Published in final edited form as:

Neuroscience. 2010 December 29; 171(4): 1256–1264. doi:10.1016/j.neuroscience.2010.10.029.

TUMOR NECROSIS FACTOR-LIKE WEAK INDUCER OF APOPTOSIS (TWEAK) AND FIBROBLAST GROWTH FACTOR-INDUCIBLE 14 (Fn14) MEDIATE CEREBRAL ISCHEMIA-INDUCED POLY(ADP-RIBOSE) POLYMERASE-1 ACTIVATION AND NEURONAL DEATH

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Abstract

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its receptor Fibroblast growth factor-inducible 14 (Fn14) are expressed in neurons. Here we demonstrate that TWEAK induces a dose-dependent increase in neuronal death and that this effect is independent of TNF- α and mediated by NF- κ B pathway activation. Incubation with TWEAK induces apoptotic cell death in wild-type (Wt) but not in Fn14 deficient (Fn14^{-/-}) neurons. Intracerebral injection of TWEAK induces accumulation of poly(ADP-ribose) polymers (PAR) in Wt but not in Fn14^{-/-} mice. Exposure to oxygen-glucose deprivation (OGD) conditions increases TWEAK and Fn14 mRNA expression in Wt neurons, and decreases cell survival in Wt but not in Fn14^{-/-} or TWEAK deficient (TWEAK^{-/-}) neurons. Experimental middle cerebral artery occlusion (MCAO) increases the expression of TWEAK and Fn14 mRNA and active caspase-3, and the cleavage of poly(ADP-ribose)polymerase-1 with accumulation of PAR in the ischemic area in Wt but not Fn14^{-/-} mice. Together, these results suggest a model where in response to hypoxia/ischemia the interaction between TWEAK and Fn14 in neurons induces PARP-1 activation with accumulation of PAR polymers and cell death via NF- κ B pathway activation. This is a novel pathway for hypoxia/ischemia-induced TWEAK-mediated cell death and a potential therapeutic target for ischemic stroke.

Keywords

Cerebral ischemia; Tumor necrosis factor-like weak inducer of apoptosis (TWEAK); Fibroblast growth factor-inducible 14 (Fn14); Poly(ADP-ribose)polymerase-1 (PARP-1); Poly(ADP-ribose) polymers (PAR)

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1.0. Introduction

Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) is a member of the tumor necrosis factor superfamily of cytokines (Chicheportiche et al., 1997). Fibroblast growth factor-inducible 14 (Fn14) is the receptor for TWEAK (Wiley et al., 2001) and binding of TWEAK to Fn14 activates the NF- κ B signal transduction pathway [Reviewed in Winkles JA, (Winkles, 2008)], which has been linked to cell death during cerebral ischemia (Schneider et al., 1999a). TWEAK has been reported to stimulate cell proliferation (Lynch et al., 1999; Harada et al., 2002; Donohue et al., 2003), migration (Harada et al., 2002; Tran et al., 2003; Donohue et al., 2003) and differentiation (Polek et al., 2003), as well as the expression of pro-inflammatory molecules (Chicheportiche et al., 1997; Saas et al., 2000; Harada et al., 2002; Chicheportiche et al., 2002; Xu et al., 2004; Kim et al., 2004; Jin et al., 2004).

TWEAK was initially described as a poor cytotoxic agent that induces cell death in several tumor cell lines in conjunction with other sensitizers [Reviewed in Winkles JA, (Winkles, 2008)]. Since then, multiple mechanisms for TWEAK-induced cell death have been described, including caspase-dependent apoptosis and cathepsin-mediated necrosis (Nakayama et al., 2002). Importantly, it has been proposed that TWEAK-induced cell death is mediated by the interaction between TNF- α and TNF receptor 1 (TNFR1) (Schneider et al., 1999b).

Ischemic stroke is the third cause of mortality and a leading cause of disability in the world (Lloyd-Jones et al., 2009). The onset of the ischemic insult is followed by increase in the expression of pro-inflammatory cytokines in the ischemic tissue, which has been associated with neuronal death and poor outcome. Accordingly, it has been reported that experimental middle cerebral artery occlusion (MCAO) in mice (Petrovita et al., 2004; Yepes et al., 2005) and ischemic stroke in humans (Inta et al., 2008) increase the expression of TWEAK and Fn14 in the ischemic tissue, and that inhibition of TWEAK activity either with an Fn14-Fc decoy receptor (Yepes et al., 2005; Polavarapu et al., 2005) or anti-TWEAK neutralizing antibodies (Petrovita et al., 2004) or genetic deficiency of Fn14, decreases the volume of the ischemic lesion and protects the integrity and barrier function of the blood-brain barrier (Haile et al., 2010; Zhang et al., 2007b).

Poly(ADP-ribose)polymerase-1 (PARP-1) is a 114 kDa nuclear chromatin-associated protein that catalyzes the transfer of ADP-ribose units onto glutamic acid residues on nuclear proteins (Hassa et al., 2001). Because PARP-1 is the main substrate for caspase-3 (van Wijk and Hageman, 2005), PARP-1 cleavage is a marker for apoptotic cell death. During cerebral ischemia PARP-1 becomes highly activated leading to the accumulation of poly(ADP-ribose) polymers (PAR) and cell death (van Wijk and Hageman, 2005). Thus, accumulation of PAR is considered as a surrogate marker of PARP-1 activation. Importantly, genetic deficiency of PARP-1 is associated with resistance to hypoxia-induced cell death and decrease in the volume of the ischemic lesion following MCAO (Eliasson et al., 1997).

In the work presented here we demonstrate that the interaction between TWEAK and Fn14 induces apoptotic neuronal death. This effect is independent of endogenous TNF- α and instead is associated with PARP-1 activation, caspase-3 cleavage, and the accumulation of PAR in the ischemic area. This is a novel pathway for cerebral hypoxia/ischemia-induced TWEAK-mediated cell death and a potential new target for the treatment of patients with ischemic stroke.

2.0. Experimental procedures

2.1. Animal model of cerebral ischemia

Murine strains were TWEAK deficient (TWEAK^{-/-}) and Fn14 deficient (Fn14^{-/-}) mice, and their corresponding wild-type (Wt) controls on a C57BL/6J genetic background, kindly provided by Dr. Kyungmin Hahm (Biogen Idec Inc, Cambridge Massachusetts). All procedures were approved by the Emory University Institutional Animal Care and Use Committee. Mice were anesthetized with 4% chloral hydrate (400 mg/kg IP). The rectal and masseter muscle temperatures were controlled at 37°C with a homeothermic blanket. The middle cerebral artery was exposed and occluded with a 6-0 silk suture advanced from the common carotid artery into the middle cerebral artery as described elsewhere (An et al., 2008). After 30 minutes of ischemia the suture was withdrawn and the animals were reperused. Cerebral perfusion (CP) in the distribution of the middle cerebral artery was monitored throughout the surgical procedure with a laser Doppler (Perimed Inc., North Royalton, OH), and only animals with a > 70% decrease in CP after occlusion and complete recovery after suture removal were included in this study. Heart rate, systolic, diastolic and mean arterial blood pressure were controlled with an IITC 229 System (IITC-Lice Science; Woodland Hills, CA).

2.2. Neuronal cultures, exposure to oxygen-glucose deprivation (OGD) conditions, and determination of neuronal survival and apoptotic cell death

Cortical neurons were cultured from E19 Wt, TWEAK^{-/-} and Fn14^{-/-} mice as described elsewhere (Zhang et al., 2007a). Briefly, the cerebral cortex was dissected, transferred into Hanks' balanced salt solution containing 100 units/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES, and incubated in trypsin containing 0.02% DNase at 37°C for 15 min. Tissue was then triturated, and the supernatant was resuspended in B27-supplemented neurobasal medium containing 2 mM l-glutamine and plated onto 0.1 mg/ml poly-l-lysine-coated wells. To study the effect of TWEAK and TNF-α on neuronal death, cortical neurons were incubated with either TWEAK 0–300 ng/ml (R & D Systems; Minneapolis, MN), or TNF-α 10 ng/ml (R & D Systems). A subgroup of neurons was incubated with 10 µM of the NF-κB inhibitor BAY 11-7085 (Alexis Biochemicals; Plymouth Meeting, PA) followed 30 minutes later by treatment with TWEAK 300 ng/ml. At that dose, BAY 11-7085 is an efficient inhibitor of cytokine-induced IκBα phosphorylation (Scaife et al., 2002). A sub-set of cells was incubated with anti-mouse TNFR1 neutralizing antibodies 100 µg/ml (R & D Systems) in combination with either TNF-α 10 ng/ml or TWEAK 300 ng/ml. To study the effect of endogenous TWEAK and Fn14 on hypoxic cell death, Wt, TWEAK^{-/-} and Fn14^{-/-} neurons were either left untreated or incubated with TWEAK 300 ng/ml and exposed to oxygen-glucose deprivation (OGD) conditions (< 0.1% oxygen) in an anabiotic chamber (Don Whitley Scientific; Frederick, MD) for 55 minutes, as previously described (Polavarapu et al., 2007). In previous studies we found that 55 minutes of exposure to OGD conditions induced cell death in approximately 50% of the neurons in culture (data not shown). In each case, cell survival was determined 24 hours later with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (ATCC; Manassas, VA), following manufacturer's instructions. Results are expressed as a percentage of cell survival observed in neurons cultured from each strain of mice and maintained under normoxic conditions. Each experiment was performed in cultures from three different animals and each observation was repeated 8–15 times. To study the effect of TWEAK on apoptotic cell death, Wt and Fn14^{-/-} neurons were incubated with either TWEAK 0–300 ng/ml or vehicle (control), followed 24 hours later by determination of terminal dUTP nick-end labeling (TUNEL) staining with the ApopTag Plus Fluorescein *in situ* Apoptosis Detection Kit (Chemicon International; Temecula, CA) following manufacturer's instructions. To determine the number of TUNEL-positive neurons, images were digitized in

a Zeiss Axioplan 2 microscope (20-fold objective) with a Zeiss AxioCam and imported into AxioVision. Images were then viewed at 150% of the original X 20 images with an Image MetaMorph Software. The number of TUNEL-positive neurons was then expressed as percentage of the total number of DAPI-positive cells per field. Each experiment was repeated in cultures obtained from 3 different animals and each observation was repeated 6 times. Results are given as a mean percentage of number of apoptotic neurons per field.

2.3. Quantitative real-time PCR analysis

Cortical neurons cultured from Wt mice were exposed to OGD conditions for 55 minutes. Wild-type mice underwent MCAO. Sham-operated animals and neurons kept under normoxic conditions were included as controls for each experiment. Four hours after exposure to 55 minutes of OGD conditions or 0–48 hours after MCAO, cells and brains were harvested. Total RNA was isolated using the RNeasy mini kit (Qiagen; Valencia, CA) according to the manufacturer's instructions and equal amounts of RNA were taken for cDNA synthesis using High-capacity cDNA Kit (Applied Biosystems; Foster City, CA). Real-time quantitative PCR analysis for TWEAK and Fn14 was performed using TaqMan Gene Expression Assays (Applied Biosystems; Foster City, CA) with forward and reverse primers as well as an internal probe also purchased from Applied Biosystems. Polymerase chain reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each observation was repeated 8 times.

2.4. Immunohistochemistry and definition of Areas of Interest (AOI)

Twenty four hours after MCAO Wt and Fn14^{-/-} mice were transcardially perfused with PBS during 10 minutes. Brains were harvested and 10 µm brain sections were stained with a monoclonal antibody that detects poly(ADP-ribose) polymers (PAR, 1:1000 dilution; Alexis; San Diego, CA). Sections were co-stained with 4'-6-Diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO). Each coronal section was then divided into 16 square areas (150 mm² each one) that involved the necrotic core and the area of ischemic penumbra, and comparable areas in the non ischemic hemisphere. Two areas of interest (AOI) were chosen in the boundaries between the ischemic penumbra and necrotic core (AOI-1 and AOI-3), whereas a third zone was located in the necrotic core (AOI-2). Each experiment was repeated 3 times.

2.5. Western blot analysis

Wt neurons were incubated 24 hours under normoxic conditions with either vehicle control or TWEAK 300 ng/ml, or with a combination of TWEAK and the PARP-1 inhibitor BSI-201 25 µM (Selleck Chemicals; Houston, Texas). Wt mice were either intracortically injected with 2 µl of TWEAK (1 µg/µl) or vehicle control at bregma: – 1 mm, mediolateral: 3 mm and dorsoventral: 3 mm (Paxinos and Franklin, 2001), or subjected to MCAO. Twenty four hours after treatment with TWEAK or 6 and 24 hours after MCAO brains were harvested and homogenized in RIPA lysis buffer and protein concentration was determined with the BCA protein assay (Thermo Scientific) followed by loading of 16 µg of total protein for SDS-page electrophoresis and immunoblotting with antibodies directed against either an 89 kDa fragment resultant of PARP-1 cleavage (PARP-1; BD Pharmingen; Franklin Lakes, NJ), or un-cleaved PARP-1 (Santa Cruz Biotechnology Inc; Santa Cruz, CA), or p-IKBA (Cell Signaling Technology; Danvers, MA), or PAR (Alexis), or cleaved caspase-3 (Cell Signalling Technology). Each observation was repeated 3 times. The intensity of the band was measured with the NIH Image Analyzer System.

2.6. Statistical analysis

Values are expressed as percentage or mean $\pm$ SD when appropriate. Statistical tests included the T-test followed by the Wilcoxon signed-ranked test. *P* values of less than 0.05 were considered significant.

3.0 Results

3.1. TWEAK induces cell death via NF- κ B pathway activation

To study the effect of TWEAK on neuronal cell death, wild-type (Wt) cortical neurons were incubated with progressive concentrations of rTWEAK (0–300 ng/ml) followed 24 hours later by determination of cell survival with the MTT assay. Our results indicate that incubation with TWEAK induces a dose-dependent decrease in cortical neuronal survival to 89.29 $\pm$ 1.28 % and 62.48 $\pm$ 1.2 %, following treatment with 100 and 300 ng/ml of TWEAK, respectively (Figure 1A). It has been reported that TWEAK induces NF- κ B activation in the central nervous system (CNS) (Polavarapu et al., 2005). Thus we decided to study whether NF- κ B activation mediates the effect of TWEAK on neuronal death. Wt cortical neurons were incubated with TWEAK 300 ng/ml either alone or in combination with the NF- κ B inhibitor BAY 11-7085 10 μ M. We found that whereas neuronal survival decreased to 61.05 $\pm$ 1.2 % following incubation with TWEAK alone, this effect was abrogated by inhibition of the NF- κ B pathway (Figure 1B).

3.2. Tumor necrosis factor- α (TNF- α) does not mediate TWEAK-induced neuronal death

Because it has been reported that the interaction between TNF- α and TNF receptor 1 (TNFR1) mediates TWEAK-induced cell death (Schneider et al., 1999b), we decided to study if the observed effect of TWEAK on neuronal death was also mediated by TNF- α /TNFR1. First, we determined cell survival in Wt cortical neurons incubated with TNF- α 10 ng/ml either alone or in combination with anti-TNFR1 neutralizing antibodies 100 μ g/ml. We found that incubation with TNF- α alone decreased neuronal survival to 76.05 $\pm$ 2.91% and that this effect was abrogated by co-treatment with anti-TNFR1 antibodies (97.96 $\pm$ 8.51%; Figure 2A). Then we studied neuronal survival following incubation with TWEAK 300 ng/ml either alone or in combination with anti-TNFR1 antibodies. Our results demonstrate that TWEAK alone decreased neuronal survival to 62.53 $\pm$ 1.56%, and that this effect remained unchanged despite co-treatment with anti TNFR1 antibodies (62.91 $\pm$ 1.32 %; Figure 2B).

3.3. Fn14 mediates the effect of TWEAK on neuronal cell death

Fn14 is the cognate receptor for TWEAK [Reviewed in Winkles JA, (Winkles, 2008)]. However, because an Fn14-independent effect for TWEAK has been reported (Bover et al., 2007), we decided to study if this receptor mediates TWEAK-induced neuronal death. First, to study the possibility of an interaction between TNF- α and Fn14, we quantified cell survival in cortical neurons cultured from Fn14^{-/-} mice and incubated with TWEAK 300 ng/ml or TNF- α 10 ng/ml. We observed that treatment of Fn14^{-/-} neurons with TNF- α decreased neuronal survival to 71.76 $\pm$ 1.39 %. In contrast, treatment of Fn14^{-/-} neurons with TWEAK failed to induce a significant decrease in cell survival (97.95 $\pm$ 8.39%; Figure 3A). To further characterize these results Wt and Fn14^{-/-} neurons were incubated with progressive concentrations of TWEAK (0 – 300 ng/ml) followed 24 hours later by determination of apoptotic cell death with the TUNEL staining. Our results indicate that whereas TWEAK induces a dose-dependent increase in apoptotic cell death in Wt neurons (white bars, Figure 3B), this effect is abrogated by genetic deficiency of Fn14 (black bars, Figure 3B).

3.4. The interaction between TWEAK and Fn14 induces accumulation of poly(ADP-ribose) polymers (PAR)

Because accumulation of PAR is a marker of PARP-1 cleavage and apoptotic cell death (van Wijk and Hageman, 2005) we decided to study the effect of TWEAK on PARP-1 cleavage and PAR accumulation in neurons. Wt neurons were incubated with TWEAK followed by Western blot analysis with antibodies against either total PARP-1 or an 89 kDa fragment product of PARP-1 cleavage, or PAR. We found that incubation with TWEAK increases the expression of total (un-cleaved) PARP-1 (Figure 4A), and induces its cleavage to an 89 kDa product (Figure 4B) with subsequent accumulation of PAR in neurons (Figure 4C). To further characterize this observation, we investigated the accumulation of PAR in the brain of Wt mice intracortically injected with recombinant TWEAK. Our results indicate that treatment with TWEAK induces the accumulation of PAR in the injected area (Figure 4D), thus demonstrating that the interaction between TWEAK and Fn14 leads to PAR accumulation in the central nervous system. To determine whether PARP-1 mediates the effect of TWEAK on NF- κ B activation, we performed a Western blot analysis to detect p-IK β in Wt neurons left untreated or incubated with TWEAK either alone or in combination with the PARP-1 inhibitor BSI-201. Our results indicate that TWEAK induces NF- κ B activation in neurons and that this effect is abrogated by PARP-1 inhibition (Figure 4E). Because our experiments show that NF- κ B activation mediates TWEAK-induced cell death, we decided to investigate the role PARP-1 on TWEAK-induced cell death. Wt neurons were incubated with TWEAK alone or in combination with BSI-201 followed by a Western blot analysis with an antibody that detects cleaved caspase-3. We found that TWEAK induces caspase-3 cleavage and that this effect is attenuated by PARP-1 inhibition (Figure 4F).

3.5. The interaction between endogenous TWEAK and Fn14 mediates hypoxia-induced neuronal death

Then we used an *in vitro* model of hypoxia to investigate the effect of endogenous TWEAK and Fn14 on neuronal death. First, we determined by quantitative RT-PCR analysis the effect of exposure to oxygen-glucose deprivation (OGD) conditions on the expression of neuronal TWEAK and Fn14 mRNA. We found that compared to cultures maintained under normoxic conditions, exposure to OGD conditions for 55 minutes induced a 3 $\pm$ 1.1 and 9 $\pm$ 2.0 fold increase in TWEAK and Fn14 mRNA expression, respectively (Figure 5A). Then we quantified cell survival in neurons cultured from Wt, Fn14^{-/-} and TWEAK^{-/-} mice and exposed to 55 minutes of OGD. A sub-set of cells was treated with TWEAK 300 ng/ml. Our results indicate that exposure to OGD conditions decreases neuronal survival to 53.5 $\pm$ 3.5 % in Wt cells, and that this effect is significantly attenuated in Fn14^{-/-} and TWEAK^{-/-} cells (83.49 $\pm$ 2.42 % and 78.3 $\pm$ 1.8 %, respectively). Importantly, incubation with TWEAK 300 ng/ml decreased neuronal survival in TWEAK^{-/-} (56.67 $\pm$ 0.84 %) but not in Fn14^{-/-} cells (80 $\pm$ 1.06 %, Figure 5B).

3.6. The interaction between endogenous TWEAK and Fn14 mediates cerebral ischemia induced cell death

Then we decided to investigate the role of TWEAK/Fn14 on ischemic cell death. First, we performed a quantitative RT-PCR for TWEAK and Fn14 mRNA in the ischemic tissue of Wt mice 0–24 hours after middle cerebral artery occlusion (MCAO). Our results indicate that cerebral ischemia induces a rapid increase in TWEAK and Fn14 mRNA expression. Interestingly, the expression of TWEAK mRNA increased at 30 minutes, was maximal at 1 hour and returned to basal levels 6 hours after MCAO. In contrast, the expression of Fn14 mRNA increased 30 minutes after MCAO, peaked at 6 hours and remained elevated thereafter (Figure 6). Because our results indicate that recombinant TWEAK induces apoptotic cell death in cultured neurons; we decided to investigate whether endogenous TWEAK also induces neuronal death. First we investigated the effect of Fn14 deficiency on

cerebral ischemia-induced caspase-3 cleavage 0–24 hours after MCAO. Our results indicate that MCAO induces a progressive increase in the expression of cleaved (active) caspase-3 in the ischemic tissue of Wt but not in Fn14^{-/-} mice (Figure 7A). Because it has been reported that PARP-1 is a substrate for caspase-3 and the mediator of the effects of NF-κB activation on inflammation and cell death, we studied the effect of cerebral ischemia on the expression and cleavage of PARP-1. Wt and Fn14^{-/-} mice underwent MCAO followed 6 and 24 hours later by Western blot analysis for PARP-1 expression. Our results indicate that cerebral ischemia induces the cleavage of PARP-1 in the ischemic tissue and that this effect is significantly attenuated in Fn14^{-/-} mice (Figure 7B). Because PARP-1 activation leads to an increase in the synthesis and accumulation of polyADP-ribose (PAR) polymers, which have been associated with cell death (Eliasson et al., 1997), then we studied the accumulation of PAR polymers in the ischemic tissue of Wt and Fn14^{-/-} mice by immunohistochemistry and Western blot analysis 6 and 24 hours after MCAO. We observed that cerebral ischemia induces the accumulation of PAR polymers in each area of interest in the ischemic tissue of Wt mice, and that this effect is significantly decreased in Fn14^{-/-} mice (Figure 7C & D).

4.0. Discussion

TWEAK is a type-2 transmembrane protein that can be cleaved to function as a highly soluble cytokine that binds to Fn14, the smallest member of the TNF receptor (TNFR) superfamily. In the central nervous system (CNS) TWEAK and Fn14 are expressed mainly in endothelial cells, perivascular astrocytes, microglia and neurons (Yepes et al., 2005). Previous work has demonstrated that the interaction between TWEAK and Fn14 in the endothelial cell-astrocyte interface regulates the permeability of the blood-brain barrier (BBB) (Polavarapu et al., 2005; Haile et al., 2010). In contrast, the role of neuronal TWEAK and Fn14 is less well understood. Our work indicates that the interaction between neuronal TWEAK and Fn14 mediates hypoxia/ischemia-induced PARP-1 activation with accumulation of PAR polymers and cell death.

Studies performed in cell culture systems have identified multiple pathways for TWEAK-induced cell death (Nakayama et al., 2002). Indeed, in 1997 Chicheportiche et al (Chicheportiche et al., 1997) reported that TWEAK induces cell death in interferon-γ treated HT-20 colon carcinoma cells. Since then it has been reported that TWEAK also induces cell death via caspase-activation and lysosomal cathepsin-B-mediated necrosis. The interaction between TWEAK and Fn14 induces activation of the NF-κB signaling pathway (Polavarapu et al., 2005). However, the role of NF-κB activation on cell death has been controversial. Indeed, some studies indicate that NF-κB is protective (Yu et al., 1999), and that TWEAK-induces glioma cell survival via NF-κB pathway activation (Tran et al., 2005). In contrast, experimental work in animal models of cerebral ischemia indicates that NF-κB activation enhances ischemic neuronal death (Schneider et al., 1999a). This apparent discrepancy suggests a cell- and injury-type specific effect for the cytotoxic effect of TWEAK. Accordingly, our results indicate that the effect of TWEAK on hypoxic/ischemic neuronal death occurs via PARP-1 mediated NF-κB pathway-activation.

Fn14 is the smallest member of the TNFR superfamily. Indeed, Fn14's cytoplasmic tail is only 28 amino acids in length and lacks a death domain (DD). Several reports indicate that endogenously produced TNF-α mediates the effect on cell death induced by activation of TNFR family members lacking a DD. Accordingly, it has been reported that the interaction between endogenously produced TNF-α and its receptor TNFR1 mediates TWEAK-induced cell death in a rhabdomyosarcoma cell line, and that this effect is seemingly independent of NF-κB pathway activation (Schneider et al., 1999b). Likewise, recent work indicates that treatment with TWEAK sensitizes immortalized but not primary tumor cells to the cytotoxic

effect of TNF- α , and that this effect is associated with TWEAK-induced degradation of the cellular inhibitor of apoptosis 1 (cIAP1) and activation of the NF- κ B pathway (Vince et al., 2008). Our results show that TWEAK-induced neuronal death is mediated by NF- κ B-activation, and that this effect is independent of TNF- α /TNFR1.

Fn14 is the cognate receptor for TWEAK. However, it has been recently reported that TWEAK also interacts with CD163 (Bover et al., 2007). Our observation that TWEAK induces a dose-dependent increase in apoptotic death in Wt but not in Fn14^{-/-} neurons, indicates that TWEAK-induced neuronal death is mediated by interaction with Fn14. Additionally our studies under OGD conditions show that TWEAK and Fn14 mediate hypoxia-induced neuronal death, and that this effect is reversed by treatment of TWEAK^{-/-} but not Fn14^{-/-} cells with recombinant TWEAK.

In earlier studies we demonstrated that MCAO induces an increase in Fn14 mRNA expression in the ischemic tissue 24–48 hours after the onset of the ischemic insult. In contrast, we failed to detect an increase in TWEAK mRNA expression (Yepes et al., 2005). Because the earliest time point after MCAO evaluated in that work was 24 hours we decided to determine the effect of MCAO on TWEAK and Fn14 mRNA expression within the first 24 hours of ischemia. Our results indicate that MCAO induces a rapid increase in TWEAK mRNA expression (30 minutes after MCAO) that peaks at 1 hour and returns to baseline levels 6 hours after MCAO. This explains why in our previous studies we failed to detect a significant change in TWEAK mRNA expression. Likewise, we found that the expression of Fn14 mRNA also increases 30 minutes after MCAO but in contrast with TWEAK mRNA, it peaks 6–24 hours later.

PARP-1 is a highly conserved nuclear enzyme that is essential for the maintenance of genomic integrity. The presence of reactive oxygen species such as peroxynitrate and hydroxyl radicals induces the formation of DNA strand breaks leading to PARP-1 activation and the accumulation of PAR polymers, which are rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG). Neuronal PARP-1 activity increases upon exposure to hypoxia or excitotoxic injury (Koh et al., 2005), and PARP-1 deficiency is associated with resistance to hypoxia and glutamate-induced cell death (Eliasson et al., 1997). Additionally, PARP-1^{-/-} mice have a significant decrease in the volume of the ischemic lesion following experimental MCAO (Eliasson et al., 1997). PARP-1 is the main substrate for caspase-3 and the presence of a PARP-1 89 kDa cleaved fragment has been considered as an indicator of apoptotic death in several cell lines (D'Amours et al., 2001). The mechanism whereby the accumulation of PARP-1 induces cell death is poorly understood. However, it has been postulated that PARP-1 and PARG can determine cellular fate by influencing levels of energetic substrates. Additionally, a link between PARP-1 and NF- κ B pathway activation has been demonstrated (Chiarugi and Moskowitz, 2003). Our results indicate that cerebral ischemia induces an increase in the expression of active caspase-3 in the ischemic tissue, and that this effect is paralleled by PARP-1 cleavage and the accumulation of PAR polymers in the ischemic tissue. Importantly, this effect was attenuated by genetic deficiency of Fn14^{-/-}. Together, these observations suggest an association between TWEAK-induced neuronal death and PARP-1 activation.

In summary, the work presented here indicates that in response to hypoxia/ischemia, the interaction between endogenous TWEAK and Fn14 in neurons induces cell death via NF- κ B pathway activation. This effect is independent of TNF- α /TNFR1, and instead seems to be associated with PARP-1 and caspase-3 cleavage, and accumulation of PAR polymers. This pathway may constitute a novel therapeutic target for hypoxia/cerebral ischemia-induced neuronal death.

Acknowledgments

This work was supported in part by National Institutes of Health Grants NS-062073 and HL-095063 (to M.Y)

6.0. Glossary

TWEAK	Tumor necrosis factor-like weak inducer of apoptosis
Fn14	Fibroblast growth factor-inducible 14
PARP-1	Poly(ADP-ribose)polymerase-1
PAR	Poly(ADP-ribose) polymers

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Figure 1. TWEAK induces cell death via NF- κ B activation

A. Wild-type (Wt) cortical neurons were either left untreated (white bar) or incubated with progressive concentrations of rTWEAK (black bars) followed 24 hours later by determination of cell survival with the MTT assay. * and ** $p < 0.05$ compared to cells treated with TWEAK 0–10 ng/ml. $n = 12$ observation per condition. Lines denote SD. **B.** Mean cell survival determined with the MTT assay in Wt cortical neurons left untreated (white bar) or incubated with TWEAK either alone (black bar) or in combination with BAY 11-7085 10 μ M (dark gray bar). $n = 15$ per condition. * $p < 0.05$ when compared to other experimental groups. Lines denote SD.

Figure 2. Effect of TNF- α on TWEAK-induced cell death

A. Mean cell survival in wild-type (Wt) cortical neurons incubated with TNF- α 10 ng/ml either alone (black bar) or in combination with an anti-mouse TNFR1 neutralizing antibodies 100 μ g/ml (gray bar). n= 12 observations per condition. * p < 0.05 compared to cells treated with a combination of TNF- α and anti-mouse TNFR1 neutralizing antibodies. Lines denote SD. **B.** Mean cell survival in Wt cortical neurons left untreated (white bar) or incubated with TWEAK 300 ng/ml either alone (black bar) or in combination with anti-mouse TNFR1 antibodies 100 μ g/ml (dark gray bar). NS: non significant. Lines denote SD. Each observation was repeated 10 times.

Figure 3. Fn14 mediates TWEAK-induced neuronal death

A. Mean cell survival in $Fn14^{-/-}$ cortical neurons incubated with TWEAK 300 ng/ml (black bar) or TNF- α 10 ng/ml (gray bar). $n=10$ observation per condition. * $p < 0.05$ compared to TWEAK-treated cells. Lines denote SD. **B.** Mean percentage of TUNEL-positive Wt (white bars) and $Fn14^{-/-}$ neurons (black bars) per field following incubation with TWEAK 0–300 ng/ml. $n=6$ observations per condition. Lines denote SD. * $p < 0.05$ compared to Wt-untreated cells and to $Fn14^{-/-}$ neurons treated with TWEAK 0–300 ng/ml. ** $p < 0.05$ compared to Wt cells treated with TWEAK 0–100 ng/ml, and with $Fn14^{-/-}$ neurons treated with TWEAK 0–300 ng/ml. **C.** Representative micrograph of a Wt (panel a) and $Fn14^{-/-}$ neuronal culture (panel b) following incubation with TWEAK 300 ng/ml. Blue is DAPI. Green is TUNEL. Magnification is 20 X. The inset in a represents a 40 X magnification of a Wt culture.

Figure 4. The interaction between TWEAK and Fn14 induces PARP-1 cleavage and accumulation of Poly(ADP-ribose) polymers in vitro and in vivo

A–C. Representative Western blot analysis and quantification of the mean intensity of the band of total PARP-1 (A), cleaved PARP-1 (B) and Poly(ADP-ribose) polymers (PAR) accumulation (C) in wild-type (Wt) neurons incubated with either vehicle (control) or TWEAK 300 ng/ml. * $p < 0.05$ compared to vehicle-treated cells. **D.** Representative Western blot analysis and quantification of the mean intensity of the band of PAR accumulation in the cerebral cortex of Wt mice injected with either vehicle (control) or TWEAK. * $p < 0.05$ compared to vehicle-treated mice. **E & F.** Representative Western blot analysis and quantification of the mean intensity of the band of IKB α phosphorylation (E) and caspase 3 activation (F) in Wt neurons incubated with TWEAK either alone or in combination with the PARP-1 inhibitor BSI-201. * $p < 0.05$ compared to cells co-treated with BSI-201.

Figure 5. Effect of endogenous TWEAK and Fn14 on hypoxia-induced neuronal death
A. Mean fold increase in TWEAK (white bar) and Fn14 (black bar) mRNA expression in Wt cortical neurons 3 hours after exposure to OGD conditions. * $p < 0.05$ compared to controls maintained under normoxic conditions. $n=8$. Lines denote SD. **B.** Mean cell survival in wild-type (Wt), Fn14 deficient (Fn14^{-/-}) and TWEAK deficient (TWEAK^{-/-}) cortical neurons exposed to oxygen-glucose deprivation (OGD) conditions. A sub-group of cells was incubated with TWEAK 300 ng/ml (gray bars). $n= 11$ per condition. * $p < 0.05$ compared to Wt cells maintained under OGD conditions. ** $p < 0.05$ compared to TWEAK^{-/-} cells maintained under OGD conditions in absence of rTWEAK. Lines denote SD.

Figure 6. Cerebral ischemia induces a rapid increase in TWEAK and Fn14 mRNA expression
A & B. Mean fold increase in TWEAK (A) and Fn14 (B) mRNA expression in the ischemic tissue of wild-type mice 0–48 hours after MCAO. n=6. Lines denote SD. * p < 0.05 compared to sham operated controls at each time point.

Figure 7. Effect of Fn14 deficiency on cerebral ischemia-induced cell death

A. Representative Western blot analysis for cleaved-caspase 3 in ischemic brain extracts from Wt and Fn14^{-/-} 6 & 24 hours after MCAO. **B.** Representative Western blot analysis of poly(ADP-ribose) polymerase-1 cleavage in brain extracts from wild-type (Wt) and Fn14-deficient (Fn14^{-/-}) mice 6 & 24 hours after MCAO. **C.** Representative micrograph of poly(ADP-ribose) polymers accumulation in the ischemic tissue (area of interest-1) in Wt (panel a) and Fn14^{-/-} mice (Panel b) 24 hours after MCAO. Blue is DAPI. Green is PAR. **D.** Representative Western blot analysis of PAR accumulation in ischemic brain extracts from Wt and Fn14^{-/-} mice 0–24 hours after MCAO. Lane 1 corresponds to HeLA cells treated with H₂O₂ for 15 minutes.