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Behavior of knock-in mice with a cocaine-insensitive dopamine transporter after virogenetic restoration of cocaine sensitivity in the striatum

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ABSTRACT

Cocaine's main pharmacological actions are the inhibition of the dopamine, serotonin, and norepinephrine transporters. Its main behavioral effects are reward and locomotor stimulation, potentially leading to addiction. Using knock-in mice with a cocaine-insensitive dopamine transporter (DAT-CI mice) we have shown previously that inhibition of the dopamine transporter (DAT) is necessary for both of these behaviors. In this study, we sought to determine brain regions in which DAT inhibition by cocaine stimulates locomotor activity and/or produces reward. We used adeno-associated viral vectors to re-introduce the cocaine-sensitive wild-type DAT in specific brain regions of DAT-CI mice, which otherwise only express a cocaine-insensitive DAT globally.

Viral-mediated expression of wild-type DAT in the rostrolateral striatum restored cocaine-induced locomotor stimulation and sensitization in DAT-CI mice. In contrast, the expression of wild-type DAT in the dorsal striatum, or in the medial nucleus accumbens, did not restore cocaine-induced locomotor stimulation. These data help to determine cocaine's molecular actions and anatomical loci that cause hyperlocomotion. Interestingly, cocaine did not produce significant reward – as measured by conditioned place-preference – in any of the three cohorts of DAT-CI mice with the virus injections. Therefore, the locus or loci underlying cocaine-induced reward remain underdetermined. It is possible that multiple dopamine-related brain regions are involved in producing the robust rewarding effect of cocaine.

1. Introduction

Cocaine is an inhibitor of the dopamine (DA), norepinephrine (NE), and serotonin (5-HT) transporters (Han and Gu, 2006; Ritz et al., 1987). It is simultaneously an addictive drug with euphorigenic effects, and adverse cardiovascular and psychiatric effects (Rotheram-Fuller et al., 2007). While inhibition of each of the monoamine transporters is likely to contribute to each of cocaine's effects in some way, there has been much effort to determine the specific role of each target in producing a behavioral effect. Knock-out mice with each of the monoamine transporters deleted still self-administer cocaine, indicating that none of these targets are individually required for its rewarding effect (Hall et al., 2002). Double knock-out mice lacking the dopamine transporter (DAT) and the serotonin transporter (SERT) do not show cocaine reward, suggesting that the monoamines are mutually or redundantly involved in producing cocaine reward (Uhl et al., 2002). However, the knock-out mice may have substantial adaptive changes. In contrast, using knock-in mice with a functional yet cocaine-insensitive dopamine transporter (DAT-CI mice), we determined that DAT inhibition is necessary for cocaine's rewarding and hyperlocomotive effects (Chen et al., 2006).

Abbreviations: AAV, adeno-associated virus; CPP, conditioned place-preference; DAT, dopamine transporter; DAT-CI mice, cocaine-insensitive dopamine transporter knock-in mice; dCPu, dorsal striatum/dorsal caudate-putamen; IHC, immunohistochemistry; lCPu, lateral striatum/lateral caudate-putamen; mNAc, medial nucleus accumbens; RTI-113, 2β-Carbophenoxy-3β-(4-chlorophenyl)tropane.

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It is well established that the mesolimbic dopamine system, containing dopaminergic projections from ventral tegmental area (VTA) to the nucleus accumbens (NAc) and other forebrain structures, plays a critical role in reward/reinforcement and that most addictive drugs elevate extracellular dopamine in the NAc (Carboni et al., 1989; Cass et al., 1992; Di Chiara, 1995; Koob, 1998). It was shown that rats self-administer amphetamine directly into the NAc (Hoebel et al., 1983), however, cocaine infusion to the NAc did not produce place-conditioning (Hemby et al., 1992). Additionally, cocaine was found to be readily self-administered into the prefrontal cortex (PFC) (Goeders and Smith, 1983). Interestingly, cocaine self-administration to the dorsal, and also to the ventral striatum (nucleus accumbens), is sufficient to induce hyperlocomotion (Delfs et al., 1990; Mao and Wang, 2000). It is therefore important to directly test how cocaine inhibition of DAT in various brain regions contributes to different behavioral responses induced by cocaine.

Here, we set out to determine specifically where DAT inhibition in the brain is involved in producing cocaine reward and hyperlocomotion. We tested the function of DAT inhibition in a specific region by restoring expression of the wild-type dopamine transporter (DAT_{wt}) in DAT-CI mice using adeno-associated viruses (AAV). We injected DAT-CI mice with AAV-DAT_{wt} in the dorsal striatum (dCPu), medial nucleus accumbens (mNAc), and in both the dorsal and ventral portions of the lateral striatum (lCPu). After expression of DAT_{wt} in these regions, we exposed the DAT-CI mice to cocaine, and tested for restoration of the reward and locomotor behaviors.

2. Materials and methods

2.1. Animal subjects

In this study, knock-in mice with a cocaine-insensitive dopamine transporter (DAT-CI mice) were used, which were generated as described previously (Chen et al., 2006). These mice contain a triply mutated dopamine transporter (DAT) which is composed of the following substitutions: L104V/F105C/A109V (termed DAT_{vcv}). C57-congenic DAT-CI and wild-type littermates were generated from sibling pairings of heterozygous mice.

The animal subjects then underwent AAV injections, followed by behavioral testing, and finally by immunohistochemical analysis. A timeline of this paradigm is in Fig. 1, and the details of each method are described below.

During the course of these experiments, all mice were kept in standard housing conditions, which include ad libitum access to food/water and 12 h each of dark/light. Only male mice were used, and all mice were between 2 and 6 months of age at the time of behavioral testing. Experimental groups compared to one another were age matched. All animal procedures were approved by The Ohio State University Internal Laboratory Animal Care and Use Committee (ILACUC).

2.2. Packaging and purification of viral vectors

Recombinant adeno-associated viral vectors containing a hemagglutinin (HA)-tagged wild-type mouse dopamine transporter (AAV-DAT_{wt}) were used in this study. The vectors were prepared by the OSU viral vector core, where viruses were packaged and purified similar to procedures described elsewhere (Clark et al., 1999). Briefly, HEK293 cells were triple co-transfected via the calcium phosphate method with the following plasmids: a capsidation plasmid (AAV1 serotype), a helper plasmid, and the recombinant genome plasmid. The viruses containing wild-type mouse DAT were then isolated from the cell and media fractions of the culture by a series of ultracentrifugation and chromatography purifications. The final preparation was titered by real-time PCR and determined to be at a concentration of 2.6×10^{12} vg/mL. The virus was diluted to 2.6×10^{11} vg/mL for microinjections.

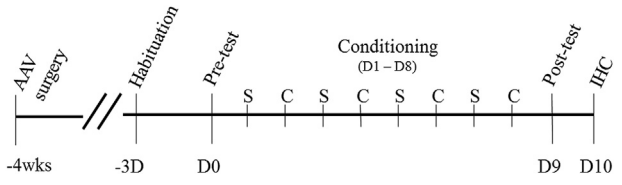

Fig. 1. Timeline and design of in-vivo experiments. The stereotaxic AAV injection surgery was performed and stable expression is reached during a four week recovery. The mice were habituated to being handled for 3 days. Then the mice went through the CPP paradigm with the pre-test on day 0 (D0), followed by four treatment/environment pairings of saline (S) and cocaine (C), and the post-test on D9. After the last behavioral test, the mice were sacrificed and processed for immunohistochemical (IHC) localization of the AAV injection site. Although the data in the Fig. 3 are presented chronologically as in this timeline, the mouse cohorts were counterbalanced for treatment order – with half receiving cocaine on day 1, and half receiving saline.

2.3. Surgeries and microinjection of viral vectors

Mice were anesthetized with a mixture of 100 mg/kg ketamine and 15 mg/kg xylazine (Sigma–Aldrich). Using aseptic surgical procedures, the mice were then fixed into a stereotaxic frame (Stoelting Co., IL.) and a small skin incision was made over the skull. The skull was made level, and the location of the bregma landmark was recorded.

The stereotaxic injection setup consisted of a Hamilton syringe and tubing system, primed with water and connected to a 33 gauge injector cannula (Plastics One, Roanoke VA). A volume of 2–4 μL of virus was injected per mouse, at a rate of 0.1–0.25 μL/min using a syringe pump.

For all behavioral studies, the AAV was injected bilaterally. Microinjections of viral vectors were carried out for three different brain regions: 1) the rostrolateral striatum/lateral caudate-putamen (lCPu), 2) the dorsal striatum/dorsal caudate-putamen (dCPu), and 3) the medial accumbens (mNAc). The coordinates targeted for each of the regions are listed in Table 1.

After infusion, the injector was left in place for 2 min, and then raised. For the lCPu region, three boli were infused along the injector's path during withdrawal, such that both ventral and dorsal striatum were targeted. The mice were sutured after the surgery and administered post-operative care for one week. Expression of the recombinant vector genome was allowed during a four week recovery. AAV does not replicate and the infected region does not spread over time. AAV-mediated expression increases gradually and then stabilizes. The four week recovery period is sufficient to reach high and stable levels of expression.

2.4. Drugs administered

All drugs administered to the mice were dissolved in a vehicle of 0.9% saline at a concentration such that 10 μL/g body weight would deliver the desired dose. Cocaine HCl was provided by the NIDA drug supply program, and administered at 10 and 20 mg/kg doses intraperitoneally (i.p.). The DAT selective inhibitor, 2β-carbophenoxy-3β-(4-chlorophenyl) tropane (Research Triangle Institute, North Carolina) was administered at 5 mg/kg i.p. This drug is hereafter referred to as RTI-113.

Ketamine and xylazine were administered for anesthesia during the stereotaxic surgeries preceding the behavioral experiments, at doses of 100 mg/kg and 15 mg/kg respectively (i.p.).

2.5. Conditioned place-preference and locomotion test

A conditioned place-preference (CPP) apparatus was used to measure cocaine-induced reward and hyperlocomotion simultaneously. The apparatus was a 12.5 cm × 42.5 cm acrylic box subdivided into the following three interconnected compartments: two side compartments (12.5 cm × 17.5 cm) and a center compartment (12.5 cm × 7.5 cm). The CPP procedure, outlined in Fig. 1, consists of a preconditioning test (D0), a cocaine/saline conditioning phase (D1–D8), and a postconditioning test (D9).

Mice were habituated to handling for three days, prior to the pre-conditioning test. On the pre-conditioning test day (D0), the three compartments were made distinct from one another by visual and tactile cues, creating three different "environments." Mice were placed into the center compartment and allowed to explore all three compartments for 30 min. Time spent in each of the three compartments, as well as the total distance traveled, were automatically recorded by the AnyMaze video tracking system (Stoelting Co.) Their preference was defined as the difference in time spent in one side compartment versus the other side. Their unconditioned (pre-existing) preference was counterbalanced in each group by designating

Table 1
Coordinates used for stereotaxic injection of AAV.

Brain region	Axis: Adjustment relative to bregma (in mm)			Approach angle
	Anterior/ posterior	Medial/ lateral	Dorsal/ ventral	
lCPu	+1.5	±1.2	−4.6	15° lateral
dCPu	0.0	±2.2	−3.3	0
mNAc	+1.5	±0.5	−4.8	18° cross midline

The stereotaxic coordinates targeted are listed in the table, in mm relative to bregma. In cases where an angle was used, trigonometric adjustments were made to the manipulanda displacements, in order to ensure that the targeted region occurred at the listed location.

individual mice to receive cocaine in either their initially preferred or initially non-preferred environment – such that the group bias was minimized.

During the conditioning phase, the entire apparatus was configured with only one of the two environmental cue sets. The mice were then administered the treatment (either cocaine or saline) corresponding with the environment for that day. Immediately after administration, they were placed the environment for 30 min, and the distance traveled was recorded. On the following day, the opposite agent was administered in the appropriate environment, and these alternations proceeded for 8 days (a total of four "pairings"). Analytical groups were counter-balanced for treatment order.

For the post-conditioning test (D9), the apparatus was configured as three-environments and the mice were tested exactly the same way as on the pre-conditioning test day. No treatments were administered during either the pre or post-conditioning tests. The "CPP score" is defined as the time spent in the cocaine-paired environment (conditioned stimulus, CS^+) during the preference tests, minus the time spent in the saline-paired environment (unconditioned stimulus, CS^-). Differences in CPP score during the pre-conditioning test (Fig. 3, gray bars) versus the post-conditioning test (Fig. 3, black or black & white bars) indicate an effect of the drug.

For some experiments, additional drugs were tested for their effects on locomotion, after the reward data were collected from the post-conditioning test. In these trials, the mice were administered the drugs in the three-environment configuration used during the preference tests. This design was used for the RTI-113 experiments presented below (Fig. 5).

2.6. Immunohistochemistry

Mice were sacrificed and perfused with 4% paraformaldehyde in order to harvest their brains, after the CPP test. Immunohistochemical localization of the injection site was performed on floating sections. The brain was sectioned coronally at 60 μm through the striatal region on a freezing microtome. The sections were stained for the HA-tagged DAT expressed by the AAV in a series of antibody and reagent incubations. All incubations were carried out at room temperature in PBS containing 0.003% triton X-100. The sections were first incubated in a blocking buffer of 1% BSA for 20 min. They were then incubated in a 1:6000 dilution of mouse monoclonal anti-HA primary antibody (Sigma). They were then incubated in a 1:1000 dilution of goat-anti mouse secondary antibody (Sigma) followed by an incubation in a 1:1000 dilution of peroxidase-conjugated mouse anti-peroxidase (Jackson Immuno Research). The sections were then washed, and the labeled tertiary was reacted with a chromogen using a nickel-intensified, glucose oxidase-catalyzed 'Diaminobenzidene (DAB) procedure' detailed elsewhere (Tian et al., 2008). The sections were mounted onto slides and visualized at 1.25x magnification on a CarlZeiss axioscope.

2.7. Fast-Scan cyclic voltammetry

In a separate experiment aimed at validating the behavioral restoration approach, DAT-CI mice were injected with AAV-DAT_{wt} in the lCPu region unilaterally. Coronal brain slices (400 μm thickness) containing the injected region were prepared. Electrically-evoked dopamine overflow – and its clearance – was measured using FSCV as described previously (Chen et al., 2006; Zhou et al., 2005). On both the AAV-injected side and the control side, a baseline reuptake curve was measured followed by a curve in the presence of 2.5 μM cocaine.

2.8. In vitro transport assay

HeLa cells (American Type Culture Collection, Rockville, MD) grown in 96-well plates were transiently transfected with plasmids containing the DAT_{wt} or DAT_{vcv} coding regions as described previously (Hill et al., 2011).

They were then incubated with a buffer containing 60 nM [^{3}H]-labeled dopamine for 10 min at room temperature. The uptake reactions were then terminated by two successive washes with the PBS/Mg/Ca buffer, and the radioactivity of the plates was determined using a TopCount (Perkin Elmer, Waltham, MA).

2.9. Statistical analysis

Statistical analyses were performed using the general linear model in SPSS (IBM; version 19). A two-way repeated-measures ANOVA was performed once for locomotor results and once for CPP results. Bonferroni post-hoc tests were then used for specific comparisons, unless there were unequal variances across groups. Dunnett's T3 tests were performed where there were unequal variances. All omnibus tests were designed to compare the test group (i.e. wild-type or AAV-injected DAT-CI mice) to uninjected DAT-CI (negative) controls. For locomotion induced by RTI-113, a two-way ANOVA was performed between lCPu and dCPu injected DAT-CI mice, followed by Scheffe's tests.

3. Results

DAT-CI mice globally express a cocaine-insensitive dopamine transporter – in place of the wild-type DAT – and they lack the basic cocaine-induced behaviors of CPP and locomotor stimulation. Selective introduction of the cocaine-sensitive DAT_{wt} would allow us to identify the minimal brain regions involved in cocaine's effects. Therefore, DAT-CI mice were intracerebrally injected with AAV-DAT_{wt} in three regions (lCPu, dCPu, mNAc) and their cocaine responses were subsequently tested.

3.1. Ex-vivo confirmation of virogenetic rescue

First, an *ex vivo* experiment was carried out to confirm the functional efficacy of reintroducing wild-type DAT into the DAT-CI mouse brain. AAV-DAT_{wt} was injected unilaterally into the striatum of DAT-CI mice. Fig. 2C shows the region of HA-tagged DAT_{wt} expression, which is present only on one side of the striatum. Using fast-scan cyclic voltammetry (FSCV), electrically-evoked DA overflow and its clearance (reflecting DA uptake) were measured in

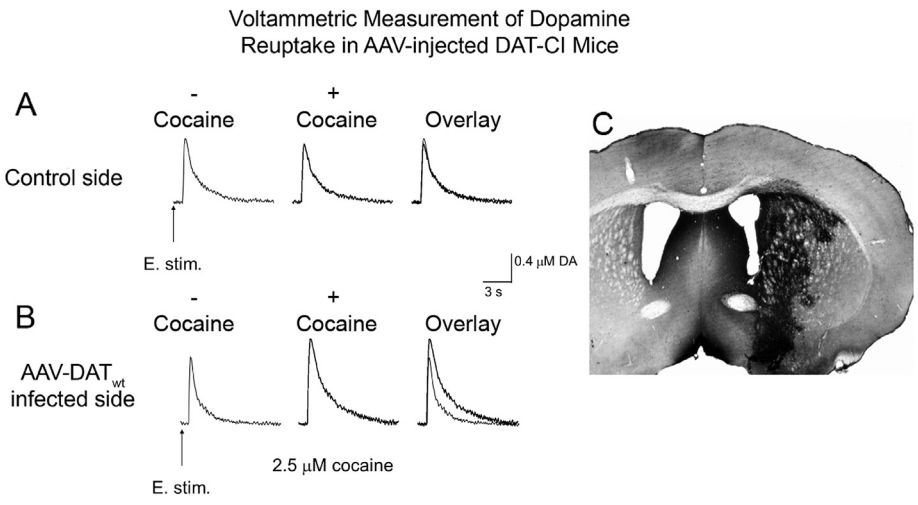

Fig. 2. Cocaine-induced dopamine overflow in brain slices from AAV-injected DAT-CI mice. Three DAT-CI mice were injected unilaterally in the striatum with AAV-DAT_{wt} and were analyzed using FSCV measurements of electrically stimulated release. For each mouse, multiple brain slices and multiple measurements were taken on the control side and AAV injected side of each brain slice – first in the absence and then in the presence of 2.5 μM cocaine. Multiple traces were recorded and representative traces are presented. On the control side of the slices, cocaine had no impact on the peak height of DA overflow or its decline (A). In contrast, cocaine increased the DA peak height and slowed its decline in regions infected by AAV-DAT_{wt} (B). Panel C shows that the HA-tagged wild-type DAT was only expressed on injected side of the brain.

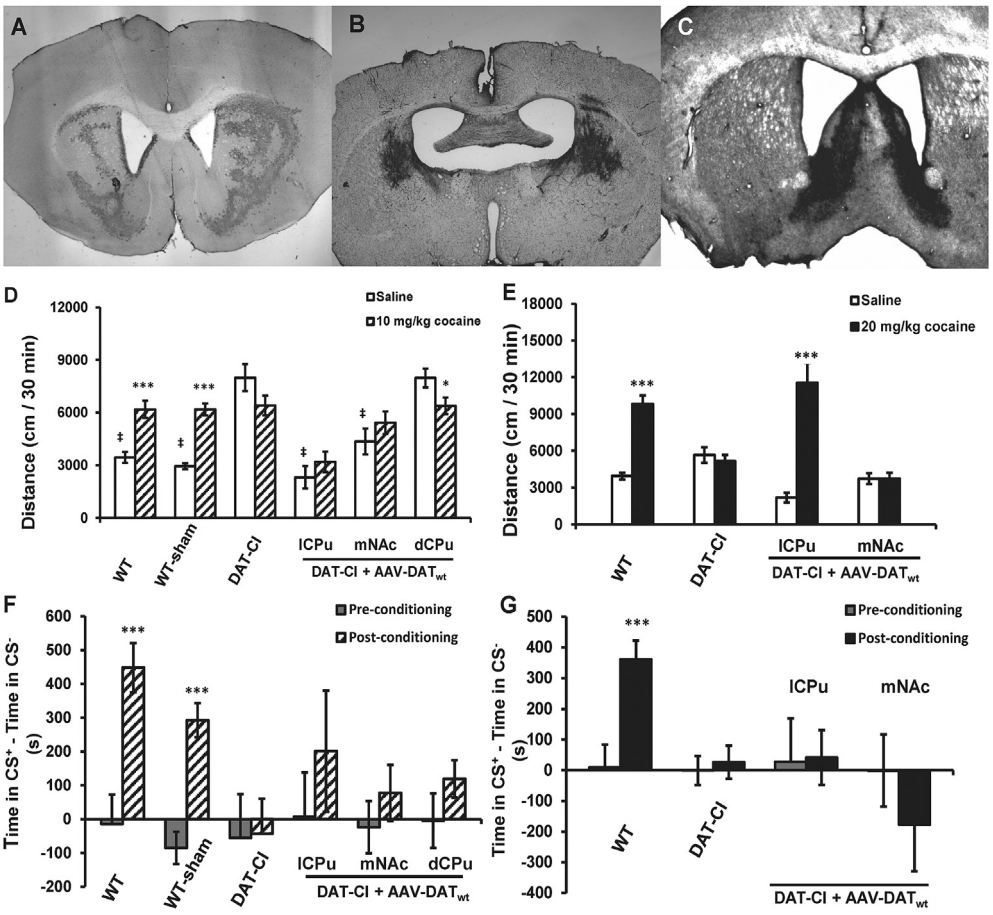

Fig. 3. Cocaine-induced locomotion and reward behaviors in AAV-injected DAT-CI mice. Depicted are data from experiments investigating the role of DAT blockade in three subregions of the striatum. Representative micrographs of coronal brain slices from DAT-CI mice with AAV vector injected into the lCPu (A, +1.5 mm relative to bregma), dCPu (B, 0 mm), and mNAc (C, +1.5 mm) show the localization and spread of AAV-mediated wild-type DAT expression. Panel D shows the locomotion after saline (open bars), or 10 mg/kg cocaine (closed bars) in wild-type (WT) mice ($n = 18$), wild-type mice with AAV-GFP injected to the lCPu (WT-sham, $n = 18$), DAT-CI mice ($n = 7$), and DAT-CI mice injected with AAV-DAT$_{wt}$ in the three subregions of striatum ($n = 8$ lCPu, 14 mNAc, 15 dCPu). In panel E, the cocaine dose was 20 mg/kg, but the sham-injected as well as the dCPu-injected cohorts were not studied ($n = 12$ WT, 8 DAT-CI, 8 lCPu, 8 mNAc). Wild-type mice and sham-injected mice display significant locomotor stimulation induced by 10 mg/kg or 20 mg/kg cocaine (saline vs cocaine; ***, $p < 0.001$), whereas DAT-CI mice do not. DAT-CI mice injected with AAV-DAT$_{wt}$ to the lCPu region have significant restoration of cocaine-induced locomotor stimulation at 20 mg/kg cocaine, relative to saline (***, $p < 0.001$). There was also a reduction of basal locomotor activity in the AAV-DAT$_{wt}$ injected mice, relative to naïve DAT-CI mice (‡, $p < 0.001$) in all groups except the dCPu-injected cohort. Panels F/G show the CPP scores collected from the same cohorts of mice as above. The CPP score is represented as the difference in the time mice spent in the cocaine compartment, minus the time they spent in the saline compartment before (solid bars) and after (striped bars) conditioning. The wild-type mice display significantly higher place-preference for the cocaine-paired chamber in the post-conditioning test relative to the pre-conditioning test (***, $p < 0.001$), at both 10 mg/kg (F) and 20 mg/kg doses (G), whereas the uninjected DAT-CI mice do not. Similar to wild-type mice, sham-injected wild-type mice display significant CPP at 10 mg/kg cocaine (saline versus cocaine; ***, $p < 0.001$). However, injection of AAV-DAT$_{wt}$ was not sufficient to restore CPP for any of the three injected DAT-CI cohorts, at either dose.

brain slices – first in the absence, then in the presence of cocaine. As shown in Fig. 2A, cocaine had no effect on the peak height of DA overflow or its decay on the uninjected side. In contrast, 2.5 μM cocaine increased the DA peak height and slowed down DA clearance (descending portion of the curve) on the side with virally-expressed DAT$_{wt}$ (Fig. 2B). These results indicate that the viral expression of DAT$_{wt}$ in the striata of DAT-CI mice is sufficient to restore cocaine-induced dopamine responses.

3.2. Behavior of AAV-injected DAT-CI mice

Restoration of behavioral effects was assessed using a CPP procedure that efficiently measures both cocaine reward and locomotor stimulation. We decided to target three different subregions of the striatum: lateral striatum/lateral caudate-putamen (lCPu), dorsal striatum/dorsal caudate-putament (dCPu), and medial nucleus accumbens (mNAc). The coordinates used to target these regions with viral injections are listed in Table 1, and the immunohistochemical localization of AAV-DAT$_{wt}$ expression in each of these regions is shown in representative micrographs (Fig. 3A–C, respectively).

Locomotor behavior was analyzed for most of these groups for responses to both 10 mg/kg and 20 mg/kg doses of cocaine (day 2 drug response versus saline on day 1). In addition to the AAV-injected groups described, naïve wild-type and DAT-CI mice, as well as wild-type mice with an AAV-sham injection (AAV-GFP expression in the lCPu region) were analyzed. Fig. 3D and E show mouse locomotion after administration of saline or 10 mg/kg cocaine (Fig. 3D) or 20 mg/kg cocaine (Fig. 3E). An omnibus two-way repeated-measures ANOVA was used on all groups at both doses – with "group" and "dose" as the between-subjects factors, and "day" (vehicle/drug) as the within-subjects factor. There was a significant main effect of drug dose ($F_{1,\ 106} = 4.978$; $p < 0.05$), as well as a significant group × dose interaction ($F_{3,\ 106} = 13.666$;

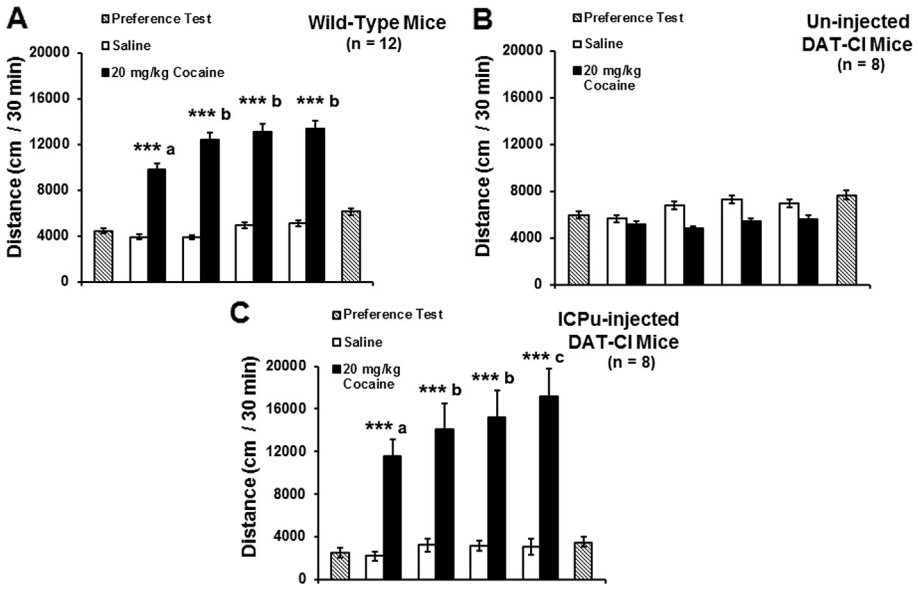

Fig. 4. Cocaine-induced locomotor sensitization in wild-type and lCPu-injected DAT-CI mice. The locomotor activities of wild-type (A), naïve DAT-CI (B), and DAT-CI mice injected with AAV-DAT_{wt} to the lCPu region (C) are shown as daily 30-min sessions throughout the CPP paradigm. The saline-induced locomotion (white bars) does not vary with time for any of the three cohorts, whereas the cocaine-induced locomotion (black bars) is significantly higher for the wild-type and lCPu-injected DAT-CI mice compared to saline (***, $p < 0.001$). Furthermore, the stimulation effect increases across days for the wild-type mice and lCPu-injected DAT-CI mice (levels of increasing parity denoted by different letters; $p < 0.05$). Striped bars indicate locomotion on the preference test days, in which no treatment was administered. The number of mice used was the same as in the 10 mg/kg experiments in Fig. 3, since these are data from the same animals.

$p < 0.001$) – indicating that the drug had an effect in an AAV-dependent manner. There was also a main effect of group ($F_{5, 106} = 9.415$; $p < 0.001$) – indicating that the AAV injection had a baseline effect. Specifically, post-hoc tests show that the lCPu-injected DAT-CI mice had significant locomotor stimulation by 20 mg/kg cocaine (***, $p < 0.001$, Fig. 3E), but not 10 mg/kg ($p = 0.085$, Fig. 3D). Furthermore, lCPu injected DAT-CI mice had a significantly lower baseline locomotion compared to DAT-CI mice (‡: $p < 0.001$, Fig. 3D). The wild type mice and AAV-GFP injected wild type mice also had lower basal locomotor activities than DAT-CI mice (‡, $p < 0.001$, Fig. 3D), due to reduced DAT function, as reported previously (O'Neill and Gu, 2013). Neither the dCPu nor the mNAc-injected DAT-CI mice showed restored locomotor responses to cocaine, however the mNAc-injected cohort had a lower (restored) basal locomotion compared to naïve DAT-CI mice (‡: $p < 0.001$). Bonferroni post-hoc tests were used, except for within AAV-injected DAT-CI cohorts – where Dunnett's T3 test was used due to inhomogeneity of variances. Fig. 3D and F show that the injection of the control AAV-GFP virus into WT mice (WT-sham) did not result in any changes in mouse behaviors as measured by the cocaine-induced hyperlocomotor and CPP test (Fig. 3D and F).

Since the cocaine was administered in the context of a conditioned place- preference (CPP) paradigm, the mice received 4 doses of cocaine during which locomotor sensitization could also be observed. Fig. 4 shows the locomotor activity of wild-type, DAT-CI, and lCPu-injected DAT-CI mice throughout the CPP test. A two-way repeated-measures ANOVA was used to analyze wild-type (or lCPu-injected DAT-CI mice, separately) versus naïve DAT-CI mice – with "group" and "treatment" as between subjects factors, and with "days" as the within-subjects factor.

For wild-type mice (Fig. 4A), there was a significant main effect of treatment (cocaine versus saline; $F_{1, 36} = 24.579$, $p < 0.001$), and of group ($F_{1, 36} = 14.070$; $p < 0.01$), as well as a significant treatment by group interaction ($F_{1, 36} = 52.075$; $p < 0.001$). Furthermore, there was a significant quadratic contrast of this interaction across days ($F_{1, 36} = 7.928$; $p < 0.01$). This indicates that wild-type mice have acute locomotor stimulation by 20 mg/kg cocaine (also shown in Fig. 3) and significant locomotor sensitization upon subsequent doses. Bonferroni-adjusted pairwise comparisons of all of the days of cocaine-induced locomotion revealed that wild-type mice exhibit locomotor stimulation by 20 mg/kg cocaine (***, $p < 0.001$) and have levels of increasing differences across days (Fig. 4A, denoted by letters; $p < 0.05$). In contrast, the pairwise comparisons show that DAT-CI mice do not have significant locomotor stimulation, or sensitization by cocaine (Fig. 4B).

Regarding sensitization in the lCPu-injected DAT-CI mice (Fig. 4C), there was a significant main effect of treatment ($F_{1, 28} = 16.641$; $p < 0.001$), and of group ($F_{1, 28} = 4.836$; $p < 0.05$), as well as a significant treatment by group interaction ($F_{1, 28} = 27.362$; $p < 0.001$). Furthermore, there was a significant linear contrast of this interaction across days ($F_{1, 28} = 5.886$; $p < 0.05$). This indicates that the lCPu-injected DAT-CI mice have acute locomotor stimulation by cocaine (also shown in Fig. 3) and significant locomotor sensitization upon subsequent doses. Bonferroni-adjusted pairwise comparisons of all of the days of cocaine-induced locomotion revealed that these mice exhibit locomotor stimulation by 20 mg/kg cocaine (***, $p < 0.001$)) as well as levels of increasing differences (Fig. 4C, denoted by letters; $p < 0.05$).

For reward, Fig. 3F and G show the results of the CPP test for 10 mg/kg and 20 mg/kg cocaine, respectively. The CPP score (time spent in cocaine compartment minus that in saline compartment) before and after conditioning are presented. A two-way repeated-measures ANOVA (with "group" and "dose" as the between-subjects factors, and "phase" as the within-subject factor) was performed. There was a significant main effect of group ($F_{5, 106} = 2.512$; $p < 0.05$), and of phase ($F_{1, 106} = 21.560$; $p < 0.001$), as well as a significant group by phase interaction ($F_{5, 106} = 7.679$; $p < 0.001$). This indicates that there is a significant, group-dependent conditioning effect by cocaine at some dose.

Post-hoc tests were performed to determine specific contrasts within each dose/group (compared to saline or to naïve DAT-CI mice). As expected, the wild-type mice exhibited robust CPP at

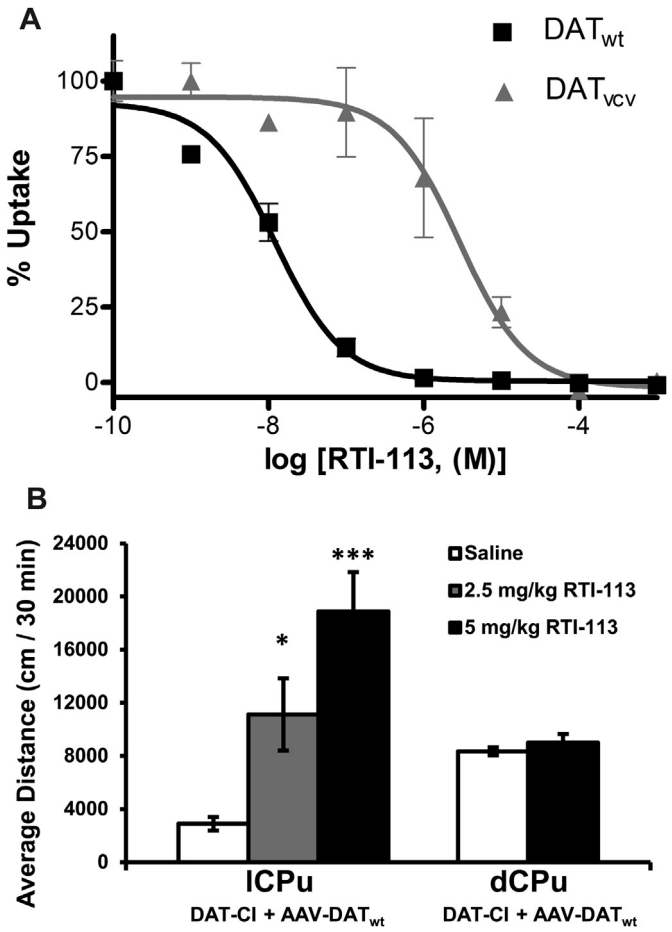

Fig. 5. Locomotor stimulation by a DAT-specific inhibitor in AAV-injected DAT-CI mice. RTI-113 is a DAT-selective cocaine analogue, and it could therefore be used to further refine the observations in the cocaine-based experiments. Here we show that RTI-113 is a potent inhibitor of wild-type DAT, but not of the mutant DAT_{vcv} (A). Transport assays in transfected cultured cells determined an approximately 200-fold higher IC_{50} for DAT_{vcv} (gray line) relative to DAT_{wt} (black line). The DAT-CI mice injected with AAV-DAT_{wt} to the lCPu region (see Fig. 3A) were also administered RTI-113 and their locomotor responses measured (B). The injected DAT-CI mice (shown at left) had significant hyperlocomotion when given both 2.5 mg/kg and 5 mg/kg doses (*, $p < 0.05$ and ***, $p < 0.001$; RTI-113 vs saline). Uninjected DAT-CI mice were confirmed to lack a behavioral response to RTI-113 in separate experiments using a different behavioral apparatus (data not shown). DAT-CI mice injected with AAV-DAT_{wt} in the lCPu region had significantly higher cocaine-induced locomotion than another non-responding group – the dCPu-injected mice (shown at right) – at the 5 mg/kg RTI-113 dose.

both 10 mg/kg (3F: ***, $p < 0.001$) and 20 mg/kg cocaine doses (3G: ***, $p < 0.001$), relative to saline. The wild-type mice with a sham injection to the lCPu retained significant CPP at 10 mg/kg cocaine (3F: ***, $p < 0.001$), relative to saline. In contrast, the DAT-CI mice injected with AAV-DAT_{wt} to the lCPu region did not have restored CPP at 10 mg/kg (3F: $p = 0.068$) or 20 mg/kg (3G: $p = 0.897$) cocaine, relative to saline – even though they did exhibit restored locomotor responses. The other AAV-DAT_{wt} injected cohorts of DAT-CI mice (mNAc and dCPu cohorts) did not exhibit significant CPP at any dose.

In the above experiments, cocaine inhibits DAT only in the viral-injected regions, but inhibits NET and SERT globally. Next, we aimed to test whether DAT-inhibition alone – and in the lCPu region alone – is sufficient to induce hyperlocomotion. We used a DAT-specific cocaine analogue (RTI-113) toward this aim (Carroll et al., 2004). Fig. 5A shows that the dopamine transporter mutant of DAT-CI mice (DAT_{vcv}) is about 200 fold less sensitive to RTI-113 than the wild-type DAT – as assayed in cultured cells. Therefore, this analogue can also be used in viral restoration studies.

We administered RTI-113 to the same cohort of lCPu-injected mice (after their cocaine tests). Fig. 5B shows the saline, or RTI-113 induced locomotion by mice with lCPu or dCPu injections. A two-way (drug × group) ANOVA was performed. There was a significant effect of RTI-113 on locomotion ($F_{2,16} = 20.020$; $p < 0.001$) compared to saline within the lCPu-injected DAT-CI cohorts. Scheffe's tests show that the locomotion induced by both 2.5 mg/kg and 5 mg/kg RTI-113 was significantly different from that of the saline dose (*, $p < 0.05$; and ***, $p < 0.001$, respectively).

Taken together, these results suggest that DAT-blockade by cocaine in the dorsal and ventral regions of the lateral striatum is sufficient to stimulate locomotion, but not sufficient to produce a significant rewarding effect in DAT-CI mice. Furthermore, cocaine inhibition of viral-expressed DAT_{wt} in the other two striatal subregions, dCPu and mNAc, was not sufficient to restore either cocaine-induced locomotor stimulation or reward.

4. Discussion

Previously, we observed that mice globally expressing a cocaine-insensitive dopamine transporter (DAT-CI mice) do not exhibit cocaine-induced locomotion or reward-related behaviors (Chen et al., 2006; Thomsen et al., 2009). DAT is expressed in many brain areas, as well as even peripheral areas. Therefore this result leads to the conclusion that DAT inhibition is necessary for these behavioral effects – either somewhere, but potentially everywhere it is expressed. In this study, we used AAV-mediated gene expression in DAT-CI mice, in order to determine if there is a minimal brain region where expression of DAT_{wt} would restore cocaine responses seen in wild-type mice. Importantly, this manipulation tests a sort of 'sufficiency' which is different in both nature and significance to our previous behavioral findings in uninjected DAT-CI mice. We hypothesized that DAT inhibition in the dCPu underlies cocaine-induced hyperlocomotion whereas DAT inhibition in the ventral striatum (mNAc) underlies cocaine reward.

Contrary to our hypothesis, we found that injection of AAV-DAT_{wt} to the dCPu region (dorsal striatum) was insufficient to restore cocaine-induced locomotion – and that injection to the mNAc region (ventral striatum) was insufficient to restore cocaine-induced reward in DAT-CI mice. In order to test whether subregions of the striatum function interactively to produce these behaviors, we added a third cohort (lCPu-injected DAT-CI mice) where the dorsal and ventral areas both express DAT_{wt}. Comparison of the dCPu and mNAc cohorts to the lCPu cohort will be helpful in discerning the cooperative function of the striatal subregions. We found that AAV-DAT_{wt} injection to the lCPu of DAT-CI mice restored the hyperlocomotor response to 20 mg/kg cocaine (Fig. 3E), as well as locomotor sensitization to cocaine (Fig. 5C). However, mice injected in this region did not display significant CPP for 10 mg/kg or 20 mg/kg cocaine. The infection of AAV vector itself may have an impact on animal behaviors. However, it is very unlikely that the restoration of specific cocaine responses is due to the nonspecific effects of virus infection. Virus infections could be disruptive and cause nonspecific losses of functions and thus might be responsible for the lack of AAV-DAT mediated restoration of CPP. However, this is also unlikely since AAV-GFP infusion in wild type mice did not have any impact on cocaine induced locomotor stimulation or cocaine reward (Fig. 3D and F). The three infusion sites were labeled mNAc, dCPu, and lCPu. Each of the infusions likely affects more than one structure – especially when considering the three dimensional spread of the virus around the infusion sites. The images were taken at the epicenter of the spread, and we observed approximate spherical symmetry in the remaining slices. The categorical labels

of mNAc, lCPu, and dCPu are not precise but should be still helpful in pointing to the affected brain regions.

Regarding the negative CPP results (Fig. 3F,G), it is possible that positive reinforcement-learning requires dopamine elevations in multiple brain regions. Reward-learning may also be more dependent on specific spatial or temporal patterns of dopamine signaling (Grieder et al., 2012) and on a certain balance between positive and negative states produced by the three monoamines. Sensitivity to these factors may be eliminated in self-infusion studies that overly-focus on single brain regions and transmitter systems in reward. For example, cocaine was found to be readily self-administered to the PFC (Goeders and Smith, 1983) – where DAT expression is low, and dopamine is transported through the NET (Mazei et al., 2002; Tanda et al., 1997). Since DAT-CI mice do not display cocaine reward, and yet have normal levels of cocaine-sensitive NET in the PFC, these studies appear to be in conflict. It is likely that global inhibition of the norepinephrine and/or serotonin transporters normally contribute to negative reward responses (Jones et al., 2010) which may nullify the reinforcing effect of NET inhibition in the PFC. These negative effects are not present in local cocaine-infusion studies in the PFC – resulting in a misleading striatum-independent and PFC-driven reward. Therefore, the negative CPP results may represent an opportunity to discover interactions between multiple regions and transporter targets of cocaine – but many more studies are necessary. Our lab is currently studying an analogous NET-CI mouse line. A double DAT/NET-CI mouse line would be instrumental in determining whether NET inhibition in the PFC underlies part of cocaine's positively rewarding effect, and in determining which regions outside of the PFC contribute to negative reward. We have shown that cocaine produces conditioned place-aversion in DAT-CI mice in which cocaine blocks NET and SERT but not DAT (O'Neill et al., 2013), suggesting that NET/SERT inhibition generally opposes the positive reward due to DAT inhibition. Intriguingly however, the striatum and dopamine have been shown to be involved in negative reward processes as well (Asaad and Eskandar, 2011; Jhou et al., 2013).

Similarly, transporter-specific inhibitors may be instrumental in dissecting the roles of DAT/NET/SERT inhibition in generating a specific behavior – especially in AAV-DAT_{wt} injected DAT-CI mice where both spatial and target specificity would be achieved. After the cocaine CPP tests, the cohort of lCPu-injected DAT-CI mice was used to test locomotor stimulation by a DAT-specific inhibitor, RTI-113. In these mice, administration of 5 mg/kg RTI-113 induced a robust locomotor stimulation (Fig. 5B) – similar to 20 mg/kg cocaine. This result suggests that DAT inhibition in the lCPu alone, without affecting SERT or NET function, is sufficient to produce hyperlocomotion.

In fact, similar to the antagonistic role of NET/SERT inhibition discussed for reward, it appears that NET or SERT inhibition also oppose DAT-mediated hyperlocomotion – since administration of RTI-113 produces more locomotor stimulation than cocaine does in these mice. These results suggest that *only* DAT-inhibition – but DAT-inhibition occurring in both the dorsal and ventral striatum simultaneously – is sufficient to restore cocaine-induced hyperlocomotion in DAT-CI mice. Apparently, restoration of DAT-inhibition separately in the ventral (mNAc) and dorsal striatum (dCPu) is not sufficient for this effect. Notably, the degree of spread and the exact localization of the dCPu injection are different than the dorsal portion of the combined (lCPu) cohort. In the dCPu cohort, the spread is very confined and more caudal than the dorsal portion of the lCPu cohort.

It is important to realize that the results from the dCPu and mNAc cohorts only suggest that DAT-inhibition in these regions is not *sufficient* to restore either behavior. This is not to say that the regions are uninvolved in cocaine-induced reward and locomotion. Both of these regions may well be areas where DAT-inhibition is *necessary* to produce a given behavioral effect. Our results would be consistent with the hypothesis that DAT-inhibition in the NAc is necessary, but not sufficient to produce cocaine reward when global NET/SERT inhibition opposes its effect. The maintenance of cocaine's global effects at the NET and SERT is a distinct advantage of our "gene-restoration" approach in DAT-CI mice over traditional intra-cranial infusion studies. In our system, specific dissection of the behavioral effects of DAT-inhibition in a given region is possible *ceteris paribus* (all else being equal).

5. Conclusions

In summary, these results strengthen the original conclusions derived from DAT-CI mice that DAT blockade is necessary for cocaine-induced reward and locomotion – since the loss of the locomotor phenotype is fully reversible. Secondly, the results demonstrate that DAT-inhibition in the rostrolateral striatum alone is sufficient for cocaine's locomotor stimulating effect. Lastly, the results suggest that multiple dopamine-related brain regions may interact to produce cocaine-induced reward. Future studies may investigate the role of DAT inhibition in the amygdala or the midbrain – since these regions are known to express DAT and to be involved in positive/negative reward responses (Yap and Miczek, 2008). Ongoing studies are also investigating the role of NET/SERT inhibition in cocaine-induced behaviors.

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