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PHARMACOLOGICAL MODIFICATIONS OF THE 6-HYDROXY-DOPA INDUCED DEGENERATION OF CENTRAL NORADRENALINE NEURONS

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Abstract—The effect of certain drugs on the nialamide + 2,4,5-trihydroxyphenylalanine (6-OH-dopa) induced degeneration of central noradrenaline (NA) neurons has been investigated by measuring the uptake *in vitro* of ^{3}H-NA in mouse brain slices. Nialamide + 6-OH-dopa treatment led to a more pronounced reduction of ^{3}H-NA uptake in the cerebral cortex than in the hypothalamus, indicating that the NA nerve terminals in the cerebral cortex are more sensitive to 6-hyroxydopamine (6-OH-DA). Reserpine was ineffective in modifying the neurotoxic effects, although a small protective effect was seen in the hypothalamus. Similar results were obtained when uptake of ^{3}H-metaraminol was measured. Both desipramine (DMI) and amphetamine could counteract the nialamide + 6-OH-dopa induced degeneration, which in the case of DMI was almost complete. Inhibition of catechol-*O*-methyl transferase (COMT) by 4-tropolone acetamide led to a small potentiating effect. There were no significant effects on the ^{3}H-DA uptake in nucleus caudatus indicating that the dopamine (DA) nerve terminals in this region were unaffected.

ALTHOUGH previous investigations have shown that systemic administration of 6-OH-dopa may cause a substantial decrease in both brain and heart NA, no conclusive neurotoxicity was shown,[1–3] similar to that seen with 6-OH-DA. This latter compound causes an acute and selective degeneration of both central and peripheral catecholamine neurons (see symposium volume edited by Malmfors and Thoenen).[4] However, recently it has been shown that 6-OH-dopa can accomplish degeneration of NA neurons, both in the central and peripheral nervous system, which is mediated via 6-OH-DA formed by decarboxylation of 6-OH-dopa.[5,6] In the central nervous system the 6-OH-dopa induced degeneration is selective for NA neurons since neither DA nor 5-hydroxytryptamine neurons were affected. Fluorescence histochemistry showed that 6-OH-dopa caused very pronounced accumulations of NA in the central NA axons which is related to the degenerative effects. In view of the possible functional implications for using 6-OH-dopa in elucidating central NA transmitter mechanisms and also as a powerful tool in the mapping of NA pathways, it was thought of interest to investigate the influence of drugs known to affect catecholamine metabolism and uptake-storage mechanisms on the 6-OH-dopa induced degeneration of central NA neurons.

MATERIALS AND METHODS

Male albino mice (Naval Medical Research Institute, Bethesda, U.S.A., 25–30 g) were used in the experiments. The animals were killed by cervical dislocation and the brains were rapidly dissected and placed in cold (4°) Krebs–Ringer bicarbonate buffer (pH 7·4). Standardized thin slices (dia. 2 mm; thickness about 0·5 mm) were made from cerebral cortex, hypothalamus and nucleus caudatus putamen

(neostriatum). The slices were preincubated for 10 min in Krebs–Ringer bicarbonate buffer, pH 7·4, containing 0·2 mg/ml ascorbic acid and saturated with 93·5% O_2–6·5% CO_2. Then ^{3}H-NA, ^{3}H-DA or ^{3}H-metaraminol was added to the incubation medium to give a final concentration of 0·1 μM and the incubation was continued for another 10 min. The incubations were carried out in a metabolic shaker, which were performed at 37° if not otherwise stated. The incubation volume was 2 ml/four brain slices. After termination of the incubation, the slices were rinsed for a few seconds in cold buffer and then dissolved in 0·5 ml soluene-100 (Packard Instrument Co.). After addition of 10 ml toluene scintillation solution, radioactivity was determined in a liquid scintillation spectrometer (Packard Tri-Carb, model 3320). Quenching was determined by recounting the vials after the addition of a standard amount of ^{3}H-toluene. The counting efficiency was 25 per cent. The uptake of radioactivity was expressed as dis/min per brain slice.

In one series of experiments, the brain slices, after incubation in 0·1 μM ^{3}H-NA or ^{3}H-DA for 10 min were extracted with 0·2 M acetic acid and the extracts analyzed by thin-layer chromatography (TLC). After centrifugation (10,000 g for 10 min) the extracts, together with appropriate reference substances, were spotted on precoated cellulose TLC plates (thickness 0·1 mm, Merck) and run in a two-dimensional system for separation of ^{3}H-catecholamine and ^{3}H-metabolites (see Fleming *et al.*[7] and Sachs and Jonsson[6]). After spraying with diazotized *p*-nitroaniline, the spots were scraped off the plates and mixed with 0·5 ml water. Radioactivity was determined after addition of 5 ml Insta-Gel® (Packard Instrument Co.).

Drugs and substances used. DL-NA-7-^{3}H-HCl, 7 Ci/mmole, ^{3}H-DA-acetate, 3·2 Ci/mmole and DL-metaraminol-7-^{3}H-HCl, 6·5 Ci/mmole were obtained from the New England Nuclear Corp.; reserpine (Serpasil®) from Ciba; nialamide–HCl (Niamid®) from Pfizer; desipramine–HCl (Petrofran®) from Geigy; (*d*-amphetamine)$_2$ SO_4 from Sigma; 4-tropolone acetamid (H 17/27) and DL-6-OH-dopa (H88/61) from AB Hässle. All drugs and substances were calculated as the free base. For injection, all drugs were dissolved in 0·9% NaCl, except 6-OH-dopa which was dissolved in a few drops of 0·1N HCl, diluted with the appropriate volume of saline (approx. pH 3), kept on ice and injected immediately.

RESULTS

The highest dose of 6-OH-dopa administered to mice for prolonged survival was 100 mg/kg (i.p.) which was repeated several times.[6] When injected as a single dose, 100 mg/kg of 6-OH-dopa did not have any apparent neurotoxic action on central NA neurons, whereas pretreatment of the animals with a potent monoamine oxidase (MAO) inhibitor (nialamide) led to degeneration of a considerable number of the NA nerve terminals in the brain[6] (Table 1). Therefore in this study the effects of reserpine, DMI, amphetamine and 4-tropolone acetamide on the 6-OH-dopa induced degeneration were investigated in mice treated with nialamide (100 mg/kg i.p.) 2 hr before the administration of 6-OH-dopa (100 mg/kg i.p. 48 hr). Since it has been shown that severe damage of the efficient "membrane pump" uptake mechanism, located at the axonal membrane of the NA neurons, is a very early sign of the degeneration process produced by 6-OH-DA, a rapid and sensitive way of obtaining quantitative information as to the degenerative effect is to measure the *in vitro* uptake of ^{3}H-catecholamine.[8] Moreover, it has been observed that both 6-OH-DA and 6-OH-dopa have an "all or

none" action with respect to neuronal damage suggesting that the reduction in neuronal ^{3}H-catecholamine uptake, seen after nialamide + 6-OH-dopa is a fairly good index of the number of nerve terminals that have degenerated.[6,8]

TABLE 1. UPTAKE OF RADIOACTIVITY IN BRAIN SLICES FROM MICE AFTER VARIOUS PHARMACOLOGICAL TREATMENTS (CONTROL GROUPS)

Treatment	Dose–time	Cerebral cortex (^{3}H-NA)	Hypothalamus (^{3}H-NA)	Nucleus caudatus (^{3}H-DA)
Untreated		12·5 ± 0·38	19·6 ± 0·49	35·1 ± 0·87
6-OH-dopa	100 mg/kg 48 hr	11·6 ± 0·48	20·9 ± 0·62	34·6 ± 1·54
Nialamide	100 mg/kg 50 hr	13·2 ± 0·48	19·6 ± 1·10	35·2 ± 2·03
Reserpine + nialamide	5 mg/kg 54 hr 100 mg/kg 50 hr + 2 hr	10·1 ± 0·52	16·5 ± 1·05	31·5 ± 1·35
Reserpine + nialamide	5 mg/kg 54 hr 100 mg/kg 2 hr	9·9 ± 0·30	17·5 ± 0·87	28·0 ± 1·24
DMI	25 mg/kg 48 hr	10·5 ± 0·45	18·1 ± 0·41	35·6 ± 1·48
Amphetamine	1 mg/kg 48 hr	12·6 ± 0·44	21·1 ± 0·82	33·2 ± 2·63
4-Tropolone-acetamide	2 × 100 mg/kg 48 hr, 1 hr interval	13·1 ± 0·82	19·2 ± 0·85	33·3 ± 1·81

The slices were preincubated in Krebs–Ringer bicarbonate buffer for 10 min and thereafter in 0·1 μM ^{3}H-NA (cerebral cortex, hypothalmus) or 0·1 μM ^{3}H-DA (nucleus caudatus) for another 10 min. Each value is the mean ± S.E.M. of six to eight determinations and expressed as n Ci/slice.

In the present investigation, tissue from three brain regions, cerebral cortex, hypothalamus and nucleus caudatus putamen have been used. The two former regions contain NA nerve terminals whereas the latter contains DA terminals. As

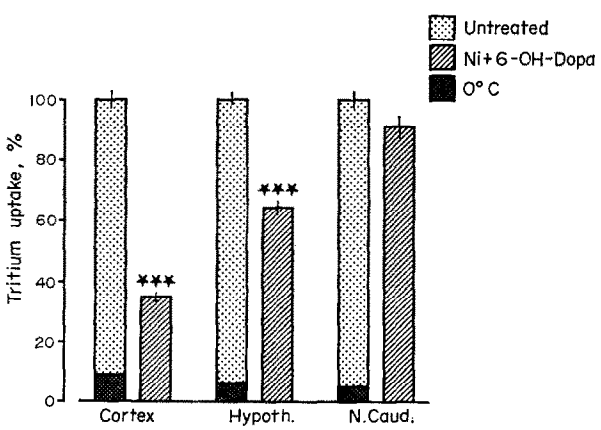

FIG. 1. Effect of nialamide (Ni) + 6-OH-dopa on the *in vitro* uptake of radioactivity in slices from cerebral cortex, hypothalamus, incubated in 0·1 μM ^{3}H-NA; nucleus caudatus putamen was incubated with ^{3}H-DA. The animals were treated with nialamide (100 mg/kg i.p.) 2 hr before 6-OH-dopa (100 mg/kg i.p. 48 hr). The values are expressed as percentages of the untreated control values (100 ± 5–8 per cent). Each value represents the mean ± S.E.M. of eight to ten determinations. The difference in values between untreated control and treatment with nialamide + 6-OH-dopa was tested with Student's *t*-test. *** $P < 0·001$.

seen in Fig. 1 nialamide + 6-OH-dopa treatment resulted in a marked reduction of radioactivity uptake in slices from cerebral cortex and hypothalamus incubated for 10 min in a medium containing 0·1 μM ^{3}H-NA. The reduction in uptake was more pronounced in slices from cerebral cortex (35 per cent of control) than in hypothalamic slices (65 per cent of control), whereas no significant effect on uptake of radioactivity was seen in caudate nucleus slices incubated in 0·1 μM ^{3}H-DA. There was a very small uptake of radioactivity in the brain slices from untreated mice after incubation in ^{3}H-catecholamine at 0°, being 5–9 per cent of the uptake at + 37° (Fig. 1). The uptake of radioactivity at 0° can be considered as mainly extraneuronal, since the active neuronal uptake is efficiently blocked at this temperature due to inhibition of the "membrane pump" mechanism (see Jonsson *et al.*[9]). The uptake of radioactivity at 37° in slices from untreated mice is thus almost exclusively localized in the catecholamine neurons. There was no difference in uptake of radioactivity at 0° in brain slices from untreated animals and animals pretreated with the various drugs used in the present investigation. Pretreatment of the mice with reserpine (5 mg/kg i.p.) 6 hr before the 6-OH-dopa treatment resulted in no clear-cut change in radioactivity uptake compared with nialamide + 6-OH-dopa treatment alone, although a small protective effect was seen in the hypothalamus ($P < 0{\cdot}01$). Omitting the nialamide treatment before the 6-OH-dopa injection had no effect on the uptake of radioactivity compared with reserpine alone (Fig. 2), indicating that reserpine does not markedly modify the action of 6-OH-dopa on NA neurons. In all groups of animals receiving reserpine, nialamide (100 mg/kg i.p.) was injected 2 hr before death in order to ensure an efficient MAO inhibition when measuring the uptake of radioactivity. After reserpine treatment MAO has to be inhibited in order to prevent intraneuronal deamination and to retain ^{3}H-NA or ^{3}H-DA taken up intraneuronally.[9,10] A similar experiment was performed using ^{3}H-metaraminol instead of

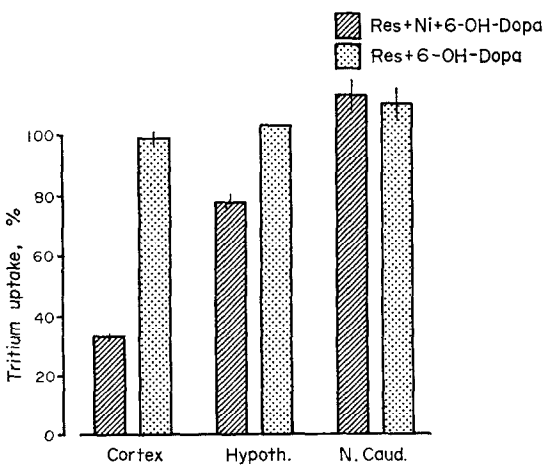

FIG. 2. Effect of reserpine (Res) on the 6-OH-dopa induced changes of the *in vitro* uptake of radioactivity in mouse brain slices incubated in 0·1 μM ^{3}H-NA or ^{3}H-DA (10 min). Reserpine (5 mg/kg i.p.) was injected 6 hr before and nialamide (100 mg/kg i.p.) 2 hr before the 6-OH-dopa (100 mg/kg i.p. 48 hr) injection. The animals received in addition another nialamide (100 mg/kg i.p.) injection 2 hr before death. The values are expressed as percentages of the respective controls where the 6-OH-dopa injection was omitted. Each value represents the mean ± S.E.M. of eight to ten determinations.

^{3}H-NA or ^{3}H-DA and the nialamide treatment 2 hr before death was omitted. Metaraminol, which is taken up in catecholamine neurons by the "membrane pump", is resistant to MAO.[11] In this experiment the results obtained were the same as those presented in Fig. 2.

Treatment with DMI, which blocks the "membrane pump" of central NA neurons but not that of DA neurons[12,13] counteracted the nialamide + 6-OH-dopa induced reduction in uptake of radioactivity in both cerebral cortex and hypothalamus (Fig. 3). DMI (25 mg/kg i.p.) was injected 30 min before the 6-OH-dopa. The uptake of radioactivity in slices from the caudate nucleus remained unchanged after this treatment. Pretreatment with DMI alone resulted in only a slight reduction of radioactivity uptake in slices from cerebral cortex and hypothalamus whereas no effect was seen in caudate nucleus (Table 1).

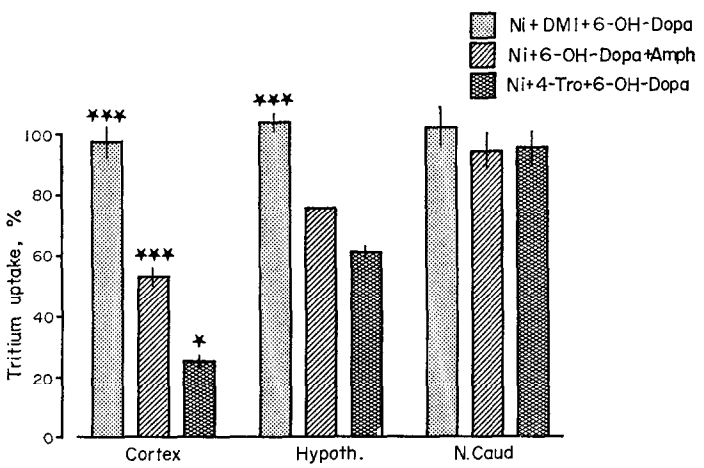

FIG. 3. Effect of DMI, amphetamine (amph) and 4-tropolone acetamide (4-Tro) on the nialamide + 6-OH-dopa (doses and times as in Fig. 1) induced changes of the *in vitro* uptake of radioactivity in mouse brain slices incubated in 0·1 μM ^{3}H-NA or ^{3}H-DA (10 min). DMI (25 mg/kg i.p.) was injected 30 min before and amphetamine (1 mg/kg i.v.) 15 min after the 6-OH-dopa injection. 4-Tropolone acetamide (100 mg/kg i.p.) was given in two doses, one dose 30 min before and the other 30 min after the 6-OH-dopa injection. The values are expressed as percentages of untreated control values (100 $\pm$ 5–8 per cent). Each value represents the mean $\pm$ S.E.M. of six to ten determinations. The difference in experimental group and treatment with nialamide + 6-OH-dopa was tested with Student's *t*-test. * $0{\cdot}05 < P > 0{\cdot}01$; *** $P < 0{\cdot}001$.

Injection of amphetamine (1 mg/kg i.v.) 15 min after 6-OH-dopa resulted in a small counteraction of the nialamide + 6-OH-dopa induced decrease in uptake of radioactivity in cerebral cortex and hypothalamus (Fig. 3). Inhibition of COMT by 4-tropolone acetamide (100 mg/kg injected 30 min before and 30 min after the 6-OH-dopa injection) resulted in a small potentiation of the nialamide + 6-OH-dopa induced change in ^{3}H-catecholamine uptake, although the effect was significant only in the cerebral cortex (Fig. 3). Neither amphetamine nor 4-tropolone acetamide itself affected the uptake of ^{3}H-catecholamines in brain slices from the various regions investigated (Table 1).

TLC analysis of the radioactivity taken up in slices from cerebral cortex, hypothalamus and caudate nucleus showed that the main part (75 per cent or more) of the total radioactivity constituted unchanged amine after all the drug treatments studied (Table 2). Although there was in some cases an increase in the relative amounts of metabolites after the pharmacological treatments, the changes were very small. It is thus evident that the radioactivity data represent almost exclusively unchanged ^{3}H-catecholamine.

TABLE 2. UNCHANGED ^{3}H-NA AND ^{3}H-DA DETERMINED BY THIN LAYER CHROMATOGRAPHY OF EXTRACTS FROM BRAIN SLICES INCUBATED *in vitro* IN ^{3}H-NA OR ^{3}H-DA AFTER VARIOUS PHARMACOLOGICAL TREATMENTS

Treatment	Cerebral cortex (% ^{3}H-NA)	Hypothalamus (% ^{3}H-NA)	Nucleus caudatus (%^{3}H-DA)
Untreated	98	91	89
Nialamide	96	94	92
Nialamide + 6-OH-dopa	81	94	95
Reserpine + nialamide*	88	87	76
Reserpine + nialamide + 6-OH-dopa*	78	92	82
Reserpine + 6-OH-dopa*	87	92	78
Nialamide + 4-tropolone acetamid + 6-OH-dopa	88	93	90
4-Tropolone acetamid	91	89	94
Nialamide + DMI + 6-OH-dopa	97	94	94
DMI	95	95	90
Nialamide + 6-OH-dopa + Amphetamine	87	92	95
Amphetamine	95	86	91

The slices were preincubated for 10 min in Krebs–Ringer bicarbonate buffer and thereafter in 0·1 μM ^{3}H-NA (cerebral cortex and hypothalamus) or 0·1 μM ^{3}H-DA (nucleus caudatus) for another 10 min. The radioactivity was extracted from the slices with 0·2 M acetic acid and the extracts analyzed by thin layer chromatography. Each value represents the mean of two to three determinations and is expressed as a percentage of the total radioactivity recovered from the chromatography plates. For dose and time see Table 1 and Figs. 1–3.

* The mice were in addition treated with nialamide (100 mg/kg i.p.) 2 hr before sacrifice, to ensure an efficient MAO inhibition.

DISCUSSION

In agreement with previously reported data it could be shown that nialamide + 6-OH-dopa treatment produced a marked reduction of the *in vitro* uptake of ^{3}H-NA in slices from cerebral cortex and hypothalamus, whereas the ^{3}H-DA uptake in caudate nucleus slices seemed almost unaffected by this treatment.[6] This reduction in ^{3}H-NA uptake is a consequence of nialamide + 6-OH-dopa inducing a damage and degeneration of NA nerve terminals in the respective brain region. This degenerative effect is

mediated via 6-OH-DA formed from 6-OH-dopa, and the potentiating effect of the MAO inhibition is due to 6-OH-DA being a substrate for this enzyme. The quantitative decrease in ^{3}H-NA uptake is probably related to a quantitatively similar reduction in the number of NA nerve terminals due to an "all or none" action of 6-OH-DA with respect to degeneration.[6,8] ^{3}H-NA uptake data indicate that the NA nerve terminals in the cerebral cortex seem to be more susceptible to the nialamide + 6-OH-dopa treatment than those in the hypothalamus. Fluorescence histochemical data also support this view (unpublished results). It is interesting to note that these two brain areas are innervated by two different ascending NA pathways.[14] The hypothalamus is innervated by an ascending ventral NA pathway, originating in cell groups in the lower brain stem, whereas the cerebral cortex is innervated by a dorsal NA pathway originating in the locus coeruleus. The terminal parts of these systems differ also in their fluorescence morphology, i.e. the varicosities in the cerebral cortex are smaller and display a weaker formaldehyde-induced NA fluorescence, indicating a lower concentration of NA. It is also known that the turn-over of endogenous NA is more rapid in cerebral cortex than in hypothalamus.[15] Thus these two morphologically and functionally different NA systems react differently to 6-OH-DA, which is the mediator of the degeneration seen after 6-OH-dopa.[6] Similar results have been obtained after the administration of 6-OH-DA to newborn animals.[16] Furthermore, it has been observed that larger amounts of 6-OH-DA are needed to produce NA denervation in the hypothalamus than in the cerebral cortex, after local application of 6-OH-DA on the ventral and dorsal NA bundle, respectively (Lidbrink, unpublished results). The reason for the difference in sensitivity to 6-OH-DA between the cerebral cortex and the hypothalamus is unknown but might be related to differences in uptake and/or metabolism of 6-OH-dopa. It is also possible that the difference in susceptibility might be related to differences in surface–volume relationships of the nerve terminals in the two brain regions.

From the present investigation it is also clear that there is a selectivity in the neurotoxic action since the uptake of ^{3}H-DA in the nigroneostriatal DA neurons was almost unaffected, pointing to these nerve terminals not being damaged. However, the selectivity seems to be dose-dependent, since after 250 mg/kg 6-OH-dopa i.p. strong retrograde catecholamine accumulations have been observed histochemically in the DA axons pointing to a degenerative effect also on these neurons. However, the animals do not survive more than 24 hr after this high dose (unpublished data). Reserpine, which blocks the ATP-Mg^{2+} dependent uptake mechanism of the amine storage granules was unable to markedly modify the degenerative action of 6-OH-dopa, although a small protective effect was seen in the hypothalamus. The present findings are in complete agreement with results obtained from mouse heart and iris after 6-OH-DA.[17] These results thus support the view that an intact granular uptake-storage mechanism is not a prerequisite for the degenerative action of 6-OH-DA, although there is evidence favouring the view that intact granules can to a certain extent help to maintain high 6-OH-DA levels intraneuronally necessary for producing degeneration.[17]

Blockade of the "membrane pump" uptake mechanism in central NA neurons, but not of that in the DA neurons,[12,13] almost completely counteracts the degenerative action induced by nialamide + 6-OH-dopa indicating that recapture of 6-OH-DA formed intraneuronally and released, and possibly also uptake of extra-neuronally

formed 6-OH-DA (cf Ref. 18), play an important role in maintaining the critical concentration of 6-OH-DA intraneuronally which is necessary for inducing degeneration.[8] A small dose of amphetamine, which releases extragranularly located catecholamines,[12] also partially prevented the action of 6-OH-dopa. Amphetamine may cause a release of 6-OH-DA formed from 6-OH-dopa, thereby decreasing the intraneuronal concentration of 6-OH-DA and reducing the number of nerve terminals undergoing degeneration. COMT-inhibition by 4-tropolone acetamide resulted in a small potentiation of the nialamide + 6-OH-dopa induced reduction of ^{3}H-NA uptake, although the effect was only significant in the cerebral cortex. This might indicate that 6-OH-dopa, like L-dopa, is to a certain extent metabolized by COMT.[19]

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