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Minocycline Provides Neuroprotection Against *N*-Methyl-D-aspartate Neurotoxicity by Inhibiting Microglia¹

Tiina M. Tikka* and Jari E. Koistinaho^{2*†}

Glutamate excitotoxicity to a large extent is mediated through activation of the *N*-methyl-D-aspartate (NMDA)-gated ion channels in several neurodegenerative diseases and ischemic stroke. Minocycline, a tetracycline derivative with antiinflammatory effects, inhibits IL-1 β -converting enzyme and inducible nitric oxide synthase up-regulation in animal models of ischemic stroke and Huntington's disease and is therapeutic in these disease animal models. Here we report that nanomolar concentrations of minocycline protect neurons in mixed spinal cord cultures against NMDA excitotoxicity. NMDA treatment alone induced microglial proliferation, which preceded neuronal death, and administration of extra microglial cells on top of these cultures enhanced the NMDA neurotoxicity. Minocycline inhibited all these responses to NMDA. Minocycline also prevented the NMDA-induced proliferation of microglial cells and the increased release of IL-1 β and nitric oxide in pure microglia cultures. Finally, minocycline inhibited the NMDA-induced activation of p38 mitogen-activated protein kinase (MAPK) in microglial cells, and a specific p38 MAPK inhibitor, but not a p44/42 MAPK inhibitor, reduced the NMDA toxicity. Together, these results suggest that microglial activation contributes to NMDA excitotoxicity and that minocycline, a tetracycline derivative, represents a potential therapeutic agent for brain diseases. *The Journal of Immunology*, 2001, 166: 7527–7533.

Activation of the *N*-methyl-D-aspartate (NMDA)³ receptors to a large extent mediates the glutamate excitotoxicity and neuronal death in several neurodegenerative diseases and ischemic stroke (1–3). Subsequent to activation of NMDA receptors occurs toxic calcium influx, which activates numerous enzymes, including neuronal NO synthase (NOS). NO is able to further increase the excitotoxicity by enhancing glutamate release from presynaptic neurons (4, 5) and inhibiting glial glutamate transporters (6, 7).

Several studies have suggested that inflammation, involving nonneuronal cells such as infiltrating leukocytes and parenchymal microglia, contributes to the delayed enlargement of ischemic brain injury (1, 2, 8), amyloid-induced neurotoxicity in Alzheimer's disease (9, 10), and demyelination in multiple sclerosis (11, 12). The key players in inflammation are glial cells, both astrocytes and microglia, which are activated in these brain diseases and in response to glutamate excitotoxicity (13–15). Activated microglia release a large variety of neurotoxins, including the free radicals hydrogen peroxide, superoxide, and NO, that also is able to nitrate proteins; glutamate and quinolinic acid; extracellular proteases; eicosanoids; and cytokines, such as TNF- α and IL-1 β , which further increase microglial proliferation and activation, thereby increasing the release of microglial toxins (8, 13, 14, 16, 17). Im-

portantly, several studies have shown that the neurotoxicity of microglial toxins is mediated through the NMDA receptor (NMDAR), involving either glutamate and quinolinic acid or a still uncharacterized NMDAR binding molecule (18–21). Because inflammation is a delayed and prolonged response to brain injury, it is regarded as a tempting pharmacological target with a potentially wide therapeutic window, unlike NMDAR antagonists that must be delivered immediately after a brain insult and have strong side effects (2, 22, 23).

We have previously shown that minocycline, a semisynthetic tetracycline derivative, is able to provide neuroprotection against global ischemia in gerbils and focal brain ischemia in rats (24, 25). In addition, minocycline delays mortality in a transgenic mouse model of Huntington's disease (26). In all these studies, the protective effect of minocycline was associated with reduced activation of inducible NOS and IL-1 β -converting enzyme, which are mainly expressed by microglia. In addition, ischemia-induced proliferation of microglia was inhibited by minocycline. In the present study, we show that NMDA-induced neuronal death involves proliferation and activation of microglial cells and that minocycline prevents completely the NMDA toxicity and the preceding activation and proliferation of microglial cells. These results support the notion that microglial activation contributes to excitotoxic neuronal death, which can be inhibited by antiinflammatory compounds, such as minocycline.

Materials and Methods

Primary spinal cord (SC) cultures

SC were dissected out from 14-day-old Wistar rat embryos (University of Kuopio, Finland), and the meninges and dorsal root ganglia were removed. Tissues were minced and trypsinized (0.25% trypsin-EDTA in 0.1 M phosphate buffer; Life Technologies, Roskilde, Denmark) for 15 min at 37°C. After centrifugation for 5 min at 800 rpm, the tissues were resuspended into DMEM (high glucose, containing 200 μ g/ml CaCl₂, 97.67 μ g/ml MgSO₄, and 30 μ g/ml glycine; Life Technologies) containing 10% FBS and 10% heat-inactivated horse serum (HS-HI), and triturated with a fire-polished Pasteur pipette. A single-cell suspension was collected, and the cell density of the suspension was counted with a Burkert hemocytometer. Cells were cultured onto poly-L-lysine-coated 96-well plates (1 \times 10⁵ cells/well) or 24-well plates (2.5 \times 10⁵ cells/well) and maintained at 37°C in a 7.5% CO₂

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³ Abbreviations used in this paper: NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; ICE, IL-1 β -converting enzyme; MAPK, mitogen-activated protein kinase; NOS, NO synthase; SC, spinal cord; HS-HI, horse serum heat-inactivated; LDH, lactate dehydrogenase.

incubator. The medium was changed on the following day to DMEM containing 5% FBS and 5% HS-HI. After 4 days *in vitro*, 5 μM cytosine β -D-arabino-furanosylcytosine (Sigma, St. Louis, MO) was added for 24 h to inhibit the growth of nonneuronal cells. This procedure results in mixed SC cultures, consisting of all neuronal populations (70%) present in the SC, astrocytes (25%), and few other nonneuronal cell types, including microglia (5%). We have previously shown that these neurons express functional glutamate receptors (27).

Primary mixed glial and microglia cultures

Cortices and midbrains were dissected out from newborn Wistar rat pups, and the meninges and blood vessels were removed. Tissues were collected into 0.1 M PBS and washed four times with cold 0.1 M PBS, homogenized mechanically without enzymes, and filtered through a 70- μm pore size nylon cell strainer (Falcon; Fisher Scientific, Pittsburgh, PA). PBS was removed by two centrifugation steps ($1000 \times g$ for 10 min at 4°C) and replaced with DMEM (low glucose, containing 200 $\mu\text{g}/\text{ml}$ CaCl₂, 97.67 $\mu\text{g}/\text{ml}$ MgSO₄, and 30 $\mu\text{g}/\text{ml}$ glycine; Life Technologies) + 10% FBS. The cells were suspended into culture medium and plated onto cell culture plates at a density of 1×10^5 cells/ mm^2 . The cultures were maintained in a humidified incubator at 37°C and 5% CO₂. The medium was changed at the second day *in vitro* and once a week thereafter. This procedure results in mixed glial cultures, consisting of dividing astrocytes and microglial cells. After 2 weeks *in vitro*, microglia was harvested once a week by carefully collecting the medium without shaking until the mixed cultures were 2–3 mo old. The purity of the microglial cultures was tested by immunocytochemical staining for OX-42, a microglia marker, and for glial fibrillary acidic protein, an astrocyte marker. Also, the cellular morphology was carefully investigated under phase contrast microscope. The purity of microglial cultures was found to be ~98%.

Cell exposure experiments

SC cultures were exposed at seventh day *in vitro* to 300 μM NMDA (Research Biochemical International, Natick, MA) for 5 min and analyzed 24 h after the onset of exposure. This NMDA treatment reduces the number of surviving neurons to 40–50%. To some cultures 20 nM minocycline (Sigma), 10 μM PD98059 (Tocris Cookson, Bristol, U.K.), a specific p44/42 mitogen-activated protein kinase (MAPK) inhibitor, or 10 μM SB203580 (Tocris Cookson), a specific p38 MAPK inhibitor, was administered 30 min before the NMDA treatment. All of the compounds were dissolved in the culture medium supplemented with 5% HS-HI, which was used alone as a 0 control. The cultures were analyzed 24 h after the onset of exposures, unless mentioned otherwise. In a set of experiments, microglial cells were cultured on top of 5-day-old SC cultures and exposed at day 7 as described above. Mixed glial cultures were exposed to 500 μM NMDA after 2 wk *in vitro*, and pure microglial cultures were exposed on the third day *in vitro*. Minocycline (200 nM) or 10 μM MK-801 (Tocris Cookson), a specific NMDAR antagonist, was administered to some cultures 30 min before exposure. All compounds were dissolved in the cell culture medium supplemented with 10% FBS, which was used alone as a 0 control.

Lactate dehydrogenase (LDH) assay

The release of LDH was measured from the culture medium using a Sigma Kinetic LDH kit. The culture medium samples were collected 24 h after the onset of excitotoxic exposures (unless mentioned otherwise), prepared cell free by centrifugation, and measured immediately with a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland) taking seven absorbance measurements during 3 min at 30-s intervals at a wavelength of 340 nm.

NO and IL-1 β assays

The production of NO was measured by measuring the released NO metabolites (nitrates and nitrites) with Griess reagent (Sigma). After a 24-h exposure, the culture medium samples were collected and prepared cell free by centrifugation. Fifty microliters of the medium were incubated with the same volume of Griess reagent at room temperature for 15 min before measuring OD₅₄₀ in a Multiskan ELISA reader (Labsystems) with appropriate standards. IL-1 β samples were prepared similar to NO samples and determined using a rat IL-1 β ELISA kit (Endogen, Woburn, MA) according to the manufacturer's instructions and a Multiskan MS ELISA reader (Labsystems).

Immunocytochemistry

The cultures were fixed with 4% paraformaldehyde in 0.1 M PBS for 20–30 min and rinsed in 0.1 M PBS. The nonspecific binding was blocked with 1% BSA and 0.3% Triton X-100 in 0.1 M PBS for 30 min at room temperature. Subsequently, the cultures were incubated with primary Abs

to neurons (mouse mAb, against neuronal nuclei; Chemicon, Temecula, CA; 1:100 dilution), to microglia (mouse mAb, OX-42, against CD11b surface Ag; Serotec, Oxford, U.K.; 1/1500 dilution), to phospho-p38 or -p42/44 MAPK (rabbit polyclonal antibody, Phospho-p38 or -p42/44 MAPK; New England Biolabs, Beverly, MA; 1/1000 and 1/250 dilution) in the blocking buffer for 48 h at 4°C. The cultures were rinsed with 0.1 M PBS, incubated with biotinylated anti-mouse IgG (Amersham, Buckinghamshire, U.K.; 1/200 dilution) or biotinylated anti-rabbit IgG (Amersham; 1/200 dilution) secondary Ab for 2 h at room temperature, and reacted with alkaline phosphatase-avidin-biotin complex (Vector Laboratories, Burlingame, CA; 1/200 dilution) for 2 h at room temperature and rinsed. The color was obtained from alkaline phosphatase substrate kit (Vector Laboratories). In a set of experiments, phospho-p38 MAPK-immunostained cultures were double-stained with OX-42 Ab (1/800 dilution) using anti-mouse IgG fluorescein (Jackson ImmunoResearch Laboratories, West Grove, PA; 1/70 dilution) as a secondary Ab. Immunoreactive cells were counted from 6–10 random fields of 4×10^{-3} mm^2 area per well and from 3–5 wells per treatment.

Proliferation assay

The thymidine analogue BrdU (Sigma) was added to the medium at 5 μM concentration. After 24 h, the cultures were fixed with 4% paraformaldehyde in 0.1 M PBS for 20–30 min and rinsed in 0.1 M PBS. The DNA was denatured by incubating the cultures first with 50% formamide in $2 \times \text{SSC}$ for 2 h at 65°C followed by 2 N HCl treatment for 30 min at 37°C. After neutralization with 0.1 M boric acid, pH 8.5, for 10 min at room temperature, the cultures were rinsed in 0.1 M PBS and incubated with a mouse monoclonal anti-BrdU (0.25 $\mu\text{g}/\text{ml}$ anti-BrdU; Boehringer Mannheim, Indianapolis, IN) in the blocking buffer at room temperature for 24 h. Otherwise, the procedure was identical with the immunocytochemistry described above. The immunoreactive cells were counted in a ratio of total cell number from 6–10 random fields of 4×10^{-3} mm^2 area per well and from 3–5 wells per treatment.

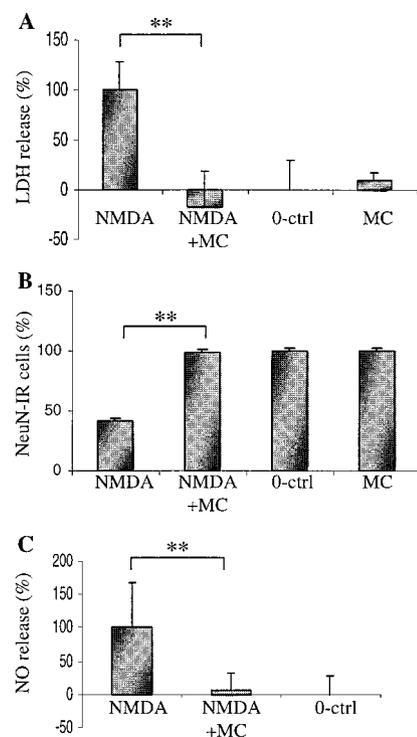


FIGURE 1. Mean (\pm SD) increase in LDH release (A), mean (\pm SD) surviving neuronal nuclei (NeuN)-immunoreactive (IR) neurons (B), and mean (\pm SD) increase in release of NO metabolites (C) in control (0-ctrl), 20 nM minocycline-treated (MC), 300 μM NMDA-treated (NMDA), and 20 nM minocycline + 300 μM NMDA-treated (NMDA + MC) rat SC cultures at 24 h after the 5-min NMDA treatment. In A and C, data have been pooled from four ($n = 12$) and in B from two independent experiments ($n = 6$). **, $p < 0.01$, ANOVA followed by Tukey's post hoc test. Minocycline treatment was able to prevent the NMDA-induced neuronal cell death and NO release.

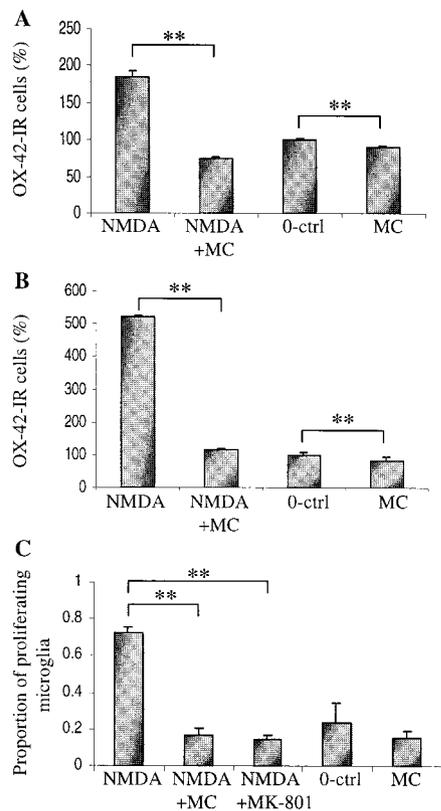


FIGURE 2. Mean (\pm SD) number of OX-42-immunoreactive (IR) microglial cells in the SC culture 24 h after 5 min exposure to 300 μ M NMDA (A), in neuron-free mixed glial culture 24 h after exposure to 500 μ M NMDA (B), and mean (\pm SD) ratio of BrdU-positive cells in pure microglial culture 24 h after 500 μ M NMDA stimulation with and without the presence of minocycline and MK-801 (C). In all cell culture preparations, stimulation with NMDA caused an increase in the number of microglial cells, which was prevented by minocycline or MK-801 treatment; $n = 8$, pooled from two independent experiments in A, $n = 3$ in B, and $n = 10$, pooled from three independent experiments in C. **, $p < 0.01$, ANOVA followed by Tukey's post hoc test. The cells were counted in a blinded manner. 0-ctrl, Control.

Statistical analysis

Data are presented as the mean \pm SD. Statistical comparisons were made by single-factor ANOVA followed by Tukey's post hoc test. Values of $p < 0.05$ were considered significant.

Results

Minocycline prevents neuronal death caused by NMDA

A 5-min exposure to 300 μ M NMDA resulted in increased LDH release from dying neurons measured 24 h after the onset of exposure (Fig. 1A). Treatment with 20 nM minocycline started 30 min before NMDA exposure was able to prevent neuronal death. The reduced neuronal loss in minocycline-treated cultures was further confirmed by counts of NeuN-stained neurons (Fig. 1B). Cell death was mainly necrotic in this cell culture model, in that no apoptotic fragmentation was seen with bisbenzimidazole staining (data not shown). The NMDA-induced neuronal death was associated with increased NO metabolite production (from 1–3 μ M to 5–20 μ M), which was inhibited by 20 nM minocycline (Fig. 1C).

NMDA stimulates microglial proliferation and activation

A 5-min exposure to 300 μ M NMDA caused a 1.9-fold increase in the number of OX-42 immunoreactive microglial cells in SC cell cultures within 24 h (Fig. 2A). Administration of 20 nM minocycline

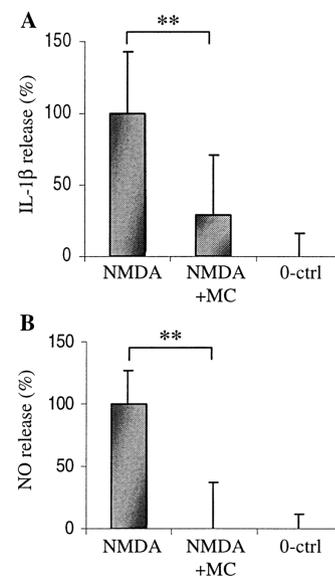


FIGURE 3. Mean (\pm SD) release of IL-1 β (A) and NO metabolites (B) in pure microglial cultures after 24 h exposure to 500 μ M NMDA treated with or without 200 nM minocycline (MC). Minocycline reduces the NMDA-induced release of IL-1 β and NO metabolites. 0-ctrl, Control. Data are pooled from two independent experiments ($n = 8$). **, $p < 0.01$, ANOVA followed by Tukey's post hoc test.

30 min before the exposure prevented the NMDA-triggered increase of microglial cells. Minocycline even slightly prevented the spontaneous proliferation of microglia. Even sublethal doses (50 μ M for 5 min) of NMDA increased the number of microglial cells (not shown), suggesting that the microglial proliferation was not secondarily due to increased neuronal death. When neuron-free mixed glial cultures were exposed to 500 μ M NMDA for 24 h, a 5.2-fold increase of OX-42-positive cells was observed, and this increase was again reduced close to basal levels by 200 nM minocycline treatment (Fig. 2B). To investigate whether microglial proliferation caused by NMDA stimulation is mediated by other glial cells, pure microglial cultures were exposed to 500 μ M NMDA for 30 min or 24 h in the presence of 5 μ M BrdU. BrdU incorporates into DNA of proliferating cells, which can be detected by anti-BrdU Ab. NMDA administered for either 30 min or 24 h caused a 2- to 4-fold increase of the microglial proliferation ratio, which was prevented by 200 nM minocycline or 10 μ M MK-801 (Fig. 2C).

Microglial cells increase cytokine release and NO synthesis on activation. Therefore, we next measured IL-1 β and NO release in pure microglial cultures. Twenty-four hours after addition of 500 μ M NMDA, increased levels of IL-1 β (from 2–4 μ M to 4–10 μ M) and NO (from 1–3 μ M to 5–20 μ M) metabolites were detected (Fig. 3). The release of both IL-1 β and NO metabolites was inhibited by 200 nM minocycline treatment before exposure. We also confirmed that significant NO release occurred both after 5 min and 24 h exposure to NMDA and that MK-801 was able to block the NO release (not shown).

Microglia enhances NMDA-mediated neuronal death

To study whether NMDA-induced microglial activation and proliferation precedes or is a consequence of neuronal death, we compared the time course of the changes in OX-42-immunoreactive cells and LDH release during a 24-h follow-up period (Fig. 4A). The number of OX-42-immunoreactive cells was increased significantly as early as 1 h after a 5-min exposure to 300 μ M NMDA and was further increased at later time points. LDH release, on the

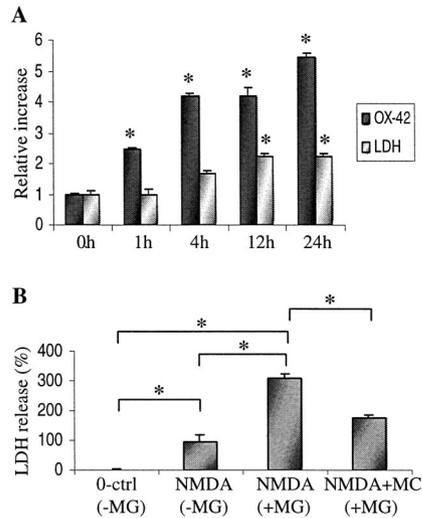


FIGURE 4. Mean (\pm SD) increases in LDH release and the number of OX-42-immunoreactive cells in SC cultures at different time points after a 5-min challenge to 300 μ M NMDA (A) and mean (\pm SD) increase in LDH release in normal SC cultures (–MG) or in SC cultures enriched with microglia (+MG) 24 h after a 5-min NMDA treatment (B). In A, $n = 6$; *, $p < 0.05$; in B, $n = 6$, pooled from two independent experiments. *, $p < 0.05$, ANOVA followed by Tukey's post hoc test. The increase in OX-42-immunoreactive cells precedes the increase in LDH release on NMDA stimulation. In B, NMDA neurotoxicity is increased by microglia enrichment, and 20 nM minocycline treatment reduced the toxicity.

other hand, was not significantly increased until 12 and 24 h after NMDA challenge (Fig. 4A), indicating that induction of CD11b surface Ag and microglial proliferation starts before neuronal death. To study further the role of microglia in NMDA excitotoxicity, microglial cells were harvested and added on top of mixed SC cultures. This procedure increases the microglia-neuron ratio from 0.071 to 0.185. Administration of extra microglia on SC cultures increased the NMDA-triggered LDH release 3-fold, which was significantly, but not completely, inhibited by minocycline (Fig. 4B).

Neuroprotection is associated with inhibition of p38 MAPK in microglial cells

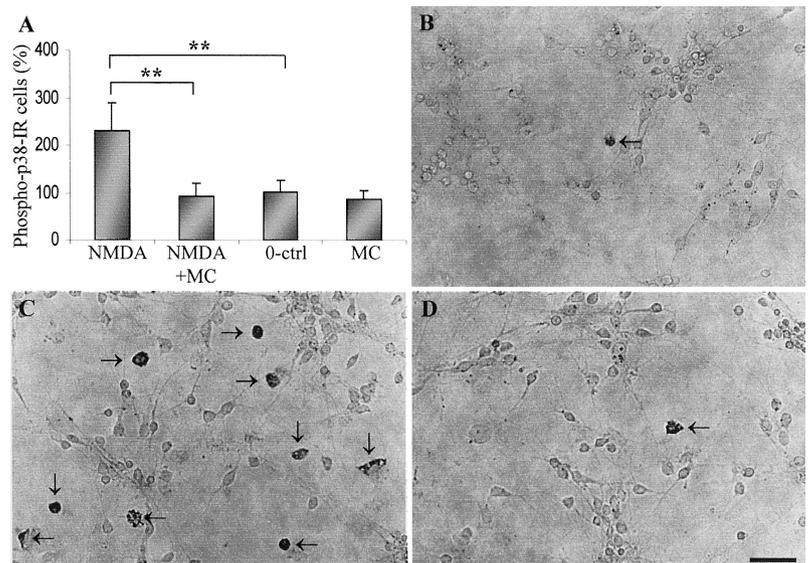
Because MAPKs have been reported to be involved in microglial activation and induction of inducible NOS, we studied whether a

10-min exposure to NMDA causes changes in activation of p38 and p44/42 MAPKs in SC cultures. Immunoblots did not reveal detectable levels of p38 MAPK (not shown), suggesting that p38 MAPK may be activated only in a minority of the cultured cells. By immunocytochemistry, the phosphorylated form of p38 MAPK was detected only in microglial cells, which was confirmed by double staining with OX-42 Ab (Fig. 6, A and B). The number of p38 MAPK-positive microglia was significantly (130%) elevated by NMDA exposure (Fig. 5). Minocycline administration (20 nM) 30 min before the onset of NMDA exposure decreased the number of p38 MAPK-immunoreactive microglial cells to the basal levels (Fig. 6, A–D). The basal levels of phosphorylated p44/42 MAPK were high in both astrocytes and neurons (Fig. 6, E–G). NMDA increased the number of p44/42 MAPK-positive neurons by 41.3% ($p < 0.005$; ANOVA, Tukey's post hoc test), but 20 nM minocycline treatment reduced the number of immunoreactive neurons by 40% (not shown). To dissociate the role of p38 MAPK activation in microglial cells and p44/42 MAPK in astrocytes and neurons, 10 μ M SB203580, a specific p38 MAPK inhibitor, or 10 μ M PD98059, a specific p44/42 MAPK inhibitor, was administered to mixed SC cultures 30 min before NMDA exposure. Inhibition of p38 MAPK but not p44/42 MAPK significantly reduced the NMDA-induced LDH (Fig. 7A). In addition, in pure microglial cultures, 10 mM SB203580 prevented NMDA-induced microglial proliferation by 95% (Fig. 7B).

Discussion

A main finding of this study was that NMDA induces a direct activation and proliferation of microglia, which precedes neuronal cell death. This activation is dependent on p38 MAPK but not p44/42 MAPK pathway and is associated with increased release of IL-1 β and NO metabolites 24 h after NMDA exposure. Our preliminary results have shown that kainate and glutamate, but not agonists of metabotropic glutamate receptors, are also able to trigger microglial activation and proliferation (T. M. Tikka, B. L. Fiebich, and J. E. Koistinaho, unpublished observations), suggesting that agonists of ionotropic glutamate receptors are potential activators of microglia. Several previous studies have suggested the involvement of microglial activation in excitotoxicity (15, 18, 28), although the mechanism is not yet fully understood. In the rat

FIGURE 5. Minocycline treatment inhibits the NMDA-induced activation of p38 MAPK in microglial cells. A, Quantification of phospho-p38 MAPK-immunoreactive (IR) microglia 10 min after stimulation with 300 μ M NMDA in SC cell culture. Data are means \pm SD pooled from three independent experiments, counted in a blinded manner ($n = 9$). **, $p < 0.01$, ANOVA followed by Tukey's post hoc test. 0-ctrl, Untreated. B–D, Representative photomicrographs demonstrating the effect of NMDA and minocycline on immunoreactivity for the phosphorylated form of p38 MAPK in SC cultures. In control cultures (B), only few microglial cells (confirmed by OX-42 double labeling) show immunoreactivity for OX-42; whereas in NMDA-treated cultures (C), a clear increase in the number of immunoreactive cells is seen. Administration of 20 nM minocycline to NMDA-treated cultures (D) blocks the induction of OX-42-immunoreactive cells. Bar, 250 μ m.



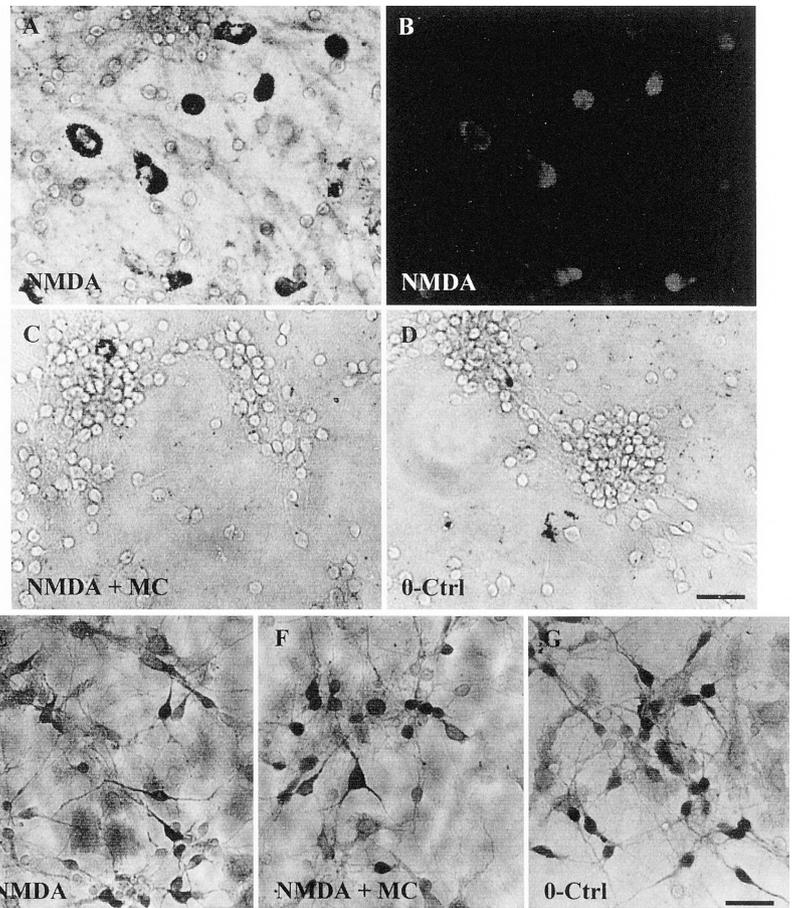


FIGURE 6. Microphotographs of mixed SC cultures stained with phospho-p38 MAPK Ab (A, C, and D), OX-42 (B), or phospho-p44/42 MAPK antibody (E–G). NMDA induced phospho-p38 MAPK immunoreactivity (A) in cells, which were double-labeled with OX-42 (B), indicating that these cells are microglia. Administration of minocycline (C) inhibited the induction of phospho-p38 MAPK which remained at a level of control cultures (D). The immunoreactivity for p44/42 MAPK was robust in both neurons and glia, and no clear differences among control (E), NMDA-treated (F), and NMDA + minocycline-treated (G) cultures were seen. Bar, 200 μm in A–D and 150 μm in E–G.

brain, reactive microglia express GluR4 and NR1 subunits, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate and NMDAR subtypes, respectively (29), and in vitro studies have demonstrated the presence of group I metabotropic (30) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate-kainate glutamate receptors in pure microglia cultures (31). Noda et al. (31) demonstrated that TNF- α release from cultured microglia is increased 2- to 3-fold 2 h after stimulation with glutamate or kainate and by 45% after stimulation with NMDA, even though only the responses to kainate and glutamate reached statistical significance. Together with the previous studies, our results support the idea that increased glutamate levels trigger microglial proliferation and activation through ionotropic receptors, including NMDAR, resulting in increased release of nitric oxide and cytokines.

The second main finding was that microglia are able to enhance the neurotoxicity of NMDA. We saw a robust increase in the number of activated microglial cells as early as 1 h after administering NMDA, whereas the LDH release, reflecting neuronal death, was significantly elevated (only after 12–24 h). In addition, culturing microglia on top of SC cultures enhanced the NMDA-induced LDH release. Several in vivo studies have reported microglial proliferation and activation early after various brain insults, including excitotoxin applications (32). It is likely that the increased number of OX-42-immunoreactive cells as soon as 1 h after NMDA stimulation is due to activation and increased density of CD11b surface Ag, whereas at later time points (12 and 24 h) increased proliferation rate may well contribute to the severalfold increase detected in the number of OX-42-immunoreactive cells. We hypothesize that activated microglia enhance NMDA neurotoxicity through increased release of IL-1 β and NO, which is supported by the previous in vivo and in vitro studies. First, application of kainate to

the hippocampus induces a rapid synthesis in IL-1 β in microglia, resulting in prolonged seizures and facilitation of glutamatergic function of the NMDARs (32), possibly by attenuating astrocytic

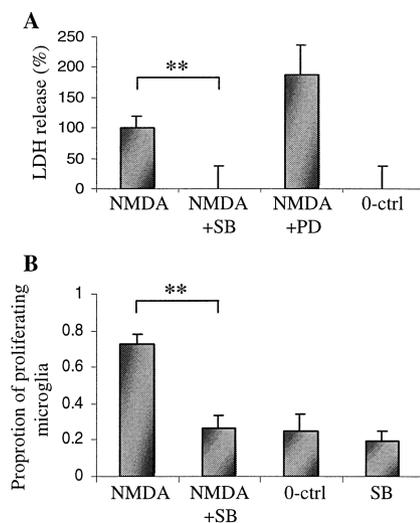


FIGURE 7. Mean (\pm SD) LDH release measured 24 h after a 5-min stimulation with 300 μM NMDA in SC cultures (A) and mean (\pm SD) proportion of proliferating microglial cells in pure microglial cultures after stimulation with 500 μM NMDA (B) were reduced by 10 μM SB203580 (SB), a specific p38 MAPK inhibitor. PD98059 (PD; 10 μM), a specific p44/42 MAPK inhibitor, did not reduce the NMDA-induced LDH release. In A, data are pooled from four independent experiments ($n = 14$); in B, they are from three independent experiments, counted in blind manner ($n = 10$). **, $p < 0.01$, ANOVA followed by Tukey's post hoc test. 0-ctrl, Control.

glutamate uptake (33) and directly enhancing NMDAR function (34). Second, blockade of IL-1 β receptors or inhibition of IL-1 β -converting enzyme provides neuroprotection against excitotoxicity and ischemia (35, 36). Third, in mixed neuronal cultures, combination of cytokines (IL-1 β + TNF- α) induces neurotoxicity, which is reduced by blocking NO production and NMDA antagonist (20). Finally, NMDA neurotoxicity is mediated through NO, and inhibitors of NOS are neuroprotective against ischemic insults (37, 38), suggesting that the additional NO released by activated microglia may well enhance NMDA-induced neuronal death. Our results stress the importance of the role of the microglia in neuronal degeneration and excitotoxicity, which is in accordance with the *in vivo* studies demonstrating that the microglial activation is necessary but not sufficient to trigger neuronal degeneration in the hippocampus after excitotoxic insult (39). Our findings do not exclude the possibility that release of other cytokines is involved in microglia-enhanced excitotoxicity or that glutamate and still uncharacterized factor(s) released from activated microglia enhance excitotoxicity by acting through NMDARs (8, 28, 40).

The third major finding of our study is the neuroprotective role of minocycline, which may be based, at least partially, on the inhibition of microglia. This microglial inhibition included prevention of microglial proliferation in mixed SC and in pure microglia cultures and reduction of NO metabolite release and cytokine production in pure microglia cultures. Moreover, we found that minocycline was able to inhibit p38 MAPK in microglia and that specific inhibition of p38 MAPK, but not p44/42 MAPK, provided neuroprotection against NMDA toxicity and prevented microglial proliferation. p38 MAPK is thought to mediate inflammatory responses in various cell types (41, 42), including microglia (17); therefore, inhibition of p38 MAPK may be beneficial in injuries involving inflammation and microglial activation. Several studies with specific inhibitors of p38 MAPK have proved to be neuroprotective and provide antiinflammatory effects (17, 43–45). Another MAPK family member, p44/42, is stimulated by extracellular mitogens and is involved in proliferation and differentiation of several cell types, thereby supporting cellular survival (46). In agreement with the survival supporting role of p44/42 MAPK, Sugino et al. (44) did not observe neuroprotection against transient brain ischemia by inhibiting p44/42 MAPK, and in the present study inhibition of p44/42 MAPK slightly increased NMDA-induced neuronal death rather than being protective. However, p44/42 has also been shown to contribute to ischemic neuronal death *in vivo* (47) and *in vitro* (48), indicating that the role of p44/42 MAPK may depend on the injury model, timing, and dosing of the inhibitor treatment.

The specificity of SB203580, a p38 MAPK inhibitor, and PD98059, a p44/42 MAPK inhibitor, has been widely studied and has been demonstrated also in cultured microglia cells (19). Importantly, Börsch-Haubold et al. (49) have shown that at 20 μ M concentrations these two inhibitors can directly inhibit cyclooxygenase-1 and -2. Because substantially lower concentrations were used in the present study and because in our cell culture model there are no detectable levels of either isoform of cyclooxygenase (27), it is very unlikely that the effects of the inhibitors were even partially unspecific. Studies by SmithKline Beecham Pharmaceuticals (Philadelphia, PA) have demonstrated that SB203580 inhibits only p38 α and p38 β isoforms (50), suggesting that p38 γ and p38 δ MAPK may not play a role in microglial activation. It remains to be studied which p38 MAPK isoforms are expressed in microglia.

Some studies have demonstrated that pretreatment with cytokines such as TNF- α and IL-1 β provides neuroprotection against

neuronal insults (51–54). However, pretreatment with sublethal injuries, including brief ischemia or oxygen/glucose deprivation, is well known to trigger tolerance against a subsequent neuronal insult, and this preconditioning pathway involves NMDARs and nitric oxide (55). It is therefore likely that, in addition to injury model, the temporal relationship of increased cytokine or NO levels and lethal neuronal insult determines, at least partially, the outcome after excitotoxic injury.

Minocycline has been used in humans for several decades with tolerable side effects (56, 57). Considering the neuroprotection achieved with minocycline in animal models of stroke (24, 25) and Huntington's disease (26), our cell culture findings suggest that minocycline may represent a potential therapeutic agent for treatment of brain diseases which involve excitotoxicity.

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