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Inhibition by dizocilpine (MK-801) of striatal dopamine release induced by MPTP and MPP^+: possible action at the dopamine transporter

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1 The NMDA-type glutamate receptor antagonist, dizocilpine (MK-801) can protect against neurotoxicity associated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its principal metabolite, the 1-methyl-4-phenylpyridinium ion (MPP^+). It has been suggested that these neurotoxic effects may be mediated by release of excitatory amino acids, but possible alternative mechanisms have been little investigated.

2 MPTP and MPP^+ (0.1–1000 μM) were tested in superfused rat striatal synaptosomes preloaded with [^{3}H]-dopamine. Both MPTP (10 μM and higher) and MPP^+ (1 μM and higher) evoked an immediate and concentration-dependent release of [^{3}H]-dopamine. The maximal effect exceeded that achievable with nicotine. For subsequent experiments, submaximal concentrations of MPTP (50 μM) and MPP^+ (10 μM) were tested.

3 MK-801 (0.1–100 μM) inhibited responses to MPTP (50 μM) and MPP^+ (10 μM) in a concentration-dependent manner. However, further tests of NMDA-type glutamate receptor involvement proved negative. Responses to MPTP or MPP^+ were unaffected by the omission of Mg^{2+} or Ca^{2+} and were not reduced by the NMDA receptor antagonists, AP-7 (200 μM) and kynurenic acid (300 μM). In this assay, N-methyl-D-aspartate (even in the absence of Mg^{2+} and with added glycine and strychnine) did not evoke [^{3}H]-dopamine release.

4 In crude membrane preparations of rat cerebral cortex, MPTP and MPP^+ inhibited high-affinity [^{3}H]-nicotine binding to nicotinic cholinoceptors (IC_{50} 1.8 μM and 26 μM, respectively).

5 [^{3}H]-dopamine release evoked by nicotine (1 μM) was blocked by the nicotinic antagonists, mecamylamine and chlorisondamine, and by MK-801 (all at 100 μM); K^+-evoked release was not affected. Release evoked by MPTP and MPP^+ was significantly attenuated by MK-801 but not by mecamylamine or chlorisondamine.

6 At a high concentration (10 μM), the selective dopamine uptake inhibitor, nomifensine, completely blocked [^{3}H]-dopamine release evoked by amphetamine 0.3 μM and MPP^+ 10 μM, attenuated responses to MPTP 50 μM and did not affect responses to 12 mM K^+. MK-801 100 μM evinced a similar profile but was less effective.

7 MK-801 inhibited [^{3}H]-dopamine uptake in striatal synaptosomes with an IC_{50} of 115 μM.

8 It is concluded that high concentrations of MK-801 inhibit the acute dopamine release evoked by MPTP and MPP^+ in synaptosomes. This antagonism may occur, at least in part, through inhibition of the cell membrane dopamine transporter. MPTP and MPP^+ also appear to interact with brain nicotinic cholinoceptors but the functional consequences of this interaction are not yet clear.

Keywords: Dizocilpine; MK-801; MPTP; MPP^+; nicotinic receptors; striatum; dopamine; excitatory amino acid receptors; N-methyl-D-aspartate

Introduction

The mechanisms by which systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) results in destruction of nigrostriatal dopamine have been elucidated in some detail (Tipton & Singer, 1993). In briefest outline, MPTP is first converted to the 1-methyl-4-phenylpyridinium ion (MPP^+) (Markey *et al.*, 1984) which enters dopaminergic terminals via the dopamine transporter (Javitch *et al.*, 1985). Inside the terminal, MPP^+ inhibits mitochondrial respiration (Vyas *et al.*, 1986) with a consequent decline in ATP levels leading to a decline of energy-dependent processes (Scotcher *et al.*, 1990; Chan *et al.*, 1991).

An excitotoxic mechanism has also been proposed, based initially on the observation that intrastriatal administration of MPP^+ can lead to a massive rise in extracellular glutamate and aspartate (Carboni *et al.*, 1990). Subsequently, this notion was supported by a report describing protective effects of excitatory amino acid receptor antagonists in rats given an intranigral infusion of MPP^+ (Turski *et al.*, 1991). However, later work has yielded mixed results, with reports of full protection (Storey *et al.*, 1992; Srivastava *et al.*, 1993), partial protection (Santiago *et al.*, 1992; Trabatabaei *et al.*, 1992; Lange *et al.*, 1993; Chan *et al.*, 1993; Brouillet & Beal, 1993), or no protection afforded by the most commonly used antagonist, dizocilpine (MK-801) (Sonsalla *et al.*, 1989; Kupsch *et al.*, 1992; Sonsalla *et al.*, 1992; Finiels-Marlier *et al.*, 1993). In general, the source of these discrepancies is not clear. However, most positive reports using MK-801 have been associated with the administration of high systemic or intracerebral doses.

MPTP, MPP^+ and MK-801 all possess additional pharmacological or toxic actions. MPTP exerts actions in the brain that do not appear to depend on conversion to MPP^+ (Wilson *et al.*, 1990; Palombo *et al.*, 1991; Duchemin *et al.*, 1992). In addition, MPTP and/or MPP^+ can damage cells that are not known to express dopamine transporters (Namura *et al.*, 1987; Turski *et al.*, 1991; Tipton & Singer, 1993). Furthermore, MPTP and MPP^+ bear a structural

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resemblance to the nicotinic receptor agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP), and MPTP binds quite potently to skeletal muscle nicotinic receptors (Hsu *et al.*, 1993). Nicotine administration can alter MPTP toxicity in mice (Janson *et al.*, 1992), but to date neither MPTP nor MPP^+ has been investigated for possible nicotinic actions in the CNS. In contrast, MK-801 has been shown to block CNS nicotinic receptors in addition to its better-known action at NMDA glutamate receptors (Ramoa *et al.*, 1990).

The aim of the present study was to identify pharmacological actions that might contribute to the neurotoxic effects of MPTP and MPP^+ or to the protective effects of MK-801. Initially, the investigation focussed on possible actions of MPTP or MPP^+ on NMDA-type glutamate or nicotinic receptors. Evidence was then obtained that high concentrations of MK-801 inhibit the cell membrane dopamine transporter. The possible implications of this action, which was reported some years ago (Snell *et al.*, 1988), has not previously been discussed in the context of MPTP toxicity.

Methods

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec), weighing 200–250 g, were maintained on a 12 h/12 h light-dark cycle. Rats were housed four per cage, and food and water were available *ad libitum*. Rats were allowed to accommodate to the housing conditions for 4 days after arrival, and were drug-naive prior to testing.

Dopamine release from superfused synaptosomes

Methods for synaptosomal preparation and measurement of [^{3}H]-dopamine release were virtually identical to those described in detail elsewhere (Clarke *et al.*, 1994). In each assay, crude synaptosomal (P2) fraction was prepared from dissected striata from 4 rats and resuspended in superfusion buffer (SB) composed of the following, in mM concentrations: NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, D-glucose 10, L-ascorbic acid 1 and pargyline 0.1 at pH 7.5. The synaptosomal preparation was preloaded with [^{3}H]-dopamine.

The superfusion apparatus comprised 32 identical channels, each having a small polypropylene retention chamber, through which superfusate was pumped at 0.4 ml min^{-1}. Each experiment included two or more assays. At the start of each assay, 100 µl of the synaptosomal suspension was introduced to each superfusion chamber. During the next 30 min, synaptosomes were superfused with SB alone or with SB containing antagonist, as appropriate. Next, 13 samples per channel were collected in consecutive 1 min intervals: after a 5 min baseline collection period, a 1 min (0.4 ml) pulse of releasing drug or SB (prepared with or without antagonist as appropriate) was given. Finally, the filters holding the synaptosomes were removed in order to measure residual radioactivity (Wallac 1410 liquid scintillation counter, LKB, Sweden). In experiments using Ca^{2+}-free medium an equivalent molar concentration of Mg^{2+} and EGTA (2.25 mM) was substituted. For Mg^{2+}-free medium an equivalent molar concentration of Na^+ was added.

In each assay, data were collected simultaneously from all 32 channels. Each assay incorporated control (SB only) channels, and treatment conditions were counterbalanced across channels and assays. We refer to the tritium released as dopamine release, since it has been established that in similar synaptosomal preparations preloaded with [^{3}H]-dopamine, tritium released by nicotinic agonists or by depolarization largely corresponds to dopamine itself (Rapier *et al.*, 1988).

For each channel, the release occurring in each 1 min collection period was calculated as a percentage of basal release, determined from the first five samples collected. Drug-evoked release was taken as the peak value that occurred in the first 3 periods after a drug challenge. This measure of drug effect was used since it is less likely to be affected by receptor desensitization than the time-averaged drug effect ('area under the curve'). Basal release was 1710 ± 70 d.p.m. min^{-1} (mean ± s.e.mean, $n = 12$ series of assays), which corresponds to approximately 3 fmol mg^{-1} of original wet tissue. Across experiments, basal release (min^{-1}) ranged from 0.8 to 1.3% of residual radioactivity collected on the tissue filters (163,000 ± 11,000 d.p.m. per filter).

[^{3}H]-dopamine uptake by striatal synaptosomes

Striatal synaptosomes were prepared as for [^{3}H]-dopamine release (see above), with the following modifications. The P2 pellet was resuspended and incubated in SB alone at 37°C for 5 min (i.e. no [^{3}H]-dopamine present), followed by centrifugation at 12,000 *g* for 5 min and resuspension in SB (0.02 mg original tissue ml^{-1}). Synaptosomes were incubated (1 mg original tissue per well) in SB for 30 min at room temperature (RT), in uncoated polystyrene, U-bottom 96 multiwell plates (Dynatech Laboratories, Chantilly, VA, U.S.A.), with or without MK801 (1–1000 µM) or nomifensine (100 µM). Synaptosomes were then cooled to 4°C for 5 min before the addition of [^{3}H]-dopamine (0.12 µM). Incubation proceeded at RT for 10 min, followed immediately by filtration and washing in an Inotech cell harvester (Biosystems International Inc., Lansing, MI, U.S.A.) at 4°C, as previously described (El-Bizri & Clarke, 1994a). Radioactivity was measured in a liquid scintillation counter (Wallac 1410; Pharmacia LKB Biotech, Sweden).

[^{3}H]-nicotine binding to tissue homogenates

Animals were decapitated, and left and right cerebral cortices were dissected on ice, pooled, and immediately weighed before storage at − 40°C. The method of tissue homogenate preparation and radioligand binding has been described in detail elsewhere (El-Bizri & Clarke, 1994a). On the day of the assay, thawed tissue was resuspended, incubated at 37°C for 5 min, centrifuged at 15,000 *g* for 20 min then resuspended in an equal volume of fresh incubation buffer (concentrations in mM: NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ (H_2O) 1.2, HEPES 20, NaOH 10). Tissue incubation was performed in uncoated polystyrene, U-bottom 96 multiwell plates (Dynatech Laboratories, Chantilly, VA, U.S.A.). The final volume of incubation in each well was 100 µl, containing the equivalent of 2 mg original wet tissue. Non-displaceable binding was determined in the presence of 10^{-5} M nicotine hydrogen tartrate. Tissue membranes were incubated with [^{3}H]-nicotine at 37°C for 20 min. Homogenates were filtered and washed, and the radioactivity counted, as previously described (El-Bizri & Clarke, 1994a).

As a check of radioligand purity (Romm *et al.*, 1990), saturation analysis of [^{3}H]-nicotine binding was performed with twenty-three concentrations of [^{3}H]-nicotine (0.1–19 nM). Inhibition binding experiments were performed under the same conditions with 1.0 nM [^{3}H]-nicotine; inhibiting drugs (MPTP, MPP^+) were added to the tissue suspension immediately before the radioligand.

Drugs

Chemicals and suppliers were as follows: [^{3}H]-dopamine ([8-^{3}H]-dopamine, specific activity 27.5 Ci $mmol^{-1}$) and [^{3}H]-nicotine (1-[N-methyl-^{3}H]-nicotine, specific activity 75.7 Ci $mmol^{-1}$) (New England Nuclear, Boston, MA, U.S.A.), l-nicotine hydrogen tartrate and N-methyl-D-aspartate (Sigma Chemical Corp., St. Louis, MO, U.S.A.), polyethyleneimine (ICN Biochemicals Canada, St. Laurent, Canada), nomifensine maleate, pargyline hydrochloride, kynurenic acid, MPTP hydrochloride and MPP^+ iodide (Research Biochemicals Inc., Natick, MA, U.S.A.), (±)-2-amino-7-phosphonoheptanoic acid (AP-7) (Cambridge Research Biochemicals, Cam-

bridge, U.K.). (+)-amphetamine sulphate (gift of SmithKline Beecham Pharma, Oakville, Canada), (+)-MK-801 HCl and mecamylamine HCl (gifts of Merck and Co., Rahway, NJ, U.S.A.), and chlorisondamine chloride (CHL) (gift of Ciba-Geigy, Summit, NJ, U.S.A.). Other chemicals and reagents were purchased from commercial sources. For superfusion, drugs were dissolved in buffer (SB).

Data analysis

Drug effects were examined by analysis of variance, using commercial software (Systat, Evanston, IL, U.S.A.). Multiple comparisons with a single control group were made with Dunnett's test (Dunnett, 1955) where variance appeared homogeneous; other multiple comparisons were made by Student's *t* test with Bonferroni's correction (Glantz, 1992). Probability values are 2-tailed. Radioligand binding data were analysed by nonlinear regression analysis (LIGAND, Elsevier Biosoft, Cambridge, U.K.).

Results

Experiment 1: Concentration-dependent effects of MPTP and MPP$^+$ on striatal [^{3}H]-dopamine release

MPTP and MPP$^+$ (0.1–100 μM) were tested separately, each in two assays. Both drugs evoked [^{3}H]-dopamine release in a concentration-dependent manner (Figure 1). Discernible effects were observed at concentrations as low as 10 μM and 1 μM, respectively. Evoked release was rapid in onset, with a latency comparable to that associated with nicotine. However, high concentrations of MPTP and MPP$^+$ produced much greater amounts of [^{3}H]-dopamine release than was obtained in parallel with nicotine which was applied at a concentration producing near maximal calcium-dependent release in this assay (El-Bizri & Clarke, 1994b).

Experiment 2: Concentration-dependent inhibition of responses to MPTP and MPP$^+$ by MK-801

Graded concentrations of MK-801 (0.01–100 μM) were tested against MPTP (50 μM) and MPP$^+$ (10 μM) in independent sets of assays (Figure 2). MK-801 reduced responses to both MPTP (MK-801 main effect: $F = 8.52$, d.f. 5,72, $P < 0.0001$) and MPP$^+$ ($F = 5.52$, d.f. 4,48, $P < 0.001$). In each case, significant effects were observed only at 10 and 100 μM. In the MPP$^+$ assays, MK-801 also affected basal release (main effect: $F = 7.38$, d.f. 5,56, $P < 0.0001$). However, the only significant change occurred at one concentration (10 μM: Dunnett's test $P < 0.0001$), and no such increase was observed in the MPTP assays (main effect: $F = 2.19$, d.f. 5,83, $P > 0.05$).

Experiment 3: Further tests of NMDA-type glutamate receptor involvement

Two other NMDA-type glutamate receptor antagonists (AP-7 200 μM and kynurenic acid 300 μM) were tested for their

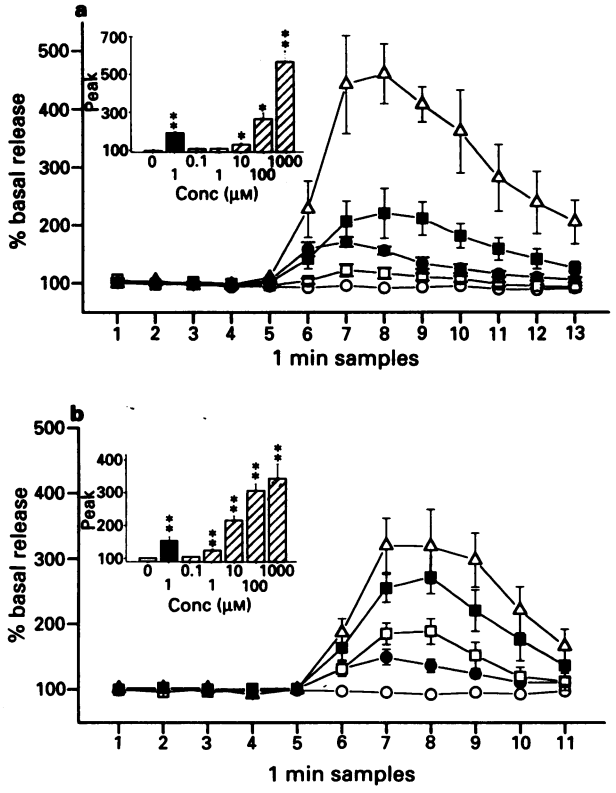

Figure 1 Effect of MPTP, MPP$^+$, and nicotine on [^{3}H]-dopamine release from striatal synaptosomes. Following superfusion with buffer for 30 min, basal release was measured for 5 min prior to the administration of a 1 min pulse of drug or buffer: nicotine 1 μM (●), buffer alone (○), and either MPTP (a) or MPP$^+$ (b) at 10 μM (□), 100 μM (■), or 1000 μM (△). The vertical axis represents the mean (± s.e.mean) release, calculated as a percentage of basal release ($n = 6$–15 channels). The inset shows the peak effect, expressed in the same way: buffer alone (open columns), nicotine (solid columns), and MPTP or MPP$^+$ (cross-hatched columns). $^*P < 0.05$, $^{**}P <$ 0.01–0.0001 vs. buffer alone (Student's *t* test with Bonferroni's correction).

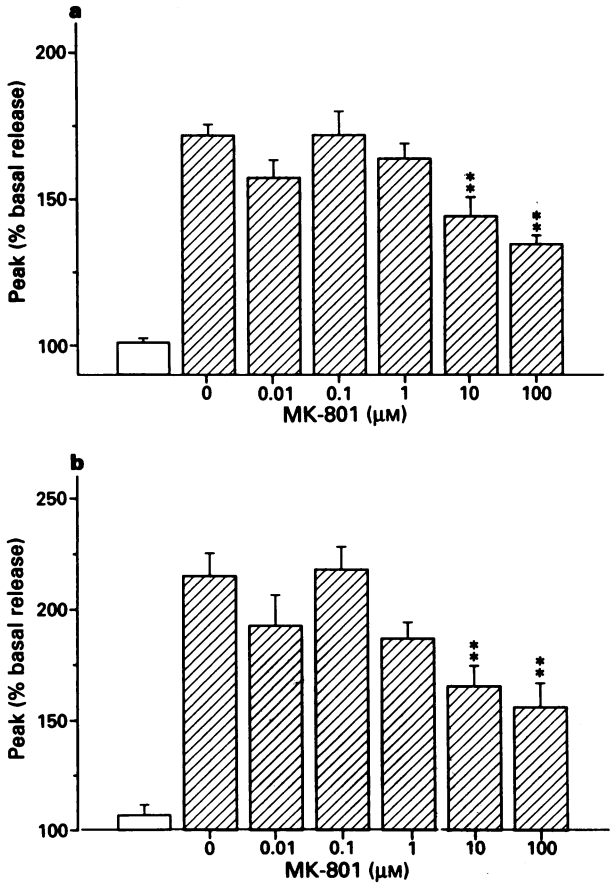

Figure 2 Effects of MK-801 (0.01–100 μM) on [^{3}H]-dopamine release evoked by a 1 min pulse of MPTP (a) or MPP$^+$ (b). Following 35 min of superfusion with MK-801 or buffer alone, synaptosomes were challenged with either MPTP 50 μM or MPP$^+$ 10 μM (cross-hatched) or with buffer alone (open columns). The vertical axis represents the mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 10$–23 and 8–16, respectively). $^{**}P < 0.01$–0.0001 vs. MPTP or MPP$^+$ alone (Dunnett's test).

ability to reduce [^{3}H]-dopamine release evoked by MPTP 50 μM and MPP$^+$ 10 μM (Figure 3). Neither antagonist was effective. Tested in parallel, MK-801 (100 μM) significantly decreased [^{3}H]-dopamine release evoked by MPP$^+$; the apparent inhibition of MPTP-evoked release was not significant ($P > 0.1$).

Several attempts were made to demonstrate NMDA-evoked [^{3}H]-dopamine release under conditions where the other secretagogues were effective. However, NMDA gave uniformly negative results, even when Mg^{2+} was omitted from the superfusion buffer (data not shown). The following challenges were tested in different assays: (1) 1 min pulse of NMDA 500 μM + glycine 1 μM ± strychnine 1 μM; (2) 1 min pulse of NMDA 100 μM + glycine 10 μM; (3) 2 min pulse of NMDA 100 μM + glycine 10 μM ± strychnine 10 μM. In each assay, NMDA failed to release [^{3}H]-dopamine significantly, although parallel treatment with another agent (K$^+$ 12 mM, nicotine 1 μM, or MPP$^+$ 10 μM) was always effective.

Release of striatal [^{3}H]-dopamine resulting from NMDA receptor activation is reported to be calcium-dependent and enhanced by omission of Mg^{2+} (see Discussion). However, neither effect was observed with [^{3}H]-dopamine release evoked by MPTP 50 μM or MPP$^+$ 10 μM (Figure 4). The apparent increase in drug-evoked release resulting from the omission of external Ca^{2+} can be attributed to a 62% reduction in basal release seen in this condition.

Experiment 4: Inhibition of [^{3}H]-nicotine binding by MPTP and MPP$^+$

Nonlinear regression analysis revealed a single saturable component of [^{3}H]-nicotine binding to rat cortical membranes ($K_D = 1.02$ nM); the Scatchard plot was correspondingly linear ($r = 0.98$). Both MPTP and MPP$^+$ inhibited [^{3}H]-nicotine binding with IC_{50}s of 1.8 and 26 μM, respectively (Figure 5).

Experiment 5: Effects of the antagonists mecamylamine, chlorisondamine and MK-801 on MPTP- and MPP$^+$-evoked [^{3}H]-dopamine release

In one set of assays, K$^+$ 12 mM and MPTP 50 μM were examined in parallel; in another set, nicotine 1 μM and MPP$^+$ 10 μM were tested in parallel (Figure 6). Mecamylamine, chlorisondamine, and MK-801 all possess nicotinic antagonist activity (see Introduction), and applied at 100 μM, all three blocked responses to 1 μM nicotine (Figure 6b). Responses to K$^+$ were not significantly altered (Figure 6a). Dopamine release evoked by MPP$^+$ was significantly attenuated by MK-801 (Dunnett's test: $P < 0.01$) but not by mecamylamine or chlorisondamine ($P > 0.2$ for both) (Figure 6b). Similarly, only MK-801 significantly reduced MPTP-induced release ($P < 0.01$; Figure 6a). Basal release was not affected by antagonist administration in either set of assays (antagonist main effect: $F < 2.31$, d.f. 3, 88, $P > 0.08$).

Experiment 6: Inhibition of responses to MPTP, MPP$^+$ and (+)-amphetamine by nomifensine and MK-801

The dopamine uptake blocker, nomifensine, was tested for its ability to inhibit evoked [^{3}H]-dopamine release. Pilot studies showed that prolonged administration of nomifensine increased basal release. To avoid this, nomifensine was not administered prior to acute challenge (the standard procedure with other antagonists), but was instead co-administered with the agonist. Nomifensine (0.1–10 μM) was first tested against

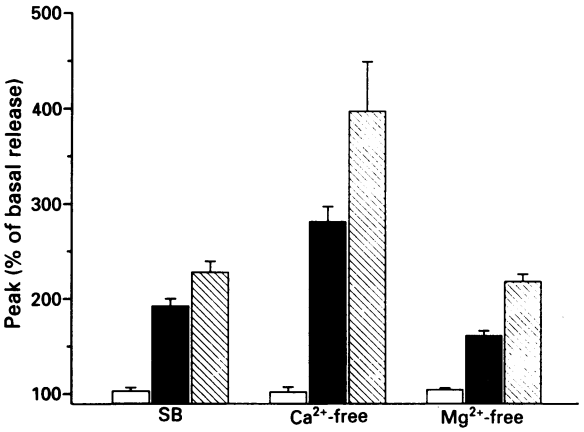

Figure 4 Effects of the omission of external calciuim or magnesium on [^{3}H]-dopamine release evoked by MPTP or MPP$^+$. Following 35 min of superfusion with superfusion buffer (SB) with or without Ca^{2+} or Mg^{2+}, synaptosomes were challenged with either SB alone (open columns), MPTP 50 μM (solid columns) or MPP$^+$ 10 μM (hatched columns). The vertical axis represents the mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 6$–10).

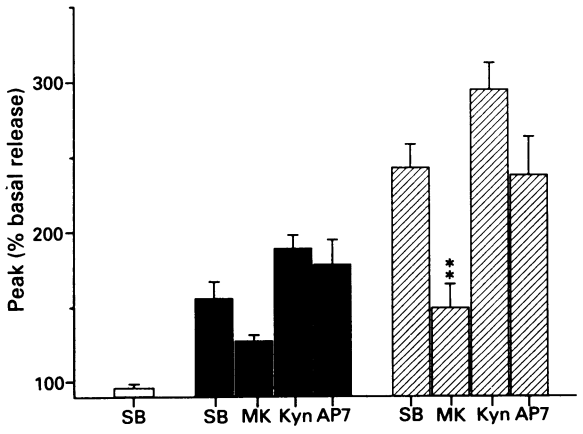

Figure 3 Effects of the glutamate receptor antagonists on [^{3}H]-dopamine release evoked by MPTP or MPP$^+$. Following 35 min of superfusion with antagonist (MK-801 100 μM, kynurenic acid (Kyn) 300 μM, or AP-7 200 μM) or with superfusion buffer alone (SB), synaptosomes were challenged with either MPTP 50 μM (solid columns) or MPP$^+$ 10 μM (hatched columns). Control channels (open columns) were superfused and challenged with SB only. The vertical axis represents the mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 6$–10). **$P < 0.002$ vs. MPP$^+$ alone (Dunnett's test).

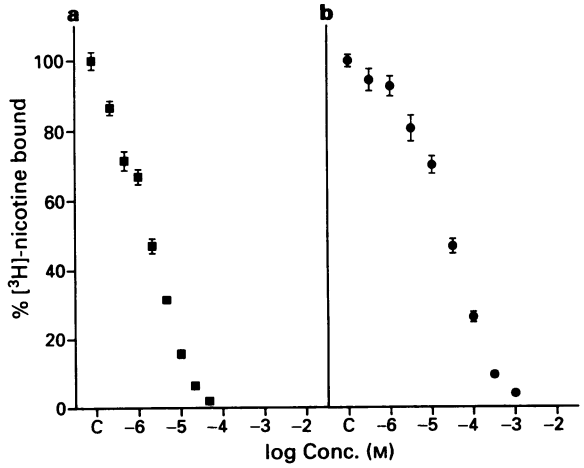

Figure 5 Effects of (a) MPTP and (b) MPP$^+$ on [^{3}H]-nicotine binding to rat cerebral cortex membranes. Displaceable binding was defined by the addition of excess non-radioactive (−)-nicotine. The vertical axis represents the mean (± s.e.mean) displaceable binding, calculated as a percentage of control (i.e. with no inhibitor present) ($n = 12$–16 wells).

K^+ 12 mM and (+)-amphetamine 0.3 μM (Table 1). The short test pulse of nomifensine did not increase [^{3}H]-dopamine release when administered alone and did not alter potassium-evoked release (nomifensine main effect: $F = 1.21$, d.f. 3, 24, $P > 0.3$). In contrast, amphetamine-induced release was attenuated in a concentration-dependent manner, with total block occurring at nomifensine 10 μM.

This concentration of nomifensine was then tested against MPTP 50 μM, MPP^+ 10 μM, (+)-amphetamine 0.3 μM, and K^+ 12 mM (Table 1). Nomifensine completely blocked MPP^+-induced release and attenuated the effect of MPTP. As before, the amphetamine effect was blocked and the K^+ effect was unchanged.

MK-801 (10 and 100 μM) was tested in parallel against (+)-amphetamine 0.3 μM, MPTP 50 μM and MPP^+ 10 μM. At 10 μM, MK-801 significantly reduced the response to MPP^+, but did not antagonize MPTP or (+)-amphetamine (Table 2). At 100 μM, MK-801 reduced responses to all three of these drugs (Table 2).

Experiment 7: Concentration-dependent inhibition of synaptosomal [^{3}H]-dopamine uptake by MK-801

In the absence of inhibitor, the mean uptake of [^{3}H]-dopamine by the synaptosomal preparation in the two assays was 52,000 d.p.m. mg^{-1} original tissue per well. Uptake was reduced by 97% by the addition of excess nomifensine (100 μM). Specific (i.e. nomifensine-sensitive) uptake was expressed as a percentage of the control condition (no inhibitor). Data normalized in this way were very similar between the two assays, and were therefore pooled. MK-801 (1–1000 μM) inhibited [^{3}H]-dopamine uptake in a concentration-dependent manner (Figure 7). A significant effect was first observed at 32 μM ($P < 0.01$), and the IC_{50} was 115 μM.

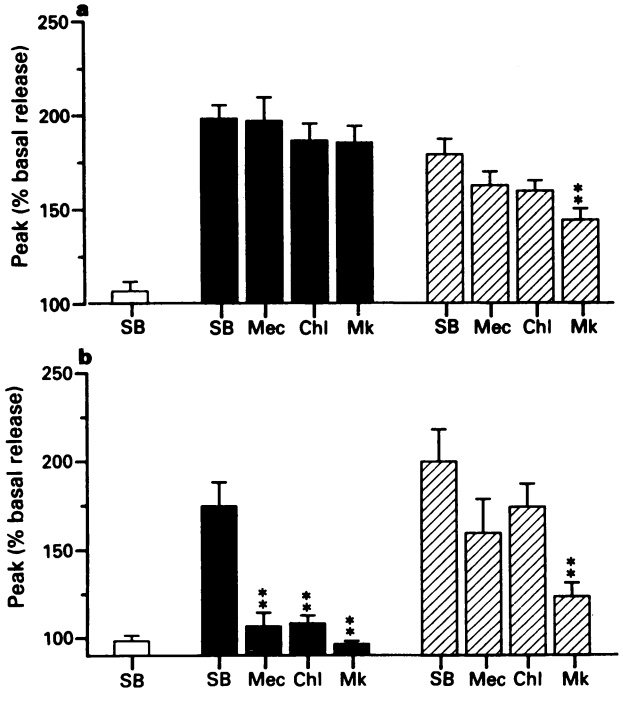

Figure 6 Effects of nicotinic antagonists on [^{3}H]-dopamine release evoked by MPTP or MPP^+. Following 35 min of superfusion with antagonist (100 μM mecamylamine (Mec), chlorisondamine (Chl), or MK-801 (MK)) or superfusion buffer alone (SB), synaptosomes were challenged with: (a) K^+ (solid columns) or MPTP 50 μM (hatched columns), or (b) nicotine 1 μM (solid columns) or MPP^+ 10 μM (hatched columns). Control channels (open columns) were superfused and challenged with SB only. The vertical axis represents the mean (± s.e.mean) peak release, calculated as a percentage of basal release ($n = 6$–15). $^{**}P < 0.01$–0.001 vs. agonist alone (MPTP or MPP^+; Dunnett's test).

Discussion

Acute administration of MPTP and MPP^+ induces striatal dopamine release both *in vivo* and from tissue slices *in vitro* (e.g. Markstein & Lahaye, 1984; Schmidt *et al.*, 1984; Pileblad *et al.*, 1984; Pileblad *et al.*, 1985; Snape *et al.*, 1988; Rollema *et al.*, 1988; Chan *et al.*, 1993). Previously, it was shown that MPTP-induced dopamine efflux from striatal slices was tetrodotoxin-insensitive (Schmidt *et al.*, 1984), but

Table 1 Drug-evoked [^{3}H]-dopamine release in the presence of the dopamine uptake blocker, nomifensine (Experiment 6)

	SB	*K^+*	*AMPH*	*MPTP*	*MPP^+*
Nomifensine					
0 μM	103 ± 2	160 ± 4	208 ± 4		
0.1 μM	103 ± 3	169 ± 10	197 ± 25		
1.0 μM	102 ± 2	151 ± 5	*158 ± 20		
10 μM	103 ± 4	161 ± 6	**109 ± 3		
0 μM	103 ± 3	148 ± 7	194 ± 4	151 ± 6	196 ± 8
10 μM	98 ± 1	160 ± 10	**103 ± 5	**118 ± 4	**105 ± 5

Drug challenge comprised a pulse of superfusion buffer (SB), K^+ 12 mM, (+)-amphetamine 0.3 μM, MPTP 50 μM, or MPP^+ 10 μM.
Evoked dopamine release is expressed as the peak effect, as a percentage of basal release. $^*P < 0.02$, $^{**}P < 0.005$ vs. drug challenge in absence of nomifensine ($n = 5$–10; Student's t test with Bonferroni's correction).

Table 2 Drug-evoked [^{3}H]-dopamine release in the presence of MK-801 (Experiment 6)

	SB	*AMPH*	*MPTP*	*MPP^+*
MK-801				
0 μM	99 ± 3	203 ± 14	164 ± 5	262 ± 6
10 μM		222 ± 12	163 ± 4	**205 ± 11
0 μM	101 ± 3	212 ± 11	155 ± 5	200 ± 9
100 μM		**159 ± 6	*132 ± 5	*158 ± 9

Drug challenge comprised a pulse of superfusion buffer (SB), (+)-amphetamine 0.3 μM, MPTP 50 μM, or MPP^+ 10 μM. Evoked dopamine release is expressed as the peak effect, as a percentage of basal release. $^*P < 0.05$, $^{**}P < 0.005$ vs. drug challenge in absence of MK-801 ($n = 7$–11; Student's t test with Bonferroni's correction).

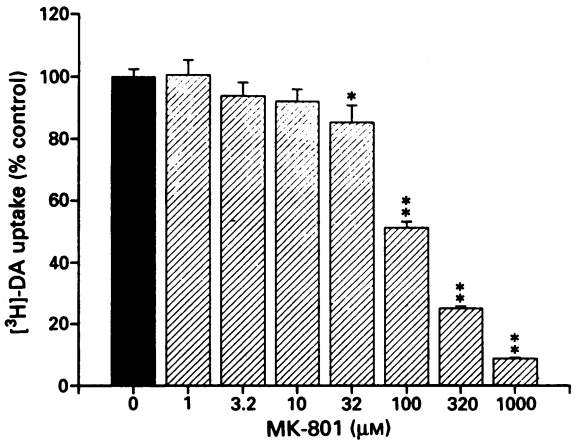

Figure 7 Inhibition of synaptosomal [^{3}H]-dopamine uptake by MK-801. Specific uptake of [^{3}H]-dopamine was defined by the addition of an excess of the selective dopamine uptake blocker, nomifensine (100 μM). The vertical axis represents the mean (± s.e.mean) specific uptake, calculated as a percentage of control (i.e. with no inhibitor present) ($n = 16$ wells). $^*P < 0.01$, $^{**}P < 0.0001$ vs. control (Dunnett's test).

such an approach cannot exclude indirect excitatory actions mediated trans-synaptically by diffusion of locally-released substances. The present study represents the first reported use of superfused synaptosomes to study this phenomenon, and provides perhaps the clearest indication to date that MPTP and MPP^+ can act directly on the dopaminergic terminals to promote dopamine release. Frequent sampling was employed, revealing a dopamine-releasing action that was very rapid, with a latency comparable to the dopamine-releasing actions of nicotine, high K^+, and amphetamine. Such a rapid action is unlikely to be due to the inhibition of mitochondrial respiration and depletion of intracellular ATP levels, a process which tends to occur more gradually after MPP^+ administration (Scotcher *et al.*, 1990; Chan *et al.*, 1991). In all of the present experiments, the monoamine oxidase inhibitor, pargyline, was included in the superfusion buffer, and thus the acute effect of MPTP did not depend on conversion to MPP^+. This observation serves to emphasize that the acute dopamine-releasing actions of MPTP and MPP^+ as studied here may not necessarily contribute to neurotoxicity.

Blockade of NMDA-type glutamate receptors

MK-801 inhibited the dopamine-releasing actions of both MPTP and MPP^+, but only at concentrations higher than those required to block NMDA receptors (Huettner & Bean, 1988; Willis *et al.*, 1991; Wamil & McLean, 1992). Additional tests of NMDA receptor involvement proved negative (Experiment 3). First, high concentrations of the potent NMDA receptor blockers AP-7 and kynurenic acid (Wong & Kemp, 1991) failed to inhibit responses to MPTP and MPP^+. Second, administration of NMDA, even at high concentrations and under conditions favouring NMDA receptor activation, did not release detectable amounts of [^{3}H]-dopamine. Third, MPTP- and MPP^+-induced [^{3}H]-dopamine release was not affected by removal of calcium or magnesium from the superfusion buffer; NMDA-induced dopamine release, where previously obtained from striatal synaptosomes, has been found to be calcium-dependent and greatly enhanced by omission of Mg^{2+} (Wang, 1991; Krebs *et al.*, 1991; Desce *et al.*, 1992).

Our inability to obtain NMDA-induced dopamine release accords with other negative reports in the literature (De Belleroche & Bradford, 1980; Carter *et al.*, 1988), but stands in contrast to the majority of published studies which are consistent with a direct releasing action of NMDA on striatal dopaminergic terminals (Snell & Johnson, 1986; Clow & Jhamandas, 1989; Wang, 1991; Krebs *et al.*, 1991; Johnson & Jeng, 1991; Desce *et al.*, 1992). Most of these positive reports were obtained with slowly-perfused synaptosomes (Krebs *et al.*, 1991; Desce *et al.*, 1992) or tissue slices treated with tetrodotoxin (Snell & Johnson, 1986; Clow & Jhamandas, 1989), in which dopamine release could conceivably be triggered indirectly via the release of some other mediator. However, this consideration does not apply to the studies of Wang (1991) and of Johnson & Jeng (1991), where NMDA-induced [^{3}H]-dopamine release was obtained from striatal synaptosomes with rapid perfusion.

Actions at nicotinic receptors

MK-801 antagonizes CNS nicotinic receptors in addition to NMDA receptors (Ramoa *et al.*, 1990; confirmed in Experiment 5). The possibility was therefore considered that MPTP and MPP^+ act at nicotinic receptors. In rat brain, a prominent population of nicotinic acetylcholine receptors is labelled with high affinity by [^{3}H]-nicotine (Clarke, 1987). Both MPTP and MPP^+ inhibited [^{3}H]-nicotine binding to rat brain membranes (Experiment 4), albeit considerably less potently than other known nicotinic agonists (Wonnacott, 1987). Nevertheless, the nicotinic receptor antagonists, mecamylamine and chlorisondamine, failed to reduce release evoked by MPTP and MPP^+ (Experiment 5), even when these antagonists were tested at concentrations more than sufficient to block nicotine-induced dopamine release (El-Bizri & Clarke, 1994b). Thus, although MPTP and MPP^+ appear to interact with brain nicotinic receptors, appreciable agonist activity was not detected at the concentrations tested.

Actions at the cell membrane dopamine transporter

Two principal mechanisms of drug-induced transmitter release have been proposed (Levi & Raiteri, 1993). The first, exemplified by depolarizing agents such as high K^+, is dependent upon external calcium but insensitive to uptake inhibitors (Raiteri *et al.*, 1979; also Experiment 6), and represents exocytotic secretion. The second mechanism, exemplified by (+)-amphetamine, has the reverse characteristics (Raiteri *et al.*, 1979; also Experiment 7), and reflects the efflux of cytosolic transmitter via its transporter. In this regard, dopamine release evoked by MPTP and MPP^+ bore at least a superficial resemblance to amphetamine: not only did the evoked release occur independently of external calcium (Experiment 3), but it was greatly reduced by nomifensine (Experiment 6) in a concentration that blocked [^{3}H]-dopamine uptake (Experiment 7). Similar results have been obtained previously in brain slices and whole animals, both in terms of calcium independence (Schmidt *et al.*, 1984; Sirinathsinghji *et al.*, 1988; Snape *et al.*, 1988; Sirinathsinghji *et al.*, 1988; Wilson *et al.*, 1991) and blockade by nomifensine (Markstein & Lahaye, 1984). However, since MPP^+ normally enters dopaminergic terminals via the dopamine transporter (Javitch *et al.*, 1985), the present observations imply either that MPP^+ acted intracellularly to release [^{3}H]-dopamine via a non-exocytotic mechanism, or that the drug acted extracellularly or intracellularly to release cytosolic dopamine. In contrast to MPP^+, MPTP-evoked dopamine release was not completely blocked by nomifensine (Experiment 6), indicating an additional, as yet unidentified mechanism of action.

The similar profile of action seen with nomifensine and MK-801 (Experiments 5 and 6) suggested that the latter might be acting at the cell membrane dopamine transporter to attenuate dopamine release evoked by MPTP and MPP^+. The results of the final experiment support this notion: MK-801 inhibited [^{3}H]-dopamine accumulation with an IC_{50} of 115 μM. As we subsequently discovered, a similar result has been reported previously but virtually ignored (Snell *et al.*, 1988). It is likely that this action of MK-801 reflects decreased dopamine influx, since in our experiments, the drug did not consistently increase basal [^{3}H]-dopamine release.

Can previous reports of protection by MK-801 be explained by block of the dopamine transporter?

Destruction of dopaminergic neurones following the administration of MPTP or MPP^+ can be completely prevented by the administration of dopamine uptake inhibitors (Ricaurte *et al.*, 1985). The finding that MK-801 inhibits the dopamine transporter therefore prompts a reconsideration of the literature on MPTP toxicity. Protection by MK-801, where obtained, has usually been associated with the administration of high doses. Thus, in the first such report (Turski *et al.*, 1991), MK-801 was co-infused with MPP^+ into the substantia nigra in a concentration of 0.25 M; transient protection also occurred after systemic administration of 3.4 mg kg^{-1}. Other studies have employed repeated systemic administration of MK-801 to rats, with mixed results. Thus, full protection against the dopamine depleting effects of intra-striatal or intra-nigral MPP^+ injection was reported after repeated administration of 5 mg kg^{-1} (6 or 12 q 4 h) (Storey *et al.*, 1992; Srivastava *et al.*, 1993), but somewhat lower doses (2.2 or 3.4 mg kg^{-1}) were ineffective (Sonsalla *et al.*, 1992; Santiago *et al.*, 1992).

Brain levels of MK-801 peak at around 10 μM and decline with a half-life of about 2 h in the rat, following a single injection of 5 mg kg^{-1}, i.p. (Vezzani *et al.*, 1989). Thus, protection against MPP^+ toxicity appears to require sustained brain concentrations of approximately 3–10 μM, a concentration-range at least one order of magnitude higher than that required to produce insurmountable, use-dependent blockade of NMDA receptors (Huettner & Bean, 1988; Willis *et al.*, 1991; Wamil & McLean, 1992). Such concentrations of MK-801 produced little inhibition of dopamine uptake (IC_{50} 115 μM) *in vitro*. Whether the drug is more potent *in vivo* or with sustained application is not known, but is a question that should not be ignored in studies where high doses of the drug are given.

If MK-801 should inhibit the cell membrane dopamine transporter *in vivo*, it would reduce MPP^+ uptake and also reduce release of cytosolic dopamine. The latter action could also be relevant to methamphetamine toxicity, which is inhibited by MK-801 by a mechanism that remains to be fully understood (Marshall *et al.*, 1993). Since it appears that the massive methamphetamine-induced release of cytosolic dopamine plays a causal role in the subsequent degeneration of dopaminergic nerve terminals (Weihmuller *et al.*, 1992; Marshall *et al.*, 1993), it will be of interest to examine the possibility that MK-801 inhibits the dopamine transporter *in vivo*.

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