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The role of tissue transglutaminase in 1-methyl-4-phenylpyridinium (MPP^+)-induced toxicity in differentiated human SH-SY5Y neuroblastoma cells

Katy E. Beck [a], Luigi A. De Girolamo [a,*], Martin Griffin [b], E. Ellen Billett [a]

[a] *School of Biomedical and Natural Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK*
[b] *School of Life and Health Sciences, Aston University, Birmingham B4 7ET, UK*

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Abstract

Tissue transglutaminase (TG2) can induce post-translational modification of proteins, resulting in protein cross-linking or incorporation of polyamines into substrates, and can also function as a signal transducing G protein. The role of TG2 in the formation of insoluble cross-links has led to its implication in some neurodegenerative conditions. Exposure of pre-differentiated SH-SY5Y cells to the Parkinsonian neurotoxin 1-methyl-4-phenylpyridinium ion (MPP^+) resulted in significant dose-dependent reductions in TG2 protein levels, measured by probing Western blots with a TG2-specific antibody. Transglutaminase (TG) transamidating activity, on the other hand, monitored by incorporation of a polyamine pseudo-substrate into cellular proteins, was increased. Inhibitors of TG (putrescine) and TG2 (R283) exacerbated MPP^+ toxicity, suggesting that activation of TG2 may promote a survival response in this toxicity paradigm.

Keywords: Parkinson's disease; MPP^+; Tissue transglutaminase; SH-SY5Y human neuroblastoma; Cell viability

Both environmental and genetic factors are implicated in the sporadic forms of Parkinson's disease (PD), a disease characterised by a profound loss of pigmented dopaminergic neurons in the substantia nigra. Associated with this loss is the presence of large eosinophilic inclusions, Lewy bodies, containing a number of proteins including α-synuclein, neurofilaments, ubiquitin and proteasomal elements. Genetic forms of the disease have suggested roles for mitochondrial impairment [30], aggregation of α-synuclein [25], and defects in the ubiquitin–proteasome system [19]. Identification of the biochemical changes that induce dopaminergic cell death in PD is crucial to the development of neuroprotective/restorative strategies. Mechanistically, toxins that reproduce PD are directed against the electron transport chain, and specifically inhibit complex 1 [3]. The most established experimental model of PD is treatment with 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), which via its active metabolite 1-methyl-4-phenylpyridinium ion (MPP^+) inhibits complex 1 and induces biochemical, pathological and symptomatic features with strong resemblance to PD [4,15,20].

Tissue transglutaminase (TG2) is a member of the transglutaminase (TG) family [14] and is endogenously expressed in the CNS where it contributes to neural development, function and regeneration [22]. Post-translational modification of proteins by TG2 causes protein cross-linking or incorporation of polyamines into proteins. The reaction is calcium dependant and inhibited by GTP [13,22]. TG2 can also function as a signal transducing G protein [11,24]. A major line of investigation relates to the role of TG2 in cell survival and death pathways. The ability of TG2 to modulate apoptotic pathways reportedly depends greatly on the type of stimulus and, more specifically, its transamidating activity which was shown to modulate apoptosis in the absence of changes in TG2 expression [27]. The ability of TG2 to catalyse the formation of insoluble protein–protein cross-links has led to its implication in neurodegenerative conditions where insoluble inclusions characterise disease pathology, including Alzheimer's disease [28] and Huntington's disease [8]. Few studies have explored the link between TG2 and PD.

Abbreviations: MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine; PD, Parkinson's disease; R283, 1,3,dimethyl-2-[(2-oxopropyl) thio] imidazolium chloride; TG, transglutaminase; TG2, tissue transglutaminase

* Corresponding author. Tel.: +44 115 848 3314; fax: +44 115 8486616.
E-mail address: luigi.de-girolamo@ntu.ac.uk (L.A. De Girolamo).

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However, it has been previously reported that TG2 can mediate α-synuclein aggregation in vitro and, significantly, TG2 catalysed ε(γ-glutamyl)-lysine cross-links were found in the halo of Lewy bodies, co-localised with α-synuclein, in PD and dementia with Lewy bodies [17]. The purpose of this study was to determine whether TG2 has a role in MPP^+ -induced toxicity.

Human SH-SY5Y neuroblastoma cells (ECACC, passages 18–20) were cultured in 90% (v/v) Dulbecco's Modified Eagles Medium/Ham's F12 (1:1), 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 200 units/ml penicillin/0.2 mg/ml streptomycin, 10% (v/v) heat inactivated foetal bovine serum then induced to differentiate for 7 days with 10 μM all-trans retinoic acid in the same medium except with 1% (v/v) FBS. Medium was exchanged on days 3/4 and 7 after which experimental studies were commenced.

Whole cell lysate was used for Western blotting with anti-TG2 (clone CUB-7402) antibody (Stratech Scientific Ltd.). Total cell protein was extracted in extraction buffer (50 mM Trizma base, pH 6.8, 150 mM NaCl, 5 mM EDTA, 1% [w/v] sodium dodecylsulphate [SDS], 2 mM PMSF, 0.2% [v/v] protease inhibitor cocktail [Sigma–Aldrich Chemical Company, UK]). Extracts were sonicated (six pulses) on ice and boiled for 1 min. Protein samples (30 μg) prepared in Lammeli's sample buffer were electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose by Western blotting and probed with anti-TG2 (1:1000). Antibody binding was detected via enhanced chemiluminescence (Amersham Biosciences, UK) in accordance with manufacturer's guidelines. Band quantification was performed using the GeneTools quantification system (Syngene, UK). To ensure equal protein loading, bands were quantified and normalised against total ERK (as opposed to actin or tubulin) due to the reported effects of MPP^+ on cytoskeletal proteins [5,29]. Total ERK levels remained constant following MPP^+ exposures during the time courses used. To assess TG2 expression by immunocytochemical analyses, cells were differentiated in permanox chamber slides over 7 days. Cells were fixed in 200 μl ice-cold 90% (v/v) methanol/TBS at −20 °C for 10 min. Cells were further permeabilised in 200 μl 0.5% (v/v) Triton X-100/PBS for 10 min at room temperature then incubated with anti-TG2 antibody (1:20) overnight at 4 °C. Antibody binding was detected with fluorescein isothiocyanate (FITC) conjugated secondary antibody (1:50). Cell nuclei were stained with propidium iodide.

TG activity in control and MPP^+ treated cells was determined using a method by Zhang et al. [33] (with modifications). Medium was supplemented with 2 mM EZ-link-5-(biotinamido) pentylamine (Perbio Science UK Ltd.), a pseudo-substrate for TG, for the final hour of incubation. Total proteins were extracted, separated by SDS-PAGE and transferred to nitrocellulose by Western blotting. TG mediated incorporation of EZ-link-5-(biotinamido) pentylamine into proteins was detected by probing nitrocellulose with neutravidin–HRP (2 μg/ml) and revealed by a choice of enhanced chemiluminescence substrates. In Fig. 2, 'ECL™ Western Blotting Analysis System' was used (Amersham Biosciences, UK), whilst in Fig. 3 'Supersignal West Dura Extended Duration Substrate' (Pierce, UK), which is more sensitive, was used.

Cell viability was assessed using the 3-[4-5-dimethylthiazol-2-y1]-2,5-diphenyl tetrazolium bromide (MTT) reduction assay for anchorage-dependent cells [9]. Viability was assayed in cells cultured in 96-well plates where 10 μl 5 mg/ml MTT/PBS was added to the culture medium (100 μl) and incubated at 37 °C in a humidified atmosphere of 95% air/5% carbon dioxide for 1 h. After this time, all medium was removed from the cells and replaced with 100 μl DMSO. The plate was then agitated on an orbital shaker to dissolve the formazan product in the remaining (intact) cells. Absorbance was read at 570 nm.

To determine the effect of MPP^+ on TG2 protein levels, pre-differentiated cells were treated with 1 and 5 mM MPP^+ (in retinoic acid containing medium) for 24 h. Following extraction, proteins were fractionated by SDS PAGE, blotted onto nitrocellulose and probed with an anti-TG2 antibody. Fig. 1A indicates that 24 h exposure to 1 and 5 mM concentrations of MPP^+ caused significant 30 and 70% reductions in TG2 expression, respectively. Immunocytochemical analysis (Fig. 1B, left panel) shows that TG2 staining in differentiated cells was generally punctate and was present throughout the cell body and axon-like processes, whilst low level staining was present in the nucleus. Flow cytometry analysis of unpermeabilised SH-SY5Y cells, using anti-TG2 antibody (clone CUB-7402), has revealed that the cells contain very low levels of extracellular TG2 (Griffin and Li, unpublished observations), consistent with the observations of Lesort et al. [21]. Following MPP^+ exposure, the staining pattern of TG2 in differentiated cells was reduced and appeared to localise predominantly around the nucleus (Fig. 1B, middle and right panels).

The ability of TG enzymes to catalyse the incorporation of polyamines into proteins was exploited to determine whether MPP^+ treatment evoked a change in TG activity. EZ-link-5-(biotinamido) pentylamine, a pseudo-substrate for TG was added to the medium where it became incorporated into cellular proteins and then visualised by Western blotting. EZ-link-5-(biotinamido) pentylamine has been used previously to measure endogenous TG activity in SH-SY5Y cells in response to the calcium-mobilising agent, maitotoxin [21]. Figs. 2 and 3A show that endogenous TG activity was increased in a dose-dependent manner following MPP^+ exposure, despite a reduction in TG2 protein levels (Fig. 1A). To determine whether polyamine incorporation was indeed TG mediated, putrescine, a competitive substrate for TG was added at a high concentration (10 mM) to the cells, prior to addition of EZ-link-5-(biotinamido) pentylamine. Amine incorporation following 1 mM MPP^+ treatment was reduced by approximately 74% in the presence of putrescine, suggesting that it be, in part, mediated by TG activity (Fig. 2A). In Fig. 2B, we show the incorporation of EZ-link-5-(biotinamido) pentylamine into proteins induced by treatment with the calcium mobilising agent, ionomycin. Cells were pre-loaded with the pseudo-substrate ± 10 mM putrescine for 1 h prior to treatment with ionomycin for 15 min. Putrescine was again found to attenuate ionomycin-induced TG activity, reducing activity by approximately 79%. It was also observed that the banding pattern following MPP^+ treatment differed to that induced by ionomycin, suggesting that TG may preferentially use different substrates depending on the insult.

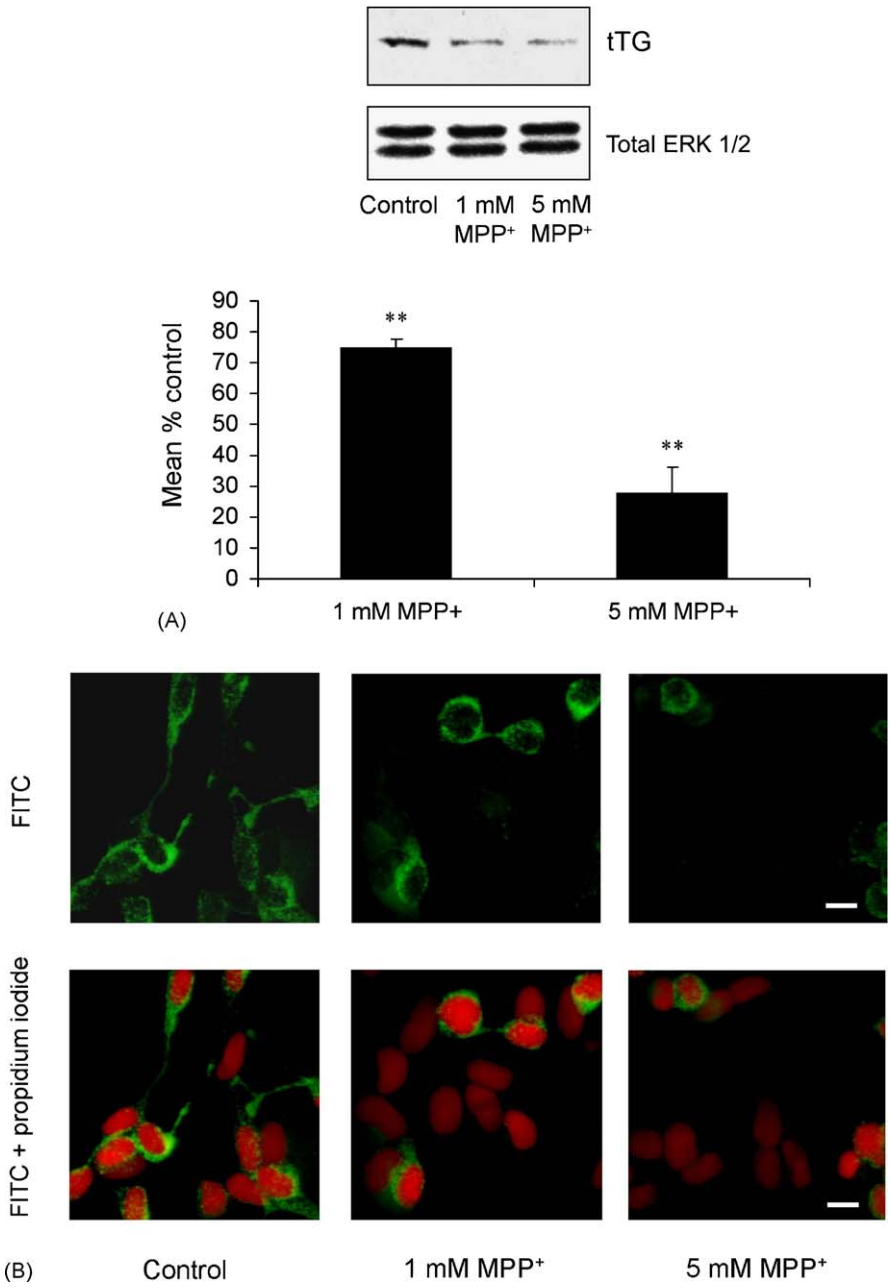

Fig. 1. TG2 expression is reduced following MPP^+ treatment. Pre-differentiated SH-SY5Y cells were treated with 1 or 5 mM MPP^+ for 24 h. TG2 was detected in cell lysates by immunoblotting (A) or by immunocytochemistry (B) with anti-TG2 antibody. For panel A, bands were quantified and corrected against total ERK for differences in protein loading. Results are presented as mean% of non-toxin treated cells (assigned 100%) ± S.E.M. for three (1 mM MPP^+) and four (5 mM MPP^+) independent experiments, respectively. Statistical analysis of control vs. MPP^+ treated cells was carried out using two-tailed *t*-tests where statistical significance was accepted at $^{**}p < 0.01$. For panel B, cells were differentiated on permanox chamber slides over 7 days. Medium was removed and replaced with fresh differentiating medium supplemented with 1 or 5 mM MPP^+. After 24 h cells were fixed and then permeabilised. Cells were probed with anti-TG2 antibody whilst nuclei are stained with propidium iodide. Confocal analysis was performed at ×400 magnification. Scale bar represents 20 μm.

To substantiate that polyamine incorporation was mediated by TG2, 1,3,dimethyl-2-[(2-oxopropyl) thio] imidazolium chloride (R283), a more specific and irreversible TG2 inhibitor, directed to the active site of the enzyme [12], was employed. Polyamine incorporation following MPP^+ treatment was dose-dependently reduced by R283 (100 and 250 μM) (Fig. 3B). Indeed, 250 μM R283 (a concentration which does not significantly affect cell viability; Fig. 4A) substantially reduced amine incorporation by 48% and 55% following treatment with 1 and 5 mM MPP^+, respectively.

Further studies sought to determine the effect of TG inhibition on MPP^+ toxicity. Using the MTT reduction assay as a determinant of viability, cells were treated with MPP^+ in conjunction with putrescine (10 mM) or R283 (ranging from 50 to 500 μM). Results show that 5 mM MPP^+ (but not 1 mM MPP^+) significantly reduced viability and that R283 alone did not affect

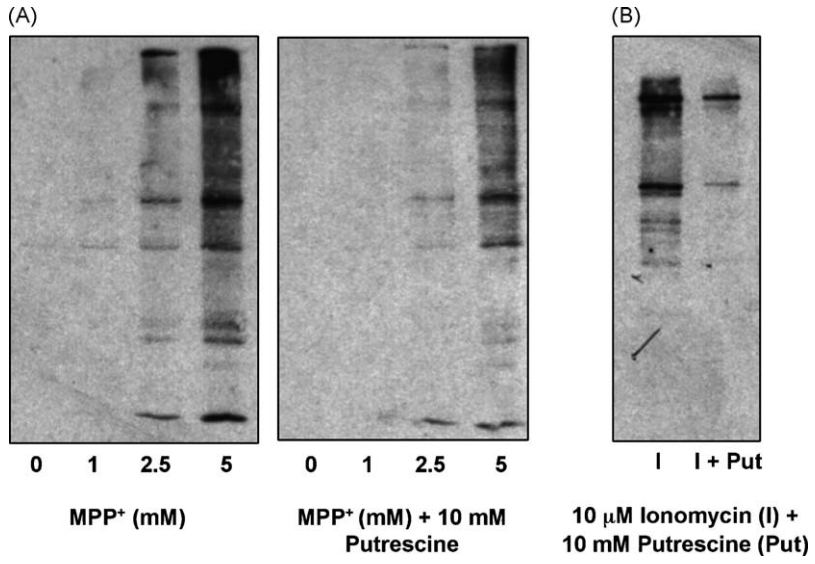

Fig. 2. TG activity is increased by MPP^+ and ionomycin treatment and is attenuated by putrescine. (A) Pre-differentiated SH-SY5Y cells were treated with $MPP^+ \pm 10$ mM putrescine for 24 h. Cells were incubated with 2 mM EZ-link-5-(biotinamido) pentylamine for the final hour. (B) Pre-differentiated cells were pre-loaded with 2 mM EZ-link-5-(biotinamido) pentylamine for 1 h prior to treatment with 10 μM ionomycin for 15 min. Sample extracts were processed together and separated on the same SDS-PAGE gel prior to transfer to nitrocellulose. TG mediated incorporation of EZ-link-5-(biotinamido) pentylamine into cells was detected by immunoblotting using neutravidin–HRP.

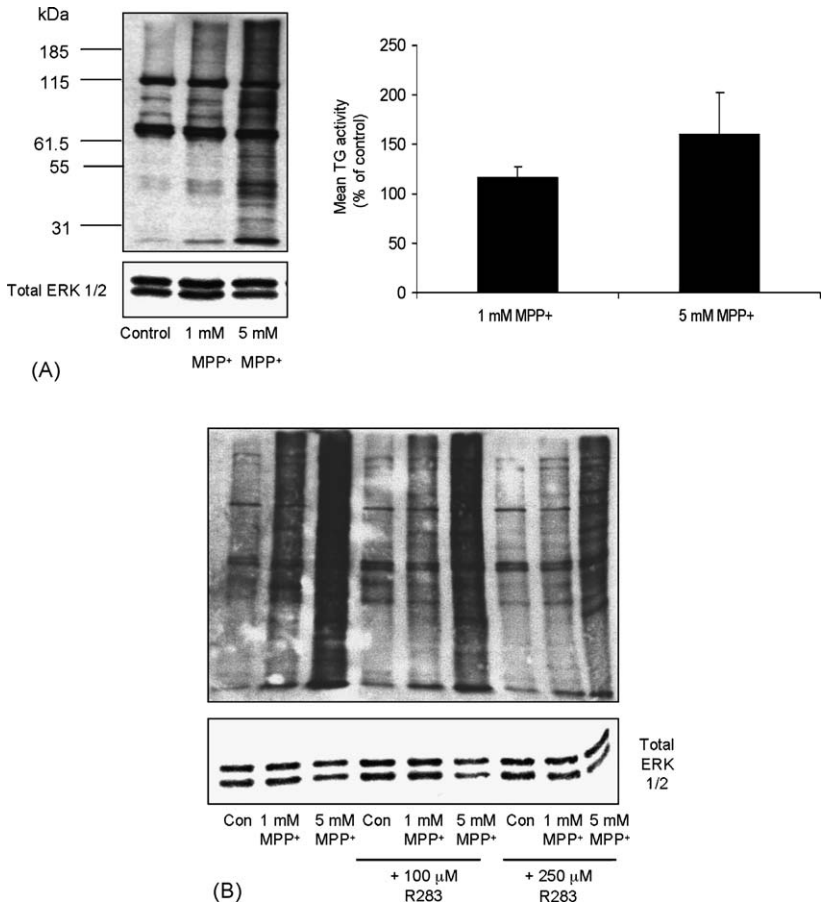

Fig. 3. Increased TG activity following MPP^+ exposure is partly mediated by TG2. Pre-differentiated SH-SY5Y cells were treated with 1 or 5 mM MPP^+ alone for 24 h (A), and in the presence or absence of R283 (B). Medium was supplemented with 2 mM EZ-link-5-(biotinamido) pentylamine for the final hour. TG mediated incorporation of EZ-link-5-(biotinamido) pentylamine into cells was detected by immunoblotting using neutravidin–HRP conjugated secondary antibody. For (A), results were quantified and corrected against total ERK for differences in protein loading. Results are presented as mean% of non-toxin treated cells (assigned 100%). Results are the mean values of four independent experiments ± S.E.M.

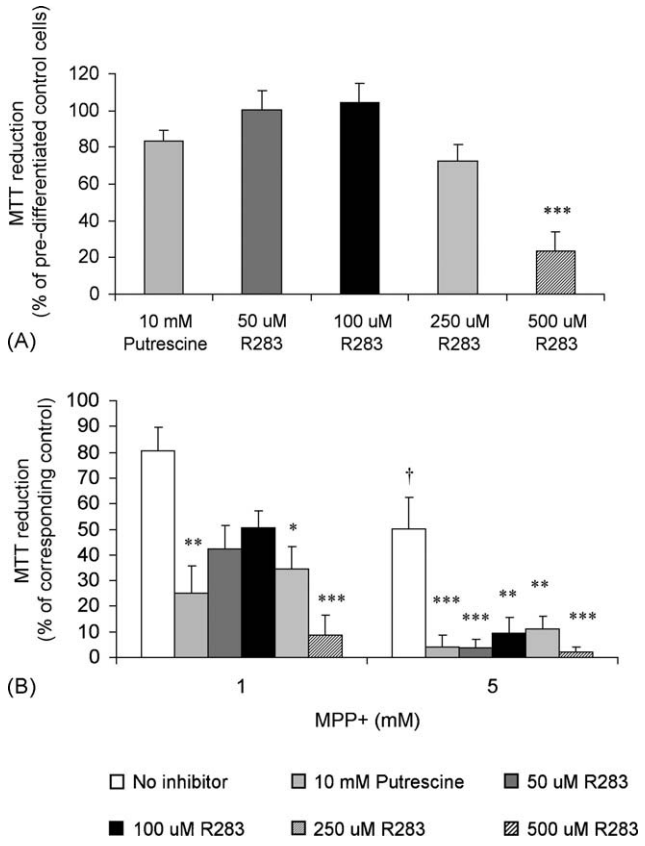

Fig. 4. (A and B) Inhibition of TG2 exacerbates MPP^+ toxicity. SH-SY5Y cells were pre-differentiated in 96-well plates. On day 7 medium was exchanged for fresh differentiation medium supplemented with putrescine or R283, 1 mM $MPP^+ \pm$ putrescine or R283, or 5 mM $MPP^+ \pm$ putrescine or R283. After 24 h, plates were assayed for viability using the MTT reduction assay. Data are expressed as mean% of cells treated with corresponding inhibitor alone (assigned 100%) ± S.E.M. Data shown are combined from six independent experiments (triplicate wells in each experiment). Statistical analysis of MPP^+ vs. MPP^+ + inhibitor treated cells was performed using one-way ANOVA with Bonferroni correction. Statistical significance was accepted at $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$. Significant toxicity induced by 5 mM MPP^+ alone compared to pre-differentiated control cells was shown ($^{\dagger}p < 0.01$).

viability except at the highest concentration (500 μM; Fig. 4A). However, both putrescine and R283 significantly exacerbated toxicity by both 1 and 5 mM MPP^+ (Fig. 4B).

Results presented propose a novel role for TG2 in MPP^+ toxicity. TG2 expression following cytotoxic MPP^+ exposure was significantly reduced; it is possible that this may be calpain-mediated since TG2 is a substrate for calpains [32] and MPTP/MPP^+ has previously been shown to increase cytosolic calcium levels, which would in turn activate calpain [7,18,29]. Indeed, treatment of mice with MPTP leads to increased calpain mediated proteolysis whilst calpain inhibition attenuated MPTP toxicity [10]. It is widely accepted that MPP^+ depletes cellular ATP levels as a consequence of complex I inhibition [6,26]. If a parallel reduction in GTP ensued, TG2 activity could increase. Indeed, it has previously been reported that mitochondrial function can lead to reduced GTP levels, associated with increased TG activity in situ [23]. We observed that TG activity was markedly increased following exposure to MPP^+ despite a reduction in TG2 protein levels; around half of this increase in TG activity was mediated by TG2, being sensitive to R283. It should be noted that uncoupling between enzyme expression and activity levels has also been reported under certain apoptotic conditions [2].

The implications of elevated TG activity in MPP^+ toxicity are as yet unclear. MPP^+ has the potential to up-regulate TG2 activity via a number of mechanisms; MPP^+ has been reported to increase ROS production [16] and increase intracellular calcium levels, both factors that would increase TG2 activity [33]. A pro-apoptotic role for TG2 was reported when transamidating activity was elevated [27]. However, in the current study, a TG2 inhibitor in combination with MPP^+ significantly exacerbated MPP^+ toxicity, suggesting that the transamidating activity of TG2 has a pro-survival role in this instance. This is in line with a study noting that the pro-survival, PI3-K pathway was necessary for retinoic acid-induced TG2 expression and activity in NIH3T3 cells [1]. Furthermore, inhibition of TG2 caused retinoic acid to activate apoptosis rather than differentiate the cells [1]. It should be noted that whilst the cell culture system used in the current project employs retinoic acid as a differentiation agent, TG inhibitors exacerbated MPP^+ toxicity at concentrations that had no effect on the viability of control cells. In addition, a recent study by Wakshlag et al. using differentiated SH-SY5Y cells has shown that the role of TG activity varies according to insult [31]. They found that whilst TG activity augments the neurotoxic effects of β-amyloid, it can also be protective against the cytotoxic effect of hydrogen peroxide, in a similar way to that found for MPP^+ in the present study. However, it remains to be determined whether the pro survival role of TG2 in our model is dependent on its subcellular localisation, whether it is solely dependent upon its transamidating function and whether the GTP hydrolysis/signal transduction activities are also involved. Identification of proteins into which TG2 had mediated incorporation of polyamine in MPP^+ treated cells would further define the exact role of TG2 in MPP^+ mediated toxicity. Whether proteins located in Lewy bodies are natural substrates would be of particular interest.

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