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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.

1. 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 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).

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

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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., 2011). 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 h 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.

2. Materials and methods

2.1. 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.

2.2. 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 h. After 5 h, 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 < 5% (data not shown).

2.3. 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 *Bgl*II and Xho1 restriction sites. The H1-shRNA (or Scr) cassette was amplified from the pSuper vector and cloned into the Hpa1/*Pac*I 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 h after transfection by centrifuging the media at 146,000 × *g* at 4 °C for 3 h. 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 h 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.

2.4. 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 h 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 h after transduction the media was fully changed. Neuronal cell viability was measured on DIV9 using the resazurin assay.

2.5. 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 min. After 30 min, 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.

2.6. 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 min at room temperature and washed three times with phosphate buffered saline (PBS). The cells were washed and stained with DAPI nuclear stain (1:1000, Molecular Probes, D-1306) for 5 min 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).

2.7. RNA isolation and cDNA synthesis

RNA was isolated from neurons transduced with Scr or shRNA TG2