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LONG-TERM EFFECTS OF REPEATED METHYLAMPHETAMINE ADMINISTRATION ON DOPAMINE AND SEROTONIN NEURONS IN THE RAT BRAIN: A REGIONAL STUDY

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SUMMARY

Repeated high doses (25 and 100 mg/kg) of methylamphetamine produce long-term depletions of both dopamine (DA) and serotonin (5-HT) in the rat brain. In the DA system, depletions are most pronounced in the neostriatum and substantia nigra, with decreased levels in these two regions being significantly correlated. Within the 5-HT system, levels are most reduced in the amygdala, frontal cortex and neostriatum. When both the DA and 5-HT depleting actions of methylamphetamine are considered, the hypothalamus stands out as one of the more resistant brain regions. The regional pattern of reduced 5-HT levels following methylamphetamine is similar to that seen after p-chloroamphetamine. After both methylamphetamine and p-chloroamphetamine, a loss of 5-HT synaptosomal uptake sites occurs. Serotonergic systems are more sensitive than DA systems to the apparent neurotoxic actions of methylamphetamine.

INTRODUCTION

Repeated administration of high doses of methylamphetamine to monkeys, guinea pigs and rats results in a large and long-lasting reduction of neostriatal DA[29,35]. In rats, the decrease in neostriatal DA content is accompanied by a long-term loss of synaptosomal DA uptake sites[34]. High doses of methylamphetamine also cause a prolonged reduction in the activity of neostriatal tyrosine hydroxylase[14,25], the rate-limiting enzyme in the DA biosynthetic pathway[20]. In addition, structural changes, pathognomonic of neuronal damage, have been noted using histofluorescent tech-

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niques in neostriatal DA terminals following continuous amphetamine administration[2]. Taken together, these findings suggest that amphetamines exert toxic effects on DA neurons in the central nervous system.

Although amphetamines are known to interact with various neurotransmitter substances in the brain, including norepinephrine (NE), DA, and 5-HT[1], little information is presently available concerning the specificity of amphetamine's neurotoxic actions. To date, it has been shown that NE neurons are not affected in the rat brain[34], as they are in the monkey[29], and that enzymatic markers for neostriatal cholinergic and GABAergic neurons are not altered by repeated methylamphetamine administration[25]. However, the status of extrastriatal DA neurons and 5-HT neurons has, to our knowledge, not yet been assessed.

In the present investigation, we examined the long-term effect of methylamphetamine intoxication on DA and 5-HT neurons in various regions of the rat brain. Since the various central DA systems do not respond in a uniform fashion to pharmacological manipulations[12,17,24], the possibility of differential sensitivity to methylamphetamine's neurotoxic actions was explored by measuring DA levels in numerous brain regions after two different methylamphetamine regimens (25 and 100 mg/kg/day $\times$ 4).

Although brain catecholaminergic neurons mediate many of the central actions of amphetamines, serotonergic neurons also seem to play a role when amphetamines are administered in high doses[19,30,33]. There is also the precedent of the halogenated amphetamines and their known ability to exert toxic effects on central 5-HT neurons[9,27]. For these reasons, the long-term effect of repeated methylamphetamine administration on cerebral 5-HT neurons was examined by measuring regional 5-HT levels and 5-HT uptake into synaptosomes three weeks after the repeated administration of high doses of methylamphetamine.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (180–200 g) obtained from the Holtzman Co. (Madison, Wisc.) were housed singly in wire-mesh cages with free access to food (Purina Lab Chow) and water. Colony room fluorescent lighting was on a 12-h light–dark cycle and ambient temperature was maintained at 22 $\pm$ 1.0 °C. Before receiving any drug, rats were given 2–3 days to adapt to their home cages. Rats were injected subcutaneously twice a day (09.00 and 17.00 h) for 4 consecutive days with methylamphetamine hydrochloride dissolved in physiological saline at a concentration of 25 mg/ml.

Two groups of rats received either 25 or 100 mg/kg/day of the methylamphetamine hydrochloride salt (molar equivalent: 133 and 532 μmol/kg/day, respectively). Dose was adjusted by changing the volume of the 25 mg/ml methylamphetamine solution that was injected. Control animals were injected with saline only. Mortality in the 25 and 100 mg/kg/day dose groups was 7 (1/15) and 43 (13/30) per cent, respectively. None of the rats receiving saline died. After drug treatment, the

mean weight for the saline, 25 and 100 mg/kg methylamphetamine groups was 230 ± 15, 208 ± 12 and 190 ± 9 g, respectively.

Rats for uptake studies were treated concomitantly with rats used for level determinations. They differed only in that some also received PCA and in that they were sacrificed 6 rather than 3 weeks after the last injection.

Regional brain dissection

Rats were decapitated, the brain was removed and placed on its dorsal surface on a cold aluminum cutting block and 5 coronal razor cuts were made. The first, which serves as a reference, was at the level of the optic chiasm (about 7020 in the König and Klippel atlas[16]). The second and third cuts were placed 1.5 and 3.0 mm anteriorly. The fourth and fifth cuts were made 4.0 and 5.5 mm posteriorly. This yielded a total of 6 brain slices. Proceeding in a rostral to caudal direction, the neocortex, neostriatal and mesolimbic area samples were obtained from the first 3 brain slices essentially as described by Heffner and Seiden[11]. The hypothalamus and amygdala samples were taken from the fourth brain slice. With the slice lying on its caudal surface, a horizontal cut was made along the anterior commissure connecting the superior aspects of the rhinal sulci. Amygdala samples (which also contain some rhinal cortex) were then isolated by extending the tips of the external capsule inferiorly and medially. The hypothalamic sample was obtained by making para-sagittal cuts along the mamillothalamic tracts and perpendicular to the horizontal portion of the anterior commissure. The substantia nigra was dissected out of the fifth brain slice with it lying on its rostral surface using the crus cerebri and medial lemniscus as landmarks. The brain stem sample was derived from the sixth brain slice. All cerebellar and cortical tissue (including hippocampus) was removed.

Promptly after dissection, each of the brain regions was wrapped in aluminum foil and stored frozen in liquid nitrogen until assay. Mean (± S.E.M.) DA and 5-HT concentrations in each of the brain regions are shown in Tables I and II. These values, expressed as ng DA/5-HT per mg of protein, agree well with previously published determinations[3,6,8].

DA and 5-HT level determinations

The DA and 5-HT content of the various brain regions was determined by cation-exchange liquid chromatography coupled with electrochemical detection as previously described by various authors[13,26], with minor modifications. The mobile phase was an acetate–citrate buffer twice the concentration of that utilized by Keller et al.[13]. A 1.0 m × 2.1 mm glass column dry-packed with Dupont Zipax SCX resin was used. Flow rate was 0.5 ml/min and the detector potential was set at + 0.72 V vs an Ag/AgCl reference electrode.

Brain parts were homogenized in 200–500 μl 0.4 N perchloric acid containing 0.5 g Na_2H_2 EDTA·$2H_2O$ and 1.0 g $Na_2S_2O_5$ per liter. Homogenates were transferred to small polypropylene tubes and immediately frozen in liquid nitrogen until assay. Twenty μl of the samples were assayed.

The amounts of DA and 5-HT in individual samples were determined by

comparing each sample's peak height with that of a standard and by multiplying by the necessary correction factors (see ref. 3, for more details)., Retention times for DA and 5-HT were 7 and 11 min, respectively.

[^{3}H]5-HT synaptosomal uptake

The in vitro uptake of [^{3}H]5-HT was studied using a modification of the Snyder and Coyle method[31] described in detail elsewhere[34]. Briefly, after decapitation, rat brains were removed and split in half by making a sagittal razor cut between the cerebral hemispheres. After discarding the cerebellum, one brain half was placed in liquid nitrogen for later 5-HT content determination; the other was immediately used for uptake studies. Brain tissue was homogenized in 9 vol. (w/v) of ice-cold 0.32 M sucrose. Under the conditions employed, [^{3}H]5-HT accumulation was linear for at least 6 min, temperature-sensitive and markedly inhibited by 0.5 μm chlorimipramine or fluoxetine but not by benztropine. Uptake activity was expressed as dpm [^{3}H]5-HT accumulated per mg tissue per 5 min after correcting for [^{3}H]5-HT accumulation at 0–4 °C.

Statistical analysis

The significance of differences between group means was assessed with a two-tailed Student's *t*-test without assuming equal group variances. The Scheffe test[28] was integrated into the statistical analysis to allow for multiple comparisons between group means. Correlations between depletions in the various brain regions were determined by linear regression. Differences were considered statistically significant only when the *P* values were less than 0.05.

Protein determinations

Protein was determined according to the method of Lowry et al.[22] or Biuret[7].

Drugs and materials

Methylamphetamine hydrochloride was supplied by the National Institute of Drug Abuse (Bethesda, Md.); 5-hydroxytryptamine creatine sulfate complex, dopamine hydrochloride, bovine serum albumin (Sigma Chemical Co., St. Louis, Mo); DL-p-chloroamphetamine hydrochloride (Regis Chemical Co., Morton Grove, Ill.); chlorimipramine (Ciba-Geigy Corp., Summit, N.J.); benztropine mesylate (Merck Sharp and Dohme, West Point, Pa); pargyline hydrochloride (Saber Labs Inc., Morton Grove, Ill.); [1,2-^{3}H(N)]5'-hydroxytryptamine creatinine sulfate (New England Nuclear, Boston, Mass.); GF/A filters (Whatman Co., Clifton, N.J.); quantafluor (Mallinckrodt Co., St. Louis, Mo.).

RESULTS

Regional DA levels after repeated methylamphetamine

Rats treated for 4 days with 25 mg/kg/day methylamphetamine are not significantly depleted of DA in any of the brain regions examined (Table I). Three

TABLE I

Regional brain DA levels in rats treated with 25 or 100 mg/kg/day methylamphetamine hydrochloride for 4 days three weeks previously

Values shown are the mean ± S.E.M. expressed in ng/mg protein.

Treatment	*Brain region*							
	Neocortex	*Mesolimbic area*	*Neostriatum*	*Septum*	*Amygdala*	*Hypothalamus*	*Substantia nigra*	*Brain stem*
Saline (n = 13)	0.28 ± 0.02	37.5 ± 2.5	53.9 ± 2.4	5.34 ± 1.07	1.56 ± 0.18	1.41 ± 0.13	2.36 ± 0.14	0.40 ± 0.02
25 mg/kg/day × 4 Methylamphetamine (n = 9)	0.29 ± 0.03	32.2 ± 3.2	44.1 ± 3.6	4.41 ± 0.77	1.72 ± 0.22	1.33 ± 0.15	1.98 ± 0.19	0.46 ± 0.03
100 mg/kg/day × 4 Methylamphetamine (n = 13)	0.22 ± 0.01*	30.3 ± 3.1*	31.5 ± 4.0*	4.13 ± 0.60	1.45 ± 0.23	1.44 ± 0.18	1.62 ± 0.13*	0.46 ± 0.03

* Significantly different from saline control ($P \leq 0.05$; two-tailed Student's *t*-test coupled with Scheffé test; group variances not assumed equal).

TABLE II

Regional brain 5-HT levels in rats treated with 25 or 100 mg/kg/day methylamphetamine hydrochloride for 4 days three weeks previously

Values shown are the mean ± S.E.M. expressed as ng/mg protein.

Treatment	*Brain region*							
	Neocortex	*Mesolimbic area*	*Neostriatum*	*Septum*	*Amygdala*	*Hypothalamus*	*Substantia nigra*	*Brain stem*
Saline (n = 13)	2.25 ± 0.05	5.75 ± 0.26	2.19 ± 0.11	2.19 ± 0.11	4.26 ± 0.13	3.05 ± 0.20	2.51 ± 0.22	3.76 ± 0.10
25 mg/kg/day × 4								
Methylamphetamine (n = 9)	1.29 ± 0.17*	5.83 ± 0.58	1.73 ± 0.22	1.92 ± 0.27	2.46 ± 0.36*	2.26 ± 0.12*	2.10 ± 0.22	2.81 ± 0.12*
100 mg/kg/day × 4								
Methylamphetamine (n = 13)	0.82 ± 0.17*	3.79 ± 0.50*	1.14 ± 0.23*	1.62 ± 0.25*	1.57 ± 0.24*	2.42 ± 0.10*	1.80 ± 0.20*	2.48 ± 0.11*

* Significantly different from saline control ($P < 0.05$; two-tailed Student's *t*-test coupled with Scheffé test; group variances not assumed equal).

weeks after the 4-day 100 mg/kg/day methylamphetamine regimen, significantly reduced DA levels are seen in the neocortex (21 ± 4%), mesolimbic area (19 ± 8%), neostriatum (42 ± 10%), and substantia nigra (35 ± 9%) (Table I). Neostriatal DA depletions are significantly correlated with depletions in the substantia nigra ($r = 0.51$; $P < 0.05$).

Regional 5-HT levels after repeated methylamphetamine

Three weeks after a 4-day regimen of 25 mg/kg/day methylamphetamine, 5-HT levels are significantly reduced in the rat neocortex (43 ± 8%), amygdala (42 ± 8%), hypothalamus (26 ± 4%), and brain stem (26 ± 3%) (Table II).

Treatment with 100 mg/kg/day × 4 methylamphetamine causes a significant reduction in the 5-HT content of all brain regions examined (Table II). The largest 5-HT depletions occur in the neocortex (64 ± 8%), neostriatum (48 ± 11%), and amygdala (63 ± 6%).

The two methylamphetamine treatments (25 and 100 mg/kg/day × 4) are equieffective in reducing hypothalamic 5-HT content. Serotonergic terminals in the mesolimbic area are only affected by the higher (100 mg/kg) daily methylamphetamine dose (Table II).

Effect of repeated methylamphetamine on 5-HT uptake

Rats treated with 25 and 100 mg/kg/day × 4 methylamphetamine and sacrificed 6 weeks later display a 29 and 51% decrease in the V_{max} of 5-HT uptake, respectively (Fig. 1). In two separate replications of this same experiment, V_{max} values were

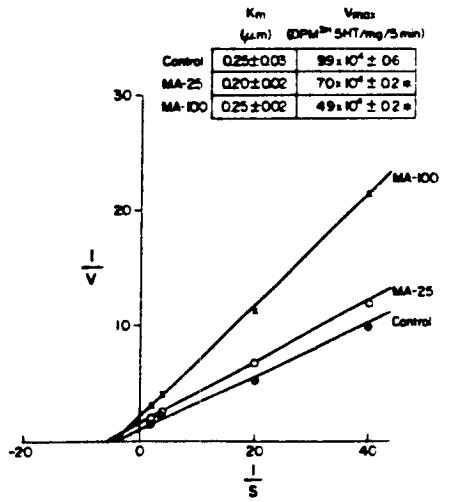

Fig. 1. Lineweaver and Burk[21] analysis of [³H]5-HT uptake by whole brain homogenates from control and methylamphetamine-treated rats. Animals were killed 6 weeks after a 4-day treatment with one of two daily doses (25 or 100 mg/kg) of methylamphetamine. [³H]5-HT uptake (V) is expressed as dpm [³H]5-HT/mg tissue/5 min × 10^{-5}. S = [³H]5-HT concentration varying from 0.05 to 0.5 μM. Results shown are from one representative experiment replicated twice. Brain 5-HT content was 79 and 42% of control for the 25 and 100 mg/kg/day × 4 rats, respectively. *Significantly different from control ($P < 0.05$).

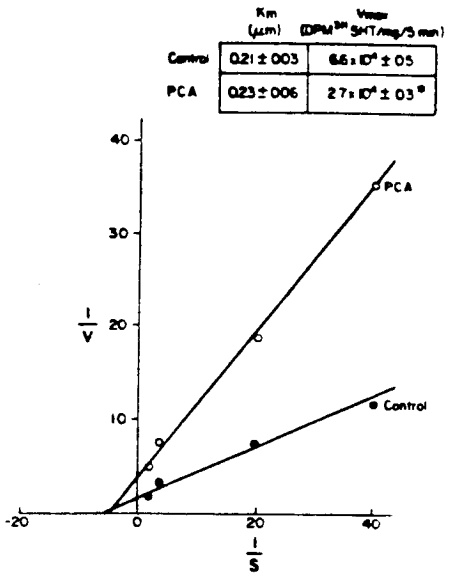

Fig. 2. Lineweaver and Burk[21] analysis of [^{3}H]5-HT uptake by whole brain homogenates from control and p-chloroamphetamine-treated rats. Animals were killed 6 weeks after a single injection of saline or p-chloroamphetamine (10 mg/kg). [^{3}H]5-HT uptake is expressed as dpm [^{3}H]5-HT/mg tissue/5 min $\times 10^{-5}$. S = [^{3}H]5-HT concentration varying from 0.05 to 0.5 μm. Results shown are from one representative experiment replicated twice. Brain 5-HT content in p-chloroamphetamine animal was 49% of control. * Significantly different from control ($P \leq 0.05$).

reduced by 19 and 59% in the first replication and by 34 and 63% in the second replication. The K_m of the 5-HT uptake system was not significantly altered by either methylamphetamine regimen (Fig. 1). Brain 5-HT content was decreased by 21 and 48% in rats treated with 25 and 100 mg/kg/day methylamphetamine for 4 days, respectively (kinetics shown in Fig. 1).

Effect of p-chloroamphetamine on synaptosomal 5-HT uptake

Six weeks after a single i.p. injection of p-chloroamphetamine (10 mg/kg) the V_{max} of whole brain synaptosomal 5-HT uptake is reduced by 59% (Fig. 2). In two replications of this same experiment, the V_{max} of 5-HT uptake was reduced by 43 and 49%. p-Chloroamphetamine does not produce a long-term change in the K_m of 5-HT uptake sites (Fig. 2). Following p-chloroamphetamine, whole brain 5-HT content is decreased by 51% (kinetics shown in Fig. 2).

DISCUSSION

The present results show that high doses of methylamphetamine produce a long-term depletion of DA not only in the neostriatum but also in three other rat brain regions: the neocortex, mesolimbic area (containing most of the nucleus accumbens and part of the olfactory tubercle), and the substantia nigra. Three weeks after

treatment with methylamphetamine DA levels in the neostriatum and substantia nigra are reduced by about 40% whereas those in the frontal cortex and mesolimbic area are decreased by approximately 20% (Table I). These results lend further support to the notion that the various central DA systems do not respond in a uniform fashion to pharmacological manipulations[12,17,24]. Also, the fact that DA depletions are largest and significantly correlated in the neostriatum and substantia nigra suggests that, of the various central DA systems, the nigrostriatal DA system is the most sensitive to the neurotoxic actions of methylamphetamine.

The finding that neither methylamphetamine regimen (25 or 100 mg/kg/day × 4) produced a long-term DA depletion in the hypothalamus is significant in two respects. First, it further attests to the relative refractoriness of tuberoinfundibular DA neurons to toxicological stress[5]. Second, it suggests the neurochemical deficits here reported are not due to a non-specific effect such as anoxia. If such an effect were at work, it is doubtful that methylamphetamine could be as anatomically and chemically selective at it is. Not only are DA levels in the hypothalamus not affected (Table I), NE levels throughout the rat brain are unchanged[34,35]. Moreover, while methylamphetamine does not affect DA neurons in the hypothalamus, it does reduce 5-HT levels in this same brain region (Table II). It seems unlikely that such selectivity could be achieved by non-specific means.

Perhaps the most significant finding of the present study is that methylamphetamine also causes prolonged changes in brain 5-HT neurons. As long as three weeks after the last methylamphetamine injection, there are substantial 5-HT depletions in various brain regions (Table II). Moreover, the V_{max} of the 5-HT uptake system is concomitantly reduced (Fig. 1). The latter effect could be due to a methylamphetamine-induced loss of 5-HT synaptosomal uptake sites. How methylamphetamine reduces both 5-HT levels and uptake site number is not yet clear. One possibility is that it does so by inducing 5-HT nerve terminal destruction. The fact that p-chloroamphetamine (CPA), a chemically related compound and a presumed serotonergic neurotoxin[9,27], causes similar neurochemical deficits (Fig. 2) supports this contention.

It is noteworthy that the 25 mg/kg/day × 4 methylamphetamine regimen did not significantly alter DA levels (Table I) but did substantially reduce the level of 5-HT in several brain regions (Table II). The same methylamphetamine treatment also reduced the number of 5-HT uptake sites (Fig. 1) but not of DA uptake sitse[34]. Hence, it would appear that 5-HT neurons are more sensitive than DA neurons to the toxic actions of methylamphetamine.

The regional pattern of long-term 5-HT depletions observed after methylamphetamine bears a certain resemblance to that seen after PCA[4,15,27]. After both drugs, brain regions severely affected include the cortex, neostriatum, septum and other portions of the limbic system. Some of the more resistant brain regions to the depleting actions of both compounds include the brain stem and hypothalamus. Though suggestive and perhaps informative, these similarities must be interpreted with caution. Differences in brain sampling, relative drug potencies and schedule of drug administration do not yet permit a rigorous comparison between the toxic actions of these two structurally related compounds.

Although most of the acute and long-term effects of methylamphetamine and PCA are similar[23,27], there are also some differences. Repeated and larger doses of methylamphetamine are needed to mimic the 5-HT neurotoxic effects produced by a single dose of PCA. However, this may be related to the fact that the half-life of PCA is about 8 times longer than that of its parent compound[18,32]. Methylamphetamine and PCA also differ in that the toxic effects of PCA appear to be restricted to 5-HT neurons[8,23,27], whereas those of methylamphetamine extend to DA neurons. This difference should be qualified, however, since when methylamphetamine is administered in lower doses, it also selectively affects 5-HT neurons (Tables I and II) and when PCA is administered in higher doses (15–20 mg/kg) it may also affect DA neurons[9,10]. Despite these differences, it may well be that amphetamines and their halogenated derivatives exert their neurotoxic actions in a similar fashion.

The precise mechanisms underlying the prolonged reduction of brain DA and 5-HT content and uptake following methylamphetamine intoxication remain to be elucidated. At present, the most parsimonious interpretation is that a loss of DA and 5-HT nerve terminals occurs. This hypothesis awaits direct histological evaluation.

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