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In vivo Gene Silencing of CD81 by lentiviral expression of siRNAs suppresses Cocaine-induced Behaviour

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***List of abbreviations*:** NAcc: Nucleus Accumbens; SNr: Substantia Nigra; VTA: Ventral Tegmental Area; CPu: Caudate Putamen, HEK293T: Human Embryonic Kidney 293T cells

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ABSTRACT

The tetraspanin CD81 is induced in the mesolimbic dopaminergic pathway after cocaine administration. To further investigate its role, a regulatable lentivirus (Lenti-CD81), bearing the *CD81* gene under the control of a tetracycline inducible promoter, and lentiviruses expressing shRNA targeted against CD81 (Lenti-CD81-shRNAs) have been prepared. Infection of HEK293T cells *in vitro* with Lenti-CD81-shRNAs resulted in 96.5% gene silencing (from qRT-PCR and immunocytochemistry). *In vivo* delivery of Lenti-CD81-shRNA into the nucleus accumbens (NAcc) or the ventral tegmental area (VTA) resulted in 91.3% and 94% silencing of endogenous CD81 respectively. Stereotaxic injection of Lenti-CD81 into these regions, resulting in CD81 overexpression, induced a 4 to 5-fold increase in locomotor activity after chronic cocaine administration, which returned to basal levels when Lenti-CD81-shRNA had been co-injected or when CD81-expression was blocked by doxycycline. Furthermore silencing endogenous CD81 *in vivo* resulted in a significant decrease in locomotor activity over controls, again suppressing cocaine-induced behaviour.

Key Words: siRNA, Cocaine, Addiction, Lentivirus, *in vivo* gene Transfer, Behaviour, CD81, Plasticity, gene knock-down

Running title: "***SILENCING CD81 SUPPRESSES BEHAVIOURAL CHANGES IN CHRONIC COCAINE ADMINISTRATION***"

INTRODUCTION

Administration of drugs of abuse induces strong molecular adaptations and plasticity within the mesolimbic dopamine (DA) system, a pathway essential for reward seeking behaviour. These adaptations underlie a complex rewiring of neural circuitry that results in the behaviours associated with addiction (Nestler 2000, Robinson and Berridge 1993). Addictive drugs (cocaine and amphetamines), depressants (ethanol) and opiate narcotics (heroin and morphine) are powerful reinforcers and produce their rewarding effects of euphoria or pleasure through an interaction with the mesolimbic DA system (Nestler 2000). Little is known about the specific targets involved in this neuroadaptation process, but it has been suggested that cocaine and other drugs of abuse may alter the morphology of neuronal dendrites and spines, the primary site of excitatory synapses in the brain, by means of inducing expression changes of surface molecules (Nestler 2000, Yue *et al.* 2002). Complex changes in expression of a number of surface axon guidance molecules have been observed upon cocaine administration, which may underlie important neuroplastic changes in the reward- and memory-related brain centres following drug action (Bahi and Dreyer, unpublished observation). Local expression changes of these cues may mediate plasticity and cytoskeleton rearrangement through mechanisms similar to synaptic targeting during development. In addition strong induction of a surface tetraspanin protein involved in cell adhesion, CD81, has been described (Michna *et al.* 2001, Halladay *et al.* 2000, Brenz-Verca *et al.* 2001). Using a regulatable lentivirus bearing the rat CD81 gene under the control of a tetracycline-inducible system, previous studies have shown that CD81 expression in the mesolimbic dopaminergic pathway contributes significantly to behavioural changes associated with chronic cocaine administration (Brenz-Verca *et al.* 2001). Overexpression of CD81 in this pathway induces a 4 to 5 fold increase in locomotor activity, which can be reversed to normal in the same animal fed doxycycline.

In this study we further characterized the role of CD81 in drug-induced behavioural changes. Cocaine-induced expression changes of CD81 were assessed under different protocols of drug administration. Lentiviruses expressing shRNA targeted against different regions of the CD81 mRNA were developed and used for silencing endogenous CD81 or overexpressed CD81 *in vivo* within the mesolimbic dopaminergic pathway. We demonstrate that stereotaxic injection of shRNA-expressing lentiviruses into the nucleus accumbens or into the ventral tegmental area results in 93-95% down-regulation of CD81 in these regions, and this effect is associated with suppression of cocaine-induced behavioural changes.

MATERIAL AND METHODS

1. Animal handling and drug administration

a.- Animal Handling: Animals used in this experiment were male Wistar rats weighing 225-250 g (BRL, Fillinsdorf, Switzerland). All animal experiments were carried out in accordance with the guidelines and regulations for Animal Experimentation, BAG, Bern, Switzerland. Animals were housed in trios in clear plastic cages with wire grid lids and kept in the animal facility maintained on a 12-h light: 12-h dark cycle (lights off at 7:00 am). Access to food and water was unrestricted.

b.- Drug administration Protocols*:** Three different protocols for Drug administration were used, as described in Figure 1. ***Acute Paradigm: Animals (n=6) were i.p. injected once with 15 mg/kg cocaine-HCl (Sigma Chemical Co., Switzerland). ***Chronic Paradigm:*** Animals (n=6) were i.p. injected daily with 15 mg/kg cocaine-HCl for a period of 15 days. ***Binge Paradigm:*** Animals (n=6) were i.p. injected with 30 mg/kg cocaine-HCl every 2 h for 4 injections.
In these three protocols control animals received 0.9 % saline i.p. injection instead of drug. 24 h after the last injection, animals were sacrificed by decapitation and the brain areas were dissected out and used for isolation of total RNA, using RiboPure Kit (Ambion, UK) followed by RNA amplification according to previous publications (Bahi *et al.* 2004, Bahi and Dreyer 2004).

c.- RNA Extraction: After sacrifice, brain regions were dissected out (NAcc and VTA), and total RNA was extracted using RiboPure Kit (Ambion, UK) including an RNase-free DNase step according to the manufacturer's protocol. Briefly, 1 ml of RNAwiz was added to 50 mg of tissue, homogenized with a Polytron homogenizer, vortexed for 30 s and incubated at room temperature for 5 min. Residual protein was removed by the addition of 0.2 ml chloroform, mixing for 30 s, incubation at room temperature for 5 min, vortexing for 30 s, and centrifugation for 15 min at 12000 g and room temperature. The aqueous phase was precipitated with 0.5 ml of absolute ethanol, filtered over a column and washed by centrifugation. The total RNA was then eluted with 0.1 ml of DEPC-treated water, incubated at 60 °C for 10 min and stored at –80 °C. RNA was quantified by spectrophotometry and its integrity verified by agarose gel electrophoresis and visualized with ethidium bromide staining.

2. Lentivirus Construction

a.- Lenti-CD81 and Lenti-GFP lentiviruses: The CD81 gene (GenBank accession no. NM_013087) 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 5 x First Strand Buffer was added, followed by 2 µl 0.1 M DTT, 10 U RNAsin (Invitrogen, Switzerland), 1 µl 200 U/µl Superscript II RNaseH- Reverse Transcriptase. The mixture was incubated at 42 °C for 3 h. To remove RNA-DNA hybrids, 2 U RNase H were added and incubated at 37 °C for 30 min. The cDNA was then PCR amplified and 6His-tagged with the two following primers: CGC **GGA TCC** GCG ATG GGG TGG AGG GCT GC as forward primer and CCG **CTC GAG** CGG TTA ATG ATG ATG ATG ATG ATG GTA CAC GGA GCT GTT CCG G as reverse primer. The forward primer contains a *BamHI* (Biolabs, Switzerland) restriction site followed by the 5' Rat CD81 cDNA specific sequence; the reverse primer contains the 3' Rat CD81 cDNA specific sequence, a 6His-Tag, a stop codon and an *XhoI* (Biolabs, Switzerland) restriction site.

The PCR product was digested with *BamHI* and *XhoI* and cloned into similar sites in pTK431 (figure 2). The pTK431 is a self-inactivating (SIN) HIV-1 vector, which contains the entire tet-off inducible system, the cPPT and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). It was generated by ligating a *BglII/BamHI* DNA fragment containing the tetracycline-regulated transactivator tTA (5') and the tetracycline inducible promoter (3') into a *BamHI* site downstream to a CMV promoter in a SIN HIV-1 vector. A control vector construct, pTK433, in which GFP expression is regulated by a tetracycline inducible promoter, was generated by cloning a *BamH I/Bgl II* DNA fragment containing the GFP gene into a *BamHI* site in pTK431. All plasmids were $CsCl_2$ purified.

b.- Construction of Lenti-CD81-shRNAs: To silence CD81 expression *in vitro* and *in vivo*, 5 targets were designed according to the CD81 mRNA sequence (GenBank accession no. NM_013087). The following targets within the CD81 sequence were selected (Figure 2), based on Hannon's design criterion (http://katahdin.cshl.org:9331/RNAi/html/rnai.html): 1st target: 907 – 927, 2nd target: 290 – 317, 3rd target: 907 – 927 (with an extra Cytosine compared to the 1st target), 4th target: 848 – 866, 5th target: 676 – 696. To each oligo, an *Xho I* restriction site was 3' added. Using the pSilencer 1.0-U6 (Ambion, UK) as a template and a U6 promoter specific forward primer containing *BamH I* restriction site GC**GGATCC**CGCTCTAGAACTAGTGC, each shRNA target was added to the mouse U6 promoter by PCR (each target contains a 3' specific U6 promoter specific primer). 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 *BamHI* and *XhoI*, cloned into similar sites in pTK431, and sequenced to verify the integrity of each construct.

c.- Lentivirus Production The vector plasmids (either pTK431-CD81-6His, pTK433-GFP or pTK431-U6-shRNAs), together with the packaging construct plasmid pΔNRF and the envelope plasmid pMDG-VSVG were co-transfected into HEK293T cells to produce the viral particles (Bahi *et al.* 2004, Naldini *et al.* 1996). The viral titres were determined by p24 antigen measurements (KPL, USA). For the *in vivo* experiments, the different viral stocks matched for viral particle content were used at 0.4 mg/ml of p24.

***d.- In vitro* Assays:** The efficiency of the Lenti-CD81-shRNAs was tested *in vitro* by infection of HEK293T cells. The day before the infection, 3 x 10^5 HEK293T cells were plated per well in six-well plates. The next day, 3 µl from lentivirus stocks were added with Polybrene (Sigma, Switzerland, at 10 µg/ml final concentration), incubated 30 min at room temperature, and the cells were plated at 37 °C for 24 h. The next day, the medium was replaced with normal growth medium and cells were left for 24 h. One part of the cells was used for RT-PCR after total RNA isolation and the remaining part was used for immunocytochemistry.

Real time PCR and CD81 mRNA Quantification: Total RNA was extracted from the HEK293T cells using RiboPure Kit (Ambion, UK) including a RNase-free DNase step, and stored at –80 °C according to the manufacturer's protocol. RNA was quantified and its integrity verified . First strand cDNA was generated from 1µg of total RNA and Oligo(dT_{12-18}) primer with the M-MLV reverse transcription kit (Invitrogen, Switzerland) in a total volume of 20 µl according to the manufacturer's protocol. To quantify the CD81 mRNA level, quantitative real-time PCR was used. Primer sets were designed to amplify 100-300-bp products, using PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).
The reverse transcription reaction was amplified (qRT--PCR was performed) with the following pairs of oligonucleotides specific for rat CD81, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-Actin. **CD81:** 5'-TGA TCC TGT TTG CCT GTG AG-3' and 5'-CAG TTG AGC GTC TCA TGG AA-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 CGT AGC CAT CC-3' and 5'-CTC TCA GCT GTG GTG GTG AA-3'. GAPDH and β-actin were used as internal controls. The quantification was performed using

the real-time PCR iCycler (BioRad, Switzerland). For PCR, 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. The following PCR program was performed: 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 following the manufacturer's instructions and checking the PCR products on 2 % agarose gel.
Values for CD81 overexpression were calculated against the signal for GAPDH (or β-actin), selected as endogenous, constitutively expressed control mRNAs for normalization. No difference was observed whether GAPDH or β-actin was used for all normalizations. The levels for NAcc or VTA samples from cocaine-injected rats were normalized to the levels for the samples from saline-treated animals within the same experiment. PCR amplifications were done at least in triplicate from 3-pooled samples isolated from separate pools of animals or cells and the PCR cycle number at which each assay target reached the threshold detection line was determined ("threshold cycles", Ct value), using the second derivative of the reaction. 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 untreated samples (Mühlhauser *et al.* 2004).

Immunocytochemistry on HEK293T Cells: after infection cells were washed twice with 1 x PBS, fixed with pre-warmed 4 % PFA for 30 min and washed again with 1 x PBS buffer. Cells were blocked for non-specific binding with 3 % normal goat serum diluted in 1 x PBS for 20 min and incubated overnight at 4 °C with mouse histidine tag antibody (MCA1396, Serotec) diluted 200 fold in 1 x PBS containing 1 % normal goat serum, 0.1 % Triton-X100. Cells were rinsed 3 times with 1 x PBS and incubated with the secondary antibody (Texas red-conjugated goat anti-mouse immunoglobulin G, Molecular Probes) diluted 1000 times in 1 % normal goat serum, 0.1 % Triton-X100 in the dark at room temperature for 3 h. Cells were washed 3 times with 1 x PBS cover-slipped with a medium containing glycerol in

1xPBS (AF1 mounting solution, Citifluor-Ltd). In all cases negative controls included omission or substitution of the primary antibody.

Fluorescence microscopy: Stained cells were observed using a multi-fluorescence microscope Axioplan 2 imaging; (Zeiss, Germany) with an x63 objective and photographed using a multi-channel camera (Axiocam, Zeiss, Germany) linked to acquisition software (Axiovision system 3.1). The fluorophore was detected with the appropriate detecting system (HAL 100). Texas red was exited by a 595 nm beam and was detected through a light path ranging from 600-660 nm.

Quantification: Cells were photographed with a x40 objective using a digital camera (Axiocam, Zeiss) attached to a multi-fluorescence microscope (Axioplan2 imaging, Zeiss). Special care was taken to place the frame in the same location on each region analyzed for each slide. Within each section, frame size was kept constant from slide to slide. The density of CD81-positive cells was calculated for each slide using the Scion software. Each image was transformed into TIFF format, normalized by subtracting the background. Then a semi-automatic cellular density measurement was undertaken by calculating total pixel density at a fixed brightness level. Separate exposures were made for each section to ensure that density measurements were reproducible. The high mean level of pixel intensity correlated to high amounts of expression of CD81 in the specific area. To each set of CD81 immunoreactivity measurements, ANOVA followed by a Scheffe F tests was used. All results are expressed as a mean ± SEM.

3. *In-vivo* Assays

a.- Stereotaxic surgery and Injection of the Lentiviral Vector: Before the operation, rats were injected with ketamine/xylazine 100 mg/kg, 10 mg/kg, i.p.). After 10 min, the surgical field was shaved, cleaned using Hibitane solution (0.5 % chlorhexidine gluconate in 70 % Ethanol) and Lacrilube was applied to eyes. Animals were placed into a stereotaxic frame (Stoelting, IL). Stereotaxic injections were performed using a 5-µl Hamilton syringe with a 33-gauge tip needle. The injections were performed in the VTA (anterior –6; lateral +/-0.5; ventral -8.2) and the NAcc (anterior +1.4; lateral +/-1.6; ventral -6.8) as calculated from bregma and the dura mera. The skin was opened with scalpel incision along the midline and 0.2 ml of Mercain (0.25 % bupivacaine hydrochloride) was applied to the wound for local analgesia. Overlying tissue was blunt dissected with sterile Q-tips to visualize bregma and the skull was delicately bored at pre-determined co-ordinates. Heating of the skull was reduced by application of sterile 0.9 % saline solution around the drilling site. Male Wistar rats were bilaterally injected into the VTA or into the NAcc with 4 µl of concentrated lentiviral stock (co-injection of 2 µl of

Lenti-CD81 or Lenti-GFP together with 2 µl of Lenti-shRNAs) slowly infused at the speed of approximately 0.2 µl/min. The cannula was then maintained in place for a further 5 min after injection and then withdrawn very slowly to prevent backflow of solution. The wound site was closed with braided silk suture. Rats were then removed from the frame, injected with a mixture of 2.5 ml of 0.9 % saline solution and Rimadyl (carprofen 2 mg/kg, s.c.) for hydratation and pain relief. Lacrilube was re-applied to eyes. The animal was returned back to the cage out of the surgery room, kept warm and off sawdust (as this can be inhaled by rats not fully conscious). Post-op diet for 3 days consisted of normal food pellets soaked in water. Rat recovery was followed up.

b.- Drug Treatment and Locomotor Activity measurement: Locomotor activity was monitored during the dark cycle. One week after surgery, each subject was weighed; the animal was brought down from the animal facility and placed immediately into the activity-monitoring cage for a 30 min baseline without drug. 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 60 min (Bahi et al., 2004).

Locomotor activity was monitored in MED-OFA-RS cages (MED Associates Inc. USA). Animals were placed in a square (43.2 cm x 43.2 cm x 30.5 cm) PVC retainer. Activity in the monitor was recorded by photobeam interruptions. A ring of 16 sensors, spaced 2.54 cm from each other, measured the "X–Y" location of the animal four times per second. 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 in this dimension. Two sets of strips were used for the "X-Y" ambulatory data input while a single set was used for the "Z" rearing data.

Speed was estimated by computing the standard deviation of the distances of the data points to their mean within a sliding time window 0.4 s wide, constructed around each data point in turn. Box size was defined with 2 photobeams of X or Y to be broken before a movement was considered ambulatory. Starting at time 0, the box was centred on the subject. When the animal moved to the outside of the box, it was considered ambulatory and the box re-centred on the subject. The subject remained ambulatory until it did not leave the last re-centred box in less than the resting delay. The travelled-distance was then calculated. Stereotypic counts were defined as the total number of beam breaks inside the box.

c.- Statistical Evaluation of Behavioural Analysis: the distance travelled was summed over each test session and analyzed by means of one-way analysis of variance (ANOVA), with the data divided into 5-min bins. Appropriate post-hoc comparisons were used to compare groups if a significant interaction was found ($P<0.05$). These comparisons were made using Fisher's least significant difference test. Independent Student's *t*-tests were used when a significant interaction was found. All these analysis were performed using SPSS software (Podhorna and Didriksen 2004, Sanchis-Segura *et al.* 2004, Okabe and Murphy 2004). The use of standard deviation is based on the analysis of the coordinates of the path. This is because estimating the speed - measured as travelled distance per min - in a given time point *ti* depends on measuring the coordinates in two time points (e.g. the difference between the coordinates at times *ti* and *ti*-1).
Within the same group, the means and standard deviations were done on measurements performed each day. No significant changes were observed in day to day recordings over the same session. The means and the standard deviations from all measurements within one session were then calculated (using values obtained from subjects of the same group at the same session) and plotted (Bahi *et al.* 2004).

4. Immunohistochemistry

At the end of the session, animals were sacrificed by decapitation. Rat brains were rapidly dissected out, frozen in isopentane upon extraction (at –30 °C for 3 min) and kept at –25 °C. Coronal sections were cut at 14 μm in a cryostat (Leitz) and placed on gelatinized glass slides, air–dried at room temperature for 20 min and kept at –25 °C until further processing. Antigens were localized using the avidin-biotin-peroxidase technique. Slices were fixed in 4 % PFA for 15 min, washed thrice with 1 x PBS. Endogenous peroxidase activity was quenched with 2 % hydrogen peroxide in water (H_2O_2) for 40 min at room temperature. Non-specific binding was blocked for 30 min at RT in 1 x PBS containing 1 % bovine serum albumin, 1 % Triton X-100 and 3 % normal goat serum. Sections were incubated overnight with mouse anti-histidine antibody (MCA1396, Serotec, 1:12000) diluted in 1 x PBS containing 1 % Triton X-100 and 1 % normal goat serum. Sections were then washed three times with 1 x PBS and incubated with the biotinylated secondary antibody (goat anti-mouse immunoglobulin G, Vector laboratories, Burlingame, 1:500) for 45 min at RT. Sections were rinsed three times for 5 min in 1 x PBS at RT, followed by avidin-biotin complex (Vector laboratories, Burlingame) in 1 x PBS solution. After three rinses in 1 x PBS, all sections were developed in 0.025 % 3-3' diaminobenzidine tetrahydrochloride (DAB) plus 0.02 % H_2O_2 for 10–15 min. Sections were then dehydrated, mounted in

permanent medium (Eukitt) and examined with a Zeiss light microscope. GFP expressing sections were cover-slipped with AF1 mounting solution (Citifluor Ltd), and directly observed using a multi-fluorescence microscope Axioplan 2 imaging (Zeiss, Germany) mounted with a multi-channel camera Axiocam (Zeiss, Germany) linked to acquisition software Axiovision 3.1 (Zeiss, Germany). In all cases negative controls included omission or substitution of the primary antibody.

RESULTS

Cocaine-induced CD81 Expression:

CD81 was found initially in a screening for genes up-regulated after cocaine administration following a "binge" protocol. In order to further characterize this observation, up-regulation of CD81 was confirmed in different paradigms of drug administration, summarized in figure 1, i.e. acute, binge and chronic treatments. The total RNA from the NAcc and the VTA of animals treated under the different paradigms (figure 1, n=6) were prepared, reverse transcribed and CD81 expression was assessed by means of quantitative real-time PCR analysis after normalization against GAPDH, β-actin and HSP-70 considered as endogenous controls (no difference was observed between all normalizations). The results are shown in figure 3. The strong up-regulation in these two brain regions observed after binge treatment could be confirmed: CD81 displays a 6.2-fold up-regulation in the NAcc and a 3.9-fold up-regulation in the VTA, in agreement with previous studies (Brenz-Verca *et al.* 2001). Furthermore, CD81 was also 2.9 to 4.0-fold up-regulated after chronic treatment in these regions. On the other hand acute treatment induced small, but significant overexpression of CD81, yielding a 1.4 fold up-regulation in the VTA and in the NAcc. Subsequent studies (locomotor activity monitoring) were then performed according to the chronic protocol, but the silencing effect of the endogenous CD81 in the NAcc and VTA was followed-up after binge treatment, which induces highest expression of endogenous CD81.

shRNA lentiviruses and *in vitro* CD81 silencing:

In order to locally knock-down CD81 expression in selected brain areas, lentivirus-based RNA interference was used. Five different shRNAs were designed, targeted against different regions of the CD81 mRNA, as displayed in figure 2 (as described in the "Materials & Methods", lentivirus construction section). Constructs bearing a U6 promoter were inserted into the transfer plasmid of the lentivirus system. Lenti-CD81-His6 (Bahi *et al.* 2004), lenti-GFP (Bahi *et al.* 2004) and lenti-CD81-shRNAs all share the same vector backbone; however lenti-CD81-shRNAs are not regulatable due to the presence of the U6 promoter. Incorporation of the tetracycline inducible system into the lentivirus vectors conferred the ability to control transgene expression *in vivo* (Kafri et al 2000, Reiser et al 2000). The tetracycline inducible system is based on two components: *(a)* the tetracycline regulated transactivator (tTA), which is a fusion protein of the tet repressor, and the transactivator domain of the herpes simplex virus protein VP16; and *(b)* the inducible promoter, which contains a minimal promoter and seven copies of the Tet operon. In the absence of tetracycline, the tTA binds and activates the inducible promoter.

Binding of tetracycline (or its more potent analogue, doxycycline) to the tTA induces conformational changes that render the tTA incapable of binding to the Tet operon and thus abolish transgene expression (Haak et al 2004). A CMV promoter located in the middle of the vector genome constitutively expresses the tTA, while the inducible promoter at the 3'end of the vector regulates the expression of the transgene on interest (Kafri et al 2000).
To assess the efficiency of the five different targets at silencing CD81, *in vitro* assays were performed in HEK293T cells co-infected with Lenti-CD81. As controls, cells co-infected with Lenti-GFP (instead of Lenti-CD81-shRNAs) and Lenti-CD81 were used. 48 h after infection, cells were harvested, total RNA was extracted and CD81 mRNA measured by means of quantitative RT-PCR. As shown in figure 4A and 4B, shRNA-3 and -5 induced 88 % and 85 % decreases in CD81 mRNA levels, respectively. The other targets, shRNA-1, -2 and –4, were less efficient at silencing CD81 *in vitro*, resulting in 62, 56 and 45 % expression of CD81 mRNA, respectively. However, when all targets were co-infected together, CD81 silencing was >96.5 % (figure 4 A and B). It should be noted that Lenti-CD81-shRNA3 and Lenti-CD81-shRNA1 target the same region of the mRNA, yet differ only very slightly in their sequence: in Lenti-CD81-shRNA3 one C has been deleted compared to Lenti-CD81-shRNA1. It is interesting to observe that this very minor change in the sequence results in much more efficient silencing of Lenti-CD81-shRNA3 compared to Lenti-CD81-shRNA1 and makes it the best among all candidates tested, whereas the other may serve as a very good negative control**.**
Protein expression was also tested by means of immunocytochemistry: cells co-transfected with the Lenti-CD81-shRNAs (all targets together) and Lenti-CD81 display 96-97 % silencing of CD81 (figure 4C and D). For *in vivo* assays, only the two most efficient Lenti-CD81-shRNA's, i.e. Lenti-CD81-shRNA-3 and Lenti-CD81-shRNA-5, were used and co-infected.

***In vivo* knock down of endogenous CD81:**

To test the *in vivo* silencing efficiency of the Lenti-CD81-shRNAs, the Lenti-CD81-shRNA-3 and Lenti-CD81-shRNA-5 were stereotaxically co-injected into the NAcc or the VTA of animals (n=6). Control animals were injected with Lenti-GFP (instead of Lenti-CD81-shRNAs) into the same areas. Endogenous CD81 is expressed at low levels in the rat brain under normal conditions, but is strongly induced in the mesolimbic dopaminergic pathway upon cocaine administration, especially after a high dose cocaine treatment ("binge" protocol, figure 3). Therefore, in order to optimally induce the expression of endogenous CD81 in the targeted areas, animals were treated according to the "binge" protocol (figure 1, 120 mg/kg cocaine in a single day), which strongly promotes CD81 expression in the mesolimbic

dopaminergic pathway (figure 3, and Brenz-Verca *et al.* 2001, Bahi *et al.* 2004). Seven days after surgery, animals were injected with 30 mg/kg of cocaine i.p. every 2 h for 4 injections and sacrificed 24 h after the last injection. Control animals, i.e. animals treated with Lenti-GFP, received cocaine injections under the same schedule. After sacrifice, the brain regions of interest were dissected out and the total RNA prepared. Quantitative Real Time-PCR was then performed on cDNA from these RNA preparations, and the mRNA levels were quantified and normalized against GAPDH considered as an endogenous control (normalization against β-actin and HSP-70 were done also and give practically the same results, data not shown). As shown in figure 5, co-injection of Lenti-CD81-shRNA-3 and –5 together resulted in 91.3% and 94% decrease in endogenous CD81 mRNA levels in the VTA and in the NAcc, respectively, compared to CD81 mRNA levels observed in control animals (infected with Lenti-GFP in the same brain areas).

Behavioural Changes induced upon CD81 expression:

Four groups of animals (n=6) were used for assessing the effects of Lenti-CD81-shRNAs on cocaine-induced behavioural changes. One group of animals was injected with the doxycycline-regulatable Lenti-CD81 into the NAcc. After surgery, animals were fed without doxycycline (normal water), enabling full expression of CD81 in the targeted area. Seven days after surgery the animals were receiving chronic cocaine i.p. (15 mg/kg) and the locomotor activity was monitored daily after drug administration. As shown in figure 6A (Session A (left panels), after cocaine administration a strong raise in locomotor activity was observed with a peak at 19,192 mm/min observed 15 min after drug administration, in agreement with previous observations (Bahi *et al.* 2004). After five days the same animals were fed doxycycline in the drinking water (inducing down-regulation of lentivirus-expressed CD81 in the targeted area), and their behaviour upon chronic cocaine administration was further evaluated for five consecutive days. Under these conditions locomotor activity dropped back almost to control levels after cocaine injection, with a peak at 6,302 mm/min (figure 6A, Session B). Five days later, doxycycline was finally removed (enabling re-expression of lentivirus-mediated CD81 in the NAcc). This restored locomotor activity back to its initial levels, with a peak at 18,852 mm/min at 45 min (figure 6A, Session C). The difference between peaks in both sessions A and C was not significant.

Another set of control animals was infected with the doxycycline-regulatable control lentivirus, Lenti-GFP, into the same brain area (NAcc) and submitted to the same cycles of chronic drug administration, with regimens consisting initially of 5 days in absence of doxycycline (Session A), followed by five days under doxycycline (Session B) and finally five days under removal of

the doxycycline (Session C). As shown in figure 6A (rightest panels), cocaine administration induced a small raise in locomotor activity, with a peak of 3,780 mm/min at 40 min. However this behaviour was independent of GFP-expression. Another control group consisting of naive animals, not treated with lentivirus, behaved like the group infected with Lenti-GFP when receiving the same doses of cocaine (data not shown). The observed locomotor activity was not significantly affected by the doxycycline regimen: locomotor activity displayed a peak at 3,647 mm/min at 40 min in the presence of doxycycline and 3,888 mm/min when doxycycline was again removed.

A third group of animals was treated by co-injection of the regulatable Lenti-CD81 (same concentration as the first group) together with (non-regulatable) Lenti-CD81-shRNA-3 and Lenti-CD81-shRNA-5. Animals were then submitted to the same regimen and drug treatment as the other groups: seven days after surgery, chronic cocaine administration was initiated in the absence of doxycycline, i.e. under conditions where lentivirus-mediated CD81 expression was achieved. As shown in figure 6A (middle left panels), in the absence of doxycycline, locomotor activity was low, with a peak at 5,105 mm/min at 45 min, corresponding to only 8 % of the locomotor activity observed under the same conditions in the absence of silencing Lenti-CD81-shRNAs; ie almost 92 % of the behavioural effect was abolished with the Lenti-CD81-shRNAs when basic locomotor activity was subtracted (Lenti-GFP infected animals) (session A, left panel). After 5 days, doxycycline was added to the regimen (session B), inducing down-regulation of exogenous, lentivirus-mediated CD81 expression, but not of lentivirus-mediated shRNA because Lenti-CD81-shRNAs are not regulated by doxycycline (due to the presence of two distinct promoters in the lentivirus construct). As shown in the panel, locomotor activity decreased further under these conditions, and reached a peak of 3,100 at 45 min, i.e. the locomotor activity under these conditions was even significantly lower (40 %) than in the corresponding GFP-treated animals. This low activity most probably corresponds to silencing of the endogenous CD81, which, in the absence of silencing shRNAs (first two groups of animals), would be strongly induced under these conditions (see figure 3, chronic treatment). As a matter of fact, the level of exogenous CD81 was decreased drastically by doxycycline. Upon removal of doxycycline five days later (session C), this group of animals displays no significant raise in locomotor activity: under these conditions lentivirus-mediated CD81 overexpression would be restored, but at the same time it was clearly silenced by the expression of Lenti-shRNAs.

A fourth group of animals was treated by co-injection of the Lenti-CD81-shRNA-3 and Lenti-CD81-shRNA-5 (same doses as previous group) together

with Lenti-GFP and submitted to the same cycles of regimen and drug administration (figure 6A, middle right panels). This control group was aimed at testing the effects of Lenti-CD81-shRNAs on expression of the endogenous protein in absence of exogenous CD81. Under all conditions and regimens, locomotor activity was slightly but significantly lower than the activity observed with the second group of (control) animals, which were treated with Lenti-CD81 only. In the initial 5-day cycle (i.e. in absence of doxycycline, session A), a peak of 3,347 mm/min was observed, significantly lower ($p<0.08$) than in the second group under the same conditions. In the presence of doxycycline (session B), a peak of 3,103 mm/min was observed, identical to the level reached by the third group of animals (3,100 mm/min) but significantly lower ($p<0.08$) than the control group, which had been injected with Lenti-GFP only (3,647 mm/min). Finally when doxycycline was removed (session C), these animals displayed no change in locomotor activity compared to the third group (3,424 mm/min versus 3,209 mm/min, respectively), but the observed activity was still significantly lower than the second group (treated with Lenti-GFP only) under the same conditions (3,888 mm/min). This decrease in activity over the control groups again should be related to silencing of endogenous CD81 and further confirms the previous observations.

A similar series of experiments was conducted on four groups of animals where the viruses were injected into the VTA, instead of the NAcc. The same results were observed, as shown in figure 6B. Cocaine-induced locomotor activity upon CD81 expression was lower, with a peak at 13,343 mm/min at 45 min, compared to 19,192 mm/min when CD81 was expressed in the NAcc. However, the decreases in locomotor activity in the presence of the Lenti-CD81-shRNAs were of the very same proportion under all conditions.

Figures 6C and 6D summarizes the changes observed under these conditions at peak activity (40 min after drug injection). As shown, under all conditions statistically significant differences ($p<0.08$) were observed between the second group (corresponding to silencing endogenous CD81) and the fourth group, i.e. where endogenous CD81 was not silenced in these brain areas. Because of low levels of endogenous CD81, differences are small but nevertheless significant. Together these data show that lentivirus-mediated gene silencing of CD81 expression in the mesolimbic dopaminergic pathway resulted in suppression of CD81-induced locomotor activity.

Expression of CD81 was assessed by immunohistochemistry at the end of each session, as shown in figure 7. Clearly lentivirus-mediated gene delivery with Lenti-CD81 results in very local gene expression in the NAcc or the VTA (figure 7A and 7C, top panels). This expression is supressed with doxycycline (Figure 7B and 7D, top panels), as expected. In addition when the CD81

silencers viruses, Lenti-CD81-shRNAs, are co-injected with Lenti-CD81 (Figure 7A-7D, lower panels), full gene knock down also results in suppression of protein expression in the infected brain area. These controls further establish the good functionality of the tools developed in this study.

DISCUSSION

For the present study we developed lentiviral-mediated delivery of shRNA *in vivo* and demonstrate the efficacy of this approach to locally reduce target CD81 expression *in vivo*. The method has been validated by several means, including *in vitro* assays and evaluation of behavioural changes *in vivo*. The ability of lentiviral vectors to transduce cells efficiently *in vivo* coupled with the efficacy of virally expressed shRNA shown here, extends the application of shRNA to viral-based therapies and *in vivo* targeting experiments that aim to define the function of specific genes.

In vivo Delivery of shRNAs in the Brain

The efficiency of shRNA at silencing gene expression *in vivo* centres around four primary challenges responsible for the downfall of the approach: *in vivo* delivery of shRNA, choice of the appropriate target, stability of the shRNA, long term expression of the shRNA (Shi 2004). So far, very few studies have reported the use of shRNA *in vivo*, because of these limitations. Its efficacy has been demonstrated *in vivo* in inhibition of expression of some disease-related targets: the vascular endothelial growth factor (Usman 2004, Filleur *et al.* 2003), caspase-8 (Zender *et al.* 2003), hepatitis B (McCaffrey *et al.* 2003) and C viruses (Usman 2004, Yokota *et al.* 2003), β-catenin (Verma *et al.* 2003), TNF-α □□□□□□□□□□□□□□□□□□□□□□□□), AGRP (Makimura *et al.* 2002), β-glucuronidase (Davidson and Paulson 2004), the receptor tyrosine kinase MuSK of neuromuscular junction (Kong *et al.* 2003) and the endogenous Fas gene expressed in adult mouse liver (Song *et al.* 2003). Delivery has been cited as one of the most important barriers to RNA-based therapy (Davidson and Paulson 2004). The effectiveness of siRNA is often lost when siRNAs are injected into animals, due to rapid clearance and degradation (Usman 2004). Recently, several breakthroughs have highlighted viruses as excellent vehicles for siRNA delivery (Zhang *et al.* 2003, Devroe and Silver 2004). Retroviruses, the transgene-delivery vector of choice for many experimental gene therapy studies, have been engineered to deliver and stably express therapeutic siRNA within cells, both *in vitro* and *in vivo* (Rubinson *et al.* 2003, Jain 2004). Delivery of siRNAs into the central nervous system (CNS) adds an additional challenge. RNAi targeting neurological disease genes can be accomplished *in vitro*, with profound effects on cellular phenotypes, but the difficult challenge lies in the translation of this technology to the CNS, primarily the problem of delivery to the brain. Therefore there has been no study so far relating the efficiency of siRNA in treating pathophysiological disorders or mood disorders i*n vivo* and studies so far are limited to target evaluation *in vitro* (Kong *et al.* 2003, Beckman *et al.* 2003). Recently another study also successfully demonstrated for the first time the use of this

technology in assessing pathophysiological conditions in the CNS, by intrathecal administration of shRNAs to down regulate P2X3 gene expression *in vivo*, and evaluation of the roles of putative pain genes in pain models (Dorn *et al.* 2004).

Our study was designed to overcome two additional difficulties: on the one hand, we aimed at silencing a gene very locally in the CNS, to prevent effects in unrelated areas, and on the other hand long-term expression of the shRNA was desired to assess behavioural changes over several weeks. With this respect, delivery of retroviral self-inactivating lentiviruses is ideal, as they transfect post-mitotic cells, including neurons, with permanent incorporation of the elements encoding for shRNA into the host genome, and will not spread away from the injection site, thus yielding to a very localised effect. This enabled the use of shRNA in combination to behavioural evaluation to assess changes induced upon gene expression *in vivo*.

Another challenge in the methodolgy lies in the design of shRNA. It is crucial that siRNA must not cause any effects *in vivo* other than those related to the knockdown of the target gene. This issue is particularly important in therapeutic applications where unwanted side effects would be undesirable (Shi 2004). Although the actual substrate specificity of individual siRNAs appears to be very high (Brummelkamp *et al.* 2002), recent studies indicate that siRNAs can tolerate single mutations located in the centre of the molecule, and up to four mutations are necessary for complete inactivation (Holen *et al.* 2002, Jacque *et al.* 2002). Thus, some mismatches can be tolerated, in particular noncanonical base pairs that are frequently found in double-stranded microRNA precursors (Mourelatos *et al.* 2002). In the present study, we found however that a single mutation in shRNA greatly affects its activity, as shown by the efficacy of shRNA3 compared to shRNA1. A further complication in the method has been pointed out from global gene expression using microarray technology to examine the specificity of siRNAs, with the interesting finding that a large number of non-targeted genes containing as few as 11 continuous nucleotides with identity to the siRNA may be affected by siRNA treatment (Jackson *et al.* 2003). Using the same approach, the overall cellular effects of siRNAs on transcription levels has been investigated (Semizarov *et al.* 2003). Similar assays of validation remain to be performed on our targets.

Application of lentiviral-based shRNA delivery *in vivo* to assess the putative role of CD81 in addiction

In this study we also confirm earlier observations establishing that the tetraspanin CD81 is induced in the mesolimbic dopaminergic pathway after cocaine administration. CD81 expression causes behavioural changes

associated with drug intake, which can be reversed by means of local gene knock-down. Applying our strategy of lentiviral-based delivery of shRNA *in vivo* to reduce CD81 expression in the mesolimbic dopaminergic pathway, we were able to demonstrate that this effect significantly reduces drug-induced locomotor activity in the rat. Stereotaxic injection of a regulatable Lenti-CD81 into the NAcc or the VTA, resulting in CD81 overexpression in these regions, induced a 4 to 5-fold increase in locomotor activity after chronic cocaine administration, which returned to basal activity when Lenti-CD81-shRNA had been co-injected or when CD81-expression was blocked by doxycycline. Furthermore *in vivo* delivery of Lenti-CD81-shRNA into these areas resulted in >90 % silencing of the endogenous CD81, which under these conditions would be induced by drug administration, yielding a significant decrease in locomotor activity as compared to controls.

The role of the tetraspanin CD81 in drug addiction is not very surprising. Proteins of the tetraspanin superfamily are scaffold proteins, which participate in the formation of plasma membrane signalling complexes, regulate signal transduction and association with extracellular matrix. Growing evidence indicates that in the nervous system they enable synaptic adaptations and plasticity, which are key processes induced by the drug. Recent evidence implicates neuronal tetraspanins in axon growth and target recognition (Fradkin *et al.* 2002). CD81 regulates neuron-induced astrocyte cell-cycle exit (Kelic *et al.* 2001) and plays a central role in the dynamic regulation of complex formation between specific G-coupled protein receptor and their G-proteins (Little *et al.* 2004). CD9, a major molecular partner of CD81 (Charrin *et al.* 2001), forms extensive complexes with other tetraspanins, integrins and other proteins (Stipp *et al.* 2001), building the tetraspanin web beneath the plasma membranes (Terada *et al.* 2004). CD9 is a paranodal component regulating paranodal junctional formation in myelinated axons (Ishibashi *et al.* 2004) and is localized to neurons early in development, coincident with a period of active axon growth for many neuronal populations (Schmidt *et al.* 1996). Tetraspanins form complexes with integrins, mainly β1 integrins (Hemler 1998); these integrins are known to mediate axon growth induced by numerous extracellular matrix proteins. Enhanced integrin-mediated neurite outgrowth in response to CD9 activation suggests a functional relationship between integrins and tetraspanins (Banerjee *et al.* 1997). Furthermore integrin association to extracellular matrix proteins, e.g. laminin-5 is regulated by EWI-2 (Stipp *et al.* 2003), a major binding partner of CD81 (Stipp *et al.* 2001). Recent evidence suggests that tetraspanins may serve as a link between integrin subunits and various intracellular signalling molecules, such as phosphatidylinositol 4-kinase and PKC (Hemler 1998, Berditchevski *et al.* 1997). CD81 is also linked to

ERK/MAPK signalling (Carloni *et al.* 2004). Tetraspanin activation can also result in increases in intracellular calcium and enhanced tyrosine kinase and phosphatase activity (Carmo and Wright 1995), each of which has been implicated in the regulation of neurite outgrowth (Keynes and Cook 1995, Desai *et al.* 1997).

As drugs of abuse induce strong plasticity and molecular adaptations which underlie a complex rewiring of neural circuitry and results in the behaviours associated with addiction, it is not surprising that a scaffolding protein such as CD81 may play a role in these processes, as clearly shown in this study. Further studies will be devoted to clarify its function.

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REFERENCES

Bahi A. and Dreyer JL. (2004) Cocaine-induced Expression Changes of Axon Guidance Molecules in the Adult Rat Brain. *Mol. Cell Neurosci.* In Press

Bahi A, Boyer F, Kafri T and Dreyer JL (2004) CD81-induced Behavioural Changes during Chronic Cocaine Administration. *In vivo* Gene Delivery with regulatable Lentivirus. *Eur J Neurosci* **19**, 1621-33

Banerjee SA, Hadjiargyrou M and Patterson PH (1997) An antibody to the tetraspan membrane protein CD9 promotes neurite formation in a partially alpha3beta1 integrin-dependent manner. *J. Neurosci*. **17**, 2756-2765.

Beckman C, Zhang Y, Hoffer BJ and Tomac AC. (2003) Short interfering RNAs (siRNAs) for reducing dopaminergic phenotypic markers. *J. Neurosci. Meth*. **131**, 51–56

Berditchevski F, Tolias KF, Wong K, Carpenter CL and Hemler ME (1997). A novel link between integrins, transmembrane-4-superfamily proteins (CD63 and CD81) and phosphatidyl-inositol-4-kinase. *J. Biol. Chem*. **272**, 2595-2598

Brenz-Verca MS, Widmer DAJ, Wagner GC and Dreyer JL (2001) Cocaine-induced Expression of the Tetraspanin CD81 and its Relation to Hypothalamic Function. *Mol Cell Neurosciences* **17**, 303-316

Brummelkamp TR, Bernards R, Agami R. (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **2**, 243–247

Carloni V, Mazzocca A and Ravichandran KS (2004) Tetraspanin CD81 is linked to ERK/MAPKinase signaling by Shc in liver tumor cells. *Oncogene* **23**, 1566–1574

Carmo AM and Wright MD (1995) Association of the transmembrane 4 superfamily molecule CD53 with a tyrosine phosphatase activity. *Eur. J. Immunol*. **25**, 2090-2095.

Charrin S, Le Naour F, Oualid M, Billard M, Faure G, Hanash SM, Boucheix C and Rubinstein E. (2001) The major CD9 and CD81 molecular partner. Identification and characterization of the complexes. *J. Biol. Chem*. **176**, 14329-14337

Davidson BL and Paulson HL. (2004) Molecular medicine for the brain:

silencing of disease genes with RNA interference. *Lancet* **3,**145-9

Desai CJ, Sun Q and Zinn K. (1997) Tyrosine phosphorylation and axon guidance: of mice and flies. *Curr. Opin. Neurobiol*. **7**, 70-74.

Devroe E and Silver PA. (2004) Therapeutic potential of retroviral RNAi vectors. *Exp Opin. Biol. Therapy*, **4**, 319 – 327

Dorn G, Patel S, Wotherspoon G, Hemmings-Mieszczak M, Barclay J, Natt FJC, Martin P, Bevan S, Fox A, Ganju P, Wishar W and Hall J. (2004) siRNA relieves chronic neuropathic pain. *Nucleic Acids Research*, **32**, 1-6

Filleur S, Courtin A, Ait-Si-Ali S, Guglielmi J, Merle, C, Harel-Bellan A, Clezardin P and Cabon F. (2003) siRNA-mediated inhibition of vascular endothelial growth factor severely limits tumor resistance to anti-angiogenic thrombospondin-1 and slows tumor vascularization and growth. *Cancer Res*., **63**, 3919-3922.

Fradkin LG, Kamphorst JT, DiAntonio A, Goodman CS and Noordermeer JN. (2002) Genome-wide analysis of the Drosophila tetraspanins reveals a subset with similar function in the formation of the embryonic synapse. *Proc. Natl. Acad. Sci. USA* **99**, 13663–13668

Haack K, Cockrell AS, Ma H, Israeli D, Ho SN, McCown TJ, Kafri T. (2004) Transactivator and structurally optimized inducible lentiviral vectors. *Mol Ther*, **10**, 585-596.

Halladay AK, Yue Y, Michna L, Widmer DAJ, Wagner GC and Zhou R. (2000) Regulation of EphB1 expression by dopamine signaling. *Mol. Brain Research* **85**, 171-178

Hemler ME. (1998) Integrin associated proteins. *Curr. Opin. Cell Biol*. **10**, 578-585.

Holen T, Amarzguioui M, Wiiger MT, Babaie E and Prydz H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. *Nucleic Acids Res*. **30**, 1757–1766

Ishibashi T, Ding L, Ikenaka K, Inoue Y, Miyado K, Mekada E and Baba H. (2004) Tetraspanin protein CD9 is a novel paranodal component regulating paranodal junctional formation. *J. Neuroscience* **24**, 96-102

Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G and Linsley PS. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol*. **21**, 635–637

Jacque JM, Triques K, and Stevenson M. (2002) Modulation of HIV-1 replication by RNA interference. *Nature* **418**, 435–438

Jain KK. (2004) RNAi and siRNA in target validation. *Drug Discovery Today* **9**, 307-9

Kafri T, van Praag H, Gage FH, Verma IM. (2000) Lentiviral vectors: regulated gene expression. *Mol Ther*, **1**, 516-521.

Kelic S, Levy S, Suarez C and Weinstein D. E. (2001) CD81 regulates neuron-induced astrocyte cell-cycle exit. *Mol. Cell. Neuroscience* **17**, 551-560

Keynes R and Cook GM. (1995) Axon guidance molecules. *Cell* **83**, 161-169

Kong XC, Barzaghi P and Ruegg MA. (2003) Inhibition of synapse assembly in mammalian muscle *in vivo* by RNA interference. *EMBO Reports* **5**, 1-6

Little KD, Hemler ME and Stipp CS. (2004) Dynamic regulation of GPCR-tetraspanin-G protein complex on intact cells: central role of CD81 in facilitating GPR56-Gαq/11 association. *Mol Biol Cell* **15**, 2375-2387

Makimura H, Mizuno TM, Mastaitis JW, Agami R and Mobbs CV. (2002) Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. *BMC Neurosci*. **3**, 18-24

McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, Wieland SF, Marion PL and Kay MA. (2003) Inhibition of hepatitis B virus in mice by RNA interference. *Nat. Biotechnol*. **21**, 639-644.

Michna L, Brenz Verca MS, Widmer DAJ, Chen SL, Rogrove J, Zhou R, Tsitsikov E, Miescher GC, J-L. Dreyer JL and Wagner GC (2001) Altered Sensitivity of CD81-deficient mice to neurobehavioural effects of cocaine. *Mol Brain Res* **90**, 68-74

Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L,

Rappsilber J, Mann M and Dreyfuss G. (2002) miRNAPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev*. **16**, 720–728

Mühlbauer M, Allard B, Bosserhoff AK, Kiessling S, Herfarth H, Rogler G, Schölmerich J, Jobin C, and Hellerbrand C. (2004) Differential Effects of Deoxycholic Acid and Taurodeoxycholic Acid on NF{kappa}B Signal Transduction and IL-8 Gene Expression in Colonic Epithelial Cells. *Am J Physiol Gastrointest Liver Physiol*. **286**, 1000-8

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM and Trono D. (1996) *In vivo* gene delivery and stable transduction of non-dividing cells by a lentiviral vector. *Science* **272**, 263–267

Nestler EJ. (2000) Genes and addiction. *Nat Genet* **26**, 277-81

Okabe C, Murphy NP. (2004) Short-term effects of the nociceptin receptor antagonist Compound B on the development of methamphetamine sensitization in mice: a behavioral and c-fos expression mapping study. *Brain Res*. **1017**, 1-12.

Podhorna J, Didriksen M. (2004) The heterozygous reeler mouse: behavioural phenotype. *Behav Brain Res*. **153**, 43-54.

Reiser J, Lai Z, Zhang XY, Brady RO. (2000) Development of multigene and regulated lentivirus vectors. *J Virol*, **74**, 10589-10599.

Robinson TE. and Berridge KC. (1993) The neural basis of drug craving: an incentive-sensitization theory of addiction. *Brain Res Brain Res Rev* **18,** 247-91

Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopina J, Rooney DL, Ihrig MM, McManus MT, Gertler FB, Scott ML, Van Parijs L. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet*. **33**:401-406.

Semirazov, D. Frost L, Sarthy A, Kroeger P, Halbert DN and Fesik SW. (2003) Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl. Acad. Sci. U.S.A*. **100**, 6347-6352.

Sanchis-Segura C, Pastor R, Aragon CM. (2004) Opposite effects of acute versus chronic naltrexone administration on ethanol-induced locomotion. *Behav Brain Res*. **153**, 61-67.

Shi Y. (2004) Mammalian RNAi for the masses. *Trends Genetics* 19, 9-12

Schmidt C, Kunemund V, Wintergerst ES, Schmitz B and Schachner M. (1996) CD9 of mouse brain is implicated in neurite outgrowth and cell migration *in vitro* and is associated with the alpha 6/beta 1 integrin and the neural adhesion molecule L1. *J. Neurosci. Res*. **43**, 12-31.

Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P and Lieberman J. (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. *Nature Med*. **9**, 347-351.

Sorensen DR, Leirdal M and Sioud M. (2003) Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J. Mol. Biol*. **327**, 761-766.

Stipp DC, Kolesnikova TV and Hemler ME. (2001) EWI-2 is a major CD9 and CD81 partner and member of a novel Ig protein subfamily. *J. Biol. Chem*. **276**, 40545-40554

Stipp CS, Kolesnikova TV and Hemler ME. (2003) EWI-2 regulates α3β1 integrin-dependent cell function on laminin-5. *J. Cell Biol* **163**, 1167-1177

Terada N, Baracskay K, Kinter M, Melrose S, Brophy PJ, Boucheix C, Bjartmar C, Kidd G and Trapp BD. (2002) The Tetraspanin protein, CD9, is expressed by progenitor cells committed to oligodendrogenesis and is linked to β1 integrin, CD81, and Tspan-2. *Glia* **40**, 350–359

Usman N. (2004) From target validation to therapeutics with RNAi *Curr. Drug Discovery* **4**, 15-19

Verma UN, Surabhi RM, Schmaltieg A, Becerra C and Gaynor RB. (2003) Small interfering RNAs directed against beta-catenin inhibit the *in vitro* and *in vivo* growth of colon cancer cells. *Clin. Cancer Res*. **9**, 1291-1300.

Yokota T, Sakamoto N, Enomoto N, Tanabe Y, Miyagishi M, Maekawa S, Yi L, Kurosaki M, Taira K, Watanabe M and Mizusawa H. (2003) Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep*. **4**, 602-608.

Yue Y, Chen ZY, Gale NW, Blair-Flynn J, Hu TJ, Yue X, Cooper M, Crockett DP, Yancopoulos GD, Tessarollo L, Zhou R. (2002) Mistargeting hippocampal axons by expression of a truncated Eph receptor. *Proc Natl Acad Sci U S A*, **99**, 10777-82.

Zhang M, McManus MT, Gertler FB, Scott ML and Van Parijs L. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA
interference. *Nature Genetics* **33**, 401-406

Zender L. Hutker S, Liedtke C, Tillmann HL, Zender S, Mundt B, Waltemathe M, Gosling T, Flemming P, Malek NP, Trautwein C, Manns MP, Kuhnel F and Kubicka S. (2003) Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc. Natl Acad. Sci. USA*, **100**, 7797-7802.

FIGURES

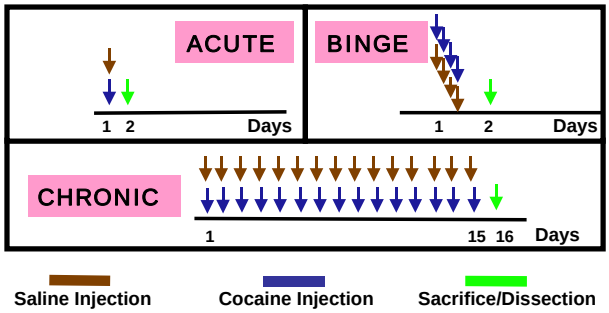

Figure 1. Drug Administration Protocols used in this study. Acute Treatment. Animals (n=6) were injected a single dose of cocaine (15 mg/kg) and sacrificed 24 h after injection. **Binge Treatment.** Animals (n=6) received four injections of high dose cocaine (30 mg/kg) and were sacrificed 24 h after the last injection. **Chronic Treatment.** Animals (n=6) received a single dose of cocaine (15 mg/kg) every day over 15 days and were sacrificed 24 h after the last injection. In each treatment (acute, binge or chronic), control animals were injected with saline solution under the same schedule.

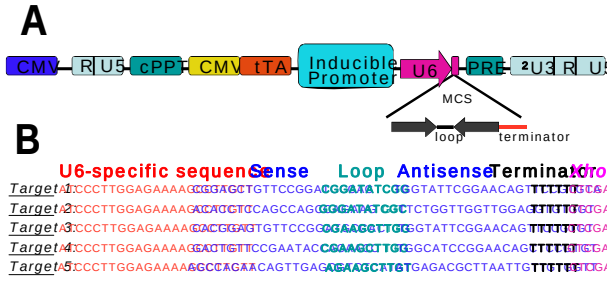

Figure 2. The Lentivirus vector-based RNA interference (Lenti-CD81-shRNAs) construct. **(A):** Generation of a hairpin siRNA directed by a Pol III promoter. An inverted repeat is inserted at the +1 position of the U6 promoter (-351 to +1). The individual motif is 19–25 nt, corresponding to the coding region of the gene of interest. The two motifs that form the inverted repeat are separated by a loop of ≈12 nt. The transcriptional termination signal of 5-6 Ts is added at the 3′ end of the inverted repeat. The resulting RNA is predicted to fold back to form a hairpin dsRNA. **(B):** Sequences of the CD81 targets used in this study. Sequences were adapted with a loop sequence to create siRNAs. The presumed transcription initiation site is located after the U6-specific sequence. Nucleotides that form the loop structure are indicated in green. The Pol III terminator is indicated in bold

black. (see materials and methods section for the exact position of each target within the CD81 sequence).

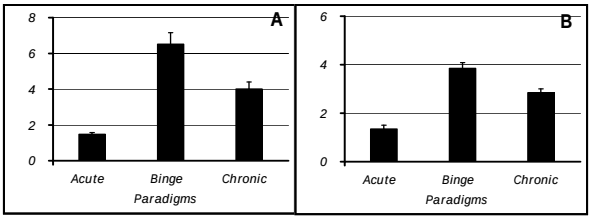

Figure 3. Cocaine-induced expression of CD81 mRNA. Real time quantitative-PCR analysis of CD81 mRNA expression changes from cocaine-treated or untreated rat brains. Animals (n=6) were administered cocaine or saline according to the different protocols described in figure 1. After sacrifice total RNA was extracted from the NAcc or the VTA and CD81 mRNA was measured by means of quantitative RT-PCR. **(A): mRNA levels in the nucleus accumbens; (B): mRNA levels in the ventral tegmental area**. Relative abundance of candidate transcripts is expressed relative to GAPDH from replicate determinations (as described in the "Materials and Methods"). After normalization, the ratios between samples from cocaine-treated and saline-treated tissues were calculated. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

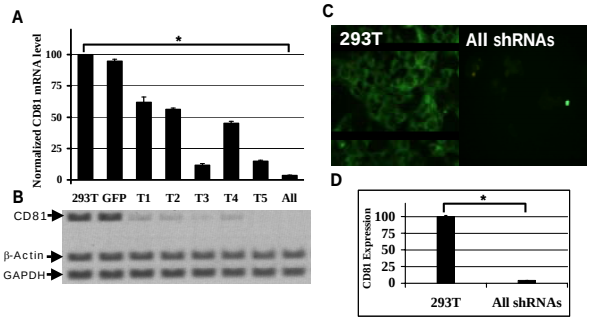

Figure 4. Expression of CD81 mRNA in HEK293T cells *in vitro* infected with Lenti-CD81 and Lenti-CD81-shRNAs: Quantification of CD81 mRNA levels was performed by means of real-time quantitative PCR **(A** & **B)** and immunohistochemistry **(C** & **D)** after HEK293T cells infection with Lenti-CD81 or/and with different Lenti-CD81-shRNAs, either separately or all targets combined together. HEK293T cells were co-infected by both constructs expressing CD81 and CD81-specific shRNAs. After 48h, culture medium was removed, one part of the cells was used for total RNA extraction, cDNA preparation and quantitative real-time PCR. **(A)**: Results from qRT-PCR after normalization against GAPDH and β-actin genes used as endogenous controls. Bars in the graphs represent means+SEM from 3 independent experiments and RNA preparations. **(B)**: PCR products were

verified by 2 % agarose gel electrophoresis and visualized with ethidium bromide staining. **(C)**: Remaining cells were used for immunocytochemistry using a mouse histidine-tag antibody to check for CD81 protein expression. **(D):** The expression level of CD81 was then quantified after background substraction.
293T: HEK293T cells infected with Lenti-CD81 alone; *GFP:* HEK293T cells infected with Lenti-GFP alone; *T 1-5:* HEK293T cells co-infected with Lenti-CD81 together with a single Lenti-CD81-shRNAs; *All:* HEK293T cells co-infected with Lenti-CD81 together with all five Lenti-CD81-shRNAs. *p<0.001.

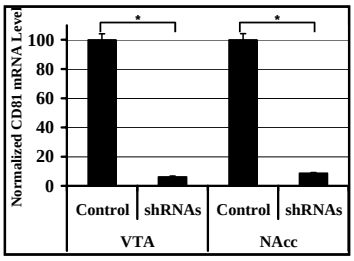

Figure 5. ***in vivo*** **silencing of endogenous CD81 in the VTA or NAcc with Lenti-CD81-shRNAs: quantification of endogenous CD81 transcripts by means of qRT-PCR**. Lenti-CD81-shRNAs or Lenti-GFP (in control animals) were bilaterally injected into the VTA or into the NAcc. Seven days after operation, each animal was injected i.p. with 30 mg/kg cocaine-HCl every 2 h for 4 injections, following the "binge" protocol (figure 1). Control animals received 0.9 % saline injections under the same schedule. The rats were sacrificed by decapitation 24 h after the last injection. The VTA and the NAcc were dissected out and used for total RNA isolation, cDNA preparation and quantitative real-time PCR using CD81 specific oligos. Results were normalized against GAPDH and β-actin genes used as endogenous controls.
VTA: Ventral Tegmentum Area; *NAcc:* Nucleus Accumbens; *GFP:* animals injected with Lenti-GFP; *shRNAs:* animals injected with Lenti-CD81-shRNAs. *p<0.001.

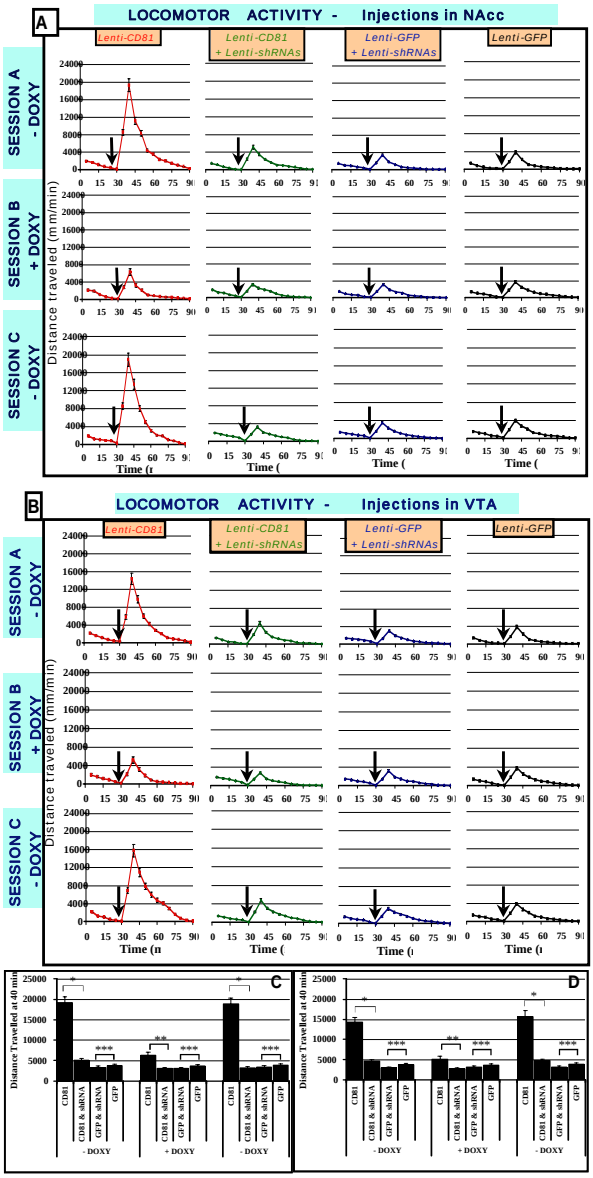

Figure 6. Effects of CD81 silencing *in vivo* on Cocaine-induced Behavioural changes. CD81 and CD81-shRNAs expression in the NAcc or VTA: Locomotor activity monitoring. Lenti-CD81 alone or Lenti-CD81 together with both Lenti-CD81-shRNA-3 and -5 were bilaterally injected into the NAcc **(Panel A)** or in the VTA **(Panel B**)(n=6). Control animals (n=6) were bilaterally injected with Lenti-GFP alone or Lenti-GFP together with Lenti-sh-RNA-3 and –5. Seven days after operation, session A (no doxycycline) was initiated and animals were chronically administered 15 mg/kg cocaine each day. Prior to daily cocaine injection, each animal was placed in the monitoring cage for 30 min until stable basal activity was measured. Cocaine (15 mg/kg, i.p.) was then injected (**Arrow**) and the animal returned to the cage for 60 min while locomotor activity monitoring was conducted. Each data point represents the average over 3 animals generated in each session. **Peak intensities of the locomotor activity measured at 45 min from the panels in A and B. Panel C** corresponds to the peak intensities in the NAcc.

Panel D corresponds to the peak intensities in the VTA. Each data point represents the average over 3 animals. Bars in the graphs represent means+SEM.
NAcc: Nucleus Accumbens; *VTA:* Ventral Tegmentum Area; *CD81:* animals injected with Lenti-CD81; *CD81 & shRNA:* animals co-injected with Lenti-CD81 and Lenti-CD81-shRNAs; *GFP & shRNA:* animals co-injected with Lenti-GFP and Lenti-CD81-shRNAs; *GFP:* animals injected with Lenti-GFP.
* p<0.001; ** p<0.05; *** p<0.08.

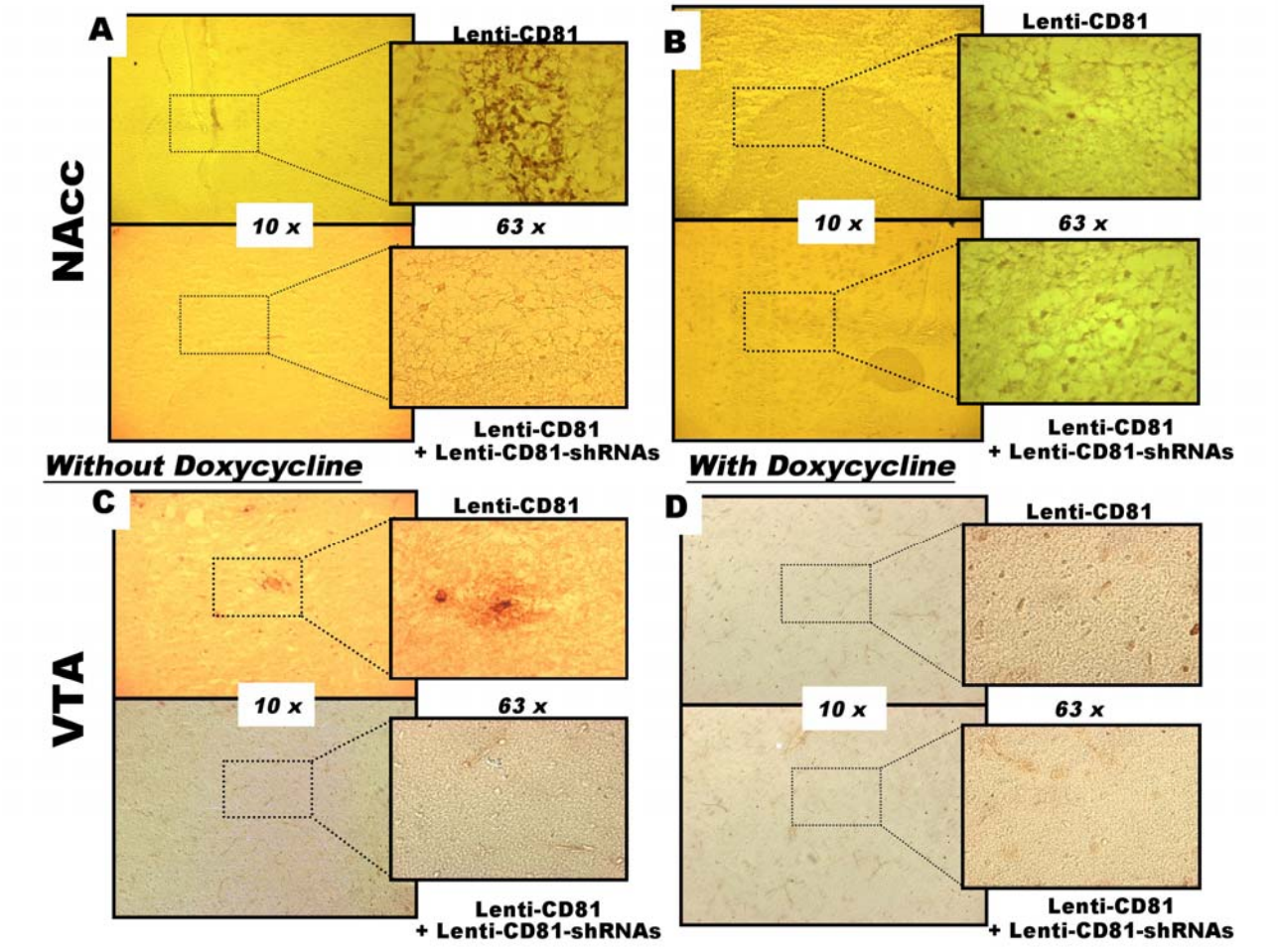

Figure 7: Immunohistochemistry of CD81 after *in vivo* transfer of Lenti-CD81 with or without Lenti-CD81-shRNAs into the NAcc ((Panel A and B) and into the VTA (Panel C and D): 2 µl of Lenti-CD81 were injected into the NAcc or VTA with and without 2 µl of Lenti-CD81-shRNAs (coordinates: see Material and Methods). Animals were given either 5 % sucrose (**Panel A and C**) or 5 % sucrose supplemented with 0.02 % doxycycline (**Panel B and D**). Animals were sacrificed before addition of doxycycline, (left panels) and at the end of the doxcycycline period (right panels) and immunohistochemistry was performed (see Methods). Slides were visualized with either 10X or 63X magnification.