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Dopamine D1 and NMDA Receptors and ERK Mediate Neuronal Morphological Changes Induced by Repeated Cocaine Administration

Zhihua Ren^{1,*}, Wei-Lun Sun^{1,*}, Hongyuan Jiao¹, Dongsheng Zhang¹, Han Kong¹, Xinkang Wang², and Ming Xu¹

¹Department of Anesthesia and Critical Care, The University of Chicago, Chicago, IL 60637

²Wyeth Research, Collegeville, PA 19426

Abstract

The development of drug addiction involves persistent cellular and molecular changes in the central nervous system. The brain dopamine and glutamate systems play key roles in mediating drug-induced neuroadaptation. Changes in dendritic morphology in medium spiny neurons (MSNs) in the nucleus accumbens (NAc) and caudate putamen (CPu) accompany drug-induced enduring behavioral and molecular changes. We have investigated the potential involvement of dopamine D1 and D2 receptors, the *N*-methyl-*D*-aspartate (NMDA) receptor, and the extracellular signal-regulated kinase (ERK) in dendritic morphological changes induced by repeated cocaine administration. We show that either a genetic mutation or pharmacological blockade of dopamine D1 receptors attenuated cocaine-induced changes in both dendritic branching and spine density of MSNs in the shell of the NAc and CPu. In contrast, antagonism of dopamine D2 receptors had no obvious effect on changes in dendritic branching but had a partial effect on changes in spine density of MSNs in these brain regions following repeated cocaine injections. Pharmacological inhibition of either NMDA receptors or ERK attenuated cocaine-induced changes in both dendritic branching and spine density of MSNs in the shell of the NAc and CPu. These results suggest that dopamine D1 and NMDA receptors and ERK contribute significantly to neuronal morphological changes induced by repeated exposure to cocaine.

Keywords

dopamine; D1 and D2 receptors; NMDA receptors; ERK; cocaine; dendrite; spine density

Drug addiction is characterized by compulsive drug seeking and taking which is long-lasting (Wise, 2000; Koob et al., 2004; Kalivas and Volkow, 2005; Hyman et al., 2006; Kauer and Malenka, 2007; Kalivas and O'Brien, 2008). Dopamine (DA) projections from the midbrain to the nucleus accumbens (NAc), caudate putamen (CPu), prefrontal cortex (PFC) and other structures play key roles in mediating the neurobiological actions of drugs of abuse (Koob,

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Correspondence: Dr. Ming Xu, Department of Anesthesia and Critical Care, The University of Chicago, Chicago, IL 60637, Tel: 773 834 7937; Fax: 773 702 4791; mxu@dacc.uchicago.edu.

*Equal Contribution

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1992). Glutamatergic inputs from the PFC to the NAc also play an essential role in mediating drug actions (Kalivas, 2004; Wolf et al., 2004; Kalivas et al., 2005). In the striatum, dopaminergic inputs terminate in the dendritic shafts whereas glutamatergic afferents form synapses at the head of dendritic spines of the medium spiny neurons (MSNs) (Sesack and Pickel, 1990). There are close interactions between these two neural systems at the cellular and molecular levels.

The enduring nature of drug addiction at the behavioral level suggests that repeated exposure to drugs of abuse leads to persistent changes in DA and glutamate receptor-mediated cell signaling and gene expression in the brain (Wolf et al., 2004; Hyman et al., 2006; Kalivas and O'Brien, 2008; McGinty et al., 2008). Genetic and pharmacological studies demonstrate that D1 receptors take part in drug-induced behaviors (Xu et al., 1994a; 1994b; 2000; Katz et al., 1999; See et al., 2001; Caine et al., 2007). Moreover, cocaine can induce D1 receptor-mediated changes in cellular signaling and gene expression such as *c-fos* and *fosB* (Zhang et al., 2002; 2004; 2005; Hyman et al., 2006; Zhang et al., 2006; Jiao et al., 2007). Genetic, imaging and pharmacological studies also suggest the involvement of D2 receptors in cocaine actions (Volkow et al., 1999; Caine et al., 2002; Berglind et al., 2006). Furthermore, the *N*-methyl-*D*-aspartate (NMDA) receptor is known to play a role in cocaine-induced neurobiological changes. Blockade or loss of function of NMDA receptors attenuates stimulant-induced behavioral sensitization (Wolf, 1998; Heusner and Palmiter, 2005; Ramsey et al., 2008). Additionally, there are functional interactions between DA and NMDA receptors such that co-activation of D1 and NMDA receptors is required for certain neuroplastic changes in the NAc (Smith-Roe and Kelley, 2000) and D1 receptor stimulation leads to PKA-dependent phosphorylation of NMDA receptors (Snyder et al., 1998; Dudman et al., 2003; Jiao et al., 2007). Cocaine also activates extracellular signal-regulated kinase (ERK) in the brain (Valjent et al., 2000; 2005; Lu et al., 2005; 2006). Cocaine-induced ERK activation is oppositely regulated by DA D1 and D3 receptors and is also dependent on NMDA receptors (Zhang et al., 2004; Jiao et al., 2007). Inhibition of the ERK signaling pathway reduces cocaine-induced behavioral responses (Pierce et al., 1999; Valjent et al., 2000). ERK has been proposed to be a convergent intracellular mediator for the activation of D1 and NMDA receptors (Valjent et al., 2005; Jiao et al., 2007).

Changes in dendritic morphology induced by repeated stimulation may represent a form of neuronal plasticity that is related to changes in behavior, synaptic connections, and gene expression (Robinson and Kolb, 2004). Increases in dendritic branching and spine density in the NAc and PFC are observed after repeated exposure to psychostimulants (Robinson and Kolb, 1997; 1999; Robinson et al., 2001; Kolb et al., 2003; Li et al., 2004; Ferrario et al., 2005). Both D1 and D2 receptor-bearing neurons exhibit changed dendritic morphology following chronic exposure to cocaine, although dendritic spine formation is only stable and long-lasting in D1 receptor-bearing neurons (Lee et al., 2006).

The NAc and CPu both contribute significantly to chronic cocaine-induced behaviors (Di Chiara et al., 2004; Ito et al., 2004; See et al., 2007). MSNs in these brain regions receive convergent inputs from both DA and glutamate projections and they exhibit robust neuroplastic changes in response to repeated exposure to cocaine and other drugs of abuse (Hyman et al., 2006; Kauer and Malenka, 2007; Kalivas and O'Brien, 2008). Despite previous studies (Norrholm et al., 2003; Lee et al., 2006; Zhang et al., 2006; Pulipparacharuvil et al., 2008; Chen et al., 2008), the molecular basis of drug-induced dendritic morphological changes remains largely unknown. We have used both genetic and pharmacological approaches to investigate the role of DA D1 and D2 receptors, NMDA receptors and ERK in changes in dendritic morphology induced by repeated cocaine administration in these two brain regions. Our results suggest that DA D1 and NMDA receptors and ERK contribute significantly to neuronal morphological changes induced by repeated exposure to cocaine.

EXPERIMENTAL PROCEDURES

Mice

The DA D1 receptor mutant mouse was generated previously (Xu et al., 1994a) and it was back-crossed from the 129Sv/C57BL6J genetic background to the C57BL6J background for three generations. Homozygous mutant and wild-type littermates were produced from heterozygous mice by in-house breeding. Genotypes of both mutant mice and wild-type mice were determined by Southern blotting (Xu et al., 1994a). Approximately equal numbers of male and female mice at an average age of 9 weeks (ranging from 7 to 12 weeks) were used in the current study. Mice were maintained in an animal facility with a 12 hour light/dark cycle, and were housed in groups of 2–4 with food and water available ad libitum. Both temperature and humidity of the housing room were controlled. All animal protocols were in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animal and were approved by the University of Chicago Institutional Animal Care and Use Committee.

Drugs and antibodies

Cocaine hydrochloride, the D1 receptor antagonist SCH 23390, the D2 receptor antagonist raclopride, and the competitive NMDA receptor antagonist CPP were obtained from Sigma (St Louis, MO) and were dissolved in saline (Brackett et al., 2000; Prinssen et al., 2004; Zhang et al., 2005). The selective MEK inhibitor SL327 (Bristol-Myers Squibb, Princeton, NJ) was dissolved in 50% dimethylsulfoxide and saline (Zhang et al., 2004). Anti-phosphorylated-ERK (p-ERK) and ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-actin and appropriate secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Treatment paradigms

For morphological studies, all mice received intraperitoneal (i.p.) injections in volumes of 1 ml/300 g of body weight daily for 28 consecutive days (Norrholm et al., 2003; Robinson and Kolb, 2004; Lee et al., 2006; Zhang et al., 2006). Injections were performed in home cages and were during the light phase of the light/dark cycle. Wild-type mice were given 20 mg/kg of cocaine or saline (n=16 and 14 each) as positive and negative controls, respectively. The cocaine dose was selected based on previous morphological studies (Li et al., 2004; Zhang et al., 2006), which was also able to induce behavioral sensitization in wild-type mice but not in D1 receptor mutant mice in the home cage (Xu et al., 2000). To evaluate the role of D1 receptors, D1 receptor mutant mice were treated daily with cocaine (20 mg/kg, n=10) or saline (n= 6). In addition, groups of wild-type mice were treated with SCH 23390 (0.5 mg/kg) 30 minutes before daily cocaine or saline injections (n=5 each). To investigate the involvement of D2 receptors, wild-type mice were pretreated with daily raclopride (2.5 mg/kg, n=5 each) 30 minutes before cocaine or saline injections. The doses and treatment time of the DA receptor antagonists were chosen based on their ability to inhibit cocaine-induced behaviors and gene expression in mice (Cabib et al., 1991; Katz et al., 1999; Prinssen et al., 2004; Zhang et al., 2005). To examine the role of NMDA receptors in chronic cocaine-induced dendritic morphological changes, CPP was used at 10 mg/kg (n = 5 each) 30 minutes before daily cocaine or saline injections. According to previous pharmacological studies (Brackett et al., 2000; Reeves et al., 2004), CPP is effective in blocking NMDA receptor activity at this dose. SL327 (50 mg/kg) was injected 15 minutes before cocaine or saline administration (n=16 and 11, respectively). The dose and timing of the SL327 treatment were based on our previous concentration-dependent studies for the effect of SL327 on the induction of p-ERK in the brain (Wang et al., 2003; Zhang et al., 2004).

Golgi-Cox impregnation

For dendritic morphological analyses, all mice were sacrificed 24 hours after the last chronic injection. This withdrawal time was chosen for three reasons: 1). to potentially identify changes in dendritic morphology associated with both D1 and D2 receptor-bearing neurons following repeated cocaine administration in mice (Lee et al., 2006); 2). to avoid potential changes associated with acute injections (Zhang et al., 2005); and 3). to compare results from the current study to several previous molecular studies (Norrholm et al., 2003; Lee et al., 2006; Zhang et al., 2006). Mice were anesthetized with sodium pentobarbital and perfused transcardially with phosphate-buffered saline (Zhang et al., 2006). The brains were removed and placed in a Golgi-Cox solution (Glaser and Van der Loos, 1981; Greenough, 1984; Kolb et al., 1998) as described previously (Robinson and Kolb, 1997; 1999; Zhang et al., 2006). Brains were processed in the dark for 14 days and subsequently in a 20% and 30% sucrose solution for 3 days. Coronal sections were produced at 80 μ m using a vibratome (Zhang et al., 2006). We used 80 μ m thickness in the current study because it exhibits excellent staining with minimal cracks and can be analyzed equally well as brain sections of 200 μ m thickness (Kolb and McClimans, 1986; Izzo et al., 1987; Gibb and Kolb, 1998). Moreover, our analyses revealed no significant differences in quantifying dendritic morphology between results using 200 μ m and 80 μ m sections (Zhang et al., 2006). Brain sections were mounted on 2% gelatin-coated slides and rinsed in distilled H₂O for 1 minute. They were then incubated in saturated NH₄OH for 15 minutes in the dark, distilled H₂O for 1 minute, a Kodak Fix solution for 15 minutes and rinsed in distilled H₂O, then dehydrated by dipping in 50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol, and xylene sequentially.

Quantification of dendritic branching and spine density

MSNs from the NAc shell and CPu were selected and traced using a Nikon Eclipse 80i microscope (Zhang et al., 2006). These brain areas were identified at 100x magnification, and MSNs from each hemisphere in both the NAc shell and CPu were drawn with a camera lucida (at 450x, zoom 8.0). Numbers of dendritic branches were determined by counting at each order away from the cell body as described (Robinson and Kolb, 1997; 1999; Zhang et al., 2006). At least 10 neurons were evaluated for each region in each hemisphere per mouse. Spine densities were calculated by tracing a length of the secondary (or greater) order dendritic segment at 1000x magnifications. The number of spines along a dendritic segment (longer than 10 μ m, range from 10–60 μ m in lengths) was counted manually on 2D images, and the exact length of the segment was calculated by using the Image J Program. On the average, 15 dendritic segments from each brain region were evaluated in each hemisphere per mouse.

Western blot analysis

To evaluate ERK activation following chronic cocaine injections, mice were treated daily with saline (n=8) or SL327 (50 mg/kg, n=16) i.p. 15 minutes before a saline or cocaine (20 mg/kg) injection for 28 days. Four experimental groups were included: cocaine (n=5), saline (n=3), SL327 (n=8) and SL327 plus cocaine (n=8). Twenty minutes after the last injection, mice were sacrificed by cervical dislocation and whole brains were removed. The NAc and CPu were dissected on an ice-cold plate and protein extracts were prepared from individual mouse brains as described (Zhang et al., 2006). Homogenates were incubated on ice for 15 minutes and centrifuged at 13,000 g for 20 minutes at 4°C. Protein concentrations were determined by the Bradford method (Zhang et al., 2006). Equal amounts of total protein (30–40 μ g) were loaded and separated by 10% SDS-PAGE as described (Zhang et al., 2006). The electrophoretically resolved proteins were transferred onto polyvinylidene difluoride membranes. After transferring, membranes were blocked for 1 hour at room temperature. Membranes were then incubated with an anti p-ERK primary antibody overnight at 4°C followed by horseradish peroxidase (HRP)-conjugated secondary antibodies for another hour. The same membranes

were then stripped and reprobed with antibodies against total ERK and actin, respectively (Zhang et al., 2004). Signals were visualized by enhanced chemiluminescence. Antibodies against ERK, p-ERK and actin were used at 1:2000 dilutions. HRP-conjugated secondary antibodies were used at 1:5000 dilutions. All Western blot analyses were performed a minimum of three times to confirm the conclusion. Western blotting results from different groups of mice were scanned. An Image J Program was used to quantify the density of each protein bands. Data were expressed as a ratio of band density of p-ERK (the sum of phosphorylation of p42 and p44) over total ERK (including both ERK1 and 2) from each sample.

Statistics

A two-way ANOVA (genetic/pharmacological model $\times$ treatment) or one-way ANOVA was used to evaluate all the dendrite branching and spine density data. One-way ANOVA was used to compare ERK activation under different treatment conditions. Bonferroni post hoc analysis was used when appropriate. In all cases, significant levels were set at $p < 0.05$.

RESULTS

The DA D1 receptor is necessary for mediating changes in dendritic branching and spine density of MSNs induced by repeated exposure to cocaine

The DA D1 receptor plays a critical role in cocaine-induced behavioral effects as well as underlying molecular changes. Dendrites and dendritic spines are a major site of drug-induced structural plasticity (Robinson and Kolb, 2004). However, the effect of D1 receptor on chronic cocaine-induced dendritic morphological changes has not been investigated. In the present study, we used both genetic and pharmacological approaches to address this issue. Previous studies indicated that following 28 days of cocaine injections, MSNs exhibit robust morphological changes in both dendritic branching and spine density (Norrholm et al., 2003; Robinson and Kolb, 2004; Lee et al., 2006; Zhang et al., 2006). Moreover, this injection paradigm likely induces behavioral sensitization (Robinson and Kolb, 1997; Xu et al., 2000) which may accelerate subsequent escalation of drug-taking behaviors (Ferrario and Robinson, 2007). Based on their importance in behavioral responses following cocaine injections (Di Chiara et al., 2004; Ito et al., 2004; See et al., 2007), we focused on the shell of the NAc and CPu in the current study. Twenty four hours after the last of 28 daily cocaine or saline injections, brain sections from D1 receptor mutant mice and wild-type littermates were analyzed. Similar to previous reports (Robinson and Kolb, 1997; 1999; Zhang et al., 2006), cocaine induced a significant increase in the number of dendrites in the shell of NAc (Fig. 1A and 1C) and CPu (Fig. 1B and 1C) in wild-type mice compared to saline injections ($p < 0.05$ in both the NAc and CPu). In contrast, cocaine failed to induce changes in dendritic branching of MSNs in the shell of NAc and CPu in D1 receptor mutant mice (Fig. 1A–C). There was no significant difference in dendritic branching of MSNs in these two brain regions between D1 receptor mutant mice and wild-type mice following saline treatment, and between D1 receptor mutant mice following cocaine or saline treatment (Fig. 1A–C, $p > 0.05$ for all comparisons). These results suggest that a functional D1 receptor is essential in mediating chronic cocaine-induced changes in dendritic branching in mice.

Although there was no obvious difference in dendritic branching in the shell of NAc and CPu between D1 receptor mutant mice and wild-type mice following repeated saline injections, D1 receptor mutant mice likely carry developmental compensations (Xu et al., 1994a; Zhang et al., 2005). We used a pharmacological method to further investigate the role of the D1 receptor in cocaine-induced changes in dendritic branching. We pretreated wild-type mice daily with the D1 receptor antagonist SCH23390 30 minutes before cocaine or saline injections for 28 consecutive days. Twenty four hours after the last injection, we analyzed dendritic branching in the brains of these mice. Similar to mice carrying a genetic mutation, pharmacological

blockade of D1 receptors in wild-type mice attenuated increases in dendritic branching in both the shell of NAc and CPu induced by repeated cocaine administration (Fig. 1A–C). No difference was found between the SCH23390 plus cocaine group and the SCH23390 plus saline group ($p > 0.05$ in both the shell of NAc and CPu). Moreover, repeated SCH23390 treatment itself did not obviously affect MSN morphology in these two brain regions (Fig. 1C). The total numbers of neurons evaluated for the shell of NAc are 345 in the wild-type cocaine group, 302 in the wild-type saline group, 210 in the D1 receptor mutant cocaine group, 129 in the D1 receptor mutant saline group, 100 in the SCH23390 plus cocaine group, and 100 in the SCH23390 plus saline group, respectively. The total numbers of neurons analyzed in the CPu are 323 in the wild-type cocaine group, 288 in the wild-type saline group, 212 in the D1 receptor mutant cocaine group, 129 in the D1 receptor mutant saline group, 100 in the SCH23390 plus cocaine group and 100 in the SCH23390 plus saline group, respectively. As additional controls, we compared dendritic branching in the shell of NAc and CPu in naïve D1 receptor mutant and wild-type mice at 8 and 34 weeks of age before and after 28 daily saline injections and no obvious differences were observed ($n=3$ each, data not shown). Together, these results suggest the importance of D1 receptors in mediating chronic cocaine-induced changes in dendritic branching in the shell of NAc and CPu.

Repeated cocaine administration also induces increases in dendritic spine density in the shell of NAc and CPu (Robinson and Kolb, 1997; 1999; Zhang et al., 2006). We used some of the same brain sections to evaluate the role of D1 receptors in mediating cocaine-induced changes in spine density in these brain regions. Following 28 days of daily treatment, cocaine induced a significant increase in spine density in MSNs in the shell of NAc and CPu in wild-type mice compared to saline treatment (Fig. 1D–F, $p < 0.05$ in both the NAc and CPu). In contrast, cocaine was no longer able to induce changes in dendritic spine density of MSNs in the shell of NAc and CPu in D1 receptor mutant mice compared to wild-type mice, and no significant difference was found between D1 receptor mutant mice following cocaine or saline treatment (Fig. 1D–F). Moreover, SCH23390 pretreatment blocked increases in spine density induced by cocaine in these brain regions in wild-type mice, while repeated SCH23390 treatment alone did not obviously affect spine density of MSNs (Fig. 1D–F). There was no significant difference in these two brain regions between D1 receptor mutant mice and wild-type mice following saline treatment. The total numbers of dendritic segments used are 418 and 411 for the NAc and CPu in the wild-type cocaine group, 458 and 456 in the wild-type saline group, 304 and 308 in the D1 receptor mutant cocaine group, 161 and 165 in the D1 receptor mutant saline group, 151 and 158 in the SCH23390 plus cocaine group, and 153 and 159 in the SCH23390 plus saline group, respectively. When we compared dendritic spine density in naïve D1 receptor mutant mice and wild-type mice at 8 and 34 weeks of age before and after 28 daily saline injections, we did not observe obvious differences in the shell of NAc and CPu in these different groups of mice ($n=3$ each, data not shown). These results suggest that the D1 receptor is also involved in chronic cocaine-induced increases in dendritic spine density in the shell of NAc and CPu.

Blockade of D2 receptors does not have obvious effects on dendritic branching but has a partial effect on changes in spine density induced by chronic cocaine administration

Previous studies implicated the importance of D2 receptors in cocaine-induced behavioral changes (Volkow *et al.*, 1999; Caine *et al.*, 2002; Berglind *et al.*, 2006). We have investigated how D2 receptors may contribute to changes in dendritic morphology using a D2 receptor antagonist. Different groups of wild-type mice were pretreated daily with raclopride 30 minutes before cocaine or saline injections for 28 consecutive days. Twenty four hours after the last injection, brain sections from these mice were analyzed. We found that raclopride at a dose sufficient to block cocaine-induced behaviors (Prinssen *et al.*, 2004) failed to attenuate increases in dendritic branching of MSNs induced by repeated cocaine treatment in the shell

of NAc and CPu (Fig. 2A–C). 100 MSNs for raclopride plus cocaine or saline, 345 and 323 MSNs for cocaine, and 302 and 288 MSNs for saline from the NAc shell and CPu per mouse group were quantified. However, raclopride pretreatment blocked increases in spine density of MSNs in the shell of NAc and partially attenuated such changes in the CPu following repeated cocaine administration (Fig. 2D–F). The total numbers of dendritic segments evaluated are 154 and 154 for the shell of NAc and CPu in the raclopride plus cocaine group, 156 and 150 in the raclopride plus saline group, 418 and 411 in the wild-type cocaine group, and 458 and 456 in the wild-type saline group. Together, these findings suggest that blockade of D2 receptors does not obviously affect dendritic branching, but has a partial effect on changes in spine density in the shell of NAc and CPu induced by chronic cocaine administration.

The NMDA receptor mediates chronic cocaine-induced morphological changes in MSNs induced by repeated cocaine administration

Based on its importance in mediating behavioral and molecular effects of cocaine (Wolf, 1998; Heusner and Palmiter, 2005; Ramsey et al., 2008), we investigated how blockade of NMDA receptors may affect dendritic morphological changes induced by repeated exposure to cocaine. Different groups of wild-type mice were given daily CPP injections 30 minutes before cocaine or saline injections for 28 days. Twenty four hours after the last injection, brain sections from these mice were analyzed. CPP attenuated chronic cocaine-induced increases in dendritic branching and spine density in both the shell of NAc and CPu (Fig. 3A–F). Repeated pretreatment of CPP itself had no obvious effects on dendritic branching and spine density of MSNs in both brain regions. The total numbers of neurons evaluated are 100 each MSNs from the NAc shell and CPu in the CPP plus cocaine group, 100 each in the CPP plus saline group, 345 and 323 in the cocaine group, and 302 and 288 in the saline group, respectively. 122 and 150 dendritic segments from the NAc shell and CPu in the CPP plus cocaine group, 149 and 150 in the CPP plus saline group, 418 and 411 in the wild-type cocaine group, and 458 and 456 in the wild-type saline group were used in the analysis. These findings suggest that NMDA receptors also contribute to chronic cocaine-induced morphological changes in MSNs in the NAc shell and CPu induced by repeated exposure to cocaine.

ERK is an intracellular mediator of chronic cocaine-induced dendritic morphological changes in MSNs

ERK is a major intracellular signal transducer following the activation of DA and NMDA receptors, and blockade of D1 and NMDA receptors can inhibit cocaine-induced ERK activation (Valjent et al., 2000; 2005; Zhang et al., 2004; Lu et al., 2006). ERK signaling is also involved in dendritic morphogenesis (Wu et al., 2001; Kumar et al., 2005). We used a selective MEK blocker, SL327, to investigate the role of ERK in mediating changes in dendritic morphology following repeated cocaine administration. Different groups of wild-type mice were given daily SL327 injections 15 minutes before cocaine or saline injections for 28 days. Twenty four hours after the last injection, brain sections from these mice were analyzed. There was no difference observed between the SL327 and saline groups in the number of dendritic branching and spine density of MSNs, suggesting that SL327 itself did not induce obvious changes in dendritic morphology in both the shell of NAc and CPu ($p > 0.05$ for all comparisons). However, SL327 blocked cocaine-induced increases in dendritic branching and spine density of MSNs in both the NAc shell and CPu (Fig. 4A–F). The total numbers of neurons evaluated are 300 and 443 MSNs from the NAc shell and CPu in the SL327 plus cocaine group, 217 and 263 in the SL327 plus saline group, 345 and 323 in the cocaine group, and 302 and 288 in the saline group, respectively. 168 and 168 dendritic segments from the NAc shell and CPu in the SL327 plus cocaine group, 293 and 305 from the SL327 plus saline group, 418 and 411 from the wild-type cocaine group, and 458 and 456 from the wild-type saline group were used in the spine density evaluations. These observations indicated that

proper ERK activation is necessary for chronic cocaine-induced changes in dendritic branching and spine density of MSNs in the NAc and CPu.

We and others previously found that an acute cocaine administration leads to a transient ERK activation and expression of downstream target genes including the cAMP-response element binding protein (CREB), c-Fos and FosB (Valjent et al., 2000; Zhang et al., 2004; Jiao et al., 2007), which can be blocked by a SL327 pretreatment (Valjent et al., 2000; Zhang et al., 2004; Jiao et al., 2007). Likewise, repeated cocaine injections for 7 consecutive days also resulted in ERK activation and changes in the expression of downstream target genes including neogenin and synaptotagmin VII via DA D1 receptors, and this induction is also blocked by daily SL327 pretreatment (Zhang et al., 2004). To further investigate the possible involvement of ERK in chronic cocaine-induced morphological changes, we treated different groups of mice with saline or cocaine in the presence or absence of SL327 once daily for 28 consecutive days. By analyzing ERK activation in the NAc and CPu of these mice, we found that repeated cocaine administration induced p-ERK in both brain regions (Fig. 5A and B, $p < 0.05$). SL327 pretreatment blocked chronic cocaine-induced ERK activation in these same brain regions when compared to the repeated saline plus cocaine group (Fig. 5A and B, $p < 0.05$ in both the NAc and CPu). Saline or SL327 treatment alone had no obvious effect on p-ERK levels in these brain regions (Fig. 5A and B, $p > 0.05$ in both the NAc and CPu). These results further support the notion that proper ERK activation is necessary for chronic cocaine-induced changes in dendritic branching and spine density of MSNs in the NAc and CPu.

DISCUSSION

Both the shell of NAc and CPu are involved in chronic cocaine-induced behaviors (Di Chiara et al., 2004; Ito et al., 2004; See et al., 2007). MSNs in the NAc and CPu receive convergent inputs from both DA and glutamate projections. One form of neuroplastic changes induced by repeated exposure to drugs of abuse is increases in dendritic branching and spine density (Robinson and Kolb, 2004). We have investigated the molecular basis of such changes by studying the role of DA D1 and D2 receptors, NMDA receptors and ERK in changes in dendritic morphology induced by repeated cocaine administration in these two brain regions. Our results suggest that DA D1 and NMDA receptors and ERK contribute significantly to neuronal morphological changes induced by repeated exposure to cocaine.

DA D1 receptors and the ERK signaling pathway are necessary for mediating changes in dendritic branching and spine density of MSNs induced by chronic exposure to cocaine

The D1 receptor plays a key role in mediating behavioral and molecular responses to both acute and repeated exposure to cocaine (Xu et al., 1994b; 2000; Zhang et al., 2002; 2004; 2005; Jiao et al., 2007; Caine et al., 2007). Changes in neuronal activity occur in MSNs in the NAc and CPu in response to exposure to drugs of abuse such as cocaine (Kauer and Malenka, 2007). Presumably, there is a series of signal transduction events from the receptor level to intracellular signaling that contributes to such cocaine-induced alterations. In addition to changes in behavior and gene expression, dendritic structural modifications may represent a form of long-term neuroplastic change via functional D1 receptors induced by cocaine treatment. To investigate this hypothesis, by using both genetic and pharmacological manipulations, we systematically compared the number of dendritic branches and the spine density in the MSN of the shell of NAc and CPu after chronic cocaine administration.

We found that repeated exposure to cocaine induced increases in dendritic branching and spine density in the MSN in both the NAc and CPu in wild-type mice (Fig. 1). In contrast, similar cocaine treatment failed to induce significant dendritic morphological changes in D1 receptor mutant mice and there was no difference between D1 receptor mutant mice and wild-type mice in dendritic branching and spine density after saline treatment (Fig. 1). In the absence of cocaine

treatment, D1 receptor mutant mice exhibit apparent normal dendritic branching and spine density compared to wild-type mice (Fig. 1). Pretreatment with a D1 receptor antagonist, SCH 23390, at a dose that is capable of inhibiting cocaine-induced locomotor activation attenuated chronic cocaine-induced morphological changes in the NAc shell and CPu (Fig. 1). These findings suggest that the D1 receptor is a critical mediator for changes in dendritic morphology induced by repeated cocaine administration.

MAPK-mediated signaling has been implicated in synaptic plasticity and learning and memory (Schaeffer and Weber, 1999). ERK signaling is also involved in dendritic morphogenesis (Wu et al., 2001; Kumar et al., 2005). Acute or repeated cocaine administration can induce ERK activation, and changes in the expression of downstream target genes, in the NAc and CPu in a D1 receptor-dependent manner (Valjent et al., 2000; Zhang et al., 2004; Jiao et al., 2007). Levels of cocaine-induced p-ERK are attenuated by the pretreatment of a D1 receptor antagonist or in D1 receptor mutant mice (Zhang et al., 2004; Jenab et al., 2005). In the present study, we found that a repeated blockade of ERK activation with SL327 attenuated increases in dendritic branching and spine density induced by repeated cocaine treatment while SL327 alone did not have an obvious effect (Fig. 4–5). Spinophilin is a protein modulating spine morphogenesis through its ability to bind to F-actin (Satoh et al., 1998; Hsieh-Wilson et al., 2003). p-ERK can phosphorylate spinophilin and increase filopodial density (Futter et al., 2005). Repeated exposure to cocaine results in an elevation of F-actin associated with filopodial formation in the NAc (Toda et al., 2006). Further, inactivation of ERK and its downstream transcriptional factor, Elk-1, attenuates spontaneous dendritic outgrowth and F-actin expression (Lavaur et al., 2007). Together, these results suggest that the D1 receptor and ERK-mediated actin remodeling may represent a major contributor to chronic cocaine-induced dendritic morphological changes in the brain.

Previous studies demonstrated that pharmacological inhibition of the activity of cyclin-dependent kinase 5 (CDK5) (Norrholm et al., 2003), genetic regulation of expression of the myocyte enhancer factor 2 (MEF2) proteins which are substrates of CDK5 (Pulipparacharuvil et al., 2008) or integrin-linked kinase (ILK) (Chen et al., 2008) attenuates chronic cocaine-induced changes in dendritic spine density in the NAc. D1 receptor stimulation can inhibit MEF2 activity (Pulipparacharuvil et al., 2008). Chronic cocaine-induced increases in dendritic spine density also correlate with increased delta-FosB accumulation mostly in D1 receptor-bearing neurons (Lee et al., 2006). A *c-fos* mutation in D1 receptor-bearing neurons attenuates chronic cocaine-induced increases in dendritic branching and spine density in the NAc and CPu (Zhang et al., 2006). *c-Fos* is also a mediator for delta-FosB induction and CDK5 activation induced by repeated cocaine administration (Zhang et al., 2006). Although how *c-Fos*, delta-FosB, CDK5 and MEF2 interact to modulate dendritic reorganization following cocaine treatment remains to be investigated, these findings further suggest that D1 receptors and ERK-mediated transcriptional mechanisms play a major role in chronic cocaine-induced dendritic morphological changes in the brain.

Blockade of D2 receptors does not have obvious effects on dendritic branching but has a partial effect on changes in spine density induced by chronic cocaine administration

We found that treatment of a D2 receptor antagonist, raclopride, at the dose sufficient to block cocaine-induced behaviors (Prinssen et al., 2004), had no obvious effects on cocaine-induced changes in dendritic branching but produced a partial effect on changes in spine density in the shell of NAc and CPu (Fig. 2). While the mechanism of this finding is still unknown, previous *in vitro* studies suggest that increases in spine density may be more sensitive than those of dendritic branching after methamphetamine or neurotrophin application (Blaesing et al., 2001; Alonso et al., 2004). It is possible that changes in spine density and dendritic branching may exhibit differential sensitivity to repeated D2 receptor antagonism. Whether the ILK and

phospho-protein kinase B signaling pathway (Kumar et al., 2005; Chen et al., 2008) underlie the differential effects of raclopride on spine density in NAc shell and CPu remains unclear. These results suggest that D2 receptors may not significantly contribute to changes in dendritic branching induced by chronic cocaine administration. It is unlikely these results are due to the dose of the D2 receptor antagonist used, since a dose of similar efficacy was chosen based on its ability to block cocaine-induced behaviors in mice (Cabib et al., 1991; Katz et al., 1999; Prinssen et al., 2004). One possible explanation for our findings is that we performed cocaine injections in the home cage. The environmental context in which stimulant drugs are administered plays a significant role in engaging different neuronal circuits (Badiani and Robinson, 2004). Cocaine given in the home cage engages primarily the D1 receptor system whereas both D1 and D2 receptor-bearing MSNs are activated when the same drug is given in a novel environment. The involvement of D2 receptors in changes in dendritic morphology when cocaine is given in a novel environment still needs to be investigated.

During the course of this study, we also used haloperidol (0.5 mg/kg) as a D2 receptor antagonist. Notably, when compared to saline-treated mice, haloperidol treatment alone induced increases in dendritic branching and spine density in the NAc shell and CPu (data not shown). This interferes with the investigation on the effect of chronic cocaine on changes in dendritic morphology. Repeated haloperidol treatment has been shown to induce morphological changes in the striatum including increasing size of neuronal soma, dendrite diameter and axon terminals, branching and spine density (Benes et al., 1985; Uranova et al., 1991; Meredith et al., 2000; Parish et al., 2002), and alter neuronal connectivity (Onn and Grace, 1995). To further clarify the role of D2 receptors in chronic cocaine-induced dendritic morphological changes, experiments with additional selective D2 receptor antagonists (and in a dose-dependent manner) and D2 receptor mutant mice will be needed. In addition, D3 receptors also contribute to cocaine-induced behavioral and molecular changes (Xu et al., 1997; Zhang et al., 2004; Liu et al., 2009). Whether D3 receptors may contribute to chronic cocaine-induced changes in dendritic morphology and whether raclopride may cross-inhibit D3 receptors remain to be investigated.

The NMDA receptor mediates chronic cocaine-induced morphological changes in MSNs induced by repeated cocaine administration

NMDA receptors have been implicated in mediating cocaine-induced neurobiological changes (Wolf, 1998; Heusner and Palmiter, 2005; Ramsey et al., 2008). Our results showed that daily pretreatment of CPP significantly blocked chronic cocaine-induced increases in dendritic branching and spine density in both the NAc shell and CPu (Fig. 3). Importantly, CPP administration alone did not induce obvious changes in dendritic morphology (Fig. 3). These results suggest the importance of NMDA receptors in cocaine-induced changes in dendritic morphology. Whether calcium influx through NMDA receptors might modulate dendritic morphology remains to be investigated. We also used MK801 (0.5 mg/kg) as an additional NMDA receptor antagonist in the current study and found that repeated administration of MK801 increased dendritic branching but not spine density in both the shell of NAc and CPu compared to saline-treated group (data not shown). This interferes with the investigation on the effect of chronic cocaine on changes in dendritic branching. MK801 administration itself is able to cause behavioral sensitization associated with DA overflow in the striatum (Wolf et al., 1993; Tzschentke and Schmidt, 2000), and may lead to an increase in neuronal activity in striatal neurons co-expressing *c-fos* (De Leonibus et al., 2002). Although our current study is based on the use of a single NMDA receptor antagonist at one dose and the underlying molecular mechanisms need to be investigated, our findings suggest that NMDA receptors also mediate chronic cocaine-induced morphological changes in MSNs in the NAc shell and CPu induced by repeated cocaine administration.

In the current study, we focused on the NAc shell and the CPu based on the importance of these brain regions in the behavioral effects of cocaine (Di Chiara et al., 2004; Ito et al., 2004; See et al. 2007). The non-contingent injection paradigm we used is known to induce changes in dendritic morphology in these brain regions (Robinson and Kolb, 1997; 1999; Kolb et al., 2003; Norrholm et al., 2003; Lee et al., 2006; Zhang et al., 2006). Li et al. (2004) reported that cocaine-induced increases in spine density in the NAc core are correlated with the development of behavioral sensitization whereas such correlation does not exist in the shell of NAc unless a high dose of the drug is used. It should be noted that this particularly study involved a novel environment and used a different species (rats) as a model system. In the current study, mice were used and injections occurred in home cages. The potential impact of these differences remains to be investigated.

We used a Golgi-Cox impregnation method (Greenough, 1984; Kolb et al., 1998) in the current study that has been used extensively to evaluate changes in dendritic morphology following repeated exposure to a variety of drugs of abuse (Robinson and Kolb, 1997; 1999; Robinson et al., 2001; Kolb et al., 2003; Norrholm et al., 2003; Ferrario et al., 2005; Zhang et al., 2006; Pulipparacharuvil et al., 2008; Chen et al., 2008) and in learning and memory (Robinson and Kolb, 2004). Although a strong correlation between measures of dendritic structure and synapses has been confirmed in many studies using electron microscopy to directly quantify synaptic density, this approach does not provide a direct measure of synapses (Robinson and Kolb, 2004). A recent study showed no change in spine density between chronic cocaine and saline treatment groups in the NAc but rather a shift from small to large diameter spines (Shen et al., 2009). This result is in contrast to those observed in the current study and reported by others showing increases in spine density in the NAc after chronic cocaine administration (Robinson and Kolb, 2004; Lee et al., 2006). Differences in treatment protocols including cocaine doses, environment and withdrawal time may contribute to the different spine density measurements in these studies. Alternatively, it may result from differences in spines being identified by DiI-labeling and Golgi-Cox impregnation. Lee et al. (2006) reported that the Golgi-Cox impregnation method and the DiI-labeling method could produce qualitatively comparable results in stimulant-induced changes in spine density.

In conclusion, using both genetic and pharmacological approaches, our current studies demonstrate that DA D1 receptors, NMDA receptors and ERK contribute significantly to neuronal morphological changes induced by repeated exposure to cocaine. These data are also in agreement with previous reports showing that D1 and NMDA receptors and ERK are necessary for cocaine-induced changes in behavior, intracellular signaling, and gene expression.

ABBREVIATIONS

(CPu)	caudate putamen
(CREB)	cAMP-response element binding protein
(CDK5)	cyclin-dependent kinase 5
(DA)	dopamine
(ERK)	extracellular signal-regulated kinase
(ILK)	integrin-linked kinase
(MSNs)	medium spiny neurons
(MEF2)	myocyte enhancer factor 2
(NMDA)	<i>N</i> -methyl- <i>D</i> -aspartate

(NAc) nucleus accumbens
(PFC) prefrontal cortex

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Fig. 1. The DA D1 receptor mediates changes in dendritic branching and spine density of MSNs in both the shell of NAc and CPu induced by chronic cocaine administration

We treated different groups of wild-type (WT) and D1 receptor mutant (D1^{-/-}) mice with cocaine (COC, 20 mg/kg, n=16 and 10 each) or saline (SAL, n=14 and 6 each) once daily for 28 consecutive days. Additional groups of wild-type mice received a D1 receptor antagonist, SCH23390 (SCH, 0.5 mg/kg) 30 minutes before the daily cocaine or saline injection (n=5 per group). **A** and **B** are representative dendritic branching drawings and bright-field confocal micrograph of Golgi-Cox impregnated images of MSNs from the shell of NAc and CPu, respectively. Scale bar in **A** indicates 30 μm. **C** statistical analyses of dendritic branching in the NAc shell and CPu. Data represent mean ± SEM of numbers of dendritic branching in MSNs. **p* < 0.05 when compared within wild-type saline-treated group. For the NAc: Treatment main effect: $F_{(1,33)} = 31.76, p < 0.001$; Model main effect: $F_{(2,33)} = 70.13, p < 0.001$; Treatment × Model: $F_{(2,33)} = 89.39, p < 0.001$. For the CPu: Treatment main effect: $F_{(1,33)} = 41.44, p < 0.001$; Model main effect: $F_{(2,33)} = 110.69, p < 0.001$; Treatment × Model: $F_{(2,33)} = 84.37, p < 0.001$. Bright-field micrographs of MSN dendritic spine images of Golgi-Cox impregnated materials from the shell of NAc (**D**) and CPu (**E**), respectively. Scale bar in **D** indicates 10 μm. **F** statistical analyses of spine density along 10 μm segments of secondary (or greater) order dendrites in the NAc shell and CPu. Data represent mean ± SEM of spine density per 10 μm of dendritic segment. **p* < 0.05 when compared to wild-type saline-treated group. For the NAc: Treatment main effect: $F_{(1,31)} = 9.46, p < 0.01$; Model main effect: $F_{(2,31)} = 30.00, p < 0.001$; Treatment × Model: $F_{(2,31)} = 14.37, p < 0.001$. For the CPu: Treatment main effect: $F_{(1,35)} = 15.51, p < 0.001$; Model main effect: $F_{(2,35)} = 28.97, p < 0.001$; Treatment × Model: $F_{(2,35)} = 20.62, p < 0.001$.

Fig. 2. Blockade of D2 receptors does not have an obvious effect on changes in dendritic branching but results in a partial effect on changes in spine density of MSNs in the shell of NAc and CPu induced by chronic cocaine administration

Wild-type mice received a raclopride (Rac, 2.5 mg/kg, n=5 each) injection 30 minutes before a daily cocaine (20 mg/kg) or saline injection for 28 consecutive days. **A** and **B** are representative dendritic branching drawings and bright-field images of MSNs in the shell of NAc and CPu, respectively. Scale bar in **A** indicates 30 μm. **C** statistical analyses of dendritic branching in the NAc shell and CPu. Data represent mean ± SEM of numbers of dendritic branching in MSNs. * $p < 0.05$ when compared to saline/saline-treated group. For the NAc $F_{(3,27)} = 30.85$, $p < 0.001$; For the CPu $F_{(3,27)} = 92.15$, $p < 0.001$. **E** and **E** are representative

dendritic spine images of MSNs from the NAc shell and CPu, respectively. Scale bar in **D** indicates 10 μm . **F** Quantification of spine density along 10 μm dendritic segment in the NAc shell and CPu. Data represent mean $\pm$ SEM of spine density per 10 μm of dendrite segment. * $p < 0.05$ when compared to saline/saline-treated animals, and # $p < 0.05$ when compared with saline/cocaine-treated group. For the NAc $F_{(3,18)} = 107.02, p < 0.001$; for the CPu $F_{(3,18)} = 92.55, p < 0.001$.

Fig. 3. NMDA receptors mediate morphological changes of MSNs induced by repeated cocaine administration

Different groups of wild-type mice received injections of either an NMDA receptor antagonist CPP (10 mg/kg, $n=4-5$ each) 30 minutes before a daily cocaine or saline injection for 28 consecutive days. **A** and **B** are representative dendritic branching drawings and bright-field micrograph of Golgi-Cox processed images of MSNs from the shell of NAc and CPu, respectively. Scale bar in **A** indicates 30 μ m. **C** statistical analyses of dendritic branching in NAc shell and CPu. Data represent mean $\pm$ SEM of numbers of dendritic branching in MSNs. * $p < 0.05$ when compared to saline/saline-treated group. For the NAc, $F_{(3,27)}=116.38$, $p < 0.001$; for the CPu, $F_{(3,27)}=121.63$, $p < 0.001$. **D** and **E** are representative dendritic spine images of

MSNs from the NAc shell and CPu, respectively. Scale bar in **D** indicates 10 μm . **F** Quantification of spine density along 10 μm dendritic segment in the NAc shell and CPu. Data represent mean $\pm$ SEM. * $p < 0.05$ when compared to saline/saline-treated animals. For the NAc, $F_{(3,24)}=14.95$, $p < 0.001$; for the CPu, $F_{(3,18)}=108.54$, $p < 0.001$.

Fig. 4. ERK is involved in chronic cocaine-induced changes in dendritic morphology in MSNs
 Wild-type mice were pretreated with a MEK inhibitor SL327 (50 mg/kg) 15 minutes prior to a daily cocaine (n=16) or saline (n=11) injection for 28 consecutive days. **A** and **B** are representative dendritic branching drawings and bright-field micrograph of Golgi-Cox processed images of MSNs from the shell of NAc and CPu, respectively. Scale bar in **A** indicates 30 μ m. **C** statistical analyses of dendritic branching in NAc shell and CPu. Data represent mean $\pm$ SEM of numbers of dendritic branching in MSNs. * p < 0.05 when compared to saline/saline-treated group. For the NAc, $F_{(3,33)}=8.53$, p < 0.001; For the CPu, $F_{(3,33)}=13.29$, p < 0.001. **D** and **E** are representative dendritic spine images of MSNs from the NAc shell and CPu, respectively. Scale bar in **D** indicates 10 μ m. **F** Quantification of spine density along 10

μm dendritic segment in the NAc shell and CPu. Data represent mean ± SEM. * $p < 0.05$ when compared to saline/saline-treated animals. For the NAc, $F_{(3,23)} = 22.25$, $p < 0.001$; For the CPu, $F_{(3,25)} = 29.24$, $p < 0.001$.

Fig. 5. Repeated cocaine administration induces ERK activation the NAc and CPu

Wild-type mice were pretreated i.p. with a selective MEK inhibitor SL327 (50 mg/kg) 15 minutes prior to a daily cocaine (20 mg/kg, n=8) or saline (n=8) injection for 28 consecutive days. Additional wild-type mice were also given an i.p. cocaine (20 mg/kg, n=5) or saline (n=3) for 28 days. Representative western blotting images for samples from **A** the NAc and **B** CPu and statistical analyses of p-ERK protein levels in these two brain regions. Data represent mean $\pm$ SEM. The protein expression level of saline control group was arbitrarily set at 1. * $p < 0.05$ when compared to saline- plus saline-treated mice. For NAc, $F_{(3,18)} = 220.54$, $p < 0.01$; for CPu, $F_{(3,18)} = 118.27$, $p < 0.05$. SAL: saline- plus saline-treated mice; COC: saline plus cocaine treatment; SL: SL327 plus saline treatment; SL/COC: SL327- plus cocaine-treated mice.