

Increased *OPRM1* DNA Methylation in Lymphocytes of Methadone-Maintained Former Heroin Addicts

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The μ -opioid receptor is the site of action of opiates and opioids. We examined whether there are differences in cytosine:guanine (CpG) dinucleotide methylation in the *OPRM1* promoter between former heroin addicts and controls. We analyzed methylation at 16 CpG dinucleotides in DNA obtained from lymphocytes of 194 Caucasian former severe heroin addicts stabilized in methadone maintenance treatment and 135 Caucasian control subjects. Direct sequencing of bisulfite-treated DNA showed that the percent methylation at two CpG sites was significantly associated with heroin addiction. The level of methylation at the -18 CpG site was 25.4% in the stabilized methadone-maintained former heroin addicts and 21.4% in controls ($p=0.0035$, generalized estimating equations (GEE); $p=0.0077$, t -test; false discovery rate (FDR) = 0.048), and the level of methylation at the $+84$ CpG dinucleotide site was 7.4% in cases and 5.6% in controls ($p=0.0095$, GEE; $p=0.0067$, t -test; FDR = 0.080). Both the -18 and the $+84$ CpG sites are located in potential Sp1 transcription factor-binding sites. Methylation of these CpG sites may lead to reduced *OPRM1* expression in the lymphocytes of these former heroin addicts. *Neuropsychopharmacology* (2009) **34**, 867–873; doi:10.1038/npp.2008.108; published online 23 July 2008

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INTRODUCTION

Chronic heroin use disrupts multiple physiological systems, contributing to addiction and relapse. Vulnerability to develop heroin addiction is due to drug-induced and environmental factors, as well as genetic factors (Kreek *et al*, 2005). Among the nongenetic factors may be integrated epigenetic factors including DNA methylation.

Cytosine methylation in genomic DNA is a common epigenetic mechanism, which occurs through the addition of a methyl group by DNA methyltransferases to cytosine residues in cytosine:guanine (CpG) dinucleotides. CpG dinucleotides are often localized in clusters referred to as 'CpG islands' (Antequera and Bird, 1993). There are approximately 45 000 CpG islands in the human genome, with many found in the promoter region of some genes, generally from upstream of the transcription start site to within the first exon (Gardiner-Garden and Frommer, 1987). DNA methylation in promoter regions is associated with decreased gene expression (Alikhani-Koopaei *et al*, 2004; Andria and Simon, 1999; Douet *et al*, 2007; Iguchi-Arigo and Schaffner, 1989; Jaenisch and Bird, 2003; Michelotti *et al*, 2007). Many transcription factor-binding

sites, such as those for Sp1, have CpG dinucleotides, and when these are methylated, they display altered binding to their cognate transcription factors (Alikhani-Koopaei *et al*, 2004; Douet *et al*, 2007; Michelotti *et al*, 2007).

Methylation has been shown to be altered by drugs of abuse. In humans, overall DNA methylation was higher in genomic DNA from lymphocytes of alcoholics than from controls (Bleich *et al*, 2006; Bonsch *et al*, 2004), with a concomitant decrease in the expression of the DNA methyltransferases DNMT-3a and DNMT-3b (Bonsch *et al*, 2006). This same group reported increased DNA methylation in the promoter region of the homocysteine-induced endoplasmic reticulum protein gene *HERP* (Bleich *et al*, 2006) and the α -synuclein gene *SNCA* in alcoholics (Bonsch *et al*, 2005). In human lymphoblast cell lines, average overall DNA methylation at the monoamine oxidase a gene promoter was found to be significantly associated point-wise, but not overall experiment-wise, with alcohol dependence and nicotine dependence in women, but not in men (Philibert *et al*, 2008). Methylation levels at 11 CpG sites were also associated point-wise, but not experiment-wise, with nicotine symptom counts. In cultured mouse cortical neurons, chronic ethanol was reported to induce demethylation of CpG islands of the NMDA receptor subunit gene *nr2b* with a concomitant increase in *nr2b* expression (Marutha Ravindran and Ticku, 2005). Methylation of the protein phosphatase 1 gene *pp1* and demethylation of the reelin gene *reln* (encoding a protein necessary for neuronal cell signaling) in the mouse

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hippocampus were reported to change after 1 h in response to fear conditioning (Miller and Sweatt, 2007). Expression of *cFos*, *bdnf*, and *cdk5* is increased by cocaine; a single cocaine injection was reported to cause chromatin remodeling at the *cFos* promoter in rat striatum, and at the *bdnf*, and *cdk5* promoters after chronic cocaine administration (Kumar *et al*, 2005). Murine studies suggest that remodeling by chronic cocaine administration was through a decrease in the histone deacetylase HDAC5 function (Renthal *et al*, 2007). In rats, cocaine administration produced decreased histone methylation in the prefrontal cortex (Black *et al*, 2006). Maternal cocaine administration in rats increased methylation at a CpG site in the promoter of the protein kinase C ϵ (*PKC ϵ*) gene in fetal heart, and decreased *PKC ϵ* mRNA and protein levels, presumably through a decrease in Ap1 transcription factor binding (Zhang *et al*, 2007). DNA methylation of the glucocorticoid receptor promoter in rats was shown to be altered in offspring by maternal behavior (Weaver *et al*, 2004). In humans, the *RELN* promoter was found to be hypermethylated, with reduced expression in the brains of schizophrenic patients (Abdolmaleky *et al*, 2005; Grayson *et al*, 2005). Mutations in the transcriptional repressor MeCP2, which binds methylated CpG dinucleotides, cause neuropsychiatric abnormalities including Rett syndrome (Chahrour and Zoghbi, 2007).

The μ -opioid receptor is the site of action of β -endorphin, morphine, and methadone (Kreek *et al*, 2005). A common variant in the *OPRM1* gene, A118G, encodes an asparagine to aspartic acid substitution. This variant was associated with vulnerability to develop heroin addiction (Bart *et al*, 2004), and was shown to alter the function of the hypothalamic–pituitary–adrenal axis (reviewed in Kreek, 2006 and Kreek *et al*, 2005).

Herein, we have examined the methylation levels of 16 CpG sites in the *OPRM1* promoter region in former severe heroin addicts in methadone maintenance pharmacotherapy and in controls. We know of no other study in humans that has reported a significant association, after correcting for multiple testing, of DNA methylation level at a specific CpG site in any gene with any addiction.

MATERIALS AND METHODS

Subjects and Phenotyping

Our sample consisted of 194 former severe heroin addicts and 135 control subjects ($N=329$), all of Caucasian

ethnicity, drawn from consecutive volunteers (January 1995–June 2007) in genetic studies conducted by the Laboratory of the Biology of Addictive Diseases at The Rockefeller University who met the inclusion criteria defined below (Table 1). Subjects were recruited from clinical resources in New York City, and from referrals, newspaper advertisements, and posted notices. All subjects gave specific consent for genetic studies and signed an informed consent approved by The Rockefeller University Hospital Institutional Review Board. Ethnicity was based on the ethnic/cultural background of the subjects, their parents, grandparents, and great-grandparents.

The Addiction Severity Index (ASI; McLellan *et al*, 1980) was administered and urine analyses were performed for multiple drugs of abuse on all subjects. Former severe heroin addicts ($N=194$) were long-term heroin addicts who met Federal guidelines for methadone maintenance treatment (1 year or more of daily multiple injections of heroin or other opiates) (Rettig and Yarmolinsky, 1995). Subjects in the control group ($N=135$) had (1) no illicit drug use for more than 6 months (except cannabis), (2) no excessive use of cannabis (three or more times per week for more than 4 years), (3) no previous history of alcohol drinking to intoxication (three or more times per week for 6 months or more), and (4) no alcohol intoxication or illicit drug use (except cannabis) in the last 30 days.

Isolation of Lymphocyte DNA

DNA was extracted from peripheral blood lymphocytes using a salting out procedure or from whole blood using the Gentra Puregene kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Determination of Percent Methylated Cytosine

Genomic DNA (300 ng) was treated with sodium bisulfite using the EZ-96 DNA Methylation Kit D5004 (Zymo Research, Orange, CA) according to the manufacturer's protocol. The final bisulfite-treated DNA was eluted in 40 μ l M-Elution Buffer.

Primers for amplifying the upstream *OPRM1* CpG island using bisulfite-treated DNA were designed using Methyl Primer Express v1.0 (Applied Biosystems, Foster City, CA) and Vector NTI Advance 10 (Invitrogen, Carlsbad, CA). Amplification was performed with 1 μ l bisulfite-treated DNA, 1 μ M of each primer (primer A: 5'-TTTTTTTTTGTTT

Table 1 Sample Categorization

Classification	Starting sample			Samples for analysis of CpG methylation		
	Male	Female	Total	Male	Female	Total
Former severe heroin addicts stabilized in methadone treatment	117	77	194	99	68	167
Controls	66	69	135	50	53	103
Total	183	146	329	149	121	270

Abbreviation: CpG, cytosine:guanine.

Samples for analysis of CpG methylation are those that had sequencing/ESME analysis carried out in both directions and had a correlation between these analyses ≥ 0.7 .

TAGTTAGG-3' and primer B: 5'-CAAATTACCATCTAAAT AAA-3'), 250 μ M each of dATP, dCTP, dGTP, and TTP, 50 mM KCl, 4 mM MgCl₂, 0.625 U AmpliTaq Gold (Applied Biosystems), and 10 mM Tris-HCl (pH 8.3) in 50 μ L. Amplification consisted of 5 min at 95°C, 40 cycles of 15 s at 95°C, 15 s at 52°C, and 30 s at 72°C, followed by a final elongation step at 72°C for 7 min. A second round of amplification was performed as above using 1 μ L of the product of the first amplification using the nested primers C: 5'-TGTAAGAAATAGTAGGAGTTGTGGTAG-3' and D: 5'-AA TAAACAAATTAACCCAAAAACC-3'. Amplification consisted of 5 min at 95°C, 40 cycles of 15 s at 95°C, 15 s at 58°C, and 30 s at 72°C, followed by a final elongation step at 72°C for 7 min.

Cloning of PCR Fragments

Amplified DNA fragments, 229 bp in size, were cloned using the TA Cloning Kit into the pCRII plasmid (Invitrogen). DNA from isolated clones was sequenced in both directions using M13 forward and reverse primers and the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Samples were run on an ABI Prism 3700 capillary sequencer (Applied Biosystems).

Direct Sequencing

Unincorporated nucleotides and primers were treated by mixing 4 μ L of the final PCR reaction mixture with 1 μ L ExoSAP-IT (USB Corp., Cleveland, OH) followed by incubation at 37°C for 30 min and 80°C for 15 min. For sequencing, 1 μ L ExoSAP-IT-treated DNA was added to 11 μ L 8 pM primer C or D. Sequencing was performed at Genewiz, Inc. (South Plainfield, NJ) on an ABI 3730 XL sequencer using the ABI 3730POP7SR basecaller (Applied Biosystems).

Epigenetic Sequencing Methylation Analysis Software

Trace files (.ab1) were analyzed using the Epigenetic Sequencing Methylation Analysis (ESME) version 3.2.1 software from Epigenomics AG (Berlin, Germany) (Lewin *et al*, 2004). The ESME software was run on a virtual machine (VMware player running on Windows XP) running Red Hat Enterprise Linux 4. The percent methylation calls by the ESME were reviewed by two independent researchers who visually inspected all the methylation calls using the associated electropherograms generated by the ESME software.

Sequence Analysis

The amplified region of *OPRM1* was analyzed for predicted transcription factor-binding sites using Transcription Element Search System (TESS; Schug and Overton, 1977).

Statistical Analysis

For each sample, for which a forward and reverse sequence file was obtained ($N=304$), a correlation value was calculated between the arcsine of methylation frequency in the forward and reverse directions. Samples with a correlation ≥ 0.7 ($N=270$) were included in the analysis of percent methylation at each CpG site (Table 1).

To determine if the mean level of methylation at each CpG dinucleotide site differed between case and control subjects, two different tests were performed. First, the mean of the forward and reverse values was calculated for each CpG site for each sample. A *t*-test was performed using these mean values to examine differences between case and control groups at each CpG site. Since there were two measurements for each individual, logistic regression using generalized estimating equations (GEE), which accounts for the correlation between the forward and reverse measurements, was also performed (Halekoh and Hojsgaard, 2006). Both these tests were two tailed.

Individual results were first evaluated for point-wise significance ($p < 0.05$). To correct for multiple testing issues, we then computed the false discovery rate (FDR) for each of the *p*-values (Benjamini and Hochberg, 1995).

RESULTS

We determined the methylation state of a CpG island in the promoter of the *OPRM1* gene in 194 former severe heroin addicts stabilized in methadone maintenance treatment and 135 controls. Two CpG islands are found from 400 nucleotides upstream to 1000 nucleotides downstream of the transcription start site (Figure 1). The first CpG island, which we studied, is located from nucleotides -93 to 27 (relative to the A of the ATG translation start site). We examined 16 CpG dinucleotides located at nucleotide -93, -90, -80, -71, -60, -50, -32, -25, -18, -14, -10, +12, +23, +27, +53, and +84 of the *OPRM1* gene. The CpG dinucleotides -18 and -14 are located in one potential Sp1-binding site, the +12 CpG site in a second binding site, and the +84 CpG dinucleotide in a third.

Comparison of Results Obtained with Two Methods

Two methods were employed to determine cytosine methylation at the CpG dinucleotides. In both methods, DNA is treated with bisulfite to convert unmethylated cytosines to uracil, leaving methylated cytosines unmodified. The region containing the first CpG island was PCR amplified, and a second round of amplification was performed using nested primers. The first method employs cloning the amplified fragments, isolating individual clones, and sequencing the amplified inserts. The second method employs direct sequencing of the amplified fragment. Trace files are processed using the ESME software (Lewin *et al*, 2004), which corrects trace files for quality problems, incomplete conversion, imbalanced or overscaled signals, and basecaller artifacts to provide quantitative measurement of cytosine methylation.

The ESME Direct Sequence Analysis Method is Correlated with Clonal Analysis

Five DNA samples chosen at random from cases and five from controls were analyzed using the cloning method. The methylation pattern of each is shown in Figure 2a. We also analyzed, with this method, five selected samples of cases previously evaluated by sequencing/ESME analysis (Figure 2b). One had the highest percent methylation at the -25 CpG site (1, Figure 2b), two had the highest

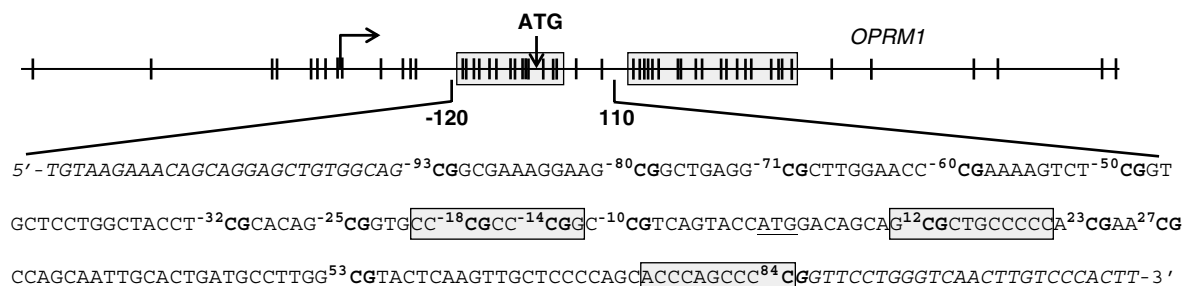


Figure 1 The *OPRM1* promoter region. Schematic of the *OPRM1* gene promoter region (from 400 nucleotides upstream to 1000 nucleotides downstream of the transcription start site) is presented in the upper diagram. The two CpG islands are boxed. The CpG dinucleotides are indicated as |. The major transcription start site (→) is located at -253 upstream of the ATG translation start site. The sequence of the amplified CpG island is displayed below with the sequences corresponding to the nested PCR primers in italics. The 16 CpG sites analyzed for cytosine methylation (bold) with their position relative to the A (chr6: 154 402 373; NCBI Build 36.1, March 2006) of the ATG translation start site (underlined) are indicated. The three putative Sp1 transcription factor-binding sites are boxed.

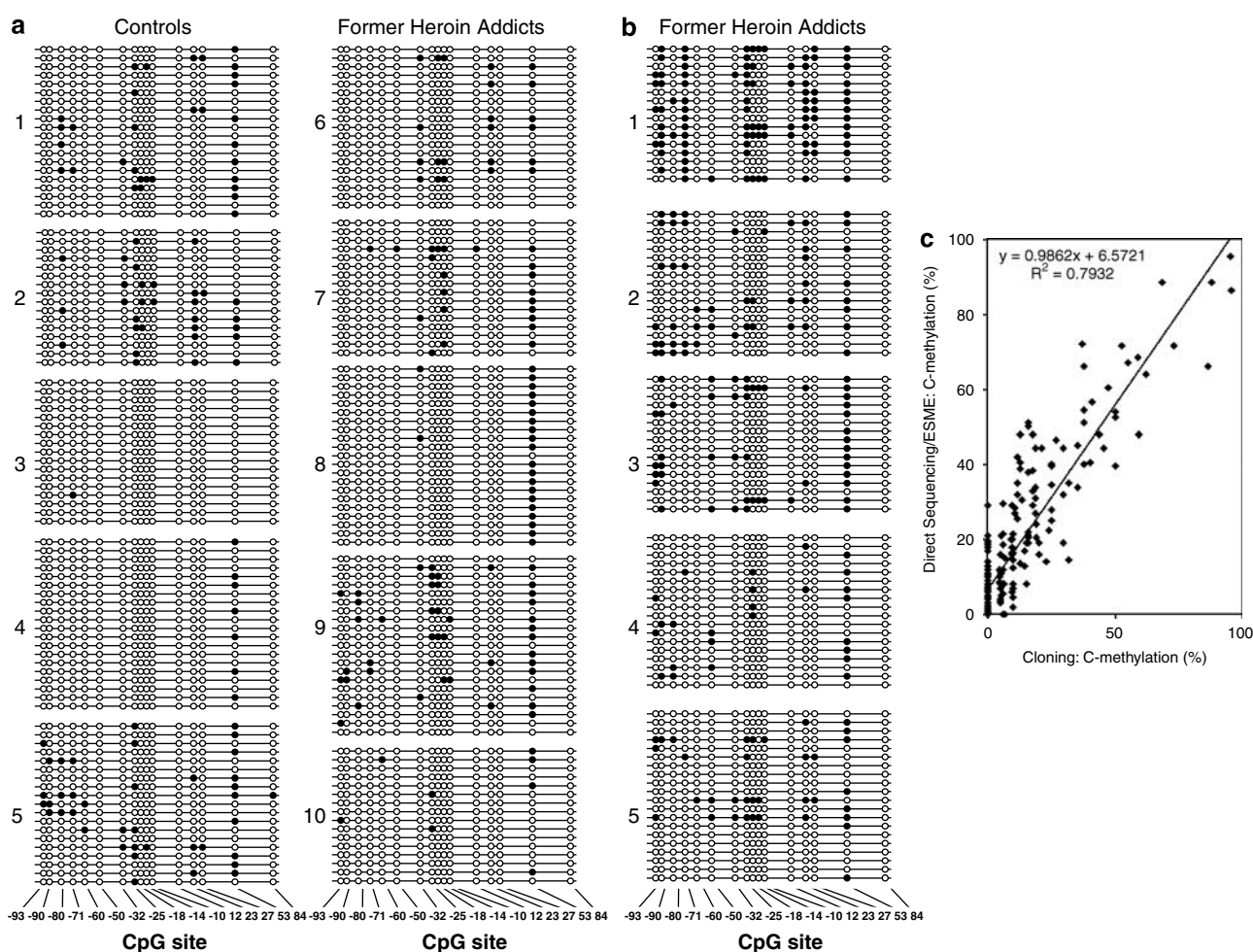


Figure 2 Methylation at the 16 CpG sites. (a) Analysis of cloned amplified bisulfite-treated DNA from five randomly selected controls and five randomly selected former heroin addicts. Solid circles are methylated CpG sites. The location of these sites is shown relative to their location in the amplified *OPRM1* region. (b) Analysis of cloned bisulfite-treated DNA from five former heroin addicts selected for varying levels of methylation. (c) Correlation of percent cytosine methylation determined by sequencing/ESME (ordinate) and by cloning (abscissa). The correlation is derived from data using clones 1, 2, 4, and 6–10 of (a), and 1–3 and 5 of (b), as these samples had a forward to reverse correlation ≥ 0.7 by sequencing/ESME analysis.

methylation at the +84 CpG site (2 and 3), one had intermediate levels of methylation across the CpG sites (4), and the final sample (5) had low levels of methylation across the sites. All 15 samples were analyzed

by sequencing/ESME analysis. Twelve samples had a forward to reverse correlation ≥ 0.7 ; for these, the correlation of the percentage of methylation was $R^2 = 0.79$ (Figure 2c).

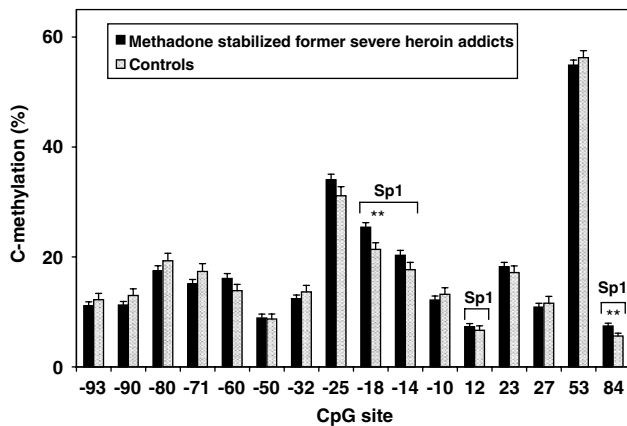


Figure 3 Percent methylation of 16 CpG dinucleotides in the *OPRM1* promoter region in former severe heroin addicts and controls. CpG sites in Sp1 binding sites are indicated. ** $p < 0.01$. Error bars represent SEM.

Former Severe Heroin Addicts have Significant Differences in CpG Methylation than Controls

A total of 167 case and 106 control samples, with a correlation of ≥ 0.7 between the forward and reverse analyses, were analyzed further (Table 1). Methadone-stabilized former heroin addicts were found to have significantly higher methylation than controls at two CpG sites (Figure 3). At the -18 CpG site, the level of methylation was 25.4% in cases and 21.4% in controls ($p = 0.0035$, GEE; $p = 0.0077$, t -test). At the $+84$ CpG site the level of methylation was 7.43% in cases and 5.62% in controls ($p = 0.0095$, GEE; $p = 0.0067$, t -test). The difference in methylation at the -18 CpG site was significant experiment-wise when evaluated by FDR (0.048), but was only point-wise significant for the $+84$ CpG site (0.080) (Benjamini and Hochberg, 1995).

DISCUSSION

This study demonstrates hypermethylation of two CpG dinucleotide sites in the *OPRM1* promoter region in former heroin addicts. Hypermethylation of the -18 and the $+204$ CpG sites in the former heroin addicts is likely to reduce *OPRM1* gene expression since DNA hypermethylation has been shown in other studies to reduce gene expression. When hypermethylated, transcription factor-binding sites have reduced binding affinity for their cognate transcription factors. Since both the -18 and the $+204$ CpG sites are in potential Sp1 binding sites, the hypermethylation may decrease binding and transactivation by Sp1. The effect of the hypermethylation at the -18 and $+204$ CpG sites in the *OPRM1* promoter region on transcription binding should be evaluated in future studies.

DNA hypermethylation of specific CpG sites in the *OPRM1* promoter region found in former heroin addicts could be the result of several independent, but by no means exclusive, mechanisms. Increased DNA methylation could be a predisposing factor for vulnerability to develop heroin addiction. Such a methylation state might be inherited through genomic imprinting. Hypermethylation also may be a result of life events occurring before trying heroin.

Finally, chronic heroin use or long-term methadone pharmacotherapy may modulate DNA methylation. Long-term heroin addiction, with its repeated cycles of on/off effects due to heroin's short half-life, may downregulate *OPRM1* expression through a negative feedback mechanism involving DNA methylation. Conversely, the relatively constant level of μ -opioid receptor ligand perfusion resulting from long-acting pharmacokinetics of methadone may increase DNA methylation. Studies of heroin addicts at entrance into methadone pharmacotherapy, and when well stabilized, should resolve this issue.

In rats, morphine withdrawal was shown to increase *oprm1* expression in the hypothalamus and striatum (Zhou *et al*, 2006). Acute and subacute cocaine administration increases *oprm1* expression in specific brain regions (Azaryan *et al*, 1998; Yuferov *et al*, 1999). Also, chronic binge cocaine administration was reported to increase μ -opioid receptor density in specific brain regions (Unterwald *et al*, 1994), and steady-state methadone administration was shown to prevent cocaine-induced conditioned place preference and to attenuate the increase of *OPRM1* mRNA in the nucleus accumbens during cocaine withdrawal (Leri *et al*, 2006). Further, in rats, the increase in μ -opioid receptor density is reported to persist for over 2 weeks following cessation of cocaine administration (Bailey *et al*, 2005), and in humans, μ -opioid receptor binding potential is increased compared to controls during cocaine abstinence (Gorelick *et al*, 2005). These effects may explain the efficacy of methadone maintenance treatment for cocaine as well as for heroin addiction (Peles *et al*, 2006). A possible mechanism of methadone pharmacotherapy is to down-regulate the increased *OPRM1* expression during heroin withdrawal, thus contributing to the reestablishment of normal μ -opioid receptor levels.

The role of methylation of the *OPRM1* gene promoter has been studied by Simon in human cell lines (Andria and Simon, 1999). SH-SY5Y cells, a neural-derived cell line expressing *OPRM1*, had low basal *OPRM1* promoter methylation. In contrast, two other neural-derived cell lines (IMR-32 and nMB cells), which do not naturally express *OPRM1*, had high methylation of the *OPRM1* promoter. Non-*OPRM1* expressing, non-neuronally derived cell lines (HEK 293, HepG2, and FS-4) had intermediate methylation. Furthermore, transient transfection experiments indicated that methylation might be involved in *OPRM1* expression (Andria and Simon, 1999). It will be of interest, in future studies, to examine the correlation of the level of *OPRM1* expression in the lymphocytes with methylation of CpG sites in the promoter region. In addition, studies should be conducted on the role of Sp1 in the regulation of transcription of unmethylated vs methylated *OPRM1* genes.

Peripheral lymphocytes, the source of genomic DNA in this study, express the *OPRM1* gene (McCarthy *et al*, 2001), and their availability greatly expands possible human studies. However, it is not known if these cells have a similar *OPRM1* methylation pattern to that in specific brain regions.

The identification of DNA methylation in heroin addiction could lead to novel pharmacotherapies for the treatment of opiate addiction. There are several pharmacological agents, such as azacitidine and valproic acid, that

could be explored in future preclinical studies on heroin and methadone effects. Azacitidine, a pyrimidine nucleoside analogue of cytidine, when metabolized and incorporated into DNA, acts as an irreversible inhibitor of DNA methyltransferases, preventing DNA methylation, with antineoplastic activity and is thought to activate tumor suppressor genes silenced by hypermethylation. Azacitidine is approved for the treatment of several subtypes of myelodysplastic syndrome, a group of diseases characterized by a disruption in the production of blood cells (Issa and Kantarjian, 2005). Valproic acid, an anticonvulsant, is a histone deacetylase inhibitor (Phiel *et al*, 2001), and histone acetylation and DNA methylation have been shown to be coupled. Valproic acid is used in the treatment of bipolar disorder and the prevention of migraine headaches. Both azacitidine and valproic acid are currently in clinical trials of the National Cancer Institute.

This is the first study to demonstrate an experiment-wise significant difference in DNA methylation at a specific CpG dinucleotide site between subjects with an addiction and controls at any gene. The identification of DNA methylation as a potential mechanism contributing to the development of heroin addiction, or as a result of heroin use or methadone pharmacotherapy, should lead to improved prevention or treatment of heroin addiction. Elucidation of the factors controlling DNA methylation may also help in understanding the role of the environment in this disease and its treatment.

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DISCLOSURE/CONFLICT OF INTEREST

All the authors, except JO, declare that, except for the income received from our primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interests. One author, JO, declares that he personally receives book royalties from the Johns Hopkins University Press and that his laboratory receives funding from Hoffmann-La Roche Inc.

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