Published in final edited form as:

Synapse. 2011 June ; 65(6): 490–496. doi:10.1002/syn.20865.

Dopamine transporter inhibition is necessary for cocaine-induced increases in dendritic spine density in the nucleus accumbens

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Abstract

Repeated exposure to cocaine produces changes in the nervous system that facilitate drug-seeking behaviors. These drug-seeking behaviors have been studied with animal models, such as cocaine-induced locomotor sensitization. Cocaine is hypothesized to induce locomotor sensitization by neural changes, including an increase in the density of spines on the dendrites of neurons in the nucleus accumbens (NAC). However, how cocaine increases dendritic spine density in the NAC has been difficult to discern because cocaine inhibits the function of multiple targets, including the transporters for dopamine, serotonin, and norepinephrine. Previously, our lab created a tool that is useful for determining how inhibiting the dopamine transporter (DAT) contributes to the effects of cocaine by generating mice that express a cocaine-insensitive DAT (DAT-CI mice). In this study, we used DAT-CI mice to determine the contribution of DAT inhibition in cocaine-induced increases in dendritic spine density in the NAC. We repeatedly injected DAT-CI mice with either cocaine or saline, and measured both dendritic spine density in the NAC and locomotor activity. Unlike wild-type mice, DAT-CI mice did not show an increase in dendritic spine density in the NAC or in locomotor activity in response to repeated injections of cocaine. These data show that cocaine-induced increases in dendritic spine density in the NAC require DAT inhibition. Thus, DAT-inhibition may play a role in mediating the long-lasting neural changes associated with drug addiction.

Keywords

knock-in mice; morphology; nucleus accumbens core; nucleus accumbens shell; locomotor sensitization

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INTRODUCTION

Individuals addicted to cocaine have difficulties quitting because they often suffer from intense cravings that can last for years. The enduring nature of these cravings is thought to be a consequence of the many long-lasting adaptations in the nervous system that are produced by cocaine (Nestler, 2001). One of the most long-lasting neural adaptations produced by cocaine is the modification of synaptic connectivity (Nestler, 2001). For example, animal models of drug abuse show that cocaine can influence synaptic connectivity by increasing the density of spines (i.e. connections) on the dendrites of neurons within many brain regions, including the nucleus accumbens (NAC) (Robinson and Kolb, 1999).

The nucleus accumbens (NAC) plays a central role in drug addiction (Koob, 1998). The NAC is commonly subdivided into two functionally and anatomically distinct areas, the shell (AcbS) and the core (AcbC) (Di Chiara et al., 2004). These two brain areas of the NAC have been shown to play dissociable roles in many behaviors related to drug abuse, such as cocaine-induced locomotor sensitization (Di Chiara, 2002).

Cocaine-induced locomotor sensitization describes the phenomenon that a dose of cocaine produces a greater increase in locomotor activity in drug-exposed animals than in drug-naïve animals (Robinson and Berridge, 1993). Since locomotor sensitization can still be detected after months of withdrawal, it has been used to study the long-lasting effects of cocaine (Markou et al., 1993); (Robinson and Berridge, 2008). The sensitizing effects of cocaine are thought to be mediated by changes in dendritic spine density in the NAC. This suggests that dendritic spine density changes in the NAC are important in long-lasting effects of cocaine abuse (Lee et al., 2006; Li et al., 2004; Nestler, 2001; Ren et al., 2010).

How cocaine exposure increases dendritic spine density in the NAC is difficult to discern because cocaine binds to and inhibits the functions of multiple targets. Cocaine binds with similarly high affinities to transporters that reuptake dopamine, norepinephrine, and serotonin (Amara and Kuhar, 1993; Ritz et al., 1987); (Han and Gu, 2006), which leads to an increase in extracellular levels of dopamine, norepinephrine, and serotonin (Reith et al., 1997).

Dopamine plays a well established role in cocaine-induced changes in dendritic spine density. For example, dopamine specific lesions created by 6-hydroxydopamine alter dendritic spine density in the NAC (Meredith et al., 1995). Also, cocaine does not increase dendritic spine density in the AcbS of mice lacking the dopamine D1 receptor or in mice pretreated with dopamine D1 receptor selective antagonists (Ren et al., 2010). Furthermore, cocaine-induced dendritic spine density changes in the NAC are found on neurons that express dopamine D1 or D2 receptors (Lee et al., 2006).

However, the findings that dopamine (DA) is involved in cocaine-induced increases in dendritic spine changes in the NAC do not necessarily implicate the dopamine transporter (DAT). Studies have shown that the norepinephrine transporter can reuptake dopamine as effectively as norepinephrine in certain regions of the brain (Moron et al., 2002). In addition, in mice with DAT completely removed, cocaine still retains the ability to increase extracellular dopamine in the NAC and produce reward (Gainetdinov et al., 1998); (Hall et al., 2002). In light of these findings, it is important to directly test the role of DAT in cocaine-induced increases in dendritic spine density in the NAC.

Our lab has previously made genetically modified mice to express a mutated DAT that can reuptake dopamine in the presence of high concentrations of cocaine (cocaine-insensitive) (Chen et al., 2006). These mice that express cocaine-insensitive DAT (DAT-CI mice) have a

functioning DAT, and therefore, should have fewer compensatory changes than DAT-knockout (DAT-KO) mice which have DAT completely removed (Gainetdinov et al., 1998); (Hall et al., 2002). Thus, the DAT-CI mouse line has been a useful tool for testing the consequences of cocaine inhibiting the reuptake function of DAT (Chen et al., 2006; Tilley et al., 2007); (Tilley et al., 2009); (Thomsen et al., 2009).

The purpose of this study was to investigate the contributions of DAT inhibition in the increase in dendritic spine density in the NAC induced by repeated exposure to cocaine. To test this, we measured the dendritic spine density within the NAC (AcbC and AcbS) of DAT-CI mice repeatedly injected with cocaine. In addition, to examine how dendritic spine density relates to locomotor activation, we assessed locomotor activity before and after repeated drug exposures.

MATERIAL AND METHODS

Animals

DAT-CI mice were generated as previously described (Chen et al., 2006) using embryonic stem cells derived from SV129J mice. These have been backcrossed to C57BL/6J for 13 generations. All mice used in this study were male, 10–15 weeks old, and were bred from heterozygous breeding pairs. Mice were weaned at 4 weeks of age, and subsequently housed 3–5 per cage, under standard conditions, with *ad libitum* access to food and water, and a 12 h light: dark cycle (lights on at 6:00 a.m.). Mice were moved to the behavioral room at least one week prior to experiments. The genotype of each mouse was determined by polymerase chain reaction analysis of tail biopsy DNA taken at 10 days of age as previously described (Chen et al., 2006). This study was approved by the Ohio State University Institutional Animal Care and Use Committee.

Drug treatment paradigm

DAT-CI and wild-type mice received either cocaine (15 mg/kg, intraperitoneal) or the vehicle (isotonic saline) on behavioral testing days (Day 1 and Day 30). On Days 2 – 27, the mice received injections in their home cage of either cocaine (30 mg/kg) or saline (10 μ L/mg) on a schedule of five consecutive days of a once-daily injection, followed by two days without an injection, for a total of 20 injections of 30 mg/kg. This drug regimen was previously shown to increase dendritic spine density on neurons in the NAC and to induce locomotor sensitization in wild-type mice (Li et al., 2004). Saline groups remained drug naïve throughout the entire experiment. All injections and experiments were conducted between 10:00 am and 12 pm. Cocaine-HCL was kindly provided by the drug supply program at the National Institute on Drug Abuse (National Institutes of Health, Bethesda, MD) and was prepared daily in 0.9% saline.

Locomotor sensitization

Locomotor activity experiments were performed in clear polyacrylic boxes (25 $\times$ 25 $\times$ 28 cm³). To habituate the animals to the testing boxes, animals were exposed to the boxes for 30 min each day for 2 days before each testing day. Locomotion was measured on the day of the first and the last injections (Days 1 and 30). On the test days, the animals were allowed to explore the boxes for 1 hour before being injected with either 15 mg/kg of cocaine (WT: n = 13, DAT-CI: n = 15) or the same volume of saline solution (WT: n = 11, DAT-CI: n = 10). A lower dose of cocaine was administered during testing days to avoid stereotypic behavior which would obscure locomotor activity interpretations. The locomotor activity was recorded for an hour before and an hour after the injection. The challenge injection was administered after 2 injection-free days (Day 30). Locomotor activity was recorded with a

video camera, and the distance traveled was calculated using Limelight software (Whitehall, PA, USA).

Tissue collection and histology

Three days after the challenge injection, all mice were sacrificed by cervical dislocation. Tissue preparation and Golgi staining were performed according to the manufacturer's instructions for the FD Rapid GolgiStain™ kit (FD Neurotechnologies, Inc., Ellicott City, MD). In brief, brains were left in vials containing Golgi solution for 9 days, followed by sucrose solution for at least 2 days before being snap frozen in a mixture of dry-ice and isopentane. Brains were cut into 50 µm sections with a cryostat. After the sections dried, they were stained with Nissl and other Golgi solutions (solution D and E). Slides were left to dry for 4–6 months before measuring dendritic spine density. Neurolucida® image analysis software (MBF Bioscience, Williston, VT) was used to quantify spine density with a 100X oil immersion objective. Ten representative neurites were assessed for each animal (5 neurites for the AcbC and 5 neurites for the AcbS). Spines were counted on dendritic segments that were distal (second order) from the cell soma and at least 40 µm in length. Spine density was defined as the number of spines per 10 µm of dendrite. Only neurons in the medial, rostral NAC (~1.34 mm anterior to bregma), AcbS or AcbC were selected (Franklin and Paxinos, 2001). This anterior coordinate was based on a previous study that showed dendritic spine density changes in the NAC of mice (Lee et al., 2006). Also, studies infusing dopamine D1 receptor selective antagonists at this coordinate attenuates behaviors associated with cocaine (Ren et al., 2010). Nissl staining was performed in conjunction with Golgi staining to facilitate the identification of the AcbS and AcbC. All neurons analyzed were completely impregnated with stain and were not obscured by other neurons. All dendritic spine density data were manually counted by an investigator who was blind to the mouse's genotype and treatment.

Statistics

The average dendritic spine densities of the 5 neurites in each region of wild-type and DAT-CI mice treated with saline or cocaine were analyzed with two-way (genotype × drug) ANOVA for each brain region. Significant main effects were further analyzed with one-way ANOVA (drug) and (genotype). Significance was defined as $P < 0.05$. All locomotor activity data were analyzed with two-way (time × group) repeated measures analysis of variance (RMANOVA, SPSS 17.0 Software, Chicago, IL). Significant main effects were further analyzed with one-way ANOVA (group) or RMANOVA (time).

RESULTS

Locomotor activity is not altered by repeated cocaine injections in DAT-CI mice

To determine whether repeated injections of cocaine alter locomotor activity in DAT-CI mice, we selected a cocaine treatment paradigm that has been reported to produce locomotor sensitization in wild type mice (Li et al., 2004); (Lee et al., 2006). The dendritic spine density experiments and the locomotor tests were performed using the same group of mice. Wild type mice were treated with either cocaine or saline for 30 days as described in the Material and Methods section. In wild-type mice (as shown in Fig. 1A), the first injection (Day 1) of cocaine (15 mg/kg, i.p.) produced higher locomotor activity than the first injection (Day 1) of saline in wild-type mice (Day 1, $F_{(1, 11)} = 7.57$, $p = <0.012$). Furthermore, the challenge injection of cocaine (15 mg/kg) administered on Day 30 after 20 cocaine treatments (30 mg/kg) caused a greater increase in locomotor activation than the first injection of cocaine ($F_{(1, 11)} = 41$, $p < 0.001$). In contrast, DAT-CI mice (Figure 1B) did not show an increase in locomotor activity after the first (Day 1) or the last injection (Day 30) of cocaine as compared to the saline injection controls. Instead, the first injection of

cocaine seemed to produce a trend towards reducing locomotor activity compared to the first injection of saline ($F_{(1,10)} = 2.578$, $p = 0.122$). To present cocaine effects more clearly, Fig. 1C shows the locomotor activities after the cocaine injection (at 75 min) subtracted from the locomotor activity after the saline injection (at 75 min) for each genotype. In wild-type mice, cocaine-induced increases in locomotor activity after repeated injections (Day 30) were higher than that after a single injection (Day 1) of cocaine (t-test, $p < 0.0001$). In contrast, in DAT-CI mice, cocaine did not increase locomotor activity on Day 1 or Day 30 (t-test, $p = 0.89$). Interestingly, the locomotor activity observed after the last (Day 30) injection of saline was greater than that after the first (Day 1) injection of saline in DAT-CI mice ($p < 0.05$). As previously reported (Chen et al., 2006), DAT-CI mice had significantly higher basal locomotor activity than wild-type mice, which has been attributed to higher levels of extracellular dopamine.

Cocaine does not increase dendritic spine density in DAT-CI mice

To determine how DAT inhibition contributes to the more long lasting changes in the brain, we measured dendritic spine density in the NAC of the animals used in the above locomotor sensitization experiments. Fig. 2A is a schema of brain areas of the NAC where neurons were sampled. Fig. 2B shows a representative neuron (at 40X) in the AcbS of wild-type mice stained with Golgi and Nissl staining procedures. Fig. 2C and 2D shows representative photomicrographs (100X) from the AcbS (Fig. 2C) and AcbC (Fig. 2D) of wild type and DAT-CI mice injected with either cocaine or saline. Two way ANOVA revealed an interaction between genotype and drug ($F_{(1, 80)} = 0.018$) in the AcbS. Following this, one way ANOVA found that repeated injections of cocaine increased dendritic spine density in the AcbS of wild-type mice as compared to repeated injections of saline (Fig. 2E, $F_{(1, 25)} = 14.6$, $p < 0.001$). The spine density in the AcbS of cocaine-treated wild-type mice was higher than the spine density in the AcbS of saline-treated DAT-CI mice ($p < 0.05$). There was no significant difference between saline-treated wild-type mice and saline treated DAT-CI in dendritic spine density in the AcbS. In contrast, cocaine did not alter dendritic spine density in the AcbS of DAT-CI mice (Fig. 2E, $F_{(1,30)} = 1.785$, $p = 0.185$). In the AcbC, two-way ANOVA found no differences in dendritic spine density as a result of drug treatments (Fig 2F, $F_{(1, 65)} = 0.688$, $p = 0.408$) or genotype ($F_{(1,80)} = 0.453$, $p = 0.502$).

DISCUSSION

We have used genetically modified knock-in mice that express cocaine insensitive DAT to directly test the role of DAT inhibition in cocaine's ability to increase the dendritic spine density of neurons within the NAC. We found that repeated exposure to cocaine failed to change dendritic spine density in the NAC of DAT-CI mice. These results suggest DAT inhibition is required for the increase in dendritic spine density in the NAC induced by cocaine. Because the norepinephrine transporter (NET) and the serotonin transporter (SERT) are still inhibited by cocaine in DAT-CI mice, these data also indicate that repeatedly inhibiting NET and SERT is insufficient to alter dendritic spine density in the NAC or change locomotor activity. These findings support the hypothesis that DAT inhibition is important in mediating the neuroadaptations underlying drug-seeking behaviors.

Our finding that locomotor sensitization was accompanied by an increase in dendritic spine density in the AcbS (but not the AcbC) of wild-type mice, suggests that the AcbS is important for cocaine-induced locomotor sensitization. There is evidence supporting a role for the AcbS in drug-induced locomotor sensitization. First, amphetamine infused into the AcbS (but not AcbC) induced robust locomotor sensitization, which was blocked by a D1 receptor antagonist pretreatment (Pierce and Kalivas, 1995). Second, extracellular levels of dopamine increased more in the AcbS than in the AcbC of drug-sensitized animals after a challenge injection (Pontieri et al., 1995). Third, electrolytic lesions to the AcbS attenuate

cocaine-induced locomotor sensitization (Brenhouse et al., 2006; Todtenkopf et al., 2002). However, there are many studies that suggest a role for the AcbC in cocaine-induced locomotor sensitization (Li et al., 2004).

We were not able to detect cocaine-induced increases in dendritic spine density in the AcbC of wild-type mice which is inconsistent with a previous study that showed increases in both the AcbS and the AcbC (Li et al., 2004). This discrepancy could be explained by several differences between the two studies. First, the animals are different; while the study by Li (2004) used rats our study used mice. Although studies from mice and rats typically support each other, there are cases in which cocaine affects the NAC of mice and rats differently. For example, it has been reported that cocaine increases glucose utilization in the NAC of rats but decreases glucose utilization in the NAC of mice (Zocchi et al., 2001). Second, the cocaine treatments are different between the two studies; in the study by Li (2004), the animals received fewer total injections (8 injections vs. 20 injections) of cocaine and had a longer drug-free period (2 weeks vs. 3 days) before tissue collection. Our data suggest that in mice the AcbS plays an important role in cocaine-induced locomotor sensitization. It is possible that the cocaine induced changes in the AcbS are more marked or easier to detect than that in the AcbC in mice.

Although there are baseline differences in locomotor activity between DAT-CI and wild-type mice, we do not believe these differences explain the observation that repeated exposure to cocaine did not increase locomotor activity in DAT-CI mice. The lack of cocaine-induced locomotor stimulation is unlikely explained by a ceiling effect because DAT-CI mice show a robust increase in locomotor activity in response to drugs other than cocaine, including amphetamine and morphine (Chen et al., 2006). Interestingly, even though DAT-CI mice have higher basal levels of locomotor activity, their dendritic spine density in the NAC was similar to wild-type mice treated with saline (Fig. 2d, 2e). This indicates that levels of basal locomotor activity are not necessarily tightly related to the density of dendritic spines in the NAC; at least it may not be observable with the test paradigm we used.

Spines on the dendrites of medium spiny neurons in the NAC are important for synaptic transmission. These spines serve as convergent centers for glutamatergic and dopaminergic neurons in the NAC (Sesack et al., 2003). While the heads of spines in the NAC primarily form synapses with glutamatergic neurons (Hering and Sheng, 2001), the shafts of spines form synapses with dopaminergic neurons (Yao et al., 2008). Furthermore, an increase in dendritic spine density is associated with long-term potentiation (Sarti et al., 2007) and a reduction in dendritic spine density (or spine shrinkage) is associated with long-term depression (Zhou et al., 2004). Thus, our data could indicate that DAT inhibition is involved in cocaine's ability to modify synaptic strength in the NAC.

In summary, we have used genetically modified knock-in mice to directly test the role of DAT inhibition in cocaine's ability to increase the dendritic spine density of neurons within the NAC. We have found that without DAT inhibition, cocaine exposure does not increase dendritic spine density in NAC. These data support the notion that DAT inhibition is required for cocaine-induced increases in dendritic spine density in the NAC. This may also suggest that DAT inhibition is involved in the long-lasting changes in the brain produced by cocaine.

Acknowledgments

This work was supported by National Institutes on Drug Abuse grant #R01DA014610 (HHG) and R01DA020124 (HHG). We would like to thank Drs. Lane Wallace and Norton Neff, Ryan Smith, Amanda Martin, Jill Green, Erik

Hill, and Brian O'Neill for providing critical discussion. We would also like to thank Pauline Chen for maintaining the mouse colony and Jason Xia for his help with immunohistochemistry.

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Fig. 1.

Effects of cocaine on locomotor activity in wild type and DAT-CI mice ($n = 12-16/\text{group}$). Locomotor activity was measured after the first (Day 1) and last (Day 30) injection of cocaine (15 mg/kg) or saline. Between the test days (days 2–27), animals received one injection per day (5 days on, 2 days off) for a total of 20 injections of cocaine (30 mg/kg). (A) In wild type mice, cocaine-induced locomotor activity was higher on Day 30 than on Day 1. (*) signifies statistically different from saline and (#) signifies statistically different from Day 1. (B) In DAT-CI mice, locomotor activity did not increase after the first (Day 1) or last (Day 30) injection of cocaine. (C) There is a significant increase in locomotor activity after cocaine injection compared to that after saline injection in wild-type mice but not in DAT-CI mice. Data are expressed as mean distance (m) traveled ($\pm$ SEM) in 15 min intervals. The arrow indicates when the injection was administered. $*P < 0.05$.

Fig. 2.

Effects of cocaine on dendritic spine density in the NAC of wild-type and DAT-CI mice (n= 8–11/group). Three days after the challenge injection of cocaine or saline, brains were processed with Golgi staining procedures. (A) Schema of a coronal section of the NAC (1.34 mm AP from bregma) showing the sampling areas (shaded areas, figure adapted from the Paxinos and Watson, 1997 atlas). (B) Photo micrograph (X100) of neurites within wild-type AcbS. The boxed area shows a region of a neurite in focus with spines that are suitable for scoring. Representative photomicrographs of neurites from WT and DAT-CI mice chronically injected with saline “Sal” or cocaine “Coc” in the AcbS (C) and AcbC (D). The spines are manually counted by a person with no knowledge of the genetic and treatment groups. The mean number of dendritic spines per 10 μm (± SEM) on medium spiny neurons of WT and DAT-CI mice in the AcbS (E) and core (F). * $P < 0.05$