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Differential Inhibition of Synaptosomal Accumulation of [^{3}H]-Monoamines by Cocaine, Tropacocaine and Amphetamine in Four Inbred Strains of Mice

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BOSY, T Z AND J A RUTH *Differential inhibition of synaptosomal accumulation of [^{3}H]-monoamines by cocaine, tropacocaine and amphetamine in four inbred strains of mice* PHARMACOL BIOCHEM BEHAV **34**(1) 165–172, 1989 —The relative ability of cocaine, tropacocaine and amphetamine to inhibit the uptake of [^{3}H]norepinephrine (NE), [^{3}H]dopamine (DA) and [^{3}H]5-hydroxytryptamine (5HT) was examined in whole brain synaptosomes from BALB, C3H, C57BL and DBA inbred mouse strains With inhibition of [^{3}H]NE uptake, synaptosomes from BALB and C57 mice were substantially more sensitive to cocaine inhibition than those from DBA or C3H Moreover, with BALB and C57 tissue, amphetamine was as potent as cocaine, whereas with C3H and DBA, amphetamine and tropacocaine were much less potent inhibitors of [^{3}H]NE uptake With respect to [^{3}H]DA accumulation, synaptosomes from BALB, C57 and DBA were equally sensitive to cocaine inhibition, while C3H synaptosomes were significantly less sensitive. In each of the four strains, amphetamine was more potent than cocaine, and tropacocaine far less potent The relative potencies of the three drugs varied significantly among the four strains With [^{3}H]5HT accumulation, synaptosomes from DBA were exquisitely sensitive to cocaine inhibition, followed by BALB and lastly by C57 and C3H In each of these strains, amphetamine and tropacocaine were equipotent at [^{3}H]5HT inhibition, and less potent than cocaine The results suggest that there are pronounced genetic differences in sensitivity to monoamine uptake inhibition by cocaine, which may arise from genetic differences in either carrier topology or other site of cocaine interaction. The results further suggest that genetic behavioral differences to cocaine and amphetamine may involve complex neurotransmitter interactions

Cocaine Amphetamine Inhibition of amine uptake C3H mouse DBA mouse C57 mouse BALB mouse Norepinephrine Dopamine 5-Hydroxytryptamine

THE use and abuse of cocaine in our society is becoming increasingly widespread, yet little is known about the genetic factors contributing to its use Genetic factors are known to contribute to the use of other psychoactive substances by humans For instance, contributing genetic factors have clearly been established in the development of alcoholism (3, 4, 13) The use of tobacco products may also be in part regulated by heritable factors (10,47)

One way of establishing the potential involvement of genetic factors in substance abuse in humans is to ascertain whether variability in acute drug sensitivity or variability in the development of drug tolerance exists in genetically-defined animal models Inbred strains of mice have been extensively used for this purpose For instance, numerous studies have shown that genetically-defined stocks of mice differ in acute sensitivity to ethanol, in the development of tolerance to ethanol, and in ethanol withdrawal and self-administration (5, 12, 17, 19, 35, 48) If genetic factors regulate the acute sensitivity and development of tolerance in laboratory animals, the possibility exists that humans may also differ in such measures because of genetically-determined factors.

We have recently demonstrated that BALB/cJ, C3H/2Ibg, C57/6Ibg and DBA/2Ibg mice differ significantly in Y-maze response following acute cocaine (44) These results suggested a pronounced influence of genetic background on cocaine response that was not due to differences in the rate of cocaine clearance from the brain.

Many of the effects of cocaine are attributable to inhibition of neuronal accumulation of dopamine (DA) (23, 34, 41, 45), norepinephrine (NE) (7, 26, 28, 43) or 5-hydroxytryptamine (5HT) (34, 42, 55) Cocaine and amphetamine have been shown to owe their reinforcing action to activation of central reward pathways (52). These reward pathways appear to be dopaminergic in nature as suggested by the antagonistic effects of dopamine

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receptor blockade on cocaine reward (11, 53, 54), and by the extinction of self-administration of cocaine following chemical denervation of DA nerve terminals in the nucleus accumbens (27, 39, 40) and ventral tegmental areas (9,37) The locomotor effects of amphetamine and cocaine are in part mediated by the same brain regions Thus, stereotaxic lesions of dopamine neurons in the caudate (6), or intranigral lesions of ascending dopamine pathways (38) greatly reduce the locomotor response to stimulants

Given that all three central nervous system monoamines have been implicated in the behavioral response to cocaine, we have examined the inhibition of synaptosomal NE, DA and 5HT accumulation by cocaine in each of the four inbred strains previously used to characterize the behavioral response to cocaine To assess the generality of any differences observed, the effects of amphetamine and tropacocaine on the synaptosomal accumulation of the three radiolabelled monoamines were also ascertained

METHOD

Animals

Male BALB/cJ, C3H/2Ibg, C57/6Ibg and DBA/2Ibg mice, 60–90 days of age, were obtained from the Core Colonies of the University of Colorado Institute For Behavioral Genetics, Boulder, CO Animals were maintained on a 12-hour light/dark cycle with food and water ad lib Animals were sacrificed by cervical dislocation

Drugs

L-[7-^{3}H(N)]-norepinephrine (20 Ci/mmol), 3,4-[7-^{3}H]-dihydroxyphenylethylamine (39 Ci/mmol) and 5-[1,2-^{3}H(N)]-hydroxytryptamine (28 Ci/mmol) were obtained from New England Nuclear Research Products, Boston, MA Cocaine HCl, d-amphetamine sulfate, desipramine hydrochloride, L-arterenol bitartrate, 3-hydroxytyramine hydrochloride, 5-hydroxytryptamine creatinine sulfate complex and tropacocaine HCl were obtained from Sigma Chemical Co , St Louis, MO All other chemicals were of reagent grade Drug solutions were prepared daily

Synaptosomes

Synaptosomes were prepared by the method of Gray and Whittaker (15) Whole brain tissue from one animal was weighed and homogenized in 4 ml of cold 0 32 M sucrose using a Potter Elvehjem teflon/glass homogenizer (7 strokes at 350 rpm) The homogenate was sequentially centrifuged at $1100 \times g$ for 10 minutes and at $12,400 \times g$ for 20 minutes The outer white portion of the resulting pellet was carefully cut away from the inner red mitochondrial portion, and was resuspended in 2 2 ml of Krebs-Henseleit buffer (4°) (20) saturated with 95% O_2/5% CO_2

[^{3}H]Amine Accumulation

The method of Bartholow *et al* (2) was employed Aliquots of synaptosomal suspension (200 µl) were added to tubes containing 1 4 ml of buffer and appropriate concentrations of competing amines, if present, also added in a volume of 200 µl The samples were preincubated for 10 minutes at 37° Two hundred µl of [^{3}H]amine (0 2 µCi, 10^{-7} M) was then added to give a final volume of 2 ml, and a final [^{3}H]amine concentration of 10^{-7} M The tubes were then incubated with shaking for 20 minutes, the time at which the accumulation of each of the monoamines was found to be maximal The tubes were then placed on ice to terminate uptake, and centrifuged at $12,400 \times g$ for 20 minutes The media samples were decanted, the tissue pellets were sonicated in 1 ml of 95% ethanol, and the protein repelleted by centrifugation Media and tissue samples were assayed for tritium content by liquid scintillation counting Minimum counting efficiency was 30% All samples were corrected for quenching Protein content of the tissue pellet following ethanol lysis was determined colorimetrically using Biuret reagent (21) The accumulation of radiolabeled amines was expressed as a tissue medium ratio (nmol of amine per mg of protein/nmol of amine per ml of medium) The effect of competing amines was then expressed as percent inhibition of uptake relative to control samples Log IC_{50} values (concentrations producing half-maximal inhibition of [^{3}H]monoamine uptake) and 95% confidence limits for each dose-response curve were determined by linear regression, using a program designed by Chris deFiebre of the Institute for Behavioral Genetics Synaptosome viability was ascertained by examining the temperature- and cocaine-sensitivity of radiolabeled NE, DA and 5HT accumulation For this purpose tissue medium ratios could be expressed as nmol of amine per g wet weight of tissue/nmol of amine per ml of medium This expression more clearly demonstrates the degree of concentration of amines by the tissue The accumulation of each radiolabeled amine was shown to be osmotically sensitive

Data Analysis

All data were analyzed using both a one-way and two-way analysis of variance (ANOVA) to determine the effects of strain, drug and drug concentration with each [^{3}H]amine system For those analyses in which significant differences were observed, the results were subjected to Newman-Keul's post hoc test

RESULTS

[^{3}H]Amine Accumulation

The synaptosomal accumulation of radiolabeled NE, DA and 5HT in each of the strains was temperature-dependent (85–90%), osmotically sensitive (85–90%), and maximal at approximately 20 minutes at 37° This is typically illustrated by the accumulation of [^{3}H]NE by BALB synaptosomes At a medium concentration of 10^{-7} M, synaptosomes accumulated NE to a tissue medium ratio of 10.6 ± 1 1 (nmol of [^{3}H]NE per g of tissue/nmol of [^{3}H]NE per ml of medium) This ratio was reduced to $1\ 78 \pm 0\ 06$ at an incubation temperature of 4°, and was reduced to $1\ 69 \pm 0\ 14$ by incubation in the presence of 10^{-5} M cocaine

Cocaine IC_{50} Values

To assist the discussion of the relative effects of cocaine, amphetamine and tropacocaine on synaptosomal monoamine accumulation, a summary of IC_{50} values for cocaine is shown in Table 1 These represent the concentrations, determined by linear regression, to produce half-maximal inhibition of [^{3}H]monoamine accumulation In each case, the 95% confidence range did not exceed 5% of the log value The significance of strain differences will be discussed with the next three figures in relation to the other uptake inhibitors

Inhibition of [^{3}H]NE Uptake

The inhibition by cocaine, amphetamine and tropacocaine of radiolabeled NE accumulation by synaptosomes from each of the four mouse strains is illustrated in Fig 1 Inhibition of [^{3}H]NE accumulation by cocaine showed a significant effect of both concentration, $F(5,141) = 268\ 55$, $p < 0\ 0001$, and strain, $F(3,141) = 16\ 071$, $p < 0\ 0001$ Newman-Keul's post hoc analysis showed

TABLE 1

COCAINE CONCENTRATIONS RESULTING IN HALF-MAXIMAL INHIBITION OF [^{3}H]-AMINE ACCUMULATION BY MOUSE BRAIN SYNAPTOSOMES

Mouse Strain	IC_{50} (M) for Inhibition of Uptake* [^{3}H]5HT	[^{3}H]DA	[^{3}H]NE
BALB	4.45×10^{-7}	1.10×10^{-7}	5.13×10^{-8}
DBA	1.41×10^{-7}	8.91×10^{-8}	1.15×10^{-7}
C57	8.13×10^{-7}	2.69×10^{-7}	6.90×10^{-8}
C3H	1.12×10^{-6}	4.68×10^{-7}	1.51×10^{-7}

*Calculated by linear regression from the dose-response data. Ninety-five percent confidence limits in each case do not exceed 5% of the log value.

that synaptosomes from BALB and C57 mice were more sensitive to cocaine (IC_{50} 5.13×10^{-8} M and 6.9×10^{-8} M, respectively) than were synaptosomes from C3H and DBA (IC_{50} 1.51×10^{-7} M and 1.15×10^{-7} M, respectively). Maximal inhibition of uptake was also greater in BALB and C57 synaptosomes (>80%) than in C3H or DBA (61% and 65%, respectively).

Comparing the relative effects of cocaine, amphetamine and tropacocaine in each of the four strains, two-way analysis of variance showed a significant difference among inhibitors in each strain [DBA $F(2,74) = 18\,495$, $p<0.0001$, C57 $F(2,75) = 35.461$, $p<0.0001$; C3H $F(2,80) = 35\,992$, $p<0.0001$; BALB $F(2,84) = 6.993$, $p<0.002$]. Newman-Keul's post hoc analysis showed that in DBA and C3H amphetamine was significantly more potent than cocaine by 8–10 fold, while in C57 and BALB amphetamine and cocaine were nearly equipotent. In each strain tropacocaine was significantly less potent than either cocaine or amphetamine, but ultimately produced the same degree of inhibition of uptake. Interestingly, concentration-response curves for inhibition of [^{3}H]NE uptake by amphetamine in the four strains are essentially superimposable. Similarly, there were no strain differences for uptake inhibition by tropacocaine.

Inhibition of [^{3}H]DA Uptake

The inhibition of [^{3}H]DA uptake by cocaine, amphetamine and tropacocaine in tissue from the four strains of mouse is shown in Fig. 2. Cocaine inhibition of [^{3}H]DA uptake in the four strains showed a significant effect of both strain, $F(3,170) = 32\,218$, $p<0.0001$, and concentration, $F(4,170) = 158\,548$, $p<0.0001$. Newman-Keul's post hoc analysis showed C3H tissue to be significantly less sensitive to cocaine inhibition of DA uptake ($IC_{50} = 4.68 \times 10^{-7}$ M) than BALB, C57 and DBA ($IC_{50} = 1.10 \times 10^{-7}$ M, 2.69×10^{-7} M and 8.91×10^{-8} M, respectively). Maximal inhibition of uptake was also significantly less in C3H synaptosomes. BALB, C57 and DBA did not significantly differ in response to cocaine inhibition of DA uptake.

Comparison of the relative effects of cocaine, tropacocaine and amphetamine showed a significant difference among inhibitors in each of the strains [DBA $F(2,97) = 19.067$, $p<0.0001$, C57 $F(2,79) = 29\,433$, $p<0.0001$, C3H. $F(2,84) = 18.100$, $p<0.0001$, BALB $F(2,75) = 52\,106$, $p<0.0001$]. In each strain amphetamine was more potent than cocaine at inhibition of [^{3}H]DA uptake. In DBA and C3H synaptosomes, amphetamine displayed a simple concentration-response relationship. In C57 and BALB, the effect of amphetamine appeared to be biphasic. In both of these strains, the effect of amphetamine was absent at a concentration of 10^{-10} M (data not shown). With the exception of the effect of low concentrations of amphetamine in C57 and BALB tissue, the concentration-response curves for amphetamine and cocaine were quite similar. In the case of DBA cocaine was intermediate in potency between amphetamine and tropacocaine. In C3H tissue, cocaine and tropacocaine did not significantly differ in potency, both being significantly less potent than amphetamine.

Inhibition of [^{3}H]5HT Accumulation

The inhibition by cocaine, amphetamine and tropacocaine of [^{3}H]5HT accumulation by synaptosomes from the four mouse strains is shown in Fig. 3. Analysis of variance demonstrated that inhibition of [^{3}H]5HT by cocaine was significantly affected by cocaine concentration, $F(4,109) = 249\,645$, $p<0.0001$, and by strain of mouse, $F(3,109) = 23\,109$, $p<0.0001$. Furthermore, a significant strain by concentration interaction was observed, $F(7,109) = 2\,294$, $p<0.05$). Newman-Keul's post hoc analysis showed DBA tissue to be significantly most sensitive to cocaine inhibition of [^{3}H]5HT accumulation ($IC_{50} = 1.41 \times 10^{-7}$ M). This was followed by BALB tissue, which was significantly less sensitive to cocaine than DBA ($IC_{50} = 4.57 \times 10^{-7}$ M). Significantly least sensitive was tissue from C3H and C57 animals ($IC_{50} = 1.12 \times 10^{-6}$ M and 8.13×10^{-7} M, respectively). Cocaine also produced a smaller maximal response in these two strains (70% vs 80% observed with synaptosomes from DBA and BALB).

A comparison of inhibition of [^{3}H]5HT accumulation by cocaine, tropacocaine and amphetamine shows in each strain a significant difference among the inhibitor [DBA $F(2,17) = 63.768$, $p<0.0001$, C57 $F(2,78) = 19\,870$, $p<0.0001$, C3H $F(2,74) = 14\,383$, $p<0.0001$, BALB $F(2,48) = 94\,623$, $p<0.0001$]. In each strain, cocaine was significantly more potent than amphetamine or tropacocaine. In each strain the dose response curves for amphetamine and tropacocaine were not significantly different.

DISCUSSION

The effect of the amine uptake inhibitors on maximal [^{3}H]-monoamine accumulation, rather than on initial rates of amine accumulation, was studied for two reasons. First, this incubation period was more physiologically relevent to the time course of behavioral effects and pharmacokinetic profile of cocaine in several species. Following intraperitoneal injection in rats, maximal brain cocaine concentrations are achieved after 15 minutes, and are maintained for 60 minutes, a period which coincides with maximal DA overflow and the time-course for behavioral effects (25). Following intranasal administration in humans, serum cocaine levels and perceived 'highs' peak at 30 minutes, and are maintained for 60 minutes (49). Thus, the pharmacological effects of cocaine involve interaction with nerve endings for periods of time sufficient to substantially perturb transmitter levels in all cellular compartments. Secondly, examination of maximal amine accumulation maximized genetic differences in sensitivity to inhibition by cocaine.

The data obtained indicate that there are genetic differences in the sensitivity of synaptosomal monoamine accumulation to inhibition by cocaine. Thus, previously described differences in behavioral responses to cocaine (44) may be in part related to genetic differences in the cocaine sensitivity of monoamine metabolism. It is significant that all three of the monoamines studied may be involved in the ultimate behavioral response to cocaine.

Cocaine has long been known to inhibit the neuronal accumulation of NE, DA and 5HT (vida supra). More recently, the

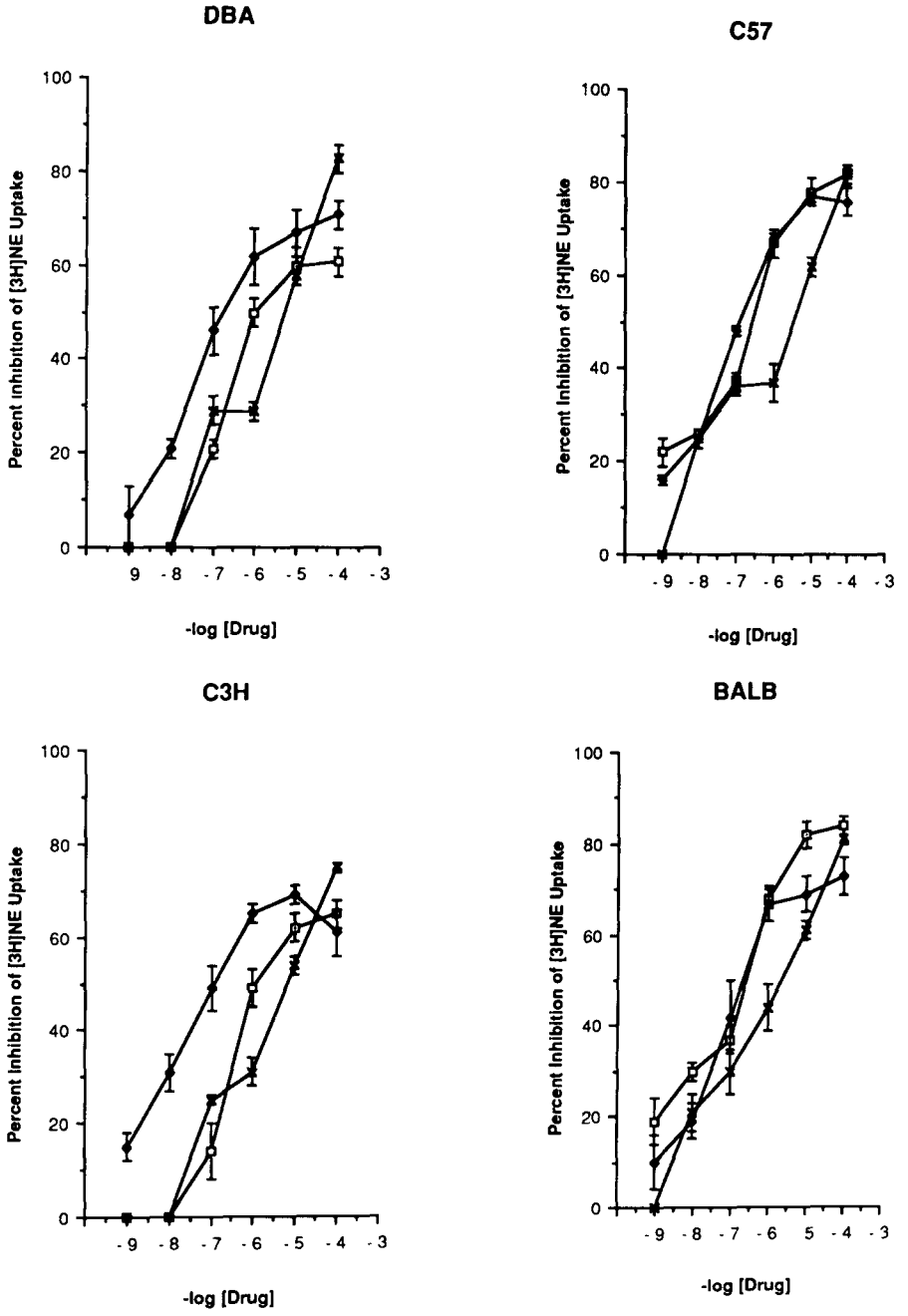

FIG 1 The inhibition of synaptosomal accumulation of [^{3}H]NE by amphetamine (●), cocaine (□) and tropacocaine (■) Whole brain synaptosomes from DBA, C57, C3H and BALB mice were incubated for 20 min at 37° with 10^{-7} M [^{3}H]NE and various concentrations of uptake inhibitors as described in the Experimental section Results are expressed as percent inhibition of [^{3}H]NE accumulation relative to control samples Mean ± SEM of 4 to 8 determinations

existence of specific cocaine binding sites in brain have been suggested (18, 30, 31, 33, 36, 46) These binding sites appear to be associated with neuronal DA and 5HT transport carriers in striatum and cortex, and may serve some regulatory function With respect to these monoamines, studies to date have most convincingly demonstrated a correlation between inhibition of neuronal DA accumulation and the locomotor response to stimulants (6,38) Inhibition of neuronal DA accumulation appears to be a major factor in the reinforcing action of cocaine and amphetamine (51), and appears to involve a specific binding site on the DA transporter (36)

However, while the behavioral sequelae of cocaine may be mediated largely by interaction of the drug with DA nerve terminals, it is clear from other studies that such effects may be modified by perturbation of noradrenergic or serotonergic neurotransmission. For instance, self administration of alcohol in rats is

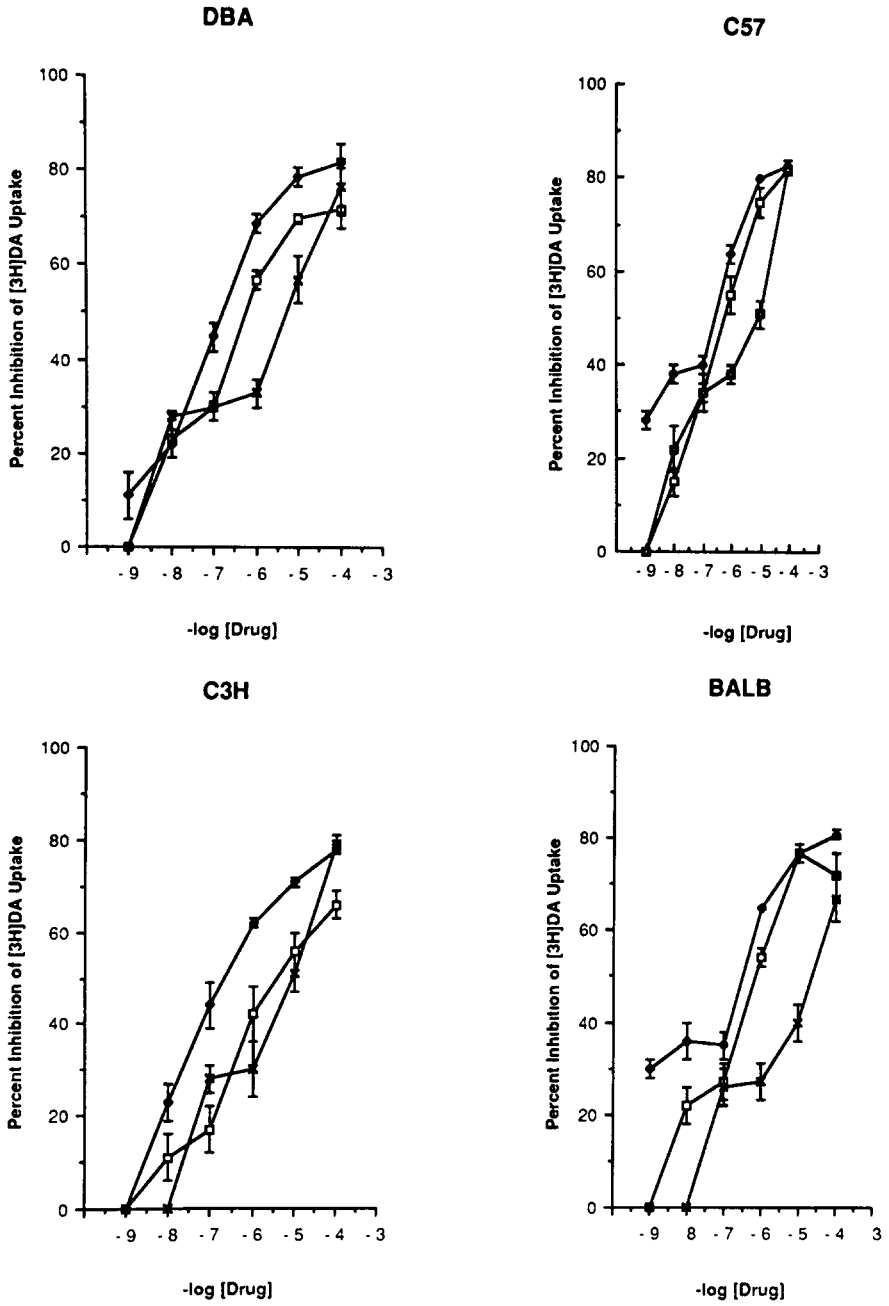

FIG 2 The inhibition of synaptosomal accumulation of [^{3}H]DA by amphetamine (●), cocaine (□) and tropacocaine (■) Whole brain synaptosomes from DBA, C57, C3H and BALB mice were incubated for 20 min at 37° with 10^{-7} M [^{3}H]DA and various concentrations of uptake inhibitors as described in the Experimental section Results are expressed as percent inhibition of [^{3}H]DA accumulation relative to control samples Mean ± SEM of 4 to 8 determinations

enhanced following destruction of serotonergic neurons (24); serotonergic mechanisms have been suggested to be operative in the self administration of amphetamine in rats (22), serotonergic mechanisms may modify apomorphine-induced stereotypy in rats (50); both noradrenergic and serotonergic mechanisms may be involved in rewarding electrical stimulation of the brain (16); noradrenergic mechanisms have been suggested in a number of DA-mediated behavioral responses (1) and in amphetamine-induced stereotypy (14) Such possibilities of multitransmitter contributions to the behavioral response to cocaine are best illustrated with strain comparisons of both neurochemical and behavioral aspects of drug action.

The result of this study demonstrate that there are significant differences in the actions of cocaine and amphetamine upon synaptosomal monoamine accumulation. In all four strains examined, amphetamine was more potent than cocaine at inhibition of

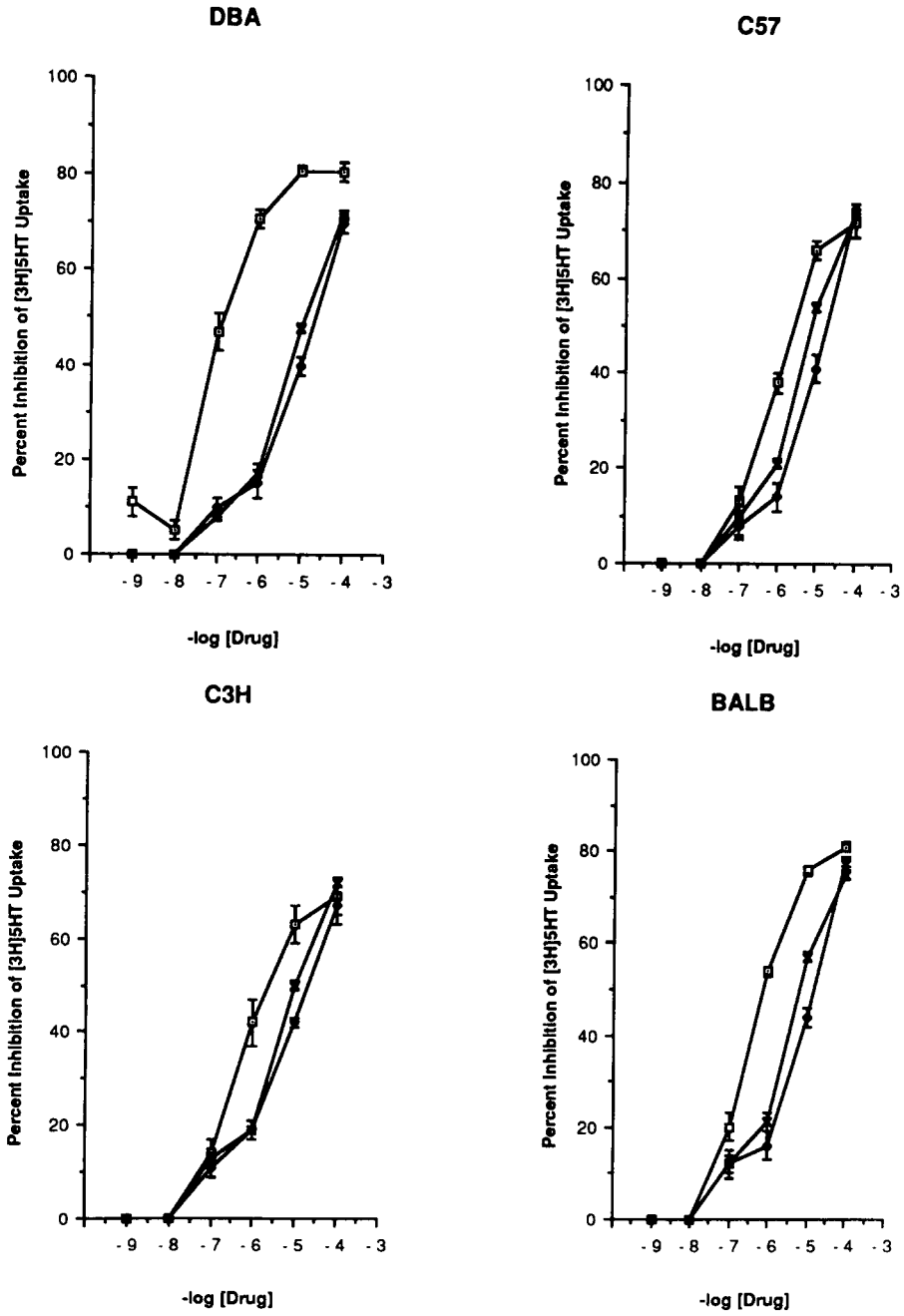

FIG 3 The inhibition of synpatosomal accumulation of [^{3}H]5HT by amphetamine (●), cocaine (□) and tropacocaine (■) Whole brain synaptosomes from DBA, C57, C3H and BALB mice were incubated for 20 min at 37° with 10^{-7} M [^{3}H]5HT and various concentrations of uptake inhibitors as described in the Experimental section Results are expressed as percent inhibition of [^{3}H]5HT accumulation relative to control samples Mean ± SEM of 4 to 8 determinations

NE and DA accumulation. Cocaine was more potent than amphetamine at inhibition of 5HT accumulation Genetic differences in sensitivity to amphetamine inhibition of amine accumulation were observed only with inhibition of [^{3}H]DA accumulation, and only at low amphetamine concentrations Amphetamine appeared to have a biphasic action in synaptosomes from C57 and BALB mice, displaying a plateau in the concentration-effect curve between 10^{-7} and 10^{-9} M This plateau was absent at 10^{-10} M amphetamine, and was not observed in studies with C3H and C57 synaptosomes The absence of substantial strain differences in the inhibition of [^{3}H]-amine accumulation by amphetamine, in contrast to cocaine, suggests either genetic differences in the topology of the monoamine carriers, or that cocaine and amphetamine interact at different sites on the amine carrier. With respect to DA in particular, Raiteri *et al* (29) have suggested that amphetamine-induced DA release, rather than inhibition of DA uptake, may be the predominant mechanism whereby amphetamine increases synaptic DA levels, thus differentiating amphetamine from cocaine

mechanistically. Our data are consistent with that view.

Since absence of the methyl ester of cocaine (tropacocaine) has been reported to result in substantial loss of activity in locomotor stimulation in mice (31), and in the ability to displace [^{3}H]cocaine from binding sites in mouse striatal and cortical membrane preparations (33), it was felt that a comparison of amphetamine, cocaine and tropacocaine would thus provide a useful array of activity for examination of monoamine uptake inhibition. If there were no genetic differences in binding site topology of the NE uptake carrier for instance, and if the competing amines were all interacting at the same site, one would expect similar profiles of activity for the three uptake inhibitors among the strains. However, cocaine and amphetamine were nearly equipotent at NE uptake inhibition in C57 and BALB synaptosomes, while in C3H tissue, cocaine was significantly less potent than amphetamine, being nearly identical in effect to tropacocaine In a similar fashion, cocaine and amphetamine were nearly identical in inhibition of [^{3}H]DA in tissue from C57 and BALB, while in C3H tissue cocaine potency more nearly resembled that of tropacocaine The results of the current study suggest either significant genetic differences in the topology of the specific monoamine carriers, or the interaction of cocaine at a site on the carrier distinct from site of amphetamine and tropacocaine interaction, and a site which displays genetic variability These data also suggest the importance of specifying genetic stock assessing relative potencies of inhibitors of monoamine uptake

While it is premature to draw conclusions relating biochemical and behavioral responses to cocaine in these animals, it is interesting to note that C57 and BALB animals, which were the strains least sensitive to the behavioral effects of cocaine (44), demonstrated the greatest sensitivity to inhibition of NE uptake by cocaine C3H animals, which were the most sensitive to the behavioral stimulation of cocaine, were the least sensitive to cocaine inhibition of DA accumulation These results underscore the importance of examining the effects of cocaine on synaptosomal amine accumulation in specific brain regions, and of correlating this biochemistry with a wider range of behavioral studies We are currently involved in such investigations

In conclusion, the results of this study suggest that genetic differences in behavioral response to cocaine may result from genetic differences in sensitivity to the effects of cocaine on monoamine dynamics. These differences in biochemical sensitivity may be the consequence of genetic differences in the site of cocaine interaction with monoamine transport sites.

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