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RESEARCH PAPER

Adenosine A_{2A} receptors are necessary and sufficient to trigger memory impairment in adult mice

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BACKGROUND AND PURPOSE

Caffeine (a non-selective adenosine receptor antagonist) prevents memory deficits in aging and Alzheimer's disease, an effect mimicked by adenosine A_{2A} receptor, but not A_1 receptor, antagonists. Hence, we investigated the effects of adenosine receptor agonists and antagonists on memory performance and scopolamine-induced memory impairment in mice.

EXPERIMENTAL APPROACH

We determined whether A_{2A} receptors are necessary for the emergence of memory impairments induced by scopolamine and whether A_{2A} receptor activation triggers memory deficits in naïve mice, using three tests to assess short-term memory, namely the object recognition task, inhibitory avoidance and modified Y-maze.

KEY RESULTS

Scopolamine (1.0 mg·kg^{-1}, i.p.) impaired short-term memory performance in all three tests and this scopolamine-induced amnesia was prevented by the A_{2A} receptor antagonist (SCH 58261, 0.1–1.0 mg·kg^{-1}, i.p.) and by the A_1 receptor antagonist (DPCPX, 0.2–5.0 mg·kg^{-1}, i.p.), except in the modified Y-maze where only SCH58261 was effective. Both antagonists were devoid of effects on memory or locomotion in naïve rats. Notably, the activation of A_{2A} receptors with CGS 21680 (0.1–0.5 mg·kg^{-1}, i.p.) before the training session was sufficient to trigger memory impairment in the three tests in naïve mice, and this effect was prevented by SCH 58261 (1.0 mg·kg^{-1}, i.p.). Furthermore, i.c.v. administration of CGS 21680 (50 nmol) also impaired recognition memory in the object recognition task.

CONCLUSIONS AND IMPLICATIONS

These results show that A_{2A} receptors are necessary and sufficient to trigger memory impairment and further suggest that A_1 receptors might also be selectively engaged to control the cholinergic-driven memory impairment.

Abbreviations

AD, Alzheimer's disease; CGS 21680, 3-[4-[2-[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl] purin-2-yl]amino]ethyl]phenyl]propanoic acid; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; M1, muscarinic receptors type 1; SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; SCO, scopolamine; veh, vehicle

Tables of Links

TARGETS	
A_1 receptor	M_1 receptor
A_{2A} receptor	

LIGANDS		
ACh	CGS 21680	Scopolamine
Adenosine	DPCPX	SCH 58261

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Adenosine plays an essential role in the maintenance of brain homeostasis mainly acting through inhibitory adenosine A_1 receptors and facilitatory adenosine A_{2A} receptors (Fredholm *et al.*, 2005). The parallel and combined effects mediated by A_1 and A_{2A} receptors control basal synaptic transmission and plasticity, respectively, and contribute to the encoding of salient information in neuronal circuits (Cunha, 2008), thus affecting different behaviours ranging from locomotion to mood (Chen *et al.*, 2013). In particular, the participation of adenosine receptors in cognitive processes has been recognized over the years, as heralded by the ability of caffeine, a non-selective adenosine receptor antagonist, to control memory performance (reviewed in Cunha and Agostinho, 2010). Thus, the acute administration of caffeine improves the performance of rodents in the object recognition task (Costa *et al.*, 2008; Botton *et al.*, 2010) and inhibitory avoidance (Angelucci *et al.*, 1999) as well as the performance of discrimination tasks in humans (Borota *et al.*, 2014). Furthermore, the chronic consumption of caffeine attenuates cognitive dysfunction observed during aging or Alzheimer's disease (AD) in humans (Eskelinen *et al.*, 2009; Cao *et al.*, 2012) and animal models (Arendash *et al.*, 2006; Dall'Igna *et al.*, 2007; Espinosa *et al.*, 2013; Laurent *et al.*, 2014). From results obtained in animal models, it was further concluded that the anti-amnesic effect of caffeine in AD was mimicked by the selective blockade of A_{2A}, but not of A_1 receptors (Dall'Igna *et al.*, 2007; Canas *et al.*, 2009).

The hypofunction of the cholinergic system is considered a trigger for memory deterioration in aging and AD (reviewed in Bartus, 2000), in accordance with the well-documented amnesic effects of the muscarinic receptor antagonist, scopolamine (reviewed in Klinkenberg and Blokland, 2010). Notably, caffeine can prevent scopolamine-induced memory impairment in rodents (Nikodijević *et al.*, 1993; Botton *et al.*, 2010) and humans (Riedel *et al.*, 1995). However, since A_{2A} receptor activation enhances, while A_1 receptors inhibit, the evoked release of ACh in the limbic cortex (Cunha *et al.*, 1994; Rodrigues *et al.*, 2008), it would be expected that blockade of A_1 rather than of A_{2A} receptors might be able to prevent cholinergic hypofunction. Thus, we compared the effects of selectively blocking A_{2A} and A_1 receptors on the impairment of memory caused by scopolamine.

Methods

Animals and housing

Adult male CF1 mice (3–4 months old), obtained from *Fundação Estadual de Produção e Pesquisa em Saúde* (Porto Alegre/RS, Brazil), were housed in standard polypropylene cages (4–5 animals per cage), under a 12/12 h light/dark cycle (lights on at 07:00 h) with food and water *ad libitum*. Independent groups of mice were used for each behavioural test, which were performed between 08:00 h and 14:00 h. All experimental procedures were approved by the ethical committee of the Federal University of Rio Grande do Sul (Proc. n° 22534) and followed the recommendations of the NIH Guide for Care and Use of Laboratory Animals and of the *Sociedade Brasileira de Neurociências e Comportamento* (SBNeC). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Drugs

1,3-Dipropyl-8-cyclopentylxanthine (DPCPX, a selective A_1R antagonist), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261, a selective A_{2A}R antagonist) and 3-[4-[2-[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid (CGS 21680, an A_{2A}R agonist) were from Tocris (São Paulo/SP, Brazil) and were prepared as 5 mM stock solutions in saline with 20% DMSO and later dissolved to the desired concentration in saline. CGS 21680 was also infused into the lateral ventricles (coordinates: AP –1, LM 1.5 and DV +1.5) in a volume of 1 µL per side. Scopolamine hydrobromide trihydrate, a non-selective muscarinic receptor antagonist, was from Sigma-Aldrich (São Paulo/SP, Brazil), and was dissolved in saline. Drugs were administered i.p. in a volume of 10 $mL \cdot kg^{-1}$ of body weight at a minimal dose of each drug, with no effects on locomotion (El Yacoubi *et al.*, 2000a,b; Botton *et al.*, 2010).

Behavioural procedures

Different groups of mice were used for each behavioural task. Mice were handled for 2 min for 4 days before beginning the behavioural tasks and transferred to the testing room one hour before the habituation session. The testing room was sound-attenuated room with a low-intensity light (15 lux)

uniformly distributed throughout the arena and a temperature of 21 ± 2°C. The experiments were monitored by two observers blind to experimental protocols and recorded with a video camera positioned above the arena. The data were collected and analysed using the ANY-Maze video-tracking system (Stoelting CO, Woods Dale, IL, USA).

Open field

Mice were exposed to an open-field arena to evaluate locomotor activity and further the object recognition task. The first day corresponded to habituation to the apparatus and the second day to training and test sessions of 90 min. The apparatus was made of black-painted Plexiglas measuring 50 × 50 cm and was surrounded by 50-cm-high walls. Each mouse was placed in the centre of the arena and the distance travelled, the time and average speed of locomotor activity in metres was recorded for 10 min. The experiments were conducted in a sound-attenuated room under low-intensity light (12 lux); activity was recorded with a video camera positioned above the arena and monitored in an adjacent room by an observer blinded to the treatment of the animals. The open-field apparatus was cleaned after the end of each session.

Novel object recognition task

The object recognition task was carried out in a grey-painted wood open field apparatus (50 × 25 × 25 cm). For the habituation session, the mice were placed in the open field 24 h before the training session and allowed to explore the arena for 10 min. The training session consisted of allowing the mouse to explore an apparatus containing two similar objects (A and A′) for 10 min. The objects were identical sized (13 cm height) glass bottles, but of different shapes and colours, and were positioned in two adjacent corners, 9 cm from the walls. Each mouse was always placed in the apparatus facing the wall. The tests sessions, after the training, were performed either for 90 min for short-term memory or for 24 h for long-term memory. In the test session, one of the objects was replaced by a novel object and the mice were allowed to explore the objects for 10 min. The apparatus and objects were cleaned with a 70% ethanol solution between trials to eliminate odor cues. The locomotor activity was measured as the total distance travelled, as the percentage of total time engaged in locomotion and as the average speed. To calculate the object discrimination ratio, we first measured the time spent exploring each object, defined as the time a mouse spent directing its nose to the object at a distance ≤2 cm and/or touching the object with the nose or forepaws; the discrimination ratio was then expressed as TN/(TN + TF) where TF is the time spent exploring familiar object and TN is the time spent exploring the novel object.

Inhibitory avoidance task

The inhibitory avoidance task was assessed in an apparatus consisting of an acrylic box (30 cm × 25 cm × 20 cm) with a floor containing parallel stainless-steel bars (1 mm diameter) spaced 1 cm apart and 25 cm platform length on the left. In the training session, animals were gently placed on the platform facing the left rear corner of the training box. When they stepped down and placed their four paws on the grid, they received a 2 s, 0.5 mA scrambled footshock and were immediately withdrawn from the training box. The test session was carried out 90 min after the training session (short-term memory) or 24 h after the training session (long-term memory). No footshock was given in the test session, and step-down latencies (180 s ceiling) were taken as a measure of retention.

Modified Y-maze

The Y-maze apparatus consisted of three arms (18 cm long, 6 cm wide and 6 cm high) made of wood covered with impermeable Formica elevated to a height of 50 cm above the floor. We used this task to assess short-term spatial memory, which is based on the innate preference of animals to explore areas that have not been previously explored. This task consisted of two trials (training and test) of 8 min each separated by an intertrial interval of 120 min. During the training trial, one arm (novel) was blocked by a removable door and mice were placed at the end of the one arm (start) facing the centre and they could chose between the start and the 'other' arm. At the end of the training trial, the mouse was removed from the maze and back housing box during the inter-trial interval (120 min). During the test trial, the novel arm was opened and the animals were once again placed at the start arm and allowed to explore freely the three arms for 8 min. The number of entries and the time spent in each arm were recorded. Entry into an arm was defined as placement of all four paws into the arm.

Experimental design

The schedules of administration of drugs and of experimental manipulations are depicted in the timelines displayed in Figure 1.

Experiment 1. Mice received a single i.p. injection of either vehicle (veh – 0.9% saline) or scopolamine (0.1, 0.3 or 1.0 $mg \cdot kg^{-1}$) 30 min before the training session. The same procedure was performed in another group of animals for DPCPX (0.2, 1.0 or 5.0 $mg \cdot kg^{-1}$, i.p.) or SCH 58261 (0.1, 0.5 or 1.0 $mg \cdot kg^{-1}$, i.p.) and for their respective controls (0.9% saline with 20% DMSO). The pharmacokinetic profile of both antagonists shows that they rapidly reach the brain parenchyma where their concentration remains at effective levels for over 120 min (Yang *et al.*, 2007; Elmenhorst *et al.*, 2013). In this experiment, each animal received only one drug in a single dose before the training session.

Experiment 2. Mice first received scopolamine (1.0 $mg \cdot kg^{-1}$, i.p.) or vehicle 60 min before the training session. After 30 min, the mice received a second injection of vehicle, DPCPX (1.0 $mg \cdot kg^{-1}$, i.p.) or SCH 58261 (0.5 $mg \cdot kg^{-1}$, i.p.). The experimental groups were as follows: vehicle (veh + veh), scopolamine (SCO + veh), scopolamine + DPCPX (SCO + DPCPX) and scopolamine + SCH 58261 (SCO + SCH). In order to minimize the number of animals and based on the data from experiment 1, we did not administer DPCPX or SCH 58261 to mice previously injected with vehicle for the novel object recognition task. In the inhibitory avoidance task and modified Y-maze, DPCPX (1.0 $mg \cdot kg^{-1}$, i.p.) or SCH 58261 (0.5 $mg \cdot kg^{-1}$, i.p.) were administered with vehicle.

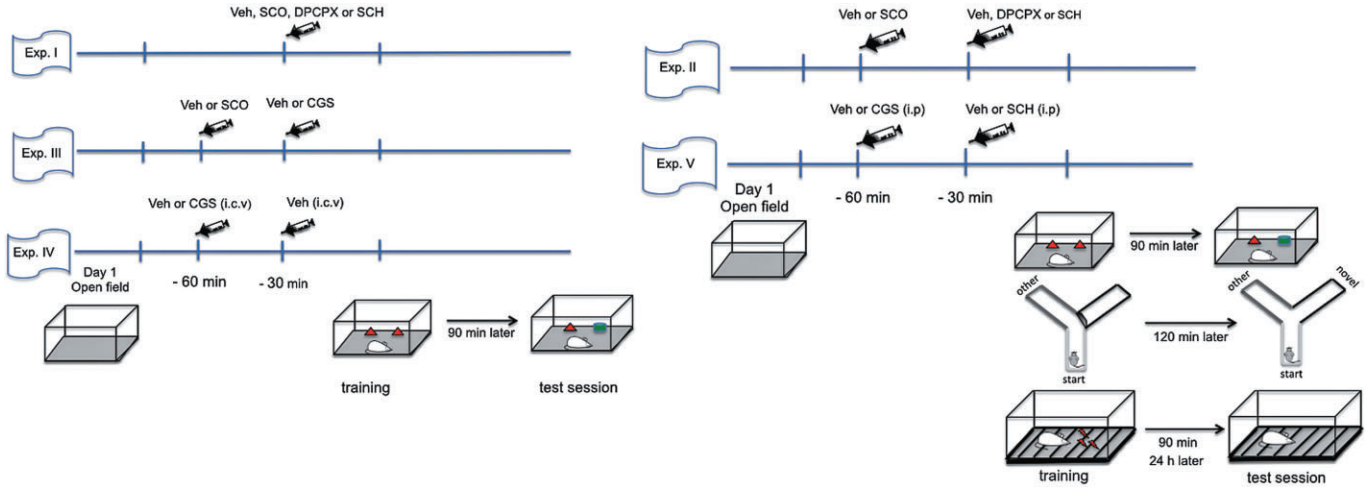

Figure 1

Schematic overview of the experimental design. In all experiments, all groups of mice were submitted to open-field analysis 24 h before the training session. In experiment 1 (Exp. 1), DPCPX (A_1 receptor antagonist, 0.2–5 mg·kg^{-1}, i.p.) or SCH 58261 (A_{2A} receptor antagonist, 0.1–1 mg·kg^{-1}, i.p.) or scopolamine (SCO; 0.1–1 mg·kg^{-1}, i.p.) was administered to different mice 30 min before training of the object recognition task. The test session was performed either 90 min or 24 h after training. In Experiment 2 (Exp. 2), scopolamine (1.0 mg·kg^{-1}, i.p.) was administered 30 min before injecting DPCPX (1.0 mg·kg^{-1}, i.p.) or SCH 58261 (0.5 mg·kg^{-1}, i.p.). The training session was carried out 30 min after and the test session was performed 90 min for object recognition and inhibitory avoidance tasks, and also 24 h for inhibitory avoidance or 120 min for the modified Y-maze task. In experiment 3 (Exp. 3), scopolamine (1.0 mg·kg^{-1}, i.p.) was administered 30 min before injecting the A_{2A} receptor agonist CGS 21680 (0.1 mg·kg^{-1}, i.p.). In experiment 4 (Exp. 4), CGS 21680 (50 nmol) was infused into the lateral ventricles (1 µL per side) 30 min before training. Test session was performed 90 min after training in the object recognition task. In experiment 5 (Exp. 5), CGS 21680 (0.1 mg·kg^{-1}, i.p.) was administered 30 min before SCH 58261 (0.5 mg·kg^{-1}, i.p.). The training session was carried out for 30 min and the test session was performed after 90 min for object recognition task, 90 min and 24 h for inhibitory avoidance task and 120 min for the modified Y-maze task.

Experiment 3. Mice received scopolamine (1.0 mg·kg^{-1}, i.p.) or vehicle 60 min before the training session. After 30 min, the mice received a second injection of either vehicle or CGS 21680 (0.1 mg·kg^{-1}, i.p.). We also tested the effects of CGS 21680 (0.05 mg·kg^{-1}, i.p.), and the groups in this experiment were vehicle (veh + veh), scopolamine (SCO + veh), CGS 21680 (veh + CGS) and scopolamine + CGS 21680 (SCO + CGS). The test session was performed only 90 min after training.

Experiment 4.

- Intracerebroventricular infusion: all surgical procedures were carried out under anaesthesia (ketamine: xylazine – 80:10 mg·kg^{-1} i.p.) and aseptic conditions. Cannulae (27-gauge) were bilaterally implanted into the lateral ventricles following coordinates: A –1.0, L ± 1.5, V –1.5, from the atlas of Paxinos and Watson. Animals were allowed to recover from surgery for 7 days. Mice received vehicle or CGS (50 nmol, 1 µL per side), 30 min before training. Infusions were carried out over 60 s with an infusion pump and cannulae were left for an additional 60 s to minimize backflow. The placement of the cannulae was verified *postmortem* 2–4 h after the end of behavioural test by staining with 1 µL of 4% methylene blue solution. Only data from animals with correct implants were analysed (95 %). The test session was performed only 90 min after the training session.
- Systemic administration: mice received CGS 21680 (0.1 mg·kg^{-1}, i.p.) or vehicle 60 min before the training session. After 30 min, the mice received either vehicle or SCH 58261 (0.5 mg·kg^{-1}, i.p.). The groups in this experiment were vehicle (veh + veh), CGS 21680 (veh + CGS) and CGS 21680 + SCH 58261 (CGS + SCH). In order to minimize the number of animals and based on the results from experiment 2, we did not administer SCH 58261 to mice previously injected with vehicle. The test session was performed only 90 min after the training session.

Statistical analysis

Data are presented as mean ± SEM. Two-way ANOVA was used to analyse the discrimination ratio with repeated measures (within-subject factor: sessions of behavioural test; between-subject factor: treatments) followed by a Tukey's multiple comparisons test. In some cases, Student's paired *t*-test was also used to analyse differences between training and test sessions within the same group. The locomotor activity and modified Y-maze task was analysed by one-way ANOVA followed by either a Tukey's multiple comparisons test or a Kruskal–Wallis test and a Dunn's multiple comparisons procedure. Data from inhibitory avoidance task are presented as median values (interquartile range) and analysed by Wilcoxon paired test for differences within groups (training vs. test sessions) and Mann–Whitney test for differences between groups. GraphPad Prism 6 software (São Paulo/SP, Brazil) was

used for statistical analysis and significance was considered as $P < 0.05$.

Results

Experiment 1

No differences were found in either the total distance travelled or the time engaged in locomotion or the average speed for each dose of DPCPX (Table 1), SCH 58261 (Table 2) or scopolamine tested (Table 3), when administered 30 min before the training session, in the object recognition task. The administration of DPCPX [$F_{(3,45)} = 2.150$; $P = 0.1071$] or SCH 58261 [$F_{(3,72)} = 1.429$; $P = 0.2413$] did not alter the discrimination ratio. In fact, all groups of mice were equally able to discriminate the novel from the familiar object with all doses of either DPCPX [$F_{(2,90)} = 42.33$; $P < 0.0001$] or SCH 58261 [$F_{(2,144)} = 26.39$; $P < 0.0001$] (Figure 2A and 2B respectively).

The highest dose of scopolamine tested, administered 30 min before the training session, impaired recognition memory. Thus, two-way ANOVA with repeated measures revealed differences between sessions [$F_{(2,84)} = 25.05$;

Table 1

Locomotor activity during the training session of object recognition task for different doses of DPCPX

	0.0 mg·kg^{-1}		0.2 mg·kg^{-1}		1.0 mg·kg^{-1}		5.0 mg·kg^{-1}			
	M	SEM	M	SEM	M	SEM	M	SEM	*F* value	*P* value
Distance travelled (m)	34.77	1.95	36.34	2.15	34.49	1.60	31.92	2.58	0.764	0.520
Time of locomotor activity (s)	489.3	7.18	486.6	10.87	500.7	8.59	443.2	25.23	3.103[a]	0.376
Average speed of locomotion (m·min^{-1})	4.25	0.21	4.48	0.25	4.14	0.20	4.31	0.21	0.435	0.29

Results from 12 to 13 animals per group.
Statistical analysis performed by one-way ANOVA or [a]Kruskal–Wallis test.
No significant differences.
M, mean.

Table 2

Locomotor activity during the training session of object recognition task for different doses of SCH58261

	0.0 mg·kg^{-1}		0.1 mg·kg^{-1}		0.5 mg·kg^{-1}		1.0 mg·kg^{-1}			
	M	SEM	M	SEM	M	SEM	M	SEM	KW value	*P* value
Distance travelled (m)	27.76	0.85	30.56	2.74	29.03	0.77	29.02	1.09	1.427	0.699
Time of locomotor activity (s)	485.6	6.52	488.2	14.50	501.8	6.56	508.5	7.61	4.024	0.259
Average speed of locomotion (m·min^{-1})	3.43	0.09	3.64	0.24	3.47	0.07	3.41	0.08	0.642	0.887

Results from 16 to 24 animals per group.
Statistical analysis performed by Kruskal–Wallis test.
M, mean.

Table 3

Locomotor activity during the training session of object recognition task for different doses of scopolamine

	0.0 mg·kg^{-1}		0.1 mg·kg^{-1}		0.3 mg·kg^{-1}		1.0 mg·kg^{-1}			
	M	SEM	M	SEM	M	SEM	M	SEM	KW value	*P* value
Distance travelled (m)	34.98	1.77	33.45	2.94	36.08	1.13	38.71	1.41	5.212	0.157
Time of locomotor activity (s)	451.3	25.80	455.6	24.41	481.7	12.36	471.2	8.78	2.352	0.503
Average speed of locomotion (m·min^{-1})	4.82	0.47	4.36	0.26	4.39	0.18	4.61	0.16	2.150	0.542

Results from 10 to 12 animals per group.
Statistical analysis performed by Kruskal–Wallis test.
M, mean.

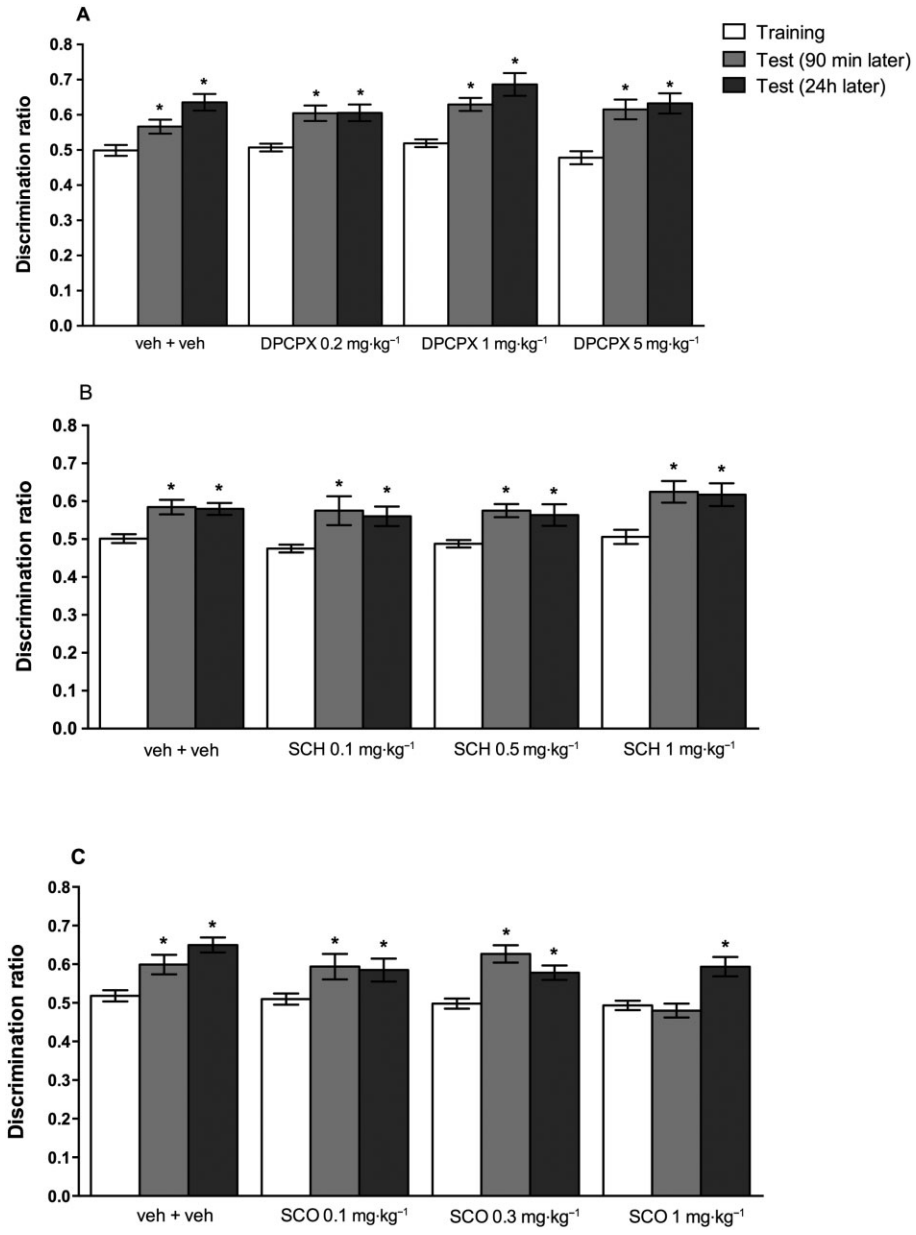

Figure 2

Dose-dependent effects of the adenosine receptor antagonists and of scopolamine (SCO) in the object recognition task (experiment 1). (A) The selective blockade of A_1 receptors by i.p. administration of DPCPX 30 min before the training session did not modify the discrimination ratio. Results are means ± SEM of n = 12–13 mice per group; *P < 0.05 versus training session. (B) The selective blockade of A_{2A} receptors by administration of SCH 58261 (SCH, i.p.) 30 min before the training session did not modify the discrimination ratio. Results are mean ± SEM of n = 16–20 mice per group; *P < 0.05 versus training session. (C) The blockade of muscarinic receptors by SCO (i.p.) 30 min before the training session only decreased the discrimination ratio at the highest tested dose and when testing was carried out 90 min after training. Results are means ± SEM of n = 10–12 mice per group; *P < 0.05 versus training session.

$P < 0.0001$], doses [$F_{(3,42)} = 3.736$; $P = 0.0182$] and interaction between sessions and doses [$F_{(6,84)} = 4.006$; $P = 0.0014$]. Mice that received vehicle and scopolamine at 0.1 and 0.3 mg·kg^{-1} were still able to discriminate the novel from the familiar object in both test sessions, whereas the administration of scopolamine (1.0 mg·kg^{-1}) impaired recognition memory when assessed 90 min after training, but not 24 h later (Figure 2C). Thus, all subsequent experiments focused only on short-term memory, with the test session performed 90 min after the training session, since this was the condition most sensitive to scopolamine.

Experiment 2

The role of A_1 or A_{2A} receptors in the scopolamine-induced memory impairment was evaluated by testing the effects of either the A_1 receptor antagonist DPCPX (1.0 mg·kg^{-1}) or of

Table 4

Locomotor activity during the training session of object recognition task for experiment 2

	veh + veh		Scop + veh		Scop + DPCPX		Scop + SCH58261			
	M	SEM	M	SEM	M	SEM	M	SEM	*F* value	*P* value
Distance travelled (m)	35.83	2.84	36.76	3.22	33.44	2.42	32.89	2.45	2.823[a]	0.4198
Time of locomotor activity (s)	482.1	12.20	487.5	37.13	484.9	18.57	476.9	16.98	2.724[a]	0.4361
Average speed of locomotion ($m \cdot min^{-1}$)	4.44	0.31	4.48	0.21	4.15	0.29	4.11	0.22	0.535	0.6610

Results from 11 animals per group.
One-way ANOVA or [a]Kruskal–Wallis test.
No significant differences.
M, mean; Scop, scopolamine; veh, vehicle.

the A_{2A} receptor antagonist SCH 58261 (0.5 $mg \cdot kg^{-1}$), administered 30 min after scopolamine (1.0 $mg \cdot kg^{-1}$), in the different memory tests. Neither DPCPX nor SCH 58261 without or with scopolamine affected locomotor activity (Table 4). By contrast, both adenosine receptor antagonists altered the effect of scopolamine on recognition memory. Two-way ANOVA with repeated measures revealed differences between sessions [$F_{(1,40)} = 51.69$; $P < 0.0001$], treatments [$F_{(3,40)} = 3.842$; $P = 0.0165$] and an interaction between sessions and treatments [$F_{(3,40)} = 3.139$; $P = 0.0357$]. Mice that received scopolamine (1.0 $mg \cdot kg^{-1}$) 60 min before training (scopolamine + veh) were not able to discriminate the novel from the familiar object. However, mice that received the A_1 receptor antagonist DPCPX (1.0 $mg \cdot kg^{-1}$) or the A_{2A} receptor antagonist SCH 58261 (0.5 $mg \cdot kg^{-1}$) 30 min after the scopolamine injection (SCO + DPCPX or SCO + SCH groups, respectively) were able to discriminate the objects (Figure 3A). In the inhibitory avoidance task, all groups presented differences between training and test sessions ($P < 0.05$), except mice treated with scopolamine ($P > 0.05$). However, the A_1 receptor antagonist DPCPX seemed more effective than the A_{2A} receptor antagonist at preventing the memory impairment caused by scopolamine (Figure 3B). The adenosine receptor antagonists had a different effect on the scopolamine-impaired memory performance in the modified Y-maze. Analysis of the percentage of exploration in three arms revealed that mice treated with vehicle spent more time exploring the novel arm compared with the other arms [$F_{(2,18)} = 18.61$; $P < 0.0001$] (Figure 3C). By contrast, mice treated with scopolamine did not show any differences ($P > 0.05$) in the time spent searching the different arms (Figure 3C). Notably, scopolamine-treated mice that received the A_{2A} receptor antagonist (SCO + SCH group) displayed again differences in the exploration of new arm when compared with the others [$F_{(2,21)} = 3.788$; $P = 0.0394$], whereas the SCO + DPCPX group still displayed no differences in the percentage of exploration of the different arms ($P > 0.05$) (Figure 3C). Finally, the antagonists were devoid of effects by themselves since both the SCH + veh [$F_{(2,15)} = 24.65$; $P < 0.0001$] and the DPCPX + veh [$F_{(2,15)} = 10.10$; $P = 0.0017$] groups spent more time searching the novel arm compared with the others.

Experiment 3

We then investigated if the activation of A_{2A} receptors further accentuates scopolamine-induced impairment of recognition memory. Thus, we administered the A_{2A} receptor agonist CGS 21680 (0.1 $mg \cdot kg^{-1}$) or vehicle 30 min after scopolamine (1.0 $mg \cdot kg^{-1}$), which was injected 60 min before training. CGS 21680 did not affect locomotor activity alone or when administered with scopolamine (Table 5). This contrasted with the decreased locomotion observed with CPA (0.3–1.0 $mg \cdot kg^{-1}$; El Yacoubi *et al.*, 2000b), which precluded testing the effect of A_1 receptor activation on the scopolamine-induced depression of recognition memory. The analysis of the effect of CGS 26180 showed that it did not significantly exacerbate the scopolamine-induced memory impairment, probably because it already caused memory impairment by itself: in fact, control mice (veh + veh group) were able to discriminate the novel from the familiar object and this was the only group displaying a significant difference between the training and test session (paired *t*-test, $t = 5.729$; $P = 0.0004$). In all other groups (SCO + veh, veh + CGS and SCO + CGS), no differences were found between sessions, suggesting that these mice were not able to discriminate the novel from the familiar object (Figure 4).

Experiment 4

In order to rule out peripheral effects of CGS 21680 as a cause for the memory impairment observed in the object recognition task, and in attempt to ensure that the effect found is predominantly central, CGS 21680 was directly infused into the lateral ventricles. As shown in Figure 5, the infusion of CGS 21680 (50 nmol) directly into the ventricles 30 min before training abolished recognition memory (Student's paired *t*-test, $t = 0.01216$; $P = 0.9906$; Figure 5).

Experiment 5

We next tested if the activation of A_{2A} receptors was sufficient to impair other types of short-term memory, beyond recognition memory, in control mice. Thus, we investigated if CGS 21680 (0.1 $mg \cdot kg^{-1}$) would impair aversive and spatial memory and if the blockade of A_{2A} receptors with SCH 58261

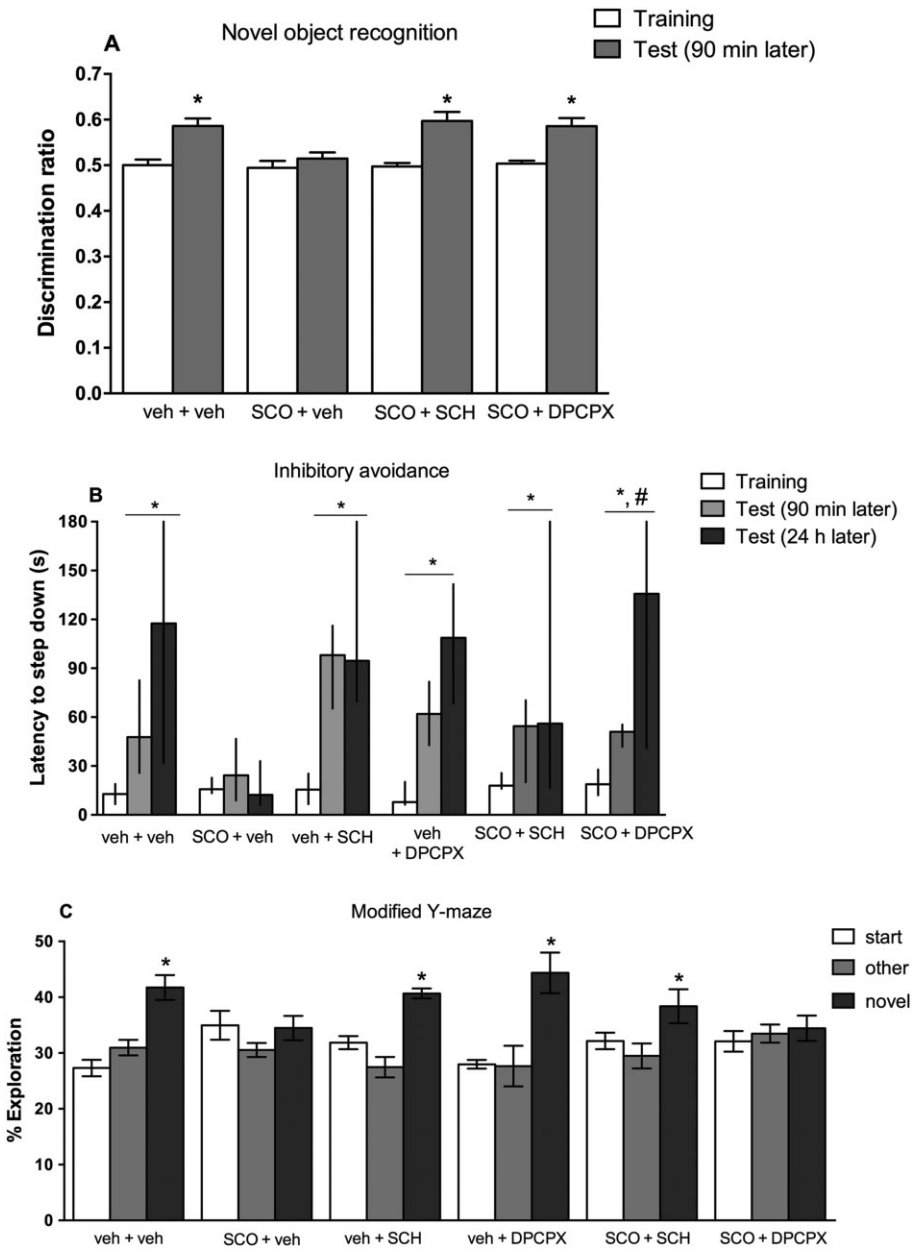

Figure 3

The selective blockade of either A_1 or A_{2A} receptors prevented the scopolamine (SCO)-induced impairment in short-term memory (experiment 2). DPCPX (A_1 receptor antagonist, 1.0 mg·kg^{-1}, i.p.) or SCH 58261 (SCH, A_{2A} receptor antagonist, 0.5 mg·kg^{-1}, i.p.) were administered 30 min after SCO (1.0 mg·kg^{-1}, i.p.), which was injected 60 min before training in three different tests, namely (A) discrimination ratio for object recognition task; data are mean ± SEM of n = 10–11 mice per group; *P < 0.05 versus training session. (B) Latencies in seconds (s) to step down from the platform in the inhibitory avoidance task; data are median and interquartile range of n = 6–10 mice per group; *P < 0.05, Wilcoxon paired t-test comparing training and test session within group; #P < 0.05, Mann–Whitney test comparing the groups SCO + veh and SCO + DPCPX in the test sessions; (C) percentage of the time of exploration in the three arms (start, other and novel) in the test trial of the modified Y-maze task; data are means ± SEM of n = 6–9 mice per group; *P < 0.05, one-way ANOVA and Tukey's multiple comparison test comparing the novel and other arms (start and other) within group. The tested groups were: veh + veh (vehicle); SCO + veh (SCO); DPCPX + veh (DPCPX) SCH + veh (SCH 58261); SCO + DPCPX (SCO + DPCPX) and SCO + SCH (SCO + SCH 58261).

(0.5 mg·kg^{-1}) would prevent the CGS 21680-induced memory impairment in the three tasks. Neither CGS 21680 nor SCH 58261 alone or in combination modified locomotor activity (Table 6). In contrast to control mice (veh + veh group), which recognized the novel from the familiar object as concluded from the differences between the training and test sessions (paired t-test, t = 7.859, P < 0.0001), the administration of CGS 21680 (0.1 mg·kg^{-1}) 60 min before the training

Table 5

Locomotor activity during the training session of object recognition task for experiment 3

	veh + veh		SCO + veh		veh + CGS21680		SCO + CGS21680			
	M	SEM	M	SEM	M	SEM	M	SEM	*F* value	*P* value
Distance travelled (m)	38.00	2.594	36.97	2.771	39.34	1.315	35.20	1.398	2.405[a]	0.4927
Time of locomotor activity (s)	407.1	28.99	380.9	22.13	428.4	23.00	375.8	30.52	0.7127	0.5499
Average speed of locomotion ($m \cdot min^{-1}$)	5.922	0.7482	6.128	0.6264	5.702	0.5255	6.059	0.5923	0.0104[a]	0.9997

Results from 8 to 16 animals per group.
One-way ANOVA or [a]Kruskal–Wallis.
No significant differences.
M, mean; Scop, scopolamine; veh, vehicle.

Table 6

Locomotor activity during the training session of object recognition task for experiment 4

	veh + veh		CGS21680 + veh		CGS21680 + SCH58261			
	M	SEM	M	SEM	M	SEM	*F* value	*P* value
Distance travelled (m)	33.06	3.182	35.12	3.640	31.88	2.891	0.2302	0.7961
Time of locomotor activity (s)	398.9	24.37	394.0	25.43	393.4	10.11	0.0524[a]	0.9741
Average speed of locomotion ($m \cdot min^{-1}$)	5.097	0.5375	5.628	0.7866	4.937	0.3934	0.3672	0.6962

One-way ANOVA (or Kruskal–Wallis). Results from 8 to 11 animals per group.
[a]Kruskal–Wallis test was used.
M, mean; veh, vehicle.

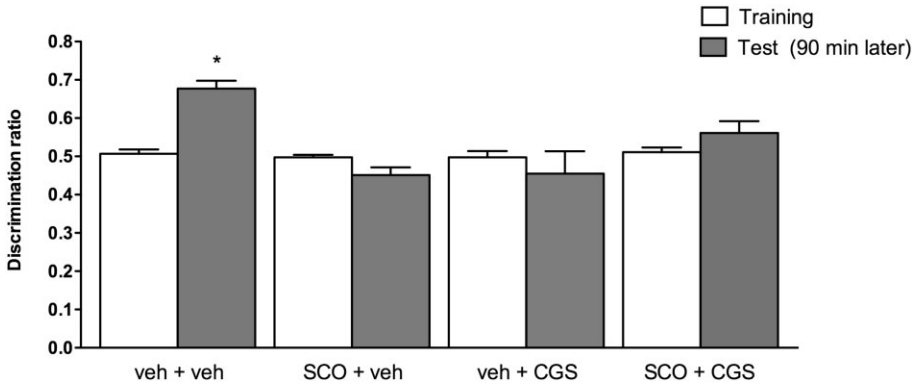

Figure 4

The activation of adenosine A_{2A} receptors did not exacerbate the scopolamine (SCO)-induced impairment of the discrimination ratio in the recognition memory test (experiment 3). The adenosine A_{2A} receptor agonist CGS 21680 (0.1 $mg \cdot kg^{-1}$) was administered i.p. 30 min before SCO (1.0 $mg \cdot kg^{-1}$, i.p.), which was injected 60 min before training. Data are means ± SEM of $n = 9–16$ mice per group; $^*P < 0.05$ versus training session.

session (CGS + veh group) impaired recognition memory, as evidenced by the lack of difference between training and test session ($P > 0.05$). Mice that received SCH 58261 (0.5 $mg \cdot kg^{-1}$) 30 min after CGS 21680 (0.1 $mg \cdot kg^{-1}$; SCH + CGS group) were again able to discriminate the novel from the familiar object, as judged by the difference between the training and test sessions (paired *t*-test, $t = 2.715$, $P = 0.0264$; Figure 6A).

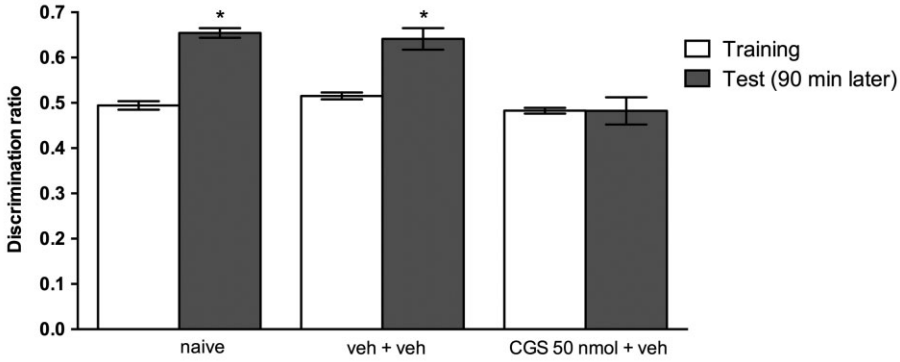

Figure 5

The i.c.v. infusion of CGS 21680 impaired the discrimination ratio in the recognition memory test (experiment 4). The adenosine A_{2A} receptor agonist CGS 21680 (50 nmol, 1 μL per side) was infused into lateral ventricles 30 min before training. Test session was carried out 90 min later. Naïve (n = 5); veh + veh (vehicle, n = 8) and CGS + veh (CGS 21680, n = 8). Data are means ± SEM *P < 0.05 versus training session.

In the inhibitory avoidance test, all groups of mice presented differences between training and both test sessions (90 min or 24 h after training; $P < 0.05$). However, CGS 21680 (0.1 mg·kg^{-1}) worsened the long-term memory performance, as testified by the different latencies measured in the test session between CGS + veh and veh + veh groups ($P < 0.05$). The selective blockade of A_{2A} receptors effectively prevented the CGS 21680-induced mnemonic deficit, as gauged by the different latencies measured in the test session between the CGS + veh and CGS + SCH groups ($P < 0.05$; Figure 6B).

In the modified Y-maze, it was observed that mice treated only with 0.1 mg·kg^{-1} CGS 21680 (CGS + veh) were the only group that did not show differences in the exploration of the novel arm when compared with the others arms [$F_{(2,18)}$ = 1.240; P = 0.3129], whereas both control mice (veh + veh) [$F_{(2,21)}$ = 36.75; P < 0.0001] and mice treated with the A_{2A} receptor antagonist, SCH 58261 before CGS 21680 (CGS + SCH) [$F_{(2,21)}$ = 3.890; P = 0.0385] spent more time exploring the novel arm than the others (Figure 6C).

Discussion

The present study shows that the selective blockade of A_{2A} receptors reproducibly prevented the scopolamine-induced impairment in short-term memory in three different behavioural tasks, which was also prevented by the A_1 receptor antagonist in most tasks. This implies that the previously observed beneficial effect of caffeine to prevent scopolamine-induced amnesia (Botton *et al.*, 2010) probably results from its dual ability to antagonize A_1 and A_{2A} receptors (Fredholm *et al.*, 1999). Furthermore, we now showed that the activation of A_{2A} receptors in naïve animals is sufficient to disrupt short-term memory in the three different tests, an effect mimicked by the direct brain activation of A_{2A} receptors. Therefore, we now provide the first integrated evidence that activation of A_{2A} receptors is necessary and sufficient for the impairment of short-term memory.

By using selective antagonists of A_1 and A_{2A} receptors that are devoid of effects in anxiety-related tests (El Yacoubi *et al.*, 2000a,b) and by controlling for their lack of effect on spontaneous locomotion, we can now conclude that the blockade of A_{2A} receptors prevents scopolamine-induced deficits in short-term recognition memory in three different behavioural paradigms. Notably, the selective blockade of A_1 receptors also attenuated the scopolamine-induced deficits in short-term recognition memory in the object recognition test and in the inhibitory avoidance test, but not in the modified Y-maze, which is the test with the lowest sensory or aversive influence. Since we have previously shown that such deficits in recognition memory are also attenuated by caffeine (Botton *et al.*, 2010), which is a mixed A_1 and A_{2A} receptor antagonist at non-toxic doses (Fredholm *et al.*, 1999), we can tentatively conclude that this prevention by caffeine of scopolamine-induced amnesia is likely to depend on both A_1 and A_{2A} receptors. This provides a conclusion different from that previously derived from the comparison of the effects of caffeine and of A_1 and A_{2A} receptors on memory impairment in different animal models of brain disease (see Cunha and Agostinho, 2010). In fact, in animal models of aging (Prediger *et al.*, 2005), AD (Dall'Igna *et al.*, 2007), attention deficit and hyperactivity disorder (Pandolfo *et al.*, 2013), early life convulsions (Cognato *et al.*, 2010) or early life stress (Batalha *et al.*, 2013), the ability of caffeine to prevent memory dysfunction is mimicked by the selective blockade of A_{2A}, but not of A_1 receptors.

Our results imply that A_1 receptors are selectively involved in this model of scopolamine-induced amnesia, in particular in tasks relying on sensory or nociceptive components (see also Suzuki *et al.*, 1993). This is in agreement with the particular importance of A_1 receptors to control the cholinergic system, which is expected to be at the core of the scopolamine-induced amnesia: in fact, A_1 receptors control the actions of muscarinic receptors (Pedata *et al.*, 1986; Oliveira *et al.*, 2002) and efficiently inhibit the release of ACh in the limbic cortex (e.g. Jackisch *et al.*, 1984; Cunha *et al.*, 1994). Accordingly, A_1 receptor antagonists can bolster the release of ACh (Pedata *et al.*, 1986; Cunha *et al.*, 1994), potentially counteracting the scopolamine-induced cholinergic

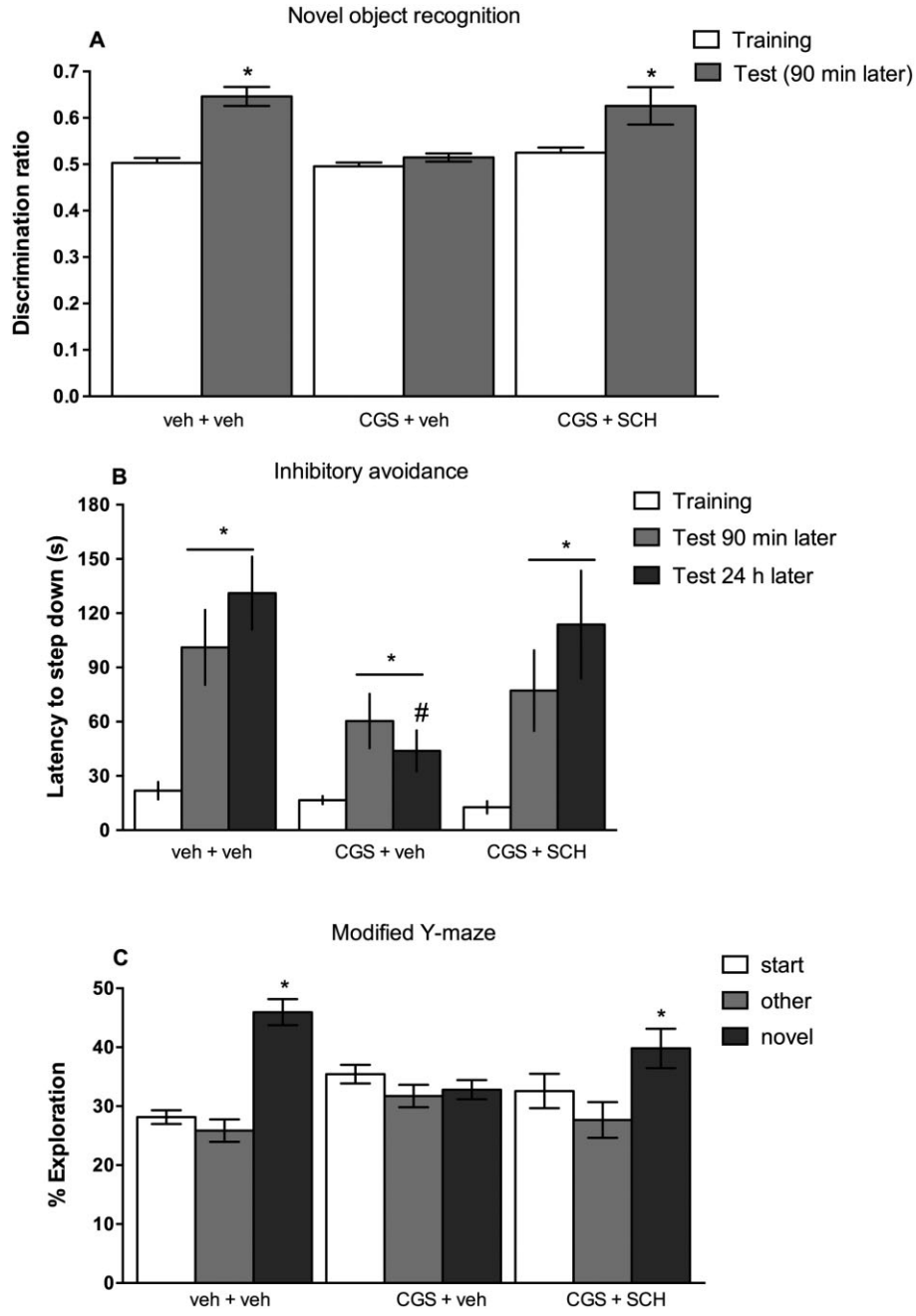

Figure 6

The blockade of adenosine A_{2A} receptors prevents the deterioration in short-term memory triggered by exposure to CGS 21680 (experiment 5). CGS 21680 (0.1 mg·kg^{-1}, i.p.) was administered 30 min before SCH 58261 (0.5 mg·kg^{-1}, i.p.), which was administered 30 min before training. CGS 21680 impaired: (A) the discrimination ratio in the recognition memory test (data are means ± SEM of n = 9–10 mice per group; *P < 0.05 vs. training session); (B) the latency in seconds (s) to step down in the inhibitory avoidance task (data are median and interquartile range of n = 6–8 mice per group; *P < 0.05, Wilcoxon paired t-test comparing training and test session within group; #P < 0.05, Mann–Whitney test comparing veh + veh and CGS + SCH group in the test sessions); (C) the percentage of the time of exploration in the three arms (start, other and novel) in the test trial of the modified Y-maze task. Data are means ± SEM of n = 7–8 mice per group; *P < 0.05 one-way ANOVA and Tukey's multiple comparison test comparing the novel and other arms within group.

hypofunction that underlies memory deficits (reviewed in Fisher, 2012).

In contrast, A_{2A} receptor antagonists inhibit the release of ACh from limbic cortical preparations (Rodrigues *et al.*, 2008), an effect that is not compatible with the beneficial effect of A_{2A} receptor antagonists to alleviate memory impairment; furthermore, other central responses triggered by the activation of muscarinic receptors, such as rapid eye

movement sleep (Marks *et al.*, 2003) or tremor (Salamone *et al.*, 2013), are prevented by A_{2A} receptor antagonists. Thus, it is most likely that the beneficial effect of A_{2A} receptor antagonists found in the present study may not result from a direct effect on the cholinergic system but might instead be related to their ability to control synaptic plasticity in cortical circuits (Rebola *et al.*, 2008; Costenla *et al.*, 2011), which is argued to be the neurophysiological basis of learning and memory (Martin *et al.*, 2000). This would explain the general ability of A_{2A} receptor antagonists to prevent memory impairment under different conditions, including upon exposure to scopolamine as now reported, especially since scopolamine might decrease LTP in slices from the hippocampus or perirhinal cortex (e.g. Hirotsu *et al.*, 1989; Warburton *et al.*, 2003; but see Tanaka *et al.*, 1989). Thus, the effect of A_{2A} receptor antagonists would be a balance between its beneficial effect normalizing synaptic plasticity and its detrimental effects on the cholinergic system. Furthermore, this hypothesis might explain a previous report using different doses of scopolamine and A_{2A} receptor antagonists that concluded that the blockade of A_{2A} receptors was not completely effective at preventing the impairment of spatial memory induced by scopolamine (Cunha *et al.*, 2008).

However, it is important to stress that the mechanism(s) underlying this beneficial effect of A_{2A} and A_1 receptor antagonists on scopolamine-induced amnesia are currently difficult to unravel. One of the reasons is essentially because the neurophysiological bases of scoplamine-induced amnesia are still unclear given that the effects of scopolamine on synaptic plasticity are not consistently observed (e.g. Hirotsu *et al.*, 1989; Warburton *et al.*, 2003; but see Tanaka *et al.*, 1989). Besides, scopolamine actually bolsters ACh release in a manner mimicked by muscarinic M_1 receptor antagonists (Vannucchi *et al.*, 1997), in contrast to the beneficial effect of muscarinic M_1 receptor agonists and acetylcholinesterase inhibitors to alleviate memory impairment (reviewed in Fisher, 2012). Furthermore, it has been observed that an injection of scopolamine into the perirhinal cortex can actually improve object recognition memory (Winters *et al.*, 2007), which argues for the involvement of circuit-mediated effects (i.e. affecting different brain regions and their connectivity) in the amnesia induced by systemic administration of scopolamine rather than single neurochemical events (i.e. restricted to a single molecular alteration in a defined brain region).

In contrast to the effectiveness of A_{2A} receptors at controlling memory dysfunction under different brain conditions, we observed that neither A_{2A} nor A_1 receptor antagonists modified recognition memory in control mice that were not challenged with scopolamine. The effect of A_1 receptor blockade on memory performance is rather controversial, with reports of beneficial (Pereira *et al.*, 2002; Mioranzza *et al.*, 2011; Harvey *et al.*, 2012) and detrimental or lack of effects (Kopf *et al.*, 1999; Giménez-Llort *et al.*, 2002; Vollert *et al.*, 2013), probably depending on the schedule of administration of the A_1 receptor antagonists and on the different dependence of the memory task on anxiety and locomotion, since the activation of A_1 receptors can cause a profound sedative effect (Snyder *et al.*, 1981; Bruns *et al.*, 1983).

As for the effect of A_{2A} receptor blockade on control rodents, a similar lack of effect of A_{2A} receptor antagonists on memory function of naïve adult animals was also reported in previous studies (Prediger *et al.*, 2005; Dall'Igna *et al.*, 2007; Cunha *et al.*, 2008; Canas *et al.*, 2009; Cognato *et al.*, 2010; Batalha *et al.*, 2013). This is confirmed in transgenic mice with A_{2A} receptor deletion, which display an unaltered reference memory (Canas *et al.*, 2009; Augusto *et al.*, 2013; but see Wang *et al.*, 2006) and an enhanced working memory performance (Zhou *et al.*, 2009; Augusto *et al.*, 2013). Thus, it seems that A_{2A} receptors are selectively engaged upon perturbation of brain function to control recognition memory and become the preferential target for the benefits of caffeine in promoting neuroprotection against the mnemonic deficits observed in experimental models of AD and aging (Prediger *et al.*, 2005; Arendash *et al.*, 2006; Dall'Igna *et al.*, 2007; Canas *et al.*, 2009; Espinosa *et al.*, 2013).

Notably, in the present study we observed that the activation of A_{2A} receptors in control mice was sufficient to trigger a memory deficit, that is, CGS 21680 attenuated short-term memory in all three behavioural tests, an effect prevented by SCH 58261. Furthermore, infusion of CGS 21680 i.c.v. mimicked the impairment in recognition memory caused by its systemic administration. CGS 21680 infused directly into the posterior cingulate cortex has previously been demonstrated to worsen memory retrieval in the inhibitory avoidance task (Pereira *et al.*, 2005). Similar deficits in memory performance in the object recognition task were observed in transgenic rats overexpressing A_{2A} receptors in the forebrain (Giménez-Llort *et al.*, 2007) and an up-regulation of limbic cortical A_{2A} receptors is observed upon memory impairment in patients (Albasanz *et al.*, 2008) and in animal models of brain disease (Cognato *et al.*, 2010; Espinosa *et al.*, 2013). Finally, we also observed that CGS 21680 did not further exacerbate the scopolamine-induced impairment in recognition memory, as this effect probably results from the full engagement of A_{2A} receptors. This body of evidence not only indicates that the activation of A_{2A} receptors is detrimental for memory performance but also prompts the hypothesis that the over-activation of A_{2A} receptors may actually be a cause of memory impairment. This makes the elucidation of the signalling mechanism operated by A_{2A} receptors particularly important (Fredholm *et al.*, 2007; Zezula and Freissmuth, 2008), as this may shed light on transducing systems critically associated with the implementation and recall of memory traits.

In conclusion, the present study establishes the involvement of both A_1 and A_{2A} receptor antagonism as likely mechanisms underlying the beneficial effect of caffeine on scopolamine-induced deficits in recognition memory. Furthermore, the present observation that A_{2A} receptor activation is sufficient to trigger memory deficits prompts the hypothesis that an over-activation of A_{2A} receptors might actually be a causative factor for the emergence of memory deficits. Overall, these results reinforce the therapeutic interest in targeting A_{2A} receptors to manage memory dysfunction.

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Author contributions

N. P., A. S. A., D. M. M., P. H. S. B., S. M., F. N., G. C. C. and C. M. L. performed the experiments and help to analyse the data. N. P., R. A. C. and L. O. P. designed the research study. N. P., R. A. C. and L. O. P. analysed the data and wrote the paper.

Conflicts of interest

The authors declare no conflicts of interest.

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