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IGF-1 and pAKT Signalling Promote Hippocampal CA1 Neuronal Survival Following Injury to Dentate Granule Cells.

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Abstract

Insulin-like growth factor-1 (IGF-1) protects neurons from apoptosis and *in vivo* offers neuroprotective support to hippocampal CA1 pyramidal neurons following ischemia or seizure. IGF-1 signals through IGF-1 receptors activating phosphatidylinositol 3-kinase (PI3K)/Akt or pMAPK pathways. IGF-1 can be induced with injury and microglia and astrocytes may serve as a source of this neurotrophic factor to promote neuronal survival. An acute systemic injection of trimethyltin (TMT; 2 mg/kg, ip) to mice induces apoptosis of dentate granule neurons within 24hrs and a differential response of microglia with ramified microglia present in the CA-1 region. Using this model, we studied the role of IGF-1 in the survival of CA-1 pyramidal neurons under conditions of altered synaptic input due to changes in the dentate gyrus. Within 24hrs of injection, IGF-1 mRNA levels were elevated in the hippocampus and IGF-1 protein detected in both astrocytes and microglia. IGF-1 was redistributed within the CA-1 neurons corresponding with an increase in cytoplasmic pAkt, elevated PKB α /Akt protein levels, and a decrease in the antagonist, Rho. pMAPK was not detected in CA-1 neurons and ERK2 showed a transient decrease followed by a significant increase, suggesting a lack of recruitment of the pMAPK signaling pathway for neuronal survival. In mice deficient for IGF-1, a similar level of apoptosis was observed in dentate granule neurons as compared to wildtype; however, TMT induced a significant level CA-1 neuronal death, further supporting a role for IGF-1 in the survival of CA-1 neurons.

Keywords

microglia; hippocampus; pyramidal neurons; trimethyltin; IGF-1; pAkt; seizure

It has been suggested that microglial responses are tailored in regional and insult-specific manners (Carson et al., 2007). Attenuation of brain inflammatory responses and microglial activation confer neuroprotection in various models of neurodegeneration (Yrjanheikki et al., 1998; Tikka et al., 2001). In contrast, there is a growing line of evidence suggesting an alternative neuroprotective role for microglia in CNS pathologies (Streit, 2002; Neumann et al., 2006; Lalancette-Hebert et al., 2007; Lamberts et al., 2009). We now know that microglia are in intimate contact with neurons for which they serve important maintenance functions, are capable of responding to subtle changes in the microenvironment, and

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dynamically survey the CNS (Kreutzberg, 1996; Davalos et al., 2005; Nimmerjahn et al., 2005; Raivich 2005).

Previous studies demonstrated that the exogenous application of microglia protects against different types of ischemic injury (Kitamura et al., 2004; Imai et al., 2007) and oxygen-glucose deprivation (Neumann et al., 2006). One possible mechanism underlying the neuroprotective actions of microglia is the production and secretion of neurotrophic factors. Proliferating microglia serve as an endogenous pool of neurotrophic and anti-apoptotic molecules. One such example is insulin like growth factor-1 (IGF-1)(Lalancette-Hebert et al., 2007) that can be induced with injury (Choi et al., 2008; O'Donnell et al., 2009).

The neurotrophic properties of IGF-1 have been demonstrated in multiple experimental systems. IGF-1 is a general survival factor for embryonic neurons in primary culture (Cheng and Mattson, 1992; Lindholm et al., 1996; Nakao et al., 1996). It can protect primary neuronal cells against a number of insults including hypoglycemia (Cheng and Mattson, 1992; Russo et al., 2004), osmotic stress (Matthews and Feldman, 1996), corticosterone (Nitta et al., 2004), and growth factor withdrawal (Russell et al., 1998). *In vivo*, IGF-1 offers neuroprotective support to hippocampal CA1 pyramidal neurons following an ischemic injury or acute seizure induction (Johnston et al., 1996; Guan et al., 1993; 1996; Zhu and Auer, 1994). The biological activity of IGF-1 is mediated by the specific high-affinity type 1 IGF receptors. Ligand binding to the receptor activates tyrosine kinase domains, which then phosphorylate various intracellular substrates leading to activation of multiple signaling pathways. Recent work suggests that the survival effect of IGF-1 is primarily due to the activation of the phosphoinositide kinase-3/protein kinase B (PI3K/Akt) pathway in cultured hippocampal (Zheng et al., 2002) and dorsal root ganglia neurons (Leininger et al., 2004). Activation of PI3K stimulates the phosphorylation of the survival kinase, Akt (Alessi et al., 1996; Delcommenne et al., 1998; Balendran et al., 1999). Activated Akt then phosphorylates multiple down stream proteins related to cell survival or cell death, such as the pro-apoptotic protein Bad (del Peso et al., 1997), p21/CIP1, MDM2, GSK3b, and FKHR. Akt is present in multiple cell types and serves as a key kinase involved in the survival promoting effects of these various factors (Dudek et al., 1997; Crowder and Freeman, 2000; Datta et al., 1999; Zheng et al. 2002) in addition to serving as a major effector of receptor tyrosine kinases to modulate cell growth, apoptosis, protein synthesis, and energy metabolism (Castellino and Durden, 2007; Riemenschneider et al., 2006; Jin et al., 2007).

Within the hippocampus, neuronal subpopulations show different vulnerabilities to cell death depending on the stressors (Kadar et al., 1998; Harry and Lefebvre d'Hellencourt, 2003). We have previously examined the dynamics of neuronal death and survival in the hippocampus using a chemical-induced model of targeted dentate granule cell apoptosis (Reuhl and Cramner, 1984; Chang et al., 1982; Bruccoleri et al., 1998; Fiedorowicz et al., 2001; Harry and Lefebvre d'Hellencourt, 2002; McPherson et al., 2003; Harry et al., 2008). An acute administration of the toxicant, trimethyltin, damages dentate granule neurons within 24–48 hrs, while sparing pyramidal neurons. Given the circuitry of the hippocampus, including the trisynaptic circuit of the dentate gyrus, CA3 pyramidal cells, and the CA1 pyramidal cells (Amaral and Witter, 1989), this model allows us to examine processes that contribute to the survival of CA1 neurons under conditions of altered synaptic input or

changes in the dentate gyrus (Johansen et al., 1987). A heterogeneous microglia response occurs within the hippocampus, and in the CA1 region, a pronounced microglia response occurs characterized by hypertrophied process bearing ramified microglia (Harry et al., 2008b). Given that microglia can produce growth factors, including IGF-1 and thus, possibly activate Akt, we examined the expression of pAkt in the CA1 neurons as a possible mechanism of neuronal survival. To further confirm a role for IGF-1, we examined neuropathology in mice deficient for IGF-1 following TMT and demonstrated death of both dentate granule neurons and CA1 pyramidal neurons in the absence of IGF-1.

Material and Methods

Murine model of dentate granule cell damage and heterogeneous microglia response in the hippocampus

To examine survival responses in CA1 neurons, we induced a localized damage to the dentate granule neurons of adolescent male mice (postnatal day 21; PND21) using the well-established trimethyltin (TMT) model. Mice were randomly assigned to experimental groups and administered a single intraperitoneal (ip) injection of either 2 mg/kg trimethyltin hydroxide (TMT, originally obtained from Alpha Products, Danvers MA) or saline (injection vol, 2 ml/kg). Tremor and mild seizure activity (Stage 3) was evident between 18 and 36 hrs (Fig. 1A). No acute morbidity or death was observed. Based upon previous studies characterizing the temporal dynamics of the injury response (Bruccoleri et al., 1998; Harry et al., 2008), mice were examined at 24, 48, and 72 hrs post-TMT. Animals were individually housed in a dual corridor, semi-barrier animal facility at 21° + 2°C with 50% + 5% humidity and 12-hr light/dark cycle. Food (autoclaved NIH 31 rodent chow) and deionized, reverse osmotic-treated water were available *ad libitum*. All procedures were conducted in compliance with NIEHS/NIH Animal Care and Use Committee.

Histopathology

Mice were anesthetized with CO₂ and decapitated. Brains were removed from the cranium, bisected in the midsagittal plane, and one hemisphere immersion-fixed in 4% paraformaldehyde/0.1M phosphate buffer (PB; pH 7.2; RNase/DNase free) overnight at room temperature (RT). Within 24 hr, the brains were rinsed with PB and either dehydrated in ethanol, embedded in paraffin, and 8-mm sections cut and collected on ProbeOn+ slides (Fisher Scientific, Norcross, GA) or cryoprotected for frozen sectioning. Sections were deparaffinized and ethanol rehydrated and stained with hematoxylin and eosin (H&E) for general cellularity. A random sampling of serial sections across mice (n=8) was stained for Fluoro-Jade B (Kanthasamy et al., 1999) or immunostained for active caspase 3 as additional indicators of neuronal insult. Following epitope retrieval, sections were blocked with 10% normal goat serum/1% BSA in PBS for 30 min then incubated with anti-rabbit IgG secondary antibodies. Rabbit polyclonal anti-active caspase 3 (AC3; 1:1000, 18 h, 4°C, #AB3623, Chemicon Intl., Temecula, CA) was visualized with goat anti-rabbit IgG AF 488 (1:1000, Invitrogen, Carlsbad, CA). Neurons were labeled with Neurotrace® blue fluorescent Nissl stain (1:500, 1hr, RT; Invitrogen). From each mouse, 6 sections at multiple planes of cut were randomly selected and the severity of cell death in dentate granule cells and CA1 pyramidal cells was scored by 3 independent observers according to an *a priori*

defined severity scale as previously described (Harry et al., 2008a). Severity scores (0–4) were assigned for dentate granule cell death based upon number and location (progressing from the inner blade to throughout the blade of the dentate) of eosin+ cells and structural characteristics of dense and collapsed chromatin. Scores of 1–2 were indicative of neuronal death occurring along the inner blade of the dentate to within a 3-cell layer width of the blades. A score of 3 indicated cell death throughout the entire blades of the dentate and a score of 4 indicated significant cell loss as indicated by the absence of distinct cellular material thus, a void in the blades of the dentate.

Glial Response

Microglia were identified on the basis of morphological criteria of size and ramification of cells stained by either rabbit polyclonal antibody to ionized calcium-binding adaptor molecule 1 (Iba1; 1:500; 1h, RT; Wako Chemicals, Richmond, VA) detected with IgG Alexafluor 488 (Invitrogen) or binding of isolectin B4 (IB₄) from *Griffonia simplicifolia* (Sigma, St. Louis, MO) as previously reported (Harry et al., 2008a,b) including pre-incubation for 30 min in 1X Automation Buffer (Biomedica Corp, Foster City, CA) containing 0.1 M CaCl₂, MgCl₂, MnCl₂, and 0.1% Triton X-100 and visualized by DAB. Astrocytes were labeled by rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:200, 1 h, RT; Dako Corp, Carpinteria, CA).

Immunohistochemical Staining for pAKT, pMAPK, IGF-1

Eight micron paraffin sections were immunostained as detailed above with either rabbit monoclonal anti-phospho-Akt (Ser473; 736E11) (Cell Signaling Technology, #3787) at a dilution of 1:50 or anti-phospho-p44/42 MAPK (Thr202/Tyr204; 20G11) Cell Signaling Technology, #4376) at 1:1000. The antisera were detected using a Vectastain Elite Rabbit kit (Vector Labs) as previously described and visualized with a DAB or AEC chromagen. 10 µm cryosections were stained with rabbit polyclonal anti-IGF1 (Santa Cruz Biotechnology; H70, #sc-9013) at 1:50 and detected using an anti-rabbit IgG-Alexafluor 594 antibody (Invitrogen). Sections were co-stained with Iba1 as described above, or rabbit anti-GFAP (Dako North America Inc, Carpinteria CA; #Z0334) at 1:250 to label astrocytes. GFAP labeled astrocytes were detected with anti-rabbit IgG-Alexafluor 488. Cell nuclei were labeled and coverslips mounted using Prolong Gold with DAPI (Invitrogen).

Microscopy

Light and fluorescence microscopy was performed with a Leica DMRBE microscope (Wetzlar, Germany) equipped with epifluorescence. Digital images were acquired using a SpotRT™ cooled, charged-couple device (CCD) camera (Diagnostic Instruments, Sterling Heights, MI) under the control of Metamorph™ software (Universal Imaging Co., Downingtown, PA). A region of interest (ROI) of the CA1 was defined and the amount of fluorescent pixels in the total area was determined by image segmentation, exclusive thresholding, and expressed as integrated fluorescence.

qPCR

Reverse transcription was performed with 2.5 µg total RNA isolated with TRIzol® (Gibco BRL, Gaithersburg, MD, using SuperScript™ II Reverse Transcriptase (Invitrogen). qPCR was carried out (Perkin Elmer ABI Prism™ 7700 Sequence Detector) using 2.5 µL cDNA as template, 1X Power SYBR® Green Master Mix (Applied Biosystems; Foster City, CA), and forward and reverse primers for IGF-1: forward: *AGAAGTCCCCGTCCCTATCG*; reverse: *GGTGTGTCTTTGGCTCACCTTT* and GAPDH: forward: *GGGAAGCTGACTGGCATGG*; reverse: *CTTCTTGATGTCATCATACTTGGCAG*. 25 µL reaction mixtures were held at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 1 min at 60°C. Amplification curves from individual qPCR reactions were generated within Sequence Detection System 1.9.1 software program. Threshold cycle values were determined and mean fold changes over the control group were calculated using the comparative C_T method (Livak and Schmittgen, 2001). GAPDH was used for normalization.

Western Blotting

Fresh hippocampi pooled from 8 mice, were sonicated in 300 µL of RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Forty micrograms protein (BCA assay, Pierce, Rockford, IL) was diluted in NuPage® LDS sample buffer containing a reducing agent (Invitrogen), held at 70°C for 10 min, subjected to electrophoresis on 4–12% NuPage® Bis-Tris gels with SeeBlue+2™ and MagicMark™ XP (Invitrogen) standards, and transferred to a 0.2 µm NuPage® PVDF membrane. Membranes were incubated overnight at 4°C with either pAkt (1:1000; Cell Signaling, Akt (1:1000; Cell Signaling, #9272), or pMAPK (1:1000; Cell Signaling, Thr202/Tyr204, 20G11, #4376). Membranes were developed with a WesternBreeze® (Invitrogen) rabbit chemiluminescent immunodetection kit. Digital images of chemiluminescent bands were captured from Kodak Biomax MR film by Image Station 440CF (Eastman Kodak, Rochester, NY) and densitometric values determined (Kodak Molecular Imaging Software; ver. 4.0).

PowerBlot™ Protein Determination

To determine levels of proteins associated with the Akt signaling pathway, data from a commercial protein PowerBlot assay was examined. Protein lysates were prepared from approximately 100 mg of hippocampal tissue 24, 48, and 72 hrs post-TMT and time matched saline controls (n=3). Protein was extracted with lysis buffer at 0.15 g wet weight of tissue/4.1 ml of boiling lysis buffer and 400 µg total protein from each sample was subjected (in triplicate) to gel electrophoresis on a preparative 5–15% gradient SDS-polyacrylamide gel. The gel was transferred to Immobilon-P nylon membrane (Millipore, Bedford, MA), subjected to immunoblotting using a manifold that isolates 45 channels across the membrane, each representing 15 µg of total protein lysate. An antibody cocktail was added to each channel for a 1-hr hybridization, rinsed and incubated for 30 min with secondary goat anti-mouse horseradish peroxidase (PowerBlot, BD, Lexington, KY). Gels were developed in Supersignal West Pico (Pierce, Rockford, IL) and quantitation of each protein band was determined from digital images of the chemiluminescence. Triplicates of each pooled sample were subjected to PowerBlot™ analysis. For each gel, data analysis

included raw and normalized digital data from each blot with changes >1.25 fold. For each analysis: Quantity = total intensity of a defined spot; Normalized Quantity = raw quantity of a spot divided by the total intensity value of all pixels in an image multiplied by 1,000,000; Ratio = normalized quantity for treated spots expressed as a ratio of the normalized quantity for the corresponding control bands to express increases or decreases in protein expression. Protein expression changes are reported in confidence levels (CL; range with 5 as highest CL and 1 as lowest) based on fold change, reproducibility, and signal intensity. Only changes at confidence levels of 5 are reported.

IGF-1 knockout mice

To examine if IGF-1 contributed to the pattern of neuronal damage in the hippocampus, homozygous IGF-I KO mice (Liu et al., 1993) were obtained by breeding heterozygous males with heterozygous females. Genotypes were confirmed by PCR of tail genomic DNA using specific oligonucleotide primers, as reported previously (Richards et al., 2001). IGF-1 homozygous male mice and wildtype controls were injected with TMT as described above and at 24 and 48 hrs post-injection, brain tissue was processed for histological evaluation by H&E staining.

RESULTS

Hippocampal Histopathology

Our lab and others have previously reported the induction of seizures and selective death of dentate granule neurons following TMT. We observed these effects in the current study (Fig. 1A). The severity and localized neuronal death in the dentate granule cell region was confirmed with H&E staining (DG; Fig. 1B,C) and the lack of neuronal death in the CA1 region was confirmed with Fluor-Jade and active caspase 3 (Fig. 2).

Microglia displayed heterogeneity in morphological responses within the hippocampus. The microglia response was consistent with previous reports in that an increase staining was seen for microglia in the dentate gyrus with cells displaying either a ramified process bearing or an amoeboid morphology (Fig. 3) depending on proximity to dying neurons. In the CA1 pyramidal cell layer, ramified process bearing microglia showed hypertrophy and thickening of processes in contact with neurons (Fig. 3).

IGF-1

To determine if IGF-1 was induced in the hippocampus following TMT, mRNA levels were determined by qRT-PCR and protein was evaluated by immunohistochemistry. mRNA levels for IGF-1 were significantly elevated in the hippocampus at 24 hrs post-TMT (Fig. 4A). Within the dentate gyrus, a sparse and diffuse punctate staining for IGF-1 was detected in both the saline control and TMT dosed animals (Fig. 4B). IGF-1 was not observed in amoeboid Iba-1+ microglia in the dentate (Fig. 4B). Within the CA1, process-bearing microglia immunopositive for IGF-1 could be detected in both control and following TMT (Fig. 4B). In the CA-1 pyramidal layer, immunostaining for IGF-1 was evident around neurons in both the saline control and TMT-dosed mice (Fig. 4C). At 48 hrs post-TMT, an increase in the staining pattern was noted with what appeared to be a collapsed staining on

the CA1 neurons (Fig. 4C) suggesting a redistribution of the protein (Fig. 4E). A localization of IGF-1 staining could be detected along GFAP+ processes (Fig 4C, higher magnification insets) and this was evident in both the controls and elevated with TMT exposure. To estimate the level of IGF-1 protein in the CA1, a region of interest (ROI) was defined and the integrated fluorescence intensity determined. No statistically significant difference was observed between the two groups (Fig. 4D). The visual interpretation of a coalesced protein would be consistent with the observation that, given the same area for the ROI, the thresholded area was decreased indicating that the area within the ROI containing pixel intensities within the thresholded range was decreased (Fig. 4E). When the integrated fluorescence was examined relative to the thresholded area there was a slight 20% increase following TMT; however, it failed to reach statistical significance (Fig. 4F).

pAKT

The IGF-1 receptor tyrosine kinase is a robust activator of Akt signaling in hippocampal neurons. Cytoplasmic activation of PI3 kinase by IGF-1 promotes Akt phosphorylation and enhances its nuclear translocation (Borgatti et al., 2000). To determine a change in the level of pAkt within the hippocampus following TMT, samples were subjected to western blot analysis. The level of total Akt showed no significant alteration with dosing; however, Akt phosphorylated at Ser473 was significantly elevated at both 24 and 48 hrs post-TMT (Fig 5A). Cyclophilin A was not altered with dosing at any of the time points examined. Activation of Akt in the cytoplasm promotes nuclear pAkt translocation critical for its antiapoptotic effects (Jackson et al., 2009). To identify the cellular localization of the protein, immunostaining was conducted. Minimal and faint staining could be detected in neurons in the control tissue however, at 48 hrs post-TMT, pAkt immunostaining was clearly present in CA1 neurons (Fig. 5B, arrow). This was characterized by a cytosolic distribution of the red AEC reaction product. To further examine changes in protein levels associated with the Akt signaling pathway, we conducted a commercial protein power blot analysis. A significant elevation was seen in the PKB alpha/Akt antibody target at 24 hrs as compared to control hippocampus. In addition, the antagonist of Akt, Rho, was significantly decreased between 24 and 72 hrs (Fig. 5C).

p42-MAPK

Since p42-MAPK can also serve as an IGF-1 receptor mediated signaling pathway for cell survival, we examined changes as a function of TMT. Western blot analysis of the hippocampus showed a transient increase in the phosphorylated MAPK at 24 and 48 hrs (Fig. 5D). While this increase in protein was detected in the hippocampus, immunohistochemical staining for pMAPK demonstrated minimal staining in the CA1 suggesting that activation of this pathway was not prominent in the CA1 pyramidal neurons (Fig. 5E). Using the PowerBlot analysis, the antibody target for ERK2 was decreased at 24 hrs followed by a significant increase at both 48 and 72 hrs (Fig. 5F).

Response in IGF-1 knockout mice

We then examined the response to TMT in IGF-1 deficient mice. At 24 hrs post-TMT, cell death was localized to the dentate granule neurons in both the IGF-1 knockout and wildtype mice and no cell death was observed in the CA1 (data not shown). At 48 hrs post-TMT,

wildtype mice continued to show no evidence of CA1 neuronal death (Fig. 7A). In the IGF-1 knockout mice, a significant level of CA1 neuronal death was observed as indicated by eosin + staining in collapsed and condensed neurons (Fig. 7B).

DISCUSSION

Over the years, TMT has been used to produce a model of hippocampal damage and has provided a significant amount of information regarding mechanisms of neuronal death and glial responses. We have now employed this model to address questions regarding mechanisms of neuronal survival. While IGF-1 has long been identified as a neurotrophic factor promoting neuronal survival, we now demonstrate a significant role for IGF-1 signaling via the Akt pathway in a differential resistance to injury in CA1 pyramidal neurons. CA1 pyramidal neurons are highly vulnerable to ischemic insults and, for that reason, have been considered as one of the more vulnerable neurons of the brain. However, in the TMT model of hippocampal injury, the CA1 pyramidal neurons are spared. This occurs with the death of the dentate granule neurons and the subsequent activation of the neural pathways between the dentate and the pyramidal cell layers. Thus, one can consider that the survival of these neurons is an active rather than passive process. An active process is supported by our previous work demonstrating unique molecular changes occurring in the CA1 region within the early stages of the TMT insult (Lefebvre d'Hellencourt and Harry, 2005), one of which was an elevation in mRNA levels for ATF3 which can prevent neuronal death via Akt activation (Nakagomi et al., 2003). Survival of CA1 neurons occurred in the presence of a high level of inflammation as indicated by elevated mRNA levels for both TNF α and IL-1 β (Lefebvre d'Hellencourt and Harry, 2005; Harry et al., 2008b). We previously demonstrated that microglia within both the DG and the CA1 region were the primary source for both TNF α and IL-1 following TMT (Brucoleri et al., 1998; Brucoleri and Harry, 2000). In these studies, the elevations in TNF α mRNA levels were consistent with the pattern of microglia response and the associated neuronal response. We reported that signaling via internalization of the TNF receptors contributed to the apoptotic death of the dentate granule neurons (Harry et al., 2008b). In the CA1 pyramidal neurons, while elevated, neither TNF receptor displayed internalization (Harry et al., 2008b). The lack of internalization of the death domain on the TNFp55 receptor (TNFR1) could possibly allowed for the TNF released by microglia to be utilized as a survival factor. It was also considered that this occurred in the presence of additional survival processes to minimize damage to the CA1 neurons. We now suggest that IGF-1 signaling via the Akt pathway serves as one such additional survival process.

IGF-1 and constitutively active PI3K suppress both TNF α induced apoptosis and ceramide generation. IGF-I protects isolated pancreas islet cells against TNF α mediated cell death via the PI3K/AKT pathway (Aikin et al., 2000). It has been proposed that the mechanism by which TNF α induces neural degeneration may also be mediated by suppression of IGF signaling and activation of PI3K (Venters et al., 2000). Alternatively, it has been proposed that IGF suppresses apoptosis by modulating the ability of TNF α to activate JNK (Nicholas et al., 2002). Inhibition of the c-Jun pathway completely reversed the ability of IGF-1 to rescue TNF α toxicity (Kenchappa et al., 2004). The work of Butts et al. (1999) suggest that IGF-1 can prolong the time of commitment to irreversible cell death and enhance the

recovery of neurons subjected to an acute apoptotic stimulus. The antiapoptotic function of IGF-1 in HT29-D4 cells is based on the enhancement of the survival pathways initiated by TNF α and mediated by p38, ERK1/2, and NF κ B, which act in concert to suppress the proapoptotic signals. These effects of IGF-1 would be consistent with the interpretation of our previous study examining the role of TNF receptor signaling in hippocampal injury (Harry et al., 2008b). IGF-1 can also block extrinsic death induced by FasL in bovine granulose cells (Quirk et al., 2000). While we previously reported that the Fas/FasL apoptotic pathway is not involved in the TMT-induced dentate neuronal injury there was an upregulation of the protein in CA1 neurons (Harry et al., 2008a). Thus, the altered staining pattern of IGF-1 in CA1 neurons may represent actions to block the Fas/FasL apoptotic pathway. In addition to the effects of IGF-1 in modulating the TNF receptor and Fas/FasL extrinsic apoptotic signaling pathways, IGF-1 offers protection against apoptosis induced by agents that cause oxidative stress. IGF-1 inhibition of neuronal apoptosis in primary hippocampal neurons is associated with decreased caspase-3 activation induced by NO donors, sodium nitroprusside, or 3- morpholin-oxynonimine. Overexpression of activated Akt significantly inhibited NO-induced cell death and expression of the kinase-defective form of Akt blocked the effects of IGF-1 (Yamaguchi et al., 2001). While an induction of iNOS has not been reported *in vivo* following TMT (Bruccoleri et al., 1998; Harry et al., 2000), apoptosis in a hippocampal cell line has been associated with an upregulation of iNOS (Zhang et al., 2006) suggesting that localized subtle levels of induction may occur for which IGF-1 and Akt could provide protection.

The serine-threonine kinase Akt exerts its anti-apoptotic effects through several downstream targets, including the pro-apoptotic Bcl-2 family member Bad, forkhead transcription factors, and cyclic AMP response element-binding protein (CREB). Akt inhibits a conformational change in the pro-apoptotic Bax protein and its translocation to mitochondria (Yamaguchi and Wang, 2001). A role for Akt mediated phosphorylation and inhibition of GSK3 α /beta and the forkhead transcription factor, FKHRL1, has been implicated in protection from oleic acid-induced apoptosis. In hippocampal neurons, activation of IGF-1 receptor activated PI3K/AKT and intracerebral injection of IGF-1 prior to ischemic insult delayed neuronal death. PI3K/Akt signaling plays a role not only in IGF-1 protection but also in protection seen with brain derived neurotrophic factor (Yamada et al., 2001) and fibroblast growth factor (Choi et al., 2008). In our previous work, the earliest changes identified in the CA1 neurons were associated with calcium signaling. In neurons, L channel mediated calcium influx can be enhanced by IGF-1 through a PI3K/Akt pathway (Blair et al., 1997; 1999; Bence-Hanulec et al., 2000). Thus, it is possible that one subsequent effect of IGF-1/pAKT signaling could be to regulate calcium-mediated responses in the CA1 neurons.

Song et al. (2003) reported that physiological levels of IGF-I function through a PI3K-dependent pathway to block several TGF β mediated responses, including gene transcription, apoptosis, and Smad3 activation. In previous work we identified by *in situ* hybridization that, with the TMT-induced damage to the dentate granule neurons, CA1 pyramidal neurons upregulated the mRNA transcript for transforming growth factor- β (TGF β). At that time we interpreted this as a neuroprotective response based upon comparison to other studies of neuronal deafferentation lesions (Lefebvre et al., 1992; Delree et al., 1993; Rogister et al.,

1993). However, an alternative interpretation is that the upregulation of IGF-1 serves to block the adverse effects of TGF β .

The juxtaposition of process bearing microglia that can serve as a pool of IGF-1 with IGF receptor enriched CA1 pyramidal neurons (Bondy et al., 1992) suggests a coordinated effort in neuronal survival. One structurally distinct difference between the hippocampal regions was the presence of reactive and activated phagocytic microglia in the DG; while, within the CA1 region, the microglia response was characterized morphologically by a ramified process bearing phenotype. Activated microglia are a source for chemokines such as the C-C chemokine, MIP-1 α , that play a significant role in nervous system inflammation and recruitment of monocytes to a site of injury (Otto et al., 2001; Rezaie et al., 2002; Ambrosini and Aloisi, 2004; Kielian, 2004). Thus, the localized amoeboid microglia contact with dentate neurons may serve to present the neuron as a target for additional extraneuronal signals and increase the likelihood of death. While, in the CA1 region, the process bearing microglia are not expressing such signals (Lefebvre d'Hellencourt and Harry, 2005) and may instead provide factors serving in a neurotrophic role for the pyramidal neurons. We propose that one such factor is IGF-1. In gerbils, IGF-1 is induced in microglia and astrocytes in the CA1 region following ischemia/reperfusion and has been proposed to serve in a paracrine/autocrine manner for the proliferation of glia for both clearing of cellular debris and the secretion of trophic factors for neuronal protection (Hwang et al., 2004). This work also showed a transient and early increase in IGF-1 in the dentate gyrus while the increase in the CA1 region occurred later with the death of pyramidal neurons. Our staining pattern for IGF-1 is similar to this work in that we observed staining along GFAP+ astrocyte processes and within process-bearing but not amoeboid microglia. The data regarding IGF-1 in process bearing microglia within the CA-1 was suggestive but given the small cell size confirmation of co-localization of the signal was difficult. Given that IGF-1 is a secreted protein, confirmation of the role of microglia in its production will require additional studies including *in situ* hybridization to determine source of the protein. However, overall the data leads us to propose that the upregulation of IGF-1 plays a substantial role in the survival of the CA1 during the death of dentate granule neurons and that the IGF-1 expression may be a component of the heterogeneity of the microglia response.

Recent studies have unveiled a growing list of Akt substrates (Nicholson and Anderson, 2002) that cooperate to prevent apoptosis and/or promote cell proliferation (Plas and Thompson, 2005; Xu et al, 2004). Thus, the activation of Akt in CA1 pyramidal neurons seen with damage to the dentate granule neurons likely represents a major endogenous mechanism for the survival of these normally vulnerable neurons. Recent work by Jackson et al. (2009) demonstrated that activated Akt was significantly reduced in the CA1 region as compared with the CA3 region. This work suggested that differential vulnerability of CA1 neurons to stressors such as ischemia/hypoxia was related to a decrease in pAkt signaling. Our data however, suggests that this may not serve as a common mechanism of vulnerability across multiple stressors. The hypoxia-inducible transcription factor-1 α (HIF-1 α) is a major downstream effector of Akt (Zhang et al., 2003; Li et al., 2008) and as HIF-1 α is also induced with ischemic injury, this may serve as one mechanism for CA1 neuronal vulnerability. Results of the current study, however, suggest that CA1 neurons can efficiently utilize the pAkt signaling pathway as a mechanism for survival against non-ischemic insults.

A distinction between the source of the insult or injury becomes important in that CA1 neurons are involved in the pathology of Alzheimer's disease (West et al., 2004) and in a mouse model of tauopathy (Spires et al., 2006) in the absence of ischemia. Thus, understanding the endogenous mechanisms that are utilized by CA1 neurons to survive within an injured environment can lead to therapeutic intervention strategies to minimize cell loss. While a number of factors can stimulate the Akt pathway, the induction of IGF-1 within the hippocampus and the damage to CA1 neurons observed in the IGF-1 knockout mice suggest a direct survival role for this growth factor. This may be due to the availability of IGF-1 from the astrocytes and resident microglia within the CA1 region thus suggesting a neuroprotective role for the localized glial response.

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Figure 1.

A. Seizure score for mice following an intraperitoneal (ip) injection of trimethyltin hydroxide (2 mg TMT /kg bwt) as compared to controls. The seizure score of 3 represents straub tail and tremors. B. Severity scores (scale 0–4) were assigned based upon number and location of eosin⁺ cells within the blades of the dentate (as described in methods and in Harry et al., 2008a). C. Representative histology of the superpyramidal blade of the dentate gyrus (DG) in saline control or 48 hrs. Neuronal death (arrows), characterized by nuclear pyknosis and karyolysis as stained by eosin (H&E). * indicates significantly different from control group (Student's t-test; N=10; $p < 0.05$).

Figure 2.

Representative histology of the dentate granule cell layer and the CA1 pyramidal cell layer in mice at 48 hrs following an injection of trimethyltin hydroxide (2 mg TMT/kg bwt). Fluoro-Jade, and Active Caspase 3 (Act. Caspase 3 - red; DAPI - blue) staining showed distinct evidence of neuronal death in the dentate with no evidence of neuronal death occurring in the CA1 cell layer. [] represent the neuronal region. Scale bar = 25 microns.

Figure 3. Representative image of ramified process-bearing microglia (*Griffonia simplicifolia* isolectin B4 (IB₄) visualized by DAB) within the CA1 region and amoeboid microglia within the dentate granule cell layer 48 hrs post-TMT as compared to the small process bearing microglia seen in controls. Scale bar = 20 microns.

Figure 4.

A. Quantitative real-time PCR (qRT-PCR) for IGF-1 in the hippocampus of controls and at 48 hrs post-TMT (2.0 mg/kg, ip.). Data are presented as fold change (mean $\pm$ SEM) *Significant difference between saline controls (n=5) and TMT dosed (n=5) mice (One-way ANOVA, $p < 0.05$). B. Representative immunofluorescent images of IGF-1 (red), DAPI (BLUE), Iba-1 for microglia (green) in the dentate gyrus and CA-1 pyramidal layer of mice injected with either saline or TMT. Scale bar = 10 microns. Arrow points to +IGF-1 staining. C. Representative immunofluorescent images of IGF-1 (red), DAPI (BLUE), GFAP (green) in the CA-1 pyramidal layer of mice injected with either saline control or TMT. Scale bar = 10 microns. Higher magnification demonstrates localization of IGF-1 along GFAP+ astrocyte processes in controls and increased at 48 hrs following an injection of TMT. Additional higher magnification of IGF-1 demonstrated the condensation of IGF-1+ on neurons cells in the CA-1 pyramidal layer. Scale bar = 10 microns, D-F. Inclusive thresholding was conducted on monochromatic red image and (D) integrated fluorescence intensity, (E) thresholded area, and (F) integrated intensity/thresholded area. E. A slight decrease in the area of the region with pixels that have intensities falling within the thresholding range.

Figure 5.

A. Representative western blot of 40µg total protein of hippocampal samples (saline control or 2.0 mg/kg TMT at 24, 48, and 72 hr). pAkt (ser 473; 1:1000; Cell Signaling) detected bands at approximately 62 kDa and approximately 56 kDa. A band was detected at approximately 50 kDa. Analysis indicated a 2-fold increase in pAkt. Total Akt antibody (1:1000, Cell Signaling) detected a band at approximately 60 kDa. Anti-cyclophilin A (18 kDa) served as an internal control and was not found to change as a function of TMT. Mark12 = molecular wt standard. B. Representative image of pAkt immunostaining (AEC) within the CA1 pyramidal neurons of control and TMT-injected mice (48 hrs; 2.0 mg/kg TMT, ip). In the control, a sparse, faint immunopositive staining could be detected within neuronal cell bodies. In the TMT dosed mouse, DAB immunoreactive product indicated prominent neuronal staining in CA1 pyramidal neurons (arrow). Scale bar = 20 microns. C. Representative changes in protein levels for PKB alpha/Akt and Rho as determined using a commercial PowerBlot Protein Gel. (Gray – increased; Black – decreased) >2-fold change in triplicate for each of 3 samples meeting high quality signals (see Methods). D. Representative western blot of 40µg total protein of hippocampal samples (saline control or 2.0 mg/kg TMT at 24, 48, and 72 hr). p42/44 MAPK (detected bands at approximately 44 kDa and approximately 40 kDa). Analysis indicated a >2-fold increase in at 24 hrs and 2-fold increase at 48 hr. Anti-cyclophilin A (18 kDa) served as an internal control and was not found to change as a function of TMT. Mark12 = molecular wt standard. E.

Representative image of pMAPK immunostaining (DAB) within the CA1 pyramidal neurons of control and TMT- injected mice (48 hrs; 2.0 mg/kg TMT, ip). In the control brain minimal immunopositive staining was evident in thin fibrous processes (arrow). In the TMT dosed mouse, DAB immunoreactive product was limited to sparse punctate staining within CA1 pyramidal neurons. Scale bar = 25 microns. F. Representative changes in protein levels for p42-MAPK as determined using a commercial PowerBlot Protein Gel. (Gray – increased; Black – decreased) >2-fold change in triplicate for each of 3 samples meeting high quality signals (see Methods).

Figure 6.

Representative hematoxylin and eosin (H&E) staining in the CA1 pyramidal cell layer in (A) wildtype (WT) and (B) IGF-1^{-/-} mice at 72 hrs post TMT injection (2.0 mg/kg, ip.). No eosin positive collapsed neurons are observed in the WT brain while (B) a significant level of CA-1 neuronal injury occurs in the IGF-1^{-/-} mice (arrow). No differences were observed between the groups 24 hrs following TMT (data not shown). Scale bar = 25μm.