

Attenuation of Neuronal Death by Peptide Inhibitors of AP-1 Activation in Acute and Delayed In Vitro Ischaemia (Oxygen/Glucose Deprivation) Models

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Abstract Using acute and delayed in vitro ischaemia models we evaluated the neuroprotective efficacy of five peptides (PYC19D-TAT, PYC35D-TAT, PYC36D-TAT, PYC38D-TAT, PYC41D-TAT) previously demonstrated to down-regulate AP-1 activation (e.g. c-Jun/c-Fos activation), and inhibit neuronal death in vitro following glutamate and kainic acid excitotoxicity. The JNK inhibitor peptide (JNKI-1D-TAT) and the TAT cell-penetrating-carrier peptide (D-TAT) were used as controls. In the acute model, all five AP-1 inhibitory peptides, JNKI-1D-TAT, and D-TAT provided neuroprotection by increasing neuronal viability from ≈ 5 to 23–53%. In the delayed model, three of the five AP-1 inhibitory peptides (PYC35D-TAT, PYC36D-TAT, PYC38D-TAT) and JNKI-1D-TAT provided neuroprotection by increasing neuronal viability from ≈ 10 to 35–80%. This study not only highlights a group of peptides with therapeutic potential, but also the need to assess putative therapeutics in multiple in vitro

models to achieve a comprehensive representation of their neuroprotective capacity.

Keywords Neuroprotection · In vitro ischaemia · AP-1 · JNKI · TAT

Introduction

The up-regulation of AP-1 has been extensively reported following cerebral ischaemia, as well as increased c-Jun and c-Fos mRNA transcription, known activator protein-1 (AP-1) complex components. Additionally, there is a positive correlation between neuronal survival and a decrease in c-Jun and c-Fos expression in vivo (Yamaguchi et al. 2006; McGahan et al. 1998; Rau et al. 2003; Domanska-Janik et al. 1999). This correlation of AP-1 activation and increased neuronal cell death is duplicated in vitro following neuronal injury, as well as the inhibition of AP-1 or c-Jun/c-Fos mRNA expression and an increase in neuronal viability (Fernandez et al. 2005; Huang et al. 2008; Dong et al. 2005; Meade et al. 2010a). To this end, we have identified five AP-1 inhibitory peptides that we have previously shown to attenuate cell death in glutamate and kainate excitotoxicity injury models (Meade et al. 2010a, 2010b).

These five AP-1 inhibitory peptides were derived from a yeast two-hybrid screen designed to isolate peptide sequences that bind the c-Jun protein and prevent AP-1 transcriptional activation. The ability of the AP-1 inhibitory peptides to reduce AP-1 transcriptional activation was confirmed by an AP-1 luciferase reporter assay. Additionally, verification of competency of the inhibitory peptides in suppression of c-Jun and c-Fos mRNA expression was established by real-time PCR (Meade et al. 2010a). With

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this in mind, in the present study we assessed the neuro-protective efficacy of the five AP-1 inhibitory peptides, using two in vitro ischaemia models that induce either acute or delayed neuronal death.

Materials and Methods

Primary Neuronal Cortical Cultures

Establishment of cortical cultures was as previously described (Meloni et al. 2001), but with some modifications; namely the use of glass wells, as we have previously shown that plastic culture wells store oxygen and therefore are not used in our models. Briefly, cortical tissue from E18-E19 Sprague-Dawley rats was dissociated in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Australia) supplemented with 1.3 mM L-cysteine, 0.9 mM NaHCO₃, 10 units/ml papain (Sigma, USA) and 50 units/ml DNase (Sigma) and washed in cold DMEM/10% horse serum. Neurons were resuspended in Neurobasal medium (NB; Invitrogen) containing 2% B27 supplement (B27; Invitrogen). Before seeding, 96-well sized glass wells (6 mm diameter, ProTech, Australia) were coated with laminin overnight (50 µl/well: Sigma). Excess laminin solution was then removed and replaced with 60 µl NB (containing 2% B27; 4% fetal bovine serum; 1% horse serum; 62.5 µM glutamate; 25 µM 2-mercaptoethanol; and 30 µg/ml streptomycin and 30 µg/ml penicillin). Neurons were plated to obtain approximately 10,000 viable neurons in 100 µl NB/2% B27 in each well on day in vitro (DIV) 11–12. Neuronal cultures were maintained in a CO₂ incubator (5% CO₂, 95% air balance, 98% humidity) at 37°C. On DIV 4 one third of the culture medium was removed and replaced with fresh NB/2% B27 containing the mitotic inhibitor, cytosine arabinofuranoside (1 µM final concentration; Sigma). On DIV 8 one half of the culture

medium was replaced with NB/2% B27. Cultures were used on DIV 11 or 12, which routinely consist of >97% neurons and 1–3% astrocytes (Meloni et al. 2001).

In Vitro Ischaemia Models

For the acute cell death model, culture medium was removed from wells and washed with 315 µl of glucose-free balanced salt solution (BSS; mM: 116 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 1 NaH₂PO₄; pH 7.0) before the addition of 60 µl BSS containing 25 mM deoxyglucose (ICN Biomedicals, USA). In vitro ischaemia was initiated by placing wells in an anaerobic incubator (Don Whately Scientific, England; atmosphere of 5% CO₂, 10% H₂ and 85% argon, 98% humidity) at 37°C, for 40 min. Upon removal from the anaerobic incubator, medium was replaced with 120 µl of 50% NB/2% N2 and 50% balanced salt solution (NB/N2:BSS) and incubated at 37°C/5% CO₂ for 24 h.

For the delayed cell death model, culture medium was removed from wells and washed with 315 µl of BSS before the addition of 60 µl BSS. In vitro ischaemia was conducted in the anaerobic incubator for 55 min. Upon removal from the anaerobic incubator, 60 µl of NB/2% N2 supplement was added to the cultures before incubation at 37°C/5% CO₂ for 24 h.

Control cultures received the same BSS wash procedures as ischaemic cultures and were resupplied with 120 µl of NB/N2:BSS before incubation at 37°C/5% CO₂. Four to eight wells were used for each treatment and control.

AP-1 Inhibitory Peptides and Control Peptides

The peptides were synthesised and HPLC purified by Mimotopes Pty Ltd (Australia) in the protease resistant D-retro-inverso form, synthesised from D-amino acids in reverse sequence (referred to as D-isomers hereafter)

Table 1 AP-1 inhibitory peptide, JNK1-ID-TAT and D-TAT control peptide amino acid sequences with corresponding peak neuronal viability percentages at optimal concentrations (µM) in both acute and delayed in vitro ischaemia models

Peptide identification	Amino acid sequence	Acute IVI		Delayed IVI	
		Most viability		Most viability	
		Conc (µM)	%	Conc (µM)	%
PYC19D-TAT	H-YIYPYAYSQNIL RRRQRRKKRG -OH	2	40	–	–
PYC35D-TAT	H-RERKSSSEIGGSRISQYAG RRRQRRKKRG -NH ₂	2	30	2	80
PYC36D-TAT	H-PKISQYGQRRRGQLGG RRRQRRKKRG -NH ₂	1	37	1	43
PYC38D-TAT	H-RHAPLARGSWRGQPQGGPQRRGQLGG RRRQRRKKRG -OH	2	53	0.5	35
PYC41D-TAT	H-LPLLRHHEQNISV RRRQRRKKRG -OH	5	23	–	–
JNK1-ID-TAT	H-TDQSRPVQPFLNLTTPRKPR RRRQRRKKRG -NH ₂	2	37	0.1	43
D-TAT	H- RRRQRRKKRG -NH ₂	5	35	–	–

Amino acid sequence that corresponds to TAT transduction domain is in bold with a glycine linker between the peptide and TAT. IVI in vitro ischaemia

(Brugidou et al. 1995) (Table 1). Peptides were fused to a truncated HIV-1 TAT_(48–57) transduction domain peptide (TAT) at the amino terminus to allow peptides to enter cells (Vives et al. 1997). A TAT-fused JNK inhibitory peptide (JNKI-1D-TAT) in the D-isoform was synthesised (Borsello et al. 2003) and also evaluated, and used as a peptide positive control. The TAT peptide (D-TAT) was also synthesised in the D-isoform and administered as a carrier control. All peptides were prepared as 100 × stocks (500 μM) in normal saline and assessed in a dose range from 0.1 to 10 μM in the acute model and 0.1 to 2 μM in the delayed model.

Peptides were added to neuronal cultures immediately prior to and/or after incubation in the anaerobic chamber. Peptides present before anaerobic incubation were added in a 5 μl BSS stock (containing deoxyglucose if in the acute model) to wells containing 55 μl of BSS. Peptides present after anaerobic incubation were added in a 20 μl NB/N2:BSS stock. A non-peptide positive control consisting of the glutamate receptor blockers 5 μM MK801/5 μM 6-cyano-7-nitroquinoxaline (MK801/CNQX) were added in a similar manner to the peptides. Controls received BSS and NB/N2:BSS vehicle solutions.

Neuronal Viability and Statistical Analysis

Eighteen to 24 h after in vitro ischaemia, neuronal cultures were examined by light microscopy for qualitative assessment of neuronal cell viability. Neuronal viability for all experiments was quantitatively measured by the 3-(4,5-dimethyliazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (Promega). MTS absorbance data were converted to reflect proportional cell viability relative to both the untreated and ischaemic controls, with the untreated control taken as 100% viability, and presented as mean ± SEM. Viability data were analysed by ANOVA, followed by post-hoc Fischer's PLSD test, with $P < 0.05$ values considered statistically significant. All assays were performed in quadruplicate with sister neuronal cultures and were repeated a minimum of three times independently.

Results

Assessment of AP-1 Inhibitory Peptides, JNKI-1D-TAT and D-TAT in the Acute In Vitro Ischaemia Model

All five AP-1 inhibitory peptides (PYC19D-TAT, PYC35D-TAT, PYC36D-TAT, PYC38D-TAT, PYC41D-TAT), JNKI-1D-TAT, and D-TAT significantly increased neuronal survival. Neuronal survival ranged from 5–10% in the in vitro ischaemic control cultures to 23–53% for the AP-1 inhibitory peptides, 37% for JNKI-1D-TAT and 35%

for D-TAT treated cultures (Table 1; Fig. 1). The peptides were neuroprotective when present after the in vitro ischaemia, and when present both during and after the in vitro ischaemic insult; but not when present solely before, solely during, before and during, or before and after (PYC36D-TAT in Fig. 2).

Assessment of AP-1 Inhibitory Peptides, JNKI-1D-TAT and D-TAT in the Delayed In Vitro Ischaemia Model

Three of the five AP-1 inhibitory peptides (PYC35D-TAT, PYC36D-TAT, PYC38D-TAT) and JNKI-1D-TAT significantly increased neuronal survival when present after in vitro ischaemia. In this model of neuronal injury D-TAT did not provide neuroprotection. Neuronal survival ranged from 10–15% in the in vitro ischaemic controls cultures, to 25–80% for the three neuroprotective AP-1 inhibitory peptides and to 43% for JNKI-1D-TAT (Table 1; Fig. 1). As mentioned, the peptides were only neuroprotective when administered after in vitro ischaemia, and were ineffective when administered solely before, during, or before and after the insult (PYC36D-TAT in Fig. 2).

Discussion

In the present study, we have shown that all five AP-1 inhibitory peptides, JNKI-1D-TAT and D-TAT were neuroprotective in an acute model and three AP-1 inhibitory peptides (PYC35D-TAT, PYC36D-TAT, PYC38D-TAT) and JNKI-1D-TAT were neuroprotective in a delayed in vitro ischaemia model. Interestingly, in the acute model the neuroprotective effect of higher concentrations of D-TAT (2–10 μM) resulted in a dose response plateau unlike the AP-1 inhibitory peptides and the JNKI-1D-TAT peptide, which generated an inverted 'U' dose response effect. The inverted 'U' indicates that the neuroprotective effects of AP-1 and JNKI-1D-TAT peptides are impeded at higher concentrations. These findings are in line with those of Cardozo et al. (2007), who demonstrated cytotoxicity of TAT conjugated peptides at concentrations from 10 μM, but not with TAT peptides alone up to 100 μM. While there is a trend for an inverted 'U' dose effect in the delayed model (specifically with PYC38D-TAT) the range of AP-1 inhibitory and JNK peptide concentrations assessed were limited to a maximum of 2 μM, thus restricting confirmation of the dampening of neuroprotective efficacy seen in the acute model at higher peptide concentrations.

Previous studies have demonstrated neuroprotective activity for the TAT peptide following glutamate and NMDA excitotoxicity, but not kainate excitotoxicity (Vaslin et al. 2009; Meade et al. 2010a; Xu et al. 2008). It has been

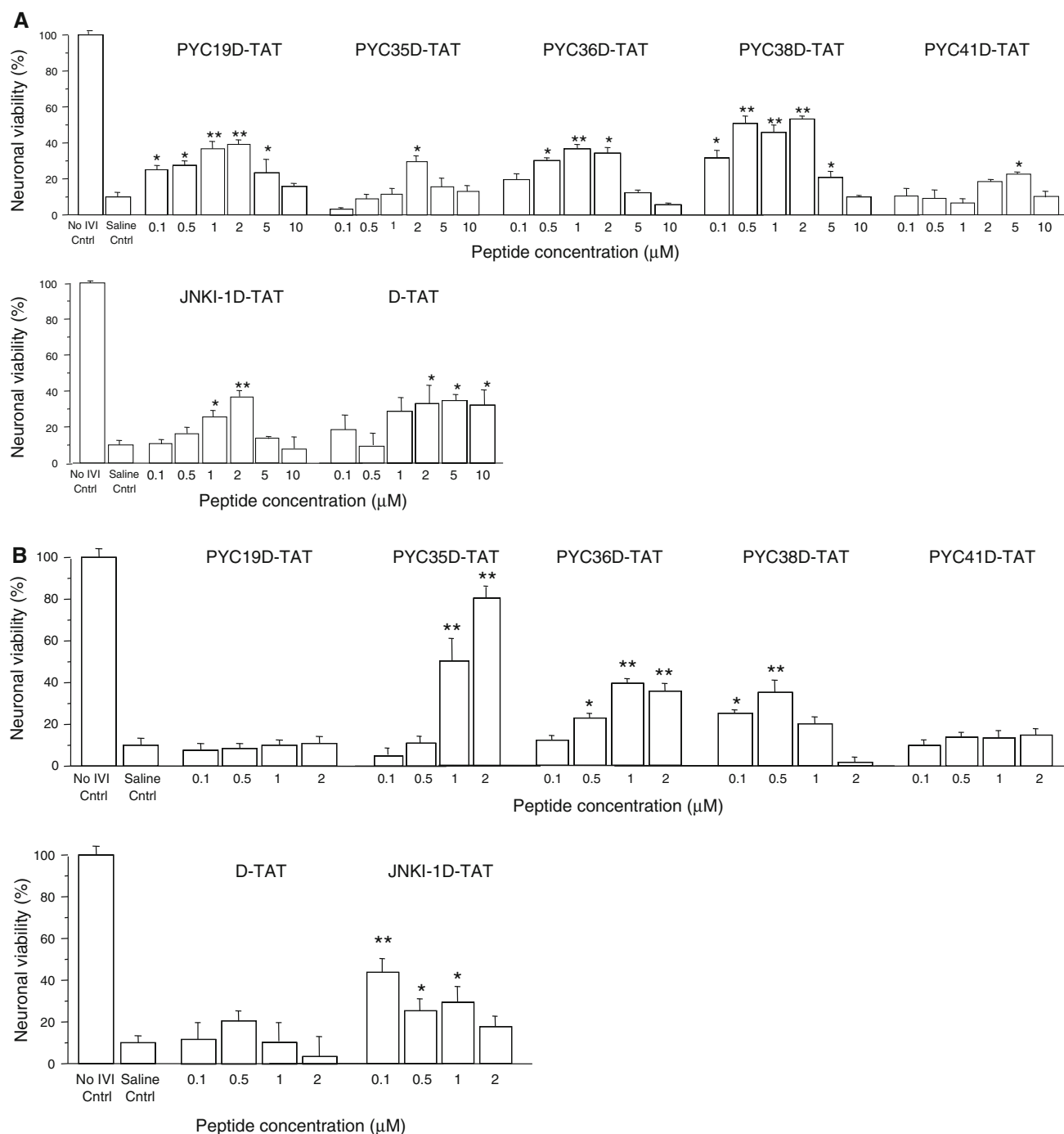


Fig. 1 Concentration response graphs for AP-1 inhibitory peptides, JNKI-1D-TAT, and D-TAT following: **a** acute in vitro ischaemia with peptides administered before and after in vitro ischaemia; and **b** delayed in vitro ischaemia with peptides administered only

post-delayed in vitro ischaemia. Neuronal viability expressed as a percentage, with no insult control taken as 100% viability (mean \pm SEM; $n = 4$; * $P < 0.05$, ** $P < 0.0001$). Cntrl control, IVI in vitro ischaemia

hypothesised that the differential neuroprotective action of the TAT peptide across injury models is via interference with NMDA receptor activation/signalling specifically (Vaslin et al. 2009). If correct, this may explain why D-TAT was neuroprotective in our acute model, but not in our delayed in vitro ischaemia model; cell death in the acute model is more

likely biased towards direct glutamate excitotoxicity mediated mechanisms (e.g. NMDA activation), whilst the delayed model is more likely biased towards indirect excitotoxicity mechanisms (e.g. ROS generation).

In the acute in vitro ischaemia model, the AP-1 inhibitory peptides (except for PYC38D-TAT), JNKI-1D-TAT,

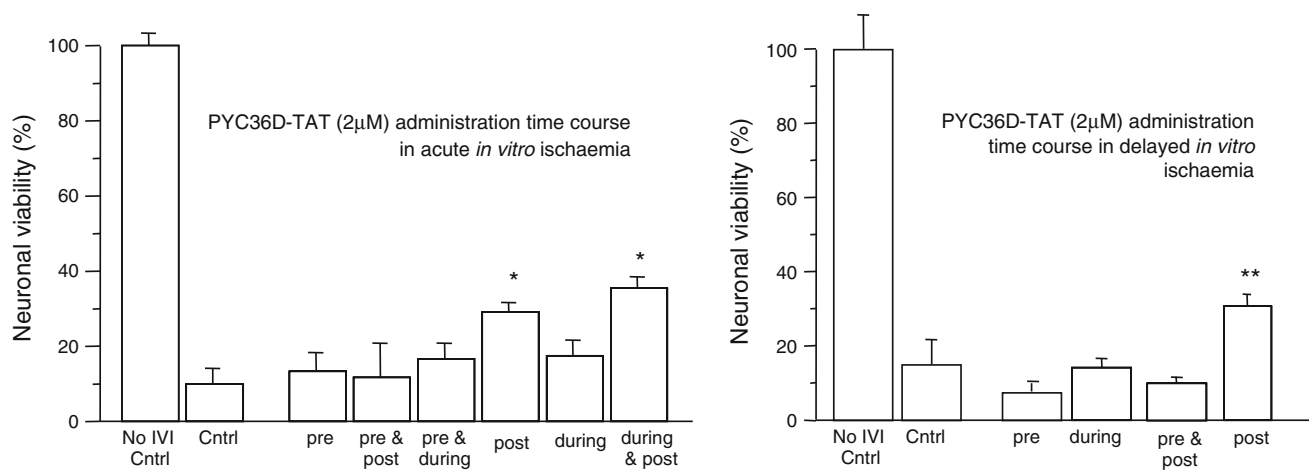


Fig. 2 Neuroprotective efficacy of PYC36D-TAT in acute and delayed *in vitro* ischaemia when administered at different time-points in relation to the insult. Neuronal viability expressed as a percentage,

with no insult control taken as 100% viability (mean \pm SEM; $n = 4$; * $P < 0.05$, ** $P < 0.0001$). *Ctrl* control, *IVI* *in vitro* ischaemia

and the D-TAT peptide displayed comparable neuroprotective efficacy. Therefore, it is possible that the neuroprotective efficacy of these AP-1 inhibitory peptides and JNKI-1D-TAT may be due, in-part or completely, to the innate neuroprotection provided by D-TAT. PYC38D-TAT is the only AP-1 inhibitory peptide that provided greater neuroprotection than D-TAT. Thus, the neuroprotection afforded by PYC38D-TAT could be the result of a combination of AP-1 inhibition and D-TAT's neuroprotective mechanism.

In the delayed *in vitro* ischaemia model the D-TAT peptide was ineffective. As such, the neuroprotection afforded by the three AP-1 inhibitory peptides (PYC35D-TAT, PYC36D-TAT, PYC38D-TAT) and JNKI-1D-TAT is likely to be due to inhibition of their respective targets of AP-1 and JNK, and not due to D-TAT. In contrast, the lack of neuroprotective efficacy of the two other AP-1 inhibitory peptides (PYC19D-TAT, PYC41D-TAT) in the delayed *in vitro* ischaemia model is suggestive that their efficacy in the acute model is due predominantly to the TAT. Alternatively, or additionally, the lack of neuroprotective efficacy may be related to differences in peptide half-life and hence cellular concentration/distribution over the time course of the injury models.

Whilst previous studies have verified the initiation and/or up-regulation of AP-1 in addition to its role in neuronal death in glucose/oxygen deprivation models (Gerlach et al. 2002; Huang et al. 2008; Whitmarsh et al. 2001) this study has highlighted the necessity for administrative time-courses for efficacious therapeutic delivery in both acute and delayed *in vitro* ischaemia models. In the acute model the peptides were only efficacious when administered during and after the *in vitro* ischaemic insult or solely after the insult, which suggests that the initiation of AP-1 driven

cell death occurs rapidly. Interestingly, in the acute model, the administration of AP-1 inhibitory peptide both before and after the insult negated the neuroprotective effect displayed when peptide was only administered after the insult. This may be due to increased intracellular concentration of the peptide affecting neuroprotective efficacy, as mentioned earlier; however, the same dampening effect would then be expected in the cultures in which the peptide was administered both during and after the insult, which was contrary to the results. This requires further exploration of peptide cellular distribution and concentrations over time to enable a better understanding of the dampening of neuroprotective efficacy.

In the delayed *in vitro* ischaemia model, the peptides were only efficacious when administered immediately after the insult, suggesting a delayed onset of AP-1 driven cell death. Hence, in the delayed model, as nuclear AP-1 activation may occur over a protracted period (Zhang et al. 2009; Hu et al. 2000) AP-1 inhibitory peptides may not be present in the nucleus at sufficient levels during the course of delayed neuronal death. This is in line with a study (Tunneemann et al. 2006) showing that the cellular distribution of TAT-fused peptides is initially high in the nucleus and subsequently decreases over time with a corresponding increase in cytosolic vesicles.

In this study, PYC35D-TAT, PYC36D-TAT and PYC38D-TAT were more efficacious than PYC19D-TAT and PYC41D-TAT, which is similar to the findings in a previous neuroprotective efficacy study using glutamate (Meade et al. 2010a). Since the amino acid sequences of the peptides vary, it is probable that their target affinity will also vary. To be effective, the AP-1 inhibitory peptides must compete not only with c-Jun homodimerising but also with c-Fos and ATF binding to c-Jun (Smeal et al. 1989).

Therefore, the efficacy of each AP-1 inhibitory peptide depends upon its individual competitive binding affinity to c-Jun relative to another c-Jun protein, c-Fos, or ATF, and upon its ability to displace c-Jun, c-Fos, and ATF from existing stable dimers.

Concluding Remarks

In conclusion, we have presented data showing that five AP-1 inhibitory peptides, JNKI-1D-TAT and the TAT peptide have neuroprotective activity in in vitro ischaemia models. In the acute in vitro ischaemia model neuroprotection may be due to the effect of AP-1 activation inhibition and/or the interaction by TAT with NMDA receptor activation/signalling. In this model there is a dampening effect of neuroprotection with increasing concentrations of peptide. However, in the delayed in vitro ischaemia model, neuroprotection appears to be mediated predominantly by AP-1 inhibition.

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