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# 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 of neurological disorders.



*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, SREBP cleavage-activating protein; SCI, spinal cord injury; sEPSC, spontaneous excitatory post-synaptic currents; siRNA, small interfering RNA; SREBP-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.

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**Contents**



## 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 neuro-damaging 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 & Pabo, 1988; Green & 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 ischaemia, spinal cord injury, traumatic brain injury, epilepsy, Parkinson's disease and Alzheimer's disease (Arthur et al., 2007; Colombo et al., 2007; Lai et al., 2005; Liu et al., 2006; Meade et al., 2009; Nagel et al., 2008). 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 ischaemia in P12 rats after intraventricular injection (Craig et al., 2011; Meade et al., 2010a, b; Vaslin et al., 2009b; Xu et al., 2008). 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

**Table 1**
Examples of commonly used cell penetrating peptides.

| Peptide | Sequence[a] | Amino acids: MW (Da) | Net charge at pH 7 |
|---|---|---|---|
| TAT | GRKKRRQRRR | 10: 1397 | 8 |
| TAT-D | rrrqrrkkrG | 10: 1397 | 8 |
| R9–R15 | RRRRRRRRR–RRRRRRRRRRRRRRR | 9: 1423–15: 2360 | 9–15 |
| Penetratin[b] | RQIKIWFQNRRMKWKK | 16: 2245 | 7 |
| Pep-1 | KETWWETWWTEWSQPKKKRKV | 21: 2848 | 3 |
| HSV-1 VP22 | DAATATRGRSAASRPTERPRAPARSASRPRRVD | 33: 3548 | 6 |
| Transportan | GWTLNSAGYLLGKINLKALAALAKKIL | 27: 2841 | 4 |
| kFGF[b] | AAVALLPAVLLALLAP | 16: 1516 | 0 |
| MAP | KLALKLALKALKAALKLA | 18: 1877 | 5 |
| MPG | GALFLGWLGAAGSTMGAPKKKRKV | 24: 2445 | 5 |

[a] 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.
[b] Penetratin is also known as antennapedia peptide and kFGF (Kaposi fibroblast growth factor) is also known as MTS (membrane translocating sequence).