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Transglutaminase inhibition protects against oxidative stress-induced neuronal death downstream of pathological ERK activation

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Abstract

Molecular deletion of transglutaminase 2 (TG2) has been shown to improve function and survival in a host of neurological conditions including stroke, Huntington's disease, and Parkinson's disease. However, unifying schemes by which these crosslinking or polyaminating enzymes participate broadly in neuronal death have yet to be presented. Unexpectedly, we found that in addition to TG2, TG1 gene expression level is significantly induced following stroke *in vivo* or due to oxidative stress *in vitro*. Forced expression of TG1 or TG2 proteins is sufficient to induce neuronal death in *Rattus novergicus* cortical neurons *in vitro*. Accordingly, molecular deletion of TG2 alone is insufficient to protect *Mus musculus* neurons from oxidative death. By contrast, structurally diverse inhibitors used at concentrations that inhibit TG1 and TG2 simultaneously are neuroprotective. These small molecules inhibit increases in neuronal transamidating activity induced by oxidative stress; they also protect neurons downstream of pathological ERK activation when added well after the onset of the death stimulus. Together, these studies suggest that multiple TG isoforms, not only TG2, participate in oxidative stress-induced cell death signaling; and that isoform non-selective inhibitors of TG will be most efficacious in combating oxidative death in neurological disorders.

Introduction

Transglutaminases (TG) are an inducible family of protein crosslinking or polyaminating enzymes that have been implicated in diverse neurological conditions. The best-studied enzyme of the family, transglutaminase 2 (gene: Tgm2; protein: TG2), is ubiquitously expressed in the human body and is the most abundant isoform in the brain.

A role for TG2 in neurodegeneration was first invoked as a crosslinker of aggregated proteins in a host of diseases associated with protein dyshomeostasis. Indeed, its transamidating activity has been shown to be upregulated at the symptomatic stage of Huntington's disease (HD) (Karpuj et al., 1999; Dedeoglu et al., 2002; Karpuj et al., 2002),

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Parkinson's disease (PD) (Gibrat et al., 2010), Alzheimer's disease (AD) (Martin et al., 2011), cerebral ischemia (Ientile et al., 2004), traumatic brain injury (Tolentino et al., 2002) and spinal cord injury (Festoff et al., 2002). To establish whether TG2 transamidating activity is causally related to neurodegeneration, structurally diverse inhibitors have been developed that inhibit the cysteine catalytic site. Intense interest in TG2 has been fueled by the success of reversible broad inhibitors (e.g. cyst(e)amine) that have shown durable and reproducible protective effects in *in vivo* models of several chronic neurodegenerative diseases such as HD (Dedeoglu et al., 2002), PD (Gibrat et al., 2010) and intracerebral hemorrhage (Okauchi et al., 2009). Indeed, Cystamine is in Phase II studies in humans with HD.

While a focus on TG2 has been validated by the therapeutic success of germline deletion of TG2 in rodent models of neurodegenerative disease (Iismaa et al., 2009), two important issues remain unsettled. First, isoform non-selective inhibitors result in therapeutic benefit beyond TG2 deletion in rodent models of HD (Bailey and Johnson, 2006), suggesting the possibility that other isoforms of the TG family can compensate for deletion of a single isoform; second, TG2 deletion does not decrease protein aggregation, suggesting a more complex role for the enzyme in stress responses (Mastroberardino et al., 2002). Indeed, current studies implicate TG2 in diverse cellular functions, including autophagosome formation (D'Eletto et al., 2009), axonal BDNF trafficking (Borrell-Pages et al., 2006), and transcriptional repression (McConoughey et al., 2010). Accordingly, the current study was designed with two specific goals: first, to elucidate the role, if any, of other TG isoforms in neuronal injury and second, to understand whether a common putative mediator of death, oxidative stress, could induce TG message levels and activity as part of a death cascade.

We report that multiple isoforms of TG are significantly induced following stroke *in vivo* or oxidative stress *in vitro*; that forced expression of transglutaminase 1 (TG1) or TG2 induces cell death in cortical neurons; and that oxidative stress-induced cell loss in cortical neurons can be rescued by isoform non-selective inhibitors.

Materials and Methods

Chemicals

Several structurally different TG inhibitors were tested in our models: Cystamine dihydrochloride (broad and reversible inhibitor Sigma); the irreversible inhibitors B003 (Boc-DON-Gln-Ile-Val-OMe, Zedira GmbH); T101 (1,3,4,5-Tetramethyl-2-[(2-oxopropyl)thio]imidazolium chloride, Zedira GmbH), D004 (1,3-Dimethyl-4,5-diphenyl-2-[(2-oxopropyl)thio]imidazolium trifluorosulfonicacid salt), and TAMRA-DON (Zedira GmbH). The MEK inhibitors U0126, SL327 and the inactive analog, U0124 (Calbiochem) were tested in our model. L-glutamic acid and its analog, L-homocysteic acid (HCA) were purchased from Sigma-Aldrich.

Transient middle cerebral artery occlusion (MCAO)

The use of animals and procedures were approved by the Institutional Animal Care and Use Committees of Weill Medical College of Cornell University. Ten to twelve week-old C57BL/6 male mice were subjected to transient ischemic by MCAO as previously described (Cho et al., 2005; Cho et al., 2007; Kim et al., 2008). Briefly, mice were anesthetized with a mixture of isoflurane/oxygen/and nitrogen. A fiber optic probe was glued to the parietal bone (2 mm posterior and 5 mm lateral to the bregma) and connected to a Laser-Doppler Flowmeter (Periflux System 5010; Perimed, Järfälla, Sweden) for continuous monitoring of cerebral blood flow (CBF) in the center of the ischemic territory. For MCAO, a 6-0 Teflon-coated black monofilament surgical suture (Doccol Co., Redland, CA) was inserted into the

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exposed external carotid artery, advanced into the internal carotid artery, and wedged into the cerebral arterial circle to obstruct the origin of the MCA for 30 min. The filament was withdrawn to allow reperfusion. Lidocaine was administered during post-ischemia as analgesics. Using a rectal probe controlled by a masterflex pump and thermistor temperature controller (Cole-Parmer, Vernon Hills, IL), animal's body temperatures was maintained at 37± 0.5°C during MCAO and 2h after post-ischemia. Only animals that exhibited greater than 80% reduction in CBF during MCAO and greater than 80% reperfusion 10 min following reperfusion were included in the study.

Tissue preparation after MCAO

Brains were excised, frozen and sectioned using an unbiased stereological sampling strategy. Infarct typically spans about 6 mm rostrocaudal, roughly from +2.8 mm to -3.8 mm bregma. To collect tissue to reflect the infarct area, the entire infarct region was cryosectioned for gene expression (4 sections at 50 μm thickness) and collected serially at 600 micron intervals. The sections were cut in half and collected for each hemisphere.

Mouse Embryonic Fibroblasts

TG2 knockout mice, designated Tgm2tm1.1Rmgr ($TG2^{-/-}$), were generated on a 129S1/Sv-ImJ background as previously described (Nanda et al., 2001). Heterozygous offspring were backcrossed to wild type C57Bl/6 (B6.Cg) mice for twelve generations to generate congenic heterozygous $TG2^{+/-}$ mice with ~99.95% B6.Cg-$TG2^{+/-}$ genomic homogeneity, respectively. These heterozygous animals were mated to generate TG2 wild type ($TG2^{+/+}$) or $TG2^{-/-}$ mice that were then set up in breeding pairs to generate $TG2^{+/+}$ or $TG2^{-/-}$ embryos for experimentation. Mouse embryonic fibroblasts (MEFs) were isolated from 13.5 days-post-coitus female and male embryos and were cultured on DMEM/10% FCS (Dulbecco's modified Eagles' medium high glucose (Invitrogen, CA, USA); 10% v/v fetal calf serum (Invitrogen); 400μM L-Glutamine (Invitrogen); 0.2U/ml penicillin (Invitrogen); 0.2mg/ml Streptomycin (Invitrogen)). Only MEFs of passage five or lower was used for experiments.

Primary neuronal and astrocyte cultures

Primary rat cortical neurons were obtained from fetal Sprague Dawley rats at embryonic day 17 (E17) as previously described (Ratan et al., 1994). Primary mouse cortical neurons were obtained from C57BL/6 $TG2^{+/-}$ mice mated to generate male and female TG2 wild type ($TG2^{+/+}$), $TG2^{-/+}$ or $TG2^{-/-}$ embryos at day 15 (E15). Single embryonic neuronal cultures were performed and their genotype was revealed by PCR. The astrocyte-neuronal cultures were obtained as previously described (Haskew-Layton et al., 2010).

Genotyping

Genomic DNA for PCR was extracted from the embryos using the DNeasy genomic DNA isolation kit (QIAGEN). The TG2 wild type alleles were detected using the primer 5'-GGAGCACACAGGCCTTATGAGCTGAAG-3'. The TG2 knockout alleles were detected using the primer 5'-CAGATAGGGATACAAGAAGCATTGAAG-3'. As a common reverse primer, we used 5'-GCCCCACAAAGGAGCAAGTGTTACTATGTC-3'.

Cell viability

For neuronal cytotoxicity studies, cortical neurons were plated at a density of 10^6 cells/ml in 96-well plates (100μl), in 6 well plates (3ml) and treated with glutamate 5mM. Specified concentrations of Cystamine, B003, T101, D004 and TAMRA-DON were added at the time of glutamate treatment. In the post-treatment experiments, TG inhibitors were added at various time points after glutamate addition (14, 17, 19 and 21 hours) and viability was assessed 24 hours later. MEF $TG2^{+/+}$ and $TG2^{-/-}$ cells were plated at 10^4 cells/ml in black

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96-well plates for 16 hours (+/- glutamate), live cells were visualized with calcein AM (Invitrogen) by a Flash Cytometer® (Trophos, France) and counted with TINA v4.8 (Trophos, France). In the astrocyte-neuronal co-cultures, astrocytes were pre-treated for 16 hours with inhibitors at various concentrations. The drugs were removed and the neurons were plated on top of the astrocytes at a concentration of 0.5×10^6 cells/ml in the presence or absence of the glutamate analog, HCA. Neuronal viability was measured 48 hr after neuronal plating by quantifying the neuronal specific marker MAP2 using a horseradish peroxidase/Amplex Red assay as previously described (Haskew-Layton et al., 2010).

RNA extraction and Real Time PCR

Total RNA was prepared from immature primary cortical neurons (E17), MEF and brain sections and reversed transcribed to cDNA with a standard protocol. The expression levels of rat and mouse Tgm1, Tgm2 were quantified by Real-Time (RT) PCR as previously described (McConoughey et al., 2010). The primers used in rat samples are the following: *Tgm1* (5'-AGAGCACCACACCGATGAGTTTGA-3' and 5'-TCCGATGAGAAGCTCAAGGGCAAT-3'); *Tgm2* (5'-GCCTTGGAACTTTGGGCAGTTTGA-3' and 5'-TCATCATTGCAGTTGACCATGCCG-3'); *β-actin* (5'-CCATTGAACACGGCATTGTCACCA-3' and 5'-GCCACACGCAGCTCATTGTAGAAA-3'). For mouse samples: Tgm1 (5'-TGTGGAGATCCTGCTCAGCTACCTA-3' and 5'-TGTCTGTGTCGTGTGCAGAGTTGA-3'); Tgm2 (5'-TTCCGGCTGACTCTGTACTTCGAG-3' and 5'-ACATTGTCCTGTTGGTCCAGCACT-3'); β-actin (5'-TGAACCCTAAGGCCAACCGTGAAA-3' and 5'-GAGTCCATCACAATGCCTGTGGTA-3'). The Comparative Ct Method was used to analyze the data from quantitative Real-Time PCR (RT-PCR). The amount of target (Tgm1 or Tgm2), normalized to an endogenous reference (beta-actin) and relative to a calibrator (Sham contralateral in Fig.1A and B; 0h treatment in Fig.1C and D; TG2+/+ in Fig.2C and D), is given by the $2^{-\Delta\Delta Ct}$ algorithm, also known as the delta-delta-Ct or ddCt algorithm. The mean Ct and standard deviation values were calculated by ABI Sequence Detection System software v1.4 (Applied Biosystem). Each sample was run in triplicate and in each experiment three or four samples per condition were analyzed. One-way ANOVA followed by Dunnett's *post hoc* test was calculated in GraphPad Software (Prism®).

Measurement of endogenous transamidating activity

For the glutamate treatments, cells were treated at different time points (4, 8 and 12 hours) and co-treated with Cystamine (100μM), B003 (200μM), D004 (50μM), T101 (50μM), U0126 (10μM), U0124 (10μM), SL327 (10μM), for 12 hours. The lysine donor, Biotin Pentylamine (BPA, 500μM) (Thermo), was added to the media at the same time. Cells were lysed in hypotonic buffer (10mM Tris-HCl pH8, 1mM KCl, 1mM $MgCl_2$). Equal amounts of proteins were loaded in a Bio-Dot™ Apparatus (Bio-Rad Laboratories). Extracellular proteins were harvested from the media and centrifuged at 21,000 × g at 4°C for 10min and then 100μl per sample was loaded in the Bio-Dot™ Apparatus. The nitrocellulose membrane was probed for streptavidin and actin (loading control). The dot densitometry was calculated with the Odyssey software (Integrated Intensity, LI-COR Bioscience).

TG1 and TG2 overexpression

Human TG2 and TG1 or EGFP sequences were introduced into cortical neurons (E17) using the Amaxa Rat neuron Nucleofector Kit (Lonza). The next day, TG2, TG1 and EGFP overexpression was confirmed by western blot and cell survival was measured by MTT.

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TG2 antibody

We produced a specific custom Rabbit monoclonal antibody (Epitomics), using recombinant mouse TG2 as immunogen. Specificity is shown in Fig.2D.

Immunoblot analysis

Protein extracts were obtained using 1% Triton buffer. Antibodies against Myc-tag (9E10, Covance), EGFP (Molecular probes), TG2 (custom made), TG1 (Abcam) and β-actin (AC-74; Sigma-Aldrich) were diluted 1:1,000, 1:2,000; 1:1,000; 1:1,000 and 1:10,000, respectively. Proteins were detected using an Odyssey infrared imaging system (LI- COR Biosciences).

Statistical analysis

Statistical analysis was conducted by two-way ANOVA followed by Bonferroni's *post hoc* test or one-way ANOVA followed by Dunnett's *post hoc* test. Statistically significant results were defined as: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. All the experiments here presented were repeated at least three times.

Results

Multiple TGs are induced following stroke in vivo or neuronal oxidative stress in vitro

TG inhibition has been shown to reduce damage in hemorrhagic (Okauchi et al., 2009) and ischemic stroke models (Tolentino et al., 2004; Hwang et al., 2009), but an analysis of how message levels of distinct TG isoforms change, following ischemic stroke, has not been performed. We analyzed a temporal profile of mRNA expression of six of the nine known TG isoforms from the ipsilateral hemisphere of rodents that underwent focal brain ischemia (MCAO; Sham, 6, 24, 72h after reperfusion). As expected, Tgm2 message levels were significantly induced in the ipsilateral hemisphere (Fig.1A). Surprisingly, however, we also found more than 100-fold increase in Tgm1, which peaked 72 hours after the onset of ischemia (Fig. 1B).

As stroke-induced damage is characterized by nitrosative (Samdani et al., 1997) and oxidative stress (Chen et al., 2011), we utilized the experimental leverage of a well established *in vitro* model in cortical neurons. In this model, glutamate simulates oxidative stress via a non-receptor mediated mechanism involving the inhibition of plasma membrane cystine transport and depletion of the versatile antioxidant glutathione (Ratan et al., 1994). Prior studies have shown the utility of this model for studying stroke pathogenesis and treatment (Siddiq et al., 2005; Langley et al., 2008). As expected, not only Tgm2, but also Tgm1 message levels were induced in a manner qualitatively similar to that found following stroke *in vivo* (Fig. 1C and D).

Conflicting data has been presented about the role of TGs in modulating cell death (Piacentini et al., 2011). However, most of the studies implicating TG2 as a positive modulator of survival have been performed in transformed cancer cells, not in primary neurons. To determine whether TG1 or TG2 are sufficient to induce cell death, we transfected immature cortical neurons with human TG1, TG2, or EGFP as control (Amaxa, Lonza). We verified expression of TG1, TG2 or EGFP in separate experiments 24h after transfection by immunoblot (Fig. 1E). Moreover, as monitored by morphological (phase contrast) or metabolic (MTT assay) criteria, overexpression of TG1 or TG2 are sufficient to induce neuronal cell death (Fig. 1F). Of note, transfection of the two isoforms together, which resulted in the same levels of each isoform as when they are expressed individually, did not enhance cell death. These results show that TG1 and TG2 are sufficient to induce

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neuronal death, and suggest that they work via a similar pathway, as their effects at this level of expression are not additive or synergistic.

Our findings suggested that TG1 and TG2 might work redundantly to ensure death of neurons following oxidative death. To establish whether TG activity is necessary for oxidative death, we took advantage of germline knockouts of TG2 ($TG2^{-/-}$). Animals deficient in this isoform have shown smaller infarct volumes in stroke (Colak and Johnson, 2012) or resistance to the deleterious effects of mutant huntingtin (Mastroberardino et al., 2002; Bailey and Johnson, 2006). However, germline knockout of TG2 was not sufficient to protect cortical neurons from oxidative stress (Fig. 2A); but its complete absence in mouse embryonic fibroblasts (cultured from $TG2^{-/-}$ mice) increased resistance to glutamate-induced oxidative stress (Fig. 2B). The ability of TG1 and TG2 to induce death of cortical neurons raised the possibility that cortical neurons were insensitive to TG2 deletion because of compensatory upregulation of TG1, whereas MEFs were sensitive to TG2 deletion because of the absence of TG1 compensation. Consistent with this model, we found that TG1 is induced in glutamate-treated cortical neurons that are $TG2^{-/-}$ (Fig. 2C) but not in glutamate-treated MEFs that are $TG2^{-/-}$ (Fig. 2D).

Intracellular transglutaminase transamidating activity is increased following oxidative stress and structurally diverse TG inhibitors protect neurons from oxidative stress-driven cell death

To establish whether TG1 and TG2 are both necessary for oxidative neuronal death, we utilized a set of structurally diverse small molecules whose commonality is that they inhibit the transamidating site cysteine in both TG1 and TG2 (Griffin et al., 2002). The use of diverse compounds makes it likely if common biological effects are observed, that these effects can be attributed to inhibition of the transamidating activity of TGs, rather than to an off-target effect of any one class of compounds. Moreover, use of small molecules allows us to target the transamidating activity without affecting TG GTPase activity or other domains. Before testing the compounds, we verified that the transamidating activity is increased following oxidative stress using biotin-labeled pentylamine (BPA), a lysine donor whose incorporation into protein has been shown to be dependent on transamidating activity (Fig. 3) (Lee et al., 1992).

We first evaluated the effect of a commonly used TG inhibitor, Cystamine that was recently shown not to have significant preference for TG2 over TG1 or TG3 in *in vitro* assays (Schaertl et al., 2010). Cystamine is the disulfide form of the free thiol, cysteamine, and it reversibly inhibits TG activity by acting as an alternative substrate for the enzyme. It is therefore considered as a pseudo-inhibitor substrate (Fig. 4A). As expected, we found that Cystamine not only inhibited TG activity (Fig. 5A and B), but also potently protected cortical neurons from oxidative death (Fig. 4B). As Cystamine has been shown to have off-target effects including caspase inhibition (Lesort et al., 2003), we tested an irreversible peptide inhibitor, B003, which has similar IC_{50}s for recombinant TG1 (1.1 μM) as TG2 (0.3 μM), but almost no activity toward caspase 3 (McConoughey et al., 2010). B003 forms a covalent bond with cysteine in the active site, and is channeled specifically to TG by the amino acids that surround the reactive DON moiety (Fig. 4A). Similar to Cystamine, B003 protected cortical neurons from oxidative death (Fig. 4C), and reduced cell associated transamidating activity (Fig. 5A and B). Together these structurally diverse, isoform non-selective TG inhibitors suggest that the transamidating activity is necessary for the death of cortical neurons in response to glutathione depletion.

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Inhibition of intracellular but not extracellular transamidating activity protects neurons from oxidative stress

By contrast to our study, a prior study in non-neural cells failed to show that B003 could inhibit cell based TG activity, raising the possibility that extracellular TG might contribute to death (Schaertl et al., 2010). Indeed, TG2 can be secreted to exert biological changes on the extracellular matrix (Belkin, 2011). To establish whether TG activity must be inhibited intracellularly, extracellularly or both to induce neuroprotection, we took advantage of two structurally similar inhibitors, D004 and T101 (Fig. 4A) where the nucleophilic thiol of the active site attacks the carbon between the sulfur and the carbonyl group leading to an irreversible TG2 acetonylation. The compounds differ in their membrane permeability. T101 has two methyl groups while D004 presents two phenyl groups. Accordingly, D004 is more hydrophobic and it tends to penetrate preferentially into cells. As expected, the hydrophilic T101 was recently shown to act only extracellularly (Antonyak et al., 2011). We tested these compounds in our system and found that only the cell permeable TG inhibitor, D004, exerted dose-dependent protection against glutamate toxicity (Fig.4D). T101 failed to inhibit intracellular activity (Fig. 5A and B) and was not able to protect neurons from glutamate toxicity at any of the concentrations tested (Fig. 4E). We conclude that Cystamine, B003, and D004 all inhibit intracellular TG in neurons to exert protection from oxidative stress. This conclusion was further reinforced by two additional observations; first, the ability to detect a fluorescently conjugated-peptide based inhibitor, TAMRA-DON, to penetrate inside neurons in a dose-dependent manner (Fig. 5C, upper panel) and the ability of this inhibitor to protect against oxidative stress at 100μM (Fig. 5C, lower panel); second, the ability of TG inhibitors to protect hippocampal neuroblasts (grown in the absence of astrocytes) from oxidative death (data not shown). Our results do not exclude the possibility that both extracellular and intracellular TG must be inhibited to protect neurons.

A recent study from our group showed that low, protective doses of hydrogen peroxide can induce a “state” change in astrocytes that facilitates their ability to protect adjacent neurons from glutathione depletion-induced death (Haskew-Layton et al., 2010). A targeted microarray demonstrated that Tgm2 was among the small number of genes whose downregulation correlated with neuroprotection (Haskew-Layton et al., 2010). To determine whether TG inhibition in astrocytes can non-cell autonomously protect neurons, we pretreated astrocytes with the isoform non-selective TG inhibitors Cystamine or B003. The inhibitors were washed off, cortical neurons were added and the co-culture was exposed to the glutamate analog, HCA. Without pretreatment with the TG inhibitors, a substantial number of neurons died in response to the glutamate analog (Fig. 6A, B and C; HCA treatments). In contrast, TG inhibition in astrocytes led to a significant sparing of the neurons (Fig. 6A, B and C; Cystamine and B003 treatments). These findings demonstrate that TG inhibition in neurons or astrocytes is sufficient to confer resistance to oxidative death.

Inhibition of the ERK pathway suppresses TG2 activation

Previous studies from our lab and others have shown that glutathione levels reach a steady state (approximately 60% below control) at 6 hours following glutamate treatment (Zaman et al., 1999). Cells become irreversibly committed to cell death much later, around 18 hours. The increase of TGs transcription and activity within 8-12 hours after glutamate exposure raised the possibility that TG inhibition, well after onset of glutamate treatment, would suppress increased TG activity and protect neurons downstream of glutathione depletion. Indeed, addition of the cell-permeant TG inhibitors (Cystamine 100μM, B003 100μM, D004 50μM) up to 14-17 hours after glutamate treatment, completely protected neurons from cell death (Fig. 7A) and this protection happened without an effect on glutathione depletion by glutamate (not shown). TG inhibitors can thus be used well after depletion of

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glutathione and still protect neurons and this may, in part, explain their effectiveness in multiple disease models. Increased TG activity well after the onset of oxidative stress and just before the cells are irreversibly committed to death lead us to ask whether TG transamidating activity lies upstream or downstream of events known to be causally related to oxidative death that occur just prior to cell death commitment. Among the signaling molecules defined, the ERK family of kinases caught our attention. Prior elegant work from DeFranco and colleagues revealed a delayed and sustained activation of ERK1/2 owing to redox mediated MKP-1 or PP2A inhibition (Ho et al., 2007). Intriguingly, prior data also suggests that ERK can activate TGs (Akimov and Belkin, 2003) and the transamidating activity may induce heterodimerization of ERK proteins into a complex in the nucleus (Lundquist and Dudek, 2006). We thus examined whether TG activity is downstream of pathological ERK activation in glutathione depletion-induced oxidative stress. To understand the relationship between TG activity and sustained ERK activation, we examined TG activity in the presence of a well-established MEK1/2 (which works upstream of ERK) inhibitor U0126, its structurally similar, but inactive analog, U0124, and a brain permeant MEK inhibitor, SL327 (Wang et al., 2003). These compounds were tested for activity (Fig. 7B) and protection (Fig. 7C) in our system. U0124 failed to block MEK (Fig. 7B) activation and failed to protect cells from oxidative stress (Fig. 7C). As expected, we found that protective concentrations of U0126 and SL327 significantly reduced TG activity and U0124 did not (Fig. 7D). Moreover, U0126 does not prevent death mediated by forced TG expression (not shown). Altogether these findings place TG activity downstream of pathological ERK activation in the oxidative stress cascade, and suggest that TG may be a distal point in the affector pathway of oxidative death.

Discussion

Most schemes for TG's role in acute and chronic neurodegeneration have centered around the ability of these enzymes to crosslink mutated and/or accumulated proteins in a host of diseases, including AD, HD, and PD (Caccamo et al., 2011). And while this model unifies diseases associated with proteotoxicity, it fails to account for the benefits of molecular or pharmacological TG deletion in ischemic (Hwang et al., 2009; Colak et al., 2011) or hemorrhagic stroke (Okauchi et al., 2009). Indeed, exciting new data on the role of TG in autophagosome formation (D'Eletto et al., 2009), in inhibiting axonal transport of growth factors such as BDNF (Borrell-Pages et al., 2006), in repressing adaptive gene expression (McConoughey et al., 2010), and influencing nuclear actin dynamics (Munsie et al., 2011) have focused attention on biological roles of these fascinating enzymes other than crosslinking. Here, we demonstrate that TG is a necessary component of oxidative stress-induced death signaling in cortical neurons (Figs. 4-6). As oxidative stress has been implicated in almost every neurological condition, the findings suggest that even in diseases characterized by proteotoxicity, that TG's major role may not be limited to crosslinking but also include cell death signaling.

Because of TG2's abundance in the brain, its transcriptional induction in a host of disease states, and the neuroprotective effects of its germline deletion, there has been almost exclusive focus on this isoform as a perpetrator of neuronal loss. Our results in a model of stroke *in vivo* and in a model of oxidative stress *in vitro* amplify recent studies by others that show that in addition to TG2, TG1 is also dramatically regulated by ischemia and/or oxidative stress. Unexpectedly, we showed that TG1 was as effective in inducing death in cortical neurons as TG2 (Fig. 1). The results help to explain the broad salutary effects of currently available small molecule inhibitors of TG including in our *in vitro* model of oxidative stress, as all of these have been shown to inhibit TG1, TG2 and other TG isoforms (Schaertl et al., 2010). They also provide one explanation why in the R6/2 mouse model of HD, isoform non-selective TG inhibition prolongs survival (19.8%) greater than germline

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deletion of TG2 (12%) (Bailey and Johnson, 2006). Future studies will examine the effect of dual TG1 and TG2 deletion on disease outcomes in models of stroke and HD.

TGs have been implicated in oxidative stress-induced death outside of the nervous system, but most of these studies, which implicate TG as an inhibitor of cell death, involve the addition of non-physiological oxidants to non-neural cells. By contrast, the model we employed in this study involves depletion of the versatile cellular antioxidant, glutathione- a theoretically more physiological way to induce an imbalance in oxidants and antioxidants above a toxic threshold, the operational definition of "oxidative stress". Indeed, exposure of enriched cultures of neurons or astrocyte-neuronal co-cultures to high concentrations of glutamate or glutamate analog (5 mM) leads to selective degeneration of neurons over 24 to 48 hours (Murphy et al., 1989; Haskew-Layton et al., 2010). In this paradigm, glutamate toxicity results not from hyperactivation of ionotropic glutamate receptors, but rather via competitive inhibition of cyst(e)ine transport, leading to diminished glutathione levels and oxidative death. Accordingly, neurons undergo antioxidant-responsive activation of an ERK-dependent, caspase-independent cell death mechanism that involves 12-lipoxygenase, tBID and AIF (Seiler et al., 2008). Of note, disrupted calcium homeostasis, which would be expected to activate TGs, has also been implicated in oxidative glutamate toxicity (Tan et al., 1998); our findings place TG activation as an event downstream of ERK signaling, but prior to cell death commitment. Indeed, ERK inhibitors cannot block death due to forced expression of TG2. In conjunction with the findings herein, recent studies from our lab (McConoughey et al., 2010) suggest that TG acts to repress prosurvival gene expression to ensure oxidative death; but our studies do not preclude a role for TG in the effector pathway of death following glutathione depletion in cortical neurons, including AIF translocation.

The *in vitro* model of oxidative death used in this study has been used by many laboratories to identify a number of targets whose inhibition protects from stroke (Siddiq et al., 2005), HD (Ferrante et al., 2003; Ferrante et al., 2004), PD (Lee et al., 2009), or AD (Sagara et al., 1998) *in vivo*, including, hypoxia inducible factor prolyl hydroxylases (Siddiq et al., 2005), class I and II HDACs (Langley et al., 2008), ATF4 (Lange et al., 2008), Sp1 (Ryu et al., 2003; Sleiman et al., 2011), Myc (Sleiman et al., 2011), and Keap (a negative regulator of Nrf2) (Smirnova et al., 2011). Indeed, we found a similar induction of Tgm1 and Tgm2 levels in cortical neurons undergoing oxidative death as we did in the ipsilateral hemisphere following stroke (Fig. 1). Although our *in vivo* studies do not distinguish neuronal versus non-neural activation of TG, we present *in vitro* data that shows that inhibition of TG in astrocytes or neurons can abrogate neuronal oxidative death (Figs 4-6).

In conclusion, our study highlights the role that oxidative stress, a putative mediator of many neurological conditions, can play in activating multiple TG isoforms downstream of pathological ERK signaling. The findings provide a unifying model for the role of TG in numerous neurological conditions, and the broad salutary effects of isoform non-selective TG inhibitors. As ERK has both physiological and pathological roles (Chu et al., 2004), we propose that TG inhibition represents a more specific way to inhibit pathological ERK signaling without affecting physiologically important ERK activation downstream of growth factor receptor activation.

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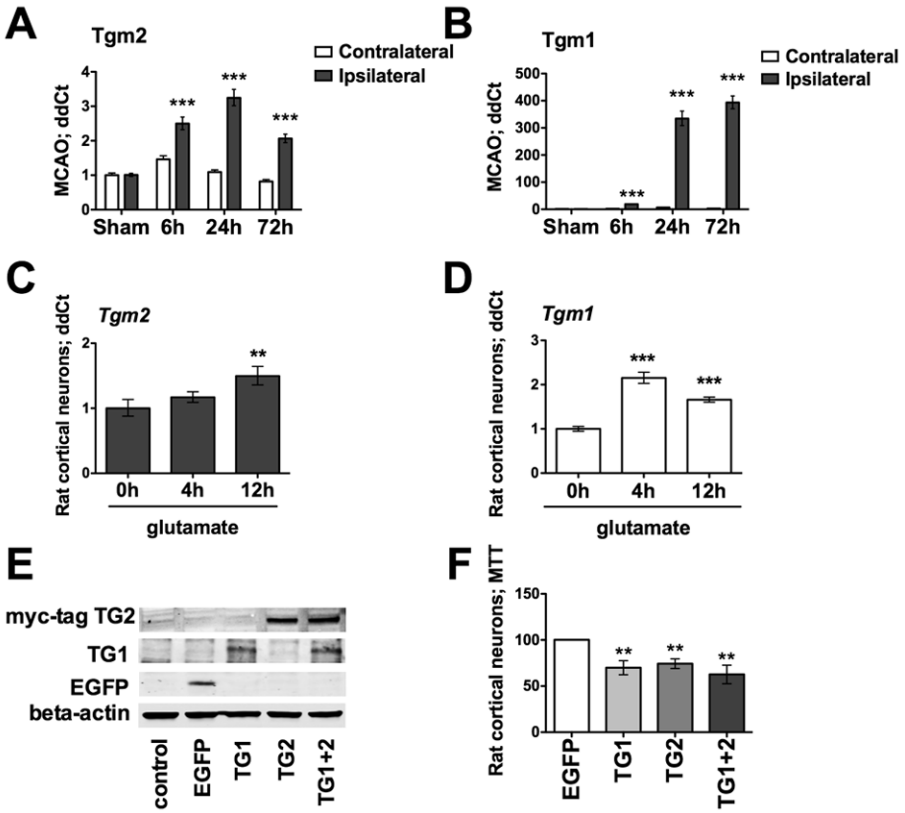


Figure 1. TG1 and TG2 mRNA levels are upregulated in a focal model of stroke (MCAO) and in an *in vitro* model of neuronal oxidative stress and they are sufficient to induce cell death

Temporal expression profile for Tgm2 (A) and Tgm1 (B) in MCAO. Both of the genes are significantly upregulated in the ipsilateral side compared to the contralateral hemisphere (***p<0.001). A similar upregulation is revealed in an *in vitro* model of oxidative stress. Tgm2 (C) and Tgm1 (D) levels are significantly upregulated 12 hrs after glutamate (5mM) exposure in rat neurons (**p<0.01; ***p<0.0001 compared to the relative glutamate 0h). Forced expression of TG1 and/or TG2 (E) exerts neuronal toxicity as revealed by MTT (F). Significant toxicity compared with EGFP expression: **p<0.01. EGFP expressing cells are calculated at 100% survival.

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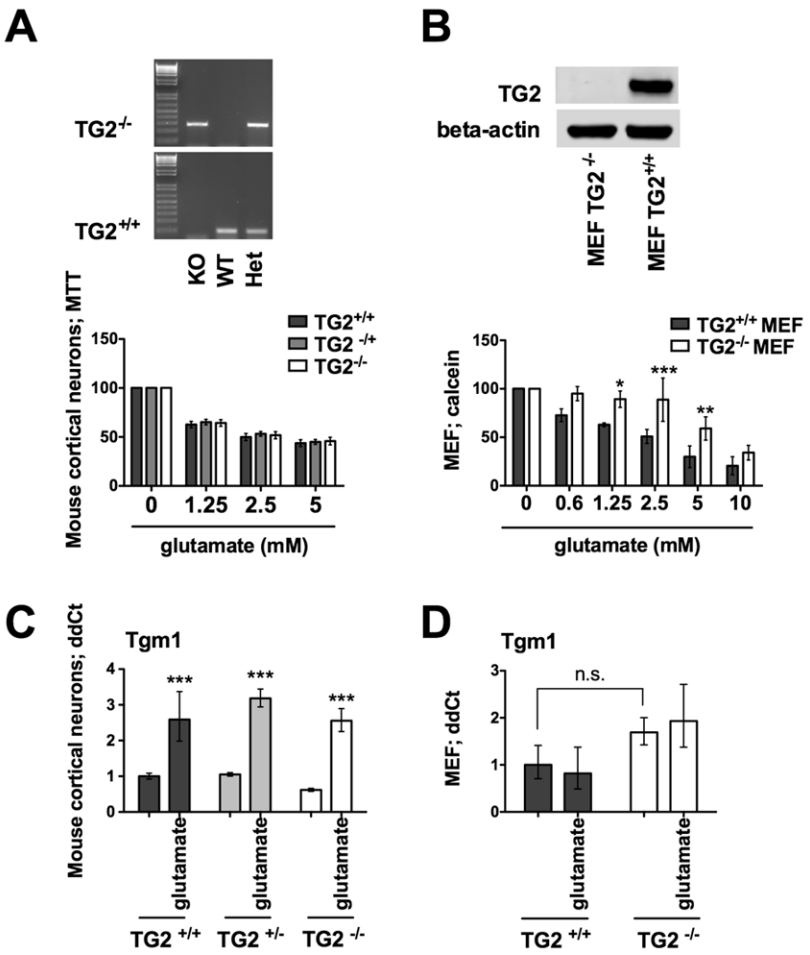


Figure 2. TG2 is necessary for oxidative death in mouse embryonic fibroblasts (MEF) but not cortical neurons. Resistance of cortical neurons to TG2 knockout is associated with compensatory upregulation of Tgm1

$TG2^{-/-}$in single embryonic neuronal cultures (A) and in MEFs (B) and relative MTT assay. TG2 deletion in MEFs significantly protects against glutamate-induced death; * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to $TG2^{+/+}$ MEFs; untreated controls are calculated at 100% of survival. Tgm1 levels are induced after 12 hours of glutamate treatment in mouse neurons (C) (*** $p<0.001$ compared to controls), but not in MEFs (D).

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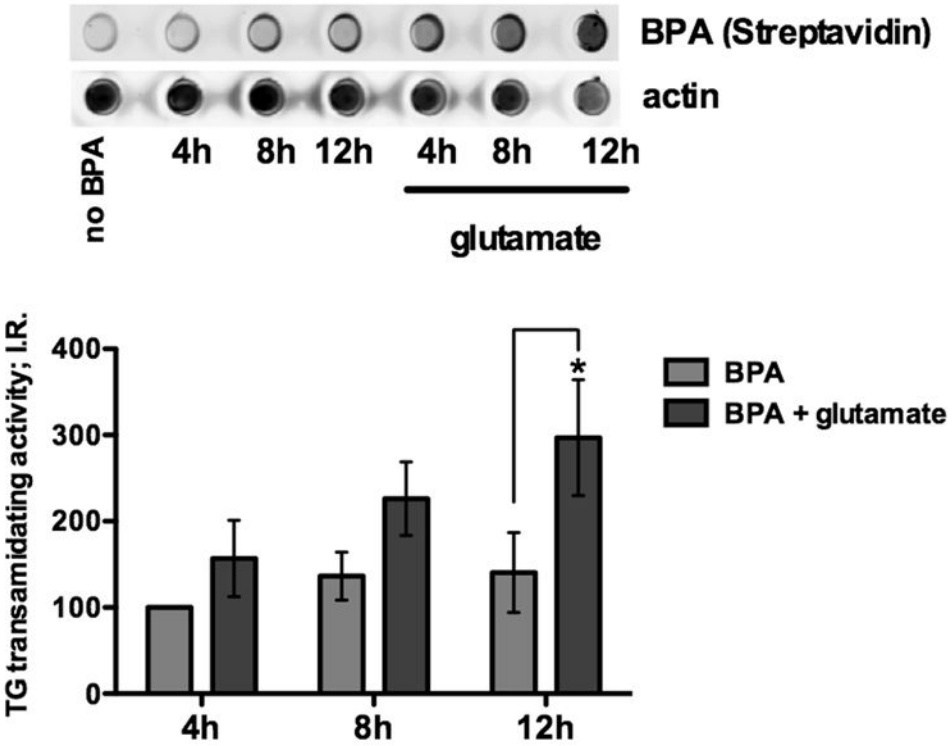


Figure 3. Intracellular TG transamidating activity is increased upon glutamate treatment

Endogenous transamidating activity is significantly increased after 12 hours of glutamate treatment compared to control by dot blot assay; ** $p<0.01$.

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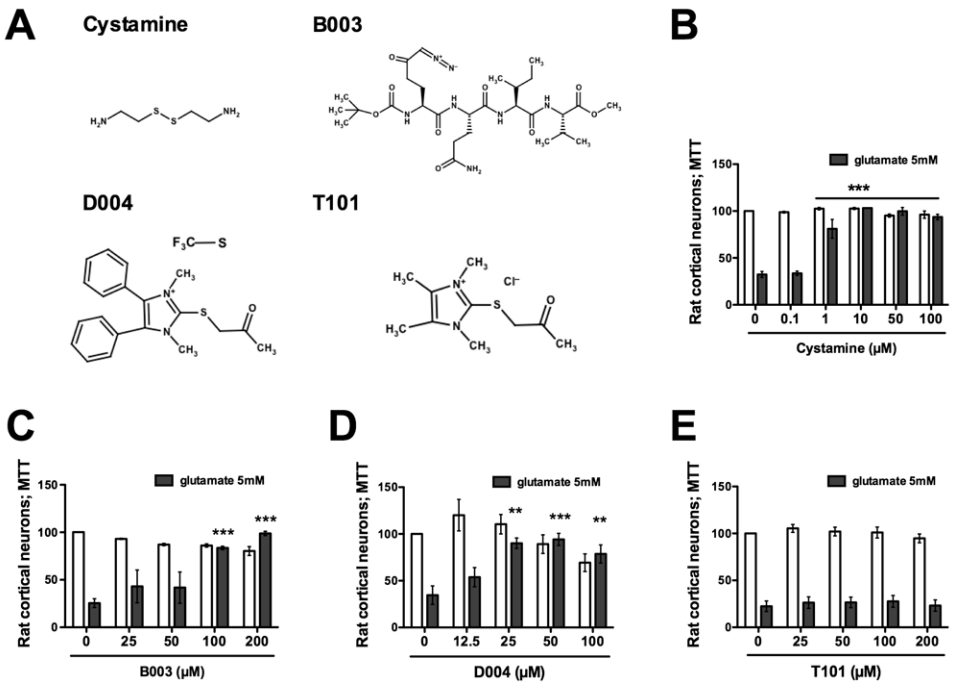


Figure 4. TG inhibition protects immature cortical neurons from oxidative stress-mediated cell death

Structures of four diverse, reversible or irreversible, isoform non-selective TG inhibitors tested in an *in vitro* model of oxidative stress (A). Cystamine (1-100μM) (B), B003 (100, 200μM) (C), and D004 (25-100μM) (D) protect primary immature cortical neurons against oxidative stress-induced cell death. T101, a cell impermeable TG inhibitor, fails to provide protection (E); ** $p<0.01$; *** $p<0.001$ compared to glutamate treatment alone; untreated controls are calculated at 100% of survival.

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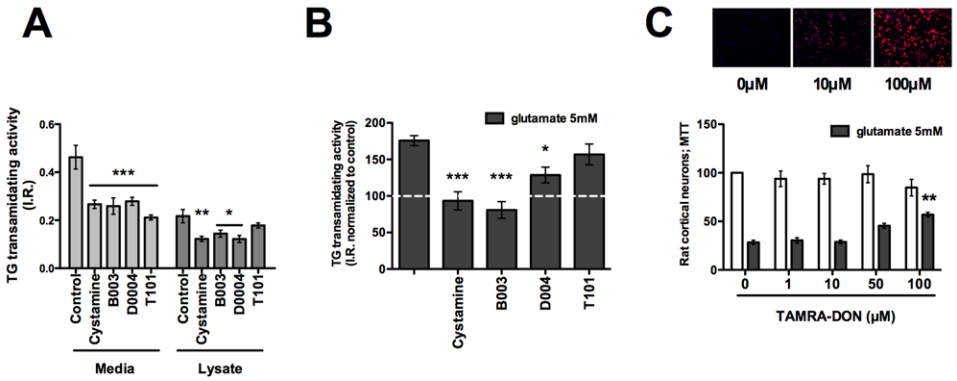


Figure 5. Inhibition of intracellular TG activity correlates with neuroprotection

All inhibitors tested are able to significantly reduce extracellular transamidating activity (A). T101 fails to inhibit intracellular TG transamidating activity (A and B). All the protective inhibitors are able to significantly downregulate TG2 activity to control levels (B). TAMRA-DON (C), a peptide-based, fluorescent inhibitor analog to B003, enters the cells (10-100μM) (upper panel) and it protects against oxidative stress (100μM) (lower panel); ** $p<0.01$ compared to glutamate treatment alone; untreated controls are calculated at 100% of survival.

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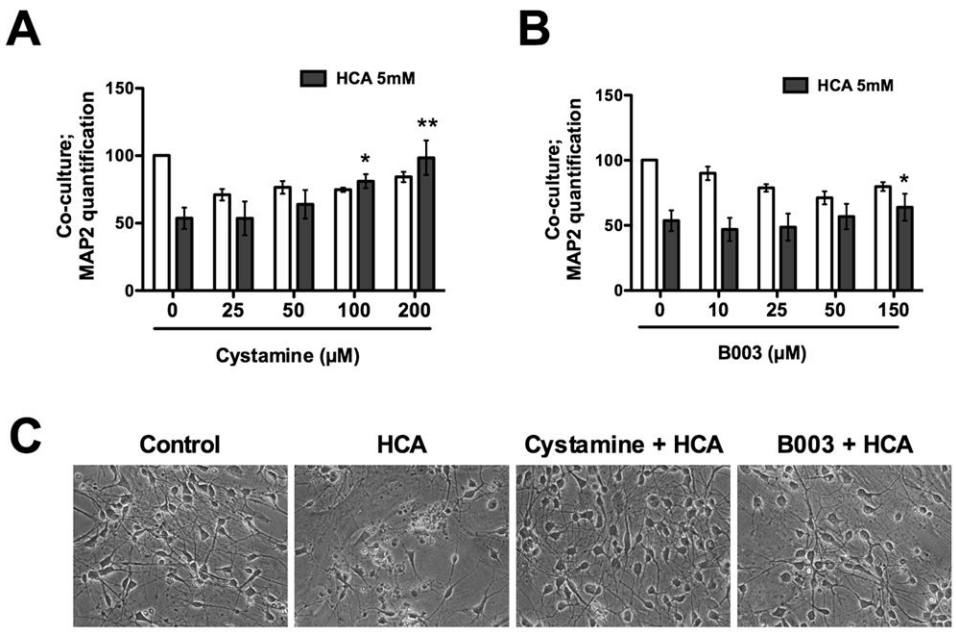


Figure 6. TG inhibition in astrocytes non-cell autonomously protects neurons

Astrocytes are treated overnight with the TG inhibitors Cystamine (A) or B003 (B). Following washoff of the inhibitors, neurons were plated +/- the glutamate analog, HCA (5mM), for 48 h; * $p<0.05$; ** $p<0.01$ neuronal survival compared to glutamate treatment alone, untreated controls are calculated at 100% of survival. (C) Representative phase contrast pictures for A and B; Cystamine 200μM and B003 150μM.

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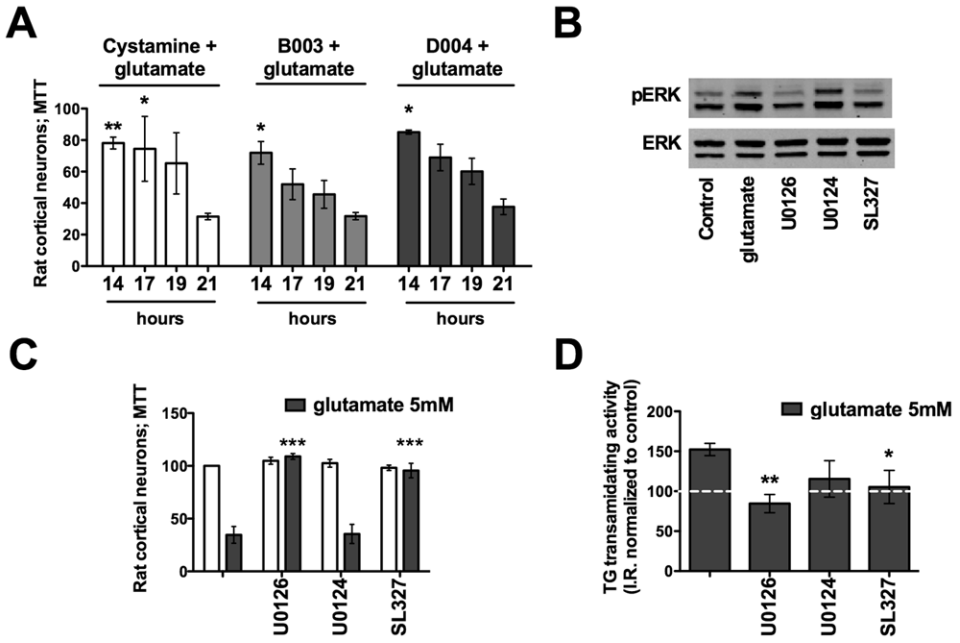


Figure 7. TG transamidating activity is a downstream target of the ERK pathway

TG inhibitors exerted protection when added up to 14-17 hours post-glutamate exposure (A). Glutamate induces pERK (B); MEK inhibitors, U0126 and SL327, block pERK activation (B) and protect against glutamate toxicity (C) by reducing TG activation to the control level (D) * $p<0.05$; ** $p<0.01$ compared to glutamate treatment alone; untreated controls are calculated at 100% of survival. U0124 is a negative control for U0126.