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Effects on elevated plus-maze behavior of exposure to caffeine during both gestation and lactation

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Eight to 9 months after exposure to gestational and lactational doses of either 26/45 (low dose) or 25/35 (high dose) mg/kg/day of maternally ingested caffeine (via drinking water), rats were observed in an elevated plus-maze. The highest level of caffeine exposure decreased total rearing and increased immobility, and increased entries and occupancy of the open arms. Differences between the open and the preferred enclosed arms in frequencies of entries, occupancy, and walking were also greatest for rats exposed to the highest level of caffeine, and adrenal gland weights relative to body weight were highest for males in this group. Although some of the results support earlier reports of long-lasting heightened emotional reactivity following perinatal caffeine exposure, others did not. It was suggested that preexperimental and testing procedures may have provided a situation less aversive than those in which perinatal caffeine effects have been observed previously. Treatment effects on open-arm behavior might have arisen from impaired spatial ability.

Chronic exposure to caffeine during gestational and/or lactational development has been shown to have a number of behavioral effects in laboratory rodents in the absence of obvious physical malformations (Nehlig & Debry, 1994; Sobotka, 1989). Of particular interest to the present study are reports of long-lasting decreases in locomotor activity and increases in responses associated with heightened emotional reactivity (e.g., Hughes & Beveridge, 1987; Sinton, Valatx, & Jouvet, 1981; West, Sobotka, Brodie, Beier, & O'Donnell, 1986). In a more recent study, Hughes and Beveridge (1991) showed that, although exposure to caffeine during either gestation or lactation separately produced long-lasting behavioral changes that were consistent with heightened emotional reactivity, exposure during both periods consecutively combined to produce even greater effects. This combined treatment also diminished male hypersensitivity to caffeine exposure during either period separately.

Although the mechanism for the behavioral effects of early exposure to caffeine is still unknown, one possibility involves long-term upregulation of adenosine receptors (Nehlig & Debry, 1994), especially since adenosine may be implicated in anxiety (Marangos & Boulenger, 1985). Pre- and early postnatal exposure to caffeine has been shown to lead to this outcome in rodents (Guillet, 1990; Guillet & Kellogg, 1991; Marangos, Boulenger, & Patel, 1984). The possible involvement of adenosine receptors in caffeine-induced emotional reactivity is supported by the observation that upregulation can occur in adult mice following exposure to either caffeine or overcrowding stress (Boulenger, Marangos, Zander, & Hanson, 1986).

Performance on the elevated plus-maze test, as developed by Pellow, Chopin, File, and Briley (1985), is widely accepted as a measure of rodent "anxiety" in the evaluation of anxiolytic and anxiogenic drug effects (see, e.g., Baldwin, Johnston, & File, 1989; Pellow, Johnston, & File, 1987; Rodgers, Johnson, Norton, & Cole, 1995). Therefore, the present study was designed to assess the combined effects of exposure to caffeine during gestation and lactation on behavior of mature rats in the elevated plus-maze, with particular attention to anxiety or fear as measured by entries and occupancy of open rather than enclosed arms (Pellow et al., 1985). Since the effects of early exposure to caffeine observed in other settings are still apparent at least 8 months after treatment (Hughes & Beveridge, 1990), the study was also designed to determine whether or not similar long-lasting effects were still detectable in 8- to 9-month-old rats tested in the elevated plus-maze.

METHOD

Subjects

The subjects were experimentally naive adult male and female Wistar albino rats that had been bred and reared for (but not tested in) an earlier study (Hughes & Beveridge, 1991) while gestationally and/or lactationally exposed to varying doses of maternally ingested caffeine. Briefly, from the 2nd or 3rd day after conception, their dams had been provided, for the rest of gestation, with ad-libitum drinking water either unadulterated or containing research-grade caffeine (1,3,7-trimethylxanthine, Sigma) at concentrations of 0.23

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or 0.38 mg/ml. These solutions were later calculated to constitute doses of 26 or 45 mg/kg/day (Hughes & Beveridge, 1991). The resulting water-exposed litters destined for the present study were fostered within 24 h after birth to other water-treated dams that continued receiving water alone during lactation. Gestationally caffeine-exposed litters were fostered to previously water-treated lactating dams who, until their foster litters were weaned, were provided with drinking solutions containing 0.115 or 0.23 mg/ml caffeine. Because lactating dams may drink nearly twice the amount of water as that drunk during gestation (Peruzzi, Lombardelli, Abbracchio, Coen, & Cattabeni, 1985), these concentrations were lower than those experienced by pregnant dams. They were later calculated to provide doses of 25 and 35 mg/kg/day. All animals had been weaned when 25 days old and kept in groups of 4 or 5 same-sex individuals in 46 × 29 × 16.5 cm high plastic cages with perforated stainless steel lids. They received ad-libitum food and water and were kept on a 12:12-h reversed light:dark cycle at an ambient temperature of 21°–23°C and relative humidity of 40%–50%.

When approximately 260 days old, 24 subjects (12 males, 12 females) were randomly chosen from the cages of animals that had not been exposed to caffeine during either gestation or lactation (control group). A further 47 rats were chosen from those that had been both gestationally and lactationally exposed to caffeine at respective maternally ingested daily doses of either 26 and 25 mg/kg (low dose, 12 males, 12 females) or 45 and 35 mg/kg (high dose, 13 males, 10 females). Equal numbers of an additional 36 untested rats (18 males, 18 females) were chosen from each of the three groups for determination of adrenal gland weights. All subjects had come from 47 control, 16 low (26 mg/kg/day), and 14 high (45 mg/kg/day) gestational dose litters. No more than 1 male and 1 female from each litter assigned to the three lactational exposure conditions (control, 25 mg/kg/day, 35 mg/kg/day) was selected for behavioral testing and adrenal weight determinations.

Apparatus

The apparatus was a wooden elevated plus-maze of a design similar to that used by Pellow et al. (1985). It had four 50 × 10 cm wide arms extending at 90° to each other from a central 15 × 15 cm platform. Two of the arms that faced each other had 25-cm-high side and end walls (enclosed arms). The remaining arms without walls constituted the open arms. The maze was elevated to a height of 1 m on a central stand. Its floor and walls were painted shiny black, and, to increase its aversiveness and thus anxiogenic potential, it was illuminated by a triangular arrangement of three unshaded incandescent 100-W ceiling lamps, 2 m apart and 1.4 m above the apparatus. The maze was positioned beneath the lamps to ensure that it was evenly illuminated in the otherwise unlit room. By means of a Toshiba Photocell Illuminometer (Model SP 1-5), average reflected light within the maze was shown to be 60 lx.

Procedure

For later identification, all subjects were marked with differently colored stock sprays. Seven days before testing, they were housed in the experimental room (floor area = 12 m^2), where they received 10 min of individual daily handling in the dimly lit room during the dark phase of their light:dark cycle. Because the rats had received virtually no handling since birth, this procedure was intended to accustom them to this relatively new experience, as well as to familiarize them with the hitherto unknown experimenter. The animals' cages were kept in enclosed compartments against one wall of the room to ensure that the occupants would be shielded from the illumination experienced by other individuals being tested.

On the 8th day, each rat was placed on the center platform and observed for exactly 6 min. Alternate rats were positioned facing different open and enclosed arms. Head and front leg entries of each arm were recorded and, every 5 sec, it was noted which arm the subject was on (occupancy) and if it was walking, rearing up on its hind legs, grooming, remaining immobile with only its vibrissae moving, or freezing. After its trial, the rat was returned to its cage and the number of fecal boli and urine spots counted before the maze was washed with a weak detergent solution. To ensure maximum activity, all testing was conducted during the dark phase of the animals' light:dark cycle with the experimenter (V.G.L.) blind as to their prior exposure to caffeine. The order of testing of subjects from the different treatment groups was randomized.

Rats chosen for adrenal weight determination were sacrificed and their adrenal glands dissected, cleaned of surrounding tissue, and the paired weights determined relative to body weight (mg/100 g body weight). Adrenals from 2 of the males exposed to the low combined dose of caffeine were discarded because of damage incurred during dissection.

RESULTS

The reproductive data derived from this study were reported previously (Hughes & Beveridge, 1991). These data revealed that gestational exposure to caffeine in drinking water at calculated doses of 0, 26, and 45 mg/kg/ day had no effect on either the proportions of successful pregnancies brought to term (90%, 80%, and 70%, respectively) or on the average number of pups per litter size (11.9 + 0.4, 12.4 + 0.9, and 13.4 + 0.4, respectively). Furthermore, there was no evidence of congenital abnormalities or differences in offspring body weight at 6 months of age (Hughes & Beveridge, 1991).

Owing to a recording error, all data on 1 female in the high-dose group were not included in any analyses. Since grooming, freezing, defecation, and urination occurred so rarely, these responses were not included either. The percentage of entries of the open arms and the total number of 5-sec observations out of 72 on which the rats were seen on each type of arm (occupancy) were calculated. The percentages of occupancies of each arm in which subjects were seen walking, rearing, and remaining immobile were also calculated. (Unlike merely counting numbers of responses emitted in each type of arm, this procedure avoided erroneous conclusions being drawn from treatment effects on the higher frequencies that would inevitability occur in the arms most often occupied.) Effects of the early caffeine treatment and sex on these measures, as well as on the total frequencies of each response, are outlined in Table 1.

Separate 3 (dose) × 2 (sex) analyses of variance (ANOVAs) revealed significant early caffeine exposure effects for total rearing [$F(2,65) = 5.67, p < .01$] and immobility [$F(2,65) = 10.86, p < .001$]. There were also significant caffeine effects for percent of entries [$F(2,65) = 8.77, p < .001$] and occupancy of each type of arm [$F(2,65) = 3.43, p < .05$]. No other measure was affected by the caffeine treatment. As shown by post hoc Dunnett's t tests ($p < .05$), the significant effects arose from less rearing but more immobility, more percentage of open-arm (and fewer closed-arm) entries and more open-arm (and less closed-arm) occupancy in rats exposed to the highest dose of caffeine. Female rats displayed higher frequencies of total arm entries [$F(1,65) = 4.54, p < .05$] and walking [$F(1,65) = 7.70, p < .01$] while on both types of

Table 1
Effects of Sex and Combined Gestational and Lactational Exposure to Caffeine on Mean Responses ($M \pm SEM$) of Adult Offspring in the Elevated Plus-Maze

	Caffeine Dose						Sex			
	Control		Low		High		Males		Females	
Measure	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*
Total Activity										
Total arm entries	25.1	1.1	24.9	1.5	25.3	1.0	23.7	1.0	26.6	0.9†
Total walking	19.1	0.7	20.4	1.1	19.4	1.2	18.1	0.8	21.3	0.7†
Total rearing	16.8	0.7	16.7	1.2	13.3	0.7*	15.1	0.6	16.2	1.0
Total immobility	29.4	1.0	30.6	1.2	35.9	1.2*	32.2	0.9	31.6	1.1
Open-Arm Behavior										
% entries	50.1	1.4	48.5	1.1	56.3	1.6*	51.5	1.2	51.6	1.3
5-sec observations in arms (occupancy)	17.9	1.2	18.3	1.4	22.4	1.4*	18.7	1.2	20.4	1.0
% observations spent walking	17.5	2.7	17.3	2.3	12.3	2.2	15.2	2.1	16.4	1.9
% observations spent rearing	1.8	0.7	3.7	1.0	1.9	0.7	2.4	0.6	2.5	0.8
% observations spent immobile	78.5	3.1	79.0	2.5	83.2	2.4	79.8	2.3	80.6	2.2
Closed-Arm Behavior										
% entries	49.9	1.4	51.5	1.1	43.7	1.6*	48.5	1.2	48.4	1.3
5-sec observations in arms (occupancy)	54.1	1.2	53.7	1.4	49.6	1.4*	53.3	1.2	79.6	1.0
% observations spent walking	29.5	1.3	32.2	2.0	33.5	2.2	28.8	1.4	34.8	1.5†
% observations spent rearing	30.9	1.6	30.7	2.6	26.2	1.5	28.0	1.3	30.7	1.9
% observations spent immobile	28.4	2.0	29.8	2.7	34.7	2.6	32.4	1.8	29.3	2.2

*Significantly different from control group, $p < .05$. †Sex difference significant, $p < .05$ or $< .01$.

arms than did males. They also spent proportionately more of their time on the enclosed arms engaged in walking than did male subjects [$F(1,65) = 9.67, p < .01$].

Differences between enclosed and open arm entries, occupancy, and percentages of observations spent walking, rearing, and immobile were calculated and can be seen in Table 2. These values were obtained by subtracting open from enclosed arm frequencies so that, the higher the value, the lower the frequency of that measure in the open relative to the enclosed arm. Within-group differences between enclosed and open-arm frequencies were determined by repeated-measures *t* tests.

All treatment groups and both sexes occupied the enclosed more often than the open arms. They also spent more of their time walking and rearing and less time immobile while on the enclosed rather than open arms. However, there was no significant difference in the number of entries of each type of arm for any group except for those rats that had experienced the highest combined dose of caffeine. Two-way ANOVAs (dose × sex) revealed significant early caffeine exposure effects for entries [$F(2,65) = 11.81, p < .01$], occupancy [$F(2,65) = 3.43, p < .05$], and percentage of observations spent walking [$F(2,65) = 3.45, p < .05$]. This effect was due to the high-dose group showing the greatest enclosed–open arm difference for entries and percentage of observations spent walking, but the smallest difference for occupancy. The dose × sex interaction was not significant for any behavioral measure.

Effects of early caffeine exposure on paired relative adrenal weights and body weights are outlined in Table 3. Because of skewed distributions of the treatment influences, the effects were nonparametrically assessed by Kruskal-Wallis one-way ANOVAs followed by Mann-Whitney *U* tests (Siegel, 1956). Adrenal relative weights were significantly affected by early caffeine exposure only in males for whom the weights were heaviest with the highest dose [$H(2) = 6.82, p < .02$]. There were no significant caffeine exposure effects on body weight for either sex.

Table 2
Effects of Sex and Combined Gestational and Lactational Exposure to Caffeine on Differences Between Closed- and Open-Arm Responses ($M \pm SEM$) of Adult Offspring in the Elevated Plus-Maze

	Caffeine Dose						Sex			
	Control		Low		High		Males		Females	
Measure	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*
Arm entries	−0.3	0.6	0.8	0.5	−3.5	0.8*†	−0.9	0.6	−1.1	0.6
Arm occupancy	36.2	2.4*	35.3	2.7*	27.2	2.8*†	34.6	2.4*	31.2	1.9*
% observations spent walking	12.0	3.1*	14.9	2.7*	21.2	2.2*†	13.6	2.0*	18.5	2.4*
% observations spent rearing	29.1	1.7*	27.0	2.7*	24.3	1.4*	25.5	1.4*	28.3	1.9*
% observations spent immobile	−50.1	3.5*	−49.3	3.3*	−48.5	2.4*	−47.4	2.4*	−51.3	2.6*

*Difference between closed- and open-arm frequencies significant, $p < .05$. † Significantly different from control group, $p < .05$.

Table 3
Effects of Combined Gestational and Lactational Exposure to Caffeine on Median Paired Relative Adrenal Weights and Body Weight for Males and Females Separately

	Caffeine Dose		
	Control	Low	High
Relative Adrenal Weights (mg/100 g body weight)			
Males	7.9	8.9	10.7*
Females	23.6	23.4	25.7
Body Weight (g)			
Males	354.1	333.0	322.9
Females	206.6	205.1	208.7

*Significantly different from control group, $p < .05$.

DISCUSSION

Clearly, combined gestational and lactational exposure to caffeine produced long-lasting effects on the behavioral development of the rats, because, as shown with open-field behavior (Hughes & Beveridge, 1990), these effects were still evident 8 or 9 months later. However, whereas the treatment decreased ambulation, walking, and rearing in an open field (Hughes & Beveridge, 1991), the highest dose of similar treatment decreased rearing but had no effect on total entries and walking in the elevated plus-maze. In line with the conclusion that activity decrements, increased defecation, and failures to emerge from a darkened chamber into a brightly lit arena reflect some anxiogenic action of early caffeine exposure (Hughes & Beveridge, 1991), the highest dose in the present study also increased total frequencies of the "anxiety-related" response, immobility (Pellow et al., 1985).

When behavior on the open arms is considered, however, a different picture emerges. If, as has been maintained by several authors (Halliday, 1966; Montgomery, 1955; Pellow et al., 1985), the extent to which rats visit and occupy the open arms of elevated mazes is inversely related to their level of fear or anxiety, then increases in these measures by the highest dose of caffeine might appear to be due to anxiolytic rather than to the anxiogenic effects of the treatment. However, it should be noted that although behavior in the elevated plus-maze has been validated as a test of anxiety (Pellow et al., 1985), it is not unusual for some treatments to have outcomes that are difficult to interpret in terms of expected changes in anxiety. For example, some anxiolytic drugs (such as buspirone, propranolol, and ritanserin) have been shown to have no effect or even anxiogenic-like effects on plus-maze responses believed to reflect rodent anxiety (Lister, 1991; Moser, 1989; Pellow et al., 1987; Rodgers et al., 1995; Wright, Heaton, Upton, & Marsden, 1992). In the present study, rats exposed to the highest dose of caffeine were also the only group that entered the open more often than the enclosed arms, as well as exhibiting the greatest difference between entries of the two arm types. But although all subjects spent less of their time walking when on the open than when on the enclosed arms, this difference was greatest for the highest dose rats, suggesting that even though they more readily ventured onto and occupied the open arms, they were more reluctant to move horizontally once committed to them. Taking into account such a possibility, along with their lower frequencies of total rearing and higher levels of total immobility, open arm behavior in the present study might not be completely inconsistent with earlier suggestions of greater emotional reactivity in rats exposed to maternally ingested caffeine during gestation and lactation either separately or combined (Hughes & Beveridge, 1987, 1990, 1991). Further support for heightened emotional reactivity following early exposure to caffeine is found in higher preferences for the black chamber in a black–white preference test (File, 1987), longer latencies of entering a conditioned aversive environment (Sinton et al., 1981; Swenson, Beckworth, Lamberty, Krebs, & Tinius, 1990), and increased proportions of rats that avoid electric shock in a step-down passive avoidance situation (West et al., 1986).

The finding that treatment with the highest dose increased relative adrenal weights in males is similar to the effects of prenatal (Hughes & Beveridge, 1987) and postnatal caffeine alone (Henry & Stephens, 1980) and is consistent with exposure to prenatal caffeine being stressful, since this outcome reflects a reaction to chronic stress (Hatch, Wiberg, Balazs, & Grice, 1963). It is also in line with reports of increased susceptibility to gastric ulceration following early caffeine exposure (Glavin & Krueger, 1985). By itself, evidence of higher adrenal weights is of little significance, but together with behavioral effects of early caffeine exposure, it further supports the view that the treatment can lead to long-lasting heightened emotional reactivity in offspring (Hughes & Beveridge, 1986a, 1987, 1990, 1991). As suggested earlier (Hughes & Beveridge, 1987), the absence of an effect on female adrenal weights was probably due to a ceiling effect resulting from the very high weights of control subjects, since prenatal caffeine effects on female adrenal growth have been observed (Hughes & Beveridge, 1990) when female control weights were lower than in either the earlier (Hughes & Beveridge, 1987) or the present studies.

It is possible that because of the opportunity to retreat to the enclosed arms, all rats in the present study found the open arms of the elevated plus-maze less aversive than the forced-choice open fields used in earlier studies (Hughes & Beveridge, 1986a, 1987, 1990, 1991). Although designed to merely decrease unfamiliarity of the experimenter, it is also likely that the daily handling of subjects in the experimental room for 1 week prior to testing further reduced the aversiveness of the overall procedure. The almost total absence of defecation and freezing would suggest that the testing situation was not markedly aversive. Therefore, perhaps the situation has to be more stressful than it may have been for pre- and early postnatal effects of caffeine on some stress-related responses (such as avoidance of plus-maze open arms) to become apparent, as occurs with acute and chronic caffeine treatment in adult humans (Cobb, 1974; Shanahan & Hughes, 1986). Alternatively, since administration of caffeine to rat pups for the 1st week of life has been shown

to impair their adult spatial learning (Zimmerberg, Carr, Scott, Lee, & Weider, 1991), it is possible that the caffeine effect on open-arm entries might have been an artifact of poorer ability in detecting the spatial location of the preferred enclosed arms, rather than to apparently lower emotional reactivity. Exposure to chronic caffeine during early development, for example, increases variability in jewel fish of spatial behavior that involves complex orientational and attentional responses (Burgess, 1982), and acute caffeine can disrupt the ability of day-old rats to orient toward a home area (Holloway, 1982). It is also possible that their greater willingness to enter the open arms may have reflected a stronger tendency for caffeine-exposed rats to explore all possible avenues of escape from the testing situation; escape responses have been suggested for what might initially appear as approach behavior in other putatively aversive environments (Hughes, 1989).

Apart from relative adrenal weights, exposure to caffeine during both gestation and lactation did not produce the sex-dependent effects (favoring males) characterizing exposure during one period or the other separately that was shown earlier (Hughes & Beveridge, 1986a, 1986b, 1990, 1991). When the drug has been experienced both gestationally and lactationally, the absence of caffeine × sex interaction effects on behavior has been ascribed to greater caffeine effects in females resulting from the longer early exposure. This was seen as reducing the males' advantage, which may have arisen from their possible testosterone-mediated hypersensitivity to caffeine influences on the developing brain (Hughes & Beveridge, 1991). The higher frequencies of total arm entries and walking in females are in line with many other reports of similar sex differences (Archer, 1975). Of incidental interest is the observation that although overall, females spent more of their time on the enclosed arms walking than did males, this difference was not apparent when the rats were on the open arms, presumably because of a type of floor effect resulting from the lower relative frequencies of walking that typified both sexes when they were on the open arms.

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