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**CURCUMIN-INDUCED DEGRADATION OF PKC δ IS ASSOCIATED WITH
ENHANCED DENTATE NCAM PSA EXPRESSION AND SPATIAL LEARNING IN
ADULT AND AGED WISTAR RATS.**

Lisa Conboy, Andrew G. Foley, Noel M. O'Boyle, Marie Lawlor, Helen C. Gallagher, Keith J.
Murphy and Ciaran M. Regan.

School of Biomolecular and Biomedical Sciences, UCD Conway Institute, University College
Dublin, Belfield, Dublin 4, IRELAND.

Corresponding author:

Prof. Ciaran Regan

UCD Conway Institute

University College Dublin

Belfield, Dublin 4, IRELAND.

Tel: +353-1-7166775

Fax: +353-1-7166920

E-mail: ciaran.regan@ucd.ie

ABBREVIATIONS: DMEM: Dulbecco's modified Eagle's medium; NCAM: neural cell adhesion molecule; PKC: protein kinase C; PSA: Poly- α 2,8-linked sialic acid; PST: polysialyltransferase; curcumin (diferuloylmethane; 1,7-bis-[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) and rottlerin (mallotoxin; 5,7-dihydroxy-2,2-dimethyl-6-[2,4,6-trihydroxy-3-methyl-5-acetylbenzyl]-8-cinnamoyl-1,2-chromine).

ABSTRACT

Polysialylation of the neural cell adhesion molecule (NCAM PSA) is necessary for the consolidation processes of hippocampus-based learning. Previously, we have found inhibition of protein kinase C delta (PKC δ) to be associated with increased polysialyltransferase activity (PST), suggesting inhibitors of this kinase might ameliorate cognitive deficits. Using a rottlerin template, a drug previously considered an inhibitor of PKC δ , we searched the CAP database with the Accelrys[®] Catalyst programme for structurally similar molecules and, using the available crystal structure of the phorbol-binding domain of PKC δ , found that diferuloylmethane (curcumin) docked effectively into the phorbol site. Curcumin increased NCAM PSA expression in cultured neuro-2A neuroblastoma cells and this was inversely related to PKC δ protein expression. Curcumin did not directly inhibit PKC δ activity but formed a tight complex with the enzyme. With increasing doses of curcumin, the Tyr¹³¹ residue of PKC δ , which is known to direct its degradation, became progressively phosphorylated and this was associated with numerous Tyr¹³¹-phospho-PKC δ fragments. Chronic administration of curcumin *in vivo* also increased the frequency of polysialylated cells in the dentate infragranular zone and significantly improved the acquisition and consolidation of a water maze spatial learning paradigm in both adult and aged cohorts of Wistar rats. These results further confirm the role of PKC δ in regulating PST and NCAM PSA expression and provide evidence that drug modulation of this system enhances the process of memory consolidation.

RUNNING TITLE: PKC δ degradation enhances NCAM PSA and cognition.

KEY WORDS: PKC δ ; PST; NCAM PSA; curcumin; spatial learning.

1. INTRODUCTION

A significant post-translational modification of the neural cell adhesion molecule (NCAM) involves the attachment of large homopolymers of α 2,8-linked polysialic acid (PSA), a modification that is specific to NCAM in the mammalian brain [1] and [2]. This post-translational modification of NCAM has been extensively argued to support structural plasticity in the adult nervous system [3], [4] and [5] and has been implicated in activity-dependent synaptic remodelling [6] and [7]. Moreover, the synaptic remodeling that accompanies task consolidation of spatial learning and avoidance conditioning paradigms [8], [9] and [10] is associated with a transient increase in the frequency of dentate granule cells expressing polysialylated NCAM at the 12 h post-training time [11], [12] and [13] and cleavage of PSA with endoneuraminidase-N impairs the development of long-term potentiation (LTP), a cellular analogue of memory and learning, and the consolidation of spatial learning and avoidance conditioning paradigms [14], [15], [16] and [17]. Intraventricular infusion of anti-PSA, when administered specifically at the 10 h post-training time, has also been demonstrated to impair the consolidation of spatial and avoidance conditioning tasks, further confirming the time-dependent functional requirement of NCAM PSA in memory formation [18]. Polysialylation of NCAM occurs in the trans-Golgi compartment and is catalyzed by two enzymes termed sialyltransferase-X (STX or ST8SiaII) [19] and [20] and polysialyltransferase (PST or ST8SiaIV) [21] and [22]. These two polysialyltransferases regulate NCAM polysialylation state differentially during development as STX is the dominant α 2,8-polysialyltransferase in the embryonic and early postnatal period whereas PST is mainly associated with α 2,8-polysialylation in the postnatal

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3 brain [23] and [24]. Pulse-labelling studies *in vitro* have indicated PSA synthesis to occur in the
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5 late-Golgi, or post-Golgi, compartment where NCAM becomes rapidly glycosylated with the
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7 newly synthesized $\alpha 2,8$ -polysialic acid [25].
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11 In general, the molecular events associated with regulation of sialyltransferase-mediated
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13 glycosylation events still remain to be fully elucidated. The proposed involvement of PKC in the
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15 regulation of PST-mediated NCAM polysialylation state stems from the observation that
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17 phorbol-12-myristate-13-acetate, which activates diacylglycerol-dependent PKC isoforms,
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19 induces a dose-dependent decrease in NCAM PSA expression in neuro-2A neuroblastoma cells
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21 and staurosporine, a pan-specific PKC inhibitor, increases NCAM polysialylation state [26]
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23 Moreover, immunoblotting procedures have demonstrated reduced PKC δ expression to be
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25 associated with the enhanced polysialylation of NCAM *in vitro* and, using a polyclonal antibody
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27 directed against a conserved 11-amino acid sequence in *E. coli* polysialyltransferase,
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29 hippocampal PKC δ has been found to form complexes with PST that increase as animals age [27]
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31 and NCAM PSA expression decreases in an exponential manner [28]. The PST associated with
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33 these complexes has also been observed to be phosphorylated on serine residues [27], a
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35 mechanism known to be associated with the inhibition of other sialyltransferases [29], [30], [31]
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37 and [32]. Phosphorylated tyrosine residues on PST are also found in PST:PKC δ
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39 immunocomplexes [27] and this may play a crucial role in regulating PKC δ expression as
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41 phosphorylation of conserved tyrosine residues in the hinged bilobal structure, typical of the PKC
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43 family of isozymes, confers a specific post-translational control on the rate of proteolytic
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45 cleavage [33] and [34].
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3 In previous studies directed to providing evidence for a role of PKC δ in the regulation of NCAM
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5 polysialylation in neuro-2A neuroblastoma cells [26], we employed rottlerin to inhibit PKC δ , as
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7 this had been reported to have an IC₅₀ value of 3-6 μ M and to be 5-17 times more potent as a
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9 PKC δ inhibitor over other PKC isozymes [35]. Neuro-2A cells exposed to rottlerin (5 μ M) in the
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11 mid-log growth phase, when NCAM PSA expression is dominant, resulted in a rapid and
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13 substantial increase in NCAM polysialylation state [26]. Moreover, exposure of the same
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15 concentration of rottlerin to cells in contact inhibition, where no NCAM PSA is expressed,
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17 resulted in PST activation and expression of polysialylated NCAM. Subsequent studies,
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19 however, have failed to confirm rottlerin to be a direct inhibitor of PKC δ [36] but that it may
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21 modulate the tyrosine phosphorylation state of PKC δ and indirectly affect enzyme activity [37].
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23 We now demonstrate curcumin to be a chemical similar of rottlerin that indirectly modulates
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25 PKC δ activity by modifying its phosphorylation state to increase the rate of degradation.
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2. MATERIALS AND METHODS

2.1 Materials

All routine laboratory chemicals, secondary antibodies, rottlerin, staurosporine and curcumin were purchased from Sigma-Aldrich, Ireland. All components of the PKC δ activity assay, including constitutively active recombinant PKC δ , were purchased from Upstate Biotechnology (Charlottesville, VA). [γ - 32 P]-ATP was purchased from Amersham Biosciences (Buckinghamshire, UK). The BCA assay was purchased from Pierce (Rockford, IL). The monoclonal antibody raised against the meningococcus group B polysaccharides (anti-PSA) was a kind gift of Prof. G. Rougon [2]. Monoclonal primary antibody to PKC δ was purchased from BD Biosciences (Oxford, UK).

2.2 *In silico* studies

Database searching with Catalyst and protein-ligand docking with Cerius2

Computational studies were carried out using a Silicon Graphics Octane workstation (SGI, Mountain View, CA). Catalyst version 3.4 (Accelrys, Cambridge, UK) was used to search the Compounds Available for Purchase (CAP) database to identify those structurally similar to rottlerin using a template structure based on its side chain (see Figure 1A). Prior to database screening, the search was restricted to drug-like molecules based on the Lipinski rules [38]. Using Cerius2 (version 4.8, Accelrys, UK), the matching compounds were rated using protein-ligand docking to the crystal structure of the PKC δ ligand binding domain which was downloaded from the Brookhaven Protein Data Bank. In preparation for docking, all organic and

inorganic cofactors, the phorbol ester ligand, as well as all water molecules, were removed from the PKC δ crystal structure. Potential PKC δ inhibitors were sequentially docked to the PKC δ crystal structure and ligand-enzyme interactions were quantified using the DOCK scoring function of the LigFit module of Cerius2. The top ranking compounds were screened in *in vitro* assays, described below, to determine their potential to modulate NCAM-PSA.

2.3 *In vitro* analysis of NCAM PSA expression

The mouse neuroblastoma cell line, neuro-2A, was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, UK) supplemented with 10% (v/v) foetal calf serum (Gibco, UK) and penicillin/streptomycin (100 mg/ml; Sigma, UK) at 37 °C in a humidified atmosphere and 9% CO₂. To investigate pharmacological modulation of PSA expression, cells were seeded on 96-well plates at a density of 10⁴ cells/cm² and grown until day 3 *in vitro* (DIV 3). On DIV3, cells were treated with either vehicle (DMSO not exceeding 1% v/v) or test PKC δ inhibitor ranging in dose from 1 μ M to 500 μ M for 5 hours. Specifically, curcumin was administered at concentrations of 1, 10, 50, 100 and 500 μ M. Cells were then fixed with 200 μ l of 4% paraformaldehyde in DMEM for 60 min at room temperature. This was replaced with undiluted fixative for a further 60 min. Non-specific binding sites were blocked with 2% (w/v) bovine serum albumin (Sigma-Aldrich, Ireland) and 2% (v/v) normal goat serum (Sigma-Aldrich, Ireland) in PBS (blocking solution; in which all antibodies were also diluted). PSA was detected using a monoclonal antibody raised against the meningococcus group B polysaccharides (anti-PSA) prepared as an ascitic fluid and diluted 1:500. Primary antibody was incubated overnight at 4 °C. Binding of the primary antibody was detected using a secondary goat anti-mouse IgG conjugated to peroxidase diluted 1: 2000 for 1 hour at room temperature. Peroxidase activity was

determined using the liquid substrate tetramethylbenzidine (Sigma-Aldrich, Ireland), which was incubated for 30 min at room temperature and the reaction was terminated by acidification with 2 M H₂SO₄. Optical density was measured in a microplate reader with a 450 nm measurement filter and 620 nm reference filter.

2.4 PKC δ activity assay

Constitutively active recombinant PKC δ was derived from Sf21 cells and assay of enzyme activity was performed according to the manufacture's instructions. In brief, 0-20 ng of recombinant PKC δ was incubated with 50 μ M PKC substrate peptide (myelin basic protein), in the presence of 200 μ M ATP, 30 mM magnesium chloride, 0.05 mg/ml phosphatidylserine, 0.005 mg/ml diacylglycerol and [γ -³²P]-ATP (Amersham Biosciences, UK) for 10 min at 30 °C. Following incubation 20 μ l of each assay was spotted onto phosphocellulose paper and the reaction terminated by washing of the phosphocellulose in 0.75% phosphoric acid for 5 minutes. Following two subsequent 5 minute washes in phosphoric acid, the samples were washed in acetone for a further 5 min. The phosphocellulose paper was dried, transferred to scintillation fluid and the radioactivity counted. Under these conditions, increasing concentrations of PKC δ were associated with increasing levels of radioactivity. Using 20 ng of recombinant PKC δ and the conditions described above, staurosporine (5 nM), rottlerin (10 μ M and 100 μ M) and curcumin (10 μ M and 500 μ M) were included in the assay and their ability to inhibit PKC δ kinase activity in a dose-dependent manner was determined. In additional experiments, the assay concentration of ATP was lowered to 40 μ M ATP to determine potential competition at the ATP binding site.

2.5 Immobilisation of curcumin on Epoxy-activated Sepharose 6B

To investigate curcumin-PKC δ interactions, curcumin was coupled to epoxy-activated Sepharose™ 6B (Sigma-Aldrich, Ireland). Using a method adapted from [39] a 1 gm aliquot of epoxy-activated Sepharose 6B beads was washed in 50 ml of dH₂O for 5 cycles to give approximately 3.5 ml of swollen beads. For coupling to the epoxy-activated beads, curcumin was prepared as a 20 mM solution in 50% dimethylformamide/0.1 M Na₂CO₃/10 mM NaOH and two volumes of this solution was mixed with one volume of swollen beads and incubated overnight at 30 °C in the dark. The following day, the curcumin-Sepharose suspension was washed 3 times with coupling buffer and the remaining non-specific binding sites were blocked by incubation with 1M ethanolamine overnight at 30 °C. Control beads were prepared by incubating the epoxy-activated Sepharose 6B with 1 M ethanolamine, pH 11. The control and curcumin-coupled beads were finally washed in 3 cycles of alternating low (0.1M acetate buffer, pH 4.0) and high (0.1M Tris-HCl buffer, pH 8, containing 0.5 M NaCl) pH buffers.

Hippocampal PKC δ was prepared by homogenising rat dentate gyrus in 100 mM Hepes buffer, pH 7.6, containing 300 mM NaCl, 1% Triton-X-100, 2 mM EDTA, 2 mM EGTA and 0.1 mM PMSF, 50mM Na₃VO₄ and 1µg/µl aprotinin as protease inhibitors (2X lysis buffer). A 250 µl aliquot of cell lysate was incubated with 20 µl of curcumin-coupled Sepharose, or control beads, with constant mixing for 3 h at 4 °C. Afterwards, the curcumin-Sepharose complex was washed twice with 500 µl of 2X lysis buffer (without protease inhibitor) containing 150 mM NaCl (low salt) followed by 500 µl of 1X lysis buffer containing 1 M NaCl (high salt). Proteins were

isolated from the bead-protein complex by resuspending and boiling the washed beads in SDS-PAGE sample buffer. PKC δ was detected by the immunoblotting procedure described below.

2.6 Immunoblot analysis of PKC δ expression

Neuro-2A cells were seeded in 75 cm² plates (10⁴ cells/cm²) and exposed to increasing concentrations of curcumin for 5 h during the mid-log growth phase, as described above. The cells were then washed 3 times in phosphate-buffered saline, scraped and pelleted by centrifugation for 10 min at 900 rpm. The pellet was solubilised in RIPA buffer (50 mM Tris, pH 8.0, containing 50 mM NaCl, 1.0 % NP-40, 0.5% sodium deoxycholate, and 0.1% SDS,) containing protease inhibitors for 30 min on ice. The supernatant was then collected by centrifugation at 3000 g and the protein concentrations determined by the BCA assay. Samples, of equal protein concentration, were boiled for 10 min in a reducing sample buffer of 70 mM Tris-HCl, pH 6.8, containing 33 mM NaCl, 1 mM EDTA, 2% (w/v) SDS, 0.01 % (w/v) bromophenol blue, 10% glycerol and 3% (v/v) dithiothreitol reducing agent (New England Biolabs, UK). Samples containing equal amounts of protein were separated on 10 % polyacrylamide minigels and electrophoretically transferred to nitrocellulose membranes and successful transfer was confirmed by ponceau S staining of the membrane (not shown). The nitrocellulose was then blocked using washing buffer (10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, and 0.05% [v/v] Tween-20) with 5% (w/v) non-fat milk powder for 1 h at room temperature. The monoclonal primary antibody to PKC δ was diluted 1:1000 in blocking buffer and incubated overnight at 4 °C with the nitrocellulose membrane. The primary antibody was then detected using goat anti-mouse IgG peroxidase conjugated secondary antibody. Following membrane washing, the immuno-complexes were detected using a chemiluminescence

peroxidase substrate and exposure of the membranes to x-ray film (Kodak, Dublin, Ireland). Equal protein loading was verified by stripping the blots and re-probing them with an anti-actin monoclonal antibody (Sigma-Aldrich, Ireland) diluted 1: 20000. The developed x-ray films were scanned using Adobe Photoshop 7.0.

2.7 Quantitative immunohistochemical analysis of NCAM PSA *in vivo*

Male Wistar rats (postnatal day 80) were obtained from the Biomedical Facility, University College Dublin, and housed singly in a 12 h light/dark cycle at $22 \pm 2^\circ\text{C}$, with *ad libitum* access to food and water. Curcumin (75 mg/kg), dissolved in a methylcellulose (0.5% w/v) vehicle, was administered daily by gavage for an 8-day period. Control animals received the methylcellulose vehicle for the same 8-day period. At 24 h after the final drug administration, the animals were sacrificed by cervical dislocation and the whole brain was quickly dissected. The brains were coated immediately in an optimal cutting temperature compound (Gurr, Poole, UK), snap-frozen in CO₂-cooled n-hexane, and stored at -80°C until required for further processing. The frequency of hippocampal polysialylated neurons was determined using PSA immunohistochemistry, as described previously [11]. Briefly, cryostat axial sections of 12 μm sections were fixed in 70% (v/v) ethanol and incubated overnight with anti-PSA ascitic fluid diluted 1:500 in phosphate buffered saline (PBS). The sections were exposed for 3 h to fluorescein-conjugated goat anti-mouse IgM diluted 1:100 in PBS and mounted in Citifluor® (Citifluor Ltd., UK), a fluorescence-enhancing medium. The total numbers of PSA-immunopositive neurons at the infragranular cell layer of the dentate gyrus, at -5.6 mm with respect to bregma, were counted in seven alternate 12 μm sections. The sections were counter-stained with propidium iodide (60 s; 40 ng/ml) (Sigma-Aldrich, Ireland) to facilitate cell identification and the use of alternate sections precluded

double counting of the 5-10 μm perikarya. Cell counts were expressed per unit area of the dentate granule cell layer (0.15mm^2) and the mean $\pm$ SEM for each treatment group calculated.

2.8 Water maze spatial learning paradigm

Separate cohorts of male Wistar rats (postnatal day 80 and postnatal day 540), sourced and maintained as described above, were used in these studies. On each of the 2 days preceding commencement of water maze studies, animals were handled and assessed in an open field arena for locomotor activity, rearing and general behaviour over a 5 min period. Each cohort of animals was treated for 8 days with 75 mg/kg curcumin by gavage. An extended 40-day treatment period did not confer any additional advantage on the parameters under investigation (data not shown). The control groups received vehicle alone. Water maze training was initiated at 24 h following the final drug administration. The water maze apparatus consisted of a large circular pool (1 m diameter, 80 cm high, water temperature $26 \pm 1^\circ\text{C}$) with a platform (11 cm diameter) submerged 1.5 cm below the water surface [12]. The water was 50 cm deep and the wall of the pool was 30 cm above the level of the water. Both the pool and the platform were constructed of black polyvinyl plastic and offered no intra-maze cues to guide escape behaviour. The experimental room contained several extra-maze visual cues. During testing the platform was hidden in the same quadrant 30 cm from the side wall. Animals were trained in 4 training sessions separated by 24 h and each session, consisting of 5 trials, was separated by an inter-trial test interval of 300 s. Each trial started with the rat facing the wall of the maze at one of three fixed points A, B, or C arranged triangularly around the pool (separated by 120°). The starting positions were used sequentially over the five trials of each training session and continuity in the

pattern of starting positions was maintained over different sessions so that all starting points were used the same number of times. Computerised tracking software (Watermaze 3.1) was used to measure relevant parameters that included swim angle, swim speed, escape latency and path length. The time taken by the rat to find the hidden platform within a 60 s criterion period was defined as the escape latency time. On the first trial, rats failing to locate the platform within the 60 s period were placed on it for 10 s. Escape latencies were measured over 5 trials in each training session. Probe trials were performed at 1 and 3 days following the final training session. In these trials the platform was removed and the time spent in each quadrant of the pool was tracked over a 60 s period.

All experimental procedures were approved by the Animal Research Ethics Committee of University College Dublin and were carried out by individuals holding the appropriate national license issued by the Department of Health.

2.9 Statistics

All statistical analysis was carried out using GraphPad Prism (version 4) software. Curcumin effects on NCAM-PSA and PKC δ expression were analysed by using Student's unpaired two-tailed *t*-test. Kinase activity assay values were analysed by ANOVA followed by Dunnett's post-hoc analysis. The effect of drug treatment and trial number on escape latency from the water maze was assessed by repeated measures ANOVA. Specific trials and probe differences were analysed using Student's unpaired two-tailed *t*-test. In all cases, *P* values less than 0.05 were considered to be significant.

3. RESULTS

3.1 Identification of curcumin as a chemical similar of rottlerin

Taking rottlerin as our template structure, we used the substructure highlighted in bold in Figure 1A to search the CAP database for structurally similar chemicals using the Accelrys® Catalyst programme. This allowed the identification of 137 compounds. This list of compounds was further refined using the Accelrys® Cerius2 modelling and simulation program to test for ligand fit to the crystallised C1B phorbol-binding domain of the PKC δ molecule (Figure 1Bi). The C1B domain of the regulatory region of PKC δ , containing the phorbol ester, was obtained from the Protein Data Base (PKC δ C1B domain RCSB PDB entry 1PTQ; [40]; Figure 1Bii). The phorbol ester was removed and the binding site defined using the site search function of Cerius2. The energy of the PKC δ crystal structure was minimised using the Dreiding 2.21 force field and energy minimised rottlerin conformers were docked to the PKC δ phorbol-binding site. The ligand-enzyme score was quantified using the 'DOCK' score of the Cerius2 LigFit module, which rated interaction energy and internal ligand energy between enzyme and ligand. DOCK scores of the 137 identified compounds with the PKC δ C1B phorbol-binding domain showed a normal distribution (Figure 1C). Within this distribution, both curcumin and rottlerin exhibited high DOCK scores of 92 and 87, respectively, indicating significant chemical similarity between these compounds. The interaction of curcumin with the PKC δ C1B phorbol-binding domain is illustrated in Figure 1Biii. Using an ELISA based assay, curcumin was also found to enhance NCAM PSA expression in a dose-dependent manner in neuro-2A neuroblastoma cells (Figure 1D). This confirmed curcumin to have the same biological activity of rottlerin, as reported previously [26], and that both compounds most likely influenced PKC δ activity in a similar

manner. The dose of curcumin required to enhance NCAM PSA expression was, however, an order of magnitude greater than that previously reported for rottlerin (5 μ M; [26]. This difference in dose-response is most likely related to the unstable nature of curcumin at neutral and basic pH both of which lead to its rapid degradation and formation of ferulic acid, a potent antioxidant [41]. Ferulic acid, however, failed to enhance NCAM PSA expression in the neuro-2A neuroblastoma cell line (data not shown) further confirming the primary role of curcumin in this effect.

3.2 Influence of curcumin and rottlerin on PKC δ kinase activity.

Given that previous studies demonstrated rottlerin to be without effect on PKC δ activity [36] and [37], we determined if curcumin similarly failed to influence enzyme activity. The *in vitro* assay employed determined the influence of curcumin on the phosphorylation rate of myelin basic protein using purified human recombinant PKC δ protein (Figure 2A) in the presence of [γ - 32 P]-ATP and phosphatidylserine and DAG lipid activators. Using this assay, the phosphorylation rate of myelin basic protein substrate was found to be directly related to the concentration of PKC δ enzyme present (Figure 2B). We further validated the assay using the non-specific PKC inhibitor staurosporine and found it to inhibit PKC δ kinase activity in a dose-dependent manner (data not shown). Staurosporine exhibited an IC₅₀ value of approximately 5nM which is in close agreement with the IC₅₀ value of 3.5nM reported previously [42]. At 5 nM, staurosporine reduced PKC δ kinase activity to 47.3 $\pm$ 9.2% of that observed in the control sample (Figure 2C). In comparison to rottlerin and curcumin, over concentration ranges of 10-100 μ M or 100-500 μ M, respectively, only staurosporine inhibited PKC δ activity (F[5,12]= 11.09; P <0.05). To further preclude the possibility that rottlerin or curcumin may be acting as competitive inhibitors at the

PKC δ ATP binding site, we determined the consequence of lowering available ATP (200 μ M to 40 μ M) on kinase activity. At this lower ATP concentration, staurosporine further reduced PKC δ kinase activity to $29.5 \pm 8.4\%$ of the control value but it was without effect on the ability of curcumin or rottlerin to inhibit PKC δ activity (Figure 2C). These results demonstrated rottlerin to be without any direct effect on PKC δ activity, confirming previous reports [36], and that the related compound curcumin similarly failed to influence enzyme activity.

3.3 Curcumin binding promotes PKC δ degradation.

Given curcumin failed to inhibit PKC δ activity, despite its high DOCK score for the phorbol-binding site of the enzyme, we determined if curcumin formed a complex with the enzyme. To achieve this, we covalently coupled curcumin to epoxy-activated Sepharose 6B, via its available hydroxyl groups, and used these beads to bait PKC δ in lysates of rat dentate gyrus solubilised with a non-ionic detergent, a procedure employed previously to identify the cellular targets of other protein kinase inhibitors [39]. Incubation of the dentate lysate with curcumin-coupled Sepharose resulted in PKC δ forming a tight linkage to curcumin. Following removal of weakly-bound proteins by a cycle of low- and high-salt wash and rigorous boiling in SDS gel sample buffer, the remaining Coomassie blue-stained electrophoretic protein profile was found to be comparable across all treatment conditions (Figure 3A; equal protein loading). Immunoblot analysis with anti-PKC δ , however, revealed the enzyme to be associated with the curcumin-coupled Sepharose 6B and not with the control Sepharose 6B (Figure 3B; lanes 1 and 2).

Moreover, the curcumin-coupled Sepharose 6B bound all of the PKC δ in the lysate as immunoblot analysis of the remainder failed to detect any enzyme immunoreactivity (Figure 3B; lane 4) and this was in contrast to the abundant PKC δ immunoreactivity observed in the lysate

following incubation with the control Sepharose 6B (Figure 3B; lane 5). Moreover, the tight association of PKC δ with curcumin appeared to exert an allosteric effect that rendered the enzyme more amenable to Tyr³¹¹ phosphorylation. Exposure of the neuro-2A neuroblastoma cell line to curcumin for a 5 h period resulted in a dose-dependent decrease in PKC δ protein expression with no effect being observed on actin protein levels (Figure 4A and B). This increase in PKC δ protein degradation was paralleled by a dose-dependent increase in the phosphorylation of the Tyr³¹¹ residue of PKC δ , accompanied by Tyr³¹¹ phosphorylated enzyme protein fragments (Figure 4C), suggesting the association of curcumin with PKC δ directed its degradation which is in agreement with previous work relating PKC δ Tyr³¹¹ phosphorylation to its increased proteolysis [34].

3.4 Curcumin enhances NCAM polysialylation state and spatial learning in adult Wistar rats.

Given the transient increase in polysialylated neurons in the hippocampal formation that accompanies consolidation of an avoidance conditioning paradigm [11] is associated with a decrease in PKC δ degradation [27], we determined if curcumin, administered *in vivo*, would similarly increase NCAM polysialylation state in the infragranular zone of the dentate gyrus of adult rats (postnatal day 80). Following daily administration of curcumin (75 mg/kg by gavage) for an 8-day period, we counted a two-fold increase in polysialylated dentate neurons ($P < 0.05$, Student's two-tailed *t*-test) (Figure 5B). Moreover, the arborisation of these polysialylated neurons in the upper granule cell layers and inner molecular layer was substantially increased, suggesting curcumin induced a substantial remodelling of the dentate neuronal architecture (Figure 5A).

Curcumin-induced change in dentate NCAM polysialylation state also correlated with improved acquisition and consolidation of the water maze spatial learning paradigm in cohorts of both adult (postnatal day 80) and aged (postnatal day 540) Wistar rats. Adult animals treated with curcumin exhibited a significant difference in task acquisition over 4 sessions ($F[1,72]=4.34$, $P=0.041$; two-way repeated measure ANOVA), as compared to the vehicle-treated controls (Figure 6A). These animals also spent significantly more time in the target quadrant during subsequent probe trials ($F[1,72]=3.9$; $P=0.05$; two-way repeated measure ANOVA) and this became significant at the 72 h recall time (Figure 6B), as judged by Bonferroni post-test and Mann-Whitney U -test ($P<0.05$). These results demonstrated curcumin treatment to exert a marked improvement on both the acquisition and consolidation of the spatial learning task. Curcumin treatment also resulted in significant improvements in both acquisition and consolidation of the water maze task in the cohort of aged animals. Drug-treated animals exhibited a superior performance to their vehicle-treated controls in task acquisition when assessed over 4 training sessions ($F[1,40]=6.7$; $P=0.01$; two-way repeated measure ANOVA) (Figure 6C). Moreover, curcumin-treatment produced a most significant effect on task recall when measured at both the 24 and 72 h probe trials (Figure 6D), as judged by a two-way repeated measure ANOVA ($F[1,44]=8.2$; $P=0.006$;) and Bonferroni post-test and Mann-Whitney U -test ($P<0.05$).

4. DISCUSSION

These studies have demonstrated increased PKC δ degradation by curcumin to be associated with an enhanced expression of NCAM polysialylation state in mouse neuro-2A neuroblastoma cells. The ability of curcumin to suppress PKC δ expression is consistent with our previous *in vitro* observations demonstrating phorbol activation and/or staurosporine inhibition of PKC δ to, respectively, suppress and/or enhance PST activity with the direct consequence of regulating the extent to which NCAM is glycosylated with extended chains of α 2,8-polysialic acid [21]. The mechanism by which curcumin induces degradation of PKC δ appears to be associated with increased phosphorylation of the Tyr³¹¹ residue which lies within the hinge region between the regulatory and catalytic domains of the enzyme. This finding agrees with previous studies which have demonstrated phosphorylation of the Tyr³¹¹ residue to first activate PKC δ and subsequently render it more susceptible to degradation [34] when the hinge region becomes accessible to proteolytic cleavage during the conformational changes associated with enzyme activation [33]. Activation of PKC δ by phorbol esters is also known to trigger their ubiquitination and degradation [43]. Moreover, the Tyr³¹¹ residue of PKC δ is flanked by a sequence that forms an optimal binding substrate for the Src family of kinases that constitutively complex with PKC δ but not other PKC isoforms, such as PKC α or PKC ϵ [44].

Chronic administration of curcumin *in vivo* was also demonstrated to enhance the polysialylation of neurons in the infragranular zone of the dentate gyrus and this included their dendritic tree which extends into the inner third of the molecular layer. α -Tocopherol, which also reduces PKC δ expression [45], has been found to similarly enhance the polysialylation of neurons in the

adult dentate gyrus and, moreover, generate greater complexity in their dendritic arbor, as evidenced by increased synaptophysin expression and synapse density in the molecular layer [46]. Upregulation of dentate NCAM PSA may also, in part, provide a basis for the neuroprotective actions attributed to curcumin [47] as chronic exposure to deprenyl, purported to slow nigral cell degeneration in Parkinson's disease [48], has been demonstrated to increase the frequency of dentate polysialylated cells [49]. Moreover, hyperthermia-induced upregulation of NCAM PSA appears to similarly protect against kainate-induced cell death [50]. Curcumin has been demonstrated to significantly increase the number of newly generated cells in the dentate infragranular zone [51], however, not all of these are polysialylated [52]. The ability of curcumin to enhance neuronal polysialylation through suppression of PKC δ activity may more likely relate to the integration of newborn cells into the dentate neuronal architecture over the 1-2 month period that follows their birth [53], [54] and [55]. This mechanism would be consistent with the transient decrease in PKC δ expression and associated increase in dentate polysialylated cell frequency that is observed at the 12h post-training time following avoidance conditioning [11], [27] and with other studies that have failed to implicate increased NCAM PSA-mediated plasticity with neurogenesis [11], [56], [17] and [50].

Chronic administration of curcumin to adult Wistar rats significantly improved their performance in both the acquisition and consolidation of the water maze spatial learning paradigm. This is not surprising given that chronic administration of curcumin produces a two-fold increase in the frequency of polysialylated dentate neurons, the numerical density of which may be directly correlated to improvement in task performance [57] and [49]. Environmental enrichment, for example, robustly improves spatial learning and memory and is associated with increased NCAM

polysialylation [58] and [49] and also a decreased expression of PKC δ [27]. Moreover, a pyrazole analogue of curcumin [59] has been demonstrated to facilitate the induction of LTP and performance in an object recognition paradigm in Wistar rats through the induction of Ca⁺⁺/calmodulin-dependent protein kinase II [60], a mechanism known to be necessary for both spatial learning and NCAM-mediated neuroplasticity [61] and [62]. Although, the dose of curcumin required to enhance NCAM polysialylation and cognition *in vivo* was relatively high (75 mg/kg), no effect on either parameter was found at a dose of 37.5 mg/kg (data not shown), it was within the range previously reported necessary to induce neuroprotective effects *in vivo* [63] and [64]. Moreover, the dose at which curcumin mediates these neuroplastic actions is likely to be exceptionally low as its bioavailability is exceedingly poor, the majority being excreted in the feces, and only small fraction of the dose penetrates the brain, estimated to be approximately 0.3% of the administered dose in mice [65] and [47]. The lack of any behavioural toxicity in these studies, and those of others in both animals and humans [66] and [67], further implies the effective dose of curcumin necessary to enhance NCAM PSA expression and cognition to be exceptionally small. This unique action of curcumin may have significant implications in facilitating memory formation as the extent of PSA activation is commensurate with the difficulty experienced with task consolidation [57].

Curcumin also significantly improved spatial learning ability in aged animals despite the significant age-dependent decrease in NCAM polysialylation state [28] and increase in PKC δ expression [27]. However, extensive NCAM polysialylation is not necessary for the acquisition and/or consolidation of behavioural paradigms in aged animals [68] and [18] and appears to relate mainly to the graded, experience-expectant development of explicit memory systems that

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3 continue to evolve into maturity [69]. Curcumin can also regulate the *in vivo* expression of
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5 several other cell signaling systems required for neuroplastic events necessary for memory
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7 formation, notably Notch-1 and NF κ B [70], [71], [72], [73] and [74]. Nevertheless, facilitation
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9 of PSA activation by curcumin, however minimal, may have implications for the treatment of
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11 neurodegenerative conditions. In Alzheimer's disease, the natural autoprotective response to age-
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13 related cognitive deficits is a small, but significant, activation of dentate polysialylated cell
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15 frequency [75] and, over the past few years, an increasing number of studies have highlighted the
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17 therapeutic potential of curcumin for the treatment of this condition [76] and [77], a suggestion
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19 further supported by its ability to reduce β -amyloid plaque burden in animal models [78].
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22 Moreover, human populations exposed to curcumin show significantly reduced incidence of
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24 Alzheimer's disease, but not ApOE polymorphisms [79], and improved cognitive ability as
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26 compared to a control group [80].
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5. REFERENCES

- [1] R. Sadoul, M. Hirn, H. Deagostini-Bazin, G. Rougon and C. Goridis, Adult and embryonic mouse neural cell adhesion molecules have different binding properties, *Nature* **30** (1983), pp. 347-349.
- [2] G. Rougon, C. Dubois, N. Buckley, J.L. Magnani and W. Zollinger, A monoclonal antibody against meningococcus group B polysaccharides distinguishes embryonic from adult N-CAM, *J Cell Biol* **103** (1986), pp. 429-437.
- [3] L. Bonfanti, PSA-NCAM in mammalian structural plasticity and neurogenesis, *Prog. Neurobiol* **80** (2006), pp. 129-164.
- [4] E. Gascon, L. Vutskits and J.Z. Kiss, Polysialic acid-neural cell adhesion molecule in brain plasticity: From synapses to integration of new neurons, *Brain Res Rev* **56** (2007), pp. 101-118.
- [5] U. Rutishauser, Polysialic acid in the plasticity of the developing and adult vertebrate nervous system, *Nat. Rev. Neurosci.* **9** (2008), pp. 26-35.
- [6] Z.S. Hoyk, A. Parducz and D.T. Theodosis, The highly sialylated isoform of the neural cell adhesion molecule is required for estradiol-induced morphological plasticity in the adult arcuate nucleus, *Eur J Neurosci* **13** (2001), pp. 649-656.

- [7] L. Stoenica, O. Senkov, R. Gerardy-Schahn, B. Weinhold, M. Schachner and A. Dityatev, *In vivo* synaptic plasticity in the dentate gyrus of mice deficient in the neural cell adhesion molecule NCAM or its polysialic acid, *Eur J Neurosci* **23** (2006), pp. 2255-2264.
- [8] A. O'Malley, C. O'Connell and C.M. Regan, Ultrastructural analysis reveals avoidance conditioning to induce a transient increase in hippocampal dentate spine density in the 6h post-training period of consolidation, *Neuroscience* **87** (1998), pp. 607-613.
- [9] A. O'Malley, C. O'Connell, K.J. Murphy and C.M. Regan, Transient spine density increases in the mid-molecular layer of the hippocampal dentate gyrus accompany consolidation of a spatial learning task in the rodent. *Neuroscience* **99** (2000), pp. 229-232.
- [10] M.D. Eyre, G. Richter-Levin, A. Avital and M.G. Stewart, Morphological changes in the hippocampal dentate gyrus synapses following spatial learning in rats are transient. *European J Neurosci* **17** (2003), pp. 1973-80
- [11] G.B. Fox, A.W. O'Connell, K.J. Murphy and C.M. Regan, Memory consolidation induces a transient and time-dependent increase in the frequency of NCAM-polysialylated cells in the adult rat hippocampus, *J Neurochem* **65** (1995), pp. 2796-2779.
- [12] K.J. Murphy, A.W. O'Connell and C.M. Regan, Repetitive and transient increases in hippocampal neural cell adhesion molecule polysialylation state following multi-trial spatial training, *J Neurochem* **67** (1996), pp. 1268-1274.

- [13] C. Sandi, J.J. Merino, M.I. Cordero, N. Krut, K.J. Murphy and C.M. Regan, Modulation of hippocampal NCAM polysialylation by stressful experiences, *Biol Psychiat* **15** (2003), pp. 599-607.
- [14] C.G. Becker, A. Artola, R. Gerardy-Schahn, T. Becker, H. Welzl and M. Schachner, The polysialic acid modification of the neural cell adhesion molecule is involved in spatial learning and hippocampal long-term potentiation, *J Neurosci Res* **45** (1996), pp. 143-152.
- [15] D. Muller, C. Wang, G. Skibo, N. Toni, H. Cremer, V. Calaora *et al.*, PSA-NCAM is required for activity-induced synaptic plasticity, *Neuron* **17** (1996), pp. 413-422.
- [16] C. Venero, A.I. Herrero, K. Touyarot, K. Cambon, M.A. López-Fernández, V. Berezin *et al.*, Hippocampal up-regulation of NCAM expression and polysialylation plays a key role on spatial memory, *Eur J Neurosci* **23** (2006), pp. 1585-95.
- [17] M.A. López-Fernández, M.-F. Montaron, E. Varea, G. Rougon, C. Venero, D.N. Abrous *et al.*, Upregulation of polysialylated neural cell adhesion molecule in dorsal hippocampus after contextual fear conditioning is involved in long-term memory formation, *J Neurosci* **27** (2007), pp. 4552-4561.
- [18] C.M. Seymour, A.G. Foley, K.J. Murphy and C.M. Regan, Intraventricular infusions of anti-NCAM PSA impair the process of consolidation of both avoidance conditioning and spatial learning paradigms in Wistar rats, *Neuroscience* (2008), In Press.

- [19] E.P. Scheidegger, L.R. Sternberg, J. Roth and J.B. Lowe, A human STX cDNA confers polysialic acid expression in mammalian cells, *J Biol Chem* **270** (1995), pp. 22685-22688
- [20] Y. Yoshida, N. Kojima, N. Kurosawa, T. Hamamoto and S. Tsuji, Molecular cloning of Sia alpha 2,3Gal beta 1,4GlcNAc alpha 2,8-sialyltransferase from mouse brain, *J Biol Chem* **270** (1995), pp. 14628-14633.
- [21] M. Eckhardt, M. Mühlenhoff, A. Bethe, J. Koopman, M. Frosch and R. Gerardy-Schahn, Molecular characterization of eukaryotic polysialyltransferase-1, *Nature* **373** (1995), pp. 715-718.
- [22] J. Nakayama, M.N. Fukuda, B. Fredette, B. Ranscht and M. Fukuda, Expression cloning of a human polysialyltransferase that forms the polysialylated neural cell adhesion molecule present in embryonic brain, *Proc Natl Acad Sci USA* **92** (1995), pp. 7031-7035.
- [23] H. Hildebrandt, C. Becker, M. Murau, R. Gerardy-Schahn and H. Rahmann, Heterogenous expression of the polysialyltransferases ST8Sia II and ST8Sia IV during postnatal rat brain development, *J Neurochem* **71** (1998), pp. 2339-2348.
- [24] E. Ong, J. Nakayama, K. Angata, L. Reyes, T. Katsuyama, Y. Arai and M. Fukuda, Developmental regulation of polysialic acid synthesis in mouse directed by two polysialyltransferases, PST and STX, *Glycobiology* **8** (1998), pp. 415-424.

[25] G. Alcaraz and G. Goridis, Biosynthesis and processing of polysialylated NCAM by AtT20 cells. *Eur J Cell Biol* **55** (1991), pp. 165-173.

[26] H.C. Gallagher, O.A. Odumeru and C.M. Regan CM, Regulation of neural cell adhesion molecule polysialylation state by cell-cell contact and protein kinase C delta, *J Neurosci Res* **61** (2000), pp. 636-645.

[27] H.C. Gallagher, K.J. Murphy, A.G. Foley and C.M. Regan, Protein kinase C delta regulates neural cell adhesion molecule polysialylation state in the rat brain, *J Neurochem* **77** (2001), pp. 425-34.

[28] G.B. Fox, N. Kennedy, and C.M. Regan, Polysialylated neural cell adhesion molecule expression by neurons and astroglia processes in the rat dentate gyrus declines dramatically with increasing age, *Int J Dev Neurosci* **13** (1995), pp. 663-72.

[29] X. Gu, U. Preuß, T. Gu and R.K. Yu, Regulation of sialyltransferase activities by phosphorylation and dephosphorylation, *J Neurochem* **64** (1995), pp. 2295-2302.

[30] L. Gao, X.-B. Gu, D.S. Yu, R.K. Yu and G. Zeng, Association of a 14-3-3 protein with CMP-NeuNAc: GM1 α 2,3-sialyltransferase, *Biochem Biophys Res Commun* **224** (1996), pp. 103-107.

- [31] J. Ma, M. Siminovic, R. Qian and K.J. Colley, Sialyltransferase isoforms are phosphorylated in the cis-medial Golgi on serine and threonine residues in their luminal sequences. *J Biol Chem* **274** (1999), pp. 8046-8052.
- [32] K.C. Breen and N. Georgopoulou, The role of protein phosphorylation in $\alpha 2,6(N)$ -sialyltransferase activity, *Biochem Biophys Res Comm* **309** (2003), pp. 32-35.
- [33] A. Kishimoto, K. Mikawa, K. Hashimoto, I. Yasuda, S. Tanaka, M. Tominaga *et al.*, Limited proteolysis of protein kinase C subspecies by calcium-dependent neutral protease (calpain), *J Biol Chem* **264** (1989), pp. 4088-4092.
- [34] R.A. Blake, P. Garcia-Paramio, P.J. Parkera and S.A. Courtneidge Src promotes PKC δ degradation, *Cell Growth Diff* **10** (1999), pp. 231-241.
- [35] M. Gschwendt, H.J. Muller, K. Kielbassa, R. Zang, W. Kittstein, G. Rincke *et al.*, Rottlerin, a novel protein kinase inhibitor, *Biochem Biophys Res Commun* **199** (1994), pp. 93-98.
- [36] S.P. Davies, H. Reddy, M. Caivano and P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, *Biochem J* **351** (2000), pp. 95-105.
- [37] S.P. Soltoff, Rottlerin: an inappropriate and ineffective inhibitor of PKC δ , *Trends Pharmacol Sci* **28** (2007), pp. 453-458.

- [38] C.A. Lipinski, F. Lombardo, B.W. Dominy and F.P. Feeney, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings, *Adv Drug Deliv Rev* **23** (1997), pp. 3–25.
- [39] K. Godl, J. Wissing, A. Kurtenbach, P. Habenberger, S. Blencke, H. Gutbrod *et al.*, An efficient proteomics method to identify the cellular targets of protein kinase inhibitors, *Proc Natl Acad Sci USA* **100** (2003), pp. 15434-15439.
- [40] G. Zhang, M.G. Kazanietz, P.M. Blumberg and J.H. Hurley, Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester, *Cell* **81** (1995) pp. 917-924.
- [41] Y.J. Wang, M.H. Pan, A.L. Cheng, L.I. Lin, Y.S. Ho, C.Y. Hsieh *et al.*, Stability of curcumin in buffer solutions and characterization of its degradation products. *J Pharm Biomed Anal* **15** (1997), pp. 1867-1876.
- [42] K. Mizuno, T.C. Saido, S. Ohno, T. Tamaoki and K. Suzuki, Staurosporine-related compounds, K252a and UCN-01, inhibit both cPKC and nPKC, *FEBS Lett* **330** (1993), pp. 114-116.
- [43] Z. Lu, D. Liu, A. Hornia, W. Devonish, M. Pagano and D.A. Foster, Activation of protein kinase C triggers its ubiquitination and degradation, *Mol Cell Biol* **18** (1998), pp. 839-845.

- [44] S.F. Steinberg, Distinctive activation mechanisms and functions for protein kinase Cdelta, *Biochem J* **384** (2004), pp. 449-459.
- J.M. Zingg and A. Azzi, Non-antioxidant activities of vitamin E, *Curr Med Chem* **11** (2004), pp. 1113-1133.
- [45] P. Ferri, T. Cecchini, P. Ambrogini, M. Betti, R. Cuppini, P. Del Grande *et al.*, α -Tocopherol affects neuronal plasticity in adult rat dentate gyrus: The possible role of PKC δ . *J Neurobiol* **66** (2006), pp. 793-810.
- [46] A. Goel, A.B. Kunnumakkara and B.B. Aggarwal, Curcumin as "Curecumin": From kitchen to clinic, *Biochem Pharmacol* **75** (2008), pp. 787-809.
- [47] M. Ebadi, S. Sharma, S. Shavali and H. El Reaey, Neuroprotective actions of selegiline, *J Neurosci Res* **67** (2002), pp. 285-289.
- [48] K.J. Murphy, A.G. Foley, A.W. O'Connell and C.M. Regan, Chronic exposure of rats to cognition enhancing drugs produces a neuroplastic response identical to that obtained by complex environment rearing, *Neuropsychopharmacology* **31** (2006), pp. 90-100.
- [49] V. Duvéau, S. Arthaud, A. Rougier and G. Le Gal le Salle, Polysialylation of NCAM is upregulated by hyperthermia and participates in heat shock preconditioning-induced neuroprotection, *Neurobiol Disease* **26** (2007), pp. 385-395.

- 1
- 2
- 3 [50] S.J. Kim, T.G. Son, H.R. Park, M. Park, M.S. Kim, H.S. Kim *et al.*, Curcumin stimulates
- 4 proliferation of embryonic neural progenitor cells and neurogenesis in the adult hippocampus, *J*
- 5 *Biol Chem* **283**, pp. 14497-14505.
- 6
- 7
- 8
- 9
- 10
- 11
- 12 [51] A.G. Foley, W.D. Hirst, H.G. Gallagher, C. Barry, J.J. Hagan, N. Upton, A.J. Hunter, F.S.
- 13 Walsh and C.M. Regan, The selective 5-HT₆ receptor antagonists SB-271046 and SB-399885
- 14 potentiate NCAM-PSA immunolabelling of dentate granule cells, but not neurogenesis, in the
- 15 hippocampal formation of mature Wistar rats, *Neuropharmacology* **54** (2008), pp. 1166-1174.
- 16
- 17
- 18
- 19
- 20
- 21
- 22
- 23
- 24 [53] S. Ge, C.H. Yang, K.S. Hsu, G.L. Ming and H. Song, A critical period for enhanced synaptic
- 25 plasticity in newly generated neurons of the adult brain. *Neuron* **54** (2007), pp. 559-566.
- 26
- 27
- 28
- 29
- 30
- 31 [54] N. Kee, C.M. Teixeira, A.H. Wang, and P.W. Frankland, Preferential incorporation of adult-
- 32 generated granule cells into spatial memory networks in the dentate gyrus, *Nat Neurosci* **10**
- 33 (2007), pp. 355-362.
- 34
- 35
- 36
- 37
- 38
- 39
- 40
- 41 [55] N. Toni, D.A. Laplagne, C. Zhao, G. Lombardi, C.E. Ribak, F.H. Gage *et al.*, Neurons born
- 42 in the adult dentate gyrus form functional synapses with target cells, *Nature Neurosci* **11** (2008),
- 43 pp. 901-907.
- 44
- 45
- 46
- 47
- 48
- 49
- 50 [56] K. Pham, B.S. McEwen, J.E. LeDoux and K. Nader, Fear learning transiently impairs
- 51 hippocampal cell proliferation, *Neuroscience* **130** (2005), pp. 17-24.
- 52
- 53
- 54
- 55
- 56
- 57
- 58
- 59
- 60
- 61
- 62
- 63
- 64
- 65

- [57] C. Sandi, M.I. Cordero, J.J. Merino, N.D. Kruyt, C.M. Regan and K.J. Murphy, Neurobiological correlates of individual differences in spatial learning ability, *Learn Mem* **11** (2004), pp. 244-252.
- [58] D. Young, P.A. Lawlor, P. Leone, M. Dragunow and M.J. During, Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. *Nature Med* **5** (1999), pp. 448-453.
- [59] Y. Liu, R. Dargusch, P. Maher, and D. Schubert, A broadly protective derivative of curcumin, *J Neurochem* **105** (2008), pp. 1336-1345.
- [60] P. Maher, T. Akaishi, D. Schubert, and K. Abe, A pyrazole derivative of curcumin enhances memory, *Neurobiol Aging* (2008), In Press.
- [61] A.J. Silva, R. Paylor, J.M. Wehner, S. Tonegawa, Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice, *Science* **257** (1992), pp. 206-211
- [62] E.J. Williams, B. Mittal, F.S. Walsh, and P. Doherty, A Ca²⁺/calmodulin kinase inhibitor, KN-62, inhibits neurite outgrowth stimulated by CAMs and FGF, *Mol Cell Neurosci* **6** (1995), pp. 69-79.

- [63] B. Jagatha, R.B. Mythri, S. Vali and M.M.S. Bharath, Curcumin treatment alleviates the effects of glutathione depletion in vitro and in vivo: Therapeutic implications for Parkinson's disease explained via in silico studies, *Free Radic Biol Med* **44** (2008), 907-917.
- [64] P.K. Shukla, V.K. Khanna, M.M. Ali, M.Y. Khan and R.C. Srimal, Anti-ischemic effect of curcumin in rat brain, *Neurochem Res* **33** (2008), pp. 1036-1043.
- [65] E.K. Ryu, Y.S. Choe, K.H. Lee, Y. Choi and B.T. Kim, Curcumin and dihydrozingerone derivatives: synthesis, radiolabelling, and evaluation for beta-amyloid plaque imaging, *J Med Chem* **49** (2006), pp. 6111-6119.
- [66] T.N. Shankar, N.V. Shanta, H.P. Ramesh, I.A. Murthy and V.S. Murthy, Toxicity studies on tumeric (*Curcuma longa*): acute toxicity studies in rats, guineapigs and monkeys, *Indian J Exp Biol* **18** (1980), pp. 73-75.
- [67] C.D. Lao, M.T. Ruffin, D. Normolle, D.D. Heath, S.I. Murray, J.M. Bailey *et al.*, Dose escalation of a curcuminoid formulation, *BMC Complement Altern Med* **6** (2006), pp. 10.
- [68] D. N. Abrous, M.F. Montaron, K.G. Petry, G. Rougon, M. Darnaudery, M. Le Moal *et al.*, Decrease in highly polysialylated neuronal cell adhesion molecules and in spatial learning during ageing are not correlated, *Brain Res* **744** (1997), pp. 285-292.

- [69] M.E. Stanton, Multiple memory systems, development and conditioning, *Behav Brain Res* **110** (2000), pp. 25-37.
- [70] A Romano, R. Freudenthal, E. Merlo, and A. Routtenberg, Evolutionarily-conserved role of the NF-kappaB transcription factor in neural plasticity and memory, *Eur J Neurosci* **24** (2006), pp. 1507-1516.
- [71] Y. Wang, S.L. Chan, L. Miele, P.J. Yao, J. Mackes, D.K. Ingram et al., Involvement of Notch signalling in hippocampal plasticity, *Proc Natl Acad Sci. USA* **101** (2004), pp. 9458-9462.
- [72] Y. Chen, W. Shu, W. Chen, Q. Wu, H. Liu and G. Cui, Curcumin, both Histone Deacetylase and p300/CBP-Specific Inhibitor, Represses the Activity of Nuclear Factor Kappa B and Notch 1 in Raji Cells, *Basic Clin Pharmacol Toxicol* **101** (2007), pp. 427-433.
- [73] L. Conboy, C.M. Seymour, M.P. Monopoli, N.C. O'Sullivan, K.J. Murphy and C.M. Regan, Notch signalling becomes transiently attenuated during long-term memory consolidation in adult Wistar rats, *Neurobiol Learn Mem* **88** (2007) pp. 342-351.
- [74] S. Shishodia, T. Singh and M.M. Chaturvedi, Modulation of transcription factors by curcumin, *Adv Exp Med Biol* **595** (2007), pp. 127-148.

- [75] M. Mikkonen, H. Soininen, T. Tapiola, I. Alafuzoff and R. Miettinen, Hippocampal plasticity in Alzheimer's disease: changes in highly polysialylated NCAM immunoreactivity in the hippocampal formation, *Eur J Neurosci* **11** (1999), pp. 1754-1764.
- [76] A. Wu, Z. Ying and F. Gomez-Pinilla, Dietary curcumin counteracts the outcome of traumatic brain injury on oxidative stress, synaptic plasticity, and cognition, *Exp Neurol* **197** (2006), pp. 309-317.
- [77] Y. Xu, B. Ku, L. Tie, H. Yao, W. Jiang, X. Ma and X. Li, Curcumin reverses the effects of chronic stress on behaviour, the HPA axis, BDNF expression and phosphorylation of CREB, *Brain Res* **1122** (2006), pp. 56-64.
- [78] A. N. Begum, M.R. Jones, G.P. Lim, T. Morihara, P. Kim, D.D. Heath *et al.*, Curcumin structure-function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease, *J Pharmacol Exp Ther* **326** (2008), pp. 196-208.
- [79] M. Ganguli, V. Chandra, M.I. Kamboh, J.M. Johnston, H.H. Dodge, B.H. Thelma *et al.*, Apolipoprotein E polymorphism and Alzheimer disease, *Arch Neurol* **57** (2000), pp. 824-830.
- [80] T.P. Ng, P.C. Chiam, T. Lee, H.C. Chua, L. Lim, and E.H. Kua, Curry consumption and cognitive function in the elderly, *Am J Epidemiol* **164** (2006), pp. 898-906.

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FIGURE LEGENDS

Figure 1: *In silico* approach to identification of curcumin as a rottlerin analogue.

The chemical structure of rottlerin and curcumin with the pharmacophore side chain in bold is shown in Panel A. Crystal structure of the PKC δ C1B binding site is shown in Figure 1Bi along with its interaction with the phorbol ester (Figure 1Bii) and curcumin (Figure 1Biii). Figure 1C shows a histogram plot of the 137 rottlerin-like molecules and their strength of their interaction, based on dock score, with the PKC δ C1B domain (Figure 1C). Figure 1D demonstrates the curcumin-induced, dose-dependent increase in PSA expression in neuro-2A neuroblastoma cells.

Figure 2: Influence of curcumin and rottlerin on recombinant PKC δ activity. Panel A illustrates the purity of the enzyme preparation (lane 1: enzyme preparation; lane 2: molecular weight markers) and Panel B shows enzyme activity with increasing concentrations of recombinant PKC δ . Panel C demonstrates inhibition of enzyme activity, in the presence of 40 μ M (filled columns) and 200 μ M (open columns) ATP, using staurosporine as a positive control. This panel also shows rottlerin and curcumin to be without effect on PKC δ activity. The data is expressed as the mean $\pm$ SEM (n=3) and values significantly different ($p<0.05$) from the vehicle-treated controls are indicated with an asterisk.

Figure 3: Association of curcumin-conjugated Sepharose-6B with PKC δ in lysates from the hippocampal dentate gyrus. Panel A shows the Coomassie blue-stained gel analysis of bead-associated protein (lanes 1 and 2) and the remaining lysate following bead incubation (lanes 4

and 5). Lane 5 shows the protein profile of the lysate. All lanes had equal protein loading. Panel B shows the immunoblot analysis of the same lanes with anti-PKC δ .

Figure 4: Influence of curcumin on PKC δ expression in neuro2A neuroblastoma cells. The immunoblot in Panel A illustrates a curcumin-induced, dose-dependent decrease in PKC δ enzyme protein and the histogram in Panel B shows the same data expressed in a semi-quantitative densitometric manner. Equal protein loading is shown with the actin immunoblot in Panel A. Panel C shows the dose-dependent increase in PKC δ tyrosine³¹¹ phosphorylated PKC δ and its fragments in the presence of curcumin and Panel D is a semi-quantitative densitometric of the same data. All data is expressed as the mean $\pm$ SEM (n=3) and values significantly different (p<0.05) from the vehicle-treated controls are indicated with an asterisk.

Figure 5: Influence of chronic curcumin administration on polysialylated cell frequency in the infragranular zone of the hippocampal dentate gyrus of the adult Wistar rat (postnatal day 80). Panel A and B form a qualitative comparison of dentate polysialylated cells (see arrow heads in Panel B) and Panel C represents their quantitative analysis. The animals received 75 mg/kg curcumin by gavage each day for 8 days. All data is expressed as the mean $\pm$ SEM (n=3) and values significantly different (p<0.05) from the vehicle-treated controls are indicated with an asterisk.

Figure 6: Influence of chronic administration of curcumin on spatial learning in adult and aged Wistar rats. Panels A and B illustrate the influence of curcumin on acquisition and consolidation, respectively, of the water maze paradigm in adult animals. Task acquisition was carried out over

four daily sessions of five trials and consolidation was assessed by determining recall at 24 h and 72 h following the last training session. Panels C and D illustrate the same data for the aged animal group. Both the adult (n=10 for both vehicle and curcumin groups) and aged (n=7 for vehicle group; n=5 for the aged group) cohorts received 75 mg/kg curcumin by gavage each day for 8 days. Significant difference in task acquisition over 4 daily sessions was determined using a two-way repeated measure ANOVA with Bonferroni *post hoc* test and Mann-Whitney *U*-test employed to determine significance at the 24 h and 72 h recall times (* $P < 0.05$ vs. vehicle).

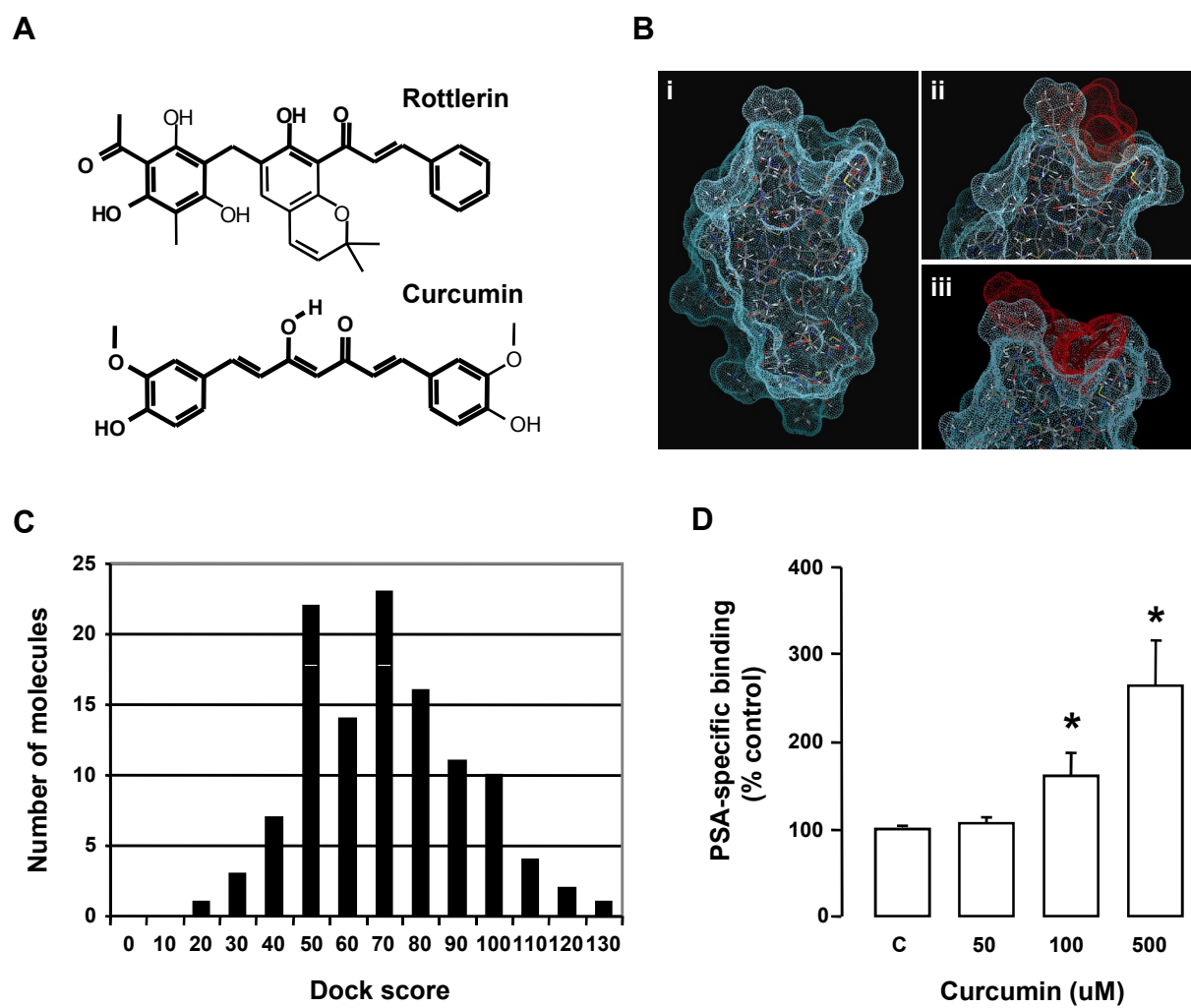


Figure 1; Conboy et al.

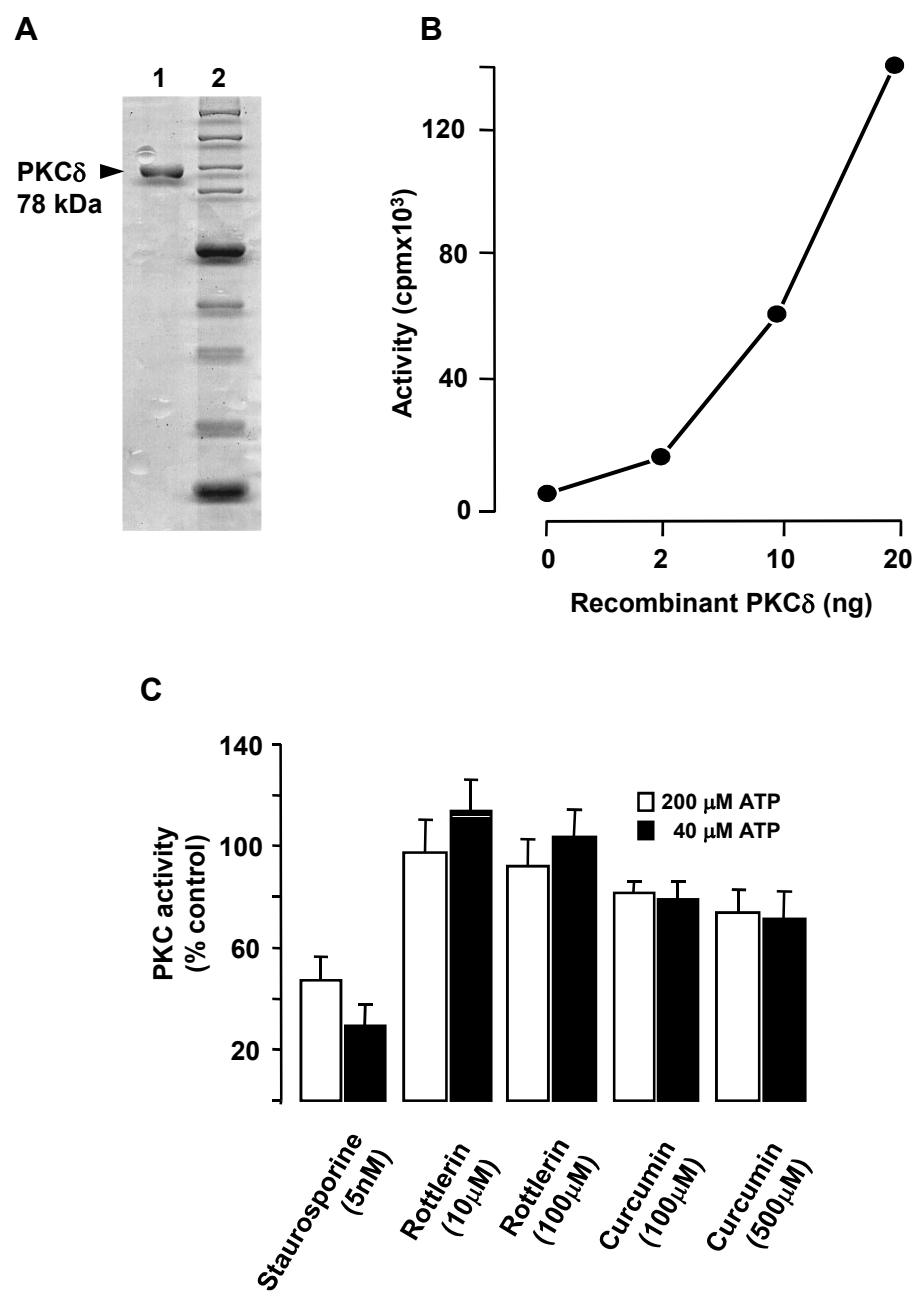


Figure 2; Conboy et al.

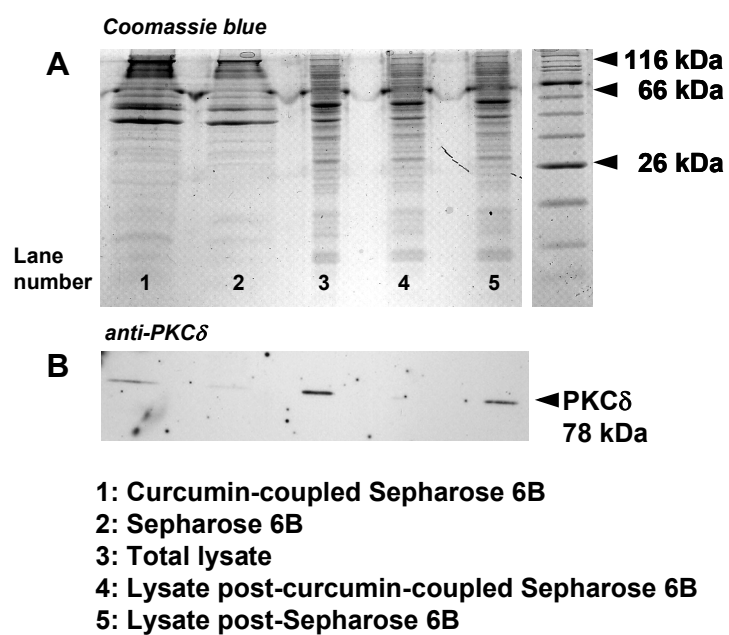


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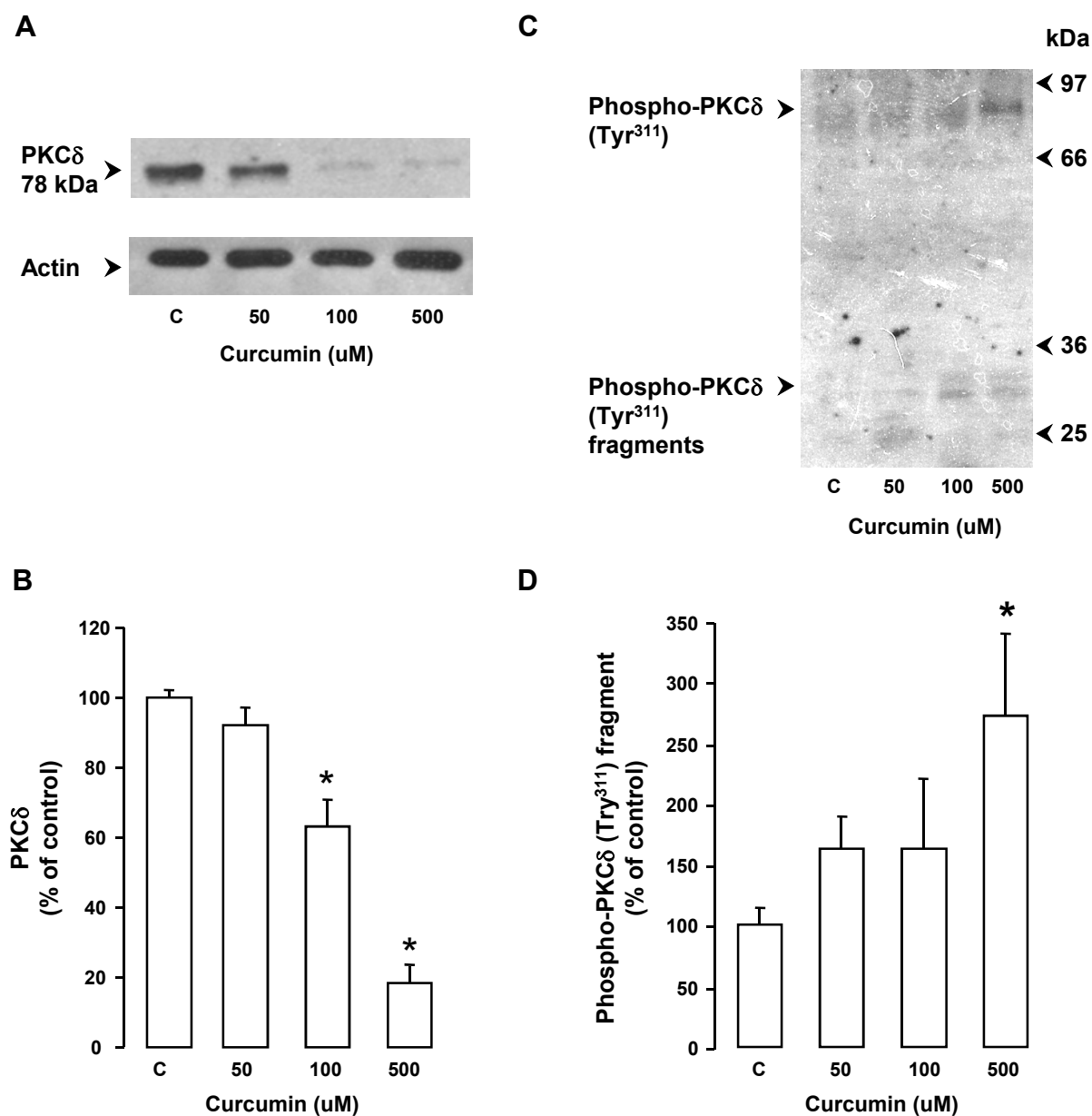


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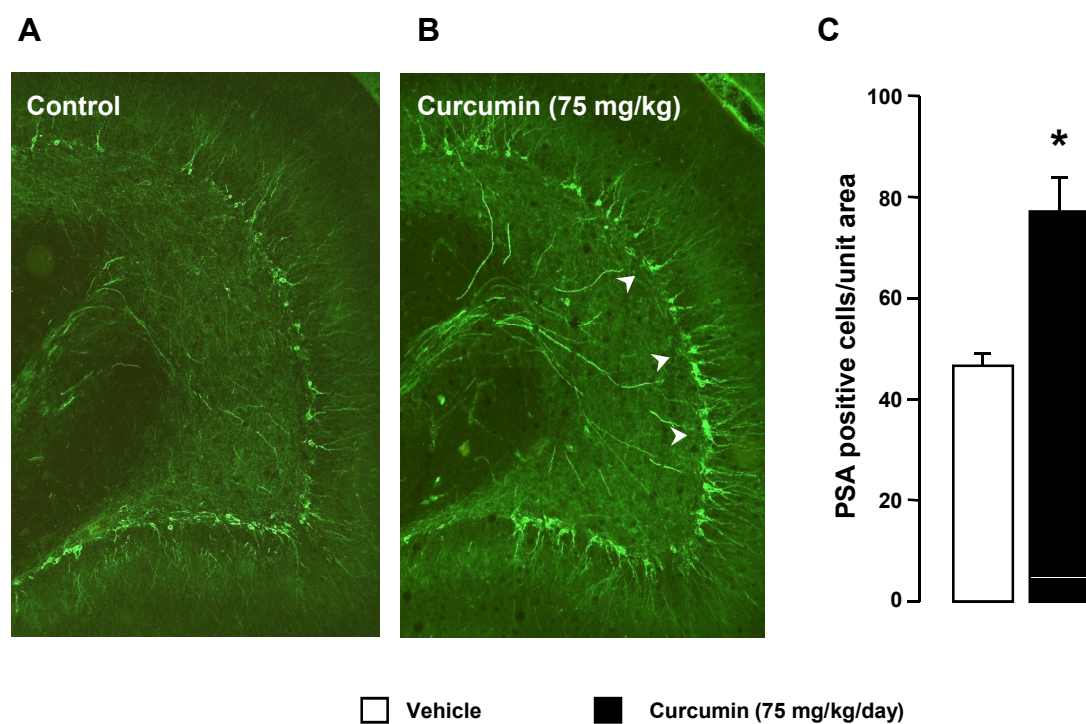


Figure 5; Conboy et al.

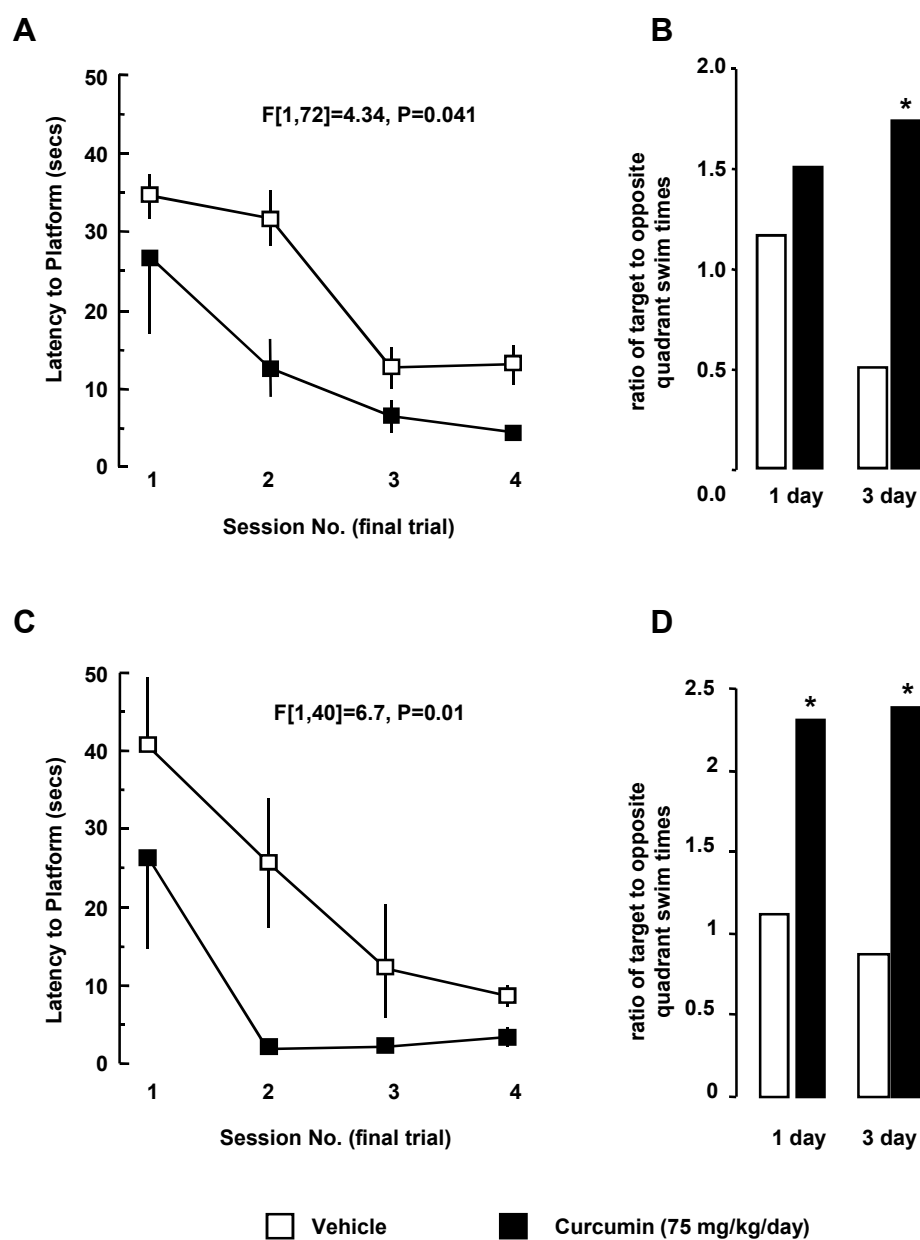


Figure 6; Conboy et al.

Curcumin increases NCAM polysialylation state through degradation of the PKC δ inhibitor of polysialyltransferase and improves spatial learning in adult and aged Wistar rats.

