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Tumor necrosis factor alpha stimulates NMDA receptor activity in mouse cortical neurons resulting in ERK-dependent death

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Abstract

Multiple cytokines are secreted in the brain during pro-inflammatory conditions and likely affect neuron survival. Previously, we demonstrated that glutamate and tumor necrosis factor alpha (TNF α) kill neurons via activation of the *N*-methyl-D-aspartate (NMDA) and TNF α receptors, respectively. This report continues characterizing the signaling cross-talk pathway initiated during this inflammation-related mechanism of death. Stimulation of mouse cortical neuron cultures with TNF α results in a transient increase in NMDA receptor-dependent calcium influx that is additive with NMDA stimulation and inhibited by pre-treatment with the NMDA receptor antagonist, DL-2-amino-5-phosphonovaleric acid, or the α -amino-3-hydroxy-5-methylisoxazole-4-pro-pionate/kainate receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione. Pre-treatment with N-type calcium channel antagonist, ω -conotoxin, or the voltage-gated sodium channel antagonist, tetrodotoxin, also prevents the TNF α -stimulated calcium influx. Combined TNF α and NMDA stimulation results in a transient increase in activity of extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs). Specific inhibition of ERKs but not JNKs is protective against TNF α and NMDA-dependent death. Death is mediated via the low-affinity TNF α receptor, TNFRII, as agonist antibodies for TNFRII but not TNFRI stimulate NMDA receptor-dependent calcium influx and death. These data demonstrate how microglial pro-inflammatory secretions including TNF α can acutely facilitate glutamate-dependent neuron death.

Keywords

mitogen-activated protein kinase; neuron death; *N*-methyl-D-aspartate; tumor necrosis factor alpha

Tumor necrosis factor alpha (TNF α) is a pleotropic cytokine capable of stimulating a variety of cell types with a diverse set of consequences (Locksley *et al.* 2001; Watts 2005). Its effects on target cells occur via binding to two receptors, CD120a (p55, TNFRI) and CD120b (p75, TNFRII) (Hehlhans and Mannel 2002). Both neurons and glia have been reported to express these receptors (Kinouchi *et al.* 1991; Dopp *et al.* 1997). The cytoplasmic domain of TNFRI contains a 'death domain' (DD) sequence of 60–80 amino acids responsible for recruiting the adaptor protein TRADD, capable of transducing diverse signaling pathways including activation of nuclear factor κ B (NF κ B) or caspases (Bradley and Pober 2001; MacEwan 2002). Although TNFRII lacks the DD sequence, it contains a

region of 78 amino acids responsible for recruiting the adaptor protein, TRAF2, allowing subsequent activation of NF κ B as well (Rothe *et al.* 1995). A conflicting body of literature has demonstrated that neuronal stimulation via either receptor mediates protection or death (Barger *et al.* 1995; Li *et al.* 2004).

In several experimental systems, TNF α stimulation mediates toxicity in the context of a pro-inflammatory milieu, suggesting that its effects are superimposed upon responses initiated by additional stimuli (Gelbard *et al.* 1993; Chao and Hu 1994; Floden *et al.* 2005). Expression and secretion of TNF α , particularly by microglia, has been observed in neurotoxic paradigms and implicated in mechanisms of neuron loss (Chao *et al.* 1995; Combs *et al.* 2001). For example, elevated central nervous system TNF α levels have been reported from multiple sclerosis, Alzheimer's disease (AD), stroke/ischemic, traumatically injured, and epileptic brains (Tchelingerian *et al.* 1993; Akiyama *et al.* 2000; Gimsa *et al.* 2000; Yin *et al.* 2003; Ravizza *et al.* 2005).

N-methyl-D-aspartate (NMDA) receptor activity-dependent death is one such response likely modulated by TNF α stimulation. NMDA receptor activity is regulated by a variety of post-translational modifications, including nitrosylation and phosphorylation (Wang and Salter 1994; Choi *et al.* 2000), which allows varying neuronal stimuli to modulate NMDA receptor-dependent calcium influx (Rostas *et al.* 1996; Yu *et al.* 1997). Increased NMDA receptor activity can facilitate the well-characterized excitotoxic death mechanism within neurons. Although this response is typically dependent upon elevated intracellular calcium levels, excitotoxic death can also require activation of members of the mitogen-activated protein (MAP) kinase family (Sato *et al.* 2000; Hughes *et al.* 2003).

Our previous work demonstrated that microglia stimulated with β -amyloid peptide secretes TNF α and glutamate to kill mouse cortical neuron cultures over a 72-h time course. Cell death was dependent upon coincident stimulation of TNF α and NMDA receptors and subsequent activation of neuronal inducible nitric oxide synthase (iNOS) (Floden *et al.* 2005). Based upon prior studies, we hypothesized that this death mechanism involved a specific cross-talk response allowing TNF α receptor stimulation to modulate NMDA receptor-dependent calcium influx and activation of MAP kinases. To determine whether a TNF α and NMDA receptor-dependent signaling cross-talk event was responsible for the death of our cultures, we have employed our same primary mouse neuron culture system treated with recombinant TNF α and NMDA and quantitated effects on NMDA receptor-dependent calcium influx, intracellular signaling responses and resultant death.

Our findings demonstrate a mechanism by which NMDA receptor activation leads to extracellular signal-regulated kinase (ERK)-dependent neuronal death in the presence of the appropriate cytokine containing environment and provide insight into a neuron loss mechanism relevant to AD and other inflammatory neurodegenerative conditions.

Materials and methods

Materials

The anti-phosphoERK antibody (pTyr-204), anti-ERK2 antibody, and affinity-purified horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For immunostaining, polyclonal anti-phosphoERK was acquired from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-c-N-terminal kinase (JNK) and anti-JNK antibodies were obtained from cell signaling. The specific JNK inhibitor (Bonny *et al.* 2001), JNK peptide inhibitor 1, β -stereoisomer (β -JNKI1) and the specific mitogen-activated protein kinase kinase (MEK) inhibitor (Favata *et al.* 1998), 1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene (UO126) were

both purchased from Alexis Biochemicals (Carlsbad, CA, USA). TNF α (cat. # 410-MT), TNFRI agonist antibody (Pollock *et al.* 2002; Soond *et al.* 2003) (cat. # AF-425-PB), brain-derived neurotrophic factor (BDNF), and interleukin-1 β (IL-1 β) were purchased from R&D Systems (Minneapolis, MN, USA). TNFRII agonist antibody (rat monoclonal HM102) (cat. # ab7369) was purchased from Novus Biologicals Inc. (Littleton, CO, USA). According to manufacturer specifications, the agonistic properties of the antibody were tested in a proliferation assay with mouse thymocytes (thymidine uptake was measured), which showed that 3 μ g/mL (HM102) leads to cell activation (0.3 and 1 μ g/mL did not lead to cell activation). Normal rat IgG (negative control for TNFRII agonist antibody) (cat. # sc-2026) was purchased from Santa Cruz Biotechnology. TNF α was resuspended in sterile phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for use. In some cases, TNF α and TNFRI and TNFRII agonist antibodies were dialyzed to remove any manufacturer contaminants before stimulation. To dialyze TNFRI agonist antibody, TNFRII agonist antibody, and TNF α , we used disposable Slide-A-Lyzer MINI Dialysis Units (3.5 K MWCO) from Pierce Biotechnology Inc. (Rockford, IL, USA) according to the manufacturer instructions in PBS containing 1% BSA at 4°C. The 6,7-dinitroquinoxaline-2,3-dione (DNQX) (cat. # 0189) was purchased from TOCRIS Bioscience (Ellisville, MO, USA). The ω -conotoxin GVIA (cat. # CA-235) was purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA). The DL-2-amino-5-phosphonovaleric acid (APV), tetrodotoxin, NMDA, glutamate, and all other materials not specified were purchased from Sigma (St Louis, MO, USA).

Tissue culture

Neurons were cultured from cortices of E16 mice (C57B1/6J). Briefly, meninges-free cortices were isolated, trypsinized and plated onto poly-L-lysine-coated (0.05 mg/mL) tissue culture wells (260 cell/mm²) for 7 days *in vitro* before use. Neurons were grown in glutamine containing Neurobasal media with B27 supplements (Life Technologies, Rockville, MD, USA) to consistently provide neuronal cultures greater than 95% pure and able to survive for at least 1 month *in vitro*. Culture purity is routinely evaluated by cell counting after immunostaining, to identify the neuronal cytoskeletal protein, microtubule-associated protein 2 (MAP2).

Cell stimulation

Neurons were stimulated for varying times with or without kinase inhibitors (UO126, D-JNKI1), tetrodotoxin, ω -conotoxin, APV, DNQX, IL-1 β , TNF α , BDNF, NMDA, TNFRI agonist antibody, TNFRII agonist antibody, or glutamate. For western blot analysis, cells were lysed as below after specified times. To perform immunohistochemical analysis, cells were fixed as described below at defined times.

Neuron viability

To assess neuronal survival, neurons were fixed following 72 h stimulation, stained using a neuron-specific anti-MAP2 antibody, and a counting grid was placed under the wells to count neuron numbers from four identical fields for each condition. MAP2-positive cells with visible nuclei and immunostained processes, at least one cell diameter in length, are counted as neurons. The average number of neurons ($\pm$ SEM) was calculated for each condition. Each experiment was performed in quadruplicate three to four times. In addition, neuronal viability was determined by measuring the cellular release of lactate dehydrogenase (LDH) from culture media of neurons (eight wells/condition) stimulated for 72 h using a commercial non-radioactive assay (Promega, Madison, WI, USA). Absorbance measurements were taken at 490 nm. Neuron viability was also determined by repetitive counting of phase bright neurons. Cells from the same field (20 $\times$ magnification), each from

six sister wells per condition were repetitively counted at 24, 48, and 72 h and averaged ($\pm$ SD).

Western blotting

Neurons were stimulated as described above, then lysed in ice-cold RIPA buffer [1% Triton, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L Na_3VO_4 , 1 mmol/L EDTA, 1 mmol/L EGTA, and 0.2 mol/L phenylmethylsulfonyl fluoride] and insoluble material was removed by centrifugation (14 000 *g*, 10 min, 4°C). Protein concentrations were quantitated by Bradford method (Bradford 1976). Proteins were resolved by 7.5% or 10% SDS – PAGE and incubated in primary antibody overnight, 4°C, followed by incubation in 2° horseradish peroxidase-conjugated antibody. Antibody binding was detected via enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Immunohistochemistry

To perform immunocytochemistry, neurons were stimulated as described above and fixed in 4% paraformaldehyde, 37°C, 30 min at selected times. Fluorescent immunodetection of active ERK was performed using anti-phosphoERK antibody. Antibody binding was visualized using Texas Red conjugated anti-rabbit secondary antibody (Santa Cruz). To visualize the nucleus, 4',6'-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA) was used. Images were captured using a Zeiss (Zeiss, Thornwood, NY, USA) LSM 510 Meta confocal microscope.

Measurement of intracellular Ca^{2+}

Primary cortical neurons were grown on cover slips (MatTek Cultureware, Ashland, MA, USA) and incubated with 2 $\mu\text{mol/L}$ Fura-2 (Molecular Probes, Eugene, OR, USA) for 45 min at 37°C (5% CO_2 –95% air). The cells were washed twice with calcium-containing buffer (10 mmol/L HEPES, 120 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L MgCl_2 , 1 mmol/L CaCl_2 , 10 mmol/L glucose, pH 7.4). Cells were then pre-treated in the calcium-containing buffer with and without drugs (30 min) and bath stimulated in the same buffer, once per culture, to assess changes in intracellular calcium levels. To measure stimulated fluorescence changes, fluorescence intensity of Fura-2-loaded cells was monitored with a CCD camera-based imaging system (Compix Inc., Cranberry, PA, USA) mounted on an Olympus XL70 inverted microscope equipped with an Olympus 40 $\times$ (1.3 NA) fluor objective. A monochromator dual wavelength enabled alternative excitation at 340 and 380 nm, whereas the emission fluorescence was monitored at 510 nm with an Okra Imaging camera (Hamamatsu Photonics, Hamamatsu, Japan). The images of multiple cells collected at each excitation wavelength were processed using the C imaging, PCI software (Compix) to provide ratios of Fura-2 fluorescence from excitation at 340 nm to that from excitation at 380 (F_{340}/F_{380}). Analog plots of the fluorescence ratio (340/380) in single cells are shown.

Statistical analysis

Experiments were performed in quadruplicate at least three times unless noted. Mean values ($\pm$ SEM) for each experiment were determined and values statistically different from controls were calculated using one-way ANOVA. The Tukey–Kramer multiple comparisons post-test was used to determine *p*-values.

Animal care and use

All procedures were reviewed and approved by the University of North Dakota Institutional Animal Care and Use Committee. Mice were housed at the Center for Biomedical Research on a 12 h light/dark cycle and were allowed food and water *ad libitum*.

Results

TNF α stimulation potentiates NMDA receptor activity and ERK-dependent neuron death

Our prior studies demonstrated that TNF α stimulation of mouse cortical neuron cultures was sufficient to induce death in the presence of non-toxic concentrations of NMDA or glutamate over a delayed time course of 72 h (Floden *et al.* 2005). To determine the nature of the signaling cross-talk response, we first assessed whether TNF α stimulation led to changes in NMDA receptor-mediated calcium influx (Fig. 1). Stimulation with TNF α alone resulted in a rapid increase in calcium influx that was inhibited via pre-treatment with the NMDA receptor antagonist, APV (Figs 1b and c). We next pre-treated neurons with an α -amino-3-hydroxy-5-methylis-oxazole-4-propionate (AMPA)/kainate receptor antagonist, DNQX (Kendrick *et al.* 1996), to determine whether the TNF α -dependent calcium influx occurred via specific modulation of only NMDA receptor activity. DNQX pre-treatment also inhibited the ability of TNF α to stimulate changes in calcium influx, suggesting that TNF α was not affected via direct modulation of NMDA receptor activity (Fig. 1c). To assess whether basal electrical activity was required for the TNF α -mediated change in NMDA receptor activity, we first pre-treated neurons with the voltage-gated sodium channel antagonist, tetrodotoxin, prior to stimulation with TNF α . Tetrodotoxin pre-treatment abolished the ability of TNF α to stimulate changes in NMDA receptor-mediated calcium influx, suggesting that neuronal electrical activity is required for TNF α effects (Fig. 1d). To further establish whether synaptic transmitter release was needed to evoke the change in calcium influx, we pre-treated the neuronal cultures with the N-type calcium channel blocker, ω -conotoxin GVIA (Horne and Kemp 1991). As predicted, ω -conotoxin prevented the TNF α -mediated calcium influx, suggesting that synaptic glutamate release was required (Fig. 1d). Interestingly, combined TNF α and NMDA stimulation did not lead to a significant increase in peak calcium influx values but rather a sustained increase in calcium influx compared with NMDA stimulation alone (Fig. 1e). Collectively, our results suggest that TNF α modulates synaptic release of glutamate to mediate an NMDA receptor-dependent calcium influx that is dependent upon initial activity of the AMPA/kainate receptors.

Based upon our observations of TNF α -mediated changes in calcium influx, we hypothesized that the cytokine would play a role in NMDA receptor-dependent toxicity. Although our cultured mouse cortical neurons at 7 days *in vitro* are relatively resistant to NMDA-dependent toxicity, our prior work has demonstrated that TNF α induces robust death of these cells in combination with NMDA receptor stimulation (Floden *et al.* 2005). To assess the role of TNF α stimulation in NMDA receptor-dependent excitotoxic death, we performed a time course survival analysis of neurons stimulated with or without TNF α and NMDA as previously described (Floden *et al.* 2005). Cells were stimulated for increasing times (0, 24, 48, and 72 h) and evaluated for cell survival. Counting live cells with morphologic characteristics of neurons from the same field showed that neurons displayed a progressive decrease in viability over 72 h in the presence of combined TNF α and NMDA stimulation (Figs 2a and b). Importantly, this is consistent with our results derived from cell counting based upon immunocytochemical detection of neurons (Floden *et al.* 2005). As a further confirmation that neurons die during combined TNF α and NMDA treatment, media aliquots were collected from cells stimulated for 72 h for quantifying cellular release of LDH (Fig. 2c). Our results confirmed that combined treatment with NMDA and TNF α is required to reduce neuronal viability in our culture system. For consistency, we relied upon immunocytochemical detection of histologically characteristic neurons and cell counting to assess neuron survival in the subsequent studies.

Although our data demonstrate a role for TNF α stimulation in NMDA receptor-dependent excitotoxic death, several studies have reported that TNF α stimulation can contribute to

either trophic or toxic support of neurons (de Bock *et al.* 1998; Carlson *et al.* 1998; Marchetti *et al.* 2004; Floden *et al.* 2005; Zou and Crews 2005). Moreover, others have demonstrated that TNF α pre-treatment of cultured neurons will condition them to resist a subsequent, toxic stressor (Houzen *et al.* 1997; Furukawa and Mattson 1998; Marchetti *et al.* 2004). To determine whether acute or prolonged TNF α stimulation was required for potentiation of the NMDA-dependent death, we pre-treated our cultures with TNF α for 24 h prior to stimulation with NMDA or glutamate (Fig. 3a). TNF α pre-treatment had no ability to condition neurons to die via subsequent stimulation with NMDA or glutamate alone, demonstrating that the neurotoxic action of TNF α involved an acute effect coincident with NMDA stimulation.

Because a variety of factors can be secreted from reactive glia, it is possible that multiple cytokines beyond TNF α will utilize a similar signaling cross-talk response to modulate NMDA receptor-dependent events. For example, it has been reported that IL-1 β stimulation also potentiates NMDA receptor-dependent death similar to our observations made using TNF α (Chao *et al.* 1995; Viviani *et al.* 2003). To determine whether the effects we observed were cytokine specific, we compared the abilities of both TNF α and IL-1 β to potentiate NMDA receptor-mediated death. Although IL-1 β was significantly toxic alone, it was unable to potentiate NMDA-dependent death at any concentration employed (Fig. 3b). On the contrary, TNF α stimulation resulted in a significant loss of neurons in a dose-dependent fashion in the presence of NMDA (Fig. 3b).

Next, we began identifying the mechanism of cell death that occurred following combined stimulation with TNF α and NMDA. As both *in vivo* and *in vitro* evidences demonstrate that ischemic and excitotoxic death can require activation of members of the MAP kinase family of serine/threonine kinases (Satoh *et al.* 2000; Sugino *et al.* 2000; Ferrer *et al.* 2001; Kuroki *et al.* 2001; Jin *et al.* 2002; Li *et al.* 2002; Noshita *et al.* 2002; Stanciu and DeFranco 2002; Hughes *et al.* 2003; Zablocka *et al.* 2003), we examined the neuron cultures for changes in activity of MAP kinases. All members of the MAP kinase family, JNK, p38 MAP kinase, and ERK have the capacity to mediate death in different paradigms (Morishima *et al.* 2001; Harper and Wilkie 2003; Hetman and Gozdz 2004). We first determined whether changes in active MAP kinase levels occurred during our toxic stimulation. Five minutes of combined TNF α and NMDA stimulation led to a rapid increase in levels of the active forms of ERKs and JNKs (Fig. 3c) but not p38 MAP kinase (data not shown). This increase was dependent upon NMDA receptor activity, as pre-treatment with 25 μ M APV prevented the increase in the active form of ERKs (Fig. 3d). More importantly, inhibition of ERK activity via maintained treatment with the MEK inhibitor, UO126, provided protection from TNF α and NMDA stimulated death in a dose-dependent fashion by 72 h (Figs 4a and b). Conversely, maintained treatment of the cultures with the JNK inhibitory peptide, D-JNKI1, had no ability to rescue neurons from TNF α and NMDA-mediated death (Figs 4c and d). Interestingly, JNK inhibition alone using the inhibitory peptide was significantly toxic to our cultures at 72 h, suggesting that a basal level of JNK activity is required for neuron viability in our system.

ERK-dependent neuron death is unique to stimulation with TNF α and NMDA

However, alteration of ERK activity is a common event in many signal transduction pathways. To assess whether increased ERK activity itself was sufficient to initiate a toxic signaling response, we examined other ERK activating stimuli for their effects on neuronal viability. Although toxic IL-1 β stimulation resulted in elevated levels of active ERKs, there was no potentiation of cell loss when the stimulation was combined with NMDA (Fig. 5). Neurons stimulated with BDNF also demonstrated elevated levels of active ERKs. In this case, ERK activity was potentiated when combined with TNF α stimulation (Fig. 6a). However, neither BDNF alone nor combined BDNF and TNF α stimulation produced a

decrease in cell viability. Instead, both treatments resulted in a significant increase in cell survival compared with unstimulated controls (Fig. 6b) consistent with the neurotrophic action of BDNF. These results collectively demonstrate that increased ERK activity itself is not toxic to our cultures but rather is a required component of the signaling response initiated by TNF α and NMDA.

To better characterize the increase in ERK activity required for TNF α and NMDA-mediated death, we next determined the temporal and spatial profile of activity following stimulation. Although non-toxic NMDA stimulation alone resulted in a prolonged increase in levels of active ERKs, the toxic TNF α and NMDA combined stimulation produced an abbreviated time course of activation (less than 30 min) (Fig. 7a). After observing this attenuation and considering the fact that ERK activity was required for death, we determined whether transient stimulation was sufficient for the delayed death we observed. As expected, transient 15 min stimulation with TNF α and NMDA was sufficient to induce neuron death by 72 h (Fig. 7b). Finally, we assessed the spatial profile of ERK activity in our stimulated neurons to determine whether kinase activity was localized to specific subcellular compartmentation during toxic stimulation (Fig. 8). Active ERK immunoreactivity was visualized following different stimulation times to establish its temporal and spatial profile. Combined TNF α and NMDA stimulated active ERK immunoreactivity within the somatodendritic region of the neurons at 5 min, which decreased after 15 min of combined stimulation. Nevertheless, the spatial staining pattern was not different from that induced by NMDA stimulation alone. Taken together, these data demonstrated that neurotoxic ERK activity appeared dependent upon a temporal rather than spatial activation pattern. Time course differences in ERK activity reportedly produce different substrate specificities and, more importantly, differences in stimulated transcriptional alteration (Stanciu and DeFranco 2002; Chu *et al.* 2004; Levinthal and DeFranco 2004; Rusanescu *et al.* 2005). Indeed, our prior work demonstrated that increased protein levels and activity of iNOS are required for combined TNF α and NMDA-dependent death (Floden *et al.* 2005).

Stimulation via TNFRII and not TNFRI mediates NMDA-dependent neuron death

As mentioned above, although the biological consequences of TNF α stimulation are typically characterized as components of TNFRI-mediated signaling events, neurons express both low- and high-affinity receptors. As both receptors have been implicated in TNF α -dependent effects on neurons, we determined which receptor subtype was required for mediating the toxicity with NMDA. Activation of the specific TNF α receptors was achieved via stimulation with subtype-specific agonist antibodies in the absence or presence of NMDA (Figs 9a and b). Agonist antibodies for TNFRII but not TNFRI resulted in increased active ERK levels and subsequent neuron death when combined with NMDA, demonstrating that the TNFRII-dependent signaling events mediated the cross-talk with the NMDA receptor (Fig. 9c). Finally, to verify that stimulation of TNFRII mediated an increase in NMDA-dependent calcium influx similar to that observed using TNF α (Fig. 1), we stimulated cells as before using the agonist antibodies for TNFRI and TNFRII (Fig. 10). As expected, TNFRII agonist antibodies stimulated a transient increase in intracellular calcium levels, which was inhibited by pre-treatment with APV, DNQX, and ω -conotoxin (Figs 10b and c). On the contrary, TNFRI agonist antibodies had no effect on altering intracellular calcium levels (Fig. 10a).

Discussion

In this study we have demonstrated that TNF α stimulation of mouse cortical neurons acts via the low-affinity TNF α receptor, TNFRII, to modulate NMDA receptor-mediated calcium influx. Stimulation with TNF α or TNFRII agonist antibodies was not toxic to the neurons. However, when TNF α or TNFRII agonist antibody stimulation was combined with

NMDA, a potent neurotoxicity was induced. More interestingly, combined TNF α and NMDA stimulation resulted in transient activation of ERKs compared with a prolonged activation observed following stimulation with NMDA alone. It is important to point out that although our data demonstrate that TNF α stimulates an NMDA receptor-dependent increase in calcium concentrations, this is likely a complex process involving several steps before the activation of the NMDA receptors. For example, we have demonstrated that AMPA/kainate receptor activity is required for the calcium concentration change as well as activity of N-type calcium channels and voltage-gated sodium channels. Collectively, these data suggest the hypothesis that TNF α stimulates synaptic glutamate release resulting in subsequent activation of AMPA receptors and membrane depolarization, leading to final NMDA receptor-dependent calcium influx. In support of this notion, prior studies have demonstrated that TNF α stimulation of dissociated neuron cultures stimulates AMPA receptor localization to the plasmalemma over a time scale of minutes in the range of the effects we observe (Ogoshi *et al.* 2005; Stellwagen and Malenka 2006). Future work is required to determine whether this AMPA receptor insertion and activation described by others is involved in the combined treatment of TNF α and NMDA, and ERK-dependent death we have observed. It is also possible that TNF α stimulation inhibits glutamate uptake by the small amount of contaminating glia in the cultures as a source of additional excitatory stimulation. Although we have not tested this hypothesis, this likely has an important role during *in vivo*, particularly, because TNF α stimulation has been reported to both increase and decrease glutamate transporter expression depending upon the paradigm (Romera *et al.* 2004; Korn *et al.* 2005).

Because of the changes we observed in active MAP kinase levels, we decided to assess the contribution of these enzymes to the resultant neuron loss. Cell death was dependent upon activation of ERKs and not JNKs, as treatment with the specific MEK inhibitor, UO126, and not the JNK inhibitory peptide, D-JNKII, was able to prevent death stimulated by combined TNF α and NMDA stimulation. Complimentary results from experiments performed using similar systems have demonstrated a crucial role for robust ERK activation during NMDA-dependent death in both neuronal cell lines and cortical neuron cultures (Stanciu and DeFranco 2002; Levinthal and DeFranco 2004). Although we observed that ERK activation was required for cell death in our system, others have reported in different culture paradigms that the MAP kinase family members, p38 and JNK, can also propagate a neurotoxic signaling response (Cao *et al.* 2004). For example, JNK activity has been implicated in NMDA receptor-dependent death in rat cortical neuron cultures (Centeno *et al.* 2006), phosphatase inhibition-associated death of rat cortical neuron cultures (Yoon *et al.* 2006), and trophic factor-dependent apoptosis in mouse dorsal root ganglia cultures (Besirli *et al.* 2005; Ben-Zvi *et al.* 2006). It is possible that the relative contribution of a particular MAP kinase to neuron death is largely dependent upon the stressor stimulus. In addition, the age of the cultures used may also represent a significant factor in kinase-dependent death. Indeed, the cortical neuron studies cited above used older culture ages than our current work. However, it has been our experience that older cultures contain a greater degree of contaminating cell types confounding possible interpretation of the data, and we have elected in the current study to use cultures at 7 days *in vitro* where our neuronal purity remains high. Collectively, these results demonstrate the broad contribution of the MAP kinase family to transducing diverse cytotoxic neuronal stimuli.

One caveat of this culture age, however, is their relative resistance to NMDA receptor-stimulated toxicity in spite of the presence of functional NMDA receptors (Fogal *et al.* 2005). Although our data demonstrate that neuron death in this paradigm is dependent upon combined TNF α and NMDA stimulation, we cannot rule out the possibility that older culture ages or neurons *in vivo* may respond differently to TNF α stimulation. Study of age-dependent effects of TNF α modulation of NMDA receptor-dependent neuronal death

represents an intriguing possibility for future effort. Indeed, TNF α expression is developmentally regulated in the brain of both rodents and humans with maximal levels appearing at approximately 10 weeks gestation or embryonic day 12 (E12), for humans and mice, respectively (Gendron *et al.* 1991; Mousa *et al.* 1999).

Extracellular signal-regulated kinase activation also has a demonstrated role in mediating a neuroprotective response. Importantly, prolonged or delayed activation of ERK appears to be critical for neuroprotection in some paradigms (Levinthal and DeFranco 2004; Levinthal and DeFranco 2005; Zhu *et al.* 2005). As suggested by these results, one explanation for the varying ability of ERK activity to mediate a toxic or protective response depends upon the temporal pattern of enzyme activation. In agreement with this hypothesis, we observed that the combined toxic TNF α and NMDA stimulation involved a transient activation of ERK when compared with that induced by non-toxic NMDA stimulation alone. Additionally, prior studies have also suggested that the spatial pattern of ERK activation can determine survival or death in neurons (Levinthal and DeFranco 2004). However, in our culture paradigm, we observed no nuclear translocation of active ERK. We did observe that active ERK immunoreactivity increased within the somatodendritic region during TNF α and NMDA combined stimulation compared with unstimulated cells. Therefore, some spatial specificity of ERK activity occurred but the substrate significance of this remains unclear.

Although we have not yet identified ERK substrate-specific phosphorylation changes, it is likely that varying kinetics of ERK activation mediate alternate transcriptional events in our cells. In fact, it is known that differing lengths and amplitudes of ERK activation regulate the phosphorylation state and stability of several immediate early genes to regulate subsequent second generation gene expression in mammalian cells (Murphy *et al.* 2002). Indeed, our prior study demonstrated that toxic TNF α and NMDA stimulation required increased protein levels and activity of iNOS (Floden *et al.* 2005). A requirement for cytotoxic protein synthesis during neuronal death processes is certainly an established mechanism of death in specific toxicity assays (Martin *et al.* 1988).

Prior studies from other groups have also documented this ability of TNF α to modulate neuron viability. An extensive body of literature exists, characterizing the pleiotropic nature of this cytokine to mediate protective (Carlson *et al.* 1998) or toxic (Nicholas *et al.* 2001; Zassler *et al.* 2003) neuron stimulations. One reason for the varying ability of TNF α to provide a neurotoxic or neuroprotective stimulus likely depends upon the relative contribution of TNFRI or TNFRII-dependent signaling to the stimulation. Although our data demonstrated that TNFRII mediates the NMDA receptor-dependent death, these observations contrast with other reports, suggesting a neurotoxic and neurotrophic role of TNFRI and TNFRII, respectively (Natoli *et al.* 1997; Yang *et al.* 2002; Li *et al.* 2004; Marchetti *et al.* 2004). One explanation for the observed differences is likely because of differing culture purities and conditions as well as the use of cells derived from receptor knockout animals in other studies. In addition, we have not yet defined the precise signaling pathway employed by TNFRII, leading to the changes in ERK activity and NMDA receptor-dependent calcium influx. However, prior studies in other cell types have implicated various MAP kinase activities in the signaling response employed by both the low- and high-affinity receptors (Natoli *et al.* 1997; Yang *et al.* 2002; Marchetti *et al.* 2004) consistent with our observations.

Although we have focused our efforts on understanding the ability of TNF α to modulate neurotransmitter receptor signaling, we are aware that the inflammatory milieu will involve multiple mediators besides glutamate and TNF α . Certainly, IL-1 β also has a demonstrated ability to increase NMDA receptor-mediated calcium influx via modulating tyrosine phosphorylation of the receptor (Viviani *et al.* 2003). In addition, IL-6 stimulation can also

increase NMDA receptor-mediated calcium influx and resultant neurotoxicity in some neuron culture paradigms (Qiu *et al.* 1995). Therefore, our data demonstrating a specific mechanism of TNF α and NMDA receptor-dependent death are only one of several toxicity pathways possibly occurring concurrently *in vivo* during inflammatory states.

Finally, it is known that NMDA receptor-expressing neurons are a population vulnerable to AD-associated loss possibly correlating with the degree of dementia (Greenamyre and Young 1989; Francis *et al.* 1993). These observations support a hypothesis of NMDA receptor-dependent excitotoxicity explaining neuron loss in AD. However, the excitotoxic hypothesis has been confounded by a lack of mechanism for excitotoxic stimulation of the NMDA receptor-expressing neurons in AD (Albin and Greenamyre 1992). Our data suggest that excitotoxic levels of glutamate are not necessary in AD brains or other pathophysiology, if physiologic levels of TNF α , such as that employed in our studies, are present (Furukawa and Mattson 1998). More importantly, brain TNF α levels are reportedly increased in several conditions, besides AD including multiple sclerosis, epilepsy and ischemia/injury (Tchelingerian *et al.* 1993; Akiyama *et al.* 2000; Gimsa *et al.* 2000; Yin *et al.* 2003; Ravizza *et al.* 2005). Therefore, our results suggest a possible mechanism by which this cytokine may potentiate loss of NMDA receptor-expressing neurons in multiple pro-inflammatory states.

Acknowledgments

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Abbreviations

AD	Alzheimer's disease
AMPA	α -amino-3-hydroxy-5-methylisoxazole-4-propionate
APV	2-amino-5-phosphopentanoic acid
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
DD	death domain
DNQX	6,7-dinitroquinoxaline-2,3-dione
ERK	extracellular signal-regulated kinase
IL-1β	interleukin-1 β
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase

LDH	lactate dehydrogenase
MAP	mitogen-activated protein
MAP2	microtubule-associated protein 2
MEK	mitogen-activated protein kinase kinase
NFκB	nuclear factor κ B
NMDA	<i>N</i> -methyl-D-aspartate
PBS	phosphate-buffered saline
TNFα	tumor necrosis factor α

Fig. 1.

TNF α stimulates increased intracellular Ca²⁺ levels in primary cortical neurons. Neurons were loaded with Fura-2 then stimulated to measure changes in Ca²⁺ influx in NMDA and TNF α -stimulated primary cortical neurons (E16, 7 days *in vitro*), in the presence or absence of APV, DNQX, ω -conotoxin, and tetrodotoxin. (a) Demonstrates representative fluorescence traces from NMDA (100 $\mu\text{mol/L}$) stimulated cells in the absence or presence of APV following pre-treatment with the drug (25 $\mu\text{mol/L}$, 30 min). (b–d) Contain representative traces from TNF α (50 ng/mL) and dialyzed TNF α stimulated cells in the absence or presence of APV (25 $\mu\text{mol/L}$), DNQX (50 $\mu\text{mol/L}$), tetrodotoxin (1 $\mu\text{mol/L}$), and ω -conotoxin (9 $\mu\text{mol/L}$), 30 min pre-treatment. (e) Shows a representative trace following dual stimulation with TNF α and NMDA. (f) Demonstrates a representative trace from cells stimulated with PBS only. (g) Represents average peak calcium influx values ($\pm$ SEM) in each set of treatments from three independent experiments. The number of cells imaged is indicated in brackets (* $p < 0.05$ from NMDA).

Fig. 2.

TNF α and NMDA stimulation leads to neuron death after 72 h. Mouse cortical neurons (E16, 7 days *in vitro*) were cultured in the absence or presence of NMDA (100 μ mol/L), and mouse TNF α (50 ng/mL). Stimuli were added to neurons for 0, 24, 48, and 72 h and neuron viability was determined. (a) Representative fields of neurons were photographed during stimulation with and without TNF α and NMDA at each time point to demonstrate morphologic changes. Sister cultures were fixed at each age and immunostained with anti-MAP2 antibody and nuclei visualized by DAPI to demonstrate representative changes in MAP2 immunoreactivity at each time point (arrowheads indicate degenerated neurons). (b) The same field of phase bright, morphologically defined neurons from six sister cultures was counted at each time point and the average number of cells ($\pm$ SD) was graphed for each condition. Data are representative of three independent experiments. (c) Media aliquots were taken from cells stimulated 72 h with or without TNF α and NMDA for 72 h and LDH concentrations were quantitated using a commercial assay. Data are representative of two independent experiments. (* p < 0.05 from control, *** p < 0.001 from control).

Fig. 3.

TNFα and glutamate/NMDA stimulated neuron death is acutely stimulated and involves MAP kinase activation. Mouse cortical neurons (E16, 7 days *in vitro*) were cultured in the absence or presence of NMDA (100 μmol/L), glutamate (25 μmol/L), mouse TNFα (1–100 ng/mL), mouse IL-1β (1–100 ng/mL), and APV (5 and 25 μmol/L). (a) Stimuli were added to neurons for 72 h with and without a 24-h pre-treatment of 50 ng/mL TNFα. (b) Neurons were stimulated with NMDA and increasing concentrations of TNFα or IL-1β for 72 h. (a, b) After treatment, cells were fixed, stained using anti-MAP2 antibody, and counted. Neurons from four fields/condition were counted in quadruplicate wells and averaged ± SEM. Graphs are representative of three independent experiments. (* $p < 0.05$ from control, ** $p < 0.001$ from control). (c, d) For western blots, stimuli were added to neurons for 5 min with and without 30 min pre-treatment with APV. Cells were lysed and proteins resolved by 10% SDS-PAGE and western blotted using anti-pERK, anti-ERK2 (loading control), anti-pJNK, and anti-JNK (loading control) antibodies. Antibody binding was visualized via enhanced chemiluminescence.

Fig. 4.

TNF α and NMDA-stimulated neuron death requires ERK activity. Mouse cortical neurons (E16, 7 days *in vitro*) were stimulated with NMDA (100 μ mol/L) and/or mouse TNF α (50 ng/mL), in the absence or presence of pre-treatment (30 min) with (a) UO126 (10, 50, and 100 nmol/L), or (c) D-JNKI1 (100, 500, and 1000 nmol/L). Stimuli were added to neurons with and without drugs for 72 h then cells were fixed, stained using anti-MAP2 antibody, and counted. Neurons from four fields/condition were counted in quadruplicate wells and averaged $\pm$ SEM. Graphs are representative of three independent experiments. (* p < 0.01 from control, ** p < 0.001 from control, *** p < 0.05 from TNF α +NMDA, **** p < 0.001 from TNF α +NMDA). For western blots, stimuli were added to neurons for 5 min following pre-treatment (30 min) with (b) UO126 (100 nmol/L) or (d) D-JNKI1 (100, 500, and 1000 nmol/L). Cells were lysed and proteins resolved by 10% SDS-PAGE and western blotted using anti-pERK, anti-ERK2 (loading control), anti-pJNK, and anti-JNK (loading control) antibodies. Antibody binding was visualized via enhanced chemiluminescence.

Fig. 5.

IL-1 β stimulation does not potentiate NMDA-dependent death and ERK activity. Mouse cortical neurons (E16, 7 days *in vitro*) were cultured in the absence or presence of NMDA (100 μ mol/L) and mouse IL-1 β (50 ng/mL). (a) For western blots, stimuli were added to neurons for 5 min then cells were lysed and proteins resolved by 10% SDS-PAGE and western blotted using anti-pERK, anti-ERK2 (loading control) antibodies. Antibody binding was visualized via enhanced chemiluminescence. (b) Stimuli were added to neurons for 72 h then cells were fixed, stained using anti-MAP2 antibody, and counted. Neurons from four fields/condition were counted in quadruplicate wells and averaged $\pm$ SEM. Graphs are representative of three independent experiments. (* $p < 0.001$ from control).

Fig. 6. TNF α and BDNF stimulation does not result in neuron death. Mouse cortical neurons (E16, 7 days *in vitro*) were cultured in the absence or presence of NMDA (100 μ mol/L), mouse TNF α (50 ng/mL) and human BDNF (50 ng/mL). (a) For western blots, stimuli were added to neurons for 5 min then cells were lysed and proteins resolved by 10% SDS-PAGE and western blotted using anti-pERK, anti-ERK2 (loading control) antibodies. Antibody binding was visualized via enhanced chemiluminescence. (b) Stimuli were added to neurons for 72 h then cells were fixed, stained using anti-MAP2 antibody, and counted. Neurons from four fields/condition were counted in quadruplicate wells and averaged $\pm$ SEM. Graphs are representative of three independent experiments. (* p < 0.01 from control, ** p < 0.001 from control, *** p < 0.001 from TNF α + NMDA).

Fig. 7.

TNF α and NMDA stimulate a transient increase in ERK activity. Mouse cortical neurons (E16, 7 days *in vitro*) were cultured in the absence or presence of NMDA (100 μ mol/L) and mouse TNF α (50 ng/mL). (a) For western blots, stimuli were added to neurons for increasing time then cells were lysed and proteins resolved by 10% SDS-PAGE and western blotted using anti-pERK, anti-ERK2 (loading control) antibodies. Antibody binding was visualized via enhanced chemiluminescence. (b) Stimuli were added to neurons for increasing times then removed and replaced with fresh Neurobasal media. Cells were fixed after 72 h then stained using anti-MAP2 antibody, and counted. Neurons from four fields/condition were counted in quadruplicate wells and averaged $\pm$ SEM. Graphs are representative of three independent experiments. (* p < 0.001 TNF α +NMDA treatment compared with paired time control).

Fig. 8.

Active ERK immunoreactivity increases after TNF α and NMDA treatment. Mouse cortical neurons were cultured for 7 days then stimulated, 5, 15, and 30 min in the absence or presence of TNF α (50 ng/mL) and NMDA (100 μ mol/L). Cells were fixed in 4% paraformaldehyde, 37°C, and then immunostained using anti-pERK antibody. Antibody binding was visualized using Texas Red conjugated secondary antibody.

Fig. 9.

Agonist antibodies for TNFRII potentiate NMDA-dependent neuronal death. Mouse cortical neurons (E16, 7 days *in vitro*) were cultured in the absence or presence of NMDA (100 $\mu\text{mol/L}$), or 0.01, 0.1, 1 $\mu\text{g/mL}$ TNFRI or TNFRII dialyzed agonist antibodies. (a) Stimuli were added to neurons for 72 h then cells were fixed, stained using anti-MAP2 antibody, and counted. Neurons from four fields/condition were counted in quadruplicate wells and averaged $\pm$ SEM. Graphs are representative of three independent experiments. (* $p < 0.001$ from control). (b) For western blots, cells were stimulated for 5 min then lysed, quantitated and proteins resolved via 10% SDS-PAGE and western blotted using anti-pERK and anti-ERK2 (loading control) antibodies. Antibody binding was visualized via enhanced chemiluminescence.

Fig. 10.

TNFRII agonist antibodies stimulate Ca^{2+} influx in primary cortical neurons. Primary cortical neurons (E16, 7 days *in vitro*) were loaded with Fura-2 and then stimulated to measure changes in Ca^{2+} influx in TNF α (50 ng/mL), TNFR-I (1 $\mu\text{g/mL}$) agonist antibody, and TNFR-II (1 lg/mL) agonist antibody stimulated cells with and without 30 min pre-treatment with APV (25 $\mu\text{mol/L}$), DNQX (50 $\mu\text{mol/L}$), or ω -conotoxin (9 $\mu\text{mol/L}$). (a) Demonstrates a representative trace from cells stimulated with TNFR-I agonist antibody, dialyzed TNFR-I agonist antibody, with and without APV pre-treatment. (b, c) Show representative traces from TNFR-II agonist antibody, dialyzed TNFR-II agonist antibody, rat IgG (isotype negative control) with and without pre-treatment with DNQX, ω -conotoxin, APV. (d) Represents average peak calcium influx values ($\pm$ SEM) in each set of treatments from three independent experiments. The number of cells imaged is indicated in brackets. (* $p < 0.05$ from TNFR-II).