ELSEVIER

European Journal of Pharmacology 322 (1997) 259–264

ejp

Effects of B vitamins on glutamate-induced neurotoxicity in retinal cultures

Katsuyuki Kaneda [a], Masashi Kikuchi [b], Satoshi Kashii [b], Yoshihito Honda [b], Takehiko Maeda [a], Shuji Kaneko [a], Akinori Akaike [a,*]

[a] *Department of Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-01, Japan*
[b] *Department of Ophthalmology and Visual Sciences, Graduate School of Medicine, Kyoto University, Kyoto 606-01, Japan*

Received 3 December 1996; accepted 20 December 1996

Abstract

The effects of B vitamins on glutamate-induced neurotoxicity were examined using primary cultures obtained from the rat retina. Cell viability was markedly reduced by a brief exposure to glutamate followed by incubation with glutamate-free media for 1 h. Glutamate cytotoxicity was reduced in the cultures that had been maintained in thiamine-, pyridoxine- or nicotinamide-containing medium before the exposure to glutamate. Glutamate cytotoxicity was also reduced by chronic application of thiamine pyrophosphate and pyridoxal phosphate, which are active coenzyme forms of thiamine and pyridoxine, respectively. By contrast, chronic application of riboflavin, pantothenate, biotin, folic acid and inositol did not affect glutamate cytotoxicity. None of the B vitamins tested had any effect on glutamate cytotoxicity when added only during the exposure to glutamate. These findings suggest that chronically applied thiamine, pyridoxine and nicotinamide protect retinal neurons against glutamate cytotoxicity.

Keywords: Vitamin B; Culture; Cytotoxicity; Glutamate; Retina

1. Introduction

B vitamins are essential for the maintenance of normal metabolic functions but are not synthesized in the body. Therefore, they must be furnished from an exogenous source. Deficiency of B vitamins such as thiamine and pyridoxine leads to peripheral and central neuropathy, such as beriberi, Wernicke's syndrome, disturbance of perception and neonatal convulsions (Marcus and Coulston, 1996).

Certain excitatory amino acids, such as glutamate, have a dual action in the retina, acting as neurotransmitters at physiologic concentrations and as toxic substances to neuronal cells when present in excess (Bresnick, 1989). Many of the neurotoxic reactions observed in hypoxic-ischemic retinal injury are now considered to be due to the cytotoxic actions of the excitatory amino acids (David et al., 1988; Mosinger and Olney, 1989; Yoon and Marmor, 1989). Moreover, we have previously demonstrated that a brief exposure to glutamate induced neuronal cell death in the retinal cultures (Kashii et al., 1994; Kikuchi et al., 1995). NMDA receptors were the predominant route of this neurotoxicity, since selective antagonists of NMDA receptors possessed protective effects against glutamate cytotoxicity.

Most studies describing neurological consequences of B vitamin deficiencies dealt with the problem at the clinical level (Marcus and Coulston, 1996), although some epidemiological studies have been performed (Giles et al., 1995). Therefore, this study was aimed to examine the effects of B vitamins in the retina at the cell level. The study was focused on the effects of thiamine (vitamin B_1) and pyridoxine (vitamin B_6) since the deficiency of these B vitamins causes peripheral and central neuropathy. To examine the neuroprotective action of vitamins, we have employed glutamate cytotoxicity since it plays an important role in retinal degeneration such as retinal ischemia (Kashii et al., 1994; Louzada-Junior et al., 1992). Moreover, no studies on the effects of B vitamins on glutamate-induced cytotoxicity in the retina have been reported. We examined the effects of B vitamins, namely, thiamine, riboflavin (vitamin B_2), pyridoxine, nicotinamide, pantothenate, biotin, folic acid and inositol, on the neuronal damage in the retina using cultured rat retinal neurons.

* Corresponding author. Tel.: (81-75) 753-4550; Fax: (81-75) 753-4579.

0014-2999/97/$17.00
PII S0014-2999(96)00997-1

2. Materials and methods

2.1. Cell culture

Primary cultures obtained from the retinae of fetal rats (17–19 days gestation) were used. The procedures have been described previously (Kashii et al., 1994; Kikuchi et al., 1995). In brief, the retinal tissues were mechanically dissociated and single-cell suspensions were plated on plastic coverslips ($(1.2–2.2) \times 10^6$ cells/dish). Cultures were incubated in Eagle's minimal essential medium (Eagle's MEM) supplemented with 10% heat-inactivated fetal bovine serum (1–8 days after plating) or 10% heat-inactivated horse serum (9–12 days after plating) containing 2 mM glutamine, 11 mM glucose (total), 24 mM $NaHCO_3$ and 10 mM HEPES. The cultures were maintained at 37°C in humidified 5% CO_2 atmosphere. After 6 days of plating, non-neuronal cells were removed by addition of 3–10 μM cytosine arabinoside. We used only those cultures maintained for 10–12 days in vitro, and only isolated cells in this study. Our previous immunocytochemical study revealed that the isolated cells in the retinal cultures mainly consisted of amacrine cells (Kashii et al., 1994). The cells located in clusters were excluded from the study since they apparently consisted of different types of cells including photoreceptors (Kashii et al., 1994). In addition, our previous electrophysiological studies using a patch-clamp technique demonstrated that all the retinal neurons tested responded to both NMDA and non-NMDA receptor agonists (Ujihara et al., 1993). Thus, according to Dixon and Copenhagen, such neurons correspond to transient amacrine cells in the tiger salamander retina (Dixon and Copenhagen, 1992).

2.2. Measurement of neurotoxicity

Neurotoxicity induced by glutamate was quantified by examining cultures under Hoffman modulation microscopy according to the methods described previously (Kashii et al., 1994; Kikuchi et al., 1995). All experiments were performed in Eagle's MEM at 37°C. Cell viability was assessed using the Trypan blue exclusion method. Cells stained with Trypan blue were regarded as nonviable. Over 200 cells were counted to determine the viability of the cell culture. The viability of the cultures was calculated as the percentage of the ratio of the number of unstained cells (viable cells) against the total number of cells counted (viable cells plus nonviable cells). In each experiment, five coverslips were used to obtain means ± S.E.M. of the cell viability.

2.3. Drug application and evaluation of drug-induced protection against glutamate cytotoxicity

Protective effects of the drugs were assessed by chronic and acute drug application (Fig. 1). To study chronic drug application, a drug was added to the incubation medium immediately after cell plating until immediately before glutamate exposure. The drugs were removed from the culture medium immediately before glutamate treatment (Fig. 1A). To assess the effects of acute drug application, a drug was added to the glutamate-containing and the following glutamate-free media without preincubation (Fig. 1B). Table 1 summarizes the drug concentrations used in the present study.

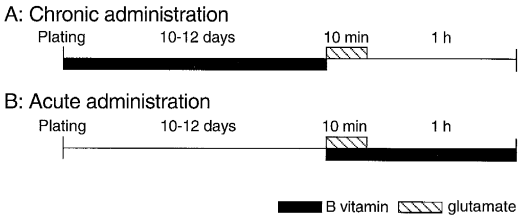

Fig. 1. Methods of B vitamin application.

We have previously reported (Kashii et al., 1994) the protection (%), which is an indicator of a drug's protective effect, calculated using the following equation: protection $(\%) = ((D - G)/(C - G)) \times 100$, in which C is the viability of non-treated cultures (control), G is the viability of glutamate-treated cultures and D is the viability of the cultures treated with a drug and glutamate.

2.4. Drugs

The following drugs were used. Monosodium L-glutamate (Nacalai Tesque), thiamine hydrochloride (Sigma), riboflavin (Sigma), pyridoxine hydrochloride (Wako), thiamine pyrophosphate chloride (Wako), pyridoxal 5-phosphate (Sigma), nicotinamide (Wako), sodium pantothenate (Wako), biotin (Wako), pteroylglutamic acid (Wako) and *myo*-inositol (Wako).

2.5. Statistics

The data were expressed as means ± S.E.M. The statistical significance of the data was determined by Dunnett's two-tailed test.

Table 1
Drug concentrations

B vitamin	Concentration (μM)	
Thiamine	10–100	(3)
Riboflavin	1–10	(0.3)
Pyridoxine	20–200	(5)
Thiamine pyrophosphate	50	(0)
Pyridoxal phosphate	200	(0)
Nicotinamide	20–200	(8)
Pantothenate	10–100	(2)
Biotin	0.2–20	(0.08)
Folic acid	10–100	(2.3)
Inositol	20–200	(10)

Values in parentheses indicate the concentration contained in Eagle's MEM.

3. Results

3.1. Effects of thiamine, riboflavin and pyridoxine

In previous experiments (Kashii et al., 1994; Kikuchi et al., 1995), marked reduction of cell viability was induced by exposing cultures to either glutamate (1 mM) or *N*-methyl-D-aspartate (NMDA) (1 mM) for 10 min followed by incubation in excitatory-amino-acid-free medium for more than 1 h. Therefore, in the present study, glutamate cytotoxicity was assessed by using the following protocol: 10 min to glutamate followed by 1 h incubation in glutamate-free medium.

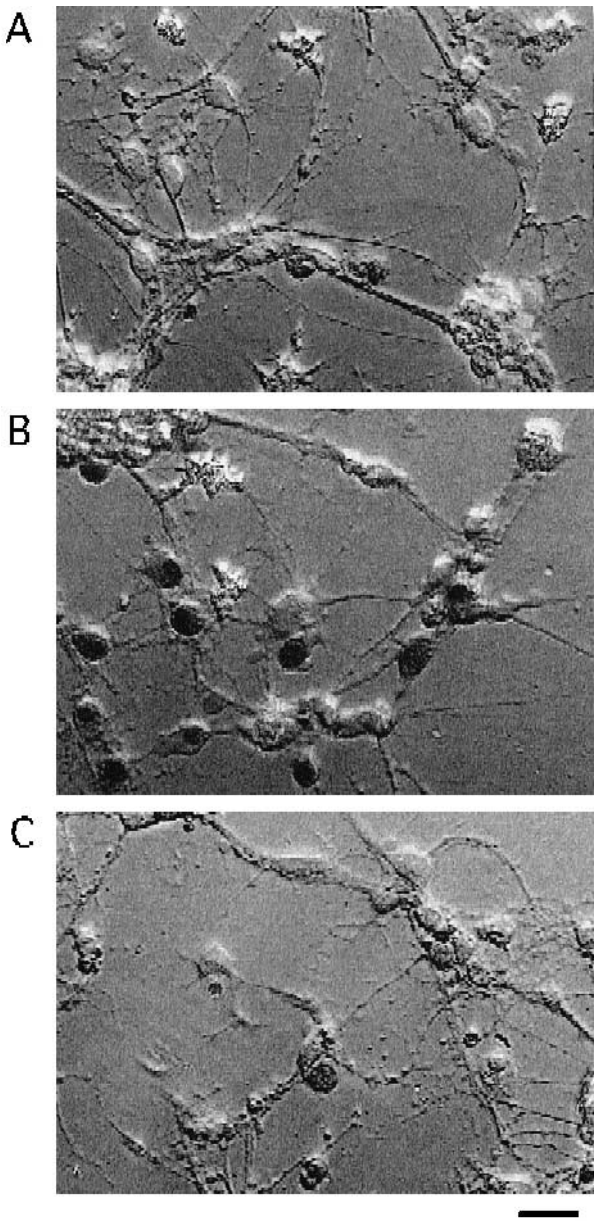

Fig. 2. Effect of chronic pyridoxine (200 μM) exposure on glutamate cytotoxicity. Culture fields were photographed after Trypan blue staining followed by formalin fixation. (A) Non-treated cells (control). (B) Cells incubated with glutamate (1 mM) for 10 min and for a further hour with glutamate-free medium. Cells were maintained in the standard medium. (C) Cells maintained in pyridoxine-containing medium and treated with glutamate (1 mM). Calibration bar = 25 μm.

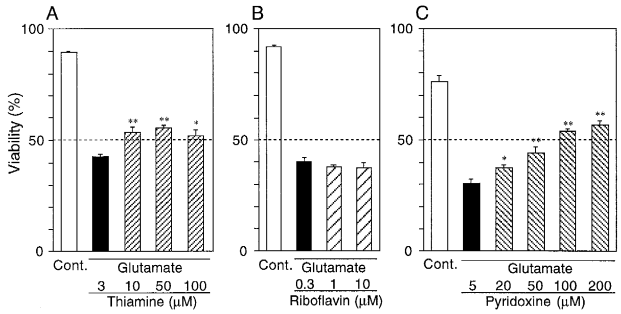

Fig. 3. Effects of chronic application of thiamine (A), riboflavin (B) or pyridoxine (C) against glutamate cytotoxicity. Cultures were exposed to glutamate (1 mM) for 10 min, and then incubated in glutamate-free medium for 1 h. * $P < 0.05$ and ** $P < 0.01$, compared with glutamate alone.

Fig. 2 shows an example of pyridoxine-induced protection against glutamate cytotoxicity. Most cells under control conditions were not stained by Trypan blue (Fig. 2A). A 10-min exposure of the cells to glutamate (1 mM), followed by a 1-h incubation, markedly increased the number of cells stained by Trypan blue (Fig. 2B). Chronic application of pyridoxine (200 μM) reduced glutamate cytotoxicity as revealed by a reduction in the number of stained cells (Fig. 2C).

Fig. 3 summarizes the effects of chronically applied thiamine, riboflavin and pyridoxine on glutamate cytotoxicity. Cultures were maintained in the drug-containing medium for 10–12 days. None of these vitamins induced significant changes in the viability or morphologic alterations of the cultured cells. Since B vitamins examined in the present study are contained in Eagle's MEM, the dosage of B vitamins was set to be 2–250 times of the concentration in the Eagle's MEM itself. In Figs. 3 and 5, the concentrations shown below the black column (glutamate alone) indicate the original concentration of each vitamin in Eagle's MEM. Chronic application of thiamine (10–100 μM) significantly reduced glutamate cytotoxicity, but its effect was not dependent on the concentration (Fig. 3A). Thiamine at 50 μM gave protection (%) of 27.5 ± 3.2%. Chronic application of riboflavin (1–10 μM) did not induce a protective action against glutamate cytotoxicity (Fig. 3B). Chronic administration of pyridoxine (20–200 μM) reduced glutamate cytotoxicity (Fig. 3C). Pyridoxine-induced neuroprotection showed marked concentration dependency. Pyridoxine at 200 μM gave protection (%) of 58.2 ± 4.0%. Incubation of the cultures with 50 μM thiamine (the most effective concentration of thiamine in chronic application) or 200 μM pyridoxine for 24 h prior to glutamate exposure did not affect glutamate cytotoxicity (Table 2). Acute administration of either thiamine (50 μM), riboflavin (10 μM) or pyridoxine (200 μM) did not affect glutamate cytotoxicity (Table 2).

Table 2
Effects of acute and subacute application of B vitamins on glutamate cytotoxicity

B vitamin	Concentration (μM)	Control	Glu	Drug + Glu
Subacute (24 h)				
Thiamine	50	89.6 ± 0.6	45.7 ± 3.6	46.0 ± 4.1
Pyridoxine	200	89.6 ± 0.6	45.7 ± 3.6	45.1 ± 1.4
Nicotinamide	200	89.6 ± 0.6	45.7 ± 3.6	46.1 ± 1.0
Acute				
Thiamine	50	85.3 ± 1.2	45.0 ± 1.3	49.2 ± 0.7
Pyridoxine	200	94.6 ± 1.1	45.7 ± 5.1	51.5 ± 5.3
Nicotinamide	200	85.3 ± 1.7	46.8 ± 2.0	50.4 ± 0.6
Riboflavin	10	94.6 ± 1.1	45.7 ± 5.1	45.3 ± 1.7
Pantothenate	100	82.6 ± 1.6	44.1 ± 1.5	45.1 ± 2.2
Biotin	20	80.1 ± 2.4	38.4 ± 4.9	43.6 ± 2.2
Folic acid	100	82.6 ± 1.6	44.1 ± 1.5	42.0 ± 2.2
Inositol	200	91.4 ± 0.6	29.6 ± 1.2	29.5 ± 2.5

Acute, a drug was added to the glutamate-containing and the subsequent glutamate-free media; Subacute, a drug was added to the medium for 24 h prior to glutamate exposure; Glu, glutamate (1 mM).

As chronic application of thiamine and pyridoxine protected against glutamate cytotoxicity, we examined the effects of their active coenzyme forms. Glutamate cytotoxicity was significantly reduced by chronic exposure to thiamine pyrophosphate (TPP), the active coenzyme form of thiamine, at 50 μM, the most effective concentration of thiamine (Fig. 4A). Moreover, chronic exposure to 200 μM pyridoxal phosphate (PLP), which is the active coenzyme form of pyridoxine, also reduced glutamate cytotoxicity (Fig. 4B).

3.2. Effects of nicotinamide, pantothenate, biotin, folic acid and inositol

Fig. 5 summarizes the effects of chronic application of nicotinamide (20–200 μM), pantothenate (10–100 μM), biotin (0.2–20 μM), folic acid (10–100 μM) and inositol (20–200 μM) on glutamate cytotoxicity. Nicotinamide at the higher concentration (200 μM) but not at the lower concentration (20 μM) significantly reduced glutamate cytotoxicity and gave protection (%) of 48.5 ± 4.5%. In contrast, none of the remaining B vitamins affected glutamate cytotoxicity. Incubating the cultures with 200 μM nicotinamide for 24 h prior to glutamate exposure did not affect glutamate cytotoxicity (Table 2). Acute administration of nicotinamide (200 μM), pantothenate (100 μM), biotin (20 μM), folic acid (100 μM) and inositol (200 μM) did not affect glutamate cytotoxicity (Table 2).

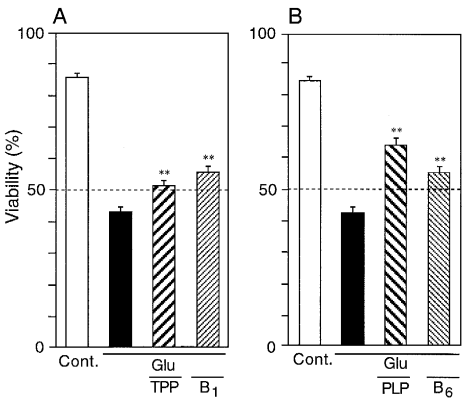

Fig. 4. Protective effects of chronic application of TPP (A) and PLP (B) against cytotoxicity induced by a brief incubation with glutamate (1 mM). (A) Cultures were maintained in either TPP (50 μM)- or thiamine (B_1, 50 μM)-containing medium prior to glutamate exposure. (B) Cultures were maintained in either PLP (200 μM)- or pyridoxine (B_6, 200 μM)-containing medium prior to glutamate exposure. ** $P < 0.01$, compared with glutamate alone.

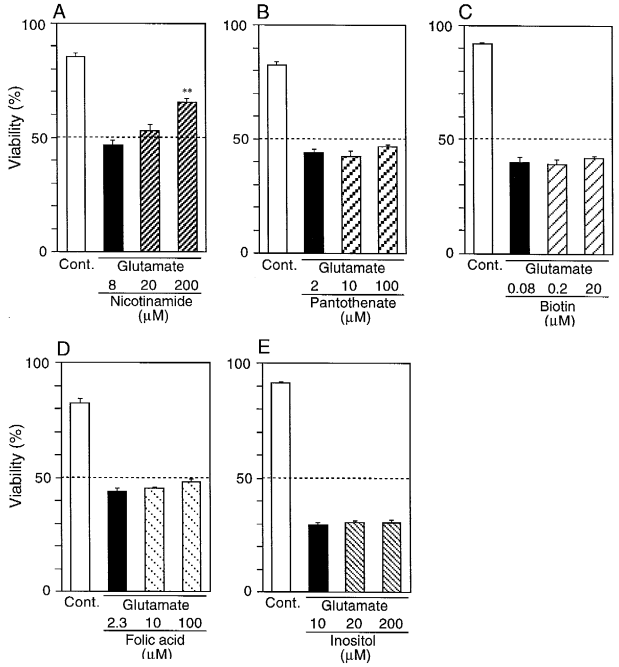

Fig. 5. Effects of chronic application of nicotinamide (A), pantothenate (B), biotin (C), folic acid (D) and inositol (E) on cytotoxicity induced by a brief incubation with glutamate (1 mM). ** $P < 0.01$, compared with glutamate alone.

4. Discussion

Brief glutamate exposure induces delayed death in cultured neurons of the cerebral cortex (Choi et al., 1987; Akaike et al., 1991) and retina (Levy and Lipton, 1990). The NMDA subtype of glutamate receptor is considered to be the predominant route of glutamate neurotoxicity, since NMDA receptor antagonists prevent neuronal degeneration in cultured cortical (Tamura et al., 1993) and retinal (El-Asrar et al., 1992; Kashii et al., 1994; Levy and Lipton, 1990) neurons. Previously both MK-801, a selective NMDA channel blocker, and CPP, a competitive NMDA receptor antagonist, inhibited glutamate cytotoxicity in retinal cultures (Kashii et al., 1994; Kikuchi et al., 1995), suggesting that the cytotoxicity induced by the brief

glutamate exposure was mediated by NMDA receptors in the cultured retinal neurons.

This study demonstrated that glutamate cytotoxicity was reduced by the chronic administration of thiamine, pyridoxine and nicotinamide. These findings indicate that thiamine, pyridoxine and nicotinamide possess protective effects against glutamate cytotoxicity in cultured rat retinal neurons. To our knowledge, this is the first evidence for the neuroprotective actions of thiamine, pyridoxine and nicotinamide against glutamate cytotoxicity in the retina. In contrast to the effects of chronic application, acute application of thiamine, pyridoxine and nicotinamide did not affect glutamate cytotoxicity. Moreover, the vitamins were removed from the medium prior to glutamate exposure in chronic application. Therefore, it is unlikely that these drugs directly interact with glutamate receptors.

Thiamine is converted to thiamine pyrophosphate (TPP), the physiologically active form of thiamine, by phosphorylation. TPP functions in carbohydrate metabolism as a coenzyme in the decarboxylation of pyruvate and α-ketoglutarate and in the utilization of pentose in the hexose monophosphate shunt (Marcus and Coulston, 1996). These reactions catalyzed by TPP-dependent enzymes are important in energy production. The impairment of energy-dependent mechanisms for maintaining membrane potential results in partial membrane depolarization which abolishes the voltage-dependent Mg^{2+} block of the NMDA receptor and permits even normal physiological concentrations of glutamate to induce abnormally prolonged and persistent inward currents (Beal et al., 1993; Henneberry et al., 1989) and neuronal degeneration (Novelli et al., 1988). In the present study, we revealed that chronic exposure to thiamine reduced glutamate cytotoxicity. Moreover, TPP also reduced glutamate cytotoxicity by chronic application. Therefore, the mechanism of thiamine-induced neuroprotection may be explained by its effects to produce energy in excess by activation of TPP-dependent enzymes.

Pyridoxine is converted to pyridoxal phosphate (PLP), the biologically active form of vitamin B_6. PLP serves a vital role in metabolism as a coenzyme for a wide variety of metabolic transformation of amino acids, including decarboxylation, transamination, and racemization, as well as for enzymatic steps in the metabolism of tryptophan, sulfur-containing amino acids, and hydroxy amino acids (Marcus and Coulston, 1996). In the present study, glutamate cytotoxicity was reduced not only by pyridoxine but also by PLP. Thus, the manifestation of pyridoxine-induced protection may be mediated by PLP which activates PLP-dependent enzymes including amino-acid metabolism.

Windebank (1985) has demonstrated that pyridoxine inhibited the outgrowth of neurites from the dorsal root ganglion (DRG) neurons in culture at a concentration of 1 mM, but causes cell death at a concentration of higher than 10 mM. The retina is within the blood–retinal barrier, but the DRG neurons are not covered by any barriers such as the blood–brain barrier. Even when systemically administered in high doses, pyridoxine would not reach the concentration of the order of 1 mM in the retinal extracellular space because pyridoxine is a water-soluble vitamin. Thus, we did not examine the effects of pyridoxine at a concentration higher than 200 μM. Chronic application of 200 μM pyridoxine did not affect the cell viability or morphology of cultured retinal neurons.

Although the mechanism of the neuroprotective action of nicotinamide remains to be determined, it may exhibit the effects as NAD or NADP because nicotinamide exerts its physiological actions after being converted to either NAD or NADP (Marcus and Coulston, 1996).

In conclusion, the present study demonstrates that chronically applied thiamine, pyridoxine and nicotinamide protect cultured retinal neurons against glutamate-induced cytotoxicity. These findings suggest the possible prophylactic and therapeutic application of thiamine, pyridoxine and nicotinamide in reducing cell death due to glutamate cytotoxicity-related diseases such as retinal ischemia. Further studies are necessary to determine the mechanism linked to the neuroprotective action of thiamine, pyridoxine or nicotinamide.

Acknowledgements

This study was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

References

Akaike, A., Y. Tamura, Y. Sato, K. Ozaki, R. Matsuoka, S. Miura and T. Yoshinaga, 1991, Cholecystokinin-induced protection of cultured cortical neurons against glutamate neurotoxicity, Brain Res. 557, 303.

Beal, M.F., B.T. Hyman and W. Koroshetz, 1993, Do defects in mitochondrial energy metabolism underlie the pathology of neurodegenerative diseases? Trends Neurosci. 16, 125.

Bresnick, G.H., 1989, Excitotoxins: a possible new mechanism for the pathogenesis of ischemic retinal damage, Arch. Ophthalmol. 107, 339.

Choi, D.W., G.M. Maulucci and A.R. Kriegstein, 1987, Glutamate neurotoxicity in cortical cell culture, J. Neurosci. 7, 357.

David, P., M. Lusky and V.I. Teichberg, 1988, Involvement of excitatory neurotransmitters in the damage produced in chick embryo retinas by anoxia and extracellular high potassium, Exp. Eye Res. 46, 657.

Dixon, D.B. and D.R. Copenhagen, 1992, Two types of glutamate receptors differentially excite amacrine cells in the tiger salamander retina, J. Physiol. (London) 449, 589.

El-Asrar, A.M., P.H. Morse, D. Maimone, E. Torczynski and A.T. Reder, 1992, MK-801 protects retinal neurons from hypoxia and the toxicity of glutamate and aspartate, Invest. Ophthalmol. Vis. Sci. 33, 3463.

Giles, W.H., S.J. Kittner, R.F. Anda, J.B. Croft and M.L. Casper, 1995, Serum folate and risk for ischemic stroke. First National Health and Nutrition Examination Survey epidemiologic follow-up study, Stroke 26, 1166.

Henneberry, R.C., A. Novelli, J.A. Cox and P.G. Lysko, 1989, Neurotoxicity at the *N*-methyl-D-aspartate receptor in energy-compromised neurons. An hypothesis for cell death in aging and disease, Ann. NY Acad. Sci. 568, 225.

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

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

Levy, D.I. and S.A. Lipton, 1990, Comparison of delayed administration of competitive and uncompetitive antagonists in preventing NMDA receptor-mediated neuronal death, Neurology 40, 852.

Louzada-Junior, P., J.J. Dias, W.F. Santos, J.J. Lachat, H.F. Bradford and J. Coutinho-Netto, 1992, Glutamate release in experimental ischaemia of the retina: an approach using microdialysis, J. Neurochem. 59, 358.

Marcus, R. and A.M. Coulston, 1996, The vitamins: water-solubule vitamins, in: Goodman and Gilman's The Pharmacological Basis of Therapeutics, 9th edn., eds. A. Goodman Gilman, J.G. Hardman, L.E. Limbird, P.B. Molinoff and R.W. Ruddon (Pergamon Press, New York, NY) p. 1547.

Mosinger, J.L. and J.W. Olney, 1989, Photothrombosis-induced ischemic neuronal degeneration in the rat retina, Exp. Neurol. 105, 110.

Novelli, A., J.A. Reilly, P.G. Lysko and R.C. Henneberry, 1988, Glutamate becomes neurotoxic via the *N*-methyl-D-aspartate receptor when intracellular energy levels are reduced, Brain Res. 451, 205.

Tamura, Y., Y. Sato, T. Yokota, A. Akaike, M. Sasa and S. Takaori, 1993, Ifenprodil prevents glutamate cytotoxicity via polyamine modulatory sites of *N*-methyl-D-aspartate receptors in cultured cortical neurons, J. Pharmacol. Exp. Ther. 265, 1017.

Ujihara, H., A. Akaike, Y. Tamura, T. Yokota, M. Sasa, S. Kashii and Y. Honda, 1993, Blockade of retinal NMDA receptors by sodium nitroprusside is probably due to nitric oxide formation, Jpn. J. Pharmacol. 61, 375.

Windebank, A.J., 1985, Neurotoxicity of pyridoxine analogs is related to coenzyme structure, Neurochem. Pathol. 3, 159.

Yoon, Y.H. and M.F. Marmor, 1989, Dextromethorphan protects retina against ischemic injury in vivo, Arch. Ophthalmol. 107, 409.