

Melatonin prevents the delayed death of hippocampal neurons induced by enhanced excitatory neurotransmission and the nitridergic pathway

STEPHEN D. SKAPER,¹ BIANCAMARIA ANCONA,* LAURA FACCI, DAVIDE FRANCESCHINI, AND PIETRO GIUSTI²

Department of Pharmacology, University of Padua, Padua, Italy; and *Biopolymer Research Center, CNR and Department of Organic Chemistry, University of Padua, Padua, Italy

ABSTRACT The mechanisms by which neurons die after stroke and status epilepticus and related neuropathological conditions are unclear, but may involve voltage-dependent Na⁺ channels, glutamate receptors, and nitric oxide (NO•). These questions were investigated using an in vitro primary cell culture model in which hippocampal pyramidal neurons undergo a gradual and delayed neurodegeneration induced by enhanced excitatory neurotransmission. When cells were treated with Mg²⁺-free, glycine-supplemented medium for a brief period (15 min) and examined 24 h later, ~30–40% of the neurons had died. Cell death could be inhibited by blockers of voltage-sensitive Na⁺ channels and by N-methyl-D-aspartate receptor antagonists. Application of either the endogenous antioxidant melatonin (EC₅₀: 19.2 ± 2.8 μM) or the NO• synthase inhibitor N^o-nitro-L-arginine after, but not during, Mg²⁺-free exposure protected against delayed neuronal death; significant neuroprotection was observed when the addition was delayed for up to 4 h. This operational time window suggests that an enduring production of NO• and reactive oxygen species from neuronal sources is responsible for delayed cell death. A role for reactive oxygen species in this injury process was strengthened by the finding that, whereas neurons cocultured with astroglia were more resistant to killing, agents capable of lowering intracellular glutathione negated this protection. Because secretion levels of melatonin are decreased with aging, reductions in this pineal hormone may place neurons at a heightened risk for damage by excitatory synaptic transmission.—Skaper, S. D., Ancona, B., Facci, L., Franceschini, D., Giusti, P. Melatonin prevents the delayed death of hippocampal neurons induced by enhanced excitatory neurotransmission and the nitridergic pathway. *FASEB J.* 12, 725–731 (1998)

Key Words: synaptic transmission · hippocampus · excitotoxicity · NO• · ROS · neuroprotection · GSH · astrocytes

SINCE THE INITIAL observation that glutamate and its analogs could kill neurons in the brain (1), it has

been suggested that endogenous glutamate may mediate neuronal death in a variety of pathological conditions, including stroke and status epilepticus (2, 3). This process has come to be known as 'excitotoxicity'. Local injection of excitatory amino acid (EAA)³ receptor agonists and stimulation of EAA afferent pathways produce a pattern of acute and chronic morphological changes in the brain similar to those found in patients with epilepsy (4, 5). Antagonists of EAA receptors possess anticonvulsant properties and prevent the morphological changes associated with seizures (6, 7). Enhanced excitatory synaptic transmission can also damage hippocampal neurons in culture via activation of N-methyl-D-aspartate (NMDA) receptors (8, 9).

Several lines of evidence suggest that reactive oxygen species (ROS) play a pivotal role in the pathogenesis of excitotoxic death (10). Neurotoxicity associated with overstimulation of NMDA receptors is thought to be mediated by an excessive Ca²⁺ influx, leading to a series of potentially neurotoxic events (11). One such event is the activation of nitric oxide synthase and the subsequent production of nitric oxide (NO•) (12); another is the stimulation of phospholipase A₂ or Ca²⁺ overload of mitochondria, leading to the generation of superoxide anion (O₂^{•-}) (13). NO• can react with O₂^{•-} to form peroxynitrite (14), which results in dose-dependent neuronal damage (15).

We used primary cultures of rat hippocampus, a brain area highly vulnerable to cerebral ischemia and

¹ Present address: SmithKline Beecham Pharmaceuticals, Neurosciences Research Department, New Frontiers Science Park North, Third Avenue, Harlow, Essex CM19 5AW, U.K. E-mail: stephen.skaper-1@sbphrd.com@INET

² Correspondence: Department of Pharmacology, L.go Meneghetti, 2, 35131 Padova, Italy. E-mail: giusti@dfem.unipd.it

³ Abbreviations: CPP, 3-((RS)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EAA, excitatory amino acid; GSH, glutathione; NMDA, N-methyl-D-aspartate; NNA, N-nitro-L-arginine; NO•, nitric oxide; PBN, N-tert-butyl-α-phenylnitrone; O₂^{•-}, superoxide anion; ROS, reactive oxygen species; TTX, tetrodotoxin.

epileptic injury, as a model to understand the roles of and relationships among Na⁺ channels, NO•, and ROS in the neurodegenerative cascade triggered by *endogenous* NMDA receptor activation. In this paradigm, placing hippocampal pyramidal neurons in a Mg²⁺-free, glycine-supplemented medium for brief periods of time increases cell death considerably 24 h later (8, 9, 16), a result of seizure-like activity (8, 9). Not unexpectedly, cell death could be inhibited by NMDA receptor antagonists and blockers of voltage-dependent Na⁺ channels applied either during or after removal of Mg²⁺. The cytoprotective antioxidant melatonin (17–19) and inhibitors of neuronal NO• synthase were neuroprotective when applied after but not during exposure to Mg²⁺-free, glycine-supplemented medium, proposing a protracted production of NO• and ROS as participants in delayed neuronal death. Astrocytes appeared to contain significant antioxidant defense stores for hippocampal neurons.

MATERIALS AND METHODS

Cell culture

Hippocampi were removed from embryonic day 18 Sprague-Dawley rat fetuses (Harlan, S. Pietro al Natisone, UD, Italy) and dissociated in culture medium as described (20). Culture medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented to contain 2 mM glutamine, 30 mM glucose, 20 mM KCl, 1 mM sodium pyruvate, 1% heat-inactivated fetal calf serum (BIO·SPA, Wedel, Germany), N1 components (100 µg/ml transferrin, 25 µg/ml insulin, 60 µM putrescine, 20 nM progesterone, 30 nM sodium selenite) (21), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cell suspension was placed in 48-well plates 9 mm in diameter (Falcon, Oxnard, Calif.) coated with 10 µg/ml poly-L-lysine (mol wt 68,000, Sigma, St. Louis, Mo.), 1×10^5 cells/cm² in 0.3 ml medium. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂-95% air. Cytosine arabinoside (5 µM) was added after 5 days to inhibit nonneuronal cell growth. Medium glucose was renewed every 7 days by adding fresh glucose to 5 mM. Cultures were used between 13 and 17 days without a change in medium.

Neurotoxicity assays

Culture-conditioned medium was removed and saved. The cultures were washed with Locke's solution (19), with or without 1 mM MgCl₂. The Mg²⁺-free Locke's solution contained 1 µM glycine. Drug treatments were carried out for 15 min (23–25°C) in Mg²⁺-free, glycine-supplemented Locke's solution in a final volume of 0.3 ml. After treatment, the hippocampal cell monolayers were washed with complete Locke's solution and returned to their original culture medium. Twenty-four hours later, cultures were fixed with 2% paraformaldehyde. Neuronal death was assessed by microscopic examination of representative fields under phase contrast optics at 200×. Cytotoxicity was evidenced by neurons that underwent disintegration in the 24 h interval after initiating treatment (8, 9, 16). Viable pyramidal-like neurons had a soma that was phase-bright and round to oval in shape, with smooth, intact neuro-

tes. Neurons were considered nonviable when they exhibited neurite fragmentation and 'beading' and when their soma exhibited swelling and vacuolation. Cell counts were made from at least five such fields in triplicate wells in three independent experiments. Control experiments showed that the loss of viable neurons assessed in this manner was proportional to the number of neurons damaged, as estimated by trypan blue staining (22).

Materials

Culture media, N1 supplements, antibiotics, cytosine arabinoside, aminoguanidine, melatonin, N^o-nitro-L-arginine (NNA), N-tert-butyl- α -phenylnitron (PBN), and tetrodotoxin (TTX) were purchased from Sigma; 3-((RS)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were from Tocris Cookson Ltd. (Bristol, U.K.).

Statistical analysis

Comparisons were made by one-way analysis of variance with the Student-Newman-Keuls post hoc test for differences between groups. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Pharmacology of neurodegeneration

Exposure of hippocampal cell cultures, which are predominantly pyramidal neurons from this age of embryonic rodent (23), to a Mg²⁺-free, glycine-supplemented solution for 15 min produced a considerable increase in cell death assessed 24 h later (**Table 1**). Comparable levels of cell death have been reported in earlier studies under similar culture conditions (9, 16). This injury very likely is a consequence of enhanced excitatory neurotransmission in a Mg²⁺-free environment (24) since TTX, which blocks voltage-gated Na⁺ channels, was protective when added either simultaneously with Mg²⁺ removal or immediately after returning the cells to a Mg²⁺-

TABLE 1. Pharmacology of delayed hippocampal pyramidal cell death after 15 min exposure to a Mg²⁺-free solution^a

Addition	% Cell death	
	Simultaneous	After
None	38.4 ± 3.9	—
MK 801 (1 µM)	0	5.3 ± 3.6*
CPP (10 µM)	0	n.d.
TTX (1 µM)	0	24.7 ± 3.3 [†]
DNQX (10 µM)	34.3 ± 5.7	43.2 ± 4.0

^a Cultures were incubated for 15 min in Mg²⁺-free/glycine-supplemented Locke's solution. Drugs were added either at this time ('simultaneous') or immediately after the cultures were returned to their original medium ('after'). Neuron survival was monitored 24 h later. Values are means ± SD (three experiments). n.d., not determined. * $P < 0.01$, [†] $P < 0.05$ vs. Mg²⁺-free alone (none).

containing medium (Table 1). Activation of NMDA receptors appeared to be a key ingredient of the injury process, as competitive (CPP) and noncompetitive (MK-801) NMDA antagonists abolished cell death when added to the Mg^{2+} -free incubation or immediately thereafter (Table 1). In contrast, the selective kainate/AMPA receptor antagonist DNQX was without effect (Table 1).

NO• and neurodegeneration

NO• has been implicated as a mediator of excitotoxicity, particularly that mediated by NMDA receptors (12). To test for the participation of NO•, hippocampal cultures were treated with the NO• synthase inhibitor NNA (100 μ M). If the inhibitor was added immediately after termination of the Mg^{2+} -free incubation, a substantial degree of neuroprotection, quantitatively similar to that produced by NMDA antagonists (e.g., MK 801), was achieved (Fig. 1, left). However, NNA was not neuroprotective if applied only during the Mg^{2+} -free exposure (Fig. 1, left). Dissociation of NNA from NO• synthase is quite slow (25), and inhibition by NNA of the enzyme is expected to be sustained well beyond its washout from the incubation medium. Moreover, significant protection was observed when addition of the NO• synthase inhibitor was delayed for up to 4 h after concluding the Mg^{2+} -free challenge (Fig. 1, right). These findings suggest that late NO• formation, rather than that occurring during or shortly after Mg^{2+} removal, was associated with neurodegeneration.

Neurodegeneration triggered by brief NMDA receptor activation could be effected by expression of the inducible form of NO• synthase, which conceivably (e.g., through activation of gene transcription) could depend on NMDA receptor activity (26). This possibility was tested pharmacologically because neuronal and inducible NO• synthase isoforms are dif-

ferentially sensitive to NNA and aminoguanidine, respectively (27). In contrast to NNA, aminoguanidine failed to inhibit cell death triggered by Mg^{2+} removal (Fig. 1, left).

Melatonin prevents death of hippocampal neurons caused by enhanced synaptic activity

NO• toxicity may arise from inhibition of mitochondrial respiration and/or reaction with $O_2^{\cdot -}$ to form the prooxidant species peroxynitrite, which can further degrade into a highly reactive and lipid-destroying hydroxyl or hydroxyl-like radical (14, 26). On this basis, a protective role for ROS scavengers would be predicted in the present injury paradigm. The pineal secretory product melatonin has antioxidant effects directed to different ROS, including hydroxyl radicals (19, 28, 29), and protects against ROS-mediated neuronal death (17, 18, 30). In contrast to conventional antioxidants, this hormone has a proposed physiological role in neuropathological settings (31). Addition of melatonin to hippocampal cultures in the absence of Mg^{2+} did not reduce cell loss (see Fig. 3, left). Melatonin, however, concentration-dependently (EC_{50} : 19.2 ± 2.8 μ M, three independent determinations) reduced neuronal death by more than 75% when added immediately after the end of the Mg^{2+} -free incubation (Fig. 2). PBN, a chemically unrelated free radical scavenger, also limited hippocampal neuron death under Mg^{2+} -free conditions when added immediately after, but not during, the period of Mg^{2+} removal (Fig. 3, left).

To investigate the time window over which melatonin neuroprotection was effective, the pineal hormone (100 μ M) was applied at various intervals after returning the Mg^{2+} -free cultures to their original medium. Significant neuroprotection was observed when addition of melatonin was delayed for up to 4 h after exposure to Mg^{2+} -free, glycine-supplemented solution, but by 6 h the effect was lost (Fig. 3, right).

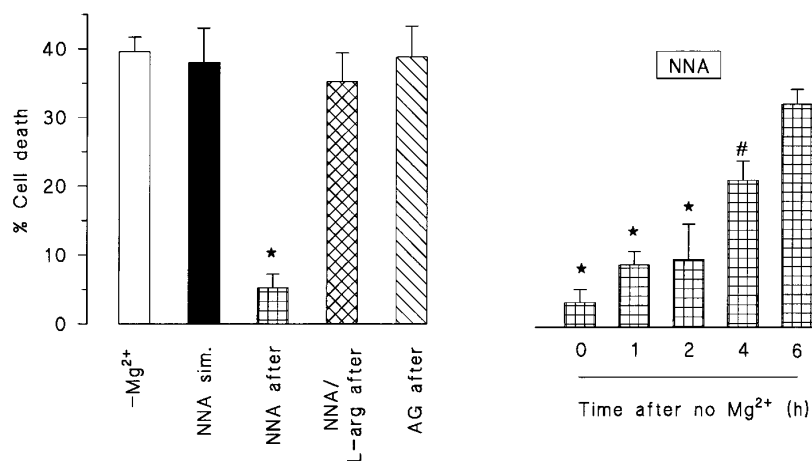


Figure 1. Inhibition of NO• synthase protects hippocampal pyramidal cells from death induced by Mg^{2+} withdrawal. Left: Cultures were exposed to Mg^{2+} -free, glycine-supplemented Locke's solution (15 min) in the presence ('sim.') or absence of NNA (100 μ M) and allowed to recover for 24 h in their original medium, without or with NNA (100 μ M) \pm L-arginine (L-Arg, 1 mM) or aminoguanidine (AG, 100 μ M), before culture viability was measured. Right: Cultures were exposed to Mg^{2+} -free, glycine-supplemented Locke's solution (15 min) and allowed to recover for various periods of time before addition of NNA (100 μ M). Culture viability was assessed after 24 h. All values are means \pm SD of three independent experiments (three culture wells/treatment per experiment). # $P < 0.05$ or * $P < 0.01$ vs. no Mg^{2+} .

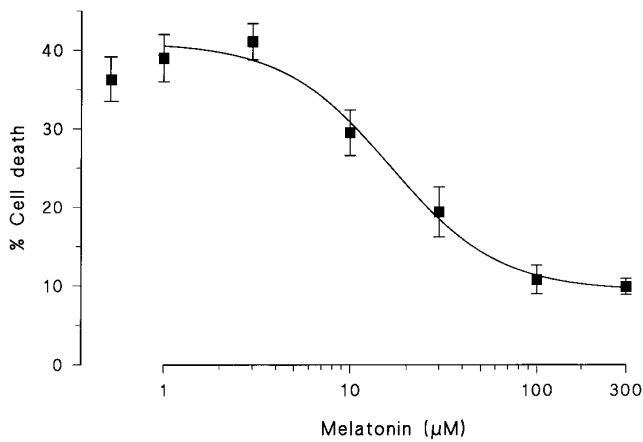


Figure 2. Melatonin concentration-dependently rescues hippocampal pyramidal cells from death induced by Mg^{2+} withdrawal. Cultures were exposed to Mg^{2+} -free, glycine-supplemented Locke's solution (15 min) and allowed to recover for 24 h in their original medium with the indicated concentrations of melatonin before culture viability was measured. Cell death in the absence of melatonin was $36.2 \pm 2.7\%$. All values are means \pm SD of three independent experiments (three culture wells/treatment per experiment).

Luzindole (30 μM), a highly specific melatonin receptor antagonist (32), did not inhibit the neuroprotective action of melatonin (30 μM) ($38.7 \pm 1.4\%$, $19.4 \pm 3.2\%$, and $15.0 \pm 5.3\%$ cell death, respectively, for no Mg^{2+} , no Mg^{2+} with delayed melatonin, or no Mg^{2+} with delayed melatonin and luzindole; $n=3$).

Astroglia and the glutathione system in neuroprotection

In the central nervous system, the antioxidant glutathione (GSH) is concentrated within glial cells, and perhaps in axons and nerve terminals, but is sparse in neuronal cell bodies (33). Glial cells have been shown to play a key role in the maintenance of neuronal GSH (34) and to protect brain neurons from oxidative stress (35–37). We therefore explored the influence of astrocytes on hippocampal neuron in-

jury induced by Mg^{2+} removal. Neuron-astroglia cocultures were generated by omission of cytosine arabinoside from the culture medium (38). Neuron death precipitated by brief omission of Mg^{2+} was markedly reduced 24 h later in astrocyte-rich cultures (Table 2). Inclusion of diethyl maleate (1 mM) to bind the free sulfhydryl groups of GSH (39) during the Mg^{2+} -free incubation significantly reduced the improvement in hippocampal neuron survival mediated by astrocytes (Table 2). Diethyl maleate had little influence on neuron vitality in Mg^{2+} -containing cultures, independent of the presence of astrocytes (Table 2). These results suggest that astrocytes act to maintain neuronal oxidant homeostasis under conditions where ROS levels become toxic. Astroglial uptake of glutamate protects cultured cortical neurons from glutamate toxicity (40). L(-)-Threo-3-hydroxyaspartic acid, a potent inhibitor of glutamate transport, however, did not antagonize the astrocyte protective effect for hippocampal neurons exposed to a Mg^{2+} -free solution (data not shown).

DISCUSSION

These studies illustrate that under Mg^{2+} -free, glycine-supplemented conditions, mature hippocampal pyramidal neurons undergo a clear decrease in cell viability over 24 h. The inhibitory effects of TTX, CPP, and MK 801 all support the view that the cell death observed is triggered by synaptically activated NMDA receptors (9, 16) and is not the result of the activation of metabotropic glutamate receptors and Ca^{2+} mobilization from intracellular stores (41). The delayed injury mechanism involved a cycle of Na^+ channel activation, NMDA-sensitive glutamate receptors, and $NO\cdot$ production from neuronal sources. Generation of $NO\cdot$ and ROS appeared to be an enduring process, as both neuronal $NO\cdot$ synthase inhibitors and the antioxidant melatonin displayed similar kinetics in protecting against delayed neu-

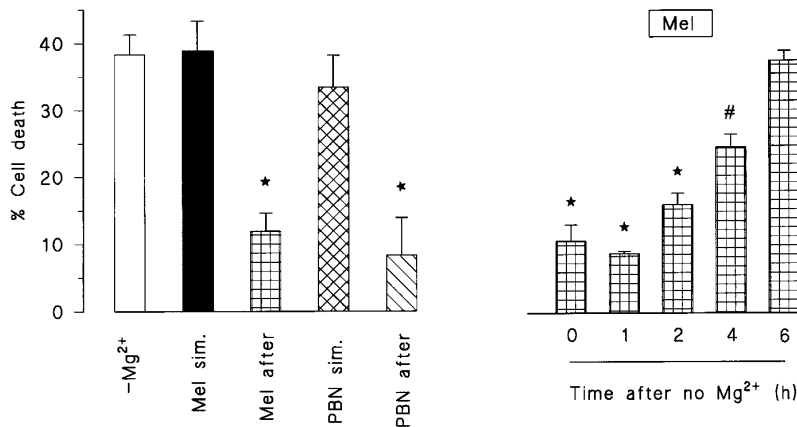


Figure 3. Kinetics of melatonin protection against hippocampal pyramidal cell death induced by Mg^{2+} withdrawal. Left: Cultures were exposed to Mg^{2+} -free, glycine-supplemented Locke's solution (15 min) in the presence ('sim.') or absence of either melatonin (Mel, 100 μM) or PBN (100 μM) and allowed to recover for 24 h in their original medium, without or with melatonin (100 μM) or PBN (100 μM), before culture viability was measured. Right: Cultures were exposed to Mg^{2+} -free, glycine-supplemented Locke's solution (15 min) and allowed to recover for various periods of time before addition of melatonin (100 μM). Culture viability was assessed after 24 h. All values are means \pm SD of three independent experiments (three culture wells/treatment per experiment). # $P < 0.05$ or * $P < 0.01$ vs. no Mg^{2+} .

TABLE 2. *Astrocytes buffer oxidative stress in hippocampal pyramidal cells incubated in Mg²⁺-free solution^a*

Culture condition	% Cell survival	
	Astrocyte-poor	Astrocyte-rich
Control	100	114.5 ± 3.4
-Mg ²⁺ /+glycine	62.1 ± 4.6	102.6 ± 5.2*
Control plus DEM (1 mM)	93.8 ± 3.5	110.8 ± 5.9
-Mg ²⁺ /+glycine plus DEM (1 mM)	51.7 ± 0.4 [†]	69.2 ± 0.9 [‡]

^a Cultures maintained in the presence ('astrocyte poor') or absence ('astrocyte-rich') of cytosine arabinoside (5 μM) were incubated 15 min in Mg²⁺-containing (control) or Mg²⁺-free/glycine-supplemented Locke's solution. In some groups, diethyl maleate (DEM, 1 mM) was added at this time. Neuron survival was monitored 24 h later. Values are means ± SD (three experiments). * $P < 0.01$ vs. the corresponding Mg²⁺-free, astrocyte-poor culture. [†] $P < 0.05$ and [‡] $P < 0.01$ vs. the corresponding Mg²⁺-free culture without DEM.

ronal death when added up to 4 h after, but not simultaneously with, removal of Mg²⁺. The data also provide support for a glial contribution in protecting neurons from oxidative stress.

NMDA receptor-mediated neurotoxicity may depend in part on the generation of NO• and O₂•⁻, which react to form peroxynitrite. This form of neurotoxicity is thought to contribute to a final common pathway of injury in a wide variety of acute and chronic neurologic disorders, including focal ischemia, trauma, epilepsy, Huntington's disease, Alzheimer's disease, and amyotrophic lateral sclerosis (42, 43). The introduction of selective pharmacological tools and the development of transgenic knockout mice specific for the different isoforms of NO• synthase have helped to clarify the role of NO• in excitotoxic brain injury (42). NO• participation in neuronal death induced by brief NMDA exposure has been observed (44, 45), although NO• synthase inhibitors were effective solely for the duration of the initial NMDA challenge (44) or during the postexposure period (45). The paradigm described here, in contrast, relies on neurotoxicity precipitated by the endogenous activation of NMDA receptors, and thus more faithfully mimics the injury process linked to excessive synaptic transmission (8). It has been reported recently that peroxynitrite and NO• donors induce neurodegeneration by eliciting autocrine excitotoxicity at NMDA receptors (46). This finding is consistent with the current data and suggests a feed-forward mechanism in which delayed neuronal death begins with Mg²⁺ removal and NMDA receptor stimulation, activation of TTX-sensitive Na⁺ channels, glutamate release, and additional NMDA receptor activation, leading ultimately to a protracted production of NO•. A similar cycle has been proposed for the delayed death of cultured striatal neurons after a brief challenge with NMDA (45).

The pineal secretory product melatonin is known to possess widespread free radical scavenging and antioxidant activities (19) and is neuroprotective in vivo and in vitro against kainic acid-induced lesions (17), GSH depletion (18), and ROS-mediated apoptotic death (30). Melatonin is believed to work via electron donation to directly detoxify free radicals such as the highly toxic hydroxyl radical, which is a probable end-product of the reaction between NO• and peroxynitrite. Melatonin is also reported to scavenge peroxynitrite (47, 48). Furthermore, melatonin limits NO•-induced lipid peroxidation (49) and inhibits cerebellar NO• synthase (50). Thus, the cytoprotective action of the pineal hormone in the present system could be attributed to one or several of these pathways. The kinetics of neuroprotection afforded by the delayed addition of melatonin closely mirror those for NO• synthase inhibitors, which lends support to this premise.

Melatonin function as a free radical scavenger and antioxidant is likely facilitated by the ease with which it crosses morphophysiological barriers (e.g., the blood-brain barrier) and enters cells and subcellular compartments (19, 51). Most studies have used pharmacological concentrations of melatonin to protect against free radical damage, although physiological levels of the indole have been shown to be beneficial in preventing the death of neuroblastoma cells exposed to the Alzheimer amyloid peptide (52). Some evidence indicates that pinealectomy, which eliminates the nighttime rise in circulating and tissue melatonin levels, exacerbates ROS-mediated tissue damage (19) and increases brain damage after focal brain stroke and excitotoxic seizures (31). The melatonin prevention of neurodegeneration linked to excitatory synaptic transmission is consistent with the last study. A decreased secretion of melatonin with aging has been documented (53), which may be exaggerated in populations with dementia (54).

The literature contains ample evidence to support a role for glial cells in antioxidant defense mechanisms (35–37). Astrocytes limited the excitotoxic neurodegeneration induced by Mg²⁺ withdrawal, and intracellular GSH-lowering agents reversed the glial protective effect. Astrocytes have a higher GSH content than neuronal cells (ref 34; M. Floreani, P. Giusti and S. D. Skaper, unpublished observations). GSH, which normally is present in high concentrations in the brain, functions as a major antioxidant in tissue defense against oxidative stress (55). Glial cells influence neuronal GSH levels in culture by effectively transporting cystine from the medium and converting it to cysteine (34). Cysteine is then released in the medium, taken up by the neurons, and used in the synthesis of GSH. A recent study suggests that the neuronal mitochondrial respiratory chain is damaged by sustained exposure to NO• and that GSH may be an important defense against such damage (56).

Melatonin can alter the activities of enzymes that improve the total antioxidative defense capacity of the organism (e.g., superoxide dismutase, glutathione peroxidase, glutathione reductase) (19). Pharmacological treatment with the pineal hormone may conceivably elicit an indirect neuroprotective action via the glial cell as well. Experiments are now planned to address this question.

In patients with epilepsy or in animal models of seizure activity, prolonged seizures (as in status epilepticus) may cause necrosis of neurons, including those in the hippocampus (4, 5). It might be predicted that drugs that control the release of glutamate or block its receptors during seizure activity would be beneficial and reduce the accompanying neuronal degeneration. The experiments reported here suggest several approaches to achieving these goals. One is the use of NMDA blockers (2). A second approach might be to use drugs that would reduce synaptic transmission (9). Finally, our results also propose the utility of examining the actions of antioxidants. Melatonin may be especially promising, as it readily enters the brain from the circulation and produces only minimal adverse effects in humans (19). The observation that melatonin deficiency can worsen seizure-induced neurodegeneration (31) strengthens this premise. FJ

This work was supported in part by 'Programma Biotecnologie: Azioni previste dalla Legge No. 95 del 29-3-1995' and 'Ricerca Sanitaria Finalizzata-Anno 1997, Regione Veneto'.

REFERENCES

- Olney, J. W. (1969) Brain lesions in an infant rhesus monkey treated with monosodium glutamate. *Science* **166**, 383-388
- Choi, D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**, 623-634
- Meldrum, B. S., and Garthwaite J. (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* **11**, 379-387
- Olney, J. W., Collins, R. C., and Sloviter, R. S. (1986) Excitotoxic mechanisms of epileptic brain damage. In *Advances in Neurology* (Delgado-Escueta, A. V., Ward, A. A., Jr., Woodbury, D. M., and Porter, R. J., eds) Vol. 44, pp. 857-877, Raven Press, New York
- Sloviter, R. S. (1987) Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science* **235**, 73-76
- Clifford, D. B., Zorumski, C. F., and Olney, J. W. (1989) Ketamine and MK-801 prevent degeneration of thalamic neurons induced by focal cortical seizures. *Exp. Neurol.* **105**, 272-279
- Croucher, M. J., Collins, J. F., and Meldrum, B. S. (1982) Anticonvulsant action of excitatory amino acid antagonists. *Science* **216**, 899-901
- Furshpan, E. J., and Potter, D. D. (1989) Seizure-like activity and cellular damage in rat hippocampal neurons in cell culture. *Neuron* **3**, 199-207
- Abele, A. E., Scholz, K. P., Scholz, W. K., and Miller, R. J. (1990) Excitotoxicity induced by enhanced excitatory neurotransmission in cultured hippocampal pyramidal neurons. *Neuron* **4**, 413-419
- Coyle, J. T., and Puttfarcken, P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262**, 689-695
- Choi, D. W. (1990) Methods for antagonizing glutamate neurotoxicity. *Cerebrovasc. Brain Metab. Rev.* **2**, 105-147
- Bredt, D. S., and Snyder S. H. (1992) Nitric oxide, a novel neuronal messenger. *Neuron* **8**, 3-11
- Chan, P. H. (1996) Role of oxidants in ischemic brain damage. *Stroke* **27**, 1124-1129
- Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87**, 1620-1624
- Lipton, S. A., Choi, Y.-B., Pan, Z.-H., Lei, S. Z., Chen, H.-S. V., Sucher, N. J., Loscalzo, J., Singel, D. J., and Stamler, J. S. (1993) A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature (London)* **364**, 626-632
- Skaper, S. D., Leon, A., and Facci, L. (1991) Ganglioside GM1 prevents death induced by excessive excitatory neurotransmission in cultured hippocampal pyramidal neurons. *Neurosci. Lett.* **126**, 98-101
- Giusti, P., Lipartiti, M., Franceschini, D., Schiavo, N., Floreani, M., and Manev, H. (1996) Neuroprotection by melatonin from kainate-induced excitotoxicity in rats. *FASEB J.* **10**, 891-896
- Floreani, M., Skaper, S. D., Facci, L., Lipartiti, M., and Giusti, P. (1997) Melatonin maintains glutathione homeostasis in kainic acid-exposed rat brain tissues. *FASEB J.* **11**, 1309-1315
- Reiter, R., Tang, L., Garcia, J. J., and Muñoz-Hoyos, A. (1997) Pharmacological actions of melatonin in oxygen radical pathophysiology. *Life Sci.* **60**, 2255-2271
- Skaper, S. D., Facci, L., Milani, D., Leon, A., and Toffano, G. (1990) Culture and use of primary and clonal neural cells. In *Methods in Neurosciences* (Conn, P. M., ed) Vol. 2, pp. 17-33, Academic Press, San Diego
- Bottenstein, J. E., Skaper, S. D., Varon, S. S., and Sato, G. H. (1980) Selective survival of neurons from chick embryo sensory ganglionic dissociates utilizing serum-free supplemented medium. *Exp. Cell Res.* **125**, 183-190
- Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. (1993) Neurodegeneration induced by β -amyloid peptides in vitro: the role of peptide assembly state. *J. Neurosci.* **13**, 1676-1687
- Banker, G. A., and Cowan, W. M. (1977) Rat hippocampal neurons in dispersed cell culture. *Brain Res.* **176**, 397-425
- Mody, I., Lambert, J. D. C., and Heinemann, U. (1987) Low extracellular magnesium induces epileptiform activity and spreading depression in rat hippocampal slices. *J. Neurophysiol.* **57**, 869-888
- Klatt, P., Schmidt, K., Brunner, F., and Mayer, B. (1994) Inhibitors of brain nitric oxide synthase. *J. Biol. Chem.* **269**, 1674-1680
- Gross, S. S., and Wolin, M. S. (1995) Nitric oxide: pathophysiological mechanisms. *Annu. Rev. Physiol.* **57**, 737-769
- Wolff, D. J., and Lubeskie, A. (1995) Aminoguanidine is an isoform-selective, mechanism-based inactivator of nitric oxide synthase. *Arch. Biochem. Biophys.* **316**, 290-301
- Tan, D. X., Chen, L. D., Poeggeler, B., Manchester, L. C., and Reiter, R. J. (1993) Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocr. J.* **1**, 57-62
- Matuszak, Z., Reszka, K., and Chignell, C. F. (1997) Reaction of melatonin and related indoles with hydroxyl radicals: EPR and spin trapping investigations. *Free Radical Biol. Med.* **23**, 367-372
- Skaper, S. D., Floreani, M., Negro, A., Facci, L., and Giusti, P. (1998) Neurotrophins rescue cerebellar granule neurons from oxidative stress-mediated apoptotic death: selective involvement of phosphatidylinositol 3-kinase and the mitogen-activated protein kinase pathway. *J. Neurochem.* In press
- Manev, H., Uz, T., Kharlamov, A., and Joo, J. Y. (1996) Increased brain damage after stroke or excitotoxic seizures in melatonin-deficient rats. *FASEB J.* **10**, 1546-1551
- Dubocovich, M. L. (1988) Luzindole (N-0774): a novel melatonin receptor antagonist. *J. Pharmacol. Exp. Ther.* **246**, 902-910
- Slivka, A., Mytilineou, C., and Cohen, G. (1987) Histochemical evaluation of glutathione in brain. *Brain Res.* **409**, 275-284
- Sagara, J., Maura, K., and Bannai, S. (1993) Maintenance of neuronal glutathione by glial cells. *J. Neurochem.* **61**, 1672-1676
- Vibulsreth, S., Hefli, F., Ginsberg, M. D., Dietrich, W. D., and Busto, R. (1987) Astrocytes protect cultured neurons from degeneration induced by anoxia. *Brain Res.* **422**, 303-311

36. Desagher, S., Glowinski, J., and Premont, J. (1996) Astrocytes protect neurons from hydrogen peroxide toxicity. *J. Neurosci.* **16**, 2553–2562
37. Hou, J.-G. G., Cohen, G., and Mytilineou, C. (1997) Basic fibroblast growth factor stimulation of glial cells protects dopamine neurons from 6-hydroxydopamine toxicity: involvement of the glutathione system. *J. Neurochem.* **69**, 76–83
38. Skaper, S. D., Facci, L., Romanello, S., and Leon, A. (1996) Mast cell activation causes delayed neurodegeneration in mixed hippocampal cultures via the nitric oxide pathway. *J. Neurochem.* **66**, 1157–1166
39. Ku, R. H., and Billings, R. E. (1986) The role of mitochondrial glutathione and cellular protein sulfhydryls in formaldehyde toxicity in glutathione-depleted rat hepatocytes. *Arch. Biochem. Biophys.* **247**, 183–189
40. Rosenberg, P. A., Amin, S., and Leitner, M. (1992) Glutamate uptake disguises neurotoxic potency of glutamate agonists in cerebral cortex in dissociated cell culture. *J. Neurosci.* **12**, 56–61
41. Murphy, S. N., and Miller, R. J. (1989) Two distinct quisqualate receptors regulate Ca^{2+} homeostasis in hippocampal neurons *in vitro*. *Mol. Pharmacol.* **35**, 671–680
42. Iadecola, C. (1997) Bright and dark sides of nitric oxide in ischemic brain injury. *Trends Neurosci.* **20**, 132–139
43. Zhang, J., and Snyder, S. H. (1995) Nitric oxide in the nervous system. *Annu. Rev. Pharmacol. Toxicol.* **35**, 213–233
44. Dawson, V. L., Dawson, T. M., Uhl, G. R., and Snyder, S. H. (1993) Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. *J. Neurosci.* **13**, 2651–2661
45. Strijbos, P. J. M., Leach, M. J., and Garthwaite, J. (1996) Vicious cycle involving Na^+ channels, glutamate release, and NMDA receptors mediates delayed neurodegeneration through nitric oxide formation. *J. Neurosci.* **16**, 5004–5013
46. Leist, M., Fava, E., Montecucco, C., and Nicotera, P. (1997) Peroxynitrite and nitric oxide donors induce neuronal apoptosis by eliciting autocrine excitotoxicity. *Eur. J. Neurosci.* **9**, 1488–1498
47. Gilad, E., Cuzzocrea, S., Zingarelli, B., Salzman, A. L., and Szabó, C. (1997) Melatonin is a scavenger of peroxynitrite. *Life Sci.* **60**, PL169–PL174
48. Cuzzocrea, S., Zingarelli, B., Gilad, E., Hake, P., Salzman, A. L., and Szabo, C. (1997) Protective effect of melatonin in carrageenan-induced models of local inflammation: relationship to its inhibitory effect on nitric oxide production and its peroxy-nitrite scavenging activity. *J. Pineal Res.* **23**, 106–116
49. Escames, G., Guerrero, J. M., Reiter, R. J., Garcia, J. J., Munoz-Hoyos, A., Ortiz, G. G., and Oh, C. S. (1997) Melatonin and vitamin E limit nitric oxide-induced lipid peroxidation in rat brain homogenates. *Neurosci. Lett.* **230**, 147–150
50. Pozo, D., Reiter, R. J., Calvo, J. R., and Guerrero, J. M. (1997) Inhibition of cerebellar nitric oxide synthase and cyclic GMP production by melatonin via complex formation with calmodulin. *J. Cell. Biochem.* **65**, 430–442
51. Menendez-Pelaez, A., Poeggeler, B., Reiter, R. J., Barlow-Walden, L. R., Pablos, M. I., and Tan, D. X. (1993) Nuclear localization of melatonin in different mammalian tissues: immunocytochemical and radioimmunoassay evidence. *J. Cell. Biochem.* **53**, 373–382
52. Pappolla, M. A., Sos, M., Omar, R. A., Bick, R. J., Hickson-Bick, D. L. M., Reiter, R. J., Efthimiopoulos, S., and Robakis, N. K. (1997) Melatonin prevents death of neuroblastoma cells exposed to the Alzheimer amyloid peptide. *J. Neurosci.* **17**, 1683–1690
53. Iguchi, H., Kato, K., and Igayashi, H. (1982) Age-dependent reduction in melatonin concentrations in healthy subjects. *J. Clin. Endocrinol. Metab.* **55**, 27–29
54. Souetre, E., Salvati, E., Krebs, B., Belugou, J. L., and Darcourt, G. (1989) Abnormal melatonin response to 5-methoxy-psoralen in dementia. *Am. J. Psychiatry* **146**, 1037–1040
55. Meister, A. (1995) Strategies for increasing cellular glutathione. In *Biothiols in Health and Disease* (Packer, L., and Cardenas, E., eds) pp. 165–188, Dekker, New York
56. Bolaños, J. P., Heales, S. J. R., Peuchen, S., Barker, J. E., Land, J. M., and Clark, J. B. (1996) Nitric oxide-mediated mitochondrial damage: a potential neuroprotective role for glutathione. *Free Radical Biol. Med.* **21**, 995–1001

*Received for publication January 9, 1998.
Revised for publication February 23, 1998.*