Effect of the Adenosine A2a Receptor Antagonist 3,7-Dimethyl-Propargylxanthine on Anxiety-like and Depression-like Behavior and Alcohol Consumption in Wistar Rats

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Background: It has been suggested that the reinforcing properties of ethanol are in part mediated via an A2 activation of cAMP/PKA signaling in the nucleus accumbens, predicting that administration of an A2a antagonist might reduce ethanol reward and consumption. We therefore examined the effect of the adenosine A2a receptor antagonist 3,7-dimethylpropargylxanthine (DMPX, 3, and 10 mg/kg intraperitoneal) on alcohol reinforcement, anxiety-related, depression, and rewarding behaviors in nonselected Wistar rats.

Methods: Operant ethanol self-administration was used for examining alcohol intake, elevated plus-maze and Vogel conflict test for anxiety-related behavior, Porsolt swim test for depression-like behavior, and conditioned place preference for examination of the rewarding properties of the drug.

Results: 3,7-Dimethylpropargylxanthine decreased lever-pressing for ethanol in a dose-dependent manner. When analyzed as percentage of pretreatment baseline, maximum suppression was approximately 60% (39 ± 7.5 vs $98 \pm 12\%$, mean $\pm$ SEM, $p = 0.017$). This effect was behaviorally specific, as no effect was found on the water lever. In agreement with previously published data, stimulation of locomotion was found (beam-breaks: 3590 ± 540 vs 2475 ± 240, 10 mg/kg vs saline, $p = 0.048$). No anxiety-modulating effects were seen in either the elevated plus-maze or the Vogel conflict test. 3,7-Dimethylpropargylxanthine was not found to have intrinsic rewarding properties in the conditioned place preference model.

Conclusions: In summary, DMPX produced a robust and behaviorally selective reduction of ethanol reinforcement, while anxiety-modulating effects were less consistent. These results bring further support to a role for adenosine in the regulation of ethanol consumption and possibly alcohol addiction/abuse, and the A2a receptor as a potential target for the treatment of alcoholism and alcohol abuse.

Key Words: Adenosine Receptor, Place Preference, Operant Self-Administration, Wistar Rat.

ADENOSINE IS A neuro-modulator in the central nervous system (CNS) and its effects are mediated via 4 different receptor subtypes: A1, A2a, A2b, and A3. In the CNS, endogenous adenosine exerts a depressant effect on neurons by reducing transmitter release from presynaptic nerve terminals and increasing potassium conductance in postsynaptic neurons (Dunwiddie and Fredholm, 1985; Dunwiddie and Haas, 1985; Fredholm and Dunwiddie, 1988). This effect is mediated via A1 receptors. A1 receptors are widely distributed throughout the brain, with high levels in the hippocampus, cerebellum, and cortex. The A2 receptors mediate excitatory effects of adenosine and have been subclassified into A2a and A2b. The A2b is a low-affinity receptor located in almost all areas of the brain (Bruns et al., 1986). The high-affinity A2a-receptor subtype is expressed in dopamine-innervated areas such as the dorsal striatum, nucleus accumbens, and olfactory tubercle (Jarvis and Williams, 1989; Jarvis et al., 1989a, 1989b; Wan et al., 1990). A2a receptors are concentrated in the striatum, particularly in the striopallidal GABAergic neurons where they are co-localized with dopamine D2 receptors (Fink et al., 1992; Pollack and Fink, 1995). Stimulation of the A2a receptor leads to a reduction in the affinity of D2 receptors for its agonists.

A wide range of compounds can affect the behavior and/or electrophysiological activity of the CNS via interactions with brain adenosine (Phillis, 1984b; Phillis et al., 1979, 1981). Central nervous system depressants, opiates, neuroleptics, and benzodiazepines inhibit adenosine uptake (Phillis, 1984a, 1984b). Ethanol has been demonstrated to

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activate adenosine A2 receptor signaling in neuronal cell culture (Gordon et al., 1986), leading to increased extracellular adenosine, in turn resulting in elevated cyclic adenosine monophosphate levels. Ethanol-induced elevation of cAMP leads to an activation of protein kinase A and translocation of its catalytic subunit (PKA-Cα) to the nucleus (Dohrman et al., 2002; Yao et al., 2002). This is followed by cAMP-dependent CRE-mediated gene transcription. It may be hypothesized that a similar mechanism mediates some of the CNS effects of ethanol (Diamond and Gordon, 1994; Diamond et al., 1991; Mailliard and Diamond, 2004). A direct behavioral link between ethanol and adenosine has been demonstrated in mice. Central administration of adenosine agonists and antagonists dose dependently accentuated and attenuated, respectively, ethanol-induced motor in-coordination (Dar, 1990). A central mechanism for interaction between ethanol and adenosine may therefore exist.

Limited data are available on the consequences of modulating A2a signaling on ethanol reinforcement in preclinical models. It has previously been reported that peripheral administration of 3,7-dimethylpropargylxanthine (DMPX) produces bimodal effects on ethanol consumption and operant responding for ethanol in Long–Evans rats. A low dose increased while a higher dose suppressed responding (Arolfo et al., 2004). In this publication, it was also demonstrated that A1 antagonists had no effect on ethanol intake and lever-pressing.

To further evaluate the A2a receptor as a putative drug target for alcoholism, we examined the effects of DMPX on an operant self-administration model in Wistar rats, and examined its effects on open field and anxiety-like behaviors. For a target to be considered as a drug-development target site, it is necessary for it to lack any intrinsic rewarding propertied. We used conditioned place preference to examine this for DMPX.

MATERIAL AND METHODS

Subjects and Drug Administration

Male Wistar rats (Charles River, Wilmington, MA) weighing 220 to 240 g at the beginning of the experiment were pair-housed with water and food available ad libitum, except where noted. The animals were maintained on a 12-hour light/dark cycle (lights on at 6:00 AM). All animal care was performed according to NIH guidelines.

The adenosine A2a-antagonist, DMPX, was obtained from Sigma Chemical Co. (St. Louis, MO). 3,7-Dimethylpropargylxanthine was dissolved in a 45% 2-hydroxypropyl-β-cyclodextrin solution in saline to a final concentration of 1.7 mg/mL. Intraperitoneal administration of 3, 10 mg/kg, or vehicle was performed 30 minutes before behavioral testing.

Ethanol Self-Administration

Operant self-administration training was performed between 10:00 AM and 3:00 PM, Monday through Friday, using a sucrose fading procedure and was performed in operant chambers housed in sound-attenuated cubicles (Med Associates Inc., Georgia, VT). Each chamber (33×30.5×33 cm) was equipped with 2 retractable levers positioned on the right wall. A recessed drinking cup was located between the levers with 2 receptacles for the solution provided by pressing either lever. Upon pressing a lever, fluid (0.1 mL) was delivered into one of the receptacles. A press at the ethanol lever led to an indicator light above the lever being switched on in addition to the delivery of a 10% (w/v, final concentration) ethanol solution. A press at the water-lever delivered 0.1 mL of tap water and no indicator light. The position of the solutions shifted sides daily to correct for any side-preference, and injections were administered on days balancing the distribution between sides. Session-start was indicated by house-light on and sessions were 30 minutes.

Elevated Plus-Maze

The elevated plus-maze is an ethological animal model of anxiety-like behavior. It is based on the conflict between the exploratory drive and fear of elevated, open areas.

The apparatus was made of black plastic with 2 open arms (50×10 cm) and 2 closed arms (50×10×45 cm) connected by a 10×10 cm central area. The maze was 50 cm above the floor and testing was performed under dim red light. Behavior was scored by an observer blind to treatment condition. At the beginning of a session, the rat was placed in the central area facing one of the open arms. The behaviors scored during the 5-minute test-time was the number of entries onto the open and closed arms, as well as the amount of time spent on each type of arm. Reported results are in the form of percent open time, which is ((time open arms)/(time open arms+time closed arms)×100%) and percent open entries ((entries open arms)/(entries open arms+entries closed arms)×100%). The total number of entries onto any arm (open+closed) was used as an indicator of general activity.

Vogel Conflict Test

The Vogel conflict test is based on the assumption that there is a conflict between the drive to drink in a thirsty animal and the fear of receiving a mild electric shock. Following a 24-hour water deprivation, animals were adapted for 12 minutes to an operant chamber (Med Associates Inc., St. Albans, VT) where they had unlimited access to a 5% glucose-solution through a drinking spout. This habituation was repeated following another 24 hours of water deprivation and after a third deprivation session, testing was performed. During the testing, subjects were allowed 3 drinking episodes before the session timer started. This was followed by a 4-minute unpunished drinking component during which drinking episodes were detected but no shock was administered. The 8-minute test period followed directly after the 4-minute unpunished component. Here, a drinking episode was accompanied by a mild electric shock through the spout and the number of accepted shocks was measured.

To control for difference in pain sensitivity, pain thresholds were measured. The pain threshold was measured by delivery of a mild shock through the grid floor of a behavioral testing chamber and observing the current at which the animal first displays a jerk, twitch, or other sudden movement in response to shock delivery. An observer blind to current strength scored behavior and shock was delivered by a control-unit operated by a second researcher.

Locomotor Activity and Open Field

Locomotor activity was measured in sound-attenuated behavioral chambers equipped with an open field (43×43 cm) with infra-red beam detectors (Med Associates Inc.). Three sets of 16 infra-red beams are used to automatically track both horizontal and vertical movements. In addition to overall activity and rest time, the automated system also gives perimeter activity and central area activity. The perimeter activity was defined as the activity measured by the outer 6 (3+3) sets of infra-red beams in the X and Y directions. The

central area was defined as beam-breaks made at the remaining central 10 beam-pairs.

Porsolt Swim Test

The swim test apparatus was a white plastic tub (diameter 34 cm; height 66 cm) filled to a level of 48 cm with 24 ± 2 °C water. A light located approximately 150 cm above the water surface illuminated it to about 20 lx. During the initial pretest exposure, rats were placed in the apparatus and allowed to freely explore for 10 minutes. Behavior was assessed the next day during a 5-minute test session. Measures included were latency to become immobile after having been placed in the apparatus, the proportion of time spent immobile, and the number of escape attempts. Escape attempts were defined as active attempts to scale the walls. Immobility was defined as the absence of active swimming and maintaining "floating" using only minor movement of the front paws.

Conditioned Place Preference

Two-compartment place preference was used (Med Associates Inc.). The 2 sides of the box have been designed to provide distinct tactile environments to maximize contextual differences. One side of the box has a wire mesh floor while the other side has a grid rod floor. The 2 compartments are connected by a manual guillotine door and are covered by hinged lids. Conditioned place preference consisted of 3 phases: habituation, conditioning, and postconditioning. During the habituation, each rat was placed in the apparatus for two 10-minute sessions and allowed free access to both chambers through the open guillotine door. The 2 sessions were run 4 hours apart and the animals were introduced into the apparatus on different sides to correct for starting point in the calculation of initial place preference. On the preconditioning day, the amount of time spent in each part of the apparatus was measured. All animals were found to have a preference for one side in the apparatus and drug-pairing was made to the nonpreferred side. During the conditioning phase, which lasted 8 days, the animals were confined to the considered compartment by closing of the guillotine door. The duration of each session was 10 minutes. On days 1, 3, 5, and 7, animals received vehicle in the preferred compartment. On days 2, 4, and 6 animals received drug in the opposite compartment. The effect of the drug treatment was analyzed during the postconditioning phase. This phase was carried out on the ninth day of trials and 24 hours after the last conditioning session. There were no preceding drug injections and the animals were in a drug-free state during this testing. As in the preconditioning phase, the guillotine door was raised and the animals were placed in the apparatus (on the side where injection of vehicle had taken place) and allowed to explore for 10 minutes. The time spent on each side was measured by an automated system (Med Associates Inc.). The data were reported in percent time spent on the drug-paired side of the apparatus.

Statistics

Statistical analyses were run using the Statistica 7.0 software. For ethanol self-administration, repeated measures ANOVA was used, followed by Tukey's HSD post hoc test. For behavioral testing, ANOVA was used, followed by Tukey's HSD post hoc test when appropriate.

RESULTS

Ethanol Consumption (Fig. 1 and Table 1)

A significant suppression of lever-pressing was seen following treatment with DMPX [repeated measures ANOVA: $F(2, 16) = 5.80$, $p = 0.013$]. This was due to a significant suppression of responding at 10 mg/kg (Tukey's HSD post hoc: $p = 0.022$ vs control). A dose-dependent decrease in responding expressed as percent of pretreatment baseline was seen [repeated measures ANOVA: $F(2, 16) = 5.33$, $p = 0.017$]. This effect was also significant at 10 mg/kg (Tukey's HSD, $p = 0.013$ vs control).

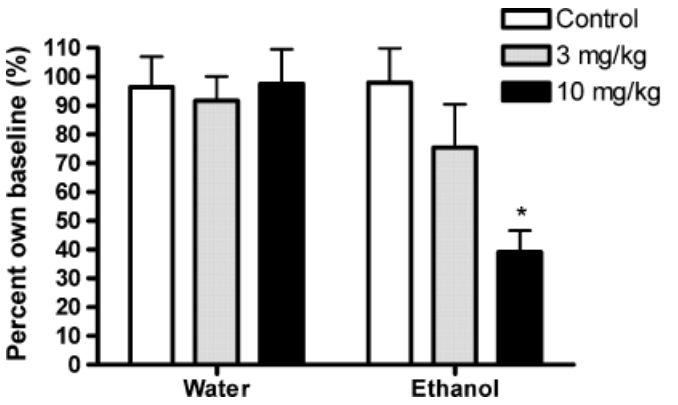

Fig. 1. Ethanol self-administration following 3,7-dimethylpropargylxanthine treatment. Animals were injected intraperitoneal 30 min before testing. Control is vehicle; low = 3 mg/kg; high = 10 mg/kg. Response rates are expressed as percent of pretreatment baseline. Responding on the ethanol panel is shown in the left part of the graph, and on the water lever to the right. $^*p < 0.05$ compared with vehicle. Absolute response rates are given in Table 1.

Anxiety-Related Behavior: The Elevated Plus-Maze (Table 2) and Vogel Conflict Test

No significant effect on exploration on the elevated plus-maze was seen following treatment with 10 mg/kg DMPX. Percent open-arm entries: $F(1, 18) = 0.53$, $p = 0.48$. Percent open arm time: $F(1, 18) = 0.24$, $p = 0.63$. Total number of entries onto any arm: $F(1, 18) = 1.89$, $p = 0.19$. No significant effect was seen in the Vogel conflict test (data not shown). Pain thresholds were not significantly affected by drug treatment (data not shown).

Locomotor Activity and Open-Field Behavior (Fig. 2)

An activating effect on locomotor behavior following 10 mg/kg DMPX was seen [$F(1, 11) = 3.89$, $p = 0.048$]. How-

Table 1. Effect of DMPX on Lever Pressing in Operant Ethanol Self-Administration Expressed as Absolute Lever Presses and the Corresponding Ethanol Intake in g/kg

Treatment	Ethanol lever	Water lever	Ethanol intake (g/kg)
Control	32.5 ± 6	16 ± 3.3	0.64 ± 0.08
3 mg/kg	31.0 ± 5	12.5 ± 2.5	0.61 ± 0.09
10 mg/kg	12.0 ± 2.5*	13 ± 3.0	0.18 ± 0.04

$^*p < 0.05$.
Data are expressed as means ± SEM ($n = 9$ per group).
DMPX, 3,7-dimethylpropargylxanthine.

Table 2. Anxiety-Related Behavior on the Elevated Plus-Maze Following DMPX Treatment (10 mg/kg, i.p., 30 Min Before Testing)

Treatment	Percent open (%)		Time on open arm (s)	Total number of entries
	Entries	Time		
Control	40 ± 9.5	43 ± 10.0	111 ± 17	11.6 ± 1.3
10 mg/kg	48 ± 6.0	37 ± 5.5	89 ± 22	9.1 ± 1.5

Data are expressed as means ± SEM ($n = 9$–10 per group).
DMPX, 3,7-dimethylpropargylxanthine, i.p., intraperitoneal.

ever, no significant differences were detected in the amount of time spent in the periphery of the open field or the number of line-crossings in the periphery (data not shown).

Porsolt Swim Test

No significant effect of DMPX was found in the Porsolt swim test (Table 3).

Conditioned Place Preference (Fig. 3)

The animals had a natural preference for one side of the apparatus (57 ± 4 vs 43 ± 2%, $p = 0.023$). Treatment with DMPX paired to the nonpreferred side did not significantly alter this preference.

DISCUSSION

We found that the adenosine A2a receptor antagonist DMPX attenuates operant responding for ethanol. This effect was behaviorally selective, because response rates on the water lever were unaffected. No significant effect of the drug on anxiety-related behavior or depression-like behavior could be detected. A locomotor stimulant effect of DMPX was detected at 10 mg/kg, which suggested possible intrinsic rewarding properties of the drug. However,

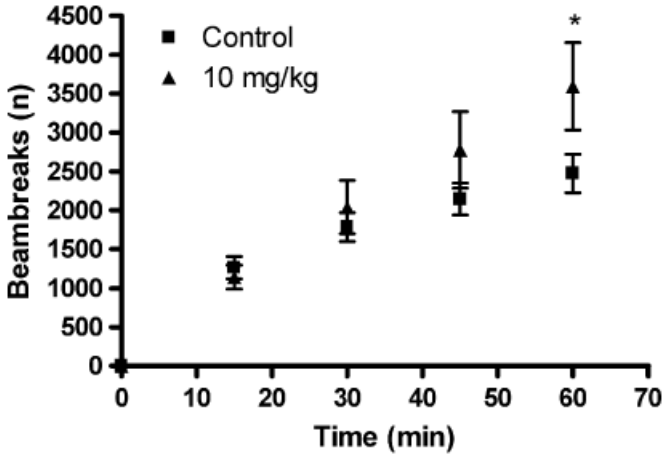

Fig. 2. Treatment with 3,7-dimethylpropargylxanthine induced a locomotor activation at 10 mg/kg, intraperitoneal. The effect was significant for accumulative activity at 60 min. $^{*}p < 0.05$.

Table 3. Effect of DMPX on Behavior in the Porsolt Swim Test, the Vogel Conflict Test, and Pain Thresholds

Test	Measure	Control	10 mg/kg
Porsolt swim test	Latency to immobility (s)	121 ± 10	133 ± 12
	Time spent immobile (s)	98 ± 13	89 ± 11
	Escape attempts (*n*)	14 ± 3	13 ± 2
Vogel conflict test	Unpunished responding (*n*)	67 ± 3	65 ± 2
	Punished responding	34 ± 2	38 ± 2
Pain thresholds	Current at jerk/twist	0.21 ± 0.02	0.19 ± 0.02

No significant differences were detected compared with controls for the dose (10 mg/kg) tested. Data are expressed as means ± SEM ($n = 9$–11 per group).
DMPX, 3,7-dimethylpropargylxanthine.

when the drug was examined in a conditioned place preference paradigm, no such properties could be detected.

The effects of DMPX on operant ethanol self-administration have previously been reported in Long–Evans rats (Arolfo et al., 2004). Here, we extend those findings to Wistar rats. The 2 lines differ significantly in their level of voluntary ethanol consumption (Gauvin et al., 1993) and in their response to benzodiazepine administration as measured on the elevated plus-maze (Onaivi et al., 1992). Long–Evans rats drink significantly less ethanol and have a higher sensitivity to the anxiolytic properties of benzodiazepines. In the Long–Evans line, DMPX had bimodal effects on ethanol self-administration, with low doses leading to an increase of ethanol-reinforced responding. This was not found in Wistar animals, in which the suppression of ethanol-reinforced responding was dose-dependent. This difference may be due to the difference in baseline drinking reported for these 2 lines. It may indicate that DMPX is more likely to consistently suppress ethanol reinforcement at high response rates, presumably reflecting higher motivation for ethanol intake.

Adenosine receptors are involved in the regulation of locomotor behavior in rodents. The A2a receptors located within the striatum are co-localized with dopamine D2 receptors. A number of studies have demonstrated that adenosine agonists produce significant inhibition of

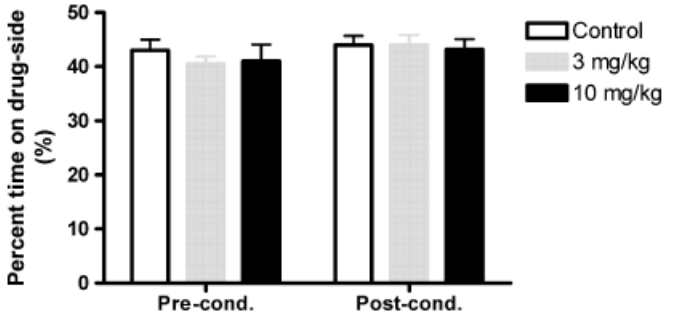

Fig. 3. 3,7-Dimethylpropargylxanthine had no intrinsic rewarding properties and did not induce place preference in the conditioned place preference paradigm. Pre-cond. refers to the place-preference naturally present in the animals before any drug administration. Post-cond. is after drug pairing has taken place.

locomotor activity in rodents (Ferre and Fuxe, 1992; Ferre et al., 1992), while adenosine antagonists such as caffeine produce locomotor activation. Performance and/or locomotor effects therefore need to be taken into consideration when interpreting the effects of DMPX on operant ethanol self-administration. However, we found no effect of DMPX on lever-pressing at the water lever. Thus, it is unlikely that the effect of this compound on ethanol self-administration is due to nonspecific locomotor activation.

An elevated locomotor activity may indicate a possible increase in positive reward induced by the administrated drug. To eliminate this, we examined the intrinsic rewarding properties of DMPX in a well-established paradigm: conditioned place preference. We found that DMPX did not induce any place preference and therefore is not in itself rewarding. This suggests that the suppression of ethanol self-administration by DMPX is not due to any intrinsic rewarding properties substituting for the rewarding properties of ethanol.

There are limited reports implicating the A2a receptor in CNS responses to ethanol or CNS regulation of ethanol intake. Adenosine A2a-null mutants have been reported to consume significantly more ethanol than their wild-type littermate controls in a 2-bottle free-choice study (Naassila et al., 2002). The mutants were also reported to display an increased anxiogenic phenotype and aggressiveness (Ledent et al., 1997). In another study, the absence of or chronic blockade of adenosine A2a receptors was shown to reduce handling-induced convulsions during ethanol-induced withdrawal (El Yacoubi et al., 2001). Our demonstration that acute administration of an A2a antagonist decreases ethanol-reinforced responding and is potentially anxiolytic contradicts these data. This may be due to compensatory developmental changes in the constitutive knockouts during development, with theoretical changes in D2 function being one possibility due to the close interaction of the 2 receptors (Franco et al., 2000; Gines et al., 2000).

In summary, we report that blockade of adenosine A2a receptors using the antagonist DMPX robustly and dose-dependently attenuates ethanol reinforcement in Wistar rats. Furthermore, the antagonist produces some degree of locomotor stimulation but is not in itself rewarding. A limitation of the present study is that the results were obtained in animals that had not been genetically selected for excessive ethanol drinking, or had a history of dependence that would lead to excessive drinking. Recent research indicates that excessive drinking and ethanol reinforcement under these conditions may be differentially sensitive to pharmacological manipulations compared with baseline levels. Our results indicate that A2a antagonists should be evaluated in these dependence-specific models. If replicated under those conditions, the blockade of adenosine A2a receptors may present a novel target for the development of treatments for alcoholism and alcohol abuse.

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