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ORIGINAL INVESTIGATION

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# The anxiogenic-like effect of caffeine in two experimental procedures measuring anxiety in the mouse is not shared by selective $A_{2A}$ adenosine receptor antagonists

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**Abstract** *Rationale:* The elevated plus-maze and the light/dark box are two established anxiety tests in rodents, which are useful to screen putative anxiogenic effects of drugs. *Objective:* Caffeine is well known to promote anxious behaviour in humans and animal models, but the precise site of action of the drug is still a matter of debate. The present study investigated whether the anxiogenic effects of caffeine observed in mice depend on the blockade of $A_{2A}$ receptor. First, the effects induced by the non-selective drug caffeine were compared with those elicited by two selective $A_{2A}$ receptor antagonists over a wide range of doses in the same experimental conditions. The effects of $A_{2A}$ or $A_1$ adenosine receptor agonists and of a selective $A_1$ adenosine receptor antagonist were also investigated. Second, wild-type and $A_{2A}$ receptor knockout mice offered another approach to delineate the role played by $A_{2A}$ receptor in caffeine's anxiogenic effects. *Methods:* Mice were exposed to the elevated plus-maze or to the light/dark box for 5 min after acute or chronic administration of tested drugs. *Results:* Caffeine acutely administered (50 or 100 mg/kg IP) induced anxiety-like effects in both procedures. Its chronic administration (50 mg/kg IP twice daily) for 1 week or consumption in the drinking water (0.3 g/l) for 8 days or 2 months were also anxiogenic in the plus-maze test. The $A_{2A}$ receptor antagonists ZM241385 (up to 60 mg/kg IP) and SCH58261 (up to 10 mg/kg IP) were devoid of acute effects in both tests. One week administration of ZM241385 (30 mg/kg IP) or SCH58261 (3 mg/kg IP) had no effects in the plus-maze test. An antagonist (DPCPX) and an agonist (CPA) at $A_1$ receptors had no acute effects on anxiety-related indices, whereas an $A_{2A}$ receptor agonist (CGS 21680) displayed non-specific motor effects in the plus-maze test. Acute administration of caffeine (50 mg/kg IP) induced no clear-cut anxiety-like effects in the plus-maze test in $A_{2A}$ receptor knockout mice that exhibited higher basal anxiety levels than wild-type mice. Chronic administration (50 mg/kg IP twice daily) for 1 week elicited less anxiety-like behaviour in $A_{2A}$ receptor knockout than in wild-type mice. *Conclusions:* Adaptative mechanisms following mutation in $A_{2A}$ receptors or their long-term blockade after chronic ingestion of caffeine may be responsible for increase proneness to anxiety. However, the short-term anxiety-like effect of caffeine in mice might not be related solely to the blockade of adenosine $A_{2A}$ receptors, since it is not shared by $A_{2A}$ selective antagonists.

**Key words** SCH58261 · ZM241385 · Adenosine · Plus-maze test · Light/dark test · $A_{2A}$ receptor knockout · $A_1$ receptor

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## Introduction

It is now well established that adenosine functions as a neuromodulator in the central nervous system acting through discrete cell-surface receptors. Adenosine receptors were recognised more than 20 years ago, in part on the base of the ability of caffeine (1,3,7-trimethylxanthine) to act as an antagonist at these receptors. Later, adenosine receptors were classified into two major subtypes called $A_1$ and $A_2$ (van Calker et al. 1979). As stated recently, the biochemical mechanism that underlies the actions of caffeine at doses achieved in normal human consumption must be activated at concentrations between barely effective doses and doses that produce toxic effects. The widely accepted mechanism that is significantly affected by the relevant doses of caffeine is binding to adenosine receptors and antagonism of the actions of agonists at these receptors. Thus, adenosine receptor antagonism is taken to be the mechanism of action of caffeine (Fredholm 1995; Fredholm et al. 1999). However, it has been also claimed

that the respiratory stimulant effects of xanthines might result from an inhibition of phosphodiesterases (Howell et al. 1997). All adenosine receptors belong to the family of G-protein coupled receptors. About 10 years ago, the first two adenosine receptors, $A_1$ and $A_{2A}$, were identified among putative G-protein coupled receptors, cloned from dog thyroid (Libert et al. 1989, 1991; Maenhaut et al. 1990). Later on, two other receptor types, $A_{2B}$ and $A_3$, were cloned (Stiles 1997). It is widely accepted that both $A_1$ and $A_2$ receptors are involved in modulating spontaneous locomotor activity (Nicodijevic et al. 1991) and the $A_{2A}$ receptor was recently shown to play a major role in the stimulant effects of caffeine (Ledent et al. 1997). The involvement of adenosine in regulating complex central functions, such as aggressiveness (Ledent et al. 1997), pain (Sawynok 1998) and in regulating emotions (Nehlig et al. 1992), has also been widely investigated in the past. However, the role of adenosine in modulating anxiety states is still under debate. The existence of interactions between adenosine and benzodiazepines, the most widely used anxiolytic agents, has been suggested in the 1980s and, for example, a down-regulation of the number of adenosine $A_2$ receptors in rat forebrain was found following chronic treatment with benzodiazepines (Hawkins et al. 1988). A more direct involvement of adenosine in regulating anxiety states has been suggested by the fact that caffeine is known to increase anxiety in humans (Greden 1974; Uhde et al. 1984; Bruce 1990). The administration of high but not low doses of caffeine leads to an increase in measures in anxiety in humans (Boulenger et al. 1987; Stern et al. 1989; Nickell and Uhde 1994–1995; Kaplan et al. 1997). The anxiogenic effects of caffeine are greater in panic disorder patients (Boulenger et al. 1984; Charney et al. 1985; Lee et al. 1988). Furthermore, there are great differences between individuals in what constitutes a high, anxiogenic dose of caffeine (Eaton and McLeod 1984; Griffiths and Woodson 1988; Stern et al. 1989; Evans and Griffiths 1992). Caffeine has been shown to promote anxiogenic-like behaviour in different animal models of anxiety following its acute administration: the social interaction test (Baldwin et al. 1989; Bhattacharya et al. 1997), the elevated plus-maze test (Pellow et al. 1985; Baldwin et al. 1989) and conflicting results have been reported in the light/dark test (Costall et al. 1989; Imaizumi et al. 1994). In addition, CGS 15943, a non-xanthine and non-selective $A_1/A_2$ adenosine antagonist, showed anxiogenic properties in the light/dark test (Griebel et al. 1991). These effects were potentially ascribed to the suppression of a tonic anxiolytic activity of endogenous adenosine at $A_1$ or $A_{2A}$ adenosine receptors. We have previously reported that adenosine $A_{2A}$ receptor-knockout mice scored higher than wild-type animals in anxiety tests (Ledent et al. 1997). This phenotype would fit with the potential development of $A_{2A}$ receptor agonists as anxiolytics (Snyder 1997). However, an anxiolytic-like activity of adenosine agonists has been difficult to prove. Earlier studies showed no significant effects of *R*-*N*$^6$-phenylisopropyladenosine (R-PIA) in the shuttle box test (Katims et al. 1983), nor did R-PIA or 5'-*N*-ethylcarboxamide adenosine (NECA) exhibit anticonflict activity in a punished responding paradigm (Commissaris et al. 1990). Two recent studies have claimed, however, that the selective activation of central $A_1$ adenosine receptors with *N*$^6$-cyclopentyladenosine (CPA) or its close analogue 2-chloro-*N*$^6$-cyclopentyladenosine (CCPA) induces anxiolytic-like behaviour (Jain et al. 1995; Florio et al. 1998). Following chronic exposure, tolerance to the acute behavioural effects of various adenosine antagonists develops. Such tolerance has been demonstrated in a variety of behavioural paradigms, including locomotor activity (Jacobson et al. 1996). Similarly, tolerance to the anxiogenic effects of caffeine has been shown using two animal models of anxiety in rats (File et al. 1988; Bhattacharya et al. 1997).

The separation between therapeutic efficacy and adverse side effects remains a challenge in the discovery and development of novel adenosine-based medicines. The first aim of the present study was to investigate the effect of selective $A_{2A}$ receptor antagonists on mouse anxiety behaviour. The elevated plus-maze and the light/dark tests were chosen because they have been extensively validated pharmacologically and behaviourally (Treit 1994). A second aim of the study was to examine the effects of caffeine following chronic injection or ingestion on the elevated plus-maze test. Finally, we designed experiments in order to investigate whether the anxiety-like effects induced by caffeine in mice is related to the blockade of the $A_{2A}$ adenosine receptor subtype, using wild-type and $A_{2A}$ receptor knockout mice.

## Materials and methods

### Animals

Male Swiss albino CD1 mice (Charles River, Saint Aubin lès Elbeuf, France) or $A_{2A}$ receptor knockout mice and their wild-type controls (Ledent et al., 1997), weighing 20–30 g were used at least after 1 week of habituation in our own facilities. Mice were housed in groups of 15–20 in Makrolon cages (38×24×18 cm) with free access to water and food (UAR, France) and kept in a ventilated room at a temperature of 21±1°C, under a 12-h light/12-h dark cycle (light on between 7 a.m. and 7 p.m.). Experiments were carried out between 9 a.m. and 7 p.m. Dose-responses curves were obtained from groups of 8–40 animals per dose with mice being randomly allocated to different groups before experiments. Mice drinking water or caffeine in their bottles were singly housed during the whole duration of experiments. Otherwise, the animals were isolated in small individual cages (27×13×13 cm) for 30 min prior testing, to facilitate habituation to the experimental environment. Each mouse was only used once for any experiment.

The procedures described comply with ethical principles and guidelines for care and use of laboratory animals adopted by the European Community, law 86/609/CCE.

### Drugs

The following drugs were purchased from RBI: caffeine (1,3,7-trimethylxanthine), CGS 21680 [2-p-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine HCl], DPCPX (8-cyclopentyl-1,3-dipropylxanthine), CPA (*N*$^6$-cyclopentyladenosine). ZM241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol) and SCH58261 (5-amino-7-(β-phenylethyl)-2-(8-furyl)pyrazolo[4,3-e]-1,2,4-triazolo-[1,5-c]pyrimidine) were gener-

ous gifts from Dr. S. Poucher (Zeneca Pharmaceuticals, Macclesfield, UK) and Dr. E. Ongini (Schering-Plough Research Institute, Milan, Italy), respectively. Except for caffeine, which was dissolved in an aqueous solution of sodium benzoate (10 mg/ml), all other compounds were dissolved in dimethyl sulphoxide (Sigma) and then diluted in Cremophor EL (Sigma) and NaCl 0.9% (final concentration: 15% DMSO and 15% Cremophor EL). The drugs were prepared fresh daily and injected IP in a volume of 10 ml/kg with control groups of mice receiving the corresponding vehicle.

In chronic caffeine-treated groups, a caffeine solution (0.3 g/l water) was provided instead of drinking water during the time prescribed by the experiment. The liquid intake was measured daily. Since some fluid may be lost in other ways than ingestion by mice, the fluid intake values are probably overestimates. The caffeine-treated and control groups consisted of ten singly housed animals each. The liquid intakes were (ml/mouse per day): controls=4.1±0.3; caffeine=4.1±0.3 in the 1 day-caffeine experiment; controls=4.7±0.1; caffeine=5.2±0.2 in the 8-day caffeine experiment; controls=5.6±0.1; caffeine=5.9±0.1 in the 60-day caffeine experiment. The body-weight changes in chronically treated mice were significant in the 8-day caffeine IP experiment (i.e. mice administered twice daily for 7 days followed by a last injection on day 8): controls=+1.1±0.4 g; caffeine=–0.2±0.3 g [$F(1,16)=7.04$, $P<0.05$] but not in other chronic treatments: controls=+0.7±0.4 g; SCH58261=+1.3±0.3 g, ZM241385=+0.6±0.4 g in the 8-day $A_{2A}$ receptor antagonists IP experiment (i.e. mice administered twice daily for 7 days followed by a last injection on day 8); or chronic ingestion experiments: controls=+2.5±0.3 g; caffeine=+2.1±0.2 g in the 8-day caffeine PO experiment; controls=+5.6±0.8 g; caffeine=+4.1±1.2 g in the 60-day caffeine PO experiment.

### Elevated plus-maze

The apparatus consisted of a wooden Greek cross, painted black, and placed 60 cm above the floor. The four arms were 18 cm long and 6 cm wide. Two opposite arms were surrounded by walls (6 cm high, closed arms) while the two others were devoid of enclosing walls (open arms). The four arms were connected by a central platform (6×6 cm). The light intensity at the level of the plus-maze was 100 lux. Mice were placed in individual cages and received drug treatments 30 min before the test. At the start of the session, the mouse was placed at the centre of the maze, head facing a closed arm. The number of entries, the time spent and the distance travelled in each arm and in the central area over a 5-min period were recorded by an automated image analysis system (Videotrack 512 system, Viewpoint, Lyon, France).

### Light/dark test

In the black and white compartments test box, a 5×5 cm opening located centrally at floor level, connected the compartments (21×15.5×25 cm high). A box was painted black and covered with a lid. The other box (not covered) was painted white and lit by a 100-W light bulb set 50 cm above the box (200 lux). Mice were placed in individual cages and received drug treatment 30 min before testing. At the start of the session, the mice were placed in the black compartment, head facing a corner. The latency of the first entry into the brightly lit white compartment, the time spent in the lit box, the number of transitions between the two compartments, the attempts at entry into the lit box followed by avoidance responses were noted during the 5-min period of the test. During observation with the aid of a mirror located above the test box, the experimenter always sat at the same place, next to the apparatus.

### Statistical analysis

Results are expressed as means±SEM. Statistics were done using SigmaStat 2.03 software. Data was tested for conformity to normality and homogeneity of variance prior to parametric analysis. Normally distributed and homogeneous data were evaluated by a parametric one-way analysis of variance (ANOVA), and a post hoc multiple comparison Newman-Keuls test was used for multiple comparison between groups in experiments involving one drug. Drug effects in the combination experiment were analysed using a two-way ANOVA. A Kruskal-Wallis one-way ANOVA on ranks combined with a Dunn's multiple comparison test was used to analyse non-parametric data. A probability level of 0.05 or smaller was used to indicate statistical significance.

## Results

### Effects of acute treatments with caffeine, SCH58261 or ZM241385 in the plus-maze test

In the plus-maze test, acute administration of caffeine (12.5–25–50–100 mg/kg IP) significantly decreased [$H(4\ df)=20.20$, $P<0.001$] the time spent by mice in the open arms from the 50 mg/kg dose and the number of entries into the open arms [$F(4,57)=9.63$, $P<0.001$] from the 25 mg/kg dose (Fig. 1, upper and middle left panels). In addition, caffeine induced biphasic changes [$F(4,57)=6.44$, $P<0.001$] in locomotor activity, reflected in the total distance travelled during the test. Indeed, post hoc Newman-Keuls test indicated that mice injected with the 12.5 mg/kg dose of caffeine travelled a significantly ($P<0.05$) larger total distance than the vehicle group and than those receiving the higher doses 25, 50 and 100 mg/kg (Fig. 1, lower left panel). SCH58261 (0.3–1-3–10 mg/kg IP) affected neither the time spent in open arms [$F(4,60)=0.84$, $P>0.05$] nor the number of entries into the open arms [$F(4,60)=0.14$, $P>0.05$]. The total distance travelled during the plus-maze test [$F(4,60)=1.96$, $P>0.05$] was also left unchanged after acute administration of SCH58261 (Fig. 1, central panel). ZM241385 (3.75–7.5–15–30–60 mg/kg IP) was also devoid of any significant effects upon the time spent in open arms of the plus-maze [$F(5,57)=0.94$, $P>0.05$], the number of entries into the open arms [$H(5\ df)=7.56$, $P>0.05$] and upon the total distance travelled [$F(5,57)=2.07$, $P>0.05$] after acute administration (Fig. 1, right panel).

### Effects of acute treatments with caffeine, SCH58261 or ZM241385 in the light/dark test

In the light/dark test, acute administration of caffeine (25–50–100 mg/kg IP) also induced anxiety-like effects (Fig. 2, left panel). Caffeine significantly decreased [$F(3,53)=3.68$, $P<0.05$] the time spent by mice in the lit box and also induced a significant decrease [$F(3,53)=14.25$, $P<0.001$] in the number of transitions between compartments at 50 and 100 mg/kg. The median values among the treatment groups for the latency of the first entry into the lit box were significantly different [$H(3\ df)=8.06$, $P=0.04$] but none of the tested doses differed from the vehicle group. The number of attempts at entry into the lit box followed by avoidance responses was significantly reduced [$F(3,53)=3.46$, $P<0.05$] by caffeine at

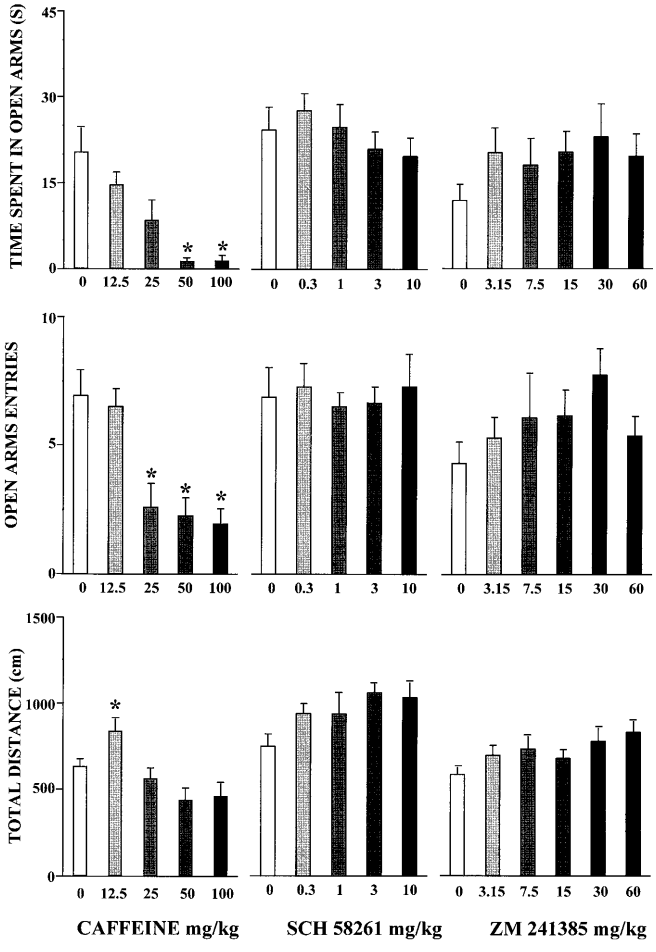


**Fig. 1** Effect of acute caffeine, SCH58261 or ZM241385 treatment on the behaviour of mice on the elevated plus-maze test. Mice were injected IP 30 min before testing with vehicle (*open bars*) or increasing doses of one of the drugs (*shaded bars*). Testing was for 5 min. Means±SEM of data from 13–14 controls and 10–13 mice in treated groups. No symbol $P>0.05$; *$P<0.05$ (one-way ANOVA followed by Newman-Keuls test)

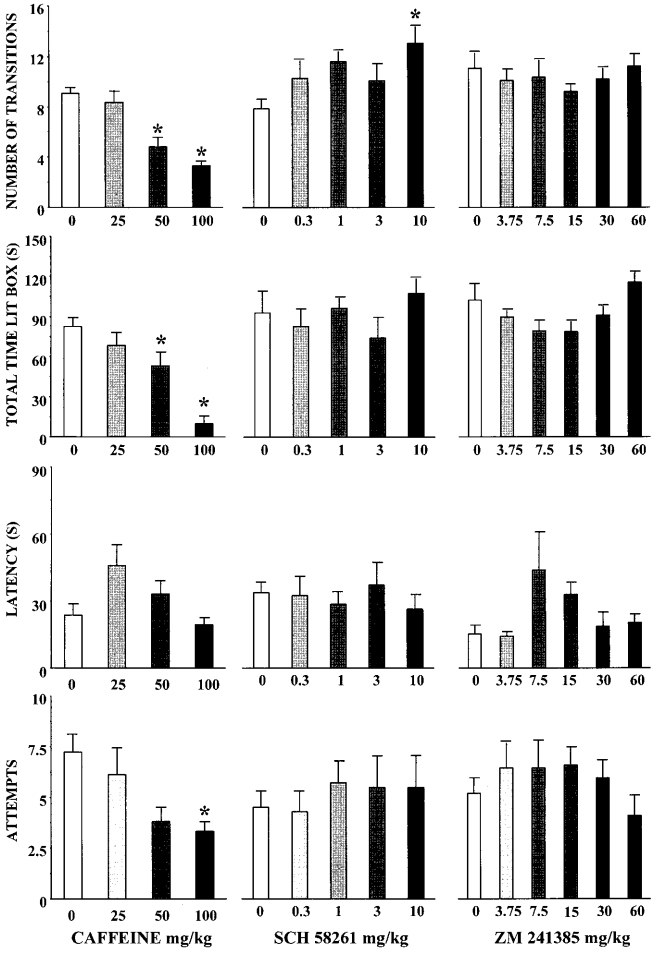


**Fig. 2** Effect of acute caffeine, SCH58261 or ZM241385 treatment on the behaviour of mice in the light/dark test. Mice were injected IP 30 min before testing with vehicle (*open bars*) or increasing doses of one of the drugs (*shaded bars*). Testing was for 5 min. Means±SEM of data from 8–17 controls and 8–16 mice in treated groups. No symbol $P>0.05$; *$P<0.05$ (one-way ANOVA followed by Newman-Keuls test)

100 mg/kg. By contrast, SCH58261 (0.3–1-3–10 mg/kg IP) only marginally modified anxiety-related measures in the light/dark test when tested up to the 10 mg/kg dose (Fig. 2, middle panel). The number of transitions between the two compartments was increased by SCH58261 [$F(4,40)=2.72$, $P<0.05$] at 10 mg/kg, but remained unchanged in all other groups. The only parameter used in the light/dark test significantly [$H(5\ df)=12.63$, $P=0.03$] influenced by an acute treatment with increasing doses of ZM241385 (3.75–7.5–15–30–60 mg/kg IP) was the latency of the first entry into the lit box. However, none of the tested doses differed from the vehicle group for this parameter (Fig. 2, right panel).

## Effects of chronic treatments with caffeine, SCH58261 or ZM241385 in the plus-maze test

When it was given to mice in the drinking water (0.3 g/l) for 24 h (from 2.00 p.m. to 2.00 p.m.), caffeine failed significantly to modify the time spent in open arms: water: 26.1±3.8 s; caffeine: 26±5.7 s [$F(1,17)=0$, $P>0.05$], the number of entries into open arms: water: 6.7±0.9; caffeine: 6±1.3 [$F(1,17)=0.18$, $P>0.05$] and the total distance travelled: water: 969±79 cm; caffeine: 758±90 cm [$F(1,17)=3.15$, $P>0.05$]. However, caffeine induced anxiety-like effects following chronic administration at 50 mg/kg IP for 8 days or after chronic ingestion (0.3 g/l) for 8 days or 60 days. Furthermore, at these chronic regimens, caffeine decreased the distance travelled by mice upon the maze during the test (Fig. 3). SCH58261 (3 mg/kg IP) and ZM241385 (30 mg/kg IP) were also administered chronically to mice twice daily for 7 days followed by a last injection on day 8, at which a plus-maze experiment was performed. Neither the time spent in open arms: vehicle=28.5±2.6 s, SCH58261= 30.1±3.6 s, ZM241385=32.8±3.6 s [$F(2,27)=0.38$, $P>0.05$], nor the number of entries into open arms: vehicle=6.6±1.1, SCH58261=8.0±1.3, ZM241385=8.0±1.0 [$F(2,27)=0.17$, $P>0.05$] were significantly modified by

**Table 1** Effects of different acute treatments with CGS 21680, CPA, DPCPX and the association DPCPX+SCH58261 on the behaviour of mice on the elevated plus-maze. Testing was for 5 min. Mice were injected IP 30 min before testing with vehicle or increasing doses of drugs. Means±SEM of data from ten to 18 mice per group

| Drugs | Doses (mg/kg) | Time spent in open arms (s) | Number of entries into open arms | Total distance (cm) |
|---|---|---|---|---|
| CGS 21680 | 0 | 12.8±3.1 | 3.9±0.7 | 543.6±34.6 |
| $A_{2A}$ agonist | 0.02 | 7.3±2.0 | 2.6±0.6 | 575.5±39.5 |
| | 0.1 | 6.3±2.5 | 2.3±0.7 | 448.5±52.8 |
| | 0.5 | 6.3±2.2 | 1.8±0.7 | 188.5±24.2* |
| | 2.5 | 0.7±0.6* | 0.1±0.1 * | 125.4±28.0* |
| CPA | 0 | 21.5±4.7 | 5.8±0.9 | 647.9±55.9 |
| $A_1$ agonist | 0.01 | 28.1±6.4 | 6.9±1.4 | 604.3±42.0 |
| | 0.03 | 24.7±3.7 | 6.8±0.9 | 652.3±35.7 |
| | 0.1 | 17.6±7.1 | 4.8±1.1 | 552.0±46.6 |
| | 0.3 | 10.6±3.1 | 3.9±0.8 | 400.6±67.2* |
| | 1 | 12.3±5.5 | 3±1 | 309.0±79.3* |
| DPCPX | 0 | 18.7±3.3 | 6±1 | 626.6±39.8 |
| $A_1$ antagonist | 0.2 | 14.9±3.8 | 4.4±0.8 | 632.0±55.6 |
| | 1 | 19.5±3.7 | 6.2±1.1 | 679.6±57.7 |
| | 5 | 19.4±3.9 | 5±1.3 | 676.5±58.7 |
| DPCPX–SCH58261 | 0–0 | 18.9±3.9 | 4.5±0.7 | 499.5±50.7 |
| $A_1$ antagonist–$A_{2A}$ antagonist | 0.2–0 | 15.2±4.8 | 4.3±0.9 | 505.5±70.8 |
| | 1–0 | 20.6±5.1 | 4.8±1 | 529.3±65.2 |
| | 0–1 | 28.1±8.2 | 6±1.4 | 606.4±46.5 |
| | 0.2–1 | 22.5±4.1 | 6.2±1.1 | 577.6±92.0 |
| | 1–1 | 23.9±6.0 | 6±1.3 | 608.4±103.9 |
| | 0–3 | 13.2±4.7 | 4.3±1.6 | 630.1±76.0 |
| | 0.2–3 | 26.8±5.9 | 7.1±1.4 | 681.2±116.8 |
| | 1–3 | 29.6±4.9 | 6.5±1.1 | 660.0±41.2 |

No symbol $P>0.05$; *$P<0.05$ (parametric one-way ANOVA or Kruskal-Wallis one-way ANOVA on ranks for experiments with only one drug and a two-way ANOVA for the association experiment)

the chronic treatment with adenosine $A_{2A}$ receptor antagonists This chronic treatment with adenosine $A_{2A}$ receptor antagonists did not either induce significant changes in the total distance travelled during the test: vehicle=956.6±93.4 cm, SCH58261=1179.4±88.7 cm, ZM241385=1245.8±88.1 cm [$F(2,27)=2.83$, $P>0.05$].

### Effects of acute treatment with an adenosine $A_1$ agonist or an adenosine $A_{2A}$ agonist in the plus-maze test

In our experimental conditions and after acute administration, the selective $A_1$ agonist CPA (0.01–0.03–0.1–0.3–1 mg/kg IP) failed to induce significant changes in the anxiety-related indices in the plus-maze test: time spent in open arms [$H(5\ df)=9.79$, $P>0.05$] or number of entries into open arms [$F(5,69)=2.07$, $P>0.05$], but decreased in a significant manner [$F(5,69)=6.13$, $P<0.001$] the index of locomotor activity (Table 1).

The selective $A_{2A}$ agonist CGS 21680 (0.02–0.1–0.5–2.5 mg/kg IP), following acute administration, reduced the time spent in the open arms [$H(4\ df)=13.12$, $P=0.01$] of the plus-maze, an effect which was marked at the highest tested dose (2.5 mg/kg). CGS 21680 also reduced the number of entries into open arms [$H(4\ df)=17.37$, $P<0.01$]. These effects appeared to be non-specific as indicated by the highly significant decrease [$F(4,65)=30.68$, $P<0.001$] in total distance travelled measured for the same animals (Table 1).

### Effects of acute treatments with an adenosine $A_1$ antagonist or a combination of $A_1$ and $A_{2A}$ antagonists in the plus-maze test

The acute IP administration of the selective $A_1$ antagonist DPCPX (0.2–1-5 mg/kg IP) had no significant effects on either the time spent in open arms [$F(3,40)=0.35$, $P>0.05$] or the number of entries into open arms [$H(3\ df)=1.5$, $P>0.05$]. The total distance travelled in the plus-maze apparatus also remained unchanged [$H(3\ df)=1.15$, $P>0.05$] at the tested doses of DPCPX (Table 1).

Combinations of adenosine antagonists, DPCPX (0.2–1 mg/kg IP, selective $A_1$ antagonist) and SCH58261 (1–3 mg/kg IP, selective $A_{2A}$ antagonist), were acutely administered in order to explore possible synergistic effects between $A_1$ and $A_{2A}$ receptor blockade upon the behaviour of mice in the plus-maze test. Two-way ANOVA for all groups of mice showed no statistically significant interaction between the effects of DPCPX and SCH58261 upon the time spent in open arms [$F(4,66)=1.24$, $P>0.05$], the number of entries into open arms [$F(4,66)=0.48$, $P>0.05$] and the total distance travelled [$F(4,66)=0.07$, $P>0.05$] in the plus-maze test. DPCPX [$F(2,66)=0.6$, $P>0.05$; $F(2,66)=0.56$, $P>0.05$] and SCH58261 [$F(2,66)=1.17$, $P>0.05$; $F(2,66)=1.5$, $P>0.05$] induced no significant effects upon either the time spent in or the number of entries into open arms of the plus-maze. Both drugs were also without motor effects as evaluated by the total distance travelled in this experiment: DPCPX [$F(2,66)=0.05$, $P>0.05$] and SCH58261 [$F(2,66)=2.8$, $P>0.05$] (Table 1).

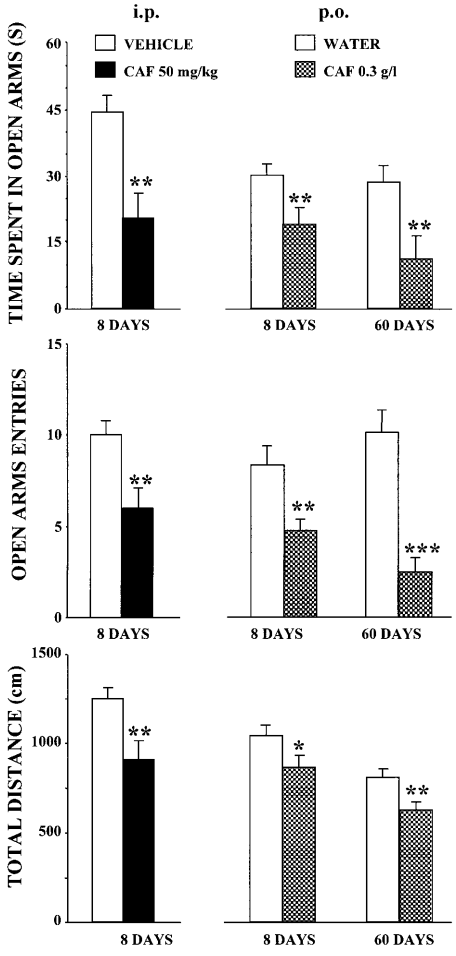


**Fig. 3** Effect of 8 or 60 days chronic caffeine treatment on the behaviour of mice on the elevated plus-maze. Testing was for 5 min. *Left panel*: mice were injected IP twice daily for 7 days and 30 min before testing with either vehicle (*open bars*) or caffeine 50 mg/kg (*black bars*). Means±SEM of data from ten controls and eight caffeine-treated mice. No symbol $P>0.05$; $^{**}P<0.01$ (ANOVA or Kruskal-Wallis). *Right panel*: mice received water (*open bars*) or caffeine solution (0.3 g/l as drinking water) for 7 or 60 days (*hatched bars*) until testing. Means±SEM of data from ten mice per groups. No symbol $P>0.05$; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (ANOVA or Kruskal-Wallis)

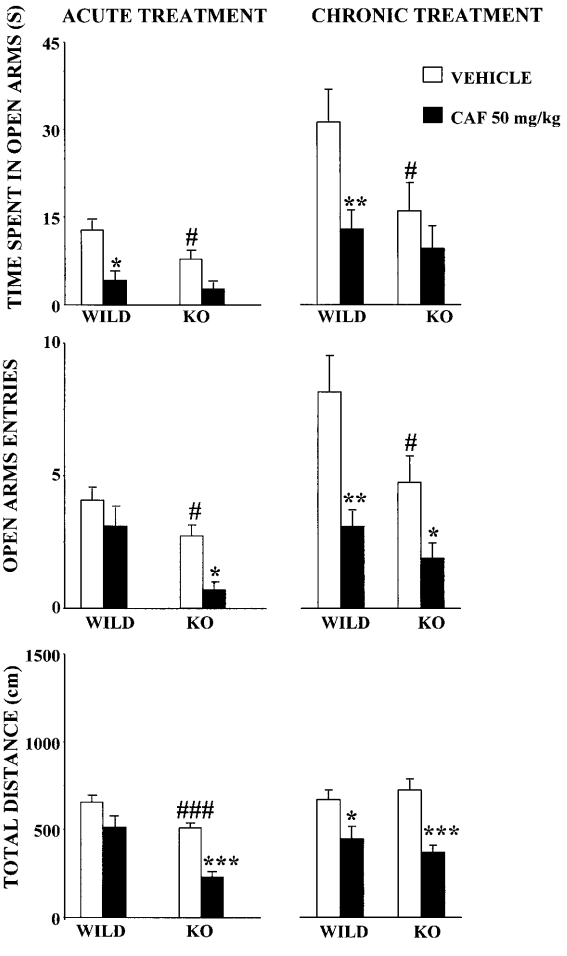


**Fig. 4** Effect of acute or chronic caffeine treatment on the behaviour of wild-type and $A_{2A}$ receptor knockout mice on the elevated plus-maze test. Testing was for 5 min. *Left panel*: mice were acutely injected IP 30 min before testing with vehicle (*open bars*) or caffeine 50 mg/kg (*hatched bars*). Means±SEM of data from 40 controls and ten caffeine-treated mice in each group. *Right panel*: mice were chronically injected IP twice daily for 7 days and 30 min before testing with vehicle (*open bars*) or caffeine 50 mg/kg (*black bars*). Means±SEM of data from nine or ten mice per group. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ as compared to respective vehicle groups; $\#P<0.05$; $\#\#\#P<0.001$ when comparing scores of knockout with wild-type mice in vehicle groups (ANOVA or Kruskal-Wallis)

## Effects of acute or chronic caffeine treatments in wild-type and $A_{2A}$ receptor knockout mice on the plus-maze test

In previous behavioural studies, $A_{2A}$ receptor knockout mice appeared to be more anxious than wild-type control mice (Ledent et al. 1997). In the present study, vehicle-treated $A_{2A}$ receptor knockout mice displayed mild but significant decreases in both the time spent in and the number of entries into open arms of the plus-maze in two different experimental conditions: i) one in which group-housed mice were removed from their cage, acutely injected (time: [$H(1\ df)=5.28$, $P<0.05$]; number: [$H(1\ df)=5.53$, $P<0.05$]) and isolated 30 min before the test; ii) a second in which mice were singly-housed, manipulated and chronically injected [time: $F(1,16)=4.44$, $P<0.05$; number: $F(1,16)=4.3$, $P<0.05$] during the 8 day-experiment. In the acute study but not in the chronic one [$F(1,16)=0.52$, $P>0.05$], vehicle-treated $A_{2A}$ receptor knockout mice travelled a smaller distance in the maze [$F(1,79)=11.4$, $P<0.01$] as compared to wild-type animals (Fig. 4).

The acute IP administration of caffeine at 50 mg/kg induced a significant decrease in the time spent in the open arms by wild-type mice [$F(1,48)=5.84$, $P<0.05$] but not by $A_{2A}$ receptor knockout mice [$H(1\ df)=2.86$, $P>0.05$]. However, the number of entries into open arms was not significantly reduced by acute caffeine in wild-type mice [$F(1,48)=1.07$, $P>0.05$], whereas this parameter was significantly modified in $A_{2A}$ receptor knockout mice [$H(1\ df)=5.04$, $P<0.05$]. In addition, there was a significant re-

duction of the total distance travelled during the experiment in the $A_{2A}$ receptor knockout caffeine-treated group [$H$(1 $df$)=17.9, $P$<0.001] but not in the wild-type caffeine-treated group [$F$(1,48)=3.21, $P$>0.05] (Fig. 4).

Finally, chronic IP administration of 50 mg/kg caffeine (twice daily for one week followed by a last injection 30 min before the experiment on day 8) affected in a dissimilar way the behaviour of wild-type and $A_{2A}$ receptor knockout mice in the plus-maze test. When compared to their respective vehicle-injected groups, caffeine induced a milder, non-significant [$F$(1,17)=1.10, $P$>0.05] decrease in the time spent in open arms in knockouts, whereas a significant [$F$(1,16)=8.12, $P$<0.01] anxiogenic-like effect was still observed in wild-type mice. However, the number of entries into open arms was significantly reduced by chronic caffeine in both wild-type mice [$H$(1 $df$)=9.53, $P$<0.01] and $A_{2A}$ receptor knockout mice [$F$(1,17)=7.37, $P$<0.05]. The locomotor activity was significantly decreased in both wild-type [$F$(1,16)=6.53, $P$<0.05] and $A_{2A}$ receptor knockout mice [$F$(1,17)=26.77, $P$<0.001], though more obviously in the latter group (Fig. 4).

## Discussion

Caffeine is a non-selective $A_1$ and $A_{2A}$ antagonist (Jacobson and van Rhee 1997). In vitro receptor binding assays performed with mouse brain tissues (Personal communication from Professor P. A. Borea and Dr. E. Ongini) have shown that its $K_i$ for the $A_{2A}$ receptor ($K_i$=20.3±1.1 µM) is slightly lower than its $K_i$ for the $A_1$ receptor ($K_i$=30.0± 2.9 µM). Following acute administration, caffeine is well known as an anxiogenic agent in rats in the social interaction test and the elevated plus-maze test (Baldwin et al. 1989; Bhattacharya et al. 1997). In the current study, we confirmed that caffeine is also an anxiogenic agent in CD1 mice after administration at high doses in two well validated models for measuring anxiety states (Pellow et al. 1985; Lister 1987; Blumstein and Crawley 1983), the elevated plus-maze and the light/dark tests.

In the elevated plus-maze, a test based on the natural aversion of rodents for open spaces (Lister 1987), caffeine decreased the time spent in the open arms, causing a significant preference for protected sections of the maze. The total distance travelled was not significantly affected when caffeine was acutely administered, a result that is believed to indicate a lack of marked sedative or motor depressant activity. Anxiogenic-like effects could not be demonstrated when caffeine was given in the drinking water (0.3 g/l) for 1 day in isolated mice. At the time at which the test occurred (1400–1500 hours), the brain concentrations of caffeine and/or metabolites had probably returned to a low level. This might have led to a degree of occupancy of adenosine receptors insufficient to induce behavioural effects. In fact, Johansson et al. (1996) using a similar protocol showed that plasma concentrations of methylxanthines are maximal at the end of the night period, when mice have been active, and very low during the afternoon.

The light/dark test is based on the conflict between the inherent tendency of mice to explore a novel environment against their natural avoidance of a brightly lighted open field (Blumstein and Crawley 1983). We found that acutely injected caffeine significantly increased the latency for entering the lit box at a medium dose and decreased the total time spent by mice in the lit box as well as the number of transitions at high doses. Broadly, similar effects of caffeine have been found by Imaizumi et al. (1994) with this procedure. These results are also in accordance with the finding that, on the contrary, anxiolytic agents such as benzodiazepines increase the number of transitions in the light/dark test (Blumstein and Crawley 1983).

The parameter “attempts at entry into the lit box followed by avoidance responses”, which includes stretch attend posture, is referred as a parameter measuring “risk assessment”. This latter concept refers to a pattern of responses invariably observed in potentially dangerous situations. Previous studies with the light/dark test demonstrated that the administration of benzodiazepines decreased aborted attempts at entry in the aversive area (Griebel et al. 1998). Regarding this ethologically derived measure, an increase in the number of aborted attempts was expected to occur in caffeine-treated mice. Unexpectedly, the drug influenced this parameter in the same way as anxiolytics. However, this effect might be non-specific, since high doses of caffeine are known to decrease locomotor activity in an actimeter (Svenningsson et al. 1995; El Yacoubi et al. 1998). Nevertheless, whether these aborted attempts may be viewed as a reliable index to measure increased anxiety remains to be demonstrated.

Long-term treatment with caffeine is known to lead to several types of adaptations (Jacobson et al. 1996). A significant degree of tolerance to the anxiogenic effects of caffeine in rats has also been reported in the social interaction test (File et al. 1988; Bhattacharya et al. 1997) or in the elevated plus-maze test (Bhattacharya et al. 1997). In contrast, in the present study, repeated injection of caffeine was found to cause a significant reduction in time spent by CD1 strain male mice in the open arms of the plus-maze. The only difference between our acute and chronic (8 days) protocols using IP injections of vehicle or caffeine is the period over which the animals are handled and injected with vehicle twice daily. This chronic handling and injection procedure is likely to be responsible for the 120% increase in time spent in open arms by chronically vehicle-injected mice as compared to naive mice in the acute experiment. In keeping with the present data, a chronic handling and injection protocol has been previously shown to reduce state anxiety in rats submitted to the elevated plus-maze (Brett and Pratt 1990). When further comparing the acute and chronic IP conditions, the decrease in time spent in and the number of entries into open arms of the plus-maze were still obvious in the chronic IP caffeine-treated (respectively 54% and 40%), although less pronounced than for acute (respectively 78% and 67%) IP caffeine-treated mice. Acutely caffeine-injected mice and chronically caffeine-injected mice exhibited the same lower (27% re-

duction) locomotor activity compared to controls, thereby introducing the prospect that the anxiogenic effects of caffeine might be less robust after chronic treatment, the bias putatively induced by the motor activity component being equal.

Similarly, in the present study, chronic ingestion of caffeine was also found to cause a significant reduction in the percentage of time spent in open arms in CD1 strain male mice. As already discussed, the "acute" 1-day ingestion of caffeine by isolated mice did not affect anxiety. The decrease in time spent in open arms and number of entries into open arms became significant after one-week caffeine ingestion (respectively 36% and 43% reduction at 8 days), and more obvious when the duration of treatment was lengthened to a period of 2 months (respectively 60% and 75% reduction at 60 days). The change in locomotor activity (about 20% reduction) observed in mice that received caffeine in their drinking water was very mild. So, it is unlikely that decreased locomotor activity may have led to a spurious increase in the anxiety index, although this possibility cannot be excluded completely. It is unknown whether this significant anxiogenic-like effect is entirely a species or strain-specific phenomenon or whether it is also linked to caffeine dosage levels attained or methodological aspects of the study. Interestingly, as pointed out by Jacobson et al. (1996), the patterns of tolerance to stimulant locomotor effects of caffeine differ in rats and CD1 mice. Indeed, chronic caffeine ingestion results even in a behavioural depression of activity in mice as verified again in this study. Interspecies variations in caffeine metabolism have been reported (Berthou et al. 1992) and one may speculate that they could contribute to this difference between species. Although caffeine is typically consumed on a daily basis, to our knowledge, only few reports mention that chronic administration of caffeine might result in tolerance to its anxiogenic effects in humans (Evans and Griffiths 1992).

CGS 21680 is an agonist with a high affinity for the $A_{2A}$ adenosine receptor and with a high selectivity versus $A_1$ receptors (Jacobson et van Rhee 1997). This compound appeared unhelpful in studying the function of $A_{2A}$ receptors in the plus-maze test since both anxiety-related and locomotor activity-related indices were greatly affected by high doses of the drug. These non-specific effects might be related to the potent hypotension induced by this drug (Casati et al. 1994; Ledent et al. 1997).

SCH58261 is a high affinity antagonist for the $A_{2A}$ adenosine receptor with a selectivity versus $A_1$ receptors of almost 500-fold in rat brain (Ongini 1997) and about 300-fold in mouse brain (personal communication from Professor P. A. Borea and Dr. E. Ongini). ZM241385 is a high affinity $A_{2A}$ receptor antagonist with a even higher selectivity: 400- to 1000-fold selective for $A_{2A}$ versus $A_1$ receptors in rat tissues (Poucher et al. 1995). However, unlike SCH58261 which is devoid of affinity for $A_{2B}$ receptors, ZM24385 was reported to be only 30- to 80-fold selective for $A_{2A}$ versus $A_{2B}$ receptor (Feoktistov and Biaggioni 1997; Ongini et al. 1999).

In the elevated plus-maze and light/dark tests in mice, the acute injection of SCH58261 or ZM241385 produced non significant effects on anxiety parameters over a wide range of doses. The $A_{2A}$ selective antagonist SCH58261 has previously been shown to display clear behavioural effects (Bertorelli et al. 1996; El Yacoubi 1998) when systemically administered in this dose-range. Furthermore, new data from our laboratory show that SCH58261 penetrates rapidly into the mouse brain and occupies a high percentage of cerebral $A_{2A}$ receptors after acute systemic administration, as demonstrated by ex vivo binding experiments using [$^3$H] SCH58261 (El Yacoubi et al., in preparation). Few data are available concerning the putative effects of the ZM241385 in the central nervous system. However, it was shown to induce weak motor stimulant effects (El Yacoubi et al. 1998) and also a neuroprotective effect in rat after systemic administration (Jones et al. 1998) suggesting that it crosses the blood-brain barrier. Following chronic systemic administration of these two antagonists, no effects on anxiety in the plus-maze test were revealed. One cannot exclude the possibility that the twice-daily administration protocol used in the present experiment might have resulted in inadequate or irregular blockade of $A_{2A}$ receptors. However, this seems unlikely, at least as far as SCH58261 is concerned. Indeed, plasma levels of SCH58261 corresponding to a fifth of the peak levels were still observed 24 h after a single administration of a low dose (1 mg/kg IP) in the rat (Ongini 1997).

Since $A_{2A}$ antagonists were devoid of anxiogenic-like effects, we were interested in further assessing the potential role of $A_1$ receptors in one of the two procedures used in the present study, namely the elevated plus-maze test.

CPA is an agonist with a high affinity for the $A_1$ subtype of adenosine receptor and with a high selectivity versus $A_{2A}$ receptors (Jacobson et van Rhee 1997). In the present study, though tested over a wide dose range (from 0.01 to 1 mg/kg), CPA was devoid of significant anxiolytic-like activity in the plus maze test, but depressed locomotor activity at high doses. This profile contrasts partially with that observed by Jain et al. (1995) in the elevated plus-maze, although administration route and time elapsed between treatment and testing were similar in both studies. Both studies show that CPA had a general depressant action at high doses and this can be related to the motor depressant effect observed after administration of selective $A_1$ adenosine agonists (Nikodijevic et al. 1991; El Yacoubi et al. 1998; Marston et al. 1998). However, a marked anxiolytic-like profile of CPA, namely a specific increase in open arm exploration at 0.01 and 0.05 mg/kg, was found by Jain et al. (1995) but not in the present study. This discrepancy could be attributed to differences in mouse strains (male ICR or MF1 mice were used by Jain et al. whereas male CD1 mice were used in the present study). However, the locomotor depressant effects produced at similar doses (0.25 or 0.3 mg/kg) in the two studies suggest another explanation. It is worth mentioning that in the Jain study, control mice spent about 15–25% of the total time in the open arms, whereas in the present situation baseline levels

barely reached 4–10%. This may indicate that basal levels of stress or anxiety in control subjects were somewhat higher in the present study, suggesting an efficacy of $A_1$ agonists to counteract mild but not severe state anxiety. This finding calls for careful attention upon experimental stress and procedures when such studies are carried out.

DPCPX is an antagonist with a high selectivity for the $A_1$ adenosine receptor versus the $A_{2A}$ receptor (Jacobson et van Rhee 1997). In vitro receptor binding assays performed with mouse brain tissues (personal communication from Professor P. A. Borea and Dr. E. Ongini) have shown that its $K_i$ for the $A_1$ receptor ($K_i$=2.8±0.2 nM) is more than 200-fold lower than its $K_i$ for the $A_{2A}$ receptor ($K_i$=613.0±24 nM). DPCPX has been demonstrated to cross effectively the blood-brain barrier (Baumgold et al. 1992; Kaplan et al. 1992). DPCPX had no effect by itself on behaviour in the plus-maze test. The lack of effect upon anxiety-related indices obtained with DPCPX in this study is in agreement with that obtained by Jain et al. (1995) with low doses (0.05–0.5 mg/kg) and in the same test, or by Griebel et al. (1991) using 0.5–2 mg/kg doses in the light/dark test. At a far higher dose (50 mg/kg), DPCPX was shown to induce decreases in all parameters studied (locomotion, rearing, time spent in the lit box) in the light/dark test in (Imaizumi et al. 1994). Given the fact that doses of DPCPX below 1 mg/kg are sufficient to fully reverse the locomotor depression induced by CPA (Marston et al. 1998), it seems likely that the behavioural effects observed in the Imaizumi study would reflect non-specific or toxic effects of the compound, unrelated to adenosinergic mechanisms. At pharmacologically relevant doses, evidence is growing that adenosine $A_1$ receptor antagonists, such as DPCPX, do not influence locomotor activity in mice (Griebel et al. 1991; Nicodijevic et al. 1991; El Yacoubi et al. 1998; Marston et al. 1998). The present data are also in line with these findings.

Overall, the present results suggest that there is no tonic activity of endogenous adenosine at $A_{2A}$ and $A_1$ adenosine receptors located in brain areas putatively involved in anxiety or that blocking this tonic activity has no effect. This is important because it raises the problem of explaining the anxiogenic-like profile of caffeine, both acutely or chronically administered. Concerning acute studies, Jain et al. (1995) put forward the hypothesis that the anxiogenic-like properties of caffeine could be due to the simultaneous blockade of $A_1$ and $A_{2A}$ receptors, since caffeine has virtually no ability to distinguish between these receptors in the central nervous system. It is unclear whether their own finding, a lack of anxiogenic profile of the xanthine DPMX (3,7-dimethyl-1-propargylxanthine), a drug 3-fold selective for the $A_{2A}$ receptor ($K_i$=16 000 nM) versus $A_1$ (Jacobson and van Rhee 1997), might be helpful in supporting the view.

The results obtained in this study provide further clues to answer this question. The acute administration of the $A_{2A}$ receptor antagonist, SCH58261 together with the $A_1$ receptor antagonist DPCPX was devoid of anxiogenic-like activity in the plus-maze test. This may suggest that the combined blockade of $A_1$ and $A_{2A}$ receptor cannot fully explain the anxiogenic effect observed following acute administration of caffeine. Although unlikely, the possibility remains, as suggested by Jain et al. (1995), that an undiscovered $A_1$ receptor subtype, different from that blocked by DPCPX, is another biological target of caffeine. This receptor would conceivably be the target of extremely (below 1 µg/kg) low doses of the agonist CCPA (2-chloro-*N*$^6$-cyclopentyladenosine) and would be blocked by a very low dose (25 µg/kg) of CPT (8-cyclopentyltheophylline), two drugs which displayed, respectively, anxiolytic- and anxiogenic-like activities in one study (Florio et al. 1998). The acute experiment performed with caffeine in wild-type and $A_{2A}$ receptor knockout mice showed that the decrease in time spent in open arms of the plus-maze was similar in the acute IP caffeine-treated wild-type (67%) and $A_{2A}$ knockout (65%) mice, as compared to their respective controls, although the drop did not reach a significant level in knockouts, possibly due to the existence of a "floor" effect. In addition, reductions in the number of entries into open arms (74%) and in the total distance travelled (55%) during the experiment were evidenced only in caffeine-treated $A_{2A}$ receptor knockout mice. These findings suggest that the blockade of $A_{2A}$ receptor may not be important in mediating the acute anxiogenic-like effects of caffeine. Further evidence is provided that caffeine becomes a depressant of locomotor activity in $A_{2A}$ receptor knockout mice (Ledent et al. 1997). Consequently, the effects upon the anxiety-related indices might have been partially confounded by the occurrence of this behavioural suppression, which could mask partially a loss of anxiogenic-like effects of caffeine in knockouts. However, in line with our suggestion, it should be remembered that acute caffeine's anxiogenic effects in the social interaction test were suggested to be mediated via an effect on noradrenergic systems in an earlier study (Baldwin and File 1989). Rolipram, a selective inhibitor of phosphodiesterase, has been shown to enhance central noradrenergic transmission in rodents (Kehr et al 1985) and to elicit anxiety-like effects in dogs (Heaslip and Evans 1995). Theoretically, an anxiety response associated with increased adrenergic tonus following administration of caffeine might result either from $A_1$ receptor blockade or phosphodiesterase inhibition. In animals, locomotor depressant effects (Snyder et al. 1981; Wachtel 1982; Choi et al. 1988; Howell et al. 1997) and hypothermia (Wachtel 1982; Durcan and Morgan 1991) have been observed following administration of selective phosphodiesterase inhibitors or of high doses of methylxanthines. Thus, an inhibitory effect of caffeine upon brain phosphodiesterase might not be completely ruled out to explain its anxiogenic effect, since caffeine already interacts with the $A_{2A}$ receptor at the very low dose of 1 mg/kg (El Yacoubi et al., in preparation).

The chronic administration or ingestion of caffeine also affected anxiety of mice in the present study. Two lines of evidence suggested that the blockade of $A_{2A}$ receptors might play a role in these effects. First, $A_{2A}$ receptor knockout mice displayed a mild increased anxiety as compared to wild-type controls in the plus-maze test (Ledent et al. 1997; this study). Second, the chronic injec-

tion of caffeine influenced in a different way the behaviour of wild-type and $A_{2A}$ receptor knockout mice in the plus-maze test. Whereas a significant anxiogenic-like effect was still observed in wild-type mice, the decrease in time spent in open arms was milder in knockout animals. This is consonant with the hypothesis that the $A_{2A}$ receptor may play a function in the anxiety state observed after chronic ingestion or treatment with caffeine in the mouse. Should have the depressant motor effects induced a bias in this study, these would have falsely accentuated the increase in anxiety index to a greater extent in the knockouts compared to the wild-type mice, as seen with the parameter number of entries into the open arms. It is worth mentioning that a recent association study of the $A_1$ and $A_{2A}$ adenosine receptor genes in panic disorder supports the hypothesis that the $A_{2A}$ receptor gene, or a locus in linkage disequilibrium with it, confers susceptibility to panic disorder (Deckert et al. 1998). Taken together, these latter findings suggest that additional studies should be carried out using new $A_{2A}$ antagonists, having an appropriate water solubility in order to perform long-lasting treatments per oral route or via the drinking water. Hopefully, such compounds would undoubtedly help to answer fully the question of the significance of the $A_{2A}$ receptor in the mechanism of the anxiogenic-like effects of caffeine.

In summary, the results of the present experiments indicate that the non-xanthine $A_{2A}$ adenosine receptor antagonists SCH58261 and ZM241385 are devoid of activity in the plus-maze and light/dark tests in mice at behaviourally active doses. By contrast, caffeine increases anxiety-related responses in both tests, both acutely and after chronic treatment. It is suggested that acute effects of this widely consumed stimulant may not be solely due to an impact upon the $A_{2A}$ receptor, but that this receptor may play a role in mediating chronic effects of the drug upon anxiety. As $A_{2A}$ antagonists have a potential for treatment of brain damage produced by Parkinson's disease or stroke (Ongini and Fredholm 1996), it will be important to confirm that their long-term administration would not induce anxiogenic side effects.

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