Activation of the cAMP/PKA/DARPP-32 Signaling Pathway is Required for Morphine Psychomotor Stimulation but not for Morphine Reward

Anders Borgkvist[1], Alessandro Usiello[1], Paul Greengard[2] and Gilberto Fisone*[1,2,3]

[1]Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden; [2]Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY, USA; [3]CEINGE, Biotecnologie avanzate, Naples, Italy

Activation of the cAMP/PKA pathway in the dopaminoceptive neurons of the striatum has been proposed to mediate the actions of various classes of drugs of abuse. Here, we show that, in the mouse nucleus accumbens and dorsal striatum, acute administration of morphine resulted in an increase in the state of phosphorylation of the dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) at Thr34, without affecting phosphorylation at Thr75. The ability of morphine to stimulate Thr34 phosphorylation was prevented by blockade of dopamine D1 receptors. DARPP-32 knockout mice and T34A DARPP-32 mutant mice displayed a lower hyperlocomotor response to a single injection of morphine than wild-type controls. In contrast, in T75A DARPP-32 mutant mice, morphine-induced psychomotor activation was indistinguishable from that of wild-type littermates. In spite of their reduced response to the acute hyperlocomotor effect of morphine, DARPP-32 knockout mice and T34A DARPP-32 mutant mice were able to develop behavioral sensitization to morphine comparable to that of wild-type controls and to display morphine conditioned place preference. These results demonstrate that dopamine D1 receptor-mediated activation of the cAMP/DARPP-32 cascade in striatal medium spiny neurons is involved in the psychomotor action, but not in the rewarding properties, of morphine.
Neuropsychopharmacology (2007) **32,** 1995–2003; doi:10.1038/sj.npp.1301321; published online 24 January 2007

Keywords: locomotor activity; behavioral sensitization; conditioned-place preference; dopamine D1 receptor; mouse

INTRODUCTION

One common property shared by drugs of abuse is their ability to promote dopaminergic transmission within the ventral striatum or nucleus accumbens (Di Chiara and Imperato, 1988). This brain region is involved in the control of motor activity and is particularly important in the generation of motivated behavior in response to addictive substances. Opioid receptor agonists, such as morphine and heroin, are known to increase the firing rate of midbrain dopaminergic neurons. This effect, which is mediated via inhibition of GABAergic interneurons within the ventral tegmental area (Johnson and North, 1992), leads to increased release of dopamine in the nucleus accumbens. Activation of dopaminergic receptors located on striatal medium spiny neurons is involved in the acute hyperloco-motor effect of morphine. Thus, the increase in locomotion

elicited by morphine is prevented by systemic administration of SCH23390, a dopamine D1 receptor antagonist (Jeziorski and White, 1995; Longoni *et al*, 1987; Serrano *et al*, 2002), and absent in dopamine D1 receptor knockout mice (Becker *et al*, 2001).

Repeated administration of morphine results in a gradual enhancement in the motor stimulant properties of this drug. This behavioral sensitization is believed to reflect some of the motivational aspects of drug addiction, such as craving and drug-seeking (Robinson and Berridge, 1993). Studies performed using dopamine D1 and D2 receptor antagonists indicate that the development of psychomotor sensitization to opioid receptor agonists is independent of dopamine (Jeziorski and White, 1995; Kalivas, 1985; Vezina and Stewart, 1989). In contrast, the expression of morphine sensitization appears to require activation of dopamine D1 receptors (Becker *et al*, 2001; Jeziorski and White, 1995).

In the striatum, activation of dopamine D1 receptors leads to G_{olf} mediated stimulation of adenylyl cyclase and increased activity of cAMP-dependent protein kinase (PKA) (Corvol *et al*, 2001; Zhuang *et al*, 2000). Phosphory-lation of the dopamine- and cAMP-regulated phospho-protein of 32 kDa (DARPP-32) at the PKA site, Thr34, results in inhibition of protein phosphatase-1 (Hemmings

*Correspondence: Dr G Fisone, Department of Neuroscience, Karolinska Institutet, Retzius väg 8, 17177 Stockholm, Sweden, Tel: +46 8 52487375, Fax: +46 8 320988, E-mail: gilberto.fisone@ki.se

et al, 1984). This prevents dephosphorylation of downstream target proteins, including glutamate receptors and voltage-dependent calcium and sodium channels (Greengard *et al*, 1999), ultimately potentiating responses produced by activation of the cAMP cascade. DARPP-32 can also be converted into an inhibitor of PKA via phosphorylation at Thr75, catalysed by cyclin-dependent kinase 5 (Bibb *et al*, 1999).

In this study, we have examined the possible involvement of DARPP-32 in the acute motor stimulant effect of morphine, and in morphine behavioral sensitization. In addition, we have used the conditioned place preference (CPP) paradigm to address the question of the possible role of DARPP-32 in the rewarding properties of morphine.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (20–30 g) were obtained from Scanbur BK (Sollentuna, Sweden). Wild-type and DARPP-32 knockout mice (Fienberg *et al*, 1998) were generated from the offspring of *DARPP-32$^{+/+}$ × DARPP-32$^{+/+}$* and *DARPP-32$^{-/-}$ × DARPP-32$^{-/-}$* mating pairs. These mating pairs were obtained from heterozygous mice, which were backcrossed for at least 20 generations on a C57BL/6 background. *DARPP-32$^{+/+}$ × DARPP-32$^{+/+}$* and *DARPP-32$^{-/-}$ × DARPP-32$^{-/-}$* mating was performed separately for no more than two generations. Mice bearing a mutation in which Thr34 or Thr75 were replaced by a nonphosphorylatable Ala (T34A and T75A mutant mice, respectively) (Svenningsson *et al*, 2003) were obtained from heterozygous animals generated from C57BL/6 × 129SV hybrids bred for one generation on a C57BL/6 background. Mice were age matched, and both female and male offspring were used. All mice were housed in groups of four to six in a colony room under standardized conditions, with lights on at 0600 (12 h light/dark cycle) and an ambient temperature of $20 \pm 0.5°C$ (40–50% relative humidity). All experiments were carried out during the light phase. All experiments were approved by the Swedish Animal Welfare Agency.

Drugs

Morphine-HCl (Apoteket AB, Stockholm, Sweden) and (R)-2,3,4,5-tetrahydro-8-chloro-3-methyl-5-phenyl-1H-3-benzazepin-7-ol (SCH 23390; a gift from Dr E Ongini, Schering-Plough, Milan, Italy) were dissolved in physiological saline (0.9% NaCl) and were administered as subcutaneous or intraperitoneal injections (1 ml/kg body weight), respectively. When mice were not treated with drug they received an equivalent volume of vehicle.

Measurement of Locomotor Activity

Mice were injected with morphine or vehicle and immediately placed in locomotor activity boxes (40 × 20 × 20 cm) under moderate illumination (50 lux). Horizontal locomotor activity was recorded using a Videotrack system from Viewpoint SA (Champagne au Mont d'Or, France) and expressed as distance covered by an animal during various periods of time. Morphine, given either acutely or chronically, induced larger motor responses in the wild-type groups used to evaluate the response of DARPP-32 knockout mice than in those used to evaluate the response of T34A and T75A DARPP-32 mutant mice. This effect is most likely attributable to the different genetic background of the strains of mice used in the experiments. DARPP-32 knockout mice and corresponding wild-type were fully backcrossed on a C57BL/6 background. In contrast, T34A and T75A DARPP-32 mutant mice had a mixed background (75% C57BL/6 and 25% 129SV).

Behavioral Sensitization Procedure

Psychomotor sensitization was induced by treating the mice for 9 consecutive days with 9 mg/kg of morphine. The animals were removed from their home cages, injected, and placed in individual cages (40 × 20 × 20 cm) for 1 h. Motor activity was monitored on various days as indicated. At the end of the sensitization procedure, wild-type mice were divided into two groups, one receiving a morphine challenge and the other receiving vehicle. The animals were killed 30 minutes after injection and the tissue was processed for biochemical studies (see below).

Morphine Conditioned Place Preference

The CPP apparatus consisted of two compartments (20 × 20 × 50 cm) with different floor textures, one smooth and the other grooved. A removable door separated the two compartments from each other. The apparatus was kept under medium-high illumination (70 lux). Tests were performed as previously reported (Acquas and Di Chiara, 1994). Each experiment consisted of three phases. Spontaneous preference was evaluated in the pre-conditioning phase, consisting of 2 daily sessions during which each mouse was given free access to both compartments of the apparatus for 15 min. The average time spent in each compartment during the pre-conditioning phase was used to determine individual unconditioned preferences. During the conditioning phase, mice were administered morphine and placed for 30 min in the 'non-preferred' compartment. On the next day, the animals received saline and were placed in the 'preferred compartment'. This procedure was repeated for 8 days so that each animal received a total of 4 injections with morphine paired to one specific compartment. Control animals were injected only with saline during the entire conditioning phase. During the post-conditioning phase, mice had free access to both compartments and the time spent by each animal in the morphine-paired compartment was recorded during 15 min. The difference in seconds between the time spent in the drug-paired compartment and that spent in the pre-conditioning test was considered as the degree of morphine-induced conditioning.

Morphine Analgesia

The hot-plate test was performed according to O'Callaghan and Holtzman (1975). Mice were placed on a platform heated to 52.5°C and the latency to paw lick or jump was recorded. A cutoff of 60 s was used. Control responses were determined for each animal before treatment. Morphine

(9 mg/kg) was injected s.c. and analgesia was monitored at 30 min after morphine injection. The antinociceptive response was calculated as a percentage of maximal possible effect (MPE), where MPE = (morphine latency−control latency)/(cutoff−control latency) × 100.

Determination of Phospho-DARPP-32

Mice were injected with vehicle or drugs and killed by decapitation after various periods of time. The heads of the animals were immediately immersed in liquid nitrogen for 6 s. The brains were then rapidly removed and placed in an ice-cold mouse brain matrix (Activational Systems Inc., Warren, MI, USA). Nucleus accumbens and dorsal striatum were dissected out from coronal sections (prepared according to (Paxinos and Franklin, 2001)); AP + 1.98 to + 0.98 and AP + 1.1 to −0.1 mm relative to bregma, for nucleus accumbens and dorsal striatum, respectively) using a dissection needle (⌀ 2 mm), sonicated in 150 μl of 1% sodium dodecylsulfate, and boiled for 10 min. Aliquots (5 μl) of the homogenate were used for protein determination. Equal amounts of protein (50 μg) from each sample were loaded onto 10% polyacrylamide gels. The proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Towbin et al, 1979). PhosphoThr34-DARPP-32 and phosphoThr75-DARPP-32 were detected using a monoclonal (Snyder et al, 1992) and a polyclonal (Bibb et al, 1999) antibody, respectively. A monoclonal antibody generated against DARPP-32, which is not phosphorylation state specific, was used to estimate the total amount of DARPP-32 (Hemmings and Greengard, 1986). Antibody binding was revealed using goat anti-mouse HRP-linked IgG (diluted 1:10 000) and the ECL immunoblotting detection system. Chemiluminescence was detected by autoradiography. Quantification of the phospho-DARPP-32 bands was done by densitometry using NIH Image (version 1.52) software.

RESULTS

Effect of Acute Administration of Morphine on DARPP-32 Phosphorylation in the Striatum

Administration of morphine (6 or 9 mg/kg, s.c.) increased the state of phosphorylation of DARPP-32 at the PKA site, Thr34, in nucleus accumbens (Figure 1a; $P < 0.01$ for morphine 6 mg/kg vs saline and $P < 0.001$ for morphine 9 mg/kg vs saline; one-way ANOVA followed by Dunnett's test; $F_{(2, 31)} = 13.03$) and dorsal striatum (Figure 1d; $P < 0.05$ for morphine 6 mg/kg vs saline and $P < 0.001$ for morphine 9 mg/kg vs saline; one-way ANOVA followed by Dunnett's test; $F_{(2, 20)} = 11.21$), without affecting phosphorylation at Thr75 (Figure 1a and d). One-way ANOVA showed a significant time-dependent increase in the phosphorylation of Thr34, which reached a maximum 30 min after morphine (9 mg/kg) administration, in both brain regions ($F_{(3, 64)} = 9.32$, $P < 0.001$ for nucleus accumbens (Figure 1b); $F_{(3, 54)} = 4.18$, $P < 0.01$ for dorsal striatum (Figure 1e)). One possible mechanism by which morphine
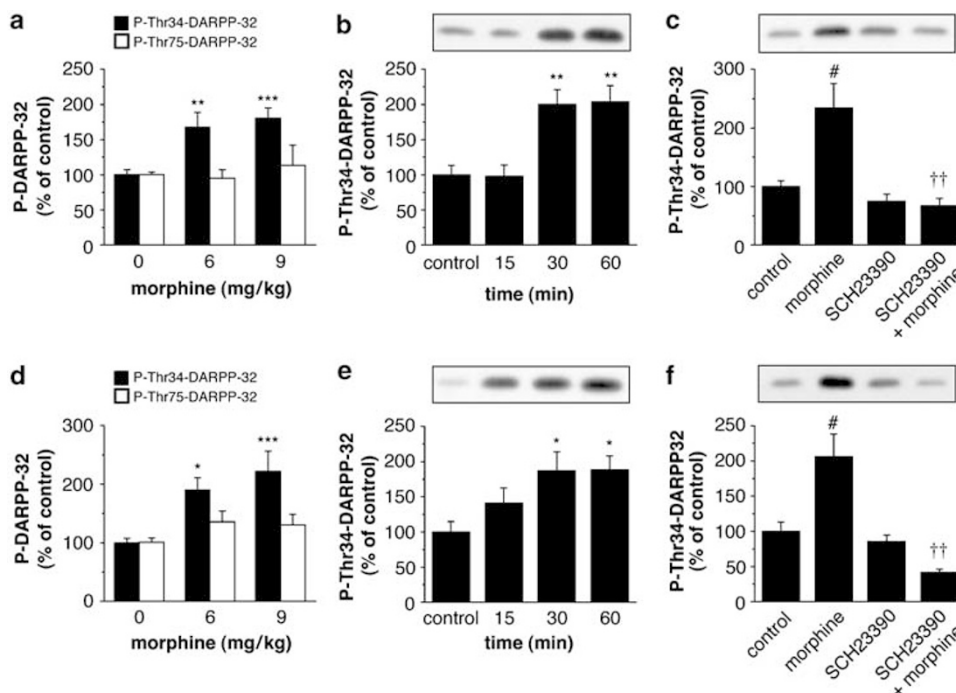

Figure 1 Morphine increases DARPP-32 phosphorylation at Thr34 in the nucleus accumbens (a–c) and dorsal striatum (d–f) via activation of dopamine D1 receptors. (a, d) Mice received a subcutaneous injection of saline or morphine (6 or 9 mg/kg) and were decapitated 30 min later. (b, e) Mice were treated with morphine (9 mg/kg) and decapitated at various times (0–60 min) after injection. (c, f) Mice were treated with morphine (9 mg/kg), SCH23390 (0.15 mg/kg, i.p.), or a combination of the two drugs and decapitated 30 min later. PhosphoThr34-DARPP-32 (filled bars) and phosphoThr75-DARPP-32 (open bars) were determined as described in Materials and methods. Upper panels show representative autoradiograms. Lower panels show summaries of data expressed as means ± SEM (n = 6–18). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs control (Dunnett's test); #$P < 0.05$ vs control and ††$P < 0.01$ vs morphine treated (Bonferroni-Dunn test).

might stimulate PKA-dependent phosphorylation of DARPP-32 involves disinhibition of midbrain dopaminergic neurons, increased dopamine release in the striatum, activation of dopamine D1 receptors and stimulation of cAMP production. Therefore, we tested the effect of SCH23390, a D1 receptor antagonist, on morphine-induced DARPP-32 phosphorylation. As shown in Figure 1c and f, the enhancement in Thr34 phosphorylation produced by morphine (9 mg/kg) was abolished by co-administration of SCH23390 (0.15 mg/kg, i.p.). Two-way ANOVA indicated a significant interaction between morphine treatment and SCH23390 treatment ($F_{(1,13)} = 8.63$, $P < 0.05$ for nucleus accumbens and $F_{(1,19)} = 9.35$ $P < 0.01$ for dorsal striatum). A significant increase in Thr34 phosphorylation was found in the group of mice treated with morphine alone ($P < 0.01$ for nucleus accumbens and $P < 0.05$ for dorsal striatum, Bonferroni-Dunn *post hoc* test).

Acute Motor Stimulant Effect of Morphine in DARPP-32 Mutant Mice

The results of the biochemical studies suggested that PKA-mediated phosphorylation of DARPP-32 could play a role in the action of morphine. We therefore tested the involvement of phosphoThr34-DARPP-32 in morphine-induced hyperlocomotion. Administration of 6 or 9 mg/kg of morphine to wild-type mice produced large increases in motor activity (Figure 2a). These effects were attenuated in DARPP-32 knockout mice (Figure 2a), T34A mutant mice (Figure 2b and c), but not in T75A mutant mice (Figure 2d and e). Two-way ANOVA of the total distance traveled by DARPP-32 knockout and wild-type mice during 120 min (Figure 2a) showed a main effect of morphine treatment ($F_{(2, 42)} = 16.51$, $P < 0.001$), a significant effect of the genotype ($F_{(1, 42)} = 7.28$, $P < 0.05$) and a significant treatment × genotype interaction ($F_{(2, 42)} = 3.47$, $P < 0.05$). Two-way ANOVA of the total distance performed by T34A mutant and wild-type littermates (Figure 2c) showed a main effect of morphine treatment ($F_{(1, 50)} = 51.50$, $P < 0.001$), a significant effect of the genotype ($F_{(1, 50)} = 5.10$, $P < 0.05$) and a significant treatment × genotype interaction ($F_{(1, 50)} = 5.59$, $P < 0.05$). In T75A mutant mice (Figure 2e) the same analysis showed main effect of morphine treatment ($F_{(1, 44)} = 60.99$, $P < 0.001$), nonsignificant effect of genotype ($F_{(1, 44)} = 0.44$, $P > 0.05$) and nonsignificant treatment × genotype interaction ($F_{(1, 44)} = 0.94$, $P > 0.05$). We next checked the possibility that the attenuation of the hyperlocomotor response to morphine observed in DARPP-32-deficient mice was due to lower expression of μ-opioid receptors. Western blotting analysis showed that the levels of μ-opioid receptors in the striata of DARPP-32 knock mice were comparable ($98.8 \pm 5.9\%$) to those found in wild-type mice.

Morphine Analgesia in DARPP-32 Knockout Mice

The possible involvement of DARPP-32 in the analgesic effect of morphine was evaluated using the hot-plate test. DARPP-32 knockout mice and wild-type mice showed indistinguishable pain thresholds. Moreover, their analgesic responses to acute administration of morphine (9 mg/kg) did not differ (MPE was 67.1 ± 12.9 for wild type mice

($n = 7$) and 79.0 ± 11.6 for DARPP-32 knock out mice ($n = 7$); $P > 0.05$, Student's *t*-test). Thus, the hyperlocomotor, but not the antinociceptive, effect produced by acute morphine depends on PKA-catalyzed phosphorylation of DARPP-32 at Thr34.

Effect of Repeated Administration of Morphine on DARPP-32 Phosphorylation

Prolonged exposure to morphine results in upregulation of the cAMP/PKA pathway in several brain regions (Nestler, 2004). We therefore examined whether repeated morphine administration is accompanied by changes in phosphorylation of DARPP-32 at the PKA site, Thr34. Mice were treated for 9 consecutive days with 9 mg/kg of morphine or saline. At the end of this period, half of the animals in each group received a challenge of morphine or saline. The animals were killed 30 min later and the levels of phosphoThr34-DARPP-32 were analyzed in tissue samples from nucleus accumbens (Figure 3a) and dorsal striatum (Figure 3b). We found that chronic treatment enhanced the ability of morphine to stimulate DARPP-32 phosphorylation. Two-way ANOVA indicated a significant interaction between morphine chronic treatment and morphine challenge ($F_{(1, 50)} = 5.04$, $P < 0.05$ for nucleus accumbens; $F_{1(1, 48)} = 24.46$, $P < 0.001$ for dorsal striatum). Blockade of dopamine D1 receptors with SCH 23390 (0.15 mg/kg) abolished the increase in phospho-Thr34-DARPP-32 produced by morphine, even after sensitization (Figure 3a and b). Repeated administration of morphine did not alter the levels of total DARPP-32 in nucleus accumbens or dorsal striatum (Figure 3c).

Comparison of Morphine Psychomotor Sensitization in DARPP-32 Mutant and Wild-Type Mice

We next examined the possibility that DARPP-32 might influence behavioral sensitization to morphine. To address this question we compared psychomotor sensitization in wild-type mice, DARPP-32 knockout mice (Figure 4a), T34A mutant mice (Figure 4b) and T75A mutant mice (Figure 4c). Two-way ANOVA with repeated measures showed a significant effect of morphine treatment in DARPP-32 knockout mice ($F_{(1, 16)} = 13.71$, $P < 0.01$) and wild-type mice ($F_{(1, 16)} = 77.07$, $P < 0.001$) and a significant treatment × days interaction ($F_{(3, 48)} = 7.86$, $P < 0.001$ for DARPP-32 knockout mice; $F_{(3, 48)} = 4.98$, $P < 0.05$ for wild-type mice). The same protocol for behavioral sensitization was used for the analysis of T34A mutant mice, T75A mutant mice and wild-type littermates (Figure 4b and c). Two-way ANOVA with repeated measures (treatment × days) showed a significant effect of morphine treatment in T34A mutant mice ($F_{(1, 43)} = 30.08$, $P < 0.001$) and wild-type mice ($F_{(1, 45)} = 46.44$, $P < 0.001$) and a significant treatment × days interaction ($F_{(4, 172)} = 3.55$, $P < 0.01$ for T34A mutant mice, and $F_{(4, 180)} = 3.37$, $P < 0.05$ for wild-type mice). Morphine treatment induced a significant increase in motor activity in T75A mutant mice ($F_{(1, 18)} = 22.01$, $P < 0.001$) and wild-type littermates ($F_{(1, 20)} = 8.04$, $P < 0.05$) and a significant treatment × days interaction ($F_{(4, 72)} = 5.62$, $P < 0.001$; $F_{(4, 80)} = 6.10$, $P < 0.001$).
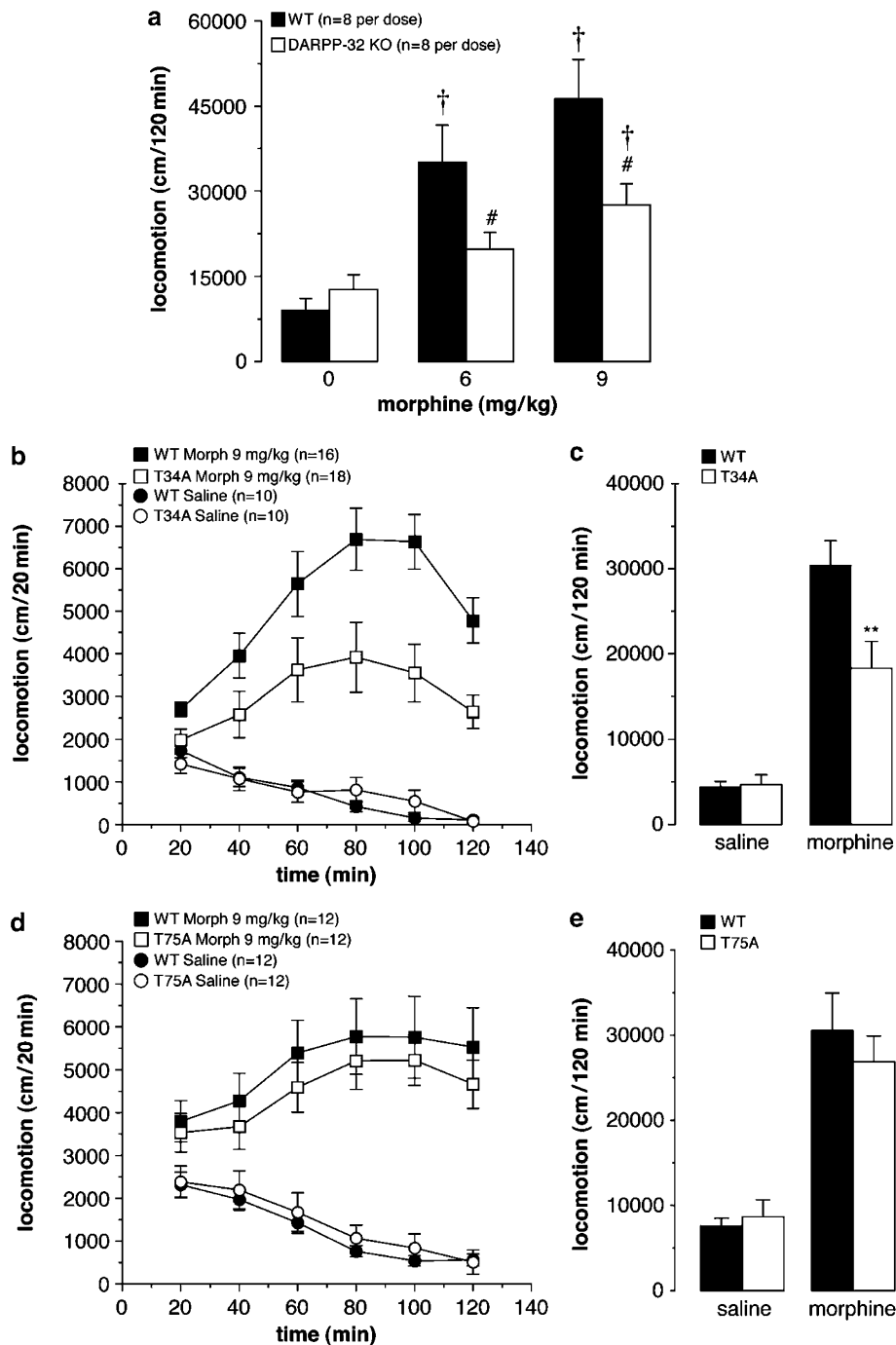
Figure 2 Morphine-induced hyperlocomotion requires phosphorylation of DARPP-32 at Thr34. DARPP-32 KO mice (a), T34A DARPP-32 mutant mice (b, c), T75A DARPP-32 mutant mice (d, e) and wild-type mice (a–e) were treated with saline or morphine and placed in individual cages. (a, c, e) Total distance covered during the 120-min period immediately following administration of saline or morphine. (b, d) Time course of the effect of morphine or vehicle on locomotion measured at 20-min intervals. Data represent means±SEM ($n = 8$–18). $^{†}P < 0.01$ vs respective control of the same genotype (Dunnett's test); $^{#}P < 0.05$ vs wild-type treated with morphine (Student's t-test); $^{**}P < 0.01$ vs morphine-treated wild-type mice (Bonferroni-Dunn test).

Altogether, these results indicated that all genotypes were able to develop psychomotor sensitization to morphine.

Morphine Conditioned Place Preference in DARPP-32 Knockout Mice

In order to assess a possible involvement of the cAMP/PKA/DARPP-32 cascade in the rewarding properties of morphine, wild-type and DARPP-32 knockout mice were analyzed for their ability to develop CPP to 3, 6, and 9 mg/kg of morphine. Two-way ANOVA of the difference in time (sec) spent in the drug associated compartment between post- and preconditioning phase revealed a significant effect of morphine treatment ($F(3, 87) = 18.61$, $P < 0.001$), no effect of genotype ($F(1, 87) = 0.14$, $P > 0.05$), and no genotype × morphine dose interaction ($F(3, 87) =$
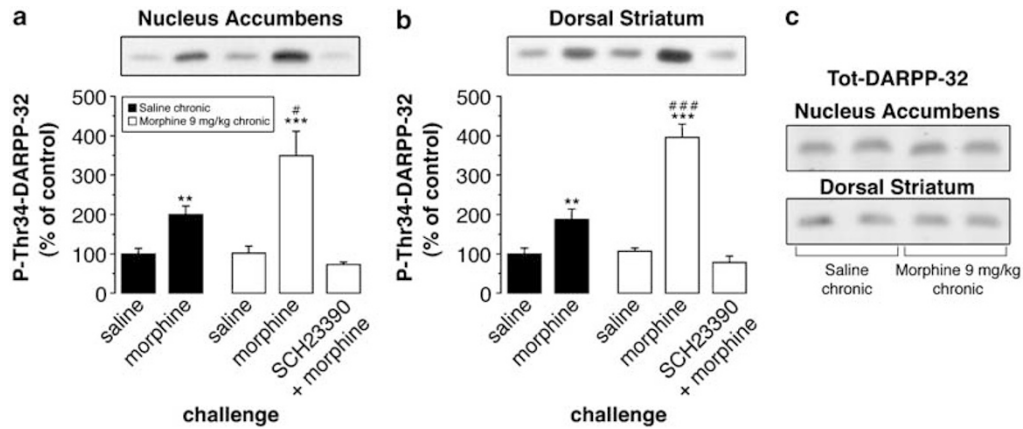
Figure 3 Effect of repeated administration of morphine on DARPP-32 phosphorylation in nucleus accumbens and dorsal striatum. Mice were treated for 9 days (once per day) with saline (filled bars) or morphine (9 mg/kg; open bars) to induce sensitization. The animals were decapitated 30 min following challenge with saline, morphine, or morphine plus SCH23390. PhosphoThr34-DARPP-32 (a, b) and total DARPP-32 (c) were determined in tissue extracts from nucleus accumbens (a, c) and dorsal striatum (b, c) as described in Materials and methods. (a, b) Upper panels show representative autoradiograms. Lower panels show summaries of data expressed as means $\pm$ SEM ($n = 5$–18). **$P < 0.01$ and ***$P < 0.001$ *vs* respective control (mice treated only with saline). #$P < 0.05$ and ###$P < 0.001$ *vs* acute morphine treatment (Bonferroni-Dunn test). (c) Chronic treatment with morphine did not affect the total levels of DARPP-32 in nucleus accumbens (upper panel) or dorsal striatum (lower panel).

0.841, $P > 0.05$). Thus, DARPP-32 knockout mice develop morphine CPP similar to wild-type animals (Figure 5).

DISCUSSION

The present study demonstrates that cAMP-dependent phosphorylation of DARPP-32 at Thr34 is involved in the hyperlocomor effect elicited by acute administration of morphine. In contrast, DARPP-32 does not appear to be required for the development of morphine psychomotor sensitization and for morphine place preference.

Increased dopamine release in the nucleus accumbens is generally regarded as a common effect elicited by various classes of drugs of abuse. Based on this notion, it has been proposed that augmented dopaminergic transmission in this brain region is a necessary step in the actions of addictive substances, including opiates (Di Chiara *et al*, 2004; Wise, 2004). There is overall consensus about the involvement of dopamine in the effect of morphine on motor activity. Dopamine-deficient mice show a dramatic reduction in morphine-induced hyperlocomotion (Hnasko *et al*, 2005). A similar suppression of the motor stimulant properties of morphine is observed after pharmacological blockade (Jeziorski and White, 1995; Longoni *et al*, 1987; Serrano *et al*, 2002) or genetic inactivation (Becker *et al*, 2001) of dopamine D1 receptors. In contrast, the increase in motor activity produced by morphine in dopamine D2 receptor knockout mice does not differ from that observed in wild-type littermates (Maldonado *et al*, 1997).

A considerable proportion of the effects exerted by dopamine on striatal medium spiny neurons depend on activation of the cAMP/PKA cascade and phosphorylation of DARPP-32. The inhibition of protein phosphatase-1 activity that occurs following PKA-catalysed phosphorylation of DARPP-32 at Thr34 acts as an amplification mechanism able to promote phosphorylation of downstream target proteins, thereby strengthening responses mediated by activation of dopamine D1 receptors (Fienberg

et al, 1998). Our results, showing that the hyperlocomotor effect of morphine is reduced in DARPP-32 knockout mice, and in T34A DARPP-32 mutant mice, demonstrate the importance of such an amplification mechanism in opiate-mediated control of motor activity. The present data also demonstrate that the ability of morphine to activate the cAMP/PKA/DARPP-32 cascade depends on activation of dopamine D1 receptors, thereby supporting the idea of a preferential involvement of these receptors in the motor stimulant properties of morphine.

Other studies have demonstrated the involvement of DARPP-32 in the acute effects of psychostimulants. Thus, the hyperlocomotor response to a single injection of cocaine, which increases DARPP-32 phosphorylation at Thr34 via activation of dopamine D1 receptors (Svenningsson *et al*, 2000), is attenuated in DARPP-32 knockout mice (Fienberg *et al*, 1998) and in T34A DARPP-32 mutant mice (Zachariou *et al*, 2006). These results are in line with the present findings on morphine and demonstrate the importance of DARPP-32 in the acute motor stimulant effects produced by addictive substances.

In a previous study, Scheggi *et al* (2004) did not observe increased phosphorylation of DARPP-32 at Thr34 in dorsal and ventral striatum of rats following acute or chronic administration of morphine. This discrepancy may be due to differences between rats and mice in their ability to respond to morphine or, more likely, to the different techniques used to extract the tissue. In fact, rapid near-freezing of the brain immediately post-mortem is critical in order to preserve the phosphorylation state of proteins (Svenningsson *et al*, 2000).

In vitro studies performed in striatal slices indicated that activation of opiate receptors reduced, rather than enhanced the state of phosphorylation of DARPP-32. In particular, it was found that Tyr-D-Ala-Gly-N-Me-Phe-glycinol (DAMGO), a μ-opioid receptor agonist, inhibited the dopamine D1 receptor-induced increase in DARPP-32 phosphorylation at Thr34 (Lindskog *et al*, 1999). This effect is attributable to the activation of μ-opioid receptors located
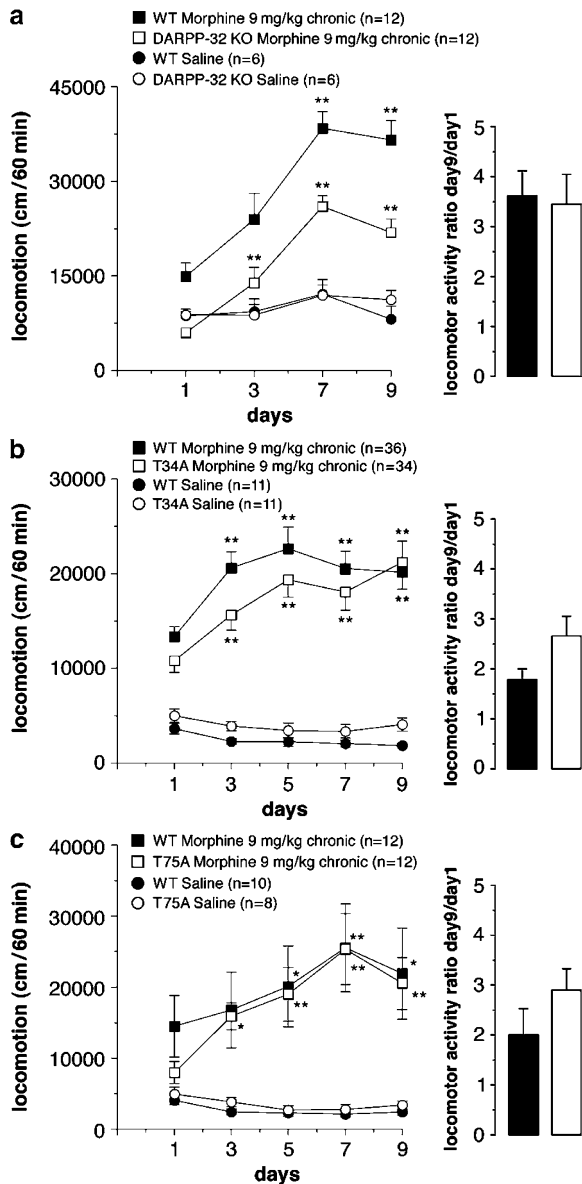
Figure 4 Comparison of morphine psychomotor sensitization in DARPP-32 mutant and wild-type mice. (a) DARPP-32 knockout mice, (b) T34A mutant mice, (c) T75A mutant mice, and (a–c) wild-type mice were treated for 9 days (once per day) with 9 mg/kg of morphine. Locomotor activity was determined for 60 min, immediately after injection, at various days as indicated. Data represent means±SEM *$P<0.05$; **$P<0.01$ vs the distance performed day 1 within the same genotype (Dunnett's test). Right panels show locomotor sensitization as ratio of locomotor activity determined at day 9 and day 1 in morphine-treated wild-type (filled bars) and mutant (open bars) mice. No significant difference was observed between wild-type and mutant strains (Student's t-test).

Figure 5 Morphine-induced place preference is unaffected in DARPP-32 knockout mice. (a) Preference scores expressed as difference between post- and pre-conditioning time spent in the compartment associated with morphine for DARPP-32 knockout (open bars) and wild-type (filled bars) animals. (b) Time spent in the drug associated compartment during pre-conditioning (filled bars) and post-conditioning testing phases (open bars). Data represent means±SEM ($n=8$–17). *$P<0.05$, **$P<0.01$ vs saline-treated animals of the same genotype (Dunnett's test).

on striatal medium spiny neurons and negatively coupled to adenylyl cyclase. The present results indicate that when administered in intact mice, morphine acts predominantly by promoting, rather that inhibiting, cAMP signaling in medium spiny neurons. This effect is most likely achieved by suppressing the inhibition of midbrain dopaminergic neurons exerted by GABAergic interneurons (Johnson and North, 1992). This leads to increased dopamine release in the striatum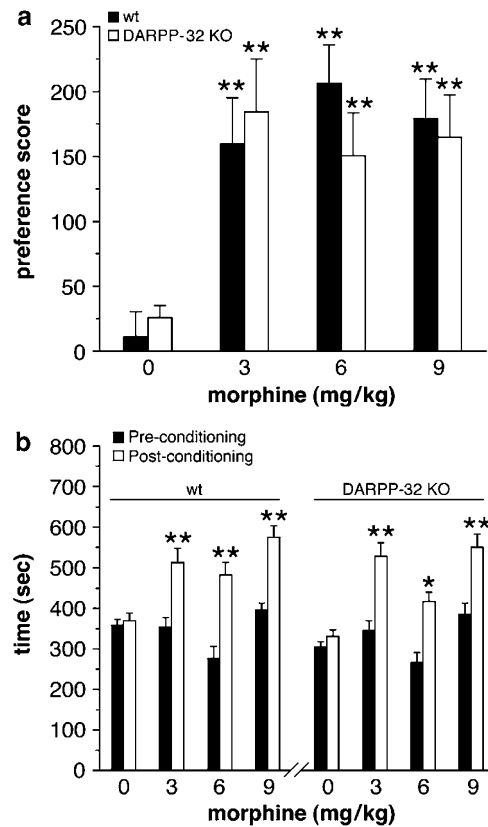, activation of dopamine D1 receptors and stimulation of the cAMP/PKA/DARPP-32 cascade. In support of this view, we found that administration of the dopamine D1 receptor antagonist, SCH23390, prevented the increase in DARPP-32 phosphorylation at Thr34 caused by morphine.

It has been reported that chronic administration of morphine increases the activity of adenylyl cyclase and PKA in the nucleus accumbens (Terwilliger et al, 1991). This effect has been attributed to reduced expression of $G_{i\alpha}$ protein, which couples μ-opioid receptors on medium spiny neurons to inhibition of adenylyl cyclase and cAMP synthesis (Terwilliger et al, 1991). The data presented in this study support the idea that repeated administration of morphine is accompanied by enhanced cAMP signaling. Thus, chronic treatment with morphine increases the ability of acute morphine to stimulate PKA-dependent phosphorylation of DARPP-32 at Thr34 in the nucleus accumbens and dorsal striatum. This change in morphine efficacy may depend on the diminished ability of striatal μ-opioid receptors to counteract the accumulation of cAMP produced by morphine via disinhibition of dopamine release and activation of dopamine D1 receptors.

The enhanced ability to stimulate DARPP-32 phosphorylation at Thr34 appears to be a unique feature associated to

chronic treatment with morphine, as repeated administration of psychostimulants has been shown to reduce rather than promote Thr34 phosphorylation (Bibb *et al*, 2001; Chen and Chen, 2005). Such biochemical sensitization, however, does not appear to mediate psychomotor sensitization. In fact, in spite of their blunted motor response to morphine, DARPP-32 null mice and T34A DARPP-32 mutant mice are fully capable of developing morphine behavioral sensitization. In line with these results, and supporting the notion that psychomotor sensitization is related to the rewarding properties of drugs of abuse, we also found that DARPP-32 knockout mice show normal morphine CPP.

The ability of cocaine to phosphorylate DARPP-32 at Thr34, which depends on dopamine D1 receptor activation (Svenningsson *et al*, 2000), is required for psychostimulant-induced sensitization (Valjent *et al*, 2005) (but see also (Zachariou *et al*, 2006)). Moreover, cocaine CPP is reduced in DARPP-32 knock out mice (Zachariou *et al*, 2002). In contrast, in the present study it is shown that morphine sensitization and CPP develop independently of dopamine D1 receptor-mediated phosphorylation of DARPP-32. These results are in agreement with previous evidence showing unaltered morphine-induced CPP in dopamine-deficient mice (Hnasko *et al*, 2005). They are also in line with the observation that opiate self-administration occurs independently of dopamine release (Pettit *et al*, 1984).

In summary, this study suggests that dopamine D1 receptor-mediated phosphorylation of DARPP-32 at Thr34 is involved in the acute psychomotor effect but not in the hedonic properties of morphine. Considering the critical involvement of the cAMP/PKA/DARPP-32 pathway in dopaminergic transmission, these results are consistent with recent evidence indicating blunted morphine-induced motor response, but intact morphine reward, in dopamine-deficient mice (Hnasko *et al*, 2005).

REFERENCES

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

Becker A, Grecksch G, Kraus J, Peters B, Schroeder H, Schulz S *et al* (2001). Loss of locomotor sensitisation in response to morphine in D1 receptor deficient mice. *Naunyn Schmiedebergs Arch Pharmacol* 363: 562–568.

Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL *et al* (2001). Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* 410: 376–380.

Bibb JA, Snyder GL, Nishi A, Yan Z, Meijer L, Fienberg AA *et al* (1999). Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. *Nature* 402: 669–671.

Chen PC, Chen JC (2005). Enhanced Cdk5 activity and p35 translocation in the ventral striatum of acute and chronic methamphetamine-treated rats. *Neuropsychopharmacology* 30: 538–549.

Corvol JC, Studler JM, Schonn JS, Girault JA, Herve D (2001). Galpha(olf) is necessary for coupling D1 and A2a receptors to adenylyl cyclase in the striatum. *J Neurochem* 76: 1585–1588.

Di Chiara G, Bassareo V, Fenu S, De Luca MA, Spina L, Cadoni C *et al* (2004). Dopamine and drug addiction: the nucleus accumbens shell connection. *Neuropharmacology* 47(Suppl 1): 227–241.

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 USA* 85: 5274–5278.

Fienberg AA, Hiroi N, Mermelstein PG, Song W, Snyder GL, Nishi A *et al* (1998). DARPP-32: regulator of the efficacy of dopaminergic neurotransmission. *Science* 281: 838–842.

Greengard P, Allen PB, Nairn A (1999). Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. *Neuron* 23: 435–447.

Hemmings HCJ, Greengard P (1986). DARPP-32, a dopamine- and adenosine 3′:5′-monophosphate-regulated phosphoprotein: regional, tissue, and phylogenetic distribution. *J Neurosci* 6: 1469–1481.

Hemmings Jr HC, Greengard P, Tung HY, Cohen P (1984). DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. *Nature* 310: 503–505.

Hnasko TS, Sotak BN, Palmiter RD (2005). Morphine reward in dopamine-deficient mice. *Nature* 438: 854–857.

Jeziorski M, White FJ (1995). Dopamine receptor antagonists prevent expression, but not development, of morphine sensitization. *Eur J Pharmacol* 275: 235–244.

Johnson SW, North RA (1992). Opioids excite dopamine neurons by hyperpolarization of local interneurons. *J Neurosci* 12: 483–488.

Kalivas PW (1985). Sensitization to repeated enkephalin administration into the ventral tegmental area of the rat. II. Involvement of the mesolimbic dopamine system. *J Pharmacol Exp Ther* 235: 544–550.

Lindskog M, Svenningsson P, Fredholm B, Greengard P, Fisone G (1999). Mu- and delta-opioid receptor agonists inhibit DARPP-32 phosphorylation in distinct populations of striatal projection neurons. *Eur J Neurosci* 11: 2182–2186.

Longoni R, Spina L, Di Chiara G (1987). Dopaminergic D-1 receptors: essential role in morphine-induced hypermotility. *Psychopharmacology (Berlin)* 93: 401–402.

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.

Nestler EJ (2004). Historical review: Molecular and cellular mechanisms of opiate and cocaine addiction. *Trends Pharmacol Sci* 25: 210–218.

O'Callaghan JP, Holtzman SG (1975). Quantification of the analgesic activity of narcotic antagonists by a modified hot-plate procedure. *J Pharmacol Exp Ther* 192: 497–505.

Paxinos G, Franklin KBJ (2001). *The Mouse Brain in Stereotaxic Coordinates*, 2nd edn. Academic Press: San Diego.

Pettit HO, Ettenberg A, Bloom FE, Koob GF (1984). Destruction of dopamine in the nucleus accumbens selectively attenuates cocaine but not heroin self-administration in rats. *Psychopharmacology (Berlin)* 84: 167–173.

Robinson TE, Berridge KC (1993). The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev* 18: 247–291.

Scheggi S, Rauggi R, Gambarana C, Tagliamonte A, De Montis MG (2004). Dopamine and cyclic AMP-regulated phosphoprotein-32 phosphorylation pattern in cocaine and morphine-sensitized rats. *J Neurochem* 90: 792–799.

Serrano A, Aguilar MA, Manzanedo C, Rodriguez-Arias M, Minarro J (2002). Effects of DA D1 and D2 antagonists on the sensitisation to the motor effects of morphine in mice. *Prog Neuropsychopharmacol Biol Psychiatry* **26**: 1263–1271.

Snyder GL, Girault JA, Chen JY, Czernik AJ, Kebabian JW, Nathanson JA *et al* (1992). Phosphorylation of DARPP-32 and protein phosphatase inhibitor-1 in rat choroid plexus: regulation by factors other than dopamine. *J Neurosci* **12**: 3071–3083.

Svenningsson P, Lindskog M, Ledent C, Parmentier M, Greengard P, Fredholm BB *et al* (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 USA* **97**: 1856–1860.

Svenningsson P, Tzavara ET, Carruthers R, Rachleff I, Wattler S, Nehls M *et al* (2003). Diverse psychotomimetics act through a common signaling pathway. *Science* **302**: 1412–1415.

Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Crain SM, Nestler EJ (1991). A general role for adaptations in G-proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. *Brain Res* **548**: 100–110.

Towbin H, Staehelin T, Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354.

Valjent E, Pascoli V, Svenningsson P, Paul S, Enslen H, Corvol JC *et al* (2005). Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. *Proc Natl Acad Sci USA* **102**: 491–496.

Vezina P, Stewart J (1989). The effect of dopamine receptor blockade on the development of sensitization to the locomotor activating effects of amphetamine and morphine. *Brain Res* **499**: 108–120.

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

Zachariou V, Benoit-Marand M, Allen PB, Ingrassia P, Fienberg AA, Gonon F *et al* (2002). Reduction of cocaine place preference in mice lacking the protein phosphatase 1 inhibitors DARPP 32 or Inhibitor 1. *Biol Psychiatry* **51**: 612–620.

Zachariou V, Sgambato-Faure V, Sasaki T, Svenningsson P, Berton O, Fienberg AA *et al* (2006). Phosphorylation of DARPP-32 at Threonine-34 is required for cocaine action. *Neuropsychopharmacology* **31**: 555–562.

Zhuang X, Belluscio L, Hen R (2000). G(olf)alpha mediates dopamine D1 receptor signaling. *J Neurosci* **20**: RC91.