OPEN

ORIGINAL ARTICLE

In silico identification and *in vivo* validation of miR-495 as a novel regulator of motivation for cocaine that targets multiple addiction-related networks in the nucleus accumbens

RM Bastle[1,3], RJ Oliver[2,3], AS Gardiner[2], NS Pentkowski[1,4], F Bolognani[2,5], AM Allan[2], T Chaudhury[1], M St. Peter[1], N Galles[1], C Smith[1], JL Neisewander[1,6] and NI Perrone-Bizzozero[2,6]

MicroRNAs (miRNAs) are important post-transcriptional regulators of gene expression and are implicated in the etiology of several neuropsychiatric disorders, including substance use disorders (SUDs). Using *in silico* genome-wide sequence analyses, we identified miR-495 as a miRNA whose predicted targets are significantly enriched in the Knowledgebase for Addiction Related Genes (ARG) database (KARG; http://karg.cbi.pku.edu.cn). This small non-coding RNA is also highly expressed within the nucleus accumbens (NAc), a pivotal brain region underlying reward and motivation. Using luciferase reporter assays, we found that miR-495 directly targeted the 3′UTRs of *Bdnf*, *Camk2a* and *Arc*. Furthermore, we measured miR-495 expression in response to acute cocaine in mice and found that it is downregulated rapidly and selectively in the NAc, along with concomitant increases in ARG expression. Lentiviral-mediated miR-495 overexpression in the NAc shell (NAcsh) not only reversed these cocaine-induced effects but also downregulated multiple ARG mRNAs in specific SUD-related biological pathways, including those that regulate synaptic plasticity. miR-495 expression was also downregulated in the NAcsh of rats following cocaine self-administration. Most importantly, we found that NAcsh miR-495 overexpression suppressed the motivation to self-administer and seek cocaine across progressive ratio, extinction and reinstatement testing, but had no effect on food reinforcement, suggesting that miR-495 selectively affects addiction-related behaviors. Overall, our *in silico* search for post-transcriptional regulators identified miR-495 as a novel regulator of multiple ARGs that have a role in modulating motivation for cocaine.

INTRODUCTION

Substance use disorder (SUD) is a chronic, debilitating condition characterized by compulsive drug use despite negative consequences and a high recurrence of relapse even after prolonged periods of abstinence.[1,2] SUD is believed to be a dysfunction of neuroplasticity,[3] whereby altered gene expression impacts neuronal function and subsequent behavior.[4] Drugs of abuse cause widespread epigenetic changes to chromatin accessibility, thereby altering the transcriptional activity of several genes.[5–7] However, less is known about the post-transcriptional processes that control mRNA dynamics and, ultimately, translation into functional proteins. Among the non-coding RNAs, microRNAs (miRNAs) have a critical role in the post-transcriptional control of a large number of transcripts. These small RNAs typically guide the RNA-induced silencing complex (RISC) through the binding of complementary sequences in the 3′ untranslated region (3′UTR) of the target mRNAs, leading to mRNA degradation or translational repression.[8] A single miRNA is predicted to target hundreds of different mRNAs, and a single mRNA can be regulated by multiple miRNAs. Therefore, dysregulation of these 'master' regulators impacts several cellular processes simultaneously and has been linked to many diseases and neurological disorders.[9–13]

Recent studies indicate that several drugs of abuse regulate miRNA expression in the nucleus accumbens (NAc) and other regions of the brain reward pathway.[14–18] In turn, *in vivo* manipulations of specific miRNAs or subsets of miRNAs alter the development of addiction-like behaviors in rodents.[18–22] Thus, we sought to identify a candidate miRNA that targets multiple addiction-related genes (ARGs) and may provide a novel mechanism for reverting drug-induced aberrant gene expression. In this study, we used bioinformatics analyses of the 3′UTRs of transcripts in the Knowledgebase of ARGs database (KARG);[23] to identify miR-495, a miRNA that targets many ARGs in regulatory networks previously implicated in SUDs. We found that miR-495 is enriched within the NAc and is downregulated by acute cocaine administration and during cocaine self-administration. Viral-mediated miR-495 overexpression not only robustly downregulated ARG expression but more importantly, diminished motivation for cocaine.

[1]School of Life Sciences, Arizona State University, Tempe, AZ, USA and [2]Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, NM, USA. Correspondence: Professor NI Perrone-Bizzozero, Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, 87131, NM, USA.
E-mail: nbizzozero@salud.unm.edu
[3]These authors contributed equally to this work
[4]Present address: Department of Psychology, University of New Mexico, Albuquerque, NM, USA.
[5]Present address: Division of Neuroscience, Ophthalmology and Rare Diseases, Roche Pharma Research and Early Development, Roche Innovation Center, Basel, Switzerland.
[6]Co-Senior Investigator Role.
Received 19 April 2016; revised 31 October 2016; accepted 14 November 2016; published online 3 January 2017

MATERIALS AND METHODS

Animals

Male 2-month-old C57BL/6J mice (Jackson Labs, Bar Harbor, ME, USA) and adult 2-month-old Sprague-Dawley rats (Charles River, San Diego, CA, USA) were maintained on a 12-h and 14/10-h reverse light/dark cycle, respectively. Animal studies were performed in accordance with NIH Animal Welfare guidelines under protocols approved by the Institutional Animal Care and Use committees at the University of New Mexico and Arizona State University.

Bioinformatics analyses

The lists for mouse, human and rat ARGs were retrieved from the KARG database (http://karg.cbi.pku.edu.cn).[23] Lists of KARG genes with evidence number scores $\geqslant 2$ (ref. 23) were used to acquire the 3′UTRs sequences from ENSEMBL BioMart. The frequencies of predicted targets of miR-495 and two previously identified addiction-related miRNAs, let-7 and miR-212,[20,21] in these KARG lists vs the respective genomes were calculated using TargetScan 6.2 (http://www.targetscan.org) conserved sites. miR-495-binding sites in the 3′UTR of the KARG/TargetScan data set were further validated using miRanda.[24]

NAc shell viral injections and cocaine self-administration, extinction and reinstatement

Rats were trained to self-administer cocaine, infused with lentiviruses (LV) containing either green fluorescent protein (GFP; LV-GFP) or GFP+miR-495 (LV-miR-495) into the NAcsh, and then tested on a fixed ratio (FR) 5 and progressive ratio (PR) schedule of cocaine reinforcement, as well as during extinction and cue and cocaine-primed reinstatement, as described in Supplementary Information.

Data analysis

Power analyses were performed to determine adequate sample sizes (PASS, NCSS software, Kaysville, UT, USA). Behavioral and biochemical measures were analyzed using Student *t*-tests or ANOVAs followed by tests for simple effects, where appropriate, using SPSS 24.0 (IBM, Armonk, NY, USA). Adjustments to degrees of freedom were made when unequal variances between groups existed (for example, Welch's correction, Huynh–Feldt correction).

Full Materials and Methods for miRNA fluorescent *in situ* hybridization, dual luciferase assays, acute cocaine treatment, reverse transcription and qPCR, western blotting, intracranial virus injections, cocaine self-administration, food reinforcement and histology are provided in the Supplementary Information.

RESULTS

All statistical values are reported in Supplementary Table 1.

In silico analyses identify miR-495 as a putative post-transcriptional regulator of addiction-related genes in the nucleus accumbens

Initial bioinformatics analyses were aimed at identifying miRNAs that target addiction-associated mRNAs expressed in the NAc. The 3′UTR sequences of mouse, human and rat gene sets of the KARG database (http://karg.cbi.pku.edu.cn) with evidence scores $\geqslant 2$ (ref. 23) were used to determine the prevalence of miRNA-binding sites predicted by TargetScan (http://www.targetscan.org). Among the ARG-targeting miRNAs, we found that miR-495, a microRNA expressed in the adult rat striatum (http://miRBase.org),[25,26] is predicted to target several ARG mRNAs, such as *Bdnf*, *Camk2a*, *Arc* and others (Supplementary Table S2). The percentage of mouse KARG genes containing conserved 3′UTR miR-495-binding sites (7%, 70 genes) is significantly higher than in the entire genome (2.5%), and similar results were obtained using human or rat KARG gene sets (83 genes, 7.7% and 55 genes, 5.6%, respectively). To confirm this method, we assessed the proportion of KARG gene targets of two miRNAs previously associated with cocaine addiction, miR-212/-132[21] and let-7.[14,20] As expected, both miRNAs targeted a higher % of KARG genes than in the genome (miR-212/-132: 2.8%; let-7: 3%), confirming the utility of this approach to identify miRNAs associated with addiction. Given that the frequency of miR-495 targets in KARG was significantly higher (~2-fold) than those for miR-212/-132 and let-7 (Figures 1a and b), it is likely that miR-495 targets may impact a wider variety of functions involved in addiction than those that were established for miR-212/-132 and let-7. Furthermore, the average evidence scores for miR-495 KARG targets were significantly higher than those for the whole KARG set, suggesting that the association of predicted miR-495 KARG targets with addiction is heavily supported by previous research (Supplementary Figure S1). Using miRanda, we further validated the presence of high affinity 3′UTR miR-495-binding sites ($\Delta G \leqslant -15$ kcal mol^{-1}) in the mouse data set, including *Bdnf* and *Camk2a* (Supplementary Table S2).[24] Fluorescent *in situ* hybridization confirmed brain-wide miR-495 expression, including the NAc and medial pre-frontal cortex (mPFC), as previously reported in human mPFC tissue (Figure 1c; scrambled locked nucleic acid control in Supplementary Figure S2).[27] Using qRT-PCR, we confirmed miR-495 expression in these regions, with the highest expression within the NAc (Figure 1d). Thus, miR-495 is a candidate regulator of a set of ARGs conserved in mammals.

miR-495 directly targets the 3′UTRs of *Bdnf*, *Camk2a* and *Arc*

To validate direct miR-495 binding to predicted target ARG mRNAs, we utilized luciferase reporter constructs containing target mRNA 3′UTRs. Due to differential poly(A) site usage, the predicted miR-495 target, *Bdnf*, is present *in vivo* as two different transcripts with a short (*Bdnf*-S) or long 3′UTR (*Bdnf*-L) produced from the same promoter. The long form contains more miR-495-binding sites (Figure 1e; http://www.targetscan.org/mmu_50/), suggesting that miR-495 preferentially regulates *Bdnf*-L. The binding sites within the 3′UTR at nucleotide (nt) positions 233, 565, 587 and 598 are highly conserved between mouse, rat and human, whereas the last binding site at nt 2487 in mouse and rat

Figure 1. miR-495 targets several ARGs and is expressed in addiction-related brain regions. (**a**) Although the frequencies of miR-495, miR-212/132 and let-7 putative targets are all enriched in the KARG database compared with the entire genome, the frequency of miR-495 targets in KARG is significantly higher (approximately two-fold) than those of miR-212/132 and let-7. ****$P < 0.0001$ vs genome, [+++]$P < 0.001$ miR-495 vs miR-212/132 and [###]$P < 0.001$ miR-495 vs let-7, two-tailed χ^2 test. (**b**) Number of genes with putative miR-495 target sites (miR-495 TG) in the mouse KARG set. (**c**) Representative images of a coronal mouse brain section where miR-495 was visualized using fluorescent *in situ* hybridization at $4 \times$ (i), with insets at $10 \times$ focusing on the PFC (ii) and the NAc (iii). Scale bars 500 μm in panel i and 200 μm in panels ii and iii. (**d**) qRT-PCR analysis of miR-495 levels in different brain regions ($n = 3$). (**e**) Schematic representation of the short and long 3′UTR transcripts of brain-derived neurotrophic factor (BDNF) including the positions of conserved and partially conserved miR-495-binding sites ($M = Mus$ *musculus*, $R = Rattus$ *norvegicus*, $H = Homo$ *sapiens*). For *in vitro* target validation, HeLa cells were transfected with a firefly luciferase reporter containing the 3′UTR of *Bdnf* and *Camk2a*. A *Renilla* vector was co-transfected with the firefly reporter. Pre-miR-495, anti-miR-495 and pre-miR miRNA precursor negative control #2 were transfected as described in Supplementary Information. The alternative 3′UTRs of BDNF (**f**), as well as the 3′UTRs of *Camk2a* and *Arc,* were assayed (**g**). $n = 4$ *$P < 0.05$, **$P < 0.01$. Error bars indicate s.e.m. ARG, addiction-related gene; ac, anterior commissure; AMY, amygdala; DH, dorsal hippocampus; D-STR, dorsal striatum; Ctx, neocortex; KARG, Knowledgebase of ARG; NAc, nucleus accumbens; PyC, pyriform cortex; OFC, orbitofrontal cortex; PFC, prefrontal cortex; VH, ventral hippocampus; VTA, ventral tegmental area.

or nt 2488 in human is partially conserved. Indeed, dual luciferase assays showed that miR-495 significantly reduced the activity of the reporter containing the 3'UTR for *Bdnf*-L by ~ 50% and for *Bdnf*-S by ~ 20% (Figure 1f). Given that these isoforms have been hypothesized to have different functions and localization within the neuron, these results suggest that miR-495 may preferentially
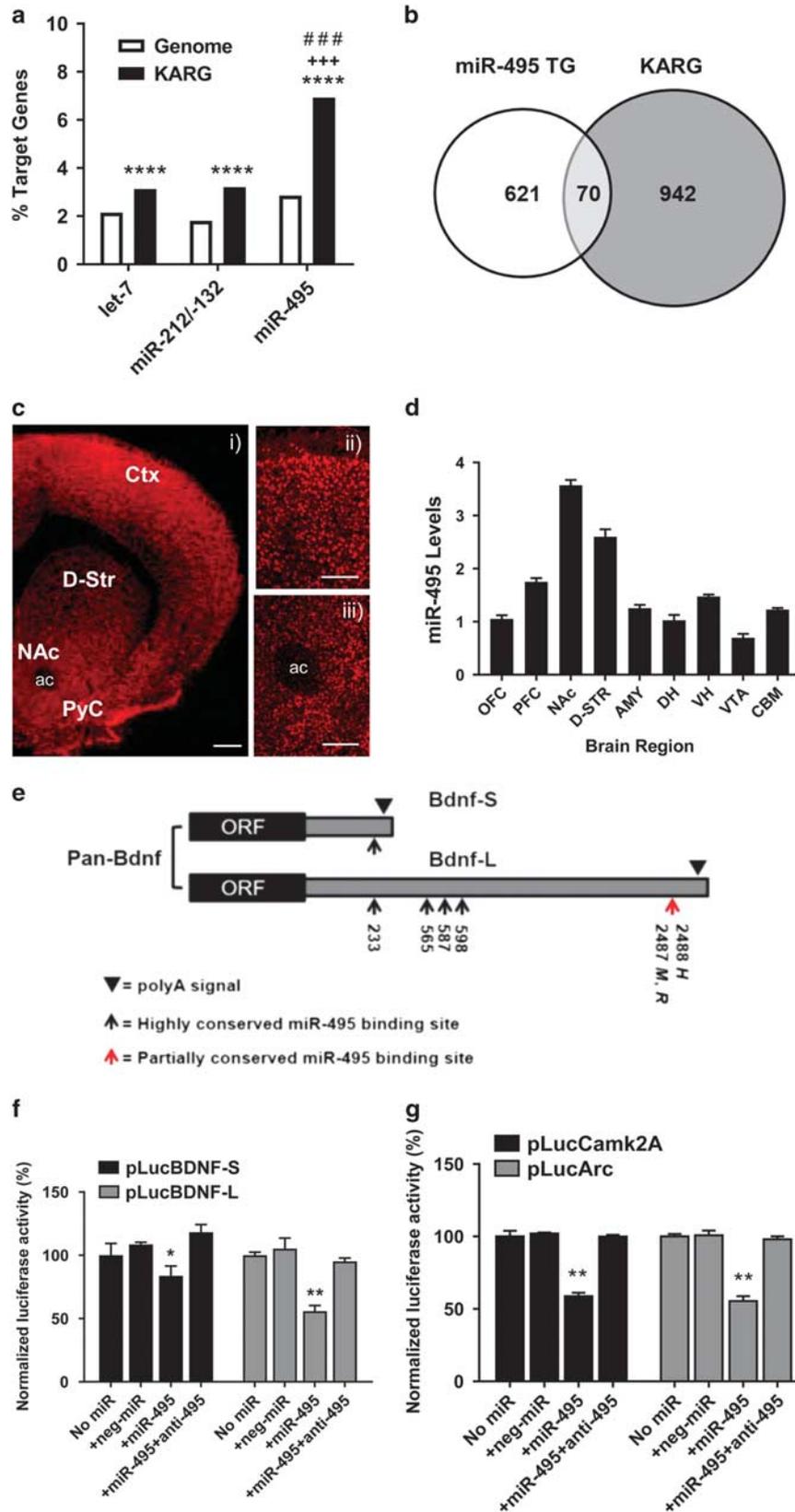

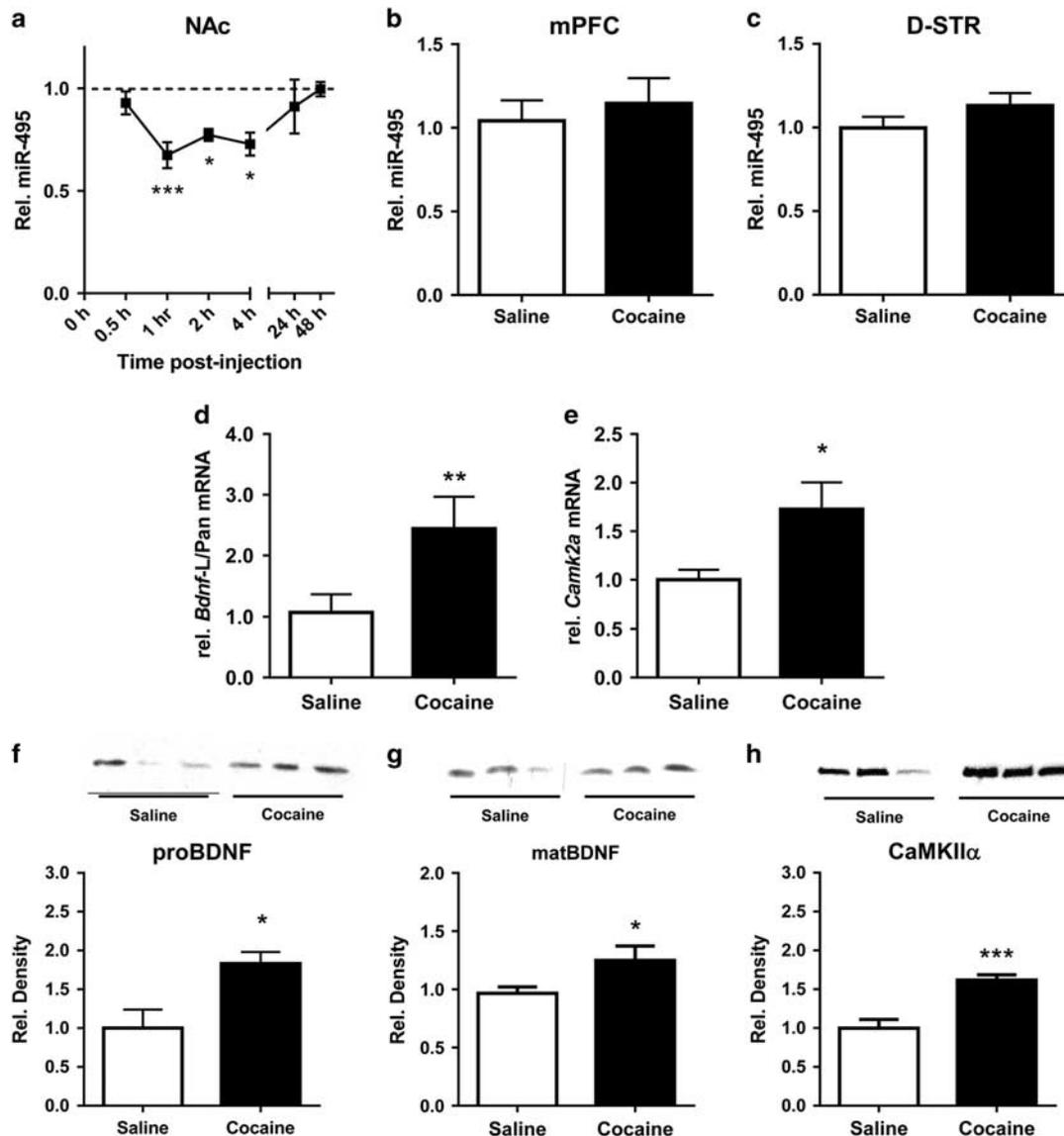**Figure 1.** For caption see page 435.

Figure 2. Acute cocaine effects on NAc miR-495 and target mRNA expression. Male C57Bl/6 mice received an acute injection of saline or cocaine (15 mg kg^{-1}, i.p.), and NAc tissue was processed for qRT-PCR and western blot. (A) NAc miR-495 levels were found to be downregulated rapidly after acute cocaine (0.5 h, $n = 5$; 1 h, $n = 10$; 2 h, $n = 4$; 4 h, $n = 6$; 24 h, $n = 6$; 48 h, $n = 5$). miR-495 expression was not altered by acute cocaine 2 h after within the mPFC (**b**); coc $n = 5$, sal $n = 6$) or D-STR (**c**); coc $n = 6$, sal $n = 8$). Acute cocaine increases expression of NAc Bdnf-L relative to pan-Bdnf nearly two-fold, as measured by qRT-PCR (**d**); coc $n = 4$, sal $n = 5$). (**e**) Acute cocaine also increased Camk2a mRNA at 2 h ($n = 5$). NAc pro-BDNF (**f**), mature BDNF (**g**) and CaMKIIα (**h**) increases in protein levels were also found by western blot 2 h post cocaine or saline injection, corrected for total protein by Coomassie Brilliant Blue staining (pro-BDNF, coc $n = 9$, sal $n = 7$; matBDNF, coc $n = 9$, sal $n = 8$; CaMKIIα $n = 6$ per group, representative blot with each lane representing individual animals; bars represent quantification of average density of each sample from duplicate blots). Error bars indicate s.e.m. *$P < 0.05$, **$P < 0.01$ vs saline. BDNF, brain-derived neurotrophic factor; D-STR, dorsal striatum; mPFC, medial prefrontal cortex; NAc, nucleus accumbens.

regulate Bdnf-L and its associated functions.[28,29] In addition, miR-495 significantly reduced the activity of reporters containing the 3'UTRs of Camk2a and Arc by ~40% and 45%, respectively (Figure 1g). All effects were blocked by anti-miR-495, and miR-495 had no effect on empty vectors (Supplementary Figure S3). These in vitro studies demonstrate that the predicted miR-495-binding sites in these ARGs are indeed functional.

miR-495 and target mRNA expression in response to acute cocaine administration
The anatomical localization and targets of miR-495 suggest that it may have a role in the post-transcriptional mechanisms

underlying addiction-related plasticity. To examine this further, we determined the effect of an acute cocaine injection (15 mg kg^{-1}, i.p.) in mice on NAc miR-495 expression at different time points. NAc miR-495 was significantly downregulated between 1 and 4 h post injection (Figure 2a). This effect was brain region-specific, as miR-495 expression was not significantly altered by cocaine 2 h post acute cocaine in the mPFC or dorsal striatum (Figures 2b and c).

Next, we assessed the expression of two luciferase-validated miR-495 targets, Bdnf and Camk2a, at the middle of this timeframe —2 h post injection. We found that both NAc Bdnf-Pan mRNA, which is the sum of Bdnf-S and Bdnf-L isoforms, and Bdnf-L mRNA

were significantly increased 2 h post injection (Supplementary Figure S4). Although this demonstrates that *Bdnf* mRNA is upregulated by acute cocaine, it does not point to the mechanism involved in this cocaine-induced upregulation. Since both *Bdnf* transcripts originate from the same promoter, differences between the two isoforms would suggest regulation at the post-transcriptional level. To evaluate the possibility, we calculated the ratio between *Bdnf*-L and *Bdnf*-Pan and found that it was significantly increased by approximately two-fold (Figure 2d), indicating that acute cocaine preferentially upregulates the long 3′ UTR variant that contains a greater number of miR-495-binding sites than the short form (Figures 1e and f). In addition, we found that both pro-BDNF and mature BDNF protein were significantly increased within the NAc 2 h after cocaine treatment (Figures 2f and g). Another luciferase-validated miR-495 target, *Camk2a*, was found to be regulated 2 h post injection within the NAc as both mRNA (Figure 2e) and protein (Figure 2h) were increased. Thus, NAc miR-495 expression is rapidly decreased by exposure to cocaine concomitantly with increased expression of its ARG targets, *Bdnf* and *Camk2a*. This inverse relationship in cocaine-induced gene expression suggests a functional link between miR-495 and its target ARGs *in vivo*.

Overexpression of miR-495 within the NAc shell reverses cocaine-induced ARG expression

To further examine the regulatory relationship between cocaine-induced NAc miR-495 downregulation and upregulation of target ARG mRNAs, we next tested whether these changes could be reversed by restoring miR-495 levels in the NAc with viral-mediated overexpression. LV encoding pri-miR-495+GFP (LV-miR-495) or GFP (LV-GFP) was infused into the NAc shell (NAcsh; Figure 3a) of male Sprague-Dawley rats who were treated 2 weeks later with saline or cocaine (15 mg kg^{-1}, i.p.). Cocaine-treated LV-GFP rats were found to express significantly lower NAc miR-495 levels compared with saline-treated LV-GFP controls (Figure 3b), replicating the cocaine-induced downregulation of NAc miR-495 in mice without lentiviral infusion (Figure 2a). LV-miR-495 rats exhibited significantly greater NAc miR-495 expression compared with LV-GFP-infused animals in either treatment group, effectively reversing the cocaine-induced decrease in miR-495 (Figure 3b). Similarly, cocaine-treated LV-GFP rats were found to express significantly higher levels of both *Bdnf* variants compared with saline-treated LV-GFP controls and LV-miR-495 animals in either drug treatment group (Figures 3c and d). Thus, the ability of miR-495 overexpression to reduce the cocaine-induced upregulation of both *Bdnf* transcripts suggests a functional link between miR-495 and its targets *in vivo*.

Pathway analysis of mRNAs downregulated by miR-495 overexpression reveals multiple regulatory networks involved in SUDs

As multiple ARGs are predicted targets of miR-495 (Supplementary Table S2), we used microarray analysis to determine the global effects of NAcsh miR-495 overexpression on mRNA expression *in vivo*. We found that 1027 mRNAs were significantly decreased after LV-miR-495 treatment. Of the previously identified 691
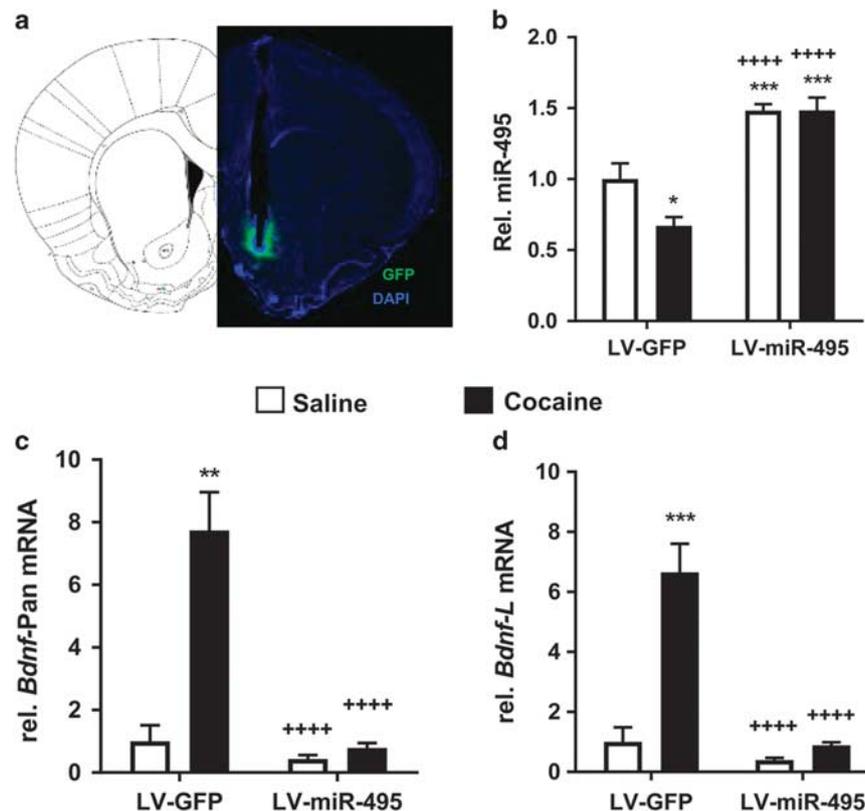

Figure 3. NAc miR-495 overexpression counteracts cocaine-induced changes in gene expression. Two weeks following infusion of either LV-GFP or LV-miR-495 into the NAcsh (**a**), rats received an injection of saline or cocaine (15 mg kg^{-1}, i.p.). LV-miR-495 blocked the cocaine-induced decrease in NAc miR-495 expression 2-h post injection (**b**) and prevented the cocaine-induced increase in NAc pan-*Bdnf* (**c**) and *Bdnf*-L (**d**) expression. LV-GFP-saline: $n = 3$; LV-miR-495-saline: $n = 5$; LV-GFP-cocaine: $n = 8$; LV-miR-495-cocaine: $n = 6$. Error bars indicate s.e.m. *$P < 0.05$, ** < 0.01, *** < 0.001 vs saline-treated LV-GFP rats and $^{++++}P < 0.0001$ vs cocaine-treated LV-GFP. LV, lentivirus; GFP, green fluorescent protein; NAc, nucleus accumbens; NAcsh, NAc shell.
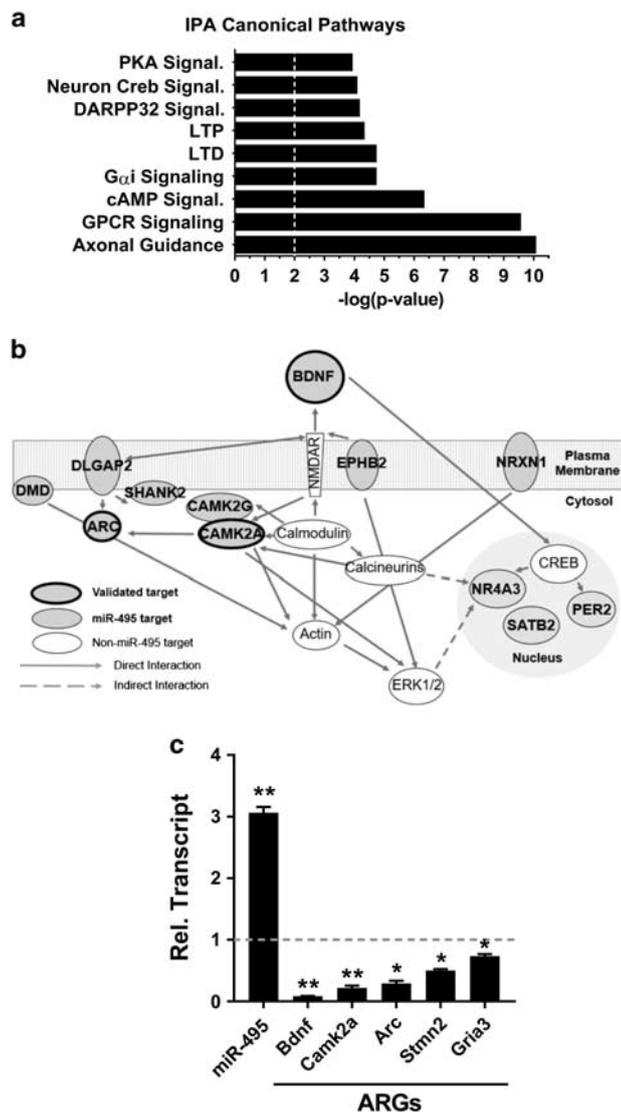
Figure 4. miR-495 regulates genes involved in several addiction-related networks. Rats were infused with either LV-GFP or LV-miR-495 into the NAcsh, and 1 week later NAc RNA was collected for both microarray and qRT-PCR analysis (*n* = 5 per group). (**a**) Top canonical pathways enriched in genes downregulated by miR-495 overexpression (vertical dotted line represents threshold, *P* < 0.01) were determined using IPA. (**b**) A graphical representation of the genes associated with the top signaling network denotes the presence of predicted and validated miR-495 targets. (**c**) LV-miR-495 infusion increased miR-495 expression and decreased expression of several ARGs compared with LV-GFP controls (dotted line), as measured by qRT-PCR. Error bars indicate s.e.m. *P* < 0.05, **P* < 0.01 compared with controls. GFP, green fluorescent protein; IPA, Ingenuity Pathway Analysis; LTD, long-term depression; LTP, long-term potentiation; LV, lentivirus; NAc, nucleus accumbens; NAcsh, NAc shell.

miR-495 targets, 76 were significantly downregulated by miR-495 overexpression, 15 of which, including *Bdnf*, *Camk2a*, *Arc*, *Gria3* and *Stmn2*, were also present in KARG (Supplementary Table S3). A complete list of miR-495 regulated transcripts on the arrays has been deposited in the Gene Expression Omnibus database (GSE85500).

Using Ingenuity Pathway Analysis, we searched for canonical pathways enriched with NAc mRNAs downregulated by miR-495 overexpression. We found multiple pathways that were previously characterized in addiction-related behavior, including GPCR

signaling, cAMP-mediated signaling, synaptic long-term depression and long-term potentiation, CREB and protein kinase A (PKA) signaling (Figure 4a). The top biological network of interacting molecules regulated by miR-495 contained 21 of the original 76 molecules (selections in Figure 4b, for complete list see Supplementary Table S4) with functions related to drug-evoked synaptic plasticity (for example, *Gria3*, *Shank2*, *Arc*, *Ephb2* and *Camk2a*), transcription factors (for example, *Satb2*, *Per2*) and chromatin remodeling (for example, *Satb2*).[6,30] Using qRT-PCR, we confirmed that miR-495 overexpression decreased selected target mRNAs (Figure 4c and Supplementary Table S3). Altogether, these results indicate that miR-495 regulates multiple target ARG mRNAs both *in vitro* (Figures 1f and g) and *in vivo*.

NAcsh miR-495 expression decreases following cocaine self-administration
Using qRT-PCR, NAc miR-495 expression was measured in rats that self-administered cocaine for either 1 or 22 days (SA1 vs SA22). The cocaine groups did not differ in total cocaine infusions during the test session (Supplementary Figure S5). Saline-yoked controls at each time point did not significantly differ in NAcsh miR-495 and were combined. NAcsh miR-495 was significantly decreased in the SA1 and SA22 group compared with the saline group (Figure 5a). Furthermore, a significant linear trend was found across time, where NAcsh miR-495 levels decreased as the number of cocaine self-administration sessions increased. Although baseline miR-495 expression in both NAc subregions are similar (Supplementary Figure S6), no effect on miR-495 expression was found in the adjacent NAc core after short or long-term self-administration (Supplementary Figure S7), demonstrating NAc subregion-specific effects.

miR-495 overexpression in the NAcsh reduces motivation to self-administer and seek cocaine
To test the role of NAc miR-495 in models of addiction-related behavior, we next examined the effect of NAcsh miR-495 overexpression on self-administration and seeking behavior. Two weeks following NAcsh infusions of LV-miR-495 or LV-GFP (Figure 5b), rats were given access to varying doses of cocaine delivered on a FR5 and PR schedule of reinforcement. NAcsh miR-495 overexpression was persistent to the end of our behavioral experiments (Figure 5c). As such, NAcsh miR-495 overexpression had no effect on responding or intake on the low-effort FR5 schedule of cocaine reinforcement (Supplementary Figure S8). However, NAcsh miR-495 overexpression significantly decreased responding and intake on the high-effort PR schedule compared with controls across all cocaine doses tested (Figures 5d and e), without effects on inactive lever pressing (Supplementary Figure S9A). The cumulative response records in Figure 5f show a lower break point in a representative LV-miR-495 rat compared with a LV-GFP rat, consistent with a decrease in motivation.

Next, we tested the effect of NAcsh miR-495 overexpression on other measures of motivation, including extinction and reinstatement. Before extinction testing, rats received >3 sessions on an FR5 schedule of cocaine reinforcement on the training dose. We found no group difference in active lever pressing during this baseline before extinction (Figure 5g). During extinction, NAcsh miR-495 overexpression decreased active lever pressing during the first 3 sessions compared with LV-GFP controls (Figure 5g), without effects on inactive lever pressing (Supplementary Figure S9B). We then tested rats for both cue and cocaine-primed reinstatement of cocaine seeking. Although the virus groups did not differ in the degree of cue reinstatement (Supplementary Figure S10), the LV-miR-495 group exhibited significantly reduced cocaine-primed reinstatement compared with the LV-GFP controls (Figure 5h), without differences in inactive lever pressing in either case (Supplementary Figure S9C and D). To confirm the effect of

miR-495 overexpression on target regulation during drug-seeking behavior, we measured the levels of *Camk2a, Bdnf*-L and *Bdnf*-Pan mRNAs in the NAcsh of rats 1 week after extinction and reinstatement experiments. As shown in Figures 5i-k the levels of these three target mRNAs were significantly decreased by

miR-495 overexpression. Collectively, these results suggest that NAcsh miR-495 overexpression decreases motivation to both self-administer and seek cocaine. The additional decrease in target gene expression suggests that the behavioral effect of miR-495 overexpression is mediated through these ARGs.
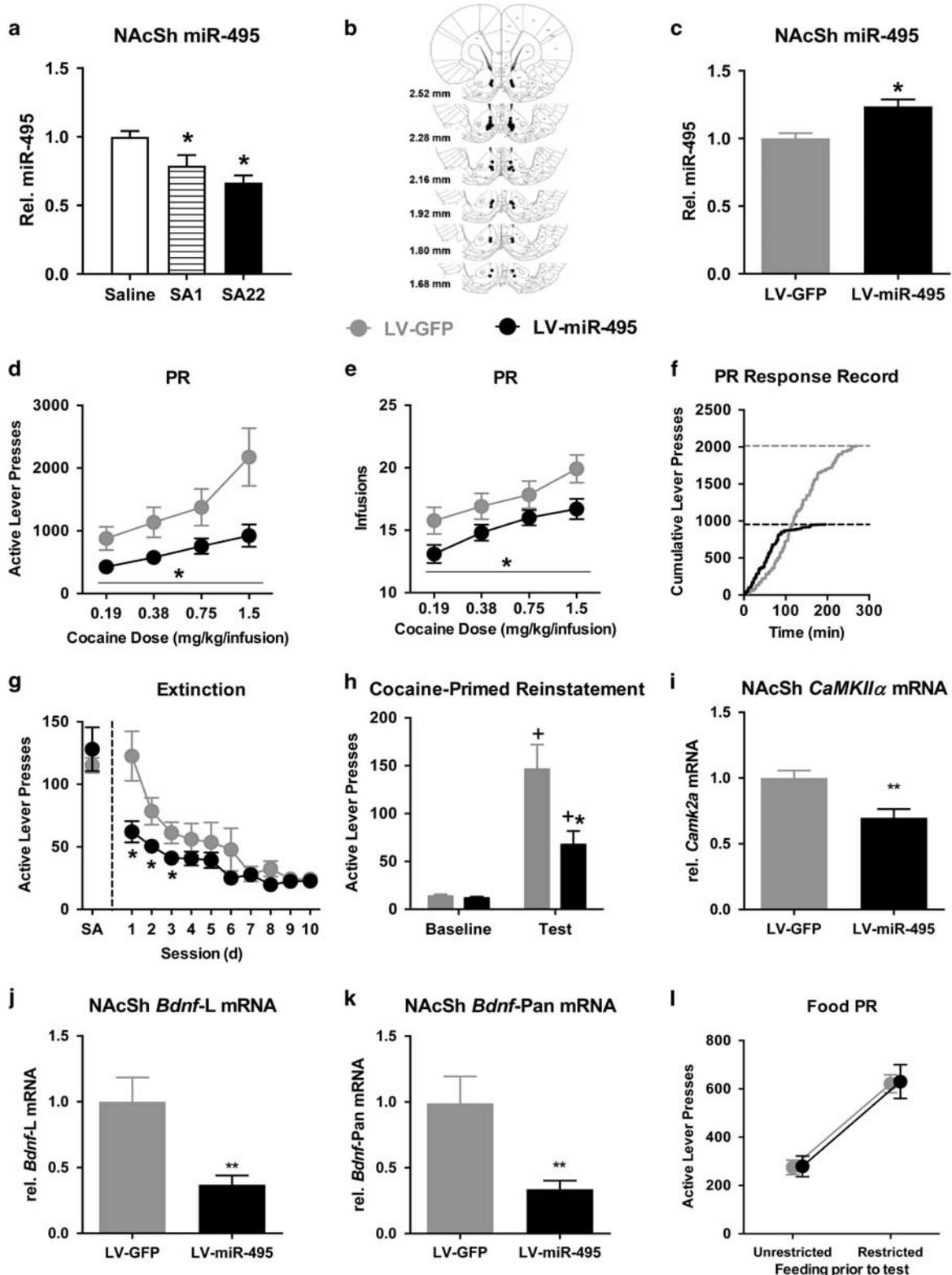
To test the specificity for cocaine and evaluate any potential locomotor-suppressing effects of miR-495 overexpression, a separate group of rats were trained to lever press for food pellets whereas receiving either LV-GFP or LV-miR-495. LV-miR-495 had no effect on responding or intake on an FR5 schedule of food reinforcement (Supplementary Figure S11). To parallel differences in motivational value of low and high cocaine doses on a PR schedule, we subjected rats to varying levels of food restriction. Rats that had been food restricted (18 g per day) exhibited higher PR measures than those that had been unrestricted, but both virus groups exhibited similar levels of motivation for food under both feeding conditions (Figure 5l and Supplementary Figure S12). This suggests a selective effect of miR-495 overexpression on motivation for cocaine.

DISCUSSION

Here we established NAc miR-495 as a novel post-transcriptional regulator of both ARG expression and motivation for cocaine. Initial bioinformatics analyses identified miR-495 as a miRNA with predicted targets enriched in the KARG database and with preferential expression in the brain reward and motivation pathway. Among these, we validated that miR-495 targeted *Bdnf* and *Camk2a* both *in vitro* and *in vivo*. Next, we found that cocaine decreased miR-495 expression along with concomitant increases in ARG targets in the NAc. When the cocaine-mediated miR-495 downregulation was blocked by lentiviral-mediated overexpression, cocaine-induced upregulation of ARG target mRNAs in the NAc was also prevented. From the miR-495 overexpression microarray analysis, we found that several of the downregulated target genes formed networks involved in receptor signaling, gene regulation and synaptic plasticity. Importantly, we found that NAc miR-495 overexpression reduced motivation to self-administer and seek cocaine, without effects on food reinforcement, suggesting that NAc miR-495 selectively regulates genes involved in motivation for cocaine. Given that motivation for drug is a key factor involved in human drug relapse, miR-495 may have translational value as a novel therapeutic target.

In contrast to other addiction-related miRNAs whose expression levels were shown to increase in response to drugs of abuse,[14,17,18,21] miR-495 expression in the NAc decreased shortly after exposure to cocaine. This rapid downregulation could be due to several factors. Although miRNAs are stable in non-neuronal cell types, some miRNAs decay at faster rates in neurons via activity-dependent processes,[31] which could be triggered by cocaine administration. The decrease in miR-495 expression may also be due to transcriptional repression. miR-495 is located within miRNA cluster B of the Dlk1-Dio3 maternally imprinted region that is under the control of Methyl-CpG-binding protein 2 (MeCP2) and other transcription factors.[32] Using *Mecp2*-null mice, Wu et al.[33] demonstrated increased expression of many of the miRNAs within this imprinted region, including miR-495. However, given that not

all of the miRNAs in this cluster are coordinately regulated,[33,34] other regulatory mechanisms such as pre-miRNA processing and/or mature miRNA stability may have a role in controlling miRNA expression profiles.[35] It is curious that we did not observe changes in miR-495 in any other addiction-related brain region, especially in those that are also innervated by ventral tegmental dopamine neurons (for example, dorsal striatum, mPFC). One possibility is that the relatively high basal expression levels of miR-495 in the NAc allowed us to detect a decrease following cocaine administration. Another possibility is that the NAc may have a more robust response to the acute and chronic effects of cocaine, resulting in decreases in miR-495 expression levels.

miRNAs has a role in fine-tuning gene expression involved in many cell signaling pathways.[5,36] Here, we established that miR-495 directly targets and regulates the ARG *Bdnf* both *in vitro* and *in vivo*. Bdnf in the NAc has been linked to several drug abuse-related behaviors, where BDNF expression levels positively correlate with cocaine reward and motivation.[19,37–40] Similarly, others have established that another miR-495 target, *Camk2a*, has a positive relationship between NAc levels and psychostimulant abuse-related behavior.[41–44] Furthermore, CaMKII has been identified *in silico* as a central node in positive feedback gene regulatory pathways involved in addiction.[23] Therefore, regulation of both of these genes by miR-495 may coordinately affect several domains of addiction-related processes. Indeed, addiction is hypothesized to be a dysfunction of neuroplasticity,[3] and both *Bdnf* and *Camk2a* genes encode for plasticity-related proteins. We found that many other miR-495 targets that we validated also form networks involved in long-term potentiation and long-term depression,[30,45,46] as well as other cell signaling cascades relevant to addiction, such as PKA signaling.[47,48] Therefore, miR-495 may act as a mechanism to fine tune the molecular response of multiple, interwoven pathways involved in the development of addiction.

Similar to acute cocaine administration, NAcsh miR-495 levels were also decreased following both brief and prolonged cocaine self-administration. It is unknown why this occurred exclusively in the NAcsh, and not the core, but this effect may be due to the differential afferent projections into these subregions or the involvement of the shell in the primary reinforcing and unconditioned effects of cocaine.[49,50] Interestingly, these effects are consistent with previous findings that both BDNF and CaMKIIα involvement in cocaine abuse-related behavior is also specific to the NAcsh, and not the core.[39,41–44,51] Furthermore, the downward trend of NAcsh miR-495 expression as cocaine self-administration experience increased may be related to the theory of incentive sensitization, where motivation to seek drug (that is, 'wanting') increases over the course of drug use.[52] Thus, decreases in NAcsh miR-495 expression may be indicative of sensitized motivation.

To test this hypothesis, we examined the effect of NAcsh miR-495 overexpression on cocaine self-administration and seeking behavior. We observed that NAcsh miR-495

Figure 5. miR-495 overexpression in the NAcsh suppresses motivation to self-administer and seek cocaine in rats. (**a**) Endogenous NAcsh miR-495 levels are suppressed following 1 and 22 days of cocaine self-administration, as measured by qRT-PCR (Saline: $n = 7$; SA1: $n = 6$; SA22: $n = 7$). *$P < 0.05$ vs saline-yoked. (**b**) Histological verification of NAcsh cannula placement. (**c**) NAcsh miR-495 levels are increased in the LV-miR-495 group compared with the LV-GFP group following behavioral testing ($n = 4$ per group). NAcsh miR-495 overexpression reduced responding (**d**) and intake (**e**) on a PR schedule of cocaine reinforcement. (**f**) Cumulative response records of representative rats tested at 1.5 mg kg^{-1} per infusion that were closest to their respective group means. Dotted line represents the total number of lever presses emitted once break point was achieved. (**g**) NAcsh miR-495 overexpression reduced cocaine-seeking behavior during the first three 1- h extinction sessions. (**h**) NAcsh miR-495 overexpression reduced cocaine-primed reinstatement during the 1- h test session (10 mg kg^{-1}, i.p.). LV-GFP: $n = 12$, LV-miR-495: $n = 10$. (**i–k**) Downregulation of *Camk2a* (LV-GFP: $n = 7$, LV-miR-495: $n = 8$, $P < 0.01$) and *Bdnf*-L and -Pan ($n = 6$ per group, $P < 0.01$) in NAcsh of miR-495 OE animals after behavioral testing. (**l**) NAcsh miR-495 overexpression had no effect on responding under a PR schedule of food reinforcement, regardless of whether the rats were food restricted (18 g per day) or not ($n = 8$ per group). Error bars indicate s.e.m. Panels C-H: *$P < 0.05$ vs LV-GFP. +$P < 0.05$ vs extinction baseline. GFP, green fluorescent protein; IPA, Ingenuity Pathway Analysis; LV, lentivirus; NAc, nucleus accumbens; NAcsh, NAc shell; PR, progressive ratio.

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overexpression decreased responding and intake when cocaine was available on the high-effort PR schedule, but did not alter intake in the low-effort FR5 schedule. PR schedules are believed to model an aspect of SUD related to an individual increasing time and energy toward drug-seeking and drug-taking behavior;[53] thus, alterations in these behaviors closely model hallmark symptoms of human addiction. Furthermore, NAc miR-495 overexpression did not alter PR measures in a similar procedure with a natural food reinforcer. This suggests that miR-495 specifically influences motivation for cocaine likely without impacting the reinforcing value of cocaine or food reinforcement/motivation. In addition, we found that NAcsh miR-495 overexpression reduced cocaine-seeking behavior during extinction and reinstatement, further supporting a selective reduction in motivation. We also confirmed that under these conditions, miR-495 overexpression resulted in decreases in *Bdnf* and *Camk2a* expression. Closely mirroring our effects, previous work has shown that NAcsh knockdown of *Camk2a* and inhibition of NAcsh CAMKII reduce PR measures and reinstatement, respectively.[43,44] Thus, CaMKIIα is an important regulatory crux of many addiction-related molecular pathways.[41,42,44,51,54] Although we did not detect a statistically significant effect during cue reinstatement ($P = 0.06$, one-tailed), the pattern is very similar. The lack of a more robust effect on cue reinstatement may highlight the more prominent role of the NAc core, rather than the shell, in regulating cue reinstatement.[55] Overall, our results suggest that miR-495 preferentially regulates a network of ARG targets involved in the incentive motivational properties of cocaine which are more critical for sustaining behavior under the high-effort PR schedule of reinforcement than under the low-effort FR5 schedule of reinforcement.

In conclusion, we demonstrate a novel *in silico* method to identify potential miRNAs that may be involved in SUD. Our findings suggest that miR-495 decreases motivation for cocaine by targeting several ARGs and regulatory pathways in the NAc involved in synaptic plasticity, PKA signaling and other pathways associated with the disorder. These results highlight the importance of moving drug abuse research from a single gene focus to biological pathways in order to better understand the complexity of the molecular networks associated with addiction. This discovery also opens new avenues for future research on the specific factors controlling cocaine-induced decreases in miR-495 and the role of miR-495 in regulating different forms of synaptic plasticity in the NAc. Most importantly, we believe this study is the first to identify a miRNA that specifically regulates the incentive motivational properties for cocaine both during active drug taking and following a period of abstinence. The latter finding is particularly compelling, as preventing relapse is a primary objective for addiction translational research. The possibility of globally targeting drug-induced changes in gene expression via miRNAs, such as miR-495, may lead to new therapeutics that shift the balance of gene regulation toward alleviating, rather than promoting, SUD-related behavior.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)