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The membrane cytoskeletal protein adducin is phosphorylated by protein kinase C in D1 neurons of the nucleus accumbens and dorsal striatum following cocaine administration

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Abstract

Repeated cocaine administration results in persistent changes in synaptic function in the mesolimbic dopamine system that are thought to be critical for the transition to addiction. Cytoskeletal rearrangement and actin dynamics are essential for this drug-dependent plasticity. Cocaine administration increases levels of F-actin in the nucleus accumbens and is associated with changes in the phosphorylation state of actin binding proteins. The adducins constitute a family of proteins that interact with actin and spectrin to maintain cellular architecture. The interaction of adducin with these cytoskeletal proteins is regulated by phosphorylation, and it is therefore expected that phosphorylation of adducin may be involved in morphological changes underlying synaptic responses to drugs of abuse including cocaine. In the current study, we characterized the regulation of adducin phosphorylation in the nucleus accumbens and dorsal striatum in response to various regimen of cocaine. Our results demonstrate that adducin is phosphorylated by PKC in medium spiny neurons that express the dopamine D1 receptor. These data indicate that adducin phosphorylation is a signaling event regulated by cocaine administration and further suggest that adducin may be involved in remodeling of neuronal cytoskeleton in response to cocaine administration.

Keywords

addiction; signaling; dopamine; D1; chelerythrine

Introduction

Behavioral responses to cocaine are thought to be a consequence of morphological changes in brain areas associated with addiction (Robinson and Kolb 2004; Shen et al. 2009). A number of changes in cytoarchitecture in the nucleus accumbens (NAc) have been identified following cocaine exposure. For example, repeated cocaine administration followed by withdrawal and a cocaine challenge can result in alterations in spine density and changes in levels of proteins associated with the actin cytoskeleton (Pulipparacharuvil et al. 2008; Shen et al. 2009). Thus, identification of molecular changes related to cytoskeletal remodeling following cocaine administration may help identify molecular mechanisms that contribute to behavioral responses leading to drug abuse.

Synaptic remodeling requires alterations of the cytoskeleton at the site of growth, but the molecular mechanisms underlying those alterations are poorly understood. A particularly

interesting family of cytoskeletal proteins is the adducins, which provide a link between signaling cascades and cell structure. Previous studies have identified changes in phosphorylation of the membrane cytoskeleton-associated protein adducin following chronic cocaine administration (Toda et al. 2006). Thus, identifying the mechanisms leading to adducin phosphorylation may be important for understanding dendritic plasticity in response to cocaine. Adducins cross-link actin filaments with spectrin, cap the barbed end of actin filaments, and bundle actin filaments (Gardner and Bennett 1987; Mische et al. 1987; Kuhlman et al. 1996). Both phosphorylation of adducins by PKC and calcium/calmodulin binding inhibit the actin-capping and spectrin-recruitment properties of adducins (Gardner and Bennett 1987; Kuhlman et al. 1996; Matsuoka et al. 1996; Matsuoka et al. 1998). Interestingly, it has been shown that synaptic plasticity and spatial learning are impaired in mice lacking beta-adducin (Rabenstein et al. 2005). This study suggests that adducin is required for the establishment of morphological changes related to synaptic plasticity.

The mammalian adducin proteins are encoded by three genes (α , β and γ) with several splice variants (Suriyapperuma et al. 2000). Functional adducin is composed of heterodimers or heterotetramers of α/β or α/γ (Dong et al. 1995; Hughes and Bennett 1995). Adducin is found throughout the brain, with high expression of α -adducin in the hippocampus and cerebellum and lower expression in cortex and striatum of rats (Seidel et al. 1995). The β subunit of adducin is enriched in brain and erythrocytes (Gilligan et al. 1999).

Adducins have been implicated in structural change in numerous cell types (Waseem and Palfrey 1988; Kaiser et al. 1989; Waseem and Palfrey 1990; Yue and Spradling 1992; Matsuoka et al. 1998; Fukata et al. 1999; Gilligan et al. 2002; Robledo et al. 2008). They are expressed throughout the brain in regions that are rich in synaptic contacts (Seidel et al. 1995), and the phosphorylated form of adducin (phospho-adducin) is detected in the dendrites of hippocampal neurons (Matsuoka et al. 1998). Thus, the adducins represent a family of proteins that are ideal candidates to establish morphological changes related to synaptic plasticity.

Adducins cap, bundle and promote spectrin binding to actin filaments in a phosphorylation-dependent manner (Gardner and Bennett 1987; Mische et al. 1987; Kuhlman et al. 1996). Indeed, several kinases including Rho kinase, PKA and PKC can phosphorylate adducin on specific consensus sites. Once adducin is phosphorylated, it is removed from the barbed end of the actin filament, allowing the filament to either polymerize or depolymerize (Matsuoka et al. 1996; Matsuoka et al. 1998). Several studies have highlighted the importance of the PKC phosphorylation site for the regulation of adducin function. For instance, adducin phosphorylation by PKC can mediate morphological changes during platelet activation (Barkalow et al. 2003). In neurons, adducin phosphorylated at PKC sites has been localized to dendritic spines, suggesting a role for adducin in cytoskeletal remodeling (Matsuoka et al. 1996). Another report suggested that pleiotrophin promotes phosphorylation of beta-adducin at PKC sites and that this could play a role in support of heterochromatin and centrioles during mitosis (Pariser et al. 2005). In *Aplysia*, ApADD (The *Aplysia* homolog of mammalian adducin) is phosphorylated by PKC in parallel with long term facilitation at sensory neuron-motor neuron synapses (Gruenbaum et al. 2003). Taken together, these data suggest that phosphorylation of adducin at the PKC consensus sites could contribute to cytoskeletal changes associated with synaptic activity.

In the current study we characterized the phosphorylation of adducin by western blotting in striatal and nucleus accumbens (NAc) homogenates and by immunohistochemistry in striatal slices in response to cocaine. A time course shows that acute cocaine administration increases adducin phosphorylation transiently, and pharmacological studies demonstrate that this event is dependent on D1 receptor signaling and activation of PKC. In addition, using transgenic mice expressing GFP only in D1-expressing neurons, we show that phosphorylation of adducin

is confined to D1-positive medium spiny neurons. These data indicate that adducin phosphorylation is a signaling event regulated by cocaine administration and suggest that adducin might be involved in remodeling of neuronal morphology associated with cocaine administration.

Materials and Methods

Animals and drug administration

Male C57BL/6J mice (8- to 12-week old) were purchased from Jackson Laboratories (Bar Harbor, ME). D1-EGFP-mice (Gensat) were backcrossed onto the C57BL/6J background for more than 5 generations. Male mice were used for all experiments. Mice were maintained in a temperature-controlled vivarium (21±21°C) under a 12:12 h light–dark cycle and housed four per cage. Food and fluid were available *ad libitum*. Mice were habituated to handling for at least 3 days before biochemical studies. Cocaine (20 mg/kg) was injected intraperitoneally and dissolved in 0.9% NaCl. *R*(+)-SCH 23390 (0.25 mg/kg; Sigma). *S*(–)-raclopride (0.25 mg/kg; Sigma) and the PKC inhibitor chelerythrin (1, 3 and 10 mg/kg; Sigma) were injected 30 min before cocaine injection. All studies were approved by the Yale University Animal Care and Use Committee and followed the NIH Guide for the Care and Use of Laboratory Animals.

Western Blotting

Mice were habituated to daily saline injections (i.p.) for 3 days preceding the experiments. For immunoblotting studies, the brain was immediately placed in liquid nitrogen (for 12 sec). The frozen brains were cut, and microdisks (diameter 1.2 mm; depth 1 mm) were punched bilaterally from the dorsal striatum and nucleus accumbens (NAc), and stored at –80°C. Microdisks were lysed by sonication in 1% (vol/vol) SDS containing 1 mM sodium orthovanadate at 100°C and maintained at this temperature for 5 min. Equal amounts of lysate (30 µg of protein) were analyzed by immunoblotting. The following antibodies were used for Western blotting: anti-phospho-adducin (mouse monoclonal, Upstate, 1/5000), β-adducin (rabbit polyclonal, Santa Cruz, 1/500); pan-adducin (rabbit polyclonal, a generous gift of Dr. D. Gilligan, University of Washington, Seattle).

Immunofluorescence

At the indicated times after drug treatment, mice were anaesthetized and perfused transcardially with 4% paraformaldehyde in 0.1 M Na₂HPO₄/NaH₂PO₄ buffer (pH 7.5) for immunofluorescence analysis. Brains were post-fixed overnight in the fixative solution used for perfusion and stored at 4°C. Sections (30 µm thick) were cut with a Vibratome (Leica) and kept at –20°C until use in a solution containing 30% (vol/vol) ethylene glycol, 30% (vol/vol) glycerol, and 0.1 M phosphate buffer. For the detection of phosphorylated proteins, 50 mM NaF was included in all buffers and incubation solutions. Floating sections were saturated for 1 h with 10% normal goat serum in Tris-buffered saline (25 mM Tris-Cl, 150 mM NaCl, pH 7.4). Sections were then rinsed three times in TBS and incubated with the phospho-adducin mouse monoclonal primary antibody raised against Ser 724 (1/200) followed by anti-mouse Cy3-conjugated secondary antibody (Amersham) (1:1000). Sections were mounted in Vectashield with 4',6-diamidino-2-phenylindole (DAPI) counterstain (Vector Laboratories). Images shown were obtained using the apotome microscope (Leica) coupled to a CCD camera. Quantitation was performed by counting the number of fluorescent cells with Image Pro Plus 4.5.0.19 software bilaterally for each region of interest in two to four brain sections per animal.

Double-fluorescence analysis of GFP and phospho-adducin immunoreactivity in adult BAC transgenic mice expressing GFP under the control of the DA-D1 receptor promoter (*drd1a*-EGFP mice, GENSAT) was performed on three different groups : group 1, mice treated once daily for 7 days with saline (sal+sal); group 2, mice treated once daily for 7 days with cocaine

(20 mg/kg) (coc+coc); group 3, mice treated for 6 days with saline followed by a single cocaine injection (20 mg/kg) on day 7. These groups were chosen to determine whether chronic cocaine treatment could increase adducin phosphorylation or whether there might be tolerance after acute injection. In addition, withdrawal from cocaine administration followed by an acute injection of cocaine was chosen to replicate paradigms used previously to identify changes in spine density following cocaine sensitization (Robinson and Kolb 2004). We therefore used this regimen to determine whether adducin phosphorylation could contribute to molecular changes previously identified in response to cocaine sensitization.

Quantitation

Images from immunofluorescence experiments were taken (20× and 10× magnification) from 20 independent fields for each experiment ($n = 5$ independent experiments for each treatment). Immunopositive cells were counted manually using Image Pro Plus 4.5.0.19 software (Media Cybernetics, Wokingham, Berkshire, UK) on all the pictures taken within an experiment.

For western blot experiments, films were scanned and analyzed using Image J software. Phospho-signal detection was measured by normalization of the density obtained from the protein of interest with the non-phosphorylated protein used as loading control (β -adducin or pan-adducin). Data are expressed as mean $\pm$ SEM.

Statistical analyses

Data were analyzed by two-way ANOVA with “treatment” (up to 6 levels) and “brain region” (2 levels) as between-subject factors. When relevant, posthoc analyses were performed by unpaired two-tailed t-tests with Bonferonni/Dunnett's corrections for multiple comparisons, but compensated only for the number of experimentally relevant comparisons in order to limit family-wise errors. In all cases, the initial value of alpha was set at 5%.

Results

Acute cocaine treatment induces adducin-phosphorylation throughout the striatum

After administration of cocaine (20 mg/kg) *in vivo*, the temporal pattern of adducin-phosphorylation was analyzed by western blot. The phosphorylation state of adducin was detected with a mouse anti-phospho-adducin antibody that specifically recognizes phospho-serine 724, which is phosphorylated by PKC in erythrocytes. In saline-treated mice, a low level of basal serine 724 phosphorylation was detected in the striatum; however, following cocaine treatment, adducin phosphorylation was rapid, and transient, reaching a peak 10 min after cocaine injection. Thirty min after cocaine administration, adducin phosphorylation had returned to basal levels (Fig. 1 A). In parallel, we checked the specificity of the anti-phospho-adducin antibody by testing it on homogenates from wild type (WT) and β -adducin knockout (KO) mice at the timepoint (10 min) and dose of cocaine (20 mg/kg) resulting in peak adducin phosphorylation. Immunoblots of striatal and NAc homogenates confirm the specificity of the phospho-specific antiserum for adducin proteins since no signal is detected in brain homogenates from β -adducin KO mice, whereas a strong signal is seen in homogenates from WT mice (Fig. 1B). While this experiment demonstrates that β -adducin is necessary for the phospho-adducin signal, we cannot rule out the possibility that the phospho-adducin antiserum also recognizes phospho- α -adducin in WT tissue, since in the absence of β -adducin, α/β heterodimers would not form and phospho- α -adducin might be lost.

Adducin-phosphorylation induced by acute cocaine administration depends on D1-dopamine receptors

We evaluated the involvement of dopaminergic receptors in cocaine-induced adducin phosphorylation at the time point of peak activation (20 mg/kg; 10 min) by western blot (Fig. 2) and immunohistochemistry (Fig. 3). In order to confirm that the anti-phospho-adducin antibody was also specific when used for immunohistochemistry in tissue sections, we first evaluated the phospho-adducin signal in striatal slices from WT and β -adducin KO mice treated with cocaine (10 min, 20 mg/kg, i.p.). No phospho-adducin staining was observed in striatal slices from β -adducin KO mice while a robust phospho-adducin signal was seen in sections from WT mice (Fig. 3A). To determine whether DA signaling is necessary for phosphorylation of adducin in response to cocaine administration, mice were treated with a selective antagonist of dopamine D1-receptors, SCH 23390 (0.25 mg/kg) or a selective antagonist of dopamine D2-receptors, raclopride (0.25 mg/kg). Western blot analyses revealed that there was an overall treatment effect ($F(5, 89) = 4.86, p = 0.006$), with no differences across the brain region considered ($F(5, 89) = 1.34, p = 0.25$). Post-hoc analyses further revealed that blockade of D1 receptors by SCH 23390 resulted in a complete inhibition of cocaine-induced adducin-phosphorylation throughout the medial striatum (Fig. 2A; $t_{19} = 0.89, p = 0.0046$) and NAc (Fig. 2B; $t_{19} = 1.07, p = 0.036$). In parallel, we counted the number of neurons immunopositive for phospho-adducin by immunohistochemistry in the different striatal subregions (medial striatum, NAc core and shell; Fig. 3A-C). Similar to what was seen in western blots, the number of neurons immunopositive for phospho-adducin was altered by the various treatments used ($F(5, 126) = 22.7, p < 0.0001$), but with a differential effect depending on the brain region measured ($F(10, 126) = 8.55, p < 0.0001$). Treatment with the D1 antagonist SCH23390 resulted in a significant decrease in phospho-adducin positive cells induced by cocaine administration in all subregions of the striatum ($F(1, 42) = 50.14, p < 0.0001$), with a difference in the size of the effect ($F(2, 42) = 18.45, p > 0.001$, mainly due to the variable levels of phospho-adducin after cocaine in the different brain regions. These results demonstrate that cocaine-induced adducin-phosphorylation is dependent on DA signaling and D1 receptor activation. Interestingly, treatment with SCH 23390 also decreased the number of neurons immunopositive for phospho-adducin in striatum and NAc under basal conditions (saline treatment; $F(1, 14) = 11.42, p = 0.0045$ and $F(1, 14) = 4.10, p = 0.025$, respectively), suggesting that D1 receptor activity may also regulate the phosphorylation state of adducin tonically (Fig. 3B-D).

The involvement of the D2 receptor subtype in cocaine-induced adducin phosphorylation was also evaluated. Western blot analyses revealed that blockade of D2 receptors by raclopride significantly inhibited cocaine-induced adducin phosphorylation in the medial striatum (Fig. 2A; $t_{19} = 0.91, p = 0.0005$), but had no effect on the phosphorylation state of adducin in the NAc (Fig. 2B $t_{19} = 1.17, p = 0.86$). In parallel with this study, we performed immunohistochemistry in the absence or presence of raclopride confirming that D2 antagonism did not alter the number of neurons immunopositive for phospho-adducin in the NAc (core ($t_{19} = 36.54, p = 0.21$) and shell ($t_{19} = 30.73, p = 0.59$)) but decreased adducin phosphorylation in the medial striatum ($t_{19} = 124.27, p < 0.0001$) (Fig 3A-C).

Taken together, these results show that D1 receptor activity is essential for cocaine-induced adducin phosphorylation, whereas the contribution of D2 receptors seems to be restricted to medial striatum.

Chronic cocaine treatment-induced adducin phosphorylation is restricted to D1R-expressing neurons in striatum and NAc

Adult BAC transgenic mice (Gong et al. 2003) expressing GFP under the control of the DA-D1 receptor promoter (*drd1a*-EGFP mice, GENSAT) were treated once daily for 7 days with

saline or cocaine (20 mg/kg), or with 6 days of saline followed by a single cocaine injection (20 mg/kg) on day 7. The number of neurons immunopositive for phospho-adducin was then counted in medial striatum, NAc core and NAc shell and co-localization with GFP in D1 receptor-positive neurons was evaluated (Fig. 4). Double-fluorescence analysis of GFP and phospho-adducin immunoreactivity in the medial striatum and NAc of mice perfused 10 min after the last saline or cocaine injection revealed that adducin phosphorylation occurred exclusively in D1R-expressing neurons in the medial striatum ($F(1, 10) = 6.21$, $p = 0.031$) and in the NAc shell ($F(1, 10) = 23.97$, $p = 0.006$) and core ($F(1, 10) = 113.42$, $p = 0.01$). Taken together with the data showing that adducin phosphorylation was blocked by a D1 antagonist, these results demonstrate that the adducin-phosphorylation induced by acute or chronic cocaine administration is dependent on D1R signaling.

Cocaine-induced adducin phosphorylation is PKC dependent

Previous studies have suggested that adducin is a substrate for PKC *in vivo* (Matsuoka et al. 1996; Matsuoka et al. 1998) and that PKC activity is required for the rewarding effects of drugs of abuse (Aujla and Beninger 2003; Harlan et al. 2004). Several pharmacological compounds can prevent PKC activity, however only chelerythrine appears to cross the brain blood barrier (Brannan et al. 2009). In order to determine whether the phosphorylation of adducin was mediated through PKC activity *in vivo*, we injected chelerythrine (1, 3 and 10 mg/kg, i.p.) before treating mice with vehicle or cocaine (20 mg/kg; i.p.). There was an overall treatment effect ($F(4, 29) = 137.33$, $p < 0.0001$) and systemic chelerythrine injection resulted in a dose-dependent attenuation of the number of neurons immunopositive for phospho-adducin following cocaine administration. The lowest dose of chelerythrine (1 mg/kg) was ineffective ($t_7 = 37.6$, $p = 0.16$) but both 3 and 10 mg/kg of chelerythrine abolished the increase in phospho-adducin induced by cocaine in the dorsal striatum ($t_7 = 53.1$, $p < 0.0001$ and $t_7 = 53.1$, $p < 0.0001$, respectively) and NAc ($t_7 = 62.9$, $p < 0.0001$ and $t_7 = 62.9$, $p < 0.0001$, respectively) (Fig. 5), demonstrating that cocaine-induced adducin phosphorylation is dependent on PKC activity.

Discussion

Long lasting neuroadaptations in dopaminergic neurotransmission are thought to underlie behavioral plasticity in response to treatment with psychostimulant drugs. Psychostimulant-induced changes in dendritic spine density in the NAc are hypothesized to contribute to drug responses (Robinson and Kolb 2004); however, the molecular mechanisms that lead to these morphological changes are not well understood. Adducin, a membrane cyto-skeletal protein is a candidate molecule that might be involved in these events. The current study demonstrates that adducin is phosphorylated in the striatum and NAc core and shell after either a single cocaine injection or chronic, daily cocaine administration *in vivo*. Adducin is phosphorylated by PKC in response to cocaine exclusively in D1-receptor expressing neurons and could therefore contribute to D1-dependent changes in neuronal activity leading to dopamine-dependent behaviors.

Adducin can be phosphorylated by several kinases that are activated by synaptic activity (Matsuoka et al. 1996; Matsuoka et al. 1998; Fukata et al. 1999). Phosphorylation by PKA or PKC, or calmodulin-binding, causes adducin to dissociate from actin filaments, whereas phosphorylation by Rho-kinase increases adducin's affinity for actin filaments. In activated platelets, adducin is phosphorylated by PKC and becomes a better substrate for calpain proteolysis, suggesting that adducin complexes play a role in maintaining the shape of resting platelets, but must be removed from actin filaments during platelet activation (Gilligan et al. 2002). It is therefore possible that adducin could act as an intermediary between synaptic signaling and the resulting cytoskeletal plasticity. Adducin phosphorylation could destabilize

the post-synaptic cytoskeleton, allowing for changes in the post-synaptic milieu to occur, and could be followed by adducin dephosphorylation leading to its reassociation with the actin network and restabilization of the cytoskeleton to stabilize new synapses. This is consistent with the lack of intermediate- and long-term synaptic plasticity in hippocampal slices and impairment of spatial learning in mice lacking β -adducin (Rabenstein et al. 2005).

Our pharmacological studies revealed a dichotomy in adducin phosphorylation between dorsal and ventral striatum. Cocaine-induced adducin phosphorylation in dorsal striatum was blocked by both D1 and D2 antagonists whereas adducin phosphorylation induced by cocaine in the NAc was only abolished by administration of a D1 antagonist, but not a D2 antagonist. The dorsal and ventral striatum are distinct structures that receive different inputs and make different efferent connections. While it is not clear how D2 receptor signaling differs between the two portions of the striatal complex, these neuroanatomic differences might explain the observed dichotomy in the sensitivity of adducin phosphorylation to dopamine receptor subtype antagonists.

The studies examining phospho-adducin levels in transgenic mice that express GFP only in neurons expressing the dopamine D1 receptor also show that basal adducin phosphorylation and phosphorylation induced by both acute and chronic cocaine administration occurs only in D1 neurons. The current findings are consistent with a previous study demonstrating a robust phospho-adducin signal in rats treated daily with cocaine or saline, withdrawn for 21 days, and challenged acutely with cocaine (Toda et al. 2006). The Toda study also demonstrated that no phospho-adducin signal could be detected following saline challenge. In the current study, we did not include a group that received cocaine chronically with an acute saline challenge following withdrawal. Thus, it is possible, based on the study by Toda and colleagues, that the phospho-adducin signal would not persist following cocaine withdrawal without the acute cocaine challenge.

It is notable that the phospho-adducin signal observed in the current study peaked at 10 min and subsequently declined, whereas Toda and colleagues identified a robust increase in phospho-adducin 45 min after injection of cocaine (Toda et al. 2006). A number of differences between the studies may explain this discrepancy. First, the previous study used rats, and the current study used mice. Mice and rats can exhibit differences in drug metabolism that might alter the time-course of cocaine response. In addition, the doses used and the length of the treatments were different between the two studies. It is therefore possible that a higher dose of cocaine (such as 30 mg/kg used in the previous study) could sustain the adducin phosphorylation signal for a longer time in mice. It should also be noted that in the rat study, western blots were performed on synaptosomes enriched in F/G actin, whereas the current analysis was performed on total homogenates of brain punches.

Several studies have identified potential molecular mechanisms underlying cocaine-dependent morphological changes. It has been reported that dendritic changes due to cocaine treatment are only stable in D1-expressing neurons (Lee et al. 2006), and these changes are associated with deterioration of the actin cytoskeleton, reduction in glutamate signaling-related proteins (Shen et al. 2009), and an increase in F-actin in the NAc (Toda et al. 2006). Indeed, cocaine can induce the phosphorylation of a number of actin binding proteins, including adducin (Toda et al. 2006). The current study is consistent with these results, and may suggest that phosphorylation of adducin on the PKC site could allow the dissociation of the spectrin-actin complex and the formation of F-actin, perhaps contributing to modulation of spine density in response to cocaine. Indeed, it has been shown that adducins cap, bundle and promote spectrin binding to actin filaments in a phosphorylation-dependent manner (Gardner and Bennett 1987; Mische et al. 1987; Kuhlman et al. 1996). These data might suggest that adducin, which

is known to cross-link the cytoskeleton and the plasma membrane (Anong et al. 2009), could help stabilize the reorganization of synaptic connectivity induced by cocaine.

Adducin is a PKC substrate (Matsuoka et al. 1996; 1998). PKC is a multigene family of at least ten isoforms. In MDCK cells adducin is phosphorylated by PKC δ (Chen et al. 2007), whereas in HeLa cells PKC α/β phosphorylates adducin (Pariser et al. 2005). Based on these studies, adducin phosphorylation can be phosphorylated by a number of PKC isoforms depending on the cell type. The identification of the adducin kinase in neurons has not been investigated. Multiple PKC isoforms (α , $\beta 1$, βII , γ , δ , ϵ , η , ζ , ν/λ) are expressed in brain (reviewed in Sacktor, 2008). PKC ζ is enriched in cortex, hippocampus, and striatum, and it is tempting to speculate that PKC ζ in neurons might be the adducin kinase. Currently, the broad PKC inhibitor chelerythrine is the most useful pharmacological compound for identifying PKC targets *in vivo* (Brennan et al. 2009) and there are no more selective pharmacological compounds that can inhibit individual PKC isoforms *in vivo*. Future studies using genetic methods may be useful in characterizing the predominant PKC subtype that acts as the adducin kinase in striatum.

In conclusion, the current study demonstrates that the plasma membrane-cytoskeleton interacting protein adducin is phosphorylated by PKC in response to cocaine. Adducin is phosphorylated after either acute or chronic cocaine treatment exclusively in D1-expressing neurons. We propose that PKC-mediated phosphorylation of adducin may stabilize structural changes induced by psychostimulants in the striatum and NAc.

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Figure 1. Time course of adducin phosphorylation in the striatum after acute cocaine injection
 Detection of phospho-adducin in striatal and NAc homogenates using an antiserum raised against phospho-serine 724 in adducin 5 min (*Coc 5*), 10 min (*Coc 10*), 20 min (*Coc 20*) and 30 min (*Coc 30*) after acute cocaine injection (20 mg/kg, i.p.). 1A, Western blot showing the time course of phospho-adducin induction after cocaine treatment (data are representative of 3-4 independent animals in each group). Quantitation of phospho-adducin compared to total adducin in each sample. 1B Immunoblots showing phospho-adducin levels in homogenates of striatum and NAc, from WT and beta-adducin KO mice after acute injection of cocaine (10 min; 20 mg/kg ip). Note the absence of phospho-adducin signal in beta-adducin KO mice compared to WT (data are representative of 3-4 independent animals in each group).

Figure 2. Cocaine-induced adducin-phosphorylation measured by western blotting is blocked by dopamine receptor antagonists

SCH 23390 (sch; 0.25 mg/kg, i.p.) or raclopride (raclo; 0.25 mg/kg, i.p.) were injected 30 min before saline (sal) or cocaine administration (coc). **A**, Immunoblots showing phospho-adducin levels in homogenates of the dorsal striatum from cocaine-injected mice (20 mg/kg; ip), in the presence or absence of dopamine receptor antagonists. **B**, Quantitation of phospho-adducin measured by Western blotting in the dorsal striatum (6-8 independent mice/group). ** $p < 0.001$ when comparing sch+coc group to sal+coc group and *** $p < 0.0001$ when comparing raclo+coc group to sal+coc group **C**, Immunoblot showing phospho-adducin in homogenates of the NAc from cocaine-injected mice (20 mg/kg, i.p.), in the presence or absence of dopamine receptor

antagonists. *D*, Quantitation of phospho-adducin measured by Western blotting in the NAc (6-8 independent mice/group). * $p < 0.05$ when compare sch+coc group to sal+coc group.

Figure 3. Cocaine-induced adducin-phosphorylation measured by immunocytochemistry in striatal slices depends on D1 receptor activity

A, Immunostaining for phospho-adducin in NAc induced by saline (sal) or cocaine (coc; 10 min, 20 mg/kg, i.p.) in striatal slices from WT and β -adducin KO mice. Note the absence of phospho-adducin staining in slices from β -adducin KO mice. B, Quantitation of phospho-adducin immunopositive neurons in medial striatum. *** $p < 0.0001$ when comparing sch+coc group to sal+coc group and *** $p < 0.0001$ when comparing raclo+coc group to sal+coc group C, Quantitation of phospho-adducin immunopositive neurons in NAc core *** $p < 0.001$ when comparing sch+coc group to sal+coc group D, Quantitation of phospho-adducin immunopositive neurons in NAc shell. *** $p < 0.001$ when comparing sch+coc group to sal+coc

group. SCH 23390 (sch; 0.25 mg/kg, i.p.) or raclopride (raclo; 0.25 mg/kg, i.p.) were injected 30 min before cocaine administration. The number of neurons immunopositive for phospho-adducin were counted in medial striatum, NAc shell and NAc core following cocaine-injection in mice (20 mg/kg; ip) in the presence or absence of DA receptor antagonists.

Figure 4. Acute or chronic cocaine administration induces adducin phosphorylation only in D1 receptor-expressing striatal neurons

A, B, C, Immunostaining for phospho-adducin (red) and EGFP fluorescence (green) in striatal subregions from *drd1a*EGFP mice (*A*, dorsal striatum; *B*, NAc core; *C*, NAc shell) 10 min after saline (sal) or cocaine administration (coc; 20 mg/kg; ip) or saline (sal). Arrowheads show phospho-adducin immunoreactivity in EGFP-positive (D1 receptor expressing) neurons. Quantitation of phospho-adducin immunoreactive/EGFP-positive (D1+) neurons in striatal subregions of *drd1a*-EGFP mice. * $p < 0.05$ when comparing sal+sal group to sal+coc and coc+coc groups in both striatal subregions.

Figure 5. Chelerythrine inhibits cocaine-induced adducin phosphorylation in the nucleus accumbens and the striatum

A,C, Phospho-adducin staining in the dorsal striatum (A) or the NAc (C), in the absence (sal) or presence of the general PKC inhibitor chelerythrine (chel; 1, 3 or 10 mg/kg). B, D, Quantitation of the number of neurons immunopositive for phospho-adducin in dorsal striatum (B) or NAc (D) in the absence or presence of chelerythrine. Data are representative of 4-5 independent mice for each group. *** $p < 0.0001$ when comparing sal+coc group to chel (3 mg)+coc group or chel (10 mg)+coc group in dorsal striatum and NAcc.