Published in final edited form as:

Cell Mol Neurobiol. 2003 June ; 23(3): 431–447.

Behavioral Characterization of Mice Lacking the A₃ Adenosine Receptor: Sensitivity to Hypoxic Neurodegeneration

Irina M. Fedorova¹, Marlene A. Jacobson², Anthony Basile^{1,3,4}, and Kenneth A. Jacobson¹

¹Molecular Recognition Section, Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, Maryland ²Department of Neuroscience, Merck Research Labs, West Point, Pennsylvania

SUMMARY

1. The potential neuroprotective actions of the A₃ adenosine receptor (A₃AR) were investigated using mice with functional deletions of the A₃AR (A₃AR^{-/-}) in behavioral assessments of analgesia, locomotion, tests predictive of depression and anxiety, and the effects of mild hypoxia on cognition and neuronal survival.
2. Untreated A₃AR^{-/-} mice were tested in standard behavioral paradigms, including activity in the open field, performance in the hot-plate, tail-flick, tail-suspension, and swim tests, and in the elevated plus maze. In addition, mice were exposed repeatedly to a hypoxic environment containing carbon monoxide (CO). The cognitive effects of this treatment were assessed using the contextual fear conditioning test. After testing, the density of pyramidal neurons in the CA1, 2, and 3 subfields of the hippocampus was determined using standard histological and morphometric techniques.
3. A₃AR^{-/-} mice showed increased locomotion in the open field test, elevated plus maze (number of arm entries) and light/dark box (number of transitions). However, they spent more time immobile in two different tests of antidepressant activity (Swim and tail suspension tests). A₃AR^{-/-} mice also showed evidence of decreased nociception in the hot-plate, but not tail-flick tests. Further, A₃AR^{-/-} mice were more vulnerable to hippocampal pyramidal neuron damage following episodes of carbon monoxide (CO)-induced hypoxia. One week after exposure to CO a moderate loss of pyramidal neurons was observed in all hippocampal subfields of both wild-type (A₃AR^{+/+}) and A₃AR^{-/-} mice. However, the extent of neuronal death in the CA2–3 subfields was less pronounced in A₃AR^{+/+} than A₃AR^{-/-} mice. This neuronal loss was accompanied by a decline in cognitive function as determined using contextual fear conditioning. These histological and cognitive changes were reproduced in wild-type mice by repeatedly administering the A₃AR-selective antagonist MRS 1523 (5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcabonyl)-6-phenylpyridine-5-carboxylate 1 mg/kg i.p.).
4. These results indicate that pharmacologic or genetic suppression of A₃AR function enhances some aspects of motor function and suppresses pain processing at supraspinal levels, while acting as a depressant in tests predictive of antidepressant action. Consistent with previous reports of the neuroprotective actions of A₃AR agonists, A₃AR^{-/-} mice show an increase in neurodegeneration in response to repeated episodes of hypoxia.

Keywords

Adenosine receptor; neurodegeneration; knockout mice; analgesia; depression

INTRODUCTION

The four subtypes of adenosine receptors (ARs), A₁, A_{2A}, A_{2B}, and A₃, are widely distributed throughout the body (Fredholm *et al.*, 2001). Activation of the G protein-coupled receptors A₁ and A₃ inhibits adenylate cyclase activity, while activation of the A_{2A} and A_{2B} subtypes stimulates this enzyme. Stimulation of A₁, A_{2B}, and A₃ subtypes also activate phospholipases (Feoktistov *et al.*, 2002; Parsons *et al.*, 2000). In the periphery, A₃AR activation by either endogenous adenosine or exogenously administered agonists appears to be cardioprotective (Parsons *et al.*, 2000), an effect mediated in cultured myocytes by phospholipase D. However, this cell-protective function of A₃AR may not be universally observed. The cardioprotective, hemodynamic, and toxic effects of A₃AR agonists appears to be species-dependent (Lasley *et al.*, 1999). In rodents, acute activation of A₃ARs increases the release of proinflammatory agents such as histamine and TNF α (Ramkumar *et al.*, 1993; Sajjadi *et al.*, 1996; Salvatore *et al.*, 2000; van Schaick *et al.*, 1996). Moreover, deletion of the A₃AR in mice enhanced adenosine-stimulated coronary blood flow (Talukder *et al.*, 2002) and generally improved cardiac tolerance to ischemia (Guo *et al.*, 2001; Harrison *et al.*, 2002). These results suggest that, in some species, inhibition of A₃AR activity may protect tissues, possibly by suppressing proinflammatory processes.

The functions of the A₃AR in the central nervous system (CNS) appear to be equally complex. The precise density and localization of A₃AR in the brain remains unclear, due in part to the absence of selective, high affinity ligands or nucleotide probes (Rivkees *et al.*, 2000). Nonetheless, there is some evidence for the expression of the A₃AR in the hippocampus, thalamus, and hypothalamus (Yaar *et al.*, 2002). Moreover, electrophysiological and biochemical evidence supports the localization of A₃AR in the rat hippocampus (Dunwiddie *et al.*, 1997; Macek *et al.*, 1998) and cortex (Brand *et al.*, 2001), where they variously exert a direct neuroinhibitory action, or cause excitation by suppressing presynaptically located mGlu III autoreceptors (Macek *et al.*, 1998). Functional evidence also supports the presence of A₃AR in the brain. The A₃AR agonist N⁶-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA) depresses behavior (Jacobson *et al.*, 1993), and chronic treatment was found to be neuroprotective in the gerbil model of ischemia (von Lubitz *et al.*, 1994).

While the lack of high affinity A₃AR specific radioligands and other pharmacological tools have impeded characterization of the localization and activity of the A₃AR in general, the function of the A₃AR in the cardiovascular system was substantially illuminated by the development of the A₃AR null (A₃AR^{-/-}) mouse. Similarly, A₃AR^{-/-} mice have the potential for providing the functional equivalent of A₃ antagonist administration by mitigating the question of A₃AR ligand specificity. Therefore, we performed behavioral and provocative functional characterizations of A₃AR^{-/-} mice in order to elucidate the function of the A₃ receptor in the CNS.

MATERIALS AND METHODS

MRS 1523 (5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcabonyl)-6-phenyl-pyridine-5-carboxylate) was purchased from Sigma-RBI (St. Louis, MO). This adenosine receptor antagonist was dissolved in 100% dimethylsulfoxide(DMSO) as a 5-mM stock solution. Injection vehicles were made by diluting the stock into Alkamuls-620, then phosphate

buffered saline, pH 7.4, to yield a final composition of 5%/20%/75%. All other chemicals were from standard commercial sources and of analytical grade.

Animals

Two genotypes of mice were used: adenosine A₃ receptor knockout (A₃AR^{-/-}) mice developed by Merck Research Laboratories, and wild-type (A₃AR^{+/+}) control mice. A description of the generation of an earlier strain of A₃AR^{-/-} mice by Merck Research Laboratories has been described (Salvatore *et al.*, 2000). These A₃AR^{-/-} mice were backcrossed against C57Bl/6 mice (Taconic Farms, Germantown, New York) for 12 generations to obtain a congenic line. Male mice (6–8 weeks of age, approximately 30 g) were used in all studies. The mice were housed in polypropylene cages with free access to food and water in an AALAC-certified vivarium. The vivarium was maintained on a 12-h light: 12-h dark cycle (lights on at 7:00) with a room temperature of 22 ± 1°C and relative humidity level of 50 ± 5%. All studies were approved by the NIH Institutional Animal Care and Use Committee and were conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

Behavioral Studies

All behavioral studies were performed between 9 A.M. and 5 P.M. in mice acclimated to the testing room.

Tests of Nociception—The sensitivity of mice to painful stimuli was assessed using the tail-flick and hot-plate tests. In the tail-flick test, the tail of an unrestrained mouse was placed under the focused beam of a halogen projection lamp. The intensity of the lamp was adjusted so that the average latency before tail movement from the beam path by A₃AR^{+/+} mice was approximately 5 s, with a cutoff latency of 10 s. The hot plate test involved the placement of mice onto a 55°C plate (Columbus Instruments, Columbus, Ohio). The latency to the first incidence of hind-paw shaking or licking was recorded.

Spontaneous Locomotor Activity—Mice were placed into the center of an open field apparatus (40 × 40 cm; Columbus Instruments, Columbus, OH) under dim lighting. Motor activity parameters (distance traveled, number of vertical and stereotypic movements, ambulating and resting times) were monitored and recorded over a 30-min period. Monitoring was begun immediately after the mice were placed in the open field activity monitor.

Anxiety Tests—The apparatus used for the light–dark transition test consisted of a cage (25 × 40 × 20 cm) divided in two by a black partition with a small central opening connecting the chambers. One chamber had white walls and was brightly illuminated, whereas the other chamber was black and dimly illuminated. Mice were placed into the lit compartment and allowed to move freely between the two chambers for 5 min. The number of transitions between the two compartments, the time spent in each chamber, and the latency to the first transition were recorded. The elevated plus-maze consisted of two open (45 × 5 cm) and two enclosed arms of the same size, with 15 cm high walls. The arms were constructed of black acrylic radiating from a central platform (5 × 5 cm) to form a plus sign. The entire apparatus was elevated 30 cm above the floor. The test was initiated by placing each mouse on the central platform facing one of the open arms. The number of entries into the open and closed arms and the time spent on the open and closed arms were recorded over a 5-min test period.

The Forced-Swim Test—Each mouse was placed individually into a Plexiglas cylinder (20 cm height × 10 cm diameter) filled with water (22°C) to a height of 10 cm (Porsolt *et*

al., 1979). The total time the mouse spent immobile was recorded over a 6-min test period. Mice were judged immobile when they ceased swimming and remained floating motionless in the water, making only those movements necessary to keep their heads above the surface of the water.

The Tail-Suspension Test—This test is based on the observation that mice suspended by their tails show alternating periods of agitation and immobility (Steru *et al.*, 1985), and is predictive of antidepressant activity. Mice were suspended using adhesive tape placed 20 mm from the tip of the tail and were kept 20 cm from the nearest object. An observer blinded to the mouse genotype determined the cumulative duration of immobility over the 6-min duration of the test.

Contextual Fear Conditioning—Contextual, hippocampus-dependent memory was assessed in mice using the conditioned fear paradigm (Sternack *et al.*, 1998). During the initial, context-conditioning phase, the animal learns to associate an aversive stimulus with the novel environment or the context in which it is placed. In this task, a novel environment (the distinct new context the animal is placed in) is paired with an electric shock administered on the training day. On the test day (24 h later), if the mouse has learned the context–shock association, then the same environmental context will elicit fear, resulting in a total lack of movement or freezing. The amount of time a mouse spends immobile or frozen on a given testing day is indicative of the degree of fear conditioning. Each mouse was placed in a test chamber (San Diego Instruments) and allowed to explore freely for 2 min. The conditioned stimulus (80 dB tone) was presented for 30 s followed by a mild (2 s, 0.5 mA) foot shock. Subsequently, the mouse was allowed to explore for 2 min. A second pairing of tone and shock was presented after this 2-min period, followed by another 1 min of exploration. The mouse was then removed from the chamber and returned to its home cage. The mouse was returned to the test chamber after 24 h for a 3-min period and the presence of freezing behavior recorded (context test). Contextual fear conditioning experiments were conducted on the seventh and eighth day after the second day of CO exposure.

Carbon Monoxide-Induced Neurodegeneration

Carbon monoxide exposure was carried out as previously described (Nabeshima *et al.*, 1991). Each mouse was placed in an acrylic cylinder (10 cm × 3 cm radius) with gas entry and exit barbs. The subject was exposed to pure CO gas until it began gasping (about 20 s after exposure at the rate of 45 mL/min), then was rapidly removed to room air. Animals were exposed for 2 days, five times a day, with 1 h between each exposure. Core body temperature was maintained at 38°C by placing the mice on a heating blanket immediately after the first CO exposure and for up to 2 h after the last exposure to avoid CO hypothermia, with subsequent blunting of hypoxic neurodegeneration (Ishimaru *et al.*, 1991).

MRS 1523 (1 mg/kg, i.p.) was administered for 5 days, with the last 2 days of administration overlapping the first 2 days of CO exposure. Control mice received vehicle.

Histology

Following the behavioral studies, mice were euthanized by AALAC-approved methods, their brains removed and placed overnight in 20% sucrose containing 0.025% DMSO. The next morning, the brain regions were blocked, and the samples flash-frozen in isopentane at −20°C. Serial 20 μm thick sections of the entire hippocampus were cut, and mounted, stained with 1% cresyl violet, destained, differentiated, and coverslipped. Morphometric analysis of CA1-3 pyramidal neurons was performed by manually counting neurons in every

fifth section of cresyl violet-stained hippocampus at 400× magnification (Kustova *et al.*, 1999). Area measures of the pyramidal neuron layer (CA1-3) of the entire hippocampus were made using digitally acquired images of hippocampal sections at 4× magnification, stacked and summed to yield the final volume measurements (Meta-Morph, Universal Imaging, Downingtown, PA).

Statistical Analysis

Data are expressed as mean ± standard error for the indicated *n*. Statistical analyses were performed using either a *t* test or one-way ANOVA followed by Bonferroni's Multiple Comparison Test (Prism, GraphPAD Software, San Diego, CA).

RESULTS

Gross physical examination of $A_3AR^{-/-}$ mice revealed an 8% greater body weight than age-matched $A_3AR^{+/+}$ mice (22.0 ± 0.4 g, *n* = 12 vs. 23.8 ± 0.4 g, *n* = 11; $A_3AR^{+/+}$ and $A_3AR^{-/-}$ respectively, *P* < 0.01, *t* test), and a 1.6% lower core body temperature ($37.3 \pm 0.1^\circ\text{C}$, *n* = 12 vs. $36.7 \pm 0.1^\circ\text{C}$, *n* = 11; $A_3AR^{+/+}$ and $A_3AR^{-/-}$ respectively, *P* < 0.01, *t* test). Moreover, $A_3AR^{-/-}$ mice demonstrated a decrease in the threshold of pain sensitivity as assessed in the hot-plate test, with a 40% increase in the latency of the hind-paw response (*P* < 0.05, *t* test, Fig. 1(A)). However, the latency to movement in the tail-flick test was unchanged (2.3 ± 0.1 s vs. 2.2 ± 0.1 s).

Locomotor activity in a novel environment was measured in the open field. $A_3AR^{-/-}$ mice showed a small but significant increase in the distance traveled in the open field (Fig. 1(B)). Interestingly, there was no significant difference between the genotypes in the number of vertical movements (Fig. 1(C)). Moreover, $A_3AR^{-/-}$ and $A_3AR^{+/+}$ mice performed equally well in the rotarod test (115 ± 20 s vs. 103 ± 21 s, *n* = 11 both groups; $A_3AR^{+/+}$ and $A_3AR^{-/-}$ respectively).

Basal levels of anxiety were assessed in the elevated plus-maze test. Although there was a trend toward increased time spent in the open arms by $A_3AR^{-/-}$ mice, this did not reach significance (Fig. 2(A)). However, the number of open- and closed-arm entries made by $A_3AR^{-/-}$ mice were significantly higher (113, 67%, respectively) than $A_3AR^{+/+}$ mice (Fig. 2(B) and (C)). $A_3AR^{-/-}$ and $A_3AR^{+/+}$ mice did not differ in the time spent in the lit compartment in the light–dark transition test (120 ± 10 s for $A_3AR^{+/+}$, *n* = 12 and 140 ± 9 s for the $A_3AR^{-/-}$ mice, *n* = 12). Nonetheless, $A_3AR^{-/-}$ mice showed a significant, 95% increase in the number of transitions between the two compartments (18.7 ± 2.0 , *n* = 12 vs. 33.2 ± 2.9 , *n* = 12; $A_3AR^{+/+}$ and $A_3AR^{-/-}$ mice, respectively, *P* < 0.01, *t* test).

The behavior of the $A_3AR^{-/-}$ and age-matched $A_3AR^{+/+}$ mice in the Porsolt forced swim test and tail suspension test was also investigated. In the tail suspension test, the $A_3AR^{-/-}$ mice spent significantly more time immobile than $A_3AR^{+/+}$ mice, consistent with an increased level of “behavioral despair” (Fig. 3(A)). Increased immobility (49%) was also observed in $A_3AR^{-/-}$ mice in the forced swim test (Fig. 3(B)).

Finally, the sensitivity of $A_3AR^{-/-}$ mice to hypoxic neurodegeneration was investigated using an in vivo model of CO-induced delayed amnesia and neuronal damage (Maurice *et al.*, 1994, 1999; Nabeshima *et al.*, 1991). The influence of CO exposure on the locomotion of $A_3AR^{-/-}$ mice was observed in the open field test. CO exposure decreased the locomotion of both genotypes of mice (Fig. 4(A)), but the relative decrease was approximately 85% greater in $A_3AR^{-/-}$ than $A_3AR^{+/+}$ mice (Fig. 4(B)). CO-induced changes in cognition were also examined using a contextual fear-conditioning paradigm. Basal, pre-CO exposure rates of contextual learning were similar in mice of both genotypes

(the ratio of the number of photobeam interruptions during the context test relative to the conditioning test was $19.6 \pm 2.3\%$ in $A_3AR^{+/+}$ mice, $n = 14$; and $19.1 \pm 1.5\%$ in $A_3AR^{-/-}$ mice, $n = 12$). In contrast, $A_3AR^{-/-}$ mice did not perform as well as $A_3AR^{+/+}$ mice after CO exposure (Fig. 5). During the conditioning phase, $A_3AR^{-/-}$ mice showed almost the same number of photobeam interruptions as $A_3AR^{+/+}$ mice, consistent with equal freezing times (Fig. 5(A), conditioning). Strikingly, $A_3AR^{-/-}$ mice interrupted the photobeams more frequently during context testing conducted 24 h after conditioning (Fig. 5(A), test-context), with their freezing time in response to shock-associated context being lower than $A_3AR^{+/+}$ mice. This difference is clearer when performance is presented as the ratio of photobeam interruptions during context testing to the number of photobeam interruptions during the conditioning period $\times 100$ (Fig. 5(B)). When the retention test was conducted 1 month after conditioning, $A_3AR^{-/-}$ mice showed a deficit in memory for the environmental context associated with an aversive stimulus. This was manifested as a 67% increase in the number of photobeam interruptions (200 ± 21 for $A_3AR^{+/+}$, $n = 8$; 340 ± 18 for $A_3AR^{-/-}$, $n = 9$, $P < 0.001$, t test). Administration of the A_3AR antagonist MRS 1523 to $A_3AR^{+/+}$ mice for 5 days (protocol 2) resulted in similar changes in contextual fear conditioning as the deletion of the A_3AR (Fig. 5), with the number of photobeam interruptions decreased during conditioning, but enhanced in context testing.

Finally, we examined whether the post-hypoxic cognitive deficits displayed by the $A_3AR^{-/-}$ mice were associated with neuronal death in the CA1-3 subfields of the hippocampus. Histological studies showed that 7 days after exposure to CO, a moderate loss of pyramidal neurons was observed in all subfields of the hippocampus. Closer examination of the CA2 and CA3 regions of the hippocampus revealed that neuronal density in the CO-exposed mice was significantly more pronounced in the $A_3AR^{-/-}$ mice than $A_3AR^{+/+}$ (Figs. 6 and 7) ($P < 0.05$, one-way ANOVA). Consistent with the enhanced loss of pyramidal neurons in $A_3AR^{-/-}$ mice, $A_3AR^{+/+}$ mice administered the A_3AR antagonist MRS 1523 showed a decrease in neuronal density in CA2–3 pyramidal neuron subfields.

DISCUSSION

The modulatory role of A_1 and A_{2A} ARs in the CNS is well established, and behavioral consequences of the deletion of these receptor subtypes have been described (Gimenez-Llort *et al.*, 2002). In contrast, the A_3AR have been implicated in a variety of peripheral organ system functions, including regulating cellular components of the immune system (Ramkumar *et al.*, 1993; Sajjadi *et al.*, 1996; Salvatore *et al.*, 2000; Shepherd *et al.*, 1996), cardiovascular function (Cerniway *et al.*, 2001; Guo *et al.*, 2001; Lasley *et al.*, 1999; van Schaick *et al.*, 1996; Zhao *et al.*, 2000), and apoptosis (Appel *et al.*, 2001; Di Iorio *et al.*, 2002). However, understanding of their function(s) in the CNS has been impeded, both by a lack of specific ligands and the low density of receptors (Rivkees *et al.*, 2000). In an attempt to understand the role of the A_3AR in CNS function, we have utilized mice with a deletion of the A_3AR receptor ($A_3AR^{-/-}$ mice) in a battery of behavioral tests. Moreover, given evidence of a cardioprotective action of the A_3AR (Parsons *et al.*, 2000), we investigated the potential influence of these receptors on neuronal survival following a hypoxic insult.

Our initial characterization of the behavioral effects of the functional deletion of the A_3AR in mice showed that $A_3AR^{-/-}$ mice manifested a decreased sensitivity to some painful stimuli, as evidenced by the increase in latency in the hot plate, but not tail-flick test. This observation reflects a decrease in the supraspinal processing and “recognition” of painful stimuli, and may be consistent with preliminary observations of the localization of A_3AR in thalamic nuclei, but not in the spinal cord (Yaar *et al.*, 2002), where they may play a role in processing nociceptive information. Interestingly, significant increases in some aspects of motor function were also observed in $A_3AR^{-/-}$ mice. Evidence for a slight increase in

locomotor activity was observed in three different tests: activity in the open field; number of arm entries in the elevated plus maze; and number of transitions in the light/dark box. This change in motor activity appears to be selective, as there was no increase in the number of vertical movements, or evidence of “ataxia,” as defined using the rotarod, and argues against a nonselective increase in motor activity resulting from the process of generating transgenic mice. While the brain region(s) involved in this hyperlocomotion remains unknown, the disinhibition of cortical neurons resulting from the deletion of the A₃AR (Brand *et al.*, 2001) may play a role, and is consistent with the substantial depression of motor activity observed after administration of the A₃AR agonist IB-MECA (Jacobson *et al.*, 1993).

In addition to the evidence for a potential role of the A₃AR in motor function and nociception, A₃AR may regulate other behavioral activities. Initial interpretation of the performance of the A₃AR^{-/-} mice in the elevated plus maze and light/dark box suggests a reduction in their level of anxiety or fearfulness, as evidenced by the increase in exploratory activity. Closer examination suggests that these results may reflect the increased motor activity of these mice, as there was no significant increase in the amount of time spent in the open arms of the plus maze, or in the illuminated side of the light/dark box. While AR antagonists have been proposed as antidepressants (El Yacoubi *et al.*, 2001), A₃AR^{-/-} mice showed an increase in the amount of time spent immobile in two tests of behavioral depression. This response is not adequately explained by a decrease in motor activity, particularly in view of the increased locomotion expressed by this genotype. A₃AR have been found to modulate hippocampal serotonin levels, possibly by regulating release and/or reuptake (Okada *et al.*, 1999). Deletion of these receptors may reduce the levels of free serotonin or catecholamines, thereby acting as a behavioral depressant and increasing immobility time in the forced-swim and tail-hang tests, which are predictive of antidepressant actions (Porsolt *et al.*, 1979; Steru *et al.*, 1985).

In contrast with the above studies, a role for the A₃AR in aspects of neuroprotection was supported by studies with the A₃AR^{-/-} mice. While the A₁ and A_{2A}ARs are associated with the protective functions of adenosine, decreasing energy demand while increasing energy supply (Jacobson, 1998), respectively, the role of the A₃AR has been more difficult to generalize, as both protective and damaging effects have been noted (Appel *et al.*, 2001; Cerniway *et al.*, 2001; Fishman *et al.*, 2002). Repetitive exposure of mice to CO gas induces a long-lasting but delayed amnesia measured 1 week after exposure (Nabeshima *et al.*, 2001), accompanied by dose-dependent death of hippocampal pyramidal neurons (Ishimaru *et al.*, 1991). This relatively mild insult was used to quantify changes in neuronal vulnerability to hypoxia that may occur in the absence of the A₃AR. Therefore, we characterized the behavior of a strain of A₃AR^{-/-} mice in a wide range of behavioral tests with or without mild ischemic damage induced by CO exposure. In the case of repeated brief exposure to CO, we found that mice lacking the A₃AR are more vulnerable than control animals to hippocampal damage following hypoxia. By histological examination 1 week after the exposure to CO, a moderate neuronal loss in all regions of the hippocampus was observed in all mice, but the degree of neuronal death in the CA2–3 subfields was more pronounced in A₃AR^{-/-} mice than in wild type. The hypoxic damage also impacted cognition, with CO-induced hypoxia causing a decline in cognitive function in a test of contextual fear conditioning that was more pronounced in A₃AR^{-/-} mice. While this increase in test-context motor activity may reflect the increase in general locomotor activity expressed by A₃AR^{-/-} mice in select environments such as the open field, plus-maze and light–dark box, there was apparently no difference between the genotypes in performance in the contextual fear conditioning test before CO exposure. Moreover, CO exposure appeared to reduce the motor activity of the A₃AR^{-/-} mice to the level of the wild-type mice. An additional factor which may explain the apparent decrease in cognitive function manifested by CO exposed A₃AR^{-/-} mice is the increased threshold for nociception indicated by the

hot-plate test. This would effectively reduce the intensity of the aversive stimulus which the environmental context is associated with, potentially slowing the rate and efficiency of learning in these mice. Nonetheless, even the evidence for changes in nociception in the $A_3AR^{-/-}$ mice is unclear, as it reflects performance in the hot plate, but not tail-flick test.

While pre-CO exposure control testing in the contextual fear conditioning assay was performed to help mitigate the influence of potentially confounding factors, additional cognitive testing using paradigms that either do not require a nociceptive input, or extensive locomotor activity (e.g., operant conditioning) may be required before any conclusions regarding the A_3AR , learning and memory in both intact and lesioned mice can be reached. This task may be eased by the observation that the apparent neuronal vulnerability observed in the $A_3AR^{-/-}$ mice was duplicated by blockade of the receptor. Repeated administration of the A_3AR -selective antagonist MRS 1523, which is selective for A_3AR in both primates and rodents, resulted in a greater degree of neuronal damage and impairment of contextual memory than after vehicle administration. The role of the A_3AR in neuroprotection and cognition may be more extensively tested in the future by chronic administration of MRS 1523 in concert with various hypoxia/ischemia lesioning paradigms, and/or with specific tests of cognition. Thus, in acute global brain ischemia it appears that either pharmacological blockade or genetic deletion of the A_3AR in the mouse is detrimental. Although the damage incurred by the CO treatment was milder than in other models of global ischemia, such as bilateral occlusion of the carotid arteries in gerbils (von Lubitz *et al.*, 1994), differences based on the presence or absence of the A_3AR were evident. This implies that the levels of adenosine in the brain during hypoxia/ischemia are sufficient to activate the A_3AR .

In summary, the $A_3AR^{-/-}$ mouse reveals a number of CNS functions where the A_3AR plays a role, including nociception, locomotion, behavioral depression, and neuroprotection. The finding that deletion of the A_3AR has a detrimental effect in a model of mild hypoxia suggests the possible use of A_3AR agonists in the treatment of ischemic, degenerative conditions of the CNS. A_3AR agonists are already under development for other clinical indications, such as antineoplastics (Fishman *et al.*, 2002). As the development of these pharmacotherapeutic agents progresses (Jacobson *et al.*, 1997), the utility of pharmacologically regulating CNS activities via the A_3AR may become more apparent.

Acknowledgments

We thank Dr John Daly and Josh Blaustein (NIDDK) for their many helpful discussions.

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Fig. 1. $A_3AR^{-/-}$ mice show alterations in nociception and locomotion. (A) $A_3AR^{-/-}$ mice showed a significant increase in the latency to lick a hind-paw in the hot-plate test ($P < 0.05$ vs. $A_3AR^{+/+}$ mice, t test, $n = 12$). (B) Spontaneous locomotor activity in the open field was significantly increased in $A_3AR^{-/-}$ mice (Genotype effect, $P < 0.01$, $F = 11.7$, 1 df, two-way ANOVA, $n = 10$). (C) No difference between the genotypes was noted in the number of vertical movements made in the open field. Each bar represents the mean $\pm$ SEM.

Fig. 2. $A_3AR^{-/-}$ mice show increased locomotor activity in the elevated plus-maze test. Although $A_3AR^{-/-}$ mice did not spend significantly more time in the open arms than $A_3AR^{+/+}$ mice (A), they made more entries into open (B) and closed arms (C). **Significantly different from $A_3AR^{+/+}$ mice, $P < 0.01$, t test. Each bar represents the mean $\pm$ SEM of $n = 11$ mice.

Fig. 3. $A_3AR^{-/-}$ mice show evidence of an increase in behavioral despair. The duration of immobility by $A_3AR^{-/-}$ mice was increased in both the tail suspension test (A) and forced swim tests (B). * $P < 0.05$, t test. Each bar represents the mean $\pm$ SEM of $n = 11$ mice.

Fig. 4.

Effect of carbon monoxide exposure on the locomotor activity of $A_3AR^{-/-}$ and $A_3AR^{+/+}$ mice. (A) CO exposure reduced the distance traveled in the open field by both $A_3AR^{-/-}$ and $A_3AR^{+/+}$ mice. *Significantly different from $A_3AR^{+/+}$ mice, $P < 0.05$. **Performance significantly different from respective pre-CO groups, $P < 0.01$. All two-way ANOVA, Bonferroni's post-hoc analysis. (B) The relative CO-induced decrease in distance traveled in the open field was greater in $A_3AR^{-/-}$ than $A_3AR^{+/+}$ mice. **Significantly different from $A_3AR^{+/+}$ mice, $P < 0.01$, t test. ($n = 9$ in both groups).

Fig. 5.

The influence of CO exposure on contextual fear conditioning. (A) The number of photobeam interruptions counted during the context testing of CO-treated mice 24 h after conditioning (Test-context Phase) was significantly higher in $A_3AR^{-/-}$ mice compared to wild-type animals. The same effect was observed in wild-type mice treated with MRS 1523 relative to vehicle treated mice. There was no significant difference between the groups during the conditioning phase. (B) The relative changes in freezing amongst the different groups is better observed as the ratio of test-context to conditioning performance. The percentage increase in photobeam interruptions was significantly increased in $A_3AR^{-/-}$ mice and wild-type mice treated with MRS 1523. * $P < 0.05$, ANOVA, Bonferroni's test, $n = 8-11$, ** $P < 0.01$, ANOVA, Bonferroni's test, $n = 11-14$.

Fig. 6.

Effect of CO treatment on hippocampal pyramidal neurons in the CA2–3 subfields. Treatment groups included naïve wild type and A₃AR^{-/-} mice, and CO-exposed animals (wild type, A₃AR^{-/-} and wild type mice after vehicle administration and after A₃R antagonist MRS 1523 administration). There was no difference in neuron density between naïve wild type and A₃AR^{-/-} mice. However, after CO treatment, all groups showed a lower neuron density than untreated mice, neuronal death after CO exposure being more pronounced in A₃AR^{-/-} mice than in wild type mice. *Significantly different from respective naïve groups, $P < 0.05$; ^aSignificantly different from CO-treated A₃AR^{+/+} mice, $P < 0.05$; **Significantly different from A₃AR^{+/+} mice, ANOVA and Tukey's test. Each bar represents the mean $\pm$ SEM of data from 6 to 12 mice.

Fig. 7. Delayed death of CA2–3 pyramidal cells in the hippocampus 7 days after CO exposure. Representative photomicrographs of 20 μ m coronal sections of cresyl violet-stained hippocampal CA2–3 subfields are shown.