HHS Public Access

Author manuscript

Mol Cell Neurosci. Author manuscript; available in PMC 2019 October 01.

Published in final edited form as:

Mol Cell Neurosci. 2018 October ; 92: 128–136. doi:10.1016/j.mcn.2018.06.007.

Depletion of Astrocytic Transglutaminase 2 Improves Injury Outcomes

Alina Monteagudo[#1], **Julianne Feola**[#2], **Heather Natola**[#2], **Changyi Ji**[3], **Christoph Pröschel**[#2,4], and **Gail V. W. Johnson**[#1,2,3,*]

[1]Department of Pharmacology and Physiology, University of Rochester, Rochester, NY 14642

[2]Department of Biomedical Genetics, University of Rochester, Rochester, NY 14642

[3]Department of Anesthesiology and Perioperative Medicine, University of Rochester, Rochester, NY 14642

[4]Stem Cell and Regenerative Medicine Institute, University of Rochester, Rochester, NY 14642 University of Rochester, Rochester, NY 14642

[#] These authors contributed equally to this work.

Abstract

Astrocytes play an indispensable role in maintaining a healthy, functional neural network in the central nervous system (CNS). A primary function of CNS astrocytes is to support the survival and function of neurons. In response to injury, astrocytes take on a reactive phenotype, which alters their molecular functions. Reactive astrocytes have been reported to be both beneficial and harmful to the CNS recovery process subsequent to injury. Understanding the molecular processes and regulatory proteins that determine the extent to which an astrocyte hinders or supports neuronal survival is important within the context of CNS repair. One protein that plays a role in modulating cellular survival is transglutaminase 2 (TG2). Global deletion of TG2 results in beneficial outcomes subsequent to *in vivo* ischemic brain injury. *Ex vivo* studies have also implicated TG2 as a negative regulator of astrocyte viability subsequent to injury. In this study we show that knocking down TG2 in astrocytes significantly increases their ability to protect neurons from oxygen glucose deprivation (OGD)/reperfusion injury. To begin to understand how deletion of TG2 in astrocytes improves their ability to protect neurons from injury, we performed transcriptome analysis of wild type and $TG2^{-/-}$ astrocytes. TG2 deletion resulted in alterations in genes involved in extracellular matrix remodeling, cell adhesion and axon growth/guidance. In addition, the majority of genes that showed increases in the $TG2^{-/-}$ astrocytes had predicted cJun/AP-1 binding motifs in their promoters. Furthermore, phospho-cJun levels were robustly elevated in $TG2^{-/-}$ astrocytes, a finding which was consistent with the increase in expression of AP-1 responsive genes. These *in vitro* data were subsequently extended into an *in vivo* model to

[*]Corresponding author at: Department of Anesthesiology, University of Rochester Medical Center, University of Rochester, 601 Elmwood Ave., Box 604, Rochester, NY 14642, USA. Tel.: +585 276 3740; fax: +585 276 2418; gail_johnsonvoll@urmc.rochester.edu.

determine whether the absence of astrocytic TG2 improves outcomes after CNS injury. Our results show that, following a spinal cord injury, scar formation is significantly attenuated in mice with astrocyte-specific TG2 deletion compared to mice expressing normal TG2 levels. Taken together, these data indicate that TG2 plays a pivotal role in mediating reactive astrocyte properties following CNS injury. Further, the data suggest that limiting the AP-1 mediated pro-survival injury response may be a contributing factor to that the detrimental effects of astrocytic TG2.

Keywords

transglutaminase 2; astrocytes; neuroprotection; CNS injury

Introduction

Astrocytes are central and indispensable players in central nervous system (CNS) function. Their contributions to neuronal survival and activity are critically important in both physiological and pathological states. A single astrocyte is well positioned to exert influence on the formation of synapses, regulate the integrity of the blood brain barrier, and modulate synaptic activity by taking up excitotoxic neurotransmitters or releasing beneficial factors to the extracellular space (reviewed in (Perez-Alvarez and Araque, 2013)). They also play a fundamental role in the response of the CNS to injury. Following an insult, astrocytes respond by becoming reactive, which involves significant changes in gene expression that result in alterations in astrocyte morphology and function (Anderson et al., 2014; Pekny and Pekna, 2014; Sofroniew and Vinters, 2010; Zamanian et al., 2012). Reactive astrocytes also contribute to the formation of a glial scar (Anderson et al., 2016; Sofroniew, 2009; Zamanian et al., 2012). Whether reactive astrocytes facilitate or hinder CNS recovery has been an area of significant investigation and debate (Liddelow et al., 2017; Sofroniew, 2009; Zamanian et al., 2012). In addition, recent studies have suggested that astrocytes are not homogenous, but rather take on either a supportive or a maladaptive phenotype depending on the type of injury or inductive signals (Liddelow et al., 2017; Zamanian et al., 2012). Although it is clear that reactive astrocytes are able to take on detrimental or beneficial phenotypes subsequent to injury, the cell intrinsic determinants that direct astrocytes towards either phenotype are not well-understood. Here, we identify transglutaminase 2 (TG2) as a modulator of the astroglial response to injury.

Transglutaminase 2 (TG2) is a multifunctional protein that is highly upregulated in reactive astrocytes in response to cell stressors (Caccamo et al., 2005; Ientile et al., 2015; van Strien et al., 2011a). TG2 has been found to play a role in mediating cell death and survival responses in numerous cell types (Eckert et al., 2014; Gundemir et al., 2012). In certain cancer cells and neurons, TG2 has been shown to promote survival responses (Eckert et al., 2015; Filiano et al., 2008; Filiano et al., 2010; Fisher et al., 2015; Mangala et al., 2007). In contrast, deletion or knockout of TG2 in astrocytes significantly increases viability subsequent to oxygen and glucose deprivation (OGD) compared to astrocytes expressing normal levels of TG2 (Colak and Johnson, 2012; Feola et al., 2017). Furthermore, $TG2^{-/-}$ astrocytes protected neurons from OGD-mediated cell death in a co-culture paradigm to a significantly greater extent than wild type (WT) astrocytes, whether or not TG2 was present

in the neurons (Colak and Johnson, 2012). These data demonstrate that TG2 expression alters the response of astrocytes to injury, as well as the effect of astrocytes on neuronal survival (Colak and Johnson, 2012). Interestingly TG2 is highly upregulated in astrocytes in response to lipopolysaccharide (LPS) treatment (Takano et al., 2010), which also results in astrocytes exhibiting a primarily maladaptive phenotype (Zamanian et al., 2012). The differential role of TG2 in promoting or hindering survival in neurons and astrocytes following an injury may in part be due to difference in intracellular localization and how the localization changes in response to stress (Quinn et al., 2018). For example, in response to hypoxic stress the nuclear levels of TG2 in neurons increases while in astrocytes it decreases (Yunes-Medina et al., 2017). Further, neurons do not externalize TG2, while astrocytes do where it becomes incorporated into the extracellular matrix and mediates astrocyte adhesion and migration (Quinn et al., 2018; van Strien et al., 2011b; Yunes-Medina et al., 2017).

Given the critical role of astrocytes in mediating neuronal survival, we now examine how TG2 affects the ability of astrocytes to protect neurons from injury-induced cell death. Using OGD/reperfusion to mimic CNS injury conditions, we show that astrocytic TG2 depletion is protective to neurons in a transwell paradigm. Whole transcriptome analysis by RNA-sequencing (RNA-seq) of WT and $TG2^{-/-}$ astrocytes revealed that deletion of TG2 resulted in the upregulation of 127 transcripts and the downregulation of 78 transcripts. A number of the transcripts upregulated in $TG2^{-/-}$ astrocytes encode proteins that are known to promote regeneration after CNS injury, including brain-derived neurotrophic factor (BDNF) (Tuvikene et al., 2016; Weishaupt et al., 2012), decorin (DCN)(Esmaeili et al., 2014; Minor et al., 2008) and A Disintegrin and Metalloprotease with Thrombospondin Motifs 6 (ADAMts6) (Gottschall and Howell, 2015). In addition, the majority of the genes that were upregulated in $TG2^{-/-}$ astrocytes were found to contain cJun/AP-1 binding motifs in their promoter region, consistent with increased levels of activated (phosphorylated) cJun in $TG2^{-/-}$ astrocytes. Overall, our findings indicate that astrocytic TG2 likely attenuates the AP-1-mediated injury response, thereby preventing astrocytes from assuming a more pro-regenerative response subsequent to CNS injury. In support of this, we demonstrated that animals with astrocyte-specific TG2 deletion (glial fibrillary acidic protein(GFAP)-Cre $^{+/-}$/$TG2^{fl/fl}$) exhibited significantly less astrocytic scarring in an *in vivo* model of CNS injury when compared to wild-type littermate controls.

Materials and Methods

Animals.

All procedures with animals were 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. $TG2^{-/-}$ mice on a C57Bl/6 background were described previously (Colak and Johnson, 2012; Nanda et al., 2001). $TG2^{fl/fl}$ mice were generously provided by Drs. R. Graham and S. Iismaa. These mice contain loxP sites flanking TG2 exons 6-8 (Nanda et al., 2001). B6.Cg-Tg(GFAP Cre)73.12Mvs/J (GFAP $Cre^{+/-}$) mice were obtained from Jackson Laboratories and have been described and characterized previously (Herrmann et al., 2008). Refer to the Supplemental Materials and Methods and Figure S1 for detailed information on the

generation and genotyping of the GFAP $Cre^{+/-}$ $TG2^{fl/fl}$ and GFAP $Cre^{+/-}$ $TG2^{fl/fl}$ mice. Timed pregnant Sprague Dawley rats were obtained from Charles River Laboratories.

Primary Cultures.

Primary astrocytes were harvested at post-natal day 0 from wild-type C57BL/6 or $TG2^{-/-}$ mouse pups as described previously (Colak and Johnson, 2012). Briefly, brains were dissected, followed by mechanical dissociation of hemispheres. Cells were plated onto culture dishes in MEM media supplemented with 10% FBS, 6 g/L glucose, 1mM sodium pyruvate and 25 μg/mL Gentamicin (Glia MEM) and maintained at 37°C/5% CO_2. The following day, the plates were shaken and rinsed to remove debris. Cells were then maintained in Glia MEM for 5-7 days until they reached confluency, upon which cells were frozen in media containing 10%FBS/10% DMSO and stored in liquid nitrogen for future use. This culturing technique yields >95% astrocytes (Colak and Johnson, 2012). For experiments, astrocytes were thawed, re-plated and maintained in Glia MEM.

Primary neurons were harvested from Sprague Dawley rat embryos at embryonic day 18 (E18) as previously described (Pallo et al., 2016). Briefly, a pregnant dam was euthanized with CO_2 followed by decapitation. Embryos were rapidly removed and decapitated, followed by dissection of the brains, hemisphere separation, and removal of the meninges. Cortices were then dissociated with 0.05% trypsin for 25 minutes at 37°C, followed by trituration with a flame-polished Pasteur pipette. Neurons were then plated at a density of 2-4 × 10^5 cells/mL in MEM containing 5% FBS, 20 mM glucose, 2 mM L-glutamine, 10 mM HEPES and 0.5% penicillin/streptomycin on 40 μg/mL poly D-lysine coted plates. After 4-5 hours, media was fully replaced with Neurobasal media supplemented with 400 μM L-glutamine and 1x B27. Neuronal experiments were begun on DIV 10.

Lentiviral Knockdown of TG2.

Human TG2 shRNA or TG2 scrambled RNA constructs in a FIGB lentiviral backbone vector were co-transfected into HEK293T cells with pMD2.G (VSVG-envelope) and psPAX2 (packaging constructs) (Gundemir et al., 2013). Transfected HEK293T cells were maintained at 33°C for 72 hours, after which virus-containing media was collected from the plates and filtered with a 0.2 μm syringe filter. Viral particles were concentrated via centrifugation at >50,000 g for 3 hours at 4°C. Viral pellets were re-suspended in 0.1% BSA in 1xPBS. For viral transduction, the culture medium of WT astrocytes was replaced with virus-containing Glia MEM. 24 hours later, this step was repeated and the following day, culture media was fully changed. On day 5 post-transduction, cells were subcultured to experimental plates. Experiments were begun on day 11 post-transduction.

OGD/Reperfusion Cell Treatment Paradigm.

Cells were rinsed once and then maintained with glucose-free Neurobasal-A media for a 90-120 minute exposure to 0.1% O_2 and 5% CO_2 at 37°C in a Coy Laboratory Products Hypoxic Chamber. For neuronal viability experiments, normoxic control cells were maintained in glucose-containing media with no added supplements, ambient O_2 levels, and 5% CO_2 for 90-120 minutes. Following OGD, all cells were placed back into glucose-

containing media and ambient O_2 levels in a humidified incubator for a 24 hour reperfusion period.

Conditioned Media Experiments.

Two separate conditioned media experiments were conducted. In the first, neurons and scrambled or TG2 shRNA-expressing astrocytes or WT or $TG2^{-/-}$ astrocytes were placed in Neurobasal media for 72 hours. Conditioned media was then collected. DIV 10 neurons were then subjected to OGD as described above, except for the reperfusion phase, in which the glucose-free Neurobasal-A media was removed from the neurons and replaced with conditioned media from either neurons or astrocytes. Neuronal viability was assessed with a resazurin assay (see below) after 24 hours.

In the second experiment, scrambled or TG2 shRNA-expressing or WT or $TG2^{-/-}$ astrocytes and neurons underwent OGD treatment for 90 minutes on neuronal DIV 10 (set 1). Conditioned media from these cells was then collected and replaced with fresh Neurobasal media. These cells were then placed back into a humidified incubator with ambient O_2 levels and 5% CO_2 for 90 minutes. During that time, fresh plates of neurons (set 2) were rinsed with glucose-free Neurobasal-A media and then maintained in OGD-conditioned media from neurons or astrocytes in set 1. Neurons in set 2 then underwent 90 minutes of OGD. Following the OGD period, conditioned media from cells in set 1 was again collected and then placed onto neurons in set 2 for the 24 hour reperfusion period, after which viability was assessed with a resazurin assay (see below).

Transwell Assay.

Scrambled and TG2 shRNA-expressing astrocytes were plated onto 6.5 mm transwell inserts with a membrane pore size of 0.4 μm at day 5 post-transduction. At neuronal DIV 7, astrocyte transwells were placed atop neurons on a 24-well plate. After 48 hours, the astrocytes and neurons underwent the OGD/reperfusion paradigm, after which the viability of the neurons was measured separately with a resazurin assay.

Resazurin Viability Assay.

A 5 mg/mL stock of resazurin was prepared in sterile ddH_2O. Resazurin was then added to each well, as well as two blank wells, at a final concentration of 50 μg/mL. Cells were then incubated for 30 minutes at 37°C, after which fluorescence was measured with a 540 nm excitation wavelength and a 590 nm emission wavelength. Blank values were subtracted from each reading and results were normalized to the normoxic control condition.

Mouse Spinal Cord Contusion Injury.

All mice were genotyped prior to surgery. GFAP $Cre^{+/-}TG2^{fl/fl}$ and GFAP $Cre^{-/-}TG2^{fl/fl}$ mice, 12-16 weeks of age were anesthetized using a combination of ketamine and xylazine given intraperitoneally. A laminectomy was performed at the thoracic (T9) level and the dura mater was exposed. The spinal cord was moderately contused bilaterally using a force-defined injury device (70 kDyn, Infinite Horizon Impactor 400, Precision Systems and Instrumentation). Muscle and skin were closed in layers. Body temperature was maintained at 37°C with a heating pad during surgery and the initial recovery period. Mice were given

buprenorphine s.c. immediately after surgery and three times daily for 3 days. Lactated Ringer's solution was administrated i.p. as needed to ensure hydration. Food and DietGel® Recovery was placed in the bottom of the cages. Following the spinal contusion mice had their bladders manually expressed every 12 hours after the injury until spontaneous voiding recurred.

Histology.

Animals were anesthetized and transcardially perfused 3 days post injury with 0.1 M PBS followed by 3.7% paraformaldehyde (PFA) in 0.1 M PBS. Dissected spinal cords were put in 20% sucrose for at least 24 hours. Mouse spinal cords were then cut into longitudinal sections from left to right. Sections were kept at −40°C until staining. On the day of staining, sections were brought to room temperature, rehydrated in 3×5 minute PBS washes, post fixed with 4% PFA for 10 minutes, blocked in HBSS with 5% bovine serum for 1 hour, and then stained with mouse anti-GFAP antibody (Sigma, cat# G3893) at 1:500 overnight at 4°C. Slides were washed 3×5 minutes in PBS, then stained with Jackson Immuno AffiniPure F(ab') Alexa Fluor® 594 anti-mouse secondary antibody, counterstained with DAPI and mounted with Southern Biotech Fluoromount. All sections used for signal quantification were processed simultaneously, using the same batch of reagents. Controls included parallel sections stained with Jackson Immuno AffiniPure F(ab') Alexa Fluor® 594 anti mouse secondary antibody only.

Images were acquired using an Olympus BX51WI equipped with MBF Bioscience Stereo Investigator (version 10) and an X-cite 120 LED epifluorescent light source. Prior to imaging and quantification, all sections were scanned to identify the brightest and dimmest signals. Exposure times were then adjusted to ensure that the brightest stain intensity did not cause pixel saturation (using the Stereo Investigator clip detect function), and anti-mouse secondary-only stained sections exhibited only minimal signal. All sections had an identical exposure time when signals were compared. For each animal, 27 images were acquired and analyzed, covering the lesion center, as well as proximal and distal regions, as well as white and grey matter regions (9 images from each of 3 sections: 3 images anterior to the lesion, 3 images centered on lesion center, and 3 images posterior to the lesion). For quantitative measures the lesion center was first identified and one image was taken in the ventral white matter, grey matter, and dorsal white matter. Then, images were taken in the same locations, 1000μm from the lesion center both rostrally and caudally for a total of nine images per section. This was repeated in two more sections with a visible lesion.

GFAP intensity was measured using ImageJ, and the background values (obtained from Jackson Immuno AffiniPure F(ab') Alexa Fluor® 594 anti-mouse secondary antibody only stained sections) were subtracted from the total image value. For grey matter measurements, the value from each grey matter image in three locations from three sections was averaged for each animal. For lesion center values, the measurement from the two white matter and the inner grey matter in the lesion center were averaged across all three sections for each animal. N=3 animals per genotype in each analysis.

RNA isolation and cDNA synthesis.

RNA was isolated from wild type or TG2$^{-/-}$ astrocytes using the RNeasy Plus Kit (QIAGEN, 74134) according to the manufacturer's instructions. cDNA was made using the 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 wild type and TG2$^{-/-}$ astrocytes. 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 mouse reference genome (GRCm38.p4) with STAR-2.4.2a (https://github.com/alexdobin/STAR). Cufflinks2.0.2 (cuffdiff2 - Running Cuffdiff) was used with the gencode M6 mouse gene annotations to perform differential expression analysis with an FDR cutoff of 0.05 (95% confidence interval). Preliminary evaluation of the RNA-seq data was carried using Ingenuity Pathway Analysis (IPA) or Enrichr (http://amp.pharm.mssm.edu/Enrichr) (Kuleshov et al., 2016). Determination of the presence of cJun/AP-1 binding motifs in the promoters was carried out using the transcription factor search function on the www.genecards.org website.

RNA Expression Analysis.

RNA was extracted and cDNA was prepared from WT and TG2$^{-/-}$ astrocytes from independent sets of samples as described above. Expression levels of each gene were determined using 10 ng cDNA for each reaction. Reverse-transcriptase quantitative polymerase chain reactions (RT-QPCR) were performed using FAM-labeled probes for BDNF (Mm04230607_s1) or decorin (DCN) (Mm00514535_m1) or VIC-labeled GAPDH (Mm99999915_g1) probe from ThermoFisher, in combination with Taqman mastermix (BioRad). For Adamts6 (PrimerBank ID 30425248a1), pro-enkephalin (Penk)(PrimerBank ID 201034a1), SPARC-related modular calciumbinding 1 (Smoc1) (PrimerBank ID 26334065a1) and GAPDH (PrimerBank ID 26328841a1) the indicated validated mouse primers from the Primer Bank (https://pga.mgh.harvard.edu/primerbank/) were used. For

these reactions the SYBR Green mix (Thermo Fisher Scientific, 4309155) was used to detect the transcript. Data were analyzed using the ΔΔCt method.

Immunoblotting.

Protein lysates WT or TG2$^{-/-}$ astrocytes were collected with RIPA buffer. Protein concentration of the samples was determined with a BCA assay. Samples were diluted in collection buffer and 2X reducing stop buffer, boiled and resolved in a 10% 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 phospho-cJun (Ser63) (Cell Signaling Technology (CST) cat # 9261), cJun (60A8)(CST cat # 9165) or α-Tubulin (CST cat# 2125) 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.

Analysis and Statistics.

For the cell culture experiments, data were analyzed using the students paired T-test. For the in vivo study the immunohistochemical data were analyzed using the students unpaired two-tailed t-test. All data were checked for normal distribution using the Shapiro-Wilk normality test.

Results

Loss of TG2 increases paracrine, neuroprotective properties of reactive astrocytes

Our lab previously demonstrated that neurons co-cultured with TG2$^{-/-}$ astrocytes exhibit enhanced viability in response to a cellular injury when compared to neurons co-cultured with WT astrocytes (Colak and Johnson, 2012). In this present study, we extended these findings to determine if direct cell-to-cell contact was required for this effect. To address this question, astrocytes expressing either TG2-shRNA or scrambled-RNA as a control were grown on inserts and maintained in the presence of neurons in a transwell system (Figure 1A, B). Transduction of astrocytes with TG2-shRNA results in a >90% reduction in TG2 levels (Feola et al., 2017). With this paradigm, factors can be freely exchanged between neurons and astrocytes for the entire duration of the experiment but in the absence of cellular contact. Both astrocytes and neurons were subjected to OGD for 90 minutes followed by a 24 hour reperfusion phase. Neuronal viability was then assessed at the end of the reperfusion period. It should be noted that this paradigm does not cause a reduction in astrocyte viability. Results showed a significant increase in the protection of neuronal viability when TG2 shRNA-expressing astrocytes were present, as compared to neurons that were incubated with scrambled control astrocytes (Figure 1B). We next determined if continuous communication between the astrocytes and neurons was required for the enhanced neuronal protection conferred by TG2 depleted astrocytes. For the next set of experiments, neurons were subjected to OGD for 90 minutes, after which the media was removed and either the neuronal conditioned media (as a control), or media conditioned by astrocytes that were

transduced with either scrambled or TG2-shRNA was added during the 24 hour reperfusion phase. Neuronal viability was then assessed at the end of the reperfusion period and data were expressed as an increase in viability relative to the neuronal conditioned media control (Figure 1C, D). Results showed no difference in viability between neurons exposed to conditioned media from scrambled or TG2 shRNA-expressing astrocytes (Figure 1D). Similar results were seen with WT astrocytes or astrocytes isolated from $TG2^{-/-}$ animals (Figure S2A). To investigate whether activation of astrocytes by OGD was sufficient to restore the neuroprotective effect of TG2-knockdown astrocytes, the conditioned media paradigm was slightly modified. This time, during OGD, neurons were exposed to media conditioned by neurons (as a control) or scrambled or TG2 shRNA-expressing astrocytes that had also undergone OGD and were then exposed to conditioned media during the 24 hour reperfusion and data were expressed as an increase in viability relative to the neuronal conditioned media control (Figure 1E, F). Results showed no difference in viability between neurons exposed to conditioned media from either scrambled or TG2 shRNA-expressing astrocytes (Figure 1F). Similarly, no differences were observed between WT or $TG2^{-/-}$ astrocytes (Figure S2B). Overall, these data indicate that loss of TG2 from astrocytes results in a neuroprotective astroglial phenotype, and that the continuous presence of astrocytes is required for the enhanced neuroprotective effect of TG2−/− astrocytes.

TG2 deletion in astrocytes favors a prosurvival phenotype

Given that TG2 depletion in astrocytes significantly enhances their survival and their ability to protect neurons from stress-induced cell death, and that TG2 regulates transcriptional process (Eckert et al., 2014; Gundemir et al., 2012), we performed transcriptome analysis by RNA-seq to provide insight into possible mechanisms. Three independent biological replicates were collected and sequenced from wildtype and $TG2^{-/-}$ astrocytes. Our analysis showed that 205 transcripts were differentially expressed between the groups (Supplemental Table 1). Among these 205 genes, 78 were downregulated in $TG2^{-/-}$ astrocytes, while 127 were upregulated, and 27 of the genes were classified as pseudogenes (Figure 2A). Preliminary analyses of the 178 identified genes that changed in TG2-depleted astrocytes indicate that there was an upregulation of pro-survival/pro-regenerative genes. For example, IPA and Enrichr (Chen et al., 2013; Kuleshov et al., 2016) analyses indicated an enrichment in genes involved in axonal outgrowth and guidance and extracellular matrix (ECM) remodeling pathways in $TG2^{-/-}$ astrocytes, among others, that characterize a prosurvival/proregenerative phenotype (Figure 2B). These results suggest that depletion of TG2 changes transcriptional activity in astrocytes.

TG2 deletion in astrocytes results in increased cJun/AP-1 activation

A previous study showed that TG2 can inhibit AP-1 activity in a human lymphoblast cell line (Ahn et al., 2008). Of the 127 genes that were upregulated in $TG2^{-/-}$ astrocytes, 98 had annotated promoters, and of these, 55% had predicted cJun/AP-1 binding motifs. Therefore, lysates from 3 biological replicates of WT and $TG2^{-/-}$ astrocytes were immunoblotted for phospho-cJun (as a measure of cJun activation state) as well as total cJun. These data showed that phospho-cJun levels were strongly upregulated in $TG2^{-/-}$ astrocytes while total cJun levels remained unchanged (Figure 3A), thus demonstrating an increase in activated cJun in $TG2^{-/-}$ astrocytes. To extend these studies, qRTPCR was performed to confirm the

transcript levels of 5 genes with predicted cJun/AP-1 binding motifs in their promotors that were shown to be upregulated in TG2$^{-/-}$ astrocytes by RNA-seq analysis. BDNF, Decorin (DCN) and Adamts6 (Esmaeili et al., 2014; Gottschall and Howell, 2015; Tuvikene et al., 2016) are involved in promoting neuronal survival and neurite outgrowth, PenK is in the mitochondrial protein processing pathway, and Smoc1 is involved in ECM organization (Figure 2B, 3B). Penk and Smoc1 were among the genes that showed the greatest fold increase in TG2$^{-/-}$ astrocytes (Figure 2A and Supplemental Table 1). qRTPCR confirmed that these 5 genes are significantly upregulated in TG2$^{-/-}$ astrocytes compared to wild type controls (Figure 3B).

Ablation of Astrocytic TG2 attenuates Astrogliosis following *In Vivo* CNS Injury

To begin to extend these *in vitro* data to an *in vivo* model, we assessed if ablation of astrocytic TG2 effects astrocytic scarring subsequent to CNS injury. GFAP Cre$^{-/-}$ TG2$^{fl/fl}$ and GFAP Cre$^{+/-}$ TG2$^{fl/fl}$ mice were subjected to a contusion spinal cord injury (SCI) and GFAP staining at the lesion site was analyzed 3 days later. Figure 4A shows representative images of GFAP staining of grey matter astrocytes in GFAP Cre$^{-/-}$ TG2$^{fl/fl}$ and GFAP Cre $^{+/-}$ TG2$^{fl/fl}$ mice. Quantitation of GFAP staining in the grey matter (Figure 4B) and lesion center (Figure 4C) revealed the GFAP expression was significantly less in the GFAP Cre$^{+/-}$ TG2$^{fl/fl}$ mice compared to GFAP Cre$^{-/-}$ TG2$^{fl/fl}$, which is indicative of a reduction in reactive astrogliosis in the mice with TG2-depleted astrocytes.

Discussion

Astrocytes support the health and function of neurons by modulating metabolites, extracellular protein expression, and other factors in the extracellular environment, particularly in the setting of a CNS injury (Barreto et al., 2011; Davies et al., 2011; Faulkner et al., 2004; Hamby and Sofroniew, 2010; Sofroniew and Vinters, 2010; Stobart and Anderson, 2013). In an OGD/reperfusion co-culture experiment, a model that can be used to mimic *in vivo* CNS injury conditions, we previously demonstrated that TG2-depleted astrocytes support the viability of neurons to a significantly greater extent than WT astrocytes (Colak and Johnson, 2012). In this study, we extended these previous findings by determining whether cellular interactions were required for the protection conferred by TG2-depleted astrocytes. Intriguingly, our results demonstrated that, although cell-cell contact was not required for the ability of TG2-depleted astrocytes to protect neurons, they must be present with the neurons throughout the OGD/reperfusion paradigm. This suggests that continuous signaling between the astrocytes and neurons is necessary in order to observe any protection conferred by astrocytic TG2 depletion in a CNS injury.

Given that TG2 has been shown to regulate transcriptional processes, we used RNA-seq as an unbiased approach to examine differences in gene expression between WT and TG2$^{-/-}$ astrocytes. Analysis of these data showed that 205 genes were significantly different in the TG2$^{-/-}$ astrocytes compared to WT astrocytes and, of these, the majority were upregulated (127). Even when a cutoff of +/−1.5 log fold change or greater was used, 143 genes were significantly different and 100 of these were upregulated (see supplemental Table 1). Many of the genes that were upregulated are involved in ECM remodeling and in supporting

neuron health and neurite outgrowth. For example, BDNF, which promotes neurite outgrowth and has been considered as a treatment for SCI (Weishaupt et al., 2012), decorin, (DCN) which is an anti-inflammatory proteoglycan that facilitates neurite outgrowth after injury (Esmaeili et al., 2014), and Adamts6, a metalloproteinase that is involved in ECM remodeling and neural plasticity (Gottschall and Howell, 2015), were all significantly upregulated in $TG2^{-/-}$ astrocytes. We also found that the majority of the transcripts that were upregulated by TG2 deletion in astrocytes had predicted cJun/AP-1 binding sites in their promoters. In agreement with these data, we found that phospho-cJun (the active form of cJun) was significantly increased in $TG2^{-/-}$ astrocytes. This finding is supported by the literature, as TG2 has been demonstrated to inhibit AP-1 activation (Ahn et al., 2008). Additionally, cJun/AP-1 signaling has been shown to be cytoprotective and to promote neurite outgrowth. For example, upregulation of cJun in Schwann cells increases the expression of multiple neurotrophic factors, including BDNF, and enhances their ability to promote neurite outgrowth (Huang et al., 2015). cJun has also been reported to be the mediator of the cytoprotective, pro-regenerative effects of BDNF (Tuvikene et al., 2016; Wu et al., 2012). These and other data indicate that in vitro the prosurvival/proregenerative phenotype of $TG2^{-/-}$ astrocytes may in part be due to increases in cJun/AP-1 activity.

To begin to understand the role of astrocytic TG2 *in vivo*, initial studies were carried out by subjecting mice in which TG2 was selectively deleted in astrocytes (GFAP $Cre^{+/-}$ $TG2^{fl/fl}$) and littermates expressing normal levels of TG2 (GFAP $Cre^{-/-}$ $TG2^{fl/fl}$) to a spinal cord contusion injury (Orr et al., 2017). Three days after the injury, GFAP immunoreactivity was analyzed at the injury site. These data indicate that expression of GFAP as a marker of astrocyte reactivity was significantly attenuated in the GFAP $Cre^{+/-}$ $TG2^{fl/fl}$ mice compared to the GFAP $Cre^{-/-}$ $TG2^{fl/fl}$ mice. Previous studies have shown that preventing astrocyte scar formation or ablating chronic astrocytic scars does not result in improved outcomes after SCI (Anderson et al., 2016). However, attenuating glial scarring following SCI has been shown to improve outcomes, indicating that glial scars can also have detrimental effects as well (Nagai et al., 2015; Sofroniew, 2009). Indeed, astrogliosis is a complex process and both promotes and hinders functional recovery following SCI (Anderson et al., 2016; Sofroniew, 2009). Nonetheless, taken together these data show that in vitro deletion of TG2 from astrocytes improves their ability to support and protect neuronal health and function.

In conclusion, the data we have presented provide evidence for a novel role for astrocytic TG2 in CNS injury. The absence of TG2 results in an enhanced ability of astrocytes to protect neurons after injury both *in vitro* and *in vivo*. Additionally, our data suggest that TG2 deleted astrocytes may be able to better support neuronal viability by increasing cJun/AP-1 activation. Although further studies are needed to elucidate the specific signaling mechanisms by which astrocytic TG2 is mediating these effects, these findings establish exciting avenues for the potential of TG2 as a therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

This work was supported by NIH grants NS065825 (GVWJ) and F31 NS081894 (JF), and the Friends of Nancy Lieberman Foundation for Spinal Cord Injury Research (CP). We also thank Dr. Robert Freeman for the use of his Coy Hypoxia Chamber and BioRad CFX96 Real Time System C1000 Thermo Cycler.

References:

Ahn JS, Kim MK, Hahn JH, Park JH, Park KH, Cho BR, Park SB, Kim DJ, 2008 Tissue transglutaminase-induced down-regulation of matrix metalloproteinase-9. Biochemical and biophysical research communications 376, 743–747. [PubMed: 18809380]

Anderson MA, Ao Y, Sofroniew MV, 2014 Heterogeneity of reactive astrocytes. Neuroscience letters 565, 23–29. [PubMed: 24361547]

Anderson MA, Burda JE, Ren Y, Ao Y, O'Shea TM, Kawaguchi R, Coppola G, Khakh BS, Deming TJ, Sofroniew MV, 2016 Astrocyte scar formation aids central nervous system axon regeneration. Nature 532, 195–200. [PubMed: 27027288]

Barreto G, White RE, Ouyang Y, Xu L, Giffard RG, 2011 Astrocytes: targets for neuroprotection in stroke. Central nervous system agents in medicinal chemistry 11, 164–173. [PubMed: 21521168]

Caccamo D, Campisi A, Curro M, Aguennouz M, Li Volti G, Avola R, Ientile R, 2005 Nuclear factor-kappab activation is associated with glutamate-evoked tissue transglutaminase up-regulation in primary astrocyte cultures. J Neurosci Res 82, 858–865. [PubMed: 16273541]

Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, Clark NR, Ma'ayan A, 2013 Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC bioinformatics 14, 128. [PubMed: 23586463]

Colak G, Johnson GV, 2012 Complete transglutaminase 2 ablation results in reduced stroke volumes and astrocytes that exhibit increased survival in response to ischemia. Neurobiol Dis 45, 1042–1050. [PubMed: 22198379]

Davies SJ, Shih CH, Noble M, Mayer-Proschel M, Davies JE, Proschel C, 2011 Transplantation of specific human astrocytes promotes functional recovery after spinal cord injury. PLoS One 6, e17328. [PubMed: 21407803]

Eckert RL, Fisher ML, Grun D, Adhikary G, Xu W, Kerr C, 2015 Transglutaminase is a tumor cell and cancer stem cell survival factor. Molecular carcinogenesis 54, 947–958. [PubMed: 26258961]

Eckert RL, Kaartinen MT, Nurminskaya M, Belkin AM, Colak G, Johnson GV, Mehta K, 2014 Transglutaminase regulation of cell function. Physiological reviews 94, 383–417. [PubMed: 24692352]

Esmaeili M, Berry M, Logan A, Ahmed Z, 2014 Decorin treatment of spinal cord injury. Neural regeneration research 9, 1653–1656. [PubMed: 25374584]

Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV, 2004 Reactive astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci 24, 2143–2155. [PubMed: 14999065]

Feola J, Barton A, Akbar A, Keillor J, Johnson GVW, 2017 Transglutaminase 2 Modulation of NF-kappaB Signaling in Astrocytes is Independent of its Ability to Mediate Astrocytic Viability in Ischemic Injury. Brain research.

Filiano AJ, Bailey CD, Tucholski J, Gundemir S, Johnson GV, 2008 Transglutaminase 2 protects against ischemic insult, interacts with HIF1beta, and attenuates HIF1 signaling. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 22, 2662–2675. [PubMed: 18375543]

Filiano AJ, Tucholski J, Dolan PJ, Colak G, Johnson GV, 2010 Transglutaminase 2 protects against ischemic stroke. Neurobiol Dis 39, 334–343. [PubMed: 20451610]

Fisher ML, Keillor JW, Xu W, Eckert RL, Kerr C, 2015 Transglutaminase Is Required for Epidermal Squamous Cell Carcinoma Stem Cell Survival. Molecular cancer research : MCR 13, 1083–1094. [PubMed: 25934691]

Gottschall PE, Howell MD, 2015 ADAMTS expression and function in central nervous system injury and disorders. Matrix biology : journal of the International Society for Matrix Biology 44–46, 70–76.

Gundemir S, Colak G, Feola J, Blouin R, Johnson GV, 2013 Transglutaminase 2 facilitates or ameliorates HIF signaling and ischemic cell death depending on its conformation and localization. Biochim Biophys Acta 1833, 1–10. [PubMed: 23085038]

Gundemir S, Colak G, Tucholski J, Johnson GV, 2012 Transglutaminase 2: a molecular Swiss army knife. Biochim Biophys Acta 1823, 406–419. [PubMed: 22015769]

Hamby ME, Sofroniew MV, 2010 Reactive astrocytes as therapeutic targets for CNS disorders. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics 7, 494–506. [PubMed: 20880511]

Herrmann JE, Imura T, Song B, Qi J, Ao Y, Nguyen TK, Korsak RA, Takeda K, Akira S, Sofroniew MV, 2008 STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. J Neurosci 28, 7231–7243. [PubMed: 18614693]

Huang L, Quan X, Liu Z, Ma T, Wu Y, Ge J, Zhu S, Yang Y, Liu L, Sun Z, Huang J, Luo Z, 2015 c-Jun gene-modified Schwann cells: upregulating multiple neurotrophic factors and promoting neurite outgrowth. Tissue engineering. Part A 21, 1409–1421.

Ientile R, Curro M, Caccamo D, 2015 Transglutaminase 2 and neuroinflammation. Amino acids 47, 19–26. [PubMed: 25398223]

Kuleshov MV, Jones MR, Rouillard AD, Fernandez NF, Duan Q, Wang Z, Koplev S, Jenkins SL, Jagodnik KM, Lachmann A, McDermott MG, Monteiro CD, Gundersen GW, Ma'ayan A, 2016 Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic acids research 44, W90–97. [PubMed: 27141961]

Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, Bennett ML, Munch AE, Chung WS, Peterson TC, Wilton DK, Frouin A, Napier BA, Panicker N, Kumar M, Buckwalter MS, Rowitch DH, Dawson VL, Dawson TM, Stevens B, Barres BA, 2017 Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541, 481–487. [PubMed: 28099414]

Mangala LS, Fok JY, Zorrilla-Calancha IR, Verma A, Mehta K, 2007 Tissue transglutaminase expression promotes cell attachment, invasion and survival in breast cancer cells. Oncogene 26, 2459–2470. [PubMed: 17043648]

Minor K, Tang X, Kahrilas G, Archibald SJ, Davies JE, Davies SJ, 2008 Decorin promotes robust axon growth on inhibitory CSPGs and myelin via a direct effect on neurons. Neurobiol Dis 32, 88–95. [PubMed: 18638554]

Nagai J, Kitamura Y, Owada K, Yamashita N, Takei K, Goshima Y, Ohshima T, 2015 Crmp4 deletion promotes recovery from spinal cord injury by neuroprotection and limited scar formation. Scientific reports 5, 8269. [PubMed: 25652774]

Nanda N, Iismaa SE, Owens WA, Husain A, Mackay F, Graham RM, 2001 Targeted inactivation of Gh/tissue transglutaminase II. J Biol Chem 276, 20673–20678. [PubMed: 11274171]

Orr MB, Simkin J, Bailey WM, Kadambi NS, McVicar AL, Veldhorst AK, Gensel JC, 2017 Compression Decreases Anatomical and Functional Recovery and Alters Inflammation after Contusive Spinal Cord Injury. J Neurotrauma 34, 2342–2352. [PubMed: 28381129]

Pallo SP, DiMaio J, Cook A, Nilsson B, Johnson GV, 2016 Mechanisms of tau and Abeta-induced excitotoxicity. Brain research 1634, 119–131. [PubMed: 26731336]

Pekny M, Pekna M, 2014 Astrocyte reactivity and reactive astrogliosis: costs and benefits. Physiological reviews 94, 1077–1098. [PubMed: 25287860]

Perez-Alvarez A, Araque A, 2013 Astrocyte-neuron interaction at tripartite synapses. Current drug targets 14, 1220–1224. [PubMed: 23621508]

Quinn BR, Yunes-Medina L, Johnson GVW, 2018 Transglutaminase 2: Friend or foe? The discordant role in neurons and astrocytes. J Neurosci Res.

Sofroniew MV, 2009 Molecular dissection of reactive astrogliosis and glial scar formation. Trends in neurosciences 32, 638–647. [PubMed: 19782411]

Sofroniew MV, Vinters HV, 2010 Astrocytes: biology and pathology. Acta neuropathologica 119, 7–35. [PubMed: 20012068]

Stobart JL, Anderson CM, 2013 Multifunctional role of astrocytes as gatekeepers of neuronal energy supply. Frontiers in cellular neuroscience 7, 38. [PubMed: 23596393]

Takano K, Shiraiwa K, Moriyama M, Nakamura Y, 2010 Transglutaminase 2 expression induced by lipopolysaccharide stimulation together with NO synthase induction in cultured astrocytes. Neurochemistry international 57, 812–818. [PubMed: 20817067]

Tuvikene J, Pruunsild P, Orav E, Esvald EE, Timmusk T, 2016 AP-1 Transcription Factors Mediate BDNF-Positive Feedback Loop in Cortical Neurons. J Neurosci 36, 1290–1305. [PubMed: 26818516]

van Strien ME, Breve JJ, Fratantoni S, Schreurs MW, Bol JG, Jongenelen CA, Drukarch B, van Dam AM, 2011a Astrocyte-derived tissue transglutaminase interacts with fibronectin: a role in astrocyte adhesion and migration? PLoS One 6, e25037. [PubMed: 21949843]

van Strien ME, Drukarch B, Bol JG, van der Valk P, van Horssen J, Gerritsen WH, Breve JJ, van Dam AM, 2011b Appearance of tissue transglutaminase in astrocytes in multiple sclerosis lesions: a role in cell adhesion and migration? Brain Pathol 21, 44–54. [PubMed: 20731658]

Weishaupt N, Blesch A, Fouad K, 2012 BDNF: the career of a multifaceted neurotrophin in spinal cord injury. Experimental neurology 238, 254–264. [PubMed: 22982152]

Wu CL, Yin JH, Hwang CS, Chen SD, Yang DY, Yang DI, 2012 c-Jun-dependent sulfiredoxin induction mediates BDNF protection against mitochondrial inhibition in rat cortical neurons. Neurobiol Dis 46, 450–462. [PubMed: 22402332]

Yunes-Medina L, Feola J, Johnson GVW, 2017 Subcellular localization patterns of transglutaminase 2 in astrocytes and neurons are differentially altered by hypoxia. Neuroreport 28, 1208–1214. [PubMed: 28957946]

Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, Barres BA, 2012 Genomic analysis of reactive astrogliosis. J Neurosci 32, 6391–6410. [PubMed: 22553043]

Highlights

- Knocking down TG2 in astrocytes increases their ability to protect neurons
- Knocking down TG2 increases phospho-cJun
- Knocking down TG2 increases expression of prosurvival genes
- Astrocytic TG2 deletion reduces scarring following spinal cord injury

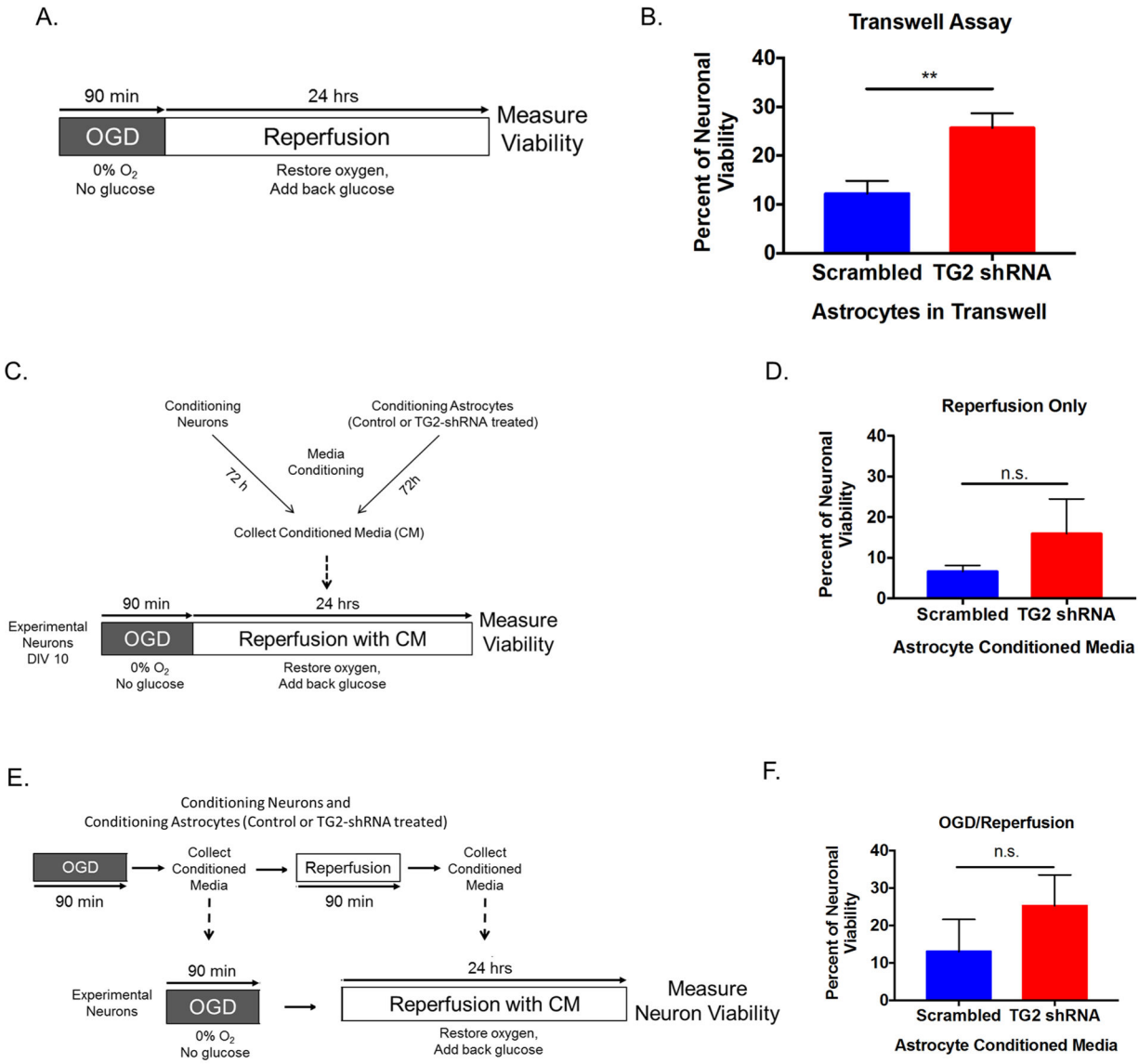

Figure 1.

TG2 depletion in astrocytes increases neuroprotection only when astrocytes and neurons are maintained together. A. Schematic of transwell treatment paradigm. **B**. Percent increase in viability of neurons incubated with scrambled or TG2 shRNA-expressing astrocytes in transwells during OGD/reperfusion relative to neurons incubated with no transwell. n=6, **$p < 0.01$. **C**. Schematic of conditioned media treatment during reperfusion only paradigm. **D**. Percent increase in viability of neurons incubated with conditioned media from scrambled and TG2 shRNA-expressing astrocytes relative to neurons incubated with neuronal conditioned media. Incubation with conditioned media occurred during the 24 hr reperfusion phase of an OGD/reperfusion paradigm, n = 6. **E**. Schematic of conditioned media treatment during OGD and reperfusion paradigm. **F.** Percent increase in viability of neurons incubated with OGD/reperfusion conditioned media from scrambled and TG2 shRNA-expressing astrocytes above neurons incubated with neuronal conditioned media, n = 4-6. All viability measurements are normalized to viability in normoxic conditions.

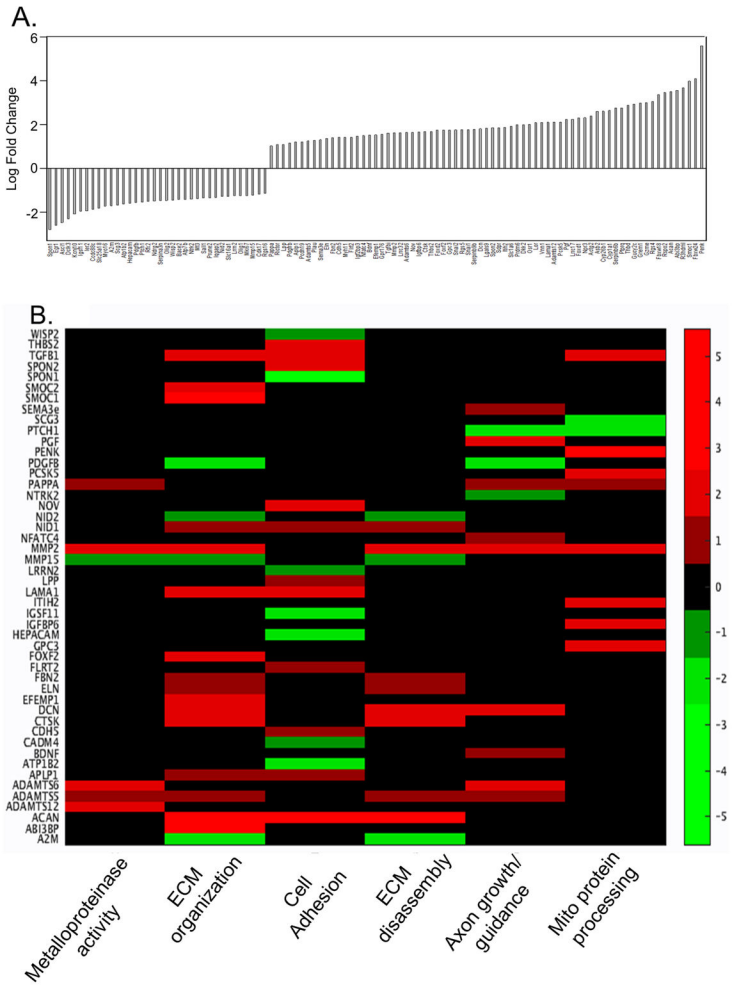

Figure 2.

Deletion of TG2 in astrocytes alters gene expression. **A**. Graphic representation of log fold changes in gene expression in TG2−/− astrocytes compared to TG2+/+ (wild type) astrocytes. **B**. Heatmap showing 6 major pathways that exhibited gene changes in TG2−/− astrocytes which could improve their ability to support neuronal survival and function after injury. Metalloproteinase activity (p=0.000292) and axon growth/guidance (p=0.0013) were identified as major pathways using IPA, and ECM organization (p=7.808e-5, adjusted p=0.004014), cell adhesion (p=7.377e-7, adjusted p=0.0002331), ECM disassembly (p=1.767e-8, adjusted p=1.375e-5) and mito processing (p=0.0001517, adjusted p=0.01967) were identified using Enrichr (Ontologies/GO Biological Processes and GO Molecular Function).

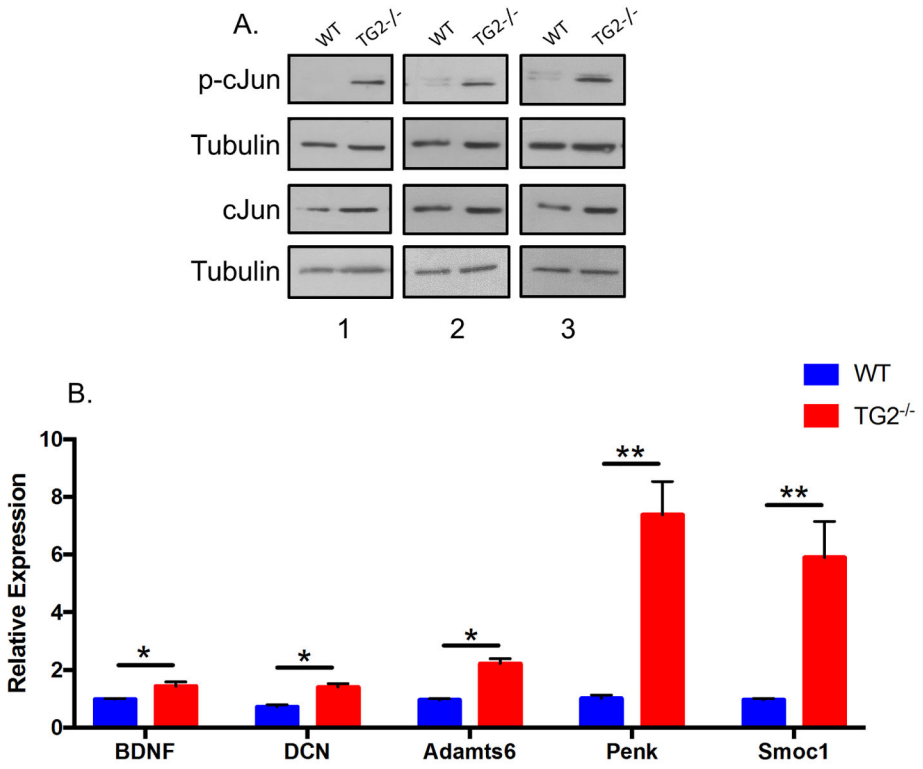

Figure 3.

Deletion of TG2 from astrocytes increases cJun activation. **A**. Immunoblots from 3 biological replicates of phospho-cJun (p-cJun) and cJun in WT and TG2−/− astrocytes. Tubulin is shown as a loading control. **B**. Representative genes with predicted cJun/AP-1 binding motifs in their promoters that are upregulated in TG2−/− astrocytes. BDNF, decorin (DCN) and Adamts6 are prosurvival/pro-regenerative genes; proenkephalin (Penk) is in the mitochondrial protein processing pathway, and Smoc1 is in the ECM organization were genes that were most robustly upregulated in the TG2−/− astrocytes by RNA-seq analysis. n=4, *p<,0.05, **p<,0.001.

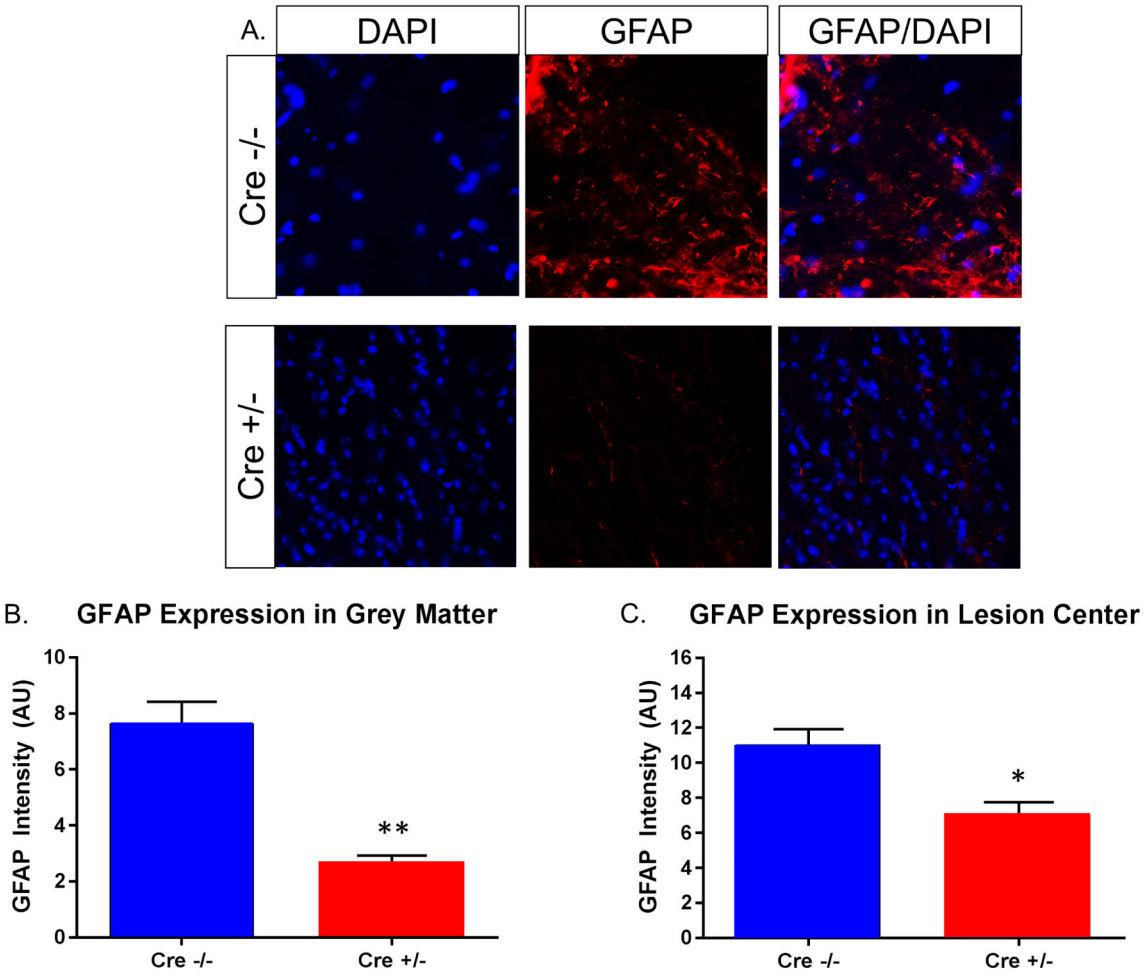

Figure 4.

GFAP Expression is significantly reduced in GFAP $Cre^{+/-}$ $TG2^{fl/fl}$ 3 days after a spinal cord contusion injury. **A.** Representative images of grey matter astrocytes showing DAPI, GFAP, and a merged image. Scale bar, 50 μm. **B**. GFAP expression was quantified by intensity in grey matter surrounding the lesion. There were significantly lower levels of GFAP staining in the GFAP $Cre^{+/-}$ $TG2^{fl/fl}$ mice compared to the GFAP $Cre^{-/-}$ $TG2^{fl/fl}$ mice. ** $p< 0.01$, n=3 animals per group. **C**. GFAP immunoreactivity at the lesion center was significantly lower in the GFAP $Cre^{+/-}$ $TG2^{fl/fl}$ mice compared to the GFAP $Cre^{-/-}$ $TG2^{fl/fl}$ mice. *$p<0.05$, =3 animals per group.