Biochemical Pharmacology, Vol. 40, No. 4, pp. 901–906, 1990.
Printed in Great Britain.
0006-2952/90 $3.00 + 0.00
Pergamon Press plc

Characterization of 1-methyl-4-[4′-amino]phenyl-1,2,3,6-tetrahydropyridine toxicity in the mouse

(*Received* 12 *September* 1989; *accepted* 12 *February* 1990)

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a systemically active neurotoxin which kills catecholamine containing cells, primarily the dopaminergic neurons which comprise the nigrostriatal system [1–5]. The toxic actions of MPTP are dependent on two steps, monoamine oxidase (MAO) mediated oxidation to the dihydropyridine intermediate ($MPDP^+$) which spontaneously forms the pyridinium (MPP^+, 1-methyl-4-phenyl-pyridinium) [6, 7], and active sequestration of MPP^+ within catecholaminergic terminals via the catecholamine uptake system [8]. Blocking either one of these steps prevents the toxic effects of MPTP [9–12].

A number of MPTP analogs have been synthesized and tested in order to define the structural requirements for toxicity. It is now clear that substitutions on the pyridine ring yield compounds with no toxicity [13], but modifications of the phenyl ring yield many analogs which retain their toxicity [14–18]. These analogs may show differing sensitivity to oxidation by the A and B forms of MAO. For example, striatal dopamine loss from administration of MPTP, 1-methyl-4-cyclohexyl-TP, and 3′-OCH_3-MPTP is blocked by MAO-B inhibitors [14, 15], whereas 2′-ethyl MPTP toxicity is inhibited slightly by prior MAO-A inhibition and the 2′-methyl-MPTP toxicity can only be prevented by pretreament with both MAO-A and -B inhibitors [19]. The toxicity of all compounds tested thus far in the mouse can be blocked by pretreatment with dopamine uptake blockers [12].

We have presented preliminary data on the toxicity of 2′, 3′, and 4′-amino analogs of MPTP, demonstrating that the 4′-amino analog reduces striatal dopamine (DA) to 20% of control 1 week after administration and that this effect is prevented by the non-selective MAO inhibitor pargyline [20]. To characterize this toxicity further we examined the effects of pretreatment with clorgyline or deprenyl, selective inhibitors of MAO-A and -B respectively, as well as the selective DA uptake inhibitor GBR 12909, on the toxicity of this MPTP analog. In addition, we examined the effects of 4′-amino MPTP 1 month after administration and characterized the anatomical changes induced by this agent. Finally, we evaluated the effect of dosing interval on 4′-amino MPTP toxicity.

Methods

Adult male C57BL/6 mice (20 g, Charles River) were used in all experiments. All injections were subcutaneous. In experiment 1, groups of mice were injected in the late afternoon with either clorgyline or deprenyl (5 mg/kg, s.c.). The next day, half the clorgyline group and half the deprenyl group were treated with 4′-amino MPTP·2HCl (prepared as described [20]) given in four doses of 75 mg/kg each at 2.5 to 3-hr intervals. Another group received the same doses of 4′-amino MPTP with no pretreatment. Animals receiving deprenyl or clorgyline alone were not treated further, and controls were untreated. Animals were killed after 3–4 weeks. In experiment 2, two groups were given four doses of 4′-amino MPTP (75 mg/kg) or left untreated as above, except that the dosing regimen was altered such that the four doses of 4′-amino MPTP were given in the morning and late afternoon on two successive days and the survival time was 1 week.

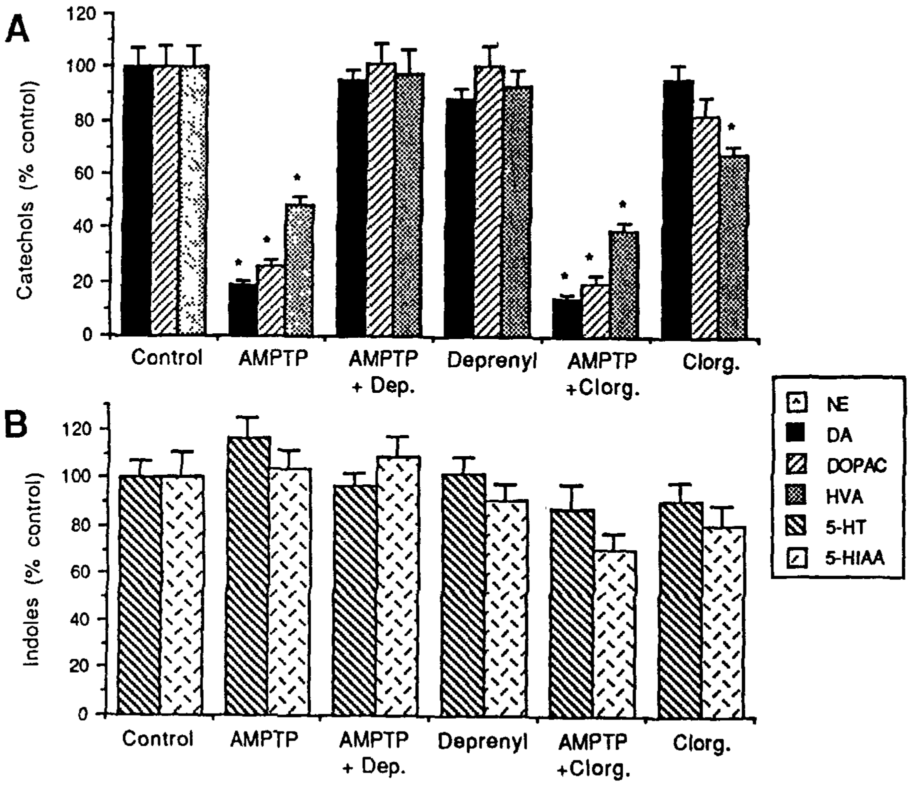
The effects of pretreatment with the selective DA uptake blocker GBR 12909 were evaluated in experiment 3. Again four injections of 4′-amino MPTP·HCl, 75 mg/kg, were given at 2.5 to 3-hr intervals, but a subset of this group was pretreated with GBR 12909 (5 mg/kg, s.c.) 15 min prior to each injection of 4′-amino MPTP. A third group received four injections of GBR 12909 only at 2.5 to 3-hr intervals, while controls were uninjected. Survival time was 2 weeks. In all experiments groups consisted of seven to ten animals.

Mice were killed by spinal dislocation followed by decapitation. The brains were removed and placed on an ice-cold petri dish for dissection. The cerebral cortices were reflected back, and both striata and both cerebral cortices were dissected free and placed in 1.5-mL plastic centrifuge tubes. When all were dissected, the samples were frozen on dry ice, and then stored at −70° until analysis. The brainstems, containing the substantia nigra and locus ceruleus, were fixed by immersion in 10% buffered formalin for subsequent histological analysis.

Frozen pairs of striata and cortices were sonicated in 0.5 or 1.0 mL, respectively, of ice-cold 0.1 M perchloric acid containing 10 μM ascorbate. Two 5-μL aliquots of each homogenate were taken for protein determination [21], and the remainder was spun at 12,000 *g* for 10 min. Aliquots of the clear supernatant fraction were loaded onto a Gilson autoinjector for the quantification of DA, dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) using a 10 cm × 1.0 mm microbore HPLC column (C18, Shandon Hypersil, 3 μm) coupled to an amperometric detector set at +0.75 V. The mobile phase consisted of 0.2 M NaH_2PO_4 containing 2.5% acetone, 50 mg/L EDTA, 120 mg/L sodium octyl sulfate (SOS), and 120 μL triethylamine (TEA) at a final pH of 3.5. Single samples from each group were run in rotating order and interspersed with a mixture of external standards. Mean control concentrations for all controls (in pg/μg protein) were: DA, 48.4 ± 3.1; DOPAC, 14.1 ± 2.57; HVA, 11.11 ± 1.21; 5-HT, 3.93 ± 0.40; 5-HIAA, 3.56 ± 0.19.

Cortical catecholamines norepinephrine (NE) and DA were determined from alumina extracts. To an aliquot of each supernatant fraction (0.5 mL) was added 5 ng of the internal standard, dihydroxybenzylamine (DHBA). To all samples were added 0.2 mL of 1.5 M Tris containing 2.0 g/L EDTA and 50 mM $NaHSO_3$, pH 8.5, followed by 10 mg of alumina. After 30 sec of vigorous vortexing the alumina was allowed to settle and the supernatant fraction was discarded. The alumina was washed three times with 1.0 mL of water, followed by elution with 85 μL of 0.1 M $HClO_4$. DHBA (5 ng) was also added to the standards which were then extracted as for samples. Samples and standards were run on the same column as above, but with a mobile phase of 0.2 M NaH_2PO_4, 50 mg/L EDTA, 50 μL/L TEA, 200 mg/L SOS, and 1.0% acetone, pH 3.8. Data were analyzed by a one-way analysis of variance followed by a Scheffe post hoc F-test (Statview 512+). Significance was determined at 95% confidence limits.

The fixed brainstems from representative animals from each group were cut at 30 μm on a freezing microtome. All sections through the substantia nigra and locus ceruleus were collected. Every third was stained with thionin, and



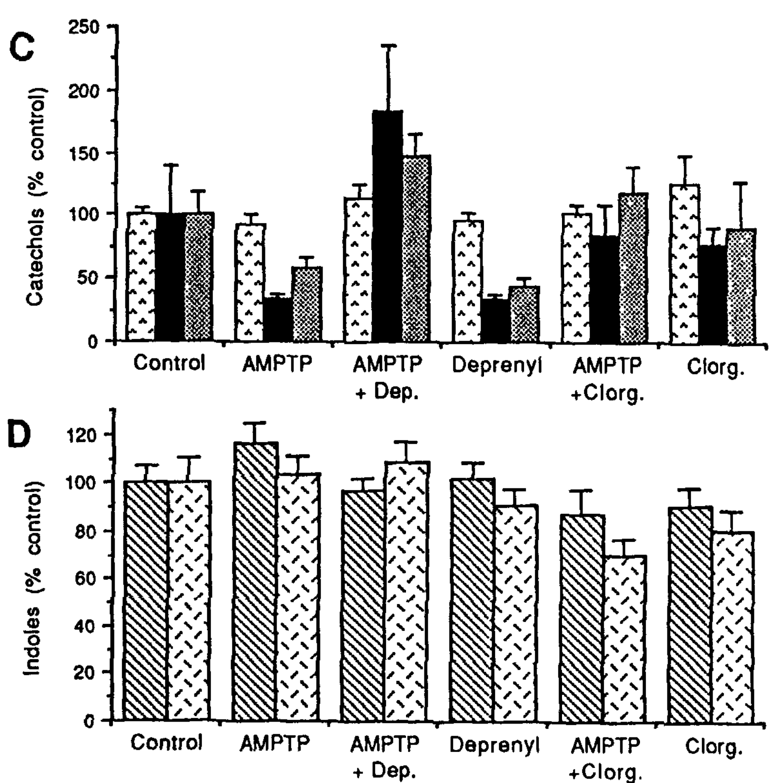


Fig. 1. Effect of deprenyl or clorgyline pretreatment on DA depletion 3 weeks following treatment with AMPTP (4'-amino MPTP·2HCl, 4 × 75 mg/kg s.c.). Key: (*) $P < 0.05$ with respect to control average. Mean control concentrations for all controls (in pg/μg protein) were: DA, 48.4 ± 3.1; DOPAC, 14.1 ± 2.57; HVA, 11.11 ± 1.21; 5-HT, 3.93 ± 0.40; and 5-HIAA, 3.56 ± 0.19.

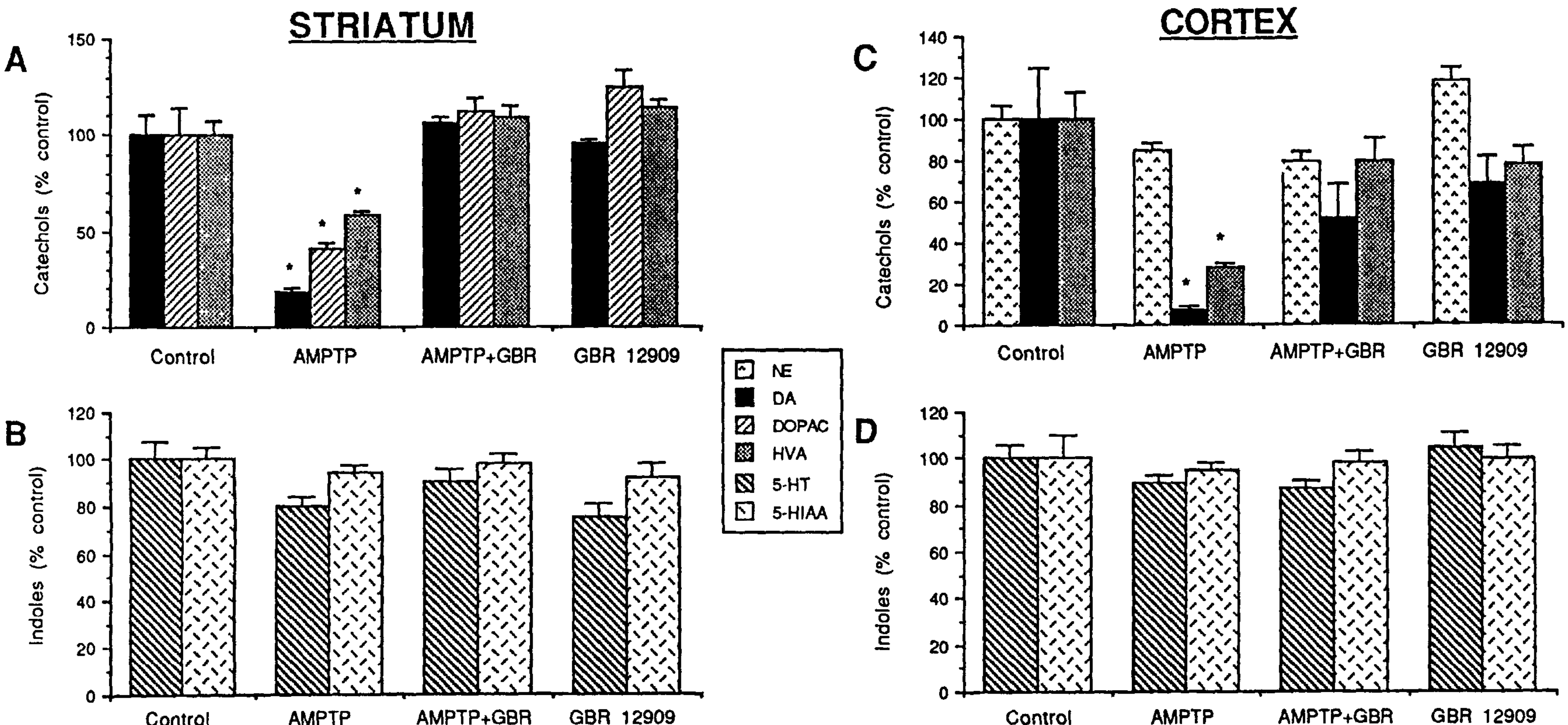


Fig. 2. Effect of pretreatment with the DA uptake inhibitor GBR 12909 on DA depletion 3 weeks following treatment with AMPTP (4′-amino MPTP·2HCl, 4 × 75 mg/kg, s.c.). Key: (*) $P < 0.05$ with respect to control average. See legend of Fig. 1 for control values.

the remaining sections were stained for tyrosine hydroxylase-like immunoreactivity using antibodies (1:2000 dilution) purchased from Eugene Tech International.

Results and Discussion

The 4'-amino analog of MPTP, when given in four doses of 75 mg/kg (s.c.), produced a pronounced loss of striatal DA (80%) and its metabolites (DOPAC and HVA) (Figs 1A and 2A), consistent with our earlier findings [20]. This depletion, evident 3 weeks after injection, was blocked completely by prior treatment with a single dose (5.0 mg/kg) of the selective MAO-B inhibitor deprenyl given approximately 17 hr before the first injection of 4'-amino MPTP (Fig. 1A). In contrast, pretreatment with clorgyline, a selective MAO-A inhibitor (5.0 mg/kg, 17 hr prior to 4'-amino MPTP) did not prevent or even blunt the degree of striatal DA depletion (Fig. 1A). Neither deprenyl nor clorgyline alone had any affect on striatal DA or its metabolites. None of the treatments altered the striatal levels of 5-HT or 5-HIAA (Fig. 1B). Cortical NE and DA concentrations were unaffected by 4'-amino MPTP (Fig. 1C) as were cortical 5-HT and 5-HIAA levels (Fig. 1D).

The interval between injections was found to have a profound effect on the potency of 4'-amino MPTP. When injected at 2.5 to 3-hr intervals, four injections of this compound produced an 80% depletion of striatal DA, but if the four injections were given at 9:00 a.m. and 4:00 p.m. on two successive days, the striatal DA loss was only about 20% (control DA, 69.4 pg/μg protein; 4'-amino MPTP, 4×75 mg/kg, 54.4 pg/μg protein).

In experiment 3, it was found that the DA uptake blocker GBR 12909, when administered 15 min prior to each of four injections of 4'-amino MPTP (75 mg/kg) completely blocked the decreases in striatal DA, DOPAC and HVA otherwise seen (Fig. 2A). The uptake blocker alone was without effect. Again, striatal 5-HT and 5-HIAA were unaffected by any of the treatments (Fig. 2B). In cortex, NE, 5-HT and 5-HIAA were not altered by any treatments (Fig. 2, panels C and D). Cortical DA was decreased significantly ($P < 0.05$) by 4'-amino MPTP, an effect blocked by GBR 12909 pretreatment.

Histological analysis revealed no evidence of cell loss (Fig. 3) within the substantia nigra following 4'-amino MPTP. Cells positive for tyrosine hydroxylase-like immunoreactivity were counted in four matching sections, and no significant differences were found between the number of cells per section in the control (Fig. 3A), 4'-amino MPTP-treated (Fig. 3B), GBR 12909/4'-amino MPTP-treated (Fig. 3C) or clorgyline/4'-amino MPTP-treated (Fig. 3D) brains. The locus ceruleus appeared unaffected by any of the treatments.

The 4'-amino analog of MPTP is very similar to MPTP in its apparent preference for MAO-B over MAO-A. Unlike some phenyl substituted analogs of MPTP which are substrates for both MAO-A and -B, the specificity seems to be complete in that the MAO-A inhibition was completely without effect on the degree of DA loss. These results also indicate that complete protection against the effects of MPTP and analogs can be achieved with minimal single doses of irreversible MAO inhibitors given well before MPTP treatment.

The degree of DA depletion observed with 4'-amino

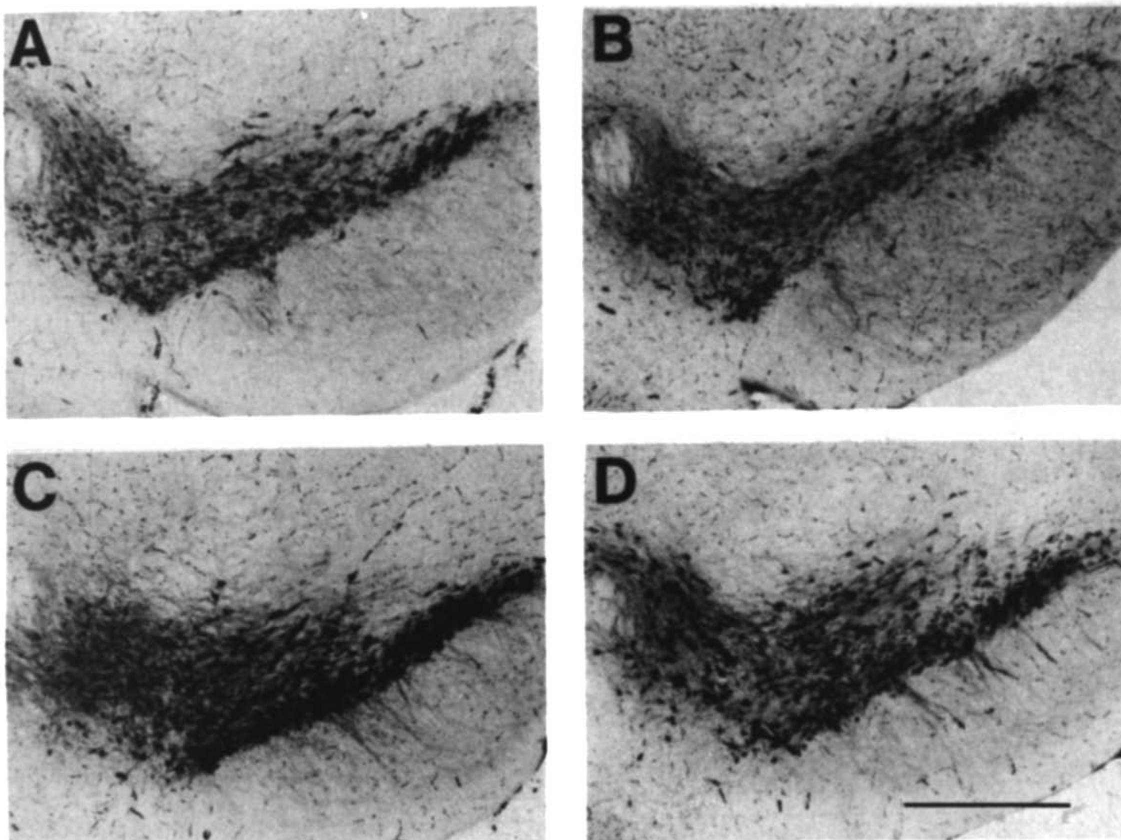


Fig. 3. Photomicrographs of tyrosine hydroxylase-like immunoreactivity in the substantia nigra pars compacta of a control mouse (A), a 4'-amino MPTP-treated mouse with no pretreatment (B), or 4'-amino MPTP-treated mice with either GBR 12909 pretreatment (C), or clorgyline pretreatment (D), killed 3–4 weeks after injection. The scale bar represents 500 μm.

MPTP was similar to that reported for MPTP [4]. The dose required (4×75 mg/kg, s.c.), however, was considerably higher. We have observed that when incubated with brain homogenates, 4′-amino MPTP produced only a small fraction of the corresponding pyridinium form as compared to MPTP (Johannessen JN, Savitt JM and Markey SP, unpublished observations). This is consistent with the finding that 4′-substituted MPTP analogs are generally poor MAO substrates [15]. Thus, formation of the active toxin may take place at a considerably lower rate. The DA depletion takes place despite this low pyridinium formation probably because the 4′-amino MPP^+ is about five times as potent as MPP^+ as an inhibitor of mitochondrial respiration.* The 4′-amino MPP^+ and 4′-acetamido MPP^+ analogs were found to be more selective and potent toxins than MPP^+ for dopaminergic neurons in fetal midbrain cultures [22, 23]. The potency of the 4′-amino substituted MPP^+ as a mitochondrial toxin may explain the uniqueness of this analog; other 4′-substituted MPTP analogs are devoid of toxicity [15].

We did not observe any appreciable cell loss following administration of 4′-amino MPTP. Reports of cell loss within the mouse substantia nigra after MPTP injections vary considerably. While some laboratories find that extensive cell loss accompanies striatal DA loss [4, 24], others do not [25, 26]. Differences in dosing regimen may explain some of these differences. Clearly in this case, extending the inter-dose interval greatly decreased the degree of DA loss. Perhaps an increase in the unit dose or a further decrease in the inter-dose interval would have yielded more pronounced effects in the substantia nigra pars compacta. It should be noted that the ability of 4′-amino MPTP to kill dopaminergic cells has been demonstrated unequivocally in preliminary experiments in the dog [27].

In summary, the 4′-amino analog of MPTP produced a striatal DA depletion which was blocked completely by pretreatment with the MAO-B inhibitor deprenyl and by the DA uptake blocker GBR 12909. Pretreatment with the MAO-A inhibitor clorgyline had no effect on the striatal DA depletion caused by 4′-amino MPTP. No effects were seen on cortical NE levels. While less potent than MPTP on a per weight basis, 4′-amino MPTP produced a degree of DA depletion comparable to that seen with MPTP and other MPTP analogs. Increasing the inter-dose interval from 3 hr to 8–16 hr abolished the toxicity of 4′-amino MPTP. Cell loss was not seen in either the substantia nigra or the locus ceruleus.

Acknowledgement—The synthesis of analogs to MPTP and MPP^+ was supported by a grant from the National Parkinson Foundation, Miami, FL, U.S.A.

Laboratory of Clinical Science
National Institute of Mental Health
Bethesda
MD 20892, U.S.A.

JAN N. JOHANNESSEN†
SANFORD P. MARKEY

REFERENCES

1. Davis GC, Williams AC, Markey SP, Ebert MH, Caine ED, Reichert CM and Kopin IJ, Chronic parkinsonism secondary to intravenous injection of meperidine analogs. *Psychiatry Res* **1**: 249–254, 1979.
2. Langston JW, Ballard P, Tetrud JW and Irwin I, Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**: 979–980, 1983.
3. Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM and Kopin IJ, A primate model of parkinsonism: Selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc Natl Acad Sci USA* **80**: 4546–4550, 1983.
4. Heikkila RE, Hess A and Duvoisin RC, Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in mice. *Science* **224**: 1451–1453, 1984.
5. Johannessen JN, Chiueh CC, Bacon JP, Garrick NA, Burns RS, Weise VK, Kopin IJ, Parisi JE and Markey SP, Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the dog: Effect of pargyline pretreatment *J Neurochem* **53**: 582–589, 1989.
6. Markey SP, Johannessen JN, Chiueh CC, Burns RS and Herkenham MA, Intraneuronal generation of a pyridinium metabolite may cause drug induced parkinsonism. *Nature* **311**: 464–467, 1984.
7. Chiba K, Trevor A and Castognoli N Jr, Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. *Biochem Biophys Res Commun* **120**: 574–578, 1984.
8. Javitch JA, D'Amato RJ, Strittmatter SM and Snyder SH, Parkinsonism-inducing neurotoxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: Uptake of the metabolite *N*-methyl-4-phenylpyridinium by DA neurons explains selective toxicity. *Proc Natl Acad Sci USA* **82**: 2173–2177, 1985.
9. Langston JW, Irwin I, Langston EB and Forno LS, Pargyline prevents MPTP-induced parkinsonism in primates. *Science* **225**: 1480–1482, 1984.
10. Heikkila RE, Manzino L, Cabbat FS and Duvoisin RC, Protection against the dopaminergic neurotoxicity of MPTP by monoamine oxidase inhibitors. *Nature* **311**: 467–469, 1984.
11. Ricaurte GA, Langston JW, DeLanney LE, Irwin I and Brooks JD, DA uptake blockers protect against the dopaminergic depleting effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in the mouse striatum. *Neurosci Lett* **59**: 259–264, 1985.
12. Mayer RA, Kindt MV and Heikkila RE, Prevention of the nigrostriatal toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by inhibitors of 3,4-dihydroxyphenylethylamine transport. *J Neurochem* **47**: 1073–1079, 1986.
13. Fries DS, de Vries J, Hazelhoff B and Horn AS. Synthesis and toxicity toward nigrostriatal DA neurons of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) analogs. *J Med Chem* **29**: 424–427, 1986.
14. Youngster SK, Saari WS and Heikkila RE, 1-Methyl-4-cyclohexyl-1,2,3,6-tetrahydropyridine (MCTP): An alicyclic MPTP-like neurotoxin. *Neurosci Lett* **79**: 151–156, 1987.
15. Youngster SK, Sonsalla PK and Heikkila RE, Evaluation of the biological activity of several analogs of the dopaminergic neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Neurochem* **48**: 929–934, 1987.
16. Wilkening D, Vernier VG, Arthaud LE, Treacy G, Kenny JP, Nickolson VJ, Clark R, Smith DH, Smith C and Boswell G, A parkinsonism-like neurological deficit in primates is caused by a novel 4-substituted piperidine. *Brain Res* **368**: 239–246, 1986.
17. Fuller RW and Hemrick-Luecke SK, Persistent depletion of striatal DA and its metabolites in mice by TMMP, an anlog of MPTP. *J Pharm Pharmacol* **39**: 667–669, 1987.
18. Fuller RW, Robertson DW and Hemrick-Luecke SK, Comparison of the effects of two 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine analogs, 1-methyl-4-(2-thienyl)-1,2,3,6-tetrahydropyridine and 1-methyl-4-(3-thienyl)-1,2,3,6-tetrahydropyridine: Effects on monoamine oxidase *in vitro* and on DA in mouse brain. *J Pharmacol Exp Ther* **240**: 415–420, 1987.

* Nicklas WJ, personal communication, cited with permission.

† Correspondence: Jan N. Johannessen, Ph.D., Laboratory of Clinical Science, Building 10/3D-40, National Institute of Mental Health, Bethesda, MD 20892.

19. Heikkila RE, Kindt MV, Sonsalla PK, Giovanni A, Youngster SK, McKeown KA and Singer TP, Importance of monoamine oxidase A in the bioactivation of neurotoxic analogs of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc Natl Acad Sci USA* **85**: 6172–6176, 1988.
20. Johannessen JN, Savit JM, Markey CJ, Bacon JP, Weisz A, Hanselman DS and Markey SP, The development of amine substituted analogs of MPTP as unique tools for the study of MPTP toxicity and Parkinson's disease. *Life Sci* **40**: 697–704, 1987.
21. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
22. Michel PP, Dandapani BK, Sanchez-Ramos J, Efange S, Pressman BC and Hefti F, Toxic effects of potential environmental neurotoxins related to 1-methyl-4-phenylpyridinium on cultured rat dopaminergic neurons. *J Pharmacol Exp Ther* **248**: 842–850, 1989.
23. Michel PP, Dandapani BK, Efange SMN and Hefti F, Potential environmental neurotoxins related to 1-methyl-4-phenylpyridinium: Selective toxicity of 1-methyl-4[4′-acetomidol]phenyl pyridinium and 1-methyl-4-cyclohexyl pyridinium for dopaminergic neurons. *Exp Neurol*, in press.
24. Sundstrom E, Stromberg I, Tetsuyuki T, Olson L and Jonsson G, Studies on the effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on central catecholamine neurons in C57BL/6 mice. Comparison with three other strains of mice. *Brain Res* **405**: 26–38, 1987.
25. Hallman H, Lange J, Olson L, Stromberg I and Jonsson G, *J Neurochem* **44**: 117–127, 1985.
26. Ricaurte GA, Langston JW, DeLanney LE, Irwin I, Peroutka SJ and Forno LS, Fate of nigrostriatal neurons in young mice given 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: A neurochemical and morphological reassessment. *Brain Res* **376**: 117–124, 1986.
27. Johannessen JN, Bacon JP, Markey CJ, Chiueh CC and Markey SP, Striatal DA depletion by an aminated analog of MPTP. *Soc Neurosci Abstr* **12**: 758–1986.

Biochemical Pharmacology, Vol. 40, No. 4, pp. 906–909, 1990.
Printed in Great Britain.
0006-2952/90 $3.00 + 0.00
© 1990. Pergamon Press plc

Postmortem stability of the $GABA_A$ receptor complex: a study using rat brain cerebrocortical membranes

(*Received* 2 *January* 1990; *accepted* 19 *April* 1990)

GABA is widely accepted as the major inhibitory neurotransmitter in the mammalian CNS. In addition, the responses to GABA which are mediated by the $GABA_A$ receptor complex are subject to modulation by several centrally acting drugs (for review, see [1]). The distribution and detailed characterization of the $GABA_A$ receptor has been extensively studied in the rat and bovine brain. However, since altered $GABA_A$ receptor function may contribute to the symptoms in several disorders of the CNS such as Parkinson's disease and Huntington's chorea [2, 3], similar detailed characterization of the receptor in human brain is desirable. A prime consideration in human postmortem studies is the stability of the molecular species to be examined. The time from death to autopsy varies considerably in human cases and could well mask any premortem changes associated with age or disease. By far the most convenient way of determining postmortem stability is the indirect method of using animal tissue although this assumes that the stability does not differ significantly between species [4]. These procedures also make the significant assumption that *in vitro* measurements with radioligands can be used to detect premortem changes in functional receptors. In the case of the $GABA_A$ receptor complex it is possible to test the functional state of the receptor complex postmortem to the extent that its putative allosteric properties can be examined using drugs known to modulate the conformation of the receptor complex. The present study reports the effect of postmortem delay on the *in vitro* modulation of the $GABA_A$ receptor by pentobarbitone in rat brain membranes.

Materials and Methods

[Methylamine-^{3}H]muscimol (sp. act. 12.2 Ci/mmol; 107 mCi/mg) was obtained from Amersham International plc (Amersham, Bucks, U.K.). Pentobarbitone (sodium salt), GABA and 5β-pregnan-3α-ol-20-one were obtained from the Sigma Chemical Company (St Louis, MO, U.S.A.). Adult male Sprague–Dawley rats (300–400 g) obtained from the departmental breeding colony within Ninewells Animal Services Unit, University of Dundee, were used. The rats were killed by stunning and cervical dislocation, and the heads were placed in a refrigerator and allowed to cool from 37° to 4° for varying time intervals up to 72 hr to simulate as far as possible the postmortem cooling curve of human cadavers. The brain was then removed and cortices dissected out and stored frozen at −20°. The period between death and refrigeration of the body was examined by leaving rat heads at room temperature after killing for various time intervals up to 6 hr.

Crude preparations of synaptic membranes were prepared from the thawed rat cortices using a procedure similar to that described by Olsen *et al.* [5]. The membranes were suspended in 50 mM tris/citrate buffer pH 7.1 containing 0.1M KCl stored frozen (−20°) prior to assay. On the day preceding the experiment, the membranes were thawed, washed once in tris buffer and dialysed for 20 hr against 1000 vol. of the same buffer.

The binding of [^{3}H]muscimol to the washed synaptic membrane preparation was performed by filtration assay using the protocol of Marangos and Crawley [6]. Aliquots of membrane suspension (equivalent to 0.15–0.25 mg protein) were incubated with the radioligand [^{3}H]muscimol (5 nM, 61 nCi/mL, final concentration), in the absence and presence of 1 mM pentobarbitone in a total vol. of 0.5 mL of 20 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl for 20 min at 0°. Non-displaceable binding was measured in the presence of 100 μM GABA. After incubation, samples were diluted with 4 mL of ice-cold assay buffer and immediately filtered on GF/B filters (Whatman) under suction. Filters were washed three times with 4 mL of buffer, dried and counted for radioactivity in 5 mL Beckman Ready Value scintillation fluid. Protein was determined by the method of Lowry *et al.* [7] with bovine serum albumin as standard.