

REGULAR RESEARCH ARTICLE

Overexpression of miR-9 in the Nucleus Accumbens Increases Oxycodone Self-Administration

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Abstract

Background: There is an urgent need to identify factors that increase vulnerability to opioid addiction to help stem the opioid epidemic and develop more efficient pharmacotherapeutics. MicroRNAs are small non-coding RNAs that regulate gene expression at a posttranscriptional level and have been implicated in chronic drug-taking in humans and in rodent models. Recent evidence has shown that chronic opioid treatment regulates the microRNA miR-9. The present study was designed to test the hypothesis that miR-9 in the nucleus accumbens potentiates oxycodone addictive-like behavior.

Methods: We utilized adeno-associated virus (AAV) to overexpress miR-9 in the nucleus accumbens of male rats and tested the effects on intravenous self-administration of the highly abused prescription opioid, oxycodone, in 1-hour short-access followed by 6-h long-access sessions, the latter of which leads to escalation of drug intake. In separate rats, we assessed the effects of nucleus accumbens miR-9 overexpression on mRNA targets including RE1-silencing transcription factor (*REST*) and dopamine D2 receptor (*DRD2*), which have been shown to be regulated by drugs of abuse.

Results: Overexpression of miR-9 in the nucleus accumbens significantly increased oxycodone self-administration compared with rats expressing a control, scrambled microRNA. Analysis of the pattern of oxycodone intake revealed that miR-9 overexpression increased “burst” episodes of intake and decreased the inter-infusion interval. Furthermore, miR-9 overexpression decreased the expression of *REST* and increased *DRD2* in the nucleus accumbens at time points that coincided with behavioral effects.

Conclusions: These results suggest that nucleus accumbens miR-9 regulates oxycodone addictive-like behavior as well as the expression of genes that are involved in drug addiction.

Keywords: microRNA, opioids, addiction, *REST*, *DRD2*

Introduction

Prescription opioid abuse has reached epidemic levels in the United States over the last decade (Compton et al., 2016). Oxycodone has become a leading drug of abuse in the United States (Van Zee, 2009). The intravenous (i.v.) drug self-administration paradigm is commonly used to model addictive-like behavior in laboratory

animals, and previous research from our laboratory demonstrated that male and female rats self-administer oxycodone in a short access self-administration paradigm (Mavrikaki et al., 2017). However, the exact neurobiological mechanisms underlying vulnerability to oxycodone addiction remain unknown.

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Significance Statement

The opioid epidemic has highlighted an urgent need to understand the neurobiological mechanisms underlying opioid abuse and the development of opioid use disorder so that effective treatments can be developed. Drugs of abuse can change the expression of hundreds of genes in the brain, and one recently discovered way this can happen is through regulation of small, non-coding RNAs called microRNAs (miRNAs). Recently, it has been shown that chronic opioid exposure regulates microRNA-9 (miR-9) in the brain. However, the role of miR-9 in addictive behavior related to opioids has not been tested. Here, we show that nucleus accumbens (NAc) miR-9 potentiates oxycodone self-administration in male rats and regulates the expression of genes known to be involved in addictive behavior. These studies are important because they link opioid exposure to specific and novel molecular changes that subsequently increase drug consumption and may contribute to the development of opioid use disorder.

MiRNAs are small non-coding RNAs that regulate gene expression at a posttranscriptional level (Anastasiadou et al., 2018). A single miRNA can regulate the expression of hundreds of genes (Sharma and Eghbali, 2014), suggesting that miRNAs could play a role in the pathophysiology of heterogeneous diseases such as psychiatric disorders. Recent evidence suggests that miRNAs are involved in acquisition of drug self-administration, levels of drug intake, drug withdrawal, reinstatement of drug taking, and other behaviors that collectively resemble addictive-like behavior in humans (Hollander et al., 2010; Tatro et al., 2013; Doura and Unterwald, 2016). MiR-9 is a brain-enriched miRNA (Sood et al., 2006; Smith et al., 2016) that is highly conserved from flies to humans (Barbano et al., 2017; Nowek et al., 2018). In humans, mature miR-9 includes both miR-9-5p and miR-9-3p (Barbano et al., 2017), which are named based on the end (5' or 3') of the precursor miRNA from which they derive (O'Brien et al., 2018). Previous research indicated that chronic morphine treatment decreased the expression of miR-9 in the prefrontal cortex in mice (Tapocik et al., 2016), whereas chronic cocaine treatment increased miR-9 expression in the nucleus accumbens (NAc) and decreased its expression in the striatum (Eipper-Mains et al., 2011). Taken together, drug-mediated regulation of miR-9 is dependent on the type of drug and on the brain region analyzed. To further understand the role of miR-9 in addictive behavior, we tested the impact of miR-9 in the NAc on oxycodone self-administration. MiR-9 has been shown to directly or indirectly regulate a number of genes implicated in reward function, including dopamine receptor D2 (DRD2) and repressor element 1-silencing transcription factor (REST) (Chandrasekar and Dreyer, 2009; Henriksson et al., 2014; Zhang et al., 2015). REST is a negative regulator of neuronal gene expression (Mozzi et al., 2017) involved in synaptic plasticity (Rodenias-Ruano et al., 2012) and has been shown to downregulate the expression of genes such as DRD2 (Sun et al., 2005) and the mu opioid receptor mu (OPRM1) (Chidambaram et al., 2017).

Drugs of abuse, including opioids, increase dopamine levels in the NAc (Di Chiara and Bassareo, 2007). Expression of DRD2 in the NAc has been shown to be increased in rats treated with progressively increasing doses of heroin (Li et al., 2017), a protocol that resembles escalation of drug intake. In addition, other studies using optogenetic stimulation suggest that activation of DRD2-expressing neurons in the NAc increases motivation for food (Soares-Cunha et al., 2016). These findings raise the possibility that increased DRD2 expression in the NAc might also increase motivation to self-administer opioids. Importantly, the miRNA-mRNA interactions are tissue specific (Sood et al., 2006), and the effect of miR-9 overexpression on REST and DRD2 in the NAc has not been examined. Taken together, we hypothesized that miR-9 overexpression would decrease REST and increase DRD2 in the NAc, contributing to an

increase in oxycodone self-administration. As such, we used AAV viral vectors to overexpress miR-9 (or a scrambled miRNA control) in the NAc—a critical component of mesolimbic reward circuitry—and assessed its contribution to the initiation and escalation of i.v. oxycodone self-administration according to previously published protocols (Schlosburg et al., 2013; Wade et al., 2015), as well as patterns of oxycodone intake, in male rats.

METHODS AND MATERIALS

Animals

Adult male (275–300 g; n = 51) Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA) were used for this study. Upon arrival, rats were group housed (3–4 rats/cage) and were acclimated for 1 week in a 12-hour-light/dark cycle with food and water ad libitum. Following surgeries and during self-administration studies, rats remained singly housed. A total of 3 rats from the drug self-administration study were excluded from the analysis because of incorrect viral infusion placement (n = 1), catheter failure (n = 1), and an abnormal accumulation of subcutaneous fluid (n = 1). All experiments were conducted during the light phase. All guidelines recommended by the Animal Care and Use Committee of McLean Hospital and by the National Institutes of Health guide for the care and use of Laboratory animals were followed.

Viral Vector Constructs

Viral vector constructs were purchased from Vector Biolabs (Malvern, PA). Adeno-associated virus (AAV), serotype 9, was packaged with enhanced Green Fluorescent Protein (eGFP)-tagged plasmids containing either miR-9 or a scrambled miRNA (a mature miRNA sequence that does not target any known mammalian mRNAs) under the control of the EF1a promoter (scAAV9-EF1a-hsa-miR-9-3-eGFP; AAV-miR-9-eGFP; stock titer: 3×10^{13} Genome Copies [GC]/mL; and scAAV9-EF1a-hsa-ctrl-miR-eGFP; AAV-ctrl-miR-eGFP; stock titer: 2.2×10^{13} GC/mL).

In Vitro Viral Vector Validation

Human embryonic kidney cells 293 (HEK293T; 2×10^6 /well) were transduced in duplicates with 2 different concentrations of AAV-ctrl-miR-eGFP (diluted to 8.8×10^{10} GC and 2.2×10^{11} GC) or AAV-miR-9-eGFP (diluted to 12×10^{10} GC and 3×10^{11} GC). Untreated cells were used as controls. At 48 hours posttransduction, eGFP expression was confirmed under a fluorescent microscope, and at 72 hours posttransduction, cells were harvested, RNA was extracted, and miR-9 expression was confirmed using quantitative real-time PCR (qRT-PCR).

Intracranial Stereotaxic Surgeries

Rats were anesthetized with a ketamine/xylazine (80/8 mg/kg, i.p.) mixture and placed in a stereotaxic instrument (Kopf). For viral vector infusions, a 1- μ L Hamilton microsyringe connected to a microsyringe pump (11 Elite Nanomite Programmable Syringe Pump, Harvard Apparatus) was placed on the stereotaxic frame. The syringe was lowered to the level of NAc at an 18° angle lateral from midline (AP: +1.6, ML: +3.5, DV: 7.7; based on the atlas of Paxinos and Watson, 2007). One μ L/hemisphere of AAV-ctrl-miR-eGFP (2.2×10^{13} GC/mL) or AAV-miR-9-eGFP (3×10^{13} GC/mL) was infused into the NAc at a rate of 200 nL/min. The injector was left in place for an additional 5 minutes postinfusion to enable diffusion of the virus.

In Vivo Viral Vector Validation

Rats infused with one μ L/hemisphere of AAV-ctrl-miR-eGFP (2.2×10^{13} GC/mL) or AAV-miR-9-eGFP (3×10^{13} GC/mL) in the NAc were killed 2, 4, or 9 weeks postinfusion. Rats were killed by decapitation and brains were snap-frozen in isopentane on dry-ice and then stored at -80°C until use. The NAc was punched from frozen brains as described in (Chartoff et al., 2016) (see Supplementary material). NAc punches from each hemisphere were stored separately. Total RNA was extracted using Trizol reagent (Ambion, Life Technologies) as described previously (Mavrikaki et al., 2016).

qRT-PCR

To verify overexpression of miR-9 following AAV-miR-9-eGFP microinfusion in the NAc and treatment in HEK293T cells, we used qRT-PCR. A total of 50 ng RNA extracted from NAc tissue was used for miRNA-specific reverse transcription using the miScript II RT kit (Qiagen). qRT-PCR was performed in a 384-well plate using miScript SYBR Green PCR kits (Qiagen) and miScript primer assays (Qiagen) for miR-9-5p, miR-9-3p, and RNU6B using an Applied Biosystems vii7 Real-Time PCR Instrument. For the HEK293T cells, miR-9-5p and miR-9-3p and RNU6B were measured using a TaqMan microRNA reverse transcription kit (Applied Biosystems; 20 ng RNA was used) and primer specific TaqMan Assays (Applied Biosystems). For the mRNA measurements, a total of 250 ng RNA extracted from the NAc and 100 ng RNA extracted from HEK293T cells were used for cDNA synthesis using an iScript cDNA synthesis kit (Biorad) and qRT-PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems) using primer sets designed for each mRNA (see Supplementary material). Primers for REST and OPRM1 were purchased from Qiagen. Expression data were analyzed according to the $2^{-\Delta\Delta\text{CT}}$ method (Schmittgen and Livak, 2008).

Intravenous Catheter Implantation

Three weeks after the viral infusion in the NAc, rats were implanted with chronic indwelling silastic (0.51 mm internal diameter) i.v. jugular catheters as described in Mavrikaki et al., 2017. Briefly, catheters were implanted into the right jugular vein, secured to the vein by nonabsorbable suture thread, and passed subcutaneously to exit dorsally from the rat's back. Catheters were flushed daily with 0.2 mL of heparinized saline (30 USP units/mL) and once per week with 0.2 mL gentamicin (10 mg/mL).

Apparatus for Oxycodone Self-Administration

Operant conditioning chambers (Med Associates, St. Albans, VT; 30.5 \times 24.1 \times 29.2 cm) were equipped with 2 retractable response

levers with cue lights above them, a house light, a counter-balanced fluid swivel and tether, and an infusion pump. The chambers were enclosed in sound-attenuated cubicles with ventilation fans. A spring-covered Tygon tube was connected to the rat's catheter through a fluid swivel to a syringe that contained oxycodone solution (0.06 mg/kg/infusion). The syringe was placed in an infusion pump located outside the chamber. A press on the active lever resulted in a 4-second-long infusion of oxycodone hydrochloride (Sigma-Aldrich) dissolved in 0.9% bacteriostatic saline. The concentration of oxycodone solution in each syringe was adjusted to the rat's body weight and updated to account for body weight changes at least once per week such that each rat received 100 μ L per infusion according to (Mavrikaki et al., 2017). MED-PC software and interfacing (MED-Associates) controlled the apparatus and data recording.

Oxycodone Self-Administration Studies

One week after surgery, rats began oxycodone self-administration training under a fixed ratio 1 (FR1) schedule of reinforcement in 1-hour sessions for 8 days (Schlosburg et al., 2013). All rats were fed ad libitum and no other manipulations were done for the first 4 days of training. On days 5 to 8, sucrose dust was sprinkled on the active lever of any rats that had received <5 infusions the day before to stimulate operant behavior at the active lever. After 8 days of training in 1-hour sessions, rats were shifted to 6-h-long access sessions for 10 days as previously described (Schlosburg et al., 2013; Wade et al., 2015). Under the FR1 schedule, each active lever press resulted in an infusion of 0.06 mg/kg oxycodone.

Self-administration trials were conducted 5 days per week. Each trial began with onset of the house light, and levers were extended to signal drug availability according to Mavrikaki et al., 2017. Each infusion (4 seconds) was signaled by the onset of a cue light over the active lever and offset of the house light. A 6-second timeout period was initiated at the start of each infusion and, during that period, any press on the active lever had no consequence. Following the timeout period, the cue light turned off and the house light turned on to signal drug availability. We recorded and analyzed total active lever presses, which included presses that led to a drug infusion and those that did not (i.e., presses during a timeout, inactive lever presses (which had no consequences), and total infusions).

To test catheter patency, once per week or more frequently if self-administration behavior rapidly and significantly changed, methohexital sodium (2 mg in 0.2 mL, i.v.) was infused and rapid response to the anesthetic effect of methohexital was assessed. Any rats that did not have patent catheters throughout testing were excluded (total of 20 rats started; 1 rat demonstrated catheter failure and was excluded from the analysis; 2 more rats underwent a second catheter surgery during the experiment and were included in the analysis).

Analysis of the Pattern of Oxycodone Intake

To assess if rats self-administer oxycodone at regular intervals, we performed an analysis of the pattern of oxycodone intake. We plotted the pattern of oxycodone infusions, active and inactive lever presses over the 6-hour session on day 10. We estimated the number of "burst" episodes on oxycodone intake (infusions) per minute by setting "burst" thresholds as a minimum of either 5, 6, 7, or 8 infusions/min. Furthermore, we analyzed the inter-infusion interval, which corresponded to the time between 2 infusion events (when infusion events exceeded 1 infusion/min).

Transcardial Perfusions and Viral Infusion Verification

At the end of the drug self-administration study, rats were injected with a lethal dose of sodium pentobarbital (130 mg/kg, i.p.) and transcardially perfused with 0.9% saline followed by 4% formaldehyde as described in (Russell et al., 2014). Brains were extracted and the AAV infusion site was confirmed by assessing the expression of the reporter, eGFP, under a fluorescent microscope (Zeiss AxioScope 2Plus). One rat was excluded from the analysis due to incorrect surgical placement of the viral infusion. All other infusions targeted the NAc (core plus shell).

Immunohistochemistry

Before the behavioral experiments, a pilot study was done in separate rats to confirm that the viral vectors infect neurons in the NAc. Male rats were infused with AAV-cntr-miR-eGFP ($n=4$ rats) and perfused 2 or 3 weeks postinfusion as described above. Coronal sections from the NAc were cut on a freezing microtome and stored in cryoprotectant at -20°C until immunohistochemistry for neuronal nuclei (NeuN, a neuron-specific protein) and glial fibrillary acidic protein (GFAP, a glia-specific protein) was performed (Russell et al., 2014) in a subset of NAc sections. Free-floating sections were rinsed 3×10 minutes in 0.01 M phosphate-buffered saline (PBS) to remove cryoprotectant, then washed 3×5 minutes in diluting buffer and blocked in 20% normal goat serum in 0.01M PBS for 1 hour at room temperature. Incubation with primary antibodies rabbit anti-GFAP (DAKO Z0334) and mouse anti-NeuN (Millipore MAB377) at 1:500 each was performed in diluting buffer at 4°C for 48 hours on a shaker. Sections were washed 3×5 minutes in 0.01 M PBS, then incubated with anti-rabbit Alexa 633 and anti-mouse Alexa 555 (1:400) for 3 hours at room temperature, followed by 3×5 -minute washes in 0.01 M PBS. Sections were mounted on Superfrost Plus slides, allowed to dry, then coverslipped in Fluoro-Gel (Electron Microscopy Sciences) and visualized with a Zeiss AxioScope 2Plus microscope using AxioVision 4.8.2 software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8. Unpaired t test (virus: 2 groups), 1-way (virus: more than 2 groups) or 2-way (time \times virus) ANOVA with (drug self-administration studies) or without (qRT-PCR) repeated measures was used when appropriate. Mixed effects models were used for the 6-hour drug self-administration study for which we had missing values in

the last 2 days for 1 rat. If significant interactions were detected, Bonferroni's post hoc tests for multiple comparisons were done. For qRT-PCR analyses of different genes, the Fisher's LSD post hoc test was used.

RESULTS

In Vitro Viral Vector Validation

To validate the viral constructs, we transduced HEK293T cells in duplicates with 1 of 2 different titers of either AAV-miR-9-eGFP (12×10^{10} and 3×10^{11} GC) or AAV-cntr-miR-eGFP (8.8×10^{10} and 2.2×10^{11} GC), and we used untreated cells as controls. We harvested cells 72 hours posttransduction and ran qRT-PCR to measure the expression of eGFP. We found that both viruses increased the expression of eGFP in HEK293T cells ($F_{(4,6)} = 132.5$, $P < .0001$; Figure 1A). Bonferroni's post hoc analysis revealed a significant increase in eGFP expression for AAV-miR-9-eGFP (12×10^{10} GC; $P = .001$), AAV-miR-9-eGFP (3×10^{11} GC; $P < .0001$), and AAV-cntr-miR-eGFP (2.2×10^{11} GC; $P = .0008$) compared with untreated control. However, the increase of eGFP expression for AAV-cntr-miR-eGFP (8.8×10^{10} GC) did not reach statistical significance ($P = .16$), although there was an 11 475-fold increase in eGFP expression compared with untreated control. To assess if AAV-miR-9-eGFP increased expression of miR-9, we ran qRT-PCR for miR-9-5p and -3p and found that AAV-miR-9-eGFP increased the expression of miR-9-5p ($F_{(4,6)} = 22.12$, $P = .001$; Figure 1B) but not miR-9-3p ($F_{(4,6)} = 2.818$, $P = .12$; Figure 1C). As expected, AAV-cntr-miR-eGFP did not change miR-9-5p expression ($P > .05$).

In Vivo Viral Vector Validation

Following in vitro validation of the viral constructs, we infused rats with one μL of AAV-ctrl-miR-eGFP (2.2×10^{13} GC/mL) to confirm viral transfection of neurons in the NAc (Figure 2A–C). We performed immunohistochemistry for the neuronal marker NeuN and glial marker GFAP and confirmed that the Efla promoter drives expression in neurons (Figure 2D). To confirm that AAV-miR-9-eGFP increases the expression of miR-9 in the NAc, we infused AAV-miR-9-eGFP and AAV-cntr-miR-eGFP in the NAc ($1 \mu\text{L}$ of AAV-miR-9-eGFP 3×10^{13} GC/mL or AAV-ctrl-miR-eGFP 2.2×10^{13} GC/mL; these titers were used for all the subsequent in vivo studies) and killed rats 2, 4, or 9 weeks postinfusion (Figure 3A). We assessed miR-9 and eGFP expression using qRT-PCR and confirmed that both AAV-miR-9-eGFP and AAV-cntr-miR-eGFP increased eGFP in the NAc. Intra-NAc AAV-miR-9-eGFP significantly increased the expression of miR-9-5p and -3p compared

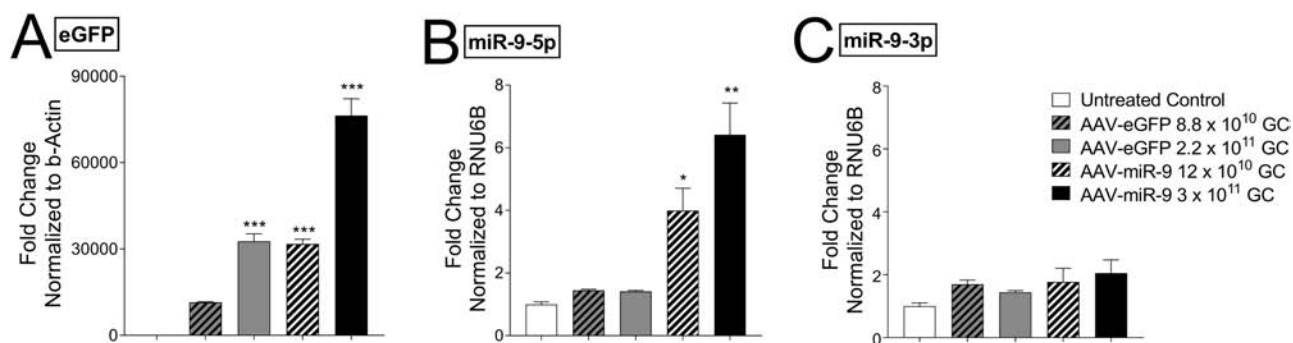


Figure 1. In vitro validation of AAV-mediated miR-9 overexpression in HEK293T cells. HEK293T cells were transduced with 2 different titers of scAAV9-EF1a-hsa-miR-9-3-eGFP (AAV-miR-9) or scAAV9-EF1a-hsa-ctrl-miR-eGFP (AAV-eGFP) and were harvested 72 hours later to measure eGFP, miR-9-5p, and miR-9-3p expression using qRT-PCR. (A) Expression of the reporter eGFP, (B) expression of miR-9-5p, and (C) expression of miR-9-3p. Data are expressed as fold change to untreated control \pm SEM; $n=2-3$ /group. Asterisks indicate significant difference compared with untreated control; * $P < .05$, ** $P < .01$, *** $P < .001$.

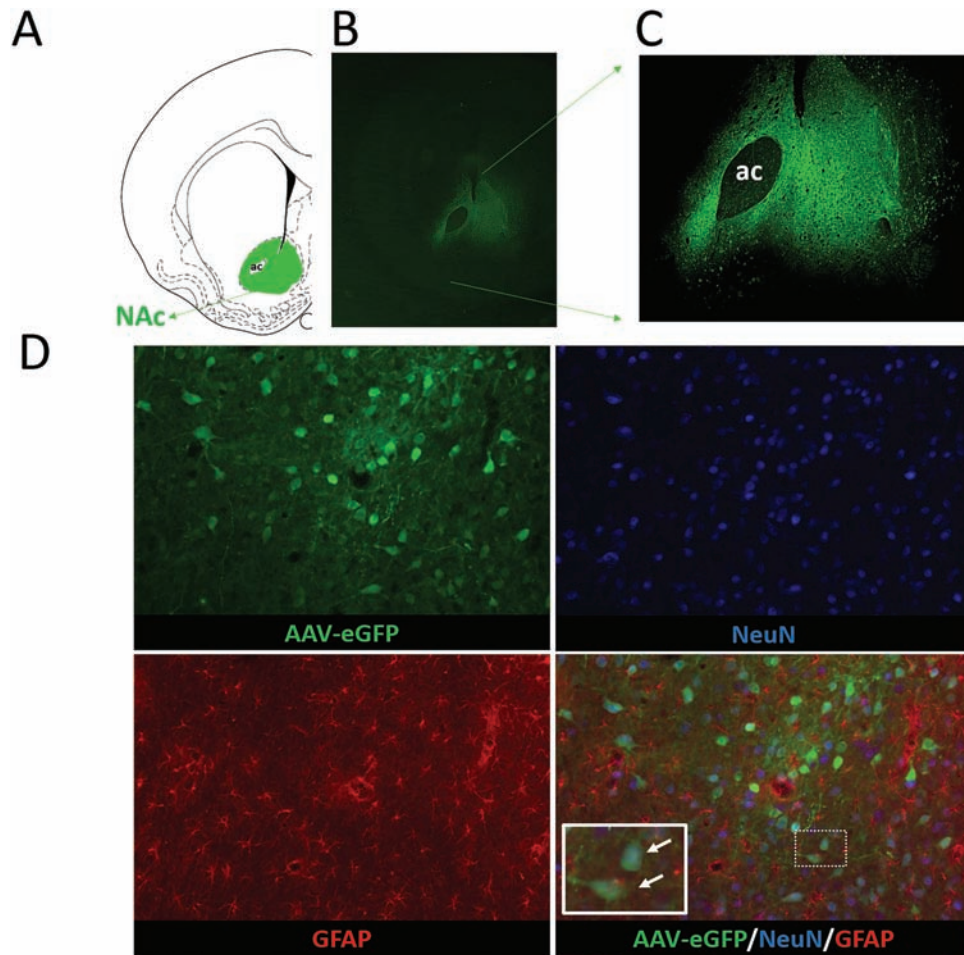


Figure 2. In vivo microinfusion of viral constructs in the nucleus accumbens (NAc). (A) Graphical representation of a coronal section of the NAc area targeted by the viral vector scAAV9-EF1a-hsa-miR-9-3-eGFP (AAV-miR-9) or scAAV9-EF1a-hsa-ctrl-miR-eGFP (AAV-eGFP). (B) Image from fluorescent microscope showing the infection site (NAc core and shell) in a rat infused with AAV-eGFP (7 weeks postinfusion) under low (1.25 \times) and (C) high (5 \times) magnification. (D) Immunohistochemistry for the neuronal marker NeuN (blue) and glial marker GFAP (red) in NAc sections obtained from a rat infused with AAV-eGFP (for AAV-eGFP see green; section obtained 3 weeks postinfusion). Merged panel shows overlap of eGFP with NeuN but not GFAP (white box and white arrows). ac, anterior commissure.

with intra-NAc AAV-cntr-miR-eGFP (main effect of virus: $F_{(1,33)} = 5.968$, $P = .02$ and $F_{(1,33)} = 6.377$, $P = .016$ respectively; main effect of time was not significant: $F_{(2,33)} = 0.80$, $P = .45$ and $F_{(2,33)} = 0.99$, $P = .37$ respectively; the interaction of virus \times time was not significant: $F_{(2,33)} = 0.80$, $P = .45$ and $F_{(2,33)} = 0.99$, $P = .37$ respectively; [Figure 3B–C](#)).

Oxycodone Self-Administration Study

In the initial self-administration training phase, rats self-administered oxycodone in 1-hour sessions for 8 days. During the 1-hour sessions, AAV-miR-9-eGFP did not significantly affect oxycodone self-administration behavior compared with AAV-cntr-miR-eGFP-infused rats (main effect of virus on infusions, $F_{(1,15)} = 0.5324$, $P > .05$; [Figure 4A](#); main effect of virus on active lever presses, $F_{(1,15)} = 0.123$, $P > .05$; [Figure 4B](#); main effect of virus on inactive lever presses, $F_{(1,15)} = 0.0003$, $P > .05$). However, the number of oxycodone infusions and active lever presses, but not inactive lever presses, increased over time (main effect of time on infusions, $F_{(7,105)} = 7.153$, $P < .0001$, [Figure 4A](#); active lever presses, $F_{(7,105)} = 5.05$, $P < .0001$, [Figure 4B](#); inactive lever presses,

$F_{(7,105)} = 0.558$, $P > .05$, [Figure 4B](#); no significant interactions of virus \times time on infusions, active or inactive lever presses, $F_{(7,105)} = 0.410$, $P > .05$, $F_{(7,105)} = 0.742$, $P > .05$, $F_{(7,105)} = 0.558$, $P > .05$ respectively), indicative of acquisition of self-administration behavior.

MiR-9 overexpression in the NAc increased oxycodone self-administration compared with control rats during the 6-hour sessions, as indicated by a significant interaction of virus \times time for oxycodone infusions ($F_{(9,133)} = 3.522$, $P = .0006$; [Figure 4C](#)) and for active lever presses ($F_{(9,133)} = 2.825$, $P = .0045$; [Figure 4D](#)). Bonferroni's post hoc analysis revealed that miR-9 overexpression significantly increased the total number of oxycodone infusions on day 6 ($P = .024$) and day 8 ($P = .019$) and the total active lever presses on day 6 ($P = .02$). MiR-9 overexpression did not affect the inactive lever presses (main effect of virus, $F_{(1,15)} = 0.417$, $P > .05$; interaction of virus \times time: $F_{(9,133)} = 1.413$, $P > .05$; main effect of time: $F_{(9,133)} = 1.820$, $P > .05$; [Figure 4D](#)). We also found that miR-9 overexpression did not affect body weight during the 6-hour sessions (main effect of virus, $F_{(1,15)} = 0.367$, $P > .05$; supplementary [Figure 1](#)), but we observed a significant decrease in body weight over time independent to the virus (main effect of time: $F_{(9,133)} = 6.591$, $P < .0001$).

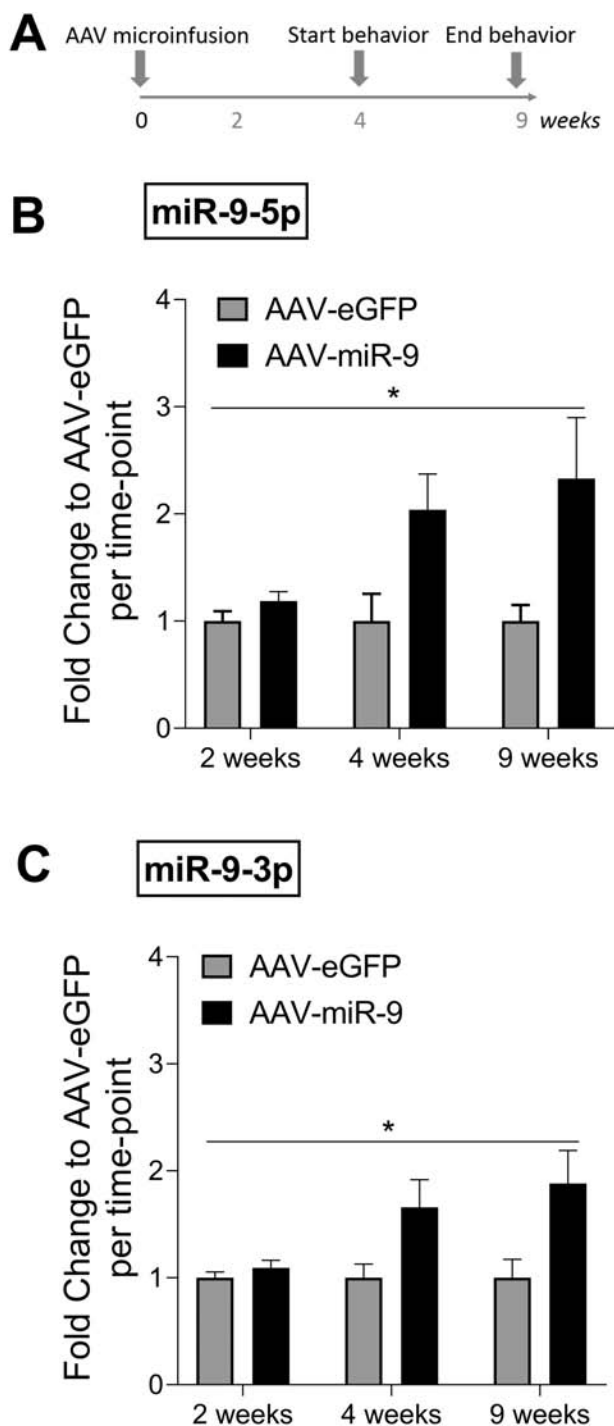


Figure 3. In vivo validation of miR-9 overexpression using viral constructs in the nucleus accumbens (NAc). Rats were infused with scAAV9-EF1a-hsa-miR-9-3-eGFP (AAV-miR-9) or scAAV9-EF1a-hsa-ctrl-miR-eGFP (AAV-eGFP) bilaterally in the NAc and were killed 2, 4, or 9 weeks postinfusion (the 4-week time point coincided with the beginning, and the 9-week time point coincided with the end, of the behavioral experiment). RNA was extracted and miR-9-5p and miR-9-3p expression were measured using qRT-PCR. (A) Experimental design. (B) Expression of miR-9-5p from extracted NAc tissue at the indicated times, and (C) expression of miR-9-3p from the same extracted NAc tissue as (B) at the indicated times. Data are normalized to RNU6B and expressed as fold change to AAV-eGFP per time-point \pm SEM, $n = 4-9$ /group. Asterisks indicate significant main effect of virus, $^*P < .05$.

Pattern of Oxycodone Intake

Previous research in rats has shown that the pattern of drug intake predicts levels of drug-seeking and loss of control over intake (Belin et al., 2009). Thus, we plotted the pattern of oxycodone infusions, active lever presses (including those during timeout), and inactive lever presses of representative rats from the AAV-miR-9-eGFP and the AAV-ctrl-miR-eGFP groups on the last day (day 10) of the 6-hour sessions (Figure 5A). These representative data were chosen by fitting the criteria of the 3rd highest intake on day 10, which approached the mean intake of each viral treatment group. We also used heat-map methodology to plot the pattern of oxycodone infusions of all rats during the 6-hour session on day 10 (Figure 5B). In general, rats from both groups self-administered oxycodone at regular intervals, with intake often occurring in “bursts” followed by a period of no drug intake. We calculated the number of “burst” episodes per minute for data on day 10 by setting “burst” thresholds as a minimum of either 5, 6, 7, or 8 infusions/min. In all cases, miR-9 overexpression increased the number of “burst” intake events ($F_{(1,56)} = 13.53$, $P = .0005$; Figure 6A). Analysis of the inter-infusion interval (the time between 2 infusion events when infusion events exceeded 1 infusion/min) revealed that miR-9 overexpression decreased the inter-infusion interval ($t_{(13)} = 2.224$, $P = .044$; Figure 6B). Specifically, miR-9 overexpression increased the number of infusions with an inter-infusion interval of 1 minute (interaction of virus \times time, $F_{(7,112)} = 2.612$, $P = .015$; posthoc for 1min: $P = .0001$; Figure 6C). The number of infusions for long inter-infusion intervals were the same between groups ($P > .05$). During the phases of regular intake, rats in both groups demonstrated repeated pressing of the active lever even during the 6-second timeout period (Figure 5A; supplementary Figure 2B), suggesting enhanced drug-seeking behavior. In contrast, there were relatively few inactive lever presses in both groups, suggesting that operant behavior was not simply due to a general increase in motor activity. Overall, we found that miR-9 overexpression in the NAc increased the total oxycodone infusions on day 10 ($t_{(14)} = 2.492$, $P = .025$; supplementary Figure 2A) and active lever presses during timeout on day 10 ($t_{(14)} = 2.3$, $P = .03$; supplementary Figure 2B) but there was no effect on inactive lever presses ($P > .05$; supplementary Figure 2C). We analyzed the results from the 1st hour of the day 10 (6 hours) session. Although we observed similar trends, miR-9 overexpression did not significantly increase oxycodone infusions or active lever presses during timeout in the 1st hour of the 6-hour session ($P > .05$; supplementary Figure 2D-F).

Effects of miR-9 Overexpression on mRNA Targets

To assess the effects of miR-9 overexpression on 2 of its mRNA targets implicated in addiction (Thanos et al., 2001; Volkow et al., 2010; Jayanthi et al., 2014), REST and DRD2, we performed qRT-PCR on NAc tissue samples from rats that were infused with AAV-miR-9-eGFP or AAV-ctrl-miR-eGFP and killed 4 or 9 weeks later. We found that miR-9 overexpression in the NAc decreased the expression of REST at 4 weeks and increased the expression of DRD2 at 9, but not 4, weeks (4 weeks: interaction of virus \times mRNA $F_{(2,32)} = 3.628$, $P = .038$; 9 weeks: interaction of virus \times mRNA $F_{(2,24)} = 5.913$, $P = .008$). There was a trend for NAc REST to be decreased in AAV-miR-9-eGFP compared with AAV-ctrl-miR-eGFP rats at 9 weeks, but it was not significant ($P = .18$; Figure 7A-B). We also measured the effects of NAc miR-9 overexpression on OPRM1, but there was no significant effect at either 4 or 9 weeks ($P > .05$; Figure 7A-B).

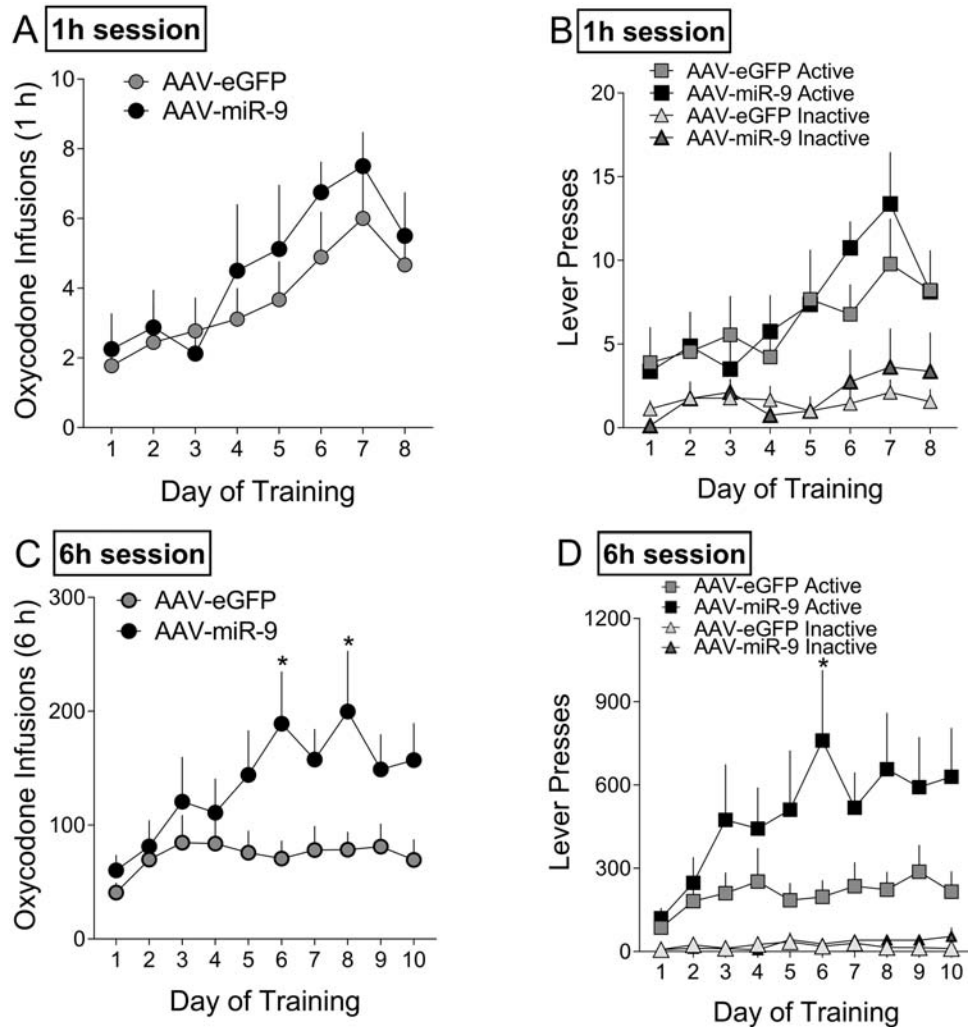


Figure 4. Effects of miR-9 overexpression on oxycodone (0.06 mg/kg/infusion on an FR1 schedule of reinforcement) self-administration. (A) Total number of oxycodone infusions in 1-hour sessions over 8 days. (B) Total number of active and inactive lever presses in 1-hour sessions over 8 days. (C) Total number of oxycodone infusions in subsequent 6-hour, long-access sessions over 10 days. (D) Total number of active and inactive lever presses in 6-hour, long-access sessions over 10 days. Data are expressed as mean \pm SEM, $n = 8-9$ /group. Asterisks indicate a significant effect compared with the corresponding AAV-eGFP, * $P < .05$.

Discussion

This study demonstrates that overexpression of miR-9 in the NAc results in escalation of oxycodone self-administration behavior under long-access conditions that do not induce escalation in control rats. Detailed pattern analysis revealed that miR-9 overexpression in the NAc increases the total number of “burst” events and decreases the inter-infusion interval. In addition, this study indicates that overexpression of miR-9 in the NAc decreases the expression of the transcriptional repressor REST and increases expression of DRD2 mRNA at time points corresponding to behavioral effects.

Our studies demonstrated that transfection of HEK293T cells with AAV-miR-9-eGFP resulted in significantly higher expression of miR-9-5p compared with miR-9-3p. These results are consistent with our *in vivo* NAc microinfusions, which demonstrated higher increases in miR-9-5p compared with miR-9-3p following treatment with AAV-miR-9-eGFP. As such, miR-9-3p might be less stable than miR-9-5p in both HEK293T cells and the NAc, suggesting that our behavioral effects are primarily due to actions of miR-9-5p. Expression of the reporter eGFP was also increased at 4 and 9 weeks postinfusion in NAc (and in HEK293T

cells), confirming that our NAc punches contained virally infused tissue. As such, we set the 4-week time point as the beginning of the behavioral experiments.

MiR-9 has been shown to be regulated by both chronic morphine and cocaine (Eipper-Mains et al., 2011; Tapocik et al., 2016). Our results indicate that rats with miR-9 overexpression in the NAc and control rats initially acquired oxycodone self-administration to a similar extent and with a similar time course in the 8-day, 1-hour acquisition phase. However, once rats were switched to the long-access (6 h/d) component of oxycodone self-administration, miR-9 overexpression in the NAc greatly increased escalation of oxycodone self-administration. This conclusion is supported by the increased total number of oxycodone infusions and active lever presses following chronic intake in the 6-hour-long access paradigm, but no difference in the first days of training in the 6-hour sessions. Escalation of drug self-administration is a widely accepted model of excessive drug intake (Jacobs et al., 2003), and according to the Diagnostic and Statistical Manual of Mental Disorders, increasing substance intake is a hallmark of addiction in humans (Edwards and Koob, 2013). Previous research has suggested that as escalation of opioid self-administration and dependence develops,

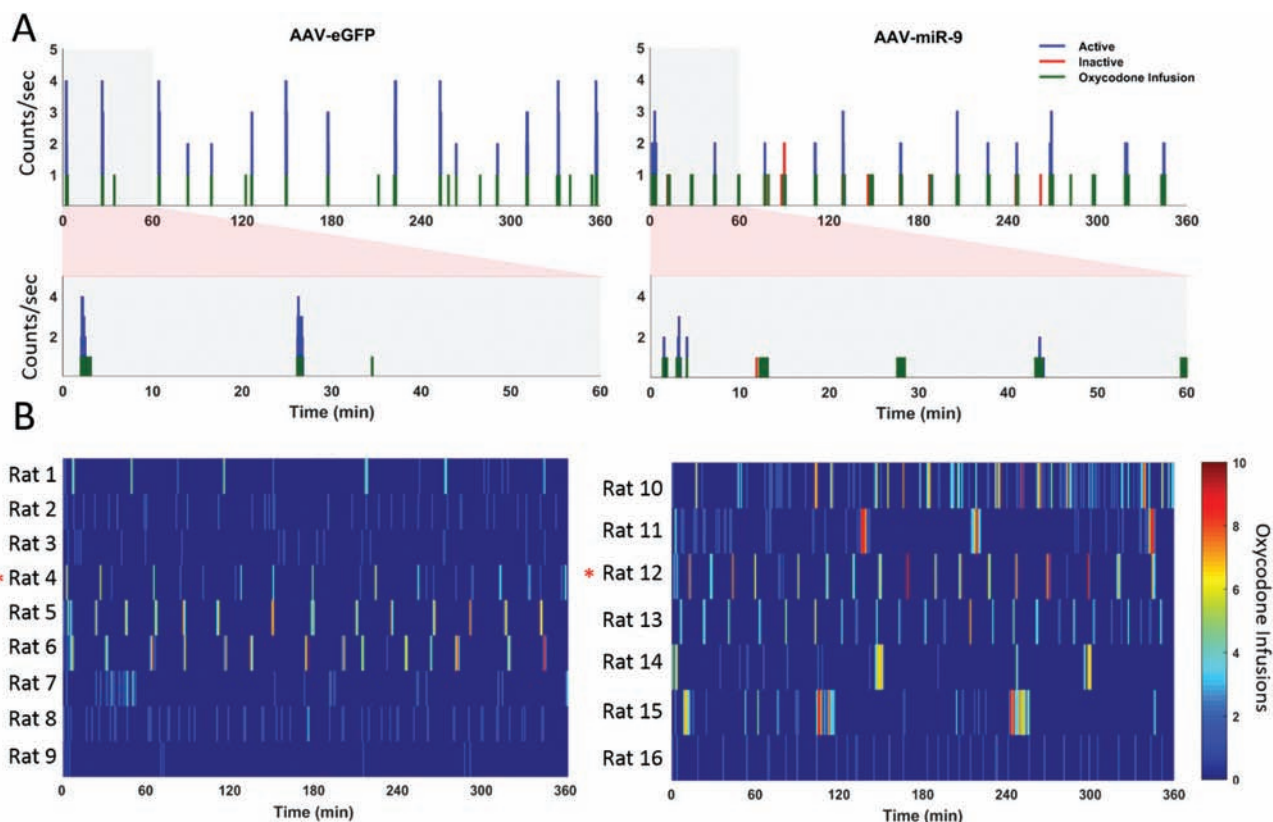


Figure 5. Pattern of oxycodone infusions following miR-9 overexpression in the NAC. (A) Pattern of oxycodone infusions (green), active lever presses (blue), and inactive lever presses (red) in 1-second intervals of representative rats from both AAV-eGFP and AAV-miR-9 groups from day 10 of the 6-hour sessions. We selected the rat from each group with the 3rd highest intake, which was close to the mean intake of the group. Grey highlighted area in each panel indicates the 1st hour, and a zoom-in is presented directly below. (B) Heatmap indicating the pattern of oxycodone infusions of all rats during the 6-hour session on day 10 in 1-minute intervals. Each row represents a rat. The red asterisk (*) represents the rat that was presented in A.

anhedonia emerges, as measured by decreased brain stimulation reward using intracranial self-stimulation (Kenny et al., 2006). As such, opioids may be self-administered to counteract anhedonia and related negative affective states (Kenny et al., 2006), consistent with the notion that the transition from drug-taking behavior to addiction is characterized by a shift from positive to negative reinforcement mechanisms (Koob and Le Moal, 2001). Other studies have suggested that the development of tolerance to reward is a primary factor in the escalation of drug intake (Wade et al., 2015), and tolerance to the rewarding effects of the drug correlates with increased intake (Ahmed et al., 2002). Furthermore, miR-9 increases the development of tolerance to the analgesic effects of opioids (Tapocik et al., 2016). Our results indicate that escalation of oxycodone self-administration does not occur in control rats (AAV-ctrl-miR-eGFP), which is in agreement with a prior study using the identical dose of oxycodone (Wade et al., 2015). In contrast, miR-9 overexpression induced robust escalation of oxycodone intake, an effect that has been previously observed with higher doses of oxycodone (0.15–0.3 mg/kg/infusion vs 0.06 mg/kg/infusion used in the present study) in a 12-hour-long access paradigm (Wade et al., 2015).

In addition to total intake and escalation of intake, we assessed the pattern of drug taking. Independent of treatment group, most rats self-administered oxycodone at regular intervals, with miR-9 overexpression increasing the total number of “burst” events and decreasing the inter-infusion interval. This “burst” pattern of intake is possibly affected by the duration of the timeout and/or the drug dose. Overall, miR-9 overexpression

increased the total number of oxycodone infusions as well as the total active lever presses during the timeout (supplementary Figure 2A–B). Our findings are consistent with those showing that active responding during the timeout (when the drug is not available) indicates persistence of “drug-seeking” and can predict addictive-like behavior. To our knowledge, previous long-access opioid self-administration studies have presented only the overall intake per session but not the pattern of intake within a session (Schlosburg et al., 2013; Wade et al., 2015). As such, our studies provide detail about the pattern of opioid intake during long-access conditions and support the involvement of miR-9 in oxycodone addictive-like behavior.

Two of the potential mRNA targets of miR-9: REST (Packer et al., 2008; Henriksson et al., 2014) and DRD2 (Zhang et al., 2015) are implicated in drug addiction (Thanos et al., 2001; Volkow et al., 2010; Jayanthi et al., 2014). In this study, miR-9 overexpression significantly decreased the expression of the mRNA encoding the transcriptional repressor REST in the NAC at 4 weeks postinfusion, but it had no effect on DRD2. At 9 weeks postinfusion, REST remained decreased and DRD2 expression was elevated. We also measured the effects of miR-9 overexpression on OPRM1 (a gene that has been shown to be regulated by REST; Chidambaran et al., 2017), hypothesizing that changes in OPRM1 expression could contribute to NAC miR-9-mediated differences in oxycodone self-administration. However, we found no significant effect on OPRM1 expression. Our results are consistent with previous studies showing that miR-9 regulates REST expression in human prefrontal cortex

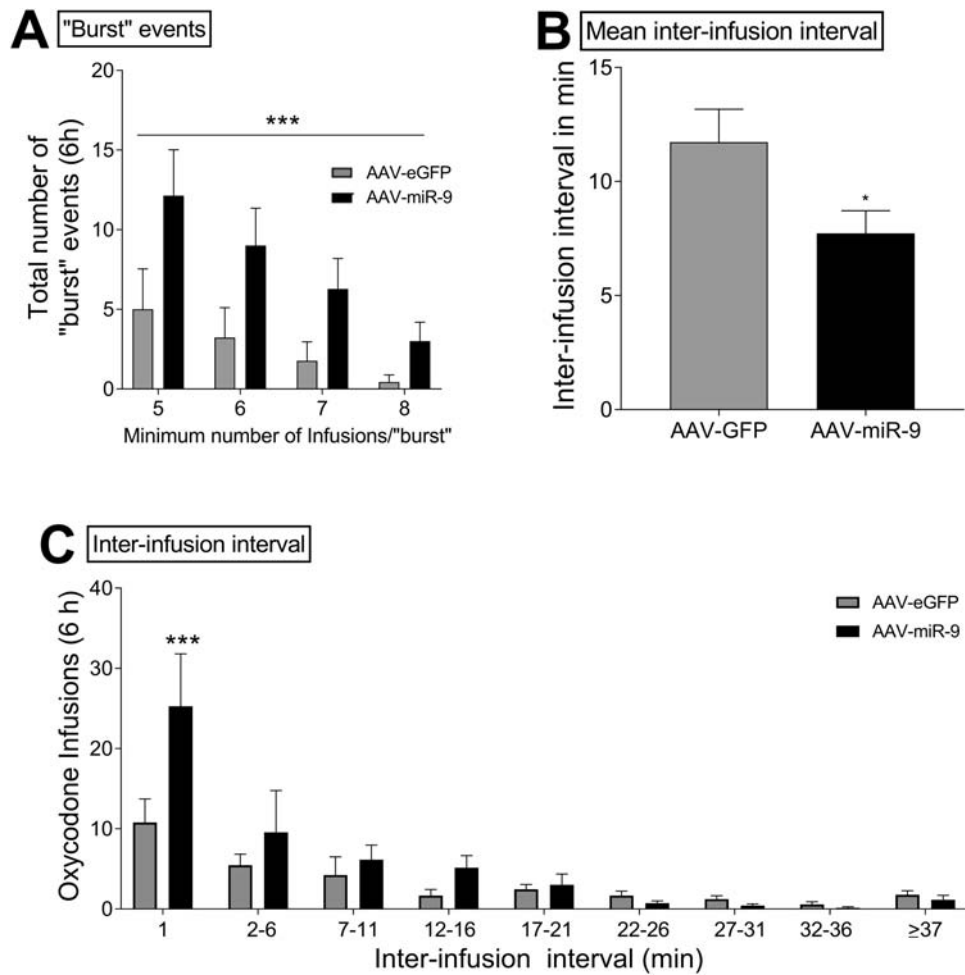


Figure 6. Quantification of the pattern of oxycodone infusions following miR-9 overexpression. (A) Total number of "burst" events during the 6-hour session on day 10; we estimated the "burst" events as minimum of 5, 6, 7, or 8 infusions/min. (B) Mean inter-infusion interval (at least 1 infusion/min was considered as infusion; "burst" events were also considered as infusion). (C) Total number of oxycodone infusions as function of the length of inter-infusion interval in AAV-eGFP and AAV-miR-9 rats. Data are expressed as mean \pm SEM, $n=7-9$ /group. Asterisks indicate significant difference compared with AAV-eGFP, * $P < .05$, *** $P < .001$.

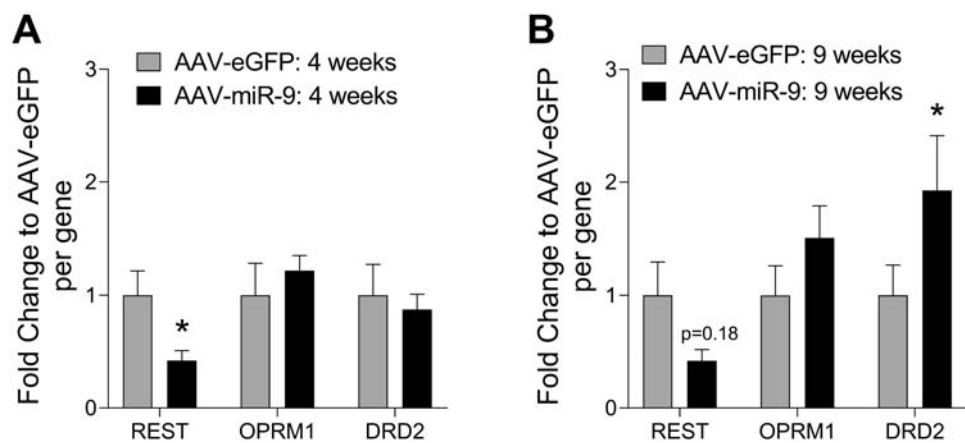


Figure 7. Effects of miR-9 on mRNA targets. Rats were bilaterally infused with scAAV9-EF1a-hsa-miR-9-3-eGFP (AAV-miR-9) or scAAV9-EF1a-hsa-ctrl-miR-eGFP (AAV-eGFP) into the NAc and were killed 4 or 9 weeks postinfusion. The NAc was dissected and REST, OPRM1, and DRD2 mRNA were measured using qRT-PCR. Effects of miR-9 overexpression on REST, OPRM1, and DRD2 mRNA are shown (A) 4 weeks postinfusion and (B) 9 weeks postinfusion. Genes of interest data are normalized to *Itm2b* and represented as fold change from the AAV-eGFP (control) rats of the corresponding gene, $n=6-10$ /group. Asterisks indicate significant effect compared with corresponding AAV-eGFP for each gene, * $P < .05$.

(Henriksson et al., 2014) and in HEK293T cells (Packer et al., 2008). Chromatin immunoprecipitation has been used to demonstrate that *DRD2* is a target gene of REST in mature hippocampus (Sun et al., 2005), although the same method has not yet been used to test REST/*DRD2* interactions in the NAc. Thus, the increase in *DRD2* mRNA following miR-9 overexpression may be indirectly explained by the downregulation of REST. Given that the effects of miR-9 overexpression in the NAc on mRNA targets were assessed in a separate line of rats and not in the rats that self-administered oxycodone, a limitation of our study is that the interaction of oxycodone intake plus miR-9 overexpression could lead to different expression levels of REST and *DRD2*. Future studies will be necessary to address this possibility.

In conclusion, the current studies directly implicate miR-9 in the NAc in the reinforcing effects of oxycodone and raise the possibility of a connection between NAc miR-9, REST, and *DRD2* expression and potentiation of addictive-like behavior. Future studies establishing a mechanistic link among these factors are essential and could ultimately lead to the development of novel treatments for opioid use disorder.

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Interest Statement

None.

References

- Ahmed SH, Kenny PJ, Koob GF, Markou A (2002) Neurobiological evidence for hedonic allostasis associated with escalating cocaine use. *Nat Neurosci* 5:625–626.
- Anastasiadou E, Jacob LS, Slack FJ (2018) Non-coding RNA networks in cancer. *Nat Rev Cancer* 18:5–18.
- Barbano R, Pasculli B, Rendina M, Fontana A, Fusilli C, Copetti M, Castellana S, Valori VM, Morrilli M, Graziano P, Luigi C, Coco M, Picardo F, Mazza T, Evron E, Murgo R, Maiello E, Esteller M, Fazio VM, Parrella P (2017) Stepwise analysis of MIR9 loci identifies mir-9-5p to be involved in oestrogen regulated pathways in breast cancer patients. *Sci Rep* 7:45283.
- Belin D, Balado E, Piazza PV, Deroche-Gamonet V (2009) Pattern of intake and drug craving predict the development of cocaine addiction-like behavior in rats. *Biol Psychiatry* 65:863–868.
- Chandrasekar V, Dreyer JL (2009) MicroRNAs mir-124, let-7d and mir-181a regulate cocaine-induced plasticity. *Mol Cell Neurosci* 42:350–362.
- Chartoff EH, Ebner SR, Sparrow A, Potter D, Baker PM, Ragozzino ME, Roitman MF (2016) Relative timing between kappa opioid receptor activation and cocaine determines the impact on reward and dopamine release. *Neuropsychopharmacology* 41:989–1002.
- Chidambaran V, Zhang X, Martin LJ, Ding L, Weirauch MT, Geisler K, Stubbeman BL, Sadhasivam S, Ji H (2017) DNA methylation at the mu-1 opioid receptor gene (*OPRM1*) promoter predicts preoperative, acute, and chronic postsurgical pain after spine fusion. *Pharmacogenomics Pers Med* 10:157–168.
- Compton WM, Jones CM, Baldwin GT (2016) Relationship between nonmedical prescription-opioid use and heroin use. *N Engl J Med* 374:154–163.
- Di Chiara G, Bassareo V (2007) Reward system and addiction: what dopamine does and doesn't do. *Curr Opin Pharmacol* 7:69–76.
- Doura MB, Unterwald EM (2016) MicroRNAs modulate interactions between stress and risk for cocaine addiction. *Front Cell Neurosci* 10:125.
- Edwards S, Koob GF (2013) Escalation of drug self-administration as a hallmark of persistent addiction liability. *Behav Pharmacol* 24:356–362.
- Eipper-Mains JE, Kiraly DD, Palakodeti D, Mains RE, Eipper BA, Graveley BR (2011) MicroRNA-seq reveals cocaine-regulated expression of striatal microRNAs. *Rna* 17:1529–1543.
- Henriksson R, Bäckman CM, Harvey BK, Kadyrova H, Bazov I, Shippenberg TS, Bakalkin G (2014) PDYN, a gene implicated in brain/mental disorders, is targeted by REST in the adult human brain. *Biochim Biophys Acta* 1839:1226–1232.
- Hollander JA, Im HI, Amelio AL, Kocerha J, Bali P, Lu Q, Willoughby D, Wahlestedt C, Conkright MD, Kenny PJ (2010) Striatal microRNA controls cocaine intake through CREB signalling. *Nature* 466:197–202.
- Jacobs EH, Smit AB, de Vries TJ, Schoffelmeer AN (2003) Neuroadaptive effects of active versus passive drug administration in addiction research. *Trends Pharmacol Sci* 24:566–573.
- Jayanthi S, McCoy MT, Chen B, Britt JP, Kourrich S, Yau HJ, Ladenheim B, Krasnova IN, Bonci A, Cadet JL (2014) Methamphetamine downregulates striatal glutamate receptors via diverse epigenetic mechanisms. *Biol Psychiatry* 76:47–56.
- Kenny PJ, Chen SA, Kitamura O, Markou A, Koob GF (2006) Conditioned withdrawal drives heroin consumption and decreases reward sensitivity. *J Neurosci* 26:5894–5900.
- Koob GF, Le Moal M (2001) Drug addiction, dysregulation of reward, and allostasis. *Neuropsychopharmacology* 24:97–129.
- Li Y, Xia B, Li R, Yin D, Liang W (2017) Changes in expression of dopamine, its receptor, and transporter in nucleus accumbens of heroin-addicted rats with brain-derived neurotrophic factor (BDNF) overexpression. *Med Sci Monit* 23:2805–2815.
- Mavrikaki M, Girardet C, Kern A, Faruzzi Brantley A, Miller CA, Macarthur H, Marks DL, Butler AA (2016) Melanocortin-3 receptors in the limbic system mediate feeding-related motivational responses during weight loss. *Mol Metab* 5:566–579.
- Mavrikaki M, Pravetoni M, Page S, Potter D, Chartoff E (2017) Oxycodone self-administration in male and female rats. *Psychopharmacology* 234:977–987.
- Mozzi A, Guerini FR, Forni D, Costa AS, Nemni R, Baglio F, Cabinio M, Riva S, Pontremoli C, Clerici M, Sironi M, Cagliani R (2017) REST, a master regulator of neurogenesis, evolved under strong positive selection in humans and in non human primates. *Sci Rep* 7:9530.
- Nowek K, Wiemer EAC, Jongen-Lavrencic M (2018) The versatile nature of mir-9/9* in human cancer. *Oncotarget* 9:20838–20854.
- O'Brien J, Hayder H, Zayed Y, Peng C (2018) Overview of microRNA biogenesis, mechanisms of actions, and circulation. *Front Endocrinol* 9:402.
- Packer AN, Xing Y, Harper SQ, Jones L, Davidson BL (2008) The bifunctional microRNA mir-9/mir-9* regulates REST and corect and is downregulated in Huntington's disease. *J Neurosci* 28:14341–14346.

- Paxinos G, Watson C (2007) *The rat brain in stereotaxic coordinates*, 6th Edition. Amsterdam, Boston: Academic Press.
- Rodenas-Ruano A, Chávez AE, Cossio MJ, Castillo PE, Zukin RS (2012) REST-dependent epigenetic remodeling promotes the developmental switch in synaptic NMDA receptors. *Nat Neurosci* 15:1382–1390.
- Russell SE, Rachlin AB, Smith KL, Muschamp J, Berry L, Zhao Z, Chartoff EH (2014) Sex differences in sensitivity to the depressive-like effects of the kappa opioid receptor agonist U-50488 in rats. *Biol Psychiatry* 76:213–222.
- Schlosburg JE, Whitfield TW Jr, Park PE, Crawford EF, George O, Vendruscolo LF, Koob GF (2013) Long-term antagonism of κ opioid receptors prevents escalation of and increased motivation for heroin intake. *J Neurosci* 33:19384–19392.
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3:1101–1108.
- Sharma S, Eghbali M (2014) Influence of sex differences on microRNA gene regulation in disease. *Biol Sex Differ* 5:3.
- Smith A, Calley J, Mathur S, Qian HR, Wu H, Farmen M, Caiment F, Bushel PR, Li J, Fisher C, Kirby P, Koenig E, Hall DG, Watson DE (2016) The rat microRNA body atlas; evaluation of the microRNA content of rat organs through deep sequencing and characterization of pancreas enriched miRNAs as biomarkers of pancreatic toxicity in the rat and dog. *BMC Genomics* 17:694.
- Soares-Cunha C, Coimbra B, David-Pereira A, Borges S, Pinto L, Costa P, Sousa N, Rodrigues AJ (2016) Activation of D2 dopamine receptor-expressing neurons in the nucleus accumbens increases motivation. *Nat Commun* 7:11829.
- Sood P, Krek A, Zavolan M, Macino G, Rajewsky N (2006) Cell-type-specific signatures of microRNAs on target mRNA expression. *Proc Natl Acad Sci U S A* 103:2746–2751.
- Sun YM, Greenway DJ, Johnson R, Street M, Belyaev ND, Deuchars J, Bee T, Wilde S, Buckley NJ (2005) Distinct profiles of REST interactions with its target genes at different stages of neuronal development. *Mol Biol Cell* 16:5630–5638.
- Tapocik JD, Ceniccola K, Mayo CL, Schwandt ML, Solomon M, Wang BD, Luu TV, Olender J, Harrigan T, Maynard TM, Elmer GI, Lee NH (2016) MicroRNAs are involved in the development of morphine-induced analgesic tolerance and regulate functionally relevant changes in *serpini1*. *Front Mol Neurosci* 9:20.
- Tatro ET, Hefler S, Shumaker-Armstrong S, Soontornniyomkij B, Yang M, Yermanos A, Wren N, Moore DJ, Achim CL (2013) Modulation of BK channel by micromi-9 in neurons after exposure to HIV and methamphetamine. *J Neuroimmune Pharmacol* 8:1210–1223.
- Thanos PK, Volkow ND, Freimuth P, Umegaki H, Ikari H, Roth G, Ingram DK, Hitzemann R (2001) Overexpression of dopamine D2 receptors reduces alcohol self-administration. *J Neurochem* 78:1094–1103.
- Van Zee A (2009) The promotion and marketing of oxycontin: commercial triumph, public health tragedy. *Am J Public Health* 99:221–227.
- Volkow ND, Wang GJ, Fowler JS, Tomasi D, Telang F, Baler R (2010) Addiction: decreased reward sensitivity and increased expectation sensitivity conspire to overwhelm the brain's control circuit. *Bioessays* 32:748–755.
- Wade CL, Vendruscolo LF, Schlosburg JE, Hernandez DO, Koob GF (2015) Compulsive-like responding for opioid analgesics in rats with extended access. *Neuropsychopharmacology* 40:421–428.
- Zhang Y, Wang Y, Wang L, Bai M, Zhang X, Zhu X (2015) Dopamine receptor D2 and Associated microRNAs are involved in stress susceptibility and resistance to escitalopram treatment. *Int J Neuropsychopharmacol* 18:1–10.