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ORIGINAL ARTICLE

Nanotherapeutic Approach for Opiate Addiction Using DARPP-32 Gene Silencing in an Animal Model of Opiate Addiction

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Abstract Opiates act on the dopaminergic system of the brain and perturb 32 kDa dopamine and adenosine 3′, 5′-monophosphate-regulated phosphoprotein (DARPP-32) function. The DARPP-32 mediated inhibition of protein phosphatase-1 (PP-1) and modulation of transcriptional factor CREB is critical to the changes in neuronal plasticity that result in behavioral responses during drug abuse. To investigate the role of DARPP-32 mediated signaling on withdrawal behavior in a rat model of opiate addiction, we used intracerebral administration of gold nanorods (GNR) complexed to DARPP-32 siRNA to silence DARPP-32 gene expression and measure its effects on the opiate withdrawal syndrome. We hypothesized that DARPP-32 siRNA will suppress the neurochemical changes underlying the withdrawal syndrome and therefore prevent conditioned place aversion by suppressing or removing the constellation of negative effects associated with withdrawal, during the conditioning procedure. Our results showed that opiate addicted animals treated with GNR-DARPP-32 siRNA nanoplex showed lack of condition place aversive behavior consequent to the downregulation of secondary effectors such as PP-1 and CREB which modify transcriptional gene regulation and consequently neuronal plasticity. Thus, nanotechnology based delivery systems could allow sustained knockdown of DARPP-32 gene expression which could be developed into a therapeutic intervention for treating drug addiction by altering reward and motivational systems and interfere with conditioned responses.

Keywords DARPP-32 · Morphine · Opiate addiction · Nanotherapy · Gold nanorods (GNR) · Condition place preference (CPP) · Periaqueductal gray area (PAG) · Ventral Tegmental area (VTA) · Behavioral response

Introduction

Long-term use of opiates leads to tolerance, sensitization and physical dependence. Opiate dependence is characterized by enhanced neuronal excitability associated with up-regulation of the cAMP second messenger system. Opiate induced dopamine receptor stimulation activates the cAMP-dependent protein kinase A (PKA) pathway, leading to induction of transcription factors and phosphorylation of many substrate proteins involved in neuronal excitability (Fienberg AA et al. 1998; Greengard et al. 1998; Guitart-Masip et al. 2006; Colvis et al. 2005). Disruptions in the dynamic balance of dopamine-receptor mediated phosphorylation and dephosphorylation cascades may lead to impaired integration of synaptic inputs, causing altered neuronal communication resulting in the induction of transcription factors and their downstream targets

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causing long-lasting changes in neuronal plasticity (Nairn et al. 2004; Takahashi et al. 2005).

An important constituent of dopaminergic activities within the brain is the 32 kDa dopamine and adenosine 3′, 5′-monophosphate-regulated phosphoprotein (DARPP-32) recognized to be critical to the pathogenesis of drug addiction (Fienberg AA et al. 1998; Greengard et al. 1998; Guitart-Masip et al. 2006). Several drugs of abuse, including opiates act on the dopaminergic system of the brain and perturb DARPP-32 function. Dopamine D1 receptor agonists increase cAMP response element-binding protein (CREB) phosphorylation indirectly by activating expression of DARPP-32 (Flores-Hernandez et al. 2002; Snyder et al. 1998: Calabresi et al. 2000). Thus, DARPP-32 is a key factor in dopamine receptor mediated regulation of gene expression. Neurotransmitters like dopamine, through direct or indirect pathways, regulate the phosphorylation of DARPP-32 that has been shown to be involved in mediating the activities of virtually all neurotransmitters (Greengard et al. 1998, 1999; Nairn et al. 2004; Svenningsson et al. 2004, 2005). DARPP-32 function can be bi-directionally regulated, depending on which threonine (Thr) residue within the protein is phosphorylated (Nishi et al. 1997). Phosphorylation at Thr^{34} by PKA converts DARPP-32 into a potent inhibitor of the multifunctional serine/threonine protein phosphatase (PP-1). Thus, the regulation of DARPP-32 can cause an inhibition of PP-1 and a concomitant dysregulation of its downstream effector proteins, glycogen synthesis kinase-3 (GSK-3), CREB, and c-Fos. CREB mediates the morphine-induced upregulation of specific components of the cAMP pathway that contribute to physical opiate dependence (Chao and Nestler 2004; Mahajan et al. 2005).

The DARPP-32 mediated inhibition of PP-1 and modulation of transcriptional factors CREB/ERK is critical to the changes in behavioral responses, which are a consequence of alterations in neuronal plasticity with structural modification of neural networks in the CNS during drug abuse. These changes in neuronal plasticity could induce and reinforce psychological dependence and susceptibility to drugs, which become progressively obdurate. Since DARPP-32 plays a central role in regulating the efficacy of dopaminergic neurotransmission, we hypothesized that sustained knockdown of DARPP-32 in drug addicts would help in the treatment of drug addiction by altering reward and motivational systems and would interfere with conditioned responses.

In the current study, we used a gene therapy approach for drug addiction, which suppressed DARPP-32 expression; this would thereby be expected to prevent relapse of substance abuse by reducing the pharmacological effects and rewarding properties of the drugs on the brain. Sustained silencing of DARPP-32 gene expression using siRNA delivered to the brain in vivo may be a new approach for the treatment of drug addiction. Recent advances in the field of nanotechnology offer an unprecedented opportunity to enhance the power of siRNA mediated gene therapy by providing both an efficient delivery system as well as targeted specificity. We used gold nanoparticles, which can bind with siRNAs forming stable complexes called nanoplexes, and these nanoplexes can overcome all the impediments associated with siRNA in the free form. Gold nanoparticles are particularly attractive for therapeutic applications due to their biocompatibility and ease of complex formation with biomolecules. We were first to report the use of gold nanorods (GNR) for the delivery of siRNA against DARPP-32 in vitro (Bonoiu et al. 2009). Further, these nanoplexes were also found to transmigrate across an in vitro model of the blood brain barrier (BBB), without compromising the integrity of the barrier, while retaining their gene silencing efficiency.

To investigate the role of DARPP-32 mediated signaling on withdrawal behavior in rats, initially we used intracerebral administration of specific siRNA nanoplexes to silence DARPP-32 gene expression and measure its effects on the opiate withdrawal syndrome. There is a constellation of aversive behavioral and physiological symptoms during the first few days of abstinence from opiate use. These symptoms are collectively referred to as “opiate withdrawal syndrome”. We hypothesize that DARPP-32 siRNA will suppress the neurochemical changes underlying the withdrawal syndrome and therefore prevent place aversion conditioning by suppressing or removing the unconditioned stimulus (i.e., the constellation of negative effects associated with withdrawal) during the conditioning procedure.

The goal of our study was to stereotaxically administer the GNR-siRNA nanoplexes into a region of the rat brain associated with expression of withdrawal behaviors, the periaqueductal gray (PAG) (Bozarth 1994; Maldonado et al. 1992). The PAG extends afferent inputs to the ventral tegmental area (VTA), a region that is important in cognition, motivation and drug addiction; thus, PAG input could modulate VTA neuronal responses to opiates (Omelchenko and Sesack 2010). Specifically, we targeted neurons in the PAG region of the brain of opiate addicted animals and evaluated behavioral responses in this rat model. We used the conditioned place avoidance (CPA) test as measure of assessing opiate withdrawal symptoms (Fig. 1). Our results showed that opiate addicted animals that did not receive DARPP-32 specific siRNA treatment avoided the side of the apparatus associated with their withdrawal symptoms, whereas the non-addicted controls and the DARPP-32 nanoplex treated groups showed no avoidance behavior. Further, the opiate addicted animals treated with DARPP-32 nanoplexes, which showed lack of CPA behavior, had decreased DARPP-32 and PP-1, but increased CREB gene expression levels. The intracellular signaling pathways of the brain offer a unique opportunity for pharmacological intervention to treat drug addiction. These results suggest that silencing DARPP-32 gene expression

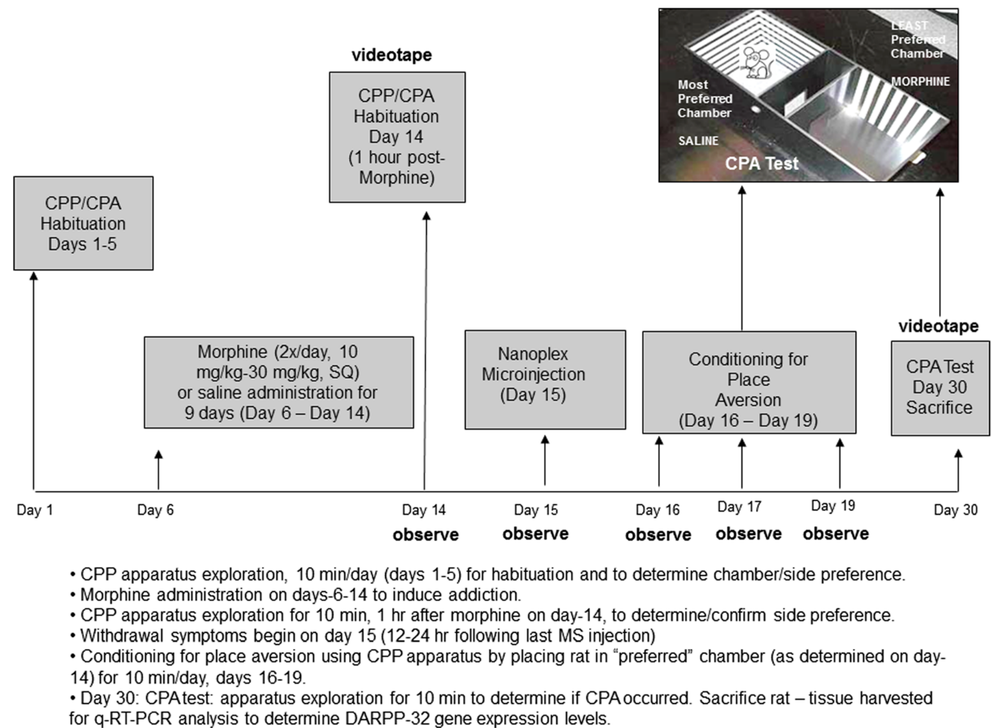

Fig. 1 Schematic of the study design

in vivo can be a novel strategy to treat and prevent addictive behavior in conjunction with behavioral therapy, thereby facilitating complete and persistent abstinence in drug addicted patients.

Materials and Methods

Synthesis of Gold Nanorods

GNRs were synthesized by the seed-mediated growth method in CTAB (cetyltrimethylammonium bromide) surfactant solution. CTAB forms rod-like micelles above its critical micelle concentration, forming the template for synthesis of GNRs. Briefly, 5 ml of 0.2 M CTAB (cetyltrimethylammonium bromide) solution was mixed with 5 ml of 0.96 mM $HAuCl_4$; 0.60 ml of ice-cold 0.01 M $NaBH_4$ was quickly added. The seed solution was vigorously stirred for 2 min, and kept at room temperature for 30 min. To this seed solution, 10 ml of 25 mM $HAuCl_4$ and 250 ml of HPLC grade water was added to 12.5 ml of 4.0 mM $AgNO_3$ solution at room temperature. 250 ml of 0.2 M CTAB and 5 ml of 0.08 M ascorbic acid was then added and gently mixed for 15 to 30 s. After the solution changed from orange to colorless, 0.6 ml of the seed solution was added to the growth solution. The mixture was left undisturbed and aged for 16–18 h at room temperature. GNRs were concentrated and separated by centrifugation. The positively charged CTAB-coated GNRs were further coated with two successive layers of polyelectrolytes, (a) the negatively charged PEDT/PSS poly (3,4ethenedioxythiophene)/-poly (styrenesulfate) and (b) the positively charged PDDAC poly (diallyldimethyl ammonium chloride). This polymeric multilayering was necessary to generate positively charged GNRs that "mask" the CTAB layer.

Nanoparticle Characterization

UV-visible absorption spectra were collected using an Agilent 8453 UV-visible spectrometer. The samples were measured against water as reference. Dynamic light scattering (Brookhaven 90PLUS with ZetaPALS option) was used to determine the size distribution. High-resolution TEM images were obtained using a JEOL model 4000EX microscope at an acceleration voltage of 400 kV. The specimens were prepared by dropping the sample onto an amorphous carbon-coated 300 mesh copper grid and allowing the solvent to evaporate. On characterization the GNR preparation had a dynamic light scattering (DLS) value of 30.2 nm, plasmon absorption wavelengths of 630 nm, and a zeta potential value of+25.6.

siRNA Sequences

The siRNA sequences for DARPP-32 (Accession #-AF464196) are: Sense- ACA CAC CAC CUU CGC UGA AAG CUG U, Antisense- ACA GCU UUC AGC GAA GGU GGU GUG U. The appropriate scrambled control siRNA sequences are, Sense- ACA CCC AUC CUC GGU

AAG ACA CUG U and Antisense- ACA GUG UCU UAC CGA GGA UGG GUG U.

Formation of the GNR-siRNA Nanoplex

The above synthesized cationic GNRs were electrostatically complexed with the DARPP-32 siRNA. Both the stock siRNA and the GNR preparations were made in Nuclease free water and complex formation was done in sterile phosphate buffered saline by gentle mixing and incubating the DARPP-32 siRNA-GNR complex for 20 mins at room temperature, prior to injection into the PAG. Prior to injection into the PAG each batch of free GNR, free siRNA (both DARPP-32 and scrambled) and various concentrations of GNR-siRNA complexes were evaluated for their ability to migrate across a 1.5 % agarose gel, and an optimal concentration of the GNR-siRNA nanoplexes was determined. The GNR-siRNA nanoplexes due to their increased size have limited mobility across the agarose gel as compared to free siRNA and GNR alone. Equal concentrations of appropriate scrambled siRNA electrostatically complexed to GNR were used as negative controls in all experiments.

Animals

Long Evans (hooded) rats (~200 g males, ~ 5 months old) obtained from Harlan Sprague–Dawley (Indianapolis, IN) were used for all experiments. The rats were housed in Laboratory Animal Facility-accredited pathogen-free quarters at 23±1 ° C, with access to food and water ad libitum. The animals were maintained on a 12 h light/dark cycle. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University at Buffalo as well as with the guidelines for the ethical treatment of animals established by the National Institute of Health. All efforts were made to ensure minimal animal suffering, as well as to use the minimum number of animals necessary to obtain reproducible results.

Animal Model of Opiate Addiction

After habituation to the condition placement apparatus (see below), rats were assigned to receive either morphine sulfate (MS) or vehicle (sterile saline) alone subcutaneously (s.c.). We used a progressive MS administration protocol to elicit dependence and mimic chronic opiate abuse (Fukunaga Y et al. 1998; Houshyar H et al. 2001; and Chu NN, et al. 2007). In this paradigm, rats received MS injections (s.c.) twice daily (12 h interval) for 8 days and once on day 9; injections began on day 6 of the experimental paradigm (Fig. 1) (incremental MS dose was 2.5 mg/kg per day from 10 mg/kg on the 1st day to 30 mg/kg on the 9th day of injections). Spontaneous withdrawal was induced by cessation of MS injections.

Physical Signs of Withdrawal

As signs of withdrawal are usually evident for up to 3–4 days post-MS cessation, the physical signs of withdrawal were observed and recorded 1 day following cessation of MS injections and continued every other day for 5 days (corresponding to days 15, 17, and 19 of the experimental paradigm; Fig. 1). Behavioral observations taken as measurement of physical signs of withdrawal included: teeth chattering, wet dog shakes, rearing, stretching, and penis licking. The rats were monitored for 10 min on days 15, 17, and 19 (prior to CPA conditioning, see below) for the above symptoms. Four rats were observed/videotaped at once using a Plexiglas apparatus consisting of 4 adjacent clear plastic chambers measuring 30 cm X 30 cm. Symptoms were counted every 2 min, respectively, and the appearance of a physical sign was scored as "1"; otherwise, a score of "0" was assigned for the 2 min period. The maximum score for any one of the signs would be "5" (25 maximum points for five symptoms). Another sign frequently observed during withdrawal is diarrhea. Since this is not 'countable', if an increase in defecation and/or diarrhea was noted, an assignment of "1" was given for the entire 10 min period. Assessment of body weight was analyzed separately.

Because rats receiving microinjection surgery (resulting in surgical staples on the head) tend to "shake" their body/head to try to dislodge the staples, we did not include the "wet dog shakes" scores in the analyses; therefore, scores are based out of a total of 20.

Conditioned Place Aversion (CPA) Procedure

CPA (environmental place aversion) is a commonly used technique to evaluate aversions for environmental stimuli that have been associated with a negative reward. The CPP/CPA apparatus consists of two main, equal sized chambers, where each differs in its visual and tactile characteristics. These distinct chambers are separated by a narrow space with a neutral platform. Animals were exposed to the apparatus during the preconditioning or habituation phase, the conditioning phase, and the testing phase of the experimental protocol (Fig. 1). In the habituation period (Day 1-Day 5), each animal was placed on the neutral platform and allowed free access to both sides of the apparatus for 10 min of exploration each day. This apparatus is used regularly in our lab and elicits an unbiased (neutral) response from the rat upon first exposure; rats show no bias for either side. On day 14 of the paradigm, 1 h after the final MS injection, a pre-test for unconditioned chamber preference was performed whereby rats were released to explore the condition placement apparatus for 10 min. While no significant preference (calculated as the ratio of the time spent in each chamber of the apparatus to the total time in the apparatus) was displayed by the animals, the chamber in which the

animal spent the most time was noted and considered the 'preferred' chamber. In the conditioning phase (days 16–19), the Plexiglas dividers remained in place and the rat was confined to one side of the apparatus – the 'preferred' chamber – for 10 min. During testing (10 min session on day 20 and day 30), the dividers in the apparatus are lifted to allow the rat access to both chambers, and the amount of time spent on each side is measured. A 2×2 (Drug History [morphine or vehicle injections] X DARPP-32 or negative control treatment) factorial design is used with no repeated measures. The dependent measure is the conditioned place aversion score (time spent in the place associated with withdrawal minus time spent in the same place during the pre-withdrawal state). It is expected that rats addicted to morphine, but not the DARPP-32 nanoplex treated addicted animals, will have a negative score demonstrating that they avoid the place associated with the opiate withdrawal period.

Intra-PAG Microinjection and siRNA-Targeted Knockdown

Animals were anesthetized with an intraperitoneal (i.p.) injection of ketamine (75 mg/kg) and xylazine (10 mg/kg) and were secured on a stereotaxic platform. Using the stereotaxic coordinates (Paxinos G and Watson C 1997) to target the dorsolateral PAG (DL-PAG): 7.8 mm posterior to bregma (anterior-posterior); 0.6 mm lateral to the midline (medial-lateral); 4.5 mm below the dura (dorsal-ventral), a hole was drilled through the skull, which received the injection using a 30G stainless steel needle on a 10 μl Hamilton syringe held by the micromanipulator on the stereotaxic apparatus. The nanoparticles containing siRNA (3 μl vol.) were injected at a rate of 0.5 μl/min. The needle remained in place for another 3 min after the injection to allow for sufficient diffusion. After this time, the needle was slowly removed. The scalp incision was closed with sterile surgical staples. Intra-DL-PAG injection of the nanoplexes contained ~0.5 nmol (0.03–1 nmol) of siRNA; this concentration is significantly less than that used for continuous microinfusion studies (120 nmol=15 mg/kg) since the injection is only expected to target neurons in the PAG region with little to no diffusion to adjacent regions, thereby necessitating less siRNA. Verification of the injection site was confirmed by gross morphology of the midbrain region at dissection.

Cell Culture

Human dopaminergic neuronal precursor (DAN) cells were obtained from Clonexpress, Inc (Gaithersburg, MD; Cat No: DAN 020). Actively growing population of cells were tested for tyrosine hydroxylase (TH) expression by immunocytochemistry. DAN cells are supplied with a proprietary growth factor supplement (DNCS) as a 100X stock solution, which is added to DMEM:F12 (50:50) containing 5 % FBS and 10 ng/ml of Basic Fibroblast Growth Factor (bFGF) (R& D Systems Cat #234-FSE-025), and 5 ng/ml of Glial Cell Line-derived Neurotrophic Factor (GDNF) (R& D Systems Cat # 212-GD-010) to make DAN cell growth medium. The cells are grown in regular tissue culture dishes and subcultured at a split ratio of 1:2 at confluence. These cells differentiate into neurons within a week, when plated on polylysine (PLL) coated plates at a density of approximately 10^4 cells per sq.cm. in DMEM/F12 (50:50) supplemented with DNC5, 5 % FBS, 10 ng/ml of bFGF, 10 ng/ml of recombinant human Epidermal Growth Factor (EGF), and 100 uM dibuturyl cAMP. Typically DAN cells are used within 2–8 passages.

RNA Extraction and Quantitative-Real Time-PCR

Upon dissection of the rat brain, the PAG region and the region of the midbrain containing the VTA, were each placed into 1 ml RNA*later* solution (Ambion) and stored at −80 °C until RNA extraction. Total RNA was extracted by an acid guanidinium-thiocynate-phenol-chloroform method as described using TRIzol reagent (Invitrogen-Life Technologies). The amount of RNA was quantitated using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop) and isolated RNA was stored at −80 °C until used.

Quantitative real-time PCR (Q-RT-PCR) was used to quantitate the relative abundance of DARPP-32, PP-1, CREB mRNA species using specific primers and the Brilliant SYBR green Q-PCR master mix from Strategene. RNA was reverse transcribed to cDNA using the reverse transcriptase kit from Promega. Relative expression of mRNA species was calculated using the comparative threshold cycle number (C_T) method (Radonic A et al. 2004). Briefly, for each sample, a difference in C_T values ($^{\Delta}C_T$) is calculated for each mRNA by taking the mean C_T of duplicate tubes and subtracting the mean C_T of the duplicate tubes for the reference RNA (β-actin) measured on an aliquot from the same RT reaction. The $^{\Delta}C_T$ for the treated sample is then subtracted from the $^{\Delta}C_T$ for the untreated control sample to generate a $^{\Delta\Delta}C_T$. The mean of these $^{\Delta\Delta}C_T$ measurements is then used to calculate the levels in the targeted cytoplamic RNA relative to the reference gene and normalized to the control as follows: Relative levels or Transcript Accumulation Index=$2^{-\Delta\Delta CT}$. This calculation assumes that all PCR reactions are working with 100 % efficiency. All PCR efficiencies were found to be >95 %; therefore, this assumption introduces minimal error into the calculations. All data were controlled for quantity of RNA input and by performing measurements on an endogenous reference gene, β-actin.

Estimation of Phospho- CREB (Ser133) Protein Levels Using ELISA

We used the commercially available InstantOne™ ELISA (eBioscience, San Diego, CA; Cat #85-86152) to accurately

measure phosphorylated CREB (Ser133) in brain tissue lysates as per manufacturers instruction. Upon dissection, rat brain tissue (VTA region)~30 mg, was placed into 500 μl of N-PER Neuronal Protein Extraction Reagent (Thermo Scientific), which is a proprietary cell lysis reagent optimized for efficient extraction of proteins from brain tissue and primary cultured neurons. Using this reagent we were able to obtain protein yields of ~75 μg of protein per mg of brain tissue. Extracted protein was stored at −80 °C until further use. The amount of protein was quantitated using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop).

Statistical Analysis

All behavioral data are expressed as means ± SEM, and gene and protein expression data are expressed as means ± SD and analyzed using SPSS v11 software. In the analysis of CPA, statistical analysis was performed using an analysis of variance (One-way ANOVA or Two-way ANOVA). F-test analysis was performed to identify significant differences among any of the group means and in case of a statistically significant F-ratio score ($p<0.05$), a second post hoc analysis using Tukey's test was done, where sets of two groups at a time were compared to specifically determine significance differences. A difference was accepted as significant when $p<0.05$.

Results

Nanoplex Formation

A schematic of the nanoplex formation using cationic GNRs that are electrostatically complexed to siRNA$^{DARPP-32}$ is shown in Fig. 2a. Transmission electron microscopy was done to determine the size of the GNR before and after complexation with siRNA$^{DARPP-32}$ (nanoplex formation). As shown in Fig. 2b, the distribution of GNRs indicates no significant aggregation of the nanoparticles before or after nanoplex formation. The binding efficiency of Cy3 labeled siRNA$^{DARPP-32}$ with GNRs was confirmed using agarose gel electrophoresis (Fig. 2c). This data indicates the complexation of Cy3 labeled siRNA$^{DARPP-32}$ with GNRs. The highest loading of GNRs with siRNA that was used for the stereotaxic injection into the PAG region of the rat brain was calculated to be 2 μg/nmol. The size of nanoplexes using dynamic light scattering is estimated at 30.2 nm, and surface charge (zeta potential) measurements of free GNRs is+25.6 mV and upon complexation with siRNA is −2.7 mV. The nanoplexes remain stable for more than 1 month post-complexation. Figure 2d shows a significant shift in the longitudinal surface resonance peak of GNR after complexation with siRNA$^{DARPP-32}$.

Release Kinetics of siRNA$^{DARPP-32}$ from the GNR-siRNA$^{DARPP-32}$ Nanoplex in Vitro

The release of siRNA molecules from the GNR-siRNA nanoplex was studied at neutral pH (~7.2) up to 2 weeks post-transfection in vitro using primary dopaminergic neural cells, so as to mimic the physiological conditions. Fluorescently labeled (6-FAM) siRNA was complexed with the GNR. Confocal microscopy was used to analyze the FAM labeled siRNA release, and releasate was collected at regular intervals and analyzed by TEM and DLS for presence and size of released siRNA-GNR nanoplexes, thus both the free siRNA (released from the nanoplex) and GNR-siRNA nanoplexes released from cells in culture were measured. The amount of siRNA released overtime post-transfection is shown in Fig. 3. The siRNA release rate is extremely low on Day 1 with less than 5 % being released in the initial 24 h, however almost 80 % of the loaded siRNA is released on Day 12 post-transfection.

Knockdown of DARPP-32 Gene Expression in Both the PAG and VTA Regions of the rat Brain Following Microinjection of GNR-DARPP-32-siRNA^{Cy3} Nanoplex

A single microinjection of GNR-DARPP-32 siRNA^{Cy3} nanoplexes was administered into the DL-PAG region of the brain, and gene silencing was determined by measuring the percentage inhibition of DARPP-32 expression using quantitative real-time PCR (Q-RT-PCR). We examined DARPP-32 gene expression both in the PAG and VTA regions of the rat brain at 5 and 15 days post administration of the GNR-DARPP-32 siRNA^{Cy3} nanoplex into the DL-PAG region of the rat brain. Previous studies have shown levels of DARPP32 (Thr34) were induced by morphine and increased in the VTA region of rats that had maintained the morphine-induced place preference (Narita M et al. 2010). Treating the morphine administered rats with the GNR-DARPP-32 siRNA^{Cy3} nanoplex resulted in a significant decrease in DARPP-32 gene expression. Data in Fig. 4 shows DARPP-32 gene expression levels in both PAG and VTA regions on days 5 and 15 post DL-PAG injection in animals administered morphine. We observed approximately 44 % suppression ($p<0.01$) of DARPP-32 gene expression in brain tissue from the PAG region at 120 h (5 days) post-DL-PAG injection ($n=5$), and sustained knockdown i.e., 48 % suppression ($p<0.01$) of DARPP-32 gene expression ($n=5$) on day 15 post-DL-PAG injection when compared to the untreated controls (no siRNA and no nanoparticle) or to rats that received the control scrambled

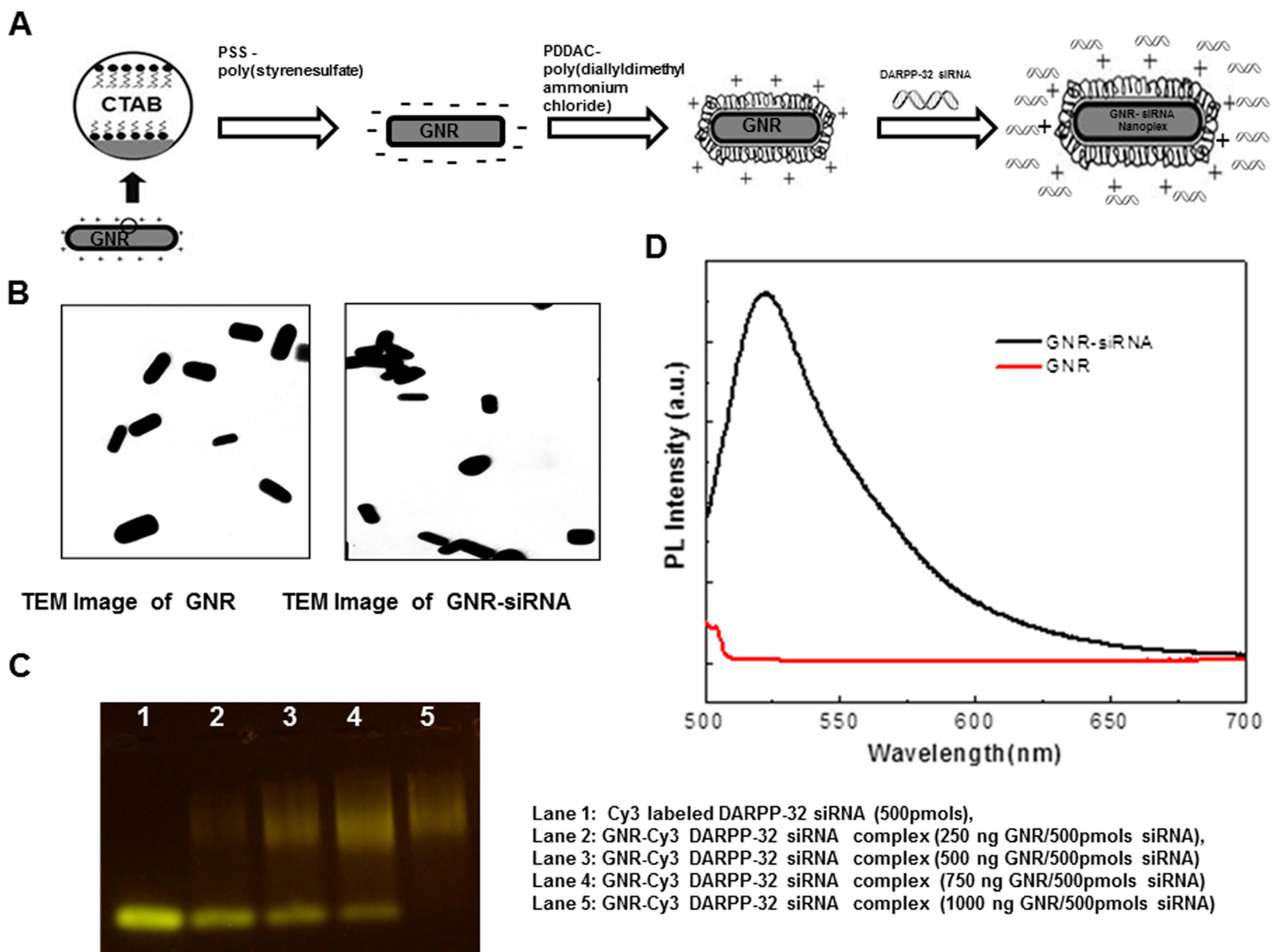

Fig. 2 GNR-siRNA nanoplex formation. **a** Schematic presentation of nanoplex formation. Cationic coated GNRs electrostatically couple with negatively charged siRNA to form stable nanoplexes. **b** The size of GNR and the GNR-siRNA nanoplex using transmission electron microscopy (*TEM*) (*i*) representative TEM image of GNR alone and (*ii*) representative TEM image GNR-siRNA nanoplex. **c**. Representative gel image that demonstrates the electrophoretic mobility of the nanoplex, which was done to determine the optimal binding efficiency of the GNR-siRNA nanoplex. Increasing amounts of GNR (250–1000 ng) were complexed to 500pmols Cy3 labeled DARPP-32 siRNA. An 1.5 % agarose gel was run for 1 h at 75 V, the electrophoretic mobility of these nanoplexes was compared to that of equivalent amount of free siRNA, and the resulting gel bands were quantified using the gel documentation system software (Bio-Rad). Lane 1: Free siRNA [Cy3 labeled DARPP-32 siRNA] (500pmols) alone; Lane 2: GNR-Cy3 labeled DARPP-32 siRNA complex (250 ng GNR/500pmols siRNA); Lane 3: GNR-Cy3 labeled DARPP-32 siRNA complex (500 ng GNR/500pmols siRNA); Lane 4: GNR-Cy3 labeled DARPP-32 siRNA complex (750 ng GNR/500pmols siRNA); Lane 5: GNR-Cy3 labeled DARPP-32 siRNA complex (1000 ng GNR/500pmols siRNA). D) Photoluminescence (*PL*) spectroscopy done to measure adsorption and emission wavelengths of GNR alone and GNR-siRNA nanoplexes. The absorption spectra were collected using a Shimadzu model 3101PC UV–vis-NIR scanning spectrophotometer over the range from 500 to 700 nm. The samples were measured against water as reference. The PL spectra were collected using a Spectrofluorometer (Fluorolog®-3, Horiba Jobin Yvon)

siRNA nanoplexes. In brain tissue from the VTA region, we observed a 32 % suppression ($p<0.05$) of DARPP-32 gene expression at 5 days ($n=7$), and a 38 % suppression ($p<0.05$) ($n=7$) on day 15 post-DL-PAG injection, as compared to untreated controls or to rats that received scrambled siRNA nanoplexes (Fig. 4). No significant differences in DARPP-32 gene expression levels were observed between untreated controls and the control scrambled siRNA nanoplexes treated rats. Statistical analysis was done using an average of the day 5 and day 15 gene expression values obtained for the untreated controls and/or the control scrambled siRNA nanoplexes treated rats.

Modulation of PP-1 and CREB Expression Following Microinjection of GNR-DARPP-32-siRNA^{Cy3} Nanoplexes

We examined the gene expression levels of secondary mediators PP-1 and CREB both in the PAG and VTA regions of the rat brain at 5 and 15 days post administration of the GNR-DARPP-32 siRNA^{Cy3} nanoplex into the DL-PAG region in the morphine addicted animals. Figure 5 shows PP-1 gene

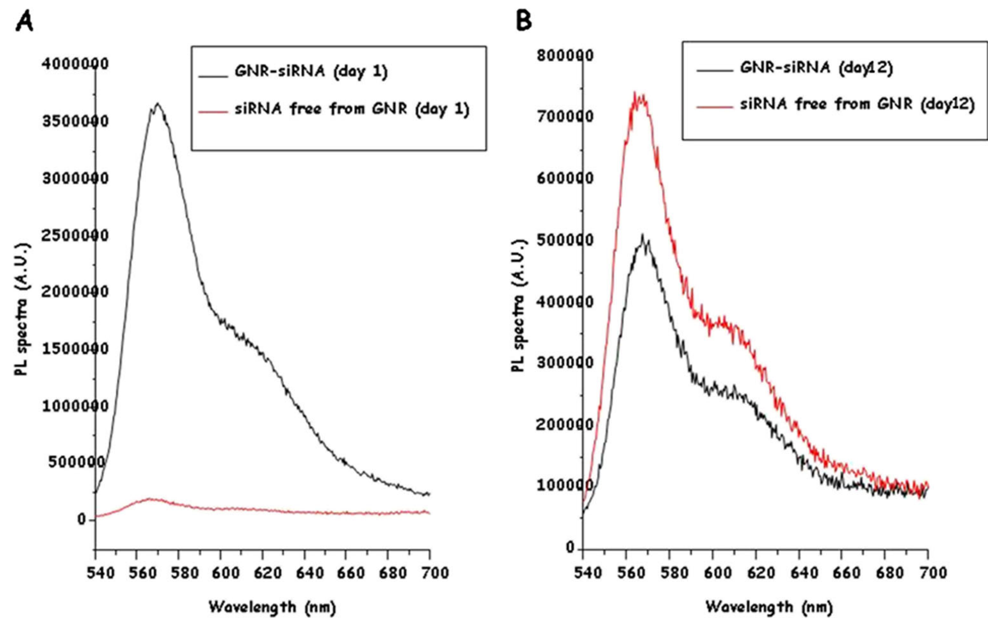

Fig. 3 Release kinetics of siRNA from GNRs. Human dopaminergic neuronal (*DAN*) cells transfected with GNR-siRNA^{Cy3} nanoplexes were monitored up to 2 weeks post-transfection to evaluate the release of siRNA^{Cy3} into the cytoplasm. Both GNR-siRNA^{Cy3} nanoplexes and free siRNA^{Cy3} (released from GNRs) were assessed at: **a** day 1 and **b** day 12 post-transfection by measuring the emission at 570 nm. Data is representative of duplicate experiments

expression levels in both PAG and VTA regions at 5 and 15 days post DL-PAG injection. We observed a 47 % suppression ($p<0.01$) of PP-1 gene expression in brain tissue from the PAG region on day 5 ($n=7$), and a 43 % suppression ($p<0.05$) ($n=7$) on day 15 post-DL-PAG injection, as compared to the untreated controls. In the VTA brain tissue, we observed a 53 % suppression ($p<0.01$) on day 5 ($n=7$), and a 58 % suppression ($p<0.01$) ($n=7$) on day 15 post-DL-PAG injection, as compared to untreated controls. Figure 6 shows CREB gene expression levels in both PAG and VTA regions at 5 and 15 days post DL-PAG injection. We observed a 59 % suppression ($p<0.001$) of CREB gene expression in brain tissue from

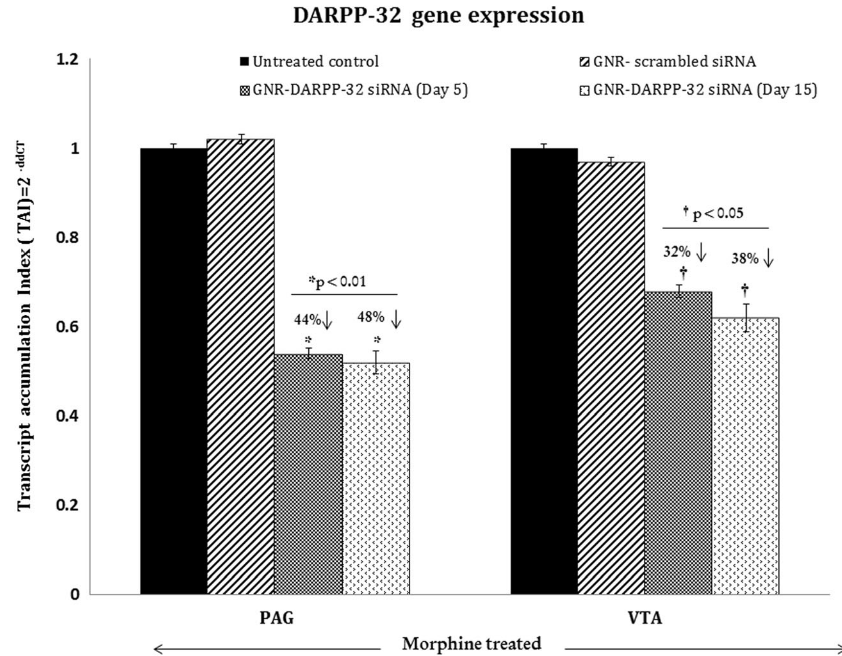

Fig. 4 Knockdown of DARPP-32 gene expression using GNR-DARPP-32 siRNA^{Cy3} in the rat brain. A single injection (3 μl; 0.5 μl/min) of GNR-DARPP-32 siRNA^{Cy3} (0.5 nmolsiRNA/3 μl volume) was administered into the DL-PAG region of the rat brain (or GNR-scrambled siRNA^{Cy3} for comparison purposes). The Q-RT-PCR data show suppression of DARPP-32 gene expression in the PAG and VTA regions of the brain at 5 and 15 days post-injection in morphine addicted animals. Statistical comparisons were made between GNR-DARPP-32 siRNA^{Cy3} treated animals and the untreated and/or scrambled siRNA nanoplex injected controls. Statistical analysis was done using an average of the day 5 and day 15 gene expression values obtained for the untreated controls and/or the control scrambled siRNA nanoplexes treated rats. Results are expressed as the mean ± SD. A p value of <0.05 is considered a statistically significant difference

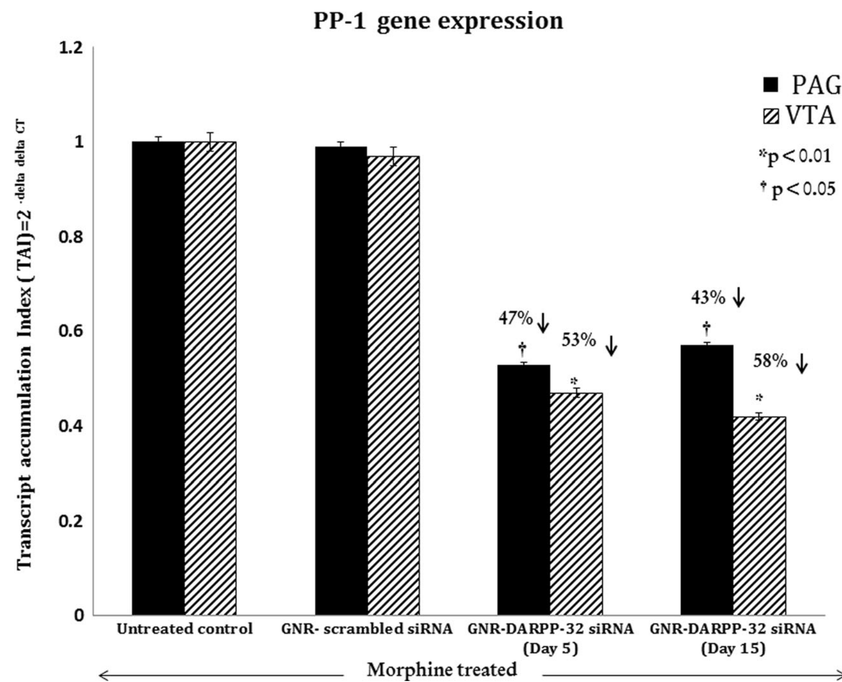

Fig. 5 PP-1 gene expression levels in the PAG and VTA brain tissue in which DARPP-32 gene expression is downregulated via DL-PAG injection of GNR-DARPP-32 siRNA^{Cy3}. The Q-RT-PCR data shows suppression of PP-1 gene expression in the PAG and VTA region at 5 and 15 days post-injection, respectively, in morphine addicted animals. Statistical comparisons were made between GNR-DARPP-32 siRNA^{Cy3} treated animals and the untreated and/or scrambled siRNA injected controls. Statistical analysis was done using an average of the day 5 and day 15 gene expression values obtained for the untreated controls and/or the control scrambled siRNA nanoplexes treated rats. Results are expressed as the mean ± SD. A p value of <0.05 is considered a statistically significant difference

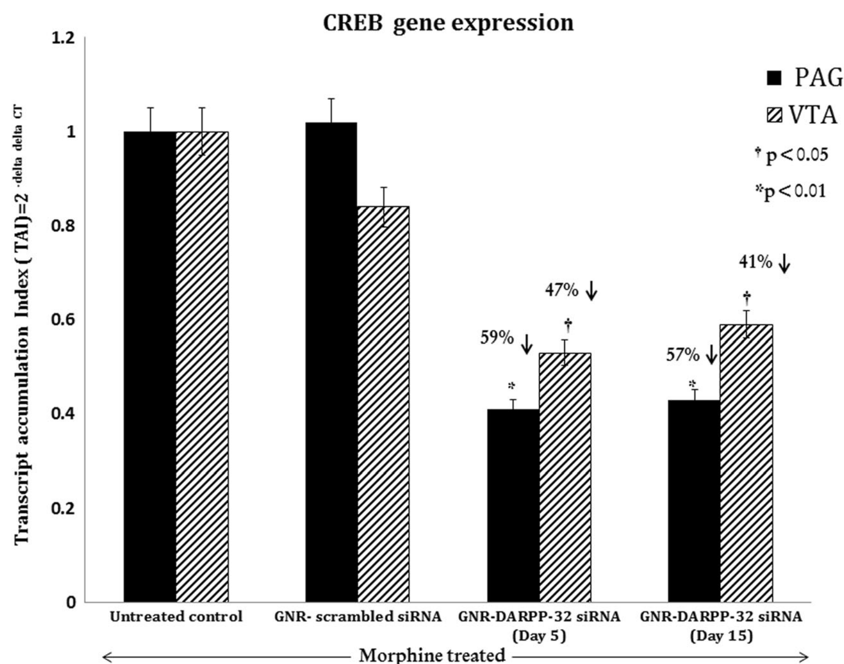

Fig. 6 CREB gene expression levels in the PAG and VTA brain tissue in which DARPP-32 gene expression is downregulated via intra-DL-PAG GNR-DARPP-32 siRNA^{Cy3} injection. The Q-RT-PCR data shows suppression of CREB gene expression in the PAG and VTA region at 5 and 15 days post-injection, respectively, in morphine addicted animals. Statistical comparisons were made between GNR-DARPP-32 siRNA^{Cy3} treated animals and the untreated and/or scrambled siRNA injected controls. Statistical analysis was done using an average of the day 5 and day 15 gene expression values obtained for the untreated controls and/or the control scrambled siRNA nanoplexes treated rats. Results are expressed as the mean ± SD. A p value of <0.05 is considered a statistically significant difference

the PAG region on day 5 (n=7), and a 57 % suppression (p<0.01) (n=7) on day 15 post-DL-PAG injection, as compared to the untreated controls -. In the VTA brain tissue, we observed a 47 % suppression (p<0.01) on day 5 (n=7), and a 41 % suppression (p<0.01) (n=7) on day 15 post-DL-PAG injection, as compared to untreated controls. No significant differences in PP-1 and CREB gene expression levels were observed between untreated controls and the control scrambled siRNA nanoplexes treated rats. Statistical analysis was done using an average of the day 5 and day 15 gene expression values obtained for the untreated controls and/or the control scrambled siRNA nanoplexes treated rats.

We evaluated the phospho-CREB levels in the protein lysates of brain tissue isolated from the PAG and the VTA region in animals above on day 5 and 15 post DL-PAG injection. Figure 7 shows data from the PAG region showing a 10-fold (p<0.01) (n=7) and a 14-fold (p<0.001) (n=7) increase in phospho-CREB levels on day 5 and 15 respectively as compared to the untreated controls . In the VTA brain tissue lysates, we observed a 6.7 fold (p<0.01) (n=7) and 7.6 fold (p<0.01) (n=7) increase in phospho-CREB (Ser 133) protein expression levels on day 5 and 15 respectively as compared to the untreated controls. Phospho-CREB levels were measured using a commercially available ELISA kit (phospho-CREB (Ser133) InstantOne™ ELISA by eBioscience; san Diego, CA; Cat # 85-86152-11). Statistical analysis was done using an average of the day 5 and day 15 gene expression values obtained for the untreated controls and/or the control scrambled siRNA nanoplexes treated rats.

Effect of Morphine on Animal Weight

Long Evans Hooded male rats were weighed every day up through day 19, and every other day thereafter until day of euthanasia. Body weight was assessed as another physical sign of morphine dependence and withdrawal. Comparisons were made between saline and morphine administered animals in all 3 groups, namely saline alone vs morphine alone, saline + GNR-scrambled siRNA vs morphine + GNR-scrambled siRNA, saline + GNR-DARPP-32 siRNA vs morphine + GNR-DARPP-32 siRNA. We observed a significant lack of weight gain in the rats during the 9 day morphine administration period (day 6–day 14, progressive schedule=10 mg/kg to 30 mg/kg; with 2.5 mg/kg incremental increases per day), and significant body weight loss in rats during the withdrawal period (days 15–17) (Fig. 8).

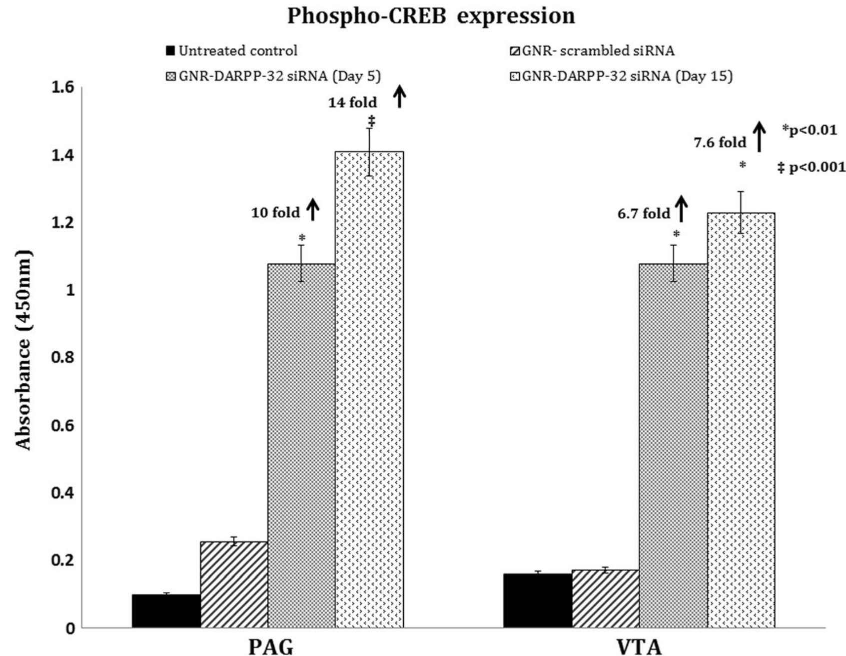

Fig. 7 phospho-CREB expression levels in tissue lysates from PAG and VTA brain tissue in which DARPP-32 gene expression is downregulated via intra-DL-PAG GNR-DARPP-32 siRNA^{Cy3} injection. phospho-CREB (Ser 133) protein expression in cell lysates were measured using a commercially available phospho-CREB (Ser133) InstantOne™ ELISA kit. Our results show a significant increase in phospho-CREB expression levels in both the PAG and VTA region at 5 and 15 days post-injection, respectively, in morphine addicted animals. Statistical comparisons were made between GNR-DARPP-32 siRNA^{Cy3} treated animals and the untreated and/or scrambled siRNA injected controls. Statistical analysis was done using an average of the day 5 and day 15 gene expression values obtained for the untreated controls and/or the control scrambled siRNA nanoplexes treated rats. Results are expressed as the mean ± SD. A p value of <0.05 is considered a statistically significant difference

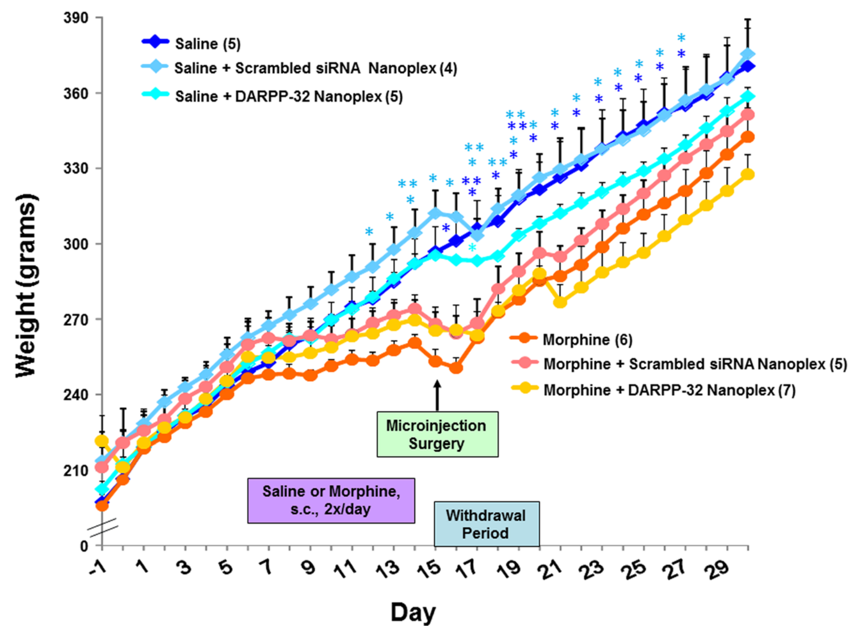

Fig. 8 Morphine administration significantly decreases body weight. Animals administered MS exhibit withdrawal symptoms and decreased weight gain. Comparisons were made between saline and morphine administered animals in all 3 groups, namely saline alone vs morphine alone, saline + GNR-scrambled siRNA vs morphine + GNR-scrambled siRNA, and saline + GNR-DARPP-32 siRNA vs morphine + GNR-DARPP-32 siRNA. A significant decrease in weight gain was observed in the rats during the 9 day morphine administration period and during the withdrawal period. Results are expressed as the mean ± SEM. Numbers in parentheses indicate number of animal in each group. A p value of <0.05 is considered a statistically significant difference

Behavioral Conditioned Place Avoidance (CPA) Testing in Long Evans Hooded Rats Administered Morphine Sulfate (MS) and Experiencing Natural Withdrawal

Figure 9 shows that the preference score of saline rats was not significantly different between the pre- and CPA tests. Cessation of MS administration induced withdrawal as observed by development of CPA. Rats that experienced morphine withdrawal spent significantly less time in their "preferred" chamber following extinction of the withdrawal symptoms as compared to saline administered rats (218±24 s vs 343±31 s, respectively, $p=0.03$).

Behavioral Scores for the Physical Signs of Withdrawal

The physical signs of morphine withdrawal were observed on days 15, 17 and 19 by rats that had received MS for 9 days followed by cessation of MS administration. Figure 10 shows the difference scores of physical signs of withdrawal in saline ($n=6$) vs morphine ($n=7$) rats on days 15, 17 and 19. Morphine addicted rats showed significant withdrawal signs as compared to the saline group on each day. Figure 11 shows the total averaged scores of physical signs of withdrawal in the animals with the microinjection of the nanoplexes into the DL-PAG region, and multiple comparisons were performed between the following groups: saline + scrambled siRNA nanoplex ($n=4$), saline + DARPP-32 siRNA ($n=5$), morphine + scrambled siRNA nanoplex ($n=5$), morphine + DARPP-32 siRNA ($n=7$) on days 15, 17 and 19, respectively. Significant withdrawal signs in the morphine + scrambled siRNA and morphine + DARPP-32 siRNA nanoplex treated animals as compared to their respective saline treated counterparts were observed on Day 19; Morphine + scrambled nanoplex vs saline + scrambled nanoplex ($p<0.05$) and Morphine + DARPP-32 nanoplex vs saline + DARPP-32 nanoplex ($p<0.05$). A p value of < 0.05 indicates a statistically significant difference.

Behavioral Effect of DARPP-32 Silencing as Evidenced by Conditioned Place Aversion

Figure 12 shows the difference score values in seconds between pre-test and CPA test and demonstrates that DL-PAG microinjection of the DARPP-32 nanoplex into morphine addicted rats approached a difference score similar to saline control groups. Unlike morphine addicted rats that demonstrated significant withdrawal-induced aversion difference scores in the condition placement test as compared to saline rats, the Morphine + DARPP-32 nanoplex group difference scores did not differ from the respective Saline + DARPP-32 nanoplex control group.

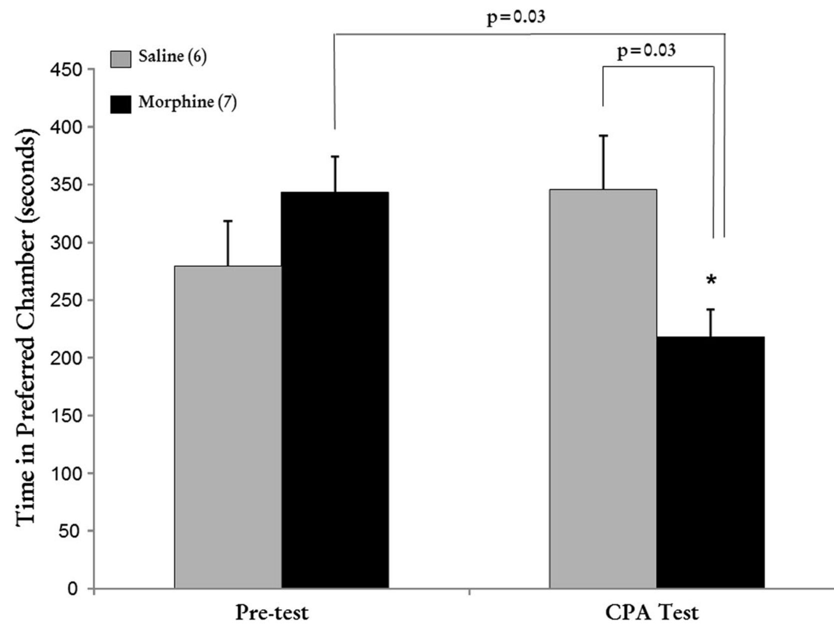

Fig. 9 Conditioned place avoidance (*CPA*) testing in Long Evans hooded rats administered morphine sulfate (*MS*) and experiencing natural withdrawal. After 5 days of habituation/pre-testing in the CPP apparatus, a progressive MS administration protocol was used to mimic chronic opiate abuse. Control rats were administered sterile saline similarly. Rats were tested for chamber preference in the CPP apparatus and were then conditioned for place aversion by placing them in the preferred chamber for 10 min each day during the withdrawal period. CPA testing was on either day-20 or day-30 of the paradigm. The preference score of saline rats was not significantly different between the pre- and CPA tests. Chronic MS administration cessation induced withdrawal as observed by development of CPA. Rats that experienced MS withdrawal spent less time in their "preferred" chamber following extinction of the withdrawal symptoms as compared to saline administered rats, * $p=0.03$, One way repeated measures ANOVA followed by Student-Newman-Keuls *post-hoc* analysis. Numbers in parentheses indicate number of animal in each group

Discussion

Increased dopamine release and augmented dopaminergic transmission in the PAG region is a common effect elicited by opiates such as morphine resulting in the activation of the cAMP/PKA/DARPP-32 signaling cascade, which modulates the levels of downstream target proteins and transcriptional regulators, thereby altering neuronal plasticity and

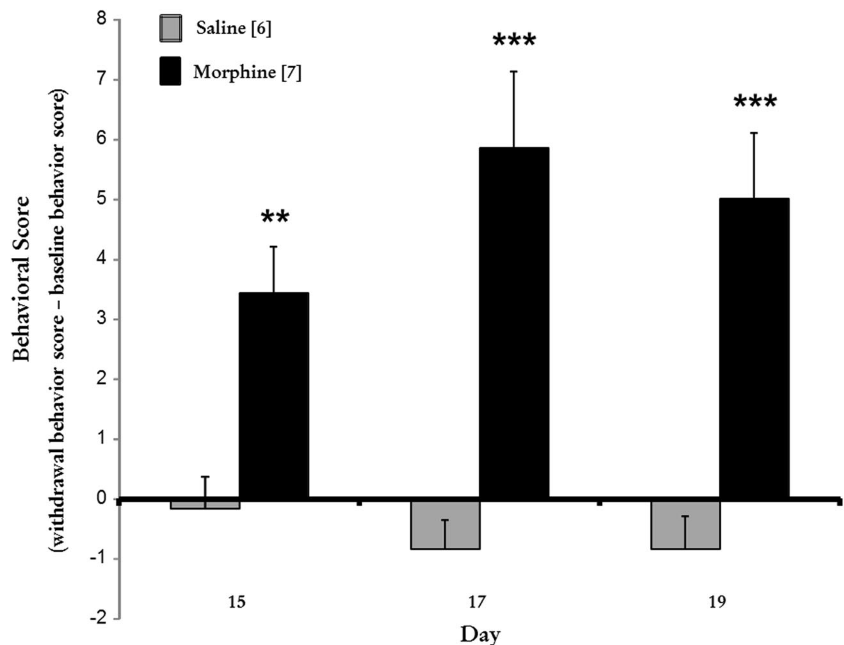

Fig. 10 Behavioral scores for the physical signs of withdrawal. The total averaged scores of physical signs of withdrawal, expressed as the difference of baseline scores from withdrawal scores in seconds, in each group are indicated. Each bar represents the mean ± SEM. **a** Student's *t*-test: ** $p<0.01$; *** $p<0.001$, as compared to the saline group at each day. Numbers in parentheses indicate number of animal in each group

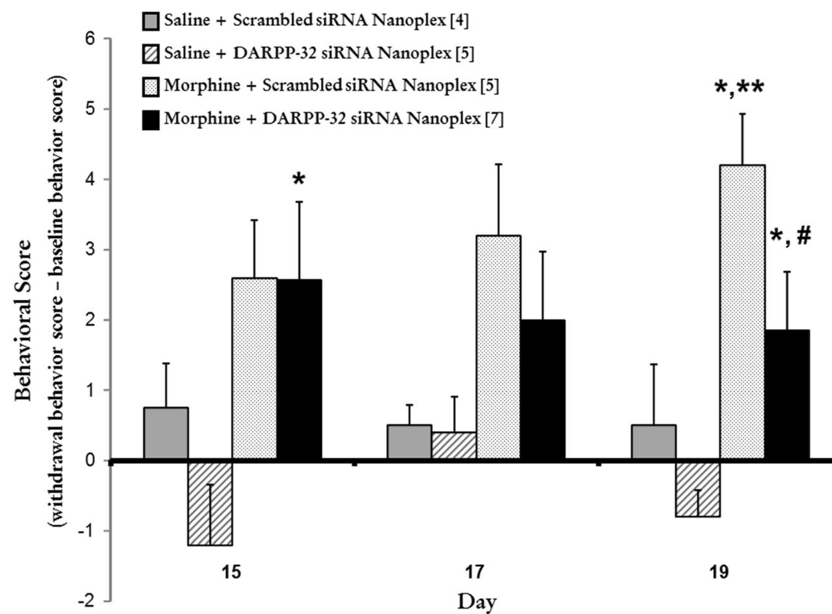

Fig. 11 DARPP-32 silencing decreased withdrawal behaviors in morphine addicted animals. Each bar represents the mean ± SEM. Numbers in parentheses indicate number of animal in each group. One-way ANOVA followed by Student-Newman-Keuls test for multiple comparison demonstrates: Day-15: * $p<0.05$, versus Saline + DARPP-32 siRNA nanoplex group; $p=0.067$, Morphine + scrambled siRNA nanoplex versus saline + DARPP-32 siRNA nanoplex group. Day-19: * $p<0.05$, Morphine + scrambled siRNA nanoplex versus saline + scrambled siRNA nanoplex, and morphine + DARPP-32 siRNA nanoplex versus Saline + DARPP-32 siRNA nanoplex; ** $p<0.01$, versus saline + DARPP-32 siRNA nanoplex group; # $p<0.05$ versus morphine + scrambled siRNA nanoplex group

consequently behavior. Modulation of the DARPP-32 signaling pathway and evaluation of the underlying behavioral changes in the opiate withdrawal syndrome have not be investigated due to methodological limitations to directly

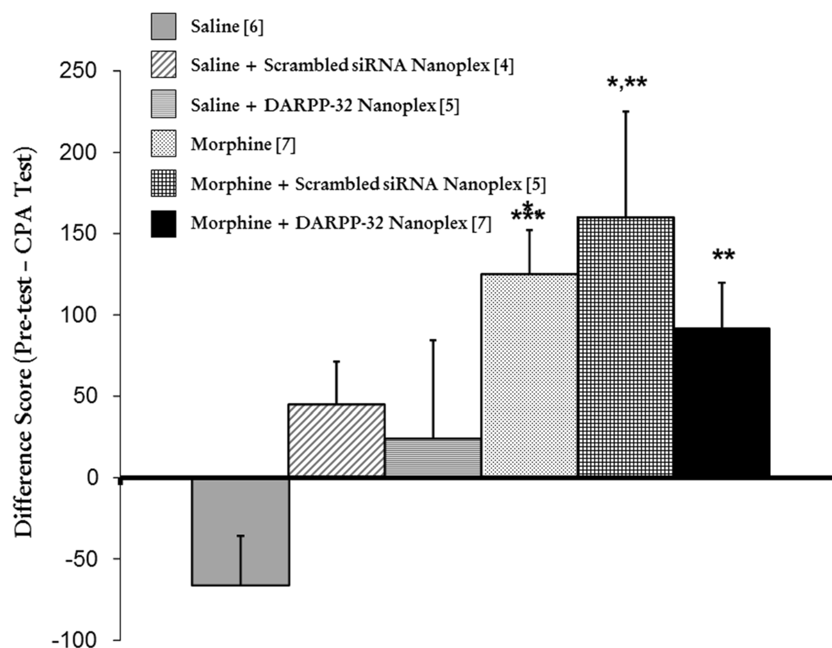

Fig. 12 The difference score (pre-test – CPA test) values in the study groups. The difference score is defined as Day 14 baseline behavior score minus withdrawal-induced aversion behavior scores, with a positive difference score reflective of aversive behavior. DARPP-32 silencing in morphine addicted animals approached a difference score similar to the saline control groups. Statistical significance was determined using a One-way ANOVA followed by Fisher LSD test for multiple comparisons: morphine alone vs saline + DARPP-32 siRNA nanoplex (* $p<0.05$), morphine alone vs saline alone (*** $p<0.001$); morphine + scrambled siRNA nanoplex vs saline + DARPP-32 siRNA nanoplex (* $p<0.05$), morphine + scrambled siRNA nanoplex vs saline group (** $p<0.01$); and morphine + DARPP-32 siRNA nanoplex vs saline group (** $p<0.01$), morphine + DARPP-32 siRNA nanoplex vs saline + DARPP-32 siRNA nanoplex (NS)

manipulating these signaling pathways. In the current study, we used DARPP-32 siRNA to downregulate DARPP-32 gene expression and evaluated if this gene silencing resulted in behavioral modification in a rat model of opiate addiction. We induced opiate withdrawal behavior in an established rat model of chronic opiate abuse, and during the initial stage of withdrawal development, we performed stereotaxic injection of nanoparticle-complexed DARPP-32 siRNA into the PAG brain region to alleviate withdrawal symptoms. Such a potential innovative therapeutic approach in addition to traditional pharmacological and psychological interventions may help break the cycle of addiction and prevent relapse to drug abuse by reducing the negative affect that occurs during withdrawal thereby preventing the recovering addict from drug seeking.

Based on our previous in vitro studies using GNR complexed to DARPP-32 siRNA using dopaminergic neuronal cultures, we observed that GNRs offer stability and protection against degradation of siRNA and that GNR–siRNA nanoplexes can effectively and efficiently knockdown gene expression for a prolonged period of time (Bonoiu et al. 2009). In this study we had demonstrated the ability of the GNR-DARPP-32 siRNA nanoplex to transverse the BBB using an in-vitro BBB model ((Bonoiu et al. 2009). In these experiments using the 2D in-vitro BBB model, wherein multiple doses of GNR-DARPP-32 siRNA are added to the upper chamber (apical end), we evaluated the percentage of DARPP-32 gene knockdown in primary human dopaminergic neuronal (DAN) cells which were grown in the lower chamber (Brain/ CNS or basolateral end). Ours results showed a 50–60 % knockdown of DARPP-32 gene expression in DAN cells, suggesting that the GNR-DARPP-32 siRNA nanoplex could transverse the BBB.

In the current study we used a single microinjection of GNR-DARPP-32 siRNA nanoplex for generating sustained knockdown of DARPP-32 gene expression in the adult rat brain, targeting the injection to the PAG, a region linked to expression of withdrawal behaviors (Bozarth 1994; Maldonado et al. 1992).

The GNRs surface is modified with cationic polyelectrolytes resulting in positively charge surface of the free GNRs, allowing electrostatic binding to the negatively charged siRNA to form a stable nanoplex that remains stable for more than 1 month post-complexation. The process of nanocomplexation can be monitored by observing a change in the peak wavelength of localized longitudinal surface plasmon resonance of the GNRs which we observed after the GNR complexed with the DARPP-32 siRNA (Fig. 2). It is the slow and sustained release of siRNA over a period of 2 weeks (Fig. 3) that allows significant suppression of DARPP-32 gene expression. We observed~60 % downregulation of DARPP-32 gene expression in brain tissue isolated from animals that were stereotaxically injected with the GNR-siRNA nanoplexes even 2 weeks post transfection (Fig. 4). The GNR-DARPP-32-siRNA produces a longer lasting knockdown that is less invasive and which is critical in the assessment of functional/behavioral studies that are done to access the effects of down regulation of DARPP-32 gene expression. Thus, GNRs complexed with siRNA can effectively and efficiently knockdown gene expression in select regions of the rat brain. A major obstacle to siRNA use in brain therapy is the need to continually perfuse the area of interest with the silencer, as free siRNA is rapidly degraded in the cytoplasm of the cell. In this case, an advantage of siRNA delivery using GNRs is the protective effect of the GNR polyelectrolyte layers on siRNA degradation, by providing a shielding effect.

We have previously shown that after stereotaxic injection into the brain, the nanoplexes appear to remain localized into the region, with little diffusion to adjacent regions (Bonoiu et al. 2011).

Chronic exposure to opiates induces plasticity in dopaminergic neurons of the PAG, which regulates morphine reward tolerance. The mesolimbic dopamine structures, including the PAG region, are involved critically in morphine-induced CPP. Elegant studies by Borgkvist A et al. (2007) have shown that DARPP-32 knockout mice and T34A DARPP-32 mutant mice were able to develop behavioral sensitization to morphine comparable to that of wild-type controls and were able to display morphine conditioned place preference. Further, Narita M et al. (2010) showed that activation of DARPP32 and CREB through dopamine D1 receptors could be implicated in the maintenance of μ-opioid-induced place preference. Since DARPP-32 plays a central role in regulating the efficacy of dopaminergic neurotransmission, we hypothesized that sustained knockdown of DARPP-32 in drug addicts would help in the treatment of drug addiction by altering reward and motivational systems and would interfere with conditioned responses. We tested this hypothesis in a rat model of opiate addiction in which DARPP-32 gene expression was downregulated using siRNA against DARPP32 delivered using GNR nanoparticles that allowed sustained release of DARPP-32 siRNA over a 2 week time period.

We also observed a significant inhibition of protein phosphatase-1 (PP-1) and CREB gene expression (Figs. 5 and 6) and an increased level of phospho-CREB protein (Fig. 7) in morphine addicted animals in whom DARPP-32 gene expression was downregulated using the GNR-DARPP-32 siRNA nanoplex as compared to untreated saline controls as well as animals treated with control scrambled siRNA nanoplexes. The inhibition of PP-1 gene expression that occurs following activation of the cAMP/PKA/DARPP-32 signaling cascade and phosphorylation of DARPP-32 at Thr34 acts as an amplification mechanism that results in phosphorylation of downstream target proteins such as the transcription factor CREB. We observed several-fold increase in phospho-CREB protein expression in cell lysates from PAG and VTA

brain tissues in which DARPP-32 has been silenced using the GNR-DARPP-32-siRNA^{Cy3} nanoplex injection into the DL-PAG region. CREB, upon phosphorylation at Ser133, activates gene expression by binding CRE elements in promoter regions of other proteins and transcription factors, which mediate neural plasticity (Berke and Hyman 2000; Hyman and Malenka 2001). Thus, our study indicates that DARPP-32 is an important regulator of CREB phosphorylation, which was corroborated by other investigators (Nairn et al. 2004; Chao and Nestler 2004; Nestler 2004) who showed that PKA-mediated phosphorylation at Thr34 resulted in increases in CREB phosphorylation via inhibition of PP- 1.

We were able to systematically show that morphine addicted animals developed withdrawal symptoms showing significant weight loss, teeth chattering, penis licking, rearing, and stretching behavior (Figs. 8, 9 and 10). We observed slightly increased weight loss in the animals treated with the DARPP-32 nanoplex post microinjection than in the saline treated and morphine treated animals, but this difference was not statistically significant as compared to scrambled siRNA nanoplex treated or saline alone or morphine alone treated control animals. We observed significant DARPP-32 gene silencing 5 days post injection of the DARPP-32 nanoplex and one of the possible reasons for the increased weight loss in this group, may be increased sensitivity to diet that results in protection against overeating and this reduced weight gain. In fact, DARPP-32 has been shown to be involved in dietary preferences, as well as in reward (Teegarden SL, et al. 2009). Another possibility could be the housing condition of the animals, i.e., after surgery, the rats were singly housed following stereotaxic injection in order to monitor food intake and they may eat less in isolation in the extended recovery phase after surgery.

We are the first to report that down regulation of DARPP-32 gene expression using a GNR-DARPP-32-siRNA^{Cy3} nanoplex in morphine addicted animals resulted in lack of CPA behavior (Figs. 11 and 12). These findings indicate that nanoplex silencing of DARPP-32 in the PAG is effective in alleviating the negative symptoms (behavior) and affect (CPA) associated with withdrawal. Morphine addicted rats not receiving DARPP-32 siRNA manifest withdrawal behavior within 24 h following cessation of MS administration as observed on day 15 of the CPA conditioning period. This was confirmed by observing the rats' behavior in the condition place apparatus (e.g., frequency of wet-dog shakes). These results indicate that the involvement of the dopaminergic system in the morphine reward mechanism is critical. The key role of the dopaminergic system in morphine reward is demonstrated by the fact that stimulation of dopamine D1 or D2 receptors facilitates the development of morphine-induced CPP (Rezayof et al. 2003). Zachariou et al. 2006 have shown that phosphorylation of DARPP-32 at Thr34 is observed in cocaine-induced CPP; and that Thr34 mutants did not develop CPP to cocaine. Morphine is known to activate PAG-NAc dopaminergic neurons (Beitner-Johnson and Nestler 1991); therefore, it is possible that morphine mediated dopaminergic D1 receptor activation can lead to increased synaptic plasticity (Yang et al. 2000; Gao et al. 2007) via the modulation of the DARPP-32 signaling pathway. This DARPP-32 signaling pathway, therefore, offers a unique opportunity for pharmacological intervention to treat drug addiction.

Several investigators have characterized CREB function and drug reward (Blendy and Maldonado 1998; Carlezon et al. 1998; Chao and Nestler 2004; Nestler 2004); however, the role of CREB in drug reward remains unclear. Decreases in CREB have been shown to mediate the rewarding properties of cocaine, amphetamine, and morphine (Carlezon et al. 1998; Walters and Blendy 2001; Barrot et al. 2002). In our study, we observed decreased CREB mRNA levels on 5 or 15 days following GNR-siRNA-DARPP-32 injection (Fig. 6), while, on the other hand we observed increased levels of phosphorylated CREB in these brain tissues (Fig. 7). Opioids regulate CREB level, its phosphorylation and binding to its corresponding response element in the promoters of several genes implicated in drug addiction. DARPP-32 phosphorylation at Thr34 results in its conversion to a potent inhibitor of protein phosphatase-1 (PP-1), which regulates the activity of CREB. Our data shows that DARPP-32 silencing results in down regulation of PP-1 gene expression and consequently decreased CREB gene expression. PP-1 is a pCREB phosphatase, and therefore PP-1 regulation can modulate CREB phosphorylation. CREB phosphorylation is specifically induced upon the expression of a sensitized response to morphine-induced conditioned place preference behavior in the brain. Acute morphine treatment leads to significant decreases in CREB phosphorylation however sustained administration of morphine (that induced morphine CPP) resulted in an increase in CREB phosphorylation as observed in our study. We and other investigators (Morón JA, et al. 2010; Narita et al. 2010) have shown increased CREB phosphorylation in response to morphine CPP and these results are consistent with a critical role for CREB in the expression of morphine-dependent conditioned behavior. At the cellular level CREB acts as convergence point for different cellular pathways namely the cAMP pathway or the calcium dependent PKC pathway. Both pathways can affect CREB expression but in different phases of opiate action. The discordance in the levels of CREB gene expression and levels of phosphorylated CREB observed in our study may be attributed to the transcriptional changes resulting from the convergence of these two distinct signaling pathways. Further, it is the CREB phosphorylation level rather than CREB levels is responsible for CREB activity (Fentzke et al. 1998). Chronic morphine treatment reduces total CREB levels and this could be considered as the compensatory mechanism to the increase in the CREB phosphorylation caused by upregulation of PKA pathway. Nye et al. (1995)

and Nestler et al. 1993, have shown that chronic morphine treated withdrawal elicited a dramatic increase in c-Fos and AP-1 binding and given the decreased total CREB level, this rapid induction apparently depended on increased CREB activity consequent of increased CREB phosphorylation. Also, CREB phosphorylation has been shown to increase CREB DNA binding activity without changing the total CREB protein level in vivo. (Yao et al. 2007; Guitart et al. 1992). Additionally, CREB expression is believed to change during the acquisition, expression, or extinction of morphine induced conditioned place preference behavior (Olson et al. 2005; Zhou and Zhu 2006).

We believe, that morphine abuse results in increased dopamine activity during periods of drug use, and activates PKA/DARPP-32/PP-1 signaling cascade leading to CREB phosphorylation in the reward pathways of the brain indicating a sustained susceptibility to neuronal plasticity. Increased CREB phosphorylation as observed in our study may initiate a negative feedback mechanism causing reduced basal dopamine activity, and in an environment of decreased DARPP-32 expression, may further contribute to lack of aversive behavioral response, which suggest a functional role for CREB in the development of morphine-induced CPP.

In conclusion, evidence from our study shows that the DARPP-32 signaling pathway plays an important role in drug abuse. We have successfully targeted the delivery of DARPP-32 siRNA into the PAG region of the rat brain using a GNR nanoparticle delivery system, and this resulted in the sustained knockdown of DARPP-32 gene expression for up to 2 weeks post-transfection. We believe that an in-depth analysis of changes in the molecular pathways of drug reward are essential to understanding the progression to compulsive drug use and abuse relapse.

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Conflict of Interest The authors do not have any conflict of interests.

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