Exhibit 8

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DOPAMINE NERVE TERMINAL DEGENERATION PRODUCED BY HIGH DOSES OF METHYLAMPHETAMINE IN THE RAT BRAIN

G. A. RICAURTE, R. W. GUILLERY, L. S. SEIDEN, C. R. SCHUSTER and R. Y. MOORE

(G.A.R., R.W.G., L.S.S. and C.R.S.) Department of Pharmacological and Physiological Sciences, and (C.R.S.) Department of Psychiatry, The University of Chicago, 947 East 58th Street, Chicago, IL 60637 (U.S.A.) and (R.Y.M.) Department of Neurology, Health Science Center, SUNY, Stony Brook, NY 11794 (U.S.A.)

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SUMMARY

Numerous recent studies indicate that when amphetamines are administered continuously or in high doses, they exert long-lasting toxic effects on dopamine (DA) neurons in the central nervous system (CNS). Specifically, it has been shown that amphetamines can decrease the content of brain DA, reduce the number of synaptosomal DA uptake sites and selectively depress the in vitro activity of neostriatal tyrosine hydroxylase (TH). To date, however, anatomical evidence of DA neuronal destruction following amphetamines has not been reported. In this study, chemical methods were used in conjunction with the Fink–Heimer method which allows for the selective silver impregnation of degenerating nerve fibers, in order to determine whether methylamphetamine, a potent psychomotor stimulant often abused by man, causes actual DA neural degeneration. It has been found that methylamphetamine induces terminal degeneration along with correlative DA neurochemical deficits in the neostriatum and nucleus accumbens; furthermore, that in cresyl violet-stained sections of the substantia nigra (SN), pars compacta, and ventral tegmental area (VTA), there is no evidence of cell body loss in rats in which 50–60% of neostriatal DA terminals have been destroyed.

INTRODUCTION

Amphetamines are indirectly acting synaptomimetic compounds which exert powerful psychomotor stimulant effects, probably through brain catecholamine-containing neurons[5,21]. The amphetamines have been used clinically in the treatment of

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childhood hyperkinesis, narcolepsy, obesity, and some types of mental depression; at times, they have also been rampantly abused[12]. We now present anatomical and corroborative chemical evidence of DA nerve terminal destruction in the rat brain following the repeated administration of methylamphetamine.

A number of recent observations have suggested that amphetamines exert toxic effects on DA neurons in the CNS. Rhesus monkeys, guinea pigs and rats treated with high doses of amphetamine or methylamphetamine display long-lasting, perhaps permanent, depletions of DA in the neostriatum[28,30,32], as well as in other brain regions[26]. In rats, a concomitant reduction in the number of neostriatal synaptosomal DA intake sites occurs[31]. The in vitro activity of rat neostriatal TH has also been found to be decreased from weeks[16,18] to as long as 110 days[7] after amphetamine administration. Moreover, catecholamine fluorescence studies have revealed structural alterations pathognomonic of neuronal damage in the neostriatum of amphetamine-treated rats[7]. Although the above-mentioned studies have clearly pointed to persistent chemical and anatomical alterations in the nigrostriatal DA system following amphetamines, they have left open the question of whether amphetamines cause actual DA neuronal destruction. The present study examined this possibility using the Fink-Heimer method. It has been found that methylamphetamine produces DA nerve terminal degeneration in the neostriatum and nucleus accumbens, but spares DA cell bodies in the pars compacta of the SN and the VTA.

MATERIALS AND METHODS

Subjects and drug treatments

Male albino rats of the Sprague–Dawley strain (Holtzman, Madison, WI), weighing 180–200 g, were housed individually with free access to food (Purina Lab Chow) and water in a colony room maintained at 22 ± 1 °C. Fluorescent lighting in the room was automatically turned on at 06.00 and off at 18.00 h. Experimental rats received 3 subcutaneous injections, 8 h apart of either 12.5 or 50 mg/kg of D-methylamphetamine hydrochloride dissolved in physiological saline at a concentration of 25 mg/ml. Dose was adjusted by varying the volume of drug solution that was injected. Control rats received only saline. Mortality in groups of rats receiving repeated doses of 12.5 or 50 mg/kg methylamphetamine hydrochloride was 5% (1/20) and 37% (31/72) respectively. Three weeks after drug treatment, the surviving rats appeared normal, although somewhat underweight (mean weight for the saline, 12.5 and 50 mg/kg groups was 248 ± 13 saline control, 243 ± 14 and 226 ± 10 g, respectively).

Neuronal degeneration studies

Rats for neuroanatomical studies of nerve fiber degeneration were killed 2 (n = 3), 4 (n = 6), or 6 (n = 3) days after the last methylamphetamine injection. An equal number of control rats was killed at each survival period. Rats were killed under sodium pentobarbital anaesthesia (40 mg/kg) by transcardial perfusion with 10% formal saline. Brains were immediately removed from the skull, stored under refrigeration in perfusion fluid for at least 1 week, then transferred to a 30%

sucrose–formalin solution. After the brains sank in the latter solution, 30 μm frozen coronal brain sections were collected in 5% formal saline and kept in cold storage for 4–7 days before being stained with silver according to procedure I of Fink and Heimer (ref. 9). This method allows for the selective silver impregnation of degenerating nerve fibers. Extreme care was always taken to stain control and experimental sections in parallel so as to control for day to day variability in the staining procedure. Silver-stained sections were mounted onto glass slides according to the method of Albrecht[1] and examined with a Zeiss light microscope by two independent observers who rated the sections according to the presence or absence of degeneration on a blind basis.

Rats for studies of cell bodies in the SN and VTA following the high dose (50 mg/kg) regimen of methylamphetamine were killed approximately 6 weeks after drug treatment in order to allow enough time for retrograde DA cell body degeneration to occur. These rats were perfused with Bouin's solution, their brains were embedded in paraffin and sections were stained with cresyl violet.

Biochemical determinations

Concentrations of dihydroxyphenylalanine (DOPA), DA, norepinephrine (NE) and dihydroxyphenylacetic acid (DOPAC) in brain tissue were determined by ion-exchange liquid chromatography coupled with electrochemical detection. The set-up for ion-exchange liquid chromatography with electrochemical detection has been described previously[17]. DOPA, DA and NE were assayed using the method of Fenn et al.[8]. DOPAC was measured with the method of Wightman et al.[33]. Level determinations were performed after the tissue has been processed through the alumina procedure of Anton and Sayre[2], as modified by Schellenberger and Gordon[29]. Acetic acid (1.0 M) was used to elute the catechols from the alumina. The recovery of DOPA, DA, NE, and DOPAC was approximately 60, 80, 80 and 45%, respectively. Rats for these studies were killed by decapitation 3 weeks after drug treatment.

Determination of neostriatal TH activity in vivo

In vivo neostriatal TH activity was determined by measuring neostriatal DOPA accumulation 30 min after inhibition of L-aromatic amino acid decarboxylase (which converts DOPA to DA) by 3-hydroxybenzylhydrazine hydrochloride (NSD-1015) (100 mg/kg, i.p.). Carlsson et al.[4] have shown that DOPA accumulation under these conditions provides an accurate estimate of in vivo neostriatal TH activity. Rats for this experiment were also killed 3 weeks after drug treatment.

Dissection

Dissection of the neostriatal tissue sample has been described previously[31].

Statistics

A two-tailed Student's *t*-test was employed to assess the significance of differences between group means.

Drugs and materials

Methylamphetamine hydrochloride was supplied by the National Institute

of Drug Abuse (Bethesda, MD); dihydroxyphenylalanine, dopamine hydrochloride, norepinephrine hydrochloride and dihydroxyphenylacetic acid were obtained from the Sigma Chemicals, St. Louis, MO. Potassium permanganate, silver nitrate, sodium thiosulfate, hydroquinine and citric acid were purchased from the J. T. Baker Chemicals, Philipsburg, NJ. Sucrose, permount, uranyl nitrate, xylenes, and formaldehyde solution were obtained from Fisher Scientific, Fair Lawn, NJ. 3-Hydroxybenzylhydrazine hydrochloride was a product of the Aldrich Chemicals, Milwaukee, WI.

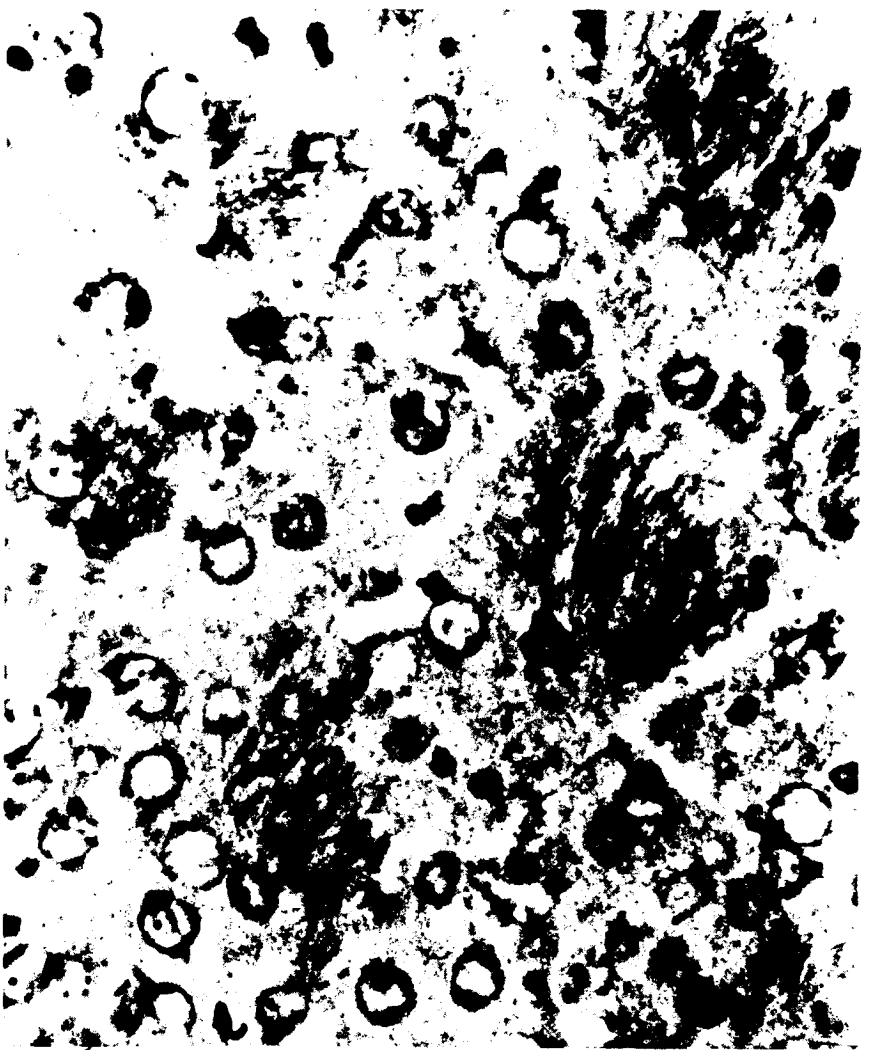

Fig. 1. Fine granular degeneration in the rat neostriatum following the high dose (50 mg/kg) regimen of methylamphetamine. Four-day survival period. Fink–Heimer method I with cresyl violet counterstain. × 1190.

Fig. 2. Absence of fine granular degeneration in the neostriatum of a rat treated with saline and killed after a 4-day survival period. Fink–Heimer method I with cresyl violet counter-stain. × 1190.

RESULTS

Neuronal degeneration

Terminals

Four days after the high dose (50 mg/kg) regimen of methylamphetamine, fine granular degeneration was found in the neostriatum of 6 out of 6 methylamphetamine-treated rats (Fig. 1). Five out of 6 matching control rats did not show any sign of neostriatal degeneration (Fig. 2). The other control rat was also judged to be a 'control, but an odd one' by one of the raters who evaluated the sections on a blind basis.

Argyrophylic degeneration was also found in the lateral portion of the nucleus accumbens. In contrast, no degeneration was found in the paraventricular nucleus and median eminence of the hypothalamus, two other brain areas which also contain DA nerve terminals[11,24], but which are resistant to the DA neurotoxic effect of methylamphetamine*. Degeneration was also absent in all other brain regions visible in the frontal plane at the level of the neostriatum (cf. 7020 in the König and Klippel atlas[19]), with the exception of the somatosensory cortex*. Only sparse fiber degeneration was found in the neostriatum of rats also treated with the high dose (50 mg/kg) methylamphetamine regimen but killed after 2 ($n = 3$) or 6 ($n = 3$) rather than 4-day survival periods.

Neuro-degenerative changes were not evidence in the neostriatum or nucleus accumbens of rats treated with the lower dose (12.5 mg/kg) regimen of methylamphetamine 4 days previously.

Cell bodies

In Nissl stained sections through the SN and VTA of rats treated with toxic doses of methylamphetamine approximately 6 weeks previously, there was no indication of neuronal loss in either the SN, pars compacta, or VTA. Indeed, in a blind study of Nissl-stained control and methylamphetamine-treated brains, the two groups could not be distinguished.

DA levels

In rats treated identically as those showing histological signs of terminal degeneration in the neostriatum and nucleus accumbens, the brain concentration of DA and its major metabolite DOPAC were decreased by 52 ± 8 and 62 ± 12%, respectively (Table I). It has been previously shown that in comparably treated rats, the level of neostriatal and mesolimbic DA is likewise reduced[26].

* In lamina III and IV of the primary somatosensory cortex, we have found a select group of neuronal perikarya that are sensitive to the toxic action of methylamphetamine. This novel form of methylamphetamine neurotoxicity will be detailed in a separate publication.

TABLE I

Effect of two methylamphetamine (MA) doses (12.5 and 50 mg/kg) given repeatedly (3 times, 8 h apart) on the brain content of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and norepinephrine (NE)

Rats were killed 3 weeks after the last drug injection. Values shown are the mean ± S.E.M. and expressed in μg/g wet tissue weight.

Group	*N*	*DA*	*DOPAC*	*NE*
Control	6	0.99 ± 0.02	0.08 ± 0.01	0.47 ± 0.02
MA 12.5	6	0.94 ± 0.03	0.07 ± 0.01	0.51 ± 0.03
MA 50	6	0.47 ± 0.08**	0.03 ± 0.01**	0.48 ± 0.02

** Significantly different from control group $P < 0.05$; two-tailed Student's *t*-test.

TABLE II

Effect of methylamphetamine treatment on in vivo neostriatal TH activity

Rats were killed 3 weeks after drug treatment.

Treatment	*N*	*Neostriatal TH activity (μg/g 30 min)*	*Decrease*
Saline	8	1.29 ± 0.11	—
Methylamphetamine (50 mg/kg × 3, 8 h apart)	8	0.62 ± 0.09*	52

* Significantly different from saline group $P < 0.05$, two-tailed Student's *t*-test.

In rats treated with the lower dose (12.5 mg/kg) regimen of methylamphetamine 3 weeks previously, the brain levels of DA and DOPAC were not found to be significantly different from those in control rats (Table I). It is to be noted that in these rats, histological signs of nerve terminal degeneration were also not found.

NE levels

Table I shows that neither the low (12.5 mg/kg) nor high (50 mg/kg) dose regimen of methylamphetamine altered the level of brain NE on a long term basis.

TH activity

In vivo TH activity was reduced by 52 ± 7% in the neostriatum of rats treated with the high dose (50 mg/kg) regimen of methylamphetamine 3 weeks previously (Table II). This decrement in TH activity is of approximately the same magnitude as the long-term neostriatal DA depletion found in comparably treated rats[26,31].

DISCUSSION

The major aim of this study was to determine whether high doses of methylamphetamine caused actual DA neuronal destruction in the rat brain. The present findings indicate that rats treated with a high dose (50 mg/kg) regimen of methylamphetamine have not only long-lasting DA depletions and fewer synaptosomal DA uptake sites, as has been reported previously[26,31,33], but also profound and persistent reductions in the in vivo activity of neurostriatal TH and in the brain concentration of DOPAC, the major metabolite of brain DA. The occurrence of these DA neurochemical deficits following methylamphetamine strongly suggests that methylamphetamine does produce DA nerve terminal destruction.

Anatomical studies of nerve fiber degeneration support this conclusion. Using the Fink–Heimer technique which allows for the selective silver-impregnation of degenerating nerve fibers, degeneration has been found in the neostriatum (Fig. 1) and lateral portion of the nucleus accumbens of rats treated 4 days previously with the high dose (50 mg/kg) regimen of methylamphetamine, a regimen known to produce long-

term DA neurochemical deficits (Tables I and II). Both the neostriatum and the nucleus accumbens are known to be innervated by DA-containing nerve terminals[11,24] and to be sensitive to methylamphetamine's DA neurotoxic effect[26] (Table I). In contrast, no evidence of nerve fiber degeneration was found in the paraventricular nucleus and median eminence of the hypothalamus, brain regions which also contain DA terminals[11,24], but which are resistant to methylamphetamine's DA neurotoxic action[26]. Worthy of note is the fact that degeneration was not found in the neostriatum of rats treated with lower dose (12.5 mg/kg) regimen of methylamphetamine, a regimen which did not produce DA neurochemical deficits (Table I). Further, only sparse neostriatal degeneration was found in rats treated with the higher dose (50 mg/kg) regimen of methylamphetamine, but killed after 2- or 6- rather than 4-day survival periods. This indicates that the time-course of methylamphetamine-induced neostriatal degeneration is comparable to that of neostriatal DA terminal degeneration produced by 6HDA[14,22], a known DA neurotoxin[3]. Thus, although it is not possible to state definitively that the degenerating terminals demonstrated with the Fink-Heimer technique are dopaminergic, their anatomical distribution, their time-course of degeneration, and the corroborative chemical evidence cited above strongly support this conclusion.

The present findings agree well with those of other investigators assessing the neurotoxic properties of amphetamines. Both Ellison et al.[7] and Hotchkiss and Gibb[16] have reported that amphetamine and methylamphetamine, when administered continuously or in high doses cause a prolonged depression in the in vitro activity of neostriatal TH. This study's finding of reduced in vivo neostriatal TH activity (Table II) is in accordance with these reports and extends them to the in vivo situation. Also, based on their finding of enlarged brightly fluorescent axons in the neostriatum of amphetamine-treated rats, Ellison et al.[7] proposed that continuous amphetamine administration damaged neostriatal DA neurons. The present finding of neostriatal terminal degeneration following methylamphetamine lends support to this notion and indicates that this damage involves actual DA terminal destruction.

Given the occurrence of DA nerve terminal destruction following methylamphetamine, the question arises as to whether the DA cell bodies in the brain stem which give rise to these terminals, are also destroyed by toxic doses of methylamphetamine. Irreversible DA cell body damage could result from either retrograde changes or from a direct cytotoxic action of methylamphetamine on DA perikarya. In Nissl-stained sections at the level of the SN, pars compacta, and VTA, there was no indication of cell body loss. This indicates that the toxic effect of methylamphetamine is largely, if not exclusively, on DA nerve terminals. Further, the preservation of DA neuronal perikarya and the partial nature of the methylamphetamine-induced DA depletion and TH depression (Tables I and II) indicate that sufficient DA terminals remain to prevent retrograde DA cell body degeneration.

With the drug regimen employed in this study, high doses of methylamphetamine were required to produce DA nerve terminal destruction. A recent report[10] suggests that this is probably related to the relatively short half-life of amphetamine in the rat brain[20]. Nevertheless, given the use of these high doses, it was important to

assess the selectivity of methylamphetamine's DA neurotoxic action. To this end, the status of central NE neurons was examined. As shown in Table I, the higher dose (50 mg/kg) regimen of methylamphetamine, the same drug regimen which caused profound DA neuronal deficits, did not alter brain NE levels. This suggests that central noradrenergic neurons are not damaged by methylamphetamine. Wagner et al.[31], after treating rats with comparably high doses of methylamphetamine, found that the regional level of brain NE in these rats was normal. This mitigates against the possibility that this study's whole brain NE analysis failed to detect a regional NE deficit. The absence of long-lasting neurochemical changes in the NE system is important for it demonstrates that the DA neurotoxic effect of methylamphetamine is selective. This selectivity is further substantiated by the fact that high doses of methylamphetamine do not alter enzymatic markers of neostriatal cholinergic or GABAergic neurons[18].

Recently, while further assessing the selectivity of methylamphetamine's neurotoxic action, we found methylamphetamine to also be toxic to central 5-HT-containing neurons. As we have reported elsewhere[26], methylamphetamine causes profound, long-lasting and dose-related reductions in the whole and regional level of brain 5-HT, in the V_{max} of synaptosomal [^{3}H]5-HT uptake and in the brain level of 5-HIAA, the major metabolite of 5-HT. These findings, coupled with the recent observation that methylamphetamine also causes a prolonged depression in the in vitro activity of tryptophan hydroxylase (TPH)[16], the rate-limiting enzyme in 5-HT biosynthesis, strongly suggests that methylamphetamine also destroys 5-HT terminals. The fact that both DA and 5-HT terminals are destroyed by methylamphetamine raises the question of whether any of the degenerating terminals demonstrated with the Fink–Heimer method in Fig. 1 are serotonergic and not dopaminergic. At the present time, it is reasonable to conclude that none of the Fink–Heimer degeneration in Fig. 1 is serotonergic since numerous investigators[6,13,15,23,27] have reported that 5-HT terminal degeneration is not demonstrable with the Fink–Heimer method.

In contrast, the demonstrability of DA terminal degeneration with this same method is well documented[14,22]. Thus, it seems reasonable to conclude that the terminal degeneration shown in Fig. 1 is indeed of DA terminals.

The observation that granular degeneration could be observed after 4 days post-treatment but not after 2 or 6 days suggests that the changes take at least 4 days to develop to the point where silver can sufficiently impregnate the degenerating terminals. Furthermore, since the DA content is lowered at 6 day survival times and as long as 8 weeks drug-free, it appears that the loss of DA fibers is permanent[31]. The degeneration appears to occur 4 days after the last injection of MA; at 6 days the degenerating fibers that can take up the silver have cleared the brain.

In summary, the data presented here provide correlative anatomical and chemical evidence of DA nerve terminal destruction without consequent retrograde DA cell body degeneration in the brain of rats previously treated with high doses of methylamphetamine. Whether similar neurodegenerative changes occur in the human brain as a result of amphetamine abuse remains to be determined. Finally, it is becoming increasingly apparent that neurotoxicity, long thought to be a unique

property of halogenated amphetamine derivatives (e.g. PCA) is, in fact, a property of the amphetamine compounds themselves.

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