TNFR2 mediated neuroprotection against glutamate induced excitotoxicity is enhanced by NMDA receptor activation: Essential role of a TNF receptor 2 mediated, PI3 kinase dependent NF-κB pathway.

Lara Marchetti, Matthias Klein, Katalin Schlett, Klaus Pfizenmaier, and Ulrich L. M. Eisel

1Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, D-70569 Stuttgart, Germany
2Xantos Biomedicine AG, Fraunhoferstrasse 22, D-82152 Martinsried, Germany
3Dept. of Physiology and Neurobiology, Eötvös Loránd University, H-1117 Budapest, Pázmány P. stny. 1/C, Hungary
4Department of Molecular Neurobiology, Rijksuniversiteit Groningen, Kerklaan 30, P.O.Box 14, 9750 AA Haren, The Netherlands

5Corresponding author: Klaus Pfizenmaier, Institute of Cell Biology and Immunology, University of Stuttgart, Allmandring 31, D-70569 Stuttgart, Germany, phone 0049 711 685 6986, fax 0049 711 685,7484, email: klaus.Pfizenmaier@izi.uni-stuttgart.de

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Summary

We have previously shown that the two TNF receptors (TNFR) exhibit antagonistic functions during neurodegenerative processes in vivo, with TNFR1 aggravating and TNFR2 reducing neuronal cell loss, respectively. In order to elucidate the neuroprotective signalling pathways of TNFR2, we investigated glutamate induced excitotoxicity in primary cortical neurons. TNF-expressing neurons from TNF transgenic mice were found to be strongly protected from glutamate-induced apoptosis. Neurons from wildtype and TNFR1$^{-/-}$ mice prestimulated with TNF or agonistic TNFR2 specific antibodies were also resistant to excitotoxicity, whereas TNFR2$^{-/-}$ neurons died upon glutamate and/or TNF exposures. Both PKB/Akt and NF-κB activation were apparent upon TNF treatment. Both, TNFR1 and TNFR2 induced the NF-κB pathway, yet with distinguishable kinetics and upstream activating components: TNFR1 induced only transient NF-κB activation, while TNFR2 facilitated long term, strictly PI3K-dependent NF-κB activation. Glutamate induced triggering of the ionotropic N-Methyl-D-Aspartate receptor was required for the enhanced and persistent, PI3K-dependent NF-κB activation by TNFR2, indicating a positive cooperation of TNF and neurotransmitter induced signal pathways. TNFR2 induced persistent NF-κB activity was essential for neuronal survival. Thus, the duration of NF-κB activation is a critical determinant for sensitivity towards excitotoxic stress and is dependent on a differential upstream signal pathway usage of the two TNFRs.

Key words: N-Methyl-D-Aspartate receptor, Nuclear Factor kappa B, PKB/Akt, Tumor Necrosis Factor, neurodegenerative diseases, apoptosis, TNFR1, TNFR2
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Introduction

Tumor Necrosis Factor (TNF) is a prominent proinflammatory mediator that has been causally associated with the pathophysiology of several acute and chronic diseases, in particular rheumatoid arthritis and Morbus Crohn (1, 2). Upregulated TNF expression has also been found in various neurodegenerative diseases such as cerebral malaria, AIDS dementia, Alzheimer’s disease, multiple sclerosis or stroke, suggesting a potential pathogenic role of TNF in these diseases as well (3-7). The membrane expressed form of TNF signals via both TNF receptors (TNFR1 and TNFR2), whereas soluble TNF - proteolytically cleaved from the membrane form- acts mainly via TNFR1 (8). Signal pathways initiated from the death domain containing TNFR1, leading to both pro- and anti-apoptotic cellular responses, have been studied in great detail (9). In contrast, there is less information about the molecular mechanisms surrounding signal pathways and cellular responses solely initiated via TNFR2, due to concomitant TNFR1 signals in normal situations. Evaluation of the physiological role of TNFR2, by large, depends on data obtained from TNFR1−/− mice.

We have recently investigated the role of TNF and its receptors in retinal ischemia and unraveled an antagonistic function of TNFR1 and TNFR2. The latter exerts neuroprotection in a phosphoinositol-3-phosphate kinase (PI3K) dependent manner, which is counterbalanced by the neurodegenerative action of TNFR1 (10). TNFR1 has also been associated with cell death of hippocampal neurons responding to TNF (11) whereas inhibition of TNFR2 expression by the use of antisense oligonucleotides sensitized neuronal-like cells towards apoptosis (12). In order to elucidate the neuroprotective signal pathways emanating from TNFR2, we have established an in vitro model of apoptosis induction of primary cortical neurons by glutamate triggered excitotoxicity, to study mechanisms controlling apoptosis sensitivity of neurons within wildtype (C57/BL6), TNF transgenic, TNFR1−/− and TNFR2−/− mice. Our data reveal, for the first time, differential activation kinetics of NF-κB and differential usage of upstream signalling components by TNFR1 and TNFR2. TNFR2, but not TNFR1, induces persistent NF-κB activation via a signalling pathway involving PI3K and PKB/Akt, which is strongly enhanced by N-methyl-D-aspartate receptor co-stimulation.

Experimental Procedures

Generation of transgenic mice with brain specific TNF expression

Transgenic mice were developed by pro-nuclear injection in general as previously described (13). A construct consisting of the murine NR2B promoter and the first three exons of the 5′ untranslated region (UTR) of the NR2B gene was fused with the Nar I site of the first exon of the murine TNF gene with replaced 3′-UTR (3′ part of exon 4 of the TNF gene) by the 3′-UTR of the human β-globin gene (14). Four mouse lines were investigated.
and showed similar TNF expression patterns differing only in the level of and duration of expression. The Expression pattern was verified by in situ hybridization as described (15). pBS II SK plasmid containing a cDNA of the murine TNF-gene was digested either with Pst I or EcoR I for synthesis of a $^{35}$S-labelled sense or antisense probe using T3 or T7 RNA polymerase.

**Primary cortical cell culture**

Cortical cultures were prepared as follows: embryonic brains were extracted (E14), the meninges removed, and cortices dissected immediately. Neurons were recovered by mechanical dissociation. Cells were plated at a density of $\sim 1 \times 10^5$ cells/cm$^2$ (96 or 6 well-plates) or 2.5 $\times 10^4$ cells/cm$^2$ (24 well-plates) on poly-L-lysine (Sigma, Deisenhofen, Germany) coated plates. Neurobasal medium with B27-supplement (Invitrogen) and 0.5 mM Glutamin with a final concentration of 0.1 mg/ml Gentamycin (Invitrogen) and 2.5 µg/ml Amphotericin B (Sigma, Deisenhofen, Germany) was used as culture medium. On the second day in vitro, cells were treated with 10 µM cytosine arabinoside for 48 hours to inhibit non-neuronal cell growth. Subsequently, the medium was completely exchanged and every third day afterwards fresh medium was added. Cells were either stimulated with increasing concentrations of glutamate for 1 hour [Figure 2B, 3A, 3C, 8C] or for 24 hours with the indicated concentrations of TNF [Figure 3B, 8A, 8B] or TNFR agonistic antibodies [Figure 4A, B and C] followed by a one hour stimulation of 250µM glutamate (Sigma, Deisenhofen, Germany) and the determination of the cell viability 24 hours later. When indicated, cells were incubated prior to the glutamate stimulus with an inhibitor of PI3K (LY294002, 25µM; Calbiochem, Germany or wortmannin, 100nm; Sigma, Germany), NMDAR (MK801, 10µM; Tocris, Germany), Caspases (ZVADfmk, 20µM; BACHEM, Germany) or NF-κB (MG-132, 20µM; Calbiochem, Germany; Geldanamycin 0,5µM Calbiochem, Germany; BAY11-7082, 20µM, Alexis Biochemicals, Germany). To determine kinetics of cell death induction, primary cortical neurons were incubated for 1 hour with 250 and 500µM glutamate and cell viability was assessed directly after the stimulus (=1hr), or 1 (=2hr), 3 (=4hr), 5 (=6hr) and 23 (=24hr) hours later [Figure 2A]. Agonistic antibodies (2µg/µl; HyCult Biotechnology, The Netherlands) were cross linked by a mouse anti-rat antibody (2µg/µl; Jackson Immuno Research, Dianova, Hamburg, Germany) to obtain optimum triggering of the TNFRs.

**Determination of Cell Viability**

Cell viability was determined by the colorimetric 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (16). Primary cortical neurons were grown in 96-well microtiter plates at a density of $6\times 10^4$ cells per well and stimulated as indicated. 10 µl of an MTT solution (1.25mg/ml) was added to each well. After 1 hour incubation, cells were lysed by adding 150 µl of isopropyl-HCl-solution (600 µl HCl/100
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ml isopropyl alcohol) for 15 minutes. The absorbance of each well was determined with an automated ELISA reader (BioRad) at 590nm with an background correction at 620nm.

**Western Blot**

Cytosolic extracts from primary cortical neurons (2x10^6) were prepared by washing twice with ice cold phosphate buffered saline (PBS), followed by the addition of 0.4 ml of the cell lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF). Lysates were incubated on ice for 20 minutes. 25 µl of 10% Nonidet P-40 was added for two minutes and nuclei were collected by centrifugation. The supernatants were boiled for 5 minutes in Laemml sample buffer (2% SDS, 5% DTT) and 50 µg of protein was separated by SDS-polyacrylamid gel electrophoresis. After transfer to a nitrocellulose membrane (Hybond ECL, Amersham, Germany), proteins were detected with a specific primary and a horseradish peroxidase conjugated secondary antibody using enhanced chemiluminescence according to the manufacturer’s instructions (LuminGlo, New England Biolabs).

Primary antibodies included were a rabbit polyclonal antibody specific for the phospho-PKB/Akt serine 473 residue (1:1000; Cell Signaling, Germany), total PKB/Akt (1:1000; Cell Signaling, Germany), IκB (1:500; Santa Cruz Biotechnology, Germany), phospho-IκB (1:1000; Cell Signaling, Germany), TNF (1:1000; BioSource, Germany) and Glutamate Receptor 1 (1:300; Chemicon, Canada).

**Immunocytochemistry and Immunohistochemistry**

Cells were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 20 minutes at room temperature (RT), permeabilized with 0.1% TritonX 100/PBS for 5 minutes and blocked with 5% goat serum in PBS. Overnight incubation with a polyclonal rabbit anti-p65 antibody (1:500; Oncogene, Germany), or rabbit anti-TNF antibody (1:1000, Santa Cruz, USA) at 4 °C was followed by a one hour incubation with a fluorescent-labeled Alexa 546 or 488 anti-rabbit antibody (1:1000; Molecular Probes, Germany) at RT. Neurons were labeled with a monoclonal neuronal-specific mouse anti-unique-β-tubulin antibody (1:2000; Babco, USA) for 1.5 h at RT and a Alexa 488 or 546 anti-mouse antibody (1:1000; Molecular Probes, Germany).

Glia cells were detected using a monoclonal mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:2000; Sigma, Germany) and a secondary Alexa 546 labeled anti-mouse antibody (1:1000; Molecular Probes, Germany). The glia cell content resulted in less than 0.5%.
Determination of apoptosis induction was assessed after fixation of stimulated (1 hour 250µM Glutamate) and unstimulated cells by incubation with an Annexin V-specific, FITC-conjugated antibody (1:20; BD Pharmingen, Germany) for 1 hr at RT followed by a 1 hr incubation with DAPI (0.1 µg/ml.) for nuclear staining.

For immunohistochemical staining, paraffin embedded sections were permeabilized with 0.02% H₂O₂-methanol for 30 minutes and blocked with 10% FCS for 20 minutes. Sections were then incubated with specific antibodies (rabbit-anti-TNF (Genzyme) 1:1000; rat-anti-mac3 (Pharmingen) 1:200; rat-anti-CD45 (Pharmingen) 1:100 in 0.1% BSA/TBS) at 4°C over night, washed, incubated with a biotin-labeled anti-rabbit IgG or anti-rat-IgG (Vector Laboratories, 1:200 in 2.5% BSA/TBS) and stained with Avidin-DAB (Sigma) according to the manufacturer’s protocol.

**Electrophoretic mobility shift assay (EMSA)**

For preparation of nuclear extracts, 2x10⁶ primary cortical neurons were washed once with cold PBS, resuspended in 0.4 ml of lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF) and then incubated on ice for 20 minutes. Subsequently, 25 µl 10% Nonidet P-40 was added for two minutes, nuclei were collected by centrifugation and resuspended in 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, shaken for 20 minutes and then centrifuged. The supernatant, containing nuclear proteins, was used for EMSA. The NF-κB consensus oligonucleotide (5'-ATCAGGGACTTTCCGCTGGGGACTTTCCG-3') was 5' labeled with [γ³²P]-ATP using polynucleotidkinase. Binding reaction was performed by incubating 10 µg of nuclear protein extracts with 4µg poly[dI-dC] in a 20µl reaction mixture containing 5 mM MgCl₂, 50 mM KCl, 5 mM HEPES pH 7.8, 0.2 mM EDTA, 5 mM DTT, and 10% glycerol. The double-stranded oligonucleotide probe (50000 cpm/µl) was added for 20 minutes at RT. Binding specificity was determined by competition with excess (100 ng) unlabeled NF-κB oligonucleotide. DNA/protein complexes were analyzed on non-denaturing 6% polyacrylamide gels and visualized by Phosphor-Imaging.

For supershift assays, nuclear extracts were preincubated with p65, p50 or cRel antibodies (1 µg/ml; Santa Cruz Biotechnology, USA) for 30 minutes at room temperature.

**Results**

**Generation and analyses of NR2B/TNF transgenic mice**

Previous transgenic mouse models resolving the role of TNF in the brain were designed to cause a widespread and often high level of TNF expression, which was typically associated with various pathologies (17-20). In
order to avoid unconditional TNF expression, we have employed the promoter of the murine N-methyl-D-aspartate (NMDA) receptor subunit NR2B to generate mice with a strictly regional and rather moderate neuronal TNF expression. The endogenous expression of NR2B subunit is restricted to forebrain regions and mainly expressed in cortical and hippocampal regions of the brain (21,22). To enforce this restricted expression pattern of TNF in transgenic mice, the 5'-UTR of the NR2B gene, which was shown to influence the expression pattern, was also included in the construct (Fig. 1A) (23,20). NR2B-directed TNF transgene expression was verified by in situ hybridization (Fig. 1B), Western blot analyses in embryonic (E14) and postnatal (P14) tissues (Fig. 1C), immuno-labeling of primary neuronal cells (Fig. 1D and E) and immuno-staining in paraffin sections (Fig. 1F, upper panel). Interestingly, among the four generated mouse lines no inflammatory responses such as infiltration or spontaneous demyelinating lesions were observed, as reported from other mouse models with ubiquitous and high level of TNF expression (17-20). Only in areas of strongest TNF expression (i.e. hippocampus and cortex) ramified microglial cells positive for CD45 or mac3, considered as activation markers, could be detected (Fig. 1E middle and lower panel). The data show that TNF transgene expression is in concordance with the expected pattern of the NR2B gene (22, 23). Moreover, immunohistochemical detection of TNF protein and microglial activation in tissue sections of transgenic but not of non-transgenic littermates, indicate functional in vivo expression of the transgene. In addition bioactive TNF could be detected on the cell membrane (Fig.1D and E) and in the supernatant of cultivated NR2B/TNF transgenic neurons (6x10^6 cells release 1ng soluble TNF in 24 hours), but not of neurons from control littermates as determined by standard bioassay (data not shown) (24, 25). Glutamate treatment had no effect on the NR2B promoter activity as determined by semi-quantitative RT-PCR of NR2B mRNA levels in wildtype neurons (data not shown).

**TNF/TNFR2 mediated signaling protects primary cortical neurons from glutamate induced cell death**

Exposure of wildtype murine primary cortical neurons to neurotoxic doses of glutamate lead to a time- and dose-dependent induction of cell death (Fig. 2A,B), which was inhibited by the caspase inhibitor ZVADfmk (Fig. 2B). Furthermore, nuclear fragmentation, Annexin V staining (Fig. 2C) and inhibition of neuronal cell death by MK801 (Fig. 3A), an inhibitor of the NMDA receptor, is indicative of NMDAR mediated excitotoxic induction of apoptosis, which is in accordance with previous studies (26). Primary cortical neurons from NR2B/TNF transgenic animals, in contrast to wildtype, TNFR1 (TNFR1^−/−) and TNFR2 deficient (TNFR2^−/−) cells, were found to be almost completely resistant to glutamate triggered excitotoxicity (Fig. 3A). In order to mimic the conditions of continuous TNF expression of TNF transgenic animals, wildtype and TNFR^−/− neuronal cells were pretreated for 24 hrs. with TNF. Under these conditions, resistance towards glutamate-induced apoptosis could
be induced in wildtype and, to an even greater extent, in TNFR1⁺⁻ neuronal cells (Fig. 3B). By contrast, TNFR2⁺⁻ neurons were not only more susceptible to glutamate-induced cell death, but also died upon TNF exposure (Fig. 3B). TNF stimulation of neuronal cultures simultaneously or subsequent to glutamate addition was found to be non-protective (data not shown). These data indicate that resistance of primary cortical neurons towards an excitotoxic stimulus requires a preceding TNF signal via TNFR2. Studying ischemia reperfusion induced damage of the retina of TNFR1⁺⁻ mice, we have recently shown that the locally applied PI3 kinase inhibitor LY294002 aggravated neuronal degeneration (10). Therefore, we have investigated the role of PI3K in the glutamate-induced in vitro excitotoxicity model. The glutamate resistance of NR2B/TNF transgenic cortical neurons (Fig. 3C) and TNF pretreated neurons of wildtype or TNFR1⁺⁻ mice (Fig. 3B) was completely abolished by LY294002. Of note, although we found that protection of wildtype and TNFR1⁺⁻ neurons required in vitro preincubation with TNF, addition of the PI3K inhibitor as little as 30 min. before the excitotoxic stimulus fully prevented the TNF dependent survival of neurons (Fig. 3B). This indicates that in order to accumulate a protective TNF response the TNFR2 mediated activation of a PI3K pathway has to occur within a narrow time span before the excitotoxic insult.

Further evidence for distinct, pro- and anti-apoptotic pathways emanating from TNFR1 and TNFR2, respectively, was obtained by the use of TNFR subtype specific agonistic antibodies (27), allowing selective stimulation of either receptor in wildtype neurons. TNFR specificity of the antibodies was verified by analyzing reactivity on TNFR1⁺⁻ and TNFR2⁺⁻ neurons (data not shown). The TNFR1 specific agonist alone elicited neuronal cell death in wildtype neurons in a dose-dependent manner and did not protect from glutamate induced apoptosis (Fig. 4A). Conversely, selective stimulation of TNFR2 had no cytotoxic effect, but instead led to an inhibition of neurotransmitter triggered excitotoxicity (Fig. 4B). Again, blocking of PI3K by LY294002 reverted the TNFR2 mediated protection (Fig. 4B), whereas the TNFR1 mediated cell death was not affected by the PI3K inhibitor (Fig. 4A). Critical involvement of PI3K signaling was confirmed by usage of wortmannin after selective TNFR2 stimulation (Fig. 4C) or TNF preincubation (data not shown).

**NMDAR signaling enhances TNFR2 induced PKB/Akt activation**

PKB/Akt is considered to be a prominent and ubiquitous downstream target protein of PI3K (28). Because of the TNF and PI3K dependent protection of cortical neurons from glutamate induced apoptosis, we investigated PKB/Akt activity during excitotoxic stimulation of untreated and TNF pretreated wildtype, TNFR1⁺⁻, TNFR2⁺⁻ as well as of NR2B/TNF transgenic mice. Western blot analyses were performed with protein lysates from cortical neurons treated with TNF and/or glutamate in the presence or absence of a specific NMDAR blocker.
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(MK801) or a selective PI3K inhibitor (LY294002). Intense and persistent PKB/Akt phosphorylation was detected in both TNFR1−/− and wildtype neurons after TNF and glutamate co-stimulation (Fig. 5A, lanes 4, 8, 12). This was partially inhibited by LY294002 in wildtype cells and completely abolished in TNFR1−/− cells (Fig. 5A, lanes 6, 10, 14). In TNFR2−/− neurons only a weak, transient PKB/Akt activation was detected upon glutamate and TNF treatment (Fig. 5A, lanes 4, 8, 12). These data show that the TNF induced, persistent PKB/Akt phosphorylation is TNFR2 mediated and PI3K-dependent. Moreover, we reveal to date unrecognized strong enhancement of this signal pathway by the glutamate activated NMDAR.

In contrast to wildtype, TNFR1−/− and TNFR2−/− neurons, substantial levels of phospho-PKB/Akt were detected in NR2B/TNF transgenic neurons without in vitro TNF treatment (Fig. 5B, lane 1), which is in accordance with endogenous TNF expression in these neurons. As expected from the above experiments, PKB/Akt phosphorylation was PI3K-dependent at all time points analyzed (Fig. 5B, lanes 4, 7, 10, 12).

NMDAR signaling increases TNFR2 induced NF-κB activation

NF-κB activation in primary cortical neurons was determined by immunofluorescence microscopy of nuclear translocated p65 (Fig. 6A) as well as by the electrophoretic mobility shift assay (EMSA; Fig. 6E and F). Lone TNF treatment of TNFR1−/− or TNFR2−/− neurons revealed characteristic differences in NF-κB activation (Fig. 6B). Nuclear NF-κB translocation after stimulation of TNFR1 reached a maximum between 20 and 40 minutes (Fig. 6B, white bars), whereas TNFR2-mediated NF-κB translocation increased gradually, peaked after 4 hours of stimulation and was still discernable after 24 hours (Fig. 6B, black bars). Wildtype neurons, which express both TNFRs, exhibited intermediate translocation kinetics (Fig. 6B, gray bars). Upon glutamate stimulation of TNF pretreated (24hrs) cultures, a strong enhancement of the residual, low level NF-κB activity could be identified by nuclear translocation (Fig. 6C) and EMSA (Fig.6E) in wildtype and in TNFR1−/− neurons, whereas TNFR2−/− neurons showed no enhancement of NF-κB activation by glutamate (Fig.6C, white bars, Fig.6E, lane 2, 4, 8). Of note, upon glutamate treatment alone no NF-κB activation was discerned (Fig. 6C; Fig. 6E, lanes 3, 7, 11). In TNF pretreated wildtype neurons, weak NF-κB activity (Fig. 6B, gray bars and Fig. 6E, lane 2) strongly increased after glutamate stimulation and persisted at elevated levels for at least four hours (Fig. 6C, gray bars and Fig. 6E, lanes 4, 8, 12). In these cells, the enhancement was strongly reduced by MK801 and LY294002 when assessed during the first two hours (Fig 6C, gray bars and Fig. 6E, lanes 5, 6, 9, 10) and NF-κB activity was no longer discernable by EMSA in LY294002 treated cultures at four hours after glutamate stimulation (Fig. 6E, lane 14). These results indicate an important contribution of a TNFR2-dependent pathway for NF-κB
activation in wildtype neurons, in particular at later time points of glutamate exposure. TNFR1\(^{\text{+}}\) neurons had comparable activation kinetics to wildtype neurons with one remarkable difference: At every time point measured, NF-κB activation was completely inhibited by LY294002 (Fig. 6C, black bars and Fig. 6E, lanes 6, 10, 14), clearly suggesting that, in cortical neuronal cells, TNFR2-mediated NF-κB activation is PI3K dependent. Moreover, virtually complete blocking of the enhancement of the TNF induced NF-κB response by MK801 discloses the essential role of NMDAR co-activation in wildtype and TNFR1\(^{\text{+}}\) neurons (Fig. 6E, lanes 5, 9, 13). By contrast, PI3K dependence of neuronal TNFR1 mediated NF-κB activation was apparently less stringent. For example, although the NF-κB response of TNF pretreated TNFR2\(^{\text{+}}\) neurons was partially LY294002 sensitive 1hr post glutamate treatment, it remained completely unaffected by LY294002 at 2hrs (Fig. 6C, Fig.6E, lanes 4 vs. 6 and 8 vs. 10). Of greater relevance, EMSA confirmed that TNFR1 mediated NF-κB activation does not persist and lacks enhancement through NMDAR activation. Similar to PKB/Akt activity, NR2B/TNF transgenic neurons displayed basal NF-κB activity due to the constitutive expression of TNF (Fig. 6D and Fig. 6F, lane 1). Additional glutamate stimulation enhanced nuclear NF-κB translocation (Fig. 6D) and its activation (Fig. 6F, lanes 2, 5, 8), which could be partially reduced by MK801 (Fig. 6D and Fig. 6F lanes 3, 6, 9) and fully abrogated by LY294002 two and four hours after glutamate stimulation (Fig. 6D and Fig. 6F, lanes 7, 10). Further, incubation of NR2B/TNF transgenic neurons with LY294002 inhibited endogenous NF-κB activation as well (Fig. 6F, lane 12), indicating that chronic TNF expression in these cells apparently imposes a bias on TNF signaling towards TNFR2 pathways. Supershift analysis using antibodies against p65, p50 and cRel identified p50 and p65 as the pre-dominant NF-κB subunits (Fig. 6G) in all mouse strains tested. Although the latter experiment is in accordance with the view that both TNFR1 and TNFR2 use the canonical NF-κB pathway (9), the differential kinetics of activation and sensitivity towards PI3K inhibitors in primary cortical neurons indicate distinct upstream signal pathway usage of the two TNFRs. TNFR1-mediated induction of NF-κB is transient, whereas TNFR2 can induce a more lasting NF-κB activation. The latter is strictly PI3K dependent and enhanced by co-stimulation of the ionotropic NMDA receptor. In contrast, the TNFR1-mediated NF-κB activation is less dependent on PI3K and not enhanced by NMDA receptor activation.

**TNFR2-dependent induction of I-κB degradation is sensitive to PI3K inhibition**

To assess the molecular level of PI3K/Akt mediated NF-κB activation, I-κB\(\alpha\) phosphorylation and subsequent degradation was analyzed in NR2B/TNF transgenic or TNF pretreated wildtype, TNFR1\(^{\text{+}}\) and TNFR2\(^{\text{+}}\) neurons. In TNFR1\(^{\text{+}}\) cells, phosphorylation and depletion of I-κB occurred earlier and was stronger as compared to
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wildtype cells (Fig. 7A, lanes 3-7). LY294002 sensitivity of I-κB phosphorylation and degradation was more pronounced in TNFR1−/− as compared to wildtype neurons (Fig. 7A, lanes 10-14). Consistent with the shorter NF-κB activation kinetics in TNFR2−/− neurons, only transient I-κB phosphorylation and degradation was observed in these cells (Fig. 7A, lanes 3-7), being largely LY204002 insensitive (Fig. 7A, lanes 10-14). NR2B/TNF transgenic neurons exhibited continuous (Fig. 7B, lane 2-6), LY294002-sensitive (Fig. 7B, lanes 8-12) I-κB phosphorylation and depletion due to the continuous expression of TNF. This finding is consistent with the shorter NF-κB activation kinetics in TNFR2−/− neurons, being largely LY204002 insensitive (Fig. 7A, lanes 10-14). NR2B/TNF transgenic neurons exhibited continuous (Fig. 7B, lane 2-6), LY294002-sensitive (Fig. 7B, lanes 8-12) I-κB phosphorylation and depletion due to the continuous expression of TNF. This finding is consistent with the basal activation of NF-κB observed in these cells (Fig. 6C and 6E). LY294002 treatment significantly raised I-κB levels and decreased the proportion of phosphorylated I-κB in NR2B/TNF transgenic neurons (Fig. 7B, lane 7), indicating permanent, PI3K dependent signaling through TNFR2. The transient, LY249002 insensitive reduction in I-κB levels observed at around 60 minutes (Fig. 7B, lane 11) likely reflects concomitant TNFR1 activity and/or other TNFR2 independent signals. To verify specific proteosomal degradation, I-κB levels were assessed in the presence of the proteosomal inhibitor MG132, which prevented the stimulus dependent reduction in I-κB protein levels in all neuronal cultures (Fig. 7 C and D).

NF-κB activation is a central mediator of neuroprotection

To scrutinize the protective role of NF-κB from excitotoxic insult of cortical neurons, we blocked NF-κB by the use of three different inhibitors known to interfere with NF-κB activation at distinct levels, i.e. assembly of the IKK complex via Geldanamycin, blocking of I-κBα phosphorylation with BAY 11-7082 and interfering with proteosomal degradation of phosphorylated I-κB via MG-132 (29, 30, 31, 32). Although MG-132, Geldanamycin and BAY 11-7082 treatment themselves showed a slight toxic effect in the in vitro neuronal culture system, all these inhibitors completely blocked TNF induced protection in TNFR1−/− and wildtype cortical neurons (Fig. 8A-C). TNFR2−/− neurons served as a negative control and revealed no influence of the applied inhibitors on the excitotoxic sensitivity of these cells (Fig. 8A-C). Likewise, resistance of NR2B/TNF neuronal cultures towards glutamate induced cell death could be abrogated by pharmacological intervention with NF-κB activation (Fig. 8D). These data provide strong evidence that NF-κB activation and thus transcription of NF-κB dependent anti-apoptotic genes plays a major role in TNF-mediated protection of neurons from excitotoxic insults.
Discussion

We have recently shown the critical involvement of TNF and its receptors in retinal ischemia, in which TNFR1 deficient animals were protected from ischemic lesions, whereas TNFR2 deficient animals developed severe pathology and enhanced neuronal loss compared to wildtype animals. These data suggested a potential antagonistic function of TNFRs with respect to neuronal survival upon exogenous stress signals and/or tissue damage (10). Here we show that the same principle applies to cortical neurons and provide evidence for the underlying mechanism of the protective signal pathway using an in vitro model of glutamate induced cell death of primary cortical neurons. Together, our previous in vivo studies (10) and the mechanistic in vitro studies presented here provide compelling evidence for a TNF-mediated anti-apoptotic pathway via TNFR2. First, a transgenic mouse line (NR2B/TNF) expressing TNF under the control of the murine NMDAR subunit NR2B promoter exerts, in vivo, a locally restricted, modest TNF activity neither causing brain inflammation nor any apparent pathological signs or neurological deficits (own unpublished data). Cortical neurons isolated from these NR2B/TNF mice were significantly protected from glutamate-induced apoptosis. Second, we have used TNF pretreatment of wildtype and TNFR deficient primary cortical neurons to mimic the phenotype observed with neurons from TNF transgenic mice and demonstrate that TNF treated neurons from wildtype and TNFR1−/− mice were significantly protected, whereas primary neurons from TNFR2−/− mice were susceptible to cell death. Third, we show by kinetic analyses of NF-κB activation and by pharmacological inhibition of signal pathway components in the TNFR knockout mouse strains that neuroprotection is mediated via a TNFR2-PI3K-Akt-NF-κB pathway, in which the duration of NF-κB activation is the critical determinant in mounting resistance towards excitotoxic insults.

The high concentration of soluble, recombinant murine TNF required to achieve significant protection in vitro is already indicative for an involvement and critical role of TNFR2, which has at 37°C a lower affinity for soluble TNF and a reduced signal competence as compared to TNFR1, thus necessitating higher TNF concentrations for signalling (33). Moreover, primary neurons derived from the TNF transgenic mouse line, which are protected from excitotoxicity, express TNF not only in its processed form, but also at the neuronal cell membrane, a prerequisite for efficient TNFR2 activation. Both, an autotropic and a juxtatropic signalling of membrane expressed TNF is conceivable. As cortical neurons coexpress both TNFRs, we conclude that under constitutive membrane TNF expression in the transgenic neurons, the neuroprotective TNFR2 pathway is dominant over TNFR1 signals. Direct support for differential roles of the two TNFRs in signalling neuronal survival comes from the use of TNFR specific agonistic antibodies, where TNFR2-, but not TNFR1-specific agonists, promoted protection from excitotoxicity. A differential role of TNFR1 and 2 has also been reported for hippocampal
neurons responding to TNF alone with TNFR1 inducing both NF-κB activation and cell death, whereas TNFR2 was found to stimulate p38 MAPK (11). However, this study did neither address the functional significance of TNFR2 mediated p38 activation nor resolve the underlying mechanism of apoptosis dominance in these cells in the presence of an apparently strong NF-κB activation (11), which is considered to play an important role in neuroprotection (34). As an example, the cellular inhibitors of apoptosis proteins (cIAPs), well-known NF-κB-target gene(s) upregulated by TNF, have been recently identified as facilitators in downregulating apoptosis sensitivity within neurons. (35, 36). The prominent role of NF-κB in protection from neurodegeneration has recently received further support from a transgenic model characterized by forebrain-specific, inducible and complete ablation of NF-κB activity by I-κB superrepressor (37). Hippocampal slice cultures from these NF-κB deficient animals presented with strongly increased lesions upon excitotoxic stress.

In accordance with NF-κB’s important role in neuroprotection, we here show that upon in vitro glutamate exposure of TNF treated cortical neurons, a PI3 kinase-dependent PKB/Akt-phosphorylation was ensued by NF-κB activation. The kinetics of both PKB/Akt and NF-κB activation support a TNFR2 initiated signal pathway to NF-κB in a PI3K-dependent manner and the involvement of PKB/Akt. A potential role of other kinases located downstream of PI3K that could contribute to NF-κB activation in response to TNF, e.g. PKCζ, cannot be formally ruled out. However, in vivo, PKCζ is apparently a tissue selective NF-κB activator, with its dominant function (with respect to NF-κB activation) in lung tissues (40). Moreover, the main action of PKCζ in the context of TNF mediated NF-κB activation has now been recognized to be at the level of RelA transactivation (38). In contrast, our data indicate that PI3K operates at a level upstream of I-κB degradation, i.e. likely at the level of IKK activation or I-κB itself as shown in other cell systems (39-43). Examples with established cell lines show that PKB/Akt may serve as an IKK kinase in response to TNF (42). More recent data indicate that IKKα is the prime target of PKB/Akt phosphorylation in the heterotrimeric IKK complex, resulting in IKKβ activation and subsequent IkBα phosphorylation (47). Our data with primary neurons reveal PKB/Akt activation in a TNFR2 specific manner. Strict PI3K dependence of TNFR2 mediated I-κB phosphorylation and activation of p50/p65 as well as neuroprotection is in full agreement with a cell context specific function of PKB/Akt in the canonical NF-κB pathway.

Of note, NF-κB activation via TNFR1-induced pathways did not protect cortical neurons from glutamate induced apoptosis, which is in accordance with data from hippocampal neurons responding to TNF only (11). Interestingly, our data reveal that, in primary neurons, TNFR1 and TNFR2 induced pathways differ with respect to the kinetics and duration of NF-κB activation , with the TNFR1 inducing a rapid, but transient NF-κB
response, and TNFR2 generating a more persistent response, still discernible after several hours when stimulated with TNF alone. As an implication, the duration of NF-κB activation is critical to achieve significant tissue protection. The usage of different upstream activators of NF-κB by TNFR1 and 2 is a likely explanation for the observed differential kinetics and duration of the NF-κB response. Indeed, it becomes increasingly apparent that the cell specific quantitative composition with signal proteins contributing to a complex and multi-regulated pathway critically determines response phenotypes. For NF-κB activation, for example, a multitude of combinatorial possibilities exist, constituting canonical and non-canonical pathways, and comprised of several potential upstream IKK activating kinases and several I-κB isoforms with differential sensitivity to degradation and feedback regulation (48). A cell’s individual composition with the relative amounts of each of these signalling molecules may not only influence the potential linkage to receptor proximal signals, but also the kinetics and duration of NF-κB activation. Our data provide a clear example that differences in the initial signal input (TNFR1 vs. TNFR2 pathway), although activating the same intracellular key molecule (NF-κB), are translated into a completely different signal output (cell death vs. survival) by virtue of a differential duration of the active state of this critical molecule.

Blocking the NMDA receptor with MK801 lead to a reduction of PKB/Akt and NF-κB activation, revealing an as of yet unknown cross-talk between a cytokine receptor, TNFR2, and the ionotropic NMDA receptor. It can be speculated that this positive cooperation, with respect to PKB/Akt and NF-κB activation, may serve as a regulative feedback to limit cytotoxic signals emanating from over-activated NMDARs. In favour of this reasoning, only persistent NF-κB activation is associated with protection from cell death. Our data show that this requires a cooperative action of TNFR2 with the ionotropic NMDA receptor, which meet above or at the level of PI3K/PKB/Akt. The molecular mechanisms of the NMDAR-TNFR2 cooperation are not yet resolved. It is apparent from sequential stimulation studies (data not shown) that the signals emanating from TNFR2 have to precede the excitotoxic insult. At first glance, this would be in accordance with strictly TNF mediated acquisition of an apoptosis resistant state of the cell via an NF-κB dependent transcriptional response without a contribution of NMDAR signals. However, pharmacological inhibition of the PI3K pathway just before glutamate stimulation not only blocked the TNFR2 dependent and NMDAR enhanced stimulation of PKB/Akt and NF-κB, but also fully abrogated protection from apoptosis. This suggests that the acute signal crosstalk between the two receptor systems is essential for establishing a protective response. We propose that sustained PKB/Akt activity ensures prolonged NF-κB activity and counterbalances the negative feedback of NF-κB dependent I-κB expression typical for TNFR1 mediated signals causing transient NF-κB activity (9). Indeed,
TNFR2 mediated neuroprotection via PI3K/NF-κB pathway

neurons protected from excitotoxicity showed consistently reduced/lower IkB levels as compared to sensitive ones. In support of our reasoning, a recent study has linked calcium-induced pathways via PI3K and Akt towards NF-κB activation in neuronal cells (51).

A critical function of other PI3K- PKB/Akt pathways in neuronal survival has been proposed, and several downstream targets aside from NF-κB, in particular Bad, potentially interfere with apoptotic signals and could be involved in neuroprotection (49). For example, calcineurin-induced Bad dephosphorylation seems to play an important role in calcium-induced apoptosis within hippocampal neurons – an effect feasible in glutamate treated cortical neurons as well (50). A dominant role for other PKB/Akt substrates, including Bad, in TNF-induced protection of cortical neurons from excitotoxicity however, seems unlikely, because selective inhibitors of NF-κB activation operating downstream of PKB/Akt, such as an -specific inhibitor of I-κBα phosphorylation (31,32) or inhibition of proteosomal I-κB degradation (30) prevented protection from glutamate induced cell death.

TNF is upregulated in a number of neurodegenerative disorders including Alzheimer’s disease, stroke, and multiple sclerosis. However, its function remained to be defined because both neurotoxic and neuroprotective effects during disease pathogenesis have been described (50-54). The TNF transgenic mouse line described here clearly shows that restricted neuronal TNF expression is a priori not harmful. To the contrary, the response phenotype of primary neurons isolated from these mice suggest that TNF expression conditions the neurons towards resistance to excitotoxic insults. Support for the in vivo relevance of a TNFR2 mediated neuroprotective pathway was previously obtained by us in a model of retinal ischemia (10). We have now preliminary evidence in a model of cerebral ischemia that NR2B/TNF transgenic mice display reduced lesions as compared to wildtype animals (Eisel and Petit, unpublished data).

In conclusion, our data presented here that TNF mediates neuroprotection via TNFR2 and NMDAR co-signalling provide new mechanistic insights and shed new light onto the role of TNF in the brain. We propose that TNF functions as an important regulatory cytokine in the CNS, with differential signaling through the two distinct TNFRs determining its contribution to degenerative and regenerative processes. Thus, depending on the type and state of disease, environmental/external factors, cellular composition of the affected tissue and intracellular availability of TNFR signaling components, TNF may function to aggravate or ameliorate disease.

A complete understanding of the differential signals that are initiated by TNFR1 and TNFR2 in neurons, and of their cross-talk with other pathways that are important for neuronal viability, is essential in order to derive therapeutically effective strategies. From our study, we conclude that selective triggering of TNFR2 could be a novel therapeutic strategy in neurodegenerative diseases, because it actively induces an anti-apoptotic state in
neurons by cooperative action with neurotransmitter receptor signals. This mechanism, elucidated here, can now be exploited for therapeutic gain in combination with or independent of drugs interfering with apoptotic signal pathways.

Acknowledgement
The authors wish to thank Horst Bluethmann for kindly providing the TNFR1 and TNFR2 knockout mice. We gratefully acknowledge Hycult Biotechnology, HBT, NL for generous supply with murine TNFR1 and TNFR2 specific antibodies. We thank Bernd Kirchherr for excellent technical assistance, Jan Bauer and Hans Lassmann for help with immunohistochemistry, and Lesley Probert for critically reading the manuscript. U.E. and K.P. were supported by grants from Volkswagen Foundation, EU Commission, Hertie Foundation, and the Deutsche Forschungsgemeinschaft, SFB 495. L.M. is a recipient of a Landesgraduiertenförderung stipend from the state of Baden-Wuerttemberg.

References
TNFR2 mediated neuroprotection via PI3K/NF-κB pathway

TNFR2 mediated neuroprotection via PI3K/NF-κB pathway


TNFR2 mediated neuroprotection via PI3K/NF-κB pathway

Figure legends

Fig. 1 Generation of NR2B/TNF transgenic mouse lines. A. Schematic depiction of the transgene construct. The promoter and 5’-UTR of the NMDA receptor subunit NR2B gene (exon 1-3 in green boxes) was fused to the NarI site of the murine TNF gene (red boxes) in exon 1, therefore replacing part of its 5’-UTR. The 3’-UTR of the murine TNF gene was replaced by the human β-globin gene (yellow box; 14) for stabilizing the transgenic message, resulting in a 4.9 kb DNA fragment, which was used for pro-nuclear injection. B. In situ hybridization. 10µm semi-thin cryostat mid-sagittal sections were hybridized with a 35S-labelled TNF-anti-sense probe to detect TNF expression in the brain of transgenic mice (three month of age). In contrast to non-transgenic controls, transgene expression could be detected in forebrain regions, mainly in the cortex and hippocampus resembling the expression pattern of the NR2B gene. C. Western Blot. Detection of TNF expression in embryonic (E14) and postnatal cortical tissue (P14) of NR2B/TNF transgenic (tg) animals in comparison to non transgenic (ntg) control D. Immunocytochemistry. TNF expression was detected by a rabbit anti-TNF-antibody (red fluorescence, Alexa 546) in in vitro cultivated cortical neurons by video microscopy. Left picture shows TNF expression alone, and the right picture shows TNF expression merged with βIII-tubulin-staining (green fluorescence, Alexa 488) to mark neuronal cell bodies. E. Confocal section of TNF- (red fluorescence, Alexa 546) and tubulin- (green fluorescence, Alexa 488) stained cortical neurons to verify membrane expression of TNF. F. Immunohistochemistry. One month old tg and ntg cortex stained for TNF in the upper panel. Staining for CD45 (middle panel) and mac3 (lower panel) indicates activated microglia in TNF transgenic cortices.

Fig. 2 Glutamate induced apoptosis in primary cortical wildtype cultures. A. 250 (□) and 500 (■) µM glutamate induces neuronal death in a time dependent manner. Cell viability was assessed at the indicated times after a 1hour glutamate stimulus. B. Dose-dependent neuronal cell death in the absence (□) and presence (■) of the caspase inhibitor ZVAD (20µM). Cell death in A and B was assessed by MTT assay [n=3] as described in materials and methods. C. Immunocytochemistry. Dapi (blue fluorescence) and Annexin V (green fluorescence) staining of in vitro cultivated primary cortical neurons. Upper panel depicts unstimulated cells. Lower panel depicts neurons after treatment for one hour with 250 µM glutamate. Glutamate treated neurons exhibit membrane localized, punctuated Annexin V staining and nuclear fragmentation (arrows).

Fig. 3 TNF mediates anti-apoptotic signals via TNFR2. A. Differences in sensitivity for glutamate induced excitotoxicity of primary cortical neurons from different mouse lines in the absence (open symbols) or presence (filled symbols) of the NMDAR blocker MK801 [□, ■ wildtype (WT); Δ, ▲ NR2B/TNF; ○, ● TNFR1−/− (R1−/−);
TNFR2 mediated neuroprotection via PI3K/NF-κB pathway.

◊, ♦ TNFR2⁻/⁻ (R2⁻/-).

B. TNF dose-dependent protection of primary neuronal cultures after exposure to excitotoxic concentrations of glutamate (1 hr, 250µM) in the absence (open symbols) or presence (filled symbols) of the LY294002 [□, ■ WT; ○, ● R1⁻/-; ◊, ♦ R2⁻/-; Δ, ▲ wildtype WT without glutamate to detect possible toxicity of the inhibitor (performed for all cell lines, shown as an example for wildtype cells). C: The PI3K inhibitor LY249002 can revert neuroprotection in NR2B/TNF transgenic neurons[Δ, ▲ NR2B/TNF; □, ■ WT; open symbols without inhibitor, filled symbols with LY249002]. Cell death in A, B and C was assessed by MTT assay [n=3] as described in materials and methods.

Fig. 4 Agonistic TNFR antibodies mimic TNF preincubation in primary cortical wildtype neurons. A. Dose-dependent toxicity of primary neuronal cultures by agonistic TNFR1 monoclonal antibody after exposure to excitotoxic concentrations of glutamate (1 hr, 250µM) in the absence (open symbols) or presence (filled symbols) of LY294002 [□, ■ wildtype (WT); Δ, ▲ wildtype (WT) without glutamate to detect possible toxicity of the inhibitor]. B. and C. Dose-dependent neuroprotection of primary neuronal cultures by agonistic TNFR2 monoclonal antibody after exposure to excitotoxic concentrations of glutamate (1 hr, 250µM) in the absence (open symbols) or presence (filled symbols) of LY294002 (B) or wortmannin (C) [□, ■ wildtype (WT); Δ, ▲ wildtype (WT) without glutamate to detect possible toxicity of the inhibitor]. Cell death in A, B and C was assessed by MTT assay [n=3] as described in materials and methods.

Fig. 5 Western blot analysis of Akt in protein lysates from primary cortical cultures of different mouse lines treated with glutamate and inhibitors of NMDAR and/or PI3K. A. Detection of phosphorylated PKB/Akt (serine 473) and total PKB/Akt protein (in order to control equal protein loading) in wildtype (WT), TNFR1⁻/⁻ (R1⁻/-) and TNFR2⁻/⁻ (R2⁻/-) neurons after TNF-, MK801- or LY294002-preincubation and glutamate stimulation as indicated B. Detection of PKB/Akt and its phosphorylated residue (serine 473) in NR2B/TNF transgenic neurons after MK801- or LY294002-preincubation and glutamate stimulation as indicated.

Fig. 6 Nuclear p65 translocation in primary cortical neurons. A. Immunocytochemistry detection of p65 (red fluorescence, Alexa 546) and βIII-tubulin (green fluorescence, Alexa 488) in primary cortical wildtype neurons. Left picture shows a TNF pretreated neuron before and the right picture after stimulation with glutamate. B. Kinetic of TNF-induced nuclear translocation of p65. [Wildtype dark gray, TNFR1⁻/⁻ black and TNFR2⁻/⁻ white bars] C. Kinetic of p65 translocation after TNF (24h)-, MK801 (30min) - or LY294002 (30min)-preincubation
and glutamate stimulation as indicated. [Wildtype dark gray, TNFR1−/− black and TNFR2−/− white bars] D. Kinetic of p65 translocation in NR2B/TNF transgenic neurons after MK801 (30min) - or LY294002 (30min)-preincubation and glutamate stimulation as indicated. Nuclear translocation in B, C and D was quantified by counting within a total of 400 cells per experiment the percentage of neuronal cells with NF-κB nuclear staining (as shown in A after treatment with TNF and glutamate). All bars depict mean +/- SD of three independent experiments and the distribution of the data sets was analyzed by 1-way ANOVA.

NF-κB activation in primary cortical neurons. E. & F. Electrophoretic mobility shift assays (EMSA) for NF-κB activity in primary cortical cultures of different mouse lines. E Nuclear extracts of wildtype (WT), TNFR1−/− (R1−/−) and TNFR2−/− (R2−/−) neurons after TNF-, MK801- or LY294002- preincubation and glutamate stimulation as indicated were incubated with an 32P-labelled NF-κB consensus oligonucleotide and analyzed as described in materials and methods F. Nuclear extracts from NR2B/TNF transgenic neurons after MK801- or LY294002-preincubation and glutamate stimulation as indicated were incubated with an 32P-labelled NF-κB consensus oligonucleotide and analyzed as described in materials and methods G. Supershift for NF-κB. Nuclear extracts of WT, R1−/−, R2−/− and NR2B/TNF transgenic neurons were preincubated with p65, p50 or cRel antibodies (1µg/ml) and analyzed as described in materials and methods.

Fig. 7 Western blot analyses of I-κb in lysates from primary cortical cultures of different mouse lines treated with glutamate and inhibitors of PI3K A. Detection of I-κb degradation and phosphorylation in primary cortical wildtype (WT), TNFR1−/− (R1−/−) and TNFR2−/− (R2−/−) neurons after TNF- or LY294002-preincubation and glutamate stimulation as indicated. B. Detection of I-κb degradation and phosphorylation in NR2B/TNF transgenic neurons after LY294002-preincubation and glutamate stimulation as indicated. Immunoblotting of the glutamate receptor 1 protein (GluR1) was performed in order to control equal protein loading for all cell lines as GluR1-levels were constant during stimulation. C. Verification of I-κb phosphorylation in the presence of the proteosomal inhibitor MG-132 in primary cortical wildtype (WT), TNFR1−/− (R1−/−), TNFR2−/− (R2−/−) and neurons after TNF-preincubation and glutamate stimulation as indicated. D. Verification of I-κb phosphorylation in the presence of the proteosomal inhibitor MG-132 in primary cortical NR2B/TNF transgenic neurons after glutamate stimulation as indicated. Equal protein loading for C and D was assessed by GluR1 immunoblotting (data not shown).
Fig. 8 NF-κB activation is important for glutamate resistance. A. TNF-mediated neuroprotection in the absence (open symbols) or presence of the proteasome inhibitor MG-132 (filled symbols). [□, ■ wildtype (WT); ○, ● TNFR1−/− (R1−/−); □, ● TNFR2−/− (R2−/−); △, ▲ wildtype (WT) without glutamate to detect possible toxicity of the inhibitor (performed for all cell lines, shown as an example for wildtype cells] B. TNF-induced resistance against glutamate induced cell death in the absence (open symbols) or presence of the HSP90 inhibitor Geldanamycin (filled symbols). [□, ■ WT; ○, ● R1−/−; □, ● R2−/−; △, ▲ WT without glutamate to detect possible toxicity of the inhibitor (performed for all cell lines, shown as an example for wildtype cells] C. TNF-mediated neuroprotection in the absence (open symbols) or presence of the I-κB phosphorylation inhibitor BAY 11-7082 (filled symbols). [□, ■ wildtype (WT); ○, ● TNFR1−/− (R1−/−); □, ● TNFR2−/− (R2−/−); △, ▲ wildtype (WT) without glutamate to detect possible toxicity of the inhibitor (performed for all cell lines, shown as an example for wildtype cells]. D. Glutamate resistance of NR2B/TNF transgenic neurons can be reverted by both MG132 and Geldanamycin. [Δ NR2B/TNF, ▲ NR2B/TNF plus MG-132, ▼ NR2B/TNF plus Geldanamycin]. Cell death in A, B, C and D was assessed by MTT assay [n=3] as described in materials and methods.
A

MTT activity [% of control]

- 250µM glutamate
- 500µM glutamate

h after glutamate stimulus

B

MTT activity [% of control]

- glutamate
- glutamate + ZVAD

glutamate [µM]

C

unstim

8µm

250µM glu

Annexin V-FITC

DAPI
Marchetti et al., figure 4

A

MTT activity [% of control]

agonistic TNFR1-mab [µg/ml]
[filled symbols with LY294002]

WT

WT + Glu

B

MTT activity [% of control]

agonistic TNFR2-mab [µg/ml]
[filled symbols with LY294002]

WT

WT + Glu

C

MTT activity [% of control]

agonistic TNFR2-mab [µg/ml]
[filled symbols with wortmannin]

WT + Glu

WT + Glu
Marchetti et al., figure 5

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Marchetti et al., figure 7

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</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>10'</td>
<td>20'</td>
</tr>
<tr>
<td></td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>40'</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>60'</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>120'</td>
</tr>
</tbody>
</table>
Marchetti et al., figure 8

A

MTT activity [% of control]

TNF [ng/ml]

[filled symbols with MG-132]

WT

WT + Glu

R1-/- + Glu

R2-/- + Glu

B

MTT activity [% of control]

TNF [ng/ml]

[filled symbols with Geldanamycin]

C

MTT activity [% of control]

TNF [ng/ml]

[filled symbols with BAY 11-7082]

D

MTT activity [% of control]

glutamate [µM]

NR2B/TNF

NR2B/TNF + MG -132

NR2B/TNF + GA

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TNF mediated neuroprotection against glutamate induced excitotoxicity is enhanced by NMDA receptor activation: Essential role of a TNF receptor 2 mediated, PI3 kinase dependent NF-κB pathway
Lara Marchetti, Matthias Klein, Katalin Schlett, Klaus Pfizenmaier and Ulrich L.M. Eisel

J. Biol. Chem. published online May 21, 2004

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