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Modulation of BK Channel by MicroRNA-9 in Neurons After Exposure to HIV and Methamphetamine

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Abstract

MicroRNAs (miR) regulate phenotype and function of neurons by binding to miR-response elements (MRE) in the 3' untranslated regions (3'UTR) of various messenger RNAs to inhibit translation. MiR expression can be induced or inhibited by environmental factors like drug exposure and viral infection, leading to changes in cellular physiology. We hypothesized that the effects of methamphetamine (MA) and human immunodeficiency virus (HIV)-infection in the brain will induce changes in miR expression, and have downstream regulatory consequences in neurons. We first used a PCR-based array to screen for differential expression of 380 miRs in frontal cortex autopsy tissues of HIV-positive MA abusers and matched controls. These results showed significantly increased expression of the neuron-specific miR-9. In vitro, we used SH-SY5Y cells, an experimental system for dopaminergic studies, to determine miR expression by quantitative PCR after exposure to MA in the presence or absence of conditioned media from HIV-infected macrophages. Again, we found that miR-9 was significantly increased compared to controls. We also examined the inwardly rectifying potassium channel, KCNMA1, which has alternative splice variants that contain an MRE to miR-9. We identified alternate 3'UTRs of KCNMA1 both in vitro and in the autopsy specimens and found differential splice variant expression of KCNMA1, operating via the increased miR-9. Our results suggest that HIV and MA-induced elevated miR-9, leading to suppression of MRE-containing splice variants of KCNMA1, which may affect neurotransmitter release in dopaminergic neurons.

Keywords

BK-channel; microRNA; microRNA-9; methamphetamine; human immunodeficiency virus; HIV; neuron; brain

1. Introduction

Despite marked improvement in survival related to current antiretroviral therapy (ART) (Fang 2007), human immunodeficiency virus (HIV)-1 often leads to neurological complications including cognitive impairment and motor dysfunction (Antinori 2007; Cysique 2009; Ellis 2010). HIV-associated neurocognitive disorders (HAND) remain prevalent, affecting up to 50% of HIV-infected individuals (Heaton 2010). In addition, drugs such as methamphetamine (MA) greatly increase the risk for infection with HIV (Colfax 2005). MA is a widely used recreational drug in North America (Durell 2008; Maxwell

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Conflict of Interest

The authors declare that they have no conflict of interest.

2008), especially among HIV-infected men (Colfax 2005; Durell 2008; Gonzales 2010). MA-abuse causes behavioral symptoms including agitation, anxiety, paranoia, psychosis, and marked increase in risk-taking or sensation-seeking activities (Derlet 1995). HIV and MA in combination appear to have additive effects on neurocognitive deficits (Letendre 2005; Basso 2000). Both MA (Cubells 1994; Sandoval 2001; Fischer 1979; Kogan 1976) and HIV infection (Sardar 1996; Agrawa 2010; Reyes 1991; Aylward 1993; Wang 2004; Chang 2008; Melrose 2008) significantly affect the dopaminergic system in the human brain.

MicroRNAs (miRs) play key roles in the development (Kosik 2006; Krichevsky 2003) and maintenance (Fukuda 2005) of the dopamine system and have diverse cellular roles (Kosik 2006; Banerjee 2009; Mikl 2010). In the striatum, the functional loss of all miRs through knockout of Dicer, the rate-limiting enzyme central to miR synthesis, lead to parkinsonian behavioral and neuroanatomical phenotypes in mice (Cuellar 2008). Furthermore, HIV infection has been shown to significantly alter the miR profile in peripheral blood mononuclear cells (Houzet 2008) and HIV-tat led to reduced expression of synaptic membrane fusion proteins via a miR (Eletto 2008). Based on these lines of evidence for a potential mechanism of neuronal dysfunction through miR in the central nervous system of HIV-positive MA abusers, we screened for differentially expressed miRs in post-mortem brain tissue, then followed up with in vitro experimentation.

We first present a case-controlled retrospective study of postmortem brain tissues from the California NeuroAIDS Tissue Network (CNTN) (Cherner 2002). *Controls* were defined as HIV-negative, drug-abuse naive individuals with age- and gender- match to cases. Two groups of *cases* were defined as follows: HIV+ individuals with a clinical diagnosis of MA-abuse within 6 months of death, and HIV+ individuals without a history of drug abuse. This study was designed to screen for candidate miRs differentially expressed in the brain of HIV + individuals with a history of MA-abuse in order to generate and test hypotheses on miR-mediated neuronal dysfunction under these conditions.

Based on results from our initial autopsy screen of 380 miRs, we hypothesized that miR-9 would be increased in dopamine neurons after exposure to MA and conditioned media from HIV-infected monocyte-derived macrophages (MDM) grown in vitro. Others have shown that miR-9 (Yuva-Aydemir 2011) is upregulated in adult neurons after exposure to alcohol (Pietrzykowski 2008; Wang 2009), but not in neural precursor cells (Sathyan 2007). This was followed by a decrease in splice variants of the KCNMA1 gene (also known as BK Channel) that contain a miR response element (MRE) which recognizes miR-9 (Pietrzykowski 2008). The KCNMA1 gene encodes the large conductance potassium transporter protein (Salkoff 2006), whose function is normally potentiated by alcohol (Butler 1993), but mRNA for more active splice variants is decreased via miR-9 (Pietrzykowski 2008). This system is hypothesized to serve as a molecular mechanism for alcohol tolerance (Pietrzykowski 2010) and we use miR inhibitors and splice-specific quantitative PCR to test the system with MA and HIV in vitro with the underlying hypothesis that it serves as a common molecular pathway for drug adaptation. While further study is necessary, we provide incremental evidence that upregulation of miR-9 and modulation of KCNMA1 in neurons is a component of drug-abuse neuronal molecular pathology. This is significant because miR-9 is increased after exposure to HIV and this process may render dopamine neurons physiologically susceptible to a drug tolerant state at the cellular level.

2. Materials and Methods

2.1. Screening for Differentially Expressed miRs

2.1.1 Study Design and Cases Selected—All human subjects provided written informed consent and studies were approved by the University of California San Diego (UCSD) Human Research Protections Program. This is a retrospective case-control study ($N = 16$) designed to identify differentially expressed miRs in the central nervous system (CNS) of HIV+ individuals and HIV+ individuals with a history of MA-abuse. Two groups of cases were defined as follows: *HIV+* ($n = 6$) and *HIV+MA* ($n = 5$), all subjects in both groups were at end-stage AIDS. Inclusion criteria for the *HIV+* group included: individuals with HIV for whom drug abuse history, neurocognitive data, virologic clinical data, and post-mortem neuropathology analysis were available. Exclusion criteria included any history drug abuse, except for nicotine; and the presence of HIV-encephalitis detected after autopsy. Inclusion criteria for the *HIV+MA* group included all the same criteria for *HIV+* plus a Psychiatric Research Interview for Substance and Mental Disorders (PRISM) (Hasin 1996; First 2000) diagnosis of drug abuse disorder and dependence for MA within six months of death and excluded polydrug abuse; e.g., cocaine and heroin. At neuropathology analysis, one subject in both the *HIV+* and *HIV+MA* groups was found to have had lymphoma; one subject in *HIV+MA* group had CMV encephalitis; and Alzheimer's Type II gliosis was described in one subject in the *HIV+MA* group. *Controls* ($n = 5$) were HIV-negative with no known history of drug abuse, no neuropathology, no Axis III medical condition, and sudden causes of death unrelated to CNS function. Table 1 summarizes all cases and controls included in the screening study. Using JMP statistical software, we estimated (Wright 1988), given our group sample sizes restricted to availability and a within-group variance of 0.25, we would have a power of 0.72 to detect between-group difference of 0.2 and power of 0.99 to detect between-group difference of 0.4 at $\alpha = 0.05$. We therefore expected to find significant differential miR expression if the difference between groups was greater than 20%. With no *a priori* expectation for directionality, we used two-way hypothesis testing to determine between-group differences with Tukey's Honestly Significant Difference test (Kramer 1956; Keselman 1977) and applied Benjamini-Hochberg correction for multiple comparisons to the associated *P*-values (Benjamini 1995).

For both the *HIV+* and the *HIV+MA* groups, we had neurocognitive and psychiatric data available according to the previously published HIV Neurobehavioral Research Center assessment battery (Woods 2004). The following deficit scores (DS) were available for correlation-testing: social information processing (SIP), verbal, learning (LEARN), memory (MEM), executive function (EXEC), working memory (WRKMEM), motor function (MOTOR), and global (GDS), a composite of all the domains. DS's are derived from demographically correct *T*-scores of cognitive and psychiatric evaluations, and range from zero to 5, higher values indicate impairment and $DS > 0.5$ is classified as "impaired." Additional psychiatric information included major depressive disorder (MDD) history, and Beck Depression inventory (BDI) score (Beck 1961; Beck 1992). Additionally, medical data available for analysis included the following: CD4 count, nadir CD4, HIV RNA in blood plasma (RNA_PLA, log-transformed copy number), HIV RNA in cerebrospinal fluid (RNA_CSF, log-transformed copy number), hepatitis C virus status (HCV), and ART history. Most recent data were used to test for correlations with miR expression only on those miRs found to be differentially expressed between groups.

2.1.2. RNA Isolation and MiR Analysis—CNS tissue samples from the frontal cortex that had been flash frozen after dissection at autopsy and stored at -80°C were supplied on dry ice. Approximately 1.5 cm^3 , weighing 100 mg, of cortical grey matter was cut from the tissue on a mortar board in dry ice and transferred to 0.6 mL ice cold MirVanaTM (Life

Technologies, Carlsbad, CA, USA) lysis-binding solution with miR stabilizing additive. Tissue was then immediately homogenized on ice at 5,000 rpm for 30 sec using a handheld tissue homogenizer equipped with a 5 mm flat bottom rotor-strator probe (Omni International, Marietta, GA, USA).

Following manufacturer's instructions for total RNA isolation, RNA was eluted in 70°C nanopure water. Quality and quantity of total RNA were determined by gel electrophoresis and UV spectroscopy. Our original study design included six subjects in each group, however one in each HIV+ and HIV+MA groups were excluded because of unacceptable RNA quality (A260/A280 ratio < 1.7 or degradation observed on gel). CDNA was synthesized in a reverse transcriptase reaction using 0.7 µg RNA with Megaplex™ Pools with preamplification kit from Life Technologies. The preamplified product was diluted in 75 µL 0.1× Tris-EDTA (pH 8.0) and stored at -20°C. For detection in real-time PCR, in low-density array (Applied Biosystems MiRNA Panel A version 2.0, part number 4398965), which detects and quantifies multiple miRs in 384-well plate format. TaqMan™ Universal PCR Master Mix without AMP Erase was used; 100 µL of reaction mixture was dispensed into each port on the array card, and RT-PCR assays carried out on an ABI2700 instrument at the UCSD Center for AIDS Research Genomics Core. Samples were run in duplicate.

For detection analysis, SDS files were imported into Applied Biosystems RQ Manager Software, automatic baseline and cycle threshold (CT) of 0.2 settings were used. The $\Delta\Delta CT$ method (Livak 2001) was used for miR quantification using the following equations:

$$\Delta CT_{miR\ i, Subject\ j} = CT_{miR\ i, Subject\ j} - CT_{EndoControl, Subject\ j} \quad 1$$

$$\Delta\Delta CT_{miR\ i, Subject\ j} = \Delta CT_{miR\ i, Subject\ j} - \Delta CT_{miR\ i, AverageControls} \quad 2$$

Data analysis and statistics were performed using the ΔCT values, and graphical representations using the $-\Delta\Delta CT$, which is mathematically equivalent to the $\log_2 RQ$ (relative quantification, also known as “fold-control”) value. For graphing, the values therefore scaled appropriately, with negative values being less than the average Control sample, and positive values being greater than the average Control sample.

2.2. Cell Culture

2.2.1. Neuronal Cell Model—Unless otherwise stated, all cell culture materials were obtained from Life Technologies. SH-SY5Y cells were grown at 5% CO₂ in DMEM/F12 in 10% fetal bovine serum on plastic 12-well plates that had been coated with 5 µg/mL laminin (Sigma-Aldrich, St. Louis, MO, USA), at a density of 10⁴ cells/well. Experiments were performed in quadruplicate after terminal differentiation following previously established protocols (Encinas 2000). Cells were differentiated to dopamine neuronal-like cells by sequential addition of 10 µM retinoic acid and 25 ng/mL brain derived neurotrophic factor (BDNF) (Promega) and serum-withdrawal for 3 days each. Differentiated SH-SY5Y cells were incubated overnight with locked nucleic acid Mircury™ LNA miR inhibitor (50 nM) (donated by Exiqon Corporation, Woburn, MA, USA) using X-treme Gene™ transfection reagent (Roche Applied Science, Indianapolis, IN, USA). For miR-9 inhibitor, product 410014-04, and for scrambled sequence negative control, product 199004-04 were used. The SH-SY5Y cells were then exposed to the experimental conditions. For MA exposure, we modeled a repeat-exposure, chronic condition paradigm. Typical blood concentrations observed for recreational MA use was 0.07–16.8 µM with a peak concentration occurring from 2.6 – 3.6 hr (Couper 2004). Cells were exposed to 10 µM MA for 3 hr, then fresh media replaced and RNA was isolated after 24 hr; or cells were exposed to 10 µM MA for 3

hr again, media replaced, and RNA isolated after 24 additional hr. This procedure was repeated until we had RNA isolated for cells exposed repeatedly to MA for 24, 48, 72, and 96 hr. For HIV exposure, the SH-SY5Y cells were exposed to 10% conditioned media from HIV-1 infected monocyte-derived macrophages (MDM) continuously for up to 24 hr.

2.2.2. In Vitro HIV Model—MDMs were maintained in vitro after isolation through differential adhesion from peripheral blood mononuclear cells (PBMC) obtained from the San Diego Blood Bank, following previously established protocols (Davies 2004). PBMCs were isolated from whole blood using 12 mL blood from each donor and Histopaque™ (Sigma-Aldrich) gradient. PBMC were seeded at 2×10^7 cells/mL onto T-75 plastic flasks in RPMI media with 7.5% human serum, after three hours non-adherent cells were washed out and remaining cells transferred to a T-25 flask and maintained at 2×10^5 cells/mL for 3 days with RPMI media in 7.5% human serum, 50 µg/mL penicillin and streptomycin, 2 mM glutamine. MDMs were infected with HIV-1 (Ba-L) by incubating cells with 3 mL stock virus at original concentration of 5 ng/mL of HIV p24 (diluted 1:3 in RPMI media) for 3 h, with gentle shaking every 15 min, after washing with Hank's Balanced Salt Solution, infected MDM were maintained for 4 weeks in vitro. The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1Ba-L from Dr. Suzanne Gartner, Dr. Mikulas Popovic, and Dr. Robert Gallo (Gartner 1986; Popovic 1989). Infection was monitored by p24 ELISA (at the UCSD Center for AIDS Research Molecular Virology Core) every 5 days following initial exposure to HIV. Low- and high- infection level cultures were maintained (at 900 pg/mL and 1,700 pg/mL of HIV-1 p24 in the supernatant, respectively). Conditioned media composed of 10% of this supernatant from the HIV-1 infected MDM were generated for exposure to neuronal cells to model HIV exposure in vitro in accordance to previously established methods (Pulliam 1991); supernatant from uninfected MDM from the same donors served as Control.

2.3. Quantitative PCR

Total RNA was isolated from SH-SY5Y cell cultures at the indicated timepoints using the MirVana RNA isolation kit following the previously mentioned protocol, except 0.3 mL lysis/binding solution per well was used with scraping. We used 10 ng input RNA following manufacturer's protocols for reverse transcriptase, real-time PCR individual TaqMan assays from Life Technologies. For miR-9, we used assay ID 00583 (recognizing the sequence UCUUGGUUAUCUAGCUGUAUGA) and for endogenous control, we computed the geometric mean of the ΔCT for RNU44 and U6 small RNAs (assay IDs 001094 and 001973, respectively).

For splice-specific quantitative PCR of the two different 3' ends, we used the Sybr Green system. Oligonucleotides were purchased from Bioneer, Inc. (Alameda, CA, USA). Real-time PCR reactions were performed at the UCSD Center for AIDS Research Genomics Core. The following primers were used: KCNMA1-v2-3-4-F: 5'-GTATGATAGTGTGCATGTGGTTGTC-3' and KCNMA1-v2-3-4-R: 5'-GGTCCGTCTGCTTATTTGCTGTTG-3'; KCNMA1-v1-F: 5'-TGAGTACCCACATGGCTAACCAGA-3' and KCNMA1-v1-R: 5'-AGGGCTGCGTGAGGTACAGT-3'. PCR assays were run in technical triplicate; in vitro experiments were performed in biological quadruplicate.

2.4. Alternative 3'UTR variants of KCNMA1

In order to analyze the 3' untranslated region (3'UTR) of the KCNMA1 gene, we followed protocols of the 3' Rapid Amplification of cDNA Ends (3'RACE) kit (Life Technologies) with a nested PCR approach (Frohman 1988). Mature messenger RNAs were reverse-transcribed using an adapter primer (AP) with 3' poly-T tail and the resultant cDNAs

subjected to nested PCR, UAP: 5'-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTT-3'. The following two "forward" gene-specific primers (GSP) were used for nested PCR, which corresponds the 3'-most exon that is present in all known splice variants: KCNMA1-GSP1: 5'-ACCACAATGCCGGCCAGTCC-3' and KCNMA1-GSP2: 5'-TCCCACTCGTCGAGTCCTCC-3'. In both reactions, for "reverse" primer, we used the abridged universal adapter primer (UAP), UAP: 5'-GGCCACGCGTCGACTAGTAC-3'. The products contained the alternative possible 3'UTR variants, which were separated by polyacrylamide gel electrophoresis in Tris-acetate-EDTA buffer; stained with SybrTM Gold (Life Technologies), and visualized under low energy UV light. Bands were excised, polyacrylamide fragments were crushed, and DNA was extracted by overnight diffusion in 0.3 mL Tris-EDTA buffer, 50°C, with shaking. After centrifuging at 1000 rpm for 10 minutes and removing supernatant containing 3'UTR splice variants, DNA was concentrated by ethanol precipitation and resuspended in 15 µL nanopure water. The 3'UTR fragments were sequenced using an Applied Biosystems 3100 Genetic Analyzer at the UCSD Center for AIDS Research Molecular Biology Core facility using the afore mentioned UAP and 9 ng input purified PCR product.

2.5. In Situ Hybridization

For qualitative (non-quantitative) assessment and visualization of the relative expression levels, cell-type specificity, and cytoarchitecture of the two variants of KCNMA1 3'UTR, we performed in situ hybridization. In order to detect the two different 3'UTR of KCNMA1 mRNA in formalin-fixed paraffin embedded (FFPE) tissue from a subset of subjects included in the original screening procedure, we used custom-designed in situ hybridization LNA probes 5'-conjugated to digoxigenin (DIG), purchased from Exiqon Corporation. FFPE slides from the frontal cortex were available in 9 of the 16 cases noted in Table 1, representing all three groups. We used the following two LNA probes: KCNMA-v1: DIG-5'-ATTACATCCATGCTTCTCCAGA-3' and KCNMA-v1-2-3: DIG-5'-AAAACCCAAAGCACCTGATA-3'.

We modified previously established protocols for in situ hybridization using DIG-labeled LNA probes on 10 µm sections of FFPE frontal cortex tissue (Nuovo 2010; Obernosterer). After deparaffinizing in xylene, sections were washed in 100% ethanol and air-dried. All aqueous solutions were diethylprocarbonate (DEPC)-treated. To unmask RNA sequences, sections were incubated with 2 µg/mL Proteinase K for 5 min at 37°C and washed in distilled water, then washed in ethanol, and air-dried. Sections were blocked by incubating In Situ Hybridization Buffer (Enzo Life Sciences, Farmingdale, NY, USA) with 500 µg/mL yeast RNA in a humidified chamber at 37°C. Probes were then diluted to 0.8 µM in hybridization buffer and 20 µL applied to the sections, which were sealed using a wax pen and coverslip, then sequentially incubated at 65°C for 30 min on a hot plate and a humidified chamber at 37°C for 15 hr. After removing coverslips, sections were washed in 0.2× SCC with 2% bovine serum albumin (BSA) pre-cooled to 4°C and a final incubation for 25 min in wash solution. For detection, alkaline phosphatase-conjugated anti-DIG F(ab) (Roche, Pleasanton, CA, USA), 1:200 dilution in 0.1 M Tris-HCl, 0.15 M NaCl, 1% BSA, pH 9.5 was applied on slides in a humidified chamber and incubated at room temperature for 30 min. Sections were washed, then equilibrated in a substrate solution without substrate (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5), then incubated in NBT/BCIP (Roche), which yielded a blue/violet precipitate. Sections were incubated for 30 min, then reaction was stopped by washing with distilled water. Sections were counterstained with pre-filtered Fast Red nuclear stain (Sigma) for 10 min, then washed in water and finally sealed and mounted using Cytoseal 60 (Electron Microscopy Science, Hatfield, PA, USA).

The stained slides were digitally scanned using a microscope slide scanner (Aperio ScanScope GL, Vista, CA, USA) equipped with a 20× objective lens (yielding a resolution of 0.5 μm^2 per pixel).

3. Results

3.1. MiR Screening in Autopsy Tissue

On the 384-well plate, six wells were dedicated to endogenous controls (four for U6, one each for RNU44 and RNU48) and two for negative controls (*A. thaliana* miR-159a and antisense to *H. sapiens* miR-155) and so 376 miR sequences were probed. Of the 376 sequences, 362 were detected in at least one sample in the tissue. Expression data are available in Supplementary Materials and were deposited to the Gene Expression Omnibus under the accession number GSE41952.

To determine the between-group differences, we performed ANOVA and Tukey HSD post-hoc test for expression levels of all 362 miRs and 260 met the criteria for significance (Benjamini-Hochberg corrected $P < 0.05$). A plot of the sorted ANOVA P -values and the Benjamini-Hochberg correction threshold is illustrated in Figure 1a. The same correction for multiple comparisons was applied to the Tukey HSD post-hoc tests, and the threshold and sorted P -values are shown in Figure 1b. A cell plot showing the relative expression of miRs, sorted by hierarchical clustering, of those significantly different based on the ANOVA is shown in Figure 1c. We have excluded those miRs that were not detected in two or more samples. These results suggest a significant impact of HIV infection on the miR repertoire in the brain, and a more modest, but present, effect of MA abuse.

These results serve as a starting point for further analysis. We took into consideration the prior evidence from the literature and available data: 1) that the miR of interest is expressed in the brain; 2) the miR is expressed in dopaminergic neurons; 3) the miR is involved in neuronal homeostasis, function, or differentiation in some way; and 4) prior evidence for some involvement in drug abuse and/or inflammation.

3.1.1. Neuropsychiatric and Clinical Correlates of miR Expression—In order to determine whether any of the neuropsychiatric or clinical data collected at the most recent visit correlated with brain expression of the miR's, we performed a multivariate correlation analysis. These data were available for participants in the UCSD HIV Neurobehavioral Research Program, which comprise both the HIV+ and HIV+MA groups ($n = 11$), but not the HIV-negative Control and the analysis therefore had 10 degrees of freedom. The Benjamini-Hochberg adjusted significance level was $\alpha < 0.00064$. We calculated the Spearman ρ correlation coefficient (with no *a priori* prediction for positive or negative correlations). The pairwise correlations, correlation coefficients, and P -values for tests meeting the criteria for significance are shown in Table 2 and all correlations are shown in the Supplemental Material.

3.2. In Vitro miR-9 Expression in Response to HIV and MA

We used an in vitro model of dopaminergic neurons to test whether HIV and MA affect miR expression in vitro. We chose three miRs that were significantly increased in the screening procedure that were known to be expressed in SH-SY5Y cells (Huang 2009), known to be expressed in the brain, and had some relevance or reference in the literature to HIV (Tatro 2010; Pacific 2012) or drug abuse (Pietrzykowski 2008; Wang 2009; Chandrasekar 2009). These were miR-9, miR-124a, and let-7d. SH-SY5Y cells were exposed to conditioned media from HIV-infected MDM at either *high* infection (1,700 pg/mL HIV p24) or *low* infection (900 pg/mL HIV p24) for up to 24 hr and RNA was isolated at various time points

for qPCR, as shown in Figure 2. MiR-9 was increased at 6 and 12 hr post-exposure to *high* HIV, but decreased at 1 and 6 hr post-exposure to *low* HIV. MiR-124 was consistently elevated after exposure to both *low* and *high* HIV through 24 hr. Like miR-9, let-7d was elevated only after exposure to *high* HIV. We used supernatant from two different cultures because of heterogeneity of in vitro virulence and wanted to determine if there was a dose effect with respect to the amount of viral particles being produced by a particular HIV-infected culture. It is more likely than downstream intracellular signaling events causing transcriptional changes in the neurons are a result of secreted factors from the infected MDM, reviewed in (Kraft-Terry 2009). These pro-inflammatory cytokines and chemokines include TNF- α , IL-1 β (Brabers 2006), CCL-17, and IL-8 (Saas 2002).

To test whether MA has an independent effect on expression of miR-9, miR-124a, and let-7d, we exposed SH-SY5Y cells to 10 μ M MA repeatedly for up to 96 hr, Figure 3. We found that all three miRs were significantly elevated. MiR-124a was increased at 24 through 96 hr. Let-7d was elevated from 48 to 96 hr. MiR-9 was increased after 48 and 72 hr.

3.3. Sequence Analysis KCNMA1 3'UTR and miR-9 Response Element

Based on evidence from the literature and our results, we investigated the potential functional consequences of miR-9 elevation. The KCNMA1 gene transcribes an alternatively spliced mRNA encoding the main, pore-forming α subunit of the BK channel, a large-conductance calcium- and voltage-activated potassium channel (also called MaxiK or Slowpoke). Previous reports have described regulation of differential splice variants of alcohol and stress - regulated exons for the BK Channel, termed *alcorex* and *strex*, respectively in the rat brain. The BK Channel has multiple polyadenylation sites within the 3'UTR, leading to 3'UTR heterogeneity. It was shown in rat cortical neurons that miR-9 controls expression of alternatively spliced BK channel mRNA variants by binding to a specific BK channel 3'UTR. We therefore hypothesized that the human BK channel would have differential splice variants with miR-9 response elements (miR-9 MRE) in the 3'UTR. Using human genome sequence databases for RefSeq Accession NG_012270.1 (Pruitt 2009), we performed a detailed analysis of the human KCNMA1 gene sequences and splice variants, and present the differential 3'UTRs in Figure 4.

Using a representative sample from the control group, we performed a 3' rapid amplification of cDNA ends (3'RACE) experiment and found two variants of the KCNMA1 3'UTR in the human frontal cortex, Figure 4a. Transcript Variants 1 and 5 (v.1 and v.5, respectively), differ only by the presence of Exon 33a in v.5, and share a common 3'UTR. V.2, v.3, v.4, and v.6 share the same coding sequence at and downstream of Exon 30, and therefore only appear as one band in the 3'RACE experiment. We will refer to these variants as 3'UTR(a), corresponding to the KCNMA1 v.1 and v.5 common 3'UTR; and 3'UTR(b), corresponding to v.2, v.3, v.4., v.6 common 3'UTR. The position of the forward primer for the 3'RACE experiment is shown in Figure 4b (black arrowhead). Sequencing revealed the presence of an miR-9 MRE in both 3'UTRs. The MRE on the longer variants, 3'UTR(a), had lower predicted minimum free energy (mfe) binding to the miR-9, -25.3 kcal/mol, which was located 85 basepairs from the stop codon. In contrast, the miR-9 MRE in 3'UTR(b) was located 1,111 basepairs from the stop codon and $mfe = -21.8$ kcal/mol.

We were interested in determining whether the two splice variants of the KCNMA1 3'UTR heteromers were differentially expressed in the human CNS. We therefore designed in situ hybridization probes that specifically recognized the two variants of the 3'UTR. The relative positions are shown in Figure 4b (black curved bar), and in detail in the Supplementary Material. Figure 5 illustrates a qualitative assessment for the distribution of KCNMA1 3'UTR(a) and KCNMA1 3'UTR(b) in the frontal cortex of representative subjects in the Control, HIV+, and HIV+MA groups. The KCNMA1 mRNA is expressed mainly in

neurons. The 3'UTR(a) is more abundant than 3'UTR(b). KCNMA1 3'UTR(a) reactivity was dramatically lower in the HIV+ frontal cortex (Figure 5b) and still lower in the HIV+MA frontal cortex, Figure 5c. Though 3'UTR(b) was less abundant than 3'UTR(a), differences between the three brains were attenuated compared to 3'UTR(a) (Figure 5 d, e, and f).

3.4. KCNMA1 Alternative 3'UTR Variant Expression in Response to MA and HIV

In order to determine whether miR-9 affects differential regulation of the BK channel variants with the two 3'UTR heteromers, we employed the in vitro dopaminergic neuronal model with exposure to HIV and MA, this time pre-incubating with LNA miR inhibitors. We designed primers for splice-specific quantitative PCR, a schematic showing relative positions of sequences recognized by the primers on the KCNMA1 3'UTRs are shown in Figure 4b and in detail in the Supplemental Material. When cells were pre-incubated with scrambled LNA sequences, exposure to both MA and HIV for 24 hr caused downregulation of both splice variants, as shown in Figure 6, black bars. The effect was more reproducible for those splice variants with 3'UTR(b), Figure 6b. For KCNMA1 3'UTR(a), treatment with HIV caused a decrease, though not statistically significant ($P = 0.07$). If the cells were pre-treated with anti-miR-9, treatment with HIV or MA the downregulation was attenuated for 3'UTR(a) (Figure 6a, grey bars). Anti-miR-9 had no effect on KCNMA 3'UTR(b) variants, Figure 6b.

4. Discussion

This study began as a retrospective autopsy project to test for differential miR expression in the HIV+ or HIV+MA brain compared to normal Controls. As a hypothesis-generating study, we identified many candidate miRs that were significantly increased or decreased compared to the normal brain (Figure 1). From a list of candidate miRs, we tested the following three: miR-9, miR-124, and let-7d; in vitro using a cell culture model for dopamine neurons with exposure to HIV and MA. These results verified the increase of all three small RNAs after exposure to the high-infection HIV (Figure 2) and MA (Figure 3). Based on recent reports in the literature, and a detailed analysis of the molecular databases, we then hypothesized that differential expression of the BK channel would be observed as a result of miR-9 upregulation under these conditions. Our results support a model whereby miR-9 can mediate drug tolerance through modulating stability of differential splice variants of the BK channel. The factors that cause changes in miR-9 expression can vary (Yuva 2011), and we present MA and HIV as two environmental factors. We then went back to human autopsy samples in order to determine whether we could observe the differential splice variants in the frontal cortex using histological analysis. We designed in situ hybridization probes specifically for the two possible 3'UTRs for the BK channel gene (KCNMA1). We found that the splice variants for the longer protein, KCNMA1 v.1 and v.6, were more abundant in the Control brain than the shorter splice variants; KCNMA1 v.2, v.3, v.4, and v.5; with decreased expression in the HIV+ and HIV+MA brains. The KCNMA1 3'UTRs were detected specifically in neurons as previously reported (Yuva 2011). In summary, our results suggest: 1) As reported elsewhere, miRs are likely to be key molecules in cellular pathways that form the CNS-physiological basis for addiction (Dreyer 2010), 2) HIV may induce changes in miR expression that make CNS neurons susceptible to drug tolerance and addiction, and 3) The human BK channel gene, KCNMA1, has splice variants that are differentially regulated by miR-9, which is elevated in neurons after exposure to HIV and MA.

Additionally, neuropsychiatric and clinical data were available for subjects in both groups of cases, HIV+ and HIV+MA-abusing, but not for controls. We tested for correlations between miR expression and neuropsychiatric domains (measures of cognition and also BDI) as well

as CD4, nadir CD4, and blood serum- and CSF- HIV RNA load. The correlations that met criteria for significance after Benjamini-Hochberg correction are listed in Table 2. While these results are preliminary, with a low sample size ($N = 11$), they serve as a starting point for hypothesis generation and testing for other molecular pathways of long-term effects of HIV infection on the CNS.

Our results are consistent with existing literature. A previous report of simian immunodeficiency virus determined that 45 miRs classified infection and several miRs had differential expression that correlated with CNS disease-associated cytokines (Witwer 2011). Our previous report with a smaller sample size also found changes in miR expression, and some of the same miR's were significantly different comparing HIV to Control (for example: miR-22, miR-122, miR-124) (Tatro 2010). Further studies are necessary to determine all of the downstream molecular consequences of the altered miR expression. The dataset published here would be useful for a predictive bioinformatics analysis to determine what common pathways may be affected by examining the predicted targets of the significantly differentially regulated miRs.

Our finding that splice variants of BK channel mRNA are differentially regulated by miR-9 in response to MA is consistent with previous reports in rat (Pietrzykowski 2008). While Pietrzykowski et al. (2008) discovered a third long splice variant in rats, our results from the 3'RACE experiment and sequencing were consistent with only two forms of the 3'UTR covering six splice variants as published in the literature under RefSeq Accession NG_012270.1 (Pruitt 2009). Since preparation of this manuscript began, three other splice variants for the gene encoding the BK channel have been described in the National Library of Medicine RefSeq database composed of only Exon 1 plus three variable Exon 2's. The function or expression of this relatively short polypeptide is not described. The primers for the 3'RACE experiment, quantitative PCR experiments, and in situ hybridization probes in our study would not have detected or been affected by these transcripts.

The BK channel (named as "Big K⁺") has the highest single-channel conductance of all the selective K⁺ channels. It can be activated either by membrane depolarization or intracellular Ca²⁺, or both synergistically; and therefore serves as an integrator of regulatory processes (Lancaster 1987; Storm 1987; Faber 2003). In neurons, the BK channel is thought to contribute to the fast phase of after-hyperpolarization potential, which is a chief determinant of the refractory period and therefore maximum firing rate (Salkoff 2006). The splice variants of this gene code for regulatory regions in the cytoplasmic C-terminal tail, reviewed in (Salkoff 2006). Pietrzykowski et al. (2008) demonstrated computationally that tetramers of the BK channel containing only the *alcorex* variant were only 15% responsive to alcohol-potential. We would therefore hypothesize, based on our results, that the electrophysiology of neurons is affected by factors contained in HIV-conditioned media (Gendelman 2009) and drugs of abuse like MA (Brady 2003; Branch 2012), mediated through transcriptional increase in miR-9. Although neuronal adaptation to drugs of abuse is multifactorial and complex, miR-9 mediated changes in BK channel isoform expression may be a common mechanism. Electrophysiology measurements from striatal neurons in miR-9 knockout or overexpression transgenes mouse models would be needed to support this conclusion.

In addition to miR-9, we showed in vitro that the expression of two other miRs, miR-124 and let-7d, is increased in SH-SY5Y cells after exposure to MA and HIV. Both of these miRs are implicated in cocaine abuse (Chandrasekar 2009). BDNF and D3-dopamine receptor (D3R) both are predicted targets of miR-124 and let-7d; and which were experimentally shown to be downregulated in response to overexpression of either miR-124 or let-7d (Chandrasekar 2009). MiR-124 is neuron enriched in the brain and stably

expressed in neuronal cells throughout the adult brain and contributes to defining cellular phenotype by regulating gene activity (Clark 2010). In a rat model of attention deficit - hyperactivity disorder, let-7d regulated the expression of galectin-3, an enhancer of cyclic AMP response element binding protein (Wu 2010). Further studies on the downstream effects of miR-124 and let-7d in HIV and MA are warranted.

We employed a “chronic” in vitro MA-exposure model, whereby the cells were exposed for 3 hr on a daily basis. The concentration of MA used, 10 μ M, was not known to cause cell death: we were interested in discovering differential miR expression and a downstream mechanism of neuronal changes that may facilitate drug tolerance or addiction. For HIV exposure in vitro we employed a conditioned-media approach; conditioning the media with supernatant from HIV-infected MDM (Pulliam 1991), using supernatants from both high and a low p24-secreting cultures. We chose this approach rather than exposing to recombinant viral envelope proteins or purified virus alone in order to recapitulate an environment of cytokines and chemokines being produced by infected MDMs and affecting intracellular signaling in the neurons and downstream miR expression. We did not include a co-exposure condition with both HIV and MA combined because we preferred to remove and re-expose the MA-containing media daily. Since the conditions alone produced similar results, this procedure would have produced an unjustifiably large volume of infectious material.

In our final experiment, we were interested in determining if HIV and MA, with or without an LNA inhibitor to miR-9, would affect expression of the BK channel splice variants of KCNMA1 3'UTR (a) and KCNMA1 3'UTR(b). When the cells were treated with a scrambled LNA sequence, both groups of splice variants were decreased on average. However, for KCNMA1 3'UTR(a), the effect was not quite significant, with $P = 0.07$; while $P < 0.01$ for KCNMA1 3'UTR(b). The non-significant result of the Student's *t* - test is likely an artifact of the large variance of the Control samples.

There is inherent limitation to retrospective autopsy studies due to a single timepoint sampling, after physiological processes have occurred. Nevertheless, there is added value in making correlations with neuropsychological and clinical outcomes in the subjects measured within six months of death, and then generating working hypotheses. While our sample size was adequate to obtain statistical significance, our cohort was relatively small ($N = 5$ Controls, $N = 6$ HIV+, $N = 5$ HIV+MA), but nonetheless, very well characterized. Our large dataset is not a definitive statement on the miR repertoire in the HIV+ brain, but rather serves as a hypothesis generating dataset for further verification and testing. It can be accessed via the National Library of Medicine Gene Expression Omnibus (GEO Access Number GSE41952).

In summary, we present here evidence for miR-9 mediated neuronal changes as a result of exposure to MA & HIV, specifically differential expression of KCNMA1 splice variants. The miR expression data from the retrospective autopsy study are available publicly via the Gene Expression Omnibus for researchers interested in pursuing further studies on miR in the HIV+ brain. As reported elsewhere (Sartor 2012), these results support a model for a molecular mechanism of a common pathway for drug adaptation in CNS and HIV susceptibility to addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MiRs are significantly differentially expressed among Control, HIV+, and HIV+MA groups

Two-hundred-and-sixty miRs met significance criteria after Benjamini-Hochberg correction (A) by ANOVA. After post-hoc Tukey HSD testing for differential expression among all possible combinations, 464 tests met criteria for significance (B). Plotted are the sorted P values (circles) and the Benjamini-Hochberg significance threshold (line). The $-\Delta\Delta CT$ values were used to sort the miRs by hierarchical clustering of correlation with one another and is illustrated by a cell plot (C) with color coding for differential expression. Any miRs that were undetected in > 1 sample were excluded. White color corresponds to the mean expression, magenta corresponds to lower, and green corresponds to higher; with color intensity proportional to magnitude of the difference. This plot demonstrates a clear effect of HIV in the miR expression in the CNS. The y-axis labels indicate the subject ID and are sorted by Group. The majority of miRs that were significantly different by ANOVA were decreased expression, with some notable exceptions like miR-9, miR-124, and let-7d, which were elevated.

Figure 2. MiR-9, miR-124, and let-7d are significantly increased in SH-SY5Y cells after exposure to 10% conditioned media from HIV-infected MDM

Terminally differentiated SH-SY5Y cells were exposed to *high* p24 (1,700 pg/mL) (black bars) and *low* p24 (900 pg/mL) (grey bars) conditioned media from HIV-1 infected MDM, and RNA isolated and quantified by Taqman qPCR for miR-9, miR-124, and let-7d; with U6 and RNU44 as endogenous controls and calibrated to the average *time 0* values. The Log (RQ) values are plotted. * ($P < 0.05$ compared to *time 0*), error bars indicate standard deviation of four independent samples.

Figure 3. MiR-9, miR-124, and let-7d are significantly increased in SH-SY5Y cells after exposure to MA

Terminally differentiated SH-SY5Y cells were exposed to 10 μ M MA for three hr repeated daily and RNA isolated in 24 hr increments. MiR-9, miR-124, and let-7d were quantified by Taqman qPCR; with U6 and RNU44 as endogenous controls and calibrated to the average *time 0* values. The Log (RQ) values are plotted. * ($P < 0.05$ compared to *time 0*), error bars indicate standard deviation of four independent samples.

Figure 4. Response elements to miR-9 in alternative splice variants of the BK channel 3'UTR
 (A) Polyacrylamide gel electrophoresis of BK channel 3'RACE and sequence analysis reveals alternate 3'UTR of the KCNMA1 mRNA. (B) Schematic constructed based on our results and reference (66). Human BK gene information lists options of exonal assembly of the 3' ends of the coding sequence and 3'UTR. Numbers in boxes represent exons. Letters represent the N-terminal amino acid sequences and (*) stop codon. Note that KCNMA1 alternative splices variants 1 and 5 (v.1 and v.5) are different lengths because v.5 contains an additional exon 33a. Variants 2, 3, 4, and 6 contain an alternate variant of exon 33 (33b), which contains a stop codon, and so they lack exon 34 and have a distinct, shorter 3'UTR from v.1 and v.5. The KCNMA1 v.1 and v.5 have a 8,270 bp 3'UTR (a) and an miR-9 MRE positioned only 85 bp from the stop codon, in contrast to the 3'UTR (b) for the other variants, where the MRE is positioned 1,111 bp from the stop codon. The black arrowhead indicates the position of the forward 3'RACE primer in exon 31. Black arrowheads illustrated in each 3'UTR denote the positions of two distinct primer pairs to quantify the two different 3'UTRs. A black bar denotes relative position of the in situ hybridization probe used to visualize the distinct KCNMA1 3'UTR variants in the human brain, shown in Figure 5. (C) Juxtaposition of the two 3'UTR variants against miR-9 at the miR-9 MRE illustrating stronger binding for v.1 and v.5 mRNA. Bases paired by Watson-Crick bond are depicted by a black line and G:U pairs depicted by two dots. The entire seed sequence, denoted by the boxes nucleotides, is aligned to the mRNA for 3'UTR (a), but not for 3'UTR (b). See Supplementary Materials for primer, probe, and MRE positions; and see also Materials and Methods for primer sequence information.

Figure 5. In situ hybridization depicting qualitative expression levels and location of the two BK channel 3'UTR variants in the human brain

Samples were obtained from a subset from those included in the initial screening procedure (Table 1). KCNMA1 3'UTR(a) is more abundant in Control frontal cortex (A) than KCNMA1 3'UTR(b) (D). In both the HIV+ (B) and HIV+MA (C) brains, KCNMA1 3'UTR(a) is less abundant. In the HIV+MA brain, KCNMA1 3'UTR(b) (F) is lower than in both the Control (D) and HIV+ (E) brains. As expected, hybridization for both variants of KCNMA1 is neuronal.

Figure 6. MiR-9 mediates downregulation of KCNMA1 3'UTR v.1-5 but not 3'UTR for v.2-3-4-6 in SH-SY5Y cells exposed to HIV and MA

Incubation of differentiated SH-SY5Y cells with HIV and MA caused a decrease in expression of KCNMA variants containing the 3'UTR(a) (A), and for 3'UTR(b) (B). Pre-incubation with Mercury LNA inhibitor specific to miR-9 stopped KCNMA1 mRNA downregulation. Black bars - cells pre-incubated with scrambled LNA sequence, grey bars - cells pre-incubated with anti-miR-9 LNA sequence. Comparisons noted in (A) $P = 0.07$; and for comparisons noted in (B) $P < 0.01$ by Student's t test.

Table 1

Age, Gender, and Ethnicity of all cases and controls included in the screening study.

Controls (n = 5)						Cases: HIV+ (n = 6)						Cases: HIV+MA (n = 5)					
<u>I.D.</u>	<u>Age</u>	<u>Gender</u>	<u>Ethnicity</u>	<u>I.D.</u>	<u>Age</u>	<u>Gender</u>	<u>Ethnicity</u>	<u>HIVVL</u>	<u>CD4</u>	<u>I.D.</u>	<u>Age</u>	<u>Gender</u>	<u>Ethnicity</u>	<u>HIVVL</u>	<u>CD4</u>		
CC191	20	M	Black	CE147 [†]	37	M	White	8.8×10 ⁵	22	CA115	37	M	White	2.8×10 ⁵	23		
CA293 [†]	26	M	White	CC125	38	M	White	7.2×10 ⁴	1	CA248	45	M	White	7.8×10 ⁵	7		
CD101	43	M	Hispanic	CC106 [†]	39	M	Hispanic	570	79	CA176 [†]	32	M	Hispanic	NA	8		
CD102 [†]	48	M	White	CC120 [†]	45	M	Black	5.0×10 ⁴	1	CA186	61	M	White	7.2×10 ⁴	28		
CA295 [†]	53	M	White	CA143	52	M	White	165	NA	CE156 [†]	35	M	Hispanic	3.3×10 ⁴	0		
				CG103 [†]	40	M	Hispanic	0	53								
Mean	38	100%M	3 W 1 B 1 H	Mean	42	100% M	3 W 1 B 2 H	1.7(3.3)×10 ⁵	53.5 (59.6)	Mean	39	100% M	3 W 2 H	2.3(3.1)×10 ⁵	13.8 (10.9)		

I.D. - Coded study identifier; HIV VL - blood viral load (copies/mL); CD4 - blood CD4 count per μ L; M - Male; W - White, H - Hispanic, B - Black.

[†] Paraffin embedded sections for these cases were available for in situ hybridization analysis.

Table 2
Neuropsychiatric and clinical correlates of miR CNS expression

Neuropsychiatric and clinical data for all HIV-positive subjects (n = 11) were obtained following the HIV Neurobehavioral Research Center battery and clinical assessment laboratory within six months of death, then compared to post-mortem expression of miR. Testing for correlation, comparing clinical parameters with measured miR levels and applying Benjamini-Hochberg correction for multiple comparisons.

Variable	by Variable	Spearman ρ	Prob> ρ
Age	hsa-miR-491-5p-4381053	-0.7954	< 0.0001
BDI	hsa-miR-199a-5p-4373272	0.7782	0.00009
	hsa-miR-302a-4378070	0.7101	0.0005
	hsa-miR-452-4395440	0.7025	0.0006
CSF_RNA_LOG	hsa-miR-125a-5p-4395309	0.7829	0.0001
	hsa-miR-145-4395389	0.766	0.0002
	hsa-miR-491-5p-4381053	0.7322	0.0006
	hsa-miR-93-4373302	0.7449	0.0004
EXEC_DS	hsa-miR-192-4373108	0.7618	< 0.0001
	hsa-miR-331-5p-4395344	0.7468	< 0.0001
	hsa-miR-598-4395179	0.7134	0.0002
GDS	hsa-miR-191-4395410	-0.6917	0.0004
LEARN_DS	hsa-let-7a-4373169	-0.6846	0.0004
	hsa-let-7b-4395446	-0.7442	< 0.0001
	hsa-miR-184-4373113	-0.7169	0.0002
	hsa-miR-383-4373018	-0.7181	0.0002
MEMORY_DS	hsa-miR-134-4373299	-0.691	0.0004
	hsa-miR-433-4373205	-0.6791	0.0005
MOTOR_DS	hsa-miR-101-4395364	-0.7072	0.0002
	hsa-miR-191-4395410	-0.6832	0.0005
PLA_RNA	hsa-miR-103-4373158	-0.6724	0.0006
	hsa-miR-125b-4373148	-0.7007	0.0003
	hsa-miR-130a-4373145	-0.6826	0.0005
	hsa-miR-26a-4395166	-0.7166	0.0002
	hsa-miR-34a-4395168	-0.845	< 0.0001
WRKMEM_DS	hsa-miR-433-4373205	-0.7636	< 0.0001