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Neonatal Caffeine Exposure Alters Developmental Sensitivity to Adenosine Receptor Ligands

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GUILLET, R. AND C. K. KELLOGG. *Neonatal caffeine exposure alters developmental sensitivity to adenosine receptor ligands.* PHARMACOL BIOCHEM BEHAV **40**(4) 811–817, 1991.—Studies were done to determine whether the apparent changes in behavioral sensitivity to adenosine receptor ligands that occur with age and with neonatal caffeine exposure were due to a change in sensitivity of the receptor for the ligand or to a more fundamental change in the receptor. Using an animal model that mimics the brain developmental period and level of caffeine exposure in human premature neonates treated with caffeine for apnea of prematurity, behavioral and neurochemical investigations were undertaken. The locomotor responses to acute challenge with caffeine (15, 30 and 60 mg/kg) and with D-phenylisopropyladenosine (D-PIA) (0.038 and 0.38 mg/kg), an adenosine receptor agonist, were measured in control and neonatally caffeine-exposed rats at 12, 15, 18, and 28 days of age. The dissociation constants (K_d) and maximal binding densities (B_{max}) for agonist binding at the adenosine A1 receptor site were determined over a similar time period. Caffeine displacement of an adenosine A1 agonist was also measured to examine in vitro sensitivity to caffeine as a function of age and neonatal caffeine exposure. Our studies demonstrated that the differential responses to adenosine receptor ligands seen as a function of both age and neonatal caffeine exposure could not be overcome by merely increasing the doses of ligand administered. In addition, the results of the binding studies indicated that changes in the adenosine receptor are occurring as a function of age in different regions of the brain of control animals and that this development is influenced by neonatal caffeine exposure.

Caffeine Adenosine receptor Cyclohexyladenosine Phenylisopropyladenosine Locomotor activity Ontogeny

CAFFEINE, an adenosine receptor antagonist, is widely used therapeutically in the treatment of apnea of prematurity. Infants born 8 to 16 weeks before term often have immature control of respiration and thus may receive relatively high doses of caffeine resulting in blood caffeine levels of 5–15 mg/l [approximately 2–10 times the blood levels measured in adult moderate coffee drinkers (3,26)] for up to 2–3 months. Although no overt, acute toxicity has been attributed to this exposure (1, 2, 21, 28), data on long-term effects are sparse (15,21). Previous research in this laboratory has demonstrated that, when given to rat pups at dosages that reproduce the blood caffeine concentrations measured in human neonates during rat brain developmental periods comparable to the 26–38-week developmental period in the human brain, early caffeine exposure does have behavioral sequelae (13). For example, whereas in the naive rat pup acute caffeine administration (at the dosage tested) did not stimulate locomotor activity until approximately 18 days of age, this locomotor response to an acute challenge with caffeine was delayed in pups that had been exposed to caffeine during the first week of life. Conversely, acute administration of D-phenylisopropyl adenosine (D-PIA), an adenosine receptor agonist, was without locomotor depressant effects in naive rats until 15 days of age but was effective at 12 days of age in pups that had received caffeine previously. Thus these initial studies suggested that the typical developmental pattern of sensitivity to an acute challenge with these two adenosine receptor ligands was disrupted in pups exposed neonatally to caffeine. The locomotor response to acute challenge with caffeine was developmentally delayed and the response to acute challenge with D-PIA was developmentally accelerated.

One of the goals of the present studies was to determine whether the apparent changes in behavioral sensitivity to adenosine receptor ligands brought about by neonatal caffeine exposure were due to a change in the sensitivity of the adenosine receptor for caffeine and D-PIA or if a fundamental change in the receptor occurred such that at a given age it was not able to respond at all to these compounds. That is, by doing dose-response studies, could an effective dose of caffeine or D-PIA be found that produced altered activity at all ages studied?

In the rat, the appearance of the A1 adenosine receptor is gradual and regionally specific (14,24). Adult receptor densities are attained in the brain stem and hypothalamus by 14 days of age and in the hippocampus, cerebellum, and cortex by 21–28 days of age. Previous studies in this laboratory (14) and by others (23) on adenosine A1 receptor binding in brain tissue from rats up to 90 days of age suggested that neonatal caffeine exposure resulted in increased specific binding of ^{3}H-cyclohexyladenosine (^{3}H-CHA) in cortex, cerebellum, and hippocampus. Saturation analysis of ^{3}H-CHA binding in the cortex demonstrated an increase in maximal A1 receptor density in this region subsequent to neonatal caffeine exposure. These previous studies examined the ontogeny of the adenosine receptor at approximately weekly intervals. However, the results of the behavioral studies indicated that such weekly analysis might miss critical changes in receptor function taking place between 14 and 21 days. By measuring behavioral consequences of acute exposure

to adenosine receptor agonists and antagonists as a function of age, particular ages of interest were targeted in the current study. Therefore, in the present study, in vitro receptor binding studies were done using brain tissue from rats at these target ages to determine whether or not adenosine A1 receptor binding affinity or receptor sensitivity to caffeine was affected by the early caffeine exposure.

METHOD

Animals

Subjects were Long Evans rats 2–28 days of age born to females obtained from Harlan Sprague-Dawley (Altamont, NY) that had been bred to in-house Long Evans males. The day of birth was considered to be postnatal day 0. Litters were adjusted to 10–12 pups each on day 1–2 and assigned to an experimental protocol (see below). Animals were housed under conditions of constant heat and humidity, on a 12-hour light cycle (lights on at 0600 h). All experiments conformed to guidelines of the University (of Rochester) Committee on Animal Resources.

Dosing Protocol

Pups 2–6 days of age were left unhandled (NH), given vehicle (V) by gavage (water, 0.05 ml/10 g body weight), or received caffeine citrate (C) by gavage as follows: 20 mg/kg on day 2 and 15 mg/kg on days 3–6, administered in a volume of 0.05 ml/10 g body weight. Injections of caffeine or vehicle were made via PE tubing attached to a 1 cc syringe by a 20-ga needle adapter. The end of the tubing was inserted into the pup's posterior pharynx and caffeine was injected slowly (over 30–60 seconds). This dose of caffeine was previously shown to result in blood caffeine levels of 5–14 mg/l over a 24-hour period (13). All pups in the same litter received the same treatment. Pups receiving caffeine or vehicle were weighed daily on days 2–6, and again on the day of locomotor activity testing. Unhandled pups were weighed only on the day of locomotor activity testing.

Locomotor Activity

Pups representing the above treatment regimens were tested for locomotor activity at 12, 15, 18 or 28 days of age. These ages were chosen to represent time of characteristic locomotor hyperactivity that occurs at 14–16 days of age (day 15), 2 days prior (12 days), 2 days post (18 days) and stable activity (28 days) (11). Testing was done in a $10'' \times 18''$ activity chamber, in a dimly lit room, in which horizontal activity was measured using photocells arranged in a grid pattern. Activity was recorded for 30 minutes as number of interruptions of a photocell beam/test session. To allow comparison across different age groups, a mean baseline (uninjected) activity for each exposure group was computed and the percent baseline activity then determined for each individual experimental value. This normalization of the data allowed comparisons between groups with different basal activity levels as a function of age and body size (5). Fifteen minutes prior to testing, animals were injected IP (0.05 ml/10 g body weight) with caffeine citrate (15, 30, or 60 mg/kg; approximately 100, 200, or 400 μmole/kg, respectively), D-phenylisopropyladenosine (D-PIA) (0.038 or 0.38 mg/kg; approximately 1 or 10 μmole/kg), or vehicle (0.9% NaCl). To determine baseline activity, an uninjected group was tested at each age. All data points represent animals (males and females combined) obtained from a minimum of 2 different litters (n = 6–13 at each age, each neonatal and each acute exposure condition). No rat received more than one acute injection.

Receptor Studies

Membrane preparation. A modification of the method of Marangos et al. (24) was used for the membrane preparation. Tissue from 6–8 pups (depending on brain size of the different age rats) from a single litter was pooled for each sample to obtain sufficient tissue for assay. A minimum of 3 pooled samples prepared from three different litters was assayed at each age (14, 18, 21 and 28 days) and neonatal exposure condition. Tissue from males and females was combined. The pups were sacrificed by rapid decapitation and their brains removed and dissected on ice into cortex, cerebellum and hippocampus. Tissue was homogenized in 25 volumes ice-cold 50 mM Tris chloride buffer, pH 7.4, with a Brinkman Polytron (setting 5 for 10 seconds) and centrifuged at $30,000 \times g$ for 20 minutes. The pellet was resuspended in 25 volumes Tris chloride buffer containing 2 U/ml calf intestinal mucosa adenosine deaminase (Sigma) and incubated for 30 minutes at 23°C to remove endogenous adenosine. The membrane suspension was then centrifuged at $30,000 \times g$ for 20 minutes, resuspended in 25 volumes cold buffer, and frozen at −70°C until assayed.

Saturation studies. Tissue samples from cortex, cerebellum, and hippocampus were prepared as described above. Equilibrium binding characteristics for the A1 adenosine receptor subtype were analyzed by determining binding over 12 concentrations of ^{3}H-cyclohexyladenosine (CHA, New England Nuclear) (0.2–40 nM), an A1 adenosine receptor agonist, for 2 hours. Nonspecific binding was determined by incubation in the presence of 10 μM L-phenylisopropyladenosine (L-PIA). Assays were terminated by filtration through Whatman GF/B filters. The filters were air dried, placed in Ecoscint A scintillation fluid (National Diagnostics), and filter-bound radioactivity determined by standard liquid scintillation spectrometry. The maximal binding density (B_{max}) and equilibrium dissociation constant (K_d) were calculated at each age and exposure condition using Scatchard transformation of the data.

Displacement studies. Tissue samples of cortex, cerebellum, and hippocampus were prepared as described above. Displacement of ^{3}H-CHA binding (1 nM) was analyzed in the presence of caffeine (10^{-2} to 10^{-6} M). Incubation, filtration, and scintillation spectrometry were performed as above. IC_{50}'s were calculated at each age and exposure condition using Hill transformation of the data. Only displacement values of 10 to 90% total specific binding (the linear part of the curve) were used in the calculations. Hill coefficients were also determined.

Protein was determined by a modification of the method of Lowry (25). All samples were assayed in duplicate for total and nonspecific binding and in triplicate for protein determination.

Data Analysis

Because there was no difference in binding parameters between nonhandled and vehicle-exposed rats, data from these two treatment groups were combined as a single control group. Similarly, there was no difference in the behavioral studies between uninjected and saline-injected controls; therefore, these two groups were also combined for further analysis. Transformed equilibrium binding data and competition binding data were analyzed using a computer-assisted curve fitting program. Behavioral (activity per test session and/or % baseline activity) and biochemical data were analyzed using analysis of variance. Main effects

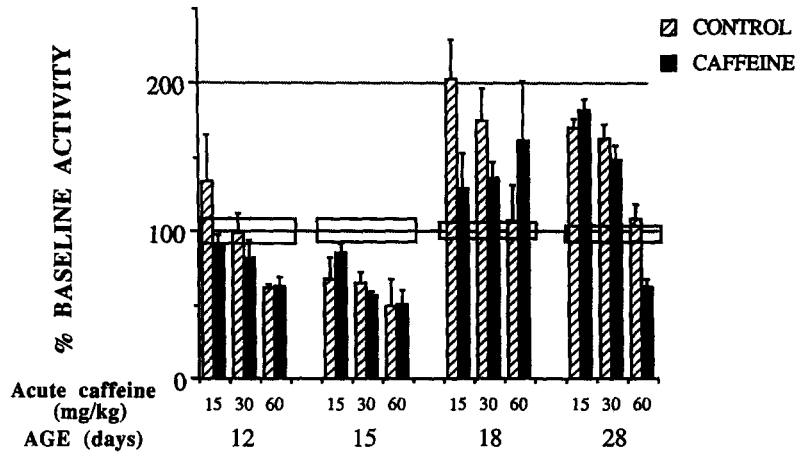

FIG. 1. Dose-response study of acute caffeine treatment as a function of age and neonatal caffeine exposure: Responsiveness expressed as % baseline activity (n = 5–13 rats at each age, neonatal exposure, acute caffeine combination). Mean baseline activity indicated by box depicting 100% ± SEM (n = 25–35 rats at each age). At 18 days of age, unlike control pups, neonatally caffeine-exposed pups do not increase their activity in response to acute caffeine injection even at high doses of caffeine. For statistical analysis, see text.

were further evaluated by post hoc Newman-Keuls comparisons. Interaction effects were probed by simple effects contrasts. Statistical significance was attributed at $p<0.05$. All data are presented as mean ± SEM.

RESULTS

Locomotor Activity

Baseline activity varied as a function of age ($p<0.005$) but not of neonatal caffeine exposure. Baseline locomotor activity approximately tripled between days 12 and 15 and remained stable thereafter (number of interruptions of a photocell beam per test session: 12-day-old rats: 1077 ± 93; 15-day-old rats: 3384 ± 225; 18-day-old rats: 3233 ± 155; 28-day-old rats: 3295 ± 191). Analyses of the data presented in Fig. 1 demonstrated that there was a significant effect of age ($p<0.0001$), neonatal exposure ($p<0.03$), and acute dose of caffeine ($p<0.0001$) on locomotor activity. In addition, there was a significant interaction of age and acute dose of caffeine ($p<0.05$) as well as an interaction of age, neonatal exposure and acute dose of caffeine ($p<0.02$). Thus, in contrast to 18-day-old control pups that responded to 15 mg/kg caffeine with a significant increase in locomotor activity ($p<0.0001$), caffeine-exposed pups showed little response to acute caffeine at that age even at 30 mg/kg. At a dosage of 60 mg/kg neither control nor caffeine-exposed pups showed a significant increase in locomotor activity at 18 days of age. By 28 days of age, both control and caffeine-exposed groups demonstrated a significant increase in locomotor activity at both 15 and 30 mg/kg acute caffeine ($p<0.0001$). There was no net effect or perhaps even a decrease in locomotor activity at 60 mg/kg caffeine.

Responses to acute injection of D-PIA at two different doses as a function of age are illustrated in Fig. 2. Statistical analysis revealed main effects of age ($p<0.0001$) and D-PIA injection ($p<0.0002$) and an interaction of age and neonatal exposure ($p<0.02$). C-exposed pups demonstrated a depressant effect of 10 μmol/kg D-PIA as early as 12 days vs. 15 days in control pups and of 1 μmol/kg D-PIA as early as 15 days vs. 28 days in controls.

Binding Studies

Preliminary studies (14) evaluating binding in cortex at weekly intervals (i.e., 14, 21, 28, etc., days of age) showed there was no effect of age on specific binding using a single concentration of ^{3}H-CHA (1 nM) in control pups. There was, however, a significant increase in specific binding in cortex after neonatal exposure to caffeine. In the present study in which 18-day-old rats were included, specific binding at 1 nM ^{3}H-CHA in cortex from 18-day-old caffeine-exposed pups was significantly lower than that measured in cortex from 14-, 21-, or 28-day-old caffeine-exposed pups as well as from 18-day-old control pups (Table 1). Analysis of variance of these data indicated that there was both a significant effect of age ($p<0.005$) and an interaction of age and neonatal exposure ($p<0.001$) on specific binding.

Previously (14), we had shown that neither K_d nor B_{max} for ^{3}H-CHA binding to cortical membranes varied significantly with age over 14–90 days. The K_d likewise did not vary as a function of neonatal exposure to the drug. However, the maximum binding density of the high-affinity A1 site was significantly increased as a function of neonatal exposure. In the current study, which included 18-day-old rats, saturation analysis of cortical tissue demonstrated a significant effect on binding affinity of age ($p<0.0001$), exposure ($p<0.0001$) and an interaction of age and exposure ($p<0.0001$) (Fig. 3A). (If K_d's from only 14-, 21-, and 28-day-old rats were considered, then, as reported previously, there was no effect of age or exposure.) There was a significant effect of neonatal caffeine exposure ($p<0.05$) but not of age on maximal receptor density in the cortex when only 14-, 21-, and 28-day-old tissue was examined. This effect was no longer statistically significant when results from 18-day-old rats were included ($p=0.09$) (Fig. 3B). Thus the decrease in specific binding observed at 1 nM ^{3}H-CHA in caffeine-exposed pups at 18 days was due primarily to a significant decrease in binding affinity (K_d control = 0.78 ± 0.07; K_d caffeine-exposed =

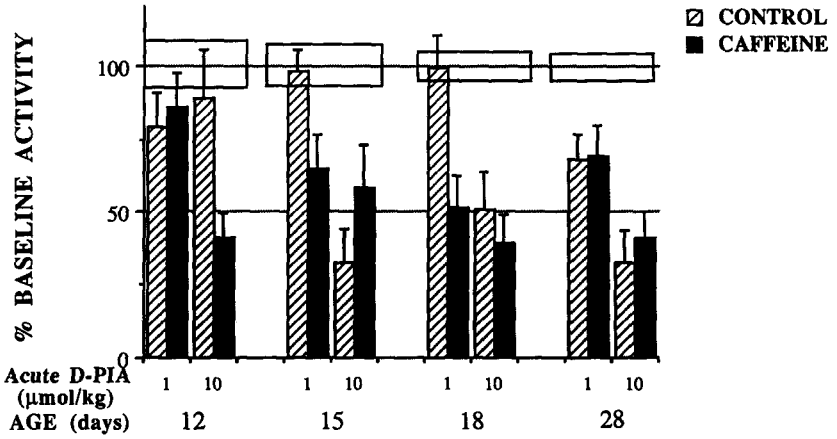

FIG. 2. Dose-response study of acute D-PIA treatment as a function of age and neonatal caffeine exposure: Responsiveness expressed as % baseline activity (n = 5–12 rats at each age, neonatal exposure, acute D-PIA combination). Mean baseline activity indicated by box depicting 100% ± SEM (n = 25–35 rats at each age). Neonatally caffeine-exposed pups demonstrate the depressant effect of 10 μmol/kg D-PIA as early as 12 days vs. 15 days in control pups and of 1 μmol/kg as early as 15 days vs. 28 days in controls. For statistical analysis, see text.

3.63 ± 0.20) at this age.

Saturation analysis of ^{3}H-CHA binding in the hippocampus and cerebellum indicated that, consistent with the increase in age seen in specific binding at 1 nM (14), there was a significant effect of age on maximal binding density in cerebellum ($p<0.002$). Affinity constants in cerebellum and hippocampus and maximal binding density in hippocampus did not vary with age. However, while earlier studies suggested that there was a significant increase in specific binding of 1 nM ^{3}H-CHA in both cerebellum and hippocampus as a result of early caffeine exposure (13), saturation analysis of ^{3}H-CHA binding did not reveal any differences in either affinity constants or receptor density for ^{3}H-CHA binding in cerebellum or hippocampus (data not shown).

Displacement Studies

In control animals, the IC_{50} for caffeine displacement of ^{3}H-CHA (1 nM) varied as a function of age in cortex ($p<0.02$) and in cerebellum ($p<0.03$) (Fig. 4). Further analysis indicated that this difference in cortical tissue could be attributed to a higher IC_{50} at 14 days of age than at older ages. In cerebellum, the IC_{50} increased from 14 to 18 days of age then decreased again from 21 to 28 days of age. There was no difference in displacement of ^{3}H-CHA by caffeine as a function of age in hippocampus from control rats. Over all ages and brain regions studied, the IC_{50} for caffeine displacement of 1 nM ^{3}H-CHA ranged from approximately 75–200 μM. Previous calculations (13) demonstrated that a dose of 15–20 mg/kg caffeine resulted in a peak blood caffeine concentration of approximately 80–90 μM. Because caffeine is widely distributed in the body water, 80–95% equilibration between plasma and brain is reached within 15–30 minutes of dosing (6, 20, 26). Thus the IC_{50} values calculated for caffeine displacement of an adenosine agonist in vitro were in the same range as the peak caffeine concentrations achieved during behavioral testing.

Early developmental exposure to caffeine had no effect on in vitro receptor sensitivity to caffeine in any of the brain regions studied.

There was no difference in Hill coefficients as a function of either age or neonatal caffeine exposure calculated for cortical, cerebellar, or hippocampal homogenates. The average Hill coefficient was 0.83 ± 0.01 in all regions.

TABLE 1

SPECIFIC BINDING (PMOLES/MG PROTEIN) IN CORTEX AT 1 nM ^{3}H-CHA AS A FUNCTION OF AGE AND NEONATAL EXPOSURE (n = 3–6 POOLED SAMPLES AT EACH AGE, NEONATAL EXPOSURE COMBINATION)

Age (days)	Control (mean ± sem)	Caffeine (mean ± sem)
14	0.0850 ± 0.0091	0.1046 ± 0.0226
18	0.0922 ± 0.0123	0.0248 ± 0.0019
21	0.1005 ± 0.0109	0.1411 ± 0.0234
28	0.0905 ± 0.0100	0.1119 ± 0.0160

DISCUSSION

Behavioral observations demonstrated an age-related sensitivity to the locomotor effects of both caffeine and D-PIA in the young control rat. Over the period of 12–18 days of age, the naive rat became differentially responsive to acute administration of these adenosine receptor ligands. Similarly, the results of the binding studies indicated that changes in the adenosine receptor were occurring over the same period in different regions of the brain in the naive rat. Furthermore, early neonatal exposure to caffeine in the rat altered both age-related behavioral and receptor development.

Caffeine, an adenosine receptor antagonist, did not stimulate locomotor activity until the third week of life in the naive rat, but D-PIA, an adenosine receptor agonist, was effective in depressing locomotor activity several days earlier. Specifically, in

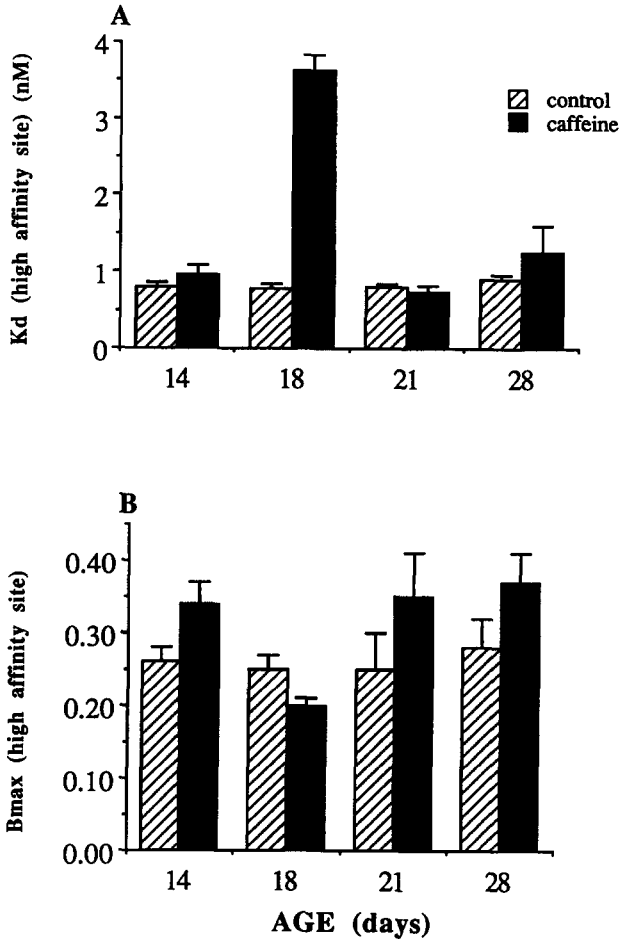

FIG. 3. Cortical adenosine A1 receptor dissociation constants (K_d) (A) and maximal binding densities (B_{max}) (B) for the high-affinity site as a function of age and neonatal caffeine exposure. K_d did not vary as a function of age in control rats. In caffeine-exposed pups, however, there was a significant increase ($p<0.0001$) in K_d in 18-day-old pups only. B_{max} did not vary as a function of age or neonatal caffeine exposure. (n = 3–4 pooled samples at each age, neonatal exposure combination.)

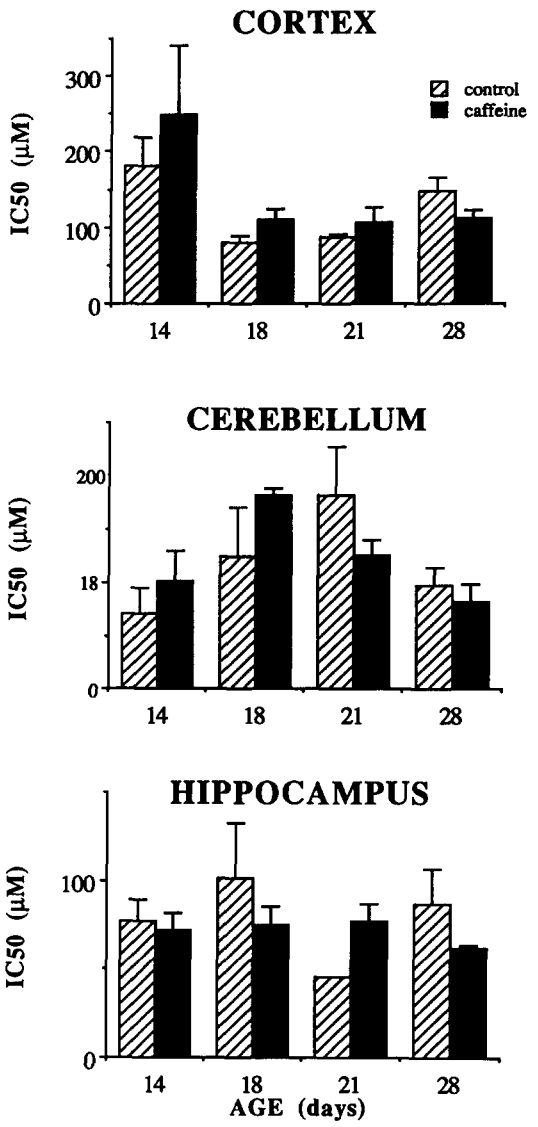

FIG. 4. IC_{50} for caffeine displacement of ^{3}H-CHA as a function of age and neonatal caffeine exposure in homogenates of cortex, cerebellum, and hippocampus. The IC_{50} varied as a function of age in cortex ($p<0.02$) and cerebellum ($p<0.03$). This difference could be attributed to a higher IC_{50} in cortex at 14 days than at older ages and to higher IC_{50}'s at 18 and 21 days than at 14 and 28 days in cerebellum. There was no effect of neonatal caffeine exposure on in vitro sensitivity to caffeine in any of these three brain regions. (n = 3–5 pooled samples at each age, neonatal exposure combination.)

the naive (control) rat, acute administration of caffeine did not produce an increase in locomotor activity until 18 days of age; 12- and 15-day-old control rats did not demonstrate an increase in locomotor activity in response to even high doses of caffeine. By 28 days of age the typical adult pattern of response to increasing doses of caffeine (4, 8, 9, 18, 20, 26, 27) was readily apparent. Maximal stimulation of locomotor activity in 28-day-old rats was observed at a caffeine dosage of 15–30 mg/kg, while administration of 60 mg/kg caffeine resulted in no stimulation or, possibly, a decrease in activity. In contrast, depression of locomotor activity by D-PIA (at a specific dose) was evident as early as 15 days of age in the naive rat. Based on these dose-response behavioral studies, it appears that the locomotor responses to caffeine, which is an antagonist with approximately equal potency at both A1 and A2 receptor subtypes, and to D-PIA, which is an agonist with a 5–10-fold potency at A1 compared with A2 receptors, develop at different rates. Since both of these ligands act at both A1 and A2 receptors, the differential appearance of responses to an agonist vs. an antagonist may be due to a difference in the ontogeny of the G-proteins that mediate the response or to a differential rate of development of the functional coupling between the receptor, G-proteins, and the second messenger, adenylate cyclase.

As previously observed (13), neonatal caffeine exposure affected later response to acute injection of both caffeine and D-PIA. Caffeine did not stimulate locomotor activity in 18-day-old rats previously exposed to caffeine (days 2–6 postnatal) as observed in control rats. The current study further demonstrated

that this lack of response did not reflect merely a decreased sensitivity to caffeine, as stimulation of activity did not appear by increasing the dose of caffeine administered. Doses as high as 4 times the original were not stimulatory in 18-day-old caffeine-exposed pups. Conversely, caffeine-exposed pups responded to acute injection of D-PIA with decreased locomotor activity beginning at an earlier age than did control pups. This apparent divergence in behavioral responsiveness may also be due to a differential effect of early caffeine exposure on the coupling between the G-proteins and stimulation (A2 receptors) or inhibition (A1 receptors) of adenylate cyclase (12).

The current study also provided data on the normal ontogeny of the adenosine receptor system in the young rat as well as on the effect of early exposure to caffeine on this development. Based on the above behavioral observations, central adenosine receptor binding parameters and sensitivity to ligands were closely examined in the 2–3-week-old rat. These experiments confirmed earlier observations (14) that during this time interval there was an age-related increase in control rats in specific binding of ^{3}H-CHA in cerebellum, hippocampus and cortex that reflected an increase in maximal receptor density. In addition, the current study demonstrated an age-dependent decrease in the IC_{50} for displacement of ^{3}H-CHA by caffeine in the cortex, such that adult values were achieved by 18 days of age in control rats. These observations suggest an increasing sensitivity of cortical tissue for caffeine as a function of age. No inferences can be made, however, on the source of this increasing sensitivity. Further studies using receptor antagonists specific for the A1 receptor may help differentiate whether this change is a function of the receptor subtype bound or receptor agonist vs. antagonist binding.

Previous research demonstrated that neonatal exposure to caffeine produced an increase in specific binding in cortex, cerebellum, and hippocampus when evaluated at weekly intervals over 7–90 days of age (14). When cortical tissue was further examined by saturation analysis at weekly intervals, this increase in specific binding at 1 nM ^{3}H-CHA was observed to reflect an increase in receptor density, with no apparent change in binding affinity for the ligand (14). However, the results of current saturation studies, which now included tissue from rats 18 days old, demonstrated a marked increase in the K_d for ^{3}H-CHA in cortex from caffeine-exposed pups as compared with the K_d in cortex from control rats at that age. Thus the inclusion of cortical tissue from 18-day-old rats in the saturation analysis markedly affected inferences concerning the ontogeny of the A1 adenosine receptor and the effect thereon of neonatal caffeine exposure. Neonatal caffeine exposure did not, however, influence in vitro caffeine displacement of ^{3}H-CHA in the any of the brain regions examined. Thus these experiments demonstrate that not only is the ontogeny of adenosine receptors regionally specific, but in vitro sensitivity of the receptors to caffeine is age dependent as well. Moreover, early neonatal exposure to caffeine can affect, also in a regionally specific manner, adenosine receptor binding parameters.

Taken together, the results of the behavioral and receptor binding experiments provide evidence on the development of the adenosine receptor system and its potential for perturbation. Changes in the animal's locomotor response to acute challenge with adenosine receptor ligands as a function of age and/or neonatal caffeine exposure are the end result of developmental changes in an extremely complex receptor system. Although the change with age seen in control animals in the in vitro sensitivity of cortical tissue to caffeine correlates well with the appearance of a behavioral response to acute caffeine administration in the young rat, and the marked increases in K_d for ^{3}H-CHA binding in the cortex from 18-day-old caffeine-exposed rats correlates well with the rats' lack of in vivo response to acute caffeine at the same age, one should not make simplistic conclusions from these observations. There may not be a one-to-one correspondence between the neurochemical and behavioral changes. Rather, the evidence suggests that the entire adenosine receptor system must be intact for normal, age-appropriate function. The data presented regarding the responses to acute injection of D-PIA, that is, that caffeine-exposed pups responded to D-PIA with decreased locomotor activity beginning at an earlier age than did control pups, cannot readily be explained by any of the measured changes in adenosine A1 receptor characteristics. Duncan and Morgan (11) have suggested that adenosine A2 receptors (localized predominantly in the striatum) may be involved in the hypomobility induced by adenosine analogues. The effect of early caffeine exposure on this receptor subtype must be carefully investigated. It is not inconceivable that caffeine, which binds to both A1 and A2 adenosine receptors, could affect the ontogeny of both receptor subtypes. Further, because of the complexity of the agonist-antagonist interactions at A1 receptors and the involvement of G-proteins (of one or more types) in the adenosine receptor system (22), the interplay of a multitude of factors may have a bearing on the ultimate behavioral responses in the developing animal.

The current study has provided insight into the normal evolution of the adenosine receptor system in the young rat. Age-related changes in receptor binding of and behavioral responses to adenosine agonists and antagonists were observed. The data suggest that, in the rat, the period from 15–18 days of age is a pivotal age in adenosine receptor system development. It is not until 18 days of age that rats normally respond in this behavioral paradigm to acute administration of both adenosine receptor agonists and antagonists. Locomotor activity of rats tested in isolation during the third week of life may reflect more complex behaviors than simple movement (7). Although caffeine will increase motoric activity (defined as any movement of the head and/or limbs) in rats as young as 1 day of age (16,17), the effects of caffeine and D-PIA on locomotor activity in rats during the third week of life may reflect actions of the drugs on arousal mechanisms as well. Thus some crucial link between receptor binding and locomotor activity (as tested in this behavioral paradigm) evolves over the third postnatal week of life. This link may be regionally specific or may represent the general phenomenon of receptor coupling with various other receptor systems or with second messengers. Further investigation is needed to elucidate these possibilities. This study also provided further evidence that early developmental exposure to caffeine over a limited period affects normal adenosine receptor development and that these effects can be demonstrated on both a biochemical and behavioral level. Furthermore, the effects of such exposure may not be demonstrable immediately, but only days to weeks following the exposure period. Even if the observed behavioral changes are themselves transient, the period during which they occur may be a developmentally critical one for functions that develop subsequently. Thus there may exist "secondary" effects. Similarly, early changes in adenosine receptor densities may have a "snowballing" effect and have far-reaching consequences that may be difficult to directly link to the original inciting event.

Extrapolation of these results to the human premature neonate administered caffeine therapeutically for the treatment of apnea of prematurity suggests that subtle behavioral effects may become apparent in the young school-aged child, a time quite remote from the original therapy. Based on comparable developmental stages, the 12–18-day-old rat is most similar developmentally to a child of 3–8 years (10). As the neurochemical bases of the effects of caffeine exposure become elucidated in

the rat, more focussed examination will be possible in children exposed to caffeine during their first few weeks of life in order to determine if, indeed, there are long-term functional effects in humans.

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