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European Journal of Pharmacology 366 (1999) 1–11

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Scopolamine prevents tolerance to the effects of caffeine on rotational behavior in 6-hydroxydopamine-denervated rats

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Received 3 November 1998; revised 2 December 1998; accepted 4 December 1998

Abstract

Continuous administration of caffeine has been shown to induce tolerance to its psychostimulant effects. In this study, using unilateral 6-hydroxydopamine nigrostriatal denervated rats, we tested the hypothesis that the muscarinic receptor antagonist, scopolamine, would prevent the tolerance to caffeine-induced contralateral rotational behavior. For that purpose we administered either caffeine (40 mg/kg) plus saline or scopolamine (5, 10 and 20 mg/kg) plus saline, as well as caffeine in combination with the various doses of scopolamine for 7 consecutive days, and measured ipsilateral and contralateral rotational behavior. The results showed that acute injections of scopolamine plus saline produced similar levels of both ipsilateral and contralateral turning, while caffeine produced more contralateral than ipsilateral turning. Tolerance to caffeine-induced contralateral turning was observed as of the second administration, while scopolamine plus saline injections did not produce significant changes in rotational behavior with repeated treatment. Scopolamine co-administered with caffeine significantly attenuated the increased contralateral turning produced by acute injections of caffeine plus saline, but significantly prevented the tolerance effects with repeated administration. These findings strongly suggest that muscarinic cholinergic processes may be involved in tolerance to caffeine-induced contralateral turning. The results are interpreted in terms of the possible interactions between dopamine, adenosine and acetylcholine neurotransmitter systems within the basal ganglia circuitry involved in motor behavior.

Keywords: Acetylcholine; Scopolamine; Turning behavior; Methylxanthine; Adenosine–dopamine receptor interaction

1. Introduction

The unilateral 6-hydroxydopamine nigrostriatal lesion rat model has been widely used as an animal model of Parkinson's disease. Direct dopamine receptor agonists, acting on supersensitive dopamine receptors in the denervated striatum, induce turning behavior contralateral to the lesioned side (Pycock, 1980; Schwarting and Huston, 1996). Methylxanthines such as caffeine, also induce strong and long-lasting contralateral turning (Herrera-Marschitz et al., 1988; Casas et al., 1989a,b), and are able to potentiate the rotational behavior induced by direct dopamine receptor agonists (Andén and Jackson, 1975; Fuxe et al., 1978; Fredholm et al., 1983). Several mechanisms of action have been proposed to explain the stimulant effects of caffeine and other methylxanthines (for review see Garrett and Griffiths, 1997). These include phosphodiesterase inhibition (Fuxe et al., 1978; Cardinali, 1980), catecholamine release (Berkowitz et al., 1970), direct action on dopamine receptors (Ungerstedt et al., 1981; Casas et al., 1988, 1989a,b; Ferré et al., 1990; Garrett and Holtzman, 1994a, 1995), direct antagonism on adenosine receptors (Snyder et al., 1981; Daly et al., 1981; Fredholm et al., 1983; Nikodijevic et al., 1993), and indirect modulation of dopamine receptors through blockade of adenosine receptors (Ferré et al., 1991b; Fredholm, 1995).

In rodents, tolerance to the locomotor activating effects of caffeine has been described (Holtzman, 1983; Finn and Holtzman, 1987), and in unilateral 6-hydroxydopamine nigrostriatal-lesioned rats contralateral rotational behavior induced by caffeine is also subject to rapid tolerance (Watanabe et al., 1982), being restored only after a long period of caffeine withdrawal (Casas et al., unpublished

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0014-2999/99/$ - see front matter
PII: S0014-2999(98)00911-X

data). The mechanisms mediating caffeine tolerance are still not completely understood. Involvement of adenosine receptor supersensitivity has been proposed (Fredholm, 1982; Boulenger et al., 1983; Chou et al., 1985; Hawkins et al., 1988), although recent evidence has been provided indicating that tolerance to the locomotor stimulating effects of caffeine (Holtzman et al., 1991), or to caffeine-induced turning (Garrett and Holtzman, 1995) cannot be explained only by adaptive changes occurring at adenosine receptor systems.

The notion that caffeine may have dopamine receptor agonist-like properties (Casas et al., 1988, 1989a,b; Ferré et al., 1990) suggested that methylxanthines could have potential antiparkinsonian activity. In this regard, theophylline has recently been tested in a short open study with parkinsonian patients, showing that it might be a useful adjuvant to the routine therapy in Parkinson's disease treatment (Mally and Stone, 1994). However, tolerance to the central nervous system stimulant effects of caffeine has also been described in humans, limiting the usefulness of methylxanthines as antiparkinsonian agents (Evans and Griffiths, 1992).

Anticholinergic drugs are also used in the treatment of Parkinson's disease, either alone or in combination with dopamine receptor agonists (Coleman, 1992). Based on the unilateral 6-hydroxydopamine nigrostriatal lesion rodent model, it has been reported that scopolamine produces moderate ipsilateral rotational behavior when injected systemically in mice (Pycock, 1980) and rats (Ungerstedt et al., 1973; Kelly and Miller, 1975). However, a more recent report showed that scopolamine did not produce any turning behavior in rats (Morelli et al., 1993a). These apparent discrepancies may be due to differences in the methodology used, including measuring apparatus, doses of scopolamine, or strain of animals. On the other hand, scopolamine can potentiate contralateral turning behavior induced by the dopamine D_1 receptor agonist, (±)-Phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol (SKF-38393), but not by the dopamine D_2 receptor agonist, *trans*-(−)-4aR-4,4a,5,6,7,8,8a,9-Octahydro-5-propyl-1*H*-pyrazolo[3,4-g]quinoline (LY- 171,555) (Morelli et al., 1993a), and either produce no effect (Pycock, 1980), or potentiate apomorphine-induced contralateral turning (Carey, 1991). Moreover, intrastriatal administration of atropine, another anticholinergic drug, reduces the inhibitory effects of the adenosine A_2 receptor agonist, 2-*p*-(2-Carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS-21680), on apomorphine-induced turning in 6-hydroxydopamine denervated rats (Vellucci et al., 1993).

As the effects of caffeine on rotational behavior might involve an A_2/D_2 interaction, and as the cholinergic system modulates the effects produced by adenosine analogs on contralateral turning induced by dopamine receptor agonists, we now investigated the effects of muscarinic receptor blockade on caffeine-induced contralateral turning. Additionally, in view of the data showing that anticholinergic drugs potentiate the effects of dopamine receptor agonists on rotational behavior, and because we found in recent studies in our laboratory that the dopamine D_2 receptor agonist, bromocriptine, administered repeatedly in combination with caffeine blocks the tolerance to caffeine-induced rotational behavior, we also tested the hypothesis that muscarinic receptor blockade would prevent the tolerance to caffeine effects in this animal model.

For that purpose, we co-administered for 7 consecutive days scopolamine in combination with caffeine to unilateral 6-hydroxydopamine nigrostriatal denervated rats previously selected for tolerance to caffeine, and measured contralateral rotational behavior during those days. In addition, to test whether the effects observed with scopolamine were centrally mediated, we also investigated the effects of methylscopolamine (a muscarinic antagonist which does not cross the blood-brain barrier) in combination with caffeine on contralateral rotational behavior.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were used in all groups. The animals were housed eight to a cage with free access to rat chow and water. They were kept in a temperature-controlled environment (21 ± 1°C) on a 12-h light/dark cycle (lights on at 8 A.M.) when they were not in experimental sessions. This experiment was carried out in compliance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) for care and use of laboratory animals.

2.2. Surgical procedure

Rats weighing 150 ± 10 g. were anaesthetized with a sodium pentobarbital solution (40 mg/kg, i.p.) and placed in a David Kopf stereotaxic frame with the incisor bar set at 2.4 mm (König and Klippel, 1963). The animals were injected unilaterally into the left hemisphere with 8 μg of 6-hydroxydopamine HCl (calculated as free base, Sigma USA) in 4 μl of physiological saline with 0.2% ascorbic acid using a Hamilton syringe with a conically shaped needle with maximum diameter 0.4 mm. The rate of infusion was 1 μl/min. The infusions were aimed at the medial forebrain bundle (A-4.4, L-1.2, V-7.8 mm, calculated from bregma and dura). This lesion has been shown to produce extensive unilateral denervation of the dopaminergic nigrostriatal system (Ungerstedt, 1971; Herrera-Marschitz and Ungerstedt, 1984).

2.3. Animal selection

In order to select the successfully denervated animals, 30 days post-surgery, all rats were challenged with a low

dose of apomorphine (0.05 mg/kg s.c) four times at one week interval between treatments. Rats showing fewer than 500 half-turns (180°) in 1 h during the last two tests with apomorphine were not included in the study. Several authors have demonstrated that at least 90% dopamine depletion is needed for this dose of apomorphine to induce contralateral rotational behavior (Hefti et al., 1980; Ungerstedt and Herrera-Marschitz, 1981; Hudson et al., 1993). In addition, in pilot studies we had observed individual variability in the development of tolerance to caffeine-induced contralateral rotational behavior (approximately 20% of all successfully denervated rats do not show tolerance to various doses of caffeine: 30, 40 and 60 mg/kg). This variability, which can possibly be attributed to genetic factors, is currently a subject of study in our laboratory. Therefore, in this study, all animals were tested for tolerance to caffeine one month before the start of the experiment in order to avoid possible confounds. Rats were given caffeine (40 mg/kg) for two consecutive days, and rotational behavior was measured. Only animals that showed at least 60% reduction in contralateral rotational behavior on the second day of treatment were used in the study.

2.4. Drugs

Scopolamine HCl, methylscopolamine HCl, apomorphine HCl, and caffeine anhydrous (Sigma, Spain) were diluted in physiological saline. All doses were calculated as free base and injected subcutaneously (s.c.) in a volume of 1 ml/kg of body weight.

2.5. General procedure

Ninety-eight well-denervated animals, also having shown tolerance to caffeine, were randomly allocated to the following groups: Group 1 received scopolamine 5 mg/kg plus caffeine 40 mg/kg ($n = 10$); Group 2 received scopolamine 10 mg/kg plus caffeine 40 mg/kg ($n = 9$), and Group 3 received scopolamine 20 mg/kg plus caffeine 40 mg/kg ($n = 8$). Group 4 received saline plus caffeine (40 mg/kg) ($n = 12$), and Group 5 received saline-saline ($n = 8$). Group 6 received methylscopolamine 5 mg/kg plus caffeine 40 mg/kg ($n = 8$), Group 7 received methylscopolamine 10 mg/kg plus caffeine 40 mg/kg ($n = 9$), and Group 8 received methylscopolamine 20 mg/kg plus caffeine 40 mg/kg ($n = 9$). Group 9 received scopolamine 5 mg/kg plus saline ($n = 8$), Group 10 received scopolamine 10 mg/kg plus saline ($n = 8$), and Group 11 received scopolamine 20 mg/kg plus saline ($n = 9$). The animals were placed individually into plastic hemispherical bowls (40 cm in diameter), attached to a harness and connected to photoelectric detectors. Following a 20-min habituation period, the rats were pretreated with either scopolamine, methylscopolamine or saline. Thirty minutes later, they received an injection of caffeine or saline, and rotational behavior was measured. Both contralateral and ipsilateral half turns (180°) were recorded for 12 h, using a computerized system (Panlab, Spain). Treatments were administered once daily for 7 consecutive days. Twelve-hour sessions were carried out in order to ensure that the entire period of caffeine action was measured.

2.6. Data analysis

Statistical comparisons were made using the SPSS/PC + computer program (SPSS, USA). The data were transformed to Log10 in order to homogenize the variance. Significance between groups for contralateral and ipsilateral rotational behavior for the 7 days of treatment was evaluated using multivariate analyses of variance (MANOVA). Post-hoc comparisons following significant interactions were made using the *t*-test implemented in the Contrast options of MANOVA in all cases. A polynomial contrast analysis was performed in order to examine the trend of contralateral rotational behavior for each group in successive days. In addition, One-way ANOVA followed by Duncan's test was used to determine differences in rotational behavior between groups whenever appropriate. The accepted level of significance for all tests was $P < 0.05$. The data are presented as uncorrected mean half-turns ± S.E.M. in 12 h.

3. Results

3.1. Previous level of tolerance to caffeine effects on contralateral rotational behavior

Descriptive data for the previous level of tolerance to caffeine effects on contralateral rotational behavior are shown in Table 1. On the first day, caffeine administration

Table 1
Previous level of tolerance to the effects of caffeine (40 mg/kg) on contralateral rotational behavior

Group	Day 1	Day 2
Group 1 ($n = 10$)	4470.80 ± 726.21	761.60 ± 80.76
Group 2 ($n = 9$)	3555.56 ± 340.44	674.22 ± 67.17
Group 3 ($n = 8$)	3893.63 ± 672.58	632.50 ± 56.01
Group 4 ($n = 12$)	4850.83 ± 663.46	654.08 ± 85.73
Group 5 ($n = 8$)	4298.63 ± 258.33	660.38 ± 79.08
Group 6 ($n = 8$)	4306.25 ± 420.22	563.38 ± 109.00
Group 7 ($n = 9$)	4497.50 ± 338.82	646.44 ± 73.04
Group 8 ($n = 9$)	5024.60 ± 680.50	552.50 ± 89.90
Group 9 ($n = 8$)	4480.40 ± 328.40	680.00 ± 88.30
Group 10 ($n = 8$)	5648.75 ± 559.10	653.90 ± 69.90
Group 11 ($n = 9$)	4583.30 ± 663.46	654.08 ± 85.73

Values are means ± S.E.M. total number of contralateral half-turns in 12 h.

(40 mg/kg) induced contralateral rotational behavior, but on the second day of administration, tolerance to the effects of caffeine was observed. No significant differences were found in contralateral rotational behavior between the different groups on the first or second day of caffeine administration.

3.2. Effects of scopolamine plus saline on ipsilateral and contralateral rotational behavior

Scopolamine plus saline produced some degree of motor activation, which was translated in the rotometers as moderate amounts of both contralateral and ipsilateral rotational behavior (see Fig. 1). For contralateral rotational behavior, statistical comparisons over the 7-day treatments showed a significant Group effect: $F(3,29) = 3.48$, $P < 0.03$, and a significant Group × Days interaction: $F(18,174) = 2.08$, $P < 0.01$. Comparisons between groups for each day of treatment revealed that, on the first day, only the doses of 10 and 20 mg/kg of scopolamine injected with saline significantly increased contralateral rotational behavior with respect to saline–saline injections ($t = 2.78$, $P < 0.02$; $t = 3.10$, $P < 0.005$, respectively). On the second day, only the dose of 10 mg/kg of scopolamine plus saline significantly increased contralateral turning with respect to saline–saline injections ($t = 3.14$, $P < 0.005$). On days 4 and 5, all doses of scopolamine significantly increased contralateral rotational behavior ($t \geq 3.18$, $P < 0.01$ in all cases), and on days 3, 6 and 7, no significant differences were observed between groups for contralateral turning behavior.

For ipsilateral rotational behavior, statistical analysis showed a significant Group effect $F(3,29) = 3.32$, $P < 0.04$, and a significant Group × Days interaction $F(18,174) = 2.17$, $P < 0.007$. Individual comparisons for each day showed that, on the first day of treatment, all doses of scopolamine plus saline significantly increased ipsilateral rotational behavior with respect to saline–saline injections ($t \geq 3.39$, $P < 0.04$, in all cases). On the second day, only the doses of 10 and 20 mg/kg of scopolamine plus saline significantly increased ipsilateral turning with respect to saline-saline injections ($t \geq 3.55$, $P < 0.04$). On days 4 and 5, all doses of scopolamine significantly increased ipsilateral rotational behavior ($t \geq 3.06$, $P < 0.05$ in all cases). On days 3, 6 and 7, no significant differences were observed between groups for ipsilateral turning behavior.

Statistical comparisons between ipsilateral and contralateral rotational behavior showed that, on the first day of treatment, only the lowest dose of scopolamine (5 mg/kg) plus saline produced significantly more ipsilateral than contralateral turns ($t = 2.44$, $P < 0.045$). With subsequent treatments (days 2 through 7), the number of ipsilateral turns were not significantly different from those of contralateral turns observed at any of the doses of scopolamine used.

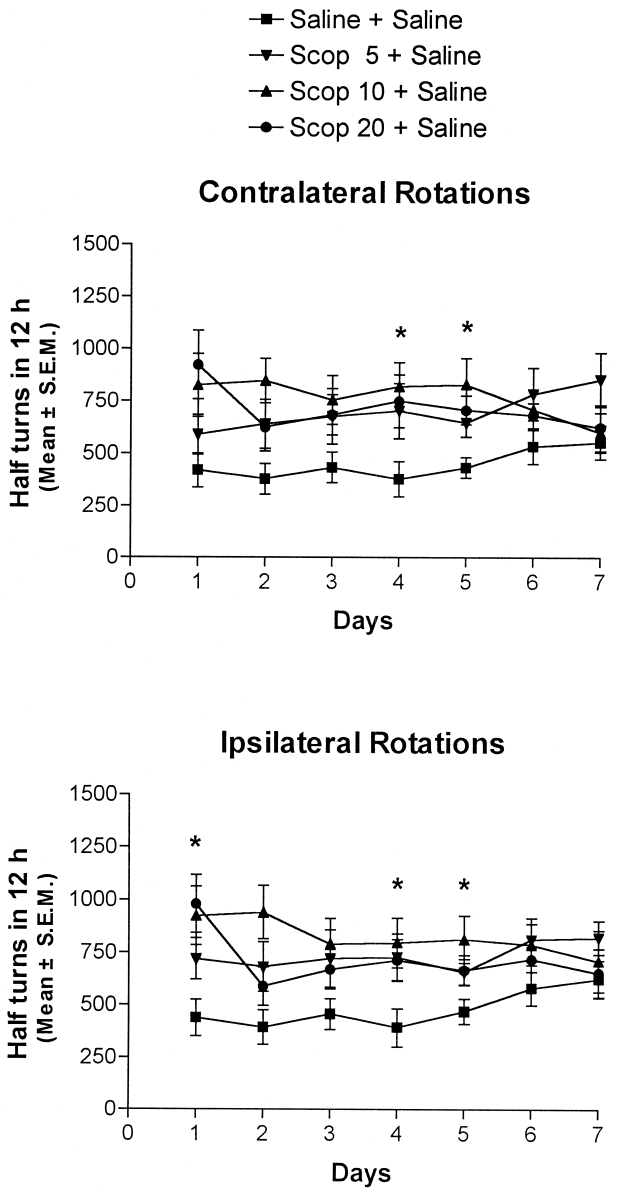

Fig. 1. Effects of repeated treatment with different doses of scopolamine (5, 10 and 20 mg/kg, s.c.) plus saline for 7 consecutive days on contralateral (upper panel), and ipsilateral (lower panel) rotational behavior in unilateral 6-hydroxydopamine-denervated rats. The asterisks (*) denote significant differences (*t*-test $P = 0.01$) between all the groups treated with scopolamine plus saline and the group treated with saline plus saline.

3.3. Effects of caffeine plus saline or in combination with scopolamine on contralateral rotational behavior

Fig. 2 (upper panel) shows the effects of caffeine plus saline or caffeine in combination with scopolamine (5, 10 and 20 mg/kg) on contralateral rotational behavior. Statistical analysis showed a significant main effect of Group $F(7,65) = 26.71$, $P < 0.001$, and a significant Group × Days interaction $F(42,390) = 8.69$, $P < 0.00$. Comparisons between groups for each day of treatment showed

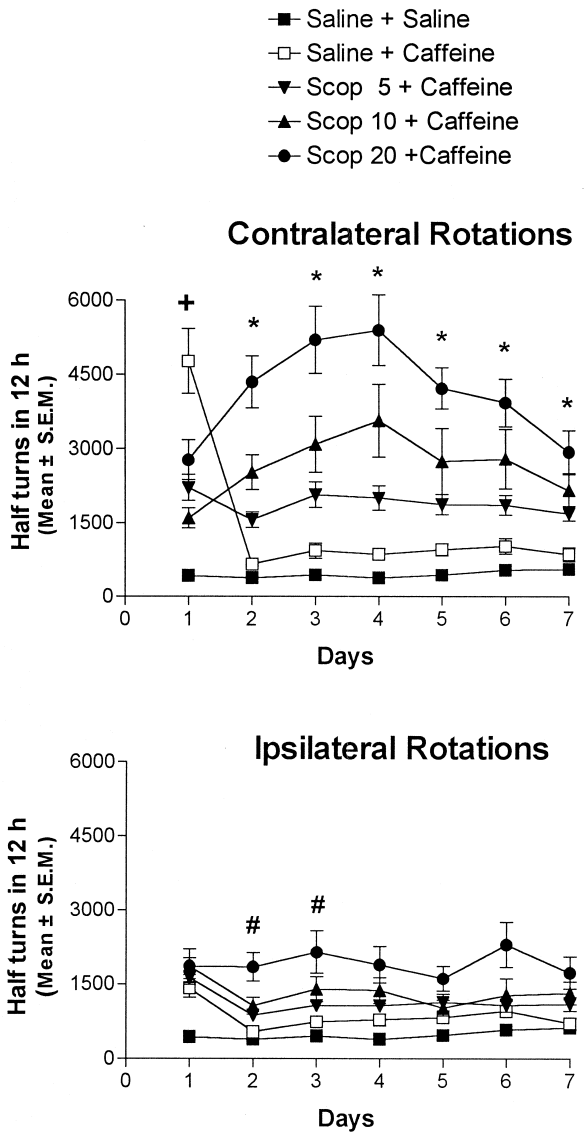

Fig. 2. Effects of repeated treatment with caffeine (40 mg/kg, s.c.) plus saline, or caffeine in combination with different doses of scopolamine (5, 10 and 20 mg/kg, s.c.) for 7 consecutive days on contralateral (upper panel), and ipsilateral (lower panel) rotational behavior in unilateral 6-hydroxydopamine-denervated rats. The plus sign (+) indicates a significant attenuation of contralateral turning by the co-administration of caffeine with different doses of scopolamine with respect to caffeine plus saline on the first day of treatment (*t*-test $P = 0.05$). The asterisks (∗) denote a significant potentiation of contralateral rotational behavior by treatment with caffeine plus all doses of scopolamine with respect to treatment with caffeine plus saline as of the second day of treatment (*t*-test $P = 0.004$). The number sign (#) denotes significant differences between the groups treated with caffeine plus the different doses of scopolamine and the group treated with caffeine plus saline on ipsilateral rotational behavior (*t*-test $P = 0.04$).

that, on the first day, all groups had significantly more contralateral rotational behavior than the group injected with saline–saline (caffeine plus saline: $t = 10.34$, $P < 0.001$; caffeine plus scopolamine 5, 10 and 20 mg/kg: $t \geq 5.74$, $P < 0.001$, in all cases). The groups injected with caffeine plus scopolamine showed significantly more contralateral turning than the groups injected with scopolamine plus saline $F(5,46) = 17.64$, $P < 0.0001$, indicating that caffeine potentiated the effects of scopolamine on the first day of treatment. However, the groups injected with scopolamine plus caffeine presented significantly less contralateral rotational behavior than the group injected with caffeine plus saline (5 mg/kg: $t = 3.06$, $P < 0.004$; 10 mg/kg: $t = 4.38$, $P < 0.001$; 20 mg/kg: $t = 1.95$, $P < 0.05$), indicating that scopolamine attenuated the acute effects of caffeine on contralateral rotational behavior. On the second day of treatment, the group injected with caffeine plus saline showed less contralateral rotational behavior than on the first day of treatment ($t = 8.43$, $P < 0.001$), revealing the development of tolerance to the effects of caffeine. In contrast, the groups injected with caffeine plus scopolamine showed significantly higher contralateral rotational behavior than the group injected with saline–saline ($t \geq 4.37$, $P < 0.001$, in all cases), and than the group injected with caffeine plus saline ($t \geq 5.74$, $P < 0.001$, in all cases), indicating that when caffeine is co-administered with scopolamine, no tolerance is observed to its effects on contralateral rotational behavior. On days 3 through 7, the group injected with caffeine plus saline still showed tolerance, and the groups injected with caffeine plus scopolamine showed significantly higher rotational behavior than the group injected with caffeine plus saline ($t \geq 3.04$, $P = 0.004$, in all cases). Significant differences between all doses of scopolamine in combination with caffeine were found only on days 2 and 4 ($t \geq 2.53$, $P = 0.004$, in all cases). On days 3, 5 and 6, the group injected with 20 mg/kg of scopolamine plus caffeine showed significantly higher rotational behavior than the groups with the other two doses of scopolamine ($t \geq 3.42$, $P = 0.003$). On day 7, no significant differences were found in the groups injected with the different doses of scopolamine.

As shown by the trend analysis, the pattern of rotational behavior on successive days was different between the group injected with caffeine plus saline and the groups injected with caffeine plus scopolamine. A significant linearly ascending trend was observed for the group injected with saline–saline ($t = 2.32$, $P < 0.002$). A significant linearly descending trend was observed for the group injected with caffeine plus saline ($t = 5.90$, $P < 0.001$), as well as a significant quadratic trend ($t = 6.86$, $P < 0.001$), indicating that caffeine produced one inflection point over the 7 day treatment, i.e., a decrease in rotational behavior as of the second day of treatment. No significant trend was found for the group injected with caffeine plus scopolamine 5 mg/kg, and a significant quadratic trend was found for the other two groups injected with scopolamine plus caffeine (10 mg/kg: $t = 3.26$, $P < 0.003$, 20 mg/kg: $t = 3.40$, $P < 0.001$), showing a maximum increase in rotational behavior on day 4, followed by a decrease, which on day 7, reached levels similar to the ones ob-

served on day 1 for scopolamine plus caffeine, but still higher than those observed for the groups injected with saline-saline, or saline caffeine on day 7.

3.4. Effects of caffeine plus saline or in combination with scopolamine on ipsilateral rotational behavior

Fig. 2 (lower panel) shows the ipsilateral rotational behavior following caffeine administered with saline or with scopolamine (5, 10 and 20 mg/kg). There was a significant Group effect $F(4,33) = 13.87$, $P < 0.001$, and a significant Group × Days interaction $F(24,198) = 2.17$, P 0.003. Individual comparisons for the first day of treatment, showed that all groups had significantly increased ipsilateral rotational behavior with respect to the group injected with saline–saline (caffeine plus saline: $t = 4.81$, $P < 0.001$; caffeine plus scopolamine 5, 10 and 20 mg/kg: $t \geq 4.38$, $P < 0.001$). The groups treated with scopolamine plus caffeine did not differ significantly from the group treated with caffeine plus saline, but showed significantly more ipsilateral turning than the groups treated with scopolamine plus saline $F(5,38) = 5.78$, $P < 0.0006$. On the second and third days, the groups treated with scopolamine plus caffeine showed significantly higher ipsilateral rotational behavior with respect to the group treated with saline–saline ($t \geq 3.18$, $P < 0.004$), and with respect to the group treated with caffeine plus saline ($t \geq 2.24$, $P < 0.04$). No significant differences were found between the group injected with caffeine plus saline and the group injected with saline–saline. On the fourth day, all the groups showed significantly higher ipsilateral turning with respect to the group treated with saline–saline ($t \geq 2.51$, $P < 0.02$), and only the groups treated with scopolamine at the dose of 10 and 20 mg/kg plus caffeine showed significantly more ipsilateral turning than did the groups treated with caffeine plus saline ($t \geq 2.51$, $P < 0.02$). On the fifth day, all the groups showed significantly higher ipsilateral turning with respect to the group treated with saline-saline ($t \geq 2.26$, $P < 0.04$), and only the group treated with scopolamine 20 mg/kg, plus caffeine showed significantly more ipsilateral turning than did the groups treated with caffeine plus saline ($t \geq 2.26$, $P < 0.04$). On days 6 and 7, the groups treated with scopolamine plus caffeine showed significantly higher ipsilateral rotational behavior with respect to the saline–saline group ($t \geq 2.24$, $P < 0.04$). No significant differences were found between the group injected with caffeine plus saline and the group injected with saline-saline. On day 6, only the group injected with the dose of 20 mg/kg of scopolamine plus caffeine showed higher ipsilateral turning than the group injected with caffeine plus saline ($t = 3.36$, $P < 0.003$), and on day 7, both 10 and 20 mg/kg of scopolamine plus caffeine produced more ipsilateral turning than seen in the group treated with caffeine plus saline ($t \geq 2.63$, $P < 0.02$).

3.5. Effects of caffeine in combination with methylscopolamine on ipsilateral and contralateral rotational behavior

Fig. 3 shows the effects of caffeine co-administered with methylscopolamine (5, 10 and 20 mg/kg) on contralateral (upper panel) and ipsilateral (lower panel) rotational behavior. Statistical analysis showed that none of the doses of methylscopolamine injected with caffeine had a significant effect on contralateral rotational behavior at any of the 7 days of treatment as compared to caffeine plus

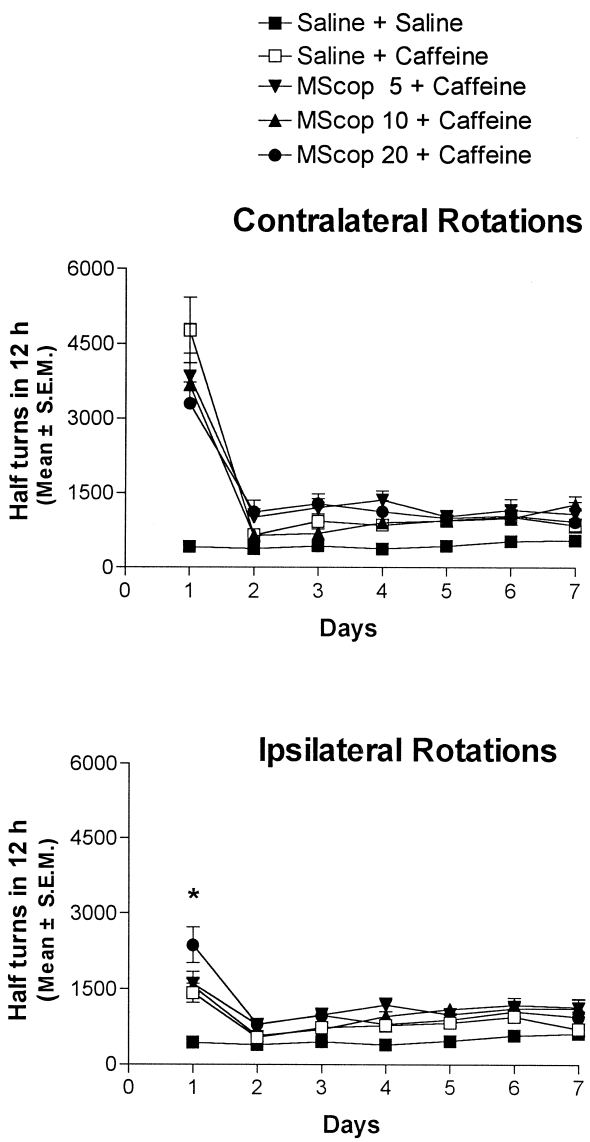

Fig. 3. Effects of repeated co-administration of caffeine (40 mg/kg) with different doses of methylscopolamine (5, 10 and 20 mg/kg, s.c.) for 7 consecutive days on contralateral (upper panel), and ipsilateral (lower panel) rotational behavior in unilateral 6-hydroxydopamine-denervated rats. The asterisk denotes a significant potentiation of ipsilateral turning on the first day of treatment with caffeine plus the dose of 20 mg/kg of methylscopolamine with respect to treatment with caffeine plus saline (Duncan, $P < 0.05$).

saline, indicating that methylscopolamine did not prevent the development of tolerance to caffeine effects on contralateral rotational behavior. For ipsilateral rotational behavior, and only on the first day of treatment, the dose of 20 mg/kg of methylscopolamine plus caffeine significantly potentiated ipsilateral turning with respect to caffeine plus saline treatment $F(3,33) = 3.01,\ P < 0.05$. The pattern of rotational behavior for the groups injected with methylscopolamine plus caffeine on successive days was similar to the one observed in the group injected with caffeine plus saline, but different from the pattern in the groups injected with scopolamine plus caffeine. All the groups injected with methylscopolamine showed a similar significant quadratic trend ($t \geq 4.90,\ P < 0.003$).

4. Discussion

The present results show that the muscarinic antagonist, scopolamine, attenuates caffeine-induced contralateral rotational behavior under acute conditions, but prevents the tolerance to caffeine-induced contralateral turning when injected repeatedly. These findings demonstrate an important role for cholinergic processes in caffeine-induced rotational behavior in unilateral 6-hydroxydopamine-lesioned rats. Several studies have shown that scopolamine can potentiate rotational behavior induced by dopamine receptor agonists such as apomorphine (Carey, 1991), or the dopamine D_1 receptor agonist, SKF 38393 (Morelli et al., 1993a), in denervated rats. In addition, the muscarinic antagonist, atropine, has been shown to reduce the inhibitory effects of the adenosine A_2 receptor agonist, CGS 21680, on apomorphine-induced contralateral turning in 6-hydroxydopamine-denervated rats (Vellucci et al., 1993). Caffeine-induced contralateral rotational behavior requires dopamine receptor supersensitivity in the denervated striatum (Herrera-Marschitz et al., 1988). It has been shown that caffeine's effects on rotational behavior could involve both dopamine D_1 and D_2 receptors (Herrera-Marschitz et al., 1988; Garrett and Holtzman, 1994a), as well as adenosine receptors (Snyder et al., 1981; Daly et al., 1981; Nikodijevic et al., 1993), or an interaction between A_2/D_2 receptors (Fuxe et al., 1993). Therefore, our interpretation of results must take into account the possible cholinergic interactions with both the dopamine and adenosine systems within the basal ganglia circuitry mediating motor behavior in denervated rats.

In the rat striatum, there are two major neuronal efferent populations controlling the activity of the basal ganglia output nuclei. One neuronal population projects directly to the substantia nigra pars reticulata and the entopeduncular nucleus, comprising what is called the direct pathway. The other neuronal population projects to the globus pallidus, which in turn projects to the subthalamic nucleus, and this structure projects to the substantia nigra and entopeduncular nucleus, comprising the indirect pathway (see Gerfen, 1992 for review). It has been proposed that normal motor activity depends on balanced neuronal activity in the direct and indirect pathways. Following unilateral 6-hydroxydopamine lesions of the nigrostriatal system, elevation of the activity in the indirect pathway, but not the direct pathway, results in increased firing of GABAergic neurons in the two output nuclei, leading to decreased behavioral activity (Gerfen et al., 1990).

Adenosine A_2 receptors are found in the GABAergic striatopallidal neurons (Schiffmann et al., 1991) where they are co-localized with dopamine D_2 receptors (Fink et al., 1992), and where they modulate the dopamine D_2 receptor-mediated inhibition of the indirect pathway. In addition, there is also evidence showing that adenosine A_{2a} receptors are localized on nerve terminals of the striatal cholinergic interneurons where they mediate stimulatory effects on the release of acetylcholine (Brown et al., 1990; Jin et al., 1993; Kurokawa et al., 1996). On the other hand, adenosine A_1 receptors are found on most GABAergic striatal neurons (Ferré et al., 1996), but are known to interact specifically with dopamine D_1 receptors (Ferré et al., 1994) which are more extensively localized on the striatonigral and striato-entopeduncular efferent neurons (Ferré et al., 1996). Adenosine A_1 receptors are also localized on cholinergic interneuron nerve terminals (Ferré et al., 1996), where they mediate inhibitory effects on the release of acetylcholine (Brown et al., 1990; Jin et al., 1993). The excitability of the direct and indirect striatal pathways can also be modulated by cholinergic activity through postsynaptic muscarinic m1 receptors expressed in striatonigral neurons that also express dopamine D_1 receptors, and in striatopallidal neurons that also express dopamine D_2 receptors, and through postsynaptic muscarinic m4 receptors expressed mostly in striatonigral neurons (Weiner et al., 1990; Gerfen, 1992). Stimulation of these receptors has been shown to modulate neuronal excitability in the two striatal populations in opposite directions, such that acetylcholine inhibits the direct pathway, and excites the indirect pathway (Bernard et al., 1993).

We found that acute systemic injections of scopolamine plus saline produced moderate amounts of both ipsilateral and contralateral rotational behavior, but no significant differences were found with respect to the magnitude of these two behaviors. These findings suggest that the effects of scopolamine on rotational behavior are probably mediated in both the denervated and the non-denervated sides. The moderate amounts of ipsilateral turning produced by scopolamine could be due to its ability to enhance the release of acetylcholine by blockade of presynaptic muscarinic m2 receptors in the intact striatum, as it has been shown that intrastriatal injections of the cholinergic agonist, carbachol, produce turning contralateral to the injection site in intact rats (McKenzie et al., 1991). Alternatively, this effect can be mediated through scopolamine's action in the intact substantia nigra. Recent behavioral

studies show that the locomotor activating effects of scopolamine are mediated through its ability to block presynaptic muscarinic receptors in the substantia nigra, disinhibiting mesopontine cholinergic neurons that can activate dopamine neurons (Mathur et al., 1997). This dopaminergic activation produced by scopolamine (Chapman et al., 1997), appears to involve the cholinergic stimulation of nicotinic receptors in the substantia nigra (Blaha and Winn, 1993; Gongora-Alfaro et al., 1996). The moderate contralateral turning produced by scopolamine probably results from blockade of postsynaptic muscarinic receptors in striatopallidal neurons in the denervated striatum, removing to some degree the overactivation in the indirect pathway.

Caffeine plus saline on the other hand, produced significantly more contralateral than ipsilateral turning, confirming previous data showing that the acute effects of caffeine are predominantly mediated in the denervated side (Herrera-Marschitz et al., 1988). However, caffeine did produce small amounts of ipsilateral turning, which may be attributed to blockade of presynaptic adenosine A_1 receptors in the non-denervated striatum modulating dopamine release at this level (Jin et al., 1993). A more recent microdialysis study showed that caffeine increases, and adenosine A_1 receptors agonists decrease dopamine release in the striatum of intact rats (Okada et al., 1996). The high levels of contralateral turning induced by caffeine may be mediated through its ability to remove the overexcitation present in the indirect pathway, thus regulating neuronal activity in the output nuclei. This situation can occur through two separate mechanisms. First, an inhibitory postsynaptic interaction has been demonstrated in the striatum between A_2/D_2 receptors (Ferré et al., 1991a), and this interaction seems to be enhanced in dopamine-denervated rats (Ferré and Fuxe, 1992). Thus, by blocking postsynaptic adenosine A_{2a} receptors on striatopallidal neurons, caffeine would stimulate a dopamine D_2 receptor-mediated inhibition of the indirect pathway. Second, by blocking presynaptic adenosine A_2 receptors located on cholinergic interneurons, caffeine may decrease the release of acetylcholine, which would also remove the muscarinic m1 receptor-mediated excitation of this pathway. In addition to the A_2/D_2 receptor interaction present in the striatum, there has been shown the existence of an inhibitory postsynaptic interaction between adenosine A_1 and dopamine D_1 receptors regulating the excitability of striato-entopeduncular neurons (Ferré et al., 1994, 1996). Therefore, caffeine may also produce contralateral turning by blocking postsynaptic adenosine A_1 receptors, promoting the dopamine D_1 receptor-mediated inhibition of the output nuclei through the direct pathway, thus counterbalancing the overactivation provided by the indirect pathway. This mechanism would be consistent with the data showing that contralateral rotational behavior can be attenuated by both dopamine D_1 and D_2 receptor antagonists (Herrera-Marschitz et al., 1988).

The co-administration of caffeine plus scopolamine potentiated the effects of scopolamine plus saline to a greater extent on contralateral rotational behavior than on ipsilateral turning. This suggests that caffeine and scopolamine may have synergistic actions in the denervated striatum, where they both remove the overexcitation of the indirect pathway, or increase the inhibition of the direct pathway. However, our results also showed that caffeine plus saline produced significantly more contralateral rotational behavior than caffeine plus scopolamine, implying that scopolamine may act in opposition to caffeine in a different area. A possible mechanism mediating this effect is one where scopolamine increases inhibitory nigral output leading to inhibition of motor output. A recent study showed that acetylcholine or carbachol increased the spontaneous [^{3}H]GABA release in the substantia nigra, and this effect was blocked by the muscarinic receptor antagonist, atropine (Kayadjanian et al., 1994). The authors concluded that the excitability of nigral efferent neurons can be modulated by acetylcholine through the activation of muscarinic receptors, probably the m4 subtype localized on striatonigral terminals. Therefore, it is possible that scopolamine, by blocking these muscarinic m4 receptors, can increase nigral activity by reducing the release of GABA, producing an attenuation of caffeine-induced contralateral turning.

With repeated administration, tolerance to caffeine-induced contralateral and ipsilateral rotational behavior was observed as of the second day of treatment. Previous studies have shown similar effects on contralateral turning with repeated injections of caffeine in denervated rats (Watanabe et al., 1982). Tolerance to the psychostimulant effects of caffeine has been linked to changes in adenosine receptors, since repeated caffeine injections produce changes in the sensitivity of both adenosine A_1 (Fredholm, 1982; Kaplan et al., 1993), and A_2 (Hawkins et al., 1988) receptors in rats. It has also been hypothesized, however, that tolerance to the effects of caffeine on locomotor activation may also involve changes in the dopamine system (Garrett and Holtzman, 1994b). In addition, it appears that the densities of acetylcholine receptors are also modified with chronic ingestion of caffeine, with reports showing both increases in muscarinic and nicotinic receptors in the mouse brain (Shi et al., 1993), and decreases in the number of cholinergic receptors in rat cortical neurons (Lin and Phillis, 1990). However, behavioral studies point to an enhancement of cholinergic function after chronic caffeine in mice, since higher doses of scopolamine are needed to stimulate open-field locomotor activity with respect to control mice (Nikodijevic et al., 1993).

The major finding in this study was that scopolamine prevented the development of tolerance to the effect of caffeine on contralateral rotational behavior that appeared with repeated administration. This effect seems to be mediated centrally, since the co-administration of caffeine with

methylscopolamine, a muscarinic receptor antagonist which does not easily cross the blood-brain barrier, did not prevent the tolerance to caffeine. The increase in contralateral turning observed with scopolamine plus caffeine as compared to caffeine plus saline, e.g., the prevention of tolerance to the effect of caffeine, is probably not related to an unspecific increase in contralateral turning induced by repeated treatment with scopolamine since no such effect was observed when scopolamine was injected with saline. These results suggest strongly that muscarinic cholinergic processes in the denervated striatum are involved in the tolerance to caffeine-induced contralateral turning. Following repeated caffeine administration, adenosine A_1 and A_2 receptors appear to be up-regulated (Fredholm, 1982; Hawkins et al., 1988; Kaplan et al., 1993). These adaptive changes may favor an agonistic action of endogenous adenosine over the antagonistic action by caffeine on these receptors. Under such circumstances, stimulation of postsynaptic adenosine A_1 and A_2 receptors located on striatonigral and striatopallidal efferent neurons, respectively, would be expected to produce effects opposite to those produced by acute injections of caffeine namely, decreased contralateral turning. Scopolamine, by blocking excitatory muscarinic m1 receptors located on striatopallidal neurons, and inhibitory muscarinic m4 receptors located on striatonigral and striato-entopeduncular neurons would act in opposition to adenosine, and prevent the observed tolerance. Additionally, tolerance to caffeine effects on contralateral turning may also involve the presynaptic modulation of acetylcholine release by endogenous adenosine through stimulation of both A_1 and A_2 receptors on cholinergic interneurons in striatum. In this case, scopolamine, by blocking postsynaptic muscarinic receptors, would antagonize the effects of increased cholinergic activity in the direct and indirect pathways, and prevent tolerance to caffeine-induced contralateral turning.

Finally, we cannot completely rule out the possibility that our results showing greater contralateral turning with scopolamine plus caffeine as compared to that with caffeine plus saline with repeated treatment were due to conditioning factors. Indeed, it has been shown that caffeine-induced (Carey, 1990), or apomorphine-induced (Silverman and Ho, 1981; Carey, 1986a,b; Casas et al., 1989c) contralateral rotational behavior can be conditioned to the specific testing environment. Additionally, there is evidence suggesting that Pavlovian conditioning can take place between scopolamine and apomorphine when they are co-administered (Carey, 1991).

The unilateral 6-hydroxydopamine nigrostriatal lesion rat model is currently used to screen potential therapeutic drugs to be introduced in the treatment of Parkinson's disease (see Schwarting and Huston, 1996, for review). Because there can be variability in the lesions produced, it is a commonly used procedure to select the successfully denervated animals by injecting low doses of apomorphine or L-DOPA (L-3,4-Dihydroxyphenylalanine) and measuring rotational behavior prior to the experimental treatments (Rouillard and Bédard, 1988; Morelli et al., 1993a, 1995; Fenu et al., 1997; Oh et al., 1997). However, it has been shown that prior experience with dopaminergic agonists may lead to persistent behavioral and physiological changes in this model, e.g., priming effects (Morelli et al., 1989, 1990, 1993b; Pollack et al., 1997). Thus, further investigation is needed to understand how the selection process interacts with subsequent drug effects. Nevertheless, the present findings have important clinical implications. It is known that the usefulness of methylxanthines in neurological disorders such as Parkinson's disease and dyskinesias is very limited owing to the rapid development of tolerance to its psychostimulant effects. Thus, it is possible that the association of methylxanthines with scopolamine would allow methylxanthines to be administered repeatedly without any observable tolerance, improving the effectiveness of these drugs in the treatment of Parkinson's disease.

Acknowledgements

We would to thank A. Rubio and S. López for excellent technical assistance and M. Lahoz for typing this manuscript. Work supported by grants from CITRAN (1991/2, 1992/2, 1993/2), CICYT PM91-0032, and Institut de Reserca Hospital Sant Pau (1995–1996).

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