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Adenosine A₁ and A_{2A} receptors are not upstream of caffeine's dopamine D₂ receptor-dependent aversive effects and dopamine-independent rewarding effects

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Abstract

Caffeine is widely consumed throughout the world, yet little is known about the mechanisms underlying its rewarding and aversive properties. We show that pharmacological antagonism of dopamine not only blocks conditioned place aversions to caffeine, but reveals dopamine blockade-induced conditioned place preferences. These aversions are mediated by the dopamine D₂ receptor since knockout mice showed conditioned place preferences to doses of caffeine that C57Bl/6 mice found aversive. Further, these aversions appear to be centrally-mediated since a quaternary analogue to caffeine failed to produce conditioned place aversions. While the adenosine A_{2A} receptor is important for caffeine's physiological effects, this receptor seems only to modulate the appetitive and aversive effects of caffeine. A_{2A} receptor knockout mice showed stronger dopamine-dependent aversions to caffeine than C57Bl/6 animals, which partially obscured the dopamine- and A_{2A} receptor-independent preferences. Additionally, the A₁ receptor, alone or in combination with the A_{2A} receptor, does not seem to be important for caffeine's rewarding or aversive effects. Finally, excitotoxic lesions of the tegmental pedunculopontine nucleus revealed that this brain region is not involved in dopamine blockade-induced caffeine reward. This data provides surprising new information on the mechanism of action of caffeine, indicating that adenosine receptors do not mediate caffeine's appetitive and aversive effects. We show that caffeine has an atypical reward mechanism, independent of the dopaminergic system and the tegmental pedunculopontine nucleus and provide additional evidence in support of a role for the dopaminergic system in aversive learning.

Keywords

alpha-flupenthixol; 8-SPT; locomotor; SCH23390; mice

Introduction

Caffeine is the most widely consumed psychoactive substance in the world (Nehlig, 1999; Ferre, 2008). 80-90 % of American adults surveyed consumed caffeinated substances

regularly (Hughes & Oliveto, 1997; Frary *et al.*, 2005), however, the neurobiological mechanisms underlying its appetitive and aversive properties remain unknown.

In humans doses of caffeine below 500mg reportedly produce a sense of well-being, wakefulness and concentration {Kaplan *et al* 1997}, while higher doses above 500mg can induce anxiety, irritability, and agitation {Nehlig, 1992}. Complementary data has been found in rats with low and high doses producing mild conditioned place preferences and robust aversions, respectively (Brockwell & Beninger 1991; Patkina & Zvartau; Bedingfield *et al.*, 1998).

The mesolimbic dopamine system, consisting of the dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), has a long history in the study of reward (Wise, 2004). This system is involved in reward processing (Kelley & Berridge, 2002; Bozarth & Wise, 1981), and learning about aversive stimuli, including nicotine (Salamone, 1994; Pezze *et al.*, 200; Laviolette & van der Kooy, 2003). This neurotransmitter system may mediate caffeine's rewarding, or aversive, effects.

The tegmental pedunculo pontine nucleus (TPP), found in the brainstem, is composed of glutamatergic, GABAergic and cholinergic neurons (Clements & Grant, 1990; Ford *et al.*, 1995). The TPP is involved in learning about appetitive stimuli (see Schultz 2002 for a review), including copulation (Kippin & van der Kooy 2003), amphetamine, morphine, and nicotine (Bechara & van der Kooy, 1989; Laviolette *et al.*, 2002) and brain stimulation (Lepore & Franklin, 1993). The TPP provides a non-dopaminergic region that may be involved in caffeine reward.

At high doses caffeine inhibits cyclic nucleotide phosphodiesterases (Smellie *et al.*, 1979) and acts on ryanodine receptors (McPherson *et al.*, 1991). At lower doses, which are consumed by humans, caffeine's physiological effects probably result from antagonism of adenosine receptors (Fredholm, 1980; Snyder, 1985). Of the four adenosine receptor subtypes, the high affinity of A₁ and A_{2A} receptors for caffeine and their tonic activity at physiological levels of adenosine (Fredholm *et al.*, 1999) suggests these are caffeine's targets (Daly & Fredholm, 1998). Linking caffeine to the dopaminergic system, within the striatum dopamine D₂ receptors interact antagonistically with A_{2A} receptors as heteromers (Ferré *et al.*, 1991; 1993; 1994), and this may also be true for A₁ and D₁ receptors (Fuxe *et al.*, 2007).

It is currently unclear whether the adenosine A₁ or A_{2A} receptor is important for the rewarding effects of caffeine. An A_{2A} receptor specific antagonist was previously implicated in caffeine preferences (Brockwell & Beninger 1996) and A_{2A} receptor knockout mice were reported to drink less caffeinated water than C57Bl/6 animals (El Yacoubi *et al.*, 2005). However, animals receiving an A₁ antagonist equated this to the experience of caffeine (Quarta *et al.*, 2004) and A₁ receptor antagonists were found to alter neurotransmitter levels similarly to caffeine (Solinas *et al.*, 2005).

Because the dopaminergic system has a long history in the study of drug reward and the adenosinergic system is a pharmacological target of caffeine, we sought to elucidate the roles of these two systems in caffeine reward and aversion.

Materials and Methods

Drugs

The drugs used in these experiments, caffeine, alpha-flupenthixol, 8-(p-sulfophenyl)theophylline (8-SPT), and N-methyl-D-aspartic acid (NMDA), were obtained from Sigma. Caffeine, alpha-flupenthixol, SCH23390 and 8-SPT were dissolved in 0.9 %

saline and injected i.p. in a volume of 1 ml/kg body weight except alpha-flupenthixol, which injected in a volume of 10 ml/kg body weight. This neuroleptic was chosen because it does not produce preferences or aversions of its own at this dose (Laviolette and van der Kooy, 2003; Dockstader, C., unpublished data) and is known to antagonize both dopamine D₁ and D₂ receptors approximately equally (Andersen, 1988; Creese *et al.*, 1976). NMDA was dissolved in a phosphate buffered saline (PBS) to yield a 0.1 M solution and titrated to pH 7.4.

Animals

Male C57Bl/6 mice (Charles River) were between 25 and 30 grams at the start of experimentation. Mice were housed four to a cage for the duration of the experiment in a climate-controlled (21°C) room with a 12-hour light (0700-1900 h)/dark cycle. Dopamine D₁ receptor knockout mice (D₁KO) and D₂ receptor knockout (D₂KO) animals were generated previously (Kelly *et al.*, 1997) and backcrossed to the C57Bl/6 background 12 times. D₁KO and D₂KO mice were between 25 and 40 grams at the beginning of experimentation; both male and female animals were used and analyses of male and female responses indicated there was no effect of gender (data not shown). Adenosine A_{2A} receptor knockout (A_{2A}KO) mice were generated as described previously (Chen *et al.*, 1999) and were backcrossed to the C57Bl/6 background 12 times. A_{2A}KO mice were between 19 and 35 grams at the beginning of experimentation. Adenosine A₁ receptor knockout (A₁KO) mice were generated as described previously (Johansson *et al.*, 1998) and backcrossed to C57Bl/6 6 times according to the Jackson Laboratory "speed congenic procedure". Adenosine A₁ receptor and adenosine A_{2A} receptor double knockout (dKO) mice were generated as described previously (Yang *et al.*, 2009), however double heterozygote matings took place at Dr. Michael Schwarzschild's lab in Boston, MA. For knockout experiments, wild type littermates were used as controls. Each mouse was used in one experiment only. All procedures were in accordance with institutional and governmental guidelines for animal care.

Place conditioning

All animals were conditioned using a standard place conditioning procedure. The conditioning apparatus consisted of a black-walled chamber (15 cm × 15 cm × 15 cm) with a smooth, black Plexiglas floor and a white-walled chamber (15 cm × 15 cm × 15 cm) with a bumpy, white floor separated by a removable partition and with a clear Plexiglas top. Immediately prior to the placement of an animal in the black environment 0.1 ml of 3 % acetic acid was wiped on the floor until dry. Animals received four 15 minute pairings of the drug with one environment and four 15 minutes pairings of saline with the other environment in an alternating fashion on consecutive days. Drug and vehicle pairings were fully counterbalanced on each conditioning day. For alpha-flupenthixol trials, animals were pretreated with an i.p. injection of either 0.8 mg/kg or 2.0 mg/kg alpha-flupenthixol 1 hour prior to conditioning and returned to their home cages. After pretreatment, conditioning was carried out as described above. For SCH23390 trials the drug was administered 30 minutes prior to conditioning and returned to their home cages. After pretreatment conditioning took place as described above. During testing, the partition between the black and white chambers was removed and mice were placed between the black and the white compartments so that they did not face either environment. This prevents bias in the initial choice to enter one side of the apparatus or the other. Animals were allowed to move freely between the compartments for 10 minutes and the time spent in each environment was recorded by the disruption of photobeams in the compartments. Testing took place one day after the final conditioning trial to ensure that they were in a drug-free state. In order to minimize the number of animals used the balance of the boxes was tested intermittently (Supplementary Figure 1). This was done by giving mice 8 saline injections (4 in each

environment) over 8 consecutive days followed by a 10 minute test to ensure that animals had no preference for either environment. The data from these saline trials was used as control data for experimental trials conducted during approximately the same time period.

Experimental groups

Mice were conditioned with seven different doses caffeine with and without alpha-flupenthixol pretreatment; each animal was used for only one experiment. All trials for the dose-response curve were run concurrently with a trial using an equivalent dose of caffeine and preceded by alpha-flupenthixol pretreatment. The doses of caffeine used in mg/kg were 0.01 mg/kg, 0.1 mg/kg, 1 mg/kg, 3 mg/kg, 10 mg/kg, 15 mg/kg, 30 mg/kg, and 100 mg/kg. For all experiments, including those with knockout mice experimental groups contained between 8 and 30 animals to ensure there was sufficient power.

Locomotor measurements

Locomotor activity was measured using six photobeams present in the conditioning apparatus: 3 in the black-walled chamber and 3 in the white-walled chamber. The disruption of a single photobeam was measured as one locomotor count. Locomotor measurements took place during the final 15 minute drug and vehicle conditioning session of each mouse. The apparatus recorded “wasted time” when no photobeams were being disrupted, which corresponded to when an animal was not entirely in one environment.

Surgical Procedures

Mice were anesthetized with isofluorane (5 % to induce, 1-2 % for maintenance) and animals were placed in a stereotaxic apparatus. Lesions of the TPP were performed bilaterally (stereotaxic coordinates AP -4.4 mm, L $\pm$ 1.1 mm, V -3.8 mm from bregma and the dura surface) by manually injecting 0.04 μ l of 0.1 M NMDA solution, or the PBS vehicle for sham surgeries, using a 1 μ l Hamilton microsyringe (Reno, NV). The infusion rate was 0.01 μ l/minute and the syringe was left in place for 1 minute following the infusion to allow for diffusion to take place. Mice were given at least 10 days to recover before conditioning sessions.

Histology

At the end of experiments, animals that had received TPP sham or NMDA lesions were deeply anesthetized with sodium pentobarbital and perfused transcardially with 30 ml of physiological saline followed by 30 ml of 4% formaldehyde. Brains were rapidly removed and stored for at least 24 hours in a 25% sucrose, 4% formaldehyde post-fixative. Brains were then flash frozen at -80°C, sliced in a freezing microtome into 40- μ m-thick sections, and mounted on gelatin-coated slides. TPP and sham lesions were verified with cresyl violet staining and light microscopy (see Figure 5B for a representative image). Only animals with lesions within the boundary of the TPP (as defined by the atlas of Hof *et al.*, 2000) were included in the analyses. Investigators were blind to the behavioral performance of the animals during lesion analyses.

Statistical analyses

Analyses of variance (ANOVA) were conducted using SPSS followed by post hoc Tukey's HSD tests and two-tailed independent t-tests where appropriate. Significance was accepted as $P < 0.05$. However when multiple t tests were conducted for analysis of the dose response curve cutoffs for statistical significant were adjusted using a standard Bonferroni Correction to control for increased risk of false positives. Error bars on figure means are standard error of the mean (SEM).

Results

Caffeine aversions depend on dopamine

The rewarding and aversive effects of caffeine were determined using an unbiased place conditioning paradigm for doses of caffeine spanning four orders of magnitude (Figure 1A). At low doses caffeine did not produce place preferences or aversions, while at doses of 10 mg/kg or above it produced conditioned place aversions. When mice were pretreated with the dopaminergic antagonist alpha-flupenthixol before conditioning with caffeine, the aversions to higher doses of caffeine were blocked and conditioned place preferences to the drug were revealed.

A two-way ANOVA indicated a significant dose by pretreatment interaction, confirming our observation that alpha-flupenthixol disrupted the higher dose aversive effects caffeine ($F_{7,243} = 5.01$, $P < 0.004$). Multiple comparison tests (with P values adjusted using a Bonferroni correction) indicated that mice showed conditioned place aversions when conditioned with 10 mg/kg ($t_{30} = 4.55$, $P < 0.004$), 15 mg/kg ($t_{13} = 4.11$, $P < 0.004$), 30 mg/kg ($t_{14} = 7.47$, $P < 0.004$), and 100 mg/kg caffeine ($t_{29} = 10.18$, $P < 0.004$). However at these same doses, when mice were pretreated with the dopaminergic antagonist alpha-flupenthixol, the aversive effects of caffeine were blocked and significant preferences were revealed at 10 mg/kg caffeine ($t_{30} = 5.24$, $P < 0.004$), 15 mg/kg ($t_7 = 4.64$, $P < 0.004$), and a trend towards a preference at 30 mg/kg caffeine ($t_{14} = 3.43$, $P = 0.004$). Animals conditioned with 3 mg/kg caffeine showed a significant preference when pretreated with alpha-flupenthixol ($t_{15} = 3.72$, $P < 0.004$) but not when untreated ($t_{15} = 0.141$, $P > 0.004$). In animals conditioned with 100 mg/kg caffeine, pretreatment with the dopaminergic antagonist blocked the aversive effects of caffeine but no significant preference was revealed ($t_{27} = 0.042$, $P > 0.004$).

To control for the possibility that the stress of receiving an injection 1 hour prior to conditioning in alpha-flupenthixol trials, a group of animals was pretreated with saline 1 hour prior to conditioning with 10 mg/kg of caffeine (Supplementary Figure 2). Mice showed aversions to caffeine equivalent in magnitude to mice not given a saline injection 1 hour prior to conditioning, indicating that the effects of pretreating with alpha-flupenthixol can be attributed to the effects of the drug and not to the stress of receiving an injection.

Caffeine's aversive effects are mediated centrally

In order to test the possibility that caffeine's aversive effects are mediated peripherally mice were conditioned with i.p. 8-SPT, a quaternary analogue to caffeine which is unable to cross the blood-brain barrier (Figure 1B). Mice did not show conditioned place aversions to the drug; the amount of time they spent in the previously saline-paired environment did not differ from the amount of time they spent in the previously 8-SPT-paired environment on test day ($t_{14} = 0.451$, $P > 0.05$). This indicates that caffeine's aversive effects result from its activities in the central nervous system.

Dopamine D₂ receptors are most important for caffeine's aversive effects

In order to confirm the surprising finding that dopamine blockade attenuated the aversive effects of caffeine and to determine which specific dopamine receptor subtypes are responsible for this effect, we conditioned dopamine D₁KO and D₂KO mice with 10 mg/kg caffeine, a dose that produces aversions in C57Bl/6 mice.

When dopamine D₁KO and D₂KO mice were conditioned with 10 mg/kg caffeine, conditioned place aversions to caffeine were blocked (Figure 2A). D₁KO mice did not show a place preference or a place aversion to caffeine while D₂KO mice showed significant

conditioned place preferences, though preferences did not maintain significance after correcting for multiple t tests. A one-way ANOVA revealed a significant effect of genotype ($F_{2,47} = 6.89$, $P < 0.05$) and a post-hoc Tukey's test indicated that the response of D_2 KO mice differed significantly from wild type littermates ($P < 0.05$), but not from D_1 KO ($P > 0.05$). The response of D_1 KO mice to caffeine did not differ significantly from that of wild type littermates ($P > 0.05$) even though difference scores in D_1 KO mice were not significantly different from zero ($t_{14} = 0.947$, $P > 0.05$) while wild type animals showed significant aversions ($t_{21} = 3.11$, $P < 0.05$). These findings confirmed our previous results implicating dopaminergic signaling in the aversive effects of caffeine. Further, these results show that the dopamine D_2 receptor, specifically, is most important in mediating caffeine's aversive properties.

D_1 KO mice did not show a place preference or place aversion to caffeine suggesting this receptor might in part mediate caffeine's rewarding effects. In order to test this, D_2 KO mice were pretreated with the D_1 receptor antagonist SCH23390 prior to conditioning with caffeine (Figure 2B). If the D_1 receptor is involved in the rewarding effects of caffeine, then we would predict that the preferences in D_2 KO mice would be diminished or attenuated. However, if the D_1 receptor is involved in the aversive effects of caffeine, then the pretreatment should have no effect or would increase the preference. At both doses of SCH23390 D_2 KO mice showed preferences to caffeine similar to those seen in non pretreated D_2 KO mice. An ANOVA indicated there was no interaction between genotype and pretreatment ($F_{2,37} = 2.06$, $P > 0.05$) and no main effect of pretreatment ($F_{2,37} = 0.926$, $P > 0.05$), but there was a main effect of genotype ($F_{1,37} = 8.45$, $P < 0.05$). This indicates, in line with our alpha-flupenthixol data, that the D_1 receptor is not involved in the rewarding effects of caffeine. The D_1 receptor, like the D_2 receptor but to a lesser extent, is involved in mediating the aversive effects of caffeine.

At a dose of 100 mg/kg caffeine, pretreatment with alpha-flupenthixol blocked the aversive effects of caffeine but failed to reveal the preferences seen at lower doses of caffeine (Figure 1A). Conditioning D_2 KO mice with 100 mg/kg revealed that at this dose of caffeine a substrate other than the D_2 receptor is responsible for caffeine's aversive effects (Figure 2C). D_1 KO and D_2 KO mice both showed aversions to this dose of caffeine and an ANOVA revealed no main effect of genotype ($F_{3,20} = 0.308$, $P > 0.05$), indicating these aversions were equivalent to those seen in their wild type littermates.

D_2 KO mice show normal locomotor stimulation to caffeine

To determine whether the well-established locomotor stimulant effects of caffeine had also been disrupted in these dopamine receptor knockout mice, we tested the locomotor effects of caffeine in D_1 KO and D_2 KO animals. The administration of 10mg/kg caffeine led to increased locomotor activity in C57BL/6, D_1 KO, and D_2 KO mice (Figure 2D). A two-way ANOVA indicated there was no significant genotype by caffeine interaction ($F_{2,42} = 0.322$, $P > 0.05$), indicating that all three genotypes experienced similar locomotor stimulation following caffeine administration. The ANOVA revealed a main effect of drug ($F_{1,42} = 17.8$, $P < 0.05$), and a main effect of genotype ($F_{2,42} = 5.70$, $P < 0.05$). A post hoc Tukey's test indicated that D_2 KO mice had significantly different locomotor activity than D_1 KO mice ($P < 0.05$) which was manifested as lower activity in D_2 KO mice following both saline and caffeine treatment. 45 minute locomotor trials were also conducted to determine if a longer observation would reveal genotypic differences in caffeine's locomotor stimulant effects. The findings from these trials did not differ from the findings of 15 minute trials.

These findings show that the appetitive and aversive effects of caffeine are dissociable from its locomotor stimulant effects since D_1 KO and D_2 KO mice showed altered responses to place conditioning with caffeine while maintaining locomotor stimulation to the drug. The

locomotor stimulation produced by caffeine served as a control showing that our knockout mice do not have a generalized inability to detect caffeine. On the contrary, they have a specific deficit in their ability to respond to caffeine's aversive motivational effects.

A_{2A}KO mice show strong caffeine aversions, but also dopamine blockade-induced preferences for caffeine

We next asked what substrate caffeine acts on to elicit an effect on the dopaminergic system. Because caffeine is an antagonist at adenosine receptors, we tested adenosine A_{2A}KO mice to determine whether this receptor is upstream of caffeine's dopamine-dependent aversions. We also tested whether this receptor is critical for caffeine's rewarding effects because of previous reports implicating its involvement (Brockwell & Beninger, 1996; El Yacoubi *et al.*, 2005).

Mice were conditioned with 15 mg/kg caffeine, a dose slightly higher than our previous dose of 10 mg/kg, to ensure that our C57BL/6 control group showed strong aversions. This 15 mg/kg dose did not produce stronger aversions than the 10 mg/kg dose in this particular group of C57BL/6 mice, however. When A_{2A}KO mice were conditioned with 15 mg/kg caffeine they showed aversions to caffeine slightly greater than those seen in C57BL/6 mice. However in contrast to C57BL/6 mice, pretreating A_{2A}KO mice with 0.8 mg/kg alpha-flupenthixol did not completely block the aversive effects of caffeine and reveal a preference. A_{2A}KO mice maintained conditioned place aversions to caffeine after alpha-flupenthixol pretreatment (Figure 3A). A t-test for independent samples revealed that the bigger aversions in A_{2A}KO mice to 15 mg/kg caffeine were not significantly greater than those of C57BL/6 mice ($t_{21} = 1.30$, $P > 0.05$). However, comparing A_{2A}KO mice and C57BL/6 mice when both groups had been pretreated with 0.8 mg/kg alpha-flupenthixol before conditioning indicated that A_{2A}KO mice showed a significantly different response than C57BL/6 mice ($t_{21} = 3.39$, $P < 0.05$). A_{2A}KO mice showed small aversions while C57BL/6 mice showed conditioned place preferences to caffeine. Perhaps the greater aversive effects of caffeine in A_{2A}KO mice partially obscure the dopamine blockade induced rewarding effects of caffeine.

We next conditioned A_{2A}KO mice and C57BL/6 mice with a lower dose of caffeine, 10 mg/kg, and a higher dose of neuroleptic, 2 mg/kg, to test whether our A_{2A}KO mice were showing a stronger aversive response to caffeine and determine more conclusively whether these A_{2A}KO mice were deficient in caffeine reward. A_{2A}KO mice showed aversions to 10 mg/kg caffeine that were slightly larger but statistically equivalent to those seen in C57BL/6 mice. In addition, when they were pretreated with a higher dose of neuroleptic our A_{2A}KO mice showed non significant preferences to caffeine just as the C57BL/6 mice did (Figure 3B). T-tests for independent samples confirmed that at 10 mg/kg caffeine aversions in A_{2A}KO mice were not significantly different from those of C57BL/6 animals ($t_{17} = 0.661$, $P > 0.05$). Further, A_{2A}KO mice showed non significant preferences to caffeine when pretreated with neuroleptic that did not differ from those of C57BL/6 mice receiving the same treatment ($t_{18} = 0.123$, $P > 0.05$).

These findings rule out the possibility that the adenosine A_{2A} receptor is the receptor substrate directly mediating caffeine's dopamine-dependent aversive effects and dopamine blockade-induced rewarding effects. The adenosine A₁ receptor remains a candidate since this receptor binds caffeine with a high affinity and mediates many of caffeine's physiological effects alongside the A_{2A} receptor (Fredholm *et al.*, 1999; Daly & Fredholm, 1998).

The adenosine A_{2A} receptor mediates the locomotor effects of lower doses of caffeine

Previous reports have indicated the adenosine A_{2A} receptor alone (Ledent *et al.*, 1997), the A₁ receptor alone (Florio *et al.*, 1997), or a combination of both adenosine receptor subtypes (Kuzmin *et al.*, 2006) can stimulate locomotion. We next tested whether A_{2A}KO mice showed normal locomotor stimulation to caffeine.

C57BL/6 mice, but not A_{2A}KO, demonstrated locomotor stimulation when given 10 mg/kg caffeine. However at a dose of 15 mg/kg caffeine both genotypes showed increased locomotor activity (Figure 3C) although an ANOVA did not reveal significant interactions. The ANOVA indicated there was no 3-way interaction between drug, dose and genotype ($F_{1,40} = 0.308$, $P > 0.05$) and showed a main effect of genotype ($F_{1,40} = 22.3$, $P < 0.05$) and a main effect of drug ($F_{1,40} = 10.2$, $P < 0.05$). As with dopamine receptor knockout mice, 45 minute locomotor trials were also conducted. The findings from these trials did not differ from the findings of 15 minute trials.

The finding that A_{2A}KO mice show increased locomotor activity following administration of 15 mg/kg caffeine, although it appears smaller than what is observed in wild type littermates, rules out the idea that the A_{2A} receptor is independently responsible for caffeine's locomotor stimulant effects. While it does seem to play an important role at lower doses of caffeine, there is clearly a threshold above which other substrates come into play. While these findings cannot speak to the type of relationship between these substrates, whether they interact additively or synergistically as indicated by Kuzmin *et al.* (2006), they do strongly suggest a substrate in addition to the A_{2A} receptor through which caffeine acts to increase locomotor activity. Previous work suggests this additional substrate may be the adenosine A₁ receptor (Florio *et al.*, 1997; Kuzmin *et al.*, 2006).

When both C57BL/6 mice and A_{2A}KO mice were pretreated with 0.8 mg/kg alpha-flupenthixol they experienced locomotor depression relative to their baseline locomotor activity in the absence of neuroleptic (dotted line in Figure 3D). In C57BL/6 mice, this locomotor depression was partially counteracted by treatment with 15 mg/kg caffeine in C57BL/6 but not A_{2A}KO mice although an ANOVA revealed no interaction between drug and genotype ($F_{1,20} = 2.90$, $P > 0.05$). A main effect of drug treatment was observed ($F_{1,20} = 6.54$, $P < 0.05$).

A₁KO and dKO mice show normal conditioned place aversions and dopamine blockade-induced preferences to caffeine

Animals in a drug discrimination task were previously shown to find treatment with an A₁ antagonist to be similar to the experience of caffeine (Quarta *et al.*, 2004). As a result we tested A₁KO mice to see if this receptor is involved in caffeine reward or aversion. Different groups of A₁KO mice were conditioned with 10 mg/kg caffeine with and without neuroleptic pretreatment (Figure 4A). A₁KO mice showed aversions to 10 mg/kg caffeine that were similar in magnitude to the aversions of their wild type littermates, the aversions seen by both groups did not withstand corrections for multiple testing ($P > 0.05$). A₁KO mice also showed preferences that were similar to those of wild type littermates when conditioned with 10 mg/kg caffeine after pretreatment with 0.8 mg/kg alpha-flupenthixol. Preferences seen in these two groups did not withstand corrections for multiple testing ($P > 0.05$). A two-way ANOVA revealed only a main effect of pretreatment ($F_{1,22} = 16.26$, $P < 0.05$). Interaction ($F_{1,22} = 1.479$, $P > 0.05$) and genotype effects ($F_{1,22} = 0.050$, $P > 0.05$) were not significant confirming that the aversive and rewarding effects of caffeine in A₁KO mice did not differ from those of wild type littermates. Surprisingly, this indicates that the adenosine A₁ receptor, in addition to the adenosine A_{2A} receptor, is not upstream of

caffeine's dopamine-mediated aversive effects or caffeine's dopamine blockade-induced preferences.

To test the possibility that the adenosine A₁ and A_{2A} receptors perform overlapping functions in signaling caffeine aversions or preferences we conditioned different groups of adenosine A₁ and A_{2A} receptor double knockout mice (dKO) with 10 mg/kg caffeine in the presence and absence of 0.8 mg/kg alpha-flupenthixol pretreatment. A two-way ANOVA revealed a main effect of pretreatment ($F_{1,28} = 10.54$, $P < 0.05$) but no genotype by pretreatment interaction ($F_{1,28} = 0.001$, $P > 0.05$) or main effect of genotype ($F_{1,28} = 0.507$, $P > 0.05$), indicating that neither the A₁ or the A_{2A} receptor alone or in combination are critical for mediating the aversive or rewarding effects of caffeine.

A₁KO mice show normal locomotor responses to caffeine

A_{2A}KO mice did not show locomotor stimulation when administered 10 mg/kg caffeine but showed a small increase in locomotor activity when given 15 mg/kg caffeine. This suggests the adenosine A_{2A} receptor is important for caffeine-induced locomotor stimulation at lower doses so we tested the possibility that the adenosine A₁ receptor mediates caffeine's additional A_{2A}-independent locomotor effects.

A₁KO mice conditioned with caffeine showed increases in locomotor activity similar to what was seen in wild type littermates (Figure 4B), ruling out the possibility that the A₁ receptor acts in addition to the A_{2A} receptor to mediate the locomotor response to caffeine. An ANOVA showed that there was no interaction between drug and genotype ($F_{1,22} = 0.997$, $P > 0.05$) and confirmed there was no main effect of genotype on locomotor count ($F_{1,22} = 0.076$, $P > 0.05$). A main effect of drug was observed ($F_{1,22} = 20.25$, $P < 0.05$).

The tegmental pedunculo pontine nucleus is not involved in caffeine's dopamine blockade-induced rewarding effects

When one of caffeine's well-established pharmacological targets, the A_{2A} receptor, failed to be involved in caffeine's dopamine blockade-induced rewarding effects, we next looked to the TPP since it is implicated in the rewarding effects of natural rewards (Kippin & van der Kooy, 2003; Stefurak & van der Kooy, 1994) and drugs (Bechara & van der Kooy, 1989; Laviolette *et al.*, 2002).

C57BL/6 mice given sham TPP lesions developed conditioned place aversions to 10 mg/kg caffeine, and these aversions were unaltered in mice with excitotoxic lesions of the TPP. Additionally, C57BL/6 mice with sham TPP lesions showed conditioned place preferences to caffeine when pretreated with neuroleptic, and these place preferences were not disrupted in mice with excitotoxic TPP lesions (Figure 5A). Comparing sham-lesioned and TPP-lesioned mice confirmed that lesioning the TPP had no effect on caffeine place aversions ($t_{24} = 0.267$, $P > 0.05$). A comparison of sham-lesioned and TPP-lesioned mice pretreated with neuroleptic before conditioning indicated that lesions of the TPP had no effect on the dopamine blockade-induced rewarding effects of caffeine ($t_{22} = 1.04$, $P > 0.05$).

The functional effectiveness of these lesions was verified by conditioning lesioned mice with morphine since TPP lesions have been previously shown to block morphine reward in naive animals (Bechara & van der Kooy, 1989). Similar TPP lesions were shown to block morphine conditioned place preferences in mice (Ting-A-Kee *et al.*, 2009). This shows that our inability to block caffeine reward and aversion was likely because the TPP is not involved in these effects and not because of ineffective lesions.

Discussion

The current data indicates that caffeine has aversive effects at high doses and neither rewarding nor unpleasant effects at low doses. Previous work in rats has indicated that caffeine induces mild preferences at low doses (Brockwell *et al.*, 1991; Bedingfield *et al.*, 1998; Patkina & Zvartau, 1998) and aversions at high doses (Brockwell *et al.*, 1991; Patkina & Zvartau, 1998). While the low-dose preferences previously observed seem to contradict the current results, caffeine is not reported to be a robust reinforcer in animals (Nehlig 1999). Indeed the rewarding effects of caffeine seen by Brockwell *et al.* (1991) were at one dose and small. This is similar to our current data; the lower doses of caffeine on our dose-response curve are weakly, but non-significantly rewarding. Also consistent, the rewarding effects of caffeine in humans are mild or absent in individuals with limited caffeine experience (Griffiths & Woodson, 1988; Nehlig 1999). Caffeine reward is often primarily attributed to the relief of abstinence-induced withdrawal in chronic users (Tinley *et al.*, 2004; James & Rogers, 2005). Further, a drinking study in rats suggests that tolerance develops to the initial aversive effects of caffeine following repeated exposure (Myers & Izbic, 2006), suggesting it may be easier to observe reward in animals with previous caffeine exposure. These data indicate that our dose-response curve is in line with previous data from animals and humans and that future studies where animals are treated chronically with caffeine before conditioning may have greater success in generating conditioned place preferences.

Previous work has shown that the dopaminergic system is involved in signaling the aversive effects of stimuli. Blockade of the dopaminergic system with alpha-flupenthixol was found to inhibit the negative effects of naloxone-precipitated morphine withdrawal (Santi and Parker, 2001). Antagonism of the dopamine D₂ receptor with haloperidol was found to inhibit conditioned place aversions to the benzodiazepine receptor inverse agonist FG 7142 (Di Scala and Sandner, 1989). Further, mice with a knockout of the long form of the dopamine D₂ receptor displayed a deficit in learning about aversive electrical stimuli (Smith *et al.* 2002) while D₂KO mice do not show place aversions to high doses of ethanol (Ting-A-Kee *et al.* 2009). The aversive effects of nicotine can also be blocked by systemic or intra-VTA alpha-flupenthixol, and place preferences can be revealed at some doses (Laviolette & van der Kooy, 2003). Additionally, place preferences to morphine, diazepam and nicotine in addition to place aversions to picrotoxin, naloxone, phencyclidine and lithium can be blocked by the dopamine D₁ receptor antagonist SCH23390 (Acquas *et al.* 1989; 1994).

It has been suggested that dopamine is not involved in signaling the appetitive aspects of stimuli but rather the motivational importance of stimuli, regardless of whether they are rewarding or aversive (Acquas *et al.* 1989). However, learning about motivationally relevant stimuli continues under conditions of dopamine blockade, as illustrated by the data in our dose response curve (Figure 1). While dopamine is involved in appetitive and aversive neurotransmission related to several different classes of stimuli, there seem to be redundant mechanisms involved. The dopaminergic system is likely to be one of a few systems engaged in the important process of signaling motivationally relevant stimuli in the brain.

Dopamine is present in the peripheral nervous system; however, the aversive effects of caffeine appear to be mediated centrally. A quaternary analogue of caffeine, 8-SPT, which was administered peripherally and is unable to cross the blood-brain barrier, did not induce a conditioned place aversion. Further, it is likely that the 10 mg/kg dose of 8-SPT chosen inhibited adenosine receptors sufficiently to induce an aversive response. Previous work has shown that a given dose of 8-SPT inhibits adenosine receptors to a greater degree than the same dose of theophylline (Tao & Abdel-Rahman, 1993), a methylxanthine with very

similar binding properties to caffeine (Ukeda *et al.*, 1986a, b). These data indicate that caffeine's aversive effects result from its actions in the central nervous system.

While D₂KO mice have lower basal locomotor activity than C57Bl/6 animals and this probably reduces exploration of the apparatus during conditioning sessions, it is unlikely that this would decrease the ability of knockout animals to learn place preferences or aversions. In fact, animals pretreated with the neuroleptic alpha-flupenthixol show dramatically reduced locomotor activity during conditioning sessions, however these animals learn robust place preferences (Figure 1A) and aversions to caffeine. This demonstrates that exploration of the apparatus is not necessary in order for an animal to associate the pleasurable and aversive effects of a drug with the environment it was experienced in.

The duration of conditioning sessions and time between drug injection and the initiation of conditioning can dramatically alter the outcome of place conditioning under some circumstances (See Tzschentke, 2007 for a review). Because caffeine is rapidly and almost completely absorbed from the gut it is likely that conditioning animals immediately after the drug injection or close to it would yield results representative of the maximum level of circulating drug. In support of our paradigm, our group previously conditioned animals for 15 minutes following a 15 minute delay after the i.p. caffeine injection. Conditioned place aversions to caffeine were still observed using this paradigm and were equal in magnitude to those observed when no delay was used (data not shown). Because the aversion seen when conditioning was conducted after a 15 minute delay was not significantly stronger, conditioning animals immediately after an i.p. injection with caffeine seems to provide enough time for the majority of drug absorption to take place.

We show by lesioning the TPP that this brainstem nucleus is not involved in dopamine blockade-induced caffeine reward. These findings suggest the mechanisms underlying caffeine's rewarding effects are different from those of other recreational drugs. Previous reports have indicated the A_{2A} receptor is a pharmacological target of caffeine, indicating this receptor might be the mediator of caffeine's rewarding or aversive effects. We have shown, surprisingly, that this receptor is not the upstream receptor mediating caffeine's dopamine-dependent aversive effects or caffeine's rewarding effects. However, the loss of the A_{2A} receptor may increase caffeine's aversive effects.

The finding that caffeine exerts reward that does not depend on either dopamine or the TPP is interesting given that few studies have reported reward processes that are independent of these two well-established systems. Under conditions of dopamine blockade, the aversions to caffeine are blocked and preferences for the drug are revealed. These data suggest that the injection of caffeine affects different and dissociable neurobiological systems that are responsible for its aversive and rewarding motivational effects. At doses where dopamine-blockade begins to reveal preferences, caffeine is known to increase the activity of multiple regions of the central nervous system (Nehlig *et al.*, 1984). While lower doses of caffeine do not induce activation of the nucleus accumbens, this region is activated at doses of 10 mg/kg or above (Nehlig *et al.*, 1984). It seems possible then that caffeine is activating the nucleus accumbens by a dopamine independent mechanism to induce its rewarding effects. Cocaine reward has been shown to occur under conditions of dopamine blockade (Spyraki *et al.*, 1982) and also when the TPP has been lesioned (Parker & van der Kooy, 1995). Further, mice unable to synthesize dopamine, called dopamine-deficient mice, display conditioned place preferences for cocaine (Lavin *et al.*, 2005). These preferences depend on the serotonergic system and dopaminergic neurons, but not on the neurotransmitter dopamine itself (Hnasko *et al.*, 2007). It has been suggested that activation of dopamine neurons may normally lead to corelease of dopamine with neuropeptides and other neurotransmitters

including glutamate; these additional transmitters may be involved in the conditioned place preferences to cocaine (Lavin *et al.*, 2005). A similar mechanism, independent of dopamine, may be at play in the pleasurable effects of caffeine.

Few previous studies have focused on the role of the adenosine A_{2A} receptor in caffeine's appetitive and aversive effects, however two studies have implicated the A_{2A} receptor in caffeine reward. Previous work showed that A_{2A}KO mice consumed less caffeinated water than C57BL/6 animals (El Yacoubi *et al.*, 2005). However the A_{2A}KO mice also consumed less caffeinated solution than caffeine-free water suggesting that the A_{2A}KO animals may not lack a preference, but may have been avoiding caffeine. The interpretation that A_{2A}KO mice were avoiding caffeine, and are perhaps more sensitive to its aversive effects than C57BL/6 animals, is in line with the current results. A second study showed that the A_{2A}-selective antagonist CGS 15943A, and not an A₁ receptor antagonist, produced place preferences in rats (Brockwell & Beninger, 1996). Surprisingly, despite widespread data indicating the high affinity of the A_{2A} and A₁ receptor for caffeine (Fredholm *et al.*, 1999) and their importance for caffeine's physiological effects (Daly & Fredholm, 1998), our results do not support a role for A_{2A} or A₁ receptors in the rewarding or aversive effects of caffeine. Additionally, using dKO mice with no functional adenosine A₁ or A_{2A} receptors, we tested the possibility that these two receptors both act to signal caffeine aversion or reward. If this were the case we would predict that in the absence of one receptor caffeine aversions or preferences would persist due to signaling via the other receptor. However the dKO showed caffeine aversions and dopamine blockade-induced preferences that were equivalent to those seen in C57BL/6 mice. This indicates that adenosine A₁ and A_{2A} receptors do not perform redundant functions in signaling caffeine aversions and preferences and provides strong evidence that caffeine acts on a substrate other than the adenosine A₁ or A_{2A} receptors to produce its aversive and rewarding effects. However it remains unclear, that if not the adenosine A₁ and A_{2A} receptor, then what molecules or receptors signal caffeine's aversive and appetitive properties, because previous work has shown that other pharmacological targets of caffeine, the A_{2B} and A₃ adenosine receptors, are activated under primarily pathophysiological conditions (Fredholm *et al.*, 2001) and have a low affinity for caffeine, respectively (Fredholm *et al.*, 2001; Solinas *et al.*, 2005).

Interesting in the present study is the finding that a lower dose of neuroleptic failed to reveal a preference for 15 mg/kg caffeine in A_{2A}KO mice, but antagonized the aversive effects of caffeine in C57BL/6 mice and revealed caffeine preferences in these animals (Figure 3B). Adenosine A_{2A} receptors and dopamine D₂ receptors interact antagonistically in striatal medium spiny neurons at a direct level on the cell surface, and also indirectly at the level of second messengers (Ferre *et al.*, 1991; Ferre *et al.*, 1993; Chen *et al.*, 2001; Herve *et al.*, 2001; Schiffmann *et al.*, 1991). This suggests that when the A_{2A} receptor is absent, dopaminergic signaling may be modified. Indeed previous studies have shown that in A_{2A}KO mice dopamine D₂ receptor mRNA is increased and binding is altered (Short *et al.*, 2006) which could lead to an increase in caffeine's aversive effects. In agreement with this idea, our lower dose of neuroleptic failed to completely block the aversive effects of 15 mg/kg caffeine, while at a higher dose of neuroleptic the aversion to caffeine was blocked and preferences equivalent to those in C57BL/6 mice were revealed.

The current locomotor data in A_{2A}KO mice offers an additional behavioral correlate of this antagonistic interaction between A_{2A} and D₂ receptors. Both C57BL/6 mice and A_{2A}KO mice showed locomotor depression when treated with a neuroleptic (Figure 3D). When given caffeine, C57BL/6 mice showed a partial rescue of locomotor activity while A_{2A}KO mice did not. Similar to the opposing roles the A_{2A} and D₂ receptor exert on caffeine's rewarding and aversive effects, antagonism of the dopaminergic system decreases caffeine's locomotor effects, while antagonism of the A_{2A} receptor stimulates locomotion. The result is

the complete absence of caffeine-induced locomotor stimulation when the dopaminergic system is inactive and the A_{2A} receptor cannot be antagonized, as in A_{2A}KO mice treated with neuroleptic.

Also interesting is the fact that D₂KO mice showed locomotor stimulation to caffeine equivalent to what was seen in C57Bl/6 animals. This indicates first that the D₂ receptor is not critical for caffeine-induced locomotor stimulation, and additionally that heteromers of the A_{2A} and D₂ receptors, which have different properties than either receptor in isolation (Ferre, 2008), are also not required. A_{2A} receptors and pathways downstream of them seem to be most important for the locomotor effects of caffeine.

Our findings provide new information about the mechanism of action of caffeine, the most widely consumed psychoactive substance in the world (Nehlig, 1999). The present results indicate that caffeine has an atypical reward mechanism in that its rewarding effects are independent of dopamine and the TPP. Further, we have shown that A_{2A}KO and A₁KO mice can display conditioned place aversions and dopamine-blockade induced preferences to caffeine, ruling out each of these receptor as the substrate upstream of caffeine's appetitive and aversive motivational effects. The difference between caffeine's reward mechanism and that of other recreational drugs may underlie its weaker addictive properties. Further characterizing caffeine's mechanism of reward may not only lead to the elucidation of novel reward mechanisms, but also help to focus in on the actions of classical drugs of abuse that are critical for inducing addictive behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Acquas E, Carboni E, Leone P, Di Chiara G. SCH23390 blocks drug-conditioned place-preference and place-aversion: anhedonia (lack of reward) or apathy (lack of motivation) after dopamine-receptor blockade? *Psychopharmacology*. 1989; 99:151–155. [PubMed: 2572027]
- Acquas E, Di Chiara G. D1 receptor blockade stereospecifically impairs acquisition of drug-conditioned place preference and aversion. *Behav. Pharmacol.* 1994; 5:555–569. [PubMed: 11224235]
- Andersen PH. Comparison of the pharmacological characteristics of [3H]raclopride and [3H]SCH 23390 binding to dopamine receptors in vivo in mouse brain. *Eur. J. Pharmacol.* 1988; 146:113–120. [PubMed: 2895008]
- Bechara A, van der Kooy D. The tegmental pedunculopontine nucleus – a brain-stem output of the limbic system critical for the conditioned place preferences produced by morphine and amphetamine. *J. Neurosci.* 1989; 9:3400–3409. [PubMed: 2795130]
- Bedingfield JB, King DA, Holloway FA. Cocaine and caffeine: conditioned place preference, locomotor activity, and additivity. *Pharmacol. Biochem. Be.* 1998; 61:291–296.
- Bozarth M, Wise R. Intra-cranical self-administration of morphine into the ventral tegmental area in rats. *Life Sci.* 1981; 28:551–555. [PubMed: 7207031]
- Brockwell N, Beninger R. The differential role of A1 and A2 adenosine receptor subtypes in locomotor activity and place conditioning in rats. *Behav. Pharmacol.* 1996; 7:373–383. [PubMed: 11224431]

- Brockwell NT, Eikelboom R, Beninger RJ. Caffeine-induced place and taste conditioning: production of dose-dependent preference and aversion. *Pharmacol. Biochem. Be.* 1991; 38:513–517.
- Chen JF, Beilstein M, Xu YH, Turner TJ, Moratalla R, Standaert DG, Aloyo VJ, Fink JS, Schwarzschild MA. Selective attenuation of psychostimulant-induced behavioral responses in mice lacking A(2A) adenosine receptors. *Neuroscience.* 2000; 97:195–204. [PubMed: 10771351]
- Chen JF, Huang ZH, Ma JY, Zhu JM, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA. A2A adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J. Neurosci.* 1999; 19:9192–9200. [PubMed: 10531422]
- Chen JF, Moratalla R, Impagnatiello F, Grandy DK, Cuellar B, Rubinstein M, Beilstein MA, Hackett E, Fink JS, Low MJ, Ongini E, Schwarzschild MA. The role of the D2 dopamine receptor (D2R) in A(2A) adenosine receptor (A(2A)R)-mediated behavioral and cellular responses as revealed by A(2A) and D-2 receptor knockout mice. *Proc. Nat. Acad. Sci. USA.* 2001; 98:1970–1975. [PubMed: 11172060]
- Clements J, Grant S. Glutamate-like immunoreactivity in neurons of the laterodorsal tegmental and pedunculopontine nuclei in the rat. *Neurosci. Lett.* 1990; 120:70–73. [PubMed: 2293096]
- Creese I, Burt D, Snyder S. Dopamine receptor-binding predicts clinical and pharmacological potencies of anti-schizophrenic drugs. *Science.* 1976; 92:481–483. [PubMed: 3854]
- Daly J, Fredholm B. Caffeine - an atypical drug of dependence. *Drug Alcohol Depen.* 1998; 51:199–206.
- Di Scala G, Sandner G. Conditioned place aversion produced by FG 7142 is attenuated by haloperidol. *Psychopharmacology.* 1989; 99:176–180. [PubMed: 2508151]
- El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois J. Reduced appetite for caffeine in adenosine A(2A) receptor knockout mice. *Eur. J. Pharmacol.* 2005; 519:290–291. [PubMed: 16129427]
- Ferre S, Voneuler G, Johansson B, Fredholm B, Fuxe K. Stimulation of high-affinity adenosine-A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proc. Nat. Acad. Sci., USA.* 1991; 88:7238–7241. [PubMed: 1678519]
- Ferre S, Snaprud P, Fuxe K. Opposing actions of an adenosine-A2 receptor agonist and a GTP analog on the regulation of dopamine-D2 receptors in rat neostriatal membranes. *Eur. J. Pharmacol.* 1993; 244:311–315. [PubMed: 8458403]
- Ferre S, Popoli P, Gimenezllo L, Finnman UB, Martinez E, Decarolis AS, Fuxe K. Postsynaptic antagonistic interaction between adenosine A(1) and dopamine D-1 receptors. *NeuroReport.* 1994; 6:73–76. [PubMed: 7703433]
- Ferre S. An update on the mechanisms of the psychostimulant effects of caffeine. *J. Neurochem.* 2008; 105:1067–1079. [PubMed: 18088379]
- Florio C, Rosati A, Traversa U, Vertua R. Inhibitory and excitatory effects of adenosine antagonists on spontaneous locomotor activity in mice. *Life Sci.* 1997; 60:1477–1486. [PubMed: 9126868]
- Ford B, Holmes C, Mainville L, Jones B. GABAergic neurons in the rat pontomesencephalic tegmentum - codistribution with cholinergic and other tegmental neurons projecting to the posterior lateral hypothalamus. *J. Comp. Neurol.* 1995; 363:177–196. [PubMed: 8642069]
- Frary CD, Johnson RK, Wang MQ. Food sources and intakes of caffeine in the diets of persons in the United States. *J. Am. Diet. Assoc.* 2005; 105:110–113. [PubMed: 15635355]
- Fredholm BB. Are methylxanthine effects due to antagonism of endogenous adenosine? *Trends Pharmacol. Sci.* 1980; 1:129–132.
- Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.* 1999; 51:83–133. [PubMed: 10049999]
- Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.* 2001; 53:527–552. [PubMed: 11734617]
- Fuxe K, Ferré S, Genedani S, Franco R, Agnati LF. Adenosine receptor-dopamine receptor interactions in the basal ganglia and their relevance for brain function. *Physiol. Behav.* 2007; 92:210–217. [PubMed: 17572452]

- Griebel G, Saffroysspittler M, Misslin R, Remmy D, Vogel E, Bourguignon JJ. Comparison of the behavioural effects of an adenosine A1/A2-receptor antagonist, CGS 15943A, and an A1-selective antagonist, DPCPX. *Psychopharmacology*. 1991; 103:541–544. [PubMed: 2062988]
- Griffiths RR, Woodson PP. Reinforcing effects of caffeine in humans. *J. Pharmacol. Exp. Ther.* 1988; 246:21–28. [PubMed: 3392652]
- Hervé D, Le Moine C, Corvol JC, Belluscio L, Ledent C, Fienberg AA, Jaber M, Studler JM, Girault JA. G alpha(olf) levels are regulated by receptor usage and control dopamine and adenosine action in the striatum. *J. Neurosci.* 2001; 21:4390–4399. [PubMed: 11404425]
- Hnasko TS, Sotak BN, Palmiter RD. Cocaine-conditioned place preference by dopamine-deficient mice is mediated by serotonin. *J. Neurosci.* 2007; 24:12484–12488. [PubMed: 18003826]
- Hof, PR.; Young, WG.; Bloom, FE.; Belichenko, PV.; Celio, MR. *Comparative Cytoarchitectonic Atlas of the C57Bl/6 and 129/Sv Mouse Brains*. Elsevier Science; San Diego, CA: 2000.
- Hughes J, Oliveto A. A systematic survey of caffeine intake in vermont. *Exp. Clin. Psychopharm.* 1997; 5:393–398.
- James JE, Rogers PJ. Effects of caffeine on performance and mood: withdrawal reversal is the most plausible explanation. *Psychopharmacology*. 2005; 182:1–8. [PubMed: 16001109]
- Johansson B, Halldner L, Dunwiddie TV, Masino SA, Poelchen W, Giménez-Llort L, Escorihuela RM, Fernandez-Teruel A, Wiesenfeld-Hallin Z, Xu XJ, Hardemark A, Betscholtz C, Herlenius E, Fredholm BB. Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. *Proc. Nat. Acad. Sci. USA*. 2001; 98:9407–9412. [PubMed: 11470917]
- Kaplan GB, Greenblatt DJ, Ehrenberg BL, Goddard JE, Cotrea MM, Harmatz JS, Shader RI. Dose-dependent pharmacokinetics and psychomotor effects of caffeine in humans. *J. Clin. Pharmacol.* 1997; 37:693–703. [PubMed: 9378841]
- Kelley AE, Berridge KC. The neuroscience of natural rewards: relevance to addictive drugs. *J. Neurosci.* 2002; 22:3306–3311. [PubMed: 11978804]
- Kelly MA, Rubinstein M, Asa SL, Zhang G, Saez C, Bunzow JR, Allen RG, Hnasko R, Ben-Jonathan N, Grandy DK, Low MJ. Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. *Neuron*. 1997; 19:103–113. [PubMed: 9247267]
- Kippin T, van der Kooy D. Excitotoxic lesions of the tegmental pedunculopontine nucleus impair copulation in naive male rats and block the rewarding effects of copulation in experienced male rats. *Eur. J. Neurosci.* 2003; 18:2581–2591. [PubMed: 14622159]
- Kuzmin A, Johansson B, Gimenez L, Ogren S, Fredholm B. Combination of adenosine A(1) and A(2A) receptor blocking agents induces caffeine-like locomotor stimulation in mice. *Eur. Neuropsychopharm.* 2006; 16:129–136.
- Lavin A, Nogueira L, Lapish CC, Wightman RM, Phillips PEM, Seamans JK. Mesocortical dopamine neurons operate in distinct temporal domains using multimodal signaling. *J. Neurosci.* 2005; 25:5013–5023. [PubMed: 15901782]
- Laviolette S, Alexson T, van der Kooy D. Lesions of the tegmental pedunculopontine nucleus block the rewarding effects and reveal the aversive effects of nicotine in the ventral tegmental area. *J. Neurosci.* 2002; 22:8653–8660. [PubMed: 12351739]
- Laviolette S, van der Kooy D. Blockade of mesolimbic dopamine transmission dramatically increases sensitivity to the rewarding effects of nicotine in the ventral tegmental area. *Mol. Psychiatr.* 2003; 8:50–59.
- Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen J-J, Costentin J, Heath JK, Vassart G, Parmentier M. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A(2A) receptor. *Nature*. 1997; 388:674–678. [PubMed: 9262401]
- Lepore M, Franklin KBJ. N-methyl-D-aspartate lesions of the pedunculopontine nucleus block acquisition and impair maintenance of responding reinforced with brain stimulation. *Neuroscience*. 1993; 71:147–155. [PubMed: 8407141]
- McPherson PS, Kim YK, Valdivia H, Knudson CM, Takekura H, Franzini-Armstrong C, Coronado R, Campbell KP. The brain ryanodine receptor: a caffeine-sensitive calcium release channel. *Neuron*. 1991; 7:17–25. [PubMed: 1648939]

- Myers KP, Izbicki EV. Reinforcing and aversive effects of caffeine measured by flavor preference conditioning in caffeine-naïve and caffeine acclimated rats. *Physiol. Behav.* 2006; 88:585–596. [PubMed: 16806323]
- Nehlig A. Are we dependent upon coffee and caffeine? A review on human and animal data. *Neurosci. Biobehav. R.* 1999; 23:563–576.
- Nehlig A, Lucignani G, Kadekaro M, Porrino LJ, Sokoloff L. Effects of acute administration of caffeine on local cerebral glucose utilization in the rat. *Eur. J. Pharmacol.* 1984; 101:91–100. [PubMed: 6086367]
- Parker JL, van der Kooy D. Tegmental pedunculo-pontine nucleus lesions do not block cocaine reward. *Pharmacol. Biochem. Behav.* 1995; 52:77–83. [PubMed: 7501683]
- Patkina NA, Zvartau EE. Caffeine place conditioning in rats: comparison with cocaine and ethanol. *Eur. Neuropsychopharm.* 1998; 8:287–291.
- Quarta D, Borycz J, Solinas M, Patkar K, Hockemeyer J, Ciruela F, Lluís C, Franco R, Woods AS, Goldberg SR, Ferre S. Adenosine receptor-mediated modulation of dopamine release in the nucleus accumbens depends on glutamate neurotransmission and N-methyl-D-aspartate receptor stimulation. *J. Neurochem.* 2004; 91:873–880. [PubMed: 15525341]
- Salamone JD. The involvement of nucleus accumbens dopamine in appetitive and aversive motivation. *Behav. Brain Res.* 1994; 61:117–133. [PubMed: 8037860]
- Santi AN, Parker LA. The dopamine antagonist, alpha-flupenthixol, interferes with naloxone-induced place aversion learning, but not with acute opiate dependence in rats. *Pharmacol. Biochem. Behav.* 2001; 70:193–197. [PubMed: 11701188]
- Schiffmann S, Jacobs O, Vanderhaeghen J. Striatal restricted adenosine-A2 receptor (Rdc8) is expressed by enkephalin but not by substance-P neurons - an in situ hybridization histochemistry study. *J. Neurochem.* 1991; 57:1062–1067. [PubMed: 1713612]
- Schultz W. Getting formal with dopamine and reward. *Neuron.* 2002; 36:241–263. [PubMed: 12383780]
- Short JL, Ledent C, Borrelli E, Drago J, Lawrence AJ. Genetic interdependence of adenosine and dopamine receptors: evidence from receptor knockout mice. *Neuroscience.* 2006; 139:661–670. [PubMed: 16476524]
- Smith JW, Fetsko LA, Xu R, Wang Y. Dopamine D2L receptor knockout mice display deficits in positive and negative reinforcing properties of morphine and in avoidance learning. *Neuroscience.* 2002; 113:755–765. [PubMed: 12182883]
- Solinas M, Ferré S, Antoniou K, Quarta D, Justinova Z, Hockemeyer J, Pappas LA, Segal PN, Wertheim C, Muller CE, Goldberg SR. Involvement of adenosine A1 receptors in the discriminative-stimulus effects of caffeine in rats. *Psychopharmacology.* 2005; 179:576–586. [PubMed: 15696333]
- Smellie FW, Davis CW, Daly JW, Wells JN. Alkylxanthines: inhibition of adenosine-elicited accumulation of cyclic AMP in brain slices and of brain phosphodiesterase activity. *Life Sci.* 1979; 24:2475–2482. [PubMed: 225626]
- Snyder SH. Adenosine as a neuromodulator. *Ann. Rev. Neurosci.* 1985; 8:103–124. [PubMed: 2858998]
- Spyraki C, Fibiger HC, Phillips AG. Cocaine-induced place preference conditioning: lack of effects of neuroleptics and 6-hydroxydopamine lesions. *Brain Res.* 1982; 253:195–203. [PubMed: 6817851]
- Stefurak TL, van der Kooy D. Tegmental pedunculo-pontine lesions in rats decrease saccharin's rewarding effects but not its memory-improving effect. *Behav. Neurosci.* 1994; 108:972–980. [PubMed: 7826519]
- Tao S, Abdel-Rahman AA. Neuronal and cardiovascular responses to adenosine microinjection into the nucleus tractus solitarius. *Brain Res. Bull.* 1993; 32:407–417. [PubMed: 8106126]
- Ting-A-Kee R, Dockstader C, Heinmiller A, Grieder T, van der Kooy D. GABA(A) receptors mediate the opposing roles of dopamine and the tegmental pedunculo-pontine nucleus in the motivational effects of ethanol. *Eur. J. Neurosci.* 2009; 29:1235–1244. [PubMed: 19302158]
- Tinley EM, Durlach PJ, Yeomans MR. How habitual caffeine consumption and dose influence flavour preference conditioning with caffeine. *Physiol. Behav.* 2004; 82:317–324. [PubMed: 15276794]

- Tzschentke TM. Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addict. Biol.* 2007; 12:227–462. [PubMed: 17678505]
- Ukena D, Jacobson KA, Padgett WL, Ayala C, Shamim MT, Kirk KL, Olsson RO, Daly JW. Analogs of caffeine antagonists with selectivity for A2 adenosine receptors. *Life Sci.* 1986a; 39:743–750. [PubMed: 3016449]
- Ukena D, Jacobson KA, Padgett WL, Ayala C, Shamim MT, Kirk KL, Olsson RO, Daly J. Species differences in structure-activity relationships of adenosine agonists and xanthine antagonists at brain A1 adenosine receptors. *FEBS Lett.* 1986b; 209:122–128. [PubMed: 3803571]
- Wise R. Dopamine, learning and motivation. *Nat. Rev. Neurosci.* 2004; 5:483–494. [PubMed: 15152198]
- Yang JN, Bjorklund O, Lindstrom-Tornqvist K, Lindgren E, Eriksson TM, Kahlstrom J, Chen JF, Schwarzschild MA, Tobler I, Fredholm BB. Mice heterozygous for both A1 and A2A adenosine receptor genes show similarities to mice given long-term caffeine. *J. Appl. Physiol.* 2009; 106:631–639. [PubMed: 19036889]

Figure 1.

Caffeine has dopamine independent rewarding effects and dopamine-dependent aversive effects that are mediated in the central nervous system.

(A) Non-pretreated mice showed significant aversions to caffeine at doses of 10, 15, 30, and 100 mg/kg, respectively. Animals pretreated with 0.8 mg/kg alpha-flupentixol showed significant preferences for caffeine at doses of 3, 10, 15, and 30 mg/kg. Open markers represent non-pretreated animals and filled markers represent alpha-flupentixol pretreated groups. Data points represent mean difference scores $\pm$ S.E.M. * indicates $P < 0.05$ for comparison of difference scores to zero.

(B) Caffeine's aversive effects are centrally-mediated. C57BL/6 mice treated i.p. with 8-SPT, a caffeine analogue unable to cross the blood-brain barrier, did not show conditioned place aversions to the drug. Bars represent the mean amount of time spent in the drug-paired and the saline-paired environments on test day $\pm$ S.E.M. NS indicates $P > 0.05$.

Figure 2.

Caffeine's dopamine dependent aversive depends on the dopamine D₂ and D₁ receptor, however the D₂ receptor is not involved in the locomotor stimulant effect of caffeine.

(A) C57BL/6 mice conditioned with 10 mg/kg caffeine showed significant aversions while D₁KO mice and D₂KO mice did not. D₂KO mice demonstrated preferences for this dose of caffeine, while D₁KO did not show an aversion or a preference. Bars represent mean difference scores $\pm$ S.E.M. * indicates $P < 0.05$ for comparison of difference scores to zero that were corrected for multiple t tests.

(B) The dopamine D₁ receptor does not mediate the rewarding effects of caffeine. D₂KO mice show a conditioned place preference to 10 mg/kg caffeine, which their wild type littermates find aversive. When D₂KO mice were pretreated with the dopamine D₁ receptor antagonist SCH23390 their preferences to caffeine did not diminish, indicating the D₁ receptor is not the substrate of caffeine reward. Bars represent mean difference scores and error bars are SEM. NS indicates no main effect of pretreatment ($P > 0.05$) and * indicates a main effect of genotype ($P < 0.05$).

(C) A non-dopaminergic substrate mediates the aversive effects of 100 mg/kg caffeine. D₁KO mice and D₂KO mice show conditioned place aversions equivalent to those seen in wild type littermates when given 100 mg/kg caffeine despite the fact that D₁KO mice did not show a preference or an aversion and D₂KO mice show conditioned place preferences to 10 mg/kg caffeine. Bars represent mean difference scores and error bars are SEM. NS indicates no main effect of genotype on difference scores.

(D) C57BL/6, D₁KO and D₂KO mice all showed equivalent locomotor stimulation after 10 mg/kg i.p. caffeine. Locomotor activity in D₂KO mice was significantly lower than locomotor activity in D₁KO mice. Bars represent mean locomotor counts for saline and caffeine trials $\pm$ S.E.M. * indicates a significant difference between activity of D₁KO and D₂KO mice ($P < 0.05$).

Figure 3.

The adenosine A_{2A} receptor is not directly involved in the aversive or rewarding effects of caffeine but is critical for caffeine's locomotor stimulant effects.

(A) Both C57BL/6 and A_{2A}KO mice showed similar aversions to 10 mg/kg i.p. caffeine. Pretreating C57BL/6 and A_{2A}KO mice with 2.0mg/kg alpha-flupenthixol blocked the aversive effects of caffeine and revealed preferences in both genotypes. Bars represent mean difference scores $\pm$ S.E.M. Non-pretreated and pretreated A_{2A}KO groups did not differ from their respective C57BL/6 control groups.

(B) Both C57BL/6 and A_{2A}KO mice showed similar aversions to 15 mg/kg i.p. caffeine. Pretreating C57BL/6, but not A_{2A}KO mice, with 0.8 mg/kg alpha-flupenthixol blocked the aversive effects of caffeine and revealed a preference for caffeine. A_{2A}KO mice pretreated with 0.8 mg/kg alpha-flupenthixol showed an aversion to caffeine that differed from the response seen in pretreated C57BL/6 mice. Bars represent mean difference scores $\pm$ S.E.M. * indicates $P < 0.05$ for comparison of neuroleptic-pretreated C57BL/6 mice to pretreated A_{2A}KO mice.

(C) C57BL/6 but not A_{2A}KO mice showed locomotor stimulation when administered 10 mg/kg caffeine i.p., however both genotypes showed a locomotor response to the 15 mg/kg dose of caffeine. There was a significant effect of drug treatment and genotype (both $P < 0.05$). Bars represent mean locomotor counts for saline and caffeine trials $\pm$ S.E.M. stats

(D) Caffeine partially counteracts the locomotor depression induced by alpha-flupenthixol in C57BL/6 but not A_{2A}KO mice. Both C57BL/6 mice and A_{2A}KO mice experienced locomotor depression relative to baseline (dotted line) when treated with 0.8 mg/kg alpha-flupenthixol. This depression was partially counteracted by treatment with 15 mg/kg caffeine in C57BL/6 but not A_{2A}KO mice. Bars represent locomotor scores when mice were given caffeine or saline after pretreatment with alpha-flupenthixol as a percentage of locomotor scores in the absence of alpha-flupenthixol. The dotted line indicates baseline locomotor activity of each genotype in the absence of neuroleptic.

Figure 4.

The adenosine A₁ receptor is not critical for the stimulant effects of caffeine and is not involved in the aversive or rewarding effects of caffeine either alone or in combination with the A_{2A} receptor.

(A) A₁KO mice showed aversions to 10 mg/kg caffeine that did not differ from those seen in wild type littermates. Knockout mice also showed preferences to 10 mg/kg caffeine when pretreated with 0.8 mg/kg alpha-flupenthixol and preferences did not differ from those seen in wild type littermates. Bars represent mean difference scores $\pm$ S.E.M.

(B) A₁KO mice show locomotor stimulation in response to caffeine equivalent to locomotor stimulation seen in wild type littermates. Bars represent mean locomotor counts $\pm$ S.E.M. * indicates a significant difference between saline and caffeine locomotor scores in A₁KO mice ($P < 0.05$) but not in wild type littermates ($P > 0.05$).

(C) dKO mice show aversions to 10 mg/kg caffeine and preferences to 10 mg/kg caffeine when pretreated with 0.8 mg/kg alpha-flupenthixol that do not differ significantly from those seen in C57BL/6 mice. Bars represent mean difference scores $\pm$ S.E.M.

Figure 5.

Lesions of the tegmental pedunculopontine nucleus in mice do not affect aversions or dopamine blockade-induced conditioned place preferences to i.p. caffeine.

(A) Both sham TPP-lesioned and TPP-lesioned mice showed aversions to 10 mg/kg i.p. caffeine. Pretreating sham TPP-lesioned and TPP-lesioned mice blocked the aversive effects of 10 mg/kg i.p. caffeine in both groups and revealed a significant preference. Bars represent mean difference scores $\pm$ S.E.M. Aversions and preferences of lesioned groups did not differ significantly from sham-lesioned controls.

(B) Coronal section of the brain; the grey rectangle indicates the region depicted in photographs and dotted lines indicate the approximate location of the tegmental pedunculopontine nucleus. Photographs on the right depict a representative bilateral sham TPP lesion (top) and an excitotoxic TPP lesion (bottom). Arrows indicate gliosis in the region of the TPP.