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# Effects of 3-O-methyl dopa on L-dopa-facilitated synthesis and efflux of dopamine from rat striatal slices

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1 The effect of L-dopa on the spontaneous and KCl-evoked efflux of dopamine from rat striatal slices, measured by high performance liquid chromatography (h.p.l.c.) with electrochemical detection (e.c.d.) was investigated in the absence and presence of 3-O-methyl dopa (OMD), an O-methylated metabolite of L-dopa.

2 The addition of exogenous L-dopa (10 $\mu$M) significantly increased both the spontaneous efflux of dopamine and that evoked by KCl.

3 In the presence of 50 $\mu$M OMD, the effects of L-dopa on the spontaneous and KCl-evoked efflux of dopamine were smaller but only the former was significantly different from that in the absence of OMD. However, the total efflux of dopamine during the overall superfusion time (70 min) including KCl depolarization was significantly lower than in the absence of OMD.

4 Analysis of tissue content after superfusion revealed that the levels of dopa and dopamine in slices superfused with L-dopa in the presence of OMD were significantly higher than those superfused with L-dopa alone.

5 The finding that OMD significantly reduced the efflux of dopamine whilst increasing its concentration in striatal slices after L-dopa superfusion could explain the reduced efficacy seen after long-term therapy with L-dopa and a peripheral dopa decarboxylase inhibitor in Parkinsonian patients when plasma and brain OMD are very high.

**Keywords:** Dopamine efflux; L-dopa; 3-O-methyl dopa; rat striatal slices

## Introduction

In the treatment of Parkinson's disease, systemically administered L-3,4-dihydroxyphenylalanine (L-dopa) is routinely given with a peripheral dopa decarboxylase inhibitor (PDI), such as carbidopa or benserazide, to reduce the dose of L-dopa required as well as some of the side-effects (e.g. vomiting) that arise from the peripheral synthesis and actions of dopamine. One consequence of the use of a concomitant PDI is that L-dopa is then predominantly metabolized by catechol *O*-methyl transferase (COMT) to 3-methoxytyrosine (or 3-O-methyl dopa, OMD), which accumulates in the plasma of Parkinsonian patients on long-term L-dopa therapy (Fabbrini *et al.*, 1987; Tohgi *et al.*, 1991). A previous *in vivo* study using microdialysis has shown that significantly higher levels of OMD also occur in the extracellular fluid of the rat striatum when L-dopa was given after benserazide (Chang & Webster, 1993). OMD was initially thought to potentiate the antiparkinsonian action of L-dopa by serving as a reservoir for it (Kuruma *et al.*, 1971), although subsequently it was shown not only to be of no therapeutic value, but actually to reduce the response to L-dopa in both animals (Gervas *et al.*, 1983) and Parkinsonian patients (Hardie, 1989). More recent studies have indicated that OMD does not compete with L-dopa for uptake into the brain, and therefore reduces its effect by an unknown mechanism within the brain (Tohgi *et al.*, 1991). Currently there is no information concerning the effect of accumulated OMD on the synthesis and/or release of dopamine within the striatum.

Brain slices have proved to be a reliable and useful *in vitro* method of examining drug effects on endogenous neurotransmitter release (Becker *et al.*, 1984) and Tyce & Rorie (1985) and Snyder & Zigmond (1990) have shown that superfusion with L-dopa increases both the spontaneous and $K^+$-induced efflux of dopamine and DOPAC (3,4-dihydroxyphenylacetic acid) from rat striatal slices. We have therefore used superfusion of striatal slices to investigate the effect of OMD on the actual synthesis and release of dopamine in rat striatum.

## Methods

All experiments were performed on male Sprague-Dawley rats (University College London, Biological Services Animal House) weighing approximately 250 g. Animals were deeply anaesthetized with halothane (2% in 95% $O_2$ and 5% $CO_2$) and killed by cervical dislocation. The brain was quickly removed and rinsed with cold (4°C) carboxygenated artificial cerebrospinal fluid (ACSF), placed on an ice-chilled Petri dish and dissected immediately. The bilateral striata were isolated, placed on a teflon platform, and cut coronally into 500 $\mu$m slices with a McIlwain tissue chopper. The slices were immediately submerged in ACSF (composition in mM: NaCl 137, KCl 2.68, $CaCl_2$ 2.20, $MgCl_2$ 1.04, $Na_2HPO_4$ 0.42. $NaHCO_3$ 11.9 and D-glucose 10; pH 7.4), which was aerated with 95% $O_2$ and 5% $CO_2$ and prewarmed to 37°C. Two slices were quickly and gently transferred onto a nylon gauze disc held in the glass chamber (5 mm diameter, 250 $\mu$l) of a superfusion apparatus and continuously superfused at 100 $\mu$l $min^{-1}$ with ACSF equilibrated with 95% $O_2$ and 5% $CO_2$ at 37°C. The superfusates were collected at 10 min intervals into microcentrifuge tubes, surrounded by ice and containing 20 $\mu$l of 1M $HClO_4$ and assayed for dopa, dopamine and DOPAC immediately or stored at −70°C for subsequent analysis. At the end of superfusion, the two slices in each chamber were transferred into a pre-weighed microcentrifuge tube containing 0.5 ml of 1M $HClO_4$, and their wet weight determined before being homogenized in 10 vol 0.5 M $HClO_4$ and centrifuged (12000*g* for 10 min). All supernatants were stored at −70°C to await assay. Concentrations of dopa, dopamine and DOPAC

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were expressed as pmol $mg^{-1}$ tissue for striatal tissue contents and in pmol $mg^{-1}$ tissue 10 $min^{-1}$ for superfusion samples.

In experiments conducted with the aim of studying the possible interference of accumulated OMD with the release of dopamine formed from exogenous L-dopa (10 $\mu$M), striatal slices were superfused with 50 $\mu$M OMD from the beginning of superfusion. The chosen concentration ratio of 5 for OMD to L-dopa mimics that found in plasma of Parkinsonian patients receiving chronic L-dopa therapy with peripheral dopa decarboxylase inhibitors (Merello *et al.*, 1994). The concentrations of L-dopa and OMD employed in this study were 10 and 50 $\mu$M, respectively. The effects of excess L-dopa (25 $\mu$M) on the spontaneous and KCl-evoked dopamine efflux were also tested.

From a dose-response study of the effect of high $K^+$ (20–100 mM KCl) on dopamine release, 40 mM KCl was chosen since it gave a significant but submaximal increase. This $K^+$-evoked release of dopamine and DOPAC was significantly reduced by replacement of $Ca^{2+}$ by $Na^+$ in the ACSF and appears to be of neuronal origin. In accord with the result obtained by Tyce & Rorie (1985) the efflux of homovanillic acid (HVA) was not significantly altered by $K^+$ stimulation or by L-dopa superfusion, and therefore data for HVA are not shown.

Estimations were performed by reverse phase-ion pair high performance liquid chromatography (h.p.l.c.) with electrochemical detection (h.p.l.c.-e.c.d.). The injection of samples onto the analytical column (Spherisorb 5ODS, 250 × 4.6 mm i.d. HPLC Technology) was controlled by an autosampler (Kontron 465) via an upstream low-volume precolumn (Upchurch, C-130-B, 20 × 2 mm i.d.). Amines were detected at a high sensitivity (0.05–0.1 pmol) analytical cell (Coulochem Model, 5100A ESA) operated at a potential of +0.3V and the results reported on an integrator (Spectra-Physics, SP4400). Standard solutions containing known amounts of the mixtures of the monoamines being studied were run several times before the start of each experiment to calibrate the integrator, and representative standards were included routinely after every 5 samples to test reliability. The mobile phase, which gave the best separation of the substances being studied, contained the following substances (mM): citric acid 60, disodium hydrogen phosphate 40, sodium heptane sulphonate 1.0, $Na_2$ EDTA 0.054 and acetonitrile 2%. It was adjusted to pH 2.60 with $HClO_4$ and filtered (Ultipor-N66 0.1 $\mu$m, Pall) before being put on-line. The solvent was degassed by an isocratic pump (SA 6410C, Severn Analytical) and delivered at a rate of 1.5 ml $min^{-1}$.

L-3,4-Dihydroxyphenylalanine (L-dopa), dopamine hydrochloride, 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-O-methyldopa (OMD) were purchased from Sigma (U.K.). All chemicals were AnalaR or AristaR HPLC grade and the amines required for standardization of the chromatography were prepared in distilled deionised water.

Results are expressed as means ± s.e.mean. Comparisons were made by Student's and paired *t* test. A *P* value $<0.05$ was considered statistically significant.

## Results

Basal effluxes of dopamine and DOPAC from slices of rat striatum gradually declined during the first 30 min of superfusion with ACSF and then became stable in each of the subsequent two 10 min fractions (in pmol $mg^{-1}$ tissue 10 $min^{-1}$; dopamine 0.15±0.07 and 0.15±0.06; DOPAC: 1.10±0.11 and 1.03±0.06, $n=7$) and remained detectable throughout a 120 min superfusion period. In the following experiments, the first sample analysed was therefore collected between 30 and 40 min after the start of superfusion. Endogenous dopa was not present in control superfusates and although it was present after superfusion with 10 $\mu$M L-dopa the efflux was not increased by $K^+$ (Figure 1).

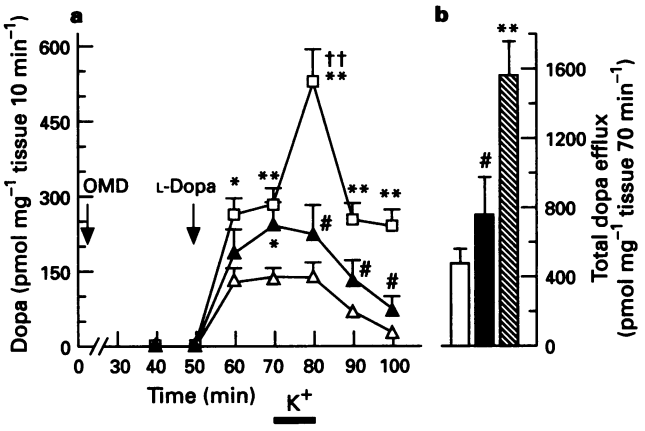


**Figure 1** Efflux of dopa from superfused striatal slices. Superfusate levels (pmol $mg^{-1}$ tissue 10 $min^{-1}$, a) or total effluxes from 30–100 min (pmol $mg^{-1}$ tissue 70 $min^{-1}$, b) are shown. Slices were superfused with 10 $\mu$M (△, open column) or 25 $\mu$M (□, hatched column) L-dopa or with 10 $\mu$M L-dopa plus 50 $\mu$M 3-O-methyl dopa (OMD, ▲, solid column). The arrows indicate the times that OMD and L-dopa were introduced into the superfusate and the horizontal bar the application of 40 mM KCl. Each value is the mean ± s.e.mean of 6 to 8 experiments. $^*P<0.05$ and $^{**}P<0.01$ significant difference from 10 $\mu$M L-dopa alone (△ or open column); $^{\#}P<0.05$ versus 25 $\mu$M L-dopa (□ or hatched column) using Student's *t* test; $^{\dagger\dagger}P<0.01$ significant difference from pre $K^+$ value (□, at 70 min) paired *t* test.

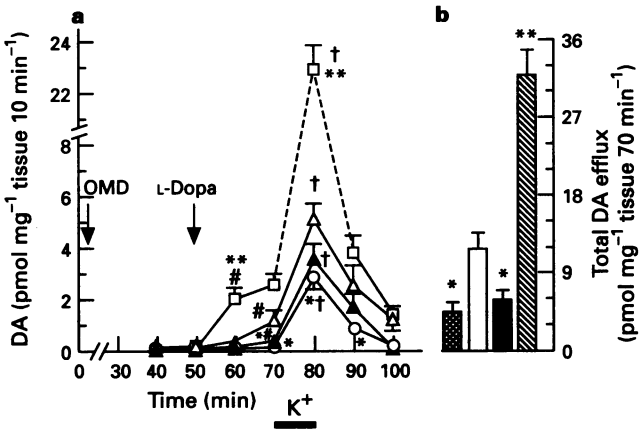


**Figure 2** The effect of 3-O-methyl dopa (OMD) on the spontaneous and KCl evoked release of dopamine (DA) from rat striatal slices superfused with L-dopa. Results show the overflow of dopamine (a, pmol $mg^{-1}$ tissue 10 $min^{-1}$) and the total dopamine effluxes (b, pmol $mg^{-1}$ tissue 70 $min^{-1}$) from striatal slices under control ACSF superfusion (○, cross hatched column), with 25 $\mu$M L-dopa (□, hatched column) or with 10 $\mu$M L-dopa either alone (△, open column) or in the presence of OMD (▲, solid column). The arrows indicate the times that OMD and L-dopa were introduced into the superfusate and the horizontal bar the application of 40 mM KCl. Each value is the mean ± s.e.mean of 6 to 11 experiments. *, $P<0.05$; **, $P<0.01$ significant difference from 10 $\mu$M L-dopa alone (△ or open column), Student's *t* test; #, and all subsequent time points (except at 100 min after 10 $\mu$M L-dopa with 50 $\mu$M OMD), $P<0.05$ significant difference from pre L-dopa superfusion, and †, $P<0.01$ significant difference from pre $K^+$ value using paired *t* test.

The addition of 10 $\mu$M L-dopa to the superfusate resulted in significant increases in the spontaneous efflux of dopamine (from 0.13±0.05 to 1.17±0.41, $n=8$, $P<0.05$) (Figure 2) and DOPAC (from 1.43±0.13 to 11.48±2.22, $n=8$, $P<0.01$) (Figure 3). In the presence of 10 $\mu$M L-dopa, the effect of potassium (40 mM) on dopamine release was significantly facilitated ($P<0.05$); however, there was no additional depolarization-induced increase in DOPAC efflux. The total

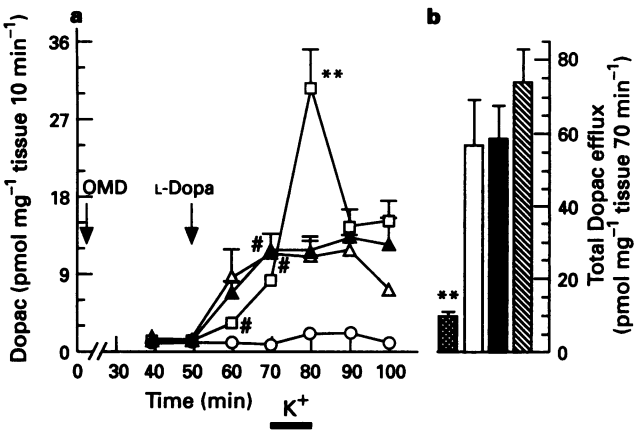


**Figure 3** The effect of 3-O-methyl dopa (OMD) on the spontaneous and KCl evoked release of DOPAC from rat striatal slices superfused with L-dopa. Results show the overflow of DOPAC (a, pmol mg$^{-1}$ tissue 10 min$^{-1}$) and the total DOPAC effluxes (b, pmol mg$^{-1}$ tissue 70 min$^{-1}$) from striatal slices under control ACSF superfusion (○, cross hatched column), with 25 μM L-dopa (□, hatched column) or with 10 μM L-dopa either alone (△, open column) or in the presence of OMD (▲, solid column). The arrows indicate the times that OMD and L-dopa were introduced into the superfusate and the horizontal bar the application of 40 mM KCl. Each value is the mean ± s.e.mean of 6 to 11 experiments, **, $P<0.01$ significant difference from 10 μM L-dopa alone (△ and open column) using Student's $t$ test; # and all subsequent time points, $P<0.01$ significant difference from pre L-dopa superfusion using paired $t$ test.

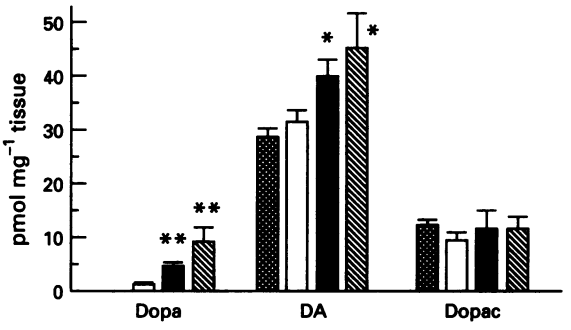


**Figure 4** Effects of 3-O-methyl dopa (OMD) and L-dopa superfusion on tissue contents of dopa, dopamine (DA) and DOPAC in striatal slices. Slices had been superfused with ACSF (cross hatched column) or with 10 μM L-dopa in the absence (open column) or presence (solid column) of 50 μM OMD or with 25 μM L-dopa (hatched column). Each value is the mean ± s.e.mean of 6 to 11 experiments. *, $P<0.05$ and **, $P<0.01$ significant difference from 10 μM L-dopa alone using Student's $t$ test.

effluxes of dopamine and DOPAC during the overall superfusion time, including depolarization were also significantly increased.

After 30 min superfusion with ACSF containing 50 μM OMD, the spontaneous effluxes of dopamine and DOPAC were lower than without OMD, although these reductions did not reach statistical significance. Twenty minutes after the addition of 10 μM L-dopa, the subsequently increased spontaneous efflux of dopamine was significantly less ($P<0.05$) than that in the absence of OMD and that evoked by 40 mM KCl was also reduced but not significantly (Figure 2). The total efflux of dopamine during the overall superfusion time, including depolarization was, however, significantly lower than when L-dopa was added alone (5.84 ± 1.02, $n=6$ vs 11.70 ± 1.87, $n=8$, $P<0.05$). In contrast, the effect of L-dopa on DOPAC efflux was not modified by OMD. The overall dopa efflux was higher in the presence of OMD although this only reached statistical significance 20 min after the addition of L-dopa (Figure 1).

As OMD increased the basal level of dopa in the superfusate it was just possible that the reduction in dopamine efflux resulted from the elevated dopa rather than the presence of OMD. Slices were therefore superfused with 25 μM instead of 10 μM L-dopa. This significantly elevated the efflux of dopa to a level (283 ± 34) just above that achieved with 10 μM L-dopa plus 50 μM OMD (244 ± 44) (Figure 1) but the spontaneous release of dopamine was still increased significantly above basal (from 0.06 ± 0.03 to 2.57 ± 0.44, $n=6$, $P<0.05$) (Figure 2). In fact after this higher concentration of L-dopa, 40 mM KCl evoked a release of dopa and DOPAC (not seen with 10 μM L-dopa alone or with OMD) (Figures 1 and 3) and a much greater release of dopamine (Figure 2).

No dopa was present in striatal slices after superfusion with ACSF alone (plus K$^+$ pulse) but became detectable after 10 μM L-dopa and increased stepwise and significantly after 10 μM with 50 μM OMD ($P<0.01$) and 25 μM ($P<0.01$) as in superfusates (Figure 4). In contrast to superfusates, 10 μM L-dopa did not significantly increase the slice concentration of dopamine but did so in the presence of OMD (50 μM). The higher concentration of L-dopa (25 μM) further increased dopamine concentration ($P<0.05$). DOPAC levels were not increased by any procedure.

## Discussion

The results presented here reveal that OMD attenuates the L-dopa-facilitated efflux of dopamine from superfused slices of rat striatum. It was observed that OMD reduced the maximal L-dopa-augmented spontaneous and K$^+$-stimulated release of dopamine and although only the former reached significance at any time, the total dopamine efflux during the overall superfusion time with 10 μM L-dopa, including K$^+$ depolarization, was significantly reduced by OMD (Figure 2). This could mean that OMD either inhibits the synthesis of dopamine inside the neurone or attenuates its release from nerve terminals, but, since the tissue levels of dopa and dopamine increased significantly after superfusion with L-dopa in the presence of OMD (Figure 4) it appears to be the release, rather than the synthesis, of dopamine which is inhibited by OMD.

Since more dopa appeared in the superfusate in the presence than in the absence of 50 μM OMD (Figure 1) after 10 μM L-dopa, the reduction in K$^+$-evoked dopamine efflux in the presence of OMD could have arisen not from the presence of OMD, as concluded above, but in some way from the higher dopa level. A higher concentration (25 μM) of L-dopa was therefore applied to the slices which produced a level of dopa in the superfusate before KCl-stimulation and in the tissue contents of dopa, dopamine and DOPAC after superfusion and depolarization, which were similar to those after 10 μM L-dopa plus 50 μM OMD. Under these conditions, increases in both the spontaneous and K$^+$-evoked release of dopamine were significantly higher ($P<0.01$) than in the presence of 50 μM OMD, which indicates that it is the OMD rather than the increased levels of dopa that reduced the release of dopamine. In the presence of 25 μM L-dopa a K$^+$-evoked efflux of dopa was obtained which accords with the observations of Goshima *et al.* (1988). This may be due to the fact that the extra dopa taken up by the nerve terminals (Figure 4) in the presence of the higher superfusion concentration is released from the cytoplasm by the depolarization or it could be in vesicles and released as a transmitter (Misu & Goshima, 1993). There was also a significant K$^+$-evoked release of DOPAC after 25 μM L-dopa although in this instance its tissue level had not been elevated and therefore it seems more likely to be the metabolic product of released dopamine, although we cannot assume that the tissue level at the end of the superfusion accurately reflects that just before the K$^+$ pulse.

It has been suggested by Elverfors & Nissbrandt (1992) that in the striatum the rate of dopamine synthesis is more effec-

tively regulated by terminal dopamine autoreceptors than by neuronal firing. It has also been recognized that presynaptic autoinhibition of dopamine release occurs locally at the terminal level and is mediated by an autoreceptor of the $D_2$ type located on the terminals themselves (Starke *et al.*, 1989; Suaud-Chagny *et al.*, 1991). Since many transmitters and neuroactive substances modify neurotransmission via presynaptic receptors (Suaud-Chagny *et al.*, 1991; Patel *et al.*, 1992), it is possible that OMD exerts its inhibitory effects on dopamine release by activation of prejunctional (dopamine) receptors. This possibility was not eliminated by studies with a $D_2$ antagonist but the structure of OMD is not in keeping with that generally expected of a $D_2$ agonist. The significantly increased tissue content of dopa in the presence of OMD eliminates the possibility that OMD competes with L-dopa for entry into the nerve terminals although it is not impossible that some OMD is actually demethylated to dopa (Bartholini *et al.*, 1972), or even released. Since OMD was not tested against $K^+$-induced dopamine release alone, i.e. in the absence of added L-dopa, a direct effect on the release mechanism remains possible.

In conclusion, the significant reduction in dopamine efflux from striatal slices after L-dopa superfusion in the presence of OMD is the first direct demonstration that release of dopamine can actually be inhibited by OMD. These results may have some relevance to the decreased therapeutic efficacy of L-dopa as often encountered during chronic L-dopa therapy. Although the precise nature of the inhibitory effect of OMD on dopamine release remains to be determined, its effect could be even more important in patients with Parkinson's disease with reduced striatal dopamine function provided dopamine is still being synthesized from dopa in, and released from, nerve terminals.

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