## SUPPLEMENTARY METHODS

**Effect of miRNA regulation on the reinstatement of cocaine CPP by lentiviral miRNA regulation in naive animals previously subjected to cocaine CPP:**

In a second set of experiments, four additional groups of rats (n=12) were used to study the effect of LV-miRNA injection on reinstatement after prior conditioning of the un-operated animals to cocaine CPP. After the establishment of cocaine CPP, the first three additional groups were stereotaxically injected bilaterally in the NAc with either LV-miR-124 or LV-miR-124-Sil or the control LV-GFP and the fourth group was un-operated naive animals. The groups were then further subdivided into two subgroups (n=6), based on pairing-treatment: the control saline-paired subgroup (n=6) received only 0.9% saline (1 ml/kg i.p.) injections throughout the experiment and the cocaine-paired subgroup (n=6) received saline/cocaine (20 mg/kg i.p.) on alternate days during the conditioning period of CPP. After the rats recovered from surgery, they were subjected to extinction for cocaine CPP. Once the complete extinction was established, animals were subjected to reinstatement of CPP. The day after the last extinction session, all groups (n=12/group) received a priming injection of 0.9% saline (1 ml/kg i.p.) and free access to both compartments (20 min), to monitor the amount of time the rat spent in each chamber. This was followed 24 h later with a priming injection of low dose cocaine (2 mg/kg i.p.) with a 20 min session to monitor the reinstatement of the CPP in the different groups (Bahi et al., 2008a, b).

**Quantification of mature miRNA in naive animals subjected to cocaine CPP by real-time qRT-PCR:** RNA samples from the NAc region of the naive animal group (n=16) subjected to cocaine CPP, extinction and reinstatement were used for quantifying the differential expression of the selected miRNAs. Expression profiling of mature miRNA was performed as mentioned before (see methods) using specific RT-primers (Taqman® MicroRNA Assay, Applied Biosystem, UK). Comparisons were made between cocaine and saline groups, and significance was calculated using two-way ANOVA followed by Bonferroni post hoc tests and the level of statistical significance was set at $P < 0.05$. Data were expressed as means ±SEM.

## SUPPLEMENTARY FIGURES AND TABLES LEGEND

**Figure S1. (A) Schematic representation of the miRNA-silencer construct used in the study.** Four perfect match miRNA-recognition elements (pMREs) (red) for each specific miRNAs were inserted into the 3' UTR region of the EGFP (green) construct and cloned into the LV-vector for expressing the doxycycline regulatable expression of individual miRNA-Silencers *in vivo*. **(B)** Mature miRNA sequences of the developmentally regulated miRNAs, miR-124 and let-7d with highlighted four nucleotides sequence similarity (red square) in their seed sequence (underlined) and a 3 nucleotide homology adjacent to the seed. The Venn diagram shows the target genes of the respective miRNAs including the significant number of shared target genes common to both the miR-124 and let-7d based on target prediction by miRanda algorithm.

**Figure S2.**

**Effect of LV-miRNA mediated regulation in the saline-paired groups in Cocaine-CPP:**

**(A)** Post-training extinction data of saline-paired animals from the different LV-groups. Regulation of miRNA expression in the NAc does not affect the rate of cocaine-CPP extinction in the saline –paired groups. Ten groups of animals (n=12/group) were pre-tested, trained and tested for CPP as displayed in Fig. 1. After the CPP recording on the 10th day, rats were subjected over 12 days to CPP extinction in 20 min daily sessions with full chamber access but no injections (see methods). **(B)** LV-miRNA regulation in the NAc has no effect on the reinstatement of CPP after saline priming injection. After the CPP recording and establishment of extinction, animals were subjected to priming injections of 0.9% saline (1ml/kg i.p.) and place preference was recorded for 20 min (see methods). **(C)** LV-miR-124 regulation after cocaine CPP has no effect on the reinstatement of CPP after saline priming. Four groups of animals (n=12) were

subjected to cocaine CPP. Immediately after the establishment of CPP, three groups were stereotaxically injected with either LV-miR-124, or miR-124-Sil or control GFP, a fourth retained as naive un-operated animals. Values represent mean ±S.E.M.

**Figure S3. Regulation of mature miRNA in the NAc of naive animals after CPP, reinstatement and extinction.** Samples from the NAc of naive rats were analyzed at the end of the behavioral studies and used for quantification of mature miRNA levels (supplementary methods). Expression levels of mature miRNAs were calculated relative to U6 snRNA levels (see methods) and represented as fold change compared to the control saline group. *P<0.05; **P<0.01; ***P<0.001 represents values significantly different from saline group by two way ANOVA, Bonferroni post hoc tests.

**Table S1. Gene regulation of cocaine-dependent direct and indirect targets, after LV-mediated miRNA expression or silencing in the NAc.** Representation of target genes modified based on qRT-PCR and Western analysis. Arrows represents genes up-regulated (↑) or genes suppressed (↓) after LV-miRNA regulation. CREB: cAMP-responsive element Binding Protein; pCREB: Phosphorylated (serine 133) cAMP-responsive element Binding Protein; MOR1: μ-opioid receptor-1; DAT: Dopamine transporter; uPA: Urokinase plasminogen activator; GRIA2: ionotropic glutamate AMPA-receptor-2; EphB1: Eph-B1 tyrosine kinase receptor; Drd3: Dopamine D3 receptor; Per2: Period Homologue 2; MeCP2: methyl CpG binding protein 2. FosB: immediate early gene; Δfos: truncated splice variant of FosB; 7MYT1: 7 zinc finger Myelin transcription factor 1; PTBP1: Polypyrimidine tract binding protein 1; PTBP2: Neural polypyrimidine tract binding protein 1.

**Table S2. Properties of Genes in Chronic Cocaine Administration.**

(Abbreviations as in Table S1)

**Supplementary Table S1. Regulation of Cocaine-dependent target Genes observed after either expression or silencing of the selected miRNAs *in vivo* (based on qRT-PCR and Western analysis).**

| **Expression of microRNA-mediated direct or indirect Target Genes** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **miRNA groups** | **Regulated Cocaine-responsive Genes** | | | | | | |
| miR-124 | DAT ↑ | uPA ↑ | EphB1↓ | Ptbp1 ↓ | Drd3 | FosB | CREB |
| let-7d | MOR1 ↓ | Drd3 ↓ | DAT ↑ | 7MYT1 ↓ | FosB | CREB | uPA |
| miR-181a | DAT ↓ | Drd3 ↓ | MeCP2 ↑ | 7MYT1 ↓ | Per2 ↓ | CREB | FosB |
| miR-124-Sil | CREB ↑ | FosB ↓ | Δfos ↑ | Ptbp2 ↓ | MeCP2 ↓ | BDNF ↓ | GRIA2 ↓ |
| let-7d-Sil | pCREB ↑ | MOR1 ↑ | FosB↑ | Δfos ↑ | Drd3 | Per2 ↓ | CREB |
| miR-181a-Sil | Per2 ↑ | MOR1 ↓ | MeCP2 ↓ | DAT ↑ | MeCP2 ↓ | uPA ↓ | CREB |

## TABLE S2: Properties of Genes in Chronic Cocaine Administration

| Gene | Properties | Effects of miR-181a | Effect of miR-124 | Effect of Let-7d | Conclusion |
|---|---|---|---|---|---|
| **MOR1** | μ-opioid receptor (MOR1) plays a crucial neuromodulatory role in the behavioral effects of cocaine. An inhibition of MOR1 attenuates cocaine-induced behavioral sensitization and conditioned reward in mice [S1]. | LV-miR-181a expression caused no significant changes in the MOR1, whereas silencing of miR-181a resulted in a significant reduction (~ 34%) in MOR1 protein levels. | | Let-7d directly targets MOR1 and our results show that LV-let-7d expression results in a ~5-fold decrease in the MOR1 protein levels, whereas silencing of let-7d results in a significant increase (~ 35%) in MOR1 protein levels. | The results suggests that let-7d directly inhibits MOR1, resulting in attenuation to cocaine and opioid tolerance and reward, whereas miR-181a acts in an opposing manner enhancing MOR1 levels and behavioral sensitization to cocaine. |
| **DAD3R** | Dopamine D3 receptors are upregulated in the NAc of human cocaine abusers [S2-S4], and in rats after cocaine self-administration [S5] or cocaine cue-induced hyperlocomotion [S6]. D3-selective antagonists inhibit cocaine-primed reinstatement [S7], suggesting that the D3 receptor mediates the incentive motivational effects of cocaine. | | | LV-let-7d expression results in a significant reduction in the mRNA levels and a ~4 fold decrease in DAD3R protein levels. | Let-7d mediated regulation of D3 receptors may be functionally related to changes in propensity for cocaine-seeking behavior. |

| | | | | | |
|---|---|---|---|---|---|
| **DAD2R** | Reduction in DAD2R expression is linked to higher risk of developing addictive behaviors [S8-S10]. | LV- mediated miR-181a regulation causes downregulation of DAD2R | | | Decreased DAD2R levels and related neural adaptations may contribute to the development of addictive behaviors. |
| **ΔFosB** | Splice variant of FosB, accumulates with repeated exposure to cocaine and overexpression of ΔFosB in the NAc increases the rewarding effects of cocaine [S11] by differentially regulating trancription of target genes specified in drug-induced behaviors [S12-S15]. | | Strongly induced upon miR-124 silencing | Strongly induced upon let-7d silencing | The induction of ΔFosB after miR-124 and let-7d silencing in NAc might be one of the key molecular mechanisms by which greater salience to cocaine CPP is achieved |
| **DAT** | Cocaine in the brain mainly binds to the dpopamine transporter (DAT and the serotonine transporter (SERT) (S16). Overexpression of DAT in the NAc, affects cocaine-induced behavior by modifying synaptic levels of dopamine [S17]. | Significant ~4 fold decrease in DAT protein levels after miR-181a expression in the NAc | expression of miR-124 results in significantly increased DAT protein levels | expression of let-7d results in significantly increased DAT protein levels | DAT is not a direct target of the miRNAs used in this study. DAT induction by miR-124 and let-7d or its decrease by miR-181a might be a major mechanism through which these miRNA affect behavior upon cocaine administration. |

| | | | | | |
|---|---|---|---|---|---|
| **UPA** | Urokinase plasminogen activator (UPA) is an extracellular serine protease and plays a role in extracellular matrix degradation and dendritic spine dynamics and neurite extension during synaptic plasticity [S18-S19]. UPA plays a major role in cocaine-mediated plasticity and associated behavioral changes [S20]. | | Induction of UPA expression after miR-124 expression | | Activation of UPA expression after miR-124 suggests that miR-124 regulation involves activation of extracellular matrix remodeling. |
| **EphB1** | EphB1 is induced after cocaine exposure in the nigrostriatal and mesolimbic pathways [S21-S22] and is known to inhibit growth of neurites [S21]. | | LV-miR-124 expression resulted in a decreased EphB1 protein levels | | miR-124 regulates neurite outgrowth via EphB1 regulation |
| **FosB** | Transcriptional regulation by fosB gene products plays a critical role in cocaine-induced behavioral responses [S23]. | | downregulation of FosB protein after miR-124 silencing | upregulation after let-7d silencing | Although both miRNAs attenuates cocaine CPP, their mechanisms might differ |
| **mPer2** | mPer2 mutant mice exhibited a hypersensitized response to cocaine and a strong cocaine-induced place preference, suggesting mPer2 suppression is essential for establishment of cocaine plasticity [S24]. | LV-miR-181a expression suppresses mPer2 mRNA levels and silencing of miR-181a leads to increased mPer2 levels | | | miR-181a suppression of mPer2 is essential for establishment of cocaine CPP |
| **7Myt1** | 7Myt1 attenuates the cocaine induced locomotor activity in rats and acts as a compensatory mechanism to the | LV-miR-181a expression significantly | LV-miR-124 silencing significantly | LV-let-7d expression significantly | Regulation of 7Myt1 levels might be one of the crucial component in the miRNA |

| | | | | | |
|---|---|---|---|---|---|
| | rewarding effects of cocaine [S25]. | downregulates 7Myt1 | downregulates 7Myt1 | downregulates 7Myt1 | mediated behavioral changes observed |
| **GRIA2** | Following cocaine self-administration in the PFC, ionotropic glutamate receptor subunit 2 (GRIA2) is significantly decreased in the VTA, NAc and HIP [S26]. | | LV-miR-124 silencing significantly downregulates (2 fold) GRIA2 mRNA levels. | LV- mediated let-7d regulation causes downregulation of GRIA2 levels | GRIA2 mRNA levels are unaffected by direct targeting by miR-181a, whereas miR-124 and let-7d mediated action involves decreases in GRIA2 levels |
| **GRM5 (mGluR5)** | Cocaine self-administration followed by home cage exposure reduced the mGluR5 protein in NAc shell and dorsolateral striatum [S27]. | | | | GRM5 mRNA levels remains unchanged after different LV-miRNA regulation in the NAc. |
| **MECP2** | Mecp2 is significantly induced in striatum, Pre-Frontal Ctx, and the Hippocampus after chronic cocaine [S28]. | LV-miR-181a expression strongly induces the MeCP2 mRNA levels whereas silencing miR-181a resulted in strong decrease. | LV-miR-124 silencing significantly downregulates MeCP2 mRNA levels. | LV- mediated let-7d regulation causes downregulation of MeCP2 levels | |
| **CREB** | Nuclear activation of CREB is an vital in converting short-term into long-term plasticity associated with learning and addiction and regulates various plasticity genes to mediate incentive salience and drug reward. | | LV-miR-124 silencing significantly induces CREB protein levels. | LV-let-7d silencing strongly induces the phosphorylated CREB protein levels | Suppression of miR-124 and let-7d might be crucial for the induction of CREB and pCREB after cocaine treatment for achieving the associated behavioral changes |

| | | | | | |
|---|---|---|---|---|---|
| | [S29, S30]. | | | | |
| **BDNF** | BDNF in dopamine terminal regions like NAc is induced markedly following cocaine exposure is essential for the cocaine induced behavioral changes [S31, S32]. | | LV-miR-124 silencing significantly downregulated BDNF mRNA levels. | LV- mediated let-7d regulation causes downregulation of BDNF levels | Although we have shown that BDNF a direct target of miR-124 is downregulated *in vitro*, intriguingly we found that silencing of miR-124 in vivo resulted in downregulation of BDNF. The exact mechanism behind this has to be determined in future studies. |

**References:**


S1. Hummel M, Schroeder J, Liu-Chen LY, Cowan A, Unterwald EM (2006) An antisense oligodeoxynucleotide to the mu opioid receptor attenuates cocaine-induced behavioral sensitization and reward in mice. Neuroscience.;142(2):481-91.

S2. Mash DC (1997) D3 receptor binding in human brain during cocaine overdose. Mol Psychiatry.;2(1):5-6.

S3. Segal DM, Moraes CT, Mash DC (1997) Up-regulation of D3 dopamine receptor mRNA in the nucleus accumbens of human cocaine fatalities. Brain Res Mol Brain Res.;45(2):335-9.

S4. Staley JK, Mash DC (1996) Adaptive increase in D3 dopamine receptors in the brain reward circuits of human cocaine fatalities. J Neurosci.;16(19):6100-6.

S5. Neisewander JL, Fuchs RA, Tran-Nguyen LT, Weber SM, Coffey GP, Joyce JN (2004) Increases in dopamine D3 receptor binding in rats receiving a cocaine challenge at various time points after cocaine self-administration: implications for cocaine-seeking behavior. Neuropsychopharmacology.; 29(8):1479-87.

S6. Le Foll B, Francès H, Diaz J, Schwartz JC, Sokoloff P (2002) Role of the dopamine D3 receptor in reactivity to cocaine-associated cues in mice. Eur J Neurosci.;15(12):2016-26.

S7. Vorel SR, Ashby CR Jr, Paul M, Liu X, Hayes R, Hagan JJ, Middlemiss DN, Stemp G, Gardner EL (2002) Dopamine D3 receptor antagonism inhibits cocaine-seeking and cocaine-enhanced brain reward in rats. J Neurosci.;22(21):9595-603.

S8. Klein TA, Neumann J, Reuter M, Hennig J, von Cramon DY, Ullsperger M. (2007) Genetically determined differences in learning from errors. Science;318(5856):1642-5.

S9. Noble EP (2003) D2 dopamine receptor gene in psychiatric and neurologic disorders and its phenotypes. Am J Med Genet B Neuropsychiatr Genet.;116B(1):103-25.

S10. Caine SB, Negus SS, Mello NK, Patel S, Bristow L, Kulagowski J, Vallone D, Saiardi A, Borrelli E. (2002) Role of dopamine D2-like receptors in cocaine self-administration: studies with D2 receptor mutant mice and novel

D2 receptor antagonists. J Neurosci ;22(7):2977-88.

S11. Nestler EJ (2008) Review. Transcriptional mechanisms of addiction: role of DeltaFosB. Philos Trans R Soc Lond B Biol Sci.;363(1507):3245-55.

S12. Kumar A, Choi KH, Renthal W, Tsankova NM, Theobald DE, Truong HT, Russo SJ, Laplant Q, Sasaki TS, Whistler KN, Neve RL, Self DW, Nestler EJ. (2005) Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. Neuron;48(2):303-14.

S13. Levine AA, Guan Z, Barco A, Xu S, Kandel ER, Schwartz JH. (2005) CREB-binding protein controls response to cocaine by acetylating histones at the fosB promoter in the mouse striatum. Proc Natl Acad Sci U S A;102(52):19186-91.

S14. Renthal W, Maze I, Krishnan V, Covington HE, Xiao G, Kumar A, Russo SJ, Graham A, Tsankova N, Kippin TE, Kerstetter KA, Neve RL, Haggarty SJ, McKinsey TA, Bassel-Duby R, Olson EN, Nestler EJ. (2007) Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. Neuron; 56(3):517-29.

S15. Renthal W, Nestler EJ (2008) Epigenetic mechanisms in drug addiction. Trends Mol Med.;14(8):341-50.

S16. Bannon MJ, Pruetz B, Manning-Bog AB, Whitty CJ, Michelhaugh SK, Sacchetti P, Granneman JG, Mash DC, Schmidt CJ (2002) Decreased expression of the transcription factor NURR1 in dopamine neurons of cocaine abusers. Proc Natl Acad Sci U S A.;99(9):6382-5.

S17. Adriani W, Boyer F, Gioiosa L, Macrì S, Dreyer JL, Laviola G (2009) Increased impulsive behavior and risk proneness following lentivirus-mediated dopamine transporter over-expression in rats' nucleus accumbens. Neuroscience;159(1):47-58.

S18. Fiorillo CD, Williams JT, Bonci A (1998) D1-receptor regulation of synaptic potentials in the ventral tegmental area after chronic drug treatment. Adv. Pharmacol. **42**, 1002–1005.

S19. Oray S, Majewska A, Sur M (2004) Dendritic spine dynamics are regulated by monocular deprivation and extracellular matrix degradation. Neuron **44**, 1021–1030.

S20. Bahi A, Boyer F, Kafri T, Dreyer JL (2006) Silencing urokinase in the ventral tegmental area in vivo induces changes in cocaine-induced hyperlocomotion. J Neurochem;98(5):1619-31.

S21. Yue Y, Widmer DA, Halladay AK, Cerretti DP, Wagner GC, Dreyer JL, Zhou R (1999) Specification of distinct dopaminergic neural pathways: roles of the Eph family receptor EphB1 and ligand ephrin-B2. J Neurosci.; 19(6):2090-101

S22. Bahi A, Dreyer JL (2005) Cocaine-induced expression changes of axon guidance molecules in the adult rat brain. Mol Cell Neurosci. ;28(2):275-91.

S23. Hiroi N, Brown JR, Haile CN, Ye H, Greenberg ME, Nestler EJ (1997) FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. Proc Natl Acad Sci U S A.;94(19):10397-402.

S24. Abarca C, Albrecht U, Spanagel R. (2002) Cocaine sensitization and reward are under the influence of circadian genes and rhythm. Proc Natl Acad Sci USA 99(13):9026-30.

S25. Chandrasekar V, Dreyer JL (2010) The brain-specific Neural Zinc Finger transcription factor 2b (NZF-2b/7ZFMyt1) causes suppression of cocaine-induced locomotor activity. Neurobiol Dis.;37(1):86-98.

S26. Hemby SE, Horman B, Tang W (2005) Differential regulation of ionotropic glutamate receptor subunits following cocaine self-administration. Brain Res.;1064(1-2):75-82.

S27. Ghasemzadeh MB, Vasudevan P, Mueller C, Seubert C, Mantsch JR (2009) Neuroadaptations in the cellular and postsynaptic group 1 metabotropic glutamate receptor mGluR5 and Homer proteins following extinction of cocaine self-administration. Neurosci Lett.;452(2):167-71.

S28. Cassel S, Carouge D, Gensburger C, Anglard P, Burgun C, Dietrich JB, Aunis D, Zwiller J (2006) Fluoxetine and cocaine induce the epigenetic factors MeCP2 and MBD1 in adult rat brain. Mol Pharmacol;70(2):487-92.

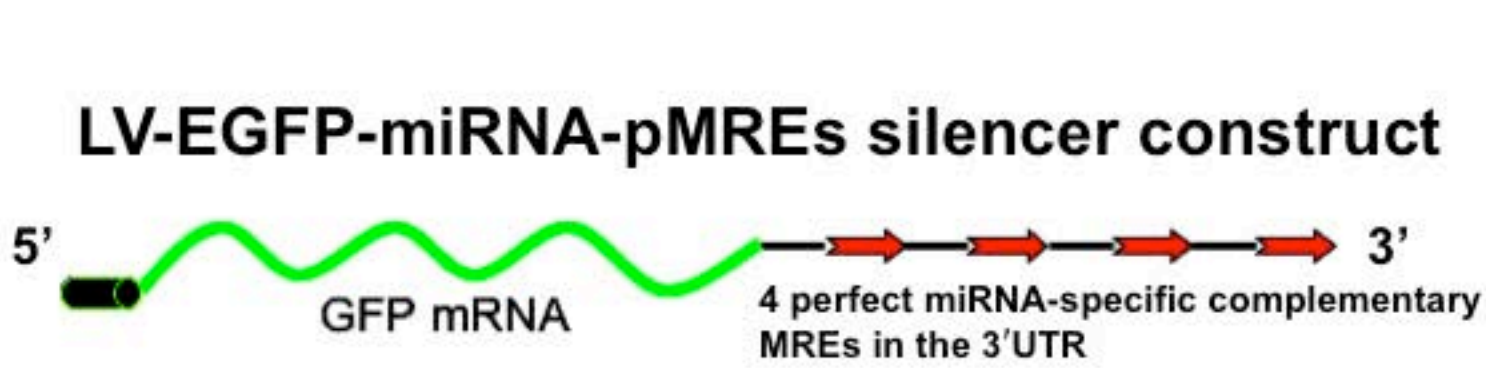
S29. Barco A, Alarcon JM, Kandel ER (2002) Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. Cell.;108(5):689-703.

S30. Carlezon WA Jr, Duman RS, Nestler EJ (2005) The many faces of CREB. Trends Neurosci.;28(8):436-45.

S31. Kalivas PW, O'Brien C. (2008) Drug addiction as a pathology of staged neuroplasticity. Neuropsychopharmacology. 33(1):166-80.

S32. Bahi A, Boyer F, Chandrasekar V, Dreyer JL. (2008) Role of accumbens BDNF and TrkB in cocaine-induced psychomotor sensitization, conditioned-place preference, and reinstatement in rats. Psychopharmacology 199(2):169-82.

A




B

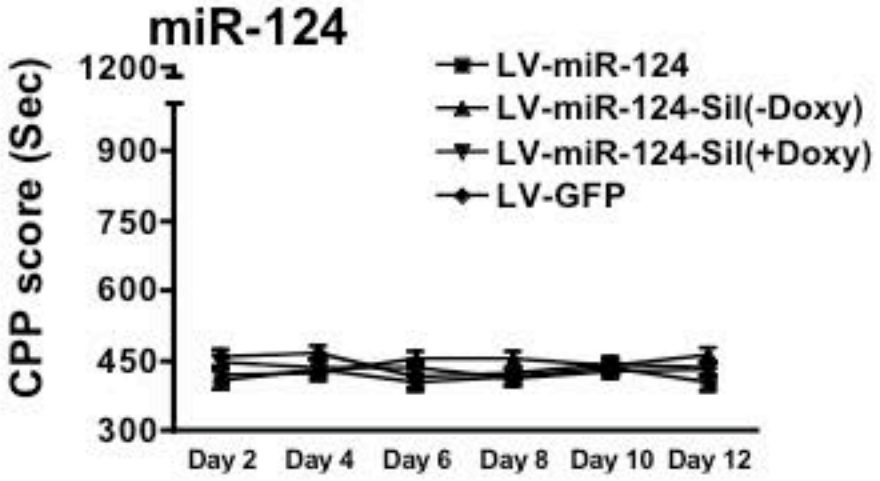
Seed sequence and shared target genes between miR-124 and let-7d predicted by miRanda

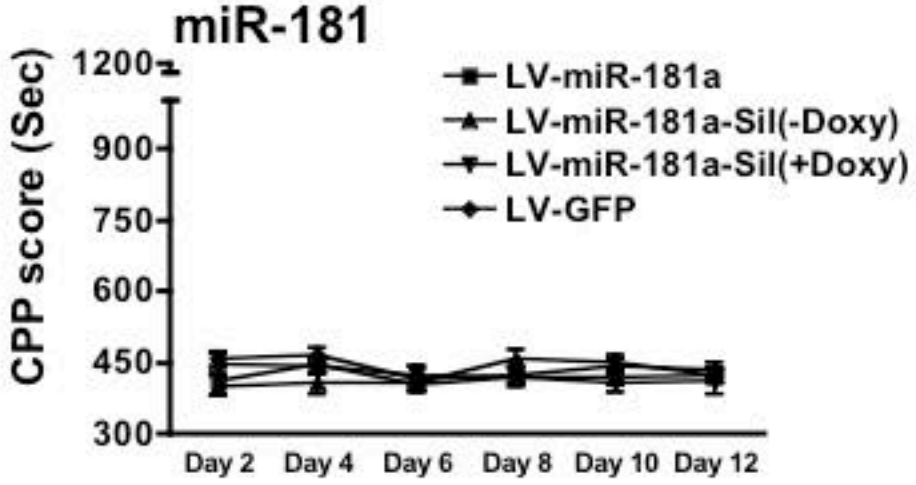
AGAGGUA GUAGGUUGCAUAGUU - let-7d
UAAGGCA CGCGGUGAAUGCC - miR-124

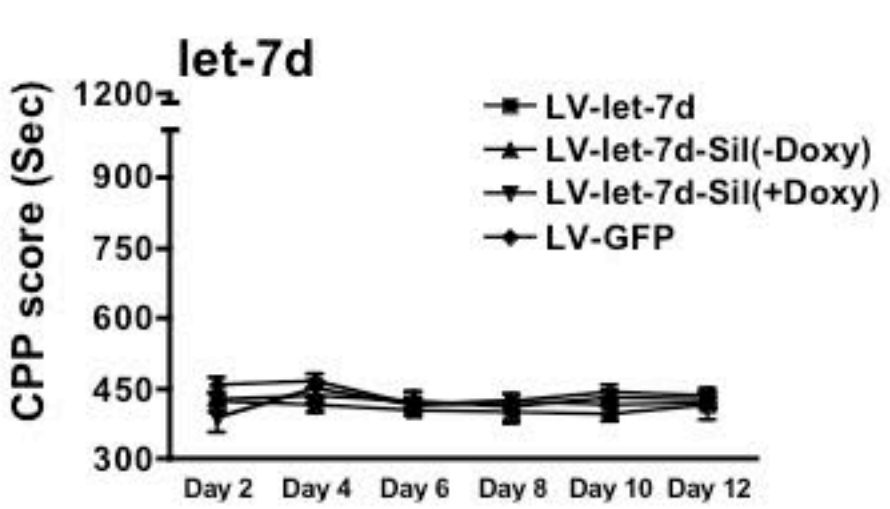

Fig. S1

# Saline Groups

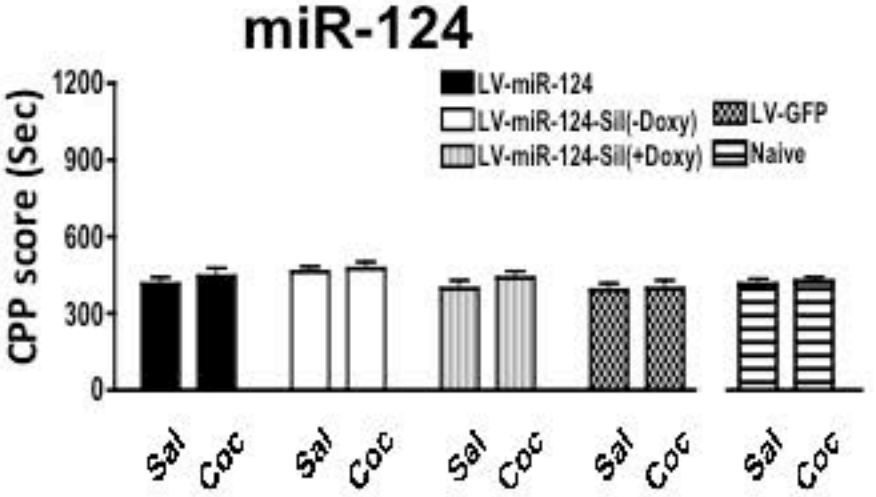
## A CPP extinction

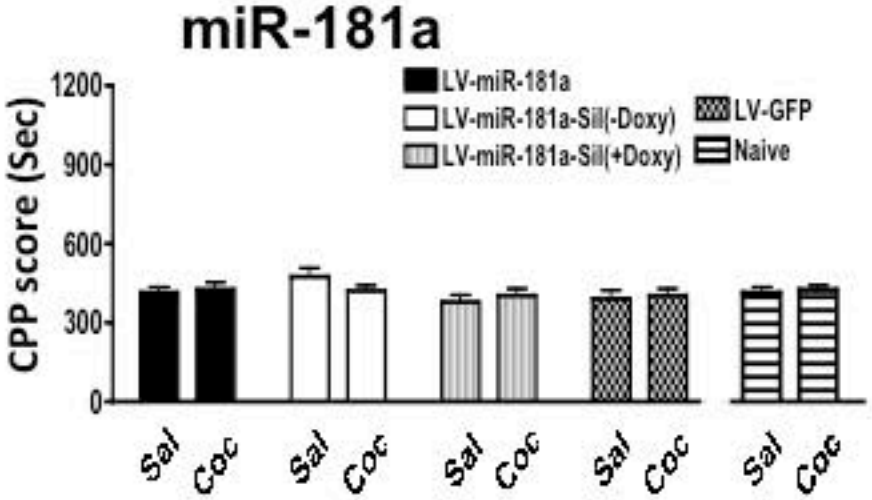


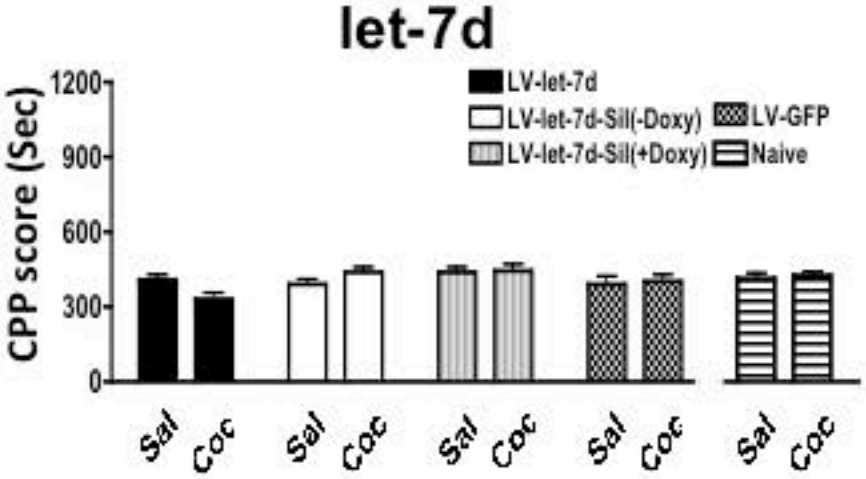




## B Reinstatement/ Priming




## C Effect miRNA-124 regulation on Cocaine CPP Reinstatement-Saline Priming

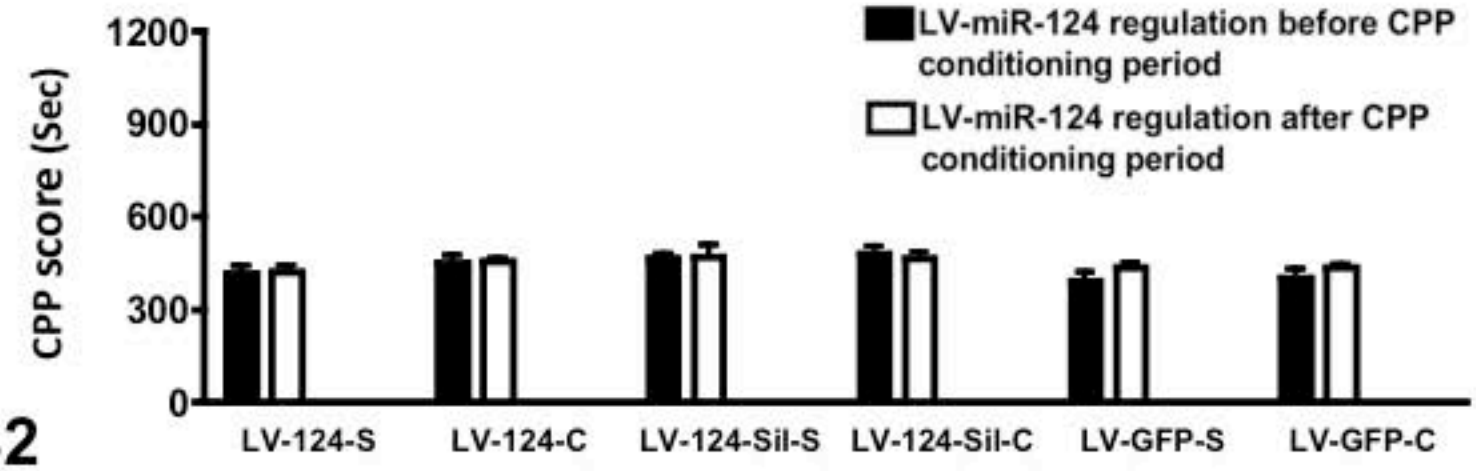



Fig. S2

## Expression of Mature miRNA in Naive Animals

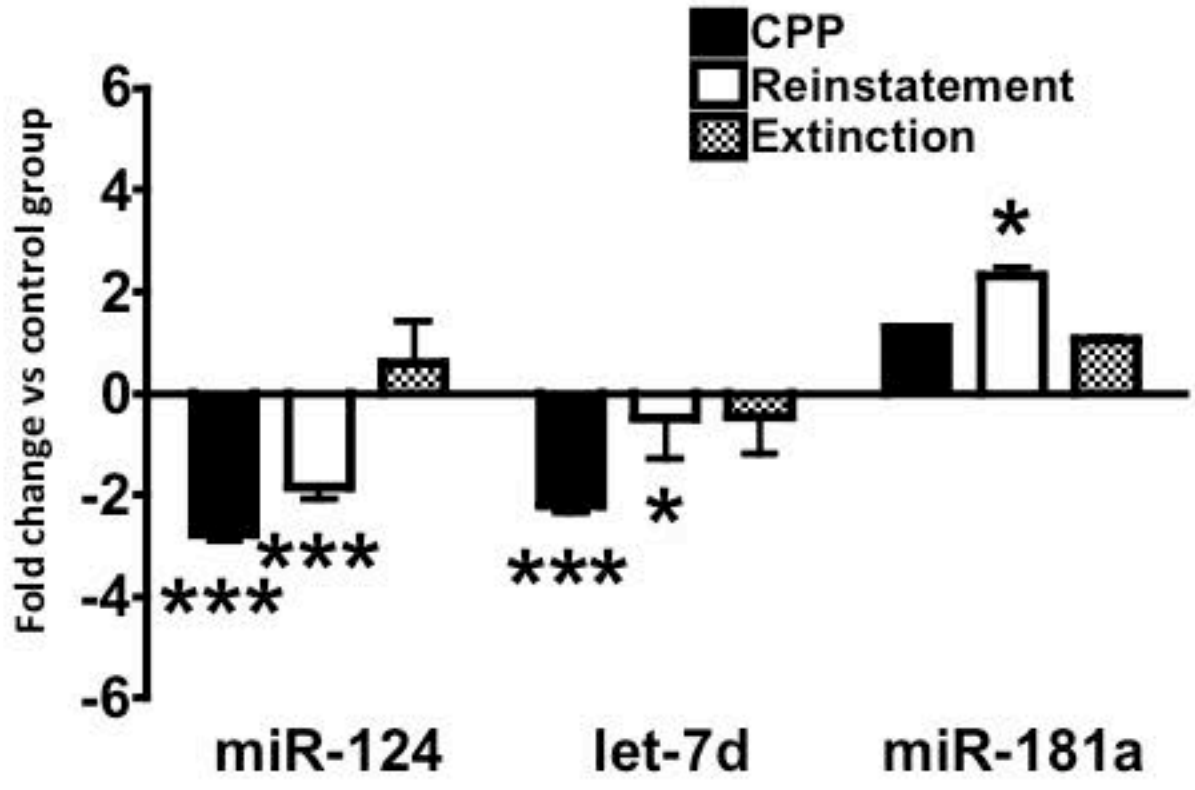



Fig. S3