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**MITOCHONDRIAL TARGETING OF CYCLOSPORIN A ENABLES SELECTIVE
INHIBITION OF CYCLOPHILIN-D AND ENHANCED CYTOPROTECTION
AFTER GLUCOSE AND OXYGEN DEPRIVATION**

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Short title: Mitochondrial-targeted cyclosporin A

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ABSTRACT

Cyclosporin A is a hydrophobic undecapeptide that inhibits cyclophilins, a family of peptidylprolyl cis-trans-isomerases. In some experimental models, cyclosporin A offers partial protection against lethal cell injury brought about by transient ischaemia; this is believed to reflect inhibition of cyclophilin-D, a mitochondrial isoform that facilitates formation of the permeability transition pore in the mitochondrial inner membrane. To evaluate this further, we have targeted cyclosporin A to mitochondria so that it becomes selective for cyclophilin-D in cells. This was achieved by conjugating the inhibitor to the lipophilic triphenylphosphonium cation, enabling its accumulation in mitochondria due to the inner membrane potential. In a cell-free system and in B50 neuroblastoma cells the novel reagent (but not cyclosporin A itself) preferentially inhibited cyclophilin-D over extramitochondrial cyclophilin-A. In hippocampal neurons, mitochondrial targeting markedly enhanced the capacity of cyclosporin A to prevent cell necrosis brought about by oxygen and glucose deprivation, but largely abolished its capacity to inhibit glutamate-induced cell death. It is concluded that cyclophilin-D has a major pathogenic role in “energy failure”, but not in glutamate excitotoxicity, where cytoprotection primarily reflects cyclosporin A interaction with extramitochondrial cyclophilins and calcineurin. Moreover, the therapeutic potential of cyclosporin A against ischaemia / reperfusion injuries not involving glutamate may be improved by mitochondrial targeting.

Keywords. Hippocampal neurons, ischaemia, glutamate toxicity, necrosis, cyclophilins, cyclosporin

Abbreviations. CsA, cyclosporin A; CyP, cyclophilin; FKBP, FK506 binding protein; DCM, dichloromethane; DMF, dimethylformamide; GST, glutathione-S transferase; I / R, ischaemia / reperfusion; LDA, lithium diisopropylamide; L-NAME, nitro-L-arginine-methyl ester; mtCsA, mitochondrial-targeted CsA; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine; NBQX, 2,3-dihydro-6-nitro-7-sulfamoylbenzoquinoline; NMDA, N-methyl-D-aspartate; OGD, oxygen and glucose deprivation; PPIase, peptidylprolyl *cis-trans*-isomerase; PT, permeability transition; PyBop, benzotriazol-1-yl-tris-pyrrolidinophosphonium hexafluorophosphate; SMBz-CsA, [Sarcosine-3(4-methylbenzoate)]CsA; THF, tetrahydrofuran; TMRE, tetramethylrhodamine ethyl ester; TPP⁺, triphenylphosphonium; $\Delta\phi_M$, mitochondrial inner membrane potential.

INTRODUCTION.

Ischaemic diseases, notably myocardial infarction and stroke, are the leading cause of death and disability throughout the world. In general, early restoration of blood flow is essential to restrict tissue damage. Paradoxically, however, as the duration of ischaemia increases, cells become adversely sensitive to restored blood supply, giving rise to additional damage on reperfusion (reperfusion injury). Resolving the critical cellular changes that underlie ischaemia/reperfusion (I/R) injury is a major goal in developing effective therapies.

A mitochondrial pore model for I/R injury was derived from two lines of evidence: First, the commonality between factors that induce formation of the permeability transition (PT) pore and recognised cellular changes in I/R, principally cellular Ca^{2+} overload, oxidative stress, and depleted adenine nucleotides [1,2]; second, inhibition of both pore formation and cell necrosis under pseudo I/R by cyclosporin A (CsA) [3-5]. The non-specific PT pore [6], estimated internal diameter 2 nm [1], allows free flow of low M_r solutes across the inner membrane. Under these conditions, mitochondria carry out ATP hydrolysis, rather than synthesis. Once ATP hydrolysis exceeds the glycolytic capacity of the cell to maintain ATP, irreversible injury leading to cell necrosis would ensue. The PT model of I/R injury is supported by whole organ and animal studies. In particular, CsA can attenuate reperfusion-induced cell necrosis in heart [7], brain [8] and other tissues. Use of 2-deoxyglucose [9] and calcein [10] reveals that the mitochondrial inner membrane does become non-selectively permeable on reperfusion, and that CsA can prevent the permeability changes. Genetic ablation of cyclophilin-D (CyP-D), a PT pore component and a target of CsA (below), markedly reduces infarct size in heart [11] and brain [12] following transient ischaemia.

CyP-D is a peptidylprolyl *cis-trans*-isomerase (PPIase), which is located in the mitochondrial matrix [13] and inhibited by CsA. CyP-D overexpression promotes pore formation [14] whereas CyP-D ablation attenuates [15]. It is believed that, under pathological conditions, CyP-D catalyses or stabilises a proline-dependent conformational switch in an inner membrane protein to form the non-selective pore. Conceivably, the CyP-D-sensitive protein is a transport protein that loses its selective transport properties when exposed to high $[\text{Ca}^{2+}]$ and oxidative stress. Reconstitution studies indicate that the adenine nucleotide translocase can deform into a non-selective pore under the influence of Ca^{2+} and CyP-D [16,17], but mitochondria from mice lacking the two translocase isoforms also display PT pore activity, albeit with much reduced Ca^{2+} sensitivity [18], and additional (alternative?) inner membrane proteins that can interact with CyP-D eg the phosphate carrier [19], have been proposed. Irrespective of the identity of the pore protein itself, however, there is little doubt that CsA inhibits PT pore formation by inhibiting CyP-D.

However, despite evidence that CyP-D has a key role in I/R injury, cytoprotection by CsA in experimental models is highly variable (and frequently marginal) and, in recent pilot trials, CsA yielded modest protection [20]. This suggests that CyP-D has a restricted pathogenic function and/or that CsA sensitivity is not a precise indicator of CyP-D involvement in cell death, possibly due to interaction with other cyclophilins. CyP-A (cytosol) and CyP-B (endoplasmic reticulum) have known links to cell viability (Discussion). In order to shed light on these issues,

we have developed a novel CsA derivative that is effectively CyP-D selective in cells by being accumulated in the mitochondrial compartment. Application of the mitochondrial-targeted CsA (mtCsA) to hippocampal neurons indicates that CyP-D has a major pathogenic role in energy failure, but only a minor role in glutamate excitotoxicity. Moreover, the cytoprotective capacity of CsA against the adverse consequences of energy failure is markedly improved by mitochondrial targeting.

MATERIALS AND METHODS

Preparation of recombinant CyP-D and CyP-A.

Recombinant rat CyP-D was prepared and purified as described previously [14]. For CyP-A, the coding sequence in rat was PCR-amplified with the addition of BamHI and EcoRI restriction sites, and cloned between the same sites of pGEX-4T-1 in E coli DH5 α cells. Transformed cells were grown for 5 hours at 21°C. The GST/CyP-A fusion protein was extracted, purified on GSH sepharose, and then cleaved with thrombin to release CyP-A. The CyP-A was purified on cation exchange (Mono-S) and gel filtration (Superdex-75) columns to give a single band on SDS-PAGE.

Interactions of cyclosporins with cyclophilins and calcineurin.

Dissociation constants for cyclophilin / cyclosporin interactions were measured as inhibitor constants, K_i . PPIase assays were conducted at 15°C in 100 mM NaCl / 20 mM Hepes (pH 7.5) using N-succinyl-alanyl-alanyl-prolyl-4-nitroanilide as test peptide as described before [14]. The peptide contains a mixture of *cis* and *trans* Ala-Pro isomers, of which only the *trans* conformer is hydrolysed by chymotrypsin at the C-terminal amide bond to release chromophore. Existing *trans* isomer is cleaved within the mixing time; further cleavage requires *cis-trans* isomerisation, which is measured. Cyclophilins were preincubated with cyclosporins for 5 min before addition of chymotrypsin and 60 μ M peptide (containing about 35 μ M *cis* peptide) to start the reaction. Cyclosporins inhibit by competing at the active site with substrate [21]. Accordingly, kinetic data were analysed by the Henderson equation for a tight binding, competitive inhibitor [22], which can be written:

$$\frac{I_o}{P} = \frac{1}{(1-P)} \cdot K_i \cdot \left(1 + \frac{S}{K_M}\right) + E_o$$

Where E_o and I_o are the total concentrations of enzyme and inhibitor (cyclosporin) respectively, K_i is the enzyme/inhibitor dissociation constant, K_M is the Michaelis constant, and S is the substrate concentration. P is the fractional inhibition, equal to $\{1 - (v_i/v_o)\}$, where v_i and v_o are the reaction velocities in the presence and absence of inhibitor, respectively. The K_M value for the *cis* peptide used (980 μ M; [21]) is much higher than its concentration in the assay (<35 μ M). Since $K_M \gg S$ the equation may be simplified:

$$\frac{I_o}{P} = \frac{1}{(1-P)} \cdot K_i + E_o \quad \text{Equation 1}$$

And plots of I_o / P against $1 / (1-P)$ are linear with slope = K_i

Interaction of cyclophilin / cyclosporin complexes with calcineurin was evaluated from inhibition of the phosphatase activity of calcineurin as measured by the release of inorganic phosphate from the RII phosphopeptide (Biomol International UK, Exeter, UK)

Experiments with isolated mitochondria.

Mitochondria were isolated from rat livers as described before [1]. PT pore opening was monitored by the associated swelling of the mitochondria [9] as measured by the decrease in absorbance at 540 nm. Mitochondria (2 mg of protein) were suspended in 3 ml of 120 mM KCl / 2 mM KH₂PO₄ / 3 mM succinate / 10 mM Hepes (pH 7.2) / 1 μM rotenone / 5 μM EGTA / recombinant CyP-A and test cyclosporins, and maintained under continuous stirring at 25°C. After 5 min, CaCl₂ was slowly infused (10 μM / min; see text) to a final concentration of 50 μM. Changes in preincubation time with CsA or mtCsA from 5 min to 2min or 10 min did not change the degree of PT pore inhibition. In a parallel incubation, mitochondria were sedimented at the time indicated (text) and the CyP-A activity of the supernatant determined (0.5 ml supernatant / ml reaction volume).

Neuronal cultures and assays.

B50 cells from a rat neuronal cell line and a clone stably overexpressing CyP-D were cultured on coverslips in DMEM (Dulbecco's minimal essential medium) containing 10% foetal calf serum as described before [14]. Uptake of TMRE was measured by incubating the cells at 25°C in basic medium (140 mM NaCl / 4 mM KCl / 24 mM Hepes (pH 7.4) / 1 mM MgSO₄ / 1 mM CaCl₂ / 1 mM KH₂PO₄ / 11 mM glucose) containing 50 nM TMRE. Fluorescence images (530 nm / >595 nm) were obtained with an Olympus IX-70 fluorescence microscope with X60 oil objective, Micromax 1401E CCD camera and Metamorph software (Universal imaging). For nitroprusside treatment, cells were incubated in basic medium containing 100 μM sodium nitroprusside for 40 min and then returned to DMEM medium. After 5 hr, cells were extracted and extracts assayed for caspase-3 activity using the fluorescent 7-amino-4-trifluoromethylcoumarin (AFC) derivative of the caspase-3 / -7 selective substrate (Ac-DEVD-AFC) as before [23]. For antisense suppression of CyP-A, cells were incubated with 1 μM phosphorothioate ODN 5'-CATGGCTTCCACAATGCT for 48 hours [23].

Hippocampal neurons were prepared from 2-4 day old Sprague Dawley rats as mixed cultures with glial cells. Dissected hippocampi were incubated in Hanks balanced salt solution (HBSS) containing 0.1% w/v trypsin for 5 min at 37°C, followed by two washes in HBSS. Hippocampi were then dissociated in HBSS containing 1 mg/ml BSA, 5% foetal calf serum and 8 mM MgCl₂. Dissociated cells were sedimented, suspended in Neurobasal A medium (NBA) supplemented with 0.5 mM glutamine, 2% B27 supplement (Gibco) and 5% foetal calf serum, seeded onto coverslips, and incubated under 95% air / 5% CO₂ in the same medium plus antimitotics mix (5-fluor-2'-deoxyuridine, uridine, 1-beta-D-arabinofuranosylcytosine, 1μM of each). Medium minus antimitotics was introduced after 3 days.

For oxygen and glucose deprivation (OGD), coverslips with hippocampal neurons were seated to form the base of a small, capped chamber mounted on the microscope stage. The chamber contained an inlet and outlet for continuous gassing, input and output tubes for changing the

incubation medium, and a heating element to maintain the temperature at 36°C. Pseudo-ischaemic conditions were imposed by omitting glucose and displacing air with N₂ in the experimental chamber. Cells were incubated under 95% N₂ / 5% CO₂ with (pregassed) 145 mM NaCl / 26 mM NaHCO₃ / 5 mM KCl / 1.8 mM CaCl₂ / 0.8 mM MgCl₂ / 4 μM ethidium homodimer / 2 μM Hoechst 33342 and cyclosporins as indicated. After 30 min the gassing was switched to 95% air / 5% CO₂ and the medium replaced with NBA medium containing 4 μM ethidium homodimer. Hippocampal neurons were identified under brightfield illumination and then correlated with their respective nuclei (just above the focal plane of glia nuclei) from Hoechst fluorescence. Necrosis was quantified from nuclear staining by ethidium homodimer. For treatment with glutamate, cultures were incubated under 95% air / 5% CO₂ in 150 mM NaCl / 5 mM KCl / 25 mM NaHCO₃ / 2.3 mM CaCl₂ / 6 mM Glucose / 5 mM Hepes (Lockes medium) containing cyclosporins (as indicated). After 10 min, 1 mM glutamate was added. After a further period (as indicated), cells were returned to NBA medium containing Hoechst 33342 and ethidium homodimer, and necrosis was quantified 15 min later.

Statistical analyses were made using a one-way ANOVA test with a post-test of Dunnett. Data are presented as means +/- SEM.

Synthesis of cyclosporin analogues.

A scheme of the syntheses is given in Figure 1.

Compound 2. [Sarcosine-3(4-methylbenzoate)]CsA, SMBz-CsA. To a stirred solution of CsA (1.00g, 0.83 mmol) in dry THF (tetrahydrofuran, 25 ml) under nitrogen at 0 °C was added dropwise fresh LDA (lithium diisopropylamide; 4.6 mmol, 2.3 ml, 2M in THF). To the resultant deep brown suspension was added, dropwise, trimethylsilylchloride (0.83 mmol, 0.1 ml) to give a clear brown solution. The mixture was stirred at 0 °C for 10 minutes. Then further LDA (7.1 mmol, 3.5 ml, 2M in THF) was added dropwise and the reaction stirred for 30 minutes at 0 °C. A solution of 4-bromomethylbenzoate (1.3 g, 5.8 mmol) in dry THF (10 ml) was added dropwise to give a pale yellow solution which was stirred for a further 1 hour at room temperature. The reaction was quenched with saturated aqueous ammonium chloride (10 ml) followed by 2M hydrochloric acid and then diluted with CH₂Cl₂ (20 ml). The separated aqueous layer was extracted with CH₂Cl₂ (2 x 20 ml). The combined organic phases were washed with 2M HCl(aq) (2 x 20 ml), saturated NH₄Cl(aq) (2 x 20 ml) and saturated NaCl (2 x 20 ml) and then dried (MgSO₄(s)). The volatiles were removed in vacuo to leave a dark brown oil residue. Purification by flash chromatography eluting with 6% methanol in CH₂Cl₂ gave a yellow solid residue (0.930 g) as a mixture of the unreacted CSA and the alkylated ester product. This was used in the next reaction without further purification.

Saponification: To a stirred solution of the yellow solid residue (0.93 g) in THF:methanol (1:1, 20 ml) at 0 °C was added dropwise a solution of LiOH·H₂O (500 mg) in water (10 ml). The reaction was allowed to gradually warm-up to room temperature over 18 hours. Then CH₂Cl₂ (20 ml) was added. The resultant solution was acidified with 2M HCl (aq) (pH = 3). The separated aqueous layer was extracted with CH₂Cl₂ (3 x 30 ml). The combined organic extracts were washed with saturated 2M HCl (aq) (2 x 30 ml) and saturated NaCl (2 x 30 ml) and then dried (MgSO₄(s)). The volatiles were removed in vacuo to leave a solid residue as a mixture of the acid (**2**, Figure 1) and unreacted CsA. The acid was separated from the CsA by flash column chromatography through an amine column eluting with a mixture of methanol:CH₂Cl₂:NH₃(aq) (1:8:1) to give the acid as a salt. After stirring the salt in CH₂Cl₂ (20 ml) and 2M HCl (aq) (20

ml) for 10 minutes, extraction by CH_2Cl_2 (3 x 20 ml), concentration, and purification by flash column chromatography gave the title compound, **2** (0.30 g, 0.24 mmol, 27 %) as a yellow solid. FAB+ve; Calc. m/z $\text{C}_{70}\text{H}_{117}\text{N}_{11}\text{O}_{14}$ (M+Na) 1358.86787, Found (M+Na) 1358.86447.

Compound 3. To a stirred solution of the acid **2** (109 mg, 0.08 mmol) in dry THF (3.0 ml) was added N-Fmoc-1,6-diaminohexane hydrobromide (68.5 mg, 0.16 mmol), PyBOP (84.5 mg, 0.16 mmol) and triethylamine (0.25 mmol, 0.4 ml) under nitrogen at room temperature and the resultant mixture was stirred for 24 hours. Then CH_2Cl_2 (5 ml) followed by saturated aqueous ammonium chloride (5 ml) were added. The mixture was extracted with CH_2Cl_2 (2 x 3 ml) and dried (MgSO_4). The volatiles were removed in vacuo to leave a brown oil residue. Purification by chromatography gave the Fmoc-protected derivative (100 mg) as a yellow solid. This was used without further purification.

Fmoc-deprotection: A solution of the Fmoc compound (100 mg) was stirred in 20% piperidine in DMF (dimethylformamide, 4 ml) under argon for 24 hours. The volatiles were removed al vacuo to leave a yellow oil. The oil was purified by flash column chromatography on silica gel eluting with 6% methanol in DCM (dichloromethane) followed by methanol:DCM: NH_3 (aq) (1:8:1) to afford the title compound **3** (70 mg, 0.05 mmol, 85%) as a yellow solid.

MS ES+ve; m/z $\text{C}_{76}\text{H}_{131}\text{N}_{13}\text{O}_{13}$ (M+1) 1435.00, (M+2) 718, Found: (M+1) 1435.53, (M+2) 718.76

Compound 4, mtCsA. To a stirred solution of the amine **3** (65 mg, 0.05 mmol) in dry THF (3 ml) under argon at room temperature was added in one portion PyBOP (35.5 mg, 0.07 mmol), 5-(carboxypentyl)triphenylphosphonium bromide (32 mg, 0.07 mmol) and triethylamine (0.15 mmol, 0.05 ml) and the resultant mixture stirred for 24 hours at room temperature. The volatiles were removed al vacuo to leave a yellow oil. The oil was purified by flash column chromatography on silica gel eluting with 6% methanol in DCM followed by methanol:DCM: NH_3 (aq) (1:8:1) to afford the title compound mtCsA, **4** (55 mg, 0.03 mmol, 70%) as a white solid.

ES+; Calc. m/z $\text{C}_{100}\text{H}_{155}\text{N}_{13}\text{O}_{14}\text{P}^+$ (M+1) 1793.8199, Found: (M+1) 1794.8270, (M+2) 898.

RESULTS

Synthesis of CsA derivatives and their interactions with cyclophilins and calcineurin.

CsA is a lipophilic cyclic undecapeptide (Figure 1). The highly conserved nature of the CsA binding site in cyclophilins offers little scope for CyP-D selective cyclosporins (Discussion). Therefore, we investigated the possibility of rendering CsA effectively CyP-D-selective in cells by targeting it to mitochondria, where CyP-D is located. The strategy was to conjugate CsA to the lipophilic triphenylphosphonium cation (TPP^+) so that the positively charged conjugate is accumulated electrophoretically by mitochondria in response to the negative-inside inner membrane potential. This principle has been widely applied in targeting antioxidants to mitochondria [24]. Seebach et.al [25] demonstrated that CsA may be specifically alkylated at the Sar-3 position if several equivalents of a strong base are used to generate a polyanion, and the same method was utilized here, as shown in Figure 1: CsA (1) was treated with lithium diisopropylamide (LDA) / trimethylsilyl chloride and then with LDA to generate the Sar-3 anion. This was alkylated with 4-bromomethylbenzoate and the crude product saponified with lithium

hydroxide to give the acid (2). The side chain was elongated by coupling with Fmoc-diaminohexane and Fmoc cleavage to yield the amine (3). This was coupled with 5-(carboxypentyl)triphenylphosphonium bromide to give the target triphenylphosphonium salt (4).

The effects of these modifications of CsA on interactions with CyP-D and CyP-A were investigated from the effects on PPIase inhibition. About 7 nM (total) CsA yielded 50% inhibition of CyP-D (Figure 2A). However, this underestimates the true CsA binding affinity since assays contained a similar concentration of CyP-D (8 nM). Accordingly, inhibition was analysed using the Henderson equation for a tight-binding inhibitor (Methods), which gave an inhibitory (dissociation) constant for CsA and CyP-D of 3 nM (inset Figure 2A). Addition to position 3 impaired binding slightly for SMBz-CsA (Figure 1), but strongly for mtCsA, where the binding affinity to CyP-D (K_i 93 nM) was about 30-fold lower than for CsA (Figure 2B). The binding affinities of CsA, SMBz-CsA and mtCsA to CyP-A (Figure 1) were similar to those with CyP-D.

In addition to inhibiting cyclophilins, CsA forms a complex with CyP-A that, in turn, inhibits the Ca^{2+} / calmodulin-dependent Ser / Thr protein phosphatase calcineurin [26] thereby enlarging considerably its sphere of action [27]. It was important, therefore, to establish how the 3-position modification affected the capacity of the complex to inhibit calcineurin. To enable comparisons, the concentrations of CsA (0.9 μ M), SMBz-CsA (1 μ M) and mtCsA (4 μ M) in the test incubations were chosen to establish the same concentrations (720 nM) of their respective complexes with CyP-A (calculated using the K_i values of CyP-A with CsA, SMBz-CsA and mtCsA; Figure 1). Figure 3 shows that, whereas CyP-A alone produced a small (20%) activation of calcineurin, the CsA / CyP-A complex (720 nM) inhibited by about 70%. In contrast, the SMBz-CsA / CyP-A and mtCsA / CyP-A complexes (720 nM) produced no inhibition. Conjugation to position 3 of the CsA ring evidently prevents formation of the ternary cyclophilin / cyclosporin / calcineurin complex which is known to require interactions between calcineurin and positions 3-7 of the ring (Discussion).

Evaluating the CyP-D selectivity of mtCsA in a mixed *in vitro* system

We first investigated whether mtCsA would select for intramitochondrial CyP-D and the PT pore, rather than extramitochondrial cyclophilins, using a test system comprising isolated mitochondria and externally-added, recombinant CyP-A. PT pore opening was induced by addition of high $[Ca^{2+}]$, and was monitored by the resultant mitochondrial swelling as the inner membrane became freely permeable to low M_r solutes. As Ca^{2+} influx into mitochondria is electrophoretic, the inner membrane potential, $\Delta\phi_M$, becomes dissipated during rapid Ca^{2+} uptake (and then restored when uptake is complete eg [14]). Since dissipation of $\Delta\phi_M$ would compromise accumulation of the positively-charged mtCsA in mitochondria, Ca^{2+} was infused slowly into the test incubations to limit the rate of Ca^{2+} uptake and thereby avoid membrane depolarisation (this was confirmed using a tetraphenylphosphonium electrode, as in Li et al, [14] and CsA to block PT pore opening; data not shown). CsA and derivatives inhibited pore opening as shown in Figures 4A, 4C and 4E. Estimation of the degrees of inhibition (from the decreases in absorbance attained at the time marked by the dashed lines), indicate that about 0.1 μ M CsA and 0.4 μ M mtCsA gave 50% inhibition of pore opening (closed symbols; Figures 4B and 4D). In the same system, CsA inhibited extramitochondrial CyP-A with a concentration profile similar

to that of the PT (Figure 4B); this was expected since CyP-D and CyP-A have similar binding affinities for CsA (Figure 1) and, being uncharged, CsA should equilibrate to the same free concentrations on either side of the inner membrane. In contrast, mtCsA inhibited PT pore formation considerably better than it inhibited CyP-A (Figure 4D), even though it binds to CyP-A and CyP-D with similar affinities (Figure 1). This indicates that mtCsA was accumulated in the mitochondrial matrix (where CyP-D is located) with respect to the external medium (containing CyP-A). SMBz-CsA gave similar inhibition profiles of the PT and CyP-A, indicating similar free [SMBz-CsA] across the inner membrane (Figure 4F).

Evaluating the CyP-D selectivity of mtCsA in intact cells.

Selectivity of mtCsA for CyP-D in intact cells was investigated using rat B50 neuroblastoma cells and a clone in which CyP-D is overexpressed about 10-fold {CyP-D(+) cells}. CyP-D overexpression did not affect CyP-A (Figure 5F). As shown previously [14], CyP-D(+) cells maintain a relatively low $\Delta\phi_M$, indicative of transient PT pore opening. Since the lowering of $\Delta\phi_M$ is caused by excessive CyP-D, restoration of $\Delta\phi_M$ to wild-type values provides an unequivocal measure of CyP-D inhibition. Changes in $\Delta\phi_M$ were monitored from the uptake of tetramethylrhodamine ethylester (TMRE), a fluorescent, lipophilic cation accumulated by mitochondria according to the magnitude of the potential. Figure 5A shows typical images of TMRE accumulated within the mitochondria of these cells. Mitochondria of the CyP-D(+) clone accumulated considerably less TMRE than wild type cells, but the difference was removed by CsA, which promoted uptake by the CyP-D(+) cells. Maximal restoration of TMRE uptake by CyP-D(+) cells was obtained with about 0.8 μM CsA (Figure 5C), 2.4 μM mtCsA (Figure 5D) and 2 μM SMBz-CsA (not shown). The same concentrations did not affect TMRE uptake by wild type cells (Figure 5B; confirmed also for SMBz-CsA). It may be concluded that about 0.8 μM CsA, 2 μM SMBz-CsA and 2.4 μM mtCsA are sufficient to inhibit CyP-D in B50 cells.

To investigate whether mtCsA inhibited CyP-D selectively ie without appreciable inhibition of CyP-A, we needed a marker of CyP-A activity. Previously, we reported that nitroprusside-induced caspase activation in B50 cells is largely prevented by CsA and by antisense suppression of CyP-A. This indicates that CsA inhibits caspase activation in this model by inhibiting CyP-A [23]. Although the specific site of action of CyP-A is unresolved, the system offers a measure of CyP-A activity. Antisense treatment decreased CyP-A expression by >85% without affecting CyP-D (Figure 5F), and antisense treatment, CSA, and SMBz-CsA all reduced nitroprusside-induced activation of caspase-3 (Figure 5E). Unlike CsA and SMBz-CsA, however, 2.4 μM mtCSA (sufficient to inhibit mitochondrial CyP-D; Figure 4D), had no significant effect on caspase activation, indicating that it is accumulated out of the cytosol, where CyP-A is located.

Ischaemia / reperfusion and glutamate-induced injury in hippocampal neurons.

Ischaemia / reperfusion (I/R) was mimicked by incubating hippocampal neurons under oxygen and glucose deprivation (OGD) for 30 min, after which glucose and O_2 were restored. To indicate the time period of OGD needed to remove O_2 sufficiently for impairment of mitochondrial electron transport, we followed TMRE loss from mitochondria of preloaded cells as an index of $\Delta\phi_M$ dissipation. TMRE was lost after about 5 min OGD indicating respiratory inhibition at this time (not shown). At the outset of each experiment, a group of hippocampal

neurons were distinguished from underlying glial cells and the same neurons were imaged at intervals thereafter. The susceptibility of neuronal cells (but not glial cells) to OGD-induced necrosis increased with days in culture, and data were obtained after culture for 24-28 days.

Following OGD, about 60% of neurons became necrotic within 90 min (Figure 6A), but mortality was approximately halved in the presence of mtCsA. Maximal protection was given with $>0.8 \mu\text{M}$ mtCsA (Figure 6B). CsA was less protective (Figure 6B). There was a relatively small protection with $0.1 \mu\text{M}$ CsA, but this was reversed at higher CsA concentrations, suggesting the existence of secondary CsA targets outside mitochondria that overrode the protection. Thus, restricting the action of CsA to mitochondria, using mtCsA, improves its protective capacity against cell necrosis brought about by a period of OGD, indicating that CyP-D and the PT are major contributors to this form of injury.

In vivo, a major component of I/R-induced neuronal injury is believed to be initiated by prolonged exposure to extracellular glutamate and overactivation of ionotropic glutamate receptors. To investigate PT involvement in glutamate toxicity, neurons were exposed to added glutamate under normoxic conditions in the presence of glucose. Following a short lag period, this led to progressive necrosis (Figure 7A). Surprisingly, the relative protective capacities of CsA and mtCsA were now reversed with respect to OGD. Whereas CsA yielded good protection, mtCsA was ineffective (Figure 7B). In a further series of experiments (Figure 8A), mtCsA did reduce necrosis to a small extent (24%, but not significant) but, again, CSA was far more effective (88% protection). Thus, restricting the action of CsA to mitochondria with mtCsA largely removes protection by CsA, indicating that CsA attenuates glutamate toxicity by interacting with extramitochondrial cyclophilins, rather than intramitochondrial CyP-D.

As expected, glutamate-induced necrosis was prevented by glutamate antagonists ie by MK-801, an NMDA (N-methyl-D-aspartate) receptor antagonist, and by NBQX, an AMPA (amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor antagonist (Figure 8A). However, glutamate antagonists had relatively little effect on OGD-induced necrosis (Figure 8B), indicating negligible contribution of glutamate toxicity under this protocol (any glutamate released from the cells during anoxia was presumably quickly diluted in the incubation medium). Thus, the involvement of extramitochondrial cyclophilins in CsA-protection correlates with glutamate toxicity, whereas their involvement in CSA-counterprotection correlates with OGD-induced necrosis. To investigate whether these effects, both protective (glutamate) and counterprotective (OGD), might involve inhibition of calcineurin by the CsA/CyP-A complex, we used FK506, which, as the FK506/FKBP complex, also inhibits calcineurin. As shown in Figure 8B, FK506 partially reversed mtCSA-protection against OGD-induced necrosis, indicating that calcineurin inhibition is counterprotective. In contrast, FK506 partially prevented glutamate-induced necrosis. Thus, calcineurin appears to have opposing roles in OGD-induced and glutamate-induced cell death.

Calcineurin dephosphorylates (activates) neuronal NO synthase, and this activation is believed to contribute to glutamate toxicity [27]. However, the NO synthase inhibitor L-NAME did not yield significant protection. Indeed, it is notable that both L-NAME and FK506 were less effective than CsA (Figure 8A). To test whether the acute effectiveness of CsA against glutamate toxicity reflected a dual action in inhibiting both calcineurin and CyP-D (and the PT

pore), we applied FK506 and mtCsA together; however this was no more effective than FK506 alone. This confirms that the inhibition of glutamate excitotoxicity by CsA is not due to inhibition of CyP-D.

The greater effectiveness of CsA than mtCsA plus FK506 in inhibiting glutamate-induced necrosis (Figure 8A) suggests that CsA-inhibition of extramitochondrial cyclophilins (PPIase activity) may also be protective. This was further investigated using SMBz-CsA, which inhibits cyclophilin PPIase activity (Figure 1), but not calcineurin (when complexed with CyP-A; Figure 3). Like CsA, SMBz-CsA permeates into mitochondria and inhibits the PT (Figure 4E), but is not accumulated by mitochondria (Figure 4F), and it enters cells where it inhibits CyP-A (Figure 5E). As shown in Figure 9, SMBz-CsA was more cytoprotective than mtCsA, consistent with some protection due to inhibition of extramitochondrial cyclophilins. Figure 9 also shows that SMBz-CsA was a better cytoprotectant than CsA following OGD and that, unlike CsA (Figure 6), protection was not reversed with increased [SMBz-CsA]. This may reflect the lack of calcineurin inhibition by SMBz-CsA, since calcineurin inhibition appears to be counterprotective in OGD-induced necrosis (mtCsA versus mtCsA plus FK506, Figure 8B). However, SMBz-CsA yielded less cytoprotection than mtCsA, suggesting that inhibition of extramitochondrial cyclophilins might also be counterprotective in OGD-induced necrosis (Figure 9). This is consistent with the fact that FK506 does not fully reverse cytoprotection by mtCsA (Figure 8B). In summary, the involvement of cyclophilins and calcineurin appears to differ between the two forms of injury: CyP-D inhibition is more protective after OGD than after glutamate, whereas inhibition of calcineurin and extramitochondrial cyclophilin(s) is more protective after glutamate than after OGD (when these interventions actually reverse protection). These differences point to different pathogenic mechanisms.

DISCUSSION

CsA is a lipophilic, cyclic undecapeptide which half-inserts, edge-on, into a cleft on the cyclophilin surface, occluding the active (PPIase) site, and forming interactions with fifteen amino acids of the cyclophilin [28]. The binding region in cyclophilins is highly conserved. In particular, apart from a single conservative substitution (Lys for Arg) the binding residues in CyP-A are all present in CyP-D, and a high resolution structure of CyP-D has confirmed that the same residues (as in CyP-A) make contact with CsA [29]. Although differences in adjacent amino acids might offer potential for selective inhibition, the CsA binding affinities of CyP-D and CyP-A are about the same (Figure 1). It is therefore difficult to envisage CyP-D selectivity based on binding affinities alone.

To address this problem, CsA was conjugated to the lipophilic cation, TPP⁺, to allow electrophoretic accumulation within mitochondria. The CyP-binding amino acids in CsA have been identified as residues 9-11 and 1-3 [28]. However, substitution at position 3 (sarcosine) does allow retention of tight binding to cyclophilins [30] (and refs therein), and the same position was utilized here for conjugation to TPP⁺. In the ternary CyP/CsA/calcineurin complex, positions 3-7 of the CsA ring form contacts with calcineurin [26,28]. As expected, therefore, the 3-position conjugates, SMBz-CsA and mtCsA, were unable to bind to calcineurin (Figure 3). The

3-position addition also reduced the binding affinity to cyclophilins (Figure 1). This reduction was marginal for SMBz-CsA, presumably reflecting steric factors, but marked in the case of mtCsA. A possible explanation lies in the fact that the predominant conformation of free CsA is quite different from that bound to cyclophilins [28] and modifications that stabilize the non-bound conformations would effectively lower the binding affinity for cyclophilins. In addition, mtCsA would be more lipophilic than CsA itself given the nature of the linking region. The TPP⁺ moiety, however, would not increase lipophilicity (octanol / buffered saline partition coefficient of triphenylmethylphosphonium = 0.35 [31]). In this connection, analyses of tritiated CsA binding to mitochondria reveal that they contain components besides CyP-D that bind CsA [32]; these components bind CsA far more weakly than CyP-D, but are present in excess. For mtCsA, bound with 30-fold lower affinity to CyP-D than CsA, the matrix free [mtCsA] needed to block the PT will be 30-fold higher than for CsA, and non-specific binding to other components will be correspondingly greater. Thus, the combination of decreased CyP-D binding affinity, increased partitioning into membrane phospholipids, and non-specific binding may explain why more mtCsA than CsA was needed to inhibit the PT (Figure 4B & 4D). Nevertheless, mtCsA displayed a good selectivity for intramitochondrial CyP-D over extramitochondrial CyP-A when tested in a mixed in vitro system (Figure 4D) and in intact B50 cells (Figure 5). The latter made use of the fact that nitroprusside-induced apoptosis in B50 cells is promoted by cytosolic CyP-A [23], but not by CyP-D [14]. In addition, although apoptosis in this system involves the release of cytochrome c from mitochondria (this laboratory, unpublished data), CyP-D and the PT do not appear to be involved in this process [33]. Thus the fact that mitochondrial targeting of CsA largely removes its capacity to inhibit apoptosis in this system (Figure 5E), is consistent with mitochondrial accumulation of the conjugate, out of the cytosol.

Cyclophilins have both positive and negative roles in cell viability. CyP-D is linked to the PT. CyP-A (cytosol) participates in the translocation of AIF (apoptosis inducing factor) to the nucleus [34], in caspase activation [23] and in protection against oxidative stress [35]. CyP-B (endoplasmic reticulum) suppresses apoptosis associated with oxidative stress and altered Ca²⁺ metabolism [36]. In addition, the CyP-A / CsA complex inhibits protein phosphatase 2B (calcineurin) which dephosphorylates proapoptotic Bad [37] and the L-type Ca²⁺ channel [38] in neurons. Calcineurin effects are avoided with some CsA derivatives eg NIM811 and Debio-025 [39], and SMBz-CsA (Figure 3), but these derivatives remain general cyclophilin inhibitors and their overall effects can be difficult to interpret. For example, SMBz-CsA protected against both glutamate- and OGD-induced necrosis (Figure 9). At face value, this could be taken to indicate similar roles of CyP-D and the PT in the two cases. However, use of the CyP-D specific mtCsA reveals that this is not the case.

The extent to which the CyP-D-dependent PT pore is the root cause of ischaemic cell death was addressed in neuronal cells, where the issue appears complex. Many models of transient ischaemia show that protection of hippocampal and other neuronal cells by CsA is equalled by FK506, suggesting that CsA-inhibition of calcineurin-mediated processes, rather than the PT, is critical (reviewed in [39]). In contrast, other studies point to a primary role of the PT [8,40,41]. Thus CsA-inhibition of both calcineurin and the PT may yield cytoprotection in different proportions depending on experimental conditions. To address this issue, we have taken account of the fact that hippocampal, and other, neuronal cells are subjected to two forms of pathogenic insult during I/R. In common with other kinds of cell, the normally high cellular energy state

(phosphorylation potential) becomes compromised in ischaemia as glycolysis and the TCA cycle are inhibited due to lack of glucose and oxygen, leading to “energy failure”. In addition, and unique to nervous tissue, active transport systems for glutamate uptake in astrocytes and neurons (normally responsible for termination of synaptic transmission) operate in reverse during energy failure leading to high extracellular glutamate and over-activation of neuronal glutamate receptors [42]. Both types of insult produce qualitatively similar changes in critical factors needed for PT pore formation. Energy-failure in ischaemia leads to losses of ATP and total adenine nucleotides and to a progressive increase in resting cytosolic Ca^{2+} , and reperfusion generates ROS (reviewed in [43]). Similarly, addition of high exogenous glutamate under aerobic conditions causes excessive Ca^{2+} influx into the cell, depletion of ATP and ADP, and oxidative stress [40,44]. Moreover, there is evidence that glutamate toxicity requires mitochondrial Ca^{2+} uptake and induces mitochondrial dysfunction ($\Delta\phi_M$ dissipation) [40,45], consistent with PT pore formation. Accordingly, it might be expected that the PT pore would contribute similarly to injury resulting from transient energy failure (alone) and from glutamate toxicity. Surprisingly however, comparisons of the cytoprotective capacities of CsA and mtCsA reveal markedly differing contributions of the PT in the two forms of pathogenesis.

Although CsA-inhibition of glutamate toxicity is frequently ascribed to inhibition of CyP-D and prevention of the PT, we obtained no indication for this, since the inhibition of glutamate-induced necrosis by CsA was largely abolished by mitochondrial targeting. This mirrors the report that genetic ablation of CyP-D has no effect on glutamate toxicity in hippocampal neurons [45]. It appears that the major site of CsA-protection against glutamate toxicity is extramitochondrial. In agreement, and in line with other studies [27,40], we obtained evidence that calcineurin plays a major part in the pathogenesis of cell necrosis induced by glutamate; in particular, FK506 was protective (Figure 8A) and SMBz-CsA was less protective than CsA (Figure 9). This may involve calcineurin-activation of neuronal NO synthase. Neuronal NO synthase is a Ca^{2+} / calmodulin-dependent enzyme, and activation of the synthase is believed to exert a prominent role in glutamate toxicity, aided by being scaffolded to the NMDA receptor mediating Ca^{2+} influx [46]. Our data are consistent with this. However, since CsA was a better inhibitor of glutamate-induced necrosis than either FK506 (calcineurin inhibition) or L-NAME (NO synthase inhibition), and the extra inhibition is not explicable by CyP-D inhibition, it appears that inhibition of extramitochondrial cyclophilins may also play a role in CsA-protection against glutamate-induced necrosis. Consistent with this, SMBz-CsA was a better cytoprotectant than mtCsA (Figure 9).

In contrast, the major site of CSA-protection against OGD-induced necrosis appears to be mitochondrial. Thus, necrosis resulting from energy failure (alone) was substantially prevented by mtCsA. CsA itself yielded relatively poor protection against OGD-induced necrosis, with a narrow concentration-range of effectiveness, similar to that found in cardiomyocytes subjected to transient OGD [4] and in perfused heart [7]. Secondary, non-mitochondrial targets of CsA presumably negate protection gained by PT inhibition as the CsA concentration is increased. These counterprotective targets may include calcineurin, since mtCsA-protection was decreased by FK506 (Figure 8B) and SMBz-CsA was a better cytoprotectant than CsA (Figure 9). Other extramitochondrial counterprotective targets cannot be ruled out, since mtCsA protection was only partially reversed by FK506 (Figure 8B), and mtCsA was a better cytoprotectant than

SMBz-CsA (Figure 9). The augmented protective capacity of CsA against OGD-induced necrosis obtained by mitochondrial targeting cannot be explained by increased inhibition of mitochondrial CyP-D, since CsA was tested at concentrations (1 μ M) sufficient to inhibit CyP-D completely (in B50 cells, 0.8 μ M total CsA completely reversed the PT activation by CyP-D overexpression; Figure 5C). Rather, enhanced protection by mtCsA must reflect relatively low [mtCsA] outside mitochondria so that non-mitochondrial, counterprotective effects of CsA are minimised. Overall, enhanced protection by mtCsA indicates that it protects against energy-failure induced necrosis by inhibiting mitochondrial CyP-D and supports the applicability of the PT pore model of pathogenesis to this form of injury.

In conclusion, we have developed a novel, mitochondrially-targeted CsA and shown that it selectively inhibits the mitochondrial CyP-D-dependent PT pore over extramitochondrial CyP-A in intact cells. Application of the targeted CSA to hippocampal neurons exposes a marked difference between the contribution of the CyP-D to necrosis induced by glutamate alone (low contribution) and that arising simply from energy failure (high). The latter protocol may be representative of I/R injury to cells in general when glutamate does not contribute. In these cases, since selective targeting of CsA to mitochondria produces better protection against transient OGD-induced cell necrosis than CsA itself, the therapeutic potential of CsA in limiting reperfusion injury, recently tested in pilot trials [20], may be improved by mitochondrial targeting.

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FIGURE LEGENDS

Figure 1. Chemical synthesis of the CsA analogues, and their binding by cyclophilins. Reagents: i) lithium diisopropylamide, trimethylsilyl chloride, 4-bromomethylbenzoate, ii) LiOH, methanol, iii) Fmoc-diaminohexane, PyBOP, iv) piperidine, DMF, v) 5-(carboxypentyl)triphenylphosphonium bromide, PyBOP. Lower box: The dissociation constants for the binding of CsA and analogues to CyP-D and CyP-A were determined as inhibitor constants as described for Figure 2. SMBz-CsA, [Sarcosine-3(4-methyl)]CsA; mtCsA, mitochondrial-targeted CsA.

Figure 2. CyP-D inhibition by CsA and mtCsA.

The PPIase activity of recombinant CyP-D was determined in the presence and absence of the given concentrations of CsA (A) and mtCsA (B). Each data point is the average of two determinations differing by <7%. Insets: data are replotted according to equation 1 (Methods) yielding values of K_i (slope).

Figure 3. Complexes of CyP-A with SMBz-CsA and mtCsA do not inhibit calcineurin.

The phosphatase activity of calcineurin was measured in the presence and absence of CyP-A, CsA, SMBz-CsA and mtCsA at concentrations giving 720 nM complexes of CyP-A with CsA and derivatives (see text).

Figure 4. mtCsA preferentially inhibits intramitochondrial CyP-D, rather than extramitochondrial CyP-A, in a mixed, in vitro system.

Isolated liver mitochondria were incubated with added recombinant CyP-A. (A,C,E) After preincubation with CsA, mtCsA or SMBz-CsA as indicated, PT pore opening was initiated by infusion of Ca^{2+} and monitored from the decrease in absorbance due to mitochondrial swelling. (B,D,F) The degrees of PT pore opening were compared from the absorbance decreases attained at the time indicated by the dashed lines, and replotted as % activity (closed symbols). In parallel incubations, mitochondria were sedimented immediately after Ca^{2+} addition and CyP-A activity in the supernatant determined (open symbols).

Figure 5. mtCsA preferentially inhibits CyP-D, rather than CyP-A, in B50 neuronal cells.

(A) Wild-type B50 cells (WT; 1,2), or a clone overexpressing CyP-D {CyP-D+ cells; 3,4} were incubated with 50 nM TMRE for 30 min (1,3) after which 1 μM CsA was added and the cells incubated for a further 30 min (2,4). (B-D) Wild type or CyP-D(+) cells were incubated in the presence and absence of the indicated concentrations of CsA and mtCsA. Whole cell fluorescence was determined at intervals (four experiments). (E) CyP-D(+) cells pretreated with CyP-A antisense oligodeoxynucleotide (AS, 42 hours), 0.8 μM CSA, 2 μM SMB-CsA or 2.4 μM mtCSA (both 10 min) were incubated with or without 100 μM nitroprusside (40 min), extracted, and assayed for caspase-3. Nitroprusside data, * $p < 0.05$ with respect to no addition (four experiments). (F) Immunoblots showing that CyP-D overexpression (CyP-D+) does not change CyP-A, and that CyP-A knockdown (AS CyP-A) does not change CyP-D.

Figure 6. Mitochondrial-targeting enhances protection of hippocampal neurons by CsA following transient oxygen and glucose deprivation.

(A) Upper two curves: Hippocampal neurons were preincubated under OGD for 30 min, and then returned to glucose-replete normoxia. When added, mtCsA was included in both anoxic and normoxic media. Lower curve: OGD was omitted. Necrotic cells were quantified from nuclear staining with live cell-impermeant ethidium homodimer. (B) Cells were incubated with CsA or mtCsA and necrosis determined after 30 min OGD / 90 min glucose-replete normoxia. 5 cell preparations; >20 cells per preparation.

Figure 7. CsA, but not mtCsA, strongly reduces glutamate toxicity in hippocampal neurons.

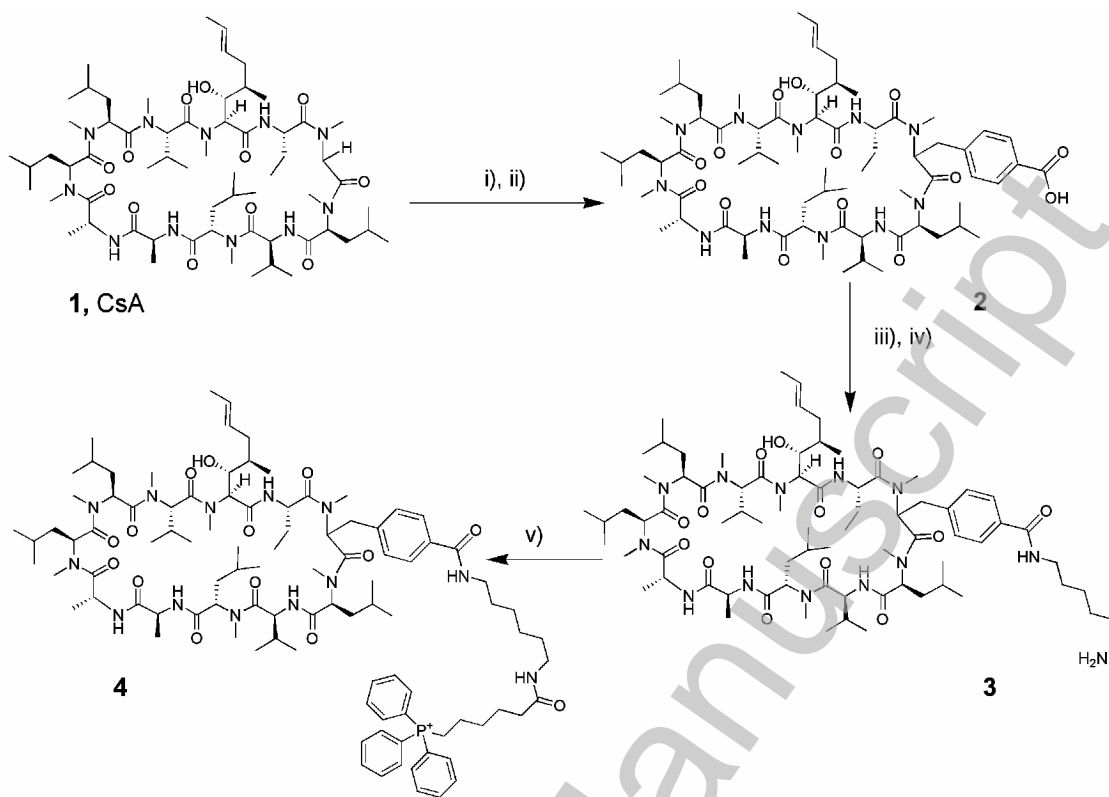
(A) Hippocampal neurons were incubated in Lockes medium with (closed symbols) and without (open symbols) 1 mM glutamate at 37°C and cell viability determined from nuclear staining with ethidium homodimer. Results from a representative single preparation. (B) Neurons were incubated in Lockes medium with CsA or mtCsA. After 10 min, 1 mM glutamate was added and cell necrosis determined 3 hours later. 5 cell preparations; >20 cells per preparation.

Figure 8. Protection against glutamate-induced and OGD-induced necrosis in hippocampal neurons.

(A) Glutamate-induced necrosis: Hippocampal neurons were incubated in Lockes medium containing either 1 μ M CsA, 1.3 μ M mtCSA, 1 μ M FK506, 250 μ M L-NAME, 50 μ M MK-801 or 100 μ M NBQX. After 10 min, 1 mM glutamate was added and cell necrosis determined 3 hours later. (B) OGD-induced necrosis: Neurons were subjected to oxygen and glucose deprivation for 30 min, and then glucose-replete normoxia for 90 min, before necrosis was quantified. Inhibitors were added to both anoxic and normoxic media (concentrations as above). n=5, >20 cells in each case. *p<0.05, **p<0.01 with respect to no addition.

Figure 9. Cytoprotection by SMBz-CsA.

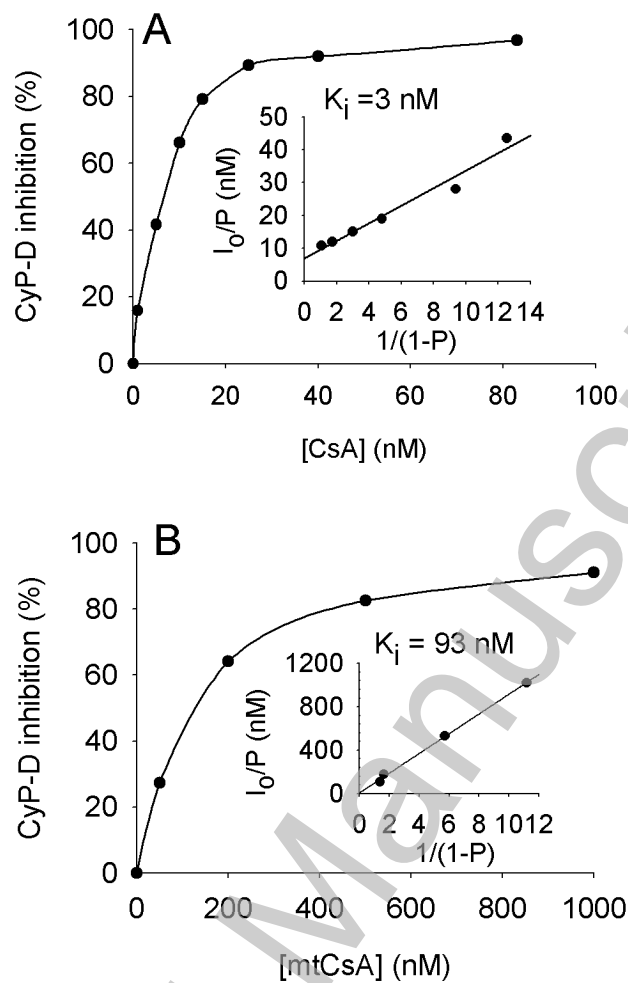
Glutamate-induced and OGD-induced necrosis in hippocampal neurons was determined as in Figure 8 in the presence of 0.3-2.0 μ M CsA, SMBz-CsA and mtCSA, as indicated. In the absence of CsA and derivatives necrosis was 34 \pm 5% (glutamate) and 64 \pm 6% (OGD). N=5, >20 cells in each case. *p<0.05 for SMBz-CsA with respect to mtCSA.

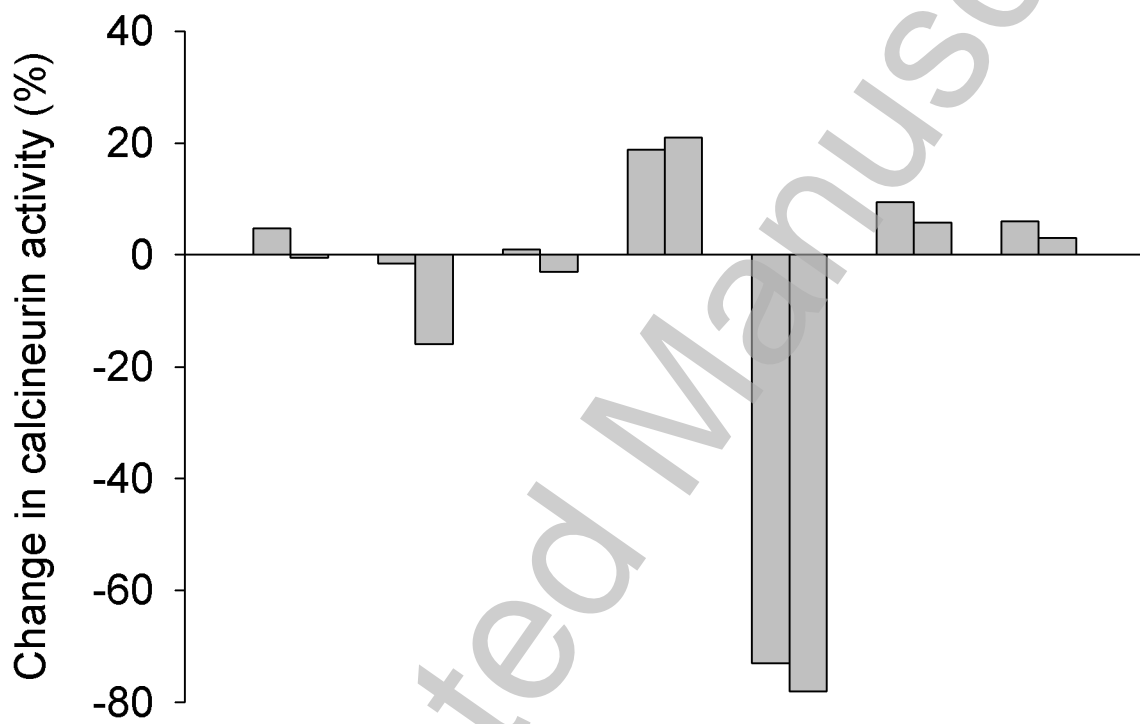


	Ki (nM)	
	CyP-D	CyP-A
1. CsA	3	4
2. SMBz-CsA	7	8
4. mtCsA	93	113

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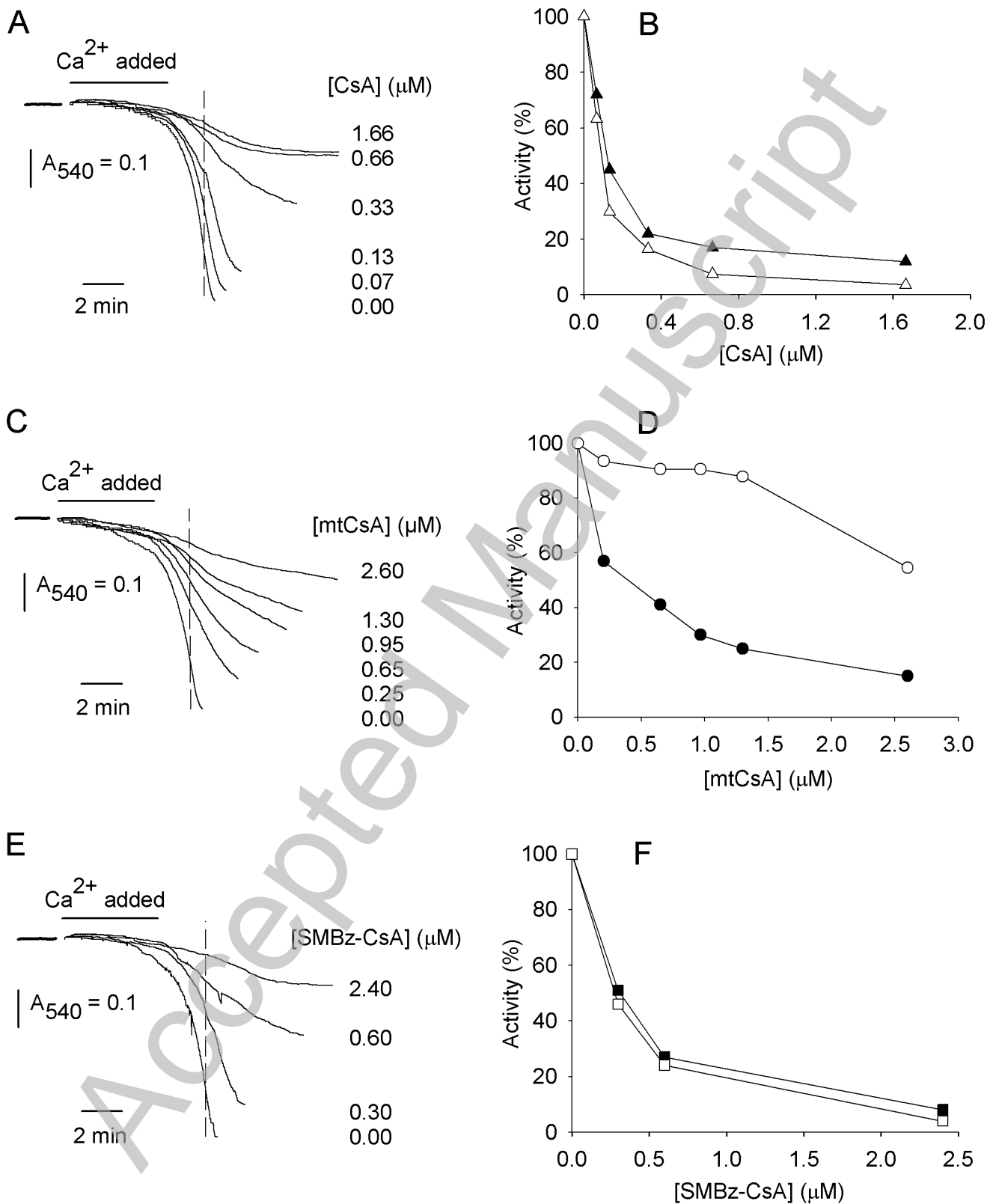
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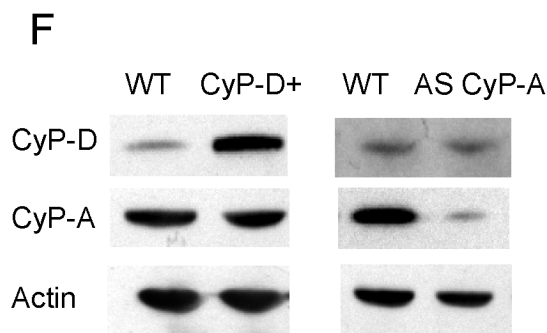
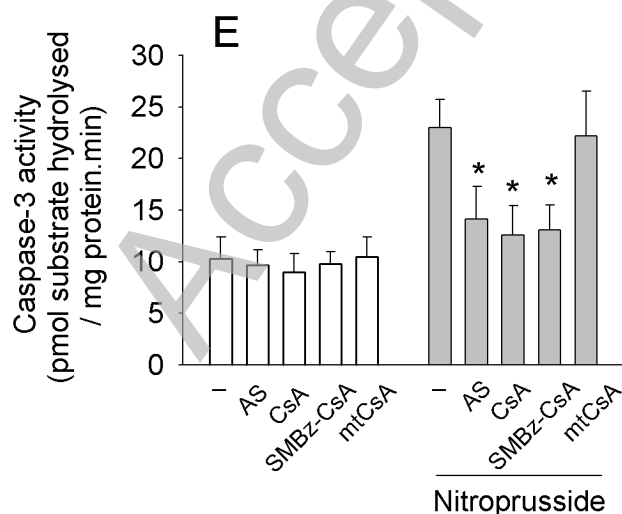
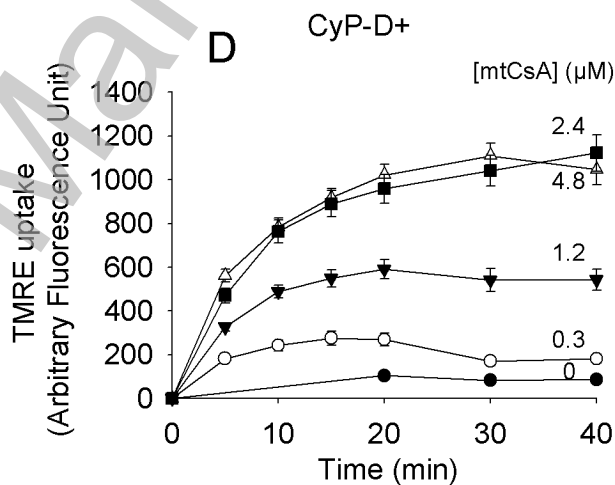
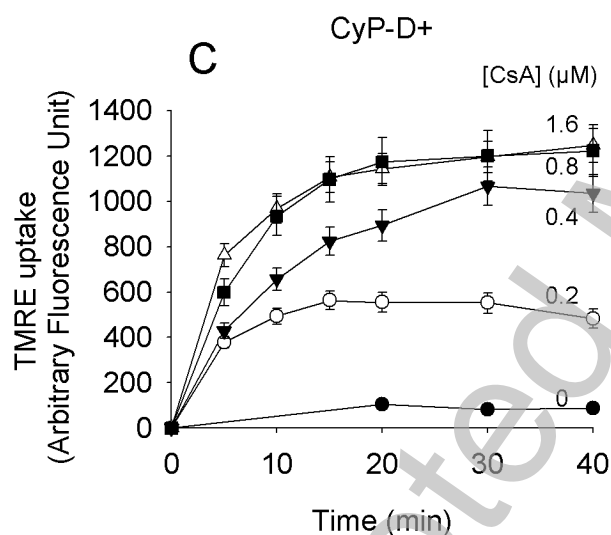
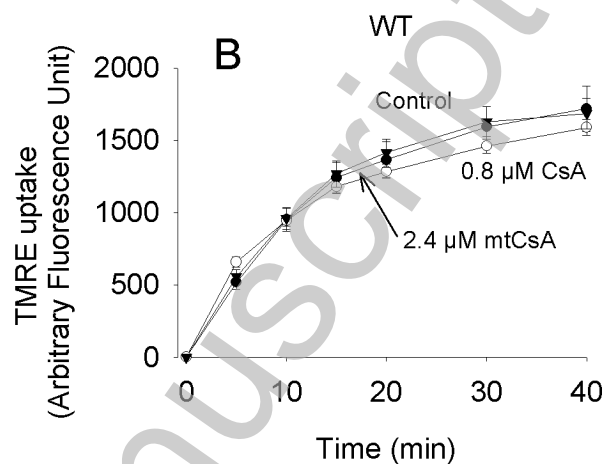
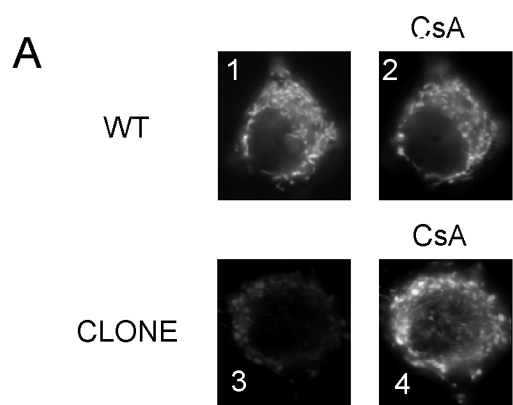


CyP-A (nM)	0	0	0	740	740	740	740
CsA (μM)	0.9	0	0	0	0.9	0	0
mtCsA (μM)	0	4	0	0	0	4	0
SMBz-CsA (μM)	0	0	1	0	0	0	1

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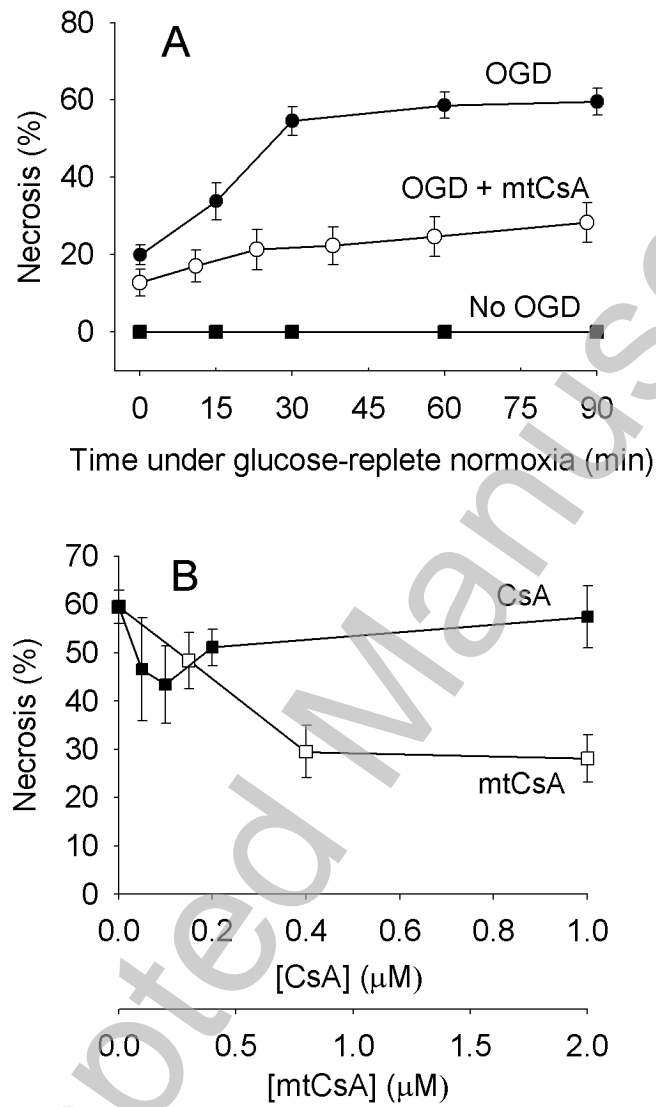


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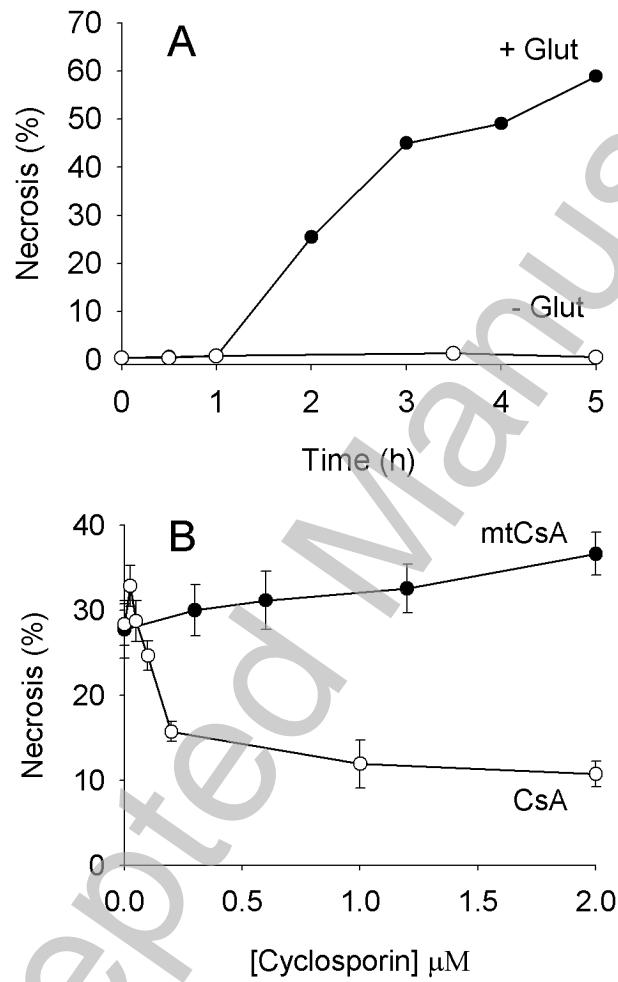
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FIG 6



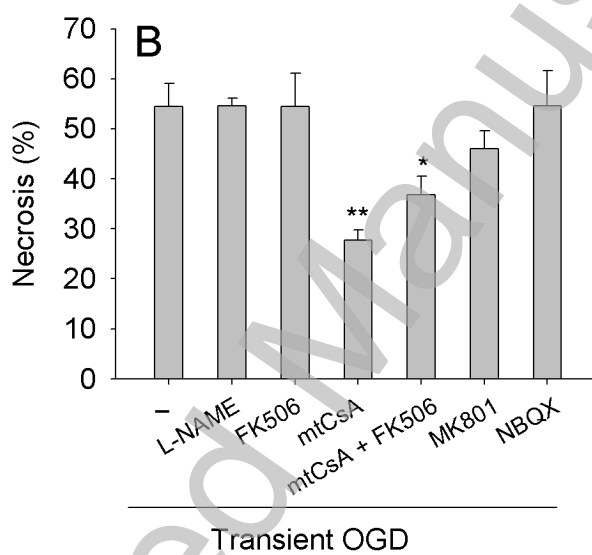
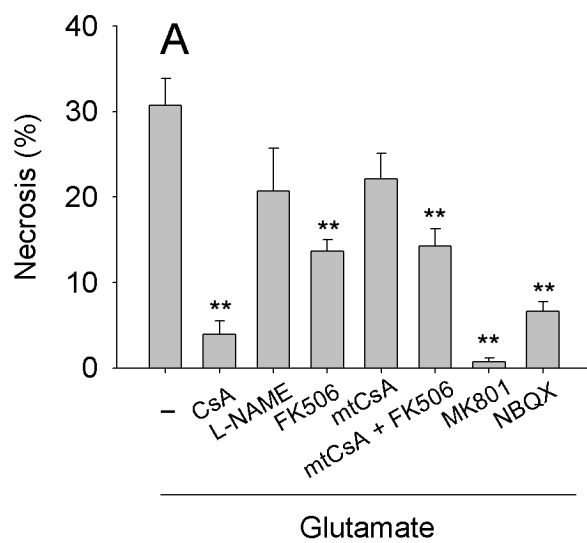
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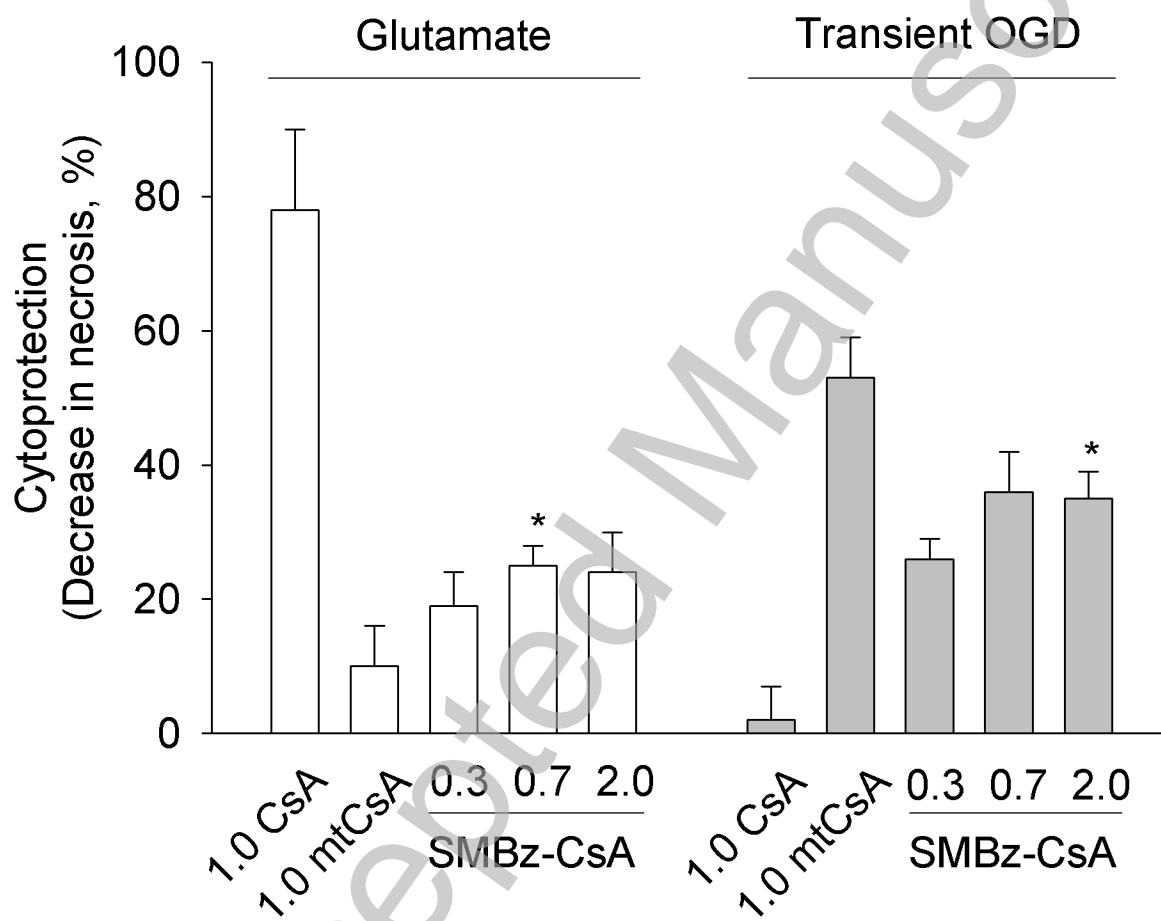
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