

## Cyclisation of Cell-Penetrating PDZ-Binding Peptides Directed to PSD95

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**Abstract:** Excitatory glutamatergic neurotransmission via N-methyl-D-aspartate receptor (NMDAR) is critical for synaptic plasticity and survival of neurons. Excessive NMDAR activity, however can cause excitotoxicity and promote cell death, underlying a potential mechanism of neurodegeneration occurring in Alzheimer's disease (AD). The regulation of calcium levels at synapses is important for pre- and post-synaptic activation in the hippocampus, where glutamate is the primary neurotransmitter, acting via metabotropic and ionotropic receptors.

### INTRODUCTION

Many biological activities are regulated through the dynamic interactions of modular protein domains (e.g., WW, SH3, SH2, PH, and PDZ) and their corresponding binding partners. Elucidation of the specificity, selectivity, and regulatory mechanisms involved in these protein-protein interactions can provide important insights into biological processes such as cell proliferation, cell polarity and cell signalling

PDZ domains are abundant protein-protein interaction modules found in various species. In the mouse genome, for example, 928 PDZ domains have been recognized in 328 proteins. PDZ domains exist in single or multiple copies or in combination with other interaction modules. From the abundance and diversity of PDZ domains in cells it is apparent that many cellular and biological functions, especially those involving signal transduction, are affected by PDZ-mediated interactions (Lee & Zheng (2010).

Recently, the postsynaptic density-95 (PSD-95/SAP90) family of cytoskeletal proteins (Topinka & Bredt 1998), which includes SAP97, SAP102 and PSD-93 (Chapsyn-110), has been suggested to mediate receptor clustering at excitatory synapses in brain. PSD-95 contains three PDZ domains, an SH3 domain and a guanylate kinase domain, suggesting that it makes a number of interactions with other proteins. Interactions between NMDA receptors and PSD-95, have been identified at several levels. The dynamic regulation of calcium ions at the synapse demands that a finely-controlled system of Ca<sup>2+</sup> transporters and Ca<sup>2+</sup> binding proteins collaborate to allow transient increases of calcium during activation of receptors, whilst over the long-term, a low resting state of Ca<sup>2+</sup> is achieved, by removing Ca<sup>2+</sup> by efflux, or by transporting Ca<sup>2+</sup> to intracellular calcium stores. The possibility arises that plasma membrane Ca<sup>2+</sup> ATPases (PMCA) are responsible for some of this fine control. One of these, PMCA2b is expressed at high levels in cerebellar Purkinje neurons. Reciprocal immunoprecipitation and GST pull-down has shown that PMCA2b interacts with the post-synaptic protein PSD95 via its COOH-terminal PDZ (PSD95/Dlg/ZO-1) binding domain (Garside et al., 2009). PSD-95 interacts with the NR2a subunit via one of its 3 PDZ domains; this leads to the possibility that binding of glutamate to NMDAR may regulate Ca<sup>2+</sup> extrusion via PMCA2b, the interactions being mediated by PSD-95 (Niethammer et al 1998). Each of the 5 protein-binding domains of PSD-95 (3 PDZ domains, an SH3 domain and a guanylate kinase domain) can bind to several other proteins, including ion channel receptors, adhesion and cytoskeleton proteins, and proteins involved in intracellular signalling, including nNOS. An additional scaffold protein NHERF2 also interacts with PMCA2b, via its C-terminus (Demarco & Streher, 2002). SHSY-5Y human neuroblastoma cells have been shown previously to express NMDA receptors, (Naarala et al., 1993; Nair et al., 1996), albeit at low levels (Sun et al),

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and thus make a useful model system for this study. Fernandes, et al. (2007) investigated the role of silencing the PMCA2b in neuronal cells, using RNAi techniques, and observed its effects on Ca<sup>2+</sup> homeostasis and cell viability. Using fluorescence ratio imaging of free intracellular Ca<sup>2+</sup>, they determined that hippocampal neurons with reduced expression of PMCA2b took longer to return to their baseline levels of intracellular Ca<sup>2+</sup>. In addition to this, they identified a greater susceptibility to various stresses, in SH-SY5Y neuroblastoma cells. This study highlights the importance of the PMCA in maintaining intracellular Ca<sup>2+</sup> concentrations, and its potential role in the pathogenesis of many diseases.

Four non-allelic genes code for mammalian PMCA 1, 2, 3, and 4 with additional isoforms arise from alternative RNA splicing. The PMCA-2 variants a and b, differ in the protein sequence C-terminal to the transmembrane domain, and create different C-terminal domains by changes in the translational reading frame. The C-terminal sequence of PMCA2b: IHSLETSL matches the consensus sequence of PDZ-binding ligands E-(T/S)-X-(V/L), where X is any amino acid, and V or L are C-terminal residues. To examine the functional importance of any interaction between PMCA2-b and PSD-95 via its PDZ domain, we synthesized the C-terminal sequence of PMCA2b, as a stabilized cyclic peptide extended with a cell penetration sequence and labelled with an internal biotin marker. PDZ-binding peptide cyclisation followed the strategy of Pischerio et al (2004) who synthesized a cyclic variant of the CRIPT PDZ-binding sequence, and suggested a 10-fold increase in binding affinity as well as increased protease resistance over the linear form. To investigate the role of a PMCA2b-PSD-95 interaction in calcium regulation and cell death, we synthesized the C-terminal sequence of PMCA2b. Pischerio et al (2004) suggested that cyclisation of a PDZ-binding peptide from CRIPT increased binding to the PDZ3 domain of PSD95 and stabilized structures against proteolytic degradation. We investigated whether a C-terminal peptide from PMCA2b would have these properties, investigating the sequence in the cyclized and linear forms.

## MATERIALS AND METHODS

Peptide chemicals were obtained from Novabiochem (Nottingham, UK) and solvents from Rathburn (Glasgow, UK). Thus, the C-terminal biotinylated peptide sequence of PMCA2B, was synthesised on solid phase in the linear form with the addition of a DArg<sub>6</sub> cell penetration sequence (Balhorn et al., 2009) and Lys-εbiotin as a marker to aid visualisation (AcRRRRRRGG (K-Biotin) SLETSL (R3) (MH<sup>+</sup>=2137.5; Calc 2137.52). The purified peptide was characterised by Maldi mass spectrometry. In order to investigate the effects of cyclisation on PDZ-binding peptides, which have previously been described by Pischerio et al (2004), an analogue of the PMCA2b sequence, in which the Ser residue at position -2 was substituted by Lys, was synthesised as a cyclised analogue (R2), incorporating β-alanine as a bridge between positions -2 (Lys) and -4 (Glu) in the sequence. Acetyl-DArg-DArg-DArg-DArg-DArg-Gly-Gly-(Lys-Biotin)-Ser-Leu-c[Glu(βAla) Thr-Lys]-Leu-COOH(R2) (MH<sup>+</sup>=2190.7; Calc 2190.5). R2 was synthesized on Leu-Peg-PS resin. Fmoc residues were coupled in four fold equivalence with with four-fold equivalents of HATU, and removal of temporary α-amino Fmoc protection, was achieved by 20% piperidine in DMF. Fmoc-ε-biotinyl lysine was incorporated at step 6 of the synthesis (position -6) to allow biotin detection of R2 by microscopy. Orthogonally protected FmocLys(ivDde) and FmocGlu (2-PhiPr) were incorporated at steps 2 and 4 of the synthesis. As a control for the cell-penetrating effect of DArg<sub>6</sub> to a cyclic PDZ-binding peptide, a cyclized peptide, without a DArg<sub>6</sub> penetration sequence, viz: Acetyl-(Lys-Biotin) Ser-Leu-c[Glu-(βAla)Thr-Lys]-Leu-COOH (R1) was synthesized. (MH<sup>+</sup>=1162.0; Calc 1157.4).

The N-termini of R2 and R1 were N-acetylated to inhibit proteolytic degradation. To cyclize the peptides, prior to final deprotection and release from resin, the ivDde group of Lys at residue 2 was removed from the peptidyl-resin by treatment with 2% (v/v) hydrazine in DMF for 3x3min. To create a bridge, Fmocβ-Ala was then coupled to the ε NH<sub>2</sub> of Lys<sub>2</sub> with PyBOP. The 2-PhiPr group on the side chain of Glu residue 4 was removed by mild treatment with 1% TFA and 0.5% ethanedithiol in dichloromethane for 4x3min, and the Fmoc group on β-Ala then removed by treatment with 20% piperidine in DMF for two periods of 5 min and 20 min. Final ring closure,

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monitored by the loss of ninhydrin colouration from a small peptidyl-resin samples, was catalysed by treatment with 4 equivalents of PyBOP in 0.9M diisopropylethylamine in DMF over a 24hr period. The peptidyl resin was washed with methanol, followed by ethylene dichloride, dried in vacuo, and then cleaved/deprotected with 98% trifluoroacetic acid containing 1% (v/v) water and 1% triisopropyl silane for 2hrs, filtered off, evaporated and triturated with diethyl ether. The residue was taken up in 0.1% trifluoroacetic acid and purified by HPLC on Vydac C8, with an acetonitrile gradient, characterised by Maldi mass spectrometry (MH=2190.5; calc 2190.42), which showed that that ring closure had been achieved. The choice of PyBOP as a cyclisation reagent was crucial to avoid guanidination (Alberichio et al, 1998).

As a comparison, the C-terminal sequence of NMDA-R2; Leu-Ser-Ser-Ile-Lys-Ser-Asp-Val-COOH, which binds a third PDZ-domain in Psd95, was synthesised as a cyclic, biotinylated analogue with a DArg6 penetration sequence, by a strategy similar to that above, giving: Ac-DArg-DArg-DArg-DArg-DArg-DArgGly-Gly-(K-Biotin)-Leu-Ser-Ser-Ile-c[Lys-( $\beta$ Ala)Ser-Asp]-Val-COOH (H2)

Measured MH was 2350.1. Calc 2349.8

## Flourescence Microscopic Studies of PDZ-Binding Peptides

The interaction of biotinylated PDZ-binding peptides with components of SHSY-5Y human neuroblastoma cells were studied by fluorecence microscopy. The presence of ionotropic, and metabotropic glutamate receptors in SHSY-5Y human neuronal cell lines has been shown by Narrala et al (1993) and Nsair et al, 1996. When confluent in 75cm<sup>2</sup> culture flasks, the SHSY-5Y cells were transferred onto glass slides in Petri dishes and incubated at 37°C in 5% CO<sub>2</sub>/95% O<sub>2</sub> for 24 hours. The media was then removed from the slides which were then washed three times with PBS containing 2% horse serum (PBS-HS) (GIBCO, Invitrogen Paisley, Scotland, UK, 16050).

A 27 $\mu$ M solution of the PDZ-binding peptides in PBS-HS was added to the cell-coated slides. The slides were then incubated at 37°C for two hours. Each slide was then washed three times with PBS-HS, and 10ml of methanol, (BDH Chemicals ltd, Poole, England 101586B), cooled to 4°C, was added to each slide. The cells in Petri dishes were incubated at 4°C for ten minutes. The methanol was subsequently removed and the cells were washed again with PBS-HS.

A 1:500 dilution of anti-PSD-95 rabbit antibody (Abcam, Cambridge, UK, AB18258) diluted to 1ml in PBS, was added to each slide and left for one hour at room temperature. The slides were then washed twice with PBS-HS. Solutions diluted with PBS-HS of 1:160 of FITC anti-rabbit IgG (Sigma, Dorset, UK, F9887) (to localize PSD-95) and 1:500 ExtrAvidin TRITC conjugate (to localize biotinylated PDZ-binding peptides), was added to the slides, which were left at room temperature for twenty minutes. The slides were then washed 3x with PBS-HS, mounted and examined using a Carl Zeiss 240M microscope with fluorescent filters. Internalization of the biotin linked to the PDZ-binding peptides was assessed using a rhodamine filter (550nm), and a GFP filter (495nm) was used to indicate the location of PSD-95.

## Intracellular Calcium

SHSY-5Y human neuroblastoma-glioma cells were grown overnight in a 96-well plate, at a starting density of 40,000 cells per well. After 24 hrs, the medium from each well was removed (to eliminate any sources of baseline fluorecence) and PDZ-binding peptides with or without buffered glutamate were added, for incubation for another 16 hrs. Intracellular calcium was measured using the Fluo-4 NW Calcium Assay Kit (Invitrogen, F36206). 100 $\mu$ l of the probenecid stock solution (250mM) to inhibit extrusion of the reagents out of cells by organic anion transporters in 10ml of HBSS was added to one vial of Fluo-4 NW dye mix, and then 100 $\mu$ l added to each well containing cells, and this was then incubated at 37°C for 30 minutes, then left at room temperature for an addition 30 minutes. Fluorecence was measured at room temperature using a bottom-read filter, for excitation at 494nm and emission at 516nm.

### RESULTS

#### Molecular Modelling

The PDZ domain is a common folded protein interaction domain that recognises and binds specific C-terminal peptides, and some internal sequences, of target proteins. PDZ domains are (1) found in a number of proteins from different species, (2) share high sequence homology, and (3) are typically composed of 80-90 amino acid residues. The domain consists of a central bent five-membered beta-sheet (coloured purple in Figure 1), surrounded by two helices (coloured green). The C-terminal target peptide binds between the C-terminal end of the second beta-strand and the top alpha-helix.

The C-terminal pentapeptide sequence of PMCa2b in a cyclic form with the sequence extended by a biotinylated Lys residue, to act as a marker, and six contiguous residues of DArg to act as a cell penetration sequence, was successfully synthesized by solid-phase Fmoc chemistry, demonstrated by close correlation of measured mass with that calculated from the structure, and its homogeneity shown by the single peak obtained on analytical HPLC. An intracellular bridge between Glu2 and Lys4 was incorporated by coupling Fmoc- $\beta$ -Ala to Glu2. After removal of the Fmoc from the peptidyl resin, by treatment with piperidine, the ring was closed by formation of a beta-lactam by treatment with excess PyBOP. Each step of the bridge formation was checked by reaction, or loss of reaction of small samples with ninhydrin. The products were >95% purity by analytical HPLC and Maldi mass spectrometry, which confirmed the cyclic structures.



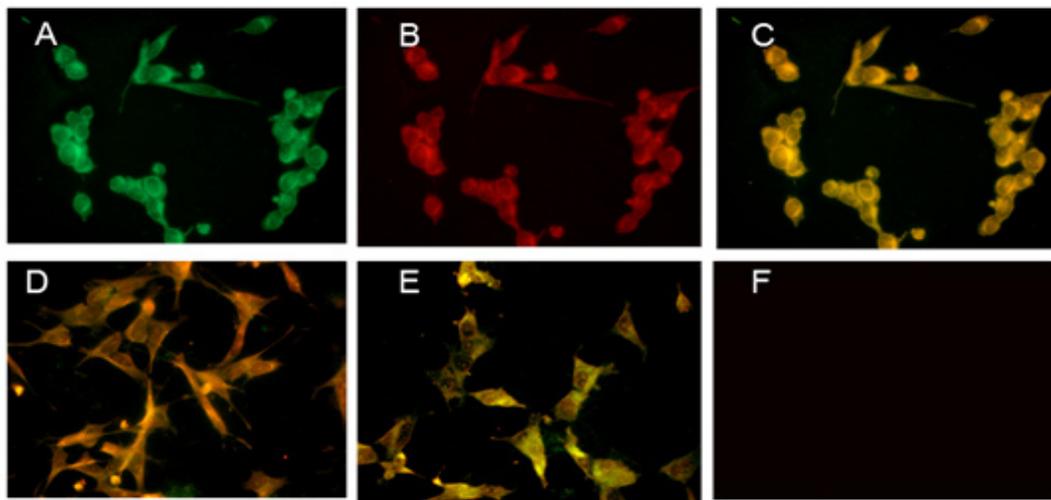
**Fig1.** A molecular model of PDZ-1 in human PSD-95 binding to a cyclic variant of the six C-terminal residues of human PMCA2b calcium channel (in orange), is derived from the pdb file of Piserchio et al, (2004), which is an NMR study of a cyclic variant of the CRIPT peptide binding to PDZ-1 of PSD-95. The model was built using Swiss-PDB-viewer using the residue substitution and energy minimisation facilities in the software. The C-terminal Leu from PMCA2b is labelled Leu6, and the bridging  $\beta$ -Ala residue BAL7. Only the 6 C-terminal residues and the bridge of the synthetic peptide R2 are modelled

#### Cell Penetration and Localization of PDZ-Binding Peptides

Both R2 and H2 (27mM), which contain DArg<sub>6</sub> cell penetration peptide sequences, were efficiently internalised into SHSY-5Y cells after 2 hours incubation (Figure 2), and readily visualised by binding of rhodamine-labelled avidin to the biotin incorporated as side-chain modifications in R2 and H2 (Fig B and E). In contrast, no intracellular staining was observed after incubation with R1, which lacked the 6xDArg sequence (Fig 2F).

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Intracellular co-localisation occurred between R2 or H2 and PSD-95 (Figs C, D and E), as stained by an anti PSD-95 rabbit antibody. Significant overlap between R2 (Fig B) and PSD-95 (Fig A) was calculated from the Pearson coefficient of 0.98, and between H2 (Fig E) and PSD-95 (Fig F) (Pearson coefficient 0.95). There was increased staining within certain regions of intracellular membranes, indicating that PSD-95, and bound peptides, may be located in the Golgi apparatus. There was sparse binding to cellular processes in the cellular extremity. To induce partial differentiation, and neurite outgrowth, SHSY-5Y cells were incubated with all-trans retinoic acid (100 $\mu$ M) for 7 days. (Pahlmann et al., 1984)) Retinoic acid induced extension of cells with neurites (Figures D and E), but little change in the distribution of R2 binding, location or levels of expression of PSD-95 and extent of co-localisation was produced (Pearson coefficient 0.97).

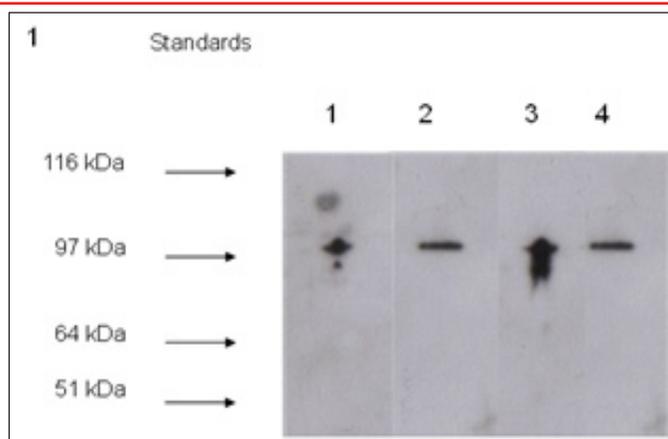


**Fig2.** Fluorescence of PDZ-binding peptides R2 and H2 and PSD-95

SHSY-5Y cells were incubated with R2 (A,B,C,D) or H2 (E) for 2 hours at 37°C in full media, or after preincubation of the cells for 7 days with retinoic acid (0.1mM) to induce differentiation (D and E), then washed with PBS plus 2% serum followed by methanol at 4°C. PSD-95 was visualised with a 1:500 dilution of PSD-95 rabbit antibody (A) (Abcam, Cambridge, UK, AB18258) and 1:160 of FITC anti-rabbit IgG and the peptides (B) with 1:500 ExtrAvidin TRITC conjugate (Sigma). Fig2 F shows lack of intracellular localization of R1, a version of R2 without the cell penetration sequence, with ExtrAvidin TRITC. Colocalisation analysis (Pearson's correlation) with retinoic-acid-treated cells revealed very similar readings (R2 A=0.971) to cells not treated with retinoic acid (0.98) suggesting no changes to the peptide binding to PSD-95.

### Characterization of PSD95 in SHSY-5Y Cells

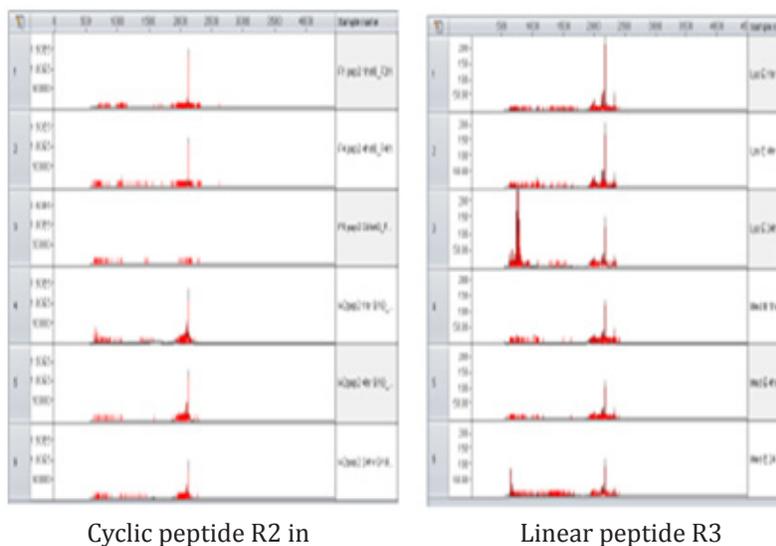
To analyse any changes to the cellular levels and molecular structure of PSD-95 caused by retinoic acid (RA) treatment, a western blot analysis of RA treated cells and control cells was performed (Figure 3). PSD-95 has a calculated molecular weight of 95kDa, equivalent to the bands seen on the western blot which illustrates the presence of PSD-95 in the SHSY-5Y cells in all conditions tested (Figure 3), with an up-regulation of PSD-95 cells treated with retinoic acid for 24 hrs (Figure 3, lane 1). This conflicts with results obtained from the immunohistochemistry, as an increase in PSD-95 staining intensity was not seen. However it is possible that because PSD-95 is partially recruited to neurites the intensity may be similar but the overall concentration of PSD-95 within the cell is greater. It is also clear that PSD-95 is further up-regulated after the longer treatment period of 120 hours (Figure 3 [lane 3]). The broadening of the band seen in lanes 1 and 3 may indicate cellular processing (eg changes in glycolipid post-translational modification) which is suggestive of changes in cell trafficking or modification during postsynaptic clustering (Topinka and Bredt, 1998) or expression of spliced variants (Niethammer et al., 1998)



**Fig3.** Western blot of PSD-95 in SHSY-5Y cells. Detergent solubilised cells (20mg) cultured without retinoic (Tracks 2 and 4) or with retinoic acid (0.1mM) for 24 hrs (Track 1) or 110 hrs (Track 3), showed a single band of 98kD molecular weight(Tracks 2&4) with increased intensity, and band broadening after retinoic acid treatment that may be due to increased expression and post-translation processing.

### Stability of PDZ-Binding Peptides

We compared the stability of the cyclic biotinylated PMCA2b peptide, R3 and its linear counterpart R2, in the presence of SHSY-5Y cells. Separation of the peptides remaining in the cell supernatants or internalized into the cells was obtained from cell supernatants of cell lysates respectively, by binding to avidin-coated plates. The bound peptides were released from the plates after washing by incubation with acetonitrile and 0.1% TFA, and amounts remaining of cyclic R2 or the linear form R3 quantitated from the mass peaks at 2189(R2) and 2137.5(R3), using Lucid (Biorad) software, are compared in Fig.5.



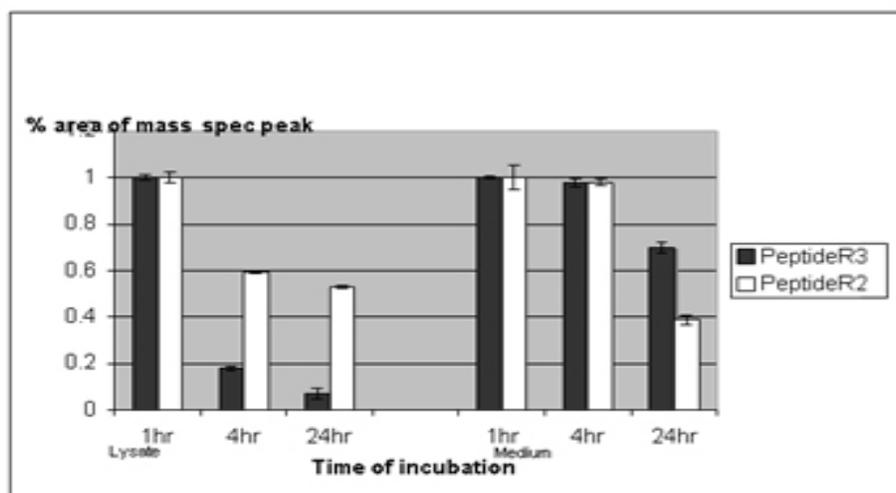
1) (1hr), 2)(4hr) and 3)(24hr) in lysate; 4(1hr) 5(4hr) and 6(24hr) in cell medium

**Fig4.** Mass spec profiles of cell-incubated PDZ-binding peptides

SHSY-5Y cells were grown to confluence, then passaged and re-suspended in 10ml of full media DMEM/F12 containing FBS (10%v/v), 1% Non-essential amino acids (Invitrogen), and 1% penicillin/streptomycin (Invitrogen), (600,000 cells/ml), then placed in 96 wells of a cell culture plate (0.1ml/well). The plate was

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incubated at 37°C overnight to allow cells to re-adhere. PDZ-binding peptides R2 or R3 at 10 $\mu$ M in total volume of full media of 0.2ml were added to the cells. After incubation for 1hr, 5hrs or 24hrs at 37°C in 5%CO<sub>2</sub>/air, media above the cells was removed, and adherent cells lysed in added RIPA buffer (Sigma-Aldrich) (0.2ml) with protease inhibitors (Roche) and EDTA 1mM. Media or cell lysates were transferred to wells of a Sigma streptavidin plate (Cat number M5432), and incubated for 2 hrs at 37°C. Media was removed and wells were washed with 200 $\mu$ l lysis buffer, 200 $\mu$ l of 50mM ammonium bicarbonate, and x3 with water. Then 70% acetonitrile/5% trifluoroacetic acid, was added and incubated for 2 hours at 37, This was removed and lyophilised, and residues were re-dissolved in 0.1% TFA(25 $\mu$ l) and spotted on a Maldi plate together with saturated  $\alpha$ -cyano-4-hydroxycinnamic acid in 70% acetonitrile/0.1% TFA, and analyzed on a Bruker Ultraflex Maldi tof mass spectrometer. Remaining peptides were quantitated using Lucid software (Biorad) after background and noise subtraction.

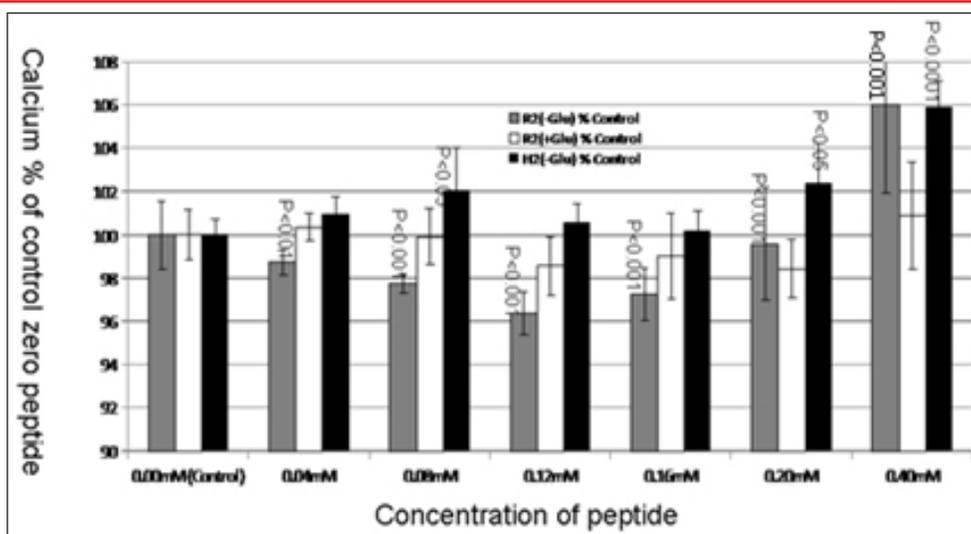


**Fig5.** Remaining biotinylated PDZ-binding peptides in cell media or lysates, after various times of incubation with cells, were extracted from avidin-coated plates and lyophilised, and residues were re-dissolved in 0.1% TFA(25 $\mu$ l) and spotted on a Maldi plate together with saturated  $\alpha$ -cyano-4-hydroxycinnamic acid in 70% acetonitrile/0.1% TFA, and analyzed on a Bruker Ultraflex Maldi tof mass spectrometer. Remaining peptides were quantitated using Lucid software (Biorad) after background and noise subtraction, and plotted as percentage of peptide/100 of that detected after 1 hour incubation. Results were obtained from triplicate cell incubations.

Results showed that the linear form of the Pdz-binding peptide from PMCA2b peptide (R3) survived in the growth medium surrounding the cells for longer than R2. However, when R3 was taken up into cells, it disappeared more rapidly in cell lysates than the cyclized form, R2. It is possible that the DArg<sub>6</sub> cell penetration sequence is more effective in R2 than in R3, but that R3 is more susceptible to intracellular proteolysis; thus it does not survive as long as the cyclic R2 in the intracellular environment that contains its target.

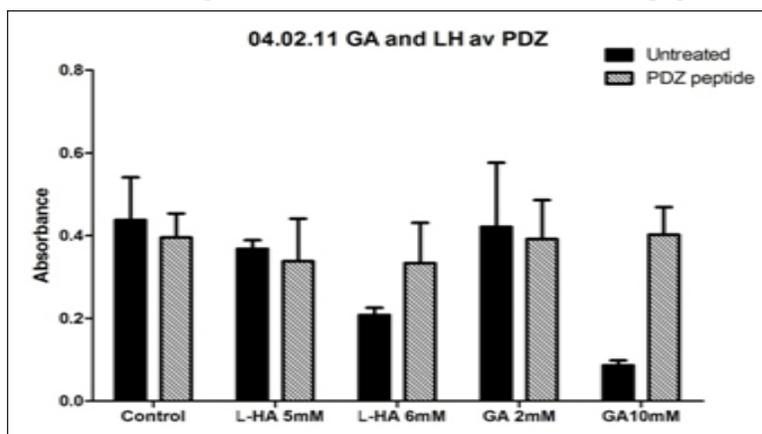
### Effects on Intracellular Calcium

The levels of intracellular calcium in cells incubated with PMCA2b PDZ-binding peptides were measured by the reagent fluo-4, which de-acetylates after import to form a fluorescent calcium adduct, which is then trapped inside cells by the presence of probenecid. Results are shown in Figure 7. R2, the PDZ-binding peptide derived from the C-terminal sequence of PMCA-2b gave rise to a biphasic response. At concentrations up to 0.12mM, R2 lowered intracellular calcium significantly in the absence of glutamate (0.02mM), whereas H2, a control PDZ-binding peptide derived from the C-terminal sequence of NMDA-R2 had no effect. At higher concentrations (ie 0.4mM) of R2 or H2, calcium levels increased, possibly due to the toxic effects of these relatively high concentrations of peptides.



**Fig6.** SHSY-5Y cells were incubated with R2 and H2, with or without buffered glutamate (20mM), in 6-well replicates for 16 hrs at 37°C. Supernatants were removed, Fluo-4 reagents were added, and cells incubated for 30 mins at 37°C followed by 30 mins at 20°C. Fluorescence was measured using a fluorescein filter at 485nm excitation and 530nm emission. Significant differences, calculated by Dunnett's multiple comparison test, compare the effects of R2 against incubated cells without peptides. ( $p < 0.001$  taken to be significant). At low concentrations of R2 (up to 0.15mM) calcium internalisation was blocked, possibly by disruption of interaction between the PNCa2b calcium channel and PSD-95. whereas at higher concentrations of both R2 and the H2 control, calcium increased significantly, possibly by the toxicity of high peptide concentrations.

SH-SY5Y neuroblastoma cells were given concentrations of R-2, in the presence and absence of 20mM Glutamate, in 6-well replicates for 24 hours. Fluo-4 NW dye solution was added to wells and fluorescence was measured using a fluorescein filter (485nm for excitation and 530nm for emission). Extent of fluorescence relates to intracellular  $Ca^{2+}$ . Error bars plotted represent standard error of the mean (SEM). Significant differences comparing the effects of Ruth-2 treated with 20mM Glutamate against the positive control (0.00 R-2, 20mM Glutamate) are marked with hashes (#). #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ . Significant differences comparing the effects of glutamate within Ruth-2 concentrations and are marked with asterisks (\*). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ . Black bars represent results obtained with a control peptide H2



**Fig7.** Absorbance at 650nm from MTT colour produced by SHSY-5Y cells treated with homocysteic acid (HA), glutamate (GA) in the presence or absence of PMCA2b-derived PDZ-binding peptide R2 (10mM).

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A 2-way ANOVA shows that there is a significant reduction of cell death by R2 in the presence of high levels of glutamate(10mM) ( $P < 0.001$ ) and homocysteic(6mM) ( $P = 0.0077$ ).

### DISCUSSION

Design of an effective PDZ-binding peptide included considerations of ability to access an intracellular target and stability in both extra-and intra-cellular environments. Cell-penetrating peptides (CPPs) are cationic peptides which, when linked to genes, proteins, or nanoparticles, facilitate the transport of these entities across cell membrane. Natural CPPs occur in basic peptide sequences (10-14aa) such as that derived from HIV-encoded TAT transactivator protein, or the penetratin protein (16aa) derived from the antennapedia protein of *Drosophila*. Synthetic arginine-rich peptides also function as CPPs (Futaki et al., 2001). We chose to incorporate a hexa-D-arginine sequence as it has previously been shown that cell penetrating peptides are stabilized by D-residues without loss of cell penetrating activity (Futaki et al, 2001). When linked N-terminal to biotinylated cyclic PDZ-binding peptides, the hexa-DArg sequence was highly effective at localizing its cargo throughout SHSY-5Y cells (Figure 2), whereas a control peptide lacking 6xDArg (figure 2F) was not visualized inside cells after the same incubation conditions.

The C-terminal sequence of PMCA2b was incorporated into a cyclic modified peptide according to the design of Piserchio et al (2004), who studied binding of a cyclic variant of CRIPT to PDZ-1 and PDZ-3 domains of PSD-95. PDZ domains have similar three-dimensional structures, consisting of antiparallel  $\beta$  sandwiches annealed by two  $\alpha$  helices and six  $\beta$  strands. The binding pocket for C-terminal sequences of PDZ-binding ligands lie between the  $\beta 2$  strand and the  $\alpha 1$  helix. Generally, PDZ-domains have been classified into three classes (I, II and III) depending on the characteristics of the  $\beta$ -strand loop and the position of the P-2 of the ligand, which bind to the region of the  $\beta 1$ :  $\beta 2$  loop. The cyclic variant of the PMCA2b C-terminal was created by replacing Ser of PMCA2b at P-2 with Lys, and cyclising the P-2Lys  $\epsilon$ -amino via a  $\beta$ -alanine bridge to the carboxyl side chain of the Glu at residue P-4. The configuration allowed the Leu residue at P0 to make important hydrophobic interactions with residues in the  $\beta$ -strand  $\beta 2$  (Figure 2), whilst Thr at residue P-3in PMCA2b continues to make hydrogen bonds with His 117 within the  $\alpha$ -1 of PDZ1. According to NMR studies of the CRIPT peptide, [Piserchio et al (2004a)], the bridging  $\beta$ -alanine residue interacts with residues in  $\alpha 2$ , and outside the PDZ-binding domain. The cyclic peptide of PMCA2B was synthesized successfully by Fmoc procedures, using orthogonal labile protecting groups on residues P-2 and P-4. Similar procedures were used to synthesize a cyclic variant (H2) of the C-terminal domain of NMDA R2, as a control. Peptides were characterized by mass spectrometry and analytical hplc.

Excitotoxicity is thought to be a common metabolic pathway in a number of neurological diseases, including stroke, epilepsy and neurodegeneration (Mark LP et al., 2001). The common pathway involves excessive activation of glutamate receptors. An interesting finding in the results recorded here, was that cell death of SHSY-5Y human neuroblastoma cells induced by 10mM glutamate, was significantly reduced by 10 $\mu$ M R2 to an extent of 72% ( $P < 0.001$ ). This supports the suggestion that the PDZ-binding peptide, R2, could exert a therapeutic role during glutamate induced excitotoxicity in the course of some human neurological disorders. Some modifications may be required to increase the affinity of the peptide for PSD-95, and to ensure its passage across the blood-brain barrier. The protective role of R2 on cell death induced by 6mM L-homocysteic acid, a brain excitotoxic analogue of glutamate (Olney et al., 1987) was not as substantial or significant ( $P > 0.5$ ) as the protection provided at 10mM glutamate, but a trend of increased cell viability was noted. As previously stated, the PDZ peptide R2 may exhibit 2 properties – neuroprotective at low concentrations due to its effect on PSD-95 binding, and neurotoxic at high concentrations.

Excessive accumulation of intracellular calcium is thought to be a key process leading to neuronal death, and stimulation of NMDA receptors. High concentrations of glutamate may open a number of channels leading to

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calcium influx. Calcium is normally maintained at low intracellular concentrations relative to extracellular calcium. Calcium is partly controlled by high-affinity plasma-membrane calcium pumps that couple calcium transport to ATP hydrolysis. Glutamate overstimulation increases intracellular calcium by directly opening calcium channels and secondarily affecting calcium homeostasis. Our results show that R2, a cyclic peptide derived from the C-terminal sequence of PMCA2b, displaces binding of the whole protein from PSD-95, with a  $K_d$  of about  $15\mu\text{M}$ . Over a range of concentrations up to 10x the  $K_d$ , R2 increases intracellular calcium in SHSY-5Y cells in culture. It is reasonable to suggest that coupling between PMCA2b and PSD-95, required for active import of calcium, is prevented when R2 displaces the binding, leading to a lowering of intracellular calcium. A consequence is the decrease produced by R2 in excitotoxic cell death seen in the presence of glutamate and homocysteic acid.

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