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The Neurotoxicity of 1-Methyl-4-Phenylpyridinium in Cultured Cerebellar Granule Cells

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Cerebellar granule cells in enriched primary culture are susceptible to the neurotoxic effects of 1-methyl-4-phenylpyridinium (MPP^+). Relatively high MPP^+ concentrations are required to elicit neurotoxic effects at early culture times, but lower concentrations of MPP^+ produce comparable neurotoxic effects at later culture times. Under identical culture conditions 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is not neurotoxic. Preincubation with the glutamate uptake blockers, DL-threo-3-hydroxyaspartic acid or dihydrokainate, or the dopaminergic uptake blocker mazindol, protects the granule cells from the cytotoxic effects of MPP^+.

Although MPTP is not neurotoxic in an enriched granule cell culture, in coculture with cerebellar astrocytes MPTP is toxic to granule cells, presumably because it is converted in astrocytes to MPP^+. Cerebellar astrocytes remain confluent and viable. The addition of pargyline to the coculture abolishes the neurotoxicity consistent with a role of MAO B in bioactivation of MPTP. The concentration of MPP^+ in the coculture medium (13 μM) was less than that required for the toxic effect in enriched neuronal cultures at earlier culture times, suggesting that an astroglial–neuronal interaction, perhaps by proximity, enhances the neurotoxicity of MPP^+. These results might explain reported effects of MPTP on some cerebellar cells in mice.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a potent neurotoxin that selectively destroys the nigrostriatal dopaminergic neurons in humans, subhuman primates, and lower animals (Burns et al., 1983; Hallman et al., 1984; Heikkila et al., 1984a; Langston et al., 1984a). Several basic features that have emerged from *in vivo* studies are (1) conversion of MPTP to MPP^+ is required for toxicity; concentrations of MPP^+ in monkey striatum reach about 50 μM (Yang et al., 1988); (2) MPTP is not neurotoxic when its conversion to MPP^+ is blocked by monoamine oxidase type B (MAO B) inhibition (Chiba et al., 1984; Cohen et al., 1984; Heikkila et al., 1984b; Langston et al., 1984b; Markey et al., 1984); (3) astrocytes or serotonergic neurons that contain MAO B (Levitt et al., 1982) are presumably the sites of the conversion process; and (4) a dopamine-uptake blocker such as mazindol protects against the neurotoxicity (Javitch et al., 1985; Ricaurte et al., 1985).

These features are the hallmarks of MPTP neurotoxicity *in vivo* and therefore serve as criteria for an *in vitro* culture system model. Although the most relevant population in which to examine this neurotoxic effect is obviously the nigrostriatal neurons, the dopamine-containing neurons in embryonic mesencephalic cultures constitute only about 1% of the total number of cells (Sanchez-Ramos et al., 1988). Therefore, we sought a more suitable culture system to study the cellular mechanisms responsible for the neurotoxic effects of MPP^+.

Although the cerebellum has not been recognized as a prominent target for MPTP neurotoxicity, several observations suggest its possible involvement: (1) Purkinje cell loss has been reported in the cerebellum of MPTP-treated mice (Takada et al., 1988), and it has been suggested that granule cells are also damaged (T. Hattori, personal communication); (2) ^{3}H-MPTP binding sites in cerebellum are equal to or greater than those found in the striatum (Bocchetta et al., 1985); (3) in monkeys given ^{14}C-MPTP, levels of ^{14}C-MPP^+ in the cerebellum were about half those in striatum and equal to those in the substantia nigra (Yang et al., 1988); (4) metabolic studies using 2-deoxyglucose demonstrated that the inferior olive was hypermetabolic, as seen in the substantia nigra; the remainder of the brain is hypometabolic (Palombo et al., 1988). Since the climbing fibers originate in the inferior olive (Palay and Chan-Palay, 1974), olivary hypermetabolism might be related to cerebellar dysfunction; (5) the tremor that develops in monkeys resembles a cerebellar, rather than a parkinsonian, tremor (C. David Marsden, personal communication).

These findings suggest that the cerebellum may in fact be another site of MPP^+ neurotoxicity. Cultures enriched in cerebellar granule cells are relatively homogeneous and have been extensively characterized. These cells take up and release D-aspartic acid, which uses the glutamate carrier, and are considered to use glutamate as their neurotransmitter (Gallo et al., 1987). Although cerebellar granule cells do not contain dopamine, preliminary studies using a coculture of granule cells and astrocytes showed that MPTP was indeed toxic to the granule cells, whereas astrocytes remained unaffected by MPTP or the MPP^+ formed in these cocultures. The characteristics of toxicity and mechanism of action of MPTP, MPP^+, and related compounds were examined in enriched cerebellar granule cell cultures or in cocultures containing granule cells and astrocytes. Cultures of enriched cerebellar granule cells, although not vulnerable to MPTP toxicity, were found to be sensitive to the neurotoxic effects of MPP^+. Furthermore, lower concentrations of MPP^+ became toxic at later times in culture.

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Materials and Methods

Culture preparation. Cocultures of cerebellar granule cells and astrocytes were prepared from 8-d-old rat pups (15–19 gm) as described previously (Novelli et al., 1988) with the exception that the suspension was diluted to a final density of 2.0×10^6 cells/ml.

Cultures enriched in granule cells were prepared in an identical fashion and were established by adding 10 μM cytosine arabinoside (Ara-C) to the neuronal culture medium after 24 hr. D-glucose (100 μl of a 100 mM solution prepared in sterile water) was added to cultures on day 7 and every fourth day thereafter. Evaporative losses from either the neuronal or the coculture medium were replaced by appropriate volumes of sterile water to maintain a 2 ml volume at all times.

Astrocytes in the neuronal culture and coculture were identified by immunoreactivity (IR) for glial fibrillary acidic protein (GFAP) (Lipsky and Silverman, 1987). In enriched neuronal cultures astrocytes represented less than 5% of the total cell population.

Primary cerebellar astrocytes were prepared as described above except that the final pellet was resuspended in basal Eagle medium supplemented with 2 mM glutamine, 10% fetal calf serum, and 100 μg/ml gentamycin. The cells were plated on uncoated Nunc plastic dishes without Ara-C. Astrocytes were >95% pure as determined by GFAP immunoreactivity.

Neuron–astrocyte cocultures and MPTP. MPTP and/or pargyline (100 μM) was added to the coculture on the second day *in vitro* (DIV 2) and medium was removed 7 d later for MPP^+ determination. MPP^+ was assayed using HPLC and IR detection according to the method of C. J. Markey (personal communication). One milliliter of media was mixed with 100 μl 50% NaOH and washed with 1 ml hexane. A 100 μl aliquot of the aqueous phase was added to 100 μl of 0.1 M $HClO_4$ containing 300 ng BPP^+ (1-butyl-4-phenylpyridinium) as internal standard. Half of the mixture (100 μl) was injected on a 0.45 cm × 15 cm, 5 μm octylsilane reverse-phase column (Ultrasphere-Octyl, Altex) using 50% sodium acetate with 0.1% triethylamine (pH 5.6) as solvent. Acetonitrile : water (35:65) containing 0.1% triethylamine was used as the eluant. MPP^+ was eluted at 3.7 min, BPP^+ at 8.9 min, and both were detected by IR absorption at 290 nm.

Determinations of MAO A and B. Activities of MAO A and B were assayed as described previously (Garrick and Murphy, 1982) and expressed as nmole substrate oxidized/min/mg protein. Determinations of MAO in either primary cerebellar astrocytes or enriched cerebellar granule cells were performed as follows: twelve 35 mm culture dishes were scraped and combined in 500 μl of a cold (4°C) buffer containing 2 mM HEPES, 1 mM EDTA, 0.2 mM DTT, and 0.001 mM PMSF, pH 8.0. Samples were stored at −70°C until assayed for MAO A or B activity and protein content (Lowry et al., 1951).

Cell fixation. Cells from the neuronal culture and coculture were fixed as follows: the medium was removed and 2 ml 95% ethanol/5% glacial acetic acid, prechilled to −20°C, was added to the culture dishes. The dishes were kept at −20°C for 30 min, the ethanol/acetic acid removed, and the cell culture dishes washed twice with PBS at room temperature. One drop of 100% glycerol was placed on each dish with a coverslip overlaid on the glycerol.

Fluorescein staining. Fluorescein staining was performed as described previously (Novelli et al., 1988). After incubation, buffer containing the fluorescein was removed, 1 ml fresh incubation buffer was added to the culture dish, and the cells were photographed under UV light microscopy.

Drug treatments. All drugs were added to the culture dishes on the days *in vitro* as indicated.

Materials. MPTP-HCl, 1-methyl-4-(4′-amino)phenyl-1,2,3,6-tetrahydropyridine hydrochloride ($p$$NH_2$MPTP-HCl), 1-methyl-4-(4′-amino)phenylpyridinium dihydrochloride ($p$$NH_2$$MPP^+$2HCl), 1-methyl-4-(3′-amino)-phenylpyridinium hydrochloride ($m$$NH_2$$MPP^+$HCl), and 1-methyl-4-phenylpyridinium iodide (MPP^+I) were kind gifts from Dr. Sanford P. Markey. 1-Methyl-4-tertiarybutyl-pyridinium iodide and mazindol were generously supplied by Dr. Richard Heikkila. DL-2-amino-5-phosphonovaleric acid (PVA), DL-threo-3-hydroxyaspartic acid (3-HA), glutamic acid, dihydrokainic acid (DHK), fluorescein diacetate (FDA), phenylmethylsulfonylfluoride (PMSF), dithiothreitol (DTT), 5-hydroxytryptamine creatinine sulfate, cytosine-1-β-D-arabinofuranoside HCl, L-glutamic acid, trypsin, soybean trypsin inhibitor, and deoxyribonuclease I were purchased from Sigma Chemicals. (+)MK-801 was purchased from Research Biochemicals. Eagle's basal medium and fetal calf serum were obtained from Grand Island Biological Company. Phenylethylamine hydrochloride, β-[ethyl-1-^{14}C] (50 mCi/mmol) was purchased from New England Nuclear Research Products. 5-Hydroxy(side chain-2-^{14}C)tryptamine creatinine sulfate (57 mCi/mmol) was purchased from Amersham International. All other chemicals were of reagent grade. Eight-day-old Sprague-Dawley rat pups (15–19 gm) were obtained from Taconic Farms.

Results

Figure 1*A* illustrates the typical appearance of the granule cell–astrocyte coculture. Numerous neurons and extensive neurite networking are prominent. Incubation of the coculture with 100 μM MPTP results in a marked decrease in the number of granule cells and a dramatic loss of neurite networking (Fig. 2*B*). In contrast, astrocytes do not exhibit any evidence of disintegration when cultured in 100 μM MPTP (Fig. 1*C*). These astrocytes will proliferate to confluence and retain their normal morphology. As shown in Figure 1*D*, addition of equimolar pargyline to MPTP-treated cocultures prevents the effects of MPTP on neurons. The MPP^+ concentration in MPTP-treated cocultures was found to be 13 μM, whereas in the cocultures treated with pargyline, the MPP^+ concentration was reduced to 5 μM.

Very different results are obtained in the enriched granule cell cultures. Figure 2*A* illustrates the typical appearance of untreated cerebellar granule cells using the vital stain fluorescein diacetate. The number of viable granule cells and the density of neurite networking in the MPTP-treated cultures (100 μM) are comparable to those of untreated neuronal cultures (Fig. 2*B*). However, incubation of the granule cells with 100 μM MPP^+ for 48 hr results in virtually total destruction of the granule cells and their neurites (Fig. 2*C*). In contrast, the brilliant fluorescence of the few cerebellar astrocytes in this culture indicates that MPP^+ has no apparent effect on these cells.

The concentration of MPP^+ required for neurotoxicity in the enriched granule cell culture is dependent on the time the neurons have been in culture (Table 1). For cells in culture for 5 d (DIV 5), 50 μM MPP^+ is required for disruption of neurite networking, while 100 μM is necessary to produce cell death within 3 d. In contrast, the neurotoxic doses of MPP^+ required for granule cells which have been in culture for 12 d (DIV 12) are 10 μM for disruption of neurites within 3 d and partial (30%) cell death after 12 d; 30 μM produces total cell death within 7 d. This enhanced sensitivity of older cultures is illustrated graphically in Figure 3: at DIV 5, 100 μM MPP^+ kills neurons in 3 d, whereas by DIV 8, the same dose of MPP^+ is neurotoxic to granule cell neurons within 24 hr.

Since MAO B is thought to be responsible for the conversion of MPTP to MPP^+, MAO activity was determined in the enriched granule cells and the primary astrocyte cultures. Cerebellar astrocyte cultures have approximately 30 times more MAO B than do enriched granule cells, whereas MAO A activity in astrocytes is approximately double that of granule cells (Table 2).

The neurotoxic specificity of the 4-phenylpyridinium species and its tetrahydropyridine derivative was determined by testing several related compounds on enriched neuronal cultures. As illustrated in Table 3, only MPP^+ and its congener, $p$$NH_2$$MPP^+$, caused neuronal death in enriched granule cell cultures. Like MPTP, $p$$NH_2$MPTP is not toxic to the enriched granule cells in culture. The pyridinium derivatives 1-methyl-4-tertiarybutylpyridinium and $m$$NH_2$$MPP^+$ are also nontoxic. Furthermore, 1-methyl-4-tertiarybutylpyridinium confers no protection against MPP^+ toxicity. Since cerebellar granule cells express the *N*-methyl-D-aspartate (NMDA) receptor (Gallo et al., 1987), 2

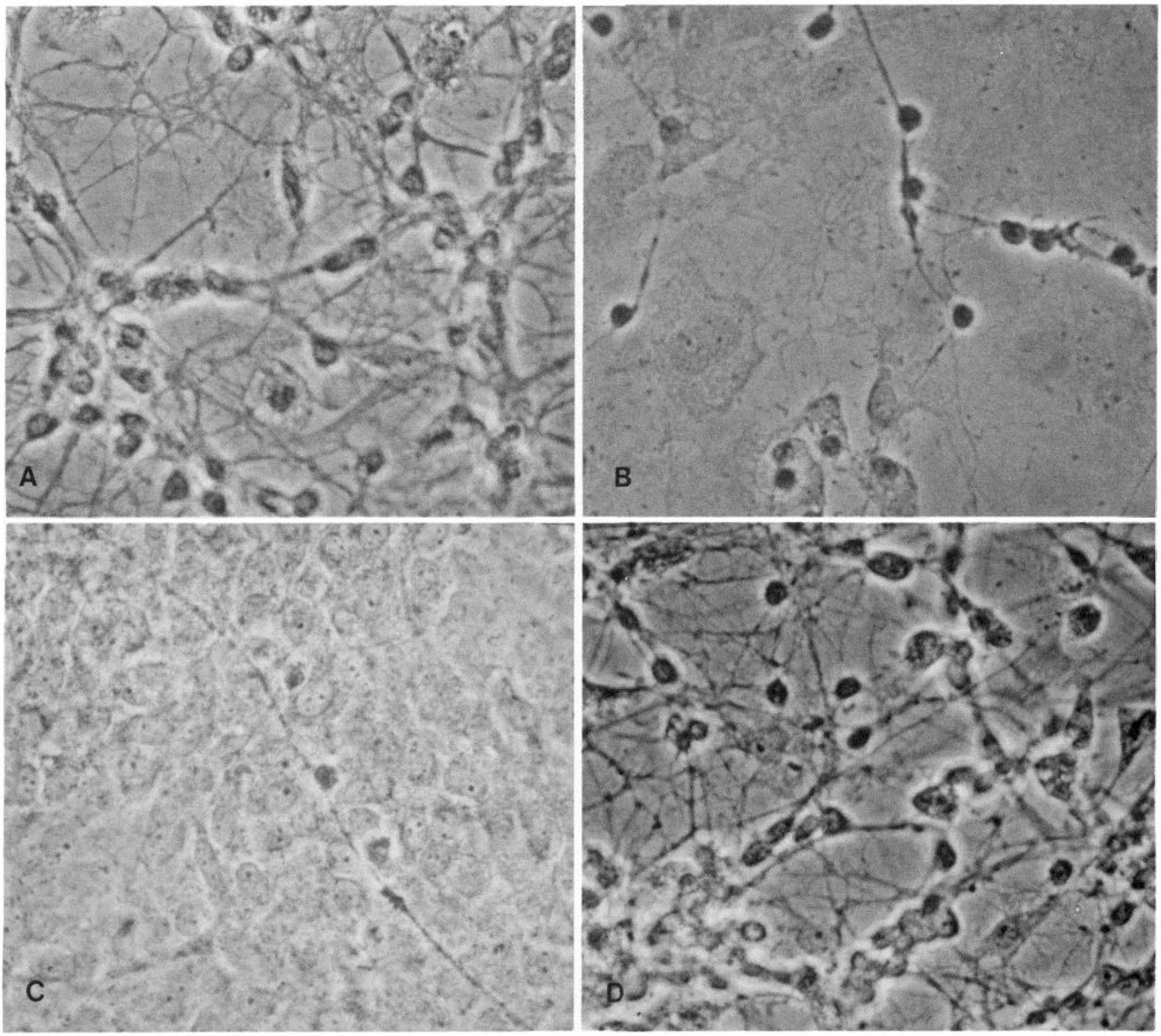

Figure 1. Treatment of the coculture containing cerebellar granule cells and astrocytes with MPTP in the presence and absence of pargyline: *A*, untreated coculture (DIV 9); *B* and *C*, coculture on DIV 9 after treatment with 100 μM MPTP for 7 d (*B*) and 9 d (*C*). *D*, Coculture on DIV 9 after treatment with 100 μM MPTP and 100 μM pargyline for 7 d (×400).

NMDA receptor antagonists, PVA and MK 801, were tested for their ability to protect against MPP^+. Neither PVA or MK-801 conferred protection.

Glutamate is considered to be the putative neurotransmitter of granule cells. The possibility that MPP^+ might be entering the cells via the glutamate carrier was tested by pretreating the enriched granule cell cultures with 3-HA, a competitive inhibitor of the glutamate uptake system (Balcar and Johnston, 1972; Balcar et al., 1977; Young et al., 1988). As shown in Figure 4, whereas MPP^+ alone results in neuronal and neurite disintegration (Fig. 4*B*), enriched granule cell cultures with 3-HA are protected against MPP^+ (Fig. 4*C*) and resemble untreated cells (Fig. 4*A*). The ability of 3 uptake antagonists to protect the enriched neuronal culture against MPP^+ (70 μM) toxicity has been quantified (Fig. 5). 3-HA (3 μM) and mazindol (3 μM) conferred complete protection and dihydrokainate (3 μM), another competitive glutamate uptake blocker, was partially effective, allowing 80% survival compared with only 20% survival with MPP^+ alone. Glutamate (100 μM), in the presence of MK-801, was less potent in protecting the cultured neurons against the neurotoxicity of MPP^+ with 60% of the neurons surviving.

Discussion

MPTP selectively destroys the nigrostriatal neurons in man, subhuman primates, and lower animals. Although the exact reasons for the selectivity are unknown, it has been proposed that the high-affinity uptake system characteristic of the dopamine neurons *in vivo* is responsible at least in part for the neurotoxicity of MPP^+ (Javitch and Snyder, 1984; Javitch et al., 1985). The clinical syndrome produced is remarkably similar to idiopathic Parkinson's disease; the selective nature of MPTP

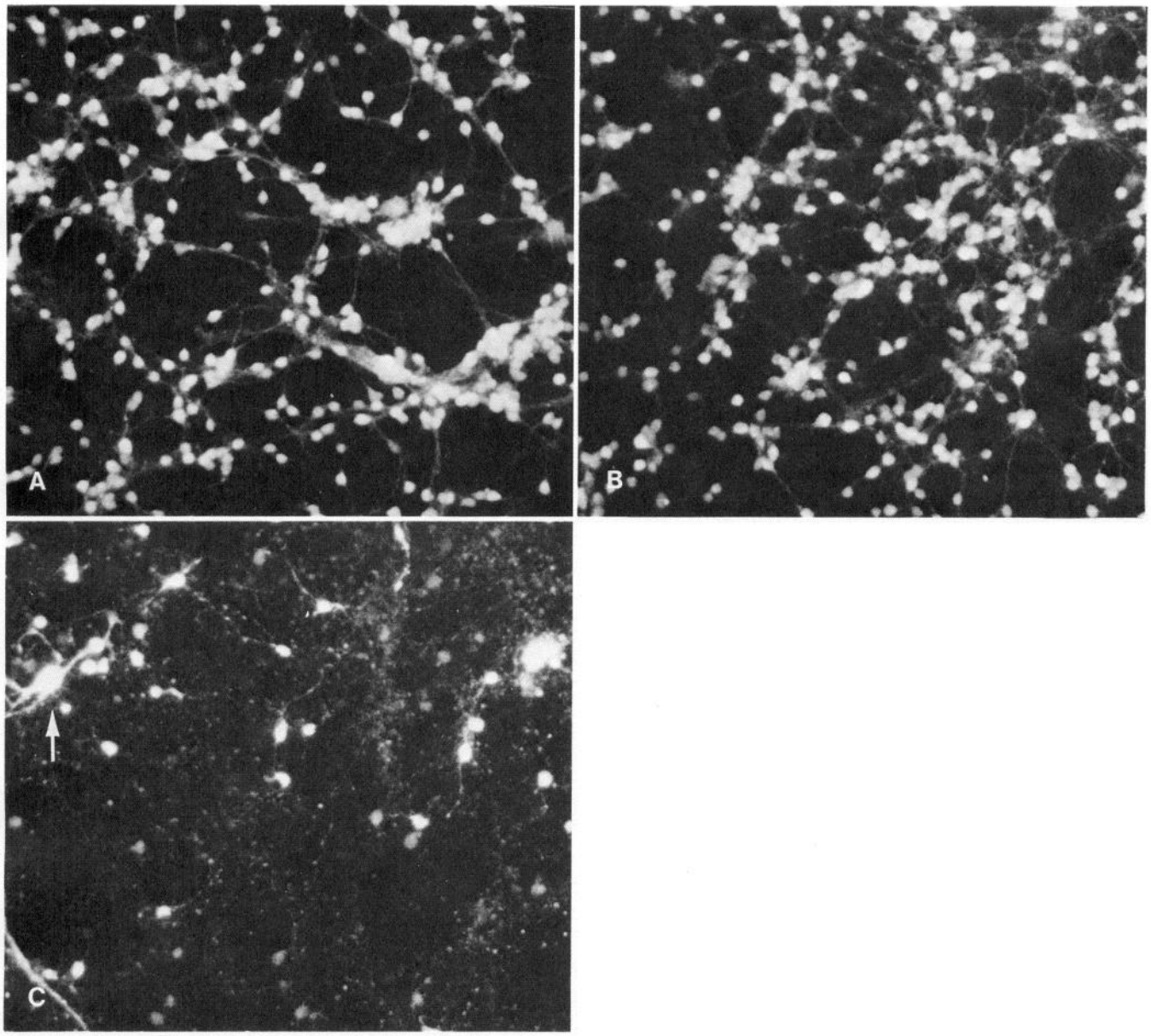

Figure 2. Fluorescein diacetate staining of enriched cerebellar granule cells in the presence of MPTP or MPP^+. *A,* Untreated cerebellar granule cells on DIV 8; *B,* cerebellar granule cells on DIV 8 after treatment with 100 μM MPTP for 48 hr; *C,* cerebellar granule cells on DIV 8 after treatment with 100 μM MPP^+ for 48 hr. *Arrow* indicates astrocyte. ×140.

has rekindled interest in the possibility that an environmental neurotoxin may be responsible for the idiopathic form of the disease. However, there is another aspect of MPTP toxicity that has not been extensively characterized: in primates an action tremor develops which is a manifestation of cerebellar dysfunction rather than the classical resting tremor characteristic of Parkinson's disease. The results obtained in the present study demonstrate that MPTP is toxic to cerebellar granule cell neurons in cocultures containing astrocytes. Furthermore, recent evidence shows loss of Purkinje cells and some granule cells in mice treated with MPTP (Takada et al., 1988; T. Hattori, personal communication). Taken together with the reported MPP^+ concentration of up to 50 μM found in cerebellum of animals treated with MPTP (Yang et al., 1988), these results suggest that loss of granule cells and their synaptic connections on Purkinje and/or Golgi cells in combination with Purkinje cell loss may contribute to the development of the tremor.

The structural specificity of MPP^+ neurotoxicity on the enriched granule cell cultures is outlined in Table 3. These results suggest that cultured granule cells can distinguish subtle structural changes in the 4-phenylpyridinium molecule. The presence of a pyridinium ring is not sufficient to produce cytotoxicity.

Our results also suggest that astroglial–neuron interactions enhance MPP^+ toxicity. The concentration of MPP^+ achieved in the cocultures is not toxic to purified granule cells at identical culture times. The MPP^+ concentration at the astrocyte–granule cell interface may be much higher than in the medium or there may be other astrocyte–neuron interactions favoring MPP^+ accumulation. The small amount of MPP^+ found in the coculture medium in the presence of pargyline is either due to incomplete

Table 1. Differential sensitivity to MPP+ of cerebellar granule cells

Conc. (μM)	Day *in vitro* MPP+ added	Day *in vitro* cells examined	Response to MPP+: Neurite disruption	Response to MPP+: Cell death (%)
10	5	8	−	−
	12	19	+	−
	12	24	+	28
30	5	8	−	−
	12	15	+	47
	12	19	+	86
50	5	8	+	−
	12	15	+	60
	12	19	+	>95
100	5	8	+	>95
	12	15	+	>95
	12	19	+	>95

Enriched cerebellar granule cell cultures were prepared and maintained as outlined in Materials and Methods. MPP+ (10–100 μM) was added on DIV 5 or 12 and the cells examined for neurotoxic effects on DIV 8, 15, 19, and 24. Neurotoxic effects ranged from disruption of neurite arborization to cell death. Percent cell death is defined according to the following formula:

$$\% \text{ cell death} = 1 - \left(\frac{[\text{surviving cells (drug-treated)}]}{[\text{surviving cells (untreated)}]} \right) \times 100.$$

Values represent the mean, in percent, of 3 comparable fields of drug-treated and untreated control cultures. In untreated control cultures, 200–300 cells were counted. Experiments were performed in triplicate using 2 different batches of granule cell neurons.

MAO inhibition or oxidation of MPTP by a pargyline-insensitive enzyme. The latter possibility is compatible with *in vivo* results: small amounts of MPP+ were found in pargyline-treated animals, but no effect was found on the nigrostriatal neurons (Markey et al., 1984). However, MAO B is the major enzyme responsible for conversion of MPTP to MPP+, and the lack of this enzyme in the enriched granule cells is presumably responsible for the absence of MPTP toxicity.

Studies performed on *in vitro* mesencephalic cultures have demonstrated that concentrations less than 10 μM are required to destroy the dopaminergic neurons, whereas higher concentrations destroy all neuronal cells (Sanchez-Ramos et al., 1988). The cultured cerebellar granule cells exhibit a differential sensitivity that is dependent on time in culture, with relatively high MPP+ concentrations required early in culture (DIV 5) and lower concentrations required when added later. The differential sensitivity of the cultured granule cell neurons to MPP+ may be related to alterations of the glutamate transporter occurring during culture which lead to enhanced uptake: it has been shown that more glutamate is taken up at later culture times compared to earlier times (Levi et al., 1984). This may be due to direct

Table 2. MAO content of cerebellar granule cells and astrocytes

Cell type	MAO A (nmol/min/mg protein)	MAO B (nmol/min/mg protein)
Granule cells	49.7	1.6
Astrocytes	116.0	50.0

MAO determinations were performed in 8-d-old enriched granule cells and in 3-week-old primary astrocytes, as described in Materials and Methods.

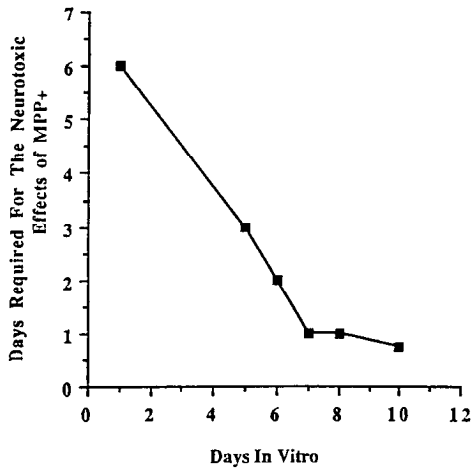

Figure 3. The time requirement for the neurotoxic effect of MPP+ on enriched cerebellar granule cells. MPP+, 100 μM, was added to the granule cells on the days *in vitro* indicated. Neurotoxicity is expressed as the length of time (d) required to kill >95% of the granule cell neurons.

activation or reflect neurite extension. The most sensitive indicator of MPP+ toxicity is neurite disruption: furthermore, MPP+ is not toxic to cultured granule cells when added after 1 d in culture (DIV 1), at which time there is very limited neurite outgrowth.

Since the neurotoxicity of MPP+ is thought to occur as a result of the uptake of MPP+ into the dopaminergic neurons, a major determinant for toxicity is the efficiency of the uptake system. The dopamine transporter takes up MPP+ very efficiently, with rapid accumulation within cultured dopaminergic neurons (Schinelli et al., 1988). Although the uptake of MPP+ into granule cells requires higher concentrations and proceeds at a slower rate compared with the dopaminergic neurons, the calculated intracellular concentration of MPP+ achieved in the granule cells (1.8 mM) following exposure to 70 μM MPP+ for 30 min (unpublished observations) is essentially identical to the calculated intracellular concentration in the dopaminergic neurons (2.5

Table 3. Neurotoxicity of various drugs on enriched cerebellar granule cells

Drug	Survival (%)	Drug	Survival (%)
MPTP	>95	MPP+	<5
pNH_2MPTP	>95	*p*NH_2MPP+	<5
1-Methyl-4-tertiary-butylpyridinium	>95	1-Methyl-4-tertiary-butylpyridinium + MPP+	<5
mNH_2MPP+	>95		
(+)MK-801	>95	(+)MK-801 + MPP+	<5
PVA	>95	PVA + MPP+	<5

Enriched cerebellar granule cells were prepared as outlined in Materials and Methods. On the fifth day of culture, drugs were added to the medium at a final concentration of 100 μM except for PVA, which was added at a final concentration of 1 mM. Percent survival was determined by counting the surviving neurons on the eighth day and comparing them to the number of neurons surviving in untreated cultures. Results are expressed as percent survival:

$$\frac{\text{number of neurons/high-power field (HPF) drug-treated}}{\text{number of neurons/HPF untreated or putative antagonist}} \times 100.$$

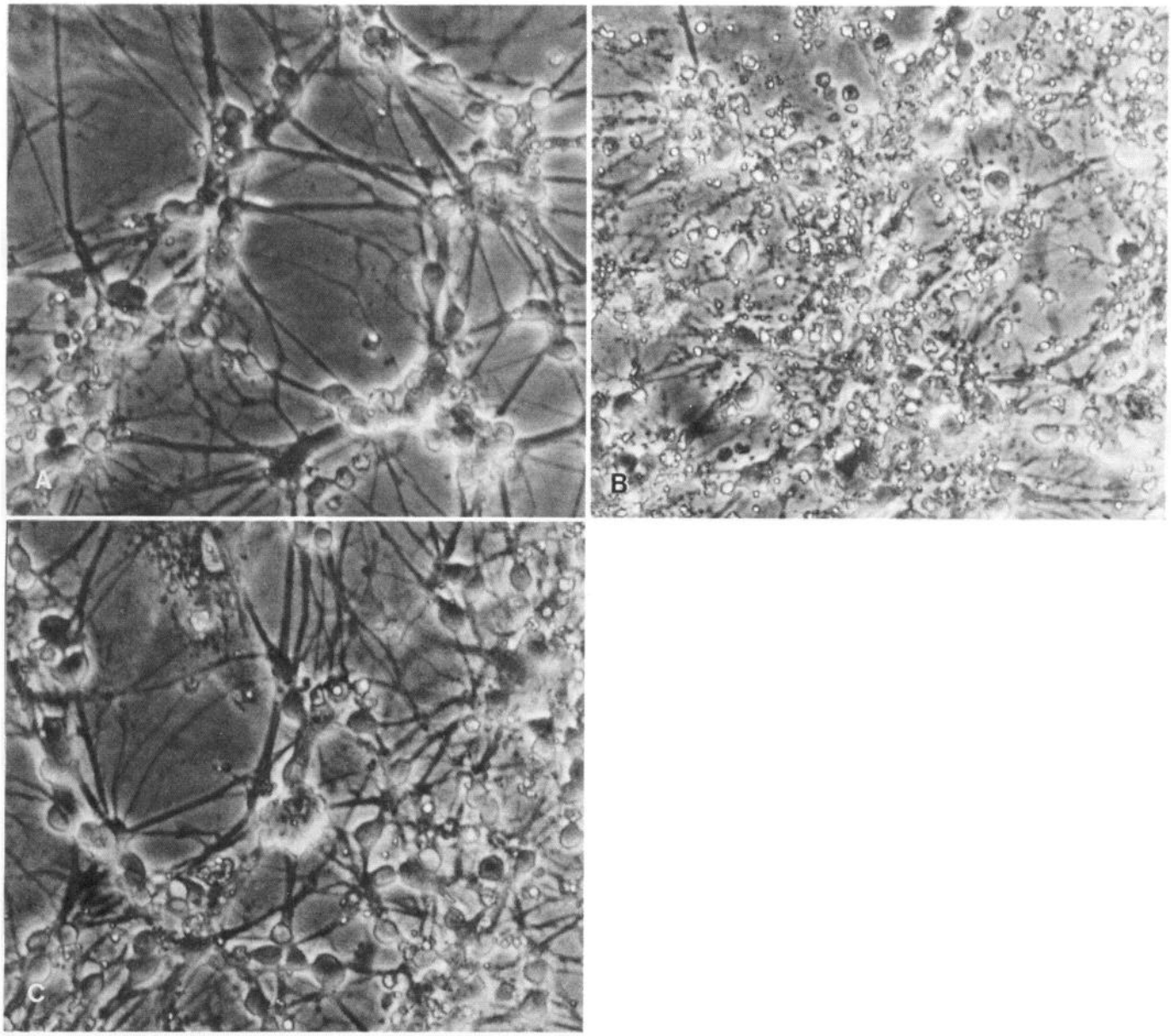

Figure 4. Protection of MPP^+-treated enriched granule cells by 3-HA. *A,* Phase-contrast micrograph of untreated cerebellar granule cells on DIV 9; *B,* cerebellar granule cells on DIV 9 after adding 70 μM MPP^+ on DIV 5; *C,* cerebellar granule cells on DIV 9 after 3-HA (3 μM) was added DIV 3 and MPP^+ (70 μM) was added DIV 5. ×250.

mM) following exposure to 50 nM for 30 min (Schinelli et al., 1988). These results suggest that efficiency of transport will determine the rate of uptake and potency of the toxin.

Our results suggest that MPP^+ enters granule cells via the glutamate transporter. Competitive glutamate uptake antagonists such as 3-HA and dihydrokainate effectively block the neurotoxic effects of MPP^+; glutamate itself was active, though less potent. The rank order of potency of the uptake antagonists to protect the neurons against MPP^+ toxicity is identical to that for inhibition of glutamate uptake in rat brain (Johnston et al., 1979). Mazindol, an established dopaminergic uptake blocker (Heikkila, 1981), is equipotent to 3-HA in protecting the cultured neurons against MPP^+ neurotoxicity. Although there is no evidence for dopamine uptake sites on granule cells, it is possible that mazindol has broader specificity in blocking transport.

A characteristic property of the granule cells is their expression of the NMDA receptor. Under certain conditions, glutamate, acting through the NMDA receptor, can be neurotoxic to cerebellar granule cells (Novelli et al., 1988). Thus, the possibility that MPP^+ causes release of glutamate from the granule cells, which then acts on the NMDA receptor to cause cell death, had to be considered. However, an NMDA receptor antagonist, PVA, and an NMDA receptor-associated channel blocker, MK-801, were ineffective in blocking the toxicity of MPP^+. These results suggest that the NMDA receptor is not involved in the neurotoxicity of MPP^+.

Several investigators have suggested that MPP^+ may be toxic

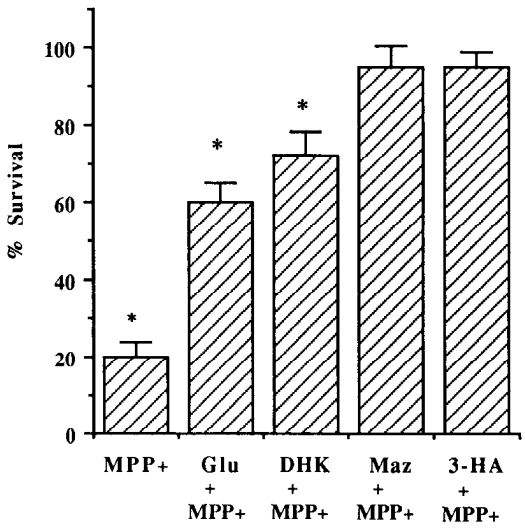

Figure 5. The ability of uptake antagonists to protect against the neurotoxicity of MPP^+. The uptake antagonist 3-HA (3 μM), dihydrokainate (3 μM), or mazindol (3 μM) was added to the culture dishes on DIV 2. On DIV 5, MPP^+ was added at a final concentration of 70 μM and the cells examined for percent survival on DIV 10. The control for each of the uptake blocker experiments consisted of the drug alone added on DIV 2. For 100% survival, approximately 300 cells were counted. The experiments designed to determine the ability of glutamate to protect the enriched neuronal culture were carried out slightly differently. Since glutamate (100 μM) added on DIV 7 or thereafter is neurotoxic to cultured granule cells via the NMDA receptor (A. Novelli, personal communication), the NMDA antagonist MK-801 was also added to the culture at a final concentration of 1 μM, a concentration which protects completely against the neurotoxic effects of glutamate. MK-801 alone afforded no protection against the neurotoxic effects of MPP^+ (see text). Therefore, glutamate (100 μM), MK-801 (1 μM), and MPP^+ (70 μM) were added to enriched neuronal culture dishes (6 dishes and 2 different neuronal batches) on DIV 7 and exposed for 72 hr (DIV 10). The controls for this experiment were culture dishes treated with glutamate (100 μM) and MK-801 (1 μM). Percent survival was determined as outlined in Table 3 except that the denominator consisted of neurons treated with the respective antagonists alone. The percent survival values are from triplicate experiments and are expressed as means ± SEM. $*p < 0.001$ compared with control using the student's 2-tailed t test.

because of its ability to interfere with mitochondrial function. MPP^+ has been shown to inhibit NADH dehydrogenase (Nicklas et al., 1985; Vyas et al., 1986; Kindt et al., 1987) and to be actively taken up (Ramsay and Singer, 1986) and concentrated in mitochondria, where it blocks NADH dehydrogenase near the Q-binding site (Ramsay et al., 1987). Heikkila et al. (1985) have suggested that mitochondrial effects of MPP^+ lead to ATP depletion, which results in destruction of the nigrostriatal neurons. Placenti et al. (1987) showed that an MPP^+ analog, 1-methyl-4-tertiarybutylpyridinium, inhibits MPP^+ uptake into mitochondria. 1-Methyl-4-tertiarybutylpyridinium was tested for its ability to protect the granule cells from a cytotoxic concentration of MPP^+: it was neither neurotoxic alone nor did it confer protection to the granule cells from the cytotoxicity of equimolar concentrations of MPP^+. If this compound does not block uptake of MPP^+ but does interfere with mitochondrial uptake of MPP^+, then the persistence of MPP^+ toxicity in the presence of 1-methyl-4-tertiarybutylpyridinium suggests that MPP^+ may not be killing the neurons solely by interfering with the generation of ATP.

In conclusion, our results suggest that MPP^+ is toxic to granule cells and that astrocytes convert MPTP to MPP^+; proximity of astrocytes to neural membranes may result in a high concentration of MPP^+ and promote neuronal damage and death. The relevance of these observations to effects of MPTP *in vivo* and to the mechanism of toxicity of MPP^+ remains to be elucidated.

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