Behavioral and biochemical responses to morphine related to its addictive properties are altered in adenosine A_{2A} receptor knockout mice

A Castañé[1#], L Wells[3], G Soria[1#], S Hourani[3], C Ledent[2], I Kitchen[3], J Opacka-Juffry[4], R Maldonado[1] and O Valverde[1*]

Running Tittle: morphine addictive properties in A_{2A} knockout mice

[1]Departament de Ciències Experimentals i de la Salut. Universitat Pompeu Fabra, Barcelone Biomedical Research Park. C/Dr. Aiguader 88, 08003 Barcelona, Spain

[2]IRIBHM, Université Libre de Bruxelles, B-1070 Bruxelles, Belgium

[3]School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey. GU2 4JA, UK

[4]School of Human and Life Sciences, Roehampton University, Holybourne Avenue, London, SW12 4JD, UK.

[*]Corresponding author:

Olga Valverde MD PhD
Grup de Recerca de Neurobiologia del Comportament
Departament de Ciències Experimentals i de la Salut
Universitat Pompeu Fabra
Barcelone Biomedical Research Park
Dr. Aiguader, 88
08003 Barcelone, Spain
olga.valverde@upf.edu

[#]Present address:

A.C.: Department of Neurochemistry and Neuropharmacology, Institut d'Investigacions Biomèdiques de Barcelona (CSIC), IDIBAPS. Rosselló, 161, 6th floor. 08036 Barcelona, Spain. G.S.: Department of Cerebral Ischemia and Neurodegeneration, Institut d'Investigacions Biomèdiques de Barcelona (CSIC), IDIBAPS. Rosselló, 161, 6th floor. 08036 Barcelona, Spain.

Abstract

Background and purpose. Purinergic system through the A_{2A} adenosine receptor regulates addiction induced by different drugs of abuse. The aim of the present study was to investigate the specific role of A_{2A} adenosine receptors in behavioral and neurochemical morphine responses related to its addictive properties.

Experimental approach. Mice lacking A_{2A} adenosine receptors and wild type littermates were used to evaluate behavioral responses induced by morphine. Antinociception was assessed using the tail-immersion and the hot-plate tests. Place conditioning paradigms were used to evaluate the rewarding effects of morphine and the dysphoric responses of morphine withdrawal. Microdialysis studies were carried out to evaluate changes in the extracellular levels of dopamine in the nucleus accumbens of A_{2A} knockout mice after morphine administration.

Key results. The acute administration of morphine induced a similar enhancement of locomotor activity and antinociceptive responses in both genotypes. However, the rewarding effects induced by morphine were completely blocked in A_{2A} knockout mice. Besides, naloxone did not induce place aversion in animals lacking the A_{2A} adenosine receptors.

Conclusions and implications. Our findings demonstrate the relevant role played by A_{2A} adenosine receptors in the addictive properties of morphine. Both, rewarding and aversive effects associated to abstinence were abolished in A_{2A} knockout mice, supporting a differential role of the A_{2A} adenosine receptor in somatic and motivational effects of morphine addiction. This study provides evidence about the role of A_{2A} adenosine receptor as a general modulator of the addictive phenomenon.

Keywords: knockout mice, A_{2A} adenosine receptors, dopamine, place conditioning, microdialysis, reward, morphine, purinergic system.

Abbreviations:

Central nervous system, CNS; dopamine, DA; high-performance liquid chromatography, HPLC; intraperitoneal, i.p.; knockout, KO; nucleus accumbens, NAc; subcutaneous, s.c.; wild-type, WT.

Introduction

Adenosine is an endogenous purine nucleoside, which acts as a neuromodulator in the central nervous system (CNS), and regulates a wide range of pathophysiological processes, such as epilepsies, sleep disorders, pain, and drug addiction (Hack & Christie, 2003). The effects of adenosine are mediated through the activation of four receptor types: A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.,* 2005). A_{2A} adenosine receptors are found at high concentrations in brain areas involved in the control of motivational responses including the olfactory tubercle, the striatum and the nucleus accumbens (NAc) (Moreau & Huber, 1999).

The generation of knockout (KO) mice with complete and specific inactivation of the A_{2A} receptor (Ledent *et al.,* 1997) provides a useful genetic model to investigate the role of these receptors on addictive processes. We have previously identified that A_{2A} gene deletion in mice modifies the behavioral effects induced by different drugs of abuse. Thus, nicotine-induced conditioned place preference was suppressed in A_{2A} KO mice (Castañé *et al.,* 2006). Additionally, both rewarding and aversive effects of Δ9-tetrahydrocannabinol were reduced, whereas the expression of rimonabant-precipitated Δ9-tetrahydrocannabinol withdrawal was reported to be attenuated in A_{2A} KO mice (Soria *et al.,* 2004). Besides, the reinforcing effects of cocaine decreased in these mutants in the self-administration paradigm (Soria *et al*., 2006).

Dopamine (DA) neurotransmission in the mesolimbic system plays a crucial role in reward processes and other addictive related behaviors (Koob, 1996; Di Chiara, 2002). Interestingly, a striatal hypodopaminergic activity was shown in A_{2A} KO mice (Dassesse *et al.,* 2001) which could account for the attenuated reward effects induced by psychostimulants observed in KO mice. In the striatum, A_{2A} adenosine receptors are

expressed on GABAergic striatopallidal neurons and are co-localized with D_2 DA receptors (Ferré *et al.,* 1997). Adenosine appears to regulate DA neurotransmission through antagonistic interactions between adenosine A_1/DA D_1 receptors and adenosine A_{2A}/DA D_2 receptors (Franco *et al.,* 2000).

Interactions between the purinergic and opioid systems have also been reported. Thus, A_{2A} adenosine receptors regulate proenkephalin gene expression in the striatum (Fink *et al.,* 1992; Schiffmann & Vanderhaeghen, 1993). A reduction of [^{3}H]deltorphin-I binding to delta receptors and an increase in [^{3}H]Cl-977 binding to kappa receptors were observed in the spinal cord of A_{2A} adenosine receptors KO mice (Bailey *et al.*, 2002) associated with functional changes in opioid antinociception. Interestingly, adenosine has been suggested to regulate pharmacological responses induced by opioids. Thus, the spinal antinociceptive effects of morphine seem to be mediated, at least in part, by the release of endogenous adenosine and subsequent activation of A_1 and A_2 receptors (Sweeney *et al.*, 1987; 1991). Adenosine also participates in the development of opioid physical dependence since the blockade of adenosine metabolism by adenosine kinase inhibitors (Kaplan & Coyle, 1998) and the administration of adenosine agonists (Kaplan & Sears, 1996) decrease the severity of morphine abstinence, whereas adenosine antagonists increase the expression of withdrawal symptoms in rats (Salem & Hope, 1997). In agreement, we have shown that the severity of morphine withdrawal was increased in mice lacking A_{2A} adenosine receptors (Berrendero *et al.,* 2003; Bailey *et al.,* 2004). However, the involvement of A_{2A} adenosine receptors in the motivational effects induced by opioids remains to be clarified.

The aim of the present study was to investigate the specific role of A_{2A} adenosine receptors in morphine-induced behavioral and neurochemical responses

related to its addictive properties. For this purpose, we have evaluated the acute locomotor and antinociceptive effects induced by morphine in mice lacking A_{2A} receptors. In addition, the rewarding properties of morphine and the aversive effects associated with morphine withdrawal were evaluated in these mutant mice by using the place conditioning paradigm. Finally, *in vivo* microdialysis studies were performed to assess if the acute effects of morphine on the extracellular levels of DA in the NAc were modified in these KO animals.

Material and Methods

Animals

Mice lacking A_{2A} adenosine receptors were generated as previously reported (Ledent *et al.*, 1997). In order to homogenize the genetic background of the mice, the first generation heterozygotes were bred for 30 generations on a CD1 background (Charles River, France) with selection for the mutant A_{2A} gene at each generation. Beginning with the 30^{th} generation of backcrossed mice, heterozygote-heterozygote matings of A_{2A} KO mice produced wild-type (WT) and KO littermates for subsequent experiments. Breeding couples were periodically renovated by crossing heterozygote mice with WT CD1 females (Charles River, France) in order to maintain a genetically diverse outbred background.

Fourteen weeks old male A_{2A} KO mice and WT littermates (30-35 g) were housed five per cage in temperature (21 ± 1ºC) and humidity (55 ± 10 %) controlled rooms, with a 12-h light/12-h dark cycle (light between 8:00 AM and 8:00 PM). For the microdialysis experiments, animals were housed three per cage. Food and water were available *ad libitum* during all experiments except during the exposure to the different behavioral paradigms. Mice were handled for one week before starting the experiments. Animal procedures were conducted in accordance with the guidelines of the UK Animals Act 1986 (Scientific Procedures), the guidelines of the European Communities Directive 86/609/EEC regulating animal research and for the behavioral experiments performed in the laboratory of Barcelona approved by the local ethical committee (CEEA-PRBB). All experiments were performed under blind conditions.

Drugs

Morphine used for behavioral studies was obtained from Ministerio de Sanidad y Consumo (Madrid, Spain). Morphine and cocaine for microdialysis experiments were purchased in Sigma Chemical Co (Dorset, U.K.). Naloxone, was purchased from Sigma Chemical Co. (Barcelona, Spain).

All the compounds were dissolved in sterile 0.9 % physiological saline.

Acute effects induced by morphine

Locomotor activity responses induced by an acute subcutaneous (s.c.) injection of morphine (5 and 10 mg/kg, s.c.) or vehicle were evaluated by using locomotor activity boxes (9 x 20 x 11 cm) (Imetronic, Bordeaux, France). The boxes were provided with two lines of photocells, one 2 cm above the floor to measure horizontal activity, and the other located 6 cm above the floor to measure vertical activity (rears), in a low luminosity environment (5 lux). Mice were habituated to the locomotor cages during 30 min for 3 consecutive days. On the day 4, mice were placed in the locomotor activity boxes immediately after morphine (5 and 10 mg/kg, s.c.) or vehicle injection, and locomotor activity was recorded during 30 min.

Antinociceptive effects induced by an acute administration of morphine (5 and 10 mg/kg, s.c.) or vehicle were evaluated 30 min after the injection by using the tail-immersion test, as previously described (Simonin *et al.*, 1998). The latency to a rapid tail-flick in the bath (50 ± 0.5ºC) was registered with a cut-off latency of 15 s in order to prevent tissue damage. Subsequently, the hot-plate test (Eddy & Leimbach, 1953) was performed 31 min after morphine (5 and 10 mg/kg, s.c.) or vehicle injection in the same experimental sequence. A glass cylinder was used to maintain the heated surface of the plate, which was kept at a temperature of 52 ± 0.5º C (Columbus Instruments,

Columbus Ohio, USA). The nociceptive threshold was evaluated measuring the licking and the jumping responses, and a cut-off of 240 s was used to prevent tissue damage.

Morphine-induced conditioned place preference

The rewarding effects of morphine were evaluated using the conditioned place preference paradigm, as previously described (Maldonado *et al.,* 1997). The apparatus consisted of two main square conditioning compartments (15 x 15 x 15 cm), with differences in texture and colors, separated by a triangular central area (Matthes *et al.*, 1996). The light intensity within the conditioning chambers was 30 lux. During the pre-conditioning phase, drug-naive mice were placed in the middle of the central area and had free access to both compartments of the apparatus for 18 min. The time spent in each compartment was recorded by computerized monitoring software (Videotrack; View Point, Lyon, France). During the conditioning phase, mice received alternating injections of morphine (5 or 10 mg/kg, s.c.) or vehicle and were immediately confined into one of the two conditioning compartments during 20 min. Three pairings were carried out with morphine and three pairings with vehicle on alternate days. Treatments were counterbalanced as closely as possible between compartments. Control animals received vehicle every day. The post-conditioning phase was conducted exactly as the pre-conditioning phase, i.e. free access to each compartment for 18 min.

Conditioned place aversion to morphine withdrawal

Dysphoric effects of morphine withdrawal were investigated using the conditioned place aversion paradigm, as previously reported (Valverde *et al.*, 1996). Naloxone-induced place aversion in morphine-dependent mice was evaluated on the same apparatus described for the previous experiment. The pre-conditioning phase (day

1) was performed in the same way as in the place preference experiment and animals were free of drugs. The day after the pre-conditioning phase was conducted, opioid dependence was induced by intraperitoneal (i.p.) administration of increasing doses of morphine (from 20 to 100 mg/kg, i.p.) twice a day at 10:00 and 19:00 h during 7 consequtive days; 20 and 20 mg/kg on day 2; 40 and 40 mg/kg on day 3; 60 and 60 mg/kg on day 4; 80 and 80 mg/kg on day 5; 100 and 100 mg/kg on day 6, 7 and 8. A control group of animals received saline by using the same injection schedule. On day 7, animals received the morning morphine (100 mg/kg, i.p.) or saline injection, and 2h later naloxone (0.05 or 0.1 mg/kg, s.c.) was administered and the animal was confined in the corresponding compartment during 15 min. On day 8, 2 h after morphine (100 mg/kg, i.p.) or saline injection, animals received saline and were then confined in the other compartment. On day 9, animals did not receive any treatment. The post-conditioning phase (day 9) was performed in the same way as in the pre-conditioning phase (free access to both compartments of the apparatus during 18 min).

Microdialysis studies

Surgery

One day prior to microdialysis, mice were anaesthetized with isofluorane (3.5-4.5%) and a microdialysis guide cannula (CMA 7, CMA microdialysis, Solna, Sweden) was stereotaxically implanted targeting the NAc. Each animal was placed on a heated mat, supported in a stereotaxic frame and a small bore hole drilled in the skull. A microdialysis guide cannula was implanted at coordinates relative to bregma and skull (anterior-posterior: +1.5 mm, laterial: -0.9 mm, depth: -4.0 mm) targeting the left NAc core-shell border. The cannula was secured to the skull with a single anchor screw and dental acrylic cement. Animals were allowed to recover until they regained their

righting reflex, and then housed individually for subsequent microdialysis. Microdialysis was carried out in freely-moving mice. Animals were equilibrated in a locomotor cage (25.4 x 25.4 x 40.64 cm) 1 h prior to the start of microdialysis. A microdialysis probe (CMA/7/7/1) was connected to a microdialysis system (CMA/120) and perfused with artificial cerebrospinal fluid (aCSF: NaCl, 145 mM; KCL, 2.8 mM; $CaCl_2$, 1.2 mM, $MgCl_2$, 1.2 mM). Under light and brief isofluorane anesthesia the probe was gently inserted into the guide cannula. The final depth of the probe relative to the skull was -5.0 mm. The probe was perfused with aCSF at a flow rate of 1 µl/min, dialysate collected for 2 h and discarded to ensure a stable basal DA level. Subsequently, five consecutive dialysate fractions (F3-7) were collected at intervals of 20 min to monitor basal DA release. Animals were then injected with 0.9% saline (5 ml/kg, s.c.), morphine (20 mg/kg, s.c.) or cocaine (20 mg/kg, s.c). Sample fractions were collected at 20 min intervals for 120 min (F8-13). All sample fractions were collected directly into 35 µl of mobile phase (NaH_2PO_4, 0.05 M; OSA, 0.8 mM; EDTA, 0.1 mM; methanol 10% (v/v), adjusted to pH 3.3 with orthophosphoric acid) and frozen immediately in dry ice. After the completion of the microdialysis procedure, mice were killed by cervical dislocation, the brains were removed and frozen in isopentane with dry ice (-20ºC to -30ºC) for verification of probe placement by histological examination.

Histology

Coronal cryostat sections (20 µm) were cut from each brain using a cryostat (Zeiss Microm 505E). Probe placement was checked visually using hematoxilin and eosin histological staining. Only data obtained from animals with correct probe tracts were used for analysis.

Dopamine Analysis

DA concentration in the dialysate was determined by high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of an ESA582 pump, ESA542 refrigerated automatic sampler, ESA5020 screening guard cell (E = +400 mV), ESA5014B dual potential coulometric microdialysis cell, CoulochemII electrochemical detector (ESA Analytical Ltd, UK) and a Waters spherisorb ODS2 (100 mm x 4.6 mm x 5 μm) analytical column (Waters Ltd, UK) protected by a guard column (Phenomenex, UK). The mobile phase (see surgery section), filtered through a 0.2 μm nylon membrane and degassed, was pumped at a flow rate of 1 ml/min. All reagents used for the mobile phase were of analytical grade. DA was detected on a dual porous graphite electrode system (first electrode reduction potential E_1 = -70 mV, working electrode oxidation potential E_2 = +340 mV). Under these conditions DA had a retention time of 9.2 min with a detection limit of 0.2 nM. Dialysate DA levels were quantified by external standard curve calibration, using peak area for quantification; data were not corrected for probe recovery.

Statistical analysis

Acute effects of morphine were compared by using two-way ANOVA (genotype and treatment as factors of variation) between subjects, followed by one-way ANOVA and Dunnet *post-hoc* comparisons when required. For the conditioned place preference and place aversion experiments, paired two-tailed Student's t-tests were made between the post-conditioning and pre-conditioning time spent in the drug paired compartment. Data from the microdialysis study (as absolute values) were analysed using a three-way ANOVA with fraction (F), genotype (G) and treatment (T) as between factors of variation.

Results

Acute effects induced by morphine

The acute administration of morphine (5 and 10 mg/kg) induced a similar enhancement of locomotor activity and antinociceptive responses in the tail-immersion and hot-plate tests in both genotypes. Two-way ANOVA revealed treatment effects in all the acute responses, but not interaction between treatment and genotype (Table 1). One-way ANOVA calculated for ambulatory movements (Fig 1a) showed a significant effect of treatment in WT ($F [2.29] = 59.414$; $p < 0.01$) and KO mice ($F [2.29] = 19.326$; $p < 0.01$). Post-hoc analysis showed differences in morphine-treated WT and KO mice at the dose of 5 ($p < 0.01$ for WT; $p < 0.05$ for KO) and 10 mg/kg ($p < 0.01$ in all the cases) when compared to saline-treated mice. One-way ANOVA calculated for total horizontal activity (Fig 1b) showed a significant effect of treatment in WT ($F [2.29] = 26.954$; $p < 0.01$) and KO mice ($F [2.29] = 12.541$; $p < 0.01$). Post-hoc analysis showed differences in morphine-treated WT and KO mice at the dose of 10 mg/kg ($p < 0.01$) when compared to saline-treated mice. One-way ANOVA calculated for vertical activity (Fig 1c) showed a significant effect of treatment in WT ($F [2.28] = 11.487$; $p < 0.01$) and KO mice ($F [2.29] = 4.188$; $p < 0.05$). Post-hoc analysis showed differences in morphine-treated WT mice at the doses of 5 and 10 mg/kg ($p < 0.01$), as well as in morphine-treated KO mice at the dose of 10 mg/kg ($p < 0.05$) when compared to saline-treated mice. No difference was revealed between genotypes.

The antinociceptive effects of morphine (5 and 10 mg/kg) were evaluated in A_{2A} KO mice and WT littermates by using the tail-immersion (tail withdrawal latency) and the hot-plate test (licking and jumping responses). Two-way ANOVA for the responses in the tail-immersion test reveled a treatment effect, without genotype effect and not interaction between treatment and genotype (Table 1). One-way ANOVA calculated for

tail withdrawal latencies showed a significant effect of treatment in WT (F [2.28] = 42.652; $p < 0.01$) and KO mice (F [2.29] = 21.192; $p < 0.05$). Post-hoc analysis showed differences in morphine-treated WT and KO mice at the doses of 5 ($p < 0.05$ for KO mice; $p < 0.01$ for WT) and 10 mg/kg ($p < 0.01$ in both genotypes) when compared to saline-treated mice (Fig 2a). Two-way ANOVA calculated for the licking latency in the hot-plate test showed a treatment effect, but not interaction between treatment and genotype (Table 1). One-way ANOVA showed a significant effect of treatment in KO mice (F [2.29] = 5.963; $p < 0.01$), but not in WT animals (F [2.29] = 3.019; n.s.). Post-hoc analysis showed differences in morphine-treated KO mice at the dose of 10 mg/kg ($p < 0.05$) when compared to saline-treated mice (Fig 2b). Two-way ANOVA calculated for the jumping responses in the hot-plate showed a treatment effect, but not genotype effect and not interaction between treatment and genotype (Table 1). Subsequent one-way ANOVA showed a significant effect of treatment in WT (F [2.29] = 287.530; $p < 0.01$) and KO mice (F [2.29] = 221.705; $p < 0.01$). Post-hoc analysis showed differences in morphine-treated WT and KO mice at the doses of 5 and 10 mg/kg ($p < 0.01$ in all the cases) when compared to saline-treated mice (Fig 2c).

Morphine-induced conditioned place preference

Rewarding responses induced by morphine (5 and 10 mg/kg) were evaluated in WT and A_{2A} KO mice using the place conditioning paradigm. One-way ANOVA revealed a similar time spent in the drug-associated compartment during the pre-conditioning phase in the different groups (F [5.86] = 1.160; n.s.), ensuring the use of an unbiased procedure (Fig 3). A significant rewarding effect of morphine was observed in WT at the both doses of morphine used (5 and 10 mg/kg), but not in mice lacking the A_{2A} adenosine receptor. Accordingly, WT mice conditioned with 5 (t [1.9] = -6.903, $p <$

0.01) and 10 mg/kg (t [1.11] = -3.186; $p < 0.01$) of morphine spent significantly more time in the drug-associated compartment during the post-conditioning phase than during the pre-conditioning phase. In contrast, A_{2A} KO mice receiving 5 and 10 mg/kg of morphine spent the same time in the drug-associated compartment during both phases (Fig 3).

Naloxone-induced place aversion in morphine-dependent mice

Naloxone-induced aversive effects in morphine-dependent mice were measured by using the place conditioning paradigm. One-way ANOVA revealed a similar time spent in the drug-associated compartment during the pre-conditioning phase in the different groups (F [11,153] = 0.233; n.s.), ensuring the use of an unbiased procedure (Fig.4). Naloxone 0.1 mg/kg (s.c.) induced a conditioned place aversion in morphine-dependent WT mice, as revealed by a significant decrease of the time spent in the drug-associated compartment during the post-conditioning vs. the pre-conditioning phase (t [1.13] = 4.410, $p < 0.01$). In contrast, morphine-dependent A_{2A} KO receiving such a dose of naloxone spent the same time in the drug-associated compartment during both phases. The administration of a lower dose of naloxone (0.05 mg/kg) did not induce aversive responses in morphine-dependent WT nor in KO animals (Fig 4).

Absence of morphine-induced effects on extracellular levels of dopamine in the nucleus accumbens

The extracellular levels of DA in the NAc were assessed by microdialysis in freely-moving WT and adenosine A_{2A} receptor KO mice ($n = 5$ for saline-treated WT mice, $n = 6$ for saline-treated KO mice, $n = 6$ for morphine-treated WT mice, $n = 7$ for morphine-treated KO mice). Basal extracellular DA levels in the NAc measured over

five dialysate fractions (F3-7) were comparable in WT (1.29 ± 0.32 nM, *n* = 11) and KO mice (1.08 ± 0.16 nM, *n* = 13) (Fig 5; genotype; *P = 0.450*, n.s.). Administration of morphine (20 mg/kg, s.c.) had no effect on extracellular DA levels in the NAc of either treated WT or KO mice compared to saline treated groups (Fig 5) despite inducing the marked behavioral responses of hyperlocomotion and straub tail (data not shown). Further studies using different probe target co-ordinates (left lateral shell of NAc anterior-posterior: +0.98 mm, laterial: -1.7 mm, depth: -5.0 mm) or prolonged time between microdialysis surgery and the day of the experiment (5 day protocol; day 1, implant cannula, day 3, implant probe, day 5, commence experiment) at two doses of morphine (10 mg/kg and 20 mg/kg) also failed to show any effect of morphine administration on extracellular DA in the NAc of WT or KO mice (data not shown). However, under the same experimental conditions, administration of cocaine (20 mg/kg, s.c.) to WT and KO mice produced a significant increase in extracellular DA levels in the NAc compared to saline-treated groups (treatment; *P < 0.001*) confirming method validation. The maximal increase of accumbal DA induced by cocaine in WT and KO mice was comparable between genotypes (268.3 % and 293.3 %, respectively) (manuscript in preparation).

Discussion

This study evaluates the participation of A_{2A} receptors in behavioral and neurochemical responses related to morphine addictive effects, including reward and the dysphoric effects associated to naloxone precipitated morphine withdrawal. We also investigated acute responses of morphine on nociception and locomotion to assure that motivational and neurochemical responses observed were not influenced by changes in acute morphine effects. Mice lacking A_{2A} receptors exhibit similar acute responses after morphine administration compared to WT littermates. However, both rewarding and aversive effects associated to morphine abstinence were completely abolished in mice lacking the A_{2A} receptors.

Deletion of A_{2A} receptors did not modify acute effects induced by morphine. A hypoalgesic phenotype was previously reported, but the thermal stimulus was stronger than that used in the present study (Ledent *et al.*, 1997). Thus, changes in basal nociceptive responses in A_{2A} KO mice are not robust when using the tail-immersion test at 50ºC, and several studies now suggest that the hypoalgesic phenotype is dependent on the intensity of the stimulus used (for review see Ferré *et al.*, 2007). Our data demonstrate that A_{2A} receptors do not participate in the acute effects induced by morphine.

Morphine-induced rewarding responses were abolished in A_{2A} KO mice. A_{2A} receptors are mainly located in striatal neurons where they interact with multiple neurotransmitter systems, being coexpressed with postsynaptic receptors in GABAergic neurons (Fink *et al.*, 1992). Adenosine regulates DA transmission through antagonistic interactions of A_{2A}/dopamine D2 receptors (Franco *et al.*, 2000), and this interaction also modulates glutamate release and thereby affects the striatal neuronal output (Tozzi

et al., 2007). The regulation of A_{2A} receptors on DA transmission is pertinent to the behavioral findings of the present study. Hence, the neurotransmitter DA has been widely implicated as a key regulator of the pharmacological actions and rewarding properties of drugs of abuse (Berridge & Robinson 1998; Spanagel & Weiss 1999; Wise, 2004). In addition, DARPP-32, which plays an obligatory role in dopaminergic transmission, was reported to be altered in mice lacking A_{2A} receptors (Svenningsson *et al.,* 2000), and a large body of evidence support a key role for DARPP-32 dependent signaling in mediating the action of multiple drugs of abuse, including caffeine and morphine (Svenningsson *et al.,* 2005). Mu-opioid receptor agonists, such as morphine, have been reported to increase DA release preferentially in the NAc by the disinhibition of γ-aminobutyric acid neurons (Di Chiara & Imperato 1988; Johnson & North 1992). Microdialysis experiments in freely-moving WT and adenosine A_{2A} receptor KO mice revealed no difference between genotypes in basal or saline-evoked extracellular DA levels in the NAc in comparison to previous reports of hypodopaminergic activity in the striatum of KO mice (Dassesse *et al.,* 2001). Despite producing recognized behavioral responses of hyperlocomotion and straub tail, (Rethy *et al.,* 1971; Gupta *et al.,* 1988) morphine up to 20 mg/kg failed to increase extracellular DA release in either the core or shell of the NAc. In contrast both genotypes displayed a robust and reliable increase in accumbal DA in response to cocaine making clear that these mice do respond to dopamimetic agents. Others have shown morphine-induced DA release in a C57/B6 mouse strain (Chefer *et al.,* 2003) and the lack of a DA response in the current study might be attributable to the outbred background strain of these mice (CD1) as previous studies have suggested that differences in the background strain of experimental models can result in a variable sensitivity to drugs of abuse (Barbaccia *et al.,* 1981; Shoaib *et al.,* 1995; He & Shippenberg 2000; Fadda *et al.,* 2005). Interestingly the CD1 strain has

been previously characterized as ethanol-avoiding (Short *et al.,* 2006). This suggests that the CD1 strain may have a differential sensitivity to drugs of abuse explaining its weak response to the neurochemical effects of morphine in the NAc.

It is known that mu-opioid receptors are responsible for morphine-induced motivational responses (Matthes *et al.,* 1996). Until now, no study has investigated the role of A_{2A} receptors related to aversive responses developed during morphine withdrawal. Interestingly, our findings demonstrate that the absence of A_{2A} receptors impair the aversive responses exhibited during abstinence. As we have shown no significant changes in mu-opioid receptor binding in brains from naloxone-precipitated withdrawn A_{2A} KO mice (Bailey *et al.,* 2004), and a lack of change in mu-opioid receptor binding also in naive A_{2A} KO mice (Bailey *et al.,* 2002), compensatory changes in the expression of opioid receptors in A_{2A} KO mice are unlikely to account for the absence of dysphoric effects related to abstinence. However, a significant increase in the level of mu-opioid receptor-stimulated [^{35}S]GTPγS binding was reported in the NAc but not in other brain structures of morphine abstinent mutant mice (Bailey *et al.,* 2004). It is therefore possible that the increase of mu-opioid receptor G-protein activity in the NAc might be a compensatory mechanism undertaken to increase levels of extracellular DA that are extremely low in A_{2A} KO mice during the morphine withdrawal syndrome. This increase in DA neurotransmission, through the enhancement of intracellular transduction mechanisms associated with mu-opioid receptor activation, could be responsible for the lack of dysphoric effects observed during morphine withdrawal in A_{2A} KO mice and could progressively appear as an adaptive process whilst physical dependence is developed.

Motor and motivational responses to opioids have been reported to be closely related (Salamone, 1996). In our case, the absence of place preference and place

aversion were not dependent on the locomotor impairment of A_{2A} KO mice as these animals preserve the motor response to morphine (Fig 1). In a previous study, using a similar procedure to induce morphine dependence, we reported an enhancement in the physical expression of the morphine withdrawal syndrome in mice lacking A_{2A} receptors as revealed by a higher global withdrawal score in KO morphine-dependent mice (Berrendero *et al.,* 2003; Bailey *et al.,* 2004). In agreement with these findings, pharmacological studies showed that A_{2A} selective adenosine receptor agonists decreased the incidence of some morphine withdrawal signs, whereas the administration of adenosine antagonists produced opposite responses (Salem & Hope, 1997; Zarrindast *et al.,* 1999). In addition, electrophysiological studies have reported that the non-selective adenosine antagonist caffeine enhances the electrical activity recorded in the nucleus paragigantocellularis (Khalili *et al.,* 2001), a brain structure which participates in the expression of the somatic expression of opioid withdrawal syndrome (Rasmussen & Aghajanian 1989).

Our results demonstrate a clear dissociation between the mechanisms involved in the motivational properties of opiates, which are related to their addictive capacities, and the somatic signs of naloxone-precipitated withdrawal syndrome, which reveal the physical component of opiate dependence. Thus, adenosine A_{2A} receptors appear to be crucial for rewarding and aversive effects of opiates and for the expression of the physical opiate dependence, modulating the motivational effects and somatic abstinence in opposite directions. Our findings also support the belief that receptors involved in addictive behaviors induced by prototypic drugs require activation of A_{2A} adenosine receptors, just as it has been shown for ethanol operant self-administration (Arolfo *et al.,* 2004). Further, we have also demonstrated that A_{2A} KO mice elicit a decreased rate of self-administration and motivation for cocaine and decrease the rewarding effects of

nicotine and Δ9-tetrahydrocannabinol (Soria *et al.*, 2004; Castañé *et al.*, 2006; Soria *et al.*, 2005). The current findings now show evidence that A_{2A} adenosine receptors are also required for the expression of morphine rewarding responses and the negative motivational effects related to morphine withdrawal syndrome. We conclude that adenosine acting through the A_{2A} adenosine receptors represent a general system for modulating addictive behaviors and support the possibility that pharmacological manipulation of these receptors may represent a new target in the management of drug addiction.

Acknowledgements: This study was supported by grants from Spanish MEC (SAF2004/0568 and SAF2007/60249) and MSC (PNSD-2006), European Communities (GENADDICT LSHM-CT-2004-005166). The Interuniversity Attraction Poles Programme (P6-14) - Belgian State - Belgian Science Policy, the Fondation Médicale Reine Elisabeth, and the Fonds National Belge de la Recherche Scientifique. Authors kindly acknowledge Dr. Don Fisher (Roehampton University) for his assistance with HPLC-EC analysis.

Legends of the Figures

Figure 1. Acute locomotor effects induced by morphine in A_{2A} KO and WT mice. Locomotor activity was evaluated during 30 min after acute morphine injection (0; 5 and 10 mg/kg, s.c.). Measurements of (a) ambulatory movements (b) total horizontal movements (ambulatory plus minor movements) and (c) vertical movements were performed. Data are expressed as mean ± SEM of locomotor activity counts (n = 10 in all groups). White columns represent WT mice and black columns represent A_{2A} adenosine receptor KO mice. ★ $p < 0.05$; ★★ $p < 0.01$ versus vehicle (Dunnet test).

Figure 2. Acute antinociceptive effects induced by morphine in A_{2A} KO and WT mice. Antinociceptive responses in the tail-immersion (a) and hot-plate (b and c) tests were measured 30 min after morphine administration (0; 5 and 10 mg/kg, s.c.). Results are expressed as mean ± SEM of latency time in seconds (n = 10 in all groups). ★ $p < 0.05$; ★★ $p < 0.01$ versus vehicle (Dunnet test).

Figure 3. Morphine-induced conditioned place preference in A_{2A} KO and WT mice. Results are expressed as mean ± S.E.M. of time spent in the drug-paired compartment (sec) during the pre-conditioning (white bars) and post-conditioning phase (black bars) in WT and KO mice after morphine 5 mg/kg (MOR 5), morphine 10 mg/kg (MOR 10) or saline (SAL) administration (SAL WT, n = 22; SAL KO, n = 22; MOR 5 WT, n = 10; MOR 5 KO, n = 9; MOR 10 WT, n = 12; MOR 10 KO, n = 12). ★★ $p < 0.01$ versus pre-conditioning phase (Student paired t-test).

Figure 4. Naloxone-induced conditioned place aversion in morphine-dependent A_{2A} KO and WT mice. Results are expressed as mean ± S.E.M. of time spent in the drug-paired compartment (sec) during the pre-conditioning (white bars) and post-conditioning phase (black bars) in saline (SAL) and morphine (MOR) treated WT and KO mice after saline (SAL), naloxone 0.05 mg/kg (NAL 0.05) or naloxone 0.1 mg/kg (NAL 0.1) administration (SAL-SAL WT, n = 11; SAL-SAL KO, n = 13; SAL-NAL 0.05 WT, n = 12; SAL-NAL 0.05 KO, n = 13; SAL-NAL 0.1 WT, n = 10; SAL-NAL 0.1 KO, n = 11; MOR-SAL WT, n = 11; MOR-SAL KO, n = 13; MOR-NAL 0.05 WT, n = 14; MOR-NAL 0.05 KO, n = 14; MOR-NAL 0.1 WT, n = 14; MOR-NAL 0.1 KO, n = 15). ★★ $p < 0.01$ versus pre-conditioning phase (Student paired t-test).

Figure 5. Effects of the systemic morphine administration on dialysate DA concentrations in the NAc of A_{2A} KO and WT mice. Morphine (20 mg/kg, s.c.) did not increase DA output in the NAc of WT (vehicle, SAL n = 5; morphine, MOR n = 6) and KO mice (vehicle, SAL n = 6; morphine, MOR n = 7) in any dialysis fraction (F8-F13). Data are percentages of pre-treatment (basal) values and are given as mean + SEM values (see methods section for details).

References

Arolfo MP, Yao L, Gordon AS, Diamond I, Janak PH (2004). Ethanol operant self-administration in rats is regulated by adenosine A2 receptors. *Alcohol Clin Exp Res* **28**: 1308-1316.

Bailey A, Davis L, Lesscher HM, Kelly MD, Ledent C, Hourani SM, Kitchen I (2004). Enhanced morphine withdrawal and micro-opioid receptor G-protein coupling in A2A adenosine receptor KO mice. *J Neurochem* **88**: 827-834.

Bailey A, Ledent C, Kelly M, Hourani SM, Kitchen I (2002). Changes in spinal delta and kappa opioid systems in mice deficient in the A2A receptor gene. *J Neurosci* **22**: 9210-9220.

Barbaccia ML, Reggiani A, Spano PF, Trabucchi M (1981). Ethanol-induced changes of dopaminergic function in three strains of mice characterized by a different population of opiate receptors. *Psychopharmacology* **74**: 260-262.

Berrendero F, Castañé A, Ledent C, Parmentier M, Maldonado R, Valverde O (2003). Increase of morphine withdrawal in mice lacking A2a receptors and no changes in CB1/A2a double KO mice. *Eur J Neurosci* **17** : 315-324.

Berridge KC, Robinson TE (1998). What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience?. *Brain Res Brain Res Rev* **28**: 309-369.

Castañé A, Soria G, Ledent C, Maldonado R, Valverde O (2006). Attenuation of nicotine-induced rewarding effects in A2A knockout mice. *Neuropharmacology* **51**: 631-640.

Chefer VI, Kieffer BL, Shippenberg TS (2003). Basal and morphine-evoked dopaminergic neurotransmission in the nucleus accumbens of MOR- and DOR-KO mice. *Eur J Neurosci* **18**: 1915-1922.

Dassesse D, Massie A, Ferrari R, Ledent C, Parmentier M, Arckens L, Zoli M, Schiffmann SN (2001). Functional striatal hypodopaminergic activity in mice lacking adenosine A(2A) receptors. *J Neurochem* **78**: 183-198.

Di Chiara G (2002). Nucleus accumbens shell and core dopamine: differential role in behavior and addiction. *Behav Brain Res* **137**: 75-114.

Di Chiara G, Imperato A (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc Natl Acad Sci U S A* **85**: 5274-5278.

Eddy NB, Leimbach D (1953). Synthetic analgesics. II. Dithienylbutenyl- and dithienylbutylamines. *J Pharmacol Exp Ther* **107**: 385-393.

Fadda P, Scherma M, Collu M, Fratta W (2005). Dopamine and serotonin release in dorsal striatum and nucleus accumbens is differentially modulated by morphine in DBA/2J and C57BL/6J mice. *Synapse* **56**: 29-38.

Ferré S, Fredholm BB, Morelli M, Popoli P, Fuxe K (1997). Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia. *Trends Neurosci* **20**: 482-487.

Ferré S, Diamond I, Goldberg SR, Yao L, Hourani SMO, Huang ZL, Urade Y, Kitchen I (2007). Adenosine A2A receptors in ventral striatum, hypothalamus and nociceptive circuitry. Implications for drug addiction, sleep and pain. *Prog Neurobiol* **83**: 332-347.

Fink JS, Weaver DR, Rivkees SA, Peterfreund RA, Pollack AE, Adler EM, Reppert SM (1992). Molecular cloning of the rat A2 adenosine receptor: selective co-expression with D2 dopamine receptors in rat striatum. *Brain Res Mol Brain Res* **14**: 186-195.

Franco R, Ferré S, Agnati L, Torvinen M, Gines S, Hillion J, Casado V, Lledo P, Zoli M, Lluis C, Fuxe K (2000). Evidence for adenosine/dopamine receptor interactions: indications for heteromerization. *Neuropsychopharmacology* **23**: S50-S59.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM (2005). Adenosine and brain function. *Int Rev Neurobiol* **63**: 191-270.

Gupta ML, Nath R, Gupta TK, Gupta GP (1988). A study of central neurotransmitter mechanisms in morphine-induced 'Straub reaction' in mice: role of central dopamine receptors. *Clin Exp Pharmacol Physiol* **15**: 727-732.

Hack SP, Christie MJ (2003). Adaptations in adenosine signaling in drug dependence: therapeutic implications. *Crit Rev Neurobiol* **15**: 235-274.

He M, Shippenberg TS (2000). Strain differences in basal and cocaine-evoked dopamine dynamics in mouse striatum. *J Pharmacol Exp Ther* **293**: 121-127.

Johnson SW, North RA (1992). Two types of neurone in the rat ventral tegmental area and their synaptic inputs. *J Physiology* **450**: 455-468.

Kaplan GB, Coyle TS (1998). Adenosine kinase inhibitors attenuate opiate withdrawal via adenosine receptor activation. *Eur J Pharmacol* **362**: 1-8.

Kaplan GB, Sears MT (1996). Adenosine receptor agonists attenuate and adenosine receptor antagonists exacerbate opiate withdrawal signs. *Psychopharmacology* **123**: 64-70.

Khalili M, Semnanian S, Fathollahi Y (2001). Caffeine increases paragigantocellularis neuronal firing rate and induces withdrawal signs in morphine-dependent rats. *Eur J Pharmacol* **412**: 239-245.

Koob GF (1996). Hedonic valence, dopamine and motivation. *Mol Psychiatry* **1**: 186-189.

Ledent C, Vaugeois JM, Schiffmann SN, Pedrazzini T, El Yacoubi M, Vanderhaeghen JJ, Costentin J, Heath JK, Vassart G, Parmentier M (1997). Aggressiveness, hypoalgesia

and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* **388**: 674-678.

Maldonado R, Saiardi A, Valverde O, Samad TA, Roques BP, Borrelli E (1997). Absence of opiate rewarding effects in mice lacking dopamine D2 receptors. *Nature* **388**: 586-589.

Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dolle P, Tzavara E, Hanoune J, Roques BP, Kieffer BL (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature* **383**: 819-823.

Moreau JL, Huber G (1999). Central adenosine A(2A) receptors: an overview. *Brain Res Brain Res Rev* **31**: 65-82.

Rasmussen K, Aghajanian GK (1989). Withdrawal-induced activation of locus coeruleus neurons in opiate-dependent rats: attenuation by lesions of the nucleus paragigantocellularis. *Brain Res* **505**: 346-350.

Rethy CR, Smith CB, Villarreal JE (1971). Effects of narcotic analgesics upon the locomotor activity and brain catecholamine content of the mouse. *J Pharmacol Exp Ther* **176**: 472-479.

Salamone JD (1996). The behavioral neurochemistry of motivation: methodological and conceptual issues in studies of the dynamic activity of nucleus accumbens dopamine. *J Neurosci Methods* **64**: 137-149.

Salem A, Hope W (1997). Effect of adenosine receptor agonists and antagonists on the expression of opiate withdrawal in rats. *Pharmacol Biochem Behav* **57**: 671-679.

Schiffmann SN, Vanderhaeghen JJ (1993). Adenosine A2 receptors regulate the gene expression of striatopallidal and striatonigral neurons. *J Neurosci* **13**: 1080-1087.

Simonin F, Valverde O, Smadja C, Slowe S, Kitchen I, Dierich A, Le Meur M, Roques BP, Maldonado R, Kieffer BL (1998). Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *EMBO J* **17**: 886-897.

Shoaib M, Spanagel R, Stohr T, Shippenberg TS (1995). Strain differences in the rewarding and dopamine-releasing effects of morphine in rats. *Psychopharmacology* **117**: 240-247

Short JL, Drago J, Lawrence AJ (2006). Comparison of ethanol preference and neurochemical measures of mesolimbic dopamine and adenosine systems across different strains of mice. *Alcohol Clin Exp Res* **30**: 606-620.

Spanagel R, Weiss F (1999). The dopamine hypothesis of reward: past and current status. *Trends Neurosci* **22**: 521-527.

Soria G, Castañé A, Berrendero F, Ledent C, Parmentier M, Maldonado R, Valverde O (2004). Adenosine A2A receptors are involved in physical dependence and place conditioning induced by THC. *Eur J Neurosci* **20**: 2203-2213.

Soria G, Castañé A, Ledent C, Parmentier M, Maldonado R, Valverde O (2006). The reinforcing efficacy of cocaine is diminished in mice lacking A_{2A} adenosine receptors. *Neuropsychopharmacology* **31**: 978-987.

Svenningsson P, Lindskog M, Ledent C, Parmentier M, Greengard P, Fredholm BB, Fisone G (2000). Regulation of the phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa in vivo by dopamine D1, dopamine D2, and adenosine A2A receptors. *Proc Natl Acad Sci U S A* **97**: 1856-1860.

Svenningsson P, Nairn AC, Greengard P (2005). DARPP-32 mediates the actions of multiple drugs of abuse. *AAPS J* **7**: E353-360.

Sweeney MI, White TD, Jhamandas KH, Sawynok J (1987). Morphine releases endogenous adenosine from the spinal cord in vivo. *Eur J Pharmacol* **141**: 169-170.

Sweeney MI, White TD, Sawynok J (1991). Intracerebroventricular morphine releases adenosine and adenosine 3',5'-cyclic monophosphate from the spinal cord via a serotonergic mechanism. *J Pharmacol Exp Ther* **259**: 1013-1018.

Tozzi A, Tscherter A, Belcastro V, Tantucci M, Costa C, Picconi B, Centonze D, Calabresi P, Borsini F (2007). Interaction of A2A adenosine and D2 dopamine receptors modulates corticostriatal glutamatergic transmission. *Neuropharmacology* **53**: 783-789.

Valverde O, Tzavara E, Hanoune J, Roques BP, Maldonado R (1996). Protein kinases in the rat nucleus accumbens are involved in the aversive component of opiate withdrawal. *Eur J Neurosci* **8**: 2671-2678.

Wise RA (2004). Dopamine, learning and motivation. *Nat Rev Neurosci* **5**: 483-494.

Zarrindast MR, Naghipour B, Roushan-zamir F, Shafaghi B (1999). Effects of adenosine receptor agents on the expression of morphine withdrawal in mice. *Eur J Pharmacol* **369**: 17-22.

Table 1. Two-way ANOVA for acute locomotor and antinociceptive responses induced by morphine in A_{2A} adenosine receptor.

	Locomotor activity						Antinociception					
	Ambulatory		Total horizontal		Vertical		Tail-immersion		Hot-plate: Licking		Hot-plate: jumping	
	F	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
Treatment	$F_{(2.54)} = 63.672$	0.01	$F_{(2.54)} = 35.977$	0.01	$F_{(2.53)} = 14.704$	0.01	$F_{(2.53)} = 50.431$	0.01	$F_{(2.54)} = 9.785$	0.01	$F_{(2.54)} = 502.15$	0.01
Genotype	$F_{(1.54)} = 9.526$	0.01	$F_{(1.54)} = 17.606$	0.05	$F_{(1.53)} = 4.523$	0.05	$F_{(1.53)} = 1.884$	n.s.	$F_{(1.54)} = 0.083$	n.s.	$F_{(1.54)} = 1.164$	n.s.
TxG	$F_{(2.54)} = 0.523$	n.s.	$F_{(2.54)} = 0.587$	n.s.	$F_{(2.53)} = 1.749$	n.s.	$F_{(2.53)} = 0.930$	n.s.	$F_{(2.54)} = 0.519$	n.s.	$F_{(2.54)} = 0.291$	n.s.

Two-way ANOVA with treatment (T) and genotype (G) as factors of variation. See Materials and Methods for details.

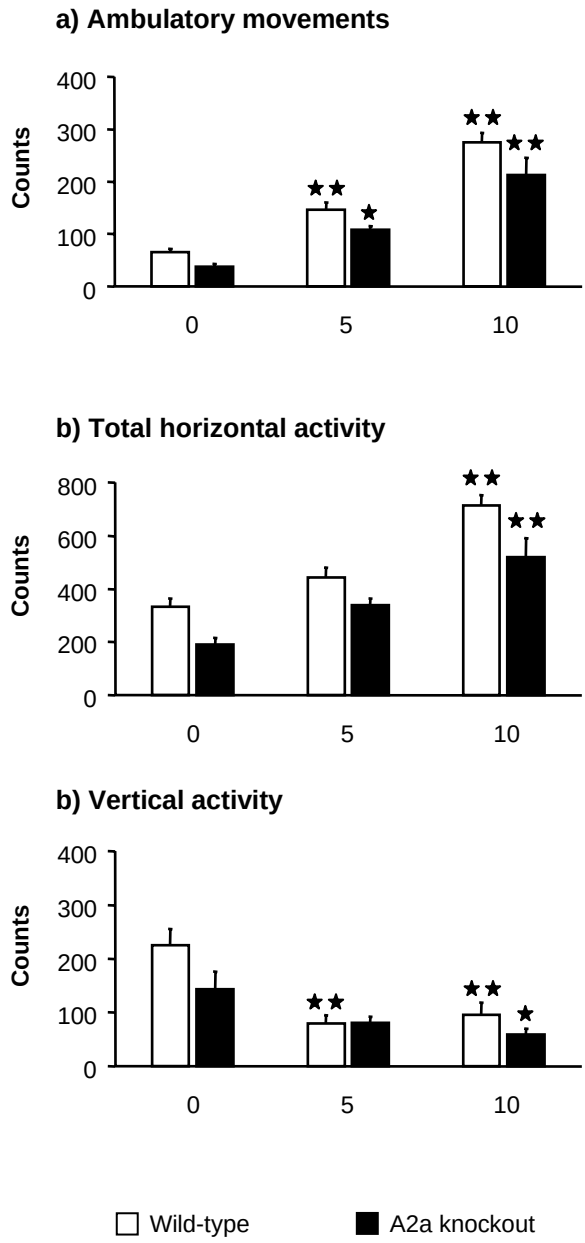

Figure 1. Castañé et al.

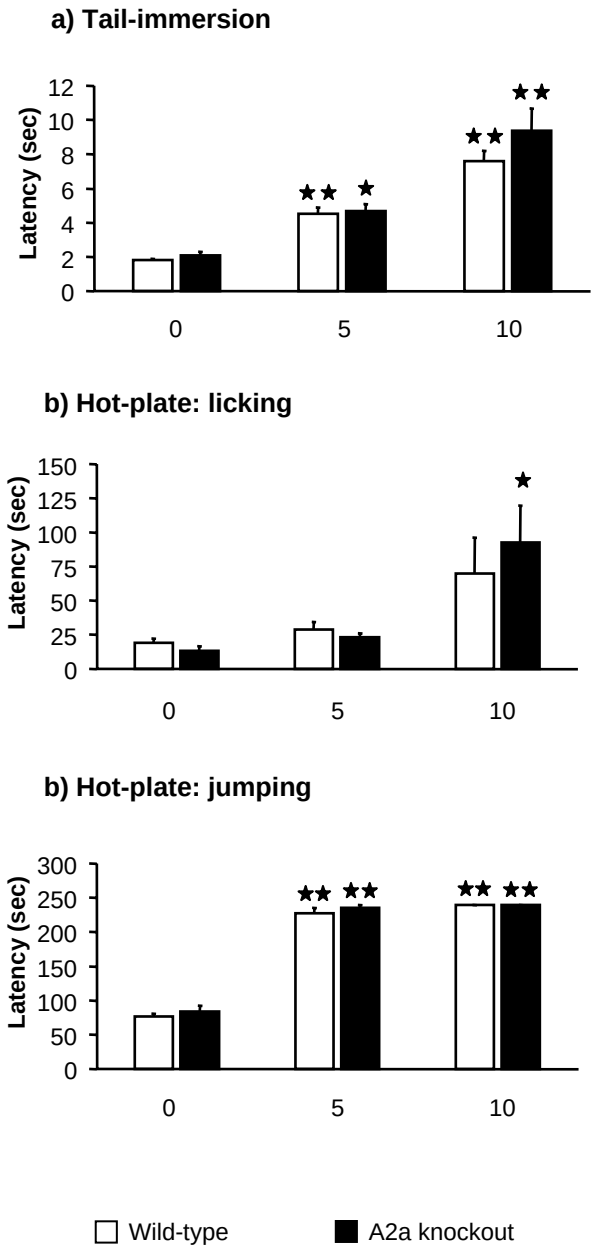

Figure 2. Castañé et al.

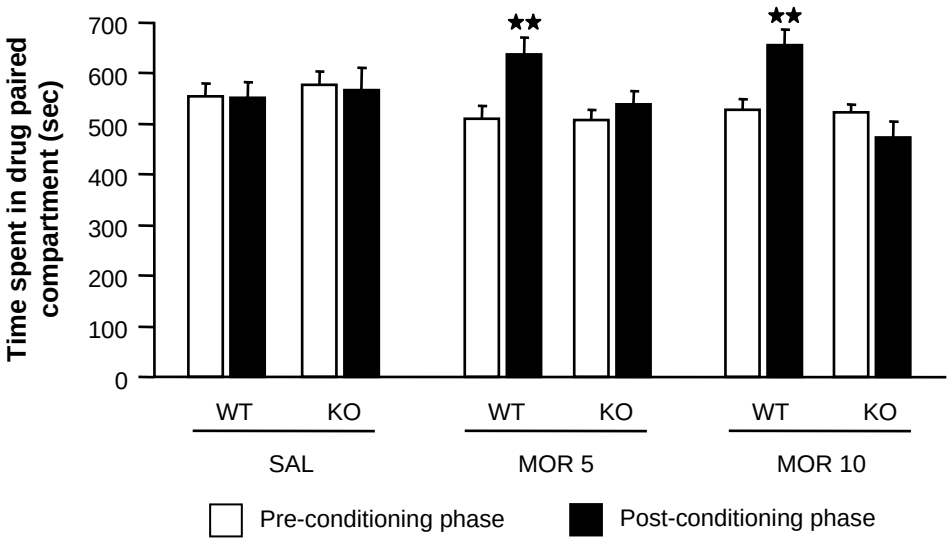

Figure 3. Castañé et al.

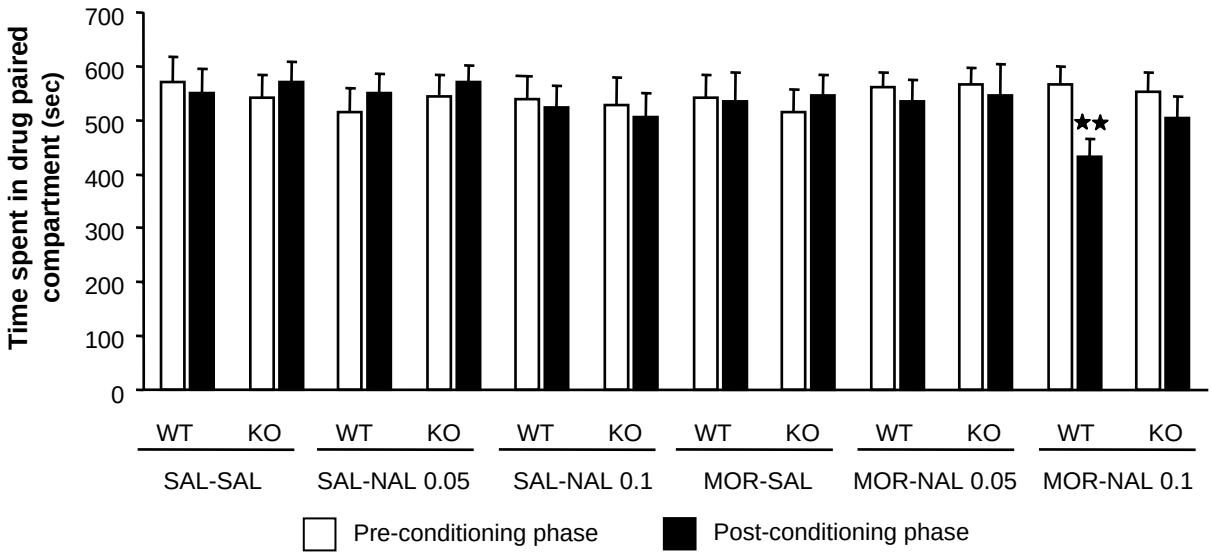

Figure 4. Castañé et al.

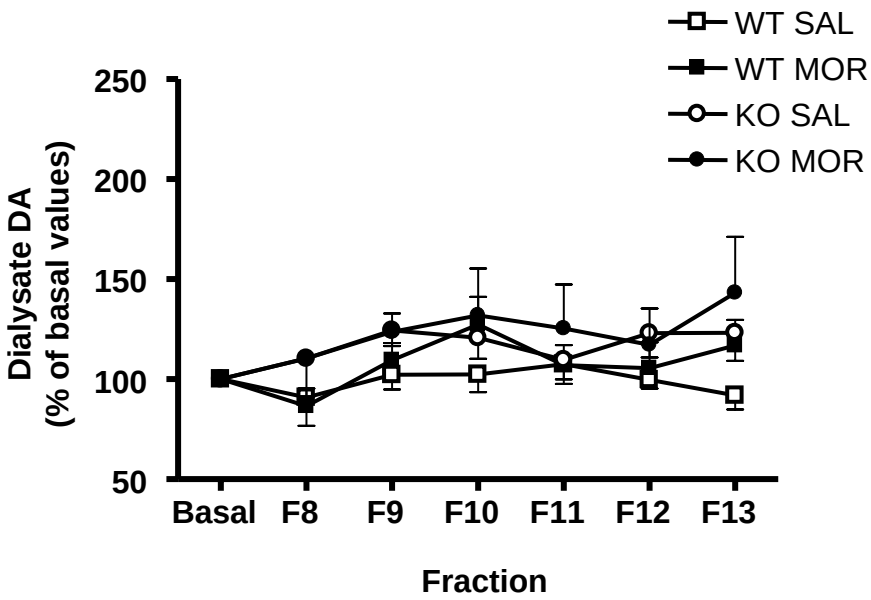

Figure 5. Castañé et al.