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Short communication

N^{ω}-Nitro-L-arginine methyl ester protects retinal neurons against *N*-methyl-D-aspartate-induced neurotoxicity in vivo

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Abstract

We investigated whether the inhibition of nitric oxide (NO) synthesis with N^{ω}-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NO synthase, affects *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity in the rat retina in vivo. A single intravitreal injection of NMDA damaged the ganglion cell layer and the inner plexiform layer without affecting the other retinal layers 7 days after injection. Intravitreal injection of (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclo-hepten-5,10-imine hydrogen maleate (MK-801) with NMDA significantly reduced NMDA-induced degeneration of the retina. NMDA-induced degeneration was also prevented by intravitreal injection of L-NAME but not of D-NAME. The protective effect of L-NAME was antagonized by L-arginine. These results suggest that NO plays an important role in NMDA-induced excitotoxic degeneration in the retina.

Keywords: Neurotoxicity; Nitric oxide (NO); NMDA (*N*-methyl-D-aspartate); N^{ω}-Nitro-L-arginine methyl ester (L-NAME); Retina

1. Introduction

It has been reported that an excess release of excitatory amino acids including glutamate occurs in ischemia, which results in the degeneration of a number of neuronal populations in the central nervous system (CNS) (Benveniste et al., 1984; Choi, 1988; Meldrum and Garthwaite, 1990). Glutamate acts as a major excitatory neurotransmitter in the vertebrate retina and has long been known to exert a neurotoxic effect on different populations of neurons in the inner retina (Lucas and Newhouse, 1957). We have previously demonstrated that *N*-methyl-D-aspartate (NMDA) receptors are the predominant route of glutamate-induced neurotoxicity in cultured retinal neurons from rats, as (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclo-hepten-5,10-imine hydrogen maleate (MK-801), a specific NMDA receptor antagonist, markedly inhibits the cell death induced by glutamate (Kashii et al., 1994; Kikuchi et al., 1995). Activation of NMDA receptors by glutamate increases the intracellular Ca^{2+} concentration. We have recently reported that nitric oxide (NO) is a key factor leading to neuronal cell death triggered by NMDA receptor stimulation in cultured rat retinal neurons (Kashii et al., 1996). Similar observations have been reported for cultured brain neurons (Dawson et al., 1993). Moreover, Huang et al. (1994) demonstrated that a lack of neuronal NO synthase activity in the brain is associated with reduced ischemic damage after middle cerebral artery occlusion by using mutant mice that do not express the gene for neuronal NO synthase. Thus, the following hypothesis has been proposed: Ca^{2+} influx through NMDA receptors activates NO synthase to produce a high concentration of NO. NO reacts with the oxygen radical superoxide, producing peroxynitrite which mediates neurotoxicity (Beckman, 1991; Dawson and Dawson, 1996). However, in the mammalian retina in vivo, there is little information on the role of NO in NMDA-induced neurotoxicity. Thus, we investigated whether NO is involved in NMDA-induced neurotoxicity in the adult rat retina in vivo.

The involvement of NO production in NMDA-induced neurotoxicity was assessed using a competitive inhibitor of NO synthase, N^{ω}-nitro-L-arginine methyl ester (L-NAME). Because detection of NO in vivo is difficult (Archer,

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1993), most of the evidence on the biological roles of NO is based on the use of pharmacological agents that inhibit the activity of NO synthase (Moncada, 1992). These agents are N^{ω}-substituted L-arginine analogues that inhibit NO synthase competitively and stereospecifically in many models (Nathan, 1992). L-NAME is one of the most widely used agents for investigating the role of NO (Iadecola et al., 1994; Traystman et al., 1995). Therefore, this study was performed to investigate whether the inhibition of NO synthase with L-NAME affects NMDA-induced neurotoxicity in the rat retina in vivo.

2. Materials and methods

2.1. Drugs

The animals were anesthetized with sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL, USA). The pupil was dilated with 1% atropine sulfate (Nacalai Tesque, Kyoto, Japan). The following drugs were dissolved in 0.1 M sterile phosphate buffer (pH 7.4) for intravitreal injection: *N*-methyl-D-aspartate (NMDA; Sigma, St. Louis, MO, USA), (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclo-hepten-5,10-imine hydrogen maleate (MK-801) (Research Biochemicals International, Natick, MA, USA), N^{ω}-nitro-L-arginine methyl ester (L-NAME; Sigma), N^{ω}-nitro-D-arginine methyl ester (D-NAME; Sigma) and L-arginine (Nacalai Tesque, Kyoto, Japan).

2.2. Animals

Experiments were performed on adult male Sprague-Dawley rats (160–220 g). Animals were housed at room temperature on a 12-h light/12-h dark cycle. Food and water were freely available.

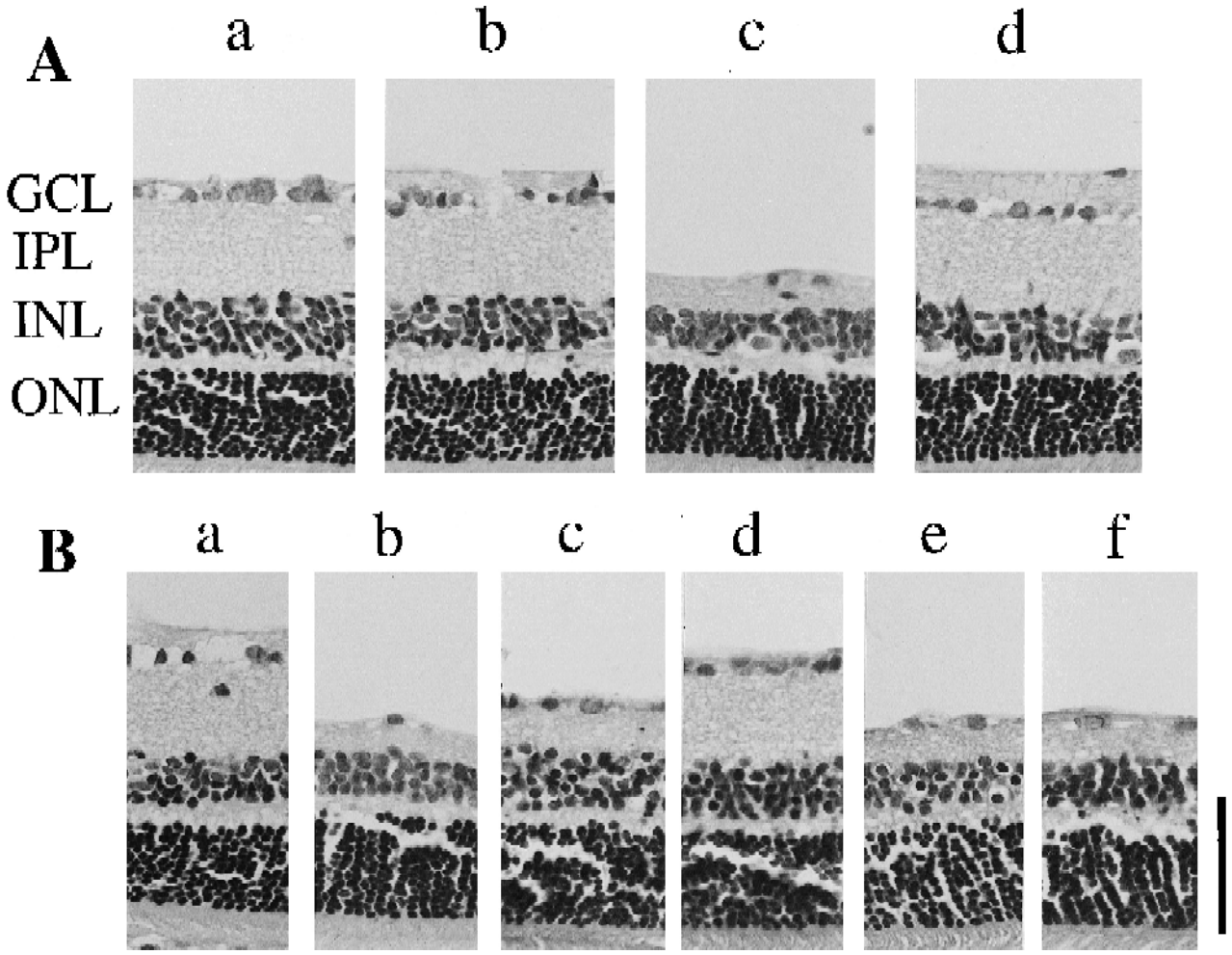

Fig. 1. Light micrographs of transverse sections of rat retinas 7 days postinjection. (A) The retina from an uninjected eye (a). The retinas following intravitreal injection of vehicle (b), 200 nmol NMDA (c) or 200 nmol NMDA and 10 nmol MK-801 (d). Note that the administration of NMDA (200 nmol) resulted in cell loss in the ganglion cell layer and a reduction in the thickness of the inner plexiform layer (c). (B) The retinas following intravitreal injection of vehicle (a) or 200 nmol NMDA (b). NMDA (200 nmol) was coinjected with 3 pmol L-NAME (c), with 3 nmol L-NAME (d), with 3 nmol D-NAME (e), or with 3 nmol L-NAME plus 300 nmol L-arginine (f). Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 50 μm.

2.3. Intravitreal injection of drugs

Rats were anesthetized with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital. Intravitreal injections were carried out using a 33-gauge needle connected to a Teflon tube with a 25-μl Hamilton syringe, following pupil dilation with 1% atropine sulfate. The 5-mm tip of the needle was inserted through the dorsal limbus of the eye under a stereoscopic microscope. Injections were completed over a period of 1 min. A single 5-μl intravitreal injection of 40 mM NMDA (corresponding to 200 nmol per injection) was administered into the right eye. Assuming that the vitreous humor volume is about 60 μl in rat eyes, the actual concentration of NMDA delivered to the retina was about 3.3 mM. The following drugs were administered with NMDA (200 nmol) in a similar fashion: MK-801 (10 nmol), L-NAME (3 pmol, 3 nmol), D-NAME (3 nmol) and L-NAME (3 nmol) plus L-arginine (300 nmol).

2.4. Histological analysis

Seven days postinjection, animals were killed and both eyes were enucleated. Eyes were immersed in fixative

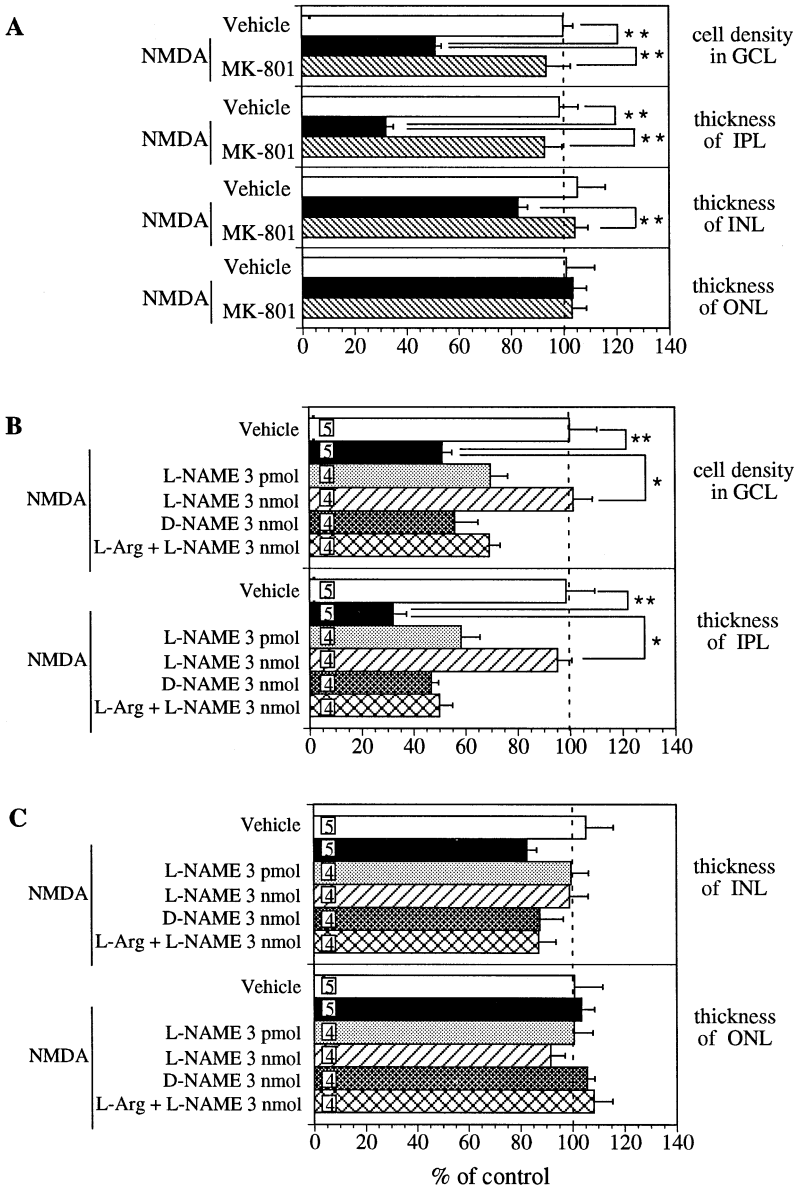

Fig. 2. Effect of MK-801 (A) and L-NAME (B,C) on NMDA-induced retinal damage 7 days postinjection. All the drugs were intravitreally injected: vehicle, NMDA (200 nmol), MK-801 (10 nmol), L-NAME (3 pmol, 3 nmol), D-NAME (3 nmol) and L-arginine (300 nmol). The degree of protection against NMDA-induced retinal damage was quantified by measuring the density (cells/mm) of cells in the ganglion cell layer (GCL) within 1 mm of the optic disk and the thickness of the inner plexiform layer (IPL), the inner nuclear layer (INL), and the outer nuclear layer (ONL) about 0.5 mm from the optic disk. Mean values for the right eye of each animal were normalized to those for the left eye (intact eye) and are shown as percentages (abscissa). * $P < 0.05$, ** $P < 0.01$.

solution containing 1% glutaraldehyde and 4% formalin in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C, followed by fixation in 10% formalin in 0.1 M phosphate buffer (pH 7.4) for at least 24 h at 4°C, followed by dehydration and paraffin embedding. Transverse sections of retinas through the optic disk (5 μm) were stained with hematoxylin and eosin.

Three slices for each eye were used for morphometric analysis. The degree of NMDA-induced retinal damage was quantified by measuring the density (cells/mm) of cells in the ganglion cell layer within 1 mm of the optic disk, and the thickness of the inner plexiform layer, the inner nuclear layer, and the outer nuclear layer about 0.5 mm from the optic disk with NIH Image freeware. Data from 3 slices were averaged for each eye. Mean values for the right eye of each animal were normalized to those for the left eye (intact eye) and are shown as a percentage.

2.5. Statistics

The data are expressed as means ± S.E.M. from 4–5 animals. The statistical significance of the data was determined by the Mann-Whitney's *U*-test.

3. Results

3.1. Neurotoxicity induced by NMDA and effect of MK-801 on NMDA-induced retinal damage

Fig. 1A shows an example of MK-801-induced protection against NMDA. No histological alterations were observed 7 days following the intravitreal injection of vehicle. The administration of NMDA (200 nmol) resulted in a loss of cells in the ganglion cell layer and a reduction in the thickness of the inner plexiform layer without affecting the thickness of the inner nuclear layer and the outer nuclear layer. To assess the specificity of the observed neurotoxic action of NMDA, the protective effect of MK-801, a non-competitive NMDA receptor antagonist, was examined. The administration of MK-801 (10 nmol) with NMDA (200 nmol) completely prevented the loss of cells in the ganglion cell layer and the reduction in the thickness of the inner plexiform layer induced by NMDA.

To quantify the degree of NMDA-induced retinal damage, morphometric analyses were performed by measuring the density (cells/mm) of cells in the ganglion cell layer within 1 mm of the optic disk and the thickness of the inner plexiform layer, the inner nuclear layer and the outer nuclear layer about 0.5 mm from the optic disk (Fig. 2A). The administration of NMDA (200 nmol) resulted in an approximately 50% loss of cells in the ganglion cell layer and an about 70% reduction in the thickness of the inner plexiform layer. Although there was a tendency for a reduction in the thickness of the inner nuclear layer, there was no significant difference between eyes treated with vehicle and those treated with NMDA. There were no obvious alterations in the thickness of the outer nuclear layer. The administration of MK-801 (10 nmol) with NMDA (200 nmol) significantly prevented the degeneration of the inner retinal layers induced by NMDA.

3.2. Effect of L-NAME on NMDA-induced retinal damage

Fig. 1B shows an example of L-NAME-induced protection against NMDA. Intravitreal injection of L-NAME (3 pmol or 3 nmol) with NMDA (200 nmol) significantly prevented the loss of cells in the ganglion cell layer and reduced the thickness of the inner plexiform layer induced by NMDA in a dose-dependent manner. D-NAME (3 nmol), an inactive enantiomer of L-NAME, had no significant effect on NMDA (200 nmol)-induced retinal damage. The protective effect of L-NAME (3 nmol) was abolished by simultaneous injection of L-arginine (300 nmol). Mean data from 4–5 animals are summarized in Fig. 2B and C. L-NAME (3 nmol) or L-arginine (300 nmol) alone did not induce any morphological changes in the retinas (data not shown).

4. Discussion

In this study, we investigated whether inhibition of NO synthesis with L-NAME, a competitive inhibitor of NO synthase, affects NMDA-induced neurotoxicity in the rat retina in vivo. NMDA was used in these experiments to avoid the reuptake and metabolism of excitotoxins by retinal cells. A single intravitreal injection of NMDA in adult rats caused the loss of cells in the ganglion cell layer and a reduction in the thickness of the inner plexiform layer 7 days after injection. The ganglion cell layer contains 2 neuronal populations: ganglion cells and displaced amacrine cells in the rat retina (Perry, 1981). The inner plexiform layer involves synapses between ganglion cells and amacrine or bipolar cells. No marked changes were observed in the more distal retinal layers such as the inner nuclear layer and the outer nuclear layer. NMDA-induced degeneration of the retina was prevented by a non-competitive NMDA receptor antagonist, MK-801, indicating that NMDA receptor stimulation is a major cause of NMDA-induced neurotoxicity in the retina. These results are consistent with those of an in vivo study by Siliprandi et al. (1992) and with those of our previous studies in vitro (Kashii et al., 1994; Kikuchi et al., 1995). Moreover, the present findings are in line with the finding that NMDA receptors exist in ganglion cells and displaced amacrine cells in the ganglion cell layer and subsets of amacrine cells in the inner nuclear layer (Brandstätter et al., 1994). Recently, Perez et al. (1995) suggested that NO synthase exists in displaced amacrine cells in the ganglion cell layer and amacrine cells in the inner nuclear layer. Therefore, it is speculated that NO is generated from L-arginine in

displaced amacrine cells having NO synthase as well as NMDA receptors, and NO diffuses to adjacent ganglion cells in the ganglion cell layer.

The involvement of NO production in NMDA-induced neurotoxicity was assessed using a competitive inhibitor of NO synthase, L-NAME. Intravitreal injection of L-NAME with NMDA completely prevented NMDA-induced degeneration such as cell loss in the ganglion cell layer and the reduced thickness of the inner plexiform layer. These findings are consistent with our previous in vitro study, which showed that N^{ω}-nitro-L-arginine (300 μM), an inhibitor of NO synthase, prevents NMDA-induced neurotoxicity in cultured retinal neurons (Kashii et al., 1996). In addition, Geyer et al. (1995) previously showed, in an in vivo study, that intravenous injection of NO synthase inhibitors protected against retinal injury induced by transient ischemia. The concentration of L-arginine in the vitreous humor is approximately 80 μM in the rat (Heinämäki et al., 1986). We injected 3 nmol L-NAME in this study. Assuming that the vitreous volume is 60 μl, then the intravitreal concentration of L-NAME was calculated to be 50 μM. Thus, the estimated ratio of the concentration of L-arginine and L-NAME is 1.6. This value is compatible with the ratio of L-arginine in the medium and an NO synthase inhibitor in our previous study with cultured retinal neurons. In that study, although the medium contained 720 μM L-arginine, 300 μM N^{ω}-nitro-L-arginine inhibited NMDA neurotoxicity (Kashii et al., 1996). Therefore, it is likely that the dose of intravitreously injected L-NAME is sufficient to have a specific effect on NO synthase. Moreover, D-NAME, an inactive enantiomer of L-NAME, had no effect on NMDA-induced retinal damage. The protective effect of L-NAME was completely abolished by simultaneous intravitreal injection of an overdose of L-arginine, the normal substrate for NO synthase. These results indicate that L-NAME-induced protection is due to its specific effect on NO synthase in the retina. Therefore, NO may play an important role in NMDA-induced excitotoxic degeneration in the retina.

References

Archer, S., 1993. Measurement of nitric oxide in biological models. FASEB J. 7, 349–360.

Beckman, J.S., 1991. The double-edged role of nitric oxide in brain function and superoxide-mediated injury. J. Dev. Physiol. 15, 53–59.

Benveniste, H., Drejer, J., Schousboe, A., Diemer, N.H., 1984. Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J. Neurochem. 43, 1369–1374.

Brandstätter, J.H., Hartveit, E., Sassoè-Pognetto, M., Wässle, H., 1994. Expression of NMDA and high-affinity kainate receptor subunit mRNAs in the adult rat retina. Eur. J. Neurosci. 6, 1100–1112.

Choi, D.W., 1988. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci. 11, 465–469.

Dawson, V.L., Dawson, T.M., 1996. Nitric oxide neurotoxicity. J. Chem. Neuroanat. 10, 179–190.

Dawson, V.L., Dawson, T.M., Bartley, D.A., Uhl, G.R., Snyder, S.H., 1993. Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. J. Neurosci. 13, 2651–2661.

Geyer, O., Almog, J., Lupu-Meiri, M., Lazar, M., Oron, Y., 1995. Nitric oxide synthase inhibitors protect rat retina against ischemic injury. FEBS Lett. 374, 399–402.

Heinämäki, A.A., Muhonen, A.S.H., Piha, R.S., 1986. Taurine and other free amino acids in the retina, vitreous, lens, iris-ciliary body, and cornea of the rat eye. Neurochem. Res. 11, 535–542.

Huang, Z., Huang, P.L., Panahian, N., Dalkara, T., Fishman, M.C., Moskowitz, M.A., 1994. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science 265, 1883–1885.

Iadecola, C., Xu, X., Zhang, F., Hu, J., El-Fakahany, E.E., 1994. Prolonged inhibition of brain nitric oxide synthase by short-term systemic administration of nitro-L-arginine methyl ester. Neurochem. Res. 19, 501–505.

Kashii, S., Takahashi, M., Mandai, M., Shimizu, H., Honda, Y., Sasa, M., Ujihara, H., Tamura, Y., Yokota, T., Akaike, A., 1994. Protective action of dopamine against glutamate neurotoxicity in the retina. Invest. Ophthalmol. Vis. Sci. 35, 685–695.

Kashii, S., Mandai, M., Kikuchi, M., Honda, Y., Tamura, Y., Kaneda, K., Akaike, A., 1996. Dual actions of nitric oxide in *N*-methyl-D-aspartate receptor-mediated neurotoxicity in cultured retinal neurons. Brain Res. 711, 93–101.

Kikuchi, M., Kashii, S., Honda, Y., Ujihara, H., Sasa, M., Tamura, Y., Akaike, A., 1995. Protective action of zinc against glutamate neurotoxicity in cultured retinal neurons. Invest. Ophthalmol. Vis. Sci. 36, 2048–2053.

Lucas, D.R., Newhouse, J.P., 1957. The toxic effect of sodium L-glutamate on the inner layers of the retina. Arch. Ophthalmol. 58, 193–201.

Meldrum, B., Garthwaite, J., 1990. Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends Pharmacol. Sci. 11, 379–387.

Moncada, S., 1992. The 1991 Ulf von Euler Lecture. The L-arginine:nitric oxide pathway. Acta Physiol. Scand. 145, 201–227.

Nathan, C., 1992. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6, 3051–3064.

Perez, M.T.R., Larsson, B., Aim, P., Andersson, K.E., Ehinger, B., 1995. Localisation of neuronal nitric oxide synthase-immunoreactivity in rat and rabbit retinas. Exp. Brain Res. 104, 207–217.

Perry, V.H., 1981. Evidence for an amacrine cell system in the ganglion cell layer of the rat retina. Neuroscience 6, 931–944.

Siliprandi, R., Canella, R., Carmignoto, G., Schiavo, N., Zanellato, A., Zanoni, R., Vantini, G., 1992. *N*-Methyl-D-aspartate-induced neurotoxicity in the adult retina. Vis. Neurosci. 8, 567–573.

Traystman, R.J., Moore, L.E., Helfaer, M.A., Davis, S., Banasiak, K., Williams, M., Hurn, P.D., 1995. Nitro-L-arginine analogues dose- and time-related nitric oxide synthase inhibition in brain. Stroke 26, 864–869.