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REVIEW

Molecular, Cellular, and Structural Mechanisms of Cocaine Addiction: A Key Role for MicroRNAs

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The rewarding properties of cocaine play a key role in establishing and maintaining the drug-taking habit. However, as exposure to cocaine increases, drug use can transition from controlled to compulsive. Importantly, very little is known about the neurobiological mechanisms that control this switch in drug use that defines addiction. MicroRNAs (miRNAs) are small non-protein coding RNA transcripts that can regulate the expression of messenger RNAs that code for proteins. Because of their highly pleiotropic nature, each miRNA has the potential to regulate hundreds or even thousands of protein-coding RNA transcripts. This property of miRNAs has generated considerable interest in their potential involvement in complex psychiatric disorders such as addiction, as each miRNA could potentially influence the many different molecular and cellular adaptations that arise in response to drug use that are hypothesized to drive the emergence of addiction. Here, we review recent evidence supporting a key role for miRNAs in the ventral striatum in regulating the rewarding and reinforcing properties of cocaine in animals with limited exposure to the drug. Moreover, we discuss evidence suggesting that miRNAs in the dorsal striatum control the escalation of drug intake in rats with extended cocaine access. These findings highlight the central role for miRNAs in drug-induced neuroplasticity in brain reward systems that drive the emergence of compulsive-like drug use in animals, and suggest that a better understanding of how miRNAs control drug intake will provide new insights into the neurobiology of drug addiction.

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INTRODUCTION

According to the 2010 National Survey on Drug Use and Health (NSDUH), approximately 13.3% of young adults reported lifetime use of cocaine, with an estimated 600 000 new users of cocaine in 2010 alone (SAaMHSA, 2010). The negative health and economic consequences of cocaine use are considerable, with the drug causing potentially fatal cerebral hemorrhage and cardiovascular events such as arrhythmias, myocardial infarction, and results in approximately US$581 million in direct health-care costs annually (Caulkins *et al*, 2002). The social consequences of cocaine use are also striking. According to the Department of Justice, in 2004 approximately 32% of State prisoners and 26% of Federal prisoners reported that they had committed their current offense under the influence of drugs (Mumola and Karberg, 2006).

In common with other substance-abuse disorders, cocaine addiction is characterized by a compulsive need to seek and take the drug, a loss of control over the amount of drug consumed, and by periods of attempted abstinence closely followed by relapse to drug-taking behavior. In a study of self-reported patterns of consumption by 111 cocaine users aged between 19 and 64 years, approximately 50% reported periods when their cocaine intake was characterized as excessive (>2.5 g per week) (Decorte, 2001), and involved consuming doses that resulted in overt signs of overdosing (Decorte, 2001). Further, it has been estimated that approximately 69% of cocaine addicts completing outpatient cocaine treatment programs relapse to their cocaine habit within 1 year, and 80% of addicts completing long-term residential cocaine treatment relapse within 1 year (Institute for Health Policy, 2001). Rates of relapse in non-treatment-seeking cocaine addicts are less clear, but are likely higher than those for treatment-seeking individuals.

There are currently no FDA-approved medications for the treatment of cocaine addiction (Kreek *et al*, 2002;

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O'Brien, 1997). Furthermore, a search of ongoing clinical trials reveals that there are no new chemical entities that have been rationally designed for the treatment of cocaine addiction currently in clinical testing; see www.clinicaltrials.gov. Importantly, cost–benefit analyses highlight the potential benefit of developing even partially effective therapeutics for the treatment of cocaine addiction. On the basis of dollar amounts, development of a cocaine medication with a modest treatment effect (10% increase in abstinence rates) would result in a benefit-to-cost ratio of approximately 1.5–5.8 (Cartwright, 2000). Hence, there is a pressing need for development of novel therapeutics with clinical utility for the treatment of cocaine addiction. A major roadblock to developing such therapeutics is our limited understanding of the underlying neurobiological mechanisms of the disorder. It is known that periods of prolonged cocaine use can trigger enduring cellular and neurochemical adaptations in brain reward systems that are hypothesized to precipitate the emergence of compulsive drug-taking (Luscher and Malenka, 2011; Nestler *et al*, 2001). However, mere drug use is not sufficient to establish addiction, as only a minority of drug users transition to addiction (Anthony *et al*, 1994), and the interval between first cocaine use and diagnosis of dependence in those cocaine users who do go on to develop addiction has been reported to be 4–12 months (Ridenour *et al*, 2006). Thus, some individuals lose control over drug intake after an extended history of drug consumption, continuing drug use in the face of escalating monetary, physical, and emotional costs. These individuals do so in spite of awareness of these negative effects (Hayaki *et al*, 2008) and a persistent desire to cut down substance use (American Psychiatric Association, 1994). In this review, we discuss the cocaine-induced changes to neural circuits that drive the appearance of compulsive drug use at the structural and cellular levels, with particular emphasis on the role of microRNAs (miRNA) in addiction.

ESCALATION OF COCAINE SELF-ADMINISTRATION UNDER EXTENDED ACCESS CONDITIONS

To understand the neurobiology of compulsive cocaine intake, it is necessary to employ an animal model that accurately recapitulates aspects of the disorder seen in human addicts. Periods of extended drug availability and resultant excessive drug consumption is likely a critical factor triggering the development of a loss of control over intake and subsequent compulsive drug seeking in humans (Ahmed, 2005; Ahmed and Koob, 1998, 2005; Kenny, 2007; Wikler, 1952). Indeed, in human drug users a sudden increase in drug availability can precipitate the transition from low to high (and increasingly uncontrolled) levels of drug use (Ahmed *et al*, 2002; Gawin and Ellinwood, 1989; Kramer *et al*, 1967). Such 'escalating' levels of drug consumption by human drug users in response to increased drug availability can be observed for most drugs of abuse (Ferri *et al*, 2001; Gawin and Ellinwood, 1989; Siegel, 1984).

The intravenous self-administration procedure is generally considered the most direct measure of the reinforcing properties of drugs of abuse in animals. In the SA procedure, experimental animals such as rats or mice are prepared with chronic indwelling intravenous catheters and trained to emit a response, typically to press a lever, to obtain intravenous drug infusions. The majority of drugs that are abused by humans are also self-administered by animals, including cocaine (Criswell and Ridings, 1983; Donny *et al*, 1995; Griffiths and Balster, 1979; Griffiths *et al*, 1981; Pickens and Thompson, 1968; Risner and Jones, 1975; Weeks, 1962; Wilson and Schuster, 1972). After the establishment of stable levels of stimulant self-administration in animals, experimenters have assessed the effects of extending drug access to 6 (Ahmed and Koob, 1998; Yokel and Pickens, 1974), 12 (Wee *et al*, 2007), or 24 h (Bozarth and Wise, 1985; Mutschler and Miczek, 1998; Tornatzky and Miczek, 2000) on patterns of consumption. Strikingly, the latter duration of access produces progressive weight loss, erratic self-administration patterns in some but not all subjects, and ultimately death in 90% of the rats within 30 days of continuous self-administration. In addition, extended access to cocaine and other addictive drugs produces a gradual escalation in daily cocaine intake (Ahmed *et al*, 2002; Ahmed and Koob, 1998). Specifically, it has been shown that animals permitted restricted (1-h) daily access to cocaine maintain regular and stable patterns of intake (Ahmed and Koob, 1998). In contrast, rats demonstrate 'escalating' levels of intake during extended daily access sessions (6–18 h per day) to cocaine (Ahmed and Koob, 1998), heroin (Ahmed *et al*, 2000), or methamphetamine (Kitamura *et al*, 2006). This apparent loss of control over intake is reminiscent of the loss of control over intake observed in human drug users during the development of dependence. Although escalation of cocaine intake is not itself a demonstration of compulsive drug intake and escalation is not necessary for the emergence of compulsive drug taking in rats (Deroche-Gamonet *et al*, 2004), it has been demonstrated that extended access to cocaine self-administration that produced escalation also resulted in resistance to contingent foot-shock punishment of cocaine seeking (Jonkman *et al*, 2012a, b), suggesting that escalation positively influences the development of compulsive cocaine seeking.

THE ROLE OF THE STRIATUM IN DRUG SELF-ADMINISTRATION

Cocaine exerts its psychomotor stimulant effects through blocking the dopamine transporter in the striatum (Giros *et al*, 1996), which increases tonic extracellular (Pontieri *et al*, 1995) and stimulus-evoked phasic dopamine levels (Gonon, 1997). The shell of the nucleus accumbens and adjacent olfactory tubercle appear to mediate the initial

rewarding effects of drugs of abuse in rodents (Barak Caine *et al*, 1995; Ikemoto, 2003), while the core of the nucleus accumbens is important for the satiating or intake-limiting effects of cocaine (Suto *et al*, 2009; Suto and Wise, 2011). The nucleus accumbens core has also been implicated in the enhancement of drug seeking by response-contingent pavlovian cues (Fuchs *et al*, 2004; Ito *et al*, 2004). Rats will not self-administer cocaine directly into the dorsal striatum, although cocaine does increase tonic extracellular dopamine levels in this structure (Di Chiara and Imperato, 1988; Ikemoto, 2003). However, the finding that the dorsolateral striatum is important for habitual control of responding for food after overtraining (Yin *et al*, 2004) led to the suggestion that the appearance of addiction after extended self-administration could be similarly mediated by the recruitment of the dorsal striatal habit system (Everitt and Robbins, 2005). Subsequent experiments showed that the dorsolateral striatum mediates the enhancement of drug seeking by response-contingent pavlovian cues after overtraining (Vanderschuren *et al*, 2005), as well as the habitual (Zapata *et al*, 2010) and punishment-resistant (Jonkman *et al*, 2012a) nature of cocaine seeking after extensive training. Surprisingly, some of the relatively early unconditioned reinforcing effects of cocaine are also regulated by the dorsal striatum (Veeneman *et al*, 2012). Thus, it is notable that the mediation of conditioned reinforcement and the rewarding effects of cocaine appear to shift from ventral to both ventral and dorsolateral striatal dependency, findings summarized in Figure 1. As such, molecular circuitries in the dorsal striatum that control the recruitment of this structure and promote its increasing responsiveness to cocaine as drug exposure increases are likely to play a key role in the emergence of addiction, and understanding these processes may reveal novel treatment strategies for compulsive cocaine use.

CELLULAR EFFECTS OF COCAINE DOSES IN THE STRIATUM: ACUTE REWARDING INJECTIONS

Cocaine is a potent pharmacological stimulus that exerts widespread effects in striatal neurons (Renthal *et al*, 2009). Cocaine administration increases synaptic dopamine levels by blocking the dopamine transporter. As noted above, subsequent increases in dopamine receptor signaling in the nucleus accumbens contribute to the reward-related effects of cocaine. There are two major families of dopamine receptor, the D1-like (D1 and D5) and D2-like (D2, D3, and D4) receptors. The D1 receptor (D1R) is mainly expressed by a distinct population of medium spiny neurons (MSNs) in the striatum that project directly to the substantia nigra pars reticulata, the so-called direct pathway neurons. The remainder of MSNs in the striatum project to the external segment of the globus pallidus and mainly express D2 receptors (D2R), the so-called indirect pathway (Deng *et al*, 2006). The D1 family of dopamine receptor are Golf-coupled G-protein coupled receptors, which when stimulated cause activation of the membrane-associated enzyme adenylyl cyclase, most prominently adenylyl cyclase 5, and resultant accumulation of the intracellular second messenger cAMP (Herve, 2011). Conversely, the D2-like receptors inhibit adenylyl cyclase activity and thereby reduce intracellular cAMP levels. Increases in cAMP in response to D1R activation engage downstream protein kinases, including protein kinase A (PKA) and extracellular signal-regulated kinase (ERK). In turn, these kinases can, among many other downstream effects, phosphorylate the transcription factor cAMP response element-binding protein (CREB) at serine residue 133 (Ser133) (Kano *et al*, 1995). CREB is a 43 kDa transcription factor belonging to the basic-leucine zipper family (Lonze and Ginty, 2002;

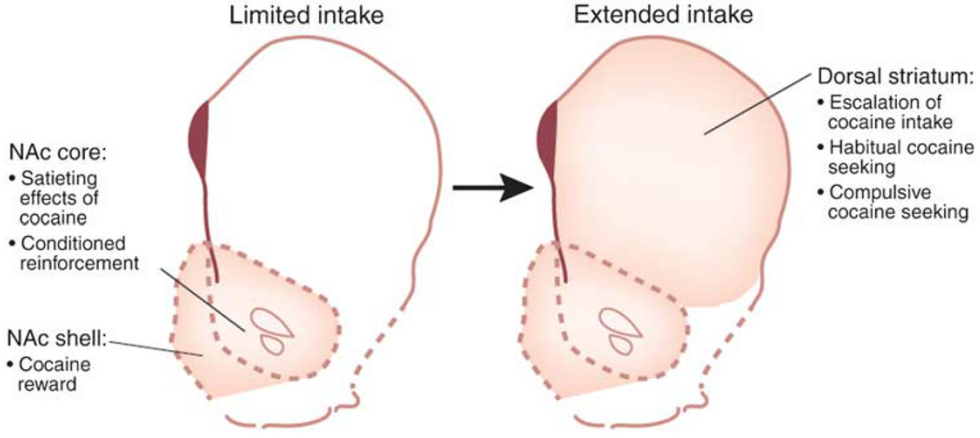

Figure 1. Subregions of the rodent anterior striatum involved in cocaine self-administration. After limited cocaine self-administration, the shell subregion of the nucleus accumbens (NAc) mediates the rewarding effects of cocaine, whereas the core of the NAc mediates cocaine's satiating effects and the conditioned reinforcing effects of drug-paired pavlovian stimuli. Extended cocaine self-administration recruits the dorsal striatum, whereas the NAc continues to play an important role in self-administration. The dorsal striatum comes to control escalation of cocaine intake, habitual cocaine seeking, and eventually continued cocaine seeking in the face of contingent foot-shock punishment. Neuroanatomical representation of the rat striatum is 1.2 mm anterior to Bregma.

Shaywitz and Greenberg, 1999), and its phosphorylation at Ser133 allows it to bind its transcriptional coactivators (Gonzalez and Montminy, 1989). In particular, CREB-regulated transcription coactivator (CRTC), also known as TORC, is a major CREB coactivator that regulates CREB transcriptional activity (Screaton *et al*, 2004). In the nucleus, p-CREB-bound CRTC recruits CREB-binding protein (CBP) and p300. Subsequently, CBP/p300 can acetylate CRTC, stabilize its activation state, and prevent its ubiquitin-mediated degradation (Liu *et al*, 2008). This association between p-CREB and CRTC is therefore required for CREB-regulated gene expression (Altarejos *et al*, 2008; Kovacs *et al*, 2007; Li *et al*, 2009; Screaton *et al*, 2004; Zhou *et al*, 2006). Deacetylation of CRTC, and concomitant inactivation of CREB signaling, is regulated by the class III histone deacetylase (HDAC) sirtuin 1 (SIRT1) (Liu *et al*, 2008). As discussed below, the CREB signaling cascades, and it signaling components, are core regulators of cocaine reward in the nucleus accumbens and the transition to compulsive cocaine use in the dorsal striatum.

It has been shown that infusion of cAMP analogs that activate PKA into the nucleus accumbens of rats caused a time-delayed increase in intravenous cocaine self-administration behavior and shifted the cocaine dose–response curve to the right (Self *et al*, 1998). This suggests that PKA activity attenuates the reinforcing properties of cocaine. Consistent with this interpretation, inhibition of PKA activity in the accumbens shifted the cocaine dose–response curve to the left, interpreted as increased sensitivity to the reinforcing properties of the drug (Self *et al*, 1998). PKA likely modulates cocaine intake through CREB signaling. Indeed, overexpression of CREB in the accumbens counteracts the rewarding effects of cocaine as measured in conditioned place preference (CPP) procedures, at least partly through increased transcription of preprodynorphin and resulting activation of the anti-reward κ-opioid receptor (Carlezon *et al*, 1998; Cole *et al*, 1995). SIRT1, which can deacetylate CRTC to inactivate CREB signaling (Liu *et al*, 2008), has been shown to positively regulate the rewarding and reinforcing properties of cocaine, as measures in CPP and self-administration procedures, respectively (Renthal *et al*, 2009). Interestingly, however, CREB activity can drive transcription of FosB and its stable truncated isoform ΔFosB (Inoue *et al*, 2004), and chronic but not acute cocaine administration causes upregulation of ΔFosB (Hope *et al*, 1994; Levine *et al*, 2005), which dimerizes with Jun family members to form the AP-1 transcription factor complex. Prolonged induction of ΔFosB increases the rewarding effects of cocaine in CPP and comes to over-ride the reward-attenuating effects of CREB (Kelz *et al*, 1999; McClung and Nestler, 2003). ΔFosB expression in turn influences the transcription of many target genes, of which increases in protein levels of cyclin-dependent kinase 5 (CDK5) (Bibb *et al*, 2001) and consequent inhibitory phosphorylation of the transcription factor myocyte enhancer factor-2 (MEF2) (Pulipparacharuvil *et al*, 2008) appear to mediate reward-attenuating effects. Conversely, activation of NF-κB transcriptional activity (Russo *et al*, 2009) or reduction in protein levels of the lysine methyltransferase G9a (Maze *et al*, 2010) both enhance the rewarding effects of cocaine in CPP, in accordance with the effect of ΔFosB induction itself. However, studies using cocaine self-administration have shown that overexpression of either CREB in the shell of the nucleus accumbens (Larson *et al*, 2011) or ΔFosB in substance P-dynorphin-containing striatal neurons (Colby *et al*, 2003) did not affect self-administration of cocaine doses typically used in such experiments, although the sensitivity to low doses was enhanced in both cases. Thus, it is currently unclear whether CREB activation in the ventral striatum would be expected to promote or protect against addiction (see section on miRNAs below also). Recent genetic advances have allowed for the separate investigation of the effects of cocaine administration on D1R- and D2R-expressing MSNs in the striatum. Although both D1R- and D2R-expressing MSNs show an acute upregulation of both ΔFosB and dendritic spine density after chronic cocaine administration, these effects were stronger and persisted 30 days into withdrawal in D1R-expressing neurons, but not in D2R-expressing neurons (Lee *et al*, 2006). Considering that D1Rs increase intracellular cAMP and CREB activation, this is consistent with a mechanism in which ΔFosB potentiates cocaine reward after chronic cocaine administration primarily in D1R MSNs through potentiation of CREB signaling.

DARPP-32 is another important effector of cocaine-induced PKA signaling. Activation of PKA through D1R causes phosphorylation of DARPP-32 on threonine 34 (Thr34), while inhibition of PKA through the D2R causes dephosphorylation at the same residue (Bateup *et al*, 2008; Nishi *et al*, 1997). When DARPP-32 is phosphorylated at Thr34, it acts as an inhibitor of protein phosphatase 1 (PP1) (Fernandez *et al*, 2006). Selective deletion of DARPP-32 in knockout mice abolished the induction of ΔFosB after chronic cocaine injections (Fienberg *et al*, 1998), although it should be noted that this deletion also produced upregulation in substance P (Hiroi *et al*, 1999). Moreover, the induction of ΔFosB after chronic cocaine injections and cocaine CPP have been shown to depend on Thr34 phosphorylation of DARPP-32 (Zachariou *et al*, 2005), and mice with genetic modification of this site are less sensitive to the reinforcing properties of intravenous self-administered cocaine compared with control mice, reflected in greater consumption of the drug when low unit doses are available for consumption and interpreted as insensitivity to cocaine reinforcement, requiring greater quantities of drug to achieve desired hedonic effects (Zachariou *et al*, 2002). In addition to the Thr32 site, the activity of DARPP-32, and hence its actions on PP1, can also be influenced by the phosphorylation status of other residues that are acted upon by various kinases and phosphatases implicated in the regulation of cocaine reward. For example, the inhibitory effects of DARPP-32 on PP1 are attenuated by CDK5-mediated phosphorylation of Thr75 (Fernandez *et al*, 2006). Further, the reinforcing properties of cocaine in mice are inversely

related to the phosphorylation status of Ser130, which is regulated by casein kinase 1 (Bibb *et al*, 1999). Finally, the dephosphorylation of DARPP-32 at the Ser97 by the cAMP-dependent activation of protein phosphatase-2A (PP2A) can facilitate the translocation of DARPP-32 to the nucleus, and this translocation was shown to be necessary for cocaine reward in the CPP procedure (Stipanovich *et al*, 2008). This action of DARPP-32 nuclear translocation on cocaine reward is likely related to relief of PP1-mediated repression of histone H3-mediated gene transcription (Stipanovich *et al*, 2008). However, the role of DARPP-32 in cocaine reward is more complex, as ERK activation is another downstream effector of DARPP-32. Activation of DARPP-32 produces phosphorylation of ERK, also likely through inhibition of PP1, as ERK is activated by cocaine in a DARPP-32-dependent manner (Valjent *et al*, 2005). This effect is specific to D1R-expressing neurons (Bertran-Gonzalez *et al*, 2008), and inhibition of MAPK/ERK kinase, which normally phosphorylates and thereby activates ERK, abolishes cocaine CPP (Valjent *et al*, 2000). Taken together, these findings suggest that the rewarding effects of cocaine through CREB, ΔFosB, SIRT1, DARPP-32, CDK5, PP2A, ERK, and various other components of the cAMP signaling cascade are mediated prominently by D1R-expressing neurons.

Cocaine administration also produces rewarding effects through striatal brain-derived neurotrophic factor (BDNF) signaling. Although there is very little BDNF mRNA in the striatum, the BDNF protein is transported to the striatum through anterograde transport in cortical and dopaminergic axons (Altar *et al*, 1997). Secreted BDNF activates tropomyosin-related kinase B (TrkB) receptors in the membrane of striatal neurons. However, the shell of the nucleus accumbens may form an exception to this scheme, as neurons in this region show a preferential enhancement of D1R/D2R co-expression (Bertran-Gonzalez *et al*, 2008), and activation of D1R/D2R heterodimers, but not activation of D1Rs or D2Rs alone, produced a rise in intracellular calcium and BDNF expression in the shell of the nucleus accumbens, but not the dorsal striatum (Hasbi *et al*, 2009). A single cocaine injection increases mRNA and protein level of BDNF (Graham *et al*, 2007) as well as phosphorylated TrkB levels (Freeman *et al*, 2003) in the shell of the nucleus accumbens, and both effects are necessary for cocaine CPP (Graham *et al*, 2009). Cocaine self-administration also produces an upregulation of BDNF and TrkB in the shell of the accumbens (Graham *et al*, 2007, 2009), and local knockdown of either BDNF or TrkB decreased cocaine self-administration, while BDNF infusions produced a modest increase in cocaine self-administration. Interestingly, the effect of TrkB knockdown on cocaine CPP could be recapitulated by D2R-expressing, but not D1R-expressing, MSN-specific TrkB deletion (Lobo *et al*, 2010). Furthermore, TrkB receptor mRNA levels are approximately fourfold higher in D2R *vs* D1R receptor-expressing MSNs across the striatum (Lobo *et al*, 2010). These findings suggest that D2R-expressing MSNs of the indirect pathway might mediate BDNF regulation of cocaine reward.

CELLULAR EFFECTS OF COCAINE DOSES IN THE STRIATUM: ESCALATION OF COCAINE SELF-ADMINISTRATION

In contrast to the initial rewarding effects of cocaine, as measured by CPP and self-administration procedures in rodents with limited histories of exposure to the drug, the cellular mechanisms in the dorsal striatum that drive escalation of cocaine intake under extended access conditions are much less well understood. In principle, the same cellular effects of acute cocaine injections on CDK5 (Bibb *et al*, 2001), MEF2 (Pulipparacharuvil *et al*, 2008), NF-κB (Ang *et al*, 2001), DARPP-32 (Nishi *et al*, 1997; Stipanovich *et al*, 2008), ERK (Valjent *et al*, 2000), and BDNF (Im *et al*, 2010; Lobo *et al*, 2010) can all be observed in the dorsal striatum, with the exception of cocaine-induced decreases in G9a (Covington *et al*, 2011). However, the functional relevance of many of these changes for escalation of cocaine self-administration remains to be determined.

Recent studies examining the escalation of cocaine intake have shown that extended access to cocaine self-administration increased levels of phosphorylated CREB in the dorsal striatum 24 h after the last cocaine self-administration session, and virus-mediated overexpression of the CREB co-activator CRTC1 (TORC1) in the dorsal striatum prevented escalation of cocaine intake, but did not affect cocaine intake under limited access conditions (Hollander *et al*, 2010). As described above, it is currently unclear whether CREB activation in the ventral striatum would be expected to promote or protect against addiction. In contrast, it seems that activation of CREB/CRTC in the dorsal striatum after extended access to cocaine is a protective homeostatic response, in line with the protective effect against cocaine reward of ventral striatal CREB activation after cocaine injection as measured by CPP (Carlezon *et al*, 1998). Extended access to cocaine also increased protein levels of BDNF and the transcriptional repressor methyl CpG-binding protein 2 (MeCP2) in the dorsal striatum 24 h after the last cocaine self-administration session, and local disruption of either MeCP2 or BDNF activity decreased cocaine intake in extended access, but not limited access conditions (Im *et al*, 2010) (see below). Conversely, overexpression of BDNF in the dorsal striatum produced an extreme escalation of cocaine intake under extended, but not limited, access conditions that produced a strong effect on the well-being of the animals. Thus, these findings support similar roles for CREB and BDNF in mediating protective and detrimental responses to cocaine, respectively, in both the dorsal and ventral striatum.

MIRNAs REGULATE COCAINE REINFORCEMENT

The above findings highlight that similar signaling cascades regulating cocaine reward in the nucleus accumbens may control escalation of cocaine intake under extended access conditions in the dorsal striatum. An important question is

how the activity of these various signaling cascades are coordinated to regulate vulnerability to cocaine addiction. As discussed in detail below, miRNAs are emerging as important regulates of gene expression that can interface between, and coordinate, activity in various intracellular signaling cascades and in neurons. As such, miRNAs are attractive candidates for regulating the rewarding properties of cocaine, and more importantly, the complex adaptive responses in the brain that occur in response to prolonged cocaine exposure that drive escalation of drug use.

miRNAs are recently discovered small (~22 nucleotides) RNA transcripts that do not code for protein, but function by repressing target mRNA translation (Im and Kenny, 2012; see also article by Schaefer *et al* (2010)). Many miRNAs have been identified (Landgraf *et al*, 2007) and each miRNA can potentially target hundreds to thousands of mRNA transcripts (John *et al*, 2004), suggesting that miRNAs play an influential regulatory role in gene expression. After transcription of miRNA genes, primary miRNA is generated that forms a characteristic hairpin secondary structure (for a recent review, see Im and Kenny (2012)). Cleavage of this hairpin by Dicer then produces mature miRNA, which is loaded into the RNA-induced silencing complex (RISC). The miRNA facilitates the interaction of the RISC complex with specific mRNA transcripts that interact with the miRNA through base pairing of the 3′-untranslated region of target mRNA transcripts with complementary sites in the miRNA, typically nucleotides 2–8 of the miRNA that are referred to as the 'seed' region (Bartel, 2009). This results in translational repression, and in some cases degradation, of target mRNA transcripts (Im *et al*, 2010). Two broad mechanisms have been suggested for the functions of miRNAs in neurons. First, it has been noted that the repressive effect of miRNA on translation is ideally suited to fulfill feedback roles in signaling cascades (Tsang *et al*, 2007), which may serve to stabilize signaling cascades by protecting against random fluctuations, and enabling stable cellular memory states (Acar *et al*, 2005). Secondly, miRNAs that are localized to dendrites may normally inhibit translation of bound mRNAs, which can be relieved upon synaptic stimulation, allowing for rapid local translation of proteins and induction of synaptic plasticity (Schratt *et al*, 2006).

Functional evidence of a role for miRNAs in cocaine reward is provided by the finding that Argonaute 2 (Ago2) knockout specifically in striatal D2R-expressing cells dramatically impaired cocaine CPP and self-administration in mice (Schaefer *et al*, 2010). Ago2 is a critical component of the RISC complex that binds miRNAs (Lingel *et al*, 2003), without which miRNAs cannot exert their effect on mRNA. In addition, the 'slicer' function of Ago2 is important for the biogenesis of a sub-population of miRNAs in the striatum (Lingel *et al*, 2003). This suggests that miRNAs in D2R-expressing neurons play a critical role in the reinforcing effects of cocaine. Further supporting a role for miRNAs in cocaine reward, chronic cocaine injections have been shown to increase miR-181a expression in the ventral striatum (Chandrasekar and Dreyer, 2009) and miR-181a overexpression increased cocaine CPP, while knockdown of miR-181a produced the opposite effect (Chandrasekar and Dreyer, 2011). A more comprehensive analysis of the effects of chronic cocaine injections on miRNA expression in the ventral striatum revealed robust upregulation (including miR-375, miR-182, miR-382, and miR-1) and moderate downregulation (including miR-31, miR2-9b, miR-344c, and miR-3106) of many miRNAs (Eipper-Mains *et al*, 2011), which would be expected to have widespread effects on gene expression.

In the dorsal striatum, chronic cocaine injections produced a decrease in miR-124a and let-7d expression and an increase in miR-181a expression, and experiments in cultured cells suggest that miR-124a downregulation may increase BDNF levels through a direct interaction (Chandrasekar and Dreyer, 2009). A comprehensive analysis of the effects of chronic cocaine injections on miRNA expression in post-synaptic density (PSD) subcellular fractions in the striatum (ventral and dorsal) revealed relatively modest upregulation (including miR-153, miR-32, miR-130b, and miR-33) and more robust downregulation (including miR-200c, miR2-125a, miR-429, and miR-370) of many miRNAs (Eipper-Mains *et al*, 2011), although the functional significance of these effects remains unexplored.

MIRNAs REGULATE THE ESCALATION OF COCAINE SELF-ADMINISTRATION

Recently, we began testing the hypothesis that miRNAs in the dorsal striatum may play a role in the escalation of cocaine self-administration after extended access. To test this hypothesis, miRNA expression profiles were first characterized in the dorsal striatum of cocaine-naïve rats and in rats with restricted (1-h) or extended (6-h) daily access to cocaine self-administration. We also assessed miRNA expression in a group of 'yoked' rats that received cocaine infusions non-contingently only when rats in the extended access group volitionally consumed the drug. We found that two miRNAs, miR-212 and miR-132, were upregulated in the dorsal striatum in the extended access rats 24 h after the last cocaine self-administration session relative to the yoked, restricted access and cocaine-naïve control groups (Hollander *et al*, 2010) (Figure 2). These two miRNAs are arrayed in tandem in the miR-212/132 cluster located on chromosome 17 in humans and chromosome 10 in rats. Moreover, both miRNAs share very close sequence homology, differing by only four nucleotides, and have identical 'seed' regions (nucleotides 2–8 of the mature miRNA sequence) that are considered critical for specifying precisely which mRNA transcripts will be targeted for repression by miRNAs (Vo *et al*, 2005). As such, both miRNAs are thought to function very similarly.

To investigate the potential role for the miR-212/132 cluster in regulating cocaine intake, our group has investigated the effects of increased miR-212 signaling in the dorsal striatum through lentivirus-mediated overexpression, on cocaine-taking behavior in rats with restricted or extended

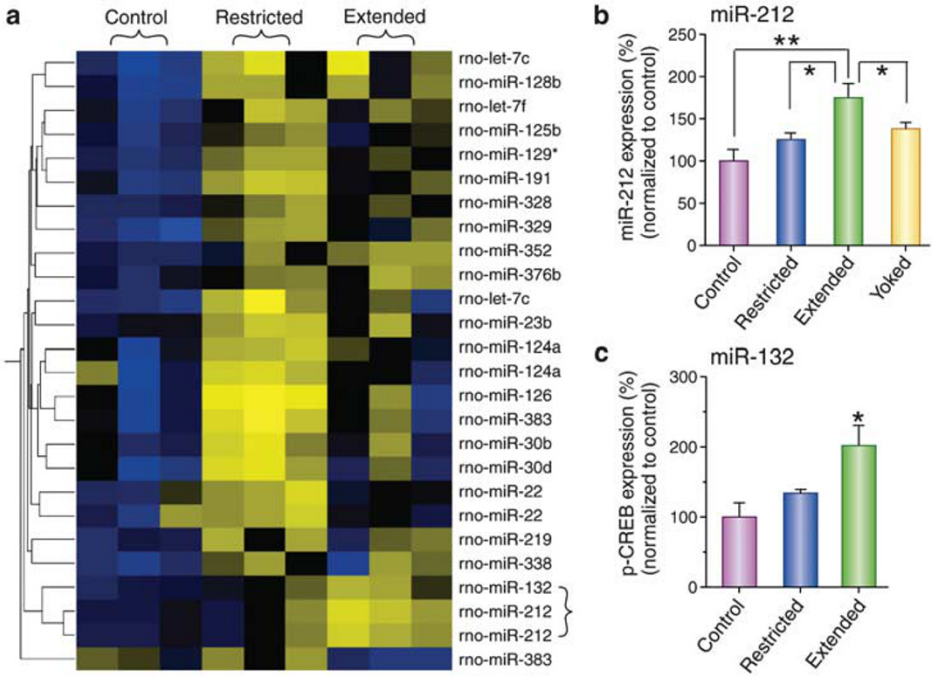

Figure 2. (a) Expression profiling of microRNAs (miRNAs) in the dorsal striatum of control, restricted, or extended access rats. Each column represents data from a cohort of two rats, with a total of six rats per group. The bracket identifies miR-212 and miR-132, the expression of which was increased only in extended access rats. (b) Taqman assay verified that striatal miR-212 levels were increased in extended access rats. (c) Taqman assay verified that striatal miR-132 levels were increased in extended access rats. *$P<0.05$, **$P<0.01$, statistically significant compared with the extended access group. This figure is reproduced with permission from Hollander *et al* (2010).

daily access to the drug (Figure 3). We found that striatal overexpression of miR-212 did not impact cocaine-taking behavior in restricted access rats (Hollander *et al*, 2010), the same access condition that did not result in increased striatal miR-212 expression (Figure 3). In contrast, striatal miR-212 overexpression strikingly reduced the motivation to consume cocaine in rats with extended access to the drug, reflected in progressively decreasing levels of cocaine intake across sessions (Hollander *et al*, 2010). This progressively increasing inhibitory effect of miR-212 on cocaine intake is opposite to the escalating rates of cocaine consumption typically observed in extended access rats. In contrast to this effect, disruption of constitutive miR-212 signaling, achieved through striatal infusion of an antisense oligonucleotide against miR-212, significantly accelerated the emergence of escalated cocaine intake in extended access rats, but did not alter intake in rats with restricted cocaine access (Hollander *et al*, 2010; Im *et al*, 2010) (Figure 3). These findings suggest that miR-212 may reverse the underlying neuroplasticity in the striatum that triggers the emergence of escalated cocaine intake in rats, and may therefore protect against the development of cocaine addiction.

CREB-MIR-212 INTERACTIONS IN THE STRIATUM CONTROL COCAINE INTAKE

miR-212 expression is increased in the striatum of rats with a history of extended access to cocaine self-administration (Hollander *et al*, 2010) and serves to reduce the motivation to further consume the drug (Figures 2 and 3). Hence, two important questions are how miR-212 expression levels are controlled in response to cocaine and how does it subsequently regulate cocaine intake? Previously, the miR-212/132 gene cluster was shown to be highly responsive to the cAMP signaling cascade and upregulated robustly by CREB (Vo *et al*, 2005; Wayman *et al*, 2008). Interestingly, levels of p-CREB were increased in the dorsal striatum of rats with extended but not restricted access to cocaine (Hollander *et al*, 2010). Hence, upregulated miR-212/132 expression detected in extended access rats likely reflects the fact that CREB signaling has been engaged in the dorsal striatum of these rats. Repressive effects of miRNAs on translation are ideally suited to regulate feedback effects onto signaling cascades to amplify or curtail the activity of networks in which they are incorporated (Tsang *et al*, 2007), As cocaine-induced increases in CREB activity are hypothesized to serve as an important compensatory response that blunts the behavioral effects of the drug (Carlezon *et al*, 1998; Dinieri *et al*, 2009; McClung and Nestler, 2003), we tested the possibility that miR-212 may feedback onto CREB to boost its signaling and thereby decrease cocaine intake. Consistent with this possibility, we found that miR-212 dramatically boosted CREB signaling in both cultured cells and in the striatum of rats (Hollander *et al*, 2010). Further analysis revealed that miR-212 could amplify CREB signaling, at least in part, through knockdown of SPRED1. As SPRED1 is a repressor of Raf1 activity, this kinase phosphorylated and thereby sensitized adenylyl cyclase

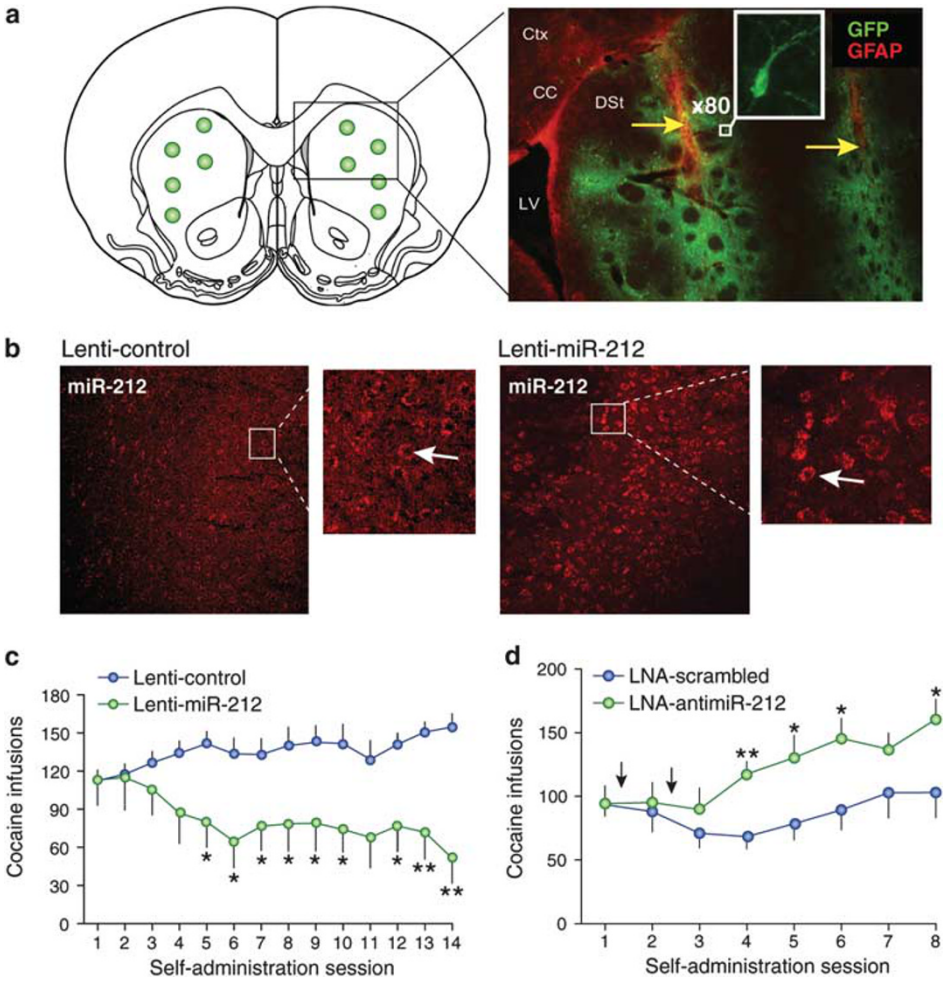

Figure 3. (a) The right panel is representative immunochemistry staining (× 10 magnification) from rats treated with a lentivirus vector to overexpress miR-212 (Lenti-miR-212) or a control lentivirus (Lenti-control). Red circles are locations at which viral infusions were targeted in the dorsal striatum. Green is green fluorescent protein (GFP) from virus; red is the astrocyte marker glial fibrillary acidic protein (GFAP). CC, corpus callosum; Ctx, cortex; DSt, dorsal striatum; LV, lateral ventricle. Yellow arrows highlight the injector track used to deliver virus. Inset is an × 80 confocal image of a virus-infected neuron. (b) Fluorescent *in situ* hybridization was used to visualize striatal miR-212 expression (shown in red) in Lenti-control and Lenti-miR-212 rats, verifying that miR-212 is constitutively expressed in the dorsal striatum and that the Lenti-miR-212 vector upregulates miR-212 expression. (c) Striatal miR-212 overexpression reverses the long-term trajectory of cocaine intake in rats with extended access. (d) A locked nucleic acid (LNA)-modified antisense oligonucleotide against miR-212 (LNA-anti-miR-212) delivered into the dorsal striatum increases cocaine intake in extended access rats. $^{*}P<0.05$, $^{**}P<0.01$. Data are presented as mean ± SEM. This figure is adapted with permission from Hollander *et al* (2010).

activity (Hollander *et al*, 2010). Sensitized adenylyl activity resulted in enhanced intracellular cAMP levels, and consequently increased levels of phosphorylated CREB in response to miR-212 (Hollander *et al*, 2010).

MECP2-MIR-212 INTERACTIONS IN THE STRIATUM ALSO CONTROL COCAINE INTAKE

Considering the important role for miR-212 in regulating responsiveness to the motivational properties of cocaine, our laboratory investigated the possibility that transcriptional machineries may limit the magnitude of increased miR-212 expression in response to cocaine and thereby increase vulnerability to addiction. Loss-of-function mutations or duplications of the *MeCP2* gene cause Rett syndrome (RTT) in humans (Amir *et al*, 1999; Van Esch *et al*, 2005), a neurodevelopmental disorder associated with severe mental retardation. MeCP2 is an epigenetic factor that binds to methylated cytosine residues of CpG dinucleotides in DNA. Upon binding to methylated DNA, MeCP2 can recruit HDACs and other transcriptional repressors to inactivate target gene expression (Nan *et al*, 1998). Previously, it was shown that repeated cocaine injections increases MeCP2 expression in the dorsal striatum and other addiction-relevant regions of the brain (Cassel *et al*, 2006). Consistent with this observation, we found that MeCP2 levels were significantly increased in the dorsal striatum of rats with extended but not restricted daily access to cocaine (Im *et al*, 2010) (Figure 4). More importantly, we found that knockdown of MeCP2 in the dorsal striatum significantly enhanced the stimulatory effects of cocaine on striatal miR-212 (and miR-132)

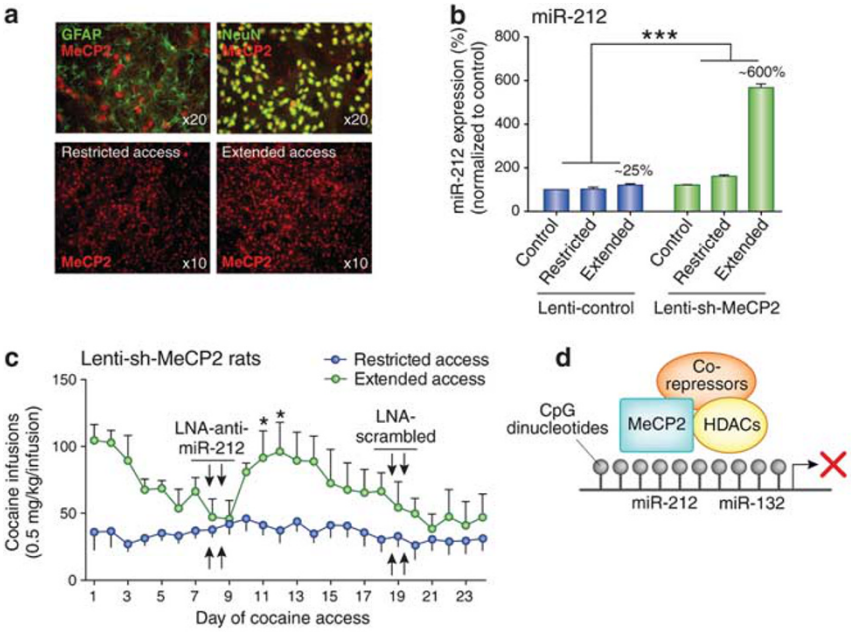

Figure 4. (a) Immunochemical detection of methyl CpG-binding protein 2 (MeCP2) in the dorsal striatum of drug-naive rats. Top left, MeCP2 (red) rarely colocalized with glial fibrillary acidic protein (GFAP; green), a marker for astrocytes. Top right, MeCP2 (red) almost exclusively colocalized with the neuronal nuclear marker NeuN (green). There was an increase in the number of MeCP2-positive cells in the dorsal striatum of rats with extended cocaine access (bottom right) compared to that in rats with restricted access (bottom left). (b) The stimulatory effects of cocaine on miR-212 expression in the dorsal striatum were dramatically increased in extended access rats treated with a lentivirus vector delivering a short hairpin interfering RNA to knockdown MeCP2 (Lenti-sh-MeCP2 rats). (c) Disruption of striatal miR-212 signaling using an antisense oligonucleotide (LNA-anti-miR-212) 'rescues' low levels of cocaine intake in Lenti-sh-MeCP2 rats with extended daily access to cocaine. (d) MeCP2 likely inhibits miR-212 (and miR-132) expression by binding to methylated CpG dinucleotides in the promoter region of the miR-212/132 gene cluster, resulting in the recruitment of histone deacetylases (HDACs) and other co-repressor proteins. *$P<0.05$, ***$P<0.001$. This figure is adapted with permission from Im *et al* (2010).

expression in rats with extended but not restricted cocaine access (Figure 4), suggesting that MeCP2 represses the miR-212/132 cluster (Figure 4). Moreover, virus-mediated knockdown of MeCP2 in the dorsal striatum of rats resulted in dramatically decreased cocaine intake in rats with extended but not restricted access, suggesting that decreased cocaine intake in extended access rats following striatal MeCP2 knockdown may be related, at least in part, to increased miR-212 expression. Consistent with this possibility, inhibition of miR-212 signaling in the dorsal striatum using an antisense oligonucleotide reversed the disrupted cocaine intake in extended access rats following MeCP2 knockdown. These findings show that MeCP2 plays a critical role in regulating cocaine intake in extended access rats, and that this action occurs, at least in part, through its repressive effects on miR-212 expression.

As noted above in the context of CREB–miR-212 interactions, miRNAs are ideally suited to regulate feedback effects onto signaling cascades into which they are incorporated (Tsang *et al*, 2007). Indeed, we found that miR-212 induced by CREB can feedback onto this transcription factor to boost its activity (Figure 5). Interestingly, miR-132 has been shown to repress MeCP2 expression in cultured mouse cortical neurons (Klein *et al*, 2007). Considering that miR-132 and miR-212 share the same seed region, we considered it likely that miR-212 may feedback onto MeCP2 to regulate its activity. Consistent with this possibility, we found that miR-212 overexpression decreased, whereas antisense oligonucleotide-mediated inhibition of miR-212 increased, MeCP2 expression levels in rat PC12 cells (Im *et al*, 2010) (Figure 5). More importantly, virus-mediated overexpression of miR-212 in the dorsal striatum of rats reduced MeCP2 levels in restricted cocaine access rats, and the magnitude of this reduction was far greater in rats with extended access. Hence, miR-212 can feedback onto MeCP2 and knockdown its expression, establishing a negative reciprocal relationship between miR-212 and MeCP2 in terms of the expression in the dorsal striatum. Intriguingly, expression of miR-212 has been found to be relatively depleted at striatal PSD extracts compared with whole-cell lysates (Eipper-Mains *et al*, 2011), strengthening the hypothesis that the effects of miR-212 on cocaine intake are due to homeostatic functions rather than regulation of synaptic translation. Reciprocal interactions between miR-212, CREB, and MeCP2 are summarized in Figure 5.

BDNF–MIR-212 INTERACTIONS IN THE STRIATUM CONTROL COCAINE INTAKE

The expression of miR-212 can be increased by CREB signaling and decreased by MeCP2 in the dorsal striatum. These opposite effects on CREB and MeCP2 on striatal miR-212 expression can decrease or increase, respectively, the likelihood of escalation of cocaine intake under extended

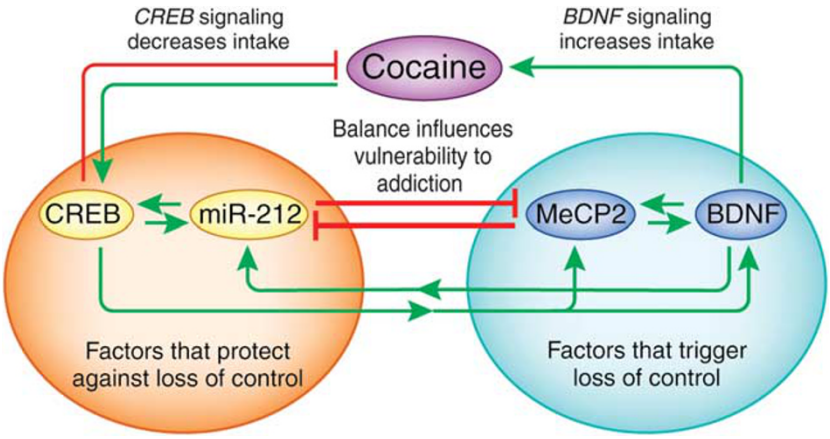

Figure 5. The complex interactions between miR-212, cAMP response element-binding protein (CREB), methyl CpG-binding protein 2 (MeCP2), and brain-derived neurotrophic factor (BDNF). The red circle implies that CREB signaling protects against the development of escalating cocaine intake, whereas the green circle implies that MeCP2-BDNF signaling promotes escalation of intake. Green arrows indicate a stimulatory relationship, whereas red lines indicate an inhibitory relationship. Cocaine is shown to activate both CREB and MeCP2-BDNF signaling, and the balance between these two pathways, coordinated by miR-212, likely regulates escalation of cocaine intake in extended access rats, and perhaps vulnerability to cocaine addiction.

access conditions in rats. An important question, then, is which downstream signaling cascades may be impacted by miR-212 signaling in the striatum to control cocaine intake. As discussed above in the context of the actions of acutely administered cocaine, BDNF in the ventral striatum is an important regulator of the reward-related actions of the drug. Interestingly, it has been well documented that brain levels of BDNF tend to correlate closely with those of MeCP2 (Chang *et al*, 2006) (Figure 5). This may appear counter-intuitive considering the fact that MeCP2 is a transcriptional repressor that generally blocks gene expression, and several putative mechanisms have been proposed to explain this positive relationship between MeCP2 and BDNF levels (for a review, see Abuhatzira *et al* (2007) and Chahrour *et al* (2008)). It has been shown that BDNF levels are reduced in the brains in *Mecp2* mutant mice (Chang *et al*, 2006), and restoring BDNF expression in the brains of these mice 'rescues' many of the RTT-like disturbances in these mice (Kondo *et al*, 2008; Larimore *et al*, 2009). Considering the role for BDNF in regulating the behavioral actions of acutely administered cocaine (Graham *et al*, 2007, 2009; Grimm *et al*, 2003; Horger *et al*, 1999; Lu *et al*, 2004, 2005), we hypothesized that miR-212 may influence cocaine intake through homeostatic interactions with MeCP2, and indirect modulation of BDNF expression levels.

We found that BDNF expression was increased in the dorsal striatum of rats with extended but not restricted access to cocaine self-administration. Furthermore, virus-mediated knockdown of MeCP2 or miR-212 overexpression, which also would be expected to reduce MeCP2 expression, decreased striatal BDNF levels (Im *et al*, 2010). Virus-mediated BDNF overexpression in the dorsal striatum resulted in a dramatic escalation of cocaine intake in rats with extended access to the drug, and the magnitude of escalated cocaine intake was far greater than that observed in control rats with extended cocaine access (Im *et al*, 2010). Conversely, inhibition of BDNF transmission in the dorsal striatum, achieved by infusing an anti-BDNF-neutralizing antibody, reduced cocaine intake in rats with extended but not restricted cocaine access. Hence, cocaine-induced increases in BDNF levels in the dorsal striatum likely drive the escalation of cocaine intake in rats with extended drug access (Figure 5). Further, the inhibitory effects of miR-212 on BDNF signaling in the striatum, likely controlled in opposite ways by CREB and MeCP2, influence vulnerability to develop escalated drug use.

The complex mechanisms by which miR-212 regulates cocaine intake are summarized in Figure 6. As can be seen, increased miR-212 levels in the dorsal striatum in response to extended cocaine access and consequent overconsumption of the drug amplifies CREB signaling through knockdown of SPRED1 and activation of Raf1 kinase. This action protects against the development of escalating levels of cocaine intake. In addition, miR-212 also knocks down MeCP2, resulting in lowered striatal levels of BDNF and decreased motivation to consume cocaine. Hence, miR-212 can coordinate the activity of at least two major signaling cascades in the dorsal striatum involved in regulating cocaine intake, and it will be interesting to determine if other signaling cascades involved in escalating cocaine taking are also regulated by miR-212. Moreover, as miR-212 protects against escalating cocaine intake, and presumably the development of cocaine addiction, it will be important to determine if other miRNAs in the dorsal striatum are involved in promoting the escalating levels of cocaine intake and the progression towards addiction.

Finally, it is important to note that when investigating the functional relevance of miR-212 and other miRNAs in brain reward systems in controlling cocaine intake, ectopic overexpression or knockdown studies are likely to impact miRNA signaling in cells not typically involved in the behavioral effects of cocaine, and thereby indirectly impact subsequent cocaine-taking behavior. Therefore, an important technical challenge for future studies will be to

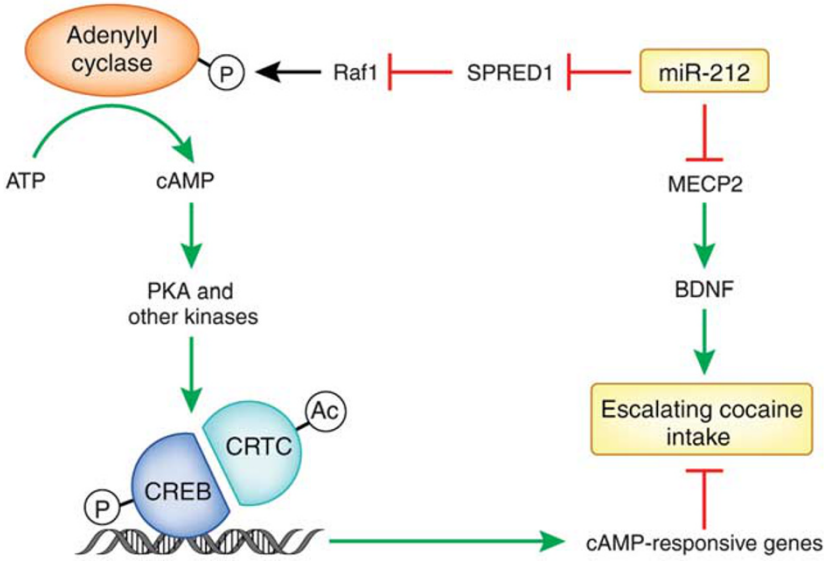

Figure 6. Detailed overview of the mechanisms by which miR-212 in the dorsal striatum controls cocaine intake in rats with extended access to the drug. Prolonged access to cocaine in rats with extended access increases levels of phosphorylated cAMP response element-binding protein (p-CREB) in the dorsal striatum, likely through activation of dopamine D1 receptors, which increases miR-212 expression (green arrow). Upregulated miR-212 can subsequently influence cocaine intake through at least two mechanisms. First, mR-212 represses expression of SPRED1, a repressor of Raf1 kinase. As a result, Raf1 can phosphorylate and sensitize the activity of membrane-bound adenylate cyclases, thereby increasing intracellular levels of the second messenger cAMP. This results in the activation of protein kinase A (PKA) and other downstream kinases, increasing the phosphorylation of CREB and its association with coactivators such as CREB-regulated transcription coactivator (CRTC). In the activated state, CRTC is acetylated, which stabilizes its association with pCREB and drives the expression of genes with a cAMP response element in their promoter, one of which is miR-212. As CREB-responsive genes are protective against the development of escalating cocaine intake, this action reduces the motivation to further consume cocaine. A second mechanism by which miR-212 can influence cocaine intake is by repressing methyl CpG-binding protein 2 (MeCP2), whose expression levels are known to correlate closely with those of brain-derived neurotrophic factor (BDNF) in the striatum. As BDNF drives cocaine intake, miR-212 knockdown of MeCP2 reduces the motivation to consume cocaine. Interestingly, MeCP2 itself can repress miR-212 expression. Hence, miR-212 can boost striatal CREB and reduce striatal MeCP2 signaling, with these two transcription factors in turn feeding back to control miR-212 expression levels. Hence, homeostatic interactions between miR-212, CREB, and MeCP2 likely control vulnerability to develop compulsive-like cocaine intake in rats with extended access to the drug.

refine experimental procedures such that miRNA activity can be controlled in discrete cell populations involved in cocaine addiction in a temporally and spatially controlled manner.

SUMMARY

Taken together, the findings reviewed here demonstrate that the ventral striatum is a critical substrate for the initial rewarding effect of cocaine, while the dorsal striatum is crucial for the escalation of cocaine intake that is observed under extended access conditions. We identify signaling cascades in the ventral striatum that regulate the rewarding effects of cocaine in rats with limited exposure to the drug, principally the cAMP signaling cascade and its various components, and discuss evidence suggesting that the development of escalation of cocaine intake reflects the transition of the control of drug intake from ventral to dorsal domains of the striatum and the progressively greater role for cAMP signaling cascades in the dorsal striatum in this regard. Finally, the findings discussed above suggest that miRNAs are key regulators of cAMP signaling in the striatum and thereby influence vulnerability to develop escalated cocaine use.

As so little is known about the functional significance of the myriad of miRNAs in addition to miR-212 whose expression in the striatum is altered by cocaine use, an important avenue for future research will be to identify those miRNAs involved in the development of cocaine addiction. It is likely that many more miRNAs in addition to miR-212 play a role in the development of compulsive cocaine intake. Furthermore, little is known about the cell types in the striatum—D1-expressing MSNs, D2-expressing MSNs, cholinergic or GABAergic interneurons—within which miRNAs exert their effects on addiction-relevant signaling cascades. Hence, characterizing the precise cellular and molecular mechanisms through which miRNAs influence cocaine intake under extended access conditions is likely to reveal fundamental new insights into the neurobiology of addiction. Hence, much more work remains to be carried out before a more complete understanding of the role of miRNAs in addiction biology is achieved.

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DISCLOSURE

Sietse Jonkman and Paul Kenny have no conflict of interest to declare.

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