The Stimulatory Action and the Development of Tolerance to Caffeine Is Associated with Alterations in Gene Expression in Specific Brain Regions

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We sought neurochemical correlates to the stimulatory action of caffeine in rats and to adaptations during development of tolerance. Acute intraperitoneal injections of caffeine (7.5 mg/kg) increased locomotion and NGFI-A mRNA, a marker of neuronal activity, in the hippocampal area CA1, but decreased NGFI-A mRNA in rostral striatum and nucleus accumbens. Rats that received caffeine (0.3 gm/l) in their drinking water for 14 d developed tolerance to the stimulatory effect of a challenge with caffeine (7.5 mg/kg) and responded with a less pronounced decrease of NGFI-A mRNA in rostral striatum and nucleus accumbens. Metabolism of caffeine to its active metabolites was increased in tolerant animals, but the total level of active metabolites in brain was not significantly altered. Thus, there are changes in caffeine metabolism after long-term caffeine treatment, but they cannot explain development of tolerance.

Caffeine-tolerant animals had downregulated levels of adenosine A_{2A} receptors and the corresponding mRNA in rostral parts of striatum, but an increased expression of adenosine A_1 receptor mRNA in the lateral amygdala. No changes in mesencephalic tyrosine hydroxylase mRNA were found in caffeine-tolerant rats.

Thus, we have identified neuronal pathways that are regulated by adenosine A_1 and/or A_{2A} receptors and are targets for the stimulatory action of caffeine. Furthermore, adaptive changes in gene expression in these brain areas were associated with the development of locomotor tolerance to caffeine.

Key words: caffeine; methylxanthines; adenosine receptors; immediate early genes; striatum; drug tolerance; locomotion; in situ *hybridization*

Caffeine is the most regularly consumed psychostimulant in the world. The average daily intake is 200–500 mg/d per person in the United States and the European countries. In laboratory animals, low doses of caffeine induce place preference (Brockwell et al., 1991) and increase motor activity (Boissier and Simon, 1965; Thithapandha et al., 1972; Snyder et al., 1981). Tolerance develops rapidly to the stimulatory effects of caffeine and its metabolite theophylline on locomotion (Holtzman and Finn, 1988; Holtzman et al., 1991; Kaplan et al., 1993; Lau and Falk, 1995). This tolerance develops regardless of whether caffeine is administered orally (Holtzman et al., 1991), intraperitoneally (Lau and Falk, 1995), or subcutaneously via mini-pumps (Kaplan et al., 1993), but no cross-tolerance with amphetamine and cocaine is seen (Holtzman and Finn, 1988).

There is some evidence that the tolerance to caffeine depends on an altered metabolism (Daly, 1993; Lau and Falk, 1995). Other studies have emphasized the importance of adaptive changes in the number of adenosine receptors for the develop-

ment of caffeine tolerance (Daly, 1993; Fredholm, 1995). Indeed, it is believed that adenosine receptor antagonism underlies the stimulatory effects of caffeine (Fredholm, 1980; Snyder et al., 1981), and several reports show that long-term administration of caffeine can cause a small increase in the number of A_1 receptors in several different brain regions (Fredholm, 1982; Ramkumar et al., 1988; Johansson et al., 1993). However, the functional role of such an upregulation is open to question (Holtzman et al., 1991; Georgiev et al., 1993; Kaplan et al., 1993), and mechanisms other than changes in A_1 receptor number probably underlie the development of caffeine tolerance. One possible target is A_{2A} receptors, which are expressed at high density in striatum and tuberculum olfactorium (Parkinson and Fredholm, 1990). Indeed, these receptors are known to be important for the stimulatory effect after acute administration of caffeine (Jacobson et al., 1993; Svenningsson et al., 1995a; 1997c; Ledent et al., 1997). We have also shown that a single dose of caffeine as well as of a selective A_{2A} receptor antagonist at behaviorally stimulating concentrations significantly decreases the expression of mRNA for the immediate early gene NGFI-A in striatum (Svenningsson et al., 1995a, 1997c). This gene is known to be regulated via cAMP response element-binding (CREB) protein (Tsai-Morris et al., 1988), a transcription factor that is involved in cocaine reward (Carlezon et al., 1998). NGFI-A is also commonly used as a marker for increased metabolic activity in neuronal pathways (Dragunow and Faull, 1989).

In the present study, we therefore investigated the development of tolerance to low doses of caffeine that produce plasma levels similar to those commonly reached in man and related that to changes in the metabolism of caffeine. To find a neurochemical

correlate we examined changes not only in adenosine receptors but also in the expression of NGFI-A mRNA.

MATERIALS AND METHODS

Animal treatments and measurements of locomotion. These experiments were approved by the regional animal ethical board. Male Sprague Dawley rats weighing 203–233 gm (ALAB, Stockholm, Sweden) were used. They were accustomed to the experimental procedure by being handled, injected with saline, and placed in the locomotion arena for 30 min on 2 consecutive days before the experiment. The locomotion arena ($68 \times 68 \times 45$ cm) is equipped with photocells at two levels for recording of horizontal (locomotion) and vertical (rearing) activity (Ericson et al., 1991). The following parameters were determined: horizontal activity (all counts recorded at the lower level of photocells), vertical activity (all counts recorded in the upper level of photocells), peripheral activity (counts recorded near the edges of the boxes, within 2.5 cm distance from the walls), forward locomotion (counts recorded at the lower level of photocells when the animal is moving in the same direction, i.e., successive interruptions of photobeams), and corner time (time spent in the corners of the boxes). Moreover, to determine locomotion in the center of the boxes, the peripheral activity was subtracted from the horizontal activity. Animals were randomly assigned to different treatment groups: animals drinking water and injected with saline ($n = 14$, referred to as water+saline) or caffeine ($n = 8$, referred to as water+caffeine), and animals drinking caffeine solution and injected with saline ($n = 8$, referred to as caffeine+saline) or caffeine ($n = 8$, referred to as caffeine+caffeine). In addition, for some caffeine-drinking animals receiving saline injections, caffeine was replaced with water as drinking fluid 14 hr before the final saline injection [$n = 6$, referred to as caffeine(withdrawal)+saline]. On the first experimental day, animals were injected intraperitoneally with saline or 7.5 mg/kg caffeine (Sigma, Labkemi, Stockholm, Sweden), and 15 min thereafter their locomotion was measured for 1 hr in the locomotion arena. After the behavioral session animals were given water containing 0.3 gm/l caffeine or water as their only source of fluid. Daily measurements of their oral intake were made, and a difference of 1.0 gm in bottle weight was assumed to represent 1.0 ml of fluid consumed. After 1 and 2 weeks, animals were again injected with saline or caffeine (7.5 mg/kg), and their motor activity was measured for 1 hr. All animals were decapitated 2.75 hr after the final behavioral session (i.e., 4 hr after the final injections).

In a follow-up experiment, animals received intraperitoneal injections of saline ($n = 8$) or caffeine at different doses (7.5, 15, 30, 50, or 100 mg/kg; $n = 5–8$) and were killed 4 hr thereafter. In addition, some animals were injected with saline ($n = 5$) or 50 mg/kg ($n = 5$) or 100 mg/kg ($n = 5$) caffeine twice daily for 2 weeks and killed 4 hr after the final injections.

Plasma levels of methylxanthines. After decapitation, trunk blood was collected from the severed neck, rapidly centrifuged, and stored at $-20°C$ until assayed for levels of methylxanthines. The procedure for the extraction and HPLC assaying of methylxanthines (caffeine, theobromine, and theophylline and/or paraxanthine) is described in detail elsewhere (Fredholm et al., 1983). The standards were obtained from Sigma (Labkemi, Stockholm, Sweden), and the very low levels of methylxanthines occasionally measured in water+saline-treated animals were considered as blank control and subtracted from values obtained in caffeine-treated animals. In an additional experiment, animals were given water containing 0.3 gm/l caffeine or water as their daily source of fluid as described above for 2 weeks and thereafter challenged with an intraperitoneal injection of caffeine, theophylline, paraxanthine, or theobromine at 15 mg/kg each. These animals were killed 4 hr after the injections, and their trunk blood, kidneys, and brains (with striata dissected out separately) were collected, and levels of methylxanthines were determined.

In situ hybridization. The brains from killed animals were rapidly dissected out and frozen at $-80°C$. Thereafter coronal cryostat sections (14 μm thick) were cut at $+3.20$, $+1.20$, $+0.48$, -0.92, -3.14, and -5.20 mm from bregma and thaw-mounted on poly-L-lysine (50 μg/ml)-coated slides. The following probes were used for *in situ* hybridization: NGFI-A, complementary to rat NGFI-A mRNA encoding amino acids 2–16 of the NGFI-A protein (Milbrandt, 1987); tyrosine hydroxylase, complementary to nucleotides 1441–1488 of the rat tyrosine hydroxylase protein (Lamouroux et al., 1982); A_1 receptor, complementary to nucleotides 985–1032 of the rat A_1 receptor (Mahan et al., 1991); and A_{2A} receptor, complementary to nucleotides 916–959 of the dog A_{2A} receptor (Schiffmann et al., 1990). All probes were radiolabeled using terminal deoxyribonucleotidyl transferase (Pharmacia LKB, Uppsala, Sweden) and α-^{35}S-

dATP (DuPont-NEN, Stockholm, Sweden) to a specific activity of $\sim 10^9$ cpm/μg. Mounted sections were hybridized in a mixture containing 50% formamide (Fluka, Buchs, Switzerland), $4\times$ sodium saline chloride, $1\times$ Denhardt's solution, 1% sarcosyl, 0.02 M $NaPO_4$, pH 7.0, 10% dextran sulfate, 0.5 mg/ml yeast tRNA (Sigma, Labkemi, Stockholm, Sweden), 0.06 M dithiothreitol, 0.1 mg/ml sheared salmon sperm DNA, and 10^7 cpm/ml of probe. After hybridization for 16 hr at 42°C, the sections were washed four times for 15 min in $1\times$ sodium saline chloride at 55°C. Thereafter they were dipped briefly in water and in 70, 95, and 99.5% ethanol, and dried. The dry sections were apposed to Hyperfilm β-max film (Amersham, Solna, Sweden) for 1–2 weeks. Some sections were thereafter dipped in NTB-3 emulsion (Kodak, Järfälla, Sweden) and exposed for 2 months.

The autoradiographic films from the *in situ* hybridization experiments were analyzed by using the Microcomputer Imaging Device system (M4, Imaging Research, Ontario, Canada). The system was calibrated with a Kodak density wedge, and the results are presented as optical density values.

Ligand binding autoradiography. Saturation experiments with [^{3}H]1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 0.05, 0.25, 0.5, 1, 2.5, 7.5, 15, and 30 nM) (120 Ci/mmol; DuPont, New England Nuclear, Stockholm, Sweden), a selective A_1 receptor antagonist, and [^{3}H]5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261; 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 nM) (68.6 Ci/mmol; a gift from Dr. Ennio Ongini, Schering-Plow, Milan, Italy), a selective A_{2A} receptor antagonist, were performed as described elsewhere (Svenningsson et al., 1997c). The nonselective adenosine receptor agonist 5′-N-ethylcarboxamidoadenosine (NECA; 100 μM) was used to define nonspecific binding at each concentration. To determine the potency of methylxanthines (caffeine, theophylline, paraxanthine, and theobromine), they were added at concentrations of 1, 3, 10, 30, 100, 300, and 1000 μM with 1 nM [^{3}H]N^6-cyclohexyladenosine (CHA; 34.4 Ci/mmol; DuPont-NEN), a selective A_1 receptor agonist, or 2 nM [^{3}H]2-[p-(2-carbonylethyl]phenethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680; 48.1 Ci/mmol; DuPont-NEN), a selective A_{2A} receptor agonist, as described in detail elsewhere (Parkinson and Fredholm, 1990; Johansson et al., 1993). It has previously been shown that caffeine, which is very water soluble and a low-affinity ligand at adenosine receptors, does not remain in the sections after the initial wash steps and therefore that caffeine remaining in the sections does not affect the results (Johansson et al., 1997). The dried sections together with plastic tritium standards (Amersham, Solna, Sweden) were apposed to ^{3}Hyperfilm (Amersham) for 5 weeks. The autoradiograms were analyzed with a Microcomputer Imaging Device system (M4). Optical density values were converted to binding density (femtomol per milligram of gray matter) using the plastic tritium standards and specific activity of the radioligands. The IC_{50} values for [^{3}H]CHA and [^{3}H]CGS 21680 were converted to K_i values according to the Cheng-Prusoff equation using the K_D values for the ligands given in the cited publications (0.32 nM for [^{3}H]CHA; 2 nM for [^{3}H]CGS 21680).

Statistics. Data obtained from the behavioral experiments were expressed as total activity counts over 60 min sessions (see Figs. 1, 2) and statistically evaluated by using either a three-way (oral water or caffeine/saline or caffeine challenge/days) ANOVA with repeated measures (days) (see Fig. 1) or a two-way (oral water or caffeine/saline or caffeine challenge) ANOVA (see Fig. 2). In all cases, *post hoc* comparisons were made by using the Tukey highest significant difference test for unequal group sample sizes (CSS:Statistical software).

Optical density values from the different treatment groups from the *in situ* hybridization experiments were statistically evaluated by using one-way ANOVAs for each region. To determine whether there were significant differences between individual treatment groups, pairwise comparisons were made using Bonferroni's test for *post hoc* comparisons (GraphPAD PRISM 2.1, San Diego, CA). Data from the autoradiographic experiments were fitted to a binding isotherm by nonlinear regression (GraphPAD PRISM 2.1).

For all statistical evaluations, $p < 0.05$ was considered significant.

RESULTS

Daily oral intake of caffeine was estimated to be 60.2 ± 1.6, 57.7 ± 1.9, and 59.4 ± 0.9 mg$\cdot$kg$^{-1}\cdot$d^{-1} for caffeine+saline-, caffeine(withdrawal)+saline-, and caffeine+caffeine-treated animals, respectively. This is lower than those reported for rats receiving 1.0 gm/l caffeine in the drinking water (Johansson et

Locomotor Activity
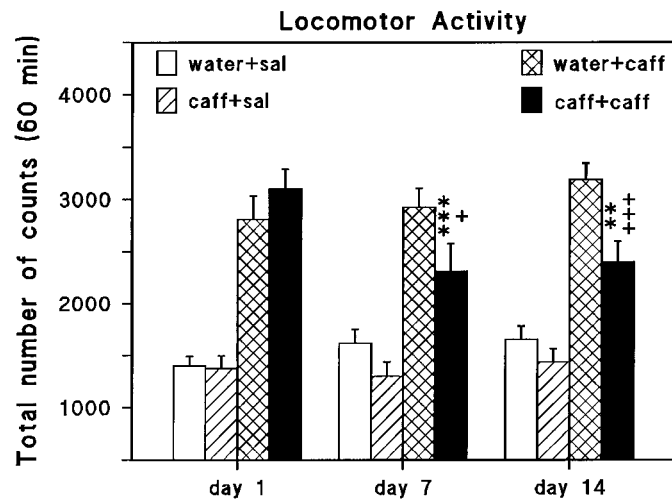
Figure 1. The development of tolerance to caffeine's ability to increase locomotor activity over a course of chronic caffeine administration. Data are expressed as mean (±SEM) of total activity counts during a 60 min session. *Asterisks* indicate a significant difference between caffeine+saline- and caffeine+caffeine-treated animals. *Plus signs* indicate a significant difference between water+caffeine- and caffeine+caffeine-treated animals. $^{+}p < 0.05$; $^{**}p < 0.01$; $^{+++},$ $^{***}p < 0.001$.

al., 1993), but similar to those reported for mice that received 0.3 gm/l caffeine in the drinking water (Johansson et al., 1997). The average daily volume of caffeine solution consumed was similar to the average daily volume of drug-free tap water that was consumed by the control group (56.3 ± 1.6, 52.9 ± 2.0, and 55.5 ± 0.6 ml/d for caffeine+saline-, caffeine(withdrawal)+saline-, and caffeine+caffeine-treated animals versus 56.9 ± 1.6 and 53.9 ± 1.8 ml/d for water+saline-and water+caffeine-treated animals). This is in contrast to the situation in rats and mice receiving 1 gm/l caffeine (Johansson et al., 1993) who consumed smaller amounts of fluid and showed a smaller weight gain than control animals. Because this relative weight loss appears to be attributable to diuresis, the results could indicate that the renal effects of 0.3 gm/l caffeine in the drinking water are limited. However, the average increase in body weight of rats receiving 0.3 gm/l of caffeine for 14 d did not differ from the average weight gain of rats in the control group (7.63 ± 0.24, 7.52 ± 0.20, and 7.60 ± 0.27 gm/d for caffeine+saline-, caffeine(withdrawal)+saline-, and caffeine+caffeine-treated animals versus 7.26 ± 0.18 and 7.18 ± 0.19 gm/d for water+saline- and water+caffeine-treated animals).

Oral administration of caffeine leads to development of tolerance to the stimulatory effect of a challenge with caffeine

Before the oral treatments with water or caffeine (0.3 gm/l), all animals that received an intraperitoneal injection of caffeine (7.5 mg/kg) responded with a marked increase in locomotion as compared with their saline-treated controls (Fig. 1). After 1 week of caffeine ingestion, tolerance, although incomplete, developed to the effect of caffeine (Fig. 1). After 2 weeks the tolerance was of a similar magnitude as after 1 week. No significant changes in locomotion were found in animals that received caffeine+saline as compared with animals treated with either water+saline (Fig. 1) or caffeine(withdrawal)+saline (results not shown).

There are both quantitative and qualitative differences in locomotion in caffeine-tolerant rats

The pattern of locomotion in animals receiving caffeine in their drinking solution (caffeine+saline) did not differ from the normal pattern observed in water+saline-treated animals. However, animals receiving water+caffeine differed significantly from water+saline-treated animals in all the examined measurements of motor activity (Fig. 2A–F). That is, the animals exhibited more forward locomotion and vertical and horizontal activity, both in the periphery and in the center of the locomotion arena, and spent less time in the corners of the arena. After 14 d of caffeine treatment the locomotion and rearing caused by a challenge with caffeine was significantly altered (Fig. 2A,C). The locomotion in the center of the arena was markedly reduced, whereas locomotion in the periphery was unaltered (Fig. 2D,F). Accordingly, caffeine+caffeine-treated animals spent more time in the corners of the arena than the water+caffeine-treated animals (Fig. 2E). Thus, tolerance to the stimulatory effect of caffeine occurred in animals chronically exposed to caffeine in their drinking fluid. However, this tolerance was incomplete because caffeine+caffeine animals showed significantly higher scores for forward locomotion and vertical and horizontal activities (but only in the periphery) than animals treated with caffeine+saline (Fig. 2A–D). No significant differences in the pattern of motor activity were found between caffeine+saline- and caffeine(withdrawal)+saline-treated animals (data not shown).

The development of tolerance to the stimulatory effect of caffeine could be attributed to pharmacokinetic adaptations and changes in the signaling via adenosine receptors and other neurotransmitter systems such as the dopaminergic system. We have tried to elucidate the role of these different possibilities.

There is an enhanced metabolism of caffeine and accumulation of active metabolites in tolerant rats

To verify the accuracy of the caffeine injections and to estimate the levels of caffeine in chronically exposed animals, trunk blood was collected after the decapitation on day 14, and the plasma levels of caffeine were determined. Because caffeine forms several active metabolites—theophylline, theobromine, and paraxanthine—we also measured the levels of these methylxanthines. As shown in Table 1, there was almost no caffeine, but there were substantial amounts of its metabolites in caffeine+saline-treated animals. Similarly, in caffeine+caffeine-treated animals the levels of caffeine were considerably lower than those of theophylline/paraxanthine and theobromine. By contrast, in water+caffeine-treated animals the level of caffeine was markedly higher than its metabolites. The plasma levels of methylxanthines were very close to the limit of detection (i.e., corresponding to those in water+saline animals) in caffeine(withdrawal)+saline animals.

These results could indicate that part of the tolerance to caffeine is caused by increased metabolism. However, the decrease in caffeine levels was accompanied by an increase in theophylline, paraxanthine, and theobromine. All of these methylxanthines act as antagonists at adenosine receptors, albeit with somewhat different affinities, so there is reason to assume that the pharmacological effects may correlate better with the summed concentrations of caffeine and its active metabolites than with the concentration of caffeine alone. We therefore determined the potency of the four methylxanthines at striatal A_1 and A_{2A} receptors by using autoradiography (Fig. 3). On the basis of this
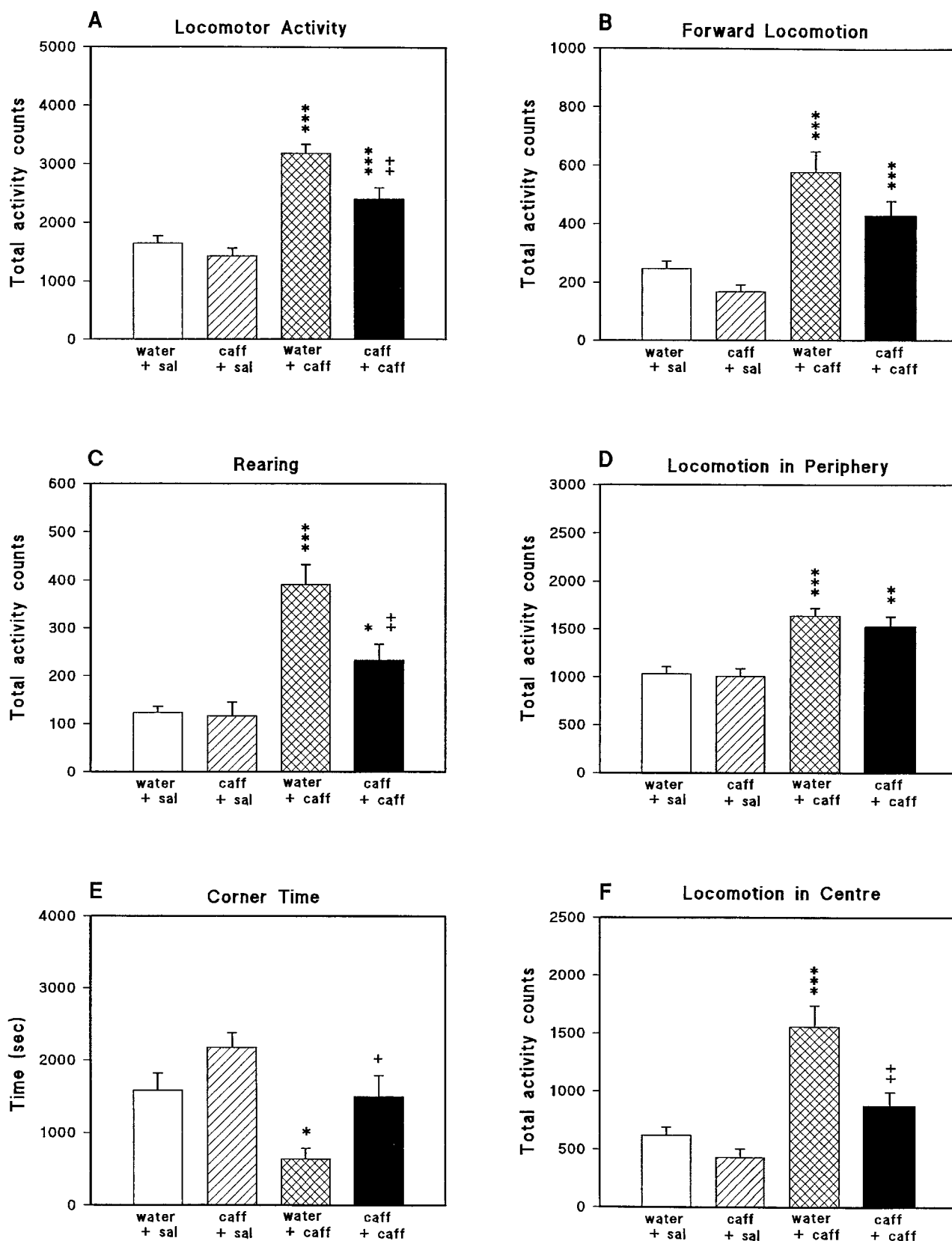
Figure 2. Effects of saline or caffeine (7.5 mg/kg) challenge on locomotor activity (*A*), forward locomotion (*B*), rearing (*C*), locomotion in periphery (*D*), corner time (*E*), and locomotion in center (*F*) in animals treated chronically with water or caffeine (0.3 gm/l) for 14 d. Data are expressed as mean (±SEM) of total activity counts during a 60 min session. *Asterisks* indicate a significant difference between caffeine+saline- and caffeine+caffeine-treated animals. *Plus signs* indicate a significant difference between water+caffeine- and caffeine+caffeine-treated animals. $^+ p < 0.05$; $^{**}p < 0.01$; $^{+++},$ $^{***}p < 0.001$.

Table 1. Plasma levels (μM; mean ± SEM) of methylxanthines after the indicated treatments

Challenge	Chronic treatment	Theobromine	Theophylline/paraxanthine	Caffeine	Total methylxanthines (weighted)
Saline	Caffeine	2.13 ± 0.22	4.35 ± 0.63	0.40 ± 0.08	11.8 ± 1.73
	Caffeine (withdrawal)	0.52 ± 0.49	0.72 ± 0.41	0.0 ± 0.0	1.64 ± 0.97
Caffeine	Water	2.57 ± 0.21	10.9 ± 0.86	14.8 ± 1.69	42.5 ± 3.73
	Caffeine	6.86 ± 0.89	17.8 ± 2.58	3.73 ± 1.11	49.6 ± 7.63

Weighted values (based on K_i values for displacement of [^{3}H]DPCPX and [^{3}H]SCH 58261 binding: caffeine, 1: theophylline/paraxanthine, 2.55; theobromine, 0.14).
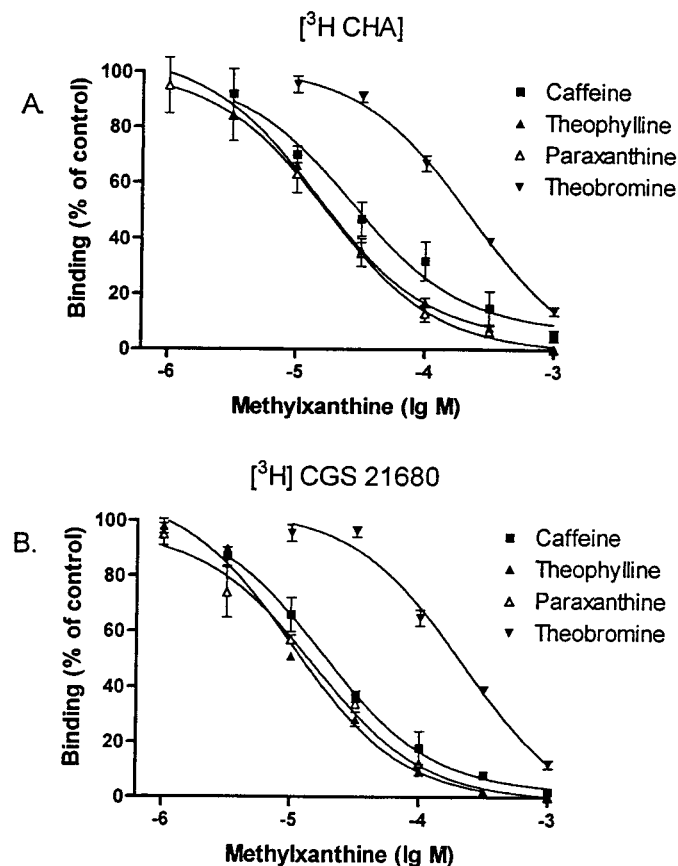
Figure 3. Potency of caffeine, theophylline, paraxanthine, and theobromine as antagonists at striatal A_1 and A_{2A} receptors. *A* shows displacement of binding of 1 nM [^{3}H]CHA from striatal A_1 receptors. Means (±SEM) from duplicate determinations from two separate experiments. *B* shows displacement of binding of 2 nM [^{3}H]CGS 21680 from striatal A_{2A} receptors. Means (±SEM) from duplicate determinations from two separate experiments. Using the K_D value for CHA determined earlier (Johansson et al., 1993), the following K_i values for A_1 receptors were calculated: caffeine 20.2 μM, theophylline 4.7 μM, paraxanthine 5.0 μM, theobromine 98 μM. Using the K_D value for CGS 21680 determined earlier (Parkinson and Fredholm, 1990), the following K_i values for A_{2A} receptors were calculated: caffeine 8.8 μM, theophylline 5.1 μM, paraxanthine 7.6 μM, theobromine 109 μM.

information, each of the methylxanthines was weighted according to its affinity for A_1 and A_{2A} receptors and thereafter added together. The weighted values were determined by using the means of K_i values for [^{3}H]CHA and [^{3}H]CGS 21680 displacement; 1 for caffeine, 2.13 for paraxanthine, 2.96 for theophylline, and 0.14 for theobromine. Calculated using this procedure, the plasma levels of weighted methylxanthines tended to be higher in caffeine+caffeine- than in the water+caffeine-treated animals (Table 1).

Caffeine penetrates the blood–brain barrier several times better than its metabolites

Previous work (Lau and Falk, 1995) has indicated that the plasma levels of methylxanthines might not correspond adequately with those in the brain. To further investigate this issue and to confirm that caffeine is metabolized more rapidly in tolerant rats, animals received water containing 0.3 gm/l caffeine or ordinary tap water as their daily source of fluid as described above for 2 weeks and were thereafter challenged with an intraperitoneal injection of either caffeine, theophylline, paraxanthine, or theobromine at 15 mg/kg each. The daily intake of water and caffeine solution was almost identical in the two groups (56.7 ± 0.57 and 54.7 ± 0.21 ml/d, respectively), leading to an amount of caffeine ingestion at 58.1 ± 0.50 mg · kg^{-1} · d^{-1} for oral caffeine-treated animals. The weight gain was also very similar: 7.90 ± 0.10 gm/d in water-treated and 7.83 ± 0.12 gm/d in caffeine-treated animals.

Each of the methylxanthines was readily transferred to the plasma from the gastrointestinal tract as well as the intraperitoneal space (Table 2). In water-drinking animals, an injection of caffeine led to high levels of caffeine in the CNS with the ratio between levels in striatum and kidney being 0.62. By contrast, the striatal levels of all the other examined methylxanthines were relatively much lower than in the kidney. The ratios striatum/kidney were 0.24 for theophylline, 0.07 for paraxanthine, and 0.25 for theobromine. Intraperitoneal injections of theophylline, paraxanthine, or theobromine also led to much higher levels in the kidney than in the striatum, and the ratios striatum/kidney were 0.26 for theophylline, 0.11 for paraxanthine, and 0.29 for theobromine. For all examined methylxanthines, levels similar to those measured in striatum were found when the remaining part of the brain (i.e., without striatum) was examined (results not shown). As in the first experiment described above, caffeine was more rapidly metabolized in caffeine-drinking than water-drinking animals (Table 2). However, the total amount of weighted methylxanthines was not significantly altered, even in striatum. The increased metabolism of caffeine after chronic exposure was unique for this methylxanthine, and none of the other examined methylxanthines were more rapidly metabolized in caffeine-drinking animals (Table 2).

Long-term treatment with caffeine causes an increase of A_1 receptor mRNA in lateral amygdala, but a decrease in A_{2A} receptors and their corresponding mRNA in striatum

The distribution of A_1 and A_{2A} receptors and their corresponding mRNAs agreed with previous studies. The brain areas in which caffeine-mediated alterations in adenosine receptors and their mRNAs were sought are indicated in Figure 4. In rats receiving oral caffeine (caffeine+saline), A_1 receptor mRNA was found to be increased in the lateral amygdala and tended to be increased in

Table 2. Levels of methylxanthines (μM) in plasma, striatum, and kidney

| Challenge	Chronic treatment	Theobromine	Paraxanthine	Theophylline	Caffeine	Total methylxanthines (weighted)
Plasma						
Caffeine	Water	2.43 ± 0.25	7.41 ± 1.76	7.54 ± 0.78	19.3 ± 5.61	57.7 ± 11.7
	Caffeine	3.04 ± 0.21	11.7 ± 3.18	9.93 ± 1.14	3.98 ± 1.05	58.8 ± 11.2
Theophylline	Water	0.25 ± 0.13	0.00 ± 0.00	55.6 ± 3.05	0.00 ± 0.00	
	Caffeine	1.25 ± 0.82	0.24 ± 0.24	49.1 ± 3.54	0.00 ± 0.00	
Paraxanthine	Water	0.00 ± 0.00	26.4 ± 5.63	1.44 ± 0.16	0.00 ± 0.00	
	Caffeine	0.79 ± 0.12	24.8 ± 2.93	1.86 ± 0.16	0.00 ± 0.00	
Theobromine	Water	14.6 ± 2.21	0.62 ± 0.36	2.29 ± 0.16	0.00 ± 0.00	
	Caffeine	10.7 ± 0.17	1.14 ± 0.24	2.95 ± 0.47	0.00 ± 0.00	
Striatum						
Caffeine	Water	0.98 ± 0.07	1.60 ± 0.57	2.04 ± 0.05	12.8 ± 3.98	22.4 ± 5.35
	Caffeine	1.22 ± 0.14	1.85 ± 0.10	2.94 ± 0.28	5.98 ± 0.68	18.8 ± 1.74
Theophylline	Water	0.02 ± 0.02	0.00 ± 0.00	12.9 ± 0.94	0.00 ± 0.00	
	Caffeine	0.23 ± 0.06	0.00 ± 0.00	12.4 ± 1.09	0.00 ± 0.00	
Paraxanthine	Water	0.00 ± 0.00	4.73 ± 0.75	0.00 ± 0.00	0.00 ± 0.00	
	Caffeine	0.23 ± 0.09	3.08 ± 1.61	0.28 ± 0.28	0.00 ± 0.00	
Theobromine	Water	4.42 ± 0.54	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
	Caffeine	4.24 ± 0.36	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	
Kidney						
Caffeine	Water	3.85 ± 0.57	24.2 ± 3.92	8.59 ± 1.00	21.0 ± 5.75	98.6 ± 17.1
	Caffeine	5.77 ± 0.37	29.9 ± 2.98	10.7 ± 0.76	6.26 ± 1.68	103 ± 10.3
Theophylline	Water	0.00 ± 0.00	0.00 ± 0.00	49.7 ± 3.43	0.00 ± 0.00	
	Caffeine	0.71 ± 0.11	2.30 ± 2.30	42.8 ± 8.80	0.00 ± 0.00	
Paraxanthine	Water	0.09 ± 0.03	43.5 ± 6.52	0.81 ± 0.33	0.00 ± 0.00	
	Caffeine	0.84 ± 0.15	37.0 ± 2.45	3.13 ± 0.08	0.00 ± 0.00	
Theobromine	Water	15.3 ± 1.80	0.00 ± 0.00	1.53 ± 0.43	0.00 ± 0.00	
	Caffeine	13.8 ± 0.41	2.10 ± 0.55	2.60 ± 0.53	0.00 ± 0.00	

Rats were given 2 weeks of oral treatment with either tap water or caffeine (0.3 g/l) and thereafter an intraperitoneal injection of 15 mg/kg caffeine, theobromine, theophylline, or paraxanthine. Animals were killed 4 hr after the injections. Weighted values (based on K_i values for displacement of [^{3}H]DPCPX and [^{3}H]SCH 58261 binding; caffeine, 1; paraxanthine, 2.13; theophylline, 2.96; theobromine, 0.14).

hippocampal areas, but remained unchanged in other examined areas as compared with their water-treated controls (water+saline) (Table 3, Fig. 5*E,F*). The increase of A_1 receptor mRNA in the lateral amygdala was more pronounced in animals from which caffeine had been withdrawn (Table 3). The presence of [^{3}H]DPCPX-binding in the lateral amygdala was established (results not shown), but because of the rather small size of this nucleus, no attempts were made to quantitate the number of binding sites. Caffeine treatment had no significant effects on binding sites for A_1 receptors, as detected by [^{3}H]DPCPX, in any of the regions where measurements could be made (Fig. 6*A*).

A significant reduction of A_{2A} receptor mRNA was detected in the rostral parts of striatum in caffeine-treated animals (caffeine+saline) as compared with rats receiving water (water+saline) (Table 3, Fig. 5*C,D*). Moreover, autoradiography revealed also a decrease of binding sites for the selective A_{2A} receptor antagonist [^{3}H]SCH 58261 in caffeine-treated animals (Figs. 5*A,B*, 6*B*). In contrast to the regulation of A_1 receptor mRNA in the lateral amygdala, the decreases of both A_{2A} receptor mRNA and protein levels returned toward control levels in animals in which caffeine had been withdrawn 14 hr earlier (caffeine(withdrawal)+saline) (Table 3, Fig. 6*B*). This rapid normalization may depend on a rapid turnover of A_{2A} receptor mRNA. Indeed, in PC 12 cells, a cell line expressing native A_{2A} receptors, A_{2A} mRNA has a short half-life (1.2 hr) (Saitoh et al., 1994).

Single or repeated intraperitoneal injections of moderate to high concentrations of caffeine downregulate A_{2A} receptor mRNA

The ability of caffeine to decrease the gene expression for A_{2A} receptors in the rostral striatum was somewhat surprising. To further examine effects of caffeine on A_{2A} receptor mRNA, animals were given acute intraperitoneal injections of saline or caffeine at several different doses (7.5, 15, 30, 50, and 100 mg/kg). A significant decrease in A_{2A} receptor mRNA was found after 50 and 100 mg/kg caffeine (Fig. 7*A*). A significant decrease was also found in animals that received 50 or 100 mg/kg caffeine twice daily for 2 weeks (Fig. 7*B*). Injections of moderate to high doses of caffeine, equivalent to 50–100 mg/kg, lead to induction of the transcription factor AP-1 in striatum (Svenningsson et al., 1995b). AP-1 is a dimer composed of the products of different *fos* and *jun* immediate early genes and is involved in the regulation of a large number of target genes (Sheng and Greenberg, 1990). Because there are binding sites for AP-1 in the promotor region of the A_{2A} receptor gene (Chu et al., 1996), we examined whether pretreatment with c-*fos* antisense oligonucleotides could inhibit caffeine-induced downregulation of this gene. For this part of the study we used slides from a previous experiment in which we confirmed that c-*fos* antisense oligonucleotides, but not the controls, reduced c-*fos* mRNA and protein (Svenningsson et al., 1997a). No effect of the c-*fos* antisense oligonucleotide on the caffeine-mediated downregulation of A_{2A} receptor mRNA was found (Fig. 7*C*).
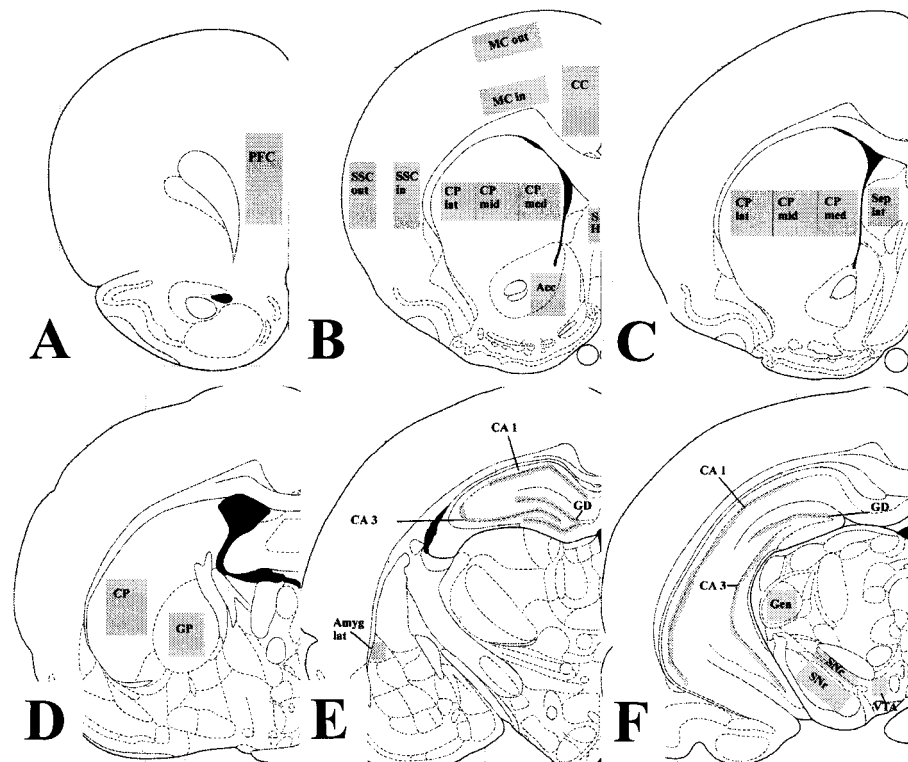
Figure 4. Quantitative measurements of gene expression and ligand binding were performed +3.20 mm (*A*), +1.20 mm (*B*), +0.48 mm (*C*), −0.92 mm (*D*), −3.14 mm (*E*), and −5.20 mm (*F*) from bregma. *Gray squares* delineate the regions examined. *Amyg lat*, Lateral amygdala; *CA 1*, field CA 1 of Ammon's horn in hippocampus; *CA 3*, field CA 3 of Ammon's horn in hippocampus; *CC*, cingulate cortex; *CP*, caudate-putamen; *GD*, gyrus dentatus; *Gen*, medial geniculate nucleus; *GP*, globus pallidus; *MC*, motor cortex; *PFC*, prefrontal cortex; *Sep lat*, lateral septum; *SH*, septohippocampal nucleus; *SNc*, substantia nigra pars compacta; *SNr*, substantia nigra pars reticulata; *SSC*, somatosensory cortex; *VTA*, ventral tegmental area.

Table 3. Optical density values (×100) of A_1 and A_{2A} receptor mRNA from the examined regions after indicated treatments

	Water+saline	Caffeine+saline	Caffeine (withdrawal) +saline
Adenosine A_1 receptor mRNA			
Nucleus accumbens	4.16 ± 0.29	4.18 ± 0.25	3.83 ± 0.37
CP lateral	4.16 ± 0.24	3.98 ± 0.18	3.89 ± 0.35
CP middle	4.29 ± 0.25	4.13 ± 0.14	4.09 ± 0.36
CP medial	3.96 ± 0.22	4.06 ± 0.16	4.03 ± 0.40
Amygdala	2.36 ± 0.13	2.97 ± 0.19[a]	3.85 ± 0.32[b]
CA 1	9.19 ± 0.60	9.22 ± 0.28	9.65 ± 0.60
Adenosine A_{2A} receptor mRNA			
Nucleus accumbens	21.5 ± 0.42	19.1 ± 1.70	19.6 ± 0.60
Rostral CP lateral	28.8 ± 0.67	24.3 ± 0.49[c]	25.6 ± 0.51
Rostral CP middle	28.1 ± 0.45	23.9 ± 0.65[c]	26.6 ± 0.31[b]
Rostral CP medial	24.7 ± 0.59	20.5 ± 0.57[c]	24.1 ± 0.30[b]

Values for caudate-putamen (CP) and the hippocampal area CA 1 are taken +1.20 mm and −3.14 mm from bregma, respectively. The actual locations of the measurements and the abbreviations used here are presented in Figure 4. One-way ANOVA followed by Bonferroni's test was used for multiple comparisons.

[a] Difference between water+saline and caffeine+saline; $p < 0.05$.

[b] Difference between caffeine+saline and caffeine (withdrawal)+saline; $p < 0.01$.

[c] Difference between water+saline and caffeine+saline; $p < 0.001$.

## Effects of chronic caffeine treatment on NGFI-A mRNA

To assess a functional correlate to the above-mentioned alterations in the levels of adenosine receptors, we examined alterations in the levels of NGFI-A mRNA. Its expression as well as that of some other immediate early genes such as c-fos is likely to correlate with neuronal activity in striatum, at least after acute treatments (Chergui et al., 1997; Gonon, 1997). However, we can only assume that there is also a correlation between neuronal activity and NGFI-A mRNA in animals treated chronically with caffeine. There was a relatively high expression of NGFI-A mRNA in striatum in water+saline-treated animals (Table 4, Fig. 8*A,E*). Nevertheless, a significant increase in the expression of this gene was detected in the rostromedial aspect of striatum and in nucleus accumbens of animals exposed to caffeine for 14 d (Table 4, Fig. 8*A,C*). Moreover, caffeine+saline-treated animals also had significantly higher levels of NGFI-A mRNA in the cingulate cortex than water+saline-treated animals. In caffeine(withdrawal)+saline-treated animals, the upregulation of NGFI-A mRNA in the striatum and the cingulate cortex had returned to control levels and tended to be normalized also in the nucleus accumbens (Table 4).

Effects of a challenge with caffeine on NGFI-A mRNA in nontolerant and tolerant animals

There was a decrease in the expression of NGFI-A mRNA throughout rostral parts of striatum and in nucleus accumbens in water+caffeine-treated animals, but an increase in the rostral part of the hippocampal area CA1 when compared with water+saline-treated animals (Table 4, Fig. 8*A,B,E,F,I,J,M,N*). The levels of NGFI-A mRNA in the somatosensory and motor parts of cerebral cortex, the lateral amygdala, and the caudal part of the hippocampal area CA 1 tended also to be higher in animals treated with water+caffeine than in those treated with water+saline (Table 4, Fig. 8*I,J*).

Similarly, in chronically caffeine-treated animals a challenge with caffeine had a tendency, albeit not significant, to reduce the expression of NGFI-A mRNA throughout rostral parts of striatum (Table 4, Fig. 8*C,D,G,H*). Furthermore, there was a trend toward an induction of NGFI-A mRNA in the rostral and caudal parts of the hippocampal area CA 1 and in the lateral amygdala (Table 4, Fig. 8*K,L,O,P*). When nontolerant (water+caffeine) and tolerant (caffeine+caffeine) animals challenged with caffeine
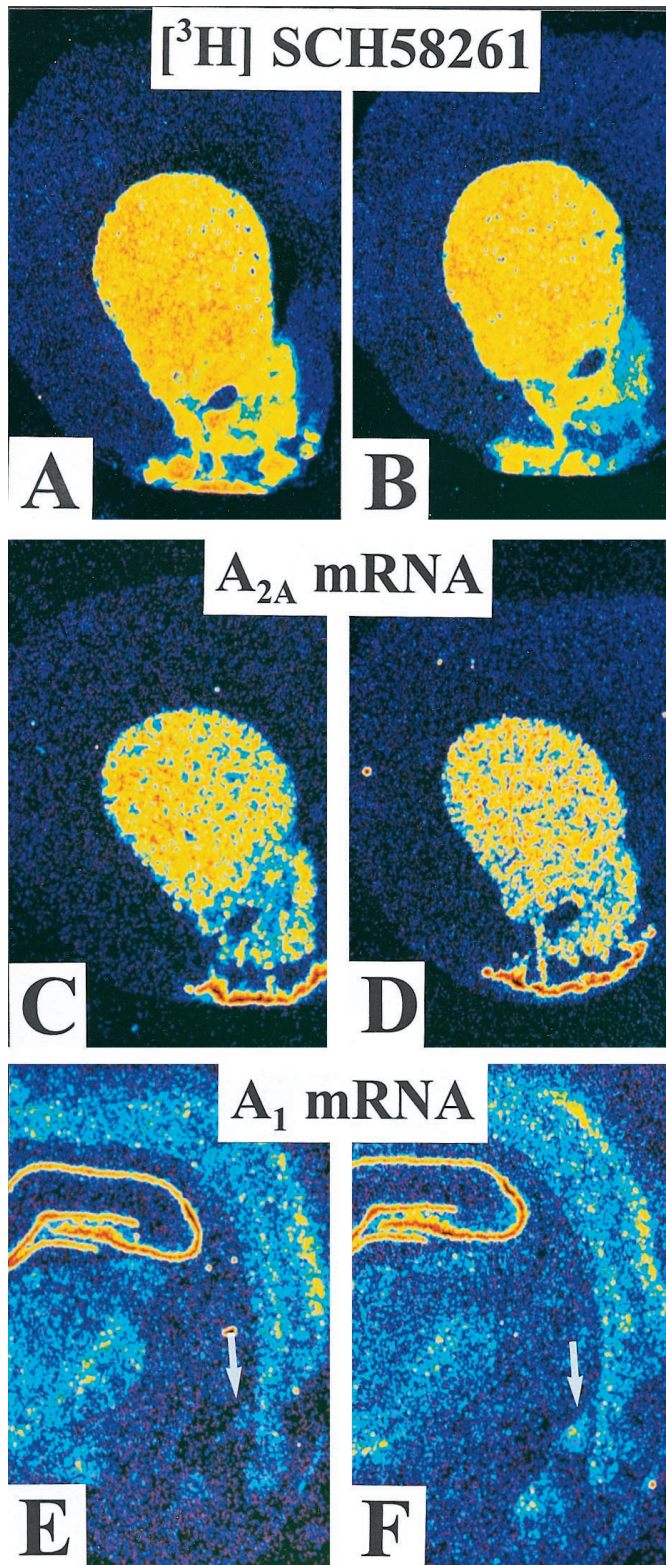
Figure 5. Color-coded photomicrographs showing binding of [³H] SCH58261 (*A, B*) and A$_{2A}$ receptor mRNA (*C, D*), +1.20 mm from bregma, in water+saline-treated (*A, C*) and caffeine+saline-treated (*B, D*) animals. *E* and *F* show A$_1$ receptor mRNA, −3.14 mm from bregma, in water+saline-treated (*E*) and caffeine+saline-treated (*F*) animals. The *white arrows* point to the lateral amygdala.

were compared, significantly lower values of NGFI-A mRNA expression in rostral parts of striatum were found in the nontolerant animals (Table 4, Fig. 8*B,D*).

There are no effects of oral caffeine treatment on tyrosine hydroxylase mRNA levels in the substantia nigra pars compacta and the ventral tegmental area

There is abundant evidence that an intact dopaminergic neurotransmission is necessary for caffeine to be stimulatory (Ferré et al., 1992). Chronic treatment with morphine and cocaine that leads to reverse tolerance (i.e., sensitization) is accompanied by increased levels of tyrosine hydroxylase in the mesencephalon (Beitner-Johnson and Nestler, 1991). To examine whether induced changes in this enzyme (which catalyzes the rate-limiting step in catecholamine synthesis) could explain the development of tolerance to caffeine, we examined expression of tyrosine hydroxylase mRNA in substantia nigra pars compacta and the ventral tegmental area in animals chronically exposed to caffeine. However, no significant differences were found between animals treated with water+saline (optical density values 1.047 ± 0.016 and 1.052 ± 0.019 for substantia nigra and the ventral tegmental area, respectively), caffeine+saline (optical density values 1.058 ± 0.011 and 1.053 ± 0.024 for substantia nigra and the ventral tegmental area, respectively) or caffeine(withdrawal)+saline (optical density values 1.036 ± 0.009 and 1.046 ± 0.015 for substantia nigra and the ventral tegmental area, respectively).

DISCUSSION

Intake of a cup of coffee yields plasma levels of caffeine between 5 and 10 μM in humans (Daly, 1993; Fredholm, 1995). This is similar to the plasma levels of caffeine measured in the present study, and the results obtained may be highly relevant for understanding adaptive changes that may occur after coffee consumption in humans.

As in the case of the development of tolerance to psychostimulatory responses of caffeine in humans (Daly, 1993), only an incomplete tolerance to the locomotor stimulatory action of caffeine was observed here. We have purposely avoided using higher doses of caffeine despite the fact that previous work (Holtzman and Finn, 1988; Lau and Falk, 1995) has suggested that the development of tolerance becomes more complete at higher doses of caffeine. We did this because effects of caffeine are biphasic: low doses of caffeine are stimulatory, whereas high doses are inhibitory. We have found previously that stimulatory doses of caffeine decrease striatal expression of immediate early genes, including NGFI-A, but that higher and depressant concentrations increase their expression (Svenningsson et al., 1995a). Thus, there is evidence that the biphasic action of caffeine on locomotion is the result of two independent mechanisms, and it is possible that chronic caffeine administration will differentially affect these mechanisms. The stimulatory effect could be masked by the inhibitory effects, if tolerance develops only to the former or if the inhibitory response becomes sensitized. Either type of adaptation could explain why tolerance to the stimulatory effect of caffeine is insurmountable under some experimental conditions (Holtzman and Finn, 1988; Holtzman et al., 1991). Because changes in NGFI-A mRNA expression after a challenge with caffeine occur in the same areas in nontolerant and tolerant animals, although to a different extent, the present data argue that tolerance to the stimulatory mechanism, rather than sensitization of the inhibitory mechanism, underlies the decreased stimulatory capacity of caffeine in long-term-treated animals. It appears that there are
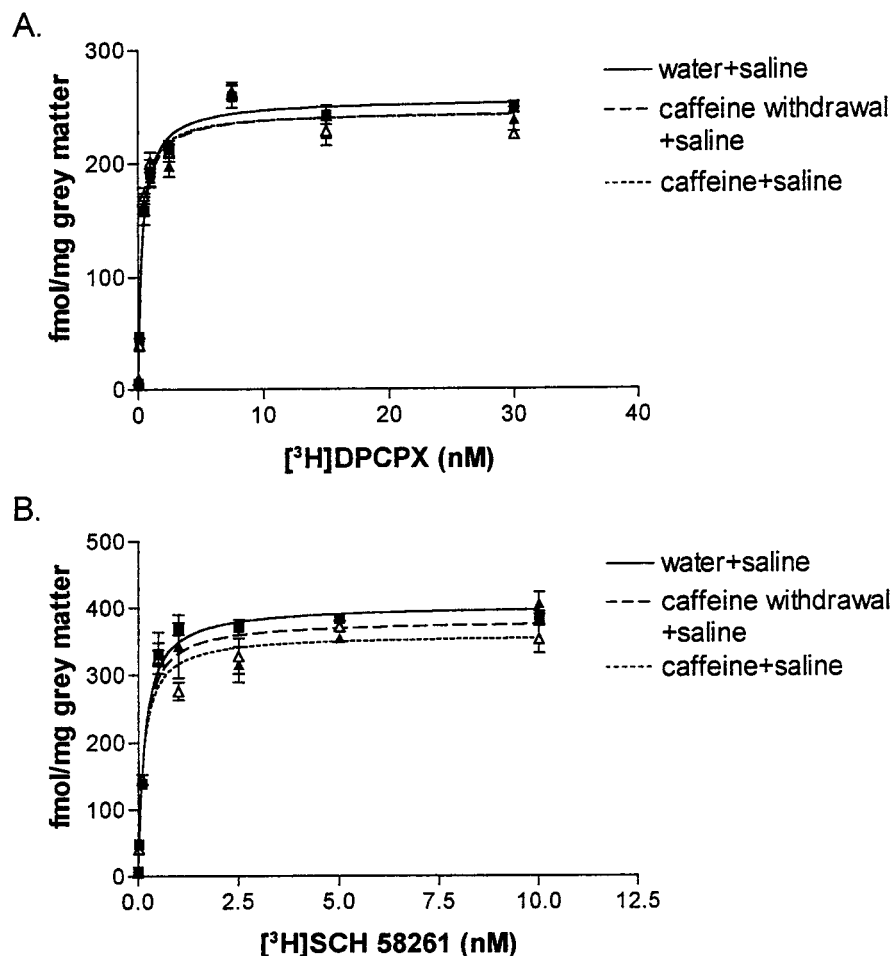
Figure 6. Saturation curves of [^{3}H]DPCPX binding in the CA 1 part of hippocampus (*A*) and of [^{3}H]SCH 58261 binding in the lateral part of rostral striatum (*B*) from animals treated with water+saline, caffeine+saline, or caffeine(withdrawal)+saline. No significant differences in the B_{max} and K_D values for [^{3}H]DPCPX-binding between the treatment groups were seen in substantia nigra pars reticulata, lateral geniculatum, or the hippocampal areas CA 1, CA 3, and gyrus dentatus. In contrast, B_{max} values were significantly lower in the rostrolateral (358 ± 10.3 fmol/gray matter) and rostromedial (341 ± 14.19 fmol/gray matter) parts of striatum in caffeine+saline- as compared with water+saline-treated animals (402 ± 7.13 and 382 ± 6.10 fmol/gray matter, respectively). No significant differences could be observed between water+saline-treated animals and caffeine(withdrawal)+saline-treated animals that showed B_{max} values of 380 ± 13.5 and 355 ± 16.4 fmol/gray matter in the rostrolateral and rostromedial parts of striatum. No significant changes in K_D values of [^{3}H]SCH 58261 (average 0.20 nM) were seen between the different treatment groups in any of the areas investigated.

specific adaptive changes in the brain after chronic caffeine administration.

The present study also shows that the peripheral metabolism of caffeine is enhanced in tolerant animals. Because two of caffeine's metabolites, theophylline and paraxanthine, bind with higher affinity than caffeine to adenosine receptors, the bioavailability of methylxanthines blocking adenosine receptors in the plasma is increased in chronically caffeine-treated animals. However, because passage through the blood–brain barrier is considerably more efficient for caffeine than its metabolites, the enhanced metabolism of caffeine in the periphery does not lead to increased amounts of active methylxanthines in the brain. Conversely, there is no decrease in the amounts of adenosine antagonists in tolerant animals, and altered metabolism cannot explain the development of behavioral tolerance.

It was originally proposed that A$_1$ receptors played an important role in the stimulatory action of caffeine (Snyder et al., 1981), and changes in neurotransmission via A$_1$ receptors may therefore be important for the development of tolerance to caffeine (see introductory remarks). However, the low doses of caffeine used here caused small or no changes in the number of A$_1$ receptors and its corresponding mRNA, and only in the lateral amygdala was there a significant upregulation of A$_1$ receptor mRNA. This increase may be related not only to the stimulatory effect of caffeine on locomotion but also to caffeine's ability to affect conditioned learning processes, especially those related to fear and anxiety (Daly, 1993; McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997).

Recent work has emphasized an important role also for striatal A$_{2A}$ receptors as a target of action for physiologically relevant concentrations of caffeine (Ferré et al., 1992; Barraco et al., 1993; Svenningsson et al., 1995a, 1997c; Ledent et al., 1997). A somewhat surprising novel finding in the present study was that long-term oral treatment with caffeine led to a reduction of A$_{2A}$ receptors and their corresponding mRNA in striatum. Furthermore, a downregulation of A$_{2A}$ receptor mRNA after both acute and repeated intraperitoneal injections with caffeine was found. The transcription factors whereby caffeine regulates expression of the A$_{2A}$ receptor gene remain to be determined. In the present study, we found no direct involvement of c-*fos*-containing AP-1.

Pharmacological principles suggest that downregulation of a receptor would lead to a smaller functional response to an agonist but not to a competitive antagonist, such as caffeine. Hence one could argue, as was done by Holtzman and coworkers (1991) in the case of A$_1$ receptors, that alterations in receptor number are not relevant to explain the tolerance to an antagonist such as caffeine. Nonetheless, the downregulation of A$_{2A}$ receptors might be relevant as a partial explanation. Frequently a decrease in the number of receptors leads to a leftward shift of the concentration–response curve for an agonist, and this was also demonstrated recently for A$_{2A}$ receptors (Arslan et al., 1999). Provided that the concentration of adenosine does not increase in the brain after long-term caffeine treatment, one would expect a smaller activation of A$_{2A}$ receptors by endogenous adenosine and hence a smaller biological effect of caffeine.

A$_{2A}$ receptors are highly expressed in striatopallidal neurons
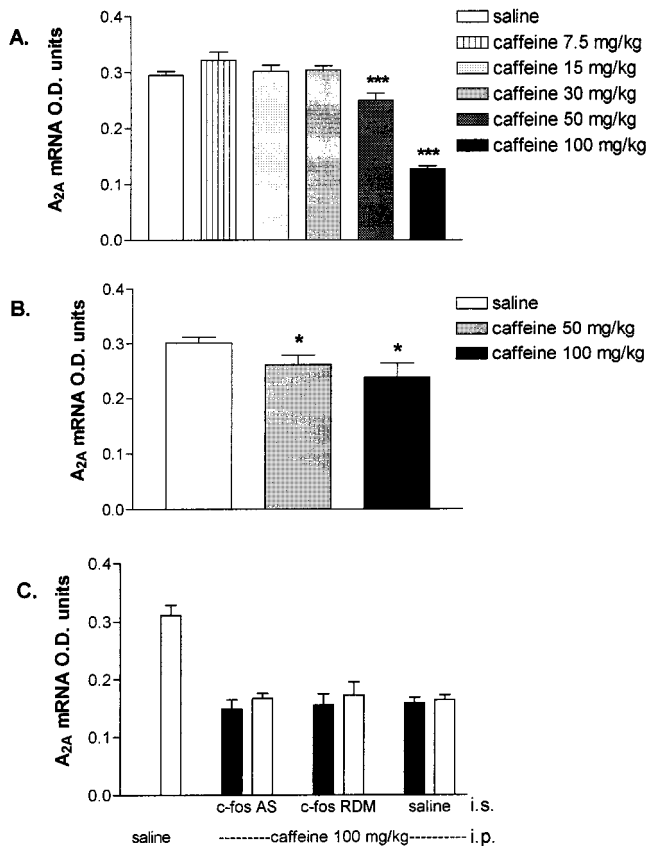
Figure 7. Histograms showing the effects of acute (*A*) or chronic (*B*) intraperitoneal administration of caffeine, at the indicated doses, on A_{2A} receptor mRNA. In *C*, the lack of effect of pretreatment with c-*fos* antisense oligonucleotide on reductions in A_{2A} receptor mRNA is shown.

where they are colocalized with dopamine D_2 receptors (Schiffmann et al., 1991; Fink et al., 1992; Schiffmann and Vanderhaeghen, 1993; Svenningsson et al., 1997b). This subpopulation of neurons is one of the two neuronal pathways from striatum; the other projects to substantia nigra–nucleus entopeduncularis (Gerfen and Wilson, 1996). Most psychostimulants, including cocaine,

amphetamine, and nicotine, increase the expression of immediate early genes such as c-*fos* and NGFI-A in striatonigral neurons (Gerfen and Wilson, 1996). By contrast, caffeine, at low doses, has no pronounced effects on immediate early gene expression in these neurons but decreases their expression in striatopallidal neurons (Svenningsson et al., 1995a, 1997c). Thus, it seems that blockade of A_{2A} receptors is crucial for the stimulatory action of caffeine and that caffeine may increase locomotion through an atypical mechanism.

The decreased ability of caffeine to reduce striatal NGFI-A mRNA in tolerant animals as compared with nontolerant animals implies that functionally relevant adaptations in striatopallidal neurons may underlie the development of tolerance to the stimulatory effects of caffeine on locomotion. Such a mechanism receives further support from the observation that an acute injection, but not repeated injections, of stimulatory doses of caffeine induces c-*fos* mRNA in globus pallidus (Svenningsson and Fredholm, 1997). Caffeine-induced pallidal c-*fos* mRNA is likely to depend, at least partly, on disinhibition of striatopallidal neurons (Svenningsson and Fredholm, 1997). Thus, behavioral tolerance to caffeine is correlated to a reduced effect of caffeine on striatopallidal neurons.

Caffeine caused also a pronounced increase in NGFI-A mRNA in the CA 1 part of the hippocampal formation and a trend toward an increase in the lateral part of amygdala. Because both of these regions contain a large number of A_1 receptors, the caffeine-mediated effects in these extrastriatal areas may reflect actions on A_1 receptor-containing neuronal pathways that could be involved in modulation by adenosine of synaptic plasticity because NGFI-A expression correlates well with the persistence of long-term potentiation in hippocampus (Cole et al., 1989; Daly, 1993). Because hippocampus and the lateral amygdala send excitatory projections to striatum, an altered neuronal activity in these areas may also be involved in the adaptive neurochemical changes in striatal gene expression that were associated with the development of locomotor tolerance in long-term caffeine-treated animals (Gerfen and Wilson, 1996; Swanson and Petrovich, 1998).

In conclusion, the present findings suggest that changes in immediate early gene expression, probably reflecting altered neuronal activity, in some specific areas correlate with the stimula-

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Table 4. Optical density values (×100) of NGFI-A mRNA from some of the examined regions after indicated treatments

	Water+saline	Water+caffeine	Caffeine+saline	Caffeine+caffeine	Caffeine+(withdrawal)+saline
Nucleus accumbens	5.78 ± 0.18^a	4.66 ± 0.24^b	6.74 ± 0.18^c	6.20 ± 0.27	6.43 ± 0.68
CP lateral	6.66 ± 0.26	5.06 ± 0.29	8.05 ± 0.52	6.09 ± 0.29	7.32 ± 1.30
CP middle	6.89 ± 0.28^a	5.33 ± 0.24	7.96 ± 0.42	6.79 ± 0.33	7.35 ± 1.06
CP medial	6.55 ± 0.25^d	4.64 ± 0.24^b	7.71 ± 0.49^c	6.55 ± 0.28	6.68 ± 0.44
SSC inner	9.11 ± 0.75	11.4 ± 3.63	9.41 ± 1.23	7.49 ± 0.55	8.27 ± 1.16
MC inner	8.53 ± 0.28	9.95 ± 0.71	9.16 ± 0.55	8.36 ± 0.39	8.82 ± 1.67
CC	11.2 ± 0.48	12.1 ± 0.58	14.8 ± 0.50^e	12.4 ± 0.90	10.7 ± 1.85^f
Amygdala	10.6 ± 2.09	13.3 ± 4.03	9.19 ± 1.27	10.8 ± 1.83	9.75 ± 1.07
CA 1	18.5 ± 0.88^a	22.1 ± 0.58	18.4 ± 0.44	21.1 ± 1.24	17.7 ± 1.00

Values for caudate-putamen and the hippocampal area CA 1 are taken +1.20 mm and −3.14 mm from bregma, respectively. The actual locations of the measurements and the abbreviations used here are presented in Figure 4. One-way ANOVA followed by Bonferroni's test was used for multiple comparisons.

[a] Difference between water+saline and water+caffeine; $p < 0.05$.

[b] Difference between water+caffeine and caffeine+caffeine; $p < 0.01$.

[c] Difference between water+saline and caffeine+saline; $p < 0.05$.

[d] Difference between water+saline and water+caffeine; $p < 0.001$.

[e] Difference between water+saline and caffeine+saline; $p < 0.01$.

[f] Difference between caffeine+saline and caffeine(withdrawal)+saline; $p < 0.05$.
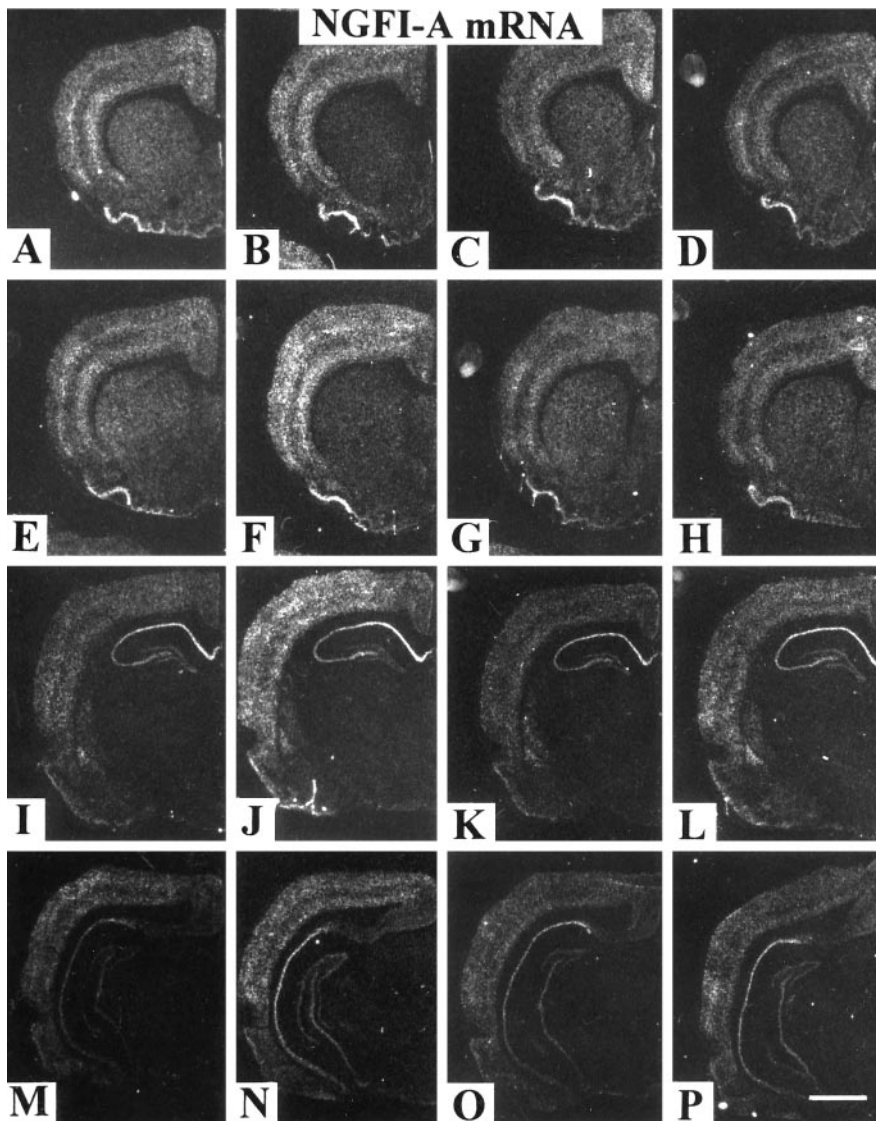
Figure 8. Dark-field photomicrographs showing NGFI-A mRNA expression at +1.20 mm (*A–D*), +0.48 mm (*E–H*), −3.14 mm (*I–L*), and −5.20 mm (*M–P*) from bregma after water+saline (*A, E, I, M*), water+caffeine (*B, F, J, N*), caffeine+saline (*C, G, K, O*), and caffeine+caffeine (*D, H, L, P*).

tory action of caffeine in nontolerant as well as tolerant animals. Alterations in gene expression in striatum are suggested to be crucial for the development of tolerance to caffeine. This conclusion is further supported by the fact that all extrastriatal regions that respond with an altered gene expression to caffeine send excitatory projections to striatum.

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