Silencing Dopamine D3-Receptor in the Nucleus Accumbens Shell in vivo induces Changes in Cocaine-induced Hyperlocomotion

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ABSTRACT
The dopamine D₃ receptor (D₃R) is an important pharmacotherapeutic target for its potential role in psychiatric disorders and drug dependence. To further explore its function in rats, a regulatable lentivirus, Lenti-D3, holding the rat D₃R cDNA has been constructed as well as three lentiviruses, Lenti-D3-Sils, expressing small hairpin RNAs, aimed at silencing D₃R expression and specifically targeted against different regions of the D₃R mRNA. In vitro Lenti-D3 expressed D₃R and could efficiently be blocked with Lenti-D3-Sils. These viruses were stereotaxically injected into the shell part of the nucleus accumbens (NAcc) and effects of passive cocaine delivery on locomotor activity were assessed. Manipulations of D₃R levels induced changes in the locomotor stimulant effects of cocaine as compared to control treatment. Suppression of DA D₃R in the NAcc by means of local knockdown (with Lenti-D3-Sils) increased locomotor stimulant effects, whereas its overexpression with Lenti-D3 drastically reduced them. The latter effects could be reversed when animals were fed doxycycline, that prevented lentiviral-mediated DA D₃R overexpression in the NAcc. Gene expression assessed by quantitative RT-PCR confirmed very efficient gene knock-down in vivo in animals treated with Lenti-D3-Sils (>93% silencing of D₃R gene). Thus D₃R expression significantly contributes to behavioral changes associated with chronic cocaine delivery.

Key Words: Dopamine D3-Receptor, Cocaine, Addiction, Plasticity, Drugs of Abuse, siRNA, Lentivirus, in vivo Gene Transfer

List of abbreviations: NAcc: Nucleus Accumbens; DA D2R: dopamine D2 receptor; DA D3R: dopamine D3 receptor; uPA: Urokinase-type plasminogen activator; HEK293T: Human Embryonic Kidney 293T cells; GFP: green fluorescent protein.
INTRODUCTION
Dopamine is a major neurotransmitter in the brain, where it plays an important role in controlling motor function and behavioral homeostasis. It exerts its effects through receptors, which, based on their structural and functional properties, are divided into ‘D₁-like’ (DA D₁R and DA D₅R) and ‘D₂-like’ (DA D₂R, DA D₃R and DA D₄R) subgroups (Strange 1993). Among them, the DA D₃R has a unique expression profile in the brain: it displays preferential localization in the mesocorticolimbic system, in regions that are thought to influence motivation and motor functions. (Levant 1998; Shafer & Levant 1998, Suzuki et al. 1998, Xu et al. 1997), and highest distributions in limbic parts of the striatum complex, including the islands of Calleja, olfactory tubercles, anterior and shell parts of the nucleus accumbens (NAcc) and the bed nucleus of stria terminalis (Bouthenet et al. 1991; Diaz et al. 1995; Landwehrmeyer et al. 1993a; Sokoloff et al. 1990). It plays a key role in several neuropsychiatric disorders including schizophrenia and addiction (Schmauss, 1996, Schmauss et al., 1993, Staley and Mash, 1996,) and rewarding properties of cocaine could be mediated specifically (at least) through interaction at the DA D₃R (Ritz et al, 1987). D₃R-selective agonists inhibit cocaine-seeking behaviour that depends upon the presentation of drug-associated cues, without having any intrinsic, primary rewarding effects (Pilla et al., 1999). Being less widely distributed than DA D₁R and DA D₂R, its restricted distribution makes it a promising target for the development of clinical compounds, reducing the risk of undesirable side effects. Distribution of DA D₃R mRNA partially overlaps with, but is different from that of DA D₂R. Moreover, the DA D₁R and DA D₃R are co-expressed in neurons, particularly in the NAcc and in the caudate putamen (Surmeier et al., 1996, Le Moine and Bloch, 1996) and can exert both opposite and analogous effects in the brain: for example, DA D₁R and DA D₃R mutant mice exhibit attenuated or enhanced locomotor activity after acute cocaine treatment, respectively, compared with wild type mice, an effect not associated with anxiety state; moreover, D₃R mutant mice exhibit enhanced behavioral sensitivity to combined injections of D₃R and D₂R agonists (Xu et al., 1997), but they exhibit cooperative effects on substance P expression in the NAcc and opposite regulatory effects on c-Fos expression in the islands of Calleja (Schwartz et al., 1998; Ridray et al., 1998). Thus D₁R and D₃R may affect neurons in either synergy or opposition according to the cell or signal generated and it has been postulated that imbalances between the levels of D₁ and D₃ receptors in the same neurons could be responsible for schizophrenic disorders (Schwartz et al., 1998).

The aim of the present study was to investigate the role of DA D₃R in cocaine-induced hyperlocomotion. For this purpose, a regulatable lentivirus holding the rat DA D₃R cDNA under the control of a tetracycline-inducible promoter was constructed together with several lentiviruses expressing DA D₃R-specific siRNAs, targeted against different regions of the DA D₃R mRNA. These viruses were tested in vitro, by means of quantitative real-time PCR and immunocytochemistry using HEK293T cells, and in vivo in rats, by stereotaxic injection of the lentiviruses into the shell part of the NAcc. Our studies show that DA D₃R expression in the NAcc significantly contributes to behavioral changes associated with chronic cocaine delivery. Overexpression of DA D₃R in this brain area induces a 50% decrease in cocaine-induced locomotor activity that can be reversed to normal in the same
animal fed doxycycline. Furthermore, local knockdown of DA D₃R in the NAcc, efficiently achieved in vivo with lentiviruses expressing DA D₃R-specific siRNAs, results in significant cocaine-induced behavioral changes.
Experimental Procedure

Animal Handling and Drug delivery Protocols:

Animals used in this experiment were male Wistar rats weighing 225-250 g (BRL, Füllinsdorf, Switzerland). All animal experiments were carried out in accordance with the guidelines and regulations for Animal Experimentation, BAG, Bern, Switzerland. The animals were housed in trios in clear plastic cages with wire grid lids. Access to food and water was unrestricted. The animals were kept in the animal facility maintained on a 12-h light: 12-h dark cycle (lights off at 07:00 am). Three different protocols for Drug administration were used: (i) Acute Paradigm: animals (n=6) were i.p. injected once with 15 mg/kg cocaine-HCl. (ii) Chronic Paradigm: animals (n=6) were i.p. injected daily with 15 mg/kg cocaine-HCl for a period of 15 days. (iii) Binge Paradigm: animals (n=6) were i.p. injected with 30 mg/kg cocaine-HCl every 2 hours for 4 injections.

In all three protocols control animals received 0.9% saline i.p. injection instead of drug under the same schedule. 24 hours after the last injection, animals were sacrificed by decapitation. The NAcc (core and shell) were dissected out and used for isolation of total RNA, using TRIzol Reagent (Invitrogen, Switzerland) followed by RNA amplification, according to previous publications (Bahi et al., 2004b, Bahi and Dreyer 2004, Kacharmina et al., 1999).

Microarray studies:

Microarray design and analysis was done according to published methods (Shalon et al., 1996). Briefly, specific oligonucleotides were synthesized with 5'-amino modification and arrayed onto QMT Aldehyde Slides (Quantifoil, Germany) and stored at room temperature for further hybridizations.

Probe design was according to previous publications (Bahi and Dreyer, 2004; Bahi et al., 2004b) and recommendations by QIAGEN™. 70mer probes were designed for 10 negative and 10 positive control genes, DA D3R and DA D2R. Oligonucleotides sequences of positive and negative controls are patented (QIAGEN™, Basel, Switzerland). The following oligonucleotides were used: DA D3R: 5'-CAA AGC CTT CCT CAA GAT CCT GTC CTG CTG AAG GAG GAG AAG AGA CCG CAC TCC TTT ACC ACT TCG AGA T-3'; DA D2R: 5'-CCA GGA TTG CCA AGT TCT TTG AGA TCC AGA CCA TGC CCA ATG GCA AAA CCC GGA CCT CCC TTA AGA CGA T-3'.

For probe preparation, each amplified RNA sample (one from control sample and one from treated sample) was reverse-transcribed in the presence of Cy3-dCTP (control samples) or Cy5-dCTP (treated samples) and purified, according to previous publications (Bahi and Dreyer 2004; Bahi et al., 2004a, b). After hybridization, slides were scanned; ratios of experimental (Cy5) versus control (Cy3) signals were normalized after background subtraction.

Data sets were further statistically analyzed and p-values evaluated using a stringent cut-off for significance. Induction or repression of a gene was defined as a minimum 1.5-fold change in its transcript level. Genes were considered to be down-regulated if the ratio of experimental vs. control was between 0.12 and 0.5 (or between -0.12 and -0.5) with P<0.01 or up-regulated if the ratio of experimental vs. control was 2 or more with P<0.01 (Bahi et al., 2004b, Bahi and Dreyer 2004).
Construction of Lenti-D3:
The DA D₃R cDNA (GenBank accession no. X53944) was amplified by reverse transcription. Briefly, 2 μg of total RNA (prepared from rat NAcc of cocaine-treated animals) were added to 1 μg Oligo-(dT)₁₂₋₁₈, 2 μl of dNTP Mix at 10 mM each and made up to 12 μl with RNase free-water. These components were mixed and heated at 65°C for 5 min, then kept on ice. To the mixture 4 μl of 5X first strand buffer was added, followed by 2 μl 0.1M DTT, 10 U RNAsin (Invitrogen, Switzerland), 1 μl 200 U/μl Superscript II RNaseH- Reverse Transcriptase, (Invitrogen, Switzerland). The mixture was incubated at 42°C for 3 hours. To remove RNA-DNA hybrids, 2 U RNase H (Invitrogen, Switzerland) were added and incubated at 37°C for 30 min. The cDNA was then PCR amplified and flagged with the two following primers: 5' GGA AGA TCT ATG GAC TAC AAG GAC GAC GAT GAC AAG GCA CCT CTG AGC CAG-3' and 5' CCG CTC GAG CGG TCA GCA GGA CAG GAT CTT-3' (restriction sites are underlined). The forward primer contains a Bgl-II restriction site followed by the flag epitope sequence (in bold) and the 5' Rat DA D₃R cDNA specific sequence, the reverse primer contains the 3' Rat D₃R cDNA specific sequence, a stop codon and an Xho-I restriction site. The PCR product was digested with Bgl-II and Xho-I and cloned into Bgl-II/Xho-I sites in pTK431. The GFP expression vector pTK433 (Bahi et al. 2004a, b) was used as a control vector.

Construction of Lenti-D₃-siRNAs:
To silence DA D₃R expression in vitro and in vivo, 3 targets were designed according to the DA D₃R mRNA sequence. The following targets within the DA D₃R sequence were selected, based on Hannon’s design criterion (http://katahdin.cshl.org:9331/RNAi/html/rnai.html): 1st target: bp 165–184, 2nd target: bp 201–220, 3rd target: bp 277–296. To each oligo, an Xho-I restriction site was added at 3' and a U6-3'-specific 10mer at 5'. Using the pSilencer 1.0-U6 (Ambion, UK) as a template and a U6 promoter-specific forward primer containing BamH-I restriction site (GCG GAT CCC GCT CTA GAA CTA GTG C), each siRNA target was added to the mouse U6 promoter by PCR. The PCR conditions were highly drastic to avoid mutations within the targets. The following PCR program was performed: 120 s at 94 °C (initial denaturation) followed by 94°C – 45 s, 64 °C – 45 s and 72 °C – 45 s repeated 35 cycles. The PCR reaction contains 4% dimethyl sulfoxide (Sigma, Switzerland). The PCR product was digested with BamH-I and Xho-I, and cloned into similar sites into pTK431, and sequenced to verify the integrity of each construct.

Lentivirus Production:
The vector plasmids (either pTK431-D3, pTK433-GFP or pTK431-U6-D₃-siRNAs), together with the packaging construct plasmid pΔNRF and the envelope plasmid pMDG-VSV-G were co-transfected into HEK293T cells to produce the viral particles (Bahi et al. 2004a, b). The viral titers were determined by p24 antigen measurements (KPL, USA). For the in vivo experiments, the different viral stocks were matched for viral particle content and used at 0.2 mg/ml of p24.

In vitro DA D₃R Transcripts Quantification Assays:
The efficiency of the Lenti-D₃ was tested in vitro by infection of HEK293T cells. 1 x 10⁵ HEK293T cells were plated per well in 6-well plates. The next day, lentiviruses were mixed with 10 μg/ml Polybrene (Sigma, Switzerland) with/without 30 ng/ml doxycycline (Sigma, Switzerland), incubated for 30 min at room temperature,
added to the cells and incubated at 37°C. After 48 h the medium was replaced with normal growth medium (with or without 30 ng/ml doxycycline) and cells were left for further 48h. Cells were then collected and used for total RNA isolation (for real-time PCR). Infections with the different lentiviruses were done as follows: a) **doxycycline regulation**: cells were co-infected using 0, 2, 4 or 8 μl of Lenti-D3 stock together with either 4 μl of Lenti-CD81-His6 (Bahi et al., 2004a) or 4 μl of Lenti-uPA-His6 (Bahi et al., 2004b) and one part of the cells was incubated in the presence of 30 ng/ml doxycycline and the other part without doxycycline; b) **in vitro silencing of DA D3R**: cells were infected with 4 μl of Lenti-D3 stock, either alone or together with: 4 μl of Lenti-GFP, 4 μl of Lenti-CD81-His6 (Bahi et al., 2004a), 4 μl of Lenti-uPA-His6 (Bahi et al., 2004b), as well as 2 μl of Lenti-D3-siRNA1, 2 μl of Lenti-D3-siRNA2, 2 μl of Lenti-D3-siRNA3, or with 2 or 4 μl of all three targets together (mix of Lenti-D3-siRNA1, Lenti-D3-siRNA2 and Lenti-D3-siRNA3).

**Stereotaxic surgery**

Stereotaxic surgery and injection of the lentiviral vectors were performed according to previous publications (Bahi et al., 2004a, b). Animals (n=9) were bilaterally injected into the shell part of the NAcc with concentrated lentiviral stock (0.2 mg/ml of p24, corresponding to 8x10⁹ IU/ml), either 2 μl of Lenti-D3 stock alone in a first group of animals, or 2 μl Lenti-D3 together with 2 μl of Lenti-D3-siRNAs mix in a second group of animals, or 2 μl Lenti-GFP in a third group of animals (control); finally, a last group of animals was infected with 2 μl Lenti-GFP together with 2 μl of Lenti-D3-siRNAs mix, with the aim to suppressing expression of endogenous DA D3R receptor upon cocaine delivery. All injections were performed bilaterally at following coordinates, as calculated from bregma and the dura mera: anterior +1.4; lateral +/-1.2; ventral -7.8 (Paxinos and Watson, 1998). After surgery animals were left for recovery during 7 days, then cocaine-treated and behavior assessed following a chronic protocol. Animals were fed 5% sucrose in drinking water for 5 days (session “A”), then 0.02% doxycycline and 5% sucrose in water for 5 further days (session “B”), then again 5% sucrose for 5 more days (session “C”).

**In vivo DA D₃R Transcripts Quantification Assays:**

At the end of each behavioral session “A”, “B” and “C”, three animals were sacrificed by decapitation to assess the efficiency of the Lenti-D3 expression in vivo; brains (n=3) were dissected out, NAcc region was isolated and total RNA was extracted using TRIzol Reagent (Invitrogen, Switzerland). RNA was used for transcripts quantification.

**Quantitative real-time PCR and quantification of DA D₃R Transcripts:**

For quantitative real-time PCR, primer sets for rat DA D₂R and DA D₃R, GAPDH, β-actin, rat uPA and rat CD81 were designed to amplify 100- to 200-bp products, using PRIMER3 software: (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Following specific primer pairs were used; **DA D₃R**: 5'-CAT TGT CTGGGT CCT GTT CCT-3' and 5'- GAC CAG CAG AGT GACGAT GA-3'. **DA D₂R**: 5'- GGG GTG ACT GTC CTT CCT TG-3' and 5'- TGG CCC TTA TTG AAA ACT GC-3'. **GAPDH**: 5'- ATG ACT CTA CCC ACG GCA AG-3' and 5'- CAT ACT CAG CAC CAG CAT CAC-3'. **β-actin**: 5'- AGC CAT GTA CTT CAG CAT CC-3' and 5'- CTC TCA GCT GTG GTG AA-3'. **uPA**: 5'- CAG ATC CGA TGC TCT TAG CC-3' and 5'- TAG AGC
CTT CTG GCC ACA CT-3'. **CD81**: 5'- TGA TCCTGT TTG CCT GTG AG-3' and 5'-
CAG TTG AGC GTC TCA TGG AA-3'. GAPDH was used as endogenous control for
normalization. Total RNA was extracted from the HEK293T cells (for **in vitro**
quantification) and from the Nacc of brains of cocaine treated animals (for **in vivo**
quantification) using TRIzol Reagent (Invitrogen, Switzerland) including an RNase-
free DNase step. RNA was quantified by spectrophotometry, and its integrity
verified by agarose gel electrophoresis as visualized with ethidium bromide staining.
First strand cDNA was generated from 2 µg of total RNA and Oligo(dT<sub>12-18</sub>) primer
with the M-MLV reverse transcription kit (Invitrogen, Switzerland) in a total volume
of 20 µl. The reaction product was used for quantitative real-time PCR using the real-
time PCR iCycler (BioRad, Switzerland). 5 µl cDNA preparation, 0.5 µM of forward
and reverse primers and 10 µl of IQ SYBR Green Supermix (Biorad, Switzerland) in
a total volume of 20 µl were applied and PCR was performed as follows: 3 min at 95°C
(initial denaturation); 20°C/sec temperature transition rate up to 95°C for 45 sec, 45
sec 62°C, repeated for 40 times (amplification). The PCR reaction was evaluated by
melting curve analysis and by checking the PCR products on 2% agarose gel.
The PCR cycle number at which each assay target reached the threshold detection
line was determined ("threshold cycles", Ct value). The Ct of each gene was
normalized against that of GAPDH or β-actin. To determine the linearity and
detection limit of the assay, cDNA samples were amplified for successive 10-fold
dilutions in a series of real-time PCRs, using duplicate assay on each dilution, so that
the correlation coefficient could be calculated from the standard curve of Ct values.
Comparisons were made between cocaine and saline groups and significance was
calculated using two-tailed Student’s *t*-test and the level of statistical significance was
set at *P*<0.05. Data were expressed as mean ± SEM. The ΔCt for each candidate was
calculated as ΔCt = [Ct (candidate) – Ct (GAPDH or β-actin)]. The relative abundance
of each target in each protocol can be calculated as the ratio between treated and
**Locomotor Activity measurement:** One week after surgery, locomotor activity was
monitored in MED-OFA-RS cages (MED Associates Inc. USA) during the dark cycle
in daily sessions over 12 days, according to previously published procedures (Bahi *et al*.,
2004a, b). The animal was injected with 0.9 % saline solution, placed into the
activity-monitoring cage for a 30 min baseline. After a 30 min period, the session
automatically paused and during this interval, each subject received cocaine-HCl (15
mg/kg, i.p.) and was then placed back into the locomotor activity-monitoring cage
for further 60 min. Statistical evaluation of behavioral analysis was performed
according to previous publications (see Bahi *et al*., 2004a, b): briefly, the activity
monitor computed the location of the animal in each of the X and Y dimensions as
the middle point between the extreme beam interruptions; speed was estimated by
computing standard deviation of the distances of data points to their mean within a
sliding time window 0.4 s wide. Within the same experimental group, means and
standard deviations were done on measurements performed each day during one
session. The *T*-test was done by comparison of the 6 animals (3 from each group) in
the same time point and the *p*-value was calculated to check for the significance of
the difference between the groups with and without doxycycline.
Results

Dopamine D₃R expression is induced by cocaine

The expression of the dopamine DA D₃R upon cocaine treatment was evaluated by means of microarray technology and quantitative real-time PCR. Microarrays were designed, and total RNA from NAcc of animals (n=6) treated under the three different paradigms were prepared, reverse transcribed and DA D₃R together with DA D₂R expression was assessed after normalization against GAPDH considered as endogenous control. Data were normalized to the values obtained from saline-treated animals with saline levels set to 1. The results are shown in figure 1. DA D₃R mRNA was induced significantly after cocaine injection in all three paradigms. Strongest up-regulation was observed after “binge” treatment (four injections of 30 mg/kg over 6h), under which conditions a 3-fold up-regulation of DA D₃R in the NAcc has been found. DA D₃R mRNA was also induced after acute and after chronic treatments, where a ~2-fold raise in expression was found in both cases. By contrast, expression of the very closely related DA D₂R was not affected by cocaine under the same conditions, and DA D₂R mRNA ratios between saline and cocaine treated animals were ~1 under all circumstances.

These expression levels could be confirmed by real-time PCR, using DA D₂R and DA D₃R specific primers. A 3.2-fold raise of DA D₃R expression after binge treatment, resp. a ~1.8- and 2.3-fold change after acute or chronic treatment. The differences observed between the two latter treatments are not significant (P> 0.22 chronic vs acute). By contrast, no change for the DA D₂R was found in all conditions. Subsequent in vivo studies were then performed according to the chronic paradigm.

Lentivirus-mediated DA D₃R expression in HEK293T cells

A tetracycline-regulatable lentivirus expressing DA D₃R (Lenti-D3) was generated (see Methods). HEK293T cells were infected with different concentrations of Lenti-D3. As controls, cells were co-infected with 4 µl of two other tetracycline-regulatable lentiviruses, Lenti-CD81-His6 (Bahi et al., 2004a) and Lenti-uPA-His6 (Bahi et al., 2004b). Transcripts levels were measured by means of quantitative real-time PCR and normalized against GAPDH used as endogenous control, with GAPDH levels set to 1. In absence of doxycycline, (figure 2A), control cells, infected with Lenti-GFP (instead of Lenti-D3) together with Lenti-CD81-His6 and Lenti-uPA-His6, displayed a basal level of DA D₃R mRNA expression of ~1.0. In Lenti-D3 infected cells, DA D₃R mRNA expression was titer dependent, and a ~2-fold, ~3.6-fold and ~7-fold increase was observed, respectively, with 2, 4 or 8 µl viral stock. Under these conditions, Lenti-CD81-His6 and Lenti-uPA-His6 also induced the expression of CD81 and uPA (~3 and 2 fold increase, respectively), but their transcripts levels were not affected by Lenti-D3 titers. Endogenous β-actin mRNA was not affected under these conditions and a constant ~2.6 ratio was found under all conditions. Likewise, DA D₂R transcripts were not induced, indicating the specificity of DA D₃R expression by Lenti-D3.

In presence of doxycycline (30 ng/ml in the culture medium during 96 h) Lenti-D3-mediated DA D₃R mRNA expression was completely abolished at lower virus concentrations (figure 2B) and DA D₃R expression reached basal levels (ratio ~1). However, cells infected at highest virus titer (8 µl of Lenti-D3) displayed residual Lenti-D3-mediated DA D₃R expression (ratio ~3), as expression blockade by 30
ng/ml doxycycline was not complete due to a slight leakiness of the CMV promoter. Under these conditions, lentivirus-mediated expression of uPA and CD81 transcripts were also fully blocked by doxycycline, in agreement with previous data (Bahì et al., 2004a and Bahì et al., 2004b) whereas DA D₃R and β-actin levels were not affected (figure2B). It should be mentioned that mRNA levels were highly correlated with flagged DA D₃R protein levels verified by means of immunohistochemistsy (data not shown).

**siRNAs-expressing lentiviruses and DA D₃R silencing in vitro**

In order to knock down DA D₃R expression lentivirus-based RNA interference was used. Three different small interfering RNAs were designed, targeted against different regions of the DA D₃R mRNA. Constructs holding a U6 promoter were inserted into the transfer plasmid of the lentivirus system and their efficiency at silencing DA D₃R was assessed *in vitro* in Lenti-D3-infected HEK293T cells (figure 3). These lentivirus constructs are not doxycycline-regulated. Target specificity was controlled by co-infections of HEK293T cells with Lenti-CD81-His6 and Lenti-uPA-His6 or with Lenti-GFP. 96h after infection, cells were harvested, total RNA was extracted and transcripts levels were measured by means of quantitative RT-PCR using specific primers for each candidate. All normalizations were performed against GAPDH. Cells co-infected with Lenti-D3, Lenti-uPA-His6 and Lenti-CD81-His6 were set-up to 100% of DA D₃R expression level, used as control (figure 3, “A”). When Lenti-GFP was also added during co-infection, no significant change of DA D₃R mRNA levels was observed and ~98% of DA D₃R transcripts were detected (figure 3, “B”), versus 100% in controls. In contrast, co-infection with Lenti-D3-siRNAs (either Lenti-D3-siRNA 1, 2, or 3) resulted in drastic DA D₃R down-regulation, yielding residual DA D₃R expression levels of 40%, 15% and 60%, respectively. (figure 3, “C”, “D” and “E”). Co-infection in presence of all three Lenti-D3-siRNAs together resulted in >93% DA D₃R mRNA knock down (figure 3, “F” and “G”). Under all conditions, the expression levels of other genes (DA D₂R, β-actin, uPA or CD81 mRNAs) were not affected by Lenti-D3-siRNAs, indicating that these targets are highly DA D₃R-specific. The expression levels of control genes were ~35%, ~65%, ~50% and ~54%, respectively. Immunohistochemistry with anti-flag antibody against flagged DA D₃R showed that protein expression highly correlates with transcripts levels (data not shown).

**In vivo DA D₃R silencing**

In order to test the effects of Lenti-D3-siRNAs *in vivo*, the shell part of rat NAcc was stereotaxically injected in three sets of animals. After surgery, animals were left for recovery during 7 days, then were administered cocaine in three 5-days sessions, under the chronic paradigm (daily injections of 15 mg/kg i.p.). The first set of animals, fed without doxycycline, received cocaine and was sacrificed after the first 5-days session; the second set received daily cocaine injection for 10 days being fed without doxycycline for 5 days, then with doxycycline added in the drinking water for another 5 day period, and animals were sacrificed; the third set of animals underwent the same schedule, but followed by a further 5-days period where doxycycline was again omitted, before animals were sacrificed. Each set consisted of 4 groups of animals (n=9) which were operated as follows: the first group (n=9) was stereotaxically injected into the shell part of the NAcc with Lenti-D3 alone, to
evaluate the effects of DA D3R overexpression in vivo; the second group (n=9) was injected in the same brain region with Lenti-D3 together with a mix of the three Lenti-D3-siRNAs, in order to assess silencing of exogenous DA D3R in vivo; the third group (n=9) was injected with a mix of the three Lenti-D3-siRNAs together with Lenti-GFP (not Lenti-D3), to check for silencing of endogenous DA D3R; the last group (n=9) was injected with Lenti-GFP alone, a mock control, to evaluate endogenous DA D3R expression. After each 5-days session, one set (n=3) of animals per group was sacrificed, brains removed, NAcc tissues were dissected out and used for total RNA extraction. After reverse transcription, mRNA expression levels were measured by means of quantitative real time-PCR. Data are shown in figure 4.

Data from the first set of animals (treated with cocaine over 5 days in absence of doxycycline, enabling local lentivirus-mediated DA D3R overexpression in the NAcc) is displayed in figure 4A, “SESSION A”. The first group of this set, infected only with Lenti-D3, displayed strong induction of DA D3R mRNA expression with a ratio of ~15 compared to GAPDH (set to 1), whereas the ratios of expression levels for DA D2R, β-actin, uPA and CD81, were ~1.5, ~1.3, ~6.3 and ~4.5, respectively. In full agreement with these data, we previously reported that chronic cocaine delivery induces uPA (6.3-fold, Bahi et al., 2004b) and CD81 (~4.2-fold, Bahi et al., 2004a) into the NAcc. The second group of this set, injected with Lenti-D3 together with a mix of Lenti-D3-siRNAs, displays drastic inhibition of DA D3R expression, resulting in a ratio of ~1.3, i.e. at least 11.5 fold inhibition (P<0.005 vs. Lenti-D3 infected animals). Lenti-D3-siRNAs had no effects at all on expression levels of DA D2R, β-actin, uPA and CD81, when compared to the first group of animals (P>0.25 vs. Lenti-D3 infected animals). In the third group of this set of animals, infected with the Lenti-D3-siRNAs and Lenti-GFP (not Lenti-D3), expression levels of all tested candidates are unchanged, except for DA D3R whose mRNA expression level was only~0.32, corresponding to silencing of the endogenous DA D3R mRNA (P<0.01 vs. Lenti-GFP infected animals). Finally, in the last group of this set, injected with Lenti-GFP, DA D3R expression was ~4.5, reflecting cocaine-mediated induction of endogenous DA D3R . This value, compared to that observed in the third group (0.32) indicates that a 93% knock down of the endogenous DA D3R has been achieved with Lenti-D3-siRNAs (P<0.01 vs. Lenti-GFP with Lenti-D3-siRNAs infected animals). In this same group, DA D2R, β-actin, uPA and CD81 mRNAs were unchanged.

Results from the second set of animals, administered cocaine chronically and fed without doxycycline over 5 days, then switched to doxycycline over 5 days (resulting in down-regulation of lentivirus-mediated DA D3R in the NAcc) are displayed in figure 4B, “SESSION B”. The first group of this set, infected with Lenti-D3, displays cocaine-mediated DA D3R induction, with a ratio of 4.4, equivalent to the ratio observed for GFP-treated animals in the previous set (cocaine-mediated endogenous induction) and much lower than the ratio observed in absence of doxycycline (15 fold increase in “Session A” for Lenti-D3 treated animals). DA D2R, β-actin, uPA and CD81 mRNAs expression were not affected by doxycycline and ratios of ~1.6, ~1.1, ~5.5, and ~3.9 respectively, similar to those observed in the previous session, were found. In the second group of this set, co-injected with Lenti-D3 together with Lenti-D3-siRNAs mix, DA D3R expression is strongly inhibited (with ratios ~0.6, compared to 1.3 observed in the corresponding group fed without doxycycline, P<0.005 vs.
Lenti-D3 infected animals), because doxycycline blocks exogenous, lentiviral-mediated DA D3R overexpression (but not the endogenously expressed DA D3R), while the silencers also block endogenous DA D3R. Under these conditions, the other targets were not modified at all ($P>0.23$ vs. Lenti-D3 infected animals). In the third group of this set, i.e. animals infected with Lenti-D3-siRNAs mix and Lenti-GFP, DA D3R expression is even lower, with a ratio $\sim0.5$ ($P<0.01$ vs. Lenti-GFP infected animals) - since no exogenous DA D3R has been generated - and again DA D2R, $\beta$-actin, uPA and CD81 were not modified ($P>0.23$ vs. Lenti-D3 infected animals). Finally in the last group of this set, infected with Lenti-GFP without Lenti-D3-siRNA, cocaine induced $\sim4.9$-fold increase in DA D3R expression (compared to GAPDH set to 1), similar to the corresponding group in session A (fed without doxycycline), whereas the ratios of the other targets remained unchanged ($P>0.25$ vs. Lenti-GFP infected animals in session A).

Figure 4C "SESSION C" displays observations from the third set of animals, which have been switched back to doxycycline-free regimen. The data coincide with observations made on the first set, which clearly establishes that cocaine-induced expression changes mediated by Lenti-D3- are reversible in a doxycycline-dependent manner. In the first group of this set (animals treated with Lenti-D3 in the shell part of the NAcc), chronic cocaine treatment again induced a $\sim15$-fold increase of DA D3R expression, identical to levels observed in the corresponding group from the first set, while DA D2R, $\beta$-actin, uPA and CD81 remained unmodified by regimen switch. In the second group of this set (animals co-infected with Lenti-D3-siRNAs and Lenti-D3), again exogenous DA D3R expression was strongly inhibited, with ratios of $\sim1.6$, while control genes remained unaffected also. In the third group of this set (animals treated with Lenti-D3-siRNAs and Lenti-GFP), DA D3R transcripts were lowest, as expected, with ratios of only $\sim0.6$, corresponding to silencing of endogenous mRNA exclusively; in this group, again, DA D2R, $\beta$-actin, uPA and CD81 mRNAs were not modified and displayed no significant difference compared to the other groups (i.e. $\sim1.7$, $\sim1$, $\sim6$ and $3.7$-fold cocaine-mediated increase, with $P>0.22$ vs Lenti-D3 infected animals). In the last group (animals treated with Lenti-GFP alone, in absence of silencers), DA D3R expression was significantly induced compared to the third group ($\sim4.5$ versus $\sim0.6$) and its level was comparable to the corresponding group from the first set. In this group, control genes were still unmodified: $\sim1.8$, $\sim2.5$, $\sim6.6$ and $\sim4$-fold increase for DA D2R, $\beta$-actin, uPA and CD81 respectively; induction of the latter two candidates by cocaine again confirms previous observations (Bahi et al., 2004a, Bahi et al., 2004b) and is independent of lentiviral-injections.

**Behavioral Changes induced upon Dopamine D3R Expression**

Four groups of animals (n=9) were used to test the effects of DA D3R expression in the shell part of the NAcc on cocaine-induced behavioural changes. Lentivirus-mediated overexpression of DA D3R and its local knock down were therefore used (figure 5).

A first group of animals (n=9), infected with Lenti-GFP, served as a control group expressing GFP in the NAcc in a doxycycline-regulated way. After surgery, animals were fed water, enabling full expression of GFP in the target area. One week after surgery, chronic cocaine delivery was started and locomotor activity was monitored. At each daily session, animals received saline injections before the habituation period
(30 min) followed by cocaine delivery (i.p. 15 mg/kg) and the locomotor activity was monitored during 60 min immediately after drug injection. As shown in the figure 5 (Session A: GFP), after saline injection animals displayed low levels of locomotor activity (with total distance traveled over that period of ~4'458 counts), but after cocaine delivery a significant induction of locomotor activity was observed with a total distance traveled during that period of ~23'638 counts (means over five days session). After five days, the same animals (n=6) were fed with doxycycline in the drinking water, and locomotor activity upon chronic cocaine delivery was further monitored for five consecutive days. Under these conditions the distance traveled after saline injection during habituation period remained unchanged (~4'630 counts) and after cocaine injection it was not significantly different, with a total activity of ~25'774 counts (P>0.23 vs. doxycycline free water-fed animals (Session A) (figure 5 Session B: GFP). After further five days, the remaining animals (n=3) were fed without doxycycline, again enabling GFP expression; during this last session, locomotor activity remained unchanged again: after saline injection it remained at ~4'590 counts and after cocaine delivery, it was comparable to the previous sessions, with a total activity of ~18'086 counts (figure 5, Session C: GFP). These behavioural activity patterns cannot be attributed to GFP-expression, because uninfected rats showed identical results (P>0.21 vs. Lenti-GFP, data not shown).

The second group of animals (n=9) was infected with the doxycycline-regulatable Lenti-D3 into the shell part of NAcc. After surgery, animals underwent the same regimen as the previous group: initially, animals (n=9) were fed water, enabling full expression of DA D3R in the targeted area and chronic cocaine delivery was started after one week together with locomotor activity monitoring. In daily sessions animals were receiving saline injections before the habituation period, followed, 30 min later, by cocaine delivery (i.p. 15 mg/kg) and locomotor activity was monitored. After saline delivery, locomotor activity remained comparable to the GFP control group (Lenti-GFP-treated animals, P>0.22) with total distance traveled over that period of ~4'450 counts; but after cocaine injection, a significant decrease in locomotor activity was observed, compared to Lenti-GFP-treated animals, with a total activity of 16'626 counts (figure 5, Session A: D3). After five days the same animals (n=6) were fed with doxycycline in the drinking water (inducing down-regulation of lentivirus-induced expression of DA D3R in the NAcc), and their behaviour upon chronic cocaine treatment was further monitored for five consecutive days. Under these conditions the distance traveled during the habituation period was unchanged (~4'326 counts, P>0.25 vs. Lenti-D3, session A) whereas after cocaine injection, a total distance of ~28'699 counts was observed (figure 5, Session B: D3). After five days under this regimen, doxycycline was removed (enabling re-expression of lentivirus-mediated DA D3R in the NAcc). Under these conditions, no changes were observed during the habituation period (total traveled distance ~4'731 counts, P>0.26 vs. Lenti-D3, session A & B), but locomotor activity returned back to its initial levels, with a total distance traveled of ~ 18'766 counts (figure 5, Session C: D3, n=3). The difference between sessions A and C was not significant (P> 0.2).

A third group of animals was treated by co-injection of the regulatable Lenti-D3 (same concentration as the first group) together with (non-regulatable) Lenti-D3-siRNAs. Animals (n=9) were then submitted to the same regimen and drug
treatment as the other groups: seven days after surgery, chronic cocaine delivery was initiated in absence of doxycycline, i.e. under conditions where lentivirus-mediated DA D₃R expression was achieved (figure 5, Session A: D₃ & siRNAs): during habituation, locomotor activity remained unchanged (~4’120 counts, \( P > 0.22 \) vs. Lenti-D3), but after cocaine delivery locomotor activity was significantly higher than in the second group under the same conditions, with a total distance traveled over that period of 24’089 counts, corresponding to ~150% the locomotor activity monitored under the same conditions in absence of silencers Lenti-D3-siRNAs (\( P < 0.05 \) vs. Lenti-D3). After five days, doxycycline was added to the regimen of animals (n=6), inducing down-regulation of exogenous, lentivirus-mediated DA D₃R expression, but not of lentivirus-mediated siRNAs, neither of endogenous induction of D₃R (figure 5, Session B: D₃ & siRNAs): locomotor activity, that remained unchanged during the habituation period (~4’656 counts, \( P > 0.26 \) vs. Lenti-D3), further increased under these conditions, and reached a total traveled distance of 38’203 counts, even significantly higher (~30%) than in corresponding GFP-treated animals (\( P < 0.05 \)). This high raise in locomotor activity most probably corresponds to silencing of the endogenous DA D₃R that, in the absence of DA D₃R-specific siRNAs, would be strongly induced by cocaine under these conditions. When doxycycline was removed from water on the same animals (n=3), enabling full expression of the Lenti-D3 in the NAcc, the locomotor activity is unmodified during the habituation period (~4’162 counts) but drops to the basal level, comparable to the second session (a total distance of 23’567 counts after cocaine delivery). The fourth group of animals (n=9), co-infected with Lenti-GFP together with Lenti-D3-siRNAs, served to assess effects from endogenous DA D₃R. In absence of doxycycline, animals (n=9) displayed the same locomotor activity during the habituation period (~4’071 counts, \( P > 0.25 \) vs. Lenti-D3), but a high locomotor activity after cocaine delivery, with a total traveled distance of ~33’343 counts (\( P < 0.05 \) vs. Lenti-GFP) (figure 5, Session A: GFP & siRNAs). When switched to doxycycline regimen (n=6), five days later, no significant change was observed during the habituation period (~4’645 count, \( P > 0.24 \) vs. Lenti-D3) but after cocaine delivery the total distance traveled was ~38’457 counts (\( P < 0.05 \) vs. Lenti-GFP) (figure 5, Session B: GFP & siRNAs). Finally, five days later, doxycycline was removed from water to the remaining animals (n=3) and the locomotor activity after saline delivery was ~4’133 counts during the habituation period and 30’878 counts after cocaine delivery (\( P < 0.05 \) vs. Lenti-GFP) (figure 5, Session C: GFP & siRNAs). The observed difference between these three sessions within this group of animals was not significant (\( P > 0.18 \)). These observed behavioural effects were highly correlated with changes of the flagged DA D₃R, as verified at protein levels by means of immunocytochemistry (data not shown).

The use of a regulatable system, described here, enables to minimize considerably the number of animals per set (n=3), since the very same animal can be used under different conditions (gene on/gene off, very locally, at will). Animals in sessions C are the very same as in sessions A; when the gene is turned on/off, behavioral data are repeated at will on the same animal (almost identical values in locomotor activity test, very good statistics, s.d.<12% even with n=3), yielding to highly reproducible
behavioural response on the very same animals in relation to local gene expression, whether before or after doxy switch. Thus data are equivalent to a group n=12 expressing the gene (sessions A and C together) compared to a group n=6 without the gene (session B). With an unregulatable system, more and larger groups (N=8 at least, per group) would be necessary to reach similar statistical correlation.
Discussion

The present study aimed at clarifying the role of local expression of DA D3R in cocaine-induced hyperlocomotion. Activation of DA D3R inhibits locomotion (Svensson et al., 1994, Sokoloff and Schwartz, 1995), in agreement with our present study, but the mechanisms remains to be established. Low doses of the DA D3R agonist, 7-OH-DPAT, preferentially activate DA D3R (Khroyan et al., 1995) and produce hypolocomotion (Daly and Waddington, 1993, Depoortere et al., 1996, Ouagazzal and Creese, 2000), while very high doses enhance locomotor activity, presumably through some action at DA D2R (Depoortere et al., 1996). Therefore the potency of DA receptor agonists in producing hypolocomotion correlates somehow with the in vitro affinity for the DA D3R than the DA D2R subtype; in mutant animals (DA D3R-/- or DA D3R+/-), however, locomotor activity is either at, below, or above that of controls (Depoortere et al., 1996), indicating that the presence of DA D3R may not be necessary for the expression of behavioral effects induced by agonists or antagonists supposedly selective for the DA D3R subtype. Alternatively, of course, possible adaptive mechanisms taking place in DA D3R-/- mice might have compensated for the absence of DA D3R (Boulay et al., 1999). Mice lacking the DA D3R display basal firing rates of DA neurons within both the substantia nigra and ventral tegmental area not different from wild-type mice (Koeltzow et al., 1998), although in vivo microdialysis studies of DA release in ventral striatum revealed higher basal levels of extracellular DA in mutant mice; thus DA D3R may not be significantly involved in DA autoreceptor function but rather participate in postsynaptically activated short-loop feedback modulation of DA release. In mutant mice, DA D2R could compensate for the loss of DA D3R, thereby masking functional loss, but the reciprocal is not true, i.e., DA D3R is unable to compensate for loss of DA D2R (Mercuri et al., 1997), suggesting that DA D2R is capable of autoregulation independently, whereas DA D3R autoregulatory activity would be dependent on concurrent DA D2R autoreceptor stimulation. As a matter of fact, lack of functional DA D3R prevents the effects of DA D3R agonists, indicating that DA D3R is necessary for DA D3R-mediated inhibition of presynaptic DA neurotransmission (Zapata and Schippenberg, 2005), in contrast to earlier data from DA D3R ko mice or pharmacological evidence that DA D3R activation regulates extracellular DA levels in the NAcc and inhibits DA release. Stimulation of DA D2R/D3R affects reward, locomotor activity and conditioned place preference; these effects are synergistically enhanced upon AMPA/kainate receptor blockade (Biondo et al., 2005) and 5-HT1b stimulation (Parson et al., 1996). Another approach to assessing DA D3R function - suppressing DA D3R expression by means of intracerebro-ventricular administration of DA D3R antisense oligodeoxynucleotide - revealed enhanced basal levels of DA synthesis in the NAcc but failed to affect the suppression of synthesis by the nonselective DA receptor agonist apomorphine (Nissbrandt et al., 1995; Tepper et al., 1997); nevertheless this approach is not well suited for assessing long term effects of gene suppression in vivo and associated behavioral changes. Because of the limitations associated to these various methods, another approach, based on regulatable lentiviral-mediated gene transfer, has been developed here. Self-inactivating lentiviruses permanently incorporate genetic elements into the infected target cells and do not diffuse away, enabling very local switch on/off of
gene expression on the very same animal for evaluating behavioral changes associated with local gene overexpression or suppression. Using these tools, our study clearly shows that suppression of DA D3R in the NAcc yields to cocaine induced hyperlocomotion (compared to controls), whereas its overexpression in the same brain area drastically reduced cocaine’s locomotor stimulant effects. Gene transfer of a mix of three siRNAs, targeting three different regions of the DA D3R mRNA, most efficiently induces gene knock-down in vivo, stably yielding >93% gene silencing. However in the present investigation only a single dose of cocaine has been studied, and only one regimen (chronic cocaine delivery by means of repeated daily treatments); accordingly, the range of conditions under which decreased and increased effects of cocaine were observed is rather narrow, and information regarding how the potency and efficacy of cocaine as a locomotor stimulant may have been altered is limited. Furthermore, a major limitation of the approach lies in its own power: the lentivirus-mediated overexpression of DA D3R is driven by a promiscuous minimal CMV promoter that infected cells in the NAcc that do not normally express DA D3R; given the complex neuroanatomical circuitry in this brain region, observed behavioral changes related to this overexpression may not reflect an exaggeration of normal DA D3R function, but may relate to compensatory mechanisms taking place under such conditions. For the same reason, expression of exogenous D3 receptor and the silencing of endogenous and exogenous D3 receptors may not have been confined to the NAcc shell area - a rather small and deeply located brain region - and we cannot rule out that some amount of lentivirus found its way to the NAcc core or other adjacent nuclei, and there contributed to the observed behavioural effects.

Our investigation has been limited here to the effects of DA D3R expression in the NAcc shell on cocaine-induced locomotor changes. Measures of cocaine-induced locomotor activity are appropriate means to reveal behavioural effects of a drug and a reasonable indication that rewarding effects may be altered similarly; however additional studies will be still necessary to establish this, using appropriate behavioural measures of reinforcement and reward, such as self-administration, ICSS or conditioned place preference. Previous studies have shown that blockade of mesolimbic DA D3R inhibits stress-induced reinstatement of cocaine seeking in rats self-administering the drug, indicating that DA D3R plays an important role in mediating stress-induced reinstatement (Xi et al., 2004). DA D3R antagonists inhibit cocaine-seeking and cocaine-enhanced brain reward in rats (Vorel et al., 2002). DA D3R blockade attenuates both the rewarding effects of cocaine and cocaine-induced drug-seeking behavior, suggesting a role for DA D3R in mediating addictive properties of cocaine. Neuroanatomical and functional investigations focusing on DA D3R have confirmed its potential role in psychiatric diseases such as drug dependence (Gurevich and Joyce, 1999, Landwehrmeyer et al., 1993b). The importance of DA D3R in addiction, in particular cocaine addiction, has been confirmed in other studies using animal models. Stimulation of DA D3R is not sufficient to modulate food-primed food-seeking behavior or alter incentive motivation for food, morphine, and/or associated cues, but it affects the perception of the rewarding value of cocaine; thus the appetitive effects of cocaine are subserved by mechanisms involving DA D3R and different, at least in part, from those of
morphine and food (Duarte et al., 2003). 7-OH-DPAT and quinpirole, selective DA D3R agonists, potently decreased cocaine self-administration in the rat at doses that were not, in themselves, reinforcing (Caine and Koob, 1993), in contrast to the DA D3R antagonist nafadotride and DA D2R agonists that modulate cocaine self-administration in correlation with their relative potencies in vitro. Together these data support the hypothesis that the DA D3R may be an important target for pharmacotherapies for cocaine abuse and dependence (Caine et al., 1997). The cue properties of cocaine may be dissociated from its locomotor activating effects, because DA D3R/D2R agonists suppress locomotor activity but produce stimulus generalization to cocaine (Garner and Baker, 1999). Furthermore, in self-administering monkeys, DA D3R agonists produce cocaine-like effects and may modulate the discriminative stimulus effects of cocaine (Lamas et al., 1996), that were attenuated by DA D2R antagonists (Spealman, 1996); in contrast, self-administration of the DA D3R agonist 7-OH-DPAT in rhesus monkeys is modified by prior cocaine exposure (Nader and Mach, 1996). We believe that the lentiviral approach developed here may help clarifying these mechanisms by means of local manipulations of receptor expression.
References


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Figure Legends

Figure 1.
Microarray and Quantitative RT-PCR analysis of Cocaine-Induced DA D3R mRNA Expression. **Panel A:** Microarrays slides were prepared as described in the Methods Section. Candidates (70mer oligos) were spotted eight-fold, together with controls (10 positive and 10 negative controls, see Methods). Animals (n=6) were administered cocaine or saline according to the different drug administration protocols “Acute” (A), “Binge” (B) or “Chronic” (C). After sacrifice, total RNA was extracted from the NAcc, hybridized as Cy3 (saline) or Cy5 (cocaine) cDNAs and DA D3R mRNA was quantified. DA D2R was used as a control. Relative abundance of candidate transcripts is expressed relative to GAPDH. Data were normalized to the values obtained from saline-treated animals with saline levels set to 1 (as described in the Methods Section). Real-time-PCR was performed with the same RNA samples and amplifications were done using specific sets of primers; after normalization, the ratios between samples from cocaine-treated and saline-treated tissues were calculated (see Methods). **Panel B:** PCR products from cDNA used for microarrays or after real-time PCR were verified by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. A: Acute; B: Binge; C: Chronic; * P < 0.05 and ** P < 0.01 vs. saline-treated group.

Figure 2.
In vitro Expression of DA D3R mRNA in HEK293T cells: Quantification by means of Real-Time PCR. HEK293T cells were infected with 0, 2, 4 or 8 µl of the Lenti-D3 together with or without 4 µl of Lenti-GFP, Lenti-CD81-Hi6 or Lenti-uPA-His6 in culture medium in presence or absence of doxycycline (30 ng/ml), as indicated. After 96h, culture medium was removed and cells were used for total RNA extraction, cDNA preparation and quantitative real-time PCR using specific oligos. Results were normalized against GAPDH (set to 1) used as endogenous control. **Panel A:** Normalized transcript levels from cells infected without doxycycline. **Panel B**: PCR products from panel A were verified by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. C: Normalized transcript levels from cells infected in presence of doxycycline. D: PCR products from panel C were verified by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. Control: non infected HEK293 cells. * P < 0.05; ** P < 0.01 and *** P < 0.005 vs. non infected cells.

Figure 3.
In vitro Expression of DA D3R mRNA in HEK293T cells infected with Lenti-D3 and Lenti-D3-siRNAs: HEK293T cells were infected with 4 µl Lenti-D3 together with or without other viruses, as indicated. After 96h, culture medium was removed and cells were used for total RNA extraction, cDNA preparation and quantitative real-time PCR using specific sets of primers. Results were normalized against GAPDH used as endogenous control. A: Lenti-D3 + Lenti-uPA-His6 + Lenti-CD81-His; B: Lenti-D3 + Lenti-GFP + Lenti-uPA-His6 + Lenti-CD81-His; C: Lenti-D3 + Lenti-D3-siRNA1 + Lenti-uPA-His6 +
Figure 4: 
*In vivo* Silencing of DA D3R in the NAcc with Lenti-D3-siRNAs: Quantification of Transcripts by means of qRT-PCR. Animals (n=9) were bilaterally injected into the shell part of the NAcc with either Lenti-D3 alone, or Lenti-D3 together with a mix of all Lenti-D3-siRNAs, or only with a mix of Lenti-D3-siRNAs (no Lenti-D3), or with Lenti-GFP (as a control). Seven days after operation, animals were chronically injected with cocaine-HCl (i.p. 15 mg/kg), while control animals received 0.9% saline injections under the same schedule. Over 5 days, animals were fed without doxycycline (*session A*, n=9), yielding full overexpression of DA D3R in the injected brain areas and locomotor activity was monitored. On day-5 (*session B*), three animals per group were sacrificed and the other animals (n=6) were fed with doxycycline (immediately after the daily injection) and behavioural activity was measured after regimen switch; then animals were kept for five further days on the same regimen (*session B*, n=6). On day-10, three animals per group were sacrificed and for the remaining animals (n=3) regimen was switched back and animals were fed without doxycycline for five days (*session C*, n=3), allowing DA D3R overexpression in the injected areas and its return to levels observed in *session A*. At the end of each session, three rats per group were sacrificed by decapitation 24h after the last injection; the NAcc’s were dissected out and used for total RNA isolation, cDNA preparation and quantitative real-time PCR using specific oligos (n=3). Results were normalized against GAPDH (set to 1). **P < 0.01 and ***P < 0.005, vs. data from Lenti-D3 infected rats.

Figure 5. 
Cocaine-induced Behavioral Changes of Locomotor Activity Monitoring upon *in vivo* Dopamine D3R Receptor Silencing in the NAcc. Four groups of animals (n=9) were bilaterally injected into the NAcc, (a) either Lenti-D3 alone, (b) Lenti-D3 together with a mix of Lenti-D3-siRNAs, (c) Lenti-GFP alone or (d) Lenti-GFP together with Lenti-D3-siRNAs. Seven days after operation, animals (n=9) were chronically treated with cocaine (15 mg/kg) each day and locomotor activity was monitored. For each session, animals were injected with saline solution prior to the habituation period and placed in the monitoring cage for 30 min until stable basal activity was measured. Cocaine (15 mg/kg, i.p.) was then injected and the animal returned to the cage for further 60 min while locomotor activity monitoring was monitored. Initially, animals were fed without doxycycline over five days, yielding full overexpression of DA D3R in the injected brain area (*Session A*, n=9); on day-5, three animals per group were sacrificed and the other animals (n=6) were fed
doxycycline for five further days (Session B, n=6); finally, on day-10, three animals per group were sacrificed and for the other animals (n=3) regimen was switched back and animals were fed without doxycycline for five days (Session C, n=3), yielding DA D3R re-expression in the injected areas, as in session A.

Values indicate means ± SE (over the five days of each session) of total counts of locomotor activity during the 30 min habituation period (saline) and during the 60 min cocaine-induced period (cocaine). * P<0.05, ** P<0.01 and *** P<0.005 vs. corresponding saline-treated rats; # P<0.05 vs. Lenti-D3 infected animals; † P<0.05 vs. Lenti-GFP infected animals; ≠ P<0.05 vs. doxycycline-fed animals (Session B).
Fig. 1

A

Normalized mRNA Levels

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MicroArray

Real-Time PCR

B

DA D3

DA D2

GAPDH

Microarray

Real-Time PCR
Fig. 2

**A**

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<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</table>

*Normalized Transcript Levels*

**B**

- Doxy

**C**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>2 ul</th>
<th>4 ul</th>
<th>8 ul</th>
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<tbody>
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<td>Lenti-GFP</td>
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<tr>
<td>Lenti-D3</td>
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<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Lenti-uPA-His6</td>
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<td>++</td>
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</tr>
<tr>
<td>Lenti-CD81-His6</td>
<td>++</td>
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<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

*Normalized Transcript Levels*

**D**

+ Doxy

DA D3 → uPA → CD81 → β-Actin → GAPDH

DA D3-Receptor, DA D2-Receptor, β-Actin, uPA, CD81
Fig. 4

**Session A: Without Doxycycline**

<table>
<thead>
<tr>
<th>Normalized mRNA level</th>
<th>Lenti-D3</th>
<th>Lenti-D3 + Lenti-D3-siRNAs</th>
<th>Lenti-D3-siRNAs</th>
<th>Lenti-GFP</th>
</tr>
</thead>
<tbody>
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</tr>
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</tr>
<tr>
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<td></td>
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</tr>
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</tr>
<tr>
<td>18</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

**Session B: With Doxycycline**

<table>
<thead>
<tr>
<th>Normalized mRNA level</th>
<th>Lenti-D3</th>
<th>Lenti-D3 + Lenti-D3-siRNAs</th>
<th>Lenti-D3-siRNAs</th>
<th>Lenti-GFP</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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**Session C: Without Doxycycline**

<table>
<thead>
<tr>
<th>Normalized mRNA level</th>
<th>Lenti-D3</th>
<th>Lenti-D3 + Lenti-D3-siRNAs</th>
<th>Lenti-D3-siRNAs</th>
<th>Lenti-GFP</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Session A: - DOXY**

- DA D3
- uPA
- CD81
- β-actin
- GAPDH

**Session B: + DOXY**

- DA D3
- uPA
- CD81
- β-actin
- GAPDH

**Session C: - DOXY**

- DA D3
- uPA
- CD81
- β-actin
- GAPDH
Fig. 5

Motility (Total Counts)

<table>
<thead>
<tr>
<th>Session A</th>
<th>Session B</th>
<th>Session C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
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</tr>
<tr>
<td>D3</td>
<td>D3 &amp; siRNAs</td>
<td>GFP</td>
</tr>
<tr>
<td>D3</td>
<td>D3 &amp; siRNAs</td>
<td>GFP</td>
</tr>
<tr>
<td>D3</td>
<td>D3 &amp; siRNAs</td>
<td>GFP</td>
</tr>
<tr>
<td>D3</td>
<td>D3 &amp; siRNAs</td>
<td>GFP</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Session A</th>
<th>Session B</th>
<th>Session C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocaine</td>
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<tr>
<td>D3</td>
<td>D3 &amp; siRNAs</td>
<td>GFP</td>
</tr>
<tr>
<td>D3</td>
<td>D3 &amp; siRNAs</td>
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<td>D3 &amp; siRNAs</td>
<td>GFP</td>
</tr>
<tr>
<td>D3</td>
<td>D3 &amp; siRNAs</td>
<td>GFP</td>
</tr>
</tbody>
</table>

**Note:** Statistical significance indicated with asterisks: *p < 0.05, **p < 0.01, ***p < 0.001.