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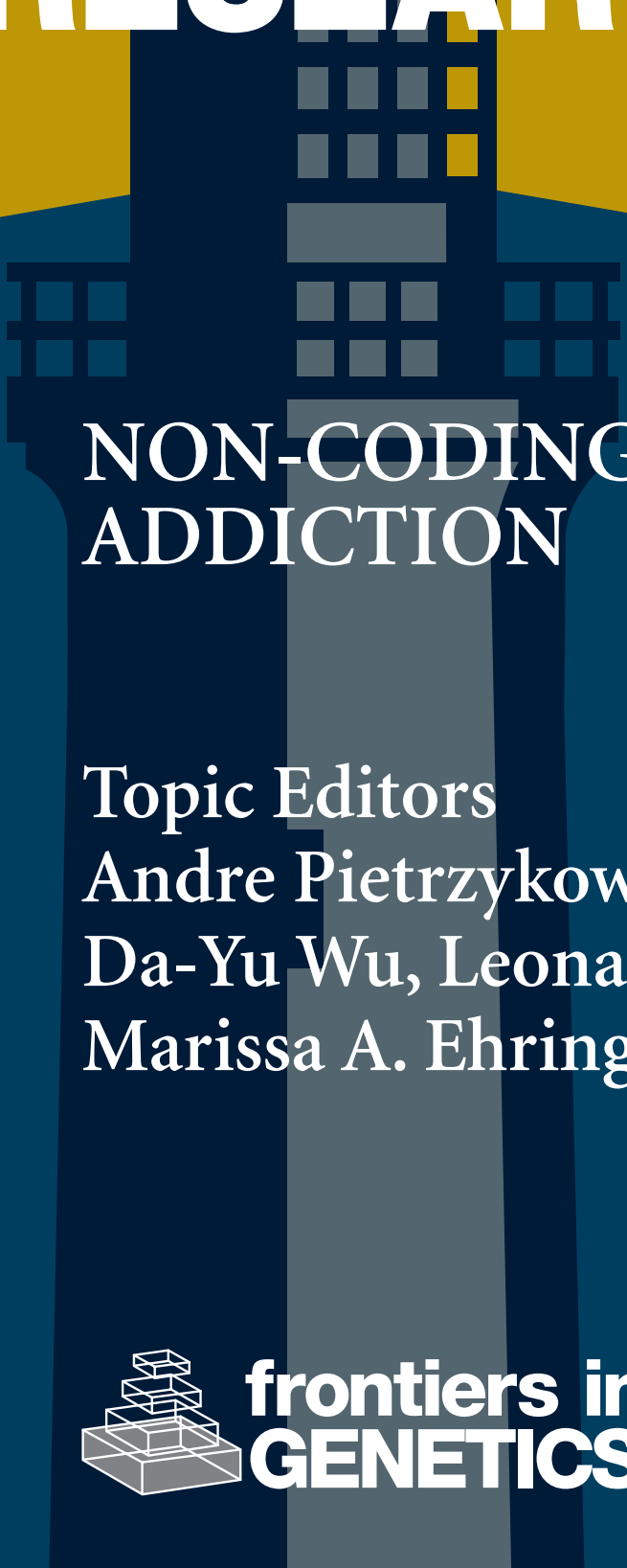

NON-CODING RNA AND ADDICTION

Topic Editors
Andre Pietrzykowski, Matthew Reilly,
Da-Yu Wu, Leonard Lipovich,
Marissa A. Ehringer and Jonathan Pollock

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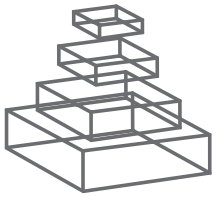

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NON-CODING RNA AND ADDICTION

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Non-coding RNA and addiction. Image created by Prof. Andrzej Pietrzykowski

Discovery of microRNAs, short, non-coding post-transcriptional regulators of gene expression, unraveled a completely new level of regulation of biological processes. Exponentially growing microRNA research provides evidence for microRNA involvement in almost every biological process, either physiological or pathological. Excitement about microRNA doesn't stop there. Since microRNAs do not encode proteins, their genes belong to a non-coding part of a genome, which was thought, up to recently, to play no role, despite the fact that non-coding elements constitute a whopping portion of human genome (99%). Therefore, fundamental role of microRNA in biology sheds some new light on mysterious genetic "dark matter". Moreover, microRNA-related pathways are attractive targets for new therapeutics.

Addictions are chronic disorders of the brain reward system. Addictions permeate history of humankind impacting profoundly whole societies and individuals. Alcohol is probably the oldest and most popular drug of abuse, with recent addition of narcotics and nicotine. However, the list is still growing: people can get addicted to "natural reinforcers" like sex or food (the latter being a particular problem in modern Western societies), or even activities (gambling, shopping, internet browsing etc).

Mechanisms of addictions are poorly understood, but at least some core molecular pathways seem to be common. Recently, several pioneering papers on role of microRNA in various

addictions have been published. They elucidate an important role of specific microRNAs in mechanisms of action of alcohol, cocaine, morphine and nicotine. This is likely just a tip of an iceberg, and many important papers will be probably coming soon defining microRNAs specific to a particular drug, and/or showing further microRNA involvement in several aspects of addiction.

Work involving microRNA is not without challenges. One particular is an accurate determination of genes targeted by microRNA. A proper selection of target genes is of great importance as it sets further research aimed to determine ramifications of microRNA regulation by a drug of abuse, including the physiological perturbations of that regulation. Lately, several bioinformatic tools have been created to address this issue.

Here, we provide an overview on microRNA, addictions and bioinformatical target selection. We will outline possible future directions of this field, emphasizing where the research should go to quickly achieve a translational level, ultimately developing novel therapeutic strategies for these debilitating diseases.

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EDITORIAL
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Coinciding revolutions: how discovery of non-coding DNA and RNA can change our understanding of addiction

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Edited by:
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> "*Omnia mutantur, nihil interit*" (Everything changes, nothing perishes)
>
> –Ovid, Metamorphoses, AD 8

The twenty-first century started with the Big Bang—the first ever sequencing of the human genome in 2000/2003 (Lander et al., 2001; Venter, 2003), and since then our scientific Universe started to expand with the speed of light. This year (2012) the Supernova exploded—the Encode project, a series of thirty papers, trashed the "junk DNA" concept. The project indicates that the vast majority (99%) of the human genome, although non-coding, is not "junk" but is biologically active and essential to maintain fundamental processes in a cell (Bernstein et al., 2012). One of the earlier stellar breakthroughs was the discovery of microRNA, a short RNA species crucial for regulation of gene expression (Lee et al., 1993). With discoveries like these, it becomes more and more obvious that our biological Universe is much more complex and dynamic than we have ever imagined. It is very probable that many biological rules and regulations still await discovery. This is particularly important for our understanding of the mechanisms underlying the development of chronic diseases, like addiction. Addiction is a persistent, neurobiological disease affecting primarily the brain's *reward system*, which consists of a circuitry of several interconnecting brain regions, including the prefrontal cortex, nucleus accumbens, ventral tegmental area, amygdala, paraventricular nucleus, and others (Koob and Le Moal, 2001). The reward system is one of the most important physiological features of the brain. We use it constantly, unconsciously, to make countless decisions essential to complete simple and complex tasks, by performing risk/benefit ratio analyses. This powerful motivator drives human and animal behavior, allows an individual to thrive and ultimately, for a species to propagate.

Some drugs target the reward system and can drastically change the behavior of individuals vulnerable to *addiction*, which is characterized as the refocusing of all activities on one goal—the continued intake of the drug. The drug's effect on the CNS is so powerful that the individual will continue to use it despite any associated adverse consequences. Neurobiological models of addiction try to explain how chronic or frequent exposure to addictive drugs hijacks the elements of the brain reward circuitry and causes transition from voluntary drug-taking to habitual and compulsive drug-seeking (Hyman and Malenka, 2001). During this transition, neuronal *adaptations* occur, which perpetuate and intensify the disease. Escalating drug intake due to *tolerance* causes incessant transition of the allostatic set-point, which fails to maintain system stability. Usually, drug addiction and alcohol addiction are described separately, but at the biological level both addictions have common core changes in the reward system through similar neuronal adaptations. However, in either type of addiction, many molecular underpinnings are still unknown.

Recently, several papers described the involvement of different non-coding RNAs, primarily microRNA, in unraveling novel molecular mechanisms of this debilitating disease. We put together this Special Issue of Frontiers in Genetics to provide our readers with a comprehensive overview of current research on non-coding RNA in both drug and alcohol addiction. We invited leaders in this dynamic new field to write a series of reviews, and other prominent scientists to share their views of this subject through a series of editorials. This special issue is divided into five chapters: alcohol, nicotine, cocaine, morphine, and bioinformatics.

Alcohol is a simple product of yeast fermentation, and is fairly easy to obtain. People have been using alcohol probably from the beginning of recorded history. Alcohol addiction (alcoholism) is the oldest and most widespread type of addiction. In this special issue, Nunez and Mayfield (2012) describe microRNA signatures in the brains of alcoholics and the process of microRNA modulation of epigenetic nuclear mechanisms. Miranda (2012) discusses teratological consequences of microRNA disregulation by alcohol during fetal development. The editorial by Reilly (2012) explains how studying microRNA can provide new perspectives and treatments of alcoholism and Fetal Alcohol Spectrum Disorder (FASD).

Nicotine is primarily inhaled during the smoking of dried tobacco leaves (*Nicotiana tabacum*), but also can be consumed in other forms. Nicotine addiction has spread quickly around the world in the post-Columbian era, becoming a major, present-day health issue in many countries. According to Maccani and Knopik (2012), detrimental effects of nicotine start very early—*in utero*. They describe in their review how maternal cigarette smoking during pregnancy alters expression of selected microRNAs in placenta. They also discuss the effect of cigarette smoking on microRNA and long non-coding RNA (lncRNA) expression in the epithelium of airways, which is important in the pathogenesis of cancer. Their review indicates that environmentally regulated epigenetic changes affect health throughout the course of one's life, while an editorial by Ehringer (2012) describes the presence of "critical periods" during which the environmental effect is particularly strong.

Morphine is the most abundant alkaloid found in opium (opiate), a product of the poppy fruit (*Papaver somniferum*). Morphine has been used for centuries to treat pain. However, morphine and related products have very potent addictive properties. Currently, addiction to morphine and related drugs is rising, mainly in affluent countries. Several microRNAs have been shown to be important in morphine actions. In this Special Issue,

Rodriquez (2012) summarizes the regulation of miR-133b by morphine and its possible involvement in morphine addiction and cancer. He and Wang (2012) describe the role of let-7, the first identified human microRNA, in regulation of morphine receptors important during the development of opioid tolerance. Zheng et al. (2012) center their review on microRNA-23b and -190. They also propose that a non-coding RNA relevant to opioid addiction can belong to one of two groups: (1) directly regulated by opioids, or (2) not-regulated by opioids but controlling pathways leading to addiction. Wood and Lipovich (2012), in their editorial, stress the importance of a systematical examination of the evolutionary conservation of microRNAs and their targets in opioid research.

Cocaine is an alkaloid obtained from the leaves of the coca plant (*Erythroxylum* family). Chewing raw coca leaves seems to be non-addictive, probably because of a very small content (<1%) of cocaine in the leaves. Coca in this form is a mild stimulant and analgesic and also a source of nutrients. However, purified cocaine has strong addictive properties. Currently, cocaine addiction is on a rise, and cocaine addicts can be found in many corners of the world. Wu (2012) in his editorial emphasizes advantages of a novel approach to study cocaine addiction using next-generation sequencing. The powerful results of this great methodology are appreciated by the review by Eipper-Mains et al. (2012). The review by Bali and Kenny (2012) describes their elegant studies implicating the regulation of CREB, MeCP2, and BDNF pathways by specific microRNAs (miR-132 and -212) in the behavioral response to cocaine.

We added a special chapter on *bioinformatics* because wet lab experimental approaches to microRNA research are initiated or complemented by bioinformatical experiments. There are several challenges to ensure synergism between next-generation techniques and bioinformatic analyses as emphasized in the editorial by Liu et al. (2012). The review by Mu and Zhang (2012) provides a comprehensive comparison of several bioinformatical resources important in addiction research. This special chapter also contains an article by Sartor et al. (2012), which draws our attention not only to the importance of microRNA but also to its "longer" brother—the lncRNA, in persistent neuronal maladaptation associated with addictive behaviors.

Research trying to understand involvement of various types of non-coding RNA in the development of addictions is at its early stages. Not all of the addictive substances have been yet screened. Moreover, an addiction can occur not only to exogenous chemicals but also to other cues like gambling, food, internet etc. Future research should look into the misregulation of gene expression by non-coding RNA in all the different types of addiction (Pietrzykowski, 2010). Nevertheless, the work discussed in this Special Issue has already left a strong legacy: there is no doubt that non-coding RNAs play absolutely essential roles in processes controlled by addictive drugs. Thus, to truly understand addictions, one must study non-coding RNA.

Research in non-coding RNA will probably increase exponentially, because we are now at a really advantageous situation in biological history—an intersection of several revolutions. *The technical revolution* provides the increasing ability to perform a deep, accurate, and fast sequencing of entire genomes. *The bioinformatical revolution* provides more and more sophisticated tools to analyze gigabytes of information delivered by sequencing. *The genome structure revolution* has started to disentangle intertwined genomic three-dimensional networks of interactions between genes and gene regulatory regions. Inevitably, we have started to experience *the conceptual revolution*: we realize that the regulation of gene expression is a multilevel interactive process, much more complex than we initially thought, and that messenger RNA can regulate each other via miRNA sequestering as proposed in the ceRNA (complementary endogenous) theory (Salmena et al., 2011), and that circulating exosomes can contain gene regulatory material, like microRNA and lncRNA, enabling a brand new cell-to-cell communication route (Valadi et al., 2007). Hopefully, these coinciding revolutions will ultimately provide fundamental breakthroughs and create radical new treatment approaches to addiction disorders, benefiting the entire society.

We hope you will find this Special issue of Frontiers in Genetics truly engaging and share our enthusiasm that we are living in one of the most exciting time in scientific history, on a cusp of a cosmic scale of discovery in biological sciences.

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Role of non-coding RNAs in the neuroadaptation to alcoholism and fetal alcohol exposure

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The control of gene expression is a fundamental process in all of biology. Understanding the mechanisms by which genes are turned off and on in a temporally and spatially mediated fashion, represents an area where major scientific advances are likely to occur in the next decade. This is because the control of the timing and location of gene expression is arguably one of the most critical regulation points in determining cellular identity and function and when gene regulation is perturbed disease states can arise. Classic gene expression control mechanisms such as *cis*-acting elements in gene promoters and *trans*-acting factors (transcription factor proteins) have been well studied. However, the recent discovery of gene expression regulation mediated by RNA molecules that are transcribed from DNA but do not code for protein, has set into motion a revolution in molecular biology. These novel RNAs are classified broadly as non-coding RNAs (ncRNAs) and include both small (microRNAs or miRNAs) and large classes (long non-coding RNAs or lncRNAs) that function to alter the expression of genes to which they bind and modify chromatin states. Yet, there remains much to be understood about the biology of ncRNAs and how this contributes to susceptibility to disease.

Like all complex traits, alcoholism is influenced by multiple genetic and environmental factors. In addition, there is significant heterogeneity found with alcoholism making it a daunting task to identify the specific genes associated with the disease. Although progress has been made in identifying some of the genetic variants, explaining the entire phenotypic variation associated with alcoholism is far from complete. Most of the recent efforts in understanding the genetic vulnerability to alcoholism have focused on genotype-phenotype associations. For example, in the last several years there has been an explosion in Genome-Wide Association Studies (GWAS), which seek to correlate common genetic variation (represented by Single Nucleotide Polymorphisms or SNPs) with a particular trait or disease. Success of GWAS has varied across different complex traits, where most studies have found that individual SNPs account for a very small proportion of the variance. This is true for GWAS studies of alcoholism and alcohol-related phenotypes, where genome-wide significance has rarely been achieved. There are several prevailing hypotheses that have been put forth to explain the small effect sizes observed with GWAS and other approaches. These include arguments for the contribution of untested rare variants, sample power issues, and "phantom heritability." It is clear, however, that new perspectives for understanding genetic vulnerability to alcoholism are warranted, and the burgeoning area of microRNAs appears to be a fruitful area of investigation. This is because determining the role of microRNAs in disease will offer both mechanistic and potential therapeutic insight.

Gene expression changes after alcohol exposure are well documented. In particular, a vast network of expression changes are found in the brain (and other tissues) following both acute and chronic alcohol exposure. These neuroadaptations are thought to underlie tolerance and dependence on alcohol as well as mediating the toxic effects of alcohol on neurodevelopment. Studies examining the effects of alcohol on microRNA expression are just beginning. Applying this new information on microRNAs to genome-wide studies of gene expression changes after alcohol exposure represents both a significant challenge and tremendous opportunity for unraveling the genetic vulnerability to alcohol dependence. In this special issue of Frontiers in Genetics two important areas relevant to microRNA biology and alcohol are presented. The first article presents recent studies on microRNAs in post-mortem human brain samples from alcoholics compared to non-alcoholics. This is the first study to profile changes in the expression of microRNAs in brain samples from alcoholics. These changes in microRNAs are correlated with previously identified gene expression changes to provide a glimpse into the possible mechanisms by which alcohol neuroadaptation alters the brain. The second article in this special issue examines the effects of fetal alcohol exposure on microRNA expression. This exciting area has important implications for understanding and treating Fetal Alcohol Syndrome and Fetal Alcohol Spectrum Disorders. Collectively, these studies highlight the emerging role of microRNAs in neurobiology in general and alcohol neuroadaptation specifically. Because microRNAs can fine-tune gene expression in subtle yet critical ways, harnessing these endogenous master regulators holds tremendous promise for the identification of novel drug targets and eventually the development of new therapeutics to treat alcoholism.

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MicroRNAs and fetal brain development: implications for ethanol teratology during the second trimester period of neurogenesis

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Maternal ethanol consumption during pregnancy can lead to a stereotypic cluster of fetal craniofacial, cardiovascular, skeletal, and neurological deficits that are collectively termed the fetal alcohol spectrum disorder (FASD). Fetal ethanol exposure is a leading non-genetic cause of mental retardation. Mechanisms underlying the etiology of ethanol teratology are varied and complex. This review will focus on the developing brain as an important and vulnerable ethanol target. Near the end of the first trimester, and during the second trimester, fetal neural stem cells (NSCs) produce most of the neurons of the adult brain, and ethanol has been shown to influence NSC renewal and maturation. We will discuss the neural developmental and teratological implications of the biogenesis and function of microRNAs (miRNAs), a class of small non-protein-coding RNAs that control the expression of gene networks by translation repression. A small but growing body of research has identified ethanol-sensitive miRNAs at different stages of NSC and brain maturation. While many miRNAs appear to be vulnerable to ethanol at specific developmental stages, a few, like the miR-9 family, appear to exhibit broad vulnerability to ethanol across multiple stages of NSC differentiation. An assessment of the regulation and function of these miRNAs provides important clues about the mechanisms that underlie fetal vulnerability to alterations in the maternal-fetal environment and yields insights into the genesis of FASD.

Keywords: microRNA, fetal alcohol spectrum disorders, FASD, miR-9, neural stem cells, cerebral cortical development

Heavy ethanol exposure during pregnancy can lead to fetal growth retardation, and to a constellation of craniofacial, cardiovascular, skeletal, and brain growth defects (Clarren et al., 1978; Clarren, 1986) that are collectively termed "Fetal Alcohol Spectrum Disorder" (FASD; Lemoine et al., 1968; Jones et al., 1973). Brain defects can include, microencephaly, the loss of the corpus callosum, and the emergence of heterotopias. FASD is a leading, non-genetic cause of mental retardation. Epidemiological studies (SAMHSA, 2009) show that 6.8% of pregnant women report continued ethanol consumption into the third trimester of pregnancy. Moreover, 2–5% of school-aged children in the US are estimated to exhibit symptoms associated with fetal ethanol exposure (May et al., 2009). Importantly, nutrition (Thomas et al., 2009; Keen et al., 2010), and drugs of abuse like nicotine (Chen et al., 1998, 1999) also contextually enhance or mitigate effects of prenatal ethanol exposure. It is important to understand the genesis of fetal vulnerability to disrupters of the maternal-fetal environment.

NEURAL STEM CELLS AND NEUROGENESIS; SECOND TRIMESTER BRAIN VULNERABILITY

The gestational period spanning the end of the first trimester through the second trimester of fetal development is a specific window of vulnerability, because during this period, neural stem cells (NSCs) produce the majority of adult neurons. The enormous rate of proliferation and maturation amplifies effects of early perturbations in the maternal-fetal environment, so that relatively minor disruptions in genetic and epigenetic instructions that guide stem cell maturation will significantly alter the structure and function of the mature brain.

CEREBRAL CORTICAL NEUROGENESIS, AN EXAMPLE OF ETHANOL VULNERABILITY

Most neurons of the adult human cerebral cortex are generated near the end of the first trimester through the second trimester of fetal development (Bystron et al., 2008). The human brain adds ∼2,500 new neurons every minute during this developmental window (Noback et al., 2005), indicating an enormous rate of NSC mitosis (mouse mitotic cycles can shorten to 8 h/cycle; Caviness et al., 1995). Newly generated neuroblasts of the ventricular zone (VZ), migrate directly to the cortical plate, or to the sub-ventricular zone (SVZ), where they may undergo additional mitosis before migrating to the cortical plate (Noctor et al., 2004).

Ethanol exposure during neurogenesis has been shown to decrease the thickness and cell proliferation rate within the VZ, while increasing the thickness and increasing proliferation within the SVZ (Miller, 1989; Miller and Nowakowski, 1991). Ethanol also disrupts the laminar organization of the emerging cortical plate (Kotkoskie and Norton, 1988), and consistent with autopsy observations in human FASD children, ethanol exposure during the period of neurogenesis also

promotes the formation of subpial heterotopias (Mooney et al., 2004).

These studies showed that the neurogenic window constituted a critical period of vulnerability, but did not specifically identify NSCs as an ethanol target. We found that ethanol exposure did not kill NSCs (Prock and Miranda, 2007), showing that ethanol is not toxic to NSCs, compared to its toxicity to maturing neurons (Luo et al., 1997; Cheema et al., 2000; Bonthius et al., 2002). Ethanol promoted neuroepithelial cell proliferation, while simultaneously depleting the numbers of cells expressing NSC markers like ABCG2, CD133, and Sca-1 (Santillano et al., 2005). Ethanol also promoted asymmetrical cell division and the appearance of radial glial-like cells (Santillano et al., 2005). Radial glia are precursors of neuroblasts that exit the VZ (Tramontin et al., 2003; Noctor et al., 2004), and also serve as a conduit for neuronal migration out of the VZ into the intermediate SVZ, and from the SVZ into the emerging cortical plate (Anton et al., 1997; Hansen et al., 2010). These data suggest a mechanism (**Figure 1**) for the premature depletion or "thinning" of the VZ and its NSCs, and the corresponding increase in thickness and cell proliferation of the SVZ that was observed in *in vivo* experiments (Miller, 1989). Moreover, as predicted from the observed appearance of radial-glia-like precursors, ethanol-treated NSCs exhibited increased migration during their early differentiation (Camarillo and Miranda, 2008), providing a mechanism for the appearance of subpial heterotopias that have been observed in the brains of FASD children and in experimental models (Mooney et al., 2004). It is likely that NSCs are heterogeneous, and that NSCs from different brain regions may exhibit varied responses to ethanol. For example, while we observed increased maturation of cerebral cortical NSCs in response to ethanol, others showed that, in neural crest models, ethanol stalls cells in G1 (Luo et al., 1999), and retards NSC maturation (Zhou et al., 2011).

These data collectively show that ethanol action on NSCs during the critical period of neurogenesis explains many neural defects associated with *in utero* ethanol exposure. A question that arises is, "*what mechanisms mediate ethanol's, effects on NSCs*?" One answer may be, "*microRNAs.*"

MicroRNAs ARE TARGETS OF ETHANOL

MicroRNAs (miRNAs) are 17–25 nucleotide long, non-protein-coding RNAs that are coded within the genomes of all plants and animals and are important for the renewal and maturation of stem cells (Yi and Fuchs, 2011). MiRNAs are initially transcribed as long primary transcripts (pri-miRNAs) and processed within the nucleus by Drosha/DGCR8 to generate shorter "pre-miRNAs" (Lee et al., 2003; Han et al., 2006). Pre-miRNAs are translocated to the cytoplasm where the Dicer enzyme complex further processes them into mature double-strand RNA molecules (Zhang et al., 2002). One strand, the "guide strand" is preferentially loaded onto

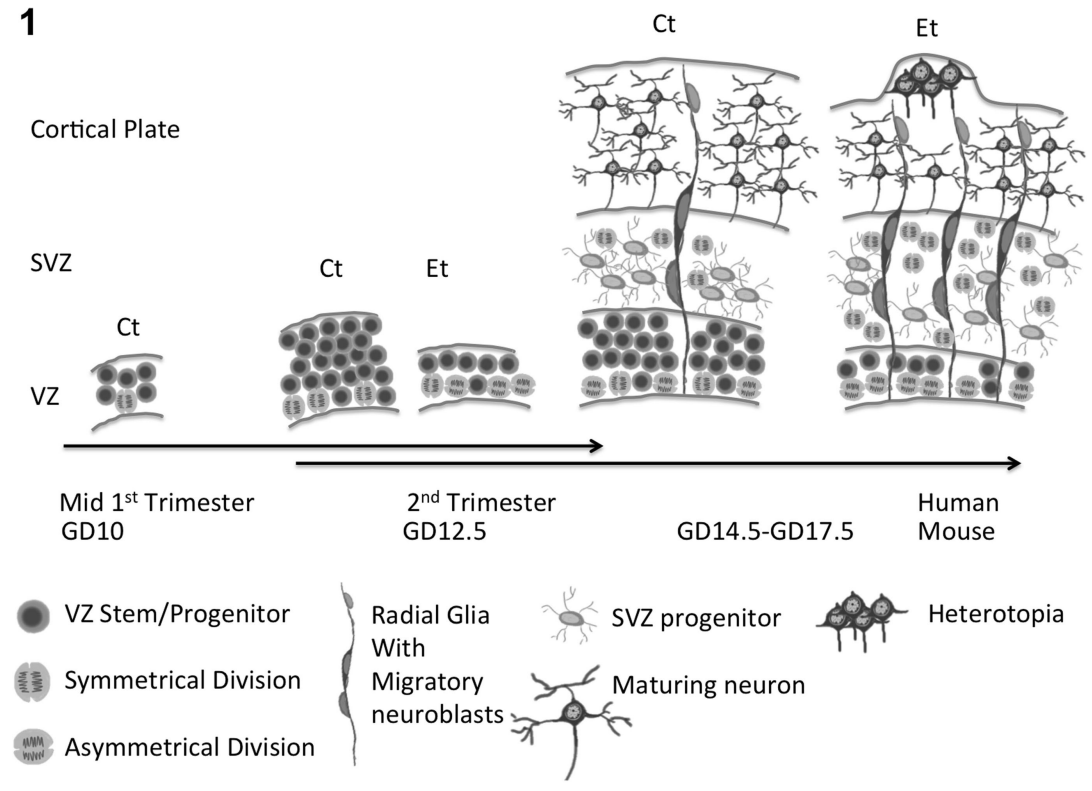

FIGURE 1 | Composite model for ethanol effects on fetal neural development. Ethanol promotes asymmetric neural stem cell (NSC) proliferation, leading to premature depletion of NSCs in the ventricular zone (VZ) and ultimately loss of reserve neuron generation capacity. The appearance of radial glial-like neuronal progenitors, supports expansion, and increased proliferation of neuronal progenitors in the sub-ventricular zone (SVZ) and increased migration of neuronal progenitor cells out of the VZ and SVZ, leading to the formation of heterotopias or displaced neuronal aggregates.

the RNA-induced silencing complex (RISC), to destabilize mRNA transcripts or repress translation (Lim et al., 2005).

In 2007, we obtained the first evidence showing that miRNAs mediate ethanol's teratogenic effects (Sathyan et al., 2007). We screened 218 mouse miRNAs for ethanol sensitivity in fetal NSCs. While 38% of sampled miRNAs were expressed in fetal NSCs, only a few, miR-9, -21, -153, -335, were significantly decreased by ethanol exposure. Subsequent reports have expanded the list of ethanol-sensitive miRNAs. Wang et al. (2009) showed that ethanol exposure between the latter half of the first trimester to the middle of the second trimester-equivalent period in mice, altered ∼3% of assessed mature miRNAs in whole brain RNA samples obtained at the end of the second trimester-equivalent period. Recently, Guo et al. (2011) showed that differentiated neurons exposed to a "chronic intermittent ethanol" paradigm exhibited significant alterations in ∼11% (42 out of 385) of detected miRNAs.

ARE SPECIFIC TYPES OF miRNAs SUSCEPTIBLE TO ETHANOL EXPOSURE?

In these early stages, it is difficult to identify unique principles that connect ethanol exposure to miRNA expression. The variety of experimental models and exposure conditions lend additional complexity to the assessment of miRNA changes. However, the accumulated data permit some conclusions.

a. Ethanol appears to influence a relatively small subset of expressed miRNAs. The apparent absence of a global effect on miRNAs suggests that ethanol does not generally target the miRNA processing machinery during development.
b. Ethanol appears to influence miRNAs that are a normal constituent of the cellular repertoire for a given stage of differentiation. None of the published screens has identified a miRNA that was uniquely expressed following ethanol exposure, but undetectable under control conditions. Therefore, ethanol is unlikely to promote lineage transformation of NSCs *via* a miRNA-dependent mechanism. For example, miR-124 which is sufficient to direct NSCs toward a neuronal lineage (Makeyev et al., 2007; Visvanathan et al., 2007) and is uniquely expressed in neurons (Smirnova et al., 2005), has not been identified as an ethanol-sensitive target in any of the published screens, whereas miR-9 which is ethanol-sensitive, is expressed by cells belonging to both neuronal and glial lineages (Smirnova et al., 2005). These data lend themselves to the prediction that ethanol regulation of miRNAs is unlikely to mediate transformation of a committed neuron to a glial cell, but may subtly bias miRNA expression to favor development along a specific lineage.
c. The numbers of ethanol-sensitive miRNAs appear to increase with developmental stage. It is possible that the sheer number of ethanol-sensitive miRNAs increases with differentiation stage (from ∼4% in our observations of fetal NSCs to ∼11% in the observations of Guo and colleagues in differentiated neurons). This increase may reflect increasing complexity and diversity of miRNAs that is recruited during neuronal maturation (Guo et al., 2011).
d. MicroRNAs exhibit developmental stage-specific sensitivity to ethanol. Because miRNAs play an important role in cell and tissue maturation, it is likely that each stage of NSC differentiation requires expression of new miRNAs. Therefore, new ethanol-sensitive miRNAs are likely to emerge as a function of tissue differentiation stage. A comparative analysis of our data on NSCs with data obtained with more differentiated cells and neural tissue (Wang et al., 2009; Guo et al., 2011) suggests that this hypothesis is generally true. MiRNAs like miR-335 and miR21 which are ethanol-sensitive in NSCs, and important for regulating NSC fate (Sathyan et al., 2007), are no longer observed to be ethanol-sensitive at the end of the second trimester-equivalent period, coincident with the depletion of NSC number, the disappearance of the VZ and the relative increase in the size of the SVZ. Instead other ethanol-sensitive miRNAs emerge, including miR-10a/10b (Wang et al., 2009), which serve as negative regulators of the Hox gene family (Woltering and Durston, 2008). The Hox family plays an important role in neuronal migration (Geisen et al., 2008), and elevated levels of miR-10 promote tumor cell invasion (another migratory behavior) by translation inhibition of HoxD10 (Liu et al., 2012). These data collectively suggest that ethanol-mediated elevations in miR-10a/b, during a critical window for neuronal migration, may dysregulate neuronal migration, whereas regulation of miR-21 and miR-335 at earlier time periods regulate NSC behavior.
e. Some miRNAs are ethanol-sensitive over multiple developmental windows. MiR-9 for example exhibits sensitivity to ethanol at multiple stages of development, from the embryo and fetus to the adult (Sathyan et al., 2007; Pietrzykowski et al., 2008; Wang et al., 2009; Balaraman et al., 2012; Tal et al., 2012). While ethanol decreased miR-9 expression in early development models in the mouse and fish (Sathyan et al., 2007; Balaraman et al., 2012; Tal et al., 2012), ethanol induces miR-9 expression at later developmental stages (Wang et al., 2009) and in the adult (Pietrzykowski et al., 2008). The functional implication of a presumptive developmental switch in ethanol regulation of miR-9 is unclear. Moreover, though miR-9 is targeted by ethanol throughout development, it is likely that downstream effects on translation will change simply because the transcriptome itself is significantly altered during NSC maturation.

ASSESSMENT OF miRNAs FOR CLUES ABOUT MECHANISMS OF ETHANOL TERATOLOGY

Tal et al. (2012) reported that zebrafish exposed to between 4 and 24 h post-fertilization (hpf) exhibited decreased miR-9. Loss of miR-9 resulted in increased daylight-related hyperactivity in juvenile fish, recapitulating increased hyperactivity in FAS children (Mattson et al., 2001). These data suggest common mechanisms for ethanol teratology emerged early in vertebrates, and that some ethanol-sensitive miRNAs mediate behavioral deficits associated with fetal alcohol spectrum disorder (FASD).

MiR-9 plays a significant role in early neural tube patterning (Leucht et al., 2008), telencephalic neurogenesis, and early differentiation (Packer et al., 2008; Shibata et al., 2008, 2011). Importantly, a murine double knockout of miR-9-2 and miR-9-3 genes (miR-9-2/-3$^{-/-}$) results in FAS-like phenotypes, fetal growth retardation and microencephaly (Shibata et al., 2011). Moreover, miR-9-2/-3$^{-/-}$ mice exhibited decreased cortical plate thickness at the end of the neurogenic period. Shibata and colleagues showed

that miR-9 acts directly as a negative regulator of Foxg1, a gene that has been shown to prevent NSC differentiation and promote proliferation (Martynoga et al., 2005). Consistent with that finding, Shibata et al. additionally observed evidence for increased NSC proliferation in GD12.5, miR-9-2/-3$^{-/-}$ mouse brain (i.e., at the beginning of the second trimester-equivalent period). This group also discovered that, during the later half of the second trimester-equivalent period (i.e., after GD16.5, or the tail-end of the neurogenic window), Foxg1 expression was no longer suppressed in miR-9-2/-3$^{-/-}$ mutant mice. They suggested that this latter refractory period for Foxg1 regulation was due to a compensatory, miR-9-depletion-mediated, increase in the expression of the RNA-binding protein, ELAVL2 which in turn, stabilized Foxg1 mRNA transcripts. These data coincide well with our findings that ethanol depleted the expression of miR-9 while promoting NSC proliferation and induced expression of ELAVL2 mRNA in murine NSCs (Santillano et al., 2005; Sathyan et al., 2007), and further suggests that miR-9 plays a critical role in early second trimester ethanol teratology in coordination with other ethanol-sensitive miRNAs (**Figures 2A,B**). Added support for miR-9 as a mediator of ethanol neuro-teratology comes from the analysis of human familial mutations of the Foxg1 locus. Mutations in cis-regulatory elements of the Foxg1 locus are associated with fetal growth retardation, microcephaly, and mental retardation (Kortum et al., 2011), i.e., features associated with FAS.

MECHANISMS OF ETHANOL AND TERATOGEN REGULATION OF miRNAs

Two early reports (Sathyan et al., 2007; Balaraman et al., 2012), suggest that $GABA_A$ receptors ($GABA_AR$) and nicotinic Acetylcholine receptors (nAChRs) mediate some ethanol effects on miRNA expression. Moreover, several of the ethanol-sensitive miRNAs are localized with chromosomal regions that are susceptible to epigenetic modification. Therefore ligand-gated ion channel receptors and epigenetic modifications are potential mediators of ethanol's effects on fetal miRNAs (**Figure 2C**).

$GABA_AR$ AND nAChRs

We previously observed that one miRNA, miR21 was regulated by ethanol in a $GABA_AR$ dependent manner, i.e., the non-competitive $GABA_AR$ antagonist, picrotoxin, prevented the ethanol-mediated decrease in miR21 (Sathyan et al., 2007). However, another miRNA, miR-335 was not regulated by a $GABA_AR$-dependent mechanism. These data suggest that $GABA_A$-dependent mechanisms selectively target a sub-population of ethanol-sensitive miRNAs. All of the tested ethanol-sensitive NSC miRNAs are

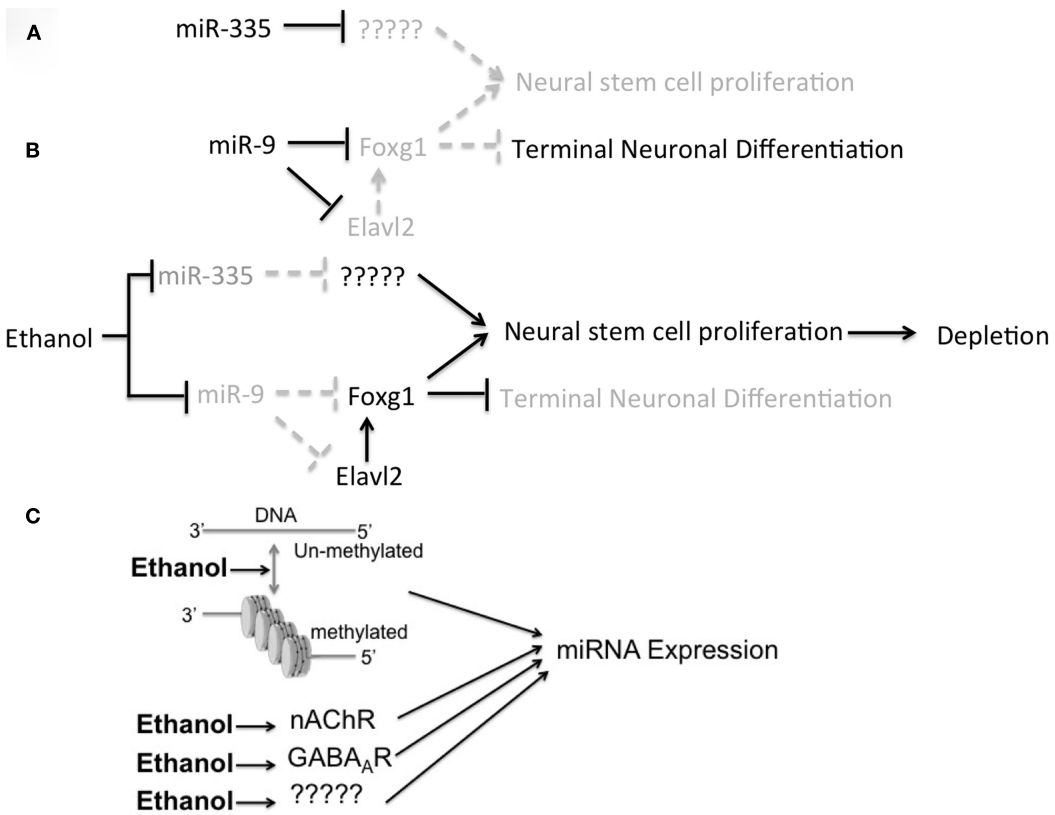

FIGURE 2 | Model for miRNA-mediated effects of ethanol on NSC maturation and ethanol regulation of miRNAs. (A) MiRNAs like miR-9 and miR-335 may repress NSC proliferation and promote maturation by repressing specific mRNA networks. **(B)** Ethanol represses these miRNAs in fetal NSCs, and would be projected to attenuate terminal neuronal maturation and aberrant NSC proliferation, ultimately depleting the NSC pool. **(C)** The collective data supports two mechanisms for ethanol control of miRNAs, i.e., by regulating the epigenetic landscape of the genome and by influencing the activity of ligand-gated ion channels like nicotinic acetylcholine receptors (nAChRs) and $GABA_A$ receptors ($GABA_ARs$).

also regulated in a nAChR-dependent manner, since the non-selective nAChR antagonist, mecamylamine attenuated the general miRNA-inducing effects of nicotine (Balaraman et al., 2012). In fact nicotine and ethanol appear to behave as functional antagonists in regulating sensitive NSC miRNAs suggesting that these agents, which are often co-abused during pregnancy (SAMHSA, 2009), may also target common miRNA-regulated gene networks. One model for miRNA regulation that emerges from these admittedly limited studies is that nAChRs serve as general regulators, while $GABA_A$Rs are more selective regulators of ethanol-sensitive NSC miRNAs. The nAChR-dependence of ethanol-sensitive miRNAs is particularly intriguing, since recent evidence shows that the dominant α4β2 nAChR isoform predominantly localizes to the endoplasmic reticulum (Richards et al., 2011), in proximity to sites for miRNA-mediated translation control. Therefore nAChRs may well share translation control with some miRNAs at the endoplasmic reticulum.

MiRNA REGULATION BY EPIGENETIC MECHANISMS

Ethanol has recently been shown to alter methylation patterns in differentiating NSCs (Zhou et al., 2011) and therefore, ethanol may regulate miRNAs by epigenetic mechanisms as well. A comparison of miR-21 and miR-335 provides important insights into the role of epigenetic mechanisms in miRNA expression. We found that these miRNAs served as functional antagonists to each other, and that miR21 was regulated by $GABA_A$R-dependent mechanisms but miR-335 was not. A comparison of the chromosome structure associated with the miR-21 and miR-335 gene loci points to an alternate mechanism for miR-335 regulation. The mammalian-specific miR-335 is coded as an intronic miRNA, within the MEST/Peg1 imprinted gene locus, which contains GC-rich regions, termed CpG islands (**Table 1**). The miR-335/Peg1 locus is part of a network of imprinted genes that controls fetal growth. Loss of this locus is associated with fetal growth retardation in a mouse model (Lefebvre et al., 1998), while hypermethylation of the paternal allele in humans is associated with the Russell–Silver syndrome (Kagami et al., 2007), characterized by growth retardation and mild mental retardation. Therefore expression of the miR-335/Peg1 locus is controlled epigenetically, whereas the miR21 locus, which is not associated with such methylation-sensitive chromatin, appears to be controlled by alternate mechanisms. Moreover, transcription at the Peg1/miR-335 locus is shut down as the growth of organs slows down with development (Lui et al., 2008; Finkielstain et al., 2009), further supporting a role in organogenesis. The Peg1/miR-335 locus has been implicated in early cortical development (Sansom et al., 2005). We showed that miR-335 is pro-apoptotic and decreases NSC proliferation, and conversely, miR-335 suppression (equivalent to the effect of ethanol exposure) resulted in increased NSC proliferation and resistance to apoptosis (Sathyan et al., 2007). Therefore, the brain effects of the miR-335 locus may also be susceptible to epigenetic programming.

Emerging evidence from the field of cancer biology shows that the three mammalian miR-9 genes are also susceptible to epigenetic programming. MiR-9-1, miR-9-2, and miR-9-3, can be independently targeted in a variety of cancers for hyper-methylation and consequently, inactivation (Trankenschuh et al., 2010; Tsai et al., 2011; Heller et al., 2012; Minor et al., 2012). Consequently, ethanol may also regulate the expression of miR-9 genes via epigenetic mechanisms (**Figure 2C**). Moreover, since ethanol appears to suppress miR-9 during early fetal development, but induce miR-9 at later developmental periods, it is possible that temporal alterations in methylation of miR-9 genes may account for the switch in ethanol's effects. A human genome map[1] of other known ethanol-sensitive miRNAs shows that several ethanol-sensitive miRNAs are either bracketed by, or proximal to CpG islands, and therefore presumptive targets for DNA methylation and epigenetic programming (**Table 1**).

CONCLUDING THOUGHTS

Teratogens including ethanol have complex effects on developing tissues and cells, making the search for cohesive principles, elusive. The significant promise of research on miRNA involvement

[1] UCSC genome browser, http://genome.ucsc.edu

Table 1 | Methylation-sensitive ethanol miRNAs

MiRNA/parent gene	miRNA locus (human)	Predicted CpG island length	Reference for ethanol sensitivity
miR-9 family			Pietrzykowski et al. (2008); Sathyan et al. (2007); Tal et al. (2012); Wang et al. (2009)
miR-9-1/C1orf61	chr1:156,390,133-156,390,221	99 bp	
miR-9-2/LINC00461/CR599257	chr5:87,962,671-87,962,757	Multiple, from 22 to 165 bp	
miR-9-3/CR612213.1-1.1	chr15:89,911,248-89,911,337	131 bp	
miR-335/MEST/Peg1	chr7:130,135,952-130,136,045	66 and 177 bp	Sathyan et al. (2007)
miR-10b	chr2:177,014,591-177,015,580	18 bp	Wang et al. (2009)
miR-339/C7orf50	chr7:1,062,569-1,062,662	40 bp	Wang et al. (2009)
miR-152/COPZ2	chr17:46,114,527-46,114,613	44 bp	Guo et al. (2011); Wang et al. (2009)
miR-503/MGC16121	chrX:133,680,358-133,680,428	40 bp	Guo et al. (2011)
miR-219-1	chr6:33,175,612-33,175,721	78 bp	Guo et al. (2011)
miR-219-2	chr9:131,154,897-131,154,993	147 bp	Guo et al. (2011)

Locations and size of presumptive CpG islands based on UCSC genome browser maps (http://genome.ucsc.edu.)

in fetal ethanol effects, is that we will finally be able to develop cohesive models for the susceptibility of developing biological systems to alterations in the maternal-fetal environment, including the effects of ethanol exposure. Moreover, since the maternal-fetal environment is often complex, and the developing fetus may be exposed to multiple prenatal stressors (teratogens, drugs of abuse, nutrition deficiencies etc.), it is likely that the miRNA profile of developing cells will be the product of such complexity. In this context, it is particularly interesting to note that drugs like ethanol and nicotine that are commonly co-abused, target an overlapping cohort of miRNAs in fetal NSCs (Balaraman et al., 2012). Clearly, research on miRNAs and teratology is in its infancy; but as outlined in this review, the possibilities for understanding and ultimately, for therapeutic intervention in teratology are enormous.

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Understanding alcoholism through microRNA signatures in brains of human alcoholics

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Advances in the fields of genomics and genetics in the last decade have identified a large number of genes that can potentially influence alcohol-drinking behavior in humans as well as animal models. Consequently, the task of identifying efficient molecular targets that could be used to develop effective therapeutics against the disease has become increasingly daunting. One of the reasons for this is the fact that each of the many alcohol-responsive genes only contributes a small effect to the overall mechanism and disease phenotype, as is characteristic of complex traits. Current research trends are hence shifting toward the analysis of gene networks rather than emphasizing individual genes. The discovery of microRNAs and their mechanisms of action on regulation of transcript level and protein translation have made evident the utility of these small non-coding RNA molecules that act as central coordinators of multiple cross-communicating cellular pathways. Cells exploit the fact that a single microRNA can target hundreds of mRNA transcripts and that a single mRNA transcript can be simultaneously targeted by distinct microRNAs, to ensure fine-tuned and/or redundant control over a large number of cellular functions. By the same token, we can use these properties of microRNAs to develop novel, targeted strategies to combat complex disorders. In this review, we will focus on recent discoveries of microRNA signatures in brain of human alcoholics supporting the hypothesis that changes in gene expression and regulation by microRNAs are responsible for long-term neuroadaptations occurring during development of alcoholism. We also discuss insights into the potential modulation of epigenetic regulators by a subset of microRNAs. Taken together, microRNA activity may be controlling many of the cellular mechanisms already known to be involved in the development of alcoholism, and suggests potential targets for the development of novel therapeutic interventions.

Keywords: human postmortem brain, alcohol dependence, alcoholism, gene expression, miRNA, non-coding RNA

INTRODUCTION

As highlighted by the World Health Organization: "The harmful use of alcohol is one of the world's leading health risks. It is a causal factor in more than 60 major types of diseases and injuries and results in approximately 2.5 million deaths each year" (World Health Organization, 2011). Alcoholism, also known as alcohol dependence, is understood as a complex, chronic brain disease with critical biological, behavioral, and socioeconomic components (Leshner, 1997). Heritable factors among the critical biological components are reported to account for about 50% of the risk for alcohol use disorders (AUD), which underscores the impact of genetic elements on disease etiology (Gorini et al., 2011). Nevertheless, the molecular mechanisms underlying genetic adaptations due to excessive alcohol consumption are not fully understood.

The chronic use of alcohol provokes long-term changes in gene and protein expression that allow neurons to adapt through homeostatic alterations in distinct signaling pathways. Among the principal transduction pathways altered by alcohol consumption are those involving receptor tyrosine kinases (which are commonly activated by interaction with growth factors such as EGF, GDNF, BDNF, and insulin among others), serine–threonine kinases (which are mostly intracellular such as PKA, PKC, ERKs, AKT, GSK3, and mTOR, but can also be receptors, e.g., TGFβR), and protein–protein interactions among scaffolding proteins and associated binding partners (examples of scaffolding proteins affected by ethanol are RACK1, PSD95, and Homer2; Ron and Messing, 2011). These signaling pathways are often implicated in the regulation of a variety of transcription factors that consequently affect the expression and activity of many genes (Miranda et al., 2010). Expression profiling studies in postmortem brains of human alcoholics have shown that the transcriptional reprogramming that takes place is brain area-specific and may reflect both preexisting differences in gene expression and alterations in response to alcohol consumption (Mayfield et al., 2008; Gorini et al., 2011). In addition, epigenetic reprogramming, primarily mediated by direct methylation of DNA and acetylation, methylation, and phosphorylation of histone proteins, appears to contribute to the altered gene expression observed in alcoholics and animal models of alcohol consumption (Miranda et al., 2010).

The discovery of microRNAs (miRNAs) and their mechanisms of action is revolutionizing our understanding of gene regulation

(Ambros, 2001; Filipowicz et al., 2008). These short (∼17–24 nucleotides long) non-coding RNAs act as post-transcriptional modulators of gene expression by binding to miRNA-recognition elements (MREs) in their target genes. This direct targeting generally results in either suppression of translation or degradation of the targeted mRNA transcript, or both (Filipowicz et al., 2008; Breving and Esquela-Kerscher, 2010). There are instances, however, when miRNAs can increase the expression of a target gene by enhancing mRNA translation (Vasudevan et al., 2007). miRNAs are highly abundant in the brain and play important roles in multiple biological processes such as neuronal differentiation (Cheng et al., 2009), brain development (Fiore et al., 2008), synapse formation and plasticity (Schratt et al., 2006), and neurodegeneration (Schaefer et al., 2007; Bushati and Cohen, 2008). miRNAs also appear to mediate the cellular adaptations induced by exposure to a number of drugs of abuse, including nicotine (Huang and Li, 2008), cocaine (Chandrasekar and Dreyer, 2009), opioids (He et al., 2010), and alcohol (Sathyan et al., 2007; Pietrzykowski et al., 2008; Miranda et al., 2010; Lewohl et al., 2011). In subsequent sections, we discuss major biological processes now known to respond to fluctuations in levels of specific miRNAs, several of which were upregulated in the brain of human alcoholics. We also uncover general patterns of miRNA regulation that may be common to a variety of addiction disorders.

ALCOHOL-INDUCED UPREGULATION OF MIRNAS IN THE HUMAN BRAIN

Our group recently published the first comprehensive study describing the impact of alcohol on microRNA levels in the brain of human alcoholics (Lewohl et al., 2011). For this, we conducted miRNA and mRNA microarray studies in prefrontal cortex (PFC) of postmortem human brain samples and developed an integrative statistical analysis highlighting differentially expressed miRNAs that inversely correlated with respective differentially expressed mRNA targets. The report uncovered a majoritarian effect of alcohol on activation of miRNA expression levels and highlighted about 35 human miRNAs that were upregulated in the alcoholic brain samples (**Table 1**). Interestingly, mRNAs that were predicted to be targeted by this group of upregulated miRNAs were significantly over-represented among the alcohol-downregulated mRNAs, while no such over-representation was detected among the set of alcohol-upregulated mRNAs. This result supports a role for miRNA-dependent inhibition of gene expression in the PFC of human alcoholics. Furthermore, we found that the alcohol-upregulated miRNAs were apparently regulating their putative target genes in a combinatorial fashion (multiple miRNAs targeting the same mRNA). Such a paradigm could be exploited by the cells to either ensure inhibition of the putative targets at any cost (redundancy) or to achieve fine-tuned regulation of specific mRNA expression levels (fine-tuning).

A recent publication by Yadav et al. (2011) described alcohol-induced changes in miRNA expression levels in human-derived neuroblastoma cells. Although the limited model used by these authors is far from reproducing the physiological environment of the alcohol-exposed human brain, their results are informative and corroborate the role of several of the upregulated miRNAs described by our group (**Table 1**, column A). Interestingly, Yadav and colleagues also found mostly upregulated miRNAs after alcohol exposure. In fact, after we subset their reported differentially expressed miRNAs for those with $p \leq 0.01$ (based on our more stringent statistical analyses of only using the lowest p values and reporting adjusted p values to account for multiple testing), only 20 upregulated miRNAs remain statistically significant. Out of these 20 upregulated miRNAs from ethanol-treated cells, six matched upregulated family members in the alcoholic human brain from our studies (miR-369-3p, miR-34c-5p, miR-203, miR-146a, miR-194, and let-7 family members, **Table 1**, column B). This is statistically highly significant ($p = 0.00022$), as it is expected that only one miRNA would be common between the two lists by chance (as empirically determined after 100,000 Monte Carlo simulations). This underscores the importance of miRNA upregulation due to alcohol exposure and suggests a consistent involvement of multiple miRNAs in mediating neuroadaptations that could potentially contribute to alcohol dependence.

Cell death-related genes were among the functionally enriched transcripts being targeted by miRNAs in our studies in PFC of human alcoholics (Liu et al., 2006b; Lewohl et al., 2011). A study from the Bakalkin group, also using postmortem PFC of human alcoholics, provides additional support for our findings. These authors found that dysregulation of the cell death machinery by inhibition of the intrinsic apoptotic pathway "may reflect molecular adaptations that counteract alcohol neurotoxicity in cells that survive after many years of alcohol exposure and withdrawal" (Johansson et al., 2008). Although the Yadav group found opposite miRNA-mediated effects inducing neuronal death in cell cultures treated with ethanol for relatively short periods of time (3–72 h), their results do not contradict the neuroadaptive phenomenon we and others observe in chronic alcoholics, but seemingly describe initial effects of acute alcohol exposure as previously documented in animal models (Heaton et al., 1999; Rajgopal et al., 2003; Nowoslawski et al., 2005). Collectively, these findings support the notion that miRNA-mediated neuroadaptations may develop after an initial, also miRNA-mediated, activation of cell death pathways that occurs during the early stages of alcohol abuse.

Our study using postmortem human brains also found that the magnitude of alcohol-related changes in miRNA levels was constrained over a narrow range of only 20–30%, with only a few miRNAs varying outside of this range (Lewohl et al., 2011). We reason that the small changes detected in miRNA, as well as mRNA expression, in PFC of human alcoholics could be due to an increased expression that is localized to a specific cellular compartment, e.g., the neuronal synapse. Such compartmentalized, enhanced differential expression would become diluted as RNA is extracted from total, unfractionated tissue. Alternatively, a larger differential expression may not be compartment-specific but cell type-specific (e.g., differential expression of immune signaling genes in glial cells or differential expression of neuronal genes in specific neuronal subtypes) and similarly become diluted when RNA is extracted from total tissue containing combined cell subpopulations. Both of these possibilities will be discussed further in the following sections.

Table 1 | Upregulated human miRNAs in alcohol-exposed tissue.

(A) Lewohl et al. (2011). Alcoholic human brain	(B) Match between (A) and Yadav et al. (2011)[†]	(C) Validated functions of family members[‡]
let-7f let-7g	miRNA family match	Neurotransmitter receptor availability, LPS-induced TLR/NFκB/IL1/TNF signaling
miR-1	–	Neurotransmitter receptor (nAChR) availability, repression of HDAC4/epigenetic gene activation
miR-7	–	Repression of α-synuclein/neurodegeneration, neurite outgrowth
miR-15b	–	Lymphocyte differentiation
miR-18a	–	Angiogenesis
miR-34c-5p	miRNA family match	Epigenetically regulated
miR-92a	–	Lymphocyte differentiation, angiogenesis, synaptic signaling, biomarker for TBI
miR-101	–	Repression of EZH2/epigenetic gene silencing
miR-135b	–	–
miR-140	–	Endocytotic recycling of neurotransmitter receptors, upregulated by nicotine and LPS
miR-144	–	–
miR-146a	miRNA match	LPS-induced TLR/NFκB/IL6 signaling
miR-152	–	TLR/IL6/IL12/TNFα/INFβ signaling, repression of DNMT1/epigenetic gene silencing, epigenetically regulated
miR-153	–	Repression of Jagged/Notch signaling, repression of α-synuclein/neurodegeneration
miR-194	miRNA match	Upregulated by LPS exposure
miR-196a	–	Virus-induced inflammation, epigenetically regulated
miR-196b	–	–
miR-203	miRNA match	JAK/STAT/IL6/INFγ signaling, repression of Bmi-1/epigenetic gene silencing, epigenetically regulated
miR-301a	–	–
miR-339-5p	–	–
miR-369-3p	miRNA match	TNFα signaling
miR-374b	–	–
miR-376c	–	–
miR-380	–	–
miR-423-5p	–	–
miR-454-3p	–	–
miR-515-3p	–	–
miR-519b-3p	–	–
miR-553	–	–
miR-580	–	–
miR-586	–	–
miR-652	–	–
miR-665	–	–
miR-802	–	–

[†] *Empirical probability of the match p = 0.00022; average number of matches/100,000 simulations = 1.04824.*

[‡] *Validated functions discussed in this review. See text for details and bibliographic references.*

HUMAN ALCOHOL-RESPONSIVE MIRNAS AND NEUROINFLAMMATION

Inflammation plays critical roles in the pathogenesis of multiple disorders of the central nervous system (CNS; Weiner and Selkoe, 2002; Hunot and Hirsch, 2003) and has recently been implicated as a mechanism of alcoholism-induced neuropathology (Crews et al., 2011; Kelley and Dantzer, 2011; Yakovleva et al., 2011). An important player eliciting activation of immune functions in the CNS in response to alcohol is Toll-like receptor (TLR) 4, which contributes to ethanol-induced activation of nuclear factor kappa B (NFκB) and consequently activates transcription of pro-inflammatory chemokines, cytokines, oxidases, and proteases. TLRs have well-established roles in pathogen detection and initiation of an innate immune response that consequently specifies adaptive responses during infection (O'Neill, 2006).

The role of innate, immune-related genes in alcohol dependency is supported by genetic association studies in humans (Pastor et al., 2000, 2005; Edenberg et al., 2008; Saiz et al., 2009), gene expression microarray studies in postmortem brains of alcoholics (Liu et al., 2006b; Ökvist et al., 2007), and transcriptome meta-analysis in rat brains (Mulligan et al., 2006). Furthermore, recent behavioral studies in mutant mice indicated that deletion of genes

involved in aspects of the neuroimmune response to alcohol (B2m, Ctss, Il1rn, Cd14, and Il6) could reduce ethanol consumption in mice (Blednov et al., 2012). Based on these and additional related findings, a hypothesis is developing in part of the alcohol research community that the neurobiology of addiction is due to altered brain signaling driven by immune signaling.

Implications of miRNAs as players in the development of the immune system and the regulation of multiple immune functions are also coming to age (Sonkoly et al., 2008; Belver et al., 2011; O'Neill et al., 2011; Turner et al., 2011). Multiple profiling studies have confirmed the hypothesis that TLR signaling can modulate miRNA expression and this generally depends on NFκB. In all cases described, TLR activation exclusively induces upregulation of miRNAs (O'Neill et al., 2011). Interestingly, this is the same effect (miRNA upregulation) we observe in our own miRNA profiling studies in brain of human alcoholics. In this section, we address a series of recent findings that uncover a potential connection between a group of upregulated miRNAs in postmortem brains of human alcoholics and neuroinflammatory processes triggered by alcohol. We summarize this evidence in a hypothetical model presented in **Figure 1**.

About 30% of the 35 miRNAs upregulated in the PFC of human alcoholics (miR-15, miR-34, miR-92, miR-140, miR-146, miR-152, miR-194, miR-196, miR-203, miR-369, let-7) are modulators of immunity and several of them appear to be involved in regulating TLR signaling. Particularly interesting is the miR-146 family, which is upregulated by bacterial endotoxin lipopolysaccharides (LPS) and controls TLR/NFκB signaling through negative feedback loops involving downregulation of IL1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) protein levels (Taganov et al., 2006; Zhao et al., 2011). TRAF2 has also being implicated as a miR-146 target (Hou et al., 2009). It was suggested that miR-146 regulatory circuit likely fine-tunes TLR and cytokine signaling, rather than totally abrogating the signal, and that expression of miR-146 may be critical in preventing excess inflammation (Sonkoly et al., 2008). Collectively, IRAK1, IRAK2, and TRAF6 represent important components of the myeloid differentiation primary-response protein 88 (MYD88)-dependent pathway for NFκB activation downstream of TLRs (O'Neill et al., 2011). Correspondingly, several reports have implicated IRAK and TRAF6 transducers in alcohol actions in the brain (Vallés et al., 2004; Guasch et al., 2007; Oliva et al., 2011).

let-7 family members, on the other hand, have been reported to directly target expression of TLR4 and regulate responsiveness to LPS (Chen et al., 2007; Androulidaki et al., 2009) and to form an epigenetic switch together with NFκB and interleukin 6 (IL6) that links inflammation to persistent cell transformation (Iliopoulos et al., 2009). The reported upregulation of let-7 miRNAs in human alcoholics could be understood as necessary to turn off expression of TLRs in glial cells and avoid excessive inflammation due to hyper-responsiveness to LPS insult. This potential regulatory loop could contribute to tolerance and dependency in chronic alcoholics. Furthermore, Blednov and colleagues recently demonstrated that LPS-triggered activation of immune signaling mediated by CD14 [a key component of the LPS-sensing co-clustering complex that also includes Hsp70, Hsp90, CXCR4, GDF5, and TLR4 (Triantafilou and Triantafilou, 2002)] promotes alcohol consumption and alters certain aspects of alcohol reward and aversion in mice (Blednov et al., 2011). Our finding that chemokine receptor CXCR4 is downregulated in brain of human alcoholics and is the most significantly miRNA-over-targeted transcript (Lewohl et al., 2011) supports a role for miRNAs in the regulation of LPS-activated TLR4-transduced immune signaling in chronic alcoholics. CXCR4 has also being recently implicated in opiate-induced hypernociception (White and Wilson, 2010) and polymorphisms in SDF1 (the CXCR4 ligand) associated with several phenotypes, including alcohol consumption (Xiao et al., 2008).

MicroRNA miR-203 is the upregulated miRNA in human alcoholics with the highest over-representation of targets among inversely correlated differentially expressed mRNA transcripts from the same samples (Lewohl et al., 2011). This miRNA has been suggested to regulate IL6 and IFNγ signaling through targeting of the Suppressor of Cytokine Signaling 3 (SOCS3; Sonkoly et al., 2007). SOCS3 is part of a negative feedback loop in cytokine signaling that inhibits the activation of transcription factor STAT3, part of the JAK–STAT signaling cascade that is the basis of the signal transduction mechanism for many cytokine receptors. Upregulation of miR-203 may hence lead to increased and/or longer inflammatory response. Furthermore, miR-203 is predicted to target above mentioned CXCR4 (component of the LPS-sensing complex), which is the alcohol-downregulated mRNA most significantly over-targeted by miRNAs in human alcoholics (Lewohl et al., 2011). These appear to represent compensatory effects that, on one hand, aim at downregulating responsiveness to the LPS insult while, on the other, aim at maintaining required levels of neuroimmune activity. These results support the notion that CXCR4 and miRNAs regulating its expression are important players modulating alcohol actions in the brain of human alcoholics.

The family miR-148/152 was recently reported to inhibit TLR-triggered MHCII expression and functional maturation of dendritic cells (DCs). By targeting CaMKIIα, miR-152 inhibited the production of cytokines, including IL12, IL6, TNFα, and IFNβ, and negatively regulated the innate response (Liu et al., 2010). Coincidently, miR-152 was also upregulated in fetal brain from mice exposed to ethanol during gestation (Wang et al., 2009). Interestingly, Kash et al. (2009) recently found a significant decrease in the total levels of CaMKIIα in the central extended amygdala after ethanol exposure in mice. CaMKIIα expression enhances the extent of desensitization of NR2B-containing NMDA receptors in heterologous cells (Sessoms-Sikes et al., 2005) and regulates ethanol sensitivity of BK channels (Liu et al., 2006a), which suggested the possibility that reduced levels of CaMKIIα could be acting in a general fashion to modulate the ethanol sensitivity of channels in the extended amygdala (Kash et al., 2009). Coincidently, another miRNA, miR-9, also implicated in controlling the activity of the TLR signaling pathway through direct targeting of the NFKB1 gene in human monocytes and neutrophils (Bazzoni et al., 2009; O'Neill et al., 2011), was demonstrated to regulate ethanol sensitivity of BK channels by selectively destabilizing splice variants that contained miR-9 recognition elements, therefore making the BK channels less sensitive to alcohol (Pietrzykowski et al., 2008). We did not detect changes in miR-9 expression in

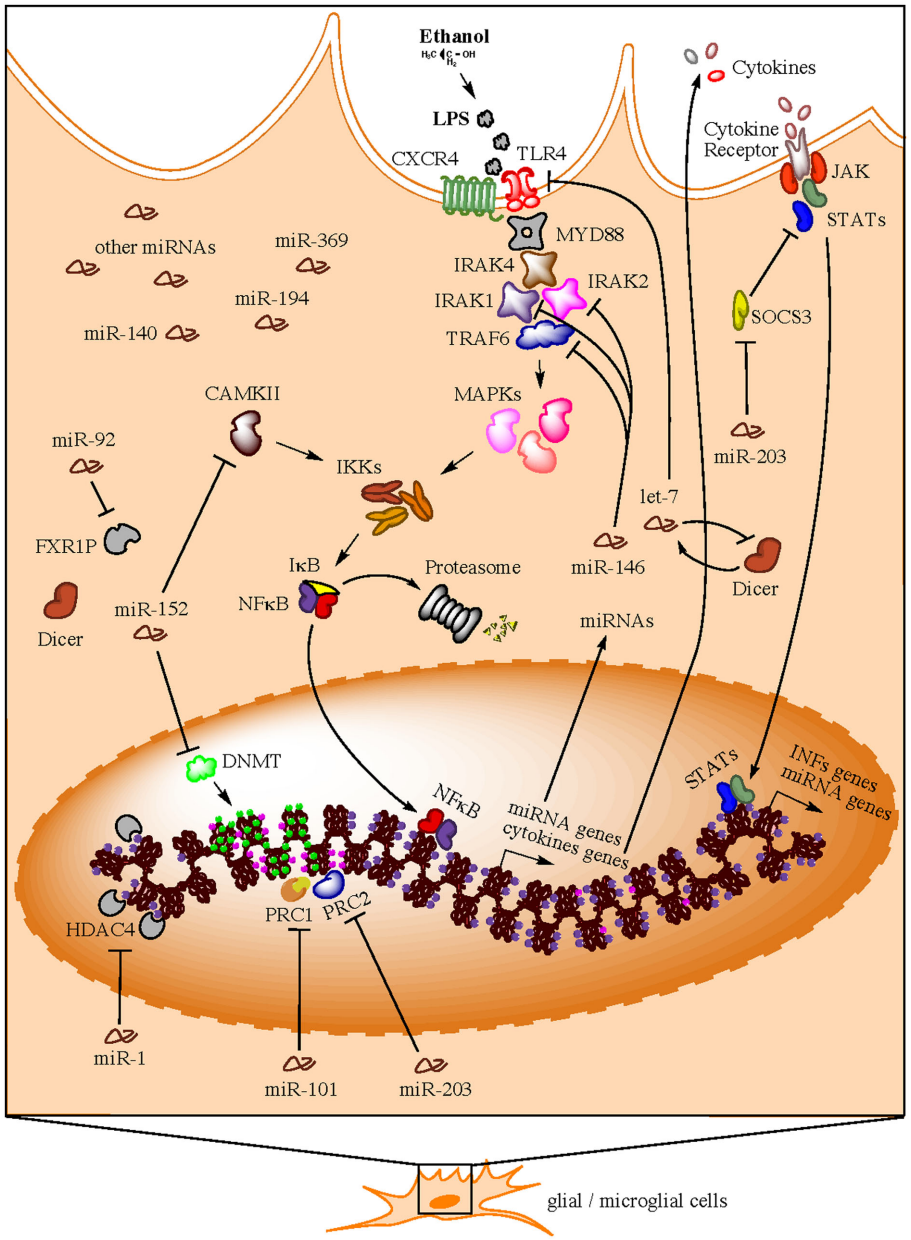

FIGURE 1 | Hypothetical model for neuroimmune-related actions of miRNAs in brain of human alcoholics (e.g., in microglia). Bacterial lipopolysaccharides (LPS) from commensal bacteria, for example, could leak into the bloodstream due to gut leakiness induced by alcohol consumption. A compromised blood brain barrier due to high ethanol concentration in the blood will consequently allow LPS to reach the brain and trigger TLR signaling cascades that activate nuclear factor kappa B (NFκB) and induce the transcription of a variety of pro-inflammatory genes (e.g., cytokines), as well as miRNA genes. Newly synthesized pro-inflammatory factors are secreted to unleash a systemic neuroinflammatory response and to maintain a positive feedback loop in same activated cell. These second round of pro-inflammatory factor production is achieved through the expression of alternative cytokine receptors in the activated cell, which signal back to the nucleus through the JAK/STAT pathway to induce production of additional pro-inflammatory factors (e.g., interferons) and miRNA genes. To avoid over-amplification of these signals and excessive inflammation due to hyper-responsiveness to LPS insult, specific miRNAs (e.g., members of the miR-146, miR-152, and let-7 families) are consequently upregulated in order to suppress TLR4/CXCR4 signaling through inhibition of multiple cascade transducers such as IL1 receptor-associated kinases (IRAKs), TNF receptor-associated factor 6 (TRAF6), and TLR4/CXCR4 themselves, among others. As a compensatory reaction, other miRNAs (e.g., miR-203) that activate the alternative JAK/STAT pathway are also upregulated in an effort to maintain the immune-activated state of the specific cell subtype while promoting a benign, contained inflammatory response. Concomitantly, immune-activated cells implement miRNA-mediated epigenetics mechanisms, such as DNA methylation and histone methylation and/or acetylation) that ensure chromatin modification and global changes in gene expression that allow for long-term homeostatic changes and cellular adaptations under the particular environmental conditions. Expectedly, miRNAs that target epigenetic factors are also activated in order to control and/or fine-tune the ongoing remodeling of the cellular epigenome.

our postmortem human brain study, but such differences among human alcoholics and animal models are plausible due to neurodevelopmental differences between species, stage of alcohol insult, and experimental protocol differences, to name a few. As suggested by O'Neill et al. (2011) miRNAs might also play a role in "controlling the switch from a strong early pro-inflammatory response to the resolution phase of the inflammatory process." This would also support a case for miRNA and mRNA expression profile discrepancies depending on the stage of the inflammatory response in the particular samples under study.

Evidence is also accumulating for novel mechanisms by which miRNAs can directly regulate mRNAs by facilitating or preventing interaction with RNA-binding proteins. An example of this that also underscores the fact that mRNA stability can be affected by environmental factors, is miR-369-3p (one of the upregulated miRNAs in human alcoholics), which associates directly with the AU-rich elements (AREs) in TNFα mRNA by base pairing and mediates translational activation of TNFα only under growth arrest conditions (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). This effect was dependent on the recruitment of the RNA-binding proteins fragile-X mental retardation-related protein 1 (FXR1) and argonaute 2 (AGo2) and could be reversed when cells were actively proliferating, in which case miR-369-3p would switch roles to represses TNFα. Discovery of such sensitive and complex switch-like regulatory mechanisms emphasizes the extreme fine-tuning capabilities encoded into the biology of miRNAs.

The specific functions of other miRNAs upregulated in human alcoholics, which have also been implicated in immune signaling, remain unknown. Among these are miR-140 and miR-194, both upregulated in *in vivo* studies in mouse lung after LPS exposure (Moschos et al., 2007); miR-92, which is upregulated in CD4+CD8+ double positive thymocytes in comparison to other stages of T lymphocyte development (Sonkoly et al., 2008); miR-15b, which is upregulated in CD8+ cells when compared with CD4+ T cells or double positive thymocytes (Sonkoly et al., 2008); and miR-196, which have sequence-predicted targets within the hepatitis C virus genomic RNA and is upregulated by antiviral cytokine INFβ (Sonkoly et al., 2008). This highlights the fact that we are just starting to understand the regulatory roles of miRNAs in general. Often, a single miRNA is found to be involved in multiple cellular functions. For example, (1) miR-92a and cluster member miR-18a block angiogenesis when overexpressed in endothelial cells (Bonauer et al., 2009; Doebele et al., 2010), (2) increased miR-92a in plasma levels in patients with traumatic brain injury (TBI) is a good biomarker for the severity of the disease (Redell et al., 2010), and (3) family member miR-92b is involved in synaptic signaling (Ceman and Saugstad, 2011). These diverse functions provide evidence for versatile and complex systemic roles of miRNAs.

HUMAN ALCOHOL-RESPONSIVE MIRNAS, NEUROTRANSMITTER SIGNALING, AND SYNAPTIC PLASTICITY

Alcohol, as well as other drugs of abuse, produce long-term changes within the brain reward circuits and these changes are thought to lead to drug tolerance, reward dysfunction, escalation of drug intake, and eventually compulsive use (Russo et al., 2009). Research on the reward circuit has been centered on dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain and their projections to the limbic system, in particular the nucleus accumbens (NAc), dorsal striatum, amygdala, hippocampus, and regions of PFC (Robison and Nestler, 2011). Nevertheless, other neurotransmitter systems seem also to contribute to brain reward responses since animals can still exhibit positive hedonic responses in the absence of dopamine (Hyman et al., 2006). Ethanol as well as opioids, cannabinoids, and nicotine are thought to produce reward partially through non-dopaminergic mechanisms, e.g., μ opioid receptors expressed on NAc neurons, which appear to bypass dopamine inputs from the VTA (Hyman et al., 2006). Studies examining the effect of selective agonist and antagonist drugs have indicated that multiple neurotransmitters, including dopamine, serotonin, acetylcholine, glutamate, GABA, and various peptides, are involved in activation of reward circuits and the production of multiple forms of neuronal plasticity that can convert drug-induced signals into long-term alterations in behavior (Bardo, 1998; Hyman et al., 2006). At the molecular level, complex gene expression mechanisms coordinate long-lasting alterations in dendrite and synapse structure and function. Among these mechanisms, the local control of mRNA translation in neuronal dendrites can account for the tight spatial regulation of plasticity at the level of individual dendrites or spines (Sutton and Schuman, 2006; Schratt, 2009). Recent evidence discussed below indicates that miRNAs play extensive roles in the regulation of these local processes and the development of addictive animal behaviors. We summarize this evidence in a hypothetical model presented in **Figure 2**.

Members of multiple miRNA families upregulated in the brain of human alcoholics have been collectively found enriched (let-7, miR-1, miR-7, miR-92, miR-135, miR-146, miR-339, miR-376, and miR-380) or depleted (miR-34, miR-101, miR-144, miR-153, miR-301, and miR-652) in rodent synapses (Lugli et al., 2008; Siegel et al., 2009; Eipper-Mains et al., 2011). miR-92 in particular, had been implicated in the regulation of FXR1P, which is an RNA-binding protein found in dendrites and involved in miRNA biogenesis (Ceman and Saugstad, 2011). Lugli et al. (2008) suggested that the miRNAs expressed within dendrites and within dendritic spines were expected to contribute to the regulation of local protein synthesis. Since the main effect of miRNA upregulation in human alcoholic brain is suspected to be downregulation of targeted-gene expression and/or inhibition of protein translation (Lewohl et al., 2011) and blockade of *de novo* protein synthesis in dendritic spines attenuates formation of long-term memory due to impairment of dendritic growth and remodeling (Schratt, 2009; Eipper-Mains et al., 2011), it might suggest that the observed alcohol upregulation of these synapse-localized miRNAs could have a major impact in the development of new addiction-promoting, long-term memories. Hence, early or accelerated upregulation of alcohol-responsive miRNAs in the brain might preempt development of addiction and could represent a potential therapeutic strategy.

miR-7 and miR-153 have both been recently implicated in the regulation of α-synuclein (Doxakis, 2010). α-synuclein plays a role in activity-dependent maintenance of SNARE complex levels in

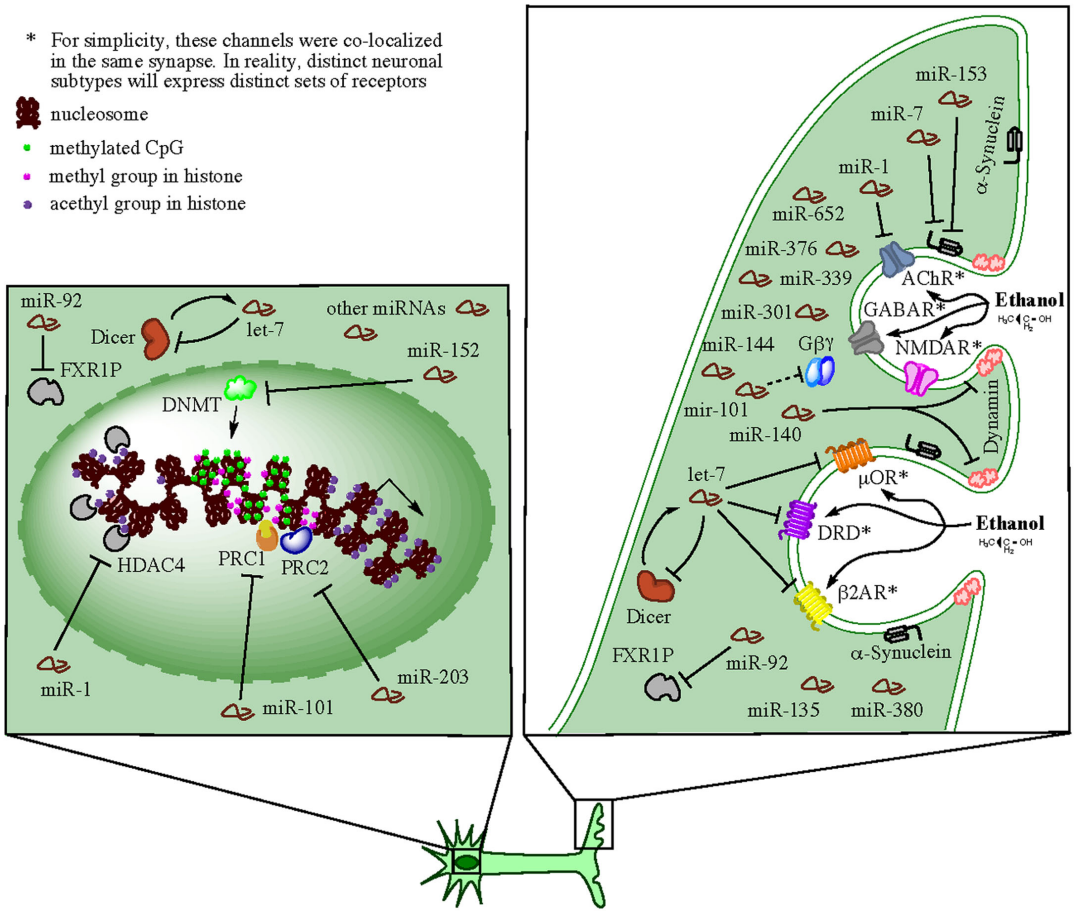

FIGURE 2 | Hypothetical model for synaptic-related actions of miRNAs in brain of human alcoholics (e.g., in neurons). Ethanol can directly or indirectly affect multiple neurotransmitter receptors at the neuronal synapse and activate reward circuits conducive to multiple forms of neuronal plasticity, which in turn, convert the drug-induced signals into long-term alterations in behavior (e.g., alcohol dependency). The receptors affected on a particular synapse depend on the neuronal subtype and the specific subset of receptors expressed. For simplicity, several receptors such as GABAR, NMDAR, AChR, μOR, DRD, and β2AR, were diagramed as co-localized on the same synapse in the cartoon, but this is probably not the case in reality. miRNAs transported to and enriched in the synapses are locally processed by resident miRNA-ribonucleoprotein (miRNP) complexes (containing Dicer and FXR1P among other factors) and exert predominately inhibitory effects on mRNA targets also present at the specific synapses. Members of miRNA families, such as let-7, miR-1, miR-101, miR-140, and several others, downregulate the activity of neurotransmitter receptors by directly targeting respective mRNAs or by interfering with synaptic endocytosis (e.g., through targeting of dynamin and α-synuclein mRNAs). In addition, synaptic miRNAs implement negative feedback loops (through targeting of dicer and FXR1 mRNA, among other miRNA biogenesis-related factors) that auto-regulate their own availability. Such feedback loops ensure a balanced homeostatic control of a variety of synaptic functions. Concomitantly, cells implement miRNA-mediated epigenetics mechanisms, such as DNA methylation and histone methylation and/or acetylation, that ensure chromatin modification and global changes in gene expression that allow for long-term homeostatic changes and cellular adaptations under the particular environmental conditions. Expectedly, miRNAs that target epigenetic factors are also activated in order to control and/or fine-tune the ongoing remodeling of the cellular epigenome.

pre-synaptic terminals (Burre et al., 2010; Greten-Harrison and Polydoro, 2010; Burgoyne and Morgan, 2011) and is involved in dopaminergic neurotransmission and neurodegenerative disorders (Doxakis, 2010). Importantly, there is a large body of evidence linking α-synuclein accumulation to alcohol dependency in humans as well as in rodents (Bönsch et al., 2005; Liang and Carr, 2006; Foroud et al., 2007; Taraskina et al., 2008; Aho et al., 2009). Furthermore, miR-7 has also been implicated in the control of neurite outgrowth *in vitro*, during neuronal differentiation of human neuroblastoma cell lines (Chen et al., 2010). On the other hand, miR-153 has been implicated in the teratogenic effects of alcohol in mice, although in this case, due to downregulation of the combinatorial function of three miRNAs (miR-21, miR-335, and miR-153). Simultaneous downregulation of these three miRNAs allowed their shared target, Jagged1 (a Notch receptor ligand), to accumulate and induce cell cycle and neuroepithelial maturation (Sathyan et al., 2007).

The Smalheiser group has found that dicer and the Ago homolog eIF2c, both involved in miRNA biogenesis, were expressed in mouse brain synaptic fractions and enriched at post-synaptic densities (PSDs; Lugli et al., 2005; Smalheiser and Lugli, 2009). The findings that NMDA and calcium stimulation of synaptoneurosomes induced calpain-dependent activation of dicer led the authors to propose a model in which (i) glutamate or other

neurotransmitter activity at synapses causes a local increase of intracellular calcium levels within the post-synaptic neuron; (ii) this calcium increase needs to be sufficient to activate calpain; (iii) calpain liberates active dicer and eIF2c from the PSDs. Furthermore, the authors postulated that the acute effects of calpain on dicer act as a highly localized, phasic, high-threshold trigger that leads to formation of small RNAs near synapses, which once formed should be relatively long-lasting and subsequently independent of ongoing dicer activity (Lugli et al., 2005). Recent studies in *Drosophila* and rodents have confirmed the importance of components of the RISC complex at the synapse, which was necessary for the establishment of certain forms of short and long-term memories in these models (Ashraf et al., 2006; Batassa et al., 2010). Remarkably, we found that dicer was downregulated in the PFC of human alcoholics and that, equal to CXCR4, it was the mRNA most significantly over-targeted by upregulated miRNAs (Lewohl et al., 2011), which suggest a relevant role for dicer-miRNA feedback loops in the mediation of and/or neuroadaptation to alcohol actions.

Several of the synaptically modulated miRNA families have been involved in the regulation of neurotransmitter signaling, which is mechanistically related to stimulation of reward circuits and the formation of associative, synapse-specific memories (Hyman et al., 2006). For example, the let-7 family, one of the miRNA families differentially expressed in PFC of human alcoholics, could be modulating the activity of a variety of neurotransmitter receptors. Several members of this family have been reported to regulate receptors such as the μ opioid receptor, the β2-adrenergic receptor, and the dopamine D3 receptor (Pillai, 2005; Chandrasekar and Dreyer, 2009; He et al., 2010). While investigating mechanisms contributing to opioid tolerance in mice, He et al. (2010) identified let-7 family members (let-7a, let-7c, and let-7g were used as representative members) as mediators of translocation and sequestration of μ opioid receptor mRNA into P-bodies, which led to translation repression without affecting the mRNA levels. A similar mechanism for repression of translational initiation of let-7 targets in human cells had previously been reported (Pillai, 2005). The μ opioid receptor is largely distributed along reward circuits, where it mediates the reinforcing activities of morphine and several non-opioid drugs such as alcohol, cannabinoids, and nicotine. The non-opioid drugs act at their own receptors [GABAA and NMDA receptors for alcohol, CB1 and CB2 receptors for cannabinoids, and nicotinic acetylcholine receptor (nAChR) for nicotine] and are likely to induce the release of endogenous opioid peptides that, in turn, activate the μ receptors. This type of receptor seems to function as a convergent molecular gate in the initiation of addictive behaviors (Contet et al., 2004). In addition, Wang et al. (2011) demonstrated that let-7f targeted β2-adrenergic receptor (β2AR) mRNA and repressed expression of the native ADRB2 protein in the human cell line H292. Interestingly, another microRNA that is also upregulated in human alcoholics, miR-15, belongs to one of the only two other groups of miRNAs that have predicted binding sites within the 3′ UTR of the β2AR mRNA (Wang et al., 2011). Recent evidence supports a role for β-adrenergic receptors in retrieval of cocaine-associated memories, mediated by norepinephrine acting at central β-adrenergic receptors in rats (Otis and Mueller, 2011). With this work, Otis and Mueller also demonstrated that propranolol, a commonly prescribed β-blocker, can be used as an adjunct to exposure therapy for the treatment of cocaine addiction. In addition, activation of β2AR also appears to be required for some of the key neurochemical changes that characterize chronic opioid administration in mice (Liang et al., 2007). Yet another let-7 family member, let-7d, was reported to be downregulated by cocaine in rat mesolimbic brain slices, and overexpression of this miRNA in hybrid NG108-15 cells downregulated the dopamine D3 receptor (Chandrasekar and Dreyer, 2009). It is well known that drugs of abuse induce persistent structural and functional changes in the mesolimbic dopaminergic system that lead to high-risk, drug-seeking behaviors, and relapse (Chandrasekar and Dreyer, 2011).

On the other hand, support for an involvement of miR-101 (another miRNA upregulated in the PFC of human alcoholics) in the modulation of GABAergic transmission in response to alcohol consumption can be found in a recent study conducted by the Tabakoff group (Saba et al., 2011). After analyzing gene expression profiles induced by alcohol-drinking in panels of recombinant and isogenic inbred mice, Saba and colleagues found that guanine nucleotide binding protein beta 1 subunit (Gnb1) was the only transcript that changed expression in all groups of high and low alcohol-drinking mice and, in addition, localized within a quantitative trait loci for alcohol consumption. Gnb1 expression level was inversely related to the level of alcohol intake. Based on differential detection of Gnb1 transcript variants, protein levels, and 3′ UTR sequence analysis, the authors suggested that miR-101 could be regulating the amount of Gβ1 protein. Gβ1 protein can initiate a cascade of intracellular signaling events leading to the internalization of GABAA and other receptors (Saba et al., 2011). Although subsequent studies investigating the direct interaction between miR-101 and the Gnb1 transcripts are necessary for validation, we are enthusiastic about the emergence of seemingly interconnected patterns spanning multiple animal species.

Additional evidence for a role of miRNAs in the regulation of endocytotic recycling of neurotransmitter receptors is found in the work of the Li group, who found that miR-140* is upregulated by nicotine treatment and directly binds to dynamin (Dnm1) mRNA to inhibit its expression (Huang and Li, 2008). Dynamin-1 is involved in scission of clathrin-coated vesicles and it is essential for endocytotic synaptic vesicle recycling only during the application of a strong or sustained stimulus when exocytosis of neurotransmitter-filled vesicles is extreme and rapid retrieval of vesicles is required to maintain the synaptic vesicle pool (Ferguson et al., 2007; Etheridge et al., 2009). Proteomic studies of synaptosomal fractions from superior frontal gyrus and occipital cortex of postmortem human brains identified dynamin-1 protein as differentially regulated between alcoholics and controls (decreased expression in alcoholics) and provided evidence for differential alteration of multiple protein isoforms in a brain region-specific manner (Etheridge et al., 2009). Furthermore, our group has demonstrated that dynamin-1 establishes a strong physical interaction with the large conductance voltage- and calcium-activated potassium channel (BKCa), another major player in alcohol actions (Gorini et al., 2010). Interestingly, multiple miRNAs upregulated in human alcoholics, including miR-101, miR-135b,

miR-140, miR-203, miR-34c-5p, miR-376, miR-454, miR-519b-3p, miR-580, and miR-586 (Lewohl et al., 2011), are predicted to target dynamin family members, which underscore the role that these proteins and respective targeting-miRNAs play in mediating alcohol actions.

Acetylcholine (ACh) could be another neurotransmitter signaling pathway regulated by miRNAs in human alcoholic brains. miR-1 is a conserved muscle-specific microRNA that regulates aspects of both pre- and post-synaptic function at neuromuscular junctions (Simon et al., 2008). Using *C. elegans* as model system, Simon et al. (2008) demonstrated that miR-1 regulates the expression level of two nAChR subunits, thereby altering muscle sensitivity to ACh. Alcohol has been reported to enhance the function of naturally expressed α4β2 nAChRs and to inhibit the activity of naturally expressed α7 nAChRs (Narahashi et al., 1999). Furthermore, activation of nAChRs was reported to selectively reduce alcohol consumption in outbred Wistar rats (Dyr et al., 1999), while mutations in transmembrane domains of α2 channel subunits can enhance actions of alcohols on neuronal nicotinic receptors (Borghese et al., 2002).

Taken together, our analysis of potential effects of alcohol-responsive miRNAs on neurotransmitter receptor-mediated signaling indicates that a variety of neurotransmitter-regulated pathways may be simultaneously changing in response to alcohol as well as other drugs of abuse, and that the activity of multiple key miRNA families may be contributing to this particular complex network of interactions. Direct effect of upregulated miRNAs (as in the cohort of human alcoholic studied by Lewohl et al., 2011) over their putative neurotransmitter receptor targets is suggestive of a neuroadaptive response to counteract the drug-induced receptor activation characteristic in multiple types of addiction. On the other hand, since receptor endocytosis contributes to signal termination and desensitization of activated G protein-coupled receptors (Finn and Whistler, 2001; Li and van der Vaart, 2011), miRNA-driven downregulation of dynamin-1, which disrupts neurotransmitter receptor endocytosis, might contribute to the addictive effects of alcohol as well as other drugs of abuse.

HUMAN ALCOHOL-RESPONSIVE MIRNAS AND EPIGENETICS

The fact that epigenetic mechanisms underlie adaptation in adult organisms has become clear thanks to an explosion in research into mechanisms by which chromatin and histone modifications impact transcriptional regulation under a variety of environmental insults. These mechanisms have been demonstrated to play a role in drug addiction, as repeated exposure to drugs of abuse induce alterations in histone acetylation, phosphorylation, and methylation levels, as well as DNA methylation levels (Maze and Nestler, 2011; Robison and Nestler, 2011; Wong et al., 2011). In alcoholic patients, a significant increase in global DNA methylation influenced by reduced levels of DNA methyl transferase 3b (DNMT3b) has been reported and a possible subsequent derangement of epigenetic control suggested (Bönsch et al., 2004, 2006). Furthermore, several genomic loci such as nerve growth factor (NGF) and pro-opiomelanocortin gene (POMC) from blood cells and prodynorphin (PDYN) from brain tissue have been found hyper-methylated in human alcoholics (Bönsch et al., 2006; Muschler et al., 2010; Heberlein et al., 2011; Taqi et al., 2011). In this section, we focus on recent studies demonstrating that miRNAs can regulate epigenetic factors and vice versa (Sato et al., 2011), highlighting, in particular, epigenetic-related miRNAs upregulated in the brain of human alcoholics. We summarize this evidence in the hypothetical models presented in **Figures 1** and **2** (see nucleus).

miR-101 was reported to specifically repress expression of EZH2, a conserved catalytic subunit within the polycomb repressor complex 2 (PRC2), which is involved in histone methylation and consequent transcriptional silencing (Varambally et al., 2008; Friedman et al., 2009). Overexpression of miR-101 markedly attenuated cell proliferation and apparently configured the histone code of cancer cells to that associated with more benign cellular phenotypes (Varambally et al., 2008). Other miRNAs commonly silenced in a variety of cancers also target factors in the epigenetic pathway; for example, miR-203 targets Bmi-1, a component of the polycomb repressor complex 1 (PRC1) that working together with PRC2, plays an important role in stabilizing epigenetic gene silencing by binding to methylated chromatin (Wellner et al., 2009; Sato et al., 2011), and miR-152 targets DNA methyltransferase DNMT1 (Tsuruta et al., 2011). All these miRNAs have been postulated as tumor suppressors (TS-miRNAs) and all have in common that they specifically downregulate factors involved in altering the epigenetic landscape of cells. These studies and several others (Rodríguez-Paredes and Esteller, 2011; Tsai and Baylin, 2011) demonstrate that when the epigenetic machinery goes awry, cells have increased chances to become malignant and develop cancers. Therefore, it is reasonable to speculate that cells and tissues in non-complicated alcoholics, as well as in any non-complicated group of individuals chronically subjected to environmental stress, should have implemented defense mechanisms such as overexpression of TS-miRNAs in order to avoid early development of malignances.

On the other hand, miR-1 has been reported to repress histone deacetylase 4 (HDAC4) and to promote differentiation of myoblasts (Chen et al., 2006). HDAC4 is highly abundant in mouse brain (Kumar et al., 2005) and its subcellular localization (nuclear or cytoplasmic) in rat hippocampal neurons is specified by neuronal activity (Chawla et al., 2003). Interestingly, Pandey et al. (2008) found that the anxiolytic effects produced by acute alcohol were associated with increased HDAC activity and reduced histone acetylation in the rat amygdala, and suggested that HDAC inhibitors may be potential therapeutic agents in treating alcohol withdrawal symptoms. Nevertheless, somewhat contradictorily, the Nestler group has reported that HDAC inhibition potentiates cocaine's behavioral effects and that reducing acetylation through viral-mediated overexpression of HDAC4 in the NAc of mice decreased cocaine reward (Kumar et al., 2005). These authors also suggested that stimulation of gene transcription may be the predominant mechanism for cocaine-induced behavioral plasticity. These drug-specific or animal model-specific cellular activities and behaviors warn us about the potential risk of extrapolating results from one model into another, but also underscore the fine-tuning capabilities of these regulatory pathways.

Another subset of miRNA families upregulated in alcoholics, including miR-1, miR-34, miR-152, miR-196, and miR-203, is

regulated by epigenetic mechanisms (Sato et al., 2011), which could partly explain how environmental factors could be contributing to disease development. The respective transcriptional units for miR-34b/c and miR-203 both contain CpG islands, and the methylation levels of these CpG islands are inversely correlated with the expression level of the mature miRNA in various cancers, including CNS tumors (Kozaki et al., 2008; Furuta et al., 2010). miR-152 and miR-196a are also silenced by hypermethylation of CpG islands on the respective DNA loci (Hoffman et al., 2009; Tsuruta et al., 2011). It is interesting to note that several of the miRNAs that target factors involved in epigenetic remodeling could be conversely regulated by epigenetic mechanisms, suggesting that miRNAs are elements of epigenetic feedback regulatory loops that fine-tune activity of the pathway.

CONCLUSION

Given the complex etiology of alcohol abuse disorders and dependence, characterized by brain-wide pathophysiological alterations under continuous crosstalk, we reason that to better understand and treat alcohol abuse, we need to develop organ and system-wide models (even if initially incomplete and speculative to some extent) with the potential to capture the dynamism in such complexity. Once we discover main dynamic relationships conducive to stable states of the disease, we may be able to design strategies to detour or reverse disease progression, akin of chemical reactions transiting through multiple equilibrium states. From the analysis and hypothetical models presented here, the notion emerges that miRNAs are efficiently being upregulated in response to alcohol insult and consequently inducing organ and system-wide homeostatic changes that produce long-term adaptation under the specific cellular environment. Remarkably, miRNAs that localize to and display activity at synapses in neurons are also capable of eliciting distinct and specific activities in other cell types, such as innate immunity-related functions in microglial cells, among others. This underscores the impact that molecular efficiency, signaling crosstalk, and cellular economy plays in the adaptation and evolution of cellular systems, all of which are concepts we have already learnt over the many years of biological research.

Due to the versatility and wide-reaching regulatory power of miRNAs, we expect the future development of novel knockdown and/or overexpression strategies targeting the activity of specific miRNAs, in an effort to prevent or even reverse addiction. Yet, many issues need to be addressed in order to better understand how miRNAs perform their functions and how they could be exploited to combat alcohol abuse. We wonder, for example, whether minor sequence variations in members of miRNA families affect their target specificity and biological function, and whether miRNA family members exist to exert redundant control over specific targets, to fine-tune their post-transcriptional regulation, or to exert a combination of both types of control. We also wonder whether we could unleash the fine-tuning regulatory potential of miRNAs by exploiting the combinatorial capability of miRNA targeting.

On the other hand, the miRNA field would immensely benefit from the development of high throughput technologies and methods for reliable characterization and validation of miRNA targets that are lagging at this time, together with the development of bioinformatic platforms capable of effectively and efficiently integrating the complex combinatorial patterns hidden under the "miRNA code." Equally thrilling will be the discovery of small molecules that could specifically interact with and modulate the availability of select miRNAs in particular tissues, which would be invaluable to the clinical and therapeutic fields.

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Big (sequencing) future of non-coding RNA research for the understanding of cocaine

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Recent advancement in genomic and genetic sequencing technology has ushered in a new era in which unprecedented amount of genomic sequence and transcript sequence data with extraordinary detail can be generated at incredibly short time. As part of this advancement, the approaches that utilize the powerful next generation sequencing technology, such as RNA-Seq, may have dramatically changed the field of non-coding RNA (ncRNA) research. Since these new approaches do not require prior knowledge of annotated transcripts for probes, theoretically the entire transcriptome of a given sample can be sequenced. This enables the detection of novel transcripts, including both protein coding and ncRNA, as well as RNA with somatic mutations and alternative splicing forms (Trapnell et al., 2009, 2010; Au et al., 2010; Griffith et al., 2010). Before this recent technological advancement, in the more traditional approaches for ncRNA studies, such as using RNA microarray technology, prior sequence knowledge of ncRNA in a cell, tissue, or an organism is required to first generate the microarray. Known RNA and RNA variants of a sample are then hybridized to the array, detected, quantified, and analyzed, while unknown RNA or RNA variants remain undetected and unanalyzed. This is especially limiting for the studying of many classes of ncRNA, such as long ncRNA (lncRNA, which are ncRNA that are longer than 200 nucleotides), due to somatic mutations, epigenetic consequences, and the possibility of secondary structures that longer RNA often take (Wang and Chang, 2011; Mercer et al., 2012; Moran et al., 2012). Some additional advantages of RNA-Seq that have been reported in studying protein coding transcripts, including the capability of detecting high dynamic range of gene expression level, and its high precision and high reproducibility (Marioni et al., 2008; Mane et al., 2009; Bradford et al., 2010; Chen et al., 2010; Twine et al., 2011; Zhang et al., 2012), will likely further ratify this new trend of ncRNA research. Nevertheless, currently microarray is still a valid technology for RNA studies in many other aspects, for its speed, specificities for targeted probing, and its low cost. For example, since statistically 5% of genes give rise to 75% of housekeeping transcripts, much effort is required in RNA-Seq to filter out high abundance transcripts before one can piece together and quantify the interested lower abundance transcripts in a typical RNA sample. Cost is another limiting factor for the migration to RNA-Seq. A typical RNA-Seq run can cost a few thousands, while a typical run for a targeted high throughput microarray costs around hundreds in dollar amount. In the foreseeable future, microarray and RNA-Seq may co-exist and each provides its own aspects of advantage.

If the size of human genome truly reflects the evolution pressure and practical genomic requirements for biological function and survival, one may conclude that the number of genes we have studied and all the facts we have discovered on genes and gene regulation is just the tip of an iceberg, because merely 1.5–2% of nucleotide (nt) bases in the human genome are transcribed into genes that code for proteins (Wolfsberg et al., 2001; The ENCODE Project Consortium, 2007), which has been the main research emphasis for genetic research for the last several decades. With the advancement of modern technologies, including RNA-Seq and microarray technology, it is clear that new information on genomes, transcripts, and their regulation will increase exponentially in the coming decades (Schulz et al., 2008; Zerbino et al., 2012). How to manage, mine, and comprehend these large data will be a real challenge. Meantime the reward can be high and the effort may be well justifiable, considering the likely impact the new knowledge will have on disease research and treatment.

A prominent member of ncRNA in brain function and psychiatric disorder is microRNA (miRNA), which is believed to be the transcripts of 1–3% of the human genome. miRNA is highly involved in brain development and plasticity at the neuronal level (Kapsimali et al., 2007; Choi et al., 2008; Cheng et al., 2009; Liu et al., 2010; Shi et al., 2010; also see reviews by Saba and Schratt, 2010; Konopka et al., 2011; Im and Kenny, 2012). This ncRNA regulates gene expression through RNA interference, RNA degradation, DNA methylation, and chromatin remodeling. As reviewed by two articles in this special issue from Kenny and Mains groups, recent discoveries of miRNA function and mechanisms in cocaine addiction have demonstrated important roles of ncRNA in this psychiatric disorder. For example, in the dorsal striatum, miR-212 was upregulated in rats addicted to cocaine and this upregulation enhances miR-212 regulated CREB signaling and counters the motivational property of cocaine (Hollander et al., 2010). Further studies found this specific function of miR-212 involves its negative homeostatic interaction with transition of acquizition period of addiction to compulsive-like increase of craving of cocaine. The downstream signaling pathway is further revealed in the same study to be through the controlling of BDNF levels in dorsal striatum (Im et al., 2010). Corroborative evidence of cocaine induced various miRNA expression and their functional role in cocaine addiction have also emerged in more and more other laboratories, complementing many aspects of our understanding (Nudelman et al.,

2010; Schaefer et al., 2010; Chandrasekar and Dreyer, 2011; Eipper-Mains et al., 2011). Particularly remarkable in one of these studies is that, using RNA-Seq approaches, cocaine is found to both upregulate and downregulate specific miRNA or family of miRNA (Eipper-Mains et al., 2011). While similar findings have been reported earlier in the nervous system (Vo et al., 2005; Krol et al., 2010; Nudelman et al., 2010), this RNA-Seq study is the first comprehensive analysis of cocaine induced changes of miRNA, and has demonstrated the advantage of next generation sequencing technology over microarray technology, which is often weakened by cross hybridization and high background signal, and critically limited by its prerequisite of prior knowledge of ncRNA identity it analyzes.

A much less tapped area in ncRNA research for the understanding of addiction of cocaine, or other substances of abuse, is the lncRNA; Wapinski and Chang, 2011; Huang et al., 2012; Jeggari et al., 2012; Moran et al., 2012. lncRNA are those ncRNA that are longer than 200 nucleotides and are oftentimes transcripts of intergenic regions between transcription clusters, or "foci," in the genome. Some earlier projects identified around 35,000 long non-coding transcripts from estimated 10,000 distinct loci in mammalian genome, often bearing signatures of protein coding mRNA, such as 5′ capping, splicing, and poly adenylation, but have no apparent open reading frame (ORF) for protein translation (Carninci, 2005, 2006; Kapranov et al., 2010). Remarkably, many more lncRNA may exist but not recognized due to the fact that the majority of lncRNA transcripts may not be poly adenylated (Cheng et al., 2005; Carninci, 2006; Kapranov et al., 2010), while many of the transcripts detection approaches rely on first hybridizing the poly adenylated region. Functionally, lncRNA have been found to have roles in epigenetic regulation, imprinting, and X-chromosome inactivation, in addition to regulation of gene transcription and translation (Mercer et al., 2009; Wang and Chang, 2011; Wapinski and Chang, 2011; Harries, 2012; Huang et al., 2012; Moran et al., 2012). Consequently, lncRNA may play significant roles in cocaine addiction. Indeed, it is likely that future work will identified high numbers of lncRNA in brain areas of the putative learning-reward-addiction neural circuits such as nucleus accumbens, and will likely shed light on important involvement of lncRNA in these brain regions for reward and addiction. How genetic and epigenetic factors interact with these lncRNA, and how changes were brought upon these lncRNA by brain activities, in terms of lncRNA mutation, expression level, dynamics, and function, may provide significant insights on the mechanisms of cocaine addiction. Because substance abuse and dependence involves substantial gene-environment interactions and epigenetic factors, the capability of RNA-Seq in detecting novel gene variations and *de novo* gene mutations also provides valuable and necessary tools for the addiction research field.

The complexity and abundance of ncRNA, especially lncRNA, mandate advanced non-traditional, large scale and high throughput transcript sequencing, and analysis. While microarray technology enjoys established base and proven utility, the next generation sequencing technology represented by RNA-Seq emerges as the future choice approach for the task because of its unbiased coverage of the entire transcriptome of the genome. In addition, RNA-Seq allows versatile experimental design for sequencing depth and adjustable detection sensitivity, using machines of different throughputs and multiplexing different numbers of samples on a sequencing lane. Furthermore, the inherited benefit of RNA-Seq (Mane et al., 2009; Bradford et al., 2010; Chen et al., 2011; Eipper-Mains et al., 2011; also see reviews in this issue), including low background noise, low error rate and low technical variance, the high accuracy of the digital nature, and the capability of detecting novel transcripts and alternative splicing forms, will definitely benefit not only ncRNA analysis, but all other forms of RNA research as well. All these will greatly enable researchers in the field of substance abuse and dependence for future discoveries and breakthroughs.

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Global approaches to the role of miRNAs in drug-induced changes in gene expression

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Neurons modulate gene expression with subcellular precision through excitation-coupled local protein synthesis, a process that is regulated in part through the involvement of microRNAs (miRNAs), a class of small non-coding RNAs. The biosynthesis of miRNAs is reviewed, with special emphasis on miRNA families, the subcellular localization of specific miRNAs in neurons, and their potential roles in the response to drugs of abuse. For over a decade, DNA microarrays have dominated genome-wide gene expression studies, revealing widespread effects of drug exposure on neuronal gene expression. We review a number of recent studies that explore the emerging role of miRNAs in the biochemical and behavioral responses to cocaine. The more powerful next-generation sequencing technology offers certain advantages and is supplanting microarrays for the analysis of complex transcriptomes. Next-generation sequencing is unparalleled in its ability to identify and quantify low-abundance transcripts without prior sequence knowledge, facilitating the accurate detection and quantification of miRNAs expressed in total tissue and miRNAs localized to postsynaptic densities (PSDs). We previously identified cocaine-responsive miRNAs, synaptically enriched and depleted miRNA families, and confirmed cocaine-induced changes in protein expression for several bioinformatically predicted target genes. The *miR-8* family was found to be highly enriched and cocaine-regulated at the PSD, where its members may modulate expression of cell adhesion molecules. An integrative approach that combines mRNA, miRNA, and protein expression profiling in combination with focused single gene studies and innovative behavioral paradigms should facilitate the development of more effective therapeutic approaches to treat addiction.

Keywords: cocaine, RNA-Seq, postsynaptic density, cell adhesion, *miR-8*, microRNAs, synaptic plasticity

miRNAs – ESSENTIAL BACKGROUND

microRNAs (miRNAs) are a class of endogenous 21–25 nucleotide small RNAs that modulate gene expression through binding to complementary sequences in the 3′-untranslated regions (3′-UTRs) of target mRNAs (Kim et al., 2009). Based on their sequences, the 672 annotated mouse miRNAs (http://www.mirbase.org) can be grouped into 253 families; there are over 1000 miRNAs in humans, and several human miRNAs not yet officially recognized in the mouse genome have been identified (Henry et al., 2011). Family members share a common seven to eight nucleotide (nt) seed sequence at their 5′-end and are thought to interact with the same target genes; the sequences of the five members of the miR-8 family are shown in **Figure 1**. Although miRNAs have only been intensively studied for the last decade, their role in regulating gene expression is now widely accepted. One to 3% of the genome is devoted to miRNAs, which are typically found in clusters in intergenic regions (Mongroo and Rustgi, 2010; Henry et al., 2011; Law and Wong, 2011). About a quarter of the known miRNAs occur within the introns of genes encoding proteins (hence "mirtrons"), in which case expression of the miRNAs is controlled by expression of the "host" gene (Mongroo and Rustgi, 2010; Law and Wong, 2011). Hundreds of miRNAs are expressed in the mature mammalian brain (Lagos-Quintana et al., 2002; Krichevsky et al., 2003; Miska et al., 2004; Landgraf et al., 2007), where they are involved in the control of synapse development and neuronal plasticity (Banerjee et al., 2009; Schratt, 2009b; Siegel et al., 2011). The binding of multiple miRNAs to sites in the 3′-UTR of a target gene can repress translation or cause mRNA degradation (**Figure 1**), with only a partial match required to alter translation. MicroRNAs are thought to target more than half of mRNAs encoded in the genome, with any one miRNA binding up to several thousand target mRNAs. Since any given target mRNA may have binding sites for over a 100 miRNAs, a consortium of miRNAs work together to regulate the translational rate and stability of that target mRNA (Mongroo and Rustgi, 2010; Henry et al., 2011; Law and Wong, 2011; Li and van der Vaart, 2011; Siegel et al., 2011). MicroRNAs mediate their effects on protein expression when packaged into the RNA-induced silencing complex? (RISC) along with several important proteins (**Figure 1**).

In the biogenesis of mature miRNAs, long primary miRNA (pri-miRNA) transcripts encoding a single miRNA or a cluster of miRNAs are first cleaved into shorter preliminary miRNA (pre-miRNA) hairpins by a complex of Drosha, an RNase III, and the cofactor DGCR8 (DiGeorge syndrome critical region gene 8;

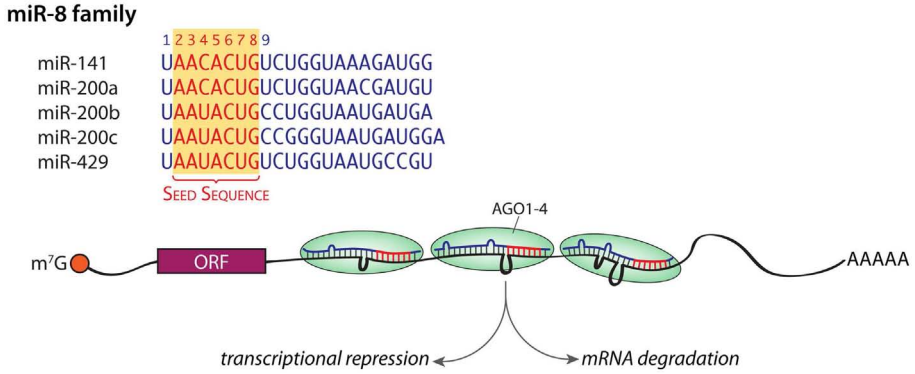

FIGURE 1 | miRNA families. The sequences of the five members of the miR-8 family are shown; the shared seed sequences are shown in red. An mRNA with RISC bound to three different loci in its 3′-UTR is shown; the net effect of these interactions can be diminished translation or mRNA degradation. m^{7}G, 7-methylguanosine in the 5′-cap; orange ball, cap-binding complex; ORF, open reading frame; AAAAA, poly A tail. Mismatches are shown as loops.

Figure 2A). About half of mammalian miRNA loci encode multiple miRNAs and are transcribed as a polycistronic transcript (Kim et al., 2009); 65 miRNA clusters encompass 45% (300) of all miRNA genes scattered throughout the mouse genome. Once cleaved from the pri-miRNA, the ∼70 nucleotide pre-miRNA is exported from the nucleus by Exportin-5. The pre-miRNA is then cleaved by Dicer, another RNase III enzyme, in complex with TRBP (HIV transactivating response RNA-binding protein or TARBP2) to make an approximately 22 nt duplex RNA. One strand of the double stranded intermediate (miRNA/miRNA* duplex) is then loaded into an Argonaute protein to form the RISC complex (Kim et al., 2009; Ender and Meister, 2010; Esteller, 2011; **Figure 2A**). The red sequence represents the mature or "guide" miRNA while the blue sequence is the "star" or "passenger" strand, which is often degraded when not loaded into a RISC. The mature miRNA/RISC targets the 3′-UTR of target mRNAs and regulates protein translation from or stability of the target mRNAs (Guo et al., 2010; Mongroo and Rustgi, 2010; Law and Wong, 2011).

Each fully assembled RISC contains an Argonaute protein (AGO1-4 in human and mouse), an enzyme similar to RNase H (Song et al., 2004), which cleaves the RNA strand of RNA-DNA hybrids. RISC also includes TRBP, the RNA helicase MOV10, a single mature miRNA, and several additional proteins (Höck and Meister, 2008; Banerjee et al., 2009; Vo et al., 2010; De and MacRae, 2011). The RISC interacts with target mRNAs via complementary base pairing interactions specified by the miRNA sequence (**Figure 2B**). AGO2 is the only catalytically active AGO family member in humans and mice; it has endonuclease ("slicer") activity and the ability to cleave target mRNAs (Kim et al., 2009). Mutant, catalytically inactive AGO2 silences translation as well as active AGO2 (Broderick et al., 2011).

miRNAs AND THEIR EMERGING ROLE IN ALCOHOL AND DRUG ABUSE

miRNAs likely participate in long-lasting forms of synaptic plasticity through the modulation of regulatory pathways that involve controlled dendritic mRNA trafficking, excitation-coupled modulation of synaptic mRNA translation, alterations to the actin cytoskeleton, neurotransmitter metabolism, and peptide hormone processing (Schratt, 2009a). They play an important role in the local control of dendritic morphology through fine-tuning translation of synaptically localized mRNAs (Schratt, 2009b). miRNAs are implicated in the pathophysiology of a variety of neuropsychiatric disorders and mental retardation syndromes, including Alzheimer's disease, Parkinson's disease (Conn et al., 2005), Huntington's disease (Martí et al., 2010), schizophrenia (Beveridge et al., 2010; Santarelli et al., 2011), bipolar disorder (Moreau et al., 2011), alcoholism (Lewohl et al., 2011), Fragile X mental retardation (Li and Jin, 2009), and Rett syndrome (Urdinguio et al., 2010; Wu et al., 2010).

A particularly intriguing example of the coordinated manner in which miRNAs alter function comes from an analysis of the effects of alcohol on expression of *miR-9*, the most prevalent miRNA in the nucleus accumbens (NAc; Pietrzykowski et al., 2008; Treistman and Martin, 2009; Eipper-Mains et al., 2011). Expression of the BK channel, a large conductance Ca^{2+} and voltage-activated K^+ channel that plays a major role in neuronal excitability, responds to alcohol in a region-specific manner and is known to play a key role in alcohol tolerance. Exposure of striatal cultures to alcohol results in a rapid (15 min) decrease in BK mRNA levels, with loss of specific splice variants. This is due to an alcohol-induced increase in *miR-9* expression, which results in degradation of BK splice variants that contain a *miR-9* binding site in their 3′-UTR (Pietrzykowski et al., 2008; Treistman and Martin, 2009). The remaining BK variants are those least affected by the presence of alcohol.

Several recent studies have investigated the role of miRNAs and AGO2 in biochemical and behavioral responses to cocaine (Chandrasekar and Dreyer, 2009, 2011; Hollander et al., 2010; Im et al., 2010; Schaefer et al., 2010; Saba et al., 2012). Using qPCR and *in situ* analysis of bioinformatically determined miRNAs, *let-7d*, *miR-124*, and *miR-181a* were identified as cocaine-regulated in rats (Chandrasekar and Dreyer, 2009, 2011). Brain derived neurotrophic factor (BDNF) expression declined in response to expression of *miR-124* while expression of *let-7d* diminished expression of a dopamine receptor (*Drd3*). Using lentiviral vectors to over-express

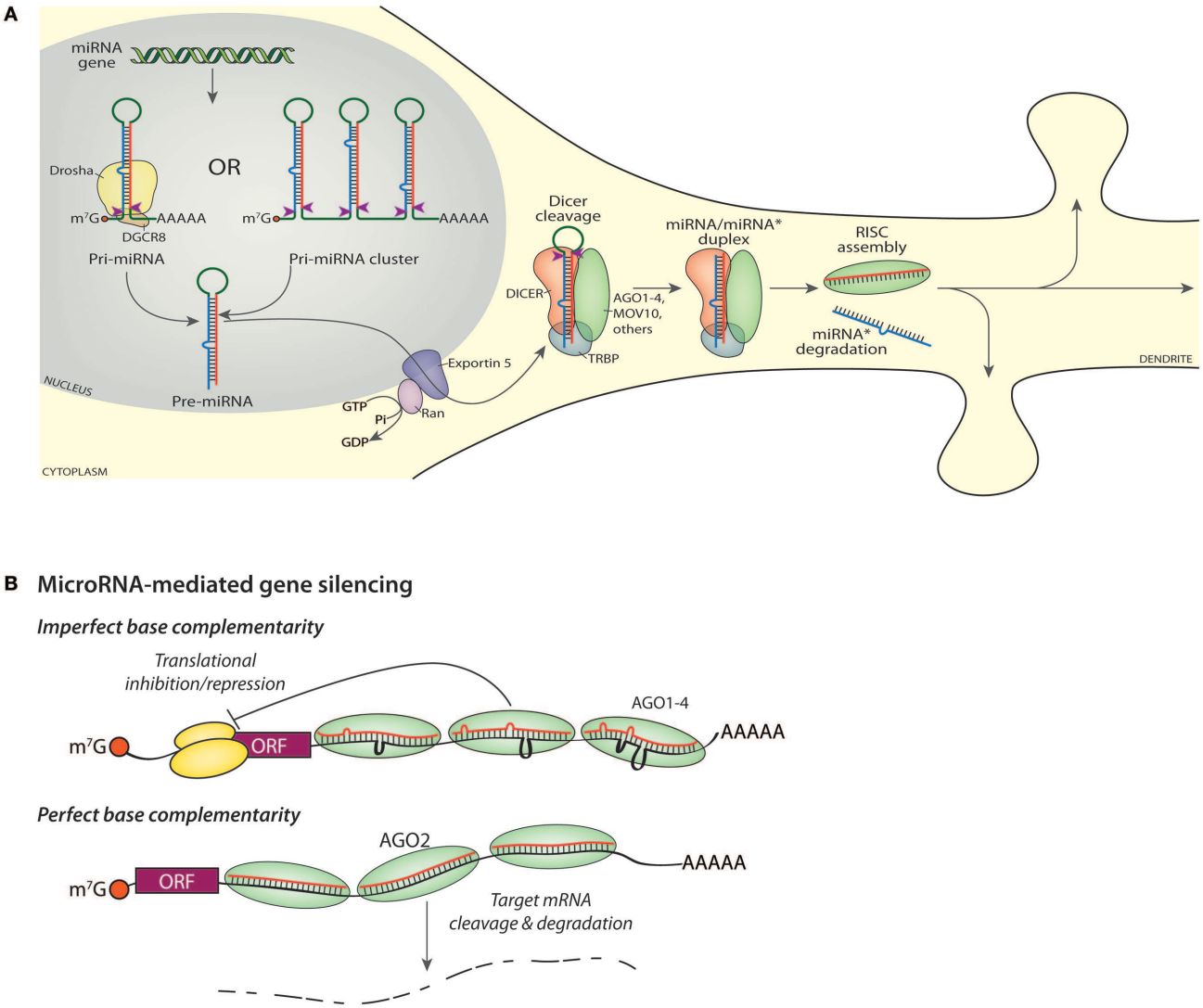

FIGURE 2 | miRNA biogenesis and gene silencing. (A) Transcription of a miRNA gene yields a capped, polyadenylated Pri-miRNA shown undergoing cleavage (red arrowheads) by Drosha (top, left). Transcription of a miRNA cluster yields a transcript encoding multiple miRNAs (top, right), each of which must be cleaved into a Pre-miRNA by Drosha. The mature or "guide" miRNA is shown in red, with the "star" or "passenger" strand shown in blue. Nuclear export of the Pre-miRNA requires Exportin-5. Cleavage of the Pre-miRNA by DICER (red arrowheads) generates a mature guide and star strand duplex; cleavage occurs in a protein complex that includes DICER, an Argonaute protein (AGO1-4), MOV10, TRBP, and additional proteins such as FMR1, DDX9, PUM2 (Schratt, 2009b; Czech and Hannon, 2011; De and MacRae, 2011). The star strand is lost from the final RISC and ultimately degraded, while the assembled RISC is available to bind to cognate RNAs and modulate protein expression. **(B)** *Imperfect base complementarity.* When the sequence of the miRNA guide strand does not perfectly match the target mRNA, translation of that mRNA is inhibited. Bound RISCs may contain any one of the four Argonaute proteins. The orange ball is the cap-binding complex; the large and small ribosomal subunits are shown in yellow. *Perfect base complementarity.* When the sequence of the miRNA guide strand matches the target mRNA perfectly, the target mRNA is sliced by AGO2.

or silence these same miRNAs in the NAc, their role in the ability of rats to exhibit conditioned place preference for cocaine was demonstrated (Chandrasekar and Dreyer, 2009, 2011). Expression of the GluA2 subunit of the AMPA receptor is diminished by the binding of *miR-181a* to a site in its 3′-UTR (Saba et al., 2012).

Two studies by the Kenny group demonstrated induction of *miR-132* and *miR-212* in dorsal striatum after 7 days of cocaine self-administration in rats and implicate *miR-212* in the behavioral and motivational response to cocaine through CREB, MeCP2, and BDNF signaling (Hollander et al., 2010; Im et al., 2010). In addition, Schaefer et al. (2010) identified an overlapping subset of cocaine-induced and AGO2-knockdown-depleted miRNAs in the NAc neurons that express the *Drd2* dopamine receptor. Depletion of AGO2 from *Drd2*-expressing neurons resulted in reduced cocaine self-administration. In a different study, array screens were used to identify 32 miRNAs whose expression increased in a similar manner in multiple regions of the mouse brain in response to repeated injections of nicotine, cocaine, or amphetamine (Lippi

et al., 2011); when these miRNAs were compared to miRNAs up-regulated at the time of synaptogenesis, the *miR-29a/b* and *miR-182/183* clusters were singled out. The ability of *miR-29a/b* to reduce mushroom spine formation in primary hippocampal neurons was then associated with its ability to diminish the expression of *Arpc3*, a component of the ARP2/3 actin nucleation complex (Lippi et al., 2011).

In addition to miRNA array analyses of postmortem brains from human alcoholics and controls (Lewohl et al., 2011), arrays have been used to identify subsets of miRNAs affected by alcohol treatment of primary mouse neuronal cultures and human neuroblastoma cell lines (Yadav et al., 2011; Guo et al., 2012). Similarly, studies targeted at specific miRNAs have been used to study the actions of morphine and other opiates in model systems such as primary neuronal cultures, neuronal cell lines, and developing zebrafish embryos (Wu et al., 2009; He et al., 2010; Sanchez-Simon et al., 2010; Zheng et al., 2010).

Together, these studies demonstrate that miRNA-mediated gene regulation plays an important role in the complex effects that chronic exposure to cocaine or other drugs of abuse have on the nervous system. However, each of these studies was targeted to a small number of candidate miRNAs or used microarrays, which are limited by cross-hybridization, high background signal, low dynamic range, and the inability to identify unknown miRNAs (Metzker, 2010). Newly developed next-generation sequencing approaches provide a more complete picture of drug-induced changes in both mRNA and miRNA expression.

SYSTEM-WIDE ANALYSIS OF GENE EXPRESSION: THE ADVANTAGES OF SEQUENCING

DNA microarrays have dominated genome-wide studies of gene expression for more than a decade, but next-generation sequencing methods are rapidly becoming the method of choice for the analysis of complex transcriptomes (Graveley, 2008). Microarrays have been instrumental in the interrogation and profiling of DNA-protein interactions, identification of single-nucleotide polymorphisms, and comparative analyses of mRNA expression (Shendure, 2008). However, microarrays are limited in a number of ways that next-generation sequencing is not. Since microarrays rely on base complementarity between the probe and the mRNA or cDNA, the technology is prone to artifacts of cross-hybridization of highly homologous genes or isoforms (Metzker, 2010). Microarrays contain a pre-defined set of oligonucleotide probes designed using existing gene annotations for organisms with known genome sequences (Graveley, 2008), whereas sequencing facilitates the identification and absolute quantification of low-abundance transcripts without prior knowledge of the sequence (Cloonan et al., 2008; Mortazavi et al., 2008; Wang et al., 2009b; Metzker, 2010). The sequencing approach has a low background because of the unambiguous nature of sequence mapping and has increased sensitivity for low-abundance transcripts (Graveley, 2008; Wang et al., 2009b). Additionally, sequencing enables the detection and investigation of alternative splicing (Cloonan et al., 2008; Mortazavi et al., 2008; Wang et al., 2008a), imprinting and allele specific expression (Wang et al., 2008b), sequence variation (Wang et al., 2009b), and RNA editing (Wahlstedt et al., 2009) much more easily and robustly than microarrays. Finally, microarrays provide far less data than current next-generation sequencing technologies, which can produce gigabases (10^9 nucleotides) of sequence from a single experiment, enabling detection of very minor transcripts (Graveley, 2008).

DNA sequencing began in earnest in 1975 (Maxam and Gilbert, 1977; Sanger et al., 1977), but the first truly high-throughput sequencing device was not introduced until the 1990s by Lynx Therapeutics. Massively parallel signature sequencing of mRNAs employed microbead arrays and involved *in vitro* cloning of cDNA templates on a monolayer of slide-fixed microscopic beads, producing "signature" sequences ranging from 16 to 20 nucleotides in length (Brenner et al., 2000). In 2005, two new methods of next-generation sequencing were introduced: pyrosequencing, or "sequencing-by-synthesis," which detects pyrophosphate release on nucleotide incorporation (Margulies et al., 2005), and multiplex "sequencing-by-ligation" of mate-paired polymerase colonies ("polonies"; Shendure et al., 2005). In 2006, Solexa introduced the Genome Analyzer, which provided up to 1 gigabase (Gb) of sequence in a single sequencing run, and in 2007, Illumina acquired Solexa, and its sequencers can generate up to 600 Gb of sequence per run (Illumina, 2011). Rapid sequencing research has exploded, as evidenced by the exponential increase in publications in the past few years, increasing from 20 papers in 2005 to nearly 1600 papers in 2011.

Next-generation sequencing of RNA requires the preparation of libraries (**Figure 3**); 1 mg of total RNA is sufficient to purify the small RNA population and prepare a library for miRNA sequencing. For sequencing of miRNAs, total RNA from tissue (such as NAc) or a subcellular fraction (such as postsynaptic densities, PSD) is subjected to size selection; gel purification can be used to isolate 18–35 nt RNAs. Adaptors are ligated to the 5′ and then 3′ ends, and then the RNAs are reverse transcribed and amplified by polymerase chain reaction (PCR). PCR products are then gel purified and sequenced for 40–50 cycles. The adaptor sequences are trimmed from the reads, and data are then aligned with annotated miRNAs (for example, from miRBase).

High-throughput sequencing of RNA is an extremely powerful tool, but it is not without limitations. The density of sequence reads varies along the length of a transcript, indicating the existence of sequence bias at some point during the library preparation or sequencing process (Fu et al., 2009; Oshlack and Wakefield, 2009). There are data showing an under-representation of AT-rich and GC-rich regions in Illumina RNA-Seq data, which is likely attributable to sequence bias during the reverse transcription and amplification steps of library preparation (Hansen et al., 2010; Levin et al., 2010; Metzker, 2010). Strand-specific sequencing, in which the sequencing adapters are ligated to the RNA fragment prior to amplification, may show a more even distribution of aligning reads along the length of the gene; this method, however, requires additional manipulation of highly labile RNA prior to its conversion to more stable cDNA (Ozsolak and Milos, 2011). Other difficulties of traditional RNA-Seq include issues of quantifying very low-abundance transcripts, problems with profiling repetitive regions of the genome, and trouble in quantifying small RNAs and RNA with a short half-life (Ozsolak and Milos, 2011). Next–next-generation (third generation) sequencing, in which single molecules are sequenced without prior amplification, is

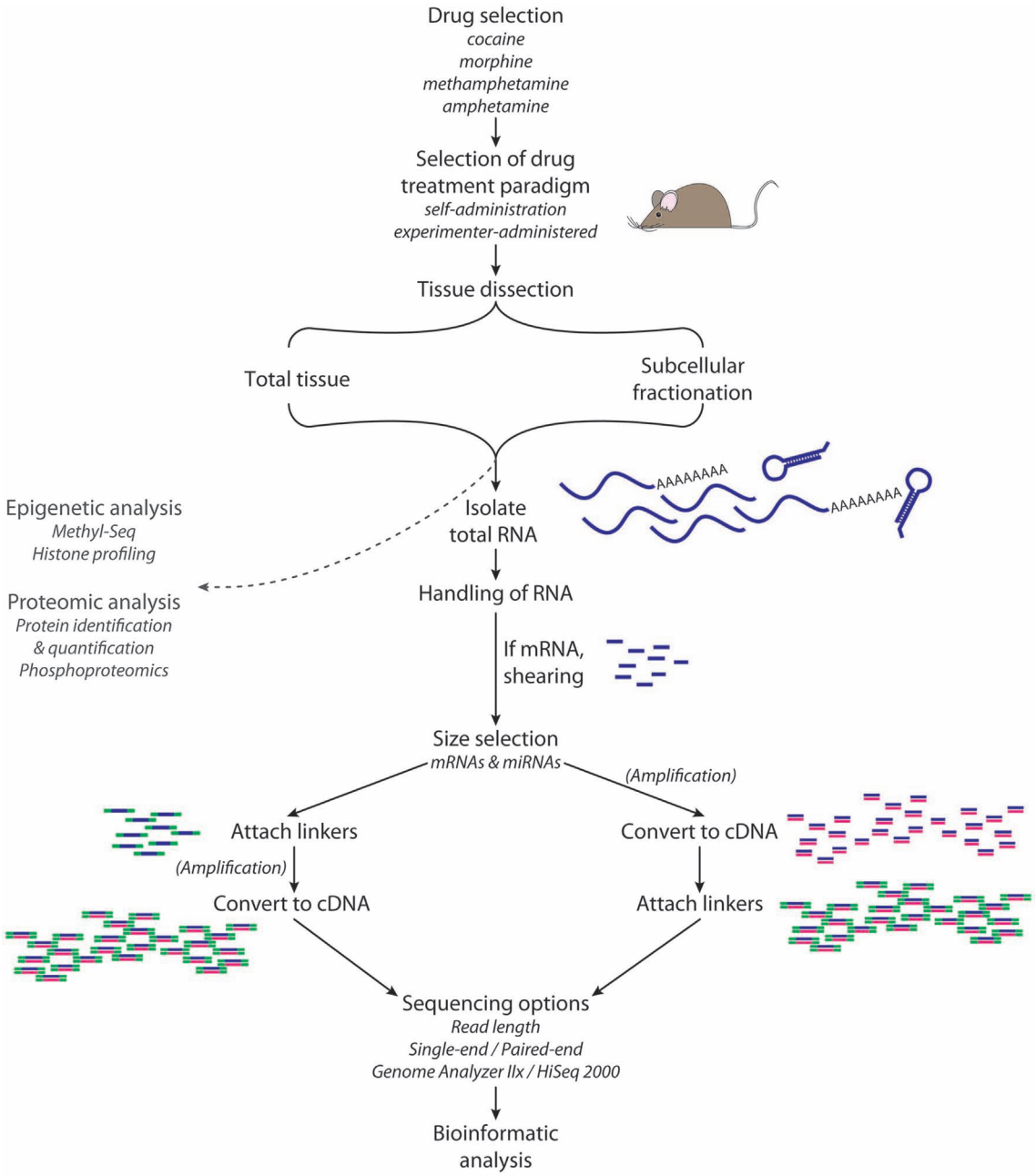

FIGURE 3 | RNA-Seq. High-throughput sequence analysis requires isolation of high quality RNA from the tissue, cell type, or subcellular organelle of interest. For the analysis of miRNAs, a size selection step yields RNA of the appropriate size (e.g., 18–35 nt); for the analysis of mRNAs, transcripts of the desired size are isolated and then sheared. A linker is attached to the 5′-end of each RNA fragment; a different linker is then attached to the 3′-end. Reverse transcription followed by PCR yields sufficient material for analysis. Adaptor sequences are computationally removed from the sequencing data, and then sequences are aligned with annotated miRNAs.

considered the future of the sequencing field (Ozsolak and Milos, 2011; Hayden, 2012).

Challenges accompany the rapidly improving technology and accumulation of datasets containing vast amounts of information. Many biologists struggle to make scientific sense of the data at hand, while many computer scientists are not equipped with the biology training to tackle the relevant questions on their own. A growing number of tools are available to assist in the analysis of high-throughput datasets, including packages for sequence alignment (Langmead et al., 2009), *de novo* genome and transcriptome assembly (Trapnell et al., 2010), analysis of alternative splicing (Trapnell et al., 2009), functional category assessment, and identification of signaling pathway enrichment (Kanehisa and Goto, 2000; Dennis et al., 2003; Thomas et al., 2003; Li et al., 2008). At

present, few "user friendly" and fully customizable packages exist for the comprehensive analysis of sequencing data.

PICKING THE SYSTEM TO STUDY

Abundant evidence, from both animals and humans, identifies the limbic system as the convergence point for the actions of all drugs of abuse (Koob and Le Moal, 2001; Nestler, 2001, 2005b; Di Chiara, 2002; Volkow et al., 2004; Wise, 2004). The mesocorticolimbic dopamine pathway, which involves a subset of all limbic structures, is activated by normal physiologic rewards such as food, drink, social interaction, and sex (Wise, 1996, 2002; Nestler, 2005a). This system includes dopaminergic projections from the ventral tegmental area (VTA) to the NAc and prefrontal cortex (PFC) as well as glutamatergic projections from the PFC to the NAc (Kauer and Malenka, 2007; **Figure 4A**). Although drugs of abuse originate from a diverse array of chemical classes and have a variety of different primary targets, their effects all ultimately converge on the dopamine neurons of the VTA (Wolf, 2006). Other neurotransmitters, including γ-aminobutyric acid (GABA), opioid peptides, serotonin, acetylcholine, and endogenous cannabinoids, are also involved in the signaling of addiction (Spangler et al., 1996; Hyman and Malenka, 2001; Ross and Peselow, 2009). The convergence of these myriad signaling systems has led many to hypothesize that the NAc is the key integration point in the rewarding effects seen with drugs of abuse (Nestler and Aghajanian, 1997; Berke and Hyman, 2000; Kalivas et al., 2005; Nestler, 2005b; Hyman et al., 2006).

Neurons modulate gene expression with subcellular precision; polyribosomes and mRNAs are found at the base of dendritic spines and are involved in localized protein synthesis (Rao and Steward, 1991; Kiebler and DesGroseillers, 2000). Individual synapses may act independently in the local control of excitation-coupled protein translation (Schratt, 2009b; Zukin et al., 2009). The morphological changes that occur in dendritic spines as a result of synaptic activity cannot occur without *de novo* protein synthesis, and a blockade of protein synthesis blunts formation of long-term memory (Schratt, 2009b). Modulating synthesis of selected synaptic proteins gives neurons the ability to fine-tune and integrate extracellular cues at single dendrites or at individual, specified synapses (Wang et al., 2009a). Subcellular fractionation is an important technique that enables the differentiation of events occurring at the synapse from those occurring in the cell soma. Despite the complex interactions between presynaptic endings and their target receptors on dendritic spines (**Figure 4B**), PSD-enriched fractions can be prepared and allow identification of postsynaptically enriched components.

miRNAs are known to play a role in modulating localized translation of mRNAs in dendrites (Kim et al., 2004). Localization of the miRNA processing enzyme DICER and the RISC component AGO2 to the PSD in mouse cortical and hippocampal slices by electron microscopy supports the possibility of local regulation (Lugli et al., 2005). Further studies identified the RISC component and RNA helicase MOV10 (homologous to Drosophila Armitage) at the synapse and established a role for RNA-induced silencing in the control of synaptic gene expression, learning, and memory (Ashraf et al., 2006; Banerjee et al., 2009; Vo et al., 2010). Through its effects on LIMK1, *miR-134*, which is synapto-dendritically localized, plays a role in the activity-dependent regulation of spine size (Schratt et al., 2006; Siegel et al., 2011). Similarly, *miR-132* and *miR-138* play a role in dendritic remodeling and synaptogenesis (Wayman et al., 2008; Siegel et al., 2009).

Sensitization is the progressive and persistent amplification of behavioral and motivational responses to a fixed dose of drug (Berke and Hyman, 2000; Hyman et al., 2006). It persists for weeks, months, and years after cessation of drug taking and is thought to play an important role in the risk of a reformed addict for relapse to drug taking behavior (Paulson et al., 1991; Castner and Goldman-Rakic, 1999; Robinson and Berridge, 2001). We therefore exposed adult male mice to a treatment paradigm (eight daily intraperitoneal injections of cocaine or saline) that reliably yielded locomotor sensitization before harvesting tissue for library preparation (**Figure 4C**). We harvested NAc 24 h after the final injection of saline or cocaine and constructed small miRNA-Seq libraries for miRNA analysis (Eipper-Mains et al., 2011).

In order to identify miRNAs localized to synapses, we prepared another cohort of saline-injected and cocaine sensitized mice; 24 h after the final injection, we purified PSD from their striata and isolated the total RNA associated with this fraction (**Figure 4B**; Eipper-Mains et al., 2011). While NAc was used to analyze the total miRNA population, striatum was used to ensure availability of sufficient PSD RNA. Sequencing libraries were prepared and analyzed as outlined in **Figure 3** (Eipper-Mains et al., 2011). As methods for library preparation improve, it will be possible to work with RNA purified from even smaller brain regions.

Sequence analysis identifies all of the RNAs present in a given sample (**Figure 5**). While miRNAs accounted for over 70% of the reads in the NAc libraries, they accounted for slightly under half of the reads in the PSD libraries. Sequences mapping to rRNAs and tRNAs were about five-times more prevalent in the PSD libraries than in the NAc libraries. Sequences that mapped to the transcriptome accounted for about 15% of the reads in both types of sample and most likely represent products of mRNA degradation isolated during size selection. Although minor components, snoRNAs, snRNAs, and SINE (short interspersed element) are present and their sequences can also be analyzed.

miRNA-Seq analysis identifies the exact miRNA species present (**Figure 6**). As shown in **Figure 2**, the opposite ends of each miRNA are generated by Drosha and DICER. While the major *miR-375* sequence identified corresponded to that predicted, multiple *miR-182* and *miR-212-3p* species (isomirs) were found in all four libraries; of note, levels of both *miR-182* and *miR-212-3p* had previously been identified as cocaine-responsive (Hollander et al., 2010; Im et al., 2010; Lippi et al., 2011). This heterogeneity has important consequences when comparing miRNA-Seq data with microarray and qPCR data, since miRNA-Seq can unambiguously identify many sequences as belonging to one miRNA (e.g., *miR-212-3p*), while qPCR and microarrays will miss most of the variants. For miRNAs with a single major species which conforms to the accepted annotation (e.g., *miR-375*), the agreement across methods should be much better, enabling firm comparisons of RNA-Seq with qPCR data (Eipper-Mains et al., 2011). Tools for analysis include websites such as http://www.microrna.org and http://www.targetscan.org.

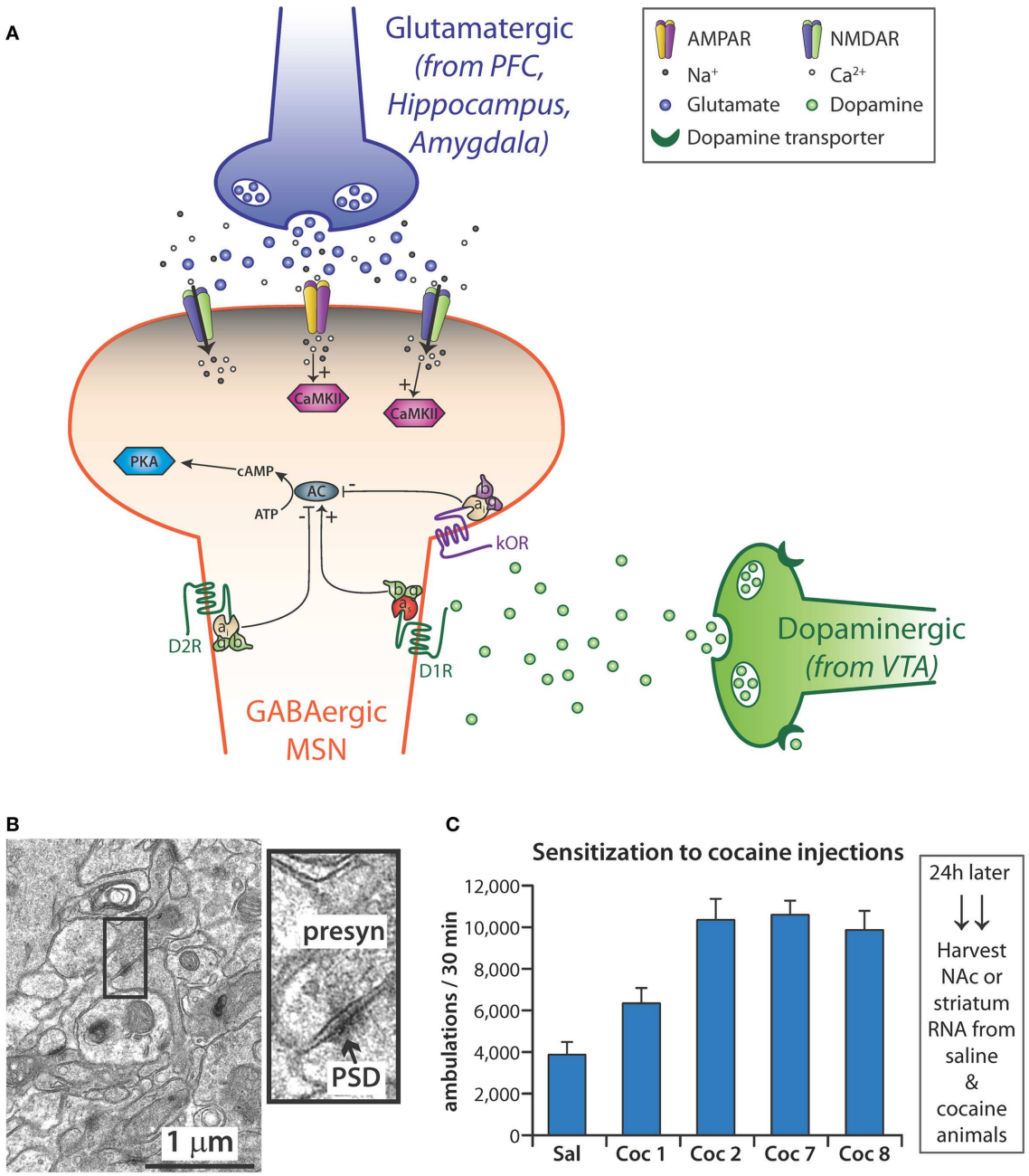

FIGURE 4 | (A) Medium spiny neurons (MSN), which use γ-NH_2-butyric acid (GABA) as their major transmitter, are the primary cell type in the nucleus accumbens (NAc). Some MSNs express mostly the D1 dopamine receptor while others express primarily the D2 dopamine receptor. Dopamine released from the endings of dopaminergic neurons in the ventral tegmental area (VTA) signals through both types of receptor, affecting the activation of adenylate cyclase (AC); reuptake of dopamine by the dopamine transporter (DAT) is blocked by cocaine. MSNs receive excitatory inputs from glutamatergic neurons in the prefrontal cortex (PFC), hippocampus, and amygdala. Activation of AMPA and NMDA receptors localized to the spine-studded dendrites of MSNs leads to the influx of Ca^{2+} and activation of CaMKII. In addition, MSNs are responsive to various neuropeptides, which bind to G-protein coupled receptors such as the κ-opioid receptor (κOR). **(B)** Standard procedures have been developed to purify postsynaptic densities (PSDs) from tissue homogenates; miRNAs enriched at the PSD are thought to allow local regulation of mRNA translation and stability. The electron micrograph (7 week mouse hippocampus) illustrates the complex system from which PSDs must be purified: presynaptic terminals can be recognized by their content of synaptic vesicles; PSDs are closely apposed to the presynaptic endings. **(C)** Sensitization to the locomotor stimulating effects of cocaine requires repeated exposure to the drug. Adult mice typically are typically given 10–20 mg cocaine/kg; locomotor activity (ambulations) is monitored for the next 30 min in an activity chamber with infrared light sensors. "Sal" is the average locomotor data for all mice given saline initially and the mice given saline throughout; Coc1 are data for mice give 10 mg/kg cocaine on day 1; Coc2 and Coc7 are data from days 2 and 7 for mice given 20 mg/kg cocaine daily for days 2–7; Coc8 are the data for the cocaine treated mice given 10 mg/kg on day 8; substantial locomotor sensitization is apparent compared to Coc1. Tissue harvested soon after drug administration allows analysis of the immediate response, while tissue harvested 24 h after final drug administration allows evaluation of the steady state response. $N = 10$ each for saline and cocaine.

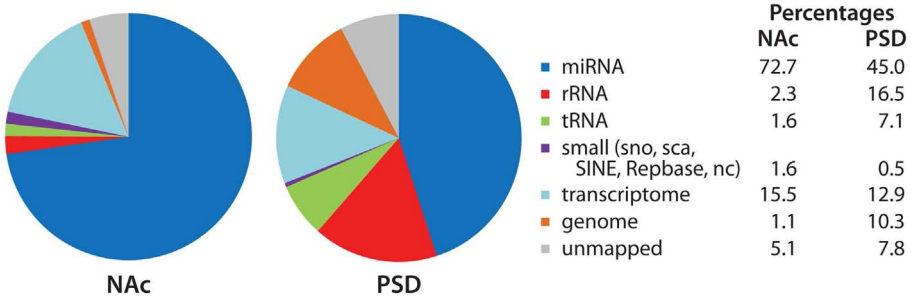

FIGURE 5 | Identity of mapped miRNA sequences in NAc and PSD libraries. Adaptor sequences were trimmed from the extracted sequence data. Bowtie (version 0.12.7; Langmead et al., 2009) was used to align sequences 18–30 nt in length to the miRBase annotated mouse microRNA database (version 16; Griffiths-Jones et al., 2006, 2008); perfect alignment was required. The Rfam, Repbase, and NON-CODE databases were used to identify sequences that aligned with annotated non-coding and repetitive elements. Sequences were then aligned to the mouse transcriptome (exons, introns, exon–exon junctions) and mouse genome. For non-miRNA alignments, 1-mismatch was allowed. Data (% total reads mapped to each category) for the NAc and PSD libraries from saline-injected animals are shown.

GLOBAL ANALYSIS REVEALS ROLE FOR miRNA CLUSTERS AND FAMILIES

In a tour de force, Lagos-Quintana et al. (2002) cloned hundreds of miRNAs from the cortex, cerebellum, and midbrain of 18.5-week-old mice. One year later, microarray technology was used to study regulation of miRNAs during neuronal development (Krichevsky et al., 2003). We chose to use ultra-high-throughput sequencing of miRNAs with the overarching goal of attaining a more complete picture of the response of the brain to chronic cocaine exposure (Eipper-Mains et al., 2011). Previous genome-wide characterizations of drug-induced gene expression focused on mRNA transcripts rather than miRNAs, were carried out using microarray technology, and often looked at other regions of the brain using different experimental paradigms (McClung and Nestler, 2008). The data reviewed here represent the first look at striatal miRNA synaptic enrichment and cocaine-regulation by deep sequencing.

Eighteen of the synaptically enriched miRNAs identified in our analysis are members of genomic miRNA clusters. For both the total lysate and PSD samples, the expression patterns of the clustered miRNAs are quite similar across the entire cluster. Especially instructive examples of clusters include the *miR-8* family clusters on chromosomes 4 and 6 (*miR-429/200a/b* and *miR-141/200c*), the *let-7* family cluster on the X chromosome (*let-7f/miR-98*) and the clusters for *miR-1/133a* (chromosome 2), *miR-182/96/183* (chromosome 6), and *miR-216a/217* (chromosome 11; **Figure 7**). In contrast, little concordance is seen across the large *miR-379-410* cluster, in which only *miR-485* is synaptically enriched (**Figure 7**).

Lugli and colleagues used microarrays to identify the subcellular localization of mature and precursor miRNAs in adult (2-month-old) mouse cortex and hippocampus. They found that synaptically enriched miRNAs were primarily expressed in evolutionarily newer species (Lugli et al., 2008). We compared our synaptic enrichment ratios to this earlier data set, revealing significant overlap between the most synaptically enriched and synaptically depleted miRNAs in our striatal samples and the previous hippocampal and cortical samples (Eipper-Mains et al., 2011). Importantly, most of the *miR-8* family (*miR-200a/b/c/429*) are markedly enriched in both datasets. In addition, *miR-182* and *miR-183* are dramatically enriched in PSDs in both datasets. By contrast, *miRs-126*, *-143*, *-145*, *-150*, and *-451* are depleted in both synaptic datasets. A separate study used microarrays to identify synaptosomally enriched and depleted miRNAs from rat P15 total forebrain samples (Siegel et al., 2009). In a direct comparison, *miRs-219-5p*, *-21*, *-377*, *-98*, *-376b*, *-218*, *-7a/b*, and *-29a* are synaptically enriched in both datasets, and in both datasets *miRs-143*, *-145*, and *-150* are depleted. The remarkable extent of agreement between these three datasets, which were obtained using different experimental platforms to analyze synaptically enriched samples prepared in different ways from multiple brain regions taken from animals of different ages, may be emblematic of the preponderance of glutamatergic synapses and additional similar properties in all of these brain regions. Whether the identities and expression levels of miRNAs differ substantially from one synapse to the next, dictated by the local microenvironment, remains to be determined.

Particularly striking was the synaptic enrichment of members of several miRNA clusters (**Figure 7**). The *miR-8* family has been studied most extensively in relation to cancer pathophysiology and has been shown to inhibit the first step of cancer metastasis, the epithelial-mesenchymal transition (Inui et al., 2010). An expression atlas of miRNAs indicates enrichment of all members of the *miR-8* family, except *miR-429*, in endocrine glands and tissues, kidney, and the reproductive system (Landgraf et al., 2007). Expression of this family of miRNAs is thought to maintain the epithelial phenotype through direct targeting of ZEB1 and ZEB2, transcriptional repressors of the cell adhesion molecule ECAD (Inui et al., 2010). Members of the cadherin family of cell surface glycoproteins mediate calcium-dependent cell–cell adhesion and have critical roles in early brain development, axonal outgrowth, and synaptogenesis (Martinek and Gaul, 1997). ECAD is present at the synapse, and antibodies which block homophilic interactions between pre- and postsynaptic ECAD attenuate induction of long-term potentiation (Tang et al., 1998).

Members of the highly conserved *let-7* family of miRNAs, with almost a dozen members in the mammalian genome and key roles in development and cancer suppression (Roush and Slack, 2008; McCarty, 2012), are also highly enriched at the PSD. The *let-7* family is highly expressed in pituitary, hypothalamus, and

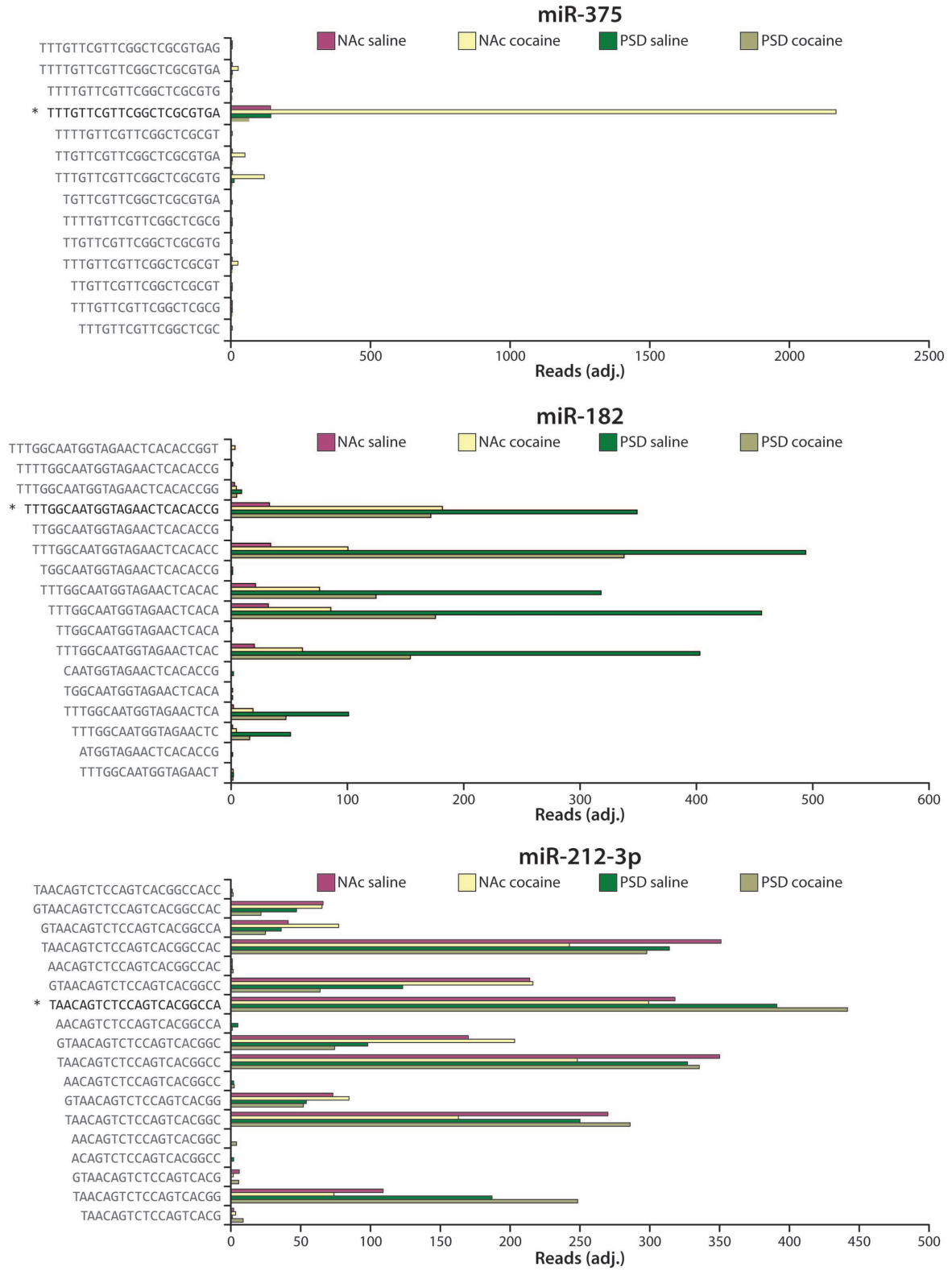

FIGURE 6 | Heterogeneity in miRNA sequence. Expression data for the four different libraries (NAc saline, NAc cocaine, PSD saline, PSD cocaine) are shown as reads (adjusted for equal total reads per sample) for sequences mapping to *miR-375* (top), *miR-182* (middle), and *miR-212-3p* (bottom). The arrowhead (➤) marks the miRBase (version 16) annotated mature miRNA sequence (Griffiths-Jones et al., 2008). Commercially available qPCR primers will only amplify the sequence marked by the arrowhead.

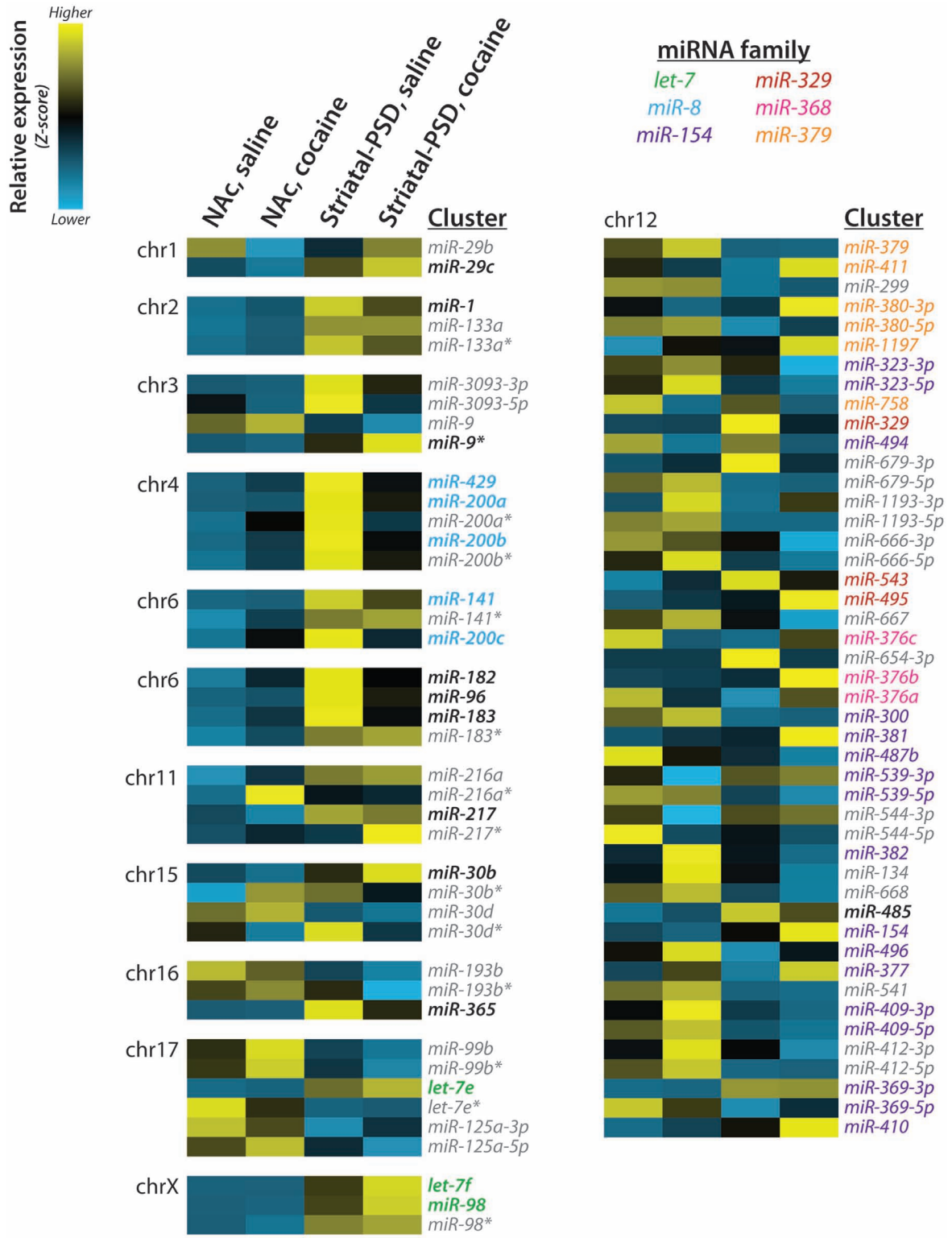

FIGURE 7 | miRNA clusters and families. Expression of striatal PSD-enriched miRNAs and other members of each genomic cluster is shown as a heat map; the chromosomal (chr) localization of each cluster is indicated. *Z*-scores were computed using normalized miRNA frequency across all samples: blue, low expression; yellow, high expression. miRNA families are indicated by text color; PSD-enriched miRNAs are indicated by bold; miRNAs not enriched at striatal PSDs and/or not belonging to the listed miR families are shown in gray italic. Reproduced from Eipper-Mains et al. (2011) with permission.

pancreatic islets (Landgraf et al., 2007), and its members play a major role in controlling insulin secretion in response to a glucose load and tissue responsiveness to insulin (Frost and Olson, 2011). The *let-7* family members localized at the PSD may participate in the control of vesicle trafficking and secretion, both of which play essential roles at the synapse. The *miR-183* family has not been studied as extensively, but clearly plays a crucial role in the development and function of sensory cells such as photoreceptors and hair cells (Li et al., 2010; Weston et al., 2011; Zhu et al., 2011).

RESPONSE TO COCAINE

Our finding that PSD levels of AGO2 protein rose in the NAc of mice sensitized to cocaine led to our analysis of the effects of cocaine on miRNA expression and localization (Eipper-Mains et al., 2011). One particularly intriguing pattern emerged from our data analysis: many of the miRNAs that were up-regulated in NAc tissue lysate after chronic cocaine were down-regulated at the synapse. Specifically, members of the *miR-8* family, which were very highly enriched in striatal PSDs, exhibited opposite changes in tissue lysate versus PSDs after cocaine.

As summarized above, several recent studies investigated miRNA regulation in the mesolimbic dopamine system after cocaine administration. The first used qPCR and *in situ* hybridization of specific bioinformatically identified miRNAs to identify three cocaine-regulated miRNAs in rats receiving cocaine for 15 days, *let-7d* (decreased in NAc), *miR-124* (decreased in NAc and dorsal striatum), and *miR-181a* (increased in NAc and dorsal striatum; Chandrasekar and Dreyer, 2009). Our data, from mice receiving cocaine for 7 days, indicate no cocaine-regulation of *let-7d* or *miR-124* and a modest increase in *miR-181a* in NAc lysates (Eipper-Mains et al., 2011). Two related studies identified induction of *miR-132* and *miR-212* in dorsal striatum after 7 days of cocaine self-administration in rats and implicate *miR-212* in the behavioral and motivational response to cocaine through CREB, MeCP2, and BDNF signaling (Hollander et al., 2010; Im et al., 2010). Our data support induction of *miR-132* in the NAc and a decrease in *miR-132* in striatal PSDs. *miR-212*, with 10 major isomirs identified using miRNA-Seq (**Figure 6**), exhibits a complex response to cocaine: tissue levels of three isomirs increase while tissue levels of five isomirs decrease and two are unchanged (Eipper-Mains et al., 2011). In the fourth study, miRNA microarrays were used to identify the overlapping subset of cocaine-induced and AGO2-knockdown-depleted miRNAs in Drd2-expressing neurons of the NAc (Schaefer et al., 2010). Direct comparison of our data to this study is not possible because the experimental paradigms differ so significantly; it is, however, interesting to note that ablation of AGO2 caused a marked reduction in the level of *miR-182* (Schaefer et al., 2010), identified in our data as cocaine-regulated and synaptically enriched. Future studies will need to address specific cell-types and subcellular localization of the entire complement of miRNAs, to determine more precisely how miRNAs are involved in the response of the brain to cocaine.

BIOINFORMATICALLY PREDICTED TARGETS

We used an established list (Suzuki et al., 2007) of PSD-enriched mRNAs to identify potential target genes for the 16 most cocaine-regulated synaptically localized miRNAs (Eipper-Mains et al., 2011). Bioinformatic analysis using miRanda yielded a mirSVR score for each gene. A striking feature of this list was the number of miRNA binding sites predicted for any given transcript. Transcripts encoding metadherin (*Mtdh*), which is also known as *AEG-1* or *Lyric*, had the most negative mirSVR score, with five sites for PSD-localized miRNAs down-regulated by cocaine and four sites for PSD-localized miRNAs up-regulated by cocaine; sites predicted for each of the PSD-enriched cocaine-regulated miRNAs are shown (**Figure 8A**).

Primarily studied for its role in cancer progression, *Mtdh* is expressed in most tissue types and has been localized to epithelial tight junctions (Hu et al., 2009). Using Western blot analysis, we first documented the presence of metadherin at the PSD, and observed a 42% increase in PSD-localized metadherin protein following chronic cocaine treatment (Eipper-Mains et al., 2011). Ectopic expression of *Mtdh* in primary human fetal astrocytes inhibits expression of the glial high-affinity glutamate transporter (*SLC1A2* or *EAAT2*; Kang et al., 2005). An expanding body of evidence implicates glutamatergic signaling in the response of the brain to cocaine (Kalivas, 2009), and more rapid removal of glutamate from the glutamatergic synapses formed by neurons of the PFC onto the medium spiny neurons of the NAc would be of functional significance.

In addition to *Mtdh*, *Pcdh8* (protocadherin 8; also known as arcadlin for activity-regulated cadherin-like protein), and *Pcdh12* (protocadherin 12) were identified as targets for synaptically localized miRNAs (Eipper-Mains et al., 2011). Expression of *Pcdh8*, which is known to be localized to synapses, is rapidly and transiently increased in response to seizures, and plays a role in long-term potentiation (Yamagata et al., 1999). A comparison of the gene expression profiles in the hippocampus of cocaine addicts versus drug-free age-matched controls identified *PCDH8* in a group of up-regulated genes, many of which are involved in regulating the extracellular matrix (Mash et al., 2007). The role of *Pcdh12* in the brain has not yet been addressed.

Ntrk2, the BDNF receptor or TrkB, was also identified as a potential target for the cocaine-regulated PSD-localized miRNAs; predicted binding sites are shown in **Figure 8A**. Prior studies have shown that the level of TrkB increases in the NAc of rats after chronic cocaine self-administration (Graham et al., 2009) and TrkB-BDNF signaling in the NAc is thought to modulate behavioral responses to cocaine (Lobo et al., 2010). Using an antibody to the BDNF receptor, we found increased expression in striatal PSDs following cocaine treatment (Eipper-Mains et al., 2011).

Two of the predicted miRNA targets, *Pcsk2* (prohormone convertase 2, PC2) and *Penk* (proenkephalin) are part of the peptidergic system, which is known to play multiple roles in addiction; predicted binding sites for cocaine-regulated PSD-enriched miRNAs are shown in **Figure 8A**. PC2, a Ca^{2+}-dependent subtilisin-like endoprotease, is the major prohormone convertase in the brain (Winsky-Sommerer et al., 2000). Expressed at relatively high levels in the striatum, PC2 is a key enzyme in the cleavage of all three opioid precursors, proenkephalin (*Penk*), prodynorphin (*Pdyn*), and proopiomelanocortin (*Pomc*), as well as CART (*Cartpt*, cocaine, and amphetamine-regulated transcript; Pan et al., 2006). Based primarily on studies of neuroendocrine cells, PC2 has been localized to the *trans*-Golgi network and secretory granules.

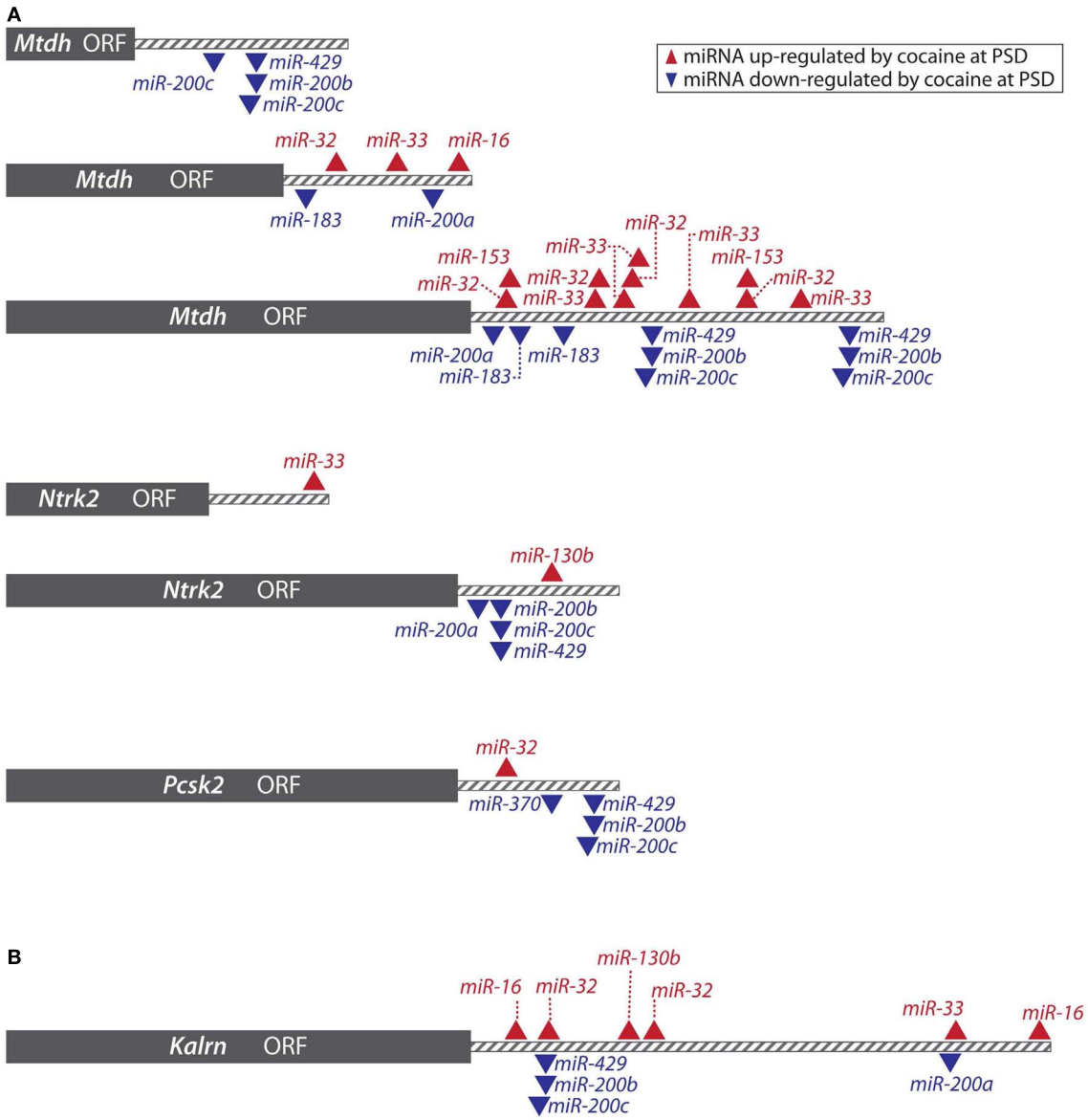

FIGURE 8 | miRanda predictions. (A) The transcripts shown are all PSD-enriched; based on miRanda analysis using the 16 most cocaine-regulated striatal PSD miRNAs, it was predicted that cocaine exposure might affect expression of these genes via regulation of miRNAs. Cocaine-regulation of metadherin, Ntrk2 (BDNF receptor), and PC2 (PCSK2) protein expression at the PSD was verified by Western blot analysis. Gray box indicates the open reading frame (ORF) for each transcript; the 3′-UTR is shown by gray stripes; arrowheads indicate miRNA binding sites predicted for miRNAs up (▲) and down (▼) regulated by cocaine. Reproduced from Eipper-Mains et al. (2011) with permission. **(B)** *Kalrn* was included in the list of synaptically enriched transcripts targeted by cocaine-regulated PSD-enriched miRNAs. Shown are the miRanda predicted binding sites for the 16 most cocaine-regulated striatal PSD miRNAs in the 3′-UTR of mouse KAL7.

Peptide-containing secretory granules are found in presynaptic axon terminals and in dendrites, at the base of spines (Winsky-Sommerer et al., 2000). Using Western blots, we confirmed the presence of PC2 at the synapse and demonstrated an increase of 67% in levels of mature PC2 at the synapse after chronic cocaine treatment (Eipper-Mains et al., 2011). PC2 is synthesized with a signal sequence that guides the nascent chain into the lumen of the secretory pathway. The signal sequence is removed co-translationally; proPC2, which is inactive, is activated by an endoproteolytic cleavage that releases an autoinhibitory N-terminal peptide (Helwig et al., 2011). Our data suggest that this entire process can occur in the vicinity of the synapse and may respond in part to synaptic activity.

Penk, another bioinformatically predicted miRNA target, is expressed primarily in the medium spiny neurons that express *Drd2* (Curran and Watson, 1995). *Penk* is involved in the behavioral response to cocaine and its mRNA and peptide levels were known to be cocaine-regulated (Przewlocka and Lason, 1995;

Daunais et al., 1997; Crespo et al., 2001). Striatal expression of CART peptides increases after cocaine exposure and is hypothesized to modulate the effects of the enhanced dopaminergic signaling in response to cocaine (Hubert et al., 2008). The increased levels of PC2 could play an essential role in their appearance.

Based on the same bioinformatic analysis, miRNA-regulated expression of Kalirin (*Kalrn*), a Rho guanine nucleotide exchange factor that regulates dendritic spine morphology and function (Ma et al., 2008), was predicted (**Figure 8B**). Cocaine-regulated expression of *Kalrn* is of special interest because of the well-documented morphological effects of chronic cocaine exposure on the mesolimbic dopaminergic pathway (Wolf, 2006). Increased dendritic spine size and density are observed in the MSNs of mice following both experimenter-administered (Robinson and Kolb, 1997, 1999; Kiraly et al., 2010a,b) and self-administered cocaine (Robinson et al., 2001). Chronic cocaine exposure is known to result in an increase in expression of Kal7, the predominant adult isoform of *Kalrn*, in the NAc (Kiraly et al., 2010a; Mains et al., 2011), and an increase in Kal7 protein at the PSD (Kiraly et al., 2010a; Mains et al., 2011). Mice lacking Kal7 fail to form additional spines in response to chronic cocaine treatment (Kiraly et al., 2010a; Mains et al., 2011). The 3′-UTR of mouse Kal7 contains multiple predicted binding sites for cocaine-regulated PSD miRNAs (**Figure 8B**).

NEXT – CONCLUSION; FUTURE STUDIES

The number of studies examining the effects of cocaine on one or a small number of genes still dwarf the number of genome-wide expression studies. These focused studies have revealed widespread changes in multiple neurotransmitter systems (dopamine, glutamate, peptidergic), in proteins controlling cytoskeletal organization and in multiple transcription factors. Examples include several dopamine receptors and the dopamine transporter, a number of ionotropic and metabotropic glutamate receptors, opiate receptors and opiate peptides, other neuropeptides, the synaptic vesicle associated proteins α-synuclein and synaptotagmin, clathrin, signaling pathway protein such as the mitogen-activated protein kinases (MAPKs), calmodulin, and Ca^{2+}/calmodulin-dependent protein kinases along with components of the machinery regulating the actin cytoskeleton (Self and Nestler, 1995; Lehrmann et al., 2003; Mash et al., 2003; McClung and Nestler, 2003; Norrholm et al., 2003; Morabito et al., 2004; Rossman et al., 2005; Benavides et al., 2007; Anderson et al., 2008; Li et al., 2008; Liu et al., 2009; Kiraly et al., 2010a,b; Mains et al., 2011).

A number of comprehensive analyses of cocaine-induced gene expression in animal models and human postmortem brain, primarily using microarray, have been published over the years. Like our global analysis of cocaine-regulated miRNA expression, these studies have further expanded the scope of targets affected by cocaine. In particular, these global approaches have consistently highlighted the effects of cocaine on components of the extracellular matrix, including cadherins, integrins, and matrix metalloproteinases (Lehrmann et al., 2003; Albertson et al., 2004; Bannon et al., 2005; Mash et al., 2007). Particularly appealing is the idea that some of the long-lived effects of cocaine may reflect changes in the extracellular matrix. Drugs designed to target multiple participants in these chronic effects of cocaine may prove to be more effective than drugs targeted to individual intracellular signaling pathways in the treatment of drug abusers.

Knockout and transgenic mouse models have been invaluable for investigations into the behavioral, biochemical, and molecular effects mediated by single genes. It is becoming increasingly clear, however, that the changes that occur as a result of drug abuse are incredibly complex and widespread, affecting multiple brain regions and cell-types in a system-wide manner. These global studies emphasize the broad effects of drug exposure on neuronal gene expression, underscoring the need for an integrative approach that combines mRNA, miRNA, and protein expression profiling in combination with focused single gene studies and innovative behavioral paradigms in the quest to uncover what drives addiction and develop integrated therapeutic approaches. With improved methodologies it will be possible to study the role of miRNAs in specific cell-types in animals self-administering drugs or undergoing withdrawal.

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MicroRNAs and drug addiction

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Drug addiction is considered a disorder of neuroplasticity in brain reward and cognition systems resulting from aberrant activation of gene expression programs in response to prolonged drug consumption. Non-coding RNAs (ncRNAs) are key regulators of almost all aspects of cellular physiology. MicroRNAs (miRNAs) are small (∼21–23 nucleotides) ncRNAs transcripts that regulate gene expression at the post-transcriptional level. Recently, miRNAs were shown to play key roles in the drug-induced remodeling of brain reward systems that likely drives the emergence of addiction. Here, we review evidence suggesting that one particular miRNA, miR-212, plays a particularly prominent role in vulnerability to cocaine addiction. We review evidence showing that miR-212 expression is increased in the dorsal striatum of rats that show compulsive-like cocaine-taking behaviors. Increases in miR-212 expression appear to protect against cocaine addiction, as virus-mediated striatal miR-212 overexpression decreases cocaine consumption in rats. Conversely, disruption of striatal miR-212 signaling using an antisense oligonucleotide increases cocaine intake. We also review data that identify two mechanisms by which miR-212 may regulate cocaine intake. First, miR-212 has been shown to amplify striatal cAMP response element binding protein (CREB) signaling through a mechanism involving activation of Raf1 kinase. Second, miR-212 was also shown to regulate cocaine intake by repressing striatal expression of methyl CpG binding protein 2 (MeCP2), consequently decreasing protein levels of brain-derived neurotrophic factor (BDNF). The concerted actions of miR-212 on striatal CREB and MeCP2/BDNF activity greatly attenuate the motivational effects of cocaine. These findings highlight the unique role for miRNAs in simultaneously controlling multiple signaling cascades implicated in addiction.

Keywords: miRNA, miR-212, MeCP2, cocaine

INTRODUCTION

Non-coding RNAs (ncRNAs) can be defined as biologically functional RNAs that do not encode proteins. This class of transcripts is characterized by the presence of an increased density of stop codons and lack of extensive open reading frames (ORFs). For decades, work on ncRNAs focused almost exclusively on transport RNAs (tRNAs) and ribosomal RNAs (rRNAs), which are key regulators of mRNA translation into encoded proteins. However, recent advances in high throughput sequencing technologies have revealed tremendous diversity in ncRNAs. Moreover, ncRNAs are playing an increasingly more recognized role in key aspects of cellular function, including the regulation of gene expression, often through novel mechanisms of action. ncRNAs have also been implicated in key cellular process such as DNA imprinting, RNA splicing, editing, transcription, mRNA degradation, and translational repression. Interestingly, analysis of ncRNA complexity through evolution reveals that the proportion of non-coding sequences in eukaryotic genomes correlates closely with the complexity of the organism, even when proportion of the protein coding genes remains relatively static across organisms (Heimberg et al., 2008).

It is currently thought that only about 2% of the human genome codes for functional proteins, yet more than 80% of transcripts encoded in the genome may have some biochemical activity. In a recent study, Djebali et al. (2012) used ultra-deep sequencing of RNAs from different cell lines and concluded that ∼75% of the genome is transcribed at some point during the cellular life cycle. These extensive studies using sequencing and sophisticated ncRNA prediction algorithms have led to the identification of thousands of ncRNAs in the human genome, and perhaps many more remain to be discovered (Majer and Booth, 2010; Yang et al., 2010).

On the basis of their length and function this heterogeneous group of transcripts can be further categorized into short ncRNAs and long ncRNAs. The short ncRNAs include microRNAs (miRNAs) and short-interfering RNAs (siRNAs), which are generally 18–25 nucleotides in length. Small RNAs (smRNAs) such as the small nucleolar RNAs (snoRNAs), smRNAs, piwi-interacting RNA (piRNAs) are between 20 and 300 nucleotides in length. The long ncRNAs (>200 nucleotides) can further be grouped into different categories on the basis of their origin (Orom and Shiekhattar, 2011) and have a broader spectrum of functions including chromatin modulation, recruitment of transcription factors, and nuclear-cytoplasmic transport (Mattick and Makunin, 2006). Indeed, there is now compelling computational and experimental evidence that many previously uncharacterized ncRNAs of unknown function, considered largely "transcriptional

noise," have key functional roles in almost all aspects of cellular physiology.

Using the high throughput *in situ* expression data from Allen Brain Atlas, Mercer et al. have recently shown that more than 800 different ncRNAs are widely expressed in the adult brain (Mercer et al., 2008). In another study, Landgraf et al. created a miRNA expression atlas by sequencing and analyzing multiple smRNAs libraries (Landgraf et al., 2007). Their data also reveals a tissue/cell specific expression of these RNAs, which correlates with *in situ* expression data. Hence, it is likely that ncRNAs play an important role in basic aspects of brain function and behavior.

MicroRNAs IN BRAIN

Amongst the different classes of ncRNAs, miRNAs are perhaps the best characterized. Since their discovery in *C. elegans* in 1993 (Lee et al., 1993), hundreds of miRNAs have been identified in different species. Initial studies demonstrated that these short (∼22 nucleotide) RNAs are mainly involved in binding to the 3′ untranslated region (3′UTR) of mRNA transcripts that share complementarity with nucleotides in their so-called seed sequence (nucleotides 2–7). Binding of miRNAs to mRNA transcripts results in post-transcription silencing of the target mRNA *via* RNA-induced silencing complex (RISC)-induced translational repression or sequestering them for storage or degradation (Bartel, 2004). However, emerging new evidence suggests that the non-seed region of miRNAs may bind to the 5′UTR or the coding sequence of target transcripts and thereby influence translation processes (Orom et al., 2008; Elcheva et al., 2009). To add further complexity, recent reports show that miRNAs can also positively regulate gene expression in some cellular contexts (Vasudevan et al., 2007; Place et al., 2008). Computational sequence analysis predicts that each miRNA can target 10–100s of mRNA transcripts (Esteller, 2011). In addition, each gene transcript can itself be targeted by potentially hundreds of miRNAs.

The brain may be a particularly prominent organ within which miRNAs play an important role in controlling gene expression and neuronal activity. In a study by Miska et al. it was established that there was considerable abundance of miRNAs in rodent and primate brain (Miska et al., 2004). Further, they developed a microarray-based method to monitor the spatiotemporal expression of miRNAs during mouse brain development and found that there was a dynamic change in miRNA expression levels during development. As a result of these new methodologies there have been more than 300 miRNAs identified in adult mouse brain. Of these brain-enriched miRNAs some are present ubiquitously whereas others are expressed in a cell specific manner (Bak et al., 2008).

Another powerful tool that improved our understanding of the functional relevance of miRNAs in neuronal tissues was the development of mice with genetic manipulations in the genes encoding Dicer and Arognaute2 (Ago2). Both of these proteins are important components of the miRNA biogenesis pathway. Dicer is an endoribonuclease that catalyzes the processing of precursor miRNA into its mature form. Characterization of Dicer1 mutants in *C. elegans* suggested that this protein plays a critical role in brain development (Grishok et al., 2001). To assess its role in mammalian brain development Bernstein et al. disrupted Dicer in mice and observed embryonic lethality with depleted number of stem cells, suggesting that this enzyme is important for development in general (Bernstein et al., 2003). Subsequently a number of groups knocked down Dicer *via* conditional gene targeting, with the findings consistent in demonstrating that ablation of dicer results in decreased miRNA expression and defects in neuronal cell differentiation and survival (Kanellopoulou et al., 2005; Murchison et al., 2005; Schaefer et al., 2007). These phenotypes could be rescued by re-expression of the gene (Kanellopoulou et al., 2005), highlighting the importance for Dicer and the smRNAs species processed by this endoribonuclease in neuronal development and function. More recently, conditional deletion of Dicer established that miRNAs expressed during neurogenesis play a role in the maintenance of neural progenitor cells, with disruption in this action contributing to defects in neuronal migration and subsequently in cortical lamination (De Pietri Tonelli et al., 2008; Kawase-Koga et al., 2009; Clovis et al., 2012).

Arognaute2, a member of the argonaute family of proteins, is the catalytically active component of the RISC that not only facilitates miRNA processing but also their regulatory actions on target mRNA transcripts (Hock and Meister, 2008). In a study by Diederichs and Haber, it was shown that overexpression of Ago2 results in increased miRNA biogenesis (Diederichs and Haber, 2007). Similar to genetic disruption of Dicer, Ago2 deletion results in embryonic lethality in mice, but conditional knock down of Ago2 reveals defects in neural tube closure and mispatterning of the anterior structure of the brain. Mouse embryonic fibroblast (MEFs) isolated from these mutant mice have reduced miRNA expression, which can be reversed by Ago2 expression (Morita et al., 2007). These findings highlight the importance of Ago2 in neuronal differentiation, brain morphogenesis, and development (Liu et al., 2004; Morita et al., 2007).

Schratt et al. found that the brain-specific miRNA, miR-134, is localized to the synaptodendritic compartment and negatively regulates the size of dendritic spines (Schratt et al., 2006). This action of miR-134 occurs through its inhibitory action on lim1 kinase expression. Another brain-enriched miRNA, miR-124, has been shown to play a critical role in the transition of progenitor neuronal cells to adult neurons by inhibiting networks of non-neuronal genes, thereby facilitating the expression of the neuronal identity (Conaco et al., 2006; Makeyev et al., 2007). Aizawa and colleagues examined the effects of double deletion of miR-9-2 and miR-9-3 on brain development in mice (Shibata et al., 2008, 2011). They found that these miR-9 family members regulate the proliferation and differentiation of neural progenitor cells in telencephalon through inhibitory actions on regulator proteins important for neurogenesis, including the homeobox protein Meis2 and the transcription factor Forkhead box protein G1 (FOXG1) (Shibata et al., 2008, 2011). Other miRNAs shown to regulate neuronal lineage commitment include members of the let-7 family and miR-125b (Leucht et al., 2008; Rybak et al., 2008). More recently, hippocampus-expressed miRNAs such as miR-134 and miR-34 have been shown to target the deacetylase sirtuin-1 (SIRT1) and thereby influence learning and memory processes (Gao et al., 2010; Zovoilis et al., 2011). In addition to their role in neuronal development and function, miRNAs may also play key role in neuronal dysfunction associated neurodegenerative diseases. For example,

miR-34c has been implicated in the cognitive impairment associated with dementia. As discussed below, miRNAs have also been implicated in drug addiction, considered by many to be an aberrant form of learning and memory.

DRUG ADDICTION AND MicroRNAs

Addiction can be defined as compulsive drug use despite negative consequences. During the last decade, multiple cellular and molecular studies have revealed significant convergence between the actions of drugs of abuse in the brain that drive the development of addiction and the molecular processes involved in learning and memory. Indeed, addiction is often conceptualized as a disorder of synaptic plasticity, and hence the cellular and molecular mechanisms involved in learning-associated synaptic plasticity and concomitant remodeling of neuronal circuits may provide an important heuristic framework to investigate the addiction process.

In a study by Schaefer et al. it was shown that cocaine-induced robust alterations in the expression of a wide-range of miRNAs in the striatum, a key brain site involved in addiction. Indeed, a subset of these miRNAs whose expression was impacted by cocaine were shown to regulate the expression levels of a wide-range of genes known to influence the motivational properties of cocaine, including *Bdnf*, *FosB* (*FBJ murine osteosarcoma viral oncogene homolog B*), and *Cdk5r1* (cyclin-dependent kinase 5 activator 1). In the same study, the effects on cocaine reinforcement of selectively ablating Ago2, the catalytic component of RISC involved in transducing the inhibitory actions of miRNAs on their target transcripts, was assessed. Specifically, Schaefer et al. investigated the effects knocking down Ago2 in medium spiny neurons (MSNs) of the striatum that express the dopamine D2 receptor (D2R) (Schaefer et al., 2010). Disruption of Ago2 in D2R MSNs resulted in dramatically reduced conditioned rewarding effects of cocaine in mice, reflected in attenuated cocaine-induced conditioned place preference (CPP). More importantly, the Ago2-D2R mutant mice also demonstrated reduced intravenous cocaine self-administration behavior across a wide-range of cocaine doses (Schaefer et al., 2010). Such downward shifts in the cocaine dose-response curve are interpreted as reduced motivation to consume the drug. Finally, Ago2 ablation in D2R MSNs dramatically decreased miRNA expression and activity in striatum. Hence, as cocaine self-administration behavior is considered the most direct measure of drug reinforcement in laboratory animals, these data provide compelling support for a key role for Ago2, and by extension miRNAs, in striatal MSNs in regulating the reinforcing properties of cocaine that drive the development of addiction (Schaefer et al., 2010).

Another interesting study performed by Eipper-Mains et al. (2011) links cocaine exposure to Ago2 induction (Eipper-Mains et al., 2011). Characterization of the subcellular fractions of the striatum shows that Ago2 is localized in synapses and is cocaine-responsive. Specifically, chronic cocaine exposure resulted in increased Ago2 mRNA and protein in the striatum, and concomitantly altered miRNA expression levels. This increase in Ago2 protein was associated with postsynaptic densities (PSDs) in striatum but not in medial prefrontal cortex (mPFC). Intriguingly, a large number of cocaine-responsive miRNAs identified so far (miR-8 family, miR-145, miR-451) can putatively target genes implicated in addiction, including TrkB receptor, which transduces the actions of BDNF in brain and play a crucial role in activity-dependent synaptic plasticity.

Chandrasekar and Dreyer identified another set of miRNAs (miR-181a, let-7d, and miR-124) whose expression is sensitive to cocaine (Chandrasekar and Dreyer, 2009). They found that chronic cocaine administration suppressed the expression of miR-124 and let-7d, but induced miR-181a in the mesolimbic dopaminergic system. The critical role of the mesolimbic dopaminergic system in addiction is well established (Koob and Volkow, 2010). *In situ* hybridization confirmed that the alterations in the expression of these miRNAs occurred in brain regions related to reward and memory. Further, *in vitro* overexpression of these miRNAs modulated the expression levels of proteins like BDNF and the dopamine D3 receptor, which have been heavily implicated in drug addiction (Heidbreder et al., 2005; Ghitza et al., 2010). In a subsequent study the same group also showed that *in vivo* modulation of these miRNAs in ventral striatum (nucleus accumbens) affects cocaine-induced place conditioning (Chandrasekar and Dreyer, 2011). These data show that cocaine can impact the expression of a range of different miRNAs depending on treatment and testing context, and support an important role for such miRNAs and their targeted mRNA transcripts in the development of drug addiction.

Alterations in dopamine signaling can lead to long-lasting neuronal adaptations that result in decreased or increased propensity for drug use. One of the proposed mechanism by which this can occur is dopamine transmission-induced alterations in the expression and subunit composition of AMPA receptors (Wolf, 2010). AMPA receptors are postsynaptic glutamate gated ion channels that mediate excitatory neurotransmission in the central nervous system. As such, AMPA receptors are core regulators of synaptic plasticity and activity-dependent remodeling of brain circuitries (Du et al., 2004; Haas et al., 2006). In a recent study by Saba et al. it was found that miR-181a expression in nucleus accumbens was increased by dopamine-mediated transmission and by the psychomotor stimulant drugs cocaine and amphetamines (Saba et al., 2012). Moreover, miR-181a was shown to repress GluA2-AMPA receptor subunit expression, and thereby modulate the magnitude of AMPA receptor clustering. Hence, miR-181a may be a key miRNA involved in drug-induced remodeling of the nucleus accumbens and greater striatal complex in response to drug exposure, thereby driving regulating the emergence of addiction. Recent evidence suggests that miRNAs also play a key role in the actions of other classes of addictive drugs, including nicotine, alcohol, and opiates; for recent review and detailed table describing specific miRNAs see Im and Kenny (2012).

THE miR-212/132 CLUSTER

The miR-132/miR-212 family of miRNAs was first identified in a genome wide search for genes responsive to the transcription factor cAMP response element binding protein (CREB) using an approach termed Serial Analysis of chromatin occupancy (SACO) (Impey et al., 2004). This family of miRNAs is highly conserved in vertebrates, is transcribed as a polycistronic primary transcript, and is highly enriched in the mature neurons of the forebrain (Marson et al., 2008; Hansen et al., 2010). Analysis of the promoter for this miRNA gene cluster reveals the presence of multiple

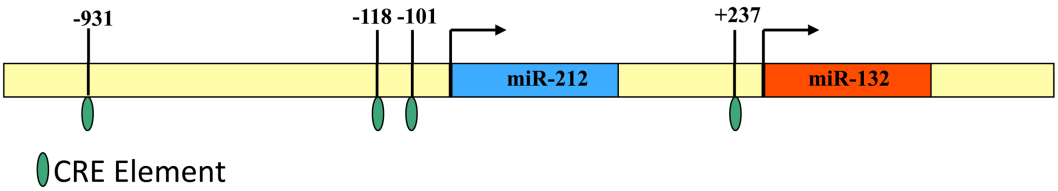

FIGURE 1 | The miR-212/132 gene cluster is located on chromosome 17 in humans, 10 in rats, and 11 in mouse. Shown are mouse/human miR-212 and miR-132 genes, with locations of CRE elements through which CREB can stimulate miR-212 and miR-132 transcription.

cAMP response element (CRE) sites and experimental evidence verifies that these miRNAs are indeed CREB inducible (Vo et al., 2005); (see **Figure 1**) Recent studies from Remenyi et al. have shown that this gene cluster in fact produces four mature miRNAs, namely miR-132, miR-132*, miR-212, and miR-212*, where miR-132* and miR-212* are encoded by the same primary transcript, but on the opposite strand, as the miR-132 and miR-212 miRNAs, respectively (Remenyi et al., 2010). Moreover, each of these four miRNAs likely have their own unique set of target mRNA transcripts. Intriguingly, even though these four miRNAs are transcribed in equal measure (by virtue of all being encoded in the same primary transcript), their relative abundance varies dramatically within various cell types including neurons, with miR-132 being far more abundant than the other three transcripts (Remenyi et al., 2010). Hence, it is likely that as yet uncharacterized mechanisms are involved in preferentially stabilizing miR-132 levels, and/or destabilizing miR-132*, miR-212, and miR-212*.

A series of studies in the last few years have demonstrated the importance of miR-132 cluster in neuronal morphogenesis and in regulating synaptic plasticity. In particular, miR-132 has been shown to increase dendritic spine complexity in both immature cortical and hippocampal neurons in part by translational inhibition of p250GAP. As p250GAP is a Rho-Rac family GTPase activating protein, this finding highlights a role for Rho, and also transducers downstream of Rac-GTPases such as PAK, in the effects of miR-132 on activity-dependent neuronal remodeling (Wayman et al., 2008; Hansen et al., 2010; Magill et al., 2010).

COCAINE INTAKE AND miR-212

Two recent studies from our group have identified a key role for miR-212, also encoded by the miR-212/132 gene cluster, in regulating compulsive-like cocaine intake in rats (Hollander et al., 2010; Im et al., 2010). As described above, the striatum is a key brain region that regulates compulsive cocaine use. The first study showed that in rats with extended access to cocaine (6 h per day) there is a ∼1.75-fold increase in both striatal miR-212 and miR-132 levels (Hollander et al., 2010). Similar increases in expression were not detected in rats that received non-contingent cocaine infusions time-locked to rats that volitionally consumed cocaine, or in rats with restricted access to cocaine (1 h per day). Further, lentivirus-mediated overexpression of miR-212 in the dorsal striatum resulted in a remarkable decrease in cocaine intake in the extended access rats compared to vector control, but overexpression did not alter cocaine intake in rats with restricted drug access (**Figure 2**). The decreased cocaine intake is related to a profound decrease in the motivational properties of the drug, as reflected by a large downward shift in the cocaine dose-response curve in the same animals. Conversely, inhibition of miR-212 signaling in the striatum, achieved by infusion of an antisense oligonucleotide, dramatically increased the motivational properties of cocaine in rats with extended, but not restricted, access to the drug (**Figure 2**). These data suggest that intrinsic or drug-induce alterations in the expression or activity of striatal miR-212 may influence vulnerability to addiction in human cocaine users.

miR-212 REGULATES COCAINE INTAKE THROUGH STRIATAL CREB SIGNALING

As noted above, expression of miR-212 is regulated by CREB. In many cases, miRNAs have been shown to influence signaling cascades that increase their expression and further modify the activity those cascades through positive or negative feedback mechanisms (Tsang et al., 2007). As CREB overexpression in ventral striatum is known to diminish the motivational properties of cocaine (Carlezon et al., 1998), we tested the hypothesis that miR-212 may regulate cocaine intake in extended access rats by amplifying striatal CREB activity through positive feedback mechanisms. Consistent with a profound stimulatory effect of miR-212 on CREB in cultured cells *in vitro*, we found that levels of CREB that was phosphorylated at serine 133 (i.e., activated CREB) was significantly increased (Hollander et al., 2010). Furthermore, forskolin-stimulated expression of the CREB-responsive gene *fos* was also increased by miR-212, as was the activity of a luciferase-based CREB reporter construct (CRE-containing element from promoter of EVX-1). Dominant negative or phosphorylation-deficient mutant forms of CREB attenuated these stimulatory effects of miR-212 on CREB signaling. More importantly, we also found that miR-212 amplified CREB signaling in the striatum *in vivo*. Specifically, we found that rats with extended access to cocaine showed increased expression of the CREB-responsive gene *Nurr1*, and that expression levels were greatly increased by miR-212 overexpression in striatum (Hollander et al., 2010).

These findings identify miR-212 as a novel cocaine-responsive gene that is up regulated in the striatum in response to cocaine overconsumption that serves to promote CREB activity through positive feedback and thereby attenuate the motivational properties of the drug. Based on these findings, we next investigated the mechanisms by which miR-212 may amplify CREB signaling. We found that miR-212 increases activity-dependent production of cAMP by sensitizing adenylyl cyclase activity. This stimulatory

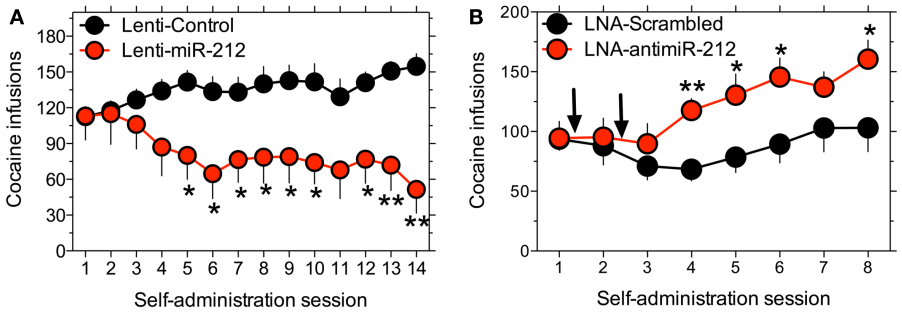

FIGURE 2 | Overexpression of miR-212 in striatum reverses the motivational properties of cocaine in rats with extended but not restricted access to cocaine. (A) Striatal miR-212 overexpression reverses the long-term trajectory of cocaine-taking behavior in rats with extended access. **(B)** Disruption of miR-212 signaling in striatum, achieved by local infusion of a locked nucleic acid (LNA) modified antisense oligonucleotide against miR-212 (LNA-antimiR-212) increases cocaine intake in extended access. Reproduced with permission from (Hollander et al., 2010).

action on adenylyl cyclase results in accumulation of phosphorylated CREB and increased activity of the core CREB co-activators CREB-regulated transcription co-activator-1 and -2 (CRTC1 and CRTC2), also known as TORC1 and TORC2. Further investigation of the mechanism by which miR-212 enhances CREB signaling revealed that the kinase Raf1 was activated by miR-212, and was found to play a key role in sensitizing adenylyl cyclase activity. Finally, computational and biochemical analysis showed that Sprouty-related EVH1 domain-containing protein 1 (SPRED1), a known negative regulator of Raf1 signaling, is a miR-212 target mRNA transcript and SPRED1 repression by miR-212 contributes to its stimulatory effects on Raf1 and CREB activity (Hollander et al., 2010).

To investigate the functional relevance of miR-212-induced amplification of CREB activity *in vivo* on the suppressive effects of this miRNA on cocaine intake, we examined the effects of overexpressing the CREB co-activator CRTC1 (TORC1) on cocaine intake in rats with restricted or extended cocaine access. It is known that CRTC overexpression increases CREB activity and that miR-212 increases CRTC1 expression (Hollander et al., 2010). Consistent with an important role for striatal CREB signaling in attenuating the motivational properties of cocaine, we found that striatal TORC1 overexpression decreased cocaine intake in extended but not restricted access rats. These findings support the hypothesis that miR-212 controls cocaine intake at least in part by amplifying the CREB-TORC signaling axis in striatum. Moreover, these findings provide compelling support for a key role for miR-212 in regulating the development of compulsive drug taking in rats, and perhaps in influencing vulnerability to cocaine addiction in human drug users.

miR-212 ALSO REGULATES COCAINE INTAKE THROUGH STRIATAL MeCP2

Considering that miR-212 expression levels may play a key role in regulating vulnerability to cocaine addiction, we next investigated the mechanisms by which baseline and cocaine-induced increases in striatal miR-212 levels are regulated. Interestingly, sequence analysis of the miR-212/132 gene cluster reveals that it is located in a CpG enriched region, which can serve as substrate for DNA methylation and gene regulation. Methyl CpG binding protein 2 (MeCP2) is known to bind to methylated DNA and can act as a gene repressor by recruiting other chromatin remodeling proteins that combine to form the so-called repressor complex (Guy et al., 2011). Based on these observations, we hypothesized that MeCP2 may regulate baseline and cocaine-induced changes in miR-212 expression in striatum and thereby influence cocaine-taking behavior. Consistent with this hypothesis, *in vitro* studies showed that knockdown of MeCP2 increased miR-212 (and miR-132) expression in cultured cells (Im et al., 2010). Similarly, pharmacologically induced disruption of DNA methyltransferase activity, which would be expected to attenuate the inhibitory activity of MeCP2 on gene expression, also increased miR-212/132 levels. Furthermore, we found that knockdown of striatal MeCP2 expression, achieved by virus-mediated delivery of a short hairpin interfering RNA (shRNA) against MeCP2, resulted in profoundly decreased cocaine intake in rats with extended but not restricted access to cocaine (**Figure 3**). Knockdown of striatal MeCP2 dramatically increased the stimulatory effects of self-administered cocaine on striatal miR-212 expression in rats with extended but not restricted access to the drug. More importantly, disruption of striatal miR-212 signaling, achieved by striatal infusion of an antisense oligonucleotide, reversed the inhibitory effects of MeCP2 knockdown on cocaine intake in extended access rats (Im et al., 2010). These findings are consistent with an inhibitory effect of MeCP2 on miR-212 expression, suggesting that MeCP2 acts as a pro-addiction transcriptional repressor that, by attenuating miR-212 expression in response to cocaine, increases vulnerability to addiction.

Intriguingly, miR-132 was previously shown to repress the expression of MeCP2 through direct interaction with the transcript 3′UTR (Klein et al., 2007). Moreover, as miR-132 and miR-212 share the same seed region, this suggests that miR-212 may similarly repress MeCP2. Based on these observations, we hypothesized that in addition to the inhibitory effects of MeCP2 on miR-212 expression described above, MeCP2 levels in turn may be repressed by miR-212. In other words, miR-212 and MeCP2 may be locked in a homeostatic relationship that serves to control miR-212 expression level and thereby

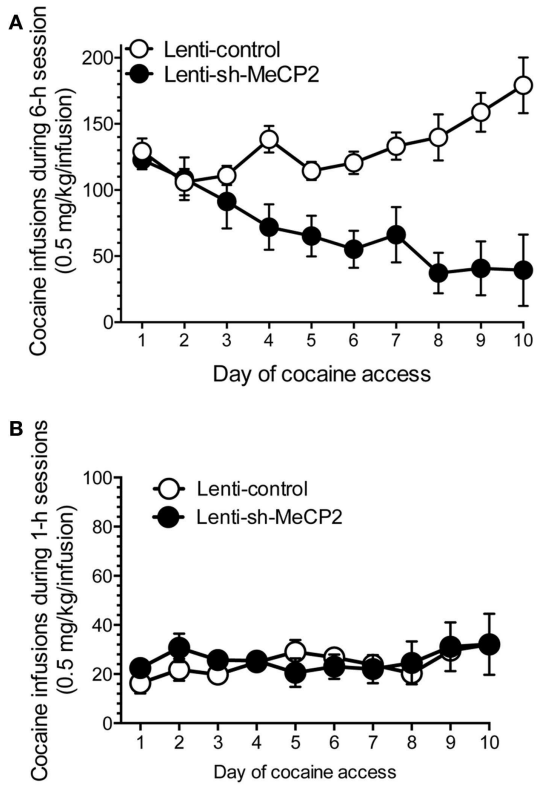

FIGURE 3 | Knockdown of MeCP2 in striatum reverses the motivational properties of cocaine in rats with extended but not restricted access to cocaine. (A) Lentivirus-mediated knockdown of MeCP2 in the striatum reverses the escalating cocaine intake typically seen in rats with extended access to cocaine. **(B)** In contract, MeCP2 knockdown does not alter cocaine intake in rats with restricted daily access to the drug. Reproduced with permission from (Im et al., 2010).

influence vulnerability to cocaine addiction. Consistent with this hypothesis, we found that miR-212 overexpression profoundly decreased MeCP2 expression in cultured cells and in the striatum *in vivo* (Im et al., 2010). Hence, homeostatic interactions between miR-212 and MeCP2 may determine vulnerability to cocaine addiction.

ROLE FOR BDNF IN REGULATING THE ACTIONS OF STRIATAL miR-212 ON COCAINE INTAKE

It has been reported that the levels of MeCP2 are closely related to those of, BDNF (Chang et al., 2006). However, the complex mechanisms by which MeCP2 regulates BDNF levels remain unclear. BDNF in the striatum is known to increase the motivational properties of cocaine (Horger et al., 1999; Schoenbaum et al., 2007). We therefore hypothesized that miR-212-MeCP2 interactions may regulate cocaine intake by influencing levels of BDNF in striatum. We observed that virus-mediated MeCP2 knockdown or miR-212 expression (which decreases MeCP2 levels) in striatum reduced BDNF expression. Moreover, virus-mediated increases in BDNF expression in striatum increased cocaine intake in rats with extended but not restricted access. Conversely, disruption of striatal BDNF signaling using a neutralizing antibody reduced cocaine intake in extended but restricted access rats (Im et al., 2010). These findings suggest that miR-212-MeCP2 interactions may determine expression levels of BDNF in striatum, which in turn regulates the motivational properties of cocaine.

NON-NEURONAL ROLES OF miR-212

Besides playing a critical role in drug-induced neuroplasticity relevant to addiction, there is emerging evidence that miR-212 is also involved in a host of other biological and pathophysiological processes. Indeed, miR-212 expression is deregulated in various cancers and its expression has been correlated to disease progression. In pancreatic carcinomas it has been shown that miR-212 targets the tumor suppressor retinoblastoma (Rb1) (Park et al., 2011), whereas the methyl binding protein (MeCP2) seems to be the main target in some of the gastric tumors (Wada et al., 2010). Incoronato et al. showed that miR-212 targets phosphoprotein enriched in diabetes (PED), a wide spectrum anti-apoptotic protein, and thereby plays an important role in tumor suppression (Incoronato et al., 2010). In a follow up study the same group also demonstrated that the expression on miR-212 in lung carcinomas is regulated by histone modifications (Incoronato et al., 2011). This suggests that epigenetic mechanisms may play an important role in regulating miRNA expression in different cancers and other biological processes.

Besides cancer, miR-212 has also been implicated in regulating organogenesis by playing a key role in modulating epithelial stromal interactions (Ucar et al., 2010). In addition, miR-212 expression can be regulated by various hormones, adding another layer of complexity to their regulation and role in development and disease (Godoy et al., 2011). Studies from Turrini et al. reveal that miR-212 may mediate drug resistance by targeting the ABC efflux transporter (Turrini et al., 2012). Two recent reports implicate the importance of the miR-132/212 family in cardiovascular development and disorders. In the first study, smRNAs deep sequencing analysis in vascular smooth muscle cells show that the miR-132/miR-212 cluster is induced by the hormone angiotensin II and by targeting PTEN, increases the expression of the gene MCP1 (Monocyte chemotactic protein 1), a key regulator of cardiovascular disorders (Jin et al., 2012). In the other study, Ucar et al. demonstrate that these miRNAs activate calcineurin signaling in cardiomyocytes by targeting the transcription factor Foxo3, and thus play an important role in cardiac hypertrophy (Ucar et al., 2012).

CONCLUSION

Taken together, these studies highlight the fact that miR-212 plays a critical role in fine-tuning transcriptional and neuroplastic responses to drugs of abuse. Specifically, we found that miR-212 can control cocaine intake through two complementary mechanisms: amplifying CREB signaling and reducing MeCP2/BDNF transmission in striatum.

Since miR-212 and miR-132 share the same seed region it is widely believed that they target the same mRNAs. However, only a few putative miR-212/132 targets have been verified experimentally, which include MeCP2, Rb1and HB-EGF. There is increasing evidence available now showing that the 5′ and 3′ regions of the miRNAs can form the basis of differential target

recognition despite having the identical seed region (Brennecke et al., 2005; Jalvy-Delvaille et al., 2012). There is also a possibility that differential expression or availability of these members can also regulate gene expression in a differential manner. Future studies in this respect could further help in improving our understanding of how miR-212 and miR-132 regulate different neuronal and non-neuronal functions or if these two miRNAs are functionally redundant? More generally, these findings highlight the novel role for miRNAs in addiction, and suggest that other ncRNAs may also play important roles in the disorder.

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MicroRNAs in opioid addiction: elucidating evolution

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Three reviews in the Frontiers Research Topic "Non-Coding RNA and Addiction" (He and Wang, 2012; Rodriguez, 2012; Zheng et al., 2012), grouped under the chapter "MicroRNAs and Morphine," focus on the contribution of microRNAs to opioid abuse. Although animal models have been fundamental to our understanding of addiction pathways, the assumption that microRNAs implicated in opioid tolerance – and their binding sites in mRNAs – are conserved in mammalian evolution was not examined by the authors. Inspired by recent reports which highlight a surprising lack of evolutionary conservation in non-coding RNA genes, in this perspective we use public genome, annotation, and transcriptome datasets to verify microRNA host gene, mature microRNA, and microRNA binding site conservation at key loci functional in opioid addiction. We reveal a complex evolutionary landscape in which certain directional regulatory edges of the microRNA–mRNA hub-and-spoke network lack pan-mammalian conservation.

Keywords: microRNA, morphine, evolution, TargetScan, OPRM1

INTRODUCTION

Although microRNAs are not the most abundant class of non-coding RNAs in mammalian systems, numbering merely 2,042 mature sequences[1] compared to the more than 9,000 long non-coding RNA genes in humans (Derrien et al., 2012), microRNAs represent a key category of post-transcriptional suppressors, and their global significance in gene expression regulation is firmly established (Bartel, 2009). Nevertheless, evolutionary conservation of protein-coding genes exceeds that of non-coding RNA genes (Derrien et al., 2012). Mammalian microRNA host genes are structurally heterogeneous and highly complex, with variable localization of the mature microRNA sequences relative to key host gene structure elements such as promoters, 3′ends, exons, splice sites, and introns (Rodriguez et al., 2004). Evolutionary complexity may accompany this genomic structure complexity of microRNA host genes: a microRNA cluster functional in stem cell regulation and pluripotency resides within a non-coding RNA host gene that exists only within placental mammals and exhibits major genomic structure distinctions between closely related species (Houbaviy et al., 2005). By no means confined to early development, sequence non-conservation of microRNA-dependent regulation is increasingly apparent in the brain. Brain-expressed microRNAs and their mRNA targets functional in neural development display accelerated rates of evolutionary change along the human lineage (Somel et al., 2011), while expression differences, some of which are shaped by adaptive forces, are evident among the microRNA repertoires of human, chimpanzee, and macaque (Dannemann et al., 2012). In summary, this evidence for gene structure as well as sequence non-conservation of microRNA host genes, mature microRNAs, and microRNA:mRNA hybridization sites motivated us to systematically examine the conservation of microRNA and target sequences implicated in opioid abuse and tolerance.

[1]mirbase.org

METHODS

We used the UCSC Genome Browser[2] (hg19 human genome assembly), miRbase[3], and the NCBI website[4], accessed on May 25 and September 20, 2012.

RESULTS AND DISCUSSION

Some microRNAs prominent in the drug abuse field, as well as their host genes and mRNA targets, lack gene structure conservation and sequence conservation within mammals. Because functional genomics of drug abuse continues to depend on animal, especially rodent, models, and because of the accumulating evidence in the literature regarding non-conservation and rapid evolution of microRNA genes, it is imperative to rigorously test the assumption that the key microRNA-to-mRNA regulatory relationships implicated in human drug abuse can, in fact, be extrapolated to animal models. We tested this assumption for microRNAs from **Table 1** at three levels: (1) genomic conservation of the mature microRNA's seed (nt 2–nt 8) sequence within the microRNA host gene; (2) gene structure conservation of the microRNA host gene; and (3) genomic conservation of the microRNA's binding site in the 3′UTR of the known mRNA target from the three reviews (**Table 2**). In examining microRNA seeds and host genes, we focused only on three microRNAs (not including let-7) whose names were sufficiently specific to allow *in silico* identification of their host gene loci in the UCSC Genome Browser: miR-23b, miR-133b, and miR-190. We found that two of the three microRNAs resided within protein-coding host genes, while one host gene was non-coding and had only expressed sequence tag (EST) evidence for a conventional, capped, polyadenylated host gene transcript. We also identified substitutions in the seed sequence of all three microRNAs in mammalian species from the public

[2]genome.ucsc.edu

[3]mirbase.org

[4]www.ncbi.nlm.nih.gov

Table 1 | MicroRNAs and morphine: function and conservation – MicroRNA names, functions, and targets from the "MicroRNAs and Morphine" chapter of the Frontiers Special Topic "Non-Coding RNA and Addiction."

Name	Review	Description from review and target
miR-23b	Zheng et al. (2012)	Targets the 3′-UTR of OPRM1 mRNA and regulated the association between OPRM1 mRNA and polysomes
miR-23b	He and Wang (2012)	Could interact with the MOR 3′-UTR via a K box motif (5′-UGUGAU-3′) in SH-SY5Y and mouse P19 cells
miR-23b	Rodriguez (2012)	Involved in linking MOR expression and morphine treatment at the post-transcriptional level. Represses MOR through 3′ UTR of MOR1 in K-box motif
miR-133b	Zheng et al. (2012)	Morphine reduces miR-133b and miR-133b increases Pitx3
miR-133b	He and Wang (2012)	Decreased by morphine in zebrafish embryos
miR-133b	Rodriguez (2012)	Possible involvement in addiction through the effects of morphine, Targets Pitx-3 (Th and Dat pathway), miR-133b also down-regulates RhoA protein expression
miR-190	Zheng et al. (2012)	Negatively regulated by fentanyl; decreased expression of miR-190 leads to an increase in NeuroD protein. miR-190 binds to the 3′UTR of NeuroD mRNA and destroys it
miR-190	Rodriguez (2012)	Targets NeuroD levels, and is negatively regulated by fentanyl. miR-190 (aka miR-190a?) regulates NeuroD, a transcription factor that is known to regulate the differentiation and maturation of neurons
miR-let7	Zheng et al. (2012)	Morphine treatment affected the expression levels of miR-23b and let-7, which have binding sites on the 3′UTR of the OPRM1 mRNA and control the expression of OPRM1
miR-let7	He and Wang (2012)	Target is 3′ end of MOR mRNA. These authors experimentally validated the *in silico* prediction that members of the let-7 miR family can interact with the 3′-UTR of MOR mRNA at the predicted positions
miR-let7	Rodriguez (2012)	Works as a mediator of the movement of the mu opioid receptor (MOR) mRNA into P-bodies, which leads to translational repression of MOR mRNA. Targets MOR through 3′ UTR binding

Table 2 | MicroRNAs and morphine: function and conservation – Seed sequence, host gene, and target binding site conservation of microRNAs with known opioid abuse functions, as assessed by viewing the corresponding microRNA host genes and their mRNA targets within the genome-wide 46-species MultiZ alignment in the human UCSC Genome Browser.

MicroRNA name	MicroRNA host gene name	mRNA target of the microRNA	Conservation of microRNA host gene	Closest species to human lacking 100% miR seed conservation at the mRNA target's binding site
miR-23b	C9ORF3	IPCEF1 and OPRM1	Vertebrates, but polyA signal is primate-specific	Gorilla
miR-190	TLN2	NEUROD1	Vertebrates	Mouse
miR-133b	Novel host (ESTs: AI803441 and others)	PITX3	Vertebrates, but polyA signal is primate-specific	n/a (not detected by TargetScan)

genome-wide 46-species MultiZ alignment, suggesting that the affinity of all three microRNAs for their known targets may differ between mammalian species, unless the target UTRs co-evolve with the microRNA seeds. Gene structure conservation of the miR-190 host gene, as assessed by the conservation of the canonical 3′end polyadenylation signal AnTAAA hexamer in the 46-species alignment was also incomplete, with two of the three host genes in human apparently reliant on primate-specific polyadenylation signals (**Table 2**).

We proceeded to gage the conservation of these microRNAs' binding sites in the 3′UTRs of their known mRNA targets, by visualization of the TargetScan microRNA target prediction tool results that are represented in the "TS miRNA" track of the human UCSC Genome Browser. We focused on three target relationships, which are reported in the three review articles comprising our "MicroRNAs and Morphine" chapter of the present Research Topic: miR-23b/OPRM1, miR-190/NeuroD, and miR-133b/Pitx3. We observed the potential absence (due to a genomic deletion relative to human and/or to a genome assembly gap in the non-human species) of two microRNA binding sites at these mRNA targets in at least one non-human mammal. The miR-190 binding site of the human NeuroD1 3′UTR is not conserved in mouse, a key animal model for addiction research used to study the OPRM1-NeuroD crosstalk via miR-190 (**Figure 1A**; Rodriguez, 2012 and references therein). We could not evaluate the third site, the miR-133b-binding sequence of the Pitx 3′UTR, because this well-known relationship is not predicted by the pre-computed TargetScan results that are made available in the TS/miR track of the UCSC Genome Browser (hg19 assembly). This deficiency attests to a false-negative rate in microRNA target prediction tools

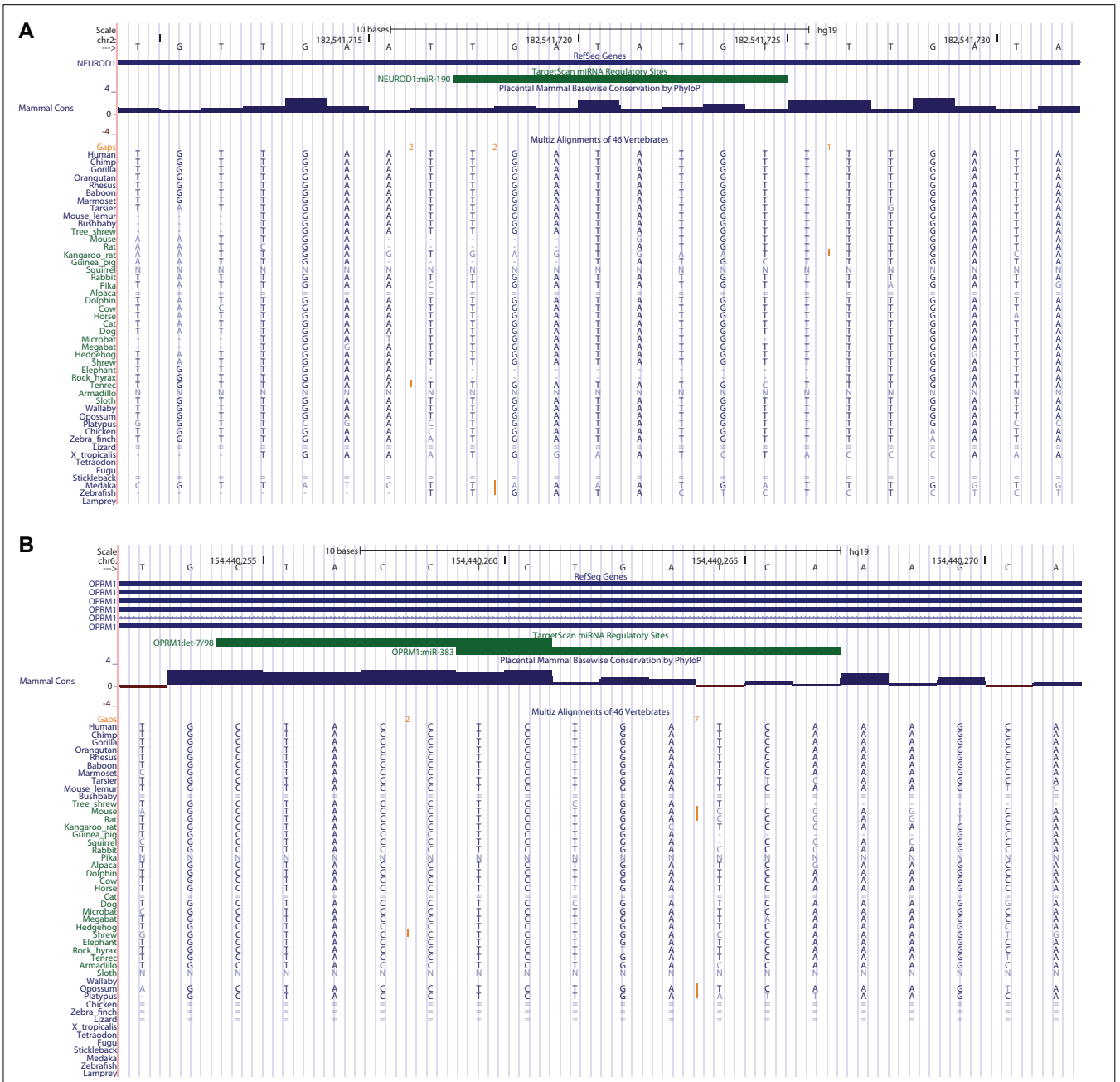

FIGURE 1 | MicroRNA binding sites at opioid addiction genes' 3′UTRs: conservation and complexity. (A) The miR-190 binding site in the human NeuroD1 3′UTR is not conserved in the mouse, according to the 46-species MultiZ sequence alignment in the UCSC Genome Browser. **(B)** Let-7 and miR-383 binding sites overlap in the OPRM1 3′UTR.

that results in the omission of some experimentally confirmed microRNA targets by these tools. Similarly, TargetScan did not detect the reported miR-23b targeting of the OPRM1 3′UTR, although, intriguingly, the neighboring gene IPCEF1 – which overlaps, in an antisense orientation, the genomic footprint of one OPRM1 splice isoform – is a miR-23b target (**Table 2**). These results summarily indicate that microRNA host gene structures, mature microRNA seed sequences, and microRNA binding sites at mRNA 3′UTRs are not always conserved in all mammals, for some of the key microRNAs and their experimentally confirmed mRNA targets in opioid abuse.

Manual annotation of microRNA:mRNA cognate sites may uncover novel regulatory relationships relevant to drug abuse transcriptomics. In the process of assessing sequence conservation at TargetScan-predicted, experimentally confirmed microRNA binding sites at loci of importance to opioid addiction, we often observed that numerous additional microRNAs – not mentioned in the literature concerning post-transcriptional regulation at

these loci – are predicted by TargetScan to regulate these same targets. In fact, 16 distinct microRNA binding sites are predicted by TargetScan in the NeuroD1 3′UTR alone. In several particularly interesting cases, two microRNA binding sites partially overlap within a single target gene's 3′UTR. This overlap implies that a given mRNA of that gene may be suppressed by either of the two microRNAs competing for the binding site, but may not be targeted by both microRNAs simultaneously. One intriguing example of these overlaps is found in the 3′UTR of OPRM1, where the experimentally validated let-7 microRNA binding site overlaps a TargetScan-predicted site for miR-383 (**Figure 1B**). This indicates the utility of UCSC Browser manual annotation in discovering possible new microRNA binding sites, which should be experimentally evaluated for function. These overlapping sites are also intriguing within the context of the competing endogenous RNA (ceRNA) hypothesis (Tay et al., 2011), since one of them in theory competes with the other for binding the same target.

CONCLUSION

We examined the evolutionary conservation of microRNA host genes and experimentally confirmed microRNA targets, focusing on validated microRNA:mRNA regulatory relationships that are central to gene expression regulation in the context of opioid addiction. We pinpointed a lack of pan-mammalian conservation at multiple levels. In particular, microRNA host genes contained primate-specific genomic structure elements, such as consensus polyadenylation signals that are absent beyond primates. At the same time, TargetScan-detected, experimentally validated microRNA seed binding sites at mRNA 3′UTRs lacked pan-mammalian conservation; certain seed matches in 3′UTRs were not even conserved in mouse. This evolutionary complexity reveals the potential limitations of animal models in which these protein-coding genes might be regulated by microRNAs differently from human. High-throughput short and long RNA sequencing should facilitate future studies of microRNA roles in drug addiction, through quantitating events such as alternate polyadenylation that are essential for making microRNA binding sites available in 3′UTRs, and through unbiased analysis of co-expression and co-localization of annotation-identified new microRNAs (e.g., miR-383) with their putative mRNA targets.

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Let-7 microRNAs and opioid tolerance

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This chapter will focus on the role of microRNAs (miRs) in regulating the actions of opioid drugs through the opioid receptors. Opioids, such as morphine, are analgesics that are used for treating many forms of acute and chronic pain. However, their chronic use is limited by undesirable effects such as opioid tolerance. The μ opioid receptor (MOR) is the primary receptor responsible for opioids' analgesia and antinociceptive tolerance. The long 3′-untranslated region (3′-UTR) of MOR mRNA is of great interest since this region may contain elements for the post-transcriptional regulation of receptor expression, such as altering the stability of mRNA, influencing translational efficiency, and controlling mRNA transport. MicroRNAs are small non-coding RNA molecules that exert their functions through base-pairing with partially complementary sequences in the 3′-UTR of target mRNAs, resulting in decreased polypeptide formation from those mRNAs. Since the discovery of the first miR, lin-4 in *Caenorhabditis elegans*, hundreds of miRs have been identified from humans to viruses, which have provided a crucial and pervasive layer of post-transcriptional gene regulation. The nervous system is a rich source of miR expression, with a diversity of miR functions in fundamental neurobiological processes including neuronal development, plasticity, metabolism, and apoptosis. Recently, the let-7 family of miRs is found to be a critical regulator of MOR function in opioid tolerance. Let-7 is the first identified human miR. Its family members are highly conserved across species in sequence and function. In the review, we will present a brief review of the opioid receptors, their regulation, and opioid tolerance as well as an overview of miRs and a perspective how miRs may interact with MOR and serve as a regulator of opioid tolerance.

Keywords: opioid, miR, epigenetics, addiction, pain

INTRODUCTION

Since first isolated from opium poppy in the early nineteenth century, morphine and related opioids remain as the most powerful analgesics to treat many forms of acute and chronic pain. However, repeated and prolonged use of opioids leads to tolerance, of which, the analgesic tolerance is the most problematic that can lead to therapeutic failure (i.e., inadequate pain control; McQuay, 1999). In some cases, even the highest tolerable dose of an opioid cannot achieve the desirable analgesic effect in patients (Wang and Wang, 2006; Harden, 2008). Development of opioid tolerance not only limits the analgesic efficacy, but the increased doses of opioids in order to counter tolerance exacerbate another problem, namely opioid addiction that is a significant medical and public health problem. Extensive efforts have been made to elucidate the mechanisms underlying opioid tolerance and drug addiction (Kieffer and Evans, 2002). Increasing evidence implicates the contribution of transcriptional and epigenetic regulation in opioid tolerance and drug addiction, such as activation and inhibition of transcription factors (Carlezon et al., 2005; Zachariou et al., 2006), modification of chromatin and DNA structure (Renthal et al., 2008; Guo et al., 2011), and induction of non-coding RNAs including microRNAs (Pietrzykowski, 2011; Robison and Nestler, 2011).

MicroRNAs (miRs) are small non-coding RNA molecules that repress target gene expression through base-pairing with partially complementary sequences in the 3′-untranslated region (3′-UTR) of target mRNAs. Owing to recent cloning, sequencing, and computational efforts, the numbers of known miRs has been rapidly increasing, and to date, there are a total of 21,643 mature miRs found across 103 species, of which 1921 miRs are found in humans (miRBase Release 18.0, November 2011[1]). With the emerging identification of miRs from humans to viruses, a crucial and pervasive layer of post-transcriptional gene regulation by miRs has been elucidated (Ambros, 2004; Taft et al., 2010). It is generally accepted that miRs serve as an important class of epigenetic regulators that participate in a variety of cellular activities. In respect to their diverse functions, miRs play an integral role in fundamental neurobiological processes including neuronal development, plasticity, metabolism, and apoptosis (Kosik, 2006). As one form of long-lasting synaptic plasticity, opioid tolerance is a particularly interesting research area to study miR-mediated cellular adaptation. In this review, we summarize recent findings on miRs in opioid tolerance, with a focus on the role of let-7 miRs in regulating opioid tolerance.

Mu OPIOID RECEPTOR AND OPIOID TOLERANCE

The μ opioid receptor (MOR), a member of the G-protein coupled receptor superfamily, is the primary receptor responsible for

[1]http://microrna.sanger.ac.uk/sequences

opioids' analgesia and antinociceptive tolerance (Matthes et al., 1996; Sora et al., 1997). Opioid tolerance may be result from opioid receptor desensitization and trafficking, which include opioid receptor down-regulation, internalization, and uncoupling from G-proteins due to chronic exposure to opioid agonists (Bailey and Connor, 2005; Martini and Whistler, 2007; Koch and Hollt, 2008; Lopez-Gimenez and Milligan, 2010). Receptor down-regulation as one of mechanisms contributing to opioid tolerance has been previously proposed (Davis et al., 1979; Tao et al., 1987; Bhargava and Gulati, 1990; Bernstein and Welch, 1998; Diaz et al., 2000). Chronic morphine treatment produced a marked decrease in brain MOR density (Davis et al., 1979; Tempel et al., 1988). Down-regulation of the high-affinity MOR site in rats has also been reported following continuous infusion of morphine (*i.t.*; Wong et al., 1996) or etorphine (*s.c.*; Tao et al., 1987). Morphine-induced MOR down-regulation was also observed in SH-SY5Y cells, with or without differentiation (Zadina et al., 1993). In addition to receptor down-regulation, chronic treatment with morphine has been shown to significantly reduce MOR-signaling in sensory neurons and brainstem nuclei (Sim et al., 1996; Johnson et al., 2006), which are in agreement with the findings that reduced receptor number and resultant reduced MOR-signaling contribute to opioid tolerance. On the other hand, there have been reports that MOR expression was not altered (Dum et al., 1979) or even up-regulated (Lewis et al., 1984) in the brain by various opioids. Some of the discrepancies may be caused by uncontrolled variables (e.g., different opioids, doses, methods of opioid treatments, anatomical regions, and times samples were taken, integrality of tissue samples before assays, and opioid receptor subtypes studied), as well as detection methods. It has been suggested that MOR down-regulation is agonist selective and depends on the agonist's intrinsic efficacy (Nishino et al., 1990; Chakrabarti et al., 1997; Chan et al., 1997; Koch and Hollt, 2008). The purity and selectivity of radiolabeled ligands – a problem not only for some early studies, but also in more recent reports employing questionable materials – used in studies are the other potential culprits for conflicting findings.

DIRECT INTERACTION BETWEEN let-7 FAMILY miRs AND MOR

The long 3′-UTR of MOR mRNA (Ide et al., 2005; Han et al., 2006) suggests that this region may contain physiologically relevant elements for regulating receptor expression by mechanisms such as miR targeting. Indeed, early research on 3′-UTR of human MOR mRNA suggested that MOR expression was increased after a 712-bp segment, immediately downstream of the stop codon, was removed (Zollner et al., 2000). Comparative bioinformatics predicted potential miRs that may interact with the human and mouse MOR (**Table 1**). Let-7 family of miRs was identified as a top candidate according to the number of putative target sites and alignment pattern. Our group experimentally validated the *in silico* prediction that members of the let-7 miR family can interact with the 3′-UTR of MOR mRNA at the predicted positions (He et al., 2010). Furthermore, downregulating let-7 with specific LNA-modified antisense oligodeoxynucleotides (LNA-let-7-AS) was found to increase MOR expression in SH-SY5Y cells, a human neuroblastoma cell line, suggesting that (1) MOR is a target of let-7; (2) expression of MOR is under constitutive suppression by let-7.

Table 1 | MicroRNA targets predicted by miRanda (http://www.microrna.org/microrna/getGeneForm.do) and ranked according to the alignment scores.

miRNA	Query target sites	Alignment score	PhastCons score
HUMAN OPRM1 3′-UTR			
hsa-miR-600	291	169	0.666615
hsa-miR-384	83	157	0.619627
hsa-miR-105	172	156	0.619627
hsa-let-7b	381	154	0.607749
hsa-let-7c	383	154	0.607749
hsa-let-7g	386	154	0.607749
hsa-let-7i	386	154	0.607749
hsa-miR-1272	261	154	0.643121
hsa-miR-642	151	154	0.619627
hsa-miR-98	386	154	0.607749
hsa-let-7f	383	153	0.607749
hsa-let-7d	386	152	0.607749
hsa-miR-18a	401	152	0.607749
hsa-miR-198	124	152	0.619627
hsa-let-7a	385	151	0.607749
hsa-miR-302a	169	151	0.619627
hsa-miR-1324	286	150	0.666615
hsa-miR-192	102	150	0.619627
hsa-miR-215	102	150	0.619627
hsa-miR-648	144	150	0.619627
hsa-miR-18b	399	149	0.607749
hsa-miR-383	389	149	0.607749
hsa-miR-526b	52	149	0.619627
hsa-miR-659	233	149	0.619627
hsa-let-7e	385	147	0.607749
hsa-miR-302b	170	147	0.619627
hsa-miR-136	166	146	0.619627
hsa-miR-431	220	145	0.619627
hsa-miR-557	216	145	0.619627
hsa-miR-1262	105	144	0.619627
hsa-miR-154	322	144	0.666615
hsa-miR-373	168	144	0.619627
hsa-miR-583	114	144	0.619627
hsa-miR-1259	271	143	0.643121
hsa-miR-135a	264	143	0.643121
hsa-miR-135b	264	143	0.643121
hsa-miR-493	4	143	0.6910845
hsa-miR-519c-5p	79	143	0.619627
hsa-miR-520d-3p	170	143	0.619627
hsa-miR-580	307	143	0.666615
hsa-miR-196a	382	142	0.607749
hsa-miR-520a-3p	172	142	0.619627
hsa-miR-577	302	142	0.666615
hsa-miR-142-3p	384	141	0.607749
hsa-miR-378	208	141	0.619627
hsa-miR-422a	207	141	0.619627
hsa-miR-1826	387	140	0.607749
hsa-miR-302c	170	140	0.619627

(Continued)

Table 1 | Continued

miRNA	Query target sites	Alignment score	PhastCons score
hsa-miR-302d	170	140	0.619627
hsa-miR-504	1	140	0.6910845
hsa-miR-521	89	140	0.619627
hsa-miR-9	234	140	0.619627
hsa-miR-942	333	140	0.666615
MOUSE OPRM1 3′-UTR			
mmu-miR-540-5p	278	168.0	0.62498
mmu-miR-540-3p	395	159.0	0.584342
mmu-miR-134	403	158.0	0.584342
mmu-miR-302c	165	158.0	0.586054
mmu-miR-302d	165	151.0	0.586054
mmu-let-7i	384	149.0	0.62498
mmu-miR-139-5p	363	149.0	0.62498
mmu-let-7g	388	148.0	0.62498
mmu-miR-199a-3p	193	148.0	0.586054
mmu-miR-199b	193	148.0	0.586054
mmu-miR-496	241	148.0	0.586054
mmu-miR-532-3p	63	148.0	0.586054
mmu-let-7a	388	147.0	0.62498
mmu-let-7f	388	147.0	0.62498
mmu-let-7c	386	146.0	0.62498
mmu-miR-486	214	146.0	0.586054
mmu-miR-741	512	146.0	0.567263
mmu-miR-383	93	144.0	0.586054
mmu-miR-695	404	144.0	0.584342
mmu-let-7b	388	143.0	0.62498
mmu-let-7e	388	143.0	0.62498
mmu-miR-188-3p	64	143.0	0.586054
mmu-miR-196b	386	143.0	0.62498
mmu-miR-302b	165	143.0	0.586054
mmu-miR-323-5p	233	143.0	0.586054
mmu-miR-466f-5p	138	143.0	0.586054
mmu-miR-98	387	143.0	0.62498
mmu-miR-298	391	142.0	0.584342
mmu-miR-339-3p	71	142.0	0.586054
mmu-miR-381	528	142.0	0.590822
mmu-miR-682	186	142.0	0.586054
mmu-let-7d	388	141.0	0.62498
mmu-miR-504	57	141.0	0.586054
mmu-miR-154	321	140.0	0.62498
mmu-miR-196a	387	140.0	0.62498
mmu-miR-340-5p	527	140.0	0.590822
mmu-miR-34b-3p	367	140.0	0.62498

In order to elucidate a physiological role of let-7 in regulating MOR and opioid tolerance, we further examined the *in vivo* relevance of let-7 miRs in cellular and animal models of opioid tolerance. For the former, SH-SY5Y cells were treated with morphine (1−10 μM for 24–48 h) to induce cellular tolerance. The expression of let-7 miRs was significantly up-regulated by chronic treatment with morphine, while the expression level of MOR was reduced as determined by the western blotting method (He et al., 2010) and the receptor radioligand binding using ^{3}H-DAMGO (He and Wang, unpublished data). Therefore, chronic morphine treatment caused the increase of let-7 and decrease of MOR expression during the development of opioid tolerance in SH-SY5Y. Furthermore, the regulation of let-7 expression by morphine occurred in a mouse model of opioid tolerance. Mice were implanted *s.c.* with a 75-mg morphine pellet (75 mg morphine pellet/mouse, *s.c.*). Brain expression of let-7 increased gradually over time after morphine treatment, temporally correlating with the development of antinociceptive tolerance to morphine. Interestingly, up-regulation of let-7 occurred in MOR-expressing cells, but not in MOR-negative cells in mice brain cortex region as determined by *in situ* hybridization (He et al., 2010). This was in agreement with the aforementioned finding that MOR is a direct target of let-7. In order to further examine a causative role of let-7 in leading to opioid tolerance, we directly targeted let-7 in the mouse model of opioid tolerance. Treatment with the let-7 inhibitor (*i.c.v.*) decreased brain let-7 levels and partially attenuated opioid antinociceptive tolerance (He et al., 2010).

Previous reports from a number of different cell lines or animal models (e.g., Brodsky et al., 1995; Johnson et al., 2006) indicated that MOR mRNA was not changed upon treatment with morphine. We also found that the total MOR transcripts were unchanged by morphine. For example, MOR mRNA remained the same in SH-SY5Y cells following chronic morphine treatment. It raised a question as to how let-7 miRs repress MOR in opioid tolerance. We confirmed that let-7 did not affect MOR mRNA stability; however, polysome-bound MOR transcript was significantly decreased. Where is MOR mRNA hiding? It turns out that it can be accumulated in P-bodies. So these intriguing data led us to propose the following regulatory mechanism where upon let-7 can regulate opioid tolerance: let-7 recruits and sequesters MOR mRNA to P-bodies that are deprived of translational machinery, effectively reducing polysome-bound MOR mRNA and leading to translation repression. A similar pathway was observed in HEK293 and HeLa cells (Pillai et al., 2005), thus translation repression may serve as a general mechanism by let-7 to regulate its target gene expression (**Figure 1**). Keep in mind that let-7 activity is turned up in opioid tolerance by up-regulation of their transcripts. Nature works in a wonder to ensure the activity of let-7, ultimately dampening the activity of opioids upon persistent activation.

POTENTIAL TARGET GENES OF let-7 IN OPIOID TOLERANCE

Let-7 was the first identified human miR. Its family members are highly conserved across species in sequence and function (Pasquinelli et al., 2000). Major roles of let-7 include the regulation of stem-cell differentiation, neuromuscular development, and cell proliferation & differentiation (Reinhart et al., 2000; Mansfield et al., 2004; Roush and Slack, 2008). Let-7 was initially identified as a heterochronic gene (Pasquinelli et al., 2000). In mammals, let-7 levels increase during embryogenesis and during brain development (Schulman et al., 2005; Wulczyn et al., 2007). Let-7 is undetectable in human and mouse embryonic stem cells, and the level of let-7 increases upon differentiation (Thomson et al., 2004, 2006; Wulczyn et al., 2007). This high expression of let-7 is then maintained in various adult tissues (Sempere et al., 2004; Thomson et al., 2004). Furthermore, let-7 is widely viewed

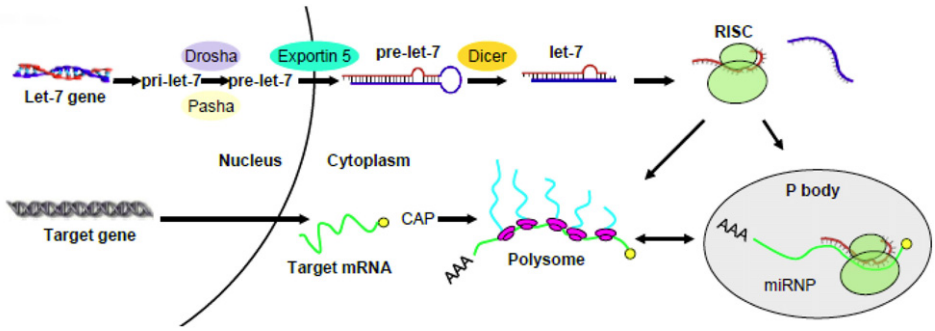

FIGURE 1 | A schematic diagram proposing a mechanism for the regulation of target gene by let-7. Pri- and pre-let-7 are produced and exported into the cytosol where the mature let-7 was incorporated into the RNA-induced silencing complex (RISC). The latter recruits and sequesters target mRNA to P-bodies that are deprived of translational machinery, effectively reducing polysome-bound mRNA and leading to translation repression (modified from He et al., 2010).

as a tumor suppressor miR (Boyerinas et al., 2010). There is a clear link between loss of let-7 expression and the development of poorly differentiated, aggressive cancers (Takamizawa et al., 2004; Dahiya et al., 2008; Childs et al., 2009). Using the computational algorithm TargetScan 6.0[2] to screen human 3′-UTR sequences containing let-7 family miRs complementary sites, 886 conserved targets, with a total of 989 conserved sites and 111 poorly conserved sites were predicted. High-mobility group AT-hook 2 (HMGA2), which is the top-scoring candidate gene on the list (**Table 2**), was confirmed as a direct let-7 target both *in vitro* and *in vivo* (Lee and Dutta, 2007; Mayr et al., 2007; Shell et al., 2007). HMGA2 is a chromatin-associated non-histone protein capable of modulating chromatin architecture and thus affecting transcription. In addition to abundant studies on HMGA2 in embryogenesis (Zhou et al., 1995; Sun et al., 2009) and tumorigenesis (Peng et al., 2008; Qian et al., 2009), it would be interesting to investigate its possible involvement in opioid tolerance as a functional target gene of let-7.

Seed pairing rules are widely used to predict functional miR target sites. MiR-mRNA recognition requires the perfect complementarity of 6-nucleotide mRNA seed sites with the 5′ end of miRs (positions 2–7; Bartel, 2009). However, when predictions based on such short complementary sequences are compared to experimental results from proteomic studies, the false-positive and false-negative rates appear to be above 50% (Easow et al., 2007; Baek et al., 2008; Mourelatos, 2008; Selbach et al., 2008). Several previous findings strongly indicated that a sizeable fraction of miR targets do not obey the "canonical" seed rule (Ha et al., 1996; Didiano and Hobert, 2006; Tay et al., 2008; Stefani and Slack, 2012). For let-7, biological studies clearly demonstrated that genetically verified let-7 targets in *Caenorhabditis elegans* contain only imperfect binding sites, with bulges or G·U wobble pairs in the seed region (Vella et al., 2004). A recent study identified a new class of miR target site nucleation bulges and an alternative mode of miR target recognition by a pivot-pairing rule (Chi et al., 2012). They proposed a transitional nucleation model in which a transitional nucleation state determines the binding of miRs to nucleation bulge mRNAs. Therefore, the identification of non-canonical let-7-mRNA interactions may lead to important breakthroughs in discovering new let-7 targets and further decipher the functional role of let-7 in opioid tolerance.

With respect to let-7 target gene identification, another important issue need to be addressed is the redundancy of let-7 family members. There are 14 and 13 different let-7 family members in mouse and human, respectively (Roush and Slack, 2008). In human, these different members, let-7a-1, 7a-2, 7a-3, 7b, 7c, 7d, 7e, f7-1, 7f-2, 7g, 7i, miR-98, and miR-202 are located on nine different chromosomes (Ruby et al., 2006). While let-7 was initially viewed as one single activity, emerging data suggest that the let-7 family contains miRs with different activities (i.e., targets). For example, it has been reported that let-7b* was highly expressed but let-7e* was drastically reduced in malignant mesothelioma (Guled et al., 2009). So the question remains as whether individual let-7 family member with its own expression pattern exerts specialized function during opioid tolerance development.

CONCLUSION AND FUTURE DIRECTIONS

Opioid tolerance, even to the analgesic actions of these drugs, is likely not caused by a single mechanism; rather an intricate neuronal circuitry involving multiple mechanisms may ultimately lead to the expression of tolerance seen in humans. Regulation of opioid tolerance, through MOR or other mechanisms, represents one of these mechanisms. What is not known is how many miRs are involved. Existing literature on miR-related mechanisms of opioid tolerance is sparse. A typical study in miR research field tends to survey transcript levels of miRs in a disease state (Zheng et al., 2010). For example, morphine-induced up-regulation of miR-15b in human monocyte-derived macrophages (Dave and Khalili, 2010), while miR-133b was decreased by morphine in zebrafish embryos (Sanchez-Simon et al., 2010). It is often extremely difficult to identify a casual relationship. Furthermore, moving from a cell line to *in vivo* relevance is another big hurdle to overcome.

In the case of let-7 miRs, it was identified as a critical regulator of both human and mouse MOR. Moreover, it was demonstrated to be relevant for both cellular opioid tolerance as well as animal models of opioid tolerance (He et al., 2010). Let-7 represents one of several miRs contributing to opioid tolerance. For example, it

[2]http://www.targetscan.org/

Table 2 | Top 100 predicted targets of let-7 miRNAs ranked by total context score (TargetScan 6.0, http://www.targetscan.org/.)

Target gene (1–50)	Total context score	Aggregate P_{CT}	Target gene (51–100)	Total context score	Aggregate P_{CT}
Hmga2	−1.08	>0.99	Map4k3	−0.46	0.96
Fignl2	−1.07	>0.99	Tmem211	−0.46	<0.1
Lin28b	−0.98	>0.99	Zfp583	−0.46	0.96
Trim71	−0.98	>0.99	Dtx2	−0.46	0.99
Zc3hav1l	−0.85	0.86	Diap2	−0.46	0.98
Fign	−0.84	>0.99	Pgrmc1	−0.46	0.9
Nr6a1	−0.8	>0.99	Pars2	−0.46	<0.1
2200002K05Rik	−0.7	>0.99	Cd200r1	−0.45	0.94
Skil	−0.67	>0.99	Abt1	−0.45	<0.1
Igdcc3	−0.65	>0.99	Acot11	−0.45	0.5
Thrsp	−0.62	<0.1	Kcnj11	−0.45	0.81
Prtg	−0.61	>0.99	Zfp248	−0.45	<0.1
Slc5a9	−0.6	0.83	Bcl2l13	−0.44	<0.1
Tgfbr1	−0.58	>0.99	Gatm	−0.44	0.95
Yod1	−0.58	>0.99	Hic2	−0.44	>0.99
Smarcad1	−0.57	0.96	Ccnj	−0.44	0.97
Gab2	−0.57	0.6	Arid3a	−0.44	>0.99
Ngly1	−0.54	<0.1	Hand1	−0.44	0.98
Kctd21	−0.54	0.98	Igf1r	−0.44	>0.99
Dna2	−0.53	<0.1	Rpusd3	−0.43	0.59
Ppp1r15b	−0.53	>0.99	Pbx3	−0.43	0.99
Nphp3	−0.53	0.94	Zmat4	−0.43	0.97
Vezt	−0.52	<0.1	Tmem164	−0.43	<0.1
Suv39h2	−0.51	0.44	Bcat1	−0.43	0.99
Gdf6	−0.5	0.98	Pxt1	−0.43	<0.1
Brwd1	−0.5	<0.1	Als2cl	−0.43	<0.1
Coil	−0.49	0.97	9930012K11Rik	−0.42	0.94
Lrig3	−0.49	0.89	Atg10	−0.42	<0.1
Hoxb1	−0.49	0.98	Limd2	−0.42	0.94
Zcchc9	−0.48	<0.1	Zfp341	−0.42	<0.1
B3gnt7	−0.48	0.76	Fam103a1	−0.42	0.87
Entpd7	−0.48	<0.1	Med28	−0.42	0.56
Acvr1c	−0.48	0.99	Smug1	−0.42	<0.1
Lrig2	−0.48	0.99	Trpm3	−0.42	<0.1
Bzw1	−0.48	>0.99	Fras1	−0.42	0.96
Gnptab	−0.48	0.98	Uhrf2	−0.41	0.86
Wdr41	−0.48	<0.1	Cdc25a	−0.41	0.8
Cdc34	−0.47	0.99	Igf2bp1	−0.41	>0.99
Slc35d2	−0.47	0.98	Cep110	−0.41	0.94
Dclre1b	−0.47	0.12	Mrps33	−0.41	<0.1
Hif1an	−0.47	0.61	Tnfsf10	−0.41	<0.1
Zfp322a	−0.47	0.82	Arhgap28	−0.41	0.98
Adrb2	−0.47	0.98	Apbb3	−0.41	0.98
Tmprss11f	−0.47	0.92	Fndc3a	−0.41	0.97
Ddx19b	−0.47	0.98	Col1a2	−0.41	0.97
Ddx19a	−0.47	0.98	Gemin7	−0.41	<0.1
Galnt1	−0.47	>0.99	Wnt9a	−0.41	0.99
Greb1l	−0.47	0.99	Igf2bp2	−0.41	0.93
Ercc6	−0.47	0.98	Tmem2	−0.41	0.98
Ddi2	−0.46	0.89	Zfp879	−0.41	0.35

was found miR-23b could interact with the MOR 3′-UTR via a K box motif (5′-UGUGAU-3′) in SH-SY5Y and mouse P19 cells (Wu et al., 2008, 2009).

While MOR is involved in the development of morphine tolerance, it is not the only target for the action of miRs in opioid tolerance (Matthes et al., 1996; Sora et al., 1997). With the increasing understanding of miRs in epigenetic regulation, further research is needed to identify other target genes of miRs (including let-7). A number of receptors, ion channels, and protein kinases, which have been implicated in opioid tolerance, such as the NMDA receptors, PKC, and CaMKII (Kieffer and Evans, 2002; Wang and Wang, 2006; Ueda and Ueda, 2009) may become potential targets for let-7.

In summary, miR-mediated mechanisms provide a novel direction toward unraveling the complex mechanisms involved in the development of opioid tolerance. These studies will enrich our knowledge on basic principles of neuronal and behavioral adaptation in opioid tolerance, and eventually lead to novel design and development of pharmacological treatments for opioid tolerance.

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Morphine and microRNA activity: is there a relation with addiction?

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When we talk about drug addiction, we are really dealing with an extremely complex system in which there still remain many unknowns and where many empty spaces or missing links are still present. Recent studies have identified changes in the expression profiles of several specific miRNAs which affect the interactions between these molecules and their targets in various illnesses, including addiction, and which may serve as valuable targets for more efficient therapies. In this review, we summarize results which clearly demonstrate that several morphine-related miRNAs have roles in the mechanisms that define addiction. In this regard, morphine has been shown to have an important role in the regulation of different miRNAs, such as miR-let-7 [which works as a mediator of the movement of the mu opioid receptor (MOR) mRNA into P-bodies, leading to translational repression], miR-23b (involved in linking MOR expression and morphine treatment at the post-transcriptional level), and miR-190 (a key post-transcriptional repressor of neurogenic differentiation, NeuroD). Fentanyl increases NeuroD levels by reducing the amount of miR-190, but morphine does not affect the levels of NeuroD. We also discuss the relationship between morphine, miRNAs, and the immune system, based on the discovery that morphine treatment of monocytes led to a decrease in several anti-HIV miRNAs (mir-28, 125b, 150, and 382). This review is centered on miR-133b and its possible involvement in addiction through the effects of morphine. We establish the importance of miR-133b as a regulatory factor by summarizing its activity in different pathological processes, especially cancer. Using the zebrafish as a research model, we discuss the relationship between mir-133b, the dopaminergic system, and morphine, considering: (1) that morphine modulates the expression of miR-133b and of its target transcript Pitx3, (2) the role of the zebrafish mu opioid receptor (zfMOR) in morphine-induced regulation of miR-133b, which depends on ERK1/2, (3) that morphine regulates miR-133b in hippocampal neurons, and (4) the role of delta opioid receptors in morphine-induced regulation of miR-133b. We conclude that the control of miR-133b levels may be a mechanism for the development of addiction to morphine, or other drugs of abuse that increase dopaminergic levels in the extracellular space. These results show that miR-133b is a possible new target for the design of new treatments against addictive disorders.

Keywords: opioid, morphine, addiction, miRNA, zebrafish, dopaminergic system, miR-133b

INTRODUCTION

OPIOIDS

Opioids are the most potent compounds known today to control pain, and are also amongst the most used drugs of abuse (Corbett et al., 2006). They bind to the three classical opioid receptors, mu (MOR), delta (DOR), and kappa (KOR).

It has been established that the MOR displays higher affinity toward morphine than the other classical opioid receptors, delta, and kappa. Also, an interaction between the mu and the DOR has been described *in vitro*, which is thought to be responsible for faster development of morphine tolerance through the mu opioid receptor (Waldhoer et al., 2004). Moreover, when DOP are knocked out, mice do not develop tolerance to morphine and do not suffer the withdrawal effect once long-term treatment with morphine is over (Fundytus et al., 1995).

After establishing the pharmacological profiles of these classical opioid receptors, investigators studied post-transductional mechanisms, such as receptor internalization and desensitization. Later, interest focused on the intracellular effectors that mediate the activation of signaling cascades. It has been suggested that these post-transductional mechanisms are closely linked to the development of opiate tolerance and dependence on opiate drugs, mainly through the same plasticity mechanisms that produce adaptive changes in neural circuitries, for example, in memory and learning (Evans, 2004).

After more than 40 years of intense research on opiates, scientists now partly understand their mechanism of action on receptors.

Many issues concerning the mechanisms of addiction still remain to be established. This can be achieved by analyzing the

different roles of the opioid receptors apart from their involvement in analgesic properties, such as their functions in developmental processes. For instance, the MOR and KOR increase neurogenesis (Kim et al., 2006), and the DOR acts as a neuroprotector (Narita et al., 2006). Also, other drugs of abuse, such as amphetamines, cocaine, or heroin, produce neuroadaptive changes in the brain that could be explained by shared gene regulatory mechanisms that lead to addiction. In this sense, opioid receptor gene regulation has been reported to be concurrently related to miRNAs and to the addiction process (Zheng et al., 2010b; Sanchez-Simon et al., 2010).

MORPHINE

There are a wide variety of opiates, classified according to their origin and/or structure. The main natural opiates are morphine, phenanthrenic alkaloids similar to morphine (codeine and thebaine), and benzylisoquinolinic alkaloids (noscapine and papaverine). Semisynthetic opiates (heroin, hydrocodone, meperidine, oxycodone, buprenorphine, and etorphine) have a morphine-like structure, whereas fully synthetic opiates (methadone, fentanyl, tramadol, metazocine, and pentazocine) display a wide range of unrelated structures that show similar pharmacological properties. Unfortunately, opiates present some undesirable side effects, such as tolerance, dependence, and addiction (Nestler et al., 1993). Thus, there is a need for novel analgesic drugs that do not have these adverse side effects, especially in light of the widespread abuse of opiates.

The opioid alkaloide *Morphine* is the main active compound in opium, the juice obtained from the seed of the poppy plant *Papaver Somniferum*, and has been used for centuries as a medical and recreational agent. It is used both by medical patients suffering acute or chronic pain, and by habitual daily abusers. Since its isolation, morphine has been used largely for pain management, although other, non-analgesic uses, including experimental depression treatments and as a cure for opium addiction, have been developed. Despite these common uses, morphine produces disruptive negative secondary effects including sleepiness or drowsiness, blurred vision, constipation, and a decrease in blood pressure and appetite. With continuous use, morphine produces physical tolerance and addiction.

Accumulating evidence has demonstrated that, upon repeated exposure to morphine, long-lasting neurochemical alterations occur in discrete brain regions. Changes in gene expression are likely to mediate these adaptations in brain neurochemistry, thereby contributing to dependence and drug addiction (Nestler, 2004). However, the key intracellular signaling molecules that participate in regulating the alterations in gene expression induced by chronic opiate exposure remain unclear.

Recent studies (Dreyer, 2010; Hollander et al., 2010), mainly based on cocaine activity, have reported a role for miRNAs in drug addiction. This opens the door to possible miRNA-mediated involvement of opioids – including morphine – in the addictive process.

ADDICTION

Addiction to drugs is a major public health problem, and represents a complex disorder with multigenic causes. Even when many humans are exposed to drugs of abuse, only some suffer from loss of control over drug use and compulsion for drug seeking and taking; factors that define the addictive situation. It is known that addiction is influenced by both the genetic constitution and the social and psychological environment in which the individual lives (Kendler et al., 2007). The pharmacological activation of brain rewards systems is largely responsible for producing addiction after drug use. Personality, genetic, and social factors are important, although drug effects in the CNS are thought to be the fundamental determinants of addiction. The genetic contribution to the risk for addiction is only close to 50% (Kendler et al., 2007), but the specific genes that are involved in the addictive process are almost completely unknown. Besides, the addictive phenotype can persist even after long periods of abstinence, implying that drugs induce long-lasting alterations in the brain that underlie addiction behaviors (for reviews on this topic, see Dreyer, 2010; Robison and Nestler, 2011).

miRNAs

MicroRNAs (miRNAs) are ∼22 nucleotide (nt) non-coding RNAs that participate in gene regulation. They bind to 3′ untranslated regions (UTRs) of their mRNA targets, inhibiting the transcripts' translation and/or destabilizing them (Valencia-Sanchez et al., 2006). Numerous studies have shown that multiple binding sites in the same 3′UTR confer much stronger regulation than single binding sites (Fang and Rajewsky, 2011). However, reporter assay experiments have suggested that miRNA targeting can also occur in coding regions (Kloosterman et al., 2004; Easow et al., 2007). Large-scale miRNA mis-expression studies also have suggested that binding in coding regions can confer regulation but are on average less effective than those in 3′UTRs (Baek et al., 2008; Selbach et al., 2008). MiRNAs have been shown to regulate the expression of many genes, including genes which function in the CNS. For example, miR-134 regulates dendritic spine morphology by controlling actin filament dynamics (Schratt et al., 2006), while miR-190 regulates neurogenic differentiation (NeuroD), a transcription factor that regulates the differentiation and maturation of neurons (Zheng et al., 2010c).

Over 3000 mature miRNAs have been identified in species ranging from plants to humans, suggesting that they have an important role in gene regulation. At present, a better understanding of miRNA biology, combined with the increasing availability of diverse sequenced genomes, have revealed many of the molecular mechanisms that underlie the evolution of miRNAs and their targets (Berezikov, 2011) Although the molecular mechanisms of miRNA activity are increasingly clear, the biological implications of miRNAs activity are not yet fully defined; functions including cell differentiation, proliferation, apoptosis, anti-viral defense, and cancer have been proposed, and, to an extent, validated.

It has recently been shown that miRNAs are highly expressed in the CNS, including the areas where opioid activity takes place: the brain and spinal cord (Dave and Khalili, 2010; He et al., 2010; Sanchez-Simon et al., 2010; Zheng et al., 2010b). Since the discovery that miRNAs are important regulators of gene expression, these molecules have been linked to biological processes such as drug addiction (He et al., 2010; Zheng et al., 2010a), pain perception (Kusuda et al., 2011), neuron development (Gao, 2010), viral

infection (Dave and Khalili, 2010; Wang et al., 2011), and opioid receptor regulation (Wu et al., 2008; Sanchez-Simon et al., 2010).

Recent experimental work demonstrates that opioids modify the expression profile of certain mRNAs in the CNS (Wu et al., 2008; He et al., 2010; Sanchez-Simon et al., 2010). Also, recent studies, including our own results, have implicated miRNAs in addiction behaviors. Most importantly, miRNAs whose expression is altered by opioids have been shown to regulate the expression of many proteins involved in the addiction pathway (Li and van der Vaart, 2011).

To understand the biological roles of miRNAs, it is essential to identify their targets. Because only a few bases of complementarity are required between miRNAs and their target sequences, mRNA targets can often be difficult to identify, computationally or experimentally. The classical model for specific miRNA target recognition by most algorithms mainly depends on (a) the detection of seed matches and (b) the thermodynamic stability of miRNA: mRNA duplexes. Different algorithms usually produce divergent results (Ambros, 2004; Bentwich, 2005; Rajewsky, 2006; Baek et al., 2008). As miRNA recognition elements are typically found in the 3′ UTR of the target gene mRNA, bioinformatics alone can identify putative targets using resources such as miRGen database. The number of putative targets for any one miRNA has increased in recent years, making the interpretation of miRNA activity more complex. A question to be asked at present is, whether there is a relationship between the different targets a miRNA binds to, and if so, what is the meaning of this situation. Further studies are need in this field in order to elucidate the meaning of the fact that a certain miRNA has different, but perhaps functionally related, target mRNAs. Improved software programs are now able to predict the targets of miRNAs in a more efficient manner, facilitating the elucidation of miRNA function. Bioinformatic predictions (Targetscan) suggest that miRNAs target at least 60% of mammalian RNAs with conserved miRNA targets (Friedman et al., 2009).

OPIOIDS AND miRNAs

miR-let-7

He et al. (2010) identified a let-7 binding site in the 3′-UTR of the MOR mRNA and found that let-7 thereby represses MOR expression. They also found that morphine significantly upregulates let-7 expression in SH-SY5Y cells and in a mouse model of opioid tolerance. Inhibition of let-7 decreased brain let-7 levels and partially attenuated opioid antinociceptive tolerance in mice. Although chronic morphine treatment did not change overall MOR transcript levels, association of polysomes with MOR mRNA declined in a let-7 -dependent manner. The miRNA let-7 works as a mediator moving MOR mRNA to P-bodies, leading to translation repression. These results suggest that let-7 plays an integral role in opioid tolerance.

miR-23b

The expression of MOR can be regulated at both the transcriptional and post-transcriptional levels. Long-term morphine treatment does not alter MOR mRNA levels (Brodsky et al., 1995), suggesting that morphine itself has no important role in the transcription of the MOR gene. Nevertheless, it has not yet been elucidated whether morphine can regulate MOR mRNA at the post-transcriptional level, by producing an interaction between *trans*-acting factors and its 3′-UTR.

Wu et al., 2008, identified miR-23b as a *trans*-acting factor that represses MOR translation efficiency through an interaction with the K box motif in the 3′-UTR of MOR1. This interaction suppresses receptor translation by inhibiting polysome-mRNA association. Later, the same group demonstrated that long-term morphine treatment increases miR-23b expression in a dose- and time-dependent manner (Wu et al., 2009). Using a translational luciferase reporter assay, these authors observed morphine-dependent suppression of reporter activity through the MOR1 3′-UTR. This finding suggests a link between MOR expression and morphine treatment at the post-transcriptional level involving miR-23b.

miR-190

Zheng et al. (2010b) have shown that fentanyl, but not morphine, increases levels of one of the targets of miR-190, NeuroD. This group also showed that by regulating NeuroD activity, mu opioid receptor agonists modulate the stability of dendritic spines. This work is discussed elsewhere in this chapter.

MORPHINE, miRNAs, AND THE IMMUNE SYSTEM

People addicted to opioids have a higher incidence of infectious diseases, and opioids exert a profound influence in immunomodulatory activity (Nair and Schwartz, 1997). In order to understand the relationship between morphine, miRNAs, and the immune system, the following features of morphine should be considered: morphine inhibits specific immunocyte activities, such as monocyte respiratory burst (Peterson et al., 1987), chemotaxis (Stefano et al., 1996), and phagocytosis (Rojavin et al., 1993). In addition, morphine induces apoptosis of macrophages and microglia (Hu et al., 2001), decreases the levels of IFN-γ and interleukin-2 in human T cells (Nyland et al., 2003), induces the expression of HIV entry coreceptors in the immune cells, and facilitates HIV replication *in vitro* (Guo et al., 2002; Li et al., 2002; Persson et al., 2003; Steele et al., 2003).

Wang et al. (2011), showed that morphine treatment in monocytes leads to a decrease in several anti-HIV miRNAs (miR-28, 125b, 150, and 382). Interestingly, these same miRNAs were correlated with the susceptibility of monocytes to HIV-1 infection. This morphine-driven decrease in anti-HIV miRNAs disappears when antagonists of the opioid receptors are used, indicating that morphine functions through its own receptors. On the other hand, type I interferon IFN-α/β, in monocytes could induce the expression of these same anti-HIV miRNAs. Other studies have also shown that type I IFNs modulate miRNA expression in several cell systems (O'Connell et al., 2007; Pedersen et al., 2007; Ohno et al., 2009), functioning as the potent inducer of miRNAs. However, morphine co-treatment with IFN-α/β in monocytes inhibited the induction of IFN-mediated anti-HIV miRNAs (Wang et al., 2011).

HIV-1 infected opiate abusers have potential to destabilize neuronal functions, and often exhibit HIV-1 associated dementia (Bell et al., 2006; Fitting et al., 2010).

Dave and Khalili, 2010 reported that in human monocyte-derived macrophages treated with morphine, miR-15b expression

levels were greatly increased. Fibroblast growth factor-2 (FGF-2), identified as a miR-15b target gene, was decreased at the protein expression levels in response to morphine. Another miRNA, miR-181b, decreased its expression levels under the same conditions. Later studies have shown that morphine induces inflammation and oxidative stress in immune cells through regulating the miR-15b and 181b, thereby contributing to expansion of the HIV-1 CNS reservoir and hence to AIDS progression.

miR-133b

miR-133 was first characterized in mice (Lagos-Quintana et al., 2002), after which homologs were characterized in several other species including invertebrates. Each species frequently encodes multiple miRNAs with identical or similar mature sequences. Three different miR-133 sequences are known: miR-133a-1, miR-133b-2, and miR-133b.

A good example of the importance of miR-133b is represented by the work of Yu et al. (2011b). They studied the function of miR-133b during zebrafish spinal cord regeneration and showed upregulation of miR-133b expression in regenerating neurons of the brainstem after transection of the spinal cord. Inhibition of miR-133b expression by antisense morpholino (MO) application resulted in impaired locomotor recovery and reduced regeneration of axons from neurons in the nucleus of the medial longitudinal fascicle, superior reticular formation, and intermediate reticular formation. They found that miR-133b targets the small GTPase RhoA, which is an inhibitor of axonal growth, as well as other neurite outgrowth-related molecules. These results indicate that miR-133b is an important determinant in spinal cord regeneration of adult zebrafish through a reduction in RhoA protein levels by direct interaction with RhoA mRNA. These authors showed that the ability of miR-133b to suppress molecules that inhibit axon regrowth may underlie the capacity for adult zebrafish to recover locomotor function after spinal cord injury (SCI).

MicroRNAs-133b plays an important role in several regulatory processes. For example, in cardiomyocytes, miR-133b serves an antiapoptotic role by inhibiting caspase-9 (Xu et al., 2007). Among its multiple targets, miR-133b down-regulates RhoA protein expression (Care et al., 2007; Chiba et al., 2009). RhoA is strongly upregulated following SCI (Conrad et al., 2005; Erschbamer et al., 2005), and inhibition of RhoA enhances regrowth of the corticospinal tract and promotes neuroprotection by decreasing the tissue damage and cavity formation that develop after SCI (Dergham et al., 2002; Fournier et al., 2003; Tanaka et al., 2004; Hoffmann et al., 2008; Holtje et al., 2009). Considering that multiple cellular and molecular pathways are regulated by miRNAs, and that the targets of miR-133b are conserved throughout development in different species from zebrafish to mammals, it could be considered that these results (such as Yu et al., 2011b) may guide the development of novel strategies for improving functional recovery after SCI in humans. The extent to which miR-133b is involved in multiple pathological phenotypes is outstanding and highly noteworthy. **Table 1** represents a summary of the involvement of miR-133b in relation to different pathological situations. Recent reports show that some miRNAs control major cancer-related signaling molecules, such as epidermal growth factor (Erkan et al., 2011), members of the p53 family (Inui et al., 2010; Ory and Ellisen, 2011), and the retinoblastoma protein (Noonan et al., 2010). In cancer, miRNAs can be divided in two separate classes: those that are tumor suppressive and those that are oncogenic. MiR-133b can participate in both systems, depending whether it is overexpressed (act as oncogenes, repressing tumor suppressor genes), or underexpressed (functioning as a tumor suppressor, negatively regulating oncogenes). **Table 1** summarizes recent research regarding the role of miR-133b in multiple pathologies, including cancers. **Table 2** lists cancers in which miR-133b exerts direct regulation.

The relationship of miR-133b with cancer is important to the topic of this chapter, since morphine and the synthetic compound

Table 1 | Involvement of miR-133b in different physiological situations.

Situation	Reference
Translational regulation of utrophin: miR-133b, related to Duchenne muscular dystrophy, mediates the repression, and confirms repression of miR-206	Basu et al. (2011)
Formation of homologue clusters with miR-206: dysregulation role	Nohata et al. (2012)
Upregulation during late stages of human, fetal muscle development	Koutsoulidou et al. (2011)
When downregulated, miR-133b may have important implications in pathogenesis of essential hypertension	Yu et al. (2011a)
Co-regulation of miR-133b with miR-206, novel biomarkers of Th 17-type immune reactions	Haas et al. (2011)
Desregulation of miR-133b is associated with overall survival and metastasis in colorectal cancer	Akcakaya et al. (2011)
Increase of miR-133b in mouse pectoralis muscle: regulation by myostatin	Rachagani et al. (2010)
Upregulated miR-133b in mouse liver by tyrosine hormone	Dong et al. (2010)
MiR-133b is upregulated on head and neck cancer	Liu et al. (2009)
Mir-133b is regulated by endurance exercise in human skeletal muscle	Nielsen et al. (2010)
Mir-133b is a biomarker of myocardial infection	D'Alessandra et al. (2010)
MiR-133b targets prosurvival molecules MCL-1 and BCL262 in lung cancer	Crawford et al. (2009)

Table 2 | Downregulation of miR-133b in different cancers.

Type of cancer	Reference
Colorectal cancer	Suzuki et al. (2011); Hu et al. (2010); Bandres et al. (2006); Sarver et al. (2009)
Bladder cancer	Song et al. (2010)
Gastric cancer	Wu et al. (2011a)
Lung cancer	Nasser et al. (2008); Crawford et al. (2009); Wu et al. (2011b)
Esophageal squamous cell carcinoma	Kano et al. (2010)
Tongue squamous cell carcinoma	Wong et al. (2008)
Head and neck squamous cell carcinoma	Nohata et al. (2011)

fentanyl are widely used as the analgesic solution for long-term pain suffering in cancer patients, and it is well known that these drugs produce addiction after long-term use. MiR-133b can act as an oncogene or as a tumor suppressor, making the interaction between this miRNA and morphine crucial in the control of a defined cancer pathology. Morphine, not only because it produces addiction, but also because of its own oncogenic effect, can be considered a negative analgesic tool in some cancer patients. Future research is needed to elucidate how to overcome this possibility.

ZEBRAFISH AS A MODEL TO STUDY THE RELATIONSHIP BETWEEN miR-133b AND MORPHINE

Although great efforts have been made on the study of the different mechanisms that are activated by the opioid system in mammalian models, many issues regarding opioid regulation remain unknown. The zebrafish (*Danio rerio*) has been used as an experimental model, not only to study genetics and development, but also to study disease-related pathways, given its easy *in vivo* manipulation. In this sense, the zebrafish can be an important tool to analyze *in vivo* the molecular mechanisms related to the activity and function of the opioid system that cannot be fully established in other models. For instance, in contrast to mammalian embryos, which develop in the uterus and are influenced by the maternal biochemical processes, zebrafish embryos develop externally, avoiding the maternal effect on these embryos. This is essential when dealing with drug exposure, as the effects observed in mammalian embryos might be due to the susceptibility of the mother and not the embryo *per se*. The study of the morphine direct effects in the embryos will provide a better understanding on the molecular mechanisms that underlie the physical and neurobehavioral defects shown in fetuses and offspring after maternal morphine consumption (Nasiraei-Moghadam et al., 2010). Also, the endogenous opioid system has been characterized in the zebrafish, and contains a mu opioid receptor (zfMOR), two DOR duplicates (zfDOR1 and zfDOR2), a kappa opioid receptor (zfKOR) and an opioid receptor like (zfORL) gene (Barrallo et al., 2000; Rodriguez et al., 2000; Alvarez et al., 2006; Pinal-Seoane et al., 2006). Hence, the presence and the existing extensive characterization of opioid receptors in zebrafish allow us to extrapolate key components of the opioid system to other biological models.

OPIOIDS AND THE DOPAMINERGIC SYSTEM

The opioid-addiction pathway has been suggested to involve the midbrain dopaminergic neurons located within ventral tegmental areas and the nucleus accumbens (NAc). The alteration of dopamine levels in this region can produce neuronal sensitization or desensitization, depending on the drug used. It has also been established that morphine increases dopamine level through the mu opioid receptor in the NAc, which may mediate reinforcing effects of morphine (Gianoulakis, 2009). Relevant to these observations, endogenous opioid peptides, such as enkephalins or dynorphins, are upregulated in the NAc after exposure to morphine, and modulate dopamine release in the midbrain (Gieryk et al., 2010).

Hence, studies on the probable opioid regulation of dopaminergic activities in zebrafish could provide insights on mammalian embryonic development during chronic exposure to drugs.

miR-133b AND THE DOPAMINERGIC SYSTEM

miR-133b, regulates the differentiation, maturation, and function of dopaminergic neurons by downregulating the transcription of its target in the dopaminergic system, the homeobox gene pitx3 (Hebert and De Strooper, 2009). Pitx3 activates the transcription of genes directly involved in the differentiation of dopaminergic neurons (**Figure 1**), such as the tyrosine hydroxylase (*th)* and the dopamine transporter (*dat*; Kim et al., 2007).

Taking the above into consideration, we analyzed the effect of morphine on the miR-133b regulatory pathway using zebrafish embryos as a model building on well-established precedents for using zebrafish to study the role of miRs in development (Schier and Giraldez, 2006). At 24 h post fertilization (hpf), the dopaminergic system begins its differentiation and the first TH- positive neurons begin to be detected at this particular developmental stage (Filippi et al., 2007). Our previous studies also indicated that at 24 hfp, the expression of zfMOR, the putative target of morphine (de Velasco et al., 2009) is increased. Therefore, the use of 24 hpf zebrafish embryos provided information on the implication of the opioid system in the maturation and differentiation of dopaminergic neurons compared to any other stages of development, and also, demonstrated that the mu opioid receptor is functional in zebrafish and has a specific role in the development of the CNS and represents a possible pathway that leads to addiction.

MORPHINE MODULATES THE EXPRESSION OF miR-133b

By means of a miRNA array, we observed a decrease in the expression of several miRNAs after embryonic exposure to morphine at three developmental stages: 16, 24, and 48 hpf. Considering the pathways in which each miRNA could be involved, we focused on miR-133b due to its reported effect on dopaminergic neurons, an essential component in drug addiction processes. Our studies were carried out in the 24 hpf embryos, when differentiation of the zebrafish CNS begins.

After validation by qPCR of our microarray results, we observed that miR-133b level was decreased in 24 hpf embryos exposed to morphine, at two different morphine concentrations; 10 and 1 nM. The antagonist naloxone did not significantly change the expression of this miR, but it could block the morphine effect. Although more selective agonists such as DAMGO or antagonists such as CTOP were not used to define the receptor involved due to the lack of affinity of such ligands for zfMOR (de Velasco et al., 2009), the effect of morphine on miR-133b level was probably mediated by the activation of zfMOR.

MORPHINE MODULATES THE EXPRESSION OF miR-133b TARGET PITX3

The transcription factor Pitx3 is a known miR-133b target. Pitx3 has been regulates the transcription of *th* and *dat*. Since miRNAs normally regulate the stability or the translation of the transcripts, by reducing miR-133b morphine should either increase the levels or the activities of these transcripts. Treatment of zebrafish embryos with 1 and 10 nM morphine increases the mRNA levels of *pitx3* and *dat* while morphine treatment decreases miR-133b level. Addition of naloxone effectively abolished the morphine-induced changes in the expression levels of miRNA-133b, *pitx3*, *th*, and *dat*,

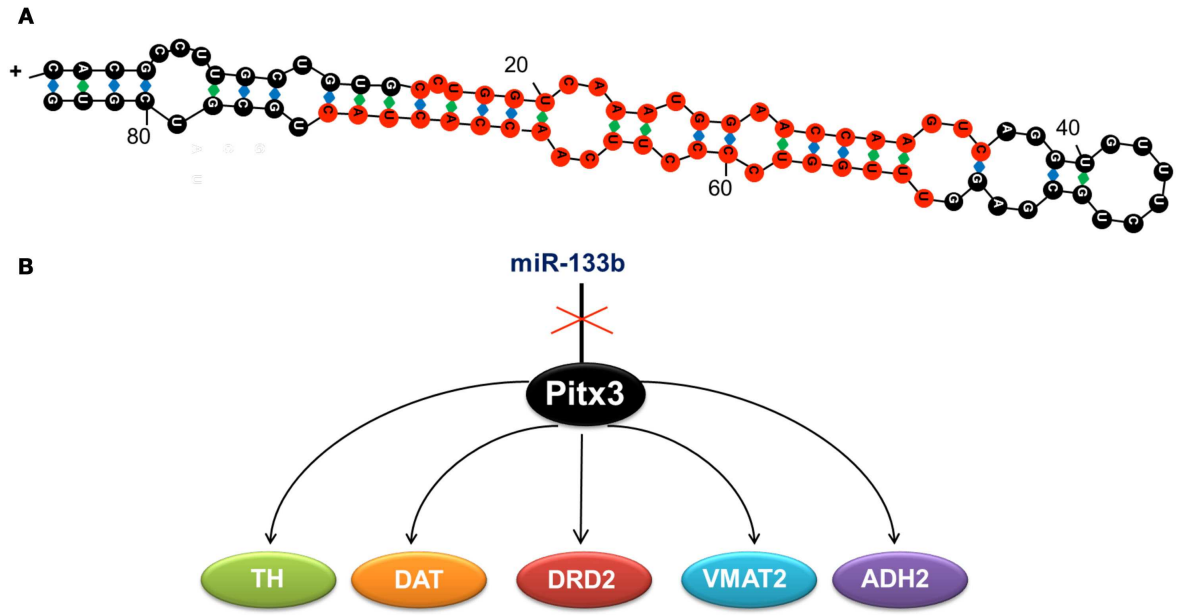

FIGURE 1 | (A) Duplex sequence of miR-133b, formed by 84 ribonucleotides. Mature miRNA is shown in red. **(B)** miR-133b inhibits the expression of transcription factor Pitx3, whose function is to activate the expression of tyrosine hydroxylase (TH), the dopamine transporter (DAT), the dopaminergic receptor (DRD2), the monoamine vesicular transporter type 2 (VMAT2), and the aldehyde deshydrogenase 2 (ADH2). These genes determine the neuronal differentiation to the dopaminergic phenotype, so that when miR-133b is expressed, the expression of the other genes is inhibited and hence, dopaminergic differentiation is blocked.

suggesting that morphine regulates the level of the dopaminergic genes via the control of miR-133b by activating zfMOR.

Although treatment of embryos with morphine clearly decreases the miR-133b level and increases Pitx3 and its targets TH and DAT levels, whether miR-133b indeed interacts with Pitx3 thereby destabilizing the transcript has not been demonstrated in zebrafish.

THE ROLE OF zFMOR IN MORPHINE-INDUCED REGULATION OF miR-133b PATHWAY

The effects of morphine on embryos are probably mediated by zfMOR, the opioid receptor that exhibits highest affinity toward morphine (de Velasco et al., 2009). In order to establish the role of zfMOR in regulating miR-133b without the availability of a zfMOR selective antagonist, we silenced (knocked down) zfMOR by morpholino oligonucleotide injection. The efficiency of the morpholino oligonucleotide to decrease the zfMOR level was determined with qRT-PCR. Injection of 0.2 μM of the morpholino oligonucleotide per embryo reduced the zfMOR transcription level by 95% (the injection of ZfMOR decreased the expression of both ZfDOR1 and ZfDOR2 by ∼2.5%, which is not statistically significant, showing the specificity of the zfMOR morpholino).

The amount of miR-133b increases within embryos when zfMOR is absent. Such an increase was not observed after the injection of a control morpholino. Furthermore, 1 or 10 nM morphine exposure did not alter the miR-133b level in embryos injected with zfMOR morpholino, while the same concentrations of morphine treatment resulted in a decrease of miR-133b levels in embryos injected with control morpholino. The increased expression in miR-133b detected in the zfMOR knock down embryos also led to a decrease of the subsequent miR-133b targets, i.e., Pitx3, TH, and DAT. Clearly, the morpholino and the opioid antagonist naloxone studies indicate zfMOR is the mediator for the morphine-induced regulation of miR-133b and its targets.

MORPHINE-INDUCED REGULATION OF THE miR-133b PATHWAY DEPENDS ON ERK1/2 ACTIVITY

Morphine, regulates multiple signaling pathways via the mammalian receptor MOR. In the rat hippocampus, morphine activates ERK1/2 and decreases the expression level of miR-190 (Zheng et al., 2010b). Whether similar signaling mechanisms are involved in morphine-induced regulation of the miR-133b pathway in zebrafish is unknown. In order to address this possibility, we used several MAPK inhibitors, such as JNK inhibitor II, SB203580 for p38 and PD98059 and U0126 for ERK1/2, to identify the signals involved in morphine-induced miR-133a regulation. The inhibition of JNK and p38 produced a significant decrease in the miR-133b level, and hence, an increase in the level of Pitx3, TH, and DAT. In contrast, the inhibition of MEK1/2 by either U0126 or PD98059 enhanced miR-133b expression, and as a consequence, it decreased the level of Pitx3, TH, and DAT transcripts (**Figure 2**).

Parallel treatment of embryos with morphine in the presence of either JNK or p38 inhibitor did not eliminate the morphine-induced decrease in the miR-133b level. Probably, by activating zfMOR, morphine, via the ERK1/2 pathway, regulates the miR-133b level in the zebrafish embryos.

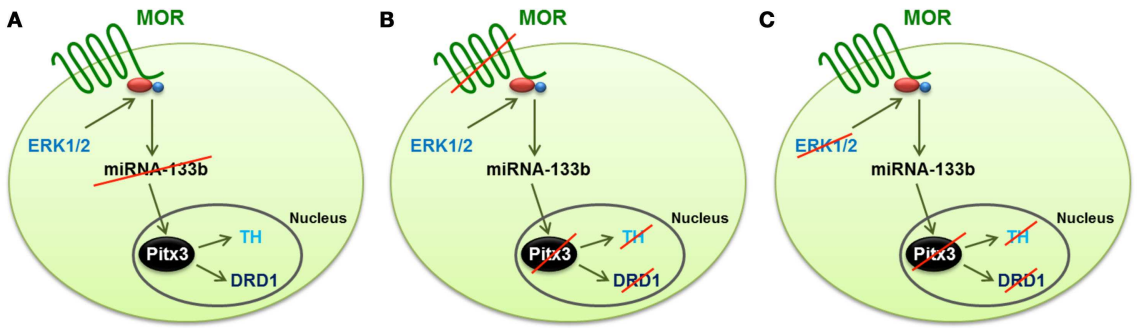

FIGURE 2 | Schematic representation of the mechanism by which morphine regulates the differentiation of dopaminergic neurons through the control of the miR-133b expression and the transcription of the genes regulated by this miRNA (Pitx3, TH, and DAT). (A) Activation of ERK1/2 signaling by MOR reduces the expression of miR-133b, and hence increases the transcription level of its target, Pitx3. This transcription factor enhances the expression of TH and DAT. **(B)** Silencing MOR by specific morpholinos produces an increase in the expression of miRNA-133b, and reduces the transcription level of Pitx3, TH, and DAT. **(C)** Inhibition of ERK1/2, even when the MOR receptor is activated by morphine, produces the same effect as knockdown of the receptor, i.e., the expression of miR-133b increases, and hence, the expression of Pitx3, TH, and DAT decreases, which reduces the level of dopaminergic neuron differentiation.

MORPHINE REGULATES miR-133b EXPRESSION IN HIPPOCAMPAL NEURONS

When using zebrafish as a research model, there is always the question of whether a mammalian counterpart for the observed results exists. In order to determine whether the observed regulation of miR-133b by zfMOR in the zebrafish embryos has any mammalian counterparts, hippocampal neurons obtained from P1 rats were treated with 100 nM morphine. Similar to previously reported studies using mature hippocampal neuron cultures from mice chronically treated with morphine, in which miRNA array and qRT-PCR studies did not reveal any effect on the miR-133b level (Persson et al., 2003), our current studies with mature, differentiated neurons (3-week culture) revealed no effect on the expression of miR-133b when treated with morphine. However, he level of miR-133b was decreased in 1-week-old neurons treated with morphine. Thus, similar to what we have observed in the zebrafish embryos, only the miR-133b level within the immature neurons was affected by morphine treatment.

miR-133b AND THE DELTA OPIOID RECEPTOR

Although the mu opioid receptor displays higher affinity toward morphine than the other classical opioid receptors, delta, and kappa, an interaction between the mu and the DORs has been described *in vitro*. This interaction is thought to be responsible for faster development of morphine tolerance via the mu opioid receptor (Waldhoer et al., 2004). Moreover, knock-out mice for the DOR do not develop tolerance to morphine and do not suffer the withdrawal effect once the long-term treatment with morphine is over (Fundytus et al., 1995). Despite knowledge acquired in the past decade on the mechanisms that define opioid activity, many issues concerning the mechanisms of addiction need to be established. These goals may be achieved by analyzing the different roles of the opioid receptors apart from their analgesic functions, such as their involvement in developmental processes. For instance, the MOR and KOR increase neurogenesis (Kim et al., 2006), and the DOR acts as a neuroprotector (Narita et al., 2006). We have found that morphine protects dopaminergic neurons against glutamate-induced neurotoxicity, and this effect is mediated by the DORs (unpublished). As the mu opioid receptor regulates dopaminergic differentiation and the DORs protect these neurons, we searched for an explanation of how the DORs relate to the adverse effects of morphine.

ROLE OF THE DORS IN MORPHINE-INDUCED REGULATION OF miR-133b PATHWAY

We have studied the role of the DORs from zebrafish in the expression of miR-133b and the genes downstream in its regulatory pathway, in order to determine the specific influence of each delta receptor duplicate as a regulator of this pathway. By knocking down each DOR duplicate, both separately and simultaneously, we demonstrated that they activate the differentiation of dopaminergic neurons. Their complete absence increases miR-133b levels and therefore, decreases the mRNA levels of the genes involved in such differentiation (*pitx3*, *th*, *and dat*). However, when only one DOR is knocked down, morphine slightly decreases the expression of miR-133b, as detected in the control embryos and embryos injected with the control morpholino. These results suggest that when one DOR is not present, the other one functionally complements its role. Partial functional complementation may explain why, although some changes are observed regarding the expression of miR-133b and its related genes, these changes are not constant in untreated and morphine-treated embryos. In contrast, when both DORs are silenced, the effect produced is similar to that observed when the mu opioid receptor is knocked down, suggesting that both types of receptors, mu and delta, are involved in the differentiation of dopaminergic neurons.

CONCLUSION

At present, evidence for the involvement of miRNAs in drug addiction is markedly increasing (Schaefer et al., 2007; Dreyer, 2010; Hollander et al., 2010), although these reports are mainly associated with cocaine. Aside from what we discussed in this review, there has been no direct report concerning opioid addiction.

Within the context of the dopaminergic system's role in addictive disorders, including addiction to morphine (Flores et al., 2004; Leggio et al., 2009), we have established a pathway that may account for the observed morphine-induced increase in dopamine production (Gianoulakis, 2009). By modulating miR-133b regulatory pathways, and hence, dopaminergic differentiation, zfMOR has a specific role in the CNS and is capable of regulating transcription through miRNAs.

Our results lead us to conclude that the consequences of maternal morphine intake on the fetus could take place through the intracellular pathways of miR-133b and Pitx3. As our results suggest, neonate abstinence syndrome might be caused by the alteration in dopaminergic differentiation, induced by morphine. In addition, inhibition of ERK1/2 shows that, the closer the treatment to the timing of early CNS differentiation, the greater is the effect of this inhibition on the expression levels of the genes involved in the maturation and differentiation of dopaminergic neurons.

These data point out the importance of the developmental stage at which embryos are exposed to drugs, as exposure at different stages varies the impact of such drugs on the embryo's development. Thus the control of miR-133b level could be a possible mechanism responsible for the development of addiction to morphine or to other drugs of abuse that increase dopamine levels in the extracellular space. These results show for the first time that the miR-133b is a possible new target for the design of new treatments against addictive disorders.

The differences in the effect of morphine on miR-133b expression of in 1-week and 3-week rat neurons demonstrate that morphine induces differentiation by decreasing the expression of this particular miRNA only in the immature neurons. Therefore, the effects of morphine consumption during pregnancy may impact neuronal differentiation, through inducing changes in miR-133b expression. These results also confirm that in mammals, morphine has the same effect as in the zebrafish in neuronal differentiation through miR-133b.

FUTURE PERSPECTIVES

There is increasing evidence that miRNAs have a role in the control and development of many diseases: cancer (He et al., 2005; Tavazoie et al., 2008, Garzon et al., 2009), cardiovascular diseases (Zhao et al., 2005), autoimmune diseases (Sonkoly et al., 2007), neurodegenerative diseases (Fiore et al., 2008), and numerous others. In clinical practice, miRNAs can be useful as both diagnostic markers and predictors of therapeutic response (Garzon et al., 2009). Further research is warranted to elucidate the interaction between different miRNAs in order to analyze the possible therapeutic value that these post-transcriptional regulators have. The number of targets predicted for each miRNA is enormous; for example, using miR and a software, 1704 targets for miR-133b have been found. Even in this case, where we have proven that miR-133b is involved in the regulation of dopaminergic neurons, we need to find out if other physiological systems are also involved. If we can confirm different miRNAs to specifically regulate different targets, and if these miRNAs can cross-talk producing a functional result, then it is possible that therapeutic agents can be designed to rationally and specifically target the entire discovered complexity of the system. Currently, while inducing or repressing a single miRNA represents a promising therapeutic strategy for specific diseases, we can not say that every miRNA known, by itself, could be a potential therapeutic agent.

The therapeutic value of miRNAs is dictated in part by the fact that miRNAs that are upregulated in different diseases can be targeted using anti-miRNAs (antisense oligonucleotides with specific modifications; Rossbach, 2010). These microRNA inhibitors (antagomiRs) demonstrate that therapeutic targeting of miRs is possible, although these inhibitors have not yet been explored in the specific context of curing drug addiction. Besides this inhibitory therapeutic approach, indirect methods, such as downregulation of specific miRNA biogenesis pathways, could also serve as therapeutics. In the opposite scenario, when miRNAs downregulation is responsible for an abnormal function, as is the case with miRNAs downregulated in tumors, a possible therapeutic approach could be to restore mature miRNA levels in the abnormal tissue.

Accordingly, considering our results relating morphine, the mu opiod receptor, and the dopaminergic system to the miRNA miR-133b, it might be possible to design a system that could control the addiction process in which these four entities are involved, perhaps by rescuing miR-133b levels with the intention of dampening the expression of the downstream targets that positively impact dopaminergic differentiation and opioid addiction. Further research is needed to elucidate the role, and the therapeutic relevance, of miRNAs in the complexity of the addiction pathway.

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Non-coding RNAs regulating morphine function: with emphasis on the *in vivo* and *in vitro* functions of miR-190

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Non-coding RNAs (ncRNAs), especially microRNAs, are reported to be involved in a variety of biological processes, including several processes related to drug addiction. It has been suggested that the biological functions of opioids, one typical type of addictive drugs, are regulated by ncRNAs. In the current review, we examine a variety of mechanisms through which ncRNAs could regulate μ-opioid receptor (OPRM1) activities and thereby contribute to the development of opioid addiction. Using miR-23b as an example, we present the possible ways in which ncRNA-mediated regulation of OPRM1 expression could impact opioid addiction. Using miR-190 as an example, we demonstrate the critical roles played by ncRNAs in the signal cascade from receptor to systemic responses, including the possible modulation of adult neurogenesis and *in vivo* contextual memory. After discussing the possible targets of ncRNAs involved in the development of opioid addiction, we summarize the mechanisms underlying the interaction between ncRNAs and opioid addiction and present suggestions for further study.

Keywords: microRNA, miR-190, non-coding RNA, opioid addiction

INTRODUCTION TO ncRNAs

The central dogma – DNA transcription results in RNA production and RNA translation results in protein production – places RNA as an intermediate between gene and protein. It has been proposed that the information storage and catalytic functions of RNA have been transferred to DNA and protein, respectively, during evolution, however, the publication of the alanine tRNA structure in 1965 suggested that translation into protein was not the only function of an RNA molecule (Holley et al., 1965). Thus, non-coding RNA (ncRNAs, also named non-protein-coding RNA or non-messenger RNA) and mRNA are now considered to be two distinct parts of the RNA world.

For many years, tRNAs and rRNAs were the only known ncRNAs. However, more ncRNAs have been discovered recently, including small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and long ncRNAs (Huttenhofer et al., 2005; Kurth and Mochizuki, 2009).

With the exception of long ncRNAs, ncRNAs are typically categorized by their function. For example, piRNAs form RNA-protein complexes with piwi proteins to induce epigenetic and post-transcriptional gene silencing in germ line cells (Girard et al., 2006). miRNAs bind to target mRNA via complementary sequences, usually resulting in translational repression or target degradation (Zeng and Cullen, 2003; Zeng, 2006; Bartel, 2009). Long ncRNAs are arbitrarily considered to be longer than 200 nucleotides. Although the functions of long ncRNAs require further investigation, they may function as signals, decoys, guides, or scaffolds (Kapranov et al., 2007; Mercer et al., 2009; Wang and Chang, 2011), and may be involved in drug addiction (Michelhaugh et al., 2011). In general, ncRNAs have been reported to affect multiple aspects of gene expression in a variety of biological processes (Shivdasani, 2006; Bueno et al., 2008; Ivey and Srivastava, 2010; Kaikkonen et al., 2011), including the development of several diseases, such as cancer, autism, Alzheimer's disease and drug addiction (Talebizadeh et al., 2008; Dreyer, 2010; Ferdin et al., 2010; Satoh, 2010). The current review discusses the involvement of ncRNAs in opioid addiction.

INTRODUCTION TO OPIOID ADDICTION

Alcohol, nicotine, caffeine, and opioids are the classic addictive drugs. Addiction to such drugs has been historically viewed from two aspects: (1) compulsive drug-taking and drug-seeking behavior is acquired because of the rewarding and reinforcement properties of the drug; and (2) drug withdrawal episodes enhance the drug's incentive value to such an extent that compulsive drug-seeking and drug-taking takes over the behavioral repertoire. Although there are common pathways for drug addiction, the ability of these individual drugs to activate distinct signaling cascades and induce specific biological responses through binding to different receptors indicate that addiction to these drugs occurs through diverse mechanisms. In the current review, we focus on the probable influence of ncRNAs on opioid addiction (Nestler and Aghajanian, 1997).

The history of opioid addiction can be traced back to the first use of morphine, one of the most efficacious and oldest drugs in the treatment of moderate to severe pain. There are also other opioids used in clinic or laboratory: fentanyl, oxycodone, etorphine, and [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO). Although opioid addiction is considered to be a complex process involving genetic/epigenetic, cellular, and molecular regulation of neuronal plasticity and drug-related contextual memory, opioid receptors are definitely the major determinants in the addiction process because the receptors are the initiation sites of all opioid function. Since the first discovery of the opioid receptor in 1973 by scientists (Pert and Snyder, 1973), four members in the family of opioid receptors have been identified: μ-opioid receptor (OPRM1), δ-opioid receptor (OPRD1), κ-opioid receptor (OPRK1), and nociceptin receptor (ORL1). OPRM1 and OPRM1-related pathways are discussed in the current review.

OPIOID ADDICTION AND ncRNAs

Although there has been no reported exploration of how ncRNAs regulate opioid addiction, several miRNAs have been suggested to interact with the signaling pathway downstream of OPRM1 and possibly be involved in the opioid addiction process, as reviewed by Dreyer and Li (Dreyer, 2010; Li and van der Vaart, 2011).

As summarized in **Table 1**, by inducing a decrease in miR-133b and a subsequent increase in the expression of Pitx3, morphine regulated dopaminergic neuron differentiation in zebra fish embryos (Sanchez-Simon et al., 2010). Significant changes in the expression of 26 miRNAs, including miR-15b and miR-181b, were identified between morphine-treated and control human monocyte-derived macrophages (Dave and Khalili, 2010). A let-7 binding site was identified in the 3′-UTR of OPRM1 mRNA, and decreasing the brain level of let-7 partially attenuated the tolerance induced by morphine (He et al., 2010). In the previous reports from our laboratory, long-term morphine treatment increased the expression of miR-20a, miR-23b, miR-224, miR-331, and miR-365 in both mice hippocampi and primary cultures of rat hippocampal neurons. Long-term fentanyl treatment increased the expression of miR-224, miR-331, and miR-365, but decreased the expression of miR-184, miR-190, and miR-301 (Wu et al., 2009; Zheng et al., 2010d). The agonist-selective regulation of miR-190 and its target, neurogenic differentiation 1 (NeuroD), resulted in differential influences over the dendritic spine stability of hippocampal neurons (Zheng et al., 2010c). In addition, miR-23b targeted the 3′-UTR of OPRM1 mRNA and regulated the association between OPRM1 mRNA and polysomes (Wu et al., 2008, 2009). Five long ncRNAs were up-regulated in the nucleus accumbens of heroin abusers when compared with drug-free control subjects (Michelhaugh et al., 2011). The ncRNAs listed in **Table 1** may contribute to the development of opioid addiction by affecting different aspects of opioid signaling, though further confirmation is still required.

ncRNAs MAY CONTRIBUTE TO OPIOID ADDICTION BY CONTROLLING RECEPTOR EXPRESSION

On the one hand, opioid treatment affects the expression of several miRNAs: morphine treatment affected the expression levels of miR-23b and let-7, which have binding sites on the 3′-UTR of the OPRM1 mRNA and control the expression of OPRM1 (Wu et al., 2008, 2009; He et al., 2010). On the other hand, the expression level of opioid receptor, especially membrane receptor, is essential for receptor signaling or even opioid addiction. Some OPRM1 agonists, like etorphine and DAMGO, induce receptor internalization and subsequent loss of membrane receptor after acute treatment (El Kouhen et al., 2001; Eisinger and Schulz, 2005). Although debate still exists, receptor internalization correlates closely with signaling desensitization *in vitro* (Koch et al., 2001; Qiu et al., 2003) and analgesia tolerance *in vivo* (Zuo, 2005; Narita et al., 2006). In addition, OPRM1 down-regulation has been observed after chronic treatment with morphine (Davis et al., 1979) and has been considered as one mechanism for the development of opioid tolerance (Tao et al., 1987; Bhargava and Gulati, 1990). Since tolerance is linked with addiction, it is still reasonable to suggest the involvement of receptor down-regulation in opioid addiction. Thus, the signaling cascade from opioid to the expression of several

Table 1 | Non-coding RNAs involved in opioid signaling.

ncRNAs involved	Phenomena	Reference
let-7	Morphine increases the expression of let-7, which binds to the 3′-UTR of OPRM1 mRNA. Decreasing let-7 impaired morphine-induced tolerance	He et al. (2010)
miR-15b, miR-181b, plus 24 additional miRNAs	These 26 miRNAs were regulated in morphine-treated human monocyte-derived macrophages	Dave and Khalili (2010)
miR-20a, miR-184, miR-224, miR-301, miR-331, and miR-365	Morphine increases the expression of miR-20a, miR-23b, miR-224, miR-331, and miR-365. Fentanyl increases the expression of miR-224, miR-331, and miR-365, but decreases the expression of miR-184, miR-190, and miR-301	Zheng et al. (2010d)
miR-23b	Morphine increases the expression of miR-23b, which can regulate the expression of OPRM1	Wu et al. (2008, 2009)
miR-133b	Morphine decreases the expression of miR-133b in zebrafish embryos, which subsequently affects dopaminergic neuron differentiation	Sanchez-Simon et al. (2010)
miR-190	Fentanyl decreases the expression of miR-190, which subsequently affects the NeuroD-related pathways	Zheng et al. (2010a,c,d)
MIAT, MEG3, NEAT1, NEAT2, and EMX2OS	These five long ncRNAs are up-regulated in the nucleus accumbens of heroin abusers	Michelhaugh et al. (2011)

ncRNAs and then to OPRM1 expression may be a mechanism for opioid addiction.

There have been numerous studies of the promoter region and UTR of OPRM1 (Min et al., 1994; Kraus et al., 1995; Shigeta et al., 2008). Two miRNAs have been reported to bind the 3′-UTR of OPRM1 mRNA and regulate the expression of OPRM1. Let-7 bound to the 399–405 region in 3′-UTR of the human OPRM1 mRNA and the 402–408 region in the 3′-UTR of mouse OPRM1 mRNA. It also impaired the association between OPRM1 mRNA and polysomes (He et al., 2010). In our laboratory, the K box in the 3′-UTR of the OPRM1 mRNA (3805–3812 bp downstream from the stop codon) was identified to be a negative *cis*-acting element (Wu et al., 2008). Since, in *Drosophila*, K box interacts with miR-2 and miR-16, which have seed sequences homologous to that of miR-23b (Kimura et al., 2004; Kokkola et al., 2005), we assessed the ability of miR-23b to regulate OPRM1 expression. Down-regulation of miR-23b expression increased the endogenous level of OPRM1 protein in NS20Y cells (Wu et al., 2008). In order to determine the involvement of miR-23b in the signaling cascade of OPRM1, we also tested the expression of miR-23b after morphine treatment. Morphine treatment increased the expression of miR-23b in an exogenous system (N2A cells stably expressing OPRM1) as well as an endogenous system (SHSY5Y and NMB cells; Wu et al., 2009). Although transcriptional regulation of OPRM1 mRNA is limited during opioid addiction since OPRM1 mRNA level does not change after morphine treatment (Brodsky et al., 1995), the post-transcriptional regulation of receptor expression should be studied in depth.

Let-7 and miR-23b are definitely not the only ncRNAs that regulate the expression of OPRM1. Additional ncRNAs can be identified via bioinformatics methods, microarray studies, or other experimental procedures. Basing future studies on the current understanding of ncRNAs, it will not be difficult to explore the mechanisms through which the identified ncRNAs regulate OPRM1 expression. However, it will be difficult to explore the roles played by these ncRNAs in opioid addiction. One of the most reasonable studies will be to determine whether opioid treatment can affect the expression of these miRNAs, as with the studies on let-7 and miR-23b.

ncRNAs MAY CONTRIBUTE TO OPIOID ADDICTION VIA miR-190-RELATED PATHWAYS

Addiction is highly related to changes in neuronal activity and involves a number of brain nuclei, thus, modulating neuronal circuitry should be one possible mechanism through which ncRNAs regulate opioid addiction (Di Chiara et al., 2004; Kelley, 2004; Koob, 2009). Since neuronal circuitry is a large and complex topic and ncRNAs can affect the expression of many proteins within the neuronal circuitry (Bartel, 2004; Kosik, 2006), the current discussion focuses on the signaling cascade surrounding miR-190.

Using microarray analysis, we determined the opioid-induced changes in the expression profiles of miRNAs in primary cultures of hippocampal neurons and in mice hippocampi (Zheng et al., 2010d). Two opioids, morphine and fentanyl, were used in our studies, because of their different characteristics in inducing receptor internalization, receptor phosphorylation, and receptor desensitization (Keith et al., 1996; Zhang et al., 1998; Chu et al., 2010; Zheng et al., 2011). The two opioids induced similar changes in the expression of miR-224, miR-331, and miR-365, but had agonist-selective effects on the expression of miR-20a, miR-184, miR-190, and miR-301 (Zheng et al., 2010d). In our several other reports, we delineated both the upstream and downstream signaling pathways of miR-190 (Zheng et al., 2010a,b,c, 2011).

The signaling cascades surrounding miR-190 are summarized in **Figure 1**. Fentanyl, but not morphine, uses the β-arrestin pathway to induce extracellular signal-regulated kinase (ERK) phosphorylation and leads to the nuclear translocation of phosphorylated ERK. Phosphorylated ERK decreases transcription of Talin2 by affecting the phosphorylation status of Yin Yang 1 (YY1) and the subsequent interaction between YY1 and Talin2 promoter (Zheng et al., 2008, 2010a). Since miR-190 is located in the intron of Talin2, fentanyl treatment decreases the expression of miR-190. Since morphine induces ERK phosphorylation in a PKC-dependent manner, morphine treatment does not affect the expression of miR-190, an example of agonist-selective regulation on miR-190 expression.

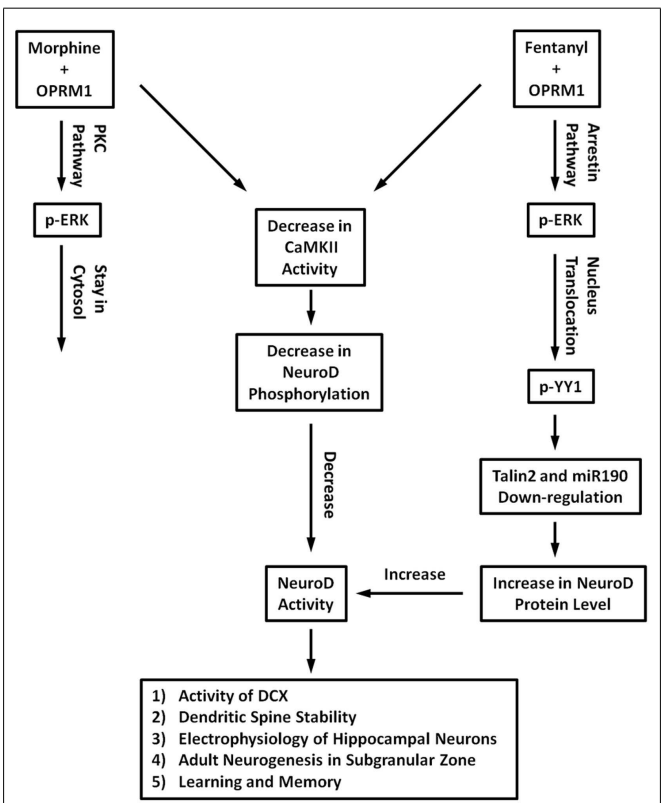

FIGURE 1 | Schematic illustration of signaling pathways surrounding miR-190. Morphine uses PKC pathway to induce ERK phosphorylation, fentanyl induces ERK phosphorylation in a β-arrestin-dependent manner. Fentanyl decreases miR-190 expression via β-arrestin pathway, YY1, and talin2. Fentanyl-increased NeuroD protein level was mediated by miR-190. Since both morphine and fentanyl impaired CaMKIIα activity, there is agonist-selective regulation on NeuroD activity. Morphine decreases NeuroD activity, but fentanyl keeps it at basal level. The activity of NeuroD may contribute to DCX expression, dendritic spine stability, neuron functions, adult neurogenesis, learning, and memory.

miR-190 binds to the 3′-UTR of NeuroD mRNA and induces a decrease of NeuroD protein (Zheng et al., 2010d). NeuroD activity is influenced by both the NeuroD protein level and calcium/calmodulin-dependent protein kinase IIα (CaMKIIα)-mediated phosphorylation on serine 336 (Gaudilliere et al., 2004). Since both morphine and fentanyl decrease the activity of CaMKIIα, the two opioids regulate NeuroD activity differentially (Zheng et al., 2010c). On the one hand, after fentanyl treatment, decreased expression of miR-190 leads to an increase in NeuroD protein, which counteracts the reduced activity of CaMKIIα. Thus, fentanyl maintains NeuroD activity close to basal levels. On the other hand, after morphine treatment, miR-190 expression is not affected, but the reduced activity of CaMKIIα leads to the impaired activity of NeuroD. Thus, morphine decreases NeuroD activity (Zheng et al., 2010c).

Since NeuroD is critical for neuronal morphology (Gaudilliere et al., 2004), morphine and fentanyl differentially regulate dendritic spine stability and the electrophysiology of hippocampal neurons (Liao et al., 2007a,b; Zheng et al., 2010c). Furthermore, due to the involvement of NeuroD and its target, Doublecortin (DCX), in adult neurogenesis in the subgranular zone of dentate gyrus (Cho and Tsai, 2004; Hevner et al., 2006; von Bohlen Und Halbach, 2007), there may exist a connection between agonist-selective signaling, opioid addiction, and ncRNA expression (Eisch and Harburg, 2006; Leuner et al., 2006).

To characterize the possible connection between miR-190-related signaling pathways and opioid addiction, we categorized the factors mentioned above into three types: (1) those related to agonist-dependent signaling of OPRM1 including β-arrestin, ERK, and YY1; (2) those expressed in the central nervous system with close relation with miR-190, including CaMKIIα and Talin2; and (3) NeuroD and NeuroD-related biological processes, including dendritic spine stability, adult neurogenesis, contextual learning, and memory.

Agonist-selective signaling

The first several factors discussed here are closely related to agonist-selective signaling. Different from the "intrinsic efficacy" concept, which suggests that each agonist activates the signaling pathways under one particular receptor with similar efficacies (Furchgott, 1966; Kenakin, 1995), agonist-selective signaling theory suggests that agonists may activate different signaling pathways differentially (Urban et al., 2007; Drake et al., 2008). Although no direct evidence links agonist-selective signaling with opioid addiction, the opioids do have differing abilities to induce tolerance (Duttaroy and Yoburn, 1995).

Extracellular signal-regulated kinase phosphorylation may be involved in opioid addiction. The ERK pathway has been reported to regulate learning and memory, which in turn relate to drug addiction (Mazzucchelli et al., 2002). In addition, addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in the mouse brain (Valjent et al., 2004). The possible involvement of the ERK pathway in opioid addiction is further supported by the roles of ERK in opioid reward (Liu et al., 2007), opioid withdrawal (Asensio et al., 2006; Li et al., 2010), and opioid-induced conditioned place preference (Valjent et al., 2006; Lin et al., 2010).

Agonist-selective ERK phosphorylation may be involved in opioid addiction. Two pathways can be used by G protein-coupled receptors (GPCRs) to mediate ERK phosphorylation: the PKC/PKA pathway and the β-arrestin pathway, as demonstrated by siRNA and kinase inhibitor studies (Shenoy et al., 2006; DeWire et al., 2007; Violin and Lefkowitz, 2007). The selectivity of agonists for these two pathways is indicated by the abilities of agonists to use the pathways to induce ERK phosphorylation (Azzi et al., 2003; Kohout et al., 2004; Shenoy et al., 2006). For OPRM1, morphine acts through the PKC pathway to induce ERK phosphorylation, and the phosphorylated ERK remains in the cytosol. Etorphine and fentanyl prefer to use the β-arrestin pathway to induce ERK phosphorylation, and the phosphorylated ERK translocates into nucleus (Zheng et al., 2008). Since the differences among opioids in inducing ERK phosphorylation are consistent with their differences in inducing receptor phosphorylation (Zhang et al., 1998; Zheng et al., 2010b, 2011), receptor internalization (Keith et al., 1996; Koch et al., 2005), and receptor desensitization (Johnson et al., 2006; Chu et al., 2008, 2010), agonist-selective ERK phosphorylation may be involved in opioid addiction.

How ERK phosphorylation and agonist-selective ERK phosphorylation contribute to opioid addiction is still unclear. Whether miR-190, which is downstream of ERK pathway, is involved is also not clear. However, miR-190 is not likely to be the only ncRNA whose expression is regulated by ERK pathway. In addition, it is likely that there additional ncRNAs that regulate the expression of ERK or related factors like PKC and β-arrestin. Thus, further investigation is required to define the connection between agonist-selective signaling and opioid addiction.

Neuronal related factors

The sequence of miR-190 is conserved between human, mouse, and rat, and is located in the intronic regions of the gene encoding Talin2 in each genome (Griffiths-Jones et al., 2008). Similar to other miRNAs located within the intronic regions (Rodriguez et al., 2004), the expression of miR-190 is regulated by Talin2 promoter activity, at least in part (Zheng et al., 2010d). Although Talin2 has not been well studied, its functions can be predicted by the reports on its homolog, Talin1. Talin1 is considered to be an integrin-associated cytoskeletal protein and induces conformational changes in integrin that increase the affinity of its extracellular domains for ligand (Calderwood, 2004; Critchley, 2009). Talin2 is similar in amino acid sequence to Talin1 (74% identity and 86% similarity). Talin1 expression is highest in the heart and lowest in the brain, as measured in both mouse and human (Ben-Yosef and Francomano, 1999; Monkley et al., 2001). The Talin2 expression pattern is different from that of Talin1, with highest expression in heart and the second highest expression in brain (Monkley et al., 2001). Considering their sequence similarity and differential expression patterns, Talin2 may execute the function of Talin1 in brain. Thus, the opioid-induced modulation of Talin2 transcription may affect cell adhesion to the extracellular matrix, which may subsequently influence neuronal circuitry and opioid addiction. These functions indicate that Talin2 is a potential start for ncRNA-mediated regulation of opioid addiction.

CaMKIIα activation is one of OPRM1's downstream signaling pathways (Lou et al., 1999). The activity of CaMKIIα increases

after acute morphine treatment, gradually returns to basal levels after chronic morphine treatment, and reaches to a high level if naloxone is used to precipitate the opiate withdrawal (Lou et al., 1999). As an important factor in central nervous system, CaMKIIα is essential for synaptic transmission and dendritic morphology (Colbran and Brown, 2004), and its phosphorylation is associated with microtubule stabilization and dendrite formation (Vaillant et al., 2002; Okamoto et al., 2009). In addition, CaMKIIα activation has been reported to be a mediator for the development of opioid tolerance (Fan et al., 1999; Shukla et al., 2006). The CAMKIIα signaling pathway has been considered a common pathway for drug addiction (Steiner et al., 2007; Anderson et al., 2008; Li et al., 2008).

NeuroD

Neurogenic differentiation 1 is critical for the development of both the central nervous and endocrine systems. NeuroD null mice have difficulty surviving after birth without insulin supplement. Surviving mice have defects in the cerebella and hippocampi (Cho and Tsai, 2004). NeuroD is an important transcription factor during adult neurogenesis in the subgranular zone of the hippocampus (von Bohlen Und Halbach, 2007), and is expressed at high levels during the differentiation and migration of neuronal stem cells. In addition to supporting the formation of new neuronal circuitry, NeuroD also contributes to the stability of existing circuitry. CaMKIIα-mediated NeuroD phosphorylation supports the formation and maintenance of dendritic morphology in cerebellar granule neurons (Gaudilliere et al., 2004). Reducing NeuroD activity with miR-190 or CaMKIIα inhibitors impairs the stability of dendritic spines in hippocampal neurons (Zheng et al., 2010c). Therefore, by regulating the neuronal circuitry, NeuroD may contribute to opioid addiction.

In addition to NeuroD itself, its target doublecortin (DCX) is also a critical factor in adult neurogenesis (Seo et al., 2007; von Bohlen Und Halbach, 2007). Since addictive drugs such as alcohol, morphine, and cocaine regulate adult neurogenesis in the hippocampus (Eisch et al., 2000; Nixon, 2006; Noonan et al., 2010), a connection between adult neurogenesis and drug addiction has been suggested (Eisch and Harburg, 2006; Canales, 2007). This hypothesis is supported by studies on learning and memory which are connected to both neurogenesis (Leuner et al., 2006; Deng et al., 2010) and drug addiction (Davis and Gould, 2008; Robbins et al., 2008). In addition, miR-19b and miR-124 have been reported to target NeuroD (Liu et al., 2011; Zhang et al., 2011), thus, these two ncRNAs may regulate opioid addiction by affecting NeuroD-related pathways.

ncRNAs MAY CONTRIBUTE TO OPIOID ADDICTION BY AFFECTING OTHER BIOLOGICAL PROCESSES

Above we discuss how factors related to miR-190 may contribute to opioid addiction. Other miRNAs, including miR-133b and let-7, will be described in depth in other reviews in this issue. However, not all miRNAs identified in the published reports have been well studied. For example, 24 miRNAs, in addition to miR-15b and miR-181b, were identified in a comparison of miRNA expression in morphine-treated versus control samples (Dave and Khalili, 2010). We also identified several miRNAs whose expression changed significantly after morphine or fentanyl treatment in primary hippocampal cultures and mouse brain (Zheng et al., 2010d). Although the majority of these miRNAs relate to cell proliferation or cancer development, it was demonstrated that several miRNAs are involved in opioid addiction.

For example, miR-26b targets the 3′-UTR of brain-derived neurotrophic factor (BDNF) mRNA (Caputo et al., 2011). BDNF is a neurotrophin that is an essential part of neuronal development and plasticity (Greenberg et al., 2009). A study on alcohol and cocaine also demonstrated BDNF's central role in drug addiction (Janak et al., 2006; McGinty et al., 2010). The expression of miR-132 in neuronal cultures is up-regulated by BDNF (Numakawa et al., 2011), and miR-132 mediates the regulation of BDNF on glutamate receptors (Kawashima et al., 2010). Other studies have revealed the interaction between miR-132 and other addiction-related pathways, including CREB and ERK signaling cascades (Remenyi et al., 2010), dendritic spine morphology and synaptic physiology (Edbauer et al., 2010; Mellios et al., 2011), and the integration of newborn neurons into the adult dentate gyrus (Luikart et al., 2011). miR-301 affects the ERK and CREB pathways by targeting EMOX2 (Cao et al., 2010).

SUMMARY

To further explore the possible mechanisms through which ncRNAs contribute to opioid addiction, a simplified schematic illustration is provided in **Figure 2**. The binding of an opioid to OPRM1 leads to various biological responses, some of which ultimately lead to addiction. The ncRNAs involved in opioid addiction can be classified into two categories: (1) ncRNAs whose expression are regulated by opioids and mediate one or several biological responses that lead to addiction, and (2) ncRNAs whose expression are not regulated by opioids, but support the cascade from receptor to biological responses and then to addiction. The ncRNAs listed in **Table 1** should belong to the first category if their contribution to opioid addiction is confirmed in future investigations, since not all ncRNAs regulated by opioids contribute to the development of addiction. The number of ncRNAs in the second category can be larger than those in the first, since the development of opioid addiction requires a large number of biological steps. Unfortunately, reports on these ncRNAs are limited, possibly because they are not regulated by opioids and thus have relatively lower significance in this field.

In further studies of the relationship between ncRNAs and opioid addiction, the key point will be to confirm the involvement of ncRNAs in the development of opioid addiction. One common method for confirmation is monitoring the development of opioid addiction after modulating the expression of a specific ncRNA. The categorization of ncRNAs mentioned above will be also useful when using the involved ncRNAs as targets to control the development of opioid addiction. Counteracting opioid-induced changes in the expression of ncRNAs in category 1 may lead to fewer side effects than blocking opioid tolerance by modulating the expression of ncRNAs in category 2.

As mentioned above, most functions of ncRNAs target different aspects of gene expression, thus, controlling the expression of essential factors in opioid signaling cascades or neuronal circuitry is one of the most likely mechanisms for regulation of opioid addiction by ncRNAs. In the sections above, we discussed several

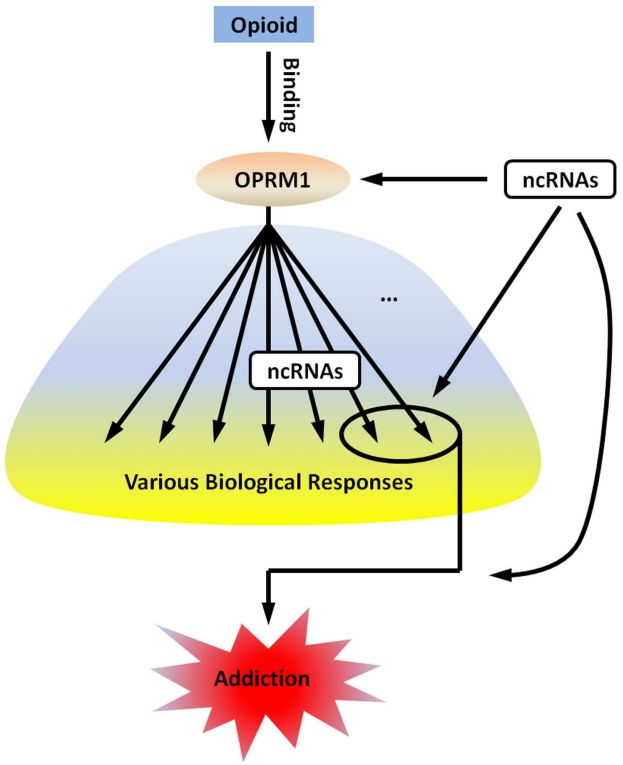

FIGURE 2 | The possible mechanisms through which ncRNAs contribute to opioid addiction. The binding of an opioid to OPRM1 leads to various biological responses, some of which ultimately lead to addiction. The ncRNAs involved in opioid addiction can be classified into two categories: (1) ncRNAs whose expression are regulated by opioids and mediate one or several biological responses that lead to addiction; and (2) ncRNAs whose expression are not regulated by opioids, but support the cascade from receptor to biological responses and then to addiction.

potential targets or target groups, including OPRM1 itself, factors related to agonist-selective signaling, and factors related to neuronal circuitry. As mentioned above, morphine treatment affected the expression levels of miR-23b and let-7, which have binding sites on the 3′-UTR of the OPRM1 mRNA. Since modulating let-7 expression could regulate morphine tolerance, it is reasonable to suggest that modulating miR-23b expression could lead to the similar effects. Furthermore, the other reported functions of miR-23b, like down-regulation of the very low lipoprotein receptor and activation of TGFβ1/Smad3 signaling pathway, may also influence opioid addiction (Ouda et al., 2011; Yuan et al., 2011).

Because of the multiple targets that may be impacted by a single ncRNA, as well as the complex and interrelated regulation of ncRNAs and their targets, it is difficult to identify the actual physiological role that each ncRNA exhibits in drug addiction processes. However, our studies of miR-190, which was identified in microarray analyses rather than bioinformatics, and the central transcription factor it regulates, NeuroD, will reveal interesting aspects of the role of ncRNAs in drug addiction.

By no means is miR-190-mediated regulation of NeuroD activities the definitive "the" physiological function of this miRNA. Rather, its activities will depend on the state of the neuronal cells in which it is expressed. For example, in addition to NeuroD, miR-190 can interact with other transcription factors that regulate neurogenesis, such as Pax6 (Zheng et al., 2010d). Pax6 expression is high in neural progenitor cells but low in differentiated neurons (Bel-Vialar et al., 2007; Kallur et al., 2008). Therefore, one can imagine that an addictive drug such as morphine, by regulating a single ncRNA level such as miR-190, will have differential effects on cellular response, whether it is proliferation or differentiation, and will be dependent on the network of transcripts being expressed during the drug treatment.

Furthermore, because ncRNAs are end products of transcript maturation, one expects that different neurons, different neural circuitry, and different brain regions will exhibit differential regulation of ncRNAs by the same addictive drugs. Such complex patterns of ncRNA regulation could have significant impact on the overall responses to addictive drugs. Our study on miR-190 regulation of NeuroD activities leading to dendritic morphology changes is only the beginning of a very complex and elaborate process by which addictive drugs such as morphine could alter neural activities and, eventually, behavioral outcome.

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Connecting ncRNA cigarette smoking studies with tobacco use behaviors and health outcomes

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Many studies have demonstrated that smoking behavior is influenced by both genetic and environmental factors, and that these influences can change over time and during development (Swan et al., 1996; Han et al., 1999; Koopmans et al., 1999; Maes et al., 1999; Stallings et al., 1999; True et al., 1999; Kendler et al., 2000; McGue et al., 2000; Hopfer et al., 2001, 2003; Rhee et al., 2003). For example, most studies comparing heritability of age of initiation of smoking and regular use patterns suggest that age of initiation is more likely influenced by environmental factors, while progression to regular use and addiction are more heritable (Han et al., 1999; Koopmans et al., 1999; Stallings et al., 1999; McGue et al., 2000; Rhee et al., 2003). Until recently, most studies of smoking behaviors aimed at understanding mechanisms have been forced to examine genetic and environmental influences separately, because little was known about genetic features that were modified by environmental factors. Within recent years, two major areas of research have exploded to provide possible novel mechanisms that may help explain why behavioral disorders such as substance dependence "runs in families" beyond the separate entities of environmental exposure and common genes. The first is epigenetic reprogramming, whereby long-lasting changes to the DNA such as methylation on CpG islands (Kim et al., 2009) and histone tail modifications (Berger, 2007) are conferred upon environmental exposures. The second is the involvement of ncRNAs in regulation of gene expression (Li and van der Vaart, 2011), and the topic of the review article by Maccani and Knopik (2012).

Despite decreasing rates of smoking over the last several decades, maternal smoking during pregnancy (MSP) remains a major public health problem (Mathews, 2001). MSP has been associated with many behavioral problems including irritability in neonatal infants (Stroud et al., 2009), attention behavioral problems in children (Fried et al., 1992), disruptive behaviors in teenagers (Wakschlag et al., 2011), and increased risk of tobacco dependence in young adults (Buka et al., 2003). Furthermore, secondhand smoke (SHS) exposure during childhood has been associated with adverse behavioral and cognitive outcomes (reviewed in Niaura et al., 2001; Shenassa et al., 2003; Herrmann et al., 2008). Animal studies provide more evidence that *in utero* exposure to nicotine directly affects offspring behaviors. For example, mice exposed to nicotine or alcohol *in utero* showed impairment in anxiety and learning behaviors (Li and Wang, 2004). These findings have been replicated in an elegant study of nicotine exposure throughout early development where mice were allowed to orally self-administer nicotine during gestation and lactation. Exposed pups showed dramatic differences for multiple behaviors including nicotine self-administration, social interactions, and performance on a forced swim test (Chistyakov et al., 2010). However, as reviewed recently by Winzer-Serhan (2008), other animal studies examining the effects of nicotine, which does not contain all of the ingredients in tobacco, have been contradictory. As discussed by Maccani and Knopik, specific miRNAs were downregulated in placental cell lines exposed to nicotine and benzo[a]pyrene, but different miRNAs were dysregulated in lung and airway epithelium tissue. In general, the few studies examining the effects of various aspects of cigarette smoking on miRNA expression have revealed that responses are likely to be highly complex, with tissue, temporal, and type of exposure leading to differential responses.

As we consider possible effects of environmental exposure on long-lasting biological mechanisms such as epigenetic reprogramming and ncRNA regulation, it is important to remember that smoking may exert its effects in different ways throughout one's life course, including *in utero*, early childhood, adolescence, young adulthood, and adulthood. Most of the mechanistic work to date has examined *in utero* smoking exposure, but there is evidence for a continuum of effect. Most smokers begin smoking during adolescence, and several studies have shown that earlier age of initiation is associated with increased risk for later dependence (Khuder et al., 1999; Lando et al., 1999; Hu et al., 2006; Palmer et al., 2009). Interestingly, there is a genetic example that associations between *CHRN* genes (encoding the nicotinic receptor subunit genes) and nicotine dependence, may differ between subjects who started smoking early and those with later onset (Schlaepfer et al., 2008; Weiss et al., 2008; Ducci et al., 2011; Hartz et al., in press). The association between *CHRN* genes and tobacco behaviors is one of the most widely replicated findings among substance abuse genetics (Amos et al., 2008; Bierut et al., 2008; Thorgeirsson et al., 2008, 2010; Saccone et al., 2010), so this developmental aspect is of high interest. These results suggest the possibility of a "critical period" during adolescence where environmental effects may have stronger effects on certain genetic mechanisms.

In addition, many animal studies have shown that the nicotinic acetylcholine receptors (nAChRs) are likely to be targets for nicotine's effects during prenatal tobacco exposure. Numerous studies have shown upregulation of certain nAChR subtypes (e.g., $\alpha 2\beta 4$-containing) in various brain regions following postnatal (Marks et al., 1992; Lain et al., 2005) and prenatal exposure (Navarro et al., 1989; Popke et al., 1997; Tizabi et al., 1997). These findings have been replicated in rats (Lv et al., 2008). However, the global nAChR response to nicotine is complex, because certain receptor subtypes (e.g., $\alpha 6$-containing) are downregulated specifically in dopaminergic brain regions (Chen et al., 2005). The nAChRs are ligand-gated ion channels containing a central cation pore that act as the primary target for nicotine and the endogenous agonist acetylcholine.

nAChRs have been shown to activate release of dopamine, and are likely important in mediating the rewarding properties of abused drugs (Salminen et al., 2004; Gotti et al., 2006). More recently, work using a mouse model has shown that the effects of early nicotine exposure on later behaviors is "primarily due to the neuropharmacological effects of the drug and not due to effects of exposure on maternal behavior," and that the nAChRs play a critical role in mediating these effects (Heath et al., 2010a,b). Therefore, the nAChRs represent a possible key modulator of how MSP has long-term behavioral effects on offspring in both human and animal studies. Given the strong genetic associations between the *CHRN* genes and smoking behaviors, possible miRNA binding sites within these genes might represent feasible targets for study and intervention in the future.

In conclusion, the role of ncRNA in smoking exposure responses is in its infancy. As reported by Maccani and Knopik, fewer than 10 studies have been conducted in this area to date, but it is a high priority topic so we can expect to see new studies published frequently. Most of these studies are likely to be conducted using cell lines and animal models. Since not all ncRNAs are conserved across mammals, it will be important to foster collaborations and cross-talk between basic science investigators and human geneticists and clinicians to integrate the knowledge for greatest potential impact for human health.

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Cigarette smoke exposure-associated alterations to non-coding RNA

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Environmental exposures vary by timing, severity, and frequency and may have a number of deleterious effects throughout the life course. The period of *in utero* development, for example, is one of the most crucial stages of development during which adverse environmental exposures can both alter the growth and development of the fetus as well as lead to aberrant fetal programming, increasing disease risk. During fetal development and beyond, the plethora of exposures, including nutrients, drugs, stress, and trauma, influence health, development, and survival. Recent research in environmental epigenetics has investigated the roles of environmental exposures in influencing epigenetic modes of gene regulation during pregnancy and at various stages of life. Many relatively common environmental exposures, such as cigarette smoking, alcohol consumption, and drug use, may have consequences for the expression and function of non-coding RNA (ncRNA), important post-transcriptional regulators of gene expression. A number of ncRNA have been discovered, including microRNA (miRNA), Piwi-interacting RNA (piRNA), and long non-coding RNA (long ncRNA). The best-characterized species of ncRNA are miRNA, the mature forms of which are ∼22 nucleotides in length and capable of post-transcriptionally regulating target mRNA utilizing mechanisms based largely on the degree of complementarity between miRNA and target mRNA. Because miRNA can still negatively regulate gene expression when imperfectly base-paired with a target mRNA, a single miRNA can have a large number of potential mRNA targets and can regulate many different biological processes critical for health and development. The following review analyzes the current literature detailing links between cigarette smoke exposure and aberrant expression and function of ncRNA, assesses how such alterations may have consequences throughout the life course, and proposes future directions for this intriguing field of research.

Keywords: cigarette smoke, epigenetics, non-coding RNA, miRNA

GENE–ENVIRONMENT INTERACTIONS AND ENVIRONMENTAL EXPOSURES

The interaction of genes and the environment, often hypothesized to be through epigenetic mechanisms, modulates an organism's reproductive fitness, response to external stimuli, and health. Throughout the life course, a number of exposures can influence an individual's development, health, and overall quality of life. Environmental exposures vary by timing, severity, and frequency and may have a number of deleterious effects throughout the life course. The period of *in utero* development, for example, is a crucial stage during which adverse environmental exposures can both alter the growth and development of the fetus as well as lead to aberrant fetal programming, increasing disease risk. During fetal development and beyond, the plethora of exposures, including nutrients, drugs, stress, and trauma, influence health, development, and survival. Recent research in environmental epigenetics has investigated the roles of environmental exposures in influencing epigenetic modes of gene regulation (Reamon-Buettner et al., 2008), and a special focus of this work has been on determining the effects of cigarette smoking on epigenetic mechanisms, such as non-coding RNA (ncRNA), and resulting downstream consequences as a result of this harmful exposure.

CIGARETTE SMOKING

One of the most common, potentially hazardous environmental exposures that negatively influences health and development is cigarette smoke exposure. The CDC has reported that almost 21% of adults in the United States smoked cigarettes in 2009, a number equaling ∼46 million people (CDC, 2010). In contrast to the number of studies reporting a decrease in the overall prevalence of smoking in women in the United States in the past 20 years, other reports have found that the prevalence of smoking in young pregnant women has increased (Jaakkola et al., 2001; CDC, 2004; Mohsin and Bauman, 2005). Conservative estimates report that 12–15% of women smoke while pregnant (Cnattingius, 2004; Goodwin et al., 2007), and it is likely that far greater numbers are exposed to secondhand (or passive) cigarette smoke during various periods of their pregnancies. Exposure to maternal cigarette smoking while *in utero* is associated with an increased risk for respiratory disease (Cook and Strachan, 1999), an increased

risk for cancer later in life (Doherty et al., 2009), and a number of deleterious neurobehavioral outcomes in infancy and beyond (Olds et al., 1994; Huijbregts et al., 2007; Knopik, 2009; Kiechl-Kohlendorfer et al., 2010). When one combines these estimates of babies who experienced *in utero* exposure to cigarette smoke with the numbers of adults who smoke cigarettes, one realizes that the number of individuals exposed to cigarette smoke during at least one period of their lives is quite large and far greater than what might be expected at first consideration.

Previous work has found over 4,000 chemicals in a cigarette, including nicotine, benzo[a]pyrene, and carbon monoxide, and more than 40 of these chemicals have been established as known carcinogens (Thielen et al., 2008). Cigarette smoking has been linked to a number of diseases and disabling conditions, including heart disease and lung diseases (CDC, 2008). Furthermore, for every individual who dies from a disease associated with smoking, 20 more people battle at least one major illness attributable to smoking (CDC, 2003). Several studies have singled out tobacco use as the world's leading preventable cause of death (CDC, 2002, 2003, 2008). By some estimates, up to five million deaths worldwide can be attributed to smoking and current trend data predicts that tobacco use will lead to more than eight million deaths a year by 2030 (WHO, 2008). In the USA, tobacco use has been attributed to 20% of deaths per year which equates to ∼443,000 deaths annually, and ∼49,000 of these have been attributed to secondhand smoke exposure (CDC, 2008). On average, smokers die 13–14 years earlier than non-smokers (CDC, 2002), suggesting that cigarette smoking and cigarette smoke exposure leads to increased morbidity and mortality.

Recent research has broadened investigations of the effects of cigarette smoke exposure into determining the effects of not only primary (or "mainstream") cigarette smoke but also environmental (also called "secondhand," "passive," or "sidestream") cigarette smoke. Sidestream cigarette smoke comprises the major component of environmental cigarette smoke and is defined as smoke which goes into the air directly from a burning cigarette (as opposed to smoke which is directly inhaled ("mainstream" smoke) from the burning cigarette; NIOSH, 1991). A variety of studies have suggested that components of sidestream or environmental cigarette smoke (ECS) differ from those in mainstream smoke in both component type and concentration, with some investigators concluding that ECS may be as harmful or even more harmful than mainstream smoke (Schick and Glantz, 2005). Work is ongoing to investigate the potentially harmful effects of both mainstream and sidestream cigarette smoke.

Taken collectively, these data suggest that cigarette smoking and cigarette smoke exposure throughout the life course is one of the most common hazardous exposures, and more recently, work has suggested that epigenetic mechanisms may be especially responsive to environmental exposures such as cigarette smoke.

EPIGENETICS: MODE BY WHICH ENVIRONMENTAL EXPOSURES INFLUENCE HEALTH OUTCOMES

By definition, epigenetics is the field of research which studies changes in gene expression not caused by changes in the sequence of DNA (Bird, 2007). The emergence of a subfield of epigenetics, called "environmental epigenetics" (Reamon-Buettner et al., 2008), focuses on studying modes of epigenetic regulation with the greater understanding that environmental exposures may affect such modes of epigenetic regulation as well. Throughout the life course, epigenetic mechanisms may be the mode by which environmental exposures influence the development, health, and survival of the individual. Research in both model systems and human cohorts has suggested that a variety of environmental exposures have consequences for gene expression through epigenetic modes of gene regulation. Particular focus has been placed on four modes of epigenetic regulation, namely DNA methylation, imprinting, histone modifications, and ncRNA-mediated gene regulation (Crane-Godreau et al., 2009). While the study of DNA methylation remains the most widely studied mode of epigenetic regulation, the study of ncRNA-mediated gene regulation has gained significant attention over the past two decades.

NON-CODING RNA

Since the earliest discoveries of RNA as a product of the transcription of DNA, many have hypothesized that RNA may not only act as the intermediate step on the pathway to protein but may also have a degree of regulatory activity itself. Of the species of ncRNA, the three best-characterized forms are microRNA (miRNA), Piwi-interacting RNA (piRNA), and long non-coding RNA (long ncRNA), with miRNA garnering especially great attention.

microRNA

In the early 1990s, two small regulatory RNAs, known as *lin-4* and *let-7* were shown to control the timing of larval development in *C. elegans* (Lee et al., 1993; Reinhart et al., 2000). These small ncRNAs, initially called "*lin-4* and *let-7* RNAs," have since been determined to be members of a class of endogenous RNAs found in a number of species, including worms, flies, and mammals and since have been renamed "microRNAs (miRNAs)" (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Additional studies suggested that these small ncRNAs capable of gene regulation are found in plants, mammals, green algae, and viruses (Griffiths-Jones et al., 2008). Depending on the species and particular form of ncRNA, names of these small ncRNA vary. miRNA are a distinct class of ncRNA and differ from other species of ncRNA in both how they are formed as well as their particular mechanism of action. miRNA are formed from precursor transcripts which fold back on themselves, forming hairpin structures.

As reviewed previously (Maccani and Marsit, 2009), miRNA are initially transcribed by RNA Polymerase II as part of longer transcripts termed primary miRNAs (pri-miRNAs) which include 5′ caps and 3′ poly(A) tails (Lee et al., 2002; Smalheiser, 2003; Cai et al., 2004). A hairpin is then formed from the miRNA portion of the pri-miRNA (Lee et al., 2002). The double-stranded RNA (dsRNA)-RNA-specific ribonuclease Drosha then digests the pri-miRNA and releases the hairpin which, post-Drosha processing, is referred to as a precursor-miRNA (pre-miRNA; Lee et al., 2003) and is ∼70–75 nucleotides in length (Lee et al., 2003; Yi et al., 2003). pre-miRNA is subsequently exported from the nucleus to the cytoplasm by a complex containing Exportin-5 (Exp-5; Yi et al., 2003; Lund et al., 2004) and in the cytoplasm, is cleaved by Dicer (Lee et al., 2003; Yi et al., 2003) into a dsRNA with short 3′ overhangs at the ends (Lund et al., 2004). After cleavage by Dicer, the

Dicer-processed dsRNA must separate into two strands, and the single-stranded mature miRNA has to associate with the RNA-induced silencing complex (RISC). The RISC traffics the mature miRNA to its mRNA target where it effectively silences it (Hutvagner and Zamore, 2002). Additionally, work has revealed that determination of the active strand in the post-Dicer-processed dsRNA is directly related to the stability of dsRNA ends (Khvorova et al., 2003; Schwarz et al., 2003); more specifically, the strand with a less-stable base pairing of the two to four nucleotides at the 5′ end of the duplex tends to associate with the RISC and becomes the active miRNA which can be trafficked to its mRNA target to effectively post-transcriptionally silence that target (Schwarz et al., 2003). miRNA biosynthesis, as well as the post-transcriptional silencing of target mRNA, is summarized in **Figure 1**.

Work has suggested that miRNA regulate gene expression post-transcriptionally by base pairing to a target mRNA. The specific mechanism of miRNA-mediated gene regulation depends on a variety of factors, the most important of which appears to be the degree of complementarity between the miRNA sequence and the target mRNA sequence (Lagos-Quintana et al., 2001)

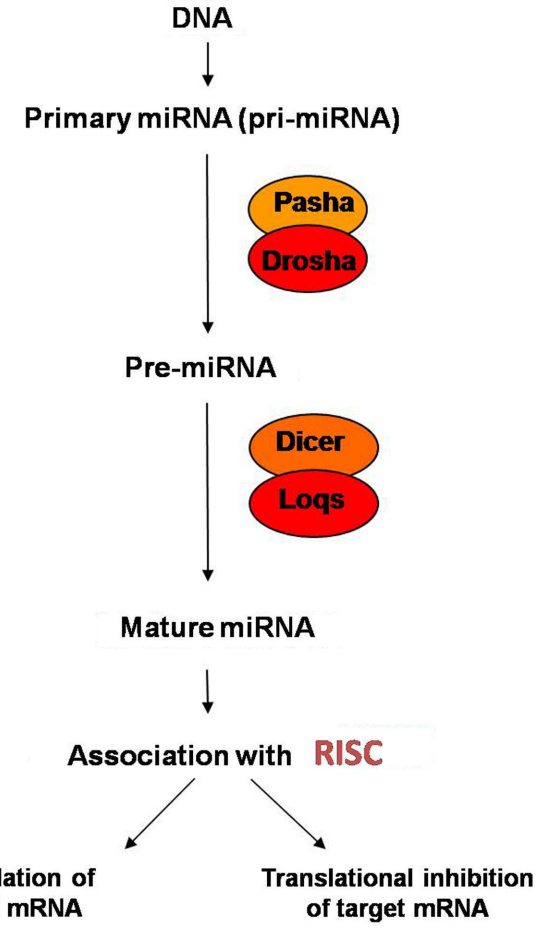

FIGURE 1 | Summary of miRNA biosynthesis and post-transcriptional silencing of target mRNA.

As described above, the mature miRNA's active strand associates with the Argonaute protein of the RISC and the RISC traffics the miRNA to its target mRNA (Hutvagner and Zamore, 2002; Mourelatos et al., 2002) where it post-transcriptionally regulates gene expression. Generally, a miRNA with perfect complementarity to the sequence of its target mRNA will lead to degradation of the mRNA transcript by a mechanism of Argonaute-catalyzed mRNA cleavage (Hutvagner and Zamore, 2002; Song et al., 2004; Yekta et al., 2004). A miRNA with imperfect sequence complementarity to its target mRNA will result in repression of translation of the target mRNA by blocking or altering the function of translational machinery (Lagos-Quintana et al., 2001) through mechanisms including the inhibition of translation initiation and poly(A) shortening (Filipowicz et al., 2008). Other observations have even suggested that miRNA can utilize a combination of both translational repression and mRNA degradation as the mechanism for their post-transcriptional gene regulation (Lim et al., 2005). Since partial or imperfect complementarity of a miRNA to a target mRNA can lead to translational repression which can effectively silence a gene, a single miRNA has the capability of regulating a large number of genes (Du and Zamore, 2007). By their mechanisms of negative regulation, miRNA have been shown to exhibit tissue-specific expression and function and play a role in regulating a wide range of biological processes, including differentiation, proliferation, apoptosis, and stress responses (Crane-Godreau et al., 2009).

PIWI-INTERACTING RNA

Another class of ncRNA that has recently been discovered is the class of piRNA. Compared to the miRNA and Argonaute protein complex, piRNAs associate with PIWI proteins to form RNA–protein complexes capable of gene regulation (Thomson and Lin, 2009). Several thousand piRNA, ∼24–32 nucleotides in length, have been discovered in zebrafish, *Drosophila*, and mammals. The majority of piRNA seem to be generated from a relatively small number of long single-stranded RNA precursors which are frequently encoded in repetitive intergenic sequences. PIWI proteins, and seemingly the piRNA which associate with them, are important for ensuring a number of key processes during germline development, including germline determination, spermiogenesis, and the silencing of transposons (Thomson and Lin, 2009). In mammals, piRNA have been detected in testes and ovaries but data suggest they may only be required for proper development in males (Aravin et al., 2006; Siomi et al., 2011).

In contrast with the relatively advanced understanding of the biogenesis of miRNA, the biogenesis of piRNA is not well understood. Recent work has suggested that in the case of pachytene piRNA, piRNA whose expression and function are important during the pachytene phase of meiosis, piRNA precursors are transcribed in a type of primary processing pathway (Brennecke et al., 2007; Seto et al., 2007; Aravin et al., 2008). Other work has suggested a type of "ping–pong" mechanism of biogenesis in which primary piRNA identify their mRNA targets and result in PIWI protein recruitment. This leads to the cleavage of the primary transcript at a location 10 nucleotides from the 5′ end of the primary piRNA, ultimately producing the secondary piRNA (Brennecke et al., 2007). Current data suggest that one or both of

these proposed mechanisms explains the biogenesis of piRNA in a species-specific fashion with some degree of conservation across species (Faehnle and Joshua-Tor, 2007; Das et al., 2008; Klattenhoff and Theurkauf, 2008; Unhavaithaya et al., 2009).

In terms of biological importance, piRNA have been implicated in the silencing of transposons, and many piRNA are antisense to sequences of transposons (Malone and Hannon, 2009). In mammals, piRNA have been suggested to be most essential during embryonic development (Aravin et al., 2008), and in many species, data reveal that piRNA are required for spermatogenesis (Grimson et al., 2008). A more detailed characterization of both the biogenesis and mechanisms of action of piRNA will be valuable in further revealing how these small, but important, ncRNA function to regulate key periods of development.

LONG ncRNA

Long non-coding RNAs are longer than 200 nucleotides, and this characterization distinguishes long ncRNA from other forms of ncRNA, such as miRNA or piRNA. As has been described above, many forms of small ncRNA, such as miRNA or piRNA, show relatively strong conservation across species (Bentwich et al., 2005); long ncRNA, however, have been shown to lack such relatively strong conservation, leading to conclusions by early investigations that such long ncRNA were non-functional (Brosius, 2005; Struhl, 2007), theories which have been largely dismissed following subsequent analyses. Even two of the most well-characterized long ncRNA, Air and Xist, exhibit relatively poor conservation (Nesterova et al., 2001) but their roles are crucial to an organism's survival. As of late 2011, well over 100 long ncRNA have been characterized and annotated on lncRNAdb, a database which provides annotations of eukaryotic long ncRNA (Amaral et al., 2011).

One of the most well-characterized long ncRNAs is Xist (Wutz, 2007), and Xist has been shown to play a key role in X-chromosome inactivation in female placental mammals (Wutz, 2007). During the period of embryonic stem cell differentiation, Xist is expressed from the future inactive X chromosome and coats what then becomes the inactive X chromosome. Following expression of Xist, irreversible chromatin modifications including loss of histone (H3K9) acetylation and H3K4 methylation and establishment of repressive H4 hypoacetylation and H3K27 trimethylation which, along with other modifications, lead to the silencing of X-linked genes (Morey et al., 2004; Wutz, 2007). Collectively, the long ncRNA "Xist" and other ncRNAs expressed from Xist loci effectively allow only one X chromosome to be active in female placental mammals.

Research focused on understanding the roles of long ncRNA in health and disease is ongoing. Work to determine associations of dysregulated expression of long ncRNA with various tumors, as well as tumor differentiation, continues and will elucidate these important relationships (Pibouin et al., 2002; Reis et al., 2004; Fu et al., 2006; Lin et al., 2007). Specific roles of long ncRNA in driving or preventing tumorigenesis, however, have been largely undetermined. Future work focusing on enhanced understanding of how these long ncRNA are involved in regulating key cell processes throughout the life course, as well as how these long ncRNA are responsive to environmental exposures, such as cigarette smoke, will be key to better elucidating the function of long ncRNA.

CIGARETTE SMOKE-ASSOCIATED EFFECTS ON ncRNA

The influence of cigarette smoking and the effects of components of cigarette smoke on miRNA expression and function have been the most heavily studied aspects of research motivated to characterize the effects of cigarette smoke exposure on ncRNA. As of manuscript preparation, no studies have been published assessing associations of cigarette smoking or direct effects of components of cigarette smoke on piRNA, and relatively few studies have investigated the influence of smoke exposure on dysregulating long ncRNA expression. Since exposure to mainstream and sidestream cigarette smoke remains relatively common and potentially hazardous, investigations into effects on miRNA, piRNA, and long ncRNA may be crucial to elucidating currently poorly understood mechanisms. Such understanding may further explain how components of cigarette smoke may be mediating their effects through piRNA, long ncRNA, and other less-characterized species of ncRNA.

MATERNAL CIGARETTE SMOKING DURING PREGNANCY AND ABERRANT EXPRESSION OF PLACENTAL miRNA

During fetal development, the placenta is of utmost importance to ensuring the proper growth and development of the fetus; it provides the fetus with nutrients, aids in the elimination of waste products, and protects the fetus from a variety of environmental toxicants (Crane-Godreau et al., 2009; Maccani et al., 2010). Recently, Maccani et al. (2010) used a candidate miRNA approach to investigate how maternal cigarette smoking during pregnancy might be associated with differential expression of miRNA in the placenta. Maccani and colleagues' data suggested that maternal cigarette smoking during pregnancy is associated with the downregulation of *miR-16, miR-21,* and *miR-146a.* In an attempt to further explore which components of cigarette smoke might modulate specific miRNA, Maccani and colleagues exposed three human placental cell lines from different stages of placental development to nicotine and benzo[a]pyrene, two components of cigarette smoke, and assessed candidate miRNA expression. The three human placental cell lines used were first trimester villous 3A cells, first trimester extravillous HTR8 cells, and the third trimester extravillous TCL-1 cells; the three cell lines represent different periods and aspects of placental development and were selected to further investigate how different components of cigarette smoke might dysregulate miRNA expression in different placental cell types (Maccani et al., 2010). Data suggested that *miR-146a* was downregulated in TCL-1 cells treated with nicotine and benzo[a]pyrene, suggesting that this particular miRNA may be particularly sensitive to agents of cellular stress and to these two components of cigarette smoke (Maccani et al., 2010; Maccani and Marsit, 2011).

As has been reviewed elsewhere (Maccani and Marsit, 2011), Maccani and colleagues' observations were limited by a relatively small set of samples ($n = 25$) and a lack of data regarding the duration of cigarette smoking during pregnancy or frequency of use, as well as more extensive environmental exposure information. Despite these limitations, Maccani and colleagues' findings comprise an important first step in investigating associations between maternal cigarette smoking during pregnancy and miRNA expression in the placenta. Future work is needed to

investigate associations between maternal cigarette smoking during pregnancy and aberrant miRNA expression in a larger cohort of samples with more extensive exposure information (Maccani et al., 2010; Maccani and Marsit, 2011).

CIGARETTE SMOKING AND ABERRANT EXPRESSION OF LUNG miRNA

Xi et al. (2010) investigated how cigarette smoke condensate (CSC) alters miRNA expression and function in normal human respiratory epithelial cells and lung cancer cells. For their experiments, Xi and colleagues prepared CSC from Kentucky Reference 3R4F research blend cigarettes (Liu et al., 2010) and resuspended the condensate at a concentration of 1 mg tar/mL in RPMI. They found that exposure of cells to this CSC increased the expression of miR-31 in both normal respiratory epithelial cells and in lung cancer cells (Xi et al., 2010). Further results suggested that overexpression of miR-31 led to increased proliferation and tumorigenicity in lung cancer cells, whereas knockdown of miR-31 expression significantly inhibited growth of lung cancer cells (Xi et al., 2010). These findings suggest that miR-31 is both responsive to cigarette smoke exposure in normal respiratory epithelia and lung cancer cells and may play a role as an oncomir, or a miRNA whose expression and function may promote the development of cancer, in the pathway leading to carcinogenesis in the lung (Xi et al., 2010).

CIGARETTE SMOKING AND DYSREGULATED miRNA EXPRESSION IN HUMAN AIRWAY EPITHELIUM

Schembri et al. (2009) investigated whole-genome miRNA expression in bronchial airway epithelium from current or never smokers ($n = 20$). They found that 28 miRNA were differentially expressed and a majority of these differentially expressed miRNA were downregulated in the airway epithelium of smokers. Furthermore, they investigated one candidate miRNA shown to be dysregulated due to smoke exposure, miR-218, by exposing primary bronchial epithelial cells to CSC, finding that such exposure downregulates miR-218 expression levels (Schembri et al., 2009). While Schembri and colleagues' sample set was limited in size, their work comprised an important first step in determining how miRNA may be a mechanism by which smoking dysregulates bronchial airway gene expression, potentially leading to downstream smoking-associated disease risk.

ENVIRONMENTAL CIGARETTE SMOKE AND ABERRANT EXPRESSION OF miRNA

While a number of studies have focused on the influences of primary cigarette smoking on miRNA expression and function, work is also being done to characterize the effects of ECS (also called "passive," "secondhand," or "sidestream" cigarette smoke) on miRNA expression. Three studies characterizing the effects of ECS on miRNA expression in the lung and liver were conducted by Izzotti et al. (2009, 2010a,b).

Izzotti et al. (2009) investigated the effects of ECS exposure on miRNA expression in the lungs of rats exposed to ECS for 28 days. They found that the most greatly downregulated miRNA belonged to miRNA families previously shown to regulate a number of key biological processes, including stress response, proliferation, angiogenesis, apoptosis, and others. Furthermore, they found that ECS exposure resulted in the upregulation of 2.9% of genes and 9.7% of proteins in the same tissue, suggesting that the ECS exposure-induced downregulation of miRNA may, in part, be leading to the increased protein levels of genes whose mRNA would have otherwise been targeted for post-transcriptional regulation.

In their two 2010 studies, Izzotti and colleagues exposed either neonatal mice (Izzotti et al., 2010b) or rats (Izzotti et al., 2010a) to ECS but also to chemopreventive agents and measured miRNA expression in the lung and the liver following exposure. Their data suggested that ECS dysregulated miRNA expression in lung and had a variety of mixed effects in liver. Phenethyl isothiocyanate and budesonide exposure was observed to protect the lung from ECS-induced dysregulated miRNA expression but had adverse effects in the liver (Izzotti et al., 2010b). Exposure to chemopreventive agents *N*-acetylcysteine, oltipraz, indole-3-carbinol, 5-6-benzoflavone, and phenethyl isothiocyanate (as single exposures or in combinations) were shown to attenuate alterations in lung attributed to ECS (Izzotti et al., 2010a). Collectively, their findings further underscore the utility of miRNA profiles in various tissues as potential key tools for analyzing both protective and adverse effects of chemopreventive agents in mitigating the effects of exposure to ECS. Future work utilizing bioinformatically informed target prediction approaches may prove essential to better characterizing potential pathways dysregulated by aberrant miRNA expression and respective target mRNA expression resulting from ECS. Future investigations into the molecular mechanisms by which ECS elicits downstream effects will be important for both understanding the effects of this potentially hazardous exposure as well as designing novel therapeutics to treat and improve outcome following exposure.

Cigarette smoke exposure-associated dysregulation of long ncRNA

Investigations into cigarette smoke exposure-associated dysregulation of miRNA have been relatively extensive compared to the few studies analyzing such alterations of long ncRNA. Silva et al. (2010) used whole-genome tiling arrays to investigate the upregulation of non-coding transcripts greater than 300 nucleotides in length in normal human bronchial epithelial cells (NHBE) exposed to the tobacco carcinogen nicotine-derived nitrosamine ketone (NNK), a component of tobacco smoke which can result in DNA damage. They found 12 long stress-induced non-coding transcripts (LSINCTs) which were upregulated in NHBE cells exposed to NNK. Follow-up analysis showed that several of these transcripts exhibit increased expression in many lung cancer and breast cancer cell lines. Analysis is ongoing to better characterize the functional aspects of these LSINCTs dysregulated by NNK. Other investigations have included using RNA-Seq technology to explore effects of smoking and lung cancer on the transcriptome of the airway (Beane et al., 2011) and characterizing the dysregulated monoallelic expression of the H19 gene in cigarette smokers' airway epithelium (Kaplan et al., 2003). These investigations all provide important first steps in characterizing the potential impacts of cigarette smoke exposure on long ncRNA; more extensive research will need to be conducted in the future to more comprehensively describe the effects of cigarette smoke exposure on long ncRNA in a variety of tissues.

CONCLUSION AND FUTURE DIRECTIONS

Taken collectively, the body of literature describing our understanding of the effects of cigarette smoke on ncRNA expression and function is very much in its infancy. Studies to date have been relatively small in scope, have considered a relatively limited number of target tissues (i.e., placenta, lung, liver, and airway epithelial cells), and examined various types of cigarette smoke exposure (e.g., primary, passive/secondhand, and prenatal). While the variety in exposure types is informative and provides preliminary results, the relatively small sample sizes and limited number of studies overall make it clear that there is much more to be done in order to more definitively elucidate the effects of cigarette smoke (of any type) on ncRNA expression and the later downstream effects on behavioral health and other medical conditions.

The possible influence of epigenetic modification, including ncRNA expression, due to exposure to cigarette smoke of any kind (i.e., passive or secondhand, primary, or during pregnancy) on behavioral health and other medical conditions is manifold. As outlined above, the three best-characterized forms of ncRNA are microRNA (miRNA), piRNA, and long ncRNA, with miRNA garnering particularly great attention. In fact, at the time of the preparation of this review, there were virtually no published data on exposure-associated alterations to piRNA and relatively few studies on cigarette smoke-associated alterations to long ncRNA. Thus, unanswered questions include, but are not limited to, (i) the effects of cigarette smoke on piRNA and long ncRNA in multiple tissues, in animals and in humans, (ii) the effects of cigarette smoke on miRNA in the brain which might alter critical neurobehavioral circuitry in the developing brain or the adult brain, (iii) the influence of smoke exposure on ncRNA in asthma, lung cancer or other medical outcomes, and (iv) intergenerational transmission of smoking-related ncRNA changes (i.e., grandmaternal smoking influences which may affect germline cells and those epigenetic changes which may escape reprogramming during development). Use of animal models in mutually informative translational research (especially in inbred lines where genetic background is held constant) may further triangulate our ability as a field to investigate these issues (Knopik et al., Under Review).

It is clear, even from the relative paucity of research in this area, that ncRNA are biologically relevant and play an important role in the disease process. ncRNA alterations may also provide valuable information about therapeutic interventions. Yet, how do we align this information with the current state of science? One part of this answer lies in the research or clinical question of interest. If the question is whether cigarette smoke exposure, whether mainstream or sidestream, alters ncRNA expression and leads to the development of cancer cells, then the tissues of interest may indeed be relatively easily accessible. Animal models can be used to investigate ncRNA alterations in multiple tissues of interest, such as the lung, the esophagus, salivary glands, and bladder, but as with all model systems, consideration must be taken in study design regarding differential degrees of conservation of particular ncRNA across species. These preclinical models can be examined alongside human data, where biopsies of particular tissues may be available. However, if the outcome of interest is behavioral or psychiatric in nature (e.g., addiction, executive function, impulsive behavior, response to stress), the approach is less clear. Ideally, to consider the role of cigarette smoke exposure and ncRNA alterations on subsequent behavior, one would want to examine brain tissue. In humans, this is unavailable unless one considers post-mortem tissue; however, even then there is then the question of whether such investigations might be confounded due to potential ncRNA alterations associated with cause of death. There is considerable debate about the utility of blood as a biomarker for gene expression in brain and other tissues (Tsuang et al., 2005; Tian et al., 2009; Shivapurkar and Gazdar, 2010; Kukreja et al., 2011). In the search for a biomarker with clinical utility, blood does have certain advantages. Blood is an accessible tissue that can be relatively easily obtained, and while not a perfect representation of what might be expressed in brain, it can provide useful information for screening purposes. In either scenario, whether the health outcome is more psychological or somatic in nature, an additional question lies in what aspect of cigarette smoke leads to epigenetic alterations: more specifically, is it nicotine or one of the 4000+ other xenobiotics (e.g., foreign substances) found in cigarette smoke – or one of the multitude of complex mixtures of these xenobiotics – which are most responsible for leading to epigenetic alterations? Carefully examining this piece of the equation will also be key to developing a better understanding of how components of cigarette smoke alter ncRNA expression and function, but also the utility of using such cigarette smoke-modulated ncRNA in diagnostic and therapeutic interventions.

In summary, increasing attention to the study of ncRNA and to "environmental epigenetics" (Reamon-Buettner et al., 2008) has inspired more researchers to embark on work to better understand how environmental exposures, such as cigarette smoke, affect ncRNA expression and function. Recommendations for future research include using both human cohorts and model systems to more comprehensively determine how the type, timing, frequency, duration, and degree of cigarette smoke exposure may alter miRNA, piRNA, and long ncRNA expression and function in a variety of tissues, thereby having the power to alter a number of health and developmental processes. The relative dearth of data demonstrating the effects of cigarette smoke on piRNA and relatively limited number of studies investigating the impact of cigarette smoke on long ncRNA underscore the need for future research to better describe such potentially hazardous effects of cigarette smoke on these two species of ncRNA and the processes they regulate. Hypothesis-generating approaches, such as microarray technology, when used in tandem with gold-standard validation approaches, such as Real-Time PCR, will be important for developing more agnostic study designs for discovering how ncRNA individually and collectively may be responsive to cigarette smoke exposure. Use of target prediction strategies combining *in silico* target prediction analysis with empirical target prediction confirmation (i.e., via Western blot) will enable researchers to better streamline their efforts to discover currently unknown targets of miRNA. Tools for predicting potential targets of piRNA and long ncRNA remain in early development and such bioinformatic tools will prove especially useful for further determining the functions of piRNA and long ncRNA, especially in the context of harmful environmental exposures. This

will not only enhance understanding of how these harmful exposures impact health but may suggest the utility of ncRNA as both therapeutic targets and biomarkers for determining treatment efficacy. Together, these advances will be crucial for determining how alterations to the expression and function of ncRNA may be important modes by which environmental exposures, such as cigarette smoke, influence health outcomes throughout the life course.

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Bioinformatic challenges of big data in non-coding RNA research

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Recent technological developments have brought forth a new era of RNA research in which large sets of data are collected rapidly using the high-throughput next generation sequencing technology. Growing evidence suggests that only around 5% of nucleotides in the mammalian genomes are transcribed into protein-coding RNA, and large amount of transcripts are non-protein-coding RNA (ncRNA). During the last decade, much information has been generated from the studies of one type of ncRNA, namely microRNA (miRNA, the ncRNA of 19–25 nucleotides). miRNA modulates the expression of target genes through repression of mRNA translation or mRNA degradation. Its dysregulation has been implicated in various biological disorders and human diseases. Meanwhile, the long-non-coding RNA (lncRNA, the ncRNA that have 200 or more nucleotides) has recently emerged to catch significant attention. lncRNA is involved in chromatin modification, epigenetic regulation, transcription control, and pre- and post-translational mRNA processing. The functions of lncRNA are believed to be associated with development, imprinting, mental and psychiatric disorders, and tumor growth.

Bioinformatics is a pivotal component of this new RNA research revolution. It utilizes mathematical models and computer simulations to form, extract and analyze RNA data, and to search new ncRNA gene sequences and predict their targets. Assumptions in this computational modeling are derived from the observations that ncRNAs are produced following step-wise processes from precursors to functional end products. Based on miRNA biogenesis, criteria in searching for new miRNAs from sequencing data include that the precursors fold into a stable stem-loop structure, mature miRNAs are found on one arm of the stem, and these sequences are usually evolutionarily conserved (Lim et al., 2003). Target prediction algorithms take into considerations stability of miRNA-mRNA duplex, accessibility of secondary structure, nucleotide content in and around the putative target sites, and position of seed-complementary sites within the mRNA transcript.

Prior to the high-throughput sequencing techniques, computational programs were developed to search for new miRNAs based on attainable sequence data. These methods used one of the following approaches (Mendes et al., 2009): filter-based approaches, which identified small high-quality sets of conserved miRNA candidates; machine learning methods, which determined initial set of candidates with stem-loops structures, and target-centered approaches, which identify short conserved motifs in the 3′UTRs of protein-coding genes (Xie et al., 2005). Even though these algorithms were developed before the high-throughput sequencing era, they establish strong bases for bioinformatic analyses of big sequencing data; new ncRNAs and targets continue to be cataloged into many databases with sufficient annotations available to the public.

High-throughput sequencing techniques and deep sequencing (or RNA-Seq) have offered much improved avenue for ncRNA discovery (Lu et al., 2005), by searching genomic sequences for evidence of hairpin structures and then determine if sequencing read aligned to these structures mimic miRNA processing byproducts (Friedlander et al., 2008), or using a regularized least-squares classification algorithm to mine miRNAs from smRNA-seq data (Lu et al., 2009) to perform genome-wide multiple sequence alignments (MSAs). At the same time, through adaptation of the latest biochemical approaches to miRNA target finding, it is possible to identify miRSNPs with greater accuracy and explain the association of certain miRNA-affecting polymorphisms with disease phenotypes (Wilbert and Yeo, 2011).

Even though bioinformatic-based methods for the identification of new ncRNA and their targets have become more sophisticated and required less CPU time, there are gaps and challenges that need to be addressed to justify their biological relevancy: cross-platform validation of genomic and transcriptional sequence data, cross-algorithm validation of search engines, and development of more accurate models for ncRNA function in regard to biological environment and diseases. For example, high-throughput sequencing of small RNA results in an output file of short sequence (often termed short-reads or reads) accompanied by a quality score for each nucleotide in each sequence. Because of the high sensitivity of the technique, the "raw" data will also contain sequencing primers and contaminants which can potentially produce sequence bias that requires more sophisticated computational approaches to sieve out miRNA transcripts (Mendes et al., 2009) and cross-platform validations. There are currently at least 45 sequence formats; the most widespread data formats being those used by the major sequence database: EMBL, GenBank, SwissProt, and PIR. The lack of standardization in sequence formats not only hampers the feasibility for

cross-platform comparison of existing data (Farazi et al., 2011), but also discourages the expansion of sequence data sharing for initial and value-added secondary analysis. In addition, currently available algorithms have employed different approaches dictated by the algorithm developers and may or may not be reproducible using a different approach. Cross-examination between the solutions derived from different algorithms is needed. Another complexity in ncRNA data analysis is that most of the software is primarily at a command-line level and not user-friendly to the end-users.

Computational approaches developed so far make extensive use of evolutionary conservation information either to predict ncRNA genes or ncRNA-target associations, sometimes ignoring the subtle rules presiding ncRNA biogenesis and target specificity. Thus, approaches combining high-throughput sequencing biochemical techniques and bioinformatic analyses that emphasizes the synergy of genome-wide approaches are essential (Mendes et al., 2009). Furthermore, most lncRNA are under lower sequence constraints than protein-coding genes and lack conserved secondary structures like the pre-miRNAs, making it hard to predict computationally. In addition, since complex diseases can be affected by a number of ncRNAs rather than a single ncRNA, and ncRNA often operates in highly complex regulatory networks (Kargul and Laurent, 2011), it is a multi-dimensional challenge to identify ncRNA interactions at a system-wide level, and analyze the roles of ncRNA in disease and disorders in the ncRNA–ncRNA synergistic network (Xu et al., 2011). Lastly, careful interpretations of data with molecular validations are critical for ensuring acceptance of bioinformatic methods in the ncRNA research community. With knowledge gained from bioinformatic analyses of exponentially increasing massive ncRNA data, many issues remain to be addressed on the functional significance and how genetic variations of ncRNA plays important roles in disease processes.

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Bioinformatic resources of microRNA sequences, gene targets, and genetic variation

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Variation in quantitative gene expression has been observed in natural populations and associated with various complex traits/phenotypes such as risks for common diseases and drug response. MicroRNAs (miRNAs), a family of small, non-coding RNA molecules, have been demonstrated to be an important class of gene regulators that mostly down-regulate gene expression at the post-transcriptional level. A comprehensive and reliable catalogue of miRNAs and miRNA gene targets is critical to understanding the gene regulatory networks. Though experimental approaches have been used to identify many miRNAs and their gene targets, due to cost and efficiency, currently miRNA and target identification still largely relies on computational algorithms. We reviewed several widely used bioinformatic resources of miRNA sequences and gene targets that take advantage of the unique characteristics of miRNA–mRNA interactions, experimental validation, as well as the integration of sequence-based evidence and microarray expression data. Furthermore, given the importance of miRNAs in regulating gene expression, elucidating expression quantitative trait loci involved with miRSNPs or miR-polymorphisms will help improve our understanding of complex traits. We reviewed the available resources of miRNA genetic variation, and the current progress (e.g., the 1000 Genomes Project) in detailing the genetic variation in miRNA-related single nucleotide polymorphisms (SNPs). We also provided our perspectives of the potential impact of next-generation sequencing on the research of miRNAs, gene targets, and miRSNPs. These bioinformatic resources may help interpret experimental and association study results, thus enhancing our knowledge of the dynamic gene regulatory networks and the physiological pathways for complex traits/phenotypes. Prospectively, these bioinformatic resources of miRNAs will need to address the challenges raised by the application of next-generation sequencing in miRNA research.

Keywords: microRNA, gene expression, gene regulation, genetic variation, single nucleotide polymorphism, RNA-sequencing

INTRODUCTION

Alterations in gene expression, a quantitative phenotype, have been implicated in a variety of human diseases and traits, as well as response to therapeutic treatments (Zhang and Dolan, 2009). The variation of gene expression, therefore, could potentially explain the phenotypic variations (e.g., susceptibility to complex diseases, and drug response) among individuals and human populations. For example, differentially expressed genes enriched in ubiquitin proteasome and Parkinson's disease pathways were found be associated with coffee consumption, a model for addictive behavior (Amin et al., 2011). In contrast, based on a cell-based pharmacogenomic discovery model using the HapMap Project (HapMap, 2003, 2005) lymphoblastoid cell line (LCL) samples, whole-genome gene expression profiling (i.e., transcriptome profiling; Zhang et al., 2008, 2009) has allowed identification of gene expression phenotypes associated with the cytoxicities to anticancer drugs, such as etoposide (Huang et al., 2007a), daunorubicin (Huang et al., 2008a), carboplatin (Huang et al., 2008b), cisplatin (Huang et al., 2007b), and Ara-C (cytarabine arabinoside; Hartford et al., 2009). In addition, significant variation in gene expression has been observed among individuals from the same human population, as well as among individuals from different ethnic background (Cheung et al., 2003a,b; Stranger et al., 2005, 2007a,b; Spielman et al., 2007; Storey et al., 2007; Duan et al., 2008; Zhang et al., 2008). For example, a substantial proportion of human genes (e.g., genes related to immune response) have been found to be differentially expressed between individuals of African and Northern/Western European ancestry (Zhang et al., 2008). Particularly, common genetic variants including single nucleotide polymorphisms (SNPs) and copy number variants (CNVs) have been identified to contribute to the variation in gene expression (e.g., through expression quantitative trait loci, eQTLs) both within and between human populations (Cheung et al., 2003a,b; Stranger et al., 2005, 2007a,b; Spielman et al., 2007; Storey et al., 2007; Duan et al., 2008; Zhang et al., 2008). Besides genetic factors, non-genetic factors such as environmental exposure, life style, diet

may play at least as great a role as genetic divergence in modulating gene expression variation in humans (Idaghdour et al., 2008).

During the past decade, epigenetic mechanisms (e.g., DNA methylation, histone modifications) have begun to be appreciated as playing critical roles in regulating gene expression, and affecting complex traits including risks for common diseases. Recent process in biomedical research emphasizes the need to move beyond protein-coding genes and highlights the fact that continued investigation of non-coding RNAs (ncRNAs) will be necessary for a comprehensive understanding of human traits and diseases (Taft et al., 2010; Kaikkonen et al., 2011). Notably, microRNAs (miRNAs), a family of small, ncRNAs molecules, have been demonstrated to be an important class of gene regulators that mostly downregulate gene expression at the post-transcriptional level (Ambros, 2001; Bartel, 2004). Dysregulation of miRNAs and some other ncRNAs is being found to have relevance to tumorigenesis, neurological, cardiovascular, developmental, respiratory, and other diseases, as well as individual response to drugs (Zhang and Dolan, 2010; Esteller, 2011; Zhou et al., 2011). For example, dysregulation of miRNAs in brains has been observed after exposure to addictive drugs like cocaine (Eipper-Mains et al., 2011), while upregulation of some miRNAs have been identified in human alcoholics (Lewohl et al., 2011). In addition, pharmacogenomic studies have related miRNAs as an important mechanism for regulating genes responsible for drug response (Zhang and Dolan, 2010; Huang et al., 2011). A comprehensive understanding of miRNAs and other functional ncRNAs will be necessary for future integration of various "omics" data for elucidating the underpinning mechanisms of complex diseases, phenotypes, and traits including the fundamental problem of gene expression regulation.

Given the critical roles of miRNAs in gene regulation and human diseases, it is imperative to have a comprehensive and reliable catalog of miRNA sequences (e.g., mature miRNAs, pre-, and pri-miRNAs) and miRNA gene targets. In addition to experimental approaches, computational algorithms have been developed for predicting miRNA sequences and their potential gene targets. To allow user-friendly queries, there are publicly available, web-based resources for miRNA sequences and gene targets. The current bioinformatic resources of miRNAs provide miRNA sequences and their gene targets from both experimental approaches and computational algorithms (*in silico* predictions). Furthermore, SNPs within the sequences of human miRNAs and their gene targets have been shown to have impact on various phenotypes and diseases such as cancer, blood pressure, and drug resistance (Mishra et al., 2007; Sethupathy et al., 2007; Landi et al., 2008). The polymorphisms in miRNA (miRSNPs) appear to have a differing effect on gene and protein expression, thus representing a novel type of genetic variation that may influence complex traits such as the risks of certain human diseases (Chen et al., 2008). With the advances in genotyping and sequencing technologies, genetic variation data on miRSNPs have become available for analyses in elucidating the mechanisms of miRNAs and complex phenotypes/traits. Therefore, we reviewed several publicly available miRNA-related (particularly, in humans) bioinformatic resources that accommodate miRNA sequences, miRNA gene targets, and miRSNPs (**Table 1**). Prospectively, these bioinformatic resources of miRNAs will need to address the challenges raised by the emerging applications of novel technologies (e.g., next-generation sequencing) in miRNA research.

miRBASE – A DATABASE OF miRNA SEQUENCES

Since its launch as the MicroRNA Registry (Griffiths-Jones, 2004), the miRBase database[1] (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008; Kozomara and Griffiths-Jones, 2011) has evolved into a central portal for miRNA sequences and other relevant information. The miRBase is a searchable database of published mature miRNA sequences, together with their predicted source hairpin precursors and annotations relating to their discovery, structure, and function (Griffiths-Jones et al., 2006). The miRBase contains miRNA sequence information from two fundamentally different sources: (1) experimentally verified mature miRNAs; and (2) miRNA sequences that are predicted homologs of miRNAs verified in a related organism (Griffiths-Jones et al., 2006). Each entry

[1]http://www.mirbase.org/

Table 1 | Some widely used bioinformatic resources of microRNA sequences, gene targets and genetic variation.

Contents	Database	Data source/type of evidence	URL
MicroRNA sequences	miRBase	Computational prediction, sequencing, and experimental verification	http://www.mirbase.org/
	Ensemble	Comprehensive genome browser	http://www.ensembl.org/
	UCSC genome browser	Comprehensive genome browser	http://genome.ucsc.edu/
MicroRNA targets	MicroCosm	Computational prediction	http://www.ebi.ac.uk/enright-srv/microcosm/
	TargetScan		http://www.targetscan.org/
	PicTar		http://pictar.mdc-berlin.de/
	ExprTarget	Computational prediction and microarray	http://www.scandb.org/apps/microrna/
	miRDB		http://mirdb.org/
	microRNA.org		http://www.microrna.org/
	TarBase	Experimental validation	http://diana.cslab.ece.ntua.gr/tarbase/
	miRecords	Comprehensive database	http://mirecords.biolead.org/
MicroRNA variation	miRvar	Literature	http://genome.igib.res.in/mirlovd/

in the miRBase database represents a predicted hairpin portion of a miRNA transcript (termed "mir" in the database), with information on the location and sequence of the mature miRNA sequence (termed "miR"; Ambros et al., 2003).

The current miRBase Release 18 (November, 2011) contains 18,226 entries representing hairpin precursor miRNAs, expressing 21,643 mature miRNA products, in 168 species. Particularly, there are 1,527 human miRNA sequences provided bythe miRBase (accessed on December 1, 2011). The miRBase also provides links of miRNAs to potential gene targets predicted by MicroCosm, TargetScan (Lewis et al., 2003), and PicTar (Krek et al., 2005), which are described in the following section. Both hairpin and mature sequences are available for searching and browsing, and entries can also be retrieved by name (e.g., "let-7" or "miR-36"), keyword (e.g. "human"), references, and annotation. Queries can also be performed by genomic location, tissue expression (e.g., adenosquamous cell, embryonic stem cell for humans) and sequence. For searches by sequence, users can perform either BLASTN or SSEARCH to search against the intact precursor sequences or just the mature miRNAs. All sequence and annotation data served at the miRBase are also available for bulk download. In addition, novel miRNA sequences can be submitted to miRBase by researchers, therefore, the miRBase acts as a central depository of miRNA sequences. Besides the miRBase website, other major genome databases such as the Ensemble[2] and UCSC Genome Browser[3] (Kent et al., 2002) also provide access to miRNA sequences and annotations. For example, the Ensemble Genome Browser can be used to obtain miRNA sequences that have been predicted by BLASTN of genomic sequence slices against miRBase sequences (Guttman et al., 2009).

RESOURCES OF miRNA GENE TARGETS

Given the critical roles of miRNAs in regulating gene expression and cellular functions, a comprehensive and reliable catalog of miRNA gene targets will benefit the biomedical research community, for example, by enabling the construction of gene regulatory networks, as well as gene-set or pathway-based analyses. Though molecular biology approaches may be used to experimentally determine gene targets for miRNAs, the lack of high throughput techniques and the relatively high cost associated with experimental approaches limit their use in identifying all of miRNA gene targets. Therefore, to date, only some gene targets of a small number of the potential >1000 human miRNAs have been confirmed experimentally. Another major challenge with miRNA gene target identification is that an individual miRNA may regulate multiple mRNAs, and in contrast, an individual gene may also be regulated by multiple miRNAs, thus representing a complex network of miRNA–mRNA interactions. In lieu of experimental approaches, several computational approaches have been developed for genome-wide prediction of miRNA gene targets by taking advantage of the properties of miRNAs' binding to gene targets. In animals, miRNAs typically bind to the 3′-UTRs (untranslated regions) of their target mRNAs to form miRNA–mRNA duplexes, leading to regulatory repression of translation (Lai et al., 2004), although the exact mechanism is not completely clear. Therefore, these approaches commonly depend on either miRNA–mRNA complementarity: e.g., miRanda (Enright et al., 2003; Griffiths-Jones et al., 2008), TargetScan (Lewis et al., 2003), or miRNA–mRNA duplex thermodynamics: e.g., PicTar (Krek et al., 2005). Notably, pair-wise comparisons of the miRanda scores (Enright et al., 2003; Griffiths-Jones et al., 2008), PicTar scores (Krek et al., 2005), and TargetScan scores (Lewis et al., 2003) were performed to evaluate the correlations between these computational algorithms (Gamazon et al., 2010). In general, these computational algorithms appeared to generate a significant number of overlapping miRNA gene targets between each pair of algorithms (Gamazon et al., 2010): 21,590 between miRanda (Enright et al., 2003; Griffiths-Jones et al., 2008) and TargetScan (Lewis et al., 2003), 2,465 between miRanda (Enright et al., 2003; Griffiths-Jones et al., 2008) and PicTar (Krek et al., 2005), as well as 8,707 between TargetScan (Lewis et al., 2003) and PicTar (Krek et al., 2005). Using TarBase (Papadopoulos et al., 2009), a database of experimentally supported miRNA targets, as gold standard, an receiver operating characteristic (ROC) analysis on each of the above-mentioned computational algorithms would seem to suggest that TargetScan (Lewis et al., 2003) may yield slightly better performance than the other computational methods (Gamazon et al., 2010). Recently, with the availability of whole-genome microarray data, approaches that aim to integrate both microarray data and sequence-based evidence have also been proposed to predict miRNA targets (e.g., ExprTarget; Gamazon et al., 2010). However, caution must be exercised in the interpretation of these results, given each prediction approach may be biased toward particular characteristics of miRNA–mRNA interactions, possibly a reason why some of these approaches may not provide consistent predictions. For example, since gene expression can be tissue-specific, results from integrative approaches utilizing both sequence-based evidence and microarray data, therefore, could be biased toward genes expressed in a particular tissue (e.g., the LCLs). Regarding bioinformatic resources, there are several widely used web-based databases that provide access to those computationally predicted miRNA gene targets, experimentally supported miRNA gene targets and miRNA gene targets derived from integrated approaches.

MICROCOSM TARGETS

The MicroCosm Targets is a web resource (formerly the miRBase Targets)[4] containing computationally predicted targets for miRNAs across many species including humans. The current Version 5 database contains detected relationships for 851 human miRNAs and ∼35,000 transcripts (accessed on December 1, 2011). The miRNA sequences were from the miRBase; Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008; Kozomara and Griffiths-Jones, 2011) and most genomic sequences from Ensembl (see text footnote 2). The miRanda algorithm (Enright et al., 2003; Griffiths-Jones et al., 2008) was used to identify potential binding sites for a given miRNA in genomic sequences. Particularly, the current Version 5 of the MicroCosm Targets database used dynamic programming alignment to identify highly complementary miRNA–mRNA pairs which are scored between 0 (i.e., no

[2]http://www.ensembl.org/
[3]http://genome.ucsc.edu/
[4]http://www.ebi.ac.uk/enright-srv/microcosm/

complementarity) and 100 (i.e., complete complementarity). The miRanda algorithm (Enright et al., 2003; Griffiths-Jones et al., 2008) uses a weighted scoring system and rewards complementarity at the 5′ end of miRNA. *P*-values are also calculated following statistical model proposed by Rehmsmeier et al. (2004). Since miRanda requires strict complementarity at the seed region (i.e., the 5′ end of miRNA) of miRNA–mRNA pairs, this algorithm does not allow alignments where more than one base in the seed region is not complementary to a target mRNA. Furthermore, gene targets selected by miRanda (Enright et al., 2003; Griffiths-Jones et al., 2008) were passed through the Vienna RNA folding routines (Hofacker, 2003; Gruber et al., 2008) for evaluating the property of thermodynamic stability. MicroCosm Targets supports queries for individual miRNAs, individual genes, gene ontology (GO; Ashburner et al., 2000) classes (e.g., cellular component, biological process) in a selected genome (e.g., the human genome), as well as batch queries. The MicroCosm Targets supports bulk download of the complete dataset.

TARGETSCAN

TargetScan[5] (Lewis et al., 2003) is a web resource that provided computationally predicted miRNA gene targets by searching for the presence of conserved 8 and 7 mer sites that match the seed region of each miRNA for a variety of species. TargetScanHuman (Release 6.0, November, 2011) is a sub-database for predicting human miRNA gene targets based on the TargetScan approach (Lewis et al., 2003). The current TargetScanHuman Release 6.0 contains ∼18,000 mRNAs, corresponding to ∼30,000 transcripts (accessed on December 1, 2011). As an option, TargetScanHuman also provides non-conserved sites and sites with mismatches in the seed region that are compensated by conserved 3′ pairing (i.e., conserved 3′-compensatory sites; Friedman et al., 2009). The TargetScan predictions are ranked based on the predicted efficacy of targeting (Grimson et al., 2007; Garcia et al., 2011) and by their probability of conserved targeting (i.e., P_{CT}; Friedman et al., 2009), which reflects the Bayesian estimate of the probability that a site is conserved due to selective maintenance of miRNA targeting rather than by chance or any other reason not pertinent to miRNA targeting. TargetScan supports bulk download of the complete dataset.

PICTAR

PicTar[6] (Krek et al., 2005), a searchable website provides details (e.g., 3′-UTR alignments with computationally predicted sites, links to various public databases) of human miRNA targets that are not conserved but co-expressed (i.e., the miRNA and mRNA expressed in the same tissue; Chen and Rajewsky, 2006), as well as miRNA target predictions in vertebrates (Krek et al., 2005), *Drosophila* (Grun et al., 2005), and nematode species (Lall et al., 2006). PicTar (Krek et al., 2005) uses a probabilistic model to compute the likelihood that sequences are miRNA target sites when compared to the 3′-UTR background. *In vivo* experimental validation suggests a high degree of accuracy and sensitivity for the PicTar algorithm in flies (Stark et al., 2005). PicTar (Krek et al., 2005) supports queries and bulk download for more than 13,000 co-expressed human microRNA target predictions (Chen and Rajewsky, 2006) between 31 unique human miRNAs and ∼5,000 transcripts in multiple tissues (accessed on December 1, 2011).

TARBASE

In contrast to those databases for computationally predicted miRNA gene targets, TarBase[7] (Papadopoulos et al., 2009) v5.0 (accessed on December 1, 2011) houses a manually curated collection of more than 1,300 experimentally supported miRNA target interactions in a variety of species including human, mouse, and several other model organisms. TarBase (Papadopoulos et al., 2009) supports queries for individual miRNAs and genes in a selected organism. For humans, the current v5.0 database contains 1,094 miRNA–mRNA relationships (>100 miRNAs and ∼900 transcripts) that are supported by experimental evidence. For each miRNA–mRNA relationship, TarBase (Papadopoulos et al., 2009) provides the type of experimental support (e.g., microarray, pSILAC – pulsed stable isotope labeling by amino acids), detailed information on miRNA and mRNA target, as well as the scientific references.

EXPRTARGET

The efficacy of computational approaches to locate and rank potential genomic binding sites is supported by the relatively high degree of miRNA complementarity to experimentally determined binding sites (Maziere and Enright, 2007). In contrast, ExprTarget[8] (Gamazon et al., 2010) provides a comprehensive catalog of miRNA targets supported by both sequence-based evidence and microarray gene expression association. Particularly, ExprTarget (Gamazon et al., 2010) combines miRNA (Huang et al., 2011) and exon array expression data (Zhang et al., 2009; mRNA-level) on 117 unrelated HapMap CEU (Caucasians from Utah, USA) and YRI (Yoruba people from Ibadan, Nigeria) LCL samples, thus providing an expression-corroborated catalog of miRNA targets (225 expressed miRNAs in LCLs and ∼9,000 transcripts) that were also predicted using the miRanda algorithm (Ambros, 2001; Enright et al., 2003; Griffiths-Jones et al., 2008). ExprTarget (Gamazon et al., 2010) will be expanded to accommodate miRNA target predictions in other human tissues such as liver.

miRDB

miRDB[9] (Wang, 2008; Wang and El Naqa, 2008), is an online database for predicted miRNA targets in animals using an algorithm called MirTarget2, which was developed by analyzing thousands of genes impacted by miRNAs with an support vector machine (SVM) algorithm and a microarray training dataset. Particularly, by systematically analyzing public microarray data, statistically significant features that are important to gene target downregulation were identified (Wang and El Naqa, 2008). For human miRNAs, the current miRDB v4.0 (accessed on January 10, 2012) contains 1,919 miRNAs and more than >16,000 unique gene targets. About half of the predicted human miRNA target sites are not conserved in

[5]http://www.targetscan.org/
[6]http://pictar.mdc-berlin.de/
[7]http://diana.cslab.ece.ntua.gr/tarbase/
[8]http://www.scandb.org/apps/microrna/
[9]http://mirdb.org/

other organisms (Wang, 2008; Wang and El Naqa, 2008). MirTarget2 has been validated with independent experimental data for its improved performance on predicting miRNA downregulated gene targets (Wang and El Naqa, 2008). miRDB (Wang, 2008; Wang and El Naqa, 2008) supports queries for individual targets or miRNAs, as well as bulk download of the complete dataset.

microRNA.org

The microRNA.org website[10] (John et al., 2004; Landgraf et al., 2007; Betel et al., 2008) is a comprehensive resource of miRNA target predictions, target downregulation scores, and experimentally observed expression patterns. Particularly, the microRNA.org target predictions are based on a development of the miRanda algorithm (Enright et al., 2003; Griffiths-Jones et al., 2008), which incorporates current biological knowledge on target rules and on the use of a compendium of mammalian miRNAs. The miRNA expression profiles were derived from a comprehensive sequencing project of mammalian tissues and cell lines of normal and disease origin (John et al., 2004; Landgraf et al., 2007; Betel et al., 2008). The current version of the microRNA.org database (released in August, 2010) contains predicted interactions between 249 human miRNAs and more than 19,000 human genes (accessed on December 1, 2011). The microRNA.org miRNA target predictions and expression data (John et al., 2004; Landgraf et al., 2007; Betel et al., 2008) are also available as tab-delimited files for bulk downloads.

miRECORDS

miRecords[11] (Xiao et al., 2009) is a comprehensive resource for animal miRNA–mRNA interactions from some of the above-mentioned algorithms and experimental approaches. miRecords (Xiao et al., 2009) consists of two components: (1) Validated Targets component – a large, high-quality database of experimentally validated miRNA targets resulting from manual literature curation; and (2) The Predicted Targets component – an integration of predicted miRNA targets produced by some established miRNA target prediction programs: e.g., miRanda (Enright et al., 2003; Griffiths-Jones et al., 2008) and TargetScan (Lewis et al., 2003). miRecords (Xiao et al., 2009) allows queries of individual miRNAs in a selected organism. The search results provide detailed evidence for miRNA–mRNA interactions from a variety of algorithms and databases. The current Validated Targets component of miRecords (Xiao et al., 2009; released in November, 2010) hosts the interactions between 384 miRNAs and more than 1,000 target genes in nine animal species. miRecords (Xiao et al., 2009) supports bulk download of the complete list of validated gene targets collected in the database.

MiRSNPs – NOVEL PLAYERS IN COMPLEX TRAITS

Genomic variations including germ line or somatic mutations may count for miRNA abnormal expression by altering their biogenesis and/or affect the ability of miRNAs to bind to their target mRNAs. For example, aberrant allele frequencies of the SNPs located in miRNA target sites were found to be potentially associated with human diseases (e.g., cancer; Yu et al., 2007; Landi et al., 2008). With the availability of comprehensive genetic variation data: e.g., the International HapMap Project (HapMap, 2003, 2005) and the 1000 Genomes Project (1000 Genomes Consortium, 2010) genotypic data, studies of miRSNPs or miR-polymorphisms have begun to shed novel light to the distribution and potential biological/clinical implication of these polymorphisms. For example, using the HapMap Phase 1/2 genotypic data (HapMap, 2003, 2005) on the CEU and YRI, a genome-scale search for the regulatory polymorphisms in the loci of pre-miRNAs and their gene targets demonstrated 187 SNPs in the pre-miRNAs, 497 consensus SNPs in the seed-matching 3-UTR of target genes, 385 CNVs harboring pre-miRNA precursors and 9 CNVs covering important miRNA processing genes (Duan et al., 2009), indicating potential roles of miR-polymorphisms in regulating miRNAs and their gene targets. Future studies that take advantage of more comprehensive genotypic data such as those from the 1000 Genomes Project (1000 Genomes Consortium, 2010) and other sequencing efforts will help generate a more comprehensive list of miR-polymorphisms, thus facilitating future association studies between the genetic polymorphisms in miRNA targets/pre-miRNAs and disease susceptibility or therapeutic outcome.

Since the research of miRSNPs is a relatively new field in miRNA research, especially relative to the research of miRNA gene targets, currently there are few bioinformatic tools or resources that specifically devote to miRSNPs. Efforts such as the miRvar database (Bhartiya et al., 2011) may prove valuable to the miRNA research community for integrating this important type of genetic variation in biomedical research.

miRVAR – A DATABASE FOR GENOMIC VARIATIONS IN miRNAs

miRvar[12] (Bhartiya et al., 2011) is a comprehensive database for genomic variations in miRNAs. This database contains curated genetic variations in miRNA loci in the human genome. Particularly, the miRNA genetic variation data is made available on the Leiden Open (source) Variation Database (LOVD) platform to provide ease of aggregation and analysis. For each genetic variant in a particular miRNA, miRvar (Bhartiya et al., 2011) provides detailed information and links to other publicly available resources such as miRBase (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008; Kozomara and Griffiths-Jones, 2011), miRecords (Xiao et al., 2009), UCSC Genome Browser (Kent et al., 2002). In addition, miRvar (Bhartiya et al., 2011) is open for community curation efforts.

CONCLUSION

Since the discovery of miRNAs a decade ago, significant progress has been made in identifying both miRNAs and their gene targets. A number of bioinformatic resources of miRNAs have emerged to accommodate the needs for the miRNA research community. Given the unique properties of these small ncRNA molecules, in addition to experimental approaches, the identification of miRNAs and their gene targets have substantially relied upon computational and systems biology approaches. The current bioinformatic resources of miRNAs have greatly facilitated

[10] http://www.microrna.org/
[11] http://mirecords.biolead.org/
[12] http://genome.igib.res.in/mirlovd/

the research of miRNAs and their gene targets, as well as the integration of miRNAs into other "omics" (e.g., transcriptomics, proteomics, metabolomics) studies in elucidating the networks of complex phenotypes (e.g., gene expression, individual response to drugs) as well as the pathogenesis of human diseases. However, a big challenge facing these current bioinformatic resources is the integration of various resources, not only with the miRNA resources (e.g., the miRNA gene target prediction databases/approaches), but also with other relevant resources to facilitate the next stage of systems biomedicine research. For example, the integration of miRNA prediction databases with the PharmGKB[13] (McDonagh et al., 2011), which provides the published genetic variants implicated in drug response, may help elucidate the role of the interactions between genetic and non-genetic factors (e.g., epigenetic factors including miRNAs) in determining individual drug response, thus facilitating the realization of personalized medical care.

The current knowledge of miRNA and their interactions with gene targets have been largely uncovered by experimental approaches, microarray-based studies, and computational algorithms. During the past several years, with the advancement of the next-generation sequencing technologies (e.g., the Illumina HiSeq Sequencing System, Roche 454 GS System; Mardis, 2008), it is now possible to perform RNA-sequencing to comprehensively catalog miRNAs in cells under various phenotypic contexts or disease statuses, thus enhancing our knowledge of the distribution, abundance and roles of miRNAs in real-time cellular environments. For example, deep sequencing platforms have revealed unexpected complexity in relation to miRNAs, including 5′ and 3′-end-length heterogeneity and RNA editing (Vaz et al., 2012). Notably, the majority of the evidence supporting miRNA annotations are now coming from deep sequencing experiments, as recorded in the miRbase (Kozomara and Griffiths-Jones, 2011). Though analysis tools such as miRDeep (Friedlander et al., 2008) have facilitated the current miRNA-targeted sequencing studies (Dhahbi et al., 2011), however, challenges remain to be addressed for properly analyzing these data, particularly the difficulties in aligning short reads from RNA-sequencing projects to profile miRNAs in various cellular environments, as well as distinguishing true miRNAs from fragments of other transcripts (Kozomara and Griffiths-Jones, 2011). The availability of these large-scale sequencing data, therefore, underscores the importance of data analysis tools and bioinformatic resources that enable users to efficiently analyze the unprecedented amounts of RNA-sequencing data. Notably, bioinformatic tools such as deepBase[14] (Yang et al., 2010) have begun to emerge with the ambitious attempt to provide a universal platform for mapping, storage, retrieval, analysis, integration, annotation, mining and visualization of next-generation sequencing data on small and long ncRNAs including miRNAs. In addition, new research resources such as the Sanger Institute mirKO (Prosser et al., 2011), a library of mouse embryonic stem cell clones with deletions for the "majority" of known miRNAs may prove to be invaluable for the rigorous investigation of miRNA function. Furthermore, data from sequencing efforts such as the 1000 Genomes Project (1000 Genomes Consortium, 2010) may greatly improve our understanding of miRSNPs and their potential contribution to complex phenotypes/traits. Given the fast-evolving technologies in the general biomedical research, future development of bioinformatic resources of miRNAs would need to address significant challenges that remain to be met for better integration and utilization of these tremendous resources for the benefit of the miRNA research community.

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[13]http://www.pharmgkb.org/

[14]http://deepbase.sysu.edu.cn/

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The emerging role of non-coding RNAs in drug addiction

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Prolonged drug use causes long-lasting neuroadaptations in reward-related brain areas that contribute to addiction. Despite significant amount of research dedicated to understanding the underlying mechanisms of addiction, the molecular underpinnings remain unclear. At the same time, much of the pervasive transcription that encompasses the human genome occurs in the nervous system and contributes to its heterogeneity and complexity. Recent evidence suggests that non-coding RNAs (ncRNAs) play an important and dynamic role in transcriptional regulation, epigenetic signaling, stress response, and plasticity in the nervous system. Dysregulation of ncRNAs are thought to contribute to many, and perhaps all, neurological disorders, including addiction. Here, we review recent insights in the functional relevance of ncRNAs, including both microRNAs (miRNAs), and long non-coding RNAs, and then illustrate specific examples of ncRNA regulation in the context of drug addiction. We conclude that ncRNAs are importantly involved in the persistent neuroadaptations associated with addiction-related behaviors, and that therapies that target specific ncRNAs may represent new avenues for the treatment of drug addiction.

Keywords: addiction, long non-coding RNA, microRNA, epigenetic, lncRNA, miRNA

INTRODUCTION

Evolutionary processes in the human lineage have coupled expanding genome complexity with the acquisition, processing, and distribution of ever increasing amounts of information (Sempere et al., 2006; Mattick, 2007; Berezikov, 2011). Non-coding RNAs (ncRNAs) occupy the center stage of this expanding genome complexity, with the ratio of non-coding genomic sequence to protein coding sequence growing more than 10-fold as primates evolved from simple multicellular organisms (Taft et al., 2007). ncRNAs occupy critical nodes and edges in a majority of physiological networks, frequently participating in feedback loops, stability, and fined-tuned regulatory control. The unique information processing features of ncRNAs permit them to transduce information through heterogeneous molecular machineries with relatively less energy cost than protein networks alone, making them particularly useful in the limited and thermally constrained real estate of the human central nervous system.

In light of these observations, it is not surprising that non-exonic transcripts (those mapping to regions of the genome outside annotated protein coding genes) comprise 2/3 of the total, non-ribosomal, non-mitochondrial RNA in the human brain (Kapranov et al., 2010). Highly articulated nervous system expression of miRNAs (Lagos-Quintana et al., 2002; Schratt et al., 2006; Makeyev et al., 2007) and long non-coding RNAs (lncRNAs; Mercer et al., 2008; Ponjavic et al., 2009) point to the involvement of ncRNAs in key aspects of nervous system homeostasis and plasticity. The ability of addictive stimuli to perturb and disrupt these functions suggests a broad based involvement of ncRNAs in the loss of functional coherence associated with chronic drug use (Chandrasekar and Dreyer, 2009; Hollander et al., 2010). In this review, we consider the participation of ncRNAs in the key nervous system molecular machineries affected by addiction, and the potential mechanisms of their involvement in the dimensions of this complex disorder.

INVOLVEMENT OF ncRNAs IN NEUROPLASTICITY AND LEARNING

Emerging evidence suggests that miRNAs and their processing machinery play a critical role in neuroplasticity by regulating protein dynamics in the synapse (Edbauer et al., 2010; Lippi et al., 2011; Siegel et al., 2011; Saba et al., 2012). Given that addiction is considered a maladaptive form of neuroplasticity, identifying miRNAs important in such neuroadaptations may lead to novel insights in addiction research. Demonstrating a role for miRNAs in neuroplasticity, Schratt et al. (2006) showed that miR-134 expression in the dendrites of developing hippocampal neurons is critically involved in dendritic spine formation and plasticity by inhibiting Lim domain-containing protein kinase 1, an important regulator of actin filament dynamics. Following this seminal report, other studies revealed that miR-138, miR-132, and miR-125b play an essential role in dendritic spine formation (Siegel et al., 2009; Edbauer et al., 2010). The role of miRNAs in synapse are not restricted to the those listed above, as recent reports have identified numerous dendritic- and synaptic-enriched miRNAs (Lugli et al., 2008; Eipper-Mains et al., 2011; Saba et al., 2012). Although most of the research thus far has focused on miRNAs, there is some evidence that suggests lncRNAs are also involved in synaptic plasticity. For instance, BC1 and BC200 are lncRNAs that are localized in post-synaptic dendritic compartments where they regulate local gene expression by controlling the activity of specific transcription factors (Lin et al., 2008). Other lncRNAs are

also localized in the synapse and neurite extensions (French et al., 2001; Mercer et al., 2008), but their functional role in plasticity remains poorly understood.

In neurons, activity-dependent regulation of miRNAs also contributes extensively to neuroplasticity. Using the marine snail *Aplysia*, Rajasethupathy et al. (2009) demonstrated that serotonin-induced activation of sensory neurons caused a significant decrease in miR-124, leading to increased cAMP response element-binding (CREB) expression and induction of long-term facilitation. Knockdown of miR-124 also robustly increased serotonin-induced synaptic plasticity and CREB expression, revealing a functional association between miR-124 activity and CREB expression. Additional *in vivo* studies have demonstrated activity-dependent up regulation of miR-132 and miR-212 following induction of LTP in hippocampal neurons, an increase that was dependent on metabotropic glutamate receptor activation (Wibrand et al., 2010). Consistent with the role of miR-132 in neuronal activation, enhanced expression of miR-132 was observed in a number of behavioral paradigms, such as contextual fear conditioning, odorant exposure, and acute cocaine treatment (Nudelman et al., 2010). In addition, over expressing miR-132 enhances neuronal activity in cortical and hippocampal neurons (Cheng et al., 2007), possibly through miR-132-mediated inhibition of p250GAP, a protein associated with dendritic plasticity (Wayman et al., 2008).

The ability of ncRNAs to regulate transcription factors and chromatin remodeling proteins represent additional mechanisms to influence long-term neuroadaptations involved in memory formation. For example, miR-324 and miR-369, two miRNAs implicated in cocaine-induced neuroplasticity (Schaefer et al., 2010), have been shown to modulate transcription factors (MEF2 and FosB) important in reward-related learning and memory (Hiroi et al., 1997; Pulipparacharuvil et al., 2008). Additionally, miR-132 regulation of chromatin remodeling factors methyl CpG binding protein 2 (MeCP2), p300, and Jumonji and ARID domain protein 1 A (JARID1A) in the suprachiasmatic nucleus is important in neuroadaptations associated with circadian rhythm (Alvarez-Saavedra et al., 2011). Although no study to date has examined lncRNAs in learning and memory paradigms, lncRNAs could potentially be involved in long-term neuroadaptations, as they have been shown to regulate transcriptional factors, DNA methylation, and histone modifications (Rinn et al., 2007; Houseley et al., 2008; Yu et al., 2008; Khalil et al., 2009).

The studies reviewed above clearly show the importance of miRNAs (and potentially lncRNAs) in synaptic plasticity and learning and memory. However, many questions remain for future studies to better understand the role of ncRNAs in neuroplasticity. For example, the relationship between ncRNA-mediated plasticity and psychiatric disorders, such as addiction, has raised a number of intriguing questions: Are the same ncRNAs that are involved in plasticity also involved in addiction, or are other addiction-related ncRNAs involved, whose functions have yet to be determined? What are the specific targets of ncRNAs and how do these interactions contribute to the neuroadaptations associated with addiction? These are just a few questions that are currently being addressed to understand the complex relationship between ncRNA-mediated plasticity and addiction.

EMERGING ROLE FOR miRNAs IN ADDICTION

Drugs of abuse induce persistent changes in neuroplasticity by usurping gene regulatory mechanisms, in turn leading to addiction. Given the role of miRNAs in gene regulation and synaptic plasticity, recent studies have begun to examine the involvement of miRNAs in response to drugs of abuse (**Table 1**). Here, we highlight some of the recent findings that illustrate the importance of miRNAs in drug-induced synaptic plasticity, drug-seeking behaviors, and tolerance to several abused drugs.

COCAINE AND AMPHETAMINE

To investigate the role of miRNAs in cocaine addiction, Chandrasekar and Dreyer (2009) utilized *in silico* prediction models to identify miRNAs that potentially regulate cocaine-associated genes and discovered a strong prediction for miR-124, let-7d, and miR-181a. Further *in vivo* studies showed that expression of miR-181a is increased and miR-124 and let-7d are decreased in striatum of rats with a history of chronic cocaine exposure. Subsequent behavioral studies revealed that over expression of miR-124 and let-7d in the nucleus accumbens reduced cocaine conditioned place preference (CPP), whereas over expression of miR-181a enhanced cocaine preference (Chandrasekar and Dreyer, 2011). These effects on cocaine CPP were inversed when expression of the aforementioned miRNAs were inhibited. Interestingly, it was found that altering expression of these miRNAs in the nucleus accumbens modulated the expression of many addiction-related genes (Chandrasekar and Dreyer, 2009). For example, overexpression of miR-124 and let-7d increased dopamine transporter, whereas miR-181a over expression decreased it. Because the dopamine transporter is the primary target of cocaine and is importantly involved in cocaine CPP (Tilley et al., 2009), the observed behavioral changes are likely reflected, in part, by miRNA-regulation of the dopamine transporter. Notably, the expression of several other addiction-related genes, such as Brain-derived neurotrophic (BDNF), CREB, MeCP2, and ΔFosB were also regulated by these miRNAs, thus illustrating the widespread effects on addiction-related gene networks in response to changes in miRNA levels.

Using an extended access model of cocaine self-administration, Hollander et al. (2010) examined the role of miRNAs in regulating compulsive-like cocaine intake. In this study, dorsal striatal miR-212 levels were found to be significantly elevated in rats with a history of extended access to cocaine, but not in rats with short-access to cocaine or in rats receiving non-contingent cocaine exposure (yoked control). Further investigation showed that over expression or knockdown of miR-212 in the dorsal striatum decreased or enhanced cocaine self-administration under extended access conditions, respectively, suggesting that striatal miR-212 is involved in an adaptive response to inhibit escalation of cocaine intake. Reduced motivation to consume cocaine was attributed in part by miR-212-mediated upregulation of CREB, a known antagonistic regulator of cocaine reward (Carlezon et al., 1998). Subsequent studies from the same laboratory revealed that MeCP2 regulates cocaine intake via a homeostatic interaction with miR-212 to influence cocaine-mediated effects on striatal BDNF (Im et al., 2010). By revealing an important miRNA-mediated epigenetic mechanism involved in drug-seeking behaviors, this study raises a number of interesting possibilities because epigenetic

Table 1 | ncRNAs regulated by drugs of abuse (green = upregulation, red = downregulation).

Drug	ncRNAs	Mechanism(s) involved	Reference
miRNAs			
Cocaine	miR-212	Elevated following extended access to cocaine; targets CREB; interaction with MeCP2 and influences BDNF expression	Hollander et al. (2010), Im et al. (2010)
	miR-124, let-7d, miR-181a	Targets BDNF, DAT, CREB, mGluR5, FosB; involved in cocaine CPP	Chandrasekar and Dreyer (2009, 2011)
	miR-181a	Enriched in NAc synapse, regulates GluA2 expression	Saba et al. (2012)
	Ago2	Ago2 knockout in striatal D2R neurons reduces cocaine self-administration.	Schaefer et al. (2010)
	miR-8, miR-7, miR-142, and let-7 families	Upregulated in striatal PSD	Eipper-Mains et al. (2011)
	miR-200c, miR125a-5p, miR-429, miR-370, miR-183, miR-200b, miR-770-5p, miR200a	Downregulated in striatal PSD	Eipper-Mains et al. (2011)
Nicotine	miR-140	Regulates dynamin-1 expression	Huang and Li (2009b)
	miR-504	Regulates DRD1 expression	Huang and Li (2009a)
		Upregulated in hippocampus	Lippi et al. (2011)
Alcohol	miR-9	Downregulates BK channels	Pietrzykowski et al. (2008)
	miR-497, miR-302b	Involved in ethanol-induced neuronal death; targets BCL2 and cyclin D2	Yadav et al. (2011)
Opioids	miR-23b	Regulates mOR expression	Wu et al. (2009)
	let-7	Regulates mOR expression	He et al. (2010)
	miR-190	Downregulated by mOR activation via ERK signaling; targets NeuroD	Zheng et al. (2010)
	miR-133b	Morphine-induced downregulation causes enhanced Pitx3 expression	Sanchez-Simon et al. (2010)
lncRNAs			
Heroin	MIAT, MEG3, NEAT1, NEAT2	Upregulated in NAc of heroin abusers	Michelhaugh et al. (2011)
Cocaine	MIAT, MEG3, NEAT2, EMX2O	Upregulated in NAc of cocaine abusers	Michelhaugh et al. (2011)

BCL2, B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; CREB, cAMP response element-binding; CPP, conditioned place preference; DAT, dopamine transporter; DRD1, Dopamine receptor D1; D2R, dopamine receptor D2; ERK, Extracellular signal-regulated kinase; FosB, FBJ murine osteosarcoma viral oncogene homolog B; mGluR5, Metabotropic glutamate receptor 5; mOR, mu opioid receptor; NAc, nucleus accumbens; Pitx3, Pituitary homeobox 3; PSD, post-synaptic density.

factors associated with addiction have recently been the focus of intense investigation (Robison and Nestler, 2011).

Recent studies have examined synaptic expression of miRNAs in response to cocaine. By isolating striatal post-synaptic densities (PSD), Eipper-Mains et al. (2011) identified more than two dozen miRNAs that were significantly altered following chronic cocaine treatment. Interestingly, many of the PSD miRNAs affected by cocaine were found to be members of one of four families (miR-8, miR-7, miR-142, and let-7 families), suggesting that cocaine influences the expression of similar miRNAs with shared synaptic targets. In a similar study, Saba et al. (2012) utilized microarray screening to identify nine enriched and seven depleted miRNAs in the synaptodendritic compartment of the nucleus accumbens. They also revealed that miR-181a, one of the synaptically enriched miRNAs, is increased in reward-related brain regions following cocaine and amphetamine exposure, and miR-181a regulates synaptic plasticity by altering AMPA receptor subunit (GluA2) expression (Saba et al., 2012). Additional reports examining the role of miRNAs in drug-induced synaptic plasticity determined that miR-29a/b was significantly upregulated in addiction-related brain regions in mice with a history of cocaine or amphetamine exposure and plays an pivotal role in synaptic structure and function *in vitro* (Lippi et al., 2011).

Enzymes that regulate miRNA processing also appear to play a functional role in cocaine addiction. A study by Schaefer et al. (2010) revealed that knockout of argonaute 2 (Ago2, a protein important in miRNA processing) in accumbal dopamine 2 receptor expressing neurons significantly attenuated cocaine self-administration. Further investigation revealed that Ago2 regulates expression of numerous miRNAs in the striatum, and many of the Ago2-dependent miRNAs were predicted to target genes important in cocaine addiction. Consistent with the role of Ago2 in cocaine addiction, Eipper-Mains et al. (2011) showed that chronic cocaine exposure elevates Ago2 expression in the striatum. Dicer,

an enzyme responsible for producing mature miRNAs, may also be necessary in cocaine addiction, as previous studies have shown that manipulation of this enzyme affects learning and memory (Konopka et al., 2010) and miRNA expression in striatum (Cuellar et al., 2008).

NICOTINE

Utilizing miRNA microarray approach, Huang and Li identified 25 miRNAs that were altered in PC12 cells following nicotine exposure. They also found that miR-140 binds and reduces expression of dynamin-1 (Huang and Li, 2009b), a GTPase that has previously been shown to be important in nicotine dependence (Hwang and Li, 2006). The same research group showed that miR-504 targets a specific dopamine receptor D1 gene containing a single nucleotide polymorphism that has been associated with nicotine dependence (Huang and Li, 2009a). Interestingly, by increasing D1 receptor expression, miR-504 may promote nicotine intake by enhancing dopamine signaling. Finally, chronic injections of nicotine increased several miRNAs in mouse hippocampus, prefrontal cortex, limbic forebrain, and midbrain (Lippi et al., 2011), indicating that nicotine has broad effects on miRNA expression in several addiction-related brain areas, though the implications are not yet clear.

ALCOHOL

In a seminal set of studies, Pietrzykowski et al. (2008) revealed that alcohol upregulates miR-9 in rat striatum and supraoptic nucleus, two regions important in alcohol tolerance. The increase in miR-9 was found to contribute to alcohol tolerance by preferentially targeting BK channel mRNA isoforms that are sensitive to alcohol. BK channels, large conductance calcium, and voltage-activated potassium channels, are important in neuronal excitability, firing frequency, and neurotransmitter release and have been one of the best described targets for alcohol tolerance (for review see Treistman and Martin, 2009). Thus, miR-9-induced destabilization of alcohol sensitive BK channels likely contributes to alcohol tolerance and addiction by promoting the expression of more tolerant BK channel isoforms. Interestingly, the role of miR-9 in alcohol dependence may not be limited to tolerance, as miR-9 was also found to target other genes that have been implicated in addiction, such as dopamine receptor D2 and histone deacetylase 5 (Pietrzykowski et al., 2008).

Other alcohol-related studies have identified miRNAs involved in ethanol dependence. Guo et al. reported differential miRNA expression patterns following chronic ethanol exposure and ethanol removal in primary cortical neuron cultures. These results may indicate that different stages of alcohol addiction (maintenance, withdrawal, etc.) have distinct miRNA expression profiles (Guo et al., 2011), information that could be important for the development of new therapeutics to treat alcohol addiction. In another study, miR-497 and miR-302b were found to be involved in ethanol-induced neuronal cell death following chronic ethanol exposure, thereby providing a possible link between miRNAs and neuronal loss associated with chronic alcohol abuse (Yadav et al., 2011). In addition, recent studies using human post-mortem tissue, revealed that 35 miRNAs were significantly upregulated in the prefrontal cortex in alcoholics (Lewohl et al., 2011), again suggesting that chronic alcohol has widespread affects on miRNA expression reward-related brain areas. The development of artificial miRNAs may be a novel approach to treating alcoholism and other forms of addiction, as one recent study showed that targeting neurokinin-1 receptor gene with an artificial miRNA significantly reduced alcohol consumption in mice (Baek et al., 2010).

OPIOIDS

Similar to alcohol, specific miRNAs have been implicated in opioid tolerance and addiction. miRNAs, let-7, and miR-23b suppress mu opioid receptor mRNA expression following long-term morphine treatment (Wu et al., 2009; He et al., 2010), demonstrating a new mechanism that might play an important role in morphine tolerance. In addition, the mu opioid receptor agonist, fentanyl, but not morphine, downregulates miR-190 expression via extracellular signal-regulated kinase (ERK) signaling (Zheng et al., 2010), indicating that specific mu opioid receptor agonists have differential influence on miRNA expression.

SUMMARY

The studies highlighted in this section illustrate a wide range of miRNA-mediated mechanisms involved in addiction. With their ability to regulate addiction-related gene networks, drug-induced plasticity, drug-seeking behaviors, and drug tolerance, miRNAs are ideal therapeutic targets for the treatment of addiction. However, much more research in this nascent field is needed to reveal miRNA targets and mechanisms that contribute importantly to the addicted state. Thus, it seems clear that future studies will reveal ever more complex and intriguing properties of these key ncRNAs in addiction.

POTENTIAL ROLE FOR LONG NON-CODING RNAs IN ADDICTION

Recent large-scale genomic studies have revealed that lncRNAs are one of the most abundant classes of ncRNAs (Jia et al., 2010; Kapranov et al., 2010). Additionally, lncRNAs have been implicated in a number of important cellular processes including gene transcription, RNA processing, and chromatin modifications (Wang and Chang, 2011). Although lncRNAs are highly expressed in the brain (Mercer et al., 2008; Belgard et al., 2011), they remain poorly characterized in this context and their role in addiction is unclear. In an attempt to determine whether lncRNAs are differently expressed in response to chronic drug use, a recent study by Michelhaugh et al. (2011) identified lncRNAs that were altered in heroin abusers by mining existing Affymetrix datasets. Of the 23 lncRNAs identified, MIAT, MEG3, NEAT1, and NEAT2 were upregulated in the nucleus accumbens of heroin abusers compared to control subjects. Preliminary reports from the same research group also found that NEAT2, MIAT, MEG3, and EMX2OS are elevated in the nucleus accumbens of cocaine abusers (Michelhaugh et al., 2011), suggesting similar aberrations in lncRNA expression in response to different drugs of abuse. The lncRNAs listed above have been implicated in a range of cellular processes including cAMP signaling (Zhao et al., 2006), GABA neuron neurogenesis (Mercer et al., 2010), and regulation of genes associated with synaptic plasticity (Bernard et al., 2010), but the functional role of these lncRNAs in addiction remains unknown. Although these initial findings are

intriguing, only a small number of lncRNAs were examined, suggesting the need for more comprehensive analysis of transcriptome changes during these critical events, with emphasis on specific cell types and locus specific complexity of lncRNA expression during these changes.

Natural antisense transcripts (NATs), a subset of lncRNAs, are transcripts derived from the opposite strand of many protein coding (sense) genes. NATs bind to sense RNA and/or proteins to regulate transcription and translation. Recently, we demonstrated that BDNF, a gene known to be involved in addiction, is controlled by a conserved long non-coding antisense RNA transcript (BDNF-AS; Modarresi et al., 2012). BDNF-AS suppresses BDNF mRNA expression by altering chromatin structure at the BDNF gene locus. Inhibiting BDNF-AS by siRNA or other methods robustly increased BDNF mRNA and protein expression and enhanced neuronal outgrowth. Given the important role of BDNF in cocaine addiction (for review see Ghitza et al., 2010), it would be interesting to determine if BDNF-AS is dysregulated in response to chronic drug use and contributes to drug-seeking behaviors. Other addiction-related NATs have also been identified (Zhang et al., 2007), but their role in addiction-related neuroadaptations and behaviors merits further research.

Although initial studies have identified a potential involvement of lncRNAs in addiction, many additional questions remain – whether specific lncRNAs are necessary for drug-seeking behaviors, whether different drugs of abuse affect different lncRNAs, whether specific lncRNAs are preferentially expressed in reward-related brain areas, and whether differences across species exist (mice vs. rats vs. humans). Thus, a major research goal is to address these important unanswered questions in order to understand the underlying mechanisms of abused drugs and to identify useful targets for the treatment of addiction.

EPIGENETIC-ncRNA INTERACTIONS: POTENTIAL INVOLVEMENT IN DRUG-INDUCED NEUROADAPTATIONS

The epigenome consists of DNA methylation and several modifications (acetylation, methylation, phosphorylation, etc.) to specific amino acid residues on histone proteins. Chromatin-modifying complexes play an important role in transcriptional regulation by adding or removing covalent modifications to histone proteins. Several key chromatin-modifying proteins have recently been implicated in neuroadaptations associated with addiction. For example, specific enzymes responsible for histone acetylation, methylation, and DNA methylation in reward-related brain areas are critically involved in cocaine addiction (LaPlant et al., 2010; Maze et al., 2010; Wang et al., 2010). However, it has been largely unclear how these proteins target specific regions of the genome, given that the majority of chromatin-modifying proteins lack DNA binding capacity.

Increasing evidence now indicates that chromatin-modifying complexes are directed to their sites of action by lncRNAs. Therefore, it is possible that lncRNAs play an important role in addiction by regulating epigenetic processes. Although no study has examined lncRNA-mediated epigenetic mechanisms in the context of addiction, we speculate that such interactions are important (**Figure 1**), given that several addiction-related epigenetic factors associate with lncRNAs in tissues outside of the brain

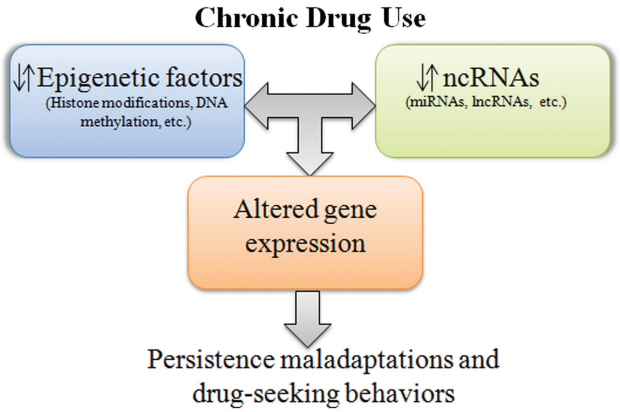

FIGURE 1 | Simplified representation of ncRNA–epigenetic interactions in response to chronic drug use. Long-term use of drugs alters expression of ncRNAs, epigenetic factors, and the interactions between these processes, causing persistent perturbations in gene expression. Chronic aberrations in gene expression are thought to evoke maladaptive neuroadaptations associated with addiction.

(Khalil et al., 2009). For example, in the placenta, lncRNAs Air, and Kcnq1ot1 regulate histone methylation by interacting with the histone methyltransferase G9a (Nagano et al., 2008; Pandey et al., 2008), an epigenetic enzyme that is downregulated in the accumbens following chronic cocaine exposure and is important in cocaine-related behaviors (Maze et al., 2010). Additionally, lncRNA-mediated regulation of histone acetylation, methylation, or DNA methylation, key modifications important in cocaine addiction (for review see, Maze and Nestler, 2011), have also been reported (Rinn et al., 2007; Houseley et al., 2008; Yu et al., 2008; Yap et al., 2010). Interestingly, not only can lncRNAs influence the activity of chromatin-modifying complexes, but evidence now indicates that alterations in epigenetic processes can alter the expression of lncRNAs (Johnson et al., 2009). Whether these specific interactions are important in the brain during drug-seeking behaviors, however, remains to be investigated.

MicroRNAs also interact with epigenetic factors important in addiction. As previously described, Im et al. (2010) found that miR-212 influences cocaine seeking by inhibiting MeCP2 in the dorsal striatum. Other epigenetic enzymes that have been implicated in addiction, such as DNA methyltransferase 3A, histone deacetylases 4, and sirtuin 1, are also regulated by specific miRNAs (Chen et al., 2006; Fabbri et al., 2007; Gao et al., 2010), but the significance of these associations in addiction-related behaviors is unknown.

Although the full spectrum of ncRNA-epigenetic associations in the CNS has yet to be seen, these interactions appear to play an essential role in fine-tuning gene expression and proper brain functioning. In drug addiction, it is possible that chronic drug use leads to long-lasting aberrations in ncRNA-mediated epigenetic mechanisms that lead to persistent drug-seeking behaviors. However, additional research is needed to determine the molecular underpinnings involved in ncRNA-epigenetic interactions in the brain and if these interactions contribute importantly to the addicted state.

FUTURE DIRECTIONS

Although a growing number of reports have implicated miRNAs in addiction-related neuroadaptations, future studies are needed to determine if lncRNAs also play a critical role in drug-seeking behaviors. Given that lncRNAs are the most abundant ncRNA in the brain and critically involved in an array of cellular processes, identifying specific lncRNAs that are regulated by drugs of abuse is likely to be a valuable approach for revealing the underlying mechanisms of addiction. With the recent development of new technologies, such as capture hybridization analysis of RNA targets (CHART) and Chromatin Isolation by RNA Purification (ChIRP; Chu et al., 2011; Simon et al., 2011), it is now possible to identify novel lncRNA-DNA or lncRNA-protein interactions involved in addiction, and future studies using these techniques will determine if these interactions are altered following prolonged drug use. In addition, as recent studies indicate that lncRNAs are differentially expressed in certain brain regions (Mercer et al., 2008; Belgard et al., 2011), identifying preferential expression of specific lncRNAs in reward-related brain areas might lead to new targets for the treatment of addiction. Finally, new therapeutic strategies and delivery approaches that target RNAs are now being explored (Bitko and Barik, 2007; Wood et al., 2007; Hung et al., 2011). Thus, using these new techniques to target ncRNAs holds great potential for treating several psychiatric disorders, including addiction.

CONCLUSION

Studies over the last several years have established a broad functional context for ncRNAs in the computational matrix of the nervous system. At the cellular level, nervous system signaling networks involve small RNAs at the synapse, where they regulate activity-dependent mRNA translation, and in turn, learning and memory-related plasticity. At the same time, in the nucleus, long RNAs function to provide temporal and spatial information to an array of epigenetic signaling systems. Chronic drug use likely perturbs these networks during the process of addiction in ways that cause a loss of plasticity and in turn establish barriers to the return to homeostasis.

Yet, the complexity of the nervous system suggests that additional layers of ncRNA-mediated events likely occur during the process of addiction. For example, recent evidence suggests that lncRNAs can serve as decoys or storage locations for small RNAs (Tay et al., 2011), in effect competing with targets for the occupancy of effector small RNAs and modulating their downstream effects (Salmena et al., 2011). In neurons this process could function together with anterograde and retrograde transport of RNA–protein vesicles, providing the potential for a link between small RNA mediated translational control at the synapse, and lncRNA-mediated chromatin signaling in the nucleus. Stresses such as repeated increases in intracellular Ca^{++} levels could reduce the performance of vesicle trafficking and lead to the progressive decoupling of such ncRNA-mediated systems relatively early in the process of addictive maladaptations.

Non-coding RNAs may also play a role in signaling between cells. Circulating exosomes and microvesicles contain many RNA species, and have the ability to traffic these RNAs from one cell type to another (Smalheiser, 2007; Dinger et al., 2008), even supporting the metastatic environment of the soma in some types of cancer. While not yet documented in the context of the nervous system, vesicle based transfer of ncRNAs could offer an additional dimension of intercellular communication in the nervous system. Such vesicles could mediate signaling between neurons and glia, for example, in response to impending cytotoxicity or other stress events. Thus, the versatility of ncRNA-based information processing provides many still unexplored avenues for function in the nervous system and involvement in the stress responses that lead to addictive maladaptations.

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