Fall 11-15-2017

Post-Transcriptional Regulation of Addiction-Related Behaviors: Opposing Roles of Nucleus Accumbens miR-495 and HuD

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BY

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A.B. Biological Sciences, University of Chicago, 2011

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DISSERTATION

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

In Biomedical Sciences

The University of New Mexico

Albuquerque, New Mexico

Dec., 2017

ACKNOWLEDGEMENTS

I would like to give credit to everyone that has helped me along this journey. First, my committee of studies including Drs. Carlos Fernando Valenzuela, Andrea Allan, Jonathan Brigman, and Derek Hamilton. Your input has been incredibly valuable for my development. I would also like to thank the other graduate students for their moral and scientific, especially Dr. Ryan Bastle that helped me with setting up lentiviral infusions. Everyone needs the support of their family and friends, and I am no different. Most importantly, Dr. Nora Perrone-Bizzozero has been integral to my development as a scientist. She has helped me through thick and thin, and has read all of my very rough drafts before turning them into gold. Finally, I would like to thank all of the scientists that came before me, allowing all of us to build on their knowledge and continue searching for the truth.

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ABSTRACT

Substance use Disorders (SUDs) are characterized by chronic relapse after periods without symptoms. This has been hypothesized to stem from persistent alterations in corticolimbic circuit function and structure caused by drug-induced alterations in addiction-related gene (ARG) expression. Transcriptional and epigenetic regulation of ARGs and addiction-like behaviors have been well characterized but the role of post-transcriptional regulation is an understudied, yet promising field. RNA binding proteins (RBPs) are one post-transcriptional regulator of mRNA stability. HuD is a neuronal specific RBP that stabilizes mRNAs and is regulated by neuronal activity and cocaine. Another type of post-transcriptional regulator, microRNAs (miRNAs), are non-coding RNAs that target specific mRNAs for degradation or translational repression. Since both RBPs and miRNAs target the 3'UTR, this opens the possibility that these two classes of

molecules could compete for access to a specific recognition site. We have found that miR-495 and HuD target a set of shared mRNAs via binding to the same GUUUGUUUG sequence. Many of these shared targets, including *Bdnf* and *Camk2a* mRNAs have been implicated in addiction and are considered ARGs. This led to my hypothesis that cocaine CPP differentially regulates HuD and miR-495 leading to a synergistic increase in ARG expression and addiction-like behaviors. We found that miR-495 was significantly decreased within the NAc while HuD protein and mRNA was significantly increased. CaMKIIα and BDNF mRNA and protein levels were increased in a similar fashion. Overexpression of HuD or miR-495 caused opposite effects on ARG expression and CPP behavior. Finally, to determine the in vivo capability of these two regulators to compete for behavior, we infused LV-miR-495 in HuD_{OE} mice and trained them in CPP. We found that HuD_{OE} + LV-miR-495 completely blocked the development of CPP compared to LV-GFP controls as well as diminishing expression of shared target mRNA and protein. This suggests that miR-495 and HuD have bidirectional roles in the regulation of CPP behavior. Further research on the role of post-transcriptional competition of shared targets on cellular dynamics and behavior may inform new pharmacological treatments that tip the balance of this post-transcriptional competition mechanism in the favor of remission.

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CHAPTER 1: INTRODUCTION

1a. Overview of Substance Use Disorders

Substance use disorders (SUDs), or more colloquially known as drug addiction, are a significant societal burden both financially, incurring an estimated $193 billion in costs (www.justice.gov/ndic, 2011), and emotionally devastating to patients and their families. In opposition to more controlled, casual drug use, SUDs are characterized by uncontrollable bouts of drug consumption despite negative life consequences. Patients with this disorder appear to lose interest in normal prosocial activities, instead directing their energy towards the pursuit of these substances. Since addiction is a chronic, relapsing condition, this disorder has been notoriously difficult to manage. The complete understanding of the molecular, physiological, and behavioral mechanisms involved in the disorder is necessary for the discovery and implementation of more effective treatments.

The mesolimbic dopamine system is the most well studied and implicated circuit associated with the disorder. Naturally, this neurocircuit may have evolved as an information-processing center to direct behavior towards environmentally important stimuli predicting the presence of vital resources, e.g. food and water. Synaptic plasticity within these regions are implicated in associative learning of these stimuli to guide behavior necessary to receive these rewarding resources. Depending on the frequency and accuracy that a particular stimulus may predict a natural reward, goal-directed behavior towards this stimulus with the intention of obtaining the reward may become habitual.

Thus, if an exogenous substance were to recruit this same neurocircuitry this may elicit similar goal-directed behavior towards obtaining these compounds.

Structurally, drugs of abuse are highly varied and have many specific protein targets within the body (for a review of pharmacological mechanisms of drugs of abuse see, (Lüscher *et al.*, 2006). However, all pharmacological mechanisms of drugs of abuse cause excessive dopamine release in the ventral tegmental area and nucleus accumbens (**Figure. 1.1**). Not only are these regions involved in this disorder, they also appear to be crucial mediators of appropriate goal-directed behavior towards natural rewards. Dopaminergic projections from VTA neurons are important in regulating motivation to perform integral, evolutionarily-conserved behaviors such as feeding and reproduction (Kelley *et al.*, 2002; Bromberg-Martin *et al.*, 2010). Drug-elicited synaptic plasticity also occurs within this region, possibly through similar mechanisms as natural rewards. Mirroring a classical symptom of addiction, the synaptic alterations induced by drugs of abuse inhibit the ability of other non-drug related experiences to elicit synaptic plasticity (Kolb *et al.*, 2003; Hamilton *et al.*, 2005). As such, it has been hypothesized that drugs of abuse cause a heightened form of plasticity-dependent associative memory leading to habit formation and addiction-like behaviors (Stuber *et al.*, 2008).

1b. Animal models of Substance Use Disorders

Animal models of human SUDs, termed addiction-related behaviors, are necessary to fully dissect the mechanisms of this disorder. Although we cannot study many of the intricacies of the human aspects of these disorders, anthropomorphized

rodent behavior has enabled the study of the long lasting constitutive changes and the transient processes that are related to

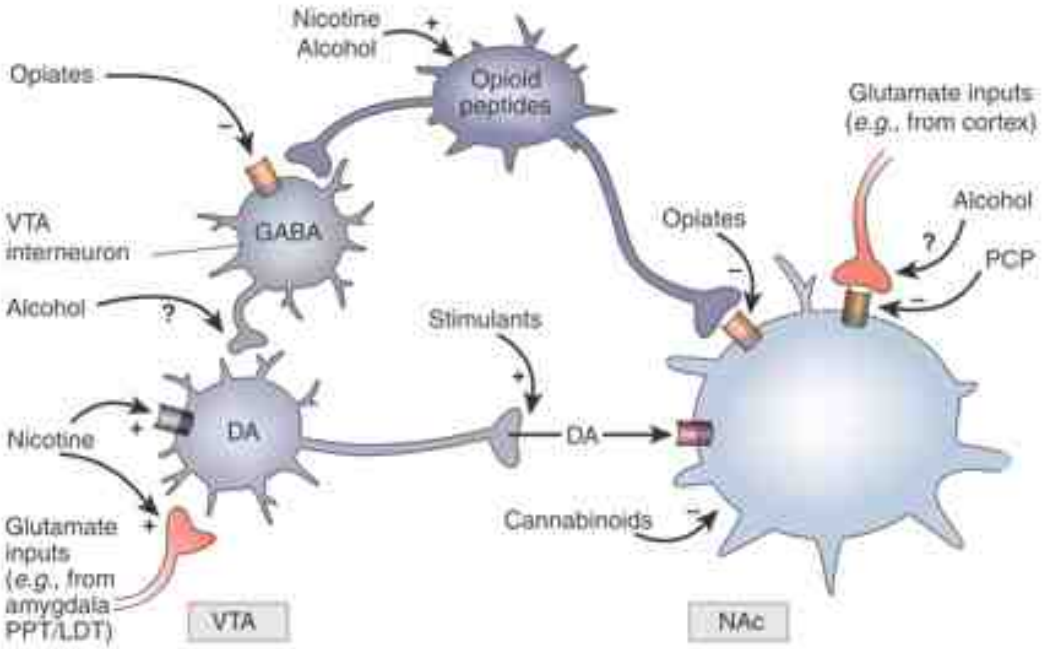

Fig 1.1. Drugs of abuse cause dopamine release within the Nucleus Accumbens. Through various interactions with specific cell types and receptors, drugs of abuse all cause influx of dopamine within the NAc during intoxication. See Nestler, 2005 for original publication.

addiction (Scofield *et al.*, 2016). As such, there are three main addiction-related behaviors, locomotor sensitization, conditioned place preference (CPP), and self-administration (SA). From these three main forms of addiction-related behavior, more specific models of various aspects of human SUDs arise. These behaviors have been found to involve encoding of contextual or cue associations, but only CPP and SA are truly forms of Pavlovian conditioning. The encoding of these previous neutral stimuli with the unconditioned stimulus of the subjective effects of the drug is a common aspect of addiction-related behaviors in experimental animals and in human SUDs (Vezina *et al.*, 2009; Mayo *et al.*, 2013). Overall, we can extrapolate features of addiction-related behaviors in animal models to certain behaviors found in human SUDs.

Drugs of abuse are a form of reward. As such, rewards in general can be "liked" and "wanted." Thus, there are differences in neural systems that drive either of these processes. After repeated exposure to drugs of abuse, the stimuli that are associated with availability of the drug drive behavior or become more "salient". This phenomenon has been termed "incentive sensitization" (Robinson *et al.*, 1993). This set of behavior is also found in human patients with substance use disorders. This manifests itself as stimuli such as drug paraphernalia or drug-paired environments become more stimulating to these patients and drive their motivation towards acquisition of these drugs. This feature of SUDs is modeled in the most basic forms of animal models of addiction-related behaviors. Animals trained in these behavioral procedures will seek out drug-paired cues, energize their drug-related responding in a cue-triggered fashion, and will work to be presented these cues in the absence of drugs. The procedures that generate these three

characteristics of incentive sensitization and how they are measured will be discussed below.

Locomotor sensitization is the elevation of drug-induced locomotor activity compared to the first drug administration, usually following repeated administration of the drug with or without a withdrawal period (Vezina *et al.*, 2009). This behavior is correlated with an increase in dopamine influx within the NAc, suggesting that this may be a mechanism involved in this phenotype (Kalivas *et al.*, 1990). In contrast to dopamine, enhanced NAc glutamate influx requires the presence of previously paired environmental cues (Bell *et al.*, 2000). Bell and colleagues also found that drug-paired, cue-induced glutamate release coincides with the enhanced drug-induced dopamine release. Though this was posited to be a mechanism for cue-induced craving in humans, this was not the case, as dopamine was not elevated in humans and non-human primates (Bradberry, 2007). Another caveat is that locomotor sensitization requires acute exposure to the drug in the final stage of testing. This adds the confound of the effect of the drug itself in the study of distinct mechanisms. In either case, locomotor sensitization is a translatable behavioral procedure that may be involved in drug-cue pairing found in human SUDs.

A more translatable model for drug-cue pairing is CPP. This is a classical form of Pavlovian conditioning, in which the drug and vehicle are non-contingently administered in the presence of two distinct environmental contexts (Tzschentke, 2007). Over time, animals will pair this conditioned, environmental stimulus with the drug but not the vehicle. This is assessed by allowing the animal to explore both environmental contexts, in which the animal will seek out the drug-paired context in the absence of the drug itself.

Due to the volitional aspect of the procedure, it allows researchers to determine the motivational value of seeking out these drug-paired cues. As such, this can be thought of as a model to study motivation to acquire drugs or seek drugs. Additionally, this pairing can be extinguished by exposure to both contexts in the absence of drugs, thus leading to no preference to either context. If the animal is given the drug, it will reinvigorate seeking for the previously drug paired environmental context (Mueller *et al.*, 2000). Thus, this CPP can be expanded to study the underlying mechanisms that mirror drug craving and relapse. As mentioned previously, humans can also exhibit CPP behavior in experimental contexts (Mayo *et al.*, 2013). Overall, the motivation to acquire drugs or seek out drug-associated contexts are important aspects of human SUDs.

Finally, the most translatable model used to study addiction is self-administration of drugs (Roberts *et al.*, 2007). In this model, an intravenous catheter will infuse cocaine into the animal once it has completed a specific behavioral response, usually pressing an active lever in opposition to an inactive lever that produces no effect. Usually, this is done following a reinforcement schedule, in which the animal must complete the "correct" instrumental response a certain number of times before the reinforcer is dispensed. Once the animal has reached a determined set of criteria of correct lever presses for drug, the animal has "acquired" SA. This can be combined with a "correct" response cue, such as a light, to study Pavlovian association between this conditioned stimulus cue and the unconditioned drug stimulus (Carroll *et al.*, 1993). Presentation of this cue can facilitate the learning of this instrumental responses, termed Pavlovian-instrumental transfer (Hall *et al.*, 2001). Overall, acquisition of SA is comparable to humans learning to take the drug.

In a progressive ratio schedule of reinforcement, the reinforcement schedule can be made to progressively increase the number of correct responses required for the reinforcer to be dispensed. Once the animal reaches an established criterion of reinforcer received, the animal is termed to have reached a "break point." This allows for experimenters to measure the reinforcing efficacy of the drug, determined as the maximum amount of effort the animal will undergo to maintain SA of the drug. Thus, the maintenance of this behavior is comparable to the effort required for stable drug use in humans.

In standard SA, animals are given a limited period to administer the drug. However, when animals are given extended access to drugs of abuse in a self-administration model, they undergo a specific behavioral pattern termed escalation of drug use. This is hypothesized to be due to a change from goal-directed behavior towards compulsive drug use, possibly due to less cortical control and more striatal control (Kalivas *et al.*, 2005). Uncontrollable, compulsive drug use is an important facet in the human condition.

Mechanisms of abstinence can be assessed through removal of the drug. This can be done by removal of the reinforcer for any instrumental action, termed extinction, or forced abstinence through leaving the animal within its home cage. Although in both models of abstinence, extinction requires new learning of the non-availability of the reinforcer. This leads to the diminishment of active lever presses. In contrast, forced abstinence recruits a separate phenomenon, incubation of craving. Animals that have undergone forced abstinence will increase their active lever presses in response to reintroduction of the operant chamber (Grimm *et al.*, 2003). In the extinction model

active lever pressing can be reinvigorated through a non-contingent priming dose of the drug, drug-associated cue, or stress. Similar to CPP, but more translatable to human SUDs, these behaviors are measures of drug seeking. Overall, both reinstatement after extinction and incubation of craving can be thought of as different models of relapse in humans.

1c. Nucleus Accumbens plasticity associated with addiction-related behaviors

Although drug use is required for the development of addiction, it is most certainly insufficient to cause this disorder. For example, around 176.6 million, of Americans ages 12 and up drink alcohol but only 17 million are classified as having an alcohol use disorder (SAMSHA, 2013). Drugs are metabolized and eventually cleared from the body, thus, their pharmacological effect is not sufficient. In addition, SUDs are classically characterized by chronic relapse after periods without symptoms (McLellan *et al.*, 2000). This may occur many years after a patient's last reported use of a drug, suggesting that some stable functional alteration has occurred within the brain. Repeated drug exposure is required for an accumulation of molecular events that alter plasticity within specific regions affecting the functioning of the entire circuit. These synaptic alterations are persistent and similar to the chronic relapse state of patients with SUDs, suggesting that reversing these processes may prevent relapse (McLellan *et al.*, 2000; Shen *et al.*, 2009). Additionally, they appear to be protein-synthesis dependent forms of plasticity (Hernandez *et al.*, 2002; Scheyer *et al.*, 2014). Thus, the molecular mechanisms regulating gene expression may play a role in the etiology of this disorder, specifically

drug-induced alterations in addiction-related gene (ARG) expression leads to persistent modifications in region-specific synaptic plasticity.

Long-term synaptic plasticity can take the form of potentiation (LTP) or depression (LTD) of synaptic strength. Nearly every form of plasticity has been found to occur within the NAc during various drug exposures or addiction-related behavioral paradigms (Scofield *et al.*, 2016). This may be due to different neuronal subtypes within the region, such as dopamine receptor D1- or D2-containing medium spiny neurons (MSNs), which are projection neurons and represent 95% of the neurons in the NAc, and small number of interneurons. To add to this complexity, these neurons have different downstream projections or upstream afferents. Thus, increased or decreased synaptic strength may be required to cause orchestrated changes in other brain regions and ultimately behavior.

1d. Molecular mechanisms of addiction-related behavior associated plasticity

Regulation of ARGs can occur at many different steps. The DNA in the genome is organized into small compact three-dimensional units assembled by histones into nucleosomes, which form the structure of chromatin. As such, basal transcription is necessarily stagnant in the absence of regulation of gene accessibility. Modifications to the accessibility of genes, by regulation of these structures is termed epigenetic regulation and has also been found to be an integral component in the development of addiction. Given that the epigenetic control of drug addiction has been elegantly reviewed in previous works (Robison *et al.*, 2011; Tuesta *et al.*, 2014; Bastle *et al.*, 2016; Cadet *et al.*,

2016), this section will focus on other aspects of gene expression control starting at the level of transcription.

i. Transcriptional Regulation of Addiction-Related Genes

Transcriptional regulation occurs through transcription factors, which respond to intracellular changes leading to association with cis-regulatory elements (CREs) in the promoter of target genes.

a. The Fos family transcription factors are differentially altered by drugs of abuse and contribute to addiction-related plasticity and behavior

Although altered transcription of various genes had been suggested by earlier work (E W Fleming *et al.*, 1981; Eugene W Fleming *et al.*, 1981), it was not until the discovery that morphine caused increased striatal expression of, c-*Fos*, that it was shown that transcription factors may regulate this drug-induced gene expression (Chang *et al.*, 1988). Although first found to be regulated by acute morphine treatment, it was found that other drugs of abuse cause specific, dopamine-dependent, alterations in the expression of this transcription factor within addiction-related regions (Graybiel *et al.*, 1990; Persico *et al.*, 1993). Important for the study of relapse, it was found that c-*Fos* was upregulated in mesolimbic structures following withdrawal, suggesting that it may play a role in the intense drug craving present in this disorder (Hayward *et al.*, 1990). c-FOS is part of the *Fos* family of transcription factors, including FosB and ΔFosB, which all heterodimerize with Jun to form the AP-1 complex (Milde-Langosch, 2005). This

complex then associates with its specific CRE, the AP-1 binding site, leading to increased transcription of genes downstream of these sequences.

Fos genes are considered immediate early genes (IEG), or factors that are basally expressed at low levels but elevated rapidly by specific stimuli (first reviewed in the context of neuronal cells (Sheng *et al.*, 1990). Since it was upregulated by other plasticity and memory inducing events, it was hypothesized that c-*Fos*, and possibly other IEGs, may alter gene expression involved in memory formation (Curran *et al.*, 1987). As expected, upregulation of c-*Fos*, *FosB*, *ΔFosB* and *Jun* lead to increased AP-1 mediated transcription within addiction-related regions such as the NAc after acute drug administration (Hope *et al.*, 1992). Due to its regional specificity, it was hypothesized that alterations in these genes may provide a mechanism for reorganization of functional interactions between these regions in addiction (Moratalla *et al.*, 1996). Although c-*Fos* is rapidly upregulated and returns to basal levels quickly, AP-1 binding continued in the absence of elevated c-*Fos* mRNA or protein expression suggesting that other *Fos* proteins may replace c-*Fos* and accumulate during chronic use of drugs. This may provide a molecular trace of chronic drug use in the absence of drug itself. This tolerance to initial c-*Fos* upregulation appears to be drug agnostic, as acute alcohol exposure blocked the previously observed acute cocaine upregulation of c-*Fos*, also suggesting that this molecular trace persists for the use of different drugs (Torres, 1994).

To more specifically test the role of specific transcription factors in addiction, transgenic mice with specific mutations were required. The first experiment to utilize this approach used a transgenic mouse with a mutant *fosB* gene that prevented the production of ΔFOSB. It is important to consider that this mutant *fosB* gene was not targeted to any

specific cell type or even to the brain, but throughout the body. These mutant mice showed attenuated chronic induction of AP-1 binding activity, suggesting that the previously observed AP-1 binding without c-*Fos* upregulation was probably due to ΔFOSB (Hiroi *et al.*, 1997). Additionally, this study was the first to link transcriptional regulation with altered addiction-like behavior. They used CPP to determine that *fosB* mutant mice showed increased CPP compared to wild-type controls, suggesting that normal ΔFOSB/AP-1 binding might be involved in a compensatory or adaptive response to drugs. *Fos* family members have also been found to be upregulated after exposure to drug-paired cues in the absence of the drug itself, suggesting this CPP effect may be due to the interaction between drug and cues soliciting the availability of the drug, an importance facet in addiction research (Schroeder *et al.*, 2000).

Although certain molecular factors within one set of cells may cause increased addiction-like behavior or plasticity, it may cause an opposite effect on these measures in another set. Cellular specificity is an important facet of experimental neuroscience. In an experiment complementary to that performed by Schroeder and colleagues, researchers developed an inducible system to specifically overexpress *ΔFosB* within D1 neurons in the striatum (Kelz *et al.*, 1999). Neurons within the striatum are generally GABAergic medium spiny neurons that mostly express D1 or D2 dopamine receptors. D1 receptors are G-stimulatory (Gs) G-protein coupled receptors (GPCRs), which upon activation increase the activity of the target neuron, leading to increased GABA release. Conversely, D2 receptors are coupled to G-inhibitory (Gi) and are inhibited by dopamine binding which lead to the opposite effect on these neurons. Since drugs of abuse increase

dopamine levels within the striatum, both D1 and D2 containing neurons play important but dissociable roles in the development of addiction.

Targeted *ΔFosB* overexpression in D1 neurons showed a similar effect on increased CPP behavior at low doses (5 and 15 mg/kg), but normal CPP behavior at higher doses (20 mg/kg). This suggests that that cocaine-induced *ΔFosB* expression within these cells is necessary and sufficient to promote addiction-like behaviors. Furthermore, GABAergic inhibition from these cells may play a role in CPP behavior. Finally, this is physiologically relevant, as drugs of abuse and to a lesser extent natural rewards cause upregulation of *ΔFosB* in this specific neuronal subtype (Nye *et al.*, 1995; Moratalla *et al.*, 1996). In a similar experiment, non-specific deletion of *ΔFosB* throughout the body caused the same behavioral alteration, increased CPP behavior to lower doses of cocaine (Hiroi *et al.*, 1997). Although this finding may seem contradictory, when taken together with previous data, this points to the existence of other regions or possibly neuronal subtypes that act in opposition to D1 neurons. Following up on this, researchers overexpressed *ΔFosB* within D2 expressing neurons in the striatum produced no effects, illustrating the cellular specificity of these drug-induced alterations (Zachariou *et al.*, 2006). Thus, *ΔFosB* within these regions and cells may not play a role, or possibly inhibit, the plasticity required for the development of these behaviors.

To study the role that *ΔFosB* may play in the motivation to consume drugs, researchers utilized PR in a SA model. Animals with a similar inducible *ΔFosB* genotype as used by Kelz and colleagues showed increased motivation to self-administer cocaine, as they were found to have an increased PR (Colby *et al.*, 2003). This was also the first

experiment to show that this alteration specifically affected motivation towards drugs and not naturally rewards such as a food pellet.

Transcription factors elicit changes in expression of genes, thus this work suggests that there must be some downstream targets of the FOS transcription factor that are mediating these effects on addiction-related behavior. Kelz and colleagues first described a downstream target that may alter plasticity involved in these behavioral alterations (Kelz *et al.*, 1999). Striatal neurons are normally quiescent, even with dopamine input. Thus, glutamate is required to alter their firing. Speaking to integral role of glutamate to MSN activity, it has been found to be an important neurotransmitter in addiction-like behaviors (reviewed in (van Huijstee *et al.*, 2015). Kelz and colleagues found that *ΔFosB* overexpression caused an increase in a specific AMPAR subunit, GluR2 which could lead to altered plasticity (Kauer *et al.*, 2007). Additionally, they could recapitulate their effects on behavior by viral mediated overexpression of this subunit in the striatum. Overall, many ΔFOSB targets are genes encoding proteins that appear to be previously linked to addiction-like behaviors such as CDK5 and NFKB.

b. CREB, a transcription factor well established in plasticity involved in learning and memory, plays a role in addiction

Drugs of abuse can cause acute regulation of molecular signaling cascades that can modify transcription factor activity. For example, many drugs of abuse can induce acute upregulation of the second messenger cAMP within many addiction-associated brain regions (Cole *et al.*, 1995; Carlezon *et al.*, 1998; Berke *et al.*, 2000; Nestler, 2001; Shaw-Lutchman *et al.*, 2002, 2003; Walters *et al.*, 2003). cAMP response element

binding protein (CREB) is phosphorylated in response to cAMP upregulation by a kinase sensitive to cAMP, protein kinase A (PKA; (Shaywitz *et al.*, 1999; Mayr *et al.*, 2001) as well as by CaMK and other kinases (Sheng *et al.*, 1991). The role of CREB in other forms of plasticity mediating memory have been well elucidated (Mayr *et al.*, 2001). Once phosphorylated CREB can enter the nucleus and bind to its response element, the cyclic AMP response element (CRE). CREB was first implicated in drug-induced alterations in response to acute and chronic morphine treatment within the locus coeruleus, another region in the reward system projecting to the NAc and containing the cell bodies of noradrenergic neurons (Guitart *et al.*, 1992). Later it was found that chronic, but not acute, morphine caused a decrease in CREB within the NAc (Widnell *et al.*, 1996). Cocaine self-administration was found to be sensitive to PKA inhibition, an upstream regulator of CREB, by increasing lever pressing (Self *et al.*, 1998b). Following this experiment, it was later found that NAc overexpression of CREB caused a decrease in cocaine-CPP behavior (Carlezon *et al.*, 1998). Conversely, overexpression of a mutant CREB containing a single point mutation, alanine for serine at residue 133, that prevents its phosphorylation and blocks its ability to induced transcription acting as a dominant negative form of CREB, caused an increase in cocaine-CPP behavior. Further correlating the role of this transcription factor in the association of drug cues, researchers found that re-exposure to the original drug-paired environment in CPP without drug caused increased phosphorylation of CREB (Tropea *et al.*, 2008). Highlighting the differences of behavioral tasks, it was shown that NAc overexpression of CREB caused an increase in self-administration behavior (Larson *et al.*, 2011). This gene has also been studied in human patients. Researchers found that opioid-dependent patients were more likely to

have a polymorphism in a protein that associates with CREB, CREB binding protein, within a region associated with its activation by phosphorylation (Kumar *et al.*, 2011). Altogether, it appears that CREB expression is involved in many aspects of addiction-related behavior.

CREB is a nuclear transcription factor that binds to CRE upstream of target genes. CREB regulation has been found to cause upregulation of ARG mRNA such as c-*fos*, *Fosb*, *Bdnf*, and *TrkB* (for example in methamphetamine self-administration, (Krasnova *et al.*, 2013). BDNF and its receptor TrkB have a very long history in the study of addiction-related behaviors as well as general plasticity occurring within the brain (Li and Marina E Wolf, 2015). Further implicating CREB as a regulator of plasticity, it has been found to be involved in late phase forms of long-term potentiation (LTP; (Barco *et al.*, 2002). As mentioned before, NAc neurons are normally quiescent and require glutamate activation with or without dopamine stimulation to fire. CREB has been associated with the regulation of this feature of NAc neuronal membrane excitability (Huang *et al.*, 2008). It was found that increased CREB activity leads to enhanced NMDAR mediated currents, an important receptor for causing action potentials as well as for learning & memory. This regulation increases NAc neuronal firing probability through enhancement of the membrane excitability. This may partially explain the effect of NAc CREB on behaviors such as self-administration. Thus, CREB regulates the expression of many genes associated with plasticity and addiction-related behaviors.

CREB and ΔFOSB are very well documented addiction-related transcription factors but, undoubtedly, there are many more transcriptional factors that alter addiction-related plasticity and ultimately behavior. Myocyte enhancing factor 2 (MEF2) was found

to be involved in cocaine-induced structural plasticity (Pulipparacharuvil *et al.*, 2008). Interestingly, it was necessary and sufficient to increase the number of dendritic spines, or synapses. Structural plasticity or the alteration of the physical synaptic shape has been associated with electrophysiological measurements of plasticity. Additionally, it was found that MEF2 regulates a wide network of plasticity-associated genes allowing for further exploration into its role in addiction-related plasticity and behavior (Flavell *et al.*, 2008). Overall, transcriptional regulation of ARG expression can lead to modifications in drug-evoked synaptic plasticity and behavior (Persico *et al.*, 1996; Nestler, 2012)s.

ii. Post-transcriptional regulation of plasticity and addiction-related gene mRNA can promote addiction-like behaviors.

Post-transcriptional regulation occurs after mRNA transcription but before the mRNA is translated into functional protein. Post-transcriptional regulation takes many forms such as mRNA processing, splicing, editing, and stability. mRNA stability has been the most well characterized post-transcriptional mechanism in plasticity, especially in addiction. Additionally, it appears that mRNA stability may be especially important in brain, as nearly 20% of brain expressed genes have been predicted to be controlled through this mechanism (Bolognani *et al.*, 2010).

a. miRNAs destabilize specific mRNAs which could lead to diminished expression of genes involved in addiction

Direct binding of factors to discrete recognition sites can influence mRNA stability. One group of factors involved in this type of mRNA stability are small non-coding RNAs, termed microRNAs (miRNAs). miRNAs are ~20-22 oligonucleotide molecules that are encoded within the genome and make up nearly 2-3% of transcribed genes, but are not translated into protein. miRNAs target specific mRNAs through binding of complementary sequences within the miRNA, termed seed regions, to the 3' untranslated region (UTR) of the mRNA. Association of miRNAs with the 5'UTR of mRNA has also been discovered (Panda *et al.*, 2014) leading to different cellular effects, but most studies within the brain have focused on the 3'UTR. Accordingly, the rest of this section will focus on this mechanism.

Although the miRNA may associate directly with the mRNA target, the regulatory effects of this binding are mediated through a large, supporting protein complex, the RNA induced silencing complex or RISC. Through associating with the RISC, a miRNA can target specific mRNAs for degradation or translational repression (Bartel, 2004). This complex contains a multitude of auxiliary proteins, with the most important being Argonaute (Ago; (Sontheimer, 2005). Ago is the component most directly involved in regulating mRNA. There are many Ago isoforms, but generally, they are separated into those containing nuclease "slicer" activity and those without. Thus, Ago is important for the well-characterized miRNA induced destruction of mRNA.

mRNAs may have numerous sites complementary for multiple miRNAs. This suggests that a single miRNA targets many mRNAs, affecting a myriad of cellular processes and pathways. miRNAs may regulate a network of plasticity-associated genes. For example, miR-9-3p was found to diminish *Dmd* and *Sap97* mRNA (Sim *et al.*, 2016).

These genes are negatively associated with LTP expression, thus miRNA induced silencing of these mRNAs would reverse this effect. A hippocampal LTP dependent behavior, Morris water maze, was also found to be regulated by the expression of this miRNA. Similar to a group of plasticity- and addiction-associated transcription factors, miRNA expression patterns appear to be similar to IEGs in their temporal and activity dependent regulation. As demonstrated before, many of these IEGs play integral roles in general plasticity and addiction-related plasticity. Similar to IEGs, miRNAs have been associated with neural development, synaptic plasticity, and even behavior.

The role of miRNA induced post-transcriptional regulation in addiction was first studied in the regulation of the large-conductance-calcium-and-voltage-activated potassium channel (BK). This channel is integral to plasticity by affecting excitability, firing, and transmitter release from neurons (Storm, 1990). It is also one of the many protein targets of alcohol within the brain, for the most part potentiating its conductance (Butler *et al.*, 1993). This channel has been well studied in its role in tolerance to alcohol in the striatum, requiring larger amounts to exert similar effects (Pietrzykowski *et al.*, 2004). This seems to be mediated by decreased BK channel expression in the membrane. A single gene can produce multiple mRNAs with the same coding region but with different 3'UTRs through the inclusion or exclusion of different exons (Legendre *et al.*, 2006). Research has shown that the alpha subunit of this BK channel is alternatively spliced both in the coding region and 3' UTR in response to neuronal activity (Xie *et al.*, 1998; Pietrzykowski *et al.*, 2004). These researchers also found that alcohol caused a decrease in one specific BK mRNA that has a miR-9 binding site in its 3' UTR. Given that this isoform of the BK channel mRNA is the most abundant in the striatum and miR-

9 expression is increased by alcohol exposure, alcohol tolerance seems to be mediated at least in part by this miRNA (Pietrzykowski *et al.*, 2004). As mentioned before, miRNAs target many mRNAs that contain a complementary sequence in the 3'UTR. Thus, these authors also proposed that miR-9 targets many other mRNAs such as the beta subunit of the GABA receptor, *Gabrb2*, and the Dopamine 2 Receptor, *D2r*. Overall, this suggests that alcohol mediated upregulation of miR-9 leads to the development of an alcohol-specific regulation of mRNAs associated with plasticity and addiction.

Subsequent studies showed that miR-212 was upregulated in the dorsal striatum of these animals after extended access (Im *et al.*, 2010). These researchers found that upregulation of miR-212 was associated with potentiated CREB signaling, possibly through upstream cAMP pathways. RAF1 potentiates adenylyl cyclase activity, leading to increased cAMP production. Various other factors can enhance or repress this action. One of the targets of miR-212 is Sprout-related, EVH1 domain containing 1 (SPRED1), a corepressor of RAF1. Thus, extended access to cocaine-induced upregulation of miR-212 led to decreased SPRED1 levels, leading to increased cAMP production and CREB activity leading to increased miR-212 expression. When miR-212 was overexpressed within the dorsal striatum, rats in extended access showed decreased levels of cocaine infusions. With this, miR-212 upregulation was associated with an increase in CREB expression. Oppositely, blocking of miR-212 by a locked nucleic acid (LNA) caused an increase in cocaine infusions. Overall, this suggests miR-212 activates a compensatory and protective pathway that leads to enhanced CREB signaling.

b. RNA binding proteins bind to mRNAs and can bidirectionally regulate addiction-associated mRNA translation into protein

RNA binding proteins (RBPs) are another group of regulators of mRNA stability and translation. Some RBPs recognize discrete sequences in the 3'UTR, such as the AU-rich instability conferring elements (AREs; (Bakheet *et al.*, 2006). As with miRNAs, there is potential for a single RBP to affect multiple downstream processes leading to miRNAs and RBP to be termed "master switches" of gene regulation (Deschênes-Furry *et al.*, 2006; Keene, 2007). Fragile x mental retardation protein (FMRP) is an RBP that regulates the translation of nearly 850 brain mRNAs, many associated with synaptic function (Darnell *et al.*, 2011). One of its regulatory targets are the group I metabotropic glutamate receptors mGluR1 and mGluR5 (Ceman *et al.*, 2003; Narayanan *et al.*, 2007; Nalavadi *et al.*, 2012). These receptors have been associated with cocaine-evoked behaviors (Chiamulera *et al.*, 2001; Olsen *et al.*, 2010). Thus, Smith and colleagues tested the role of FMRP in addiction-related behaviors (Smith *et al.*, 2014). They found that *Fmr1* KO mice were deficient in cocaine locomotor sensitization and conditioned place preference. The effect on conditioned place preference was reversed by genetic reduction in mGluR5, suggesting that FMRP's effect on drug-evoked behaviors was due to mGluR5 activity. Finally, they found that FMRP caused a reduction in cocaine-induced structural plasticity suggesting that these behavioral deficits may be caused by this aberrant plasticity. Overall, post-transcriptional regulation may emerge as an important player tying drug-evoked molecular alterations with behavior and will be discussed extensively in this work.

c. mRNA editing can alter the sequence or expression of an addiction-related mRNA leading to alterations in addiction-related plasticity and behavior

mRNA editing is another post-transcriptional regulatory mechanism. The most common mechanism for RNA editing is through covalent modifications of a specific nucleotide, without the alteration of the original DNA encoded gene. This would generate multiple alternative forms of a protein which may be important in different cellular contexts.

The most common mRNA modification leading to editing is the removal of an amino group and its replacement with an oxygen, termed deamination. This occurs on the N6 position of adenosine nucleotides, converting this nucleotide into an inosine. This position directly contributes to conventional Watson-Crick base pairing, thus allowing inosine to pair with cytosine similar to guanosine. Translationally, this leads to the original adenosine to be read as a guanine, effecting causing an A-->G site-directed mutagenesis. This modification is catalyzed by adenosine deaminase that acts on RNA (ADAR) enzymes (Bass *et al.*, 1988; Wagnert *et al.*, 1989; Kim, Garner, *et al.*, 1994; Kim, Wang, *et al.*, 1994; Melcher *et al.*, 1996).

Specific mRNA editing in a neurophysiological context was first described in the GluRA2 subunit of AMPAR (Lomeli *et al.*, 1994). As mentioned before, editing of mRNA is a rapid mechanism to generate multiple alternative forms of a protein depending on environmental circumstances. These receptors are integral to excitatory synaptic signaling, especially in plasticity. Thus, AMPAR editing generates channels that are impermeable to calcium and display faster recovery rates from desensitization. In

terms of drug-induced plasticity, a recent study demonstrated that GluA2 editing by ADAR2 in the NAc of rats regulates cocaine seeking (Schmidt *et al.*, 2015). These researchers studied reinstatement, a specific stage of drug self-administration (Roberts *et al.*, 2007). Animals first need to learn the required instrumental behavior to receive a reward in self-administration. When the animal reaches an experimenter determined criterion, then the animal is said to have "acquired" self-administration behavior. If the animal is then presented with the same context and levers, but rewards are withdrawn, the animal will eventually override the previously learned reward driven behavior. Eventually, the animal will no longer perform the task required for the reward, which is termed extinction. Finally, when an animal is exposed to cues associated with the reward or the reward itself, the animal will reinstate its behavior to receive the reward. Reinstatement is thought to be analogous to relapse in human addiction patients. In this study they found that abstinence from cocaine was associated with a decrease in NAc edited calcium-impermeable AMPAR as well as ADAR2. Next, Schmidt and colleagues overexpressed ADAR2 within the NAc. They found that this diminished cocaine-primed reinstatement of self-administration behavior, suggesting that ADAR2 within the NAc is important for the regulation of relapse-like behavior. Overall, there are many avenues for mRNA editing in drug-induced plasticity and addiction-like behaviors (Rosenthal, 2015).

Another post-transcriptional mRNA modification is the covalent modification of adenosine to N6-methyladenosine (m6A). This modification is carried out by a methyltransferase complex, containing WTAP, METTL14, and KIAA1429. Hence, this complex is usually thought of as the "writers" of m6A modifications. In opposition to the activity of this complex, enzymes have been discovered that promote the demethylation

of mRNA, or the "erasers" of these modifications. These modifications have been studied for many years as a stable, unalterable modification. However, with the discovery of m6A demethylases this shifted opinion to suggest that this modification is dynamic and thus could be regulated by physiological conditions (reviewed in (Cao *et al.*, 2016). For example, it was reported that regulation of m6A was important to the normal functioning of the circadian rhythm in mammals (Fustin *et al.*, 2013). Thus, m6A is physiologically relevant in behavior.

The first m6A demethylase to be discovered was the fat mass and obesity associated protein (*Fto*). As the name suggests, it was identified as the strongest genetic variation to predispose patients to obesity (Frayling *et al.*, 2007). At the opening of this chapter, evidence was presented to show that addiction-related neurocircuitry originally evolved to elicit goal-directed behavior towards food, suggesting this gene may also be involved in other forms of goal-directed behavior. The first study to link these findings was performed in alcoholic patients, finding that a polymorphism in the *Fto* gene was associated with obesity (rs9939609) was inversely correlated with general alcohol consumption, measures of alcohol dependence, as well as cigarette use (Sobczyk-Kopciol *et al.*, 2011).

Later work found that this gene is expressed in dopaminergic neurons within the midbrain and is increased by acute cocaine administration (20 mg/kg; (Hess *et al.*, 2013). Cocaine, and to a lesser extent amphetamines, inhibit the dopamine transporter (DAT), leading to increased synaptic dopamine. This in turn leads to hyperactivation of dopamine receptors on both sides of the synapse. The presynaptic terminal contains D2 and D3 receptors, which are inhibitory GPCRs. Thus, the presynaptic dopaminergic

neuron will show increased suppression of firing in response to cocaine. In the next set of experiments, these researchers found that Fto deficient dopaminergic neurons showed diminished cocaine-induced suppression of firing rate, suggesting that signaling through D2/D3 receptors is regulated by *Fto*. As expected, it was found that protein expression of these downstream targets (DRD3, GIRK2, and NMDAR1) were reduced by *Fto* deficiency. Functionally, *Fto* deficiency led to a reduction in GIRK currents, possibly detailing a mechanism for the diminished cocaine-induced suppression of firing rate in dopaminergic neurons. Finally, *Fto* deficient mice showed attenuated acute cocaine-induced locomotor activity, suggesting that these genetic and electrophysiological measures translate into this behavioral modification.

As shown here, there are many avenues for regulation of ARGs at the transcriptional or the post-transcriptional level. These regulatory mechanisms all have been implicated in the development of this disorder and thus may be involved in the vulnerability of some individuals to substance use disorders. Understanding the regulation of addiction-related genes and their impact on synaptic plasticity and behavior may inform new pharmacological treatments that reverse these aberrant drug-evoked forms of plasticity in the favor of remission.

1e. Rationale for Nucleus Accumbens HuD and miR-495 involvement in addiction-related processes

As mentioned previously, post-transcriptional regulation takes many forms such as mRNA processing, splicing, editing, stability, and translation. Previous research in our

laboratory has found that nearly 20% of brain expressed genes may be controlled through mRNA stability (Bolognani *et al.*, 2010). mRNA stability can be influenced by direct binding of factors to discrete recognition sites. miRNAs are one such group and target specific mRNAs through complementary regions within the miRNA, termed seed regions, and the 3' untranslated region (UTR) of the mRNA. Through associating with the RNA-inducible silencing complex (RISC), a miRNA can target specific mRNAs for degradation or translational repression (Bartel, 2004). Although one miRNA has one specific seed region to bind to mRNAs, mRNAs may have numerous sites complementary for multiple miRNAs. This suggests that a single miRNA targets many mRNAs, affecting a myriad of cellular processes and pathways. As posited in the above sections, many characteristics of miRNAs make them appealing targets for disorders such as SUDs. Thus, we sought to identify a candidate miRNA that targets multiple addiction-related genes (ARGs) and may provide a novel mechanism for - reverting drug-induced aberrant gene expression.

RBPs are another group of regulators of mRNA stability. They too recognize discrete sequences in the 3'UTR, such as the AU-rich instability conferring elements (AREs; (Bakheet *et al.*, 2006). As with miRNAs, there is potential for a single RBP to affect multiple downstream processes leading to miRNAs and RBP to be termed "master switches" of gene regulation (Deschênes-Furry *et al.*, 2006; Keene, 2007). This is especially important in the brain, as around 15-20% of brain specific transcripts contain AREs suggesting these genes are regulated by this type of post-transcriptional regulation (Bolognani et al., 2010). One such family of RBPs are the Hu, or Embryonic Lethal Abnormal Vision like (ELAV-L) proteins. Hu proteins are primarily brain and neuronal

specific and are homologous to the *Drosophila* protein ELAV (Szabo *et al.*, 1991; Yao *et al.*, 1993). Similar to miRNAs, HuD expression and function is activity dependent, suggesting it may regulate IEGs and other activity dependent genes associated with plasticity (Tiruchinapalli, Ehlers, *et al.*, 2008; Bolognani *et al.*, 2010; Vanevski *et al.*, 2015). This activity dependence is compounded by evidence that this regulation of HuD leads to association with plasticity associated mRNAs such as *Homer1a* and *Camk2a*. Recent studies indicate that HuD also plays a role in translation and that PKC-mediated phosphorylation of HuD increases local translation of BDNF, suggesting that HuD may be involved in protein synthesis-dependent forms of plasticity. HuD is localized to the dendritic compartment of hippocampal neurons (Bolognani *et al.*, 2004), where it has been implicated in activity-dependent synaptic tagging controlling CamkIIα mRNA localization and translation (Sosanya *et al.*, 2015a). In confirmation of this idea, our laboratory has shown that HuD is critical for stabilizing U-rich containing mRNAs during neuronal development and synaptic plasticity such as those encoding BDNF and CaMKIIα (work from our laboratory reviewed in (Perrone-Bizzozero *et al.*, 2002; Bolognani *et al.*, 2008, 2010; Allen *et al.*, 2013; Sosanya *et al.*, 2015a; Vanevski *et al.*, 2015). Moreover, HuD has been associated with hippocampal-dependent learning (Bolognani *et al.*, 2004; Pascale *et al.*, 2004; Bolognani, Qiu, *et al.*, 2007) and seizures (Bolognani, Qiu, *et al.*, 2007), suggesting it may be involved in other activity-dependent regulatory networks. Thus, HuD is a critical regulator of multiple plasticity associated genes, thus suggesting that it may be involved in addiction-related altered gene expression, plasticity and ultimately behavior.

Since many learning paradigms have been found to be protein synthesis-dependent, RBPs that influence translation, such as HuD, may be involved. Substance use disorders, and their animal model cognate, addiction-like behaviors, are aberrant forms of learning that have been hypothesized to arise from similar molecular and electrophysiological processes as those found in other forms of associative learning and memory (Alkon *et al.*, 1991; Ann E. Kelley, 2004; Hyman, 2005; Hyman *et al.*, 2006; Koob *et al.*, 2010). The encoding of environmental cues predicting reward-availability is a feature shared by both natural and drug-induced forms of learning and memory. These strong associations with cues are thought to be involved in the chronic relapsing state of patients with substance use disorders (Childress *et al.*, 1999). CPP is an animal model useful for studying learning and motivation acquired through associations between drug effects and environmental cues present during the drug experience (see section 1b for a more detailed explanation). Acquisition and expression of CPP is thought to recruit protein-synthesis dependent mechanisms (Kuo *et al.*, 2007; Robinson *et al.*, 2007), which are critical for forming the drug-cue associations that motivate approach and contact with the cues (i.e., a model of conditioned drug-seeking behavior). Thus an RBP that regulates mRNA levels and translation before or during translation may play an integral role in SUDs.

Since both RBPs and miRNAs target the 3'UTR, this opens the possibility that these two classes of molecules could compete for access to a specific recognition site (Jing *et al.*, 2005; Bhattacharyya *et al.*, 2006; van Kouwenhove *et al.*, 2011). We have found a specific miRNA, miR-495, and HuD target a set of shared mRNAs through the same GUUUGUUUG sequence (Bolognani *et al.*, 2010); Gardiner *et al., In prep.*). Many

of these shared targets, including *Bdnf* and *Camk2a* have been implicated in addiction (Knowledgebase of addiction related genes, KARG, (Li *et al.*, 2008). With this, we hypothesized that the competition between miR-495 and HuD could inhibit or promote the expression of ARG mRNA, respectively.

Alterations in dendritic and spine structural plasticity have been documented in learning & memory, exposures to drugs of abuse, as well as addiction-related behavioral paradigms (in general, (Alvarez *et al.*, 2007); in addiction (Russo *et al.*, 2010). Alterations in these measures occur in other experience dependent situations, suggesting that structural plasticity is a learning phenomenon (reviewed in (Alvarez *et al.*, 2007). Possibly mirroring a classical symptom of addiction, these structural alterations induced by drugs of abuse inhibit the ability of other non-drug related experiences to elicit structural plasticity (Kolb *et al.*, 2003; Hamilton *et al.*, 2005). These structural alterations are very persistent similar to the chronically relapsing state of patients with addiction and suggests that reversing these structural alterations may prevent this chronic relapsing condition (McLellan *et al.*, 2000; Shen *et al.*, 2009). Additionally, multiple addiction-related behaviors are associated with alterations in these measures. For example, place preference to cocaine, but not home cage treatment, was correlated with NAcSh increases in spine density, mostly by increases in thin spines (Marie *et al.*, 2012). This suggests that drug-evoked structural alterations of neurons within discrete regions may be involved in addiction-related behaviors, especially in the NAcSh during CPP. More importantly, in the context of my proposal, two of the shared targets of HuD and miR-495, BDNF and CamKIIα, have been implicated both in CPP and in the increase in spine density and change in spine morphology associated with these behaviors (Jourdain *et al.*, 2003;

Okamoto *et al.*, 2007; Bahi *et al.*, 2008; Orefice *et al.*, 2013; Robison *et al.*, 2013; Yagishita *et al.*, 2014). Thus, this suggests that miR-495 and HuD differentially affect plasticity associated with the learning and memory found in addiction-related behaviors.

1f. Hypothesis

Combined, this led to the hypothesis that cocaine CPP differentially regulates HuD and miR-495 leading to a synergistic increase in ARG expression and addiction-like behaviors. This was tested utilizing two specific aims:

1. Test the hypothesis that HuD, miR-495 and targets within the NAc are differentially regulated by cocaine CPP training

A) Determine expression of HuD and miR-495 and two of their shared target mRNAs (*Bdnf* and *CamK2a*) after conditioned place preference (CPP) by qRT-PCR

B) Western blots

2. Test the hypothesis that miR-495 and HuD have opposite roles in CPP

A) LV mediated OE of miR-495

B) Transgenic HuD Overexpressing animals (HuD_{OE})

C) Potential rescue of the increased CPP in HuD_{OE} mice by LV-miR-495.

Although the focus of this work is on the role of HuD and miR-495 within the context of cocaine CPP, the findings related to this hypothesis as well as other supporting data illustrating the role that HuD and miR-495 may play in addiction-related behaviors and plasticity in general will be discussed in this work.

CHAPTER 2 *In silico* identification and *in vivo* validation of miR-495 as a novel regulator of motivation for cocaine that targets multiple addiction-related networks in the nucleus accumbens

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Abstract

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

Keywords: microRNA, addiction, BDNF, Camk2a, self-administration

Introduction

Substance use disorder (SUD) is a chronic, debilitating condition characterized by compulsive drug use despite negative consequences and a high recurrence of relapse even after prolonged periods of abstinence (Leshner, 1997). SUD is believed to be a dysfunction of neuroplasticity (Kalivas *et al.*, 2008), whereby altered gene expression impacts neuronal function and subsequent behavior (McClung *et al.*, 2008). Drugs of abuse cause widespread epigenetic changes to chromatin accessibility, thereby altering the transcriptional activity of several genes (Robison *et al.*, 2011; Feng *et al.*, 2013; Rogge *et al.*, 2013). However, less is known about the post-transcriptional processes that control mRNA dynamics and, ultimately, translation into functional proteins. Among the non-coding RNAs, microRNAs (miRNAs) play a critical role in the post-transcriptional control of a large number of transcripts. These small RNAs typically guide the RNA-induced silencing complex (RISC) through the binding of complementary sequences in the 3' untranslated region (3'UTR) of the target mRNAs, leading to mRNA degradation or translational repression (Bartel, 2004). A single miRNA is predicted to target hundreds of different mRNAs, and a single mRNA can be regulated by multiple miRNAs. Therefore, dysregulation of these "master" regulators impacts several cellular processes simultaneously and has been linked to many diseases and neurological disorder (Kloosterman *et al.*, 2006; Miller *et al.*, 2010; Im *et al.*, 2012; Sartor *et al.*, 2012; Bali *et al.*, 2013).

Recent studies indicate that several drugs of abuse regulate miRNA expression in the nucleus accumbens (NAc) and other regions of the brain reward pathway (Chandrasekar *et al.*, 2009; Nudelman *et al.*, 2009; Schaefer *et al.*, 2010; Eipper-Mains *et al.*, 2011; Guo

et al., 2012). In turn, in vivo manipulations of specific miRNAs or subsets of miRNAs alter the development of addiction-like behaviors in rodents (Bahi *et al.*, 2008; Hollander *et al.*, 2010; Schaefer *et al.*, 2010; Chandrasekar *et al.*, 2011; Tapocik *et al.*, 2014). Thus, we sought to identify a candidate miRNA that targets multiple addiction-related genes (ARGs) and may provide a novel mechanism for - reverting drug-induced aberrant gene expression. In this study, we used bioinformatics analyses of the 3' untranslated regions (3'UTRs) of transcripts in the Knowledgebase of Addiction-Related Genes database (KARG;(Li *et al.*, 2008) to identify miR-495, a miRNA that targets many ARGs in regulatory networks previously implicated in SUDs. We found that miR-495 is enriched within the NAc and is downregulated by acute cocaine administration and during cocaine self-administration. Viral-mediated miR-495 overexpression not only robustly downregulated ARG expression but more importantly, diminished motivation for cocaine.

Materials and Methods

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

Bioinformatics analyses. The lists for mouse, human, and rat ARGs were retrieved from the KARG database (http://karg.cbi.pku.edu.cn, (Li *et al.*, 2008). Lists of KARG genes with evidence number scores ≥2 (Li *et al.*, 2008) were used to acquire the 3'UTRs sequences from ENSEMBL BioMart. The frequencies of predicted targets of miR-495 and two previously identified addiction-related miRNAs, let-7 and miR-212 (Hollander *et al.*, 2010; Chandrasekar *et al.*, 2011), in these KARG lists vs. the respective genomes were calculated using TargetScan 6.2 (http://www.targetscan.org) conserved sites. miR-495 binding sites in the 3'UTR of the KARG/TargetScan dataset were further validated using miRanda (Enright *et al.*, 2003).

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

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

Full materials and methods for miRNA fluorescent in situ hybridization, dual luciferase assays, acute cocaine treatment, reverse transcription and qPCR, western blotting, intracranial virus injections, cocaine self-administration, food reinforcement, and histology are provided in Appendix C.

Results

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

Initial bioinformatics analyses were aimed at identifying miRNAs that target addiction-associated mRNAs expressed in the NAc. The 3'UTR sequences of mouse, human, and rat gene sets of the KARG database (http://karg.cbi.pku.edu.cn) with evidence scores ≥2 (Li *et al.*, 2008) were used to determine the prevalence of miRNA binding sites predicted by TargetScan (http://www.targetscan.org). Among the ARG-targeting miRNAs, we found that miR-495, a microRNA expressed in the adult rat striatum (http://miRBase.org, (Landgraf *et al.*, 2007; Kozomara *et al.*, 2014), is predicted to target several ARG mRNAs, such as *Bdnf*, *Camk2a*, *Arc*, and others (**Table AC.1**). The percentage of mouse KARG genes containing conserved 3'UTR miR-495 binding sites (7%, 70 genes) is significantly ($p < 0.0001$) higher than in the entire genome (2.5%), and similar results were obtained using human or rat KARG gene sets (83 genes, 7.7% and 55 genes, 5.6 %, respectively). To confirm this method, we assessed the proportion of KARG gene targets of two miRNAs previously associated with cocaine addiction, miR-212/-132 (Hollander *et al.*, 2010) and let-7 (Chandrasekar *et al.*, 2009, 2011). As expected, both miRNAs targeted a higher % of KARG genes than in the genome (miR-212/-132: 2.8%, $p < 0.0001$; let-7: 3%, $p < 0.0001$), confirming the utility of this approach to identify miRNAs associated with addiction. Given that the frequency of miR-495 targets in KARG was significantly higher ($p < 0.001$; ~2-fold) than those for miR212/-132 and let-7 (**Figure 2.1*A*, *B***), it is likely that miR-495 targets may impact a

wider variety of functions involved in addiction than those that were established for miR-212/-132 and let-7. Furthermore, the average evidence scores for miR-495 KARG targets were significantly higher than those for the whole KARG set, suggesting the association of predicted miR-495 KARG targets with addiction is heavily supported by previous research (**Figure AA.1, Mann-Whitney U,** U = 36189). Using miRanda, we further validated the presence of high affinity 3'UTR miR-495 binding sites ($\Delta G \geq -15$ kcal/mol) in the mouse dataset, including *Bdnf* and *Camk2a* (**Table AC.1**; (Enright *et al.*, 2003). Fluorescent in situ hybridization (FISH) confirmed brain-wide miR-495 expression, including the NAc and medial prefrontal cortex (mPFC), as previously reported in human mPFC tissue (**Figure 2.1*C***; scrambled locked nucleic acid (LNA) control in **Figure AC.2**; (Mellios *et al.*, 2008). Using qRT-PCR, we confirmed miR-495 expression in these regions, with the highest expression within the NAc (**Figure 2.1D**). Thus, miR-495 is a candidate regulator of a set of ARGs conserved in mammals.

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

To validate direct miR-495 binding to predicted target ARG mRNAs, we utilized luciferase reporter constructs containing target mRNA 3'UTRs. Due to differential poly(A) site usage, the predicted miR-495 target, *Bdnf*, is present in vivo as two different transcripts with a short (*Bdnf*-S) or long 3'UTR (*Bdnf*-L) produced from the same promoter. The long form contains more miR-495 binding sites (**Figure 2.1*E***; http://www.targetscan.org/mmu_50/), suggesting that miR-495 preferentially regulates *Bdnf*-L. The binding sites within the 3'UTR at nucleotide (nt) positions 233, 565, 587,

and 598 are highly conserved between mouse, rat and human, while the last binding site at nt 2487 in mouse and rat or nt 2488 in human is partially conserved. Indeed, dual-luciferase assays showed that miR-495 significantly reduced the activity of the reporter containing the 3'UTR for *Bdnf*-L by ~50% and for *Bdnf*-S by ~20% (**Figure 2.1*F***; $F_{3,7}$ = 39.4, $p < 0.01$ for *Bdnf*-L; $F_{3,7} = 9.2$, $p < 0.05$ for *Bdnf*-S). Given that these isoforms have been hypothesized to have different functions and localization within the neuron, these results suggest that miR-495 may preferentially regulate *Bdnf*-L and its associated functions (An *et al.*, 2008). Additionally, miR-495 significantly reduced the activity of reporters containing the 3'UTRs of *Camk2a* and *Arc* by ~40% and 45%, respectively (**Figure 2.1*G***; $F_{3,7}$ = 156.3, $p < 0.01$ for *Camk2a*; $F_{3,7} = 137.0$, $p < 0.01$ for *Arc*). All effects were blocked by anti-miR-495, and miR-495 had no effect on empty vectors (**Figure AC.3**; $F_{3,7} = 1.1$, p = ns). These in vitro studies demonstrate that the predicted miR-495 binding sites in these ARGs are indeed functional.

miR-495 and target mRNA expression in response to acute cocaine administration

The anatomical localization and targets of miR-495 suggest that it may play a role in the post-transcriptional mechanisms underlying addiction-related plasticity. To examine this further, we determined the effect of an acute cocaine injection (15 mg/kg, i.p.) in mice on NAc miR-495 expression at different time points. NAc miR-495 was significantly downregulated between 1-4 h post-injection (**Figure 2.2*A***; $F_{6,42} = 4.4$, $p < 0.01$; 1h ps < 0.001, 2h ps < 0.05, 4h ps < 0.05). This effect was brain region-specific, as miR-495 expression was not significantly altered by cocaine 2 h post-acute cocaine in the

mPFC or dorsal striatum (**Figure 2.2*B*, *C***; t(9) = 0.53, p = ns for mPFC; t(12) = 1.3, p = ns).

Next, we assessed the expression of two luciferase validated miR-495 targets, *Bdnf* and *Camk2a*, at the middle of this timeframe: 2 h post-injection. We found that both NAc *Bdnf*-Pan mRNA, which is the sum of *Bdnf*-S and *Bdnf*-L isoforms, and *Bdnf*-L mRNA were significantly increased 2 h post-injection (**Figure AC.4;** t(7) = 5, $p < 0.01$ for *Bdnf*-L; t(8) = 3.5, $p < 0.01$ for *Bdnf*-Pan). Although this demonstrates that *Bdnf* mRNA is upregulated by acute cocaine, it does not point to the mechanism involved in this cocaine-induced upregulation. Since both *Bdnf* transcripts originate from the same promoter, differences between the two isoforms would suggest regulation at the post-transcriptional level. To evaluate the possibility, we calculated the ratio between *Bdnf*-L and *Bdnf*-Pan and found that it was significantly increased by ~two-fold (**Figure 2.2*D***; t(7) = 2.4, $p < 0.05$), indicating that acute cocaine preferentially upregulates the long 3' UTR variant that contains a greater number of miR-495 binding sites than the short form (**Figure 2.1*E*, *F***). Additionally, we found that both proBDNF and mature BDNF protein were significantly increased within the NAc 2 h after cocaine treatment (**Figure 2.2*F*, *G***; t(12) = 2.8, $p < 0.05$ for proBDNF, t(13) = 2.4, $p < 0.05$ for matBDNF). Another luciferase-validated miR-495 target, *Camk2a*, was found to be regulated 2 h post-injection within the NAc as both mRNA (**Figure 2.2*E***; t(10) = 2.8, $p < 0.05$) and protein (**Figure 2.2*H***; t(10) = 4.8, $p < 0.001$) were increased. Thus, NAc miR-495 expression is rapidly decreased by exposure to cocaine concomitantly with increased expression of its ARG targets, *Bdnf* and *Camk2a*. This inverse relationship in cocaine-induced gene expression suggests a functional link between miR-495 and its target ARGs in vivo.

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

To further examine the regulatory relationship between cocaine-induced NAc miR-495 downregulation and upregulation of target ARG mRNAs, we next tested whether these changes could be reversed by restoring miR-495 levels in the NAc with viral-mediated overexpression. Lentivirus (LV) encoding pri-miR-495+GFP (LV-miR-495) or GFP (LV-GFP) was infused into the NAc shell (NAcsh; **Figure 2.3*A***) of male Sprague-Dawley rats who were treated 2 weeks later with saline or cocaine (15 mg/kg, i.p.). Cocaine-treated LV-GFP rats were found to express significantly lower NAc miR-495 levels compared to saline-treated LV-GFP controls (**Figure 2.3*B***; Virus, $F_{1,33} = 4.5$, $p < 0.05$; Treatment, $F_{1,33} = 70.1$, $p < 0.0001$; Virus x Treatment, $F_{1,33} = 4.6$, $p < 0.05$; Tukey HSD, $q(33) = 4.2$, $p < 0.05$ LV-GFP saline vs. LV-GFP cocaine), replicating the cocaine-induced downregulation of NAc miR-495 in mice without lentiviral infusion (**Figure 2.2*A***). LV-miR-495 rats exhibited significantly greater NAc miR-495 expression compared to LV-GFP-infused animals in either treatment group, effectively reversing the cocaine-induced decrease in miR-495 (**Figure 2.3*B;*** Tukey HSD, LV-GFP saline vs. LV-miR-495 saline $q(33) = 6.1$, $p < 0.001$; LV-GFP Cocaine vs. LV-miR-495 Cocaine $q(33) = 10.7$, $p < 0.0001$). Similarly, cocaine-treated LV-GFP rats were found to express significantly higher levels of both *Bdnf* variants compared to saline treated LV-GFP controls and LV-miR-495 animals in either drug treatment group (**Figure 2.3*C*, *D***; For *Bdnf*-Pan, Virus, $F_{1,18} = 14.2$, $p < 0.01$; Treatment, $F_{1,18} = 12.5$, $p < 0.01$; Virus x

Treatment, $F_{1,18} = 10.2$, $p < 0.01$; Tukey HSD, LV-GFP saline vs. LV-GFP cocaine, $q(18) = 6.32$, $p < 0.01$; LV-GFP cocaine vs. LV-miR-495 saline, $q(18) = 8.2$, $p < 0.0001$; LV-GFP cocaine vs. LV-miR-495 cocaine, $q(18) = 8.3$, $p < 0.0001$; For *Bdnf*-L, Virus, $F_{1,18} = 15.9$, $p < 0.01$; Treatment, $F_{1,18} = 17.0$, $p < 0.001$; Virus x Treatment, $F_{1,18} = 11.2$, $p < 0.001$; Tukey HSD, LV-GFP saline vs. LV-GFP cocaine, $q(18) = 7.0$, $p < 0.01$; LV-GFP cocaine vs. LV-miR-495 saline, $q(18) = 9.1$, $p < 0.0001$; LV-GFP cocaine vs. LV-miR-495 cocaine, $q(18) = 8.9$, $p < 0.0001$). Thus, the ability of miR-495 overexpression to reduce the cocaine-induced upregulation of both *Bdnf* transcripts suggests a functional link between miR-495 and its targets in vivo.

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

Since multiple ARGs are predicted targets of miR-495 (**Table AC.2**), we used microarray analysis to determine the global effects of NAcsh miR-495 overexpression on mRNA expression in vivo. We found that 1027 mRNAs were significantly decreased after LV-miR-495 treatment. Of the previously identified 691 miR-495 targets, 76 were significantly downregulated by miR-495 overexpression, 15 of which, including *Bdnf*, *Camk2a*, *Arc*, *Gria3* and *Stmn2*, were also present in KARG (**Table AC.2**). A complete list of miR-495 regulated transcripts on the arrays has been deposited in the Gene Expression Omnibus (GEO) database (GSE85500).

Using Ingenuity Pathway Analysis (IPA), we searched for canonical pathways enriched with NAc mRNAs downregulated by miR-495 overexpression. We found

multiple pathways that were previously characterized in addiction-related behavior, including GPCR signaling, cAMP-mediated signaling, synaptic long-term depression (LTD) and potentiation (LTP), CREB, and PKA signaling (**Figure 2.4*A***). The top biological network of interacting molecules regulated by miR-495 contained 21 of the original 76 molecules (selections in **Figure 2.4*B***, for complete list see **Table AC.3**) with functions related to drug-evoked synaptic plasticity (e.g., *Gria3*, *Shank2*, *Arc*, *Ephb2*, *Camk2a*), transcription factors (e.g., *Satb2*, *Per2*), and chromatin remodeling (e.g., *Satb2*). Using qRT-PCR, we confirmed miR-495 overexpression decreased selected target mRNAs (**Figure 2.4*C***, **Table AC.2**). Altogether, these results indicate that miR-495 regulates multiple target ARG mRNAs both in vitro (**Figure 2.1*F*, *G***) and in vivo.

NAcsh miR-495 expression decreases following cocaine self-administration

Using qRT-PCR, NAc miR-495 expression was measured in rats that self-administered cocaine for either 1 or 22 days (SA1 vs. SA22). The cocaine groups did not differ in total cocaine infusions during the test session (**Figure AC.5**; $t(6) = 0.46$, p = ns with Welch's correction). Saline-yoked controls at each time point did not significantly differ in NAcsh miR-495 and were combined. NAcsh miR-495 was significantly decreased in the SA1 and SA22 group compared to the saline group (**Figure 2.5*A***; $F_{2,17} = 9.3$, $p < 0.01$). Furthermore, a significant linear trend was found across time, where NAcsh miR-495 levels decreased as the number of cocaine self-administration sessions increased. Although baseline miR-495 expression in both NAc subregions are similar (**Figure AC.6**; $t(15) = 0.3$, p = ns), no effect on miR-495 expression was found in the

adjacent NAc core after short or long-term self-administration (**Figure AC.7**; $F_{2,20}$ = 0.47, p = ns), demonstrating NAc subregion-specific effects.

miR-495 overexpression in the NAcsh reduces motivation to self-administer and seek cocaine

To test the role of NAc miR-495 in models of addiction-related behavior, we next examined the effect of NAcsh miR-495 overexpression on self-administration and seeking behavior. Two weeks following NAcsh infusions of LV-miR-495 or LV-GFP (**Figure 2.5*B***), rats were given access to varying doses of cocaine delivered on a FR5 and PR schedule of reinforcement. NAcsh miR-495 overexpression was persistent to the end of our behavioral experiments (**Figure 2.5*C***; $t(6) = 3.7$, $p < 0.05$). As such, NAcsh miR-495 overexpression had no effect on responding or intake on the low effort FR5 schedule of cocaine reinforcement (**Figure AC.8**; Virus, F1,20 = 1.1, p = ns). However, NAcsh miR-495 overexpression significantly decreased responding and intake on the high effort PR schedule compared to controls across all cocaine doses tested (**Figure 3.5*D*, *E***; Responding, Virus, $F_{1,20} = 7.14$, $p < 0.05$; Dose, $F_{3,60} = 9$, $p < 0.01$; Virus x Dose, $F_{3,60}$ = 2.2, p = ns; Intake, Virus, $F_{1,20} = 5.5$, $p < 0.05$; Dose, $F_{2.2,44.9} = 13.7$, $p < 0.01$; Virus x Dose, $F_{2.2,44.9} = 0.65$, p = ns), without effects on inactive lever pressing (**Figure AC.9*A***). The cumulative response records in **Figure 2.5*F*** show a lower break point in a representative LV-miR-495 rat compared to a LV-GFP rat, consistent with a decrease in motivation.

Next, we tested the effect of NAcsh miR-495 overexpression on other measures of motivation, including extinction and reinstatement. Before extinction testing, rats received >3 sessions on an FR5 schedule of cocaine reinforcement on the training dose. We found no group difference in active lever pressing during this baseline before extinction (**Figure 2.5*G***; $t(17) = 0.7$, p = ns). During extinction, NAcsh miR-495 overexpression decreased active lever pressing during the first 3 sessions compared to LV-GFP controls (**Figure 2.5*G***), without effects on inactive lever pressing (**Figure AC.9*B***). We then tested rats for both cue and cocaine-primed reinstatement of cocaine seeking. While the virus groups did not differ in the degree of cue reinstatement (**Figure AC.10**), the LV-miR-495 group exhibited significantly reduced cocaine-primed reinstatement compared to the LV-GFP controls (**Figure 2.5*H***; Virus, $F_{1,20} = 6.6$, $p < 0.05$; Day, $F_{1,20} = 9$, $p < 0.001$; Virus x Dose, $F_{1,20} = 6.3$, $p < 0.05$), without differences in inactive lever pressing in either case (**Figure AC9.*C*, *D***). To confirm the effect of miR-495 overexpression on target regulation during drug-seeking behavior, we measured the levels of *Camk2a*, *Bdnf*-L, and *Bdnf*-Pan mRNAs in the NAcsh of rats 1 week after extinction and reinstatement experiments. As shown in **Figures 2.5 *I-K*** the levels of these three target mRNAs were significantly decreased by miR-495 OE (*Camk2a*, $t(12) = 3.3$, $p < 0.01$; *Bdnf*-L, $t(10) = 3.2$, $p < 0.01$; *Bdnf*-Pan $t(11) = 3.2$, $p < 0.01$). Collectively, these results suggest that NAcsh miR-495 overexpression decreases motivation to both self-administer and seek cocaine. The additional decrease in target gene expression suggests that the behavioral effect of miR-495 overexpression is mediated through these ARGs.

To test the specificity for cocaine and evaluate any potential locomotor-suppressing effects of miR-495 overexpression, a separate group of rats were trained to lever press for food pellets while receiving either LV-GFP or LV-miR-495. LV-miR-495 had no effect on responding or intake on an FR5 schedule of food reinforcement (**Figure AC.11**; Active lever, Virus, $F_{1,14} = 1.16$, p = ns; Day, $F_{6,84} = 4.08$, $p < 0.01$; Virus x Day, $F_{6,84} = 0.5$, p = ns). To parallel differences in motivational value of low and high cocaine doses on a PR schedule, we subjected rats to varying levels of food restriction. Rats that had been food-restricted (18g/day) exhibited higher PR measures than those that had been unrestricted, but both virus groups exhibited similar levels of motivation for food under both feeding conditions (**Figure 2.5*L***, **Figure AC.12**; For active lever, Virus, $F_{1,14} = 0.02$, p = ns; Food Restriction, $F_{1,14} = 55.26$, $p < 0.001$; Virus x Food Restriction, $F_{1,14} = 0.003$, p = ns). This suggests a selective effect of miR-495 overexpression on motivation for cocaine.

Discussion

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

In contrast to other addiction-related miRNAs whose expression levels were shown to increase in response to drugs of abuse (Chandrasekar *et al.*, 2009; Nudelman *et al.*, 2009; Hollander *et al.*, 2010; Schaefer *et al.*, 2010), miR-495 expression in the NAc decreased shortly after exposure to cocaine. This rapid downregulation 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 administration. The decrease in miR-495 expression may also be due to transcriptional repression. miR-495 is located within miRNA cluster B of the Dlk1-Dio3 maternally imprinted region that is under the control of Methyl-CpG-binding protein 2 (MeCP2) and other transcription factors (Benetatos *et al.*, 2013). Using Mecp2-null mice, Wu and colleagues (Wu *et al.*, 2010) demonstrated increased expression of many of the miRNAs within this imprinted region, including miR-495. However, given that not all of the miRNAs in this cluster are coordinately regulated (Wu *et al.*, 2010; Lempiäinen *et al.*, 2013), 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). It is curious that we did not observe changes in miR-495 in any other addiction-related brain region, especially in those that are also innervated by ventral tegmental dopamine neurons (e.g., dorsal striatum, mPFC). One possibility is that the relatively high basal expression levels of miR-495 in the NAc allowed us to detect a decrease following cocaine administration. Another possibility is that the NAc may have a more robust response to the acute and chronic effects of cocaine resulting in decreases in miR-495 expression levels.

miRNAs play a role in fine-tuning gene expression involved in many cell signaling pathways (Schratt, 2009; Feng *et al.*, 2013). Here, we established that miR-495 directly targets and regulates the ARG *Bdnf* both in vitro and in vivo. *Bdnf* in the NAc has been linked to several drug abuse-related behaviors, where BDNF expression levels positively correlate with cocaine reward and motivation (Horger *et al.*, 1999; Hall *et al.*, 2003; Graham *et al.*, 2007, 2009; Bahi *et al.*, 2008). Similarly, others have established

that another miR-495 target, *Camk2a*, has a positive relationship between NAc levels and psychostimulant abuse-related behavior (Anderson *et al.*, 2008; J. A. Loweth *et al.*, 2010a; Wang *et al.*, 2010; Kourrich *et al.*, 2012a). Furthermore, CaMKII has been identified in silico as a central node in positive feedback gene regulatory pathways involved in addiction (Li *et al.*, 2008). Therefore, regulation of both genes by miR-495 may coordinately affect several domains of addiction-related processes. Indeed, addiction is hypothesized to be a dysfunction of neuroplasticity (Kalivas *et al.*, 2008), and both *Bdnf* and *Camk2a* genes encode for plasticity-related proteins. We found that many other miR-495 targets we validated also form networks involved in LTP and LTD (Kauer *et al.*, 2007; Kasanetz *et al.*, 2010; Russo *et al.*, 2010), as well as other cell signaling cascades relevant to addiction, such as PKA signaling Self, Boudreau (Self *et al.*, 1998b; Boudreau *et al.*, 2009). Therefore, miR-495 may act as a mechanism to fine tune the molecular response of multiple, interwoven pathways involved in the development of addiction.

Similar to acute cocaine administration, NAcsh miR-495 levels were also decreased following both brief and prolonged cocaine self-administration. It is unknown why this occurred exclusively in the NAc shell, and not the core, but this effect may be due to the differential afferent projections into these subregions or the involvement of the shell in the primary reinforcing and unconditioned effects of cocaine (Ito *et al.*, 2004; Yager *et al.*, 2015). Interestingly, these effects are consistent with previous findings that both BDNF and CaMKIIα involvement in cocaine abuse-related behavior is also specific to the NAcsh, and not the core (Graham *et al.*, 2007; Anderson *et al.*, 2008; J. A. Loweth *et al.*, 2010b; Wang *et al.*, 2010; Kourrich *et al.*, 2012a; Robison *et al.*, 2013). Furthermore, the downward trend of NAcsh miR-495 expression as cocaine self-

administration experience increased may be related to the theory of incentive sensitization, where motivation to seek drug (i.e. 'wanting') increases over the course of drug use (Robinson *et al.*, 1993). Thus, decreases in NAcsh miR-495 expression may be indicative of sensitized motivation.

To test this hypothesis, we examined the effect of NAcsh miR-495 overexpression on cocaine self-administration and seeking behavior. We observed that NAcsh miR-495 overexpression decreased responding and intake when cocaine was available on the high effort PR schedule, but did not alter intake in the low effort FR5 schedule. PR schedules are believed to model an aspect of SUD related to an individual increasing time and energy toward drug-seeking and -taking behavior (Roberts *et al.*, 2007), thus alterations in these behaviors closely model hallmark symptoms of human addiction. Furthermore, NAc miR-495 overexpression did not alter PR measures in a similar procedure with a natural food reinforcer. This suggests that miR-495 specifically influences motivation for cocaine likely without impacting the reinforcing value of cocaine or food reinforcement/motivation. Additionally, we found that NAcsh miR-495 overexpression reduced cocaine-seeking behavior during extinction and reinstatement, further supporting a selective reduction in motivation. We also confirmed that under these conditions, miR-495 overexpression resulted in decreases in *Bdnf* and *Camk2a* expression. Closely mirroring our effects, previous work has shown that NAcsh knockdown of *Camk2a* and inhibition of NAcsh CAMKII reduces PR measures and reinstatement, respectively (Anderson *et al.*, 2008; Wang *et al.*, 2010). Thus, CaMKIIα is an important regulatory crux of many addiction-related molecular pathways (Wu *et al.*, 2001; Anderson *et al.*, 2008; J. A. Loweth *et al.*, 2010; Kourrich *et al.*, 2012a; Robison *et al.*, 2013). Although

we did not detect a statistically significant effect during cue reinstatement ($p = 0.06$, one-tailed), the pattern is very similar. The lack of a more robust effect on cue reinstatement may highlight the more prominent role of the NAc core, rather than the shell, in regulating cue reinstatement. Overall, our results suggest that miR-495 preferentially regulates a network of ARG targets involved in the incentive motivational properties of cocaine which are more critical for sustaining behavior under the high-effort PR schedule of reinforcement than under the low-effort FR5 schedule of reinforcement.

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

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by NIDA grants 1R01DA034097 (N.P.B. & J.L.N.), F31DA035069 (R.M.B.), and T32AA014127, F31DA041214 (R.J.O.). We are grateful to Dr. Yue Feng at Emory University for providing the luciferase-*Bdnf* 3' UTR constructs. We also wish to thank Timothy Cheung, Nora Dado, Peter Kufahl, Sayuri Nixon, Karen Mooneyhan, Jason Newbern, Natalie Peartree, Erika Perez, Lara Pockros, Justine Saavedra, and Jennifer Taylor for their technical assistance.

See Appendix C for Supplementary information.

Figures

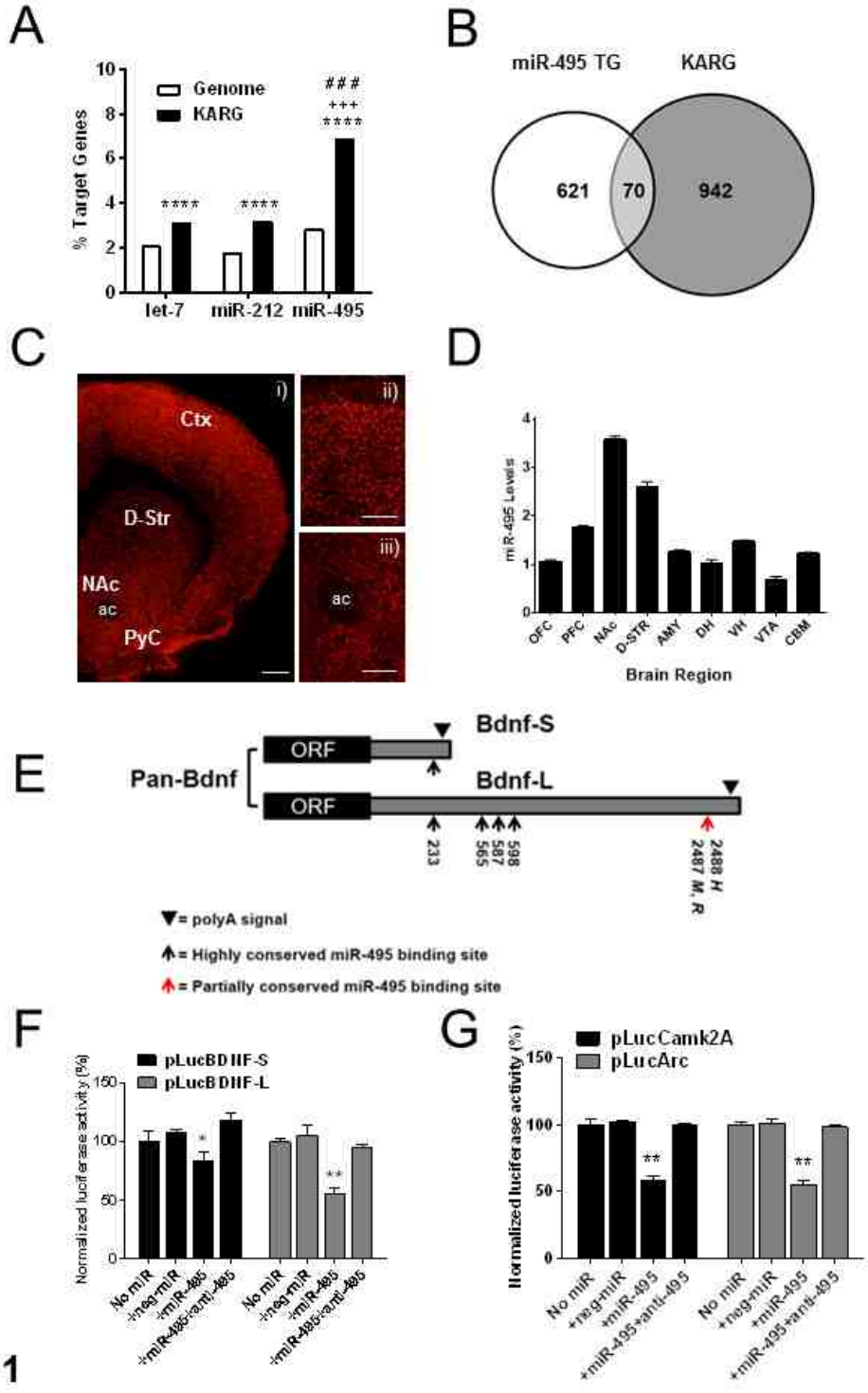


Fig. 1

Fig. 2.1. miR-495 targets several addiction-related genes (ARGs) and is expressed in addiction-related brain regions. (*A*) Although the frequencies of miR-495, miR-212/132 and let-7 putative targets are all enriched in the KARG database compared to the entire genome, the frequency of miR-495 targets in KARG is significantly higher (~2-

fold) than those of miR-212/132 and let-7. ****$p < 0.0001$ vs. genome, $^{+++}p < 0.001$ miR-495 vs. miR-212/132 and $^{\#\#\#}$ p< 0.001 miR-495 vs. let-7, two-tailed Chi square test. (*B*) Number of genes with putative miR-495 target sites (miR-495 TG) in the mouse KARG set. (*C*) Representative images of a coronal mouse brain section where miR-495 was visualized using fluorescent *in situ* hybridization at 4x (*i*), with insets at 10x focusing on the PFC (*ii*) and the NAc (*iii*). Scale bars 500 μm in panel *i* and 200 μm in *ii* and *iii*. (*D*) qRT-PCR analysis of miR-495 levels in different brain regions (n = 3). (*E*) Schematic representation of the short and long 3'UTR transcripts of BDNF including the positions of conserved and partially conserved miR-495 binding sites (*M = Mus musculus*, *R = Rattus norvegicus*, *H = Homo sapiens*). For *in vitro* target validation, HeLa cells were transfected with a firefly luciferase reporter containing the 3'UTR of *Bdnf* and *Camk2a*. A *Renilla* vector was co-transfected with the firefly reporter. Pre-miR-495, anti-miR-495 and pre-miR™ miRNA precursor negative control #2 were transfected as described in Supplementary Information. The alternative 3'UTRs of BDNF (*F*), as well as the 3'UTRs of *Camk2a* and *Arc,* were assayed (*G*). n = 4 *$p < 0.05$, **$p < 0.01$. Error bars indicate SEM. Ctx = neocortex, PyC = pyriform cortex, ac = anterior commissure. OFC = orbitofrontal cortex, PFC = prefrontal cortex, NAc = nucleus accumbens, D-STR = dorsal striatum, AMY = amygdala, DH = dorsal hippocampus, VH = ventral hippocampus, VTA = ventral tegmental area, CBM = cerebellum.

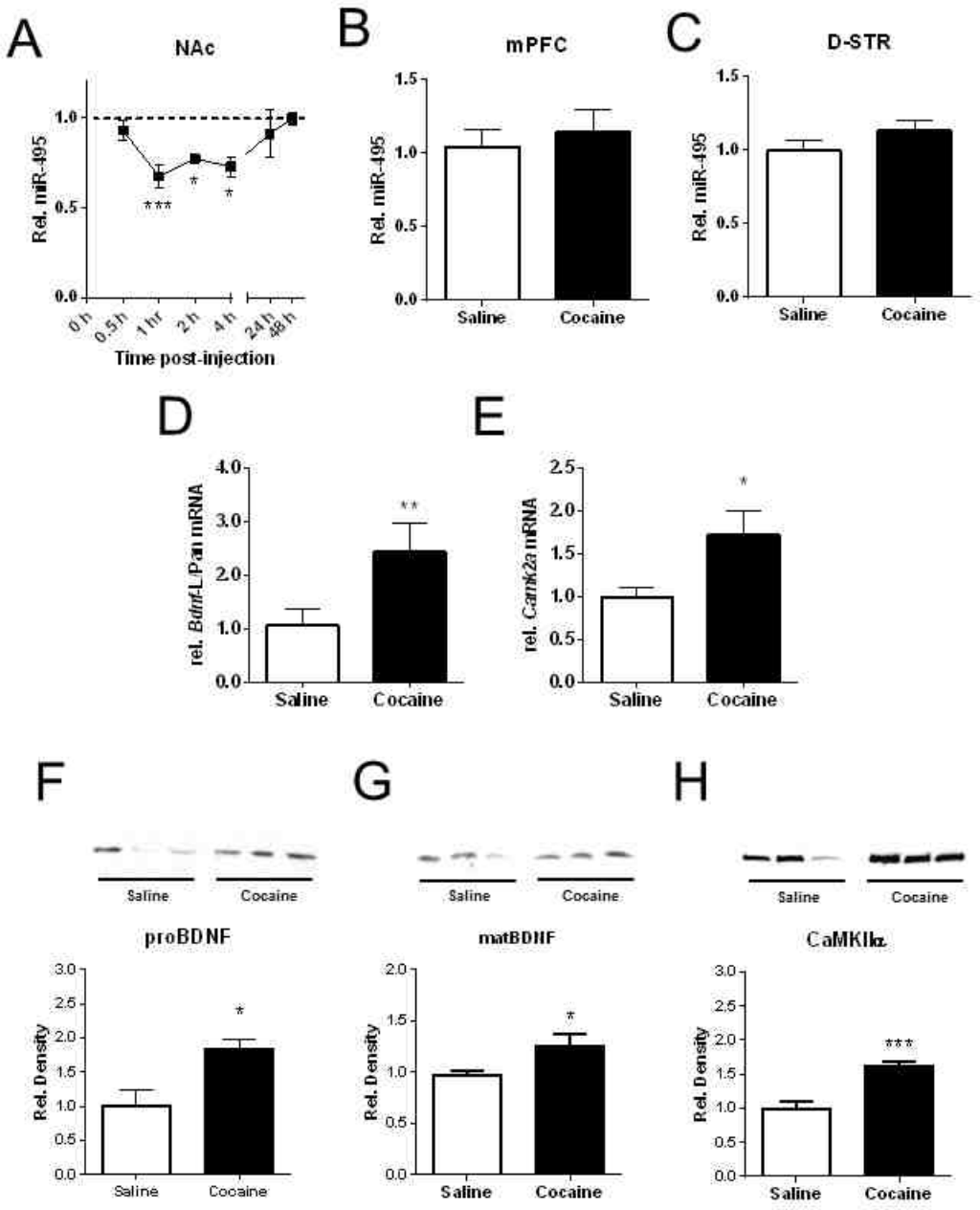


Fig. 2

Fig. 2.2. Acute cocaine effects on NAc miR-495 and target mRNA expression. Male C57Bl/6 mice received an acute injection of saline or cocaine (15 mg/kg, i.p.) and NAc tissue was processed for qRT-PCR and Western blot. (*A*) NAc miR-495 levels were

found to be downregulated rapidly after acute cocaine (0.5 h, n = 5; 1 h, n = 10; 2 h, n = 4; 4 h, n = 6; 24 h, n = 6; 48 h, n = 5). miR-495 expression was not altered by acute cocaine 2h after within the medial prefrontal cortex (B; mPFC; coc n = 5, sal n = 6) or dorsal striatum (C; DS; coc n = 6, sal n = 8). Acute cocaine increases expression of NAc *Bdnf*-L relative to pan-*Bdnf* nearly two-fold, as measured by qRT-PCR (*D*; coc n = 4, sal n = 5). (*E*) Acute cocaine also increased *Camk2a* mRNA at 2 h (n = 5). NAc pro-BDNF (*F*), mature BDNF (*G*) and CaMKIIα (*H*) increases in protein levels were also found by Western blot 2 h post-cocaine or saline injection, corrected for total protein by Coomassie Brilliant Blue staining (proBDNF, coc n = 9, sal n = 7; matBDNF, coc n = 9, sal n = 8; CaMKIIα n = 6/group, representative blot with each lane representing individual animals; bars represent quantification of average density of each sample from duplicate blots). Error bars indicate SEM. *$p < 0.05$, **$p < 0.01$ vs. saline.

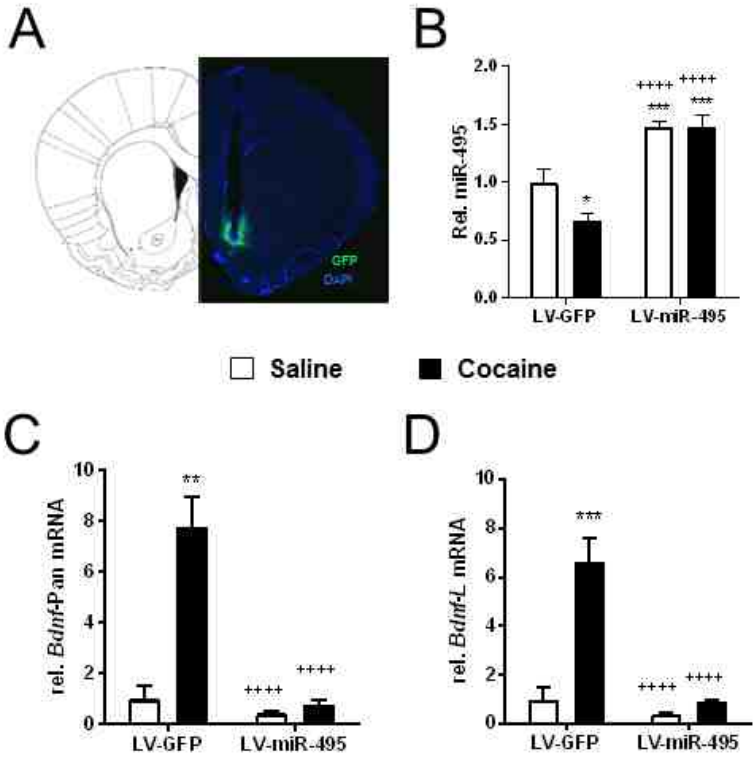
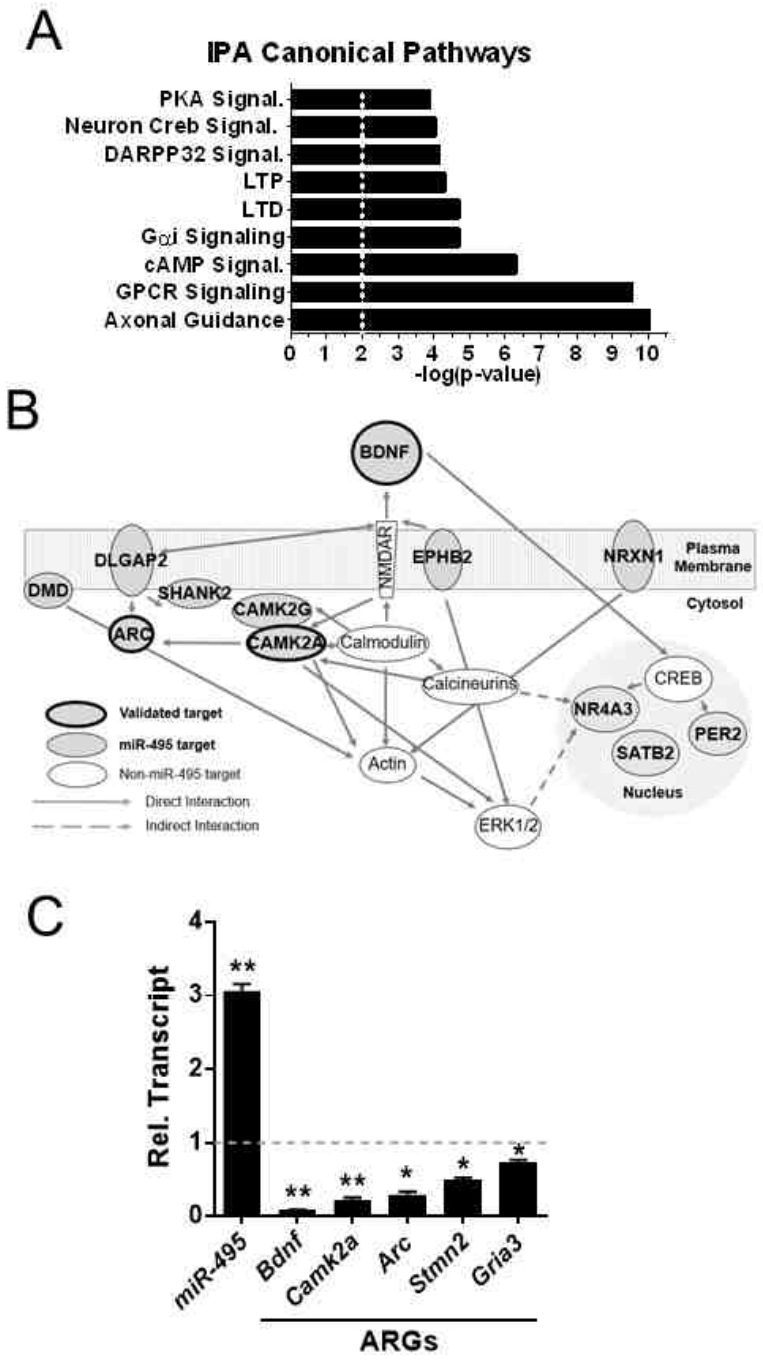


Fig. 2.3. NAc miR-495 overexpression counteracts cocaine-induced changes in gene expression. Two weeks following infusion of either LV-GFP or LV-miR-495 into the NAcsh (*A*), rats received an injection of saline or cocaine (15 mg/kg, i.p.). LV-miR-495 blocked the cocaine-induced decrease in NAc miR-495 expression 2-h post-injection (*B*) and prevented the cocaine-induced increase in NAc pan-*Bdnf* (C) and *Bdnf*-L (*D*) expression. LV-GFP-saline: n = 3; LV-miR-495-saline: n = 5; LV-GFP-cocaine: n = 8; LV-miR-495-cocaine: n = 6. Error bars indicate SEM. *p < 0.05, ** < 0.01, *** < 0.001 vs. saline treated LV-GFP rats and $^{++++}$ p < 0.0001 vs. cocaine treated LV-GFP.


A
IPA Canonical Pathways
PKA Signal.
Neuron Creb Signal.
DARPP32 Signal.
LTP
LTD
Gαi Signaling
cAMP Signal.
GPCR Signaling
Axonal Guidance
0 1 2 3 4 5 6 7 8 9 10
-log(p-value)
B
BDNF
NMDAR
EPHB2
NRXN1
Plasma Membrane
Cytosol
DLGAP2
DMD
SHANK2
CAMK2G
ARC
CAMK2A
Calmodulin
Calcineurins
CREB
NR4A3
PER2
SATB2
Nucleus
Actin
ERK1/2
Validated target
miR-495 target
Non-miR-495 target
Direct Interaction
Indirect Interaction
C
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0
1
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miR-495
Bdnf
Camk2a
Arc
Stmn2
Gria3
ARGs

Fig. 4

Fig. 2.4. miR-495 regulates genes involved in several addiction-related networks. Rats were infused with either LV-GFP or LV-miR-495 into the NAcsh and 1 week later NAc RNA was collected for both microarray and qRT-PCR analysis (n = 5/group). (*A*) Top canonical pathways enriched in genes downregulated by miR-495 overexpression (vertical dotted line represents threshold, $p < 0.01$) were determined using Ingenuity Pathway Analysis (IPA). (*B*) A graphical representation of the genes associated with the top signaling network denotes the presence of predicted and validated miR-495 targets. (*C*) LV-miR-495 infusion increased miR-495 expression and decreased expression of several ARGs compared to LV-GFP controls (dotted line), as measured by qRT-PCR. Error bars indicate SEM. $*p < 0.05$, $**p < 0.01$ compared to controls.

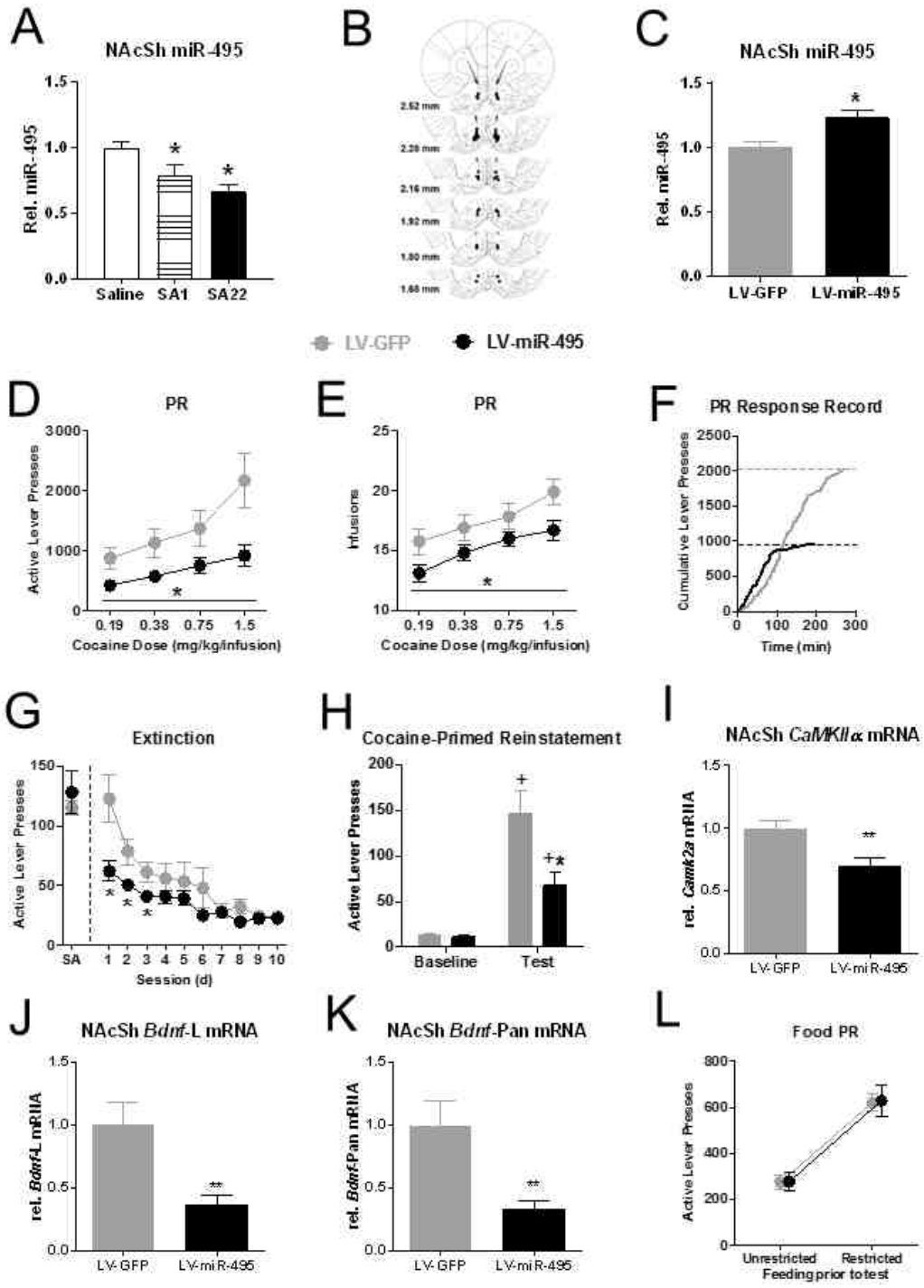
A
NAcSh miR-495
Rel. miR-495
Saline
SA1
SA22
B
2.52 mm
2.28 mm
2.16 mm
1.92 mm
1.80 mm
1.68 mm
C
NAcSh miR-495
Rel. miR-495
LV-GFP
LV-miR-495
LV-GFP
LV-miR-495
D
PR
Active Lever Presses
Cocaine Dose (mg/kg/infusion)
E
PR
Infusions
Cocaine Dose (mg/kg/infusion)
F
PR Response Record
Cumulative Lever Presses
Time (min)
G
Extinction
Active Lever Presses
SA
Session (d)
H
Cocaine-Primed Reinstatement
Active Lever Presses
Baseline
Test
I
NAcSh CaMKIIα mRNA
rel. Camk2a mRNA
LV-GFP
LV-miR-495
J
NAcSh Bdnf-L mRNA
rel. Bdnf-L mRNA
LV-GFP
LV-miR-495
K
NAcSh Bdnf-Pan mRNA
rel. Bdnf-Pan mRNA
LV-GFP
LV-miR-495
L
Food PR
Active Lever Presses
Unrestricted
Restricted
Feeding prior to test

Fig. 5

Fig. 2.5. miR-495 overexpression in the NAcsh suppresses motivation to self-administer and seek cocaine in rats. (A) Endogenous NAcsh miR-495 levels are suppressed following 1 and 22 days of cocaine self-administration, as measured by qRT-PCR (Saline: n = 7; SA1: n = 6; SA22: n = 7). *$p < 0.05$ vs. Saline-yoked. (*B)* Histological verification of NAcsh cannula placement. (*C*) NAcsh miR-495 levels are increased in the LV-miR-495 group compared to the LV-GFP group following behavioral testing (n = 4/group). NAcsh miR-495 overexpression reduced responding (*D*) and intake (*E*) on a PR schedule of cocaine reinforcement. (*F*) Cumulative response records of representative rats tested at 1.5 mg/kg/infusion that were closest to their respective group means. Dotted line represents the total number of lever presses emitted once break point was achieved. (*G*) NAcsh miR-495 overexpression reduced cocaine-seeking behavior during the first three 1-h extinction sessions. (*H*) NAcsh miR-495 overexpression reduced cocaine-primed reinstatement during the 1-h test session (10 mg/kg, i.p.). LV-GFP: n = 12, LV-miR-495: n = 10. (*I-K*) Downregulation of *Camk2a* (LV-GFP: n = 7, LV miR-495: n = 8, $p < 0.01$) and *Bdnf*-L and -Pan (n = 6/group, $p < 0.01$) in NAcsh of miR-495 OE animals after behavioral testing. (*L*) NAcsh miR-495 overexpression had no effect on responding under a PR schedule of food reinforcement, regardless of whether the rats were food restricted (18g/day) or not (n = 8/group). Error bars indicate SEM. Panels C-H: *$p < 0.05$ vs. LV-GFP. $^{+}p < 0.05$ vs. extinction baseline.

CHAPTER 3: Neuronal RNA-binding protein HuD regulates addiction-related gene expression and behavior

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Key words: RNA binding protein, HuD, post-transcriptional regulation, Nucleus Accumbens, CPP, CaMKIIα, BDNF

Abstract

The neuronal RNA-binding protein HuD is involved in synaptic plasticity and learning and memory mechanisms. These effects are thought to be due to HuD-mediated stabilization and translation of target mRNAs associated with plasticity. To investigate the potential role of HuD in drug addiction, we first used bioinformatic prediction algorithms together with microarray analyses to search for specific genes and functional networks upregulated within the forebrain of HuD overexpressing mice (HuD_{OE}). When this set was further limited to genes in the Knowledgebase of Addiction-related gene databases (KARG) that contain predicted HuD-binding sites in their 3' untranslated regions (3' UTR), we found that HuD regulates networks that have been associated with addiction-like behavior. These genes included *Bdnf* and *Camk2a*, two previously validated HuD targets. Since addiction is hypothesized to be a disorder stemming from altered gene expression causing aberrant plasticity, we sought to test the role of HuD in cocaine conditioned placed preference (CPP), a model of addiction-related behaviors. HuD mRNA and protein were upregulated by CPP within the nucleus accumbens (NAc) of wild type C57BL/6J mice. These changes were associated with increased expression of *Bdnf* and *Camk2a* mRNA and protein. To test this further, we trained HuD_{OE} animals and wild type littermates in CPP and found that HuD_{OE} mice showed increased cocaine CPP compared to controls. This was also associated with elevated expression of HuD target mRNAs and proteins, CaMKIIα and BDNF. These findings suggest HuD involvement in addiction-related behaviors such as cocaine conditioning and seeking, through increased plasticity-related gene expression.

Introduction

Post-transcriptional mechanisms affect gene expression at multiple levels, including the control of mRNA stability or ability of recently transcribed mRNA to be translated into functional protein (Bevilacqua *et al.*, 2003). This set of regulatory mechanisms may be even more integral to neuronal physiology because many neuronal proteins have a variety of isoforms generated through alternative splicing, the neuronal structure requires long-distance transport of mRNAs, and de novo protein synthesis can occur away from the cell body (Lee *et al.*, 2003; Lipscombe, 2005; Sutton *et al.*, 2006; Hengst *et al.*, 2007). Given the simultaneous influence over mRNA processing, stability and protein translation, it is not surprising that post-transcriptional regulation plays a role in many behaviors (Dracheva *et al.*, 2009; Im *et al.*, 2010; Lin *et al.*, 2011; Tan *et al.*, 2013; Smith *et al.*, 2014; Tapocik *et al.*, 2014; Schmidt *et al.*, 2015; Bastle *et al.*, 2017; Yan *et al.*, 2017).

HuD is a neuronal RNA binding protein (RBP; (Perrone-Bizzozero *et al.*, 2002) that stabilizes specific target mRNAs, such as those encoding BDNF and CaMKIIα, through direct interaction with their 3'UTRs (Bolognani *et al.*, 2010; Allen *et al.*, 2013; Sosanya *et al.*, 2015). HuD is localized to the dendritic compartment of hippocampal neurons (Bolognani *et al.*, 2004), where it has been implicated in activity-dependent synaptic tagging controlling CamkIIα mRNA localization and translation (Sosanya *et al.*, 2015). Additionally, neuronal activity regulates HuD expression, localization, and association with plasticity associated mRNAs such as *Homer1a* and *Camk2a* (Tiruchinapalli, Ehlers, *et al.*, 2008). Recent studies indicate that HuD also plays a role in translation (Fukao *et al.*, 2009) and that PKC-mediated phosphorylation of HuD increases

local translation of BDNF (Vanevski *et al.*, 2015), suggesting that HuD may be involved in protein synthesis-dependent forms of plasticity. Additionally, HuD can regulate alternative RNA processing and localization these mRNA transcripts (Sosanya, Peggy P.C. Huang, *et al.*, 2013). Therefore, HuD is involved in nearly every level of post-transcriptional regulation. Moreover, HuD has been associated with hippocampal-dependent learning and seizures (Bolognani *et al.*, 2004; Pascale *et al.*, 2004; Bolognani, Tanner, *et al.*, 2007), suggesting it may be involved in other activity-dependent regulatory networks. A single RBP, such as HuD, can associate with a number of target mRNAs via binding to specific nucleotide sequences in the 3' untranslated regions (3'UTRs). In fact, HuD has been predicted to bind about 20% of the mouse forebrain transcripts (Bolognani *et al.*, 2010). Thus, HuD and related RBPs have been termed "master switches" of gene regulation (Deschênes-Furry *et al.*, 2006; Keene, 2007).

Substance use disorders, and their animal model cognate, addiction-like behaviors, are aberrant forms of learning that have been hypothesized to arise from similar molecular and electrophysiological processes as those found in other forms of associative learning and memory (Alkon *et al.*, 1991; Ann E Kelley, 2004; Hyman, 2005; Hyman *et al.*, 2006; Koob *et al.*, 2010). The encoding of environmental cues predicting reward-availability is a feature shared by both natural and drug-induced forms of learning and memory. These strong associations with cues are thought to be involved in the chronic relapsing state of patients with substance use disorders (Childress *et al.*, 1999). Drug-conditioned place preference (CPP) is an animal model useful for studying learning and motivation acquired through associations between drug effects and environmental cues present during the drug experience. Acquisition and expression of CPP is thought to

recruit protein-synthesis dependent mechanisms (Kuo *et al.*, 2007; Robinson *et al.*, 2007), which are critical for forming the drug-cue associations that motivate approach and contact with the cues (i.e., a model of conditioned drug-seeking behavior; (Tzschentke, 2007). Since HuD mRNA targets have been suggested to play a role in general plasticity and learning, as well as cocaine-induced behaviors (Graham *et al.*, 2007; Anderson *et al.*, 2008), we hypothesized that HuD regulation of mRNAs, such as *Camk2a* and *Bdnf*, may play a role in cocaine-CPP.

Methods

Transgenic mice overexpressing HuD (HuD_{OE}) under the *Camk2a* promoter were generated and backcrossed to a C57BL/6J background for >20 generations (Bolognani, Qiu, *et al.*, 2007). Control animals were either wild type littermates or standard C57Bl/6 mice. Animals were maintained on a 12 h reverse light/dark cycle. Experiments were performed in accordance with NIH Animal Welfare guidelines under protocols approved by the Institutional Animal Care and Use committees at the University of New Mexico.

To identify the in vivo regulatory program induced by HuD, either directly or indirectly, RNA samples from transgenic mice overexpressing HuD and control littermates were run on Affymetrix 430 2.0 microarrays (n = 3/group). The forebrain, which includes the cerebral cortex, basal ganglia, hypothalamus, thalamus, subthalamus, and hippocampus, was isolated by removal of the olfactory bulb and cerebellum from the entire brain. RNA was extracted from this tissue using a standard Trizol isolation technique. Molecules that were upregulated by a fold change of ≥1.25 were selected as a set of transcripts regulated by HuD. Within this set, putative HuD targets were identified by searching for HuD binding sites found within the 3'UTR using Perl Biomodules as described in (Bolognani *et al.*, 2010). Briefly, to search for these motifs, we used a Perl script to search for the following sequences, allowing for one mismatch: [CG][CT][CT]TC[CT][CT]TC[TC]C[TC]C, [TG]TTTGTTT[TG][GT]TTT, and TTTTTTTTT[TA]AAA, for motifs 1, 2 and 3, respectively.

Next, the list for mouse ARGs were retrieved from the Knowledgebase of Addiction-Related Genes database (KARG; http://karg.cbi.pku.edu.cn; accessed in 2016 (Li *et al.*, 2008). High confidence molecules are designated by the database through

evidence score numbers, or number of evidences of their association with addiction from categories such as microarray data and low-throughput evidence. Thus, high confidence molecules from the mouse KARG database with evidence scores ≥2 were cross-analyzed with the identified molecules regulated by HuD.

For mRNA analyses, brains from HuD_{OE} mice were isolated and quickly frozen in isopentane cooled at -40 C° by a methanol-dry ice bath. They were then dissected using a brain matrix and the nucleus accumbens was punched using a 1.25 mm brain punch (Harris Unicore™). Total RNA was isolated and mRNAs quantified using Power SYBR Green PCR Master Mix (Life Technologies) as described previously (Bullock *et al.*, 2009), with primers designed against mouse HuD (Mansfield *et al.*, 2012) and selected target mRNAs (*Bdnf*-L: Forward TGGCCTAACAGTGTTTGCAG, Reverse GGATTTGAGTGTGGTTCTCC; *Camk2a*: Forward TATCCGCATCACTCAGTACCTG, Reverse GAACTGGACGATCTGCCATTT); and compared to a reference transcript (*Gapdh*: Forward TGTGATGGGTGTGAACCACGAGAA, Reverse GAGCCCTTCCACAATGCCAAAGTT). Relative expression was determined using the comparative 2-ΔCt method (Livak & Schmittgen 2001). A no reverse transcriptase (RT) reaction was run for each sample and none of the no-RT controls amplified. qPCR experiments were replicated on 2-3 separate occasions. Data collected from each run were averaged together.

For protein analysis, NAc tissue was homogenized with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) supplemented with fresh protease inhibitors (cOmplete™ Mini, Roche, Indianapolis, IN, # 4693159001). Lysates

were pre-cleared by centrifugation at 14,000 x g and then used for protein determination. Aliquots containing 35 µg of total protein were diluted 1:1 in 2x Laemmli Sample Buffer (Sigma, # S3401) and run on 4-15% SDS polyacrylamide gels (Bio-Rad, #4568086). Western blots were performed as previously described (Tanner *et al.*, 2008) using antibodies to CaMKIIα (1:2000; Santa Cruz, Dallas, TX, USA, # sc-13141), mature BDNF (1:1000; Icosagen, Õssu, Ülenurme, Tartumaa, Estonia, #327-100), proBDNF (1:1000; Alomone, Jersusalem, Israel, #AGP-032), HuD (1: 2000 Santa Cruz, Dallas, TX, USA, # sc-28299), or GAPDH(1:5000; Santa Cruz, Dallas, TX, USA # sc-32233). Membranes were then incubated for 1 h in either secondary goat anti-mouse HRP (1:5000; Santa Cruz, # sc-2005) or goat anti-guinea pig HRP (1:5000; Santa Cruz, # sc-2438), respectively, and were developed with standard chemiluminescent reagents and procedures (NEL103001EA, Perkin Elmer, Waltham, MA, USA). Each sample n is exactly one animal with both hemispheres pooled together. Specific bands that correspond to the correct molecular weight of the target (~14 kDa for mature BDNF, ~38 kDa for proBDNF, ~38 for GAPDH, ~50 for CaMKIIα) were quantified using densitometric analysis in ImageJ and then standardized by pixel density to the Coomassie Brilliant Blue stain of total protein as described previously (Tanner et al. 2008). Westerns were replicated 2-3 times and data were averaged together.

For CPP, 2 month old Male C57Bl/6J mice (n = 20/group) underwent training as previously described (Allan *et al.*, 2001). The conditioning apparatus is a set of two 20x20x20 Plexiglass chambers connected by a 9x10x20 cm anteroom, with the walls either entirely black or vertical black and white stripes in addition to either textured plastic or metal lattice floors to allow for multidimensional discrimination between

chambers. Experiments were performed under red light at 30-34 lux. On the first day, animals received a saline injection and were allowed to roam throughout the apparatus for 10 minutes to habituate to the chambers. Time spent in each chamber was recorded in the habituation session with an Ethovision XT8 system calibrated for C57Bl/6J mice on the chamber background. Data were recorded in the habitation session to determine if the animals inherently preferred either chamber, confirming these mice showed no preference for either chamber. Conditioning to non-contingent injections took place over the next three days, and on each day animals were confined to one of the chambers for 30 min, and 5h later were confined to the other chamber for 30 min. Mice were returned to their home cages between sessions. In one experiment, wild type mice were randomly assigned to either a conditioned group that received cocaine immediately before placement into the conditioned stimulus (Cs+) chamber and saline immediately before placement into the other (Cs-) chamber, or a control group that received saline immediately before placement into either chamber. In the subsequent experiment, wild type mice were compared to HuD_{OE} mice and all were cocaine-conditioned as above. The chamber assigned as the Cs+ was randomly assigned assuring unbiased equal numbers assigned to each chamber in each group. The session during which conditioned mice received cocaine was counterbalanced across AM/PM sessions. Finally, on the 5th day, animals were given a saline injection and allowed to freely explore the entire apparatus for 20 minutes. Using the Ethovision XT8, preference was calculated by time spent in CS+ versus CS-. The first 5 minutes of the test day was not analyzed to remove the effects of the aversive injection, such as post-injection grooming. Animals were

sacrificed 1 h following the saline injection. Behavioral and biochemical measures were analyzed using Student t tests using SPSS 24.0 (IBM, Armonk, NY, USA).

Results

Previously, it has been shown that HuD regulates a number of genes associated with memory, learning, and plasticity through stabilization and translation of the mRNA transcript (Tiruchinapalli, Ehlers, *et al.*, 2008; Bolognani *et al.*, 2010; Allen *et al.*, 2013; Sosanya *et al.*, 2015; Vanevski *et al.*, 2015). Due to the multiple sets of transcripts regulated by HuD, the entire regulatory program induced by this RBP has not been fully determined. Since we hypothesized that HuD may regulate mRNAs associated with addiction-related behaviors, we sought to identify predicted targets of HuD in the Knowledgebase of Addiction-related Genes database (KARG; http://karg.cbi.pku.edu.cn; (Li *et al.*, 2008), including those identified by expression patterns in microarray studies. These analyses (**Figure 3.1*A***) revealed that the KARG database set was significantly enriched in genes containing any of the three consensus HuD binding motifs described in forebrain neurons (***$p<0.001$ two-tailed $\chi 2$ test; (Bolognani *et al.*, 2010) in their 3'UTRs. To identify which of the predicted targets were regulated by this RBP in vivo, we used Affymetrix 430 2.0 microarrays to analyze forebrain samples from transgenic animals overexpressing HuD and control littermates (n = 3/group). Transcripts that were upregulated by a fold change of ≥1.25 relative to control mice were selected as putative HuD-regulated targets. We then used KARG to refine our list to high confidence addiction-related transcripts with evidence scores ≥2. The presence of predicted HuD binding sites in this subset was confirmed upon 3' UTR analyses as described before (Bolognani *et al.*, 2010) and in **Supplementary Table 1** (available online). From these bioinformatic analyses, we found 175 HuD-regulated genes were previously identified as ARGs (**Table 3.1**). Finally, we used Ingenuity Pathway Analysis (IPA) to identify

pathways, networks, and functions associated with this set of genes. Many of the HuD-regulated ARGs have been implicated in behavior, neurological disease, cell morphology, and psychological disorders as well as GPCR signaling, cAMP-mediated signaling, and ERK/MAPK signaling (**Figure 3.1*B***). Focusing on the 76 molecules specifically associated with behavior (**Table 3.1**), we found that these molecules were enriched in top canonical pathways associated with addiction such as GPCR signaling, cAMP-mediated signaling, and synaptic long-term potentiation (LTP) (**Figure 3.1*C***). Additionally, the top network from these HuD-regulated and behavior-associated ARGs included many key molecules in addiction-associated plasticity (complete network in **Table 3.1**; selections shown in **Figure 3.1*D***). Thus, we concluded that HuD induces a regulatory program that controls the expression of many genes associated with drug addiction.

Since these HuD-regulated transcripts were previously identified as ARGs and associated with behavior, we sought to determine whether HuD and its targets were regulated during cocaine CPP. A group of 16 C57Bl/6J male mice were trained, as previously described, with alternating cocaine or saline injections each paired with a specific chamber, respectively, to form an association between the cocaine unconditioned stimulus (US) and the environmental conditioned stimulus (CS+ or in the case of Saline, CS-). A control group of 16 mice were trained in a similar fashion with saline paired with both chambers, thus removing the associative learning between CS and US. On the test day, animals received a saline injection and were allowed to freely roam between the chambers. As described earlier, CPP was measured by time spent in the CS+ versus the CS- chamber, thus scores >1 indicate preference for the CS+ chamber while scores ~1 suggest no preference for either chamber. As expected, mice trained with cocaine and

saline showed CPP for the cocaine paired chamber (**Figure 3.2*A***; t(28.47) = 4.05, p = 0.0004, with Welch's Correction). Animals were sacrificed 1 h following the start of the test day session and brains were flash frozen for RNA and protein analyses. We focused on the NAc because this region is necessary and sufficient, specifically the shell, for cocaine CPP as determined by lesion and region-specific cocaine injection experiments (Liao *et al.*, 2000; Sellings *et al.*, 2006). We found that NAc HuD mRNA and protein was significantly increased in those animals trained with cocaine (**Figure 3.2*B*, *C***; t(10) = 2.5, p = 0.03; t(10) = 2.7, p <0.02). This suggests that NAc HuD upregulation is associated with cocaine CPP.

Given that CPP was associated with an upregulation in NAc HuD, we expected that HuD ARG targets may be upregulated as well. We assessed the expression of two well validated HuD targets, *Bdnf* and *Camk2a*. As shown in **Figures 3.2*D*** and ***E***, we found that *Camk2a* mRNA and CaMKIIα protein were both upregulated within the NAc after cocaine CPP training (t(5) = 2.8, p = 0.04; t(11) = 3.5, p = 0.005). The other validated HuD target and ARG, *Bdnf*, can be transcribed from many promoters (Timmusk *et al.*, 1993). However, at the mRNA level, differential poly(A) site usage leads to two different variants via alternative splicing. One variant is characterized by a shorter 3'UTR (*Bdnf*-S) while the other has a longer 3'UTR (*Bdnf*-L). Since *Bdnf*-S and the initial segment of *Bdnf*-L are identical, we could only reliably quantify total *Bdnf* mRNA (*Bdnf*-Pan) from *Bdnf*-L. Thus, to fully evaluate the regulation of *Bdnf* mRNA, we assessed both variants. As such, we found *Bdnf*-L and *Bdnf*-Pan mRNA were both upregulated by cocaine CPP training (**Fig. 3.2*F*, *H***; t(10) = 2.8, p = 0.02; t(11) = 2.2, p = 0.047). BDNF protein also exists in two different forms, the unprocessed proBDNF and the smaller,

processed mature BDNF (matBDNF; (Leibrock *et al.*, 1989). These two forms of protein have different receptors and cellular effects, so it is again important to assess both forms of BDNF (Zanin *et al.*, 2017). Both proBDNF and matBDNF were upregulated by cocaine CPP training within the NAc (**Fig. 3.2*G*, *I***; $t(6) = 3.8$, $p = 0.009$; $t(6) = 2.8$, $p = 0.03$). Taken together, this suggests that two independently-validated HuD ARG targets that have been previously shown to be associated with addiction-related behaviors are upregulated by cocaine CPP and associated with the upregulation of HuD after CPP training.

To confirm our microarray analyses and further dissect the role of HuD-induced upregulation of *Camk2a* and *Bdnf in vivo* specifically in the NAc, we used HuD_{OE} animals to determine if overexpression without training or drug treatment was sufficient to elicit a similar effect on these two validated targets. Analysis of HuD expression in the NAc of naïve HuD_{OE} mice revealed that both the mRNA (**Figure 3.3*A***; $t(7) = 2.6$, $p = 0.04$) and HuD protein (**Figure 3.3B**; $t(7) = 3.2$, $p = 0.01$) were overexpressed within this region. We also found that Camk2a, Bdnf-L, and Bdnf-Pan mRNA were concomitantly increased in HuD_{OE} mice (**Figures 3.3*C*, *E*,** and ***G***; $t(6) = 2.5$, $p = 0.049$; $t(6) = 2.6$, $p = 0.04$; $t(6) = 2.6$, $p = 0.04$). Additionally, NAc CaMKIIa, pro- and mat-BDNF proteins were increased (**Figures 3.4*D*, *F*,** and ***H***; $t(5) = 9.9$, $p = 0.0002$; $t(11) = 2.2$, $p = 0.049$; $t(5) = 2.7$, $p = 0.04$). This suggests that in the absence of CPP training or cocaine treatment, animals overexpressing HuD within the NAc show increased levels of HuD and its plasticity associated targets.

To test the functional role of enhanced levels of HuD, we trained HuD_{OE} mice and wild type littermates in the same cocaine CPP procedure described above. Notably,

although all groups received cocaine during conditioning, HuD_{OE} animals showed increased CPP behavior compared to wild type controls (**Figure 3.4*A***; $t(25) = 2.6$, $p = 0.02$). First, we confirmed that HuD_{OE} animals indeed had elevated HuD in the NAc, as found in **Figures 3.3*A*** and **3.3*B*** in naive animals. Although we did not detect increased expression of HuD mRNA, we did find increased HuD protein expression in HuD_{OE} animals that underwent cocaine CPP (**Figures 5*A*, *B***, $t(9) = 4.7$; $p = 0.47$; $t(5) = 3.5$, $p = 0.02$). Additionally, HuD_{OE} animals showed significantly higher expression of HuD ARG target mRNA and protein (**Figures 5*C*-*H***, $t(5) = 2.9$; $p = 0.03$; $t(5) = 3.2$, $p = 0.02$; $t(4) = 4.0$, $p = 0.02$; $t(8) = 3.8$, $p = 0.005$; $t(7) = 2.5$, $p = 0.04$; $t(4) = 3.0$, $p = 0.04$). To determine the specificity of HuD overexpression, we assessed the expression of GAPDH which is not predicted to be targeted by HuD. We found that *Gapdh* mRNA and protein were not significantly different between controls and HuD_{OE} animals (**Figures 5*I***, ***J***; $t(12) = 0.04$, $p = 0.97$; $t(7) = 0.9$, $p = 0.4$). Overall, this data suggests that HuD regulates cocaine CPP by inducing a regulatory program within the NAc associated with increased expression of two selected targets involved in CPP behavior.

Discussion

Overall, our study is the first to identify the neuronal RNA-binding protein HuD as a post-transcriptional regulator of ARG expression and addiction-related behaviors. HuD overexpression induced global alterations including multiple predicted direct HuD-regulated transcripts containing HuD binding sequences in their 3' UTRs. Many of these targets were found to be enriched in the KARG and formed regulatory networks associated with behavior. In agreement with these observations, we found that HuD was upregulated by cocaine CPP training and overexpression caused increased cocaine CPP behavior along with upregulation of two well-studied, direct HuD targets CaMKIIα and BDNF. HuD_{OE} animals that showed increased cocaine CPP behavior also were found to have increased expression of these targets within the NAc, a critical brain region associated with CPP and addiction in general. Since the encoding of cues indicative of drug exposure are required for CPP behavior and are cited as a major cause of relapse in human patients, post-transcriptional regulation involving HuD could play a role in therapeutics targeting relapse to drugs of abuse.

Our data suggests that HuD regulates multiple networks of genes associated with addiction-related behaviors. For example, ERK/MAPK signaling, which was a central node in a network significantly regulated by HuD_{OE}, has been found to be integral for CPP to other drugs (Gerdjikov *et al.*, 2004; Mizoguchi *et al.*, 2004a; Miller *et al.*, 2005). Additionally, elevated ERK signaling within other brain regions, such as subregions of the amygdala, have been found to be necessary for reactivity to cues that elicit drug seeking during withdrawal (Lu *et al.*, 2005, 2006; Thiel *et al.*, 2012). Since cue-induced

reinstatement of drug-seeking is proposed to model human drug-relapse behavior, this suggests that HuD may be involved in reward reinstatement behavior.

To assess the regulatory impact of HuD on behavior, we analyzed the expression of two direct targets of HuD (Tiruchinapalli, Ehlers, *et al.*, 2008; Allen *et al.*, 2013; Sosanya, Peggy P.C. Huang, *et al.*, 2013; Sosanya *et al.*, 2015; Vanevski *et al.*, 2015). Both CaMKIIα and BDNF have been highly associated with behavioral and addiction-related processes. For example, these two genes regulate molecular processes involved in the learning and memory of discrete spatial cues (Silva *et al.*, 1992; Falkenberg *et al.*, 1992; Linnarsson *et al.*, 1997; Cho *et al.*, 1998; Mu *et al.*, 1999; Mizuno *et al.*, 2000; Gorski *et al.*, 2003; Cirulli *et al.*, 2004; Poulsen *et al.*, 2007; McGauran *et al.*, 2008; Nakajo *et al.*, 2008; Achterberg *et al.*, 2014). Since visual spatial cues are critical in CPP behavior (Cunningham *et al.*, 2006), and these molecules have been associated with CPP for other drugs of abuse, this suggested that HuD may regulate cocaine-CPP through direct targeting of CaMKIIα and BDNF (Lu *et al.*, 2000; Sakurai *et al.*, 2007; Bahi *et al.*, 2008; Rosen *et al.*, 2015).

Although overexpression of HuD was found to facilitate cocaine CPP learning, previous work from our laboratory on the role of HuD in other learning associated behaviors suggests that its constitutive overexpression disrupts more complex learning and retention of spatial memories (Bolognani, Qiu, *et al.*, 2007). This apparent discrepancy may be due to the valence of the hedonic stimulus in drug-induced behaviors versus stress-induced behaviors such as contextual fear conditioning (CFC) or Morris water maze (MWM). Secondarily, this may be due to differences in the regions recruited in these tasks. Indeed, researchers studying the role of NR2B containing NMDARs found

that ifenprodil inhibition blocked the acquisition of morphine-induced CPP without affecting the acquisition of MWM demonstrating a delineation between drug and non-drug associated spatial learning (Ma *et al.*, 2011). Finally, temporal and spatial aspects of HuD expression and activity may play an important role in these findings.

The influence of post-transcriptional regulators in spatial learning seems to follow a bidirectional response, where both overexpression and knockout of a regulatory gene impairs spatial learning, thus, suggesting that a specific expression pattern of downstream plasticity-associated genes is required for proper learning and memory (Paradee *et al.*, 1999; Peier *et al.*, 2000). Adding further complication, these plasticity-associated genes themselves exhibit discretely regulated spatial and temporal expression. For example, while hippocampal Bdnf mRNA was upregulated at specific time points of MWM training (Kesslak *et al.*, 1998), many drugs of abuse acutely and chronically upregulate BDNF within the NAc (Filip *et al.*, 2006; Graham *et al.*, 2007; Bastle *et al.*, 2017). Additionally, BDNF is upregulated during abstinence from drug, which may contribute to "incubation of craving" (Li and Marina E. Wolf, 2015). Thus, overexpression of plasticity-associated genes such as BDNF and CaMKIIα may cause biphasic learning responses as well (Mayford *et al.*, 1996).

In conclusion, we have determined that HuD plays an important role in cocaine CPP behavior, suggesting that regulation of mRNA stability and protein translation mediated by this RBP is involved in controlling reward seeking. While further testing is required to delineate the exact function of HuD in the molecular underpinnings of this behavior, our work suggests that regulation of ARG expression mediated by HuD is a novel regulatory pathway involved in addiction-related behaviors.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by National Institutes of Health grants 1F31DA041214 to RJO, 1R01DA034097 to NPB/JLB with a supplement to RJO, and T32AA014127 to RJO

Tables

Table 3.1. HuD regulated Addiction-related genes (ARGs)

Putative HuD ARG target mRNA upregulated in HuD$_{OE}$	*Acot12, Acot2, Adarb1, Adcy8, Adcyap1, Adrb2, Aff1, Ank1, Ap2a2, Apod, Arf4, Atp6v1b2, Atp6v1e1, Bche, Bcl2, Bcl2l11, Bdnf, Bmp7, Cacng1, Calm3, Camk2a, Ccl12, Cd44, Cdh1, Cdkn1a, Cebpg, Chn2, Chrna3, Cnr1, Cnr2, Cpd, Creb1, Crem, Csk, Ddhd2, Dld, Dnajb14, Dpysl2, Drd4, Dsc2, Dusp4, Edil3, Egfr, Eif4e, Elavl4, Fgf2, Fkbp5, Foxp1, Fyn, Gap43, Gdnf, Gnaq, Gnb5, Gpm6a, Gria2, Grip1, Gsn, H2-D1, Hhex, Hint1, Hmgb2, Hspb1, Igf1, Ikbkb, Il10ra, Il16, Ipo11, Itgb1, Itpkb, Jun, Kalrn, Kcmf1, Kcna1, Kcnc1, Kcnj6, Klk6, Kmo, Ldb2, Limk1, Lims1, Lrch4, Mapk1, Mapk10, Mapk14, Mapk8, Matr3, Mbp, Mme, Mpdz, Mrpl17, Myt1, Myt1l, Ncam1, Ndst1, Ndufs4, Nedd4, Nf1, Nfasc, Nfia, Nfkbia, Nr4a3, Nsf, Ntrk3, Oprd1, Oprm1, Pdk4, Pdxk, Phtf1, Pml, Ppm1b, Ppp1cc, Ppp1r14c, Ppp1r2, Ppp2cb, Ppp2r2c, Ppp2r5c, Ppp3cb, Ppp3r2, Prkca, Prrx1, Psmb2, Ptdss2, Pten, Ptk2b, Ptprk, Rac2, Rad23b, Rad51, Ran, Rb1, Rgs12, Rgs9, Rhobtb3, Rhou, Rps6ka5, Sall3, Samhd1, Scn2b, Seh1l, Serpina3n, Sesn2, Sfpq, Sh2d5, Slc6a1, Smarcd1, Sox9, Ssx2ip, Syn2, Syp, Syt11,Tacr1, Tacstd2, Taf7, Thrb, Tiam1, Timp2, Tlk2, Tmed10, Tmod2, Tmpo, Tmprss2, Tnfrsf9, Tph1, Trib2, Trim59, Trp53inp2, Ubtf, Ugcg, Uqcrc2, Vamp3, Vnn1, Xpo1, Ywhaq, Zbtb16, Zwint*

HuD ARG targets associated with behavior	*Adarb1, Adcy8, Adcyap1, Adrb2, Apod, Arf4, Bche, Bcl2, Bdnf, Camk2a, Cdkn1a, Chrna3, Cnr1, Cnr2, Creb1, Crem, Csk, Ddhd2, Drd4, Dusp4, Eif4e, Elavl4, Fgf2, Foxp1, Fyn, Gdnf, Gnaq, Gria2, Hint1, Igf1, Ikbkb, Itgb1, Jun, Kalrn, Kcna1, Kcnc1, Kcnj6, Limk1, Mapk1, Mapk10, Mapk14, Mapk8, Mme, Ncam1, Ndufs4, Nedd4, Nf1, Nfasc, Nfkbia, Nr4a3, Ntrk3, Oprd1, Oprm1, Pml, Ppp1cc, Ppp1r14c, Ppp3cb, Prkca, Pten, Rad23b, Rb1, Rgs9, Rps6ka5, Sall3, Scn2b, Sfpq, Slc6a1, Syn2, Tacr1, Thrb, Timp2, Tmod2, Tph1, Trib2*
Top HuD regulated network associated with behavior	*Adcy, Adrb, Adrb2, Bdnf, Calcineurin Protein(S), Camk2a, Cnr2, Cofilin, Drd4, Erk1/2, F Actin, G Protein, G Protein Alphai, Gnaq, Gpcr, Gria2, Kcnj6, L-type Calcium Channel, Limk1, Nmda Receptor, Nr4a3, Oprd1, Oprm1, Pka, Pkg, Plc, Pp1 Protein Complex Group, Pp2a, Ppp1cc, Ppp3cb, Prkca, Proinsulin, Syn2, Tacr1, Trib2*

Shown are candidate HuD target mRNAs that were significantly upregulated (fold change ≥1.25 and $p<0.05$) in HuD_{OE} animals containing potential HuD binding sites in their 3' UTR as defined by (Bolognani *et al.,* 2010) and that were identified as ARG in the KARG database, suggesting that they may play a role in addiction-related processes.

Figures

Fig 1

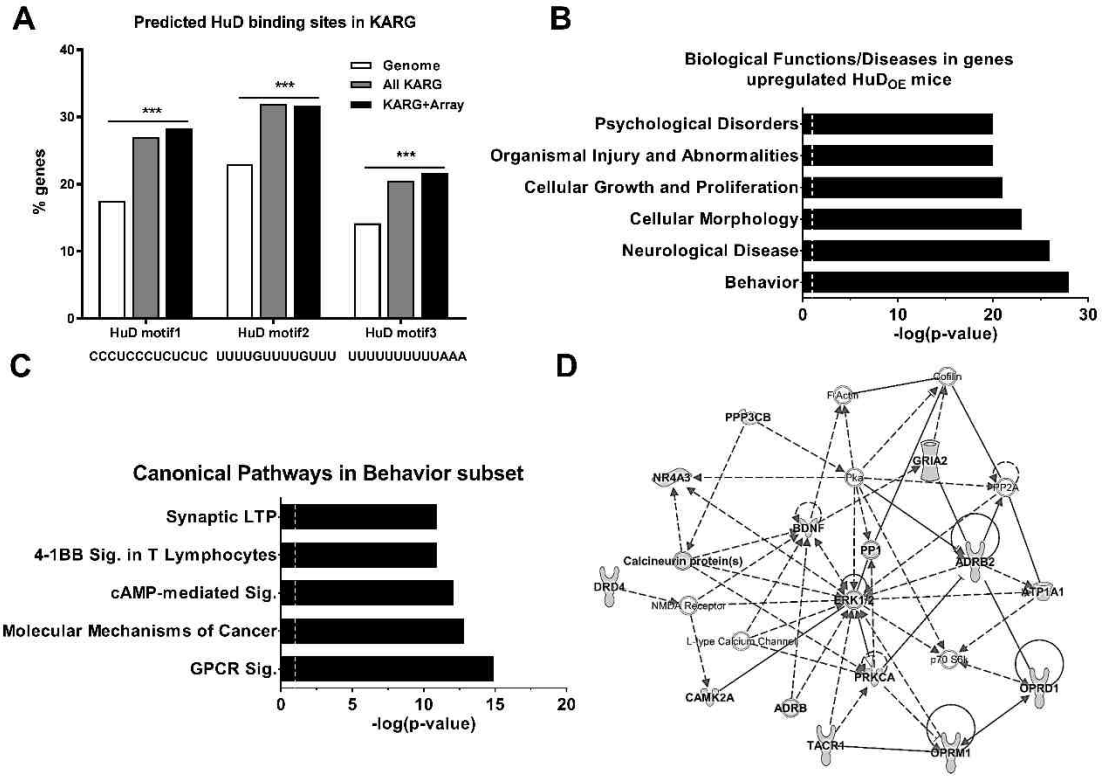

Fig 3.1: HuD regulates addiction-associated molecular networks. Predicted HuD targets were first determined by an *in silico* identification of mRNAs which contained ≥ 1 HuD binding motif(s) within the 3'UTR of mRNA from the entire mouse genome through Perl Biomolecules assessing consensus motifs of HuD binding (shown below each data set) as described by (Bolognani *et al*, 2010). (***A***) Predicted HuD targets were enriched within the KARG datasets for genetic evidence and genetic and microarray evidence compared to the entire mouse genome (*** $p<0.001$ two-tailed $\chi 2$ test). Next, *in vivo* regulated, addiction-related targets were identified using microarrays. A set of transcripts that a) were upregulated by a fold change of ≥1.25 in HuD$_{OE}$ compared to littermate control forebrain tissue, b) were present in the KARG database and c) contained HuD binding sites in the 3' UTR as described in (***A***) were selected for future analyses. (***B***) Molecules fitting these criteria were associated with functions and diseases, such as behavior in particular. (***C***) Identified mRNAs associated were behavior were more clearly organized into molecular pathways associated with addiction and formed a top network (***D***) heavily implicated in addiction-associated behaviors.

Figure 2

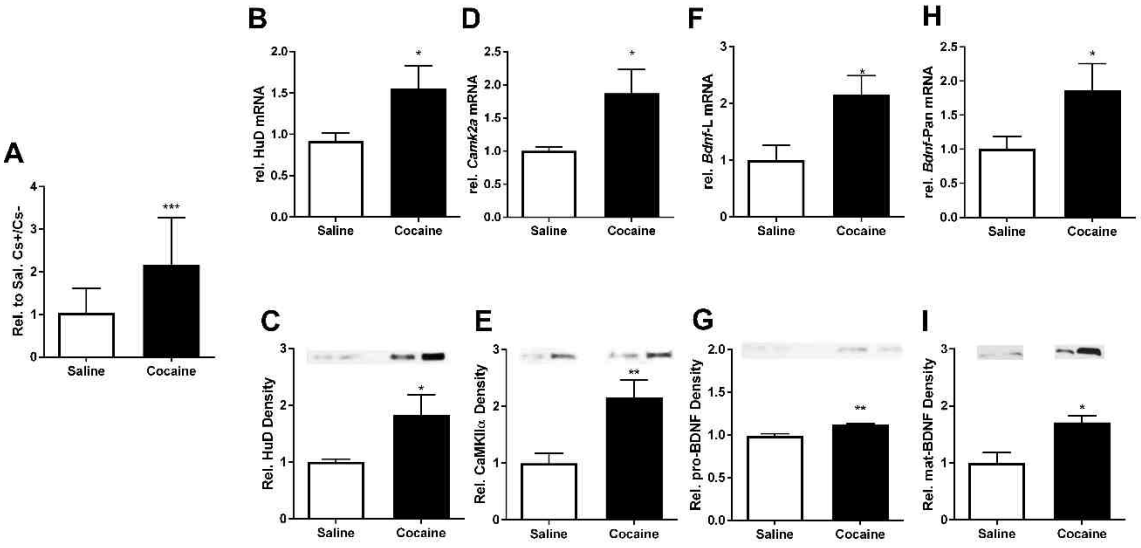

Figure 3.2: Cocaine CPP training is associated with elevated NAc HuD and two validated HuD-targets. ***A***) C57Bl/6J mice trained with 15 mg/kg cocaine show increased preference for the cocaine-paired conditioned stimulus (Cs+) compared to animals that received saline paired with both chambers (n = 20/treatment). Cocaine CPP training was associated with an increase in NAc HuD mRNA ***B***) and protein (***C***; n = 5-7/treatment). Cocaine trained animals showed elevated *Camk2a*, *Bdnf-L*, and *Bdnf-Pan* mRNA (***D, F, H***) as well as CaMKIIα, pro-BDNF, and mature-BDNF protein (***E***, ***G***, ***I*** with representative blots). n = 3-8/treatment. **p*<0.05, ***p*<0.01, ****p*<0.001, Student's t-test cocaine versus saline. Data are Means ±SEM.

Figure 3

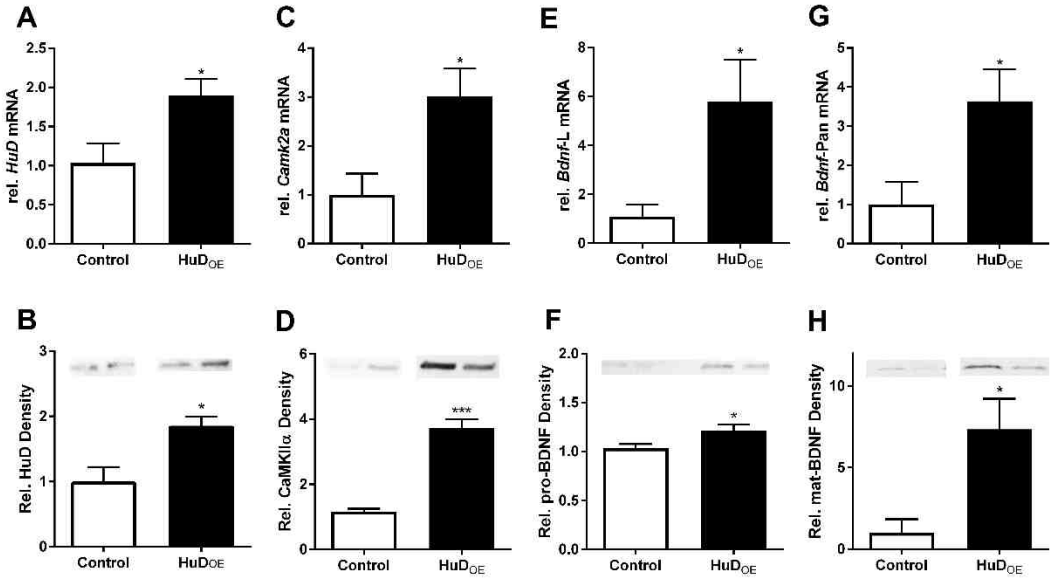

Fig 3.3: Naïve HuD_{OE} animals show increased NAc HuD and validated HuD targets. Male HuD_{OE} mice and littermate controls were sacrificed around 2 mo. of age and the NAc was dissected. HuDOE mice show increased NAc HuD mRNA (***A***) and protein (***B***) compared to controls. Following elevated HuD expression, previously validated, direct HuD targets were elevated in this region as well. Camk2a, Bdnf-L and Bdnf-Pan mRNA was increased within the NAc (***C***, ***E***, ***G***) as well as CaMKIIα, pro-BDNF, and mature-BDNF protein (***D***, ***F***, ***H***). n = 3-6/genotype. *p<0.05, **p<0.01, ***p<0.001 Student's t-test cocaine versus saline. Error bars indicate ±SEM.

Figure 4

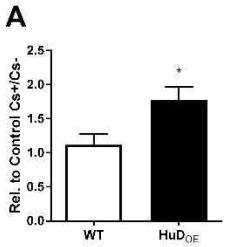

Fig 3.4: HuD$_{OE}$ animals show increased cocaine CPP. Male HuD$_{OE}$ mice and littermate controls trained with 15 mg/kg cocaine in the same CPP-induced protocol as **Fig. 3.2** except both groups were trained with cocaine. HuD$_{OE}$ mice show increased preference for the cocaine-paired Cs+ compared to littermate controls (n = 13-14/genotype). *p<0.05, Student's t-test HuD$_{OE}$ versus littermate control. Error bars indicated ±SEM.

Figure 5

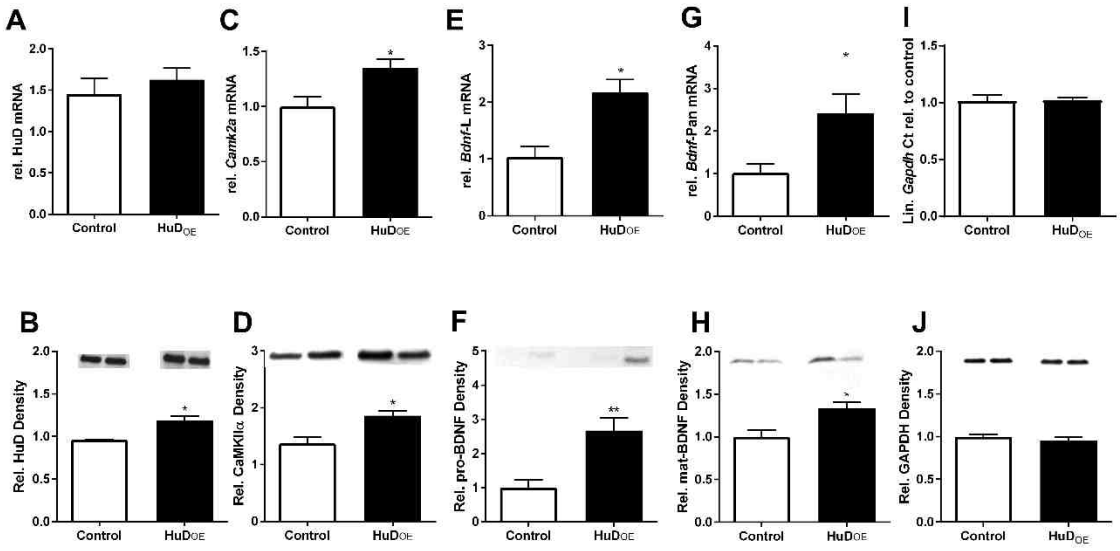

Fig 3.5: HuD$_{OE}$ animals show increased cocaine CPP-induced HuD target expression within the NAc. The NAc was harvested 1hr following initiation of the test day in the CPP protocol. HuD$_{OE}$ animals did not show an increase in *HuD* mRNA (***A***) but did show elevated HuD protein within this region (***B***). HuD$_{OE}$ animals showed elevated *Camk2a*, *Bdnf-L*, and *Bdnf-Pan* mRNA (***C, E, G***) as well as CaMKIIα, pro-BDNF, and mature-BDNF protein (***D***, ***F***, ***H*** with representative blots to the right). No significant change in GAPDH mRNA or protein (***I***, ***J***), which is not a predicted HuD target. n = 3-5/treatment. *p<0.05, **p<0.01 Student's t-test HuD$_{OE}$ versus littermate control. Error bars indicated ±SEM.

CHAPTER 4: DISCUSSION

4a. Overview

Here we established NAc miR-495 and HuD as novel post-transcriptional regulators of both ARG expression and motivation for cocaine. Initial bioinformatics analyses identified miR-495 as a miRNA with predicted targets enriched in the KARG database and with preferential expression in the brain reward and motivation pathway. Similarly, many predicted HuD targets were found to be enriched in the KARG and formed regulatory networks associated with behavior. (Everitt *et al.*, 2005)

Among these, we validated that miR-495 and HuD targeted *Bdnf* and *Camk2a* both in vitro and in vivo. Next, we found that cocaine decreased miR-495 expression along with concomitant increases in ARG targets in the NAc. When the cocaine-mediated miR-495 downregulation was blocked by lentiviral-mediated overexpression, cocaine-induced upregulation of ARG target mRNAs in the NAc was also prevented. From the miR-495 overexpression microarray analysis, we found that several of the downregulated target genes formed networks involved in receptor signaling, gene regulation, and synaptic plasticity. Similarly, HuD_{OE} induced global alterations including multiple predicted direct HuD-regulated transcripts containing HuD binding sequences in their 3' UTRs. Thus, both HuD and miR-495 regulate multiple networks of genes associated with addiction-related behaviors. For example, ERK/MAPK signaling, which was a central node in a network significantly regulated by HuD_{OE}, has been found to be integral for CPP to other drugs (Gerdjikov *et al.*, 2004; Mizoguchi *et al.*, 2004b; Miller *et al.*, 2005). Additionally, elevated ERK signaling within other brain regions, such as subregions of the amygdala, have been found to be necessary for reactivity to cues that elicit drug

seeking during withdrawal (Lu *et al.*, 2005, 2006; Thiel *et al.*, 2012). Since cue-induced reinstatement of drug-seeking is proposed to model human drug-relapse behavior, this suggests that HuD may be involved in reward reinstatement behavior.

In agreement with these observations, we found that HuD was upregulated by cocaine CPP training and overexpression caused increased cocaine CPP behavior along with upregulation of two well-studied, direct HuD targets CaMKIIα and BDNF. Concurrently, NAc miR-495 was downregulated by cocaine CPP training. HuD_{OE} animals that showed increased cocaine CPP behavior also were found to have increased expression of these targets within a critical brain region associated with CPP and addiction in general, the NAc. In opposition, NAcSh LV-miR-495 OE in C57Bl/6J mice and HuD_{OE} blocked the cocaine CPP training induced upregulation in target mRNA and protein, as well as diminished CPP. Furthermore, we found that NAc miR-495 overexpression reduced motivation to self-administer and seek cocaine, without effects on food reinforcement, suggesting that NAc miR-495 selectively regulates genes involved in motivation for cocaine. Since the encoding of cues indicative of drug exposure are required for CPP behavior and are cited as a major cause of relapse in human patients, post-transcriptional regulation involving HuD and miR-495 could play a role in therapeutics targeting relapse to drugs of abuse.

Although overexpression of HuD was found to facilitate cocaine CPP learning, previous work from our laboratory on the role of HuD in other learning associated behaviors suggests that its constitutive overexpression disrupts more complex learning and retention of spatial memories (Bolognani, Qiu, *et al.*, 2007). This apparent discrepancy may be due to the valence of the hedonic stimulus in drug-induced behaviors

versus stress-induced behaviors such as contextual fear conditioning (CFC) or Morris water maze (MWM). Secondarily, this may be due to differences in the regions recruited in these tasks. Indeed, researchers studying the role of NR2B containing NMDARs found that ifenprodil inhibition blocked the acquisition of morphine-induced CPP without affecting the acquisition of MWM demonstrating a delineation between drug and non-drug associated spatial learning (Ma *et al.*, 2011). Finally, temporal and spatial aspects of HuD expression and activity may play an important role in these findings. The influence of post-transcriptional regulators in spatial learning seems to follow a bidirectional response, where both overexpression and knockout of a regulatory gene impairs spatial learning, thus, suggesting that a specific expression pattern of downstream plasticity-associated genes is required for proper learning and memory (Paradee *et al.*, 1999; Peier, 2000). Adding further complication, these plasticity-associated genes themselves exhibit discretely regulated spatial and temporal expression. For example, while hippocampal Bdnf mRNA was upregulated at specific time points of MWM training (Kesslak *et al.*, 1998), many drugs of abuse acutely and chronically upregulate BDNF within the NAc (Filip *et al.*, 2006; Graham *et al.*, 2009; Bastle *et al.*, 2017). Additionally, BDNF is upregulated during abstinence from drug, which may contribute to "incubation of craving" (Li and Marina E Wolf, 2015). Thus, overexpression of plasticity-associated genes such as BDNF and CaMKIIα may cause biphasic learning responses as well (Mayford *et al.*, 1996).

To test further link miR-495 with other addiction-like behaviors, we examined the effect of NAcsh miR-495 overexpression on cocaine self-administration and seeking behavior. We observed that NAcsh miR-495 overexpression decreased responding and

intake when cocaine was available on the high effort PR schedule, but did not alter intake in the low effort FR5 schedule. PR schedules are believed to model an aspect of SUD related to an individual increasing time and energy toward drug-seeking and -taking behavior (Roberts *et al.*, 2007), thus alterations in these behaviors closely model hallmark symptoms of human addiction.

Furthermore, NAc miR-495 overexpression did not alter PR measures in a similar procedure with a natural food reinforcer. This suggests that miR-495 specifically influences motivation for cocaine likely without affecting the reinforcing value of cocaine or food reinforcement/motivation. Conversely, HuD regulates the molecular underpinnings of reinstatement to instrumental responding for an appetitive reward. Although HuD appears to specifically regulate reinstatement, as acquisition and extinction were not affected by upregulation of HuD. In our LV-miR-495 instrumental food response experiment we only tested the acquisition of this behavior, thus it is a possibility that HuD and miR-495 may regulate reinstatement to appetitive reward.

This behavioral effect may involve HuD-dependent stabilization of target mRNAs. CaMKIIα and BDNF mRNAs are two HuD targets that have been implicated in several forms of learning and memory. However, very little work has been done in the role that they may play in instrumental learning for an appetitive reward. There is evidence that Ser-831 on the GluR1, a CaMKIIα specific phosphorylation site, may be important for the retrieval of instrumental learning for an appetitive reward (Crombag *et al.*, 2008). This points to a shared HuD and miR-495 regulatory pathway involving CaMKIIα action in this behavior. Additionally, we found that NAcsh miR-495 overexpression reduced cocaine-seeking behavior during extinction and reinstatement,

further supporting a selective reduction in motivation. We also confirmed that under these conditions, miR-495 overexpression resulted in decreases in *Bdnf* and *Camk2a* expression. Closely mirroring our effects, previous work has shown that NAcsh knockdown of *Camk2a* and inhibition of NAcsh CAMKII reduces PR measures and reinstatement, respectively (Anderson *et al.*, 2008; Wang *et al.*, 2010). Thus, CaMKIIα is an important regulatory crux of many addiction-related molecular pathways (Wu *et al.*, 2001; Anderson *et al.*, 2008; Jessica A. Loweth *et al.*, 2010; Kourrich *et al.*, 2012b; Robison *et al.*, 2013). Although we did not detect a statistically significant effect during cue reinstatement ($p = 0.06$, one-tailed), the pattern is very similar. The lack of a more robust effect on cue reinstatement may highlight the more prominent role of the NAc core, rather than the shell, in regulating cue reinstatement (Fuchs *et al.*, 2004). Although HuD was not discretely studied in the context of self-administration, if miR-495 has an effect on these targets, HuD may very well play a role in this behavior. Overall, our results suggest that HuD and miR-495 preferentially regulates a network of ARG targets involved in the incentive motivational properties of cocaine which are more critical for sustaining behavior under the high-effort PR schedule of reinforcement than under the low-effort FR5 schedule of reinforcement.

With this work, we have shown that HuD and miR-495 play a role in the regulation of addiction-related gene expression and behavior. How drugs of abuse regulate HuD and miR-495 to elicit these changes in gene expression and behavior remains a standing question. Additionally, these ARGs have been associated with addiction-related behavior but it is unclear how this is occurring. The general hypothesis put forth by the community is that ARGs regulate features of neuronal plasticity, be it LTP or LTD, that drive

compulsive behavior found in addiction. In the remaining sections, I will address the evidence that HuD and miR-495 are regulated by factors that have been previously associated with addiction. Finally, given the data provided and the current state of literature, I will further discuss the evidence that the competition between HuD and miR-495 regulates shifts in neuronal plasticity either towards LTD or LTP from the initial set point of the neuron depending on connectivity, the neuronal genotype, and the stage of behavior or drug exposure.

4b. Limitations of this study

In general, the main limitation of these studies in its translation to the human condition is the fact that these behavioral studies have only assessed one theory of addiction presented in pre-clinical behavior. All of these behavioral models have shown that HuD and miR-495 regulate the incentive-salience of drugs, as in CPP. Though self-administration itself is very similar to the human condition, our models of self-administration only assessed the motivation to consume drugs of abuse in acquisition or after withdrawal in cocaine and cue-induced reinstatement. Thus, we have shown these behaviors are regulated by HuD and miR-495 but we have not assessed the role of these factors in other theories of SUDs. For example, other theories of addiction take into consideration the effects of intoxication compared to withdrawal states (Koob *et al.*, 2001). This idea suggests that the motivation to acquire drugs after repeated drug exposure is driven by the negative internal states that develop during withdrawal. For example, escalation of self-administration has been suggested to induce a state of tolerance. This tolerance to the positive effects of the drug has been hypothesized to be

counteracted by an increase in the negative effects of the withdrawal state. Thus far, we have shown that miR-495 and HuD play a role in discrete drug presentation models such as locomotor sensitization, CPP, and short-access self-administration procedures. We could assess the role of miR-495 and HuD in escalation to determine if these factors may also play a role in these other theories of SUDs.

A limitation in the use of HuDOE animals is the regional specificity of these behavioral effects. In profiling of these animals, we found that HuD is overexpressed in brain regions that show more regulated, lower expression of HuD. As such, cortical regions and specific regions of the hippocampus that normally have with high baseline expression of HuD have little to no elevation of HuD in transgenic animals. Conversely, regions with low baseline expression of HuD, such as the NAc, show large upregulation of HuD in transgenics. Although this may suggest that HuD is less important in these regions, we found that HuD is upregulated in this region in response to acute cocaine and cocaine CPP. Thus, this suggests that HuD in these regions have more specific, temporal HuD expression pattern in response to specific events. Thus, we proposed that this is a model to study the overexpression of HuD in regions that have low baseline expression. However, HuD may be overexpressed in other regions that may play a role in addiction-related behaviors. This could be overcome by lentiviral mediated overexpression of HuD within the NAc specifically. This approach would be tenuous to study the competition of miR-495 and HuD, since both would need to be overexpressed by a lentiviral vector. This is due to lower levels of co-infection of both viruses at a similar level. In either case, we believe our approach effectively assessed the region-specific effects of this competition,

as miR-495 OE within the NAc of transgenic animals was sufficient to cause alterations in cocaine CPP behavior.

4c. Regulation of HuD and miR-495

Although this work has delineated the various expression patterns of HuD and miR-495 in response to cocaine and behavioral training, we have not determined the mechanism for this regulation. HuD regulation through cocaine exposure can occur at many steps. Transcriptionally, HuD mRNA induction has been found to be regulated by Neurogenin2 in development (Ngn2; (Bronicki *et al.*, 2012). This positive transcriptional regulation appears to be neuronal specific, as it was not found in mesodermal cells, which express HuD at lower levels compared to developing neurons. Since HuD is an early marker of neuronal differentiation, it suggests that Ngn2 is activated early in development to induce HuD mRNA expression.

SATB1 is another transcription factor/epigenetic regulator that may influence HuD transcription. Satb1 null mice show signs that would suggest that it is an upstream regulator of HuD. For example, *Satb1* KO animals show diminished dendritic spines, altered IEG expression in response to stimulation (Balamotis *et al.*, 2012). Additionally, this factor appears to bind to upstream regulatory sequences of HuD as well as some of its targets (Balamotis *et al.*, 2012). To further complicate this matter, we discovered that Satb1 is a putative target of both miR-495 and HuD. Other studies from our laboratory have also found this link and have suggested that may be part of a positive feedback loop between SATB1 and HuD (Wang *et al.*, 2015). In this study, it was found that this

positive feedback loop was integral to neurogenesis further showing that this is mechanism is essential to normal neuronal development and processes. Although implicated in many neuronal-specific functions, there has not been any research into the role of calcium in the regulation of SATB1. Thus, SATB1 plays a role in the transcription of HuD but the role it may play in learning and memory associated with Ca^{++} transients cannot be established as of now. In conclusion, the exact mechanism and timing of HuD transcriptional regulation is uncertain, especially in the context of addiction-related behaviors. However, multiple avenues for regulation are already associated with addiction-related behaviors.

Once HuD is translated, the activation state of this protein can be modified by post-translational modifications. The most well studied of this is an activating phosphorylation site through Protein Kinase C (PKC; (Pascale *et al.*, 2005; Lim *et al.*, 2012). As assayed by mRNA decay assays, activation of PKC stimulates HuD phosphorylation leading to increased HuD/mRNA target association which was reversed by a PKC inhibitor. Specifically, Lim & Alkon assayed neurotrophic factor mRNAs such as BDNF. Not only does PKC phosphorylation of HuD induce its activation and association with mRNAs, it also promotes HuR movement into the cytoplasm and likely affects HuD localization as well (Schulz *et al.*, 2013). This is integral to many of its neuronal specific effects, as for example cytoplasmic localization is required for HuD-dependent induction of neuronal differentiation (Kasashima *et al.*, 1999). Thus, HuD activation through PKC is necessary for many of its effects within the neuron.

In opposition to phosphorylation, HuD methylation causes inhibition of its activity. This methylation is carrying out by Coactivator Associated Arginine

Methyltransferase 1 (CARM1; (Fujiwara *et al.*, 2006). In this study, Fujiwara and colleagues found that active CARM1 prevented PC12 cells from differentiation into neurons as well as processes such as neuritogenesis. This was rescued by expression of a methylation-resistant HuD. CARM1 activity is negatively regulated by phosphorylation (Higashimoto *et al.*, 2007; Feng *et al.*, 2009). In addition to their previous findings, Lim & Alkon found that HuD methylation was diminished in response to PKC stimulation, suggesting that PKC may also regulate CARM1 to disinhibit HuD as well as activating HuD. As hypothesized, CARM1 phosphorylation was increased by PKC activation. Overall, PKC appears to positively modulate HuD activity.

PKC itself has been associated with various forms of addiction-related behaviors, suggesting PKC regulation of HuD may also be involved in this disorder. PKC inhibition blocked the stereotypical acute cocaine induced locomotor response when infused into the VTA (Steketee, 1993). Acute cocaine induces LTP, increased spine density, within this brain region, suggesting it may do so through a PKC/HuD dependent process (Borgland *et al.*, 2006; Sarti *et al.*, 2007). LTP within the VTA is required for later drug-induced plasticity within the NAc and is dependent on a shared HuD/miR-495 target, CaMKII (Mameli *et al.*, 2009; Liu *et al.*, 2014). PKC inhibitor infusion blocks potentiated NMDAR-mediated early LTP which transitions into an AMPAR dependent LTP (Borgland *et al.*, 2006). This still suggests that PKC regulates these forms of plasticity, potentially through downstream effects on HuD. Additionally, VTA PKC was associated with the initiation of sensitization while PFC PKC was associated with the expression of sensitization (Steketee *et al.*, 1998). Pharmacological reversal of cocaine sensitization through co-administration of pergolide/ondansetron was found to diminish

total PKCζ and pPKCζ and λ within the NAcC and NAcSh, suggesting that PKC may be necessary for the induction of plasticity within the NAc for sensitization (Chen *et al.*, 2007). Following this, a single, systemic infusion of a zeta inhibitory particle (ZIP) blocked sensitization to cocaine (Howell *et al.*, 2014). This treatment also decreased whole brain membrane bound AMPAR suggesting it altered plasticity as well. The specificity of this effect to specific brain regions or even the specificity of ZIP itself is in question (Lee *et al.*, 2013).

PKC has also been found to play a role in CPP. Intraventricular administration of a PKC inhibitor immediately after each conditioning session blocked cocaine CPP (Cervo *et al.*, 1997). This effect was not found when infused immediately preceding the conditioning session, leading these authors to hypothesize that PKC was involved in the consolidation of the pairing between drug and environmental cues. Regional specificity was assessed later by Aujla & Beninger, when they found NAc PKC inhibition blocked amphetamine CPP (Aujla *et al.*, 2003). Atypical PKC Mζ inhibition within the NAc, but not the NAcSh, was found to block cocaine and morphine CPP expression (Li *et al.*, 2011). However, the specificity of ZIP is in question as knockout animals of PKCζ and PKMζ show normal cocaine CPP but show reduced CPP when given ZIP (Lee *et al.*, 2013). ZIP may be influencing other PKCs or other PKCs may be upregulated in response to KO. This is especially attractive as other studies focused on pan-PKC inhibition through other means than ZIP (Cervo *et al.*, 1997; Aujla *et al.*, 2003). In either case it appears that PKC inhibition blocks CPP, suggesting this effect may be due to HuD inhibition.

Finally, PKC plays a role in the SA of stimulants. D1 or D2 receptor specific stimulation in the NAcC or Sh can cause reinstatement of SA. Using this model, Ortinski and colleagues infused a PKC inhibitor within this region 10 mins before infusion of a D1 or D2 specific agonist to stimulate reinstatement. D2 specific reinstatement was blocked by infusion of a PKC inhibitor, suggesting that the downstream effects of PKC must be involved in this behavior (Ortinski *et al.*, 2015). This effect was also accompanied by a diminished evoked excitatory post-synaptic potential (eEPSP), specifically through decreased AMPAR currents, suggesting this may be due to alterations in PKC mediated plasticity.

As for miR-495, very little is known regarding the mechanisms that may link its expression to cellular events induced by cocaine and or behavioral training. In contrast to other addiction-related miRNAs whose expression levels were shown to increase in response to drugs of abuse (Chandrasekar *et al.*, 2009; Nudelman *et al.*, 2009; Hollander *et al.*, 2010; Schaefer *et al.*, 2010), miR-495 expression in the NAc decreased shortly after exposure to cocaine. This rapid downregulation 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 administration. The decrease in miR-495 expression may also be due to transcriptional repression. miR-495 is part of a large cluster of imprinted genes between delta-like homolog 1 and type III iodothyronine deiodinase genes, also known as the Dlk1-Dio3 imprinted region (Lin *et al.*, 2003; Seitz *et al.*, 2004). Within this region, are protein coding genes and non-coding RNA genes including miRNAs, small nucleolar RNAs (snoRNAs), and long non-coding RNAs. The miRNAs found in this region are

clustered into polycistronic transcripts, or a single large precursor miRNA transcript encoding several miRNAs. From this large precursor miRNA transcript, RNA processing leads to discrete miRNA strands. This miRNA cluster has been associated with various forms of neuronal function and dysfunction, suggesting that this region may play a role in other neuropsychiatric disorders (Gardiner *et al.*, 2012; I. Laufer *et al.*, 2012; Laufer *et al.*, 2013; Hollins *et al.*, 2014; Winter, 2015; Marty *et al.*, 2016).

Since these miRNA clusters appear to be transcriptionally linked together, this led many researchers to hypothesize that these miRNAs may show coordinated expression patterns. To this end, it has been found that adjacent to the Glt2/Dio3 promoter region is a site for differential methylation, suggesting that a methyl binding transcriptional regulator may regulate this site (Kernohan *et al.*, 2010). Wu and colleagues found that methyl-CpG-binding protein 2 (MeCp2) bound to methylated CpG islands upstream of these miRNA clusters through chromatin immunoprecipitation (Wu *et al.*, 2010). Using a *MeCp2* $^{-/-}$ mouse model of Rett Syndrome, they found that miRNA within these clusters were upregulated, including miR-495. They posited this was due to the inhibitory influence of MeCp2 on the transcription of these miRNA clusters. A salient feature of Rett syndrome is the dysregulation of Bdnf expression, leading to cognitive disability. Following our data, they found that many of these miRNAs directly targeted the 3'UTR of Bdnf, including miR-495 (Wu *et al.*, 2010). Further work into the mechanism of the *Mecp2* $^{-/-}$ mouse model of Rett syndrome has shown that these miRNAs may regulate ERK signaling, a shared addiction-related pathway between HuD and miR-495 (Mellios *et al.*, 2017). Thus, the *Mecp2* $^{-/-}$ mouse model of Rett syndrome recapitulates this

dysregulation of BDNF through the removed inhibitory block on these miRNA clusters leading to excessive miRNA-mediated downregulation of BDNF and ERK signaling.

If MeCP2 regulates the transcription of miRNAs associated with addiction, it suggests that it may play a role in the disorder. The role of MeCP2 in addiction was first studied in the context of escalation in the self-administration of cocaine (Im *et al.*, 2010). When animals are given extended access to drugs of abuse in a self-administration model, they undergo a specific behavioral pattern termed escalation of drug use. Uncontrollable, substantial amounts of drug use is an important facet in the human condition. With this, researchers sought out a miRNA that may be increased by this behavior. They found that miR-212, and activity-dependent miRNA regulated CREB, was upregulated in the dorsal striatum of these animals after extended access (Hollander *et al.*, 2010; Im *et al.*, 2010). This appeared to be corresponding to an increase in MeCP2, which appears to inhibit miR-212 expression but contains a miR-212 binding site within the 3'UTR. Thus, if miR-212 expression continues, it removes its own inhibitory block. These researchers found that upregulation of miR-212 was associated with potentiated CREB signaling, possibly through upstream cAMP pathways. RAF1 potentiates adenylyl cyclase activity, leading to increased cAMP production. Various other factors can enhance or repress this action. One of the targets of miR-212 is Sprout-related, EVH1 domain containing 1 (SPRED1), a corepressor of RAF1. Thus, extended access to cocaine-induced upregulation of miR-212 led to decreased SPRED1 levels, leading to increased cAMP production and CREB activity leading to increased miR-212 expression. When miR-212 was overexpressed within the dorsal striatum, rats in extended access showed decreased levels of cocaine infusions. With this, miR-212 upregulation was associated with an increase in CREB

expression. Oppositely, blocking of miR-212 by a locked nucleic acid (LNA) caused an increase in cocaine infusions. Another miRNA was found to have a similar effect on MeCP2, which then led to inhibition of heroin-seeking behavior (Yan *et al.*, 2017). Overall, this suggests that Dorsal Striatal MeCP2 may elevate addiction-related behaviors and inhibits the expression of miRNAs that block the induction of these behaviors.

MeCP2 can also be regulated by phosphorylation, leading to decreased association of this factor with methylated DNA sites (Chen *et al.*, 2003; Martinowich *et al.*, 2003; Zhou *et al.*, 2006). Furthermore, this was found to be a CaMKII dependent event and thus is associated with plasticity (Zhou *et al.*, 2006). With this, it was found that acute cocaine transiently increases the inhibitory phosphorylation of MeCP2 within the striatum, 20 minutes post-injection but not 3h (Mao *et al.*, 2011). This effect was found to be NMDAR dependent within the Dorsal Striatum, but not in the NAc. This may suggest that the mechanisms for MeCP2 regulation are different in various regions of the striatum. Since MeCP2 is an inhibitory influence on miR-495 transcription, this suggests that this effect of acute cocaine may play a role in the upregulation of miR-495.

Studies into the function of MeCP2 in addiction-related plasticity and behaviors show a wide variety of results. NAc MeCP2 OE blocked normal amphetamine CPP (Deng *et al.*, 2010). However, NAc shMeCP2 increased low dose amphetamine CPP and normal high dose CPP (Deng *et al.*, 2010). To further study this effect, Deng and colleagues utilized a hypomorphic, truncated MeCP2, MeCP2308. MeCP2308 mice showed blocked CPP for amphetamine and sucrose. Additionally, MeCP2308 mice showed blocked acute amphetamine induced structural plasticity, cFos/FosB/JunB induction but increased acute amphetamine locomotor activity. This follows with the idea

that miR-495 blocks addiction-related plasticity, thus an inhibited MeCP2 would lead to disinhibited miR-495 expression and further blockade of new drug-induced plasticity.

Although MeCP2 regulation likely plays a role in the transcriptional regulation of the polycistronic miRNA cluster containing miR-495, it would also similarly regulate the expression of the other miRNAs within this cluster. Thus, if MeCP2 was to wholly account for the cocaine-induced regulation of miR-495 then the expression of the other miRNAs from this cluster would also be sensitive to cocaine. However, given that not all of the miRNAs in this cluster are coordinately regulated (e.g., miR-376b vs. miR-495 after CPP, data not shown; (Wu *et al.*, 2010; Lempiäinen *et al.*, 2013), 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). It is curious that we did not observe changes in miR-495 in any other addiction-related brain region, especially in those that are also innervated by ventral tegmental dopamine neurons (e.g., dorsal striatum, mPFC). One possibility is that the relatively high basal expression levels of miR-495 in the NAc allowed us to detect a decrease following cocaine administration. Another possibility is that the NAc may have a more robust response to the acute and chronic effects of cocaine resulting in decreases in miR-495 expression levels. In any case, this suggests that miR-495 itself may be regulated post-transcriptionally in response to cocaine.

Although this effect is novel to exposure of drugs of abuse, researchers have found that other stimuli can induce similar, rapid shifts in the expression neuronal miRNAs (Krol *et al.*, 2010). This contrasts with slower decay rates found in non-neuronal cell types, as Krol and colleagues found miRNA decay rates were much faster in neurons

differentiated from ESCs as compared to undifferentiated control cells. This effect was found to be activity dependent as blockade by tetrodotoxin diminished the quick decay rates of selected miRNAs. Transcriptional effects could mediate activity-dependent diminished expression of miRNAs, but the rapid time course of this effect suggests it is mediated post-transcriptionally. One possibility is that specific endonucleases target discrete miRNAs for destruction. Tudor domain staphylococcal/micrococcal-like nuclease (Tudor-SN) is an evolutionarily conserved nuclease that initiates the decay of human miRNAs (Elbarbary *et al.*, 2017). The Tudor domain of Tudor-SN mediates protein-protein interactions, suggesting it could be regulated by these interactions. In this study, it was found that this domain allowed for interaction with RISC members such as Ago2 and trinucleotide repeat-containing 6A (GW182) even after RNAse treatment. This suggests that even an active miRNA, normally protected by incorporation into the RISC, could still be targeted by this endonuclease. In conjunction with this, Tudor-SN appears to target select miRNAs for destruction as opposed to any miRNAs. From personal communication with Dr. Lynne Maquat and Dr. Nora Perrone-Bizzozero, miR-495 appears to contain the correct sequence for Tudor-SN mediated destruction. Dr. Maquat also mentioned this endonuclease may Ca^{++} dependently initiate the decay of these miRNAs. Taken together, this opens the possibility that Tudor-SN could be regulated in response to plasticity-initiating events within the neuron to remove blocks to plasticity, such as miR-495.

Another nuclease that may be involved in this rapid decay of miR-495 could be the XRN exonucleases (Kai *et al.*, 2010). Although understudied in mammals and even more so in neurons, XRN exonucleases can degrade miRNAs at multiple steps of their

biogenesis. Since we analyzed mature miR-495, an XRN specific for mature microRNAs, such as XRN-2, may be mediating this effect (Chatterjee *et al.*, 2009). The mechanisms regulating miR-495 or other activity-dependently regulated neuronal miRNAs are understudied.

Based on this evidence so far, HuD activity appears to be most directly regulated first negatively by CARM1 methylation. This may hold HuD in an inactive state while allowing for miR-495 to have more influence over shared targets. Upon neuronal stimulation, PKC can phosphorylate CARM1 to inhibit HuD methylation as well as phosphorylating HuD to stimulate its activity. On the other hand, miR-495 may be rapidly degraded during neuronal activity by Tudor-SN, although other yet to be determined post-transcriptional mechanisms may also be at play. Transcriptionally, HuD is probably regulated by Ngn2 and Satb1 in response to drugs of abuse, but tying this effect with neuronal stimulation is tentative at best. MeCp2 may regulate pre-miR-495 induction, but our evidence suggests that a post-transcriptional mechanism is most likely at play. Overall, the potential regulators of HuD and miR-495 expression and function are associated with addiction-related behaviors and various forms of plasticity (see **Figure 4.1**).

4d. Downstream of HuD/miR-495: Plasticity?

Post-transcriptional regulation through RBPs and miRNAs play a role in fine-tuning gene expression involved in many cell signaling pathways (Schratt, 2009). Although we have determined that miR-495 and HuD share a regulatory network including many pathways associated with addiction, the consensus within the field is that drug-induced long-term forms of plasticity drive the behavior characteristic of addiction. Thus, since HuD and miR-495 are associated with addiction-related behavior, they must play a role in long-term forms of plasticity.

However, within the hypothesis that circuit level changes in plasticity mediate addiction-related behaviors is the notion that specific neuronal circuits are being regulated differentially to cause changes in behavior. Region specific studies may illuminate this prospect, but for the field to progress, neuronal specific studies are needed. In our study, we have shown that HuD, miR-495 and shared targets are altered within most of the mouse NAc. Although we try to target the NAcSh in viral studies and tissue punches are centered on this region, it does not negate the fact that even within this subregion is a several of neuronal phenotypes and specific afferents connecting to these specific neurons. Thus, due to the region wide effects we find in CPP induced expression (**Figure 3.2**) or genetic manipulation (**Figures 3.4, 3.5, AA1-3**), our data suggests that HuD and miR-495 are altered within this entire region in many different cell types and circuits. Furthermore, various forms of LTP and long term depression (LTD) are found within this region and are associated with addiction-related behavior. Therefore, HuD and miR-495 may not regulate a specific form of long term plasticity, either LTP or LTD but both (**Figure 4.1**).

From our in silico analyses informed by *in vivo* data, we found that many HuD and miR-495 targets form networks involved in both LTP and LTD (**Figures 2.4, 3.1**; (Kauer *et al.*, 2007; Kasanetz *et al.*, 2010; Russo *et al.*, 2010), as well as other cell signaling cascades relevant to addiction and plasticity, such as PKA signaling (Self *et al.*, 1998a; Boudreau *et al.*, 2009). Therefore, HuD and miR-495 within the entire NAc may act as a mechanism to fine tune the molecular response of multiple, interwoven pathways involved in the development of addiction. Additionally, HuD is upregulated within whole brain lysates after acute cocaine (Tiruchinapalli, Caron, *et al.*, 2008). However, HuD and miR-495 may not regulating whole brain LTP or LTD, as many of these are occurring within specific regions and circuits after cocaine.

Within the framework of the idea that post-transcriptional regulation fine-tunes a specific response, HuD and miR-495 competition could have divergent effects depending on the specific neuronal phenotype and circuit, even if net effect is increased or decreased ARG and Plasticity related gene (PRG) mRNA stability. The downstream effects of shared HuD and miR-495 targets may be sensitive to the specific neuronal stimulation that evoked HuD and miR-495 regulation as well as to initiate changes to plasticity evoked by this neuronal stimulation. For example, LTP and LTD are both are calcium sensitive processes that require similar protein machinery involved in either phenomenon (Lüscher *et al.*, 2012). Thus, instead of positing that HuD supports one form of plasticity while miR-495 promotes another, it is more congruent with our current data to hypothesize that HuD and miR-495 compete for the control of changes both forms of long term plasticity (**Figures 4.1*B*, *C***). This follows with our data as they both regulate LTD and LTP through similar targets such as BDNF and CaMKIIα. With this, our data

supports the hypothesis HuD regulation promotes changes to the plasticity found within discrete neurons in response to their specific circuitry and phenotype, while miR-495 acts as a block to these forms of plasticity. Here, this idea will be expanded upon by examining the role that a selection of shared HuD and miR-495 targets associated with both LTP and LTD could play in the formation of either of these forms of plasticity.

Although not tested for direct interaction, we found that *Gria3* mRNA was upregulated in the HuD_{OE} microarray and sensitive to miR-495 OE (**Figures 2.4** and **3.1**). *Gria3* mRNA encodes the Ca^{++} permeable GluA3 AMPAR subunit. Although much more research has been devoted to understanding the role of GluA1 and GluA2 in hippocampal LTP and LTD, respectively, very little is known about GluA3 in these processes. Especially since it was suggested from global KO studies that GluA3 has no effect on basal synaptic properties, LTP, or LTD (Meng *et al.*, 2003). Since it is Ca^{++} permeable, it suggests it must play some role in changes to neurotransmission. Additionally, GluA3 is abundant in many hippocampal synapses and is associated with synaptic dysfunction in disease states (Wu *et al.*, 2007). Very recently it was found that GluA3 promotes LTP in Purkinje cells during motor learning as well as in the hippocampus (Gutierrez-Castellanos *et al.*, 2017; Renner *et al.*, 2017). In hippocampal synapses, it was found that under basal conditions GluA2/3 containing AMPARs are low-conductance. During periods of stimulation and cAMP accumulation, GluA2/3 AMPARs become high-conductance receptors and promote downstream synaptic potentiation. This heightened cAMP production was stimulated by β-adrenergic receptors. However, this does not preclude the possibility that other GPCRs, possibly DA receptors, could be involved in a similar mechanism. Other research focused on synaptic pathologies, have

found that GluA3 is a target of Aβ which removes it from the synapse leading to synaptic and memory impairment (Reinders *et al.*, 2016). GluA3, along with other receptor mRNAs, were upregulated in the Hippocampal Dentate Gyrus region of human alcoholics, suggesting a potential role in addiction (Jin *et al.*, 2014). Supporting this, findings from prenatal alcohol exposed (PAE) animals generally show a higher propensity for addiction-related behaviors such as self-administration. Researchers found that PAE exposed animals showed higher levels of amphetamine SA induced upregulation of GluA3 in VTA neurons which was also associated with higher levels of LTP in these neurons (Hausknecht *et al.*, 2015). Thus while the potential role of GluA3 in long-term plasticity is still under investigation it does appear that GluA3 regulation is associated with LTP.

In addition to directly regulating AMPAR subunit mRNA stability, HuD and miR-495 also regulate factors that can indirectly regulate the composition of AMPARs within specific synapses. Arc is a well-studied PRG or, as termed by the synaptic tagging hypothesis, Plasticity-related product (PRP, but will be discussed further as PRG; (Redondo *et al.*, 2011). Conventionally, it is hypothesized that Arc organizes actin, as it was first discovered as a factor that co-fractionated with actin, and overexpression causes actin dependent increases in thin spines (Lyford *et al.*, 1995; Peebles *et al.*, 2010). Recently, other functions of Arc have been discovered. For example, Arc has been found to mediate AMPAR cycling associated with LTD (Waung *et al.*, 2008). Converging with the hypothesis that HuD and miR-495 regulate plasticity through altering the translation of specific PRGs, this same study found that mGluR1 dependent LTD required ARC translation but not transcription. Thus, the local translation of pre-existing dendritic Arc

mRNA was integral to the development of this form of long-term plasticity. Following with the argument that HuD and miR-495 regulate both LTP and LTD, Arc KO animals have deficits in both LTD and LTP (Plath *et al.*, 2006). More distinctly, Arc has been found to be required for late phase LTP (Guzowski *et al.*, 2000; Plath *et al.*, 2006; Messaoudi *et al.*, 2007). Additionally, Arc is required for LTP induced by BDNF, another HuD and miR-495 shared target (Messaoudi *et al.*, 2002, 2007). Therefore, HuD and miR-495 regulation of Arc mRNA could cause alterations in both LTP and LTD in response to different cellular stimuli.

Arc mRNA and protein expression has been found to be correlated with synaptic activity, as well as learning and memory (reviewed in (Korb *et al.*, 2011). As such, it has been heavily implicated in addiction-related behaviors (Fosnaugh *et al.*, 1995; Klebaur *et al.*, 2002; Freeman *et al.*, 2008; Hearing *et al.*, 2008, 2010, 2011, Lv *et al.*, 2011, 2015; Riedy *et al.*, 2013; Alaghband *et al.*, 2014; Salery *et al.*, 2017; Gao *et al.*, 2017). Acute cocaine rapidly induces striatal Arc mRNA in a D1-dependent manner (Fosnaugh *et al.*, 1995). Arc expression has also been found to be potentiated in response to the pairing of the drug to a specific environmental context, suggesting it may be involved in encoding this pairing (Klebaur *et al.*, 2002; Hearing *et al.*, 2008, 2010). Arc may also be involved in the motivation aspect of these cues, as cue-induced reinstatement increased Arc mRNA in the orbitofrontal, prelimbic, and anterior cingulate regions of the prefrontal cortex (Zavala *et al.*, 2008). Additionally, following previous research that specific ensembles of neurons are activated by addiction-related behaviors, striatal neurons expressing cfos-lacZ in response to cocaine sensitization have been found to have increased levels of Arc mRNA (Guez-Barber *et al.*, 2011). The expression of Arc is

altered within the striatum during active cocaine SA and withdrawal (Freeman *et al.*, 2008; Gao *et al.*, 2017). Arc expression has also been associated with cocaine and morphine CPP (Lv *et al.*, 2011, 2015; Alaghband *et al.*, 2014). Although well studied as a marker of activity in the context of addiction-related behaviors, very little research has gone into the downstream mechanism of Arc regulation in these behaviors. Even though Arc-induced reorganization of synaptic AMPARs has focused on LTD, it was also found that morphine CPP caused an Arc-induced increase in synaptic GluR1 associated with LTP (Lv *et al.*, 2015). Though nuclear reorganization has not well studied in plasticity, Arc may also have a nuclear function and promotes promyelocytic leukemia nuclear bodies (PML-NBs; (Irie *et al.*, 2000; Bloomer *et al.*, 2007). Recently, acute cocaine has been found to cause Arc to accumulate within the nucleus (Salery *et al.*, 2017). Furthermore, Salery and colleagues instead studied the role it may play in chromatin remodeling and it was found that Arc colocalizes with phosphorylated Ser10-histone H3. Arc overexpression was found to diminish this phosphorylation while KO animals showed decreased heterochromatin domains and enhanced pol II activity as well as cFos expression. Behaviorally, these Arc KO animals showed increased cocaine-induced sensitization and CPP, but was not directly linked to its nuclear activity. In either case, this presents another possibility for Arc-induced regulation of plasticity and addiction-related behaviors.

Another integral synaptic receptor, GluN2B or NR2B, is also regulated by HuD and miR-495. Additionally, it has been associated with many forms of addiction-related behaviors and cocaine exposure. It was first found to be upregulated during withdrawal (Loftis *et al.*, 2000). NR2B was found to interact with D2 receptors to inhibit D2 neuron

circuits (Liu *et al.*, 2006). NR2B synergistically activated ERK along with D1Rs, while inhibition of this pathway prevented cocaine-induced sensitization and CPP (Pascoli *et al.*, 2011). Finally, it has been associated with cocaine-induced generation of silent synapses within the NAc (Huang *et al.*, 2009). As one of subunits in the NMDAR complex, it has been hypothesized to regulate many forms of plasticity. Despite not being well studied in addiction-related LTP, it has been found to be associated with forms of LTP through its interaction with CaMKII (reviewed in (Coultrap *et al.*, 2012). Additionally, the ratio between NR2A/NR2B has been hypothesized to be involved in determining the threshold for LTD/LTP with higher NR2A/NR2B associated with LTP and lower with LTD (Yashiro *et al.*, 2008). With this, NR2B is another shared target of HuD and miR-495 that could play a dual role in LTD or LTP induction during addiction-related behaviors.

In addition to these ARGs, we also established that HuD and miR-495 directly targets and regulates the ARG *Bdnf* both in vitro and in vivo (HuD and miR-495 data shown in Gardiner *et al.,* In prep, only miR-495 in **Figure 2.1**). Additionally, *Bdnf* has been a well characterized target of HuD (Allen *et al.*, 2013; Vanevski *et al.*, 2015). BDNF have been highly associated with behavioral and addiction-related processes. For example, BDNF regulates molecular processes involved in the learning and memory of discrete spatial cues (Falkenberg *et al.*, 1992; Linnarsson *et al.*, 1997; Mu *et al.*, 1999; McGauran *et al.*, 2008). Since visual spatial cues are critical in CPP behavior (Cunningham *et al.*, 2006), and these molecules have been associated with CPP for other drugs of abuse, this suggested that HuD and miR-495 may regulate cocaine-CPP through direct targeting of BDNF (Bahi *et al.*, 2008). Additionally, *Bdnf* in the NAc has been

linked to several drug abuse-related behaviors, where BDNF expression levels positively correlate with cocaine reward and motivation (Horger *et al.*, 1999; Hall *et al.*, 2003; Graham *et al.*, 2007, 2009; Bahi *et al.*, 2008). Thus, HuD and miR-495 regulation of BDNF may play a role in these behavioral processes.

What is not well known is how BDNF can regulate these behavioral processes through plasticity. As posited in the beginning of this section, addiction is a disorder of neuroplasticity and thus, ARGs must affect plasticity. With this, BDNF has been associated with various forms of plasticity. *Bdnf* KO animals were first found to have an impairment in hippocampal LTP but was restored with recombinant BDNF or viral-mediated expression (Korte *et al.*, 1995, 1996; Patterson *et al.*, 1996). Additionally, BDNF appears to be released in response to LTP or immediately after its induction (Aicardi *et al.*, 2004). Additionally, its interaction with the TrkB receptor suggests that this is the post-synaptic mediator of BDNF-induced LTP (Chen *et al.*, 1999). Through this receptor, BDNF appears to upregulate protein synthesis-dependent functional and structural changes required for late phase LTP (reviewed in (Panja *et al.*, 2014). This direct effect of BDNF on LTP has led researchers to propose that TrkB receptors act as a synaptic tag and BDNF as the PRP required in LTP and memory consolidation (Lu *et al.*, 2011). As with the other shared HuD and miR-495 ARGs, BDNF may also be involved in LTD. In this case, it is that an unprocessed form of BDNF is associated with LTD, rather than the mature BDNF usually studied. Through interaction and activation of the p75NTR, Pro-BDNF has been associated with NMDAR-dependent LTD within the hippocampus (Woo *et al.*, 2005; Yang *et al.*, 2014). Additionally, LTD-induction is associated with an upregulation in proBDNF rather than BDNF through the secretion of

proteases (Nagappan *et al.*, 2009). This suggests that depending on the input, a neuron can dictate whether BDNF or proBDNF acts on a synapse leading to LTP or LTD, respectively. Finally, this LTD has been hypothesized to occur through the upregulation of NR2B NMDARs, another shared HuD and miR-495 target (Woo *et al.*, 2005). In terms of Bdnf mRNA regulation, Bdnf-L has been proposed to be associated more with proBDNF expression while Bdnf-S is more related to mature BDNF (Orefice *et al.*, 2013). Both HuD and miR-495 target these dual Bdnf mRNAs, thus could affect expression of proBDNF and matBDNF leading to different forms of long term plasticity associated with addiction-related behaviors.

Additionally, *Camk2a* has been a well characterized target of HuD (Sosanya, Peggy P C Huang, *et al.*, 2013; Sosanya *et al.*, 2015) and has been associated with behavioral and addiction-related processes. As with *Bdnf*, *Camk2a* also regulates molecular processes involved in the learning and memory of discrete spatial cues and has been found to play a role in CPP (Silva et al. 1992; Cho et al. 1998; Poulsen et al. 2007; Sakurai et al. 2007; Rosen, Zunder, et al. 2015). Others have established that CaMKIIα has a positive relationship between NAc levels and psychostimulant abuse-related behavior (Anderson *et al.*, 2008; J. A. Loweth *et al.*, 2010; Wang *et al.*, 2010; Kourrich *et al.*, 2012b). Furthermore, CaMKII has been identified in silico as a central node in positive feedback gene regulatory pathways involved in addiction (Li *et al.*, 2008). Additionally, it has been associated with LTP and LTD, possibly through differential phosphorylation (reviewed in (Coultrap *et al.*, 2012). CaMKIIα also interacts with NR2B, another HuD and miR-495 shared target ARG, to remain in an active state leading to

many of its downstream effects. Therefore, regulation of CaMKIIα by HuD and miR-495 may coordinately affect several domains of addiction-related processes.

Given this evidence that miR-495 and HuD regulate many factors associated with various forms of long term plasticity, our understanding of the effects that either of these regulators have on long term plasticity is very limited. Previous work from our laboratory found that HuD_{OE} did not cause alterations in long term potentiation within mossy fiber neurons in the hippocampus (Tanner *et al.*, 2008). These neurons did show increased paired pulse facilitation, a form of short-term pre-synaptic plasticity. Although a presynaptic form of plasticity appears to discount that HuD_{OE} regulated this, this mouse shows increased HuD within most forebrain neurons, suggesting that this presynaptic effect may have in fact been driven by HuD_{OE}. These animals were naïve and not subjected to any behavioral or drug challenges, suggesting that the effect of HuD_{OE} may require strong stimuli to cause changes to long term plasticity. The effects of miR-495 on plasticity are unknown. With the help of Dr. Manuel Mameli's group we attempted these measures. After slicing the tissue for electrophysiology he found that LV-miR-495 OE MSNs appeared to be damaged and thus were unable to determine electrophysiological measures of the effect of miR-495. Furthermore, Kate Reinhardt in Dr. Bill Shuttleworth's laboratory, found a similar effect in Bdnf *Val*/*Met* mice. After preparation of these brains for electrophysiological assays, she found that defective BDNF mice neurons appeared damaged. To add to this, LV-miR-495 OE neurons appeared damaged, missing many of their spines (data not shown). This might suggest that BDNF protects against injury during electrophysiological slicing, and miR-495 induced knockdown or defective Bdnf *Val*/*Met* variants may diminish the capacity of these neurons to survive

this insult. In conclusion, it does in fact appear that the competition between HuD and miR-495 putatively regulates plasticity but the actual effects of these molecules on plasticity is unknown.

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

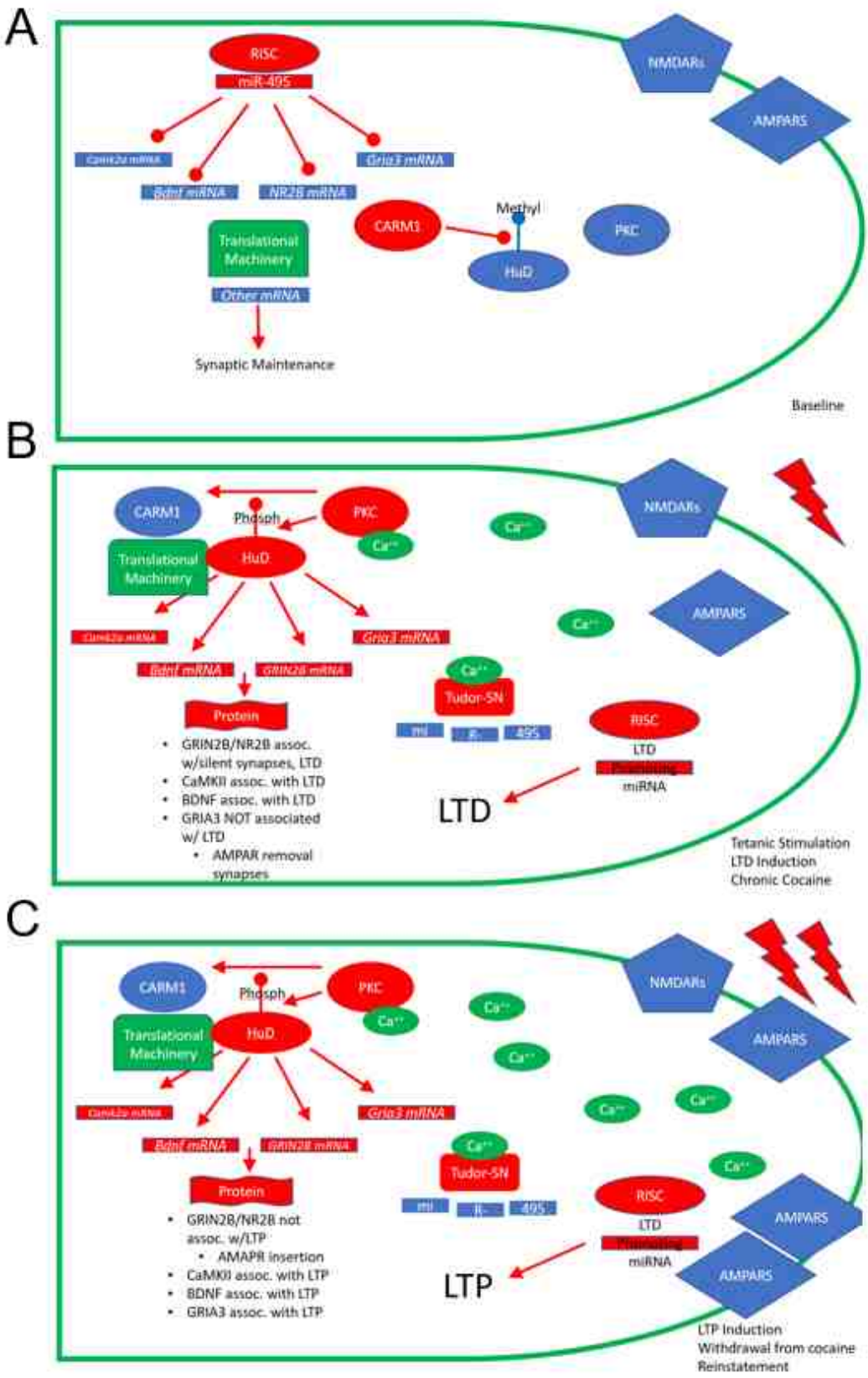
A
RISC
miR-495
NMDARs
AMPARS
Camk2a mRNA
Bdnf mRNA
NR2B mRNA
Gria3 mRNA
CARM1
Methyl
PKC
HuD
Translational Machinery
Other mRNA
Synaptic Maintenance
Baseline
B
CARM1
Phosph
PKC
Ca++
Translational Machinery
HuD
NMDARs
AMPARS
Camk2a mRNA
Gria3 mRNA
Bdnf mRNA
GRIN2B mRNA
Protein
Tudor-SN
RISC
LTD
Processing
miRNA
LTD
• GRIN2B/NR2B assoc. w/silent synapses, LTD
• CaMKII assoc. with LTD
• BDNF assoc. with LTD
• GRIA3 NOT associated w/ LTD
• AMPAR removal synapses
Tetanic Stimulation
LTD Induction
Chronic Cocaine
C
CARM1
Phosph
PKC
Ca++
Translational Machinery
HuD
NMDARs
AMPARS
Camk2a mRNA
Gria3 mRNA
Bdnf mRNA
GRIN2B mRNA
Protein
Tudor-SN
RISC
LTD
Processing
miRNA
AMPARS
AMPARS
LTP
• GRIN2B/NR2B not assoc. w/LTP
• AMAPR insertion
• CaMKII assoc. with LTP
• BDNF assoc. with LTP
• GRIA3 assoc. with LTP
LTP Induction
Withdrawal from cocaine
Reinstatement

Fig. 4.1. miR-495 and HuD compete for the regulation of ARGs associated with both forms of long-term plasticity. (***A***) miR-495 and HuD may compete for the regulation of synaptic maintenance but they may also compete for the expression of either LTD (***B***) or LTP (***C***) depending on the neuronal input. As discussed in the previous section, both LTP and LTD processes are driven by Ca^{++} which can positively regulate HuD and negatively regulate miR-495. This could lead to the increased mRNA stability and translation of ARGs. Many of these genes have dual functions in either LTD or LTP. As such, I propose that miR-495 and HuD compete for control of long-term plasticity depending on the neuronal input.

APPENDIX A: Effects of NAcsh LV-miR-495 OE in C57Bl/6 and HuD_{OE} mice on CPP behavior

First, using the same tissue processed in figure, we assessed the expression of NAc miR-495 after cocaine CPP. We found that NAc miR-495 was significantly downregulated in these same animals (data not shown, will be submitted as part of Gardiner *et al.,* In prep.; $t(13) = 2.7$, $p<0.05$). To assess whether this CPP induced alteration in miR-495 expression was specific to miR-495 or generalized to other miRNAs within the same transcriptional unit, we measured the expression of miR-376b. NAc expression of miR-376b was not altered by CPP training (data not shown; $t(9) = 0.7$, p = ns), suggesting that these alterations in miR-495 expression were specific to this miRNA and possibly occur after transcription.

Next, we aimed to specifically delineate the role of NAc miR-495, in contrast to HuD, in cocaine CPP behavior. To test this, we infused LV-miR-495 within the NAcSh of C57Bl/6J male mice 2 weeks before cocaine CPP training. Since HuD_{OE} potentiated cocaine CPP, we hypothesized that miR-495 would diminish standard C57Bl/6J mouse cocaine CPP. With this, we found that LV-miR-495 OE significantly decreased cocaine CPP compared to LV-GFP (**Figure AA.1*A***; $t(7.2) = 2.6$; $p < 0.05$, with Welch's Correction). As expected, miR-495 was upregulated nearly 1.5 fold within the LV-miR-495 treated animals (**Figure AA.1*B***; $t(15) = 2.4$; $p < 0.05$). Additionally, shared miR-495 and HuD target mRNA was significantly downregulated (Figure **AA.*C-E***; *Bdnf*-L, $t(6) = 2.8$; $p <0.05$; *Bdnf*-Pan, $t(7) = 2.5$, $p <0.05$; *Camk2a*, $t(10) = 3.3$, $p <0.01$). This suggests that NAc miR-495 negatively regulates the expression of ARGs associated with CPP as well as motivation associated with CPP behavior itself. Together, both sets of data imply

that NAc HuD and miR-495 have opposite effects on CPP-induced ARG expression and CPP behavior.

Previously, we found that miR-495 and HuD compete for a shared GUUUGUUUG region within the 3'UTR of ARGs in a luciferase assay. To test if HuD and miR-495 compete for control of ARG expression within the NAc, we infused LV-miR-495 within the NAcSh of HuD_{OE} animals. HuD_{OE} + LV-miR-495 animals were found to have increased NAc miR-495, as compared to HuD_{OE} + LV-GFP controls (**Figure AA.2*A***; $t(23) = 2.9$, $p < 0.01$). Additionally, this was combined with a significant decrease in shared ARG mRNA (**Figure AA.2*B-D***; *Bdnf*-L, $t(10.4) = 2.3$, $p < 0.05$ with Welch's Correction; *Bdnf*-Pan, $t(13.8) = 2.3$, $p < 0.05$ with Welch's Correction; *Camk2a*, $t(18) = 2.4$, $p < 0.01$). An alternative hypothesis for this effect may include miR-495 targeting of HuD, leading to diminished HuD mRNA and decreased stabilization of shared ARG mRNAs. However, we found there was no significant difference *HuD* mRNA in either viral conditions (data not shown, $t(19) = 1$, $p = ns$). This is in line with our previous luciferase experiments detailing that miR-495 does not target HuD 3'UTR luciferase reporters (data not shown, will be published in Gardiner *et al.,* In Prep.) and that miR-495 OE in SH-SY5Y cells does not decrease HuD mRNA (data not shown). Together these results add to our previous luciferase reporter findings, suggesting that HuD and miR-495 compete for expression of shared ARG mRNAs through the 3'UTR within a region associated with addiction-like behaviors. This data also suggests that this competition may be happening during addiction-related behaviors.

As found previously, cocaine CPP was associated with a decrease in NAc miR-495 and a concomitant increase in HuD mRNA and protein, as well as shared ARG target

expression (**Figure 3.2**). Additionally, we found that HuD_{OE} caused an increase in CPP in contrast to miR-495 OE, which diminished CPP (**Figure 3.4, AA.1*A***). In combination with our findings that HuD and miR-495 have a shared target GUUUGUUUG sequence in ARG 3'UTRs, this data suggests that NAc HuD and miR-495 may compete for the control of CPP-induced shared ARG expression and ultimately addiction-related behaviors. To test this hypothesis, we infused LV-miR-495 within the NAcSh of HuD_{OE} animals before they were trained in cocaine CPP. HuD_{OE} + LV-miR-495 animals showed decreased cocaine CPP in comparison to LV-GFP controls (**Figure AA.3*A***, $t(12) = 6.8$, $p < 0.0001$). As expected, HuD_{OE} animals infused with LV-miR-495 had increased miR-495 within the NAc (**Figure AA.3*B***, $t(9) = 3.8$, $p < 0.01$). Additionally, this alteration in CPP behavior was associated with decreased shared HuD and miR-495 ARG target mRNA (**Figure AA.3*C*-*E***; Bdnf-L, $t(13) = 3.6$, $p < 0.01$; Bdnf-Pan, $t(8) = 2.8$, $p < 0.05$; Camk2a, $t(8) = 4.9$, $p < 0.01$). This suggests that HuD and miR-495 compete for the expression of NAc shared ARGs in response to CPP as well as CPP behavior itself.

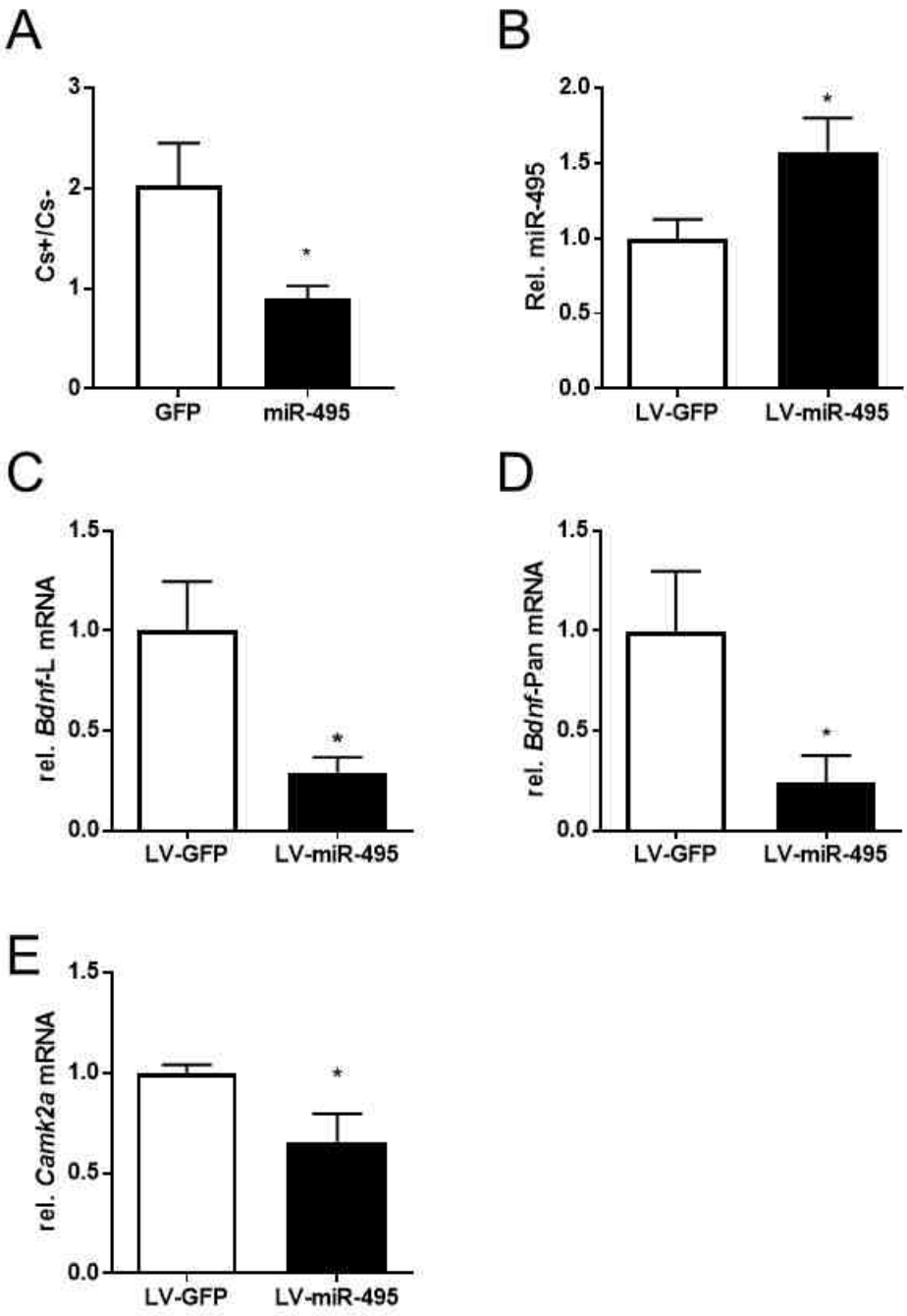

Fig. AA.1. NAcSh LV-miR-495 OE animals show decreased cocaine CPP-induced behavior and target expression within the NAc. Male C57Bl6/J mice were injected with LV-miR-495 or LV-GFP control and trained with 15 mg/kg cocaine in the same CPP-induced protocol as **Figure 3.2** except both groups were trained with cocaine. LV-

miR-495 OE decreased preference for the cocaine-paired Cs+ compared to littermate controls (n = 13-14/genotype). The NAc was harvested 1hr following initiation of the test day in the CPP protocol. LV-miR-495 OE did in fact lead to increased miR-495 within this region ***(B.)*** LV-miR-495 OE led to elevated *Camk2a*, *Bdnf-L*, and *Bdnf-Pan* mRNA (***C, D, E***) n = 3-5/treatment. **p*<0.05, ***p*<0.01 Student's t-test LV-miR-495 versus LV-GFP control. Error bars indicated ±SEM.

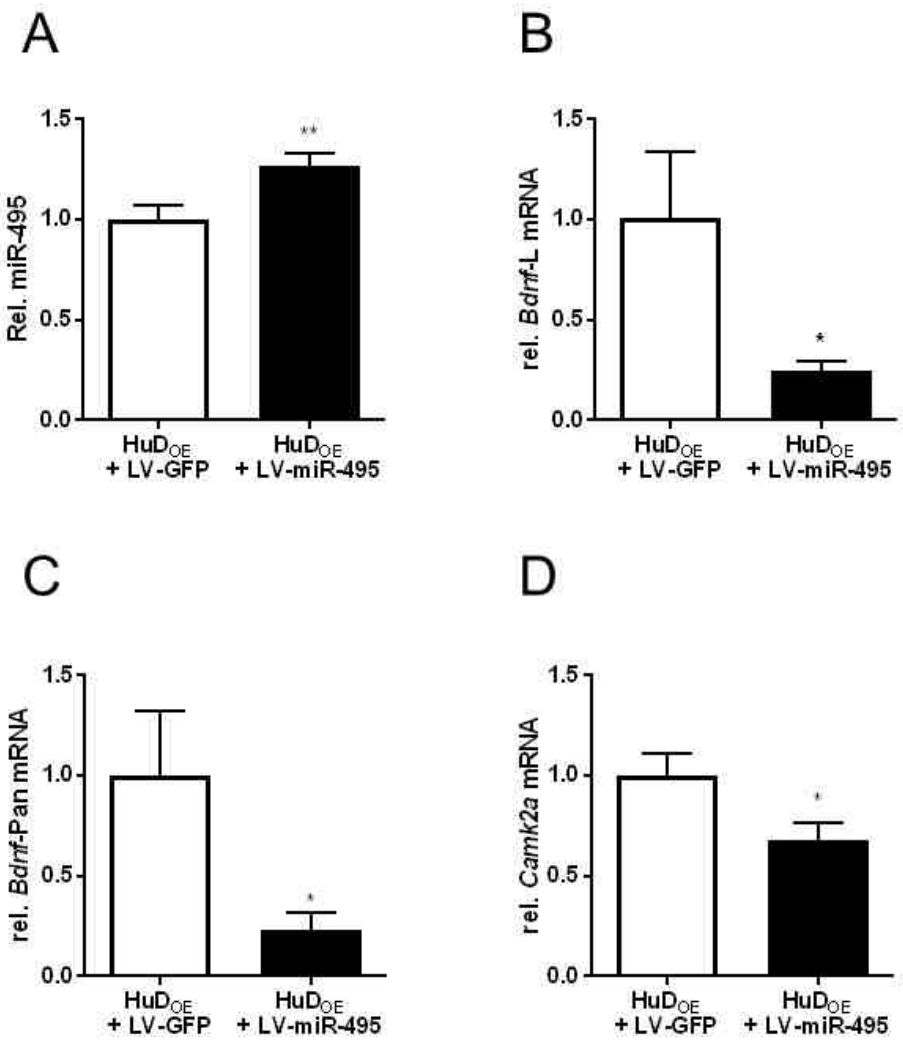

Fig. AA.2. NAcSh LV-miR-495 OE in naïve HuD_{OE} animals show decreased shared target mRNA expression. Male HuD_{OE} mice were injected with LV-miR-495 or LV-GFP control within the NAcSh. The NAc was harvested 2 weeks following viral infusion to allow for sufficient recovery and viral expression. HuD_{OE} + LV-miR-495 OE did lead to increased miR-495 within this region ***(A).*** HuD_{OE} + LV-miR-495 OE diminished *Camk2a*, *Bdnf-L*, and *Bdnf-Pan* mRNA (***B, C, D***) n = 3-5/treatment. *p<0.05, **p<0.01 Student's t-test LV-miR-495 versus LV-GFP control. Error bars indicated ±SEM.

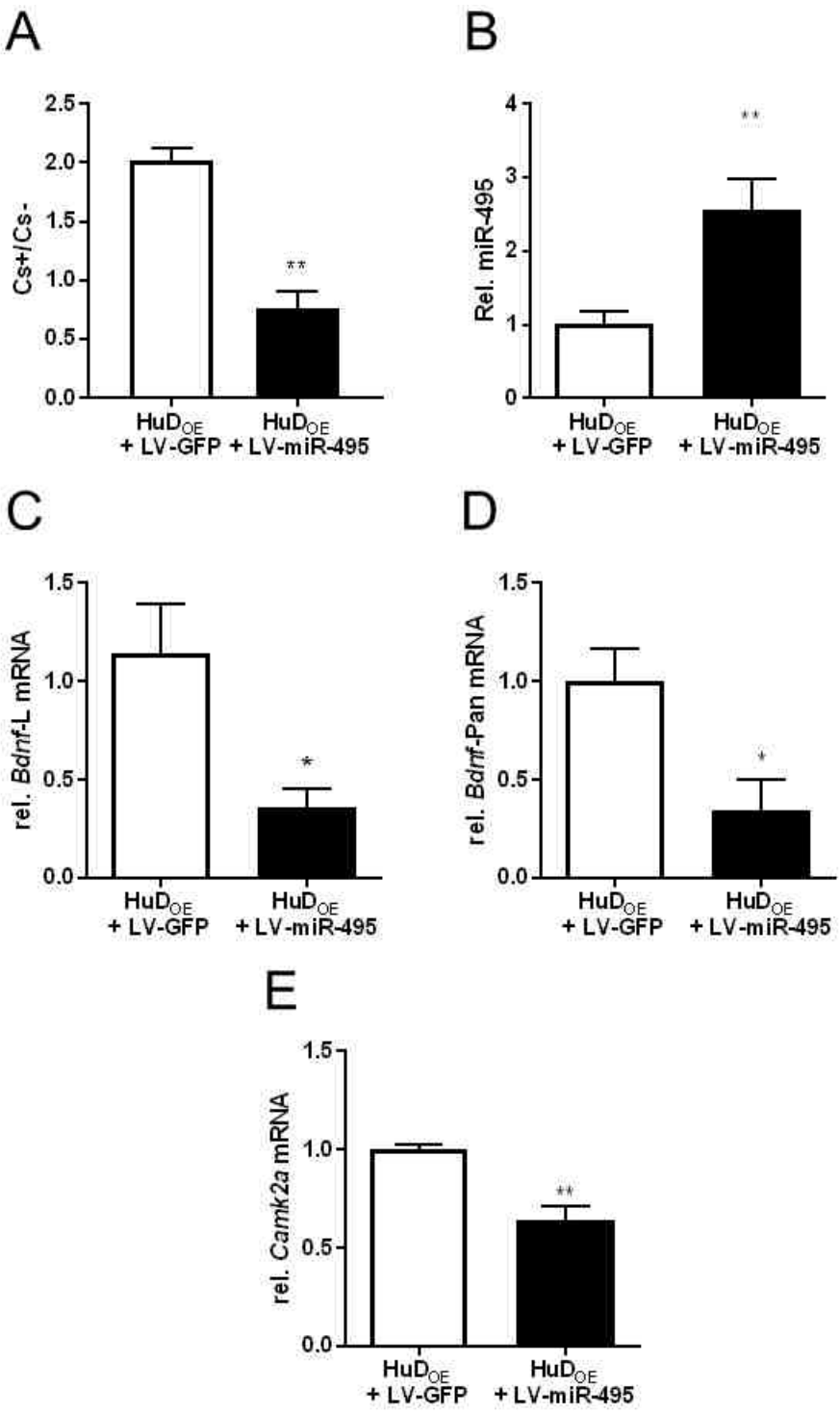

Fig. AA.3. NAcSh LV-miR-495 OE in HuD$_{OE}$ animals show decreased cocaine CPP-induced behavior and target expression within the NAc. Male HuD$_{OE}$ mice were injected with LV-miR-495 or LV-GFP control and trained with 15 mg/kg cocaine in the same CPP-induced protocol as **Figure 3.2** except both groups were trained with cocaine.

HuD_{OE} + LV-miR-495 OE decreased preference for the cocaine-paired Cs+ compared to littermate controls (n = 13-14/genotype). The NAc was harvested 1hr following initiation of the test day in the CPP protocol. HuD_{OE} + LV-miR-495 OE did in fact lead to increased miR-495 within this region ***(B)***. HuD_{OE} + LV-miR-495 OE led to diminished *Camk2a*, *Bdnf-L*, and *Bdnf-Pan* mRNA (***C, D, E***) n = 3-5/treatment. **p*<0.05, ***p*<0.01 Student's t-test LV-miR-495 versus LV-GFP control. Error bars indicated ±SEM.

APPENDIX B: Sensitization in C57 and HuD_{OE} animals

To test the role of miR-495 and HuD in addiction-related behaviors, we initially assayed the role of these factors in behavioral sensitization to cocaine. Additionally, many of the shared HuD and miR-495 targets within the NAc are associated with sensitization (Pierce *et al.*, 1998; Guillin *et al.*, 2000; Zhang *et al.*, 2007; Bahi *et al.*, 2008; Crooks *et al.*, 2010; J. A. Loweth *et al.*, 2010; Brown *et al.*, 2011; Kourrich *et al.*, 2012b; Robison *et al.*, 2013; Salery *et al.*, 2017). Mice were sensitized to cocaine (15 mg/kg, i.p.) by 5 single, daily injections of cocaine, followed by a 7-d withdrawal period before receiving a challenge injection of either saline or cocaine (15 mg/kg, i.p.) on day 12. These mice received cocaine injections within locomotor monitoring chambers, a novel environment. In contrast, we injected a separate group of animals in their home cage with 5 single, daily injections of cocaine (15 mg/kg, i.p.) which served as a subchronic home cage control group for molecular analyses. Mice given repeated injections of either cocaine or saline exhibited differential locomotor activity across time (**Figure AB.1*A***; Day, $F_{3, 12} = 15.6$, $p < 0.001$; Day x Treatment, $F_{3, 12} = 34.76$, $p < 0.001$). Cocaine treated mice exhibited an increase in locomotor behavior on days 1, 5, and 12 compared to baseline (**Figure AB.1*A***; Day, $F_{3, 5} = 21.93$, $p = 0.003$; post-hoc paired-samples t-tests, ps < 0.05), as well as an increase on day 12 compared to day 1 ($p < 0.001$), indicating locomotor sensitization had occurred. Saline controls exhibited a decrease in locomotor behavior on days 5 and 12 compared to both their baseline and day 1 (**Figure AB.1*A***; Day, $F_{3, 5} = 12.855$, $p = 0.009$; post-hoc paired-samples t-tests, ps < 0.05), suggesting these animals had habituated to the injections and/or activity

monitoring chambers. Animals were sacrificed 1 hour following the challenge injection and the NAc was dissected for molecular analyses. The mice treated subchronically within their home cage had no altered changes in NAc miR-495 expression, with levels that were comparable to saline-treated control mice (**Figure AB.1*B***; $p = 0.28$). In contrast, mice that received a sensitizing cocaine regimen had a decrease in NAc miR-495 expression 1 h after the challenge cocaine injection, compared to saline controls (**Figure AB.1*C***, $p = 0.02$). Furthermore, we calculated scores for changes in locomotion (i.e., sensitization in cocaine-treated mice and habituation in saline-treated mice) as a ratio of day12/day 1 and found that NAc miR-495 expression significantly and negatively correlated with these ratios in cocaine-treated mice, but not saline-treated mice (**Figure AB.1*D***, Cocaine: $r(4) = 0.93$, $p = 0.02$; Saline: $r(4) = 0.36$, $p = 0.26$). Additionally, we found that shared HuD and miR-495 target mRNA were increased within the NAc following behavioral sensitization to cocaine and concurrent with the decrease in NAc miR-495 (**Figure AB.1*E-G***; *Camk2a* $t(6) = 2.3$, $p = 0.05$; *Bdnf*-L $t(11) = 2.3$, $p < 0.05$; *Bdnf*-Pan $t(10) = 2.8$, $p < 0.05$). Thus, miR-495 expression within the NAc may play a role in the expression of behavioral sensitization to cocaine.

Next, we utilized HuD_{OE} mice to determine the role that HuD may play within sensitization. Since genotypes may not provide large effects for behavioral changes, we used a submaximal sensitization dose (7.5 mg/kg) to determine how HuD_{OE} may affect sensitization. Comparatively, the rest of the sensitization protocol remained unchanged from the original wild type experiment above. Since decreased NAc miR-495 was associated with increased sensitization to cocaine, we hypothesized that HuD_{OE} animals would have elevated levels of sensitization to cocaine, despite the submaximal

sensitization dose. There was a significant difference in locomotor activity across time and genotype, as well as a significant interaction between the two (**Figure AB.1*H***; Day, $F_{3, 18} = 16.6$, $p < 0.0001$; Genotype, $F_{1, 6} = 8.8$, $p < 0.05$; Day x Genotype, $F_{3, 18} = 4.8$, $p < 0.05$). Wild-type mice did not exhibit an increase in locomotor behavior on day 1 or day 5 but did have significantly elevated levels of locomotor activity on day 12 compared to baseline (**Figure AB.1*H***; Day, $F_{3, 12} = 20.1$, $p < 0.0001$; post-hoc paired-samples t-tests, baseline vs. day 5, Sidak's $ps < 0.05$; baseline vs. day 12 $ps < 0.001$), as well as an increase on day 12 compared to day 1 (Sidak's $p < 0.001$), indicating locomotor sensitization had occurred. As evidenced by the non-significant difference between baseline and day 1, these animals did not exhibit elevated acute-cocaine induced locomotor activity, as hypothesized by this submaximal dose of cocaine. In contrast, HuD_{OE} mice did exhibit an increase in locomotor behavior on day 1, in addition to days 5 and 12 compared to baseline (**Figure AB.1*H***; Day, $F_{3, 12} = 20.1$, $p < 0.0001$; Sidak's post-hoc paired-samples t-tests baseline vs. day 1, $ps < 0.01$; baseline vs. day 5, $ps < 0.05$; baseline vs. day 12 $ps < 0.001$). However, there was not a significant difference between day 12 and day 1 of cocaine treatment ($p = ns$). This suggests that although HuD_{OE} exhibited increased cocaine-induced locomotor activity in response to this submaximal dose of cocaine, they did not sensitize to cocaine.

To compare these differing effects on sensitization without affecting cocaine-induced locomotor activity, we examined the significant interaction between genotype x day (Day x Genotype, $F_{3, 18} = 4.8$, $p < 0.05$). There was no significant difference between control or HuD_{OE} in baseline locomotor measures, suggesting these mice show proper basal activity measures ($ps = ns$). Additionally, there was no significant difference

between genotypes in day 5 and day 12, suggesting that the behavioral plasticity induced by this cocaine regimen appears normal in both genotypes (p = ns). However, there was a significant difference between genotypes in the day 1 locomotor activity ($p < 0.01$). Thus, HuD_{OE} mice show an elevated locomotor response to acute cocaine. The HuD_{OE} acute cocaine locomotor response appears to be the maximal cocaine-induced locomotor response, as their day 12 locomotor response is not different from the control animals. To examine this difference further, we calculated scores for changes in cocaine-induced sensitized locomotor activity as a ratio of day12/day 1. As expected, there was a significant difference between genotypes in their sensitized response ($t(6) = 2.5$, $p < 0.05$). The mean ratio in the control group was 2.7, suggesting withdrawal induced behavioral plasticity, as characteristic of this experimental setup, had occurred in these animals. However, the mean ratio in the HuD_{OE} group was 1.1, suggesting that no such behavioral plasticity occurred in these animals. Although it is uncertain what plasticity may have occurred within the brain, we believe that the HuD_{OE} animals reached a maximal cocaine-induced locomotor activity level that could not be surpassed.

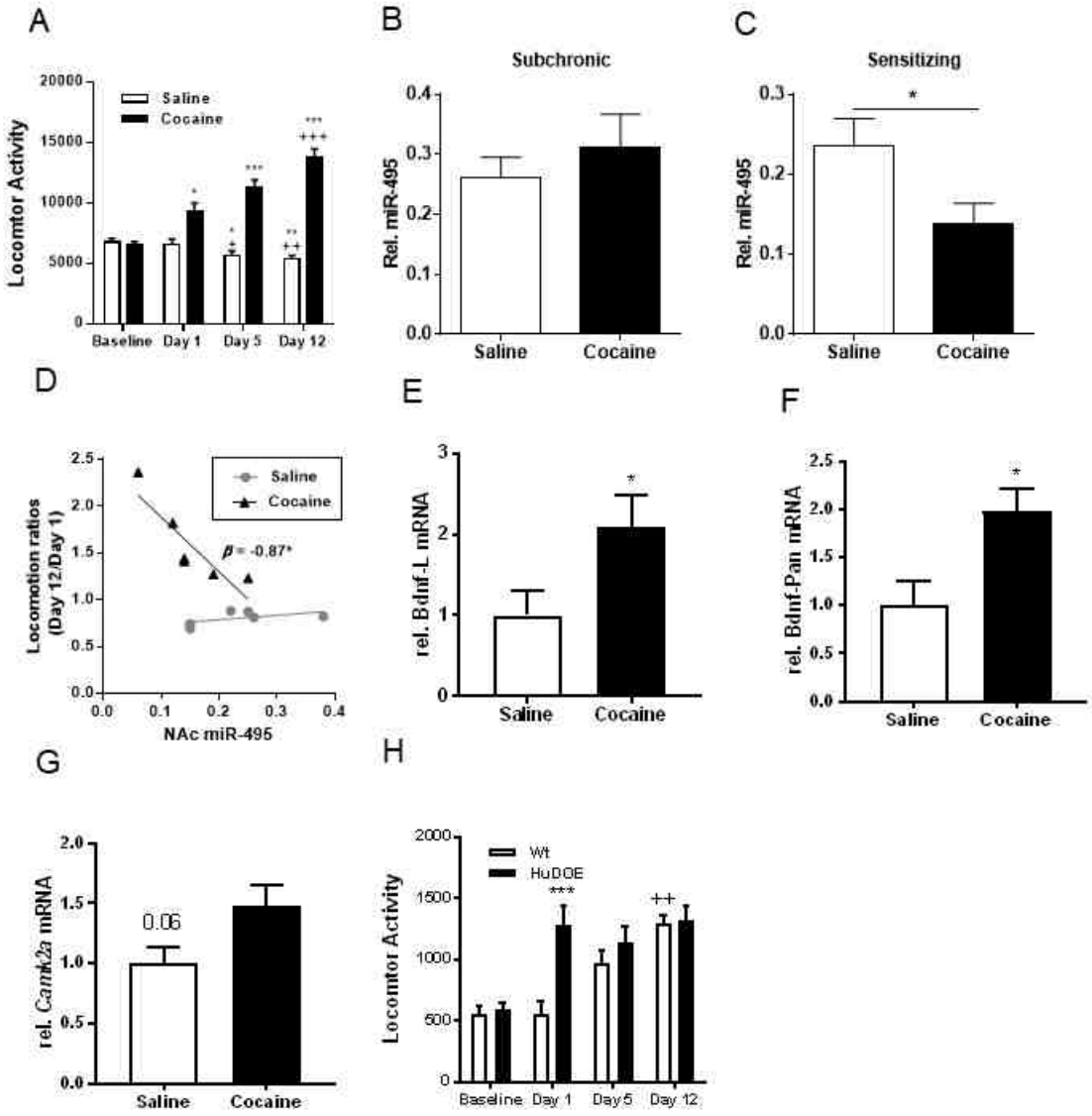

Fig. AB.1. NAc HuD and miR-495 are associated with locomotor sensitization to cocaine. (*A*) Challenge doses of saline or cocaine (15 mg/kg, *i.p.,*) in the context of an initially novel environment cause stereotyped alterations in locomotor activity defined as habituation and sensitization, respectively (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus baseline; + $p < 0.05$, ++ $p < 0.001$, +++ $p < 0.0001$ versus Day 1). (***B***) qRT-PCR analysis of miR-495 expression in the NAc 1 h following 5 daily injections (subchronic) cocaine (15 mg/kg, *i.p.,*) and saline treatments. Similar to sensitization dosing regimen without pairing to the novel locomotor chamber and without withdrawal + challenge dose. In contrast to acute cocaine administration, miR-495 levels did not decrease after subchronic cocaine treatment. (***C***) qRT-PCR analysis of miR-495 levels in the NAc 1 h following the final challenge dose of cocaine (15 mg/kg, *i.p.,*) and pairing with novel locomotor chamber. (*D*) miR-495 expression significantly and negatively correlated with locomotor sensitization scores expressed as day12/day 1 ratios. Line represents a best fit linear

function for the cocaine and saline treated groups separately, *$p < 0.05$. (E-G) Sensitization was associated with an increase in shared HuD and miR-495 targets. (H) HuDOE mice and littermate controls underwent a similar locomotor sensitization procedure as in (A), but both genotypes received cocaine but at a submaximal dose (7.5 mg/kg). *** $p < 0.001$ versus Wt at that day; ++ $p < 0.001$ versus Day 1 vs Day x within genotype). Error bars indicate SEM for (*A-C, E-H*).

APPENDIX C: Supplementary Information from miR-495 manuscript

Materials and Methods

miRNA fluorescent in situ hybridization. Cryosections (12 µm) from adult mouse brains were first fixed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to prevent miRNA loss from the tissue followed by 4% paraformaldehyde (Pena *et al.*, 2009). miR-495 and scrambled locked nucleic acid (LNA) probes (3 pmol; LNA miRCURY probe, Exiqon, Woburn, MA, USA) were labeled with digoxigenin (DIG Oligonucleotide Tailing Kit, Roche Applied Sciences, Basel, Switzerland, cat # 3353583910) and hybridized to the sections for 16 h at 55°C. After hybridization, sections were washed and then incubated in the presence of peroxidase-conjugated anti-DIG antibodies, followed by tyramide-Cy3 or fluorescein conjugates (TSA Plus Florescence Systems Kit, PerkinElmer, Waltham, MA, USA, # NEL741001KT). After subsequent washes, slides were mounted in Prolong Gold (Invitrogen, Grand Island, NY, USA, # P36930), and images were acquired on an Olympus BX-60 microscope with a DP71 CCD-digital camera (Olympus America, Center Valley, PA, USA).

Reverse transcription and qPCR. Mouse and rat brains were flash frozen and dissected using a brain matrix. Regions of interest were dissected using a 1.25 and 2 mm brain punch (Harris Unicore™). Tissue from one hemisphere was randomly chosen for RNA processing and the contralateral side was processed for protein analyses. RNA was isolated via standard Trizol® (Invitrogen, # 15596026) 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, # 4366596) 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, # 18064014) and quantified using Power SYBR Green PCR Master Mix (Life Technologies, # 4367660) with primers designed against selected targets (Mouse *Bdnf*-L: Forward TGGCCTAACAGTGTTTGCAG, Reverse GGATTTGAGTGTGGTTCTCC; Rat *Bdnf*-L: Forward GCCACTGAAATGCGACTGAA, Reverse CATTCCCCACCTCCATCTAGAC; Mouse/Rat Pan-*Bdnf*: Forward GACTCTGGAGAGCGTGAAT, Reverse CCACTCGCTAATACTGTCAC; Mouse/Rat *Camk2a*: Forward TATCCGCATCACTCAGTACCTG, Reverse GAACTGGACGATCTGCCATTT; Mouse/Rat *Arc*: Forward GGTAAGTGCCGAGCTGAGATG, Reverse CGACCTGTGCAACCCTTTC; Mouse/Rat *Gria3*: Forward GCATACACCCCTCTGGAGAA, Reverse TGAGAAGCCCTTCCATTTGA; Mouse/Rat *Stmn2*: Forward GCTACAGCTGGACCCTTCTC, Reverse GCAGGAGCAGATCAGTGACA; Mouse/Rat *Cnr1*: Forward ACCTCTTTCTCAGTCACGTTG, Reverse TGTCATTTGAGCCCACGTAG) and compared to a reference transcript (Mouse *Gapdh*: Forward TGTGATGGGTGTGAACCACGAGAA, Reverse GAGCCCTTCCACAATGCCAAAGTT; Rat *Gapdh*: Forward CCACAGTCCATGCCATCACT, Reverse GCCTGCTTCACCACCTTCTTG). Relative expression was determined using the comparative 2-ΔCt method (Livak *et al.*, 2001). A

no reverse transcriptase (RT) reaction was run for each sample and none of the no-RT controls amplified. qPCR experiments were replicated on 2-3 separate occasions. Data collected from each run were averaged together.

Dual luciferase assays. HeLa cells were transfected with a pcDNA3 Firefly luciferase reporter containing the 3'UTR of either the short or long form of *Bdnf*, termed pLuc*Bdnf*-S and pLuc*Bdnf*-L, respectively. The pcDNA3 vectors, in which the Firefly luciferase gene and the mouse *Bdnf* 3'UTRs were cloned, were a gift from Dr. Yue Feng (Allen *et al.*, 2013). Fragments of the 3'UTRs of *Camk2a* and *Arc* containing miR-495 binding-sites were cloned into the pIS0 Firefly luciferase reporter using SacI and XbaI restriction sites. The primers used were as follows: *Camk2a* 3'UTR: Forward CCGTGCTGGCTTCTTTGTTAC and Reverse CCAATCCATAGGACCAGGACTT; *Arc* 3'UTR: Forward AAGATTACAGAGAGGAGGTG and Reverse ATAAGTTTCATAGTTTTATTAACA. The pIS2 Renilla vector was co-transfected with the Firefly reporter. Pre-miR-495, anti-miR-495, and pre-miR™ miRNA precursor negative control #2 (Ambion) were obtained from Life Technologies™ and transfected at 20nM. Luciferase activity was determined using the Dual- Luciferase® Reporter Assay System (Promega Corporation, Madison, WI, USA, # E1910) and the Infinite® 200 plate reader (Tecan, Maennedorf, Switzerland).

Acute cocaine administration. C57BL/6J mice were randomly assigned to receive one injection of either saline or cocaine hydrochloride (15 mg/kg, i.p.) and were sacrificed either at 0.5 (saline: n = 3; cocaine: n = 5), 1 (saline: n = 3; cocaine: n = 10), 2 (saline: n = 3; cocaine: n = 4), 4 (saline: n = 3; cocaine: n = 6), 24 (saline: n = 3; cocaine: n = 6), or

48 h (saline: n = 3; cocaine: n = 5) post-injection. Brains were harvested, frozen in 2-methylbutane (-50^{0}C), and stored at -80^{0}C until processing.

Western blotting. NAc tissue was homogenized with lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40-) supplemented with fresh protease inhibitors (cOmplete™ Mini, Roche, Indianapolis, IN, # 4693159001). Lysates were pre-cleared by centrifugation at 14,000 x g and then used for protein determination. Aliquots containing 20 µg of total protein were diluted 1:1 in 2x Laemmli Sample Buffer (Sigma, # S3401) and run on 4-15% SDS polyacrylamide gels (Bio-Rad, #4568086). Western blots were performed as previously described (Tanner *et al.*, 2008) using antibodies to either CaMKIIα (1:2000; Santa Cruz, Dallas, TX, USA, # sc-13141), mature BDNF (1:1000; Icosagen, Õssu, Ülenurme, Tartumaa, Estonia, #327-100), or proBDNF (1:1000; Alomone, Jersusalem, Israel, #AGP-032). Membranes were then incubated for 1 h in either secondary goat anti-mouse HRP (1:5000; Santa Cruz, # sc-2005) or goat anti-guinea pig HRP (1:5000; Santa Cruz, # sc-2438), respectively, and were developed with standard chemiluminescent reagents and procedures (NEL103001EA, Perkin Elmer, Waltham, MA, USA). Each sample n is exactly one animal with both hemispheres pooled together. Specific bands that correspond to the correct molecular weight of the target (~14 kDa for mature BDNF, ~38 kDa for proBDNF, ~50 for CaMKIIα) were quantified using densiometric analysis in ImageJ and then standardized by pixel density to the Coomassie Brilliant Blue stain of total protein as described previously (Tanner *et al.*, 2008). Westerns were replicated 2-3 times and data was averaged together.

Intracranial viral injections. As described previously (Pentkowski *et al.*, 2012), 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) while rats were under isoflurane anesthesia (2-3%). Lentiviral vectors contained either green fluorescent protein (GFP; LV-GFP; 8.28 x 108 IUF/mL), which also expresses a non-targeting sequence, or GFP + pri-miR-945 (LV-miR-495; 1.32 x 109 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 Microdialysis, 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. For in vivo virus validation and microarray experiments, brains were harvested 7 days later under deep anesthesia, frozen in 2-methylbutane (-50^{0}C), and stored at -80^{0}C until processing.

NAcsh miR-495 overexpression and acute cocaine. Rats were implanted with guide cannula aimed at the NAcsh and randomly assigned to receive an infusion of either LV-GFP or LV-miR-495 (see Intracranial viral injections). Two weeks later, rats were randomly assigned to receive one injection of either saline or cocaine (15 mg/kg, i.p.; LV-GFP-saline: n = 3; LV-miR-495-saline: n = 5; LV-GFP-cocaine: n = 8; LV-miR-495-cocaine: n = 6) and were sacrificed two hours later. Brain tissue was rapidly collected as described previously.

Microarray analysis. We used Affymetrix® Rat Gene 2.0 ST arrays (Santa Clara, CA, USA) to determine changes in gene expression in the NAc from LV-miR-495 vs. LV-GFP infused rats (n = 3). Data were 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. Predicted targets of miR-495 that were downregulated by miR-495 overexpression were used for Ingenuity Pathway Analysis (Qiagen, Lenlo, Limburg, Netherlands). Results were deposited on NCBI with a deposition number GSE85500.

NAcsh miR-495 expression following cocaine self-administration. Adult, male Sprague Dawley rats (200-225g upon arrival) were used for the self-administration experiments because catheter patency can be maintained for longer periods of time compared to mice, and therefore, rats were better suited for the self-administration experiments. The rats were handled ~2 min daily for 7 days before receiving surgery for implanting intravenous (i.v.) jugular catheters as previously described (Pockros *et al.*, 2011; Pentkowski *et al.*, 2012). Briefly, the free end of the catheter had a bent metal cannula attached (Plastics One, Roanoke, VA, USA) that was bored subcutaneously and exited out an incision on top of the skull. The cannulae were affixed on top of the skull using screws and dental acrylic. The incisions were sutured and treated with a topical antibiotic. Catheters were flushed daily with a 0.1 mL solution containing heparin sodium (70 U/mL; APP Pharmaceuticals, Schaumburg, IL, USA) and Timentin (66.7 mg/mL; GlaxoSmithKline, Research Triangle Park, NC, USA). Catheter patency was tested periodically by

administering 0.05 mL of methohexital sodium (Brevital, 16.6 mg/mL, Jones Pharma, Inc., St. Louis, MI, USA), which results in rapid anesthetic effects when administered i.v.

Self-administration began 7 days post-surgery and two days prior to the first session, rats were placed on mild food restriction (16 g/day). Rats were then randomly assigned to receive either 1 or 22 d of self-administration (e.g., SA1, SA22). In order for the SA1 group (n = 6) to receive a comparable number of cocaine infusions as the SA22 group (n = 7), we established a criterion where rats were only included in the experiment if they received at least 12 infusions during the first 2-h session. Cocaine was delivered on a fixed ratio (FR) 1 schedule of cocaine reinforcement (0.9375 mg/kg/0.1mL infusion) throughout the first session, 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 = 7 total controls). Following the first session, the SA22 group went on to continue self-administration 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 across sessions depending on individual performance. Criteria for advancing to a higher ratio at the start of a session were <15% variance in the number of infusions received across 3 consecutive days. Once

rats started and stabilized on a VR5 for 3 consecutive days, they received gradual increases in daily food (i.e., 18, 20, then 22g/day for the remainder of the experiment). Based on the NAc miR-495 expression time course following acute cocaine in mice, rats in both the SA1 and SA22 groups were sacrificed one hour following the last session. One hour post-session represents 3 h from their first infusion, as well as 1 h from their last infusion, well within the 1-4 h time frame of the miR-495 decrease observed after acute cocaine administration. Brains were rapidly extracted, frozen in 2-methylbutane, and stored at -80^{0}C until processing.

NAcsh miR-495 overexpression and cocaine self-administration, extinction, and reinstatement. Adult, male Sprague Dawley rats (200-225g upon arrival; n=28) received surgery and initial self-administration training as previously described (see above, NAc miR-495 expression following cocaine self-administration), with the following exceptions: they received NAcsh intracranial guide cannulae along with jugular vein catheters, they were given food ad libitum once they stabilized on an FR5 schedule, and the cocaine training dose was 0.75 mg/kg/infusion. Once stability was reached under unrestricted food conditions, rats were infused with either LV-GFP (n = 12) or LV-miR-495 (n = 10) while under isoflurane (2-3%) anesthesia (see Intracranial viral injections). Total cocaine intake prior to receiving viral infusions was counterbalanced across groups. One week later, when lentiviral-mediated miR-495 overexpression is known to occur (Fig. 4C), 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 progressive ratio (PR) schedule of reinforcement at the same dose. The response demand

in the PR sessions increased exponentially according to the formula 5*e^(0.2n)-5 (Richardson *et al.*, 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. After completion of PR testing, the rats were switched 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 a low extinction baseline (<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. 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 to injections. 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 in the operant chamber. Lever pressing resulted in no consequences.

NAcsh miR-495 overexpression and 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, # F0021) in 30-min daily sessions. Advancement of reinforcement schedule was identical to the cocaine SA experiment, however 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; see Intracranial viral injections), where group assignment was counterbalanced for previous total food intake during training (n = 8/group). Rats were given one week of recovery, followed by one week of FR5 sessions, and then 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).

Histology. To visualize cannula placement and spread of virus, rats were perfused transcardially with 4% paraformaldehyde (PFA) following behavioral testing 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 microtome. Sections were mounted on gel-coated glass slides using Vectashield+DAPI mounting medium (Vector Labs, Burlingame, CA, USA, # H-1200). 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 and DAPI staining were overlayed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA,

USA). Either no GFP expression or >50% of GFP expression outside of the target region were considered misplaced. Six rats from the cocaine self-administration experiment and four rats from the food experiment were excluded from the study.

Figures

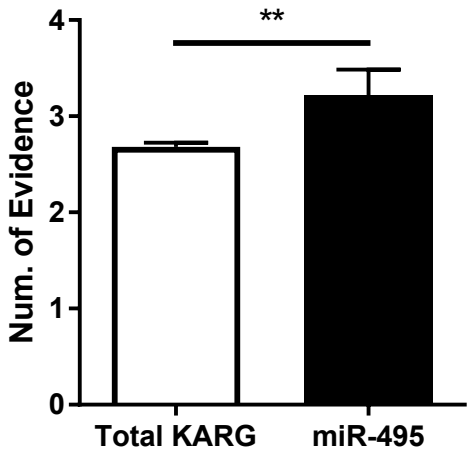

Fig AC.1. Predicted miR-495 targets from the KARG have more evidence for their involvement in addiction than the total KARG. The entire mouse KARG dataset was searched for miR-495 targets as in Fig. 1*A*, *B* and Table S1. Each gene in the dataset included a score for how many pieces of evidence linked the gene to addiction. Average evidence scores from the entire dataset in comparison to predicted miR-495 targets were compared. Mann-Whitney U, ** $p < 0.01$.

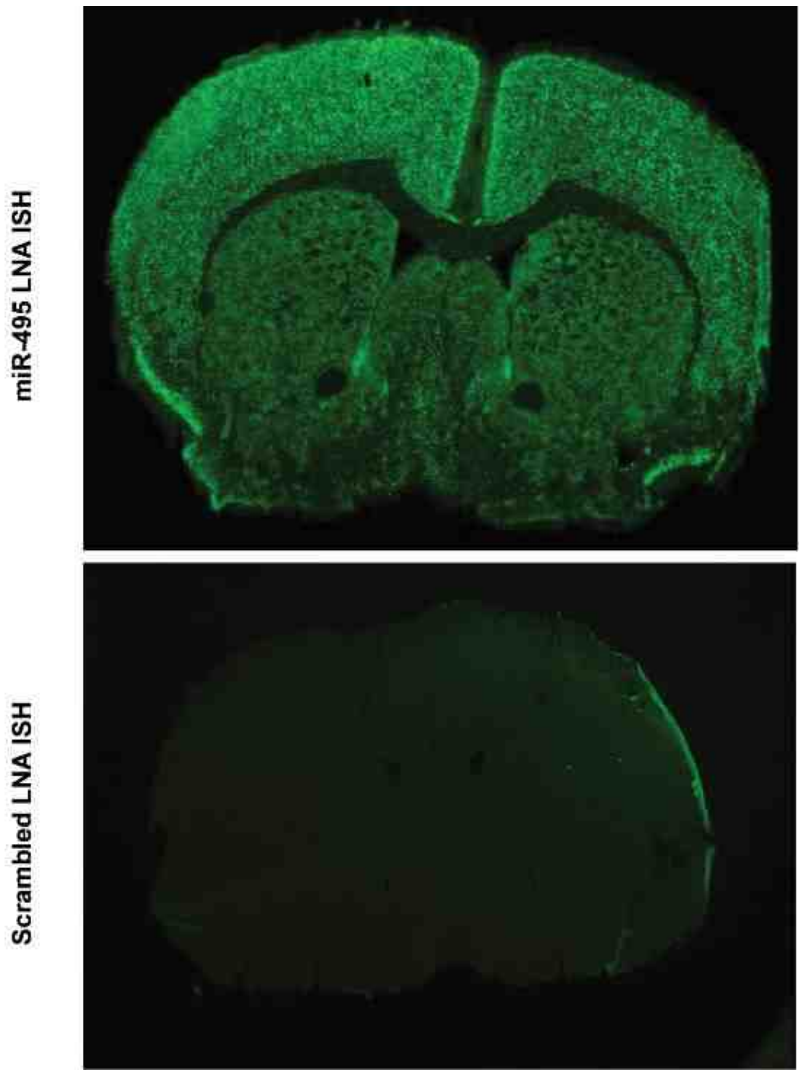

Fig AC.2. Fluorescent *in situ* hybridization of miR-495 in the mouse brain demonstrating probe-specific labeling. Sections were hybridized with miR-495 specific LNA (top panel) or scrambled (bottom panel) probes. Both images were acquired using the same exposure time.

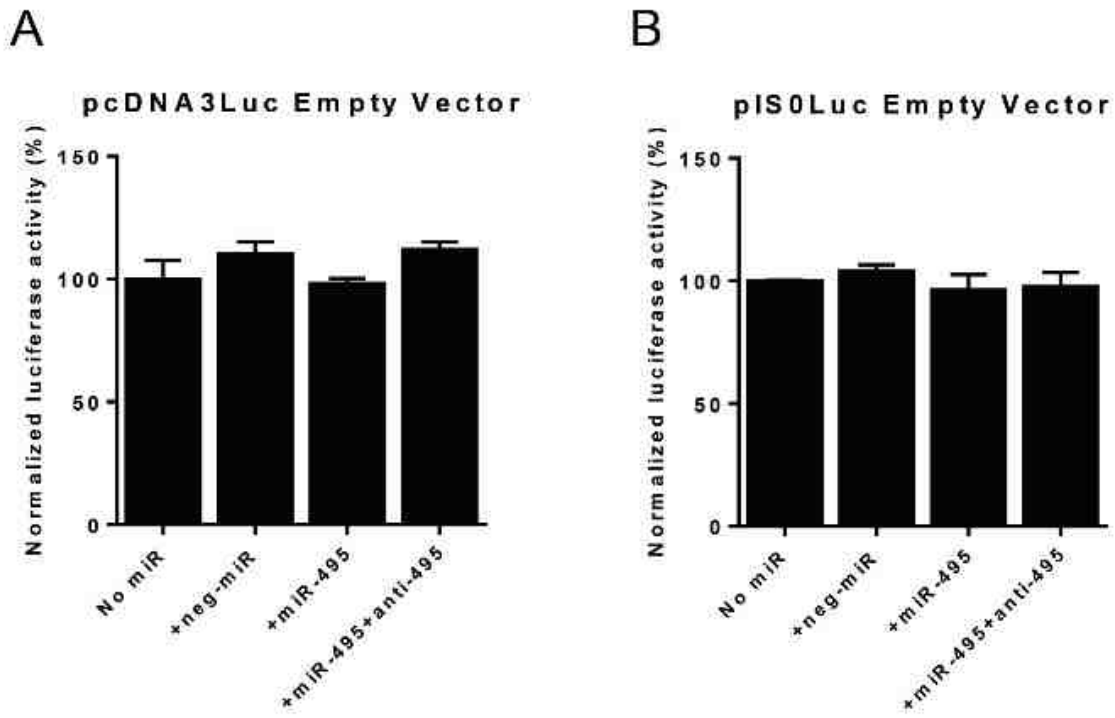

Fig AC.3: Controls for luciferase experiments. HeLa cells were transfected with a firefly luciferase reporter pcDNA3Luc empty vector (*A*) or pISOluc empty vector (*B*). A *Renilla* vector was co-transfected with the firefly vectors. Pre-miR-495 and anti-miR-495 were transfected at 20nM. Pre-miR™ miRNA precursor negative control #2 was used as a negative control. Error bars indicate SEM.

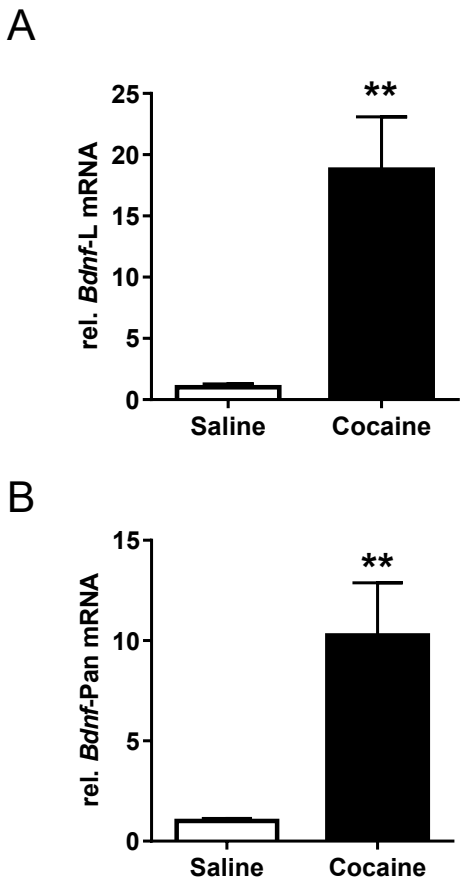

Fig AC.4. NAc *Bdnf* mRNA isoform expression 2 h following acute cocaine administration. qRT-PCR analysis of *Bdnf*-L and *Bdnf*-Pan mRNA levels in the NAc 2 h following acute saline or cocaine administration (15 mg/kg, *i.p.*; n = 8/group) in male C57Bl/6 mice. Although both *Bdnf*-L and *Bdnf*-pan mRNA within the NAc were significantly increased 2 h post-injection, the relative upregulation of *Bdnf*-was about 2-fold higher than the total *Bdnf* (*Bdnf*-pan). Error bars indicate SEM. $^{**}p < 0.01$ vs. saline.

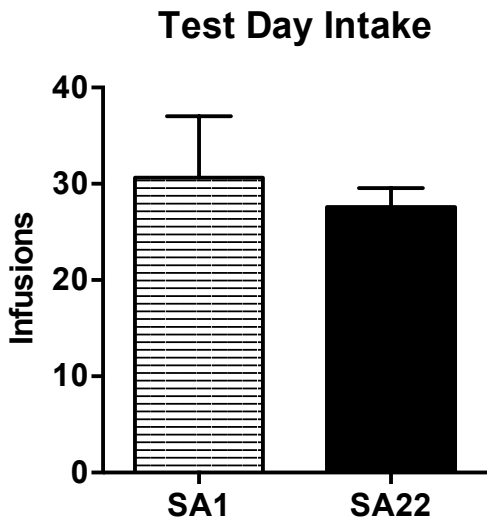

Fig AC.5: Cocaine intake during the final 2-h session in rats given either 1 or 22 sessions of cocaine self-administration. Rats were trained to lever press for cocaine (0.9375 mg/kg/0.1mL) and either received 1 (SA1; n = 6) or 22 (SA22; n = 7) 2-h sessions of cocaine self-administration. Bars indicate mean number of infusions received on the last day of self-administration. Error bars indicate SEM.

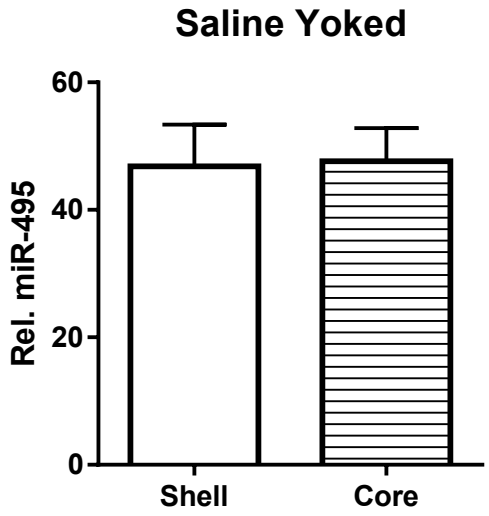

Fig AC.6: No difference in NAc shell vs. core miR-495 expression in saline-yoked animals.
Rats received saline infusions yoked to a cocaine partner who had access to cocaine (dose) on an FR5 schedule of reinforcement. NAc tissue was collected 1 h following the test session and was processed using qRT-PCR with Taqman probes for miR-495 and U6 snRNA. Bars indicate mean miR-495 levels normalized to U6. n = 7/group. Error bars indicate SEM.

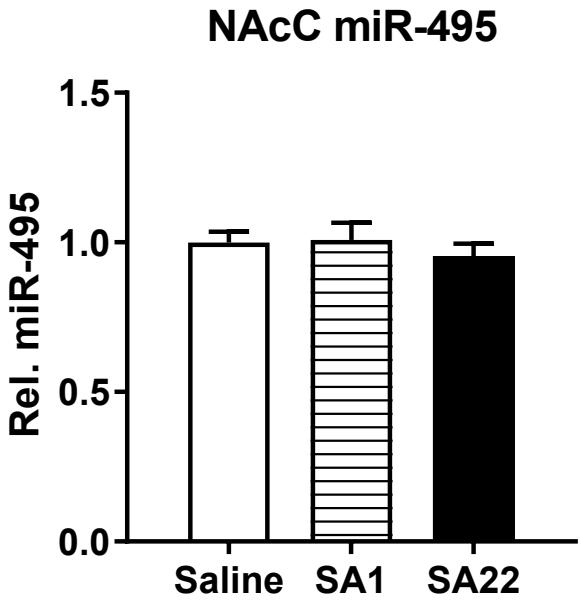

Fig AC.7: No change in NAc core miR-495 expression following cocaine self-administration. Rats were trained to lever press for cocaine (0.9375 mg/kg/0.1mL) and either received 1 (SA1; n = 6) or 22 (SA22; n = 7) days of cocaine self-administration. Controls (n = 7) received saline infusions yoked to a cocaine partner. NAc tissue was collected 1 h following the test session to determine changes in miR-495 levels as a result of cocaine self-administration. The tissue samples were processed using qRT-PCR with Taqman probes for miR-495 and U6 snRNA. Bars indicate mean miR-495 levels normalized to U6. Error bars indicate SEM.

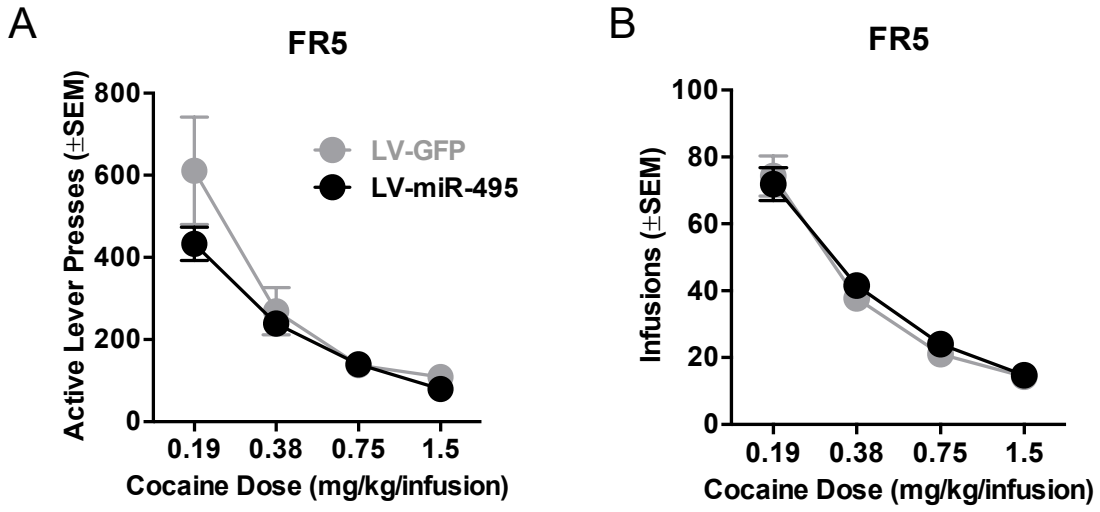

Fig AC.8: NAcsh miR-495 overexpression did not alter cocaine self-administration on a FR5 schedule. Total responses (*A*) and infusions (*B*) during 2-h sessions across cocaine doses on an FR5 schedule. LV-GFP: n = 12, LV-miR-495: n = 10. Error bars indicate SEM. FR = fixed ratio.

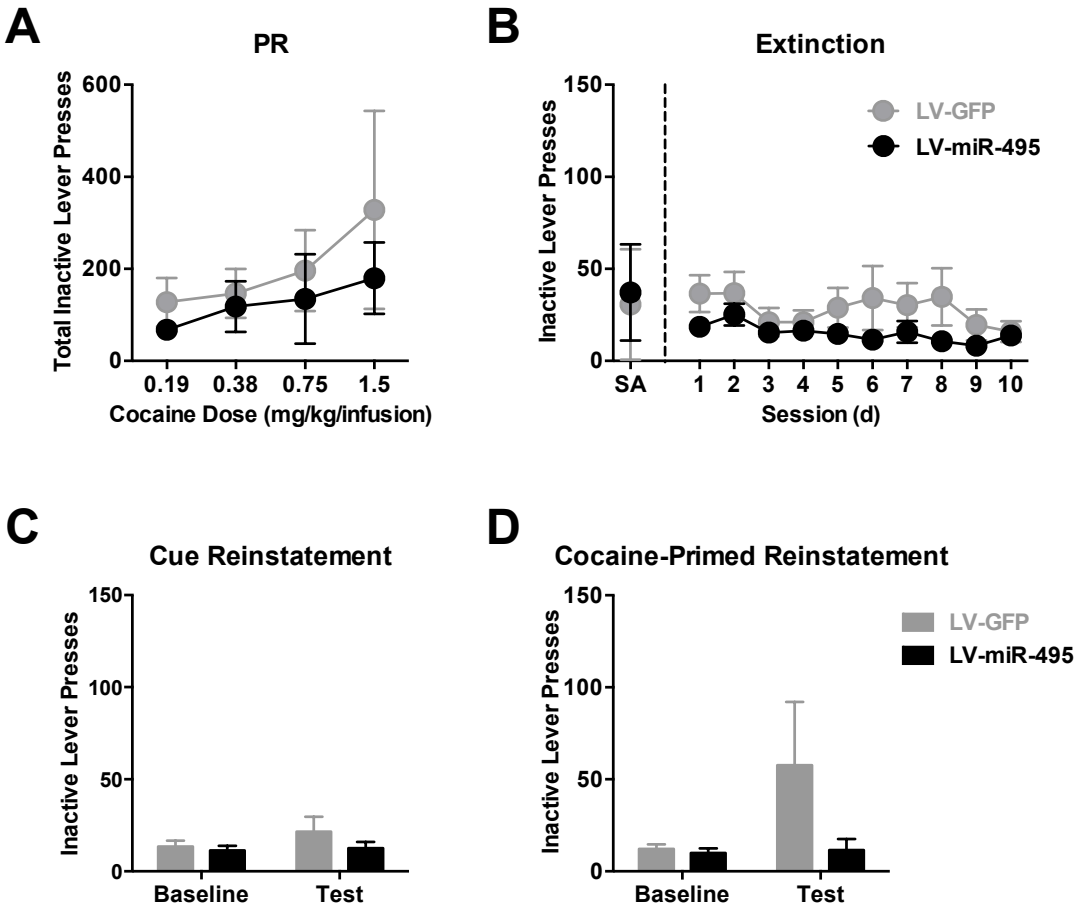

Fig AC.9: NAcsh miR-495 overexpression did not alter inactive lever presses on a PR schedule or during extinction and reinstatement of cocaine-seeking behavior. (*A*) Total inactive lever presses/session across several cocaine doses on a PR schedule. (*B*) Inactive lever presses during extinction in rats initially trained on an FR5 schedule of cocaine self-administration. During the 1-h daily extinction sessions, lever presses produced no consequences. (*C*) Inactive lever presses during the cue reinstatement test. After meeting criteria for extinction of cocaine-seeking behavior (Baseline), rats underwent cue reinstatement where cues that were previously presented response-contingently during self-administration we available on an FR1 schedule for 1 h. (*D*) Inactive lever presses during the cocaine-primed reinstatement test. After re-establishing an extinction baseline, rats received a priming injection of cocaine (10 mg/kg, i.p.) and were immediately placed into the operant conditioning chamber. They were then allowed to lever press under extinction conditions for 1 h (i.e., responses produced no scheduled consequences). LV-GFP: n = 12, LV-miR-495: n = 10. Error bars indicate SEM. PR = progressive ratio.

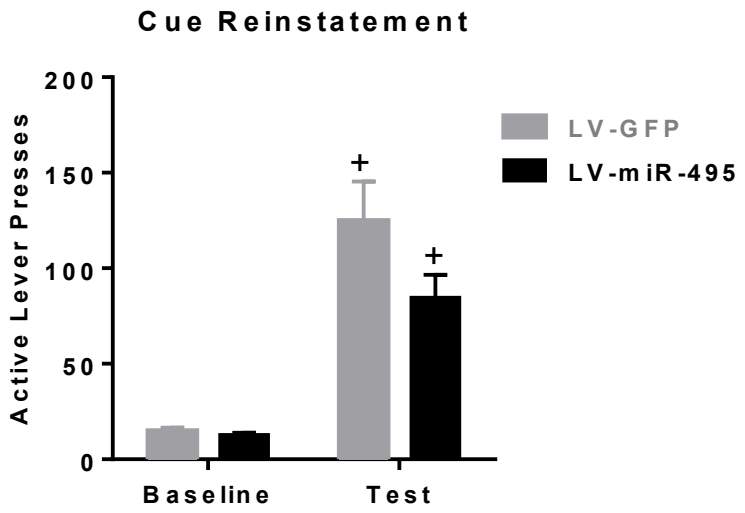

Fig AC.10: NAcsh miR-495 overexpression did not alter cue reinstatement. After meeting criteria for extinction of cocaine-seeking behavior (Baseline), rats underwent cue reinstatement where cues that were previously presented response-contingently with cocaine during self-administration sessions were available on an FR1 schedule for 1 h. LV-GFP: n = 12, LV-miR-495: n = 10. Error bars indicate SEM. + $p < 0.05$ vs. Baseline.

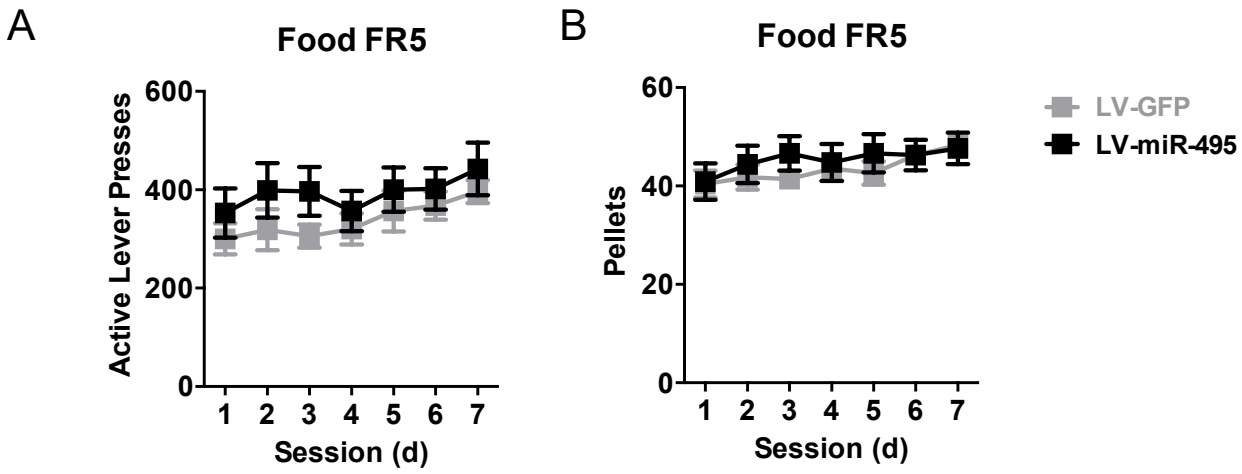

Fig AC.11: NAcsh miR-495 overexpression did not alter responses or intake of food on an FR5 schedule. Total responding (*A*) and intake (*B*) for food reinforcement on an FR5 schedule across 7 sessions. Lentiviruses were infused one week prior to the first FR5 session. n = 8/group. Error bars indicate SEM. FR = fixed ratio

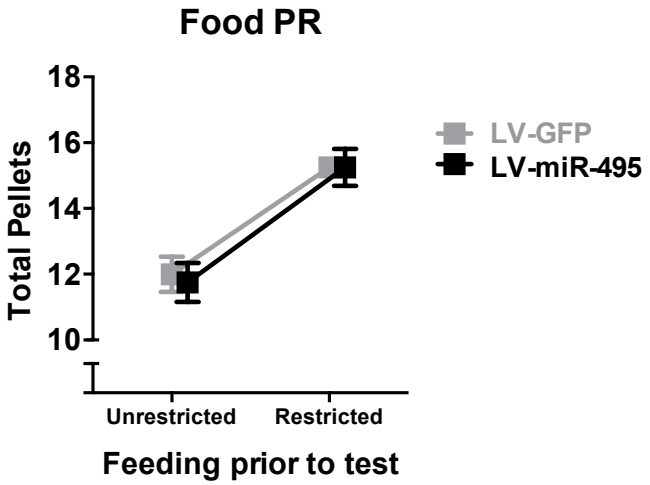

Fig AC.12: NAcsh miR-495 overexpression did not alter food intake on a PR schedule. Total responding (*A*) and infusions (*B*) for food reinforcement on a PR schedule. Rats were initially tested on restricted feeding conditions (18g/day) and then tested one week later on unrestricted feeding conditions. n = 8/group. Error bars indicate SEM. PR = progressive ratio

Tables

Table AC.1. *In silico* identification of miR-495 target mRNAs in the KARG database

Identification Method	miR-495 target mRNAs
KARG &TargetScan (TS)	*Actn2, Arc, Bcl2l1, Bdnf, Bmp7, Cacna1d, Camk2a, Cask, Cdh1, Clock, Cnbp, Cnr1, Crebbp, Ctnnd2, Ddhd2, Ddit4, Dedd, Eif5a, Fkbp5, Foxg1, Gabbr2, Gad2, Gap43, Gosr1, Hnrnph2, Hspa5, Htr2c, Igf1, Igf1r, Kat2b, Kcmf1, Mapk10, Myt1, Myt1l, Nacc2, Ndst1, Nfib, Nkx2-1, Npas2, Nptxr, Nr4a3, Per2, Pgk1, Pibf1, Ppargc1a, Ppme1, Ppp1cb, Prkar1a, Prosc, Prrc2a, Pten, Ptpra, Pura, Rab11b, Ran, S1pr3, Scg2, Sgk1, Slc14a1, Slc2a1, Snca, Sox9, Sptbn1, Srsf6, Ssx2ip, Stmn2, Tfap4, Timp2, Ube2n, Wnk1*
KARG, TS, miRanda	*Bdnf, Camk2a, Cnr1, Ctnnd2, Dusp6, Fkbp5, Hspa5, Jun, Atxn1, Sgk, Per2, Clock, Fu Tmed10, Ddit4*

Shown are candidate miR-495 target and Knowledgebase of Addiction related Genes (KARG) mRNAs that have been identified through *in silico* analyses by overlapping predicted miR-495 targets found in TargetScan (TS) with genes found in the KARG database. To more stringently identify predicted miR-495 targets, we also utilized miRanda, considering the free energy of the RNA duplex $\Delta G \leq -15$ kcal/mol.

Table AC.2. miR-495 target mRNAs downregulated by LV-miR-495 overexpression within the NAcSh

Down <0.75 and TS targets	*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*

Shown are candidate miR-495 target mRNAs found within TargetScan that were significantly downregulated (fold change ≤0.75 and $p<0.05$) by miR-495 overexpression in NAcsh. These mRNA targets are expressed within the NAcSh and many are found within the KARG, suggesting that they may play a role in addiction-related processes.

*downregulation confirmed by qRT-PCR.

Table AC.3. Molecular network highly regulated by miR-495

miR-495 targets	**Arc, Atp2b2, *Bdnf, Cacna1d, *Camk2a, Camk2g, Dlgap2, Dmd, Ephb2, *Gria3, Lhx2, Ncam2, Neurod6, Nr4a3, Nrxn1, Per2, Rap1gap2, Rimbp2, Satb2, Shank2, Tmeff2*
Interacting molecules	*Actin, AMPARs, Calcineurin proteins, Calmodulin, CaMKII, CREB, Cytochrome C, ERK1/2, HDACs, L-Type Calcium Channels, mGluR1, NMDARs, PIAS, Proinsulin*

Shown are miR-495 target mRNAs found within TargetScan that were significantly downregulated (fold change ≤0.75 and $p<0.05$) by miR-495 overexpression in NAcsh and were found to form an interacting molecular network. These mRNA targets are expressed within the NAcSh and many are found within the KARG, suggesting that they may play a role in addiction-related processes. Also shown are molecules or classes of molecules that interact with miR-495 targets. *downregulation confirmed by qRT-PCR.

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