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Depletion of transglutaminase 2 in neurons alters expression of extracellular matrix and signal transduction genes and compromises cell viability

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Abstract

The protein transglutaminase 2 (TG2) has been implicated as a modulator of neuronal viability. TG2's role in mediating cell survival processes has been suggested to involve its ability to alter transcriptional events. The goal of this study was to examine the role of TG2 in neuronal survival and to begin to delineate the pathways it regulates. We show that depletion of TG2 significantly compromises the viability of neurons in the absence of any stressors. RNA sequencing revealed that depletion of TG2 dysregulated the expression of 86 genes with 59 of these being upregulated. The genes that were upregulated by TG2 knockdown were primarily involved in extracellular matrix function, cell signaling and cytoskeleton integrity pathways. Finally, depletion of TG2 significantly reduced neurite length. These findings suggest for the first time that TG2 plays a crucial role in mediating neuronal survival through its regulation of genes involved in neurite length and maintenance.

Keywords

transglutaminase; neurons; RNA sequencing; cell survival; neurite length

Introduction

Transglutaminase 2 (TG2) is a multifaceted protein that is expressed in numerous tissues and cell types. However, its subcellular localization and function differ depending on the cell

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type. TG2 was initially identified as a calcium-dependent transamidating enzyme, but subsequent studies showed that TG2 is also a GTPase, a protein disulfide isomerase, and can function as a scaffold or linker protein (Begg et al., 2006; Chen and Mehta, 1999; Gundemir et al., 2012; Hasegawa et al., 2003). In the central nervous system (CNS) TG2 is expressed in both neurons and glial cells (Kim et al., 1999; Maggio et al., 2001). In both cell types TG2 is found predominantly in the cytosol, and in neurons nuclear localization has been clearly documented (Colak et al., 2011; Filiano et al., 2008; van Strien et al., 2011b). Nuclear TG2 may play a crucial modulatory role as studies in other cell types have demonstrated that TG2 can regulate transcriptional processes that are involved in mediating cell death, survival and differentiation (Ahn et al., 2008; Filiano et al., 2008; Gundemir et al., 2012; Gundemir et al., 2013; Mann et al., 2006).

It is becoming increasingly apparent that TG2 plays critical roles in the CNS. In astrocytes TG2 deletion facilitates survival in an oxygen and glucose deprivation (OGD) paradigm (Feola et al., 2017). However, intriguingly, the opposite seems to be true in neurons (Colak and Johnson, 2012). Expression of exogenous TG2 in neurons significantly attenuated OGD-induced cell death independent of the transamidating activity, as a transamidating inactive TG2 mutant protected to the same extent as the wild type TG2 (Filiano et al., 2008). Additionally, the ability of TG2 to facilitate cell survival appears to be dependent on its nuclear localization. In HEK cells exogenous expression of wild type or transamidating inactive forms of TG2 increased survival subsequent to OGD when the TG2 was targeted to the nucleus using a nuclear localization signal (NLS). Expression of TG2 constructs with a nuclear export sequence (NES) afforded no protective effects against OGD-induced cell death (Gundemir and Johnson, 2009). When comparing the localization of TG2 in neurons and astrocytes it was found that exposure to hypoxia resulted in a significant increase in nuclear TG2 levels in neurons, but in astrocytes just the opposite was true; nuclear levels decreased significantly with hypoxia (Yunes-Medina et al., 2017). Further, in a mouse model, overexpressing TG2 in neurons significantly decreased infarct volumes following a middle cerebral artery ligation (MCAL)-induced stroke concurrent with increases in nuclear TG2 levels (Filiano et al., 2010). These data clearly indicate that TG2 promotes neuronal survival and that it is likely dependent on its localization to the nucleus..

TG2 also plays a role in differentiation and adaptive processes in the CNS. In a model of de- and re-myelination TG2 was involved in differentiating oligodendrocyte precursor cells into mature oligodendrocytes, and this function required TG2 to be active as a transamidating enzyme (Van Strien et al., 2011a). In astrocytes TG2 plays a central role in facilitating migration, which is important in both development and injury responses (Monteagudo et al., 2017; van Strien et al., 2011c). However, the ability of TG2 to modulate differentiation or development processes in neurons has not been examined.

To begin to understand the role TG2 plays in neurons and the underlying molecular mechanisms, TG2 was knocked down in primary cortical neurons with an shRNA lentivirus and compared to controls transduced with a scrambled RNA. Surprisingly reduction of TG2, without any added stress, significantly decreased the viability of the neurons. These findings indicate that, at physiological levels, TG2 plays a pro-survival role in neurons. Given the fact that TG2 can regulate transcription, we examined how TG2 depletion affected the

transcriptome profile of neurons by using RNA-sequencing (RNA-seq). These analyses were carried out on neurons at least 24 hours before any decreases in viability were observed. RNA-seq data showed that the majority of genes upregulated when TG2 was knocked in neurons play key roles in extracellular matrix (ECM) function, cell signaling and cytoskeleton integrity pathways. These findings indicate that TG2 plays a role in maintaining the appropriate complement of genes that neurons use to properly remodel, particularly their neuritic extensions, during development but also in response to insults. This is evidenced by the finding that knockdown of TG2 significantly decreased neurite length prior to loss of viability. Overall, these findings suggest that TG2 plays a pivotal role in supporting neuronal health by maintaining an appropriate gene expression profile and providing new avenues for determining the mechanisms by which TG2 mediates these processes.

Materials and Methods

Animals

Animals were housed and euthanized in accordance with guidelines established by the University of Rochester Committee on Animal Resources. The studies were carried out with approval from the Institutional Animal Care and Use Committee. Timed-pregnant Sprague Dawley rats were obtained from Charles River Laboratories and used for preparation of primary cortical neurons.

Primary neuronal culture

At embryonic day 18 (E18) rat cortices were dissociated using trypsin and mechanical force as previously described (Filiano et al., 2008). The dissociated cells were plated in poly-D-lysine coated plates with MEM supplemented with 10% fetal bovine serum, 20 mM glucose, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin for 5 hours. After 5 hours, the medium was fully replaced with Neurobasal media with B-27 supplement and L-glutamine. Neurons were maintained in a humidified incubator at 37°C and 5% CO_2. Based on immunostaining with GFAP, the astrocyte contamination of the neuron cultures was less than 5% (data not shown).

Lentivirus preparation and transduction

The shRNA for rat TG2 is 5′-GAGCGAGATGATCTGGAAT-3′ which is identical to the shRNA for human/mouse except the final 3′ nucleotide is T in rat TG2, instead of C in human/mouse TG2 (Gundemir et al., 2013; Robitaille et al., 2008). Human TG2 in FigB (Colak et al., 2011) was made shRNA resistant by changing the underlined bases to A, T and A, respectively (see below). shRNA for TG2 and a scrambled version were synthesized, annealed and subcloned into the pSuper shuttle vector just downstream of the H1 promoter using BglII and Xho1 restriction sites. The H1-shRNA (or Scr) cassette was amplified from the pSuper vector and cloned into the Hpa1/PacI sites of the lentiviral vector FG12. Plasmids were confirmed by sequencing. The lentiviral vector backbone was a generous gift from Dr. C. Pröschel at the University of Rochester. Lentivirus was made by co-transfecting the lentiviral vector, pPAX and VSVG plasmids into 60% confluent HEK293 TN cells. The viral particles were collected 48 hours after transfection by centrifuging the media at 146,000 × g

at 4°C for 3 hours. The viral pellet was resuspended in 1% bovine serum albumin in sterile phosphate buffered saline (PBS). The resuspended virus was rapidly frozen in liquid nitrogen and stored at -80°C for up to one month. Lentivirus was added to DIV3 neurons. Within 16 hours after transduction the media was fully changed. For cell viability assays neurons were collected on DIV 9; for RNA-seq they were collected on DIV 8.

Re-expression of TG2

Lentiviruses expressing shRNA TG2, empty vector (FigB) or shRNA resistant human TG2 (hTG2) were prepared as described above. hTG2 in the lentiviral vector FigB (Colak et al., 2011) was made shRNA resistant by creating 3 mismatches (see sequence above) within the shRNA target domain that did not alter the amino acid sequence using the QuikChange Mutagenesis kit (Agilent). Neurons were prepared as described above and lentivirus expressing shRNA TG2 was added on DIV3. Within 16 hours after transduction the media was fully changed. On DIV5 the same neurons expressing TG2 shRNA were transduced with either empty vector (FigB) or shRNA resistant TG2 lentivirus. Within 16 hours after transduction the media was fully changed. Neuronal cell viability was measured on DIV9 using the resazurin assay.

Resazurin assay

The resazurin assay was performed as described previously (Colak and Johnson, 2012). Six days after transducing the neurons, resazurin was added directly to the culture media to a final concentration of 50 μg/ml. The neurons were returned to the incubator for 30 minutes. After 30 minutes, fluorescence was measured by exciting the cells at a wavelength of 540 nm and reading at 590 nm emission using a plate reader (BioTek Synergy HT Multi-Detection Microplate Reader). The fluorescence intensity was taken as proportional to the number of viable cells.

Nuclear Pyknosis Count

Neurons were plated on coverslips and transduced as described above. Six days after transduction, neurons were fixed with 4% paraformaldehyde for 10 minutes at room temperature and washed three times with phosphate buffered saline (PBS). The cells were washed and stained with DAPI nuclear stain (1:1,000, Molecular Probes, D-1306) for 5 minutes at room temperature allowing for pyknotic cell identification and quantitation. After washing the cells, the coverslips were mounted on glass with Prolong diamond Antifade Mountant (Invitrogen, P36961). The images were acquired using a Zeiss Axio Observer D1 inverted microscope with an Axiocam-XMR camera (Carl Zeiss, Hamamatsu ORCA-ER digital camera). The percentage of neurons with pyknotic nuclei (condensed or blebbing) was determined for each treatment group (shRNA TG2 or Scr transduced).

RNA isolation and cDNA synthesis

RNA was isolated from neurons transduced with Scr or shRNA TG2 virus using the RNeasy Plus Kit (QIAGEN, 74134) according to the manufacturer's instructions. cDNA was made using Verso cDNA Synthesis Kit (Thermo Scientific, AB1453B) for qPCR analyses. The RNA and cDNA were stored at -80°C.

RNA sequencing and enrichment pathway analysis

RNA-seq was performed at the University of Rochester Genomics Research Center on three biological replicate samples of RNA isolated from Scr and shRNA TG2 expressing neurons. RNA concentration was determined with the NanopDrop 1000 spectrophotometer (NanoDrop, Wilmington, DE) and RNA quality was assessed with the Agilent Bioanalyzer (Agilent, Santa Clara, CA). The TruSeq RNA Sample Preparation Kit V2 (Illumina, San Diego, CA) was used for next generation sequencing library construction per the manufacturer's protocols. Briefly, mRNA was purified from 100 ng total RNA with oligo-dT magnetic beads and fragmented. First-strand cDNA synthesis was performed with random hexamer priming followed by second-strand cDNA synthesis. End repair and 3′ adenylation was then performed on the double stranded cDNA. Illumina adaptors were ligated to both ends of the cDNA, purified by gel electrophoresis and amplified with PCR primers specific to the adaptor sequences to generate amplicons of approximately 200 - 500bp in size. The amplified libraries were hybridized to the Illumina single end flow cell and amplified using the cBot (Illumina, San Diego, CA) at a concentration of 8 pM per lane. Single end reads of 100 nt were generated for each sample. Sequenced reads were cleaned according to a rigorous pre-processing workflow (Trimmomatic-0.32) before mapping them to the rat genome (rn6) with STAR-2.4.2a (https://github.com/alexdobin/STAR). Cufflinks2.0.2 (cuffdiff2 - Running Cuffdiff) was used with the UCSC rat gene annotations to perform differential expression analysis with an FDR cutoff of 0.05 (95% confidence interval). Enrichment pathway analysis was performed on the 86 significant differentially expressed genes with the computational platform Lynx (http://lynx.ci.uchicago.edu/)(Sulakhe et al., 2014). Lynx uses the following public databases for enrichment pathway analysis: GO Molecular Function, GO Biological Process, GO Cellular Component, GO Hierarchy - Molecular Function, GO Hierarchy - Biological Process, GO Hierarchy - Cellular Component, Pubmed (Uniprot & NCBI Generif), Diseases, Tissues, Phenotype, Pathways (In-house integrated resource from pathway information from KEGG, BIOCARTA, NCI and Reactome), Uniprot Keywords, Interpro Domain, Customized Brain Connectivity Ontology, VISTA TFBS Clusters and VISTA Tissue Enhancers.

qPCR

The cDNA was diluted in DEPC treated water 1:5 and 2 μl was used per well of a 96-well plate. The SYBR Green mix (Thermo Fisher Scientific, 4309155) was used to detect transcription. Exon-exon spanning primers were made for serpinE1 (F 5′-CGTCTTCCTCCACAGCCATT -3′,R 5′-GCTGGCCCATGAAGAGGATT-3′), Itga5 (F 5′-GAAGGGACGGAGTCAGTGTG -3′, R 5′-TGAATGGTGCTGCACTGGAT-3′), Col3a1 (F 5′- TGGGCCTCAAGGTGTAAAGG -3′, R 5′-GCCCTGGATTACCATTGTTGC-3′), Loxl2 (F 5′- AGCCTATAAGCCGGAGCAAC-3′, R 5′-GTCCCACTTGTCATCGCAGA-3′), Bgn (F 5′-GAACAGTGGCTTTGAACCCG-3′, R 5′-GGTCTCAGGGAGATCTTTGGG-3′), Col6a2 (F 5′-CTTTGTCCGGACCCCCAGAT-3′, R 5′-TGTAGAAGTTCTGCTCGCCC-3′), Mmp2 (F 5′- GGGTGGTGGTCACAGCTATT-3′, R 5′-CCCAGCCAGTCCGATTTGAT-3′), Ltbp1 (F 5′-TCTCTGCAAGAACGCCAAGT-3′, R 5′-CAAAGCGGTGTTCAGTGGTG-3′), Mfap4 (F 5′- GTGGCAAGTGGACGGTTTTC-3′, R 5′-AGCCCCAGCCAGTACTCC-3′), Fn1 (F 5′- GCTAGCAGTCCTGTGCCTG-3′, R 5′-CAGCCAGGCTTGCTCTGA-3′),

Col18a1 (F 5′-TCCAGGACCTCCCTCATTCC-3′, R 5′-ATCCTTACCTGCCCAGCAGA-3′), Col1a1 (F 5′-CCGATGGATTCCAGTTCGAGT-3′, R 5′-GCTACGCTGTTCTTGCAGTG-3′), Fbln1 (F 5′-TCCATCAACGAGACCTGCTTC-3′, R 5′-TTCTCTTGGCGAAAGGTGTCT-3′) and Col5a2 (F 5′- TGATGGAGAGCCAGGTGTTC-3′, R 5′-GCCAATAGGACCCACAGAGC-3′). The data was normalized to HPRT (F 5′-ACAGGCCAGACTTTGTTGGAT -3′, R 5′-TTGCCGCTGTCTTTTAGGCT -3′). Amplification was performed using a BioRad CFX96 Real Time System C1000 Thermo Cycler. Prior to qPCR analysis all amplification products were determined to be approximately the same size – the products were not more than 30bp different in size difference and only one peak was detected in the melting curve, primer amplification efficiencies were tested by a serial dilution of a template to generate a standard curve and the primers were also used with a temperature gradient to find their optimal binding temperature. Data were analyzed using the ΔΔCt method. Data are presented was the fold change compared to the control group, neurons expressing scramble RNA.

Immunoblotting

Protein lysates from Scr and shRNA TG2 expressing neurons were collected with RIPA buffer supplemented with protease inhibitors 6 days after transduction. Protein concentration of the samples was determined by BCA assay. Samples were diluted in collection buffer and 5× reducing stop buffer, boiled and resolved in a 12% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in tris buffered saline with Tween20 (TBS-T) (20 mM Tris base, 137 mM NaCl, 0.05% Tween-20) for one hour at room temperature. After blocking, the membranes were incubated overnight at 4°C with primary antibodies against serpinE1 (1:1,000, ABclonal (www.abclonal.com), A6211), vinculin (1:1,000, Bioss Antibodies (www.biossusa.com), bs-6640R), phospho (pY397) focal adhesion kinase (FAK) (1:1,000, Bioss Antibodies, bs-3159R), TG2 (clone CUB7402, 1:5000, Abcam (www.abcam.com), ab2386, or TGMO1, 1:5000 rat monoclonal antibody (Song et al., 2013)), tubulin (1:5000, Cell Signaling Technology (www.cellsignal.com), 2125S) or GAPDH (1:5,000, MilliporeSigma (www.emdmillipore.com), MAB374) in blocking buffer. Membranes were later washed three times with TBS-T and incubated at room temperature for one hour with HRP-conjugated secondary antibody. The membranes were washed with TBS-T and visualized using an enhanced chemiluminescence reaction.

Immunocytochemistry

Neurons were plated on coverslips and transduced as described above. Six days after transduction, neurons were fixed with 4% paraformaldehyde for 10 minutes at room temperature and washed three times with PBS. The neurons were permeabilized with 0.2% Triton-X in PBS for 2 minutes and washed three times with PBS. The cells were blocked with 10% normal goat serum (NGS) for an hour and later incubated overnight at 4°C with MAP2 antibody (1:500, MilliporeSigma, M4403) in 1% NGS. The next day cells were washed three times with PBS and incubated at room temperature for 2 hours with Alexa 594 (1:400, Invitrogen, A11032) in 1% NGS. The cells were washed and stained with DAPI nuclear stain (1:1,000, Molecular Probes, D-1306) for 5 minutes at room temperature. After

washing the cells, the coverslips were mounted on glass with Prolong diamond Antifade Mountant (Invitrogen, P36961). The images were acquired using a Zeiss Axio Observer D1 inverted microscope with an Axiocam-XMR camera (Carl Zeiss, Hamamatsu ORCA-ER digital camera). The FG12 vector backbone expresses GFP under the UbiC promoter and therefore transduced neurons were GFP positive and were the ones used for quantification. MAP2 staining was used to determine neurite length by tracing with Image J and measuring the length of neurites.

Cellular treatments

Neurons were plated and transduced as described above. Four days after transduction one group of neurons was treated with serpinE1 (Sigma, A8111): 0.1 μg/ml, 1.0 μg/ml or vehicle (ddH2O). Another group of neurons was treated with a serpinE1 inhibitor (Elokdah et al., 2004), PAI-039 (Sigma): 5 μM, 10 μM, 15 μM, or vehicle (DMSO). These concentrations were chosen according to previously published work (Cho et al., 2013). The treatment lasted for 48 hours. After treatment cells were stained as described above.

Statistical analysis

A D'Agostino and Pearson test or a Shapiro-Wilk test was conducted to determine whether the data in each experiment followed a normal distribution. A t-test or one-way ANOVA with Tukey's post-hoc for multiple comparisons was used to determine statistical significance for data that followed a normal distribution. A Mann-Whitney test was used to determine statistical significance for data that did not follow a normal distribution. All experiments were conducted with at least three biological replicates. A p value of < 0.05 was considered statistically significant.

Results

TG2 depletion reduces neuronal viability

Previous data have shown that exogenously expressed TG2 facilitates neuronal survival following insults (Filiano et al., 2008, Filiano et al., 2010). In order to better understand TG2's role in neuronal survival, we transduced neuronal cultures with Scr control, or shRNA TG2 lentivirus to downregulate expression of TG2 (Figure 1A and B). Six days after transduction cell viability was measured using the resazurin assay (DIV9). TG2 depletion significantly reduced neuronal viability as measured by resazurin assay and nuclear pyknosis count (Figure 1C and D). To confirm the effects of TG2 depletion on neuron viability, cells were transduced with the shRNA TG2 or scramble RNA lentivirus on DIV3 and then on DIV5 transduced either with an empty vector lentivirus (FigB) or with one expressing shRNA resistant TG2. When cell viability was measured on DIV9 neurons transduced with shRNA TG2 and then with the TG2 lentivirus were significantly more viable than those that were subsequently transduced with an empty vector (Figure 2). These results strongly indicate that TG2 is necessary for neuronal survival under basal conditions.

TG2 depletion changes transcriptional activity in neurons

Given that TG2 depletion compromised neuron viability (Figure 1) and that TG2 regulates transcriptional process (Gundemir et al., 2012; Kumar and Mehta, 2012), we performed

RNA-seq to provide insight into the possible mechanisms by which TG2 supports neuronal cell health. Rat primary cortical neurons were transduced with lentivirus shRNA TG2 or Scr control, and RNA was collected 5 days after transduction. Three independent biological replicates were collected and sequenced. Our analysis showed that 86 genes were differentially expressed between the groups (Figure 3A). Among these 86 genes, 27 were downregulated after TG2 depletion, while 59 were upregulated (Figure 3A and Supplementary Table 1). These results suggest that depletion of TG2 changes transcriptional activity in neurons.

Extracellular matrix (ECM) function, cell signaling and cytoskeleton integrity pathways are upregulated after TG2 depletion

Pathway analysis of the differentially expressed genes showed enrichment for signal transduction, axon guidance, β1 integrin interactions, focal adhesion, ECM organization, and phosphoinositde kinase-3 (PI3K)-Akt signaling pathways (Figure 3B). The enrichment pathway analysis revealed that, of the 86 differentially expressed genes, 31 genes were identified in these pathways (Figure 3C). These results suggest that depletion of TG2 disrupts pathways involved in cytoskeleton and cellular structural integrity.

RNA sequencing results were validated by qPCR

The enrichment pathway analysis showed that a substantial number of the upregulated genes were in the ECM/focal adhesion pathway. Therefore, we validated this pathway by qPCR using the same samples sequenced for RNA-seq. The qPCR results for the all the ECM associated genes followed the same trend as shown in the RNA sequencing results (Figure 4A). These findings were further validated by performing qPCR using a new set of RNA samples collected from Scr or shRNA TG2 transduced neurons. Similar to the RNA-seq samples, the new qPCR samples showed the same trend in the fold change in gene expression (Figure 4B). It is important to note that even though the expression of some genes was not increased to the same extent in the new samples the overall trend stayed the same. In summary, these results confirm the RNA-seq results at the mRNA level.

Depletion of TG2 increases the expression of proteins involved in ECM organization

To determine if the increases in mRNA observed in the TG2 depleted neurons resulted in increases in protein levels, samples were immunoblotted for proteins in the ECM organization pathway; serpinE1 and vinculin (Halstead et al., 2010; Myers et al., 2011; Turney et al., 2016). We also immunoblotted for the phosphorylated active form of FAK, which plays a pivotal role in mediating ECM signaling, as well as neurite outgrowth and maintenance (Ivankovic-Dikic et al., 2000; Robles and Gomez, 2006). Increases in all three were detected after TG2 depletion. (Figure 5). These findings indicate that changes in gene expression caused by TG2 depletion are also accompanied by changes at the protein level.

Depletion of TG2 decreases neurite length

In order to investigate further how the depletion of TG2 affects cytoskeleton integrity, neurite length was measured in neurons expressing Scr or shRNA TG2. The depletion of TG2 significantly decreased total neurite length (Figure 6A, C). Given that serpinE1 was

highly upregulated both at the mRNA and protein levels, and data suggest that the serpineE1-tissue plasminogen activator (tPA) axis plays a role in mediating cytoskeletal remodeling and neurite outgrowth and stability (Baranes et al., 1998; De Stefano et al., 2007; Salles and Strickland, 2002), we next determined whether manipulating serpinE1 impacts neurite length. Neurons transduced with shTG2 or a Scr control were either treated with increasing concentrations of serpinE1 or PAI-039, an inhibitor of serpinE1. Neither increasing the levels of serpinE1 nor inhibiting its activity resulted in a change in the total neurite outgrowth of neurons expressing Scr or shRNA TG2 (Figure 6B).

Discussion

Early studies on the role of TG2 in the CNS were primarily in the context of neurodegenerative diseases. Given the findings that TG2 is upregulated in Alzheimer disease, Huntington disease and other neurodegenerative conditions, it was originally speculated that TG2 played a detrimental role in neurons (Kim et al., 1999; McConoughey et al., 2010; Ruan and Johnson, 2007; Tolentino et al., 2004). However, selective overexpression of TG2 in neurons decreased infarct volumes following a MCAL stroke, and in primary neurons increased expression of wild type or a transamidating inactive mutant significantly improved survival in an OGD/reperfusion model (Filiano et al., 2008; Filiano et al., 2010). These findings clearly suggested that TG2 plays a beneficial role in neurons, and this function is not dependent on TG2's transamidating activity. The localization of TG2 might play a role in neural protection since following MCAL nuclear levels of TG2 increase (Filiano et al., 2010). Other studies also strongly indicated that localization of TG2 to the nucleus was required for it to be protective in different stress paradigms, and further that this effect was transamidating independent (Gundemir and Johnson, 2009). In addition, fractionation studies showed that nuclear TG2 was predominantly localized to the chromatin fraction (Lesort et al., 1998). These data support the conceptual framework that TG2 modulates transcriptional processes that support neuronal health.

A drawback to these previous studies was that they were all done in overexpression models. Therefore, in this study we used a knockdown approach in primary neurons to begin to understand the role of physiological TG2 levels in neurons. In a previous study, we found that neurons isolated from $TG2^{-/-}$ mice and wild type mice were equivalently viable, although $TG2^{-/-}$ neurons were significantly more sensitive to OGD/reperfusion-induced cell death (Colak and Johnson, 2012). In the current study, we unexpectedly found that just knocking down TG2 in neurons significantly reduced cell viability. The discrepancy in these findings is likely due to compensatory changes in gene expression that take place in the $TG2^{-/-}$ mice during development. Indeed, this is one reason why models where the gene of interest is deleted or expressed using an inducible cre/loxP approach are becoming more prevalent (Fowler and Kenny, 2012; Heldt and Ressler, 2009).

Results from the RNA-seq data demonstrated that knockdown of TG2 resulted in significant changes in the expression of 86 genes, with the majority of these being upregulated. Maintaining the appropriate complement of genes is essential for neuronal survival. This cellular function requires a balance of transcriptional activation and repression, and these findings suggest that TG2 plays a role in maintaining this balance perhaps in part by

facilitating repression of specific genes. However, TG2 may also be involved directly or indirectly in facilitating the expression of genes that neurons depend on to maintain morphological integrity. Indeed, the pathway analysis revealed that the axon guidance, focal adhesion, ECM organization, and β1 integrin interactions pathways were impacted by depletion of TG2. All of these pathways are related to neurite outgrowth and maintenance, and could explain the phenotype resulting from TG2 depletion.

Two proteins that play key roles in neurite outgrowth and maintenance, and were downregulated in the TG2 shRNA transduced neurons, are profilin 2 and α-tubulin. Profilin 2 is an actin binding protein that enables rapid polymerization of microfilaments (Da Silva et al., 2003). Profilins have been shown to play a pivotal role in axonal growth (Wills et al., 1999) and thus decreased levels could impact neurite extension. Interesting, the transcript for α-tubulin was similarly decreased in TG2 depleted neurons which could also negatively impact neurite outgrowth and stability.

In response to decreases in the expression of genes such as profilin 2 and α-tubulin it can be speculated that there may be compensatory upregulation of other genes when TG2 levels are decreased. For example, expression of integrin α-5, a well-known binding partner of β1 integrin, was increased in TG2 depleted neurons. The α5/β1 integrin complex forms the fibronectin receptor, and fibronectin expression was also increased in the TG2 depleted neurons. This signaling complex has been shown to play a significant role in facilitating axonal outgrowth (Tonge et al. 2012), and thus could be a response to the decrease in the expression of genes such as α-tubulin and profilin 2. In support of this we also observed an increase in phospho-FAK in the TG2 depleted neurons. Integrins facilitate neurite outgrowth by activating FAK (Ivankovic-Dikic et al., 2000; Tan et al., 2011) and thus increases in phospho-FAK are a likely outcome of the increases in integrin α-5 and fibronectin in response to TG2 knockdown. Given that neuronal health is dependent on the ability of neurites to respond to external signals and appropriately remodel, it is highly likely the TG2 depletion-induced alterations in the temporal and spatial expression of genes necessary for the maintenance of neuritic function negatively affects neuron survival (Colak and Johnson 2012, Stiles and Jernigan 2010).

The transcript that showed the most robust increase in response to TG2 knock down was serpinE1, a protease inhibitor. Previous studies have shown that neuronal injury increases serpinE1 expression (Nilsson et al., 2005; Yamanaka et al., 2005). The increase in serpinE1 has been linked to a decrease in neurite growth, and treatments inhibiting serpine1 or increasing its main target, tPA, resulted in increased neurite growth (Cho et al., 2013). However, in the present study the increase or inhibition of serpinE1 did not ameliorate or exacerbate the decrease in neurite length. This may be due to the fact that the outcomes of TG2 depletion are mediated by the coordinated effects of more than one gene, and manipulating only serpinE1 was insufficient to compensate for the dysregulation; thus neurite length remained unchanged. Future studies are required to understand how the transcriptional changes elicited by TG2 knock down decrease neurite length.

Overall, this study has demonstrated for the first time that depletion of TG2 causes a loss of neuronal viability. This reduction in cell viability is accompanied by changes in

transcriptional activity which results in a disruption of cell structure integrity. This disruption causes a decrease in neurite length, which is likely an event that contributes to loss of viability. A greater understanding of TG2 ability to affect transcription will allows us to understand TG2's role in maintaining neuronal integrity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DIV	days in vitro
ECM	extracellular matrix
MCAL	middle cerebral artery ligation
NES	nuclear export signal
NLS	nuclear localization signal
OGD	oxygen and glucose deprivation
scr	scrambled
TG2	transglutaminase 2

Highlights

- Knocking down TG2 in primary neurons decreases viability
- Knocking down TG2 affects the expression of specific genes
- TG2 mediates the expression of genes that regulate ECM dynamics
- TG2 knockdown reduced neurite length

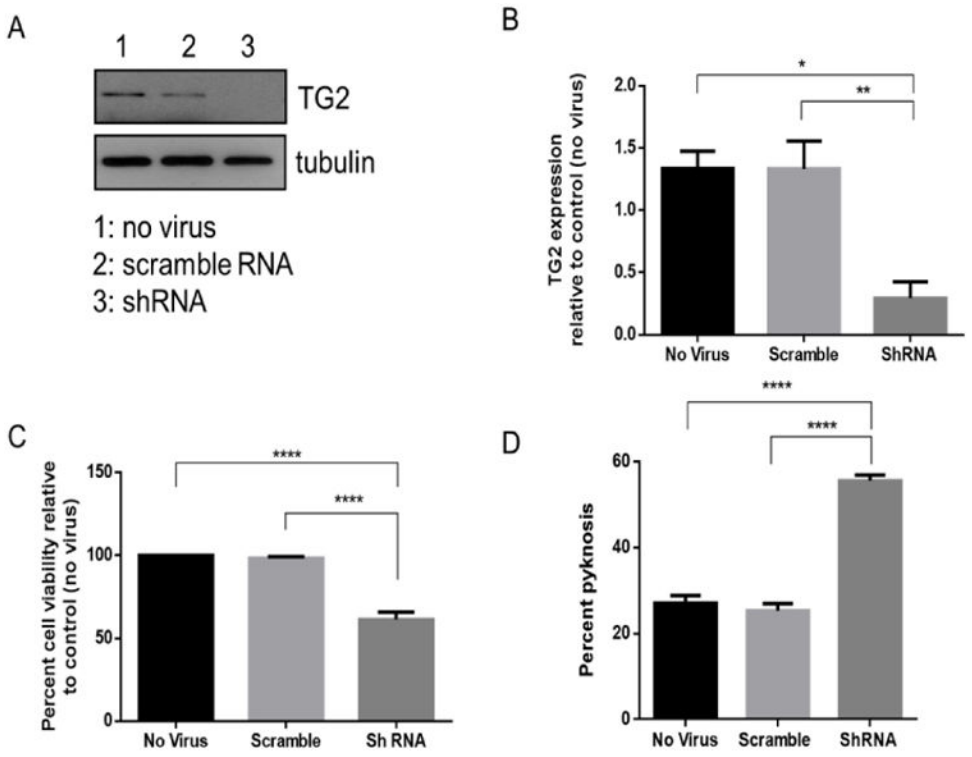

Figure 1. Downregulation of TG2 decreases neuronal viability

(A) Representative immunoblots of neurons transduced with control scramble RNA or shRNA against TG2. Tubulin was used as a loading control. (B) Quantification of (A). (n= 3-5, * $p < 0.05$, ** $p < 0.01$) (C) Neuronal cell viability was significantly reduced after 6 days transduction with shRNA compared to control as measured by the resazurin assay. (n=8, *** $p < 0.0005$) (D) Nuclear pyknosis counts are significantly higher 6 days after transduction with TG2 shRNA as compared to scramble RNA and no virus controls (n= 3-6, ~100 cells per n, **** $p < 0.0001$). Results are presented as mean ± SEM.

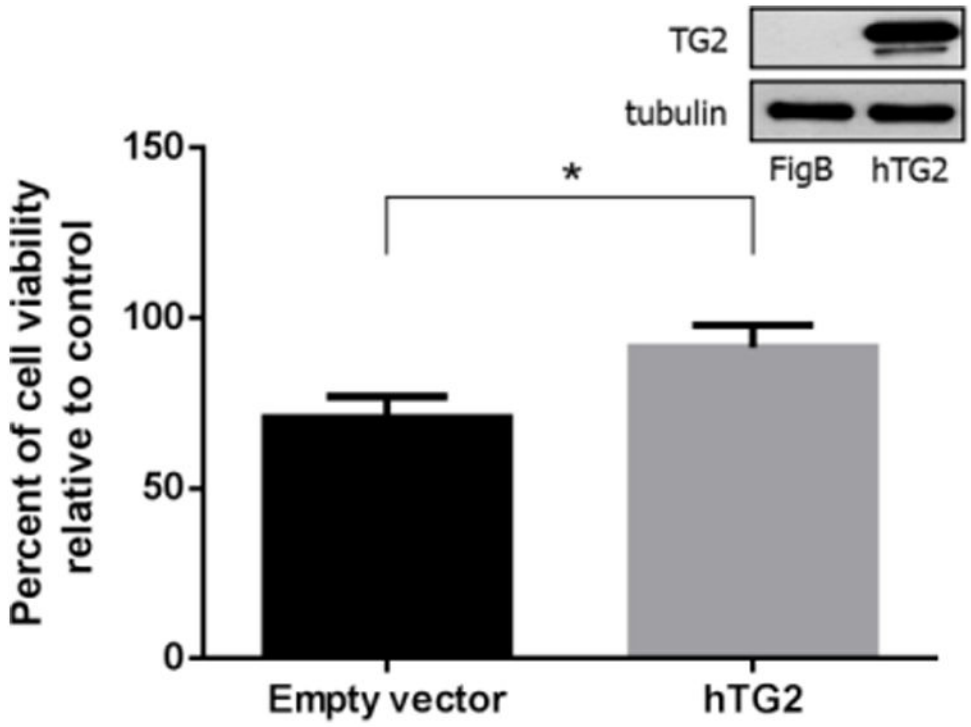

Figure 2. Re-expression of TG2 rescues cell viability

Neurons were transduced with shRNA for TG2 or scramble RNA and 2 days later transduced with either an empty vector lentivirus (FigB) or one expressing shRNA resistant TG2. Four days later neuronal viability was measured. The controls were neurons that had been initially transduced with scrambled RNA. Neuronal cell viability was significantly increased after re-expression of shRNA resistant TG2 in the neurons in which TG2 was knocked down (n= 7, * $p < 0.05$). Results are presented as mean ± SEM. The inset shows a representative immunoblot of TG2 in shRNA TG2 transduced neurons that were subsequently transduced with FigB or shRNA resistant TG2. Tubulin was used as a loading control.

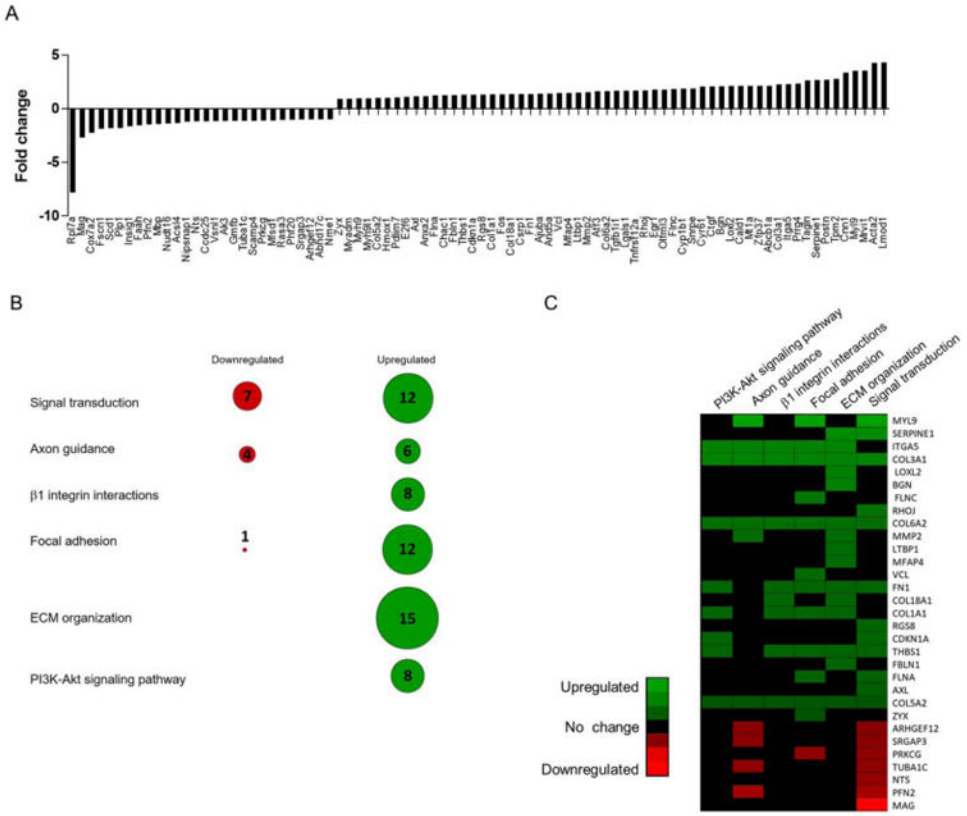

Figure 3. Downregulation of TG2 results in differentially expressed genes

(A) TG2 neuronal depletion resulted in 86 significantly differentially expressed genes compared to neurons transduced with scramble RNA. Fifty nine of these 86 genes were upregulated and 27 were downregulated. (B) Six pathways were enriched in the differentially regulated genes. The circles represent the quantity of genes expressed in each pathway. (C) Representation of differentially regulated genes and the pathways in which they are found.

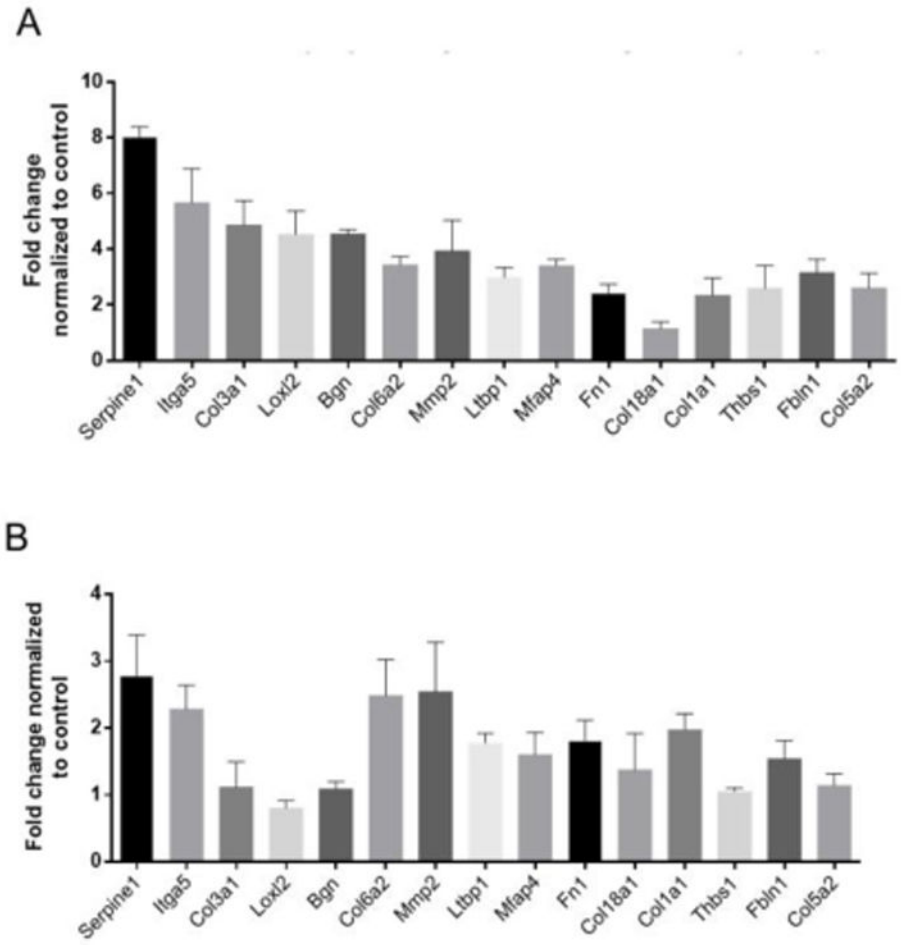

Figure 4. Validation of RNA sequencing

(A) Validation of RNA-seq results by qPCR using the same samples sequenced (n = 3). (B) Validation of RNA-seq results by qPCR of newly collected RNA samples (n = 5). Results are presented as mean ± SEM.

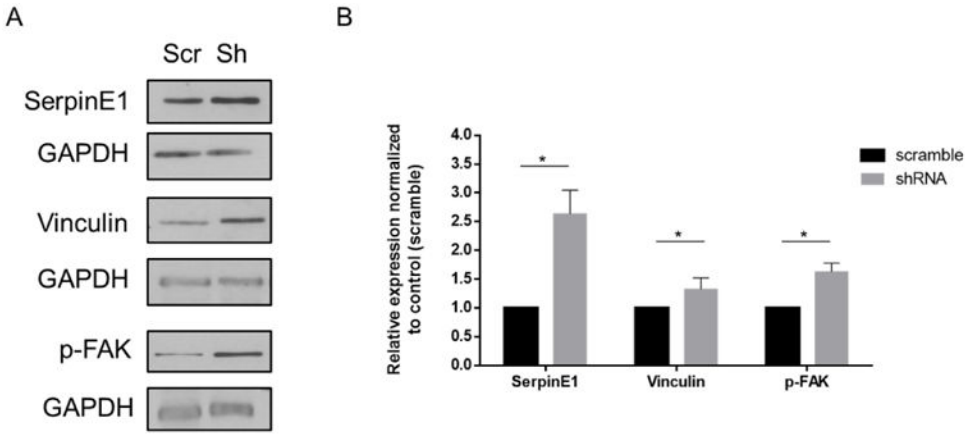

Figure 5. Downregulation of TG2 increases expression of proteins involved in ECM organization

(A) Representative immunoblots of serpinE1, vinculin and p-FAK expression in scramble RNA (Scr) and shRNA TG2 (Sh) expressing neurons. (B) Quantification of (A). Protein expression was normalized to GAPDH. Results are presented as mean ± SEM (n = 5, * $p < 0.05$).

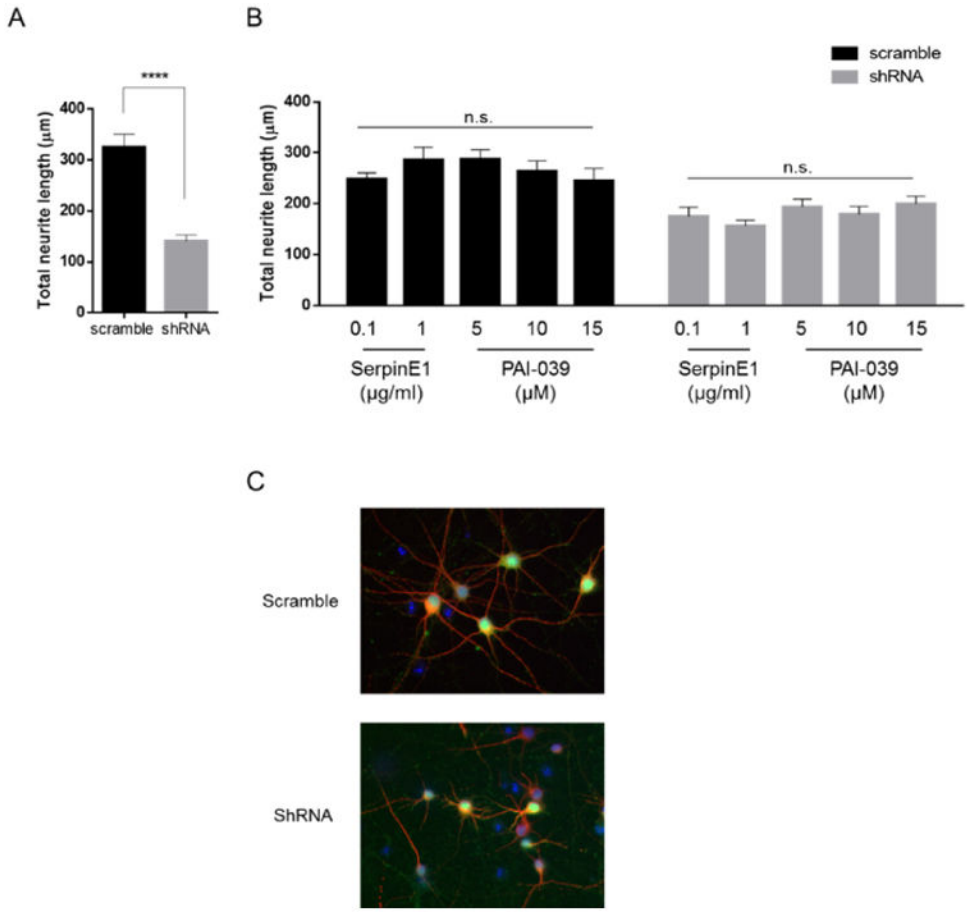

Figure 6. Downregulation of TG2 decreases total neurite length in neurons

(A) Total length of neurites from scramble and shRNA TG2 expressing neurons. (n = 3, ≥ 25 cells per n). (B) Total neurite length measured from scramble and shRNA TG2 expressing neurons treated for 48 hours with serpinE1 or PAI-039. (n = 3, ≥ 25 cells per n). (C) Representative images of neurons expressing scramble or shRNA TG2 (GFP) immunostained against MAP2 (red) and DAPI (blue). Results are presented as mean ± SEM. (**** $p < 0.0001$).