microRNAs miR-124, let-7d and miR-181a regulate Cocaine-induced Plasticity

Vijay Chandrasekar, Jean-Luc Dreyer *

Division of Biochemistry, Department of Medicine, University of Fribourg, Rue du Musée 5, Chemin du Musée 5, CH-1700 Fribourg, Switzerland

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ABSTRACT

MicroRNAs play key regulatory roles in cellular processes including neurogenesis, synapse development and plasticity in the brain. Psychostimulants induces strong neuroadaptive changes through a surfeit of gene regulatory mechanisms leading to addiction. MicroRNA profiling for identifying miRNAs regulating cocaine-induced, plasticity-related genes revealed significant regulation of a set of miRNAs upon cocaine administration, especially let-7d, miR-181a and the brain-specific miR-124. These miRNAs target many genes involved in cocaine addiction. Precursor and mature miRNA quantification by qRT-PCR showed that miR-124 and let-7d are significantly downregulated, whereas miR-181a is induced in the mesolimbic dopaminergic system under chronic cocaine administration. Results were confirmed by *in situ* hybridization, Northern blots, FISH analysis and RNase protection assay. Using lentiviral-mediated miRNA expression, we show a significant downregulation of BDNF and D3R both at mRNA and protein levels by miR-124 and let-7d, respectively. Our data suggest that miR-124, let-7d and miR-181a may be involved in a complex feedback loop with cocaine-responsive plasticity genes, highlighting the possibility that some miRNAs are key regulators of the reward circuit and may be implicated in addiction.

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Introduction

Addictive drugs (cocaine and amphetamines), depressants (ethanol) and opiate narcotics (heroin and morphine) are very powerful reinforcers and produce their rewarding effects of euphoria or pleasure through an interaction with the mesolimbic dopaminergic system (Nestler, 1997, Koob et al., 1998, Woolverton and Johnson, 1992). Important neuroplastic changes occur in the reward- and memory-related brain centers following drug action. Exposure to cocaine, both acute and chronic, blocks the dopamine transporter, preventing dopamine re-uptake and alters gene expression in midbrain dopaminergic neurons (Nestler, 2001), however the precise molecular machinery involved in this chronically relapsing disorder remains largely unclear.

Recent studies have demonstrated that post-transcriptional regulation of gene expression plays a key role in neurogenesis, synaptic plasticity and in diseases associated with the CNS. MicroRNAs, a class of small non-coding transcripts are involved in coordinating the fine-tuning of gene expression during differentiation and development of the brain (Kosik, 2006). The target mRNAs are either translationally suppressed or subjected to transcriptome degradation by miRNA based on the imperfect or perfect complementarity in the 3′ UTR of the mRNA (Kosik, 2006). The miRNAs in mature neurons display a high degree of diversity and current estimates speak of about 100 miRNAs in postmitotic neurons (Fiore and Schratt, 2007a). Enriched expression of certain miRNAs in the brain suggests that they could be involved in long-term potentiation and in regulating structural and functional aspects of synaptic plasticity, like neuronal morphogenesis and activity dependent translation during synapse formation, memory and addiction (Kosik, 2006; Fiore and Schratt, 2007b; Schratt et al., 2006; Ashraf et al., 2006; Huang and Li, 2008; Pietrzykowski et al., 2008). miRNAs in the vertebrate system predominantly inhibit productive translation of mRNAs (Schratt et al., 2006), a function that could be used to keep dendritic mRNAs dormant during transport and storage near synapses (Ashraf et al., 2006). This is further supported by recent studies demonstrating that aberrant expression of few miRNAs may cause synaptic dysfunction, leading to the etiology of different neurological disorders (Beveridge et al., 2008; Wang et al., 2008a,b; Nelson et al., 2006; Johnson et al., 2008; Niwa et al., 2008). Moreover a role of miRNA in feedback circuit has been demonstrated in midbrain dopaminergic neurons (Kim et al., 2007).

We have previously shown that cocaine treatment induces changes in expression of axon guidance molecules (Bahi and Dreyer, 2005), which may contribute to cognitive deficits associated with drug abuse. Interestingly, a number of axon guidance molecules implicated in cocaine addiction are regulated by miRNAs (John et al., 2004). Therefore expression analysis aimed at identifying miRNAs involved in cocaine-induced alteration in gene expression may provide cues to a better understanding about this reward circuit. We show in this study that miR-124 and let-7d are significantly downregulated, whereas miR-181a is strongly up-regulated after cocaine treatment. Our observation provides insight into a possible miRNA-mediated feedback loop involving miRNAs, transcription factors and other genes involved in signal transduction in cocaine-induced plasticity.

* Corresponding author. Fax: +41 26 300 9735.
E-mail address: jean-luc.dreyer@unifr.ch (J.-L. Dreyer).

Results

Computational prediction of cocaine-specific miRNAs

Screening for miRNA targeting cocaine-responsive genes was performed on the basis of lists of genes modulated upon chronic cocaine (Brenz Verca et al., 2003). For this purpose, miRNA target prediction softwares were used (http://www.microrna.org) (Betel et al., 2008). The results were later compared with other open access softwares (Griffiths-Jones et al., 2006). This comparison allowed us to narrow down the miRNAs candidates possibly involved in cocaine-induced altered gene expression, based on stringent conservation constraints, vertical (cross species) and horizontal comparisons (genes involved in same pathway). This methodology avoids over-prediction leading to false-positives and reveals a list of putative targeting miRNAs highly conserved over species and of high binding energy. From these data, we found that miR-124 (a brain enriched microRNA) and let-7d (a ubiquitous miRNA) were targeting a large number of classical cocaine up-regulated genes (Table S1, supplementary material). miR-124 has predicted target sites of high binding energy in BDNF, CREB, SEMA6A, NAC1, and neurophilin2 (Fig. 1), genes which are well established to be involved in cocaine addiction (Nestler, 2001; Bahi and Dreyer, 2005; Kalivas and Volkow, 2005). The let-7d miRNA displays overlapping with miR-124, and exhibits target sites of high binding energy in both SEMA6A and neurophilin2, as well unique sites in SEMA4C and μ_1-opioid receptor (Fig. 1). We also found that miR-181a targets at least four cocaine down-regulated genes (Yuferov et al., 2003; Toda et al., 2002) with a predicted high binding energy for phosphatidylinositol-4-kinase type-2, regulator-of-G-protein signaling-4, as well as the metabotropic glutamate receptor-5 and the ionotropic AMPA-2 glutamate receptor.

Cocaine causes altered expression of miRNA precursors

To determine whether the hypothetical differential regulation of miRNAs occurs at the precursor RNA transcription level,

Genes and predicted miRNA Targeting Site

(A)

gene	miRNA-124 Targeting site	PBE
BDNF	3' cCGUAAGUGGCGCACGGAAU 5' hsa-miR-124 II I III IIIII:III 1387:5' uGCUUGGACCUCGUGCUUUA 3' BDNF	-19.23
CREB	3' ccGUAAGUGGCGCACGGAAu 5' hsa-miR-124 IIIIII:: :IIII:II 138:5' uuCAUUCAUU-UGUGCUUUu 3' CREB1	-19.26
SEMA6A	3' ccGUA-AGUGGCGCACGGAAU 5' hsa-miR-124 III I : :IIIIIIII 1286:5' caCAUGUAAAUUUGUGCCUUA 3' SEMA6A	-16.68
SEMA6A	3' ccGUAAGUGG---CGCACGGAAu 5' hsa-miR-124 III III I:IIIIIII 2789:5' ucCAUAAACCCGAGUGUGCCUUu 3' SEMA6A	-19.96
MAP2K4	3' CCGUAAGUGGCGCACGGAAU 5' hsa-miR-124 I:I I :I :IIIIIIII: 989 :5' GCUAGUAGCAAUGUGCCUUG 3' MAP2K4	-16.48
NAC1	3' ccguaaguggCGCACGGAAU 5' hsa-miR-124 :IIIIIIII 2285:5' aaagacagggUUGUGCCUUA 3' BTBD14B	-15.09
RCOR1	3' ccguaaagUGGCGCACGGAAu 5' hsa-miR-124 I::I IIIIIII 837:5' auuuggaAUUG-GUGCCUUc 3' RCOR1	-15.1
NRP2	3' cCGUAAGUGGCGCACGGAAU 5' hsa-miR-124 I: II:I:: IIIIIII 1417:5' uGUGUUUUAUUAAGUGCCUUG 3' NRP2	-16.29
NRP2	3' ccGUAAGUG---GCGCACGGAAu 5' hsa-miR-124 :IIIIII: :I:III:III 1963:5' uuUAUUCAUGUGUGUGUGUCUUu 3' NRP2	-19.62
Integrin β1	3' ccguaAGUGGCGCACGGAAu 5' hsa-miR-124 IIII :IIIIIII 259:5' uaagguCACAUUGUGCCUUu 3' ITGB1	
Integrin β1	3' ccguaaguggcgCACGGAAu 5' hsa-miR-124 IIIIIII 1118:5' uucuuguuuuaaGUGCCUUu 3' ITGB1	
GR	3' ccguaaguggcGCACGGAAu 5' hsa-miR-124 :IIIIIII 479:5' aaaagggaaguUGUGCCUUu 3' NR3C1	

(B)

gene	Let-7d Targeting site	PBE
Opioid receptor	3' uuGAUACGUUGGAUGAUGGAGa 5' hsa-let-7d I:IIII I:- :IIIIIII 186:5' cuCGGUGCUCCUGGCUACCUCg 3' OPRM1	-21.2
DAD3R	3' uuGAUACGUUGGAUGAUGGAGa 5' hsa-let-7d II I I III: IIIIIIII 158:5' AAACA-G-AAUAUUCUACCUCc 3' DRD3	
SEMA4C	3' uUGAUACGUUGGAUGAUGGAGa 5' mmu-let-7d II I II III :IIIIIII 62:5' cAC-AGGC-ACCAGCTACCTCa 3' Sema4c	
SEMA6A	3' uUGAUACGUUGGAUGAUGGAGa 5' hsa-let-7d I:I I I: :I III:IIII 61:5' AGCAAGGUUCUCAACUGCCUCg 3' SEMA6A	-16.39
SEMA6A	3' uUGAUACGUUGGAUGAUGGAGa 5' hsa-let-7d I IIIII I III:I:III: 1200:5' uAAUAUGCUGGCUGCUACCUUg 3' SEMA6A	-21.09
MAP2K4	3' UUGAUACGUUGGA-UGAUGGAGA 5' hsa-let-7d I:II I:IIII I III:I:III 2309:5' AGCUCAGUAACAUAACUGCUUCU 3' MAP2K4	-18.43
NRP2	3' uUGAUACGUUGGAUGAUGGAGa 5' hsa-let-7d III: ::II I:IIIIII 2720:5' uACUGAAUGACACAUUACCUCc 3' NRP2	-17.03
PLAU	3' uugaUACGUUGGAUGAUGGAGA 5' hsa-let-7d II I IIII IIII:IIII 1055:5' gagaAUUCUACCGACUAUCUCU 3' PLAU	-17.11

(C)

gene	miRNA-184a Targeting site	pBE
Per2	3' uGAGUGGCUGUCGCAACUUACAA 5' mmu-miR-181a III :I I : IIIIIIIII 2962:5' uCUC-UCUUCUCUCUUGAAUGUU 3' Per2	-12.98
PI4K2B	3' uGAGUGGCUGUCGCAACUUACaa 5' hsa-miR-181a :III: III I I IIIII 1361:5' cUUCAUGGAC-GAGAUGAAUGag 3' PI4K2B	-16.99
PI4K2B	3' ugaguggcugucgcAACUUACAa 5' hsa-miR-181a IIIIIIII 115:5' uaaaagguuguauuUUGAAUGUa 3' PI4K2B	-7.842
RGS4	3' ugAGUGGCUGUCG-AACUUACAA 5' hsa-miR-181a I :I: ::I:I IIII:IIII 160:5' uaUAGCUUUUGGUGUUUGAGUGUU 3' RGS4	-15.01
GRIA2	3' UGAGUGGCUGU-CGC-AACUUACAa 5' hsa-miR-181a I III I:I:II I I IIIIIII 124:5' AGUC-CUGGCAUGGGAAUGAAUGUc 3' GRIA2	-17.32
GRM5	3' UGAGUGGCUGUCGCA--ACUUACAa 5' hsa-miR-181a I:I:II II :IIII IIIIIII 553:5' AUUUACGGA-GGCGUUGUGAAUGUg 3' GRM5	-21.27
GRM5	3' UGAGUGGCUGUCGCAACUUACAa 5' hsa-miR-181a I:IIIII I I IIIIIII 416:5' AUUCACC-A-AAC-AUGAAUGUa 3' GRM5	-16.07

Fig. 1. Predicted binding site of microRNA and the targeted genes by miRanda algorithm. (A) Computational miRNA target prediction for miR-124. (B) Computational miRNA target prediction for let-7d. (C) Computational target prediction for miR-181a. The figures show the putative miRNA-targeting site with the predicted high binding energy for each miRNA-mRNA interaction.
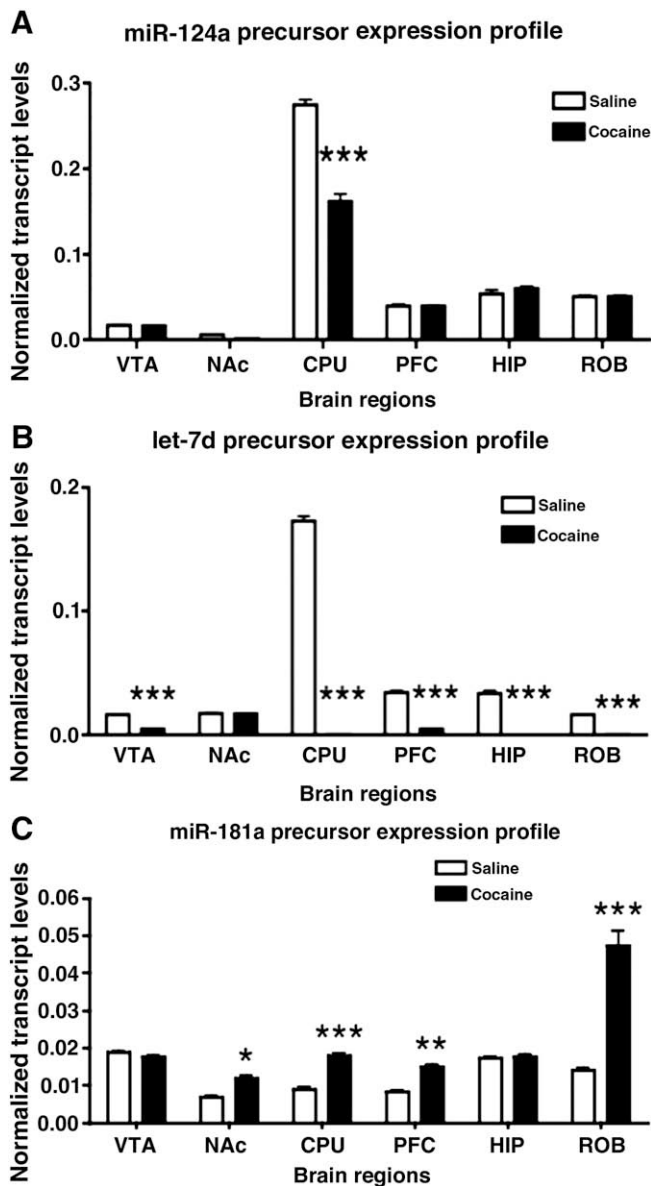
Fig. 2. Cocaine induces differential expression of miRNA precursors in different brain regions. (A) Quantification of miR-181a precursor by qRT-PCR; (B) Quantification of let-7d precursor by qRT-PCR. (C) Quantification of miR-181a precursor by quantitative RT-PCR. See Experimental methods for details. Expression levels were calculated relative to the small RNA, U6. *$P<0.05$; **$P<0.01$; ***$P<0.001$ by two-way ANOVA, Bonferroni *post hoc* tests. Normalization was done with saline-treated samples.

tegmental area (VTA), in the (CPU), in the hippocampus (HIP), in the prefrontal cortex (PFC) regions and in the rest of the brain (ROB) ($F_{(5,24)}=985.77$, $P<0.0001$), compared to the saline-treated control group. Transcript levels of precursor miR-181a were significantly elevated (Fig. 2C; $F_{(1,24)}=171.66$, $P<0.0001$) following chronic cocaine treatment in the NAc, CPU, HIP and ROB regions ($F_{(5,24)}=56.22$, $P<0.0001$), compared to the saline treatment. This clearly correlates with our initial hypothesis that cocaine modulates the expression levels of both the targeting miRNA and the targeted mRNA by a mutually dependent mechanism.

Effects of cocaine on the expression of mature miRNAs

In order to check whether the differential regulation of miRNA precursor transcripts seen after chronic cocaine administration matches with mature miRNA levels, the Taqman qRT-PCR (ABI) was
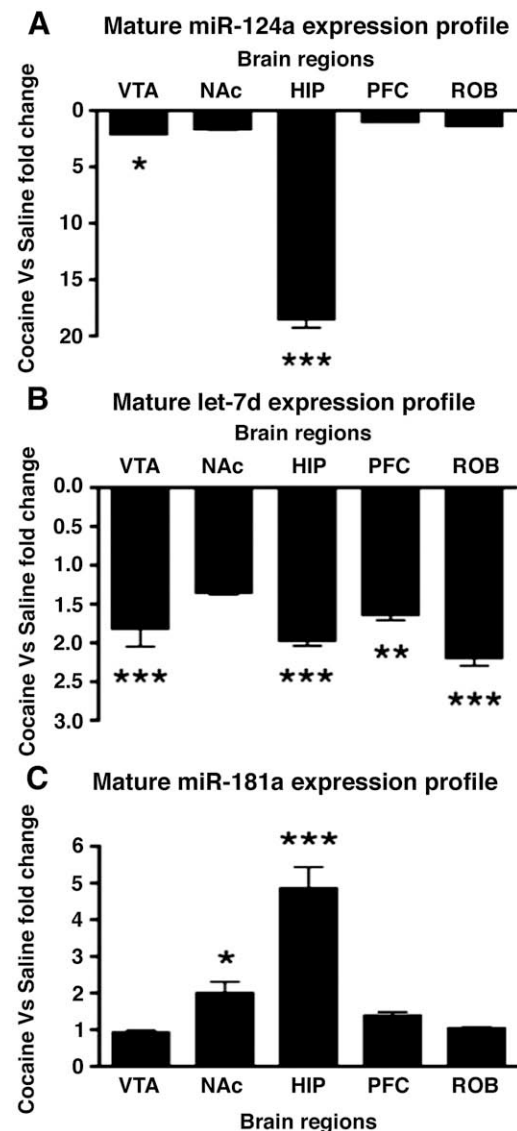

Fig. 3. Cocaine alters the expression profile of mature miRNAs in different brain regions. (A) Quantification of mature miR-124 by Taqman qRT-PCR; (B) Quantification of mature let-7d by Taqman quantitative RT-PCR; (C) Quantification of mature miR-181a by Taqman qRT-PCR. See Experimental methods for details. Expression levels were calculated relative to the small RNA, U6. For quantification of the miRNA in the NAc region normalization was done with the house-keeping gene GAPDH. *$P<0.05$; **$P<0.01$; ***$P<0.001$ by two-way ANOVA, Bonferroni *post hoc* tests. Normalization was done with saline-treated samples.

quantification by means of qRT-PCR of the selected miRNAs was performed (Schmittgen et al., 2004; Jiang et al., 2005), after chronic cocaine administration in different micro-dissected brain regions. Two groups of animals were used for the purpose (n = 6): a cocaine-treated group was injected daily with 15 mg/kg of cocaine ip. over a period of 15 days, whereas a saline group (n = 6) was injected daily with 0.9% saline. After sacrifice brains were micro-dissected and pooled for RNA isolation. Primers specific to the pre-miR-124-2, pre-miR-181a-2 and pre-let-7d precursor transcripts were normalized against the U6 snRNA. The brain-specific miR-124 (Fig. 2A; $F_{(1,24)}=77.05$, $P<0.0001$) and let-7d (Fig. 2B; $F_{(1,24)}=2772.49$, $P<0.0001$) showed a significant down-regulation. We observed a significant down-regulation of miR-124 in the caudate putamen (CPU) region ($F_{(5,24)}=79.15$, $P<0.0001$) after chronic cocaine treatment, a non-significant down-regulation in the nucleus accumbens (NAc), but no changes in the other brain regions. On the other hand, let-7d showed a drastic downregulation of the precursor molecule levels in the ventral

used to quantify the mature miRNA levels from the same RNA samples used for precursor transcript quantification. The Taqman assay is designed to specifically quantify mature miRNA levels; furthermore it has the advantage that members of the same miRNA family, differing only in 1–2 nucleotides, can be distinguished. A selected set of mature miRNAs were analyzed for differential expression (data not shown). The results showed a significant down-regulation of mature miR-124 (Fig. 3A; $F_{(1,20)} = 628.54$, $P < 0.0001$) in the VTA and a drastic change in the HIP region (>18-fold decrease; $F_{(4,20)} = 474.14$, $P < 0.0001$). Let-7d also showed a significant down-regulation (Fig. 3B; $F_{(1,10)} = 201.93$, $P < 0.0001$) in VTA, HIP, PFC and the ROB regions (Fig. 3B; $F_{(4,10)} = 6.59$, $P = 0.0073$) following cocaine treatment, when compared with the saline treatment. By contrast, mature miRNA levels of miR-181a were significantly elevated after chronic cocaine (Fig. 3C; $F_{(1,20)} = 57.12$, $P < 0.0001$), notably in the NAc and HIP regions ($F_{(4,20)} = 28.24$, $P < 0.0001$), compared to saline control treatment in the same regions. The results demonstrate a great degree of differential regulation of the mature miRNAs after exposure to cocaine.

Validation of the miRNA quantification by mirVANA technique

To validate the findings from the precursor and mature miRNA quantification by qRT-PCR, quantification of miRNA was performed by the mirVana miRNA Detection Kit (Ambion). This method has the advantage of low background, high sensitivity and multiple target detection. Since the method cannot distinguish between precursor and mature miRNA, it served as validation for overall regulation of specific miRNAs upon exposure to cocaine. The same RNA samples from chronic cocaine and from control saline tissues used for qRT-PCR were used for this quantification by mirVANA. The results again showed significant down-regulation of miR-124 (Fig. 4A; $F_{(1,12)} = 116.72$, $P < 0.0001$) and let-7d (Fig. 4B; $F_{(1,12)} = 284.07$, $P < 0.0001$), in agreement with our observations with the precursor and mature miRNA quantification in different regions of the mesolimbic system. miR-124 showed an expected significant down-regulation in the NAc, CPU and HIP, ($F_{(5,12)} = 30.49$, $P < 0.0001$), whereas let-7d was suppressed significantly in the VTA, NAc, HIP, PFC and the ROB regions ($F_{(5,12)} = 6.45$, $P = 0.0039$). The results taken on an average point of mature and precursor miRNA levels correlate very well with the data obtained from qRT-PCR studies on both the precursor and the mature forms of miRNAs, taken together. Again, by contrast, the levels of miR-181a were significantly up-regulated after chronic cocaine treatment (Fig. 4C; $F_{(1,12)} = 110.97$, $P < 0.0001$) in the NAc, CPU, and the HIP regions ($F_{(5,12)} = 26.97$, $P < 0.0001$), which validates the results obtained from precursor and mature miR-181a quantification studies by qRT-PCR.

Northern Blot analysis for miRNA regulation by Cocaine

The results from qRT-PCR were validated by northern blot analysis using total brain samples (data not shown). Results showed that mature forms of both let-7d and miR-124 were significantly down-regulated, about 2.87 and 2.92 fold, respectively, in chronic cocaine-treated samples compared to the saline control. Since the amount of input total RNA needed for efficient detection by this technique was high for small tissues like VTA etc., the technique was not performed in other brain region RNA samples.

REST expression is induced after chronic cocaine administration

REST-mediated regulation of miRNAs plays a role in determining neuronal identity (Conaco et al., 2006; Wu and Xie, 2006; Visvanathan et al., 2007) and the effect of dysregulation of this mechanism in the etiology of neurological disorders has been well established (Johnson et al., 2008). These studies have established that REST and miR-124 act in a feed-back mechanism whereby they determine the neuronal
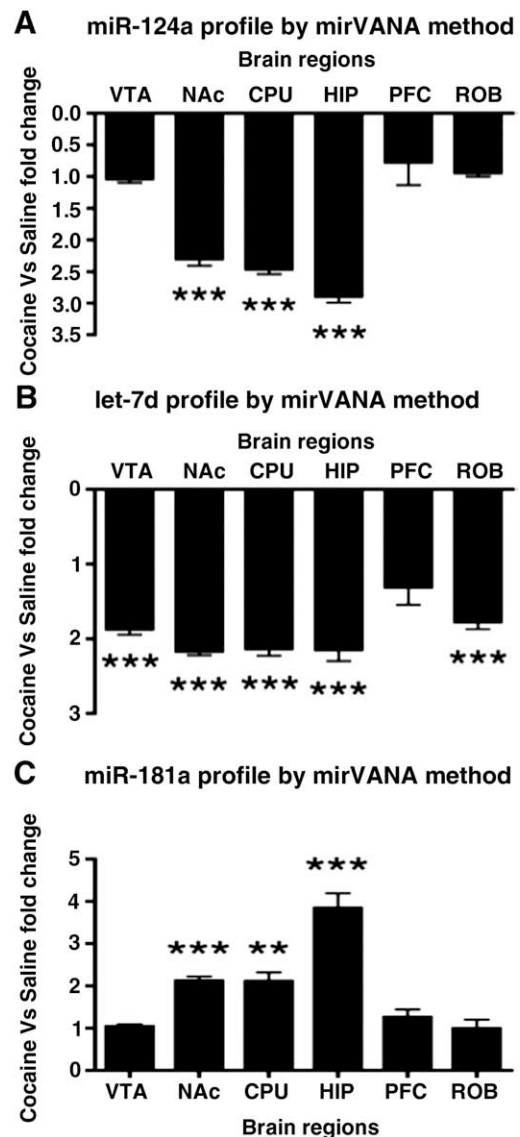

Fig. 4. Validation of qRT-PCR data by means of the mirVANA solution hybridization method. (A) Quantification of miR-124 by mirVANA method; (B) Quantification of let-7d by mirVANA method; (C) Quantification of miR-181a by mirVANA method. See Experimental methods for details. Expression levels were calculated relative to the small RNA, U6 (Ambion mirVANA kit). **$P < 0.01$; ***$P < 0.001$ by two-way ANOVA, Bonferroni post hoc tests. Normalization was done with saline-treated samples.

and non-neuronal cell phenotypes. To check whether the down-regulation of miRNAs observed with cocaine administration (especially miR-124) could be mediated by REST-mediated repression, we quantified REST mRNA levels after chronic cocaine treatment. RNA samples used for miRNA quantification were used for quantifying REST by qRT-PCR in the same brain regions. The results clearly underline the significant induction of REST mRNA after chronic cocaine treatment (Fig. 5; $F_{(1,12)} = 135.62$, $P < 0.0001$), particularly in the NAc and the CPU regions ($F_{(2,12)} = 65.98$, $P < 0.0001$). The data show significant, inversely proportional levels of REST and of miR-124 and let-7d precursor forms, especially in the CPU region, indicating possible regulatory mechanisms between these miRNAs and REST.

In situ hybridization-mediated quantification of miRNA regulation by Cocaine

In situ hybridization of the different miRNAs displayed very different expression profiles within the dopaminergic pathways.
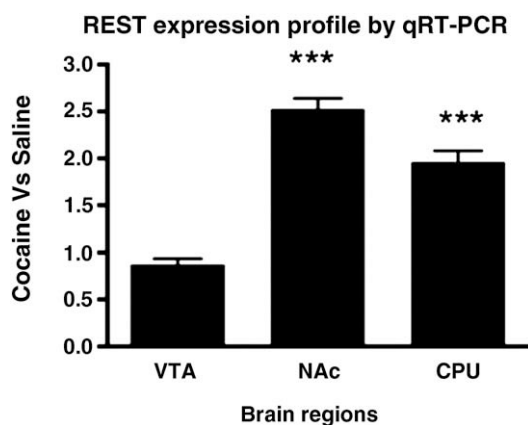
Fig. 5. Cocaine induces REST mRNA after chronic treatment. Expression levels were quantified by qRT-PCR and were calculated relative to the house keeping control genes GAPDH and cyclophilin. **$P<0.01$; ***$P<0.001$ by two-way ANOVA, Bonferroni *post hoc* tests, and normalization was done with saline-treated samples.

Data are shown in Table S2 (supplementary material) and Fig. 6. *In situ* hybridization of miR-124 revealed enriched expression mainly in the neuronal lamina both in superficial (layers I/II) and deeper (layers IV/V). Pyramidal cells and lacunosum molecular layers of the hippocampus were richly stained, but also in the anterior basomedial, the basolateral and the posteromedial cortical nuclei of the amygdala. The ventral postero-lateral part of the thalamus and, in the cortex, the parietal association cortex, S1 and the auditory cortex were also strongly stained. Strong staining was also observed in the ventral pallidum, the lateral olfactory tract and the corpus callosum. These results from *in situ* of miR-124 confirmed our findings that cocaine downregulates miR-124 (Table S2 and Fig. 6A; $F_{(1,84)} = 396.26$, $P<0.0001$) especially in the NAc (both core and shell regions); CPU (striatum); piriform cortex (Pir) and in several regions of the amygdala, particularly the anterior basomedial, the basolateral and the posteromedial cortical nuclei. In addition, hippocampal layers, mainly CA1, CA2, CA3, as well as the hippocampal pyramidal layers, but not the dentate gyrus (DG) display also strong miR-124 down-regulation upon cocaine. Finally strong downregulation of miR-124 is observed in the substantia nigra (SN) ($F_{(20,84)} = 13.52$, $P<0.0001$).

Strong staining for let-7d was observed in reticular and sub-thalamic nuclei, in mammillary nuclei, in the medial posterodorsal nuclei of the amygdala, bed nucleus of the stria terminalis, intra amygdaloid and diverse sub-regions of the amygdala. Striking differences in staining over miR-124 were observed in regions like the anterior commissure (anterior part, aca), the lateral olfactory tract and the ventral pallidum, which were very poorly stained for let-7d, unlike to miR-124 staining. We found let-7d to be very significantly down-regulated after chronic cocaine (Table S2 and Fig. 6B; $F_{(1,64)} = 345.30$, $P<0.0001$), particularly in the NAc (both core and shell regions), the VTA, the piriform cortex, the arcuate hypothalamic nuclei, as well as in CA1, CA3, DG layers of hippocampus (but not in CA2). Furthermore, strong downregulation was observed after cocaine in the SN and the amygdala (especially in the medial posterodorsal nuclei,)($F_{(15,64)} = 13.60$, $P<0.0001$).

In situ hybridization of miR-181a revealed that, unlike the other two miRNAs analyzed, the miR-181a displays strong induction after chronic cocaine treatment; staining for miR-181a was strong in regions like the alveus of the hippocampus, the anterior part of the anterior commissure, the lateral olfactory tract and the ventral pallidum, where overlapping expression pattern with miR-124 could be observed. Prominent staining was also seen in the islands of Calleja. Strong induction of miR-181a expression after chronic cocaine was observed (Table S2 and Fig. 6C; $F_{(1,56)} = 187.09$, $P<0.0001$), particularly in the NAc (both core and shell regions), the CPU (striatum), the VTA, the piriform cortex. Strong induction was

also observed in the CA1 layer of the hippocampus as well as in the DG (but not in the CA2 or CA3 layers!). This was also observed in the amygdala ($F_{(13,56)} = 10.29$, $P<0.0001$), but, in comparison to miR-124
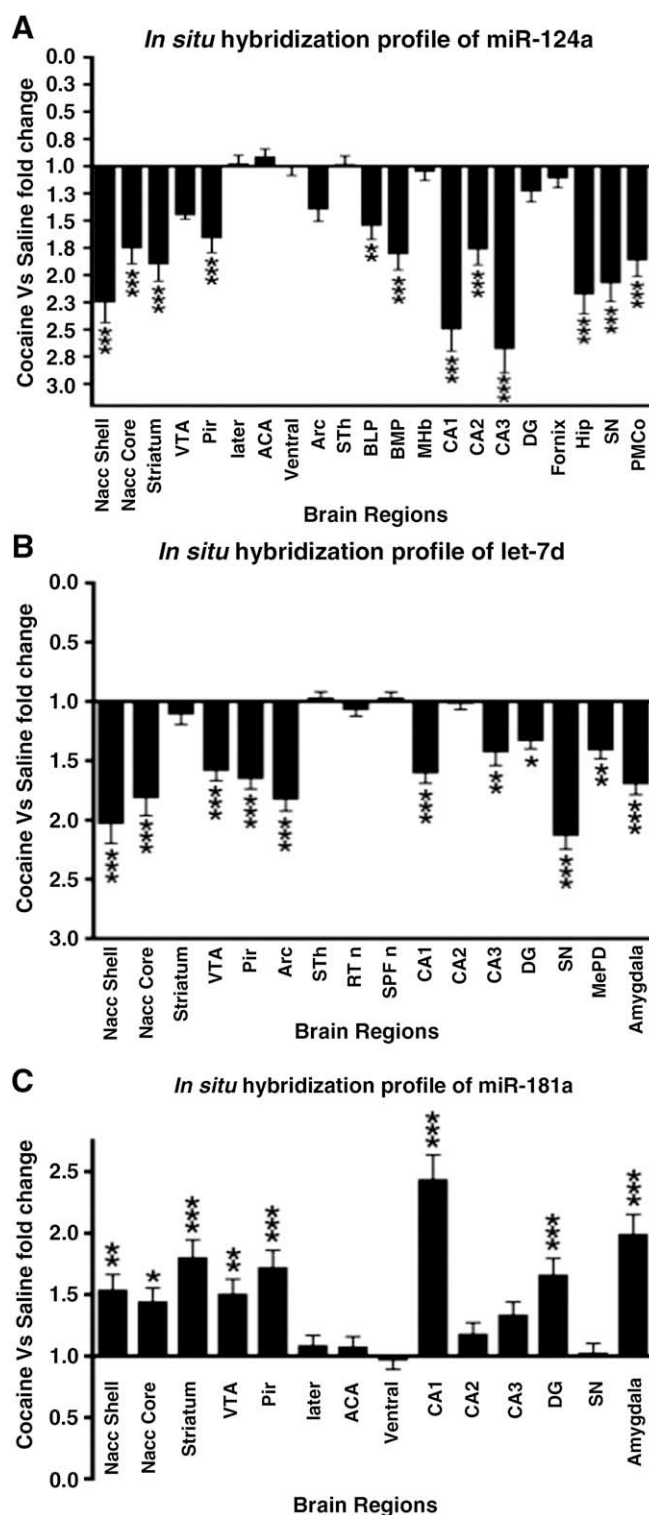

Fig. 6. Validation of differential expression of miRNAs by in situ hybridization. Serial coronal brain sections were hybridized to ^{35}S-labeled antisense RNA probes for miR-124, let-7d, miR-181a (Ambion miRVANA kit). No detectable hybridization signals were obtained with the sense probe. (A) Quantification of miR-124 by in situ hybridization; (B) Quantification of let-7d by in situ hybridization; (C) Quantification of miR-181a by in situ hybridization. See Experimental methods for details. Expression levels were calculated after proper background subtractions. *$P<0.05$; **$P<0.01$; ***$P<0.001$ by two-way ANOVA, Bonferroni *post hoc* tests. Normalization was done with saline-treated samples.

or let-7d, miR-181a staining in the amygdala was poor. These results from *in situ* hybridization correlate with the miRNA quantification studies, and enable detailed resolution for changes observed in different nuclei in the brain.

Validation of miRNA differential regulation after cocaine by FISH

Furthermore, to assess miRNA expression, LNA-based FISH analysis was performed (see Experimental methods). As expected, miR-124 was significantly downregulated in the NAc after cocaine administration (Fig. 7). The expression profile of let-7d showed a significant down-regulation in the NAc and to a lesser extent in the VTA. By contrast, there was an increase in neurons stained for miR-181a after chronic cocaine in both VTA and NAc. Together these data show that the LNA-based FISH method provides validation for the quantification studies performed on different miRNAs after chronic cocaine treatment (Figs. 3, 4, 5, 6, compared to Fig. 7).

Overexpression of miR-124, let-7d and miR-181a modulates the expression pattern of the target genes in vitro

Before proceeding to miRNA overexpression studies to quantify the miRNA-mediated regulation of target genes, the LV-miRNAs were checked by an *in vitro* assay to determine their efficiency by using sensor LV-GFP-pMREs viruses, which express 4 tandem repeats of

perfect MREs specific for the corresponding three miRNAs (see Experimental methods; Fig. S1A, supplementary material). The down-regulation of the specific GFP-sensor mRNA *in vitro* was quantified both at mRNA and protein levels to assess the efficiency of the system. By means of qRT-PCR, GFP mRNA levels were quantified after *in vitro* transfection in HEK-293 cells of the sensor LV-GFP-pMREs virus, either alone or in different concentrations of the corresponding LV-miRNAs (see Experimental methods). Significant and specific down-regulation of GFP-pMREs sensor mRNAs by their corresponding LV-miRNAs was found, that was concentration-dependent (Fig. S1B; $F_{(2,18)} = 838.60, P < 0.0001$). An average reduction in the GFP transcript levels of 49%, 56% and 69%, respectively, was observed upon co-transfection of 1 μl of LV-miR-124, LV-let-7d, and LV-miR-181a (0.2 mg/mL of p24). With co-transfection of 2 μl of the viruses an average reduction of the transcript levels of 79%, 82% and 92% for miR-124, let-7d and miR-181a, respectively, was found, suggesting the effective expression and efficient silencing potential of the LV-miRNAs of the corresponding GFP-pMREs sensors at the mRNA level. Expression changes at protein level of the GFP-pMREs sensors after specific LV-miRNAs were quantified by Western blots from protein extracts of transfected HEK-293 cells. Correlating with observed mRNA down-regulation. Significant down-regulation of GFP-pMREs sensor protein was observed for the corresponding LV-miR-124, LV-let-7d, and LV-miR-181a (Fig. S1C; $F_{(2,18)} = 223.39, P < 0.0001$) with, respectively, ≈35, 40, 50% and ≈70, 70, 78% reduction in the protein level after 1 or 2 μl co-transfection of the LV-miRNA virus. These results

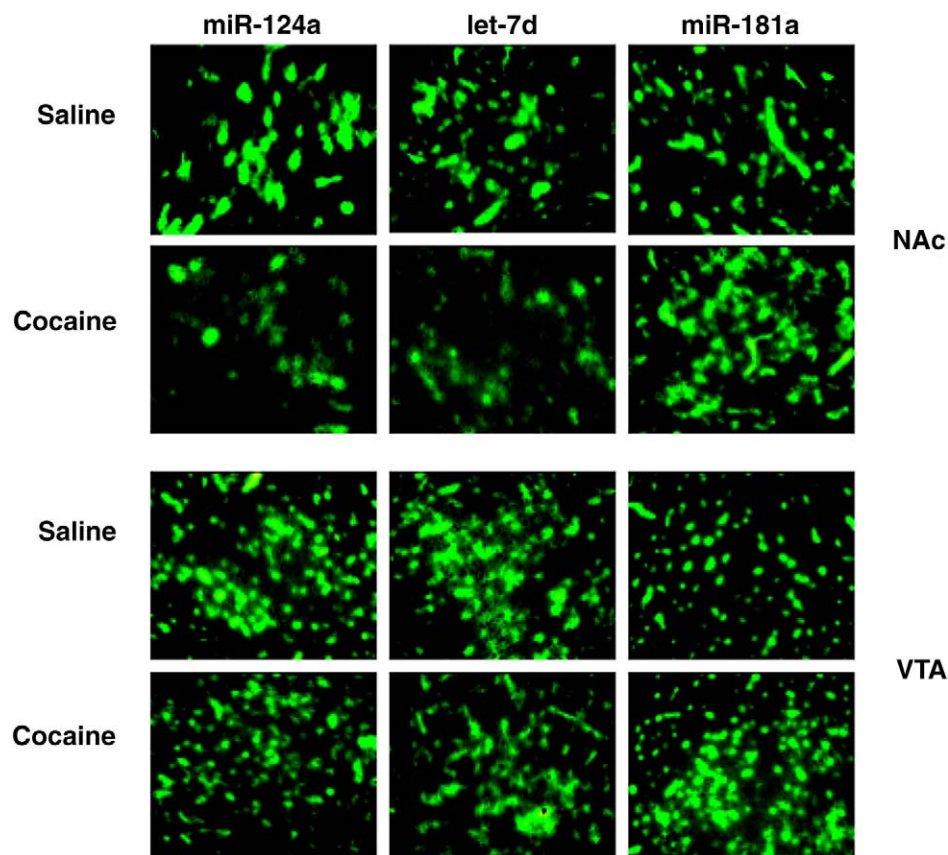**Fig. 7.** Fluorescence in situ hybridization (FISH) based miRNA detection after chronic cocaine administration. Fluorescence in situ hybridization (FISH) based miRNA detection after chronic cocaine administration. Cryosections of rat NAc and VTA regions from chronic saline- (control) and cocaine-treated animals were used for detecting miR-181a, miR-124, and let-7d using LNA probes labeled with DIG. Positive signals were visualized in green by using anti-DIG primary mouse monoclonal antibody, followed by FITC-conjugated secondary antibody. Pictures were taken with a ×40 magnification acquired using Axioplan 2 imaging (Zeiss microscope). miR-181a expression is induced significantly in both NAc and VTA after cocaine administration, whereas miR-124 and let-7d expression is significantly reduced in the NAc.

clearly show viral concentration-dependent expression of GFP-pMREs at protein and mRNA levels, and silencing effects of the corresponding target-specific LV-miRNAs. These data were also corroborated by fluorescence microscopy after co-transfection of the specific LV-miRNAs at different concentration (Fig. S1D).

To validate the relevant biological significance of the miRNA regulation upon cocaine exposure, lentiviral-mediated overexpression of the three chosen miRNAs was performed. LV-miRNA expression assay was performed *in vitro* in NG108-15 cells at different viral concentrations (see Experimental methods) and the levels of the target genes (chosen based on *in silico* predictions; Fig. 1) were analyzed. Since analyzing all the target genes of the miRNAs from the *in silico* data is beyond the scope of this study, we restricted our analysis to few genes well implicated in cocaine addiction and reward. LV-miR-124 overexpression in NG108-15 cells resulted in significant, concentration-dependent down-regulation of BDNF (brain derived neurotropic factor, a target gene of miR-124), in perfect correlation with the target prediction data. BDNF is well implicated in synaptic plasticity and plays a central role in reward and memory (Kalivas and Volkow, 2005; Nestler, 2001). A significant reduction by LV-miR-124 of BDNF mRNA levels of 22, 47, and 49% was found by qRT-PCR (Fig. 8A; $F_{(1,12)} = 265.95$, $P<0.0001$), in a viral dose dependent manner ($F_{(2,12)} = 13.45$,

$P=0.0009$). At the protein level, significant downregulation by LV-miR-124 of BDNF was also observed (Fig. 8A; $F_{(1,24)} = 23.80$, $P<0.0001$) in a viral dose dependent manner with 2 and 3 µl of virus ($F_{(2,24)} = 1.84$, $P=0.1809$). LV-let-7d transfection yielded no significant change in the BDNF mRNA (Fig. 8A) suggesting that regulation of the BDNF gene is specific to miR-124 in this paradigm of cocaine administration.

Overexpression of LV-let-7d in NG 108-15 cells resulted in a significant, concentration-dependent downregulation of the DA D3R (Dopamine D3 receptor, a target of let 7d), in full correlation with the target prediction data (Fig. 8B). DA D3R is well implicated in synaptic plasticity and our previous studies showed that DA D3R expression in the NAc significantly contributed to behavioral changes associated with chronic cocaine delivery (Bahi et al., 2005). A significant downregulation was observed from qRT-PCR with reduction in DA D3R mRNA levels of 29, 41, and 41% (Fig. 8B; $F_{(1,12)} = 222.42$, $P<0.0001$), in a LV-let-7d viral dose dependent manner ($F_{(2,12)} = 2.55$, $P=0.1198$). No significant change was observed at DA D3R mRNA level after LV-miR-124, suggesting that the suppression of DA D3R is let-7d specific. At protein levels, Western blots analysis showed a significant decrease of DA D3R (Fig. 8B; $F_{(1,24)} = 32.26$, $P<0.0001$) in a viral dose dependent manner ($F_{(2,24)} = 0.33$, $P=0.7238$) with LV-let-7d but no change with LV-miR-124.
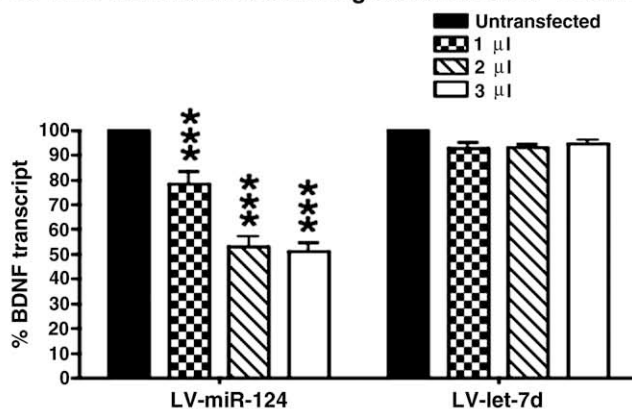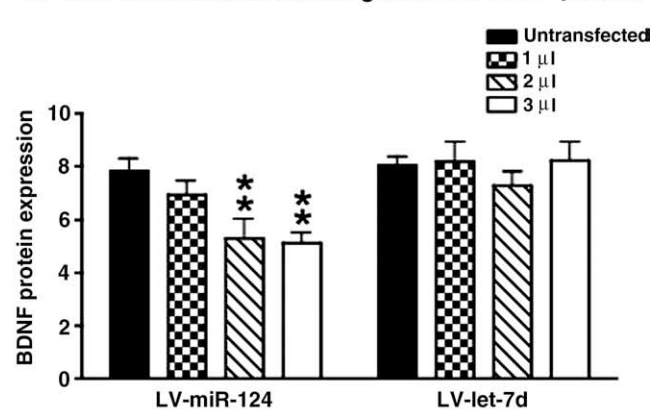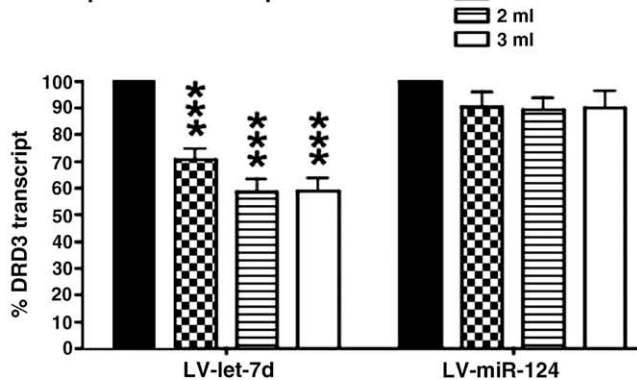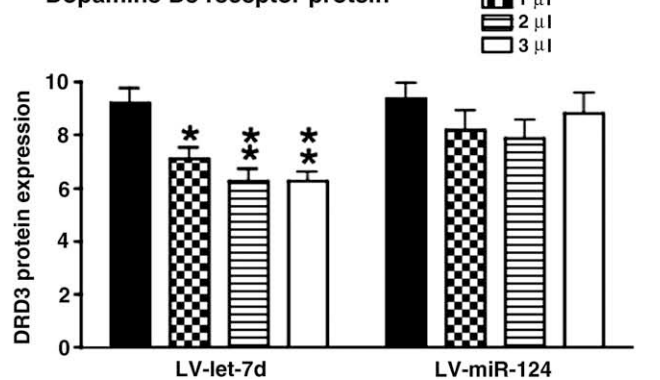

Fig. 8. Lentiviral-mediated overexpression of miRNAs causes target-specific downregulation of cocaine-responsive genes. (A) Quantification of BDNF (mRNA and protein levels by qRT-PCR and Western blot, respectively) after LV-miR-124 or LV-let-7d transfection in NG108-15 cells at different viral concentrations. (B) Quantification of DA D3R (mRNA and protein levels by qRT-PCR and Western blot, respectively) after LV-let-7d or LV-mir-124 transfection in NG 108-15 cells at different viral concentrations. NG108-15 cells were transfected with 0, 1, 2 or 3 µL of the corresponding LV-miRNAs in culture medium (See Experimental methods for details). mRNA expression levels were calculated from qRT-PCR relative to the housekeeping gene cyclophilin. Semi quantitative expression analysis for either BDNF or DA D3R was performed compared with β-actin antibody to compare the expression levels in different lentiviral-treated samples (see Experimental methods for details). Data represent means ± SEM. *P<0.05; **P<0.01; ***P<0.001 by two-way ANOVA, Bonferroni *post hoc* tests. Normalization was done with untransfected samples.

Discussion

In this study, application of bioinformatic predictions on cocaine-responsive genes revealed that several cocaine-induced genes were targeted by miR-124 and let-7d either individually or in a co-operative manner, whereas miR-181a has putative target sites on at least four cocaine-suppressed genes. Using various quantification methods, we found that cocaine indeed causes a strong induction of miR-181a in different regions of the midbrain whereas the levels of miR-124 and let-7d are significantly decreased. These data confirm recent studies demonstrating miRNA-mediated regulatory mechanisms that could be responsible for addiction to drugs of abuse (Huang and Li, 2008; Pietrzykowski et al., 2008).

Cocaine causes a differential expression of miRNAs in the precursor level. Significant downregulation of let-7d precursor is observed in many regions of the mesolimbic pathway, and miR-124 is significantly downregulated in the CPU, whereas miR-181a is significantly upregulated in the NAc, CPU and HIP regions. miRNAs with more than one precursor (miR-124 and miR-181) may be independently regulated (Krichevsky et al., 2003). Several factors can contribute to miRNAs expression (transcription, maturation, degradation) and the expression pattern of precursor and mature forms is finely tuned. In our study, we observed no significant differential expression of miR-124 at the precursor level (except in the CPU) but significant differential expression of its mature form in many regions, implying its down-regulation at processing level. By contrast strong differential expression of let-7d precursor is observed, yet subtle but significant expression changes of its mature form. The "Zip code model" (Fiore and Schratt, 2007a,b) supports this interpretation, which assumes that the processing of precursor miRNAs occurs locally in the synaptodendritic compartment. Based on these results the CPU or striatum seems to be the focal brain area for cocaine-mediated miRNA modulation at least at the precursor transcript levels.

miRNA and addiction

A growing number of studies identify the correlation between miRNA regulation and addiction. miR-9 has been implicated in neuroadaptation to alcohol (Pietrzykowski et al., 2008) and modulation of miR-140 may be involved in nicotine addiction (Huang and Li, 2008). Furthermore miR-504 mediates differential expression of the dopamine D1 receptor, which may contribute to nicotine dependence (Huang and Li, 2009). Our findings on the differential expression of three miRNAs may facilitate the understanding of pathways involved in cocaine addiction.

miR-124 is a highly conserved and exclusively neuronal miRNA (Krichevsky et al., 2003), that accounts for 25–48% of total adult brain miRNA (Lagos-Quintana et al., 2002). Its expression increases over 13-fold from E12 to E21, then remains stable in neuronal cells (Krichevsky et al., 2003). Our observation of 1.5–2.2 fold down-regulation of this enriched miRNA might have a profound effect on the targets (many cocaine-upregulated genes) and is certainly an important adaptation for drug-induced plasticity.

The miRNA let-7d is highly conserved and is involved in various temporal developmental stages (Lee and Ambros, 2001). Studies suggested that its de-regulation might play a crucial role in the etiology of several neurological disorders (Niwa et al., 2008). We found that let-7d has a putative binding site in the mu-opioid receptor and our in situ data show significant suppression of let-7d in the CA1, a region of the hippocampus that displays high expression of this receptor. Furthermore both miR-124 and let-7d target MAP kinases, which is induced after chronic cocaine in the NAc (Freeman et al., 2001). Down regulation of these miRNAs targeting MAP kinases suggest a role in cocaine-induced signal transduction pathway.

miR-181a is involved in lineage modulation determining ratio of cell types and is a positive modulator of B-cell differentiation (Chen et

al., 2004) and is also implicated in brain disorders like schizophrenia (Beveridge et al., 2008). From our studies it is induced by cocaine in the NAc and the prefrontal cortex and has putative targeting sites on mGluR5 and Homer-1, whose expression decreases upon chronic cocaine in these brain areas (Ghasemzadeh et al., 2003, 2008). Interestingly Per2 (another target of miR-181a) is induced after cocaine exposure but mPer2 mutant mice exhibit a hypersensitized response to cocaine (Spanagel et al., 2005) and show a strong cocaine-induced place preference, suggesting that development of association cues related to cocaine depends on downregulation of Per2 (Abarca et al., 2002). This is in support of our model (Fig. 9) where we suggest that miR-181a may be involved in a dynamic regulation of circadian rhythm-dependent, cocaine-mediated, differential gene regulation.

miRNA-mediated gene regulation in cocaine administration

Recent studies have discussed a feedback loop between miRNAs, REST and CREB, wherein positive regulation of CREB and negative regulation of REST results in the activity-dependent expression of miRNAs (Wu and Xie, 2006). The transcriptional repressor REST regulates neuronal gene expression and promotes neuronal fate. It is dynamically regulated during neurogenesis (Chong et al., 1995) and its expression increases in the hippocampus in response to ischaemia (Calderone et al., 2003) or in kainate-induced seizures (Palm et al., 1998). REST targets many cocaine-induced plasticity genes (synaptophysin, stathmin, BDNF) (Bruce et al., 2004) and REST-mediated regulation of these genes may therefore be a fine tuning mechanism, balancing between tolerance, sensitization and dependence. CREB, on the other hand, is a cocaine-induced transcription factor and a positive regulator of many cocaine-specific genes (Carlezon et al., 1998; Walters and Blendy, 2001), and it is a direct target of miR-124. In our study we observe an induction of REST and suppression of miR-124 (a REST target, and *vise versa*) (Johnson et al., 2008), indicating that REST probably potentiates the suppression of miRNAs observed after cocaine administration.

Alteration of dendritic spines on dopaminoceptive neurons in the NAc is an adaptive neuronal response, linked to long-lasting addictive behaviors, that results in increased spine density after chronic cocaine treatment (Lee et al., 2006). Interestingly dendritic spine size of
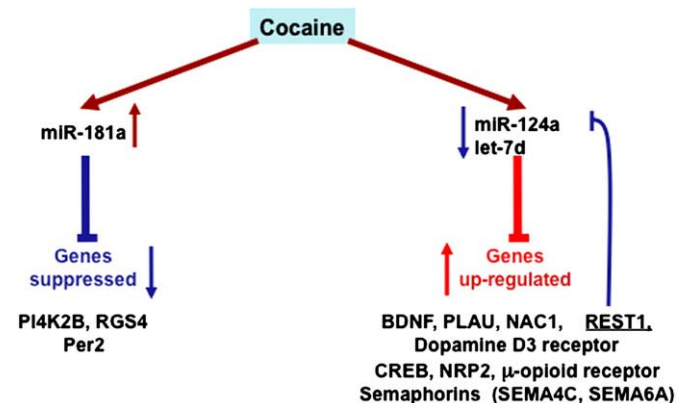

Fig. 9. Model for miRNA pathway in cocaine-induced synaptic plasticity. Several microRNAs affect the expression of many cocaine-regulated genes, and. The brain-enriched miR-124 is suppressed by chronic cocaine in the mesolimbic dopaminergic pathway (presumably by the induction of REST), which induces expression of miR-124 target genes (BDNF, integrin β1, NAC1, axon guidance molecules like SEMA6A, etc.) favoring the formation of addictive phenotype. Also downregulation of let-7d results in induction of its target genes (OPRM1, DA-D3R, SEMA6A, SEM4C, uPA). In contrast, miR-181a (also a brain-enriched miRNA) is markedly induced by cocaine, causing down-regulation of its targets (RGS4, GRM5, GRIA2, PI4K2B). Our study strongly supports the hypothesis that miR-124, let-7d and miR-181a are involved in a dynamic double negative feed-back loop, thereby regulating the differential expression of various genes in response to cocaine, which may result in the molecular adaptation leading to addiction.

hippocampal neurons is regulated by miR-134 through inhibition of Limk1, which can be relieved by BDNF, implying the important role of miRNA in synaptic plasticity (Schratt et al., 2006). BDNF is involved in cocaine-induced plasticity in reward and memory (Nestler, 2001; Bahi et al., 2008; Thomas et al., 2008) and we observed that overexpression of miR-124 results in a significant downregulation of BDNF both at mRNA and protein levels. Cocaine-induced suppression of miR-124 and thereby elevated BDNF might be crucial for the regulation of dendritic spine size. Interestingly miR-124 overexpression also results in increased expression the of transcription factor NAC1 (data not shown). Although the exact mechanism for this is yet to be determined, it is possible to speculate that de-repression of miR-124 during withdrawal to cocaine might be involved in NAC1 induction. Our observation of REST induction after chronic cocaine is contradictory to earlier findings that REST suppresses BDNF expression in Huntington's disease (Zuccato et al., 2003) and in the brain of Rett syndrome patients (Abuhatzira et al., 2007). However a truncated, neuronal-specific REST isoform, REST4, has been described which can complete the silencing effect of REST/NRSF (Tabuchi et al., 2002). We speculate that cocaine inhibits the BDNF-specific suppression of REST by inducing a REST isoform, eg. REST4; and that this mechanism is different from the miRNA suppression induced by REST.

Analyzing the expression of all the target genes of the three miRNAs under investigation after viral-mediated miRNA expression is out of the scope of this work. However we also observed a significant downregulation of DA D3 receptor upon overexpression of let-7d, both at mRNA and protein levels. But let-7d overexpression has no effect on uPA mRNA levels (data not shown), another cocaine-responsive gene (Bahi et al., 2004, 2006, 2008, Bahi and Dreyer, 2007), implying that the decreases seen in BDNF and DA D3R mRNA levels are unique and not necessarily a common phenomenon of miRNA-mediated regulation. Our GFP-sensor assay enables for sensitive and reliable *in vitro* analysis and quantification of the miRNA expression. Based on these studies, our findings of BDNF and DA D3R regulation by miR-124 and let-7d can be taken for a physiologically relevant regulation happening in the cocaine-induced alteration of gene expression.

Studies show that changes of miRNA levels do not necessarily lead to changes of abundance of the target mRNA (Tsang et al., 2007). Because of the nature of miRNA-mediated gene regulation, the repression of the targeted genes occurs mostly by translational inhibition with little effect on the targeted mRNA levels (Bartel, 2004; Bartel and Chen, 2004). Therefore the observed negative correlation between miRNA levels and their corresponding targeted mRNAs may be due to suppression of these target mRNAs either by transcriptional control or by miRNA-mediated mRNA decay (Bartel and Chen, 2004). miRNAs can indirectly regulate their own expression through double-negative feedback loops (Johnston et al, 2005). Our findings suggest that such a feedback loop also occurs upon cocaine (Fig. 9). miRNAs (124, let7d and 181a) and transcription regulators (mainly CREB, NAC1, Per2 and REST) along with various plasticity related genes (Semaphorins, BDNF), neurotransmitter receptors (DA D3R, mu-opioid receptor) and signaling proteins (MAP2K4, PI4K2B) might act in such a double negative feedback loop during the development of cocaine-induced neural plasticity. Our work clearly supports such a brain-specific modulation of miRNAs that could act by pairing to the mRNAs of their target genes to direct gene-silencing processes critical for maintenance of addiction phenotype.

Experimental methods

Animal handling

All animal experiments were carried out in accordance with the guidelines and regulations for Animal Experimentation, BAG, Bern, Switzerland. Male Wistar rats weighing 250–300 g (BRL, Fillingsdorf, Switzerland) were used for all the experiments. The animals were housed in groups (four per cage) in clear plastic cages with wire grid lids. The animals were kept on a 12-h light/dark cycle (lights off at 07.00 am), with access to food and water *ad libitum*.

Cocaine administration

Chronic cocaine administration was performed based on established methodologies (Bahi et al., 2004), allowing optimum evaluation of drug induced gene expression changes. Rats ($n = 6$) were given single daily i.p. injection of 15 mg/kg cocaine-HCl for a period of 15 days. Control animals ($n = 6$) received 0.9% saline i.p. injection under the same schedule. All animals were sacrificed by decapitation 24 h after the last injection and brain were removed and stored rapidly, either in TRIzol reagent (Invitrogen) for RNA extraction or cryopreserved in isopentene for *in situ* hybridization and FISH analysis.

Total RNA isolation

HEK-293 cells or rat brain parts (micro-dissected from cocaine- or saline-treated animals) were homogenized in TRIzol reagent (Invitrogen, Switzerland). Total RNA was extracted according to the manufacturer's instructions. RNA was quantified by spectrometry, and its integrity was verified by 2% agarose gel electrophoresis and visualized with ethidium bromide staining. RNA samples were stored at −70 °C.

Identification of cocaine-specific microRNAs

In order to identify cocaine-specific miRNAs, we analyzed a large set of known cocaine-responsive genes (data not shown) well established by various studies to be involved in cocaine addiction, to identify putative miRNAs which are targeting these genes based on stringent constraints of homology in cross species and single pathway. We used open source software for target prediction based on a development of the miRanda algorithm (http://www.microrna.org) (Betel et al., 2008). We cross compared our analysis with various other programs (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl) as described (Griffiths-Jones, 2006), (http://www.mirz.unibas.ch/ElMMo/), (http://pictar.bio.nyu.edu/cgi-bin/PicTar_vertebrate.cgi), (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), (http://www.russell.embl.de/miRNAs/) to choose miRNAs which are conserved for their targeted mRNA and to eliminate false-positive predictions.

MicroRNA lentiviral expression constructs

Lentiviral expression constructs of the miRNAs were generated by cloning a ≈450 bp fragment containing either the rat miR-124, miR-181a or let-7d precursor hairpin loops. Fragments obtained by PCR based sense-antisense strand construction (under RNA Pol III mouse U6 promoter) were cloned into pDrive cloning vector (QIAGEN). The primers were flanked by *Bam*HI and *Xho*I site in the 5′ and 3′ end, respectively. Positive clones were sequence confirmed and subcloned into pTK-431 lentiviral vector using *Bam*HI/*Xho*I sites (Bahi et al., 2004; Boyer and Dreyer, 2007).

MicroRNA lentiviral sensor constructs

miRNA recognition elements (MREs) of either miRNA-124, miR-181a or let-7d were cloned separately in form of tandem repeats consisting of four perfect complementary sequences into the 3′ region of the enhanced Green Fluorescent protein (EGFP) sequence in pTK-433 (Fig. 1A). This doxycycline-regulatable lentiviral vector enables stable expression of the sensor under the doxycycline regulatable Tet-off system. Sense oligos of ≈125 bp with the 4 perfect match MREs

sequences (pMREs), corresponding to each miRNAs, were amplified by specific primers and the PCR products were ligated into the pDrive cloning vector (QIAGEN). Positive clones were sequence confirmed and sub-cloned into the pTK-433 lentiviral vector using the BamHI/XhoI sites (Bahi and Dreyer, 2005; Boyer and Dreyer, 2007) The specific LV-EGFP-pMREs sensors corresponding to each of the three miRNAs express the EGFP under the control of a regulatable CMV promoter with Tet-Off system.

Lentivirus production

The lentiviral vector expression plasmids (either pTK431-miR-124, pTK431-let-7d or pTK431-miR-181a; or the three corresponding specific sensor constructs pTK433-GFP-miRNAs or the control vector pTK433-GFP) were co-transfected into human embryonic kidney (HEK) 293T cells—along with the packaging construct plasmid pΔNRF and the envelope plasmid pMDG-VSV-G—to produce the viral particles (Bahi et al., 2004; Boyer and Dreyer, 2007). The viral titers were determined by p24 antigen measurements (KPL, USA). For in vivo experiments, the different viral stocks were matched for viral particle content and used at 0.2 mg/mL of p24.

Quantification of miRNA precursors by real-time qRT-PCR

RNAs prepared from cocaine- and saline-treated animal brain parts were reverse transcribed using M-MLV reverse transcriptase (Promega, Switzerland). 2 μg of RNA from each sample was reverse transcribed at 42 °C for 30 min with 1 μg of Oligo(dT) or specific primers, 5× first strand buffer, 100 mM DTT, 10 mM dNTP, RNAsin (Invitrogen, Switzerland) and M-MLV. cDNA's were checked for their optimum dilution in subsequent real-time qRT-PCR reactions.

Precursors detection of miRNAs by RT was performed as described (Schmittgen et al., 2004; Jiang et al., 2005). PCR reaction mixtures included cDNAs in optimum dilution, the SYBR Green qPCR Master mix (BioRad, Switzerland), 10 μM primers, in a total reaction volume of 20 μl. Expression profiling was done with dissociation curves using iCycler (BioRad, Reinach, Switzerland). Cycling parameters were 95 °C for 4 min followed by 40 cycles of 20 °C/s temperature transition rate up to 95 °C (30 s), 62 °C (45 s), followed by melting curve analysis. All reactions were performed in triplicates with reference dye normalization (U6 SnRNA) and the median Ct (Cycle threshold) value was used for analysis. The relative abundance of each target can be calculated as the ratio between treated and untreated samples (Boyer and Dreyer, 2008).

qRT-PCR was performed with sets of primers for amplification of following miRNA precursors: miR-124-2 (5′-TCC GTG TTC ACA GCG GAC-3′; 5′-CAT TCA CCG CGT GCC TTA-3′), let-7d (5′-AGG TTG CAT AGT TTT AGG GCA-3′; 5′-AAG GCA GCA GGT CGT ATA GT-3′)′; miR-181a-2 (5′-AAC ATT CAA CGC TGT CGG T-3′; 5′-CAG TCA ACG GTC AGT GGT TT-3′); U6 SnRNA (5′-CTC GCT TCG GCA GCA CA-3′; 5′-AAC GCT TCA CGA ATT TGC GT-3′).

Quantification of mature miRNA by real-time qRT-PCR

Expression profiling of mature miRNA was performed after reverse transcription using specific RT-primers (Taqman® MicroRNA Assays Human-Early access kit, Applied Biosystem, UK) and as described by the manufacturer's guidelines. cDNA was used at a dilution of 1:50 in qRT-PCR. Typically, 15 ng of total RNA from micro-dissected tissue was used per cDNA preparation for RT. 20 μl of total reaction mixture included rat miRNA-specific probes, primer sets and the Taqman Universal PCR Master Mix. Reactions were performed on a Sequence detection system ABI 7500 (Applied Biosystem, UK). Cycling parameters were 95 °C for 5 min followed by 40 cycles of 95 °C (15 s), 60 °C (1 min). All reactions were performed in triplicates and the relative abundance of each target was calculated as the ratio between treated and untreated samples (Boyer and Dreyer, 2008).

Solution hybridization and RNase protection assay

miRNA profiling was also performed by solution hybridization using mirVana miRNA Detection kit (Ambion). miRNA were specifically transcribed and radiolabeled in vitro (α-^{32}P UTP, Amersham, UK). Probes were used at a final labeled nucleotide concentration of 3.125 μM per labeling reaction (mirVana miRNA probe Construction kit, Ambion). The experiment was performed with 4 μg of total RNA using 1–5×10^4 cpm labeled RNA probe, 5 μg yeast RNA, 10 μl hybridization buffer in 20 μl reactions and hybridized overnight at 42 °C. Thereafter RNase digestion was followed by elution of the protected miRNA-labeled probe and separation in 8 M urea denaturing PAGE, according to the manufacturer's manual. U6 snRNA was probed for usage as loading control. The gel was then exposed for autoradiography for 1 h at −80 °C.

The following primers were used for in vitro-transcription and radio-labeling of miRNA antisense probes used in mirVana miRNA detection kit (Ambion): miR-124 (5′-TTA AGG CAC GCG GTG AAT GCC ACC TGT CTC-3′); let-7d (5′-AGA GGT AGT AGG TTG CAT AGT CCT GTC TC-3′); miR-181a (5′-AAC ATT CAA CGC TGT CGG TGA GTA AAA CCT GTC TC-3′); miR-98 (5′-TGA GGT AGT AAG TTG TAT TGT TAA AAA AAA CCT GTC TC-3′); miR-133b (5′-TTT GGT CCC CTT CAA CCA GCT ATT CCT GTC TC-3′); U6 SnRNA standard (5′-TTC GGC AGC ACA TAT ACT AAA ATT GGA ACG ATA CCC TGT CTC-3′).

Northern blot analysis

Northern blot was performed as described (Lau et al., 2001). Total RNA from the micro-dissected brain parts after cocaine and saline treatment was used. 30 μg total RNA was separated on a 15% denaturing polyacrylamide gel containing 8 M urea, at 20 Watts for 75 min in 0.5× TBE. The RNA was then transferred to a Hybond-N+ membrane (Amersham Biosciences, UK) and run at 25 Volts for 30 min using 0.5× TBE as a transfer buffer. The membrane was then UV cross-linked and baked at 80 °C for 1 h. 30 pmol of LNA-modified probe (Exiqon, UK) for miR-124 and let-7d end-labeled with DIG was used for non-radioactive detection. Pre-hybridization and hybridization were carried out according to the Tm of the probes and as mentioned by the manufacturer's guidelines (Exiqon, UK) using DIG Easy Hyb-hybridization solution for DIG-labeled probes (Roche, Switzerland). After washings, the membrane was incubated with HRP-conjugated anti-DIG antibody (Abcam, UK) followed by detection with DAB substrate buffer and DAB substrate (Boehringer Mannheim GmbH) according to the manufacturer's manual. Multi-DIG labeled U6 snRNA was probed as loading control.

In situ hybridization

Frozen cryosections of cocaine- and saline-treated rat brains (from animals undergoing the same chronic paradigm used for other quantifying methods) were used for in situ hybridization. In situ was performed according to established protocols (Spanagel et al., 2005; Nelson et al., 2008) except for probe design. Specific riboprobes for miR-124, miR-181a, miR-133b, let-7d were in vitro transcribed and radiolabeled (α-^{35}S UTP, Amersham, UK) and used at a final labeled nucleotide concentration of 3.125 μM. 14 μm brain sections were postfixed in 4% phosphate-buffered para-formaldehyde and rinsed twice with phosphate-buffered saline (PBS). Sections were then acetylated twice in 0.1 M triethanolamine, washed again with PBS, and dehydrated in graded ethanol series. Sections were hybridized overnight with denatured antisense riboprobes in a humid chamber at 50 °C. They were then rinsed with SSC, treated with ribonuclease A

(Sigma, USA), rinsed with stringency washes of SSC, and dehydrated in graded ethanol series. Slices and radioactive standards were exposed for 1 week to an autoradiographic film (Hyperfilm MP, Amersham Biosciences, UK). Standards were included in each cassette to verify that the measured values of optical densities were in linear response range of the film. Densitometry analysis of hybridization signals was performed with QuantityOne software (Bio-rad). The optical density of specific signals was calculated by subtraction of the intensity of staining background area (defined as a circle of 100 µm diameter). Data were expressed as relative optical-density values (Spanagel et al., 2005).

In vitro LV-GFP-pMRE sensor assay for quantifying the efficiency of LV-miR-124, LV-let-7d, and LV-miR-181a

The efficiency of the LV-miR-124, LV-let-7d, and LV-miR-181a were tested in vitro by infection of HEK293T cells along with the corresponding LV-GFP-pMRE sensor virus. HEK293T cells were plated at $0.2–0.8 \times 10^5$ per well in 24-well plates. The next day, lentivirus stocks were mixed with 10 µg/mL Polybrene (Sigma, Switzerland), incubated for 30 min at room temperature, added to the cells, and incubated at 37 °C. After 24 h, the medium was replaced with normal growth medium supplemented with 10% fetal calf serum and 1× penicillin/streptomycin, and cells were left for a further 48 h. Cells were then collected and used for total RNA isolation (for real-time PCR) or for total protein isolation (for Western blot). The efficient silencing of the corresponding GFP-pMRE sensor both at the transcripts and protein levels were taken for assessing the lenti-miRNA efficiency. For that purpose, cells were infected with 2 µL of LV-GFP-pMRE sensor virus stock (corresponding to each specific lenti-miRNAs), either alone or together with 1 or with 2 µL of respective LV-miR-124, LV-let-7d, and LV-miR-181a viruses.

Quantification assay of the effect of lenti-miRNA overexpression on specific target genes

The effects of LV-miR-124, LV-let-7d, or LV-miR-181a on specific target genes were tested *in vitro* by infection of NG 108-15 cells (neuroblastoma × glioma hybrid cells), a cell line stably expressing the dopamine D3 receptor. NG 108-15 cells were plated at $0.2–0.8 \times 10^5$ per well in 24-well plates. Viral transfection was then performed as mentioned above for HEK-293 cells and cells were then collected and used for total RNA isolation (for real-time PCR) or for total protein isolation (for Western blot). Triplicate wells of cells were infected with virus stock corresponding to each specific LV-miRNAs, with different concentration of 1, 2 and 3 mL of respective LV-miR-124, LV-let-7d, and LV-miR-181a viruses with appropriate untransfected controls. Quantitative real-time PCR was performed for assessing the target mRNA transcript levels after LV-miRNA treatment. All reactions were performed in triplicates with reference dye normalization (β-actin or Cyclophilin) and the median Ct (Cycle threshold) value was used for analysis. The relative abundance of each target can be calculated as the ratio between treated and untreated samples (Boyer and Dreyer, 2008). qRT-PCR was performed with following sets of primers for amplification of genes: Cyclophilin (5′-GTG AGA AGG GCT TTG GCT AC-3′ and 5′-TTC TCG TCA GGA AAG CGG-3′); β-actin (5′-AGC CAT GTA CGT AGC CAT CC-3′ and 5′-CTC TCA GCT GTG GTG GTG AA-3′); BDNF (5′-GGT TCG AGA GGT CTG ACG AC-3′ and 5′-CAA AGG CAC TTG ACT GCT GA-3′); REST (5′-CGA GTT GAT GCC TGT TGG AGA C-3′ and 5′-TGC TTC AAA TAC GGG CTG GG-3′); DA D2R (5′-CAT TGT CTG GGT CCT GTT CCT-3′ and 5′-GAC CAG CAG AGT GAC GAT GA-3′); DA D3R (5′-GGG GTG ACT GTC CTG GTC TA-3′ and 5′-TGG CCC TTA TTG AAA ACT GC-3′); uPA (5′-CAG ATC CGA TGC TCT TAG CC-3′ and 5′-TAG AGC CTT CTG GCC ACA CT-3′); and for the sensor EGFP (5′-ATG GTG AGC AAG GGC GAG-3′ and 5′-GTG GTG CAG ATG AAC TTC A-3′).

Western blotting

Transfected HEK293T or NG 108-15 cells were homogenized in buffer (50 mM Tris, pH 7.5; 120 mM NaCl, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 5 mM EDTA) with a protease inhibitor mixture (1 mL/20 g of tissue, Sigma, USA). Homogenates were solubilized with 1% digitonin, followed by the addition of secondary solubilization buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet-P40, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM Na-vanadate, 1 mM phenyl-methyl-sulphonyl fluoride, 1% Triton X-100), and centrifuged at 10,000g at 4 °C for 15 min. Solubilized extracts were subjected to sodium dodecyl-sulfate-polyacrylamide gel electrophoresis. Blots were blocked with 5% non-fat dried milk dissolved in TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature, and incubated with primary antibodies for either BDNF (1: 500, ab6201, Abcam, UK) or GFP (1: 1000; G-6539, Sigma, USA) or DA D3R (1: 1000; AB1785P, Chemicon, USA) overnight at 4 °C. Blots were washed three times with TBST and incubated for 1 h with the peroxidase-conjugated secondary antibody solution. Proteins were then visualized using enhanced chemiluminescence (Millipore, Switzerland). Membranes were washed for 30 min in TBST and placed in stripping solution (25 mM glycine-HCl, pH 2.0, 1% sodium dodecyl-sulfate) for 30 min and used as described above for β-actin detection (1: 4000; ab1801, Abcam, UK). Protein band signals were estimated using Quantity one (Bio Rad, UK) software and normalized against β-actin signal.

Fluorescence in situ hybridization (FISH) using LNA probes

Frozen cryosections of cocaine- and saline-treated rat brains from chronic administration were utilised to perform FISH. The miRNA FISH protocol was followed as described (Silahtaroglu et al., 2007), but without tyramide amplification. Instead, a secondary antibody-based signal amplification of the fluorescence signal was used. The cryosections were fixed using fresh 4% (wt/vol) para-formaldehyde (PFA), then acetylated and prehybridized. This was followed by a 1- to 4-h hybridization step using a DIG-labeled LNA oligonucleotide probe complementary to the mature miRNA. The in situ signal was detected by incubation with the primary anti-DIG mouse monoclonal antibody (Abcam, UK), followed by incubation with the secondary, FITC or HRP-conjugated anti-mouse goat antibody (Abcam, UK). For imaging a conventional epi-fluorescence microscope (Axioplan-2 imaging; Zeiss, Switzerland) equipped with FITC/TRITC and DAPI filters was used and signals were detected with appropriate systems (HAL-100). Stained sections were visualized, photographed using a multichannel camera (Axio-cam, Zeiss, Switzerland) combined with acquisition software (Axiovision system 3.1, Zeiss, Switzerland).

Statistical analysis

Results were expressed as the mean ± SE. The significance of differences was determined by a two-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* tests to compare the influence of cocaine and brain regions on the expression profile of miRNAs. All the analysis was done using GraphPad PRISM (V3.0, GraphPad, San Diego, CA).

Financial disclosure

Conflict of interest
The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2009.08.009.

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