Amphetamine-induced release of dopamine from the substantia nigra in vitro

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ABSTRACT

Incubation of chopped tissue from the substantia nigra of the rat brain with d-amphetamine resulted in a significant release of [3H]dopamine into the incubation medium. This effect was observed with both exogenous [3H]dopamine previously taken up by the tissue and [3H]dopamine endogenously synthesized from L-[3,5-3H]tyrosine. The observed release was greater in magnitude when the apparent conversion of released dopamine to 3-methoxy-tyramine was taken into account. The relevance of the present results to the previously postulated self-inhibition by dopaminergic neurons of the substantia nigra pars compacta is discussed. The present data also provide support for the concept that catechol-O-methyltransferase (E.C.2.1.1.6.) is located primarily extraneuronally in brain.

INTRODUCTION

Groves et al. (1-3) have recently proposed that dopaminergic neurons of the substantia nigra, pars compacta, display a novel mode of neurohumoral regulation involving release of dopamine from dendrites to inhibit their own firing rate. The dendrites of these neurons possess numerous flourescent varicosities that contain large amounts of dopamine (4). Dopaminergic neurons of the substantia nigra are inhibited by iontophoretically applied dopamine as well as both direct and indirect-acting dopamine agonists administered systemically or locally into the substantia nigra (1-3,5,6). Geffen et al. (7) have recently provided additional support for this concept by demonstrating potassium-induced, calcium-dependent release of [3H]dopamine previously taken up by slices of nigral tissue in vitro. In addition, their data suggest that a substantial proportion of that release is from dendrites rather than cell bodies. Korf et al. (8) have reported an in vivo increase in 3,4-dihydroxyphenylacetic acid (DOPAC) in the substantia nigra following electrical stimulation of the median forebrain bundle that they attribute, in part, to increased release of dopamine from cell bodies and/or dendrites as a result of antidromic activation of dopaminergic neurons.

An important aspect of the experimental evidence for the functional role of the dopamine released from dopaminergic neurons of the substantia nigra is that localin-fusion of amphetamine produces a marked inhibition of neuronal activity in pars compacta of the substantia nigra (1-3). This effect has been attributed to amphetamine-induced release of dopamine from dopaminergic neurons

rather than, for example, a direct action of amphetamine on somatic or dendritic receptors on dopaminergic neurons. However, although recent accounts go far toward showing that dopamine may be released by dopaminergic neurons, there has yet been no biochemical evidence for amphetamine-induced release of dopamine from the substantia nigra. Such evidence has been significant in determining that amphetamine releases dopamine from terminals of the nigro-neostriatal pathway in the striatum (9-10). The purpose of the present experiments was to test the hypothesis that amphetamine releases dopamine from the substantia nigra in vitro.

METHODS

Adult male Sprague-Dawley rats (250-400 g), supplied by Simonsen, Gilroy, Calif. were killed by decapitation and the brains quickly removed. The substantia nigra was dissected bilaterally by knife cuts made just caudal to the mammillary bodies, just rostral to the pons, lateral to the interpeduncular nucleus, and along a line extending through the medial lemniscus to the lateral edge of the brain stem ventral to the medial geniculate. Included in the sample were all but the most rostral portion of the substantia nigra, pars compacta and pars reticulata, the crus cerebri, part of the medial lemniscus, the root of the oculomotor nerve, and a small triangular portion of the ventral tegmental nucleus of Tsai.

The isolated nigral tissue was lightly smeared onto the teflon stage of a McIlwain tissue chopper using a stainless steel spatula and was choppec into 0.3 mm square sections. The functional and structural integrity of this preparation has been discussed elsewhere (11). Immediately after being chopped, the tissue was transferred to a test tube containing 3 ml of an oxygenated physiological salt solution (9), pH 7.4 and 5°C, and the sections separated by agitation with a vortex mixer. Pargyline was present in the salt solution at a concentration of 2.5×10^{-4} M to inhibit monoamine oxidase activity (E.C.1.4.3.4.) (12). This medium was used throughout the experiment. The nigral tissue from six rats was combined in the same tube and centrifuged at 1OO x g for two minutes, following which the supernatant was discarded and the tissue resuspended in 3.0 ml of medium and the process repeated. The tissue was then resuspended in 2.0 ml of medium and incubated for,1O minutes at 37°C with continuous agitation in a Dubnoff metabolic shaking incubator under a steady flow of 95% O_2 : 5% CO_2. Following the incubation the tissue was centrifuged and resuspended in approximately 1.7 ml of medium containing 10 microCi

[³H]dopamine (5.6 Ci/mmole, giving a final concentration of 1 x 10-6 M dopamine). The tissue was incubated for 20 minutes as described above, after which it was centrifuged and resuspended in 3.0 ml of medium (without dopamine) twice. Following the second rinse the tissue was centrifuged and resuspended in 3.0 ml of medium once again and then incubated as described above for 20 minutes. At the conclusion of that period, the tissue was centrifuged and rinsed once with 3.0 ml of medium. The second incubation in the absence of dopamine and the numerous rinses were employed to assure that [³H]dopamine bound to extraneural or other nonspecific binding sites was reduced to a minimum prior to the addition of d-amphetamine. After the final rinse, the tissue was centrifuged and resuspended in 0.70 ml of medium. A 0.100 ml Eppendorf pipette with approximately 5 mm of the disposable tip cut off was used to divide the tissue into 8 fractions, each of which contained approximately 0.6 mg of protein. Each tissue sample was rinsed once with 1.0 ml of medium containing the appropriate concentration of d-amphetamine, centrifuged, and resuspended in 1.0 ml of the same medium. The tissue samples were then incubated as described above for 20 minutes.

At the conclusion of the incubation period the samples were centrifuged and the release medium decanted into test tubes on ice containing 2 ml of 2 N HCl and 50 microgram each of L-norepinephrine, dopamine and in some cases, 3-methoxy-tyramine (3MT : 4-hydroxy-3-methoxy-phenylethylamine). Each tissue sample was resuspended in 2 ml of medium without amphetamine, centrifuged, and the supernatant was combined with the release medium. Five drops of 2 N HCl were added to the tissue pellets which were then allowed to stand on ice for two hours. At the end of that period the tissue samples vere homogenized by hand in an all glass Dounce homogenizer (40 strokes) in 2 ml of 1 N HCl, and the homogenizing vessel was rinsed with 2 ml of 0.2 N HCl. Each homogenate and rinse was combined and centrifuged at 15,000 x g for 15 minutes. The supernatants were decanted and the pellets rinsed with 0.5 ml of 0.2 N HCl, which was then combined with the supernatant. The pellets were dissolved in 1 N HaOH for estimation of protein content (13).

The release media and tissue homogenates were adjusted to pH 6.5 with 5 N K_2CO_3 and passed over 5 x 65 mm Dowex 50-X4 columns (200-400 mesh, Na form) using peristaltic pumps adjusted to give a flow rate of 0.6 ml/min. Following the passage of 20 ml of distilled-deionized water, 1 N HCl was used to elute norepinephrine and 2 N HCl was employed to elute dopamine and 3-methoxy-tyramine in a modification of the method of Rutledge and Jonason (14). Norepinephrine was collected in fractions 6-15 (1 ml fractions) of 20 ml of 1 N HCl, following which dopamine was collected in fractions 1-10 and 3-methoxy-tyramine in fractions 11-20 of 20 ml of 2 N HCl. Thin layer

chromatography on silica gel using either 8% NaCl : 1 N NH_4OH (19:1) or chloroform : methanol : 1 N NH_4OH (65:30:5) as solvents (17,18) confirmed that more than 99% of the radioactivity recovered in the 3-methoxytyramine fraction was associated with authentic 3-methoxy-tyramine. Approximately 10% of the 3-methoxytyramine radioactivity in each sample was found in the dopamine fraction.

The 10 ml amine fractions were evaporated to dryness under vacuum in a Buchler Rotary Evapo-Mix and the amines were resuspended in 1.0 ml of 0.1 M phosphate buffer, pH 6.5, of which 0.1 ml was taken for an estimation of amine recovery by the phenol method (15). The remainder of the amine samples was transferred to vials to which were added 10 ml of dioxane fluor [100 g naphthalene and 6 g 2,5-diphenyloxazole (PPO) per liter] for counting in a Beckman LS250 liquid scintillation system. Activities were corrected for counting efficiency using the external standard method and for recovery, which averaged 71.4%, 83.5% and 69.7% for norepinephrine, dopamine, and 3-methoxytyramine, respectively.

In some experiments the chopped nigral tissue was incubated with L-[3, 5-³H]tyrosine instead of [3H]dopamine. The tissue was suspended in 1 ml of medium containing 540 microCi of L-[3,5- ³H]tyrosine (60.3 Ci/mmole, giving a final concentration of 9 microM tyrosine) and incubated as described above for 45 minutes, following which it was centrifuged and resuspended in 3 ml of fresh medium three times. The tissue was then divided and each portion incubated for 20 minutes in 1 ml of medium containing the appropriate concentration of d-amphetamine as described above. The remainder of the experimental procedure was identical to that employed in the studies using labeled dopamine except that catechols were separated from tyrosine and other noncatechols by batch adsorption onto alumina at pH 7.8 (16) prior to Dowex column chromatography.

D-amphetamine sulfate was supplied by Smith, Kline & French Laboratories, Philadelphia, Pa., and pargyline HCl was donated by Dr. A. O. Geiszler of Abbott Laboratories, North Chicago, Ill. [³H]dopamine HCl, 5.6 Ci/mmole, ring labeled, was obtained from Amersham/Searle, Arlington Heights, Ill. L-[3,5-H] tyrosine, 60.3 Ci/mmole was obtained from New England Nuclear, Boston, Mass., and was purified prior to use by aluminum oxide and Dowex 50 column chromatography. L-norepinephrine HCl, dopamine HCl, and 3-methoxytyramine HCl were obtained from the Sigma Chemical Co., St. Louis, Mo. Dowex 50 was also obtained from Sigma and was purified prior to use by repeated washing in 2 N HCl and 2 N NaOH at 90°C followed by extensive rinsing. Aluminum oxide (Woelm neutral activity grade I) was purified prior to use by heating in HCl (17). Pre-coated silica gel 60 glass TLC plates, (layer thickness 0.25 mm) were obtained from EM Labo-

ratories, Elmsford, N.Y. All other materials used were reagent grade. All drug concentrations are expressed as the concentration of free base.

The results were expressed as the percentage of the [³H]amine recovery corrected disintegrations per minute (dpm) found in the release medium, calculated with the formula (12)

$$\frac{(\text{amine dpm in media}) \times 100}{(\text{amine dpm in media}) + (\text{amine dpm in tissue})}$$

where the amine may be dopamine, dopamine plus 3-methoxytyramine, or norepinephrine.

RESULTS

Addition of amphetamine to the incubation medium led to a dose-dependent increase in the release of previously accumulated [³H]dopamine from chopped nigral tissue samples (Figure 1), which was statistically significant when tested by analysis of variance (F = 13.69, df = 4/26, p < .05). The minimum effective dose of amphetamine was 1 x 10⁻⁵ M. Our initial data on amphetamine-induced release of [3H]dopamine indicated that the amount of labeled dopamine found in the release medium was less than the amount which had disappeared from the corresponding tissue samples (which was calculated by subtracting the tissue dopamine from the level of the control). Since monoamine oxidase activity was blocked

throughout the experiment by pargyline (12), we reasoned that extraneuronal catechol-O-methyltransferase (COMT; E.C.2.1.1.6) activity could have degraded a portion of the dopamine released by amphetamine. Thus we measured the amount of [³H]3-methoxytyramine (3MT), the metabolite formed by the action of COMT on dopamine, in both medium and tissue for some of the data points. Substantial amounts of labeled 3MT were found, with almost all of this metabolite invariably present in the medium (average 89.6%, standard error of the mean 1.60%, n = 11). Furthermore, the ratio of total [³H]3MT activity to [3H]dopamine activity in the medium was approximately constant regardless of amphetamine concentration, suggesting a product-precursor relation, while that of total [³H]3MT activity to that of [³H]dopamine in the tissue increased over four fold as the amphetamine concentration was varied from 10⁻⁵ to 10⁻³ M. These data suggest that the [³H]3MT found in the medium was formed following the release of labeled dopamine from the tissue, if it is assumed that COMT was not saturated by endogenous dopamine levels present in the substantia nigra. This assumption seems reasonable in view of the high Km values of brain COMT for other catecholamines (18). Thus, a perhaps better estimate of the magnitude of the releasing action of amphetamine on nigral dopamine may be obtained by adding the recovery corrected activity of labeled dopamine and 3MT in both medium and tissue prior to calculating the percent of amine in the medium. The releasing effect of amphetamine on nigral dopamine is even greater when calculated in this manner (Figure 1).
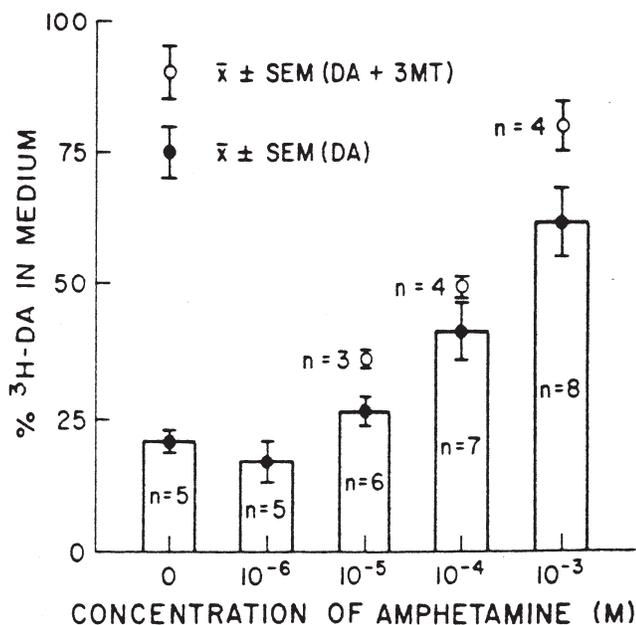
[³H]Norepinephrine was detected in chopped nigral tissue samples incubated with labeled dopamine, indicating the presence in the tissue sample of adrenergic nerve cell processes capable of converting dopamine to norepinephrine. Norepinephrine represented 68% of the total [³H]catechols recovered from samples incubated with labeled dopamine. Formation of norepinephrine from labeled precursors in the substantia nigra has been previously observed, both in vivo and in vitro (19,20). Unlike the case with labeled dopamine, however, amphetamine did not cause an increase in the percentage of labeled norepinephrine in the release medium except at the highest concentration employed (10⁻³ M). While norepinephrine release at this high amphetamine concentration was found to be statistically significant by analysis of variance (F = 4.10, df = 4/28, p < .05), the magnitude of the release effect was much less than that observed for dopamine. The percentage and one standard error of the mean of norepinephrine in the medium were 14.1 and 4.24 for controls compared to 23.2 and 3.99 in the presence of 10⁻³ M amphetamine.

5-Hydroxytryptamine (serotonin) is present in moderately high concentration in the substantia nigra and may act as a neurotransmitter there (21,22). Serotonergic nerve terminals have been shown to be capable of taking up exogenous catecholamines (23) and are sensitive to the

Fig. 1 Amphetamine-induced release of [3H]amines in the substantia nigra in vitro; open circles, [3H]dopamine; filled circles, [3H]dopamine plus [3H]3-methoxytyramine. Each point represents the mean and standard error of the mean for the number (n) of determinations.

releasing action of amphetamine in vitro (12) and in vivo (24). Therefore, in order to test the possibility that the observed amphetamine-induced release of [3H]dopamine from nigral tissue might have been mediated by serotonergic processes, we incubated some nigral tissue samples with L-[3,5-^{3}H]tyrosine instead of [^{3}H]dopamine. Since serotonergic terminals lack the ability to convert tyrosine to dopamine, any synthesis of [^{3}H]dopamine from labeled tyrosine must occur in catecholaminergic neurons. Substantial amounts of labeled dopamine were formed in our experiments, and a significant amphetamine-induced release of [^{3}H]dopamine was found at the amphetamine concentration employed (10^{-4} M). The mean observed percentages of labeled dopamine in the release medium and the standard error of the mean were 5.6 and 3.9 for controls, compared to 21.6 and 13.6 for 10^{-4} M amphetamine ($t = 3.804$, $df = 13$, $p < .05$, Student's t-test).

[^{3}H]Norepinephrine was also formed when chopped nigral tissue was incubated with labeled tyrosine, but as was the case for incubation with labeled dopamine, no amphetamine-induced release of norepinephrine was observed at 10^{-4} M amphetamine. The means and standard errors of the mean of the percentages of [^{3}H]norepinephrine in the release medium were 8.5, 4.5 and 8.1, 2.4 for no amphetamine and 10^{-4} M amphetamine, respectively.

DISCUSSION

The results of our experiments show that amphetamine releases dopamine from the substantia nigra in vitro within a concentration range nearly identical to that which has been reported for amphetamine-induced release of dopamine from the corpus striatum in vitro (12), where dopamine is believed to function as a neurotransmitter (25). The substantial release we observed was even greater in magnitude when the conversion of a portion of the released dopamine to 3MT is taken into account. Since amphetamine-induced release of catecholamines probably occurs directly into the extracellular space, as suggested by the decrease in deaminated metabolites of labeled norepinephrine following amphetamine administration in vivo (11,26), our data on 3MT formation indicate that COMT is located extraneuronally in the substantia nigra. This finding is consistent with data obtained from peripheral sympathetic neurons regarding the localization of COMT (27,28) and with some data on metabolism of catecholamines in brain. For example, the ratio of normetanephrine (4-hydroxy-3-methoxyphenyl-ethanolamine) to norepinephrine is 15 times higher if the norepinephrine is injected intraventricularly than if it is endogenously synthesized from injected dopamine (29).

The possibility that the action of amphetamine we observed could have been mediated by serotonergic nerve terminals taking up labeled dopamine and releasing it in the presence of amphetamine may be ruled out because amphetamine was also shown to be capable of releasing dopamine endogenously synthesized from tyrosine in the substantia nigra. Further, amphetamine failed to increase the release of norepinephrine above its resting level except at the highest concentration employed (10^{-3} M), and even then the effect was much smaller in magnitude than that observed for dopamine. It appears then that the observed dopamine release could not have been mediated by noradrenergic neurons. The accumulation of [^{3}H]norepinephrine in our samples was probably due to synthesis by adrenergic fibers of passage consisting predominately of the ventral noradrenergic bundle which courses through this region (30), and is unlikely to represent an amphetamine sensitive pool of norepinephrine in the substantia nigra.

Dopaminergic axonal endings do not appear to be present in the substantia nigra (21), leading to the conclusion that amphetamine-induced release of dopamine from the substantia nigra in vitro occurs from dendrites, cell bodies and/ or proximal portions of axons of dopaminergic neurons. Groves et al. (1-3) have argued that the inhibition of dopaminergic neuronal activity produced by systemically administered amphetamine or amphetamine infused locally into the substantia nigra is due to this form of amphetamine-induced release of dopamine. Thus, the inhibition produced by systemically administered amphetamine is blocked by alpha-methyl-para-tyrosine (5,6), an inhibitor of catecholamine synthesis (31), as is the inhibition produced by local infusion of amphetamine into the substantia nigra (1-3). Similarly, the amphetamine-induced depression of dopaminergic neuronal activity following systemically or locally administered amphetamine is also blocked by ipsilateral diencephalic lesions anterior to the substantia nigra (1-3,5,6). Groves et al. suggested that these lesion effects were due to transection of dopaminergic axons (1-3). However, as described here, amphetamine-induced release of dopamine from chopped nigral tissue in vitro occurs for concentrations of amphetamine of 10^{-5} M and higher, a preparation in which axons of dopaminergic neurons must be severed. Importantly, the effects of diencephalic transections on the inhibition of dopaminergic neuronal activity produced by local infusion of amphetamine in vivo are blocked at a concentration of 5×10^{-6} M, but a less marked inhibition of neuronal activity still occurs at 5×10^{-5} M (2). We believe that these results provide strong support for the theory that amphetamine acts to increase release of dopamine from dopaminergic neurons in the substantia nigra, leading to a process of self-inhibition by dopaminergic neurons (1).

ACKNOWLEDGEMENTS

REFERENCES

1. P.M. GROVES, C. WILSON, S. YOUNG, and G. REBEC, Science 190 522-529 (1975).
2. P.M. GROVES, S.J. YOUNG, and C.J. WILSON Neuropharmacology (in press).
3. P.M. GROVES, S.J. YOUNG, and C.J. WILSON Cholinergic-Monoaminergic Interactions in the Brain, Ed. L.L. Butcher, Academic Press, New York (in press).
4. A. BJORKLUND, and O. LINDVALL, Brain Res. 83 531-537 (1975).
5. G.K. AGHAJANIAN, and B.S. BUNNEY, Frontiers in Catecholamine Research, Eds. E. Usdin and S.H. Snyder, (pp. 641-648) Pergamon Press, New York (1973).
6. B.S. BUNNEY, and G.K. AGHAJANIAN, Frontiers in Catecholamine Research, Eds. E. Usdin and S.H. Snyder, (pp. 957-962) Pergamon Press, New York (1973).
7. L.B. GEFFEN, T.M. JESSEL, A.C. CUELLO, and L.L. IVERSEN, Nature 260 258-260 (1976).
8. J. KORF, M. ZIELEMAN, and B.H.C. WESTERINK, Nature 260 257-258 (1976).
9. M.J. BESSON, A. CHERAMY, P. FELTZ, and J. GLOWINSKI, Proc. Nat. Acad. Sci. USA 62 741-748 (1969).
10. C.C. CHIUEH, and K.E. MOORE, J. Pharm. Exp. Ther. 192 642-653 (1975).
11. R.J. ZIANCE, and C.O. RUTLEDGE, J. Pharm. Exp. Ther. 180 118-126 (1972).
12. A.J. AZZARO, and C.O. RUTLEDGE, Biochem. Pharmacol. 22 2801-2813 (1973).
13. O.H. LOWRY, N.J. ROSEBROUGH, A.C. FARR, and R.J. RANDALL, J. Biol. Chem. 193 265-275 (1951).
14. C.O. RUTLEDGE, and J. JONASON, J. Pharm. Exp. Ther. 157 493-502 (1967).
15. E.A. BARNESS, W.J. MELLMAN, T. TEDESCO, D.G. YOUNG, and R. NOCHO, Clin. Chem. 9 600-607 (1963).
16. M.K. SHELLENBERGER, and J.H. GORDON, Anal. Bioche. 39 356-372 (1971).
17. A.H. ANTON, and D.F. SAYRE, J. Pharm. Exp. Ther. 138 360-375 (1962).
18. G.D. ROCK, J.H. TONG, and A.D. D'IORIO, Can. J. Biochem. 48 1326-1331 (1970).
19. F. JAVOY, M. HAMON, and J. GLOWINSKI, Eur. J. Pharmacol. 10 178-188 (1970).
20. E.P. KINDWALL, and N. WEINER, J. Neurochem. 13 1523-1531 (1966).
21. J. PARIZEK, R. HASSLER, and I.J. BAK, Z. Zellforsch. 115 !37-348 (1971).
22. K. FUXE, Acta. Physiol. Scand. Suppl. 247 37-85 (1965).
23. J.G. RICHARDS, H.P. LOREZ, and J.P. TRANZER, Brain Res, 57 277-288 (1973).
24. K. FUXE, and U. UNGERSTEDT, Amphetamines and Related Compounds Eds, E. Costa and S. Garattini, (pp. 257-288) Raven Press, New York (1970).
25. K. KRNJEVIC, Advances in Neurology Yol. 9, Eds. D.B. Calne, T.N. Chase, A. Barbeau, (pp. 13-24) Raven Press, New York (1975).
26. J. GLOWINSKI, J. AXELROD, and E.E. IVERSEN, J. Pharm. Exp. Ther. 153 30-41 (1966).
27. J. AXELROD, Pharmacol. Rev. 13 95-113 (1966).
28. A.J. EISENFELD, E. LANDSBERG, and J. AXELROD, J. Pharm. Exp. Ther. 158 378-385 (1967).
29. J. GLOWINSKI, and J. AXELROD, Pharmacol. Rev. 18 775-785 (1966).
30. U. UNGERSTEDT, Acta. Physiol. Scand. Suppl. 367 1-48 (1971).
31. S. SPECTOR, A. SJOERDSMA, and S. UDENFRIEND, J. Pharmacol. Exp. Ther. 147 86-95 (1965).