Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A_1 receptor

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Caffeine is believed to act by blocking adenosine A_1 and A_{2A} receptors (A_1R, $A_{2A}R$), indicating that some A_1 receptors are tonically activated. We generated mice with a targeted disruption of the second coding exon of the A_1R ($A_1R^{-/-}$). These animals bred and gained weight normally and had a normal heart rate, blood pressure, and body temperature. In most behavioral tests they were similar to $A_1R^{+/+}$ mice, but $A_1R^{-/-}$ mice showed signs of increased anxiety. Electrophysiological recordings from hippocampal slices revealed that both adenosine-mediated inhibition and theophylline-mediated augmentation of excitatory glutamatergic neurotransmission were abolished in $A_1R^{-/-}$ mice. In $A_1R^{+/-}$ mice the potency of adenosine was halved, as was the number of A_1R. In $A_1R^{-/-}$ mice, the analgesic effect of intrathecal adenosine was lost, and thermal hyperalgesia was observed, but the analgesic effect of morphine was intact. The decrease in neuronal activity upon hypoxia was reduced both in hippocampal slices and in brainstem, and functional recovery after hypoxia was attenuated. Thus A_1Rs do not play an essential role during development, and although they significantly influence synaptic activity, they play a nonessential role in normal physiology. However, under pathophysiological conditions, including noxious stimulation and oxygen deficiency, they are important.

Adenosine acts on four cloned and pharmacologically characterized receptors, A_1, A_{2A}, A_{2B}, and A_3 (1). Adenosine is believed to play a particularly important role in hypoxia and ischemia, and there is evidence that adenosine serves to limit damage secondary to ATP loss (2, 3). However, adenosine may have important actions under more normal physiological circumstances as well. For instance, the effects of caffeine, at concentrations reached during habitual caffeine consumption, are believed to be a consequence of blockade of tonic activity at some A_1 and A_{2A} receptors (A_1R and $A_{2A}R$) (4). Studies on mice lacking $A_{2A}Rs$ show that adenosine tonically activates $A_{2A}Rs$ and that this activation has functional effects, particularly on behavior, blood pressure, and blood platelets (5). A_1Rs are more widely distributed than $A_{2A}Rs$ (4, 6), but despite extensive pharmacological studies their physiological and pathophysiological roles remain unclear. Here we show that A_1Rs mediate physiological as well as pathophysiological effects of endogenous adenosine. In particular, adenosine acts tonically to activate presynaptic and postsynaptic A_1Rs to depress synaptic transmission and to reduce nociceptive signaling. At elevated levels seen during hypoxia, adenosine acting at A_1Rs is responsible for the depression of neuronal activity, and in this situation elimination of A_1Rs results in impaired functional recovery.

Materials and Methods

Generation of A_1R Knockout Mice. A major part of the protein-coding sequence of the mouse A_1R gene (7) corresponding to exon 6 of the human A_1R gene described by Ren and Stiles (8) was cloned. The targeting construct was built (9) as illustrated in Fig. 1: a 5′ homologous segment (≈3 kb), a phosphoglycerokinase-neo cassette (obtained from J. K. Heath, Oxford University) replaced most of the protein-coding sequence of the mouse exon and a 3′ homologous segment (≈5 kb). The A_1R gene was inactivated in E14.1 embryonic stem cells. The correct integration of the mutant allele was demonstrated by Southern blotting using, inter alia, one internal and one external probe. One of the clones was used to generate chimeric mice. Male chimeras were mated to C57BL females, and $A_1R^{+/-}$ mice from this 129/OlaHsd/C57BL hybrid offspring were intercrossed to generate $A_1R^{+/+}$, $A_1R^{+/-}$, and $A_1R^{-/-}$ offspring (Fig. 1*B*). These appeared with the expected Mendelian frequency. Each experiment was carried out with siblings with different genotypes.

Receptor Autoradiography. Cryostat sections of brain and spinal cord (10 μm) were incubated with increasing (0.2–10 nM) concentrations of [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) (for A_1R) or [^{3}H]7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (for $A_{2A}R$) as described (10). The autoradiograms were analyzed with an MCID M5 system (Imaging Research, St. Catharines, Canada). Optical density was converted to density of bound ligand (in fmol/mg tissue). K_d and B_{max} values were calculated with GRAPHPAD PRISM.

Hippocampal Slice Physiology. Transverse hippocampal slices (400 μm) were prepared from 4- to 6-week-old mice and studied with conventional extracellular and whole-cell recording techniques (11, 12). Recordings were made from submerged slices incubated at 31–33°C in aerated (95% O_2, 5% CO_2) artificial cerebrospinal fluid containing 126 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 2.4 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 11 mM glucose, and 26 mM $NaHCO_3$. For recordings of field excitatory postsynaptic potential (fEPSP) responses, the recording electrode was placed in s. radiatum of the CA1 region and the stimulation electrode in s. radiatum near the border of the CA1 and CA2 regions; stimuli were delivered at 15-s intervals. Concentrated stock solutions of drugs were delivered with a calibrated syringe pump

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Abbreviations: A_1R, adenosine A_1 receptor; fEPSP, field excitatory postsynaptic potential; EPSC, excitatory postsynaptic current; R-PIA, N^6-(*R*-phenylisopropyl)adenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine.

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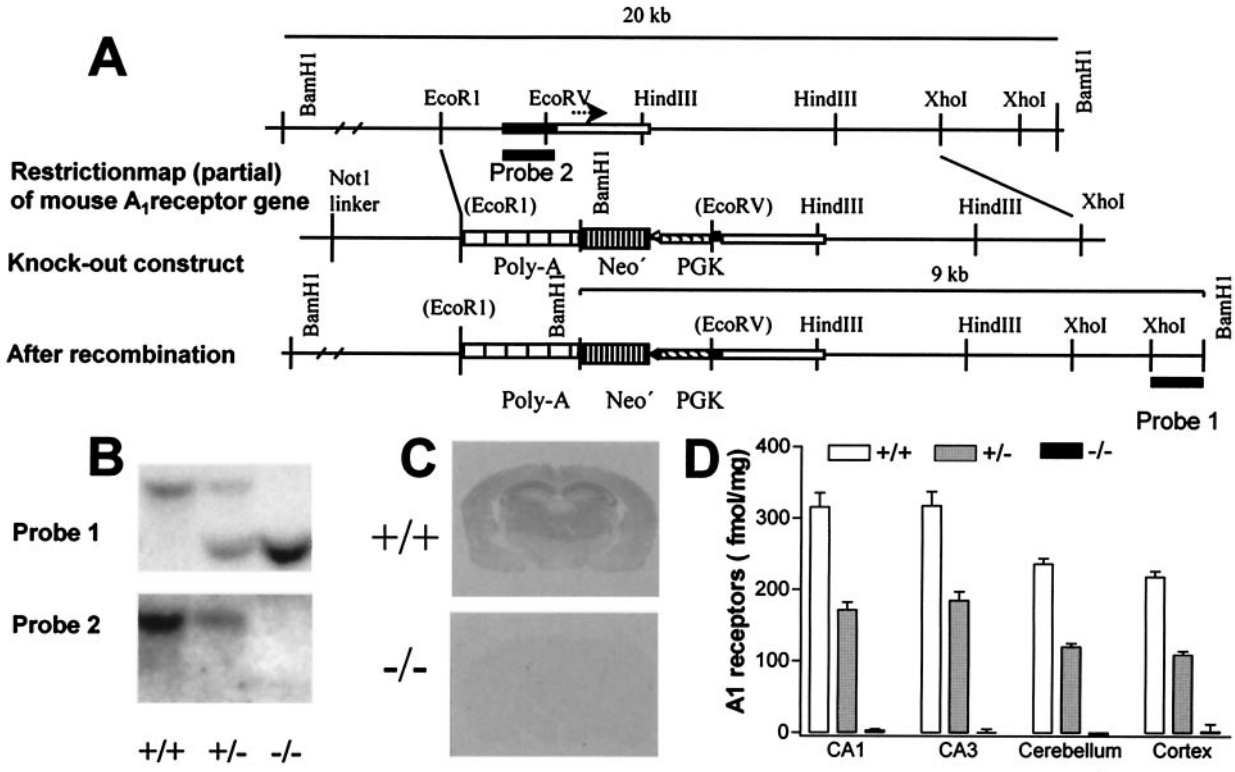

Fig. 1. (*A*) Partial restriction map of the mouse A_1R gene and organization of the targeting construct and the allele resulting from the homologous recombination. The site of interaction of two probes (1 and 2) is also shown. (*B*) Southern blots with the two probes. The upper band is at 20 kb; the lower band is at 9 kb. (*C*) Autoradiographic verification of the elimination of A_1R. [^{3}H]DPCPX (10 nM) was used. Concentrations closer to K_d resulted in undetectable binding in $A_1R^{-/-}$ mice. (*D*) Quantitation (B_{max}) of [^{3}H]DPCPX binding to measure A_1Rs in some brain regions. There were no significant differences in the affinity of DPCPX for its binding site (K_d ranged from 0.38 to 0.60 nM in different regions and genotypes).

directly into the superfusion system. Excitatory postsynaptic currents (EPSCs) were recorded from CA1 neurons with the whole-cell patch-clamp technique. Patch recording electrodes were filled with a solution containing 125 mM potassium gluconate (Fluka), 5 mM KCl, 10 mM Hepes (Fluka), 0.1 mM $CaCl_2$, 1 mM potassium-EGTA (Fluka), 2 mM $MgCl_2$, 2 mM magnesium-ATP, and 0.2 mM Tris·GTP. Series resistances ranged from 10 to 41 MΩ (average 30 ± 1.5 MΩ).

Body Temperature. The initial control core temperature was determined with a rectal thermistor probe. The animals were then injected i.p. with 0.1 mg/kg of N^6-cyclohexyladenosine in saline, returned to their home cage for 30 min, and then retested to determine the change in body temperature. All testing was done in a room maintained at 22–24°C.

Behavioral Testing. Five-month-old male mice were housed under standard conditions on a 12:12-h light–dark cycle with light onset at 1500 h. For the sensorimotor studies, animals were tested in three consecutive trials of visual placing reflex, two 20-s trials for equilibrium on the wire rod, and two 5-s trials for prehensility (13). The mice were tested between 1700 h and 1900 h. For the activity test, triads of animals were tested individually in a multicage actimeter system (25 × 25 × 25 cm each). A 24-h schedule divided into 30-min periods was studied starting at 1700 h. For the dark–light box test, mice were placed in the dark compartment (head facing the wall) and observed for 5 min. The dark compartment was 18 × 27 cm and 27 cm high, and the light compartment was 27 × 27 cm, 27 cm high, with a 20-W red bulb placed 37 cm above the floor. The two compartments were connected by a 7 × 7 cm opening. Time spent in and entries into the lit compartment were recorded (SMART; Panlab, Barcelona, Spain). The mice were tested between 1000 h and 1200 h.

Analgesia. To determine paw withdrawal threshold to mechanical stimulation, a mouse was placed in a plastic cage (10 × 8 × 7 cm) with a metal mesh floor. The plantar surfaces of both hind paws were stimulated with a set of calibrated nylon monofilaments (von Frey hairs; Stoelting) with increasing force until the mouse withdrew the limb. Each monofilament was applied five times. The withdrawal threshold was taken as the force at which the mouse withdrew the paw from at least three of five consecutive stimuli. The median value for the two paws was used. During the tail flick test the mouse was gently held in the experimenter's hand, a radiant heat source was focused 1–2 cm from the tip of the tail, and the latency to tail flick was recorded automatically. The intensity of the stimulation was adjusted so that basal tail flick latency was 3–5 s for normal C57/BL6 mice. N^6-(*R*-phenylisopropyl)adenosine (R-PIA) was administered intrathecally as described (14). A 27-gauge needle was inserted between the L5 and L6 vertebrae 15–30 min after an incision had been made through the skin over the lumbar spine. The injection (5 μl) was made with a microsyringe connected via PE 50 tubing.

Brainstem Respiratory Activity. Brainstem–spinal cord preparations were isolated under ether anesthesia from P1–P4 mice (46 preparations) as described in detail elsewhere (15, 16). Briefly, the brainstem was rostrally decerebrated between VIth cranial nerve roots and the lower border of the trapezoid body so that the pons was removed. The preparation was continuously perfused in a 2-ml chamber with artificial cerebrospinal fluid: 128 mM NaCl, 3.3 mM KCl, 0.8 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM

$MgSO_4$, 26 mM $NaHCO_3$, and 30 mM glucose at 28°C (flow rate, 3–4 ml/min). The solution was continuously equilibrated with 95% O_2 (or N_2) and 5% CO_2 to pH 7.4. Respiratory-like activity corresponding to the inspiratory rhythm was monitored at the C4 ventral root through a glass suction electrode and recorded (1–5 kHz) and analyzed offline.

Results

The lack of adenosine A_1Rs was confirmed by *in situ* hybridization (not shown) and by the absence of high-affinity binding sites for the selective A_1R antagonist DPCPX (Fig. 1*C*). The number of A_1Rs was determined in several areas of the brain and spinal cord by quantitative autoradiography with [^{3}H]DPCPX as the ligand. In all areas examined the number of receptors was twice as high in $A_1R^{+/+}$ as in $A_1R^{+/-}$ mice and essentially zero in $A_1R^{-/-}$ mice (Fig. 1*D*). There were no adaptive changes in the number of A_{2A}Rs in striatum (B_{max}, 284 ± 10 fmol/mg gray matter in $A_1R^{+/+}$, 282 ± 10 in $A_1R^{+/-}$, and 268 ± 10 in $A_1R^{-/-}$ mice; mean ± SEM, $n = 6$).

$A_1R^{-/-}$ mice appeared to be normal and bred normally. The body mass was similar in $A_1R^{+/+}$, $A_1R^{+/-}$, and $A_1R^{-/-}$ mice from birth until at least 5 months of age, when male mice weighed 40.5 ± 1.5 g ($A_1R^{-/-}$) and 37.3 ± 1.8 g ($A_1R^{+/+}$). The animals showed normal blood pressure and heart rate (not shown). Body temperature was similar in all genotypes. However, the hypothermia induced by i.p. administration of A_1R agonist N^6-cyclohexyladenosine (0.1 mg/kg) in $A_1R^{+/+}$ animals (−3.4 ± 0.16°C) was reduced to −2.0 ± 0.55°C in $A_1R^{+/-}$ mice and was eliminated (−0.08 ± 0.39°C) in $A_1R^{-/-}$ mice.

Adenosine inhibits excitatory synaptic transmission in several brain regions, including hippocampus, via both presynaptic and postsynaptic mechanisms (17). Because of the high density of A_1Rs in the CA1 and CA3 regions of hippocampus (Fig. 1*D*), the ability of adenosine to modulate glutamatergic synaptic transmission in this region was examined. Adenosine had no effect on fEPSPs in hippocampus slices from $A_1R^{-/-}$ animals (Fig. 2*A*), and the concentration of adenosine required to decrease fEPSPs by 50% was increased from 37 ± 1.4 μM for slices from $A_1R^{+/+}$ animals to 73 ± 1.2 μM for slices from $A_1R^{+/-}$ mice ($P < 0.001$). The rightward shift of the dose–response curve was thus of the same magnitude as the decrease in receptor number. There was no difference in the maximal response. Similar estimates could not be made for responses evoked from slices from the $A_1R^{-/-}$ mice because inhibition of the fEPSP was never observed. A small increase was observed in the fEPSP response from the $A_1R^{-/-}$ mice (12.7% increase at 500 μM adenosine), but this increase could not be blocked by the nonselective adenosine antagonist 8-*p*-sulfophenyl theophylline. No significant increase was observed at lower concentrations of adenosine, where possible activation of A_{2A}Rs might be expected.

Similar results were obtained when EPSCs were recorded with whole-cell patch electrodes; i.e., the inhibitory effect of adenosine was completely lost in slices from $A_1R^{-/-}$ animals (Fig. 2*B*). As in rat hippocampal slices, both nonselective and A_1R-selective antagonists significantly increased fEPSPs and EPSCs in slices from $A_1R^{+/+}$ mice, and this facilitatory effect was abolished in slices from $A_1R^{-/-}$ mice (Fig. 2*C*). This finding suggests that under basal conditions in hippocampus, the only modulatory effects on glutamatergic transmission exerted by endogenous adenosine are mediated via A_1Rs.

In the hippocampus, adenosine also hyperpolarizes neurons via activation of a G protein-coupled K^+ channel (17). As with the presynaptic effects of adenosine, the effect of adenosine on the holding current was completely eliminated in $A_1R^{-/-}$ slices (Fig. 2*C*). Because A_1Rs and γ-aminobutyric acid type B receptors can both inhibit synaptic transmission and activate the same population of K^+ channels, we attempted to determine whether there were compensatory changes in γ-aminobutyric acid type B receptor sensitivity that might offset the loss of the tonic A_1 inhibition. However, neither the presynaptic nor the postsynaptic effects of the γ-aminobutyric acid type B agonist baclofen were enhanced and, if anything, were reduced in $A_1R^{-/-}$ mice (Fig. 2 *B* and *C*).

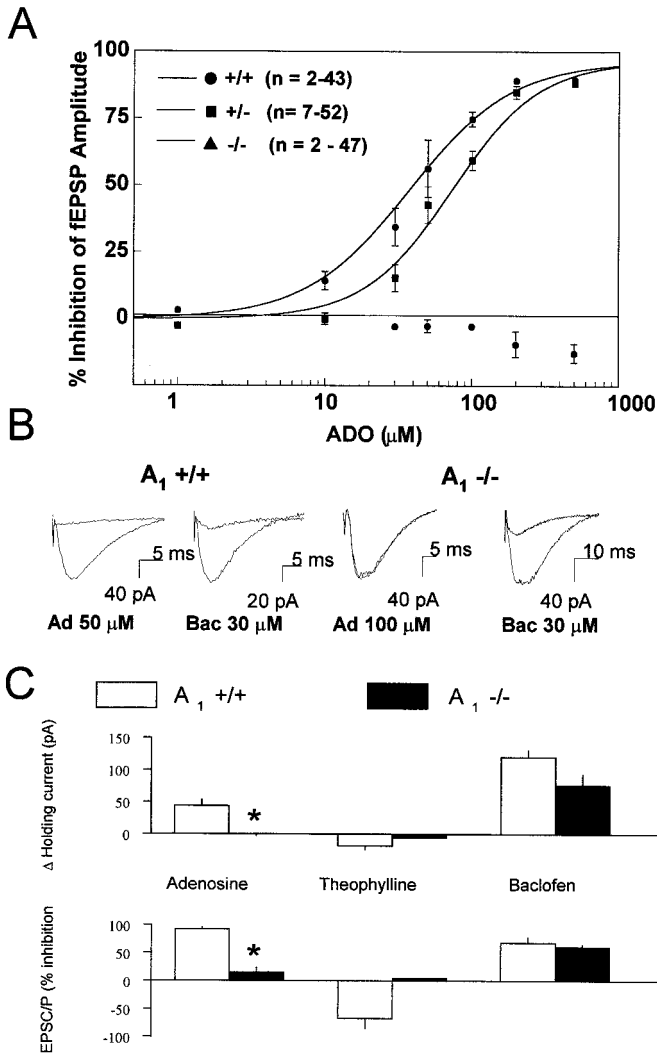

Fig. 2. (*A*) Inhibition of hippocampal field EPSPs by increasing concentrations of adenosine in slices from $A_1R^{+/+}$, $A_1R^{+/-}$, and $A_1R^{-/-}$ mice. Each point represents the mean ± SEM inhibition of the fEPSP response evoked from hippocampal slices (n = number of slices tested at each concentration). Because desensitization was never observed in any line of mice, cumulative concentrations of adenosine were tested on individual slices. Sigmoidal dose–response curves were obtained with the GRAPHPAD INPLOT program. Calculated EC_{50} values were 37 ± 1.4 μM and 73 ± 1.2 μM for the $A_1R^{+/+}$ and $A_1R^{+/-}$ mice, respectively ($P < 0.001$), but there were no significant differences in the E_{max} values or Hill slopes. (*B*) Whole-cell recordings of EPSCs made from hippocampal CA1 pyramidal neurons with whole-cell patch electrodes. Basic physiological properties of these neurons did not differ significantly in any of the mice. Resting membrane potentials were −61.1 mV and −60.4 mV in the $A_1R^{+/+}$ and $A_1R^{-/-}$ mice, respectively, and the input resistances determined from hyperpolarizing voltage steps were 144 ± 8.6 MΩ and 160 ± 20 MΩ, respectively. (Scale bar, 5 ms/50 pA for records 1, 3, and 4; 5 ms/25 pA for record 2.) (*C*) Summary of effects of adenosine, baclofen, and the nonselective adenosine receptor antagonist theophylline on the holding current (*Upper*) and EPSC amplitude (*Lower*). Slices were superfused with adenosine (50 or 100 μM), theophylline (250 μM), and baclofen (30 and 50 μM). These concentrations produced near-maximal effects on EPSCs and holding currents. Between 1 and 12 cells were tested in each condition. * indicates a significant difference between $A_1R^{+/+}$ and $A_1R^{-/-}$, $P < 0.05$.

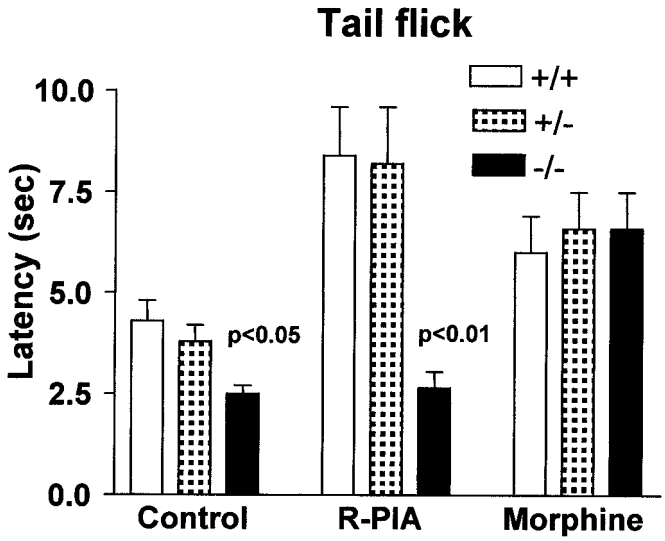

Fig. 3. Behavioral effects of A_1Rs. Thermal nociception was measured by a tail-flick test under basal conditions, after intrathecal administration of R-PIA (0.75 μg) or after i.p. administration of morphine (5 mg/kg). *P* values refer to comparison with $A_1R^{+/+}$ or $A_1R^{+/-}$ mice with ANOVA followed by a Fisher probable least-squares difference test. For baseline $F_{(2,32)} = 3.532$, $P < 0.05$; for R-PIA effects $F_{(2,16)} = 8.309$, $P < 0.01$.

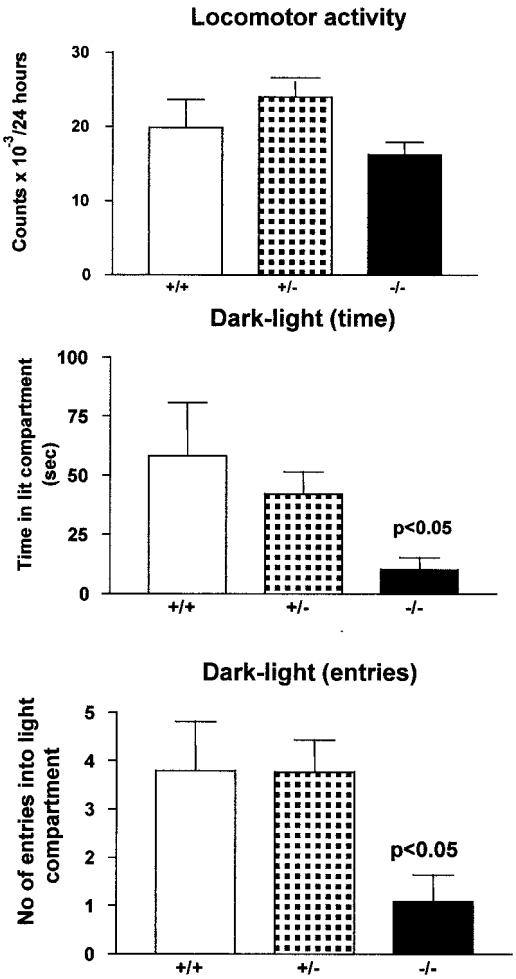

Fig. 4. Behavioral effects of disruption of A_1Rs. (*Top*) Lack of change (ANOVA $F_{(2,41)} = 0.949$, not significant) in 24-h motor activity in mice with a targeted disruption of A_1Rs. (*Middle*) In the dark–light test of anxiety the $A_1R^{-/-}$ mice spent less time in the lit compartment (mean ± SEM) than the $A_1R^{+/-}$ and $A_1R^{+/+}$ mice (ANOVA $F_{(2,41)} = 3.765$, $P < 0.05$; versus both $A_1R^{+/-}$ and $A_1R^{+/+}$, Duncan's multiple range test; after sqrt transformation for reducing heterogeneity of variances). (*Bottom*) The number of entries into the lit compartment (mean ± SEM) was also reduced in $A_1R^{-/-}$ compared with both $A_1R^{+/-}$ and $A_1R^{+/+}$ mice (ANOVA $F_{(2,41)} = 3.654$, $P < 0.05$; Duncan's multiple range test).

A role for adenosine as an endogenous analgesic substance has long been suspected (18). A_1Rs were abundant in mouse spinal cord, with the highest levels in the outer lamina of the dorsal horns (results not shown), where the density of receptors was close to that observed in hippocampus. In contrast, the distribution in the spinal cord of A_1R mRNA was more uniform, perhaps indicating that many of the A_1Rs in the dorsal horn are located on nerve terminals. The antinociceptive effect of intrathecal administration of an adenosine analogue R-PIA was completely abolished in the $A_1R^{-/-}$ animals (Fig. 3), suggesting that A_1Rs are responsible for the analgesic effects of intrathecally administered adenosine analogues. Although it has frequently been suggested that A_1Rs participate in the antinociceptive effects of opioids (19), the effect of morphine was unaltered. The $A_1R^{-/-}$ mice reacted faster to thermal pain than did wild-type or heterozygous mice (Fig. 3), but this increase was not matched by an increased sensitivity to mechanical stimulation (not shown). These results suggest that endogenous adenosine acting at A_1Rs decreases nociception mediated via C fibers.

Caffeine produces many behavioral effects, presumably secondary to adenosine receptor blockade (4). Behavioral tests to evaluate sensorimotor reflexes revealed no differences between $A_1R^{-/-}$ or $A_1R^{+/-}$ compared with $A_1R^{+/+}$. These included visual placing reflex (means ± SEM, score = 3 ± 0 in all genotypes), equilibrium (wire rod test: means ± SEM, $A_1R^{+/+}$ 31.6 ± 3.7 s; $A_1R^{+/-}$ 25.8 ± 3.0 s; $A_1R^{-/-}$ 24.1 ± 3.7 s; ANOVA $F_{(2,41)} = 0.967$, not significant), and prehensility (means ± SEM, $A_1R^{+/+}$ 9.5 ± 0.4 s; $A_1R^{+/-}$ 9.7 ± 0.2 s; $A_1R^{-/-}$ 9.8 ± 0.1 s; ANOVA $F_{(2,41)} = 0.305$, not significant). Similarly, there were no differences in total activity over a 24-h period (Fig. 4*A*). However, anxiety-related behavior in the dark–light box test was increased in the $A_1R^{-/-}$: they showed a significant reduction in the number of entries into as well as the total time spent in the lit compartment compared with $A_1R^{+/-}$ and $A_1R^{+/+}$ (Fig. 4 *B* and *C*). No evidence for increased anxiety was obtained in $A_1R^{+/-}$ mice.

Brain levels of adenosine are markedly elevated during hypoxia (20), and there is good evidence that adenosine protects against hypoxic/ischemic cell damage (2, 3, 21, 22). In hippocampal slices hypoxia markedly inhibited glutamatergic neurotransmission (Fig. 5*A*), consistent with the role proposed for A_1Rs based on results in rats (23). This reduction in fEPSP responses occurred significantly more slowly in $A_1R^{-/-}$ mice, and the magnitude of the inhibition was greatly attenuated as well. Furthermore, after the reintroduction of oxygenated medium the fEPSP responses in control mice recovered fully, whereas responses from A_1 $R^{-/-}$ animals remained significantly depressed.

Hypoxia also affects respiration. In the immature neonatal respiratory system hypoxia results in an initial increase in ventilation and then a depression of ventilation below baseline levels. This *in vivo* response can be studied in the deafferented brainstem *in vitro* (24). It has been suggested that adenosine contributes to this depression by acting on brainstem neurons (25). This suggestion provides a rationale for the use of methylxanthines to reduce the number of neonatal apneas. In an *in vitro* brainstem spinal cord preparation from $A_1R^{+/+}$ mice (15, 16), hypoxia induced a transient (1–2 min) increase in C4

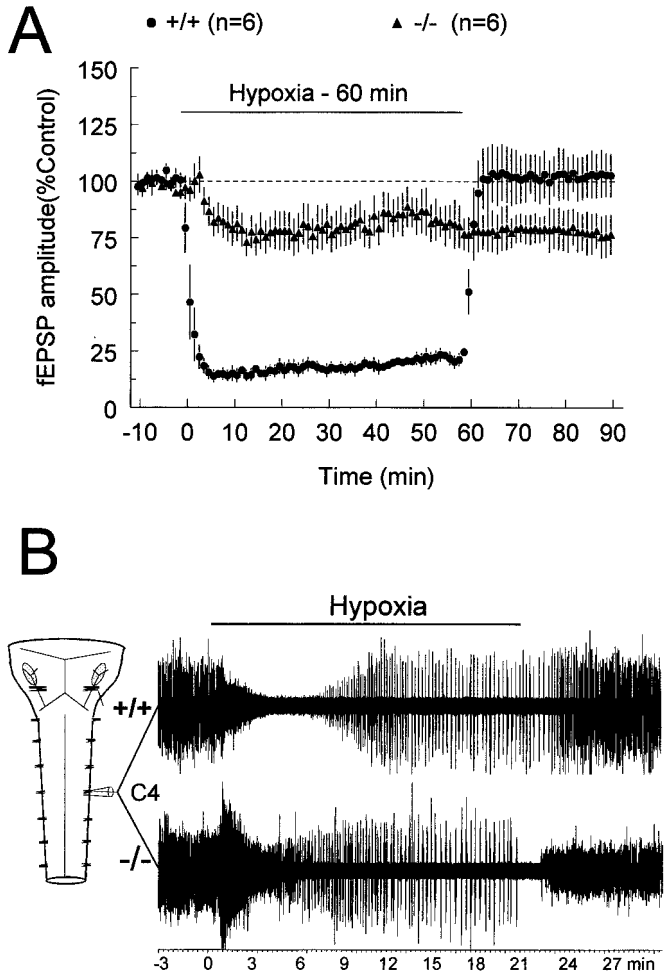

Fig. 5. Mice lacking A_1Rs show a reduced response to hypoxia/anoxia. (*A*) Perfusion of hippocampal slices with medium equilibrated with 95% N_2/5% CO_2 leads to a rapid decrease in synaptic transmission in $A_1R^{+/+}$ mice. In $A_1R^{-/-}$ mice the depression is delayed and reduced in magnitude, and there is no recovery after 60 min of incubation in hypoxic buffer. (*B*) Recording of C4 respiratory activity in isolated superfused brainstem spinal cord from 3-day-old $A_1R^{+/+}$ and $A_1R^{-/-}$ mice, before, during, and after switching to a medium equilibrated with 95% N_2/5% CO_2 for 20 min. The preparation is shown at the left. The respiratory output decreases within 3 min in $A_1R^{+/+}$ preparations and is fully recovered when oxygenated medium is reintroduced. In $A_1R^{-/-}$ the depression is significantly delayed ($P < 0.01$) and full recovery is not achieved ($P < 0.01$, ANOVA repeated measures design).

discharges followed by a decrease in C4 respiratory activity until either a slow respiratory rhythm (gasps) developed or respiratory discharge activity ceased (24). In $A_1R^{-/-}$ preparations the respiratory output was significantly greater at 3 and 5 min after the onset of hypoxia ($P < 0.05$ and $P < 0.01$, respectively, ANOVA; $n = 15$ $A_1R^{+/+}$ and 6 $A_1R^{-/-}$ siblings), and the subsequent slowing of the rhythm occurred later than in $A_1R^{+/+}$ (Fig. 5*B*). Reintroduction of oxygenated medium led to full recovery of respiratory rhythm and brainstem neuronal activity in $A_1R^{+/+}$ (data not shown). However, one-third of the preparations from $A_1R^{-/-}$ mice did not recover respiratory activity at all, and the remaining preparations did not recover fully.

Discussion

The present paper describes characteristics of a mouse line with a targeted disruption of A_1Rs. The elimination of A_1Rs was confirmed by Southern blot, *in situ* hybridization, quantitative autoradiography, and several functional tests. Specific binding of the xanthine derivative DPCPX was completely lost in the A_1R knockout mice. This loss of binding has the important implication that this drug is indeed highly selective for A_1Rs (1), despite the fact that it also binds to A_{2B}Rs (26). Hence, our results lend credence to a substantial body of pharmacological evidence and to results on the distribution of A_1Rs based on DPCPX binding (27).

The functional studies confirm and extend our knowledge of the roles played by A_1Rs. For example, the known ability of adenosine analogues to decrease body temperature (28) can now be ascribed to an action on A_1Rs. Furthermore, the magnitude of the effect appears to be related to receptor number. Similarly, the effects of adenosine on excitatory transmission in the hippocampus can be ascribed totally to A_1Rs. Thus, these results suggest that the reported effects of A_2Rs and A_3Rs (29, 30) in hippocampus must reflect either the limited selectivity of the available pharmacological tools or the possibility that these other adenosine receptors act indirectly by modulating activity at A_1Rs.

Our results show that adenosine analogues exert their analgesic effects in the spinal cord by acting at A_1Rs. Furthermore, the fact that the knockout animals exhibited hyperalgesia provides strong evidence that these receptors play a modulatory role during nociception. It is interesting that mice lacking A_{2A}Rs were hypoalgesic, probably an effect mediated via peripheral receptors (5). Thus, depending on the site of action and the receptor activated, adenosine may exert very different effects on pain. This conclusion could partly explain why caffeine has some analgesic effects in some, but not all, types of pain (4, 31). The results also suggest that the A_1R may be a target for the development of antinociceptive drugs.

Given that caffeine appears to act predominantly as an antagonist at A_1Rs and A_{2A}Rs, the essential normality of mice lacking either of the targets (ref. 5, present data) is reassuring for coffee drinkers—particularly if one considers the fact that an amount of caffeine corresponding to 3–6 cups of coffee per day is likely to bind to and inhibit half of the adenosine receptors (4). This inhibition would produce a situation virtually identical to that in the $A_1R^{+/-}$ mice described here, which have half the number of A_1Rs and a doubling of the dose of adenosine required to produce a given effect compared with control animals. These animals were very close to normal in the tests performed. One potential concern is, however, that not only $A_1R^{-/-}$ mice, but also $A_{2A}R^{-/-}$ mice (5) show signs of increased anxiety. The effect of dual blockade by high doses of caffeine could be very pronounced. This pronounced blockade is consistent with the observation that high doses of caffeine, which would presumably block most adenosine receptors, are anxiogenic in animals and humans (4), but that low doses are not.

As noted above, adenosine is generally believed to protect tissues (not least nervous tissue) against negative consequences of hypoxia or ischemia. Our results support this contention and assign a particularly important role to A_1Rs. Thus, adenosine acting at A_1Rs appears to play a critically important role in mediating the neuronal depression in hippocampus and brainstem. Our data also suggest that the survival after a hypoxic challenge may be reduced if A_1Rs are absent or blocked. This possibility has not been studied extensively in the present study, and further investigation is necessary. We have preliminary *in vivo* data showing that immature mice subjected to a brief anoxic period fare much worse if they lack A_1Rs (E.H., A.H., B.J., and B.B.F., preliminary observations). It has been found that aminophylline treatment in neonatal mice decreases anoxic survival by decreasing the time of gasping (32). Thus, A_1Rs seem to be necessary for the initial hypoxic depression of breathing activity, and during prolonged hypoxia their absence decreases the survival rate.

There have been concerns that caffeine consumption during pregnancy might lead to developmental problems, but more recent data indicate that such problems occur only at very high doses of caffeine (33–35), and the negative effects may be maternal rather than on the offspring. The present data suggest, however, that endogenous adenosine acting at A_1Rs is very important in affording neuronal protection against hypoxia/anoxia. Thus the use of methylxanthines in doses that completely block A_1Rs may be hazardous in hypoxic human newborns.

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