Cellular/Molecular

Excitotoxic Death of Retinal Neurons *In Vivo* Occurs via a Non-Cell-Autonomous Mechanism

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The central hypothesis of excitotoxicity is that excessive stimulation of neuronal NMDA-sensitive glutamate receptors is harmful to neurons and contributes to a variety of neurological disorders. Glial cells have been proposed to participate in excitotoxic neuronal loss, but their precise role is defined poorly. In this *in vivo* study, we show that NMDA induces profound nuclear factor κB (NF-κB) activation in Müller glia but not in retinal neurons. Intriguingly, NMDA-induced death of retinal neurons is effectively blocked by inhibitors of NF-κB activity. We demonstrate that tumor necrosis factor α (TNFα) protein produced in Müller glial cells via an NMDA-induced NF-κB-dependent pathway plays a crucial role in excitotoxic loss of retinal neurons. This cell loss occurs mainly through a TNFα-dependent increase in Ca^{2+}-permeable AMPA receptors on susceptible neurons. Thus, our data reveal a novel non-cell-autonomous mechanism by which glial cells can profoundly exacerbate neuronal death following excitotoxic injury.

Introduction

Glutamate is the predominant excitatory neurotransmitter in many regions of the CNS. Elevation of endogenous glutamate and activation of glutamate receptors contribute to a variety of acute and chronic neurological disorders, including hypoxic–ischemic brain injury (stroke), trauma, seizures, and various forms of dementia and neurodegeneration (Kalia et al., 2008). In the retina, excess glutamate has been proposed to underlie common neurodegenerative disorders, including glaucoma and retinal artery occlusion (Hare et al., 2001, 2004; Casson, 2006; Seki and Lipton, 2008). The central hypothesis for excitotoxic injury is that excess glutamate binds to cell surface NMDA receptors on neurons, triggers massive Ca^{2+} influx, and activates proapoptotic signaling cascades. However, NMDA antagonists have consistently failed in clinical trials (Lee et al., 1999; Ikonomidou and Turski, 2002; Kemp and McKernan, 2002), suggesting that other mechanisms contribute to the devastating excitotoxic damage that occurs *in vivo*.

Neighboring non-neuronal cells may facilitate neuronal damage after excitotoxic injury. For example, activated microglia and astrocytes produce proinflammatory cytokines after excitotoxic injury (Wood, 1995; Barone and Feuerstein, 1999; Jeon et al., 2008; Vogt et al., 2008). Tissue plasminogen activator derived from microglia promotes excitotoxin-induced neuronal death in the hippocampus (Tsirka et al., 1995), whereas inhibition of microglial activation and proliferation has been shown to reduce excitotoxic injury (Tikka et al., 2001). Collectively, these data support a model in which excitotoxic brain damage activates glial cells, which then enhance neuronal death. However, the molecular mechanisms by which glial cells regulate excitotoxic loss of neurons have not yet been defined. A primary aim of this study was to investigate whether non-neuronal cells play a role in excitotoxic injury in the adult retina and, if so, to define the molecular pathways by which they influence neuronal death *in vivo*.

A potential effector of injury in the CNS is the nuclear factor κB (NF-κB). In non-neuronal cells, NF-κB activates transcription of antiapoptotic genes and production of cytokines and chemokines that mediate inflammation (Hoffmann and Baltimore, 2006). NF-κB also plays important roles in the nervous system, both promoting neuronal survival and facilitating synaptic function (Mattson and Camandola, 2001; Meffert and Baltimore, 2005). The role of NF-κB in neuropathological settings, however, has been controversial. Several studies have indicated that NF-κB is neuroprotective after injury (Maggirwar et al., 1998; Hamanoue et al., 1999; Bhakar et al., 2002), whereas others have shown that NF-κB activation facilitates neuronal damage (Schneider et al., 1999; Schwaninger et al., 2006).

In this study, we report that the extensive death of retinal neurons induced by NMDA is effectively blocked with pharmacological inhibitors of NF-κB activity. Using a transgenic NF-κB reporter mouse, we unequivocally identify the cellular site of NMDA-induced NF-κB activation as Müller glial cells. Further-
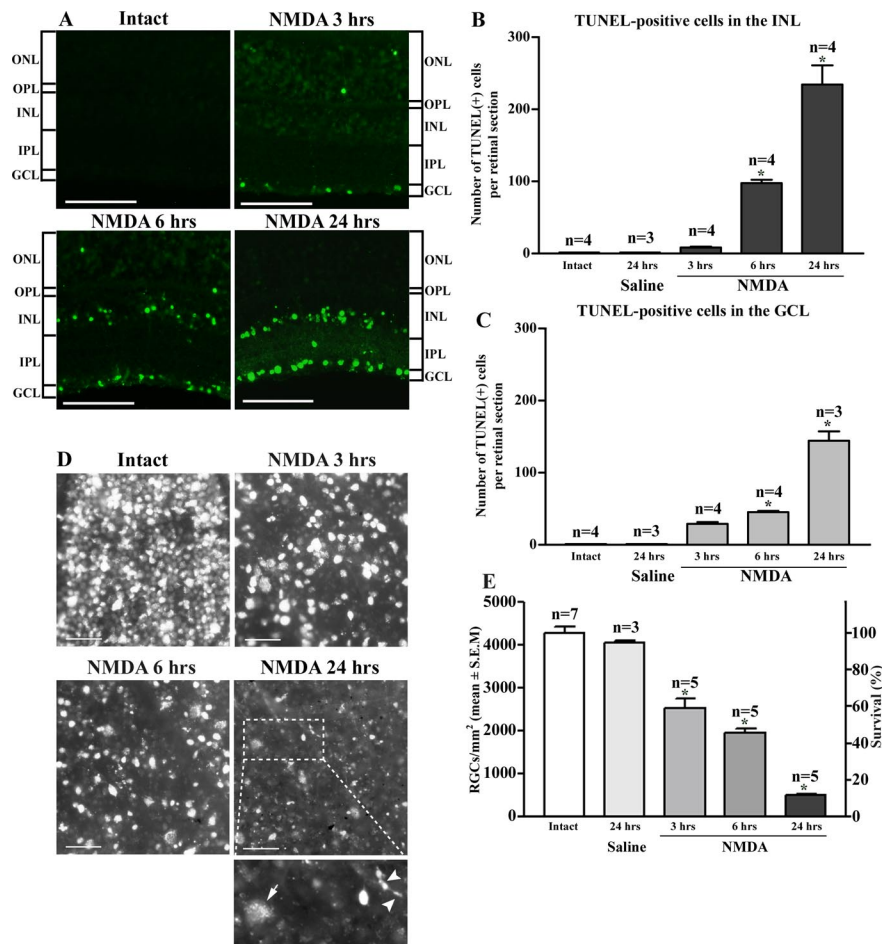
Figure 1. Retinal neurons die following intraocular injection of NMDA. **A**, Fluorescent microscopy images of retinal sections from intact or NMDA-treated eyes following TUNEL assay. **B**, **C**, Quantification of TUNEL$^+$ cells in the inner nuclear layer (**B**) and ganglion cell layer (**C**) demonstrated a striking increase in the number of apoptotic cells in the inner retina. **D**, Retinal whole mounts following retrograde labeling with Fluorogold were examined to assess the density of retinal ganglion cells in intact and NMDA-treated retinas. Inset shows an example of the morphological difference between retinal ganglion cells (arrow) and fluorogold-labeled microglia (arrowheads). **E**, Quantitative analysis of the number of retinal ganglion cells that survived at 3, 6, and 24 h after NMDA treatment. The density of retinal ganglion cells in intact, uninjured retinas is shown as reference (100% survival; ☐). Values are expressed as mean ± SEM (ANOVA, Tukey's multiple comparison test; *$p < 0.001$). ONL, Outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars, 100 μm (**A**), 50 μm (**D**).

more, we demonstrate that NF-κB-dependent tumor necrosis factor α (TNFα) production by Müller cells promotes retinal ganglion cell death. Finally, we show that Müller cell-derived TNFα leads to neuronal loss by increasing cell surface expression of Ca^{2+}-permeable AMPA receptors (AMPARs). Our data reveal a novel non-cell-autonomous pathway by which retinal glia can exacerbate neuronal death following excitotoxic injury.

Materials and Methods

Experimental animals

Experimental procedures were carried out on C57BL/6 transgenic or wild-type littermate control mice in accordance with the policies on the Use of Animals in Neuroscience Research and the Canadian Council on Animal Care guidelines (Olfert et al., 1993). The NF-κB reporter mice contain an NF-κB-responsive minigene composed of three tandem HIV-derived κB binding repeats placed upstream of a minimal SV40 promoter and drive expression of an *Escherichia coli* β-galactosidase (β-gal) gene tagged with a nuclear localization sequence (Bhakar et al., 2002). TNFα knock-out mice were kindly provided by Dr. D. Stellwagen (Montreal General Hospital, McGill University, Montreal, QC, Canada). The num-

ber of animals used in each experiment (*n*) is shown on each graph and/or in the figure legend.

Intraocular injections

NMDA (20 mM; Sigma) was injected into the vitreous chamber of the left eye using a 10 μl Hamilton syringe adapted with a 32 gauge glass microneedle (total volume, 2 μl). The vitreous fluid in adult mice is estimated to range from 5 to 20 μl (Remtulla and Hallett, 1985; Sharma et al., 2005; Yu and Cringle, 2006); therefore, the concentration of NMDA that reaches retinal ganglion cells is 2–8 mM. This amount of NMDA allows us to compare our results with previous studies using similar NMDA concentrations (Shimazawa et al., 2005; Ito et al., 2008; Nakazawa et al., 2008).

NMDA was injected alone or in combination with cell-permeable recombinant SN50 peptide (100 μg/ml; Biomol), SN50M (100 μg/ml; Biomol), PS1145 (500 μM; a kind gift from Millennium Pharmaceuticals), MK-801 (1 mM; Sigma), Etanercept (Enbril; 25 μg/μl; Immunex), z-IETD-fmk (50 μM; Biovision), GYKI 52466 (500 μM; Tocris), or IEM 13460 (500 μM; Sigma). Eyes injected with vehicle (PBS), control Fc, or noninjected intact eyes served as controls. Intraocular injections were performed under general anesthesia (2% isoflurane/oxygen mixture, 0.8 L/min). The needle tip was inserted into the superior hemisphere of the eye, at a 45° angle through the sclera into the vitreous body. This route of administration avoided retinal detachment or injury to eye structures, including the iris and lens, which release factors that induce neuronal survival (Mansour-Robaey et al., 1994; Leon et al., 2000; Fischer et al., 2001). Surgical glue (Indermill; Tyco Health Care) was used to seal the injection site.

β-gal detection

Mice were perfused transcardially with 0.8% glutaraldehyde, and the eyes were immediately dissected out. Tissue was incubated overnight at 37°C in staining solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (Invitrogen). Tissue was embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles), and retinal cryosections (16 μm) were collected onto gelatin-coated slides and examined under light microscopy.

Retinal immunohistochemistry

Mice were perfused with 4% paraformaldehyde, and retinal sections were prepared as above. Tissue sections were first incubated in 3% BSA and 0.3% Triton X-100 (Sigma) to block nonspecific binding, then incubated with primary antibody (Ab) (see list below) overnight at 4°C, followed by incubation with secondary Ab at room temperature. Slides were mounted with SlowFade (Invitrogen) and visualized on a Zeiss Axioskop 2 Plus microscope. Primary Abs used were β-gal (6.6 μg/ml; Millipore Bioscience Research Reagents), cellular retinaldehyde-binding protein (1:1000; a gift from J. C. Saari, University of Washington, Seattle, WA), protein kinase C (5 μg/ml; BD Biosciences), calretinin (2 μg/ml; Millipore Bioscience Research Reagents), glial fibrillary acidic protein (2.5 μg/ml; Millipore Bioscience Research Reagents), and macrophage/monocytes (MAC-1; 1:2; a gift from S. David, McGill University, Montreal, QC, Canada), and TNFα (0.4 μg/ml; Millipore Bioscience Research Reagents). The secondary Abs used were sheep anti-mouse IgG (1 μg/ml; FITC conjugate; Sigma) or anti-rabbit IgG (1 μg/ml; Cy3; Jackson ImmunoResearch Laboratories).

Immunoblot analysis
Retinas were homogenized in lysis buffer and centrifuged at 10,000 rpm for 5 min, and supernatants were collected and analyzed by SDS poly-acrylamide gels and immunoblotting. Membranes were incubated with TNFα Ab (0.2 μg/ml; Millipore Bioscience Research Reagents) or β-actin (0.5 μg/ml; Sigma), washed in Tris-buffered saline–Tween 20, and then incubated with peroxidase-linked secondary Ab (0.5 μg/ml; GE Healthcare). Protein signals were detected using a chemiluminescence reagent (ECL; GE Healthcare) followed by exposure of blots to X-OMAT (Kodak) imaging film.

Cell survival assays and quantification
Terminal dUTP nick end labeling. The terminal dUTP nick end labeling (TUNEL) assay (Gavrieli et al., 1992) was performed using the *In Situ* Apoptosis Detection Kit (Millipore Bioscience Research Reagents) on retinal cryosections following the manufacturer's instructions. TUNEL-positive cells in the entire retinal section were counted on 16 sections per eye.

Retrograde labeling. Fluorogold (2%; Fluorochrome) in 0.9% NaCl was applied to the superior colliculus as described previously (Sapieha et al., 2005). The maximum number of retinal ganglion cells is observed after 7 d of Fluorogold application, and it is stable for several weeks thereafter (Berkelaar et al., 1994; Mansour-Robaey et al., 1994; Peinado-Ramón et al., 1996; Cheng et al., 2002). Therefore, procedures were performed 1 week after Fluorogold application. Mice were perfused with 4% paraformaldehyde; the eyes were dissected and flat-mounted vitreal side up on glass sides. Fluorogold-positive retinal ganglion cells were counted in 12 retinal zones: three areas in each eye quadrant located at 0.5, 1.0, and 1.5 mm from the optic nerve head were examined (Sapieha et al., 2005), corresponding to a total area of 0.5 mm^2.

Identification of Ca^{2+}-permeable AMPARs
Ca^{2+}-permeable AMPARs were identified using the Co^{2+} staining technique (Osswald et al., 2007). Retinas were dissected, cut in small pieces, and incubated for 30 min in oxygenated assay buffer (5 mM KCl, 2 mM MgCl, 12 mM glucose, 20 mM bicarbonate, 139 mM sucrose, 57.5 mM NaCl, and 0.75 mM CaCl$_2$). The tissue was then incubated in 5 mM CoCl$_2$ and 10 mM L-glutamic acid in the presence or absence of AMPA receptor blockers GYKI 52466 (40 μM; Tocris) or IEM 13460 (100 μM, Sigma) for 15 min. Co^{2+} was precipitated with 0.24% ammonium sulfide, and the retina was then fixed in 0.8% glutaraldehyde and 20 μm sections were prepared. Silver enhancement of the Co^{2+} sulfide precipitate was performed using the Intense kit (GE Healthcare), then retinal sections were rinsed and mounted, and photomicrographs were captured with a Zeiss Axioplan 2 imaging microscope.

Statistical analyses
Statistical analyses were performed using one-way ANOVA, followed by nonparametric test (Tukey's multiple comparison test) or by Student's *t* test, as indicated in legends.

Results
NMDA induces NF-κB-dependent death of retinal neurons
Retinal neurons, particularly amacrine and retinal ganglion cells, are exquisitely sensitive to excitotoxicity (Siliprandi et al., 1993; Kikuchi et al., 1995; Manabe and Lipton, 2003). To dissect the signaling pathways that contribute to excitotoxic injury in the retina, we first established the time course of neuronal loss following NMDA intraocular injection. Two complementary techniques were used for quantification of neuronal cell death: TUNEL labeling on retinal cross sections, and Fluorogold retrograde labeling of retinal ganglion cells visualized on retinal whole mounts.

Figure 1*A–C* shows that TUNEL$^+$ cells were first detected 3 h after NMDA exposure, and their number increased markedly by 6 and 24 h after NMDA injection. TUNEL staining was observed in the inner nuclear layer, in which amacrine cells are found, and in the ganglion cell layer, in which retinal ganglion cells and displaced amacrine cells are located. Fluorogold retrograde label-
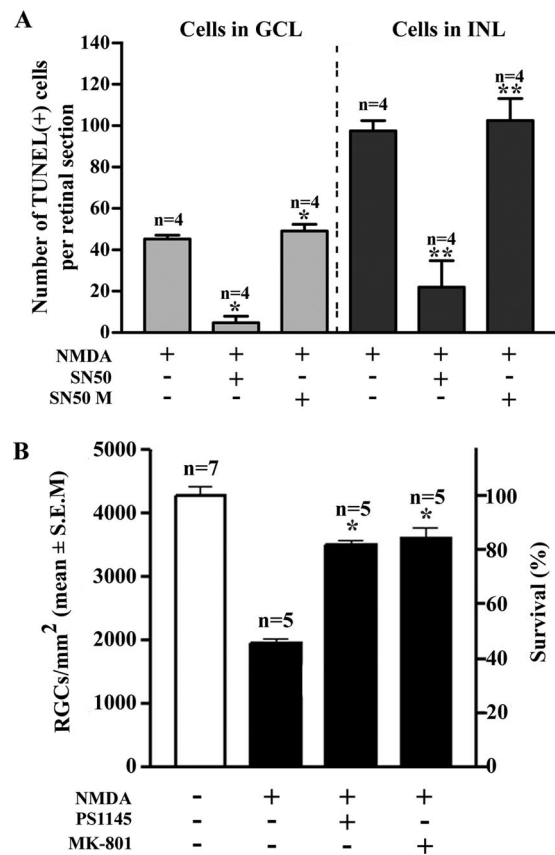

Figure 2. NMDA-induced neuronal death is NF-κB dependent. *A*, Quantification of TUNEL$^+$ cells in the inner nuclear layer (INL) or ganglion cell layer (GCL) at 6 h after treatment with NMDA alone, a mixture of NMDA and SN50, or NMDA and inactive SN50M peptide. In the presence of SN50, but not SN50M, there was a ninefold and fourfold reduction in the number of TUNEL$^+$ cells in the ganglion cell layer and INL, respectively. Values are expressed as mean ± SEM (ANOVA; Tukey's multiple comparison test; *p < 0.05; **p < 0.001). *B*, Treatment with PS1145, a small molecule inhibitor of IKK2, promoted retinal ganglion cell neuroprotection in the presence of NMDA (ANOVA; Tukey's multiple comparison test; *p < 0.001). The neuroprotective effect of PS1145 was comparable to that of MK-801, a noncompetitive antagonist of NMDA receptors (ANOVA; Tukey's multiple comparison test; *p < 0.001). Values are expressed as retinal ganglion cell densities (RGCs/mm^2; mean ± SEM) and are compared with intact retinas (100% survival; ☐).

ing, performed to assess retinal ganglion cell viability, showed that NMDA injection resulted in rapid loss of these neurons: 50% of retinal ganglion cells remained at 6 h after NMDA exposure, while <10% survived at 24 h post-NMDA treatment (Fig. 1*D,E*). Microglia and macrophages, which may have incorporated Fluorogold (Fluorochrome) after phagocytosis of dying retinal ganglion cells, were excluded based on morphological criteria. Microglia invariably have smaller cell size, visible process ramifications and lack axons (Thanos, 1991; Kacza and Seeger, 1997) (Fig. 1*D* inset, arrowheads). Although NMDA was administered in the dorsal (superior) retina, loss of Fluorogold-labeled retinal ganglion cells was similar between dorsal, ventral, nasal, and temporal retinal quadrants (ANOVA; p > 0.05; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The apparent higher number of TUNEL$^+$ cells compared with Fluorogold$^+$ cells in the ganglion cell layer (Fig. 1*A,D*; 24 h) likely reflects that this layer is composed of retinal ganglion cells and displaced amacrine cells in ~1:1 ratio (Perry, 1981). Therefore, TUNEL$^+$ cells in the ganglion cell layer represent both retinal ganglion cells as well as displaced amacrine cells, whereas Fluorogold labels only retinal ganglion cells.
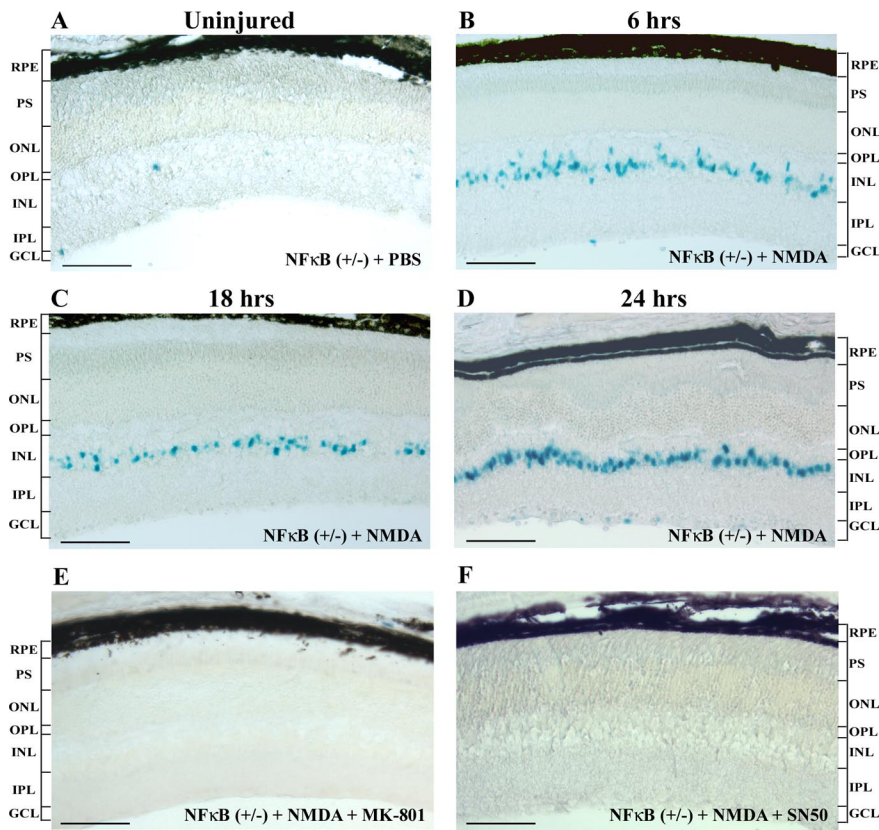
Figure 3. NF-κB is activated in the inner nuclear layer following exposure to NMDA. The cellular localization of NMDA-induced NF-κB activity in the adult retina was examined in NF-κB reporter mice. *A–D*, Almost no NF-κB reporter activity was detected in the PBS-treated, uninjured retina (n = 3) (*A*), whereas retinas examined at 6 h (*B*), 18 h (*C*), and 24 h (*D*) after NMDA injection showed a striking increase in β-gal activity in the inner nuclear layer (n = 4–6/group). *E*, *F*, The NF-κB transcriptional activity invoked by NMDA was completely blocked with the NMDA receptor antagonist, MK-801 (n = 4) (*E*), and with the NF-κB inhibitor SN50 (*F*, n = 4). RPE, Retinal pigment epithelium; PS, photoreceptor segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars, 100 μm.

NF-κB transcriptional activity has been reported to promote proapoptotic or antiapoptotic responses following CNS injury (Maggirwar et al., 1998; Hamanoue et al., 1999; Schneider et al., 1999; Bhakar et al., 2002; Schwaninger et al., 2006), but the molecular basis for these divergent effects remains unknown. To determine whether NF-κB activity influenced NMDA-induced neuronal death in the retina, we asked whether SN50, a cell-permeable peptide that blocks nuclear translocation of NF-κB subunits, altered levels of NMDA-induced cell loss. Intriguingly, coadministration of SN50 with NMDA sharply reduced the number of TUNEL$^+$ cells, both in the ganglion cell layer and inner nuclear layer (Fig. 2*A*). In contrast, SN50M, a mutant form of SN50 that does not block NF-κB nuclear translocation, had no effect on NMDA-induced TUNEL labeling.

Induction of canonical NF-κB signaling requires activation of IκB kinase (IKK)1 and IKK2, kinases that phosphorylate IκB family members (Schmid and Birbach, 2008). IKK2 often plays an obligatory role in canonical NF-κB signaling; thus, we asked whether PS1145, a small-molecule inhibitor of IKK2, altered retinal ganglion cell death induced by NMDA. Figure 2*B* shows that PS1145 elicited a remarkable inhibition of NMDA-induced retinal ganglion cell death, equivalent to that evoked by SN50 or by the noncompetitive NMDA receptor antagonist, MK801. Therefore, NF-κB is strongly activated in the retina following NMDA exposure and inhibitors that block NF-κB transcriptional activity, by different mechanisms, rescue neurons challenged with NMDA. We conclude that NF-κB activation plays a crucial role in NMDA-induced loss of retinal neurons.

NMDA induces NF-κB activity in Müller glia but not in retinal neurons

To determine the cellular localization of NMDA-induced NF-κB activity in the adult retina, we used a well-characterized reporter mouse in which NF-κB-responsive elements drive expression of nuclear β-gal (Bhakar et al., 2002). In the PBS-treated, uninjured retina, almost no NF-κB reporter activity was detected (Fig. 3*A*). In contrast, retinas examined at 6, 18, and 24 h after NMDA injection showed a striking increase in β-gal activity within the inner nuclear layer (Fig. 3*B*–*D*). The induction of NF-κB in the retina invoked by NMDA was completely blocked by the NMDA receptor antagonist, MK-801 (Fig. 3*E*), and with the NF-κB inhibitor SN50 (Fig. 3*F*). These results indicate that the β-gal activity detected in the inner nuclear layer reflects an NF-κB transcriptional response mediated through activation of NMDA receptors.

The inner nuclear layer of the retina consists of interneurons (amacrine, bipolar, and horizontal cells) and Müller glial cells. To determine which of these cell types exhibit NMDA-induced NF-κB activation, retina derived from NF-κB reporter mice were stained using antibodies for β-gal and for retinal cell-type markers. After intravitreal injection of NMDA, β-gal-expressing cells were consistently positive for cellular retinaldehyde-binding protein (CRALBP), a specific Müller cell marker (Fig. 4*A*–*C*). In contrast, β-gal staining was never found in cells positive for markers of bipolar and amacrine cells, including protein kinase C (Fig. 4*D*–*F*) or calretinin (Fig. 4*G*–*I*), respectively. We conclude that Müller glia is the only cell type within the inner nuclear layer that undergoes NMDA-induced NF-κB activation.

NMDA exposure induced β-gal expression in a very small number of cells within the ganglion cell layer in the NF-κB reporter mice (Fig. 3*B*–*D*). In addition to retinal ganglion cells, this region contains astrocytes, macrophages, and displaced amacrine cells. The few β-gal-labeled cells observed in this region were invariably positive for cell-specific markers of astrocytes (GFAP, Fig. 4*J*–*L*) or macrophages (MAC-1, Fig. 4*M*–*O*). β-gal staining was never observed in retinal ganglion cells retrogradely labeled with Fluorogold (Fig. 4*P*–*R*) or in calretinin-positive displaced amacrine cells (data not shown). These data demonstrate that NMDA induces NF-κB activity in Müller glia but not in retinal neurons. This finding led us to test the hypothesis that a Müller cell-derived factor, regulated by NF-κB, acts in a non-cell-autonomous manner to facilitate neuronal death following NMDA exposure.

TNFα produced by Müller cells promotes NMDA-dependent neuronal loss

TNFα transcription is strongly regulated by NF-κB activity (Shakhov et al., 1990; Hiscott et al., 1993; Mori and Prager, 1996), and TNFα added exogenously can increase death of retinal neurons (Tezel and Wax, 2000; Nakazawa et al., 2006; Berger et al., 2008). We therefore tested whether Müller cell-derived TNFα acts as an endogenous factor that facilitates NMDA-dependent neuronal death *in*

vivo. Figure 5*A* shows that TNFα protein was undetectable in the adult, intact retina, but its levels increased dramatically after NMDA exposure. The NMDA-induced increase in TNFα expression was completely blocked by the NF-κB inhibitor SN50, indicating that activation of NF-κB plays a central role in NMDA-dependent expression of this cytokine. Similarly, TNFα was not detected in the uninjured retina by immunocytochemistry (Fig. 5*B*) but was clearly visualized after NMDA injection in presumptive Müller cell bodies and processes in the inner retina (Fig. 5*C*). Moreover, NMDA-induced accumulation of TNFα immunoreactivity was blocked by the NF-κB inhibitor SN50 (Fig. 5*D*). Colabeling experiments using the Müller cell-specific marker CRALBP confirmed that Müller cells selectively upregulate expression of TNFα in the presence of NMDA (Fig. 5*E–G*). We conclude that Müller glial cells are the main cellular source of TNFα production in the retina following NMDA exposure.

To test whether Müller cell-derived TNFα facilitates excitotoxic death of retinal neurons, we examined whether the extent of NMDA-induced retinal ganglion cell loss was attenuated in the absence of TNFα. Our data show striking survival of retinal ganglion cells in TNFα knock-out mice at 6 h after NMDA exposure: 76% of these neurons remained alive compared with only 42% in wild-type littermate controls ($p <$ 0.001) (Fig. 5*H*). Although the total number of retinal ganglion cells in adult TNFα null mice treated only with vehicle was similar to that in wild-type mice (Fig. 5*H*), it is conceivable that increased neuronal survival following NMDA injection in TNFα null mice reflects a chronic effect of TNFα deprivation. As an alternative approach, we blocked TNFα action in the retina using Etanercept, a soluble Fc fusion protein that contains the extracellular ligand-binding domain of TNFα receptor (TNFR)2 that is used clinically for blockade of TNFα in humans with rheumatoid arthritis or psoriasis (Fantuzzi et al., 2008; Lyudmila, 2008). Figure 5*H* shows that Etanercept (Immunex), but not control Fc, protected retinal ganglion cells from NMDA-induced death. Etanercept protected 75% of these neurons from NMDA-induced death compared with only ∼45% in Fc-treated or vehicle-treated controls ($p <$ 0.001). We conclude that TNFα derived from Müller glia acts in a non-cell-autonomous manner to promote NMDA-dependent neuronal death in the retina.

Endogenous TNFα exacerbates excitotoxicity by increasing neuronal Ca²⁺-permeable AMPARs

We then asked what mechanisms are used by endogenous TNFα released from Müller cells to exacerbate NMDA-dependent neu-
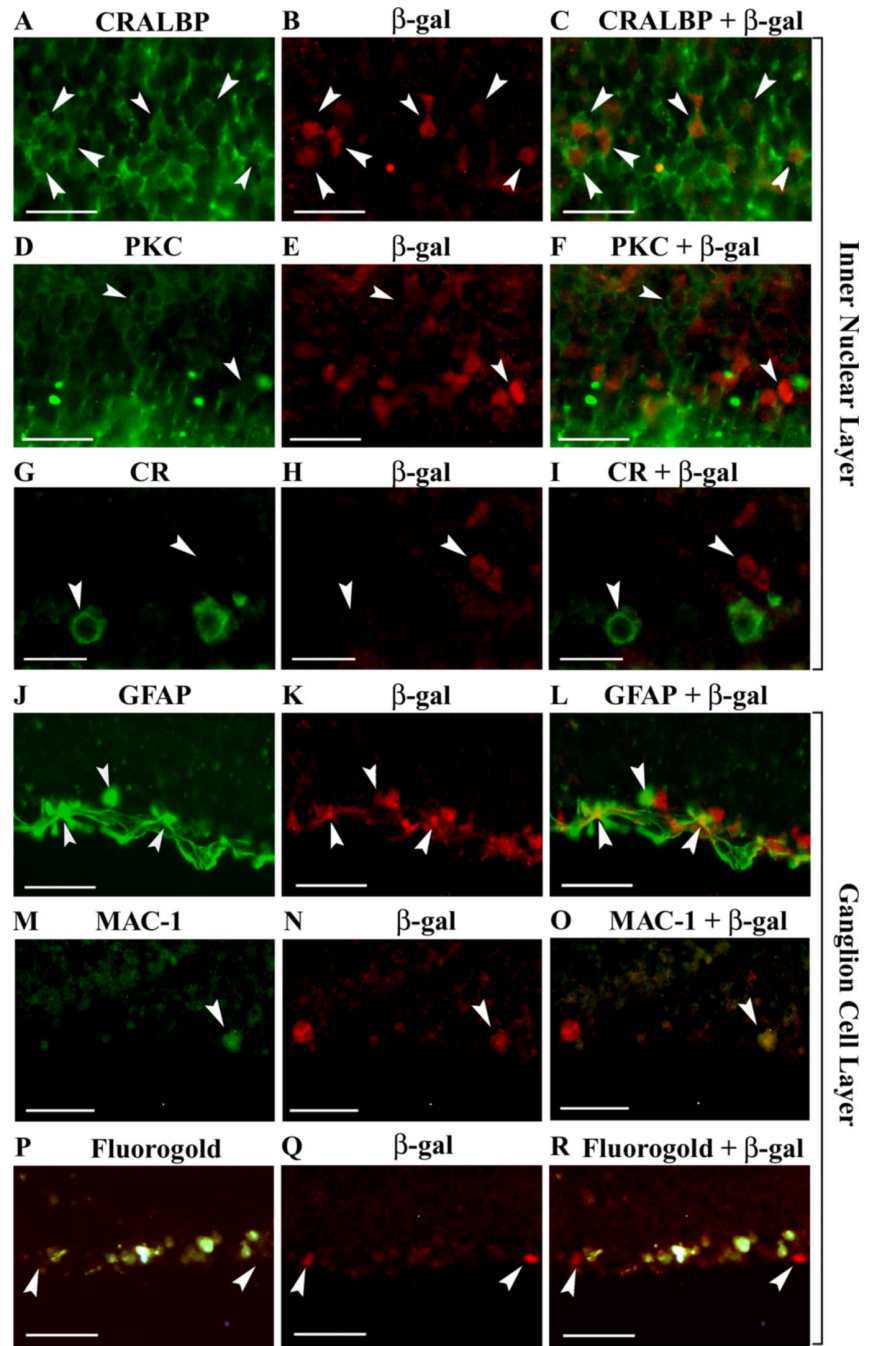

Figure 4. NMDA induces NF-κB activity in Müller glia but not in retinal neurons. Double immunohistochemistry using a β-gal Ab and specific markers for each retinal cell type was carried out on retinal sections at 24 h after NMDA injection. In the inner nuclear layer, cells positive for CRALBP, a Müller cell-specific marker, were always positive for β-gal (*A–C*). No colocalization was found between β-gal and bipolar cell markers, including protein kinase C (PKC) (*D–F*, arrows), or amacrine cell markers, including calretinin (CR) (*G–I*, arrows). The few β-gal-labeled cells observed in the ganglion cell layer were invariably positive for markers of astrocytes (GFAP) (*J–L*) or macrophages/microglia (MAC-1) (*M–O*). β-gal staining was never observed in retinal ganglion cells retrogradely labeled with Fluorogold (*P–R*) or in displaced amacrine cells (data not shown). All scale bars, 50 μm, except *G–I*, 12.5 μm.

ronal loss in the retina. Engagement of the TNFR1 receptor can activate caspase-8 to induce apoptosis (Thorburn, 2004). We therefore tested whether inhibition of caspase-8 activation reduced NMDA-mediated excitotoxicity. Blockade of caspase 8 activity using z-IETD-fmk, a potent caspase 8 inhibitor previously shown to effectively inhibit caspase 8 *in vivo* at the concentrations used here (Weishaupt et al., 2003), had no effect on NMDA-
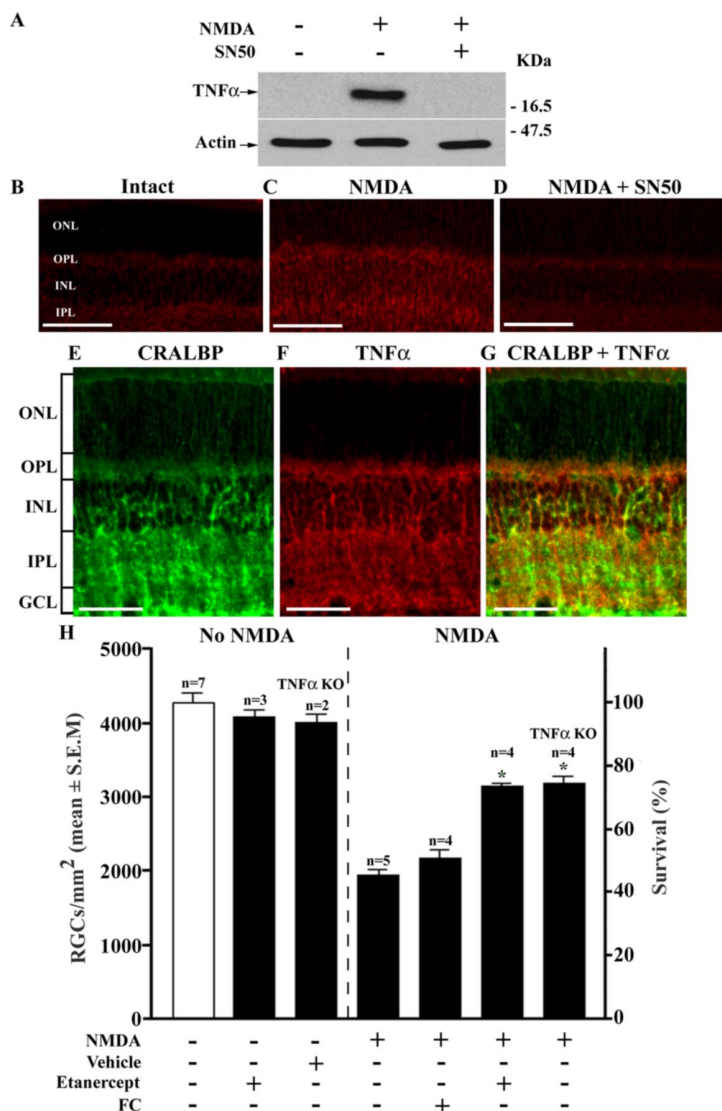
Figure 5. TNFα produced by Müller glia promotes NMDA-dependent neuronal loss. **A**, Western blot analysis of retinal homogenates show that TNFα protein levels were low in the uninjured retina and increased following NMDA intraocular injection. NMDA-induced increase in TNFα protein was blocked with the NF-κB inhibitor SN50. **B**, **C**, By immunohistochemistry, TNFα was not detected in the uninjured retina (**B**) but was clearly visualized after NMDA injection (**C**) in presumptive Müller cell bodies and processes in the inner retina. **D**, NMDA-induced TNFα immunoreactivity was blocked by the NF-κB inhibitor SN50. **E–G**, Colabeling experiments using the Müller cell-specific marker CRALBP confirmed that TNFα is upregulated in Müller glia in the presence of NMDA. Scale bars, 100 μm (**B–D**), 50 μm (**E–G**). **H**, Striking neuroprotection after NMDA exposure was observed in TNFα knock-out mice or in the presence of the TNFα blocker Etanercept, whereas no survival was observed in wild-type littermate or Fc-treated controls (ANOVA; *$p < 0.001$). Values are expressed as retinal ganglion cell densities (RGCs/mm^2; mean $\pm$ SEM) and are compared with intact retinas (100% survival, $\square$).

induced retinal ganglion cell death (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). z-IETD-fmk, by itself, was not toxic and did not promote retinal ganglion cell death. These findings indicate that TNFα-dependent neuronal loss occurs via an alternative pathway.

Exogenous TNFα has been shown to induce selective cell surface expression of Ca^{2+}-permeable AMPARs (Beattie et al., 2002; Ogoshi et al., 2005; Stellwagen et al., 2005), and their expression has been correlated with enhanced neuronal death in models of ischemia and excitotoxicity (Hermann et al., 2001; Noh et al., 2005; Ferguson et al., 2008; Leonoudakis et al., 2008). We tested the hypothesis that TNFα produced by Müller glia increases cell surface Ca^{2+}-permeable AMPARs on retinal neurons, thus rendering them more susceptible to excitotoxicity. Ca^{2+}-permeable

AMPARs transport divalent cations such as cobalt (Co^{2+}); therefore, we used an *in situ* Co^{2+} staining technique to identify retinal cells that express Ca^{2+}-permeable AMPARs. In the absence of NMDA, Co^{2+} accumulation was restricted to cells in the inner nuclear and ganglion cell layers (Fig. 6A) previously identified as horizontal and AII amacrine cells (Osswald et al., 2007) as well as ganglion cells (Zhang et al., 1995). Following NMDA exposure, both amacrine and retinal ganglion cells accumulated Co^{2+}, indicating that Ca^{2+} permeability in these cells was markedly enhanced when the retina was challenged with NMDA (Fig. 6B). To test whether TNFα was required for NMDA-induced increase of Ca^{2+}-permeable AMPARs on retinal neurons, NMDA was coadministered with the TNFα antagonist Etanercept. Figure 6C shows that Etanercept effectively diminished the NMDA-induced Co^{2+} uptake.

To confirm that increased Co^{2+} accumulation in retinal neurons occurs via Ca^{2+}-permeable AMPARs and not other glutamate receptors, retinas were incubated with GYKI, a noncompetitive AMPAR-selective antagonist, and IEM, a channel blocker selective for Ca^{2+}-permeable non-NMDA receptors (i.e., AMPA and kainate receptors). Figure 6, D and E, shows that NMDA-induced Co^{2+} accumulation in these neurons was blocked with IEM and GYKI, indicating that NMDA-induced Co^{2+} accumulation is due to increased flux of divalent cations exclusively through AMPARs. Finally, we tested whether TNFα-mediated increase in neuronal Ca^{2+} permeability influenced neuronal death following NMDA exposure. Figure 6F shows that retinal ganglion cells were markedly protected from NMDA-induced death in the presence of either IEM (63% survival) or GYKI (76% survival) with respect to control retinas (ANOVA; $p < 0.001$). The neuronal survival afforded by AMPAR blockers was similar to that observed in TNFα null mice or following treatment with the TNFα antagonist Etanercept (Fig. 5H). We conclude that Müller cell-derived TNFα plays a crucial role in the NMDA-dependent elaboration of Ca^{2+}-permeable AMPARs and, ultimately, excitotoxic death of retinal neurons.

Discussion

This study reports four major findings. First, NMDA exposure causes induction of NF-κB activity in Müller glia, but not neurons, in the adult retina. Second, the death of amacrine and retinal ganglion cells induced by NMDA is sharply attenuated when Müller cell NF-κB activation is blocked. Third, TNFα is the target of NF-κB activity in Müller cells and is required for NMDA-induced loss of retinal neurons. Fourth, increased levels of Ca^{2+}-

permeable AMPARs evoked by Müller cell-derived TNFα is a crucial mechanism that renders retinal neurons susceptible to excitotoxic injury.

The accessibility and defined cellular architecture of the retina, together with the exquisite sensitivity of retinal neurons to NMDA, make it an ideal system to elucidate the contribution of NF-κB to excitotoxicity. We showed that NF-κB is activated in Müller glia following NMDA exposure. The effect of NMDA on NF-κB activation in Müller glia was blocked by MK-801, confirming that NMDA receptors are involved in this response. NMDA receptors have been described on dissociated Müller cells from human (Puro et al., 1996) and chick retinas (Lamas et al., 2005), and *in situ* hybridization studies have demonstrated expression of NMDA receptors in the rat inner plexiform layer where Müller cells reside (Brandstätter et al., 1994; Hartveit et al., 1994; Fletcher et al., 2000). The functional properties of glial and neuronal NMDA receptors are thought to be different (Lamas et al., 2005); it is thus possible that NMDA receptors with different oligomeric structures differ in their ability to activate NF-κB. Alternatively, NMDA-dependent NF-κB activation in retinal glia may be indirect and results from a stimulating factor produced by neurons. Candidates for this effect are neuronal release of ATP and activation of glial P2Y receptors (Metea and Newman, 2006) and glutamate, which is released in high amounts by stressed neurons (Fujimoto et al., 2004). Regarding the latter possibility, previous studies have shown that occupancy of metabotropic glutamate receptors on glia can induce NF-κB activation (Caccamo et al., 2005a,b; Kaushal and Schlichter, 2008; Sitcheran et al., 2008).

Blockade of NF-κB activity in Müller cells dramatically reduced amacrine and retinal ganglion cell death, indicating that a Müller cell-derived factor plays a crucial role in NMDA-induced excitotoxicity. We focused on TNFα as a major effector in this pathway because the TNFα gene is tightly regulated by NF-κB, and previous studies have indicated that TNFα can facilitate excitotoxic damage (Chao and Hu, 1994; Hermann et al., 2001; Bernardino et al., 2005; Noh et al., 2005; Ferguson et al., 2008; Kaushal and Schlichter, 2008; Leonoudakis et al., 2008). We showed that TNFα in Müller glia was dramatically increased by NMDA exposure, and our loss of function experiments confirmed that TNFα plays an obligatory role in the loss of retinal neurons induced by NMDA. These results are consistent with a previous study showing that retinal ganglion cells lacking TNFR1 are protected following optic nerve crush (Tezel et al., 2004). Interestingly, TNFα and
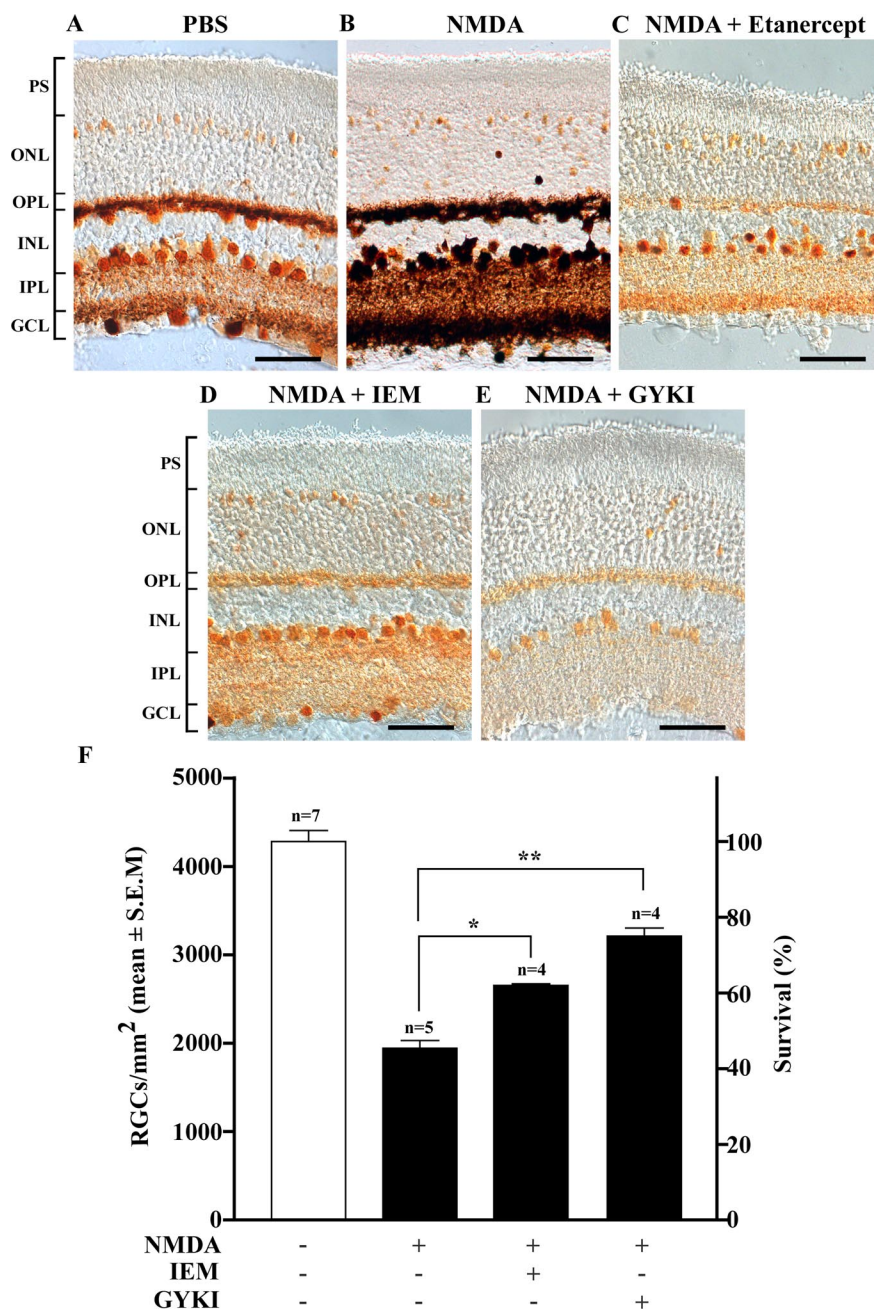

Figure 6. TNFα exacerbates excitotoxicity by increasing neuronal Ca^{2+}-permeable AMPARs. **A**, In the absence of NMDA, Co^{2+} accumulation was restricted to a few cells in the inner nuclear and ganglion cell layers. **B**, Following NMDA exposure, both amacrine and retinal ganglion cells accumulated Co^{2+}, indicating that Ca^{2+} permeability in these cells was markedly enhanced. **C–E**, The TNFα antagonist Etanercept (**C**), as well as the AMPAR blockers IEM-1460 (**D**) and GYKI (**E**) effectively reduced NMDA-induced Co^{2+} uptake. Scale bars, 40 μm. **F**, Retinal ganglion cells were protected from NMDA-induced death in the presence of either IEM-1460 or GYKI, with respect to control retinas (ANOVA; *$p < 0.05$; **$p < 0.001$). Values are expressed as retinal ganglion cell densities (RGCs/mm²; mean ± SEM) and are compared with intact retinas (100% survival, □).

TNFR1 have been shown to be upregulated in human donor eyes with glaucoma (Yan et al., 2000; Yuan and Neufeld, 2000; Tezel et al., 2001), diabetic retinopathy, and age-related macular degeneration (Armstrong et al., 1998; Oh et al., 1999); in addition, TNFα gene polymorphisms have been found in patients with primary open angle glaucoma (Lin et al., 2003; Funayama et al., 2004), supporting a crucial role for TNFα in ocular diseases.

How does NF-κB-mediated production of TNFα by Müller glia kills retinal neurons? Ligand binding to TNFR1 triggers ap-

optosis by induction of caspase-8 activation, which in turn activates caspase-3 and promotes cell death (Boldin et al., 1996; Hsu et al., 1996). Previous studies have shown that TNFα released from microglia can kill primary cortical neurons via a caspase-8-dependent mechanism (Velier et al., 1999; Kaushal and Schlichter, 2008); however, we found that caspase-8 inhibition had no effect on NMDA-induced apoptosis of retinal neurons *in vivo*. Nevertheless, apoptosis is still a major cell death mechanism in this model based on the widespread TUNEL labeling observed by us and others (Manabe and Lipton, 2003; Nakazawa et al., 2005) and is likely to involve induction of the intrinsic mitochondrial pathway and activation of caspase-3.

Our data support an alternative mechanism that invokes TNFα-mediated increase of Ca^{2+}-permeable AMPARs on neuronal cell surfaces. A series of elegant *in vitro* studies demonstrated that glia-derived TNFα selectively stimulates cell surface insertion of Ca^{2+}-permeable AMPARs, while simultaneously decreasing surface $GABA_A$ receptors, a process required for rapid control of synaptic strength at excitatory synapses (Beattie et al., 2002; Stellwagen et al., 2005; Stellwagen and Malenka, 2006). Ca^{2+}-permeable AMPARs have been proposed as mediators of excitotoxic cell death (Kwak and Weiss, 2006; Liu and Zukin, 2007). Recent studies have shown that exogenous TNFα may contribute to excitotoxicity by increasing cell surface levels of Ca^{2+}-permeable AMPARs in primary hippocampal neurons and spinal cord (Ferguson et al., 2008; Leonoudakis et al., 2008), but the cellular source and mechanisms that lead to TNFα production were not addressed in these studies. We provide the first *in vivo* evidence that Müller glia-derived TNFα exacerbates excitotoxic damage by increasing surface levels of Ca^{2+}-permeable AMPARs in retinal neurons.

Our data support a model in which non-cell-autonomous signaling events play a major role in NMDA-triggered excitotoxicity in the retina. We demonstrate that NMDA-dependent activation of NF-κB in Müller glial cells induces production of endogenous glia-derived TNFα, which in turn renders retinal neurons highly sensitive to excitotoxicity by increasing their surface levels of Ca^{2+}-permeable AMPARs. Our loss-of-function experiments show that this non-cell-autonomous mechanism accounts for >60% of the NMDA-induced neuronal loss in the retina. Given the prevalence of NMDA receptors on retinal neurons (Zhang and Diamond, 2006), direct action of NMDA may play a modest, yet tangible, role in excitotoxicity in this system. However, our findings are a departure from the traditional paradigm of excitotoxic damage in which the death of neurons was entirely attributed to excessive Ca^{2+} influx via neuronal NMDA receptors. Instead, our data indicate that glial regulation of neuronal AMPAR distribution via TNFα is a central requirement for neuronal loss in retinal excitotoxicity.

There is strong evidence that glutamate, the endogenous neurotransmitter involved in excitotoxicity, increases intracellular Ca^{2+} in retinal ganglion cells primarily via NMDA receptors (Hartwick et al., 2008) raising the question: how do AMPARs mediate neuronal death? Interestingly, AMPARs are highly permeable to Zn^{2+}, whereas NMDA receptors are blocked by this cation. Zn^{2+}, known to accumulate in hippocampal neurons following ischemia and before the onset of cell death (Koh et al., 1996; Lee et al., 2003), is extremely toxic for neurons (Sensi et al., 1999, 2003; Jiang et al., 2001; Kim and Koh, 2002). The elucidation of the precise role of Zn^{2+} in retinal ganglion cell death will be an interesting topic for future studies.

Our results are unexpected because Müller cells have been traditionally attributed a neuroprotective role under physiological conditions (Bringmann et al., 2006). Indeed, one of the normal functions of Müller cells is the modulation of synaptic transmission and neuronal excitability in the mammalian retina (Newman, 2004). It is possible that the injury-induced effects reported here represent the end of a physiological continuum; it will be interesting to determine if the low levels of TNFα produced by Müller cells in the uninjured, adult retina alters synaptic transmission through AMPAR-dependent mechanisms.

The failure of NMDA receptor antagonists in clinical trials (Lee et al., 1999; Ikonomidou and Turski, 2002; Kemp and McKernan, 2002; Kalia et al., 2008) presupposes that other molecular mechanisms play a critical role following the initial excitotoxic insult. Our study provides new insight into these events *in vivo* by demonstrating a new role for NF-κB in the injured CNS. Furthermore, we identify TNFα as a key glial-to-neuron signal that exacerbates excitotoxicity through modulation of Ca^{2+}-permeable AMPARs. The precise delineation of the molecular events that occur in glia versus those in neurons, as shown here, should be useful for the design of novel therapeutic interventions applicable to neurodegenerative diseases and ischemia.

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