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# MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212

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## Abstract

The X-linked transcriptional repressor methyl CpG binding protein 2 (MeCP2), known for its role in the neurodevelopmental disorder Rett syndrome, is emerging as an important regulator of neuroplasticity in post-mitotic neurons. Cocaine addiction is commonly viewed as a disorder of neuroplasticity, but the potential involvement of MeCP2 has not been explored. Here we identify a key role for MeCP2 in the dorsal striatum in the escalating cocaine intake seen in rats with extended access to the drug, a process that mimics the increasingly uncontrolled cocaine use seen in human addicts. MeCP2 regulates cocaine intake through homeostatic interactions with microRNA-212 (miR-212) to control the effects of cocaine on striatal brain-derived neurotrophic factor (BDNF) levels. These data suggest that homeostatic interactions between MeCP2 and miR-212 in dorsal striatum may play an important role in regulating vulnerability to cocaine addiction.

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MeCP2 is a transcription factor that binds to methylated cytosine residues of CpG dinucleotides in DNA, recruiting histone deacetylases (HDACs) and other transcriptional repressors to “silence” target genes 1. Loss-of-function mutations or duplications of the MeCP2 gene cause Rett syndrome (RTT) 2, 3, a neurodevelopmental disorder associated with severe mental retardation. Several lines of evidence suggest that MeCP2 may also play a role in drug addiction. First, repeated cocaine injections increase MeCP2 expression in addiction-relevant regions of the brain, particularly in the dorsal striatum 4. Second, drug-induced neuroplasticity in brain reward circuitries is thought to underlie addiction 5, and MeCP2 is emerging as a key regulator of many basic aspects of neuronal plasticity in post-mitotic neurons 6. Third, the development of compulsive drug-taking is hypothesized to reflect migration of behavioral control from ventral to dorsal domains of the striatum that are less subject to executive control 7. RTT is characterized by compulsive behaviors, including bruxism and athetosis, with autonomous motoric behaviors considered a necessary diagnostic for the disorder 8. These habitual behaviors are related to dysfunction in dorsal striatal activity in RTT patients 8, 9, suggesting that MeCP2 may play a role in compulsion-related striatal plasticity.

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**Author Contributions**

H-I.I., J.A,H. and PB conducted all experiments. H-I.I. and P.J.K. designed the experiments and wrote the manuscript.

MicroRNAs (miRNAs) are a class of non-protein coding RNA transcripts that regulate gene expression at the post-transcriptional level. miRNAs control gene expression by binding to complementary sequences (miRNA response elements; MREs) in the 3′ untranslated region (3′ UTR) of target mRNA transcripts to facilitate their degradation and/or inhibit their translation 10. We recently reported that expression of miR-212is increased in the dorsal striatum of rats with extended but not restricted daily access to intravenous cocaine self-administration 11. Overexpression of miR-212 in the dorsal striatum decreased, whereas its knockdown increased cocaine intake in rats with extended but not restricted drug access 11, suggesting that increased striatal miR-212 levels is a counter adaptive response to cocaine overconsumption. Interestingly, miR-212 decreases MeCP2 levels in human gastric carcinoma (GC) cell lines 12, and the closely related miRNA, miR-132, repressesMeCP2 in cultured mouse cortical neurons 13. Conversely, the miR-212 gene is located within a genomic region enriched with CpG dinucleotides 14, termed a CpG island, and subsets of genes within CpG islands are subject to transcriptional repression by MeCP2 15. Thus, it is an intriguing possibility that MeCP2 and miR-212 may be locked in an inhibitory homeostatic relationship in the dorsal striatum, and that interactions between both may in influence cocaine-taking behavior.

MeCP2 levels are closely correlated with those of brain-derived neurotrophic factor (BDNF) in the brain 16, although the underlying dynamics of this complex relationship remain unclear 17, 18. MeCP2 overexpression in cultured mouse cortical neurons increases BDNF expression 13, whereas brain levels of BDNF are reduced in *Mecp2* mutant mice 16. Restoring BDNF in the brains of *Mecp2* mutant mice ameliorates many of their RTT-like physiological and behavioral deficits 19, 20. BDNF also contributes to the actions of cocaine. For example, BDNF infused into the nucleus accumbens (NAcc) increases sensitivity to the psychomotor stimulant effects of cocaine 21, and induces a long-lasting increase in cocaine self-administration behavior in rats 22. Conversely, targeted disruption of the BDNF gene in NAcc decreases cocaine self-administration in mice 23. BDNF concentrations gradually increase in midbrain dopamine and amygdalar regions after cessation of cocaine self-administration in rats 24, 25. Such increases in BDNF levels, and consequent activation of the downstream ERK signaling cascade, may underlie the progressively greater motivation to seek cocaine during periods of increasing drug abstinence 25, 26, a phenomenon termed “incubation of craving”. It is noteworthy that BDNF transmission in the prefrontal cortex (PFC) decreases cocaine-seeking behavior 27, and in the striatum may inhibit ethanol intake 28, suggesting that in some cases BDNF transmission can decrease drug-seeking behaviors. Here, we tested the hypothesis that homeostatic interactions between MeCP2 and miR-212 in the dorsal striatum may regulate the effects of self-administered cocaine on striatal BDNF expression and thereby influence the propensity to develop compulsive-like responding for the drug.

## Results

### Striatal MeCP2 knockdown decreases cocaine intake

We found that MeCP2 is abundantly expressed almost exclusively in the nuclei of NeuN-positive neurons in the rat dorsal striatum, with little expression in GFAP-positive astrocytes

(Fig. 1a). MeCP2 expression was increased in dorsal striatum (Fig. 1) and decreased in the prefrontal cortex (PFC; Supplementary Fig. 1) in rats with extended (6-h) but not restricted (1-h) daily access to cocaine self-administration (0.5 mg/kg/infusion)for 7 consecutive days compared with cocaine-naïve control rats, when assessed 24-h after the final self-administration session. In contrast, MeCP2 was upregulated in hippocampus similarly in both restricted and extended access rats, and was unaltered by cocaine in the cerebellum(Supplementary Fig. 1).

To investigate the role for striatal MeCP2 in regulating cocaine intake, we designed and validated a lentivirus to deliver a short-hairpin interfering RNA to knockdown MeCP2 (Lenti-sh-MeCP2; Fig. 2a, Supplementary Figs. 2 and 3). In extended access rats treated with an empty lentivirus vector (Lenti-controlrats), we observed a compulsive-like escalation of intake similar toprevious reports 11, 29 (Fig. 2b). Strikingly, knockdown of striatal MeCP2 reversed the trajectory of drug-taking behavior such that Lenti-sh-MeCP2 rats with extended access initially consumed the same high levels of cocaine as Lenti-control rats, but their intake progressively decreased across sessions(Fig. 2b). Upward and downward shifts in the cocaine dose-response (D-R) curve reflect increases and decreases, respectively, in the motivation to consume the drug 11, 29, 30. When the unit dose of cocaine available for self-administration was varied in these rats, we found that the cocaine D-Rcurve was shifted downward in Lenti-sh-MeCP2 rats with extended access compared with Lenti-control rats (Fig. 2c). In fact, the D-R curve was entirely flat in the Lenti-sh-MeCP2 rats, indicating that their motivation to consume cocaine was almost completely abolished. Cocaine intake across various doses was similar in Lenti-sh-MeCP2 and Lenti-controls under restricted access conditions (Fig. 2d; Supplementary Fig. 4). Thus, MeCP2 knockdown decreases the motivation to consume cocaine, but only in rats with extended access to the drug. Because Lenti-control and Lenti-sh-MeCP2 rats displayed similar high rates of responding for food rewards under the same reinforcement schedule (fixed ratio 5) used for cocaine self-administration (Supplementary Fig. 5), this effect was not related to deficits in task performance.

## MeCP2-miR-212 interactions control cocaine intake

Next, we tested whether MeCP2 may influence cocaine intake through regulation of striatalmiR-212 expression. We found that expression of miR-212, and the closely neighboring miR-132, was increased in HEK-293 cells following knockdown of MeCP2 (Fig. 3a). Further, the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-aza-dC), which decreases the methylation status of DNA and thereby reduces the inhibitory influence of MeCP2 on gene expression 12, also increased miR-212/132 expression (Fig. 3b). In replication of recent findings from our laboratory 11, miR-212 and miR-132 levels were upregulated by ~25% and ~40%, respectively, in the dorsal striatum of the Lenti-control rats with extended cocaine access when measured 24-h after the last self-administration session(Fig. 3c,d; tissues from rats shown in Fig. 2). This effect of cocaine was dramatically increased by striatal MeCP2 knockdown (Fig. 3c,d). Moreover, a locked nucleic acid (LNA)-modified antisense oligonucleotide (LNA-antimiR-212), which disrupts miR-212 signaling without affecting the actions of miR-132 or other miRNAs 11, “rescued” the decreased cocaine intake in the extended access Lenti-sh-MeCP2 rats without altering intake

in the restricted access group (Fig. 3e). Previously we found thatmiR-212 decreases cocaine intake, at least in part, through amplification of striatal CREB signaling 11. Expression of c-fos, a known CREB-responsive gene, was upregulated by ~30% in Lenti-controlrats with extended access (Fig. 3f), and this effect was approximately 10-fold greater in the MeCP2 knockdown rats (Fig. 3f). Striatal MeCP2 therefore attenuates cocaine-induced increases in striatal miR-212 expression, thereby limiting the stimulatory effects of miR-212onstriatal CREB signaling.

## miR-212 inhibits striatal MeCP2 expression

Next, we tested whether miR-212 may repress striatal MeCP2 expression. Alternative polyadenylation sites can give rise to at least four different MeCP2 mRNA transcripts, each with different sized 3′UTRs 31. The two most prominent transcripts are those with short (~1.8kb)or long (~10 kb) 3′UTRs. The long but not the short transcript is abundantly expressed in brain and is known to be regulated by miR-132 13, and contains a putative MRE for miR-212 12. We found that rat PC12 cells express both the long and short forms of MeCP2transcripts, and that miR-212 selectively reduced levels of the long but not the short form(Fig. 4a). Consistent with these data, total protein levels of MeCP2 were also reduced in PC12 cells overexpressing miR-212 (Fig. 4b). Conversely, inhibition of miR-212 signaling in PC12 cells using the LNA-antimiR-212 oligonucleotide increased expression of the long form of MeCP2 (Fig. 4c). When we overexpressed miR-212 in the dorsal striatum using a lentivirus vector(Lenti-miR-212; Fig. 4d), we found that MeCP2 levels were reduced restricted access rats, and that the magnitude of this knockdown was even greater in extended access rats(Fig. 4e, f). Furthermore, we replicated our previous finding that cocaine intake is markedly lower in Lenti-miR-212 rats with extended but not restricted cocaine access (Supplementary Fig. 6), effects identical to those described above for Lenti-sh-MeCP2. These findings identify a negative homeostatic relationship between MeCP2 and miR-212in dorsal striatum similar to that previously identified between MeCP2and miR-132 in cortical neurons 13.

## MeCP2-miR-212 interactions control cocaine effects on BDNF

Next, we tested whether MeCP2-miR-212 interactions may regulate the effects of cocaine on striatal BDNF levels. First, BDNF levels were upregulated in dorsal striatum of extended access rats, when measured 24-h after the last cocaine session (Fig. 5a). BDNF levels were unaltered in the hippocampus and cerebellum (Supplementary Fig. 7), and were similarly unaltered in the NAcc and PFC even though MeCP2 was downregulated in these areas(Supplementary Fig. 1). Second, we found that striatal BDNF levels were significantly reduced in Lenti-sh-MeCP2 rats with restricted or extended access to cocaine (Fig. 5b), and in those that remained cocaine-naïve (Supplementary Fig. 8). Third, striatal BDNF levels were slightly reduced under restricted access conditions in Lenti-miR-212 rats, and this inhibitory effect was far greater under extended access conditions (Fig. 5c), data closely mirroring the actions of miR-212 on striatal MeCP2 levels(Fig. 4e,f). Bioinformatics analysis (http://www.targetscan.org/) failed to reveal a putative binding site for miR-212 in the 3′UTR of BDNF. Further, miR-212 did not alter expression of a reporter construct in which the entire BDNF 3′UTR was fused to a GFP cassette 32 (Supplementary Fig. 9), even when the cells were stimulated with forskolin (10 μM) to trigger potential activity-dependent

interactions. It is therefore unlikely that miR-212 directly represses BDNF expression. Instead miR-212 likely regulates BDNF levels through an indirect mechanism involving MeCP2 knockdown. Hence, MeCP2 and miR-212 regulate striatal BDNF levels in an opposite manner in cocaine self-administering rats, suggesting that homeostatic interactions between these factors control the magnitude by which cocaine increases striatal BDNF expression.

### BDNF facilitates compulsive cocaine-taking behavior

Next, we investigated the behavioral relevance of BDNF transmission in the dorsal striatum in controlling cocaine intake. Specifically, we used a lentivirus vector to overexpress BDNF (Lenti-BDNF; Fig. 6a and Supplementary Fig. 10) and examined cocaine intake under restricted and extended access conditions. We found that Lenti-control and Lenti-BDNF rats initially consumed the same number of cocaine infusions under extended access conditions (Fig. 6b). However, the Lenti-BDNF rats consumed progressively more cocaine than the Lenti-control rats across sessions and their intake escalated at a more rapid rate (Fig. 6b). After approximately 16 consecutive extended access sessions there was an abrupt and dramatic increase in cocaine consumption in the Lenti-BDNF rats, such that they consumed almost 100 infusions (~1.5 fold increase; 50 mg/kg cocaine) more than the Lenti-control rats during each session. After 3 consecutive sessions of this high intake we stopped the experiment because of fears of drug overdose and an obvious deterioration in the well-being of the animals (loss of body weight; increased agitation and reactivity to environmental noise or sound stimuli; repetitive face scratching resulting in bleeding and injury). The cocaine D-R curve was also shifted upward in the Lenti-BDNF rats compared with the Lenti-controls with extended access (Fig. 6c). Cocaine intake did not differ between Lenti-control and Lenti-BDNF rats with restricted cocaine access (Supplementary Fig. 11), and the cocaine D-R curve was similarly unaltered (Supplementary Fig. 11). Also, we found no evidence for behavioral abnormalities (e.g., weight loss, agitation and self-injury) in the Lenti-BDNF rats with restricted cocaine access. Lenti-control and Lenti-BDNF rats did not differ in their responding for food rewards under the same reinforcement schedule used for cocaine self-administration (Supplementary Fig. 11), demonstrating that effects of BDNF overexpression on cocaine intake were not secondary to alterations in task performance. When we assessed BDNF levels in the above rats 24-h after the last self-administration session, we again found that BDNF expression was increased in the dorsal striatum of Lenti-control rats with extended cocaine access compared to those with restricted access (Fig. 6d). Moreover, there was a general increase in striatal BDNF levels in the Lenti-BDNF rats confirming that the virus was functional, with the highest levels seen in the Lenti-BDNF rats with extended cocaine access that had consumed by far the highest levels of cocaine (Fig. 6d).

A concern related to virus use in striatum is that retrograde transport back to cortical areas may occur, resulting in behavioral effects that are independent of the striatum and related to virus-induced alterations in cortical gene expression. We observed low levels of GFP-positive cell bodies dispersed throughout the PFC of Lenti-sh-MeCP2 rats (Supplementary Fig. 12), an area rostral to the dorsal striatum and sites of virus injection. Importantly, decreases in cortical MeCP2 levels were not detected in these rats (Supplementary Fig. 13).

Similarly, alterations in cortical BDNF levels were not detected in Lenti-BDNF rats (Supplementary Fig. 13). It is therefore possible that lentivirus vectors may undergo low levels of retrograde transport away from injection sites in the striatum, but this phenomenon is unlikely to contribute to the behavioral effects reported here.

Finally, we investigated the role for endogenous striatal BDNF transmission in regulating cocaine intake. Specifically, we disrupted BDNF transmission in the dorsal striatum using an anti-BDNF neutralizing antibody known to reduce BDNF transmission in rat brain 33, and assessed cocaine intake under restricted and extended access conditions. The anti-BDNF antibody decreased cocaine intake in extended but not restricted access rats(Fig. 7), whereas IgG control injections had no effects on cocaine intake (Fig. 7). Endogenous BDNF transmission in the dorsal striatum therefore regulates cocaine intake under extended but not restricted access conditions.

## Discussion

Extended access to cocaine can trigger compulsive-like increases in the motivation to consume the drug, reflected in escalating intake and an upward shift in the cocaine dose-response curve 29. Little is currently known about the molecular mechanisms that control the transition from controlled to compulsive cocaine intake. Here, we show that MeCP2 and miR-212 are locked in a state of negative homeostatic coupling in the dorsal striatum, where they regulate the effects of self-administered cocaine on striatal BDNF expression in an opposite manner. Elevating BDNF levels in the dorsal striatum triggered an apparent loss of control over intake in rats with extended drug access, whereas disrupting striatal BDNF transmission reduced cocaine intake under extended access conditions. Thus, the dynamic balance between MeCP2 and miR-212 expression levels in the dorsal striatum, and factors that can influence this balance, are likely to play a crucial role in establishing vulnerability to develop compulsive cocaine-taking behaviors.

### MeCP2 controls cocaine intake

Striatal MeCP2 expression was upregulated in extended but not restricted access rats. Moreover, striatal MeCP2 knockdown profoundly decreased cocaine intake and rendered the cocaine D-R curve almost entirely flat in extended but not restricted access rats, suggesting that motivation to consume cocaine was almost completely abolished in these rats. Striatal MeCP2 may therefore play a critical role in regulating the increasing motivation to consume cocaine observed in rats under extended access conditions. A concern related to MeCP2 knockdown in adult brain is the possibility that RTT-like behavioral disturbances may emerge, and a decrease in cocaine intake may be secondary to these behavioral deficits 34. Consistent with previous reports involving RNA interference-mediated knockdown of MeCP2 in rat brain 35, we did not observe RTT-like behavioral disturbances or deficits in behavioral performance following striatal MeCP2 knockdown. This may reflect the fact that MeCP2 deficiency specifically in GABAergic cells in the forebrain may account for the motor deficits associated with RTT syndrome 36, a cell population that was not targeted in our studies.

It is interesting to note that MeCP2 knockdown impacted cocaine intake in extended but not restricted access rats. This may relate to a progressively more important role for the dorsal striatum in regulating drug-taking behavior as drug exposure increases. Recently it was shown that repeatedly engaging in drug-taking and drug-seeking behaviors over prolonged periods of time engages “spiraling loops” of connectivity between the ventral striatum, midbrain dopamine neurons and ever more dorsal striatal domains 37. That is, as drug-taking becomes more established and habitual, behavioral control transitions from ventral to dorsal striatal domains through striato-nigral-striatal loops 37, such that dopaminergic transmission in the dorsal striatum becomes ever more pronounced. Our data suggest that behavioral control of drug-taking transitions rapidly to the dorsal striatum in extended access rats, reflected by the fact that MeCP2 knockdown begins to influence drug-taking behavior after just a few (~3–4) extended access sessions, whereas cocaine-taking behavior is not influenced by these experimental manipulations in restricted access rats.

## MeCP2 acts through homeostatic interactions with miR-212

Because MeCP2 is a transcriptional repressor 1, 38, we sought to identify MeCP2-targeted genes that may explain its complex actions on cocaine-taking behavior. We recently reported that striatal miR-212 expression was increased in extended but not restricted access rats 11. Moreover, striatal miR-212 overexpression decreased cocaine intake and flattened the cocaine D-R curve in extended but not restricted access rats 11, effects identical to striatal MeCP2 knockdown. We found that striatal MeCP2 knockdown potentiated the stimulatory effects of cocaine on striatal miR-212 (and miR-132) expression in extended access rats. Moreover, inhibition of striatal miR-212 signaling using an antisense oligonucleotide “rescued” the decreased cocaine intake seen in MeCP2 knockdown rats with extended access. MeCP2 therefore controls cocaine intake, and may influence vulnerability to cocaine addiction, by regulating the stimulatory effects of the drug on striatal miR-212 expression.

In addition to repressing miR-212 expression, MeCP2 in turn is repressed by miR-212. Thus, MeCP2 and miR-212 are locked in a state of negative homeostatic balance in the striatum similar to that reported between MeCP2 and miR-132 in mouse cortical neurons 13. Considering that miR-212 and miR-132 share close sequence homology and similar homeostatic relationships with MeCP2, it will be important to determine the role of miR-132 in regulating cocaine intake. Intriguingly, this reciprocal interaction was dependent upon the level of access to cocaine. For example, striatal MeCP2 knockdown did not alter miR-212 expression in cocaine–naïve or restricted access rats, but potentiated the increased miR-212 expression detected in extended access rats. Similarly, striatal miR-212 overexpression moderately reduced striatal MeCP2 expression in restricted access rats, but markedly reduced expression in extended access rats. Interactions between MeCP2 and miR-212 are therefore activity-dependent indorsal striatum. This could be explained by spatial segregation of miR-212 and MeCP2 transcripts in neurons under basal conditions, with direct interactions only occurring upon repeated cocaine overconsumption. Alternatively, it is possible that an RNA binding protein (RBP) may mask the miR-212 MRE on MeCP2 transcripts under basal conditions, with cocaine overconsumption dissociating this putative RBP from MeCP2 transcripts.

## MeCP2-miR-212 interactions control striatal BDNF levels

Striatal BDNF transmission is known to increase sensitivity to the motivational properties of cocaine 21–24, 39, 40. Similar to MeCP2, striatal BDNF expression was increased in extended but not restricted access rats. Moreover, we found that MeCP2 expression was positively correlated, whereas miR-212 expression was negatively correlated, with striatal BDNF levels. BDNF overexpression in dorsal striatum triggered an apparent loss of control over the amount of drug consumed in extended access rats, but did not alter cocaine intake in restricted access rats. Conversely, disruption of local BDNF transmission in the dorsal striatum using an anti-BDNF neutralizing antibody significantly decreased cocaine intake in extended access but not restricted access rats. These findings demonstrate that MeCP2-miR-212 homeostatic interactions control the effects of cocaine on striatal BDNF levels, and that cocaine-induced increases in striatal BDNF likely play a key role in the emergence of compulsive-like responding for cocaine in extended access rats.

This raises the important question of precisely how MeCP2-miR-212 interactions impact striatal BDNF levels. BDNF can be synthesized locally in the striatum in an activity-dependent manner, and drugs of abuse can stimulate local BDNF production 28, 41–43. Our findings suggest that repeated cocaine overconsumption in extended access rats triggers *de novo* production of BDNF locally in the dorsal striatum. Moreover, MeCP2 may facilitate this process by inhibiting the expression of repressors that block BDNF transcription such as REST (RE1 silencing transcription factor; also known as NRSF) 18. In the same manner, miR-212 could decrease striatal BDNF levels by knocking down MeCP2 expression, thereby disinhibiting repressors of BDNF transcription. An alternate mechanism to explain these actions is the recent finding that MeCP2 may serve as a necessary co-activator of CREB activity at the promoters of a subset of CREB-responsive genes, including *BDNF* 17. In this manner, MeCP2 levels may determine the stimulatory effects of CREB signaling on BDNF production in striatum. Whatever the underlying mechanisms, our data demonstrate that MeCP2 and miR-212 exert opposite effects on striatal BDNF levels, and suggest that homeostatic interactions between these two factors may play a key role in determining vulnerability to cocaine addiction.

## MeCP2-miR-212 interplay may regulate addiction vulnerability

The notion that homeostatic interactions between MeCP2 and miR-212 control the motivational properties of cocaine may help resolve a number of curious aspects of cocaine-taking behavior. In particular, given that striatal CREB and miR-212 signaling is engaged by cocaine overconsumption, and feed forward interactions between both factors can limit drug intake, it is something of a paradox that the motivation to consume cocaine can still increase in extended access rats, reflected in escalating intake across sessions. Indeed, we found that cocaine intake escalated in extended access rats even though miR-212 expression was upregulated by ~1.75-fold under this access condition 11, although it is important to point out that disruption of endogenous miR-212 signaling markedly accelerated escalation of intake 11. This suggests that pro-addiction neuroplastic responses to cocaine can oppose the protective effects of miR-212 and CREB. Important in this regard, cocaine-induced increases in MeCP2 blunted the responsiveness of miR-212 expression to cocaine, with striatal miR-212 levels increased ~6-fold in rats with extended cocaine access following

MeCP2 knockdown. Striatal MeCP2 signaling therefore represents a pro-addiction response that facilitates the emergence of compulsive drug use by attenuating the otherwise large increases in miR-212 expression that would be observed in response to cocaine overconsumption, thereby limiting the protective effects of miR-212. This finding highlights the complexity of miR-212 signaling in striatum, in which it can powerfully counteract the motivational properties of cocaine, yet itself is negatively regulated by a transcription factor that may increase the behavioral actions of cocaine. Based on these findings, it is likely that factors influencing MeCP2-miR-212homeostasis have a profound influence on vulnerability to addiction. Specifically, factors that shift this dynamic balance in the favor of MeCP2, perhaps by increasing the methylation status of the miR-212 gene promoter, may increase vulnerability. Conversely, factors that shift the balance toward miR-212, perhaps through demethylation of its promoter or inhibition of MeCP2 signaling, may decrease vulnerability to addiction.

Finally, the above findings suggest that BDNF signaling in dorsal striatum plays a key role in facilitating the transition from controlled to compulsive cocaine-taking behavior, and that miR-212 may decrease cocaine intake through knockdown of striatal MeCP2 resulting in lowered BDNF levels. Importantly, we recently reported that miR-212 amplifies striatal CREB signaling in a Raf-1 kinase-dependent manner 11. Given that increased striatal CREB signaling reduces cocaine intake under extended but not restricted access conditions 11, we proposed that miR-212 controls cocaine intake through a stimulatory effect on striatal CREB signaling 11. Indeed, overexpression of CREB or the essential co-activator TORC (transducer of regulated CREB; also known as CRTC) in NAcc or dorsal striatum attenuates the motivational properties of cocaine and can increase aversive-like responses to the drug 11, 44. Conversely, disruption of striatal CREB signaling increases sensitivity to cocaine reward 44–47. CREB-induced increases inNMDA receptor subunits 48 and sodium channels 49, 50 or decreased potassium channel expression, is hypothesized to counteract the motivational properties of cocaine by enhancing excitability of striatal medium spiny neurons (MSN), cells that are inhibited by cocaine. In addition, stimulatory effects of CREB on dynorphin 44 or G-protein receptor kinase-3 (GRK3) 45 may also counter the motivational properties of cocaine. Thus, it appears that miR-212 may serve as a fulcrum in the striatum linking the actions of cocaine on CREB and BDNF signaling, simultaneously increasing CREB signaling whilst decreasing BDNF expression, and thereby limiting cocaine intake. Indeed, when we knocked down striatal MeCP2in extended access rats, we found that miR-212 expression was increased concomitant with a decrease in BDNF expression and significantly enhanced striatal CREB signaling (reflected in increased cFos mRNA expression). Considering that CREB is known to increase BDNF 43 and MeCP2 expression 13, our data further suggest that miR-212 serves to “filter” CREB signaling such that its impact on genes that protect against cocaine are maximized, whilst its stimulatory actions on genes that may enhance the actions of cocaine are minimized. This interpretation reconciles the fact that CREB is positively coupled to MeCP2 and BDNF expression, and yet CREB and MeCP2/BDNF signaling have opposite effects on cocaine-taking. More generally, this highlights the fact that miR-212, and perhaps many other miRNAs, are uniquely positioned to fine-tune transcriptional and neuroplastic responses to drugs of abuse. Indeed, the concerted actions of miR-212 on CREB, MeCP2and BDNF, and perhaps on

many other addiction-relevant signaling cascades, suggest that miR-212 may be a key focal point in controlling cocaine-induced striatal neuroplasticity and vulnerability to addiction.

## Methods

### Animals

Male Wistar rats (Charles River Laboratories, Raleigh, NC) weighing 300–320 g were housed in groups of 1–2 per cage in a temperature-controlled vivarium on a 12-h reverse light/dark cycle (lights off at 7:00 a.m.). Food and water were available *ad libitum* except when training animals to perform the operant response to receive food rewards, when animals were restricted to 20 g chow per day. Behavioral testing occurred during the dark portion of the light/dark cycle. All procedures were conducted in adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Scripps Florida.

### Cocaine self-administration

Rats were anesthetized by inhalation of 1–3% isoflurane in oxygen, and surgically prepared with silastic catheters in the jugular vein. The catheter was passed subcutaneously to a polyethylene assembly mounted on the animal's back. Rats were permitted at least 7 days recovery before behavioral training commenced. Animals were food restricted (20 g per day; 3–4 days) then trained to respond on an "active" lever for food pellets (45 mg; 60 min sessions) under a fixed ratio 5 time-out 20 sec (FR5TO20) schedule of reinforcement. Rats were also presented with an "inactive" lever during training and testing sessions, responses on which were recorded but were without scheduled consequence (data not presented). Rats responded for food until stable intake was achieved, defined as >80 pellets per 1 h session. Rats then responded for cocaine on the FR5TO20 sec reinforcement schedule during 1 h daily testing sessions for at least 7 consecutive days. Cocaine hydrochloride was supplied by the National Institute on Drug Abuse (NIDA), and dissolved in sterile saline solution (0.9% w/v). Each cocaine infusion earned resulted in the delivery of 0.5 mg/kg/infusion cocaine (0.1 ml injection volume delivered over 4-sec), and initiated a 20-sec time-out period signaled by a light cue located above the active lever during which responding on the lever was without consequence. After training to self-administer cocaine as described above, two balanced groups of rats were formed that consumed similar amounts of cocaine. One group of rats continued to respond for cocaine infusions during 1-h daily testing session (restricted access), and the other responded for cocaine during 6-h daily sessions (extended access). When required as part of the experimental design, control groups of rats were surgically prepared with IV catheters and trained to respond for food reinforcement as described above, but remained cocaine-naïve for the duration of the experiment. To determine the cocaine dose-response curve, the unit dose of cocaine available for self-administration was adjusted upward or downward during 3-hour testing sessions every other day between regular 6-h self-administration sessions; for detailed description of procedure see 29. Doses of cocaine were tested once, and in the following order: 0.5, 0.0625, 0.25, 0.125, and 0 mg/kg/infusion.

### Intracerebral injection procedures

For intra-dorsal striatal administration of lentivirus vectors (Lenti-controls; Lenti-sh-MeCP2; Lenti-miR-212 or Lenti-BDNF), rats were first anaesthetized by inhalation of 1–3% isoflurane in oxygen and positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA). The animals' scalp was carefully shaved and cleaned, and a ~1 cm rostro-caudal incision was made to expose the underlying skull. A total of 5 viral injections (1 μl per injection; viral supernatant concentrations ranged from $3 \times 10^7$–$5 \times 10^9$ infection units/ml) were delivered into each side of the striatum, for a total of 10 striatal injections per rat. The viruses were directed toward medial and lateral portions of the dorsal striatum. Medial injection sites were according to the following stereotaxic coordinates (all surgeries were in the flat skull position): anterior/posterior (AP): 1.20 mm from Bregma; medial/lateral (ML): ±2.00 mm from midline; dorsal/ventral (DV): −5.0 and −3.8 mm below dura. Lateral injection sites were according to the following stereotaxic coordinates: AP: 1.20 mm from Bregma; ML: ±3.25 mm from midline; DV: −6.5, −5.5 and −4.5 mm below dura. To deliver the virus, a small hole was drilled through the skull at the ML coordinate, and a stainless steel injector (32 gauge, 14mm in length) was lowered to the most ventral injection site. The viral supernatant injection was delivered over 60 sec. After the infusion, the injector was left in place for an additional 60 sec. The injector was then raised to the next more dorsal injection site, and the injection procedure repeated. After the final virus injection, the drill holes in the skull were filled with dental acrylic, the scalp sutured, and the incision site treated with antibiotic ointment.

For intrastriatal administration of LNA oligonucleotides or anti-BDNF neutralizing antibody, chronic indwelling intracerebral cannula were first implanted above the dorsal striatum. Briefly, rats were anaesthetized by inhalation of 1–3% isoflurane in oxygen and positioned in a stereotaxic frame (Kopf Instruments). Bilateral stainless steel guide cannulae (23 gauge, 12 mm in length) were implanted 2.0 mm above the most dorsal injection site in the dorsal striatum according to the following stereotaxic coordinates: AP, 1.20 mm from bregma; ML, ± 3.25 mm from midline; DV, 2.40 mm from dura. Four stainless steel skull screws and dental acrylic held the cannulae in place. Cannulae were kept patent using 12 mm long stainless steel stylets. The oligonucleotides or antibodies were delivered on two consecutive days. On each day, animals were gently restrained after their daily cocaine self-administration session, and received a total of three oligonucleotide (1 μl per injection; 25 μM concentration) or antibody (1 μl per injection; 100 μg/ml concentration) injections into each side of the dorsal striatum (a total of six striatal injections per rat per day). A stainless steel injector (32 gauge, 16 mm in length) was lowered into the most ventral injection site. The molecule was delivered over 60 s. After the infusion, the injector was left in place for a further 60 s. The injector was then raised 1 mm to the next more dorsal injection site, and the injection procedure repeated. After the final LNA injection, the 12 mm long stylet was re-inserted into the cannula.

### Real-time PCR

All real-time-PCR analyses of gene and miRNA expression levels were performed using stock primers and miRNA assays commercially available from Applied Biosystems (ABI). For all reactions, 10 ng of total RNA for miRNA, and 2μg for mRNA analysis was reverse-

transcribed using miRNA-specific primers (ABI), and primers to GAPDH or small nuclear RNA (snoRNA) as endogenous control for protein-coding genes or miRNAs, respectively. The protocol followed the manufacturer's specifications. All reactions were normalized to the endogenous control, and comparison between groups made using the method of $2^{-\Delta\Delta Ct}$.

### Reverse transcription PCR

PCR was carried out according to standard procedures. The primers used to detect the short or long form of MeCP2 were: Long 3′UTR Forward: 5′-GCA GAG ATA TTT GTA GGC CC-3′; Long 3′UTR Reverse: 5′-GCA CAC ATT GAG TAA CAG TCC TGG-3′. Short 3′UTR Forward: 5′-AAG GAG CCA GCT AAG ACT CA-3′; Short 3′UTR Reverse: 5′-TTG TCA GAG CCC TAC CCA-3′.

### Immunoblotting and immunochemistry

Immunoblotting and immunochemistry were carried out according to standard procedures. For immunochemistry, rats were deeply anesthetized and then perfused transcardially with cold 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4. Brains were post-fixed in 4% PFA overnight and stored in 30% sucrose in PBS. Brains were sectioned (30 μm) in the coronal plane that incorporated the dorsal striatum (typical range: +1.7 mm to −0.4 mm from bregma) on a cryostat (HM 505 E, Microm, Walldorf, Germany) kept at −20°C. For immunoblotting, brain areas of interest were collected from slices and placed in Eppendorf tubes and stored at −80°C until protein extraction. In all cases, brain tissues were harvested 24-h after the last self-administration session. Tissues were sonicated in 1X RIPA buffer. Protein content was determined using the Bio-Rad DC Protein Assay kit. Protein samples were seperated by gel electrophoresis, and proteins transferred to a nitrocellulose membrane (NC membrane, Invitrogen iBlot system). Mark high molecular weight pre-stained standards (BIO-RAD) were also run on each gel. Nonspecific binding sites on the membranes were blocked by 5% nonfat dry milk in TBS and 0.1% Tween 20 (TBS-T). Blots were incubated in primary antibody in PBS-T, washed and then incubated in secondary antibody. Blots were washed and immunological detection was carried out using Super Signal Chemiluminescent Substrate (Thermo Scientific). Antibodies were stripped from the blots, and the blots probed for anti-β-actin or GAPDH (Sigma or Santa Cruz). Primary antibodies used were: anti-copGFP (Axxora; Cat# AB502); anti-chicken GFP (Abcam; Cat# ab13970-100); anti-GFAP (Covance, SMI-22R; Cat# SMI-22R); anti-BDNF (Santa Cruz for Blotting Cat#SC-546; and Millipore for Immunochemistry Cat#AB1513); anti-MeCP2 (Millipore; Cat#07-013). In cases were bands have been cropped from immunoblots and incorporated into images, it is important to note that all samples were run at the same time and on the same gel. For immunochemistry, sections were mounted on Superfrost Plus slides (Fischer Scientific, Pittsburg, PA), dehydrated, and coverslipped. Sections were visualized by using a BX61 (Olympus) fluorescence microscope at 2×, 10× and 20×. For cell counting, the percentage of immunoreactive cells was calculated from counts on at least 800 cells by an investigator blinded to the identity of the samples.

### Generation of concentrated lentivirus vectors

For MeCP2 knockdown, a 70 nucleotide short hairpin interfering RNA was designed using the Genscript, Inc. online construct builder (see Supplementary Fig. 2 for shRNA sequence).

The shRNA was cloned into the pRNAT-U6.2/Lenti virus expression vector from Genscript. For BDNF overexpression, the BDNF full-length cDNA was purchased from Open Biosystems (Clone ID; 7319966), and BDNF was cloned into the pCDF1 lentivirus expression vector from System Biosciences, Inc. For Lenti-miR-212 production, the pMIF-copGFP-rno-miR-212 construct was purchased from System Biosciences Inc. In all cases, control vectors were identical to the expression constructs, but without the gene insert. Lentivirus particles were packaged using the Invitrogen ViraPower™ Lentiviral Expression System. Virus was then concentrated and titered according to manufacturers' instructions, and stored at −80°C in 10 μl aliquots in cold phosphate-buffered saline, until use.

### Oligonucleotides

The LNA-antimiR-212 and LNA-scrambled molecules were purchased from Exiqon Inc, and were synthesized as unconjugated oligonucleotides with a phosphodiester backbone, see ref. 11.

### Neutralizing antibody

The anti-BDNF neutralizing antibody (20 μg) and control IgG were purchased from Calbiochem (#GF35L). The anti-BDNF antibody was a mouse monoclonal. Lyophilized antibody (or immunoglobulin control) was resuspended sterile 20 mM Tris-saline (20 mM Tris containing 0.15 M NaCl;pH 7.4), then diluted in 1ml of sterile PBS (pH 7.4) to yield a final concentration of 100 μg/ml.

### Statistical Analyses

All data were analyzed by one- or two-way repeated-measures analysis of variance (ANOVA). Significant main or interaction effects were followed by Bonforroni *post-tests* or Newman-Keuls *post-hoc* tests as appropriate. All statistical analyses were performed using Graphpad Prism software. The level of significance was set at 0.05.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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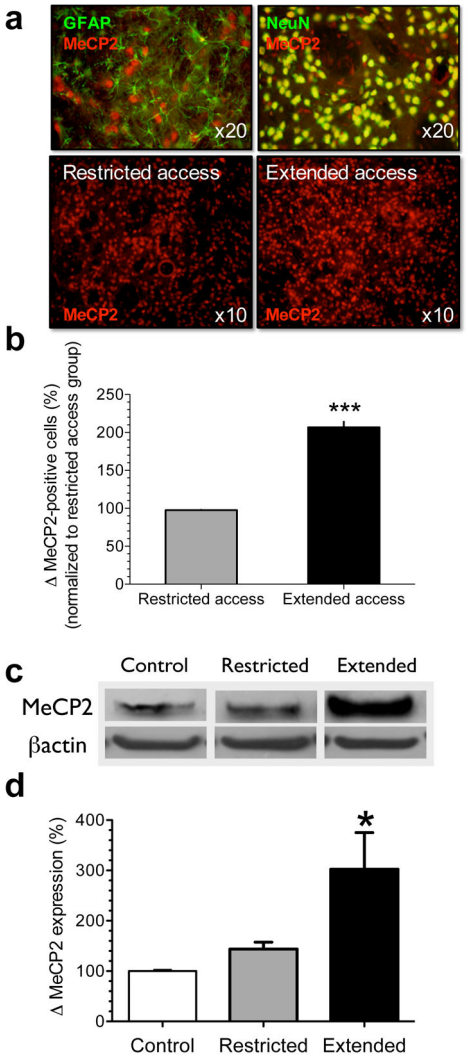


**Figure 1. Increased striatal MeCP2 expression in extended access rats**

**(a)** Immunochemical detection of MeCP2 in the dorsal striatum of drug-naïve rats. *Left upper panel*, MeCP2 (shown in red) rarely co-localized with glial fibrillary acidic protein (GFAP; shown in green), a marker for astrocytes. *Right upper panel*, MeCP2 (in red) was almost exclusively co-localized with the neuronal nuclear marker NeuN (shown in green). There was an increase in the number of MeCP2-positive cells in the dorsal striatum of rats with extended cocaine access *(lower right panel)* compared to rats with restricted access *(lower left panel)*. **(b)** Relative numbers of MeCP2-positive cells shown in the lower left and right panels above. ***$P<0.001$, t-test. **(c)** Representative immunoblot demonstrating that MeCP2 expression is increased in the dorsal striatum of rats with extended cocaine access compared with restricted access or drug-naïve control rats. **(d)** Relative amounts of MeCP2 in dorsal striatum was quantified by densitometry. *$P<0.05$ compared with control, post-hoc comparison after significant one-way ANOVA. In all cases, $n=6$ rats per group, and error bars are given as s.e.m.

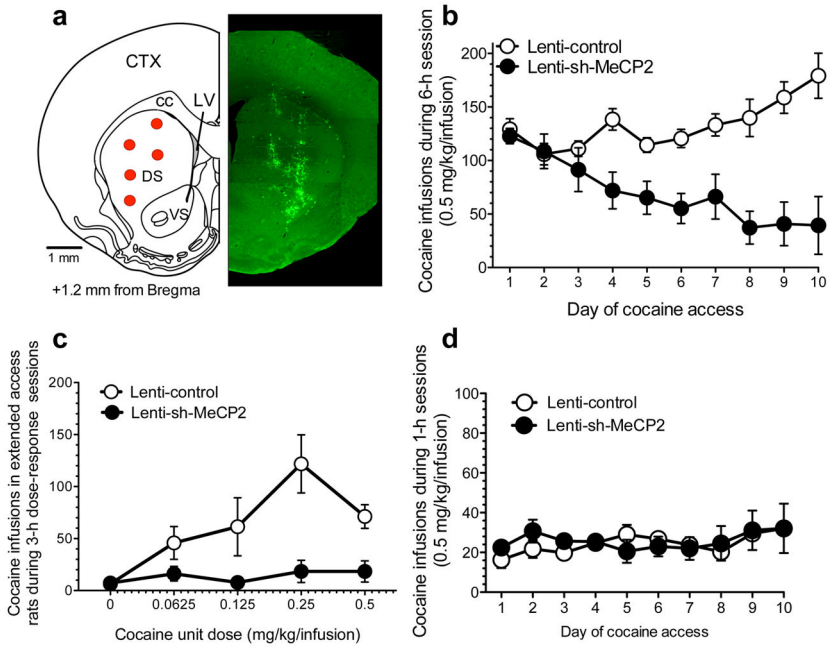


**Figure 2. Dissociable effects of MeCP2 knockdown on cocaine intake**

**(a)** The left panel is a graphical representation of the dorsal striatum and surrounding brain structures. Red circles represent the locations at which the Lenti-sh-MeCP2 virus infusions were targeted. The right panel is a representative immunochemistry staining from the brain of a Lenti-sh-MeCP2 rat. Green staining is GFP from virus; CTX, cortex; cc, corpus callosum; LV, lateral ventricle; DS, dorsal striatum; VS, ventral striatum. **(b)** Lentivirus-mediated knockdown of MeCP2 in the dorsal striatum blocks the development of escalated cocaine intake and reverses the long-term trajectory of cocaine-taking behavior in rats with extended access (Two-way ANOVA, Virus: $F_{9,72}$=7.3, P<0.0001; Virus × Session: $F_{1,8}$= 19.6, P=0.05). **(c)** MeCP2 knockdown flattens the cocaine dose-response curve in rats with extended cocaine access (Two-way ANOVA, Virus: $F_{1,8}$=11.6, P<0.01; Dose: $F_{4,32}$=4.7, P<0.005; Virus × Dose: $F_{4,32}$= 6.7, P<0.005). **(d)** MeCP2 knockdown did not alter cocaine intake in rats with restricted access to the drug. In all cases, *n*=6 rats per group, and error bars are given as s.e.m.

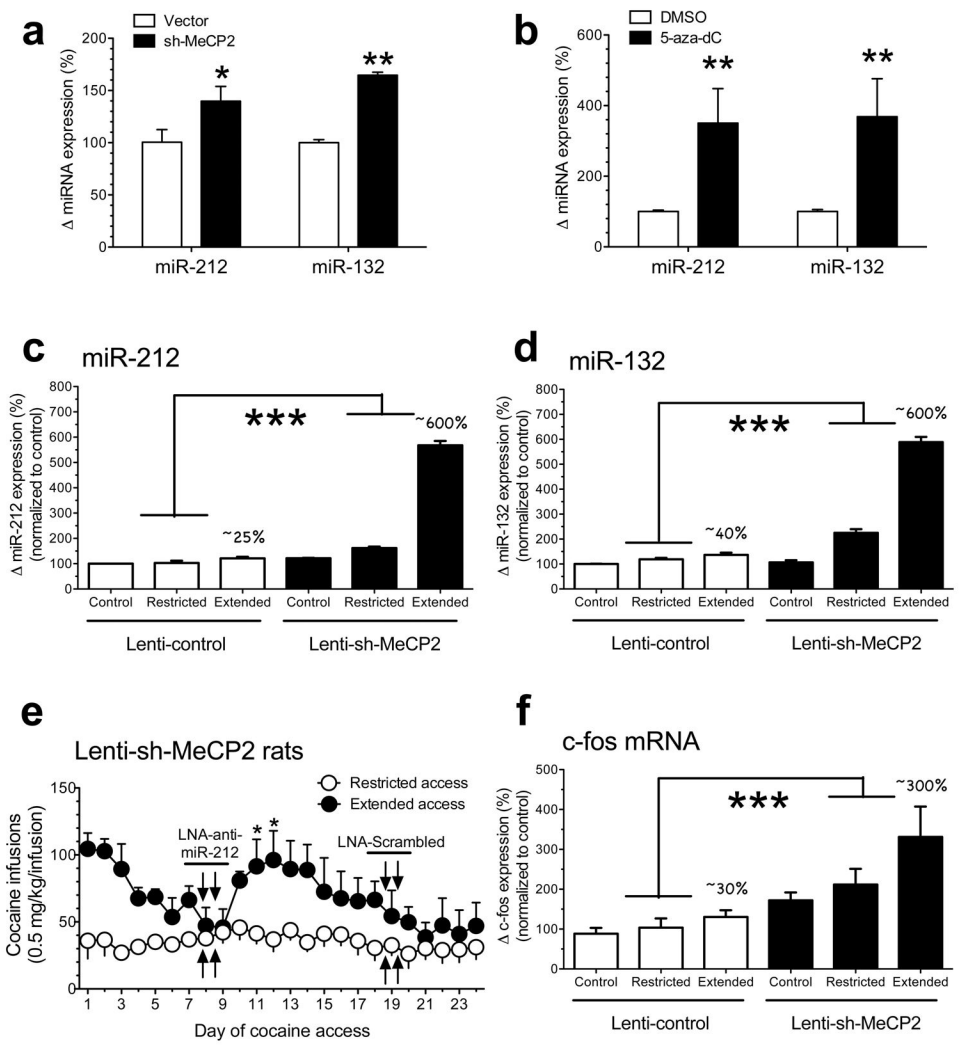


**Figure 3. MeCP2 blunts the effects of cocaine on microRNA-212 expression**

**(a)** Knockdown of MeCP2 in HEK-293 cells increases expression levels of miR-212 and miR-132. *P<0.05, **p<0.01, t-test compared with vector-transfected cells. **(b)** The DNA methyltransferase inhibitor 5-aza-dCincreases expression levels of miR-212 and miR-132 in HEK cells. **P<0.01, t-test compared with vector-transfected cells. **(c)** Lentivirus-mediated knockdown of MeCP2 expression in the dorsal striatum potentiates the stimulatory effects of cocaine on miR-212 expression (Two-way ANOVA, Virus: $F_{1,12}$=607.0, P<0.0001; Access: $F_{2,12}$=438.6, P<0.0001; Virus × Access: $F_{2,12}$= 363.5, ***P<0.0001). **(d)** Knockdown of MeCP2 also potentiates the stimulatory effects of cocaine on miR-132 expression in dorsal striatum (Virus: $F_{1,12}$=387.3, P<0.0001; Access: $F_{2,12}$=263.3, P<0.0001; Virus × Access: $F_{2,12}$= 198.9, ***P<0.0001). **(e)** Disruption of striatal miR-212 signaling using an antisense oligonucleotide (LNA-antimiR-212) “rescues” low levels of cocaine intake in Lenti-sh-MeCP2 rats with extended access. Striatal infusion of a control oligonucleotide(LNA-Scrambled) did not alter cocaine in restricted or extended access Lenti-sh-MeCP2 rats. *P<0.05, compared with intake on access day 9 (the session after the final LNA-antimiR-212 injection). **(f)** Lentivirus-mediated knockdown of MeCP2 in dorsal striatum

potentiates the stimulatory effects of cocaine on the CREB-responsive gene c-fos (Virus: $F_{1,12}$=20.9, P<0.0001, ***P<0.0001; Access: $F_{2,12}$=4.4, P<0.05). In all cases, samples were run in triplicate for *in vitro* studies, there were *n*=6 rats per group for *in vivo* studies, and error bars are given as s.e.m.

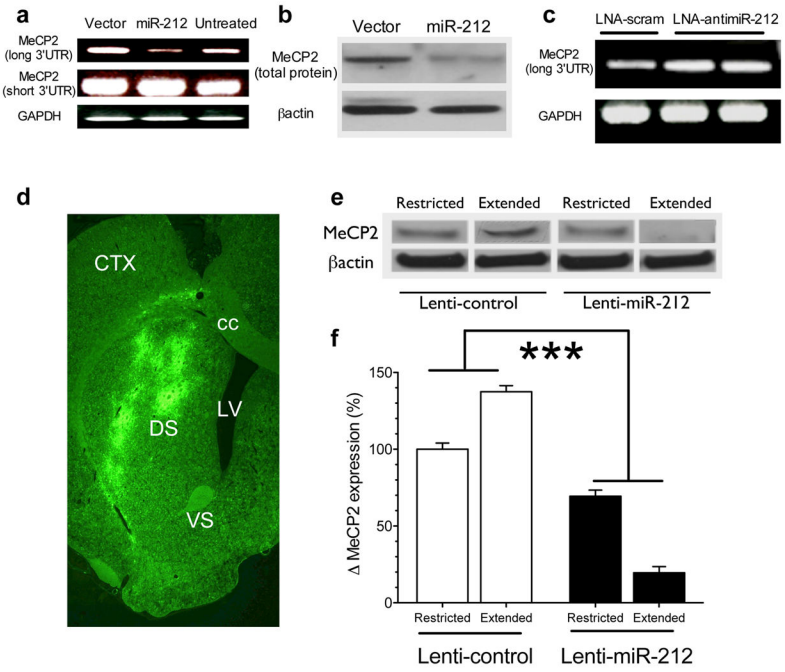


**Figure 4. MicroRNA-212 represses MeCP2**

**(a)** Using reverse transcription PCR and primers designed to selectively detect the long or short form of MeCP2, we found that miR-212 overexpression in rat PC12 cells resulted in a selective reduction in expression of the long MeCP2 transcript. **(b)** Representative immunoblot demonstrating that total protein levels of MeCP2 were decreased in PC12 cells following overexpression of miR-212. **(c)** LNA-antimiR-212 increased expression of the long MeCP2 transcript. **(d)** Representative GFP immunochemistry staining from the brain of a Lenti-miR-212 rat. **(e)** Representative immunoblot demonstrating dorsal striatal expression of MeCP2 in Lenti-control and Lenti-miR-212 rats with restricted or extended access to cocaine self-administration. **(f)** Relative amounts of MeCP2 in dorsal striatum was quantified by densitometry (Two-way ANOVA, Access: $F_{1,18}$=219.4, P<0.0001; Virus × Access: $F_{1,18}$= 75.9, ***P<0.0001). In all cases, samples were run in triplicate for *in vitro* studies, there were *n*=6 rats per group for *in vivo* studies, and error bars are given as s.e.m.

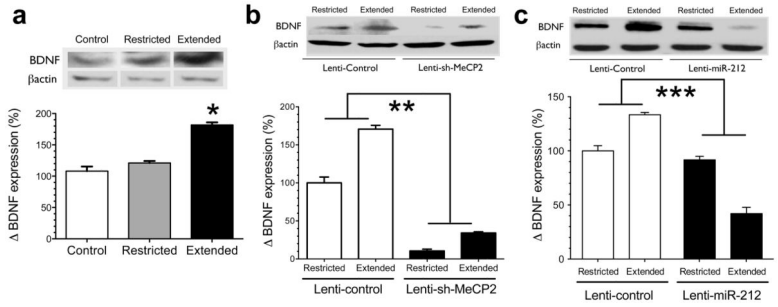


**Figure 5. MeCP2-microRNA-212 interplay controls striatal BDNF expression**

**(a)** BDNF expression is increased in the dorsal striatum of rats with extended access to cocaine self-administration. *Upper panel*, Representative immunoblot demonstrating dorsal striatal expression of BDNF in cocaine-naïve control rats, and in rats with restricted or extended access to cocaine self-administration. *Lower panel*, Relative amounts of BDNF in dorsal striatum was quantified by densitometry. **P*<0.05, post-hoc comparison after significant one-way ANOVA. **(b)** Knockdown of MeCP2 in dorsal striatum decreases BDNF expression in cocaine self-administering rats. *Upper panel*, Representative immunoblot demonstrating dorsal striatal BDNF expression in Lenti-control and Lenti-sh-MeCP2 rats with restricted or extended access to cocaine self-administration. *Lower panel*, Relative amounts of BDNF in dorsal striatum was quantified by densitometry (Two-way ANOVA, Virus: $F_{1,8}$=556.9, P<0.0001; Access: $F_{1,8}$=97.0, P<0.0001; Virus × Dose: $F_{1,8}$=24.2, **P=0.005). **(c)** Overexpression of miR-212 in dorsal striatum decreases BDNF expression in cocaine self-administering rats. *Upper panel*, Representative immunoblot demonstrating dorsal striatal BDNF expression in Lenti-control and Lenti-miR-212 rats with restricted or extended access to cocaine self-administration. *Lower panel*, Relative amounts of BDNF in dorsal striatum was quantified by densitometry (Two-way ANOVA, Access:$F_{1,8}$=137.8, P<0.0001; Virus × Dose: $F_{1,8}$=95.8, ***P=0.001). In all cases, *n*=6 rats per group and error bars are given as s.e.m.

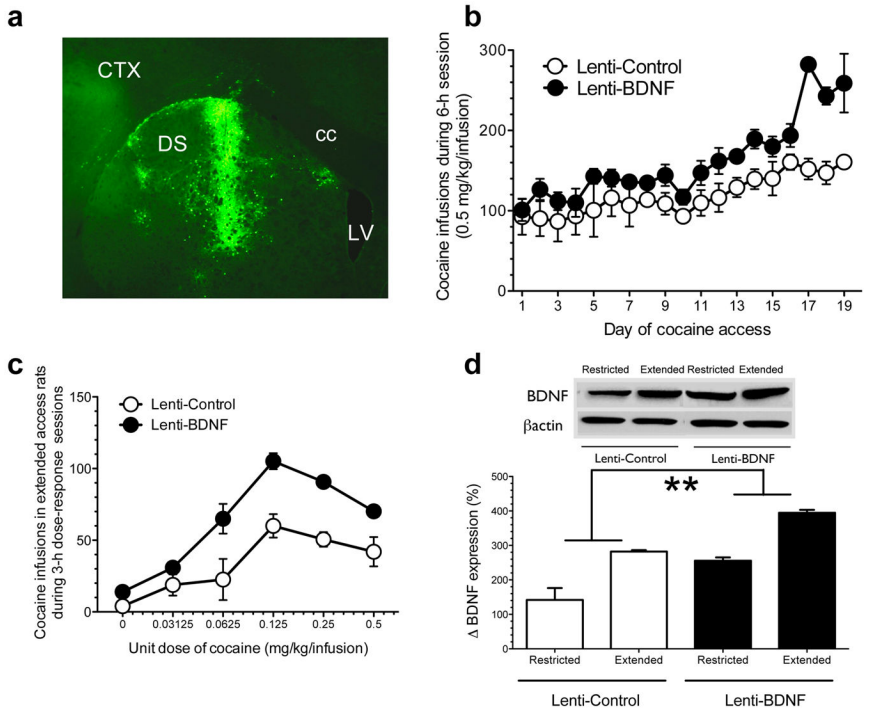


**Figure 6. Enhanced BDNF expression triggers compulsive cocaine intake**

**(a)** Representative GFP immunochemistry staining from the brain of a Lenti-BDNF rat. **(b)** Striatal BDNF overexpression accelerates the development of escalated cocaine intake and precipitates a compulsive-like loss of control over intake (Two-way ANOVA, Virus: $F_{1,8}$=28.1, P<0.0001; Session: $F_{18,144}$=10.8, P<0.0001; Virus × Session: $F_{18,144}$= 1.9, P=0.005). **(c)** BDNF overexpression shifts the cocaine dose-response curve upward in rats with extended access to the drug (Virus: $F_{1,9}$=31.4, P<0.0005; Dose: $F_{5,45}$=35.9, P<0.0001; Virus × Dose: $F_{5,45}$= 2.87, P<0.05). **(d)** BDNF expression in the dorsal striatum in Lenti-control and Lenti-BDNF rats with restricted or extended access to the drug. *Upper panel*, Representative immunoblot demonstrating dorsal striatal BDNF expression in Lenti-control and Lenti-BDNF rats with restricted or extended access to cocaine self-administration. *Lower panel*, Relative amounts of BDNF in dorsal striatum was quantified by densitometry (Two-way ANOVA, Virus: $F_{1,8}$=37.6, **P<0.0005; Access: $F_{1,8}$=57.3, P<0.0001). In all cases, *n*=6 rats per group and error bars are given as s.e.m.

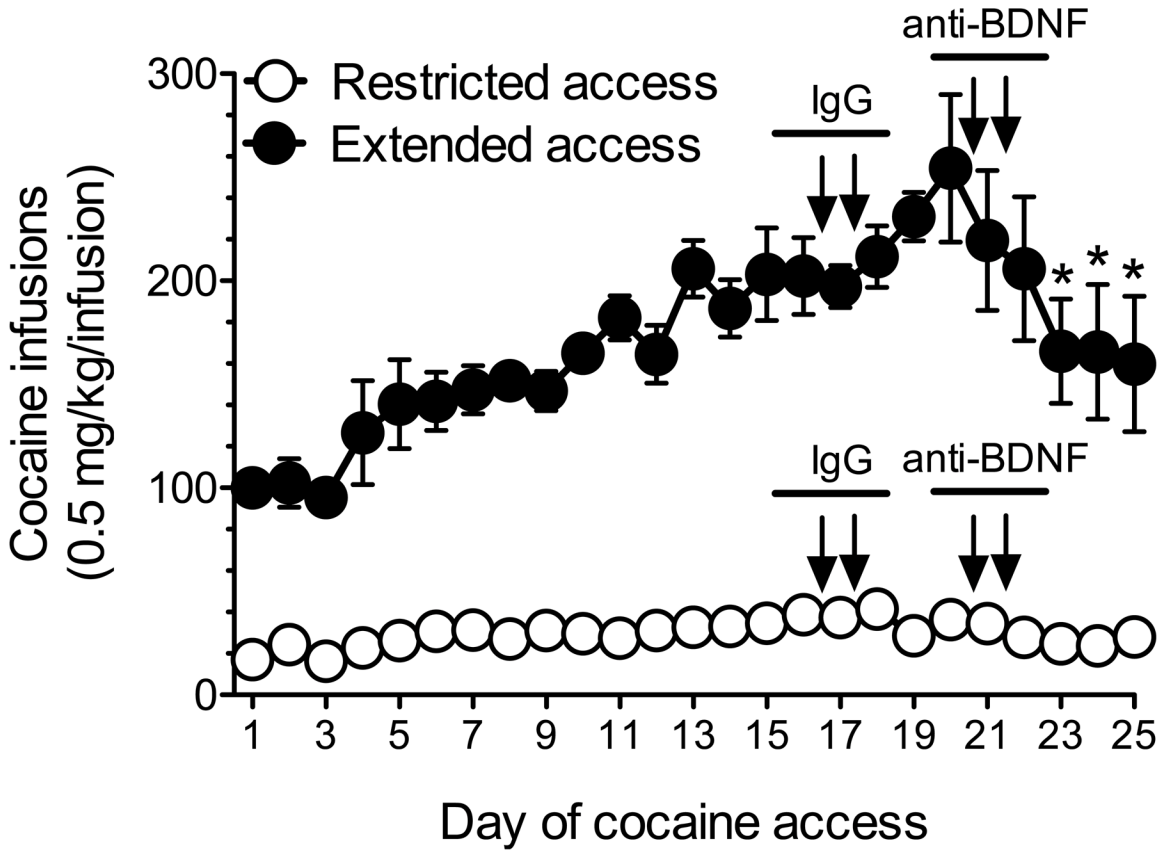


**Figure 7. Disruption of endogenous BDNF transmission decreases cocaine intake**

Disruption of endogenous BDNF signaling in dorsal striatum using an anti-BDNF neutralizing antibody decreases cocaine intake in rats with extended but not restricted access to cocaine. Control IgG infusions into the dorsal striatum did not alter cocaine in the restricted or extended access rats. Two-factor ANOVA of cocaine intake data on access days 20–25 was carried out: Access: $F_{1,10}$=41.8, P<0.0001; Session: $F_{5,50}$=3.5, P<0.01; *P<0.05, compared with intake on access day 20 (the session before the first anti-BDNF antibody infusion). There was an *n*=6 rats per group and error bars are given as s.e.m.