IV: 1h after MCAO IV: 2h after MCAO	500, 1000 or 1500nmol/kg 250nmol/kg	No No	Si et al., 2012
	Mouse: SCI	IP: 6h after SCI	2800nmol/kg	Yes	Repici et al., 2012
	Rat (W) 7d: 2h hypoxia + CCAO	IP: 0, 3 or 6h or 0 & 3h after HI IP: 0h after HI (scrambled)	2550nmol/kg	Yes. No, 0 & 3h No, scrambled	Nijboer et al., 2013

[1] JNKI-1-TATD peptide used in study unless indicated. Peptides synthesised using D-amino acids are represented in lowercase, TATD = rrqrrkkrgy, TAT = YGRKKRRQRR. [2] W: Wistar, SD: Spargue Dawley, SH: Spontaneously Hypertensive tMCAO: transient middle cerebral artery occlusion; pMCAO: permanent middle cerebral artery occlusion, tGCI: transient global cerebral ischaemia; 4VO: 4 vessel occlusion, TBI: traumatic brain injury, CCAO: common carotid artery occlusion, HI: hypoxia-ischaemia, KA: kainic acid, ICH: intracranial haemorrhage, SCI: spinal cord injury. [3] ICV: intracerebroventricularly; IP: intraperitoneally; IV intravenously. [4] Neuroprotection: reduced infarct volume, tissue injury or neuronal cell death.

Table 4
Studies using CBD3 peptide fused to TAT or R9 in CNS disorder models.

Peptide name & sequence[1]	Injury model[2]	Route & treatment schedule[3]	Dose	Neuroprotection or reduced pain[4]	Study
TAT-CBD3: TAT-ARSRLAELRGVPRGL**, TAT-CRMP-2: TAT-**GVPRGLYDGVCEV or **R9-CBD3**: R9-ARSRLAELRGVPRGL					
TAT-CRMP-2	Mouse: pMCAO	ICV: before MCAO	10mg	Yes (6 h endpoint)	Bu et al., 2011
	Rat (SD) Pain; SC formalin in hind-paw Pain; capsaicin in eye Pain; IP ddC	 SC: before formalin Eye: before capsaicin IP: after ddC	 20µl: 3, 30 or 100µM 40µl: 3, 30 or 100µM 313nmol/kg	 Yes. No 3µM Yes. No 3µM Yes. No scrambled	Brittain et al., 2011b
Control: scrambled; TAT-WEAKEMLYFEALVIE					
	Mouse: TBI	IP: 5min after TBI	6400nmol/kg	Yes	Brittain et al., 2011a
	Rat (SD): tMCAO; 120min	IV: 1h before MCAO or 15min before reperfusion	6400nmol/kg	Yes	Brittain et al., 2012
TAT-CBD3-A6K Control: TAT-CBD3-reverse	Rat (SD): Pain; IP d4T	IP; after d4T	3300 or 9600 nmol/kg (TAT-CBD3) 3300nmol/kg (TAT-CBD3-A6K) 3300nmol/kg (CBD3-reverse)	Yes Yes No	Piekaraz et al., 2012
TAT-CBD3-G14F TAT-CBD3, TAT-CBD3-G14F Control: TAT-CBD3-reverse	Rat (SD) Migraine; capsicin nasal mucosa Pain; IP d4T	 Dural surface: before capsaicin IP; 7 days after d4T	 50µl: 10µM (TAT-CBD3-G14F) 3300nmol/kg (TAT-CBD3) 3300nmol/kg (TAT-CBD3-G14F) 3300nmol/kg (CBD3-reverse)	 Yes Yes Yes No	Ripsch et al., 2012
TAT-CBD3, R9-CBD3, TAT-CBD3-A6K R9-CBD3	Rat (SD): Pain: reverse transcriptase inhibitor or tibial nerve injury	Details not provide	Details not provide	Yes, R9-CBD3 > TAT-CBD3-A6K > TAT-CBD3	Feldmam & Khana, 2013 (review article)
R9-CBD3	Rat (SD) Pain; IP ddC Pain; tibial nerve injury	 IP: after ddC IP: after nerve injury	 33, 330 or 3300nmol/kg 33, 330 or 3300nmol/kg	 Yes, 3300nmol/kg Yes, all doses	Ju et al., 2013

[1] TAT-CBD3 peptide used in study unless indicated. TAT = YGRKKRRQRRR, TAT-CBD3-A6K**:** TAT-ARSRLKELRGVPRGL**,** TAT-CBD3-G14F: TAT-ARSRLKELRGVPRFL. [2] SD = Spargue Dawley, pMCAO: permanent middle cerebral artery occlusion tMCAO = transient middle cerebral artery occlusion, ddC: 2′-3′-dideoxycytidine, d4T: 2′,3′-didehydro-2′,3′-dideoxythymidine, IP: intraperitoneally. [3] ICV: intracerebroventricularly, SC: subcutaneously, IV intravenously TBI: traumatic brain injury. . [4] Neuroprotection: reduced infarct volume or tissue injury.

Table 5
Studies using peptides fused to TAT (or TAT & R9 alone) in cerebral ischaemia/stroke and other CNS injury models.

Peptide name & sequence[1]	Proposed target[2]	Injury Model[3]	Route & treatment schedule[4]	Dose	Neuroprotection[5]	Study
TAT-NR2B$_{ct}$ KKNRNKLRRQHSY Control: NRRRNSKLQHKKY	DAPK1	Mouse: tMCAO; 60min	IV: 1h before or 1h after MCAO	3060nmol/kg	Yes. No scrambled control	Tu et al., 2010
TAT-NR2Bct-CTM KKNRNKLRRQHSY -KFERQKILDQRFFE	DAPK1	Rat (SD): tMCAO; 60min	IV: 1h after reperfusion	2010nmol/kg	Yes	Fan et al., 2014
TAT-p53DM RVCACPGRDRRT Control: CCPGECVRTRRR	DAPK1	Mouse: tMCAO; 60min	IV: 2h after reperfusion IV: 5h after reperfusion	360nmol/kg 360, 720, 1080, 1440 or 1800nmol/kg	Yes. No, control Yes. No, control	Wang et al., 2014
AIP (no CPP) KKALRRQEAVDAL	CaMKII	Rat (SD): NMDA retinal injections	Intravitreal injection: at the NMDA exposure	3000ng (2 nmol)	Yes	Goebel, 2009
TAT-CN21 KRPPKLGQIGRSKRVVIEDDR		Mouse: tMCAO; 60min	IV: at time of reperfusion	250nmol/kg	Yes	Vest et al., 2010
PYC36-TAT GGLQGRRRQGYQSIKP	AP-1	Rat (SH): tMCAO; 90 min	IV: 10min after MCAO	7.6, 76 and 255nmol/kg	No	Gow et al., 2011
PYC36-TAT GGLQGRRRQGYQSIKP	AP-1	Rat (SD): pMCAO	IV: 1h after MCAO IV: 2h after MCAO	500, 1000 or 1500nmol/kg 250nmol/kg	No No	Si et al., 2012
TAT-PYC36-HP GGLQGRRRQGYQSIKP- CLEVSRKNC	AP-1	Rat (SD): pMCAO	IV: 2h after MCAO	250nmol/kg	No	Si et al., 2012
TAT-GluR6 RLPGKETMA Control: RLPGKAADD	GluR6	Rat (SD): 4VO; 15min	ICV: 40min before 4VO	100,000ng	Yes. No control	Pei et al., 2006
		Rat (SD): 4VO; 15min	ICV: 40min before 4VO	100,000ng	Yes. No control	Zhang et al., 2007
TAT-mGluR1 VIKPLTKSYQGSGK	mGluR1α	Mouse: kainic acid; SC 30mg/kg	IP: 90min before kainic acid	16406nmol/kg	Yes	Xu et al., 2007
		Rat (SD) P7: 2.5h hypoxia + CCAO	IP: 1h before hypoxia	49,220nmol/kg	Yes	Zhou et al., 2009
TAT-K13 KEIVSRNKRRYQED Contol:GPEETSEKVENGS	PTEN	Rat (SD): tMCAO; 90min	IV: 30min or 4.5h after reperfusion	3125nmol/kg	Yes	Zhang et al., 2013
TAT-Indip GEPHKFKREW Control: GEPHRFRREW	SREBP-1	Rat (SD): tMCAO; 90min	IV: 45min before MCAO or 30min after reperfusion	2885nmol/kg	Yes	Taghibiglou et al., 2009
TAT-CN21 KRPPKLGQIGRSKRVVIEDDR	CaMKII	Mouse: tMCAO; 60min	IV: at time of reperfusion	250nmol/kg	Yes	Vest et al., 2010

TAT-D rrrqrrkkrg	N/A	Rat (SD) P12: pMCAO	ICV: just after MCAO IP: 6h after MCAO	15.7 or 100ng 71.6 or 716nmol/kg	Yes No	Vaslin et al., 2009b
	N/A	Rat (SD): pMCAO	IV: 2h after MCAO	250nmol/kg	No	Si et al., 2012
D-TAT-GESV yagqwgesv Control scrambled peptide:YAGQWGASA	NOS1AP	Rat (SD) P7: 2.5h hypoxia + CCAO	ICV: after carotid occlusion	100ng	Yes	Li et al., 2013

Table 5 cont.

Peptide name & sequence	Proposed target	Injury Model	Route & treatment schedule	Dose	Neuroprotection	Study
TAT-NBD TALDWSLQTE	NF-kB	Rat (W) P7: 2h hypoxia + CCAO	IP: 0 and 3h after HI	7394nmol/kg	Yes	Van der Kooij et al., 2010
TAT-SAB$_{KIM1}$ GFESLSVPSPLDLSGRRVVAPP	JNK	Rat (SD): 6-OHDA	Mid-forebrain bundle: at time of 6-OHDA injection	Mid-forebrain bundle: 12,600ng	Yes	Chambers et al., 2013
TAT-ψεHSP90 PKDNEER Control: TAT peptide	PKCε	Mouse: tMCAO; 60min	IP: 15mim before MCAO and 3min before reperfusion	414nmol/kg	Yes	Sun et al., 2013
δSV1-1-TAT SFNSYELGSL Control: YGRKKRRQRRR (note: δSV1-1 fused to TAT via cysteine S-S bond)	δPKC	Rat (SD): tMCAO; 120min	ICA: immediately before MCAO or immediately after reperfusion IP: 1 or 6h post-reperfusion	21nmol/kg 70nmol/kg	No, before MCAO. Yes after reperfusion Yes	Bright et al., 2004
		Rat (SD) tMCAO; 120min Rat (Dahl): tMCAO; 90min	IP: at onset of reperfusion SC: for 4-5d before MCAO and 24h after MCAO	70nmol/kg 1mM; 5µl/h	Yes; cerebral microvascular Yes	Bright et al., 2007
TAT-ISP DMAEHMERLKANDSLKLSQEYESI	PTPσ	Rat (SD): SCI (contusive)	SC: 24h after SCI, daily thereafter for 49d	2.5nmol/kg (500µl/5µM)	Yes, improved recovery	Lang et al., 2015
R9D rrrrrrrr	N/A	Rat (SD): pMCAO	IV: 30min after MCAO	1000nmol/kg	Yes	Meloni et al., 2015

[1] Peptides synthesised using D-amino acids are represented in lowercase, TATD = rrqrrkkrgy, TAT = YGRKKRRQRR. [2] DAPK1: death-associated protein kinase 1 protein , CaMKII: calcium/calmodulin-dependent protein kinase II, AP-1: activator protein 1, GluR6-9c: glutamate receptor 6, mGluR1α: metabotropic glutamate receptor 1α, PTEN: Phosphatase and tensin homolog, SREBP-1: Sterol Regulatory Element-Binding Protein-1, NO1AP: nitric oxide synthase 1 adaptor protein, NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells, JNK: c-Jun N-terminal kinase, PKCε: Protein kinase Cε, δPKC: protein kinase C δ, PTPσ: Protein tyrosine phosphatases σ. [3] W: Wistar, SD: Spargue Dawley, SH: Spontaneously Hypertensive tMCAO: transient middle cerebral artery occlusion; pMCAO: permanent middle cerebral artery occlusion, tGCI: transient global cerebral ischaemia; 4VO: 4 vessel occlusion, TBI: traumatic brain injury, CCAO: common carotid artery occlusion (HI: hypoxia-ischaemia), ICH: intracranial haemorrhage, SCI: spinal cord injury, 6-OHDA: 6-hydroxydopamine, SC: subcutaneously. [4] ICV: intracerebroventricularly; IP: intraperitoneally; IV intravenously. [5] Neuroprotection: reduced infarct volume, tissue injury or neuronal cell death.

Table 6
Studies using arginine-rich peptides in CNS injury models.

Peptide & sequence[1]	Injury Model[2]	Route & treatment schedule[3]	Dose	Neuroprotection[4]	Study
APOE receptor binding domain					
COG133 LRVRLASHLRKLRKRLL	Mouse: Inflammation; IV LPS	IV: At time of LPS	2765nmol/kg	Reduced serum & brain inflammatory markers (TNF, IL-6)	Lynch et al., 2003
	Rat (W) P7: CCAO/2h hypoxia	ICV: before hypoxia	100 to 5000ng	Yes	McAdoo et al., 2005
	Mouse: TBI; contusion/diffuse	IV: 30min post-TBI	92 or 184nmol/kg	Yes	Lynch et al., 2005
	Mouse: MS; EAE	IP: Every other day starting on day 2 & ending 30d post-immunisation	COG133: 469nmolkg COG112: 214nmol/kg	Yes, reduced symptoms; COG112 > COG133	Li et al., 2006
COG112: Penetratin-COG133	*Drosophlia* (APP transgenic): Alzheirmer's disease	Injected in abdomen; days 2, 5, 9, 13, 17, 21, 25 &29	0.2µl: concentration not provided	Yes, COG133 & COG112 reduced neurodegeneration	Sarantseva et al., 2009
	Mouse: TBI; contusion/diffuse	IV: 60min post-TBI	461, 1843 or 3687nmo/kg	No	Laskowitz et al., 2007
COG1410 ASAibLRKLAibKRLL	Mouse: SAH	IV: Immediately after SAH, & at 12h intervals for 3d	425 or 850nmol/kg	Yes, reduced vasospasm, improved survival & behaviour	Gao et al., 2006
	Mouse: TBI; contusion/diffuse	IV: 30min post-TBI	285 or 570nmol/kg	Yes, 570nmol/kg. No, 285nmol/kg	Hoane et al., 2007
Control: ASAibLRKLAibKR	Mouse: TBI; contusion/diffuse	IV: 120min post-TBI	213 or 426nmol/kg	Yes, No control peptide (507nmol/kg)	Laskowitz et al., 2007
	Mouse: TBI; contusion/diffuse	IV: 30min & 24h post-TBI	570nmol/kg	Yes, histologically & functionally	Hoane et al., 2009
	Rat (SD): tMCAO; 90min	IV: 30min post-reperfusion	570nmol/kg	Yes, histologically & functionally	Tukhovskaya et al., 2009
	Mouse: TBI; fluid percussion	IV: 2 & 4h post-TBI, & then at 24, 48 & 72h	710nmol/kg	Yes, Histologically & functionally	Kaufman et al., 2010
	Mouse: ICH	IV: 30min IV: 30min, 1, 2 or 4h after ICH, & continued daily for 5d	355, 710, 1420 or 2840nmol/kg 1420nmol/kg	Yes, functionally Yes, functionally. No reduction in haematoma volume	Laskowitz et al., 2012
	Mouse: TBI; cortical impact	IV: 30min post-TBI, then every 24h for 3 or 7d	710nmol/kg	Yes, reduced axonal APP immunoreactivity & microglia activation. No reduction of peri-contusional white matter volume	Jiang & Brody, 2012
Dynorphine A 1-13 YGGFLRRIRPKLK	Rat (SD): tMCAO; 90min	ICV: infusion started 1h before MCAO & continued for 4h	30µL: 1604, 16040 or 160400ng (1, 10 or 100 nmol)	Yes, 16040, 16040 nmol. No, 1604ng	Kao et al., 2008
APP APP96-110 NWCKRGRKQCKTHPH	Mouse: TBI; cortical impact	ICV: 30min post-TBI	2µl: 25µM	Yes	Corrigan et al., 2014

[1] Penetratin = RQIKIWFQNRRMKWKK, Aib = 2-Aminoisobutyric acid, APP: amyloid precusor protein. [2] W: Wistar, SD: Spargue Dawley, tMCAO: transient middle cerebral artery occlusion; TBI: traumatic brain injury, CCAO: common carotid artery occlusion, ICH: intracranial haemorrhage, MS: multiple sclerosis, EAE: Experimental autoimmune encephalomyelitis, IV intravenously, LPS: lipopolysaccharide. [3] ICV: intracerebroventricularly; IP: intraperitoneally. [4] Neuroprotection: reduced infarct volume, neural tissue injury, neuronal cell death or improved functional outcomes.