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Effects of Postnatal Caffeine Exposure Through Dam's Milk Upon Weanling Rats

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GULLBERG, E. I., F. FERRELL AND H. D. CHRISTENSEN. *Effects of postnatal caffeine exposure through dam's milk upon weanling rats.* PHARMACOL BIOCHEM BEHAV 24(6) 1695–1701, 1986.—The effects of postnatal caffeine exposure received through mother's milk were examined in rat pups by administering 0.0125 or 0.05% caffeine solution to the dams throughout lactation. Offspring of caffeine-treated dams showed significantly earlier onset of auditory startle and air righting reflexes. No direct effects of treatment were observed on eight measures of open field activity. At weaning, high-dose caffeine pups exhibited a transient increase in caffeine acceptance in two-bottle taste preference tests and showed cyclic exaggerations in caffeine preference-aversion functions which persisted throughout the 22-day postweaning test period. The caffeine levels in the dams' milk had a 30-fold daily fluctuation reflecting the fluid ingestion pattern related to the light-dark cycle and caffeine elimination rate. Caffeine concentrations in the brains of the pups varied between 0.01 and 9.0 μg/g.

Caffeine Early experience Taste preference Flavor cues Developmental reflexes
Locomotor activity Rat High performance liquid chromatography

CAFFEINE, a pharmacologically active component of many commonly consumed beverages and nonprescription medications, has received interest as a teratogen in fetal rats [14,35]. It can cause behavioral abnormalities in the offspring when administered to the pregnant dam [7, 23, 39]. Caffeine has been cited as a possible risk factor for pregnancy complications when consumed in coffee by pregnant women [6, 28, 43], and in a 1980 position statement, the Food and Drug Administration advised pregnant women to eliminate or limit their consumption of caffeine-containing products [20]. No recommendations were made at that time to breast feeding mothers, yet the number of women breast feeding their infants has risen substantially over the past three decades [29,33]. Because caffeine enters breast milk, it is of interest to study the effects of caffeine ingestions during lactation unconfounded by caffeine exposure during pregnancy [4, 9, 42]. Newborns eliminate caffeine more slowly than adults [2] and theoretically might ingest potentially harmful concentrations through nursing. Of additional interest is the possibility that caffeine in breast milk might impart sensory cues which affect the infant's subsequent food habit development [30]. Exposure to specific dietary components early in life has been shown in several species to enhance preference for them [18], and chemical cues in the diet eaten by a lactating mammal and transmitted to the young through her milk can influence the offspring's food choices during weaning [8,22]. Conversely, learned aversions have been described in animals [21] and in human infants [16, 25, 38] when flavor cues received through nursing were associated with alimentary upsets.

Some limitations of developmental studies on caffeine employing rat models have been use of extremely high doses, administration through methods such as gavage or injection, which tend to deliver the drug in a bolus dose compared to the normal ingestive route, failure to separate effects of prenatal and postnatal caffeine exposure, or combinations of these [7, 13, 14, 23, 24, 39]. The present experiments were undertaken to examine behavioral and neurological effects on offspring of caffeine-treated rats in which the caffeine exposure was limited to lactation. Entry of caffeine into the maternal milk and into plasma and brain of dams and offspring was verified by high performance liquid chromatography.

EXPERIMENT 1: EFFECTS OF CAFFEINE EXPOSURE THROUGH DAM'S MILK ON DEVELOPMENTAL MEASURES AND POSTWEANING CAFFEINE PREFERENCE

METHOD

Animals

Subjects were offspring of 18 Sprague-Dawley derived rats (Simonsen Laboratories, Gilroy, CA 95020). Dams were obtained 3–4 days before due date, housed individually in maternity cages, and allowed to deliver normally. Within twenty-four hours of parturition (Day 0) each litter was culled to six animals, to include three males and three females when possible. Each dam was housed with her litter until Day 21, when pups were weaned. Pups and dams were then

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TABLE 1

FLUID AND CAFFEINE CONSUMPTION OF DAMS DURING CONTINUOUS CAFFEINE EXPOSURE IN DRINKING WATER

Group	Week of Lactation* 1	2	3
Control (distilled deionized water)			
Total Fluid (ml)	41.8 ± 10.3	58.3 ± 7.8	71.4 ± 11.9
Total Caffeine (mg)	—	—	—
Lo Caf (0.125 g/1,000 ml)			
Total Fluid (ml)	44.5 ± 15.4	59.6 ± 11.7	68.7 ± 14.6
Total Caffeine (mg)	5.6 ± 1.9	7.5 ± 1.5	8.6 ± 1.8
Hi Caf (0.5 g/1,000 ml)			
Total Fluid (ml)	42.5 ± 16.4	58.4 ± 18.6	72.4 ± 26.1
Total Caffeine (mg)	21.2 ± 8.2	29.2 ± 9.3	36.2 ± 13.1

Values are mean ± S.E.M.
*Daily consumption averaged over seven-day intervals.

housed individually in wire bottom cages for three weeks of taste preference testing. Throughout the six-week experiment animals were fed ad lib Rat Chow 5012 (Ralston Purina Co., St. Louis, MO 63164) and were maintained on a 14:10 light-dark cycle. Pups were weighed on Day 1, Day 3 and every third day thereafter. Body weights, developmental measures and activity tests were recorded by animals' treatment, litter and sex.

Caffeine Administration

After delivering, the 18 dams were randomly assigned to one of three groups: control, low dose caffeine (Lo Caf) or high dose caffeine (Hi Caf). Control dams received ad lib access to deionized water. Dams in the two experimental groups received ad lib caffeine solutions as their sole source of fluid. Drinking bottles were equipped with nondrip, double ball bearing stainless steel spouts (Atco Mfg. Co., Napa, CA 94558) and positioned throughout the study so that the pups did not have direct access to them. Lo Caf and Hi Caf dams received 0.125 g/1,000 ml and 0.5 g/1,000 ml, respectively, of anhydrous caffeine USP (Sigma Chemical Co., St. Louis, MO 63178) dissolved in deionized water. Those concentrations were chosen for comparative purposes and correspond to low and high caffeine levels used in a large study conducted at the Food and Drug Administration [39] which examined perinatal caffeine exposure via maternal intake. The 24-hour fluid intake of each dam was measured daily.

Procedures

Auditory startle. The typical rat pup is functionally deaf until the thirteenth day after birth, at which time a sudden noise will regularly elicit a startle reaction [5]. Beginning on Day 10, each pup was tested daily. Each animal was placed individually on a table and a ball point pen was used to make a standardized clicking sound about five inches above and behind the pup. Each pup received three trials daily. The auditory startle response was judged to be present if at least two positive responses occurred. Tests were terminated for a litter when all six pups in that litter exhibited the response.

Eye opening. Beginning on Day 11, drawings were made daily of the stage of eye opening of each animal. The first day on which both eyes were fully opened is reported here.

Air righting reflex. Beginning on Day 14, each pup was tested daily until all the members of its litter had demonstrated the reflex for two successive days. Each animal was held supine by fore- and hindlimbs, twelve inches above a three-inch thick foam pad. One experimenter released the animal while a second observed and scored. Each animal received three daily trials in which the reflex was arbitrarily defined as present if all four of the subject's feet touched the foam pad at once in two or more of the three trials. The scorer did not know the treatment group of the litters.

Open field activity. Each animal was tested on Day 18 in an apparatus consisting of a 40 cm Plexiglas circle surrounded by a 20 cm high cardboard wall. The floor was marked with four concentric circles, with the three outer circles divided into thirds, fifths and sevenths, respectively, resulting in sixteen spaces of equal area. The light source was located directly overhead to avoid shadows. The Plexiglas was cleaned with isopropyl alcohol and allowed to dry before each animal's test to minimize the possible influence of olfactory cues. Each pup was placed in the central circle and timed for three minutes with a stop watch. The observer who timed the animal recorded the following: latency (sec) to leave center of circle; total number of rearings; number of unassisted rearings (without use of front paws); seconds spent grooming per 3-min interval; total grooming events; total urinations and total defecations. A second observer, who did not know which treatment group was being examined, counted the number of lines crossed by the animal's front and rear feet.

Taste preference tests. After weaning on Day 21, all animals were housed individually in suspended wire bottom cages and preference tests were conducted for pups and dams over a 22-day period. Preference for 0.002 M (~0.0039%) caffeine solution was measured using a 24-hr two-bottle choice technique [26] in which each animal was presented with two drinking bottles, one containing the caffeine (test) solution dissolved in deionized water; the other containing only deionized water (the standard). The caffeine solution was placed either on the right or the left side of the

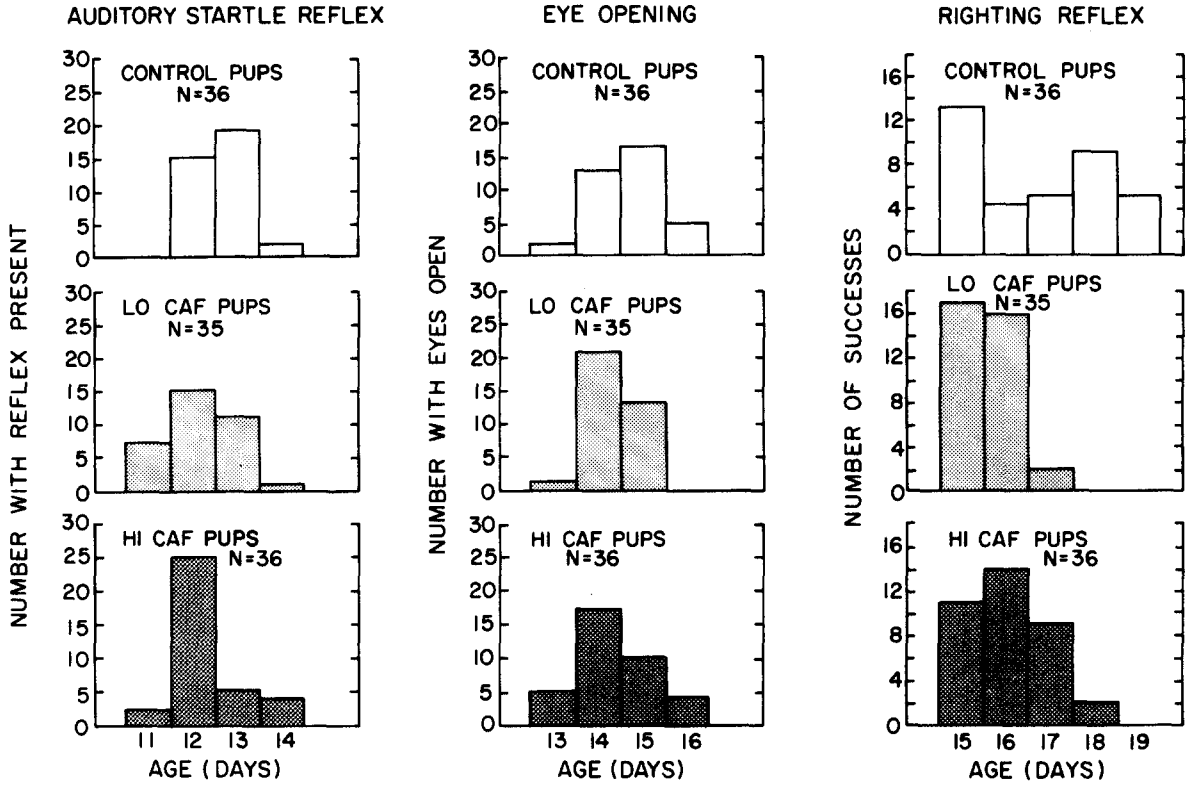

FIG. 1. Effects of caffeine treatment upon time of onset of developmental reflexes and eye opening.

cage front for the first 24 hr, after which the positions of the test and of the standard were reversed. Every 48 hr the bottles were washed and refilled with fresh solutions. The initial placement of each test bottle during a 24-hr testing session was altered in a right-left-left-right . . . sequence in an attempt to counterbalance the effects of possible position preferences [45]. The preference ratio, or 'percent preference' score, exhibited by each rat for the test solution during each 24-hr session was computed by dividing the g of test solution consumed by the total g of fluid consumed (test + standard) and multiplying the quotient by 100. Twenty-two such 24-hr tests were conducted.

Statistical Analyses

The effect of caffeine treatment upon onset of the auditory startle response, eye opening and development of the righting reflex were examined using Chi square tests. Significance levels were adjusted by the Bonferroni method [19] whenever multiple comparisons were made. Two-way analysis of variance was employed to compare effects of caffeine treatment upon activity measures. All taste preference scores were normalized using the logit transformation [3] in which logit preference score, L, = 1 n[p/1−p], where p is the ratio of test fluid consumed to total fluid consumed. Preference data were analyzed using multivariate analysis.

RESULTS

The dams' fluid consumption increased during lactation in all groups (Table 1). Addition of caffeine to drinking water *per se* did not influence fluid intake. Whereas the estimated caffeine consumption was approximately 23 mg/kg/day for the Lo Caf dams and 94 mg/kg/day for the Hi Caf dams,

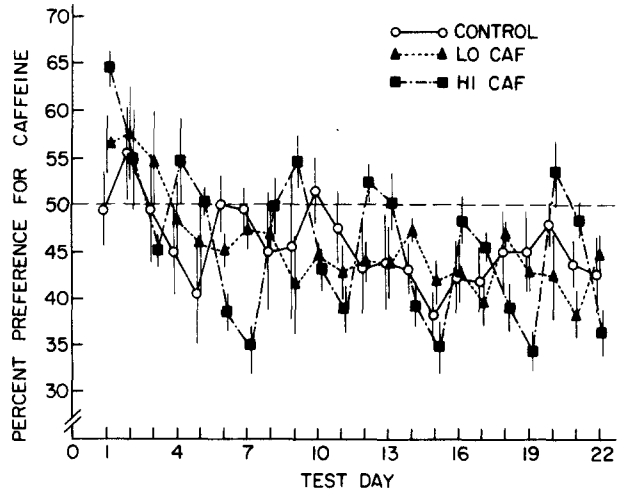

FIG. 2. Preference for caffeine solution. Each data point represents the mean (±1 SEM) of six litter means within a treatment condition.

because of the progressive increase in fluid consumption, it reached levels as high as 30 mg/kg/day and 135 mg/kg/day in the two groups, respectively. Hi Caf dams showed marked day-to-day variation in fluid intake. Maternal body weights were not affected by either of the dose levels of caffeine used. Postnatal caffeine exposure through dams' drinking fluid exerted slight, statistically nonsignificant effects of body weight of nursing pups. Control and Hi Caf pups weighed less than Lo Caf pups for the first nine days after birth, but by weaning Control pups had the highest average

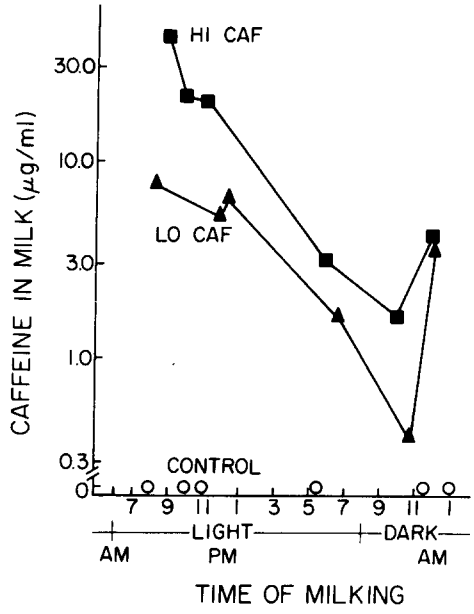

FIG. 3. Caffeine concentrations in milk of Hi Caf dams (0.5 g/1,000 ml; filled squares), Lo Caf dams (0.125 g/1,000 ml; filled triangles) and Control dams (open circles) as a function of the time of day.

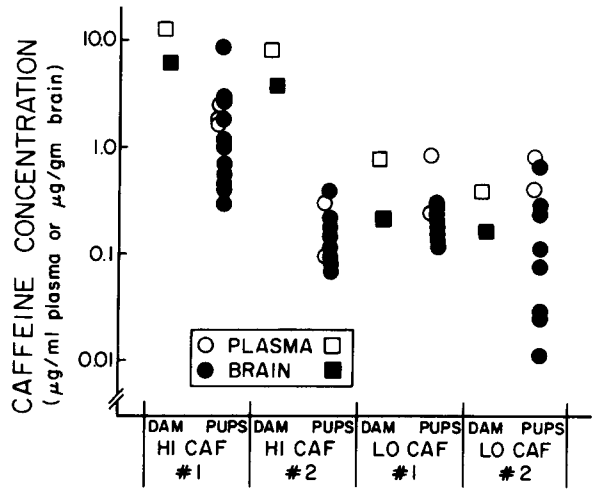

FIG. 4. Caffeine concentrations in plasma (open figures) and brain (filled figures) of Hi Caf and Lo Caf dams (squares) and their pups (circles).

body weight and Hi Caf pups had the lowest. Male pups in each group weighed more than females. Sex differences were significant by postnatal Day 24 in Lo Caf ($p<0.025$) and Hi Caf ($p<0.025$) animals and by Day 30 in Controls ($p<0.005$).

Developmental Measures

The effects of caffeine treatment upon time of onset of the auditory startle reflex, eye opening and development of the air righting reflex are shown in Fig. 1. Caffeine exerted a dose-related effect upon onset of the auditory startle response. The Control group exhibited the response later than the Lo Caf group (ns) and the Hi Caf group, $\chi^2(3)=13.32$; $p<0.05$. Eye opening showed directional trends although none were significant. Lo Caf and Hi Caf pups opened their eyes slightly sooner than Control animals. Treatment also affected the air righting reflex. Controls exhibited this reflex later than the Lo Caf group, $\chi^2(4)=22.99$; $p<0.01$, and the Hi Caf group, $\chi^2(4)=16.32$; $p<0.05$. In this case, the effects of dose were reversed, as low dose animals were able to right themselves slightly sooner than their high dose counterparts.

Open Field Activity

No significant treatment effect or sex effect was found for any activity measure employed. A treatment by sex interaction was present for number of defecations. Frequency of defecation of female, but not male pups, increased directly with caffeine level of their dams, $F(2,101)=3.26$; $p<0.05$.

Taste Preference Tests

Caffeine exposure during the nursing period produced an overall effect on postweaning caffeine preference, $F(22,65)=1.76$; $p<0.05$, and group by sex interaction was present, $F(22,65)=1.99$; $p<0.025$. On the first day of preference testing, Hi Caf, but not Lo Caf pups, preferred caffeine solution to water more than did Controls, $F(2,99)=3.76$; $p<0.05$. First-day mean preferences were 50%, 56% and 64% in Control, Lo Caf and Hi Caf animals, respectively (Fig. 2). Acceptance of caffeine relative to water declined in all three treatment groups over the 21 subsequent days of preference testing. Hi Caf animals showed a cyclic pattern of preference-rejection of caffeine. That is, compared to Lo Caf and Control animals, they not only exhibited exaggerated preferences on some days (Fig. 2; see Days 4, 9, 12, 16 and 20) compared to the other groups, but also showed more extreme rejections (Fig. 2; Days 7, 15, 19 and 22). No differences in caffeine preference were found among treatment groups for the dams.

EXPERIMENT 2: ANALYSIS OF CAFFEINE CONCENTRATIONS IN MILK, PLASMA AND BRAIN

METHOD

Animals

Six pregnant Sprague-Dawley derived rats were obtained to investigate caffeine concentrations in dam's milk. They and their litters were subsequently used to examine caffeine levels in plasma and brain tissue. Animals were purchased from the same source used in Experiment 1, and treatment conditions replicated those producing developmental effects and taste preference patterns reported in that study.

Milk Collection

On Days 7, 14 and 20 of lactation, each dam was milked by gentle hand massage after having been lightly anesthetized with ether and injected with oxytocin (Sigma Chemical Co., St. Louis, MO 63178) for milk let-down. Rats consume over 85% of their fluid during the dark cycle [36, 40, 49] and have an estimated half life ($t_{1/2}$) for caffeine of about 2 hr [12, 32, 41]. Thus, care was taken to obtain either a morning, midafternoon or late evening sample from each

dam over the three days on which milking occurred. Only one sample was collected per animal on a single day. An average volume of 0.5–1.0 ml per animal was collected at each milking. Milk samples were frozen at −4°C until analysis using high performance liquid chromatography.

Plasma and Brain Collection

Blood samples were taken by cardiac puncture with heparinized syringes and brains were removed from exsanguinated animals between noon and 2 p.m. on Day 21 of lactation. Plasma from pups within litters was pooled within groups of three or four animals. Plasma was separated by centrigugation (3,000 × g, 20 min). Samples were stored at −4°C until HPLC analysis.

HPLC Determination of Caffeine Concentrations

For all samples, the liquid chromatographic caffeine analysis was performed using a Waters ALC/GPC-204 liquid chromatograph, u.v. detector 254 nm, μBondapak C_{18} column and a mobile phase of 10% acetonitrile: 0.01 M sodium acetate, pH 4.0 [10]. The milk and plasma samples were prepared by mixing with 20% acetonitrile in sodium acetate buffer, centrifugation and injection of the supernatant. Caffeine from brain tissue was extracted with chloroform.

RESULTS

Caffeine Concentrations in Milk

Caffeine levels in dams' milk are shown in Fig. 3. No caffeine was present in milk of Control animals. Caffeine levels ranged from 0.39 μg/ml–7.36 μg/ml and from 1.63 μg/ml to 41.81 μg/ml in the milk of Lo Caf and Hi Caf dams, respectively. Within each treatment group, the caffeine concentration in milk was dependent upon the time during the light-dark cycle at which the dams were milked. Maximum caffeine concentrations were found when milking occurred about 7 a.m. Values declined throughout the day and began rising again after the lights were turned off at 8:00 p.m.

Caffeine Concentrations in Plasma and Brain

While there was a clear difference in the plasma and brain caffeine concentration between the Hi Caf and Lo Caf dams (Fig. 4), the brain caffeine concentrations of their pups showed overlap with wide variance. The pups' plasma concentrations observed at this midday time point varied from about 0.1 to 4.0 μg/ml.

DISCUSSION

The dams' fluid consumption increased as lactation progressed, a finding similar to that reported by Sobotka *et al.* [39], who used identical caffeine concentrations but with an exposure period which encompassed gestation and lactation, and in keeping with results observed by Concannon *et al.* [15], in dams postnatally exposed to 0.05% caffeine solution. This increase was not specific to the caffeine-treated animals, as reported by Butcher *et al.* [7], whose rats received chronic exposure to caffeinated fluid beginning 60 days before breeding and continuing throughout gestation and lactation. Like Concannon *et al.* who used 0.05% caffeine, we failed to see significant reductions in pups' body weights as a result of caffeine exposure at 0.0125% or 0.05% restricted to the postnatal period. Sobotka and colleagues reported body weight reductions at those concentrations, and Butcher *et al.* found that 0.056% caffeine administered to dams throughout pregnancy and lactation, either as caffeine solution or in the form of brewed coffee, resulted in lower pup body weights. Caffeine elimination has been demonstrated in humans [1,11] and baboons [11] to be considerably slower during pregnancy than postpartum. Perhaps caffeine exposure during pregnancy is required to obtain the pup body weight differences observed by other researchers. Decreased body weight 24 hr after birth, for example, had been reported in pups whose mother consumed 0.05% caffeine solution during pregnancy [24].

A more important question is the amount of caffeine consumed by the pups. On the basis of the current study, the caffeine concentrations at the behavioral testing times were quite low, 0.01 to 10 μg/ml. Caffeine concentration in the pup will be determined by caffeine concentration in the dam's milk, amount ingested when nursing occurs plus the rate at which the pup eliminates the ingested caffeine. The adult rat has a caffeine plasma elimination half life of about 2 to 3 hours [12,41]. Caffeine elimination from milk in other species is similar to that in plasma with about 50 to 100% the concentration at any time [4, 9, 42]. Although not a kinetic study, the milk values found are suggestive that the half life of caffeine in milk of the lactating rat is also 2–3 hours. The newborn rat has extremely limited capacity to metabolize caffeine, which increases with age to about half the adult level at 15 days, equal at 21 days, and 125% at 30 days [46]. The rate of metabolism is the prime factor in determining caffeine clearance. The rat, including the lactating one, consumes 85–95% of its daily fluid intake during the dark period [36,40]; however, the lactating rat spends more time with the litter during the light period, which suggests that milk intake is increased during this period. Caffeine administered at a dosage of 1 mg/kg to any species will result in a peak plasma concentration of about 1 μg/ml, with an individual range of at least 0.5–2.0 μg/ml [17]. Thus, if the dams in the two treatment groups had consumed their fluid as a bolus dose at one point during the night, peak concentrations to about 35 and 140 μg/ml, respectively, would have been anticipated. Measurement of milk caffeine levels of 7 to 40 μg/ml in the morning are quite reasonable considering both the consumption interval and the dams' clearance rate.

Rat pups consume the higher portion of their milk during the day, at least between Days 4 and 17 of lactation [27]. When they have access to food, they switch to a nocturnal food and drinking pattern at about Day 19, although milk consumption will continue if permitted until about Day 26. Since water was not available to the pups in this study until weaning, their sole source of fluid was the dams' milk. Considering all the kinetic parameters, both the range and concentration measured in the plasma and brain of the pups are reasonable. Their caffeine exposure falls within the range for the human population drinking decaffeinated (control) to 10–15 cups of caffeinated coffee daily.

Postnatal caffeine treatment led to significantly earlier demonstration of an auditory startle in our Hi Caf rats, whereas perinatal exposure [7] did not have a significant effect upon this reflex. Also in contrast with earlier studies employing pre- and postnatal caffeine exposure, eye opening was slightly accelerated in a non-dose related manner in our animals. Perinatal exposure appeared to delay eye opening somewhat in one study [39], while another found no significant differences [7]. Unfortunately, testing for appearance of the air righting reflex was begun too late. On Day 15, our first

test day, 13 of 36 control animals, or 36%, were already able to right themselves (Fig. 1). Righting tests are sensitive to several treatments, but differences are not long lasting and testing should be begun before the response matures in the controls [37]. The study on which we based that portion of our experiment used Long-Evans rats [34], which apparently tend to develop the reflex later than Sprague-Dawley animals. Despite the late start in testing, air righting was the developmental reflex most affected by postnatal caffeine exposure and caffeine accelerated its appearance. Earlier perinatal studies examined surface righting rather than air righting, so their results are not directly comparable to ours. One found no effect at any dose level [39] while the other reported a significant delay only in the animals receiving high caffeine levels in the form of brewed coffee [7].

Subjective observation of the pups in their maternity boxes showed that caffeine-exposed animals were more active. Our open field tests, conducted on Day 18, did not reveal significant differences. Butcher and coworkers observed increased activity in caffeine-treated pups, whereas Concannon *et al.*, using different activity measures, reported hypoactivity at 15 and 20 days in caffeine-exposed pups. Some of the variation in the behavior response of the pups might have been due to the kinetic status of the caffeine at the time of measurement. The absolute level of caffeine may not be as critical as periodic changes in the concentration. Measured caffeine responses correlate fairly well with the rise, but not the decline, in plasma levels [11, 31, 47, 48]. Since behavioral measures are exquisitely sensitive to postnatal maternal influences [37], the caffeine could have made subtle changes in the dams that were reflected in the pups' behavioral changes.

Our study represents the first to examine postweaning caffeine preferences in rat pups whose caffeine exposure was restricted to the nursing period and received exclusively through the milk of dams ingesting caffeine solutions. The lower concentration employed, considered on a body weight basis, approximates that ingested by a heavy consumer of caffeinated products. It had virtually no effect on the developing rat. A short term enhancement in preference for caffeine solution, presented in a two-bottle choice situation, was observed in Hi Caf animals, but it was limited to the initial 24-hr preference test administered immediately after weaning and separation from the dams. Whereas substances tasting bitter to humans are usually strongly rejected by animals, early weaned guinea pigs exhibit a temporary increase in acceptance of bitter sucrose-octa-acetate solution if it is presented as their sole drinking fluid for the first eight days after weaning [45]. Similarly, for their first meals of solid food, weanling rats actively seek and preferentially ingest the diet consumed by their mother during the nursing period, even if that diet is relatively unpalatable compared to others available to them [22]. These and other studies [18] suggest that early experience with a flavor, even an unpalatable one, might increase subsequent acceptance of that flavor, although this effect was particularly transient in the present study.

Perhaps our most intriguing and unexpected observation was the occurrence in Hi Caf pups of pronounced swings in amplitude in regularly occuring cycles of the caffeine taste preference-aversion function (Fig. 2). Possibly Hi Caf animals, through chronic exposure to higher caffeine levels while nursing, suffered aversive symptoms upon withdrawal from their caffeine source at weaning, and consequently ingested comparatively higher quantities of caffeine solution when it was made available to them during the initial taste preference test. Preweanling exposure of Hi Caf pups to higher concentrations of caffeine's bitter taste and/or aversive postingestional consequences might have resulted in more extreme rejection by this group on some later tests. Hi Caf dams exhibited extreme rejection of their caffeine solution on some days of lactation. Their trends had a cyclic pattern, but with a slightly shorter period than that shown by their pups. One might speculate that the pups developed their postweaning patterns from prior exposure to cyclic levels in their dams' milk during lactation. In adult rats a period of forced caffeine consumption produces a subsequent increase in free-choice caffeine intake but a decreased preference for a flavor associated with the forced caffeine consumption [44]. These data suggest that enhanced caffeine intake following caffeine exposure is related to its drug properties and despite its taste. Whatever the mechanisms influencing pups' cyclic preference patterns, the effect was enduring, and was as pronounced when preference tests were terminated after three weeks as it was when they were begun. Understanding of the preference-aversion function, particularly if it could be related to neonatal exposure, might be of clinical importance. Preference for xanthines, and thereby, compliance if required for therapeutic reasons, is observed to vary among siblings.

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