MicroRNA Regulation of Addiction-Related Gene Expression and Motivation for Cocaine in Rats

by

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ABSTRACT

MicroRNAs are small, non-coding transcripts that post-transcriptionally regulate expression of multiple genes. Recently microRNAs have been linked to the etiology of neuropsychiatric disorders, including drug addiction. Following genome-wide sequence analyses, microRNA-495 (miR-495) was found to target several genes within the Knowledgebase of Addiction-Related Genes (KARG) database and to be highly expressed in the nucleus accumbens (NAc), a pivotal brain region involved in reward and motivation. The central hypothesis of this dissertation is that NAc miR-495 regulates drug abuse-related behavior by targeting several addiction-related genes (ARGs). I tested this hypothesis in two ways: 1) by examining the effects of viral-mediated miR-495 overexpression or inhibition in the NAc of rats on cocaine abuse-related behaviors and gene expression, and 2) by examining changes in NAc miR-495 and ARG expression as a result of brief (i.e., 1 day) or prolonged (i.e., 22 days) cocaine self-administration. I found that behavioral measures known to be sensitive to motivation for cocaine were attenuated by NAc miR-495 overexpression, including resistance to extinction of cocaine conditioned place preference (CPP), cocaine self-administration on a high effort progressive ratio schedule of reinforcement, and cocaine-seeking behavior during both extinction and cocaine-primed reinstatement. These effects appeared specific to cocaine, as there was no effect of NAc miR-495 overexpression on a progressive ratio schedule of food reinforcement. In contrast, behavioral measures known to be sensitive to cocaine reward were not altered, including expression of cocaine CPP and cocaine self-administration under a low effort FR5 schedule of reinforcement. Importantly, the effects were accompanied by decreases in NAc ARG expression, consistent with my hypothesis.

In further support, I found that NAc miR-495 levels were reduced and ARG levels were increased in rats following prolonged, but not brief, cocaine self-administration experience. Surprisingly, inhibition of NAc miR-495 expression also decreased both cocaine-seeking behavior during extinction and NAc ARG expression, which may reflect compensatory changes or unexplained complexities in miR-495 regulatory effects. Collectively, the findings suggest that NAc miR-495 regulates ARG expression involved in motivation for cocaine. Therefore, using microRNAs as tools to target several ARGs simultaneously may be useful for future development of addiction therapeutics.

DEDICATION

To all of my teachers, mentors, and colleagues over the years,

for inspiring me, challenging me,

and giving me the confidence to achieve my goals.

To my entire family and close friends,

for providing unrelenting and vital support along the way.

To my mom, dad, and sister

for always being proud of me and encouraging me to follow my dreams

Finally, I would like to dedicate this dissertation

in memory of the late Kyle Kassman.

Your passion for research was unparalleled,

and your love for family was never-ending.

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CHAPTER 1

GENERAL OVERVIEW

Drug addiction is a chronic, debilitating condition that leads to psychological, physical, and social distress. In the clinical field, addiction is referred to as substance use disorder (SUD) that falls along a continuum, from mild to severe, and is characterized by excessive drug use, persistent desire or craving for drug, excessive time spent attempting to acquire drug, or continued use despite negative consequences. SUD is a pervasive problem in the US, where the costs to society are staggering (e.g., $193 billion in 2007; www.justice.gov/ndic, 2011). Therefore, much research is devoted to examining the neural mechanisms underlying drug reinforcement and motivation in order to find novel therapeutics for SUDs.

Drugs of abuse exert their reinforcing effect primarily by activating mesocorticolimbic dopamine neurons that originate in the ventral tegmental area (VTA) and project to the nucleus accumbens (NAc), prefrontal cortex (PFC), amygdala, and hippocampus (Feltenstein & See, 2008; Pierce & Kumaresan, 2006). Psychostimulants, and in particular cocaine, act as potent reinforcers due to their ability to increase synaptic monoamines levels through blockade of presynaptic reuptake transporters. Although this action appears to be involved in the initial reinforcing phase of drug use, the persistent compulsion to procure drugs that characterizes dependence suggests that more enduring changes occur within this reward circuitry (Kalivas & Volkow, 2005). Indeed, drugs of abuse alter gene expression, resulting in changes in synaptic plasticity and efficacy (Hyman, Malenka, & Nestler, 2006; Kauer & Malenka, 2007). Such changes are thought to underlie the transition from recreational to compulsive drug use (Hyman & Malenka,

2001; Kalivas, 2009). While work in this area has focused on changes at the transcriptional level (e.g. DNA methylation, chromatin remodeling, transcription factors, etc.) (LaPlant & Nestler, 2011; Renthal & Nestler, 2009), little is known about post-transcriptional mechanisms that also contribute to altered addiction-related gene (ARG) expression.

MicroRNAs

Before the 1990s, non-protein-coding RNA was often referred to as "junk DNA"; believed to have little relevance to biological function. A growing body of research over the past 25 years has shown that non-coding RNAs have pivotal roles in almost every cellular process investigated (Kloosterman & Plasterk, 2006). One class of non-coding RNA that has received much attention is microRNAs (miRNAs), which are highly conserved RNAs that regulate gene expression post-transcriptionally. miRNA genes reside in the genome in several forms, either as a single miRNA gene with its own promoter, in a cluster of several miRNAs that are transcribed together, or intronic genes that are transcribed with a host protein-coding gene (Berezikov, 2011). Once transcribed, the resulting pri-miRNA transcript folds and binds to itself imperfectly, producing a stem-loop hairpin structure (Fig. 1). The RNAse III endonuclease, Drosha, trims the double-stranded stem portion of the pri-miRNA, producing a smaller hairpin known as a pre-miRNA. Pre-miRNAs are then transported out of the nucleus into the cytoplasm, where the loop portion of the pre-miRNA cleaved by the RNAse III enzyme Dicer (Bartel, 2004). Now as a double-stranded RNA molecule, (i.e., miRNA-miRNA* duplex), it is unbound and one strand joins with several proteins to form the miRNA-induced silencing complex (miRISC), while the passenger strand (i.e., miRNA*) is

typically degraded. The miRISC-associated miRNA (~22 nucleotides) binds to complementary sequences in the 3' untranslated region (UTR) of a target mRNA where miRISC can cause translational repression, deadenylation, or endonucleolytic cleavage of the target mRNA, thus preventing its expression (Filipowicz, Bhattacharyya, & Sonenberg, 2008). Importantly, the mature miRNA needs only ~6-8 complementary nucleotides for which to base pair with the 3' UTR of the target mRNA (i.e., seed region), so one miRNA can target several hundreds of different mRNAs in a given cell (Bartel, 2004). Changes in miRNA expression can therefore lead to widespread changes in gene expression and alteration in several cellular signaling cascades.

MicroRNAs and Drug Abuse

Given that drugs of abuse produce widespread changes in protein-coding gene expression (Maze et al., 2010; Renthal et al., 2007), finding candidate miRNAs that target a large proportion of addiction-related genes (ARGs) may provide a novel tool for normalizing drug-induced aberrant gene expression. One approach to finding candidate addiction-related miRNAs is to examine miRNA expression changes within brain regions implicated in addiction following varying levels of drug exposure. Recent studies indicate that several drugs of abuse regulate miRNA expression in the NAc and other regions of the brain reward pathway (Chandrasekar & Dreyer, 2009; Eipper-Mains et al., 2011; Guo, Chen, Carreon, & Qiang, 2012; Nudelman et al., 2010; Schaefer et al., 2010; Tapocik et al., 2014). More specifically, Hollander and colleagues (2010) found that rats given extended (6h/day), but not restricted (2h/day), access to cocaine self-administration exhibited upregulation of miR-212 in the dorsal striatum, a region involved in establishing habitual behavior (Everitt & Robbins, 2005). miR-212 directly targets

methyl-CpG binding protein 2 (MeCP2) and decreases in striatal MeCP2 are associated with decreases in compulsive drug-taking behavior (Im, Hollander, Bali, & Kenny, 2010). Therefore, upregulation of miR-212 may be involved in countering cocaine's effects through suppression of MeCP2. Additionally, alcohol-dependent rats exhibit increases in miR-206 expression in the medial PFC (mPFC; Tapocik et al., 2014), a brain region involved in executive control of drug-seeking behavior (Kalivas & Volkow, 2005). miR-206 directly targets and suppresses brain-derived neurotrophic factor (BDNF) expression in the PFC (Tapocik et al., 2014), where decreases in BDNF in this region are associated with increases in motivation for cocaine (Sadri-Vakili et al., 2010). This suggests that increases in miR-206 likely promote the development of alcohol dependency through suppression of BDNF. Collectively, these findings provide evidence that miRNAs are altered by drug exposure and can be involved in either the pro- or anti-addiction-related processes, depending on the brain region and target genes.

Identification of miR-495 as a Putative Addiction-Related miRNA

In order to find other candidate addiction-related miRNAs, we used an *in silico* approach to find miRNAs that target several addiction-related genes (ARGs) and that are highly expressed in brain reward-related regions. Initial bioinformatics analyses investigated the prevalence of miRNA binding sites within the 3'UTR of ARGs taken from the Knowledgebase of Addiction-Related Gene (KARG; http://karg.cbi.pku.edu.cn) database. Genes within KARG that have conserved miRNA-binding sites were selected using TargetScan (http://www.targetscan.org). Among the miRNAs that target ARGs, we found that miR-495 is expressed in the adult rat striatum (http://miRBase.org, Kozomara & Griffiths-Jones, 2014; Landgraf et al., 2007) and its predicted targets include *Bdnf*,

Camk2a, and *Arc* mRNAs, all of which have been implicated in drug addiction (Li, Mao, & Wei, 2008; McGinty, Whitfield, & Berglind, 2010; Pandey et al., 2008). The percentage of KARG genes containing conserved miR-495 binding sites in their 3'UTRs is higher than in the entire genome (Oliver, Gardiner, Bastle, Neisewander, & Perrone-Bizzozero, 2014). Approximately 7% of the mouse KARG genes (70 genes) are predicted to have highly conserved binding sites for miR-495 and similar results were obtained using the human or rat KARG gene sets (7.7% and 5.6 %, respectively). In comparison, another miRNA associated with cocaine addiction, let-7 (Chandrasekar & Dreyer, 2009, 2011), was predicted to target only ~3% of the mouse KARG genes and ~2.5% of the entire genome. Thus, miR-495 is a conserved, predicted regulator of ARGs in mammals.

Subsequent studies used a combination of fluorescent *in situ* hybridization (FISH) and qRT-PCR to validate the striatal expression of miR-495 and its distribution in other brain regions. miR-495 expressing cells were localized to the NAc, dorsal striatum, pyriform and neocortex (Oliver et al., 2014), with the latter consistent with miR-495 expression in the human PFC (Mellios, Huang, Grigorenko, Rogaev, & Akbarian, 2008). Using qRT-PCR, we found that miR-495 was expressed in several brain regions and that the highest expression levels were in the NAc (Oliver et al., 2014). Interestingly, acute cocaine administration in mice reduced miR-495 1 h post-injection selectively in the NAc, but not in the mPFC, orbitofrontal cortex, or dorsal striatum (Oliver et al., 2014). This downregulation was rapid and transient, where cocaine downregulated NAc miR-495 expression 1-4 h post-injection, but miR-495 levels returned to baseline levels the following day. Furthermore, this downregulation corresponded with increases in target

ARG expression, suggesting a potential regulatory role. Indeed, dual luciferase assays validated that miR-495 directly binds to the 3'UTR of *Bdnf*, *Arc*, and *Camk2a* mRNA (Oliver et al., 2014).

Bdnf is present *in vivo* as two different transcripts produced from the same promoter. Due to differential polyA site usage, this mRNA exists with a short (*Bdnf*-S) or a long 3'UTR (*Bdnf*-L) variant. Interestingly, the long 3' UTR variant is predominantly localized in dendrites, rapidly translated following neuronal activation, and subsequently regulates LTP (An et al., 2008; Lau et al., 2010). Furthermore, the long form contains a greater number of miR-495 binding sites than the short form (http://www.targetscan.org/mmu_50/), suggesting that miR-495 would preferentially alter the stability and/or translation of *Bdnf*-L versus *Bdnf*-S. miR-495 also targets specific subunits of CaMKII (*Camk2a*), where the alpha subunit has a greater role in promoting drug abuse-related behavior than other subunits (Kourrich, Klug, Mayford, & Thomas, 2012; Loweth et al., 2010; Wang et al., 2010). Thus, miR-495 is a lead addiction-related miRNA candidate due to its 1) ability to target several ARGs, 2) its high expression in the NAc, and 3) its regulation in the NAc by cocaine exposure.

Aims of Research

This dissertation research examined the hypothesis that NAc miR-495 regulates cocaine abuse-related behavior through targeting several ARGs. I initially tested this hypothesis by examining the effects of viral-mediated NAc miR-495 overexpression (OE) on cocaine's acute rewarding properties in rats, specifically during expression and extinction of cocaine conditioned place preference (CPP). Next, I examined changes in NAc miR-495 and ARG expression as a result of brief (i.e. 1 day) or prolonged (i.e., 22

days) cocaine self-administration. I then examined the effect NAc miR-495 OE and inhibition on cocaine SA for the following measures: 1) low and high effort schedules of reinforcement, 2) extinction and reinstatement of cocaine-seeking behavior, and 3) ARG expression. Subsequent studies examined the effect of NAc miR-495 OE on food reinforcement in order to test the specificity of cocaine effects.

CHAPTER 2

EFFECTS OF NUCLEUS ACCUMBENS MIR-495 OVEREXPRESSION ON COCAINE-INDUCED GENE EXPRESSION AND COCAINE CONDITIONED PLACE PREFERENCE

Epigenetic mechanisms serve as mediators of environmental effects on gene expression, whereby external stimuli can turn gene expression on and off. Acute administration of drug of abuse results in widespread changes in gene expression in the brain, particularly in the nucleus accumbens (NAc) of rodents (Maze et al., 2010; Robison & Nestler, 2011). While much research has focused on epigenetic regulation at the transcriptional level in response to drugs of abuse (e.g., DNA and histone modifications) (McQuown & Wood, 2010; Renthal & Nestler, 2008, 2009), relatively fewer studies have examined post-transcriptional mechanisms of gene regulation. MicroRNAs (miRNAs) are small, non-coding transcripts that post-transcriptionally suppress gene expression through base pairing with target mRNAs (Bartel, 2004). Given that one miRNA can target several different mRNAs within a given cell, miRNAs are poised to fine-tune gene expression in multiple cellular pathways that are activated following environmental perturbations.

Given the widespread expression of miRNAs in the brain, miRNAs have been shown to play a key role in neural development and function (Kosik, 2006; Wang, Kwon, & Tsai, 2012). Interestingly, the time scale for transcriptional activation of some miRNAs mimic those of immediate early genes (Nudelman et al., 2010; Oliver et al., 2014), suggesting they also play a role in fast regulatory processes, including synaptic plasticity (Aksoy-Aksel, Zampa, & Schratt, 2014). Indeed, several miRNAs have been

implicated in processes relating to learning and memory (Wang et al., 2012). While miRNAs have been linked to several disease states and disorders (Im & Kenny, 2012), only recently have researchers begun to examine the relationship between miRNAs and drug addiction (Li, Zhao, Wang, & Yang, 2011).

Previous work has linked changes in miRNA expression with cocaine exposure and reward (Chandrasekar & Dreyer, 2009, 2011; Eipper-Mains et al., 2011; Nudelman et al., 2010). For instance, Chandrasekar & Dreyer (2011) found that viral-mediated manipulation of NAc miR-124, miR-181a, and let-7 expression altered expression and extinction of cocaine CPP. In order to establish CPP, an animal experiences a drug state while confined to one compartment of an apparatus and a neutral state while confined to an alternate compartment during conditioning, resulting in a shift in the animal's preference for the drug-paired compartment when given free-access to both compartments. Classical conditioning processes govern these effects, where the animal learns to associate the internal rewarding drug experience (i.e., unconditioned stimulus) with the external environmental stimuli (i.e., conditioned stimulus). The results from Chandrasekar & Dreyer (2011) suggest that these miRNAs regulate genes involved in drug reward learning and motivation.

We previously identified miR-495 as a potential regulator of addiction-related genes based on three findings: 1) it is enriched in the NAc, 2) it directly targets the 3'UTR of *Bdnf* and *Arc,* and 3) it is downregulated by acute cocaine (Oliver et al., 2014; Perrone-Bizzozero et al., 2009). *Bdnf* is critical for development and maintenance of synaptic structure and function (McAllister, Katz, & Lo, 1999; Poo, 2001), while *Arc* interacts with cytoskeleton proteins involved in spine formation and regulates AMPA

receptor endocytosis (Bramham, Worley, Moore, & Guzowski, 2008; Shepherd et al., 2006). Both genes are upregulated in the NAc following acute drug administration (Graham et al., 2007; Loweth et al., 2010; Samaha, Mallet, Ferguson, Gonon, & Robinson, 2004); therefore, these genes may be involved in the initial plasticity that occurs following drug administration. Indeed, downregulation of *Bdnf* and *Arc* expression in the NAc impairs expression of drug CPP (Graham et al., 2009; Lv, Xu, Han, & Cui, 2011).

Along with cocaine-induced decreases in NAc miR-495, we previously found that acute cocaine administration in mice increases NAc *Bdnf* expression at a similar time point (i.e., 2 h post injection) (Oliver et al., 2014). Therefore, we initially sought to counter downregulation of NAc miR-495 and upregulation of *Bdnf* expression by using a viral-mediated overexpression (OE) approach in rats. We chose to target the shell subregion of the NAc (NAcSh) for miR-495 OE based on data that acute cocaine increases *Bdnf* expression in the NAcSh, but not the core, of rats (Graham et al., 2007). Furthermore, we tested the effect of NAcSh miR-495 OE on the initial rewarding effects using cocaine CPP (Experiment 2b). Given the established roles of target ARGs in the NAc on drug CPP, we predicted that NAcSh miR-495 OE would decrease cocaine CPP, as well as target gene expression.

Methods

Animals

Male Sprague–Dawley rats (Charles River, San Diego, CA) (N=33; 200-225g) were individually housed in a climate-controlled facility with a 14-h dark/light cycle (lights off at 7 AM) with *ad libitum* access to food and water. Housing and care were

conducted in accordance with the 8th edition of the Guide for the Care and Use of Laboratory Animals (Committee, 2011). Prior to surgery, animals were acclimated to handling (~2 min/day) for 7 days.

Intracranial viral injections

Animals in both experiments were placed under isoflurane anesthesia (2-3%) and positioned in a stereotactic frame (Kopf, Tujunga, CA, USA). An incision was made on the head (~2 cm) and skin was separated to expose the top of the skull. Holes were drilled in reference to bregma and virus was infused bilaterally through guide cannula aimed at the nucleus accumbens shell (NAcsh; +1.6 mm AP, ±1.1 mm ML, -6.8 mm DV from Bregma; Paxinos & Watson, 2007). Lentiviral vectors contained either green fluorescent protein (GFP; LV-GFP; 8.28 x 10^8 IUF/mL), which also expresses a non-targeting sequence, or GFP + pri-miR-945 (LV-miR-495; 1.32 x 10^9 IUF/mL; System Biosciences Inc. Mountain View, CA, USA). The infusions (2 µl/side) were given at a rate of 0.2 µL/min through a 30-gauge injector (Plastics One, Roanoke, VA, USA) connected via Tygon microbore tubing (Norton Performance Plastics, Akron, OH, USA) to a 25-µL syringe (Hamilton Co., Reno, NV, USA) housed in an infusion pump (CMA Microdialyis, North Chelmsford, MA, USA). Injectors extended 1 mm below guide cannula and were left in place for 10 min post-infusion to allow for virus diffusion.

Acute cocaine administration

For the acute cocaine experiment, rats (n = 22) were infused with their respective virus, counterbalanced by weight. Two weeks later, rats were treated with an acute injection of either saline (1 mL/kg) or cocaine (15 mg/kg, i.p.) and sacrificed 2 h later. The cocaine dose and sacrifice time were chosen based on previous data showing

cocaine-induced decreases in miR-495 and increases in *Bdnf* mRNA and protein expression in mice at this time point (Oliver et al., 2014). Brains were rapidly collected, frozen in 2-methylbutane (-50˚C), and stored at -80˚C until further processing.

CPP Apparatus

Conditioning took place in rectangular Plexiglas chambers as previously described (Peartree et al., 2012). Each chamber contained a removable solid partition that separated the chamber into two equal-sized compartments, each measuring 35 × 24 × 31 cm high. One compartment had corncob bedding beneath a wire 1 × 1 cm grid floor and alternating black and white vertical stripes on the walls. The other compartment had pine-scented bedding beneath a parallel bar floor (5 mm diameter) and alternating black and white horizontal stripes on the walls. On the pre- and post-conditioning test days, the removable center partition of the apparatus was replaced by a similar partition that contained an opening in the center (28 × 6 cm), allowing the rats free-access to the adjacent compartments simultaneously. A rectangular tower measuring 70 × 24 × 74 cm high of clear Plexiglas was used as an extension of the apparatus to prevent the rats from escaping from the chamber while maintaining the ability to record their behavior via an overhanging video camera. The conditioning room was dimly lit with two overhead lamps, each containing a 25-Watt light bulb providing equal light distribution for each conditioning chamber. Unpublished data from our laboratory established that adult experimentally naïve rats showed no preference for a particular compartment (i.e., unbiased apparatus). A camera (Panasonic WV-CP284, color CCTV, Suzhou, China) used to record testing sessions was mounted 101 cm above the center of the apparatus. A WinTV 350 personal video recorder (Hauppage, NJ, USA) captured live

video and encoded it to MPEG streams. A modified version of TopScan Software (Clever Sys., Inc. Reston, VA, USA) used the orientation of an animal's body parts to track its location, which yielded measures of time spent in each compartment.

Baseline Preference

One week following viral infusions, rats (n = 17) in the CPP experiment were placed individually into their assigned CPP apparatus where they had free access to both compartments for a 15-min habituation period. This procedure was repeated across the next 2 consecutive days with the starting compartment counterbalanced across days and the time spent in each compartment recorded to assess initial baseline preference. The tracking software determined the location of the rat's head. Time spent in each compartment was averaged across the two baseline tests to determine each rat's initial side preference. Rats that failed to demonstrate at least five compartment crossovers during either baseline day were excluded from the experiments due to inadequate environmental exploration.

Conditioning, Testing, and Extinction

Conditioning sessions took place over 4 consecutive days, where rats were confined either to the initially non-preferred side of the apparatus for 30 min following a cocaine injection (15 mg/kg, i.p.) or were confined in the initially preferred side of the apparatus following a saline injection (1 mL/kg). These session types alternated across consecutive days (e.g., cocaine-saline-cocaine-saline). Whether the first conditioning session was on the initially preferred or non-preferred side was counterbalanced across groups. On the day following the last conditioning session, all rats were given a 15-min place preference test. On the three days following the test day, rats were placed in the box

with access to both sides of the apparatus, but received no injections prior to placement (i.e., extinction). The day following the last extinction session, rats were sacrificed and brains were rapidly collected, frozen in 2-methylbutane (-50˚C), and stored at -80˚C until further processing.

qRT-PCR

NAc tissue was dissected using a 2 mm brain punch (Harris Unicore™). RNA was isolated *via* standard Trizol® (Invitrogen) extraction followed by ethanol precipitation in the presence of glycogen to recover both mRNA and small RNAs. Starting with 15 ng of purified RNA, cDNA was prepared using the Taqman® MicroRNA Reverse transcription kit (Applied Biosciences, Grand Island, NY, USA) and Taqman® MicroRNA Assay primers (Life Technologies, Grand Island, NY, USA) for miR-495 and U6. For mRNA qPCR, cDNA was prepared using the SuperScript II First-Strand Synthesis system (Life Technologies) and quantified using *Power* SYBR Green PCR Master Mix (Life Technologies) with primers designed against selected targets (*Bdnf-L*: Forward GCCACTGAAATGCGACTGAA, Reverse CATTCCCCACCTCCATCTAGAC; *Bdnf-Pan*: Forward GACTCTGGAGAGCGTGAAT, Reverse CCACTCGCTAATACTGTCAC; *Arc*: Forward GGTAAGTGCCGAGCTGAGATG, Reverse CGACCTGTGCAACCCTTTC) and compared to a control transcript (*Gapdh*: Forward CCACAGTCCATGCCATCACT, Reverse GCCTGCTTCACCACCTTCTTG). *Bdnf*-Pan refers to the total sum of *Bdnf* mRNA that includes both *Bdnf*-L and *Bdnf*-S mRNA. Relative expression was determined using the comparative $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001).

Data Analysis

The acute cocaine experiment was analyzed using a two-way ANOVA with Drug (saline vs. cocaine) and Virus (LV-GFP vs. LV-miR-495) as between-subjects factors. The CPP experiment was analyzed using a mixed-factor two-way ANOVA with Day (baseline, test, extinction) as a within-subjects factor and Virus as a between-subjects factor. Significant interactions for both experiments were followed by tests for simple effects. Comparisons of interest (test day vs. extinction day 3) were analyzed using paired-sample *t*-tests with Bonferroni correction for multiple comparisons (alpha level/number of comparisons). qRT-PCR data were analyzed using student *t*-tests. Results were considered statistically significant if $p < 0.05$.

Results

Acute Cocaine Experiment

We found that NAcSh miR-495 OE blocked the cocaine-induced decrease in NAc miR-495 expression (Virus x Drug treatment: $F_{(1, 18)} = 5.19$, $p < 0.05$; Virus: $F_{(1, 18)} = 8.59$, $p < 0.01$; Drug treatment: $F_{(1, 18)} = 4.80$, $p < 0.05$). While cocaine significantly reduced NAc miR-495 expression in LV-GFP controls, rats that received LV-miR-495 and cocaine had significantly greater NAc miR-495 levels than the LV-GFP-cocaine group and did not differ from the LV-miR-495 group that received saline (Fig. 2; tests for simple effects, ps < 0.05). LV-miR-495 also blocked the cocaine-induced increase in *Bdnf*-Pan mRNA expression (Virus x Drug treatment: $F_{(1, 13)} = 7.20$, $p < 0.05$; Virus: $F_{(1, 13)} = 19.21$, $p < 0.001$; Drug treatment: $F_{(1, 13)} = 14.20$, $p < 0.01$), where cocaine increased *Bdnf*-pan mRNA expression in LV-GFP controls and the LV-miR-495-cocaine group had significantly lower NAc *Bdnf*-Pan mRNA levels than the LV-GFP-cocaine group and did

not differ from the LV-miR-495-saline group (Fig. 3A; tests for simple effects, *p*s < 0.05). Although we did not detect a cocaine-induced decrease in *Bdnf*-L mRNA expression in the LV-GFP groups, LV-miR-495 produced an overall decrease in NAc *Bdnf*-L mRNA expression (Fig. 3B; Virus x Drug treatment: $F_{(1, 16)} = 2.46$, *not significant*; Virus: $F_{(1, 16)} = 25.16$, $p < 0.001$; Drug treatment: $F_{(1, 16)} = 1.24$, *n.s.*).

CPP Experiment

We found that both virus groups exhibited significant cocaine CPP expression, but did not differ from each other during either baseline or test day (Fig. 4; Day: $F_{(1, 15)} = 25.98$, $p < 0.0001$; Virus: $F_{(1, 15)} = 0.11$, *n.s.;* Day x Virus: $F_{(1, 16)} = 0.11$, *n.s.*). While both groups had a significant trend toward extinguishing cocaine CPP by the third day (Fig. 5; Day: $F_{(3, 45)} = 2.56$, $p = 0.067$; Virus: $F_{(1, 14)} = 0.87$, *n.s.;* Day x Virus: $F_{(3, 45)} = 0.36$, *n.s.*), only the LV-miR-495 group had a significant decrease in CPP on extinction day 3 compared to test day (LV-GFP: $t_7 = 0.69$, *post-hoc* Bonferroni, *n.s.*; LV-miR-495: $t_7 = 2.98$, Bonferroni, $p < 0.025$,). Furthermore, the LV-miR-495 group exhibited a significant linear trend in CPP across extinction ($F_{(1, 8)} = 6.73$, $p < 0.05$), whereas the LV-GFP group had no significant trend ($F_{(1, 7)} = 0.45$, *n.s.*), indicating the LV-miR-495 group was decreasing across days, while the LV-GFP group remain the same. Following testing, the LV-miR-495 group exhibited greater NAc miR-495 levels (Fig. 6A; $t_{11} = 2.34$, $p < 0.05$) and reduced *Bdnf*-L (Fig. 6B; $t_{12} = 3.18$, $p < 0.01$), *Bdnf*-Pan (Fig. 6C; $t_{12} = 2.92$, $p < 0.05$), and *Arc* (Fig. 6D; $t_{12} = 4.96$, $p < 0.01$) mRNA compared to the LV-GFP group.

Discussion

In this chapter, we found that NAcSh miR-495 OE facilitated extinction of CPP, but did not affect CPP expression. These effects were accompanied by miR-495 OE-

induced decreases in NAc *Bdnf* and *Arc* mRNA. Furthermore, we found that NAcSh miR-495 OE was able to prevent cocaine-induced downregulation and upregulation of miR-495 and *Bdnf* mRNA, respectively. Our findings suggest that miR-495 OE is able to prevent some cocaine-induced changes in gene expression and has effects on specific aspects of cocaine reward-related behavior.

We considered several explanations for the selective effect of miR-495 expression on extinction, but not initial expression, of cocaine CPP. First, the lack of an effect on expression of CPP is not likely due to a floor effect because we used a fairly high cocaine dose during conditioning (15 mg/kg, i.p.) in order to produce a robust CPP. Furthermore, we observed a decrease in the initial magnitude of CPP across extinction in the miR-495 OE group. It was also unlikely that the virus did not produce adequate OE because we validated that miR-495 OE occurred in these animals following behavioral testing and we have previously shown that this viral vector can increase miR-495 expression at the time point when conditioning and testing had occurred (i.e., 1 week post-infusion) (Bastle et al., 2014). One possibility of a selective effect on extinction is the length of time elapsed since the last cocaine injection, given that we have found that motivation for drug increases with longer periods of abstinence (Tran-Nguyen et al., 1998). Thus, if NAcSh miR-495 OE produces its effects by reducing motivation for cocaine, then one might expect to see no effect initially (i.e., initial CPP test day) but to observe attenuated CPP at later time points (i.e., extinction test days). Although the present findings are on a smaller time scale, previous research has found that both NAc BDNF expression and motivation for cocaine increase as the withdrawal period increases (i.e., incubation effect) (Grimm et

al., 2003; Li et al., 2013). Thus, it is possible that miR-495 OE may attenuate expression of cocaine CPP if testing is delayed to a time when motivation to seek cocaine is higher.

Another possibility is that NAcSh miR-495 OE is impacting learning, such that the CPP was not as well established and therefore extinguished more rapidly. For instance, *Arc* is a plasticity-related gene that is involved in several forms of learning, including memory consolidation (Guzowski et al., 2000; Plath et al., 2006). *Arc* knockdown produces deficits in recall of spatial and fear memories (Guzowski et al., 2000; Ploski et al., 2008). Perhaps NAcSh miR-495 OE reduced *Arc* expression to a level that weakened the establishment of memory for the drug-associated context. Either way, NAcSh miR-495 OE accelerates the rate of extinction compared to controls and this may have overlapping and/or distinct effects on drug motivation and learning and memory.

We chose to target the NAcSh for miR-495 OE because cocaine selectively upregulates *Bdnf* expression in this subregion, and in turn, manipulation of *Bdnf* signaling in the NAcSh alters cocaine abuse-related behavior (Graham et al., 2007). The NAcSh is involved in extinction of drug seeking, where inactivation by AMPA receptor antagonists or GABA agonists increases responding during extinction (Millan & McNally, 2011; Peters, LaLumiere, & Kalivas, 2008). This suggests that activity in the NAcSh is engaged during inhibition of responding for drug during extinction. The NAcSh receives inputs from several regions involved in extinction learning, such as the basolateral amygdala (BLA), infralimbic prefrontal cortex (IL), and dorsal hippocampus (DH). The glutamatergic projections from the BLA and IL to the NAcSh are recruited during extinction and inactivation of this pathway reduces extinction learning (Hamlin, Clemens, Choi, & McNally, 2009; Millan & McNally, 2011; Peters et al., 2008). The DH is

involved in forming associations between contextual and rewarding stimuli (Meyers, Zavala, Speer, & Neisewander, 2006). Therefore, DH projections to the NAcSh may be recruited during CPP extinction, where information about the lack of reward in a particular context is integrated in the DH and sent to the NAcSh to inhibit responding. Output regions of the NAcSh may also be involved in these effects. Indeed, the NAcSh GABAergic neurons project to the lateral hypothalamus (LH) and inhibit this region during extinction and NAcSh inactivation increases drug seeking via disinhibition of LH activity (Millan, Furlong, & McNally, 2010). Collectively, our findings agree with previous work that establishes a role of the NAcSh in extinction of drug seeking. Thus, miR-495 OE may alter the activity of NAcSh neurons and their associated circuitry to enhance extinction processes.

In addition to *Bdnf* and *Arc*, both *Camk2a* and *Cnr1* are other miR-495 targets that might be involved in our behavioral effects. *Camk2a* encodes for calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα), which is another plasticity-related gene whose target substrates include AMPA and NMDA receptors (Colbran & Brown, 2004). miR-495 directly targets the 3'UTR of *Camk2a* (Oliver et al., 2014) and NAcSh miR-495 OE downregulates *Camk2a* mRNA (Table 1) (Bastle et al., 2014). Transgenic mice expressing autonomously-active striatal CaMKIIα exhibit increases in cocaine-induced locomotor activity and CPP (Kourrich et al., 2012). Furthermore, viral-mediated increases in NAcSh CaMKIIα also increase drug-induced locomotion (Loweth et al., 2010), suggesting this molecule has a facilitating effect on drug abuse-related behavior. *Cnr1* encodes for the cannabinoid receptor type 1 (CB_1R), which are G protein-coupled receptors functioning as presynaptic heteroreceptors on both

glutamatergic and GABAergic neurons; thereby activation of CB_1R inhibits presynaptic release. We previously found through microarray analysis that *Cnr1* mRNA is downregulated in the NAc by miR-495 OE (Table 1). Similar to downregulation, intra-accumbal CB1 antagonist administration facilitates extinction learning in rats (Khaleghzadeh-Ahangar & Haghparast, 2015). While ultrastructural localization studies in the NAc have shown CB_1R expression on the soma and dendrites of GABAergic interneurons, they are also present on glutamatergic cortico-striatal terminals in the NAc (Pickel, Chan, Kearn, & Mackie, 2006). Therefore, pharmacological blockade would not be specific to one cell type versus the other. Similarly, if our effects were *Cnr1*-mediated, we would need to determine whether they were occurring through local GABAergic interneurons or through GABAergic projection neuron where CB_1R would be expressed on terminals in output regions of the NAc. Future research into the differential roles of efferent versus local NAc CB_1R action on drug-related extinction processes is warranted.

Our finding that miR-495 OE decreased *Bdnf* and *Arc* mRNA expression in NAcSh when measured following behavioral testing conflicts with previous work demonstrating that downregulation of these genes impairs drug CPP expression (Bahi, Boyer, Chandrasekar, & Dreyer, 2008; Lv et al., 2011). One possibility for our lack of an effect on CPP expression is the degree to which miR-495 OE is downregulating the measured ARGs. Although we observed a >50% reduction in mRNA, perhaps knockout and antisense procedures produce greater reductions in functional protein output. Also, we cannot rule out that miR-495 OE may be reducing expression of other genes that produce opposite effects on drug CPP. Indeed, our microarray results showed that NAc miR-495 OE also decreases *Per2* mRNA expression (Table 1). *Per2* encodes for the

period circadian clock 2 (Per2) protein which has a critical role in circadian rhythms (Rosenwasser, 2010). Interestingly, *Per2* is upregulated in the striatum 30 min following repeated cocaine administration (Uz et al., 2005), and knockout mice exhibit increased cocaine-induced hyperlocomotion (Abarca, Albrecht, & Spanagel, 2002) and alcohol intake (Spanagel et al., 2005). Thus, *Per2* expression may counter cocaine's action and downregulation may produce an addiction-like phenotype. Therefore, the balance between miR-495 OE-induced repression of genes that inhibit versus promote CPP expression may have not been tipped toward the former enough to regulate this aspect of CPP.

In conclusion, we demonstrated that NAcSh miR-495 OE blocks cocaine-induced gene expression and facilitates extinction of cocaine CPP. These results suggest that although miR-495 may not alter the rewarding effects of cocaine given that CPP expression was not altered, increasing NAcSh miR-495 may target genes involved in learning to inhibit reward-seeking behavior and/or motivation evoked by the cocaine-associated context. This may give insight into the possible use of microRNAs as tools to control gene expression associated with specific aspects of drug abuse-related behavior.

CHAPTER 3

PROLONGED COCAINE SELF-ADMINISTRATION DOWNREGULATED MIR-495 IN THE NUCLEUS ACCUMBENS

Although several models exist to examine drug abuse-related behavior following experimenter-delivered drug administration (e.g., locomotor sensitization, CPP, etc.), the self-administration (SA) model is considered the "gold standard" because it has high face, construct, and predictive validity for modeling human addiction (Panlilio & Goldberg, 2007), although predictive validity is difficult to establish due to relatively few effective compounds available to treat addiction. This is especially the case for psychostimulants where no FDA-approved treatment exists. In the SA model, an animal learns to perform an operant response in order to receive a drug reinforcer, thereby requiring volitional behavior in contrast to experimenter-delivered drug administration models. Furthermore, previous studies have found that molecular factors in the NAc, such as CREB signaling and MeCP2 (Carlezon et al., 1998; Deng et al., 2010; Larson et al., 2011; Lewis & Olive, 2014), have differential functions in drug abuse-related behavior depending on whether the drug is experimenter-delivered versus self-administered. Thus, it is important to validate the relevance of drug-induced changes in gene expression profiles to addiction with SA models.

Similar to experimenter-delivered cocaine administration, levels of *Bdnf*, *Camk2a*, and *Arc* are all upregulated in the NAc following cocaine SA (Graham et al., 2007; Wang et al., 2010; Zavala, Osredkar, Joyce, & Neisewander, 2008). Interestingly, upregulation of *Bdnf* and *Camk2a* expression occurs in the NAc shell, but not the core (Graham et al., 2007; Wang et al., 2010). These region-specific effects on gene

expression may speak to the different roles that the NAc core and shell play in drug reinforcement. For instance, the shell is involved in processing the unconditioned reinforcing effects of drugs, whereas the core is involved in processing the conditioned reinforcing effects (Kalivas & Volkow, 2005; Yager, Garcia, Wunsch, & Ferguson, 2015). Given that *Bdnf*, *Camk2a*, and *Arc* are all plasticity-related genes, their expression in the NAc shell may be altered during cocaine SA as the incentive value of cocaine presumably increases with increasing periods of exposure to drug (Robinson & Berridge, 1993).

While acute cocaine downregulates NAc miR-495 (Chapter 2), we found that varying degrees of experimenter-delivered cocaine administration results in dynamic changes in NAc miR-495 expression. For instance, repeated cocaine injections in mice across 5 days does not alter NAc miR-495 levels (Oliver et al., 2013). However, a priming injection after a 1-week withdrawal period from repeated daily injections (5 days) reduced NAc miR-495 expression to a similar extent as acute administration (Oliver et al., 2013). Therefore, NAc miR-495 downregulation is dependent on both the degree and timing of cocaine exposure.

Here, we sought to test the effects of varying degrees of cocaine SA on NAc miR-495 expression. We chose to measure expression in the NAc core and shell following 1 or 22 days of cocaine SA in order to compare different cocaine SA histories. We also measured ARG expression in order to examine if levels would correspond to any changes in NAc miR-495 expression. We hypothesized that cocaine SA would produce differential gene expression depending on SA history, and that these effects may be specific to the NAc core or shell.

Methods

Animals

Male Sprague–Dawley rats (Charles River, San Diego, CA) (N=33; 200-225g) were individually housed in a climate-controlled facility with a 14-h dark/light cycle (lights off at 7 AM). The rats had *ad libitum* access water throughout the experiments and initially they had *ad libitum* access to food as well. Housing and care were conducted in accordance with the 8th ed. Guide for the Care and Use of Laboratory Animals (Committee, 2011). Prior to surgery, animals were acclimated to handling (~2 min/day) for 7 days.

Surgery

Catheters were constructed from Silastic tubing (10 cm in length, inner diameter 0.012, outer diameter 0.025 inches, Dow Corning, Midland, MI, USA) connected to a 22 gauge nonferrous metal cannula encased within a plastic screw connector (Plastics One, Roanoke, VA, USA). The free end of the catheter was implanted into the jugular vein and other end was secured to the skull with dental acrylic cement under isoflurane anesthesia (2-3%) as described previously (Zavala, Biswas, Harlan, & Neisewander, 2007). Incisions were sutured and treated with a topical antibiotic and the rats were given buprenorphine (0.05 mg/kg, s.c.) analgesia and an anti-inflammatory agent (meloxicam; 1 mg/kg, s.c.). Patency of the catheters was maintained throughout the experiment by daily flushing with 0.1 ml timentin (66.67 mg/mL; bioWORLD, Dublin, OH) in saline solution containing 70 units/mL heparin sodium. Catheter patency was tested periodically with 0.8 g methohexital sodium (Brevital, Sigma, St. Louis, MO), a dose that produces rapid loss

of muscle tone only when administered i.v. Rats were given 7 recovery days in their home cages and were handled and weighed daily.

Apparatus

Training and testing were conducted in Plexiglas operant conditioning chambers (20 × 28 × 20 cm) equipped with a food pellet dispenser and a food well located between two levers mounted on the front panel (Med Associates, St. Albans, VT). A cue light was mounted above one lever, a tone generator (500 Hz, 10 dB above background noise) was mounted on the side wall near a lever and a house light was mounted on the rear wall opposite the levers. The lever below the cue light and nearest to the tone generator was designated as the active lever. Each conditioning chamber was housed within its own ventilated, sound-attenuating cabinet. An infusion pump containing a 10-ml syringe was located outside of the cabinet. Tygon tubing connected to the syringe was attached to a liquid swivel (Instech, Plymouth Meeting, PA) suspended above the operant conditioning chamber. The outlet of the swivel was fastened to the catheter via Tygon tubing that ran through a metal spring leash (Plastics One). The leash fastened onto the plastic screw of the cannula that was anchored on the animal's head.

Cocaine Self-Administration

Self-administration (SA) began 7 days post-surgery. Two days prior to the first session, rats were placed on mild food restriction (16 g/day) to facilitate lever pressing. Rats were then randomly assigned to receive either 1 (n = 11) or 22 days (n = 12) of SA (e.g., SA1, SA22). In order to define acquisition of SA, we established a criterion where rats were only included in the experiment if they received at least 12 infusions within the first session. Rats were given a minimum of 2 h and a maximum of 8 h to meet this

criterion and the session was terminated for a given rat once the criterion was met. During the first session, cocaine was delivered on a fixed ratio (FR) 1 schedule of cocaine reinforcement (0.9375 mg/kg/0.1mL infusion), with the relatively high cocaine dose chosen to increase rates of acquisition during the first session. Completion of the required schedule resulted in activation of both a cue light above the active lever and a tone stimulus, and 1 s later the infusion pump was activated for 6 s. The cues and infusion pump were then terminated concurrently with illumination of the house light for a 20-s timeout period during which lever presses were recorded but had no consequences. Lever presses on an inactive control lever were also recorded but had no consequences. Controls in both the SA1 and SA22 groups received saline infusions and cues yoked to a cocaine partner, but lever presses produced no consequences (n = 11). Following the first session, the SA22 group and their saline-yoked controls went on to continue SA training, which consisted of daily 2-h sessions (6 days/week; 0.9375 mg/kg/infusion) that began on an FR1 schedule and progressed to a VR5 schedule of cocaine reinforcement. Once rats stabilized on the VR5 for 3 consecutive days with <15% variance in the number of infusions received across days, they received gradual increases in daily food rations (i.e., 18, 20, then 22g/day for the remainder of the experiment). Rats in both the SA1 and SA22 groups were sacrificed one hour following the last session. This time frame was chosen based on our previous time course of NAc miR-495 expression following acute cocaine administration in mice (Oliver et al., 2014). Brains were rapidly extracted, frozen in 2-methylbutane, and stored at -80°C until further processing.

qRT-PCR

Procedures were identical to Chapter 2, with the exception that the NAc core and

shell were dissected separately using 1.25 mm and 2 mm brain punches (Harris Unicore™; Fig. 7) and the additional use of primers for *Camk2a* mRNA (Forward TATCCGCATCACTCAGTACCTG, Reverse GAACTGGACGATCTGCCATTT).

Western Blot

NAc tissue punches from the contralateral hemisphere from the hemisphere that was used for RNA (counterbalanced across groups) was placed in a neuronal protein extraction reagent (N-PER, G-Biosciences, USA) containing a protease inhibitor cocktail and 1 mM EDTA. Tissue was homogenized with a polypropylene pestle and a Branson sonicator (Danbury, CT), and then centrifuged at 10,000 x g at 4°C for 10 min. The supernatant was then stored at -80°C until further analysis. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA). For immunoblot analysis, samples (30 µg of protein per lane) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 4-20% precast gels (BioRad, Hercules, CA, USA). Proteins were transferred to nitrocellulose membranes, pre-blocked with StartingBlock ™ blocking buffer (ThermoFisher Scientific) for 30 min before overnight incubation with the following primary polyclonal antibodies: mouse anti-CaMKIIα (Santa Cruz, sc-13141, Dallas, TX, USA; 1:1000 dilution), rabbit anti-Arc (Synaptic Systems, 156 003, Goettingen, Germany; 1:2000), and as a loading control, mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, ab8245, Cambridge, MA, USA; 1:10,000 dilution). Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated donkey anti-mouse (Abcam, ab6789; 1:10,000 dilution) and anti-rabbit IgG antisera (Abcam, ab6721; 1:10,000 dilution) for 2 h, and immunoreactive bands were detected by enhanced chemilluminescence (Perkin Elmer,

Waltham, MA, USA) and exposure to Kodak Bio-Max films. Developed films were scanned and analyzed using ImageJ software. For each sample, the optical density of the target gene band was divided by the corresponding GAPDH band in order to yield a target gene/GAPDH ratio.

Data Analysis

Behavioral data and saline qRT-PCR data were analyzed using independent-samples student *t*-tests. qRT-PCR data were analyzed using a one-way ANOVA with *post-hoc* Tukey tests. Significant interactions for both experiments were followed by tests for simple effects. Comparisons of interest for ARG expression (Saline & SA1 vs. SA22, based on miR-495 data) were analyzed using independent-samples *t*-tests with Bonferroni correction for multiple comparisons (alpha level/number of comparisons). Otherwise, results were considered statistically significant if $p < 0.05$.

Results

The two cocaine SA groups did not differ in the total number of responses performed (Fig. 8A, B; $t_{21} = 1.38$, *n.s.*) or cocaine infusions received (Fig. 8C, D; $t_{21} = 1.13$, *n.s.*) during their respective test sessions. For qRT-PCR, saline-yoked controls were included at each time point, but did not significantly differ from one another for NAc miR-495 or ARG expression (data not shown; range from $t_{5\text{-}9} = 0.09\text{-}0.95$, all *n.s.*), so they were combined into one group. We found that NAcSh miR-495 expression was significantly reduced compared to both the Saline and SA1 groups (Fig. 9; $F_{2, 19} = 8.61$, $p < 0.01$; *post-hoc* Tukey, *p*s < 0.05), but no difference was found between the Saline and SA1 group (Tukey, *n.s.*). No differences in NAc core (NAcC) miR-495 expression were observed across groups (Fig. 10; $F_{2, 25} = 0.33$, *n.s.*).

Upon measuring ARG expression in the NAcSh, we found no change in *Bdnf*-L (Fig. 11A; $F_{2, 27} = 0.81$, *n.s.*), or *Bdnf*-Pan (Fig. 11B; $F_{2, 27} = 0.87$, *n.s.*) mRNA. Although we found no difference between SA1 and Saline groups in *Arc* mRNA ($F_{2, 25} = 2.82$, $p = 0.08$, *post-hoc* Bonferroni, *n.s.*), we found an increase in the SA22 group compared to the Saline group (Fig. 12A; Bonferroni, $p \leq 0.025$). Furthermore, we found a significant linear trend across all groups ($p < 0.05$), indicating *Arc* mRNA levels were increasing as cocaine exposure increased. However, we found no difference in Arc protein across groups (Fig. 12B; $F_{2, 31} = 1.01$, *n.s.*). For *Camk2a* mRNA and protein, neither cocaine SA group differed from the Saline group, but the SA22 group showed significantly increased levels compared to the SA1 group (Fig. 13A, B; mRNA: $F_{2, 25} = 2.92$, $p = 0.07$, Bonferroni, $p = 0.03$ for SA1 vs. SA22; Protein: $F_{2, 30} = 3.89$, $p < 0.05$, Tukey, $p < 0.05$ for SA1 vs. SA22). Both *Camk2a* mRNA and protein had significant quadratic trends across all groups (ps < 0.05), indicating a U-shaped curve of *Camk2a* expression as cocaine exposure increased.

Discussion

Here we found that prolonged, but not brief, cocaine SA decreases NAcSh miR-495 expression. This effect was region-specific, as there were no group differences in the NAcC. The difference in miR-495 across the cocaine SA groups is not likely due to differences in cocaine exposure on test day, as both SA groups had similar intake on their respective test days. We also found that NAcSh *Arc* mRNA expression was increased in a similar pattern as the miR-495 decrease across cocaine SA groups. In contrast, there were no group differences in *Bdnf* mRNA in NAcSh and *Camk2a* levels in this region appeared to decrease slightly during initial cocaine exposure and then normalized

following prolonged cocaine SA. The latter is suggested based on the significant decrease in *Camk2a* mRNA in SA1 relative to SA22 and because although neither SA group differed from saline controls, the SA22 group better approximated saline control levels of *Camk2a* than the SA1 group. These data suggest miR-495 and ARG expression are regulated in a time- and region-specific manner during cocaine SA.

Although the NAc core and shell are situated in close proximity, our selective effects in the NAc shell versus the core are not surprising given how these subregions differ in circuitry and morphology. While both the core and shell receive inputs from dopaminergic neurons originating in the ventral tegmental area (VTA) and glutamatergic neurons originating from the prefrontal cortex (PFC), amygdala, and hippocampus, the shell receives input specifically from the IL subregion of the medial PFC and the core receives input from the prelimbic (PrL) cortex (Yager et al., 2015). Also, while both regions send projections to the ventral pallidum and substantia nigra, these projections are to different subregions within these areas. Furthermore, the shell sends additional projections to the ventral tegmental area, lateral hypothalamus, and extended amygdala, while the core sends projections to the entopeduncular nucleus (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991). The predominant projection neurons from the NAc are GABAergic medium spiny neurons (MSNs) and the dendritic morphology of MSNs slightly differs across NAc subregions. For instance, the shell has a lower density of dendritic spines, less terminal segments, and less branch segments than the core (Meredith, Agolia, Arts, Groenewegen, & Zahm, 1992). As such, the shell MSNs have greater input resistance and attenuated negative resting membrane potential compared to those of the core (Pennartz, Dolleman-Van der Weel, & Lopes da Silva, 1992).

NAc MSNs can often be divided into two populations that predominantly express either dopamine D1 receptors (D1R) or D2Rs. These receptors have functionally distinct actions following stimulation, where D1R stimulation increases adenylyl cyclase and cAMP signaling, while D2R activation inhibits these effects (Neves, Ram, & Iyengar, 2002). Interestingly, some neurons in the NAc co-express both D1Rs and D2Rs. While the core appears to have greater segregation of D1R- and D2R-expressing neurons, as only 5% of the neurons co-express both receptor subtypes, 17% of the shell neurons co-express both receptors (Bertran-Gonzalez et al., 2008). Collectively, the circuitry, morphology, and receptor profile of these two subregions differ and may give insight into the mechanism underlying selective cocaine-induced decreases in NAcSh miR-495 expression.

In accordance with our previous findings that acute cocaine administration downregulates miR-495 in both mice and rats (Oliver et al., 2014; Perrone-Bizzozero et al., 2009), the present experiment found that cocaine SA also downregulated miR-495 in NAcSh. These suppressive effects are in contrast with other addiction-related miRNAs whose expression levels were shown to increase in response to drugs of abuse (Chandrasekar & Dreyer, 2009; Hollander et al., 2010; Nudelman et al., 2010; Schaefer et al., 2010). The downregulation in NAcSh miR-495 expression could be due to several factors. While miRNAs are stable in non-neuronal cell types, some miRNAs decay at faster rates in neurons via activity-dependent processes (Krol *et al*., 2010), which could be triggered by cocaine. 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 (Benetatos *et al*., 2013). Using *Mecp2*-null mice, Wu and colleagues (2010) demonstrated increased expression of many of the miRNAs within this imprinted region, including miR-495. Furthermore, cocaine SA increases striatal MeCP2 (Im et al., 2010). It is important to note that not all of the miRNAs in this cluster are coordinately regulated by MeCP2 knockout (Lempiainen et al., 2013; Wu et al., 2010), suggesting that other regulatory mechanisms, such as pre-miRNA processing and/or mature miRNA stability, may play a role in controlling miRNA expression profiles (Joilin et al., 2014).

Unexpectedly, we observed relatively little change in ARG expression following cocaine SA. Previous research has shown that repeated cocaine self-administration increases both *Bdnf* and *Camk2a* expression in the NAc shell (Graham et al., 2007; Wang et al., 2010). It is important to note that these effects were found in tissue that was collected either immediately or 30 min after the last self-administration session. Given that the present study collected tissue 1 h following the session, we may have missed the window to detect an increase in expression of these genes. Although we found no changes in *Bdnf* mRNA across groups, *Camk2a* mRNA and protein levels slightly decreased in the SA1 group and then returned to levels more comparable to saline following prolonged cocaine self-administration. *Arc*, on the other hand, exhibited expression levels that more closely resembled the inverse pattern of the NAcSh miR-495 effects where increasing the history of cocaine SA increased *Arc* mRNA levels. We previously found that NAcSh *Arc* mRNA is unchanged immediately following the last of 20 consecutive cocaine SA sessions (i.e., prolonged exposure) (Neisewander, Dickey, Shepard, Barnes, & Hammer, 2008), suggesting the increases are time-dependent.

Collectively, these data suggest that the expression patterns of ARG target genes do not perfectly coincide with one another, most likely because multiple mechanisms are involved in regulating ARGs.

Although we found that prolonged, but not brief, cocaine SA decreases NAcSh miR-495 expression, other factors besides the length of cocaine history that may have contributed to these effects. While both groups received a comparable number of cocaine infusions on their respective test days, the infusion rate somewhat differed between the two groups. While the SA22 group had established stable lever-pressing behavior over several weeks, some rats in the SA1 group took close to 8 h in order to receive our established minimum of 12 infusions. Indeed, rate of administration has previously shown to produce differential changes in gene expression, where faster infusion rates (5 vs. 100 sec) produce greater increases in NAcSh *Arc* mRNA expression (Samaha et al., 2004). Additionally, the SA1 group had an extra degree of novelty on their test day compared to the SA22 group, where they received their first exposure to the levers and the cues that coincided with cocaine infusions. It is unlikely that novelty influenced the findings, however, since we pre-exposed all rats to the chambers on each of two consecutive days before the first day of SA, albeit without the levers present. Furthermore, we did include saline-yoked controls that were also exposed to novelty on the test day, but did not find any difference between the controls and the SA1 groups.

Given that NAcSh miR-495 OE was found to facilitate extinction of cocaine CPP (Chapter 2), this suggests that miR-495 may be involved in motivation for cocaine. According to Robinson and Berridge's (1993) theory of incentive sensitization, two dissociable processes in the form of 'liking' and 'wanting' change over the course of

addiction. While the initial hedonistic value of the drug-taking experience (i.e. 'liking') appears to decrease or remain stable over time, the motivation to seek out drug (i.e. 'wanting') increases and can persist even after extended periods of discontinued drug use (Lamb et al., 1991; Robinson & Berridge, 1993, 2008). Therefore, it is reasonable to suggest that rats that received prolonged cocaine SA were more motivated for cocaine than those that received brief exposure and decreases in NAcSh miR-495 expression may be indicative of increased motivation.

Collectively, our results suggest that miR-495 is regulated in the NAc in a time- and region-specific manner where miR-495 is selectively downregulated in the NAcSh following prolonged cocaine SA. Given our previous findings with countering cocaine's effect on miR-495 and ARG expression using the lentiviral-mediated OE approach (Chapter 2a), this information will provide direction into where and when manipulation of miR-495 may be most useful to test its function in cocaine SA.

CHAPTER 4

EFFECT OF NUCLEUS ACCUMBENS MIR-495 OVEREXPRESSION ON COCAINE SELF-ADMINISTRATION

The SA model offers advantages, in addition to its strong validity, in allowing researchers to capture specific aspects of drug-taking behavior by using different schedules of reinforcement. Low fixed ratio (FR) schedules are typically used to test the effect of a manipulation on the sensitivity to the reinforcing effects of drugs across several doses (i.e., dose-response curve). Manipulations that shift the dose-response curve to the left and/or upward are interpreted as an increase in sensitivity/reinforcement, whereas shifts to the right and/or downward are regarded as a decrease in sensitivity/reinforcement (Ahmed & Koob, 1998). Another common FR procedure used for tapping into aspects of addiction is the extended access model, where animals are given extended (6h/day), but not restricted (2h/day), access to daily SA on an FR schedule. This model is believed to produce a behavioral phenotype that mimics the escalation of drug intake observed in human drug addicts (Everitt & Robbins, 2005).

The progressive ratio (PR) schedule, on the other hand, is a high effort, high demand schedule where the response requirement for each successive reinforcer increases exponentially across a single session. Effects on PR measures are believed to reflect not only reinforcing efficacy of a drug, but also how hard an animal is willing to work for a drug reinforcer (Richardson & Roberts, 1996). In other words, the PR schedule requires an animal to assign incentive/motivational value to the drug that is indicated by the "break point." Typically, break point is defined operationally as the final schedule completed before meeting a criterion used to determine that the animal is no longer

motivated to continue exerting effort for a drug reinforcer (e.g., 1 h elapses without completing the next ratio requirement). In terms of clinical translation, this schedule may highlight the SUD symptom that involves spending increasing amounts of time and effort to obtain drug (Roberts, Morgan, & Liu, 2007). Indeed, Roberts et al. (2007) have argued that the PR schedule is especially suited to tap into addiction-like phenotypes in rodents. Therefore, examining both low and high ratio schedules during SA can give a clearer picture of what processes are being affected by an experimental manipulation.

In addition to active drug-taking behavior during SA, the extinction/reinstatement model has been useful in examining the incentive motivation to seek drug when the drug is no longer available. Following SA, animals undergo extinction training where they are placed back into the SA chamber and the levers no longer produce consequences. High rates of responding occur during the first few sessions, operationally defined as seeking behavior in the absence of drug. Over time, the animal learns that the levers no longer produce drug and will decrease the rate of responding. Then during reinstatement testing, stimuli previously associated with drug (e.g., drug-paired cues, drug priming injection, etc.) are presented and the rate of responding increases significantly compared to the low extinction baseline. This latter procedure allows researchers to examine the underlying neural mechanisms of individual factors known to elicit drug-seeking behavior, thereby identifying potential interventions aimed at attenuating drug craving in humans addicts (Markou et al., 1993).

Recent evidence has shown that manipulating miRNA expression in the brain can alter SA behavior through suppressing target ARG expression. Hollander and colleagues (2010) found that viral-mediated OE of dorsal striatal miR-212 prevents escalation of

cocaine SA (Hollander et al., 2010). This effect appears to be driven by miR-212-induced suppression of MeCP2 expression (Im et al., 2010). Furthermore, alcohol-dependent rats exhibit increases in miR-206 in the mPFC, a brain region involved in executive control of drug-seeking behavior (Kalivas & Volkow, 2005). Viral-mediated OE in mPFC miR-206 creates an alcohol-dependent phenotype in rats (Tapocik et al., 2014), which is mediated in part by miR-206-targeted suppression of BDNF (Tapocik et al., 2014). Indeed, decreases in BDNF in the mPFC are associated with increasing motivation to self-administer cocaine on a PR schedule (Sadri-Vakili et al., 2010). These examples provide evidence that altering levels of miRNAs in specific brain regions can alter facets of drug SA through regulating target ARG expression.

ARG targets of miR-495, *Bdnf* and *Camk2a,* both have facilitating roles in cocaine SA and seeking behavior (Anderson et al., 2008; Graham et al., 2007; Loweth et al., 2010; Wang et al., 2010). Specifically, knockdown of both NAc *Bdnf* and *Camk2a* decreases cocaine SA (Graham et al., 2007; Wang et al., 2010). Given that 1) the effects of *Camk2a* were found in the NAcSh, 2) NAcSh miR-495 OE decreases expression of both *Bdnf* and *Camk2a* expression (Table 1) (Bastle et al., 2014), and 3) miR-495 expression is selectively downregulated in the NAcSh following prolonged cocaine SA (Chapter 3), we predicted that NAcSh miR-495 OE would decrease levels of these genes and decrease cocaine SA. We tested these effects across both FR5 and PR schedules of cocaine SA, as well as during extinction and cue and cocaine-primed reinstatement. We also tested the specificity of our cocaine effects by using a non-drug food reinforcer.

Methods

Animals

Male Sprague–Dawley rats (Charles River, San Diego, CA) (200-225g) were individually housed in a climate-controlled facility with a 14-h dark/light cycle (lights off at 7 AM) with *ad libitum* access to food and water unless otherwise specified in the procedures. Housing and care were conducted in accordance with the 8th ed. Guide for the Care and Use of Laboratory Animals (Committee, 2011). Prior to surgery, animals were acclimated to handling (~2 min/day) for 7 days.

Surgery and Intracranial Viral Infusions

Procedures for catheter and stereotactic surgeries and intracranial viral infusions were identical to those described in Chapter 2 and 3, with the exception that animals from the food reinforcement experiment did not receive catheter surgery.

Experiment 4a: Cocaine Self-Administration

Cocaine SA training began 5-7 days post-surgery. Two days prior to the first training session, rats (n = 21) were placed on mild food restriction (18-22 g/day) that was maintained throughout the entire experiment. Training sessions (4 h/day; 6 days/week) began on a fixed ratio (FR) 1 schedule of lever presses for cocaine reinforcement (0.75 mg/kg/0.1mL, i.v.). The reinforcement schedule was set at FR1 for the first 5 sessions and then advanced daily from an FR1 to FR5 once rats received at least 20 infusions/2-h session. Once SA infusion rates stabilized, defined as less than 10% variability per session across 3 consecutive days with no upward or downward trends (16–22 sessions), rats were given 30-min access to varying cocaine doses presented in ascending order (0.0, 0.1, 0.32 and 1.0 mg/kg/0.1 mL, i.v.) on a FR5 schedule with a 10-min timeout period

between doses (Barrett, Miller, Dohrmann, & Caine, 2004). Within-session dose-response training occurred every 2 days, alternating with access to the training dose of cocaine for 2-hour sessions. Once rats stabilized on the within-session dose-response for 3 consecutive days with <15% variance in the number of infusions received across days, they were infused with either LV-GFP or LV-miR-495 (System Biosciences Inc.) while under isoflurane (2-3%) anesthesia. Total cocaine intake prior to receiving viral infusions was counterbalanced across groups. Beginning the following day, rats received three daily FR5 sessions on the training dose before beginning the test phase in order to allow time for the pre-miRNA encoded by the lentiviral construct to reach peak expression, as observed with similar constructs (Deroose et al., 2006). Rats were then tested on the within-session dose-response and were placed back on the training dose for the next two days. In order to further examine effects on an FR5 dose-response, rats were then given between-session access to varying doses of cocaine with each of the test doses (0.0, 0.03, 0.1, 0.3, 1.0 mg/kg/0.1mL, FR5) available in ascending order during 2-h tests. Additional FR5 sessions on the training dose occurred between each test dose until responding was again stable across 2 sessions. Following the FR5 dose-response test phase, rats were given two FR5 sessions before testing on a PR schedule of reinforcement where the response demand increased exponentially according to the formula 5*e^(0.2n)-5 (Richardson & Roberts, 1996), with n representing the number of reinforcers the rat received in the session (i.e., the progressive response requirement was 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, etc). The last ratio achieved after failing to attain a reinforcer in 1 h was defined as the break point. Two cocaine doses were tested under the PR schedule (0.375 & 0.75 mg/kg/0.1mL) in an ascending order with two

intervening FR5 sessions between the doses. Total lever pressing, intake, and break points were measured and slopes of the linear dose-response PR curve were calculated using the formula $(Y_2-Y_1)/(0.75-0.375)$. Following testing, rats were sacrificed and brain tissue was collected to verify cannula placement using cresyl violet staining.

Experiment 4b: Cocaine Self-Administration, Extinction, Reinstatement

Rats (n = 28) were trained in a similar manner as those in Experiment 4a, except they were given food *ad libitum* once they stabilized on an FR5 schedule. Once stability was reached under unrestricted food conditions, rats were infused with either LV-GFP or LV-miR-495 into the NAcSh as described previously. Total cocaine intake prior to receiving viral infusions was counterbalanced across groups. One week following viral infusions, rats received six daily FR5 sessions (0.75 mg/kg/infusion, i.v.). Once their intake was stable across 3 consecutive sessions on the training dose, rats were tested on a PR schedule of reinforcement at the same dose. After completion of PR testing, the rats would switch to the next dose and the same steps were taken to stabilize on an FR5 schedule and then test on a PR schedule at the respective cocaine dose. The doses were administered in a pseudorandom order: 0.75, 0.375, 1.5, and 0.1875 mg/kg/infusion.

Following the final PR test, rats were placed on the training dose (0.75 mg/kg/infusion) on an FR5 schedule of reinforcement for at least 3 sessions. Then rats received daily 1-h extinction sessions for at least 10 days, where lever presses produced no consequences. Once each rat achieved an extinction criterion of <20% of highest response during extinction or <20 responses in a session for 3 consecutive sessions, they were tested for cue reinstatement in a 1-h test session. On the test day, the light and tone cues were presented response-contingently on an FR1 schedule and were presented non-

contingently only if the rat did not press the active lever within the first 5 min of the test session. Rats were then re-stabilized under extinction conditions before testing for cocaine-primed reinstatement. The day before testing, rats were injected with saline (1 mL/kg) to acclimate them to being injected. Rats did not reinstate lever pressing following saline injections (data not shown). The next day rats were tested for cocaine-primed reinstatement by receiving a cocaine injection (10 mg/kg, i.p.) prior to being placed into the operant conditioning chamber. Lever pressing during the 1-h cocaine-primed reinstatement test produced no consequences. Following testing, one half of the rats were transcardially perfused with 4% paraformaldehyde and brain tissue was collected for GFP histology and immunohistochemistry. The other half the rats were sacrificed and brain tissue was rapidly extracted, frozen using 2-methylbutane (-50˚C), and stored at -80˚C until further processing.

Experiment 4c: Food Reinforcement

Rats (n = 20) were handled daily for 1 week prior to receiving training to lever press for food reinforcement (45 mg pellet, BioServ, Frenchtown, NJ, USA) in 30-min daily sessions, initially on an FR1 schedule of reinforcement. Advancement of the reinforcement schedule was identical to the procedure used in the cocaine SA experiments, except that rats were maintained on 16 g of rat chow/day until stabilized on an FR5 schedule, at which point they received 18 g/day (i.e., restricted). Once stable on an FR5 schedule, rats were infused with their assigned virus (LV-GFP or LV-miR-495) into the NAcSh, where group assignment was counterbalanced for previous total food intake during training. Rats were given one week of recovery, followed by one week of daily FR5 sessions, and then they received a PR test. Following testing, rats received

food *ad libitum* for the remainder of the experiment. To demonstrate varying degrees of motivation for food, rats were tested one week later on a PR schedule following daily exposure to food *ad libitum* (i.e., unrestricted). Following testing, rats were transcardially perfused with 4% paraformaldehyde and brain tissue was collected for GFP histology and immunohistochemistry.

Histology and Immunohistochemistry

For Experiment 4a, the harvested brains were sectioned using a cryostat (-20°C). The sections were mounted on gel-coated slides and stained with cresyl violet to determine cannula placement. Only rats with correct placements were included in the final analysis.

For GFP histology and double-label immunohistochemistry, rats from Experiment 4b and 4c were perfused transcardially with 4% paraformaldehyde (PFA) and their brains were post-fixed in PFA for 24-h, followed by serial increases in sucrose solutions (15%, then 30%). Brains were then frozen at -80°C until sectioned at 40 μm using a cryostat (-20°C). For GFP histology, sections were immediately mounted on gel-coated glass slides using Vectashield+DAPI mounting medium (Vector Labs, Burlingame, CA, USA). For immunohistochemistry, free-floating sections were washed in 0.1 M phosphate buffered solution (PBS), then incubated in 50 mM ammonium chloride for 30 min, 3% normal goat serum (NGS) for 2 h, and mouse anti-NeuN (1:1000; MAB377, EMD Millipore, Darmstadt, Germany) or rabbit anti-GFAP (1:1000; AB7260, Abcam, Cambridge, MA, USA) antibodies in a solution containing 0.1% bovine serum albumin, 0.2% Triton X-100, and 2% NGS for 48 h. Following incubation in the primary antibody, sections were washed and incubated in Alexa Fluor 555 anti-mouse (1:1000; A31570, Life

Technologies, Grand Island, NY, USA) or Alexa Fluor 555 anti-rabbit (1:1000; A31572, Life Technologies, Grand Island, NY, USA) secondary antibody for 1 h, then washed and mounted on gel-coated glass slides using Vectashield+DAPI mounting medium (Vector Labs). Images of the sections were captured using a Hamamatsu Digital Camera (Hamamatsu City, Japan) attached to an Olympus BX53 microscope at 20x magnification and automated stitching software (cellSens Dimension, Olympus, Center Valley, PA, USA). GFP, DAPI, and either NeuN or GFAP staining were overlayed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA).

qRT-PCR and Western Blot

Procedures were identical to those described in Chapters 2 and 3.

Data Analysis

Pre-virus intake and qRT-PCR data was analyzed using independent-samples student *t*-tests. SA data was analyzed using a mixed-factor two-way ANOVA with Dose, Day, and Food Restriction condition as within-subjects factors and Virus as a between-subjects factor. Significant interactions for both experiments were followed by tests for simple effects. Comparisons of interest were analyzed using *t*-tests with Bonferroni correction for multiple comparisons (alpha level/number of comparisons). Results were considered statistically significant if $p < 0.05$.

Results

Effects on an FR5 schedule of cocaine reinforcement

The within-session FR5 dose-response curve in Experiment 4a showed a non-significant trend toward a slight shift to the right in the LV-miR-495 group compared to the LV-GFP group (Fig. 14; Dose: $F_{(3, 30)} = 31.45$, $p < 0.0001$; Virus: $F_{(1, 10)} = 0.02$, *n.s.*;

Dose x Virus: $F_{(3, 30)} = 1.78$, *n.s.*). When the data were analyzed as a change in infusion rates compared to baseline there was a significant interaction between Dose and Virus (Dose: $F_{(3, 30)} = 4.75$, $p < 0.01$; Virus: $F_{(1, 10)} = 0.93$, *n.s.*; Dose x Virus: $F_{(3, 30)} = 2.95$, $p < 0.05$. Subsequent *post-hoc* tests for simple effects revealed that when the 0.32 mg/kg dose of cocaine was available, the LV-miR-495 group showed an increase in infusions earned compared to the LV-GFP group (Fig. 15). Furthermore, trend analysis revealed that the LV-miR-495 group had a significant cubic trend for the difference-from-baseline curve ($F_{(1, 5)} = 14.58$, $p < 0.05$), whereas the LV-GFP group had no such trend (*n.s.*). Subsequent testing on a between-session cocaine dose-response revealed that the cocaine self-administration dose-effect curve was again slightly shifted to the right in the LV-miR-495 group compared to the LV-GFP group, where the LV-miR-495 group had decreased intake at the low 0.032 cocaine dose when measuring total infusions (Fig. 16A; Dose: $F_{(4, 60)} = 61.65$, $p < 0.0001$; Virus: $F_{(1, 15)} = 0.23$, *n.s.*; Dose x Virus: $F_{(4, 60)} = 2.23$, $p = 0.08$; *post-hoc* Bonferroni, $p < 0.05$) and increased intake at the 1.0 mg/kg cocaine dose when measuring total mg/kg (Fig. 16B; Dose: $F_{(3, 45)} = 320.7$, $p < 0.0001$; Virus: $F_{(1, 15)} = 1.26$, *n.s.*; Dose x Virus: $F_{(3, 45)} = 4.10$, $p < 0.05$; tests for simple effects, $p < 0.05$).

During FR5 testing in Experiment 4b and 4c, NAcSh miR-495 OE had no effect on intake across cocaine doses (Fig. 17; Dose: $F_{(3, 60)} = 176.97$, $p < 0.01$; Virus: $F_{(1, 20)} = 0.02$, *n.s.*; Virus x Dose: $F_{(3, 60)} = 0.85$, *n.s.*) or food intake (Fig. 18; Day: $F_{(6, 84)} = 8.30$, $p < 0.0001$; Virus: $F_{(1, 14)} = 0.23$, *n.s.*; Day x Virus: $F_{(3, 60)} = 1.83$, *n.s.*).

Effects on a PR schedule of cocaine reinforcement

In Experiment 4a where we tested PR measures on two cocaine doses (0.375 and 0.75 mg/kg/infusion), we found that the LV-GFP group exhibited a dose-dependent

increase in responding and intake at the 0.75 versus the 0.375 mg/kg dose, whereas the LV-miR-495 group did not exhibit an increase in any of the measures (Fig. 19A, B; Dose: active lever presses, intake, $F_{(1, 14)} = 19.77, 25.64$, ps < 0.001, respectively; Virus: active lever presses, intake, $F_{(1, 14)} = 0.52, 0.31$, *n.s.*, respectively; Dose x Virus: active lever presses, intake, $F_{(1, 14)} = 5.75, 6.41$, ps < 0.05, respectively, tests for simple effects, LV-GFP, ps < 0.05, LV-miR-495, *n.s.*). Furthermore, by calculating the slope of the dose-response curves, we found that the LV-GFP group had a significantly higher slope than the LV-miR-495 (Fig. 19C, D; active lever presses, intake, $t_{(15)} = 2.40, 2.53$, ps < 0.05), suggesting that the OE of miR-495 attenuated motivation for cocaine. The representative cumulative response records in Figure 20 depict a lower break point in the LV-miR-495 group compared to the LV-GFP group for the higher 0.75 mg/kg dose. We found no effect on inactive lever pressing (Fig. 21; Dose, $F_{(1, 10)} = 1.19$; Virus, $F_{(1, 10)} = 0.002$; Dose x Virus, $F_{(1, 10)} = 1.69$, all *n.s.*).

Our results from Experiment 4b support our PR findings in Experiment 4a where we found that NAcSh miR-495 OE decreased responding and intake on a PR schedule compared to LV-GFP controls, regardless of cocaine dose (Fig. 22; Dose: active lever pressing, intake $F_{(3, 60)} = 8.98, 13.70$, ps < 0.01, respectively; Virus: active lever pressing, intake, $F_{(1, 20)} = 7.14, 5.55$, ps < 0.05 respectively; Virus x Dose: active lever pressing, intake, $F_{(3, 60)} = 2.23, 0.65$, *n.s.*, respectively). We found no effect on inactive lever pressing (Fig. 23; Dose, $F_{(3, 60)} = 1.33$; Virus, $F_{(1, 20)} = 0.71$; Dose x Virus, $F_{(3, 60)} = 0.17$, all *n.s.*).

For Experiment 4c where we measured food reinforcement on a PR schedule, we subjected rats to varying levels of food restriction to parallel differences in motivational

value obtained using low and high cocaine doses on a PR schedule. Similar to increasing the incentive value for cocaine by increasing the dose, food has increased incentive value in hungry rats compared to sated rats. Indeed, we found that rats that had been food-restricted (18g/day) exhibited higher responding and intake than those that had been unrestricted (Fig. 24; Food Restriction: active lever pressing, intake, $F_{(1, 14)} = 55.26$, 45.97, ps < 0.001, respectively). Importantly, both groups exhibited similar levels of motivation for food under both feeding conditions (Fig. 24; Virus: active lever pressing, intake, $F_{(1, 14)} = 0.02$, 0.06, *n.s.*, respectively; Virus x Food Restriction: active lever pressing, intake, $F_{(1, 14)} = 0.003$, 0.06, *n.s.*, respectively). This finding suggests a selective effect of miR-495 OE on motivation for cocaine. We also found no effect on inactive lever pressing (Fig. 25; Food Restriction, $F_{(1, 14)} = 0.24$, *n.s.*; Virus: $F_{(1, 14)} = 0.01$, *n.s.*; Food Restriction x Virus, $F_{(1, 14)} = 5.62$, $p < 0.05$, tests for simple effects, all *n.s.*).

Effects on extinction and reinstatement

Rats in Experiment 4b went on to undergo testing for extinction and cue and cocaine-primed reinstatement. Before extinction testing, rats received at least 3 sessions on an FR5 schedule of cocaine reinforcement on the training dose. We found no group difference in active lever pressing during these baseline sessions before extinction (Fig. 26; $t_{17} = 0.70$, *n.s.*). During extinction, NAcSh miR-495 OE decreased active lever pressing during the first 3 sessions compared to LV-GFP controls (Fig. 26; Day: $F_{(9, 180)} = 19.36$, $p < 0.001$; Virus: $F_{(1, 20)} = 3.19$, $p = 0.09$; Virus x Day: $F_{(9, 180)} = 3.17$, $p < 0.01$, *post-hoc* tests for simple effects, ps < 0.05). We found no group differences for inactive lever pressing during extinction (Fig. 27; Day: $F_{(9, 180)} = 2.90$, $p < 0.05$; Virus: $F_{(1, 20)} = 1.46$, *n.s.*; Day x Virus, $F_{(9, 180)} = 1.48$, *n.s.*). We then tested rats for both cue and cocaine-

primed reinstatement. While both virus groups showed a similar degree of cue reinstatement (Fig. 28A; Day: $F_{(1, 20)} = 56.17$, $p < 0.001$; Virus: $F_{(1, 20)} = 2.54$, *n.s.*; Day x Virus, $F_{(1, 20)} = 2.48$, *n.s.*), the LV-miR-495 group exhibited reduced cocaine-primed reinstatement compared to the LV-GFP controls (Fig. 28B; Day: $F_{(1, 20)} = 38.35$, $p < 0.001$; Virus: $F_{(1, 20)} = 6.55$, $p < 0.05$; Virus x Day: $F_{(1, 20)} = 6.32$, $p < 0.05$, *post-hoc* tests for simples effects, $p < 0.05$). No group differences were found for inactive lever pressing during reinstatement testing (Fig. 29; Cue Day, $F_{(1, 20)} = 1.73$; Cue Virus, $F_{(1, 20)} = 0.73$; Cue Dose x Virus, $F_{(1, 20)} = 0.93$; Coc Day, $F_{(1, 20)} = 1.55$; Coc Virus, $F_{(1, 20)} = 1.55$; Coc Dose x Virus, $F_{(1, 20)} = 1.35$, all *n.s.*). Collectively, these results suggest that NAcSh miR-495 OE decreases motivation to both self-administer and seek cocaine.

Effects on ARG expression

Following testing, qRT-PCR and Western blot analysis was used to measure levels of miR-495 and ARG expression. We verified that the LV-miR-495 group exhibited greater NAcSh miR-495 expression than the LV-GFP group (Fig. 30; $t_6 = 3.70$, $p < 0.05$). We found that miR-495 OE reduced NAcSh *Arc* mRNA (Fig. 31A; $t_8 = 1.95$, $p < 0.05$, one-tailed), but not protein (Fig. 31B; $t_6 = 1.06$, *n.s.*). miR-495 OE also reduced NAcSh *Camk2a* mRNA and protein (Fig. 32; mRNA, protein, $t_8 = 1.63, 2.23$, $p \leq 0.05$, one-tailed, respectively). miR-495 OE had no effect on NAcSh *Bdnf* mRNA expression (Fig. 33; *Bdnf*-L, *Bdnf*-Pan, $t_8 = 1.27, 1.00$, *n.s.*, respectively).

Histology and Immunohistochemistry

Only rats with >50% of GFP expression within the NAcSh were included in the final analysis (Fig. 34). Twelve rats (Experiment 4a, n = 2; Experiment 4b, n = 6; Experiment 4c, n = 4) were excluded due cannula and virus misplacement. We also found

through double-labeling immunohistochemistry that GFP co-labels preferentially with the neuronal marker, NeuN, and not the astrocyte marker, GFAP (Fig. 35).

Discussion

Here we find that NAcSh miR-495 OE decreases several measures of motivation for cocaine, including SA on a PR schedule of reinforcement, extinction responding, and cocaine-primed reinstatement. We also examined whether the same manipulation altered motivation for food on a PR schedule and found no effect, suggesting our motivational effects were specific to cocaine. Furthermore, we found that increasing NAcSh miR-495 levels decreased levels of *Camk2a* and *Arc* expression, suggesting possible molecular mechanisms underlying our behavioral effects.

During cocaine SA, we utilized both low FR5 and effortful PR schedules of reinforcement to examine whether miR-495 had an effect on reinforcement and/or motivation to self-administer cocaine. We observed slight changes in the FR5 dose-response curve (Experiment 4a), where the LV-miR-495 group's curve was slightly shifted to the right, suggesting decreased sensitivity/reinforcement. However, upon further examining this effect in a different group of animals (Experiment 4b), we did not observe any change on the FR5 schedule. Collectively, these findings suggest miR-495 had little to no impact on the reinforcing properties of cocaine. We did observe consistent decreases in responding and intake in the LV-miR-495 group when cocaine was available on the high effort PR schedule. These findings are believed to model an aspect of SUD related to an individual increasing time and energy spent on drug-seeking and drug-taking behavior (Roberts et al., 2007). In order to rule out impairment in motor activity required to press a high number of times on PR schedule, we used a similar procedure with a

natural food reinforcer. We found no reduction in PR measures for food reinforcement, suggesting that our effects were not due to impairment in high rates of responding and that our effects on motivation were specific to cocaine.

We further tested motivation for cocaine during extinction and reinstatement to examine if miR-495 OE would suppress seeking behavior in the absence of cocaine. We found that NAcSh miR-495 OE reduced active lever presses during the first three sessions of extinction, where cocaine-seeking behavior was at its highest. We also found decreases in cocaine-primed reinstatement following miR-495 OE. Although we didn't detect a statistically significant effect during cue reinstatement, the pattern is similar. The lack of the effect on cue reinstatement may also highlight the more prominent role of the NAc core in regulating cue reinstatement (Fuchs, Evans, Parker, & See, 2004). Collectively, our results suggest motivation to both self-administer and seek cocaine was blunted by NAcSh miR-495 OE.

We chose to target the NAcSh for miR-495 OE given our previous finding that prolonged cocaine SA decreases miR-495 in the shell, but not the core (see Chapter 3). Furthermore, both *Bdnf* and *Camk2a* facilitate cocaine abuse-related behaviors in this subregion (Anderson et al., 2008; Graham et al., 2007; Wang et al., 2010). Although we didn't detect a miR-495 OE-induced decrease in *Bdnf* mRNA in the present study, we have previously shown that NAcSh miR-495 OE decreases both *Bdnf*-L and *Bdnf*-Pan mRNA in the NAc (See Chapter 2). Similarly, increases in endogenous miR-495 expression in *Mecp2*-null mice are associated with diminished *Bdnf* expression (Wu et al., 2010). The inverse relationship between miR-495 and BDNF is also observed in the human prefrontal cortex (Mellios et al., 2008) and in the hippocampus of adolescent rats

upon binge-like alcohol exposure (Prins, Przybycien-Szymanska, Rao, & Pak, 2014). Furthermore, knockdown of local *Bdnf* in the NAc using a Cre-Lox recombination approach decreases cocaine self-administration in mice (Graham et al., 2007). It should be noted that these effects occurred on an FR schedule of cocaine reinforcement. Alternatively, shRNA-mediated knockdown of *Camk2a* in the NAcSh decreases cocaine self-administration on a PR, but not FR schedule, closely mirroring our effects (Wang et al., 2010). Due to CaMKII's pervasive role in several signaling pathways, our effects may also involve miR-495-induced downregulation of CaMKII and its target activity. For instance, CaMKII has previously been implicated in SUD-related processes through its ability to regulate phosphorylation and stability of ΔFosB (Robison et al., 2013), trafficking of GluR1 subunit-containing AMPA receptors (Anderson et al., 2008; Loweth et al., 2010), CREB activity (Loweth et al., 2010; Wu & McMurray, 2001), and A-type K^+ currents (Kourrich et al., 2012). Most importantly, CaMKII has been identified *in silico* as a common, central node in positive feedback gene regulatory pathways involved with drug addiction (Li et al., 2008). These feedback loops have been hypothesized to further strengthen and perpetuate the molecular underpinnings of addiction, thus suggesting that CaMKII signaling is critical in this disorder.

Given that several miR-495 targets are plasticity-related genes, miR-495 OE is likely altering plasticity-related activity of infected NAcSh neurons, thereby altering drug motivational processes. Indeed, the NAcSh plays a role in inhibition of drug seeking, but not food seeking (Millan & McNally, 2011; Peters et al., 2008; Sutton et al., 2003). While the NAcSh may be recruited for drug and non-drug reinforcement, the plasticity occurring during drug reinforcement may be distinct from non-drug reinforcers. This is

supported by work demonstrating that distinct populations of neurons are active during drug versus non-drug goal-directed behavior (Cameron & Carelli, 2012; Carelli, Ijames, & Crumling, 2000). Furthermore, previous work has shown that manipulations of NAc *Bdnf* and *Camk2a* levels alter drug-, but not food-, seeking behavior (Graham et al., 2007; Wang et al., 2010), suggesting these are indeed "addiction"-related genes that specifically control drug abuse-related processes. Consistent with this line of reasoning, we found that NAcSh miR-495 OE had no impact on motivation to seek food reinforcement, even though these genes were also downregulated. Therefore, NAcSh miR-495 OE may be reversing the drug-induced plasticity involved in motivation for cocaine that is not shared by natural reinforcers.

Another factor that may relate to our effects is the cell-type specific functions of NAc neurons. As mentioned previously, the NAc contains neurons that either predominantly express D1R or D2Rs. Recent work has shown that optogenetic stimulation of D2R-containing neurons in the NAc prevents compulsive cocaine SA in mice, while D1R stimulation has no effect (Bock et al., 2013). Furthermore, cell type-specific loss of BDNF signaling or the histone methyltransferase G9a in either D1R or D2R has opposite effects on cocaine reward CPP (Lobo et al., 2010; Maze et al., 2014). In general, loss of BDNF signaling disinhibits the cell, leading to increases in activity. Similar to the findings from Bock and colleagues (2103), loss of BDNF in D2R-containing neurons leads to decreases in cocaine CPP by increasing activity of these cells (Lobo et al., 2010). Under normal conditions, G9a suppresses gene expression to control developmental cell-type specification (Maze et al., 2014). Loss of G9a in D2R neurons results in increased gene expression profiles, anatomical circuitry, and

electrophysiological properties that resemble D1R neurons, which leads to increases in cocaine reward CPP (Maze et al., 2014). Additionally, ΔFosB overexpression in D1R, but not D2R, neurons increases *Camk2a* expression and this leads to increases in cocaine reward CPP (Kelz et al., 1999; Robison et al., 2013). These examples provide evidence for cell-type specific effects within the same brain region. Given that our viral manipulation likely infected NAc neurons without preference for either D1R- versus D2R-containing neurons, our effects may change depending on the specific cell type that miR-495 is altered. Based on these previous findings, our manipulation may have increased D2R neuronal activity and/or suppressed D1R activity. Further work into this area is warranted.

In conclusion, we demonstrated that NAcSh miR-495 OE reduces motivation to both self-administer and seek cocaine, but not food reinforcement. These findings open new avenues for future research on the specific genes and pathways responsible for the reductions in motivation, the role of miR-495 in regulating different forms of synaptic plasticity in the NAc, and whether specific cell types affected by miR-495 in the NAc may be driving the distinct neural circuits underlying drug motivation. 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.

CHAPTER 5

EFFECT OF NUCLEUS ACCUMBENS MIR-495 INHIBITION ON COCAINE SELF-ADMINISTRATION

While overexpression of miRNAs provides information on how reducing target gene expression impacts drug abuse-related behavior, miRNA inhibition provides information on how disinhibiting and increasing target gene expression may produce opposite behavioral effects. Since a given mRNA is regulated by several different miRNAs (Doench & Sharp, 2004), inhibiting one miRNA may not disinhibit all target mRNA expression. One factor that increases the degree miRNA-mediated regulation is the number of binding sites in the 3'UTR, where a greater number increases the magnitude and probability of repression (Brennecke, Stark, Russell, & Cohen, 2005). Indeed, miR-495 has greater binding efficacy and repression for *Bdnf*-L (5 sites) over *Bdnf*-S (1 site) (Oliver et al., 2014). In addition to number of binding sites, several other factors influence miRNA-mRNA interactions, including thermodynamic stability, conserved seed regions across species, and G-U wobbles (Brennecke et al., 2005; Doench & Sharp, 2004; Stark, Brennecke, Russell, & Cohen, 2003). Therefore, the subset of mRNAs that are disinhibited by miRNA inhibition suggests that expression of those particular mRNAs is governed strongly by that specific miRNA.

Several methods have been used to inhibit miRNA action, including antisense oligonucleotides, gene knockout, and sequestering transcripts (e.g., sponges) (Ruberti, Barbato, & Cogoni, 2012). Antisense oligonucleotides (ASO) are small strands of complementary nucleic acids that will bind to the target sequence and "inactivate it" or prevent it from being expressed. Several chemical modifications can be made to ASOs in

order to increase stability, affinity, and cellular and tissue uptake. For example, locked nucleic acid (LNA) ASOs are particularly effective at miRNA suppression due to additions of a 2', 4' methylene bridge in the ribose. LNAs targeting miR-212 have been shown to produce opposite behavioral effects on cocaine SA as OE (Hollander et al., 2010). One disadvantage of this method is that it requires repeated administrations directly into tissues of interest (e.g., brain) in order to get long-lasting inhibition. Another approach is to knockout miRNA genes from the genome using Cre-Lox recombination or CRISPR-Cas9 gene editing technology. Both procedures silence target gene expression by excising specific DNA sequences. One disadvantage is that several miRNA genes reside within clusters in the DNA. Therefore, knocking out one miRNA gene may affect the transcriptional processing and ultimate expression of the other miRNAs in the cluster. For instance, miR-495 is located within the miRNA cluster B of the Dlk1-Dio3 maternally imprinted region (Benetatos et al., 2013). Finally, sequestering transcripts is another way to inhibit endogenous miRNA action. This approach involves synthesizing a protein-coding transcript with a 3'UTR that contains several adjacent binding sites for a particular miRNA of interest (i.e., sponge) (Ebert, Neilson, & Sharp, 2007). Once transfected into cells, endogenous miRNAs will bind to the sponge transcript and prevent miRNA:target mRNA interaction. One of the advantages of this approach is that the transcript can be packaged into a viral vector in order to transfect and express a sponge construct for long periods of time. However, as with all of these approaches, target gene expression changes need to be validated.

In order to directly compare the effects miR-495 OE on cocaine abuse-related behaviors (Chapter 4) to those produced by miR-495 knockdown, we created a sponge

construct that could be packaged into a lentiviral vector for long-term miR-495 inhibition. The sponge construct contained six miR-495 binding sites in the 3'UTR. We first verified that this manipulation effectively prevented miR-495-induced repression of reporter activity *in vitro* (data not shown). Given that increases in target gene expression, such as *Bdnf* and *Camk2a*, in the NAc lead to increases in cocaine abuse-related behavior (Bahi et al., 2008; Loweth et al., 2010), we predicted that sponge-mediated miR-495 inhibition would increase motivation to self-administer and seek cocaine through disinhibition of target ARG expression. Here, we tested the effect of the miR-495 sponge on cocaine reinforcement under FR5 and PR schedules, and during extinction and reinstatement of cocaine-seeking behavior.

Methods

Procedures for animals, surgery, intracranial viral infusions, cocaine self-administration, extinction, reinstatement, histology and immunohistochemistry, qRT-PCR, Western blot and data analysis were identical to those described for Chapter 4. The only exception was that rats were infused with either LV-GFP or LV-miR-495-sponge (LV-Sponge), which expressed a GFP reporter that contained 6 miR-495 binding sites in the 3'UTR. The complementary base pairing included a bulge (mismatched pairing) so that endogenous miR-495 would not result in endonucleolytic cleavage of the sponge transcript.

Results

Effects on FR5 and PR schedule of cocaine reinforcement

LV-sponge had no effect on intake across cocaine doses on an FR5 schedule (Fig. 36; Dose: $F_{(3, 63)} = 150.80$, $p < 0.01$; Virus: $F_{(1, 21)} = 0.32$, *n.s.*; Virus x Dose: $F_{(3, 63)} = 0.43$, *n.s.*). LV-Sponge also had no effect on responding or intake on a PR schedule (Fig. 37; Dose: active lever pressing, intake $F_{(3, 63)} = 9.62, 13.50$, ps < 0.01, respectively; Virus: active lever pressing, intake, $F_{(1, 21)} = 1.69, 0.50$, *n.s.,* respectively; Virus x Dose: active lever pressing, intake, $F_{(3, 63)} = 1.95, 0.96$, *n.s.*, respectively), as well as no effect on inactive lever pressing (Fig. 38; Dose, $F_{(3, 63)} = 1.57$; Virus, $F_{(1, 21)} = 0.49$; Dose x Virus, $F_{(3, 63)} = 0.15$, all *n.s.*).

Effects on extinction and reinstatement

We found no group difference in active lever pressing during the baseline before extinction (Fig. 39; $t_{18} = 1.48$, *n.s.*). Interestingly, the LV-Sponge group exhibited decreased active lever pressing during the first extinction session compared to LV-GFP controls (Fig. 39; Day: $F_{(9, 189)} = 22.13$, $p < 0.001$; Virus: $F_{(1, 21)} = 2.50$, *n.s.*; Day x Virus: $F_{(9, 189)} = 5.03$, $p \leq 0.05$, *post-hoc* tests for simple effects, $p < 0.05$). We found no group differences for inactive lever pressing during extinction (Fig. 40; Day: $F_{(9, 189)} = 1.01$; Virus: $F_{(1, 21)} = 0.06$; Day x Virus, $F_{(9, 189)} = 1.30$, all *n.s.*). We then tested rats for both cue and cocaine-primed reinstatement. While there was no significant difference between virus groups in the degree of cue reinstatement (Fig. 41A; Day: $F_{(1, 21)} = 72.29$, $p < 0.001$; Virus: $F_{(1, 21)} = 0.83$, *n.s.*; Day x Virus, $F_{(1, 21)} = 0.66$, *n.s.*), the LV-Sponge group exhibited a trend toward a reduction in cocaine-primed reinstatement compared to the LV-GFP controls (Fig. 41B; Day: $F_{(1, 21)} = 45.32$, $p < 0.001$; Virus: $F_{(1, 21)} = 3.05$, $p =$

0.10; Virus x Day: $F_{(1, 21)} = 3.47$, $p = 0.08$, *post-hoc* Bonferroni, test day, $p = 0.09$). No group differences were found for inactive lever pressing during reinstatement testing (Fig. 42; Cue Day, $F_{(1, 21)} = 0.18$; Cue Virus, $F_{(1, 21)} = 0.22$; Cue Dose x Virus, $F_{(1, 21)} = 1.09$; Coc Day, $F_{(1, 21)} = 2.76$; Coc Virus, $F_{(1, 21)} = 0.03$; Coc Dose x Virus, $F_{(1, 21)} = 0.002$, all *n.s.*). Collectively, these results suggest that blockade of endogenous miR-495 slightly decreases motivation to seek cocaine.

Effects on ARG expression

Following testing, qRT-PCR and Western blot analysis were used to measure levels of miR-495 and ARG expression. We found no differences in NAcSh miR-495 (Fig. 43; $t_7 = 1.01$, *n.s.*), *Camk2a* (Fig. 44; mRNA, protein, $t_{10} = 0.69$, 0.44, *n.s.*, respectively), or *Bdnf* (Fig. 45; *Bdnf*-L, *Bdnf*-Pan, $t_{10} = 1.36$, 1.31, *n.s.*, respectively) expression between the LV-GFP and LV-Sponge groups. Interestingly, we found that LV-Sponge exhibited reduced NAcSh *Arc* mRNA (Fig. 46A; $t_{10} = 1.83$, $p < 0.05$, one-tailed), but not protein (Fig. 46B; $t_6 = 1.02$, *n.s.*).

Discussion

Here we find that inhibition of NAcSh miR-495 slightly reduces motivation to seek cocaine, evident as a decrease in cocaine-seeking behavior during the first extinction session. However, this manipulation failed to alter SA on either reinforcement schedule tested. Furthermore, reinstatement of cocaine seeking by either cues or cocaine priming did not differ between virus groups, although there was a non-significant trend toward a decrease in cocaine-primed reinstatement. The behavioral effects observed are contrary to our hypothesis that LV-sponge would increase motivation for cocaine; however, post-

mortem measures of ARG expression revealed a decrease in *Arc* mRNA in the LV-sponge group compared to LV-GFP controls, but no change in miR-495 and other ARGs.

Although we observed no differences in miR-495 expression between the two virus groups, this is likely because sponge constructs typically sequester, and not degrade, target miRNAs (Ebert & Sharp, 2010). The mechanism underlying sponge-mediated reduction in *Arc* expression, however, is unknown, but could be due to several factors. One possibility is that since the binding sites for miR-495 are AU rich, this may result in other molecules binding to similar sequences and being sequestered by the sponge transcript. Indeed, several RNA-binding proteins (RBP) preferentially bind to AU-rich elements (ARE) in the 3'UTR of mRNAs (e.g., HuR, HuD, KSRP, etc.). Since several RBPs stabilize bound mRNAs and increase expression, sequestering these RBPs could leave target transcripts more vulnerable to degradation processes. Similarly, miRNA inhibition-mediated increases in specific target genes will create competition among transcripts for RBPs. One target gene may receive preferential stabilization, while others will be left vulnerable. Recent work has shown that *Camk2a* and *Kv1.1* mRNA can act as a "sponge" for the RPB HuD, depending on the level of mTOR signaling (Sosanya et al., 2015; Sosanya et al., 2013). Therefore, sponge-mediated increases in certain miR-495 target genes may leave *Arc* mRNA vulnerable to degradation. Additional work is needed to directly test this hypothesis.

Interestingly, some of the changes observed with LV-sponge are similar to those observed previously with miR-495 OE. Both manipulations decreased cocaine-seeking behavior during extinction and *Arc* mRNA expression. In contrast, miR-495 OE, but not LV-sponge, also reduced responding on a progressive ratio schedule of cocaine

reinforcement and cocaine-primed reinstatement of extinguished cocaine-seeking behavior, as well as reduced *Camk2a* and *Bdnf* expression. Much work has demonstrated a role of *Camk2a* in drug abuse-related behavior. Bi-directional regulation of NAc *Camk2a* levels and activity produces opposite effects on psychostimulant-induced behavior (Anderson et al., 2008; Loweth et al., 2010; Wang et al., 2010), where *Camk2a* expression facilitates abuse-related behavior. Therefore, the greater suppression of motivation for cocaine following OE compared to LV-sponge may be due to *Camk2a* downregulation.

Much less is known about *Arc*'s role in motivation for drug, but previous work has shown that antisense-mediated *Arc* knockdown in the NAcSh reduces the expression of morphine CPP (Lv et al., 2011). In contrast, the same manipulation in the dorsal striatum increases cocaine-seeking behavior during extinction (Hearing, Schwendt, & McGinty, 2011). Therefore, *Arc*'s role in drug abuse-related behavior may be region-specific. In addition, *Arc* mRNA is upregulated in several brain regions following tests for contextual- and cue-induced reinstatement of cocaine-seeking behavior, including in the mPFC, orbitofrontal cortex, NAc, and dorsal striatum (Hearing, Miller, See, & McGinty, 2008; Hearing, See, & McGinty, 2008; Zavala et al., 2008). More specifically, upregulation in the NAc occurs following context-induced reinstatement where cocaine-seeking behavior correlates with *Arc* mRNA expression (Hearing, See, et al., 2008). While *Arc* mRNA is upregulated in the mPFC following cue reinstatement, no change occurs in the NAcSh (Zavala et al., 2008). These findings may relate to our more selective behavioral effects on extinction versus cue reinstatement, where *Arc* appears to

be recruited in the NAcSh during drug-seeking behavior that is evoked by contextual stimuli (e.g., extinction) rather than discrete cues (e.g., cue reinstatement).

Arc is an effector immediate early gene (IEG) that is rapidly transported to dendrites following transcription (Dynes & Steward, 2012; Link et al., 1995; Lyford et al., 1995; Steward, Wallace, Lyford, & Worley, 1998), where it can undergo local translation and participate in structural and synaptic plasticity (Korb & Finkbeiner, 2011). Specifically, Arc protein regulates spine density, spine type, long-term potentiation (LTP) and depression (LTD), and homeostatic plasticity (Bramham et al., 2008). Although Arc has several roles in synaptic plasticity, one common consequence of Arc knockdown is impairment in AMPA receptor endocytosis, leading to increased surface AMPA receptor expression (Chowdhury et al., 2006). This leads to impairment in LTD, where neurons have increased firing rates (Shepherd et al., 2006). Given that activity in the NAcSh is associated with inhibiting drug-seeking behavior during extinction, perhaps downregulation of *Arc* expression leads to increased surface AMPA receptor expression and activity of viral-infected NAcSh neurons, thereby leading to decreases in drug-seeking behavior during extinction. Indeed, viral-mediated upregulation of GluR1 and GluR2 AMPA receptor expression in the NAcSh facilitates extinction of cocaine-, but not sucrose-, seeking behavior (Sutton et al., 2003). Future work into the role of NAcSh *Arc* in extinction of cocaine-seeking behavior is warranted.

In conclusion, we found that our attempt to inhibit miR-495 expression in the NAcSh with LV-sponge failed to increase cocaine abuse-related behaviors and ARG expression, but instead attenuated cocaine-seeking behavior during extinction, as well as *Arc* mRNA expression in NAcSh. Although unexpected, these data support our overall

hypothesis that downregulating ARG expression reduces cocaine abuse-related behavior. Importantly, given that miR-495 OE is more effective than the sponge construct at reducing both ARG expression and motivation for cocaine, this further supports the idea of using miRNAs at tools for targeting several genes simultaneously for greater regulation of drug abuse-related behavior.

CHAPTER 6

CONCLUDING REMARKS

This dissertation aimed to test the hypothesis that NAc miR-495 regulates cocaine abuse-related behavior by targeting ARG expression. The main findings that support this hypothesis include: 1) NAcSh miR-495 OE attenuated motivation for cocaine during extinction of cocaine CPP, cocaine SA on a PR schedule of reinforcement, and cocaine-seeking behavior during both extinction and cocaine-primed reinstatement, 2) NAcSh miR-495 OE decreased ARG expression, 3) NAcSh miR-495 OE had no effect on food reinforcement on a PR schedule or the rewarding properties of cocaine as measured by expression of cocaine CPP and cocaine SA on an FR5 schedule of reinforcement, 4) NAc miR-495 levels were reduced and ARG levels were increased in rats following prolonged, but not brief, cocaine SA, and 5) sequestration of NAc miR-495 expression unexpectedly decreased both cocaine-seeking behavior during extinction and expression of the ARG, *Arc*, in the NAcSh. Collectively, these exciting findings demonstrate miR-495 as a novel target for reducing addiction-like phenotypes.

Candidate Mechanisms

The NAcSh was chosen as our target region for miR-495 manipulation based on the region-specificity of its predicted ARG targets and their known effects in this region on cocaine abuse-related behaviors, as well as the NAc miR-495 expression profile following cocaine SA (Chapter 3). Previous work has established a role of the NAcSh in regulating drug-seeking behavior. While some have found that pharmacological inhibition increases drug seeking during extinction (Di Ciano, Robbins, & Everitt, 2008; Millan et al., 2010; Peters et al., 2008), others have shown reductions in cocaine-primed

reinstatement of drug seeking (Famous et al., 2008). These discrepancies may be due to NAcSh activity regulating drug abuse-related processes across different behavioral domains. Specifically, extinction normally inhibits drug seeking such that inactivation of the NAcSh prevents this effect, resulting in an increase in the behavior. In contrast, cocaine priming normally facilitates drug seeking such that inactivation of the NAcSh attenuates the behavior. Perhaps NAcSh miR-495 OE reduced cocaine-seeking behavior during extinction by facilitating extinction learning, whereas the same manipulation reduced cocaine-primed reinstatement of cocaine seeking by attenuating the motivational effects of cocaine. Although further research is needed to understand the psychological processes responsible for the behavior effects observed, the findings suggest that the molecular consequences of miR-495 OE underlie these behavioral changes.

Arc is involved in AMPA-receptor endocytosis (Chowdhury et al., 2006); thereby reductions in *Arc* expression are associated with increases in surface AMPA receptor expression and activity (Shepherd et al., 2006). Extinction also increases AMPA receptor expression in the NAcSh and viral-mediated increases in NAcSh GluR1/2 expression enhance extinction of cocaine-seeking behavior (Sutton et al., 2003). Therefore, the facilitated extinction of cocaine-seeking behavior and reductions in *Arc* expression in both the LV-miR-495 and LV-Sponge groups may be due to decreases in *Arc*-mediated AMPA receptor endocytosis.

CaMKII is involved in the insertion of GluR1 into NAcSh synapses and blocking this effect decreases cocaine-primed reinstatement (Anderson et al., 2008). We observed reductions in *Camk2a* expression and cocaine-primed reinstatement in the LV-miR-495 group, but not in the LV-Sponge group, consistent with the idea that *Camk2a* expression

may be involved in cocaine-primed reinstatement. This idea is further supported by our observation that cocaine SA on a PR schedule of cocaine reinforcement was attenuated in the LV-miR-495 group, but not in LV-Sponge group, consistent with previous work demonstrating that NAcSh *Camk2a* knockdown decreases cocaine SA on a PR schedule (Wang et al., 2010). While their roles may be more or less pronounced depending on the type of behavioral test (i.e., PR, extinction, reinstatement), both *Arc* and *Camk2a* may be working in concert to alter NAcSh activity to suppress motivation for cocaine across several domains of behavior. Future work is needed to directly test these hypotheses.

The role of *Bdnf* in our effects is less clear, as we did not observe a significant change in expression following either miR-495 OE or inhibition (Chapter 4, 5). Furthermore, previous work has primarily linked reductions in NAc *Bdnf* expression with decreases in cocaine CPP expression and cocaine SA on an FR5 schedule of reinforcement (Bahi et al., 2008; Graham et al., 2007), both of which were unaffected by NAcSh miR-495 OE. Therefore, the potential role of NAcSh *Bdnf* in our effects on motivation for cocaine is currently unknown.

Limitations of the present research

Our findings are exciting as they are the first to identify a miRNA involved in regulating motivation to seek cocaine. However, future work is needed to explore the generalizability of our effects, specifically across drug class, sex, and age. Our finding that NAcSh miR-495 OE specifically decreases motivation for cocaine, but not food, needs to be investigated for other drugs of abuse. Nearly every drug of abuse increases dopamine levels in the NAc (Hyman et al., 2006), but the mechanism for this effect differs across drug classes. Furthermore, ARGs can have opposing roles on drug abuse-

related behavior depending on the drug class. For example, manipulation of VTA BDNF signaling has opposite effects on cocaine and morphine abuse-related behavior (Graham et al., 2009; Koo et al., 2012; Lu, Dempsey, Liu, Bossert, & Shaham, 2004). Given that polydrug abuse is common, future work aimed at testing the effects of miR-495 on other drugs of abuse is warranted.

Sex differences in drug abuse research exist both in animal and human studies (Carroll & Anker, 2010). In animal studies, female rodents predominantly exhibit higher reinforcement and motivation for drugs across multiples phases of dependence (for review, see Carroll & Anker, 2010). Similarly, women tend to transition from initiation to treatment quicker and have greater psychological, physical, and work-related complications associated with drug use than men (Hernandez-Avila, Rounsaville, & Kranzler, 2004). Additionally, recent work has shown that the transcriptional profiles in the NAc differ considerably across male and female rodents following chronic variable stress (Hodes et al., 2015), a known predictor of drug abuse liability (Enoch, 2011). Given that all of the previous research on the role miRNAs and drug abuse-related behavior has been performed in adult males, research is needed to examine these effects in females.

Drug use is often initiated during adolescence (Breslau & Peterson, 1996; Kandel & Logan, 1984) and adolescent rodents are typically more sensitive to the rewarding and reinforcing effects of drug (O'Dell, 2009; Wong, Ford, Pagels, McCutcheon, & Marinelli, 2013). Neurobiological differences in the brain reward circuitry exist between adolescents and adults, including differences in basal dopamine levels (Matthews, Bondi, Torres, & Moghaddam, 2013; Stansfield & Kirstein, 2005; Walker et al., 2010),

dopamine innervation between midbrain and striatal regions (Haycock et al., 2003), and D1/D2 receptor expression (Meng, Ozawa, Itoh, & Takashima, 1999; Teicher, Andersen, & Hostetter, 1995). Therefore, drug-induced gene expression changes likely differ depending on the age of the organism. For instance, heroin seeking produces attenuated immediate early gene expression (i.e., Fos protein) in the mPFC of adolescents compared to adults (Doherty, Cooke, & Frantz, 2013). Given that drug abuse often begins in adolescence, the anti-motivational effects of NAc miR-495 need to be tested at this developmental stage when rodents and humans are particularly vulnerable to drug effects.

Future Directions

Effect of Environmental Enrichment and Abstinence on miRNA Expression

We have previously found that animals housed in enriched environments (EE; e.g., groups of 4-6 rats, novel toys, exercise, etc.) exhibit dramatically reduced cocaine-seeking behavior compared to animals in isolated housing conditions (Thiel et al., 2012; Thiel, Sanabria, Pentkowski, & Neisewander, 2009). Furthermore, EE reduces the incubation effect, where cocaine-seeking behavior increases as the withdrawal period increases (Grimm et al., 2003; Tran-Nguyen et al., 1998). Given that these manipulations produce vastly different levels of motivation for cocaine, miR-495 may underlie these changes. We are in the process of performing RNA-seq on brain tissue from animals trained to cocaine self-administration who were subsequently housed in isolation versus EE during a long versus short period of forced abstinence. This study replicated our previous findings that motivation to seek cocaine varies greatly across groups, providing a means to examine correlations between miRNA levels, including miR495, and motivation for cocaine. This study will provide information on the natural alterations in

miRNA expression that accompany changes in drug-seeking behavior and may point to novel miRNAs involved in drug motivation.

Competition between miR-495 and HuD

In addition to miRNAs, other molecules post-transcriptionally regulate gene expression. As mentioned previously, RNA-binding proteins (RPBs) can also bind to 3'UTRs of target mRNAs affecting their stability and expression levels. For instance, HuD, also known as ELAV4, is a neuron-specific RBP that binds to AU-rich elements on mRNAs and stabilizes target gene expression (Allen et al., 2013; Sosanya et al., 2015; Sosanya et al., 2013; Tsai, Cansino, Kohn, Neve, & Perrone-Bizzozero, 1997). Given that the seed region for miR-495 bindings sites is also AU-rich, HuD competes for control of ARG expression *in vitro,* where miR-495 decreases and HuD increases ARG expression (Gardiner, Oliver, & Perrone-Bizzozero, 2013). In contrast to miR-495, cocaine increases HuD expression in the NAc (Oliver, Gardiner, Brigman, Allan, & Perrone-Bizzozero, 2015). Furthermore, transgenic mice with HuD overexpression in forebrain neurons exhibit increases in cocaine CPP (Oliver et al., 2015). This suggests that HuD may stabilize target ARG expression and promote cocaine abuse-related behaviors. Examining the competition between miRNAs and RBPs in drug abuse-related behavior is a very exciting and relatively unexplored area in the field.

miRNAs as Biomarkers for Disease

Given their widespread role in gene regulation, miRNAs are attractive targets for treating complex neuropsychiatric disorders that are not caused by one single gene, but multiple interacting cellular processes (Goldman, Oroszi, & Ducci, 2005; Li et al., 2008). One exciting new field in the study of miRNAs and disease is using miRNA expression

profiles as biomarkers for susceptibility to neuropsychiatric disorders, including drug abuse. Small RNAs can be secreted from the tissue of origin and circulate in peripheral body fluids, such as plasma or cerebrospinal fluid (Redis, Calin, Yang, You, & Calin, 2012). Circulating miRNAs can travel in exosomes and/or bind to and travel with high-density lipoproteins, which increase their stability (Kosaka et al., 2010; Vickers, Palmisano, Shoucri, Shamburek, & Remaley, 2011). One challenge in using miRNAs as biomarkers is relating the changes in circulating miRNA levels with those occurring in the brain, although some evidence suggests these can be related (Drusco et al., 2015; Liu et al., 2010). Research has already begun to examine circulating miRNAs in the context of drug abuse (Beech et al., 2014; Gardiner et al., 2015). Although this field is in its relative infancy, circulating miRNAs have exciting potential for use as biomarkers that can characterize disease progression and severity, as well as point to novel and perhaps individualized treatments.

Conclusion

This dissertation supports the hypothesis that NAc miR-495 regulates motivation for cocaine through targeting several ARGs. Potential mechanisms underlying these effects include altering the synaptic plasticity and activity of NAcSh neurocircuitry through targeting ARG expression, thereby reducing the motivation to seek cocaine. Future lines of work will explore: 1) the competition between miRNAs and other post-transcriptional regulators of gene expression, 2) the influence of other mediating factors in drug abuse on miRNA expression (e.g, drug class, sex, age, social environment, etc.), and 3) the diagnostic value of miRNAs as biomarkers for addiction-like phenotypes. The findings from this dissertation point to an exciting new avenue in understanding the

influence of microRNAs on the development, expression, and possible treatment for SUDs.

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APPENDIX A

TABLES

Down <0.75 and TS	***Adrbk2, *Arc, Arhgap5, Atp2b2, Bcl11a, *Bdnf, Cacna1d, *Camk2a, Camk2g, Clmn, Cnr1, Cpeb2, Cpne4, Dcaf6, Dlgap2, Dmd, Dpp10, Drp2, Dyrk2, Ephb2, Fat4, Gabbr2, Gpr22, Gpr3, *Gria3, Hivep2, Kctd16, Lancl2, Large, Lhx2, Limch1, Lrrc57, Mapk10, Mast4, Mcf2l, Nacc2, Nat8l, Ncam2, Neurod6, Nfib, Nmt2, Nptxr, Nr4a3, Nrxn1, Ntm, Pcdh9, Pde4d, Per2, Pgr, Pitpnm3, Pou6f1, Ppargc1a, Ppme1, Prickle1, Psme3, Ptprd, Pura, R3hdm2, Rap1gap2, Rimbp2, Rims4, Satb1, Satb2, Scn8a, Shank2, Slit1, Smad9, Sox5, *Stmn2, Tcf4, Tgfb2, Tmeff2, Tox, Usp32, Xylt1, Zfhx4***
Down <0.75, TS targets, KARG	***Arc, *Bdnf, Cacna1d, *Camk2a, Cnr1, Gabbr2, Mapk10, Nacc2, Nfib, Nr4a3, Per2, Ppargc1a, Ppme1, Pura, *Stmn2***

Table 1. Microarray Results of Putative miR-495 Targets Downregulated by miR-495 OE. Rats were infused with either LV-GFP or LV-miR-495 and NAc tissue was collected one week later. Affymetrix® Rat Gene 2.0 ST arrays (Santa Clara, CA, USA) were used to determine changes in gene expression (n = 3/ group). Data was normalized using Robust multi-array average (RMA) and significant changes in expression were determined with MeV4_9_0 software (Boston, MA, USA) using a *t*-test with Bonferroni corrections and permutation validations. (Top) Candidate miR-495 target mRNAs found within TargetScan that were significantly downregulated (fold change ≤0.75 and $p<0.05$) by miR-495 OE in NAcSh. (Bottom) Downregulated miR-495 target mRNAs also found within the KARG database. * downregulation confirmed by qRT-PCR.

APPENDIX B

FIGURES

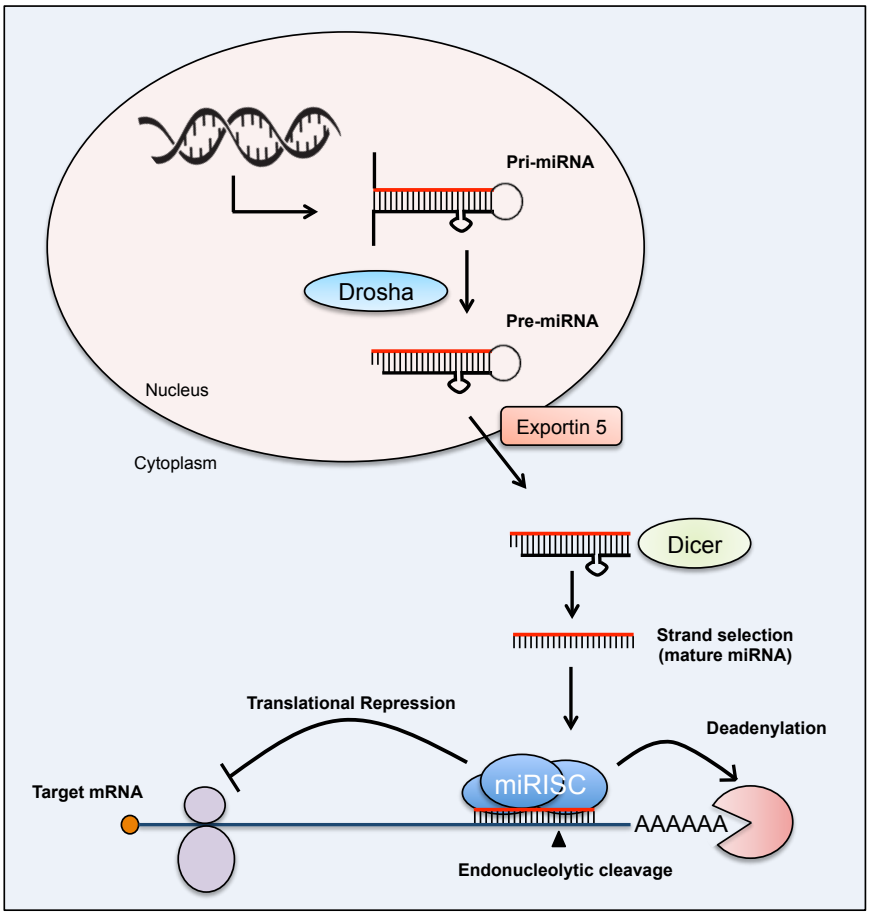

Figure 1. MicroRNA Biogenesis and Function. MicroRNAs are transcribed similarly to protein-coding RNAs, except they form a stem-loop structure following transcription (i.e, pri-miRNA). The enzyme, Drosha, trims the ends of the stem (i.e., pre-miRNA) to prepare for exportation from the nucleus via Exportin 5. Once in the cytoplasm, Dicer cleaves the loop of the pre-miRNA, producing a double-stranded RNA. One strand (i.e., mature miRNA) is incorporated into the miRNA-induced silencing complex (miRISC). Upon binding to a complementary sequence in the 3' untranslated region of a target mRNA, either translational repression, deadenylation, or endonucleolytic cleavage may occur. All three mechanisms lead to decreases in resulting protein.

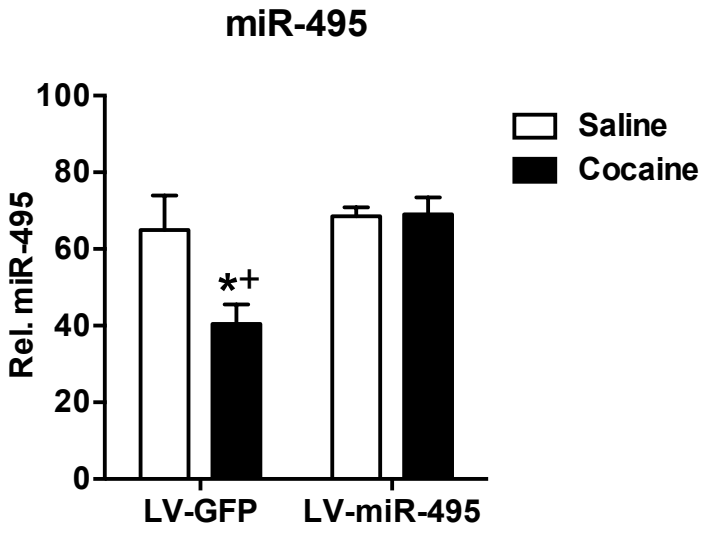

Figure 2. NAc Shell miR-495 Overexpression Blocked Cocaine-Induced Downregulation of NAc miR-495. Rats were infused with either LV-GFP or LV-miR-495 into the NAcSh. Two weeks later, rats received an injection of saline (1 mL/kg, i.p.) or cocaine (15 mg/kg, i.p.) and were sacrificed 2 h later (n = 5-6/group). NAc tissue was collected and processed for qRT-PCR using Taqman® primers for miR-495 or U6 snRNA (control). Rel. miR-495 levels were normalized to U6. Error bars indicate S.E.M. * different from LV-GFP-saline, $^{+}$ different from LV-miR-495-cocaine, *p*s < 0.05.

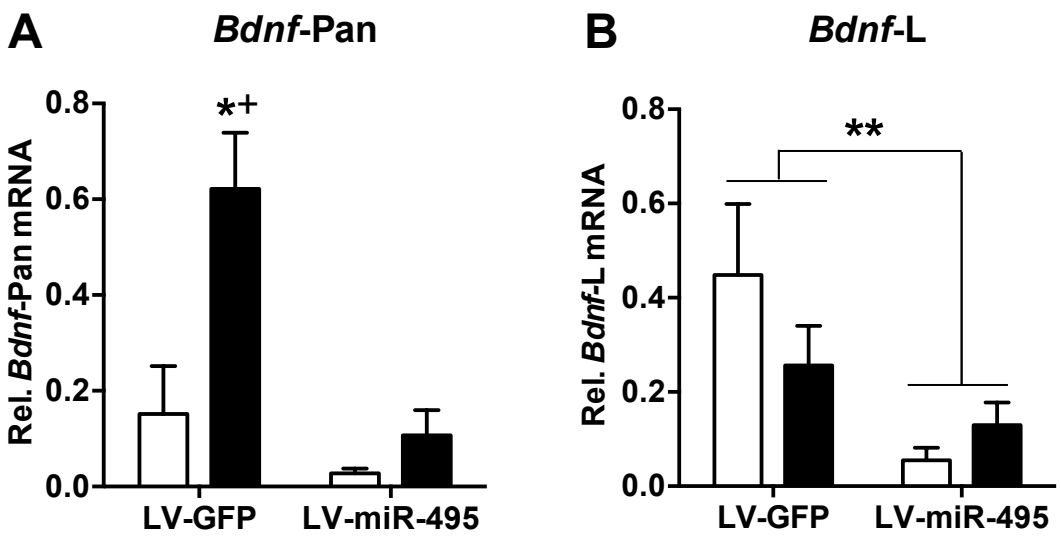

Figure 3. NAc Shell miR-495 Overexpression Reduced NAc *Bdnf* mRNA. Rats were infused with either LV-GFP or LV-miR-495 into the NAcSh. Two weeks later, rats received an injection of saline (1 mL/kg, i.p.) or cocaine (15 mg/kg, i.p.) and were sacrificed 2 h later (n = 5-6/group). NAc tissue was collected and processed for qRT-PCR using primers for *Bdnf*-Pan (A), *Bdnf*-L (B) or *Gapdh* mRNA (control). Rel. *Bdnf* mRNA levels were normalized to GAPDH. Error bars indicate S.E.M. * different from LV-GFP-saline, $^{+}$ different from LV-miR-495-cocaine, *p*s < 0.05, ** $p < 0.01$.

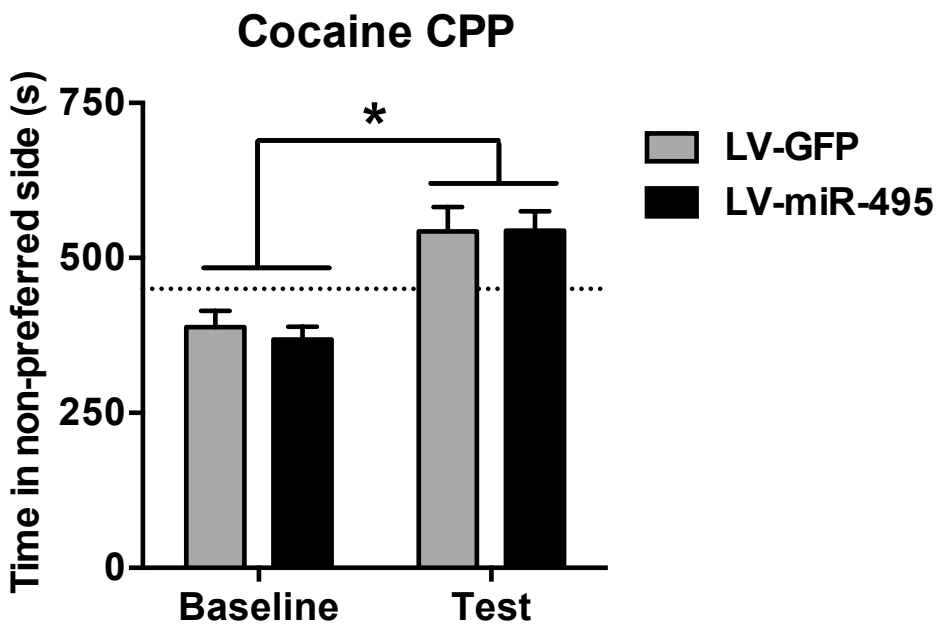

Figure 4. NAc Shell miR-495 Overexpression Had No Effect on Cocaine CPP Expression. Rats were infused with either LV-GFP (n =8) or LV-miR-495 (n = 9) into the NAcSh. One week later, rats were conditioned with cocaine (15 mg/kg, i.p.) on the initially non-preferred side that was established during baseline. Saline was conditioned on the initially preferred side. All rats received 4 conditioning sessions that alternated between cocaine and saline (a total of 2 cocaine and 2 saline conditioning sessions). Each 30-min conditioning session was given once/day. The day following the last conditioning session, the rats were place in the CPP apparatus and allowed access to both sides for 15 min. Dashed line represents 50% of total session length. Error bars indicate S.E.M. * main effect of day, $p < 0.05$.

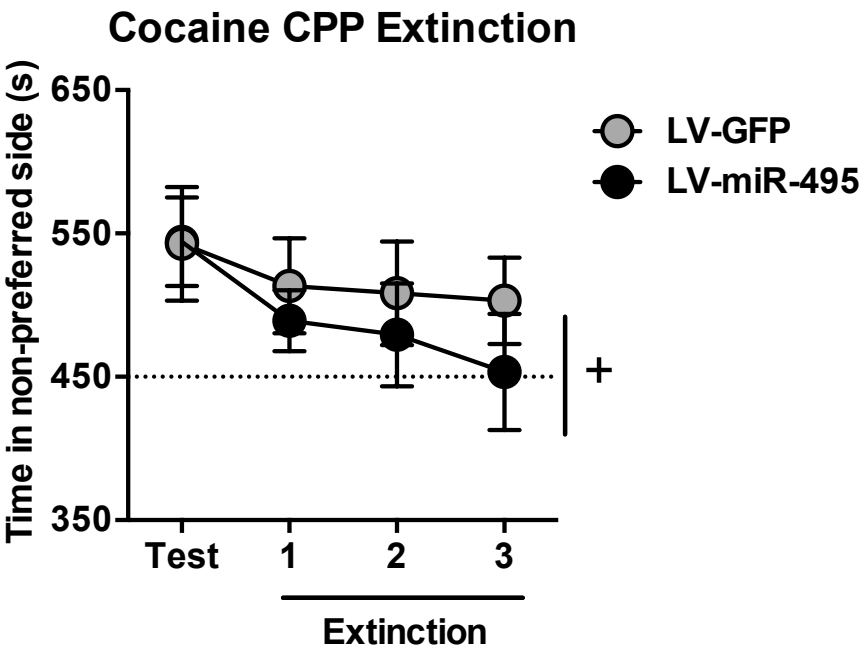

Figure 5. NAc Shell miR-495 Overexpression Facilitated Cocaine CPP Extinction. Following test day, rats were placed back in the CPP apparatus for 15-min extinction sessions where they were allowed free access between both sides of the chamber (n = 8-9/group). Dashed line represents 50% of total session length. Error bars indicate S.E.M. $^{+}$ Test day vs. Ext. 3 for the LV-miR-495 group, $p < 0.025$, Bonferroni correction.

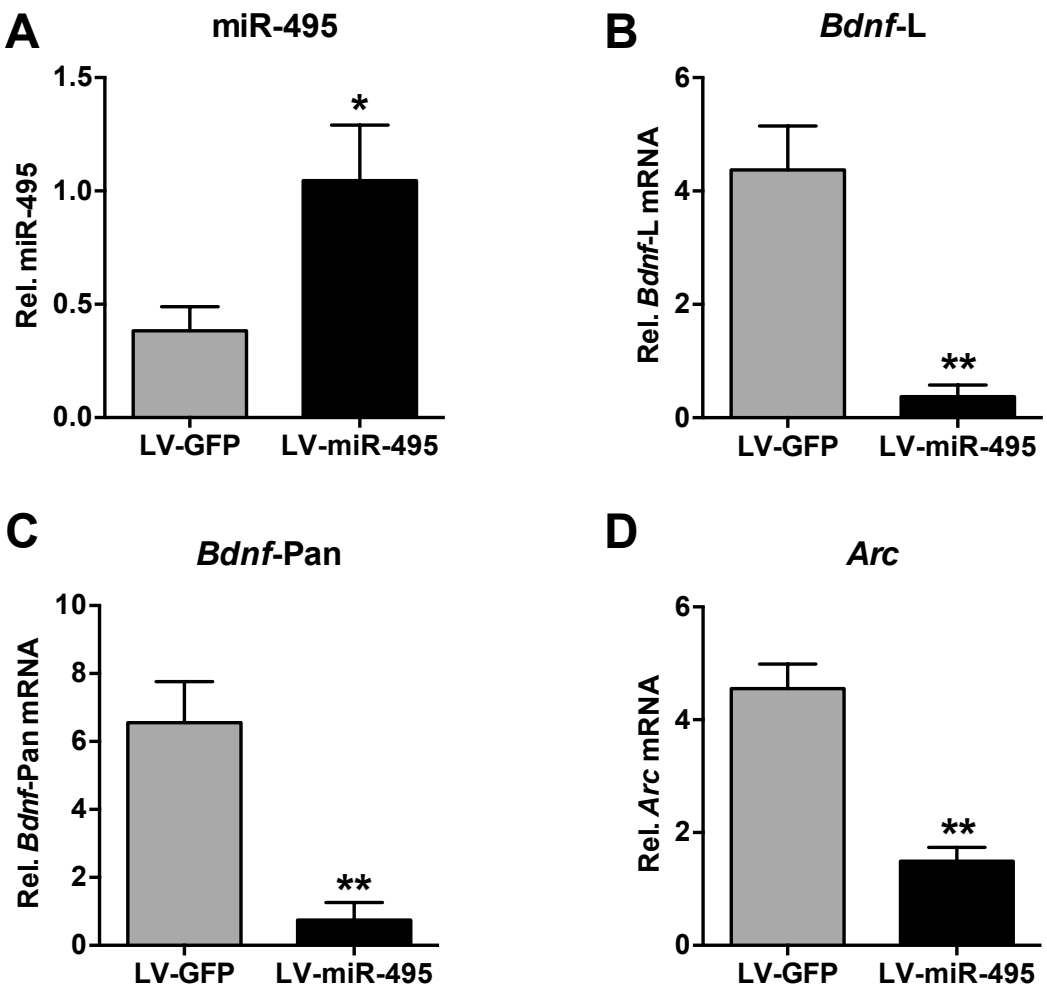

Figure 6. NAc Shell miR-495 Overexpression Increased NAc miR-495 and Decreased NAc *Bdnf* mRNA Expression. One day following the last extinction session, rats were sacrificed and NAc tissue was collected and processed for qRT-PCR using Taqman® primers for miR-495 (A) or U6 snRNA (control) and primers for *Bdnf*-L (B), *Bdnf*-Pan (C) or GAPDH mRNA (control). Relative miR-495 levels were normalized to U6 and relative *Bdnf* mRNA levels were normalized to GAPDH. n = 8-9/group. Error bars indicate S.E.M. * different from LV-GFP group, $p < 0.05$, ** $p < 0.01$.

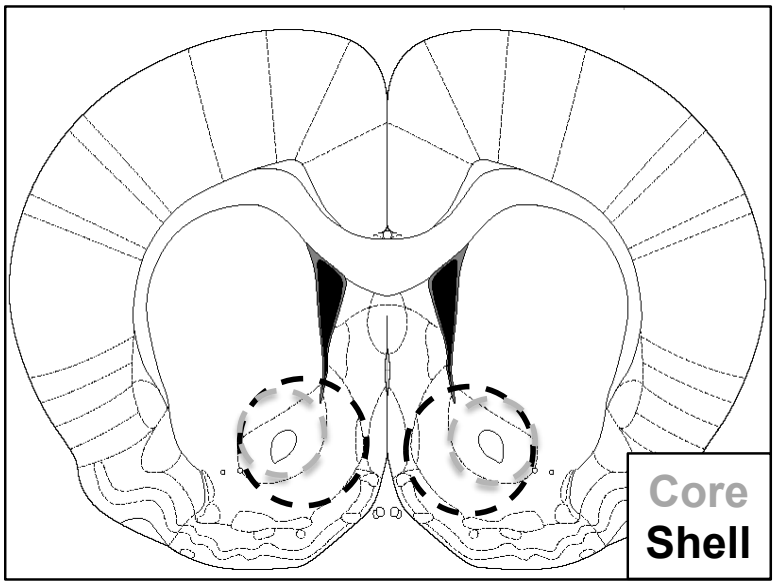

Figure 7. Tissue Punch Method for NAc Core and Shell Collection. Frozen brains were placed in a rat brain matrix, sliced into 2 mm thick sections using razor blades, and punches were taken that corresponded to the landmarks near the NAc (e.g., anterior commissure). First, the core (gray dashed circle) was collected with a 1.25 mm tissue punch, and then the shell (black dashed circle) was collected with a 2 mm tissue punch.

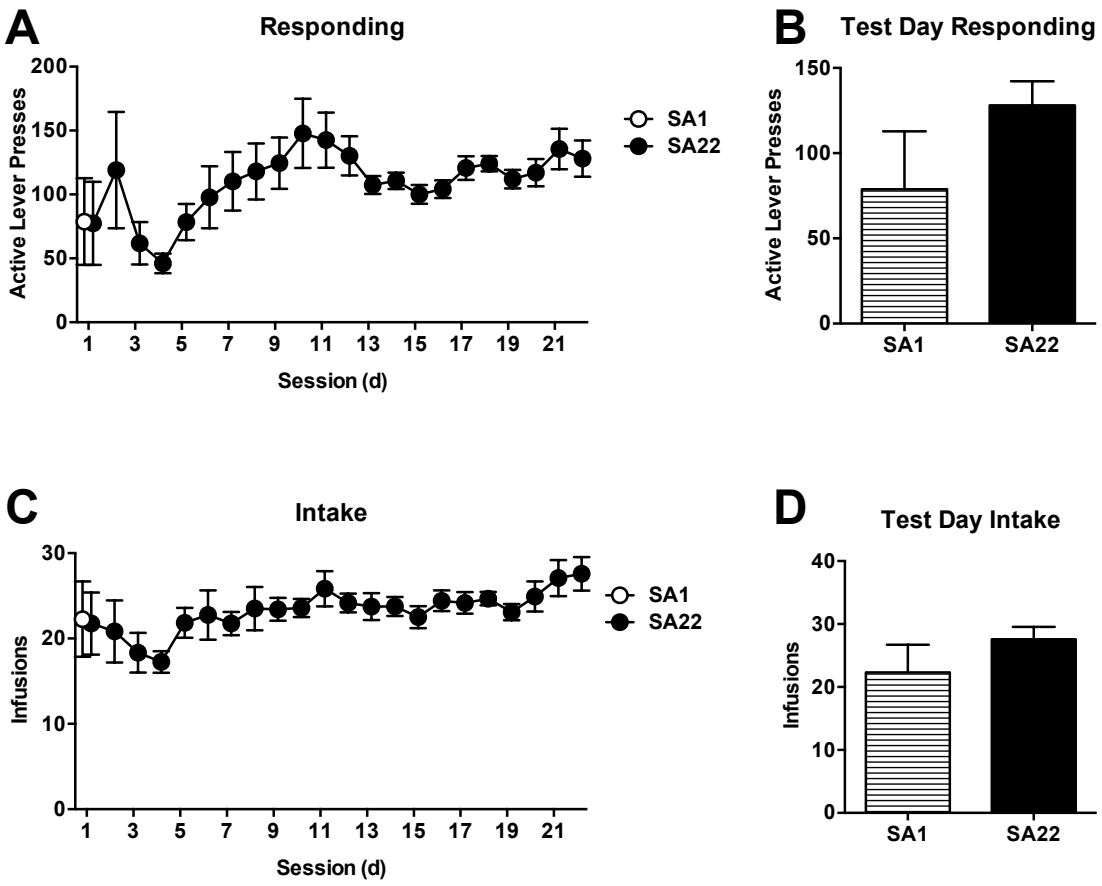

Figure 8. Self-Administration Behavior Across Different Levels of Training. Rats were randomly assigned to receive either 1 (SA1; n = 11) or 22 d (SA22; n = 12) of cocaine SA training (0.9375 mg/kg/infusion, i.v.). Test day refers to the day that the respective groups were sacrificed where the SA1 group was sacrificed 1 h following the first session, and the SA22 group was sacrificed 1 h following the 22nd session (A, C). There was no difference between the two groups in the total number of active lever presses (B) or number of cocaine infusions received (D). Error bars indicate S.E.M.

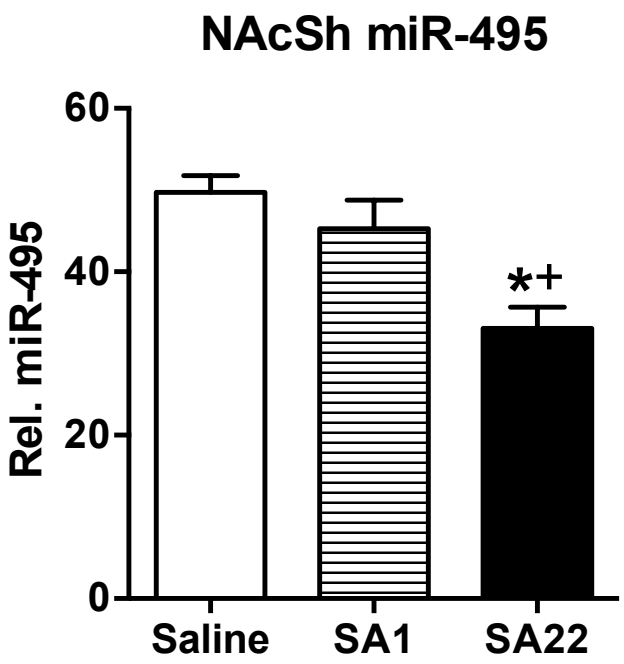

Figure 9. Prolonged, but Not Brief, Cocaine Self-Administration Decreases NAc Shell miR-495 Expression. Rats received either 1 or 22 d of cocaine SA (0.9375 mg/kg, i.v.; n = 11-12/group). Controls received saline infusions yoked to a cocaine partner (n = 11). One hour following the last SA session, rats were sacrificed and NAcSh tissue was collected and processed for qRT-PCR using Taqman® primers for miR-495 or U6 snRNA (control). Rel. miR-495 levels were normalized to U6. Error bars indicate S.E.M. * different from Saline, $^{+}$ different from SA1 group, Tukey *p*s < 0.05.

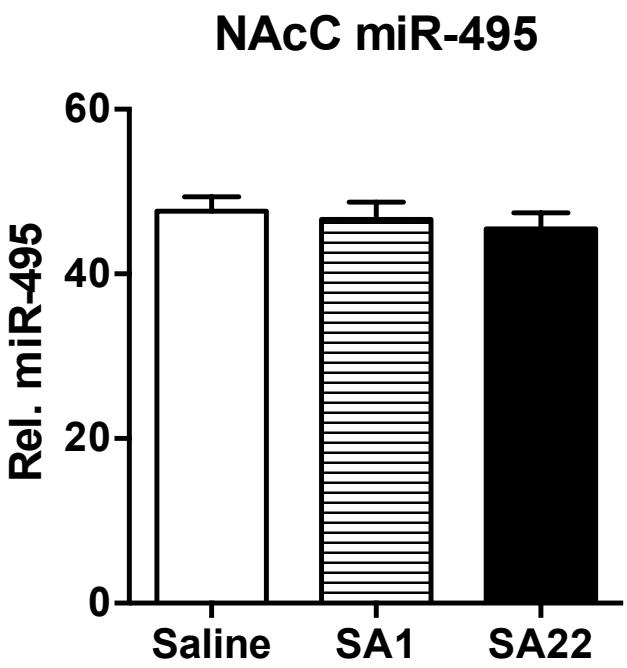

Figure 10. Cocaine Self-Administration Had No Effect on NAc Core miR-495 Expression. Rats received either 1 or 22 d of cocaine SA (0.9375 mg/kg, i.v.; n = 11-12/group). Controls received saline infusions yoked to a cocaine partner (n = 11). One hour following the last SA session, rats were sacrificed and NAcC tissue was collected and processed for qRT-PCR using Taqman® primers for miR-495 or U6 snRNA (control). Rel. miR-495 levels were normalized to U6. Error bars indicate S.E.M.

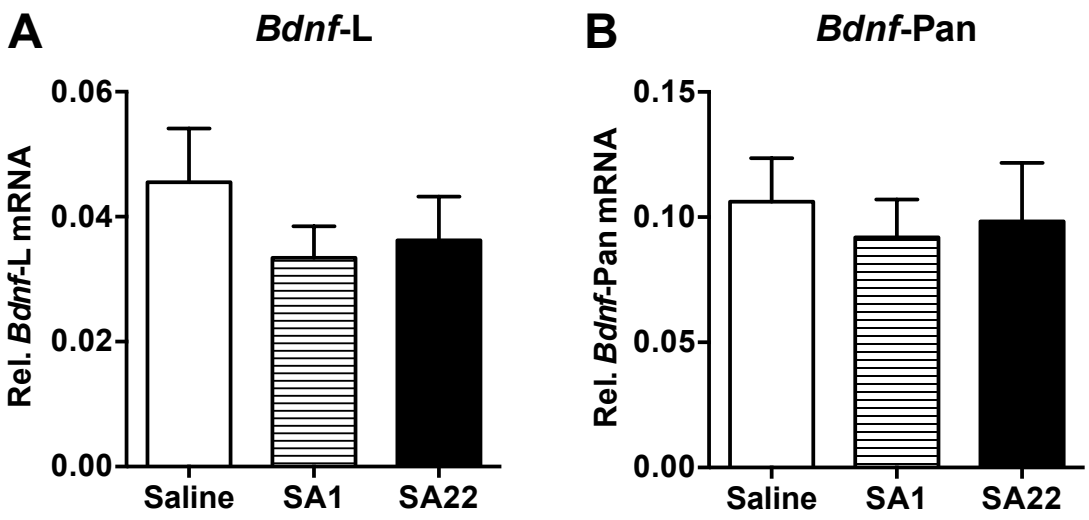

Figure 11. Cocaine Self-Administration Had No Effect on NAc Shell *Bdnf* mRNA Expression. Rats received either 1 or 22 d of cocaine SA (0.9375 mg/kg, i.v.; n = 11-12/group). Controls received saline infusions yoked to a cocaine partner (n = 11). One hour following the last SA session, rats were sacrificed and NAcSh tissue was collected and processed for qRT-PCR using primers for *Bdnf*-L (A), *Bdnf*-Pan (B) or *Gapdh* mRNA (control). Rel. *Bdnf* mRNA levels were normalized to *Gapdh*. Error bars indicate S.E.M.

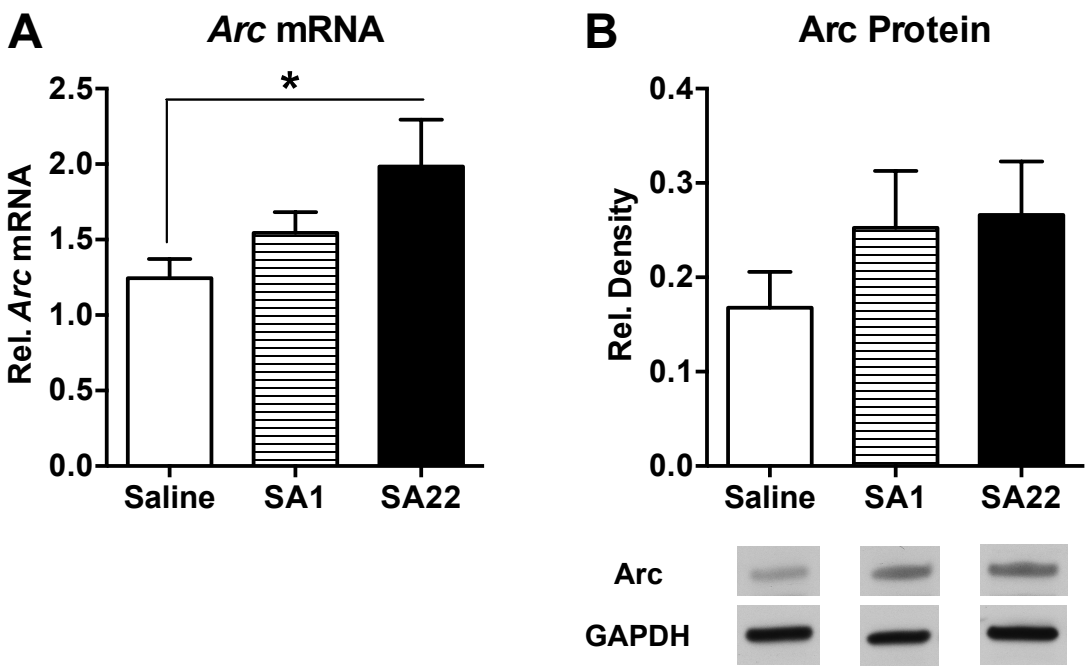

Figure 12. Prolonged, but Not Brief, Cocaine Self-Administration Increased NAc Shell *Arc* mRNA. Rats received either 1 or 22 d of cocaine SA (0.9375 mg/kg, i.v.; n = 11-12/group). Controls received saline infusions yoked to a cocaine partner (n = 11). One hour following the last SA session, rats were sacrificed and NAcSh tissue was collected and processed for qRT-PCR using primers for *Arc* (A) or *Gapdh* mRNA (control). Rel. *Bdnf* mRNA levels were normalized to *Gapdh*. (B) Arc protein was quantified using Western blot and normalized to GAPDH protein. Representative blots are depicted for each group. Error bars indicate S.E.M. * Bonferroni correction, $p \leq 0.025$.

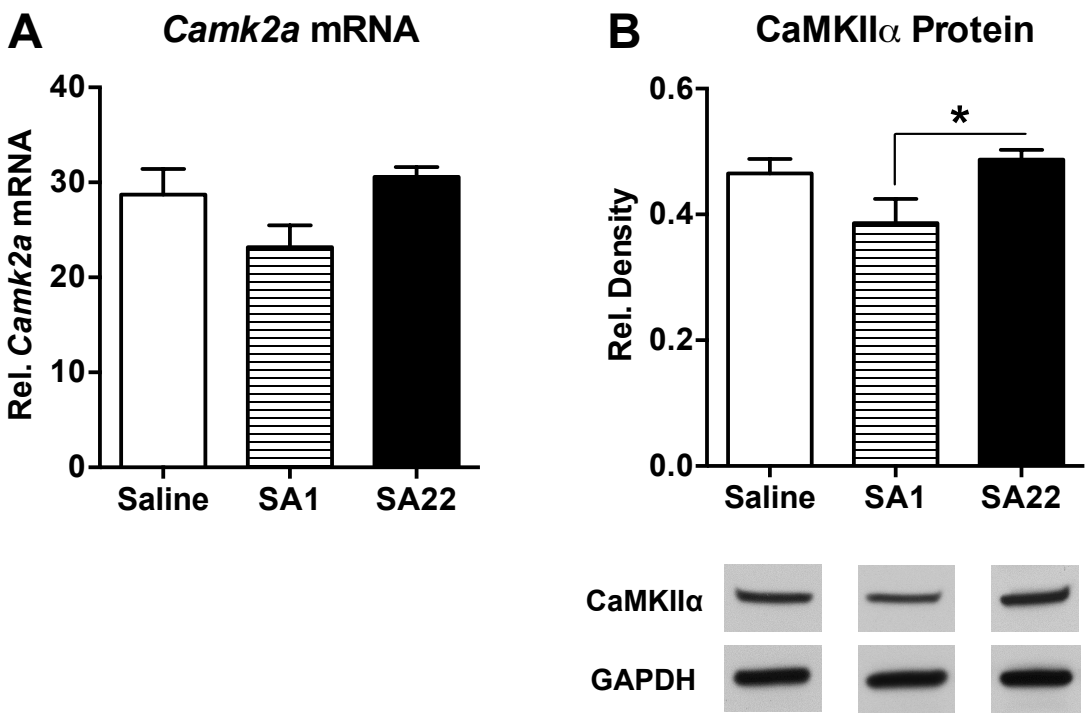

Figure 13. Effect of Cocaine Self-Administration on NAc Shell *Camk2a* Expression. Rats received either 1 or 22 d of cocaine SA (0.9375 mg/kg, i.v.; n = 11-12/group). Controls received saline infusions yoked to a cocaine partner (n = 11). One hour following the last SA session, rats were sacrificed and NAcsh tissue was collected and processed for qRT-PCR using primers for *Camk2a* (A) or *Gapdh* mRNA (control). Rel. *Camk2a* mRNA levels were normalized to *Gapdh*. (B) CaMKIIα protein was quantified using Western blot and normalized to GAPDH protein. Representative blots are depicted for each group. Error bars indicate S.E.M. * Tukey, $p < 0.05$.

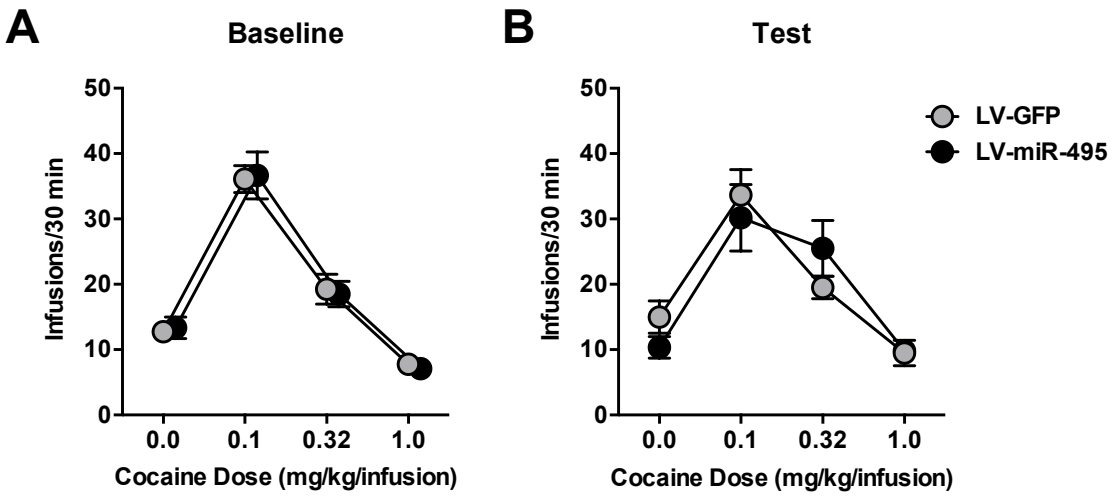

Figure 14. NAc Shell miR-495 Overexpression Slightly Shifted the Within-Session FR5 Dose-Response Curve to the Right. Rats were initially trained on within-session dose-response sessions on an FR5 schedule where each dose (0.0, 0.1, 0.32. and 1.0 mg/kg/infusion) was presented for 30 min each in an ascending order. Once stability was achieved (A), rats were infused with either LV-GFP or LV-miR-495 (n = 8-9/group). (B) Four days later, rats were tested on the within-session dose-response for one session. Error bars indicate S.E.M.

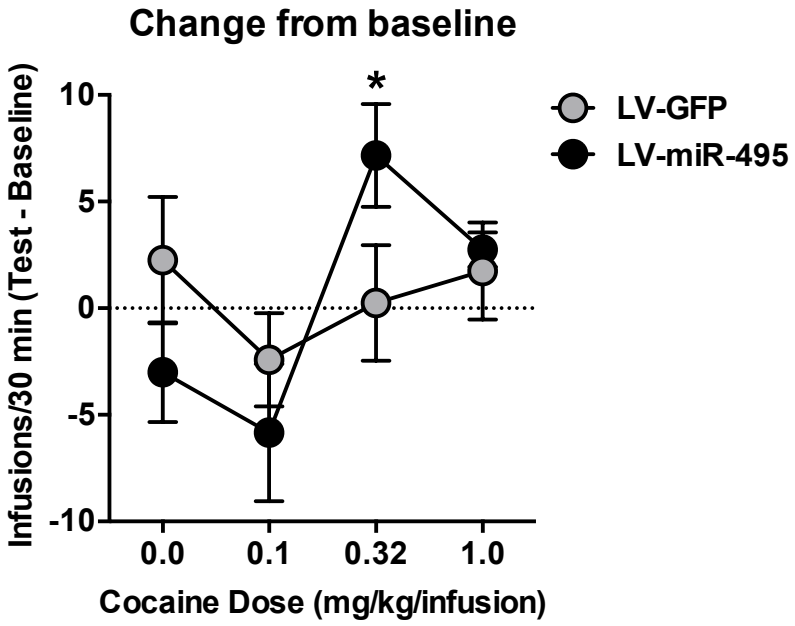

Figure 15. Change from Baseline During the Within-Session FR5 Dose-Response Curve. Rats were initially trained on within-session dose-response sessions on an FR5 schedule where each dose (0.0, 0.1, 0.32. and 1.0 mg/kg/infusion) was presented for 30 min each in an ascending order. Once stability was achieved, rats were infused with either LV-GFP or LV-miR-495 (n = 8-9/group). Four days later, rats were tested on the within-session dose-response for one session. Error bars indicate S.E.M. * different from LV-GFP, $p < 0.05$.

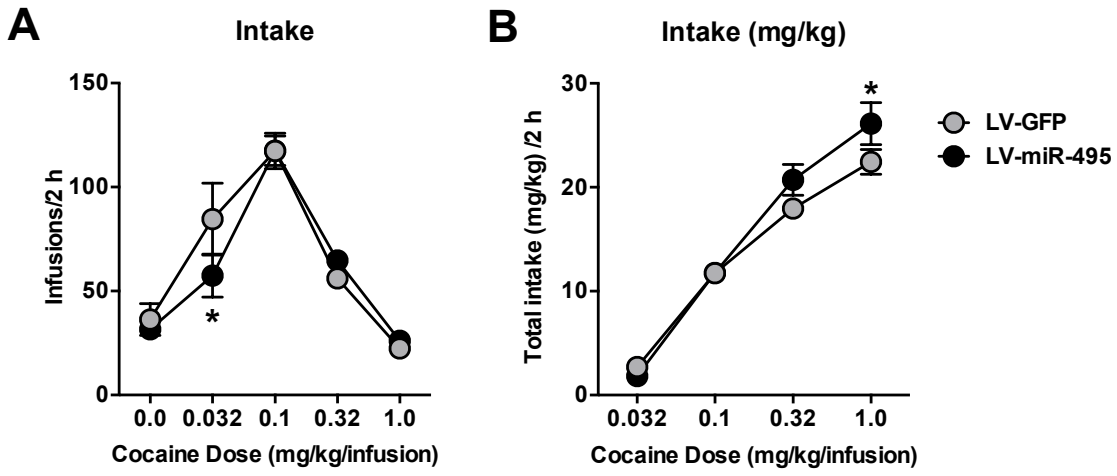

Figure 16. NAc Shell miR-495 Overexpression Slightly Shifted the Between-Session FR5 Dose-Response Curve to the Right. One week following virus infusions (n = 8-9/group), rats received cocaine doses (0.0, 0.032, 0.1, 0.032, 1.0 mg/kg/infusion) during 2-h sessions in an ascending order where both total infusions (A) and intake (B; mg/kg) were measured. Error bars indicate S.E.M. * different from LV-GFP, $p < 0.05$.

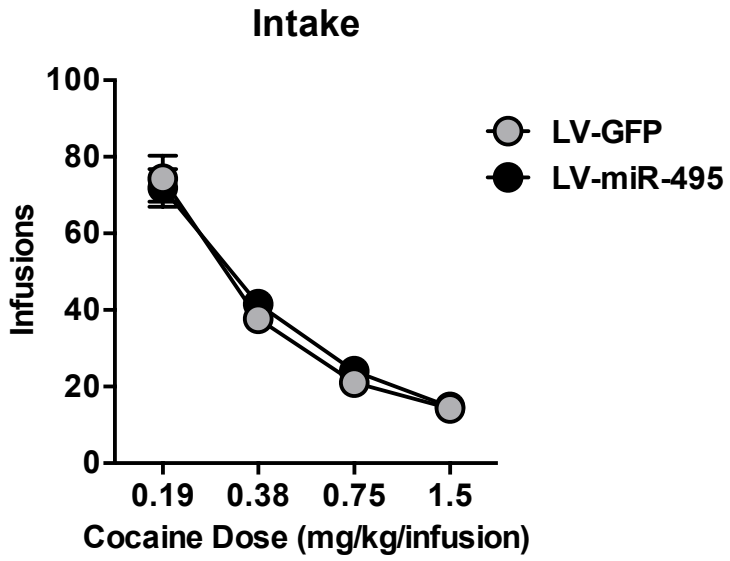

Figure 17. NAc Shell miR-495 Overexpression Had No Effect on a Between-Session FR5 Dose-Response Curve. Two weeks following virus infusions (n = 10-12/group), rats received cocaine doses on an FR5 schedule of reinforcement during 2-h sessions in a pseudorandom order (0.75, 0.38, 1.5, 0.19 mg/kg/infusion). Infusions were averaged across 3 sessions once rats achieved stability (< 15% variability). Error bars indicate S.E.M.

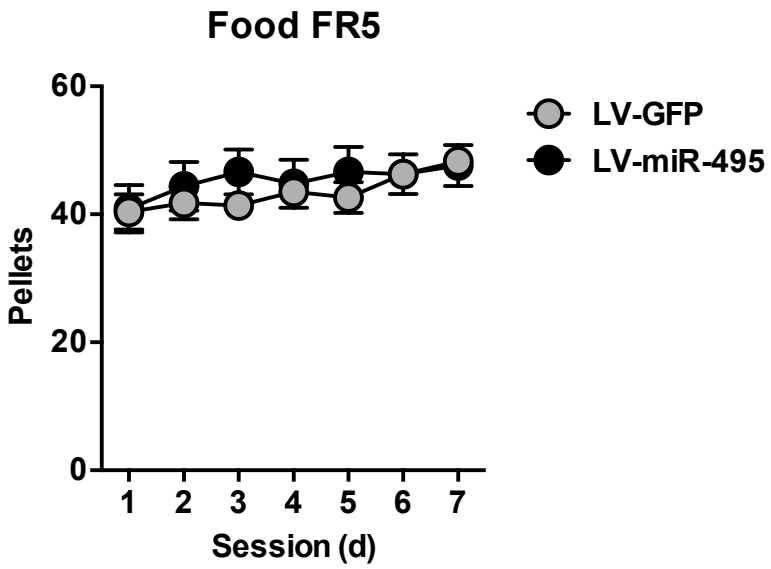

Figure 18. NAc Shell miR-495 Overexpression Had No Effect on Food Intake on an FR5 Schedule. One weeks following virus infusions (n = 8/group), rats received food reinforcement on an FR5 schedule of reinforcement during 30-min sessions for 7 sessions. Error bars indicate S.E.M.

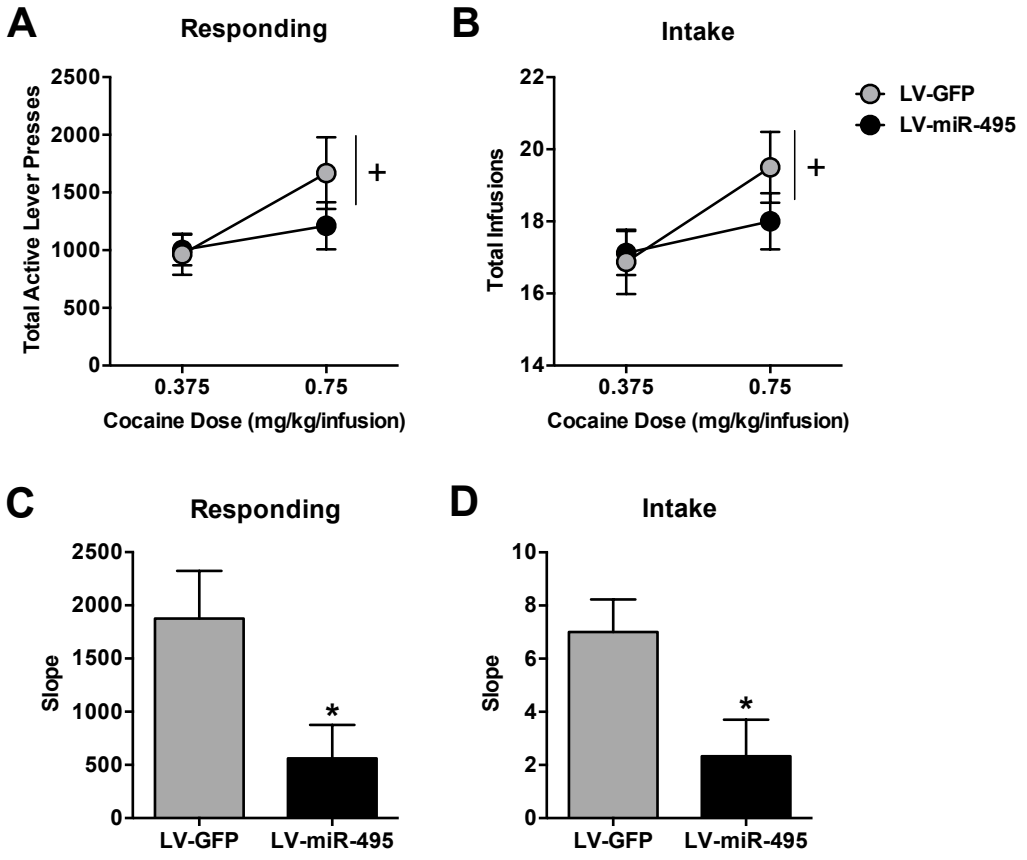

Figure 19. NAc Shell miR-495 Overexpression Reduced PR Measures. Rats (n = 8-9/group) received cocaine doses (0.375, 0.75 mg/kg/infusion) on a PR schedule of reinforcement in an ascending order where both total active lever presses (A) and intake (B) were measured. Dose-effect curve slopes $[(Y_2-Y_1)/(0.75-0.375)]$ were measured for active lever presses (C) and intake (D). Error bars indicate S.E.M. $^{+}$ different from the 0.375 dose in LV-GFP group, $p < 0.05$. * different from LV-GFP, $p < 0.05$.

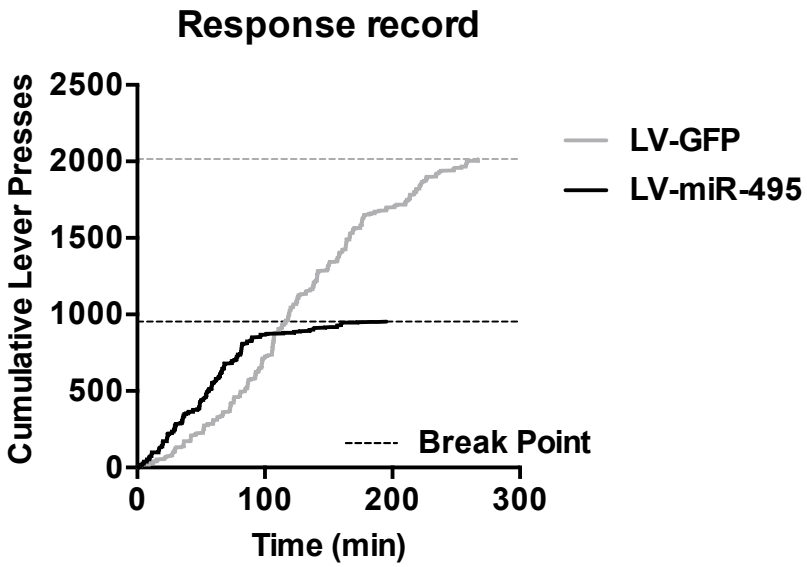

Figure 20. NAc Shell miR-495 Overexpression Reduced Break Points on a PR Schedule. Cumulative response record for representative rats in the LV-GFP and LV-miR-495 groups that were closest to their respective group mean at the 0.75 mg/kg/infusion dose on a PR schedule of cocaine reinforcement. Dotted line represents break point, where rats went 1 h without receiving a cocaine infusion.

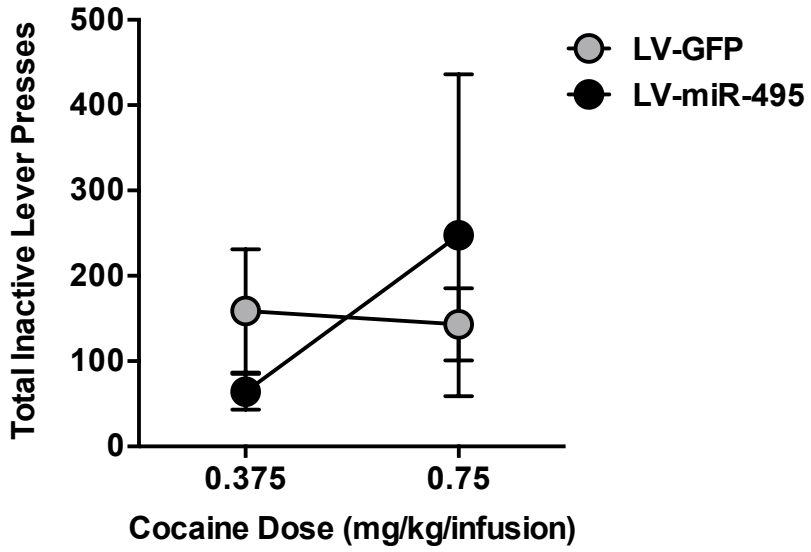

Figure 21. NAc Shell miR-495 Overexpression Had No Effect on Inactive Lever Presses on a PR Schedule. Rats (n = 8-9/group) received cocaine doses (0.375, 0.75 mg/kg/infusion) on a PR schedule of reinforcement in an ascending order. Error bars indicate S.E.M.

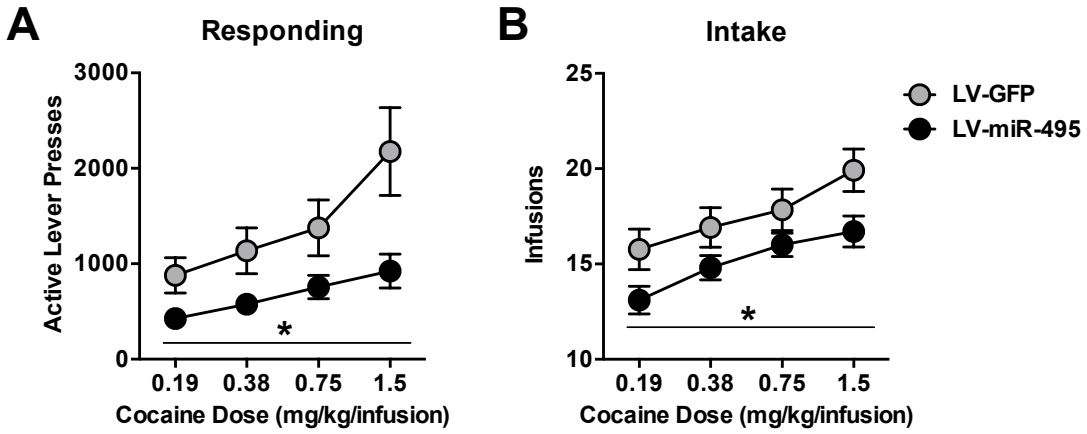

Figure 22. NAc Shell miR-495 Overexpression Reduced Responding and Intake on a PR Schedule. Rats (n = 10-12/group) received cocaine doses on a PR schedule of reinforcement in a pseudorandom order (0.75, 0.38, 1.5, 0.19 mg/kg/infusion) where both total active lever presses (A) and intake (B) were measured. Error bars indicate S.E.M. * different from LV-GFP group, $p < 0.05$.

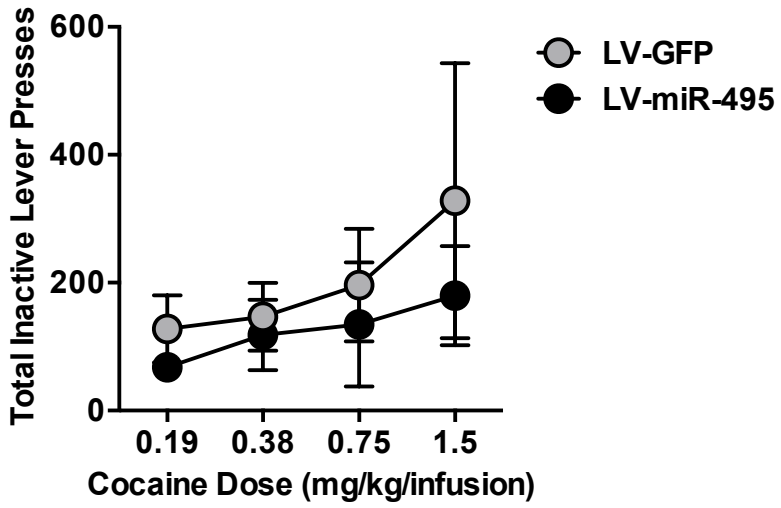

Figure 23. NAc Shell miR-495 Overexpression Had No Effect on Inactive Lever Presses on a PR Schedule. Rats (n = 10-12/group) received cocaine doses on a PR schedule of reinforcement in a pseudorandom order (0.75, 0.38, 1.5, 0.19 mg/kg/infusion). Error bars indicate S.E.M.

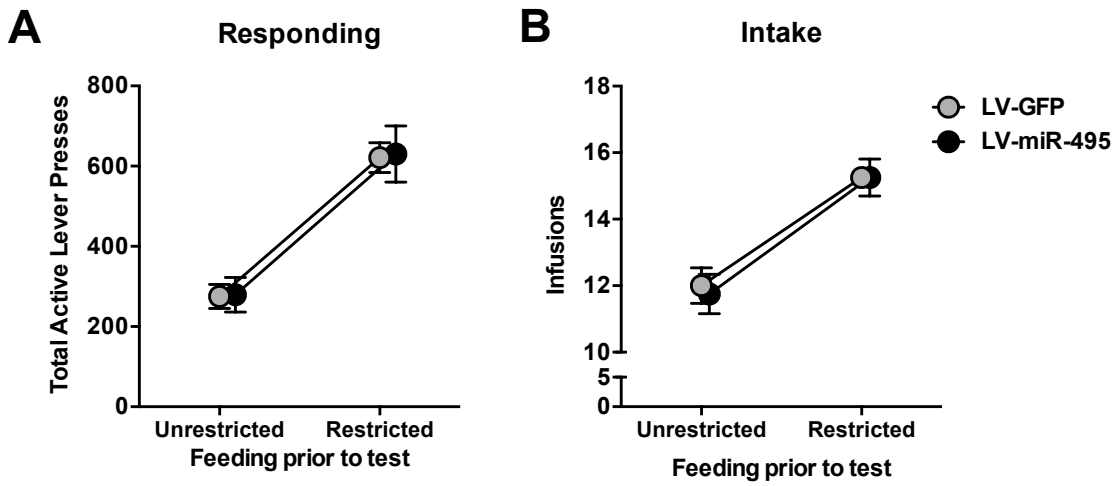

Figure 24. NAc Shell miR-495 Overexpression Had No Effect on Responding or Intake on a PR Schedule of Food Reinforcement. Total responding (A) and infusions (B) for food reinforcement were measured on a PR schedule. Rats (n = 8/group) were initially tested on restricted feeding conditions (18g/day) and then tested one week later on unrestricted feeding conditions. Error bars indicate S.E.M.

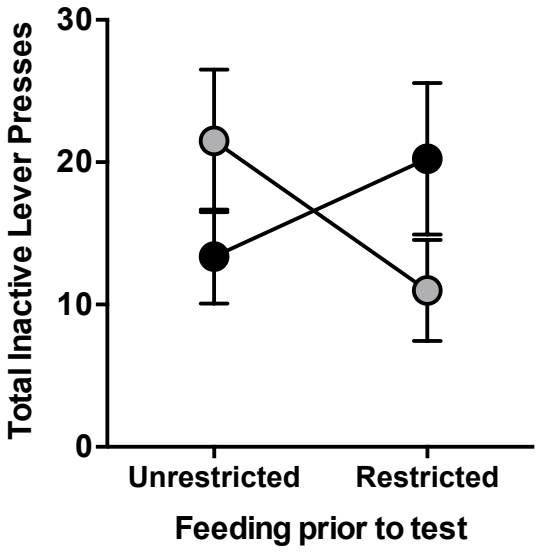

Figure 25. NAc Shell miR-495 Overexpression Had No Effect on Inactive Lever Presses on a PR Schedule of Food Reinforcement. Rats (n = 8/group) were initially tested on restricted feeding conditions (18g/day) and then tested one week later on unrestricted feeding conditions. Error bars indicate S.E.M.

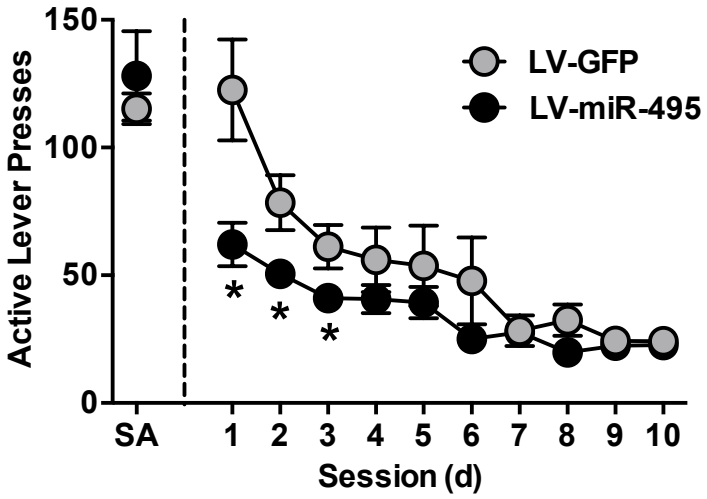

Figure 26. NAc Shell miR-495 Overexpression Reduced Responding During Extinction. Rats (n = 10-12/group) were initially given >3 sessions on an FR5 schedule of cocaine self-administration (SA) to establish an SA baseline. During the 1-h daily extinction sessions, lever presses produced no consequences. Error bars indicate S.E.M. * different from LV-GFP, $p < 0.05$.

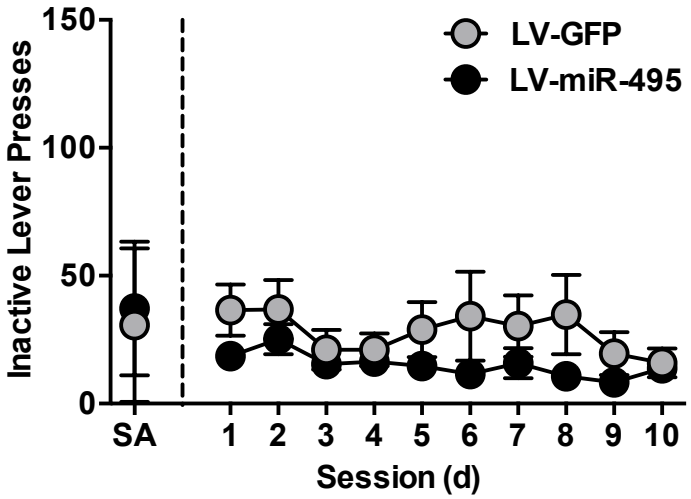

Figure 27. NAc Shell miR-495 Overexpression Had No Effect on Inactive Lever Pressing During Extinction. Rats (n = 10-12/group) were initially given >3 sessions on an FR5 schedule of cocaine self-administration (SA) to establish an SA baseline. During the 1-h daily extinction sessions, lever presses produced no consequences. Error bars indicate S.E.M.

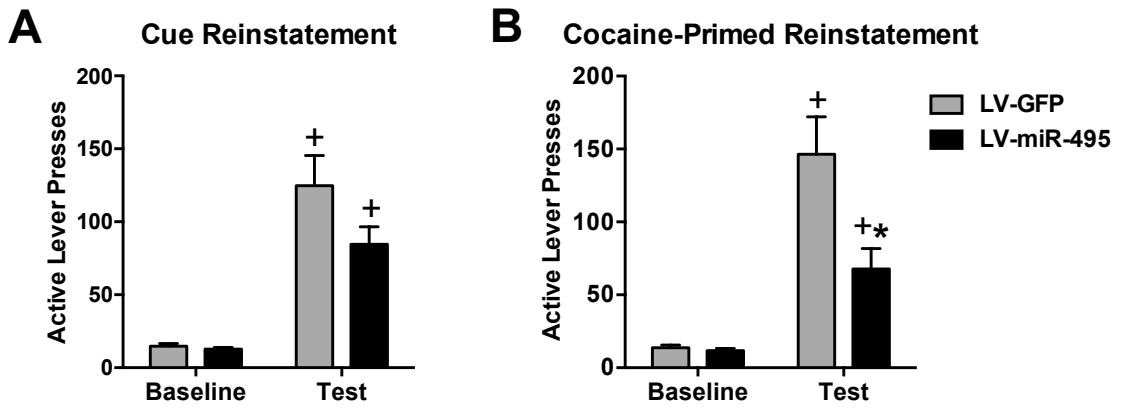

Figure 28. NAc Shell miR-495 Overexpression Reduced Cocaine-Primed Reinstatement. (A) Following >3 sessions with low extinction responding (i.e., Baseline), rats (n = 10-12/group) underwent cue reinstatement where cues that were previously presented response-contingently during self-administration we available on an FR1 schedule for 1 h. (B) After re-establishing an extinction baseline, rats (n = 10-12/group) received a priming injection of cocaine (10 mg/kg, i.p.) and were immediately place in the operant chamber and were allowed to lever press under extinction conditions for 1 h. Error bars indicate S.E.M. $^{+}$ different from Baseline, $p < 0.05$. * different from LV-GFP, $p < 0.05$.

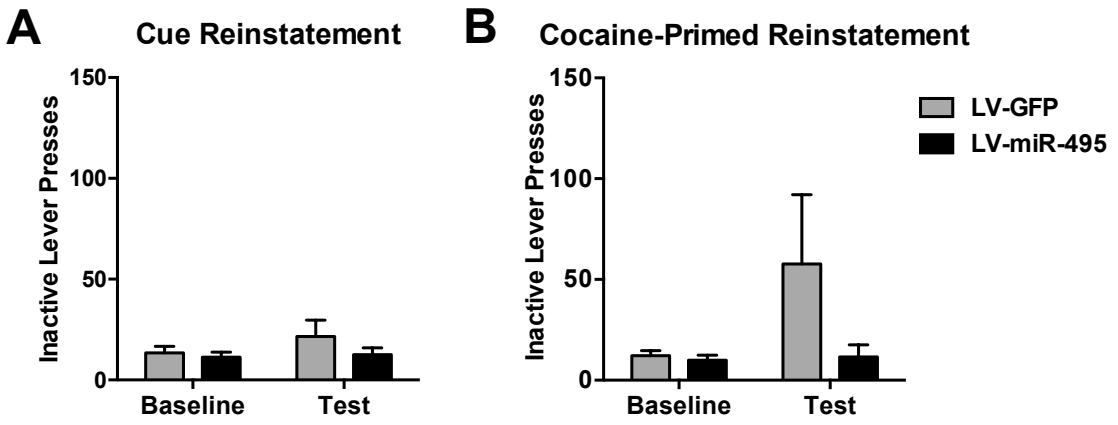

Figure 29. NAc Shell miR-495 Overexpression Had No Effect on Inactive Lever Pressing During Reinstatement. (A) Following >3 sessions with low extinction responding (Baseline), rats (n = 10-12/group) underwent cue reinstatement where cues that were previously presented response-contingently during self-administration we available on an FR1 schedule for 1 h. (B) After re-establishing an extinction baseline, rats received a priming injection of cocaine (10 mg/kg, i.p.) and were immediately place in the operant chamber and were allowed to lever press under extinction conditions for 1 h. Error bars indicate S.E.M.

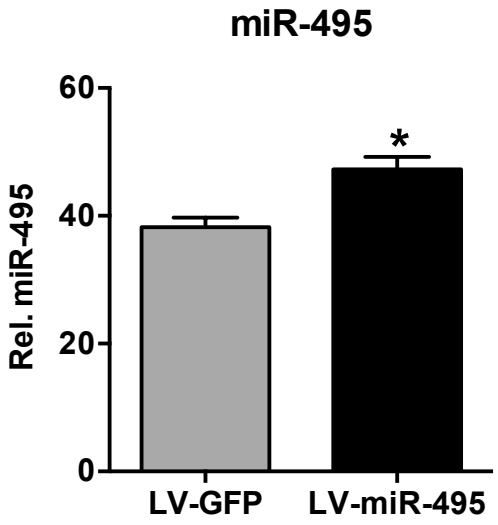

Figure 30. NAc Shell miR-495 Overexpression Increased NAc Shell miR-495 Expression. Following behavioral testing, NAcSh tissue (n = 5-7/group) was collected and processed for qRT-PCR using Taqman® primers for miR-495 or U6 snRNA (control). Rel. miR-495 levels were normalized to U6. Error bars indicate S.E.M. * different from LV-GFP, $p < 0.05$.

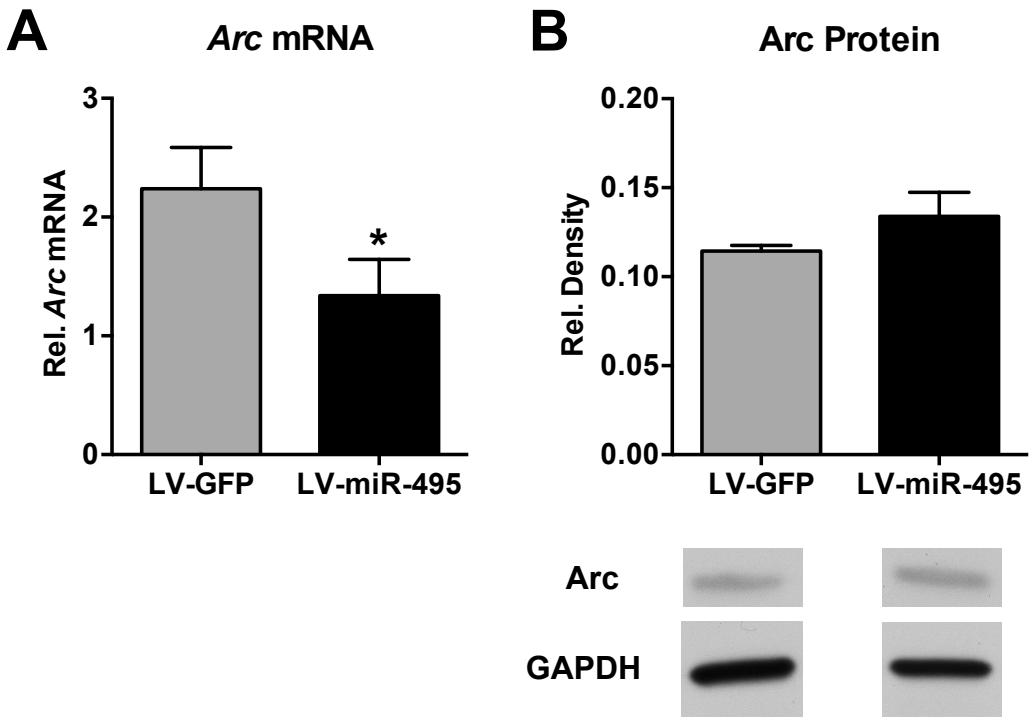

Figure 31. NAc Shell miR-495 Overexpression Reduced NAc Shell *Arc* mRNA. NAcSh tissue (n = 5-7/group) was collected following behavioral testing and processed for qRT-PCR using primers for *Arc* (A) or *Gapdh* mRNA (control). Rel. *Arc* mRNA levels were normalized to *Gapdh*. (B) Arc protein was quantified using Western blot and normalized to GAPDH protein. Representative blots are depicted for each group. Error bars indicate S.E.M. * different from Saline, $p < 0.05$.

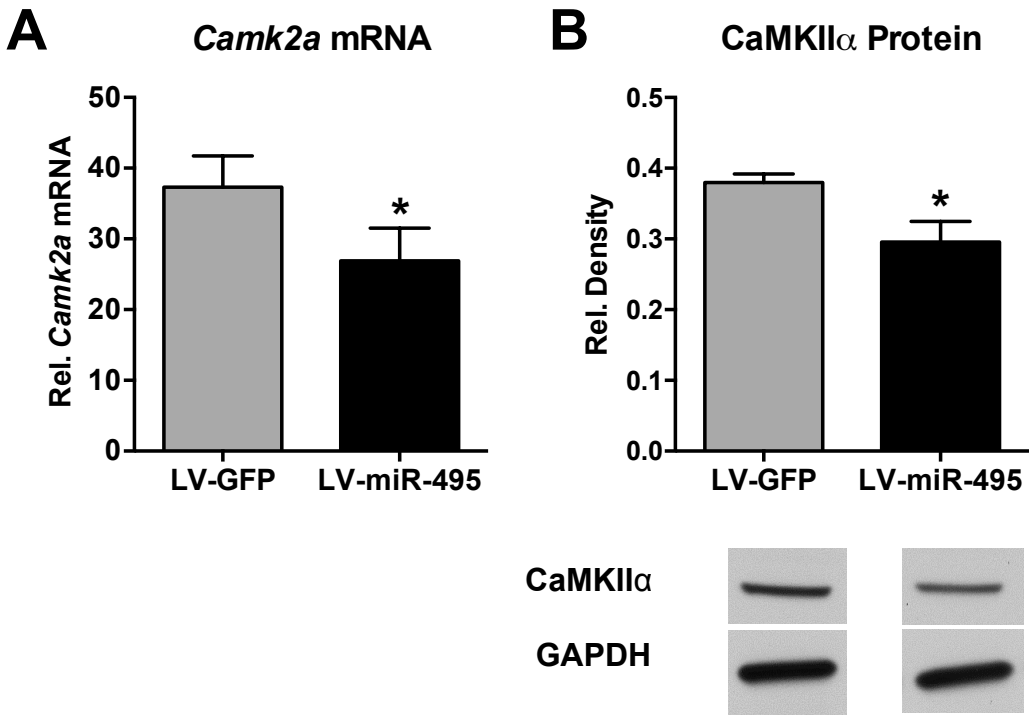

Figure 32. NAc Shell miR-495 Overexpression Reduced NAc Shell *Camk2a* Expression. NAcSh tissue (n = 5-7/group) was collected following behavioral testing and processed for qRT-PCR using primers for *Camk2a* (A) or *Gapdh* mRNA (control). Rel. *Camk2a* mRNA levels were normalized to *Gapdh*. (B) CaMKIIα protein was quantified using Western blot and normalized to GAPDH protein. Representative blots are depicted for each group. Error bars indicate S.E.M. * different from LV-GFP, $p < 0.05$.

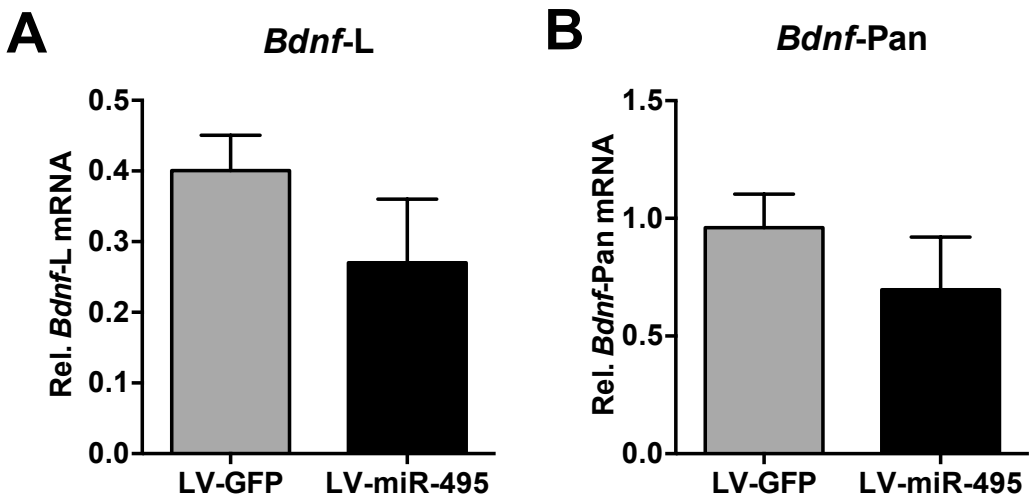

Figure 33. NAc Shell miR-495 Overexpression Had No Effect on NAc Shell *Bdnf* mRNA Expression. NAcSh tissue (n = 5-7/group) was collected following behavioral testing and was processed for qRT-PCR using primers for *Bdnf*-L (A), *Bdnf*-Pan (B) or *Gapdh* mRNA (control). Rel. *Bdnf* mRNA levels were normalized to *Gapdh*. Error bars indicate S.E.M.

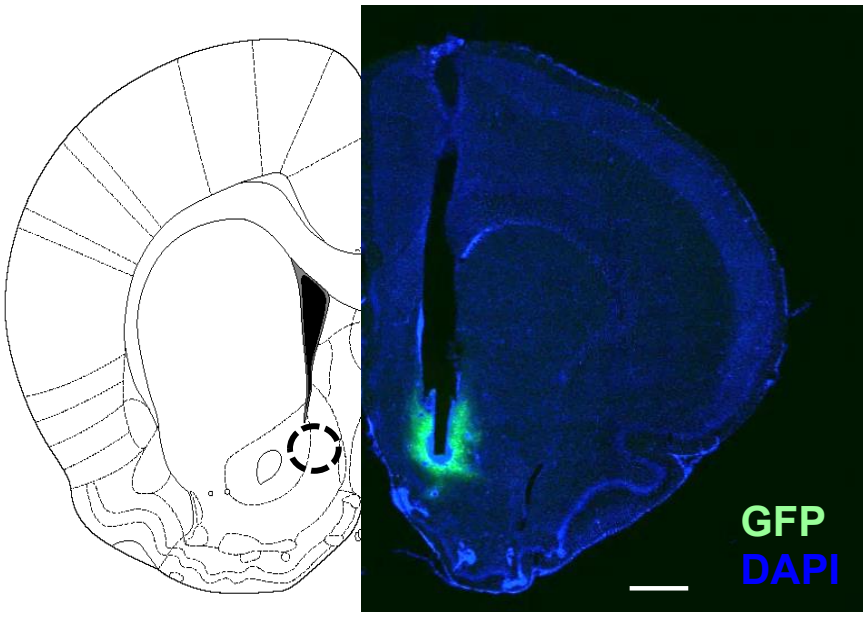

Figure 34. Verification of Viral Infusion Placement. The left panel is a schematic of the target nucleus accumbens shell (NAcSh) taken from the rat brain atlas (Paxinos & Watson, 2005) at +1.6mm from bregma. The right panel is a photomicrograph of a representative infusion site with viral-expressed GFP (green) and DAPI (blue) staining. Scale bar = 1 mm.

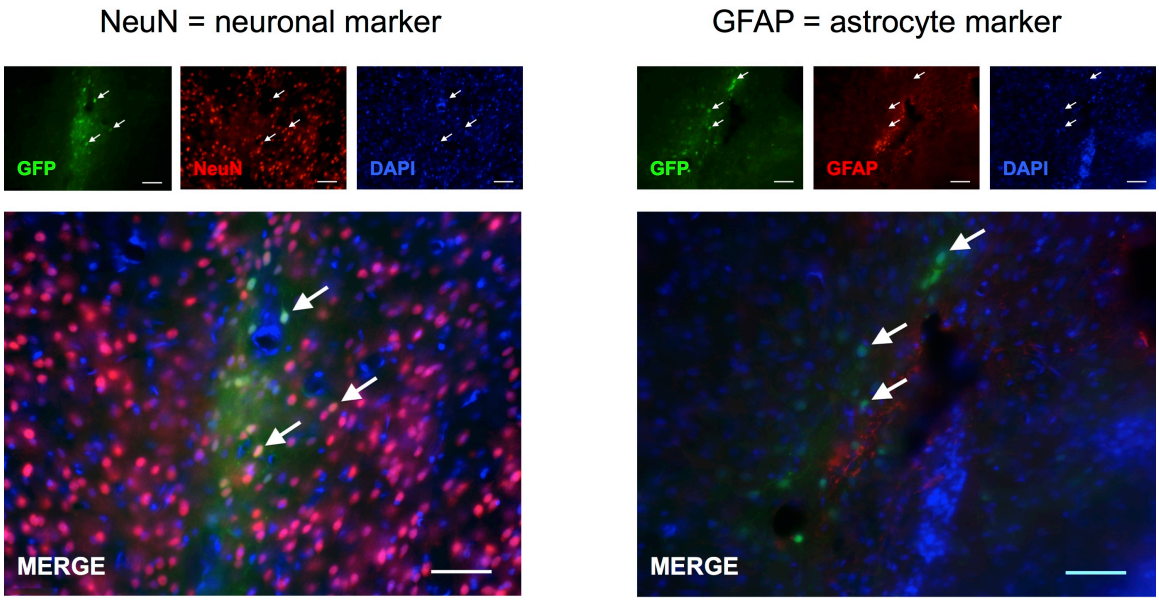

Figure 35. Lentiviral Vector Preferentially Co-Labels with Neurons. Double-label immunohistochemistry for GFP (green) and neuronal marker NeuN (Red, left panel) or astrocyte marker GFAP (Red, right panel). Cell bodies labeled with DAPI (blue). Scale bar = 50 µm.

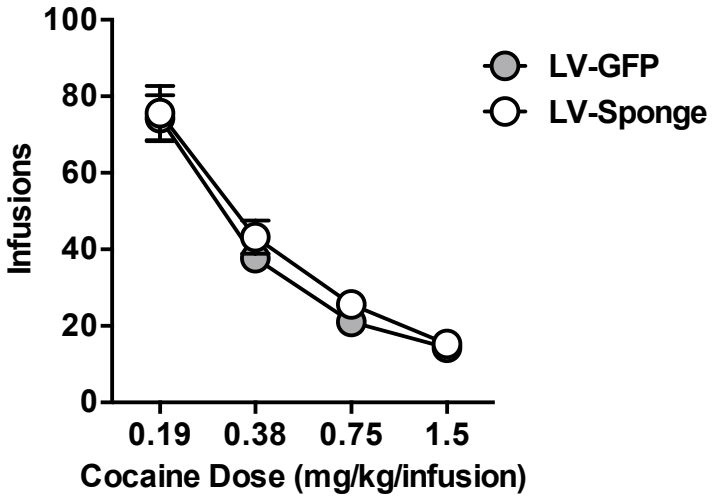

Figure 36. NAc Shell miR-495 Inhibition Had No Effect on a Between-Session FR5 Dose-Response Curve. Two weeks following virus infusions (n = 11-12/group), rats received cocaine doses on an FR5 schedule of reinforcement during 2-h sessions in a pseudorandom order (0.75, 0.38, 1.5, 0.19 mg/kg/infusion). Infusions were averaged across 3 sessions once rats achieved stability (< 15% variability). Error bars indicate S.E.M.

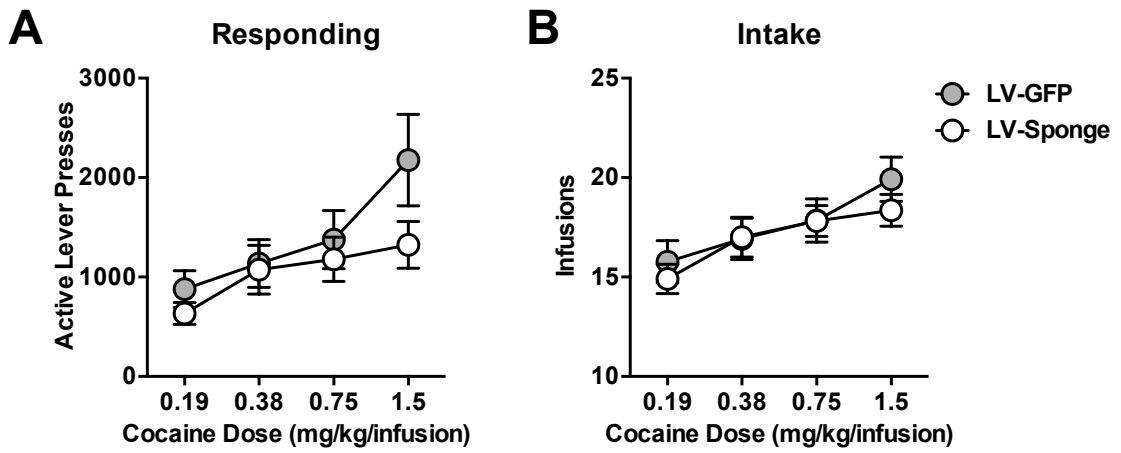

Figure 37. NAc Shell miR-495 Inhibition Had No Effect on Responding and Intake on a PR Schedule. Rats (n = 11-12/group) received cocaine doses on a PR schedule of reinforcement in a pseudorandom order (0.75, 0.38, 1.5, 0.19 mg/kg/infusion) where both total active lever presses (A) and intake (B) were measured. Error bars indicate S.E.M.

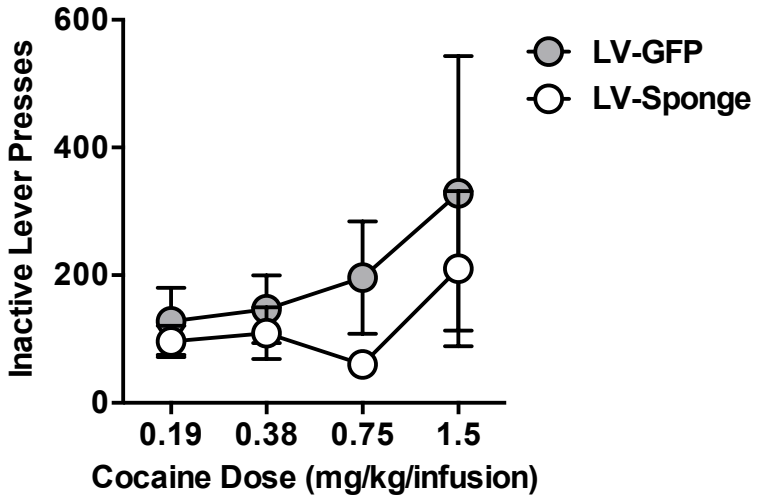

Figure 38. NAc Shell miR-495 Inhibition Had No Effect on Inactive Lever Presses on a PR Schedule. Rats (n = 11-12/group) received cocaine doses on a PR schedule of reinforcement in a pseudorandom order (0.75, 0.38, 1.5, 0.19 mg/kg/infusion). Error bars indicate S.E.M.

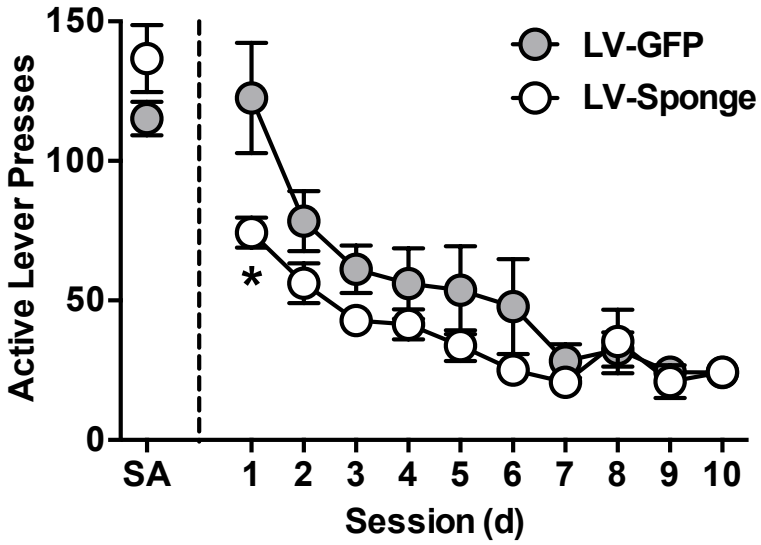

Figure 39. NAc Shell miR-495 Inhibition Reduced Responding During Extinction. Rats (n = 11-12/group) were initially given >3 sessions on an FR5 schedule of cocaine self-administration (SA) to establish an SA baseline. During the 1-h daily extinction sessions, lever presses produced no consequences. Error bars indicate S.E.M. * different from LV-GFP, $p < 0.05$.

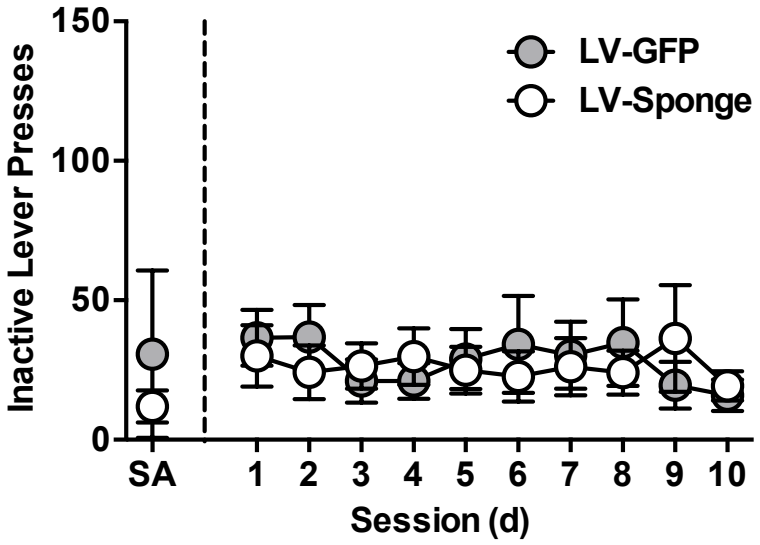

Figure 40. NAc Shell miR-495 Inhibition Had No Effect on Inactive Lever Pressing During Extinction. Rats (n = 11-12/group) were initially given >3 sessions on an FR5 schedule of cocaine self-administration (SA) to establish an SA baseline. During the 1-h daily extinction sessions, lever presses produced no consequences. Error bars indicate S.E.M.

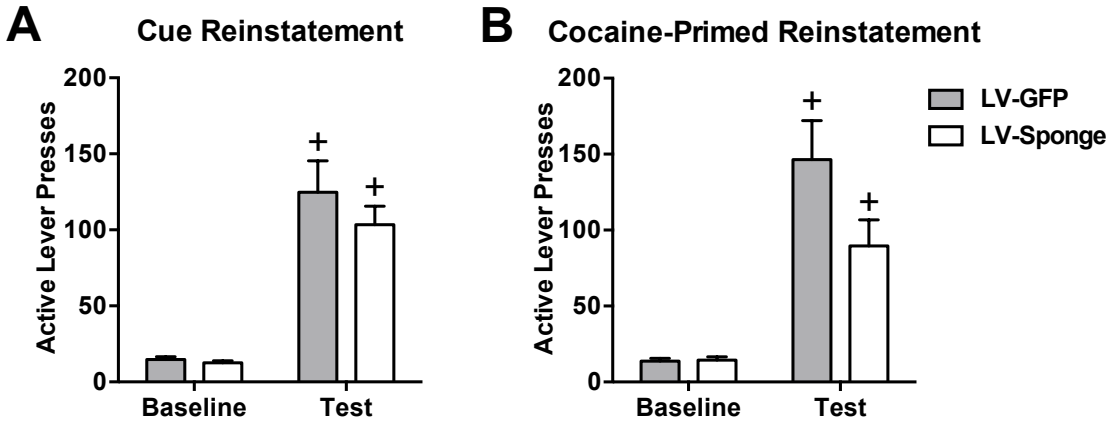

Figure 41. NAc Shell miR-495 Inhibition Had No Effect on Reinstatement. (A) Following >3 sessions with low extinction responding (i.e., Baseline), rats (n = 11-12/group) underwent cue reinstatement where cues that were previously presented response-contingently during self-administration we available on an FR1 schedule for 1 h. (B) After re-establishing an extinction baseline, rats (n = 11-12/group) received a priming injection of cocaine (10 mg/kg, i.p.) and were immediately place in the operant chamber and were allowed to lever press under extinction conditions for 1 h. Error bars indicate S.E.M. $^{+}$ different from Baseline, $p < 0.05$.

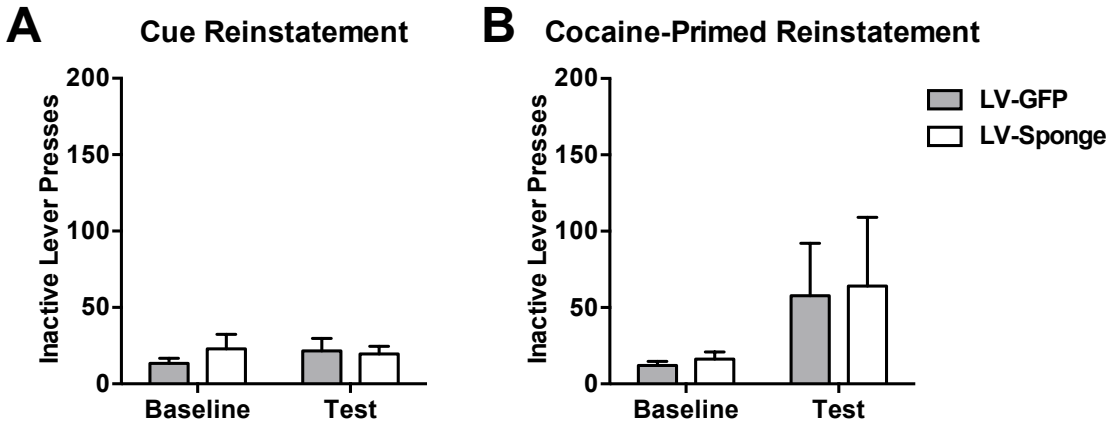

Figure 42. NAc Shell miR-495 Inhibition Had No Effect on Inactive Lever Pressing During Reinstatement. (A) Following >3 sessions with low extinction responding (Baseline), rats (n = 11-12/group) underwent cue reinstatement where cues that were previously presented response-contingently during self-administration we available on an FR1 schedule for 1 h. (B) After re-establishing an extinction baseline, rats (n = 11-12/group) received a priming injection of cocaine (10 mg/kg, i.p.) and were immediately place in the operant chamber and were allowed to lever press under extinction conditions for 1 h. Error bars indicate S.E.M.

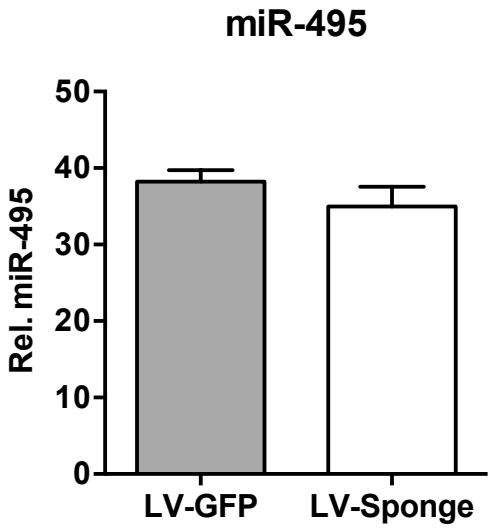

Figure 43. NAc Shell miR-495 Inhibition Had No Effect on NAc Shell miR-495 Expression. Following behavioral testing, NAcSh tissue (n = 5-7/group) was collected and processed for qRT-PCR using Taqman® primers for miR-495 or U6 snRNA (control). Rel. miR-495 levels were normalized to U6. Error bars indicate S.E.M.

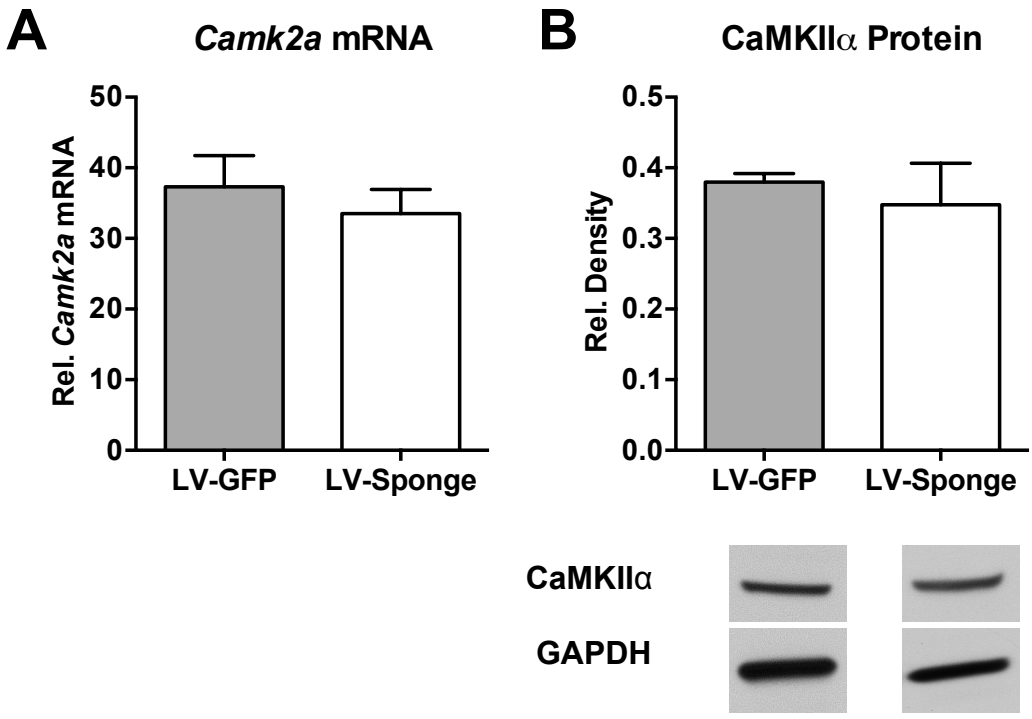

Figure 44. NAc Shell miR-495 Inhibition Had No Effect on NAc Shell *Camk2a* Expression. NAcSh tissue (n = 5-7/group) was collected following behavioral testing and processed for qRT-PCR using primers for *Camk2a* (A) or *Gapdh* mRNA (control). Rel. *Camk2a* mRNA levels were normalized to *Gapdh*. (B) CaMKIIα protein was quantified using Western blot and normalized to GAPDH protein. Representative blots are depicted for each group. Error bars indicate S.E.M.

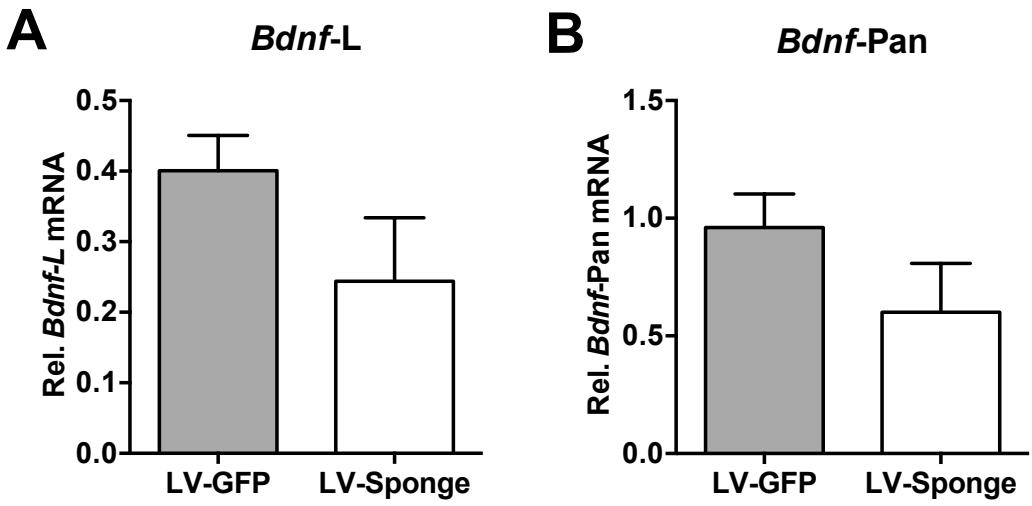

Figure 45. NAc Shell miR-495 Inhibition Had No Effect on NAc Shell *Bdnf* mRNA Expression. NAcsh tissue (n = 5-7/group) was collected following behavioral testing and was processed for qRT-PCR using primers for *Bdnf*-L (A), *Bdnf*-Pan (B) or *Gapdh* mRNA (control). Rel. *Bdnf* mRNA levels were normalized to *Gapdh*. Error bars indicate S.E.M.

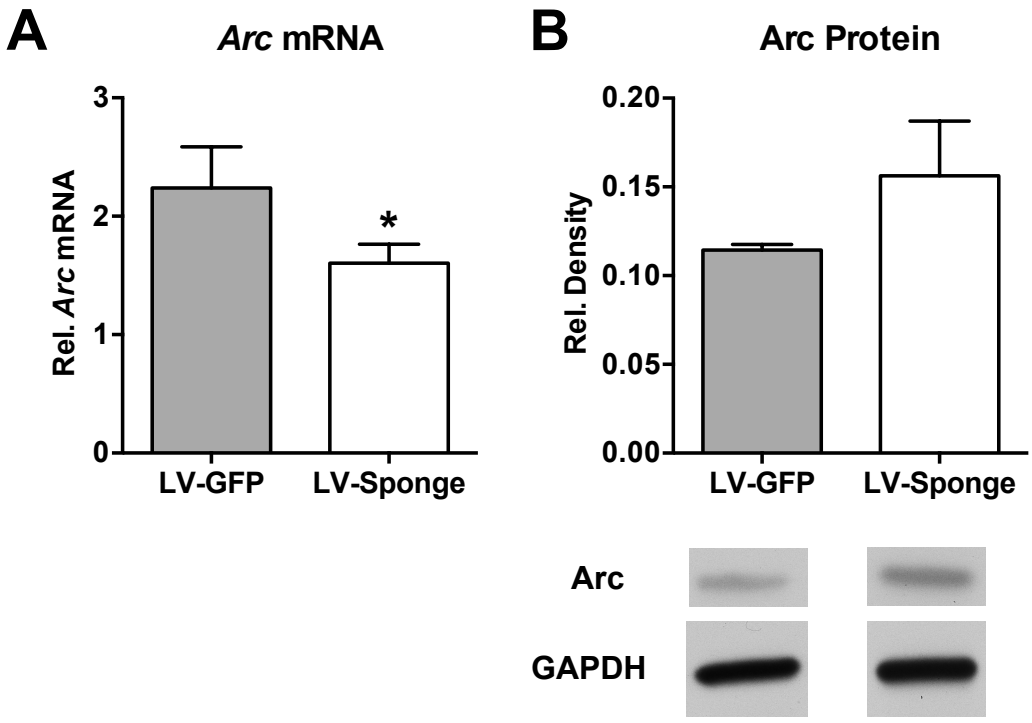

Figure 46. NAc Shell miR-495 Inhibition Reduced NAc Shell *Arc* mRNA. NAcSh tissue (n = 5-7/group) was collected following behavioral testing and processed for qRT-PCR using primers for *Arc* (A) or *Gapdh* mRNA (control). Rel. *Arc* mRNA levels were normalized to *Gapdh*. (B) Arc protein was quantified using Western blot and normalized to GAPDH protein. Representative blots are depicted for each group. Error bars indicate S.E.M. * different from Saline, $p < 0.05$.

APPENDIX C

LIST OF ABBREVIATIONS

ARG: addiction-related gene
Arc: activity-regulated cytoskeleton-associated protein
Bdnf: brain-derived neurotrophic factor
Bdnf-L: brain-derived neurotrophic factor, long 3'UTR variant
Bdnf-Pan: brain-derived neurotrophic factor, sum of long and short 3'UTR variants
Bdnf-S: brain-derived neurotrophic factor, short 3'UTR variant
Camk2a: calcium/calmodulin-dependent protein kinase type 2, alpha subunit
Cnr1: cannabinoid receptor type 1
CPP: conditioned place preference
CREB: cAMP response element binding protein
FR: fixed ratio
Gapdh: glyceraldehyde 3-phosphate dehydrogenase
GFP: green fluorescent protein
GluR: glutamate receptor
KARG: Knowledgebase of Addiction-Related Genes
LV: lentivirus
MeCP2: methyl-CpG binding protein 2
miRNA: microRNA
mPFC: medial prefrontal cortex
NAc: nucleus accumbens
NAcC: nucleus accumbens core
NAcSh: nucleus accumbens shell
OE: overexpression
Per2: period circadian clock 2
PR: progressive ratio
RPB: RNA-binding protein
SA: self-administration
SUD: substance use disorder
U6 snRNA: U6 small nuclear RNA
UTR: untranslated region
VTA: ventral tegmental area

APPENDIX D

CIRRICULUM VITAE

RYAN MICHAEL BASTLE

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EDUCATION

Present **Ph.D. Neuroscience**, Arizona State University, Tempe, AZ.
Dissertation topic: "MicroRNA regulation of addiction-related gene and motivation for cocaine in rats"
Committee: Janet Neisewander (Chair), Jason Newbern, Ella Nikulina, Nora Perrone-Bizzozero, and Federico Sanabria

2012 **M.A. Psychology** (Behavioral Neuroscience), Arizona State University, Tempe, AZ.
Masters Thesis: "Novel cues reinstate cocaine-seeking behavior and induce Fos protein as effectively as conditioned cues"
Committee: Janet Neisewander (Chair), M. Foster Olive, and Federico Sanabria

2009 **B.S. Psychology**, Minor: Mathematics, University of Minnesota Duluth, MN

AWARDS & HONORS

National

- Ruth L. Kirschstein F31 National Research Service Award Individual Predoctoral Fellowship (2013-2016), National Institute on Drug Abuse (NIDA)
- Achievement Rewards for College Scientists (ARCS) Scholarship (2014-2015), ARCS Foundation, Inc.
- Co-author of "Hot Topic" Press Release (2013), Society for Neuroscience, titled "miR-495, a post-transcriptional link between gene expression and the behavioral effects of cocaine"
- Co-author of "Hot Topic" Press Release (2013), Society for Neuroscience, titled "BDNF over-expression in the ventral tegmental area potentiates intermittent social defeat stress-induced escalation of cocaine self-administration"
- Travel Award (2013), International Behavioral Neuroscience Society
- Travel Award (2012), NIDA/INSERM

Arizona State University

- Travel Award (2015), Graduate and Professional Student Association (GPSA)
- CLAS Graduate Excellence Award (2014), Arizona State University
- Travel Award (2011), GPSA
- Travel Award (2011), GPSA
- Research Enhancement Award (2009-2010)

University of Minnesota, Duluth

- B.S. conferred Summa Cum Laude (2009)
- B.S. conferred Departmental Honors (2009)

- Undergraduate Research Opportunities Program grant (2008-2009), School of Pharmacy
- Undergraduate Research Opportunities Program grant (2007-2008), Department of Psychology
- CEHSP Dean's List (2004-2009)
- George C. Martin, Jr. Scholarship (2004-2008)
- Academic Scholarship (2004)

PROFESSIONAL POSITIONS

Arizona State University

2012-present	Graduate Research Assistant, School of Life Sciences
2012	Graduate Teaching Assistant, School of Life Sciences
2011-2012	Graduate Teaching Assistant, Department of Psychology
2009-2012	Graduate Research Assistant, Department of Psychology

University of Minnesota, Duluth

2008-2009	Undergraduate Research Assistant, School of Pharmacy
2006-2009	Undergraduate Teaching Assistant, Department of Psychology
2006-2008	Undergraduate Research Assistant, Department of Psychology

PROFESSIONAL AFFILIATIONS

- Society for Neuroscience
- International Behavioral Neuroscience Society
- College on Problems of Drug Dependence
- ARCS Foundation, Inc.
- Psi Chi National Honors Society

PUBLICATIONS AND MANUSCRIPTS UNDER REVIEW

Bastle, R.M., Oliver, R.J., Gardiner, A.S., Pentkowski, N.S., Bolognani, F., Allan, A.M., Chaudhury, T., St. Peter, M., Galles, N. Smith, C.D., Neisewander, J.L., Perrone-Bizzozero, N.I. Nucleus accumbens-enriched miR-495 regulates motivation for cocaine by targeting multiple addiction-related genes. *Under review*.

Bastle, R.M. & Neisewander, J.L. (in review). Epigenetics and Drug Abuse, *Substance Abuse*. InTech. Rijeka, Croatia.

Wang, J.*, Bastle, R.M.*, Bass, C.E., Hammer, Jr., R.P., Neisewander, J.L., Nikulina, E.M. Overexpression of BDNF in the ventral tegmental area enhances binge cocaine self-administration in rats exposed to repeated social defeat. *Under review*. *Equal contribution

Lewis C.R., Bastle, R.M., Manning, T., Himes, S., Fennig, P., Conrad, P., Hess, L., Pagni, B., Matekel, C., Olive, M.F. Interactions between early life stress, nucleus accumbens MeCP2 Expression, and methamphetamine intake behavior in male rats

Pentkowski, N.S., Harder, B.G., Brunwasser, S.J., Bastle, R.M., Peartree, N.A., Yanamandra, K., Adams, M.A., Der-Ghazarian, T., Neisewander, J.L. (2014). Pharmacological evidence for an abstinence-induced switch in $5HT_{1B}$ receptor modulation of cocaine self-administration and cocaine-seeking behavior. *ACS Chemical Neuroscience*, *5* (3), 168-176.

Bastle, R.M., Kufahl, P.R., Turk, M.N., Weber, S.M., Pentkowski, N.S., Thiel, K.J., Neisewander, J. L. (2012). Novel cues reinstate cocaine-seeking behavior and induce Fos protein as effectively as conditioned cues. *Neuropsychopharmacology, 37* (9), 2109-2120.

MANUSCRIPTS IN PREPARATION

Peartree, N.A., Bastle, R.M., Williams, A., Chandler, K.N., Goenaga, J., Hood, L.E., Neisewander, J.L. Fos expression after exposure to social and nicotine rewards and reward-conditioned environments.

MEETING PRESENTATIONS AND ABSTRACTS

Bastle, R.M., Pentkowski, N.S., Chaudhury, T., St. Peter, M. Smith, C.D., Galles, N., Leslie, K.R., Oliver R.J., Gardiner, A.S., Perrone-Bizzozero, N.I., Neisewander, J.L. (October 2015) Blockade of cocaine-induced gene expression and reinstatement by nucleus accumbens miR-495. **45th Annual Society for Neuroscience Meeting Abstract**, Chicago, IL.

Bastle, R.M., Pentkowski, N.S., Chaudhury, T., St. Peter, M. Smith, C.D., Galles, N., Leslie, K.R., Oliver R.J., Gardiner, A.S., Perrone-Bizzozero, N.I., Neisewander, J.L. (June 2015) Viral-mediated overexpression of miR-495 in the nucleus accumbens shell reduces addiction-related gene expression and motivation for cocaine. **International Behavioral Neuroscience Society Meeting Abstract**, Victoria, British Columbia, Canada.

Bastle, R.M., Pentkowski, N.S., Oliver R.J., Gardiner, A.S., Smith, C.D., Taylor, J.M., Galles, N., Perrone-Bizzozero, N.I., Neisewander, J.L. (December 2014) Viral-mediated overexpression of miR-495 in the nucleus accumbens shell reduces motivation for cocaine. **53rd Annual American College of Neuropsychopharmacology Meeting Abstract**, Phoenix, AZ.

Bastle, R.M., Pentkowski, N.S., Smith, C.D., Chaudhury, T., Leslie, K.R., Oliver, R.J., Gardiner, A.S., Perrone-Bizzozero, N.I., Neisewander, J.L. (November 2014) Overexpression of miR-495 in the nucleus accumbens shell decreases cocaine, but not food, intake and seeking behavior. **44th Annual Society for Neuroscience Meeting Abstract**, Washington, D.C.

Oliver, R.J., Gardiner, A.S., Bastle, R.M., Neisewander, J.L., Perrone-Bizzozero, N.I. (November 2014) Regulation of miR-495 and addiction-related mRNAs following exposure to cocaine. **44th Annual Society for Neuroscience Meeting Abstract**, Washington, D.C.

Bastle, R.M., Oliver, R.J., Pentkowski, N.S., Gardiner, A.S., Perrone-Bizzozero, N.I., Neisewander, J.L. (June 2014) Overexpression of miR-495 in nucleus accumbens attenuates cocaine intake on a progressive ratio schedule of reinforcement. **76th Annual College on Problems of Drug Dependence Meeting Abstract**, San Juan, PR.

Wang, J., Bastle, R.M., Bass, C.E., Hammer Jr., RP, Neisewander, J.L., Nikulina, E.M. (November 2013) BDNF over-expression in the ventral tegmental area potentiates intermittent social defeat stress-induced escalation of cocaine self-administration**. 43rd Annual Society for Neuroscience Meeting Abstract**, San Diego, CA.

Oliver, R.J., Bastle, R.M., Gardiner, A.S., Wright, C., Saavedra, J.L., Pentkowski, N. S., Allan, A. M., Neisewander, J.L., Perrone-Bizzozero, N.I. (November 2013) miR-495, a post-transcriptional link between gene expression and the behavioral effects of cocaine. **43rd Annual Society for Neuroscience Meeting Abstract**, San Diego, CA.

Bastle, R.M., Pentkowski, N.S., Turk, M.N., Adams, M.D., Berger, A.L., Dado, N., Smith, K., Hammer, R.P., Jr., Perrone-Bizzozero, N., Neisewander, J.L. (June 2013) Regulation of *arc* through miR-495 as a potential mediator of cocaine motivation and extinction learning.

International Behavioral Neuroscience Society Meeting Travel Awardee Presentation, Malahide, Ireland.

Bastle, R.M., Pentkowski, N.S., Turk, M.N., Adams, M.D., Berger, A.L., Perrone-Bizzozero, N., Neisewander, J.L. (October 2012) The role of a microRNA, miR-495, in regulating target gene expression and cocaine self-administration in rats. **42nd Annual Society for Neuroscience Meeting Abstract**, New Orleans, LA.

Wang, J., Bastle, R.M., Terwilliger, E.F., Neisewander, J.L., Hammer, R.P., Jr., Nikulina, E.M. (October 2012) BDNF overexpression in ventral tegmental area: escalation of cocaine self-administration and elevated ΔFosB expression in rat nucleus accumbens. **42nd Annual Society for Neuroscience Meeting Abstract**, New Orleans, LA.

Pentkowski, N.S. Harder, B., Brunwasser, S., Yanamandra, K., Bastle, R.M., Der-Ghazarian, T., Adams, M.D., Alba, J., Neisewander, J.L. (October 2012) The effects of 5-HT1B receptors on motivation for cocaine vary depending on length of abstinence. **42nd Annual Society for Neuroscience Meeting Abstract**, New Orleans, LA.

Bastle, R.M., Kufahl, P.R., Turk, M.N., Pentkowski, N.S., Thiel, K.J., Weber, S.M., & Neisewander, J. L. (November 2011) Novel and conditioned stimuli reinstate extinguished reward-seeking behavior and induce similar patterns of Fos expression. **41st Annual Society for Neuroscience Meeting Abstract**, Washington D.C.

Bastle, R.M., Dickey, E.D., Thiel, K.J., Pentkowski, N.S., Hammer, Jr., R.P., & Neisewander, J.L. (June 2011) Region-specific changes in *zif268* mRNA following cocaine self-administration, abstinence, and extinction training. **73rd Annual College on Problems of Drug Dependence Meeting Abstract**, Hollywood, FL.

Bastle, R.M., Weber, S.M., Sanabria, F., Cheung, T.H., & Neisewander, J.L. (November 2010) Contextual and discrete cue contributions to renewed cocaine-seeking behavior. **40th Annual Society for Neuroscience Meeting Abstract***,* San Diego, CA.

Bastle, R.M. & Einat, H. (April 2009) AMPA receptor modulators: A possible new target for mood stabilizing agents*.* **21st Annual National Conference for Undergraduate Research Meeting Abstract**, La Crosse, WI.

Bastle, R.M. & Gordon, R.A. (March 2009) Enhancing performance by delaying tasks: Positive procrastination and its relationship to behavioral and claimed self-handicapping. **9th Annual Society for Personality and Social Psychology Meeting Abstract**, Tampa, FL.

Bastle, R.M. & Gordon, R.A. (May 2008) Active procrastination and defensive pessimism: Adaptive strategies for goal attainment*.* **6th Annual Twin Ports Undergraduate Psychology Conference Symposium Presentation**, Duluth, MN.

Bastle, R.M. & Gordon, R.A. (April 2008) Active procrastination and defensive pessimism: Adaptive strategies for goal attainment*.* **79th Annual Midwest Psychological Association Meeting Abstract**, Chicago, IL.

COLLOQUIA AND INVITED PRESENTATIONS

"Nucleus Accumbens miR-495 as a novel target for the anti-motivational effects of cocaine" (March 2016) School of Life Sciences, Arizona State University, Tempe, AZ

"MicroRNA Regulation of Addiction-Related Gene Expression and Motivation for Cocaine in Rats" (January 2015) Neuroscience Symposium, Arizona State University, Tempe, AZ

"Size Really Doesn't Matter: MicroRNA Regulation of Addiction-Related Genes and Cocaine Intake in Rats" (October 2013) School of Life Sciences, Arizona State University, Tempe, AZ

"The Role of MicroRNAs in Post-Transcriptional Regulation of Addiction-Related Genes" (February 2012) Department of Psychology, Arizona State University, Tempe, AZ

"The Impact of Novel Stimuli on Cue-Elicited Responding and Immediate Early Gene Expression" (April 2011) Department of Psychology, Arizona State University, Tempe, AZ

"Contextual and Discrete Cue Contributions to the Renewal of Cocaine-Seeking Behavior" (May 2010) Department of Psychology, Arizona State University, Tempe, AZ

"Active Procrastination and Defensive Pessimism: Adaptive Strategies for Goal Attainment" (May 2008) Twin Ports Undergraduate Psychology Symposium, Tempe, AZ

TECHNICAL SKILLS

Behavioral

- Subcutaneous and intraperitoneal injection of pharmacological agents
- Intravenous drug self-administration
- Drug conditioned place preference
- Intracranial drug administration
- Spontaneous and drug-induced locomotor activity
- Forced Swim Test
- Sweet solution preference
- Light/dark box
- Resident/intruder

Molecular

- Tissue sectioning (microtome, cryostat, micro punch, vibratome)
- Cresyl violet staining
- Immunohistochemistry (single and double labeling; chromogenic and fluorescent)
- *In situ* hybridization histochemistry (autoradiography and fluorescent)
- RNA/protein isolation and quantification
- qRT-PCR
- Western Blot
- Brightfield and epifluorescent microscopy
- Cell counting
- Densitometry

Surgical

- Jugular vein catheterization with head mount
- Stereotactic surgery, including viral-mediated gene transfer (mice and rats)
- Transcardial perfusions
- Trunk blood collection
- Rapid brain extraction

TEACHING AND TRAINING EXPERIENCE

Teaching Assistantships

2013 Lab Instructor, *Animal Physiology*, Arizona State University

2012 Teaching Assistant, *Interpersonal Influence*, Arizona State University
2012 Teaching Assistant, *Introduction to Statistics*, Arizona State University
2011 Teaching Assistant, *Memory and Cognition*, Arizona State University
2011 Lab Instructor, *Research Methods*, Arizona State University
2011 Lab Instructor, *Research Methods*, Arizona State University
2009 Teaching Assistant, *Social Psychology*, University of Minnesota, Duluth
2008 Teaching Assistant, *Social Psychology*, University of Minnesota, Duluth
2008 Teaching Assistant, *General Psychology*, University of Minnesota, Duluth
2007 Teaching Assistant, *General Psychology*, University of Minnesota, Duluth
2006 Teaching Assistant, *Abnormal Psychology*, University of Minnesota, Duluth

Undergraduate/High School Student Research Training/Supervision

Arizona State University

Mari Turk*	Matthew Adams	Khaliliah Smith+	Nicholas Galles*
Heather Koch*	Stephanie Kost	Colton Smith	Kenneth Leslie
Alyssa Jensen	Nicholas DeFelice	Trisha Chaudhury∆*	Madeleine St. Peter*
William Toy	Nora Dado	Angela Neuckranz	Katie Olson∆
Lauren Hood	Jeremy Kleiman	Jennifer Taylor	Juliette Venault•
Hanna Molla+	Samantha Scott+		

* Barrett Honors College student
∆ high school student
\+ PREP scholar
• French exchange scholar

Technician Research Training/Supervision

Arizona State University

Matthew Adams, M.D.
John Paul Bonadonna, B.S.
Suzanne Weber, B.S.

Graduate Student/Postdoctoral Fellow Research Training/Supervision

Arizona State University

Candace Lewis, Ph.D.	Nathan Pentkowski, Ph.D.	Junshi Wang, Ph.D.
Amy Louriaux, Ph.D.	Lara Pockros, Ph.D.	
Geeta Negi, Ph.D..	Gregory Powell, Ph.D.	
Natalie Peartree, Ph.D.	Thomas Sebastian, Ph.D.	

University of Minnesota, Duluth

Tonya Meinerding, B.S. Darcie Sackett, B.S.

PROFESSIONAL ACTIVITIES

Committee Service

2016 Committee Member, Honors Thesis for Trisha Chaudhury, Arizona State University
2015 Committee Member, Honors Thesis for Nicholas Galles, Arizona State University
2014 Committee Member, Honors Thesis for Jeffrey Lynn, Arizona State University
2011 Committee Member, Honors Thesis for Mari Turk, Arizona State University

Community Service

2016 Science Fair Judge, Chandler High School

2014 Brain Fair, Monte Vista Elementary School

2012 Homecoming Week Psychology Booth, Arizona State University

2010-2012 Brain Fair, Arizona State University

Scientific Service

2015-16 Reviewer for journal article submission in *Cellular and Molecular Neurobiology*

2014 Reviewer for journal article submission in *Behavioral Pharmacology*

2013 Reviewer for journal article submission in *Progress in Neuropsychopharmacology and Biological Psychiatry*

REFERENCES

Janet Neisewander	480-965-0209	Janet.Neisewander@asu.edu
Nora Perrone-Bizzozero	505-272-1165	nbizzozero@salud.umn.edu
Ella Nikulina	602-827-2168	nikulina@email.arizona.edu
Federico Sanabria	480-965-4687	Federico.Sanabria@asu.edu