



ELSEVIER

available at [www.sciencedirect.com](http://www.sciencedirect.com)[www.elsevier.com/locate/brainres](http://www.elsevier.com/locate/brainres)BRAIN  
RESEARCH

## Research Report

# AP-1 inhibitory peptides attenuate *in vitro* cortical neuronal cell death induced by kainic acid

Amanda J. Meade<sup>a</sup>, Bruno P. Meloni<sup>a,b,\*</sup>, Frank L. Mastaglia<sup>a</sup>,  
Paul M. Watt<sup>c</sup>, Neville W. Knuckey<sup>a,b</sup>

<sup>a</sup>Centre for Neuromuscular and Neurological Disorders, The University of Western Australia and Australian Neuromuscular Research Institute, QEII Medical Centre, Nedlands, WA, Australia

<sup>b</sup>Department of Neurosurgery, Sir Charles Gairdner Hospital, Nedlands, WA, Australia

<sup>c</sup>Phylogica Ltd and Telethon Institute for Child Health Research, The University of Western Australia, Australia

## ARTICLE INFO

## Article history:

Accepted 1 September 2010

Available online 15 September 2010

## Keywords:

Neuroprotection

Excitotoxicity

Phylomer peptide

TAT

c-Jun

JNKI

## ABSTRACT

This study has assessed the neuroprotective efficacy of five AP-1 inhibitory peptides in an *in vitro* excitotoxicity model. The five AP-1 inhibitory peptides and controls of the JNK inhibitor peptide (JNKI-1D-TAT) and TAT cell-penetrating-peptide were administered to primary cortical neuronal cultures prior to kainic acid exposure. All five AP-1 inhibitory peptides and JNKI-1D-TAT provided significant neuroprotection from kainic acid induced neuronal cell death. Kainic acid exposure induced caspase and calpain activation in neuronal cultures, with caspase-induced cleavage of  $\alpha$ -fodrin reduced by administration of the AP-1 inhibitory peptides. Sequence analysis of the AP-1 inhibitory peptides did not reveal the presence of any secondary structures; however two peptides shared 66% amino-acid sequence homology. As a result, truncated sequences were designed and synthesised to identify the active region of the peptides. All truncated peptides were significantly neuroprotective following kainic acid and glutamate exposure. We have shown for the first time the neuroprotective efficacy of full-length and truncated AP-1 inhibitory peptides in kainic acid and glutamate neuronal excitotoxicity models. The identification of therapeutic targets, such as the AP-1 complex, is an important step for the development of pharmaceuticals to reduce neuronal loss in disorders with a prevalence of excitotoxic cell death such as epilepsy, cerebral ischaemia, and traumatic brain injury.

Crown Copyright © 2010 Published by Elsevier B.V. All rights reserved.

## 1. Introduction

The progressive neuronal cell loss with subsequent epileptic seizures is an area of particular clinical relevance as currently

there is no neuroprotective therapy to inhibit neuronal cell death. In order to study epileptic cell death processes, seizure induced excitotoxicity has been modelled in small animals, such as rats and mice, and in neuronal cultures by kainic acid

\* Corresponding author. Australian Neuromuscular Research Institute, A Block, 4th Floor, QEII Medical Centre, Verdun St, Nedlands WA 6009, Australia. Fax: +61 8 9346 3487.

E-mail address: [meloni@cyllene.uwa.edu.au](mailto:meloni@cyllene.uwa.edu.au) (B.P. Meloni).

Abbreviations: AP-1, activating protein-1; CNQX, 6-cyano-7-nitroquinoxaline; DIV, day *in vitro*; HRP, horseradish peroxidase; JNK, c-Jun N-terminal kinase; JNKI, JNK inhibitor; MTS, 3-(4,5-dimethyliazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; NB, neurobasal; PBS/T, phosphate-buffered saline/Tween; PI, propidium iodide; SDS, sodium dodecyl sulfate

0006-8993/\$ – see front matter. Crown Copyright © 2010 Published by Elsevier B.V. All rights reserved.

doi:10.1016/j.brainres.2010.09.007

administration (Nadler, 1981; Choi et al., 1987; Ben-Ari and Cossart, 2000). Kainic acid induced neuronal death is associated with increased intracellular sodium and calcium (Hoyt et al., 1998; Niebauer and Gruenthal, 1999), activation of MAP kinases (p38 and JNK1) (Jeon et al., 2000), activation and/or increased expression of pro-apoptotic AP-1 transcription factors (c-Jun and c-Fos) (Morgan et al., 1987; Mielke et al., 1999), bax (Liu et al., 2001), proteolytic enzymes (e.g., calpain and caspases) (Gillardon et al., 1997; Schauwecker, 2000; Manno et al., 2007), and cytochrome c release from the mitochondria (Liu et al., 2001).

Despite the identification of cell-damaging processes in seizure induced excitotoxic neuronal injury, there has been no translation into neuroprotective treatments. To this end, a potential therapeutic target that warrants further investigation to inhibit seizure induced neuronal cell death is the AP-1 transcription factor. Previous studies using *in vitro* and *in vivo* kainic acid excitotoxicity models have shown that increased AP-1 activation (c-Jun and/or c-Fos activation/expression) is associated with increased neuronal cell death and that inhibiting activation is associated with decreased neuronal death (Troy et al., 2001; Fahrig et al., 2005; Fernandez et al., 2005; Pugazhenthhi et al., 2006; Wang et al., 2008). Therefore, in this study, we have assessed the potential for inhibition of AP-1 activation to reduce neuronal death in cortical neuronal cultures exposed to kainic acid.

To inhibit AP-1 activation we used five TAT-peptide conjugated AP-1 inhibitory peptides, which 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 previously 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., 2010). With this in mind, we assessed the neuroprotective efficacy of the five AP-1 inhibitory peptides (PYC19D-TAT, PYC35D-TAT, PYC36D-TAT, PYC38D-TAT, and PYC41D-TAT) and the control peptides JNKI-1D-TAT and D-TAT in an *in vitro* model of kainic acid excitotoxicity.

## 2. Results

### 2.1. Neuronal morphology following kainic acid exposure

Acute exposure of neurons to kainic acid induces cell death that is morphologically representative of both necrosis and apoptosis, though with a greater proportion of necrosis (Fig. 1). Light microscopy revealed that from 15 min after the 45-minute kainic acid exposure, the majority of dying neurons appeared swollen and rounded with easily visible shrunken/condensed nuclei (fried egg appearance). These are generally accepted as indicators of necrosis. The proportion of neurons with PI staining nuclei increased slightly after the 15-minute to 2-hour time points following kainic acid exposure. At the 3-hour time point, over 50% of cells displayed PI stained nuclei, with the majority displaying condensed nuclei, and a small number displaying pyknotic or fragmented nuclei. Nuclear

pyknosis and fragmentation are generally regarded as morphological markers of apoptosis.

### 2.2. Neuroprotective efficacy of AP-1 inhibitory peptides following kainic acid exposure

All five AP-1 inhibitory peptides (PYC19D-TAT, PYC35D-TAT, PYC36D-TAT, PYC38D-TAT, and PYC41D-TAT) and the JNK inhibitory peptide (JNKI-1D-TAT) showed significant neuroprotection, in a concentration-dependent manner with a range of protection from 35% to 75% (Table 1 and Fig. 2). Peptide PYC36D-TAT presented the greatest efficacy and potency with an  $IC_{50}$  value of 5.1  $\mu$ M (Table 1). The TAT-D peptide did not provide neuroprotection from kainic acid exposure (Fig. 2).

### 2.3. Sequence homology of AP-1 inhibitory peptides and peptide truncation

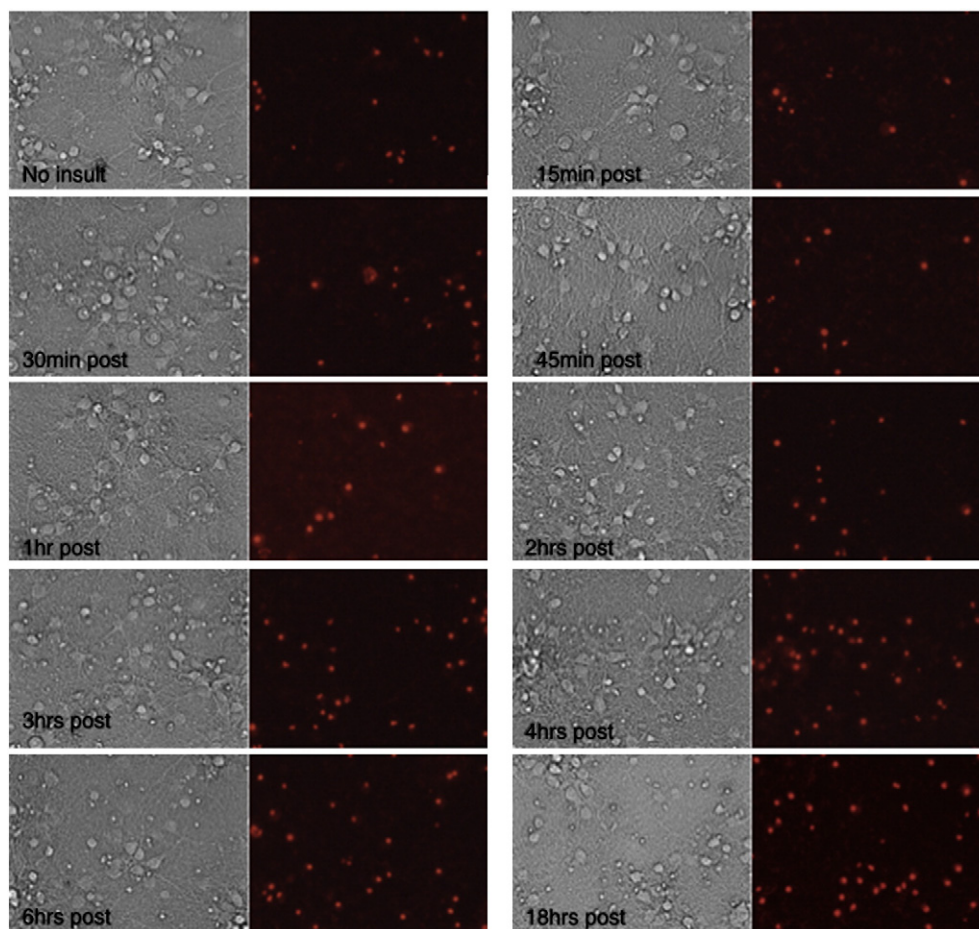
Algorithms to predict potential structures from the AP-1 inhibitory peptide amino-acid sequences did not reveal evidence of leucine zippers, coils or areas of significant hydrophobicity. Sequence alignment of the five AP-1 inhibitory peptides revealed homology evident between peptides PYC36D-TAT and PYC38D-TAT (Fig. 3). Peptides PYC36 and PYC38, which are 15 and 38 amino acids in length respectively, shared identical amino-acid residues at eight positions and amino acids with similar properties at two positions, such as the hydrophilic residues arginine and glutamine, or the polar uncharged amino-acid residues serine and proline. Due to the high level of amino-acid sequence similarity (~66%) between the two peptides, and the fact that PYC36D-TAT is 10 residues shorter than PYC38D-TAT and still neuroprotective, eight truncated consensus peptides were synthesised (AM1D-TAT to AM8D-TAT; Fig. 3 and Table 1) from the two peptides. These 8 smaller peptides were subsequently evaluated in both the kainic acid and glutamate excitotoxicity models.

### 2.4. Neuroprotective efficacy of truncated AP-1 inhibitory peptides following kainic acid and glutamate exposure

All eight truncated peptides provided neuroprotection following kainic acid ranging from 35% to 70%, and glutamate exposure ranging from 44% to 92% (Table 1, Figs. 2 and 4). In the kainic acid exposure model,  $IC_{50}$  values ranged from 1.7  $\mu$ M for AM6D-TAT to >15  $\mu$ M for peptides AM2D-TAT and AM5D-TAT. In the glutamate exposure model,  $IC_{50}$  values ranged from 0.8  $\mu$ M for AM3D-TAT to 9.6  $\mu$ M for peptide AM7D-TAT. As we have reported previously, the TAT-D peptide at high concentrations (>5  $\mu$ M) provided significant neuroprotection following glutamate exposure.

### 2.5. Western blot analysis of $\alpha$ -fodrin cleavage following kainic acid exposure

The activation of calpain and caspases in the kainic acid model was determined by Western analysis of  $\alpha$ -fodrin cleavage products; calpain generates 150 and 145 kDa products whilst caspases generate 150 kDa and 120 kDa products. Following the exposure of neuronal cultures to



**Fig. 1 – Time course photomicrographs of neurons following kainic acid exposure (200  $\mu$ M for 45 min) under light and fluorescence microscopy; morphological features and propidium iodide staining of dying neurons suggest the occurrence of both necrotic and apoptotic forms of cell death. The time points stated commence at the conclusion of exposure to kainic acid.**

kainic acid, Western analysis revealed 150, 145 and 120 kDa  $\alpha$ -fodrin cleavage products signifying activated calpain and caspases (Fig. 5). The one-hour post-kainic acid exposure time point was chosen to assess whether the five full-length and eight truncated AP-1 inhibitory peptides could reduce calpain and caspase  $\alpha$ -fodrin cleavage products. Western analysis suggested that all the truncated AP-1 inhibitory peptides, except AM2D-TAT reduced  $\alpha$ -fodrin caspase cleavage (reduced 120 kDa fragment); however statistical significance was only reached for AM1D-TAT, when data from multiple blots was combined. There was no reduction in calpain generated  $\alpha$ -fodrin cleavage products by any of the AP-1 inhibitory peptides following kainic acid exposure (Fig. 5).

### 3. Discussion

In the present study, we have shown that five AP-1 inhibitory peptides (PYC19D-TAT, PYC35D-TAT, PYC36D-TAT, PYC38D-TAT, and PYC41D-TAT) reduced neuronal death in a kainic acid excitotoxicity model. In a previous

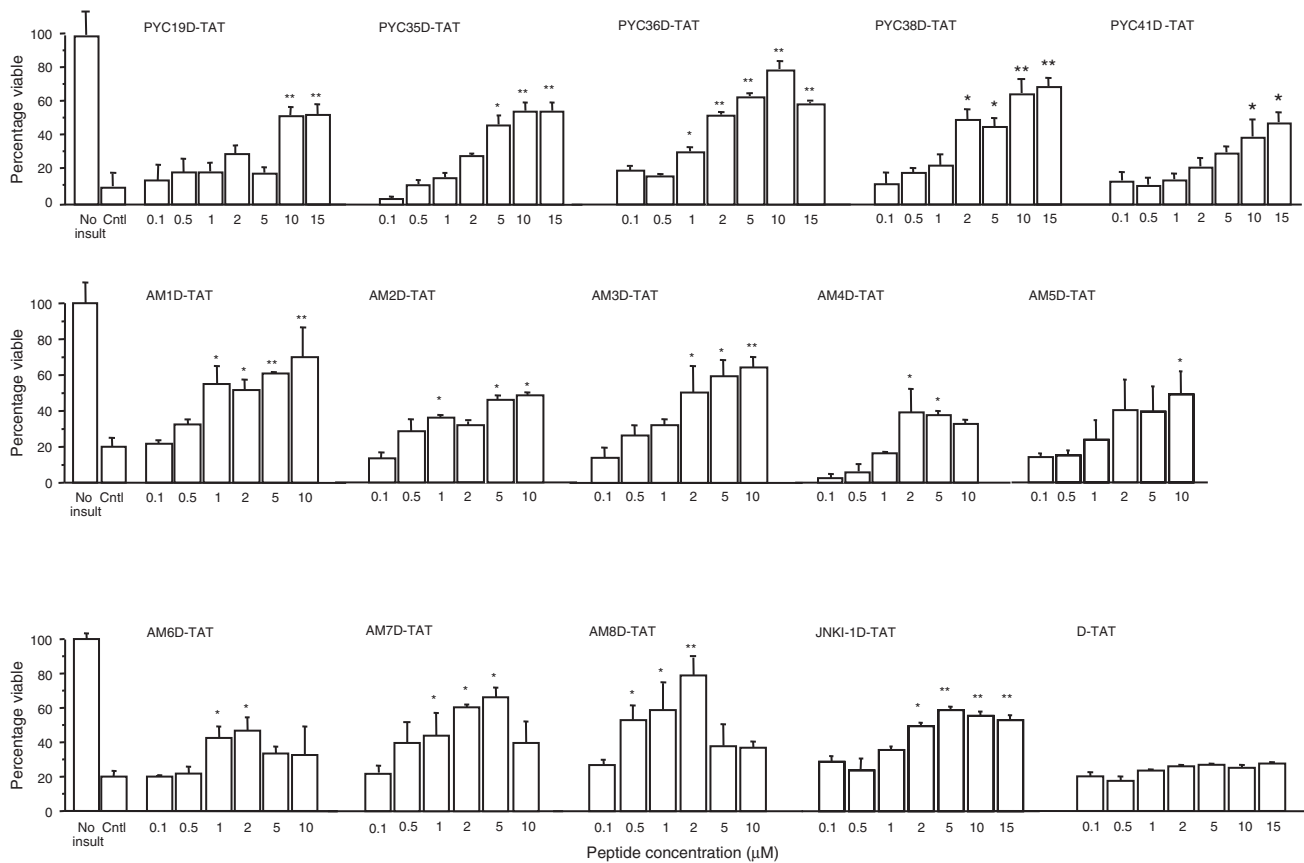
study we showed that the same five peptides can also reduce neuronal death in glutamate excitotoxicity model (Meade et al., 2010), with peptides PYC36D-TAT and PYC38D-TAT providing the greatest neuroprotection against glutamate excitotoxicity, akin to the results seen here with kainic acid. In addition, in this study, we have presented eight truncated peptides derived PYC36D-TAT and PYC38D-TAT that reduce neuronal death in both the kainic acid and glutamate excitotoxicity models.

Whilst it is becoming clearer in the current literature (Blomgren et al., 2007; Degterev and Yuan, 2008; Gill and Perez-Polo, 2008), and from our own observations, that both models are not restricted to a single mode of cell death, there does appear to be a bias towards the necrotic form of cell death in these models our past and current findings show a neuroprotective effect with AP-1 inhibitory peptides in the kainic acid and glutamate models correlating with the ability of these AP-1 inhibitory peptides to significantly down-regulate c-Jun mRNA (Meade et al., 2010), thus indicating that the peptides appear to be blocking AP-1 mediated necrotic and apoptotic cell death pathways.

**Table 1 – IC<sub>50</sub> and percentage neuronal viability at most effective dose for of AP-1 inhibitory, truncated, JNK1-ID-TAT and D-TAT peptides in kainic acid and glutamate excitotoxicity models. Amino- acid sequence that corresponds to TAT transduction domain is in bold with a glycine linker between the peptide and TAT. Underlined amino-acid letter code indicates D-isoform of the amino acid.**

Peptide identification	Amino-acid sequence	Derivative	Kainic acid exposure		Glutamate exposure	
			IC <sub>50</sub> (μM) (95% CI)	Viability (±SE): at the most effective dose	IC <sub>50</sub> (μM) (95% CI)	Viability (±SE): at the most effective dose
PYC19D-TAT	H-YIYPYAYSQNILGRRRQRRKRG-OH	Phylomer	>15 (11.4–98.0)	53% (±5.9): 15 μM	2.6 (1.9–3.5)	76% (±5.7): 10 μM
PYC35D-TAT	H-RERKSSSEIGGSRISQYAGRRRQRRKRG-NH2	Phylomer	6.7 (5.1–8.9)	56% (±4.4%): 10 μM	8.5 (5.5–13.1)	56% (±4.4): 10 μM
PYC36D-TAT	H-PKISQYQRRRQQLGRRRQRRKRG-NH2	Phylomer	5.1 (4.3–6.2)	75% (±5.8%): 10 μM	1.1 (0.7–1.5)	82% (±2.6): 5 μM
PYC38D-TAT	H-RHAPLARGSWRGPOQPQRRRQRRKRG-OH	Phylomer	9.7 (5.6–16.7)	68% (±4.1%): 15 μM	2.2 (1.4–3.4)	83% (±1.3): 5 μM
PYC41D-TAT	H-LPLLRRHHEQNIISVGRRRRQRRKRG-OH	Phylomer	>15 (14.9 to >100)	47% (±4.4%): 15 μM	>15 (8.3–41.8)	41% (±5.5): 10 μM
<i>Truncated peptides</i>						
AM1D-TAT	H-GORRRQQLGRRRQRRKRG-NH2	PYC36D-TAT	3.8 (1.9–7.6)	70% (±16.3%): 10 μM	1.1 (0.8–1.4)	88% (±3.7): 5 μM
AM2D-TAT	H-PGQPQPQRRRQQLGRRRQRRKRG-NH2	PYC36D-TAT and PYC38D-TAT	>15 (16.9 to >100)	48% (±1.9%): 10 μM	4.8 (3.8–6.0)	62% (±3.9): 10 μM
AM3D-TAT	H-PKISQYGRRRQRRKRG-NH2	PYC36D-TAT	6.2 (2.9–12.9)	65% (±6.1%): 10 μM	0.8 (0.51–1.3)	92% (±7.8): 15 μM
AM4D-TAT	H-RHAPLARGSWRGPOQPQRRRQRRKRG-NH2	PYC38D-TAT	13.9 (3.7–52.6)	40% (±13.3%): 2 μM	1.1 (0.6–2.2)	82% (±9.0): 15 μM
AM5D-TAT	H-GQLGRRRQRRKRG-NH2	AM1D-TAT and AM2D-TAT	>15 (0.8 to >100)	37% (±15.3%): 2 μM	1.4 (0.4–4.5)	81% (±24.0): 15 μM
AM6D-TAT	H-GQRRRQRRRQRRKRG-NH2	AM1D-TAT and AM2D-TAT	1.7 (1.1–2.8)	65% (±1.0%): 2 μM	2.4 (1.0–5.6)	65% (±5.1): 10 μM
AM7D-TAT	H-SQYGRRRQRRKRG-NH2	AM3D-TAT	3.4 (0.4–29.0)	61% (±24.7%): 2 μM	9.6 (5.0–18.5)	73% (±9.1): 15 μM
AM8D-TAT	H-PKIGRRRQRRKRG-NH2	AM3D-TAT	2.2 (0.8–5.8)	68% (±7.6%): 2 μM	1.1 (0.7–1.6)	88% (±6.5): 10 μM
<i>Control peptides</i>						
JNK1-ID-TAT	H-TDQSRVQPFLLNLTTTPRKPPRRRQRRKRG-NH2	JNK interacting protein	6.5 (4.2–9.8)	52% (±2.3%): 5 μM	2.1 (1.7–2.7)	81% (±1.2): 10 μM
D-TAT <sup>a</sup>	H-RRRQRRKRG-NH2	HIV-Tat	N/A	N/A	>15 (N/A)	25% (±4.4): 10 μM

<sup>a</sup> N/A=as D-TAT was either ineffective (kainic acid) or only mildly effective (glutamate) values are not applicable.



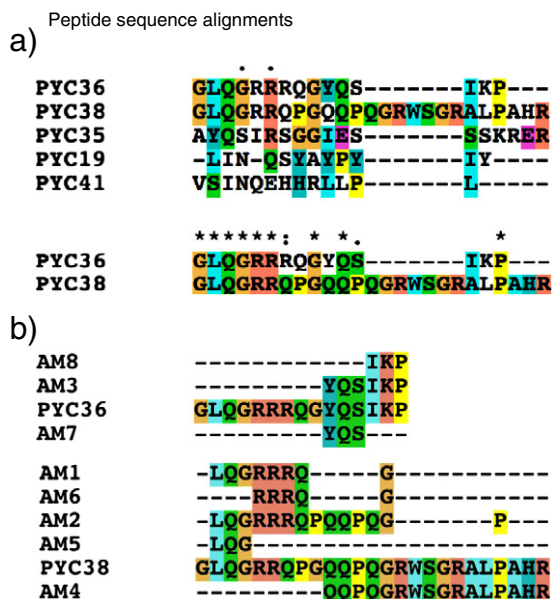
**Fig. 2 – Concentration response graphs for primary cortical neurons administered with AP-1 inhibitory peptides (PYC19D-TAT, PYC35D-TAT, PYC36D-TAT, PYC38D-TAT, and PYC41D-TAT), truncated peptides (AM1D-TAT, AM2D-TAT, AM3D-TAT, AM4D-TAT, AM5D-TAT, AM6D-TAT, AM7D-TAT, and AM8D-TAT), or peptide controls D-TAT and JNKI-1D-TAT, following kainic acid exposure (200  $\mu$ M for 45 min). MTS data was taken 18 h post-kainic acid and was expressed as percentage neuronal viability with no insult control taken as 100% viability (mean  $\pm$  SEM; n = 4; \*p < 0.05; \*\*p < 0.0001).**

Our findings showing that both calpain and caspases are activated in neuronal cultures following exposure to kainic acid is in line with previous studies (Bi et al., 1996; Liu et al., 2001). Interestingly, in the present study we observed that following kainic acid exposure, the truncated AP-1 inhibitory peptides reduced caspase activation, but there was no visible reduction in calpain activation. This is in contrast to our previous study in which the AP-1 peptides reduced glutamate induced calpain activation in neuronal cultures. The differential inhibition of caspases and calpain by the AP-1 inhibitory peptides across the two models of neuronal excitotoxicity was unexpected. This disparity may be due to the activation of calpain following kainic acid by a pathway independent of c-Jun. Whilst beyond the scope of this study, this may warrant further investigation.

The difference in activated cellular pathways between models, even though the cell death modes appear similar, may in part explain the overall increased neuroprotective capabilities of the AP-1 inhibitory peptides in the glutamate excitotoxicity model over that of the kainic acid excitotoxicity model. In both the kainic acid and glutamate excitotoxicity models, the full-length AP-1 inhibitory peptide PYC36D-TAT had the greatest efficacy and potency, and PYC41D-TAT was the least efficacious and potent (Table 1). The efficacy of the other peptides (PYC19D-TAT,

PYC35D-TAT, PYC38D-TAT, and JNKI-1D-TAT) in the two models was less consistent. For example, PYC19D-TAT and JNKI-1D-TAT were highly potent in the glutamate model ( $IC_{50}$ : 2.6 and 2.1  $\mu$ M respectively), but only mildly so in the kainic acid model ( $IC_{50}$ : >15  $\mu$ M and 6.5  $\mu$ M respectively). With respect to the truncated peptides, a similar pattern of efficacy and potency was observed, with some peptides being highly effective in both the kainic acid and glutamate models (AM1D-TAT and AM8D-TAT), whilst others displayed differential efficacy in the two models (AM2D-TAT and AM7D-TAT).

The potent neuroprotective efficacy of some of the truncated peptides was surprising as several consisted of only 3–6 amino acids conjugated to the TAT peptide (AM3D-TAT, AM5D-TAT, AM6D-TAT, AM7D-TAT, and AM8D-TAT). In some cases, the truncated peptides provided a 3–4 fold greater efficacy than that of the derived full-length peptide (AM8D-TAT). Whilst we and others (Wei et al., 2008; Vaslin et al., 2009) have shown that the TAT peptide has endogenous neuroprotective activity following glutamate/NMDA excitotoxicity, and therefore may be responsible for some of the neuroprotection demonstrated in these models by the AP-1 inhibitory peptides, particularly in the glutamate excitotoxicity model, it is unlikely that the neuroprotective effects observed with the truncated peptides (and full-



**Fig. 3** – Clustal X alignment of a) AP-1 inhibitory peptide sequences and b) truncated AP-1 inhibitory sequences against PYC36D-TAT or PYC38D-TAT, without inclusion of the TAT sequence. Legend: colours indicate amino-acid residue properties, hydrophobic are blue, hydrophobic tendency are light blue, hydrophilic are green, basic are red, acidic are purple, glycine is orange, and proline is yellow (Thompson et al., 1994); ‘\*’ indicates position containing fully conserved residues, ‘:’ indicates position containing strong group of amino acids, and ‘.’ indicates position containing weak group of amino acids.

length peptides) following kainic acid exposure are entirely associated with TAT. We have shown that the control neuronal cultures treated with the TAT peptide (0.1–15 μM; Fig. 2) and exposed to kainic acid did not display any significant increase in neuronal viability. However, it is possible that some of the AP-1 inhibitory peptides and/or truncated peptides may be enhancing the neuroprotective efficacy of TAT in the glutamate model, especially in regard to the peptides which appear to be an extension of the TAT amino-acid sequence (AM6: GQRRR; TAT: RRRQRRKKRG).

It is likely that the diversity in neuroprotective efficacy in the full-length and truncated AP-1 inhibitory peptides is due to differences in one or more of the following: i) peptide c-Jun binding affinity, ii) peptide half-life/susceptibility to intracellular proteases, or iii) cellular/nuclear uptake. With respect to binding affinity, as the amino-sequence varies between the peptides it is likely that their affinity to c-Jun will differ. In addition, AP-1 activation involves both c-Jun homodimerisation, and c-Jun heterodimerisation with c-Fos and ATF (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 c-Jun protein binding to c-Fos or ATF, and upon its ability to displace c-Jun homo- and heterodimers.

In conclusion, effective screening of potential neuroprotective therapeutics is enhanced if evaluation occurs in

multiple cell death models relevant to the disorder being targeted. In this study, we have shown for the first time the neuroprotective efficacy of full-length and truncated AP-1 inhibitory peptides in kainic acid and glutamate neuronal excitotoxicity models. Hence, the identification of therapeutic targets, such as these AP-1 inhibitory peptides, is an important step for the development of pharmaceuticals to reduce neuronal loss and improve neurological outcome in patients following acute brain injuries such as epilepsy, stroke, and traumatic brain injury.

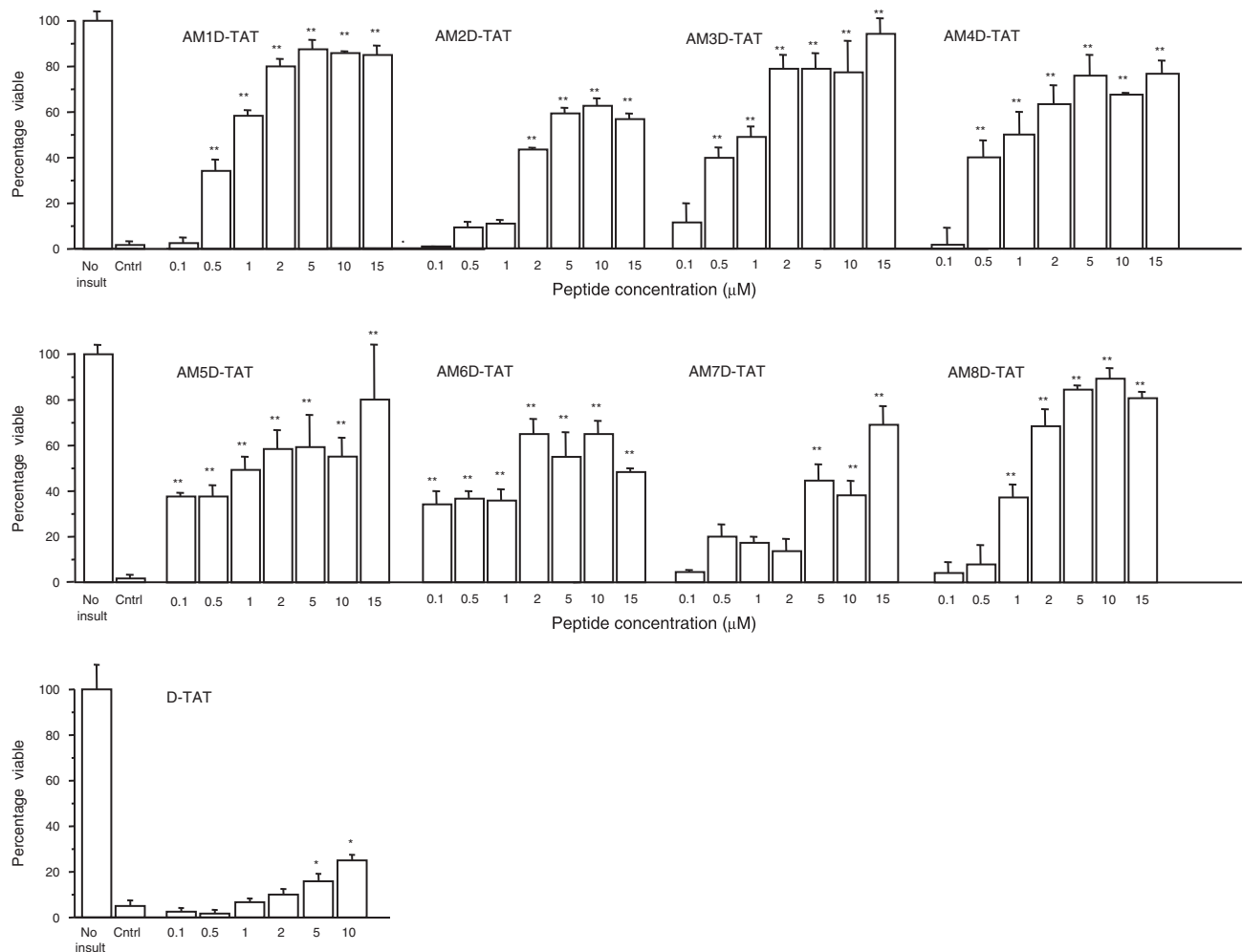
## 4. Experimental procedures

### 4.1. Primary neuronal cortical cultures

Establishment of cortical cultures was as previously described (Meloni et al., 2001). 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 NaHCO<sub>3</sub>, 10 U/mL papain (Sigma, USA) and 50 U/mL DNase (Sigma) and washed in cold DMEM/10% horse serum. Neurons were resuspended in Neurobasal (NB; Invitrogen) containing 2% B27 supplement (B27; Invitrogen). Before seeding 96-well plastic plates (ProSci-Tech, Australia) were coated with poly-D-lysine (50 μg/mL; 70–150 K; Sigma) and incubated overnight at room temperature. Excess poly-D-lysine solution was then removed and replaced with 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 for each well of a 96-well plate on days *in vitro* (DIV) 11–12. Neuronal cultures were maintained in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 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 after which time they routinely consist of >97% neurons and 1–3% astrocytes (Meloni et al., 2001).

### 4.2. Kainic acid and glutamate excitotoxicity models

For comparative purposes a glutamate excitotoxicity model was also used for some experiments. To induce excitotoxicity in the cortical neuronal cultures (96-well plate format) 50 μL of conditioned media (media in which the neurons were previously maintained) containing kainic acid (Sigma) or L-glutamate (Sigma) was added to culture wells containing 50 μL of conditioned media (200 μM final kainic acid concentration and 100 μM final L-glutamate concentration). Cultures were incubated at 37 °C in the CO<sub>2</sub> incubator for 45 min for kainic acid, and 5 min for glutamate, after which time the media was replaced with 100 μL of 50% NB/2% N2 and 50% balance salt solution (NB/N2:BSS). Peptides were added to wells 15 min prior to kainic acid or glutamate exposure. A non-peptide positive control consisting of the NMDA and non-NMDA receptor blockers 5 μM MK801/5 μM 6-cyano-7-nitroquinoxaline (MK801/CNQX) were added in



**Fig. 4 – Concentration response graphs for primary cortical neurons administered with truncated AP-1 inhibitory peptides (AM1D-TAT, AM2D-TAT, AM3D-TAT, AM4D-TAT, AM5D-TAT, AM6D-TAT, AM7D-TAT, and AM8D-TAT), and peptide control D-TAT, following glutamate exposure (100 μM for 5 min). MTS data was taken 18 h post-glutamate exposure and was expressed as percentage neuronal viability with no insult control taken as 100% viability (mean ± SEM; n=4; \*p<0.05; \*\*p<0.0001).**

a similar manner to the peptides prior to kainic acid or glutamate exposure. The kainic acid and glutamate treated controls and untreated controls received media additions with and without kainic acid or glutamate respectively.

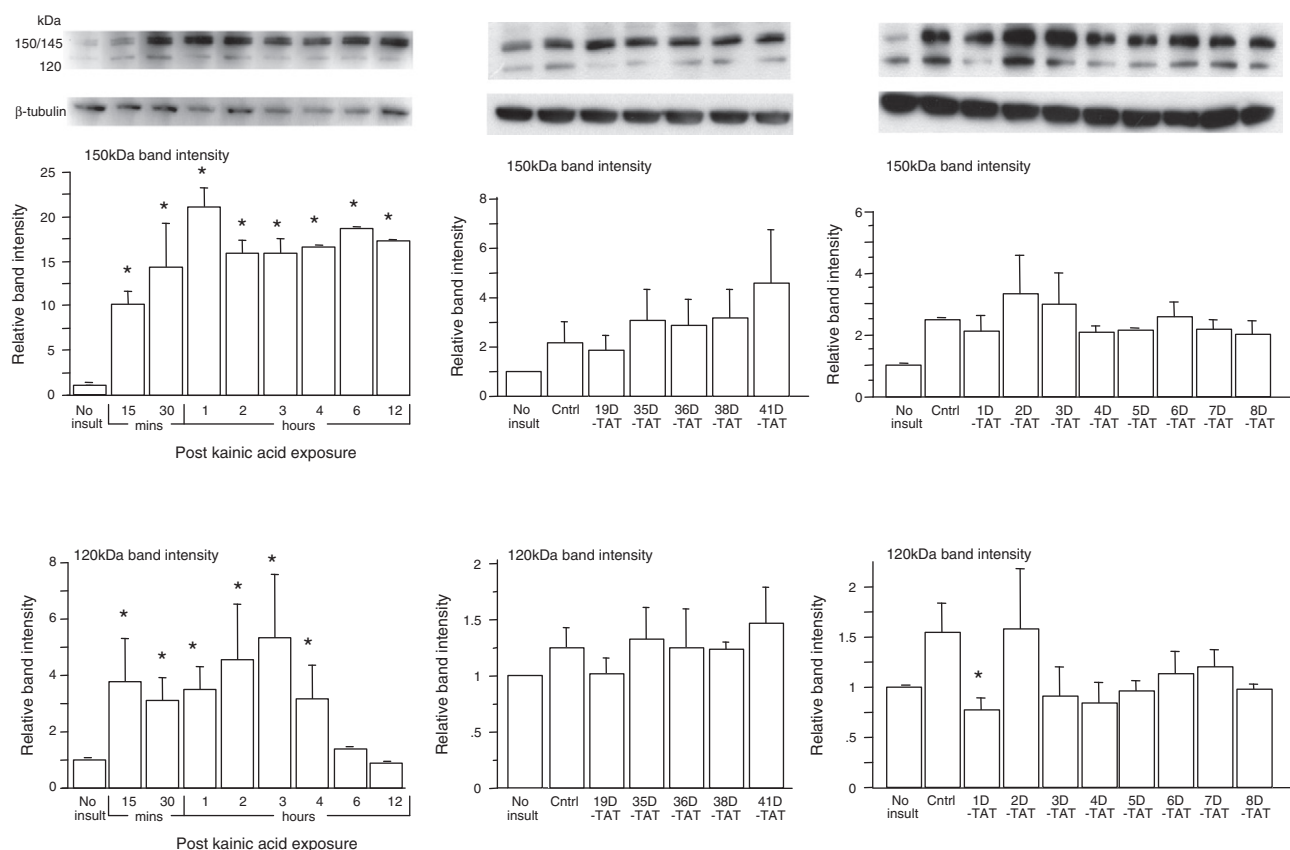
#### 4.3. AP-1 inhibitory peptides and control peptides

The peptides were synthesised and HPLC purified (<95% purity) 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) (Brugidou et al., 1995) (Table 1) (Borsello et al., 2003) amino terminus to allow peptides to enter cells (Vives et al., 1997; Patel et al., 2007). A TAT-fused JNK inhibitory peptide (JNKI-1D-TAT) in the D-isomer was synthesised (Borsello et al., 2003) and evaluated, and used as a peptide positive control. The TAT peptide (D-TAT) was also synthesised in the D-isomer 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 15 μM. Peptide sequences were analysed to determine homology using the multiple alignment program

Clustal X (Larkin et al., 2007); primary and secondary structure determination was performed using 2ZIP (Bornberg-Bauer et al., 1998) and Jpred (Cole et al., 2008).

#### 4.4. Neuronal viability and statistical analysis

Eighteen hours after kainic acid or glutamate exposure, neuronal cultures were examined by light microscopy for qualitative assessment of neuronal cell viability. Neuronal viability was quantitatively measured by 3-(4,5-dimethyliazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (Promega, Australia). The MTS assay measures the mitochondrial conversion of the tetrazolium salt to a water-soluble brown formazan salt, which is detected spectrophotometrically at 490 nm. MTS absorbance data were converted to reflect proportional cell viability relative to both the untreated and kainic acid treated 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. IC<sub>50</sub> values were determined from



**Fig. 5 – Western blot analysis of  $\alpha$ -fodrin cleavage in primary cortical neurons a) following exposure to kainic acid in a time response; b) 1 h following kainic acid exposure with the addition of AP-1 inhibitory peptides (5  $\mu$ M); c) 1 h following kainic acid exposure with the addition of truncated AP-1 inhibitory peptides (5  $\mu$ M). Cleavage of  $\alpha$ -fodrin by caspases results in bands at 150 and 120 kDa, and by calpain results in bands at 150 and 145 kDa. Blots presented are single representative blots, whilst graphs are derived from the averaged band intensities for all blots performed. Bands for protein of interest are normalised against  $\beta$ -tubulin (mean  $\pm$  SEM;  $n=3$ ; \* $p<0.05$ ).**

concentration response curves, where the concentration of the peptide enabled neuronal viability to reach 60% for kainic acid and 52% for glutamate insults. These percentages were determined as being half way between maximal cell viability (no insult control) and minimal cell viability (kainic acid/glutamate control). All assays were performed in quadruplicate with sister neuronal cultures and repeated a minimum of three times independently.

For the morphological time-course, 15 min to 18 h following kainic acid exposure, propidium iodide (PI) staining (0.05  $\mu$ g/mL final concentration) was added 15 min prior to visualisation by fluorescence microscopy. Image acquisition was performed using an Olympus IX70 inverted microscope fitted with a digital camera (DP70; Olympus) under software control (DP controller; Olympus).

#### 4.5. Western blot analysis for $\alpha$ -fodrin cleavage following kainic acid exposure

For protein extraction, cultured cells were lysed in buffer [50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), 0.2% deoxycholic acid, containing Complete protein inhibitor (Roche, USA)], vortexed briefly,

and clarified by centrifugation at 4  $^{\circ}$ C. Protein concentrations were determined by the Bradford assay (Bio-Rad). Equivalent amounts of protein (5–10  $\mu$ g per lane) were loaded and separated on 10% SDS-polyacrylamide Bis-Tris minigels, (Invitrogen) and transferred to PVDF membrane. Membranes were blocked in phosphate-buffered saline/0.5% Tween 20 (PBS/T) containing 5% skim milk for 1 h at room temperature before washing in PBS/T and PBS. Membranes were incubated at 4  $^{\circ}$ C overnight in PBS/T containing 1 mg/mL ovalbumin and primary antibody, and incubated in blocking solution containing horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Protein bands were detected using ECL Plus (Amersham, United Kingdom), visualized by exposure to X-ray film (Hyperfilm; Amersham), and scanned and quantified in Image J. As required, membranes were incubated for 10 min at room temperature in stripping solution (Chemicon, USA) prior to immunodetection of control proteins. Primary antibodies used were mouse monoclonal anti- $\alpha$ -fodrin (1:500; MP Biomedicals, USA) antibody and mouse monoclonal anti- $\beta$ -tubulin (1:1000; BD Pharmingen, USA). Secondary antibody was sheep anti-mouse IgG (1:10,000; Amersham). These experiments were repeated three times independently and data analysed by ANOVA, followed by post-

hoc Fischer's PLSD test, with  $p < 0.05$  values considered statistically significant, and presented as mean  $\pm$  SEM.

## Disclosure

Authors A and D have received support from Phylogica, however Phylogica was not involved in either the experimental design or the analysis of data arising from this study. The remaining authors have no conflicts of interest. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

## Acknowledgments

The authors would like to thank Dr Nadia Milech, Ms Kate Thomas, Ms Joanne Chieng and Mr Tom Hamilton for their technical support and Dr Kym Campbell for expert editing. This work was supported by a Neurotrauma Research Project Grant, The University of Western Australia and Phylogica Ltd.

## REFERENCES

- Ben-Ari, Y., Cossart, R., 2000. Kainate, a double agent that generates seizures: two decades of progress. *Trends Neurosci.* 23 (11), 580–587.
- Bi, X., Chang, V., et al., 1996. Regional distribution and time-course of calpain activation following kainate-induced seizure activity in adult rat brain. *Brain Res.* 726 (1–2), 98–108.
- Blomgren, K., Leist, M., et al., 2007. Pathological apoptosis in the developing brain. *Apoptosis* 12 (5), 993–1010.
- Bornberg-Bauer, E., Rivals, E., et al., 1998. Computational approaches to identify leucine zippers. *Nucleic Acids Res.* 26 (11), 2740–2746.
- Borsello, T., Clarke, P.G., et al., 2003. A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat. Med.* 9 (9), 1180–1186.
- Brugidou, J., Legrand, C., et al., 1995. The retro-inverso form of a homeobox-derived short peptide is rapidly internalised by cultured neurones: a new basis for an efficient intracellular delivery system. *Biochem. Biophys. Res. Commun.* 214 (2), 685–693.
- Choi, D.W., Maulucci-Gedde, M., et al., 1987. Glutamate neurotoxicity in cortical cell culture. *J. Neurosci.* 7 (2), 357–368.
- Cole, C., Barber, J.D., et al., 2008. The Jpred 3 secondary structure prediction server. *Nucleic Acids Res.* 36 (Web Server issue), W197–W201.
- Degterev, A., Yuan, J., 2008. Expansion and evolution of cell death programmes. *Nat. Rev. Mol. Cell Biol.* 9 (5), 378–390.
- Fahrig, T., Gerlach, I., et al., 2005. A synthetic derivative of the natural product rocaglaol is a potent inhibitor of cytokine-mediated signaling and shows neuroprotective activity *in vitro* and in animal models of Parkinson's disease and traumatic brain injury. *Mol. Pharmacol.* 67 (5), 1544–1555.
- Fernandez, M., Pirondi, S., et al., 2005. Role of c-Fos protein on glutamate toxicity in primary neural hippocampal cells. *J. Neurosci. Res.* 82 (1), 115–125.
- Gill, M.B., Perez-Polo, J.R., 2008. Hypoxia ischemia-mediated cell death in neonatal rat brain. *Neurochem. Res.* 33 (12), 2379–2389.
- Gillardon, F., Bottiger, B., et al., 1997. Activation of CPP-32 protease in hippocampal neurons following ischemia and epilepsy. *Brain Res. Mol. Brain Res.* 50 (1–2), 16–22.
- Hoyt, K.R., Stout, A.K., et al., 1998. The role of intracellular Na<sup>+</sup> and mitochondria in buffering of kainate-induced intracellular free Ca<sup>2+</sup> changes in rat forebrain neurones. *J. Physiol.* 509 (Pt 1), 103–116.
- Jeon, S.H., Kim, Y.S., et al., 2000. Activation of JNK and p38 in rat hippocampus after kainic acid induced seizure. *Exp. Mol. Med.* 32 (4), 227–230.
- Larkin, M.A., Blackshields, G., et al., 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23 (21), 2947–2948.
- Liu, W., Liu, R., et al., 2001. Kainate excitotoxicity in organotypic hippocampal slice cultures: evidence for multiple apoptotic pathways. *Brain Res.* 916 (1–2), 239–248.
- Manno, I., Antonucci, F., et al., 2007. BoNT/E prevents seizure-induced activation of caspase 3 in the rat hippocampus. *NeuroReport* 18 (4), 373–376.
- Meade, A.J., Meloni, B.P., et al., 2010. AP-1 inhibitory peptides are neuroprotective following acute glutamate excitotoxicity in primary cortical neuronal cultures. *J. Neurochem.* 112 (1), 258–270.
- Meloni, B.P., Majda, B.T., et al., 2001. Establishment of neuronal *in vitro* models of ischemia in 96-well microtiter strip-plates that result in acute, progressive and delayed neuronal death. *Neuroscience* 108 (1), 17–26.
- Mielke, K., Brecht, S., et al., 1999. Activity and expression of JNK1, p38 and ERK kinases, c-Jun N-terminal phosphorylation, and c-jun promoter binding in the adult rat brain following kainate-induced seizures. *Neuroscience* 91 (2), 471–483.
- Morgan, J.I., Cohen, D.R., et al., 1987. Mapping patterns of c-fos expression in the central nervous system after seizure. *Science* 237 (4811), 192–197.
- Nadler, J.V., 1981. Minireview. Kainic acid as a tool for the study of temporal lobe epilepsy. *Life Sci.* 29 (20), 2031–2042.
- Niebauer, M., Gruenthal, M., 1999. Neuroprotective effects of early vs. late administration of dantrolene in experimental status epilepticus. *Neuropharmacology* 38 (9), 1343–1348.
- Patel, L.N., Zaro, J.L., et al., 2007. Cell penetrating peptides: intracellular pathways and pharmaceutical perspectives. *Pharm. Res.* 24 (11), 1977–1992 Epub 2007 Apr 1919.
- Pugazhenthii, S., Phansalkar, K., et al., 2006. Differential regulation of c-jun and CREB by acrolein and 4-hydroxynonenal. *Free Radic. Biol. Med.* 40 (1), 21–34.
- Schauwecker, P.E., 2000. Seizure-induced neuronal death is associated with induction of c-Jun N-terminal kinase and is dependent on genetic background. *Brain Res.* 884 (1–2), 116–128.
- Smeal, T., Angel, P., et al., 1989. Different requirements for formation of Jun: Jun and Jun: Fos complexes. *Genes Dev.* 3 (12B), 2091–2100.
- Thompson, J.D., Higgins, D.G., et al., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22 (22), 4673–4680.
- Troy, C.M., Rabacchi, S.A., et al., 2001. beta-Amyloid-induced neuronal apoptosis requires c-Jun N-terminal kinase activation. *J. Neurochem.* 77 (1), 157–164.
- Vaslin, A., Rummel, C., et al., 2009. Unconjugated TAT carrier peptide protects against excitotoxicity. *Neurotox. Res.* 15 (2), 123–126.
- Vives, E., Brodin, P., et al., 1997. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* 272 (25), 16010–16017.
- Wang, L., Jing, W., et al., 2008. Glutamate-induced c-Jun expression in neuronal PC12 cells: the effects of ketamine and propofol. *J. Neurosurg. Anesthesiol.* 20 (2), 124–130.
- Wei, X., Miou, Z., et al., 2008. Neuroprotection by cell permeable TAT-mGluR1 peptide in ischemia: synergy between carrier and cargo sequences. *Neuroscientist* 14 (5), 409–414.