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Transglutaminase 2 Modulation of NF- κ B Signaling in Astrocytes is Independent of its Ability to Mediate Astrocytic Viability in Ischemic Injury

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Abstract

Transglutaminase 2 (TG2) is a multifunctional protein that can contribute to cell death and cell survival processes in a variety of disease contexts. Within the brain, TG2 has been shown to promote cell death in ischemic injury when expressed in astrocytes (Colak and Johnson, 2012). However, the specific functions and characteristics of astrocytic TG2 that mediate this effect are largely unknown. Therefore, the goal of this study was to investigate the role of astrocytic TG2 in mediating cellular viability processes in the context of ischemic injury, with a specific focus on its contributions to intracellular signaling cascades. We show that, in response to oxygen/glucose deprivation (OGD), acute lentiviral-mediated knockdown of TG2, as well as inhibition with an irreversible TG2 inhibitor, enhances cell survival. We also show that TG2 depletion increases nuclear factor- κ B (NF- κ B) signaling, whereas inhibition reduces NF- κ B activity. Despite its clear contribution to NF- κ B signaling, however, TG2 modulation of NF- κ B signaling is not likely to be a major contributor to its ability to mediate astrocytic viability in this context. Overall, the results of this study provide insight into the role of TG2 in astrocytes and suggest possible avenues for future study of the relationship between astrocytic TG2 and ischemic injury.

Keywords

Transglutaminase 2; ischemia; NF- κ B; astrocyte; viability; p38; JNK; ERK1/2; transamidation; oxygen/glucose deprivation

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1. Introduction ¹

The multifunctional protein transglutaminase 2 (TG2) has long been implicated in cellular viability processes (Chhabra et al., 2009; Colak and Johnson, 2012; Filiano et al., 2008; Filiano et al., 2010; Gundemir and Johnson, 2009; Gundemir et al., 2013; Ientile et al., 2004; Milakovic et al., 2004). The disease model, the cell type in which its expressed and its subcellular localization can all affect its specific role in mediating cell death and survival, thus rendering TG2 a diverse and complex contributor to cellular viability (Colak et al., 2011; Colak and Johnson, 2012; Gundemir and Johnson, 2009; Milakovic et al., 2004). TG2 belongs to a class of enzymes known as the transglutaminases. TG2 is unique in this class of proteins, as it is ubiquitously expressed in all cell types within the brain, and can be found in most subcellular compartments (Espitia Pinzon et al., 2014; Grosso and Mouradian, 2012; Hilton et al., 1997; Hwang et al., 2009; Kim et al., 1999; Lesort et al., 1998; Lesort et al., 1999; Lorand and Graham, 2003; Mastroberardino et al., 2006; Piacentini et al., 2002; Piacentini et al., 2014; van Strien et al., 2011a; van Strien et al., 2011b).

The most commonly studied function of TG2 is its role as a transamidation enzyme, which typically involves the catalysis of the formation of an isopeptide linkage between the γ -carboxamide group of a glutamine residue and the ϵ -amino group of a lysine residue (Gundemir et al., 2012). Aside from this function, TG2 has also been shown to exhibit a wide array of additional roles, including scaffolding, GTPase activity and kinase and protein disulfide isomerase functionalities (Akimov et al., 2000; Belkin, 2011; Gundemir et al., 2012; Mastroberardino et al., 2006; Nurminskaya and Belkin, 2012). Its transamidation function in particular has been implicated in cell death processes in some disease models (Gundemir and Johnson, 2009; Ientile et al., 2004; Milakovic et al., 2004; Tucholski and Johnson, 2002). In other cases, however, this enzymatic function has not been shown to play a role at all (Filiano et al., 2008).

Given its multifunctionality, TG2 has been shown to modulate several signaling pathways that are involved in cell death and survival. In cell culture models, TG2 has been shown to contribute to the activation of members of the mitogen activated protein kinase (MAPK) family (Belkin et al., 2005; Cecil and Terkeltaub, 2008; Dardik and Inbal, 2006; Robitaille et al., 2004; Robitaille et al., 2008; Telci et al., 2008; Yen et al., 2014). MAPKs are a set of kinases that are activated in a sequential phosphorylation cascade by upstream kinases (Cargnello and Roux, 2011). The MAPKs, JNK, p38 and ERK1/2, in turn phosphorylate a number of targets, including other kinases and transcription factors (Cargnello and Roux, 2011). MAPK signaling has been implicated in a variety of cellular processes, many of which are involved in modulating cell health in response to cellular stressors (Chen et al., 2009; Dong et al., 2009; Jiang et al., 2012; Legos et al., 2001; Lin et al., 2014; Liu et al., 2014; Okami et al., 2013; Roy Choudhury et al., 2014; Thompson and Van Eldik, 2009).

Another signaling cascade that TG2 can regulate, and that has been shown to mediate cell death and survival, is the nuclear factor- κ B (NF- κ B) signaling pathway. NF- κ B is a transcription factor composed of two subunits that can induce transcription of numerous pro-

¹Abbreviations: TG2, transglutaminase 2; OGD, oxygen/glucose deprivation; MAPK, mitogen activated protein kinase

cell death and pro-survival genes (Mincheva-Tasheva and Soler, 2013). Under basal conditions, NF- κ B is sequestered in the cytosol by its inhibitor, I κ B. In response to a cellular stressor I κ B becomes phosphorylated and subsequently targeted for degradation, allowing the NF- κ B subunits to freely translocate to the nucleus (Mincheva-Tasheva and Soler, 2013). The particular activating stimulus and the specific subunits that are activated all determine the role that NF- κ B plays in mediating cell death or survival (Mattson and Camandola, 2001; Mincheva-Tasheva and Soler, 2013; Pizzi et al., 2009; Ridder and Schwaninger, 2009; Zhang et al., 2005). TG2 has been shown to enhance NF- κ B signaling in tumor cell lines, which leads to an increase in their survival (Cao et al., 2008; Han et al., 2014; Jang et al., 2010; Mann et al., 2006; Yakubov et al., 2013). It is thought that its role in activating signaling is either by facilitating its translocation to the nucleus or inducing upstream signaling via an extracellular mechanism (Han et al., 2014; Mann et al., 2006; Yakubov et al., 2013).

An illustration of the diverse and complex contributions of TG2 to cellular viability can be seen in the case of ischemic injury. In a transient ischemic stroke model, TG2 expression and enzymatic activity were increased in regions of damage, which initially led to the hypothesis that TG2 might be enhancing cell death in this model (Ientile et al., 2004). In a mouse model of permanent ischemic injury, however, the overexpression of TG2 specifically in neurons reduced ischemic stroke damage (Filiano et al., 2010). In another study, mice with constitutive TG2 knockout in all tissues also exhibited a reduction in ischemic stroke damage (Colak and Johnson, 2012). This effect was further examined in primary neurons and astrocytes generated from these mice. TG2 depletion in neurons was detrimental to their survival following oxygen/glucose deprivation (OGD), which aligned with the previous study, in which neuronal overexpression of TG2 was protective (Colak and Johnson, 2012; Filiano et al., 2010). On the other hand, TG2 depletion in astrocytes enhanced their survival in OGD (Colak and Johnson, 2012). Furthermore, TG2 depleted astrocytes protected neurons from OGD-mediated cell death, whether or not TG2 was present in the neurons (Colak and Johnson, 2012). These data demonstrated that TG2 in astrocytes is not only detrimental to astrocytic survival, but also to neuronal survival, and that astrocytic TG2 may be a key modulator of ischemic cell death in an ischemic injury model (Colak and Johnson, 2012).

Astrocytic TG2 expression has been shown to be induced by cell stress-relevant stimuli, including mediators of oxidative stress and inflammatory cytokines (Monsonogo et al., 1997; Takano et al., 2010; van Strien et al., 2011a). Furthermore, astrocytic TG2 has been shown to contribute to adhesion and migration in response to inflammatory stimuli (van Strien et al., 2011a). The specific role of astrocytic TG2 in mediating cellular viability, however, has not yet been explored. Given the extensive influence that the astrocytic response to pathological stimuli can exert on the spread of damage in an ischemic injury (Chen and Swanson, 2003; Filous and Silver, 2016; Ricci et al., 2009), the goal of this study was to begin to investigate possible functions and characteristics of TG2 in astrocytes that may contribute to its ability to modulate viability. Using ischemia as a model for cellular stress, we examined viability following acute TG2 depletion and inhibition. We also investigated its role in modulating MAPK activation and NF- κ B transcriptional activity. Finally, using an adenoviral-mediated approach to modulate NF- κ B activity, we examined whether or not the

ability of TG2 to modulate NF- κ B activity is responsible for its ability to mediate cellular viability. The results of this study highlight novel roles for TG2 within astrocytes and lay the foundation for future investigations into its ability to mediate cellular viability in response to a pathological insult.

2. Results

2.1 TG2 depletion and treatment with the irreversible TG2 inhibitor VA4 increases astrocytic survival in response to an ischemic challenge

To gain a better understanding of the role of astrocytic TG2 in ischemic injury, a lentiviral-mediated knockdown approach was established using shRNA against TG2 (TG2 shRNA) (Figure 1A–B). Astrocytes transduced with TG2 shRNA were significantly more viable following OGD than astrocytes transduced with a scrambled RNA lentivirus (Figure 1C). To determine whether the protective effect of TG2 depletion was due to the loss of its transamidation activity, primary WT astrocytes were treated with 0, 5 or 10 μ M VA4, an irreversible inhibitor of TG2 that holds TG2 in its open conformation, inhibits its transamidation activity and ablates its GTP-binding ability (Gundemir et al., 2017). These concentrations were not toxic to astrocytes under basal conditions and previously have been shown to effectively inhibit TG2 transamidating activity in situ (Gundemir et al., 2017; Monteagudo et al., 2017). Relative to vehicle treatment, both 5 and 10 μ M VA4 increased the viability of WT astrocytes exposed to OGD (Figure 1D), indicating that the protective effect of TG2 depletion may be due, at least in part, to loss of its transamidation activity and/or to loss of its ability to bind GTP.

2.2 Constitutive TG2 depletion but not acute TG2 knockdown reduces p38 activation

To begin to understand how TG2 modulates cellular injury in astrocytes, it is imperative to investigate its modulation of cell death and survival signaling cascades. Phosphorylation and, therefore, activation of ERK1/2, p38 and JNK have been shown to increase in astrocytes exposed to OGD (Dong et al., 2009). To determine if TG2 modulates basal MAPK activation to affect astrocytic cell death, total and phosphorylated levels of p38, JNK and ERK1/2 were examined in cell lysates from primary astrocytes harvested from WT and TG2^{-/-} mice under basal culturing conditions. Levels of phosphorylated p38 were reduced in TG2^{-/-} astrocytes relative to WT astrocytes (Figure 2A–B), but there were no differences in total levels of p38, nor were there differences in total or phosphorylated levels of JNK or ERK1/2 (Figure 2A–F). In addition, there were no differences in phosphorylated levels of p38 between WT and TG2^{-/-} astrocytes when exposed to two or four hours of OGD (Figure S1).

To confirm that TG2 depletion modulates basal p38 activation in astrocytes, the same experiment was carried out in WT astrocytes transduced with scrambled or TG2 shRNA lentiviruses. In this case, basal phosphorylation of p38 was not different between TG2 shRNA-expressing astrocytes and astrocytes expressing scrambled control (Figure 2G–H). In a previous study we demonstrated that TG2^{-/-} astrocytes are significantly more viable following OGD than WT astrocytes (Colak and Johnson, 2012). This finding was confirmed in the current study, as knocking down TG2 in astrocytes with shRNA resulted in

significantly increased viability following OGD compared to WT astrocytes. Taken together, these data suggest that the mechanism by which TG2 modulates astrocytic viability is likely not due to its role in mediating MAPK activation.

2.3 TG2 depletion increases NF- κ B activity

Previous work has demonstrated a role for TG2 in modulating NF- κ B signaling (Cao et al., 2008; Han et al., 2014; Jang et al., 2010; Mann et al., 2006; Yakubov et al., 2013). To determine whether or not TG2 modulates NF- κ B activity in astrocytes, WT and TG2^{-/-} astrocytes were transfected with an NF- κ B luciferase reporter construct and then subjected to 8 or 10 hours of serum starvation followed by measurement of NF- κ B activity. TG2^{-/-} astrocytes exhibited an increase in NF- κ B reporter activation relative to WT astrocytes (Figure 3A). The same effect was observed following acute TG2 depletion, as there was an increase in NF- κ B reporter activity in astrocytes transduced with TG2 shRNA relative to astrocytes transduced with scrambled control (Figure 3B). Total and phosphorylated levels of I κ B did not differ between WT and TG2^{-/-} astrocytes after exposure to 10 hours of serum starvation (not shown), suggesting that TG2 depletion does not alter I κ B expression or activity.

To determine whether the effect of TG2 depletion on NF- κ B activity was still present in response to an ischemia-relevant stressor, NF- κ B reporter activity was assessed after 12 hours of serum starvation/normoxia or serum starvation/hypoxia. Under both normoxic and hypoxic conditions, NF- κ B reporter activity was still increased in TG2 shRNA-expressing astrocytes (Figure 3C), suggesting that the presence of TG2 in astrocytes attenuates NF- κ B signaling. This was further confirmed when WT and TG2^{-/-} astrocytes were exposed to hypoxia, as NF- κ B reporter activity was enhanced in TG2^{-/-} astrocytes using the same paradigm (data not shown).

2.4 Treatment with an irreversible TG2 transamidation inhibitor reduces NF- κ B activity

Given that previous studies in cancer cell lines have shown that TG2 facilitates NF- κ B activation in a transamidation activity and/or GTP-binding-dependent manner (Gundemir et al., 2017; Mann et al., 2006; Yakubov et al., 2013), we sought to determine whether inhibition of transamidation activity and GTP binding in our model would affect NF- κ B activation. NF- κ B luciferase reporter activity was measured in WT astrocytes treated with DMSO or 10 μ M VA4. In contrast to what was observed with TG2 deletion or after lentiviral-mediated TG2 depletion, inhibition of transamidation activity/GTP binding with VA4 led to a small but significant reduction in NF- κ B reporter activity (Figure 3D). Taken together, these results indicate that astrocytic TG2 prevents NF- κ B activation and that treatment with an irreversible inhibitor of the transamidation and GTP binding activity of TG2 further exacerbates its effect.

2.5 Astrocytic TG2 depletion has no effect on the expression of key NF- κ B subunits

To determine whether TG2 modulates the expression or modification of specific NF- κ B subunits, we measured the expression of total levels of p65, p50 and p105, the precursor to p50, in TG2 shRNA and scrambled-expressing astrocytes. These particular subunits were chosen given their enriched expression in astrocytes and high propensity for

heterodimerization (Mattson and Camandola, 2001; Mincheva-Tasheva and Soler, 2013). Furthermore, expression and activation of these subunits has been implicated in brain injury (Gabriel et al., 1999; Mattson and Camandola, 2001; Mincheva-Tasheva and Soler, 2013; Pennypacker et al., 2000). Phosphorylated levels of p65 (P-p65) were also assessed, as phosphorylation regulates several transcriptional functions of p65 (Mincheva-Tasheva and Soler, 2013). In protein lysates collected from TG2 shRNA and scrambled-expressing astrocytes, there was no difference in total or phosphorylated levels of p65 (Figure 4A–B). Furthermore, there was no difference in basal levels of p105 and p50 between TG2 shRNA and scrambled-expressing astrocytes (Figure 4C–D). Therefore, TG2-mediated suppression of NF- κ B transcriptional activity under basal conditions is likely not mediated by increased expression of the predominant NF- κ B subunits.

2.6 The ability of TG2 to modulate NF- κ B activity does not play a large role in its ability to modulate cellular injury

To determine if the increase in NF- κ B transcriptional activity in TG2-depleted astrocytes is responsible for the protection observed in response to a cellular stressor, NF- κ B activity was modulated using an adenoviral-mediated approach in astrocytes exposed to OGD. We also exposed astrocytes to an oxidative stress paradigm, which is another ischemia-relevant cellular stressor that is particularly common during the reperfusion phase of an ischemic injury (Mergenthaler et al., 2004).

WT and TG2^{-/-} astrocytes were first transduced with control or mutant I κ B (mt I κ B) adenovirus. The mt I κ B adenovirus, which has previously been used in primary astrocytes (Sanchez et al., 2003), encodes for a dominant negative form of I κ B that is unable to be phosphorylated and targeted for degradation, thus inhibiting NF- κ B signaling (Sanchez et al., 2003). Expression of mt I κ B (Figure 5A) reduced NF- κ B luciferase reporter activity in WT and TG2^{-/-} astrocytes to <1% of control (Figure 5B). Transduced TG2^{-/-} astrocytes were then exposed to OGD or tert-butyl hydroperoxide (tBOOH). In WT and TG2^{-/-} astrocytes, there were no significant differences in viability between astrocytes transduced with control and mt I κ B adenovirus in either paradigm (Figure 5C–F).

NF- κ B signaling was also modulated in astrocytes by overexpressing the p65 subunit. Overexpression of p65 (Figure 6A) in WT and TG2^{-/-} astrocytes increased NF- κ B luciferase reporter activity five-to-sevenfold over astrocytes transduced with a control adenovirus (Figure 6B). Under control conditions, p65 overexpression significantly reduced the viability of WT astrocytes, an effect that was no longer present upon exposure to tBOOH (Figure 6C). Intriguingly, p65 overexpression did lead to a very slight but significant increase in the viability of WT astrocytes exposed to OGD (Figure 6E). In TG2^{-/-} astrocytes, overexpression of p65 had no effect on their viability in response to tBOOH or OGD (Figure 6D,F). These findings suggest that increasing NF- κ B activity via p65 overexpression in WT astrocytes, which have lower endogenous NF- κ B activation than TG2^{-/-} astrocytes, may have a small protective effect in response to various cellular stressors. Overall, however, the lack of effect seen with mt I κ B expression indicates that NF- κ B signaling may not be a major pathway by which TG2 modulates astrocytic viability.

3. Discussion

Prior to these studies, the role of astrocytic TG2 in mediating cellular viability was largely unexplored. Several others have examined astrocytic TG2 in different disease models (van Strien et al., 2011a; van Strien et al., 2011b), but to the best of our knowledge, our group was the first to examine the role of astrocytic TG2 in ischemic injury. Our previous study demonstrated that TG2 knockout in astrocytes not only enhanced the viability of the astrocytes themselves, but also that of neurons in co-cultures (Colak and Johnson, 2012). Thus, an understanding of the mechanisms by which TG2 modulates astrocytic viability is a necessary step in understanding ischemic injury progression as a whole.

In this study, we demonstrated that lentiviral-mediated knockdown of TG2 enhances astrocytic viability in response to OGD, as does inhibition of TG2's transamidation activity and GTP binding ability with the irreversible inhibitor VA4. Transamidation activity has often been associated with enhanced apoptotic cell death in response to various stress stimuli (Colak et al., 2011; Gundemir and Johnson, 2009; Tucholski and Johnson, 2002). Several studies, however, have provided evidence that the conformational state, rather than transamidation activity of TG2, may be responsible for enhanced cell death (Colak et al., 2011; Gundemir et al., 2012). For TG2 to exhibit its transamidation activity, a catalytic triad of amino acids must be exposed within the catalytic core domain of the protein (Pinkas et al., 2007). This occurs when several calcium binding sites on the protein are occupied, which shifts the protein into its open conformation (Pinkas et al., 2007). A function of TG2 that inhibits its transamidation activity is GTP binding, which occurs when TG2 is in its closed conformation (Liu et al., 2002). The inhibitor used in these studies, VA4, binds TG2 at the catalytic site, thus holding it in its open conformation and preventing it from exhibiting its transamidation activity and from binding GTP/GDP (Gundemir et al., 2017). Thus, we can hypothesize that the protective effect that was observed with VA4 treatment may be due to loss of TG2's transamidation activity, inhibition of GTP/GDP binding by TG2, or to a scaffolding function that is attributed specifically to the open conformation of TG2 (Gundemir et al., 2017; Pinkas et al., 2007). Further studies are required to delineate the mechanism for this protective effect.

Intriguingly, TG2 depletion and treatment with VA4 had opposing effects on its ability to modulate NF- κ B signaling. Both constitutive knockout and acute lentiviral-mediated knockdown enhanced NF- κ B activity, which, to the best of our knowledge, is the first time that this has been demonstrated in any cell type. In contrast, inhibition of transamidation activity with VA4 slightly reduced NF- κ B activity, which aligns with previous studies in other cell types that have demonstrated that the transamidation activity of TG2 is required for its ability to activate NF- κ B (Gundemir et al., 2017; Mann et al., 2006; Yakubov et al., 2013). Thus, our findings indicate that TG2 modulation of NF- κ B activity in astrocytes may be due to its conformation, rather than its activity state, as both transamidating activity and GTP/GDP binding are lost when TG2 is depleted and when it is bound by VA4. We can thus hypothesize that TG2 may be acting as a scaffold to either directly or indirectly inhibit NF- κ B activity, and that this scaffolding property is enhanced when it is held in its open conformation following treatment with VA4. Further studies are required, however, to confirm this possibility.

Despite the fact that TG2 modulates NF- κ B signaling in astrocytes, this function does not appear to be a key contributor to its ability to mediate astrocytic viability in response to ischemia-relevant stressors. We showed that, while overexpression of p65 led to a very slight increase in viability in WT astrocytes exposed to OGD, inhibition of NF- κ B activity by mt I κ B expression did not alter viability in either cell type. Furthermore, although TG2 depletion and VA4 treatment yielded the same effect on cellular viability, they had opposing effects on NF- κ B activity.

The protective effect of TG2 depletion on astrocytes is also not likely due to its ability to modulate MAPK signaling, as basal p38 phosphorylation is reduced in TG2^{-/-} astrocytes, but not in astrocytes with lentiviral-mediated knockdown of TG2. Constitutive TG2 knockout likely mediates an array of long-term signaling changes, which, according to our data, includes a reduction in p38 MAPK activation. The main difference between the TG2 knockout astrocytes and the acute knockdown model is that TG2 is constitutively deleted in the knockout astrocytes. Therefore, any compensatory changes that are due to early TG2 gene deletion are not likely to have occurred in astrocytes transduced with the TG2 shRNA. An additional consideration is that the knockdown model, although quite efficient, does not result in complete deletion of TG2, which could contribute to differences in the knockout and knockdown models. Despite these differences, however, we have shown that acute knockdown yields the same effect on astrocytic viability as constitutive knockout (Colak and Johnson, 2012). Although it is possible that constitutive TG2 knockout and acute TG2 knockdown enhance astrocytic viability in different ways, we do not believe that TG2 modulation of MAPK signaling is a major contributor to astrocytic viability in response to OGD, as we observed no differences in p38 phosphorylation between WT and TG2^{-/-} astrocytes after 2 and 4 hours of OGD.

Changes in MAPK and NF- κ B signaling could, however, influence other cellular processes that may be important in the overall context of ischemic injury (Cargnello and Roux, 2011; Mattson and Camandola, 2001; Mincheva-Tasheva and Soler, 2013). Astrocytic contributions to damage after an ischemic injury are not only attributed to a loss of their physiological functions but can also be due to new injury-specific behaviors that occur in response to a stressor, such as the formation of the glial scar and cytokine and growth factor production (Filous and Silver, 2016; Ricci et al., 2009). Therefore, rather than playing a role in mediating astrocytic viability, TG2 modulation of signaling processes in astrocytes could be contributing instead to their ability to support neuronal viability. Future investigations into the effect of astrocytic TG2 on other injury-specific processes, such as inflammation, are required to examine this possibility.

4. Conclusion

The contributions of TG2 to cellular viability and intracellular signaling in response to cellular stressors are complex. We show, for the first time, that acute TG2 depletion and treatment with an irreversible TG2 inhibitor protect astrocytes from OGD-mediated cell death, but that these manipulations have opposing effects on NF- κ B activity within astrocytes. These studies have thus contributed to our understanding of this complex protein

within astrocytes and provide a foundation for continued investigations into the functions and characteristics of TG2 within astrocytes.

5. Experimental Procedure

5.1 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. Wild-type (WT) C57Bl/6 and TG2^{-/-} mice, which are on a C57Bl/6 background, were obtained from breeding colonies at the University of Rochester. TG2^{-/-} mice have been previously described (Colak and Johnson, 2012; Nanda et al., 2001).

5.2 Primary Culture

Primary astrocytes were harvested at post-natal day 0 from WT or TG2^{-/-} mouse pups as previously described (Colak and Johnson, 2012). Briefly, cortical hemispheres were separated and meninges were removed following brain dissection. Hemispheres were then mechanically dissociated and plated onto culture dishes in MEM media supplemented with 10% fetal bovine serum (FBS), 6 g/L glucose, 1 mM sodium pyruvate and 25 µg/mL Gentamicin (Glia MEM). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The following day, the plates were shaken to remove nonadherent cells and the remaining adherent cells were then rinsed once with fresh MEM media. 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 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. For the maintenance of astrocyte cultures, a full media change with fresh Glia MEM was performed every other day.

5.3 Lentiviral mediated knockdown of TG2

TG2 shRNA or a scrambled RNA control in a FIGB lentiviral backbone vector (Gundemir et al., 2013) were co-transfected into HEK293T cells with pMD2.G (VSVG-envelope) and psPAX2 (packaging constructs) as previously described (Colak et al., 2011). The transfection was performed with PolyJet Transfection reagent (SignaGen) and was done according to the manufacturer's instructions. 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 resuspended in 0.1% bovine serum albumin (BSA) in 1× phosphate buffered saline (PBS). TG2 shRNA has previously been used by our group to knockdown the expression of TG2 (Gundemir et al., 2013).

For viral transduction, WT astrocytes were plated onto 60 mm culture dishes. After 48 hours, culture medium was replaced with virus-containing Glia MEM. 24 hours later, this step was repeated and the following day cells underwent a complete media change. On day 5 post-transduction, cells were sub-cultured to experimental plates. Experiments were begun on day 11 post-transduction.

5.4 Cell Treatment Paradigms

For hypoxic treatment, cells were transferred to serum free MEM media supplemented with 6 g/L glucose, 1 mM sodium pyruvate and 25 µg/mL Gentamicin (SF Glia MEM) and placed in a Coy Laboratory Products Hypoxic Chamber maintained at 37°C with 0.1–0.2% O₂ and 5% CO₂. Normoxic control cells were transferred to SF Glia MEM and maintained in a humidified incubator with ambient O₂ levels and 5% CO₂.

For oxygen/glucose deprivation (OGD), cells were rinsed once with glucose-free, serum-free DMEM supplemented with 25 µg/mL Gentamicin (OGD DMEM) and then maintained in OGD DMEM. Cells were then placed in a Coy Laboratory Products Hypoxic Chamber maintained at 37°C with 0.1% O₂ and 5% CO₂. Normoxic control cells were also rinsed once with OGD DMEM and were then maintained in serum-free, glucose-containing DMEM supplemented with 25 µg/mL Gentamicin (Normoxic DMEM). These cells were then placed in a humidified incubator with ambient O₂ levels and 5% CO₂.

For experiments with tert-butyl hydroperoxide (tBOOH), a 2-mM solution was made up in SF Glia MEM from a stock solution of 70% tBOOH (TCI America). Astrocytes were rinsed once with SF Glia MEM and then treated for 3 hours with 2 mM tBOOH or vehicle in SF Glia MEM.

For experiments with VA4 (Gundemir et al., 2017), a stock solution of 25 mM VA4 in DMSO was further diluted to 5 or 10 µM for treatment of cells. For OGD experiments, VA4 was diluted in OGD DMEM or Normoxic DMEM and added to the cells for the duration of the experiment. For NF-κB luciferase experiments (see below), VA4 was diluted in Glia MEM and added to cells 12 hours pre-transfection and again 4 hours post-transfection. At 24 hours post-transfection, VA4 was diluted in SF Glia MEM and added to the cells during serum starvation.

5.5 Calcein AM Viability Assay

The Calcein AM viability assay was performed as previously described (Gundemir et al., 2013). The stock concentration of Calcein AM was 200 µM in DMSO. The Calcein AM was then added directly into the culture media in each well to a final concentration of 2 µM. Calcein AM was also added to two blank wells as a control. Cells were incubated with the dye for 30 minutes, after which fluorescence was measured with a 485-nm excitation wavelength and a 530-nm emission wavelength on a BioTek Instruments Fluorescent Plate Reader. Blank values were subtracted from each reading and results were normalized to the control condition for each experiment.

5.6 Immunoblotting

Cultured astrocytes were rinsed with 1× PBS and then lysed in 1× RIPA buffer with phosphatase and protease inhibitors (250 mM Tris-HCl, pH 7.4, 750 mM NaCl, 5% Triton X-100, 5 mM EDTA, 25% glycerol, 0.4% sodium dodecyl sulfate, 20 mM NaF, 2 mM Na₃VO₄, 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL pepstatin and 5 µg/mL leupeptin). The lysate was then sonicated and centrifuged at > 15,000 g for 15 minutes at 4°C. The pellet

was then discarded and protein concentration in the supernatant was determined with a bicinchoninic acid (BCA) assay.

Lysate samples were diluted in 1× RIPA buffer with protease and phosphatase inhibitors and 5× reducing stop buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue) at a final concentration of 1 mg/mL. Samples were then boiled and resolved on 10–12% polyacrylamide gels, followed by transfer to nitrocellulose membranes. Membranes were then blocked in 5% nonfat dry milk in TBS-T (20 mM Tris base, 137 mM NaCl, 0.05% Tween-20, pH 7.4) for one hour. After blocking, blots were incubated overnight with primary antibodies for p38 (1:5000; Cell Signaling Technology; rabbit polyclonal), P-p38 (1:500; Thermo Scientific; rabbit monoclonal), JNK (1:5000; Cell Signaling Technology; rabbit polyclonal), P-JNK (1:1000; Cell Signaling Technology; rabbit monoclonal), ERK1/2 (1:5000; Thermo Scientific; rabbit polyclonal), P-ERK1/2 (1:1000; Thermo Scientific; rabbit polyclonal), TG2 (1:2500; TGM01; rat monoclonal (Song et al., 2013)) or α -Tubulin (1:5000, Cell Signaling Technology; rabbit monoclonal) in 5% nonfat dry milk in TBS-T buffer or primary antibodies against p65 or P-p65 (1:1000, Cell Signaling Technology; rabbit monoclonal) or p105/p50 (1:2000, Cell Signaling Technology; rabbit monoclonal) in 5% BSA in TBS-T buffer. Membranes were then washed with TBS-T and incubated for 1 hour with HRP-conjugated secondary antibodies. After incubation with secondary antibody, membranes were washed and visualized with the use of an electrochemiluminescence reaction.

5.7 Luciferase Reporter Assay

The NF- κ B luciferase reporter construct, which contains NF- κ B response elements in the promoter region for the firefly luciferase gene, and a promoterless-null Renilla construct were generously provided by Dr. Sanjay Maggirwar. The NF- κ B luciferase reporter has previously been used to measure NF- κ B luciferase activity (Kieba et al., 2015). Constructs were co-transfected into astrocytes with PolyJet transfection reagent as per the manufacturer's instructions. Four hours later, a complete media change was performed. 24 hours post-transfection, astrocytes were placed into SF Glia MEM for 8–12 hours. After serum starvation, wells were rinsed with 1× PBS and plates were stored at -80°C until measurement of luciferase activity.

Luciferase and Renilla measurements were taken using the Dual-Luciferase Reporter Assay System (Promega). The assay was carried out according to the manufacturer's instructions. Luciferase measurements for each sample were normalized to its Renilla measurement. Final results were normalized to control measurements for each experiment.

5.8 Adenovirus Preparation and Transduction

Mutant I κ B (mt I κ B), p65 and control adenoviruses were generously provided by Dr. Sanjay Maggirwar (Sanchez et al., 2003). Propagation of the adenovirus was conducted in a manner similar to what has been described previously (He et al., 1998). 2 μL of viral stock was diluted in 1× PBS and added to a single well on a 24-well plate of HEK293 cells for 90 minutes, after which 1 mL DMEM with 5% FBS was added. Cells were left to incubate for 5 days and were then collected and subjected to three freeze/thaw cycles. Cellular debris was

then pelleted and the supernatant was collected. 500 μ L of viral supernatant was then added to 5×10^6 HEK293 cells on a 10-cm plate and incubated for 90 minutes, after which 9 mL of DMEM with 5% FBS was added. Cells were incubated for 72 hours and were then collected and subjected to three freeze/thaw cycles. Cellular debris was pelleted and the supernatant was collected. 4 mL viral supernatant was then added to each of four 15-cm plates containing 8×10^6 cells and incubated for 90 minutes, after which 24 mL DMEM with 5% FBS was added. Cells were incubated for 72 hours and were then collected, pelleted and resuspended in 400 μ L 1 $\times$ PBS per plate. Cells then underwent three freeze/thaw cycles after which debris was pelleted and viral supernatant was collected and stored for future use. After the final propagation step, viral titer was determined using the Adenovector Rapid Titer Kit (Applied Biological Materials). For experiments, primary WT and TG2^{-/-} astrocytes were transduced with 1×10^6 ifu (for mt I κ B experiments) or 1×10^7 ifu (for p65 experiments) upon reaching about 70% confluency. Experiments were begun 12 hours post-transduction.

5.9 Statistical Analysis

To determine whether the data in each experiment followed a normal distribution, a D'Agostino & Pearson test or a Shapiro-Wilk test was conducted. Statistical significance for data that followed a normal distribution was determined with an unpaired t-test or a One-way ANOVA with Tukey's post-hoc for multiple comparisons. Statistical significance for data that did not follow a normal distribution was determined with a Mann-Whitney test or a Kruskal-Wallis test with Dunn's post-hoc for multiple comparisons. All experiments were conducted with a minimum of three biological replicates (i.e. three separate primary cultures). In all cases, except for quantitation of immunoblots, each replicate was in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- In astrocytes transglutaminase 2 (TG2) depletion or inhibition enhances survival
- Astrocytic TG2 depletion increases nuclear factor- κ B (NF- κ B) signaling
- Inhibition of astrocytic TG2 reduces NF κ B signaling
- Modulation of NF κ B activity by TG2 is unlikely to impact astrocyte viability.
- TG2 conformation likely plays a role in mediating cell survival pathways

Figure 1. Astrocytic TG2 depletion or inhibition of its transamidation activity increases cellular viability in response to OGD

(A) Representative immunoblot of lentiviral-mediated knockdown of TG2. Astrocytes were transduced with either TG2 shRNA or a scrambled control lentivirus. (B) Quantification of lentiviral-mediated knockdown of TG2. TG2 expression was normalized to tubulin and is presented relative to TG2 expression in scrambled-expressing astrocytes (n = 10). (C) Cellular viability of scrambled or TG2 shRNA-expressing astrocytes exposed to 6–12 hours of OGD. Data are normalized to the viability of scrambled-expressing astrocytes in normoxia (n = 40). (D) Cellular viability of WT Astrocytes incubated with 0, 5 or 10 μ M VA4 and exposed to 12–14 hours of OGD (n = 12). Results are shown as mean $\pm$ SEM. ***p < 0.001, ** p < 0.01.

Figure 2. Phosphorylated p38 levels are reduced when TG2 is constitutively knocked out, but not after acute knockdown

(A–F) Representative immunoblots and quantification of total and phosphorylated levels of p38 (A–B, $n = 4$), ERK1/2 (C–D, $n = 4$) and JNK1/2 (E–F, $n = 3$) in WT and TG2^{-/-} astrocytes. (G–H) Representative immunoblots and quantification of total and phosphorylated levels of p38 in WT astrocytes expressing either TG2 shRNA or a scrambled control ($n = 5$). All bands were normalized to tubulin and protein expression is normalized to expression in either WT astrocytes (A–F) or scrambled-expressing astrocytes (G–H). Lines

separating lanes in representative immunoblot images (A, C & E) indicate removal of intervening lanes. Results are shown as mean $\pm$ SEM. ** $p < 0.01$, n.s.- not significant.

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Figure 3. TG2 depletion and inhibition of its transamidation activity have opposing effects on NF-κB luciferase reporter activity

(A–B) NF-κB luciferase reporter activity in WT and TG2^{-/-} astrocytes (A, n = 12) or WT astrocytes transduced with TG2 shRNA or a scrambled control lentivirus (B, n = 15–18) after 8 or 10 hours of serum starvation. Data are normalized to WT (A) or scrambled (B) as a control. (C) NF-κB luciferase reporter activity in WT astrocytes transduced with TG2 shRNA or a scrambled control lentivirus after 12 hours of serum starvation (normoxia) or serum starvation with hypoxia. Data are normalized to scrambled normoxia as a control (n = 12). (D) NF-κB luciferase reporter activity in WT astrocytes treated with vehicle or 10 μM VA4 and exposed to 12 hours of serum starvation. Data are normalized to luciferase measurements obtained from vehicle treated cells (n = 18). All NF-κB luciferase reporter measurements were normalized to a Renilla control reporter that was cotransfected with the NF-κB luciferase reporter. Results are shown as mean ± SEM. ** p < 0.01, *** p < 0.001, **** p < 0.0001.

Figure 4. TG2 depletion has no effect on the expression level of NF- κ B subunits

(A–B) Representative immunoblot and quantification of total and phosphorylated levels of the p65 subunit in astrocytes transduced with TG2 shRNA or a scrambled control lentivirus ($n = 5–7$). (C–D) Representative immunoblot and quantification of total levels of p50 or its precursor, p105 ($n = 5$). Lines separating lanes in the representative immunoblot in (A) indicate removal of intervening lanes. Results are shown as mean $\pm$ SEM. n.s. - not significant.

Figure 5. Inhibition of NF-κB with a dominant negative form of IκB has no effect on astrocytic viability in response to ischemia-relevant stressors

(A) Immunoblot of WT astrocytes transduced with control or mt IκB adenoviruses for 12 hours. (B) NF-κB luciferase reporter activity in WT and TG2^{-/-} astrocytes transduced with control or mt IκB adenoviruses. Data are normalized to control-expressing astrocytes (n = 4–5). (C–D) Cellular viability in WT (C) or TG2^{-/-} (D) astrocytes transduced with control or mt IκB adenoviruses and exposed to either vehicle or 2 mM tBOOH for 3 hours (n = 18–21). (E–F) Cellular viability in WT (E) or TG2^{-/-} (F) astrocytes transduced with control or mt IκB adenoviruses and exposed to 24–32 hours of OGD. Data are presented as fold c

hange in cellular viability relative to the viability of astrocytes transduced with control adenovirus (n = 18). Results are shown as mean $\pm$ SEM. **** p < 0.0001, ** p < 0.01, n.s. – not significant.

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Figure 6. Overexpression of the NF-κB subunit p65 led to a slight increase in cellular viability in WT but not TG2^{-/-} astrocytes exposed to OGD

(A) Immunoblot of WT astrocytes transduced with control or p65 adenoviruses for 12 hours. (B) NF-κB luciferase reporter activity in WT and TG2^{-/-} astrocytes transduced with control or p65 adenoviruses. Data are normalized to control-expressing astrocytes (n = 3–5). (C–D) Cellular viability in WT (C) or TG2^{-/-} (D) astrocytes transduced with control or p65 adenoviruses and exposed to either vehicle or 2 mM tBOOH for 3 hours (n = 18–21). (E–F) Cellular viability in WT (E) or TG2^{-/-} (F) astrocytes transduced with control or p65 adenoviruses and exposed to 24–32 hours of OGD. Data are presented as fold change in

cellular viability relative to the viability of astrocytes transduced with control adenovirus (n = 12–18). Results are shown as mean $\pm$ SEM. ** p < 0.01, *** p < 0.001, **** p < 0.0001.

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