Reducing Ventral Tegmental Dopamine D2 Receptor Expression Selectively Boosts Incentive Motivation

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INTRODUCTION

Both obesity and substance addiction are enormous socioeconomic and public health problems (European Commission, 2007; Gustavsson et al., 2011). Estimates are that 27 million people around the world are addicted to illicit drugs, and 76 million are addicted to alcohol (United Nations Office on Drugs and Crime, 2012). Indeed, substance addiction has been calculated to be the most financially costly of all major neuropsychiatric disorders (Effertz and Mann, 2013). Obesity prevalence is increasing and its comorbidities, ie, type 2 diabetes, cardiovascular disease, and cancer are a major cause of death in the western world (Flegal et al., 2010). Interestingly, it has been suggested that there is overlap in the neural and behavioral processes of these disorders, as addiction-like processes may underlie certain forms of obesity (Gearhardt and Corbin, 2009; Volkow et al., 2013; Potenza, 2014). Moreover, addictive behaviors have also been proposed to play a role in the psychopathology of eating disorders that do not necessarily lead to obesity such as bulimia nervosa and binge eating disorder (Kessler et al., 2013; Gearhardt et al., 2014).

All addictive substances directly or indirectly target the mesolimbic dopamine (DA) system (Di Chiara and Imperato, 1988; Nestler, 2005), and DA signaling has also been implicated in the (maladaptive) intake of palatable food (Baik, 2013; Meye and Adan, 2014). The cell bodies of the mesolimbic DA system are located within the midbrain ventral tegmental area (VTA). These DA neurons project throughout the forebrain, and the most dense projection reaches the ventral striatum (nucleus accumbens (NAcc) core, shell and olfactory tubercle). Five types of DA receptors have been identified. These can be divided into two classes: the DA receptor D1-like (D1 and D5) which activate adenylyl cyclase and the DA receptor D2-like (D2, D3 and D4) which have an inhibitory influence on this enzyme (Sibley and Monsma, 1992). Here, we focus on the DA D2 receptor (D2R) which is expressed post-synaptically on (among others) GABAergic medium spiny neurons in the striatum, as well as pre-synaptically and somatodendritically on DA neurons, where they act as auto-inhibitory receptors (Usiello et al., 2000). Stimulation of the D2R on midbrain DA neurons inhibits firing as well as DA production and release (Aghajanian and Bunney, 1977; Tepper et al., 1997; Anzalone et al., 2012).

Altered mesolimbic dopamine signaling has been widely implicated in addictive behavior. For the most part, this work has focused on dopamine within the striatum, but there is emerging evidence for a role of the auto-inhibitory, somatodendritic dopamine D2 receptor (D2R) in the ventral tegmental area (VTA) in addiction. Thus, decreased midbrain D2R expression has been implicated in addiction in humans. Moreover, knockout of the gene encoding the D2R receptor (Drd2) in dopamine neurons has been shown to enhance the locomotor response to cocaine in mice. Therefore, we here tested the hypothesis that decreasing D2R expression in the VTA of adult rats, using shRNA knockdown, promotes addiction-like behavior in rats responding for cocaine or palatable food. Rats with decreased VTA D2R expression showed markedly increased motivation for both sucrose and cocaine under a progressive ratio schedule of reinforcement, but the acquisition or maintenance of cocaine self-administration were not affected. They also displayed enhanced cocaine-induced locomotor activity, but no change in basal locomotion. This robust increase in incentive motivation was behaviorally specific, as we did not observe any differences in fixed ratio responding, extinction responding, reinstatement or conditioned suppression of cocaine, and sucrose seeking. We conclude that VTA D2R knockdown results in increased incentive motivation, but does not directly promote other aspects of addiction-like behavior.

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In the striatum, neurons expressing the D2R are thought to work in concert with DA D1 receptor-expressing neurons to mediate action selection, salience, motivation, and incentive learning (Robinson and Berridge, 1993; Isomura et al., 2009; Salamone et al., 2009).

Both the pre- and post-synaptic D2R have been associated with addictive behavior. Decreased D2R availability in the striatum is associated with drug addiction and severe obesity (Wang et al., 2001; de Weijer et al., 2011; Volkow et al., 2011) (but see Eisenstein et al., 2013). Decreased somatodendritic D2R availability has recently been involved in novelty seeking and impulsivity in humans (Zald et al., 2008; Buckholtz et al., 2010) and rodents (Tournier et al., 2013). These character traits have been associated with drug addiction (Jupp and Dalley, 2014) and obesity (Nederkoorn et al., 2007). The notion that somatodendritic D2R play a role in addictive behavior is also supported by animal studies. Thus, rats that exhibit enhanced cocaine self-administration show sub-sensitivity of D2 somatodendritic autoreceptors (Marinelli and White, 2000). Likewise, mice lacking the D2 autoreceptor display elevated DA release and are hypersensitive to the psychomotor effect of cocaine (Bello et al., 2011), and midbrain D2R downregulation has been shown to be involved in the effects of cocaine on plasticity of VTA glutamate signaling (Madhavan et al., 2013). In addition, amphetamine self-administration as well as prolonged exposure to cocaine results in decreased midbrain D2R sensitivity (Henry et al., 1989; Calipari et al., 2014b). Conversely, administration of the D2R agonist quinpirole into the VTA inhibits cocaine-induced reinstatement of cocaine seeking (Xue et al., 2011). Taken together, these data indicate that decreased midbrain D2R availability promotes addictive behavior.

Previously, a DA cell-type-specific approach has been used to ablate the D2R in DA neurons in mice. This resulted in an enhanced locomotor response to cocaine and increased motivation for sucrose (Bello et al., 2011). As this approach ablated the D2R in all midbrain DA neurons, it remains unclear whether these behavioral effects were mediated by altered function of DA neurons in the VTA or the substantia nigra. Furthermore, constitutive absence of the D2R throughout development may evoke compensatory functional adaptations. Here, we therefore opted for a shRNA-mediated approach to reduce the expression of the D2R specifically in the VTA of adult animals. As previous experiments have suggested a role for decreased midbrain D2R availability in addiction, we hypothesized that the knockdown of VTA D2R promotes behaviors associated with addiction, i.e., high motivation, resistance to punishment, and high vulnerability to relapse (see Deroche-Gamonet et al., 2004)). First, we tested the psychomotor effect of a low dose of cocaine. Next, animals were trained to self-administer sucrose or cocaine, and we probed their motivation for sucrose or cocaine under a progressive ratio (PR) schedule of reinforcement. Subsequently, we investigated conditioned suppression as a model of compulsive cocaine seeking (Vanderschuren and Everitt, 2004). Last, we investigated the propensity of animals to reinstate sucrose or cocaine seeking after extinction by priming with sucrose, cocaine, or a cocaine-associated cue, as a model for relapse.

MATERIALS AND METHODS

Animals

Six-week-old male Wistar rats (Charles River, Sulzfeld, Germany) weighing 150–200 g at the start of the experiment were individually housed in makrolon cages (37.8 × 21.7 × 18.0 cm) under a reversed 12:12 light/dark cycle (lights on at 19:00 h). They had ad libitum access to chow and water and a wooden block was provided as home cage enrichment. All experiments were approved by the Animal Ethics Committee of Utrecht University and were conducted in agreement with Dutch laws (Wet op de Dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Production of the D2R shRNA

Four sequences were selected based on homology to Rat D2R mRNA (NM_012547.1). To prevent aspecific binding, we assessed the sequence using the NCBI Basic Local Alignment Search Tool. These were: 1: CATCGTCACCTGCTGGTCTA, 2: CAACCTGAAGACACCACTCAA, 3: TGGTGGGTAAGTGGAATCTTA, and 4: AGGATTCACTGTGACATCTTT. Hairpins were designed and cloned into an miR155-based precursor, which was located in the first intron of enhanced green fluorescent protein (GFP) (Du et al., 2006). The construct containing enhanced GFP and the artificial pre-mRNA was then cloned into an AAV vector behind the enhanced synapsin promoter (White et al., 2011). In this way, the expression of the shRNA would be driven by RNA polymerase II. We opted for this strategy because U6 or H1 promoter-driven (polymerase II-mediated) shRNA expression has been shown to have toxic effects (van Gestel et al., 2014), which we do not observe using the current strategy. In the case of van Gestel et al. (2014), damage was assessed by staining for tyrosine hydroxylase (TH) as well as for miR-124 using an LNA in situ hybridization procedure. We observed proper miR-124 and TH expression in animals injected with the D2R knockdown or the control vector. Ex vivo testing on a dual luciferase assay (Du et al., 2006) showed that sequence 2 and 3 resulted in the most efficient knockdown (Figure 1a). These two sequences were therefore selected for in vivo testing in a pilot experiment (n = 12), in which both altered the sensitivity to cocaine (see below). For the final experiments in this paper, we selected sequence 2, as it showed a slightly larger effect in the pilot experiment. Animals injected with the D2R knockdown vector were compared with animals injected with a control vector of equal length that was targeted at a gene (luciferase) that does not have an equivalent in mammals. The sequence of the control shRNA was: AAAGCAATTGTTCAGGAACC.

Surgery

Rats were anesthetized with Hypnorm (0.315 mg/kg fentanyl, 10 mg/kg fluanisone intramuscular, Janssen Pharmaceutica, Beerse, Belgium) and supplemented with Hypnorm as needed. Rats allocated to cocaine self-administration experiments were implanted with a single intravenous catheter into the right jugular vein aimed at the left vena cava. Catheters (Camcaths, Cambridge, UK) consisted of a 22 g cannula attached to silastic tubing (0.012 ID) and fixed to nylon mesh. The mesh end of the catheter was sutured subcutaneously (s.c.)
on the dorsum. Next, the animals were placed in a stereotaxic apparatus (David Kopf), and 1 μl of a solution containing AAV vector was injected bilaterally into the VTA (coordinates relative to bregma: anteroposterior −5.40, mediolateral ±2.20, dorsoventral −8.90). Carprofen (50 mg/kg, s.c.) was administrated once before and twice after surgery. Gentamycin (5 mg/kg, s.c.) was administered before surgery and for 5 days post surgery. Animals were allowed 7–9 days to recover from surgery.

Cocaine-Induced Locomotor Activity
Locomotor activity was assessed as described previously (Veeneman et al., 2011). Animals were first habituated to the testing apparatus (plastic boxes measuring 50 × 33 × 40 cm, l × w × h). Horizontal locomotor activity was registered using a camera positioned approximately 2 m above the setup. The data were recorded and analyzed using a video tracking system (Ethovision, Noldus, Wageningen, the Netherlands). A session started with a 15 min habituation period. Next, the animals received an intraperitoneal (i.p.) injection of saline or cocaine (cocaine HCl, Bufa BV, Uitgeest, the Netherlands; 5 mg/kg or 10 mg/kg dissolved in saline). Locomotor activity was measured for 30 min. All locomotor experiments were performed during the light phase of the day/night cycle. The animals for this experiment were used for the sucrose self-administration experiments before testing for cocaine-induced locomotion, which was assessed 5 weeks after surgery.

Operant Conditioning Apparatus
Rats were trained in operant conditioning chambers (30.5 × 24.1 × 21.0 cm; Med Associates, St. Albans, VT, USA). The chambers were placed in light- and sound-attenuating cubicles equipped with a ventilation fan. Each chamber was equipped with two 4.8-cm-wide retractable levers, placed 11.7 cm apart and 6.0 cm from the grid floor. The assignment of the left and right lever as active and inactive lever (see below) was counterbalanced across rats. A cue light (28 V, 100 mA) was present above each lever and a house light (28 V, 100 mA) was located on the opposite wall. Sucrose pellets (45 mg; 5TUL, TestDiet, USA) were delivered to a receptacle between the two levers. Cocaine infusions were controlled by an infusion pump (PHM-100-3-33; Med Associates) placed on top of the cubicles. During the cocaine self-administration sessions, polyethylene tubing ran from the syringe placed in the infusion pump via a swivel to the cannula on the subjects’ back; in the operant chamber, tubing was shielded with a metal spring. Sucrose and cocaine self-administration experiments were conducted in identical chambers. The operant testing apparatus was controlled by MED-PC (version IV) Research Control & Data Acquisition System software.
Self-administration sessions were carried out once daily, between 9 AM and 6 PM, for 5–7 days a week.

Sucrose Self-Administration

Animals were trained to respond for sucrose as described previously (la Fleur et al., 2007). Operant sessions lasted 1 h during which the availability of the reward was signaled to the animal by illumination of the house light. Pressing the active lever resulted in the delivery of a sucrose pellet, the illumination of the cue light above the active lever for 5 s and retraction of the levers. After a 20-s time-out period, the levers were reintroduced and the house light illuminated, signaling the start of a new cycle. Pressing on the inactive lever was without scheduled consequences. Animals were trained to respond for sucrose under a fixed-ratio (FR) 1 schedule of reinforcement, meaning that each active lever press resulted in the delivery of one sucrose pellet (45 mg).

After acquisition of sucrose self-administration under this schedule, the response requirement was increased to five lever presses (ie, an FR5 schedule of reinforcement). Subsequently, we assessed the motivation for sucrose under a PR schedule of reinforcement, in which the response requirement was progressively increased after each obtained reward (1, 2, 4, 6, 9, 12, 15, 20, 25, etc.; Richardson and Roberts, 1996). A PR session ended after the animal failed to obtain a reward within 30 min. In this experiment, we assessed FR and PR responding both before and after D2R knockdown; PR testing commenced 2 weeks after virus injection. After the final PR session (the animals received six sessions in total), animals received two more FR5 sessions before extinction. Extinction responding was assessed under identical circumstances as FR responding, except that responding on either lever was without scheduled consequences. Animals received 10 30-min extinction sessions followed by a reinstatement session that was identical to an extinction session, except that three sucrose pellets were delivered at the beginning of the session. All operant behavior for sucrose was assessed in the same cohort of animals (n = 23). During the experimental period, animals received ad libitum chow in the home cage.

Cocaine Self-Administration

A separate cohort of 37 rats was used for the cocaine self-administration experiments. Cocaine self-administration experiments, conducted as previously described (Veeningen et al., 2012a; 2012b), started 2 weeks after surgery. Cocaine HCl (Bupa BV, Uitgeest, The Netherlands) was dissolved in saline. The first 2 weeks consisted of acquisition of self-administration under an FR1 schedule of reinforcement, followed by 1 week of testing under a PR schedule of reinforcement (which occurred 4 weeks after virus injection).

To assess the sensitivity of the animals to acquire cocaine self-administration, we used a unit dose (0.083 mg/infusion) that was threefold lower than our usual training dose during the first five self-administration sessions, followed by five sessions in which our usual unit dose of cocaine was available, ie, 0.25 mg/infusion (Baarndse et al., 2014). Self-administration training started under a FR1 schedule of reinforcement, in which responding on the lever resulted in delivery of a cocaine infusion (16.7 μl/s, during 6 s), retraction of the levers, and the illumination of a cue light above the active lever for 6 s. This was followed by a 20-s time-out period during which the levers remained retracted and both the cue and house light were turned off. A new cycle was then started by insertion of the lever. These FR1 sessions lasted for 60 min. After FR1 responding had stabilized (approximately 10 sessions), a PR schedule of reinforcement was introduced, in which the response requirement increased progressively after each obtained reward (1, 2, 4, 6, 9, 12, 15, 20, 25, etc.; Richardson and Roberts, 1996), and reward delivery was followed by a 10-min time-out period to minimize the influence of cocaine-induced psychomotor effects on responding for the next infusion. Animals were tested for three PR sessions. Next, the animals were trained under a heterogeneous seeking-taking chain schedule of reinforcement (Olmstead et al., 2000; Vanderschuren and Everitt, 2004; Veeningen et al., 2012b; Limpens et al., 2014) with a random interval (RI) of 120 s on the seeking link (ST(RI-120)). These seeking-taking sessions started with the introduction of a new lever (‘seeking lever’) and the illumination of the house light. The first press on the seeking lever initiated the RI and pressing this lever was without consequences until the RI had elapsed. When the RI had elapsed, pressing the seeking lever resulted in retraction of the seeking lever and insertion of the taking lever. Next, responding on the taking lever (under the FR1 schedule of reinforcement) resulted in an infusion with cocaine, illumination of the cue light, retraction of the taking lever, and the switching-off of the house light. This was followed by a 10-min time-out period to minimize the influence of cocaine-induced psychomotor effects on responding for the next infusion. After the time-out period, a new cycle started with the reintroduction of the seeking lever and the illumination of the house light. When the rats had acquired the task under a RI of 2 s, the RI was progressively increased between sessions until animals had acquired the task under an RI of 120 s. The program automatically ended after 2 h or if animals had obtained 10 rewards, whichever occurred first.

The conditioned suppression procedure consisted of a conditioning phase in which a subgroup of rats (n = 26, 12 control and 14 D2R knockdown) learned to associate a 85 dB, 2900 Hz tone (CS) with footshock (0.35 mA, 1 s, 20 presentations) in a different environment, as described before (Vanderschuren and Everitt, 2004; Limpens et al., 2014). A control group of animals (n = 7, 4 control and 3 D2R knockdown) was subjected to the same procedure but without the delivery of footshocks. Rats were then presented with the seeking lever in the operant-conditioning chamber. We assessed the amount of seeking lever responses made during seven 2-min blocks. Two-minute intervals in which the tone CS was presented (CS-ON interval) were alternated with 2-min intervals where the tone CS was absent (CS-OFF interval). The total number of lever presses made during presentation of the CS was taken as the outcome measure.

Cocaine was not available during conditioned suppression sessions. Subsequently, the rats were exposed to daily 1-h extinction sessions in which taking lever responses were without scheduled consequences. This continued until the rats made less than 30 responses for three consecutive days. We then assessed reinstatement following an i.p. injection of cocaine (5–10 mg/kg, i.p.) or following the presentation of the cocaine associated cue-light and retraction of the levers.
contingent with a response on the taking lever. After each 1-h reinstatement session, rats received extinction sessions until they made less than 30 responses in a session.

Tissue Preparation

Animals were killed by i.p. injection of pentobarbital. For qPCR experiments, the brains were removed, quickly frozen on dry ice and stored at −80 °C. For immunohistochemistry experiments, animals were given an intracardial perfusion with cold 4% PFA in PBS. After dissection, the brains were post-fixed for 24 h in 4% PFA in PBS and then stored in 30% sucrose in PBS at 4 °C until ready for immunohistochemistry.

Quantitative Polymerase Chain Reaction

Fresh frozen brains were thawed in PBS and the VTA was quickly dissected and dissolved in Trizol (Invitrogen). RNA was obtained using phenol-chloroform extraction followed by ethanol precipitation. All samples were diluted to a concentration of 100 ng/μl, 1 μl of which was used per qPCR measurement. qPCR was performed using QantiTect SYBR Green RT-PCR kit (QIAGEN) according to the manufacturer’s instruction. The following primers were used: Drd2-forward: CTTGCGTGTACCTGCTTG; Drd2-reverse: CACACGTTCCAGGATGCTT; beta-actin-forward: CGTTGCAACATCCGTAAGACC; beta-actin-reverse: TAGAGCCACCAATCCACACA.

Immunohistochemistry

Perfused brains were cut at 40 μm and stored in 30% sucrose and 0.02% NaAz in PBS until ready for processing. Sections were then washed in PBS and incubated in blocking buffer (1% normal goat serum, 0.2% Triton X) and incubated overnight at 4 °C with 1:500 chicken anti-GFP (Abcam ab13970) and rabbit anti-TH (1:500, Millipore, USA). GFP was visualized with Alexa-488-labeled goat anti-chicken and TH with Alexa-594-labeled goat anti-rabbit (both Molecular Probes, USA, 1:500). After washing in PBS, sections were mounted and embedded in FluorSave (Merck Millipore).

Statistics

Data were analyzed using Prism 6 (Graphpad Software). Where appropriate (ie, cocaine locomotion, sucrose self-administration, and cocaine-induced reinstatement), the D2R knockdown group was compared with the control group using a two-way repeated measures ANOVA with cocaine dose or before/after surgery as within-subjects factor. Significant main effects were then followed up with Sidak correction. Where appropriate (ie, cocaine locomotion, sucrose self-administration, and cocaine-induced reinstatement), the D2R knockdown group was compared with the control group using a two-way repeated measures ANOVA with cocaine dose or before/after surgery as within-subjects factor. Significant main effects were then followed up with Sidak correction.

RESULTS

Knockdown of the D2R

To validate knockdown, a fusion construct of renilla-D2R cDNA was transfected into human embryonic kidney cells. These cells where then transfected with one of four shRNA sequences targeted at D2R mRNA or a control sequence. The data were corrected for transfection efficiency based on a co-transfection with luciferase. We observed efficient knockdown in all cases (Figure 1a). On the basis of this and a pilot experiment that assessed cocaine-induced locomotor activity (data not shown), we selected sequence 2 for subsequent experiments. Knockdown of D2R mRNA was assessed by qPCR on VTA dissections from animals after completion of the sucrose or cocaine self-administration experiment (n = 25) (Figure 1b–d). D2R and TH mRNA quantification was normalized to beta-actin mRNA. We observed no difference in TH expression (ΔCT mean difference: −0.145 ± 0.269, t = 0.541, df = 23, P = 0.59) and an increase in ΔCT value for D2R in the D2R knockdown group (ΔCT mean difference: 1.00 ± 0.272, t = 3.680, df = 23, P = 0.001). This corresponds to ±50% knockdown. We observed comparable knockdown in a cohort (n = 3) that was killed 4 weeks after surgery (Supplementary Figure 1). Efficient virus expression was validated using immunohistochemistry against TH and GFP (Figure 1e). GFP expression was confined to the VTA in all cases (n = 48).

Cocaine-Induced Locomotor Activity

We validated the knockdown behaviorally by determining cocaine-induced locomotor activity (Vanderschuren et al, 2006; Bello et al, 2011). We found that animals with D2R knockdown were hypersensitive to a low dose of cocaine (F(1, 60) knockdown = 11.80, P = 0.001) (Figure 2a). Animals with D2R knockdown showed an increase in locomotor activity after injection with both 5 mg/kg (t = 2.508, df = 60, P = 0.044) and 10 mg/kg cocaine (t = 2.509, df = 60, P = 0.044). We observed a trend towards increase in locomotor activity after D2R knockdown during the 15-min habituation period (F(1, 60) knockdown = 3.697, P = 0.059) but there was no effect of D2R knockdown on locomotor activity following a saline injection (t = 0.897, df = 60, P = 0.754). The increase in locomotor activity started within 5 min after i.p. injection and lasted for at least 30 min (Figure 2b–d).

Sucrose Self-Administration

To investigate the effect of VTA D2R knockdown on sucrose self-administration, rats (n = 23) were tested on FR and PR for sucrose before and after virus injection. We found an increase in FR responding in both groups after surgery (F(1, 21) surgery × knockdown = 11.80, P = 0.001), which may reflect increased sucrose appetite in older and therefore heavier animals. We did not observe a difference in FR responding between the groups (F(1, 19) knockdown = 0.922, P = 0.349). Data from two animals were excluded because of a malfunctioning operant chamber (Figure 3a). However, D2R knockdown animals made approximately twice as many responses under a PR schedule of reinforcement (F(1, 21) surgery × knockdown = 8.929, P = 0.007) (Figure 3b). VTA D2R knockdown did not affect inactive lever presses (F(1, 21) surgery × knockdown = 1.388, P = 0.253) (Figure 3c). Next, we assessed responding under extinction and sucrose-primed reinstatement. We did not observe a difference between the two groups, neither under extinction (F(1, 21) knockdown = 0.076, P = 0.785) (Figure 3d).
nor during sucrose-primed reinstatement ($F_{(1, 21)}^{\text{reinstatement}} = 5.304, P = 0.032$; $F_{(1,21)}^{\text{knockdown}} = 0.167, P = 0.687$) (Figure 3e).

Cocaine Self-Administration
We first tested the acquisition of cocaine self-administration during five sessions using a low unit dose of cocaine (ie, 0.083 mg per infusion; Baarendse et al, 2014) (Figure 4a). During acquisition, we did not observe a difference between the two groups ($F_{(1,19)}^{\text{knockdown}} = 0.0137, P = 0.908$), nor did we observe a difference in responding for cocaine during the five subsequent FR1 sessions using our usual unit dose (ie, 0.25 mg per infusion; Veeneman et al, 2012a; 2012b; Baarendse et al, 2014) ($t = 0.068, df = 35, P = 0.95$)(Figure 4b). There was also no difference between the groups in the loading dose of cocaine (ie, the initial amount of cocaine the animals take to bring their blood cocaine concentration up to a certain level at the beginning of the self-administration session) (Supplementary Figure 2). However, the D2R knockdown group made about twice as many active lever presses under a PR schedule of reinforcement ($t = 2.608, df = 28, P = 0.014$)(Figure 4c).

Conditioned Suppression of Cocaine Seeking
In order to assess compulsive cocaine seeking, we employed a conditioned suppression model as previously used (Vanderschuren and Everitt, 2004). Figure 4d describes the total number of lever presses during presentation of the CS. We observed profound suppression of cocaine seeking during presentation of the footshock-associated CS, but no differences in responding between the groups ($F_{(1,29)}^{\text{conditioning}} = 31.03, P = 0.001$; $F_{(1,29)}^{\text{knockdown}} = 2.691, P = 0.29$; $F_{(1,29)}^{\text{conditioning} \times \text{knockdown}} = 1.631, P = 0.4112$) (Figure 4d).

Reinstatement of Cocaine Seeking
Significant cocaine-induced reinstatement was observed following injection of either 5 mg/kg or 10 mg/kg cocaine, but VTA D2R knockdown did not change reinstatement of cocaine seeking ($F_{(2,50)}^{\text{cage dose}} = 9.90, P = 0.0002$; $F_{(1,25)}^{\text{knockdown}} = 0.11, P = 0.738$) (Figure 4e). Response-contingent presentation of the cocaine-associated cue-light in combination with retraction of the levers lead to reinstatement of cocaine-seeking, but there was no effect of VTA D2R knockdown ($F_{(1,25)}^{\text{cue}} = 55.20, P < 0.0001$; $F_{(1,25)}^{\text{knockdown}} = 0.001, P = 0.980$) (Figure 4f).

DISCUSSION
Previous experiments have suggested that decreased D2R availability in the midbrain contributes to addiction-like behavior (Marinelli and White, 2000; Zald et al, 2008; Buckholtz et al, 2010; Bello et al, 2011; Madhavan et al, 2013). Moreover, obese mice have been shown to have desensitized D2 autoreceptors (Koyama et al, 2014). To investigate whether decreased expression of the D2R in the VTA promotes addictive behavior directed at food or drugs, we used shRNA-mediated knockdown of the D2R. VTA D2R knockdown increased the psychomotor response to a low dose of cocaine and increased the motivation to respond for

**Figure 2** D2R knockdown animals show an enhanced locomotor response to a low dose of cocaine. (a) Total distance moved in the 30 min following an injection with saline or cocaine. The same data are displayed in blocks of 5 min for saline (b), 5 mg/kg cocaine (c) or 10 mg/kg cocaine (d). Each session started with a 15-min habituation period, the end of which is indicated by the vertical dotted line. D2R-KD = D2R knockdown. Data represent means±SEM *p < 0.05.
sucrose or cocaine. However, there were no differences in sucrose or cocaine self-administration under a FR schedule of reinforcement, conditioned suppression of cocaine seeking, or reinstatement of sucrose or cocaine seeking. These data show that VTA D2R downregulation renders animals more motivated to work for a reward, but that other aspects of addictive behavior are not affected. A caveat of this study is that D2R knockdown was assessed by quantifying D2R mRNA expression 4 weeks after virus injection (Supplementary Figure 1) and at the end of the study (Figure 1). Clearly, mRNA expression is only an indirect measure of D2R function. Furthermore, two of the behavioral experiments (ie, responding for sucrose under a PR schedule of reinforcement, acquisition of cocaine self-administration) were performed 2 weeks after virus injection. Although behavioral effects of gene expression knockdown in the brain have been demonstrated within days after virus injection (eg, Tung et al, 2010), we cannot completely exclude that the increase in responding for sucrose under a PR schedule was caused by an off-target effect of the D2R knockdown vector, or that the lack of an effect on the acquisition of cocaine self-administration was the result of insufficient D2R knockdown. The results should be interpreted with this limitation in mind.

Downregulation of VTA D2R increased the psychomotor response to a low dose of cocaine. This is in accordance with previous work, in which the D2R was ablated in all DA neurons (Bello et al, 2011). Cocaine increases the synaptic DA concentration by blocking the DA transporter (Ritz and Kuhar, 1989) and in turn, DA inhibits firing of DA neurons by binding to the somatodendritic D2R (Zhou et al, 2006). Decreased D2R expression on DA neurons is therefore likely to impair this feedback mechanism, providing a plausible explanation for our results. Indeed, it has previously been shown that decreased D2R autoreceptor activity or Drd2 knockout in DA neurons results in increased DA release in the striatum as assessed with fast scan cyclic voltammetry (Bello et al, 2011; Calipari et al, 2014b). Importantly, psychostimulant drugs evoke stereotyped behavior at higher doses than those that induce psychomotor hyperactivity (Sahakian et al, 1975; Kuczenski et al, 1991; Flagel and Robinson, 2007), but only at very high doses of cocaine (typically at 30 mg/kg and higher) does stereotyped behavior interfere with the expression of psychomotor activity. Interestingly, we observed in pilot experiments that after treatment with cocaine doses > 15 mg/kg, horizontal locomotor activity was reduced in D2R knockdown animals compared with controls, which may be the result of an increase in stereotyped behavior. We chose not to include these data in the manuscript, however, because our test setup measures horizontal locomotion, so any referral to stereotyped behavior would be speculative. Interestingly, we did not find a change in locomotor activity after a saline injection, or in inactive lever presses in the self-administration experiments. This result diverges from the findings with Drd2 autoreceptor knockout mice, which did show
increased basal locomotor activity (Bello et al., 2011). Apart from the species difference, the different findings may be explained by the location of the knockdown, which, in our case, was confined to the VTA. Thus, the effects of Bello et al. may be the result of the absence of the D2R in the substantia nigra, which is known to play a role in novelty-induced exploration (Schiemann et al., 2012). Alternatively, in our study, knockdown resulted in a 50% reduction in D2R expression, which is likely to evoke more modest behavioral effects than a complete knockout of D2R. We would also like to emphasize that in our study, the D2R knockdown was induced in adult animals, whereas in the study of Bello et al. (2011), the Drd2 gene was constitutively absent throughout the development of the animals, which may also have induced compensatory functional changes. A limitation of the current approach is that the vector used does not selectively transfect dopamine neurons, but other neuron types as well. Although they form a minority, there are GABAergic neurons in the VTA that are responsive to a D2R agonist (Margolis et al., 2006), and project to, eg, the PFC, amygdala, and NAcc (Swanson, 1982; Margolis et al., 2008).

Likewise, there are glutamatergic neurons that are sensitive to a D2R agonist in the medial VTA (Hnasko et al., 2012). Although a contribution of VTA GABAergic or glutamatergic neurons to the behavioral changes observed could therefore not be excluded, the available literature strongly suggests a role for VTA DA, as described below. We observed that downregulation of the D2R in the VTA results in increased motivation to respond for both sucrose and cocaine without changing responding under a FR schedule of reinforcement. This is consistent with the well-established notion that mesolimbic DA mediates incentive motivation and willingness to work for rewards, especially when the effort requirement is high (Salamone and Correa, 2012). The striking difference in how FR responding (which may be more related to reward intake than to incentive motivation) and PR responding (widely accepted as an index of incentive motivation) are affected by VTA D2R knockdown fits well into a large literature that shows markedly different neural substrates of reward consumption vs processes related to motivation and willingness to perform effort (Berridge et al., 2009), whereby DA has been primarily implicated in the

Figure 4 Cocaine self-administration under FR and PR schedules of reinforcement, conditioned suppression, extinction, and reinstatement. (a) Rats acquired cocaine self-administration using a unit dose of 0.083 mg per infusion for five sessions, followed by five sessions in which 0.25 mg per infusion was available. Acquisition of cocaine self-administration was not altered by VTA D2R knockdown. (b) Cocaine intake as assessed under a FR1 schedule (2 h session) did not differ between the groups. The data represent the average cocaine intake during session 6–10 in Figure 4a. (c) When tested under a PR schedule (average of three sessions), D2R knockdown animals made significantly more active lever presses. (d) Cocaine seeking was suppressed by the presentation of a footshock-associated CS. There was no difference in the magnitude of suppression between control and D2R knockdown animals. Data represent the total number of lever presses during CS presentation. (e) Cocaine-primed reinstatement of cocaine seeking. Both 5 and 10 mg/kg cocaine induced significant reinstatement of responding in both experimental groups. (f) Cue-induced reinstatement. Presentation of the cocaine-paired cues induced reinstatement in both groups. All reinstatement sessions lasted 1 h. D2R-KD=D2R knockdown. Data represent group means±SEM *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
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