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Maternal Caffeine Intake Has Minor Effects on Adenosine Receptor Ontogeny in the Rat Brain

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ABSTRACT

Maternal caffeine intake has been suggested to influence the offspring. We have studied the effects of maternal caffeine intake on adenosine and GABA receptors, targets for caffeine, during development of the rat brain. Caffeine (0.3 g/L) was added to the drinking water of rat dams during pregnancy and early postnatal life. Adenosine A_1 and A_{2A} and $GABA_A$ receptor development was studied using receptor autoradiography and *in situ* hybridization. Pups were examined on embryonic d 14 (E14), E18, E21, 2 h after birth (P2h), P24h, postnatal d 3 (P3), P7, P14, and P21. Adenosine A_1 receptor mRNA was detected at E14 and receptors at E18. A_1 mRNA levels increased from the level reached at E18 between P3 and P14 (maximally a doubling), whereas A_1 receptors, studied by [^{3}H]-1,3-dipropyl-8-cyclopentyl xanthine binding, increased later and to a much larger extent (about 10-fold) postnatally. Caffeine treatment had no significant effect on adenosine A_1 receptors or on A_1 receptor mRNA. A_{2A} mRNA had reached adult levels by E18, whereas receptor levels were low or undetectable before birth and increased dramatically until P14. Caffeine did not influence A_{2A} receptors or A_{2A} receptor mRNA at any stage during development. [^{3}H]-flunitrazepam binding, representing $GABA_A$ receptors, showed large regional variations during ontogeny, but there were no clear differences between the caffeine-exposed and the nonexposed pups. Thus, exposure to a low dose of caffeine during gestation and postnatal life had only minor effects on development of adenosine A_1 and A_{2A} receptors and $GABA_A$ receptors in the rat brain. **(*Pediatr Res* 48: 177–183, 2000)**

Abbreviations

E, embryonic day
P, hours (h) or days after birth
DPCPX, 1,3-dipropyl-8-cyclopentyl xanthine
CGS 21680, 2-[*p*-(2-carbonylethyl)phenylethylamino]-5′-N-ethylcarboxamidoadenosine
SCH 58261, 5-amino-7-(2-phenylethyl)2-(2-furyl)-pyrazolo-[4,3-e]-1,2, 4-triazolo[1,5-c]pyrimidine
R-PIA, R-N^6-phenylisopropyladenosine
NECA, 5′-N-ethylcarboxamidoadenosine

Caffeine is the most widely consumed psychoactive substance. Whereas the use of alcohol and tobacco are usually limited during pregnancy, beverages containing caffeine are consumed at a normal or near normal rate (1). Negative effects on fertility and birthweight, risk for prematurity, and congenital malformations have been demonstrated in offspring of animals given high doses of caffeine (for review see Ref. 2), but there is no conclusive evidence that normal human consumption has teratological consequences. Epidemiologic studies have shown a dose-dependent reduction in birth weight when mothers consume more than seven cups of coffee a day (3, 4). Prenatal caffeine intake has been described to result in behavioral hyperactivity in developing rodents (5–7).

The only known biochemical effect of caffeine in the brain in concentrations relevant to daily intake of coffee is blockade of adenosine A_1 and A_{2A} receptors (8). Both A_1 and A_{2A} receptors are present at birth in the rat (9–11), but the major development in terms of density and coupling to second messenger-forming systems occurs postnatally (12, 13). Secondarily to actions on adenosine receptors there are effects on dopaminergic transmission (for review see Ref. 14, 15) and it is known that alterations in dopaminergic transmission may result in developmental changes (16). In addition, there is evidence that caffeine can directly and indirectly influence GABAergic neurotransmission (8, 15). Studies on the effects of methylxanthines on benzodiazepine receptors are contradictory. An increased number of benzodiazepine binding sites in the adult mouse brain after chronic administration of a high dose of caffeine has been reported (17, 18), but other investigators have shown no change at all (19, 20) or altered function of the receptor (21).

Caffeine given in doses similar to those resulting in long-term behavioral changes has been reported to alter the postnatal

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development of adenosine A_1 receptors (22–24). However, adaptive effects of low doses of caffeine have not been described. We have earlier shown that caffeine (0.3 g/L) in the drinking water given to the rat dam during gestation and lactation produces plasma concentrations in the pups of 0.4–2 mg/L and that this treatment leads to reduced susceptibility to hypoxic brain ischemia on postnatal d 7 (25), a stage when the rat CNS maturity is thought to correspond to that of a near-term human fetus (for review see Ref. 26).

The present study was designed to examine the influence of chronic pre- and postnatal treatment with a dose of caffeine similar to that used by humans on development of adenosine A_1 and A_{2A} receptors and their corresponding mRNA as well as on benzodiazepine binding sites representing in the rat pup brain.

METHODS

Treatment. The experiments, which followed the European Community regulations, were approved by the regional animal ethics committee. Forty-four Wistar rats and their litters were used. Dams ($n = 23$) were given caffeine in the drinking water (0.3 g/L), which was exchanged every third day to fresh solutions, from embryonic d (E) 2 throughout gestation and postnatal life. Twenty-one dams received ordinary tap water. The daily intake of water was measured in all litters. The day when a vaginal plug was found was designated E0. Rat brains were examined at E14, E18, E21, exactly 2 h and 24 h after vaginal delivery (P2h, P24h), and at postnatal d 3 (P3), P7, P14, and P21. From each treatment group 6 animals (from two different litters) were used for *in situ* hybridization and receptor binding studies.

Plasma concentrations of caffeine. Trunk blood from fifteen animals was collected in heparinized plastic tubes and centrifuged. Plasma concentrations of caffeine and its metabolites theophylline, theobromine, and paraxanthine were analyzed using high pressure liquid chromatography as described (27).

Sections. From embryos and pups up to P3, the whole head was collected, whereas in older animals, the brain was rapidly dissected out. Heads and brains were frozen on dry ice and stored at −80°C. Sagittal sections from the left hemisphere were cut on a Leitz cryostat. Sections were collected from the lateral part of olfactory bulb toward midline. For *in situ* hybridization, 14-μm thick sections were thaw-mounted on poly-L-lysine (50 μg/mL) coated slides. For receptor autoradiography, 14-μm thick sections were thaw-mounted on gelatin-coated slides. Specimens from different ages were processed in the same *in situ* hybridization and receptor binding runs to allow comparison of signals and binding density.

Receptor autoradiography. Receptor density was determined using receptor autoradiography with the adenosine A_1 receptor antagonist [^{3}H]-1,3-dipropyl-8-cyclopentyl xanthine (DPCPX) (0.5 nM) (28), the adenosine A_{2A} receptor agonist [^{3}H]-2-[*p*-(2-carbonylethyl)phenylethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680) (2 nM) (29), the adenosine A_{2A} receptor antagonist [^{3}H]-5-amino-7-(2-phenylethyl)2-(2-furyl)-pyrazolo-[4,3-e]-1,2, 4-triazolo[1,5-c]pyrimidine (SCH 58261) (0.2 nM) (30), and the $GABA_A$ receptor agonist [^{3}H]-N-methyl-flunitrazepam (flunitrazepam) (1 nM) (20). Nonspecific binding was determined using R-PIA (100 μM) (DPCPX-binding) and 2-chloroadenosine (100 μM) (CGS 21680 binding), NECA (50 μM) (SCH 58261 binding), and diazepam (5 μM) (flunitrazepam binding). Ten-micrometer-thick sections were preincubated in 170 mM Tris-HCl buffer containing 1 mM EDTA and 2 U/mL adenosine deaminase at 37° for 30 min. Sections were then washed twice for 10 min at 23° in 170 mM Tris-HCl buffer. Incubations were performed for 2 h at 23° in 170 mM Tris-HCl buffer containing DPCPX (120 Ci/mmol, 0.5 nM), CGS 21680 (42.1 Ci/mmol, 2 nM), or SCH 58261 (0.2 nM) and 2 U/mL adenosine deaminase. In the experiments with DPCPX, 1 mM $MgCl_2$ was added to preincubation and to incubation buffer. The incubation with DPCPX was done in the presence of 100 μM guanosine triphosphate (GTP) to convert all the receptors to the low-affinity state for agonists and thereby remove all endogenous adenosine (28). Sections were then washed twice for 5 min each in ice-cold Tris-HCl, dipped three times in ice-cold distilled water, and dried at 4° over a strong fan. Slides were exposed to [^{3}H]-sensitive film with [^{3}H] microscales for 4–8 wk. $GABA_A$ receptor density was determined using receptor autoradiography with flunitrazepam (1 nM). Nonspecific binding was determined using diazepam (5 μM). Ten-micrometer-thick sections were preincubated in 170 mM Tris-HCl buffer at 4° for 30 min. Incubation was performed for 1 h at 4° in 170 mM Tris-HCl buffer containing N-methyl-flunitrazepam (85 Ci/mmol, 1.0 nM). Sections were then washed twice for 1 min each in Tris-HCl at 4°, dipped in ice-cold distilled water, and dried at 4° over a strong fan. Slides were exposed to [^{3}H]-sensitive film with [^{3}H] microscales for 4–8 wk.

In situ *hybridization.* The 48-mer A_1 adenosine receptor probe was complementary to nucleotides 985-1032 of the rat A_1 receptor (31). The 44-mer A_{2A} probe was complementary to nucleotides 916–959 of the dog RDC8 cDNA (32). The adenosine receptor probes have been tested for specificity (33, 34). The oligodeoxyribonucleotides were radiolabeled using terminal deoxyribonucleotidyl transferase and [^{35}S] dATP to a specific activity of about 10^9 cpm/μg. Slide-mounted sections were hybridized in a cocktail containing 50% formamide, 4×SSC, 1×Denhardt's solution, 1% sarcosyl, 0.02 M $NaPO_4$ (pH 7.0), 10% dextran sulfate, 0.06 M dithiothreitol, 0.5 mg/mL sheared salmon sperm DNA, and 10^7 cpm/mL of probe. After hybridization for 15 h at 42°, the sections were washed four times, 15 min, in 1×SSC at 55° (A_1 probe) or 45° (A_{2A} probe), then dipped briefly in water and 70%, 95%, and 99.5% ethanol, and air-dried. Finally, the sections were apposed to Hyperfilm β-max for 1–4 wk.

Drugs and chemicals. Caffeine was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]-DPCPX (120 mCi/mmol; 1,3-dipropyl-8-cyclopentyl xanthine) and [^{3}H]-CGS 21680 (42.6 Ci/mmol; 2-[*p*-(2-carboxy-ethyl)phenylethylamino]-5′-*N*-ethylcarboxamido adenosine) were from New England Nuclear-DuPont (Stockholm, Sweden), [^{3}H]-SCH 58261 (5-amino-7-(2-phenylethyl)2-(2-furyl)-pyrazolo-[4,3-e]-1,2, 4-triazolo[1,5-c]pyrimidine) was a kind gift from Schering-Plough (Milan, Italy), and [^{3}H]-N-methyl-flunitrazepam was from Amersham (Stockholm, Sweden). Adenosine deaminase

and NECA were obtained from Boehringer Mannheim Scandinavia AB (Bromma, Sweden). The oligonucleotide probes were synthesized by Scandinavian Gene Systems (Köping, Sweden), [^{3}H]-film, terminal deoxyribonucleotidyl transferase, [^{35}S]-dATP, Hyperfilm β-max, and [^{3}H] microscales were from Amersham. R-PIA and 2-chloroadenosine were obtained from Sigma Chemical Co. (Stockholm, Sweden). Formamide was from Fluka (Buchs, Switzerland). All other chemicals were purchased from Merck (Spånga, Sweden).

Image analysis. Analysis of receptor expression and binding was performed using a computerized image analysis system (Imaging Research Systems, St Catherines, Ontario, Canada). Relative optic density of expression or binding was measured in autoradiograms and amounts of receptor-bound radioactivity of the specific brain regions were determined using [^{3}H] microscale standards. Specific binding was calculated by subtraction of the OD values in sections where nonspecific binding was determined. Different regions of the brain in the prenatal rats were identified using the atlas by Altman and Bayer (35) and in postnatal rats using atlases by Sherwood and Timiras (36) and Paxinos and Watson (37).

Statistics. Results are given as mean ±SEM. Weight and fecundity were compared between the groups using the t test. Water intake was compared between caffeine and control group using the t test and multivariate ANOVA, repeated measures design with Scheffe's F post hoc test using procedures in the JMP statistics package by SAS (Cary, NC, U.S.A.). The results from quantitative receptor autoradiography and *in situ* hybridization in each specific brain region were analyzed by two-way ANOVA (Graph Pad Prism; SYSTAT). All measurements were done on sections from 5 or 6 animals and each brain was treated as one observation by averaging density values of each region studied. Statistical hypotheses were considered significant if $p < 0.05$.

RESULTS

Caffeine ingestion did not alter weight gain during first week of pregnancy (20.5 ± 0.9% in caffeine treated group and 19.0 ± 0.9% in controls), fecundity (10.88 ± 0.55 pups and 10.91 ± 0.59 pups, respectively) or birth weight (6.0 ± 0.31 g and 5.5 ± 0.22 g, respectively). Litters that received caffeine drank more than control litters at all postnatal time points studied ($p < 0.05$), but differences were small (1–8 mL per litter per day). The increased water intake in the caffeine group might be explained by the diuretic actions of xanthine adenosine antagonists (38). Plasma concentrations of caffeine and the metabolites theophylline, paraxanthine, and theobromine were measured on P7 and are presented in Table 1.

Table 1. *Plasma concentrations of methylxanthines on P7*

Methylxanthine (mg/L) mean ± SEM	Controls (n = 5)	Caffeine-treated group (n = 10) (0.3 g/L)
Caffeine	0.00 ± 0.00	0.85 ± 0.04
Theophylline	0.01 ± 0.01	0.04 ± 0.019
Paraxanthine	0.00 ± 0.00	0.09 ± 0.07
Theobromine	0.00 ± 0.00	0.01 ± 0.01

Adenosine A_1 Receptor mRNA and [^{3}H]-DPCPX Binding

We found that adenosine A_1 receptor mRNA was present on E14 in low levels in the neuroepithelium, in agreement with previous studies (9, 11), but we could not detect significant [^{3}H]-DPCPX binding until E18. On E18 both adenosine A_1 receptor mRNA and [^{3}H]-DPCPX binding was present in most structures in the brain (Fig. 1). On E21, the distribution of A_1 mRNA, and DPCPX binding resembled that seen in the adult rat brain, but levels were lower (Figs. 1 and 2). There were no differences in adenosine receptors in the brains of pups decapitated shortly after vaginal delivery (2 or 24 h) compared with levels on E21 (Fig. 2).

In the *cerebral cortex*, both A_1 receptor mRNA and [^{3}H]-DPCPX binding were detected on E18. Whereas mRNA levels were 40% of levels on P21, only a small amount of receptor protein was detected (8% of levels on P21). There was a clear elevation of both mRNA and receptor protein levels between P3 and P7 (Fig. 2). Caffeine-treated fetuses were found to have the same levels of both mRNA and receptor protein as controls. However at 24 h (P24h) and 7 d after birth (P7), [^{3}H]-DPCPX binding was significantly higher in caffeine-exposed pups than in controls (31%, $p = 0.044$ and 15%, $p = 0.033$, respectively). A_1 mRNA in caffeine group was not altered at any time point studied compared with controls.

In cerebellar cortex, the development of A_1 receptors was clearly delayed compared with other regions. Both A_1 mRNA

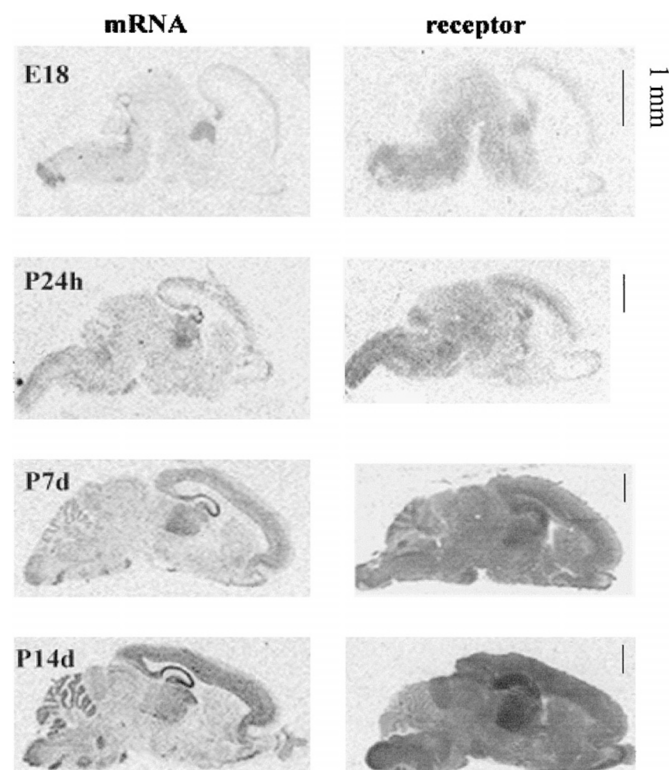

Figure 1. Film autoradiograms generated from *in situ* hybridization (left part) and receptor autoradiography (right part) showing adenosine A_1 mRNA expression and A_1 receptor binding at different stages during early development: E18, P24h, P7, and P14. Sagittal sections are shown. *In situ* hybridization was performed using an oligonucleotide probe and receptor binding was determined using [^{3}H]-DPCPX-binding (0.5 nM). Nonspecific binding was determined and was equal to background.

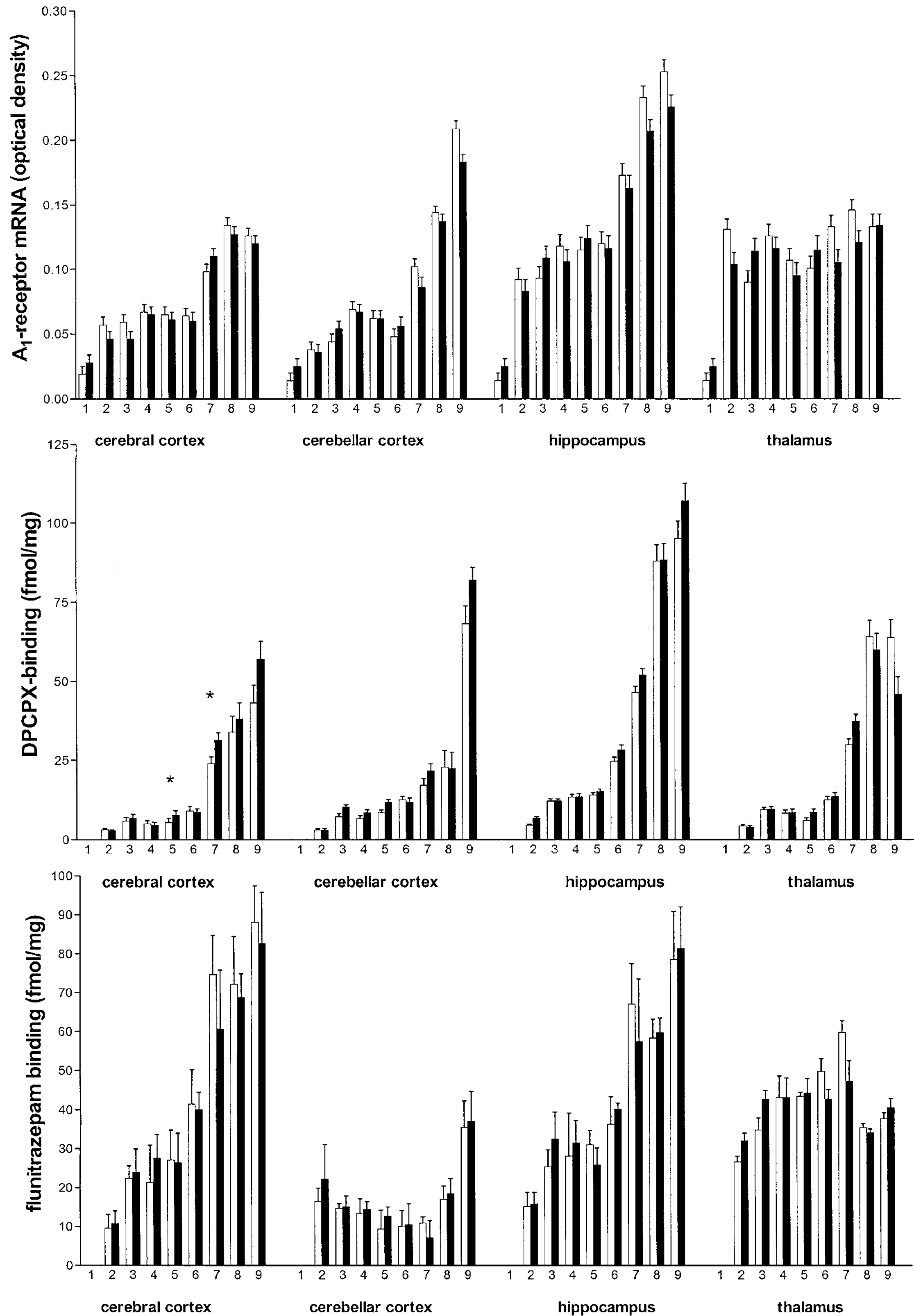

Figure 2. Regional pre- and early postnatal time course of adenosine A_1 mRNA expression (upper panels), [^{3}H]-DPCPX binding (middle panels), and binding of [^{3}H]-flunitrazepam (lower panels) in the brain in controls (white bars) and low-dose caffeine-treated (black bars) rat fetuses (1 = E14, 2 = E18, 3 = E21) and pups (4 = P2h, 5 = P24h, 6 = P3, 7 = P7, 8 = P14, and 9 = P21). In cortex, measurements include all cortex layers; hippocampal measurements include CA1, CA3, and dentate gyrus. Measurements on E14 were not performed in each region, but represent a "whole brain" value. Mean and SEM of groups with $n = 6$. * represents a p value < 0.05 when comparing controls and caffeine-treated pups. In all regions there was a highly significant ($p < 0.001$) age-related change for A_1 mRNA expression, [^{3}H]-DPCPX binding, and [^{3}H]-flunitrazepam.

and [^{3}H]-DPCPX binding could be detected on E18. The A_1 mRNA was down-regulated on P3, but increased thereafter and the major development of receptor protein took place between P14 and P21 (Fig. 2). A_1 mRNA was found in the granular and Purkinje cell layer and [^{3}H]-DPCPX binding in the molecular cell layer, in agreement with previous studies (39), and this distribution was detected on P14. In the caffeine group, [^{3}H]-DPCPX binding and A_1 mRNA was not altered at any specific time point studied, compared with controls.

A_1 mRNA and [^{3}H]-DPCPX binding were higher in hippocampus than in all other regions at all time points studied (Fig. 2). Receptor protein increased earliest in this region, and on P7, the distribution of A_1 mRNA and [^{3}H]-DPCPX binding in the hippocampus was qualitatively the same as in the oldest animals studied here. There were no statistically significant differences in [^{3}H]-DPCPX binding, but A_1 mRNA levels were slightly lower in the caffeine-treated animals than in controls. A significant difference was observed if the data on P3–P21 were pooled (6% to 12%, $p = 0.009$).

Total A_1 mRNA amounts in thalamus reached adult levels already on E18, but the development of receptor binding had a time course similar to that in most A_1 receptor-rich areas. No differences were seen between the caffeine-treated animals and controls.

A_{2A}mRNA and Receptor Binding

A_{2A} mRNA was diffusely distributed all over the brain on E14 (not shown) in agreement with previous findings (10), but binding of the A_{2A} receptor ligands [^{3}H]-CGS 21680 and [^{3}H]-SCH 58261 could not be detected at this stage. From E18 and onward, A_{2A} mRNA was expressed in the caudate putamen at relatively high levels (Fig. 3). [^{3}H]-CGS 21680 binding was present from E21 and [^{3}H]-SCH 58261 binding was detected from P3 in the caudate putamen (Fig. 3) in low amounts and binding with both ligands increased mainly between P3 and P14 (Fig. 4). A_{2A} mRNA and receptor protein were also found in the olfactory tubercle (Fig. 3), but no measurements were made there.

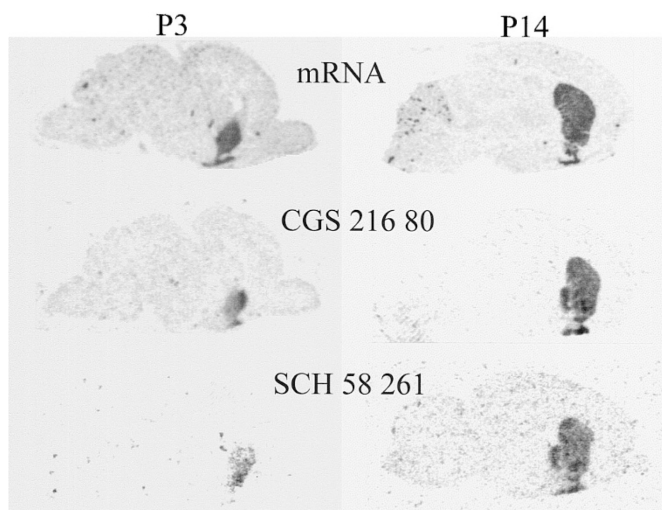

Figure 3. Film autoradiograms generated from *in situ* hybridization with an oligonucleotide probe (upper panels) and receptor autoradiography (with agonist [^{3}H]-CGS 21680, 2 nM (middle panel) or antagonist [^{3}H]-SCH 58261, 0.2 nM (lower panel)) showing adenosine A_{2A} mRNA and receptors on P3 (left panels) and P14 (right panels). Nonspecific agonist and antagonist binding was determined and was in both cases equal to background.

Whereas previous studies on the effect of low doses of caffeine have not indicated any clear-cut effects on A_1 receptors, recent results do show a decrease in A_{2A} receptors and the corresponding mRNA, which is related to the known behavioral tolerance (40). There was no significant difference in development of A_{2A} mRNA or receptor binding between pups subjected to caffeine treatment and controls, and there were no differences in adenosine A_{2A} receptors shortly after birth.

$GABA_A$ Receptor Binding

The $GABA_A$ receptor ontogeny in different brain regions was studied using [^{3}H]-flunitrazepam binding and the results are shown in Fig. 2. As previously reported (41), [^{3}H]-flunitrazepam binding sites could be detected on E14 in pons and medulla (not shown). Binding in cerebral cortex, cerebellar

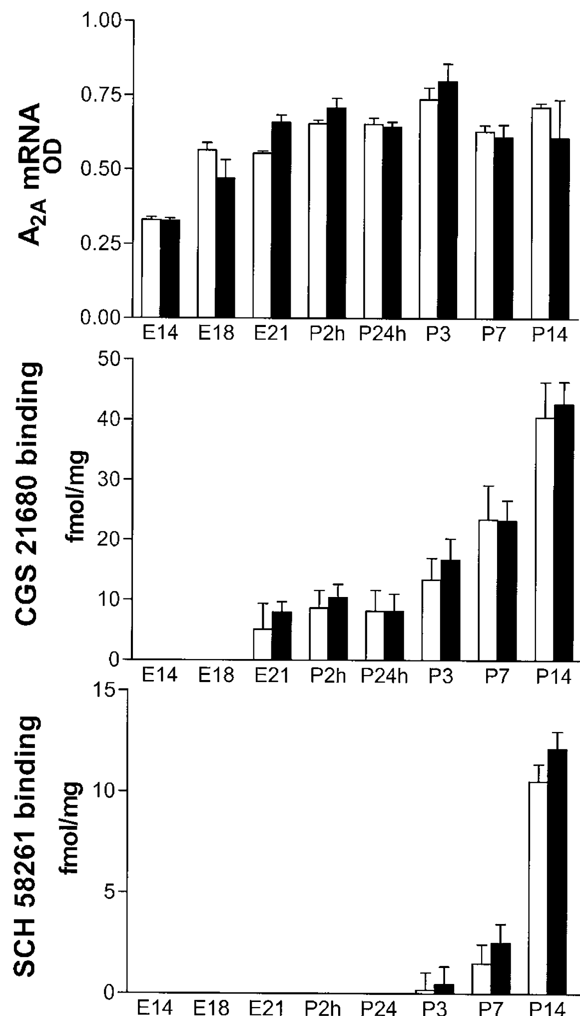

Figure 4. Adenosine A_{2A} mRNA expression (upper panel), agonist (middle panel), and antagonist (lower panel) binding to A_{2A} receptors in caudate putamen during pre- and early postnatal development in controls (white bars) and caffeine-treated (black bars) fetuses and pups. All measurements made on consecutive sagittal sections. For further details see legend to Figure 3. Mean and SEM of groups with $n = 6$. For each panel, there was a highly significant ($p < 0.001$) age-related change.

cortex, hippocampus, and olfactory bulb was detected on E18 and in striatum on E21. There were no significant differences between controls and caffeine-treated animals at any specific time point or in any region.

DISCUSSION

The major finding of this study is that administration of caffeine in doses that resemble those consumed by humans does not significantly influence the development of receptors that are known or believed to be affected by this drug. These results are in apparent contrast to several previous studies mentioned at the beginning of this article, where caffeine has been reported to modify adenosine receptors and/or behavior.

A possible explanation is the difference in doses. For example, in neonatal pups who received 80 mg/kg/d of caffeine, behavioral effects were observed (7). This dose is some 10–20 times higher than that ingested by the pups in the present study, who received the caffeine *via* the breast milk. In another study behavioral effects were observed in the offspring when mouse dams received more than 80 mg/kg/d during pregnancy (6). In the present study the dose of caffeine given was low. It gives plasma concentrations in rat pups comparable to those seen in the umbilical cord plasma in newborns of moderately (up to 3 cups a day) coffee-drinking human mothers (0.5–2 mg/L) (42). The plasma levels of the pups also resembled the concentrations in breast-fed infants of caffeine-consuming mothers (43, 44).

Perinatal treatment with 6- to 12-fold higher doses of caffeine than those used here induces up-regulation of A_1 receptors in the postnatal and adult (P14–90) rat brain (19, 22, 23, 45). However, with the more relevant dosage used here little or no change in A_1 receptors was seen. We cannot exclude the possibility that there are some changes in some regions, but we can conclude that if they occur, they are small. In adult rodents there is good evidence that high doses of caffeine do produce increases in A_1 receptors, that are not accompanied by changes in A_1 receptor mRNA (33). When lower doses are given no changes are observed, however. The present data considered together with the results of previous studies hence suggest that adaptive changes in adenosine A_1 receptors are strongly dependent on the dose of caffeine given, not only in adults but also in young animals.

Thus, some of the reported long-term adaptive changes in behavior or in adenosine receptors may represent a high dose phenomenon. It is well known that the behavioral effects of caffeine, at least in mature animals, are biphasic: low doses are behaviorally stimulant, whereas higher doses produce an inhibited motor behavior (see Ref. 8). When caffeine is given in a low dose, it is unlikely that other receptors than adenosine A_1 and A_{2A} receptors are directly affected (8). By contrast, higher doses can affect other targets including phosphodiesterases and benzodiazepine receptors.

The present results have confirmed that the primary targets for low-dose caffeine, *i.e.* the adenosine A_1 and A_{2A} receptors, are poorly developed in the immature rat brain. Beautiful studies on the prenatal development of mRNA of these receptors have already been published (9–11); however, the postnatal development of mRNA has not been studied in detail before. Both the absolute magnitude of the binding and the magnitude of the postnatal increase agree with a previous study on rat forebrain (24). Although A_1 mRNA was present already on E14, and A_1 receptor binding was apparent on E18 in agreement with previous studies (9, 10), the number of A_1 receptors is almost 10 times lower at birth than in the mature animal. Moreover, part of the binding detected at the earliest times might represent A_{2B} receptors, because DPCPX has a high affinity also to these receptors (46). The development of A_{2A} receptors has also been described (10, 12, 47) using *in situ* hybridization and [^{3}H]-CGS 21680 binding (but not [^{3}H]-SCH 58261 binding), and our results essentially agree with these extensive studies. However, although in the present study [^{3}H]-CGS 21680 binding revealed A_{2A} receptors on E21 in caudate putamen, nucleus accumbens, and olfactory tubercle, [^{3}H]-SCH 58261 binding was not detected until P3. It is possible that part of the [^{3}H]-CGS 21680 binding reflects sites other than A_{2A} receptors. Indeed, previous studies have shown that [^{3}H]-CGS 21680 may bind to other binding sites, especially in cerebral cortex and hippocampus, than the A_{2A} receptor (48), whereas [^{3}H]-SCH 58261 binds selectively to A_{2A} receptors (30).

Although A_1 and A_{2A} receptors are present at birth in the rat (9–11), the major development in terms of density and coupling to second messenger-forming systems occurs postnatally (12, 13). This is highlighted in a difference between the present data and those of Rivkees in the magnitude of the increase in A_1 receptors from birth to adulthood. Whereas we found a 5- to 10-fold increase, Rivkees *et al.* reported a doubling (9). This might be explained by the fact that in the previous study GTP was not added to the binding assay and that, therefore, mainly A_1 receptors not coupled to G-proteins were detected. In our study, where GTP was added, both coupled and uncoupled receptors are detected, and in adult animals at least twice as many receptors are detected in the presence of GTP. This therefore suggests that in the immature brain few A_1 receptors are coupled to G proteins. Furthermore, we have other results using GTPγS binding indicating that the A_1 receptors that are present are poorly coupled to G proteins (Ådén U, unpublished observation).

Therefore, we believe that a reason for a lack of major effect on, for example, adenosine receptors in the brain of animals receiving low doses of caffeine pre- and postnatally is that the primary targets for caffeine action are poorly developed both in number and in coupling to effector proteins. It must be borne in mind that the situation may be different in tissues outside the brain. Indeed, there is evidence that the cardiac adenosine A_1 receptors, for example, are well developed at birth (9). It is possible that maternal caffeine intake may affect other tissues than those studied here. It is also conceivable that effects on tissues outside the CNS may influence brain development.

The changes in benzodiazepine binding sites during early development were much less pronounced than in the case of the adenosine receptors. No significant up-regulation of [^{3}H]-flunitrazepam binding was seen after perinatal caffeine exposure, at least at the doses used in the present study. As noted above, previous studies on adult animals have given variable

results. Again these may be related to the dose of caffeine inasmuch as direct effects of caffeine on benzodiazepine receptors require 40–100 times higher plasma concentrations than those observed here (see Ref. 8 for references). They are also higher than those measured in adults after normal human daily consumption of caffeine-containing beverages. Nonetheless, benzodiazepine receptors may be a target for high dose caffeine given perinatally.

In summary, the present results indicate that perinatal treatment with caffeine in doses that correspond to human consumption produces minimal changes in A_1, A_{2A}, and $GABA_A$ receptors in cortex, hippocampus, striatum, and cerebellum. Although this is a negative finding it is potentially important because adaptive changes in these receptors have been linked to changes in the behavior of the offspring of coffee-consuming mothers. It is also suggested that the reason for the lack of effect is that in the immature brain the primary targets for caffeine given in low doses are poorly developed. Whereas the previous studies have raised concerns about maternal caffeine consumption, the present results may be reassuring for pregnant and breast-feeding human mothers who drink coffee in moderation.

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