Pharmacology Biochemistry & Behavior, Vol. 28, pp. 179–185. © Pergamon Journals Ltd., 1987. Printed in the U.S.A. 0091-3057/87 $3.00 + .00

Effects of Caffeine and L-Phenylisopropyladenosine on Locomotor Activity of Mice[1]

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Received 7 April 1986

BUCKHOLTZ, N. S. AND L. D. MIDDAUGH. *Effects of caffeine and L-phenylisopropyladenosine on locomotor activity of mice*. PHARMACOL BIOCHEM BEHAV **28**(2) 179–185, 1987.—C57BL/6J and DBA/2J mice were used to determine if possible differences in the behavioral response to caffeine might be related to differences in A_1 adenosine receptors. Caffeine stimulated locomotor activity of both strains, but the dose-response relationship and time course of drug action differed according to strain. Although their response to caffeine differed, the strains did not differ in response to the A_1 adenosine agonist L-phenylisopropyladenosine (PIA) nor in the binding of the A_1 agonist [^{3}H]N^6-cyclohexyladenosine (CHA) in various brain regions. Thus, the behavioral differences in response to caffeine could not be accounted for by differences in adenosine binding. Of alternative mechanisms, strain differences in A_2 receptors appear to be the most promising.

Caffeine Adenosine receptors L-PIA Locomotor activity Inbred mice

THE stimulatory effects of caffeine are well documented [33] and the compound is used for this effect in a variety of beverages. Recent interest in the mechanism of action of caffeine has been the result of reports that it and other methylxanthines have an antagonistic action on brain adenosine receptors [10, 13, 29, 30]. Neurophysiologically, adenosine inhibits the spontaneous firing of most central neurons, and the inhibition can be blocked by caffeine and another methylxanthine, theophylline [13,29]. Neurochemically, adenosine blocks the potassium-stimulated release of norepinephrine from cortex, and this is antagonized by theophylline [17]. More direct evidence for the involvement of adenosine receptors in the effects of methylxanthines comes from *in vitro* receptor binding studies. These studies indicate that the potency of caffeine and other methylxanthines for displacing radioactively labeled adenosine analogues is greater than their potency for displacing ligands from receptors of common neurotransmitters [4,26].

In moderate doses, caffeine and other methylxanthines stimulate locomotor activity in rodents [32, 34, 35], whereas adenosine agonists such as L-phenylisopropyladenosine (L-PIA), N^6-cyclohexyladenosine (CHA) and chloroadenosine depress activity [13, 32, 36]. Furthermore, the potencies of methylxanthines in stimulating locomotor activity reportedly correlate fairly well with their potencies in adenosine receptor competitive binding studies [32]. The hypothesis generated from the above studies is that the central nervous system excitatory properties of the methylxanthines may be mediated by an antagonism of the depressant effects of endogenously released adenosine [31].

The above evidence suggests that the acute behavioral effects of caffeine could be mediated by adenosine receptor blockade. Whether genetic differences in adenosine receptor numbers or affinity might account for the substantial individual variation in reaction to caffeine frequently reported [15,25] remains to be tested.

The study reported here consists of experiments to evaluate the role of A_1 adenosine receptors in accounting for genetic differences in reaction to acute caffeine. To evaluate whether possible genetic differences in these receptors might account for variation in the behavioral response to caffeine, we used two inbred strains of mice, DBA/2J and C57BL/6J, which have a number of documented neurochemical and behavioral differences [18] and were previously reported to respond differently to caffeine [6]. Strain difference in response to caffeine and to the adenosine agonist L-PIA, as well as adenosine receptor binding, was assessed.

METHOD

Subjects

Male C57BL/6J (C57) and DBA/2J (DBA) mice were re-

[1]This work was supported in part by NIMH Grant No. MH37747.

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TABLE 1

LOCOMOTOR ACTIVITY OF C57 AND DBA MICE AT 15-MIN INTERVALS FOLLOWING CAFFEINE INJECTIONS

Post-Injection Interval (Min)	0.00 (N=18) Mean ± SE	1.25 (N=7) Mean ± SE	2.50 (N=7) Mean ± SE	5.00 (N=14) Mean ± SE	10.00 (N=6) Mean ± SE	20.00 (N=9) Mean ± SE	40.00 (N=6) Mean ± SE
			C57 Caffeine Dose (mg/kg)				
0–15	163 ± 9	182 ± 12	237 ± 17	241 ± 12	245 ± 28	237 ± 23	204 ± 40
16–30	111 ± 7	120 ± 17	139 ± 17	168 ± 10	192 ± 24	178 ± 24	148 ± 44
31–45	98 ± 8	109 ± 14	130 ± 12	170 ± 13	187 ± 18	181 ± 21	156 ± 60
46–60	89 ± 9	99 ± 13	125 ± 12	142 ± 11	167 ± 20	159 ± 23	167 ± 52
Total[a]	461 ± 28	510 ± 44	631 ± 51	721 ± 42*	791 ± 81*	755 ± 82*	675 ± 151
Post-Injection Interval (Min)	0.00 (N=19) Mean ± SE	1.25 (N=8) Mean ± SE	2.50 (N=8) Mean ± SE	5.00 (N=15) Mean ± SE	10.00 (N=6) Mean ± SE	20.00 (N=9) Mean ± SE	40.00 (N=6) Mean ± SE
			DBA Caffeine Dose (mg/kg)				
0–15	126 ± 8	157 ± 11	193 ± 16	195 ± 9	200 ± 14	201 ± 12	166 ± 19
16–30	77 ± 6	87 ± 7	110 ± 15	138 ± 8	166 ± 13	179 ± 12	135 ± 18
31–45	56 ± 4	71 ± 5	94 ± 8	116 ± 8	152 ± 21	172 ± 11	136 ± 18
46–60	34 ± 5	53 ± 3	81 ± 5	106 ± 10	124 ± 19	163 ± 12	126 ± 16
Total[b]	293 ± 17	368 ± 17	478 ± 39*	555 ± 29*	642 ± 63*	715 ± 37*	563 ± 37*

[a] $F(6,59)=3.059$, $p=0.11$; Asterisks indicate statistically confirmed elevation above controls via Dunnett's test.
[b] $F(6,64)=16.517$, $p<0.001$; Asterisks indicate statistically confirmed elevation above saline controls via Dunnett's test.

ceived at about six weeks of age from Jackson Laboratories. They were maintained 4/cage in our colony room adjacent to the behavioral laboratory under conditions of constant temperature (23°C), a 12 hr light-dark cycle, and ad lib access to food and water for at least two weeks before testing procedures were initiated.

Behavioral Testing Apparatus and Testing Procedures

Mice were tested individually in one of three activity runways enclosed in sound and light controlled, ventilated boxes. The runways were constructed of clear Plexiglas and were oval-shaped with inside and outside wall dimensions of 19×33 and 31×45 cm. The runway floor was 6 cm wide and the walls were 18 cm high. Photocells and infrared light sources were located at four equidistant points around the runway at a height of 1.25 cm above the floor. Interruption of the light source was detected, amplified, and recorded to define one unit of locomotor activity. Electronic programming was arranged such that an adjacent photocell needed to be activated before reactivation of any given photocell. Thus, a unit of activity was equivalent to locomotion of approximately 32 cm. Activity counts were cumulated and printed out at 15-min intervals.

On the day of testing, mice were removed from their home cage in the colony room, placed individually in a small cage, brought to the testing room and injected with the appropriate drug. They were immediately placed individually into one of three activity runways and tested for one hr in a dark environment. A different strain was tested each day between 0800 and 1200 hr. The individual treatment conditions were equally distributed across the three runways. The runways were cleaned of fecal boli, wiped with Windex, and dried after each test to eliminate olfactory cues on subsequent tests.

Behavioral Experiments

In the caffeine experiment, mice were injected with either 1.25, 2.5, 5, 10, 20, or 40 mg/kg caffeine (anhydrous, Sigma, dissolved in 0.9% saline) or 0.9% saline. In the experiment to assess L-PIA, mice received 0.025, 0.05, or 0.1 mg/kg L-PIA or 0.9% saline. L-PIA (Boehringer-Mannheim) was dissolved initially in a small amount of 0.06 N HCl and diluted to the appropriate volume with 0.9% saline. All injections were given intraperitoneally in a volume of 0.1 ml/10 g body weight.

Adenosine Receptor Binding

Membrane preparation. Brains were dissected into cortex, hippocampus, striatum and cerebellum. Brain tissue was homogenized for 15 sec in 20 volumes of ice-cold Tris-HCl (50 mM, pH 7.4 at room temperature) using a Tekmar Tissumizer (setting 6.5). The homogenate was centrifuged at 30,000 × g for 20 min. The pellet was resuspended in 20 vol Tris-HCl, incubated at room temperature for 30 min with 2 U/ml adenosine deaminase (Sigma, Type III) and centrifuged at 30,000 × g for 30 min. The pellet was resuspended in 10 vol Tris-HCl and frozen at −70°C until used in the binding assay.

Binding assay. Binding was done in triplicate in 13×100 mm glass tubes containing 100 μl [^{3}H]CHA (13.5 Ci/mmol,

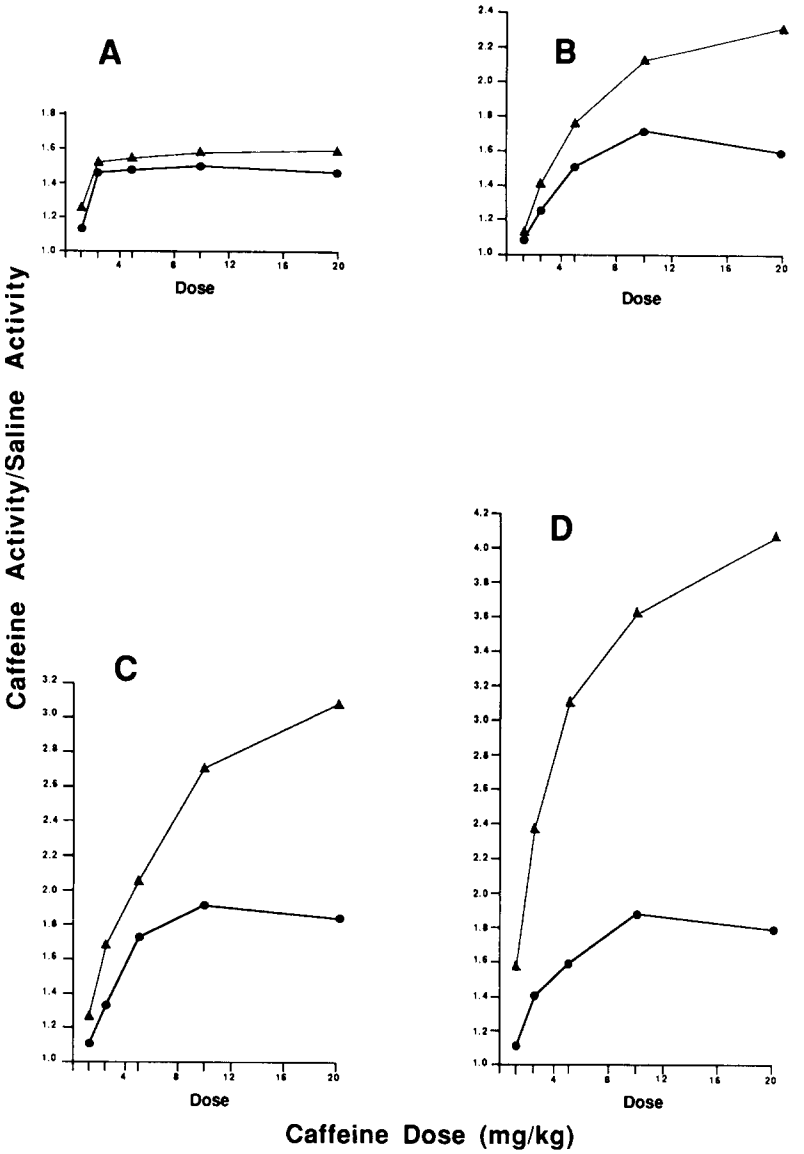

FIG. 1. Effect of caffeine (1.25, 2.5, 5.0, 10.0 and 20 mg/kg) on locomotor activity of C57 (●) and DBA (▲) mice at 15 (A), 30 (B), 45 (C) or 60 (D) min after IP injections. Activity is expressed as mean ratios of activity counts under drug conditions to counts under the saline vehicle condition.

New England Nuclear), 100 l Tris-HCl buffer or 20 M (final concentration) unlabeled CHA (Calbiochem) to define nonspecific binding. 100 μl membrane preparation, and Tris-HCl to reach a total volume of 1.0 ml. In binding experiments using only one concentration of [^{3}H]CHA, 2.0 nM (final concentration) was used, and in saturation studies, seven concentrations ranging from 0.1–20.0 nM were used. Specific binding at 2.0 nM [^{3}H]CHA averaged 92%. Tubes were incubated for 2 hr at room temperature, and the reaction was terminated by adding 3.0 ml ice-cold Tris-HCl (50 mM, pH 7.0 at room temperature) followed by vacuum filtration over Whatman GF/B filters and three further 3.0 ml Tris-HCl washes. Filters were placed in scintillation vials, air-dried overnight, 10 ml Scintiverse E (Fisher) added, extracted for 24 hr, and counted in a Beckman LS-350 scintillation system at an efficiency of approximately 34%.

Data Analysis

Data were analyzed using analyses of variance (ANOVA) programs for the Apple IIe (ANOVA II, Human Systems Dynamics, Cupertino, CA) according to procedures of Winer [37].

RESULTS

Effect of Caffeine

Mean locomotor activity scores at 15-min intervals and the total scores over the one hr testing period for C57 and DBA mice injected with saline or one of the six caffeine doses are shown in Table 1. Inspection of this table suggests that caffeine stimulated locomotor activity of both mouse strains and that C57 mice had higher activity levels than did DBA mice. An ANOVA of the total activity scores across

TABLE 2

LOCOMOTOR ACTIVITY OF C57 AND DBA MICE AT 15-MIN INTERVALS FOLLOWING L-PHENYLISOPROPYLADENOSINE (L-PIA) INJECTIONS

Post-Injection Interval (Min)	0 Mean ± SE	0.025 Mean ± SE	0.05 Mean ± SE	0.10 Mean ± SE
		C57 (N=6) L-PIA Dose (mg/kg)		
0–15	158 ± 15	126 ± 16	88 ± 17	43 ± 6
16–30	105 ± 16	86 ± 11	25 ± 14	2 ± 8
31–45	85 ± 10	93 ± 14	38 ± 16	0
46–60	70 ± 10	77 ± 14	54 ± 15	5 ± 5
Total	418 ± 37	382 ± 47	205 ± 56	50 ± 4
		DBA (N=6)		
0–15	138 ± 14	139 ± 12	92 ± 13	45 ± 8
16–30	82 ± 8	94 ± 8	26 ± 14	2 ± 1
31–45	63 ± 5	74 ± 8	36 ± 17	0
46–60	42 ± 8	60 ± 11	58 ± 15	11 ± 5
Total	325 ± 21	367 ± 31	212 ± 55	58 ± 9

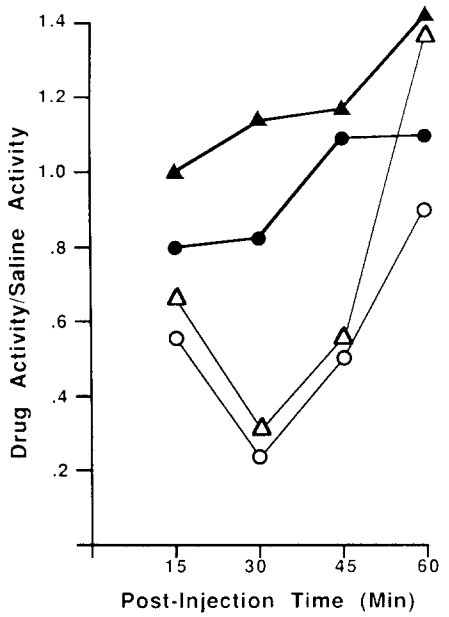

FIG. 2. Effects of L-phenylisopropyladenosine (0.025 mg/kg, filled symbols; 0.05 mg/kg, open symbols) on locomotor activity of C57 (●, ○) and DBA (▲, △) mice at 15-min intervals. Data points are as described for Fig. 1.

doses provides statistical confirmation for this observation [Strain: $F(1,123)=20.615$, $p<0.001$; Dose: $F(6,123)=12.156$, $p<0.001$]. Subsequent ANOVAs within each strain and Dunnett's tests comparing each treated group to its control indicated that C57 mice were stimulated by doses of 5, 10, and 20 mg/kg, whereas DBA mice were stimulated by all but the 1.25 mg/kg dose. Since basal activity level frequently influences drug effects on motor activity, data from saline controls, which index basal activity level in this experiment, were analyzed separately. An analysis of variance (ANOVA) on these data established that DBA controls were less active than C57 controls, $F(1,34)=27.588$, $p<0.001$; however, there was no statistically confirmed evidence that the two strains had different rates of declining activity across the test period. Because of the difference in control data, the caffeine data for the two strains were normalized by calculating ratios of the activity scores for each drug-injected animal to the mean activity score for its appropriate control group for each of the four 15-min intervals of testing. These ratios were then subjected to a Strain × Dose × Time ANOVA. The data for the 40 mg/kg dose were eliminated from the analysis because they were highly variable and clearly indicated no additional stimulation for either strain. In fact, some of the animals injected with the dose had reduced activity.

The group means for these ratios are plotted as a function of dose for each 15-min interval of testing in Fig. 1 (ratios >1 indicate elevation above saline controls). Inspection of these graphs and the results of the ANOVA indicate that caffeine produced a dose-related increase in activity, $F(4,79)=12.981$, $p<0.001$, that the degree of stimulation varied across time, $F(3,237)=85.707$, $p<0.001$, and that compared to their respective controls, the degree of stimulation was greater for DBA than C57 mice, $F(1,79)=43.043$, $p<0.001$. In addition, the degree of caffeine stimulation depended upon the interaction of Strain × Dose × Time, $F(12,237)=4.864$, $p<0.001$. Because of this interaction, the data were analyzed separately within each time period and in cases with significant interactions, an analysis of the simple main effects was completed [37].

The results of these analyses indicated that caffeine produced dose-related increases in activity at all four time periods. The strain difference in caffeine-induced stimulation became significant during the second 15-min block, $F(1,79)=10.936$, $p<0.001$, and continued for the remaining two periods. Finally, there was a Strain × Dose interaction during the third, $F(4,79)=2.669$, $p=0.036$, and fourth, $F(4,79)=6.256$, $p<0.001$, periods. Subsequent analysis of the simple main effects for these time periods indicated that the

TABLE 3
[^{3}H]CHA BINDING (fmol/mg PROTEIN) IN BRAIN REGIONS OF C57 AND DBA MICE

	[^{3}H]CHA Binding (fmol/mg protein)		
Strain	Hippocampus Mean ± SE	Striatum Mean ± SE	Cortex Mean ± SE
C57 (N=3)	453.5 ± 79.0	398.1 ± 34.5	374.4 ± 39.8
DBA (N=3)	414.1 ± 43.2	354.2 ± 33.2	360.7 ± 16.1

strains differed at the two highest doses during the third 15-min interval and at all but the lowest dose during the last 15-min interval.

Effect of L-PIA

Activity data across time after injection of vehicle or the three doses of PIA are summarized in Table 2. As indicated in this table, activity declined with increasing doses of PIA and the 0.1 mg/kg dose virtually eliminated activity after the first 15 min. An ANOVA on vehicle control data again established that DBA mice were less active than C57 mice, $F(1,11)=4.850$, $p<0.05$, but that the rate of declining activity across time was not different for the two strains. The effect of the two lower doses of L-PIA on activity of the two strains across time is shown in Fig. 2 as ratios of drug to saline activity scores as described above for the caffeine experiment. The 0.1 mg/kg dose is not shown and was not included in an ANOVA on the ratios since it completely eliminated the activity of most mice. The ANOVA supports the observations noted in Fig. 2. Clearly, activity varied as a function of Dose, $F(1,20)=8.206$, $p=0.009$, Time, $F(3,60)=18.256$, $p<0.001$, and their interaction, $F(3,60)=5.769$, $p=0.001$. It is important to note, however, that the strains did not differ in their reaction to the compound. The time course of drug action is interesting in that the effect was transient, being near maximum at 30 min after injection and absent by 60 min after injection.

[^{3}H]CHA Binding

There was no strain difference in [^{3}H]CHA binding in cortex by Scatchard analysis (n=3/strain). B_{max}s (mean±S.E.) for DBA and C57 were 1027±84 and 1007±56 fmol/mg protein, respectively and Kds were 3.6±0.6 and 3.5±0.4 nM, respectively. Binding at a 2.0 nM [^{3}H]CHA concentration was also determined in hippocampus, striatum and cortex (Table 3). An ANOVA on these data indicated no difference between the two strains. Regional difference in [^{3}H]CHA was marginally significant, $F(2,8)=3.519$, $p=0.079$. An ANOVA and Duncan's multiple range test on the strains combined indicated greater binding in hippocampus than in either cortex or striatum.

DISCUSSION

In agreement with previous studies, caffeine produced dose-related increases in activity. Although caffeine stimulated both strains, compared to C57 mice DBA mice were stimulated by lower doses of the drug, showed a greater degree of stimulation by the higher doses and had a longer time course of drug action. The wider range for stimulatory caffeine doses in DBA compared to C57 mice noted in the present study is consistent with another recent report [20]. In that study, both strains were stimulated by a 10 mg/kg dose but only the DBAs were stimulated by 3.2 and 32 mg/kg doses. Several additional studies using other mouse strains indicate stimulation thresholds for caffeine at doses between 2.5 and 6 mg/kg [12,19]. The previous report [32] that low caffeine doses (1 or 2 mg/kg) reduced activity of mice was not confirmed by the present study. The observed attenuation of stimulation noted for the 40 mg/kg dose, however, does confirm other reports for higher caffeine doses [12,29]. Compared to their respective vehicle controls, caffeine produced a greater increase in the activity of DBA than of C57 mice and the strain difference increased over the one-hr test period. Some of this difference is due to the lower basal activity level for DBA control mice. As previously reported [20], DBA control mice in the present study were less active than their C57 counterparts. By analyzing the ratios of drug activity to control activity, it was apparent that caffeine produced a much greater stimulatory effect in DBA than C57 mice and that the strain difference increased with time and dose. As a side issue, it is apparent from this study that strain differences in non-drug basal activity levels, as well as complete time courses of drug action, must be considered to properly evaluate strain differences in reaction to drugs.

The A_1 adenosine agonist, L-PIA, reduced locomotor activity by about 50% at the 0.05 mg/kg dose and completely eliminated activity at the 0.1 mg/kg dose. The time of maximum effect was near 30 min after injection, with a return to control values by the end of the 60-min testing session. Similar dose and time-related effects have been previously reported [13, 19, 32, 36]. In a low dose range of 0.004–0.02 mg/kg, L-PIA reportedly increases locomotor activity, but higher doses within the range of the present study, 0.04–0.08 mg/kg, reduced activity [19]. In the present experiment, some mice had elevated activity at early periods after injection of the 0.025 mg/kg dose. This apparent stimulation however was not supported statistically.

The observed activity reduction produced by the 0.05 and 0.1 mg/kg L-PIA doses was probably not a consequence of sedation since the animals were responsive to tactile stimulation. This type of "quiet alertness" has been noted by others [32,33]. The L-PIA induced activity reduction appears to be centrally mediated [1] and is unrelated to hypothermia [13] or to changes in blood pressure or heart rate [2,19].

Other direct or indirect adenosine agonists including the direct agonists CHA and 2-chloroadenosine [8, 13, 32] and the adenosine deaminase inhibitor EHNA [22] also reduce locomotor activity. In addition, two adenosine uptake inhibitors have been reported to potentiate the effects of adenosine-induced locomotor activity reduction without having effects themselves [9].

The strain differences in reaction to caffeine injections observed in the present study were not observed for L-PIA regardless of dose or post-injection time. The presence of strain differences in the reaction to caffeine in the absence of any apparent difference in A_1 adenosine receptors has also been reported for SWR and CBA mice [28]. Thus, the hypothesis that strain differences in A_1 adenosine receptors might mediate the strain differences in reaction to caffeine has not been confirmed by either study.

We found no differences between untreated DBA and C57 mice in [^{3}H]CHA binding in any of the brain areas we investigated. The relative degree of binding at the 2.0 nM concentration among the areas is consistent with rat data showing

the highest binding in hippocampus with approximately equivalent binding in cerebellum, caudate and cortex [24].

The evidence discussed above suggests that strain differences in A_1 receptors, as evidenced by differences in the behavioral responses to L-PIA or the binding of [^{3}H]CHA, are unlikely candidates to account for the strain differences in the behavioral response to caffeine. Potential alternative mechanisms include: (1) a differential drug distribution or metabolism, (2) other A_1 receptor subtypes or configurations, or (3) A_2 receptors.

We did not measure caffeine levels in our study; however, other reports indicate that differences in plasma or brain concentrations of caffeine could not account for extreme differences in the behavioral effects of the drug in other strains [5,28]. The fact that activity was elevated for a longer time in DBA than C57 mice, however, suggests that tissue concentrations of the drug should be examined.

Regarding the possibility that different A_1 receptor subtypes might mediate the strain difference in reaction to caffeine, it has been shown that the A_1 receptor agonist [^{3}H]CHA and the antagonist [^{3}H]1,3-diethyl-8-phenylxanthine (DPX) are differentially regulated by guanine nucleotides, cations and sodium [16], have species differences in binding [23] and exhibit differential sensitivity to caffeine *in vitro* [21] and *in vivo* [3]. These findings indicate that the two ligands bind either to different A_1 receptor subtypes or to different configurations of the same receptor [31]. Since we did not assess [^{3}H]DPX binding, we may have missed possible strain differences in this A_1 receptor subtype.

In addition to its action on A_1 receptors, caffeine interacts with A_2 receptors with about the same potency [10,27]. In fact, some evidence suggests that A_2 receptors are more important than A_1 receptors in mediating the stimulatory effects of caffeine. A_2 receptors are found in the striatum [10,38] and the role of this structure in motor activity is well documented. In addition, IP injections of caffeine have been reported to block the depressant effects of the adenosine A_2 agonist 5'-N-ethyl-carboxamidoadenosine (NECA) [7]. This compound has also been reported to produce strain-related changes in the behavior of animals having a differential response to caffeine [29]. Thus, a strain difference in A_2 receptors appears to remain a viable mechanism for the strain differences in the behavioral reaction to caffeine.

In summary, the present study confirms previous reports on the stimulatory effects of caffeine and has demonstrated genetic differences in response to the drug as indicated by its different effects on inbred strains of mice. These experiments also help establish the dose and time conditions under which the strain difference in response to caffein occur. Neither the [3[H]CHA binding experiments nor the response of the two strains to the A_1 receptor agonist, L-PIA, supported the hypothesis that the strain differences in reaction to caffeine are mediated by differences in systems involving the A_1 adenosine receptor.

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