

# Phosphorylation of DARPP-32 at Threonine-34 is Required for Cocaine Action

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Mice lacking DARPP-32, a striatal-enriched phosphoprotein, show abnormal behavioral and biochemical responses to cocaine, but the role of individual phosphorylation sites in DARPP-32 in these responses is unknown. We show here that mutation of Thr-34 in DARPP-32 mimicked the behavioral phenotype of the constitutive DARPP-32 knockout in cocaine-induced place conditioning, locomotor activity, and sensitization paradigms. In contrast, mutations of Thr-75 did not affect conditioned place preference or the acute locomotor response to cocaine, but DARPP-32 Thr-75 mutants showed no locomotor sensitization in response to repeated cocaine administration. Consistent with these behavioral findings, we found that cocaine regulation of gene expression in striatum, including the acute induction of the immediate early genes *c-fos* and *arc* (activity-regulated cytoskeletal-associated gene), was abolished in DARPP-32 Thr-34 mutants, but not in Thr-75 mutants. Similarly, induction of the transcription factor  $\Delta$ FosB in the ventral striatum (nucleus accumbens) by chronic cocaine was diminished by the Thr-34, but not the Thr-75, mutation. These findings highlight distinct roles of the Thr-34 and Thr-75 phosphorylation sites of DARPP-32 in mediating short- and long-term behavioral and biochemical actions of cocaine.

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## INTRODUCTION

Striatal function is highly regulated by DARPP-32, a 32 kDa phosphoprotein enriched in medium spiny neurons in dorsal and ventral striatum (Greengard, 2001). This protein has a complex mechanism of action, with both protein phosphatase and protein kinase regulatory properties. Upon protein kinase A (PKA) phosphorylation at Thr-34, DARPP-32 becomes a potent inhibitor of protein phosphatase 1 (PP1) (Hemmings *et al*, 1984). On the other hand, phosphorylation of DARPP-32 at Thr-75 by cyclin-dependent kinase-5 converts it into an inhibitor of PKA (Bibb *et al*, 1999). Through such actions, DARPP-32 appears to integrate the physiological effects of multiple extracellular and intracellular signals in striatum.

DARPP-32 plays a critical role in regulating dopamine signaling in striatum (Greengard, 2001), and dopamine signaling is critical in mediating the actions of psychostimulants such as cocaine and amphetamine (Nestler, 2001). Recent evidence indicates that mice lacking DARPP-32 show abnormal responses to psychostimulants. Loss of DARPP-32 in mice reduces an animal's sensitivity to the acute locomotor activating and rewarding effects of cocaine, and also blunts cocaine's ability to induce the transcription factor *c-Fos* acutely and the related transcription factor  $\Delta$ FosB (including all of its isoforms) chronically (Fienberg *et al*, 1998; Hiroi *et al*, 1999; Zachariou *et al*, 2002). In contrast, locomotor sensitization to repeated cocaine exposure is paradoxically enhanced in the DARPP-32 knockout mice (Hiroi *et al*, 1999).

However, the contribution of the regulation of PP1 or PKA by DARPP-32 in mediating these effects of cocaine is unknown. In the present study, using recently generated DARPP-32 mutant mice (Svenningsson *et al*, 2003), in which either Thr-34 or Thr-75 was mutated to a nonphosphorylatable residue, alanine, we address this important question. We characterize the influence of each mutation on behavioral responses to cocaine. We also investigate the importance of each phosphorylation site in cocaine's ability to regulate gene expression in striatum, including the induction of the transcription factors *c-Fos* and  $\Delta$ FosB. In

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addition, given the likely importance of changes in the neuronal cytoskeleton in molecular adaptations to psychomotor stimulants (Nestler and Aghajanian, 1997; Noble *et al*, 2000; Robinson and Berridge 2001; Robinson and Kolb, 2004), we investigated the expression of Arc, a cytoskeletal-related protein. Of interest, Arc is induced in striatum by acute cocaine (Fosnaugh *et al*, 1995; Tan *et al*, 2000) or chronic morphine (Marie-Claire *et al*, 2004) and is critically involved in the regulation of synaptic plasticity (for a review, see Steward and Worley, 2002). The results obtained highlight distinct roles of the Thr-34 and Thr-75 sites of DARPP-32 in mediating short- and long-term behavioral and biochemical actions of psychostimulants.

## MATERIALS AND METHODS

### Animals

Homozygous mutant and wild-type mice were generated from heterozygous matings for each mouse line. All of the mutants were generated on a 129SV background; the phosphomutant lines were backcrossed for two generations to a C57Bl/6 background, while the DARPP-32 knockout line was fully backcrossed to this background. Separate groups of male mice were used for each behavioral and biochemical assay.

### Conditioned Place Preference

The place preference apparatus used in this study has been described before (Rahman *et al*, 2003). Briefly, the apparatus consists of two large chambers with distinct wall patterns and floor textures separated by a smaller chamber. On day 1, baseline preference was monitored for 20 min. For the next 3 days, mice were conditioned to one chamber with saline in the morning and to the opposite chamber with cocaine in the afternoon. On the test day (day 5), conditioned preference was monitored for 20 min. No preference for one of the sides was observed for any of the lines used at baseline. Owing to the different genetic backgrounds of the various mouse lines, threshold cocaine doses were first determined in each behavioral paradigm in wild-type animals for each line.

### Locomotor Activity Assays

Acute locomotor activity assays were performed in an automated system as previously described (Rahman *et al*, 2003). During habituation, mice were injected i.p. with saline and placed in the locomotor activity cages for 30 min for 2 consecutive days. Baseline locomotor activity was monitored on day 3 for 30 min after a saline injection, followed by another 30 min session of cocaine administration (20 mg/kg i.p.). For locomotor sensitization assays, locomotor activity was monitored for 30 min after an i.p. saline injection (days 1–3), then after daily cocaine injections (20 mg/kg i.p.; days 4–8).

### Tissue Preparation for *In Situ* Hybridization

Mice were killed 1 h after injection of saline or cocaine and their brains were fixed by intracardiac perfusion of 4%

paraformaldehyde in 0.1 M phosphate buffer, pH 7.5. Brains were removed and postfixed in the same fixative solution for 2 h, washed overnight in 0.1 M phosphate buffer containing 15% sucrose, and then frozen. Rostro-caudal series of coronal sections (20  $\mu$ m) were cut on a microtome and then kept in a solution containing 30% ethylene glycol, 30% glycerol, 0.1 M phosphate buffer, and 0.1% diethyl pyrocarbonate (DEPC) at 20°C until they were processed for *in situ* hybridization.

### *In Situ* Hybridization

<sup>35</sup>S-radiolabeled antisense riboprobes were used in this study. The Arc probe containing the entire 3232 bases for arc cDNA was subcloned into pBluescriptKS, linearized after *Eco*RI digestion and transcribed with T7 RNA polymerase. The DARPP-32 antisense probe (500 bp fragment of the coding sequence of rat DARPP-32 cDNA subcloned into pBluescriptKS) was linearized after *Xba*I digestion and transcribed with T3 RNA polymerase. The c-fos antisense probe was generated from a 2100 bp cDNA fragment encoding the full-length c-fos gene (Curran *et al*, 1987), subcloned into pBluescriptKS, linearized with *Sac*II and transcribed with T7 RNA polymerase followed by alkaline phosphatase digestion to generate 300 bp fragments. The transcription reaction contained 6  $\mu$ l [<sup>35</sup>S]UTP (25 mCi/ml; NEN Life Sciences), 250  $\mu$ M ATP, CTP, and GTP, and unlabeled UTP (10.5  $\mu$ M); the reaction mixture was incubated at 37°C for 2 h. After DNase I digestion for 15 min, the labeled RNA was purified on microspin G25 columns (Amersham Pharmacia Biotech). Free-floating sections (4–6 per animal) were mounted on SuperFrost slides in RNase-free conditions. Once the sections were dried, *in situ* hybridization was performed at 55°C in humid chambers for 16 h according to a previous protocol (Sgambato *et al*, 1998). After coverslips were removed in 4  $\times$  SSC and 10 mM DTT, the slides were washed in the same solution for 1 h at room temperature and then in 50% formamide, 10 mM Tris-HCl, pH 8, 75 mM NaCl, and 2.5 mM EDTA. Sections were treated with RNase A (20  $\mu$ g/ml; Sigma) in 400 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 50 mM EDTA for 1 h at 37°C and then were rinsed for 15 min at 60°C in 2  $\times$  SSC, followed by 0.1  $\times$  SSC. After dehydration, the sections were air-dried and exposed to Biomax-MR films (Kodak). Densitometric measurements of mRNA signals were obtained from autoradiograms using the NIH Image J 1.29 software after subtraction of background value. The region used for quantitation was the dorsal striatum at rostral levels (ie anterior relative to Bregma comprised between 0.8 and 0.4 mm according to the atlas of Franklin and Paxinos (1997)). Data are expressed as means  $\pm$  SEM. Statistical analysis used ANOVA with multiple comparisons (Newman-Keuls multiple comparison test).

### $\Delta$ FosB Immunohistochemistry

At 18–24 h after their last treatment, animals were deeply anesthetized with chloral hydrate and perfused intracardially with 200 ml of 0.01 M phosphate-buffered saline (PBS) pH 7.4, followed by 400 ml of 4% paraformaldehyde in 0.01 M PBS. Brains were removed and stored for 12 h in 4%

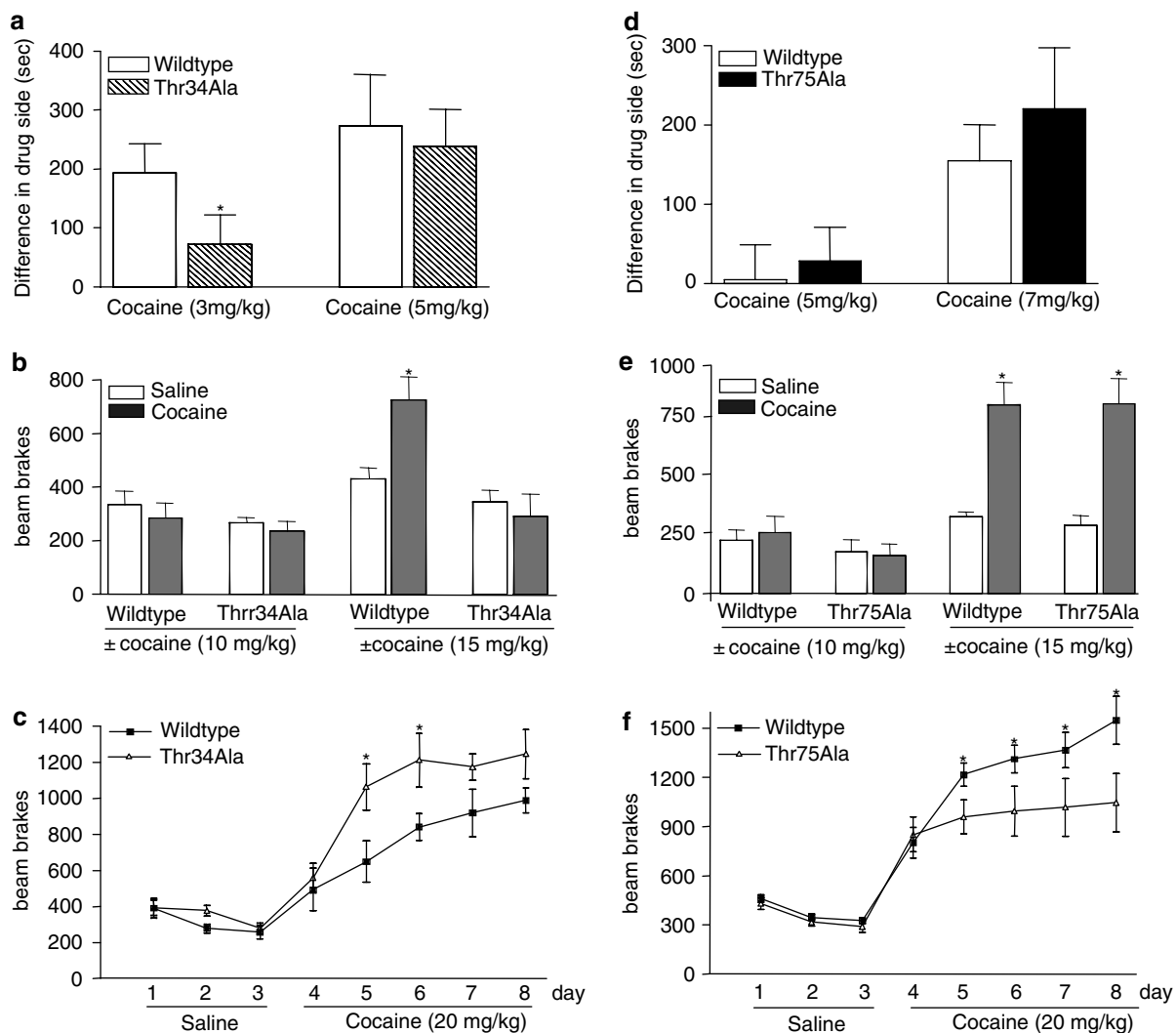
paraformaldehyde at 4°C. The next morning, brains were transferred to a 20% glycerol in 0.01 M PBS solution for cryoprotection. Coronal sections (30 µm) were cut on a freezing microtome (Leica, Bannockburn, IL) and then processed for immunohistochemistry. FosB/ $\Delta$ FosB immunoreactivity was detected using a rabbit polyclonal antibody raised against an N-terminal region of FosB/ $\Delta$ FosB (SC-7203, Santa Cruz Biotechnology, Santa Cruz, CA). FosB staining was revealed by use of a fluorescent secondary antibody. Slices were incubated for 30 min in 0.1% Triton X-100 and 3% normal donkey serum to minimize nonspecific labeling. Tissue sections were then incubated overnight at room temperature in 1% normal donkey serum, 0.1% Triton X-100, and anti-FosB antibody (SC-7203; 1:500). Sections were washed, placed for 1.5 h in a 1:200 dilution of CY2-labeled donkey anti-rabbit IgG (Jackson immunoresearch). The total number of cells immunoreactive for FosB/ $\Delta$ FosB was counted in the NAc (core and shell) by an

experimenter blind to the experimental conditions. The analysis was performed using a Leeds microscope equipped with an x-y-z stage connected to a personal computer running Bioquant Nova (Bioquant Image Analysis, Nashville, TN).

## RESULTS

### Behavioral Responses to Cocaine in DARPP-32 Mutant Mice

DARPP-32 Thr-34 mutants were first analyzed for their behavioral responses to cocaine. As shown in Figure 1a, mutation of Thr-34 to Ala decreases sensitivity to cocaine's rewarding effects in the place preference paradigm, as mutant mice required higher doses of cocaine to show significant place conditioning compared to their wild-type littermates. The Thr-34 mutant mice also showed reduced



**Figure 1** Behavioral responses to cocaine in Thr-34 and Thr-75 DARPP-32 mutant mice. Thr-34 mutants showed (a) reduced sensitivity to cocaine place conditioning as they develop place preference to higher doses than their wild-type littermates ( $n = 4-7$ ), (b) reduced acute locomotor responses to cocaine ( $n = 4$ ), but (c) enhanced locomotor sensitization to repeated cocaine ( $n = 7-9$ ). In contrast, Thr-75 mutants showed (d) normal cocaine place conditioning ( $n = 6-7$ ) and (e) acute locomotor responses to cocaine ( $n = 4$ ), but (f) reduced locomotor sensitization (response on day 8 vs day 4) to repeated cocaine ( $n = 7$  per group). Data are expressed as mean  $\pm$  SEM, \* $p < 0.05$  between genotypes over dose (for place conditioning and locomotor activity) or between genotypes over treatment day (for locomotor sensitization) by ANOVA followed by PLSD test. Note that dose responses to cocaine differed in the wild-type mice for the Thr-34 and Thr-75 mutants due to the somewhat different backgrounds of the lines.

sensitivity to the acute locomotor effects of cocaine. Cocaine at 10 mg/kg had no effect on locomotor activity in either wild-type or Thr-34 mutant mice, while cocaine at 15 mg/kg increased locomotor activity in wild-type mice, but not in Thr-34 mutant mice (Figure 1b). At the dose of 20 mg/kg, cocaine increased locomotor activity to the same level in both wild-type and Thr-34 mutant mice (data not shown). However, this high dose of cocaine (20 mg/kg) caused an abnormal pattern of sensitization to repeated cocaine administration in the Thr-34 mutants, with a maximal increase in locomotor activity reached upon the second exposure to the drug (Figure 1c).

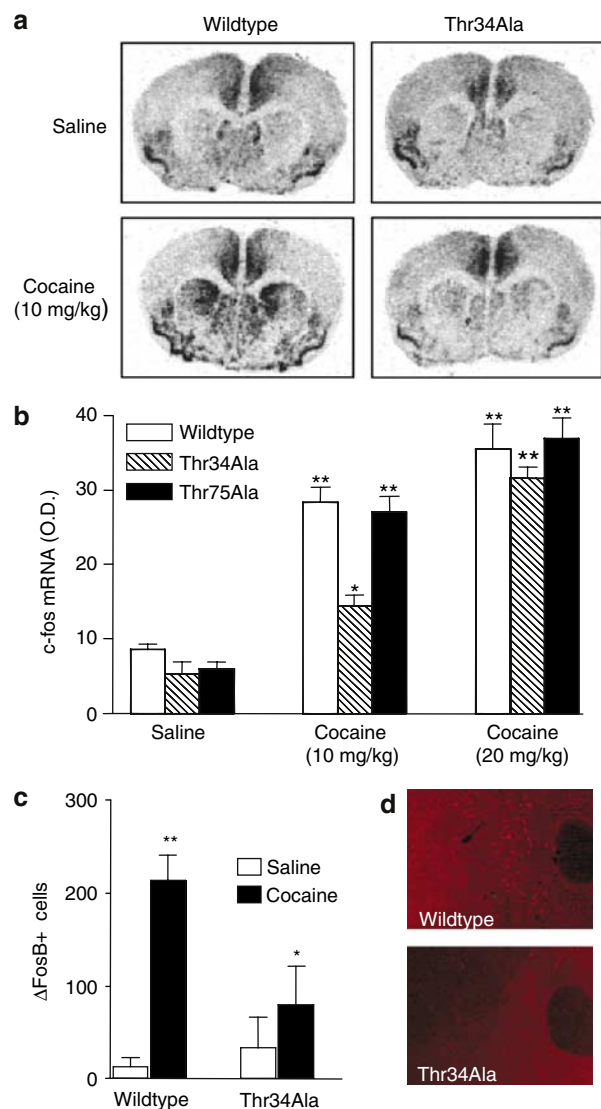
Very different results were obtained in the DARPP-32 Thr-75 mutants. Loss of Thr-75 had no influence on the animal's sensitivity to the rewarding effects of cocaine as measured in the place preference paradigm (Figure 1d), nor was there any difference in locomotor responses induced by acute injection of the drug (Figure 1e). However, the DARPP-32 Thr-75 mutants showed no locomotor sensitization in response to repeated cocaine administration (Figure 1f), a phenotype opposite to that seen in the Thr-34 mutants. These behavioral findings are consistent with the opposing effects of DARPP-32 Thr-34 and Thr-75 phosphorylation on PKA signaling in striatum (Bibb *et al*, 1999; Greengard 2001).

### Induction of Fos Family Transcription Factors in DARPP-32 Mutant Mice

Acute exposure to cocaine causes the rapid and transient induction of c-Fos in the nucleus accumbens and dorsal striatum, while repeated cocaine administration selectively induces  $\Delta$ FosB in these regions (Nestler *et al*, 2001). The latter protein has been related to some of the longer-lasting effects of cocaine (Nestler *et al*, 2001; McClung *et al*, 2004). Interestingly, the ability of an acute cocaine injection (10 mg/kg) to induce *c-fos* (Figure 2) or *fosb* (data not shown) mRNA in the nucleus accumbens was significantly attenuated in the Thr-34 mutant mice, while such induction was normal in the Thr-75 mutant mice (Figure 2a and b). At a higher dose (20 mg/kg), cocaine induced *c-fos* expression in the Thr-34 mutants to levels only slightly lower than their wild-type controls. Importantly, induction of  $\Delta$ FosB in the nucleus accumbens by chronic cocaine administration was reduced dramatically in the Thr-34 mutant mice (Figure 2c and d), but was not significantly affected in Thr-75 mutant mice (number of  $\Delta$ FosB-positive cells: wild-type saline =  $29.6 \pm 11.6$ , wild-type cocaine =  $150 \pm 21.8$ , mutant saline =  $25 \pm 12$ , mutant cocaine =  $124 \pm 20.8$ ,  $n = 4-5$ ).

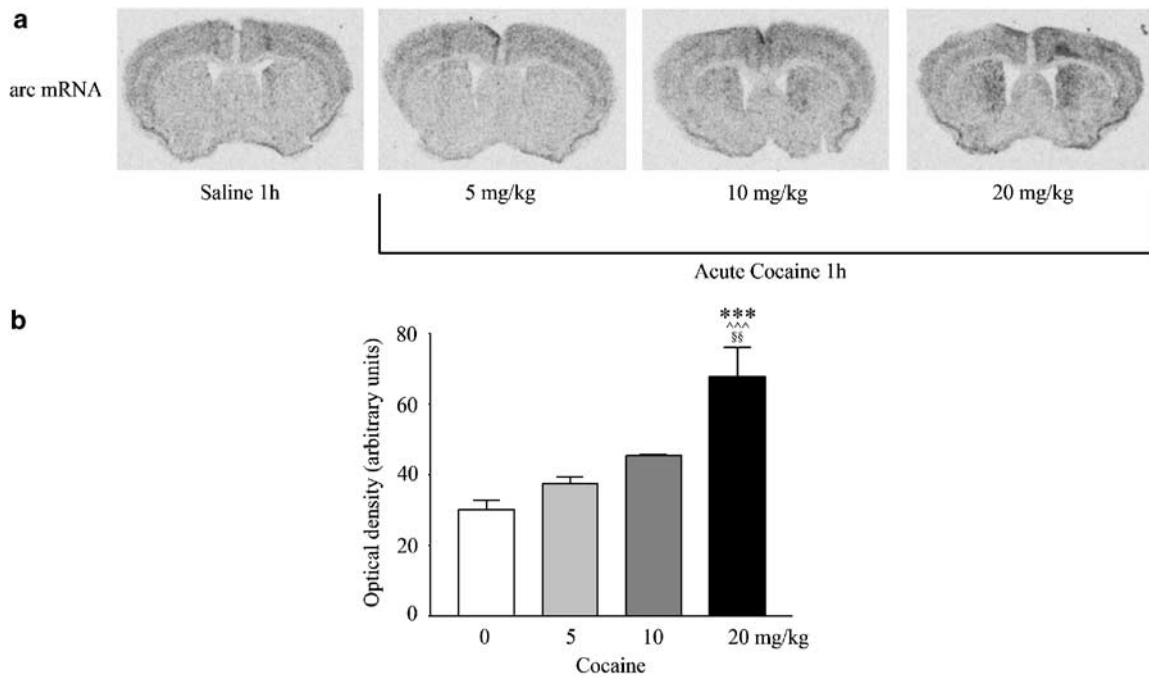
### Induction of Arc in DARPP-32 Mutant Mice

We next investigated the effect of cocaine on *arc* gene expression. *Arc* mRNA is expressed at relatively low levels constitutively (Figure 3a). Using treatment parameters (20 mg/kg, 1 h) that elicit robust activation of other immediate early genes like *c-fos*, we found that cocaine also induced *arc* mRNA in wild-type mice (Figure 3a and b) throughout most of the dorsal striatum at rostral levels, and in a large centromedial region at caudal levels. In the nucleus accumbens, *arc* induction was much lower in magnitude than in the dorsal striatum. These results are in



**Figure 2** Induction of *c-fos* and  $\Delta$ FosB in Thr-34 and Thr-75 DARPP-32 mutant mice. Thr-34 mutants showed attenuated induction of *c-fos* mRNA after acute cocaine injection (a, b; 20 mg/kg i.p.,  $n = 5$  per group), whereas Thr-75 mice showed normal *c-fos* responses (b). Data for nucleus accumbens are expressed as mean  $\pm$  SEM, \* $p < 0.05$  between genotypes, \*\* $p < 0.05$  between cocaine and saline, by ANOVA followed by PLSD. Thr-34 mutants also show attenuated  $\Delta$ FosB induction after repeated cocaine administration (c, d; 20 mg/kg i.p. daily for 7 days,  $n = 5-6$  per group). Data for nucleus accumbens are expressed as mean  $\pm$  SEM, \* $p < 0.05$  between genotypes, \*\* $p < 0.05$  between cocaine and saline ANOVA followed by PLSD test.

agreement with previous data obtained in the rat (Tan *et al*, 2000). Characterization of this response revealed that mRNA levels were elevated within 15 min of cocaine administration (data not shown). In assessing the effect of various doses of cocaine, we found that smaller responses were elicited following injections of 10 mg/kg, with no effect detected at a lower dose (5 mg/kg) (Figure 3a). The response elicited by 20 mg/kg appeared to be near maximal, as increasing the dose to 40 mg/kg elicited a similar response (data not shown). These results are in agreement with previous data obtained in the rat (Fosnaugh *et al*, 1995).



**Figure 3** Cocaine-driven induction of *arc* mRNA in the striatum of wild-type mice is dose dependent. (a) Mice were killed 1 h after a single i.p. injection with saline or cocaine at 5, 10, or 20 mg/kg. Brain sections (5–6 sections per mouse,  $n=4$ –5 per group) were then processed in parallel for *in situ* hybridization of *arc* mRNA. (b) Data for dorsal striatum are expressed as mean  $\pm$  SEM. Statistical significance was assessed by ANOVA with multiple comparisons (Newman–Keuls multiple comparison test): \*\*\* $p < 0.001$  when compared to injection with saline; ^^^ $p < 0.01$  when compared to injection with cocaine at 5 mg/kg; \$\$ $p < 0.01$  when compared to injection with cocaine at 10 mg/kg.

Cocaine-driven *arc* induction was next analyzed in DARPP-32 knockout mice (Figure 4a). Adjacent brain sections of saline- or cocaine-treated wild-type or knockout mice were processed in parallel for *in situ* hybridization of DARPP-32 (Figure 4a, upper panels) and *arc* (lower panels) mRNAs. As expected, *arc* mRNA was significantly induced by acute cocaine bilaterally in the dorsal striatum of wild-type mice (Figure 4a), but this induction was completely lost in the DARPP-32 knockout mice (Figure 4a). These data strongly suggest that Arc is a target of DARPP-32-mediated signaling in striatal neurons. Using DARPP-32 Thr-34 (Figure 4b) and Thr-75 (Figure 4c) mutants, we investigated the role of each phosphorylation site in the regulation of *arc* mRNA induction. As shown in Figure 4b, the induction of *arc* mRNA by cocaine was significantly reduced in the dorsal striatum of Thr-34-mutant mice, but normal in Thr-75 mutant mice (Figure 4c).

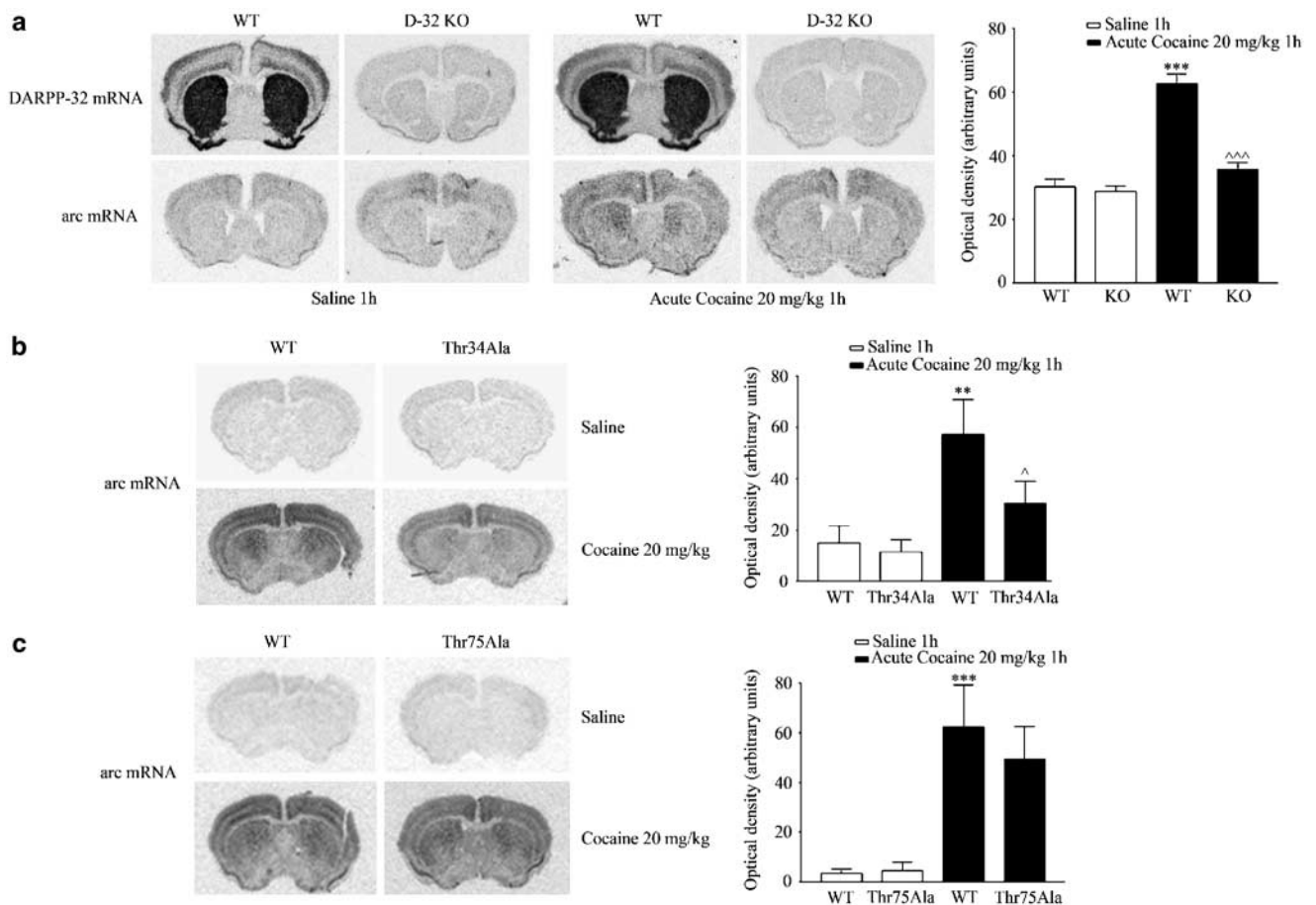
## DISCUSSION

We examined the role of Thr-34 and Thr-75 DARPP-32 phosphorylation sites in behavioral and biochemical responses to cocaine. The results obtained reveal distinct roles for these two sites in DARPP-32 in mediating short- and long-term actions of this psychostimulant drug. The findings from studies using the Thr-34 mutant mice highlight the importance of the role of DARPP-32 and its inhibition of PP1 in mediating acute actions of cocaine. The results obtained also reveal unexpected roles for the Thr-34

and Thr-75 sites in mediating certain sustained actions of cocaine.

DARPP-32 Thr-34 mutant mice show reduced sensitivity to the rewarding and acute locomotor activating effects of cocaine. Moreover, cocaine-dependent regulation of gene expression in the striatum of the Thr-34 mutant mice generally parallels the behavioral consequences of this mutation. The induction of *c-fos* mRNA in striatum caused by acute cocaine is lost in the Thr-34 mutant mice. Similarly, the induction of  $\Delta$ FosB in the nucleus accumbens caused by chronic cocaine is lost in the Thr-34 mutant mice. Notably, loss of Thr-34 in DARPP-32 results in a behavioral and biochemical phenotype with respect to acute and chronic cocaine administration that is the same as that observed in DARPP-32 knockout mice (Fienberg *et al*, 1998; Hiroi *et al*, 1999; Snyder *et al*, 2000). Sustained induction of  $\Delta$ FosB in the nucleus accumbens following repeated exposure to cocaine or other drugs of abuse leads to numerous neuronal adaptations via regulation of the expression of a wide range of gene targets (Nestler *et al*, 2001; McClung and Nestler, 2003). The loss of  $\Delta$ FosB induction could account for the reduction in rewarding responses to cocaine seen in the DARPP-32 Thr-34 and knockout mice, since direct blockade of  $\Delta$ FosB activity results in a similar phenotype (Peakman *et al*, 2003).

These findings support an essential role of PKA phosphorylation of DARPP-32 at Thr-34 in cocaine action. DARPP-32 is likely to play a critical role in amplifying the actions of D1-receptor/cAMP signaling to ensure robust activation of downstream signaling targets. Important targets are likely to include ERK and subsequent regulation



**Figure 4** Induction of *arc* mRNA in DARPP-32 KO mice and in Thr-34 and Thr-75 DARPP-32 mutant mice. (a, upper panels) DARPP-32 mRNA; (a, lower panels; b, c) *arc* mRNA. DARPP-32 KO (a,  $n = 5$  per group) and Thr-34 (b,  $n = 5$  per group) mutant mice showed attenuated induction of *arc* mRNA after acute cocaine injection (20 mg/kg i.p.), whereas Thr-75 mutants (c,  $n = 3-4$  per group) show normal levels of induction. Five to six brain sections per mouse were used for these studies. Data for dorsal striatum are expressed as mean  $\pm$  SEM. Statistical significance was assessed by ANOVA with multiple comparisons (Newman-Keuls multiple comparison test): \*\*\* $p < 0.001$  or \*\* $p < 0.01$  when compared to injection with saline in the wild-type (WT) mice; ^^^ $p < 0.001$  or ^ $p < 0.05$  when compared to injection with cocaine at 20 mg/kg in the WT mice.

of CREB-dependent transcription. In support of this, recent studies have found that Thr-34 of DARPP-32 is necessary for the activation of ERK by several drugs of abuse, including cocaine (Valjent *et al*, 2005). In this respect, phosphorylation of Thr-34 of DARPP-32 and inhibition of PP1 is required for normal acute responses to cocaine, including its acute behavioral effects and its initial regulation of gene expression. DARPP-32 is also apparently required for the slower accumulation of  $\Delta$ FosB, which is seen following chronic cocaine administration (Nestler *et al*, 2001).

Interestingly, the Thr-34 mutant mice show an abnormal pattern of locomotor sensitization to repeated cocaine exposure, with maximal sensitization occurring more rapidly compared to wild-type mice. The same pattern of locomotor sensitization was also observed in DARPP-32 knockout mice (Fienberg *et al*, 1998; Hiroi *et al*, 1999). However, the loss of  $\Delta$ FosB induction seen in the DARPP-32 Thr-34 and knockout mice cannot explain the accelerated locomotor sensitization seen in these mice, since increased  $\Delta$ FosB levels enhance locomotor responses to cocaine and

blockade of  $\Delta$ FosB has no effect (Kelz *et al*, 1999; Peakman *et al*, 2003). The molecular basis for the phenomenon of accelerated locomotor sensitization to cocaine observed in the DARPP-32 Thr-34 and knockout mice is not known at this time. These observations raise the possibility that, in addition to its role in signal amplification being coupled to positive aspects of D1/cAMP signaling, phosphorylation of Thr-34 plays an additional negative feedback role. Alternatively, since the locomotor sensitizing vs rewarding effects of cocaine are likely mediated by complex striatal and nonstriatal networks, DARPP-32 and inhibition of PP1 may have functionally opposing roles in these various networks.

The rewarding and locomotor activating effects of cocaine are not affected by deletion of the Thr-75 phosphorylation site of DARPP-32. Our previous studies showed that acute inhibition of DARPP-32 phosphorylation at Thr-75 achieved by local infusion of a Cdk-5 inhibitor into the nucleus accumbens caused increased locomotor sensitization (Bibb *et al*, 1999). These and other studies supported the hypothesis that Cdk-5, and phosphorylation of Thr-75

in DARPP-32, play a negative-feedback role in the actions of cocaine, likely via the ability of Thr-75 DARPP-32 to inhibit PKA and its phosphorylation of DARPP-32 at Thr-34 among other substrates. Removal of the inhibitory Thr-75 site in the mutant mice would be expected to alter the set-point of PKA- and DARPP-32 Thr-34-dependent signaling, and to lead to an increased sensitivity to cocaine. Notably, in direct contrast to the Thr-34 mutant mice, the Thr-75 mutant mice exhibited no locomotor sensitization to repeated cocaine. This discrepancy could be due to the different effect of acute vs constitutive loss of Thr-75 in the two situations. It also could relate to the fact that the Thr-75 mutation affects DARPP-32 function in all cells in which the protein is expressed, with different consequences for sensitization occurring in regions other than the nucleus accumbens. While further work is needed to study these possibilities, it is likely that the molecular basis for the effect of Thr-75 on locomotor sensitization results from an unknown negative feedback function of DARPP-32, as discussed above for the Thr-34 mice. In any event, the present results further support antagonistic properties of Thr-34 and Thr-75 phosphorylation in the actions of DARPP-32.

Consistent with our findings on *c-fos* induction, cocaine-driven induction of *arc* mRNA was also abolished in DARPP-32 knockout mice, as well as in DARPP-32 Thr-34 mutants, but not in Thr-75 mutants. Arc is highly enriched in neuronal dendrites where its distribution parallels that of F-actin (Lyford *et al*, 1995), and where it interacts with cytoskeletal proteins such as MAP-2 (Fujimoto *et al*, 2004). Arc is unique among immediate early genes, in that its mRNA rapidly localizes to discrete dendritic regions that have received direct synaptic stimulation (Steward *et al*, 1998; Steward and Worley, 2002). Moreover, Arc protein is required for long-term potentiation as well as certain forms of memory (Guzowski *et al*, 2000; Plath *et al*, 2001; Ying *et al*, 2002), and promotes CaM/kinase II-dependent neurite extension (Donai *et al*, 2003). Our results add Arc as an important downstream molecular target for DARPP-32, and provide further support for a role for DARPP-32 in the regulation of synaptic plasticity in the basal ganglia.

Together, our findings support an essential role of PKA phosphorylation of DARPP-32 at Thr-34 in cocaine action. Our hypothesis is that PKA phosphorylation of DARPP-32, and its subsequent inhibition of PP1, is required for normal acute responses to dopamine signals. Such responses mediate cocaine's acute behavioral effects and its ability to induce *c-fos*, *fosb*, and *arc* initially. These responses are also required for the accumulation of  $\Delta$ FosB, which is seen after chronic cocaine (Nestler *et al*, 2001). In contrast, the absence of DARPP-32's PP1 inhibitory activity leads to differential adaptations to repeated perturbations in dopamine signaling, with the ultimate result of enhanced locomotor sensitization to chronic cocaine. We hypothesize that loss of DARPP-32's PKA inhibitory activity, through mutation of Thr-75, leads to opposing adaptations and to inhibition of locomotor sensitization, even though it does not affect acute dopamine signaling. While further work is needed to understand the molecular basis of such abnormal adaptations, these results highlight the complex and central role of DARPP-32 in mediating behavioral and molecular plasticity to cocaine.

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