Biochemical Pharmacology, Vol. 27, pp. 1497–1501.

0006-2952/78/0515-1497/$02.00/0

EFFECTS OF RACEMIC, (S)- AND (R)-METHYLENEDIOXYAMPHETAMINE ON SYNAPTOSOMAL UPTAKE AND RELEASE OF TRITIATED NOREPINEPHRINE*

GERALD M. MARQUARDT,† VICTOR DISTEFANO and LYDIA L. LING

Department of Pharmacology and Toxicology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, U.S.A.

(*Received* 31 *May* 1977; *accepted* 3 *October* 1977)

Abstract—Racemic, (S)- and (R)-methylenedioxyamphetamine (MDA) were potent competitive inhibitors of [^{3}H]norepinephrine (NE) uptake by rat hypothalamic synaptosomes. (S)- and (R)-α-methyldopamine were extremely potent competitive inhibitors of [^{3}H]norepinephrine uptake, establishing these compounds as the most potent inhibitors of brain NE uptake reported to date. Racemic, (S)- and (R)-MDA were slightly less potent as releasing agents than (±)-, (+)- and (−)amphetamine respectively. (S)- and (R)-α-methyldopamine were the most potent releasing agents examined.

(S)- and (R)-methylenedioxyamphetamine (MDA) have been shown to produce different pharmacological effects both peripherally and centrally.‡ The actions of (±)-, (S)- and (R)-MDA on the uptake and release of [^{3}H]norepinephrine (NE) in synaptosomal preparations were examined to determine whether differential effects of (±)-, (S)- and (R)-MDA could be observed. Such observations might provide insight into the mechanisms responsible for the differential effects of these drugs on blood pressure, behavior and toxicity.

Racemic MDA is metabolized *in vitro* [1] and *in vivo* [2] to α-methyldopamine (α-MeDA). The effects of α-MeDA isomers on the uptake and release of [^{3}H]NE in synaptosomal preparations, therefore, were also examined.

MATERIALS AND METHODS

[^{3}H]*NE uptake by rat hypothalamic synaptosomes*

Preparation of synaptosomes. Synaptosomes (P2 fraction) were prepared by a modification of the method of Gray and Whittaker [3]. Male Sprague–Dawley rats, weighing 150–200 g, were decapitated, and the hypothalamus of each animal was rapidly dissected from other brain tissue on dental wax by the method of Glowinski and Iversen [4]. After washing in 0.32 M sucrose, the tissue was blotted dry, weighed, and homogenized in 0.32 M sucrose (10 ml/g of tissue) by 5–6 strokes of a Teflon pestle in a Potter–Elvehjem homogenizer. All procedures were carried out at 0–4°.

The homogenate was transferred to chilled polypropylene centrifuge tubes and centrifuged at 1000 *g* for 10 min to sediment cellular debris. The supernatant fraction was decanted and centrifuged at 11,000 *g* for 20 min to pellet the synaptosomes. The pellet was resuspended in the same volume of 0.32 M sucrose and then centrifuged at 11,000 *g* for an additional 20 min to remove free NE and other substances that might interfere with the transport of NE across the synaptosomal plasma membrane (e.g. dopamine) [5]. This pellet was then resuspended in the same volume of 0.32 M sucrose to prepare the synaptosomal suspension used in the uptake experiments.

Incubation conditions. Fifty μl of normal saline (containing the appropriate concentration of drug) was added to 1.90 ml of incubation medium [containing 5.90 mM KCl, 1.20 mM $MgSO_4$, 1.28 mM $CaCl_2$, 112.20 mM NaCl, 31.10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, sodium salt (Na TES), 18.90 mM TES, 1.14 mM ascorbic acid, 0.06 mM Na_2EDTA, and 12.50 μM nialamide] in 15-ml polypropylene beakers. One hundred μl of the synaptosomal suspension was added to each sample and preincubated at pH 7.40 for 5 min at 37°. Incubations were started by the addition of 10 μl [^{3}H]NE (*l*-noradrenaline; New England Nuclear Corp; sp. act., 3.8 Ci/m-mole; this solution was diluted to 20 μCi/ml of [^{3}H]NE), resulting in a final NE concentration of 1.0 or 2.0×10^{-7} M.

Incubations were terminated 5 min later by filtration through 0.45 μm Millipore filters (type HAWP, Millipore Corp.). Filters were then washed with 5 ml of normal saline containing 1% BSA at 22°. The filters were dried in glass scintillation vials heated to 100° for 30 min. After the vials had cooled, 10 ml of Bray's solution [6] was added to each sample and the radioactivity was determined in a Packard Tri-Carb model 3385 liquid scintillation spectrometer.

* Supported in part by Grants GM-00032 and GM-15190 from the United States Public Health Service.

† Present address: Environmental Protection Agency, Metabolic Effects Branch, OPP/CED, 401 M Street S.W., Washington, DC 20460.

‡ G. M. Marquardt, V. DiStefano and L. L. Ling, *Topical and Applied Pharmacology* (in Press).

The counting efficiency was monitored by internal and external standards. The protein concentrations of the synaptosomal suspensions were determined by the method of Lowry *et al.* [7].

α-MeNE (330 μM) or metaraminol (150 μM) was included in some incubations to estimate the amount of [^{3}H]NE entering the synaptosomes by diffusion (approximately 20 per cent of the total radioactivity taken up during control incubations). The radioactivity of these diffusion blanks was routinely subtracted from that of samples not incubated with either α-MeNE or metaraminol to estimate the quantity of [^{3}H]NE taken up by synaptosomes by nondiffusional processes. The percentage of total radioactivity representing unchanged [^{3}H]NE was estimated by the method of Baldessarini and Vogt [8].

Subcellular distribution of the [^{3}H]*NE.* Several incubations containing 1.0 ml of the synaptosomal suspension in a total volume of 20.6 ml were terminated by pouring the contents of each beaker into chilled polypropylene centrifuge tubes and centrifuging at 11,000 *g* for 20 min (at 0–4°). The supernatant fraction was discarded; the pellet was washed with 1.0 ml of 0.32 M sucrose and then resuspended in 1.0 ml of 0.32 M sucrose. Two hundred μl of this suspension was then layered on top of 3.6 ml of a linear sucrose density gradient (freshly prepared by linearly diluting 1.60 M sucrose with 0.32 M sucrose using a Buchler monostaltic pump and a Buchler Autodensiflow) and centrifuged for 2 hr (at 0–4°) in a preparative ultracentrifuge with an SW65L Ti rotor. Four-drop fractions were then removed with a monostaltic pump and an Autodensiflow apparatus and collected in scintillation vials. The radioactivity of each fraction was determined after the addition of 10 ml of Bray's solution.

[^{3}H]*NE release from synaptosomes*

Preloading synaptosomes with [^{3}*H*]*NE.* Five ml of the synaptosomal suspension was transferred to polypropylene centrifuge tubes and incubated, with shaking, at 37° for 5 min. Fifty μl [^{3}H]NE (final NE concentration of 10^{-6} M) was then added and the incubation was continued for an additional 15 min. Samples were centrifuged at 15,000 *g* for 15 min. The supernatant fraction was discarded and the pellet resuspended in the same volume of incubation medium. This procedure was repeated three times to remove unbound and nonspecifically bound [^{3}H]NE.

Incubation conditions. Unless otherwise stated, 50 μl of the preloaded synaptosomal suspension was added to 0.95 ml of incubation medium (containing the appropriate concentration of drug) and incubated in 15-ml polypropylene beakers with shaking, at 37° for 30 min.

Incubations were terminated by filtration through 0.45 μm Millipore filters. Filters were then washed with 5 ml of incubation medium at 22°. The filters were placed in glass scintillation vials and the radioactivity was determined in 10 ml of Bray's solution.

In some experiments $CaCl_2$ was omitted from the incubation medium, incubations were carried out at 2, 22 and 42°, [^{3}H]inulin (5×10^{-5} M) was substituted for [^{3}H]NE (10^{-6} M) during the initial incubation, or the preloaded synaptosomes were incubated for 10 or 20 min.

Expression of results. The rate of uptake of [^{3}H]NE by synaptosomes is expressed as pmoles [^{3}H]NE/mg of synaptosomal protein/5 min; the K_i for NE uptake inhibitors was determined by the method of Dixon [9]. Release of [^{3}H]NE from synaptosomes was calculated as follows [10]:

$$\% \text{ Release} = \frac{\text{tissue dis./min (control)} - \text{tissue dis./min (experimental)}}{\text{tissue dis./min (control)}}$$

This method of calculating per cent release accounts for the spontaneous loss of [^{3}H]NE from synaptosomes during incubations. The per cent release was then plotted vs the log of drug concentration. The largest difference between any value and the mean of quadruplicate samples did not exceed 2.5 per cent in any experiment. The results of each experiment are expressed, therefore, as the mean of quadruplicate samples.

RESULTS AND DISCUSSION

Synaptosomal uptake studies

The uptake of [^{3}H]NE by hypothalamic synaptosomes was linear with time (for 30 min) and protein concentration (0.8 to 3.6 mg protein). The results of other control experiments demonstrated that uptake was markedly temperature dependent, had an absolute requirement for sodium, and was ouabain-sensitive. These findings are characteristic of synaptosomal NE uptake [11]. Over 90 per cent of the [^{3}H]NE taken up by synaptosomes during a 5-min incubation remained as the unchanged catecholamine. Subcellular distribution studies demonstrated that the [^{3}H]NE taken up by synaptosomes was concentrated in a particulate fraction which sedimented in 1.0 to 1.2 M sucrose (this fraction contained approximately 50 per cent of the total radioactivity applied to the gradient). This region has been found to concentrate hypothalamic synaptosomes and coincides with the distribution of potassium and lactic acid dehydrogenase [12]. These findings indicate that the uptake of [^{3}H]NE is a synaptosomal transport process.

The rate of [^{3}H]NE uptake by rat hypothalamic synaptosomes was determined at various NE concentrations (10^{-7} to 10^{-6} M). An Eadie–Hofstee plot of these data revealed the following kinetic parameters: $K_m = 1.91 \times 10^{-7}$ M; $V_{max} = 35.65$ pmoles [^{3}H]NE/mg of synaptosomal protein/5 min. These values are characteristic of a high affinity uptake process [5].

The potency of inhibitors of [^{3}H]NE was determined by plotting the reciprocal of [^{3}H]NE uptake ($1/v$) against the concentration of drug (Fig. 1). The intersection of the two lines gives K_i directly. For competitive inhibitors, the interaction occurs at a height equivalent to $1/V_{max}$ (Fig. 1A); for noncompetitive inhibitors, the intersection occurs at a point on the base line (Fig. 1B). The K_i and the type of inhibition were determined by this method for

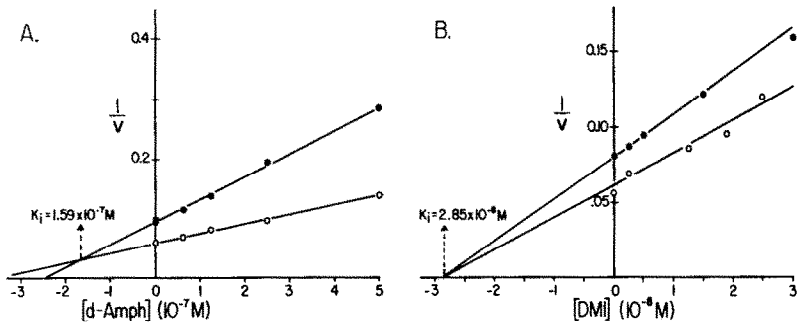

Fig. 1. Dixon plots [9] of the reciprocal of the rate of [^{3}H]NE uptake by rat hypothalamic synaptosomes ($1/v$) vs inhibitor concentration demonstrating the graphic estimation of K_i for the competitive inhibitor (+)-amphetamine (A) and for the noncompetitive inhibitor desmethylimipramine (DMI) (B). The rate of [^{3}H]NE uptake by synaptosomes, v, is expressed in pmoles [^{3}H]NE/mg of synaptosomal protein/5 min. Each point represents the mean of quadruplicate incubations. The largest difference between any value and the mean of the quadruplicate samples did not exceed 2.5 per cent in any experiment. Key: (●) represents mean values determined during incubation of an NE concentration of 1.0×10^{-7} M; (○) represents mean values obtained when the NE concentration was 2.0×10^{-7} M.

each drug investigated. (S)- and (R)-MDA are competitive inhibitors of NE uptake and are slightly less potent than (+)- and (−)-amphetamine respectively (Table 1). Racemic MDA was more potent than either of its isomers. This unusual finding was reproduced in four experiments.

The potency of (±)-, (S)- and (R)-MDA as inhibitors of the neuronal reuptake of NE suggests that this action may be at least partly responsible for some of the sympathomimetic effects produced by these drugs. (S)- and (R)-α-MeDA were also potent competitive inhibitors of [^{3}H]NE uptake (K_i = 1.21 and 4.45×10^{-9} M respectively). Heretofore, neither enantiomer of α-MeDA has been investigated as an inhibitor of [^{3}H]NE uptake, but the effects of (±)-α-MeDA on the uptake of [^{3}H]NE by noradrenergic neurons in the isolated rat heart have been examined by Burgen and Iversen [13]. In that system, the K_i of (±)-α-MeDA was 1.80×10^{-7} M. Desmethylimipramine (DMI), reported to be the most potent inhibitor of [^{3}H]NE uptake in rat hypothalamic synaptosomes [9], was found to be a potent, noncompetitive inhibitor of ^{3}H-uptake, with a K_i of 2.85×10^{-8} M. This value is approximately the same as that reported by other investigators [11, 14].

Table 1. Inhibition of the synaptosomal uptake of [^{3}H]NE by (±)-, (S)- and (R)-MDA and other drugs*

Type of inhibition	Drug	K_i(M)
Competitive	(S)-MDA	4.53×10^{-7}
	(R)-MDA	1.41×10^{-6}
	(+)-MDA	3.41×10^{-7}
	(+)-Amphetamine	1.59×10^{-7}
	(−)-Amphetamine	4.46×10^{-7}
	(±)-Amphetamine	2.13×10^{-7}
	(S)-α-MeDA	1.21×10^{-9}
	(R)-α-MeDA	4.45×10^{-9}
	(±)-MMDA-2	2.96×10^{-6}
Noncompetitive	Ouabain	3.27×10^{-6}
	Desmethylimipramine	2.85×10^{-8}

* Rat hypothalamic synaptosomes were incubated with 1.0 or 2.0×10^{-7} M [^{3}H]NE at 37° for 5 min in the presence or absence of drug, as described in the text. The type of inhibition and the K_i were estimated by the method of Dixon [9], as described in Fig. 1.

Synaptosomal release studies

Time course and temperature dependence of drug-induced release of [^{3}H]*NE by synaptosomes.* The spontaneous efflux of [^{3}H]NE from the control (non-drug-treated) preparations was greatest during the first 10 min of incubation; this spontaneous efflux of [^{3}H]NE became linear during the final 20 min of incubation. Racemic, (S)- and (R)-MDA released [^{3}H]NE from preloaded synaptosomes; this drug-induced release of [^{3}H]NE was linear for at least 30 min. [^{3}H]NE release by α-MeDA and amphetamine isomers was also linear with time.

Release of [^{3}H]NE by various drugs was markedly temperature dependent (Fig. 2). The release of [^{3}H]NE from synaptosomes by potassium (12.5 to 50.0 mM) was also temperature dependent. Potassium is thought to cause the release of [^{3}H]NE by depolarizing synaptosomal membranes, thus mimicking neurotransmitter release at nerve terminals [5].

Drug-induced release of [^{3}H]*NE: calcium dependence and dose–response relationships.* The dose–response curves for [^{3}H]NE release from synaptosomes elicited by various drugs are illustrated in Fig. 3. When Ca^{2+} (1.28 mM) was omitted from the incubation medium, the dose–response curves for (±)-, (S)- and (R)-MDA (Fig. 3A), (S)- and (R)-α-MeDA (Fig. 3B), and (±)-, (+)- and (−)-amphetamine (Fig. 3C) were influenced only slightly. On the other hand, the dose–response curve for potassium (Fig. 3C) was markedly shifted to the right. These results indicate that the release of synaptosomal NE by the amphetamine-like drugs tested is not significantly affected by the absence of Ca^{2+}. However, K-mediated release of NE, which is thought to mimic nerve stimulation, demonstrates a high degree of Ca^{2+} dependence.

(S)- and (R)-α-MeDA, which were potent inhibitors of [^{3}H]NE uptake by synaptosomes, were also potent releasing agents (Fig. 3B); (±)-2-methoxy-

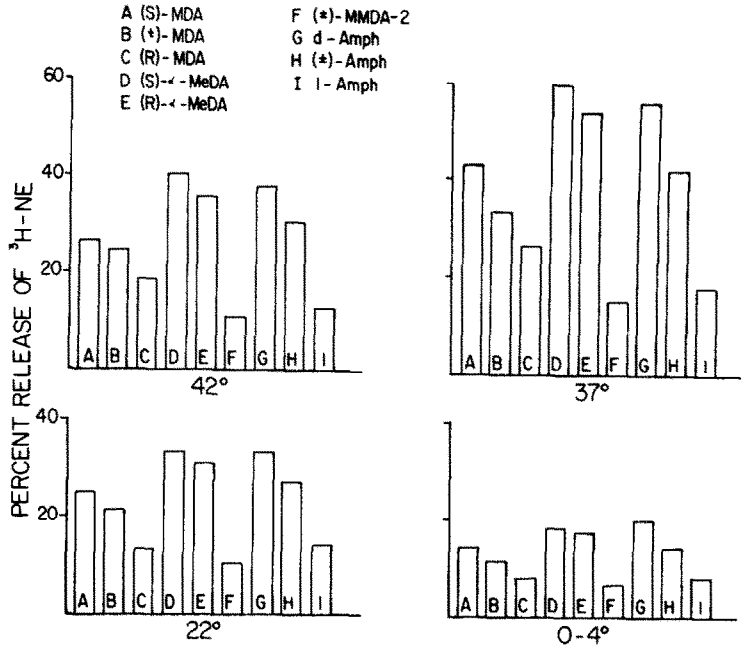

Fig. 2. Drug-induced release of [³H]NE from preloaded synaptosomes at incubation temperatures of 0–4, 22, 37 and 42°. Release experiments were carried out as described in the text. Each value represents the mean of quadruplicate incubations. All drugs were present at concentrations of 10^{-3} M.

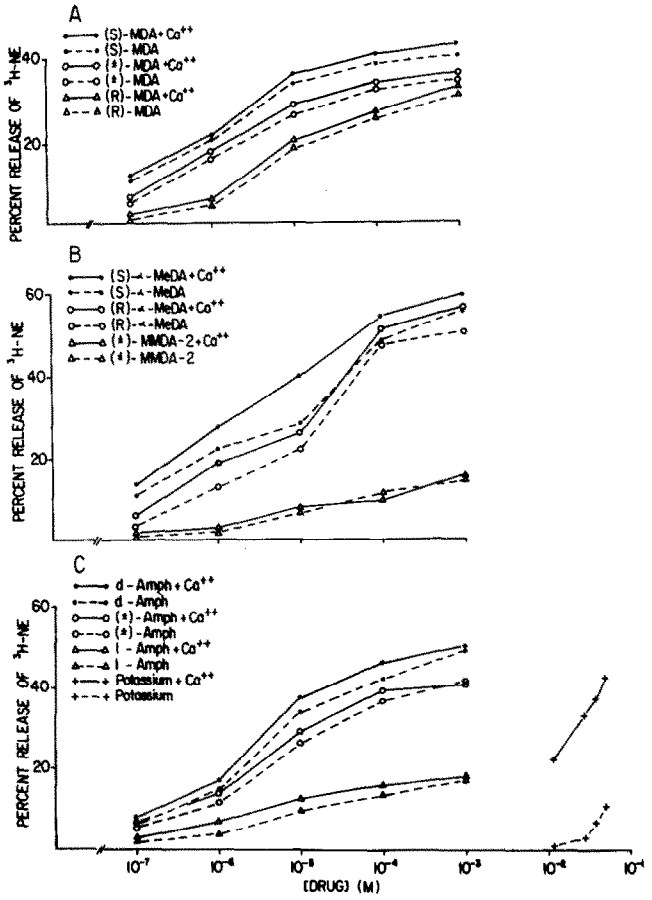

Fig. 3. Dose–response curves for drug-induced release of [³H]NE from preloaded synaptosomes in the presence or absence of 1.28 mM $CaCl_2$. Each point represents the mean of quadruplicate incubations.

4,5-methylenedioxyamphetamine [(±)-MMDA-2], an MDA analogue, was the least potent releasing agent examined (Fig. 3B). (S)- and (R)-MDA were nearly as potent as the corresponding isomers of amphetamine, both in blocking the synaptosomal uptake of NE and in causing the release of this neurotransmitter from preloaded synaptosomes. These findings suggest that the block of uptake and the release of NE may be at least partly responsible for some of the sympathomimetic effects produced by these drugs.

(S)- and (R)-α-MeDA were shown to be extremely potent inhibitors of NE uptake and stimulators of NE release from synaptosomes. The demonstration of the extreme potencies of the MDA metabolites, (S)- and (R)-α-MeDA, as inhibitors of synaptosomal NE uptake and stimulators of NE release from synaptosomes suggests a role for these substances in the late central actions of MDA [15]. This idea is further substantiated by the demonstration of the presence of these metabolites in the brain 6–24 hr after MDA treatment [2].

REFERENCES

1. G. M. Marquardt and V. DiStefano, *Life Sci.* **15**, 1603 (1974).
2. G. M. Marquardt, V. DiStefano and L. L. Ling, *Biochem. Pharmac.* **27**, 1503 (1978).
3. E. G. Gray and V. P. Whittaker, *J. Anat.* **96**, 79 (1962).
4. J. Glowinski and L. L. Iversen, *J. Neurochem.* **13**, 655 (1966).
5. G. Levi and M. Raiteri, *Brain Res.* **57**, 165 (1973).
6. G. A. Bray, *Analyt. Biochem.* **1**, 279 (1960).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
8. R. J. Baldessarini and M. Vogt, *J. Neurochem.* **18**, 951 (1971).
9. D. M. Dixon, *Biochem. J.* **55**, 170 (1953).
10. R. I. Heikkila, H. Orlansky, C. Mytilineou and G. Cohen, *J. Pharmac. exp. Ther.* **193**, 47 (1975).
11. L. L. Iversen, in *Drugs and Transport Processes* (Ed. B. A. Callingham), p. 275. University Park Press, Baltimore (1974).
12. V. P. Whittaker, in *The Handbook of Neurochemistry* (Ed. A. Lajtha), Vol. II, p. 327. Plenum Press, New York (1969).
13. A. V. S. Burgen and L. L. Iversen, *Br. J. Pharmac. Chemother.* **25**, 34 (1965).
14. A. S. Paintal, *Physiol. Rev.* **53**, 159 (1973).
15. G. M. Marquardt, V. DiStefano and L. L. Ling, in *The Psychopharmacology of Hallucinogens* (Eds. R. Willette and R. Stillman), Chap. 7. Pergamon Press, New York (1977).