EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

*European Journal of Neuroscience, Vol. 32, pp. 143–154, 2010* doi:10.1111/j.1460-9568.2010.07247.x

BEHAVIORAL NEUROSCIENCE

# Adenosine $A_1$ and $A_{2A}$ receptors are not upstream of caffeine's dopamine $D_2$ receptor-dependent aversive effects and dopamine-independent rewarding effects

Jessica E. Sturgess,[1] Ryan A. Ting-A-Kee,[2] Dominik Podbielski,[3] Laurie H. L. Sellings,[1] Jiang-Fan Chen[4] and Derek van der Kooy[1,2]

[1]Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada
[2]Institute of Medical Science, University of Toronto, Toronto, ON, Canada
[3]Department of Ophthalmology and Vision Sciences, University of Toronto, Toronto, ON, Canada
[4]Department of Neurology, Boston University School of Medicine, Boston, MA, USA

*Keywords*: 8-SPT, α-flupenthixol, locomotor, mice, SCH23390

## Abstract

Caffeine is widely consumed throughout the world, but little is known about the mechanisms underlying its rewarding and aversive properties. We show that pharmacological antagonism of dopamine not only blocks conditioned place aversion to caffeine, but also reveals dopamine blockade-induced conditioned place preferences. These aversive effects are mediated by the dopamine $D_2$ receptor, as knockout mice showed conditioned place preferences in response to doses of caffeine that C57Bl/6 mice found aversive. Furthermore, these aversive responses appear to be centrally mediated, as a quaternary analog of caffeine failed to produce conditioned place aversion. Although 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 aversive responses to caffeine than did C57Bl/6 mice, which partially obscured the dopamine-independent and $A_{2A}$ receptor-independent preferences. Additionally, the $A_1$ 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. These data provide 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.

## Introduction

Caffeine is the most widely consumed psychoactive substance in the world (Nehlig, 1999; Ferre, 2008). Eighty to ninety per cent of US 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 500 mg reportedly produce a sense of well-being, wakefulness, and concentration (Kaplan *et al.*, 1997), whereas higher doses, above 500 mg, can induce anxiety, irritability, and agitation (Nehlig *et al.*, 1992). Similar findings have been made in rats, with low and high doses producing mild conditioned place preferences and robust aversive effects, respectively (Brockwell *et al.*, 1991; Bedingfield *et al.*, 1998; Patkina & Zvartau, 1998).

*Correspondence*: Jessica E. Sturgess, as above.
E-mail: jessica.sturgess@utoronto.ca

*Received 4 November 2009, revised 8 March 2010, accepted 30 March 2010*

The mesolimbic dopamine system, consisting of the dopaminergic projections from the ventral tegmental area to the nucleus accumbens (NAc), has a long history in the study of reward (Wise, 2004). This system is involved in reward processing (Bozarth & Wise, 1981; Kelley & Berridge, 2002), and learning about aversive stimuli, including nicotine (Salamone, 1994; Pezze *et al.*, 2001; Laviolette & van der Kooy, 2003). This neurotransmitter system may mediate caffeine's rewarding, or aversive, effects.

The tegmental pedunculopontine 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 $A_1$ and $A_{2A}$ receptors for caffeine and their tonic activity at physiological levels of adenosine (Fredholm *et al.*, 1999) suggest that these are caffeine's targets (Daly & Fredholm, 1998). Linking caffeine to the dopaminergic system, within the striatum dopamine $D_2$ receptors interact antagonistically with $A_{2A}$ receptors as heteromers (Ferre *et al.*, 1991, 1993, 1994), and this may also be true for $A_1$ and $D_1$ receptors (Fuxe *et al.*, 2007).

It is currently unclear whether the adenosine $A_1$ 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 mice (El Yacoubi *et al.*, 2005). However, mice receiving an $A_1$ antagonist equated this with the experience of caffeine (Quarta *et al.*, 2004), and $A_1$ 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's rewarding and aversive effects.

## Materials and methods

### Drugs

The drugs used in these experiments – caffeine, α-flupenthixol, 8-(*p*-sulfophenyl)theophylline (8-SPT), and *N*-methyl-D-aspartic acid (NMDA) – were obtained from Sigma (Sigma-Aldrich, Oakville, Ontario, Canada). Caffeine, α-flupenthixol, SCH23390 and 8-SPT were dissolved in 0.9% saline and injected intraperitoneally in a volume of 1 mL/kg body weight, except for α-flupenthixol, which was injected in a volume of 10 mL/kg body weight. This neuroleptic was chosen because it does not produce preferences or aversive effects of its own at this dose (Laviolette & van der Kooy, 2003; Dockstader, C., unpublished data) and it is known to antagonize both dopamine $D_1$ and $D_2$ receptors approximately equally (Creese *et al.*, 1976; Andersen, 1988). NMDA was dissolved in phosphate-buffered saline 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 g 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-h light (07:00–19:00 h)/dark cycle. Dopamine $D_1$ receptor knockout ($D_1$KO) mice and $D_2$ receptor knockout ($D_2$KO) mice were generated previously (Kelly *et al.*, 1997) and backcrossed to the C57Bl/6 background 12 times. $D_1$KO and $D_2$KO mice were between 25 and 40 g at the start of experimentation; both male and female mice were used, and analyses of male and female responses indicated that 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 g at the start of experimentation. Adenosine $A_1$ receptor knockout ($A_1$KO) mice were generated as described previously (Johansson *et al.*, 2001) and backcrossed to the C57Bl/6 background six times, according to the Jackson Laboratory 'speed congenic procedure'. Adenosine $A_1$ 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 M. Schwarzschild's laboratory in Boston (MA, USA). 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 (Faculty Advisory Committee on Animal Services, University of Toronto).

### Place conditioning

All mice were conditioned using a standard place conditioning procedure. The conditioning apparatus consisted of a black-walled chamber (15 × 15 × 15 cm) with a smooth, black Plexiglas floor and a white-walled chamber (15 × 15 × 15 cm) with a bumpy, white floor separated by a removable partition and with a clear Plexiglas top. Immediately prior to the placement of a mouse in the black environment, 0.1 mL of 3% acetic acid was wiped on the floor until dry. Mice received four 15-min pairings of the drug with one environment and four 15-min 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 α-flupenthixol trials, mice were pretreated with an intraperitoneal injection of either 0.8 mg/kg or 2.0 mg/kg α-flupenthixol 1 h 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 min prior to conditioning, and mice were 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 decision to enter one side of the apparatus or the other. Mice were allowed to move freely between the compartments for 10 min, and the time spent in each environment was recorded by the disruption of photobeams in the compartments. Testing took place 1 day after the final conditioning trial to ensure that the mice were in a drug-free state. In order to minimize the number of mice used, the balance of the boxes was tested intermittently (Fig. S1). This was done by giving mice eight saline injections (four in each environment) over eight consecutive days, and then applying a 10-min test to ensure that animals had no preference for either environment. The data from these saline trials were used as control data for experimental trials conducted during approximately the same time period.

### Experimental groups

Mice were conditioned with seven different doses of caffeine with and without α-flupenthixol pretreatment; each mouse 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 α-flupenthixol pretreatment. The doses of caffeine used were 0.01, 0.1, 1, 3, 10, 15, 30 and 100 mg/kg. For all experiments, including those with knockout mice, experimental groups contained between eight and 30 animals to ensure that there was sufficient power.

### Locomotor measurements

Locomotor activity was measured using six photobeams present in the conditioning apparatus: three in the black-walled chamber and three 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-min drug and vehicle conditioning session of each mouse. The apparatus recorded 'wasted time' when no photobeams were being disrupted, which corresponded to when a mouse was not entirely in one environment.

### *Surgical procedures*

Mice were anesthetized with isofluorane (5% to induce, 1–2% for maintenance), and placed in a stereotaxic apparatus. Lesions of the TPP were performed bilaterally (stereotaxic coordinates: anteroposterior −4.4 mm, lateral ±1.1 mm and ventral –3.8 mm from bregma and the dura surface) by manually injecting 0.04 $\mu$L of 0.1 M NMDA solution, or the phosphate-buffered saline vehicle for sham operations, using a 1-$\mu$L Hamilton microsyringe (Reno, NV, USA). The infusion rate was 0.01 $\mu$L/min, and the syringe was left in place for 1 min 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 the experiments, animals that had received TPP sham or NMDA lesions were deeply anesthetized with 54.7 mg/100 mL sodium pentobarbital in a dose of 0.1 mL per 30 g 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 h in a 25% sucrose/4% formaldehyde post-fixative. Brains were then flash frozen at −80°C, sliced in a freezing microtome into 40-$\mu$m-thick sections, and mounted on gelatin-coated slides. TPP and sham lesions were verified with cresyl violet staining and light microscopy (see Fig. 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*

ANOVAs were conducted using SPSS (SPSS, IBM, Chicago, Illinois, USA) 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 significance were adjusted using a standard Bonferroni correction to control for increased risk of false positives. Error bars on figure means are standard errors of the mean.

## Results

### *Caffeine's aversive effects 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 (Fig. 1A). At low doses, caffeine did not produce place preferences or aversive effects, whereas at doses of 10 mg/kg or above, it produced conditioned place aversion. When mice were pretreated with the dopaminergic antagonist α-flupenthixol before conditioning with caffeine, the aversive responses to higher doses of caffeine were blocked and conditioned place preferences to the drug were revealed.

A two-way ANOVA indicated a significant dose × pretreatment interaction, confirming our observation that α-flupenthixol disrupted the higher-dose aversive effects of 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 aversion 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 ($t_{29} = 10.18$, $P < 0.004$) caffeine. However, at these same doses, when mice were pretreated with the dopaminergic antagonist α-flupenthixol, the aversive effects of caffeine were blocked, and significant preferences were revealed at 10 mg/kg ($t_{30} = 5.24$, $P < 0.004$) and 15 mg/kg ($t_7 = 4.64$, $P < 0.004$) caffeine, and a trend towards a preference was seen at 30 mg/kg caffeine ($t_{14} = 3.43$, $P = 0.004$). Mice conditioned with 3 mg/kg caffeine showed signif-

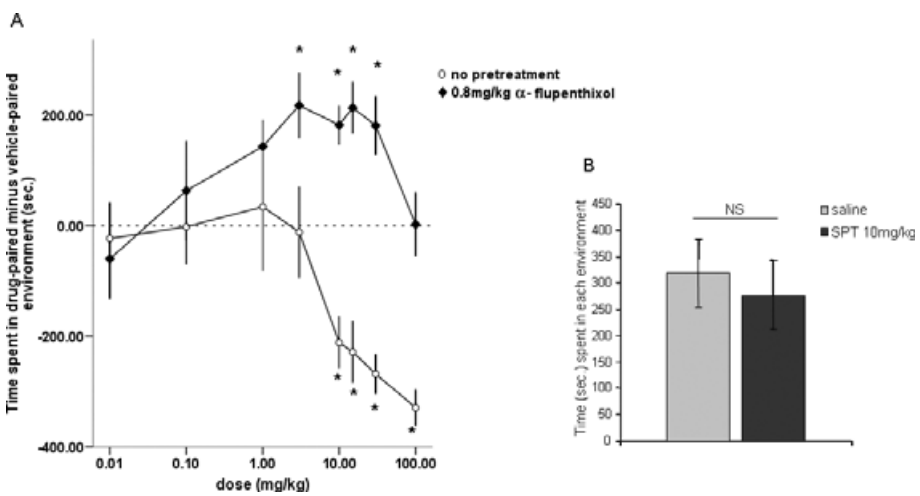


FIG. 1. Caffeine has dopamine-independent rewarding effects and dopamine-dependent aversive effects that are mediated in the central nervous system. (A) Non-pretreated mice showed significant aversive responses to caffeine at doses of 10, 15, 30 and 100 mg/kg, respectively. Animals pretreated with 0.8 mg/kg α-flupenthixol showed significant preferences for caffeine at doses of 3, 10, 15 and 30 mg/kg. Open symbols represent non-pretreated animals, and filled symbols represent α-flupenthixol-pretreated groups. Data points represent mean difference scores ± standard errors of the mean (SEMs). *$P < 0.05$ for comparison of difference scores with zero. (B) Caffeine's aversive effects are centrally mediated. C57BL/6 mice treated intraperitoneally with 8-(*p*-sulfophenyl)theophylline (8-SPT), a caffeine analog that is unable to cross the blood–brain barrier, did not show conditioned place aversion to the drug. Bars represent the mean amounts of time spent in the drug-paired and the saline-paired environments on the test day ± SEMs. NS, not significant ($P > 0.05$).

icant preferences when pretreated with α-flupenthixol ($t_{15} = 3.72$, $P < 0.004$) but not when unpretreated ($t_{15} = 0.141$, $P > 0.004$). In mice conditioned with 100 mg/kg caffeine, pretreatment with the dopaminergic antagonist blocked the aversive effects of caffeine, but no significant preferences were revealed ($t_{27} = 0.042$, $P > 0.004$).

To control for the possibility that the stress of receiving an injection 1 h prior to conditioning in α-flupenthixol trials affected the preferences and aversions to caffeine during conditioning, a group of mice was pretreated with saline 1 h prior to conditioning with 10 mg/kg caffeine (Fig. S2). Mice showed aversive responses to caffeine that were equivalent in magnitude to those shown by mice not given a saline injection 1 h prior to conditioning, indicating that the effects of pretreatment with α-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 intraperitoneal 8-SPT, a quaternary analog of caffeine that is unable to cross the blood–brain barrier (Fig. 1B). Mice did not show conditioned place aversion to the drug; the amount of time that they spent in the previously saline-paired environment did not differ from the amount of time that they spent in the previously 8-SPT-paired environment on the 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 $D_2$ 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 $D_1$KO and $D_2$KO mice with 10 mg/kg caffeine, a dose that produces aversive effects in C57Bl/6 mice.

When dopamine $D_1$KO and $D_2$KO mice were conditioned with 10 mg/kg caffeine, conditioned place aversion to caffeine was blocked (Fig. 2A). $D_1$KO mice did not show a place preference or a place aversion to caffeine, whereas $D_2$KO mice showed significant conditioned place preferences, although the preferences did not maintain significance after correction 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 test indicated that the response of $D_2$KO mice differed significantly from that of wild-type littermates ($P < 0.05$), but not from

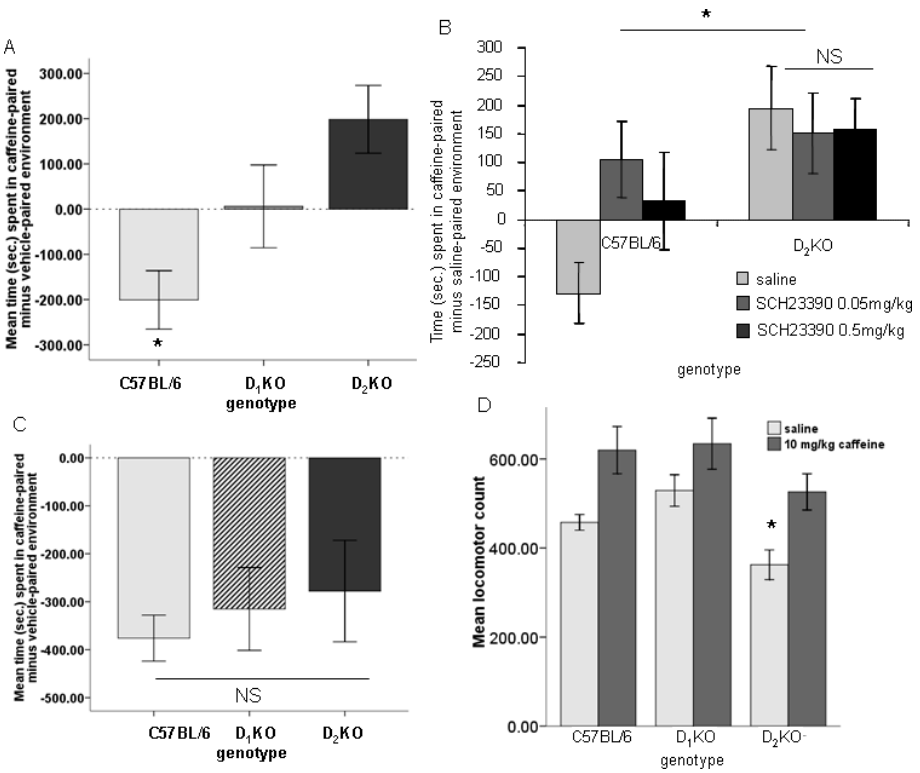


FIG. 2. Caffeine's dopamine-dependent aversive effects depend on the dopamine $D_2$ and $D_1$ receptors; however, the $D_2$ receptor is not involved in the locomotor stimulant effect of caffeine. (A) C57BL/6 mice conditioned with 10 mg/kg caffeine showed significant aversion, whereas dopamine $D_1$ receptor knockout ($D_1$KO) and dopamine $D_2$ receptor knockout ($D_2$KO) mice did not. $D_2$KO mice demonstrated preferences for this dose of caffeine, whereas D1KO mice did not show aversive effects or preferences. Bars represent mean difference scores ± standard errors of the mean (SEMs). *$P < 0.05$ for comparison of difference scores with zero that were corrected for multiple *t*-tests. (B) The dopamine $D_1$ receptor does not mediate the rewarding effects of caffeine. $D_2$KO mice show a conditioned place preference for 10 mg/kg caffeine, which their wild-type littermates find aversive. When $D_2$KO mice were pretreated with the dopamine $D_1$ receptor antagonist SCH23390, their preferences for caffeine did not diminish, indicating that the $D_1$ receptor is not the substrate of caffeine reward. Bars represent mean difference scores, and error bars are SEMs. NS, no main effect of pretreatment ($P > 0.05$); *main effect of genotype ($P < 0.05$). (C) A non-dopaminergic substrate mediates the aversive effects of 100 mg/kg caffeine. $D_1$KO and $D_2$KO mice show conditioned place aversion equivalent to that seen in wild-type littermates when given 100 mg/kg caffeine, despite the fact that $D_1$KO mice did not show preferences or aversive effects, and $D_2$KO mice show conditioned place preferences. Bars represent mean difference scores, and error bars are SEMs. NS, no main effect of genotype on difference scores. (D) C57BL/6, $D_1$KO and $D_2$KO mice all showed equivalent locomotor stimulation after 10 mg/kg intraperitoneal caffeine. Locomotor activity in $D_2$KO mice was significantly lower than that in D1KO mice. Bars represent mean locomotor counts for saline and caffeine trials ± SEMs. *Significant difference between the activities of $D_1$KO and $D_2$KO mice ($P < 0.05$).

that of $D_1$KO mice ($P > 0.05$). 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 ($t_{14} = 0.947$, $P > 0.05$), whereas wild-type mice showed significant aversive effects ($t_{21} = 3.11$, $P < 0.05$). These findings confirmed our previous results implicating dopaminergic signaling in the aversive effects of caffeine. Furthermore, 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 place preference or place aversion to caffeine, suggesting that this receptor might, in part, mediate caffeine's rewarding effects. In order to test this, we pretreated $D_2$KO mice with the $D_1$ receptor antagonist SCH23390 prior to conditioning with caffeine (Fig. 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 for caffeine similar to those seen in non-pretreated $D_2$KO mice. An ANOVA indicated that 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 α-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.

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

### *$D_2$KO mice show normal locomotor stimulation by 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 mice. The administration of 10 mg/kg caffeine led to increased locomotor activity in C57BL/6, $D_1$KO and $D_2$KO mice (Fig. 2D). A two-way ANOVA indicated that there was no significant genotype × 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 test indicated that $D_2$KO mice had significantly different locomotor activity than $D_1$KO mice ($P < 0.05$), manifested as lower activity in $D_2$KO mice following both saline and caffeine treatment. Forty-five-minute locomotor trials were also conducted to determine whether a longer observation period would reveal genotypic differences in caffeine's locomotor stimulant effects. The findings from these trials did not differ from the findings from 15-min trials.

These findings show that the appetitive and aversive effects of caffeine are dissociable from its locomotor stimulant effects, as $D_1$KO and $D_2$KO mice showed altered responses to place conditioning with caffeine while maintaining locomotor stimulation by 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 aversive responses to caffeine, but also dopamine blockade-induced preferences for caffeine*

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

Mice were conditioned with 15 mg/kg caffeine, a dose that is slightly higher than our previous dose of 10 mg/kg, to ensure that our C57BL/6 control group showed strong aversive effects. However, this 15 mg/kg dose did not produce stronger aversive effects than the 10 mg/kg dose in this particular group of C57BL/6 mice. When $A_{2A}$KO mice were conditioned with 15 mg/kg caffeine, they showed aversion to caffeine that was slightly greater than that seen in C57BL/6 mice. However, in contrast to what was seen in C57BL/6 mice, pretreatment of $A_{2A}$KO mice with 0.8 mg/kg α-flupenthixol did not completely block the aversive effects of caffeine and reveal a preference. $A_{2A}$KO mice maintained conditioned place aversion to caffeine after α-flupenthixol pretreatment (Fig. 3A). A *t*-test for independent samples revealed that the largest aversive effects in $A_{2A}$KO mice in response to 15 mg/kg caffeine were not significantly greater than those in C57BL/6 mice ($t_{21} = 1.30$, $P > 0.05$). However, comparison of $A_{2A}$KO mice and C57BL/6 mice when both groups had been pretreated with 0.8 mg/kg α-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 aversive effects, whereas C57BL/6 mice showed conditioned place preferences in response 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 stronger aversion to caffeine and determine more conclusively whether these $A_{2A}$KO mice were deficient in caffeine reward. $A_{2A}$KO mice showed aversive responses 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 for caffeine just as the C57BL/6 mice did (Fig. 3B). Independent-sample *t*-tests confirmed that, at 10 mg/kg, the aversive effects of caffeine in $A_{2A}$KO mice were not significantly different from those of C57BL/6 mice ($t_{17} = 0.661$, $P > 0.05$). Furthermore, when they were pretreated with neuroleptic, $A_{2A}$KO mice showed non-significant preferences for caffeine 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 that directly mediates caffeine's dopamine-dependent aversive effects and dopamine blockade-induced rewarding effects. The adenosine $A_1$ receptor remains a candidate, as this receptor binds caffeine with high affinity and mediates many of

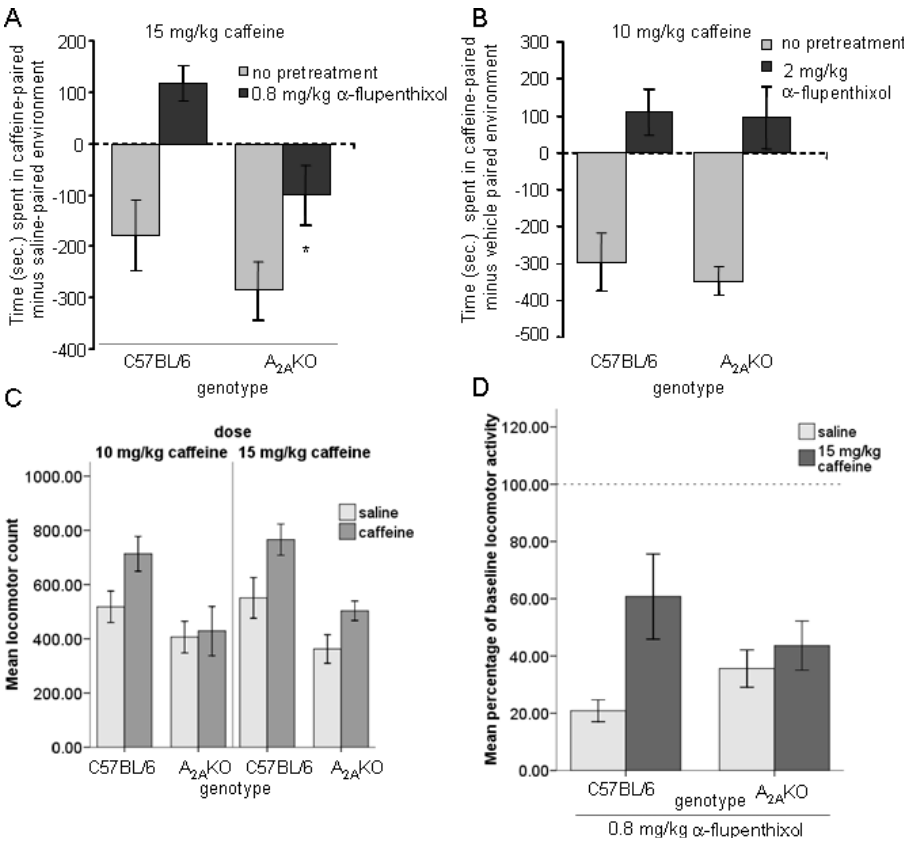


FIG. 3. The adenosine $A_{2A}$ receptor is not directly involved in the aversive or rewarding effects of caffeine, but is critical for caffeine's locomotor stimulant effects. (A) C57BL/6 and adenosine $A_{2A}$ receptor knockout ($A_{2A}$KO) mice showed similar aversive responses to 10 mg/kg intraperitoneal caffeine. Pretreating C57BL/6 and $A_{2A}$KO mice with 2.0 mg/kg α-flupenthixol blocked the aversive effects of caffeine and revealed preferences in both genotypes. Bars represent mean difference scores ± standard errors of the mean (SEMs). Non-pretreated and pretreated $A_{2A}$KO groups did not differ from their respective C57BL/6 control groups. (B) C57BL/6 and $A_{2A}$KO mice showed similar aversive responses to 15 mg/kg intraperitoneal caffeine. Pretreating C57BL/6, but not $A_{2A}$KO, mice with 0.8 mg/kg α-flupenthixol blocked the aversive effects of caffeine and revealed a preference for caffeine. $A_{2A}$KO mice pretreated with 0.8 mg/kg α-flupenthixol showed aversion to caffeine that differed from the response seen in pretreated C57BL/6 mice. Bars represent mean difference scores ± SEMs. *$P < 0.05$ for comparison of neuroleptic-pretreated C57BL/6 mice with pretreated $A_{2A}$KO mice. (C) C57BL/6 but not $A_{2A}$KO mice showed locomotor stimulation when given 10 mg/kg intraperitoneal caffeine; 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 ± SEMs. (D) Caffeine partially counteracts the locomotor depression induced by α-flupenthixol in C57BL/6 but not $A_{2A}$KO mice. Both C57BL/6 and $A_{2A}$KO mice experienced locomotor depression relative to baseline (dotted line) when treated with 0.8 mg/kg α-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 α-flupenthixol as a percentage of locomotor scores in the absence of α-flupenthixol. The dotted line indicates the baseline locomotor activity of each genotype in the absence of neuroleptic.

caffeine's physiological effects alongside the $A_{2A}$ receptor (Daly & Fredholm, 1998; Fredholm *et al.*, 1999).

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

Previous reports have indicated that the adenosine $A_{2A}$ receptor alone (Ledent *et al.*, 1997), the $A_1$ 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 by caffeine.

C57BL/6 mice, but not $A_{2A}$KO mice, demonstrated locomotor stimulation when given 10 mg/kg caffeine. However, at a dose of 15 mg/kg caffeine, both genotypes showed increased locomotor activity (Fig. 3C), although an ANOVA did not reveal significant interactions. The ANOVA indicated that there was no three-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-min locomotor trials were conducted. The findings from these trials did not differ from the findings from 15-min trials.

The finding that $A_{2A}$KO mice show increased locomotor activity following administration of 15 mg/kg caffeine, although it appears to be 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. Although 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. These findings do not reveal the type of relationship between these substrates, namely whether they interact additively or synergistically, as indicated by Kuzmin *et al.* (2006), but they do strongly suggest a substrate in addition to the $A_{2A}$ receptor through which caffeine acts to increase locomotor activity. Previous work suggests that this additional substrate may be the adenosine $A_1$ 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 α-flupenthixol, they experienced locomotor depression relative to their baseline locomotor activity in the absence of neuroleptic (dotted line in Fig. 3D). 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 ($F_{1,20} = 6.54$, $P < 0.05$).

### *$A_1$KO and dKO mice show normal conditioned place aversion to and dopamine blockade-induced preferences for caffeine*

Animals in a drug discrimination task were previously shown to respond to treatment with an $A_1$ antagonist and to treatment with caffeine in similar ways (Quarta *et al.*, 2004). Therefore, we tested $A_1$KO mice to determine whether this receptor is involved in caffeine reward or aversion. Different groups of $A_1$KO mice were conditioned with 10 mg/kg caffeine with and without neuroleptic pretreatment (Fig. 4A). $A_1$KO mice showed aversive responses to 10 mg/kg caffeine that were similar in magnitude to those of their wild-type littermates; the aversive effects seen in both groups did not withstand corrections for multiple testing ($P > 0.05$). $A_1$KO 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 α-flupenthixol. The 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 ($F_{1,22} = 0.050$, $P > 0.05$) effects were not significant, confirming that the aversive and rewarding effects of caffeine in $A_1$KO mice did not differ from those in wild-type littermates. Surprisingly, this indicates that the adenosine $A_1$ receptor, in addition to the adenosine $A_{2A}$ receptor, is not upstream of caffeine's dopamine-mediated aversive effects or dopamine blockade-induced preferences for caffeine.

To test the possibility that the adenosine $A_1$ and $A_{2A}$ receptors perform overlapping functions in signaling aversive responses to or preferences for caffeine, we conditioned different groups of adenosine $A_1$ and $A_{2A}$ receptor dKO mice with 10 mg/kg caffeine in the presence and absence of 0.8 mg/kg α-flupenthixol pretreatment. A two-way ANOVA revealed a main effect of pretreatment ($F_{1,28} = 10.54$, $P < 0.05$) but no genotype × 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 $A_1$ receptor alone, the $A_{2A}$ receptor alone nor both of them in combination are critical for mediating the aversive or rewarding effects of caffeine.

### *$A_1$KO mice show normal locomotor responses to caffeine*

$A_{2A}$KO mice did not show locomotor stimulation when given 10 mg/kg caffeine, but showed a small increase in locomotor activity when given 15 mg/kg caffeine. This suggests that the adenosine $A_{2A}$ receptor is important for caffeine-induced locomotor stimulation at lower doses, so we tested the possibility that the adenosine $A_1$ receptor mediates caffeine's additional $A_{2A}$-independent locomotor effects.

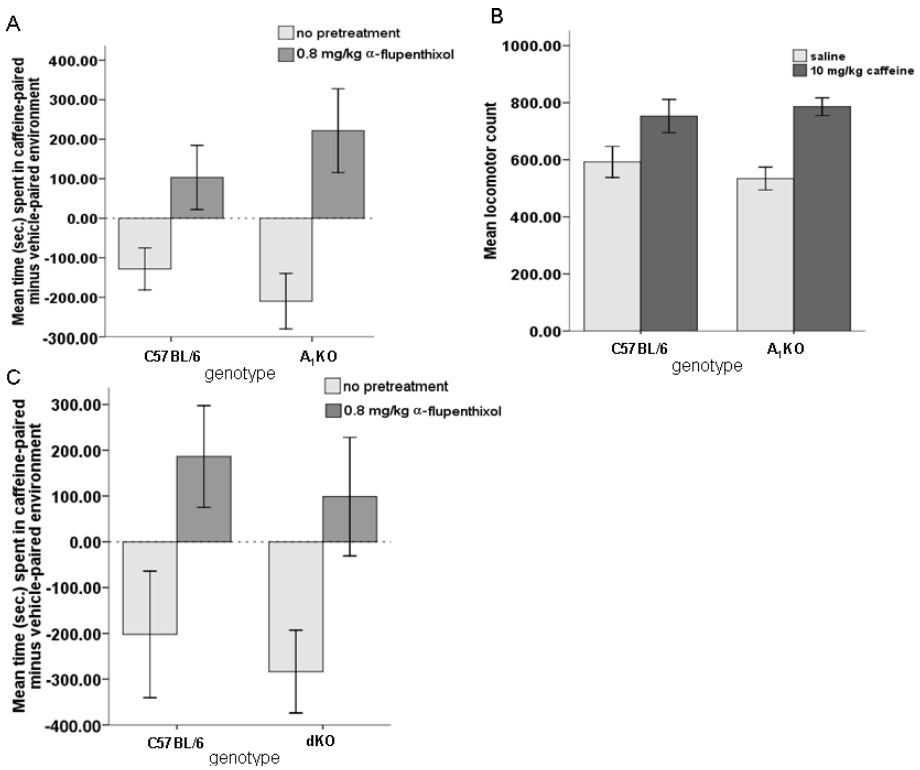


FIG. 4. The adenosine $A_1$ receptor is not critical for the stimulant effects of caffeine and is not involved in the aversive or rewarding effects of caffeine, either alone or in combination with the $A_{2A}$ receptor. (A) Adenosine $A_1$ receptor knockout ($A_1$KO) mice showed aversive responses to 10 mg/kg caffeine that did not differ from that seen in wild-type littermates. Knockout mice also showed preferences for 10 mg/kg caffeine when pretreated with 0.8 mg/kg α-flupenthixol, and preferences did not differ from those seen in wild-type littermates. Bars represent mean difference scores ± standard errors of the mean (SEMs). (B) $A_1$KO mice show locomotor stimulation in response to caffeine that is equivalent to the locomotor stimulation seen in wild-type littermates. Bars represent mean locomotor counts ± SEMs. *Significant difference between saline and caffeine locomotor scores in $A_1$KO mice ($P < 0.05$) but not in wild-type littermates ($P > 0.05$). (C) Double knockout (dKO) mice show aversive responses to 10 mg/kg caffeine and preferences for 10 mg/kg caffeine when pretreated with 0.8 mg/kg α-flupenthixol that do not differ significantly from those seen in C57BL/6 mice. Bars represent mean difference scores ± SEMs.

$A_1$KO mice conditioned with caffeine showed increases in locomotor activity similar to those seen in wild-type littermates (Fig. 4B), ruling out the possibility that the $A_1$ 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 that 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 TPP is not involved in caffeine's dopamine blockade-induced rewarding effects*

When one of caffeine's well-established pharmacological targets, the $A_{2A}$ receptor, was shown not to be involved in caffeine's dopamine blockade-induced rewarding effects, we next looked to the TPP, as it is implicated in the rewarding effects of natural rewards (Stefurak & van der Kooy, 1994; Kippin & van der Kooy, 2003) and drugs (Bechara & van der Kooy, 1989; Laviolette *et al.*, 2002).

C57BL/6 mice given sham TPP lesions developed conditioned place aversion to 10 mg/kg caffeine, and this aversion was unaltered in mice with excitotoxic lesions of the TPP. Additionally, C57BL/6 mice with sham TPP lesions showed conditioned place preferences in response to caffeine when pretreated with neuroleptic, and these place preferences were not disrupted in mice with excitotoxic TPP lesions (Fig. 5A). Comparison of sham-lesioned and TPP-lesioned mice confirmed that lesioning the TPP had no effect on caffeine place aversion ($t_{24} = 0.267$, $P > 0.05$). 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, as 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 probably because the TPP is not involved in these effects and not because of ineffective lesions.

## Discussion

The current data indicate 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 aversive effects at high doses (Brockwell *et al.*, 1991; Patkina & Zvartau, 1998). Although 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 small and occurred with one dose. These findings are similar to our current data; the lower doses of caffeine on our dose–response curve are weakly, but non-significantly, rewarding. Also consistent with our data is the fact that 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). Furthermore, a drinking study in rats suggests that tolerance develops to the initial aversive effects of caffeine following repeated exposure (Myers & Izbicki, 2006), suggesting that 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 in which 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 α-flupenthixol was found to inhibit the negative effects of naloxone-precipitated morphine withdrawal (Santi & Parker, 2001). Antagonism of the dopamine $D_2$ receptor with haloperidol was found to inhibit conditioned place aversion to the benzodiazepine receptor inverse agonist FG 7142 (Di Scala &

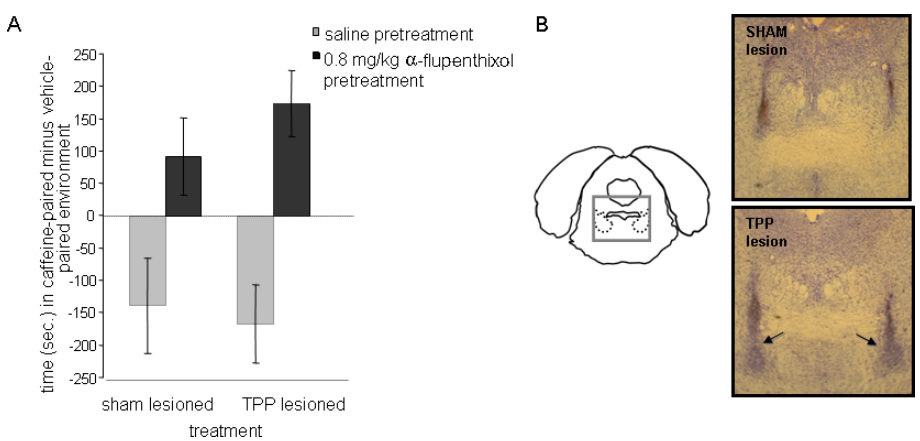


FIG. 5. Lesions of the tegmental pedunculopontine nucleus (TPP) in mice do not affect aversion or dopamine blockade-induced conditioned place preference in response to intraperitoneal caffeine. (A) Both sham TPP-lesioned and TPP-lesioned mice showed aversion to 10 mg/kg intraperitoneal caffeine. Pretreating sham TPP-lesioned and TPP-lesioned mice blocked the aversive effects of 10 mg/kg intraperitoneal caffeine in both groups and revealed significant preferences. Bars represent mean difference scores ± standard errors of the mean. The aversive responses and preference of lesioned groups did not differ significantly from those of sham-lesioned controls. (B) Coronal section of the brain; the gray rectangle indicates the region depicted in the photographs, and dotted lines indicate the approximate location of the TPP. The photographs on the right show a representative bilateral sham TPP lesion (top) and an excitotoxic TPP lesion (bottom). Arrows indicate gliosis in the region of the TPP.

Sandner, 1989). Furthermore, mice with knockout of the long form of the dopamine $D_2$ receptor displayed a deficit in learning about aversive electrical stimuli (Smith *et al.*, 2002), and $D_2$KO mice do not show place aversion to high doses of ethanol (Ting-A-Kee *et al.*, 2009). The aversive effects of nicotine can also be blocked by systemic or intra-ventral tegmental area α-flupenthixol, and place preferences can be seen at some doses (Laviolette & van der Kooy, 2003). Additionally, place preferences in response to morphine, diazepam, and nicotine, in addition to place aversion to picrotoxin, naloxone, phencyclidine, and lithium, can be blocked by the dopamine $D_1$ receptor antagonist SCH23390 (Acquas *et al.*, 1989, Acquas & Di Chiara, 1994).

It has been suggested that dopamine is involved in signaling not 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 (Fig. 1). Although 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 analog of caffeine, 8-SPT, which was administered peripherally and is unable to cross the blood–brain barrier, did not induce conditioned place aversion. Furthermore, 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 (Ukena *et al.*, 1986a,b). These data indicate that caffeine's aversive effects result from its actions in the central nervous system.

Although $D_2$KO mice have lower basal locomotor activity than C57Bl/6 mice, 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 preference or aversion. In fact, mice pretreated with the neuroleptic α-flupenthixol show dramatically reduced locomotor activity during conditioning sessions; however, these animals learn robust place preferences (Fig. 1A) and aversive responses 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 in which it was experienced.

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 min following a 15-min delay after the intraperitoneal caffeine injection. Conditioned place aversion to caffeine was still observed with this paradigm, and was equal in magnitude to that observed when no delay was used (data not shown). Because the aversion seen when conditioning was conducted after a 15-min delay was not significantly stronger, conditioning animals immediately after an intraperitoneal injection with caffeine seems to provides enough time for the majority of drug absorption to take place.

We have shown by lesioning the TPP that this brainstem nucleus is not involved in dopamine blockade-induced caffeine reward. This finding suggests that the mechanisms underlying caffeine's rewarding effects are different from those for other recreational drugs. Previous reports have indicated that the $A_{2A}$ receptor is a pharmacological target of caffeine, indicating that 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 provides a 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 aversive effects of caffeine are blocked and preferences for the drug are seen. These data suggest that the injection of caffeine affects different and dissociable neurobiological systems that are responsible for its aversive and rewarding motivational effects. With doses at which 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). Whereas lower doses of caffeine do not induce activation of the NAc, this region is activated at doses of 10 mg/kg or above (Nehlig *et al.*, 1984). It seems possible, then, that caffeine activates the NAc 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). Furthermore, 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 co-release of dopamine with neuropeptides and other neurotransmitters, including glutamate; these additional transmitters may be involved in the conditioned place preferences for 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 did 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 mice 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 are C57BL/6 mice, is in line with the current results. A second study showed that the $A_{2A}$-selective antagonist CGS 15943A, and not an $A_1$ receptor antagonist, produced place preferences in rats (Brockwell & Beninger, 1996). Surprisingly, despite widespread data indicating the high affinity of the $A_{2A}$ and $A_1$ receptors 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 $A_1$ receptors in the rewarding or aversive effects of caffeine. Additionally, using dKO mice with no functional adenosine $A_1$ 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's aversive effects or caffeine preferences would persist because of signaling via the other receptor. However, the dKO

mice showed caffeine aversion and dopamine blockade-induced preferences that were equivalent to those seen in C57BL/6 mice. This indicates that adenosine $A_1$ and $A_{2A}$ receptors do not perform redundant functions in signaling caffeine aversion and preferences, and provides strong evidence that caffeine acts on a substrate other than the adenosine $A_1$ or $A_{2A}$ receptors to produce its aversive and rewarding effects. However, it remains unclear whether, if they are not the adenosine $A_1$ and $A_{2A}$ receptors, 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 $A_3$ 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).

An interesting finding of the present study is 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 (Fig. 3B). Adenosine $A_{2A}$ receptors and dopamine $D_2$ 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, 1993; Schiffmann *et al.*, 1991; Chen *et al.*, 2001; Hervé *et al.*, 2001). 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, the level of dopamine $D_2$ 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, whereas at a higher dose of neuroleptic the aversion to caffeine was blocked and preferences equivalent to those in C57BL/6 mice were seen.

The current locomotor data obtained in $A_{2A}$KO mice offer an additional behavioral correlate of this antagonistic interaction between $A_{2A}$ and $D_2$ receptors. Both C57BL/6 and $A_{2A}$KO mice showed locomotor depression when treated with a neuroleptic (Fig. 3D). When given caffeine, C57BL/6 mice showed a partial rescue of locomotor activity, whereas $A_{2A}$KO mice did not. Similar to the opposing effects that the $A_{2A}$ and $D_2$ receptors exert on caffeine's rewarding and aversive effects, antagonism of the dopaminergic system decreases caffeine's locomotor effects, whereas 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 $D_2$KO mice showed locomotor stimulation by caffeine equivalent to what was seen in C57Bl/6 mice. This indicates, first, that the $D_2$ receptor is not critical for caffeine-induced locomotor stimulation, and additionally that heteromers of the $A_{2A}$ and $D_2$ 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 on 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. Furthermore, we have shown that $A_{2A}$KO and $A_1$KO mice can display conditioned place aversion and dopamine blockade-induced preferences in response to caffeine, ruling out each of these receptors 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 characterization of caffeine's mechanism of reward may not only lead to the elucidation of novel reward mechanisms, but also help us to focus on the actions of classic drugs of abuse that are critical for inducing addictive behavior.

## Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Mice do not have a preference for either conditioning environment.

Fig. S2. The effects of pretreatment are caused by the drug and not the stress of injection.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset by Wiley-Blackwell. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

## Acknowledgements

We wish to thank Michael Schwarzschild for his generous gift of adenosine $A_{2A}$ and $A_1$ receptor knockout mice. This research was supported by grants from the Canadian Institutes for Health Research, and was also supported in part by NIH grant DA019362.

## Abbreviations

$A_1$KO, adenosine $A_1$ receptor knockout; $A_{2A}$KO, adenosine $A_{2A}$ receptor knockout; dKO, double knockout; $D_1$KO, dopamine $D_1$ receptor knockout; $D_2$KO, dopamine $D_2$ receptor knockout; NAc, nucleus accumbens; NMDA, *N*-methyl-D-aspartic acid; TTP, tegmental pedunculopontine nucleus; 8-SPT, 8-(*p*-sulfophenyl)theophylline.

## References

Acquas, E. & Di Chiara, G. (1994) D1 receptor blockade stereospecifically impairs acquisition of drug-conditioned place preference and aversion. *Behav. Pharmacol.*, **5**, 555–569.

Acquas, E., Carboni, E., Leone, P. & Di Chiara, G. (1989) SCH23390 blocks drug-conditioned place-preference and place-aversion: anhedonia (lack of reward) or apathy (lack of motivation) after dopamine-receptor blockade? *Psychopharmacology*, **99**, 151–155.

Andersen, P.H. (1988) Comparison of the pharmacological characteristics of [3H]raclopride and [3H]SCH 23390 binding to dopamine receptors in vivo in mouse brain. *Eur. J. Pharmacol.*, **146**, 113–120.

Bechara, A. & van der Kooy, D. (1989) 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.*, **9**, 3400–3409.

Bedingfield, J.B., King, D.A. & Holloway, F.A. (1998) Cocaine and caffeine: conditioned place preference, locomotor activity, and additivity. *Pharmacol. Biochem. Behav.*, **61**, 291–296.

Bozarth, M. & Wise, R. (1981) Intra-cranical self-administration of morphine into the ventral tegmental area in rats. *Life Sci.*, **28**, 551–555.

Brockwell, N. & Beninger, R. (1996) The differential role of A1 and A2 adenosine receptor subtypes in locomotor activity and place conditioning in rats. *Behav. Pharmacol.*, **7**, 373–383.

Brockwell, N.T., Eikelboom, R. & Beninger, R.J. (1991) Caffeine-induced place and taste conditioning: production of dose-dependent preference and aversion. *Pharmacol. Biochem. Behav.*, **38**, 513–517.

Chen, J.F., Huang, Z.H., Ma, J.Y., Zhu, J.M., Moratalla, R., Standaert, D., Moskowitz, M.A., Fink, J.S. & Schwarzschild, M.A. (1999) A2A adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J. Neurosci.*, **19**, 9192–9200.

Chen, J.F., Moratalla, R., Impagnatiello, F., Grandy, D.K., Cuellar, B., Rubinstein, M., Beilstein, M.A., Hackett, E., Fink, J.S., Low, M.J., Ongini, E. & Schwarzschild, M.A. (2001) 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. Natl Acad. Sci. USA*, **98**, 1970–1975.

Clements, J. & Grant, S. (1990) Glutamate-like immunoreactivity in neurons of the laterodorsal tegmental and pedunculopontine nuclei in the rat. *Neurosci. Lett.*, **120**, 70–73.

Creese, I., Burt, D. & Snyder, S. (1976) Dopamine receptor-binding predicts clinical and pharmacological potencies of anti-schizophrenic drugs. *Science*, **92**, 481–483.

Daly, J. & Fredholm, B. (1998) Caffeine – an atypical drug of dependence. *Drug Alcohol Depend.*, **51**, 199–206.

Di Scala, G. & Sandner, G. (1989) Conditioned place aversion produced by FG 7142 is attenuated by haloperidol. *Psychopharmacology*, **99**, 176–180.

El Yacoubi, M., Ledent, C., Parmentier, M., Costentin, J. & Vaugeois, J. (2005) Reduced appetite for caffeine in adenosine A(2A) receptor knockout mice. *Eur. J. Pharmacol.*, **519**, 290–291.

Ferre, S. (2008) An update on the mechanisms of the psychostimulant effects of caffeine. *J. Neurochem.*, **105**, 1067–1079.

Ferre, S., Voneuler, G., Johansson, B., Fredholm, B. & Fuxe, K. (1991) Stimulation of high-affnity adenosine-A2 receptors decreases the affnity of dopamine D2 receptors in rat striatal membranes. *Proc. Natl Acad. Sci. USA*, **88**, 7238–7241.

Ferre, S., Snaprud, P. & Fuxe, K. (1993) 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.*, **244**, 311–315.

Ferre, S., Popoli, P., Gimenezllort, L., Finnman, U.B., Martinez, E., Decarolis, A.S. & Fuxe, K. (1994) Postsynaptic antagonistic interaction between adenosine A(1) and dopamine D-1 receptors. *NeuroReport*, **6**, 73–76.

Florio, C., Rosati, A., Traversa, U. & Vertua, R. (1997) Inhibitory and excitatory effects of adenosine antagonists on spontaneous locomotor activity in mice. *Life Sci.*, **60**, 1477–1486.

Ford, B., Holmes, C., Mainville, L. & Jones, B. (1995) GABAergic neurons in the rat pontomesencephalic tegmentum – codistribution with cholinergic and other tegmental neurons projecting to the posterior lateral hypothalamus. *J. Comp. Neurol.*, **363**, 177–196.

Frary, C.D., Johnson, R.K. & Wang, M.Q. (2005) Food sources and intakes of caffeine in the diets of persons in the United States. *J. Am. Diet. Assoc.*, **105**, 110–113.

Fredholm, B.B. (1980) Are methylxanthine effects due to antagonism of endogenous adenosine? *Trends Pharmacol. Sci.*, **1**, 129–132.

Fredholm, B.B., Battig, K., Holmen, J., Nehlig, A. & Zvartau, E.E. (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol. Rev.*, **51**, 83–133.

Fredholm, B.B., Ijzerman, A.P., Jacobson, K.A., Klotz, K.N. & Linden, J. (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol. Rev.*, **53**, 527–552.

Fuxe, K., Ferré, S., Genedani, S., Franco, R. & Agnati, L.F. (2007) Adenosine receptor–dopamine receptor interactions in the basal ganglia and their relevance for brain function. *Physiol. Behav.*, **92**, 210–217.

Griffiths, R.R. & Woodson, P.P. (1988) Reinforcing effects of caffeine in humans. *J. Pharmacol. Exp. Ther.*, **246**, 21–28.

Hervé, D., Le Moine, C., Corvol, J.C., Belluscio, L., Ledent, C., Fienberg, A.A., Jaber, M., Studler, J.M. & Girault, J.A. (2001) G alpha(olf) levels are regulated by receptor usage and control dopamine and adenosine action in the striatum. *J. Neurosci.*, **21**, 4390–4399.

Hnasko, T.S., Sotak, B.N. & Palmiter, R.D. (2007) Cocaine-conditioned place preference by dopamine-deficient mice is mediated by serotonin. *J. Neurosci.*, **24**, 12484–12488.

Hof, P.R., Young, W.G., Bloom, F.E., Belichenko, P.V. & Celio, M.R. (2000) *Comparative Cytoarchitectonic Atlas of the C57Bl/6 and 129/Sv Mouse Brains*. Elsevier Science, San Diego, CA.

Hughes, J. & Oliveto, A. (1997) A systematic survey of caffeine intake in vermont. *Exp. Clin. Psychopharmacol.*, **5**, 393–398.

James, J.E. & Rogers, P.J. (2005) Effects of caffeine on performance and mood: withdrawal reversal is the most plausible explanation. *Psychopharmacology*, **182**, 1–8.

Johansson, B., Halldner, L., Dunwiddie, T.V., Masino, S.A., Poelchen, W., Giménez-Llort, L., Escorihuela, R.M., Fernandez-Teruel, A., Wiesenfeld-Hallin, Z., Xu, X.J., Hardemark, A., Betscholtz, C., Herlenius, E. & Fredholm, B.B. (2001) Hyperalgesia, anxiety, and decreased hypoxic neuroprotection in mice lacking the adenosine A1 receptor. *Proc. Natl Acad. Sci. USA*, **98**, 9407–9412.

Kaplan, G.B., Greenblatt, D.J., Ehrenberg, B.L., Goddard, J.E., Cotrea, M.M., Harmatz, J.S. & Shader, R.I. (1997) Dose-dependent pharmacokinetics and psychomotor effects of caffeine in humans. *J. Clin. Pharmacol.*, **37**, 693–703.

Kelley, A.E. & Berridge, K.C. (2002) The neuroscience of natural rewards: relevance to addictive drugs. *J. Neurosci.*, **22**, 3306–3311.

Kelly, M.A., Rubinstein, M., Asa, S.L., Zhang, G., Saez, C., Bunzow, J.R., Allen, R.G., Hnasko, R., Ben-Jonathan, N., Grandy, D.K. & Low, M.J. (1997) Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. *Neuron*, **19**, 103–113.

Kippin, T. & van der Kooy, D. (2003) 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.*, **18**, 2581–2591.

Kuzmin, A., Johansson, B., Gimenez, L., Ogren, S. & Fredholm, B. (2006) Combination of adenosine A(1) and A(2A) receptor blocking agents induces caffeine-like locomotor stimulation in mice. *Eur. Neuropsychopharmacol.*, **16**, 129–136.

Lavin, A., Nogueira, L., Lapish, C.C., Wightman, R.M., Phillips, P.E.M. & Seamans, J.K. (2005) Mesocortical dopamine neurons operate in distinct temporal domains using multimodal signaling. *J. Neurosci.*, **25**, 5013–5023.

Laviolette, S. & van der Kooy, D. (2003) Blockade of mesolimbic dopamine transmission dramatically increases sensitivity to the rewarding effects of nicotine in the ventral tegmental area. *Mol. Psychiatry*, **8**, 50–59.

Laviolette, S., Alexson, T. & van der Kooy, D. (2002) Lesions of the tegmental pedunculopontine nucleus block the rewarding effects and reveal the aversive effects of nicotine in the ventral tegmental area. *J. Neurosci.*, **22**, 8653–8660.

Ledent, C., Vaugeois, J.M., Schiffmann, S.N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J.-J., Costentin, J., Heath, J.K., Vassart, G. & Parmentier, M. (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A(2A) receptor. *Nature*, **388**, 674–678.

Lepore, M. & Franklin, K.B.J. (1993) N-methyl-D-aspartate lesions of the pedunculopontine nucleus block acquisition and impair maintenance of responding reinforced with brain stimulation. *Neuroscience*, **71**, 147–155.

McPherson, P.S., Kim, Y.K., Valdivia, H., Knudson, C.M., Takekura, H., Franzini-Armstrong, C., Coronado, R. & Campbell, K.P. (1991) The brain ryanodine receptor: a caffeine-sensitive calcium release channel. *Neuron*, **7**, 17–25.

Myers, K.P. & Izbicki, E.V. (2006) Reinforcing and aversive effects of caffeine measured by flavor preference conditioning in caffeine-naïve and caffeine acclimated rats. *Physiol. Behav.*, **88**, 585–596.

Nehlig, A. (1999) Are we dependent upon coffee and caffeine? A review on human and animal data *Neurosci. Biobehav. Rev.*, **23**, 563–576.

Nehlig, A., Daval, J.L. & Debry, G. (1992) Caffeine and the central nervous system: mechanism of action, biochemical, metabolic and psychostimulant effects. *Brain Rs. Rev.*, **17**, 139–170.

Nehlig, A., Lucignani, G., Kadekaro, M., Porrino, L.J. & Sokoloff, L. (1984) Effects of acute administration of caffeine on local cerebral glucose utilization in the rat. *Eur. J. Pharmacol.*, **101**, 91–100.

Parker, J.L. & van der Kooy, D. (1995) Tegmental pedunculopontine nucleus lesions do not block cocaine reward. *Pharmacol. Biochem. Behav.*, **52**, 77–83.

Patkina, N.A. & Zvartau, E.E. (1998) Caffeine place conditioning in rats: comparison with cocaine and ethanol. *Eur. Neuropsychopharmacol.*, **8**, 287–291.

Pezze, M.A., Heidbreder, C.A., Feldon, J. & Murphy, C.A. (2001) Selective responding of nucleus accumbens core and shell dopamine to aversively conditioned contextual and discrete stimuli. *Neuroscience*, **108**, 91–102.

Quarta, D., Borycz, J., Solinas, M., Patkar, K., Hockemeyer, J., Ciruela, F., Lluis, C., Franco, R., Woods, A.S., Goldberg, S.R. & Ferre, S. (2004) Adenosine receptor-mediated modulation of dopamine release in the nucleus accumbens depends on gluatamate neurotransmission and N-methyl-D-aspartate receptor stimulation. *J. Neurochem.*, **91**, 873–880.

Salamone, J.D. (1994) The involvement of nucleus accumbens dopamine in appetitive and aversive motivation. *Behav. Brain Res.*, **61**, 117–133.

Santi, A.N. & Parker, L.A. (2001) The dopamine antagonist, alpha-flupenthixol, interferes with naloxone-induced place aversion learning, but not with acute opiate dependence in rats. *Pharmacol. Biochem. Behav.*, **70**, 193–197.

Schiffmann, S., Jacobs, O. & Vanderhaeghen, J. (1991) Striatal restricted adenosine-A2 receptor (Rdc8) is expressed by enkephalin but not by

substance-P neurons – an in situ hybridization histochemistry study. *J. Neurochem.*, **57**, 1062–1067.
Schultz, W. (2002) Getting formal with dopamine and reward. *Neuron*, **36**, 241–263.
Short, J.L., Ledent, C., Borrelli, E., Drago, J. & Lawrence, A.J. (2006) Genetic interdependence of adenosine and dopamine receptors: evidence from receptor knockout mice. *Neuroscience*, **139**, 661–670.
Smellie, F.W., Davis, C.W., Daly, J.W. & Wells, J.N. (1979) Alkylxanthines: inhibition of adenosine-elicited accumulation of cyclic AMP in brain slices and of brain phosphodiesterase activity. *Life Sci.*, **24**, 2475–2482.
Smith, J.W., Fetsko, L.A., Xu, R. & Wang, Y. (2002) Dopamine D2L receptor knockout mice display deficits in positive and negative reinforcing properties of morphine and in avoidance learning. *Neuroscience*, **113**, 755–765.
Snyder, S.H. (1985) Adenosine as a neuromodulator. *Annu. Rev. Neurosci.*, **8**, 103–124.
Solinas, M., Ferré, S., Antoniou, K., Quarta, D., Justinova, Z., Hockemeyer, J., Pappas, L.A., Segal, P.N., Wertheim, C., Muller, C.E. & Goldberg, S.R. (2005) Involvement of adenosine A1 receptors in the discriminative-stimulus effects of caffeine in rats. *Psychopharmacology*, **179**, 576–586.
Spyraki, C., Fibiger, H.C. & Phillips, A.G. (1982) Cocaine-induced place preference conditioning: lack of effects of neuroleptics and 6-hydroxydopamine lesions. *Brain Res.*, **253**, 195–203.
Stefurak, T.L. & van der Kooy, D. (1994) Tegmental pedunculopontine lesions in rats decrease saccharin's rewarding effects but not its memory-improving effect. *Behav. Neurosci.*, **108**, 972–980.
Tao, S. & Abdel-Rahman, A.A. (1993) Neuronal and cardiovascular responses to adenosine microinjection into the nucleus tractus solitarius. *Brain Res. Bull.*, **32**, 407–417.
Ting-A-Kee, R., Dockstader, C., Heinmiller, A., Grieder, T. & van der Kooy, D. (2009) GABA(A) receptors mediate the opposing roles of dopamine and the tegmental pedunculopontine nucleus in the motivational effects of ethanol. *Eur. J. Neurosci.*, **29**, 1235–1244.
Tinley, E.M., Durlach, P.J. & Yeomans, M.R. (2004) How habitual caffeine consumption and dose influence flavour preference conditioning with caffeine. *Physiol. Behav.*, **82**, 317–324.
Tzschentke, T.M. (2007) Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addict. Biol.*, **12**, 227–462.
Ukena, D., Jacobson, K.A., Padgett, W.L., Ayala, C., Shamim, M.T., Kirk, K.L., Olsson, R.O. & Daly, J.W. (1986a) Analogs of caffeine antagonists with selectivity for A2 adenosine receptors. *Life Sci.*, **39**, 743–750.
Ukena, D., Jacobson, K.A., Padgett, W.L., Ayala, C., Shamim, M.T., Kirk, K.L., Olsson, R.O. & Daly, J. (1986b) Species differences in structure–activity relationships of adenosine agonists and xanthine antagonists at brain A1 adenosine receptors. *FEBS Lett.*, **209**, 122–128.
Wise, R. (2004) Dopamine, learning and motivation. *Nat. Rev. Neurosci.*, **5**, 483–494.
Yang, J.N., Bjorklund, O., Lindstrom-Tornqvist, K., Lindgren, E., Eriksson, T.M., Kahlstrom, J., Chen, J.F., Schwarzschild, M.A., Tobler, I. & Fredholm, B.B. (2009) Mice heterozygous for both A1 and A2A adenosine receptor genes show similarities to mice given long-term caffeine. *J. Appl. Physiol.*, **106**, 631–639.