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An *in vitro* model of 1-methyl-4-phenyl-pyridinium (MPP+) toxicity: incubation of rabbit caudate nucleus slices with MPP+ followed by biochemical and functional analysis

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1 Slices of rabbit caudate nucleus were preincubated for up to 24 h *in vitro* in the presence of the neurotoxic compound 1-methyl-4-phenyl-pyridinium (MPP+). Subsequently the levels of endogenous monoamines in the slices were determined by h.p.l.c. with electrochemical detection. MPP+, in concentrations higher than 32 nM significantly diminished the dopamine levels within the slices in a concentration- and time-dependent manner; at 32 μM the depletion was more than 95%. The concentration of the major metabolite of dopamine, dihydroxyphenyl acetic acid (DOPAC) was decreased at concentrations of MPP+ that did not alter dopamine levels. Thus, MPP+ increased the dopamine/DOPAC ratio.

2 In contrast, both 5-hydroxytryptamine (5-HT) levels and 5-HT/5-hydroxyindolacetic acid (5-HIAA) ratios were increased at nanomolar concentrations of MPP+. 5-HT was significantly reduced only at 32 μM.

3 The dopamine uptake inhibitor nomifensine reduced the depletory effect of MPP+ on dopamine and DOPAC content.

4 Following 24 h pretreatment with MPP+, the uptake of [^{3}H]-dopamine into rabbit caudate nucleus slices was either enhanced (at 0.32 μM, 1 μM and 3.2 μM MPP+) or reduced (at 32 μM MPP+).

5 Preincubation of slices with 10 μM MPP+ for only 1 h increased their ^{3}H-labelling (in contrast to 24 h pretreatment) whereas after 9 h no net increase was detectable. After 1 and 9 h MPP+ pretreatment, much less deaminated metabolites of [^{3}H]-dopamine were found in the incubation medium of MPP+ treated slices than in the medium of control slices. These findings suggest that MPP+ strongly inhibits the enzyme monoamine oxidase (MAO) within dopaminergic (and 5-hydroxytryptaminergic) terminals before destroying them.

6 To validate the proposed *in vitro* model functionally, the electrically evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh) was investigated in MPP+ treated slices and controls. MPP+ reduced both the facilitatory effect of the D_2-receptor antagonist domperidone and the inhibitory effect of the catecholamine uptake inhibitor nomifensine on [^{3}H]-ACh release; effects compatible with a diminished inhibitory dopaminergic input on cholinergic neurones.

7 These findings also show that the terminal region of dopaminergic neurones, the caudate nucleus, is a site for MPP+ toxicity. The present *in vitro* model may be useful for investigating the effects of MPP+ and its interaction with other drugs under defined conditions.

Introduction

The sequence of events leading from administration of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to selective destruction of nigral dopaminergic neurones in primates has been suggested to involve the following steps: MPTP is converted to the neurotoxic compound 1-methyl-4-phenyl-pyridinium (MPP+) in glial cells or 5-hydroxytryptaminergic terminals that contain monoamine

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oxidase B (MAO-B) and closely surround the dopaminergic neurones of the substantia nigra and the caudate nucleus. Following release from the MAO-B containing cells, MPP+ is accumulated via the dopamine uptake pump into and concentrated within dopaminergic neurones (Snyder & D'Amato, 1986). In favour of the role of the substantia nigra in the pathogenesis it was assumed that within pigmented cell bodies of nigral neurones MPP+ was bound to neuromelanin and gradually released in a depot-like fashion, maintaining a toxic intracellular concentration of MPP+. However, since monoamine uptake is more prominent in nerve endings than in cell bodies (Snyder & D'Amato, 1986), the dopaminergic terminal field in the striatum rather than the cell bodies of the substantia nigra could be a primary target for MPP+ toxicity.

In the present investigation we studied the effects of long term incubation with MPP+ in caudate tissue of the rabbit *in vitro* in order to establish clear concentration-effect relationships and to provide a more accessible and less expensive experimental model than that of MPTP-induced Parkinsonism in primates *in vivo*. Acute dopamine releasing effects of MPTP and of MPP+ in rat CNS tissue have already been described (Schmidt *et al.*, 1984; Markstein & Lahaye, 1985; Rollema *et al.*, 1986a). However, acute effects may not reflect adequately the toxic effects of MPP+ on the dopaminergic system *in vivo* since, ultimately, the destruction of dopaminergic cells leads to MPTP-induced Parkinsonism.

The analysis of subacute effects of MPTP or MPP+ on cultured neuronal cells (Mytilineou & Cohen, 1984) also represents an incomplete model of MPP+ toxicity, since postsynaptic effects of the impaired dopaminergic neurotransmission cannot be investigated in cell cultures. In the striatum, the dopaminergic system is known to inhibit cholinergic transmission. If the present model of MPP+ toxicity is valid functionally and reflects an impaired function of dopaminergic neurotransmission, reduced postsynaptic effects on [^{3}H]-acetylcholine ([^{3}H]-ACh) release in the caudate nucleus should be evident (Hertting *et al.*, 1980).

Some of the present results were presented at the seventh European Winter Conference on Brain Research, Val Thorens, France, March 1987.

Methods

Rabbits of either sex weighing 1.7–2.5 kg were decapitated. The brain was quickly removed and the caudate nuclei bluntly detached from their surroundings. Slices of the head of the caudate nucleus (0.3 mm thick, 3–4 mm diameter, 1.5–2.5 mg wet weight) were prepared using a tissue chopper. These slices were transferred to dishes and treated with different concentrations of MPP+, dissolved in 10 ml of a modified Krebs-Henseleit buffer, for different time intervals up to 24 h at 37°C. During this incubation they were supplied superficially with 5% CO_2/95% O_2 within a closed incubation chamber rotating slowly. The composition of the incubation medium was (mmol^{-1}): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03.

Endogenous monoamines and deaminated metabolites

After preincubation with or without MPP+, about 10 to 20 slices, i.e. the content of one of 6–10 dishes, were rinsed, weighed and prepared for determination of endogenous monoamines and metabolites (i.e. dopamine and its major metabolite dihydroxyphenyl acetic acid (DOPAC), noradrenaline (NA) and 5-hydroxytryptamine (5-HT) and its deaminated metabolite 5-hydroxyindolacetic acid (5-HIAA)) by high performance liquid chromatography (h.p.l.c.) with electrochemical detection (Starke *et al.*, 1981). The interaction between MPP+ and other substances was evaluated by estimation of endogenous monoamines after co-incubation of slices with the drugs and MPP+.

Accumulation of [^{3}H]-dopamine and tritiated metabolites

Slices were pretreated with or without MPP+ at different concentrations up to 24 h and then incubated for 30 min in the presence of [^{3}H]-dopamine (0.1 μM, 27 Ci mmol^{-1}) in a volume of 4 ml. After labelling, the supernatant was analysed for the unmetabolized transmitter and deaminated metabolites. Subsequently, a 30 min period of discontinuous washing was applied: after 0, 10 and 20 min the slices were washed once with medium followed by re-addition of 10 ml fresh medium. At the end of the washing period each slice was dissolved in 0.5 ml Soluene-350 (Packard Instruments, Frankfurt, FRG) for tritium determination (see Feuerstein *et al.*, 1986).

Separation of [^{3}H]-dopamine from its deaminated metabolites in supernatant samples was performed using the separation method described by Steppeler *et al.* (1982). The recovery from standard samples of [^{3}H]-dopamine was about 90% (Feuerstein *et al.*, 1986).

Superfusion experiments

Rabbit caudate nucleus slices with or without MPP+ (10 μM) pretreatment for 1 h were incubated

with [^{3}H]-choline, superfused and electrically stimulated. The electrically evoked overflow of tritium following preincubation with [^{3}H]-choline reflects release of [^{3}H]-ACh (Hertting *et al.*, 1980). Briefly, following pretreatment, the slices were incubated for 30 min in the presence of [^{3}H]-choline (0.1 μM, 80 Ci mmol^{-1}). They were then rinsed, transferred to glass superfusion chambers, superfused with medium (prewarmed to 37°C and saturated with 5% CO_2/95% O_2, pH 7.4) at a rate of 1 ml min^{-1}. After 30 min of continuous washing the superfusate was collected in 5 min samples for tritium determination by liquid scintillation counting.

During superfusion the slices were electrically stimulated twice (for 2 min each; rectangular unipolar pulses 2 ms, 3 Hz; 5 V cm^{-1}, 24 mA) after 45 min (S_1) and 75 min (S_2). Drugs to be tested were added to the medium from 15 min before S_2 onwards. None of them affected basal tritium outflow. When domperidone was added before S_2, nomifensine (1 μM) was present in the medium throughout the superfusion. Controls without drugs added before S_2 were always run in parallel with drug experiments. At the end, the slices were removed from the chambers and dissolved in Soluene-350 for tritium determination.

Evaluation and statistics

In superfusion experiments, the fractional rate of tritium outflow 5 min^{-1} and the stimulation evoked overflow of tritium are expressed as % of the tritium content of the tissue at the onset of the respective stimulation period. Drug effects were evaluated by calculating the ratio (S_2/S_1) of the evoked overflow in the two stimulation periods (Feuerstein *et al.*, 1986).

All results (endogenous monoamines and metabolites, tritium contents of accumulation experiments, and S_2/S_1 ratios) are expressed as mean ± s.e.mean. The existence of differences between the means of treatments and their corresponding controls or between treatment values was tested with a one-way analysis of variance; the preconditions of this analysis of variance were proved using Bartlett's test with a minimum level of significance of 10% to refute the null hypothesis (homogeneity of variances, goodness-of-fit for normal distribution). Student's *t* test (two-tailed) was used subsequently to determine the significance of differences between the groups of treatments, employing the error mean square of the analysis of variance to an estimate of the standard deviation of all means. When results are expressed as % of corresponding controls, the law of error propagation was respected for calculation of s.e.means.

Drugs used

[Methyl-^{3}H]-choline and [7-^{3}H(N)]-dopamine were obtained from NEN, Dreieich, F.R.G. The following drugs were kindly provided or acquired as follows: 1-methyl-4-phenyl-pyridinium iodide (MPP+; ANAWA, Wangen, Switzerland); nomifensine hydrogen maleate (Hoechst, Frankfurt, F.R.G.); domperidone (Janssen, Beerse, Belgium). All other chemicals were of analytical grade from commercial sources.

Stock solutions of drugs were freshly prepared in water, 0.001 M HCl or medium for each experiment with the exception of domperidone which was dissolved in 10 mM citric acid.

Results

Endogenous monoamines and metabolites

Figures 1 and 2 illustrate the effects of preincubation of rabbit caudate nucleus slices with MPP+ at different concentrations for 24 h on the levels of endogenous dopamine, 5-HT and their deaminated metabolites. The monoamine content of control slices after 24 h incubation was not significantly different from that of 'fresh' slices (Table 1). MPP+ (3.2–320 nM) failed to change the dopamine level of caudate nucleus slices whereas the dopamine/DOPAC ratio was significantly enhanced. (At micromolar concentrations of MPP+, it was no longer possible to calculate reliable dopamine/DOPAC ratios, since DOPAC values were often below the detection limit of the method). MPP+ (0.1–32 μM) diminished the dopamine content of the slices in a concentration-dependent manner (Figure 1).

In contrast to the marked effects of MPP+ on the levels of dopamine, the concentrations of NA in caudate nucleus slices were not affected over the whole concentration range applied (Table 1). However, MPP+ (10 μM) diminished the NA content of hippocampal slices pretreated analogously for 24 h from 245.7 ± 3.7 ng g^{-1}; $n = 8$, to 11.2 ± 0.7 ng g^{-1}; $n = 8$ ($P < 0.001$). 5-HT levels were changed in a biphasic manner: they were enhanced at concentrations of 3.2–320 nM, but significantly reduced at the highest MPP+ concentration of 32 μM (Figure 2). Furthermore, MPP+ (3.2–320 nM) significantly increased the ratio of 5-HT/5-HIAA. Treatment with 1 μM MPP+, however, diminished this ratio (Figure 2, insert). As observed with the dopamine/DOPAC ratio, at higher micromolar concentrations the effect of MPP+ on the 5-HT/5-HIAA ratio was not measurable since 5-HIAA values were often below the detection limit of the method.

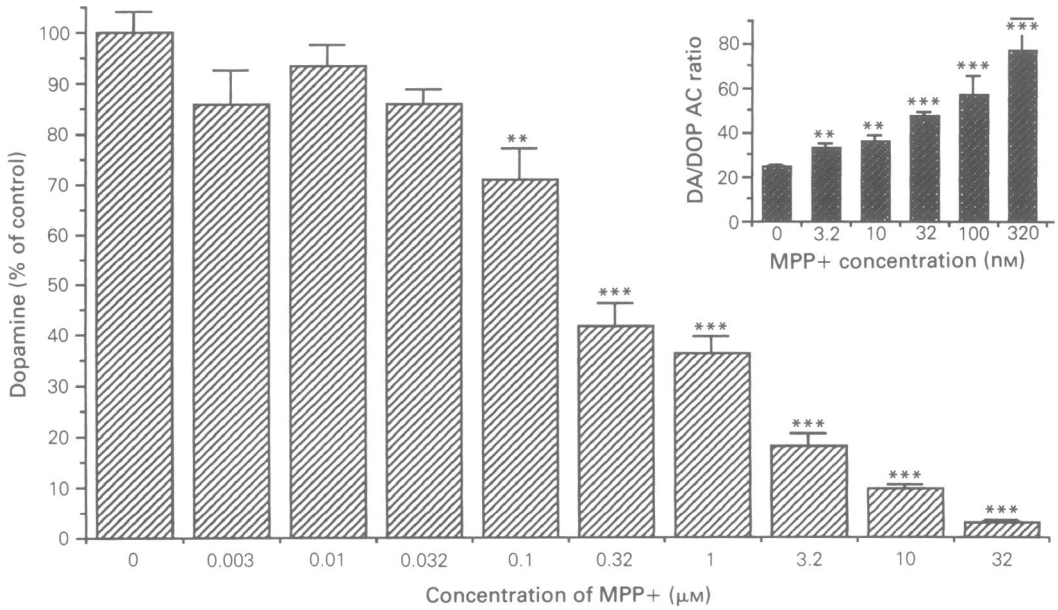

Figure 1 Effects of 1-methyl-4-phenyl-pyridinium (MPP+) on the endogenous content of dopamine (DA) and dihydroxyphenyl acetic acid (DOPAC) in caudate nucleus slices of the rabbit. The slices were incubated for 24 h in a modified Krebs-Henseleit buffer containing various concentrations of MPP+ and supplied with oxygen. Endogenous dopamine and DOPAC levels within the slices were determined by h.p.l.c. with electrochemical detection. Each column represents the mean and vertical bars s.e.mean of dopamine values ($ng\,g^{-1}$), expressed as % of corresponding controls incubated without MPP+ ($n = 7$–14 for each group). The insert shows the dopamine/DOPAC ratios at nanomolar concentrations of MPP+, all of which differed significantly from the respective control ratio, in contrast to the effect on dopamine levels within this concentration range. $^{**}P < 0.01$; $^{***}P < 0.001$; significantly different from control.

Figure 3 illustrates the effect of the application interval of MPP+ on endogenous dopamine levels. MPP+ (1 or 10 μM) was either absent or present throughout the respective time intervals. Subsequently, all slices were further incubated up to a total incubation time of 24 h. When present for 1 h MPP+ (10 μM) strongly diminished the levels of dopamine measured after further incubation of the slices in the absence of drug. The dopamine content determined immediately after 1 h MPP+ (10 μM) treatment without further incubation was $1853 \pm 139\,ng\,g^{-1}$; $n = 8$ (compare to the 1 h value of Figure 3). Apart from the information about the time-dependency of the effects of MPP+, the experimental approach of Figure 3 was used to determine the concentration of the neurotoxin that, present only for the first hour of 24 h, markedly diminished the dopamine content of caudate nucleus slices mea-

Table 1 Endogenous monoamine content of rabbit caudate nucleus slices before incubation and after 24 h incubation in the absence and presence of 1-methyl-4-phenyl-pyridinium (MPP+; 32 μM)

Period of incubation (h)	*Dopamine* ($ng\,g^{-1}$)	*5-HT* ($ng\,g^{-1}$)	*NA* ($ng\,g^{-1}$)
0	6710 ± 450, n = 7	229 ± 41, n = 12	65 ± 9, n = 11
24 (without MPP+)	6990 ± 375, n = 15	307 ± 36, n = 15	53 ± 11, n = 8
24 (with 32 μM MPP+)	163 ± 32, n = 8	18 ± 4, n = 8	68 ± 10, n = 8

The slices were prepared for determination of endogenous monoamines without preincubation, after incubation in medium for 24 h, or after incubation in medium containing 32 μM MPP+ for 24 h.

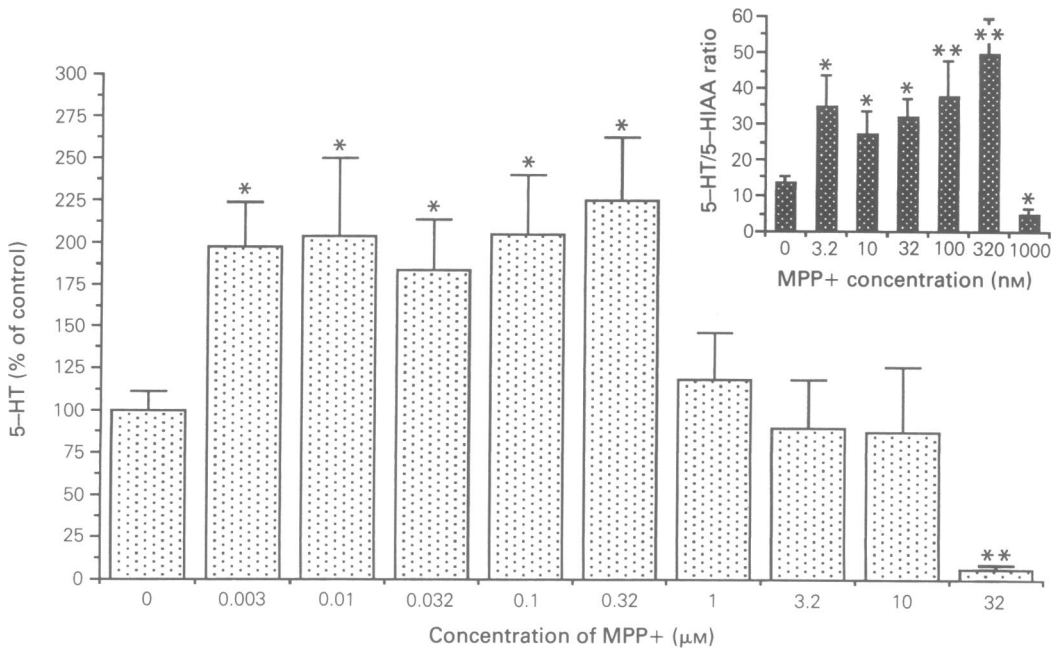

Figure 2 Effects of 1-methyl-4-phenyl-pyridinium (MPP+) on the endogenous 5-hydroxytryptamine (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) content of caudate nucleus slices of the rabbit. The slices were incubated for 24 h in a modified Krebs-Henseleit buffer containing various concentrations of MPP+ and supplied with oxygen. 5-HT and 5-HIAA levels within the slices were determined by h.p.l.c. with electrochemical detection. Each column represents the mean and vertical bars s.e.mean of 5-HT values ($ng\,g^{-1}$), expressed as % of corresponding controls incubated without MPP+ ($n = 7$–14 for each group). The coefficient of variation of 5-HT measurements was considerably higher than that of dopamine determinations (compare s.e.mean bars in Figures 1 and 2). The insert shows the 5-HT/5-HIAA ratios at nanomolar concentrations of MPP+, all of which differed significantly from the respective control ratio. $^{*}P < 0.05$; $^{**}P < 0.01$; significantly different from control.

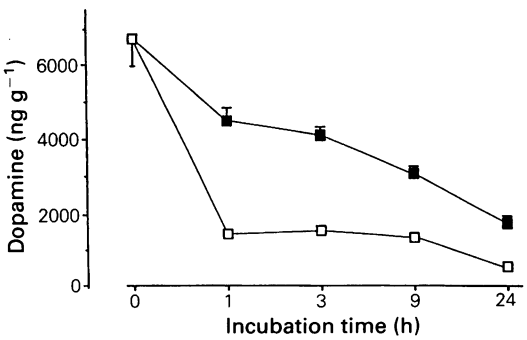

Figure 3 Effect of the application interval of 1-methyl-4-phenyl-pyridinium (MPP+) at 1 μM (■) and 10 μM (□) on the levels of endogenous dopamine in caudate nucleus slices. MPP+ was either absent (0 h) or present from the beginning and during the respective time interval of 1, 3, 9 and 24 h, as indicated. Subsequently all slices were further incubated up to a total incubation time of 24 h. Other conditions were the same as those described in the legend of Figure 1. All values significantly differed from the value at 0 h ($P < 0.01$).

sured after a further incubation of 23 h. Thus, it became possible to test the interactions of MPP+ with the catecholamine uptake inhibitor nomifensine.

When present for 24 h, nomifensine even at high concentrations inhibited, but did not completely abolish the uptake of substrates for the dopamine uptake pump (results not shown). Therefore, the interactions of nomifensine with MPP+ were studied as follows: nomifensine was present in the incubation medium for the whole incubation period of 24 h, whereas MPP+ (10 μM) was present only during the first hour of the 24 h incubation. The dopamine content of control slices incubated for 24 h with nomifensine (10 μM) was $6769 \pm 539\,ng\,g^{-1}$; $n = 8$, and did not differ from that of untreated slices ($6990 \pm 375\,ng\,g^{-1}$; $n = 15$). The reduction by MPP+ of the dopamine content in slices incubated in nomifensine-containing medium was compared with the effect of MPP+ alone, given for the first of 24 h. Nomifensine (10 μM) reduced the depletory effects of MPP+ on the dopamine and DOPAC

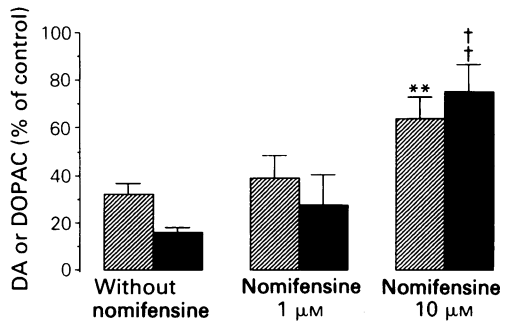

Figure 4 Effects of nomifensine on the depletory action of 1-methyl-4-phenyl-pyridinium (MPP+) on the concentrations of endogenous dopamine (DA; hatched columns) and dihydroxyphenyl acetic acid (DOPAC; solid columns) of caudate nucleus slices. Nomifensine (1 or 10 μM) was present in the incubation medium for the whole incubation period of 24 h, whereas MPP+ (10 μM) was present only during the first hour. Results are expressed as % of respective controls treated without MPP+. The reduction of the endogenous concentrations of dopamine and DOPAC by MPP+ of slices incubated in the presence of nomifensine at the concentrations indicated was compared to the effects of MPP+ alone. $^{**}P < 0.01$, comparison of dopamine contents; $\dagger\dagger P < 0.01$, comparison of DOPAC contents. Further conditions were similar to those described in the legend of Figure 1.

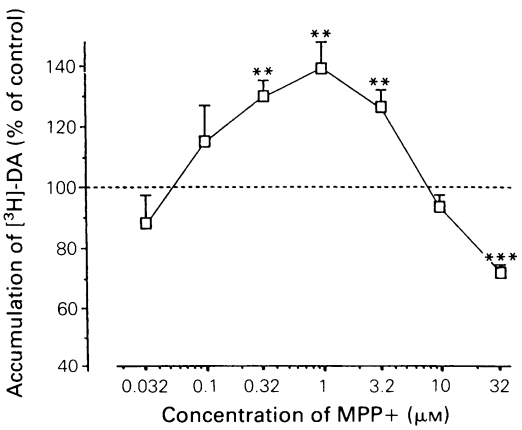

Figure 5 Effects of 1-methyl-4-phenyl-pyridinium (MPP+) pretreatment on the accumulation of [^{3}H]-dopamine (DA) in rabbit caudate nucleus slices preincubated for 24 h without or with various concentrations of MPP+. Following preincubation the slices were further incubated for 30 min in medium containing [^{3}H]-dopamine (0.1 μM). After discontinuous washing the tritium content of MPP+ pretreated slices was compared to that of corresponding control slices (n = 8–14 for each group). $^{**}P < 0.01$; $^{***}P < 0.001$; significantly different from 100% representing the ^{3}H-labelling of control slices.

content of caudate nucleus: it significantly increased dopamine and DOPAC levels as compared to controls treated with MPP+ in the absence of nomifensine (Figure 4).

[^2H]-dopamine accumulation and metabolism

MPP+ both enhanced and reduced [^{3}H]-dopamine accumulation in a concentration-dependent manner: MPP+ pretreatment for 24 h at low concentrations (0.1 μM, 0.32 μM, 1 μM and 3.2 μM) significantly enhanced [^{3}H]-dopamine accumulation in caudate nucleus slices. MPP+ pretreatment was ineffective at 10 μM, but at 32 μM it strongly reduced the accumulation of tritium (Figure 5). The accumulation of [^{3}H]-dopamine in fresh slices did not differ significantly from the nomifensine sensitive [^{3}H]-dopamine uptake of slices preincubated for 24 h in physiological buffer only (not shown). Deaminated ^{3}H-metabolites in the supernatant of slices pretreated with MPP+ for 1 or 9 h were reduced following preincubation with 10 μM MPP+ for both preincubation intervals compared to control slices (Figure 6a). After 1 h preincubation with 10 μM MPP+, the total tritium content in the medium was reduced

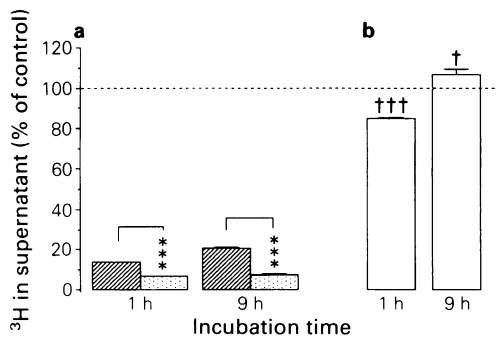

Figure 6 Effects of 1-methyl-4-phenyl-pyridinium (MPP+) pretreatment of rabbit caudate nucleus slices on the concentration of tritium compounds within the supernatant of these slices following incubation with [^{3}H]-dopamine. Slices were preincubated for 1 h or 9 h without or with MPP+ (10 μM). Subsequently the slices were further incubated for 30 min in medium containing [^{3}H]-dopamine (0.1 μM). After ^{3}H labelling of the slices, the tritium content of the supernatant of MPP+ pretreated slices was compared to that of untreated control slices. (a) Columns represent the mean of deaminated ^{3}H-metabolites (as % of the total tritium content of the supernatant) of control slices (hatched) and of slices pretreated with MPP+ (stippled). (n = 4 for each group). $^{***}P < 0.001$. (b) Open columns represent the total tritium content of the supernatant of MPP+ pretreated slices, as compared to the total tritium content of the supernatant of controls (100%) (n = 4 for each group). $\dagger P < 0.05$; $\dagger\dagger\dagger P < 0.001$. Vertical bars represent s.e.mean.

compared to the supernatant of control slices (Figure 6b). Consequently, the tritium content of MPP+ treated slices was significantly ($P < 0.01$) enhanced by about 17% (not shown). However, after 9 h pretreatment with 10 μM MPP+, more tritium was found in the supernatant of MPP+ treated slices than in the incubation medium of corresponding control slices (Figure 6b), whereas the ^{3}H-content of MPP+ treated slices or controls was no longer significantly different (not shown).

Evoked release of tritium from brain slices pretreated with or without MPP+ and then incubated with [^{3}H]-choline

In caudate nucleus slices preincubated for 1 h without MPP+, then incubated with [^{3}H]-choline, superfused and electrically stimulated twice, the dopamine reuptake inhibitor nomifensine markedly diminished, whereas the D_2-receptor antagonist domperidone greatly enhanced the evoked tritium overflow, which is assumed to represent the release of ACh (see Methods). Both the inhibitory effect of nomifensine and the facilitatory effect of domperidone were reduced, when the slices were pretreated for 1 h with MPP+ (10 μM; Figure 7).

Qualitatively similar results were obtained using caudate nucleus slices after 24 h pretreatment with or without MPP+ (10 μM; not shown). However, the pretreatment procedure of 24 h markedly reduced the evoked tritium overflow, expressed as % of tissue tritium, since, due to the preceding preincubation, a considerable amount of [^{3}H]-choline was incorporated into the membranes of the tissue preparation. Thus, the tissue tritium content no longer accurately represented the vesicular storage pool of [^{3}H]-ACh in cholinergic neurones.

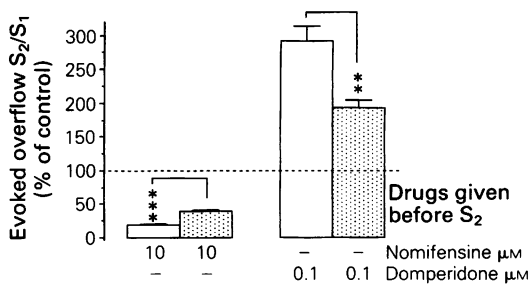

Figure 7 Effects of 1-methyl-4-phenyl-pyridinium (MPP+) pretreatment of caudate nucleus slices on the evoked overflow of tritium from slices incubated with [^{3}H]-choline, superfused and electrically stimulated twice (S_1, S_2). The slices were preincubated for 1 h in the absence (open columns) or presence of MPP+ (10 μM) (stippled columns). Nomifensine or domperidone were added to the superfusion medium 15 min before S_2. In the case of domperidone given before S_2, nomifensine (10 μM) was present throughout the superfusion in order to increase the endogenous tone of dopamine. Ordinate scale: ratio between the overflow of tritium evoked by S_2 and the overflow evoked by S_1 (S_2/S_1), expressed as % of corresponding control ratios, when no drug was added before S_2. The tritium overflow of S_1 (expressed as % of tissue tritium) in the absence of nomifensine during superfusion amounted to $4.93 \pm 0.35\%$ ($n = 9$) in slices pretreated in the absence of MPP+ and to $6.16 \pm 0.48\%$ ($n = 9$) in MPP+ pretreated slices (significantly different from each other, $P < 0.05$). In the presence of nomifensine throughout the superfusion the mean of the respective S_1 values of slices pretreated in the absence of MPP+ was $1.24 \pm 0.03\%$ ($n = 18$). The preincubation with MPP+ significantly ($P < 0.001$) increased the S_1 values obtained in the presence of nomifensine to $2.90 \pm 0.10\%$ ($n = 17$). Each column represents the mean and vertical bars indicate s.e.mean. ($n = 6$ for each group). $^{**}P < 0.01$; $^{***}P < 0.001$.

Discussion

The aim of the present investigation was to establish an easily accessible *in vitro* model for the neurotoxicity of MPP+. This model shows the following main features: (1) MPP+ selectively and potently affects the transmitter content of dopaminergic and much less of 5-hydroxytryptaminergic nerve terminals. (2) [^{3}H]-dopamine accumulation studies indicate that MPP+ pretreatment probably destroys dopaminergic nerve terminals. (3) The decrease in endogenous dopamine content can be demonstrated in the functional model of dopamine modulated striatal ACh release.

Several observations indicate that, even after a long preincubation period, the functional integrity of brain slices persists. Firstly, the similarity between the accumulation of [^{3}H]-dopamine in fresh and in long-term pretreated slices supports the assumption of an intact dopamine uptake pump which is known to be highly energy-dependent. Secondly, Allgaier *et al.* (1987) have shown that a preincubation period of 18 h did not abolish the release of NA from slices of the rabbit hippocampus. Our own results of the receptor modulated release of 5-HT from caudate nucleus slices preincubated from 20–24 h in physiological medium and then labelled with [^{3}H]-5-HT confirms the intact functional status of the action potential-induced exocytotic release of the transmitter (unpublished results).

Selectivity of effects of MPP+ on endogenous monoamines

The rather selective toxic effect of MPP+ on dopaminergic neurones represents the basis of the animal models of Parkinson's disease using MPTP *in vivo*

(Burns et al., 1983). In the present *in vitro* model MPP+ from 3.2 nM up to 32 μM markedly changed the dopaminergic and, in a different way, also the 5-hydroxytryptaminergic system. In contrast, endogenous NA of caudate nucleus slices remained unchanged whereas preincubation with MPP+ (10 μM) for 24 h strongly diminished the NA content of hippocampal slices. The rate of accumulation of MPP+ in preparations of synaptosomes containing predominantly noradrenergic terminals was shown to amount to only 10–20% of that in striatal synaptosomes (Chiba et al., 1985; Javitch et al., 1985). Thus, it may be assumed that MPP+ did not enter noradrenergic terminals in the caudate nucleus. An alternative explanation for the lack of effect on striatal NA levels is that a MAO inhibitory effect and an effect on the release of NA or the destruction of NA terminals compensate for each other. This does not seem to occur over the large concentration range of MPP+ studied. However, Namura et al. (1987) showed that MPP+, injected directly into the locus coeruleus, destroyed noradrenergic neurones. The reasons for the differences observed in the hippocampal region (NA rich) and in the caudate nucleus (NA poor) are currently being investigated in our laboratory. In the caudate nucleus at least, the effects of MPP+ seem to be restricted mainly to dopaminergic and to 5-hydroxytryptaminergic neurones.

At low nanomolar concentrations the neurotoxin MPP+ enhanced the dopamine/DOPAC ratio without changing the levels of dopamine, whereas at micromolar concentrations it reduced the content of endogenous dopamine in a concentration-dependent manner (Figure 1). These observations may be explained by two opposing effects of MPP+: MAO inhibition and enhanced dopamine release. At nanomolar concentrations, these opposite actions may cancel each other out, thus leaving the dopamine content unchanged. Also MPTP has been shown to induce dopamine release (Schmidt et al., 1984; Markstein & Lahaye, 1985), an effect which may be considered as an amphetamine-like action (Rollema et al., 1986b).

Inhibition of intraneuronal MAO by both the parent compound MPTP and by MPP+ has been described by Markstein & Lahaye (1985) and by Arai et al. (1986), respectively. With respect to the subtypes of the enzyme, it was shown that MPP+ potently and competitively inhibits MAO-A, which therefore does not significantly contribute to the conversion of MPTP to MPP+. The B form of the enzyme which appears to be only slightly inhibited by MPP+ (Fritz et al., 1985), plays the predominant role in the bioactivation of MPTP (Singer et al., 1987). However, in the light of the present findings, i.e. the increase of both endogenous 5-HT and the 5-HT/5-HIAA ratio by nanomolar concentrations of the neurotoxin (Figure 2), the ability of MPP+ to inhibit MAO in 5-hydroxytryptaminergic terminals may be of greater importance than suggested by Singer et al. (1987).

The present investigation shows that, at relatively high concentrations, MPP+ may also damage 5-hydroxytryptaminergic terminals. At such high concentrations of MPP+, its toxic properties appear to overcome the increase in 5-HT content produced by MAO inhibition. After being formed within 5-hydroxytryptaminergic terminals or glial cells, the neurotoxin MPP+ is thought to leave them (Snyder & D'Amato, 1986) and therefore may also re-enter 5-HT terminals. This view is further supported by the similarity between the 5-HT and dopamine uptake carrier (Feuerstein et al., 1986), since MPP+ is a substrate for the dopamine transporter (Snyder & D'Amato, 1986). Thus, MPP+, by product inhibition of the enzyme which catalyses its *in vivo* formation from MPTP, may increase endogenous 5-HT at low concentrations, whereas destruction of 5HT nerve endings may take place at higher concentrations. The *in vitro* finding of increased 5-HT levels following MPP+ is supported by the *in vivo* observation that this neurotoxin causes behavioural effects linked to 5-HT activation (Namura et al., 1987).

Since uptake of MPP+ into dopaminergic neurones precedes its neurotoxic action (see Introduction), the toxicity of MPP+ should be diminished by inhibiting its uptake into dopaminergic terminals with nomifensine. In fact, the present investigation also showed that the *in vitro* effects of MPP+ in caudate nucleus slices can be reduced by nomifensine. This observation underlines the possible importance of the MPP+ uptake occurring in the terminal field of the nigral dopaminergic neurone, the caudate nucleus, for the systemic toxicity of MPTP. Antidromic transport to and storage in the nigral cell bodies may occur before the loss of nigral cells.

Effects of MPP+ on [^{3}H]-dopamine accumulation

The efficiency of the dopamine uptake system in striatal slices can be regarded as a sensitive marker for the integrity and function of dopaminergic nerve terminals in this tissue. Therefore, the accumulation of [^{3}H]-dopamine within MPP+ pretreated slices was analysed in order to estimate the amount of surviving dopaminergic terminals following incubation. [^{3}H]-dopamine accumulation was enhanced at low concentrations of MPP+ (Figure 5). The most likely explanation for this increase in ^{3}H-content of striatal slices induced by low concentrations of MPP+ is again an inhibition of the enzyme MAO within dopaminergic terminals, as suggested above. Inhibition of MAO leads to increased intraterminal mono-

amine concentrations, which cannot cross the terminal membrane as easily as the deaminated metabolites: hence the loss of [^{3}H]-dopamine from the tissue during incubation and washout procedures is decreased. Therefore, the amount of [^{3}H]-dopamine accumulated as a quantitative index of the density of dopaminergic terminals after MPTP or MPP+ treatment (Mytilineous & Cohen, 1984) would be corrected for by the opposing effect which is due to MAO inhibition: The bell shaped curve of [^{3}H]-dopamine accumulation at increasing concentrations of MPP+ therefore reflects the sum of the decrease in the number of dopaminergic terminals and the increased [^{3}H]-dopamine retention following MAO inhibition.

The MAO inhibitory property of MPP+ is further supported by the reduction of deaminated ^{3}H-metabolites in the supernatant of slices incubated with [^{3}H]-dopamine subsequent to pretreatment with MPP+ (Figure 6a). MAO inhibition by MPP+ may also precede its destructive effects on nerve terminals, since 1 h of pretreatment with MPP+ reduced the total tritium content in the [^{3}H]-dopamine incubation medium, and, consequently, increased the tritium content of slices pretreated with MPP+, whereas the opposite was true after 9 h of pretreatment with MPP+ (Figure 6b). Thus, dependent on the concentration or the duration of action, a positive or negative net effect of MPP+ on [^{3}H]-dopamine accumulation in caudate nucleus slices was obtained.

Functional validation of the in vitro *model of MPP+ toxicity*

It is well known that cholinergic transmission in the striatum is strongly influenced by the firing of nigrostriatal dopaminergic neurones. The loss of these neurones underlies the overactivity of cholinergic striatal neurones in Parkinsonism. We have shown earlier that, in rabbit caudate nucleus slices, an increase in the endogenous tone of dopamine following inhibition of its reuptake by nomifensine potently reduced, whereas blockade of dopamine receptors on cholinergic neurones with neuroleptics enhanced ACh release (Hertting *et al.*, 1980).

The present observations show that in the MPP+ *in vitro* model, in close parallelism to the reduced dopaminergic input into the striatal cholinergic system in Parkinsonian patients, a disinhibition of cholinergic neurotransmission occurs. Both in the absence and presence of nomifensine throughout superfusion, the release of ACh evoked by electrical stimulation was significantly higher in slices pretreated with MPP+ (see S_1 values in the legend of Figure 7). Furthermore, MPP+ pretreatment diminished both the inhibitory effect of nomifensine and the facilitatory, i.e. disinhibitory, effect of the D_2-receptor antagonist domperidone on the electrically evoked [^{3}H]-ACh release (Figure 7). Thus, by reducing the dopaminergic input, MPP+ impaired the dopaminergic modulation of the cholinergic transmission within the caudate nucleus slices.

General conclusions

In summary, the present investigation shows that, besides the dopaminergic cell bodies in the substantia nigra, the terminal field of dopaminergic neurones within the caudate nucleus may be an additional target for MPP+ toxicity. It should be noted that the MAO inhibitory properties of MPP+ are detectable at low concentrations and presumably precede its neurotoxic effect on terminals. The functional relevance of the present *in vitro* model becomes evident from the impaired dopaminergic modulation of electrically evoked [^{3}H]-ACh release following MPP+ treatment. This *in vitro* effect closely corresponds to a disinhibited cholinergic function in Parkinsonian patients.

Thus, the present model allows the investigation of MPP+ toxicity under defined, reproducible conditions *in vitro*, for instance, the mode of action of MPP+-like compounds, the role of neuromelanin in MPP+ toxicity in the terminal field of nigral dopaminergic neurones and further cellular mechanisms of MPP+ toxicity. Some of these questions are already under investigation in our laboratory.

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References

ALLGAIER, C., HERTTING, G., HUANG, H.Y. & JACKISCH, R. (1987). Protein kinase C activation and α_2-autoreceptor-modulated release of noradrenaline. *Br. J. Pharmacol.*, **92**, 161–172.

ARAI, Y., KINEMUCHI, H., HAMAMICHI, N., SATHOH, N., TADANO, T. & KISARA, K. (1986). Inhibition of rat brain amino oxidase by some analogues of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and 1-methyl-4-phenyl pyridinium ion. *Neurosci. Lett.*, **66**, 43–48.

BURNS, R.S., CHIUEH, C.C., MARKEY, S.P., EBERT, M.H., JACOBOWITZ, D.M. & KOPIN, I.J. (1983). A primate model of parkinsonism: selective destruction of dopaminergic neurones in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 4546–4550.

CHIBA, K., TREVOR, A. & CASTAGNOLI, N. Jr. (1984). Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. *Biochem. Biophys. Res. Comm.*, **120**, 574–578.

CHIBA, K., TREVOR, A. & CASTAGNOLI, N. Jr. (1985). Active uptake of MPP+, a metabolite of MPTP, by brain synaptosomes. *Biochem. Biophys. Res. Comm.*, **128**, 1228–1232.

FEUERSTEIN, T.J., HERTTING, G., LUPP, A. & NEUFANG, B. (1986). False labelling of dopaminergic terminals in the rabbit caudate nucleus: uptake and release of [^{3}H]-5-hydroxytryptamine. *Br. J. Pharmacol.*, **88**, 677–684.

FRITZ, R.R., ABELL, C.W., PATEL, N.T., GESSNER, W. & BROSSI, A. (1985). Metabolism of the neurotoxin MPTP by human liver monoamine oxidase B. *FEBS Lett.*, **186**, 224–227.

HERTTING, G., ZUMSTEIN, A., JACKISCH, R., HOFFMANN, I. & STARKE, K. (1980). Modulation by endogenous dopamine of the release of acetylcholine in the caudate nucleus of the rabbit. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **315**, 111–117.

JAVITCH, J.A., D'AMATO, R.J., STRITTMATTER, S.M. & SNYDER, S.H. (1985). Parkinsonism inducing neurotoxin, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4-phenylpyridine by dopamine neurones explains selective toxicity. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 2173–2177.

MARKSTEIN, R. & LAHAYE, D. (1985). Neurochemical investigations in vitro with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in preparations of rat brain. *Eur. J. Pharmacol.*, **106**, 301–311.

MYTILINEOU, C. & COHEN, G. (1984). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine destroys dopamine neurones in explants of rat embryo mesencephalon. *Science*, **225**, 529–531.

NAMURA, I., DOUILLET, P., SUN, C.J., PERT, A., COHEN, R.M. & CHIUEH, C.C. (1987). MPP+ (1-methyl-4-phenylpyridine) is a neurotoxin to dopamine-, norepinephrine- and serotonin-containing neurones. *Eur. J. Pharmacol.*, **136**, 31–37.

ROLLEMA, H., DAMSMA, G., HORN, A.S., DE VRIES, J.B. & WESTERINK, B.H.C. (1986a). Brain dialysis in conscious rats reveals an instantaneous massive release of striatal dopamine in response to MPP+. *Eur. J. Pharmacol.*, **126**, 345–346.

ROLLEMA, H., DE VRIES, J.B., WESTERINK, B.H.C., VAN PUTTEN, F.M.S. & HORN, A.S. (1986b). Failure to detect 6-hydroxydopamine in rat striatum after the dopamine releasing drugs dexamphetamine, methylamphetamine and MPTP. *Eur. J. Pharmacol.*, **132**, 65–69.

SCHMIDT, C.J., MATSUDA, L.A. & GIBB, J.W. (1984). In vitro release of tritiated monoamines from rat CNS tissue by the neurotoxic compound 1-methyl-phenyl-tetrahydropyridine. *Eur. J. Pharmacol.*, **103**, 255–260.

SINGER, T.P., CASTAGNOLI, N., RAMSAY, R.R. & TREVOR, A.J. (1987). Biochemical events in the development of parkinsonism induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J. Neurochem.*, **1**, 1–8.

SNYDER, S.H. & D'AMATO, R.J. (1986). MPTP: a neurotoxin relevant to the pathophysiology of parkinson's disease. *Neurology*, **36**, 250–258.

STARKE, K., HEDLER, L. & STEPPELER, A. (1981). Metabolism of endogenous and exogenous noradrenaline in guinea pig atria. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **317**, 193–198.

STEPPELER, A., DÖRING, C., HEDLER, L. & STARKE, K. (1982). Effect of amezinium on the release and catabolism of ^{3}H-monoamines in brain slices. *Biochem. Pharmacol.*, **31**, 2395–2402.

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