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SHORT COMMUNICATION
Mice lacking the adenosine A_1 receptor are anxious and aggressive, but are normal learners with reduced muscle strength and survival rate

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Abstract

Behavioural assessment of mice lacking adenosine A_1 receptors (A_1Rs) showed reduced activity in some phases of the light–dark cycle, reduced exploratory behaviour in the open-field and in the hole-board, increased anxiety in the plus maze and dark-light box and increased aggressiveness in the resident-intruder test. No differences were found in spatial reference and working memory in several Morris water maze tasks. Both mutant mice had reduced muscle strength and survival rate. These results confirm the involvement of adenosine in motor activity, exploratory behaviour, anxiety and aggressiveness. A_1Rs also appear to play a critical role in ageing-related deterioration.

Introduction

Adenosine is involved in an array of functions in the central nervous system (Dunwiddie & Masino, 2001) but despite extensive pharmacological studies, the behavioural roles of its receptor subtypes remain unclear. The antagonists like caffeine promote wakefulness, disrupt normal sleep (Strecker *et al*., 2000), and their biphasic stimulant effects on motor activity (Daly & Fredholm, 1998), mainly through adenosine A_{2A} receptors (A_{2A}Rs), may be also mediated through A_1 receptors (A_1Rs; Ferré *et al*., 2001). Chronic administration of high doses of caffeine induce aggressive behaviour in rats and increase nervousness and irritability in man (Fredholm *et al*., 1999) whereas adenosine analogues counteract induced aggressiveness in mice (Palmour *et al*., 1989). Mice lacking the A_{2A}Rs show increased aggressiveness and anxiety (Ledent *et al*., 1997) although a preferential role of A_1Rs in the modulation of anxiety is suggested (i.e. Florio *et al*., 1998). A_1Rs and A_{2A}Rs might also mediate hippocampal long-term potentiation (Arai *et al*., 1990). A_1Rs agonists and antagonists cause, respectively, impairment or facilitation of learning and memory (i.e. Suzuki *et al*., 1993) but the opposite effects have been reported after chronic administration (VonLubitz *et al*., 1993) or acute high doses (Zarrindast & Shafaghi, 1994) of antagonists. The present study was aimed at characterizing some relevant aspects of the behavioural phenotype of A_1R knockout mice (Johansson *et al*., 2001) related to those functions where A_1Rs seem to be involved.

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Materials and methods

Animals

Mice lacking the second encoding exon of the A_1R were the offspring of $A_1R^{+/-}$ mice on a 50% C57BL, 50% 129/OlaHsd background, that in turn were derived from matings of male chimeric $A_1R^{-/-}$ mice with normal C57BL females. Male littermates were maintained (Macrolon, 57 × 35 × 19 cm) under standard laboratory conditions (food and water *ad lib,* 22 ± 2 °C; inverse 12 h light : 12 h dark cycles beginning at 15.00 h) and assessed in a series of tests (see Table 1; Costall *et al*., 1989; Palmour *et al*., 1989; Escorihuela *et al*., 1995; Crawley *et al*., 1997) used as a first screen for behavioural abnormalities in mutant mice. Animals were weighed before each test and survival was recorded daily. Animals showing signs of illness or weight loss were excluded of the tests. Exploratory and anxiety-like behaviours were tested under dim red light from 10.00 to 12.00 h, circadian activity during one whole light–dark (LD) period, and the rest of the tests from 17.00 to 21.00 h under dim white light. Except otherwise indicated, all the behavioural variables were recorded by a video-computerized tracking system (SMART, Panlab S.L., Barcelona, Spain). The research was conducted in accordance with 86/609/EEC regarding the care and use of animals for experimental procedures.

Wire hang test

In the wire hang test, a horizontal wire (diameter 2 mm, length 40 cm) divided into eight segments was suspended 80 cm above a padded table. The animal was allowed to cling in the middle of the wire with its forepaws for one 60 s trial. Motor coordination was measured as the number of segments crossed, and muscle strength as the time until falling off the wire.

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TABLE 1. Behavioural assessment of mice lacking the A_1Rs

	Age (weeks)	$A_1R^{+/+}$	$A_1R^{+/-}$	$A_1R^{-/-}$
A. MOTOR FUNCTION	20	(*n* = 10)	(*n* = 24)	(*n* = 13)
Wire hang test				
Motor coordination (segments)		4.0 ± 0.9	3.5 ± 0.7	2.4 ± 0.5
Muscular strength (latency in s)		56.0 ± 4.0	40.2 ± 4.9*	35.9 ± 6.5*
B. CIRCADIAN MOTOR ACTIVITY	20–21	(*n* = 10)	(*n* = 24)	(*n* = 13)
Peak of nocturnal activity (counts)		4298 ± 975	4215 ± 414	2389 ± 255[a,b]
C. EXPLORATORY BEHAVIOUR				
Open-field test	23	(*n* = 10)	(*n* = 24)	(*n* = 13)
Number of rearings		8.0 ± 3.60	15.8 ± 3.1	4.2 ± 2.0[b]
Total distance (cm)		1093 ± 220.9	1277.9 ± 150.7	947.7 ± 184.2
Hole-board test	28	(*n* = 10)	(*n* = 24)	(*n* = 13)
Number of head-dips		19.7 ± 2.4	22.6 ± 2.6	14.2 ± 3.2[a,b]
Time head-dipping (s)		17.2 ± 2.1	23.7 ± 2.9	12.2 ± 2.5[b]
Total distance (cm)		421.5 ± 90.8	598.2 ± 79.7	438.7 ± 70.4
D. ANXIETY-LIKE BEHAVIOURS				
Elevated plus-maze test	24	(*n* = 10)	(*n* = 21)	(*n* = 11)
Number of entries in the OA (OAE)		3.8 ± 0.9	4.4 ± 0.7	5.3 ± 1.1
Number of entries in the EA (EAE)		5.9 ± 1.4	8.9 ± 1.1	9.4 ± 1.6
Time in the OA (TOA) (s)		120 ± 41.0	120.3 ± 22.6	99.8 ± 15.7
Time in the EA (TEA) (s)		174.8 ± 40.0	241.0 ± 21.8	251.9 ± 25.1[a]
Dark-light box test	29	(*n* = 10)	(*n* = 22)	(*n* = 11)
Latency dark-light (s)		102.3 ± 34.6	77.4 ± 21.8	187.3 ± 43.0
Number of crossings		8.1 ± 2.1	8.2 ± 1.3	2.6 ± 1.2[a,b]
Distance index		27.9 ± 7.1	31.1 ± 4.7	14.0 ± 5.6[a,b]
Time index		10.5 ± 3.6	8.5 ± 1.3	4.5 ± 2.1[a,b]
E. AGGRESSIVE BEHAVIOUR				
Resident–intruder test	49	(*n* = 10)	(*n* = 14)	(*n* = 11)
Attack latency (s)		142.8 ± 18.0	166.9 ± 7.7	108.0 ± 20.6[b]

Results are means ± SEM. *$P < 0.05$ vs. $A_1R^{+/+}$ (Student's *t*-test). [a]$P < 0.05$ vs. $A_1R^{+/+}$ and [b]$P < 0.05$ vs. $A_1R^{+/-}$ (Duncan's test).

Circadian motor activity test

Three mice per day (one of each genotype) were tested for 23 consecutive hours (beginning at 17.00 h, periods of 30 min) in a multicage activity meter system (three cages simultaneously, Sensor Unit PANLAB 0603, Panlab, S.L., Barcelona, Spain) set to measure horizontal and vertical motor activity. Testing cages (Macrolon, 35 × 35 × 25 cm), slightly different from the home cage, contained clean sawdust and had food and water available.

Open-field and hole-board tests

The open-field (55 × 55 × 25 cm high) and the hole-board (32 × 32 × 30 cm) were woodwork white boxes. Mice were placed in the centre of the apparatus and observed for 5 min. Exploratory behaviour was measured as the number of head-dips and time spent head-dipping on each of the four holes (3 cm diameter) equally spaced in the floor of the hole-board and rearings in the open-field. In both tests, distances and defaecations were also recorded.

Plus-maze test

The plus-maze (woodwork, black Plexiglass) consisted of two enclosed arms (EA, 30 × 5 × 15 cm, transparent walls) and two open arms (OA; 30 × 5 cm) forming a square cross with a 5 × 5 cm square centrepiece. The apparatus was elevated 40 cm above the floor. The animal was placed in the centre of the plus-maze facing one of the open arms. The number of entries (all four paws) into, the time spent, and the distance covered in each arm were recorded for 7.5 min.

Dark–light box test

The dark–light box (Panlab, S.L., Barcelona, Spain) consisted of two compartments (black, 270 × 180 × 270 mm; white, 270 × 270 × 270 mm) connected by an opening (70 × 70 mm). A slight illumination of the white box (red 20 W bulb) was chosen to facilitate wild-type animals entering the white compartment. The mice were introduced into the black compartment and observed for 5 min. Latency to enter (all four paws) into the lit compartment, number of crossings, time spent and distance covered in the lit compartment were recorded. The ratios of time and distance over the number of entries (time and distance indexes, respectively) into the lit compartment were calculated.

Morris water maze tests

Five paradigms in the Morris water maze were carried out. Mice (35–38 weeks old) were trained to locate a hidden platform (16 cm diameter, 28 cm height, 2 cm below the water surface) in a circular pool (home-made, 140 cm diameter, 60 cm height, 24 °C opaque water) located in a test room with distal visual cues. Mice failing to find the platform were placed on it for 20 s, the same period as the successful animals.

Days 1–5, place learning

This invoved four trial sessions per day, with trials spaced 15 min apart. The mouse was gently released (facing the wall) from one

randomly selected starting point (N, S, E or W) and allowed to swim until escaped onto the platform (always in the middle of the S quadrant). At the end of session 5, the platform was removed from the maze and the mice performed a 'probe trial' of 60 s.

Days 6–7, reversal

Four trials of 60 s/day; the platform was placed opposite its location during the place learning task.

Days 8–9, cue learning

Four trials of 60 s/day; the platform was elevated 1 cm above the water level and its new W position was indicated by a visible striped flag (5 × 8 × 15 cm), whereas extra maze cues were hidden by two black panels around the pool.

Annulus crossings, the time spent and distance travelled in each quadrant were measured in the probe trial. In the other tasks, the escape latency and the distance travelled were recorded.

Resident–intruder aggression test

Seven $A_1R^{+/-}$ mice were left group-housed (2–3 per cage) to be used as intruders in one 3 min resident–intruder aggression test per day against an unfamiliar resident (mice isolated for 6 weeks in a Macrolon, 23 × 23 × 15 cm, cage). Attack (resident biting or physically assaulting the intruder) latency was measured.

Results and discussion

In a previous study (Johansson *et al*., 2001), we reported that $A_1R^{-/-}$ bred and gained weight normally. These animals did not show deficits in visual placing reflex, equilibrium and prehensility but showed increased anxiety, lowered pain thresholds and an altered response to hypoxia. Here we present additional results from a battery of well-characterized paradigms selected to assess the relevance of A_1Rs in several behavioural functions.

Both mutant mice showed normal motor coordination but a reduced muscle strength ($P < 0.05$, Table 1A), which would agree with results of A_1R activation improving the skeletal muscle cell function *in vitro* (Reading & Barclay, 2001).

No differences in overall spontaneous motor activity were detected along a 23 h LD period (Fig. 1). However, a biphasic 'genotype' effect during the low activity phase of the light cycle was found (ANOVA, $F_{2,44} = 4.02$, $P < 0.05$) with increased activity in the $A_1R^{+/-}$ and decreased in the $A_1R^{-/-}$. The peak of nocturnal activity (02.30–04.00 h) was also decreased in the $A_1R^{-/-}$ mice (MANOVA, 'genotype per interval' effect, $F_{46,1012} = 1.397$, $P < 0.05$; Table 1B). These results would be congruent with the effect of moderate doses of caffeine promoting wakefulness and disrupting normal sleep (Strecker *et al*., 2000) and the fact that A_1Rs are likely to be involved in regulating the sleep–wake rhythms in animals (e.g. Elliot *et al*., 2001).

The $A_1R^{-/-}$ mice showed a decrease in exploratory behaviour (Table 1C), i.e. rearings in the open-field (ANOVA, 'genotype' effect: $F_{2,44} = 3.455$, $P < 0.05$), number of head-dips ($F_{2,44} = 4.346$, $P < 0.05$) and time spent head-dipping ($F_{2,44} = 4.155$, $P < 0.05$) in the hole-board but such an effect was not related to differences in motor activity in those tests (Table 1C). As changes in novelty-induced exploratory behaviours may be modified by anxiolytic and anxiogenic compounds (Crawley *et al*., 1997), these results could reflect an anxiogenic state in $A_1R^{-/-}$ mice. Accordingly, a trend for higher preference for the EA (TEA) was shown by $A_1R^{-/-}$ animals (ANOVA, $F_{2,39} = 2.948$, $P = 0.064$; $P < 0.05$ $A_1R^{-/-}$ vs. $A_1R^{+/-}$)

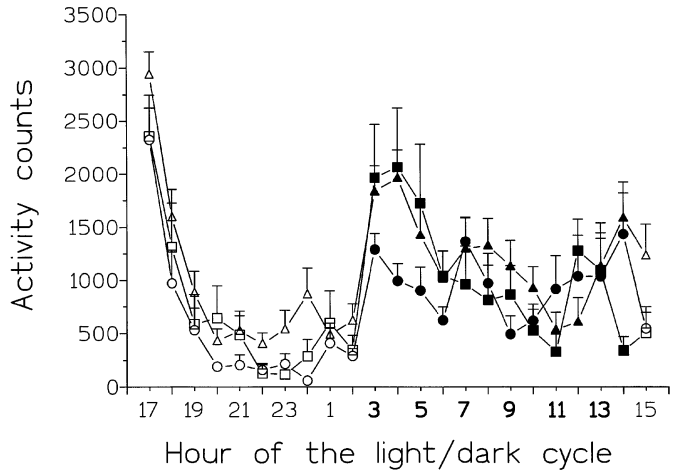

FIG. 1. Spontaneous motor activity during a 23 h light : dark cycle. Results are mean ± SEM. Symbols: open, light period; closed, dark period; square, $A_1R^{+/+}$, $n = 10$; triangle, $A_1R^{+/-}$, $n = 24$; circle, $A_1R^{-/-}$, $n = 13$.

whereas no differences in entries were seen in the two types of arms. Also, the $A_1R^{-/-}$ mice exhibited a higher latency to enter into the light compartment of the dark–light box (ANOVA, 'genotype' effect: $F_{2,40} = 3.379$, $P < 0.05$), made less crossings ($F_{2,40} = 4.743$, $P < 0.05$) and performed the shortest and least exploratory visits as indicated by the time ('genotype': $F_{2,40} = 3.334$, $P < 0.05$) and distance indexes ($F_{2,40} = 3.304$, $P < 0.05$; Table 1D). Therefore, both $A_{2A}R^{-/-}$ (Ledent *et al*., 1997) and $A_1R^{-/-}$ mice exhibit increased anxiety consistent with the well-known pronounced anxiogenic effects of high doses of caffeine, which will presumably block most of both adenosine receptors (Fredholm *et al*., 1999), but low doses do not.

Enhanced aggressive behaviour found in $A_{2A}R^{-/-}$ isolated male mice (Ledent *et al*., 1997) was also observed in our $A_1R^{-/-}$ isolated animals (ANOVA, 'genotype' effect: $F_{2,32} = 3.9$, $P < 0.05$; Table 1E) in agreement with the decrease of offensive behaviour induced by selective stimulation of A_1Rs (Navarro *et al*., 2000). These results suggest that both adenosine receptor subtypes are involved in the effects of adenosine on aggressiveness.

No differences between genotypes appeared in any of the Morris water maze tasks. All the mice showed identical good acquisition patterns in place learning (MANOVA, 'day' effect: $F_{4,150} = 84{,}705$, $P < 0.001$) and reversal (MANOVA, 'day': $F_{1,36} = 59{,}401$, $P < 0.001$), a maximum efficiency to reach the platform in the cue learning (MANOVA, 'day': $F_{1,36} = 6{,}616$, $P < 0.05$) and equally higher preference for the trained quadrant in the probe trial (Duncan's test, $P < 0.05$ vs. other quadrants). Compensatory mechanisms triggered during development of the mutant mice could explain the discrepancy with the reported impaired performance induced by chronic administration of A_1R antagonists (VonLubitz *et al*., 1993). Working memory was also evaluated using the 'repeated acquisition paradigm' in the Morris water maze (Whishaw, 1985) and showed no differences among the three genotypes (data not shown) in agreement with Hooper *et al*. (1996), suggesting that endogenous adenosine would not mediate working memory processes.

Most of the tests were performed at the beginning of the survival curve (Fig. 2) and no differences were found in the weight of the animals. The reduction or complete loss of receptors entailed an earlier drop in the viability of the animals, which was more severe in

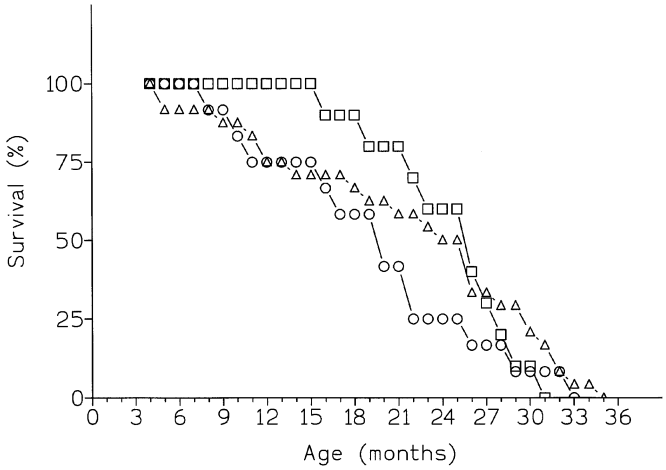

FIG. 2. Survival curves. Symbols and number of animals at the starting point as in Fig. 1.

the $A_1R^{-/-}$ animals. All of the $A_1R^{+/+}$ mice survived until the age of 15 months whereas the $A_1R^{+/-}$ and $A_1R^{-/-}$ groups decreased to 71% and 75%, respectively (Chi-square, 2, 33.672, $P < 0.001$). In most of the animals, regardless of the genotype, spinal kyphosis and sudden dramatic body weight losses preceded death. The fast increase in the mortality rate of mutant mice suggests that impairment or naturally occurring ageing physiopathological processes might be advanced. Adenosine protects against the negative consequences of hypoxia or ischaemia through A_1Rs (Fredholm, 1996) and our previous results with hippocampal slices and brainstem respiratory activity (Johansson *et al*., 2001) showed reduced functional recovery and decrease in the survival rate in both A_1Rs mutant groups. In addition, adenosine regulation of excitatory activity was impaired or completely lacking in these animals ($A_1R^{+/-}$ and $A_1R^{-/-}$, respectively). Finally, the fact that adenosine regulates the energy supply/demand balance in the tissues and that A_1Rs are likely to play an important role in the normal physiology of cardiovascular, hepatic and renal systems (Bruns, 1991) could also account for the observed reduced survival. In this sense, disturbances in the renal system have already been reported in these animals (Brown *et al*., 2001).

In summary, the present work has confirmed and extended our knowledge on the functional roles proposed for adenosine acting at A_1Rs: reduction of muscular resistance and exploratory behaviour; increase of anxiety and aggressiveness; and no deficits on spatial reference and working memory. The results also alert us to the role of A_1Rs in survival.

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