and B. J. Huntley, *Agroanimalia* **3**, 23 (1971)]; 7. *Aepyceros melampus* (2, 2) [J. D. Skinner, *Zool. Afr.* **6**, 75 (1971)]; 8. *Oreamnos americanus* (4, 4) [R. E. Henderson and B. W. O'Gara, *J. Wildl. Manage.* **42**, 921 (1978)]; 9. *Cervus elaphus* (14, 14) [B. Mitchell, D. McCowan, I. A. Nicholson, *J. Zool.* **180**, 107 (1976)]; 10. *Ovis canadensis* (3, 3) [unpublished museum records, University of Montana, Missoula]; 11. *Tragelaphus strepsiceros* (4, 4) [J. D. Skinner and B. J. Huntley, *Zool. Afr.* **6**, 293 (1971)]; *T. angasi* (3, 3) [J. L. P. L. Tello and R. G. Van Gelder, *Bull. Am. Mus. Nat. Hist.* **155**, 323 (1975)]; 12. *Kobus defassa* (19, 10) [C. A. Spinage, *J. Reprod. Fertil.* **18**, 445 (1969); H. P. Ledger, *East Afr. Wildl. J.* **2**, 159 (1964)]; 13. *Alces alces* (10, 97) [J. M. Peek, *J. Wildl. Manage.* **26**, 360 (1962); P. Schladweiler and D. R. Stevens, *J. Mammal.* **54**, 772 (1973)]; 14. *Bison bonasus* (1, 1) [K. Swiezynski, *Acta Theriol.* **13**, 511 (1968); J. Gill, *ibid.*, p. 499]; 15. *Giraffa camelopardalis* (15, 18) [A. J. Hall-Martin, J. D. Skinner, B. J. Hopkins, *J. Reprod. Fertil.* **52**, 1 (1978); A. J. Hall-Martin, *J. Wildl. Manage.* **41**, 740 (1977)].
13. R. R. Sokal and F. J. Rohlf, *Biometry* (Freeman, San Francisco, 1981), p. 231.
14. I thank J. Alcock, F. Allendorf, J. Byers, R. Hutto, D. Janzen, D. Jenni, J. McAuliffe, L. Metzgar, B. O'Gara, R. Redmond, A. Rutberg, A. Sheldon, and P. Watson for advice and criticism, and B. Hogg and P. Sykes for aid in data collection. Supported by the Montana Department of Fish, Wildlife, and Parks, the Wildlife Management Institute, the National Rifle Association, the Boone and Crocket Club, the Montana Cooperative Wildlife Research Unit, the University of Montana Zoology Department, and the U.S. Fish and Wildlife Service.

7 November 1983; accepted 21 March 1984

1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Destroys Dopamine Neurons in Explants of Rat Embryo Mesencephalon

Abstract. *Explants of embryonic rat mesencephalon were grown in organotypic culture. Addition of 10 μM 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to the culture medium for 4 to 7 days resulted in loss of dopamine cell bodies and fiber outgrowths, as observed by fluorescence histochemistry. At the same time, the cultures showed decreased uptake of tritium-labeled dopamine. However, no signs of generalized toxicity were evident when the explant cultures were viewed by light and phase-contrast microscopy. These results show that MPTP exerts a relatively selective destructive action in dopamine neurons in vitro, similar to the action observed in humans and monkeys in vivo. Pargyline (10 μM), a monoamine oxidase inhibitor, protected the dopamine neurons in the explants. Organotypic cultures provide an experimental model for the study of the properties of MPTP in vitro.*

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a syndrome indistinguishable from Parkinson's disease in humans and monkeys. Several instances of parkinsonism were reported in human subjects after self-administration of illicit samples of meperidine analogs that were contaminated with MPTP (*1, 2*). Subsequent studies with monkeys (*3, 4*) showed that intravenous or intraperitoneal administration of MPTP resulted in destruction of the dopaminergic cell bodies in the substantia nigra and loss of dopaminergic innervation in the caudate-putamen. Loss of cell bodies in the substantia nigra was also reported for one human subject (*1*). Cellular destruction appears to be specific for nigrostriatal neurons; noradrenergic and serotonergic neurons are spared.

Other animal species, including the rat, cat, and guinea pig (*5*), respond to MPTP either mildly or not at all, despite injection of higher doses of the drug. Thus, the monkey has served best as a model for the study of the degenerative effects of MPTP. In an effort to provide a more accessible and less expensive experimental model, we have evaluated the response of rat substantia nigra neurons in explant organotypic cultures exposed in vitro to MPTP. We now report that exposure of the cultures to MPTP resulted in destruction of dopamine neurons and that there was no other evidence of toxicity to the cultures as a whole.

Explant cultures were established from embryonic rat midbrain (15 to 16 days of gestation) and grown on cover slips coated with collagen in Maximow chambers with double cover slip assemblies (*6*). The ventral midbrain area from a single embryo was used on each cover slip. In such cultures the dopaminergic neurons grow, extend processes, and exhibit some of the normal characteristics of dopaminergic neurons in vivo (*7*). The neurons synthesize dopamine, and homovanillic acid, a dopamine metabolite, accumulates in the medium. In addition, a high-affinity uptake and storage system for tritium-labeled dopamine is present.

On day 8 in vitro, MPTP (10 μ*M*) was incorporated into the feeding medium (*6*) and applied to the cultures. The feeding medium was changed thereafter twice weekly. Control cultures were fed with medium that did not contain MPTP. The cultures were exposed to MPTP for either 4 or 7 days and analyzed subsequently at either 24 hours or 5 days after the drug was withdrawn. Phase-contrast light microscopy of both living and fixed cultures, as well as catecholamine histofluorescence and the uptake of [^{3}H]dopamine, were used to assess the effects of the drug on the cultures.

No generalized toxic effect of MPTP was observed. A large number of axons were present in the outgrowth of cultures treated with MPTP, and their appearance did not differ from untreated cultures of the same age (Fig. 1, A and B). Cultures treated with MPTP contained fewer neurons within the explants, but the surviving neurons appeared normal (Fig. 1, C and D). To identify the dopamine neurons within the explants, we incubated cultures with α-methylnorepi-

Table 1. Uptake of [^{3}H]dopamine by cultures of ventral midbrain from rat embryos after exposure to MPTP (10 μ*M*) for 4 to 7 days. In each experiment, three to four cultures were used for each treatment protocol; with one exception, results are pooled from two experiments. Pargyline was present, where indicated, at 10 μ*M*. Uptake was measured at 37°C for 10 minutes with 10 n*M* [^{3}H]dopamine (New England Nuclear; 45.4 Ci/mmol).

Treatment	Accumulation of [^{3}H]dopamine (picomoles per culture)			
	Measured 1 day after cessation of treatment		Measured 5 days after cessation of treatment	
	Mean ± standard error of the mean (*n*)	Percent of control	Mean ± standard error of the mean (*n*)	Percent of control
	*Experiment 1**			
Control	193 ± 29 (4)	100	340 ± 88 (6)	100
MPTP (4 days)	118 ± 13 (4)	61	119 ± 16 (6)	35
	Experiment 2†			
Control	214 ± 19 (8)	100	411 ± 70 (6)	100
MPTP (7 days)	55 ± 10 (8)	26	72 ± 25 (8)	18
	Experiment 3			
Control			491 ± 104 (8)	100
Pargyline			451 ± 42 (8)	92
MPTP (7 days)			72 ± 20 (8)	15‡
Pargyline + MPTP (7 days)			283 ± 37 (8)	58§

*For comparison of experimental and control cultures, $P < 0.05$ (two-tailed Student's *t*-test). †For comparison of experimental and control cultures, $P < 0.001$ (two-tailed Student's *t*-test). ‡$P < 0.001$ (Student-Newman-Keuls test for comparison of MPTP-treated and control culture). §$P < 0.025$ (Student-Newman-Keuls test for comparison of pargyline plus MPTP to MPTP alone).

nephrine (10 μ*M* for 30 minutes at 37°C) and subsequently treated them by either the Falck-Hillarp method (moist formaldehyde vapor) or the glyoxylic acid method for the fluorescence-visualization of catecholamines (*8*). During incubation with α-methylnorepinephrine, the cell bodies and processes of the dopamine neurons filled with a false transmitter that could be visualized even if the endogenous catecholamines were depleted by the drug. When viewed under the fluorescence microscope, control cultures contained numerous fluorescing neuronal somata within the explant (Fig. 1E), with fluorescing nerve fibers extended around it (Fig. 1G). After treatment with MPTP, the number of fluorescing cell bodies within the explants decreased (Fig. 1F), and the decrease in the number of fluorescing fibers in the outgrowth was even greater (Fig. 1H). The effects were more pronounced in cultures treated with MPTP for 7 days compared to those treated for 4 days. The loss of cell bodies indicated destruction of dopamine neurons by MPTP.

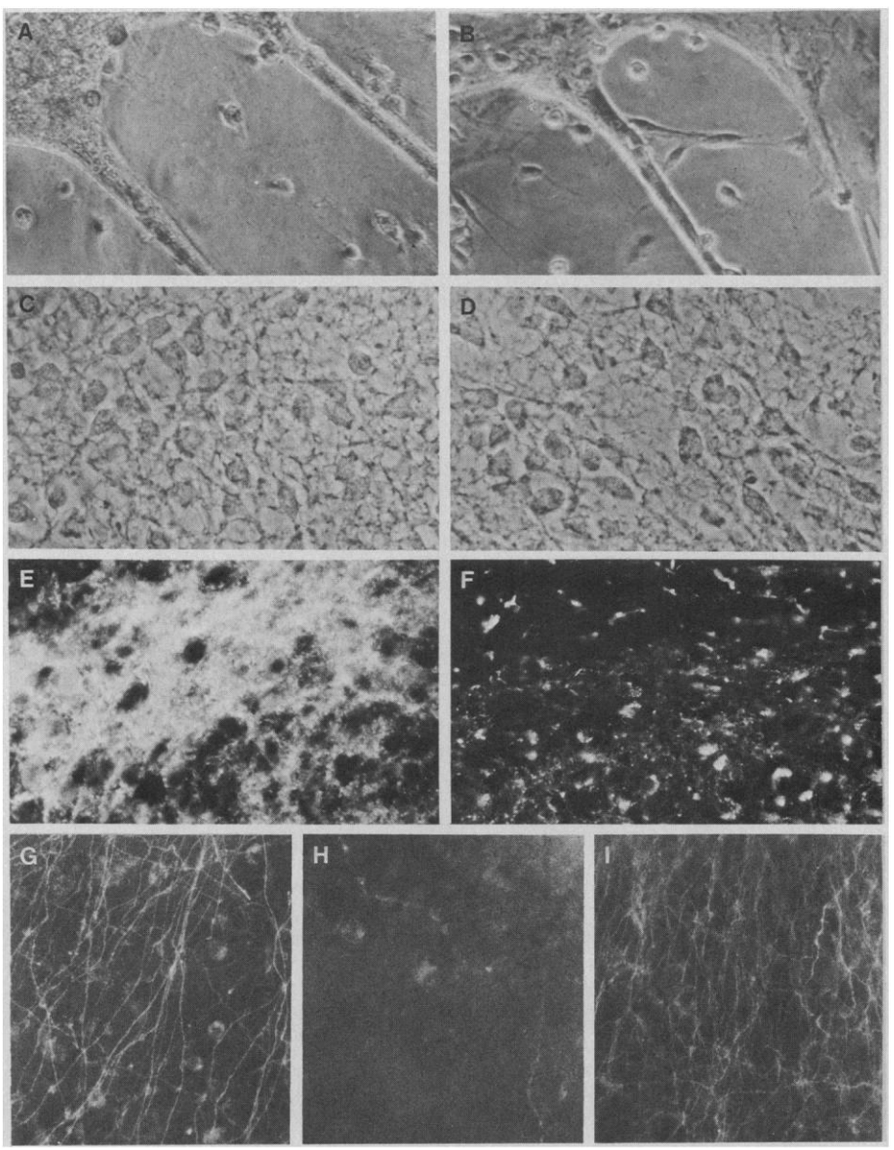

Fig. 1. (A and B) Phase-contrast photomicrographs of living cultures showing bundles of fibers emanating from the main explants. (A) Control culture; (B) culture treated with MPTP for 7 days. No generalized cytotoxic effect can be seen after MPTP treatment (×210). (C and D) Phase-contrast photomicrographs of sections through the explants where the neuronal somata are located. The cultures were fixed in 4 percent paraformaldehyde. (C) Control culture; (D) culture treated with MPTP for 7 days. No generalized cytotoxic effect can be seen after MPTP treatment (×370). (E and F) Fluorescence photomicrographs of cultures incubated with 10 μ*M* α-methylnorepinephrine for 30 minutes at 37°C and subsequently freeze-dried and treated according to the Falck-Hillarp method for the histochemical visualization of catecholamines. A plexus of fluorescing varicose fibers and several fluorescing neuronal somata are present in the control culture (E). After 7 days of MPTP treatment (F), a severe loss of both fluorescing neurons and fibers occurs within the explant. Some of the fluorescence in the explant represents accumulation of pigment which has an orange autofluorescence (×300). (G, H, and I) Fluorescence photomicrographs of the outgrowth from cultures incubated with α-methylnorepinephrine (as in E and F) and subsequently treated by the glyoxylic acid method for the visualization of catecholamines. A plexus of fluorescing fibers is present in the outgrowth from a control culture (G). Only a few faintly fluorescing fibers survive after 7 days treatment with MPTP (H). When the cultures are treated with 10 μ*M* pargyline for 24 hours and the pargyline is maintained in the culture fluid medium during the subsequent 7 days of exposure to MPTP, many of the fibers are spared from destruction (I) (×300).

We used uptake of [^{3}H]dopamine as a quantitative index of the dopaminergic axons and terminals present in the cultures. After exposure of the cultures to MPTP for 4 days (Table 1), the uptake of [^{3}H]dopamine was reduced to 61 percent of the control in measurements made 24 hours after the end of drug treatment. To determine whether the reduction in uptake of [^{3}H]dopamine was due to a direct inhibitory effect of the drug on the axonal membrane transport system, we repeated the measurements on other cultures 5 days after the end of drug treatment. During this time, the cultures were fed twice with fresh medium. At 5 days, the decrease in the uptake of [^{3}H]dopamine by the cultures treated with MPTP was even greater (namely, uptake was only 35 percent of control). This difference, however, was not due to further reduction of [^{3}H]dopamine uptake by the treated cultures but rather to increased uptake by the control cultures as a result of normal growth. In cultures exposed to MPTP for 7 days (Table 1), the uptake of [^{3}H]dopamine was reduced to 26 percent of control when measured 24 hours after removal of the drug and to 18 percent of control when measured after 5 days. Again, the apparent augmentation in effect indicated by measurements made after 5 days was due to an increase in uptake by the control cultures (that is, continued growth).

In some experiments pargyline, a monoamine oxidase inhibitor, was added to the feeding medium during incubation with MPTP. If the presence of MPTP increases intraneuronal turnover of dopamine, then hydrogen peroxide, which is a cellular toxin generated during the oxidative deamination of dopamine, could play an important role in the molecular mechanism of neuronal injury (*9*). Pargyline (10 μ*M*) was added to the cultures 24 hours before addition of MPTP, and both drugs were present during the subsequent incubation for 7 days. Pargyline exerted a significant protective action on dopamine neurons (Table 1). After 7 days of treatment with MPTP, [^{3}H]dopamine uptake was 15 percent of

control, indicating destruction of 85 percent of the dopamine nerve terminals. However, in the presence of pargyline uptake was reduced to only 58 percent of the untreated control (or 63 percent of the corresponding pargyline control). At the same time, catecholamine histofluorescence showed a healthy outgrowth of fluorescing fibers around the explants in cultures exposed to pargyline plus MPTP (Fig. 1I), while cultures treated with MPTP alone had few fluorescing fibers (Fig. 1H). No significant difference in uptake was seen between control cultures and cultures treated with pargyline alone (Table 1). The protective action seen with pargyline is consistent with the notion that the metabolism of dopamine by monoamine oxidase plays a significant role in the cytotoxic mechanism, but further studies are required to verify such an interpretation.

A direct comparison between the concentrations of MPTP effective in the monkey model in vivo and those used in our studies in vitro is difficult to make. Such factors as tissue distribution and rate of metabolism in vivo and stability in the feeding medium in vitro remain to be examined. As an approximation, however, the amounts of MPTP injected intravenously into rhesus monkeys (*3*) were in the range 0.15 to 0.69 mg per kilogram of body weight, which corresponds to average whole-body concentrations of 1 to 4 μM, for each daily injection over the 5- to 9-day injection schedule. In another study (*4*), squirrel monkeys received repeated intraperitoneal injections in the range 0.5 to 3.0 mg per kilogram of body weight, which corresponds to average whole-body concentrations of 3 to 17 μM. The concentration we selected for investigation in vitro was 10 μM with exposure for 4 to 7 days, which is in a reasonably comparable range.

Our results show that the relative insensitivity of rat brain in vivo to MPTP administered parenterally cannot be ascribed to failure of the tissue to produce a toxic metabolite. Embryonic rat mesencephalon in organotypic culture showed a marked loss of dopamine cell bodies and outgrowths, comparable to changes observed in vivo with monkeys. Organotypic cultures provide a good experimental model for the study of MPTP and its toxic properties on nigrostriatal neurons.

CATHERINE MYTILINEOU*
GERALD COHEN
Department of Neurology and *Division of Neurobiology, Mount Sinai School of Medicine of the City University of New York, New York 10029*

References and Notes

1. G. C. Davis, A. C. Williams, S. P. Markey, M. H. Ebert, E. D. Caine, C. M. Reichart, I. J. Kopin, *Psychiatr. Res.* **1**, 249 (1979).
2. J. W. Langston, P. Ballard, J. W. Tetrud, I. Irwin, *Science* **219**, 979 (1983).
3. R. S. Burns *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4546 (1983).
4. J. W. Langston, L. S. Forno, C. S. Rebert, I. Irwin, *Brain Res.* **292**, 390 (1984).
5. G. Kolata, *Science* **220**, 705 (1983); C. C. Chiueh *et al.*, *Pharmacologist* **25**, 131 (1983); R. S. Burns, S. P. Markey, J. M. Phillips, C. C. Chiueh, *Can. J. Neurol. Sci.* **11**, 166 (1984). A recent publication has described some success with mice [H. Hallman, L. Olson, G. Jonsson, *Eur. J. Pharmacol.* **97**, 133 (1984)]. In addition, several recent abstracts also report various degrees of success with mice or rats [R. E. Heikkila *et al.*, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 743 (1984); R. A. Wallace, R. Boldry, T. Schmittgen, D. Miller, N. Uretsky, *ibid.*, p. 585; L. Sterenka, L. N. Polite, K. W. Perry, R. W. Fuller, *ibid.*, p. 587].
6. The feeding medium for the cultures consisted of 33 percent human placental serum, 10 percent chick embryo extract, and 57 percent Earl's minimum essential medium (Gibco) supplemented with 3.4 mM glucose. MPTP (Aldrich) was dissolved in 0.1M HCl and subsequently diluted to one-thousandth of its original volume in the feeding medium. Equivalent volumes of 0.1M HCl were added to the control culture medium (without MPTP). The *p*H of the feeding medium was unchanged by this dilution of HCl.
7. Rat dopamine neurons in explant culture exhibit neurochemical characteristics similar to those of the dog [see C. Mytilineou, G. Cohen, D. Dembiec-Cohen, M. Van Woert, E. Hwang, *J. Neural Transm. Suppl.* **19**, 37 (1983)].
8. B. Falck, N.-A. Hillarp, G. Thieme, A. Thorp, *J. Histochem. Cytochem.* **10**, 348 (1962); W. O. Whetsell, Jr., C. Mytilineou, J. Shen, M. Yahr, *J. Neural Transm.* **52**, 149 (1981).
9. G. Cohen, *J. Neural Transm. Suppl.* **19**, 89 (1983).
10. Supported in part by U.S. Public Health Service grant NS-11631 and by a grant from the American Parkinson Disease Association (C.M.). We thank J. Shen for technical assistance.
* To whom correspondence should be addressed at the Department of Neurology (Ann. 14-70), Mount Sinai School of Medicine, One Gustave Levy Place, New York 10029.

21 March 1984; accepted 15 May 1984

Brain Factor Control of Sex Pheromone Production in the Female Corn Earworm Moth

Abstract. *Sex pheromone production in the female corn earworm moth* Heliothis zea *is controlled by a hormonal substance produced in the female's brain. It is present in the brain in scotophase as well as photophase, but it is released into the hemolymph to stimulate pheromone production only in the scotophase. The stimulatory activity was also detected in the brains of male corn earworm moths and of other moths.*

The female corn earworm moth *Heliothis zea* (Boddie) produces a sex pheromone during scotophase from a glandular area at its abdominal tip to attract and sexually activate a mate (*1*). We report the discovery of a hormonal substance in the brain of *H. zea* that controls production of the sex pheromone in the female. When females were ligated between head and thorax, causing isolation of the brain from the rest of the body, they were incapable of pheromone production. However, ligated females could be stimulated to produce pheromone by injection of a saline extract of the adult insect's brain. The stimulatory substance was found in brains of females in both the scotophase and the photophase, but it was detected in hemolymph only during the scotophase. Results indicate that the hormonal factor is a peptide produced in the female's brain and released under photoperiodic control into the hemolymph, thereby stimulating the pheromone gland to produce sex pheromone. This stimulatory activity is not restricted to extracts of the female brain; extracts of the male corn earworm brain and those of other moth species also triggered pheromone production in the female gland.

Table 1. Effect of ligation and subsequent injection of nervous tissue homogenates on the extractable sex pheromone in female *Heliothis zea*. Females were ligated with cotton thread between the head and thorax 1 hour after emergence. Means followed by the same letter are not statistically different from one another according to Duncan's new multiple range test ($P = 0.05$). BR, brain; CA, corpora allata; CC, corpora cardiaca; TG, thoracic ganglion; *Z*-11-HDAL, (*Z*)-11-hexadecenal.

Treatment	*Z*-11-HDAL ($\bar{x}$ ng per female ± S.E.M.)
Unligated*	122.93 ± 14.17 b
Ligated*	0.57 ± 0.15 e
+ 30 μl of saline*	0.93 ± 0.09 e
+ BR*	131.55 ± 26.55 b
+ BR and CC*	139.27 ± 19.73 b
+ BR (tissue-free)†	189.74 ± 23.13 a
+ CC†	13.56 ± 1.87 c
+ TG†	3.43 ± 0.52 d
+ CA†	0.52 ± 0.06 e

*$n = 10$. †$n = 5$.

Earlier efforts to elucidate physiologic factors that control female sex pheromone production in insects indicated that, in cockroaches, the corpora allata (CA) played some role (*2*), and studies of two species of moths were inconclusive (*3*). In a study of the gypsy moth *Lymantria dispar* (L.) removal of the brain in the pupal stage was reported to prevent pheromone release in 100 percent of the resulting females, but 79 percent of the brain-deficient females were observed to "call" (that is, to extend the terminal