

Mice Lacking the Adenosine A₁ Receptor Have Normal Spatial Learning and Plasticity in the CA1 Region of the Hippocampus, But They Habituate More Slowly

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ABSTRACT Using mice with a targeted disruption of the adenosine A₁ receptor (A₁R), we examined the role of A₁Rs in hippocampal long-term potentiation (LTP), long-term depression (LTD), and memory formation. Recordings from the Shaffer collateral–CA1 pathway of hippocampal slices from adult mice showed no differences between theta burst and tetanic stimulation-induced LTP in adenosine A₁ receptor knockout (A₁R^{-/-}), heterozygote (A₁R^{+/-}), and wildtype (A₁R^{+/+}) mice. However, paired pulse facilitation was impaired significantly in A₁R^{-/-} slices as compared to A₁R^{+/+} slices. LTD in the CA1 region was unaffected by the genetic manipulation. The three genotypes showed similar memory acquisition patterns when assessed for spatial reference and working memory in the Morris water maze tasks at 9 months of age. However, 10 months later A₁R^{-/-} mice showed some deficits in the 6-arm radial tunnel maze test. The latter appeared, however, not due to memory deficits but to decreased habituation to the test environment. Taken together, we observe normal spatial learning and memory and hippocampal CA1 synaptic plasticity in adult adenosine A₁R knockout mice, but find modifications in arousal-related processes, including habituation, in this knockout model. **Synapse 57:8–16, 2005.** ©2005 Wiley-Liss, Inc.

INTRODUCTION

Adenosine A₁ receptors (A₁Rs) are abundant in brain and a large proportion of these receptors are found on glutamatergic nerve endings, where they regulate transmitter release (Fredholm and Dunwiddie, 1988; Lupica et al., 1992; Johansson et al., 1993; Dunwiddie and Masino, 2001). The hippocampal formation is enriched with A₁Rs (Fastbom et al., 1987; Svenningsson et al., 1997) and these receptors may be involved in the development and consolidation of long-term potentiation (LTP) (Arai et al., 1990; de Mendonca and Ribeiro, 1990; Alzheimer et al., 1991; Forghani and Krnjevic, 1995) and in induction of long-term depression (LTD) (de Mendonca et al., 1997; Bon and Garthwaite, 2002; Moore et al., 2003). Selective A₁R agonists and antagonists have been reported, respectively, to impair and to

facilitate learning and memory in passive-avoidance tasks (e.g., Normile et al., 1991; Suzuki et al., 1993; Pereira et al., 2002) and spatial learning in mazes (e.g., Suzuki et al., 1993; Von Lubitz et al., 1993), and have been proposed as therapy in cognitive disorders (reviewed by Erfurth and Schmauss, 1995). However, if

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given at high doses, antagonists produce an impairment (Zarrindast and Shafaghi, 1994), perhaps reflecting a biphasic action. Furthermore, chronic administration of selective A₁R agonists and antagonists may have effects on learning and memory that are opposite the effects elicited by acute administration of the same drugs (Von Lubitz et al., 1993; Hauber and Bareiss, 2001).

Many of these findings rely on the use of pharmacological agents that may be incompletely selective. Therefore, it is interesting that several recent articles have reported little or no effect of deletion of the A₁R on learning and memory (Giménez-Llort et al., 2002; Lang et al., 2003), despite the fact that at least some forms of LTP are strongly affected (Moore et al., 2003). In the present study the role of A₁R in these processes has therefore been reexamined.

MATERIALS AND METHODS

Animals and general procedure

Mice lacking the second encoding exon of the adenosine A₁R were generated as described previously (Johansson et al., 2001). They were the offspring of A₁R^{+/-} mice on a 50% C57BL/6, 50% 129/OlaHsd background, which in turn were derived from matings of male chimeric A₁R^{-/-} mice with normal C57BL/6 females. A total of 71 male animals were used: 10 A₁R^{+/+}, 24 A₁R^{+/-}, and 13 A₁R^{-/-} mice were used in behavioral experiments, and 12 A₁R^{+/+}, 3 A₁R^{+/-}, and 9 A₁R^{-/-} mice were used in electrophysiological experiments. Multiple hippocampal slices from each animal were used for electrophysiological recordings.

Electrophysiology

Adult mice between 6 and 18 months were used for electrophysiological experiments. Hippocampal slices 400 μm thick were obtained acutely using standard procedures (Masino and Dunwiddie, 1999). Briefly, the hippocampus was removed rapidly and placed initially in ice-cold oxygenated (95% O₂/5% CO₂) aCSF buffer containing (in mM): NaCl 126.0, KCl 3.0, MgSO₄ 1.5, CaCl₂ 2.4, NaH₂PO₄ 1.2, NaHCO₃ 25.9, and D-glucose 11.0. Slices were allowed to recover for

a minimum of 90 min at 32.5°C, and slices were then placed on a nylon net in a submerged recording chamber superfused with oxygenated aCSF at 2 ml/min. Recordings were made in the CA1 region by stimulating the Schaffer collateral pathway every 30 sec and recording the fEPSP response from the stratum radiatum. Stimulation was performed using a bipolar stimulating electrode and recordings were made with a glass micropipette filled with 3 M NaCl. Baseline recordings were established at ~50% of the maximum fEPSP response.

On each recording day, after a stable baseline had been established and before any stimulation protocol, 100 μM adenosine was applied transiently to a slice from each animal. This adenosine application assessed functionally the genotype of each animal and in each case confirmed the genotype determined by PCR analysis. Previous studies demonstrated that adenosine has no effect on synaptic transmission in hippocampal slices from A₁R^{-/-} animals, has an intermediate effect in slices A₁R^{+/-} animals (due to the increased EC₅₀ for adenosine), and has the expected profound inhibitory effect in slices from A₁R^{+/+} animals (Johansson et al., 2001; Masino et al., 2002).

We used standard long-term potentiation (LTP) and long-term depression (LTD) stimulation protocols to induce synaptic plasticity (Bliss and Collingridge, 1993; Dudek and Bear, 1992; Dunwiddie and Lynch, 1978). LTP was induced with one of two protocols: tetanic or theta burst stimulation. The tetanic LTP stimulation protocol was 100 Hz for 1 sec, repeated three times at 10-sec intervals. The theta burst stimulation pattern was delivered at 5 Hz. Each burst contained four pulses at 200 Hz. This burst stimulation pattern was delivered 10 times at 5 Hz and was also repeated three times. LTD was induced with continuous 1-Hz stimulation for 900 sec. Other than during LTP or LTD induction, the fEPSP was assessed every 30 sec throughout the experiment. In a subset of experiments, a paired-pulse stimulation (pulse interval 70 ms) was delivered every 30 sec to assess ongoing paired-pulse facilitation. The fEPSP slope was quantified and any differences between the genotypes were analyzed with Student's *t*-test comparisons. Statistical significance was considered at *P* < 0.05.

Abbreviations

ARs	adenosine receptors
A ₁ Rs	adenosine A ₁ receptor subtype
A ₁ R ^{+/+}	wildtype mice for the adenosine A ₁ receptor subtype
A ₁ R ^{+/-}	heterozygote mice for the adenosine A ₁ receptor subtype
A ₁ R ^{-/-}	knockout mice for the adenosine A ₁ receptors subtype
CHA	N(6)-cyclohexyladenosine
CPX	cyclopentyl-1,3-dipropylxanthine
fEPSP	field excitatory post synaptic potential
LTD	long-term depression
LTP	long-term potentiation

Behavioral testing

A 1-month adaptation period to the animal maintenance facilities of the laboratory in Barcelona was allowed prior to any test. The litters were housed at a maximum of four animals per cage in standard plastic type Macrolon[®] IV cages (57 × 35 × 19 cm, with 2 l of wood cuttings as bedding) up to the time of the test. They were maintained at an ambient room temperature of 22 ± 2°C with 60 ± 10% relative humidity and an inverse 12-h light/dark (LD) schedule with

lights on at 15:00 h. The animals had lab chow and tap water ad libitum until the moment of the test. Behavioral screening started at 5 months of age (see Giménez-Llort et al., 2002) and when the animals were 9 months old they were successively confronted with several learning and memory tests: place learning for reference memory, removal, reversal, cue learning, and place learning for working memory in the Morris water maze, over weeks 35–38, and a six-arm radial tunnel maze during week 74. The experiments were performed under dim white light during their light phase of the LD cycle (17:00–21:00 h).

The research was conducted in compliance with the Spanish legislation on “Protection of Animals Used for Experimental and Other Scientific Purposes” and in accordance with the European Communities Council Directive (86/609/EEC) on this subject.

Morris water maze tests

Animals were tested in a series of five different paradigms in the Morris water maze consisting of five sessions of place learning for reference memory (days 1–5), one session of removal (day 5), two sessions of reversal (days 6–7), two sessions of cue learning (days 8–9) (Morris, 1984; Escorihuela et al., 1998), and two sets of four sessions of place learning for working memory (days 14–17 and days 21–24) (Whishaw, 1985).

The testing apparatus consisted of a circular pool (diameter: 140 cm, height: 60 cm), filled to a depth of 29 cm with 24°C water rendered opaque with milk. Water was replaced every day. Orientation within the room was made possible by the presence of a door, PC, and water taps, etc., which were visible from the pool. Four points equally spaced around the perimeter of the tank were arbitrarily designated to serve as starting locations. On this basis, the tank was virtually divided into four equal quadrants.

On days 1–5, place learning consisted of four trial sessions per day, with trials spaced 15 min apart. In each trial the mouse was gently lowered into the water with the head facing the wall and released by the experimenter from one starting point (randomly selected: north, south, east, or west). The animals were allowed to swim until they located the platform (16 cm diameter, 28 cm height) submerged 2 cm in a fixed position (southwest quadrant and 18 cm away from the wall). Mice that failed to find the platform within 60 sec were placed on it for 20 sec, the same period as allowed for the successful animals. In session 5, after the fourth trial of place learning, the platform was removed from the maze and the mice performed an extra test (removal). They were released from the east starting point and allowed to swim for 60 sec. On days 6 and 7, the Morris water maze paradigm was modified to the reversal task.

The platform was placed opposite its location during the place-learning task (in the center of the northeast quadrant) and the test followed the same procedure with four trials per day for 2 consecutive days. On days 8 and 9, the animals were tested for cue learning, in which the platform was placed in the northwest and elevated 1 cm above the water level with its position indicated by a visible cue, a tube 10 cm high protruding from one angle of the platform and supporting a black and white striped panel (5 × 8 cm). Moreover, two black panels were placed around the pool to prevent subjects using most of the cues outside the maze. The cue learning consisted of four trials per day for 2 consecutive days.

In the last paradigm, the animals were tested for working memory in a “repeated acquisition paradigm” consisting of four consecutive trials per day in two sets of four consecutive days (14–17 and 21–24). In these place-learning acquisitions the animals were placed at the same starting point (randomly selected: north, south, east, or west) in all four consecutive trials of one session and allowed to swim until they located the platform submerged in a fixed position which was randomly changed every day (set 1, days 14–17: in the middle of the west, in the middle of the east, in the center of the pool, and 12 cm away from the northern starting point, respectively; set 2, days 21–24: close to the center, in the center, 12 cm away from the south starting point, 12 cm away from the northwestern starting point, respectively). Mice that failed to find the platform within 90 sec were placed on it for 20 sec (the same period of time as was allowed the successful animals) and thereafter they were immediately retested. Several fixed room cues were constantly visible from the pool.

Behavior was evaluated by both direct observation and analysis of videotape recorded images by two independent observers unaware of the animal's genotype. During each trial of all those tasks (except for removal), the escape latency, measured with a stopwatch, was recorded. All trials were videotaped for the retrospective computerized measurement of the path lengths. A tracking system (SMART, Panlab, Barcelona, Spain) connected to a video camera placed above the behavioral apparatus recorded the animal's behavior and enabled computerized measurement of the distance traveled by the animal during the test.

The average swimming speed (cm/s) of the mice during the trials was calculated by dividing the total distance the mice traveled by the length of the recording session. The place strategy of the mice in the removal, the time spent in each of the four quadrants, the distance traveled along them, and the number of crossings over the old platform position (annulus crossings) were measured retrospectively by means of the computerized tracking system.

Six-arm radial tunnel maze test

A six-arm radial maze was configured by means of barriers in a hexagonal maze (extension 1.4 m, tunnels 8 cm wide and 9 cm high) (Fitzgerald et al., 1988). Each radial main arm of the maze leads to a T-shaped branching point with a blind alley on the right and a long angled alley on the left. Forty-two infrared photocell units were uniformly distributed throughout the tunnels and interfaced to an IBM XT computer. Entry into each of the six radial main arms and to the short, blind alley was thus measured. The ceiling and side walls were fitted together to form a unit that could be lifted from the floor to permit easy removal of a subject and subsequent cleaning. Before testing the animals were familiarized with the experimental room for at least 4 min. Then the animals were introduced into the maze by a ceiling door at the center, the door was closed, and data recording started. After 6 min, each trial was terminated automatically and the maze floor was cleaned. This procedure was repeated for 5 consecutive days. From each session the following behavioral parameters were obtained from individual animals: total number of photobeams interrupted (locomotor activity); the number of different arms explored; the number of short, blind alleys explored; and the number of reentries (repetitions) into arms before all six arms had been visited.

Behavioral statistics

Results are expressed as mean \pm SEM. Differences between genotype were analyzed by one-way ANOVA followed by post-hoc Duncan's test comparisons. Differences between genotype and genotype \times interval interactions in the different Morris water maze tests and the six-arm radial tunnel maze were analyzed by ANOVA for repeated measures. Specific differences between two of the genotypes were analyzed with Student's *t*-test comparisons, and differences between two sessions of the same test with paired *t*-test. In all cases, statistical significance was considered at $P < 0.05$.

RESULTS

Slice experiments

We found that elimination of A₁Rs clearly affected synaptic physiology in hippocampus, confirming earlier results (Johansson et al., 2001; Masino et al., 2002). As noted previously, hippocampal slices from A₁R^{-/-} mice were insensitive to the application of exogenous adenosine. In addition, A₁R^{-/-} slices showed essentially no paired-pulse facilitation, a presynaptic phenomenon augmented by activity of the adenosine A₁ receptor. At an interpulse interval of 70 ms, the paired-pulse ratio (fEPSP response to the second

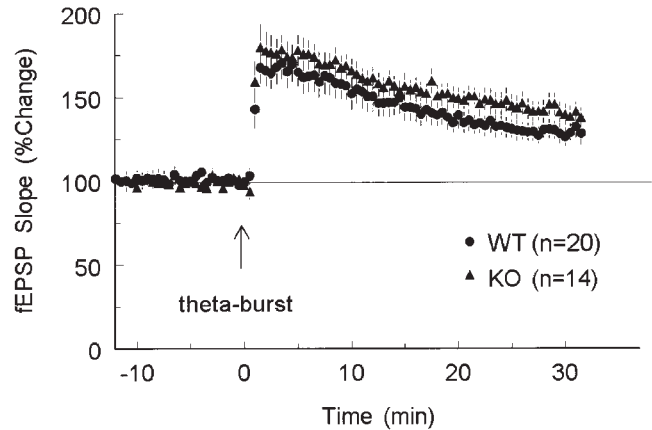


Fig. 1. Theta burst LTP was unaffected in A₁R^{-/-} slices. After establishing a stable baseline fEPSP, theta burst stimulation (see Materials and Methods) was given as indicated by upward arrow. Both genotypes showed significant potentiation of the fEPSP slope. However, the magnitude of the potentiation was not different between the A₁R^{-/-} (KO) and the A₁R^{+/+} (WT) slices. Slices were obtained from animals between 6 and 18 months old. No effect of genotype was observed at any age, but LTP magnitude did decrease with age (see Results for details).

pulse/fEPSP response to the first pulse) was significantly higher for A₁R^{+/+} slices than A₁R^{-/-} slices, where it was nearly abolished (1.33 ± 0.07 vs. 1.09 ± 0.04 , respectively, $n = 7$, $P < 0.02$). The paired pulse ratio for A₁R^{+/-} slices was intermediate at 1.24 ± 0.11 ($n = 5$).

Despite these differences between the genotypes in a measure of presynaptic function, there were no significant differences in the amplitude of LTP induced with theta burst stimulation (Fig. 1) or tetanic stimulation (data not shown). In general, when the data were analyzed and grouped according to age, theta burst LTP was impaired significantly in hippocampal slices from older (15 months) vs. younger (9 months) A₁R^{-/-} mice (fEPSP slope at 35 min: 113.4 ± 4.86 at 15 months, $n = 5$, vs. 141.4 ± 10.7 at 9 months, $n = 6$, $P < 0.05$) and was impaired in a very similar manner in slices from the A₁R^{+/+} mice (117.5 ± 11.8 at 15 months, $n = 6$, vs. 144.5 ± 6.5 at 9 months, $n = 6$, $P < 0.05$). There were no major differences between the genotypes in LTD (Fig. 2), but there does appear to be a greater augmentation and diminution of the fEPSP in the A₁R^{-/-} knockout slices during the initiation and termination of the stimulation protocol, respectively.

Morris water maze tests

The one-way analyses of swimming speed over the different sessions and paradigms in the Morris water maze indicated no differences between genotypes. The distance traveled was used as the main measure of spatial learning, since it has been shown to reflect

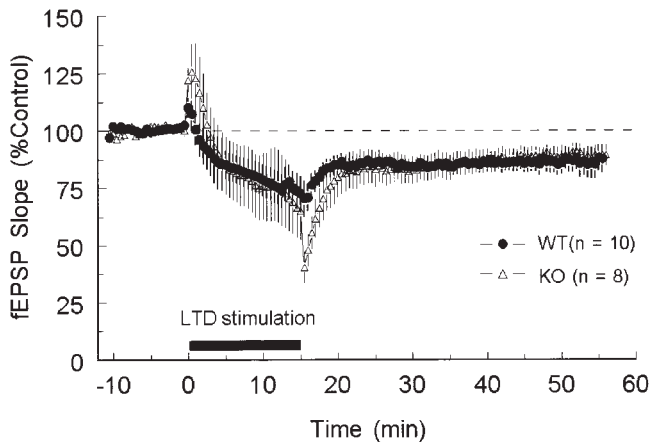


Fig. 2. LTD was unaffected in $A_1R^{-/-}$ slices. After establishing a stable baseline fEPSP, we induced LTD with 1-Hz stimulation for 900 sec where indicated on the x-axis. Slices were obtained from animals 6–9 months old. Both genotypes showed some depression, but there was no long-term difference between the $A_1R^{-/-}$ (KO) and the $A_1R^{+/+}$ (WT) slices. The initial augmentation of the fEPSP in the knockout slices at the onset of LTD stimulation could be due to the loss of A_1R -mediated inhibition.

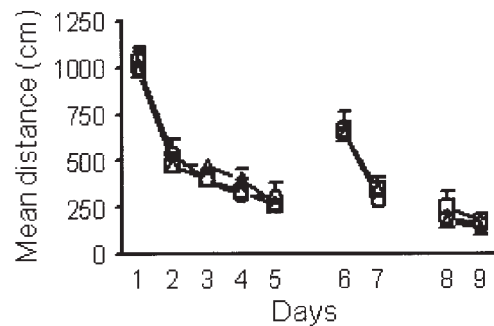
spatial learning performance more accurately than the frequently used escape latency measure (Gerlai and Clayton, 1999).

The three groups of mice showed similar acquisition patterns with distances (averaged for the four trials of each session) decreasing over sessions of place-learning and reversal tasks, as well as a maximum efficiency to reach the platform in the cue learning task, already observed in the first cue session (Fig. 3A). No differences between genotypes appeared in any of the variables recorded during the removal session. Thus, all the groups covered similar distances in the pool (mean \pm SEM, $A_1R^{+/+}$ 1290 \pm 58 cm; $A_1R^{+/-}$ 1161 \pm 48 cm; $A_1R^{-/-}$ 1226 \pm 32 cm) with similar number of annulus crossings (mean \pm SEM, $A_1R^{+/+}$ 4.33 \pm 0.53; $A_1R^{+/-}$ 4.63 \pm 0.58; $A_1R^{-/-}$ 5.62 \pm 0.9). In all the groups greater preference was shown for the trained quadrant (P) compared to the opposite one (O) as measured by the time (Fig. 3B) and the percentage of distance in these quadrants vs. the total ($A_1R^{+/+}$ 35% in P vs. 15% in O; $A_1R^{+/-}$ 37% in P vs. 17% in O; $A_1R^{-/-}$ 40% in P vs. 17% in O; in post-hoc Duncan's comparison, quadrant P different from quadrant O, $P < 0.05$, in all cases). In the two sets of place learning to assess working memory, no genotype differences were found in the distance covered in each trial (Fig. 3C; to simplify, only the first set is presented) or in the comparisons between the first and each of the following trials within each session, (2, 3, or 4; not shown).

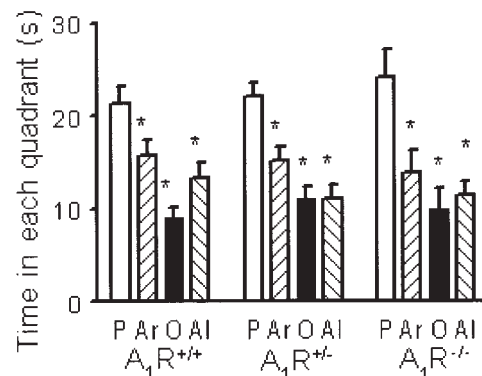
Six-arm radial tunnel maze test

Upon initial testing in the six-arm radial tunnel maze, no genotype differences were found in the total

A. PLACE TASK, REVERSAL & CUE



B. REMOVAL



C. WORKING MEMORY

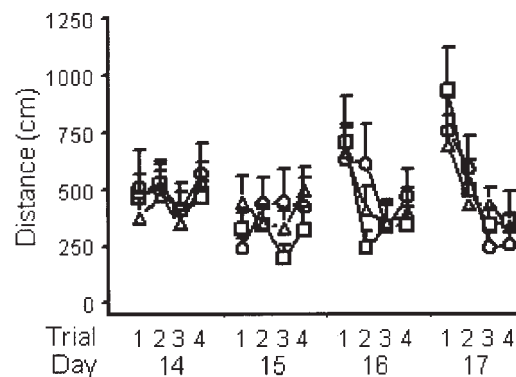


Fig. 3. Learning and memory in several tasks in the Morris water maze. Square symbol: $A_1R^{+/+}$, $n = 9$; triangle symbol: $A_1R^{+/-}$, $n = 19$; circle symbol: $A_1R^{-/-}$, $n = 13$. Results are mean \pm SEM. **A:** Place task, reversal, and cue: Mean total distance covered to reach the platform in the four trials per session during the place learning (days 1–5), reversal (days 6 and 7), and cue (days 8 and 9) tasks. **B:** Removal: Time spent in P, the trained quadrant where the platform was previously located; Ar, the adjacent right; O, opposite and Al, adjacent left quadrants during the free swim trial (session 5, day 5). * $P < 0.05$ vs. P, trained quadrant (Duncan's test). **C:** Working memory: Mean total distance covered to reach the platform in each of the four consecutive trials of sessions in the second place-learning task for working memory (days 14–17).

activity or in the number of explored arms, reentries, or explored blind alleys, as illustrated in Figure 4. Repeated testing in the six-arm radial tunnel maze

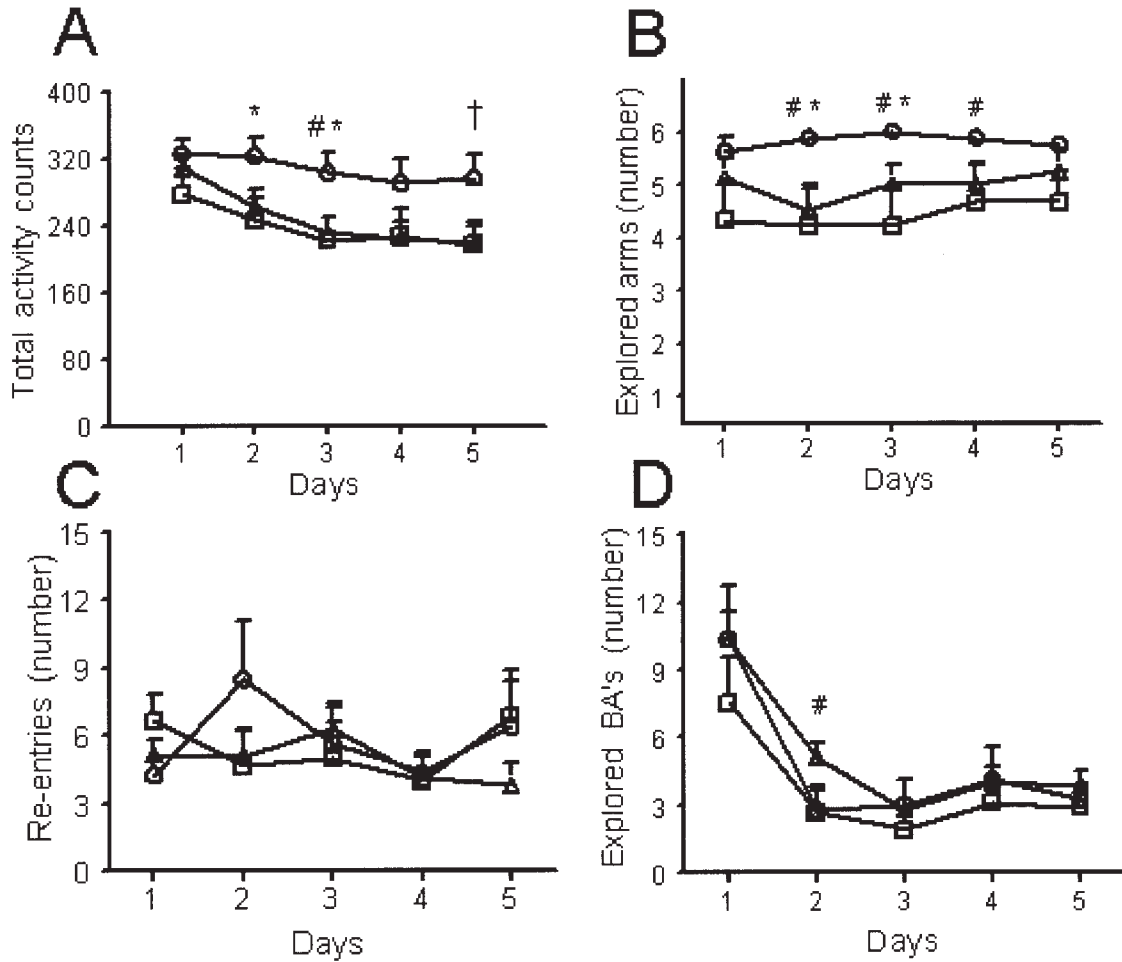


Fig. 4. Learning and memory in a six-arm radial tunnel maze repeated test (days 1-5). Square symbol: $A_1R^{+/+}$, $n = 9$; triangle symbol: $A_1R^{+/-}$, $n = 18$; circle symbol: $A_1R^{-/-}$, $n = 8$. Results are mean \pm SEM. **A:** Total activity. **B:** Number of explored arms. **C:** Number of reentries to already explored arms. **D:** Number of explored blind alleys. * $P < 0.05$ vs. the $A_1R^{+/+}$, # $P < 0.05$ vs. the $A_1R^{+/-}$ (Student's t -test), † $P < 0.05$ day 5 vs. day 1 (paired t -test).

resulted in decreased activity over the sessions of the test as revealed by a “day” effect (MANOVA, $F(4,122) = 8.822$, $P < 0.001$). As compared to the other two genotypes, $A_1R^{-/-}$ mice showed the slowest habituation process (Fig. 4A) as indicated by a lower decrease of activity between days 1 and 5 (paired t -test, $P < 0.05$ in $A_1R^{+/+}$ and $A_1R^{+/-}$ but n.s. in $A_1R^{-/-}$) and higher activity levels on days 2–3 (Student's t -test, $A_1R^{-/-}$ different from $A_1R^{+/+}$ on days 2 and 3, $A_1R^{-/-}$ different from $A_1R^{+/-}$ on day 3, $P < 0.05$ in all cases). Number of explored arms (Fig. 4B) and reentries (Fig. 4C) remained constant in all the groups over the different days, although $A_1R^{-/-}$ animals explored more different arms than the other two groups (Student's t -test, $A_1R^{-/-}$ different from $A_1R^{+/+}$ on days 2, 3, and 4; $A_1R^{-/-}$ different from $A_1R^{+/-}$ on days 2 and 3, $P < 0.05$ in all cases). A “day” effect was found in the number of explored blind alleys (Fig. 4D, MANOVA, $F(4,122) = 25.604$, $P < 0.001$) with a fast

decrease from the first to the second and following days.

DISCUSSION

Herein we characterized LTP and LTD in adenosine A_1 receptor knockout vs. wildtype hippocampal slices, and performed a series of behavioral evaluations aimed at testing hippocampal-dependent learning and memory. We found that elimination of A_1R s did not modify LTP or LTD when explored using standard protocols and recording conditions and learning and memory was unaltered in the behavioral tests performed. Given the major modulatory actions of adenosine and the prevalence of synaptic plasticity in the hippocampal slice, and the role of the hippocampus in learning, particularly spatial learning, we expected to see deficits in the $A_1R^{-/-}$ mice. Despite the lack of genotypic differences in long-term synaptic plasticity,

we found the presynaptic phenomenon of paired pulse facilitation decreased in the $A_1R^{+/-}$ slices and nearly eliminated in the $A_1R^{-/-}$ slices. In addition, we observed reduced habituation in the knockout mice when they were tested in an ethologically relevant six-arm radial tunnel maze.

In these experiments we examined LTP and LTD in the CA3–CA1 pathway. Here, LTP is believed largely due to postsynaptic mechanisms (Malenka and Nicoll, 1999). However, in the mossy fiber–CA3 synapse, the endogenous inhibitory influence of the adenosine A_1R is larger than in CA1, and LTP is largely presynaptic in nature. It was recently shown that LTP in the mossy fiber pathway is virtually abolished when adenosine A_1R s are eliminated (Moore et al., 2003). The predominant functional effects of the adenosine A_1R on synaptic transmission are presynaptic, as judged by pharmacological experiments (see Fredholm and Dunwiddie, 1988; Dunwiddie and Masino, 2001). Indeed, the major impact of the adenosine A_1R knockout is on a presynaptic form of long-term synaptic plasticity. As noted, we did find the presynaptic phenomenon of paired-pulse facilitation reduced significantly in the knockout hippocampal slices.

As we assessed the functional consequences of extracellular adenosine electrophysiologically, we do not know if extracellular levels of adenosine are altered, or if the regulation of adenosine release under various conditions is modified by the knockout. We have shown that synaptic transmission in knockout hippocampal slices is not inhibited, even by large quantities of adenosine (Johansson et al., 2001). However, information about the regulation and release of adenosine in this knockout model is important to obtain, and could help to resolve postulated mechanisms such as adenosine A_1 receptor-regulated adenosine release (Sinclair et al., 2000).

Several paradigms in the Morris water maze were designed to assess in detail two categories of learning and memory, namely, spatial reference memory and working memory. Appropriate performance of the water maze tasks requires predominantly spatial reference memory, that is, the information to solve the problem (i.e., finding the hidden platform) endures over the 15-min intertrial time or the 24 h between sessions (Olton et al., 1979). In contrast, in the place-learning task with a “repeated acquisition paradigm,” the four trials of each session are administered consecutively (Whishaw, 1985). In this procedure, working memory, that is, information persisting only for a short period of time (Olton et al., 1979), can also be tested.

No differences between genotypes could be detected in spatial reference memory or the standard place-learning task. In all the groups the performance curves showed that major acquisition was attained on the first day, as evidenced by the reduction of the dis-

tances covered during the following days. Escape latencies were also reduced.

The removal trial assesses the accuracy of spatial reference memory more reliably than the standard place-learning task since it requires memory of the precise location of the platform. In addition, it is largely independent of swimming abilities and speed. The equal distributions of navigation preferences shown by the three groups of mice emphasized the results of the previous place-learning task and demonstrated a strong similarity in the searching strategy (i.e., the preference for the platform quadrant in contrast to the opposite quadrant).

As a result of previous experience in a similar task, all groups showed faster acquisition in the first reversal session (day 6) as compared to the very first performance in the maze (day 1). However, the unexpected change in the usual platform position (north-east instead of southwest) introduced a certain degree of difficulty to the test since animals needed more time and covered more distance before finding the new position as compared to the last sessions of the previous tasks (days 2–5). Once again, the three genotypes showed identical acquisition and retention, with an improved performance being already evident in the second session (day 7).

Since indirect action of adenosine on attention and wakefulness could contribute to its effects on learning and memory, a cue-learning task was administered with the intramaze cue facilitating the localization of the hidden platform and also reducing possible confounds of motivational or sensorimotor differences. In this task, the performance was again similar in all the groups from the first cued session. Together, these results indicate that none of the groups had deficits in spatial reference memory as measured by these tasks in the water maze. In addition, they did not have sensory, motivational, or motor problems, as shown by the swim speed and the cued task.

The second place-learning task (days 14–17 and 21–24) consisted of a “repeated acquisition paradigm” with the four trials of each session administered consecutively to assess working memory. No differences could be detected, in agreement with the results reported by Hooper et al. (1996), where the A_1R selective agonist CPA prevented working memory deficits induced by scopolamine but failed to modify working memory alone, suggesting that endogenous adenosine does not normally participate in working memory processes.

$A_1R^{+/-}$ and $A_1R^{-/-}$ mice would mimic a life-long administration of medium and high doses of selective adenosine A_1R antagonist, respectively. The absence of deficits observed in the Morris water maze thus indicates that such life-long administration does not cause any major effects on memory. This is in apparent contrast to a study where chronic administration

of the selective A₁R antagonist CPX was shown to impair performance in the water maze (Von Lubitz et al., 1993), even though no effect was observed after acute administration. In a recent report (2003), Lang et al. also confirmed that the deletion of A₁R does not affect spatial performance during both training and probe trials in a place navigation task in the water maze, although their A₁R^{-/-} mice showed increased wall hugging, a strategy choice suggested to be a result of their emotional instability.

To further examine learning and memory, the six-arm radial tunnel maze test was included in the behavioral battery. The structure of this dark tunnel maze, which allows animals to move freely and to explore spontaneously, reproduces the main features of mouse burrows in the wild (Bättig, 1983) and provides a more ethological context than other devices. In our study, all the animals showed similar high exploratory activity in their first experience in the maze. During the 6 min of the first test, they visited most of the six arms, made some reentries, and explored several short, blind alleys. These results may be in contrast to the higher and lower activities observed in A₁R^{+/-} and A₁R^{-/-}, respectively, in the open-field and the hole-board tests (see Giménez-Llort et al., 2002). Nevertheless, the exploratory behavior measured in those tests is mixed with a certain level of anxiety/emotionality which depends, for instance, on how different the apparatus is from the natural environment, the level of illumination, and the open space. In fact, no differences in exploratory activity were found during the first hours in activity meter cages (see Giménez-Llort et al., 2002), which were very similar to their own home-cages. In the same way, the dark tunnel maze resembling the natural mouse burrows would allow us to measure the exploratory behavior without (or with less) interference of anxiety/emotionality.

The number of reentries into previously visited arms is considered to correspond to the retracing errors in more conventional 8- or 16-arm mazes. The choice between long-angled arms and blind alleys at the T-intersections provides a measure of working memory similar to that of standard T-maze, and have thus been interpreted in terms of changes of within-session working memory (Fitzgerald et al., 1988). The lack of differences between genotypes in these behavioral parameters would agree with the results obtained in the repeated acquisition paradigm in the Morris water maze, where no working memory differences were found.

In all the groups, repeated testing over the days increased the exploratory efficiency as revealed by the fast reduction in the number of blind alley visits, providing evidence of between-session reference memory (Fitzgerald et al., 1988). In this regard, it is also worth noting that the number of reentries and

the number of different arms explored was constant over the tests, independent of the decrease in total activity.

The total activity was reduced successively in parallel with the decrease in novelty. However, in the A₁R^{-/-} mice the decrease was slower and they explored a larger number of arms than the other genotypes, perhaps reflecting a lower ability to become habituated to the maze, which could be related to certain differences in long-term reference memory as well. These results agree with those reporting that rats exposed to caffeine do not make fewer errors and do not decrease their latency in mazes of varying complexity, although exploration is stimulated (reviewed in Nehlig et al., 1992). Overall, the assessment of spatial learning and memory in the six-arm radial tunnel maze indicates that all the groups show similar working memory abilities, and that further studies should focus on the reduced habituation to a known environment. It has been pointed out that many of the results of caffeine on higher cognitive functions might be explained by reduced habituation, and hence to a maintained arousal level (reviewed in Nehlig et al., 1992). In these studies, habituation is reported to be decreased in both animals (Olivera et al., 1990) and man (Davidson and Smith, 1991). We also observed that both the time-dependent decrease in exploratory motor behavior when mice are exposed to a test box, and the reduction in overall activity level upon repeated exposure to that environment, are somewhat diminished in A₁R^{-/-} mice as compared to wildtype (Halldner et al., 2004).

In conclusion, the behavioral assessment of spatial learning and memory of mice lacking adenosine A₁R has shown that no deficits on spatial reference and working memory could be found in Morris water maze tests or in the six-arm radial tunnel maze. Furthermore, LTP (and LTD) in the CA1 region appear normal in the adult adenosine A₁ receptor knockout mice. Further electrophysiological studies focused on frequency-specific plasticity phenomena, maximal inducible plasticity, or other recording parameters may yet reveal differences between the genotypes. Most importantly, though, radial tunnel maze experiments provide evidence for the involvement of A₁R_s in maintaining arousal, as knockout animals show reduced habituation to this known environment.

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