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Lack of tolerance to motor stimulant effects of a selective adenosine A_{2A} receptor antagonist

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Abstract

It is well known that tolerance develops to the actions of caffeine, which acts as an antagonist on adenosine A_1 and A_{2A} receptors. Since selective adenosine A_{2A} antagonists have been proposed as adjuncts to 3,4-dihydroxyphenylalanine (L-DOPA) therapy in Parkinson's disease we wanted to examine if tolerance also develops to the selective A_{2A} receptor antagonist 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo [1,5-*c*]pyrimidine (SCH 58261). SCH 58261 (0.1 and 7.5 mg/kg) increased basal locomotion and the motor stimulation afforded by apomorphine. Neither effect was subject to tolerance following long-term treatment with the same doses given intraperitoneally twice daily. There were no adaptive changes in A_1 and A_{2A} adenosine receptors or their corresponding messenger RNA or in dopamine D_1 or D_2 receptors. These results demonstrate that the tolerance that develops to caffeine is not secondary to its inhibition of adenosine A_{2A} receptors. The results also offer hope that long-term treatment with an adenosine A_{2A} receptor antagonist may be possible in man.

Keywords: Adenosine receptor; Locomotion; Dopamine receptor; Caffeine; In situ hybridization; Autoradiography

1. Introduction

In one model of Parkinson's disease, the model of rotation in animals with unilateral lesions of the nigrostriatal dopamine pathway, the classical adenosine receptor antagonists theophylline and caffeine enhance the effect of 3,4-dihydroxyphenylalanine (L-DOPA) (Fuxe and Ungerstedt, 1974). This enhancement was originally attributed to phosphodiesterase inhibition, but this was later doubted (Fredholm et al., 1976), and inhibition of the actions of endogenous adenosine was instead proposed as the likely mechanism (Fredholm, 1979). More recently, antagonism of adenosine A_{2A} receptors has been directly implicated as the cause of the potentiated turning behavior by xanthines (Pinna et al., 1996; Fenu et al., 1997).

Adenosine A_{2A} receptors are abundantly expressed in striatum of rat, mouse, monkey and man (Ongini and Fredholm, 1996; Svenningsson et al., 1999a). They are co-localized with dopamine D_2 receptors in medium-sized striatopallidal spiny output neurons throughout striatum, and it is known that the two receptors have mutually antagonistic effects (Ferré et al., 1992, 1997; Svenningsson et al., 1999a). Thus, adenosine A_{2A} receptor antagonists mimic several of the effects of D_2 receptor agonists both behaviorally and neurochemically (Svenningsson et al., 1999a).

It has therefore been suggested that adenosine A_{2A} receptor antagonists may be useful in the treatment of Parkinson's disease (Ferré et al., 1992; Mally and Stone, 1996; Ongini and Fredholm, 1996; Richardson et al., 1997). Early tests of the concept of adenosine antagonists as treatment of Parkinson's disease using xanthine derivatives failed (Shoulson and Chase, 1975), but later tests with lower doses of these drugs did show some improvement in Parkinsonian patients (Mally and Stone, 1994). A recent epidemiological study revealed a robust negative association between risk of Parkinson's disease and caf-

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feine intake (Ross et al., 2000). In a monkey model, a selective adenosine A_{2A} receptor antagonist could dose-dependently reverse 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced motor disability (Kanda et al., 1998).

If therapy with adenosine A_{2A} receptor antagonists is going to be successful the effect must be maintained during long-term treatment. It is well known that several of the effects of the non-selective adenosine receptor antagonist caffeine exhibit tolerance that develops over the course of a few days of administration in both animals and man (Fredholm et al., 1999). The mechanisms underlying this tolerance to caffeine were recently investigated (Svenningsson et al., 1999b). The behavioral tolerance was accompanied by less pronounced changes in immediate early gene expression in rostral striatum and nucleus accumbens than after acute caffeine administration (Svenningsson et al., 1995, 1997), suggesting that neuronal activity is reduced in parallel with behavioral stimulation. Furthermore, long-term treatment with caffeine led to a small, but statistically significant decrease in the number of adenosine A_{2A} receptors. We therefore examined if long-term treatment with a selective adenosine A_{2A} receptor antagonist, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo [1,5-*c*]-pyrimidine (SCH 58261) (Zocchi et al., 1996; Fredholm et al., 1998) would cause adaptive changes in adenosine receptors and other neurochemical markers or if its ability to enhance the locomotor stimulant effect of a dopaminergic agonist would be maintained.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats ($n = 29$, first series; $n = 30$, second series) were purchased from B&K Universal (Sweden). They were kept three in each cage in a 12-h dark–light cycle, lights on at 6:00 a.m. The rats had free access to food and water throughout the experiment except during the measurement of locomotor activity. At the time of the locomotor assay the animals weighed between 270 and 340 g. Before sacrifice by decapitation the animals were anesthetized with carbon dioxide. All efforts were made to reduce animal suffering and the number of animals used, and the experimental procedures were approved by the Regional Animal Ethics Committee.

2.2. Drug treatment

In a first series, animals were divided into six different treatment groups: caffeine 7.5 mg/kg chronic, SCH 58261 at 0.1, 1.0 or 7.5 mg/kg chronic, SCH 58261 at 7.5 mg/kg acute, and vehicle, in order to study changes in receptors and immediate early genes after acute and long-term treatment with SCH 58261 and chronic caffeine treatment. Chronically treated animals were injected with the indicated drug or vehicle twice daily for 14 days. All rats were killed by decapitation 4 h after the last injection. The brain was dissected out and directly frozen on dry ice and kept at −80°C until sectioned.

In a second series, the locomotor effects in combination with neurochemical effects of acute and long-term SCH 58261 were studied. The animals were divided in five groups: vehicle, 0.1 or 7.5 mg/kg SCH 58261 (acutely administered), and 0.1 or 7.5 mg/kg SCH 58261 (chronically administered). Chronically treated animals were injected twice daily (once in the morning, once in the late afternoon) for 14 or 15 days before testing. In all registrations of locomotion the animals were transferred to the experimental room at least 30 min before start. On experimental day 1, the animals were habituated to the boxes for 30 min in the morning and 30 min in the afternoon, during which the locomotion of each rat was recorded. On experimental day 2, the rats were injected either with vehicle, 0.1 or 7.5 mg/kg SCH 58261 before the third 30-min habituation period. Thereafter, all animals were injected with 0.5 mg/kg apomorphine, and their motor activity recorded for a 60-min session. Ninety minutes after the apomorphine injection the animals were anesthetized with CO_2 and decapitated. The brains were dissected out and directly frozen on dry ice and kept at −80°C until sectioned.

All drug injections were made intraperitoneally in a volume of 5 ml/kg. SCH 58261 was dissolved by sonication in saline with 8% Tween 80, 0.02 or 1.5 mg/ml. Drug doses were chosen to obtain a span from a low SCH 58261 dose, 0.1 mg/kg, which has been shown to have a neuroprotective effect (Monopoli et al., 1998), to a high dose, 7.5 mg/kg, to assure a robust behavioral effect (see Pinna et al., 1996; Svenningsson et al., 1997). Apomorphine was dissolved in saline containing 0.1% ascorbic acid.

2.3. Registration of locomotor activity

The motor activity was measured in a 680 × 680 × 450 mm box with 16 photocells in both horizontal directions, divided into levels of eight photocells each. The breaking of the light beams was recorded as counts by a computer (Kungsbacka Mät och Reglerteknik, Kungsbacka, Sweden) during 5-min periods. According to the order in which beams were broken, the activity was divided into different parameters: (a) total horizontal activity, (b) forward locomotion, and (c) vertical activity (referred to as rearing).

2.4. Receptor autoradiography

Serial frontal sections of 14 μm were made approximately +1.7/+1.2 from Bregma and approximately −1.3/−1.7 from Bregma and thaw-mounted on gelatine treated slides. These were kept at −20°C until used.

2.4.1. Dopamine D_1 receptors

Dopamine D_1 receptors were examined using binding of the D_1 antagonist [^{3}H]-*R*(+)-7-chloro-8-hydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride ([^{3}H]SCH 23390; specific activity 87.0 Ci/mmol, New England Nuclear). The slides were dried for 60 min at room temperature, and thereafter pre-incubated at room temperature for 10 min in the same buffer as during incubation (see below) but without radioligand. Slides were incubated at room temperature for 150 min covered with a buffer (pH 7.4) containing 25 mM Tris, 100 mM NaCl, 1 mM $MgCl_2$, 1 μM pargylin and 0.001% ascorbic acid (to prevent generation of toxic dopamine metabolites) as well as 20 nM mianserin (to block binding to 5-HT_2 receptors), 0.5% ethanol and 1 nM [^{3}H]SCH 23390. For controls of non-specific binding 10 μM butaclamol was added. After incubation, the slides were washed for 10 min in the same buffer at 4°C without [^{3}H]SCH 23390, ethanol and mianserin, and then briefly dipped three times in ice cold distilled water. The sections were dried with a fan at 4°C overnight. Finally, X-ray film was exposed to the slides for 4 weeks.

2.4.2. Dopamine D_2 receptors

Dopamine D_2 receptors were studied using binding of the D_2 antagonist [^{3}H]raclopride (specific activity 74.0 Ci/mmol, NEN). After first being dried for 60 min at 4°C, slides were pre-incubated twice for 5 min at 4°C in 50 mM Tris–HCl (pH 7.4). Thereafter, the slides were incubated at room temperature for 60 min covered with a buffer (pH 7.4) containing 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 5 nM [^{3}H]raclopride. Non-specific binding was determined with 1 μM butaclamol. After incubation the slides were washed twice for 5 min in 50 mM Tris–HCl at 4°C, and then briefly dipped three times in ice-cold distilled water. The sections were dried with a fan at 4°C overnight. Finally, X-ray film was exposed to the slides for 3 weeks.

2.4.3. Adenosine A_{2A} receptors

Adenosine A_{2A} receptors were studied using the agonist [^{3}H]2-[4-(2-*p*-carboxyethyl)phenyl-amino]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS 21680; specific activity 109.0 Ci/mmol, NEN) and the antagonist [^{3}H]SCH 58261 (specific activity 68.6 Ci/mmol, NEN). After first being dried for 60 min at room temperature, slides were pre-incubated for 30 min at room temperature in a buffer (pH 7.4) containing 170 mM Tris–HCl, 2 U/ml adenosine deaminase and 1 mM EDTA to eliminate endogenous adenosine. The sections were then prewashed twice for 10 min at room temperature in 170 mM Tris–HCl with (for CGS 21680) or without (for SCH 58261) 10 mM $MgCl_2$. Thereafter, the slides were incubated at room temperature for 120 min covered with a buffer containing 170 mM Tris–HCl, 10 mM $MgCl_2$ (only for CGS 21680), 2 U/ml adenosine deaminase, and 2 nM [^{3}H]CGS 21680 or 0.3 nM [^{3}H]SCH 58261. Non-specific binding was determined with 20 μM 2-chloroadenosine (for CGS 21680) or 50 μM 5′-*N*-ethylcarboxamidoadenosine (NECA) (for SCH 58261). After incubation the slides were washed twice for 5 min in 170 mM Tris–HCl at 4°C and then briefly dipped three times in ice cold distilled water. The sections were dried with a fan at 4°C overnight. Finally, X-ray film was exposed to the slides for 3 (SCH 58261) or 5 (CGS 21680) weeks.

2.4.4. Adenosine A_1 receptors

Adenosine A_1 receptors were studied using the antagonist [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX; specific activity 109.0 Ci/mmol, NEN) in the presence of GTP. Essentially the same procedure as above was used but slides were pre-incubated for 30 min at 37°C in a buffer (pH 7.4) containing 170 mM Tris–HCl, 2 U/ml adenosine deaminase and 1 mM EDTA to eliminate endogenous adenosine, and then prewashed twice for 10 min at room temperature in 170 mM Tris–HCl with 1 mM $MgCl_2$. Thereafter, the slides were incubated at room temperature for 120 min covered with a buffer containing 170 mM Tris–HCl, 1 mM $MgCl_2$, 2 U/ml adenosine deaminase, 0.5 nM [^{3}H]DPCPX and 100 μM GTP. Non-specific binding was determined with 20 μM *R*-phenylisopropyladenosine (R-PIA).

2.5. In situ hybridization

Serial frontal sections of 14 μm were made approximately +1.7/+1.2 from Bregma and approximately −1.3/−1.7 from Bregma and thaw-mounted on poly-L-lysine coated slides. These were kept at −20°C until used.

In situ hybridization was performed using [^{35}S]-end-labeled oligo probes complementary to mRNA for adenosine A_1 and A_{2A} receptors and for the immediate early genes nerve growth factor-induced clone A (NGFI-A), JunB, and c-*fos*. Unlabeled probe was incubated for 1–1.5 h at 37°C with 40 μCi [^{35}S]dATP (10 mCi/ml, Amersham), reaction buffer (Amersham), and 24 U terminal transferase (Amersham). Probe was purified on a nucleic acid purification column (NENSORB 20; NEN, Boston, MA). Finally, 10 mM dithiothreitol was added to the probe. Slides were incubated in a hybridization cocktail containing 50% deionized formamide (Fluka, Buchs, Switzerland), 4× standard saline citrate (SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 1× Denhardt's solution, 1% sarcosyl, 0.02 M $NaPO_4$ (pH 7.0), 10% dextran sulphate, 0.06 M dithiothreitol, 0.1 mg/ml sheared salmon sperm DNA and labeled probe for approximately 16 h at 42°C. The sections were then washed for 1 h in 1× SSC at 56°C for A_1, NGFI-A, JunB and c-Fos probes, and at 45°C for A_{2A} probe, then dipped for 60 s in water followed by 70%, 95% and 99.5% ethanol. Slides were air-dried and apposed to Hyperfilm βmax (Amersham) for 3–7 weeks.

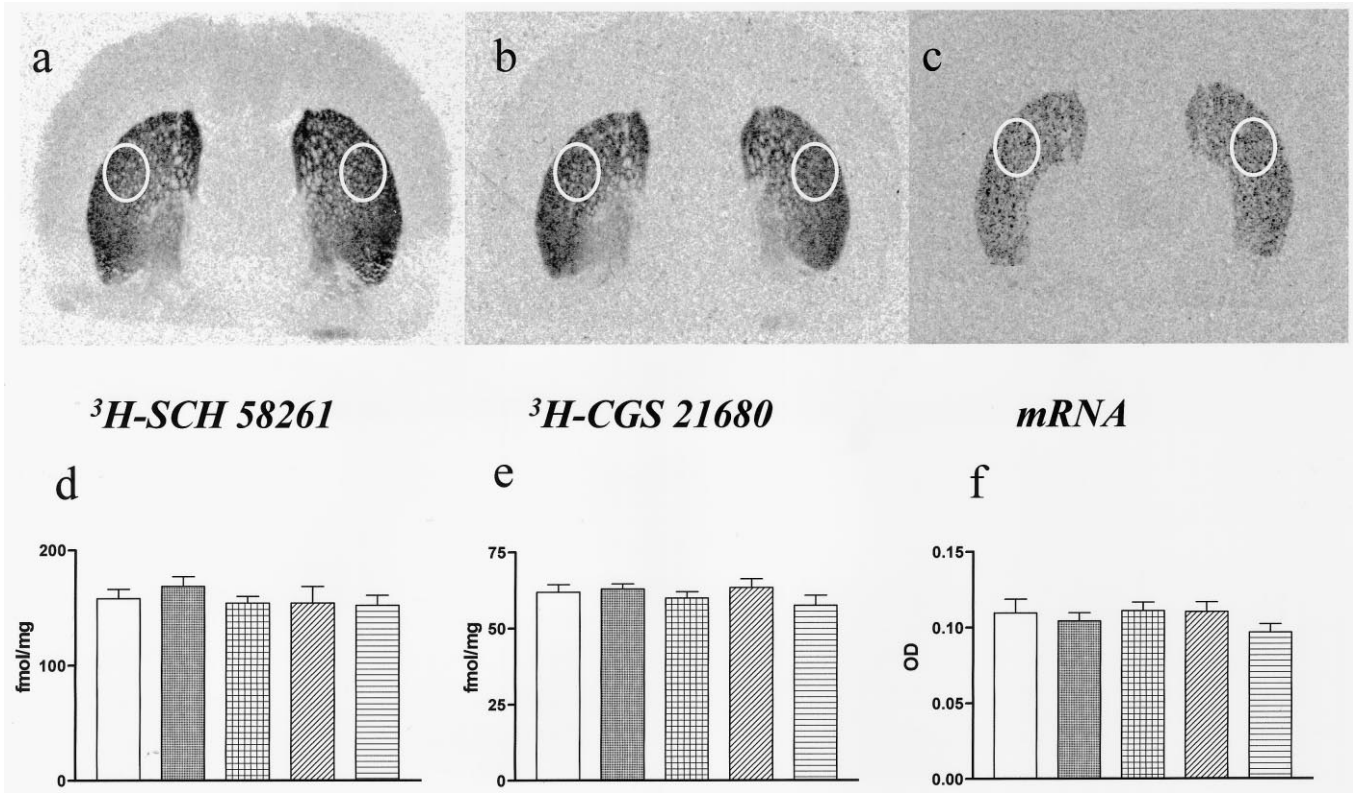

Fig. 1. Acute and long-term treatment with SCH 58261 does not cause any change in the expression of the adenosine A_{2A} receptor and its mRNA. The three upper panels (a, b, c) show the distribution of adenosine A_{2A} receptors (a, b) and mRNA (c). (a) Autoradiogram showing binding of the antagonist [^{3}H]SCH 58261. (b) Autoradiogram showing the binding of the agonist [^{3}H]CGS 21680. (c) Autoradiogram showing the distribution of adenosine A_{2A} receptor mRNA. Note the difference in distribution of the receptor (striatum and globus pallidus) and its mRNA (only in striatum). The lower panels (d, e, f) show the level of A_{2A} binding in fmol/mg (d, e), and A_{2A} mRNA expression in optical density (f) in each group in dorsal caudate putamen. The sample sites are indicated by circles in (a), (b) and (c). Open bar represents animals treated with vehicle, stippled bar 0.1 mg/kg acute SCH 58261, cross-hatched bar 7.5 mg/kg acute SCH 58261, diagonal striped bar 0.1 mg/kg chronic SCH 58261, and horizontal striped bar 7.5 mg/kg chronic SCH 58261. Values are means ± S.E.M of six determinations.

2.6. Analysis of autoradiograms and in situ hybridizations

Autoradiograms and in situ hybridizations were analyzed with a Micro-computer Imaging device (Imaging Research, Canada). For autoradiograms, optical densities were converted to density of bound ligand (fmol/mg protein) using autoradiographic [^{3}H]-micro-scale standards (Amersham). Specific binding was obtained by subtracting non-specific binding from total binding. For in situ hybridization, optical densities were measured using the same imaging device calibrated with a Kodak density wedge.

2.7. Statistics

The in situ and receptor autoradiography data were analyzed with one-way analysis of variance (ANOVA) tests using Newman–Keul's Multiple Comparison Test as post hoc test. The behavioral data were analyzed with a two-way ANOVA test (treatment × time) or a one-way ANOVA with a Bonferroni test for selected pairs of means as post hoc test.

3. Results

3.1. Adenosine and dopamine receptors and receptor mRNA

Long-term treatment with caffeine has previously been shown to cause a decrease in adenosine A_{2A} receptors (when low doses of caffeine are given orally) accompanied by a decrease in the corresponding mRNA (Svenningsson et al., 1999b), or (when given in higher doses by injection) an increase in the number of adenosine A_1 receptors without changes in mRNA expression (Johansson et al., 1993). However, in the present experiments, and as seen in Fig. 1, there were no changes in the binding of an adenosine A_{2A} receptor antagonist (Fig. 1a,d), agonist (Fig. 1b,e) or in adenosine A_{2A} receptor mRNA (Fig. 1c,f) after acute or long-term treatment with the adenosine A_{2A} receptor antagonist SCH 58261. The distribution of receptors (Fig. 1a,b) and of the corresponding mRNA (Fig. 1c) is consistent with the receptors in the projection area globus pallidus being located on the terminals of the striatopallidal neurons (see Svenningsson et al., 1999a). In no part of striatum (or pallidum) could any significant effect of either acute or chronic SCH 58261 be detected, even though a tendency towards a decreased binding was observed with caffeine given chronically at the dose 7.5 mg/kg twice daily (data not shown). Similarly, and as indicated in Fig. 2, there was no detectable effect of acute or long-term treatment with SCH 58261 on A_1 receptors or the corresponding mRNA in cerebral cortex. Adenosine A_1 receptors in nucleus accumbens core and shell and caudate–putamen were similarly unaffected (not shown).

There were no changes in binding of the dopamine D_1 receptor antagonist [^{3}H]SCH 23390 or the dopamine D_2 receptor antagonist [^{3}H]raclopride in any group compared to controls, neither in the anterior caudate putamen, the posterior caudate putamen, the accumbens, nor in the cortex (results not shown).

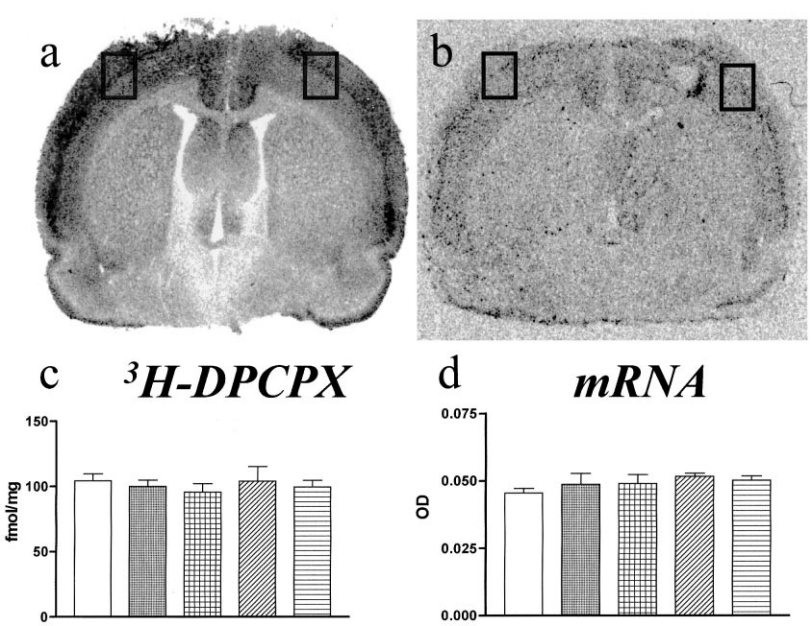

Fig. 2. Acute and long-term treatment with SCH 58261 does not cause any change in the expression of the adenosine A_1 receptor and its mRNA. Adenosine A_1 receptors as shown by (a) [^{3}H]DPCPX autoradiography, (b) in situ hybridization. Bar graph (c) shows the level of adenosine A_1 receptor binding and (d) the level of receptor mRNA in ventral cortex in different groups of animals. The sample sites are indicated by squares in (a) and (b). Open bar represents animals treated with vehicle, stippled bar 0.1 mg/kg acute SCH 58261, cross-hatched bar 7.5 mg/kg acute SCH 58261, diagonal striped bar 0.1 mg/kg chronic SCH 58261, and horizontal striped bar 7.5 mg/kg chronic SCH 58261. Values are means ± S.E.M of six determinations.

3.2. Behavioral studies

In the second series of experiments, in a different set of animals, the effect of SCH 58261 alone and in combination with the dopamine receptor agonist apomorphine was examined. During the first day of habituation (habituation 1 and 2) the only animals that received drug treatment were the chronically SCH 58261-treated rats, who had their regular injections in the morning before habituation sessions and received the second daily dose in the evening after habituation. In all sessions, including the habituation periods, there was, as expected, a time-dependent decrease in horizontal activity in all groups (including vehicle-treated rats). The overall activity was higher during the first (Fig. 3a) than during the second habituation period.

During the second habituation session, the horizontal activity (defined as total counts in the lower row of light beams) was significantly increased ($P = 0.0041$ and $P < 0.0001$) in both groups of animals receiving chronic SCH 58261 (0.1 and 7.5 mg/kg SCH 58261, respectively) (Fig. 3b). The same was true for rearing (defined as the breaking of upper row beams when the animal rises on its back paws) ($P = 0.0054$ and $P = 0.0035$; not shown). This difference was not detected in the first habituation period, probably because of the high motor activity in all groups due to the novel environment.

The next day, after acute SCH 58261 injection, the horizontal activity was significantly increased ($P = 0.0009$

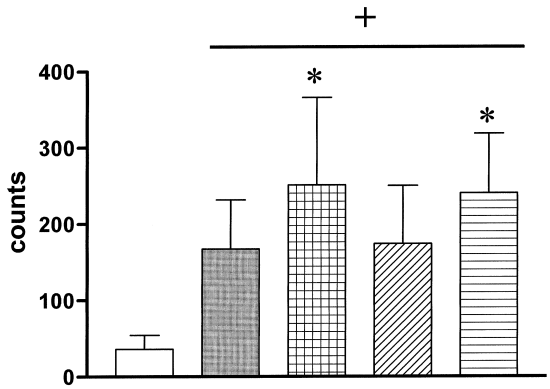

Fig. 4. No tolerance to the locomotor-stimulant effect of acute SCH 58261 after long-term SCH 58261 treatment. The difference in activity count during the last 15 min of the second habituation and after SCH 58261/vehicle injection are given (means ± S.E.M.; $n = 5–6$ for each group). First column, open bars — saline injected animals; Second column, stippled bars — animals receiving an injection of 0.1 mg/kg SCH 58261 but no prior drug treatment; Third column, cross-hatched bars — animals receiving an injection of 7.5 mg/kg SCH 58261 but no prior drug treatment; Fourth column, diagonal striped bars — animals receiving an injection of 0.1 mg/kg SCH 58261 after 14 days prior treatment with the same dose twice daily; Fifth column, horizontally striped bars — animals receiving an injection of 7.5 mg/kg SCH 58261 after 14 days prior treatment with the same dose twice daily. One-way ANOVA showed a significant ($P < 0.05$) difference when the control group was included (+). However, no significant differences were observed between the groups that received SCH 58261. A pairwise comparison showed a significant increase in locomotor activity after 7.5 mg/kg SCH 58261 injection in both acutely and chronically treated animals ($P < 0.05$) compared to control (indicated by *).

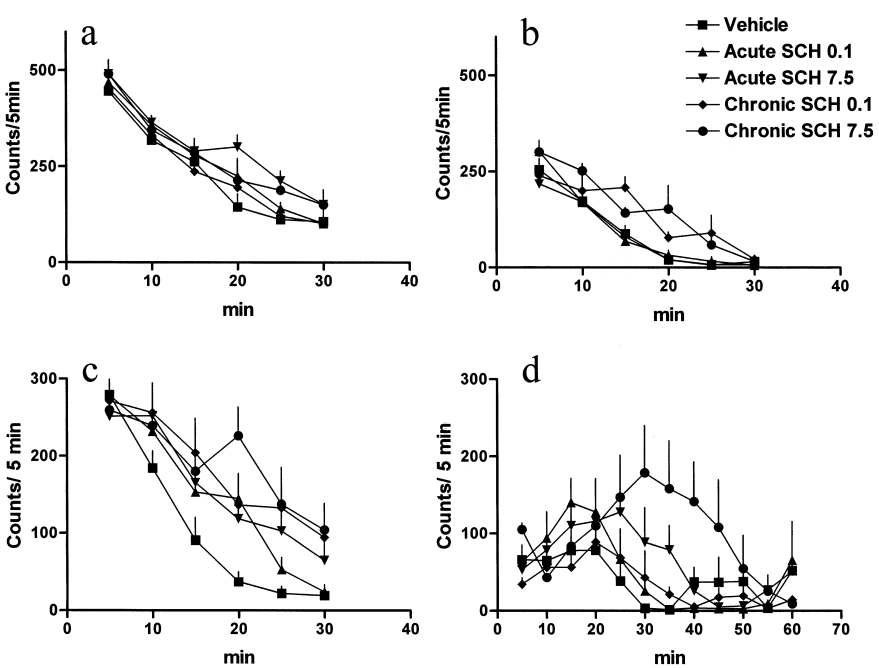

Fig. 3. Locomotor responses of rats given apomorphine after acute or long-term administration of SCH 58261. Horizontal activity in the different groups during the first (a) and second (b) habituation periods. Before the third period (c) vehicle, 0.1 or 7.5 mg/kg SCH 58261 was injected, showing the acute effect on horizontal activity of SCH 58261 in both chronically treated and untreated animals. In panel (d), horizontal activity was measured for 60 min after injection of 0.5 mg/kg apomorphine. For further explanations, see text. Note that the scale is different between day 1 (periods a and b) and day 2 (periods c and d). All values are means + S.E.M. from each 5-min period; $n = 5–6$.

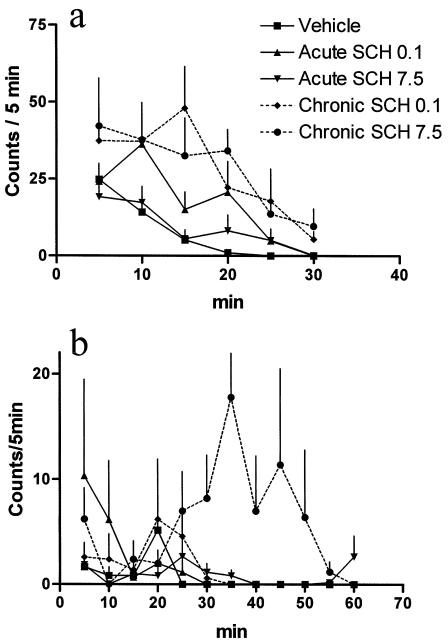

Fig. 5. Effect of SCH 58261 and apomorphine on rearing in rats. Rearing (i.e., vertical motor activity) was measured simultaneously with horizontal activity as presented in Fig. 3. Panel (a) shows the rearing activity in the different groups during the third habituation session directly after injection of vehicle, 0.1 or 7.5 mg/kg SCH 58261. An acute effect on rearing with 0.1 mg/kg SCH 58261 is seen in both chronically treated and untreated rats, and with 7.5 mg/kg SCH 58261 in chronically treated animals. Panel (b) shows rearing measured for 60 min after the injection of 0.5 mg/kg apomorphine given directly after the session shown in panel (a). All values are means + S.E.M. from each 5-min period; $n = 5–6$.

and $P = 0.0048$ for 0.1 and 7.5 mg/kg, respectively) in a third test period (Fig. 3c). An essentially similar increase was also seen when instead of total locomotion the forward locomotion (defined as counts following each other in the same direction) was studied (results not shown).

In animals that received no drug the total locomotor activity was virtually identical between the third test session and the second habituation period, and this agrees with previous experience. The chronically SCH 58261-treated animals were more active than untreated controls, particularly during the latter part of the test period. We wanted to examine the effect of the acute dose in these animals, and to that end, we compared the motor scores for the three last 5-min episodes of the second habituation period with the same periods during the recording after acute SCH 58261 administration (Fig. 4). By this means we were able to examine the effect of the acute administration SCH 58261 in the presence of an underlying motor activation. The acute effect of SCH 58261 alone on rat behavior has been evaluated previously: SCH 58261 has been shown to significantly increase locomotion at 15–50 and 70–75 min after an injection of 3.75 mg/kg (Svenningsson et al., 1997).

At the end of this test session, the animals were taken out of the box, injected with apomorphine in a low dose, and immediately returned to the box. Previously it has been shown that a saline injection at this time causes a minor increase in the motor activity during the first 5 min that progressively abates and disappears over the following 15 min (e.g., Svenningsson et al., 1997). In this study, the injection of apomorphine resulted in a transient increase of the horizontal activity that peaked at 15–20 min. This increase was significantly higher ($P < 0.0001$, $n = 5$) in the chronically 7.5 mg/kg treated group compared to vehicle, and the peak also seems to appear later (at 30 min) than in the other groups. The same tendency for a prolongation of the response was seen in the group of 7.5 mg/kg acutely treated animals (Fig. 3d).

When SCH 58261 was administered before the third behavioral session the rearing parameter was also increased (Fig. 5a). The increase reached statistical significance (ANOVA) in both the chronically treated groups ($P < 0.0001$) and in the group acutely treated with 0.1 mg/kg SCH 58261 ($P = 0.0055$). Subsequent administration of apomorphine caused a marked increased in rearing in the animals treated chronically with 7.5 mg/kg SCH 58261 ($P < 0.0001$, $n = 5$) (Fig. 5b).

3.3. Immediate early gene mRNA expression

There were no differences in the basal expression of NGFI-A, c-*fos* or *jun*B in any region examined after acute

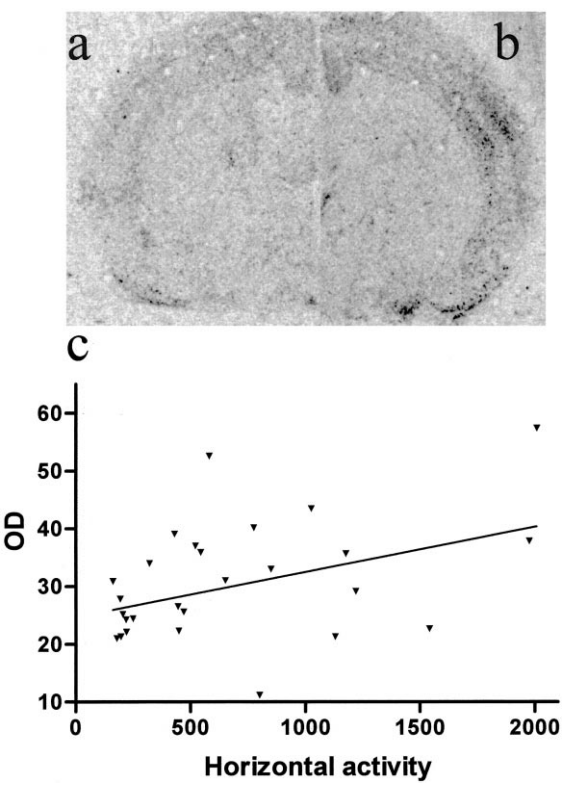

Fig. 6. SCH 58261 increases cortical c-*fos* expression after apomorphine. c-*fos* mRNA expression after apomorphine (a) without pretreatment (vehicle) and (b) after chronic pretreatment with 7.5 mg/kg SCH 58261. Panel (c) shows the correlation between c-*fos* measured in cingulate cortex and the motor activity.

or long-term treatment with SCH 58261 in either dose used (data not shown). However, long-term treatment with SCH 58261 at either 0.1 (not shown) or 7.5 mg/kg (Fig. 6) did increase the expression of c-*fos* mRNA in motor cortex after apomorphine. The cortical expression of c-*fos* mRNA may reflect the motor activation, as a weak positive ($P = 0.048$; assuming Gaussian distribution) correlation was observed between the horizontal activity induced by apomorphine and the c-*fos* expression in the cingulate cortex.

4. Discussion

The major conclusion of this study is that tolerance does not develop to the locomotor effects of the selective adenosine A_{2A} receptor antagonist SCH 58261. Thus, this selective antagonist is different in this respect from the non-selective adenosine receptor antagonist caffeine, to which tolerance develops rapidly (see Holtzman and Finn, 1988; Fredholm et al., 1999; Svenningsson et al., 1999b). Our conclusion is based on two studies, one neurochemical and one behavioral.

4.1. No adaptive changes in adenosine or dopamine receptors following long-term treatment with a selective A_{2A} antagonist

It has long been known that higher doses of non-selective adenosine receptor antagonists such as caffeine and theophylline cause an up-regulation of the number of adenosine A_1 receptors (Fredholm, 1982; Lupica et al., 1991; Johansson et al., 1993). This effect was originally thought to mediate the development of tolerance, but that interpretation has later been doubted, partly because administration of these drugs in low doses, which do produce tolerance, does not cause adaptive changes in adenosine A_1 receptors (Johansson et al., 1996; Svenningsson et al., 1999b), and partly for other reasons (see also Garrett and Holtzman, 1995).

More recently, chronic low dose caffeine treatment was shown to result in a down-regulation of A_{2A} receptors and the corresponding mRNA in rostral striatum (Svenningsson et al., 1999b). This was postulated to be one reason for tolerance, since a smaller number of receptors means that endogenous adenosine produces a lower degree of receptor activation. This in turn could mean that the antagonist will produce a smaller effect (Svenningsson et al., 1999b).

A major finding of the present study is therefore that acute or long-term treatment with the selective adenosine A_{2A} receptor antagonist SCH 58261 does not cause any measurable changes in adenosine A_1 or A_{2A} receptors, either at a high or a low dose. There were also no adaptive changes in the dopamine D_1 or D_2 receptors. Thus, the selective antagonism of A_{2A} receptors cannot mimic the adaptive changes in receptor expression that have been associated with tolerance to a non-selective adenosine receptor antagonist. Furthermore, these results show that antagonism of adenosine A_{2A} receptors is not the cause of such changes following long-term administration of caffeine or theophylline.

4.2. No adaptive changes in locomotor stimulation following long-term treatment with a selective A_{2A} antagonist

The selective adenosine A_{2A} receptor antagonist SCH 58261 has been previously shown to induce a weak locomotor stimulant effect in rats (Svenningsson et al., 1997) and in CD1 mice (El Yacoubi et al., 2000). This was confirmed in the present study. Thus, a selective adenosine A_{2A} receptor antagonist can produce a motor stimulant effect just as a non-selective adenosine receptor antagonist such as caffeine (see Svenningsson et al., 1997). Blockade of adenosine A_{2A} receptors is essential to produce locomotor stimulation, as shown by the observation that caffeine does not induce motor activity in adenosine A_{2A} receptor knock-out mice (Ledent et al., 1997). In fact, in these animals a dose-dependent depressant effect is produced by caffeine (El Yacoubi et al., 2000).

Although there is rapid development of tolerance to the locomotor stimulant effect of caffeine, we find no evidence that a similar tolerance develops to the selective adenosine A_{2A} receptor antagonist SCH 58261. Animals injected with the drug for 14 days prior to the experiment and given the latest injection of SCH 58261 in the morning showed a higher motor activity during the afternoon. This demonstrates that the drug produces a persistent motor enhancement. A single injection of the adenosine A_{2A} receptor antagonist given 30 min before the test session caused a clear increase in motor activity, both in animals that were drug naive and in animals that had received prior treatment. This increase appeared dose-dependent as it was statistically significant in animals given the high dose (7.5 mg/kg), but not in animals given the low dose. The total motor activity tended to be higher in the animals that had received the drug repeatedly beforehand than in the animals that received no prior drug treatment. When the persistent motor activation seen after long-term drug treatment was subtracted, the effect of the single acute dose of the adenosine A_{2A} receptor antagonist appeared similar to that seen in drug naive animals. In particular, there was no evidence that the motor stimulant effect of SCH 58261 was smaller in animals chronically treated with the drug.

4.3. The motor stimulant effect of apomorphine is not reduced by long-term treatment with an adenosine A_{2A} receptor antagonist

Caffeine tolerance is accompanied by tolerance to selective dopamine D_1 and D_2 receptor agonists, but little tolerance to combined treatment with D_1 and D_2 agonists (Garrett and Holtzman, 1994) or to cocaine or am-

phetamine (Holtzman, 1983; Finn and Holtzman, 1986). Here, we show that long-term treatment with the adenosine A_{2A} receptor antagonist SCH 58261 instead, if anything, enhanced the motor stimulant effects of a low dose of the dopamine receptor agonist apomorphine. The fact that the rearing parameter was elevated after apomorphine injection in SCH 58261-treated compared to vehicle-treated animals, indicates that the enhanced motor activity induced by the drug combinations represents a normal behavior, and that it is not of the stereotyped nature seen for example in rats given amphetamine.

The dose of apomorphine used in this study produced only a minimal increase in horizontal or vertical motor activity per se. Particularly in the animals that had received long-term treatment with SCH 58261 in a high dose, apomorphine produced a large, long-lasting motor stimulation. The fact that the enhancement was largest in the animals chronically treated with 7.5 mg/kg SCH 58261 might be due to accumulation of the drug. However, the acute SCH 58261 effect was not increased in these animals. Thus, these results suggest a sensitization phenomenon as an explanation. In this context, it is interesting to note that 6-hydroxydopamine-lesioned rats do not respond with rotational behavior on caffeine, unless they are primed with apomorphine or other dopamine receptor agonists (Fenu and Morelli, 1998). Conversely, the rotation behavior induced by non-selective adenosine receptor antagonists is blocked by dopamine receptor antagonists, and actions of dopamine receptor agonists are enhanced by adenosine A_{2A} receptor blockade (see Ferré et al., 1992; Fenu and Morelli, 1998). Thus, the adenosine A_{2A} and dopamine receptors in several behavioral aspects seem to have opposite functions in the striatum. Furthermore, there are long-term adaptive changes.

The increase in the apomorphine-induced locomotion in the present study was accompanied by an increase in c-*fos* mRNA expression in cortical areas. There was also a weak positive correlation between locomotor behavior and c-*fos* expression. There were, however, no clear-cut changes in immediate early gene expression in basal ganglia following long-term treatment with the A_{2A} antagonist per se, even though such changes have been observed following long-term caffeine treatment (Johansson et al., 1994; Svenningsson and Fredholm, 1997; Svenningsson et al., 1999b). This could indicate that sensitization to the effects of apomorphine occurs not in the primary targets of drug action but depends on changes in the output structures.

Selective A_{2A} antagonists might be developed as novel treatment of Parkinsonism. The idea that non-selective adenosine receptor antagonists such as caffeine and theophylline might be used in treatment of Parkinson's disease was suggested many years ago (see Fuxe and Ungerstedt, 1974; Fredholm et al., 1976; Jarvis and Williams, 1987). The fact that tolerance rapidly develops to these non-selective antagonists has argued against the success of such therapy. The finding that there is no tolerance development to the locomotor stimulating effect of SCH 58261, however, indicates that such selective drugs could possibly be used as a long-term supplement to L-DOPA in treatment of Parkinson's disease. How this would affect the inevitable on–off phenomenon after long-term L-DOPA treatment remains to be examined, and it is uncertain if this can be done in animals. An adenosine A_{2A} receptor blocking agent could perhaps also be used in early stages of Parkinson's disease in younger patients, in order to prolong an efficient treatment without L-DOPA. In this case an excessive motor stimulant effect is rather unlikely, since the main effect of the adenosine A_{2A} receptor antagonist seemed to be to enhance dopamine receptor-mediated stimulation.

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