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microRNA regulation related to the protective effects of environmental enrichment against cocaine-seeking behavior

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Abstract

Background: MicroRNAs (miRNAs) are "master post-transcriptional regulators" of gene expression. Here we investigate miRNAs involved in the incentive motivation for cocaine elicited by exposure to cocaine-associated cues.

Methods: We conducted NanoString nCounter analyses of microRNA expression in the nucleus accumbens shell of male rats that had been tested for cue reactivity in a previous study. These rats had been trained to self-administer cocaine while living in isolate housing, then during a subsequent 21-day forced abstinence period they either stayed under isolate housing or switched to environmental enrichment (EE), as this EE intervention is known to decrease cocaine seeking. This allowed us to create groups of "high" and "low" cocaine seekers using a median split of cocaine-seeking behavior.

Results: Differential expression analysis across high- and low-seekers identified 33 microRNAs that were differentially expressed in the nucleus accumbens shell. Predicted mRNA targets of these microRNAs are implicated in synaptic plasticity, neuronal signaling, and neuroinflammation signaling, and many are known addiction-related genes. Of the 33 differentially-expressed

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Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.drugalcdep.2021.108585.

microRNAs, 8 were specifically downregulated in the low-seeking group and another set of 8 had expression levels that were significantly correlated with cocaine-seeking behavior.

Conclusion: These findings not only confirm the involvement of previously identified microRNAs (e.g., miR-212, miR-495) but also reveal novel microRNAs (e.g., miR-3557, miR-377) that alter, or are altered by, processes associated with cocaine-seeking behavior. Further research examining the mechanisms involved in these microRNA changes and their effects on signaling may reveal novel therapeutic targets for attenuating drug craving.

Keywords

Drug abuse; Substance use disorders; Gene expression; miRNA; Isolation stress; Motivation

1. Introduction

Psychostimulant abuse is a significant, ongoing problem in the U.S with devastating economic and social costs to drug abusers and their communities (National Drug Intelligence Center, 2011; National Institute on Drug Abuse, 2015; Pomara et al., 2012). Cocaine use disorders (CUDs) in particular are a serious issue, as cocaine-related deaths have increased substantially over the past few decades, even as general use has declined (Center for Behavioral Health Statistics and Quality, 2015; McCall Jones et al., 2017; National Institute on Drug Abuse, 2020). Unfortunately, there are few treatment options that are effective in promoting long-term abstinence from drug use, especially psychostimulant use. Consequently, 40–60 % of drug users relapse within the first year of abstinence (National Institute on Drug Abuse, 2018). This is in part because drug-associated cues elicit drug craving that strengthens over prolonged abstinence, leaving those with CUDs vulnerable to relapse despite efforts to cease drug use (Dackis and O'Brien, 2001; Gawin and Kleber, 1986; Neisewander et al., 2000). For example, cues such as a crack pipe or crack house acquire conditioned stimulus effects which can trigger craving and relapse (Ciccocioppo and Martin-Fardon, 2004; Conklin and Tiffany, 2002; Ehrman et al., 1992; Weiss et al., 2001). Motivational effects of cocaine-conditioned cues persist even after months without drug use in animal models of drug-seeking, a phenomenon referred to as incubation of craving (Grimm et al., 2001; Tran-Nguyen et al., 1998). Thus, treatments that reduce cue-elicited craving are needed to promote long-term abstinence.

In both animals and humans, various forms of enrichment are effective in attenuating cocaine-related behaviors throughout the abstinence-relapse cycle (Lynch et al., 2013; Vannan et al., 2018). Typically, environmental enrichment (EE) in animal models consists of social housing in small groups that are given access to novel toys and/or exercise equipment. Importantly, EE is effective in reducing drug-seeking behavior when given as an intervention during abstinence, as measured in cocaine conditioned place preference and operant behavior animal models (Chauvet et al., 2012; Ma et al., 2016a; Solinas et al., 2008; Thiel et al., 2011, 2010, 2009). Thus, EE can be used as a tool experimentally to create groups of animals with differing levels of cocaine-seeking behavior.

There is growing interest in the epigenetics of drug abuse, including the role of microRNAs (miRNAs). In general, mammalian miRNAs post-transcriptionally silence gene expression

by the imperfect base-pairing of nucleotides at positions 2–8 in the 5′ end of the miRNA (widely referred to as the "seed sequence") and other miRNA sequences to the 3′ untranslated regions (UTRs) of target mRNAs (Bartel, 2009; Schirle et al., 2014). Because hundreds to thousands of genes have miRNA target sequences in the 3′UTRs, miRNAs function as "master regulators" of gene expression (Plotnikova et al., 2019). The capacity of miRNAs to manipulate and alter gene expression has made this class of RNAs an exciting avenue for finding new therapeutic targets for CUDs treatment development. So far, several miRNAs have been shown to play a role in the motivational processes underlying CUDs, including the let-7 family (Chandrasekar and Dreyer, 2011, 2009), miR-212 (Hollander et al., 2010; Im et al., 2010), miR-495 (Bastle et al., 2018), and others (Kenny, 2014). It is likely that many miRNAs that contribute to resilience or susceptibility to CUDs have not yet been identified. Furthermore, many previous studies have examined the NAc as a whole, including both the core and shell, yet these subregions interface differently with corticolimbic inputs and play different roles in cocaine-seeking behavior. For instance, the NAc shell receives input from the basolateral amygdala, and this pathway is involved in processing incentive salience of cocaine-associated cues (Ma et al., 2016b; Millan and McNally, 2011).

The present study employed NanoString nCounter analysis to identify miRNAs that are differentially-expressed in the NAc shell in rats with "high" vs. "low" levels of cocaine-seeking behavior. Tissue was harvested from a subset of rats utilized in a previous experiment (Powell et al., 2020) that had confirmed that rats with a history of cocaine self-administration exhibit less operant responding reinforced by cocaine-associated light/tone cues when they were housed in EE for 21 days of abstinence than when housed in isolation (ISO) [90.25 ± 20.96 and 207.4 ± 33.55 mean responses ± SEM, respectively (n = 15–16/group)]. Here, we took advantage of the varying degrees of cue-elicited motivation for cocaine across a subset of the animals from this study to explore miRNAs as possible mediators of cue-elicited cocaine-seeking behavior.

2. Methods

2.1. Animals and tissue collection

All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal care and Use Committee at Arizona State University. Male Sprague-Dawley rats (N = 12) used in a previous study (Powell et al., 2020) were sacrificed by isoflurane overdose immediately after a 1-h test for cocaine cue reactivity. In the previous study, single-housed rats had been trained to self-administer cocaine (0.75 mg/kg, IV) delivered response-contingently with light and tone cues. After ≥15, 2-h sessions of training, rats underwent 21 days of forced abstinence, either remaining in single-housing or switching to an enriched environment with 3–5 cage mates, a running wheel, tubes, toys to enhance novelty, and extra nesting materials. Upon completion of forced abstinence, animals were placed back into the self-administration chamber with the cues, but not cocaine, available response-contingently (i.e., cue reactivity test) (Acosta et al., 2008; Kufahl et al., 2009). The number of times that a rat pressed the lever resulting in cue presentations without cocaine delivery was used as a

measure of cocaine-seeking behavior and is thought to reflect the degree of incentive motivation for cocaine elicited by the cues. Within 5 min of completing the test, brains were harvested and rapidly frozen in 2-methylbutane that was placed on dry ice to achieve a temperature of approximately −20 °C. Later, 2 mm coronal sections containing the NAc shell were excised using a brain matrix to place razor blades at the appropriate location on the ventral surface of the brain for capturing the NAc in the tissue section. Tissue punches were then taken containing the NAc core and anterior commissure (1 mm diameter). Secondary punches (2 mm diameter) were taken containing the NAc shell using the previously punched location of the core as a landmark. RNA was isolated from the NAc shell samples using the standard Trizol method as performed previously (Bastle et al., 2018). Samples (100–150 ng of RNA) were then analyzed for miRNA expression using the Nanostring® nCounter Rat miRNA Expression Assay Kit v1.5 at the University of Arizona Genetics Core. The panel quantifies expression of 423 rat miRNAs in version v1.5, slightly fewer than the 496 listed in the miRBase *Rattus norvegicus* miRNA database.

2.2. Bioinformatics analyses

Nanostring nCounter analysis provided expression levels of miRNAs as raw counts for each miRNA in the sample. 100 ng total RNA was used in a multiplexed reaction to anneal specific miRNA tags followed by ligation and enzymatic purification to remove excess unincorporated tags in the assay, using the manufacturer protocol. Sequence specific fluorescent reporter probes and biotinylated capture probes were hybridized to ligated target nucleic acid complexes overnight at 65 °C for >12 h, followed by a series of automated washes and immobilization onto a streptavidin lined cartridge for data collection. Digital images from the cartridges were obtained over 4 h with the nCounter Digital Analyzer (CCD camera and microscope objective lens) using 555 FOV data resolution. Digital counts were tabulated and exported as comma separated values.

Differential expression analysis was performed using the R package “limma” (Ritchie et al., 2015) to identify differences in miRNA expression between the high- and low-seeking groups. Briefly, normalization factors were calculated to scale the raw library sizes. Raw counts were converted to counts per million reads (CPM) for each miRNA, and miRNAs with very low expression (CPM < 30) were filtered out, which removed 68 of 420 miRNAs before the analysis. Weighted least squares were calculated for each miRNA and sample, then a linear model was fit. Contrasts were performed on the fitted linear model to compare expression of each miRNA between high- and low-seeking groups based on log2 fold change values.

TargetScan 7.2, a miRNA target predictor, was used to determine possible mRNA targets of the differentially-expressed miRNAs (http://www.targetscan.org/vert_72) (Agarwal et al., 2015). TargetScan predicts targets based on the miRNA’s seed sequence, as well as conserved sites on mRNAs that fully or partially match this sequence. For some miRNAs, the available data on the predicted mRNA targets applied not to a single miRNA, but to a miRNA family. For example, mir-3573-5p is part of the miR-423-5p/3573-5p family and predicted targets for all miRNAs in this family are shared. To obtain accurate TargetScan predictions, MIMAT accession numbers provided by Nanostring were used to determine

whether each mature miRNA originated from the 5′ or 3′ arm of its precursor. Prior literature does not always follow this convention; thus, in this paper, the 3p/5p label is only included when discussing results of the current study exclusive of comparisons to other work, or when prior authors have made the miRNA designations clear.

Because some miRNAs were upregulated, while others were downregulated, predicted mRNA targets were given “impact scores” to signify the levels of up- and downregulation that might result from the miRNA changes. The predicted targets and impact scores were input into IPA (version 51963813; QIAGEN Inc.) to identify significantly regulated pathways (Krämer et al., 2014). We then compared the TargetScan predicted mRNA targets of the differentially-expressed miRNAs to the known addiction-related genes in the Knowledgebase of Addiction-Related Genes (KARG) database (http://karg.cbi.pku.edu.cn) (Li et al., 2008). At the time of analysis, the rat KARG contained 1135 genes, of which 347 had an evidence score of 2 or more (were supported by 2 or more lines of evidence), and these were the only genes included in the analysis.

2.3. miRNA validation with RT-qPCR

Leftover RNA from the same samples used for the Nanostring analyses was used to validate select miRNAs. For each sample, approximately 45 ng of purified RNA were used to prepare cDNA using the Taqman® Advanced MicroRNA cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA, # A28007), Taqman® Advanced MicroRNA Assay primers (Life Technologies, Grand Island, NY, USA) for miR-376c-3p, miR-107-3p, and miR-212-3p, and Taqman® MicroRNA Assay primer for the control transcript U6. cDNA for each sample was diluted 1:100 with nuclease-free water, then run in triplicate for each miRNA and U6. Relative expression was determined using the comparative $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4. Statistical analysis

Rats were divided into groups based on median split of cocaine-seeking behavior. Statistical calculations were performed in GraphPad Prism 8, or R 3.6 (R Core Team, 2019). Linear regressions were used to analyze the correlation between miRNA levels (CPM) and cocaine-seeking behavior. For differential expression analysis, p-values and false discovery rates (FDR) using the Benjamini-Hochberg method were calculated using the R package “limma” (Ritchie et al., 2015). The statistical threshold for all tests was $p < 0.05$.

2.5. Data availability

Nanostring data are deposited in the Gene Expression Omnibus (GSE153524). R code used for data analyses are available at https://gitlab.com/neisewander_asu/vannan-powell-2020.

3. Results

3.1. Differentially-expressed miRNAs in the NAc shell correlate with cocaine-seeking behavior

The high and low cocaine-seeking groups (n = 6/group) derived from the median split of cocaine seeking values were significantly different in their cocaine-seeking behavior [t(10) =

3.452, p = 0.0062, Fig. 1A]. Cocaine-seeking values aligned well with housing condition: the “high” cocaine-seeking group consisted of 83.3 % (5) ISO and 16.7 % (1) EE rats and vice versa for the “low” cocaine-seeking group. In total, expression levels of 75 miRNAs were significantly correlated with cocaine-seeking behavior (Supplementary Table 1), although not all of these miRNAs were differentially expressed in the low vs. high seeking groups. Analysis of Nanostring counts using limma identified 33 miRNAs that were differentially expressed in the NAc shell in animals that displayed high versus low cocaine-seeking behavior (Table 1). Of these, 8 were downregulated and 25 were upregulated (Fold Change >1 and <1 on Table 1, respectively) in the low-seeking group relative to the high-seeking group. For Table 1, log2 fold change values have been converted to linear values where Fold Change = 2^(log2 values). In addition, 8 of the 33 miRNAs had expression levels that correlated with cocaine-seeking behavior, 5 positively and 3 negatively (Fig. 1C).

Nanostring results for miR-376c-3p, miR-107-3p, and miR-212-3p, which were all elevated in low-seeking animals, were validated using RT-qPCR (Supplementary Fig. 1). Subsequent t-tests including all rats (n = 6 of each group) did not show significant differences in expression between high- and low-seeking groups for these miRNAs. However, separating the seeking groups into quartiles (including only the 3 highest and 3 lowest cocaine-seeking animals), revealed significantly higher expression in the low-seeking group for miR-376c-3p [t(4) = 3.05, p = 0.0379, Suppl. Fig. 1A] and miR-107-3p [t(4) = 2.81, p = 0.0481, Suppl. Fig. 1B]. Although thevalues for miR-212-3p did not quite meet the threshold for significance after separating animals into quartiles [t(4) = 2.63, p = 0.0583], they did correlate significantly with behavior overall ([F(1,10) = 6.16, p = 0.0324]) (Suppl. Fig. 1C).

3.2. Predicted mRNA targets of the differentially-expressed miRNAs

TargetScan 7.2 was utilized to identify predicted mRNA targets of the differentially-expressed miRNAs for the rat, which were then utilized in subsequent analyses. The number of predicted targets varied for each miRNA and ranged from 11 (miR-487b-3p) to 3,972 (miR-3557-5p).

To create a list for input into IPA, first we determined the overall impact of our miRNAs on the list of predicted targets (mRNAs) relative to the low-seeking condition. If a miRNA was upregulated in low cocaine-seeking animals, its predicted targets were given a score of −1, as they would be downregulated by that miRNA, whereas targets of downregulated miRNAs were given a score of +1. For example, the mRNA *Nuclear factor I B (Nfib)* is a predicted target of 16 differentially-expressed miRNAs, of which 3 were downregulated in the low-seeking group (+3) and 13 of which were upregulated (−13), leading to a total impact score of −10 (Supplementary Table 2). We began with 9,761 predicted targets, which comprised 23.76 % of the transcribed genes in the tissue analyzed. Because more miRNAs were upregulated rather than downregulated in the low-seeking condition, predicted targets were more likely to have a negative impact score, and negative impact scores were greater in magnitude: 6,456 targets had a negative impact score (range: −1 to −14); 3,305 had a positive or 0 impact score (range: 0 to +3.) Because IPA analysis is more robust with smaller lists of genes, we prioritized candidates based on impact score. Only mRNA targets that

were mapped to IPA and had an impact score of −3 or lower and +1 or higher were included, reducing the list to 3,600 predicted mRNA targets.

The majority of mRNAs were predicted targets of only 1 or 2 differentially-expressed miRNAs (6,128; 62.8 %). However, 9 mRNAs were targets of 15 or more miRNAs: *Zinc finger and BTB domain containing 20 (Zbtb20)*, *Nuclear factor of activated T-cells 5 (Nfat5)*, *Argonaute RNA-induced silencing complex (RISC) component 1 (Ago1)*, *Nfib*, *Protein quaking (Qki)*, *Phosphodiesterase 3A (Pde3a)*, *Retinoic acid receptor-related orphan receptor B (Rorb)*, *Transcription factor 4 (Tcf4)*, and *Kruppel like factor 7 (Klf7)*, with impact scores ranging from −8 to −14. In total, 136 mRNAs (1.39 %) were predicted targets of at least 10 differentially-expressed miRNAs.

IPA revealed many significant pathways, Including *Wnt/β-catenin Signaling*, *Synaptogenesis Signaling Pathway*, *Axonal Guidance Signaling*, *Dopamine/DARPP34 Feedback in cAMP Signaling*, *CREB Signaling in Neurons*, and *ERK5 Signaling*, among others (Fig. 2A, Supplementary Table 3). Significant Diseases and Functions were also provided by IPA (Fig. 2B, Supplementary Table 4) and included: *Learning* in the category *Behavior*; *Development of Neurons* and *Morphogenesis of Neurons* in the categories *Cellular Development* and *Cellular Growth and Proliferation*; *Migration of Neurons* in the category *Cellular Movement*; *Transcription of RNA* and *Expression of RNA* in the category *Gene Expression* (Supplementary Table 9); and *Neurotransmission* in the category *Nervous System Development and Function* (Supplementary Tables 5–10). Including *Wnt/β-catenin Signaling*, *Synaptogenesis Signaling Pathway*, *Axonal Guidance Signaling*, *Dopamine/DARPP34 Feedback in cAMP Signaling*, *CREB Signaling in Neurons*, and *ERK5 Signaling*, among others (Fig. 2A, Supplementary Table 3).

3.3. miRNAs and addiction-related genes

For each miRNA, the number of predicted targets found in TargetScan was also compiled and then cross-referenced to addiction-related genes in the KARG database (Table 2). Three miRNAs, miR-3557-5p, miR-377-3p, and miR-337-3p, targeted a large percentage of KARG (between 17.00 % and 24.21 %). Together, the putative targets of significant miRNAs covered 205 (59.1 %) of the 347 KARG genes included in our analysis. Several addiction-related mRNAs were predicted to be targets of at least 10 differentially-expressed miRNAs: *Nuclear factor I A (Nfia), Nfib, Circadian locomoter output cycles protein kaput (Clock), Ataxin 1 (Atxn1), cyclic AMP (cAMP)-responsive element binding protein 1 (Creb1)*, *Zinc finger and BTB domain containing 16 (Zbtb16), Purine rich element binding protein A (Pura),* and *Gamma-aminobutyric acid (GABA) type B receptor subunit 2 (Gabbr2)*, potentially indicative of their key role in cocaine-seeking and drug motivation (Supplementary Table 11). Notably, all these mRNAs were predicted to be downregulated in the low-seeking group compared to high-seeking, with impact scores between −4 *(Zbtb16)* and −11 *(Atxn1)* (Supplementary Table 2).

4. Discussion

NanoString nCounter analysis of the NAc shell of male rats with a history of cocaine self-administration that were tested for cocaine-seeking behavior after 21 days of abstinence

identified 33 miRNAs displaying differential expression in the “high” and “low” cocaine-seeking groups. Of these, expression of 8 miRNAs correlated significantly with cocaine-seeking behavior. Predicted mRNA targets of the 33 miRNAs were analyzed using IPA, which revealed several significant pathways including *Synaptogenesis* and *Opioid Signaling*. Cross-reference of TargetScan and the KARG database showed that many of these miRNAs were predicted to target mRNAs of addiction-related genes.

Many of the differentially-expressed miRNAs identified in this study have been previously implicated in drug abuse, including miR-29a, miR-16, miR-495, mir-376c, miR-329, miR-138, miR-137, miR-337, miR-125b, miR-212, miR-130b, miR-221, and miR-132 (Bastle et al., 2018; Dave and Khalili, 2010; Eipper-Mains et al., 2011; Hollander et al., 2010; Im and Kenny, 2012; Lippi et al., 2011; Schaefer et al., 2010; Shin et al., 2010). Interestingly, these miRNAs all had greater expression in the low-seeking group compared to the high-seeking group. Of these, miR-212, a CREB-induced activity-dependent miRNA in the same family as miR-132, is perhaps the best-studied in the addiction field. For example, Sadakierska-Chudy et al. found that 2-h daily access to cocaine increases both miR-212 and miR-132 in the dorsal striatum compared to saline-yoked controls, and this increase is persistent, lasting 10 days into subsequent extinction training (Sadakierska-Chudy et al., 2017). In addition, Hollander et al. demonstrated that miR-212 and miR-132 may be involved in the transition from casual to compulsive drug use, as both are upregulated in the dorsal striatum following extended (6-h) daily cocaine self-administration compared to cocaine-naïve rats (Hollander et al., 2010). They also found that striatal miR-212 overexpression reduces compulsive-like cocaine-taking behavior during extended access (6-h daily sessions), while knockdown produces more compulsive cocaine consumption. Due to the close relatedness of miR-132 to miR-212, the authors suggest miR-132 may play a similar role in compulsive cocaine-taking. In the present study, the increased expression of miR-212 and miR-132 in the NAc shell of rats with low cocaine-seeking behavior suggests that these miRNAs are protective against motivation for cocaine. Together, it appears that striatal miR-212 and miR-132 expression may shield against two defining phases of CUDs: transition to an addicted-like phenotype as well as craving during protracted abstinence.

Among the miRNAs previously associated with motivation for cocaine is miR-495, a miRNA that we identified because its levels decrease during cocaine self-administration and its over-expression in the NAc shell attenuates motivation for cocaine (Bastle et al., 2018). We have shown that miR-495 regulates expression of multiple addiction-related genes including *Brain derived neurotrophic factor (Bdnf)*, *Calcium-calmodulin activated protein kinase IIα* (*Camk2a)* and *Activity-regulated cytoskeleton-associated protein (Arc)* among other mRNAs such as *Per2* and *Gria3* (Bastle et al., 2018). Here, we found that miR-495 has higher expression in low-seeking animals, supporting our prior research that suggests upregulation of miR-495 is protective against motivation for drugs of abuse.

To identify novel candidate miRNAs that may regulate cocaine-related behavior in this study we cross-referenced TargetScan and KARG. Of the 33 differentially-expressed miRNAs, miR-3557-5p, miR-377-3p, and miR-337-3p were predicted to target particularly high percentages of the KARG database (>17.00 %, up to 24.14 %). Of these 3, miR-3557-5p and

miR-377-3p have not been studied in substance abuse or psychiatric illness to our knowledge, suggesting they may be novel targets for addiction research. However, miRNAs that were predicted to target a large percentage of KARG also had many TargetScan predicted targets (>2000). Caution is needed when the number of predicted targets is so large, due to an increased likelihood of false positives and limitations of these databases. By contrast, miRNAs with fewer than 200 predicted targets (miR-346-5p, miR-483-3p, miR-193a-3p, mir-3573-5p, miR-376c-3p, miR-652-3p, miR-487b-3p, miR-409-5p) had little overlap with the KARG database (under 2.02 %).

Among the miRNAs predicted to target expression of a large number of addiction-related genes, miR-337-3p has been the subject of prior drug abuse research. Here, we found that miR-337-3p has significantly higher expression in the low-seeking group, suggesting it may be protective against cocaine-seeking behavior. However, in striatal *Dopamine Receptor 2* (*Drd2*)-expressing neurons, miR-337-3p is upregulated after acute cocaine injection in mice (Schaefer et al., 2010), suggesting that this miRNA may be associated with the initial neurobiological changes after drug exposure. Similarly, miR-376c, miR-138, and miR-137, which all have significantly higher expression in the low-seeking group in our study, are also upregulated in striatal *Drd2*-expressing neurons after acute cocaine injection (Schaefer et al., 2010). Therefore, these miRNAs may either protect against or facilitate addictive behaviors depending on factors such as previous drug experience. Other factors that may contribute to these seemingly contradictory results include differences in the brain region studied and cell-type specificity.

Several addiction studies have identified the importance of let-7 miRNAs, particularly let-7d (Chandrasekar and Dreyer, 2011, 2009; He et al., 2010; Hollander et al., 2010), although these miRNAs have few addiction-related targets according to KARG (5.48 %). For example, let-7d expression is decreased in regions of the mesolimbic reward pathway including the NAc core and shell, striatum, and ventral tegmental area after 15 days of daily cocaine injections compared to saline controls (Chandrasekar and Dreyer, 2009), whereas overexpression of let-7d in the NAc attenuates cocaine conditioned place preference (Chandrasekar and Dreyer, 2011). Here, we demonstrate that another let-7 family member, let-7a, has higher expression in low-seeking animals and displays a significant negative correlation with cocaine-seeking. Together, these data suggest let-7 miRNAs may modulate different aspects of cocaine-related behavior. Although TargetScan assumes the same predicted targets for all let-7 miRNAs, differences in their biogenesis and expression patterns may contribute to distinct roles in neuronal function and thus drug abuse (Roush and Slack, 2008).

Many of the miRNAs identified here have been explored primarily in relation to other psychiatric illnesses, such as schizophrenia and depression, which have high comorbidity with substance abuse (Batel, 2000; Kessler et al., 2005; Kosten et al., 1998; Paykel et al., 2005). For example, miR-16, miR-495, miR-652, miR-107, miR-138, and miR-137 have been linked to schizophrenia (Beveridge et al., 2010; Moreau et al., 2011; Ripke et al., 2011; Santarelli et al., 2011; Wright et al., 2013) and were all upregulated in the low-seeking group compared to the high-seeking animals in the present study. Similarly, miR-16 has been implicated in depression, and is believed to underlie the therapeutic effects of the

antidepressant fluoextine through targeted downregulation of the serotonin transporter (SERT) mRNA (*Slc6a4*) (Baudry et al., 2010). Importantly, antidepressants that blocks serotonin reuptake through SERT, encoded by the gene *Slc6a4*, are effective in reducing cocaine-seeking and -taking in some preclinical models (Baker et al., 2001; Burmeister et al., 2003; Harris et al., 2001; Richardson and Roberts, 1991).This suggests that miR-16-5p, which was found here to have higher expression in low-seeking rats, may be therapeutic for treating addiction by reducing depressive symptoms. The overlap of miRNAs implicated in addiction and comorbid psychiatric illnesses may help inform treatments for those suffering from addiction occurring in conjunction with, or exacerbated by, other conditions.

We utilized IPA to uncover pathways that are potentially regulated by the 33 differentially-expressed miRNAs by inputting the miRNAs' predicted mRNA targets and the estimated impact of the miRNAs on their expression (i.e. impact score). This analysis revealed several pathways, including *Axonal Guidance Signaling*, *Opioid Signaling*, and *ERK5 signaling*, that are potentially regulated by the differentially-expressed miRNAs. Many of these pathways have previously been implicated in addiction, including *WNT/β-catenin signaling* (Cuesta and Pacchioni, 2017) and *Neuroinflammation signaling pathway* (Clark et al., 2013). These results also validate our recent RNA-seq analysis of the NAc shell in animals that underwent the same training and testing procedures as the present study, except that in addition to EE and ISO housing, animals also underwent different lengths of forced abstinence (1 or 21 days) (Powell et al., 2020). We found that contrasting EE and ISO animals given 21 days of abstinence, similar to the present study, implicated several of the pathways found here, including *Synaptogenesis Signaling*, *Reelin Signaling*, *Neuroinflammation Signaling*, *Synaptic Long-Term Potentiation*, and *CREB Signaling in Neurons*, and that *Bdnf*, a widely studied addiction gene, (Li and Wolf, 2015) was a top upstream regulator of this comparison. In the present study, 7 miRNAs are predicted to target *Bdnf* with an impact score of −7. IPA also revealed significant functions of the predicted targets including *Dendritic Growth/Branching* and *Morphology of Dendritic Spines*, which support prior research that several of our miRNAs of interest, including miR-29a, miR-329, miR-137 and miR-132, regulate dendritic spine formation and morphology (Impey et al., 2010; Lippi et al., 2011; Smrt et al., 2010). Indeed, our IPA results both validate and expand on current knowledge by implicating several miRNAs in cue-elicited cocaine-seeking behavior.

Of the pathways identified in the current study, CREB signaling is one of the most highly studied in addiction (Carlezon et al., 2005; Gomez et al., 2015; Krasnova et al., 2016; Kreibich et al., 2009; Larson et al., 2011; Mattson et al., 2005). As mentioned earlier, increased miR-212 expression is related to reduced compulsive-like cocaine-taking behavior, which is thought to involve increasing CREB signaling and decreasing MeCP2/BDNF signaling (Hollander et al., 2010; Im et al., 2010). Similarly, our prior study suggested *CREB Signaling in Neurons* is an important mechanism underlying cocaine-seeking behavior, and found that *Creb1* was a top upstream regulator of the differentially-expressed RNAs in the contrast between EE and ISO animals with 21 days of abstinence (Powell et al., 2020). Here, we found that 11 of the 33 differentially-expressed miRNAs in high- vs. low-cocaine seeking animals are predicted to target *Creb1* (impact score = −7), suggesting that these miRNAs may be important regulators of the pathways identified in our previous study.

A caveat of the median split used to examine the effects of housing environment during forced abstinence in the present study is that animals at the outer range of the housing groups showed nearly the same lever pressing during the cue reactivity tests, as can be seen in Fig. 1A. We considered using a quartile analysis, which includes only the 3 animals with the greatest and least cue reactivity (i.e., 6 total animals representing the first and fourth quartiles of the total dataset), however, a drawback to this approach is the loss of power due to smaller sample size, reducing the capacity to find potentially relevant changes in miRNA expression. Our follow up analyses showing significant correlations of cocaine-seeking behavior with expression of 75 individual miRNAs, including 8 of the 33 miRNAs that are differentially expressed between “low” and “high” reactivity groups, mitigated concern with our approach. Furthermore, a preliminary comparison to the quartile analysis showed 11 differentially-expressed miRNAs, 4 of which are shared with the original analysis (miR-346-5p, miR-193a-3p, miR-3573-3p, and miR-107-3p). These 4 miRNAs, along with 4 of the 7 uniquely identified miRNAs (miR-301b-3p, miR-3561-3p, miR-3558-3p, and miR-208b-3p), are significantly correlated with behavior. We also used the quartile analysis in our follow-up RT-qPCRvalidation experiments, which confirmed increased expression of miR-276c and miR-107-3p in the low-seeking group relative to high-seeking animals, and additionally showed a significant correlation of miR-212-3p expression with cocaine-seeking behavior with all 12 animals. Finally, Nanostring nCounter employs dual probes and hybridization to directly measure target molecules without the bias of amplification-dependent techniques (e.g. RT-qPCR) (Eastel et al., 2019), and though not as sensitive as small RNA-seq, Nanostring displays improved detection of lowly-expressed miRNAs that might be missed by techniques such as microarrays (Eastel et al., 2019; Kulkarni, 2011). This is especially important for detecting disease biomarkers, which may be present in low abundance (Foye et al., 2017). Indeed, for the present study, all 8 differentially-expressed miRNAs that were elevated in the high-seeking group displayed low CPM values. Still, Nanostring is a proprietary platform, which somewhat limits flexibility and future use. In addition, the specificity of the assay depends largely on the design of the probes (Eastel et al., 2019); here, however, we used a pre-designed assay from Nanostring that has been widely used by other researchers (e.g. Chaudhuri et al., 2018; Mellios et al., 2018; Murphy et al., 2014), bolstering confidence in our approach.

5. Conclusion

In this study, we identified 33 miRNAs that are differentially-expressed in rats displaying high versus low cocaine-seeking behavior. Although this study focused on motivation for cocaine during abstinence, it is possible that the miRNAs identified may be relevant to other aspects of CUDs, as others have linked miRNAs to acute cocaine exposure (Bastle et al., 2018), escalation of cocaine self-administration (Hollander et al., 2010), and cocaine CPP (Chandrasekar and Dreyer, 2011; Viola et al., 2016). Understanding the role of these miRNAs in motivation for cocaine may lead to novel treatments as currently pioneered in the cancer field, where some miRNA therapeutics have even advanced to clinical trials (Bonneau et al., 2019; Wahid et al., 2010). Further research on the role of miRNAs in CUDs will aid in understanding the underlying mechanisms involved and position the field to capitalize on the knowledge for development of treatments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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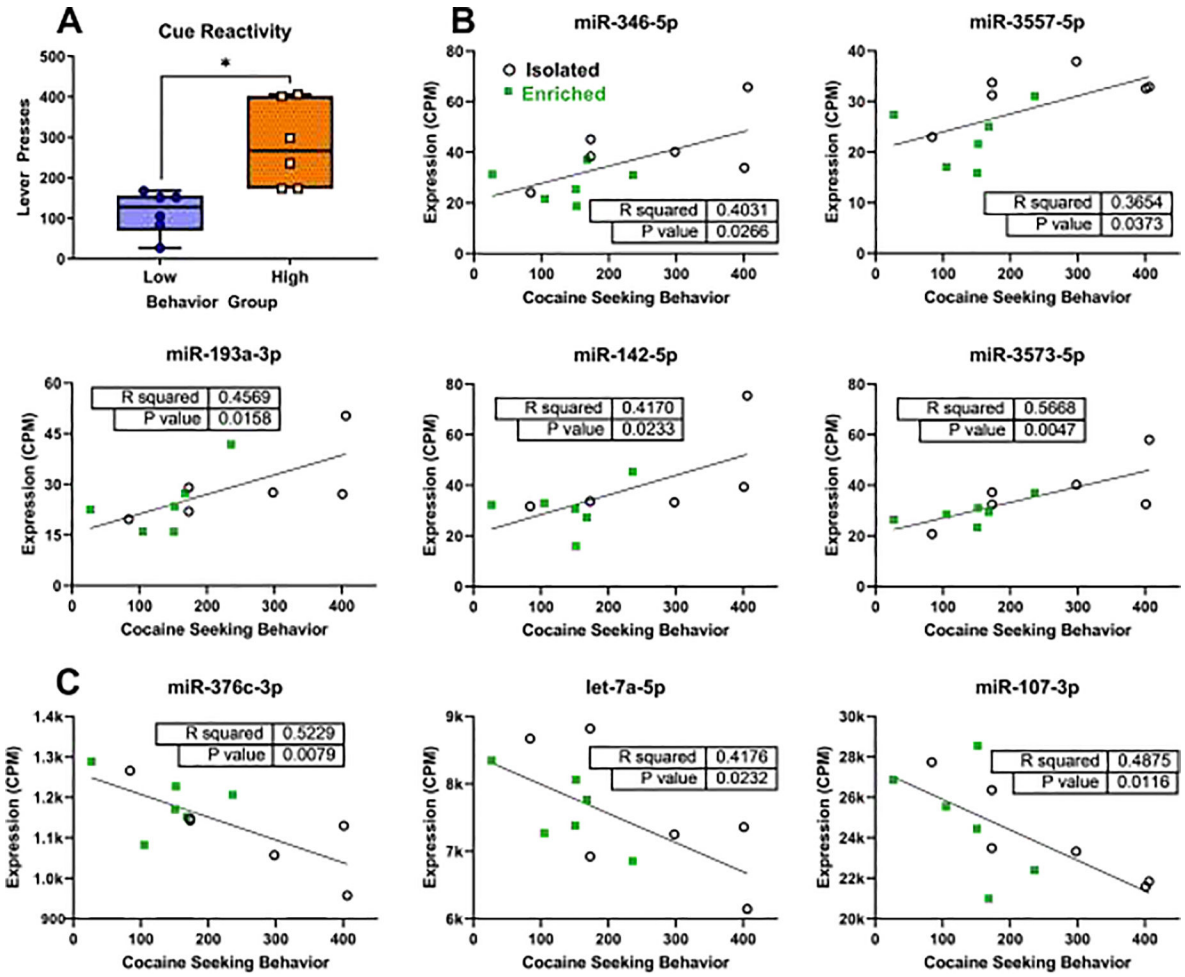

Fig. 1.

Correlation of miRNA expression levels and the number of active lever presses during the cue reactivity test in each animal. (A). Separation of groups of the low- (blue) and high-seeking (orange) groups determined by median split of active lever presses. Boxes indicate median and quartiles; whiskers indicate minimum and maximum. * indicates difference from low-seeking group, $p < 0.05$, independent samples *t*-test. Positive (B) and negative (C) Pearson correlations of miRNA expression with cocaine-seeking behavior, as measured by active lever presses during the cue reactivity test. Isolated and enriched animals are depicted with open black circles and green squares, respectively. Only miRNAs that had both significantly different expression between high- and low-seeking groups and that correlated significantly with behavior (table inset on each graph) are shown here. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

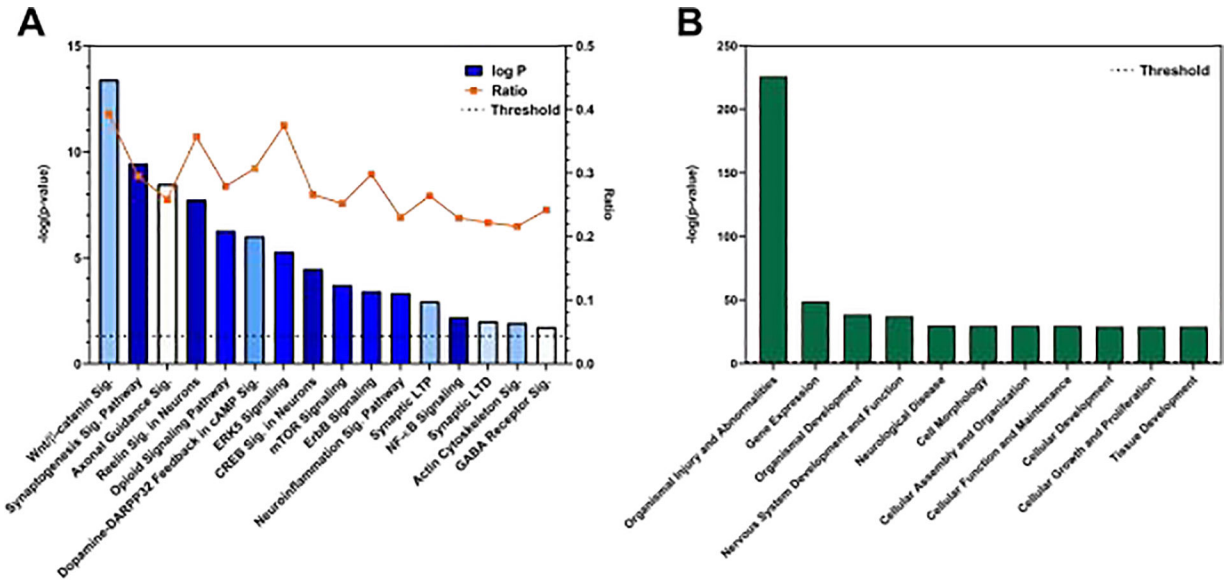

Fig. 2.

Summary of pathway analysis using IPA. Panels depict several pathways (A) and diseases and functions (B) related to the predicted targets of the differentially-expressed miRNAs. Threshold levels indicate −[log (p = 0.05)]. Bar colors represent the ranges of the z-score calculated by IPA. Darker shades indicate z-scores farther from zero, and blue indicates a negative z-score. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 1:

Differentially-expressed miRNAs between the high- and low-seeking groups.

miRNA	High-Seeking (CPM)[1]	Low-Seeking (CPM)	Fold Change[2]	P-Value	FDR[3]
miRNAs with higher expression in high cocaine-seeking animals					
miR-463-3p	37.04	23.26	1.447	0.0188	0.3885
miR-346-5p	42.42	26.40	1.439	0.0114	0.3291
miR-483-3p	45.76	29.62	1.431	0.0036	0.3108
miR-3557-5p	33.24	21.68	1.417	0.0074	0.3108
miR-193a-3p	32.98	20.81	1.390	0.0275	0.4400
miR-133a-3p	68.68	46.95	1.378	0.0133	0.3291
miR-142-5p	43.47	28.52	1.342	0.0385	0.4906
mir-3573-5p	39.58	26.59	1.331	0.0204	0.3993
miRNAs with higher expression in low cocaine-seeking animals					
miR-29a-3p	68099.60	70947.29	0.878	0.0360	0.4868
miR-16-5p	10815.91	11290.58	0.875	0.0465	0.5104
miR-93-5p	306.12	324.61	0.864	0.0326	0.4783
miR-495-3p	6727.51	7266.45	0.850	0.0422	0.4906
miR-376c-3p	1106.98	1197.90	0.845	0.0289	0.4429
miR-410-3p[a]	2722.19	2973.03	0.841	0.0239	0.4205
miR-329-3p	5361.27	5863.05	0.834	0.0432	0.4906
let-7a-5p	7226.04	7915.63	0.833	0.0258	0.4320
miR-652-3p	734.49	800.55	0.832	0.0486	0.5104
miR-377-3p	290.24	320.87	0.832	0.0418	0.4906
miR-107-3p	23171.08	25691.54	0.828	0.0150	0.3291
miR-138-5p	1727.38	1915.73	0.822	0.0225	0.4175
miR-487b-3p	4030.70	4628.25	0.802	0.0066	0.3108
miR-344b-1-3p/miR-344b-2-3p[a]	357.45	411.75	0.793	0.0026	0.3108
miR-128-3p	1644.39	1883.53	0.791	0.0346	0.4868
miR-137-3p	2451.13	2888.88	0.790	0.0147	0.3291
miR-323-3p	1155.50	1336.43	0.790	0.0088	0.3108
miR-337-3p	317.90	377.92	0.776	0.0051	0.3108
miR-125b-3p	1341.30	1589.26	0.772	0.0125	0.3291
miR-409-5p	326.65	389.53	0.771	0.0049	0.3108
miR-218a-5p	6391.04	7740.13	0.759	0.0393	0.4906
miR-212-3p[b]	354.77	448.44	0.728	0.0078	0.3108
miR-130b-3p	1279.74	1616.11	0.719	0.0104	0.3291
miR-221-3p	862.09	1078.44	0.718	0.0087	0.3108
miR-132-3p[b]	25643.46	32814.25	0.710	0.0188	0.3885

[a, b] Indicate miRNAs in the same family.

[1] miRNA expression levels were calculated as counts per million (CPM) and where available, 3p or 5p designations are included.

[2]Fold change is an estimate of the effect derived from the log2 fold changes from “limma”, which were then converted to linear values with the equation 2^(log2 value). Values <1 signifies higher expression in high-seeking animals, and values >1 signifies higher expression in low-seeking animals.

[3]FDR = false discovery rate.

Table 2:

Overlap of the predicted mRNA targets for each miRNA in TargetScan and KARG databases.

miRNA	TargetScan[1]	KARG[1]	% KARG[2]
miRNAs with higher expression in high cocaine-seeking animals			
miR-463-3p	1396	26	7.49 %
miR-346-5p	114	6	1.73 %
miR-483-3p	79	4	1.15 %
miR-3557-5p	3972	84	24.21 %
miR-193a-3p	190	5	1.44 %
miR-133a-3p	549	25	7.20 %
miR-142-5p	704	21	6.05 %
mir-3573-5p	129^	6	1.73 %
miRNAs with higher expression in low cocaine-seeking animals			
miR-29a-3p	1013	32	9.22 %
miR-16-5p	1090^	30	8.65 %
miR-93-5p	1056^	29	8.36 %
miR-495-3p	616	22	6.34 %
miR-376c-3p	188^	7	2.02 %
miR-410-3p[a]	497^	14	4.03 %
miR-329-3p	255^	7	2.02 %
let-7a-5p	1022^	19	5.48 %
miR-652-3p	14	1	0.29 %
miR-377-3p	2217	59	17.00 %
miR-107-3p	558^	22	6.34 %
miR-138-5p	537	16	4.61 %
miR-487b-3p	11	1	0.29 %
miR-344b-1-3p/miR-344b-2-3p[a]	497^	14	4.03 %
miR-128-3p	950	25	7.20 %
miR-137-3p	1019	26	7.49 %
miR-323-3p	357	10	2.88 %
miR-337-3p	3243	65	18.73 %
miR-125b-3p	443	9	2.59 %
miR-409-5p	100	2	0.58 %
miR-218a-5p	869	26	7.49 %
miR-212-3p[b]	360^	14	4.03 %
miR-130b-3p	748^	19	5.48 %
miR-221-3p	366^	14	4.03 %
miR-132-3p[b]	360^	14	4.03 %

[^] Represents miRNAs that were present on TargetScan as miRNA families, not individual miRNAs. miRNAs in the same family share their seed sequence and thus the same predicted miRNA targets.

[a, b] Indicate miRNAs in the same family.

[1] Values are the number of predicted mRNA targets found in each respective database.

[2] Percentage of KARG that the predicted targets comprise. Colors indicate miRNAs relative to the other group.